

Studies on the Effect of Cryopreservation on Gene Expression in Zebrafish Blastomeres

Chia-Hsin Lin

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Studies on the effect of cryopreservation on gene expression in zebrafish blastomeres

by

CHIA-HSIN LIN

A thesis submitted to the University of Bedfordshire in accordance with the requirements for the degree of Doctor of Philosophy

Institute of Research in the Applied Natural Sciences
University of Bedfordshire
The LIRANS
250 Butterfield
Great Marlings
Luton, Bedfordshire
LU2 8DL UK

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STUDIES ON THE EFFECT OF CRYOPRESERVATION ON GENE EXPRESSION IN ZEBRAFISH BLASTOMERES

CHIA-HSIN LIN

ABSTRACT

Cryopreservation is now a common practice in the fields of aquaculture, conservation and biomedicine. The success of cryopreservation is usually analysed in terms of cell survival, although there are other potential adverse effects that don't necessarily result in cell death. These include DNA damage, which could result in altered gene expression. The aim of this study is to discover if cryopreservation has an impact on gene expression using zebrafish (*Danio rerio*) as the model organism. As the whole embryo cannot yet be successfully cryopreserved, isolated blastomeres from the embryos were used to investigate the impact of cryo-treatment on gene expression.

This study sets out firstly to determine an optimum cryopreservation protocol for 50% epiboly blastomeres (Epiboly displaces the blastoderm margin to 50% of the distance between the animal and vegetal pole). Blastomeres had the highest survival level (70.2 ± 3.2%) when a mixture of 1.5 M dimethyl sulfoxide (DMSO) and 0.1 M sucrose were used as cryoprotectants. As quantitative analysis of gene expression using real-time PCR requires the use of a housekeeping gene as an internal control to normalize date, the second study aimed to identify and validate housekeeping genes for use in cryopreservation studies of zebrafish blastomeres. Seven potential housekeeping genes were analysed across a range of embryo stages and isolated blastomeres using the GeNorm and NormFinder software packages. Results indicated β-actin and EF1α housekeeping genes to be the most appropriate for cryopreservation studies on zebrafish embryos and blastomeres, and these housekeeping genes were used in the third study, the effect of cryopreservation on Pax gene expression. The results indicated that the trends (profile changes) in expression of Pax2a and Pax5 occurred to a lesser extent in frozen-thawed blastomeres than in fresh blastomeres whilst the opposite was true for Pax8. The trends in expression of Pax2b were delayed in frozen-thawed blastomeres compared to fresh blastomeres. Cryopreservation can therefore disrupt normal gene expression patterns in zebrafish embryos which could have a detrimental effect on embryo development.

This is the first study on the stability of housekeeping and transcription factor genes in chilled and cryopreserved embryonic cells of the zebrafish. This work will significantly enhance future studies investigating the impact of cryopreservation on gene expression in zebrafish embryos.

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LIST OF ABRREVIATIONS

ACTB	actin beta
AFP	antifreeze protein
ANOVA	analysis of variance
ATP	adenosine triphosphate
Bax	BCL2-associated X
Bcl	B-cell lymphoma
CD9	CD9 molecule
cDNA	complementary deoxyribonucleic acid
CirpB	cold-inducible RNA binding protein B
CNS	central nervous system
CPA	cryoprotectant
CT	cycle number
CuSOD	superoxide dismutase enzyme
DEPC	diethylpyrocarbonate
Dlx	distal-less homeobox
DMSO	dimethy sufoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
EG	ethylene glycol
FBS	fetal bovine serum
FDA	fluorescein diacetate
Fgf	fibroblast growth factor
GLUT	glucose transporter
HKG	housekeeping gene
hpf	hours post fertilization
HPRT	hypoxanthine-guanine phosphoribosyl transferase
HSP	heat-shock protein
IVF	*in vitro* fertilisation
LN_2	liquid nitrogen
MBT	midblastula transition

METH	methanol
MnSOD	manganese superoxide dismutase
MPF	maturation promoting factor
MSS	minimum sample size
Myb	myeloblastosis
NOEC	no observed effect concentration
OP	octapeptide
OPS	open pulled straw method
p53	protein 53
Pax	paired box
PBS	phosphate buffered saline
PCR	reverse transcriptase polymerase chain reaction
PEG	poly-ethyleneglycols
PG	propylene glycol
PI	propidium iodide
RAG	recombination-activating
Rbm	RNA binding motif
RNA	ribonucleic acid
RPL4	Ribosomal protein L4
RT-PCR	reverse transcriptase polymerase chain reaction
SAGE	serial analysis of gene expression
SCSA	the sperm chromatin structure assay
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
Sox	SRY-box containing
TB	trypan blue
TM	melting temperature
tRNA	transfer ribonucleic acid
Trp	transformation related protein
TSC	tuberous sclerosis
TTF	thyroid transcription factor

CHAPTER 1: INTRODUCTION

1.1 Aim of the research and significance

Correct gene expression is crucial for embryo development. Any abnormal gene expression may lead to irreversible damage. Cryopreservation is a complicated procedure involving physical and chemical factors that expose cells to extreme conditions. Therefore, it is important to determine whether cryopreservation protocols could adversely effect gene expression.

Fish embryos and oocytes have still not been successfully cryopreserved with the main problems being identified as their large size, high yolk content and complex membrane systems (Zhang and Rawson, 1995, 1996). In order to investigate the effects of cryo-treatments on fish embryos, it was only possible to use embryos in chilling studies and to undertake full cryopreservation, storage and recovery with isolated blastomeres. Blastomere cryopreservation is an alternative technique, as the blastomeres have the full genetic compliment and retain pluripotency (Ho and Kimmel, 1993; Kusuda et al, 2004). There are several reports of cryopreservation of fish blastomeres (Kopeika et al., 2005; Kusuda et al., 2002; Strussmann, 1999; Calvi and Maisse, 1999; Harvy, 1983). However, only one report incorporated cryopreserved blastomeres into recipient embryos for chimeric fish experiment (Kusuda et al., 2004). In their results, cryopreserved blastomeres retain pluripotency but the ratio of a primordial germ cell differentiated from a cryopreserved blastomere was very low.

Several studies on the effect of cryopreservation at a molecular level such as DNA damage and gene expression reported instances where no adverse effect of cryopreservation was observed (Hamamah et al., 1990; Stachowiak et al., 2007); however a second group, found there were indications of negative effects (Succu et al., 2008; Uechi et al., 1997; Tachataki et al., 2003). These negative effects might subsequently be corrected or repaired during further growth and development (Succu et al., 2007). Studies on the alteration of gene expression as a result of

cryopreservation may explain some of the differences in viability between fresh and post-thaw cells. Moreover, alteration of gene expression may also lead to major defects in the brain, ear, eye and kidneys (Torres et al., 1995, 1996; Favor et al., 1996).

The zebrafish has been used widely as a vertebrate model in cellular, developmental and genetic research and the findings of such research informs medical research. This present programme of research seeks to study gene expression using this popular model species. The overall aim of the present work was to investigate the varying freezing parameters relating to cryopreservation in order to develop a successful protocol for the cryopreservation of isolated blastomeres and to use this protocol to discover if cryopreservation had an impact on blastomere gene expression.

1.2 Cryobiology

Cryobiology is generally associated with the physics of freezing, the phase change of water in both extracellular and intracellular environments at sub-zero temperatures and the effects of cold on biochemical and physiological processes. It applies to all classes of living cells, tissues, organs or organisms. It is important to understanding the mechanisms of cell cryoinjury and cell responses to environment change during the cryopreservation process and to develop the optimal procedures preventing cryoinjury for the long-term cryopreservation of living cells. In this chapter, five major aspects of cryobiology have been described: chilling injury, freezing injury, cryoprotectants, controlled slow freezing and vitrification.

1.2.1 Chilling injury

Chilling injury is associated with low temperatures without the formation of ice. In general, some cells are sensitive to temperature changes in this range, causing a high percentage of the cells to become irreversibly damaged. When cells are cooled from room temperature to -5°C or lower, without freezing, they can undergo irreversible loss of viability (Watson, 1995). Chilling injury to cells can be divided

into two types: direct chilling injury (cold shock) and indirect chilling injury (Morris and Watson, 1984).

1.2.1.1 Direct chilling injury (cold shock)

Direct chilling injury is cooling rate dependent and occurs mainly at fast cooling rates. Cold shock is related to lipid phase transitions that can result in changes in the membrane permeability to ions such as K^+ and $Ca2^+$ and is likely to produce leaky membranes and cause severe damage to cells (Hammerstedt et al., 1990; Parks and Lynch, 1992; Drobnis et al., 1993). The effects of direct chilling injury on survival and metabolism of cells have been well recognised. Early studies showed that cold shock significantly reduced the respiratory activity of ram semen (Chang and Walton, 1940). In studies of bull semen, cold shock produced a marked loss of motility, respiratory activity and glycolysis (Hancock 1952; Blackshaw and Salisbury, 1957).

1.2.1.2 Indirect chilling injury

Indirect chilling injury is observed when there is a relatively long exposure period at the reduced temperatures and is not related to cooling rate. Studies on mammalian embryos or oocytes showed that cooling rate had little effect on their survival. However, chilling injury may develop very rapidly (Martino et al., 1996) and a similar result was obtained in the early stage insect embryos (Mazur et al., 1992). It affected the development of *Drosophila* embryos and the chilling caused injury at low temperatures (Mazur et al., 1992). Low temperature may affect the structure and function of proteins by decreasing the rate of enzyme activity (Morris and Clarke, 1983). The disorder of metabolic and enzymatic processes increases with decreasing temperature. In addition, the properties of membrane are altered during a reduction in temperature. Some cells can change their lipid composition to reverse phase change behaviour but some cells can not and this leads to the long-term phase separation (Morris and Clarke, 1983).

1.2.2 Freezing injury

Freezing injury occurs at temperatures below freezing point and following ice formation. There are several potential factors involved in freezing injury to animal cells including pH fluctuation, extracellular ice formation, intracellular ice formation, osmotic effects and solution or volume effects.

1.2.2.1 pH fluctuation

During cryopreservation, the pH value of biological solution changes, this fluctuation is caused by solution effect or addition of cryoprotectants (CPA; Xu et al., 2003). The pH fluctuation affects the properties of the cells causing temporary or permanent cryoinjury during the course of freezing. The pH fluctuation inside the cell can cause a variety of physiological problems such as the separation of cytoskeleton, fragmentation of plasma membranes, protein denaturation and contortion of a normal cell cycle (Dube and Eckberg, 1996; Fagotto and Maxfield, 1994; Xu et al., 2003).

1.2.2.2 Extracellular ice formation

Extracellular ice formation occurs as cells are cooled slowly, water migrates out of cells and ice forms in the extracellular space. It has been shown that extracellular ice is compatible with cell survival (Farrant, 1977; Griffiths et al., 1979). However, biological systems will be subjected to a series of stresses arising from the formation of the extracellular ice including osmotic effects, solution effects and volume effects. Excessive extracellular ice can also cause mechanical damage of cells due to crushing.

1.2.2.3 Intracellular ice formation

If cells are cooled too rapidly, water does not have enough time to migrate out of

cells and intracellular ice will form. The cause of intracellular ice formation may be damage to the structure of plasma membrane by mechanical puncture and disarrangement of cytoskeleton (Muldrew and McGann, 1990; Mazur, 1977). There is a risk of recrystallisation and emergence of intracellular ice during thawing (Bank, 1973).

