THE DEVELOPMENT OF A CELL-BASED ASSAY FOR QUALITY CONTROL IN HUMAN *IN VITRO* FERTILISATION

by

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Declaration

No part of this thesis has been submitted in support of an application for any degree or qualification of Canterbury Christ Church University or any other University or institute of learning.

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Abbreviations

4E-BP - eIF4E binding protein

α-KG - α-ketoglutarate

αMEM - alpha minimum essential medium

AMP - adenosine monophosphate

AMPK - AMP-activated protein kinase

ART - assisted reproductive technique

ATP - adenosine triphosphate

BLM - blastocyst medium

CDX2 - caudal type homeobox 2

CE - Conformité Européene

CHO - Chinese hamster ovary

CIRP - cold-inducible RNA-binding protein

CLM - cleavage medium

CSP - cold shock protein

DMSO - dimethyl sulfoxide

EB - embryoid body

EC - embryonic carcinoma cells

EIF - eukaryotic initiation factor

EPL - early primitive ectoderm-like cells

ER - endoplasmic reticulum

ES - embryonic stem cells

EST - embryonic stem cell test

$FADH_2$ - flavin adenine dinucleotide

FCS - fetal calf serum

FLM - fertilisation medium

GMP - guanosine monophosphate

hCG - human chorionic gonadotropin

HSA - human serum albumin

HTS - high throughput screening

ICM - inner cell mass

ICSI - intracytoplasmic sperm injection

IU - international units

IVF - *in vitro* fertilization

LDH - lactate dehydrogenase

MEA - mouse embryo assay

mTOR - mechanistic target of rapamycin

MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NADH - nicotinamide adenine dinucleotide

ORB - oocyte retrieval buffer

PBS - phosphate-buffered saline

PMSG - pregnant mare serum gonadotropin

qHTS - quantitative high throughput screening

RA - retinoic acid

raptor - regulatory-associated protein of mTOR

RBM3 - RNA-binding motif protein 3

RBP - RNA-binding protein

REDOX - reduction-oxidative

rHA - recombinant human albumin

rictor - rapamycin-insensitive companion of mTOR

ROS - reactive oxygen species

rP - recombinant protein

S/B - signal-to-background ratio

S/N - signal-to-noise ratio

S6K - ribosomal S6 kinase

SG - stress granule

SOM - simplex optimization medium

TCA - the citric acid cycle

Abstract

Quality control systems are critical to improving laboratory practices and contribute significantly to success in human *in vitro* fertilisation (IVF). The mouse embryo assay (MEA) is commonly used in production quality control, as well as in product research and development. Despite its popularity there are some well-known issues associated with it; these include, but are not limited to, insensitivity to sub-optimal conditions, ethical issues due to animal use and variation in end-point interpretation between operators. The aim of this thesis was to therefore develop a cell-based assay that could facilitate research and development whilst addressing the issues presented above.

Firstly, using the pluripotent P19 embryonal carcinoma cell line, 2D and 3D cell-based assays were developed and optimised, and their response to culture perturbations such as varying osmolality and energy source levels were tested. Secondly, given that mammalian cells are generally resistant to changes in culture condition, characteristic features of the mammalian cold stress response, mTOR inhibitor rapamycin and AMPK inhibitor dorsomorphin were incorporated as potential tools for decreasing cellular resistance to altering culture conditions. The P19 assay demonstrated sensitivity to changes in energy substrate availability when treated with rapamycin and dorsomorphin, and was subsequently used to systematically screen different media formulations in order to identify the ranges of pyruvate, glutamine and calcium lactate concentrations required for optimum cell growth. Lastly, the developed 2D assay and the MEA were treated with the same products and their responses were directly compared to highlight similarities, and to identify differences, between the two assays. These assays/comparisons showed that the P19 assay identified differences in human IVF culture media that were not identified by the MEA.

The P19 assay may therefore represent a sensitive alternative to the MEA that can detect discrete changes in culture conditions and indicate the extent to which different energy substrates affect cell growth and proliferation. It is important to note that the two assays differ in their robustness and the P19 assay responded differently to repeat experiments with the same treatment. Overall, this novel P19 assay may provide an effective means of validating and testing products used in IVF whilst reducing animal use in research and development.

1. Introduction

1.1. Assisted reproduction in humans

Since the birth of Louise Brown, the first child conceived from successful *in vitro* fertilisation (IVF) in 1978 (Steptoe and Edwards, 1978), more than eight million children have been born globally as a result of assisted reproductive technologies (Wyns *et al.*, 2020). This success is attributed to many factors (see Table 1.1.), with the most significant deriving from the diversity of treatment options available (Roesner and Strowitzki, 2017; Abdelazim *et al.*, 2018; Braam *et al.*, 2021), as well as improvements in laboratory practices. One example of this is the introduction of the intracytoplasmic sperm injection (ICSI) technique (Palermo *et al.*, 1992), the injection of a single spermatozoon into an oocyte. This was initially used to address severe male infertility, particularly in patients with a low sperm count, but in many countries this has now replaced conventional IVF as the most commonly used assisted reproductive technique (ART) (Esteves *et al.*, 2018). This is unsurprising given that ICSI bypasses the standard oocyte-spermatozoa interactions required for fertilisation (Zemyarska, 2019), thereby providing applications that extend beyond male-factor infertility. Such applications include the prevention of the transmission of paternal infections such as HIV and hepatitis (Cito *et al.*, 2019), the facilitation of preimplantation genetic screening through the prevention of paternal DNA contamination from extraneous sperm that typically attaches to the zona pellucida during conventional IVF (Thornhill *et al.*, 2005; Van Landuyt *et al.*, 2005), and also its use in donor programmes to maximise the chances of fertilisation with frozen oocytes and spermatozoa (Fishel *et al.*, 2000).

Table 1.1. Treatment options that have contributed the success of assisted reproductive technologies over the last 40 years. Additional treatments (also referred to as supplements) have proven to be effective in improving success rates and are subsequently used to enhance traditional IVF methods.

Treatment Option	Description	Reference
Artificial oocyte activation	Gamete (oocyte and spermatozoa) fusion initiates a succession of oscillations in the intracellular concentration of calcium within the oocyte. This prompts a series of events that are collectively known as oocyte activation (see section 1.2.2.). Where there is a defect in oocyte activation, calcium ionophore is used to pump extracellular Ca^{2+} against the concentration gradient, increasing the concentration of Ca^{2+} in the oocyte plasma and ultimately activating the oocyte.	(Borges *et al.*, 2009; Ebner *et al.*, 2012; Ramadan *et al.*, 2012; Murugesu *et al.*, 2017; Saleh *et al.*, 2020; Xu *et al.*, 2021)
Assisted hatching	Assisted hatching refers to a technique that artificially thins or breaches the zona pellucida of the embryo. This improves the capacity of the embryo to implant to the endometrium lining of the uterus.	(Cohen *et al.*, 1992; Hammadeh, Fischer-Hammadeh and Ali, 2011)
Embryo culture medium	See section 1.1.	
Endometrial scratching	Endometrial scratching is the process of obtaining an endometrial biopsy sample with a sampler. The resulting endometrial injury or 'scratch' facilitates embryo implantation by inflammatory and immunologic mechanisms.	(Nastri *et al.*, 2013; Lensen *et al.*, 2019; Van Hoogenhuijze *et al.*, 2021)
Immunological testing	Reproductive failure has been associated with abnormal autoimmune function with increased levels uterine Natural Killer (NK) cells identified as the route cause. Immunological profiling is used to assess changes in uterine NK cells over the menstrual cycle.	(Putowski *et al.*, 2004; Gnainsky *et al.*, 2010; Granot, Gnainsky and Dekel, 2012)
Intracytoplasmic morphologic sperm injection (IMSI)	Given that sperm morphology plays a key role in determining fertility potential, IMSI uses an inverted light microscope to observe sperm at a high magnification (x6000) as opposed to the standard power microscopes typically used in IVF and ICSI.	(Bartoov, Berkovitz and Eltes, 2009; De Vos *et al.*, 2013)
Intracytoplasmic sperm injection (ICSI)	See section 1.1.	
Pre-implantation genetic testing for aneuploidy (PGT-A)	PGT-A involves genetic screening of trophectoderm cells from the blastocyst for the presence of normal numbers of chromosomes. Euploid embryos are subsequently selected for transfer.	(Vaiarelli *et al.*, 2016; Bracewell-Milnes *et al.*, 2021)
Time lapse imaging	See section 1.4.5.	

Improvements in culture conditions in the IVF laboratory have also played a significant role in success rates, specifically when considering the development of culture media. Its commercialisation has not only increased the standard of practice within IVF labs but has also changed some of the options for clinical practice. Until the early 1990s, human serum obtained from donors was the most commonly used protein source in IVF media, even though embryotoxic factors were often found in the serum (Leung, Gronow and Kellow, 1984; Léveillé, Carnegie and Tanphaichitr, 1992; Dokras *et al.*, 1993), and the risk of transmitting infections was not wholly eliminated. An example of such infection was seen in 1988, when a group of 128 women were exposed to the hepatitis B virus through supplementation of contaminated donor serum to media used in IVF treatment (Alberda *et al.*, 1989). The active virus was subsequently detected in 79 of these women with another three women becoming infected carriers. Going forward, to minimise risk, treatment centres then chose to use maternal serum obtained directly from patients for their own treatment. Interestingly however, certain patients were found to have unknown embryotoxic factors in their serum and consequently experienced failed cycles (Shaw *et al.*, 1987). These findings were also observed in a study by Léveillé and colleagues where some maternal sera supported embryonic development in mice, whilst others did not (Léveillé, Carnegie and Tanphaichitr, 1992). Furthermore, it was found that serum from women with unexplained infertility inhibited embryo growth in both mice and humans (Dokras *et al.*, 1993). At present, the majority of commercially available IVF media is supplemented with purified preparations of recombinant human albumin (rHA), which presents little to no risk of disease transmission diseases whilst supporting embryo and blastocyst development with an efficiency similar to that of serum obtained from donors (Bungum, Humaidan and Bungum, 2002).

Embryo culture media was first developed in 1973 (Hoppe and Pitts, 1973) by modifying Earle's balanced salt solution or Ham's F10 and T6, and in 1981, conversion into biological media was achieved by supplementation with maternal serum (Edwards, 1981). In later years, Menezo *et al.* created more advanced solutions by adding a mix of the 21 essential and non-

essential amino acids so that supplementation with serum was no longer essential (Menezo, Testart and Perrone, 1984). Lawitts and Biggers took this a step further and determined the optimal concentration of each media component, resulting in the formulation of Simplex Optimization Medium (SOM), which has since been modified to produce one-step media that is used as an alternative to sequential media to culture human zygotes to the blastocyst stage (Lawitts and Biggers, 1992; Mascarenhas *et al.*, 2021).

1.2. Physiology of embryo development

1.2.1. Fertilisation

Embryo development begins with fertilisation, where two haploid gametes each containing 23 chromosomes come together to produce a zygote (Leese, 1988). The functional roles and contributions of male and female gametes differ significantly. Whilst both oocytes and sperm contribute their genetic material to the resulting zygote, oocytes also provide cellular organelles and the nutrition required, to support early-stage embryo development (Stuppia *et al.*, 2015; Holt and Fazeli, 2016; Bradley and Swann, 2019). There are also striking dimensional differences between the two gametes; for instance, an oocyte is typically more than twice the length of a spermatozoon, with a volumetric ratio of 2000:1 (Koh and Marcos, 2015; García-Vázquez *et al.*, 2016). The physical process of fertilisation requires a single spermatozoon to penetrate the cells surrounding the oocyte to reach specific surface receptors on the oocyte membrane (Leese, 1988). For this purpose, the acrosomal cap contains digestive enzymes such as hyaluronidase, which break down the bonds between adjacent follicular cells, particularly the corona radiata of the cumulus layer (Talbot, 1985; Hoshi *et al.*, 1993; Hirohashi and Yanagimachi, 2018). The spermatozoon must bind to sperm receptors within the zona pellucida to induce the rupture of the acrosomal cap and subsequently release hyaluronidase and acrosin (another proteolytic enzyme), which digest a path through the zona pellucida towards the surface of the oocyte (Wassarman, 1987; Benoff, 1997). As these events occur, the sperm and oocyte membranes fuse to trigger oocyte activation (Tesarik, Sousa and Testart, 1994). Regardless of the number of spermatozoa that break through the cumulus

layer, for normal embryo development only a single spermatozoon will accomplish fertilisation and activate the oocyte (Neri *et al.*, 2014) (Figure 1.1).

1.2.2. Oocyte activation

Oocyte activation involves a series of changes in the metabolic activation of the oocyte (Yeste *et al.*, 2016; Kashir *et al.*, 2017) and is accompanied by the depolarisation of the oocyte membrane as a result of increased permeability to sodium ions (Carvacho *et al.*, 2018). The entry of sodium ions causes the release of calcium ions from the smooth endoplasmic reticulum. This sudden rise in Ca^{2+} levels activates signalling cascades that induce exocytosis of vesicles within the oocyte membrane (Abbott and Ducibella, 2001). This process, also referred to as the cortical reaction, releases enzymes that inactivate the sperm receptors and harden the zona pellucida to prevent polyspermy (fertilisation by more than one sperm), which would otherwise create a zygote incapable of normal development (Gwatkin, 1977; Cheeseman *et al.*, 2016). Meiosis II and the formation of a second polar body is also completed (Cooper, 2000; Larson *et al.*, 2010), in addition to the activation of enzymes that cause rapid increases in intracellular metabolic rates (Miao and Williams, 2012). The cytoplasm contains a large number of mRNA strands that are used to synthesise proteins required for embryo development to proceed (Miao and Williams, 2012). After oocyte activation and the completion of meiosis, the remaining nuclear material within the ovum reorganises itself to form the female pronucleus (Wakayama, Hayashi and Ogura, 1997; Schultz, 2002) while the nucleus of the spermatozoon swells to form the male pronucleus (Yanagimachi, 2005). The male pronucleus then migrates towards the centre of the cell and the two pronuclei fuse in a process known as amphimixis (Niklas and Kutschera, 2014). The activated oocyte is subsequently referred to a zygote, containing 46 chromosomes (Clift and Schuh, 2013). The chromosomes line up along the metaphase plate as the fertilised ovum prepares to divide (Larson *et al.*, 2010; Clift and Schuh, 2013); this marks the beginning of the cleavage stage, a series of cell divisions that produce an ever-increasing number of smaller and smaller daughter cells called blastomeres (Downie, 1978).

1.2.3. Cleavage and blastocyst formation

The first cleavage occurs 30 hours after fertilisation and subdivides the cytoplasm of the zygote to produce a presumptive zygote consisting of two blastomeres (Nogueira *et al.*, 2000). As illustrated by Figure 1.1, subsequent divisions occur at 10 – 12 hour intervals, and whilst the blastomeres divide simultaneously, the timing becomes less predictable as the number of blastomeres increases. After three days of cleavage, the pre-embryo becomes a solid ball of cells known as the morula, typically reaching the uterus on day four of development (Gardner, 1990). Over the subsequent two days, the blastomeres are no longer identical in size and shape and form a hollow ball with an inner cavity known as the blastocyst (Gardner, Lane and Schoolcraft, 2002). The outer layer of cells that separate the inner cavity from the surrounding environment are called trophoblasts and provide the developing embryo with nutrients. A second group of cells, the inner cell mass (ICM) form a cluster at one end of the blastocyst and, with time, will go on to form the foetus (Bongso *et al.*, 1994; Wang *et al.*, 2018). During blastocyst formation, enzymes released by trophoblasts erode a hole through the zona pellucida, which then shed in a process known as hatching (Lopata and Hay, 1989). The blastocyst is now freely exposed to the glycogen-rich content of the uterine cavity, whereas during the previous days, the pre-embryo and early blastocyst had been dependent on nutrients from the low-glucose surroundings of the fallopian tube (Amso *et al.*, 1994). Implantation begins when the surface of the blastocyst closest to the inner cell mass touches and adheres to the uterine lining. At the point of contact, the trophoblasts divide rapidly to produce several layers, and the cells closest to the interior of the blastocyst form a layer of cellular trophoblasts, while the plasma membranes that separate the trophoblasts disappear, creating an outer layer (the syncytial trophoblast) of cytoplasm containing multiple nuclei (Su and Fazleabas, 2015; Roland *et al.*, 2016). The syncytial trophoblast erodes a path through the uterine lining by secreting hyaluronidase, which dissolves bonds between uterine epithelial cells (Schatten and Constantinescu, 2007), just as hyaluronidase released by spermatozoa dissolves the connections between cells of the corona radiata. At first, the erosion creates a

gap in the uterine lining, but migration and divisions of maternal epithelial cells soon repair the surface, and by day ten, the blastocyst loses contact with the uterine cavity, and further development occurs within the confinement of the endometrium (Tabibzadeh and Babaknia, 1995).

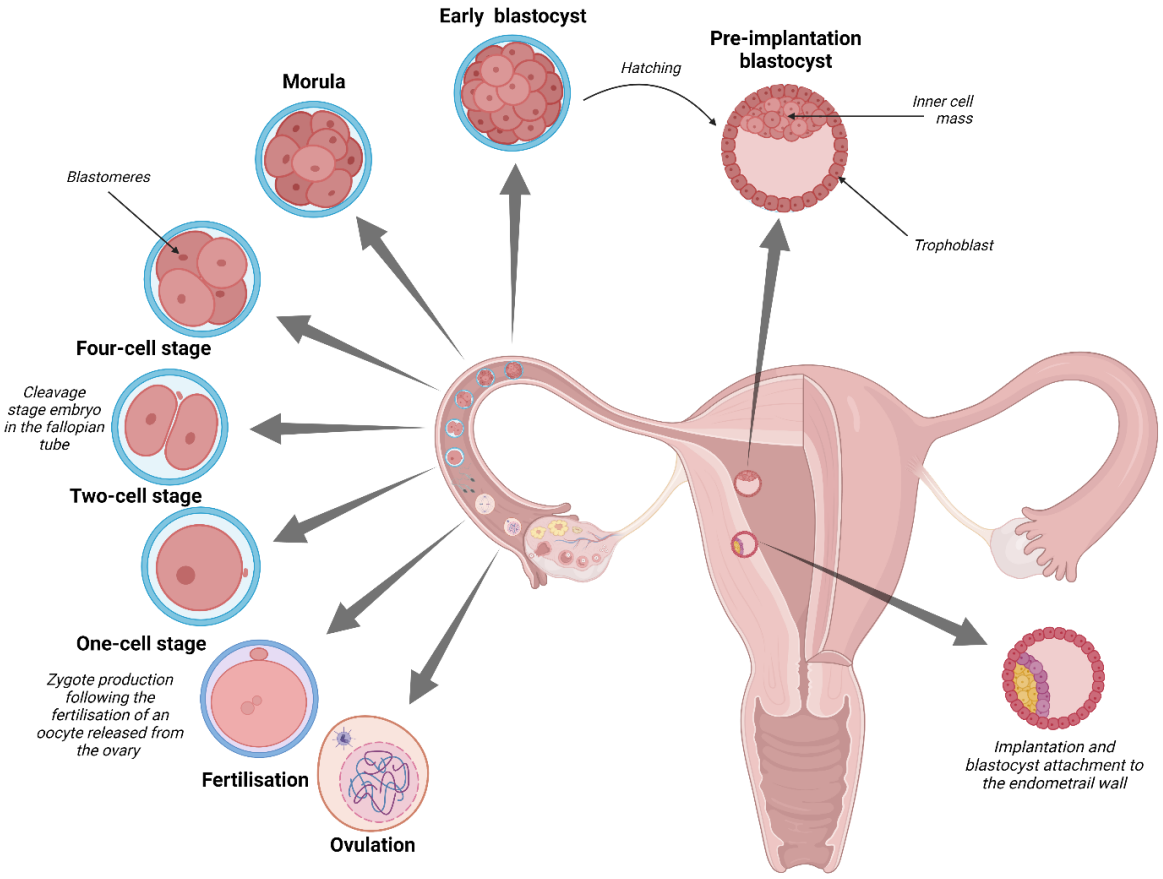


Figure 1.1. Oocyte and embryo development in the fallopian tube and uterus. In human females, a mature oocyte is released each month from the ovary during ovulation. Each oocyte has the potential to grow and acquire the capacity to be fertilised to form a zygote embryo; however, the mid-cycle LH surge from the pituitary glands triggers the release of the most mature oocyte. The mature oocyte is viable for 12 – 48 hours and if fertilised upon contact with active sperm, becomes a zygote. In its primitive stage, the zygote develops in the fallopian tube whilst on its way to the endometrium. The trophoblast cells that form the outer layer of the blastocyst invade the endometrial wall and form an attachment known as the trophoblast invasion. Image inspired by (Clift and Schuh, 2013; Robker, Hennebold and Russell, 2018). Created with BioRender.com.

1.2.4. Gastrulation and germ layer formation

On day 12, a third layer begins to form through the process of gastrulation. Here, the embryo transforms from the spherical dimension of the blastocyst into a multi-dimensional structure known as a gastrula (Solnica-Krezel and Sepich, 2012; Ghimire *et al.*, 2021). Cells in specific

areas of the surface move towards a central line known as the primitive streak, where they leave the surface and move between the two existing layers, creating three distinct embryonic layers (endoderm, mesoderm and ectoderm) called germ layers as shown on Figure 1.2 (Muhr and Ackerman, 2020). The ectoderm consists of superficial cells that do not migrate into the interior of the ICM, while the endoderm consists of cells adjacent to the ICM. Unsurprisingly, given that it makes up the middle layer, the mesoderm consists of the layer of migrating cells between the ectoderm and endoderm (D'Amour *et al.*, 2005; Muhr and Ackerman, 2020). These structures correspond to the development of specific primitive systems during organogenesis, the process of organ formation.

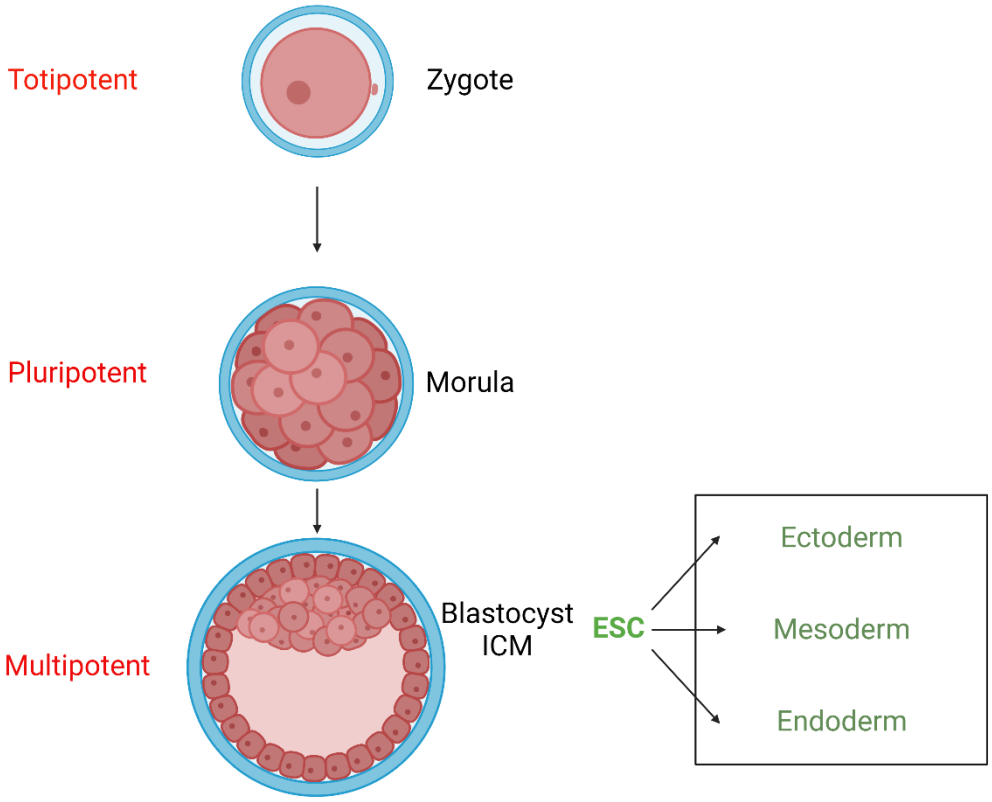


Figure 1.2. Schematic representation of blastocyst development and gastrulation. The zygote is defined as totipotent because it can give rise to any cell type or organism. Morula stage embryos are defined as pluripotent and retain the capacity to differentiate into all three primary germ layers. - Multipotent cells at the blastocyst stage retain the capacity to develop into more than one cell type but are more limited in their differentiating abilities than pluripotent cells (Wobus and Boheler, 2005). Created with BioRender.com.

1.3. The needs of a growing embryo

Reports on the metabolism of preimplantation embryos have demonstrated that the metabolic needs of each stage of early embryonic development differ from each other. As illustrated by Figure 1.1, following successful oocyte fertilisation, embryos in the cleaving process develop in the fallopian tube, whereas the natural environment for later stage embryos is in the endometrium (Morbeck, Krisher and Herrick, 2014). As such, in many IVF labs sequential media is used to provide the necessary nutrients to mimic these changing conditions. Alternative to sequential culture is continuous culture with one-step media, where a balance of substrates is used to support the embryo throughout the whole *in vitro* culture period. This method minimises interruption to embryos and maximises the benefits of time-lapse microscopy (see section 1.4.5) and is therefore becoming more prevalent throughout the clinical IVF sector (Fabozzi *et al.*, 2020; Moutos, Verma and Phelps, 2020). Both culture methods require pyruvate, lactate, and glucose which serve as primary sources of energy during early embryonic development and provide essential nutrients throughout preimplantation development (Gruber and Klein, 2011; Morbeck, Krisher and Herrick, 2014). For this reason, they are the most variable components between various types of human IVF media, with the base salts and other components tending to stay the same. Given that energy sources are critical to successful embryo development, it would be important to define methods that rapidly identify changes in their *in vitro* concentrations. This way, optimum conditions for growth at each stage of embryo development could be maintained by adjusting the concentration of necessary components. There are however very few studies that explore the practical ways in which this can be achieved.

1.3.1. Pyruvate

Pyruvate is endogenously produced during glycolysis in all eukaryotic cells (including gametes), and functions as an additional source of carbon. As such, pyruvate is commonly added to culture media as an extra source of energy (Bergemann *et al.*, 2019). As a metabolic intermediate, pyruvate is transported from the cytoplasm into the mitochondria, where it serves

as the master fuel input, controlling citric acid cycle carbon flux (Gray, Tompkins and Taylor, 2014). Pyruvate is oxidised to acetyl CoA which functions as a master regulator of metabolism due to its intersection with metabolic pathways and transformations. Cells monitor the levels of acetyl CoA as an indicator of their metabolic state and promote or obstruct its utilisation in lipid synthesis (Shi and Tu, 2015). Citric acid cycle products such as reduced nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$) feed the electron transport chain to produce 32 – 36 molecules of adenosine triphosphate (ATP), which serve as the primary energy currency for cellular processes (Figure 1.3). Thus, pyruvate is critical for generating mitochondrial ATP and driving major biosynthetic pathways in the cell (Magistretti and Allaman, 2018). Pyruvate also acts as an antioxidant through its ability to scavenge hydrogen peroxide molecules, which decarboxylases pyruvate molecules into acetic acid, carbon dioxide and water, without any oxygen release. This protects cells from oxidative stress triggered by reactive oxygen species (ROS), preventing tissue damage from haemorrhagic shock, brain damage and ischemia-reperfusion injury in humans (Korkmaz *et al.*, 2017).

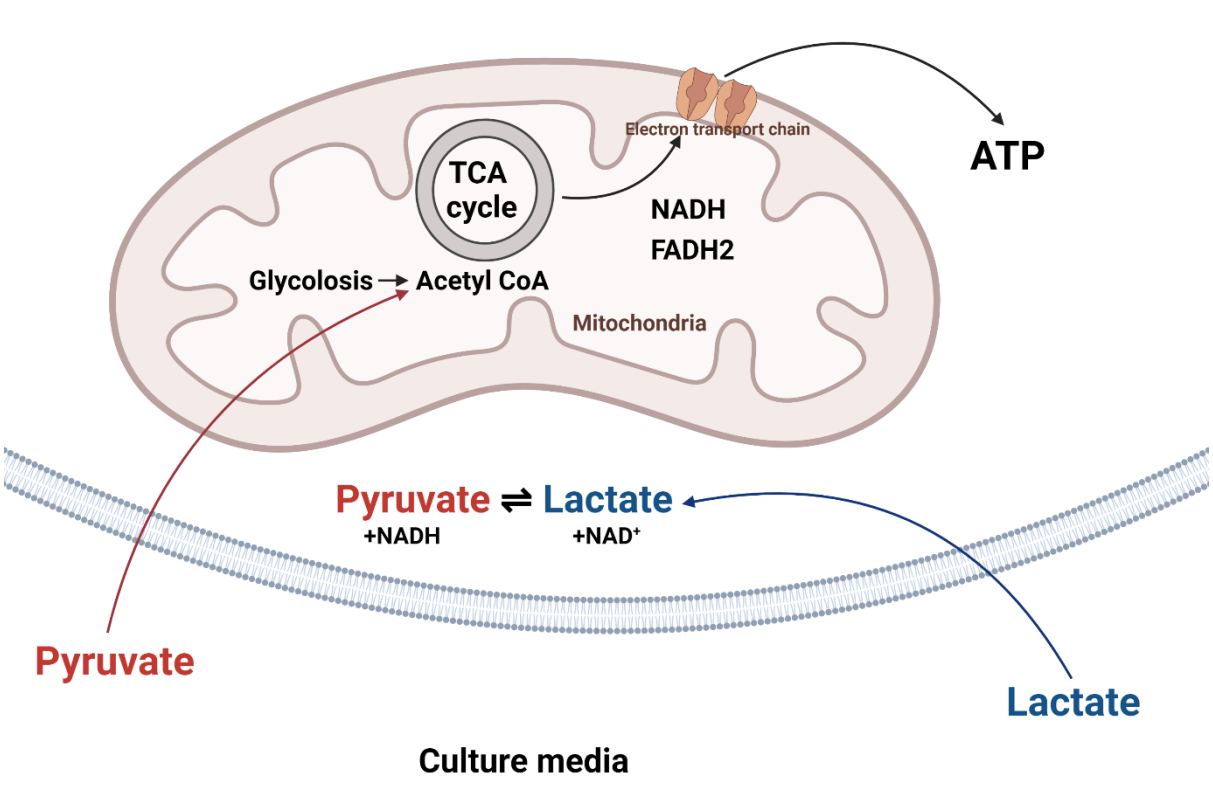


Figure 1.3. Pyruvate metabolism in the oocyte. Both pyruvate and lactate are transported into the oocyte from culture media *in vitro*, or from surrounding follicular fluid *in vivo*. Pyruvate is transported into the mitochondria and oxidised to acetyl CoA, which enters the citric acid cycle (TCA) to undergo a series of chemical reactions alongside reducing factors NADH and $FADH_2$. Electrons are transferred along a series of proteins bound to the inner membrane of the mitochondria whilst reducing oxygen to water in order to drive ATP production. Lactate and lactate-derived pyruvate are not taken up into the mitochondria for ATP production; however, these play a significant role in redox balance through NADH generation (Image inspired by Bradley and Swann, 2019). Created with BioRender.com.

In vitro studies of early-stage human embryos have established that pyruvate is imperative for the cleavage of one-cell embryos in the fallopian tube and is also the primary energy substrate throughout development from the morula stage to the blastocyst stage, with the growing embryo transitioning from the fallopian tube into the uterus (Dumollard *et al.*, 2008; Swain, 2019). Other studies have demonstrated that human embryos that arrest in culture consume approximately 20% less pyruvate on day two than those that progress to the blastocyst stage, indicating the vital support pyruvate provides for successful embryonic development. In mice and humans, oocytes and early embryos have an absolute requirement for pyruvate as an energy source, rather than glucose and only at the four to eight-cell stage can glucose, as a

sole substrate, support embryo development in culture (Hardy *et al.*, 1989; Gott *et al.*, 1990). The transition of preimplantation human embryos from a preference for pyruvate to glucose during early development is reflected by low glucose and high pyruvate and lactate in the human oviduct (Absalón-Medina, Butler and Gilbert, 2014), but high glucose and low pyruvate in the uterus (Gardner *et al.*, 1996; Morbeck, Baumann and Oglesbee, 2017; Ermisch *et al.*, 2020).

1.3.2. Lactate

Lactate is also a critical metabolite formed in the absence or presence of oxygen through aerobic glycolysis in eukaryotic cells. Upon entry into cells through monocarboxylate transporters and conversion by lactate dehydrogenase (LDH) to pyruvate, it is oxidised in the mitochondria, through the electron transport chain, to produce ATP (Hashimoto *et al.*, 2007). Aside from its role as an energy substrate, lactate is also a signalling factor for various types of cells within different tissues; for example, it promotes transcriptional changes that induce biogenesis of the mitochondria (Tauffenberger *et al.*, 2019). It is also known to support tumour cells, which rely on aerobic glycolysis by promoting cells migration, angiogenesis and expression of pro-survival factors such as HIF1α (Haas *et al.*, 2015). As shown in Figure 1.3, lactate is easily converted into pyruvate by oxidation with NAD+ in the cytosol, through a reaction catalysed by LDH, and subsequently fed into the citric acid cycle alongside other pyruvate molecules (Dumollard *et al.*, 2007). It can also be converted into glucose by the processes of glycolysis in the mitochondria and gluconeogenesis in the liver (Rabinowitz and Enerbäck, 2020), and for these reasons lactate is the most abundant metabolite in human IVF culture media, which typically contains a lactate to pyruvate ratio of one: five (Summers and Biggers, 2003). The lactate-pyruvate conversion and its associated consumption and production of NADH and NAD+ play a critical role in regulating cellular metabolism and maintaining the appropriate intracellular reduction-oxidative (REDOX) balance. Abnormal metabolism and REDOX states are detrimental to cells and are likely to cause deficiencies in embryo viability and IVF outcomes (Morbeck *et al.*, 2017).

1.3.3. Glutamine

Glutamine is another vital substrate often found in culture media, and is an essential source of reduced nitrogen for biosynthetic reactions, and similar to pyruvate, is an essential source of carbon for replenishing the citric acid cycle. It is the most predominant amino acid in humans, accounting for approximately 20% of the total amino acids in blood plasma and is by far the most abundant amino acid in the cerebrospinal fluid (Häberle *et al.*, 2006). Glutamine plays a central role in nitrogen metabolism for many cell systems as the main storage and transport form of both glutamate and ammonia while serving as a precursor to nucleotides and lipid synthesis through reductive carboxylation (Cluntun *et al.*, 2017). In purine biosynthesis, two molecules of nitrogen from glutamine are consumed in the biosynthesis of inosine monophosphate, which gives rise to both adenosine monophosphate (AMP) and guanosine monophosphate (GMP). Likewise, the initiating step of pyrimidine biosynthesis involves condensation of glutamine-derived nitrogen with bicarbonate and ATP to generate carbamoyl phosphate (Zhang, Pavlova and Thompson, 2017). Aside from its role as a nitrogen donor, glutamine is associated with the influx of carbon into the citric acid cycle, and citrate generated from this process is exported into the cytoplasm, where it is converted to acetyl-CoA. This is used to produce ATP while serving as a precursor for the biosynthesis of fatty acids and cholesterol (DeBerardinis *et al.*, 2007). The first step of glutamine catabolism is its conversion to glutamate, which is catalysed by glutamine aminotransferases or mitochondrial glutaminases. Glutamine-derived glutamate has several essential roles within proliferating cells, including its role as an energy source during protein synthesis, secretion from the cell in exchange for other nutrients, incorporation into the antioxidant glutathione and conversion into α-ketoglutarate (α-KG) for citric acid cycle anaplerosis. The formation of α-KG is catalysed by glutamate dehydrogenases, which releases ammonia as a by-product or transaminases, which transfers the amine-nitrogen of glutamate onto an α-keto acid to generate another amino acid (Cluntun *et al.*, 2017). As a critical intermediate of the citric acid cycle, glutamine-derived α-KG is believed to be the key determinant of cellular glutamine dependency (Figure 1.4). In culture, many mammalian cells fail to proliferate or even survive in the absence of

glutamine which is surprising given that cells possess the necessary machinery to synthesise it from the condensation reaction between glutamate and ammonia, catalysed by glutamine synthetases in an ATP-dependent manner (Wise and Thompson, 2010; Zhang, Pavlova and Thompson, 2017). Recently, asparagine, the amino acid most structurally similar to glutamine, was found to be sufficient to reduce glutamine-depletion-induced apoptosis in MYC (proto-oncogene and transcription Factor)-transformed cells, indicative of the dependency of these cells on glutamine anaplerosis (Wise *et al.*, 2008). These responses are attributed to the demands of various glutamine-utilising enzymes and the insufficiency of *de novo* biosynthesis of glutamine in accommodating these processes (Häberle *et al.*, 2006).

1.3.4. Glucose

Pyruvate, lactate and glutamine are undoubtedly essential energy sources in cell metabolism; however, glucose is the most extensively documented energy source typically used to supplement mammalian culture media (Navale, 2019). This is unsurprising considering that it is the principal source of ATP in mammalian cells (Donnelly and Finlay, 2015). As illustrated in Figure 1.4, glucose is initially metabolised in the cytoplasm via glycolysis, which involves a series of enzymatic steps that produce two molecules of ATP per molecule of glucose. Pyruvate, which is the end-product of glycolysis, is then oxidised to CO_2 by the citric acid cycle following transportation into the mitochondria. The reduced NADH and $FADH_2$ molecules generated from this fuel oxidative phosphorylation by feeding electrons into complex I and II of the electron transport chain within the inner mitochondrial membrane, reducing O_2 to H_2O (Zhao *et al.*, 2019). Protons produced during this process are translocated from the inner to the outer mitochondrial membrane, and the resulting proton gradient is used to drive ATP synthase, an enzyme that converts ADP to ATP, to generate 32 – 36 molecules per glucose molecule (as mentioned in section 1.3.1). In anaerobic conditions where there is no oxygen available to facilitate oxidative phosphorylation by the electron transport chain, cells take up large amounts of glucose that is metabolised to pyruvate by a highly active glycolytic pathway (Warburg, 1956), and convert it to lactate, which is then secreted from the cell.

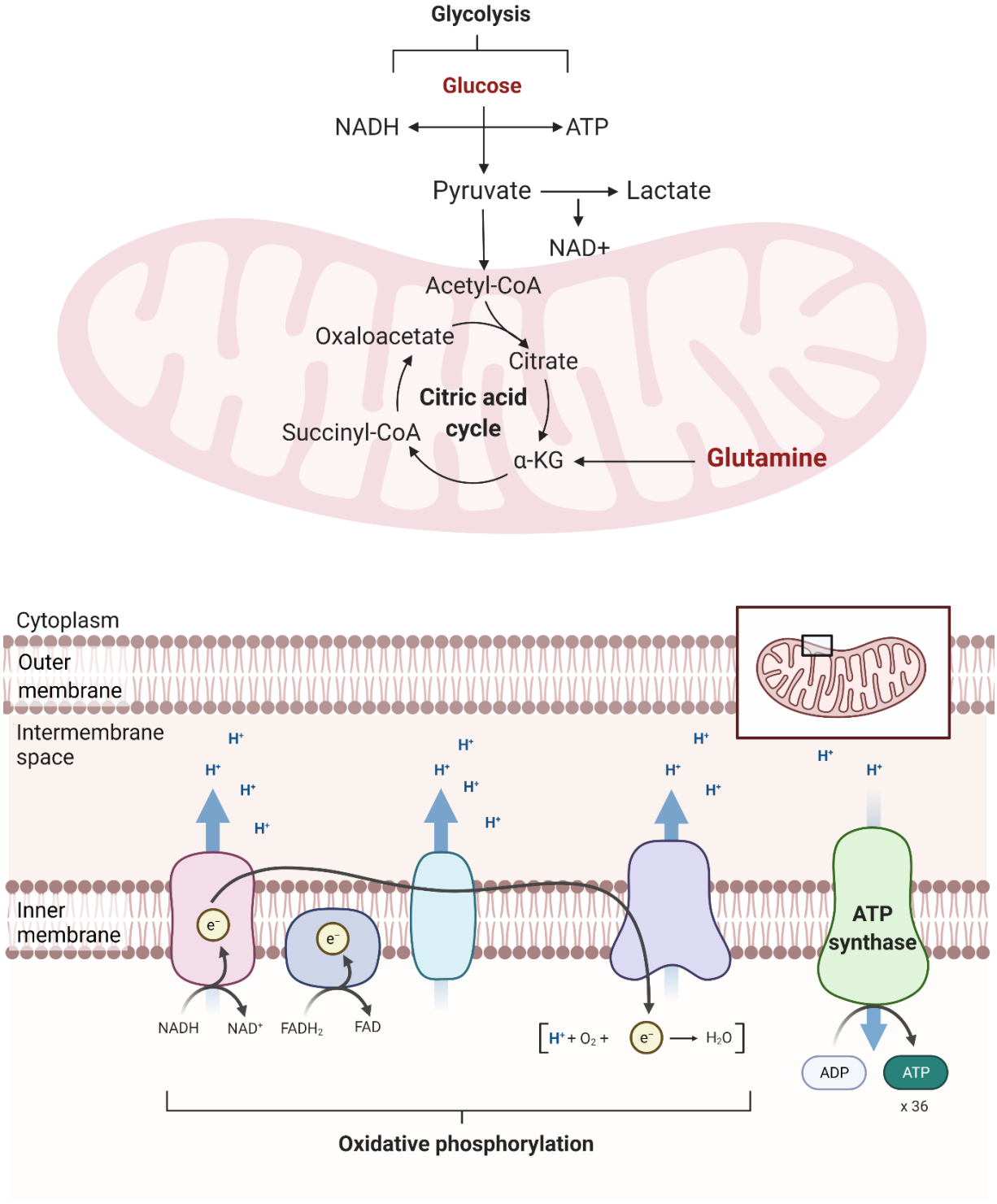


Figure 1.4. ATP production by glycolysis, the citric acid cycle and oxidative phosphorylation. The first step of glycolysis involves a series of enzymatic reactions that generate pyruvate. Depending on cellular activity and oxygen availability, pyruvate can have multiple fates within the cell. It can be completely oxidised to CO_2 in the mitochondria for the production of ATP or under anaerobic conditions, be converted to lactate which is secreted out of the cell. Pyruvate is also converted to lactate during aerobic glycolysis (Image inspired by Donnelly and Finlay, 2015). Created with BioRender.com.

In mammals, the cumulus cells that surround the oocyte are glycolytically active during oocyte maturation; they take up glucose and generate pyruvate, the preferred metabolic substrate of mammalian oocytes (Morbeck *et al.*, 2017). This is because the pentose phosphate pathway, which forms part of the glycolytic pathway, plays a significant role in glucose utilisation during meiotic maturation in oocytes and early embryos. As discussed above, metabolised via oxidative phosphorylation, pyruvate, and lactate are the primary energy substrates in early embryos. Following entry into the relatively anaerobic environment of the uterus, embryos switch to glycolysis as their primary mode of metabolism. Early studies by Barbehenn and colleagues indicated that mouse and hamster cleavage stage embryos experience a development block at the two-cell stage when cultured with media containing glucose (Barbehenn, Wales and Lowry, 1974; Schini and Bavister, 1988). More recent work by Gardner and Lane demonstrated that this glucose block could be effectively alleviated by adding amino acids to culture media (Gardner and Lane, 1996). Although glucose is not an absolute requirement for pre-compaction development in mice (it is instead used for post-compaction and blastocyst development), these observations support the rationale for including glucose in embryo culture media to provide more energy for metabolically active compacting embryos and blastocysts.

1.4. Quality control in human IVF

The exact formulation of commercially available human embryo culture media is generally only known by the manufacturers (Mantikou *et al.*, 2013; Sunde *et al.*, 2016), and although a list of components is provided as part of the product manual, details of the exact concentrations of each component are not included (Morbeck *et al.*, 2014), therefore making it impossible to replicate products in a standard research laboratory setting. Whilst the commercialisation of culture media has enhanced the standard of clinical and laboratory practises, it has created significant marketing competition (Karamalegos and Boiton, 1999), and commercial interests have caused companies to withhold full disclosure of the composition of their IVF media (Biggers, 2000; Chronopoulou and Harper, 2015; Sunde *et al.*, 2016). It is therefore

understandable that regulatory bodies that govern human IVF practices have implemented measures that force manufacturers to assess the quality of their products and report their findings before distribution (White and Woodward, 2020).

IVF media are classified as class III medical devices under the remit of the Medicines and Healthcare products Regulatory Agency (Chronopoulou and Harper, 2015) and therefore require Conformité Européene (CE) approval before use in the clinical laboratory. Interestingly, other consumables used in the production of IVF media such as disposable plasticware (petri dishes, well dishes, serological pipettes, and centrifuge tubes) are not under the same remit even though they also potentially impair the viability of embryos and gametes by the potential introduction of *in vitro* toxins to cell culture (Grimm *et al.*, 2018; Delaroche *et al.*, 2020). Generally, disposable plastics are not toxic to cells, however changes that occur during sterilisation, packaging, and storage can release breakdown products such as hydrocarbons and volatile organic compounds (Śladowski *et al.*, 2008). Other toxins can be derived from additives used to facilitate the transformation of polymers used as the basis of plastics to increase their stability (Olivieri *et al.*, 2012). Similarly, mineral oil is essential in embryo culture. This is derived from crude oil and consequently contains aromatics and unsaturated hydrocarbons that are susceptible to peroxidation and free radical formation (Otsuki, Nagai and Chiba, 2007; Morbeck and Leonard, 2012). These are known toxins that directly impact embryo development and IVF outcomes, even after their safety has been approved by manufacturers (Hughes *et al.*, 2010). The chemical nature of mineral oil makes it hazardous, yet in spite of this, it is one of the most widely used reagents in IVF laboratories because it plays a critical role in preventing medium evaporation during embryo incubation. Given that such substances adversely affect embryo development, it would be beneficial to develop methods that allow end-users to further scrutinise IVF products, even after they have been CE approved. This would ensue greater trust in the safety of reagents and equipment, and subsequently laboratory processes. Current quality control methods however are

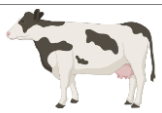
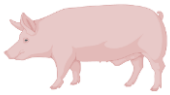
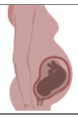
expensive and impractical for standard human IVF laboratory settings where animal use are typically restricted to prevent the spread of disease.

1.4.1. Bioassays for IVF quality control

Bioassays are a widely-used and accepted means of assessing toxicity in IVF media and laboratory supplies, including embryo transfer catheters and additional media components like albumin (Punt-Van Der Zalm *et al.*, 2009). The most documented of these assays use one-cell and two-cell mouse embryos, zona-free mouse embryos, mouse hybridoma cells, human fibroblast cells or hamster or human sperm (Fleming, Pratt and Braude, 1987; Bavister and Andrews, 1988; Davidson *et al.*, 1988; Bertheussen *et al.*, 1989; Gorrill *et al.*, 1991; Gilbert *et al.*, 2016). To date, one-cell and two-cell mouse embryo assays (MEAs) are considered to be the gold standard for toxicity testing in IVF labs, and as such are routinely used by manufacturers of IVF media and equipment to screen products before distribution, despite the fact that numerous studies argue that mouse embryo development is not directly comparable to that in humans (Naaktgeboren, 1987; Montoro *et al.*, 1990; Dumoulin *et al.*, 1991; Van Den Bergh, 1996). In the past, bovine and porcine models were considered as possible candidates for reducing the large number of laboratory animals used for quality control in IVF since oocytes can be obtained from the leftover ovaries in abattoirs (Santos, Schoevers and Roelen, 2014). As shown in Table 1.1, there are clear similarities between the development of oocytes and embryos in mice, pigs, cows and humans; however, generating bovine and porcine embryos *in vitro* presents numerous challenges. Firstly, the origin of abattoir derived ovaries is often unknown, making it impossible to obtain information on age, reproductive status and possible reproductive disorders affecting the animal that they came from. Secondly, as highlighted in Table 1.1, bovine embryos develop relatively slowly compared to porcine, murine and human embryos (Hasler, 2000; Morotti *et al.*, 2017). Furthermore, porcine embryos are challenging to generate and morphologically assess because of their high lipid density (Rath, Niemann and Torres, 1995), making it difficult to observe signs of successful fertilisation and subsequent development. Given that the use of human embryos in such

assays is not an option for ethical reasons, mouse embryos are currently the most suitable option for assessing materials that come into contact with human embryos, predominantly because their rapid development meets the commercial needs of fast-paced IVF laboratories (Harlow and Quinn, 1982).

Table 1.2. Differences between murine, porcine, bovine and human oocyte and embryo development. The average oocyte maturation time is indicative of the lipid content; thus, porcine oocytes have a relatively higher lipid density in comparison to murine oocytes. Overall, porcine and bovine embryos have a longer average period of development than that of murine and human embryos (Harlow and Quinn, 1982; Braude, Bolton and Moore, 1988; Prather, 1993; Laurincik, Rath and Niemann, 1994; Durinzi, Saniga and Lanzendorf, 1995; Papaioannou and Ebert, 1995; Rath, Niemann and Torres, 1995; Bouniol-Baly *et al.*, 1997; Otoi *et al.*, 1997; Anderson *et al.*, 1999; Rizos *et al.*, 2002; Sanfins *et al.*, 2003). Table inspired by (Santos, Schoevers and Roelen, 2014).

	Oocyte maturation (hours)	Zygote to two-cell embryo (hours)	Two-cell embryo to blastocyst (days)	Hatched blastocyst (days)	Developmental stage at time of embryonic genome activation
	14	12	2.5	1	Two-cell embryo
	40-44	32	4-5	2	Four-cell embryo
	20-24	36	4-5	2	Eight-cell embryo
	24-40	24-30	3-5	1	Four-cell embryo

1.4.2. The mouse embryo assay

The generation of large quantities of high quality oocytes for producing the embryos used in MEAs is made possible by superovulation, a hormonal treatment that causes rapid maturation of preovulatory follicles in mammals (Hoogenkamp and Lewing, 1982). In most studies, young female mice (21 to 27 days old) are superovulated by intraperitoneal injection with 5 international units (IU) of Pregnant Mare Serum Gonadotropin (PMSG), followed by a subsequent injection with 5 IU of human Chorionic Gonadotropin (hCG) 47 to 48 hours later (Behringer *et al.*, 2004). Similar to FSH, PMSG binds to granulosa cells in antral follicles to

stimulate oocyte maturation (Bevers *et al.*, 1989; Tirpan *et al.*, 2019) whilst hCG ensures the ongoing production of progesterone by the corpus luteum, to permit further follicular development (Shikone *et al.*, 1996). As illustrated in Figure 1.5, *in vivo* produced embryos are obtained by housing superovulated female mice in a cage with stud males and euthanizing them with CO_2 upon successful mating (evidenced by plug detection). Embryos are collected from the dissected oviducts (ampulla) after 24 hours for one-cell embryos and 36 hours for two-cell embryos (Yildiz *et al.*, 2007; Leonard *et al.*, 2013). For *in vitro* embryos, superovulated mice are sacrificed 12-13 hours after hCG administration and oocytes obtained from the dissected oviducts are placed in micro drops containing sperm from the vas deferens and cauda epididymis of adult male mice. These are cultured overnight at 37°C and 5% CO_2 and the resulting embryos are considered viable if there are two visible polar bodies after 24 hours (Gilbert *et al.*, 2016). The MEA is performed by submerging mouse embryos in test medium with a mineral oil overlay, and monitoring development for 96 hours (U.S. Food and Drug Administration, 2019) although some centres extend this period of assessment to 120 hours for greater accuracy (Wolff *et al.*, 2013). Throughout this time, embryo morphology is assessed, and the media is considered suitable and safe for clinical use if 80% or more of the embryos achieve early or late blastocyst formation (Figure 1.6).