1.2.2.4 Osmotic effect

During slow freezing, ice crystals are formed first in the solution surrounding the cells, resulting in increasing concentrations of solutes in the bathing medium. Because of the difference in osmotic pressure inside and outside the cells, water continues to move out of the cells as long as the imbalance in solute concentration remains. Osmotic stresses then occur which lead to a rupture in the plasma membrane due to cell shrinkage and allows extracellular ice to propagate into the cytoplasm (Muldrew and McGann, 1990).

1.2.2.5 Solution or volume effect

When the freezing rate is too slow, the concentration of intracellular solutes rises to very high levels due to osmotic effect and cause a cryoinjury as a result of concentration of solutes, termed ‘solution effects’. This was characterized collectively by Mazur et al. (1972). There is some evidence that concentrated solutes caused denaturation of lipoproteins and enzyme (Jaenicke, 1981). Solution effects were also linked to cell volume, when cell volume decrease beyond the minimum volume during freezing, the ‘volume effects’ occurs (Pegg and Diaper, 1989, 1991). The injury is a consequence of damage that develops when cells are unable to shrink osmotically below a certain level in an attempt to achieve osmotic equilibrium (Meryman, 1968).

1.2.3 Cryoprotective agents

Cryoprotectants are substances that provide protection against harmful effects during cryopreservation or long term storage in the liquid nitrogen (LN_2). Cryoprotectants are normally highly soluble to water and have relatively low toxicity to cells. Cryoprotectants are classified into two categories, permeating and non-permeating cryoprotectants.

1.2.3.1 Permeating cryoprotectants

The permeating cryoprotectants e.g. dimethyl sulfoxide (DMSO; CH_3SOCH_3), methanol (METH; CH_3OH), Propylene glycol (PG; $HOCOCH_3CH_2OH$) and Ethylene glycol (EG; $HOCH_2CH_2OH$) are low molecular weight chemicals that can penetrate the cell membrane and provide protective action both intracellularly and extracellularly. Permeating cryoprotectants are essential for dehydrating cells and remove intracellular water and lowering the freezing point (Shepard et al., 1976). DMSO is the most widely used cryoprotectant and has been used in cryopreservation of many cell types, tissues and organs. It has been reported to be a membrane modifier and plastifier (Orvar et al., 2000) so that the membrane becomes less brittle and less likely to be disrupted (Zeron et al., 2002). DMSO has been widely used as a cryoprotectant for the cryopreservation of many species of fish sperm (Tsvetkova et al., 1996; Ritar and Campet, 2000; Cabrita et al., 1998) and in the freezing and long-term storage of fish blastomeres (Harvey, 1983; Strussmann et al., 1999; Kusuda et al., 2002, 2004; Cardona-Costa and Garcia-Ximenez, 2007). Methanol has been shown to be the most effective cryoprotectant for embryo and oocytes of zebrafish and other species (Zhang et al., 1993, 2005; Walsh et al., 2004; Liu et al., 2003; Rall et al., 1984; Hagedorn et al., 1996). Methanol was also found to be an effective cryoprotectant for fish sperm of several species (Tiersch et al., 1994; Lahnsteiner et al., 1997; Urbanyi et al., 2003). PG and EG are similar compounds and they have also been reported as effective cryoprotectants for vitrifying mammalian embryos (Rall, 1987; Scheffen et al., 1986) and oocytes (Ko and Threlfall, 1988; Leibo and Oda, 1993; Kasai et al., 1990).

1.2.3.2 Non-permeating cryoprotectants

The non-permeating cryoprotectants e.g. poly-ethyleneglycols (PEG) and various sugars (glucose, fructose, sorbitol, mannitol, sucrose, trehalose and raffinose) are high molecular weight agents that can not enter cells and act extracellularly. Non-permeating cryoprotectants are key ingredients of cell cryopreservation protocols and contribute to enhance vitrification of the solutions, stabilize proteins and membranes and prevent progressive ice formation (Fahy, 1986; Fahy et al., 1984). Sugars serve as an osmotic buffer, reducing osmotic shock on warming by reducing the speed and magnitude of cell swelling (Liebermann et al., 2003). Kuleshova et al. (1999) estimated the role of the sugars listed above at 0, 0.5 and 1M in the vitrification system. They suggested that monosaccharides may be better than disaccharides currently used in vitrification protocols, due to their low toxicity to embryo and ability to form glass at lower total solute concentrations.

1.2.3.3 Cryoprotectant supplements

Cryoprotectant such as glucose, sucrose and trehalose used at lower concentrations (less than 0.5mol/l) can help the dehydration of cells and lessen the time of exposure to the more toxic permeable cryoprotectants (Yuksel et al., 2002). Liu et al. (2000) reported that cryoprotective supplement caused a marked increase in survival after freezing and thawing. Sucrose is widely used as an extracellular cryoprotectant, often in combination with permeating cryoprotectants. Using sucrose as cryoprotective supplement provides an osmotic buffer, they were successfully used for the cryopreservation of fish sperm of several species such as African catfish (Steyn and Van Vuren, 1987; Urbányi et al., 1999) and various sturgeon species (Tsvetkova et al., 1996; Glogowski et al., 2002). It has been reported that trehalose is very effective as a cryoprotectant supplement for vitrification of oocytes (Dinnyes et al., 2000; Begin et al., 2003). The supplementation of glucose was successfully used for the cryopreservation of sperm of several other fish species such as turbot, grass carp (Zhang and Liu, 1991) and catfish (Carolsfeld et al., 2003).

1.2.3.4 Toxicity of cryoprotectants

In general, cryoprotectants in high concentrations can be toxic to cells (Fahy, 1986; Fahy et al., 1990a; Arnaud and Pegg, 1988; Pegg and Arnaud, 1988). It has been found that DMSO is more toxic than other cryoprotectants for zebrafish embryos (Zhang et al, 1993; Lahnsteiner, 2008) at 22 °C, but it is less toxic for oyster embryos (Cook et al., 1993). High concentration of cryoprotectants may cause osmotic stress to the cells, disarrangement of membrane bilayers and denaturation of proteins (Fahy, 1986; Arnaud and Pegg, 1988; Pegg and Arnaud, 1988; Arakawa et al., 1990; Fahy et al., 1990a). Although cryoprotectants are toxic at high concentrations, their toxicity can be reduced by stepwise additions at low temperatures and reducing periods of exposure (Agca et al., 1998). Cryoprotectant toxicities may become negligible if the cryoprotectants can be used at a low enough temperature. Unfortunately, cryoprotectants become increasingly viscous at low temperature, reducing their capacity to diffuse into cells requiring long exposure times to achieve tissue saturation at low temperature.

1.2.4 Freezing protocol

Cryopreservation is a process where cells, tissue and organisms can be preserved by freezing and storage at low temperatures for a long period usually in liquid nitrogen (-196 °C). At this temperature any biological activity is effectively stopped and normal cellular chemical reactions do not occur, as kinetic energy levels are too low to allow the necessary molecular motion. Two cryopreservation methods have been used: controlled slow freezing and vitrification.

1.2.4.1 Controlled slow freezing

Control slow freezing involves the use of low concentrations of cryoprotectants in the cell culture medium, and low freezing rates, typically achieved by use of a programmable freezer. Controlled slow freezing involves rate-controlled freezing to

minimize ice-crystal formation inside cells, prevent osmotic effects and with low concentrations of cryoprotectant, the effects of osmotic shock and toxicity of the cryoprotectant (Bryant, 1995). Currently mammalian oocyte and embryo controlled slow freezing protocols have been successful with human, mouse and cattle embryos (Trounson and Bongso, 1996; Kaufman et al., 1995; Stachecki et al., 2000) and controlled slow freezing procedures have also been successful in fish blastomeres of several species (Calvi and Maisse, 1998; Strussmann et al., 1999; Kusuda et al., 2002).

1.2.4.1.1 Freezing rate

During the slow freezing period, the freezing rates must be strictly controlled. Too low a freezing rate can damage cells through solution effects, whilst too high a freezing rate favours intracellular ice formation, which is often lethal to cells (Mazur, 1984; Shirakashi and Tanasawa, 1998). The optimum freezing rate is slow enough to allow a sufficient degree of cell dehydration during freezing which in combination with cryoprotectants, prevents intracellular ice formation (Konc, 2005; Mazur et al., 1977). However, the freezing rate has to be fast enough to avoid over-exposure to cryoprotectants and concentrated salt solutions (Muldrew and McGann, 1990). The optimal freezing rate is cell type specific. In general, fish sperm require no complicated freezing protocols and freezing rates between 20 to 40 °C/min have been used successfully in sperm preservation (Tiersch 2001; Liu et al., 2006; Huangab et al., 2004). For cryopreservation of fish oocyte and embryos, slow freezing at freezing rates as low as 0.07 ºC/min, 0.3 ºC/min and 0.5 ºC/min have been used (Zhang et al., 1989; Zhang et al., 1993). These freezing rates are in the similar range for cryopreservation of mammalian oocytes and embryos (Bass et al., 2004; Visintin et al., 2000; Karlsson et al., 1996; Stachecki et al., 1998; Carroll et al., 1993; Czlonkowska et al., 1991). Successful cryopreservation protocols for fish blastomeres have used freezing rates around or slower than 1 °C/min (Kusuda et al., 2002, 2004; Strussmann et al., 1999; Harvey, 1983; Calivi and Maisse, 1998, 1999).

1.2.4.1.2 Seeding

An ice seeding procedure is used for inducing ice formation under controlled conditions. The purpose of ice seeding is to avoid excessive supercooling of the sample. Avoiding supercooling can lessen the risk of intracellular ice formation and reduces the probability of lethal intracellular freezing (Holmstrup and Zachariassen, 1996). The latent heat is the amount of energy in the form of heat released or absorbed by a substance during a change of phase (Mazur, 1977). Normally, as ice crystals form within the medium, the resulting latent heat of crystallization would cause the temperature of the sample to rise back to the freezing point. Seeding to induce extracellular ice formation minimises the occurrence of latent heat. Several studies have shown that extracellular ice seeding temperature can affect survival of mammalian cells such as red blood cells (Diller, 1975), granulocytes (Schwartz and Diller, 1984), hepatocytes (Toner et al., 1992) and mouse embryos (Toner et al., 1993). It is common for cryoprotectant concentrations of 1M to require seeding at a temperature of -5 ºC. For each additional 1M increase in cryoprotectant concentration, the seeding temperature is normally reduced by 2.5 ºC. However, it is common to use an ice seeding temperature of -7 ºC for 1.5 molar concentrations of cryoprotectants (Stachecki and Willadsen, 2000; Yi et al., 2001; Salle et al., 1999; Lassalle et al., 1985; Carroll et al., 1993). For 2 M concentration of cryoprotectants seeding at -7.5 ºC is common, although seeding temperatures of -6 ºC (Bass et al., 2004) and -5.5 ºC (Zhang et al., 1989) have been used. For 3 M concentration of methanol, seeding temperature -10 ºC has been successfully applied for cryopreservation of sheep embryos (Czlonkowska et al., 1991).

1.2.4.1.3 Thawing

The thawing step is as important as the freezing steps in successful cryopreservation. The types of damage that can occur during freezing can also occur during thawing. The most common method for sample thawing is by plunging the sample into a water bath at temperatures between 20-40°C (Lahnsteiner et al., 1995). Cell damage caused by thawing is normally due to the reformation of intracellular ice crystals. Therefore, it is important to thaw samples rapidly to minimize the

recrystalisation (Farrant et al., 1977). Moreover, the optimum thawing rates for fish sperm and embryos vary. For example, a slow thawing rate of 8°C/min was reported as optimal for common carp embryos (Zhang et al., 1989), an intermediate thawing rate of 43°C/min was preferred for zebrafish blastoderm (Harvey, 1983), and a fast thawing rate of 300°C/min resulted in best survival for zebrafish embryos (Zhang et al., 1993).

1.2.4.1.4 Removal of cryoprotectant

Removal of cryoprotectant swiftly and with minor cell volume changes after thawing is an important consideration (Wessel and Ball, 2004). One step removal of cryoprotectant is attributed to osmotic damage of cytoplasmic membranes (Lieberman et al., 2002). The function of stepwise removal of cryoprotectant is to lessen osmotic shock after thawing. It has also been shown that six-step dilution provides improved results over two-step dilution in human blastocysts (Cho et al., 2002).

1.2.4.2 Vitrification

Vitrification is the approach that uses high concentrations (5-7 M) of cryoprotectants with high cooling/warming rates for cryopreservation of cells and organs at extremely low temperatures without ice formation. It is the process of converting a material into a glass-like amorphous solid, free of any crystalline structure. When materials are vitrified, the formation of ice is prevented by the presence of high concentration of chemicals. Vitrification prevents damage associated with ice formation, cellular osmotic dehydration and shrinkage during slow freezing, intracellular ice formation and destructive intracellular ice recrystallisation (Rall and Fahy 1985). Vitrification has been recognised as the only survival mechanism for maintaining the viability of hydrated cells and tissues in the low temperature of liquid nitrogen by avoiding crystallization on rapid freezing (Sakai, 1990). Study on zebrafish blastomeres using vitrification showed that the highest blastomere survival was 93.4% with 5M DMSO (Cardona-Costa and García-Ximénez, 2007).

1.3 Applications of cryopreservation

Cryopreservation is now common practice in many biomedical technology applications including assisted reproduction and *in vitro* fertilisation (IVF) treatments in humans; storage of clinical materials such as cornea, skin, blood cells (Routledge and Armitage, 2003; Villalba et al., 1996; Farrugia et al., 1993), stem cell lines (Sandhya et al., 2005), gametes and embryos and cell and deoxyribonucleic acid (DNA) banking in genomic research (Lubzens et al., 1997). Cryosurgery or tissue destruction by controlled freezing has been investigated as a possible surgical intervention in the treatment of many diseases such as prostate and liver cancer. Cryopreservation method has been developed for blood cells, blood stem cells and blood corpuscles. Frozen blood cells are being used for various diagnostic and clinical purposes, for example, cryopreserved erythrocytes are used for rare blood group and antibody problems; cryopreserved lymphocytes and monocytes is now a routine procedure for clinical laboratory testing (Sputtek et al., 2007) and cryopreserved haematopoietic stem cells are used successfully for the treatment of leukaemia and plasma-cell tumours.

Cryopreservation is a long-term storage technique to store genetic material for extended periods of time at a relatively low cost (Withers, 1991; Engelmann, 1991). Germplasm cryopreservation includes storage of sperm, eggs, and embryos and contributes directly to animal breeding programmes. The establishment of germplasm banks using cryopreservation technology can contribute to conservation and extant populations in the future. Since the first successful cryopreservation of bull semen (Polge et al., 1949), cryopreserved bull semen has been used to propagate rare and endangered species using assisted reproduction techniques. Every year, more than 25 million cows are artificially inseminated with frozen-thawed bull semen (Foote, 1975) and many bovine calves have been produced using the transfer of cryopreserved embryos into cows (Mapletoft, 2005). Cryogene banks may also contain tissues, cultured cell lines, DNA and serum samples. Mice, and sheep have been generated from frozen-thawed pieces of ovary that have been replaced in a female and stimulated to ovulation (Candy et al., 2000; Gosden et al., 1994), the principle of

testicular cell freezing and transplantation has been demonstrated and is currently used for human male infertility (Clouthier et al., 1996). Significant efforts are being made on non-mammalian species using cryobiology techniques. Cryopreservation of aquatic sperms are relatively common in the breeding and management of fish, but cryopreservation of oocytes and embryos of aquatic species have not been successful (see section 1.3.2 and 1.3.3), except for the eastern oyster (*Crassostrea virginica*) (Paniagua-Chavez et al., 1998).

1.3.1 Cryopreservation of fish sperm

The major advantage of fish sperm cryopreservation is the establishment of cryobanks, which allow the storage and dissemination of valuable genetic stock and to bridge gaps in the availability of gametes for assisted reproduction (Palase and Mapletoft, 1996). Cryopreservation of fish sperm has been successful in more than 200 species (Kopeika et al., 2007; Tiersch et al., 2007), and techniques of sperm management have been established in some freshwater (Bokor et al., 2007; Babiak et al., 1995) and marine fish species (van der Straten et al., 2006; Suquet et al., 2000). A 95% fertilization and hatching rate for common carp has been obtained using cryopreserved sperm and these results are not significantly different from fresh sperm (Magyary et al., 1996). Spermatozoa have successfully been cryopreserved in many salmonid species (Lahnsteiner, 2000). Tilapias are among the exotic fishes that are successfully cultured in Taiwan; they have been cryopreserved successfully and produced 40-80% motility with cryoprotectant DMSO (Chao et al., 1987). Sperm of more than 30 marine fish species have been cryopreserved successfully (Suquet et al., 2000; Gwo, 2000; van der Straten et al., 2006). Generally, high survival and fertilization capacity was obtained in frozen-thawed spermatozoa when compared to freshwater species (Drokin, 1993; Gwo, 2000).

1.3.2 Cryopreservation of fish oocytes

Oocyte cryopreservation is potentially the best way to preserve female fertility. Cryopreservation of fish oocytes has been recently studied (Isayeva et al., 2004;

Plachinta et al., 2004; Zhang et al., 2005). Developing methods for cryopreservation of oocytes requires screening of potential cryoprotectant treatments, evaluation of tolerance to chilling, determination of the appropriate rate of freezing to cryogenic temperatures and rate of thawing. Viability assessment methods of oocytes with trypan blue (TB), fluorescein diacetate (FDA) + propidium iodide (PI) and adenosine triphosphate (ATP) content assay have been offered for quick assessment of viability (Plachinta et al., 2004; Zampolla et al., 2006; Guan et al., 2008). A functional test based on *in vitro* maturation followed by germinal vesicle breakdown (GVBD) has also been shown effective for late stage III oocyte (Plachinta et al., 2004).

The permeability of the zebrafish oocyte membrane to water and cryoprotectants has been studied (Zhang et al., 2005), and membrane permeability was shown to decrease with temperature and permeability was generally lower than those obtained from sea urchin eggs (Adam et al., 2003) but higher than those obtained with immature medaka oocyte (Valdez et al., 2005) or zebrafish embryos (Zhang and Rawson, 1998). Studies on chilling sensitivity of zebrafish oocytes showed that those oocytes were very sensitive to chilling and their survival decreased with decreasing temperature (Isayeve et al., 2004). Cryopreservation studies on zebrafish oocytes (Guan et al., 2008) showed that although oocytes viability obtained immediately after freeze-thawing was relatively high with 88% using TB staining, oocytes viability decreased to 29.5% after 2 h incubation at 22 °C. The study also showed that the ATP level in oocytes decreased significantly after thawing and all oocytes became translucent. High chilling sensitivity (Tsai et al., 2007) and low membrane permeability (Guan et al., 2008) of zebrafish oocytes are major obstacles to the development of a successful protocol for their cryopreservation.

1.3.3 Cryopreservation of fish embryos

Cryopreservation of embryos has become an integral part of assisted reproduction. Successful cryopreservation of embryos is important because the biodiversity of both paternal and maternal genomes will be preserved. While cryopreservation techniques have been largely established for mammalian embryos, successful cryopreservation of intact fish embryos has not yet been achieved. Factors limiting fish embryo

cryopreservation include their compartmental structure, high chilling sensitivity, low membrane permeability and their large size which gives a low surface area to volume ratio (Zhang and Rawson, 1995). Chilling sensitivity has been reported for many species of fish embryos (Maddock, 1974; Haga, 1982; Dinnyes et al., 1998; Cloud et al., 1988; Liu et al., 1993; Zhang and Rawson., 1995., Zhang et al., 2003; Isayeva et al., 2004). Those studies demonstrated the later stages (after 50% epiboly) are less sensitive to chilling, but chilling sensitivity increases significantly as the temperature falls below zero. Cryoprotectant toxicity follows a similar pattern to chilling sensitivity with later stages being less sensitive to cryoprotectant toxicity (Zhang et al., 2005; Zhang and Rawson, 1993; Liu et al., 1993; Suzuki et al., 1995). Several studies have determined membrane permeability for zebrafish embryos (Liu et al., 2001, Zhang and Rawson, 1998; Hagedorn et al., 1997) and membrane permeability to water and most cryoprotectants was shown to be low (Zhang and Rawson, 1996, 1998). Studies on cryopreservation of zebrafish embryos demonstrated 8% embryo survival in 2M methanol at -25 °C, however, no embryo survival were observed when freezing to -30 °C or below (Zhang et al., 1993).

1.3.4 Cryopreservation of fish blastomeres

Blastomeres are the cells produced as the result of cell division and cleavage in the fertilized egg. They are pluripotent having the ability to differentiate into any of the three germ layers. Cryopreservation of blastomeres can maintain the genetic diversity of both nuclear genome and mitochondrial DNA (Harvey, 1983; Nilsson and Cloud, 1992). Blastomeres from the early embryos of fish still retain pluripotency (Ho and Kimmel, 1993), and their cryopreservation may be a promising approach to preserve the genotypes of zygotes and reconstitution of the organism. Cryopreservation of blastomeres has been successful in several fish species. In the first reported studies, Harvey (1983) used a two-step freezing procedure, with ice-seeding at -6°C, and cooling to -25°C, followed by immersion in liquid nitrogen. The survival rate of 84.8% was obtained after cryopreservation of 50% epiboly zebrafish blastomeres. However, freezing rates were not controlled and the results obtained from a very small sample size of 3 embryos and 270 cells. Rainbow trout blastomeres have been cryopreserved using controlled slow freezing procedures with

a survival of 95% (Calvi and Maisse, 1998). It has been reported that the controlled slow freezing protocol adopted for rainbow trout was successfully applied to carp blastomere with survivals of 94% and 96% (Calvi and Maisse, 1999). Lower survival rates of cryopreserved blastomeres using controlled slow freezing have also been reported for other fish species such as whiting (20%), medaka (34%), pejerrey (67%) and chum salmon (59%) (Strussmann et al., 1999; Kusuda et al., 2002). Vitrification of zebrafish blastomeres was studied more recently and the highest blastomere survival was 93.4% (Cardona-Costa and Garcia-Ximenez, 2007).