1.4.3. Mouse IVF success rates

Fertilisation rates in mice are dependent on numerous factors including the weight and age of the female donor (Beaumont and Smith, 1975), and these differ considerably between strains (Takeo and Nakagata, 2015). For instance, the optimal weight range for superovulation is between 10.5 to 14.4 grams in C57BL/6 mice and 16.5 to 18.5 grams in B6D2F1 mice whereas FVB donors do not respond as well to superovulation as other strains regardless of their age and weight (Takeo and Nakagata, 2015). The success of fertilisation is also dependent on the dose of PMSG and hCG administered, with C57BL/6NHsd mice generally producing more oocytes when treated with 2.5 IU PMSG as opposed to the standard dose of 5 IU PMSG (Takeo and Nakagata, 2015). Surprisingly, considering its effect on the overall success of mice

IVF, development of new, strain-specific superovulation protocols have been limited and most studies tend to treat female mice of the same age with equal amounts of PMSG and hCG (H. Wang *et al.*, 2001). The challenge with this is that increased numbers of female donors are sacrificed to generate the requisite number of oocytes for research and quality control in human IVF if a particular strain responds poorly to superovulation. Subsequently, despite the fact that embryos generated from F_1 hybrid mice have greater success in embryo development than those produced from inbred or random bred strains (Harlow and Quinn, 1982), and are therefore the standard for quality control testing in human IVF, it is recommended that embryos from outbred mice are used (Khan *et al.*, 2013). This is because they generally respond well to standard doses of PMSG and hCG and are more sensitive to toxins and alterations in culture conditions, than inbred and F_1 hybrid strains (Khan *et al.*, 2013; Takeo and Nakagata, 2015). Overall, *in vivo* and *in vitro* fertilisation success in mice can range from 32% to 86%, depending on the factors mentioned above (Takeo and Nakagata, 2015).

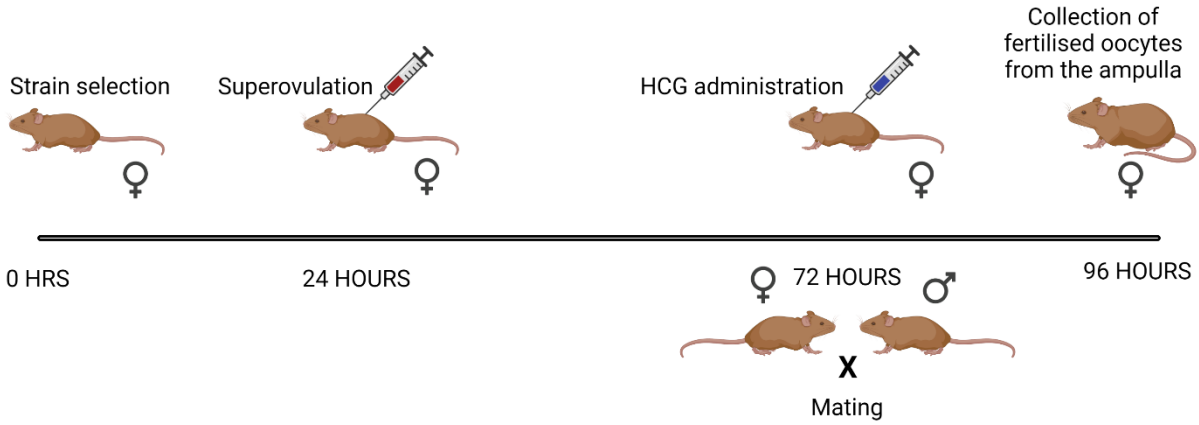


Figure 1.5. Schematic representation of steps involved in obtaining embryos from mice for use in IVF quality control. Before mating, hybrid mouse strains such as the CBA/B6 are super ovulated by intraperitoneal injection with 5 IU of PMSG on day one, followed by a second injection of hCG to stimulate the release of oocytes on day three. Male and female mice of the same hybrid are then placed in a cage for mating and on day four, female mice are checked for the presence of a copulation plug as evidence of successful mating. To obtain the fertilised oocytes, female mice are euthanized by CO_2 inhalation , the oviducts are removed and the cumulus-oocyte complexes are extracted from the ampulla (Gilbert *et al.*, 2016; U.S. Food and Drug Administration, 2019). Created with BioRender.com.

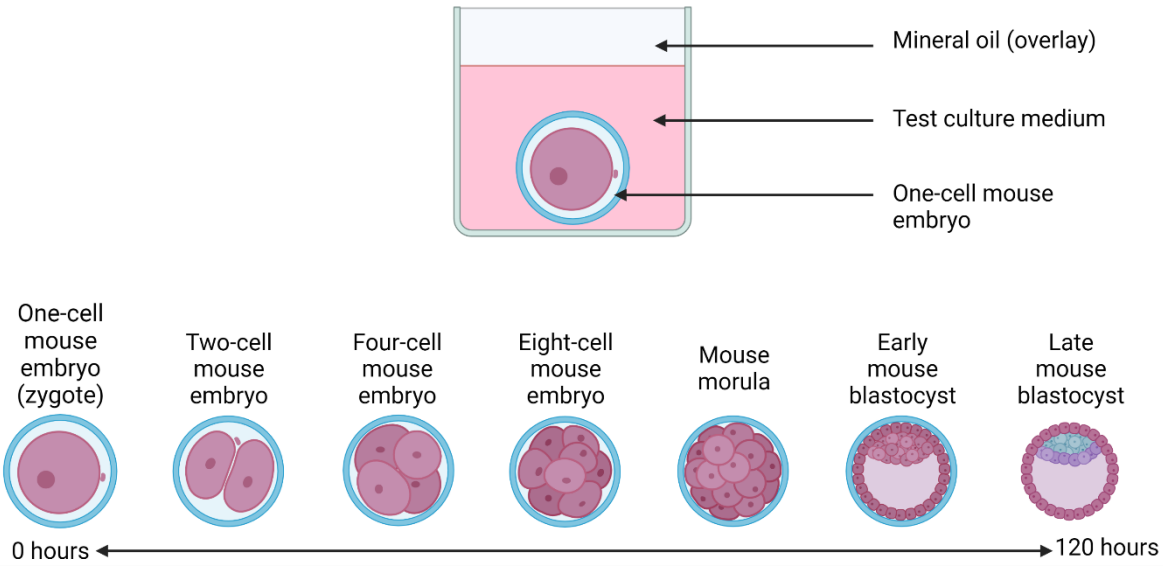


Figure 1.6. Quality control of human embryo culture media. Fertilised oocytes obtained from mice are observed under a microscope for two pronuclei as evidence of successful fertilisation. These are then cultured in test medium with an oil overlay and development rates are evaluated after 96 hours or after 120 hours for extended MEAs (Punt-Van Der Zalm *et al.*, 2009). Created with BioRender.com.

1.4.4. Challenges associated with the mouse embryo assay

Despite its popularity, there are some concerns over the use of MEAs for toxicity testing in IVF laboratories. Firstly, manufacturers and clinical laboratories have reported different approaches to quality control and toxicity testing of IVF media, and the lack of standard practices may result in interpretational disparities between laboratories and technicians. The strain of mouse (outbred, inbred or hybrid), the number and origin (fresh or frozen/thawed) of the embryos, as well as their developmental stage (one-cell or two-cell), differs from one manufacturer to another, and these parameters influence the sensitivity of the test (Davidson *et al.*, 1988; Gardner *et al.*, 2005; Punt-Van Der Zalm *et al.*, 2009; Khan *et al.*, 2013; Wolff *et al.*, 2013). Secondly, previous studies tended to report toxicity of laboratory materials without identifying the specific toxin; however, more recent reports of treatment with known or suspected toxins like cumene hydroperoxide, a peroxide surrogate that accumulates in mineral oil have consistently demonstrated that the one-cell MEA is superior to the two-cell MEA (Otsuki, Nagai and Chiba, 2007; Hughes *et al.*, 2010; Morbeck *et al.*, 2010; Wolff *et al.*, 2013). Despite this evidence, the two-cell system is still commonly used by many manufacturers and numerous IVF laboratories (Zhang *et al.*, 2009; Hughes *et al.*, 2010; Gao *et al.*, 2017). Furthermore, studies on the variation in sensitivity between different strains of mice have provided evidence that current standards of using inbred or F_1 hybrid MEA may not be the most sensitive method (Khan *et al.*, 2013; Wolff *et al.*, 2013). For instance, a study by Wolff *et al*., highlighted that embryos from CF_1 outbred mice were four times more sensitive to cumene hydroperoxide than other inbred strains, detecting concentrations as low as 2 µM (Wolff *et al.*, 2013). Discussions on the reproductive potential of F_1 hybrid female mice and the ambiguity caused by its dependency on housing conditions such as the light-dark schedule, temperature and noise, as well as seasonal influences, have served as justification for continuous use of inbred strains; making it difficult to standardise MEA procedures (Gardner *et al.*, 2005; Punt-Van Der Zalm *et al.*, 2009). Lastly, more recent studies on the suitability of mice as animal models indicate that the timing of the first three cell division cycles in human embryos are vital in determining the developmental potential of the embryo and could serve

as a robust quality control tool (Ajduk and Zernicka-Goetz, 2013; Gardner *et al.*, 2015; Mancini *et al.*, 2020). At present, this information cannot be determined from MEA methods because they do not assess the pronuclear or cleavage stage of embryo development. In fact, MEAs are most commonly criticised for only indicating extreme differences in toxicity without providing information on more subtle alterations in culture conditions (Wolff *et al.*, 2013; Gilbert *et al.*, 2016). Further to this, there is evidence of mouse embryos developing under adverse culture conditions, to the extent that media previously considered suitable for IVF use by the manufacturer was recalled following in-house toxicity testing (Morbeck *et al.*, 2014). Considering that over the years, discoveries made through MEA findings have been intimately linked to success in human IVF, it is important that these challenges are directly addressed in order to improve quality control processes. However, previous studies have tended to focus on alternative solutions as opposed to tackling the existing ones.

1.4.5. Improvements to the mouse embryo assay

Suggestions for improved toxicity testing include incorporating fluorescent molecular biomarkers for transcription factors caudal type homeobox 2 (*CDX2*) and *Pou5f1* (previously known as *OCT4*) which are respectively expressed in the trophectoderm and ICM of human and mice embryos during early stage development (Bou *et al.*, 2016). This method, the mouse embryo genetic assay (MEGA) is believed to provide greater insight into the development of mouse embryos to the blastocyst stage as opposed to the single endpoint observation experienced with current protocols (Gilbert *et al.*, 2016; Zhang *et al.*, 2016). These early developmental genes are differentially expressed at the two-cell stage when gene expression profiles switch from the maternal pattern to the zygotic pattern (zygotic gene activation) and exhibit specific expression patterns (Gilbert *et al.*, 2016). *Pou5f1,* which is highly expressed at the beginning of the four- to eight-cell stage of embryo development, is a well-described transcription factor known for its role in embryo pluripotency (Kehler *et al.*, 2004; Loh *et al.*, 2006; Shi and Jin, 2010) and *CDX2* is another transcription factor that signals the initial stages

of cell lineage differentiation in mice and is typically expressed in eight- to 16-cell embryos and the trophectoderm (Beck *et al.*, 1995; Jedrusik *et al.*, 2008). *Pou5f1* and *CDX2* are co-expressed in preimplantation mouse embryos and form a complex for repression of their target genes in embryonic stem cells (Niwa *et al.*, 2005). For example, during development from the morula to the blastocyst stage, coordinated repression of *Pou5f1* by *CDX2* takes place, as a regulatory complex formed with *CDX2* suppresses expression of *Pou5f1* in the trophectoderm. Conversely, the expression of *Pou5f1* is maintained within the ICM in the absence of *CDX2* (Wang *et al.*, 2010). Overall, transgenic mouse technology provides an advantage by generating embryos that express fluorescent reporter proteins under the control of regulatory elements controlled by *Pou5f1* and *CDX2* (Gilbert *et al.*, 2016), and as such, provides more sensitive detection of potential toxins. However, these methods are not widely used because they require costly equipment which may not be universally available or realistically used for quality control testing (Tokoro *et al.*, 2015).

Time-lapse imaging and morphokinetic analysis of cell division during embryo development have also been suggested as more sensitive quality control measures that enable the observation of key developmental markers such as polar body extrusion, pronuclear formation, cleavage timings and patterns, as well as enabling identification of fragmentation throughout the development process, as reviewed by (Mandawala *et al.*, 2016). A series of snapshots of the developing embryos are taken at set time intervals (which can be adjusted to accommodate specific research needs), and these images are collated by a software pre-installed software within the time-lapse device. This method allows continuous monitoring of developing embryos in an undisturbed culture environment whilst highlighting abnormal cleavage patterns and fragmentation (Dolinko *et al.*, 2017). Initially used in 1929 to monitor the development of rabbit embryos (Lewis and Gregory, 1929), time-lapse imaging has successfully identified key features associated with early development in zebrafish (Distel and Köster, 2007) *Caenorhabditis elegans* (Boyd, Hajjar and O'Connell, 2011) as well as human embryos (Conaghan *et al.*, 2013). However, whilst useful from a clinical and research

standpoint, time-lapse embryo imaging is an expensive and time-consuming option that has not been conclusively shown to provide clinically significant information on the suitability of culture media (Wolff *et al.*, 2013).

Lastly, the extended MEA, which involves assessing blastocyst formation after 120 hours (as opposed to 96 hours) has been put forward as an inexpensive improvement to standard MEA protocols. In fact, it has been found to be as sensitive to toxins as the time-lapse imaging and morphokinetics analysis assay described above (Wolff *et al.*, 2013; Ainsworth, Fredrickson and Morbeck, 2017). However, although well-received, this extended version is not mandatory (U.S. Food and Drug Administration, 2019), and the 96-hour incubation period is still the most common practice for media manufacturers and IVF laboratories.

1.5. 2D cell culture systems

Primary cell cultures and permanent cell lines provide an alternative method for detecting toxicity in IVF media. The use of traditional cell culture (or 2D culture) is well-established for investigating the mechanisms of disease, pharmacological activity and protein production, and as such, it is used in preclinical research (Breslin and O'Driscoll, 2013). For example, Chinese hamster ovary (CHO) cells are the most widely-used mammalian hosts in biological and medical research, particularly for expressing human therapeutic proteins like FSH and LH (Yoon *et al.*, 2006; Jayapal *et al.*, 2007; Du and Webb, 2011). Since the approval of their use by the US food and drug administration (FDA) in 1987, approximately 70% of recombinant therapeutic proteins are produced in CHO cell systems with a market value of over £15 billion per annum (Jayapal *et al.*, 2007; Du and Webb, 2011; Kaplon *et al.*, 2020). Although they require expensive culture media and grow relatively slowly in comparison to *Escherichia coli* and yeast (Yamano-Adachi *et al.*, 2020), CHO cells allow more accurate protein assembly and folding so that these processes are similar to that of human cells, making them suitable models

of key steps associated with protein synthesis in humans (Omasa *et al.*, 2010). Subsequently, for proteins whose activities are closely linked to posttranslational modification, such as erythropoietin, CHO cells are suitable production hosts. CHO cells also synthesise extremely large molecules like blood factor VIII as discussed in section 1.8.

In adherent 2D cultures, cells are grown as a monolayer in a culture flask or petri dish and cultivated after an adequate level of proliferation has occurred (Duval *et al.*, 2017). This method has contributed significantly to biological research and its application in quality control in IVF has demonstrated that cell culture systems are susceptible to media toxicity (Bertheussen *et al.*, 1989; Breslin and O'Driscoll, 2013). A challenge here however, is that studies tend to use cell lines established from specialised somatic cells that do not exhibit the properties of early embryonic development, particularly differentiation, making them unsuitable for detecting toxicity within embryo culture environments (Bertheussen *et al.*, 1989).

Fortunately, more recent studies have used pluripotent human or mouse embryonic stem cells, which have the capacity to differentiate into all three primary germ layers (Festag, Viertel, Steinberg and Sehner, 2007). An example of this is the embryonic stem cell test (EST), an *in vitro* assay designed to assess the teratogenic nature of drugs and substances that come into contact with *in vivo* embryos. It was developed in Berlin by the Centre for Documentation and Evaluation of Alternative Methods to Animal Experiments in 1997 and validated by the European Centre for the Validation of Alternative Methods in 2011 (Genschow *et al.*, 2004; Seiler and Spielmann, 2011). Based on the ability of immortalised murine cell lines 3T3 fibroblasts and embryonic stem cells from the D3 line to differentiate into contraction cardiomyocytes in embryo culture environments, it was deemed the most promising *in vitro* assay for determining embryotoxicity in compounds (Spielmann *et al.*, 1997). Here, the cytotoxicity of cells following treatment with test substances are determined by the 3-(4,5-

dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (see section 1.6), a widely used viability test. Using a statistical predication model, the endpoint readings are used to determine the toxic nature of the substance. Although beneficial in the pharmaceutical industry for predicting toxicity, the challenge with such a model is that it was initially designed for use in drug development where adverse effects on embryonic development are amongst the most harmful side-effects of drugs (Scholz *et al.*, 1999; Spielmann and Liebsch, 2001; Buesen *et al.*, 2009; Marx-Stoelting *et al.*, 2009; Riebeling *et al.*, 2011). It therefore does not consider characteristics and features that are specific to IVF products.

1.5.1. 3D cell culture systems

Given the importance of cell culture in scientific research, many advances have been made to further mimic *in vivo* environments in *in vitro* systems. The use of three-dimensional (3D) cell culture has emerged as a novel approach that share more similarities to *in vivo* conditions than 2D culture systems; 3D cell cultures better reflect the distribution of nutrients, metabolites, catabolites, and oxygen *in vivo* (Wu *et al.*, 2018). In stem cell biology where most advances are based on mouse embryogenesis, 3D cultures enhance the differentiation capability of stem and progenitor cells. For instance, progenitor cells derived from salivary glands can differentiate into hepatocytes and pancreatic lineages only in 3D spheroidal culture but not in 2D adherent culture (Okumura *et al.*, 2003).

1.5.1.1. Embryonic stem cell culture systems

A fertilised oocyte has the capacity to generate an organism because of its totipotency which is retained until the eight-cell stage of embryo development (Condic, 2014). Successively, cell differentiation in humans and mice gives rise to the blastocyst, composed of the outer trophoblast cells and the undifferentiated ICM, as discussed in section 1.2.3. These cells are pluripotent and retain the ability to develop into all cell types, as shown in Figure 1.2. Following transplantation of ICM cells (often referred to as embryonic stem (ES) cells) to extra uterine

sites of appropriate mouse strains (Figure 1.7), benign teratomas or malignant teratocarcinomas are produced (Stevens, 1970; Evans, 1972). In its 3D form, embryonic carcinoma (EC) cells derived from teratomas or teratocarcinomas retain the capacity to differentiate to produce derivatives of all three germ layers; the ectoderm, mesoderm and endoderm. Importantly, ECs have demonstrated an ability to participate in embryonic development when introduced into the ICM of early embryos and subsequently present a powerful tool for studying early embryonic development (Bradley *et al.*, 1984). On the other hand, some studies have highlighted some of the shortcomings associated with such a system. Firstly, ECs lose some of their differentiating abilities, although this can be overcome by incubation with chemical inducers such as dimethyl sulfoxide (DMSO) (Bernstine *et al.*, 1973; Nicolas *et al.*, 1975). The underlying mechanisms are however not fully understood (Jasmin *et al.*, 2010; Whaley *et al.*, 2021). Secondly, maintaining their undifferentiated state relies on cultivation with a feeder layer which provides self-renewing signals to the cells (Taru Sharma *et al.*, 2012). Lastly, ECs are not consistent in their colonisation of the germline when transferred into blastocysts suggesting that EC cells cannot retain their pluripotent capacity during early embryonic development, but undergo cellular changes during the transient tumorigenic state *in vivo* (Mintz and Illmensee, 1975; Wobus and Boheler, 2005). To avoid these alterations, researchers advise that cells from the ICM are directly cultured *in vivo* rather than injecting them into peritoneal cavities for teratocarcinoma formation. In 1981 Evans and Kaufman successfully cultivated pluripotent cell lines from mouse blastocysts using a feeder layer of mouse embryonic fibroblasts (Evans and Kaufman, 1981), while Martin produced a medium expressly conditioned to promote EC growth (Martin, 1981). Subsequently, ECs could be maintained *in vitro* without losing their ability to differentiate. In later studies, the pluripotency of EC cells was assessed *in vivo* by introducing them into mouse blastocysts, and the resulting mass demonstrated that these cells could contribute to all cell lineages, including the germline (Bradley *et al.*, 1984).

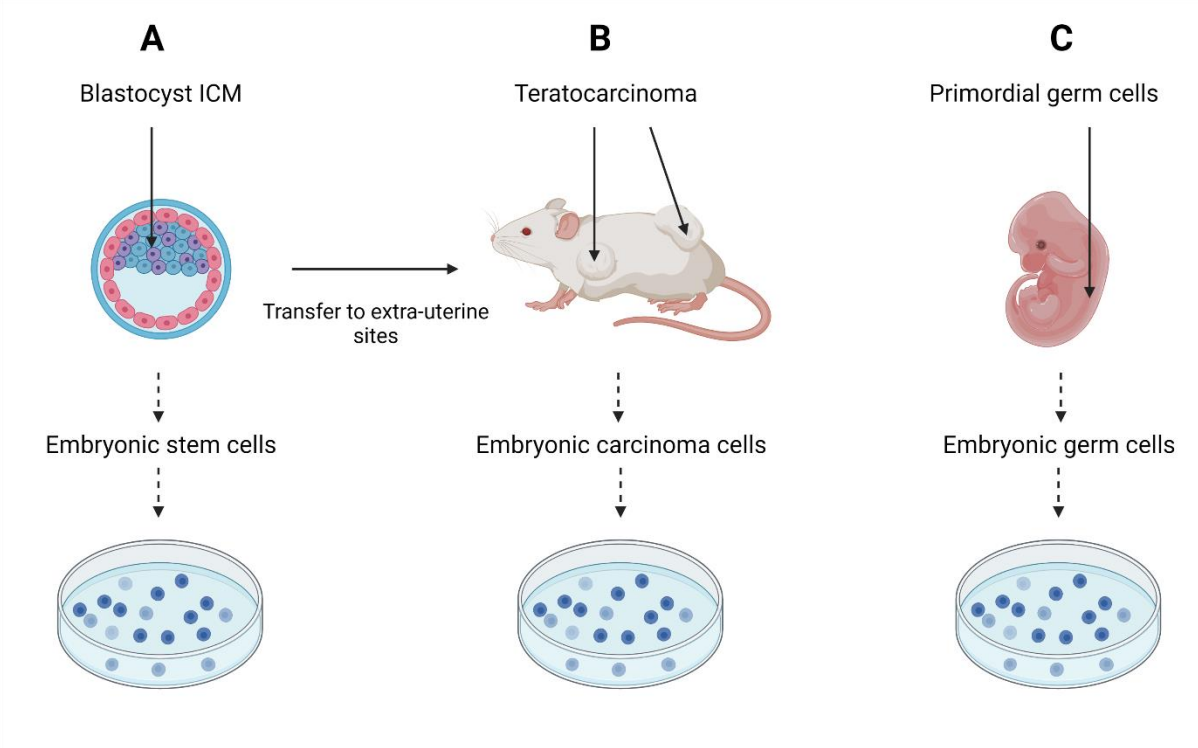


Figure 1.7. Schematic diagram of the developmental origin of pluripotent embryonic stem cell lines in mice. Shown are the derivation of ESCs (A), EC cells (B) and EGCs from different embryonic stages of the mouse (C). EC cells are derived from teratocarcinomas that originate from blastocysts transplanted in extrauterine sites within the mouse. EGCs are cultured from PGCs isolated from the genital ridges of day 9 – 12 embryos. Image inspired by (Wobus and Boheler, 2005). Created with BioRender.com.

1.5.1.2. Spheroids

In general, research has found that cells in 2D culture differ physiologically from cells in 3D culture, and that numerous cell lines exhibit different gene expression levels and different drug sensitivity levels between 2D and 3D culture models (Shaw, Wrobel and Brugge, 2004; Loessner *et al.*, 2010). The additional dimension in 3D cultures results in differences in cellular responses. Not only are there differences in the spatial organisation of cell surface receptors involved in interactions with surrounding cells, but there is also physical constraint impacting which cells can communicate with each other (Adcock *et al.*, 2015). These spatial and physical differences in 3D culture also affect the signal transduction from the extracellular environments into the intracellular environment of cells and ultimately influence gene expression patterns (Zietarska *et al.*, 2007; Lee, Cuddihy and Kotov, 2008; Shield *et al.*, 2009). There are several different kinds of 3D cell culture methods, such as cell spheroid formation (Edmondson *et al.*,

2014; Ravi, V. Paramesh, *et al.*, 2015), cells seeded into moulds (Vunjak Novakovic, Eschenhagen and Mummery, 2014), cells seeded onto hydrogels (Dosh *et al.*, 2017), matrices (Blazeski, Kostecki and Tung, 2015) and cellular sheets (Shimizu *et al.*, 2002), with cell spheroids being the most widely-used of these in both research and industry to date (Haycock, 2011; Zuppinger, 2016; Zanoni *et al.*, 2020). For example, in drug discovery, spheroids that mimic vascularisation of ovarian tumours have been used to study the performance of the first FDA approved nanomedicine drug, Doxil® (Singh *et al.*, 2020). Furthermore, spheroids formed by prostate cancer cell lines VCaP and LNCaP have been used to successfully define the efficacy of the preclinical anticancer drug MLN924 while it was being evaluated in clinical trials for treatment of hematologic malignancies and solid tumours (Mittler *et al.*, 2017).

On the other hand, 3D bioprinting allows complex structures to be created through the integration of cells and natural materials like glycosaminoglycan (Da Silva *et al.*, 2020). These can be used to produce extra cellular matrices that mimic 3D scaffolds and serve as grafts for replacing damaged tissues and organs, as well as drug delivery vehicles in the cell (Mazza *et al.*, 2015). An example of this is the use of wild non-mulberry tropical tasar silk gland fibroin protein as a 3D scaffold for biotechnological and tissue engineering applications (Mandal and Kundu, 2008). Furthermore, leaching of salts and sugars, and phase separation and freeze drying has been used to introduce porous architecture into 3D scaffold, to create blood vessel substitutes for the treatment of cardiovascular disease such as coronary artery disease (Sarkar *et al.*, 2006). However, for clinical implementation, these techniques have to satisfy specific criteria including *in vivo* compatibility, biodegradability, pore structure for vascularisation, non-toxic degradation of by products, and an ability to facilitate cell attachment, proliferation and differentiation as reviewed by (Da Silva *et al.*, 2020). Overall, further development is required for such a structure to be deemed a suitable *in vitro* model of mammalian biochemical and biological activity.

Despite efforts to develop robust 3D cell culture systems that can be used for drug discovery and toxicity testing, this is still an evolving field (Adcock *et al.*, 2015; Duval *et al.*, 2017). To date, there are no published studies on the use of 3D cell cultures in IVF medium development and quality control, although some investigations have compared cellular responses in 3D pluripotent cell models to early mouse embryos (Gordeeva and Gordeev, 2021). These however have not been sufficient, and the conformity of morphological and functional characteristics of 3D cell systems and their responses to environmental perturbations remain unclear. Consequently, to confirm their effectiveness in research and development and their validity as predictors of embryotoxic potency *in vivo*, further efforts have to be made to improve the experimental design of 3D cell-based assays (Sittampalam *et al.*, 2015; Booij, Price and Danen, 2019).

1.5.1.3. Embryoid bodies

As described in section 1.5.1.2, aggregations of somatic cells are known as spheroids (Nunes *et al.*, 2019), and aggregates of embryonic stem cells are referred to as embryoid bodies (EBs) (Brickman and Serup, 2017). EBs have been used in numerous studies as *in vitro* models for evaluating early extraembryonic tissue formation and for promoting multicellular interactions that consist of ectodermal, mesodermal, and endodermal tissues that have led to cell differentiation during embryogenesis (Diomede *et al.*, 2018). Differentiation is induced by culturing aggregates of EC cells in the absence of the self-renewal signals provided by the feeder layer of LIF. Coculture with stromal cell line activity and adherent monolayer cultures in the absence of LIF have also been used to induce EB differentiating *in vitro* (Ying *et al.*, 2003). Initially, an outer layer of endoderm-like cells forms within the EB when differentiation begins, followed by the development of an ectodermal layer and subsequent specification of mesodermal cells. Transfer of these EBs to tissue culture plates allows continued differentiation into a variety of specialised cell types, including cardiac, smooth, and skeletal muscle, and pancreatic, hepatic, cartilage, neuronal or glial cells. The expression of tissue-

specific genes and proteins in EC cells during *in vitro* differentiation indicates that early processes of *in vivo* development into ectoderm, mesoderm and endoderm lineages are recapitulated *in vitro* (Leaky *et al.*, 1999). Another 3D model often used to study differentiation events in mammals are aggregates of early primitive ectoderm-like (EPL) cells, which are also derived from the inner cell mass of mouse embryos. Unlike EC cells, these cells require adherent culture in a medium conditioned by human hepatocellular carcinoma HepG2cells (MEDII-CM) (Rathjen *et al.*, 1999, 2003). EPL cells exhibit properties consistent with the primitive ectoderm of mouse embryos but do not participate in embryogenesis following blastocyst injection (Heo *et al.*, 2005). They do, however, allow the modelling of early differentiation events without genetic modification. The aggregation of EPL cells into EBs results in a loss of visceral endoderm and neuroectoderm differentiation, but on the other hand, late primitive ectodermal, parietal endodermal and mesodermal cells develop. This suggests that the EPL EB differentiation model may be suitable for studying mesoderm development *in vitro* but not ectoderm and endoderm differentiation due to the lack of ectoderm lineage formation in these EBs (Rathjen *et al.*, 2002). Overall, EBs are of significant importance for investigating the embryotoxic properties of teratogens such as retinoic acid (RA) and alcohol. When applied during the various stages of EB formation, RA significantly affects the differentiation of EC cells in a time and concentration dependent manner; for instance, high concentrations of RA applied during early EB development induce the differentiation of neurons, while lower concentrations applied at later EB stages promote the differentiation of skeletal and cardiac muscle cells (Wobus *et al.*, 1994). The underlying mechanisms are however not fully understood.

As illustrated in Figure 1.8, EBs can be generated through various means, the most common being those that involve cell culture in untreated plates or dishes to prevent adherence to the surface (Desbaillets *et al.*, 2000; Dang *et al.*, 2001). The petri dish has been used to generate EBs since 1985, and these are typically used to initiate differentiation of ECs into differentiated cell types such as neural progenitors, vascular cells, cardiomyocytes and hepatic cells

(Shafaroudi *et al.*, 2016). Cells do not attach to the plastic surface but instead spontaneously adhere to one another to form heterogeneous aggregates. Although popular, the disadvantage of such a technique is that the heterogeneously sized EBs quickly lose synchrony in differentiation, preventing consistency between experiments. Consequently, round-bottomed 96-well plates and small conical tubes have become essential tools for producing EBs from a pre-determined number of cells, allowing for regularity in EB size (Ng *et al.*, 2005). The low-adherence nature of the plate forces cells to accumulate at the bottom of each well, promoting cell-to-cell contact and cell aggregation. Moreover, the formation of one EB per well allows spent media to be replaced without removing cells from the suspension and allows size and development to be directly monitored by microscopic analysis. This also means that experiments can be conducted by treatment and analysis of each well without having to move or disrupt the EBs.

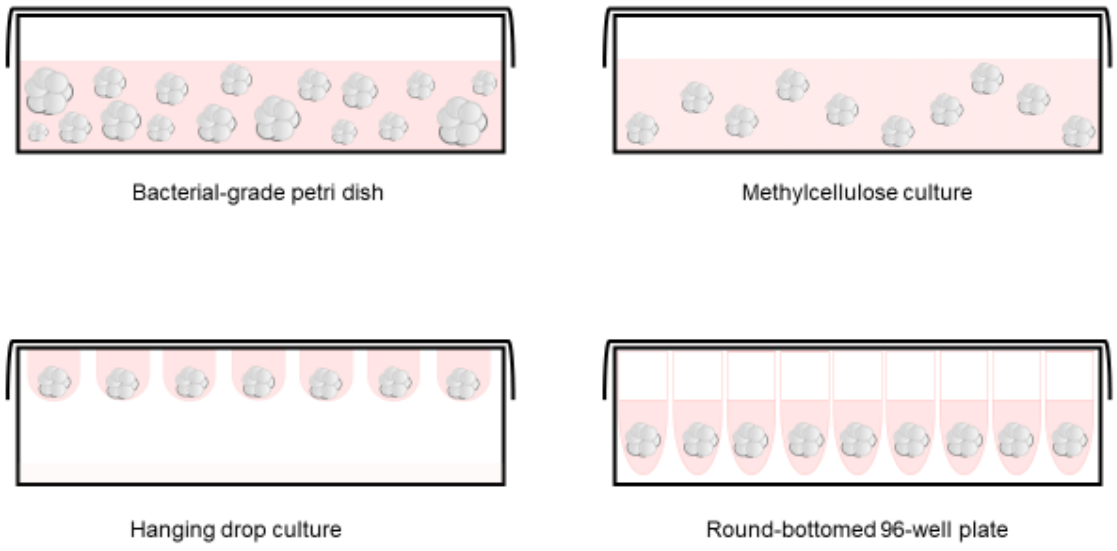


Figure 1.8. Schematic representation of the most common methods used to generate embryoid bodies. Under standard incubation conditions and in the absence of a feeder layer, P19 cells differentiate spontaneously to form spherical three-dimensional structures that can differentiate into germ layers. Created with BioRender.com.

1.5.2. P19 embryonal carcinoma cells

The P19 embryonal carcinoma cell line represents one of the most widely studied pluripotent cell lines in cell biology. It is often used as an *in vitro* model system to investigate embryonic development and early developmental events because of its ability to express derivatives of all three embryonic germ cell layers (Marikawa *et al.*, 2009; Jasmin *et al.*, 2010). Although initially isolated from teratocarcinoma of normal embryos, they are now derived from teratomas induced in the testes of adult C3H/He mice. Teratomas typically contain an array of somatic, extraembryonic and embryonal carcinoma cells, and although benign, they become malignant when they grow in the testis of humans and mice (Gudjonsson and Magnusson, 2005). The term teratocarcinoma is generally used when tumours contain both EC cells and teratoma components. An early study of teratomas and teratocarcinomas demonstrated that these tumours can be experimentally induced in a limited strain of mice by explanting genital ridges of foetuses to ectopic sites (Figure 1.7) (Stevens, 1964).

Similar to other ECs and ESCs, P19 cells are developmentally pluripotent and differentiate in a comparable manner to normal embryonic cells (Jones-Villeneuve *et al.*, 1982; McBurney, 1993; Okuda *et al.*, 1998). Their immortal state also allows for an unlimited amount of material to be produced for research. Furthermore, they grow even in the absence of irradiated feeder cells, and maintain an undifferentiated state until they are induced to do so by manipulation of culture conditions (McBurney, 1993). In suspension culture, embryonic stem cells spontaneously form EBs (as described in section 1.4.1). Complex interactions between heterologous P19 cells within EBs induce differentiation of these stem cells into derivatives of the germ layers, although the position of cells within spatially distinctive environments within the structure ultimately governs their fate in a regulated manner. For instance, the P19 cells at the periphery of the embryoid body tend to differentiate into primitive endodermal cells, while the cells at the centre usually differentiate into primitive ectodermal cells (Hamazaki,

2004). As shown in Figure 1.9, these aggregates grow until they reach a specific size, at which point, passive diffusion can no longer provide nutrients for the cells within the core, nor can waste products adequately diffuse out of the structure. The gradients in oxygen and nutrients and the absence of sufficient detoxification result in growth arrest and development of necrosis (Wartenberg, 2001). Given that P19 cells within EBs differentiate and subsequently reflect some of the events associated with germ layer formation in embryos, it would be useful to determine its suitability as an alternative to mouse embryos, so that animal use in IVF quality control can be reduced. There are however no studies that directly compare growth and development in embryoid bodies to that of early stage embryos.

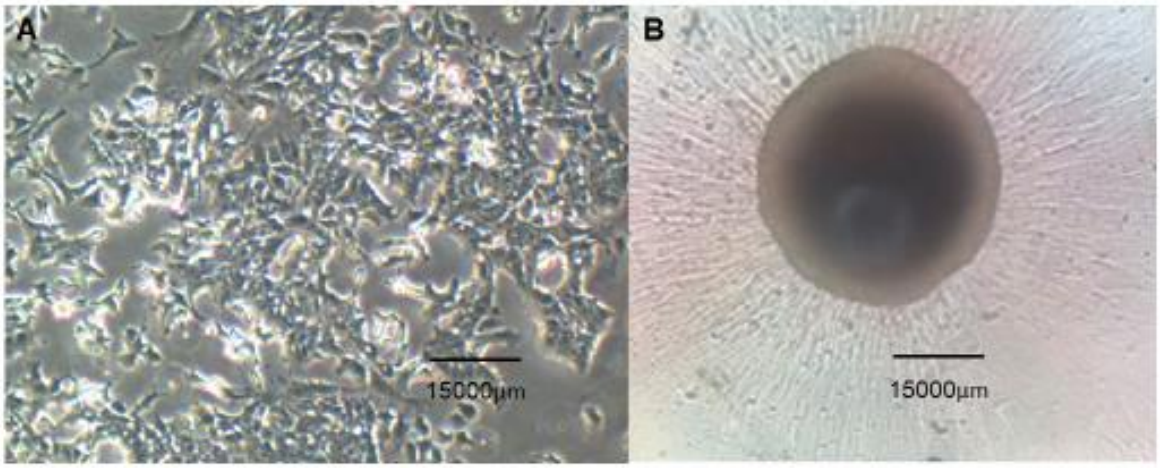


Figure 1.9. P19 embryonic carcinoma cells in 2D form and 3D form. In tissue culture treated dishes cells form a monolayer and proliferate until the surface area of the container is covered (A). There is also a change in morphology from single cells into the fibroblast-like structure pictured above. When cultured in untreated dishes, the cell aggregate form 3D structures known as embryoid bodies (B) which grow until they are unable to obtain nutrient and oxygen, and subsequently begin to perish.

1.6. High throughput screening

The term 'high throughput screening (HTS) is used to describe the process of applying automated equipment to the rapid testing of thousands to millions of samples for biological activity within a model organism (usually mammalian cell lines such as CHO or HeLa, *C. elegans* and zebrafish), at the molecular level (Attene-Ramos, Austin and Xia, 2014). In the pharmaceutical and biotechnology industries, samples are typically screened for substances

such as chemical mixtures, natural product extracts, oligonucleotides and antibodies to identify compounds which can be used as starting points for medicinal optimisation during drug discovery and development (Gordon *et al.*, 1994; Gold, 1995; Chang, Kim and Kwon, 2016; Rasul, 2018). Historically, enzymes and receptors including kinases, ion channels, proteases, nuclear receptors and G-protein coupled receptors were the most studied targets identified by HTS as reviewed by (Gribbon and Sewing, 2003; Hüll, Morstein and Trauner, 2018). However, advances in instrumentation and assay development technologies have made it possible to evaluate other biological functions using more sophisticated assays that assess protein-protein, protein-DNA and protein-RNA interactions (Espina *et al.*, 2004; Mao *et al.*, 2006; Wheeler, Van Nostrand and Yeo, 2018). More complex cell-based assays such as those that focus on signal transduction pathways, transcriptional regulation, protein expression, pre-mRNA splicing events and epigenetics have also been developed a result of the development of luciferase and beta-lactamase dyes (Li *et al.*, 2011). In more recent times, 'high content' cellular imaging assays have become common and enable the identification of compounds with efficacy in a variety of cellular phenotypes including apoptosis (Chan *et al.*, 2013; Martin *et al.*, 2014), protein distribution, neurite outgrowth and disease-relevant phenotypes (Zanella, Lorens and Link, 2010). Quantitative high throughput screening (qHTS), has emerged from the development of these methods, and consists of testing compounds at multiple concentrations using a HTS platform (Attene-Ramos, Austin and Xia, 2014). Concentration response curves are subsequently generated for each molecule tested immediately after the screen is performed (Cho *et al.*, 2008). Given that HTS has significantly contributed to successful drug discovery in the pharmaceutical industry, it would be important to determine whether these same tools could be used to improve quality control practices in the IVF industry. However as discussed in section 1.5, previous attempts to use cell culture systems for quality control in IVF have failed so it could be assumed that the implementation of HTS methods would be futile and wasteful. Nonetheless, no studies have explored the potential benefits of HTS systems in human IVF quality assessments.

1.7. Cell viability assays

1.7.1. The resazurin assay

Central to the success of HTS is the use of 96-, 384-, 1,536-, and 3,456-well plates as these allow multiple experiments to be carried out simultaneously in a uniform format (Bajorath, 2002). Coupled with fluorescence and bioluminescence based assays (the most commonly used measurement methods in HTS), these techniques demonstrate high sensitivity, good tolerance to interference, fast signalling speed and a non-destructive way of tracking and analysing targets (Fang *et al.*, 2019). Especially suitable for HTS is resazurin (7-Hydroxy-3H-phenoxazin-3-one 10-oxide), a phenoxazin-3-one dye that has been used since the 1950s to assess bacterial and yeast contamination in biological fluids and also to measure the viability of human sperm (Erb and Ehlers, 1950; Zalata *et al.*, 1998; O'Brien *et al.*, 2000). In 1993, the resazurin assay was commercialised as a cell viability test, and coined as Alamar blue, and has since been used to assess cytotoxicity in various cells types including fibroblasts, mouse lymphocytes and neuronal cells (Ansar Ahmed, Gogal and Walsh, 1994; White, DiCaprio and Greenberg, 1996; Voytik-Harbin *et al.*, 1998). It is also used in the bioengineering and tissue engineering industries as a measure of cell viability (Ren *et al.*, 2015). As illustrated in Figure 1.10 and 1.11, when added to cell cultures, the oxidised form of resazurin which is blue and weakly fluorescent, is internalised by cells and irreversibly reduced to highly fluorescent pink resorufin. With time, resorufin is excreted outside the cells and into the media, resulting in a visible colour change from blue to pink. Subsequently, the rate of reduction based on colour change reflects the number of viable cells.

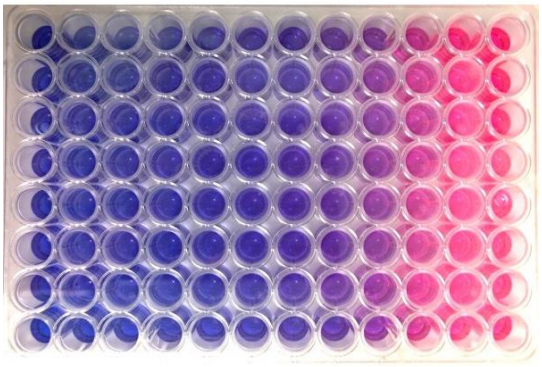


Figure 1.10. P19 cells treated with decreasing concentrations of resazurin solution (left to right). Resazurin is blue and weakly fluorescent but upon reduction by live cells, becomes pink and highly fluorescent resorufin, indicative of cell proliferation. Resorufin production is the result of the action of several enzymes in the mitochondria, cytosol and microsome.

For several years, it was suggested that resazurin reduction occurred as a result of mitochondrial enzyme activity, but some studies have demonstrated the involvement of cytosolic enzymes such as diaphorases (Matsumoto *et al.*, 1990). Decreased resazurin reduction, therefore, indicates an impairment in cellular metabolism as well as in mitochondrial function. In a subsequent reversible reaction, resorufin is further reduced to colourless and non-fluorescent hydroresorufin as shown in Figure 1.11, which interferes with measurements of resorufin levels, so it is advised that experimental parameters such as the incubation time and resazurin concentration are optimised beforehand to avoid aberrant and inaccurate results (Perrot *et al.*, 2003). This is also critical in ensuring that sufficient concentrations of the substrate and resazurin are present for the entire duration of the assay so that the rate at which resazurin is reduced to resorufin remains constant (Uzarski *et al.*, 2017).

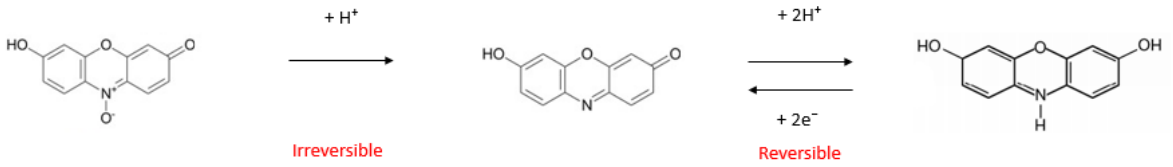


Figure 1.11. Chemical structure of resazurin in various forms. Oxidised resazurin is blue, its irreversibly reduced form resorufin is pink and reversibly reduced dihydroresorufin is colourless (Image inspired by Riss *et al.*, 2004; Uzarski *et al.*, 2017). Created with BioRender.com.

The MTT assay is another widely-used quantitative colorimetric assay based on the capacity of viable cells to reduce MTT to formazan crystals through activity in the mitochondria (Mosmann, 1983; Vega-Avila and Pugsley, 2017). The tetrazolium salt, which is slightly yellow, is converted to an insoluble purple crystal through cleavage of the tetrazolium ring by succinate dehydrogenase within the mitochondria. As formazan is impermeable to cell membranes, it accumulates in healthy cells; therefore, the amount of formazan produced is directly proportional to viable cell numbers. It can be solubilised by organic solvents such as Isopropanol or DMSO; however, the reductase activity responsible for formazan formation is highly dependent on the concentration of intracellular of NADH and NADPH, so care must be taken to ensure that exhausted cell culture media used to dilute the organic solvents do not lead to inaccurate absorbance readings.

1.7.2. Differences between the MTT and resazurin assay

Several studies have indicated comparable features between resazurin and MTT in monitoring cell viability (O'Brien *et al.*, 2000; Neufeld *et al.*, 2018; Zheng *et al.*, 2019), however, as shown in Table 1.2, resazurin offers some distinct advantages. It is non-toxic to cells and particularly advantageous in studies involving the use of scarce patient-specific cells. It is still advised that the incubation period is kept to a minimum as high resazurin concentrations or prolonged incubation may interrupt normal metabolic activity in cells (Xiao *et al.*, 2010; Pace and Burg, 2013). Furthermore, unlike MTT which can only be detected colorimetrically, resazurin offers extra versatility because results can be determined qualitatively by visual colour change or quantitatively with absorbance or fluorescence-based instruments. Resazurin also accommodates continuous cell viability monitoring, although this should be maintained within a four-hour (or less) incubation period to avoid the toxic effect of hydroresorufin (Pace and Burg, 2013). On the other hand, resazurin also presents its own setbacks and these have been thoroughly discussed (O'Brien *et al.*, 2000; Breznan *et al.*, 2015). It can be reduced by components within the cell culture medium such as ascorbic acid, cysteine or Dithiothreitol,

giving rise to background interference and inaccurate interpretation of cell viability. The rate of reduction of resazurin is also affected by the presence of any protein within the cell culture media. For both assays, studies show that they are susceptible to interference from test compounds such as including nanomaterials (Breznan *et al.*, 2015; Neufeld *et al.*, 2018) but these can be overridden by stringent optimisation of experiments prior to investigations.

Table 1.3. Comparison between MTT and resazurin as indicators of cell viability. The advantages and disadvantages of each are summarised by information obtained from (Willard, Merrit and Dean, 1974; Mosmann, 1983; Maeda and Matsu-Ura, 2001; Ren *et al.*, 2015).

MTT		Resazurin	
Advantages	**Disadvantages**	**Advantages**	**Disadvantages**
The most widely-used cell viability assay and therefore substantially documented	Insolubility of formazan crystals generated by aggregated cells can lead to high well to well variability	Allows qualitative and quantitative measurement of cell viability	Large numbers of cells and extended incubation times may cause the reduction rate of resazurin to decrease resulting in underestimation of cell number
Known to be cell specific and requires little or no optimization	Variable background due to protein precipitation may cause inaccurate interpretation of results	Less toxic to cells than MTT and non-toxic to laboratory personnel	Hyper-reduction of resorufin to non-fluorescent hydroresorufin causes underestimation of cell activity
	Potentially carcinogenic	Allows continuous cell viability monitoring and kinetic study.	Toxic to cells when incubation periods exceeds 4 hours (cell specific)
	Susceptible to interferences from test compounds	Less costly than MTT	Can be reduced by components of cell culture media
			Susceptible to interferences from test compounds

1.7.3. The Z' factor

Technological advancements in science have not only improved the quality of HTS, but has also highlighted the importance of data analysis. Since its introduction in 1999, the Z' factor criterion has become the most widely used parameter for evaluating and validating the quality of HTS experiments, with the publication by Zhang *et al*., being the most cited paper of its kind in the drug discovery industry. Based on statistics that model the distribution of variables within a dataset, the Z' factor combines the information of both location and scale of the distributions of sample signal and background and provides a better representation of the assay quality than traditionally used signal-to-background or signal-to-noise ratio alone. As a reference point, values ≥ 0.5 and ≤ 1 are deemed acceptable, and values < 0 indicate a poor-quality assay with a small separation band (the difference between negative and positive controls).

Large laboratories including the National Institutes of Health Chemical Genomics Centre (http://ncgc.nih.gov) and the National Screening Laboratory for the Regional Centres of Excellence in Biodefense and Emerging Infectious Diseases (http://nsrb.med.harvard.edu) highly recommend assessing the Z' factor of assays prior to its use on a wide-scale, and more recently, Iversen *et al* conducted a simulation study and compared the performance of the Z' factor to that of the signal window and assay variability ratio and recommended the Z' factor as a preferred assay performance measure (Iversen *et al.*, 2006). This is because the signal window and assay variability ratio are based solely on positive and negative control data whereas the Z' factor factors in the overall distribution of all datasets (Iversen *et al.*, 2006).

1.8. Cell culture and hypothermic stress

Recombinant protein (rP) yield is of great importance in the pharmaceutical industry for the mass production of medicines (Masterton and Smales, 2014). Examples of such medicines include the human growth hormone substitutes, Valtropin and Humatrope (Hepner *et al.* 2005 and Pavlovic *et al.* 2008), and insulin used in the treatment of diabetes (Kapitza *et al.* 2017and Sandow *et al.* 2015), as well as in the development of novel therapeutics. Extensive research has been undertaken to improve the volumetric productivity of mammalian cells (Al-Fageeh *et al.*, 2006), and this has led to a better understanding of gene expression, growth and metabolism, and apoptosis delay in mammalian cells. In 2004, the extended batch culture was deemed to be the most high-yielding protein process whereby medium components are added in semi-continuous batches; however, perfused protein production takes this a step further, and waste medium is withdrawn from culture and replaced with equal volumes of fresh medium. The production of anti-haemophilic factor VIII, the largest protein (2,332 amino acids) produced by perfusion technology, is an example of this, and approximately 150g of this product is produced each year in industry (Wurm, 2004; Orlova *et al.*, 2013). Changes in media formulation have also improved protein yield, and replacing glucose with mannose as a source carbon has been found to increase the volumetric rate of production of recombinant proteins by 30% compared to a glucose control (Berrios *et al.*, 2011). At present, there is a great deal

of interest in the use of reduced temperature (hypothermia) cultivation of mammalian cells, for enhanced production of rP. Changes in protein yield and the changes associated with hypothermic incubation are complicated; however, it is clear that the general response to hypothermia includes global attenuation of transcription and translation, whilst proteins that are essential to cell survival are selectively upregulated (Sonna *et al.*, 2013; Eskla *et al.*, 2018). As reviewed in (Adjirackor, Harvey and Harvey, 2020), protein synthesis is one of the most energy-consuming cellular processes, requiring approximately 30-40% of accessible energy and achieving the correct tertiary and quaternary structure of newly synthesised proteins is a crucial challenge, with the accumulation of misfolded proteins posing a severe risk to cell viability. It is therefore unsurprising that transcription and translation are globally attenuated in response to hypothermic incubation. Cell culture systems are sometimes manipulated by stressors such as cold stress, and therapeutics that modulate their subsequent response, in order to determine the role of specific proteins and structures. In turn, these alterations enable investigation of the various ways in which cells respond to changes in culture conditions. This is particularly useful for altering culture conditions to improve recombinant protein yield (Al-Fageeh *et al.*, 2006) as described in section 1.6, and for inducing known cellular responses in research. Considering that traditional cell culture systems often fail to identify toxicity in IVF because cell survival has been too high (Bertheussen *et al.*, 1989), reducing cellular resistance to stress could provide a novel tool for making cell-based assays more applicable to quality control in IVF. This is under the condition that the mechanisms that govern cold stress are well-understood and easily controlled, to reduce the ability of cells to withstand the detrimental effects of suboptimal culture conditions.