1.4 Gene expression

Gene expression is the transfer of information from inheritable DNA to cellular function through the production of Ribonucleic acid (RNA) and protein (Figure 1.1). This occurs through the processes of transcription and translation. Regulation of gene expression gives the cell control over structure and function, and is the basis for cellular differentiation, morphogenesis and the versatility and adaptability of any organism.

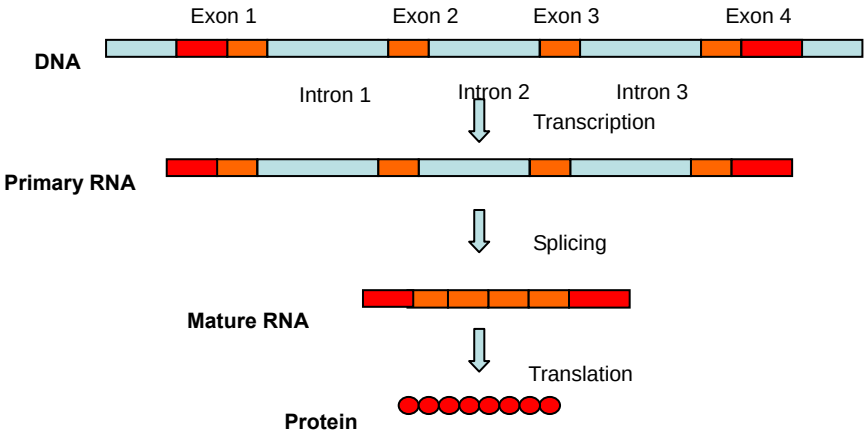


Figure 1.1 Simplified overview of gene structure and expression

1.4.1 Transcription

Transcription produces an RNA copy of a gene which is known as a primary transcript. DNA and RNA polymerase are the two most important elements in transcription. DNA is the template for the RNA product and transcription is carried out by RNA polymerase. These two components must get together in order for transcription to begin. All the primary transcripts produced in the nucleus must go through processing steps to produce functional RNA molecules. The processing includes synthesis of the cap, excision of introns and splicing of exons, and polyadenylation. In the process of capping, a modified guanine is attached to the 5' end of the primary transcripts. Then, introns are cut out of primary transcripts and exons are joined together to form the mature RNA (Figure 1.1). Adenine nucleotides are added to the end in the process of polyadenylation.

1.4.2 Translation

Translation is the process by which the mature RNA is converted into protein. Translation is divided into three phases which are initiation (a special transfer ribonucleic acid (tRNA) for initiation), elongation (adding amino acids to the growing chain) and termination (release of the finished polypeptide). After transcription, transfer RNAs add amino acids in the order determined by the sequence of the RNA transcript. One end of a tRNA is bonded to a specific amino acid. The other contains an anticodon that can pair with the corresponding codon in the mRNA. Ribosomes provide the environment for the process of stringing together amino acids to make a protein. They are the protein synthesis factories.

1.4.3 Gene expression assays

In order to study the process of gene expression, techniques for measuring how gene activity patterns vary within the developing organism are needed. Since most

genes encode messenger RNAs which are translated into proteins, expression can be assessed at either the RNA or the protein level. Several methods are commonly used for quantification of transcription such as serial analysis of gene expression (SAGE), microarray analysis, northern blotting, in situ hybridization, RNAse protection assays and real-time RT-PCR.

1.4.3.1 SAGE (Serial analysis of gene expression)

SAGE is a sequencing-based method and can be used to profile a whole mRNA transcriptome based on a single RNA sample without requiring any prior knowledge of the transcripts to be assayed. A transcript is identified uniquely by a short sequence tag (10-14bp). The sequence tag is obtained form a unique position within each transcript. Moreover, all the sequence tags can be connected together and can be cloned and sequenced. The observations are not based on hybridization, which result in more qualitative and digital values. Briefly, the SAGE procedure is to isolate the mRNA from sample and then extract a small sequence from a defined position of each mRNA. These small sequences were connected together to become a long chain. These chains were cloned into a vector which can be taken up by bacteria. These chains were sequenced using modern high-throughput DNA sequencers. Thereafter, the small sequence tags were counted by computer system (Velculescu et al., 1995).

1.4.3.2 Microarray analysis

Microarray experiments can evaluate tens of thousands of preselected mRNA targets at a time, at considerably lower cost than SAGE. Microarray analysis allows scientists to detect thousands of genes in a small sample simultaneously and to analyze the expression of those genes (Ushizawa et al., 2004). Microarrays are usually glass or silicone chips that have thousands to millions different DNA clones or oligonucleotide probes on them. Each clone or probe is located in a precise spot on the array and corresponds to a certain gene transcript. Microarrays have been used to examine global changes in mRNA levels across different settings (Cox et al., 2005).

1.4.3.3 Northern blotting (Northern hybridization)

Northern blotting is the only method providing information about mRNA size, alternative splicing and the integrity of an RNA sample (Parker & Barnes 1999). Northern blotting involves several steps. Total RNA or mRNA is size-separated by agarose gel electrophoresis and transferred onto a nylon membrane. The RNA is then detected with isotopic or non-isotopic labeled complementary DNA or RNA probe (During, 1993; Trayhurn, 1996).

1.4.3.4 *In situ* hybridization

In situ hybridization uses a labelled antisense mRNA probe to hybridize with the mRNA in the sample which gives more detailed information of gene expression. Although *In situ* hybridization is complex, it allows localisation of transcripts to specific cells within a tissue (Pinhasov et al., 2004; Bustin 2000). The labelled probe can be visualized, thus allowing the concerned mRNA sequence to be also visualized. However, *In situ* hybridization can only give qualitative results, whether the gene is being transcribed or not in the sample, but the level of gene expression can not be directly determined.

1.4.3.5 Conventional Reverse transcriptase polymerase chain reaction (RT-PCR)

The polymerase chain reaction is a technique which allows the amplification of a particular DNA sequence which is replicated again and again to produce numerous copies (Saiki et al., 1988). A temperature cycling profile for a PCR reaction is shown in Figure 1.2. In order to achieve successful amplification, the process needs a pair of oligonucleotide primers which are complementary to the target DNA sequences (Newton and Graham, 1997). The design of the primers is generally carried out using some simple rules as described below. The primers are designed 1) to be complementary to the template DNA; 2) around 20 nucleotides in length (Bustin, 2000); 3) to have similar number of each of the four bases; 4) to avoid repetitive

stretches of polypurines and polypyrimidines; 5) to avoid secondary structure (self-complementarity) particularly in the 3' region to prevent primer dimers; 6) so that the distance between the primers when hybridized to the target DNA is less than 1000bp (400 bp for real time RT-PCR, Bustin et al., 1999); 7) across exon-exon junction; 8) to be a similar melting temperature (TM) (TM = 0.41 x（%G+C）+ 64.9-(600/n)) (Sambrook et al., 1989).

As RNA cannot be a template for PCR, the first step in an RT-PCR assay is to reverse RNA into complementary DNA. This resulting complementary deoxyribonucleic acid (cDNA) can be used in a PCR reaction. The process of reverse transcription firstly is to denature RNA secondary structure. When the primer anneal to the RNA, other components including deoxyribonucleotide triphosphates (dNTPs), RNase inhibitor, reverse transcriptase and RT buffer start the reaction. Finally, the temperature increase to inactivate the enzyme. Three types of primers, oligo (dT) primers, random (hexamer) primers and gene specific primers are commonly used for RT reaction.The success of reverse transcription depends on the RNA quality and the enzyme used for the reverse transcription (Shimomaye and Salvato, 1989).

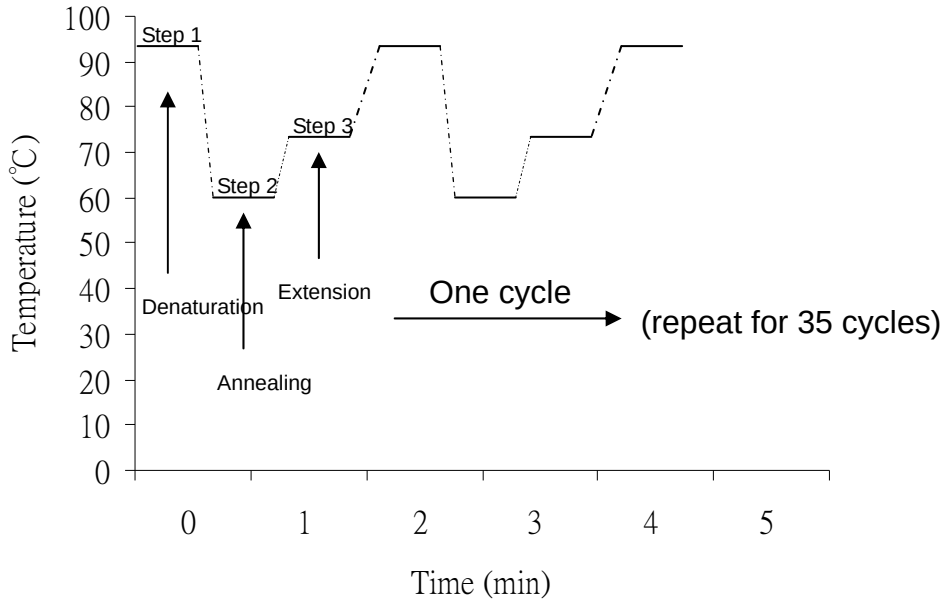


Figure 1.2 The profile of PCR temperature cycling. Step 1: Heat to denature the double-stranded DNA in the presence of primers, the four dNTPs, PCR buffer and a DNA polymerase in the range 93-100°C. Step 2: Lower the temperature to 50-65°C to anneal the primers to the denatured template. Step 3: DNA polymerase extends the primers at 72°C. Step 1 to Step 3 is one cycle of PCR and the process is usually repeated 35 times.

1.4.3.6 Quantitative RT-PCR

As RNAse protection assays, in situ hybridization, northern blotting are relatively low sensitivity (Melton et al., 1984), an ideal strategy is using quantitative real-time PCR methods to measure the amplification of the target molecule continuously during a PCR. Real-time RT-PCR is the most accurate and sensitive way for quantification among all the quantitive methods (Wang and Brown, 1999). Moreover, Real-time RT-PCR analysis allows quantification of small transcripts and changes in gene expression (Pfaffl, 2001; Bustin, 2002). The whole process of real time RT-PCR includes RNA extraction, reverse transcription and finally running real time PCR.

1.4.3.6.1 Techniques of Real time RT-PCR

Real time PCR allows the researchers to detect and quantify the increase in the amount of DNA as it is amplified in real time. Several different methods are available for determining the amount of PCR product present at the end of each cycle, but all rely on measuring the amount of a fluorescent molecule that is associated with each newly synthesized molecule. Molecular beacons, DNA-binding dyes, hybridization probes and hydrolysis probes are currently four available techniques and detect amplified product with about the same sensitivity (Wittwer et al., 1997).

Molecular beacons are single-stranded oligonucleotide probes in a stem-and-loop structure (Tyagi and Kramer, 1996). Usually, the beacon has a fluorescent reporter dye bound to its 5' nucleotide and a quenching dye attached to its 3'nucleotide. When the molecular beacon is attached to a target sequence the quencher is separated from the fluorophore and a fluorescence signal is observed (Broude, 2005). The disadvantages with molecular beacons are the high cost to construct a DNA chip that consists of a large number of different molecular beacon probes, tedious procedures in probe synthesis and purification (Nutiu an Li, 2002) and not stable in the existence of DNA polymerases with exonuclease activities (Broude, 2005).