1.8.1. Hypothermia

Hypothermia in humans is generally defined as a core temperature of 35°C and below (Niazi and Lewis, 1958), and temperatures below this point interrupt metabolic processes that occur within human cells in culture (Frink *et al.*, 2012). In common with the general response to

external stressors seen more broadly in eukaryotes, human cells respond to hypothermia by altering the lipid composition of the bilayer to withstand the decreased membrane fluidity caused by low temperatures and by modifying proteins (Hofmann *et al.*, 2012; Young *et al.*, 2013). The changes in protein synthesis involve attenuating global protein synthesis through the downregulation of key initiation and elongation factors whilst upregulating the expression of a small group of proteins, cold shock proteins (Roobol *et al.*, 2009). Interestingly, the cellular response to mild hypothermia differs from that of more severe hypothermic conditions, supporting the notion that cells continue to grow and proliferate at moderately low temperatures and undergo growth arrest at more severe temperature (Al-Fageeh and Smales 2006). For instance, *Saccharomyces cerevisiae* responds to mild cold shock (10-18°C) by upregulating translational machinery and Tip1, Tir1, Tir3 and Nsr1, genes associated with transcription, although global protein production during these conditions is reduced (Homma, Iwahashi and Komatsu, 2003). More severe forms of cold stress (< 10°C) cause growth arrest and induce upregulation of an alternative set of proteins that synthesise trehalose to sustain cell viability (Homma, Iwahashi and Komatsu, 2003; Kandror *et al.*, 2004) (Table 1.3). A critical factor in the cellular response to hypothermia is the rates of temperature change experienced during both cooling and warming. For instance, mammalian cells respond to hypothermia faster and at a less drastic temperature reduction (25 - 35°C) than most organisms (Roobol *et al.*, 2009). Rapid changes limit the ability of cells to respond with potentially protective changes and instead shifts the response such that it is instead focused on the repair and mitigation of damage associated with the temperatures experienced.

Table 1.4. Summary of stress granule formation and expression of cold-shock proteins in response to mild cold stress and severe cold stress in mammalian cells and *Saccharomyces cerevisiae.* Cells continue to proliferate with mild forms of hypothermia but undergo growth arrest with more severe forms of cold stress (Adjirackor, Harvey and Harvey, 2020).

	Mammalian cells	**Yeast (*S. cerevisiae*)**
Mild cold stress (25˚C to 35˚C in mammalian cells) (10°C to 18°C in yeast)	No stress granule formation RBM3 and CIRP expressed	No stress granule formation Tip1, Tir1, Tir2, and Nsr1 expressed Hsp12 and Hsp26 expressed
Severe cold stress (0˚C to 10˚C in mammalian cells) (<10°C in yeast)	Stress granules strongly induced at 10˚C (no stress granule formation at 4˚C) No RBM3 and CIRP expressed	Stress granules induced at 10˚C Stress granules strongly induced at 0˚C Tps1, Tps2, NTH1 and CCT expressed Hsp12, Hsp42, Hsp104 and Ssa4 expressed

1.8.2. Cold shock proteins

The two cold shock proteins (CSP) identified in mammalian cells are Cold-inducible RNA-binding motif protein 3 (RBM3) and cold-inducible RNA-binding protein (CIRP). Both proteins are RNA-binding, with conserved glycine-rich domains which categorises them as members of the family of glycine-rich proteins (Al-Fageeh and Smales 2006; Zhu, Bü Hrer, and Wellmann 2016). CIRP was the first CSP to be identified in mammalian cells, however, far less is known about its molecular mechanisms than RBM3. Both proteins are transcriptionally upregulated during mild cold stress (32°C to 34°C) and function as regulators of translational reprogramming by binding to the 5'-UTR or 3'-UTR of mRNA molecules and facilitating their response to environmental signals (Nishiyama *et al.*, 1997; Fujita *et al.*, 2017). In mammalian cells at 32°C, mRNA molecules bind to RBM3 and CIRP in the nucleus and cytoplasm and form a complex known as ribonucleoprotein particles (mRNPs) (Reineke and Lloyd, 2015). The RBP composition determines the fate of these mRNPs, and some play a crucial role in inducing the immediate translation of the attached mRNA whilst others are transported to specific subcellular regions for storage or localised translation (Smart *et al.*, 2007).

Studies by Dresios *et al.* (Dresios *et al.*, 2005) demonstrate increased RBM3 expression and binding to 60S ribosomal subunits in cells cultured at 32˚C, indicating the significant role cold stress proteins play during cell culture at reduced temperatures. This results in elevated levels of 80S polysomes consistent with the increased association of mRNA and ribosomal subunits and translation initiation. In addition, RBM3 regulates eIF2α activity during cold stress by inhibiting PERK dependent eIF2α phosphorylation (further explained in section 1.6.3.) and also by interacting with components within stress granules to modulate transcriptional and translational processes (Zhu *et al.*, 2016). Although RBM3 may facilitate protein synthesis during cold stress, it has a relatively small impact on polysome profiles, and its association with only a subset of 60S ribosomal subunits make it unlikely that its ribosomal binding solely accounts for its effect on translation initiation (Dresios *et al.*, 2005; Logan and Storey, 2020). Global protein synthesis remains suppressed in cells exposed to cold stress, but RBM3 and CIRP positively affect protein synthesis by preventing a more drastic reduction in protein synthesis.

Whilst the exact mechanisms are not fully understood, RBM3 and CIRP are known to mediate neuroprotection during hypothermia by increasing resistance to neural apoptosis (Chip *et al.*, 2011). In studies of cortical organotypic slice cultures obtained from mice, blocking the RBM3 over-expression that occurs immediately after mild cold stress diminishes the protective effect of hypothermia, and inducing RBM3 expression increases resistance against the induction of apoptosis. RBM3 and CIRP also have an essential role in maintaining testicular function in human testis, where temperatures are typically maintained between 2˚C and 8˚C lower than average body temperature (Danno *et al.*, 2000). In animal models the surgical induction of cryptorchidism, a condition where the testis fail to descend from the abdominal region to the scrotum, disrupts spermatogenesis and infertility (Bergh and Söder, 2007). Studies suggest that this occurs because the expression of RBM3 and CIRP is restricted to cells subjected to mild cold stress only, so although the events are not fully known, RBM3 and CIRP positively affect testicular function and fertility.

1.8.3. Stress granule formation and eIF2α phosphorylation

In vitro studies of cellular responses to hypothermia confirm the presence of cytosolic SGs, which are by-products of translation arrest and polysome disassembly (Kramer *et al.*, 2008). Initially observed in mammalian cells, SGs have been found in numerous eukaryotes, including yeast and plants (Kedersha *et al.*, 1999; Weber, Nover and Fauth, 2008). It is however not known if they are formed in all eukaryotic cells (Hofmann *et al.*, 2012). The exact function of SGs remains unclear, but it is hypothesised that they play a crucial role in sequestering proteins and modulating signalling cascades required for cell survival (Aulas, Fay, *et al.*, 2017). Several studies have investigated the composition of SGs, with these studies showing that they contain a complex mix of proteins and RNAs, as summarised in Table 1.4.

Table 1.5. Components of stress granules that form within mammalian and yeast cells upon exposure to moderate hypothermia (25 - 35°C). The molecules listed are orthologues of the two eukaryotic organisms and perform interrelated functions (Adjirackor, Harvey and Harvey, 2020).

Mammalian cells: African green monkey COS7 kidney, Mouse embryonic fibroblasts, Du145, HeLa, Huh7		**Yeast: *S. cerevisiae***	
eIF3B **eIF4A** **eIF4E** **eIF4G** **eIF2α** **Poly(A) mRNA** **Poly(A) binding protein PABP**	**Eukaryotic initiation factors**	eIF4E eIF4GI eIF4GII Poly (A) binding protein PABP	**Eukaryotic initiation factors**
G3BP	**Ras GTPase-activating binding-protein**	Pub1	**Yeast orthologue of mammalian TIA 1**
HuR **TIA 1** **TIA R**	**RNA binding proteins**	Ngr1	**Yeast orthologue of mammalian TIA R**
Ataxin-2	**Stress granule assembly protein**	Pbp1	**Yeast orthologue of mammalian Ataxin-2**
40S ribosomal subunit		Hrp1 Gbp2	**Nuclear cytoplasmic binding-proteins**
		Nrp1 Ygr250c Eap1	**RNA binding proteins**

In mammalian cells, SG formation is initiated by phosphorylation of the α subunit of eukaryotic initiation factor 2 (eIF2α) (Anderson, Kedersha and Ivanov, 2015). In the presence of GTP, eIF2α transports initiator tRNA methionine ($tRNAi^{met}$) to the site of translation initiation on the 40S small ribosomal subunit, and alongside eIF3 and eIF4, form the pre-initiation complex. Phosphorylation of eIF2α on serine 51 inhibits GTD/GTP exchange by eIF3 and prevents further eIF2-GTP-$tRNAi^{met}$ formation, leading to failure of $tRNAi^{met}$ delivery to the site of translation initiation (Hofmann *et al.*, 2012; Aulas, MM, *et al.*, 2017). In mammalian cells, eIF2α phosphorylation occurs through four known kinases, each activated by specific yet overlapping cellular stresses (Li *et al.*, 2006). The double-stranded RNA (ds-RNA) dependent kinase (PKR) is activated by ds-RNA synthesised during viral replication and participates in anti-viral mechanisms through eIFα phosphorylation and suppression of protein synthesis resulting in reduced viral replication (Reineke and Lloyd, 2015). Heme-regulated inhibitor (HRI) is expressed in erythroid cells and facilitate the production of suitable quantities of globin required for erythrocyte formation (Chefalo *et al.*, 1998). PERK responds to unfolded proteins within the ER by inducing reduced protein production thereby reducing the burden of additional protein substrates for ER folding (Yan *et al.*, 2002). PERK activation also occurs under hypothermic or hypoxic culture conditions, or when cellular energy levels are depleted (Bi *et al.*, 2005; Hofmann *et al.*, 2012). Lastly, GCN2 which is activated by uncharged tRNAs during amino acid starvation, induces phosphorylation of eIF2α in response to lack of available substrates (Ye *et al.*, 2015). Despite the core role of eIF2α, poly (A) binding proteins (PABP), small ribosomal subunits, mRNA molecules, and translation initiation factor eIF2, eIF3, eIF4E and eIF4G, also contribute to SG formation during hypothermic incubation.

1.8.4. Mechanistic target of rapamycin and control of cell growth

The mechanistic target of rapamycin, mTOR, previously known as mammalian target of rapamycin is a serine/threonine protein kinase that plays a central role in the regulation of cell growth and proliferation, protein degradation and lipid metabolism (Reiling and Sabatini, 2006; Ben-Sahra and Manning, 2017). Its yeast orthologue, TOR was first discovered by mutations

in *Saccharomyces cerevisiae* that induced resistance to the growth inhibitory properties of antifungal macrolide rapamycin (Heitman, Movva and Hall, 1991). Since then, its kinase activity has been associated with anabolic pathways such as protein synthesis, ribosome production, lipogenesis and nucleotide synthesis, all of which are crucial to cell and tissue growth (Peterson *et al.*, 2011; Xie, Wang and Proud, 2016). It also suppresses cellular autophagy by inhibiting its activation and suppressing the production of lysosomes, the organelle in which autophagy occurs (Thoreen *et al.*, 2009). The mTOR protein contains numerous sub-domains with highly conserved genetic sequences. In human, mice and rat, mTOR shares a 95% amino acid sequence homology, indicating comparable or similar cellular functions between species (Giles and Albitar, 2005). mTOR forms part of the PI3K-related protein kinase family (PIKK) which act as regulatory enzymes during DNA damage, DNA repair and DNA recombination. Its catalytic domain is highly homologous to the lipid kinase domain of phosphoinositide 3-kinase (PI3K) which interacts with several subunits to form two distinct complexes; mTOR1 and mTOR2. The complexes participate in different pathways and recognise distinct substrates through unique mTOR-interacting proteins (Guertin *et al.*, 2017). Both complexes contain the mTOR catalytic subunit, mLST8, also known as GβL, DEPTOR and the Tti1 and/Tel2 complex. The regulatory-associated protein of mTOR (raptor) is exclusive to rapamycin sensitive mTOR1 and functions as both a scaffolding protein and a bridging protein that connects downstream substrate targets to the mTOR kinase domain whilst enhancing its phosphorylation activity (Sampath and Ntambi, 2005; Aylett *et al.*, 2016). Germline disruption of raptor in mice results in embryonic lethality at the implantation stage. In fact, the ICM and trophoblast of blastocysts obtained from mice deficient in raptor fail to expand in culture and eventually die, highlighting the importance of mTOR and protein synthesis in early embryonic development (Gangloff *et al.*, 2004).

Conversely, rapamycin-insensitive companion of mTOR (rictor) is associated with mTOR2 only (Guertin *et al.*, 2017). In eukaryotic organisms, mTORC2 phosphorylates and activates Akt (PKB) which is also involved in cell proliferation, growth, survival, and metabolism in

accordance with its environmental cues (Bai and Jiang, 2010). When activated by membrane-bound receptors, PI3K catalyses the conversion of phosphatidylinositol (4,5)-biphosphate (PIP2) to phosphatidylinositol (3,4,5)-triphosphate (PIP3) which binds to Akt to induce dimerisation and exposure of it catalytic domain (Shukla *et al.*, 2007). In order be fully active, Akt must be phosphorylated at two critical residues, THR308 and SER473. THR308 is known to be phosphorylated by upstream kinase PDK1 but the identity of the kinase responsible for SER473 phosphorylation has been elusive until recently. Several candidates including PDK1, integrin-linked kinase (ILK) and Akt itself were previously proposed, but there is compelling evidence that rictor phosphorylates Akt at SER473 (Maira *et al.*, 2008). Prolonged treatment of cells with rapamycin inhibits Akt phosphorylation as newly synthesised mTOR components are sequestered. This interferes with the assembly of mTOR2, thereby inhibiting the activity of rictor within the cell (Sarbassov *et al.*, 2005).

Translation initiation is a rate-determining step involved in protein production and is controlled through two downstream substrates of mTOR1; S6K and 4E-BP, both of which are regulated through rapamycin-sensitive phosphorylation. Active mRNA molecules possess a 5'terminal 7-methylguanosine cap which is required for translation. This is recognised and bound by cap binding protein eIF4E, which associates with scaffold protein eIF4G and RNA helicase eIF4A to form the eIF4F complex. Changes in the rate of translation are intimately linked with changes in the eIF4F activity and actively growing cells produce higher levels of eIF4F (Giles and Albitar, 2005). Alongside pre-initiation complex eIF2α-GTP-tRNAi^{met} and eIF3, eIF4F delivers the mRNA molecule to the 40S ribosomal subunit in preparation of initiation and also interacts with PABP to circularise the mRNA molecule between the 5'terminal and 3' poly (A) tail (Thoreen *et al.*, 2009). 4E-BP interrupts this process by binding to same region eIF4E as eIF4G, thereby preventing eIF4E and eIF4G interacting and engaging in translation initiation complexes. Although there are three known mammalian variants of 4E-BPs but 4E-BP1 is the most characterised (Wang *et al.*, 2001). Under normal conditions, 4E-BP1 is phosphorylated

by mTOR which decreases its affinity for eIF4E, making it available to form part of the eI4F complex (Connolly *et al.*, 2006).

To date, there are two known variants of mammalian S6K (S6K1 and S6K2), both of which exhibit comparable control mechanisms (Pende *et al.*, 2004). It was previously assumed that S6K control of translation occurred through rapamycin sensitive phosphorylation of the 40S ribosomal protein S6 however this has been disputed. S6K1 knockout mice exhibit a smaller cell size phenotype than that seen in S6K2, suggesting a link between translational control and S6K1, but not S6K2 (Marke, Sloane and Ryan, 2005). Under normal cellular conditions, S6K1 is phosphorylated at residue THR389, through its association with mTOR. This generates a docking site for further phosphorylation by phosphoinositide-dependent kinase 1 (PDK1), within the activation loop at residue THR229, resulting in complete activation of S6K1 (Holz *et al.*, 2005). Its two substrates, ribosomal protein, a component of the 40S ribosomal subunit and mRNA helicase eIF4B are phosphorylated by its activation in preparation for translation initiation. eIF4B is an mRNA binding protein with no autonomous catalytic activity, but it enhances the affinity of eIF4A to ATP and mRNA, which increases its helicase activity (Raught *et al.*, 2004). These interactions are sensitive to rapamycin and environmental changes thus hypothermia induces mTOR1 inactivation and disassociation from S6K1, preventing its phosphorylation and subsequent activation of eIF4B and the 40S ribosomal subunit. S6K1 remains bound to pre-initiation complex eIF2α-GTP-tRNAi^{met} and eIF3, which acts as a scaffold through which mTOR regulates S6K1 and 4E-BP activity. Previous studies have associated low eIF4A helicase activity, and decreased mRNA binding with the absence of eIF4B (Bordeleau *et al.*, 2005). It can therefore be assumed that phosphorylated eIF4B and eIF4A have shared functions although this is yet to be fully investigated. S6K1 inactivity leads to inefficient translation of mRNA encoding proteins involved in ribosome biogenesis which is necessary for enhanced protein synthesis.

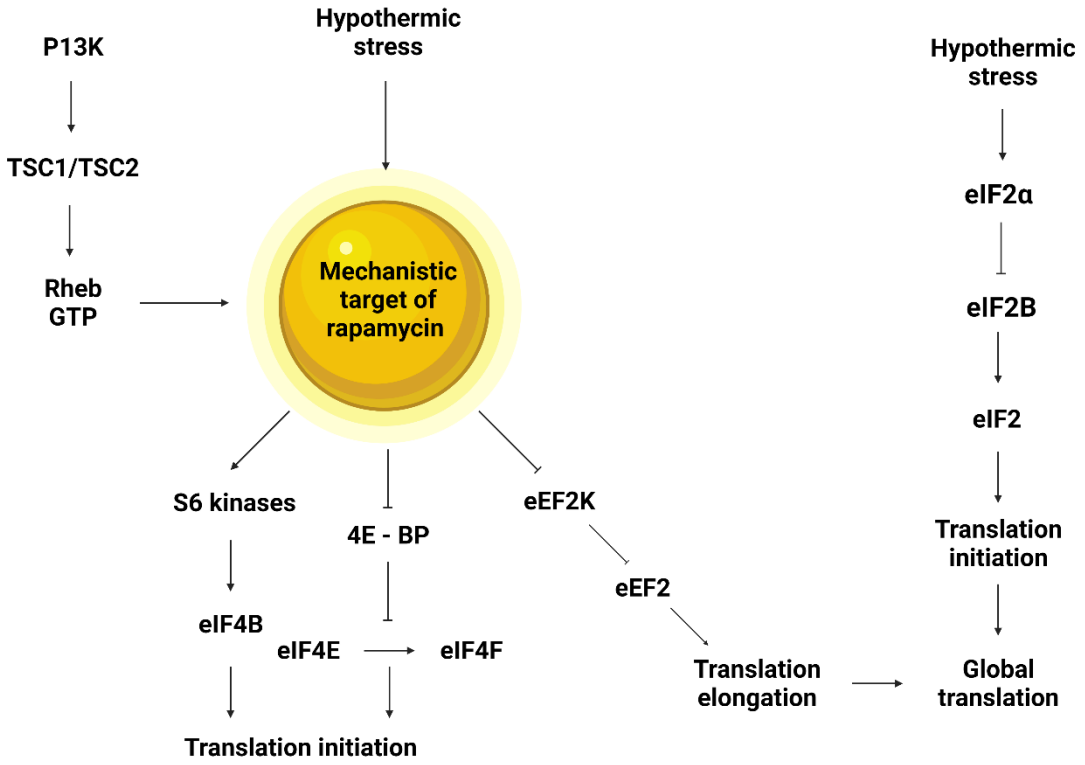


Figure 1.12. Mechanistic target of rapamycin (mTOR) and eIF2α control of transcription factors involved in translation initiation and elongation. Hypothermia reduces mTOR signalling and protein synthesis is subsequently stalled to preserve intracellular resources (Adjirackor, Harvey and Harvey, 2020).

Mammalian cells also require eukaryotic elongation factors eEF1 (eEF1A and eEF1B) and eEF2 to facilitate the elongation phase of translation. Cellular activity of mTOR is subject to phosphorylation, although eEF1 is phosphorylated by protein kinase C whilst eEF2 is controlled by downstream regulation of mTOR signalling (Browne and Proud 2002). eEF1A participates in translation elongation by binding to GTP and interacting with amino-acyl tRNA whilst eEF2 promotes translocation as the ribosome moves along the mRNA molecule by the equivalent of one codon (Frank *et al.*, 2007). GTP-bound eEF1A transports aminoacyl-tRNA molecules to the site of peptide formation whilst eEF2 exposes the mRNA codon. Complementary base pairing between mRNA codons and tRNA anticodons causes disassociation of eEF1A, translocation from the A-site to the P-site and transportation of charged aminoacyl-tRNA molecules to the vacant A-site (Chefalo *et al.*, 1998; Yan *et al.*, 2002). During hypothermic incubation, eEF2 is deactivated by phosphorylation at threonine

56 causing decreased rates of translation elongation. This process is catalysed by a highly specific enzyme that does not belong to the main protein kinase super family but to another small group of enzymes with primary sequences that have no similarity to other protein kinases (Wang 2006). The regulation of eEF2 kinase (eEF2K) through mechanisms induced by cold stress is not fully understood but it is known to be involved in mTOR signalling through phosphorylation at SER366, SER78, SER359 and THR56 by PDK1-dependent protein kinases, p70 S6k and p90RSK(Wang *et al.*, 2001; Knight *et al.*, 2015).

1.8.5. Adenosine monophosphate activated kinase and control of cell growth

Adenosine monophosphate activated protein kinase (AMPK) is also a key regulator of cell growth and proliferation, and lipid metabolism. As the main sensor of cellular energy in all eukaryotic cells, it is activated in response to stress by sensing increases in AMP to ATP or ADP to ATP ratios, and restores energy balance by inhibiting anabolic processes that consume ATP while promoting catabolic processes that generate ATP (Hardie, 2011). Moreover, the activity of AMPK is extensively regulated by multiple upstream signals making AMPK a central regulator for coordinating intracellular metabolism with specific energy demands.

Structurally, AMPK is a heterotrimeric enzyme comprised of a catalytic (α) subunit and two regulatory (β and γ) subunits (Figure 1.13), all of which are encoded by separate genes. In mammals, the α subunit is encoded by two isoforms, and the β and γ subunits are encoded by two or three isoforms (Garcia and Shaw, 2017). The expression levels of the AMPK isoforms vary across tissues, leading to diverse subunit combinations in different cell types; however, AMPKα1, AMPKβ1, and AMPKγ1 are ubiquitously expressed while other isoforms show a more restricted expression pattern (Ross, Mackintosh and Grahame, 2016).

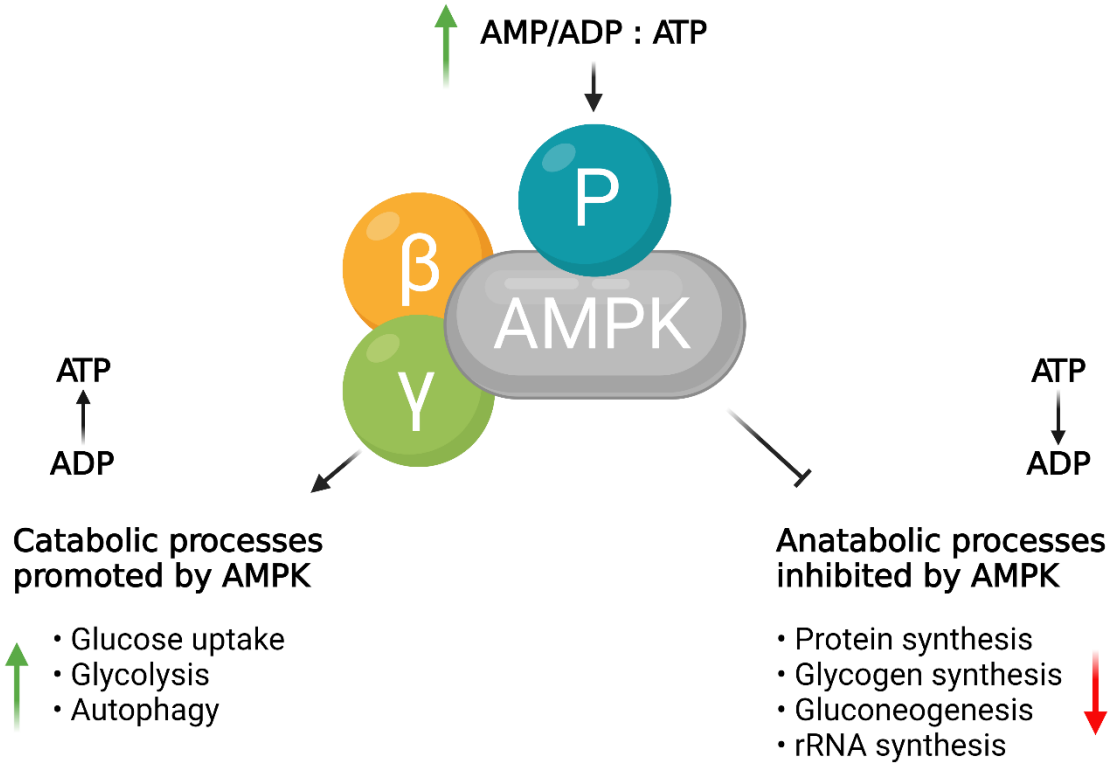


Figure 1.13 Metabolic consequences of AMPK activation. The regulation of AMPK is nucleotide dependent resulting from changes in AMP to ATP or ADP to ATP ratios that are caused by various environmental stressors. In response, AMPK restores ATP levels by acutely inhibiting ATP-consuming biosynthetic pathways while simultaneously activating catabolic pathways that regenerate ATP through the breakdown of macromolecules (Image inspired by Garcia and Shaw, 2017). Created with BioRender.com.

During normal cellular activity, cells maintain an ATP to ADP ratio of 10:1; however, low oxygen and nutrient levels cause increased AMP concentrations as a result of increased adenylate kinase activity (2ADP ⇌ ATP + AMP) (Hardie, 2011). When activated, AMPK limits energy-consuming processes such as glycolysis and fatty acid oxidation, to preserve energy. AMPK also regulates translation through inhibition of eEF2 and mTOR substrates 4E-BP1 and S6K (Leprivier *et al.*, 2013). Furthermore, elevated AMP levels and activated AMPK regulate translation through repression of mTOR through the TSC2 complex and inhibition of 4E-BP and eEF2 (Connolly *et al.*, 2006). In general, mTOR and AMPK controlled mechanisms are interlinked and collaborate to regulate cell growth (González *et al.*, 2020). AMPK signals lack of nutrients or insufficient ATP and inhibits growth in response, whereas mTOR indicates nutrient availability and promotes cell growth.

1.9. Pharmaceutical induction of cellular response to culture conditions

Rapamycin, also known as Sirolimus is the most documented inhibitor of mTOR, from which its name is derived. Most known mTOR substrates were discovered and validated using rapamycin as a pharmacological probe, so it is a valuable tool for investigating protein synthesis and cellular response in relation to mTOR function (Thoreen *et al.*, 2009). Rapamycin was initially identified as an antifungal agent but has since been developed as an immunosuppressant and anticancer drug. Although cellular response to rapamycin varies between cell lines, it is generally known to interrupt growth and proliferation through changes in translation, resulting in reduced cell size (Thoreen and Sabatini, 2009). When bound to intracellular protein FKBP12, rapamycin allosterically binds to the FRB domain of mTOR1 and inhibits its phosphorylating activity through mechanisms that have not been fully elucidated. The S6K kinases (S6K1 and S6K2) and translational inhibitor 4E-BP, both of which mediate significant links between mTOR and components of the translation machinery have been identified as specific targets of rapamycin. S6K activity is potently inhibited throughout the duration of rapamycin treatment but 4E-BP activity is restored after 1-3 hours, indicating differential regulation of the two substrates; however, prolonged rapamycin treatment hyper-activates the PI3K pathway which is upstream of mTOR1 and may therefore overcome the partial inhibition caused by rapamycin (Choo *et al.*, 2008; Thoreen *et al.*, 2009). Although rapamycin is a highly selective inhibitor of mTOR, there are disparities in the pharmacological effects between different cell lines. *In-vitro* studies have identified an interesting pattern of cellular responses where there is either complete sensitivity or profound resistance. Relatively high concentrations of rapamycin (≥ 10μM) are required to supress mTOR activity and in most cases, not all activity is completely inhibited (Geoerger *et al.*, 2001; Xie, Wang and Proud, 2016). Some studies have suggested that these effects occur as a result of the inability of rapamycin to inhibit mTOR2 activity and Akt phosphorylation; however, recent findings suggest that mTOR1 function is dependent on rapamycin resistance, which may account for the mTOR activity and phosphorylation of associated proteins observed in rapamycin treated cells (Thoreen *et al.*, 2009). In spite of findings, rapamycin is still an attractive candidate for

mTOR inhibition. It exhibits minimal systematic toxicity in animal and human cells and is also highly lipophilic and subsequently able to penetrate through most blood-tissue barriers. It is also specific to mTOR, thereby eliminating the risk of crossover inhibition of other lipid and protein kinases (Geoerger *et al.*, 2001).

As shown in Table 1.5, rapamycin analogues such as temsirolimus, everolimus and ridaforolimus have been developed as alternative mTOR inhibitors. Although they are structurally similar to rapamycin, these compounds have been designed to have increased hydrophilic ability to enable intravenous administration in oncology treatment (Vignot *et al.*, 2005). Some of the analogues are referred to as ATP-competitive inhibitors and exhibit a much lower half-maximal inhibitory concentration against mTOR activity than rapamycin (Xie, Wang and Proud, 2016). Unlike the temporary inhibitory effect seen in rapamycin treated cells, ATP-competitive inhibitors continuously inhibit both mTOR complexes and cause significant decrease in phosphorylation of all its substrates. This leads to more severe inhibition of cap dependent translation than observed in cells treated with rapamycin (Chresta *et al.*, 2010). ATP-competitive inhibitors are smaller in size than the FKBP12-rapamycin complex and are consequently likely to access mTOR targeting sites more easily (Thoreen *et al.*, 2009). In spite of its effectiveness in supressing mTOR activity, there are concerns over the widespread use of ATP-competitive inhibitors in mammalian cell lines because mTOR signalling is required for normal cellular function and its complete inhibition presents detrimental health issues in cells such as stagnated growth. Furthermore, mTOR and PI3K share a high degree of homology within their catalytic domains and ATP-competitive inhibitors inhibit both kinases with similar potency, indicating that these are not reliable compounds for selective inhibition of mTOR.

Table 1.6. Commonly used first-generation rapalogs (rapamycin analogues) and ATP-competitive inhibitors of mTOR.

Compound name	Target system	Effect	Reference
AZD8055	mTOR1 and mTOR2 (ATP competitive) *First drug to inhibit both mTOR1 and mTOR2	Inhibits phosphorylation of mTOR1 targets p70S6K and 4E-BP1 as well as mTOR2 (Akt), resulting in reduced cap-dependent translation.	Geoerger et al., 2001 Chresta et al., 2009
BGT226	mTOR1 and mTOR2	Inhibits phosphorylation of Akt, p70S6K and 4E-BP1.	Simioni et al., 2015
CC-223	mTOR1 and mTOR2	Inhibits phosphorylation of mTOR1 targets S6 ribosomal protein and 4E-BP1, and mTOR2 substrates Akt.	Mortensen et al., 2015
Chrysophanic Acid	mTOR1 and mTOR2	Reduces phosphorylation of S6K and Akt.	Lee et al., 2011
Omipalisib	mTOR1 and mTOR2	Inhibits phosphorylation of Akt at SER473	Knight et al., 2010
Palomid 529	mTOR1 and mTOR2	Inhibits phosphorylation of Akt at SER473 and ribosomal protein S6.	Xu et al., 2017
PF-04691502	mTOR1 and mTOR2	Inhibits activation of PI3K and mTOR downstream targets including Akt, p70S6K and 4E-BP1.	Yuan et al., 2011
PP121	mTOR1 and mTOR2	Directly inhibits PI3K and blocks phosphorylation of Akt, p70S6K and ribosomal protein S6.	Che et al., 2014
Ridaforolimus	mTOR1	It inhibits phosphorylation of both S6K and 4E-BP1 and causes increased phosphorylation of Akt at SER473 and THR308.	Rivera et al., 2011
Sapanisertib	mTOR1 and mTOR2	Inhibits phosphorylation of mTOR1 substrates S6K and 4E-BP1, and mTOR2 substrate Akt at SER473	Hsieh et al., 2012
Torin 1	mTOR1 and mTOR2	Inhibits phosphorylation of mTOR1 and mTOR2 substrates through a rapamycin resistant mechanism.	Thoreen et al., 2009
Torin 2	mTOR1 and mTOR2	Inhibits both mTOR1 and mTOR2 activity. Blocks phosphorylation of S6K1 at T389 and Akt at S473.	SImioni et al., 2014
Vistusertib	mTOR1 and mTOR2	Decreases 4E-BP1 phosphorylation at THR37 and THR46. It inhibits translation initiation and decreases global protein synthesis. Inhibits phosphorylation of mTOR2 substrate Akt at SER473.	Zheng et al., 2014

Alternative to rapamycin inhibition is AMPK inhibition, which can be achieved through medium supplementation with dorsomorphin (also known as Compound C) in *in vitro* experiments. Initially discovered as an AMPK inhibitor from a screen of a 10000 library by Merck Scientists, it is also known as the first selective inhibitor of bone morphogenic protein (BMP) signalling (Hao *et al.*, 2008; Yu *et al.*, 2008). It was named dorsomorphin due to the dorsoventral patterning defects in zebrafish usually seen in BMP-pathway mutant embryos (Dasgupta and Seibel, 2018). Despites its ability to inhibit *in vivo* and *in vitro* proliferation (Knight *et al.*, 2015), the mechanism that govern AMPK inhibition by dorsomorphin is not as well documented as that of mTOR inhibition by rapamycin. Nonetheless, it has proved to be a potent cytotoxic

agent that inhibits proliferation in various cell types through multiple AMPK dependant mechanisms (Liu *et al.*, 2014).

1.10. Thesis rationale

With the above in mind, and when considering the importance of the MEA in IVF quality control, it is surprising that there are still uncertainties regarding its use. Whilst it has had an invaluable contribution to our understanding of embryo development in mammals, particularly in humans, it also presents many challenges that are yet to be addressed. Some of these challenges, particularly those associated with the lack of standardisation, have been addressed by the development of MEGA, time-lapse imaging and extended incubation as detailed in section 1.4.5; however, ethical issues (including extensive animal use) and the regulatory guidance to use the MEA remains. A proposed alternative is the use of pluripotent cell lines, which are often used in research to induce germ layer formation and in this way, mimic one of the most significant events in early-stage embryos as described in section 1.2.4. Cells lines are not typically used in IVF research and development because, despite its drawbacks, the MEA has proven to be a more sensitive tool in the identification of suboptimal culture conditions. As mentioned in section 1.4, previous IVF studies that involved cell lines tended to use somatic cell lines that are not characteristic of early embryonic events and are generally more resistant to suboptimal conditions than mouse embryos. Given its ability to differentiate into the three germ layers and then all cell types, use of the P19 cell line presents an opportunity to assess the dynamic, constantly developing nature of embryos without sacrificing animals.

As described in section 1.5, with the evolution of drug discovery processes in the pharmaceutical industry, HTS tools have significantly improved, but have yet to be adapted for use in IVF. Nonetheless, these have the potential to create reproducible methods in IVF that reduce the cost of quality control without the need to sacrifice animals. Furthermore, considering that concerns surrounding the use of cell lines in IVF involve their inability to

indicate suboptimal culture conditions, our understanding of the mechanisms that control cellular response to suboptimal conditions and the ability to manipulate these responses through pharmacological induction provides an opportunity to alter how cells respond to environmental perturbations.

Many publications have identified the components in embryo culture media that contribute to successful embryonic development, however the exact concentration of each component is unknown to end-point users outside of the research and development community. What is known however is that the energy requirements of embryos change as they develop, and this is reflected in most commercially available culture media. Therefore, it is of great importance to develop an alternative to the MEA that adequately reflects the embryonic response to changes in energy source availability. Given the sparsity of studies on the effects of pyruvate, lactate and glutamine supplementation in culture media, in comparison to studies that focus on glucose metabolism, there is a need to further assess the impact of various energy sources on cellular development. At the same time, such information could inform research and development in the IVF industry to improve media used to generate embryos.

1.11. Specific aims

With the above in mind, the specific aims of this thesis were:

1) To develop and optimise a 2D cell-based assay with pluripotent abilities that mimics early embryonic development in mammals, and to assess the ability of mammalian cells to respond to suboptimal environmental changes, particularly fluctuating concentrations of exogenous metabolic substrates;

2) To develop and optimise a 3D cell-based assay with pluripotent abilities that mimics early embryonic development in mammals, and to assess the contributions of

exogenous metabolic substrates in the supply of energy and subsequently, embryoid body growth;

3) To test the hypothesis that manipulating intracellular responses to environmental stressors such as hypothermic incubation increases the ability of mammalian cells to respond to the contributions of exogenous metabolic substrates;

4) To use the cell-based assays developed in specific aims (1) and (2) to identify suboptimal embryo culture media with a view to improve current research and development practices in the IVF sector.

2. Materials and methods

All reagents and plasticware were purchased from Thermo Fisher Scientific unless otherwise stated. Gems® products were provided by Genea Biomedx.

2.1. Routine cell maintenance

P19 embryonal carcinoma cells (European Collection of Cell Cultures; ECACC 95102107) were received at passage nine and immediately isolated from preservation medium by centrifugation at 150xg for 15 minutes. The supernatant was removed with a serological pipette, and the pellet was re-suspended in 1 ml of alpha minimum essential medium (αMEM; Gibco). As shown in Figure 2.1, cells were maintained in 75 cm² culture flasks with 20 ml of αMEM containing 1% penicillin-streptomycin solution (Gibco) and supplemented with 10% heat-inactivated fetal calf serum (FCS; Gibco), under culture conditions of 5% CO_2 and 37°C (standard culture conditions). When 80-90% confluent, cells were split and sub-cultured at a seeding density of 1x10⁶ cells/75cm² flask.

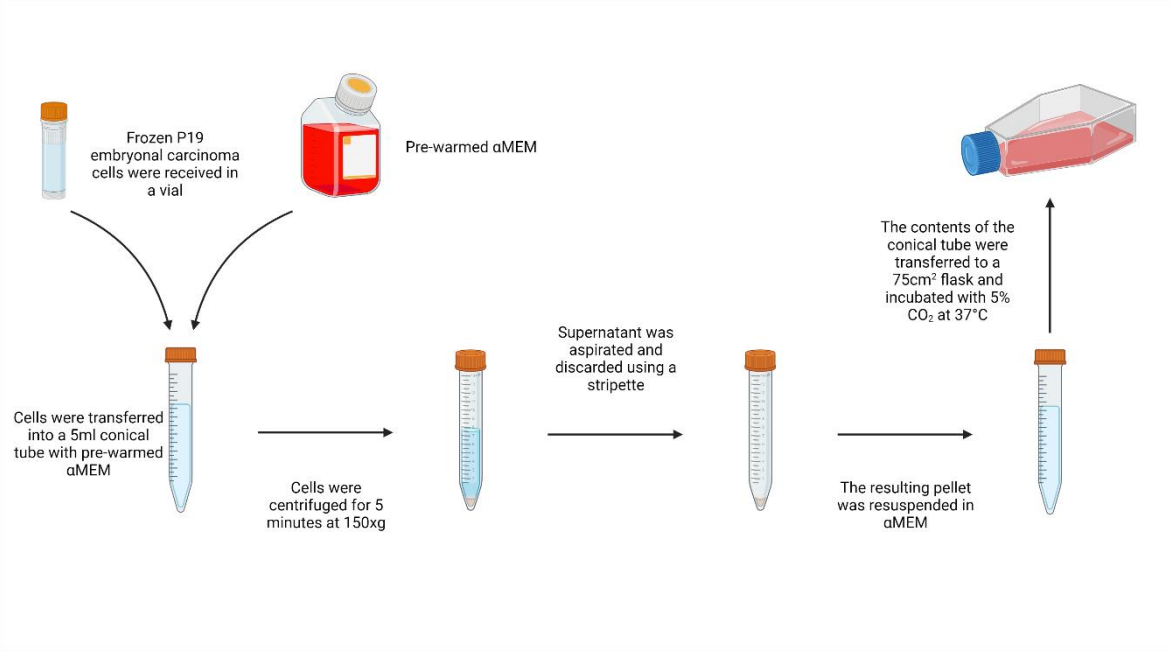


Figure 2.1. Schematic showing the steps involved in preparing P19 embryonal carcinoma cells for culture. Cells were received in a cryovial, separated from preservation medium by centrifugation and maintained in a flask with complete growth medium. Created with BioRender.com.

2.2. Scale-up and cell banking

In order to maintain consistency between experiments and to ensure material availability, an in-house cell bank was established. Each subsequent experiment was conducted using cells from this bank, allowing direct comparison of test substances whilst eliminating variability from different cell passages. The use of this cell bank also ensured that cells were preserved at a low passage number, thereby preventing inconsistent differentiation which may occur as a result of culturing at a high passage number (> 25) (Seiler and Spielmann, 2011).

As shown by Figure 2.2, cells were detached from confluent cultures by adding 2 ml of trypsin-EDTA (0.25%) solution (Gibco) to flasks, followed by five minutes of incubation at standard conditions, with gentle manual shaking every thirty seconds. Trypsin activity was stopped by adding 5ml of complete αMEM. The cell suspension was then centrifuged at 150xg for five minutes, and the resulting pellet was re-suspended in 1ml of αMEM and mixed thoroughly by repeated pipetting. To preserve cells, cryoprotectant made up of 90% FCS and 10% dimethyl sulfoxide (DMSO; Thermo Fisher Scientific) was added at a volume ratio of 1:1, and 2 ml aliquots were transferred into cryovials. These vials were then placed in an isopropanol progressive freezing container (Nalgene Mr Frosty; Sigma Aldrich) at -80°C for overnight incubation and immersed in liquid nitrogen after 24 hours (Chen *et al.*, 2007; Baust, Gao and Baust, 2009).

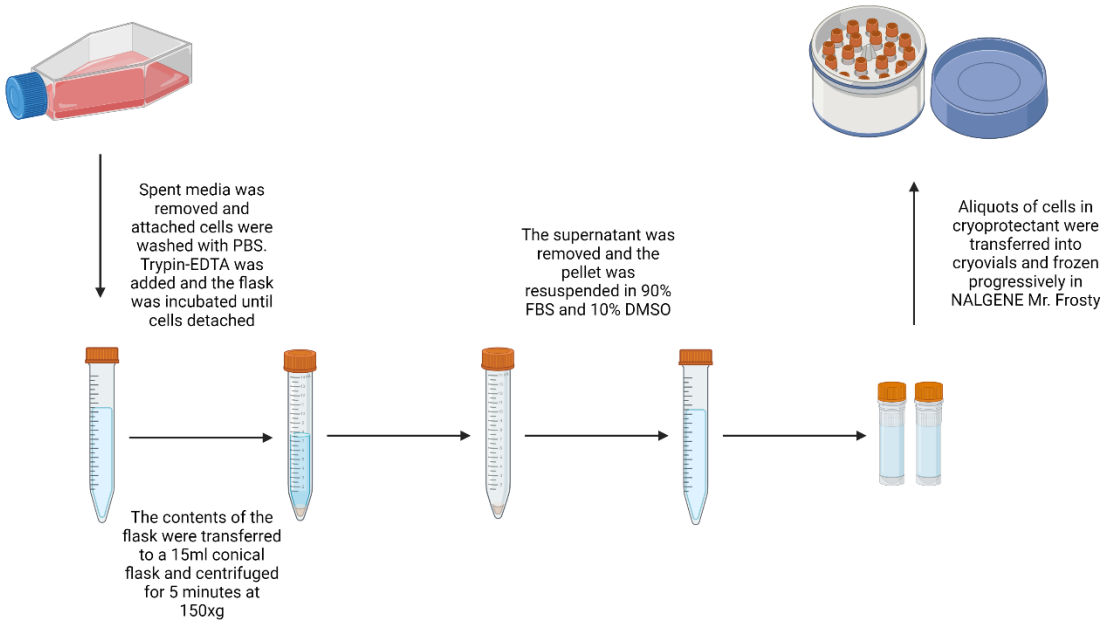


Figure 2.2. Sub-culturing and preparation of P19 cells for cryopreservation. A slow-freezing method involving a DMSO-based cryoprotectant and a progressive freezing container stored at -80°C was used, followed by submersion in liquid nitrogen for long term storage. The progressive freezing container allows for cells to be frozen at a rate of 1°C/minute. Created with BioRender.com.

2.3. Determination of a positive control

2.3.1. DMSO

Negative and positive controls were established in order to assess cellular function at both extremes of the activity spectrum. The negative control (also known as the background measure) indicates regular cellular activity, assuming there are no changes in variables whereas the positive control produces a known response as a result of changes within a variable. αMEM provides optimum growth conditions for P19 cells and was subsequently deemed suitable to be used as the negative control. Conversely, dimethyl sulfoxide (DMSO) is an organic solvent that interrupts cell metabolism and causes damage to the membrane when administered in high concentration, and was consequently chosen as the positive control. There are few studies on the maximum non-toxic usable concentration of DMSO, and this differs considerably between cell types. For example, in isolated rat intestinal tissue, 5% DMSO is the highest concentration that can be used without causing damage to the cell

membrane whereas the Caco2/TC7 cell line has a maximum tolerance of 1% DMSO (Da Violante *et al.*, 2002).

Cells were maintained and harvested from confluent cultures (as described in section 2.1) and brought to a final concentration of 10^5 cells/ml by supplementation with αMEM. The suspension was poured into a 50 ml pipette basin and dispensed into the wells of 96-well plates using a multichannel pipette. To achieve a density of 1 x 10^4 cells/well, 100 µl of cell suspension was seeded in each well and the plate was incubated at standard culture conditions for 24 hours to allow cell attachment as described in Putnam, Bombick and Doolittle, 2002. Spent media was removed and replaced with 50 µl of increasing concentrations of DMSO (0%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40% and 100%) in replicates of six wells overnight. Wells were emptied, washed with phosphate-buffered saline (PBS; Gibco) and 20µl of 5mg/ml MTT solution was added as shown in Figure 2.3 (see section 2.4).

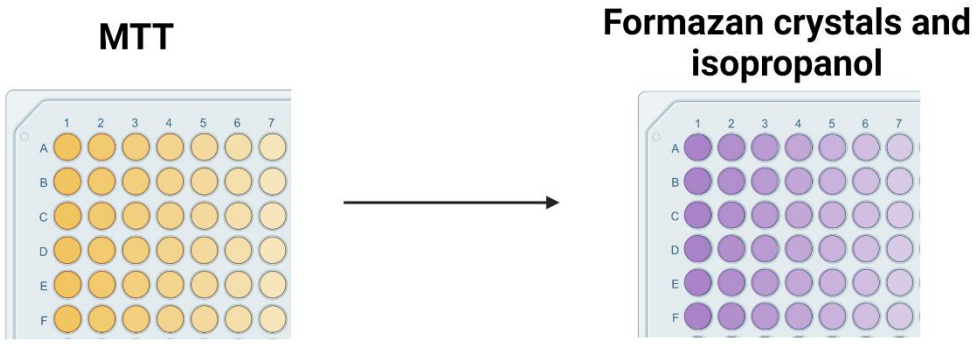


Figure 2.3. The cytotoxic effects of increasing DMSO concentrations on P19 embryonal carcinoma cells. Illustrated by the colour intensity of MTT (yellow) and formazan (purple), increasing concentrations of DMSO led to increased cell death, resulting in reduced conversion of MTT to formazan and increased well transparency. Created with BioRender.com.

2.4. Cell viability assay

2.4.1. MTT assay

MTT (Sigma-Aldrich) was prepared by dissolving the product in PBS to a final concentration of 0.5 mg/ml (0.5 g of MTT in 10 ml of PBS). For cell viability testing, 20 µl/well was added to culture assays, followed by two hours at standard culture conditions. Resulting formazan crystals were dissolved in 100 µl of isopropanol in each well and the optical densities and absorbance were determined using a plate reader (FLUOstar Galaxy, BMG LABTECH GmbH, Ortenberg) at an absorbance wavelength of 540 nm. Absorbance readings were quantified using OMEGA software version 5.10.R2 (BMG LABTECH GmbH, Ortenberg).

2.4.2. Resazurin assay

To determine the optimum resazurin concentration for this study, cells were seeded in a 96-well plate at increasing densities (2.5×10^4, 5.0×10^4, 1.0×10^5 and 2.0×10^5). Spent media was then removed after 24 hours and replaced with 100 µl of αMEM or 100 µl of 25% DMSO in alternating rows as illustrated by Figure 2.4. A stock solution of 160 mM resazurin solution (4 mg of resazurin sodium salt in 10 ml of PBS) was prepared, filter sterilised using a 0.2mm syringe filter and serially diluted with αMEM to produce 40 µM, 80 µM, 160 µM and 350 µM solutions. After two hours of incubation with αMEM and 25% DMSO, wells were emptied, washed with PBS and replaced with 50 µl of resazurin solution. Resorufin fluorescence was recorded every 15 minutes for 1 hour, then every 30 minutes for four hours using the same plate reader as above at an excitation wavelength of 544 nm, and emission wavelength of 590 nm (these parameters will be referred to as standard parameters from this point on). Fluorescence readings were quantified using the same software as above.

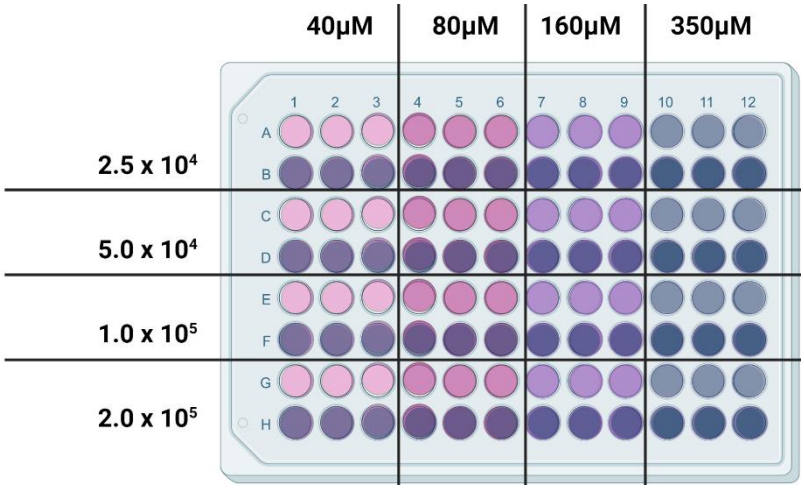


Figure 2.4. Illustration of the optimisation of resazurin concentration and cell density. P19 embryonal carcinoma cells were seeded at increasing densities and treated with complete αMEM or 25% DMSO for two hours. Resazurin reduction was observed and measured over the course of five hours. Treatment with 25% DMSO resulted in highly compromised cell viability and cells were unable to reduce resazurin to resorufin; hence there was no colour change in these wells. There was increased resazurin reduction with increasing cell density and colour change from blue to fluorescent pink occurred at a faster rate with the 40 µM and 80 µM treatments than with the 160 µM and 350 µM treatments. Created with BioRender.com.

2.4.3. RealTime-Glo® MT cell viability assay

RealTime-Glo® was prepared by adding 10 µl of NanoLuc® luciferase (Promega) and 10 µl of cell-permeant substrate MT (Promega) to 10 ml of complete αMEM. For cell viability testing, 50 µl/well were added to culture assays, followed by incubation at standard culture conditions for one hour. As shown in Figure 2.5, viable cells reduced MT to generate a substrate that diffuses into the surrounding medium, where it is used by NanoLuc® luciferase to produce a luminescent signal. Resulting luminescence were determined using the same plate reader and software as mentioned above at a standard setting with one second intervals between measurements.

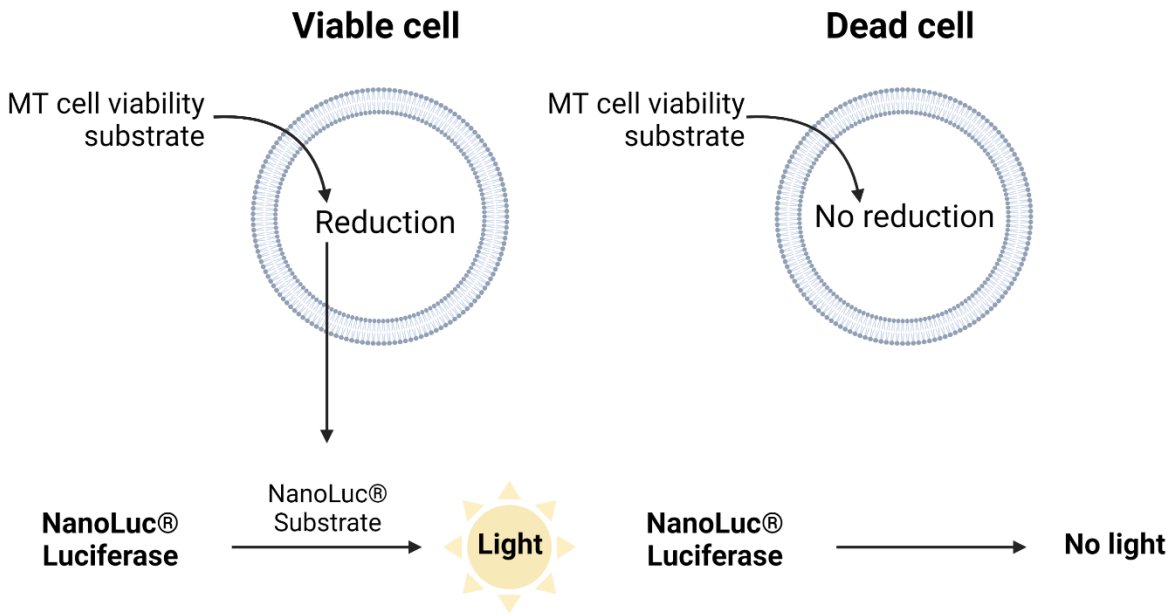


Figure 2.5. RealTime-Glo® MT cell viability assay. NanoLuc® luciferase and MT cell viability substrates are added to culture media, where MT cell viability substrate is reduced to a NanoLuc® substrate by metabolically active cells. The NanoLuc® substrate diffuses from cells into the surrounding culture media and is used by NanoLuc® enzyme to produce a luminescent signal. The signal emitted is directly linked to the number of viable cells. Dead cells do not reduce the substrate and therefore produce no signal. Created with BioRender.com.

2.5. Evaluation of assays

2.5.1. Signal-to-noise ratio and signal-to-background ratio

The signal-to-noise ratio (S/N) and the signal-to-background ratio (S/B) were evaluated to determine the quality of the assays. Both S/N and S/B are an indication of the degree to which a signal can be regarded as different from the background noise, i.e., the measure of any signal emitted when there is no cellular activity. They differ in their inclusion of data variation, as shown by the formulae below. The mean background signal was determined by treating the wells of a 96-well plate with 25% DMSO followed by signal detection with MTT or resazurin solution, in the absence of P19 cells.

$$\text{Signal-to-noise} = \frac{\text{mean signal} - \text{mean background signal}}{\text{standard deviation of background signal}}$$

$$\text{Signal-to-background} = \frac{\text{mean signal}}{\text{mean background signal}}$$

2.5.2. Z' Evaluation

2.5.2.1. Z' Evaluation for 2D assay

The Z' factor was determined in order to quantify the suitability of assays of this nature for high-throughput screening (HTS). Cells were maintained and harvested from confluent cultures as described in section 2.1, and brought to a concentration of 10^6 cells/ml by supplementation with αMEM. To estimate the proportion of live cells, 20 µl of cell suspension was incubated with 20 µl of 0.4% trypan blue solution (Gibco) for five minutes in an Eppendorf tube; viable cells exclude the dye from their cytoplasm while those with compromised membrane integrity do not and appear blue. Two cell counts were performed using the improved Neubauer haemocytometer (Blaubrand; Sigma Aldrich) and the ratio of trypan blue stained (dead) cells over the total number of cells were found to be consistently over 95%. Cells were seeded at a density of 1.0 x 10^5/well in a 96-well plate and incubated for 24 hours under standard culture conditions to allow cell attachment. Spent media was removed and

replaced with 50 µl of 25% DMSO in αMEM or 50 µl of complete αMEM. The plate layout was designed to reduce systematic and random errors that may affect different parts of the plate unevenly. Wells were emptied after 24 hours of treatment, washed with 100 µl of PBS and 50 µl of 160 µM resazurin solution added. Resorufin fluorescence was determined every hour for four hours at standard parameters as per section 2.4.2. The suitability of MTT as a measure of cell viability was investigated by repeating the above and replacing resazurin solution with 20 µl of 5 mg/ml MTT solution for two hours. To dissolve formazan crystals, 100 µl of isopropanol was added to each well and the absorbance was determined as described in section 2.4.1.

2.5.2.2. Z' Evaluation for 3D assay

For 3D assays, cells were maintained and harvested from confluent cultures as described in section 2.5.2.1 and brought to a final concentration of 10^4 cells/ml by supplementation with αMEM. Cells were seeded at a density of 1.0 x 10^3/well in 2x ultra-low attachment round-bottom 96-well plates (Corning) and incubated for 24 hours under standard culture conditions to allow embryoid body formation. Spent media was removed and replaced with 50 µl of 25% DMSO in αMEM or 50 µl of complete αMEM. After 48 hours, 50 µl of 160 µM resazurin solution was added to one plate, and 50 µl of RealTime-Glo® was added to the other. Resorufin fluorescence was determined every hour for four hours at standard parameters as described in section 2.4.2. Luciferase luminescence was determined after one hour by the same plate reader as per section 2.4.3.

2.6. Cell culture and assay preparation

2.6.1. 2D assay

Cells were maintained and harvested from confluent cultures as described in section 2.1 and brought to a final concentration of 10^6 cells/ml by supplementation with αMEM. For 2D assays,

the proportion of live cells was estimated as described in section 2.5.2.1 then the cell suspension was poured into a 50 ml pipette basin, and 100 µl was dispensed into the inner wells of 96-well plates using a multi-channel pipette. Plates were incubated under standard culture conditions for 24 hours to allow cell attachment and to achieve a density $1x10^5$ cells/well. Over-confluence in plates incubated for 96 hours was prevented by lowering the seeding density to $1.25x10^4$/well, as previous cultures indicate a doubling time of 12 hours. Spent media was removed after 24 hours and replaced with the various media formulations listed in table 2.1. All treatments were replicated in 12 wells unless otherwise stated, with the 36 outer wells of each plate treated with 100µl of 25% DMSO in the absence of cells, to detect background signal of resazurin and systematic errors (Spielmann *et al.*, 1997). Remaining wells were treated with 100 µl of complete αMEM as a negative control and 100 µl of 25% DMSO as a positive control. Spent media was replenished after 48 hours to provide cells with sufficient nutrients. These methods are summarised in Figure 2.6.

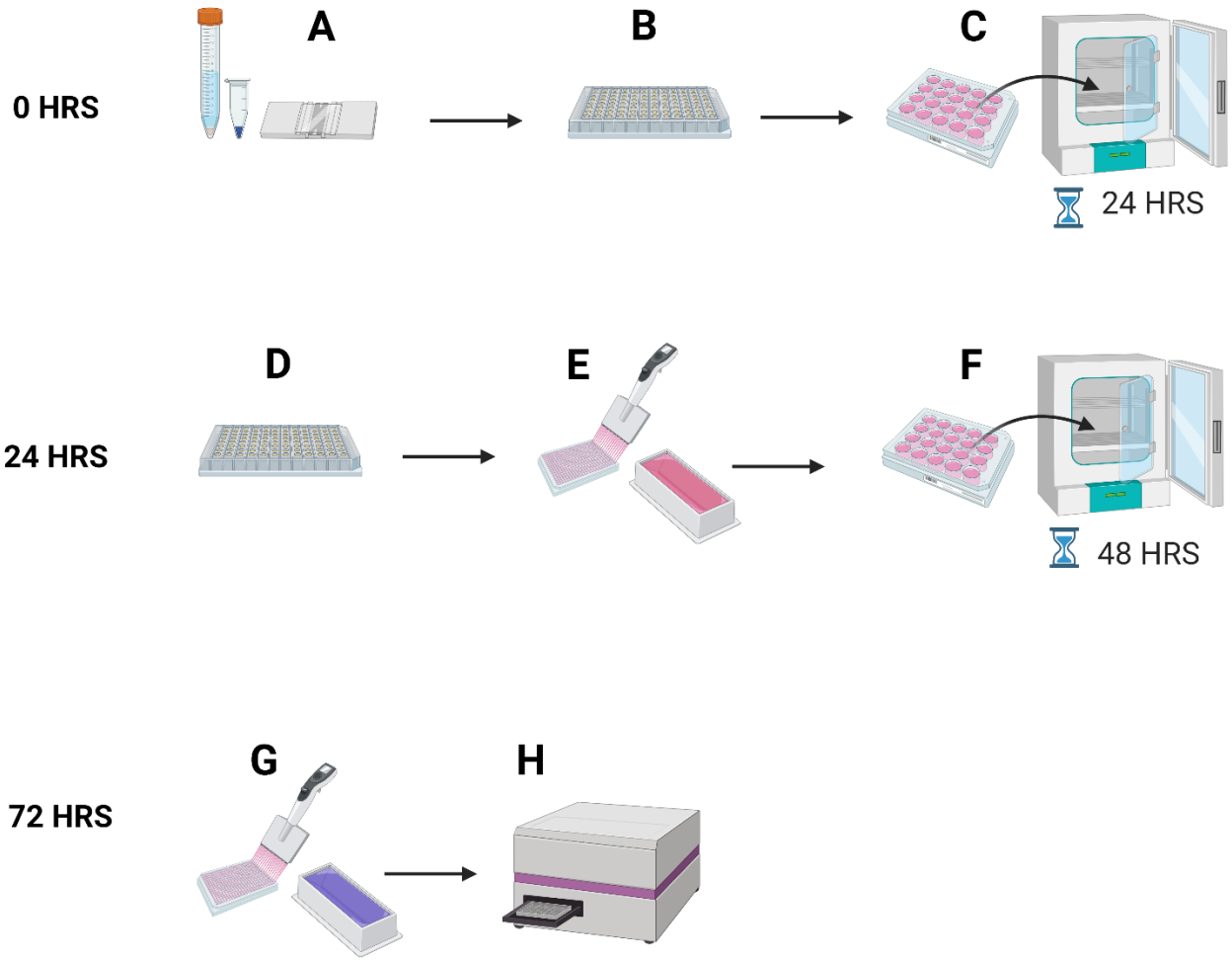


Figure 2.6. Overview of the steps involved in the 2D and 3D assay. P19 embryonal carcinoma cells were harvested from confluent cultures and the proportion of live cells were determined by a 0.4% trypan blue dye (A). Cells were seeded into 96-well plates (B) and incubated for 24 hours to allow cell attachment (C), after which, test substances replaced spent media (D & E). A 48-hour incubation period was allowed (F) then cells were treated with MTT, resazurin solution or RealTime-Glo® MT (G). Absorbance, fluorescence or luminescence were quantified using a plate reader (H). Created with BioRender.com.

2.6.2. 3D assay

Cells were maintained and harvested from confluent cultures as described in section 2.1 and brought to a concentration of 10^4 cells/ml by supplementation with αMEM. For 3D assays, the proportion of live cells were estimated as described in section 2.5.2.1. Following this, the suspension was poured into a 50 ml pipette basin and dispensed into the wells of ultra-low attachment round-bottom 96-well plates using a multichannel pipette. 100 µl of cell suspension was seeded in each well to achieve a density of 1.0 x 10^3 cells/well. As shown by Figure 2.7,

plates were ultra-centrifuged at 250xg for five minutes and incubated at standard culture conditions to aid embryoid body (EB) formation. Spent media was removed after 24 hours and replaced with the various media formulations listed below. All treatments were replicated in 24 wells, with remaining wells treated with 100 µl of complete αMEM as a negative control and 100 µl of 25% DMSO as a positive control. Spent media was replenished after 48 hours to provide cells with sufficient nutrients. EB dimension and area were determined using a digital microscope camera (AmScope MU500) and EBs with an area of < 2000 µm² were not included in the quantification as per earlier studies by (Jasmin *et al.*, 2010).

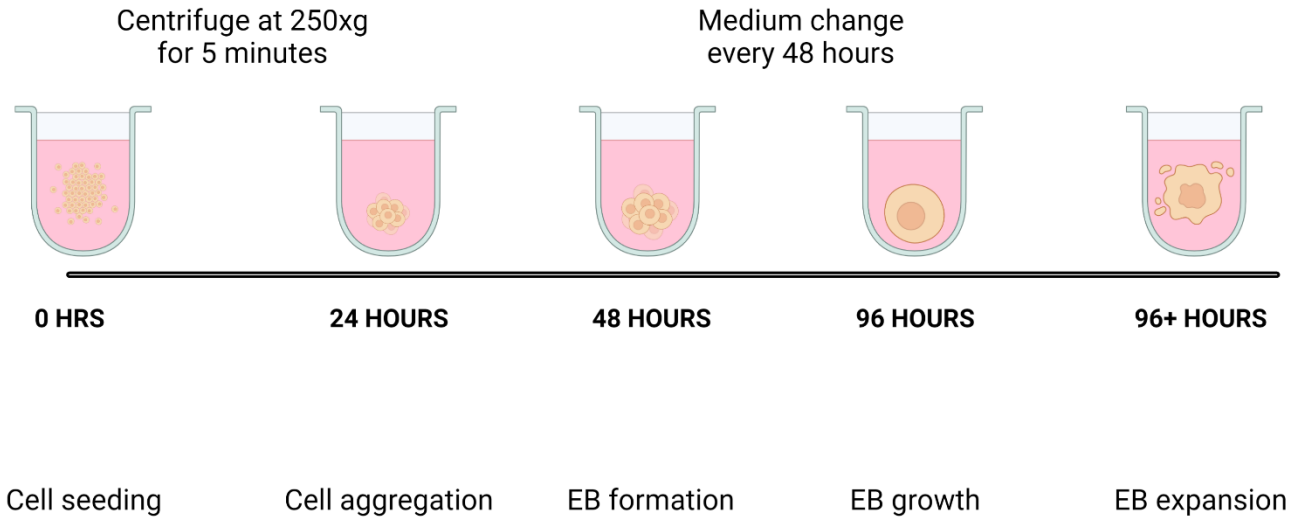


Figure 2.7. Schematic representation of the typical process of EB formation. Droplets of P19 embryonic carcinoma cell-medium suspension were dispensed into the untreated wells of round-bottomed 96-well plates, followed by incubation for 24 hours to allow EB formation. The formed EBs were treated with the Gems® media suite (human oocyte retrieval buffer, fertilisation medium, cleavage medium and blastocyst medium) as well as increasing concentrations of pyruvate and calcium lactate in blastocyst media for up to 96 hours. Created with BioRender.com.

2.7. Preparation of test substances

Freshly made ORB and BLM were also prepared from stock solutions of base salts (sodium chloride, potassium chloride, potassium phosphate monobasic, magnesium sulphate heptahydrate), amino acids (taurine, glycine, L-carnitine) and vitamins (sodium L-ascorbate,

folic acid, cobalamin, D-Pantothenic acid hemicalcium salt) in Milli-Q water. 100µl of each stock solution was mixed with sodium bicarbonate, HEPES, EDTA disodium salt dihydrate, essential and non-essential amino acids, human serum albumin (HSA) and gentamicin sulphate, filtered using a 0.2mm syringe filter and the osmolality and pH were adjusted to 270 mOsmol/kg and pH 7.41 by adding more Milli-Q water or sodium chloride, or hydrochloric acid or sodium hydroxide.

2.8. Rapamycin and dorsomorphin optimisation

Stock solutions of 10 mM mTOR inhibitor rapamycin (Sigma Aldrich; 50 mg of rapamycin in 5 ml of DMSO) and 10 M AMPK inhibitor dorsomorphin (Sigma Aldrich; 20 mg of dorsomorphin in 5 ml of DMSO) were prepared, filter sterilised using a 0.2 mm syringe filter and serially diluted with αMEM to produce increasing concentrations of rapamycin (0, 0.0002, 0.01, 0.02, 0.1, 0.2, 1, and 2 mM) and dorsomorphin (0, 0.01, 0.02, 0.1, 0.2, 1, 2 and 10 mM) solutions. Spent media was removed from the 96 well plates and replaced with 100 µl of increasing concentrations of rapamycin and dorsomorphin in replicates of 48 wells for 18 hours.

2.8.1. Hypothermic incubation

Cold stress was induced by placing treated plates in an unstirred water bath at 15°C for 18 hours, followed by incubation at standard culture conditions for a further 30 hours. Preliminary experiments (not included in this thesis) indicated that cells responded to cold stress when induced at 15°C for at least 18 hours before or after incubation at standard culture conditions for 30 hours. Wells were emptied after 48 hours of treatment, washed with PBS and 50 µl of 160 µM resazurin solution was added for four hours as described in section 2.4.2.

2.9. Treatments

2D and 3D assays were treated with the various test products listed below. Some were obtained directly from Genea Biomedx whilst others were made in house using the formulations provided by the same company. The concentrations of the various components within these products are protected by a non-disclosure agreement and therefore cannot be disclosed in this thesis. To maintain stability, medium was not kept for longer than 72 hours at 37°C. As advised by the research and development team at Genea Biomedx, this timeframe ensures that sodium bicarbonate, alanyl glutamine and human serum albumin do not completely decompose before use.

Table 2.1. A list of test products used to treat P19 embryonal carcinoma cells in their 2D and 3D form. Treatments were obtained directly from Genea Biomedx or produced in-house using the formulations provided by the same company. Response to various types of human IVF media, osmolality and increasing concentrations of energy sources were assessed, as well as the effects of mTOR inhibitor rapamycin and AMPK inhibitor dorsomorphin.

Treatment	Type of assay
Human embryo culture media: Cellular response to human IVF media was assessed by treatment with human ORB, FLM, CLM and BLM for 48 and 96 hours, as per industrial practice (U.S. Food and Drug Administration 2019). Following a standard MEA protocol (provided by Genea Biomedx), wells treated with ORB, CLM and BLM were emptied after 48 hours and replaced with fresh CLM whilst spent FLM was replaced with fresh FLM. Negative, positive and blank controls were also replenished to maintain consistency.	2D and 3D assay
Increasing media osmolality: Cellular response to changes in osmolality was assessed by treatment with blastocyst media and increasing concentrations of sodium chloride (500, 250, 229, 208, 188, 167, 146, 125, 0.00025, 0.00001875, 0.0000125, 0.000009375, 0.00000625 mM) for 48 hours.	2D assay
Absence of energy sources: Cellular response to the absence of individual energy sources was assessed by treating cells with blastocyst media with no pyruvate; no glutamine; no calcium lactate; and no pyruvate, glutamine or calcium lactate for 48 hours.	2D assay
Mono and dipeptide glutamine: Cellular response to changes in glutamine composition was assessed by treating cells with blastocyst media supplemented with L-glutamine and alanyl glutamine for 48 hours.	2D assay

Table 2.1 continued. A list of test products used to treat P19 embryonal carcinoma cells in their 2D and 3D form. Treatments were obtained directly from Genea Biomedx or produced in-house using the formulations provided by the same company. Response to various types of human IVF media, osmolality and increasing concentrations of energy sources were assessed, as well as the effects of mTOR inhibitor rapamycin and AMPK inhibitor dorsomorphin.

Treatment	Type of assay
***Absence of energy sources and supplementation of vitamins*:** Cellular response to the effects of increased concentrations of vitamins in the absence of energy sources was assessed by treating cells with blastocyst media with no pyruvate; no glutamine; no calcium lactate; and no pyruvate, glutamine, or calcium lactate, supplemented with a double dosage of sodium L-ascorbate, folic acid and cobalamin for 48 hours.	2D assay
***Pyruvate concentration*:** The effects of increasing concentrations of pyruvate on EB growth were assessed by treatment with blastocyst media containing glutamine and calcium lactate, supplemented with increasing amounts of sodium pyruvate (0, 0.1, 0.2, 0.3, 0.4, and 0.5 mM).	3D assay
Pyruvate concentration and rapamycin: Cellular response to the effects of increasing concentrations of pyruvate was assessed by treatment with blastocyst media containing glutamine and calcium lactate, supplemented with increasing amounts of sodium pyruvate (0, 0.1, 0.2, 0.3, 0.4, 0.5, 5.0 and 10.0 mM). Each sample was used to treat 24 wells, and 12 of these were supplemented with rapamycin to a final concentration of 20 μM.	2D assay
Pyruvate concentration and dorsomorphin: As above with dorsomorphin supplementation to a final concentration of 200 μM.	2D assay
Glutamine concentration and rapamycin: Cellular response to the effects of increasing concentrations of glutamine was assessed by treatment with blastocyst media containing pyruvate and calcium lactate, supplemented with increasing amounts of glutamine (0, 0.1, 0.2, 0.3, 0.4, 0.5, 5.0 and 10.0 mM). Each sample was used to treat 24 wells, and 12 of these were supplemented with rapamycin to a final concentration of 20 μM.	2D assay
Glutamine concentration and dorsomorphin: As above with dorsomorphin supplementation to a final concentration of 200 μM.	2D assay
***Calcium lactate concentration*:** The effects of increasing concentrations of pyruvate on EB growth were assessed by treatment with blastocyst media containing pyruvate and glutamine, supplemented with increasing amounts of calcium lactate (0, 1, 2, 3, 4 and 5 mM).	3D assay

Table 2.1 continued. A list of test products used to treat P19 embryonal carcinoma cells in their 2D and 3D form. Treatments were obtained directly from Genea Biomedx or produced in-house using the formulations provided by the same company. Response to various types of human IVF media, osmolality and increasing concentrations of energy sources were assessed, as well as the effects of mTOR inhibitor rapamycin and AMPK inhibitor dorsomorphin.

Treatment	Type of assay
Calcium lactate concentration and rapamycin: Cellular response to the effects of increasing concentrations of calcium lactate was assessed by treatment with blastocyst media containing pyruvate and glutamine, supplemented with increasing amounts of calcium lactate (0, 1, 2, 3, 4, 5, 10.0 and 20.0 mM). Each sample was used to treat 24 wells, and 12 of these were supplemented with rapamycin to a final concentration of 20 µM.	2D assay
Calcium lactate concentration and dorsomorphin: As above with dorsomorphin supplementation to a final concentration of 200 µM.	2D assay
Pyruvate, calcium lactate and glutamine concentrations: Cellular response to the effects of varying concentrations of energy sources was assessed by treatment with blastocyst media containing increasing concentrations of sodium pyruvate (0, 0.1, 0.2, 0.3, 0.4 and 0.5 mM); L-glutamine (0, 0.1, 0.2, 0.3, 0.4 and 5.0 mM); and calcium lactate (0, 1, 2, 3, 4 and 5.0 mM).	2D assay
Pyruvate, calcium lactate and glutamine concentrations, with rapamycin: Cellular response to the effects of varying concentrations of energy sources was assessed by treatment with blastocyst media containing increasing concentrations of sodium pyruvate (0, 0.1, 0.2, 0.3, 0.4 and 0.5 mM); L-glutamine (0, 0.1, 0.2, 0.3, 0.4 and 5.0 mM); and calcium lactate (0, 1, 2, 3, 4 and 5.0 mM), supplemented with rapamycin to a final concentration of 20 µM.	2D assay
Pyruvate, calcium lactate and glutamine concentrations, with dorsomorphin: As above with dorsomorphin supplementation to a final concentration of 200 µM.	2D assay
Pyruvate, calcium lactate and glutamine concentrations, with rapamycin and dorsomorphin: The combined effects of rapamycin and dorsomorphin on cellular response to varying concentrations of energy sources was assessed by treatment with blastocyst media containing increasing concentrations of sodium pyruvate (0, 0.1, 0.2, 0.3, 0.4 and 0.5 mM); L-glutamine (0, 0.1, 0.2, 0.3, 0.4 and 5.0 mM); and calcium lactate (0, 1, 2, 3, 4 and 5.0 mM), supplemented with rapamycin and dorsomorphin to a final concentration of 20 µM and 200 µM respectively.	2D assay
Geri culture media**:** Cellular response to the effects of variation in Geri medium formulation was assessed by treatment with two versions of Gems® Geri culture media for 96 hours, as per industrial practice (U.S. Food and Drug Administration 2019). Wells were emptied after 48 hours and replaced with fresh Geri medium. Negative, positive, and blank controls were also replenished to maintain consistency.	2D assay and MEA

2.10. Mouse embryo assay

2.10.1. Preparing the dishes

The negative control was prepared using Geri media that had previously passed mouse embryo assay (MEA) screening (conducted by Genea Biomedx), whilst the positive control was produced by adding a section from a surgical latex glove to a falcon tube containing 1 ml of the same media. Whilst there is not a standard positive control for quality assessment in IVF (Dubin, Bornstein and Gong, 1995), given its effect on embryo development, powdered surgical latex gloves are often used by embryologists to introduce toxicity in media (Critchlow *et al.*, 1989; Lierman *et al.*, 2007; Boone *et al.*, 2010). 1 ml of each test product and the controls were added to the individual wells of one four-well dish (Nunc) the day before testing to allow the media to equilibrate at standard culture conditions, and 4 ml of CLM were equilibrated in another four-well dish on the morning of testing to prepare the embryos for treatment.

2.10.2. Preparation of mouse embryos

Drops of 4x 500 µl warming solution one® (1.0 M trehalose; Genea Biomedx) and pre-equilibrated CLM were dispensed into a 100 mm petri dish lid and brought to 37°C by incubation at standard culture conditions for 10 minutes, while 4x 500µl drops of warming solution two® (0.5 M trehalose; Genea Biomedx) and three® (M91 cryopreservation buffer and HSA (2% w/v); Genea Biomedx) were brought to ambient room temperature in a separate petri dish. C57BL/6NCrl mouse embryos (Charles River Laboratories) vitrified in SAGE vitrification solution were received in straws and thawed by exposure to ambient room temperature for two minutes. The heat seals of each straw were removed using a pair of scissors, and a plastic stylet (Charles River Laboratories) was used to push the remaining plug downwards to expel the contents of the straw into the CLM drops (one straw/500 µl drop) as shown in Figure 2.8. To ensure the straws were sufficiently emptied, the number of embryos in each drop was counted and the cotton plugs from the straws were removed and examined

using an inverted microscope (Olympus). Embryos were washed by gentle pipetting in CLM using a stripper pipette and 145 µm stripper

tip (Cooper Surgical) and moved to warming solution one® for 60 seconds (20 embryos/drop). Embryos were subsequently moved to warming solution two® and three® for three and five minutes respectively. For all warming solutions, embryos were released at the bottom of the drop in a 'cushion' and the medium was allowed to slowly dissipate. Some embryos floated to the top but were not moved to reduce the risk of mechanical damage.

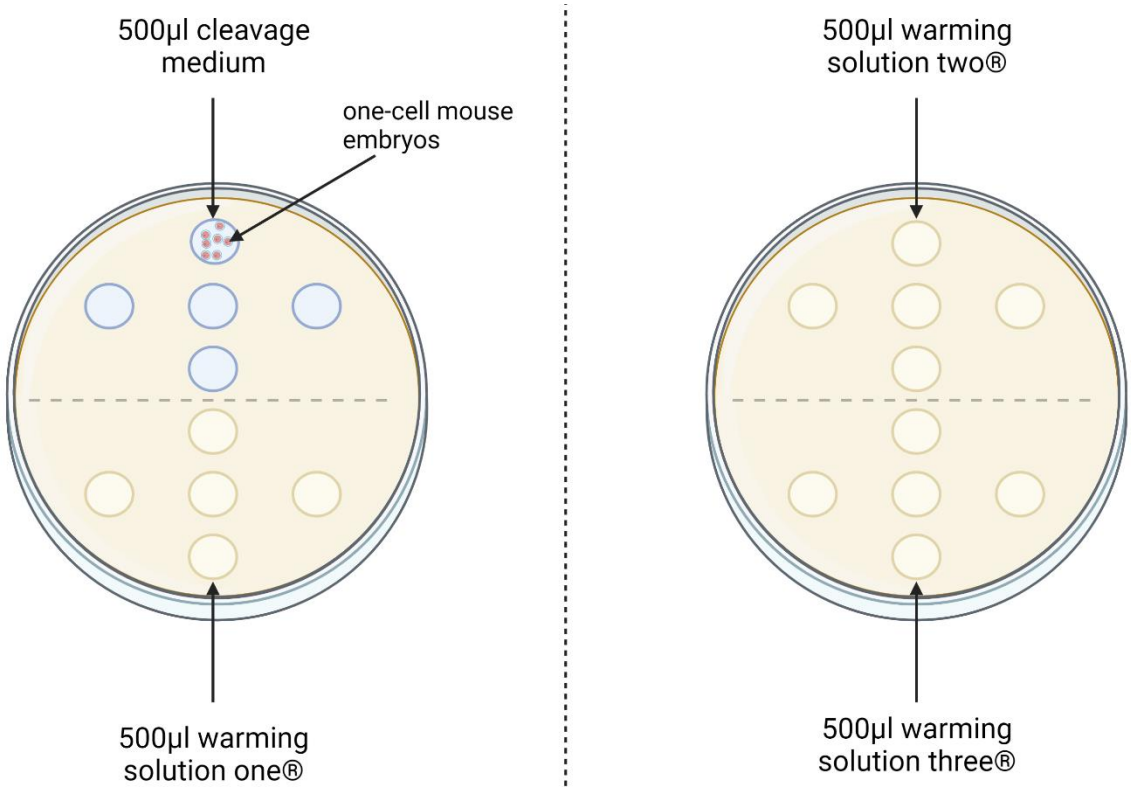


Figure 2.8. Schematic representation of dishes prepared for mouse embryo thawing. Drops of cleavage medium and warming solution one® were brought to 37°C in a petri dish lid by incubation at standard culture conditions (left). Warming solution two and three® were brought to ambient room temperature in a separate petri dish (right) and these were used to prepare mouse embryos for incubation with unknown test substances. Created with BioRender.com.

2.10.3. Running the mouse embryo assay

A stripper pipette (CooperSurgical) and 145 µm stripper tip (CooperSurgical) were used to fill the 16 microwells of Geri dishes with 2 µl of pre-equilibrated test or control media (one dish/sample), ensuring no air bubbles were introduced. The stripper tip was replaced and used to transfer one embryo to each microwell. Culture wells were filled with 80 µl of its respective

test or control media, and wash wells were filled with 80 µl of CLM. 4 ml of mineral oil was added to cover the drops to prevent media evaporation, and dishes were placed in the Geri chambers at standard culture conditions for 96 hours, with images captured every five minutes.

2.11. Statistical analysis

For both 2D and 3D assays, data analysis was performed in R (R Core Team, 2020) accessed via RStudio (RStudio Team, 2020). Normal distribution was determined by visualising the spread of residuals on a histogram and statistical analysis were performed by ANOVA, with Tukey HSD used as a *post hoc* test, and values of $p < 0.05$ were considered to be statistically significant. Positive and negative controls were used to assess the quality of the assay, but these data were not included in the analyses. For the 3D assay, quantitative analysis of EB dimension and area were determined using AmScope (AmScope Incorporated (2003-2016) software version x64 3.7.7934) and these data were uploaded into RStudio where statistical analysis were performed by ANOVA and Tukey HSD *post hoc* test. Mouse embryo development was quantified by data obtained from the morphokinetics analysis of time-lapse incubation by Geri.

3. Thesis aim one: To develop and optimise a 2D cell-based assay with pluripotent abilities that mimic early embryonic development in mammals and to assess the ability of mammalian cells to respond to suboptimal environmental changes, particularly fluctuating concentrations of exogenous metabolic substrates.

3.1. Background

Quality control systems that effectively identify toxicity in culture media and examine the robustness of clinical procedures contribute significantly to the success of *in vitro* fertilisation (IVF). Although deemed to be the gold standard for quality assessment (Mestres *et al.*, 2019), the mouse embryo assay (MEA) is challenging for both ethical and scientific reasons. To this end, investigators have resorted to using somatic cell lines such as HeLa cells, mouse hybridoma and neuroblastoma cells, and human fibroblast and sarcoma cells (Bertheussen *et al.*, 1989; Vijayasankaran *et al.*, 2010; Kastl *et al.*, 2017) to assess media quality. Two dimensional (2D) cell cultures represent both a well-established method, and a convenient *in vitro* model for studying the physiology and biochemistry of mammalian cells (Antoni *et al.*, 2015). Their use typically involves growing adherent eukaryotic cells that form a monolayer of attached cells, with a nutrient rich medium as a source of nutrition, and incubation at body temperature (37°C). Incubation at a temperature of 37°C and a relative humidity of 95% supports cell metabolism through enzyme activation, and prevents increased osmolality caused by medium evaporation and condensation (Triaud *et al.*, 2003; Chatterjee *et al.*, 2015). Gas exchange is delicately maintained through the relationship between sodium carbonate ($NaHCO_3$) in culture media and CO_2 in the incubator, which is conventionally set to 5 – 6% (Potter and DeMarse, 2001). Successively, intracellular pH is controlled by reversible reactions catalysed by carbonic anhydrases, and gaseous CO_2 reacts with H_2O to form H_2CO_3 which rapidly disassociates into HCO_3^- and H^+ ions (Figure 3.1.)(Auslä nder *et al.*, 2014). Should a homeostatic defect occur, the adverse effects of elevated CO_2 on mammalian cells include inhibited growth and nutrient utilisation, alterations in cell metabolism and protein

production, and aberrant protein processing (DeZengotita *et al*. 1998; Kimura and Miller 1996; Pattison *et al*. 2000).

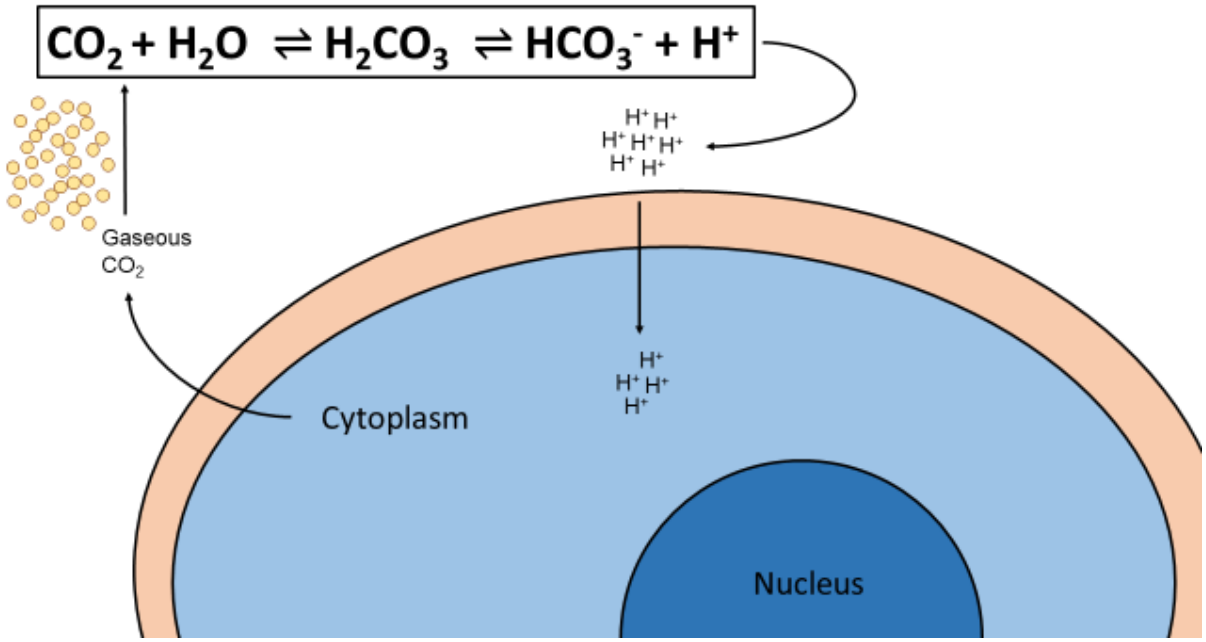


Figure 3.1. Dissolved CO_2 in an equilibrium with H^+ and HCO_3^-. Intracellular pH is delicately maintained through reversible reactions catalysed by carbonic anhydrases, and CO_2 reacts with H_2O to form H_2CO_3 which rapidly disassociates into HCO_3^- and H^+ ions. Base addition causes the equilibrium reaction to be driven farther to the right, increasing intracellular osmolality with CO_2 whilst acid addition drives the reaction to left, resulting in increased protein damage from elevated H^+ levels.

Over the last century, cell culture technology has been of fundamental importance to the pharmaceutical industry, where drug efficacy and toxicity are routinely assessed using mammalian cells. They are also widely-used in the IVF industry where success rates are directly linked to culture conditions (Breslin and O'Driscoll, 2013; Antoni *et al.*, 2015; Souza *et al.*, 2018). Culture medium is undoubtedly one of the most important features of cell culture technology and through a synergistic relationship, its development depends on 2D assays to indicate specific nutritional needs of different cell types. For instance, George O. Gey established the widely used HeLa cell line from a tissue of a patient with uterine cervical cancer (Strong *et al.*, 2014), and its emergence meant it was no longer necessary to obtain cells from tissues, but instead, that assays could be performed using the same homogenous population of cells, thereby allowing the differences in the effects of various media formulations to be

precisely quantified (Yao and Asayama, 2017). Alternatively, following successful growth of Chinese hamster ovary (CHO) cells in synthetic F-12 medium in 1965 (Ham, 1965), its components were used to develop chemically defined Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) medium, which is the most widely used medium for cell culture today (Kuwae et al. 2018).

Improvements in cell culture techniques and the refinement of processes for culture medium production has led to the development of a disciplined devoted to the design of optimised assays for detecting compounds that can be used as potential targets in therapeutics (Oldenburg *et al.,* 1998 and Stevens *et al.*, 1998). In a typical experiment, samples are assayed alongside reference controls that indicate cell viability at two extremes of the activity range. The negative control (sometimes referred to as the background) represents cellular activity under standard conditions, whilst the positive control demonstrates a known response as a result of a specific change (i.e., cell death). Prior to use at an industrial-scale, cell-based assays are assessed for robustness, sensitivity and accuracy to ensure results draw meaningful conclusions that are repeatable and reliable (Sittampalam *et al.*, 1997). The Z' factor provides a measure of assay robustness using statistics that model the distribution of variables within a particular dataset (Zhang, Chung and Oldenburg, 1999). By taking the dynamic range into account (the difference between the negative and positive controls), as well as the variation associated with a specific dataset and its reference controls, the Z' factor indicates the sensitivity of assays to changes in data variability. With optimised methods and tight control of data variability, assays with narrow dynamic ranges yield satisfactory Z' factors and are subsequently regarded as high-quality assays.

Given the differences between cell types, and the high variability inherent with the use of 2D cell culture systems (Rebuzzini *et al.*, 2016), and that somatic cell lines do not exhibit

properties of early embryonic development (such as differentiation) (Bertheussen *et al.*, 1989), the development of an embryonic carcinoma cell-based assay for assessing the impact of suboptimal culture conditions would, for the first time, provide a suitable tool for quality control and product research in assisted reproduction.

With this in mind,

The specific objectives of this chapter were to:

1) Design and optimise a 2D cell-based assay for identifying markers that can be changed to improve the quality of human blastocyst culture media;

2) Define an acceptable dynamic range for undertaking a systematic screen of components within human blastocyst culture media, to create novel methods that can be used to validate human blastocyst culture media;

3) Test the hypothesis that a cell-based assay can detect common perturbations in culture conditions including changes in osmolality and energy substrate availability.

3.2. Materials and methods

Negative and positive controls were established as described in section 2.3.1, and the resazurin concentration and initial seeding density for P19 embryonal carcinoma cells were simultaneously optimised as described in section 2.4.2. The Z' factor was determined to quantify the quality of the proposed cell-based assay as described in section 2.5.2.1 and a suitable Z' factor was obtained (section 3.3.1.). Assays and test media were subsequently prepared as per section 2.6.1 and 2.7 respectively, and P19 cells were treated with the following options from Table 2.1 (human embryo culture media, increasing media osmolality, absence of energy sources, mono and dipeptide glutamine, absence of energy sources and supplementation of vitamins). The proportion of live cells were determined as described in section 2.4.2, and the data obtained were analysed as per section 2.10.

3.3. Results

3.3.1. Determination of a positive control

Treatment with increasing concentrations of DMSO demonstrated its cytotoxic effects and subsequent contributions to cell damage and reduced cell proliferation. Active cells convert MTT into purple formazan crystals that are dissolved in isopropanol, so the colour intensity of the dissolved crystals is an indication of cell viability. Average fold change values (the difference between the no DMSO treatment and each consecutive DMSO treatment) also indicated an increasing difference between the no DMSO and increasing DMSO treatments, as a result of decreasing optical densities from absorbance readings (Figure 3.2). As shown in Table 3.1 and Figure 3.2, treatment with 25% DMSO was found to be the lowest concentration to cause ≤70% cell death and was therefore identified as a suitable positive control. An arbitrary survival rate of ≤30% for the positive control was deemed suitable for assays of this nature.

Table 3.1. The cytotoxic effects of increasing DMSO concentrations on P19 embryonal carcinoma cells. Shown are the mean and standard deviation absorbance values obtained from treatment of P19 cells with increasing DMSO concentrations for 18 hours. Percentage cell viability was calculated under the assumption that there was no cell death with the 0% DMSO treatment. Formazan crystal formation showed that > 50% of cell viability was lost with the 20% DMSO treatment, and although significantly reduced, there was a degree of cell survival with the 100% DMSO treatment.

DMSO (% V/V)	Absorbance (OD_{540}) (Mean ± SD)	Proportion of live cells (%)
0%	0.780 ± 0.423	100.0
5%	0.677 ± 0.319	87
10%	0.592 ± 0.269	76
15%	0.380 ± 0.153	49
20%	0.260 ± 0.082	33
25%	0.225 ± 0.073	29
30%	0.222 ± 0.070	28
35%	0.207 ± 0.068	27
40%	0.192 ± 0.052	25
100%	0.149 ± 0.039	19

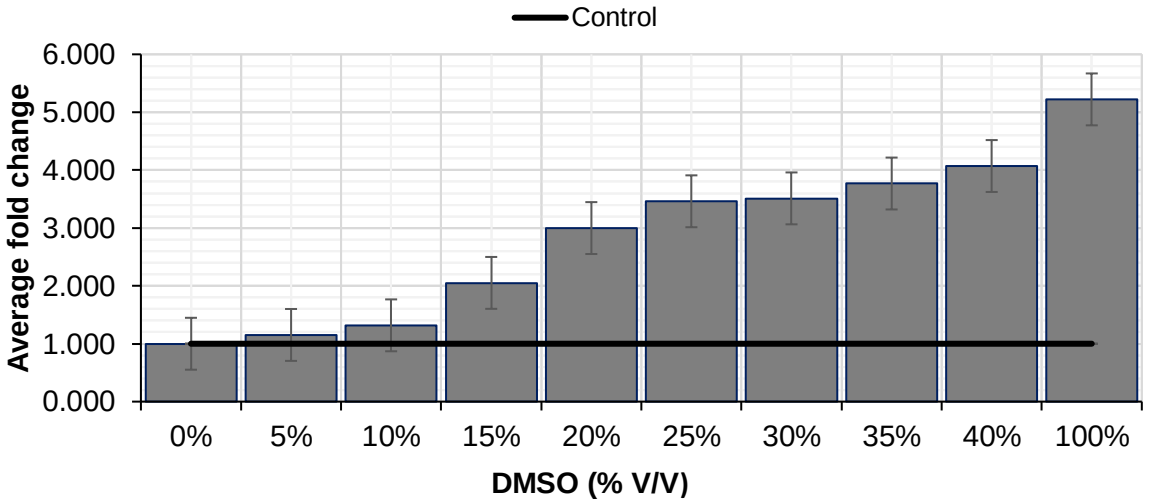


Figure 3.2. The cytotoxic effects of increasing DMSO concentrations on P19 embryonal carcinoma cells. Shown are the average fold change values (the difference between each treatment and the 0% DMSO treatment) following treatment of P19 cells with increasing DMSO concentrations for 18 hours. The proportion of live cells decreased with increasing concentrations of DMSO, resulting in an increasing difference between the no DMSO treatment and increasing DMSO concentrations.

3.3.2. Resazurin assay

The colour change from blue to fluorescent pink occurred at a faster rate with the 40 µM and 80 µM resazurin treatments; however, hydroresorufin build-up was less likely to occur with the 160 µM and 350 µM resazurin treatments (see section 1.7), as evidenced by the less drastic reduction in average fold change (Figure 3.3). The average fold change was calculated by dividing the average fluorescence of live cells (αMEM treatment) by that of dead cells (25% DMSO treatment), and the values in green are indicative of a large window between the negative and positive controls whereas the values in red represent a smaller difference between controls. A one hour incubation period with resazurin was required before a noticeable fold change was observed at all concentrations, and higher resazurin concentrations (160 µM and 350 µM) required extended incubation periods before a noticeable colour change was observed. Wells seeded with $1.0x10^5$ cells produced a significant fold change with all resazurin concentrations; however, incubation with 40 µM and 80 µM resazurin showed that P19 cells rapidly metabolise resazurin to resorufin, resulting in further reduction to hydroresorufin and a subsequent reduction in average fold change. Wells

seeded with $2.0x10^5$ cells consistently produced a relatively low average fold change, suggesting a narrow window between control treatments, caused by cellular activity within the positive control at this density. This suggests that treatment with 25% DMSO does not cause sufficient cell death when the seeding density is above $1.0x10^5$ cells. Overall, the 160 μM treatment produced a high fold change and hydroresorufin build-up was less likely to occur as shown by the less drastic reduction in fold change (Figure 3.3). It was subsequently deemed as a suitable resazurin concentration for subsequent assays.

40 µM

Time	2.5 x 10^4	5.0 x 10^4	1.0 x 10^5	2.0 x 10^5
0	1.00	1.10	1.26	1.32
15	1.30	1.56	1.83	2.06
30	1.71	2.10	2.61	2.53
45	2.05	2.58	3.22	3.00
60	2.60	3.23	3.85	3.50
90	3.28	3.81	4.31	3.83
120	4.06	4.26	4.78	4.01
150	4.48	4.65	4.98	4.02
180	4.63	4.71	4.91	3.86
210	4.72	4.53	4.64	3.63
240	4.65	4.16	4.36	3.39
270	4.46	3.86	3.98	3.10
300	4.35	3.71	3.78	2.91

80 µM

Time	2.5 x 10^4	5.0 x 10^4	1.0 x 10^5	2.0 x 10^5
0	0.96	1.19	1.37	1.26
15	1.38	1.78	2.36	2.29
30	1.99	2.34	3.20	3.53
45	2.45	3.15	3.84	4.25
60	3.23	3.95	4.51	4.65
90	4.11	4.82	5.16	4.95
120	5.02	5.81	5.78	5.12
150	5.57	6.03	5.87	5.18
180	6.05	6.02	5.78	5.16
210	6.16	5.73	5.38	4.96
240	6.02	5.26	4.98	4.55
270	5.63	4.73	4.46	4.04
300	5.24	4.37	4.04	3.65

160 µM

Time	2.5 x 10^4	5.0 x 10^4	1.0 x 10^5	2.0 x 10^5
0	0.97	1.21	1.35	1.42
15	1.46	1.86	2.21	2.39
30	2.09	2.73	3.13	3.15
45	2.73	3.61	3.94	4.07
60	3.54	4.32	4.88	4.86
90	4.72	5.57	6.08	5.75
120	5.74	6.72	7.21	6.25
150	6.20	7.29	7.70	6.32
180	6.08	7.62	8.00	6.04
210	5.81	7.26	7.81	5.80
240	5.61	6.61	7.16	5.35
270	5.25	6.15	6.71	4.96
300	4.99	5.98	6.53	4.59

350 µM

Time	2.5 x 10^4	5.0 x 10^4	1.0 x 10^5	2.0 x 10^5
0	1.02	1.34	1.45	1.58
15	1.80	2.19	2.37	2.56
30	2.64	3.37	3.35	3.96
45	3.33	4.50	5.39	5.48
60	4.07	5.37	6.29	6.59
90	4.93	6.56	7.68	7.28
120	5.58	7.00	8.26	7.69
150	6.32	7.34	8.87	8.12
180	6.66	8.09	9.06	8.13
210	6.88	8.43	9.13	8.09
240	6.98	8.51	9.29	7.92
270	7.01	8.30	9.15	7.81
300	6.96	8.17	8.98	7.50

Figure 3.3. Optimisation of resazurin concentration and cell density. P19 embryonal carcinoma cells were seeded at increasing densities and treated with complete αMEM or 25% DMSO for two hours. Resazurin reduction was observed and measured over the course of five hours because prolonged incubation has detrimental effects on cell health. The average fold change was calculated by dividing the average fluorescence of live cells (negative control; αMEM) by that of dead cells (positive control; 25% DMSO). The values in green are indicative of a large window between the negative and positive controls whilst the values in red represent a smaller difference between controls. Wells seeded with $1.0x10^5$ cells produced a high fold change with all resazurin concentrations but incubation with 40 µM and 80 µM resazurin indicated that P19 cells rapidly metabolise resazurin to resorufin, resulting in further reduction to hydroresorufin and a subsequent reduction in average fold change.

3.3.3 Z' Evaluation

Fluorescence and absorbance values, and the formula below (equation 1) were used to determine the Z' factor of the negative and positive controls used in the assays described in this chapter (Table 3.2).

(1) $1 - \frac{3\delta\rho + 3\sigma\eta}{I\mu\rho - \mu\eta I}$ (σ = standard deviation, μ = mean, ρ = positive control, η = negative control)

A

	1	2	3	4	5	6	7	8	9	10	11	12
A	113885	115934	115799	114155	113156	114097	4856	4823	4743	4735	4609	4622
B	111453	114710	114435	115103	115292	114971	5568	5366	5077	4992	4987	4691
C	108349	111779	111631	113518	112788	112347	5008	4891	4914	4750	4579	4550
D	110378	112285	111746	111382	110930	114136	4796	4731	4607	4380	4519	4763
E	4614	4946	4954	4782	4859	5120	114248	116289	115265	115944	115121	102138
F	4970	4508	4674	4720	4722	4967	113143	113204	113805	114659	113248	110992
G	4362	4803	4881	4571	4620	4667	112325	112900	113287	114377	113658	115311
H	4399	4595	4696	4795	4840	4865	113282	113766	110033	110053	111653	109316

B

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.346	1.478	1.287	1.303	1.274	1.420	0.175	0.154	0.201	0.108	0.199	0.200
B	1.106	1.497	1.014	1.511	2.389	1.436	0.126	0.120	0.172	0.190	0.220	0.172
C	0.906	1.079	1.035	2.147	2.317	1.900	0.177	0.163	0.159	0.190	0.151	0.198
D	1.036	1.643	2.227	2.383	1.768	1.458	0.169	0.117	0.155	0.177	0.169	0.180
E	0.170	0.209	0.165	0.232	0.123	0.172	2.124	2.282	1.857	1.571	1.456	1.621
F	0.122	0.123	0.111	0.197	0.171	0.108	1.446	1.383	1.331	1.111	1.183	1.553
G	0.106	0.089	0.180	0.141	0.133	0.241	2.357	1.980	1.798	1.189	1.073	1.282
H	0.194	0.161	0.203	0.118	0.084	0.201	1.310	1.272	1.308	1.328	1.354	1.188

Figure 3.4. Resazurin (resorufin) fluorescence readings of Z' assays after 24 hours of incubation with 25% DMSO and αMEM, and a further four hours of incubation with 160 µM resazurin solution (A) and absorbance readings after two hours of incubation with 5 mg/ml MTT(B). Values shaded in blue are indicative of wells treated with 25% DMSO (positive control) and those shaded in pink represent treatment with complete αMEM (negative control).

Table 3.2. Z' factor of MTT and resazurin assays. P19 cells were treated with 25% DMSO and complete αMEM for 24 hours. The resazurin assay was observed every hour over a four hour period, and one end-point reading was determined from the MTT assay.

Cell Viability Method	Treatment Incubation Period	Z' Factor
Resazurin	24 hours	0.883 (1 hour) 0.923 (2 hours) 0.926 (3 hours) 0.926 (4 hours)
MTT	24 hours	0.100 (N/A)

The Z' values obtained from the resazurin assay (> 0.5) indicated that complete αMEM and 25% DMSO were suitable controls for P19 cell-based assays that require a treatment period of 24 hours or more. It was determined that the MTT was not an appropriate viability test for an assay of this kind, as the Z' factor indicated low robustness (> 0.5), hence making it unsuitable for high throughput screening.

3.3.4. P19 embryonal carcinoma cells respond differently to various types of human embryo culture media

Treatment with different types of human IVF culture media indicated that P19 cells respond to variation in media composition and grow best in fertilisation media and oocyte retrieval buffer, whilst cell viability was significantly compromised in cleavage media after 48 hours of standard incubation (Figure 3.5 top panel, $F = 41.08$, $df = 3$, $p < 0.001$). *Post hoc* comparison indicated significant differences (Tukey HSD, $p < 0.001$) between treatments, but not between the oocyte retrieval buffer and blastocyst media treatments ($p = 0.98$). Assessed by resorufin fluorescence values, growth and proliferation reduced with extended incubation (96 hours), however, cells responded to the various media formulations (Figure 3.5 bottom panel, $F = 98.08$, $df = 3$, $p < 0.001$), with *post hoc* comparisons indicating significant differences between

treatments (Tukey HSD, $p < 0.001$), except the oocyte retrieval buffer and cleavage media treatments ($p = 0.25$).