Hybridization probe pairs is sequence-specific, dye-labeled oligonucleotides, used in real-time PCR approaches (Froehlich and Geulen, 2008). The probe sequences are selected to bind to the target sequences on the amplified DNA fragment in a head-to-tail orientation, therefore bringing the two dyes in close proximity. The energy emitted excites the acceptor dye attached to the second hybridization probe, which then emits fluorescence at a different wavelength.

Hydrolysis probes (also called TaqMan probes) bind to the PCR template and have a fluorogenic reporter dye on one end and a quencher dye on the other end. When the template is being copied, the reporter dye is cleaved from the 5' end by the DNA polymerase. As the reporter is cleaved from more and more probe molecules, it can no longer be quenched by the quencher dye and the fluorescence signal increased.

SYBR Green is the most widely used DNA binding dye for real time PCR (Wittwer et al., 1997; Bengtsson et al., 2003) due to lower cost than molecular probes and the ability to test multiple genes quickly without designing multiple probes. DNA-binding dyes can bind double-stranded DNA but not to single-stranded DNA and release bright fluorescence. The fluorescence signal can be observed during the polymerisation step of PCR, but decreases when the DNA is denatured. Fluorescence measurements are collected at the end of each elongation step and increase with cycle number. Melting curve analysis can be performed to identify primer-dimers and other nonspecific amplification products after the amplification that has been amplified (Ririe et al., 1997). In melting curve analysis, the temperature is gradually increased and the fluorescence is monitored. The dye is released when the temperature is reached that melts the DNA, therefore fluorescence decreases.

1.5 Paired box genes

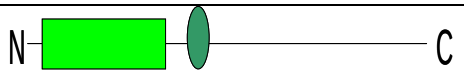
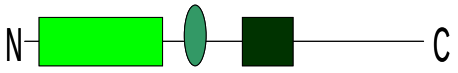
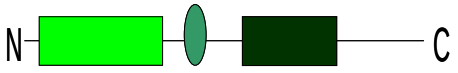
Paired box (Pax) genes are known transcription factors and were first described in drosophila (Bopp et al., 1986). They have also been found in zebrafish, frog, chick, mouse and human (Wehr and Gruss, 1996). Transcription factors are important regulators of gene expression in cells as they make proteins which bind with DNA to activate or suppress transcription of particular genes (Chau et al., 2002).

1.5.1 Pax gene family

Pax genes have been classified into four groups (Table 1.1) based on their sequence similarity, expression pattern and protein structures in mammals (Noll, 1993; Mansouri et al., 1996; Chi and Epstein, 2002; Dahl et al., 1997). Pax2, Pax5 and Pax8 belong to group two, and contain a paired box domain, octapeptide (OP) motif and the partial homeo-box (Czerny et al., 1997). The paired box is a highly conserved DNA region, encoding a DNA-binding protein domain of 128 amino acids (Deutsch and Gruss, 1991). Pfeffer et al. (1998) found two closely related Pax2 proteins which were

named Pax2.1 (Pax2a) and Pax2.2 (Pax2b) in zebrafish. These two genes were about 80% identical at the nucleotide level. In zebrafish, several gene families have contained extra members compared to mammals such as the hox, pou, otx, msx, dlx, brn and hedgehog genes (Pfeffer et al., 1998).

Table 1.1 The structure and expression domain of Pax genes (Kwak, 2006). Diagrams show schematic locations of functional domains in each group. Light green boxes show paired box domains. Green ovals indicate octapeptide domains and dark rectangular boxes indicate homeo-box. The dark green square box indicates the partial homeo-box.

	Gene	Structure	Expression domain
		Paired Box *OP* *Homeo Box*	
Group1	Pax1		Skeleton, Thymus
	Pax9		Skeleton, Cranio-facial, Tooth
Group2	Pax2		Central nervous system (CNS), Kidney, Ear
	Pax5		CNS, B cell, Ear
	Pax8		CNS, Kidney, Thyroid, Ear
Group3	Pax4		Pancreas
	Pax6		CNS, Eye, Pancreas
Group4	Pax3		CNS, Neural crest, Skeletal muscle
	Pax7		CNS, Neural crest, Skeletal muscle

1.5.2 The function of Pax genes in development and organogenesis

The zebrafish Pax2a gene can be found on chromosome 13 and is expressed in the midbrain-hindbrain boundary region, optic stalk, otic system, pronephros, nephric ducts and specific interneurons of the hindbrain and spinal cord which is close to the expression pattern of the mammalian pax2 gene (Krauss et al., 1991). Pax2a is the only pax member that has been observed during nephron patterning events (Majumdar et al., 2000). Pax2b can be found on chromosome 12. Beside nephric system, Pax 2b gene is expressed in all Pax2a expression domains. However, the level of Pax2a expression is greater than Pax2b. The timing of Pax2b expression is always later than

Pax2a (Pfeffer et al., 1998). However, activation of both genes is co-ordinated at the 12-somite stage in the hindbrain and spinal cord (Pfeffer et al., 1998). Terzic et al. (1998) demonstrate that the differentiation of neural tube, eye, ear and kidneys were mediated by Pax2 gene at defined stages of human development. Lack of Pax2 expression is associated with major defects in the brain, ear, eye and kidneys (Torres et al., 1995, 1996; Favor et al., 1996).

Pax5 can be found on chromosome 1 and is not expressed in the optic and nephric system of zebrafish. It is expressed in the hindbrain and spinal cord transiently and weakly (Pfeffer et al., 1998). However, the expression of Pax5 is not found in the mouse otic region (Adams et al., 1992). Adams et al. (1992) demonstrated that Pax5 is uniquely expressed throughout B-lymphopoiesis. Mice lacking Pax5 fail to develop B-lymphocytes (Urbánek et al., 1994; Mansouri et al., 1996). In hematopoiesis, the function of Pax5 is well described and highlighted in its clinical importance (Rolink et al., 1999; Schaniel et al., 2002).

Pfeffer et al. (1998) demonstrated Pax8 was highly expressed at the 80% epiboly stage in zebrafish. Pax8 can be found on chromosome 5. It has been expressed in the central nervous system and is later regulated by Pax2a (Kwak, 2006). In contrast to the zebrafish, Pax8 expression has not been found in the otic placode in the mouse embryo. However, Pax8 was found in pre-otic cells during the gastrulation period and uniquely expressed during thyroid gland development in mouse (Plachov et al., 1990). As a result, defective Pax8 results in deficient development of the thyroid gland in mice (Mansouri et al., 1998) and hypothyroidism in humans (Maccia et al., 1998). Pax8 and Pax2 both are also important requlators in kidney development in humans (Narlis et al., 2007).

Pax genes have been found to play an important role in cancer growth. For example, Muratovska et al. (2003) demonstrated the expression of Pax gene is needed for the cancer cell growth and about 90% of the cancer cell lines expressed Pax genes. They also found Pax2 was expressed in a high proportion of primary tumors in breast, ovarian, lung, colon, prostate and lymphoma. The exact function of Pax genes in cancer is not totally understood yet. However, abnormal regulations of interactions such as interaction of Pax2, Pax5 and Pax8 gene with protein 53 (p53, Stuart et al.,

1995) and B-cell lymphoma 2 (Bcl-2, Hewitt et al., 1997) could be a reason for various cancers.

1.5.3 The genetic interactions of Pax genes

Several studies have been reported on genetic interactions of Pax genes (Hans et al., 2004; Solomon et al., 2003; Aghaalaei et al., 2007; Di Palma et al., 2003; Kishi et al., 2002). During otic placode induction in zebrafish, Fibroblast growth factor 3 (Fgf3) and Fgf8 genes express from the hindbrain with Foxil gene to induce Pax8 at 85% epiboly. Then, Pax8 expression regulates the expression of SRY-box containing 9a (Sox9a) which maintains expression of a second pair of competence factors, distal-less homeobox 3b (Dlx3b) and Dlx4b. Pax2a expression at the 3 somite stage requires Fgf3, Fgf8, Pax8, Dlx3b and Dlx4b regulation (Hans et al., 2004). Pax2a gene can maintain its own expression and give a feedback through Sox9a and Dlx3b. Pax8 and Sox9b also help to maintain this pathway (Hans et al., 2004; Solomon et al., 2003). In agreement with these reports, Foxil also plays an important role in ear development in medaka (Aghaalaei et al., 2007). However, Aghaalaei's paper also reported Fgf8 can activate Foxil, but not Pax8 which is different from zebrafish. As mentioned earlier, Pax8 was the only Pax gene expressed during thyroid gland development (Plachov et al., 1990). Thyroid transcription factor 1 (TTF-1) is another important gene also expressed in the thyroid tissue and cooperates with Pax8 to impact thyroid-specific gene expression (Di Palma et al., 2003). Similarly, cooperation of Pax5 with c-Myb activates the recombination-activating 2 (RAG-2) promoter in immature B cells in mouse (Kishi et al., 2002).

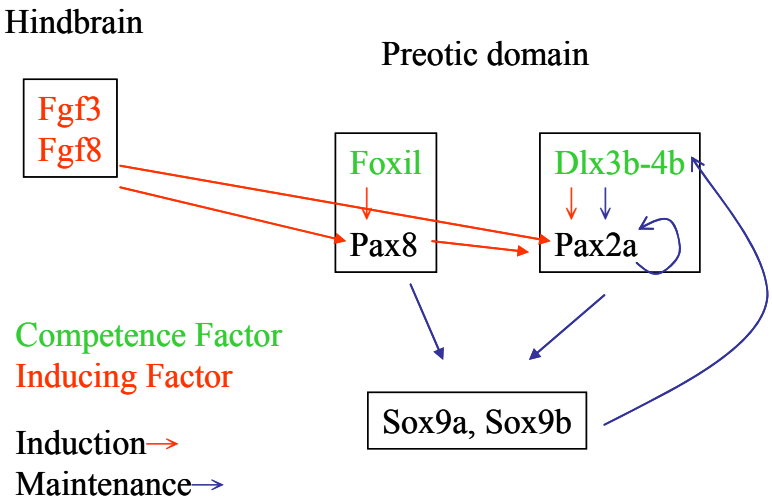


Figure 1.3 Pax8 and Pax2a function synergistically in otic specification, downstream of the Foxi1 and Dlx3b transcription factors (Hans et al., 2004).

1.6 The effect of cryopreservation on genome

1.6.1 The effect of cryopreservation on spermatozoa

Spermatozoa are the reproductive cells of males. The function of spermatozoa is to donate the paternal genome to the oocyte. Spermatozoan penetration and activation of oocyte triggers embryo development. During fertilization, the egg cell destroys mitochondria of sperm, as a result only maternal mitochondrial DNA remains (Aurelio et al., 2004). The structure of spermatozoa has been reported for about 300 fish species from more than 100 families (Matos et al., 2002) and sperm DNA is at least sixfold more condensed than somatic cells (Ward and Coffey, 1991). From the earliest work on sperm cryopreservation, the main standard for sperm viability has been motility and ability to fertilise the mature egg (Rana and McAndrew, 1989; Tiersch, 2001). However, if cryopreservation is to be used for routine storage of genetic material from different species, studies on the effect of cryopreservation at the molecular level must to be done to make sure that the process does not cause detrimental changes (Chao and Liao 2001).