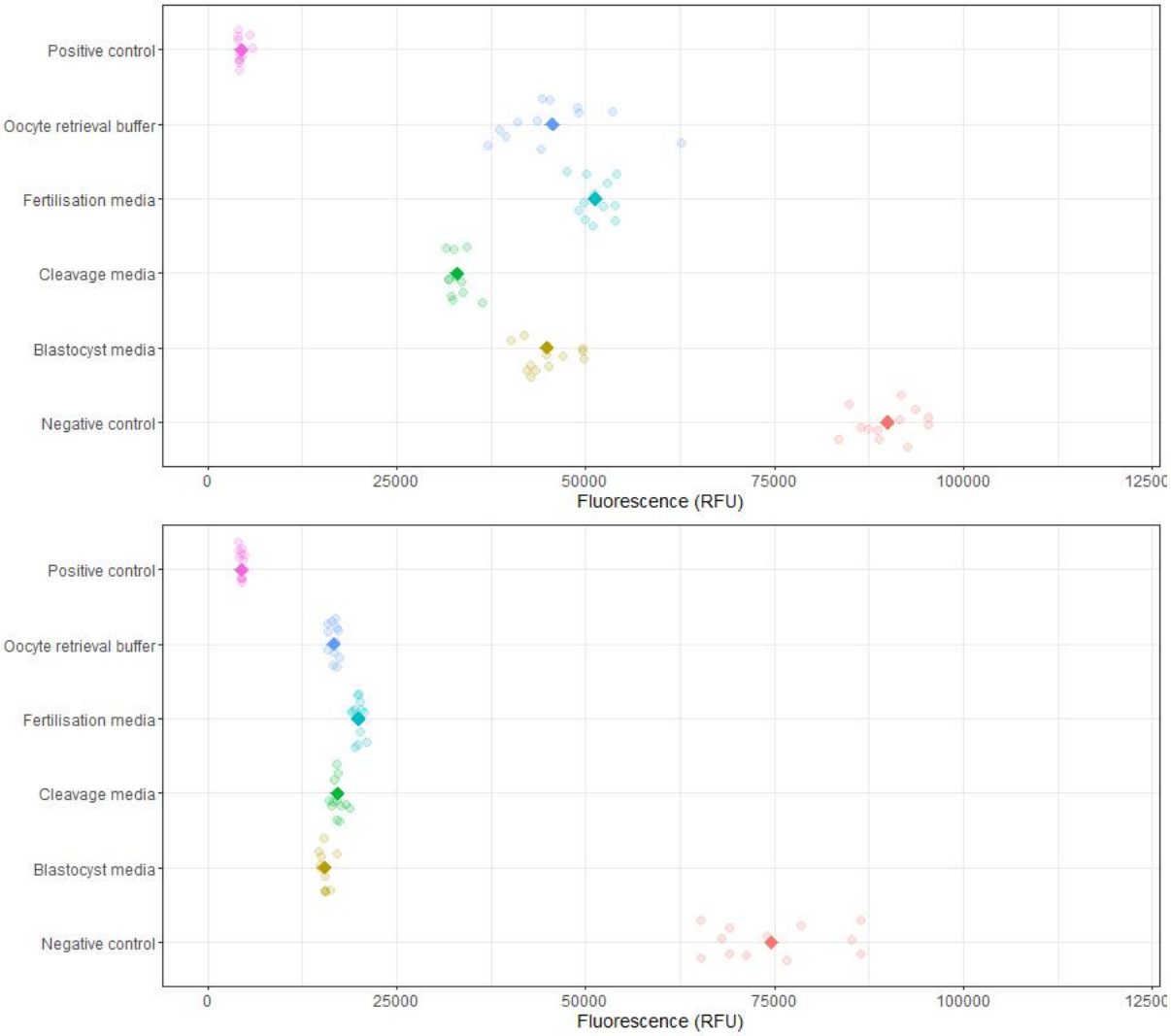


Figure 3.5. P19 embryonal carcinoma cells respond to differently to various types of human embryo culture media. Resorufin fluorescence values for replicate wells of P19 cells treated with human oocyte retrieval buffer, fertilisation medium, cleavage medium and blastocyst medium for 48 (top) and 96 hours (bottom). Complete αMEM was used as a negative control and 25% DMSO was used as a positive control. The diamond symbols show the mean value for each group. Pairwise comparisons indicate significant differences between fluorescence values obtained from the various treatments after 48 hours, except oocyte retrieval buffer and blastocyst media treatments ($p = 0.975$, *post hoc* test), and significant differences between treatments after 96 hours, except oocyte retrieval buffer and fertilisation media treatments ($p = 0.253$, *post hoc* test).

3.3.5. P19 embryonal carcinoma cells respond to changes in media osmolality

Treatment with increasing concentrations of sodium chloride in blastocyst media indicated that P19 cells respond to the toxic effects of high osmolality (Figure 3.6 top panel, $F = 589.9$, $df = 8$, $p < 0.001$), with *post hoc* comparisons indicating no significant differences (Tukey HSD, $p = 0.997$) between cell death (positive control treatment) and the various additional sodium chloride treatments (125 mM to 500 mM). Furthermore, P19 cells responded to low increments in sodium chloride (Figure 3.6 bottom panel, $F = 51.15$, $df = 5$, $p < 0.001$), with *post hoc* comparisons indicating significant differences (Tukey HSD, $p < 0.001$) between cell death and treatment with ≤ 0.0000125 mM, but not ≥ 0.00001875 mM ($p = 0.994$). It is also clear that although treatment with 0.000009375 mM and 0.0000125 mM did not cause cell death, growth and proliferation were significantly compromised ($p < 0.001$ for *post hoc* comparison with no additional sodium chloride treatment).

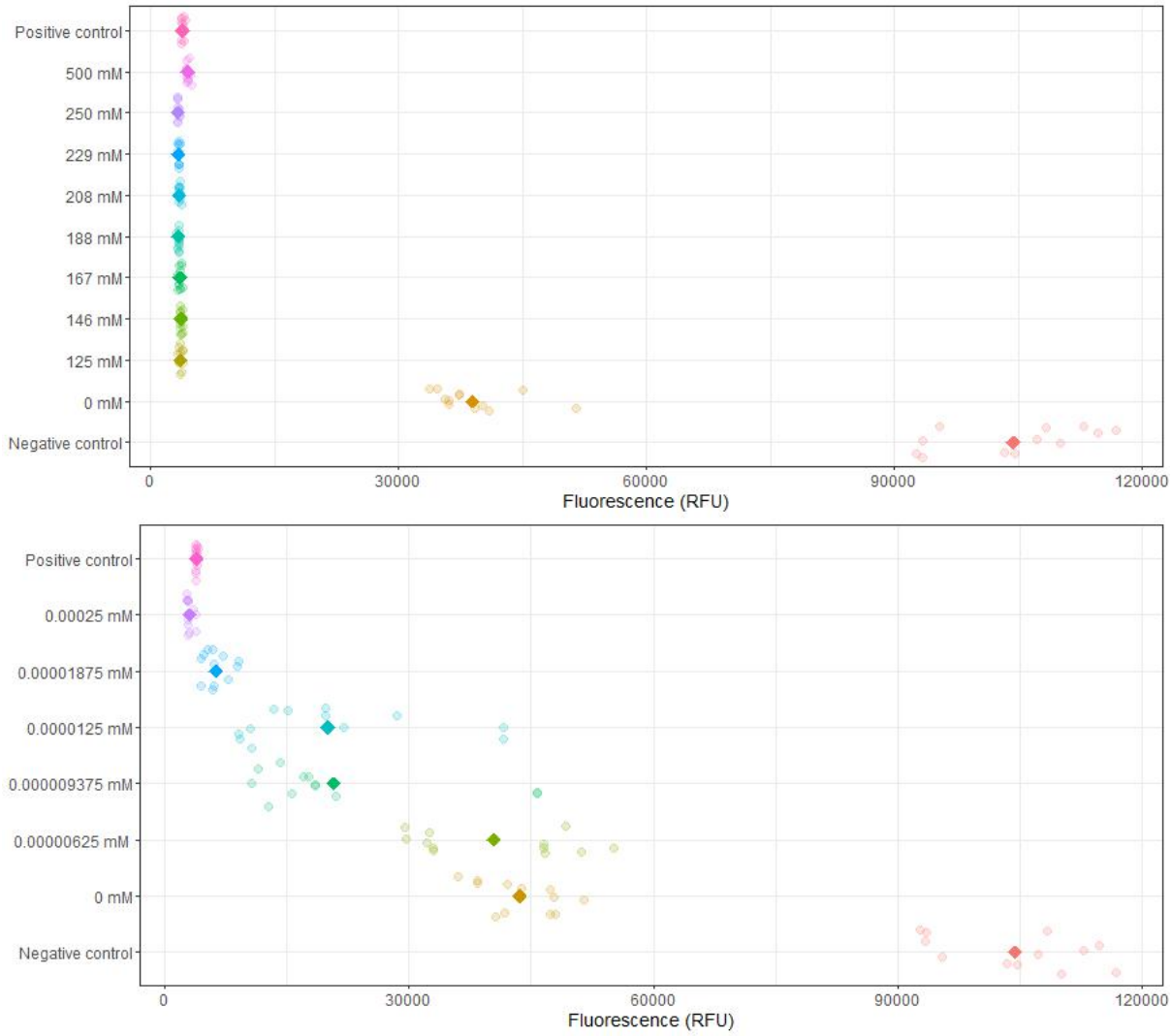


Figure 3.6. P19 embryonal carcinoma cells respond to changes in media osmolality. Resorufin fluorescence values for replicate wells of P19 cells treated with blastocyst media supplemented with increasing concentrations of sodium chloride (500 mM to 125 mM (top) and 0.00025 mM to 0.00000625 mM (bottom)). Complete αMEM was used as a negative control and 25% DMSO was used as a positive control. The diamond symbols show the mean value for each group. Assessed by resorufin fluorescence, increased sodium chloride concentration caused significant cell death or reduced cell growth, indicative of the sensitivity of P19 cells to changes in osmolality. Cellular response to treatment with 125 mM and 0.00025 mM sodium chloride was also assessed and found to cause significant cell death. Pairwise comparisons indicate that the fluorescence values from all groups differed significantly from that obtained from treatment with no additional sodium chloride ($p < 0.001$, *post hoc* test), except for the 0.00000625 mM treatment.

3.3.6. P19 embryonal carcinoma cells respond to the elimination of energy substrates but not to changes in glutamine composition.

Removal of different energy sources indicated that P19 cells respond to the absence of calcium lactate and glutamine, but not to the removal of pyruvate (Figure 3.7 top panel, $F = 151.6$, $df = 4$, $p < 0.001$), with *post hoc* comparisons indicating significant differences between

complete blastocyst media and the no calcium lactate and no glutamine treatments (Tukey HSD, $p < 0.05$ for both), but not with the no pyruvate treatment ($p = 0.1689$). Conversely, independent repeat experiments suggested that P19 cells do not respond to the removal of glutamine (Figure 3.7 centre panel, F = 16.77, $df = 4$, $p < 0.001$; Figure 3.7.bottom panel, F = 31.52, $df = 4$, $p < 0.001$), with *post hoc* comparisons indicating no significant differences between complete blastocyst media and the no glutamine treatments (Tukey HSD, $p > 0.05$). This indicates that P19 cells may not consistently respond to glutamine in the same manner. As expected, given the effect of removing calcium lactate, the removal of all three energy sources also reduced cell growth as assessed by resorufin fluorescence values (Tukey HSD, $p < 0.05$ for *post hoc* comparison with the blastocyst media). Response to the removal of pyruvate, calcium lactate and all three energy sources remained consistent between repeat experiments, with *post hoc* comparisons indicating no significant differences (Tukey HSD, $p > 0.05$) between complete blastocyst media and the removal of pyruvate, and also between the positive control (cell death) and cells lacking pyruvate, glutamine and calcium lactate.

Media supplementation with mono and dipeptide glutamine showed that P19 cells do not respond to changes in the arrangement of glutamine molecules (Figure 3.8 top panel, F = 9.388, $df = 3$, $p < 0.001$), with *post hoc* comparison indicating no significant differences between L-glutamine and alanyl glutamine treatments (Tukey HSD, $p = 0.156$). These responses differed significantly from that of treatment with no glutamine ($p > 0.05$ for both); however, the alanyl glutamine treatment differed significantly from the gems blastocyst media treatment ($p < 0.05$) whilst the L-glutamine treatment did not ($p = 0.156$). Given that gems blastocyst media and the alanyl glutamine media are made from the same components, differences between the two treatments is indicative of increased cell viability with freshly-made media, as opposed to commercially-manufactured media. This notion is reinforced by *post hoc* comparison of gems blastocyst media and the no glutamine treatment, which demonstrated comparable cellular responses (Tukey HSD, $p = 0.234$). Independent repeat

experiments confirmed that cellular response to L-glutamine does not differ from that of alanyl-glutamine (Figure 3.8 centre panel, F = 43.80, *df* = 3, $p < 0.001$; Figure 3.4 bottom panel, F = 49.15, *df* = 3, $p < 0.001$), with *post hoc* comparison indicating no significant differences (Tukey HSD, $p > 0.05$) between L-glutamine and alanyl glutamine treatments. Furthermore, response to gems blastocyst media differed significantly from that of L-glutamine treatment, but not alanyl glutamine, indicating that cells respond differently to various batches of blastocyst media. All treatments differed significantly (Tukey HSD, $p < 0.05$) from no glutamine treatments, indicating that P19 cells respond to the removal of glutamine with some variation between batches of media.

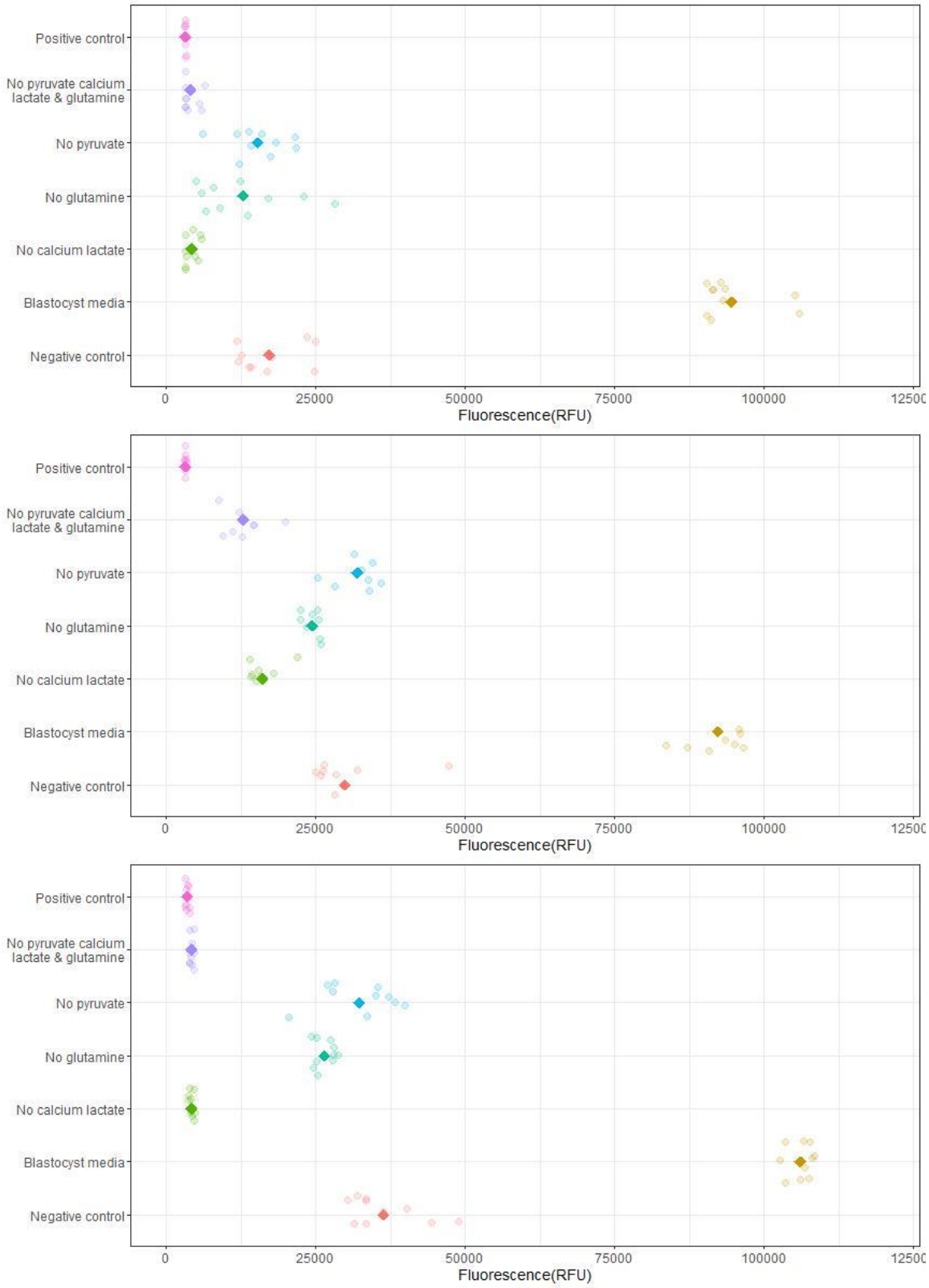


Figure 3.7. P19 embryonal carcinoma cells respond to the elimination of energy substrates. Resorufin fluorescence values for replicate wells of P19 cells treated with complete blastocyst media, blastocyst media without: glutamine; pyruvate; calcium lactate; pyruvate, calcium lactate and glutamine for 48 hours, through three independent experiments. Complete αMEM was used as a negative control and 25% DMSO was used as a positive control. The diamond symbol shows the mean value for each group. Pairwise comparisons indicate that the fluorescence values from all groups, except for the no calcium lactate treatment, differ significantly from that obtained from cells lacking pyruvate, calcium lactate and glutamine ($p < 0.05$, *post hoc* tests).

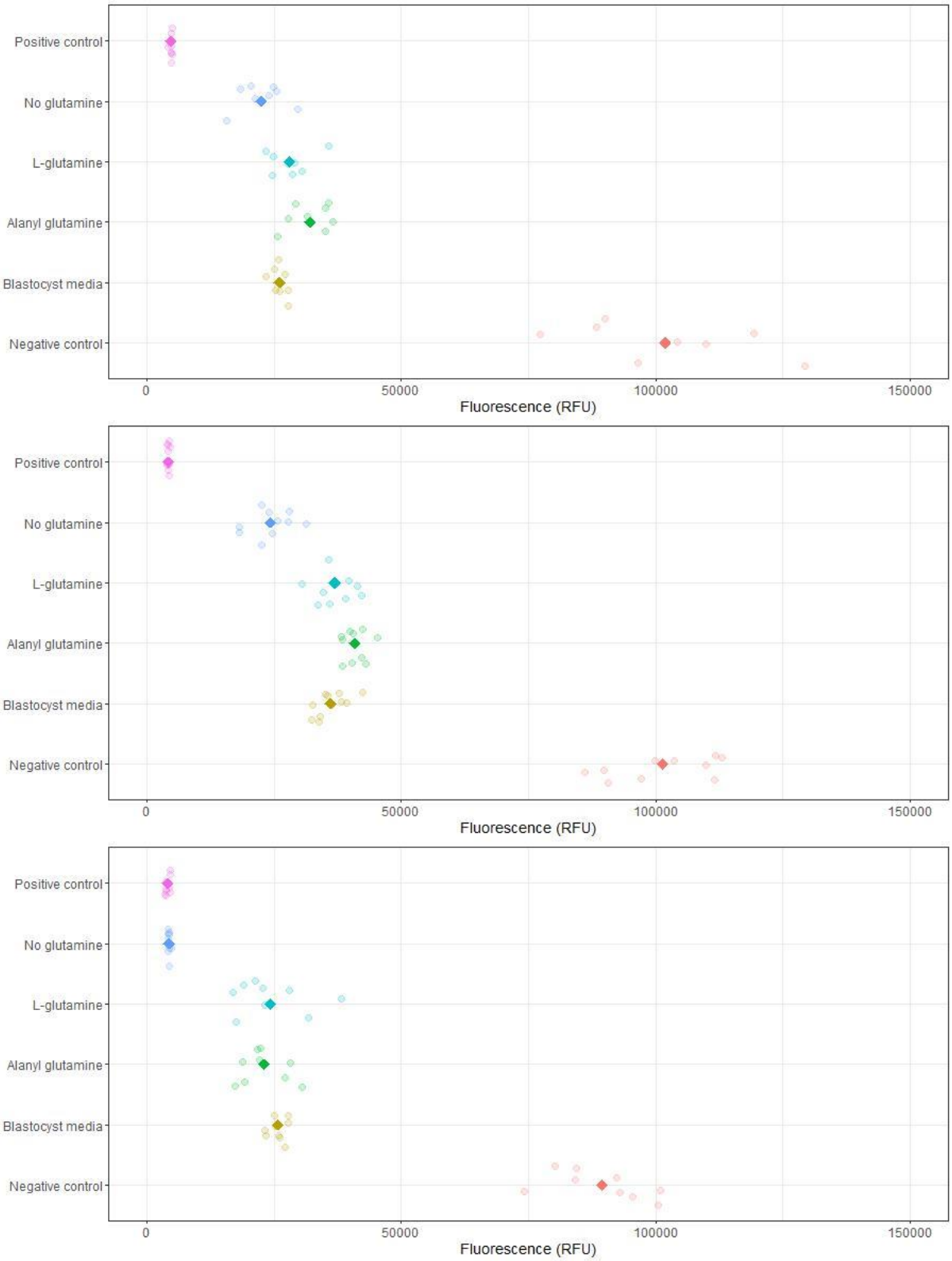


Figure 3.8. P19 embryonal carcinoma cells do not respond to changes in glutamine composition. Resorufin values for replicate wells of P19 cells treated with, Gems blastocyst media, blastocyst media containing L-glutamine or alanyl glutamine and blastocyst media without glutamine for 48 hours. Complete αMEM was used as a negative control and 25% DMSO was used as a positive control. The diamond symbols show the mean value for each group. Pairwise comparisons indicate that though the mean fluorescence of L-glutamine and alanyl glutamine treatments do not differ significantly ($p > 0.05$, *post hoc* test), both treatments differ significantly from that obtained from treatments with no glutamine ($p < 0.001$, *post hoc* test).

3.3.7. P19 embryonal carcinoma cells respond to differences between aged and freshly made culture media.

Treatment with expired gems media showed that aged media does not impact cell viability (Figure 3.9, F = 0.398, *df* = 4, *p* = 0.53), with *post hoc* comparisons indicating no significant difference (Tukey HSD, *p* > 0.05) between the response to aged and within date cleavage media, oocyte retrieval buffer, and blastocyst media.

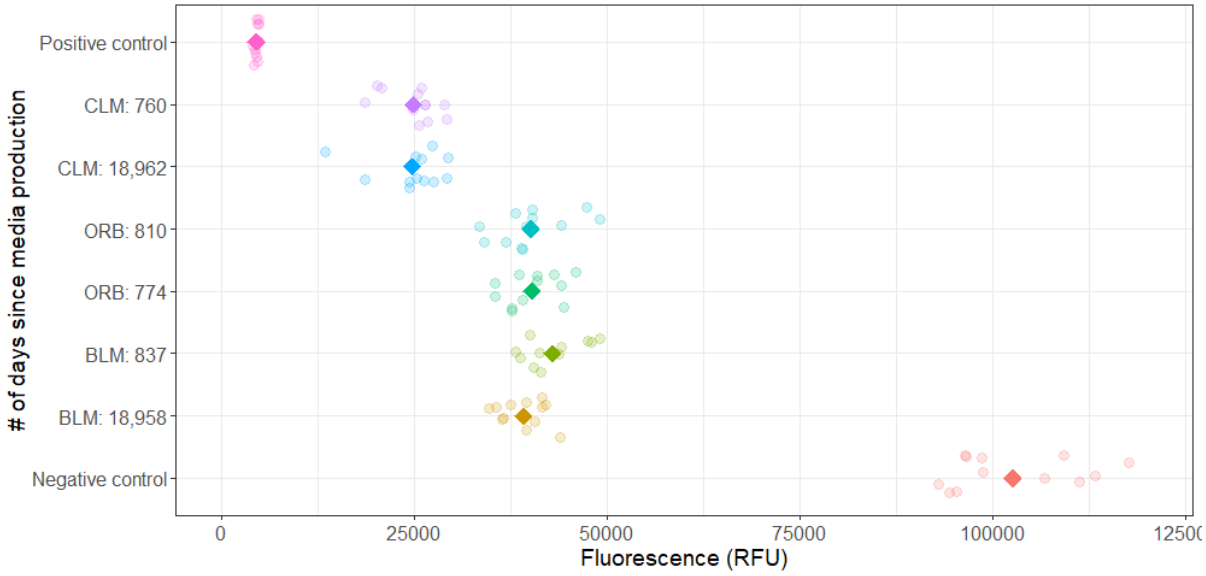


Figure 3.9. P19 embryonal carcinoma cells respond to differences between aged and freshly made culture media. Resorufin values for replicate wells of P19 cells treated with expired and within date cleavage media, oocyte retrieval buffer and blastocyst media for 48 hours. Complete αMEM was used as a negative control and 25% DMSO was used as a positive control. Pairwise comparisons indicate that the fluorescence values obtained from treatment with expired media did not differ significantly from that of within date media (*p* > 0.05, *post hoc* test for all treatments).

3.3.8. The effects of increased vitamin concentrations do not mask the response of P19 embryonal carcinoma cells to the elimination of energy substrates

Treatment with increasing doses of sodium L-ascorbate, folic acid and cobalamin showed that P19 cell growth is not affected by increases in vitamin availability (Figure 3.10, F = 4.267, *df* = 4, *p* = 0.0045), with *post hoc* comparisons indicating no significant differences (Tukey HSD, *p* > 0.05) between treatments, except the no vitamins and 3.0 dosage treatments. Conversely, the removal of pyruvate, glutamine and calcium lactate and subsequent supplementation with

a double dose of vitamins suggested that cellular response to energy substrate availability is masked by the effects of increased vitamin concentrations (Figure 3.11 top panel, F = 16.38, *df* = 1, $p < 0.001$), with *post hoc* comparisons indicating significant differences (Tukey HSD, $p < 0.05$) between complete blastocyst media and all increased vitamin dosage treatments. The no pyruvate and no glutamine treatments differed significantly ($p < 0.05$) from their increased vitamin counterparts, whereas the no calcium lactate treatment did not ($p > 0.05$), highlighting the ineffectiveness of vitamins in controlling cell death caused by the removal of calcium lactate. Consequently, the effect of removing all three energy sources was not supressed by increased vitamin availability. Independent repeat experiments indicated that increased vitamin concentrations do not interfere with cellular response to the removal of energy sources (Figure 3.11 middle panel, F = 0.04, *df* = 1, $p = 0.84$; Figure 3.7 bottom panel, F = 0.87, *df* = 1, $p = 0.35$), with *post hoc* comparisons indicating significant differences (Tukey HSD, $p > 0.05$) between complete blastocyst media (standard dose of vitamins) and the no calcium lactate treatment, but not the no pyruvate and no glutamine treatments ($p > 0.05$ for both), with and without a double dose of vitamins. There were no significant differences between treatments and their increased vitamin counterparts, indicating that vitamins are unable to increase cell growth in the absence of energy sources.

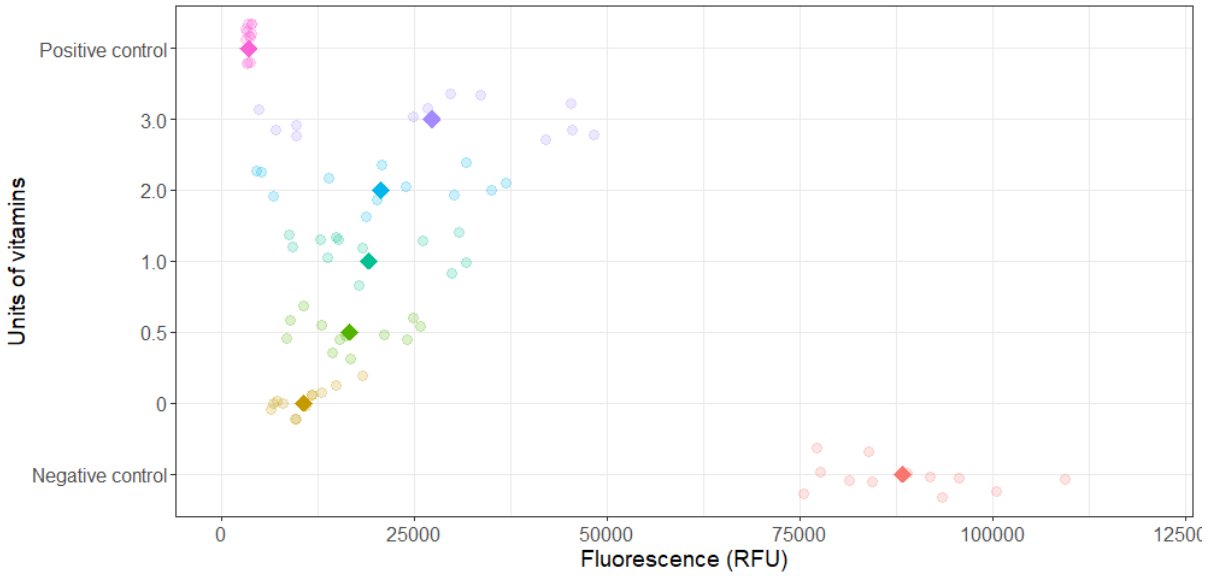


Figure 3.10. The effects of increased vitamin concentrations on P19 embryonal carcinoma cell growth. Resorufin values for replicate wells of P19 cells treated with increasing doses of sodium L-ascorbate, folic acid and cobalamin for 48 hours. Complete αMEM was used as a negative control and 25% DMSO was used as a positive control. The diamond symbols show the mean value for each group. Pairwise comparisons indicate that P19 cell growth was significantly affected by treatment with supplemented vitamins only when it exceeded three units ($p < 0.001$, *post hoc* comparison to the no vitamins treatment); otherwise, P19 cells did not respond to additional doses of vitamin ($p > 0.05$, *post hoc* comparison to the no vitamins treatment).

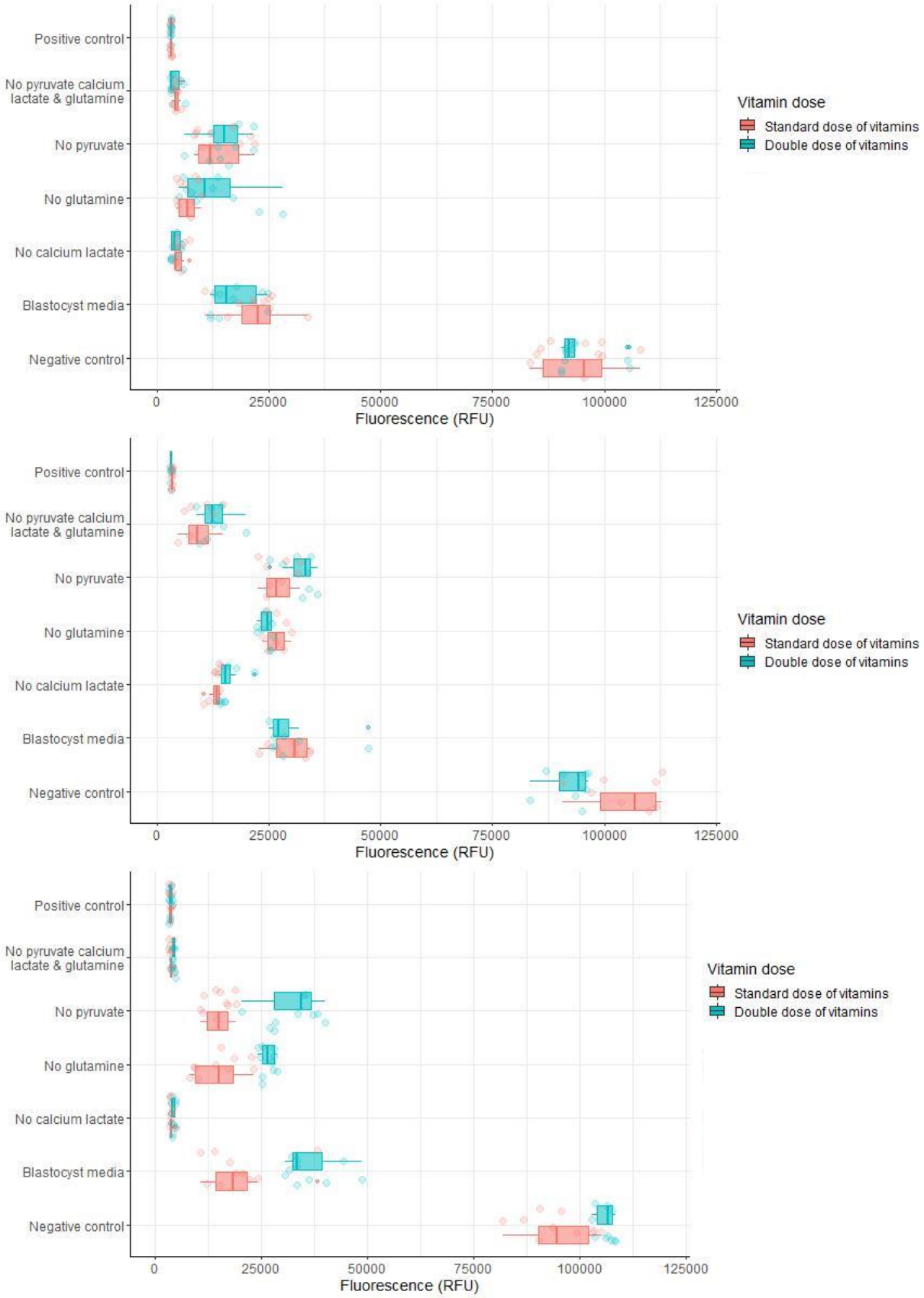


Figure 3.11. The effects of increased vitamin concentrations do not mask the response of P19 embryonal carcinoma cells to the elimination of energy substrates. Boxplot of resorufin fluorescence values for replicate wells of P19 cells treated with increasing doses of sodium L-ascorbate,

folic acid and cobalamin for 48 hours. Cells were also treated with complete blastocyst media, blastocyst media without: glutamine; pyruvate; calcium lactate; pyruvate, calcium lactate and glutamine, with and without a double dose of vitamins for 48 hours. Complete αMEM was used as a negative control and 25% DMSO was used as a positive control. Pairwise comparisons indicate that fluorescence values obtained from treatment with additional vitamins did not differ significantly from that obtained from cells treated with a standard dose of vitamins ($p > 0.05$, *post hoc* tests).

3.4. Discussion

The main objective of this chapter was to investigate the sensitivity of P19 cells to increasing osmolality, energy substrate availability and changes in media composition, given that these are some of the most common perturbations associated with human embryo culture (Davidson *et al.*, 1988; Morbeck *et al.*, 2014; Lin *et al.*, 2017). A positive control was initially established using MTT for viability testing, in order to assess the effects of increasing DMSO concentrations on cellular activity. MTT was chosen because the optimum conditions for its use had been previously established by other studies and no further optimisation was required; therefore, it could be instantly used while other forms of viability testing, specifically resazurin, were optimised (Mosmann, 1983). The absorbance values in Table 3.1 show that P19 cells became increasingly susceptible to cellular damage with increasing DMSO concentrations, resulting in reduced mitochondrial and cytosolic enzyme activity (Yuan *et al.*, 2014). Formazan formation was subsequently reduced, and this was confirmed by significantly reduced optical densities with increasing DMSO concentrations. For studies with the P19 cells, 25% DMSO was considered a suitable positive control because in comparison to the 0% DMSO treatment, near maximal cell death was achieved at this concentration so it would be unnecessary to use a higher concentration of DMSO. Moreover, treatment with 25% DMSO allowed a window of difference between the positive and negative control that was significant enough to determine optimum cell density and resazurin concentration for further experiments. Once negative and positive controls were established, the Z' factor was determined in order to quantify their suitability for assays. Positive and negative controls were evenly spread across the top, bottom, left and right of the plate to reduce to the impact of systematic and random error. Edge effects (the possibility that some areas of the 96-well plate could produce different results due

to faster rates of evaporation, altering cell viability or treatment concentrations), were also taken into account (Zhang *et al.* 1999). The Z' factor obtained for the resazurin-based assay was deemed suitable prior to experimental design and implementation, whereas the MTT-based alternative proved to be an unsuitable means of viability testing for assays, confirming that fluorescence based methods are indeed a more sensitive means of detecting cell viability, than absorbance-based readings (O'Brien *et al.*, 2000).

Over the last two decades, resazurin has been increasingly documented as a viable tool for determining *in vitro* cell proliferation and cytotoxicity. Its reduced form resorufin is measured qualitatively by a visual colour change or quantitatively by fluorescence-based readings and these measurements are recorded in a kinetic manner or as a final reading (O'Brien *et al.*, 2000). To date, no studies have investigated the high throughput screening of IVF media using EC cell lines and resazurin solution, so it was necessary to optimise conditions before use. Current literature refers to the use of various concentrations of resazurin, ranging from 44 µM to 700 µM (Barilli *et al.*, 2008; Borra *et al.*, 2009), whilst other studies use ready-made resazurin kits like Alamar Blue which has a concentration of 440 µM to 560 µM (O'Brien *et al.*, 2000). This chapter examined resazurin activity at various concentrations starting with 40 µM as per previous studies by (Barilli *et al.*, 2008). From the heat maps generated in Figure 3.3, it was established that resazurin requires at least a one hour incubation period before a noticeable difference between the negative and positive control groups were observed. This supports the notion that resazurin reduction to resorufin does not occur instantaneously but rather throughout a specific time frame (O'Brien *et al.*, 2000). The most significant window between the negative and positive control was observed with cells seeded at a density of $1.0x10^5$, and the low fold change observed with a seeding density of $2.0x10^5$ suggests that 25% DMSO was not sufficient in killing cells at such a high density. This could have been validated by the use of an apoptosis assay that detects and quantifies the cellular events associated with programed cell death. Treatment with 40 µM resazurin is suitable for assessing proliferation over three hours with a seeding density of $\geq 1.0x10^4$ because extended

incubation caused a decline in fold change values as resorufin was reversibly reduced to colourless hydroresorufin, thereby interrupting the fluorescence emitted by resorufin. These observations are consistent with findings from the 80 µM treatment, although the fold change values increased with increasing seeding densities. Overall, the average fold change increased with increasing concentrations of resazurin, and resazurin concentrations of 160 µM and above produced increasing fold change values for at least four hours. High concentrations of resazurin can, however, introduce toxicity *in vitro* during prolonged incubation periods (Pace and Burg, 2013); therefore, without compromising the quality of the assay, the lowest concentration of resazurin required should be used. Subsequently, for future studies, a seeding density of $1.0x10^5$/well and 160 µM of resazurin was deemed suitable for producing an adequate fold change whilst allowing an incubation period of at least four hours.

For these assays, blastocyst media was chosen as a suitable test product because of its ability to yield high rates of cell growth, and also because of the versatility of its composition, meaning that it could be manipulated to realise cellular responses to altering levels of various components. The response of P19 cells to the various treatments were compared to that of complete αMEM and 25% DMSO, to quantify the amount of cell growth or death produced by each treatment. P19 cells sensed differences between various types of IVF culture media and responded accordingly. Although cell growth rates were reduced in comparison to the negative control, treatment with the Gems® media suite was not particularly toxic to P19 cells, and significant proliferation was observed. Notably, cleavage media contains low glucose concentrations in comparison to oocyte retrieval buffer, fertilisation and blastocyst media as reflected by the observed reduction in cell viability after 48 hours of incubation. The same observations were made as cleavage media replaced oocyte retrieval buffer and blastocyst media after two hours and 27 hours, respectively, in subsequent experiments. Here, the assay was designed to replicate key features of the mouse embryo assay protocol; hence, cells were cultured in cleavage media for the majority of the 96 hour incubation period, except the fertilisation media treatment which was left unchanged. Although there was significant

variation between the different IVF media treatments, cell viability was considerably reduced as a result of extended incubation with cleavage media.

Next, findings demonstrated similarities between treatment with increased concentrations of sodium chloride and that of 25% DMSO, suggesting that P19 cells are effected by miniscule change in media osmolality. The osmolality of commercially available media naturally fluctuates as air movement within flow hoods causes' evaporation, and also as cultured dishes are prepared on heated work surfaces. Intracellular mechanisms that protect cells from the harmful effects of high osmolality are activated (Richards *et al*. 2010 and Van Winkle *et al*. 1990); however, the standard osmolality range for mammalian culture media is 260 – 320 mOsmol/kg (Lin *et al.*, 2017). In these studies, P19 cells survived the 0.0000125 mM treatment with an osmolality of 332 mOsmol/kg but perished with the 0.00001875 mM treatment and an osmolality of 341 mOsmol/kg, suggesting that cell death does not increase significantly with increasing osmolality. Instead, cells survive until culture conditions reach a cell-specific, toxic level of osmolality, which in the case of P19 cells is 333 - 341 mOsmol/kg.

Consistent with previous studies (Wise *et al.*, 2008; Magistretti and Allaman, 2018; Bergemann *et al.*, 2019; Tauffenberger *et al.*, 2019), cell viability was significantly impacted by the absence of calcium lactate and glutamine, but not pyruvate as it is naturally synthesised during glycolysis, meaning its presence in culture media serves as an additional source of energy rather than an essential requirement. Conversely, supplementation of calcium lactate in culture media is critical to cell survival because it is oxidised in the mitochondria to produce large quantities of ATP (Tauffenberger *et al.*, 2019). Here, it was found that P19 cells were particularly sensitive to the removal of calcium lactate, highlighting its significance in cellular development, in comparison to cells lacking pyruvate and glutamine (Figure 3.2). On the other hand, the concentration of calcium lactate in blastocyst media is typically approximately ten times higher than that of pyruvate and glutamine, so its removal was likely to have a profound

effect on cell growth, considering it makes up a significant proportion of total energy substrate availability in complete blastocyst media. The removal of glutamine significantly impacted cell viability, but there was some degree of cell growth, supporting previous ideas that glutamine is a non-essential amino acid at standard culture conditions; however, cells use alternative sources of nitrogen or synthesise their own when glutamine availability is limited (Wise *et al.*, 2008). Studies by (Kleijkers *et al.*, 2016) advice that culture media is supplemented with dipeptide glutamine such as alanyl glutamine or glycyl-glutamine, as oppose to monopeptide L-glutamine, which has a different half-life and is more likely to induce ammonium build-up *in vitro* resulting in reduced cell growth. P19 cells responded to mono and dipeptide glutamine in the same manner, demonstrating that its growth was not hindered by ammonium, though other non-observable detrimental changes such as DNA damage could have occurred. Future studies may wish to explore the effects of ammonium on the structural integrity of extracellular receptors and internal organelles, in order to confirm the superiority of dipeptide glutamine over its monopeptide form.

Differences in commercially available and in-house produced blastocyst media were explored, and the effects of aged media were assessed in order to determine whether P19 cells had a preference for freshly made media. In reference to its use in clinics, it is important to understand the potential effects of using expired media, and the impact it could have on embryo development, in case this were to occur in error although this is unlikely. The shelf-life is typically determined by the manufacturer during production and printed on the label of the product. Assessed by a range of Gems® media with different expiration dates, cell viability was unaffected by expired media and the age of media was not found to be of significance in the response of P19 cells to various media formulations. Removal of energy sources and supplementation with a double dose of vitamins demonstrated the extent to which P19 cells depend on energy substrate availability and confirmed that abundant vitamin supply does not make up for the absence of energy sources. This may be linked to the fact that macronutrients

(energy substrates) serve as building blocks for protein synthesis whereas micronutrients (vitamins) enhance cell growth and aid DNA metabolic pathways; thus, the role of both nutrients are mutually exclusive (Arigony *et al.*, 2013).

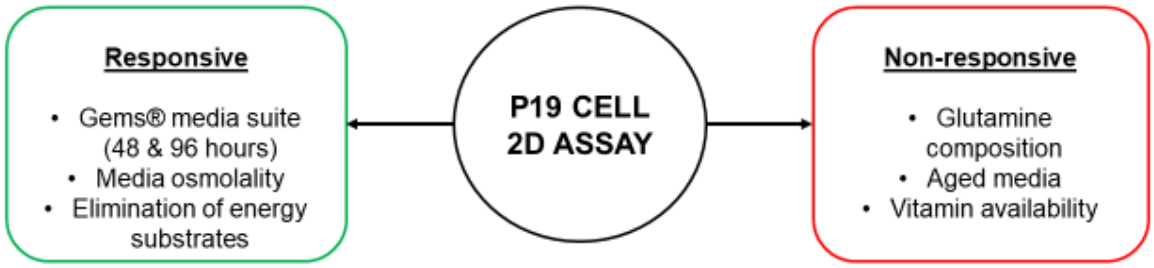


Figure 3.12. Summary of P19 embryonal carcinoma response to treatment with Gems® media suite, changes in osmolality, energy substrate availability, glutamine composition and aged-media, for 48 hours. Assessed by resorufin fluorescence in their 2D form, P19 cells responded to the treatments listed in the green box but not to those listed in the red box. Positive (25% DMSO) and negative controls (αMEM) were used to measure cellular response at two extremes of the activity spectrum, but these data were not included in the analyses.

In conclusion, the assay described here was sensitive to some of the most common perturbations associated with human embryo culture including fluctuating osmolality and reduced energy substrate availability, but P19 cells did not respond to the different glutamine compositions, aged media, or double doses of vitamins administered in independent repeat experiments despite responding its absence (Figure 3.12). This is not particularly concerning, given that human embryos are unlikely to encounter altering glutamine composition or expired media outside of a research and development setting. Direct comparison with findings from the MEA in chapter six will further determine its sensitivity; however, these assays are still of significance for identifying sub-optimal content in IVF media. The subsequent chapters focus on identifying cellular response to more discrete changes in culture media.

4. Thesis aim two: To develop and optimise a 3D cell-based assay with pluripotent abilities that mimic early embryonic development in mammals and to assess the contributions of exogenous metabolic substrates in the supply of energy and subsequently, embryoid body growth.

4.1. Background

Embryoid bodies (EB) have been popular *in vitro* models of early-stage mouse embryos for over three decades and are regarded as versatile biological systems that are able to be used as genetic models of diseases in humans and mice while expressing important molecular markers such as the stage-specific embryonic antigen (Wobus *et al.*, 1984). Subsequent advances in research have led to the implementation of cell replacement therapy and, amongst other ground-breaking treatments, neural cells derived from mouse embryonic stem cells (ESC) have been used to successfully treat defective neurons in the central nervous system of another mouse (Brüstle *et al.*, 1999; Mcdonald *et al.*, 1999). Initially described as aggregates of ESCs, EBs gained prominence in 1981 following the derivation of karyotypically normal ESCs from mouse blastocysts (Evans and Kaufman 1981; Martin 1981). When removed from a blastocyst, ESCs are usually in the process of making lineage choices that would lead to them either forming the trophectoderm, the extraembryonic tissues that support the growing, or the primitive endoderm and epiblast which will progress to form the inner cell mass (ICM) (Brickman and Serup, 2017). ESCs are therefore referred to as pluripotent cells because of their ability to differentiate into somatic lineages and germ layers when induced to do so *in vivo* by blastocyst injection, or by teratoma formation *in vitro* (Fu *et al.*, 2020).

Although the use of ESCs was popularised in 1981, the ability of other cell types to form organised heterogeneous aggregates when injected intraperitoneally into mice was observed in the early 1950s, as reviewed by Brickman and Serup (2017). Depending on the availability of necessary progenitor cells, teratocarcinomas subcutaneously grafted in mice formed

malignant or benign teratomas (Pierce *et al.* 1960). These undifferentiated tumour forming cells resemble embryonic cells (EC), and when they mature they comprise of well-differentiated tissues of the three germ layers (Kleinsmith and Pierce, 1964). In fact, the first ESC cell lines were established following comparative studies of teratocarcinomas and early embryos where the formation of EBs was used to demonstrate similarities between ESCs and EC cells, particularly their pluripotent abilities (Martin and Evans, 1975). In later years, the pluripotent nature of mouse ESCs was confirmed by injecting them into mammalian embryos and transferring them into pseudo pregnant mice females to allow them to develop to full-term (Bradley *et al.*, 1984). These experiments found descendants of ESCs in all lineages, including the germline, causing blastocyst injection to become the gold standard for demonstrating pluripotency (Doetschman *et al.*, 1985). *In vitro* differentiation of ESCs has since become a powerful tool for studying lineage specification and commitment. It enables a level of experimental accessibility that is not possible with *in vivo* studies, and when combined with modern molecular tools for lineage labelling and cell sorting, the *in vitro* production of different cell types from ESCs can provide unlimited amounts of intermediates in differentiation such as neural progenitors that are difficult, if not impossible, to obtain from embryos (Ying *et al.* 2003; D'Amour *et al.* 2005).

The formation of EBs makes up an essential first step in the differentiation of many types of stem cells, and protocols for directed differentiation of neural cells and cardiomyocytes rely on this initial step (Skerjanc, 1999; Desbaillets *et al.*, 2000; Doevendans *et al.*, 2000; Shafaroudi *et al.*, 2016). In recent times, EBs have shown a high degree of self-organisation, mimicking some of the three-dimensional early embryonic developmental aspects, although with some important exceptions (Zeevaert, Mabrouk, *et al.*, 2020). Differentiation of ESCs and ECs into EBs can be achieved through different procedures, however, the most common method involves transferring cells from self-renewing culture conditions to culture in suspension within liquid media or methylcellulose supplemented media in bacterial grade petri dishes (Liyang *et al.*, 2014). ESCs and ECs do not adhere to the culture dish under these conditions but rather

form EBs composed of differentiating cells. Although simple, this approach produces heterogeneous EBs that vary in size and shape, with EB size significantly impacting the lineages generated. For example, when small EBs are incubated with activin, they form the endoderm layer, whilst larger ones form the mesoderm layer (Livigni *et al.*, 2009). Another popular technique used to generate EBs involves the culture of cells in 'hanging drops'. Sedimentation of the cells at the bottom of the hanging drop and the resulting close association of ESCs or ECs promote EB formation whilst allowing the size to be controlled by regulating the number of cells placed in each drop (Spelke *et al.*, 2011). Once formed, these EBs can be transferred to standard suspension cultures in bacterial dishes to complete development. Finally, EBs can be generated by seeding ESCs or induced pluripotent stem cell suspension in untreated micro-well plates. This is an easy and standardised approach for producing large numbers of EBs uniform in size and shape, making differentiation experiments reproducible (Hwang *et al.*, 2009; Spelke *et al.*, 2011). Typically, EBs have an outer layer of primitive endoderm, an inner layer of primitive ectoderm, a basement membrane separating these and a central cavity that resembles the proamniotic cavity (Shen and Leder, 1992). The primitive ectoderm differentiates to generate derivatives of definitive ectoderm, mesoderm and endoderm (Wobus *et al.*, 1984; Doetschman *et al.*, 1985; Keller *et al.*, 1993). EBs are also categorised according to a specific developmental stage; simple (day 2-4), cavitating (day 4-5) or larger cavitated EBs (day 8+) (Abe *et al.*, 1996; Kurosawa, 2007).

There are structural and functional similarities between EBs and embryos; however, EBs are produced at a low throughput and with low efficiency. Consequently, there is a need for the methods that increase control and throughput to be improved, whilst allowing for the direct assessment of development. Such a model would allow direct comparison to MEAs and offer an alternative means of quality testing in IVF laboratories.

With the above information in mind, the specific objectives of this chapter were therefore to:

1) Design and optimise a robust 3D cell-based assay for assessing the quality of human culture media used in IVF laboratories;

2) Define an acceptable dynamic range for using a 3D cell-based assay by undertaking a systematic screen of components within human blastocyst culture media;

3) Determine the response of the 3D cell-based assay mentioned in (1) to various human embryo culture media formulations and altering concentrations of pyruvate and calcium lactate in human blastocyst media.

4.2. Materials and methods

Similar to the assay described in chapter three, the Z' factor of the 3D cell-based assay was determined as per section 2.5.2.2. Once a suitable Z' factor was obtained, the 3D assay was prepared as described in section 2.6.2, followed by the preparation of test substances as described in section 2.7. The resulting embryoid bodies were treated with human embryo culture media containing increasing concentrations of pyruvate and of calcium lactate as detailed in Table 2.1. Embryoid body size and viability were determined at the end of the treatment period as described by section 2.4.3 and 2.6.2.