There is evidence that cryopreservation causes DNA damage of spermatozoa in

human (Donnelly et al., 2001a, 2001b; Hammadeh et al., 2001; Royere et al., 1988, 1991), monkey (Li et al., 2007), livestock (Fraser and Strzezek, 2004; Peris et al., 2004) and fish species (Labbe et al., 2001; Zilli et al 2003; Fisher et al., 1994). Comet assay is a popular method for detecting DNA damage being less labour intensive, more rapid and less expensive than other methods. The resulting image shows the damaged DNA is separated from the intact DNA and resembles a comet with a head and tail after electrophoresis and staining. Labbe et al. (2001) demonstrated the effect of cryopreservation on DNA stability of rainbow trout sperm by using this method. The results showed that freezing only slightly affected sperm DNA stability but significantly increased the percentage of nuclei damage. This result was different from a later report on a different species by Zilli et al. (2003) who demonstrated significant damage at DNA level in sea bass sperm with the same assay. Similar results were obtained by Donnelly et al. (2001a) who found sperm DNA of infertile men to be affected by cryopreservation. Interestingly, this case does not appear on fertile men. It is possible that the semen from fertile men has better resistance to cryopreservation due to the presence of antioxidants protecting against cryodamage (Lewis et al., 1995). However, there is evidence that even the DNA integrity of sperm from healthy men can be affected by the freezing procedure (Donnelly et al., 2001b).

The process of cryopreservation can destabilize the chromatin and further increase DNA susceptibility to denaturation. Indeed, this result is already evidenced in human (Spanó et al., 1999) and ram (Peris et al., 2004) sperm. The sperm chromatin structure assay (SCSA) which is a flow cytometric technique was used in these studies. SCSA uses the metachromatic properties of acridine orange to stain sperm. When acridine orange binds with intact DNA (double-stranded), a green fluorescence is seen. Red fluorescence emits when binding is to denatured DNA (single-stranded). The studies showed that sperm quality degenerates after the freeze-thaw protocol due to DNA damage. Although the susceptibility of ram sperm DNA to denature *in situ* had no effect of cryopreservation, significant DNA damage appeared after 3 hours of incubation (Peris et al., 2004). A similar result was also obtained on isolated erythrocytes and leukocytes of largemouth bass (Fisher et al., 1994). In addition, human and porcine spermatozoa have also been compared using acridine orange staining after cryopreservation. Hamamah et al. (1990) demonstrated that the cryopreservation procedure affected the percentage of DNA in human sperm but did

not appear in boar. It is possible that the boar DNA-protamine complex is more anti-freezing or relate to the quality of sperm between human and porcine (Kopeika, 2007).

To date, there is no information about the effect of cryopreservation on gene expression in sperm of any species. However, few reports are available on the effect of the freezing protocol on cellular proteins. In human (Cao et al., 2003) and boar (Huang et al., 1999), heat-shock protein 90 (HSP 90) has been described. The results demonstrated that HSP 90 decrease substantially in boar sperm after cryopreservation when analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot. Cao et al. (2003) found significantly decreased levels of HSP 90 after cryopreservation of human sperm. These results might be associated with a reduction in frozen-thawed sperm motility. Surface proteins are involved in the steps leading to fertilisation (McLesley et al, 1998), and Lessard et al. (2000) concluded that P25b surface protein may be degraded during cryopreservation resulting in a reduction of fertility in bull sperm. Proteins analysis of fish sperm (Zilli et al., 2005), demonstrated degradation of protein after cryopreservation found in sea bass sperm using two-dimensional polyacrylamide gel electrophoresis. This could explain the lower sperm motility and hatching rate of embryos derived from cryopreserved sperm.

1.6.2 The effect of cryopreservation on oocytes

The oocyte contributes not only the maternal nuclear genome, but also the mitochondrial genome and nutrients to the zygote and embryo. Cryopreservation of fish oocytes has not yet been successful (see section 1.3.2.). However, cryopreservation of mammalian oocytes by controlled slow freezing and vitrification is currently conducted successfully, producing live offspring (Glenister et al., 1987; Nobuya, 2005; Al-Hasani et al., 1989; Vincent et al 1989; Chen, 1986; Van Uem et al., 1987; Fuku et al., 1992).

Although cryopreservation of mammalian oocytes has been successful, the effectiveness of oocyte cryopreservation in some species including human is still low.

Recently, DNA damage in the oocyte has been found after cryopreservation and this could be one of the reasons for reduced developmental ability of cryopreserved oocytes (Stachowiak et al., 2007; Men et al., 2003a, 2003b; Demirci et al., 2002). Stachowiak et al. (2007) compared three different vitrification methods on the levels of DNA integrity using comet assay. The authors found both minimum sample size (MSS) vitrification methods were safe for cryopreserving bovine oocytes. However, open pulled straw method (OPS) resulted in a significant level of DNA damage. These results are different from the results presented by Men et al. (2003), who demonstrated that oocyte DNA exhibited different degrees of damage following similar freezing methods. The different results between these two papers might be due to some differences between vitrification procedures. Also Stachowiak et al. (2007) removed granulosa cells from oocytes prior to cryopreservation but Men et al. (2003) left granulosa cells on the surface of oocytes. However, to have sufficient saturation of oocytes by cryoprotectants for successful vitrification, granulose cells had better to be removed (Papis, 1996). So far, there is no publication on the effect of cryopreservation on DNA damage in fish oocytes.

Some studies have been undertaken on mRNA and protein levels affected by cryopreservation. Succu et al. (2007) was the first group to analyse the effect of vitrification on maturation promoting factor (MPF) using three different cryodevices (open pulled straws, cryoloops and cryotops). Cryoinjures in ovine oocytes are capable of reversal after a period of time of thawing. Although MPF was affected by the different cryodevices, it was restored after 2 hours of culture in the open pulled straw and cryoloop groups but not in the cryotop group. In the following year, Succu et al. (2008) reported a quantitative assay of development related genes (beta actin, H2A.Z histone, Poli A Polimerase, Heat shock protein 90β, $P34^{cdc2}$, Cyclin b, Na/K-ATPase and Type I cadherin) in frozen-thawed ovine oocytes to determine the potential influences of vitrification method on the oocyte mRNA abundance. The results show that freezing procedures lower the ovine oocyte mRNA levels. Similarly, a decrease in CD9 molecule (CD9) expression was reported in frozen-thawed mouse oocytes (Wen et al., 2007). These data provide an early signal of quality in association with the poor developmental capacity of oocytes after cryopreservation.

1.6.3 The effect of cryopreservation on embryos

Most sperm and oocyte studies investigated the effects of cryopreservation on DNA damage. There is evidence that cryopreservation could lead to DNA damage in embryos (Takagi et al., 1996; Park et al., 2006) and embryonic cells (Kopeika et al, 2005). These results indicate that cryopreservation procedures cause embryo damage and result in DNA fragmentation and decreasing developmental ability.

As maternal mRNA and proteins from the oocyte support the earlier cleavages of the embryo, studies on specific gene expression during embryonic development are very important. Successful cryopreservation of human embryos has been reported and the thawed embryos have the same implantation ability as fresh embryo at the same stage (Burns et al., 1999; Edgar et al., 2000). There is a problem of limited number of human embryos for research, however, studies on gene expression in frozen-thawed embryos can offer valuable information. Tachataki et al. (2003) used real time RT-PCR to analyse the expression pattern of the tuberous sclerosis TSC2 gene during preimplantation development of human embryos. The results of gene expression obtained in this study are similar to the results reported for the mouse housekeeping gene (β-actin), heat shock protein gene (Hsp 70), genes related to oxidative stress (MnSOD and CuSOD), cold stress (CirpB, Rbm3), cell-cycle arrest (Trp53) (Boonkusol et al., 2006), apoptosis (Bax, Bcl2 and p53) (Dhali et al., 2007) and glucose transporter (GLUT1) (Uechi et al., 1997). These studies concluded that cryopreservation does affect the normal pattern of gene expression during embryonic development although cryopreservation has different levels of effect on these genes as exemplified by upregulation of stress-related and GLUT1 genes and the downregulation of apoptosis and TSC2 gene.

There have been no reported studies on the effect of cryopreservation on mRNA abundance in fish species. This might be due to fish embryos not having been successfully cryopreserved (see section 1.3.3). However, investigations of the activity of cytoplasmic enzymes (lactate dehydrogenase and glucose-6-phosphate dehydrogenase) have been conducted on zebrafish and turbot embryos even with zero survival rates after cryopreservation (Robles et al., 2004). Their results showed significantly lower enzymatic activity linked to cell rupture due to ice crystal

formation during vitrification. Similar results were found in wood frog organs (Cowan and Storey, 2001) and insects (Storey et al., 1991). Some researchers even tried to find certain genes which have the potential to improve the successful cryopreservation of fish embryos such as antifreeze protein (AFP) (Young and Fletcher, 2008; Robles et al., 2005) and Aquaporin-3 gene (Hagedorn et al., 2002). AFP can protect cell membranes from cold induced damage while Aquaporin-3 gene increased membrane permeability to water and cryoprotectant.

As mentioned in section 1.3.4, an alternative way to maintain full genetic complement is cryopreservation of fish blastomeres. Blastomeres have been successfully cryopreserved in many fish species (Harvey, 1983; Nilsson and Cloud, 1992; Calvi and Maisse, 1998, 1999; Cardona and Garcia-Ximenez, 2007; Kusuda et al., 2004, 2002; Strussmann et al., 1999). There has been no reported studies on the effect of cryopreservation on DNA, mRNA levels and protein expression. A study on cryopreservation of zebrafish blastomeres demonstrated on increased frequency of mutations in the mtDNA (Kopeika et al, 2005). However, whether such mutations affect overall function of cells and have any consequences for the future cell development requires further study.

1.6.4 The effect of cryopreservation on tissues and cells

Cryopreservation of ovarian tissue is a viable alternative to cryopreservation of oocytes or embryos in human and animals. Grafts of cryopreserved ovarian tissue have resulted in live-born mice (Carroll and Gosden, 1993; Parrott, 1960), sheep (Gosden et al., 1994) and human (Donnez et al., 2004). However, the expression of heat shock proteins, DNA-damage-inducible protein 45 and death-related apoptosis genes were significantly increased in mouse ovarian tissue after cryopreservation (Liu et al., 2003). This work suggested that although competent follicles and mature oocytes were yielded by freezing techniques, they need to be further refined. Interestingly, cryopreservation procedure did not affect apoptotic cell death proteins (Bcl-2 and p53) in porcine ovarian tissue (Hussein et al., 2006).

The effect of cryopreservation on somatic cells has been studied. Some reports

demonstrate that cryopreservation has no negative effect on cells (Sperling and Zeindl, 1983; Ashwood-Smith 1985; Pearson et al., 1990) but some do (Fisher et al., 1994; Deneys et al., 1999; Fairbairn et al., 1994; Ohnishi et al., 1977; Tanaka et al., 1979). These studies were carried out using different subjects. Although it is difficult to compare between the results of these studies, some results are worthy of discussion.