4.3. Results

4.3.1. Z' Evaluation

The Z' factors were determined using fluorescence and luminescence values obtained from EB culture with the negative and positive controls (Figure 4.1). Findings indicated that αMEM and 25% DMSO were appropriate controls for 3D P19 cell-based assays after 48 hours of treatment, with resazurin reduction as an indication of cell viability (Z' factor = 0.571). Similarly, NanoLuc® luciferase proved to be an accurate viability test for 3D assays, with the Z' factor indicating excellent robustness (Z' factor = 0.613), making it suitable for high throughput screening.

A

	1	2	3	4	5	6	7	8	9	10	11	12
A	50641	49179	58002	53480	51450	52128	7630	7575	7458	7962	7799	8035
B	53239	48994	51697	59792	63264	60431	6902	7038	7595	7914	7986	8092
C	43922	47463	48912	52114	49408	54985	7364	6735	7700	8066	8068	8320
D	43290	48621	54065	55147	57270	62121	8099	8177	8122	8998	8903	9300
E	7497	7349	7292	7369	7335	7364	53234	53484	44322	46677	51425	55800
F	7448	7299	7265	7392	7369	7359	47640	51864	47792	62374	58370	62592
G	7517	7342	7284	7350	7351	7403	51443	50495	46404	46980	47335	48740
H	7824	7637	7684	7716	7729	7723	50332	44662	52491	62001	66068	67507

B

	1	2	3	4	5	6	7	8	9	10	11	12
A	2280	2692	2601	2289	2414	2937	197	110	175	165	186	111
B	2452	2851	2657	2861	2391	2196	169	135	120	198	135	146
C	2692	2943	2770	2691	2629	2234	160	112	174	199	193	118
D	2416	2646	2793	2958	2687	2359	168	137	111	103	156	104
E	121	176	182	132	153	114	2821	2624	2586	2447	2628	2605
F	116	170	191	145	180	182	2133	2993	2375	2239	2994	2284
G	119	135	155	188	190	101	2203	2913	2558	2414	2846	2134
H	157	143	137	117	132	126	2113	2150	2885	2109	2140	2994

Figure 4.1. Resazurin (resorufin) fluorescence readings of Z' assays after 48 hours of incubation with 25% DMSO and αMEM, and a further four hours of incubation with 160 µM resazurin solution (A) and luminescence readings after one hour of incubation with NanoLuc® luciferase (B). Values shaded in blue and dark yellow are indicative of wells treated with 25% DMSO (positive control) whilst those shaded in pink and pale yellow represent treatment with complete αMEM (negative control).

4.3.2. P19 EBs respond to changes in the composition of human embryo culture media

As shown by Figure 4.2, P19 EBs were produced 24 hours before treatment in the negative control as P19 cells were unable to form aggregates in the Gems® media suite. P19 EB growth in ORB, FLM, CLM, BLM (Figure 4.3) and the negative (Figure 4.2) and positive controls were monitored over the course of 96 hours and, for all treatments except the positive control, EBs expanded after 24 hours of incubation with a layer of differentiating cells forming at the periphery of the structure. Cells within the core structure became dark and uneven after 48 hours, whilst the outer structure appeared pale and more uniform. These physical traits were consistent with EBs on day three although all structures increased in size. After 96 hours, P19 EBs cultured in the negative control had darkened and the outer layer disintegrated, causing cells to leak into the surrounding media. Conversely, P19 EBs cultured in the Gems® media suite maintained their structural integrity despite increasing necrosis within the core structure (Figure 4.3). Determined by size and volume, P19 EBs grew the most in blastocyst media and the least in fertilisation media after 24 hours (Figure 4.4 $F = 668.70$, $df = 2$, $p < 0.001$), with *post hoc* comparisons indicating significant differences between blastocyst media and fertilisation and cleavage media treatments (Tukey HSD, $p < 0.001$), but not the fertilisation and cleavage media treatments ($p = 0.28$). These findings are consistent with P19 EB growth after 48 hours (Figure 4.4 $F = 8.14$, $df = 2$, $p < 0.001$), although *post hoc* comparisons indicated significant differences (Tukey HSD, $p < 0.001$) between fertilisation media and blastocyst and cleavage media treatments, but not cleavage and blastocyst media treatments ($p = 0.84$). Conversely, accelerated EB growth with cleavage media, and reduced EB growth with fertilisation media were observed after 72 hours of culture (Figure 4.4 $F = 11.38$, $df = 2$, $p < 0.001$), with *post hoc* comparisons indicating significant differences (Tukey HSD, $p < 0.001$) between these treatments, but not the cleavage and blastocyst media treatments ($p = 0.72$). After 96 hours of treatment, resorufin fluorescence, luciferase luminescence and EB size all showed that P19 EBs responded best to cleavage media and least to blastocyst media (Figure

4.4 F = 13.87, *df* = 4, $p < 0.001$) and *post hoc* comparisons indicated significant differences ($p < 0.05$) between treatments, but not the fertilisation and blastocyst media treatments ($p = 0.11$).

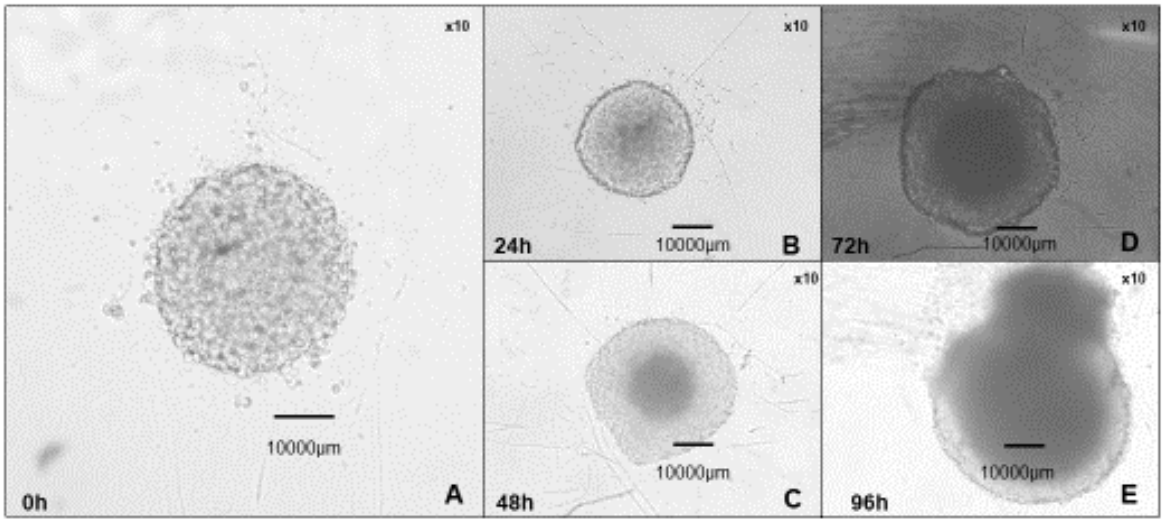


Figure 4.2. P19 EBs cultured in complete αMEM for 96 hours. Prior to treatment, P19 cells were cultured in a non-tissue culture treated 96-well plate for 24 hours to allow EB formation (A). The dark circle surrounding EBs after 24 hours is believed to be a sign of early differentiation (B). After 48 hours, cells within the core structure begin to perish (C & D) and leak out of the structure into the surrounding culture environment after 96 hours (E). All images captured at a magnification of x10.

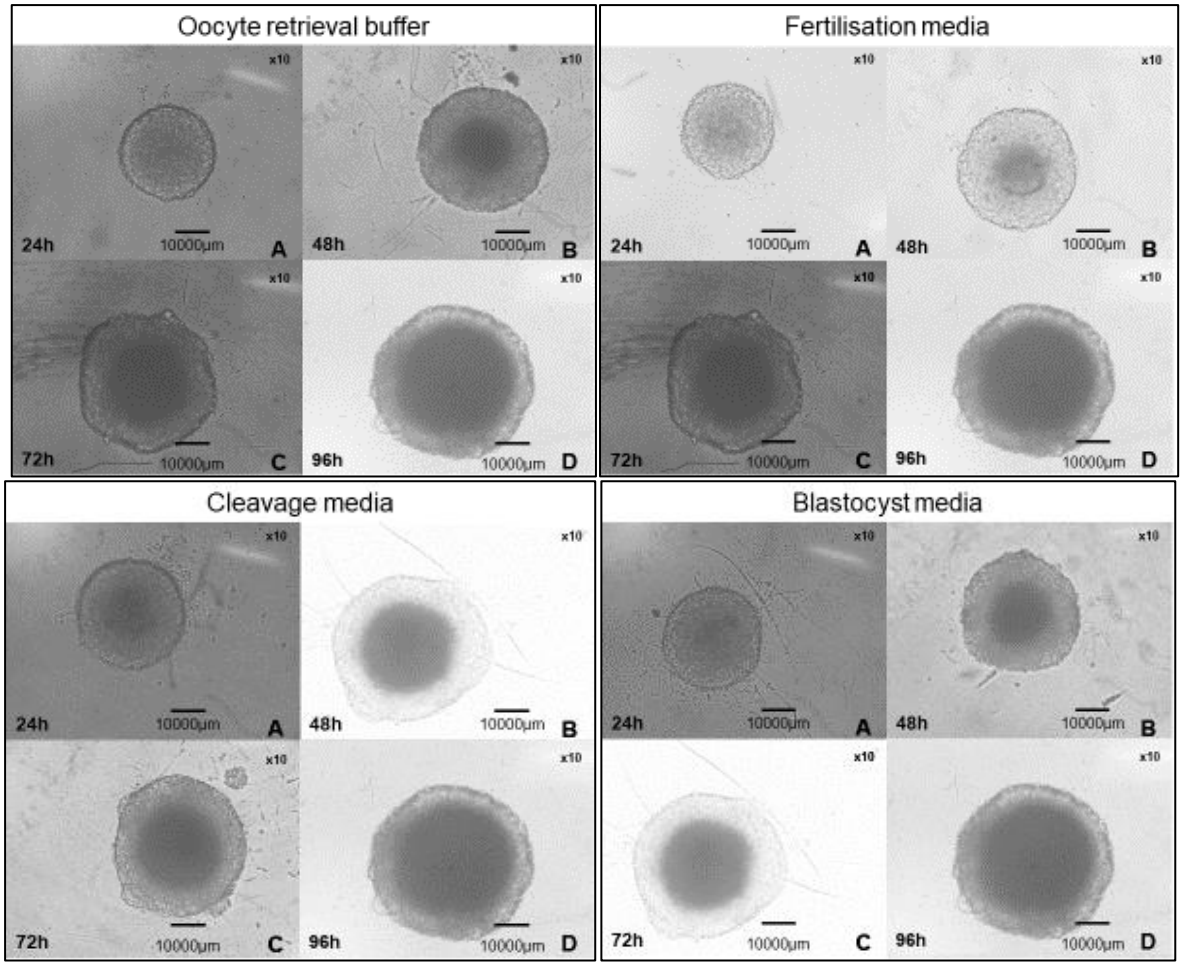


Figure 4.3. P19 EBs cultured in Gems® media suite (oocyte retrieval buffer; fertilisation, cleavage and blastocyst media) for 96 hours. Prior to treatment, P19 cells were cultured in a non-tissue culture treated 96-well plate for 24 hours to allow EB formation. The dark circle surrounding EBs after 24 hours are believed to be a sign of early differentiation (A) and after 48 hours, cells within the core structure begin to perish (C & D). Pictures were taken at a magnification of x10 and these represent repeat wells of each treatment.

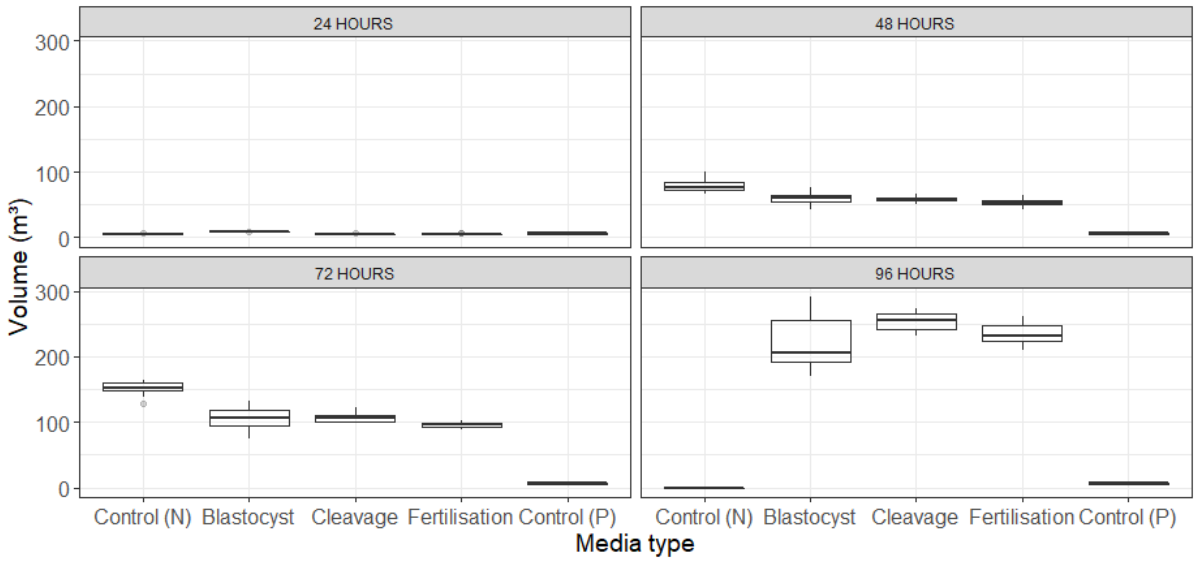


Figure 4.4. P19 EBs respond to changes in the composition of human embryo culture media. Boxplots showing P19 EB size and volume following treatment with fertilisation, cleavage and blastocyst media. There were significant differences in EB size between treatment groups. The most extensive EB growth was observed in blastocyst media over the first 48 hours, and in cleavage media over the next 48 hours. Complete αMEM was used as a negative control, and 25% DMSO in αMEM was used as a positive control.

4.3.3. P19 EBs respond to increasing concentrations of sodium pyruvate and calcium lactate

Assessed by luminescence from NanoLuc® luciferase reduction, treatment with increasing concentrations of sodium pyruvate in blastocyst media showed that P19 EBs were sensitive to pyruvate availability (Figure 4.5 $F = 47.67$, $df = 5$, $p < 0.001$), with *post hoc* comparison indicating significant differences between complete blastocyst media (0.2 mM pyruvate) and the 0.1 mM and 0.2 mM treatments (Tukey HSD, $p < 0.05$), but not the other treatments. Similarly, EB size and volume responded to the various treatments after 96 hours (Figure 4.6 $F = 60.82$, $df = 5$, $p < 0.001$), although *post hoc* comparison indicated significant differences (Tukey HSD, $p < 0.05$) between complete blastocyst media and the 0.5 mM treatments, but not the other treatments. Treatment with increasing concentrations of calcium lactate showed that EBs are sensitive to calcium lactate availability (Figure 4.5 $F = 111.2$, $df = 5$, $p < 0.001$), with *post hoc* comparison indicating significant differences (Tukey HSD, $p < 0.05$) between complete blastocyst media and all treatments except the 0.1 mM treatment ($p = 0.66$).

Assessment of EB size and volume showed that P19 EBs respond to changes in calcium lactate concentration after 96 hours (Figure 4.6 F = 4.57, *df* = 5, $p < 0.001$), although *post hoc* comparison indicated no significant differences (Tukey HSD, $p > 0.05$) between complete blastocyst media and all other treatments (Figure 4.5).

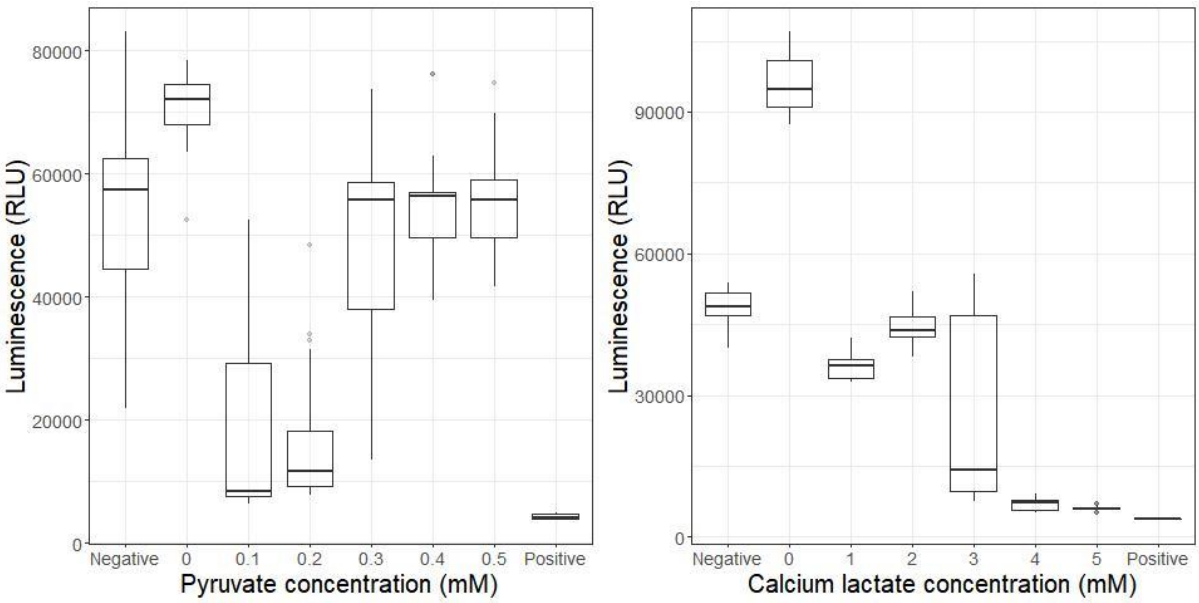


Figure 4.5. P19 EBs respond to increasing concentrations of sodium pyruvate and calcium lactate. Boxplots showing luminescence values for treatment of P19 cells treated with blastocyst media containing increasing concentrations of sodium pyruvate (left) and calcium lactate (right) for 48 hours. Complete αMEM was used as a negative control, and 25% DMSO in αMEM was used as a positive control. *Post hoc* comparison indicated that the complete blastocyst media treatment differed significantly from the 0.1 mM and 0.2 mM pyruvate treatments only, but differed significantly from all calcium lactate treatments, except the 0.1 mM calcium lactate treatment (Tukey HSD, $p < 0.05$).

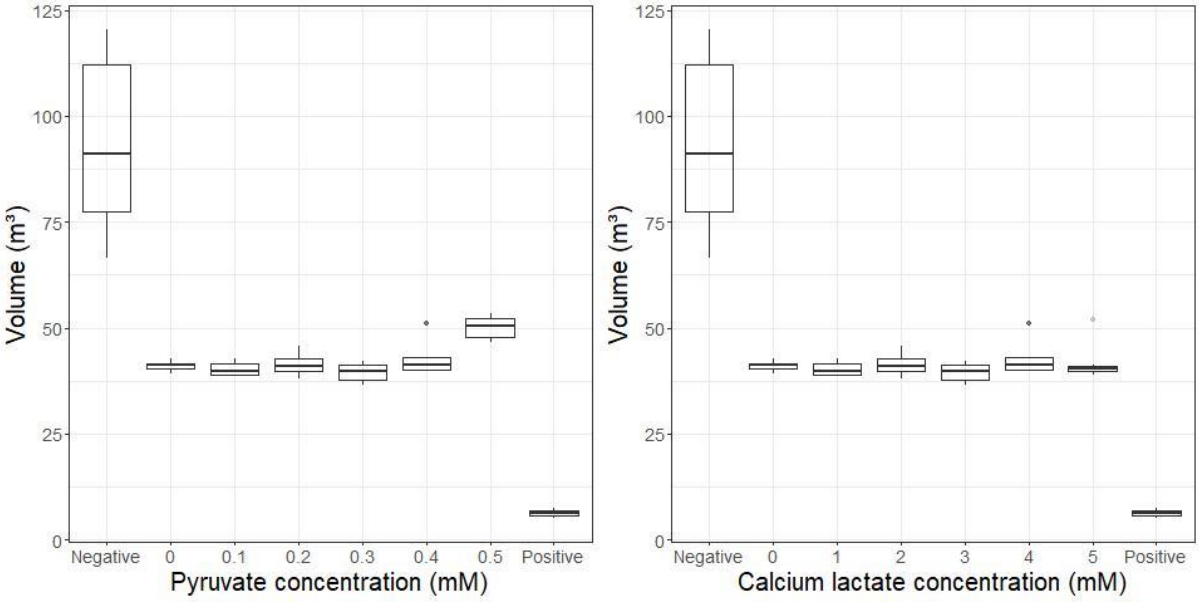


Figure 4.6. P19 EBs respond to increasing concentrations of sodium pyruvate and calcium lactate. Boxplots showing P19 EB volume following treatment with blastocyst media containing increasing sodium pyruvate concentrations (left) and calcium lactate (right) for 96 hours. There were significant differences between the size of EBs cultured in complete blastocyst media and those cultured in 0.5 mM pyruvate, and also between the various calcium lactate treatments (Tukey HSD, $p < 0.05$).

4.4. Discussion

Over the last decade, there has been significant interest in 3D cell-based assays as potential models for assay development as 3D culture systems retain organ specific functions better than their 2D counterparts. Subsequently, they better reflect the distribution of nutrients, metabolites and oxygen *in vivo* (Piccinini *et al.*, 2017; Kapałczyńska *et al.*, 2018). The advantages of 2D cultures over 3D ones however, include the low-cost maintenance of cells, and functional viability performance (Duval *et al.*, 2017; Agrawal *et al.*, 2020); but on the other hand, cell-to-cell and cell-to-extracellular environment interactions are not represented as they would be in an embryo or organ (Ravi, V Paramesh, *et al.*, 2015). This chapter's main objective was to determine whether cell-to-cell interactions in a 3D structure make them more responsive to alterations in culture conditions than 2D cultures. The Z' factor of the P19 3D cell-based assay was determined to be 0.571 with resazurin reduction as an indication of cell viability, and 0.613 with NanoLuc® luciferase, demonstrating enhanced sensitivity with luminescence-based viability assays. Notably, the fluorescence readings and subsequent Z' factor obtained from the 2D assay was much higher than that obtained from the 3D assay suggesting greater robustness and a wider difference between the negative and positive controls with the 2D assay. This makes the 2D assay a more sensitive screening tool. The response of P19 EBs to the various treatments were compared to that of complete αMEM and 25% DMSO, to quantify the amount of growth, or death of EBs produced by each treatment. Wells were seeded with 1,000 cells per well because EBs generated with higher seeding densities formed dark residues within the core structures, that were possibly early signs of necrosis. Moreover, the walls of EBs seeded with more than 1,000 cells per well appeared to perforate after 48 hours, causing cells to leak from the core structure and into the surrounding culture environment. EBs seeded with 500 cells also maintained their structure for more than 48 hours, however, as per chapter three, the aim was to choose a seeding density that emitted sufficient fluorescence to create the most significant possible fold change. Consequently,

1,000 cells per embryoid body was deemed to be the most suitable seeding density for use in this study.

In contrast to previous studies where cells cultured in 3D displayed different responses to those cultured in 2D (Cushing and Anseth, 2007; Langhans, 2018), here P19 EBs responded to the various IVF media treatments in a similar way to the 2D assay (section 3.4.), and EB size also accurately represented differences in cellular response to the various media formulations. Notably, daily assessment of EB size made it possible to monitor EB development over an extended period instead of the one endpoint reading, commonly used in 2D assays. This form of assessment demonstrated the changing needs of growing cells by showing that EBs grew best in blastocyst media within the first 48 hours but that growth rate accelerated in cleavage media over the next 48 hours. Furthermore, it became evident that although blastocyst media successfully supported EB growth in the first 48 hours, it was not a suitable source of nutrients for ≥ 72 hour old EBs. Resazurin and NanoLuc® luciferase proved to be suitable forms of viability testing by accurately indicating that after 96 hours, EBs grew best in cleavage media and worst in blastocyst media. Notably, the viability measurements indicated that luminescence units are a more sensitive measure of cell viability than fluorescence so NanoLuc® luciferase was chosen as the preferred viability test in subsequent experiments.

Previous studies have suggested that 2D cultures respond better to different treatments than 3D cultures (Bonnans *et al.* 2014; Lv *et al.* 2017), because compounds target certain receptors on the surface of cells, and the structure and spatial arrangement of 2D structures allow more frequent binding to specific receptors, thereby eliciting desired responses (Lancaster and Knoblich, 2014; Langhans, 2018). In contrast to this notion, here EB viability and size were significantly impacted by pyruvate concentration in blastocyst media, even though it is

naturally synthesis in cells during glycolysis. It is possible that in their 3D form, P19 cells metabolise pyruvate at a higher rate than they do in their 2D form and subsequently require more significant amounts than those produced within the cell, making them dependant and more responsive to extracellular pyruvate. This could be verified by assessing and comparing ATP production in both cell structures. Alternatively, metabolic profiling has demonstrated that cells that make-up the protective outer layer of 3D structures respond better to treatments than cells within the core structure (Russell *et al.*, 2017). This is because cells that make up the internal structure do not have direct access to extracellular nutrients, so it is possible that this makes them more responsive to pyruvate availability, than cells in 2D culture. Furthermore, it has been seen that lower intracellular pH levels reduces the ability of cells to respond to different treatments (Lancaster and Knoblich, 2014) but the difference in shape between 2D and 3D cell structures causes a difference in local pH levels within the cells. This favours 3D cell culture because cells in this structure have greater depth and therefore accommodate more efficient pH balance (Lancaster and Knoblich, 2014).

Consistent with the 2D assay, EBs responded to changes in calcium lactate concentration in blastocyst media, although luminescence measurements better reflected this, instead of EB size. This is unsurprising considering that 3D cells are usually in different cell stages (much like *in vivo* cells), whereas cells cultured in 2D are often at the same cell stage (Langhans, 2018) meaning that significantly higher rates of proliferation occur within the outer region of 3D structures. To verify this, differentiation and proliferation of EBs could be characterised by using molecular markers to identify the unique expression patterns of stage specific embryonic transcription factors like *Pou5f1* (Chakritbudsabong *et al.*, 2018). Effective response to treatment requires continuous proliferation (Bonnans *et al*. 2014), and considering that P19 cells are naturally responsive to calcium lactate availability (section 3.4.), the increased rate of proliferation observed within growing EBs is likely to result in more significant response to different calcium lactate treatments than 2D cultures. Also, as described in chapter three,

calcium lactate concentration is approximately ten times higher than that of other energy sources; thus, its reduction or increase significantly impacts cell growth.

Overall, the data presented here suggest that luminescence measurements better identified EB response to changes in culture conditions than EB size and volume. Nonetheless, the latter enabled kinetic assessment of EB formation, as opposed to an endpoint reading. When compared to the 2D assay, it is obvious that P19 EBs share more structural and morphological similarities to the MEA, suggesting that they have the potential to be a useful model. On the other hand, 3D cultures can be overly complicated because they require extended culture and are more time-consuming and expensive than 2D assays. There are also significantly fewer commercially available cell viability tests for 3D assays, so a high degree of optimisation would be needed to ensure high performance and reproducibility. As a result, the next chapter in this thesis makes use of the 2D assay described in chapter three, to undertake a systematic screen of P19 response to energy sources pyruvate, glutamine, and calcium lactate to improve on current protocols used to develop and validate human IVF media. Even so, it can be concluded that the 3D assay shares structural similarities with the MEA, and in the future methods could be further developed to mimic the structural changes that occur within the mouse embryo.

5. Thesis aim three: To test the hypothesis that manipulating intracellular responses to environmental stressors such as hypothermia increases the ability of mammalian cells to respond to the contributions of exogenous metabolic substrates.

5.1. Background

In the biopharmaceutical industry, recombinant protein (rP) yield is of great importance for the mass production of medicines such as the human growth hormone substitutes Valtropin and Humatrope (Hepner *et al.* 2005 and Pavlovic *et al.* 2008), and also for insulin production in the treatment of diabetes (Sandow *et al.*, 2015 and Kapitza *et al.*, 2017). Extensive research has been undertaken to improve volumetric productivity of mammalian cells (Al-Fageeh *et al.*, 2006) and there is currently a great deal of interest in the use of reduced temperature (hypothermic) cultivation of mammalian cells for enhanced rP production. Changes in protein yield and the changes associated with hypothermic incubation are complicated; however, it is clear that the general response to hypothermia includes global attenuation of transcription and translation, whilst proteins that are essential to cell survival are selectively upregulated (Eskla *et al.* 2018 and Sonna *et al.* 2013). Cellular response to mild hypothermia is different to the response to severe hypothermic conditions as cells continue to grow and proliferate at moderately low temperatures, but undergo growth arrest at more severe temperatures (Al-Fageeh and Smales 2006). *Saccharomyces cerevisiae* for example, responds to mild cold shock (10-18°C) by upregulating translational machinery and Tip1, Tir1, Tir3 and Nsr1, genes associated with transcription (see Table 1.3), but global protein production during these conditions is reduced (Homma *et al.* 2003). More severe forms of cold stress (<10°C) cause growth arrest and induce upregulation of an alternative set of proteins that synthesise trehalose to sustain cell viability (Homma *et al*. 2004).

Cold shock proteins RNA-binding motif protein 3 (RBM3) and cold-inducible RNA-binding protein (CIRP) are transcriptionally upregulated during mild hypothermia (32°C to 34°C) and function as regulators of translational reprogramming by binding to the 5'-UTR or 3'-UTR of mRNA molecules and facilitating their response to environmental signals (Fujita *et al.* 2017 and Nishiyama *et al.* 1997). In mammalian cells at 32°C, mRNA molecules bind to RBM3 and CIRP in the nucleus and cytoplasm and form mRNPs (Reineke and Lloyd, 2015) which play a crucial role in inducing immediate translation of the attached mRNA whilst others are transported to specific subcellular regions for storage or localised translation (Smart *et al.*, 2007). Whilst the exact mechanisms are not fully understood, RBM3 and CIRP are known to mediate neuroprotection during hypothermia by increasing resistance to neural apoptosis and also by inducing upregulation of reticulon 3 which aids synaptic plasticity and synapse formation (Chip *et al.*, 2011; Bastide *et al.*, 2017).

Hypothermic conditions also induce cytosolic stress granules (SGs) in mammalian cells, which are by-products of translation arrest and polysome disassembly (Kramer *et al.*, 2008). Although the exact function of SGs are unclear, they play a key role in sequestering proteins and modulating signalling cascades required for cell survival (Aulas, Fay, *et al.*, 2017). A number of studies have investigated the composition of SGs and have shown that they contain a complex mix of proteins and RNAs, as summarised in Table 1.4. In mammalian cells, SG formation is initiated by phosphorylation of the α subunit of eIF2α (Anderson *et al.* 2015). In the presence of GTP, eIF2α transports $tRNAi^{met}$ to the site of translation initiation on the 40S small ribosomal subunit, and alongside eIF3 and eIF4, form the pre-initiation complex. Phosphorylation of eIF2α on serine 51 inhibits GTD/GTP exchange by eIF3 and prevents further eIF2-GTP-$tRNAi^{met}$ formation which leads to failure of $tRNAi^{met}$ delivery to the site of translation initiation (Hofmann *et al.* 2012 and Aulas *et al.* 2016).

Numerous studies confirm that mammalian cells cultured *in vitro* at hypothermic temperatures improve product quality and yield; however, the cell-specific and volumetric effects vary between target proteins and cell lines (Al-Fageeh *et al.*, 2006). In spite of our increased understanding of cellular responses to cold stress, and reports indicating that changes in mammalian interferon-gamma mRNA levels during culture at reduced temperatures are responsible for increased protein yield (Underhill and Smales, 2007), the underlying mechanisms involved in increased protein production are poorly understood.

Cell culture systems are often manipulated by therapeutics to induce a known response that enables investigation of the various ways cells respond to changes in culture conditions, and also to determine the role of cellular proteins and structures. Rapamycin, also known as Sirolimus is an inhibitor of mechanistic target of rapamycin (mTOR) which plays a central role in the regulation of cell proliferation and stress-specific mechanisms through changes in translation (Reiling and Sabatini, 2006 and Ben-Sahra and Manning, 2017). Dorsomorphin is a commonly used immunosuppressant used to block the activity of AMP-activated protein kinase (AMPK), an evolutionary conserved energy sensor found in mammalian cells (Yu *et al.*, 2008 and Dasgupta and Seibel, 2018). Considering that previous efforts to develop cell-based assays for quality control in *in vitro* fertilization (IVF) have failed (Bertheussen *et al.*, 1989), because cell survival has been too high to identify the effects of reagents and equipment, reducing cellular resistance to stress by blocking survival mechanisms could amplify cellular response to more subtle changes in culture conditions.

The effects of cold stress and pharmacological inhibition by rapamycin and dorsomorphin have the potential to be used to reduce the resistance of mammalian cells to variability in culture conditions. Such stressors therefore have the potential to be used to increase cell sensitivity to variation in medium formulation.

With the above in mind, the specific objectives of this chapter were:

1) To evaluate the response of P19 cells on the removal of sodium pyruvate, L-glutamine and calcium lactate from human embryo culture media;

2) To assess the effects of mTOR and AMPK inhibition on cellular response to fluctuating concentrations of energy source in standard incubation conditions;

3) To assess the effects of mTOR and AMPK inhibition on cellular response to fluctuating concentrations of energy sources and hypothermia;

4) To test the hypothesis that P19 cells respond more strongly to subtle changes in culture media when mTOR or AMPK are inhibited or when exposed to hypothermia, than they do in standard incubation conditions.

5.2. Methods

The 2D assay was prepared as per section 2.6.1 and used to determine optimum rapamycin and dorsomorphin concentrations as described in section 2.8. Preliminary experiments (not included in this thesis) indicated that cells responded to cold stress when induced at 15°C for at least 18 hours before or after incubation at standard culture conditions for 30 hours. Consequently, cold stress was induced as described in section 2.8.1, followed by cell viability testing as described in section 2.4.2. For experiments, 12 x 96-well plates and test substances were prepared as described in section 2.6.1 and 2.7 respectively, and cells were treated with the following options from Table 2.1; absence of energy sources, pyruvate concentration and rapamycin, pyruvate concentration and dorsomorphin, glutamine concentration and rapamycin, glutamine concentration and dorsomorphin, lactate concentration and rapamycin, lactate concentration and dorsomorphin. BLM was prepared as per section 2.7 and supplemented with two of the three energy sources; sodium pyruvate, calcium lactate or glutamine Sigma Aldrich, to allow for the assessment of varying concentrations of the third energy source. Cold stress was induced as described in section 2.8.1 and following a total incubation period of 48 hours, cell viability was determined as described in section 2.4.2. These assays were repeated three times using different batches of test substances and P19 cells (passage 16 – 18). The suitability of this assay for high throughput screening was confirmed by a Z' prime factor of 0.8 (see section 3.3.1.), and a blank control was included to determine the baseline fluorescence of resorufin as described in section 4.2.1.

5.3. Results

5.3.1. Rapamycin and dorsomorphin optimisation

Treatment with the various rapamycin concentrations show that mTOR inhibition had an effect on cell growth and proliferation, with P19 cell viability altering as the concentration of rapamycin increased under standard incubation (Figure 5.1, ANOVA, $F = 15146$, $df = 7, 376$, $p < 0.001$) and also with hypothermic culture (Figure 5.1, ANOVA, $F = 5077$, $df = 7, 376$, $p < 0.001$). The 0 mM rapamycin treatment shows that P19 cell growth was hindered by cold stress in the absence of rapamycin compared to cell growth with the same treatment at standard incubation, with *post hoc* analysis indicating significant differences (Figure 5.1; *post hoc* comparison $p < 0.001$) between the two treatments. Treatment with increasing concentrations of rapamycin demonstrated that cold stress and mTOR inhibition caused cell growth to increase until rapamycin concentration exceeded 0.02 mM, then cell growth was inhibited under both incubation conditions, as evidenced by significantly reduced resazurin fluorescence (Figure 5.1; *post hoc* comparison with 0 mM rapamycin at standard incubation $p < 0.001$). Increased cell growth with hypothermic incubation and rapamycin was perhaps due to the prevention of energy preservation leading to uninterrupted metabolism and cell growth. Unsurprisingly, given that low resazurin fluorescence is indicative of cell death, there were no significant differences between viable cells cultivated under standard and hypothermic incubation when rapamycin concentration surpassed 0.02 mM.

Treatment with increasing concentrations of dorsomorphin demonstrate that P19 cells also respond to varying concentrations of dorsomorphin under standard incubation (Figure 5.2, ANOVA, $F = 3320$, $df = 7, 376$, $p < 0.001$) and with hypothermic culture (Figure 5.2, ANOVA, $F = 1448$, $df = 7, 376$, $p < 0.0001$). Tukey HSD *post hoc* comparisons indicated significant differences ($p < 0.001$) between treatments with the various dorsomorphin concentrations at standard incubation conditions, with the exception of the no dorsomorphin and 0.01 mM treatment ($p = 0.97$), and significant differences between the same treatments under

hypothermic incubation, with the exception of the no dorsomorphin and 0.1 mM treatment (p = 0.99). Similar to the rapamycin treatments, the 0 mM dorsomorphin treatment shows that P19 cell growth was hindered by cold stress in the absence of dorsomorphin compared to cell growth with dorsomorphin at standard incubation, with *post hoc* analysis indicating significant differences (p < 0.001) between the two culture conditions. Assessed by significantly low resazurin fluorescence (Figure 5.2; *post hoc* comparison with 0 mM dorsomorphin at standard incubation p < 0.001), cell growth was inhibited when dorsomorphin concentrations exceeded 1mM under both culture conditions, and there were no significant differences between standard and hypothermic incubation.

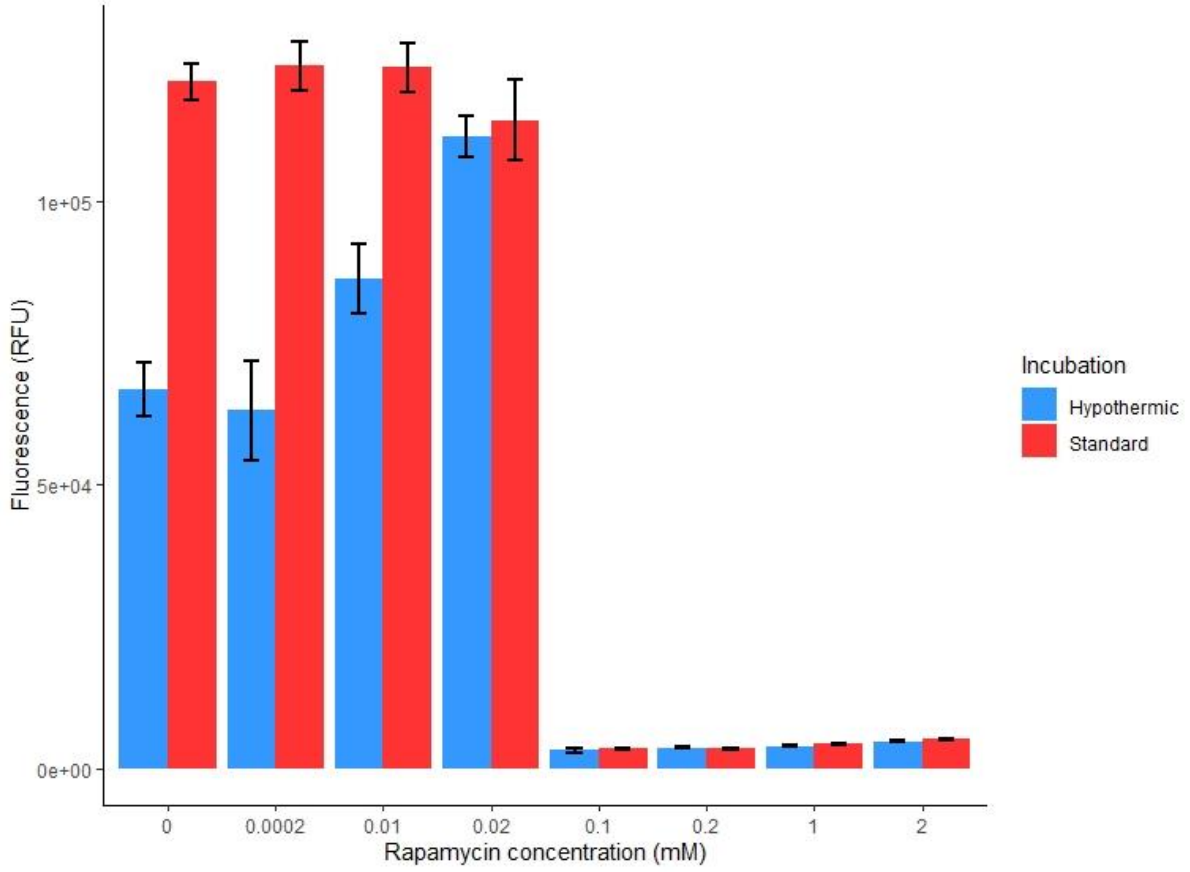


Figure 5.1. Effects of increasing concentrations of rapamycin on P19 cell viability. Mean resorufin fluorescence values for treatment of P19 cells with increasing concentrations of rapamycin for 48 hours at standard incubation conditions, and with cold stress. Whilst treatment with ≥ 0.1 mM rapamycin were highly toxic to cells, rapamycin appeared to block the arrested cell growth caused by cold stress when administered at a concentration of 0.01 mM – 0.02 mM (p < 0.001 for *post hoc* comparison with 0 mM rapamycin under hypothermic incubation).

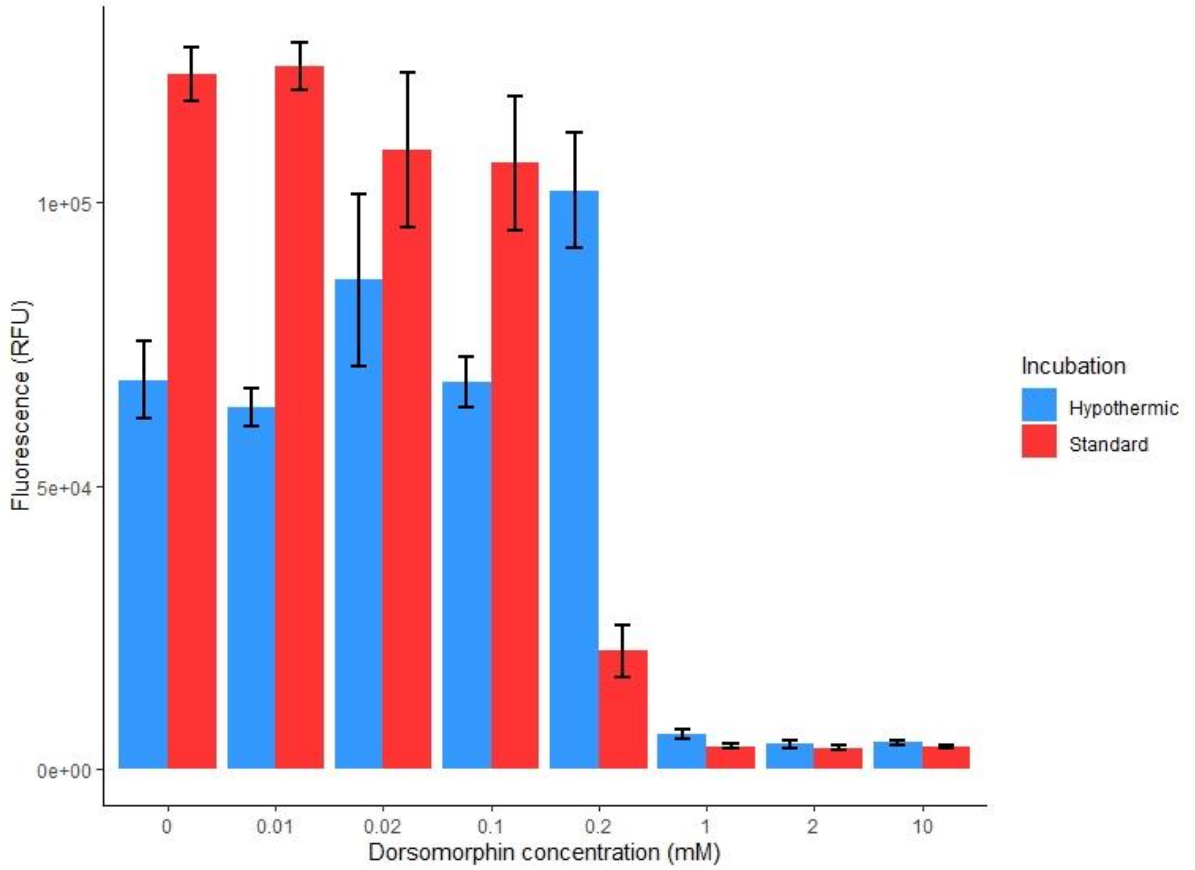


Figure 5.2. Effects of increasing concentrations of dorsomorphin on P19 cell viability. Mean resorufin fluorescence values for treatment of P19 cells with increasing concentrations of dorsomorphin for 48 hours at standard incubation conditions, and with cold stress. 0.2 mM dorsomorphin appeared to block the arrested cell growth caused by cold stress ($p < 0.001$ for *post hoc* comparison to the 0 mM dorsomorphin treatment under hypothermic incubation), whilst inhibiting cell growth at standard incubation conditions ($p < 0.001$ for *post hoc* comparison to the 0 mM dorsomorphin treatment at standard incubation).

5.3.2. P19 embryonal carcinoma cells respond to the elimination of energy substrates

Removal of different energy sources indicated that P19 cells respond to changes in calcium lactate, but not to the removal of pyruvate and glutamine (Figure 5.3, *Kruskal-Wallis,* H = 58.74, *df* = 6, $p < 0.0001$), with *post hoc* comparisons indicating significant differences ($p < 0.05$) between complete blastocyst media and the no calcium lactate treatment, but not with the no pyruvate and no glutamine treatments ($p > 0.05$ for both). As expected, given the effect of removing calcium lactate, the removal of all three energy sources also reduced cell growth

as assessed by resorufin fluorescence values ($p < 0.05$ for *post hoc* comparison with the blastocyst media).

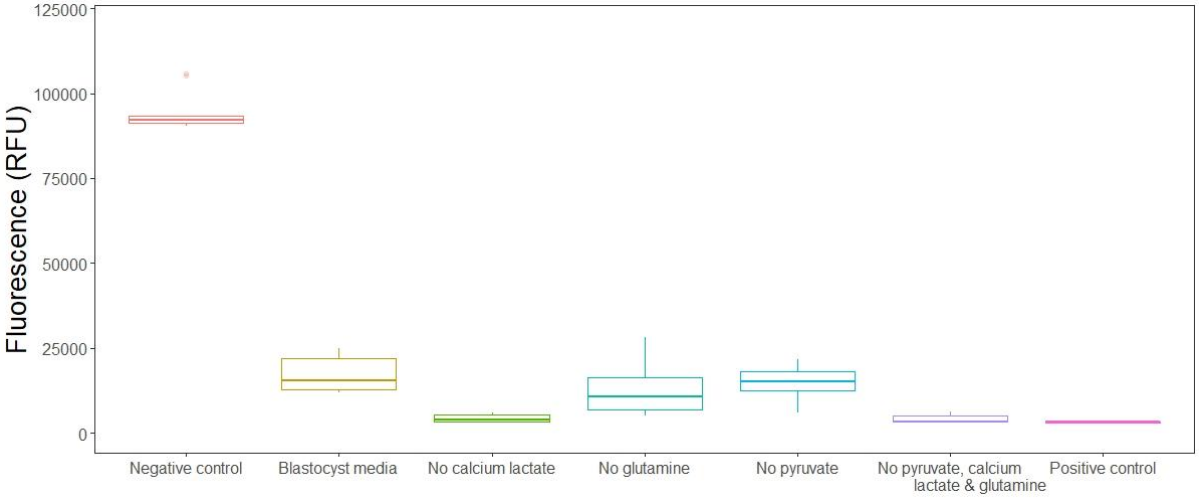


Figure 5.3. P19 embryonal carcinoma cells respond to the elimination of energy substrates. Boxplots of resorufin fluorescence values for replicate wells of P19 cells treated with complete blastocyst media, blastocyst media without: glutamine; pyruvate; calcium lactate; pyruvate, calcium lactate and glutamine for 48 hours. Complete αMEM was used as a negative control and 25% DMSO was used as a positive control. Median values for each group are shown by the solid symbol. Pairwise comparisons indicate that the median fluorescence of all groups, except for the no calcium lactate treatment, differ significantly from that obtained from cells lacking pyruvate, calcium lactate and glutamine ($p < 0.05$, *post hoc* tests).

5.3.3. Cold stress and rapamycin affect the response of P19 embryonal carcinoma cells to increasing concentrations of sodium pyruvate.

Analysis of resorufin values indicate that at standard culture conditions, P19 cell viability was affected by changes in pyruvate availability (PYRUVATE; $F = 22.37$; $df = 7, 248$; $p < 0.0001$) and by treatment with rapamycin (RAPAMYCIN; $F = 15.71$; $df = 1, 254$; $p < 0.0001$), with *post hoc* comparisons indicating significant differences ($p < 0.05$) between complete blastocyst media (0.2 Mm pyruvate), the no pyruvate and ≥ 5.0 mM pyruvate treatments, but not the ≤ 5.0 mM pyruvate treatments, both in the absence and presence of rapamycin (Figure 5.4). An interaction between pyruvate concentration and rapamycin was found (PYRUVATE X RAPAMYCIN; $F = 66.35$; $df = 15, 240$; $p < 0.0001$), with this deriving from the reduced cell viability seen at low pyruvate concentrations in the absence of rapamycin (Figure 5.4). Under hypothermic conditions, P19 cell viability was affected by minor changes in pyruvate

availability (PYRUVATE; F = 85.82; *df* = 7, 248; $p < 0.0001$), but not treatment with rapamycin (RAPAMYCIN; F = 0.005; *df* = 1, 254; $p = 0.941$), although *post hoc* comparisons indicated significant differences between complete blastocyst media, the no pyruvate and ≥ 0.4 mM pyruvate treatments, but not the 0.1 mM, 0.2 mM and 0.3 mM pyruvate treatments in the presence of rapamycin. This shows that P19 cells were responsive to minor changes in pyruvate concentrations with cold stress and rapamycin. On the other hand, *post hoc* comparisons indicated significant differences between complete blastocyst media and the 0.4 mM and ≥ 5.0 mM treatments, but not the no pyruvate, 0.1 mM, 0.3 mM and 0.5 mM pyruvate treatments in the absence of rapamycin, highlighting that P19 cells were more responsive to minor increases in pyruvate concentrations under hypothermic incubation, with rapamycin further enhancing this response (Figure 5.4). This is perhaps due to the uninterrupted cell metabolism associated with mTOR inhibition and subsequent utilisation of internal resources, creating a need for external pyruvate. Under these conditions, there was no interaction between pyruvate concentration and rapamycin (PYRUVATE X RAPAMYCIN; F = 42.99; *df* = 15, 240; $p < 0.0001$), suggesting that treatment with rapamycin had a greater effect on cellular response to pyruvate availability than cold stress did. Overall, major increases in pyruvate concentrations significantly impacted cell viability at standard culture conditions, but minor increases did not. Furthermore, cold stress caused cells to respond to the ≤ 0.5 mM pyruvate treatments ($p < 0.05$ for *post hoc* comparison with the blastocyst media) indicating that P19 cells are more sensitive to minor increases in pyruvate availability when subject to cold stress. At standard culture conditions, rapamycin blocked the decreased cell growth observed at low concentrations of pyruvate, although neither the decreased growth nor blocking of decreased growth were observed under hypothermic conditions. Cold stress also resulted in increased cell growth at higher pyruvate concentrations (Figure 5.4).

5.3.4. Cold stress affects the response of P19 embryonal carcinoma cells to increasing concentrations of sodium pyruvate but dorsomorphin does not.

Repeat experiments confirmed that P19 cell viability was affected by changes in pyruvate availability (PYRUVATE; F = 211.10; *df* = 7, 504; $p < 0.0001$) and by treatment with dorsomorphin (DORSOMORPHIN; F = 27.56; *df* = 1, 510; $p < 0.0001$); however, *post hoc* comparisons indicated significant differences ($p < 0.05$) between complete blastocyst media and all treatments in the absence of dorsomorphin, but not the ≤ 5. 0mM pyruvate treatments (with the exception of the no pyruvate treatment), in the presence of dorsomorphin (Figure 5.5). An interaction between pyruvate concentration and dorsomorphin was found (PYRUVATE X DORSOMORPHIN; F = 596; *df* = 15, 496; $p < 0.0001$), with this resulting from the increased cell viability observed at high pyruvate concentrations in the absence of dorsomorphin, in comparison to treatment in the absence of dorsomorphin (Figure 5.5). Consistent with treatment at standard conditions, P19 cell viability was affected by changes in pyruvate availability (PYRUVATE; F = 130; *df* = 7, 504; $p < 0.0001$) and by treatment with dorsomorphin (DORSOMORPHIN; F = 127.2; *df* = 1, 510; $p < 0.0001$) under hypothermic conditions, with *post hoc* comparisons indicating significant differences between complete blastocyst media and all treatments except the 0.1 mM pyruvate treatment in the absence of dorsomorphin, and significant differences between the ≥ 5.0 mM pyruvate treatments in the presence of dorsomorphin. There was an interaction between pyruvate concentration and dorsomorphin (PYRUVATE X DORSOMORPHIN; F = 639.9; *df* = 15, 496; $p < 0.0001$), resulting mainly from the increased cell viability observed at low pyruvate concentrations in the absence of dorsomorphin (Figure 5.5). These data suggest that both cold stress and treatment with dorsomorphin had an effect on cellular response to pyruvate availability but P19 cells were less responsive to minor increases in pyruvate concentration in the presence of dorsomorphin. Furthermore, dorsomorphin appears to cause increased cell growth at low pyruvate concentrations under hypothermic conditions, but not at standard culture conditions.

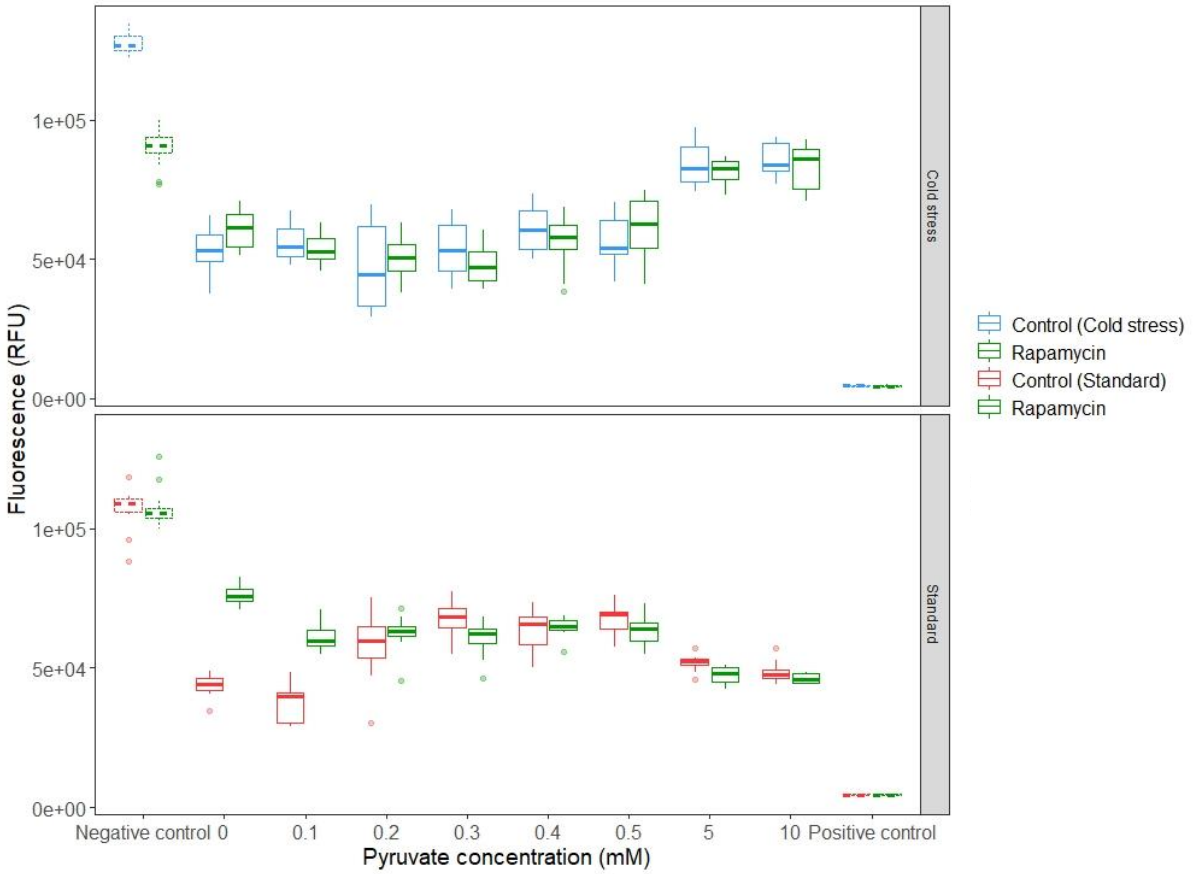


Figure 5.4. P19 embryonal carcinoma cells respond to minor increases sodium pyruvate concentration in the presence of cold stress and rapamycin. Boxplots of resorufin fluorescence values for replicate wells of P19 cells treated with blastocyst media containing increasing concentrations of sodium pyruvate for 48 hours. The effects of cold stress and rapamycin on cellular response to increasing pyruvate concentrations are also shown as resorufin values. Complete αMEM was used as a negative control, and 25% DMSO in αMEM was used as a positive control (dashed-lined boxplots). There is evidence to support an interaction between cellular response to treatments, rapamycin and cold stress.

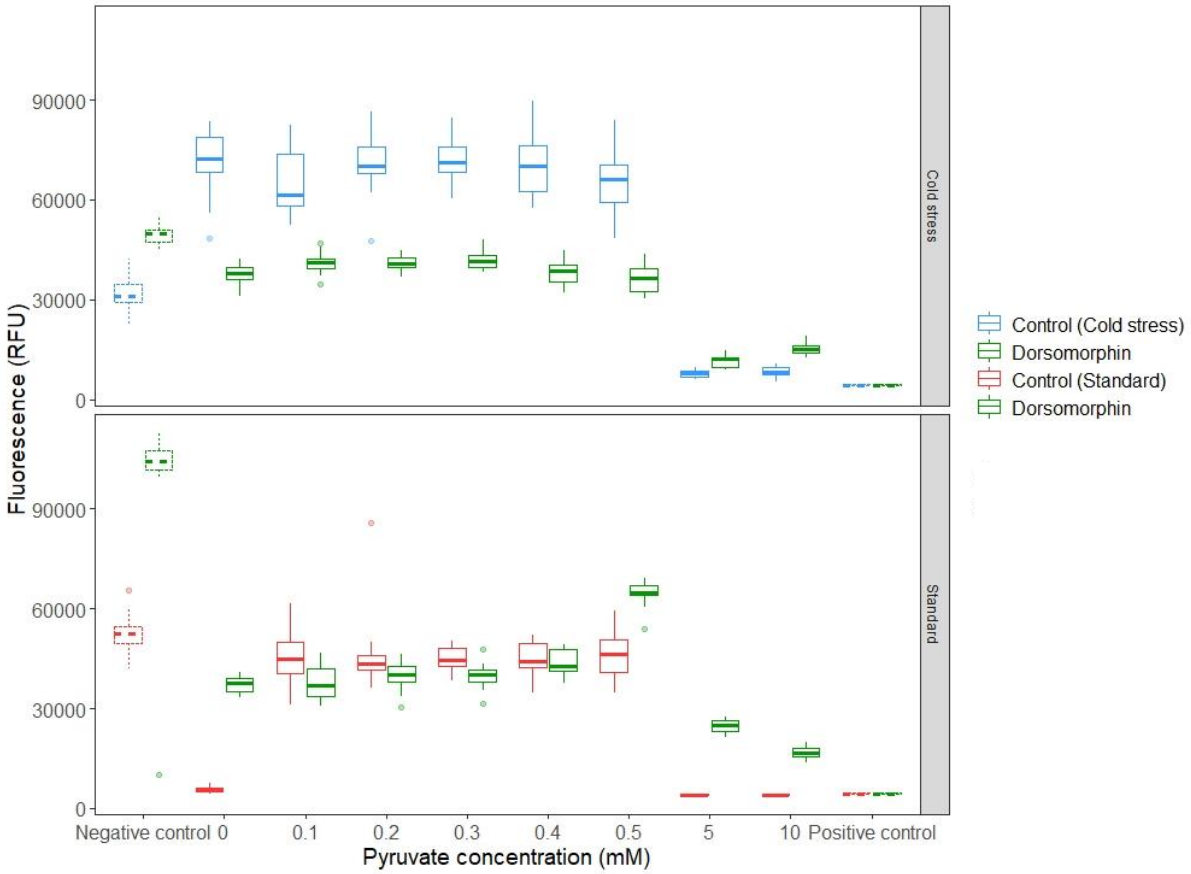


Figure 5.5. P19 embryonal carcinoma cells do not respond to minor increases in sodium pyruvate concentration in the presence of dorsomorphin. Boxplot of resorufin fluorescence values for replicate wells of P19 cells treated with blastocyst media containing increasing concentrations of sodium pyruvate for 48 hours. The effects of cold stress and dorsomorphin on cellular response to increasing pyruvate concentrations are also shown as resorufin values. Complete αMEM was used as a negative control, and 25% DMSO in αMEM was used as a positive control (dashed-lined boxplots). Dorsomorphin prevented P19 cells from responding to minor increases in pyruvate but cold stress did not.

5.3.5. Cold stress and rapamycin do not affect the response of P19 embryonal carcinoma cells to increasing concentrations of alanyl glutamine.

P19 cells responded to major changes in glutamine concentration (GLUTAMINE; F = 1202; *df* = 7, 248; $p < 0.0001$), but not to treatment with rapamycin (RAPAMYCIN; F = 0.411; *df* = 1, 254; $p = 0.522$) at standard culture conditions, with *post hoc* comparisons indicating significant differences ($p < 0.05$) between complete blastocyst media and the ≥ 5.0 mM glutamine treatments, but not the ≤ 5.0 mM glutamine treatments, both in the absence and presence of

rapamycin (Figure 5.6). There was no interaction between glutamine concentration and rapamycin (GLUTAMINE X RAPAMYCIN; F = 754.80; *df* = 15, 240; $p < 0.0001$), indicating that treatment with rapamycin had no effect on cellular response to glutamine availability. Under hypothermic conditions, P19 cell viability was affected by changes in glutamine availability (GLUTAMINE; F = 58.64; *df* = 7, 248; $p < 0.0001$) and treatment with rapamycin (RAPAMYCIN; F = 5.926; *df* = 1, 254; $p = 0.0156$), with *post hoc* comparisons indicating significant differences ($p < 0.05$) between complete blastocyst media and the ≥ 5.0 mM glutamine treatments, but not the ≤ 5.0 mM glutamine treatments, both in the absence and presence of rapamycin. There was no interaction between glutamine concentration and rapamycin (GLUTAMINE X RAPAMYCIN; F = 30.64; *df* = 15, 240; $p < 0.0001$), indicating that treatment with rapamycin had no effect on cellular response to glutamine availability, even with cold stress. Furthermore, cold stress appears to block the decreased cell growth observed at high concentrations of glutamine, suggesting that hypothermic culture conditions cause cells to become less sensitive to changes in glutamine availability; however, at both conditions, cells responded to major increases in glutamine concentration.

5.3.6. Cold stress and dorsomorphin do not affect the response of P19 embryonal carcinoma cells to increasing concentrations of alanyl glutamine.

Repeat experiments confirm that P19 cells responded to changes in glutamine concentration (GLUTAMINE; F = 131.40; *df* = 7, 504; $p < 0.0001$) and treatment with dorsomorphin (DORSOMORPHIN; F = 32.21; *df* = 1, 510; $p < 0.0001$) at standard culture conditions, with *post hoc* comparisons indicating significant differences ($p < 0.05$) between complete blastocyst media and all treatments except the 0.1 mM glutamine treatment both in the absence and presence of dorsomorphin (Figure 5.7). There was an interaction between glutamine concentration and dorsomorphin (GLUTAMINE X DORSOMORPHIN; F = 249.40; *df* = 15, 496; $p <$

0.0001), indicating that treatment with dorsomorphin had an effect on cellular response to glutamine availability. Under hypothermic conditions, P19 cells responded to changes in glutamine concentration (GLUTAMINE; F = 169.60; *df* = 7, 504; *p* < 0.0001), but not treatment with dorsomorphin (DORSOMORPHIN; F = 7.831; *df* = 1, 510; *p* = 0.0533), with *post hoc* comparisons indicating significant differences between complete blastocyst media and all treatments except the no glutamine treatment in the absence of dorsomorphin, but significant differences between the ≥ 5.0 mM pyruvate treatments in the presence of dorsomorphin. There was no interaction between glutamine concentration and dorsomorphin (GLUTAMINE X DORSOMORPHIN; F = 152.50; *df* = 15, 496; *p* < 0.0001), confirming that treatment with dorsomorphin did not have an effect on cellular response to glutamine availability. Cold stress appears to block the effects of dorsomorphin on cellular response to higher glutamine concentrations, suggesting that hypothermic culture conditions and dorsomorphin may cause cells to become less sensitive to changes in glutamine availability.

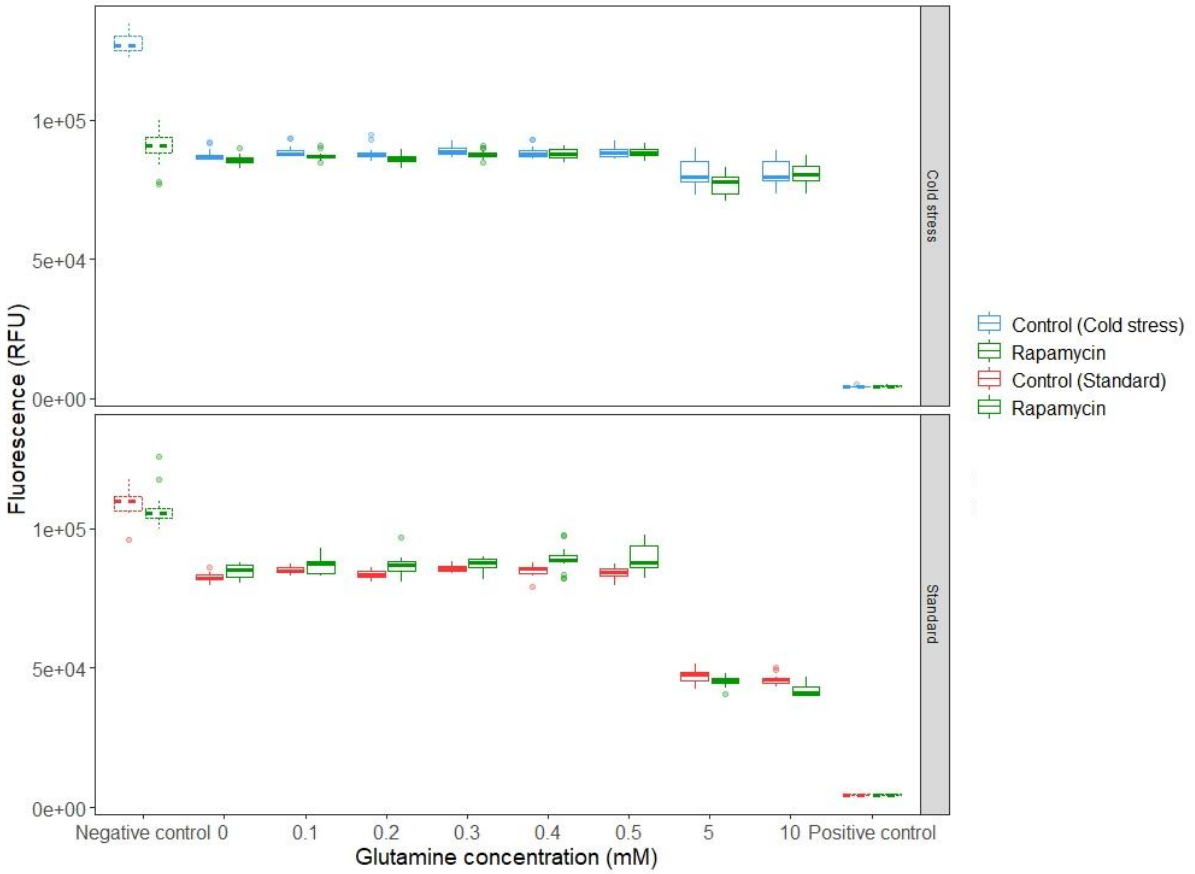


Figure 5.6. P19 embryonal carcinoma cells do not respond to minor increases alanyl glutamine concentration but respond to major increases, at all incubation conditions. Boxplots of resorufin fluorescence values for replicate wells of P19 cells treated with blastocyst media containing increasing concentrations of alanyl glutamine for 48 hours. The effects of cold stress and rapamycin on cellular response to increasing pyruvate concentrations are also shown as resorufin values. Complete αMEM was used as a negative control, and 25% DMSO in αMEM was used as a positive control (dashed-lined boxplots). There is no evidence to support an interaction between cellular responses to treatments, rapamycin or cold stress.

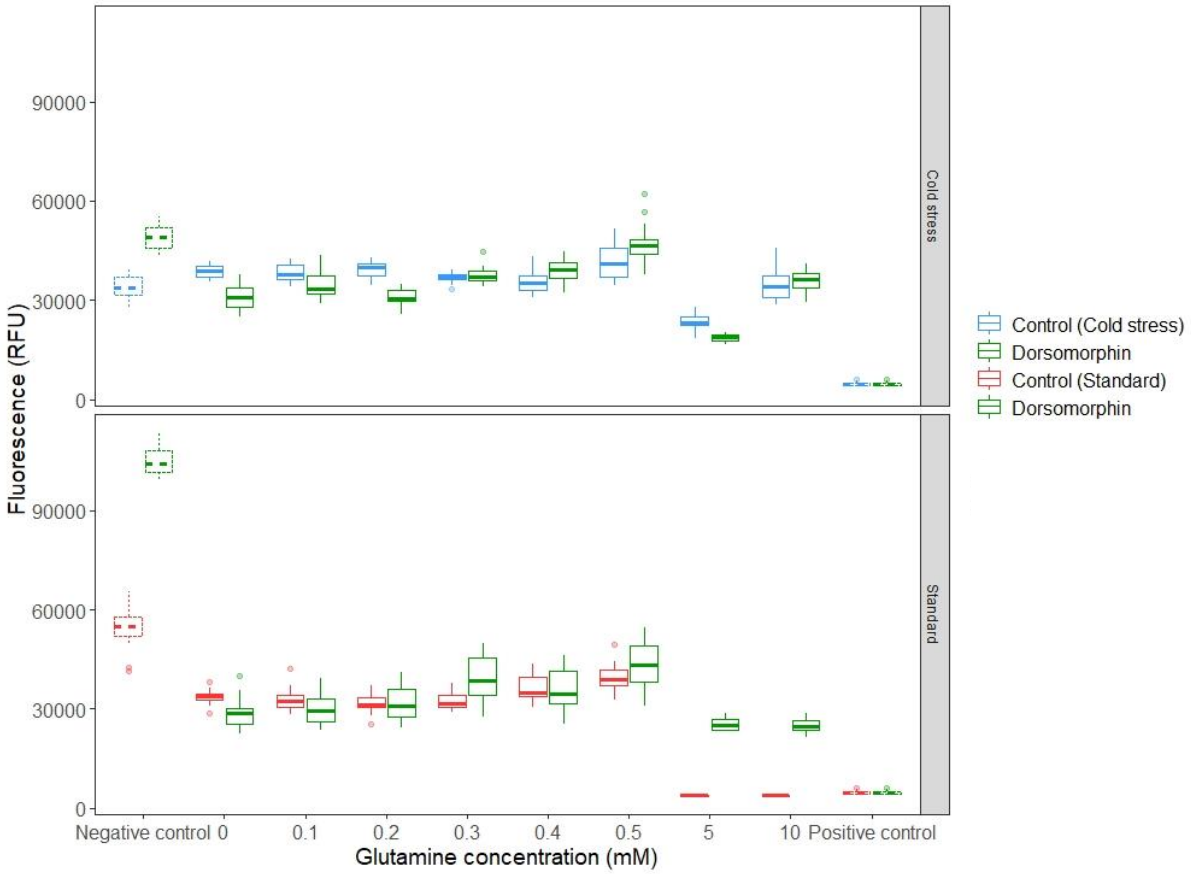


Figure 5.7. Cold stress and dorsomorphin affect the response of P19 embryonal carcinoma cells to increasing concentrations of sodium pyruvate. Boxplots of resorufin fluorescence values for replicate wells of P19 cells treated with blastocyst media containing increasing concentrations of alanyl glutamine for 48 hours. The effects of cold stress and dorsomorphin on cellular response to increasing pyruvate concentrations are also shown as resorufin values. Complete αMEM was used as a negative control, and 25% DMSO in αMEM was used as a positive control (dashed-lined boxplots). There is no evidence to support an interaction between cellular responses to treatments and cold stress or dorsomorphin.

5.3.7. Cold stress and rapamycin do not affect the response of P19 embryonal carcinoma cells to increasing concentrations of calcium lactate.

Exposure to increasing concentrations of calcium lactate at standard culture conditions showed that P19 cells responded to calcium lactate availability (CALCIUM LACTATE; F = 200.6; *df* = 7, 248; $p < 0.0001$), but not to rapamycin (RAPAMYCIN; F = 0.354; *df* = 1, 254; $p = 0.553$), with *post hoc* comparisons indicating significant differences between all treatment groups both in the absence and presence of rapamycin (Figure 5.8). There was no interaction between calcium lactate concentration and rapamycin (CALCIUM LACTATE X RAPAMYCIN; F = 131.70; *df* = 15, 240; $p < 0.0001$), further highlighting that treatment with rapamycin had no effect on cellular response to calcium lactate availability. Consistent with the data above, P19 cell viability was affected by changes in calcium lactate availability (CALCIUM LACTATE; F = 149.4; *df* = 7, 248; $p < 0.0001$), but not treatment with rapamycin (RAPAMYCIN; F = 0.652; *df* = 1, 254; $p = 0.42$) under hypothermic conditions, with *post hoc* comparisons indicating significant differences ($p < 0.05$) between all treatments groups both in the absence and presence of rapamycin. Again, there was no interaction between calcium lactate concentration and rapamycin (CALCIUM LACTATE X RAPAMYCIN; F = 106.20; *df* = 15, 240; $p < 0.0001$), demonstrating the natural sensitivity of P19 cells to changes in calcium lactate concentrations, so much so that neither hypothermic incubation nor treatment with rapamycin could impact cellular response to calcium lactate availability.

5.3.8. Cold stress and dorsomorphin do not affect the response of P19 embryonal carcinoma cells to increasing concentrations of calcium lactate.

Repeat experiments indicate that P19 cells responded to calcium lactate availability (CALCIUM LACTATE; F = 409.60; *df* = 7, 504; $p < 0.0001$), but not to treatment with dorsomorphin (DORSOMORPHIN; F = 0.003; *df* = 1, 510; $p = 0.959$) at standard culture conditions; however,

post hoc comparisons indicated significant differences ($p < 0.05$) between complete blastocyst media, the no calcium lactate and ≥ 5.0 mM calcium lactate treatments in the absence of dorsomorphin, but significant differences complete blastocyst media and all treatment groups in the presence of dorsomorphin (Figure 5.9). An interaction between calcium lactate concentration and dorsomorphin was found (CALCIUM LACTATE X DORSOMORPHIN; F = 1599; *df* = 15, 496; $p < 0.0001$), indicating that treatment with dorsomorphin had no effect on cellular response to calcium lactate availability. Under hypothermic conditions, P19 cells responded to calcium lactate availability (CALCIUM LACTATE; F = 1112; *df* = 7, 504; $p < 0.0001$) and treatment with dorsomorphin (DORSOMORPHIN; F = 6.095; *df* = 1, 510; $p = 0.0139$), with *post hoc* comparison indicating significant differences between complete blastocyst media, the no calcium lactate and ≥ 5.0 mM calcium lactate treatments, but not the other treatments in the absence and presence of dorsomorphin (Figure 5.9). There was no interaction between calcium lactate concentration and dorsomorphin (CALCIUM LACTATE X DORSOMORPHIN; F = 814.1; *df* = 15, 496; $p < 0.0001$), suggesting that cold stress could have reduced the sensitivity of P19 cells to changes in calcium lactate availability. Furthermore, cold stress blocks the effect of dorsomorphin on cellular response to changes in calcium lactate availability.

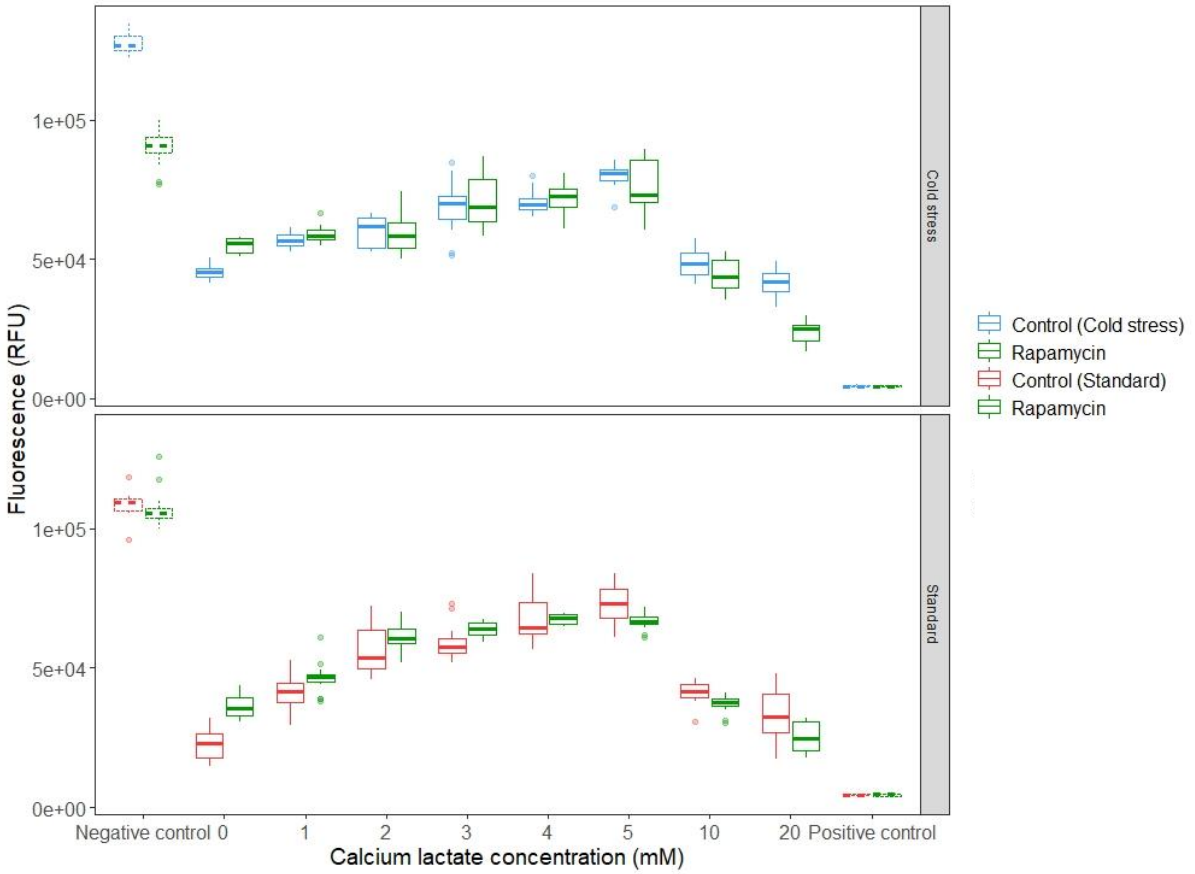


Figure 5.8. P19 embryonal carcinoma cells respond to increasing calcium lactate concentrations at all incubation conditions. Boxplots of resorufin fluorescence values for replicate wells of P19 cells treated with blastocyst media containing increasing concentrations of calcium lactate for 48 hours. The effects of cold stress and rapamycin on cellular response to increasing calcium lactate concentrations are also shown as resorufin values. Complete αMEM was used as a negative control, and 25% DMSO in αMEM was used as a positive control (dashed-lined boxplots). There is no evidence to support an interaction between cell reduction in response to treatments, rapamycin and cold stress.

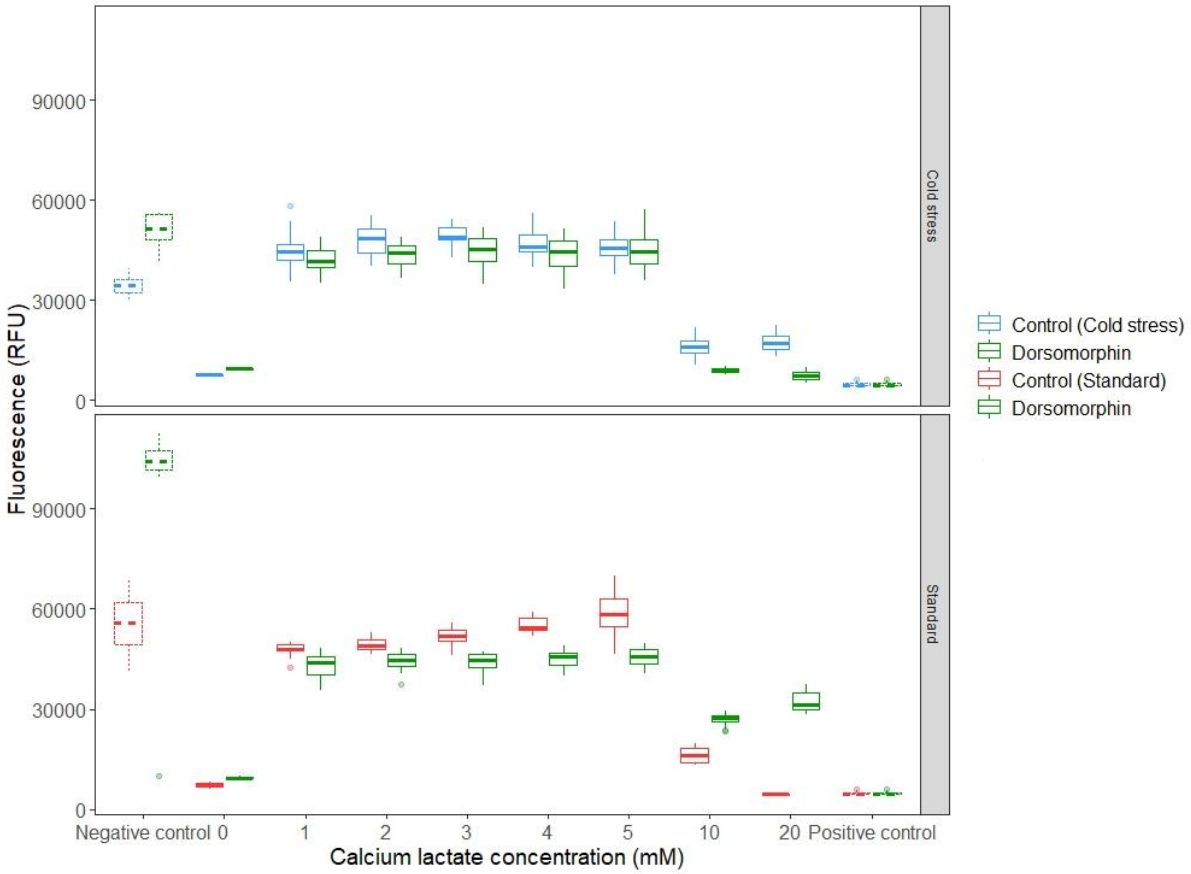


Figure 5.9. Cold stress and dorsomorphin do not affect the response of P19 embryonal carcinoma cells to increasing concentrations of calcium lactate. Boxplot of resorufin fluorescence values for replicate wells of P19 cells treated with blastocyst media containing increasing concentrations of calcium lactate for 48 hours. The effects of cold stress and dorsomorphin on cellular response to increasing calcium lactate concentrations are also shown as resorufin values. Complete αMEM was used as a negative control, and 25% DMSO in αMEM was used as a positive control (dashed-lined boxplots). Dorsomorphin affects cellular response to changes in calcium lactate concentrations, but cold stress does not.

5.4. Discussion

In this chapter, hypothermic incubation, rapamycin and dorsomorphin were investigated as potential tools for refining cellular response to fluctuating concentrations of energy sources in embryo culture media. The overall aim of this was to improve the assay developed in chapter three in order to provide a competitive alternative to the widely used MEA. Rapamycin is an attractive candidate for mTOR inhibition because it exhibits minimal systematic toxicity in both animal and human cells, it is highly lipophilic, is able to penetrate tissue barriers and is also highly specific to mTOR, thereby eliminating the risk of crossover inhibition of other lipid and protein kinases (Feldman and Shokat, 2010 and Kon *et al.*, 2021). Dorsomorphin is also considered to be a suitable inhibitor, and the most commonly used AMPK inhibitor for cell-based assays (Viollet *et al.*, 2010; Dasgupta and Seibel, 2018; Dite *et al.*, 2018; Hurley *et al.*, 2021). Prior to assaying the test products, the optimum working concentrations of both rapamycin and dorsomorphin were pre-determined to ensure that intracellular response to external stressors were stalled without interfering with normal cell function or viability; in order to allow accurate assessment of the test products.

Pyruvate is naturally synthesised during glycolysis in mammalian cells, meaning that its presence in culture media serves as an additional source of energy rather than it being an essential requirement (Abusalamah *et al.* 2020). Accordingly, here it was found that cell growth was not consistently impacted by its absence or low availability (Figure 5.4). Treatment with small incremental increases in pyruvate did not result in significant cell growth, but exposure to more than 0.5 mM pyruvate caused compromised cell viability at standard culture conditions. Previous studies have found that cells initiate moderate cold stress responses when temperatures fall to between 25°C and 35°C, compared with those observed at 10°C or less, suggesting that cells actively grow between 25°C and 35°C (Al-Fageeh and Smales, 2006); this means that cell proliferation may have occurred under the conditions (15°C) applied here. Cells subjected to cold stress in the absence of rapamycin undergo growth arrest

initiated by decreased metabolism, and although these are useful for prolonging cell survival and protecting cells from mechanical damage, this suggests that it causes cells to become less responsive to changes in culture conditions (Al-Fageeh and Smales, 2006; Roobol *et al.*, 2009). Cellular response to the various pyruvate treatments was not reflective of the typical effects of rapamycin, *i.e.*, growth inhibition as a result of uncontrolled protein synthesis (Barilli *et al.*, 2008; Hofmann *et al.*, 2012; Ozates *et al.*, 2021). On the other hand, response to the negative control shows that cell viability was indeed compromised with cold stress and rapamycin. Overall, the impact of different levels of pyruvate on P19 cells was more prominent in the presence of cold stress and rapamycin than at standard incubation, indicating that inhibition of adaptive responses to cold stress enhances cellular response to changes in pyruvate concentrations. Repeat experiments demonstrated the effects of batch-to-batch variation, with P19 cells responding to minor increases in pyruvate when treated with a different batch of blastocyst media. This suggests that the components within blastocyst media directly affect cellular response to fluctuating levels of extracellular pyruvate, and that P19 cells do indeed respond to changes in many of the components within blastocyst media, even if their response to changes in pyruvate is comparably insignificant. In contrast to treatment with rapamycin, P19 cells were less sensitive to minor increases in pyruvate concentration when treated with dorsomorphin at standard incubation conditions and under hypothermic conditions, perhaps because inhibition of AMPK and the subsequent prevention of limited energy-consumption (Leprivier *et al.*, 2013 and Bressan and Saghatelyan, 2020) resulted in available intracellular pyruvate, as a result of uninterrupted glycolysis, causing cells to be less dependent on pyruvate outside the cell.

Glutamine is also a vital source of nutrition for metabolic function and protein synthesis (Cruzat *et al.*, 2018), providing a source of carbon and reduced nitrogen for biosynthetic pathways (Kodama and Nakayama, 2020 and Kodama *et al.*, 2020). Before 1990, it was considered to be a non-essential amino acid (Lacey and Wilmore; Pavlova *et al.*, 2018) and was therefore

made in small amounts to maintain growth or nitrogen balance in mammalians systems. More recent studies have however highlighted additional functions of amino acids, meaning that glutamine is now considered to be conditionally essential during cellular stress, although the exact mechanism underlying this is not fully understood (Wernerman, 2008; Watford, 2016; Leguina-Ruzzi and Cariqueo, 2017). Similar to the pyruvate treatments, cell viability was not significantly impacted by absent or low concentrations of glutamine. This supports previous conclusions that under standard culture conditions, glutamine is a non-essential amino acid, and cells can depend on alternative sources of nitrogen or synthesise their own when glutamine availability is limited. Treatment with ≥ 5 mM glutamine resulted in compromised cell viability at both incubation conditions, meaning that the detrimental effects of elevated glutamine levels is not attributed to the effects of cold stress or rapamycin. Rather, the ≥ 5 mM glutamine treatments caused increased osmolality (≥ 356 mOsmol/kg), thereby introducing another form of *in vitro* toxicity.

Comparable to the pyruvate treatments, cold stress and rapamycin prevented cells from responding to minor alterations in glutamine concentration, confirming that the adaptive response to cold stress prevent cells from accurately responding to changes in culture environment. In repeat experiments, P19 cells responded to minor increases in glutamine concentration when treated with a second batch of blastocyst media, and consistent with the rapamycin treatment, cells were less responsive to minor changes in glutamine concentrations with cold stress, in the presence of dorsomorphin. It is possible that the combination of AMPK inhibition and lack of controlled energy-consumption associated with hypothermic incubation caused cells to synthesise glutamine from the citric acid cycle and ammonium, as they do in the presence of stressors (Wise *et al.* 2008), thereby making them less responsive to glutamine outside the cell.

Lastly, consistent with previous studies (Malhotra and Kaufman, 2007; Wohnsland *et al.*, 2010; Martínez-González *et al.*, 2011), it was found that the removal of calcium lactate was detrimental to P19 cell survival. This is unsurprising considering that calcium lactate promotes mitochondrial biogenesis which is undoubtedly the most vital resource required for energy production, because lactate is oxidised in the mitochondria to produce significant proportions of total intracellular ATP (Tauffenberger *et al.*, 2019). These studies indicated that cells are sensitive to increasing concentrations of calcium lactate, further highlighting its significance in cellular development. This response differed from that of pyruvate and glutamine, where minor increases in concentration did not always result in increased growth and proliferation under standard incubation. It is however important to note that increments in calcium lactate concentration were ten times higher than that of pyruvate and glutamine concentrations, but this is reflective of the relative concentration of each of these substrates in IVF culture media. Cell viability was highest with cold stress and rapamycin, meaning under these conditions cells were least impacted by the lack of, and by low concentrations of calcium lactate, therefore confirming that reduced temperatures inhibits the ability of P19 cells in responding to changing culture conditions. There was a positive correlation between increasing calcium lactate concentrations and cell viability, but no indication of increased assay sensitivity with cold stress and rapamycin. Findings also indicated the extent to which each calcium lactate concentration affected cell function. It is apparent that although cells thrived in the presence of abundant calcium lactate availability, increased concentrations of other substrates may be required to support adequate cell development with the more than 5 mM calcium lactate treatments. As with the pyruvate and glutamine treatments, response to repeated assays demonstrated the inconsistencies associated with batch-top-batch variation, with data indicating conflicting responses of P19 cells to varying calcium lactate concentrations. In contrast to the pyruvate and glutamine treatments, P19 cell were more responsive to changes in calcium lactate concentrations in the presence of dorsomorphin, suggesting that AMPK inhibition causes cells to metabolise calcium lactate at higher rate than that of pyruvate and glutamine, making cells dependent on extracellular calcium lactate when intracellular stores

have been depleted. Consistent with the other experiments, cold stress and its associated mechanisms cause cells to become less responsive to changes in calcium lactate availability.

As summarised by Figure 5.10., cellular responses to changes in pyruvate concentration were most significant with cold stress and mTOR inhibition, with P19 cells responding to minor increases in pyruvate availability exclusively under hypothermic conditions but also with hypothermic incubation and rapamycin. Conversely, the effect of AMPK inhibition by dorsomorphin was counterproductive and P19 cells were less responsive to fluctuating pyruvate concentrations with dorsomorphin treatment at standard and hypothermic incubation. Cellular responses to glutamine were least affected by cold stress and mTOR and AMPK inhibition, with the most significant finding deriving from the reduced response to fluctuating levels caused by dorsomorphin treatment at standard incubation. Although these findings suggest that AMPK inhibition reduces the ability of cells to respond to fluctuating energy sources, treatment with the various calcium lactate formulations and dorsomorphin at standard incubation showed that P19 cells were more responsive to calcium lactate availability with AMPK inhibition. This demonstrates that AMPK plays a key role in the energy sensory machinery of cells and is particularly affected by calcium lactate availability which serves as the most significant substrate in intracellular ATP production (Sonveaux *et al.*, 2008; Knight *et al.*, 2015).

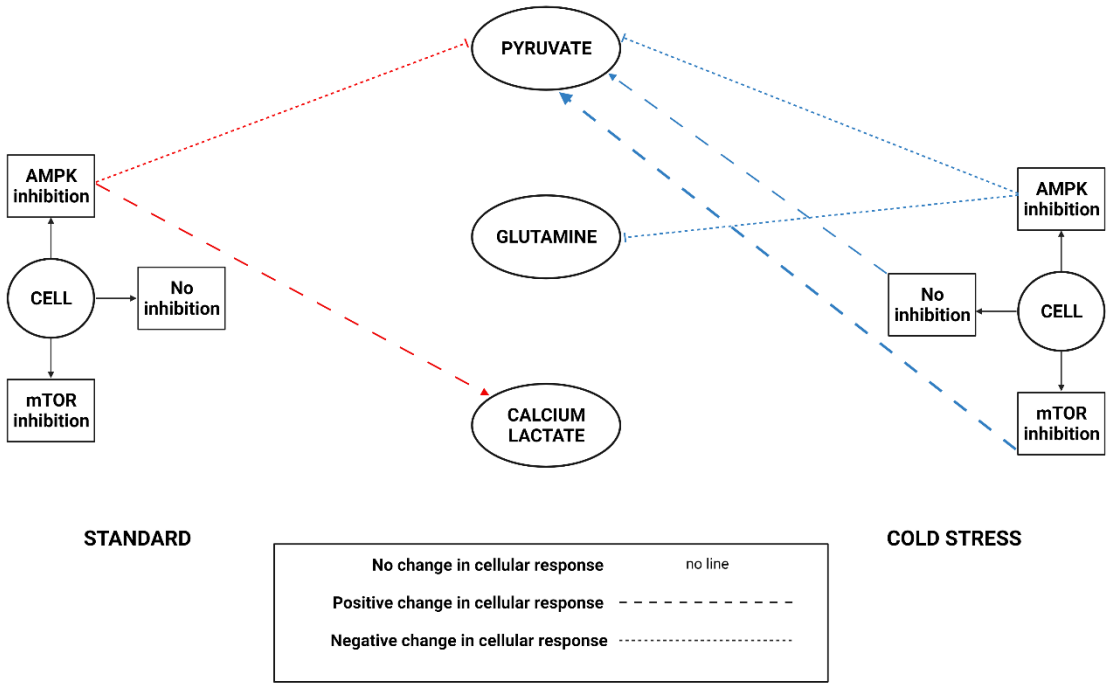


Figure 5.10. Schematic representation of the effects of mTOR and AMPK inhibition on the response of P19 embryonal carcinoma cells to fluctuating concentrations of energy sources at standard incubation (left) and with cold stress (right) as shown in this thesis. Absent lines indicate comparative responses to fluctuating energy sources, as that shown by the no mTOR and AMPK treatments at standard incubation. Dashed lines show the conditions that induced increased response to various treatments whilst dotted lines highlight the conditions that reduced response to fluctuating energy sources. Red lines represent incubation at standard culture conditions and blue lines represent hypothermic incubation. Cold stress and AMPK inhibition had a greater effect on cellular response to fluctuating energy sources than standard incubation and mTOR inhibition.

In conclusion, the P19 assay described here is sensitive to large changes in culture conditions, but less able to detect minor changes. Exposing cells to cold stress in the presence of rapamycin resulted in improved cellular responses to perturbation in media composition. On the other hand, given the response other mammalian cell lines have to mTOR inhibition (Kaur and Sharma, 2017; Tee, 2018) it is not surprising that treatment with rapamycin in the absence of a cold stress significantly reduced cell growth. Previous studies on the depletion of nutrients by mouse and human embryos suggest that reduced metabolic rates are consistent with viable phenotypes (Leese *et al.*, 2008; Ermisch *et al.*, 2020), which explains why cold stress responses could result in high cell viability. The effects of dorsomorphin alternated between the different energy sources, suggesting that it is easier to manipulate cellular response through treatment with rapamycin, as opposed to dorsomorphin. This can be attributed to the

fact that dorsomorphin is an inhibitor of bone morphogenic protein signalling in embryonic stem cells (Yu *et al.*, 2008), as well as AMPK signalling, whereas the inhibitory effects of rapamycin is exclusive to mTOR. Overall, these studies indicate the extent to which cell viability is impacted by each energy substrate, for instance, cellular response to the absence of calcium lactate is comparable to cell death, whilst adequate cell growth and proliferation occurred in the absence of pyruvate and glutamine. Importantly, cell sensitivity was amplified by exposure to cold stress, rapamycin and to some effect, dorsomorphin; as such this offers a novel tool that could be used to manipulate cellular response to various culture conditions.

Given that these results indicated both rapamycin and dorsomorphin alter the ability of P19 cells to respond to changes in energy substrate availability, the next chapter in this thesis further explored the effects of rapamycin, dorsomorphin, and their combination on cellular response to changes in culture medium. It was expected that the combined effects of mTOR and AMPK inhibition would cause P19 cells to indicate more subtle changes in culture conditions than those described in this chapter.

6. Thesis aim four: To use the cell-based assay developed in chapter three to identify suboptimal embryo culture media with a view to improve current research and development practices in the IVF sector.

6.1. Background

Improvements to culture media have been of great importance to the increased success seen in human IVF over the last two decades (Chronopoulou and Harper, 2015). As described in section 1.4.1, media quality control directly impacts IVF success, and bioassays are a widely used and well-accepted means of assessing this. The most common bioassays used in the IVF industry include one and two-cell mouse embryos, zona-free mouse embryos, mouse hybridoma cells and human fibroblast cells (Fleming, Pratt and Braude, 1987; Bavister and Andrews, 1988; Davidson *et al.*, 1988; Bertheussen *et al.*, 1989; Gorrill *et al.*, 1991; Gilbert *et al.*, 2016); however, the one-cell mouse embryo assays (MEAs) represent the gold standard for quality assessment (Ainsworth et al. 2015; Gilbert et al. 2016; Herrick et al. 2016). MEAs have however been criticised for only indicating extreme differences in media quality (Ainsworth et al. 2015; Gilbert et al. 2016; Herrick et al. 2016), with mouse embryos often able to develop in suboptimal culture media (Morbeck, Krisher and Herrick, 2014). Moreover, studies show variation in assay sensitivity between embryos from different strains of mice and suggest that F1 hybrid embryos, the current industry standard, may not be the most sensitive (Khan *et al.*, 2013). It is also recommended that the timing of the first three cell division cycles is useful in determining the developmental potential of the early embryo (Cruz *et al.*, 2012). This information cannot however be determined from current MEA methods as the assay does not assess any pre-blastocyst stage events, providing only an endpoint analysis of blastocyst development after 96 hours of incubation (U.S. Food and Drug Administration, 2019). On the other hand, analysis of the MEA sample size (which is typically 21 embryos per test) shows that a high degree of confidence can be given to the effect size; that is, its ability to detect an effect of any magnitude (Wolff *et al.*, 2013; Van Osselaer, 2018; Ledolter and Kardon, 2020).

Primary cell cultures and immortal cell lines provide an alternative to the MEA for detecting toxicity in IVF media. It is well known that cells in culture can be particularly sensitive to media toxicity (Spielmann *et al.*, 1997); however, previous studies have used cell lines established from specialised somatic cells. These do not exhibit properties of early embryonic development such as differentiation and are therefore unsuitable for detecting toxicity in embryo culture environments (Bertheussen *et al.*, 1989). More recent studies have involved the use of pluripotent embryonic stem cells of human or mouse origin, which can differentiate into the three primary germ layers (Festag, Viertel, Steinberg and Vitro, 2007). For example, the embryonic stem cell test (EST), an *in vitro* assay designed to assess the teratogenic nature of drugs and substances that may come into contact with *in vivo* embryos has been thoroughly investigated (Spielmann *et al.*, 1997; Seiler and Spielmann, 2011). Given however that the EST was designed for use in drug development, where adverse effects on embryonic development are often amongst the most harmful side-effects of drugs, it is not deemed suitable for use with IVF products, where the detection of more subtle effects is vital. A novel, highly sensitive alternative to the MEA and EST is therefore of great importance, for improved quality control in IVF laboratories.

Highly conserved signalling pathways controlled by mammalian target of rapamycin (mTOR) and AMP activated protein kinase (AMPK) are central to cellular metabolism and cell proliferation. As described in section 1.9, mTOR inhibitor rapamycin and AMPK inhibitor dorsomorphin represent two of the most documented protein kinase inhibitors. The two inhibitors are typically used in separate experiments; however, previous studies reveal fundamental, bi-directional regulation between the two metabolic signalling networks, and pharmacological inhibition of mTOR signalling leads to AMPK activation in the absence of increased AMP:ATP ratios and under nutrient stress conditions (González *et al.*, 2020; Ling *et al.*, 2020). These effects have been associated with growth inhibition in yeast and human cell cultures. Previous attempts to use cell culture systems for quality control in IVF have failed because cell survival has been too high and therefore not indicative of the true nature of

products tested (Bertheussen *et al.*, 1989). As discussed in chapter five, for the first time two kinase inhibitors were incorporated into the same cell culture system and were shown to reduce cellular resistance to suboptimal conditions, providing a solution for improving previous quality control systems.

The previous chapters described optimising and using 2D and 3D cell-based assays to assess the impact of suboptimal culture conditions on cellular growth, and determining P19 cell sensitivity to treatment in the absence of vital energy sources and increasing concentrations of substrates, in the presence and absence of rapamycin and dorsomorphin.

With this in mind, the specific objectives of this chapter were to:

1) Design an experiment to systematically screen various formulations of blastocyst media, to identify the optimum concentrations of sodium pyruvate, L-glutamine and calcium lactate for P19 cell growth.

2) Further analyse the effects of mTOR and AMPK inhibition on cellular response to the various embryo culture media formulations.

3) Compare the findings obtained from the P19 2D cell-based assay with those obtained from the MEA and identify similarities and differences between the two assays.

6.2. Materials and methods

To identify the optimum concentrations of sodium pyruvate, L-glutamine and calcium lactate for P19 cell growth, 54 x 96 well plates and test substances were prepared as described in sections 2.6.1 and 2.7 respectively. Cells were treated with increasing concentrations of pyruvate, calcium lactate and glutamine, and also with rapamycin, dorsomorphin and a combination of rapamycin and dorsomorphin, as detailed in Table 2.1. Cell viability was determined after 48 hours of treatment as described in section 2.4.2. In a second set of experiments, the MEA and cell-based assays were prepared as described in sections 2.9.1, 2.9.2 and 2.6.1, and treated with Geri medium as detailed in Table 2.1 and section 2.9.3. For the cell-based assay, cell viability was determined as described in section 2.4.2, and the data obtained were analysed as per section 2.10.

6.3. Results

6.3.1. P19 embryonal carcinoma cells respond to pharmacological induction by rapamycin and dorsomorphin, and indicate the optimum concentrations of sodium pyruvate and L-glutamine for human blastocyst media.