The frequency of chromosomal aberrations in skin fibroblasts increased with duration of cell preservation in liquid nitrogen. Cells treated with DMSO only displayed an increased number of chromosomal and chromatid breaks and translocations without cryopreservation (Polianskaya et al., 1990). Several studies showed that the effect of cryopreservation on the genome is dependent on the repair system of bacteria and skin fibroblasts (Ohnishi et al., 1977; Tanaka et al., 1979). These results suggested that the repair system might be one of the reasons for the different species in cryoresistance. It has been also shown that cryopreserved lymphocytes were unable to repair hydrogen peroxide-induced DNA strand breaks (Duthie et al., 2002).

1.7 Use of zebrafish (*Danio rerio*) as a model system

The zebrafish is a tropical fresh water fish originating from southeast Himalayan region (Mayden et al., 2007). The fish can be reared at a wide range of temperatures (15.5-43.3 ºC) and pH (6.6-9.2). They are recognised as having many advantages as experimental animals for biological research (Westerfield, 1993) including the combination of low cost and large-fertilized embryos (about 0.75 mm) produced on a daily basis, rapid embryonic development, short life history (Hisaoka and Battle, 1958), transparent embryos developing outside the mother's body, easy handle and observe under a microscope.

Zebrafish embryos develop organs that are similar to those in humans, including central nervous system, pancreas, and thymus and quickly form blood vessels and beating heart, and zebrafish are rapidly becoming an important model for transgenic and mutagenic studies in human health and disease.

In environmental sciences, the zebrafish embryo has been promoted to develop an acute fish toxicity test for environmental risk assessment (Nagel, 2002; Scholz et al., 2008). Acute fish toxicity tests are required for the testing of chemicals, pesticides, biocides and pharmaceuticals for environmental risk assessment (Nagel, 2002; Scholz et al., 2008).

1.7.1 Structure of zebrafish embryos

The zebrafish embryo is surrounded by a chorion and perivitelline space. (Figure 1.3). The chorion is approximately 1.5-2.0 μm thick (Hart and Collins, 1983), and has roles in diffusive exchange of gases, providing physical protection (Stehr and Hawkes, 1979; Grierson and Neville, 1981), as a flexible filter for transport of some materials (Toshimore and Yasuzumi, 1976) and protection against micro-organisms (Schoots et al., 1982). The space between the chorion and vitelline membrane is called perivitelline space. The formation of perivitelline fluid is brought about by the release of colloidal protein into the perivitelline space, bringing water from the external medium. The developing embryo has two compartments: blastoderm and yolk. The blastoderm is the developing part of the embryo, comprising embryonic cells (blastomeres) and the first cleavage occurs 40 min after fertilization. The role of the yolk is to provide nutrients to the blastomeres (Dasgupta and Singh, 1981; Kimmel and Law, 1985). The yolk syncytial layer is below the blastomeres and covering the yolk. The yolk syncytial layer forms at the time of the 10th cell division by a collapse of a group of blastomeres into the yolk (Kimmel and Law, 1985).

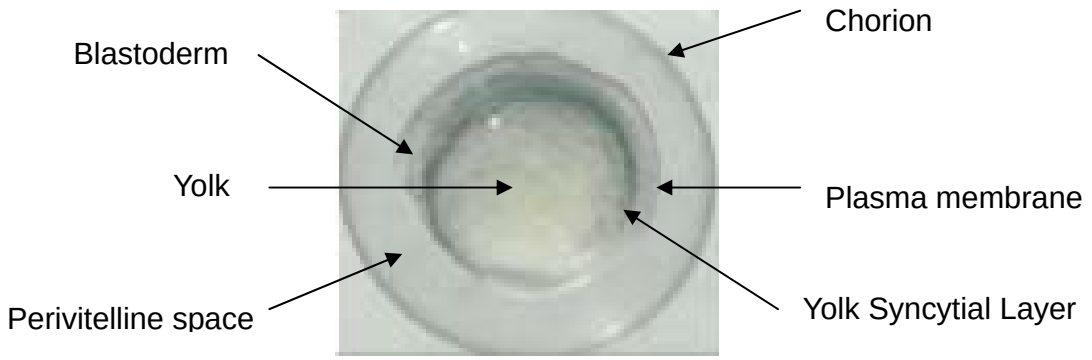


Figure 1.4 6-somite stage of zebrafish embryo (modified form Hagedorn et al., 1996)

1.7.2 Development of zebrafish embryos

The timing of development stages of the embryo is temperature dependant. Hatching is approximately 4 days after fertilisation at 26°C. The yolk disappears and is absorbed by the larvae after approximately 11 days (Hisaoka and Battle, 1958). Kimmel et al. (1995) described the development stages of zygote, cleavage, blastula, gastrula, segmentation, pharyngula and hatching. Brief descriptions of all the embryo stages are given in Table 1.2. Zebrafish blastomeres were selected from seven selected developmental stages of zebrafish embryos (Oblong, 30% epiboly, 50% epiboly, 75% epiboly, 100% epiboly, 6 somite and 20 somite stages) for use in the present study.

Table 1.2 Stages of zebrafish embryo development at 28.5°C (Kimmel et al., 1995).

Stage (hours post fertilization)	Description
Zygote Period (0-0.75 h) 1-cell (0.2 h)	Cytoplasm streams toward animal pole to form the blastodisc
Cleavage Period (0.75-2.2 h) 2-cell (0.75 h), 4-cell (1 h), 8-cell (1.25 h), 16-cell (1.5 h), 32-cell (1.75 h), 64-cell (2 h)	The first 6 cleavages occur. The blastomeres divide synchronously at about 15 minute intervals.
Blastula Period (2.25-5.25 h) 128-cell (2.25 h), 256-cell (2.5 h), 512-cell (2.75 h), 1000-cell (3 h), High (3.3 h), Oblong (3.7 h), Sphere (4 h), Dome (4.3 h), 30%-epiboly (4.7 h)	Midblastula transition occurs at the 10th cleavage. At this division, cell membranes do not form between cells of the bottom, marginal, row of blastomeres, and thereafter, it develops into the yolk syncytial layer of the yolk cell. After midblastula, transition cell divisions are asynchronous. Margin reaches 30% epiboly.
Gastula period (5.25-10 h) 50%-epiboly (5.25 h), Germ-ring(5.7 h), Shield (6 h), 75%-epiboly (8 h), 90%-epiboly (9 h), Bud (10 h)	Morphogenetic movements of involution, convergence, and extension form the epiblast, hypoblast, and embryonic axis through to the end of epiboly.
Segmentation Period (10-24 h) 1-somite (10.3 h), 6-somite (12 h), 14-somite (16 h), 20-somite (19 h), 26-somite (22 h)	Somites, pharyngeal arch primordia, and neuromeres develop; primary organogenesis; earliest movements; the tail appears.
Pharyngula period (24-48 h) Prim-5 (24 h), Prim-11 (30 h), Prim-23 (36 h), Pec-ridge (42 h)	Phylotypic-stage embryo; body axis straightens from its early curvature about the yolk sac; circulation, pigmentation, and fins begin development.
Hatching period (48-72 h) Pec-fin (60 h), Protruding-mouth (72 h)	Completion of rapid morphogenesis of primary organ systems; cartilage development in head and pectoral fin; hatching occurs asynchronously. At 72 h, swim bladder inflates; food-seeking and active avoidance behaviours.

1.8 Intended approaches of the present study

The aim of the study was to determine whether cryopreservation caused any change in gene expression of isolated blastomeres. The study can be divided into three main parts.

(a) Cryopreservation of zebrafish blastomeres by controlled slow freezing.

For successful cryopreservation with controlled slow freezing it was essential to determine the optimal freezing regime. The study set out to develop a protocol for controlled slow freezing for zebrafish blastomeres. This optimal protocol then served as the basis for further gene expression experiments. Several critical variables such as toxicity of cryoprotectants, freezing rates and cryoprotectant supplements were examined. The results are presented in Chapter 3.

(b) Identification of suitable housekeeping genes for use in gene expression studies of zebrafish embryos and blastomeres following cryopreservation.

Housekeeping genes are commonly used as internal control for the quantification of genes of interest in gene expression studies (Sturzenbaurn and Kille., 2001; Bustin, 2002). To date, there has been no systematic study for selecting suitable housekeeping genes in zebrafish embryos and blastomeres after cryopreservation. The aim of the study set out to assess seven different housekeeping genes for their potential use as internal controls to normalize for expression of genes of interest in experimental treatments with chilling in zebrafish embryos and freezing in isolated blastomeres. Results on this study are presented in Chapter 4.

(c) Effect of chilling and cryopreservation on expression of Pax genes in zebrafish embryos and blastomeres.

The optimal cryo-protocol was used to investigate potential possible influence of freezing on mRNA levels, a study was carried out to compare the pattern of expression of four transcription factor genes (Pax2a, Pax2b, Pax5 and Pax8) in fresh and chilled embryos as well as in fresh and frozen-thaw blastomeres using sensitive quantitative real time PCR. Results on this study are presented in Chapter 5.

CHAPTER 2: MATERIALS AND METHODS

2.1 Introduction

Three main areas of study were covered by the project: cryopreservation of zebrafish blastomeres by controlled slow cooling; identification of suitable housekeeping genes for use in gene expression studies of zebrafish embryos and blastomeres following cryopreservation; effect of chilling and cryopreservation on the expression of Pax genes in zebrafish embryos and blastomeres. All the experimental work was carried out in the laboratories at LIRANS - Institute of Research in Applied Natural Sciences, University of Bedfordshire, UK.

Information on the chemicals used in the present study is given in Appendix A. Fresh aqueous solutions were prepared in deionised water shortly before their use. If necessary, solutions were stored in a fridge (4 °C) or freezer (-20 °C).