Assessed by resorufin fluorescence, P19 cells responded to changes in pyruvate and glutamine concentrations (PYRUVATE & GLUTAMINE; $F = 82.38$; $df = 35, 1692$; $p < 0.0001$), and to mTOR and AMPK inhibition (KINASE INHIBITORS; $F = 31.97$; $df = 3, 1724$; $p < 0.0001$), with *post hoc* comparisons indicating significant differences ($p < 0.05$) between the no kinase inhibitor treatment and the dorsomorphin, and rapamycin and dorsomorphin combination treatments, but not the rapamycin treatment ($p = 0.991$). There was an interaction between cellular response to pyruvate and glutamine concentration, and treatment with the kinase inhibitors (PYRUVATE & GLUTAMINE X KINASE INHIBITORS; $F = 56.34$; $df = 143, 1584$; $p < 0.0001$), indicating that response to fluctuating pyruvate and glutamine levels, and mTOR and AMPK inhibition are linked. Further comparisons indicated that in the absence of kinase inhibitors, P19 cells did not respond to variations in pyruvate availability ($p > 0.05$ for *post hoc* comparison with the no energy sources formulation), but responded to increasing levels of glutamine, provided the formulation contained at least 0.1 mM pyruvate. There were no significant differences between the ≥ 0.1 mM pyruvate and ≥ 0.1 mM glutamine treatments; however, cell viability was significantly reduced by the 0.5 mM pyruvate and ≥ 0.3 mM glutamine treatments. Similarly, P19 cells did not respond to pyruvate fluctuations, but responded to glutamine fluctuations when treated with rapamycin; however, cell growth increased in the absence of pyruvate with the ≥ 0.2 mM glutamine treatments. Furthermore, cell growth was increased by treatment in the absence of glutamine with formulations containing ≥ 0.4 mM pyruvate, and there were no significant differences between these and treatment with ≥ 0.1 mM pyruvate and ≥ 0.1 mM glutamine. Conversely, treatment with dorsomorphin caused cells to respond to changes in both pyruvate and glutamine

concentrations, with significant cell growth occurring with the 0.1 mM pyruvate and 0.1 mM glutamine treatment ($p < 0.05$ for *post hoc* comparison with the no energy sources formulation). Cell growth declined with the ≥ 0.2 mM pyruvate and 0.5 mM glutamine treatments; however, optimum cell growth was observed with the 0.1mM pyruvate and 0.5mM glutamine, and the 0.5 mM pyruvate and 0.1 mM glutamine treatments, indicating that P19 cells accommodate high concentrations of pyruvate under low glutamine concentrations, and vice versa. Finally, as expected, given the effects of rapamycin and dorsomorphin, P19 cells responded to fluctuations in pyruvate and glutamine concentrations when treated with the rapamycin and dorsomorphin combination, with significant cell growth observed when cells were treated with ≤ 0.2mM pyruvate and ≥ 0.3mM glutamine. Cell viability was detrimentally affected by the 0.5 mM pyruvate and 0.5 mM glutamine treatment ($p > 0.05$ for *post hoc* comparison with the no energy sources formulation), suggesting that the dual effects of rapamycin and dorsomorphin cause P19 cells to be less accommodating of changes in pyruvate and glutamine concentration (Figure 6.1).

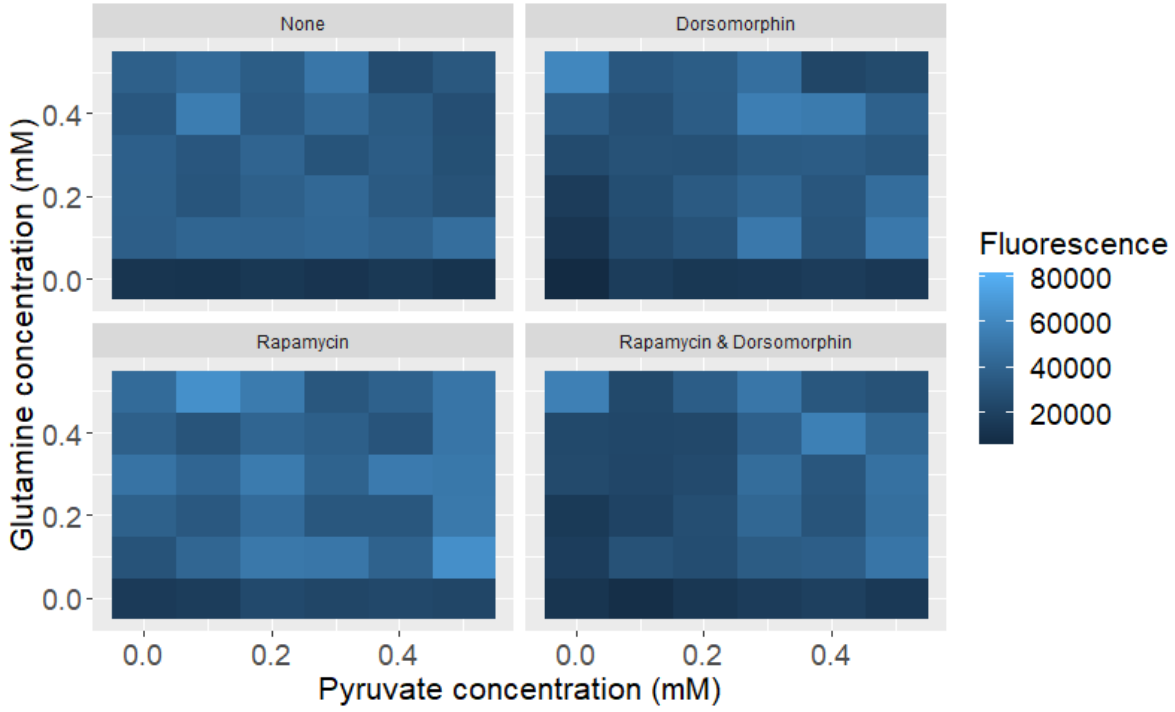


Figure 6.1. P19 embryonal carcinoma cells respond to pharmacological induction by rapamycin and dorsomorphin and indicate the optimum concentrations of sodium pyruvate and L-glutamine for human blastocyst media. Resorufin fluorescence values for replicate wells of P19 cells treated with various blastocyst media formulations containing increasing concentrations of pyruvate and glutamine for 48 hours. The effects of rapamycin, dorsomorphin and a combination of rapamycin and dorsomorphin are also shown. Treatment with rapamycin and dorsomorphin caused significantly reduced cell growth ($p < 0.05$ for *post hoc* comparison with the standard treatment), but indicated the optimum concentrations of pyruvate and glutamine for P19 cell growth (the light blue boxes in D; 0 mM pyruvate and 0.5 mM glutamine, and 0.4 mM pyruvate and 0.4 mM glutamine).

6.3.2. P19 embryonal carcinoma cells respond to pharmacological induction by dorsomorphin and indicate the optimum concentrations of sodium pyruvate and calcium lactate for human blastocyst media.

P19 cells responded to changes in pyruvate and calcium lactate concentrations (PYRUVATE & CALCIUM LACTATE; $F = 382.1$; $df = 35, 1692$; $p < 0.0001$), and to mTOR and AMPK inhibition (KINASE INHIBITORS; $F = 30.76$; $df = 3, 1724$; $p < 0.0001$), with *post hoc* comparisons indicating significant differences ($p < 0.0001$) between the no kinase inhibitor treatment and the dorsomorphin, and rapamycin and dorsomorphin combination treatments, but not the rapamycin treatment ($p = 0.839$)(Figure 6.2). There was an interaction between cellular response to pyruvate and calcium lactate concentration, and treatment with kinase inhibitors (PYRUVATE & CALCIUM LACTATE X KINASE INHIBITORS; $F = 301.3$; $df = 143, 1584$; $p < 0.0001$), confirming that the response to changes in energy source availability, and mTOR and AMPK inhibition are linked. As expected, given the effects of pyruvate and calcium lactate in previous experiments (chapter five), P19 cells responded to changes in pyruvate concentration when treated with the formulations containing calcium lactate, but responded to changes in calcium lactate availability in the absence of pyruvate, with the no kinase inhibitor treatment and also with rapamycin. *Post hoc* comparison indicated significant differences ($p < 0.05$) between all treatment groups except the 0.1 mM pyruvate and 1.0 mM calcium lactate, and the 0.2 mM pyruvate and 2.0 mM calcium lactate treatments, suggesting that both treatments generate similar proportions of intracellular ATP. Treatment with dorsomorphin and the rapamycin and

dorsomorphin combination caused cells to respond to changes in pyruvate availability in the absence of calcium lactate, but not in its presence, and there were significant differences between all treatment groups except the 0.1 mM pyruvate and 1.0 mM calcium lactate, 0.2 mM pyruvate and 2.0 mM calcium lactate, and 0.0 mM pyruvate and 4.0 mM calcium lactate treatment, indicating that 4.0 mM calcium lactate provides sufficient ATP, to mask the effects of the removal of pyruvate. Finally, cell viability was reduced by treatment with 0.5 mM pyruvate and 5.0 mM calcium lactate, in the presence and absence of kinase inhibitors.

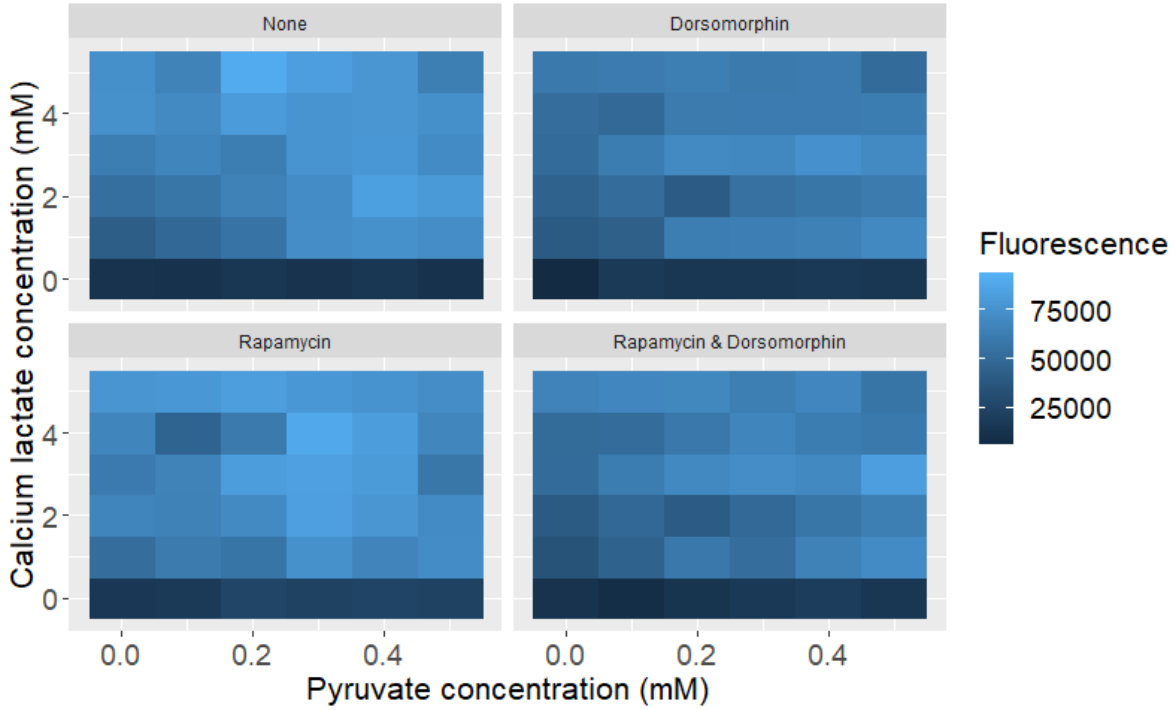


Figure 6.2. P19 embryonal carcinoma cells respond to pharmacological induction by dorsomorphin and indicate the optimum concentrations of sodium pyruvate and calcium lactate for human blastocyst media. Resorufin fluorescence values for replicate wells of P19 cells treated with various blastocyst media formulations containing increasing concentrations of pyruvate and calcium lactate for 48 hours. The effects of rapamycin, dorsomorphin and a combination of rapamycin and dorsomorphin are also shown. P19 cells were naturally sensitive to variations in calcium lactate concentration, with and without rapamycin and dorsomorphin treatment, but kinase inhibition was required to induce mechanisms that cause cells to respond to variations in pyruvate concentration.

6.3.3. P19 embryonal carcinoma cells respond to pharmacological induction by rapamycin and dorsomorphin and indicate the optimum concentrations of L-glutamine and calcium lactate for human blastocyst media.

P19 cells responded to changes in glutamine and calcium lactate concentrations (GLUTAMINE & CALCIUM LACTATE; $F = 103.8$; $df = 35, 1692$; $p < 0.0001$), and to mTOR and AMPK inhibition (KINASE INHIBITORS; $F = 42.59$; $df = 3, 1724$; $p < 0.0001$), with *post hoc* comparisons indicating significant differences ($p < 0.0001$) between the no kinase inhibitor treatment and the dorsomorphin, and rapamycin and dorsomorphin combination treatments, but not the rapamycin treatment ($p = 0.998$)(Figure 6.3). Again, there was an interaction between cellular response to pyruvate and calcium lactate concentration, and treatment with kinase inhibitors (GLUTAMINE X CALCIUM LACTATE X KINASE INHIBITORS; $F = 58.07$; $df = 143, 1584$; $p < 0.0001$), indicating that the response to changes in glutamine and calcium lactate availability, and mTOR and AMPK inhibition are linked. *Post hoc* analysis indicated no significant differences between the 1 and 3 mM calcium lactate treatments in the presence and absence of kinase inhibitors, and reduced cell viability with the 0.1 and 0.3 mM pyruvate formulations when cells were treated with rapamycin and dorsomorphin. Treatment in the absence and presence of rapamycin caused significantly reduced cell growth with 0.5 mM glutamine and ≥ 2 mM calcium lactate formulations whereas treatment with dorsomorphin caused cells to increase in viability with the 0.5 mM glutamine and 5 mM calcium lactate. Overall, cells at the highest rate when treated with low concentrations of glutamine (0.1 and 0.3 mM) and high concentrations of calcium lactate (4 and 5 mM), and there were no significant differences ($p > 0.05$) between these treatment groups.

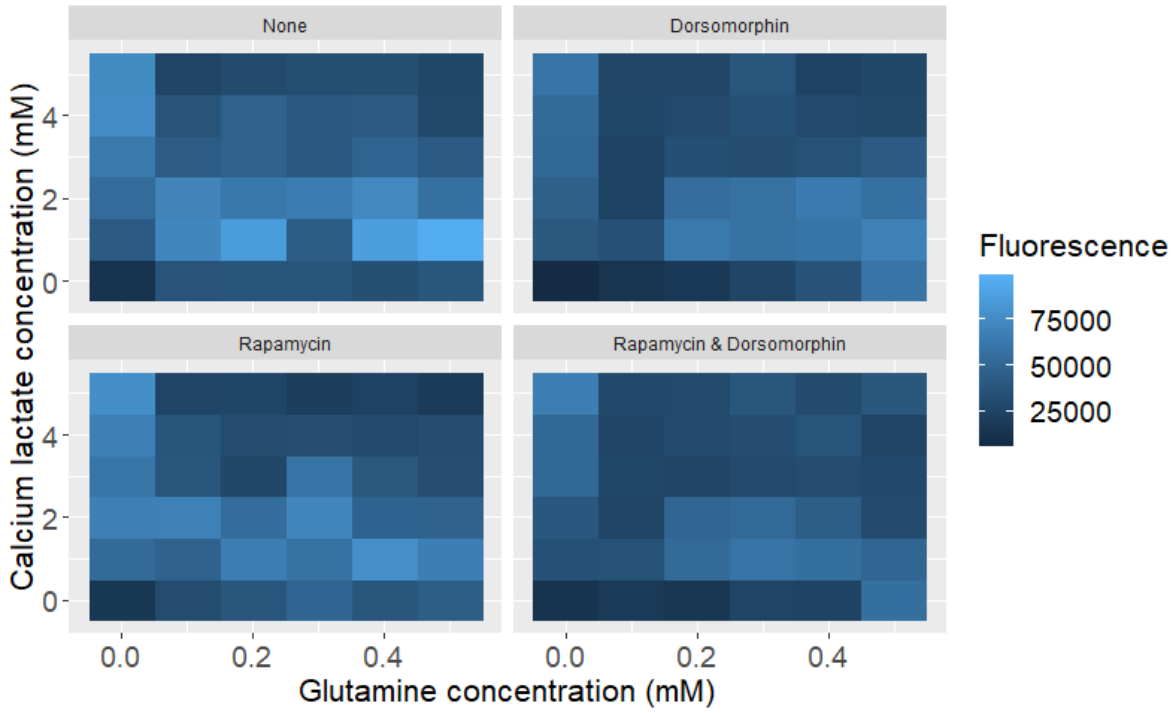


Figure 6.3. P19 embryonal carcinoma cells respond to pharmacological induction by rapamycin and dorsomorphin, and indicate the optimum concentrations of L-glutamine and calcium lactate for human blastocyst media. Resorufin fluorescence values for replicate wells of P19 cells treated with various blastocyst media formulations containing increasing concentrations of glutamine and calcium lactate for 48 hours. The effects of rapamycin, dorsomorphin and a combination of rapamycin and dorsomorphin are also shown. P19 cells were naturally sensitive to variations in calcium lactate concentration, with and without rapamycin and dorsomorphin treatment, but kinase inhibition was required to induce mechanisms that cause cells to respond to variations in glutamine concentration.

6.3.4. P19 embryonal carcinoma cells responded to different Geri media formulations following treatment with rapamycin and dorsomorphin, but the MEA did not.

As can be seen in Figures 6.4 and Figure 6.5 A, C57BL/6NCrl mouse embryos developed into blastocysts after 96 hours of treatment with the negative control and both Geri media formulations (A & B), but not with the positive control, with overall blastocyst development rates of 17% for the negative control (one of six viable embryos), 36% for the media A treatment (four of 11 viable embryos), 58% for the media B treatment (seven of 12 viable embryos) and 0% for the positive control treatment (zero of five viable embryos). Embryo development was particularly advanced with the media B treatment where five out of the seven blastocysts progressed to the hatching stage (see section 1.2.3), however Pearson's Chi-

squared test indicated no significant differences in embryo development (to the blastocyst stage) between the two treatments (Chi-squared = 0.14; *df* = 1; *p* = 0.71). Conversely, P19 cells with inhibited mTOR and AMPK activity responded differently to the two Geri medium formulations (Figure 6.5 B, F = 1519; *df* = 1; *p* < 0.0001). Assessed by the colour change from blue to fluorescent pink associated with resazurin reduction, P19 cells grew best with the formulation B treatment in comparison to treatment with formulation A. Given that formulation B contained increased concentrations of amino acids, specifically L-Carnitine, these results indicate that these components play a critical role in P19 cell growth.

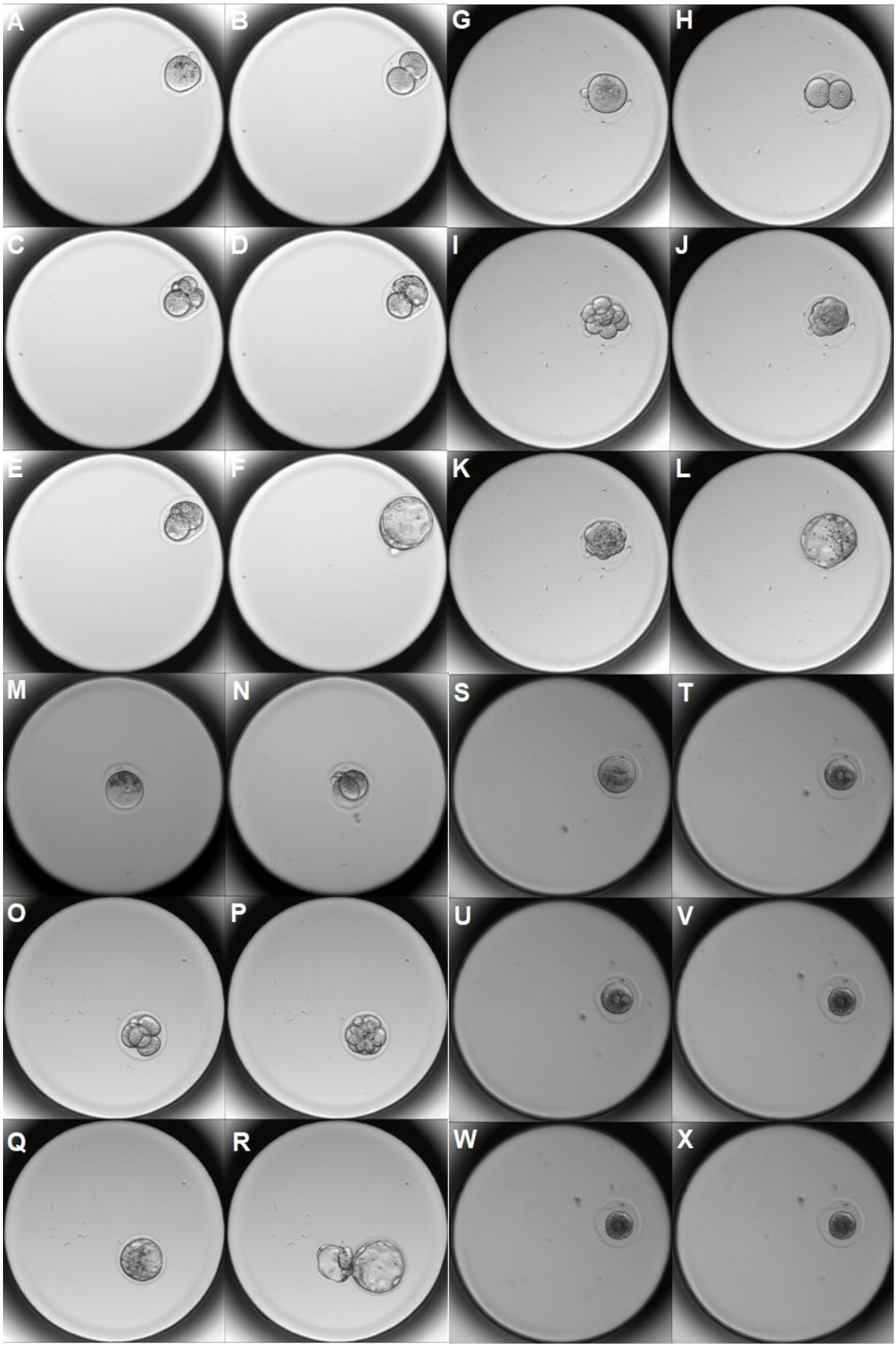

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Figure 6.4. Mouse embryo response to different Geri media formulations. Shown are images of C57BL/6NCrl mouse embryos treated with αMEM as a negative control (image A – F), contaminated Geri medium as a positive control (image S – X), and formulations A (image G – L) and B (image M – R) for 96 hours in a Geri time lapse incubator. Images were captured intermittently and the key developmental stages (two-cell, morula and blastocyst stages) are shown above. Embryos treated with the positive control failed to develop and underwent growth arrest at the one-cell stage.

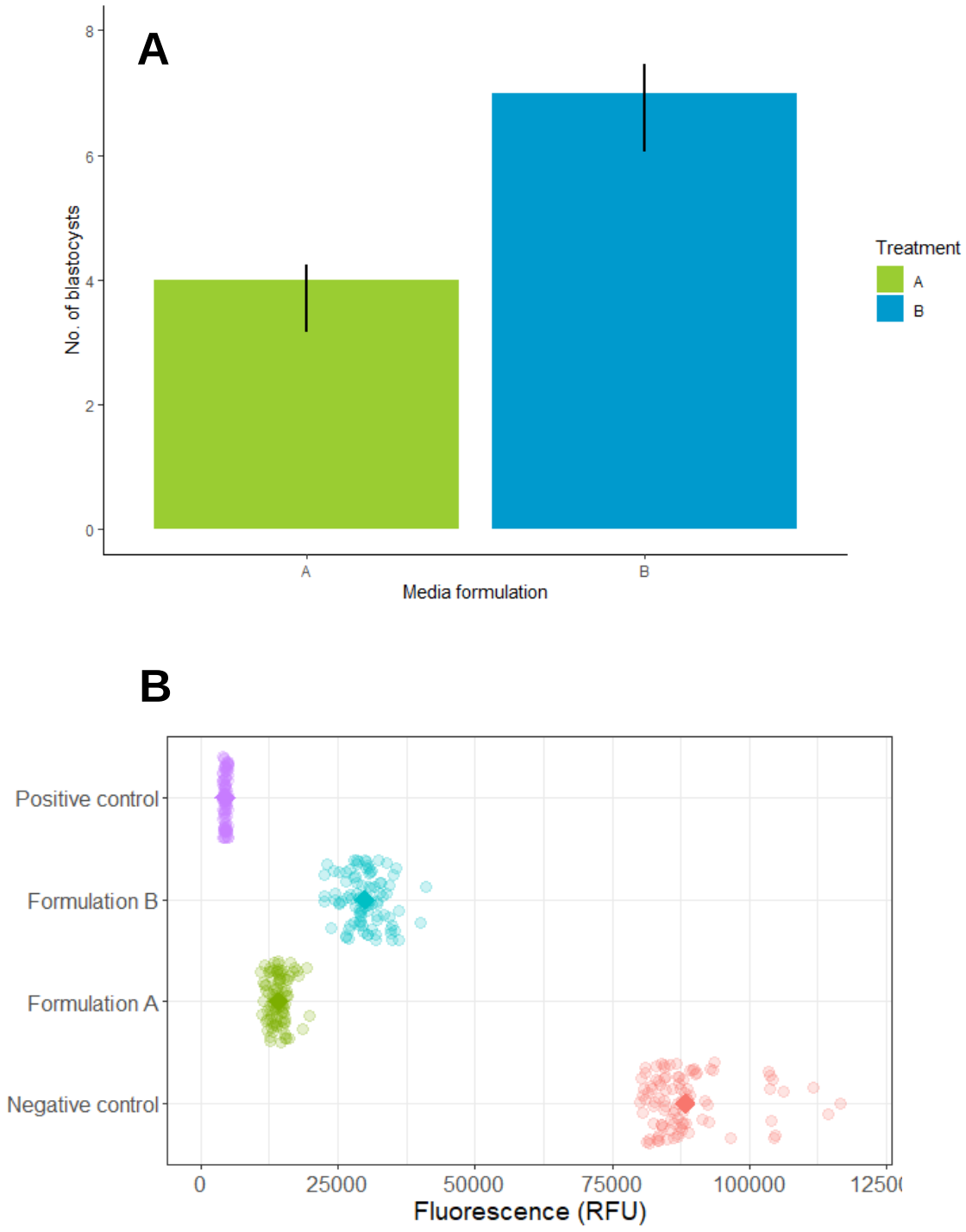

Figure 6.5. P19 embryonal carcinoma cells responded to different Geri media formulations following treatment with rapamycin and dorsomorphin, but the MEA did not. Illustrated in panel (A) are the total number of C57BL/6NCrl mouse embryos that developed to the blastocyst stage when treated with two formulations of Geri media for 96 hours (Each bar is indicative of the proportion). No

significant differences in embryo development were detected between the two treatments at the 95% confidence interval (Blastocyst development; Chi-squared = 0.14; *df* = 1; *p* = 0.71). Panel (B) shows resorufin fluorescence values for replicate wells of P19 cells treated with different Geri media formulations for 48 hours. Complete αMEM was used as a negative control and 25% DMSO was used as a positive control. Median values for each group are shown by the solid symbol. Pairwise comparisons indicate that the median fluorescence of both treatment groups differ significantly from the controls and from each other ($p < 0.05$, *post hoc* tests).

6.4. Discussion

Previous chapters explored the natural sensitivity of P19 cells to changes in media formulation, osmolality, energy sources and vitamin supplementation, as well as the effects of hypothermic incubation and mTOR and AMPK inhibition. Here, previous findings were used to systematically screen 108 different media formulations in order to characterise the optimum concentration of pyruvate, glutamine, and calcium lactate for human blastocyst culture medium. As previously mentioned in chapter three, blastocyst media was chosen as a suitable test product because it yields high rates of cell growth and is versatile in its composition, allowing it to be easily manipulated. Furthermore, the concentration of base components tends to stay the same between various types of IVF media, whereas the concentrations of glucose, pyruvate, glutamine, and calcium lactate differ considerably to accommodate the changing needs of the developing embryo. Consequently, when developing an assay for the purpose of assessing the quality of IVF media, it is important to consider its response to various energy sources. Numerous studies on the effects of glucose on embryo development already exist (Schini and Bavister, 1988; De Silva, 1998; de Lima *et al.*, 2020), but that there are very few on the effects of pyruvate, glutamine and calcium lactate, despite their well-known roles in media, these components were focussed on here. A statistical framework was also implemented to assess the effects of the various energy sources and kinase inhibitors as well as their various interactions. Hypothermic induction was not used as a tool for increasing assay sensitivity because chapter five indicated that the growth arrest often associated with cold stress (Roobol *et al.*, 2009; Adjirackor, Harvey and Harvey, 2020) reduces the ability of cells to respond to minor changes in pyruvate and glutamine.

In general, P19 cells demonstrate an ability to respond to alterations in culture conditions, even without pharmacological manipulation. Initial analysis suggested that mTOR and AMPK inhibition did not affect cellular response to alterations in media formulation; however, treatment with a combination of rapamycin and dorsomorphin amplified cell sensitivity to alterations in energy source availability. Previous studies typically applied one protein kinase inhibitor, with rapamycin being the most common of these. Here however, two kinase inhibitors were simultaneously used to treat cells, providing new insight on cellular response to kinase inhibition (Heitman, Movva and Hall, 1991; Geoerger *et al.*, 2001; Yu *et al.*, 2008; Chresta *et al.*, 2010). Response to the no calcium lactate formulations indicated that P19 cells require mTOR and AMPK inhibition in order to respond to minor increases in both pyruvate and glutamine (Figure 6.1). This is perhaps due to the fact that stress increases glycolysis through AMPK activation (Queiroz *et al.*, 2014). Subsequently, in addition to inhibiting the cells' ability to monitor ATP consumption (Ke *et al.*, 2018), treatment with dorsomorphin and the resulting AMPK inhibition reduces intracellular pyruvate production (a by-product of reduced glycolysis). Particularly with environmental perturbations, the cells' dependency on extracellular pyruvate is therefore likely to increase. In addition, treatment with rapamycin and dorsomorphin indicated a window for the minimum and maximum amounts of pyruvate and glutamine required for optimum cell growth whilst demonstrating enhanced sensitivity to increased osmolality (evidenced by cellular response to more than 3 mM pyruvate and/or glutamine), providing a means of determining exact product concentration for human IVF media development when using P19 cells as a model.

Consistent with observations made in chapter five, P19 cells were highly sensitive to changes in calcium lactate availability, thus, responses to the various calcium lactate formulations provided sufficient insight, even without mTOR and AMPK inhibition. The rapamycin and dorsomorphin treatments, however, indicate similarities in intracellular ATP production

between the various treatments, and also the amount of calcium lactate required for optimum P19 cell growth. Again, this is perhaps the result of the cells' inability to monitor energy expenditure, so once intracellular energy sources stores have been used up, cells become dependent on extracellular supplies, making it possible to determine the most suitable formulation for survival and growth.

Treatment with the different Geri media formulations showed that P19 cells are sensitive to minute changes in culture conditions. Despite the exact formulation of the treatments used here being unknown, they were both variants of the same type of medium. Using the same treatments, the MEA were simultaneously conducted, but the response differed from that of the 2D assay. Statistical analysis showed that the 2D assay identified differences between the two treatments, but the MEA did not. On the other hand, formulation B seemed to be the best media for cell proliferation in both assays in comparison to treatment with formulation A, resulting in increased P19 cell growth and hatched mouse blastocysts. Specific to the MEA, approximately one third of the embryos had at least two sperm cells attached to the zona pellucida, indicating possible incidences of polyspermy during fertilisation (Yanagimachi, 2005; Cheeseman *et al.*, 2016). Therefore, the low blastocyst development rates observed with all treatments could be attributed to abnormal embryo development as opposed to poor medium quality. Furthermore, because the food and drug administration recommends a minimum of 21 mouse embryos for each test article (Food and drug administration (US), 2021) whereas here, ≥12 mouse embryos were used to test each product, it was important to consider the effects of sample size. It is known that the power of a study increases as its sample size increases (Suresh and Chandrashekara, 2012); however, a large number of embryos would be required to identify differences of the scale seen in the 2D assay, many more than would be required to meet the FDA recommendations.

Here, the benefits of the P19 2D assay are highlighted, because as well as providing numerical indicators of cell growth and proliferation, the associated methods can be repeated quickly without incurring major additional costs. On the other hand, the MEA offers distinct advantages over the P19 2D assay including its ability to monitor the changing morphology of embryos over the entire treatment period (Wolff *et al.*, 2013; Gilbert *et al.*, 2016); a feature that is impossible to mimic with the P19 2D assay. It also indicates the effects of various media components at key developmental stages such as the cleavage and embryo hatching stage. In this way, the MEA may offer early signs of product quality, which is again, a feature that is challenging, if not impossible to mimic with the P19 2D assay. Importantly the P19 2D assay showed differences between media formulations that were not seen in the MEA. Given that the developed P19 2D assay aimed to mimic MEA response to treatments, these varying responses suggest that the two assays are not directly comparable (at least in their responses to non-sequential IVF media). To conclude, the P19 2D assay designed specifically for use within the IVF industry, is the first of its kind and responds to changes in human embryo culture media formulation. Importantly the P19 2D assay identifies differences not seen in the MEA. The effects of mTOR and AMPK inhibition further enhances cellular response to changes in culture conditions. Although valuable for its ability to repeatedly reflect the quality of human IVF media in a cost-effective manner, particularly in relation to identifying optimum concentrations of energy sources, the P19 2D assay is, at present, unable to mimic the structural changes that occur within the developing embryo, or to identify the nutritional needs of the embryo at various stages of development. Arguably however, standard MEA protocols do not provide this information either because decisions on the quality of the products tested is based on one-end point reading obtained at the end of the 96 hour incubation period (U.S. Food and Drug Administration, 2019). Further to this, Geri time-lapse imaging was used to monitor embryo development throughout the 96-hour treatment period. This is however not standard practice, meaning that these data reflect a more advanced version of the MEA. Overall, it can be concluded that although the P19 2D assay can identify differences between various media types and environmental conditions in a research environment, it is not directly

comparable to the standard MEA. In order for the 2D assay to accurately identify the formulation of media most suitable for promoting embryo development, more work needs to be done to automate both processes so that both assays can be run at a very large scale. For the P19 2D assay, this could be achieved by developing methods where the P19 cells are pre-prepared in well-plates and kept refrigerated or frozen until use, then cells could be revived by thawing at room temperature and treated with various batched of medium, followed by viability testing with resazurin solution.

7. General discussion

In this thesis, methods for assaying P19 embryonal carcinoma cells were optimised to assess the quality of human embryo culture media. This work aimed to address a critical need in the IVF industry as quality control contributes significantly to the success of human *in vitro* fertilisation (IVF). Despite representing the most established means to date of quality assessment in IVF, use of the mouse embryo assay (MEA) presents numerous challenges for both research and the industry. The work presented in this thesis makes significant contributions towards alleviating some of these issues and, for the first time, a cell-line with the ability to differentiate has been used to assess and refine commercially available blastocyst media. Importantly, as a model of mammalian development, these cells have the potential to improve current practise whilst alleviating some key research issues which could impact the success of human IVF.

In general, this thesis was successful in achieving its aims as follows:

- The P19 cell line was primed to form 2D and 3D assays, which were developed through a series of methods that identified appropriate controls and established suitable viability tests whilst defining the quality of the assay through Z' factor evaluation. Findings indicate that these assays repeatedly and successfully modelled early embryonic development, as evidenced by the various ways in which P19 cells responded to the different embryo culture media formulations and environmental perturbations (specific aims one and two).

- The effects of exogenous metabolic substrates on the proliferation rates of mammalian cells were investigated by exposing P19 cells to increasing concentrations of pyruvate, glutamine and calcium lactate, separately (chapter three, four and five) and in tandem

with one another (chapter six). In contrast to current literature (Tauffenberger *et al.*, 2019), the data obtained suggests that P19 cells were unaffected by the removal of pyruvate and glutamine, but experienced significantly reduced growth in the absence of calcium lactate (specific aims one and two).

- Mammalian cells were pharmacologically induced by rapamycin and dorsomorphin to block survival mechanisms induced by cold stress and to increase their resistance to suboptimal culture conditions. In this state, the cellular response to various embryo culture medium formulations were compared to cells treated with the same formulation with no rapamycin and dorsomorphin, and with incubation at standard culture conditions. The results from chapter five indicate that the resistance of P19 cells to the removal, or low concentrations of energy sources was reduced by a combination of rapamycin and dorsomorphin, and culture at standard incubation, but not cold stress. For the first time, the issue of cell survival being too high to assess the quality of embryo culture media has been addressed and has the potential to change the ways in which quality control in IVF is conducted (specific aims three and four).

- The concentrations of pyruvate, glutamine and calcium lactate required for optimum P19 cell growth were identified for the first time, highlighting the point at which the supplementation of energy sources introduces toxicity (specific aim four).

7.1. A novel pluripotent cell-based assay for quality control in IVF

The work presented in this thesis was mainly driven by the need to reduce industry's reliance on mouse embryos, especially the quantities required for quality control within the IVF research and development industry. Moreover, these studies sought to fill in some of the gaps in the literature by addressing cellular response to hypothermia which is less understood than other forms of cellular stress (Al-Fageeh and Smales, 2006; Swindell, Huebner and Weber,

2007). This was achieved by subjecting P19 cells to cold stress and modulating the cell's adaptive response so that translation regulation was blocked, and cell survival was reduced. In turn, these effects successfully reduced cellular resistance to environmental perturbations, making them more sensitive to changes in culture media. The findings from this thesis come together to show that the P19 2D assay, and to some extent the 3D assay, have the potential to reduce animal use in research and development, whilst reducing technical procedures and costs associated with traditional quality control measures. As discussed in chapter three, key features from the MEA protocol were incorporated into the design and implementation of the novel assay, in order to highlight their potential similarities. Practical comparisons were not made at this stage due to the high costs associated with the running of the MEA. Consequently, theoretical comparisons based on current literature were made instead (U.S. Food and Drug Administration, 2019; Esfandiari and Gubista, 2020). P19 cells responded the various formulations of embryo culture media, whilst indicating its preference for fertilisation media. In contrast to previous studies where 2D cultures have displayed different responses to 3D cultures (Cushing and Anseth, 2007; Langhans, 2018), chapter four describes how embryoid body response to embryo culture media was similar to that of the 2D assay. Perhaps the pluripotent and differentiating nature of P19 cells cause them to respond to fluctuating culture environments irrespective of their physical form (Desbaillets *et al.*, 2000), and despite the fact that structurally, EBs mirror growing embryos better than 2D assays (Guo *et al.*, 2019; Sagy *et al.*, 2019; Zeevaert, Elsafi Mabrouk, *et al.*, 2020; Gordeeva and Gordeev, 2021). 3D culture with the EBs had an advantage over the 2D assay because the study was designed to allow daily assessment of EB size over an extended period, as opposed to the one endpoint reading associated with the 2D version. On the other hand, the 2D assay was cheaper and easier to optimise, and the fluorescence and luminescence values generated indicated that data obtained from the 2D assay were more centrally distributed than that of the 3D assay, and therefore more reflective of the effects of various treatments. It could be argued that the daily assessments of EB size as a stand-alone method reflects those applied to the MEA where mouse embryo development is monitored over the 96-hour incubation period; however,

given that the darkened cells within the core EB were possible signs of necrosis (Wartenberg *et al.*, 2001; Takayama *et al.*, 2011), it was necessary to determine cell viability in addition to EB growth and size, to ensure that dead cells did not form part of the final assessment.

In chapter five, cold stress, rapamycin and dorsomorphin were used to decrease the resistance of P19 cells to environmental perturbations. This was achieved through a convergence of methods that blocked cell survival mechanisms whilst cells were exposed to hypothermic stress, then cellular response to various concentrations of pyruvate, glutamine and calcium lactate were determined. Similar to findings from chapter three, cells were unaffected by the removal or low concentrations of pyruvate and glutamine but responded to the removal of calcium lactate. The effects of elevated concentrations of energy source in relation to increased osmolality was also addressed, further highlighting the need to maintain a specific concentration range for each media component. In addition, a method for reducing cellular resistance to altering culture conditions was identified for the first time. Surprisingly, given that cold stress is marked by attenuated protein synthesis (Roobol *et al.*, 2009; Hofmann *et al.*, 2012), hypothermic incubation resulted in accelerated cell metabolism. It was subsequently decided that cold stress alone could not make P19 cells more responsive to suboptimal culture conditions. On the other hand, it was evident that treatment with the rapamycin and dorsomorphin combination significantly reduced cellular resistance to altering culture conditions. This caused cells to respond to media with reduced concentrations of energy sources, which they had previously demonstrated resistance to. In chapter six, a combination of rapamycin and dorsomorphin were used to successfully amplify cell sensitivity to alterations in 108 different media formulation and for the first time, cellular response to treatments were directly compared to MEA responses to the same treatments. These studies showed that P19 response to the various test substances reflected the nature of test product better than MEA findings. In this way, although useful in a research setting for identifying differences between test products, the P19 assay is not directly comparable to the MEA

because the two assays responded differently to treatments. Furthermore, considering its response to various treatments, the P19 assay can be described as sensitive, but not as robust. A robust assay is one that remains unaffected by small but deliberate changes in test conditions (Cowan, 2013) however, as described in chapter three, P19 cells responded differently to independent repeat experiment with the same treatments. This does not discredit the benefits of using the P19 assay given that cells repeatedly responded to changes in various types of media. However, assay robustness directly impacts reproducibility and must be further optimised before the P19 assay is used on a wider-scale to validate test products.

7.2. The wider scope of this work, and the implications of using a cell-based assay in IVF quality control

Having established the benefits of using a mammalian cell-based assay over the MEA, certain considerations should be made before either the P19 2D assay described here, or an alternative, could be used on a wider-scale. Firstly, given that MEAs have been used in research and development and also in quality control since as early as the late 1940s (Hammond, 1949), it would, at least at first, be best to use such an assay alongside the MEA. This way, end-point users would be given the opportunity to train and become accustomed to using a cell-based assay. Secondly, unlike the introduction of ICSI and other accessory IVF methods developed in the 1990s (Cohen *et al.*, 1992; Palermo *et al.*, 1992), modern day embryology practice requires systems and processes to be scrutinised for their health and safety implications as well as their success rates before they can be fully implemented for use in an embryology laboratory (Choucair, Younis and Hourani, 2021). Subsequently, the 2D assay would have to undergo further assessment by relevant regulatory bodies in order for it to be approved, and even after that, it would have to gain acceptance from the wider embryology community. Lastly, careful consideration would have to be given as to how these methods could be standardised. For instance, in order to maintain consistency between different operating centres, cells of the same passage number would have to be used in each

experimental run; meaning that a suitable passage number would have to be pre-established. The embryonic stem cell test (EST) which is similar in design to the assay described here (Spielmann *et al.*, 1997) is conducted using cells at a passage number of ≤ 25, and these are considered to be relatively young cells. Nonetheless, it is important to consider whether ≤ 25 is a suitable passage number for this particular assay, or whether cells of a lower passage will produce more precise, more conclusive results and improve assay robustness.

7.3. Practical considerations of the use of cell-based assays in IVF quality control

Whilst considering some of the more practical implications of implementing a 2D cell-based assay for quality control in IVF, it is also important to acknowledge some of the concerns that may arise. First of all, for this assay, the P19 cells are arranged in a 2D format which is in direct contrast to the spherical nature of developing embryos. Studies demonstrate that increasing the dimensionality of extracellular matrix surrounding cells significantly impacts cell proliferation, differentiation and cell survival (Baker and Chen, 2012; Gauvin *et al.*, 2012; Bonnier *et al.*, 2015). In general, these discoveries suggest that 3D systems should be applied whenever possible; however, the platform of choice is dictated by the specific process of interest, and in this case, the need to incorporate easy-to-use, robust HTS methods means that the 2D assay is a suitable option. Furthermore, 2D cell culture approaches can still recapitulate *in vivo* behaviour for many bioactivities (Duval *et al.*, 2017) and with new technological advances offering new capabilities for this platform, a 3D system is not always absolutely necessary.

Another potential concern, which stems from previous studies, is the fact that the suitability of mouse embryos as models for studying cellular dysfunction in human embryos have been questioned (Winston and Johnson, 1992; Quinn and Horstman, 1998), yet here, a cell line derived from mice was used. Whilst these studies address important issues associated with

MEA use, particularly those associated with lack of standardisation and costs, concerns over the use of a model that does not directly reflect early human development could remain. A possible solution to this would be the application of a human induced pluripotent stem cell line (hiPSC) which involves the reprogramming of human somatic cells to a pluripotent stage through ectopic expression of specific transcription factors (De Souza Fernandez, De Souza Fernandez and Mencalha, 2013). The potential issue with this option however, is that cell culture methods such as traditional flat culture systems impose significant limitations on its expansion, differentiation efficiency and ability to form EBs (Guo *et al.*, 2020). Furthermore, differentiation from hiPSCs to target cells is relatively complex, time-consuming and is often associated chromosomal abnormalities (Chen *et al.*, 2014). These issues could however be tackled by developing methods for streamlining processes for culturing hiPSCs on large scale.

Lastly, considering that cell culture methods are rapidly evolving with technological advances (Langhans, 2018; Rothbauer, Zirath and Ertl, 2018), the implementation of a 2D cell culture system could result in constantly changing code of practices. Conversely, MEA methods have remained the same over the years despite the fact that solutions for more advanced versions (*i.e.* MEGA and the extended MEA) have been suggested (Wolff *et al.*, 2013; Gilbert *et al.*, 2016). Implementing stringent guidelines would support some level of stability in respect to standardising protocols; however, careful consideration should be given to ensure that these guidelines do not inhibit innovation and corrective action plans.

7.4. Potential future studies

The evidence presented in this thesis provides novel insights into the various ways in which quality control in IVF can be improved or made easier, whilst addressing some critical issues discussed in the literature; however, findings also highlighted areas that could benefit from further research. For example, the P19 cell-based assays (2D and 3D) work and are sensitive

to test products, however, further automation is required to improve assay robustness and to allow the assays to be run at a large scale. Although the data obtained here demonstrates a level of consistency between repeat experiments, there are likely to be high levels of variation between research sites. The assays could therefore be improved by adapting it to run in an automated way that includes liquid handling for rapid dispensing of the various treatments, or by developing a means for it to be used as a standard test kit. Automation would be ideal to allow the assay to realise its potential as a valuable research and development tool, whilst a test kit would represent a more viable way to lower MEA use.

Next, the P19 cell line used in these studies has the ability to form EBs and can be chemically induced to differentiate into all three germ layers, however, there are some limitations with its 3D structure. Firstly, the cells are teratocarcinomas and consequently grow very quickly, even when the seeding density is low (≥ 500 cells/well). This prevents study over an extended period of time because cells within the core structure undergo necrosis and begin to perish, causing them to leak from the internal structure into the surrounding culture media. The use of cell lines with lower doubling times could allow study of more gradual growth over an extended period of time, which may be a more sensitive means of assessing media quality. Secondly, attempts to freeze P19 EBs were unsuccessful because the structures were not robust, and their morphology were subsequently impacted by the freeze-thaw process. This may be unique to P19 EBs, and as such an alternative cell line could give rise to more robust structures, which could allow mass production of EBs that can be frozen and thawed for use in quality control, as an alternative to the MEA. Previous studies have used EBs derived from human embryonic stem cells (BG01V cell line) to address some micro-environmental issues, particularly focusing on how the number of cells in culture determine access to nutrients and oxygen concentration and in turn influence differentiation patterns (Lach *et al.*, 2018). Other studies have used porcine induced pluripotent stem cell lines VSMUi001-A and VSMUi001-D to induce differentiation into all three germ layers, using homogeneity in EB size and shape as an indicator of successful differentiation (Chakritbudsabong *et al.*, 2018). A comparative

study of these cell lines (which are not teratocarcinomas) and how they can be used to determine the quality of IVF media would be of great value. This study could employ the use of other cell lines that exhibit properties of early-stage embryos (i.e., that have the ability to differentiate into all three germ layers), and explore how they can be used to determine the quality of IVF media.

In addition, a monitoring strategy based on direct measurements of dissolved oxygen and pH could provide further insights into the effects of various test products on cell growth. Oxygen is necessary for respiration and is absorbed by cells for metabolic processes like protein synthesis. It decreases with increased biomass and the subsequent lactic acid build up reduces intracellular pH levels which leads to decreased cell growth and function (Naciri, Kuystermans and Al-Rubeai, 2008). Intracellular pH regulates many cellular processes including enzymatic reactions, cell division, differentiation, cytoskeleton establishment and mitochondrial localisation so its internal and external regulation is fundamental to cell growth (Roos and Boron, 1981; Gatimel *et al.*, 2020). Buffers such as phenol red are added to culture media to enable assessment of acidity by eye however more quantitative methods that involve electrochemical probes exist (Hansen, Dawson and Brockbank, 1994; Michl, Park and Swietach, 2019). Monitoring the levels of dissolved oxygen and pH could provide more extensive insight into the effects of various treatments on metabolic activity. It would also be beneficial to assess P19 response to smaller increases in rapamycin and dorsomorphin than those explored in chapter five, given that the optimum concentrations established in preliminary experiments did not consistently impact cellular response to test products. Higher concentrations than those administered in these studies could result in more sensitive responses to test products, particularly the various pyruvate and glutamine treatments. Establishing working concentrations of rapamycin and dorsomorphin that reduce cellular resistance to stress without causing cell death would allow future experiments to be conducted with one kinase inhibitors (as opposed to the two used in these studies), thereby reducing overall procedural costs.

Furthermore, as mentioned above, P19 cells have the ability to be chemically induced to differentiate into all three germ layers, and a number of studies have focused on the underlying mechanisms behind this process. Previous studies have also developed novel methods for uniform embryoid body formation including the use of round-bottomed 96-well plates or conical tubes (Kurosawa, 2007). A suitable next step would focus on quantifying the level of gene expression that occurs within each germ layer in different cell lines, and whether this information could be used to indicate the quality of various media formulations. Cells that form part of the mesoderm layer are preferentially expressed by early stage P19 embryoid bodies but there is no evidence to suggest that this occurs with embryoid bodies developed from other cell lines (Choi *et al.*, 2004, 2015). It would also be beneficial to determine whether there is a 'preferential' pattern of gene expression observed when embryoid bodies are cultured in optimised media and compare this to changes that may occur with suboptimal media quality. This work would determine if the expression levels of specific genes could be used as reporters for media quality.

Lastly, more extensive comparisons between the P19 assay and the MEA have to be made in order to better understand the needs of the developing embryo and how these relate to findings from the P19 assay. Despite demonstrating sensitivity to the various treatments and conditions presented in this thesis, P19 assay responses must align with that of the MEA for it to be deemed as a suitable replacement. This refers to its robustness (as opposed to its proven sensitivity) and ability to repeatedly produce a desired outcome. Section 5.1 describes how previous MEA studies have failed to identify toxic culture conditions causing some researchers to render it insensitive however it has played a central role in developing the various forms of IVF media used in successful treatments today. Subsequently, its replacement should align with and improve current practises as opposed to producing completely different outcomes. Further studies should therefore explore shared responses between the two assays and how these can improve the quality of currently used reagents and equipment.

7.5. Concluding remarks

With an increasing prevalence of infertility and a growing demand for fertility services globally, there is now a greater focus on quality control systems in human IVF than there has been previously. The work in this thesis considered a model that could improve current IVF quality control practices and also started to provide solutions for reducing animal use through the establishment of an in-house cell bank (it was not necessary to order more than one cell line throughout this thesis). Furthermore, the commercial needs of the research and development industry has meant that products are still tested using the MEA, despite the fact that there are ethical issues associated with it; however, the optimisation of the assay developed here has the potential to directly alleviate some of these concerns. It is therefore possible that these methods could be integrated into quality control practices in IVF, under the condition that they are successfully scaled for wider-scale use. It would also have to undergo some scrutiny by the research and development industry before it can be accepted; however, discussions like these encourage the industry to pay attention to quality control issues that have been not been fully addressed, and provide an opportunity for feasible solutions to be considered.

8. References

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9. Publications and other research activities arising from this thesis

9.1. Publications

Adjirackor, N. A., Harvey, K. E. and Harvey, S. C. (2020) 'Eukaryotic response to hypothermia in relation to integrated stress responses', *Cell Stress and Chaperones*, 25(6), pp. 833–846. doi: 10.1007/s12192-020-01135-8

9.2. Manuscripts in preparation

Adjirackor, N. A., Beckitt, T., Harvey, K. E. and Harvey, S. C. 'The development of a novel pluripotent cell based assay for human IVF media quality control'

9.3. Presentation

Oral presentation, Postgraduate Symposium, University of Kent, Canterbury, UK, July 2019, Cell-based assay for the development and validation of IVF media.

Oral presentation, Postgraduate Forum in Human and Life Sciences, Canterbury Christ Church University, Canterbury, UK, June 2020. P19 cellular response to human embryo culture media is comparable to the mouse embryo assay.

Poster presentation, European Society of Human Reproduction and Embryology, July 2020. 'The development of a stem cell-assay for quality control testing in IVF'.