2.2 General methods

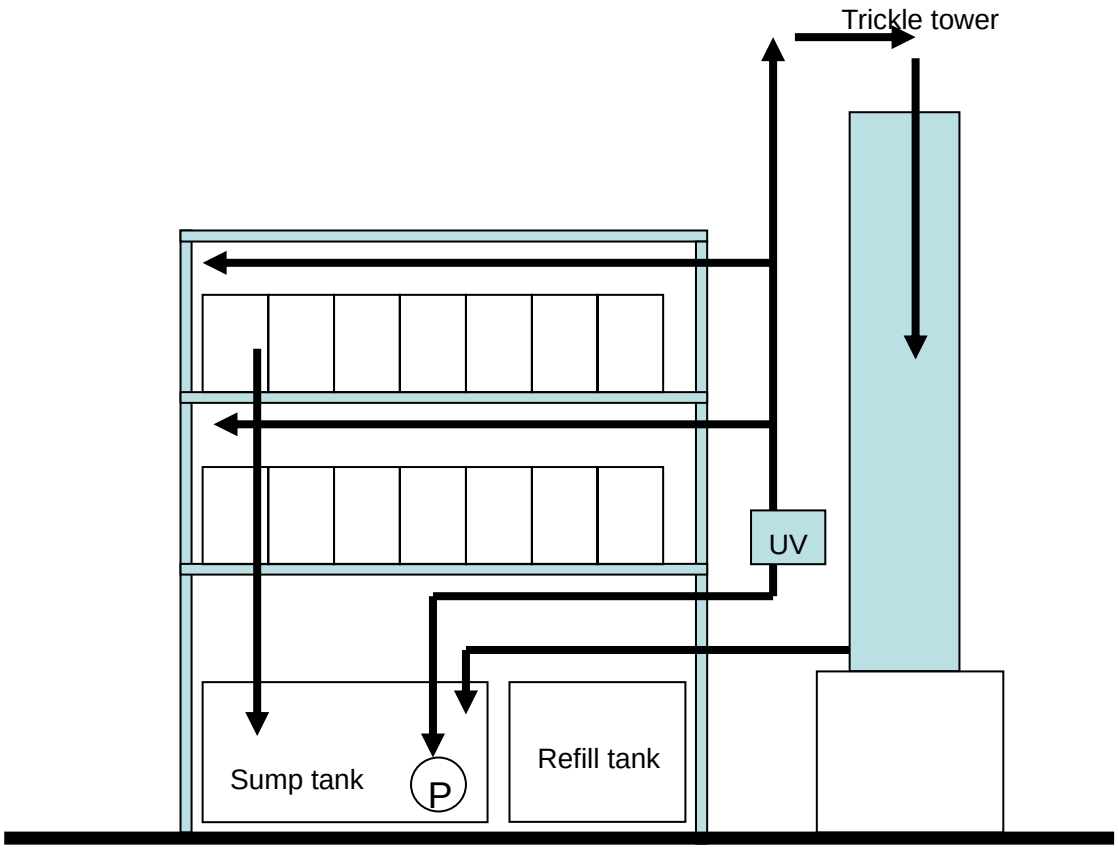
2.2.1 Maintenance of zebrafish

2.2.1.1 General information

Adult zebrafish (*Danio rerio*) 12-14 weeks old were obtained from Aquascape Ltd. (Birmingham, UK). They were maintained in 45 L (30x30x60 cm) glass fish tanks at 28°C with approximately 50 fish per tank and a controlled day-night cycle of 12 hr light/12 hr dark. A quarter of the tank water was replaced twice per week. Tap water aged for at least 2 days was used as tank water with added sea salts (0.25 g/L). The tanks were constantly aerated and filtration of the water in the tanks was carried out by using an electric pump connected to an upturned funnel which was surrounded by filter floss in a beaker (1 L) immersed in the fish tank. The funnel and floss were held in position by a layer of smooth gravel. Water was pulled through the gravel and floss

by suction effect generated by the rising air. Water circulating system was also used to maintain zebrafish (see Figure 2.1). It held approximately 30 fish per tank (35x17x26 cm). The water was prefiltered through a carbon filter sited to the right of the trickle tower. Water automatically entered the refill tank from sump tank which contain filter bags, the heaters and the main pump to supply water to the system via the UV filter. Filter bags and activated carbon in prefilter were checked annually.

Figure 2.1 Zebrafish system in LIRANS



2.2.1.2 Feeding

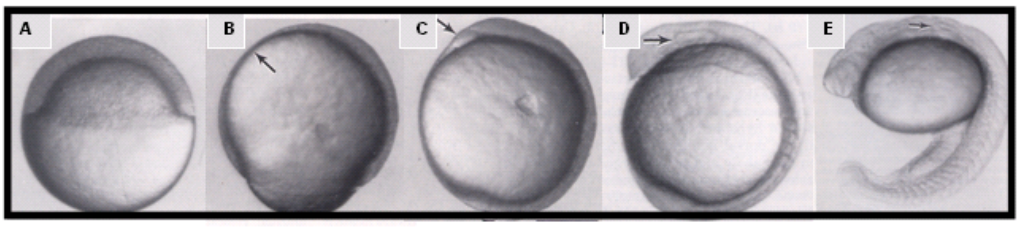
Fish were fed three times per day with Tetra brand dry flake food (ingredients: processed fish and fish derivatives, cereals, yeast, vegetable protein extracts, molluscs and crustaceans, oils and fats, derivatives of vegetable origin, algae, various sugars contains permitted colorants). Flake food was pulverized finely by hand. In order not

to over feed the fish, the maximum amount of food fed to the fish was the amount that could be eaten within five minutes. An afternoon feed of live adult brine shrimp (*Artemia salini*) was also given. Brine shrimps were specially prepared in sea-water-filled, aerated hatcheries (52.5 g of sea salts in 1.5 litres of deionised water). During weekends and holidays, automatic fish feeders (Fish Mate F14 Aquarium Fish Feeder) were used.

2.2.1.3 Breeding and embryo collecting

Females and males were kept together in the ratio of 1:2-3. A glass tray covered with a plastic net and plastic grass was placed at the bottom of the tank to trap eggs. The eggs were removed the following morning since spawning was induced by the first light of the morning. Pairs of male and female or trios of one female and two males swam closely together and the male pressed and curved his body around the female and fertilised her eggs as they were laid. The eggs fell through the net and were kept away from the cannibalistic parents. Embryos collected in the morning were then placed in tank water at 28°C and kept incubated until they had developed to the required stages (Figure 2.2)

Figure 2.2 Determination of the developmental stage of zebrafish embryos. Embryo developmental stages were determined by light microscopy according to the criteria based on corresponding morphological features described by Kimmel et al. (1995).



A) 50% epiboly (early gastrula; 5.3 hours post fertilization (hpf), B) 75% epiboly (mid-late gastrula; 8 hpf), C) 100% epiboly (late gastrulation; 9.5 hpf), D) 6-somite (early segmentation; 12 hpf) and E) 20-somite (late segmentation; 20 hpf)

2.2.1.4 Collection of blastomeres

Intact embryos at different development stages were washed with phosphate buffered saline (PBS) and placed in 6-well tissue culture dishes. The blastoderm was removed by mechanical separation using pipette suction and forceps. The blastoderm suspension was washed twice with PBS (centrifuged for 10min at 3000 rpm). After the last centrifugation the supernatant was discarded and pellet was resuspended in the PBS buffer for treatment (Kopeika *et al*., 2005).

2.2.1.5 Blastomere culture

The fresh and frozen-thawed blastomeres isolated from 50% epiboly stage embryos were washed (centrifuge for 5min at 3000 rpm) three times in washing medium (10% PBS, 400 µg/ml gentamycin, 200U/ml penicillin and 2.5 mg/ml amphotericine B). After the last centrifugation the supernatant was discarded and the pellet was resuspended in the culture medium (50% Leibowitz's L-15 medium, 25mM Hepes, 5mM $NaHCO_3$, 50µg/ml gentamycin, 2mM L-glutamine, 200 µg/ml penicillin, 2.5 mg/ml amphotericine B and 2% Ultroser, pH 7.5) and seeded into a single well of a 24- well culture plate. The culture medium was filter sterilised, and stored frozen (-20°C) before use.

2.3 Cryopreservation of zebrafish blastomeres by controlled slow cooling

2.3.1 Toxicity of cryoprotectants to zebrafish blastomeres

Four penetrating cryoprotectants, METH, DMSO, PG and EG, at four concentrations 2, 3, 4 and 5 M were investigated. For DMSO, 1 M and 1.5 M were also investigated. Toxicity trials were initiated by adding 50 µl of double-concentration cryoprotectants to microtubes containing blastomeres in 50 µl

phosphate buffered saline (PBS) and held for 30, 60, 120 and 180 min at 22°C. Control blastomeres were incubated in PBS solution under the same conditions. After incubation in cryoprotectants, viability tests were conducted using TB staining and No Observed Effect Concentrations (NOECs), the highest concentration that was found to have no statistically significant difference comparing with control ($p>0.05$), were determined.

2.3.2 Cryopreservation of zebrafish blastomeres

PBS with 10% fetal bovine serum (FBS) solution was used as a working medium for all procedures, with 1.5 M DMSO and 2 M METH, PG, and EG. Blastomeres at 50% epiboly stage were used for all freezing experiments. Blastomeres were placed in Eppendorf tubes (1.5 ml) and incubated in cryoprotectant solutions for 30min at 22°C. Blastomeres were then drawn into 0.25 ml plastic straws (9 straws per treatment) and cooled in a programmable cooler (Planer KRYO10 Series II). The following cooling protocols were used: from 22°C to seeding temperature (-6°C for DMSO and -7.5°C for METH, PG, and EG) at the rate of 1, 2 and 5°C/min (protocol 2, Table 2.1), hold for 15 min. Freezing from -6°C to -40°C at the rate of 0.3, 1 and 2°C /min (protocol 1, Table 2.1), from -40°C to -80°C at the rate of 2, 5 and 10°C /min (protocol 3, Table 2.1), hold for 10 min. Samples were then plunged into liquid nitrogen for at least 10-20 min. Frozen samples were thawed rapidly by immersing straws into a 28°C water bath for approximately 15 s. After thawing, step-wise removal of cryoprotectants was performed in Eppendorf tubes (1.5 ml) by mixing the 100µl sample with a graded series of PBS+10% FBS solutions (50, 150, 300 and 600 µl) for gradual removal of the cryoprotectant in four 5 min steps. Some samples were also plunged into liquid nitrogen after holding at -40° for 10min using the above mentioned protocol, for the study on the effect of different liquid nitrogen plunge temperatures (protocol 4, Table 2.1).

Table 2.1 Main steps in the development of a cryopreservation protocol for zebrafish blastomeres by controlled slow cooling.

Protocol 1	Protocol 2	Protocol 3	Protocol 4
22°C ↓1°C /min, -6°C or -7.5°C ↓0.3, 1 and 2/min -40°C ↓2°C /min -80°C hold for 10min ↓ LN2	22°C ↓1, 2 and 5°C /min, -6°C ↓0.3/min -40°C ↓2°C /min -80°C hold for 10min ↓ LN2	22°C ↓5°C /min, -6°C ↓0.3/min -40°C ↓2, 5 and 10°C /min -80°C hold for 10min ↓ LN2	22°C ↓5°C /min, -6°C ↓0.3/min -40°C ↓ LN2

2.3.3 Effect of additional cryoprotective supplements

The addition of sugars to a freezing solution influences the overall properties of the solution, and the properties of sugar supplement modification of a freezing solution was investigated. The results of freezing and thawing experiment revealed the optimal freezing protocol for blastomeres to be 5°C/min to -6°C hold for 15min, 0.3°C/min to -40°C, 2°C/min to -80°C, hold for 10 min, then directly plunged into liquid nitrogen using 1.5M DMSO as the cryoprotectant. In this experiment, the optimal freezing protocol was used to investigate the effect of sugar supplements on blastomere survival. Three supplements were tested - sucrose, glucose and trehalose - at three final concentrations of 0.05, 0.1 and 0.2M.

2.3.4 Viability assessment

TB staining was used to assess cell viability after cryoprotectant exposure and cryopreservation. A blastomere suspension (20 µl) was mixed with an equal volume of TB (0.4%, Sigma) solution for 3-5 min and a total of ~ 150 blastomeres were

checked and the number of unstained (surviving) and stained (dead) cells determined using a hemocytometer slide under an inverted microscope (LEICA DMIL, 100 times total power). The suvival rate was calculated by the following formula: survival rate (%) = unstained cells / (unstained cells + stained cells) x 100%. TB was also compared with another method, FDA + PI. Staining viability was assessed after 1.5M DMSO plus 0.1M sucrose treatment and the optimal freezing and thawing protocol. The efficacy of the FDA and PI methods, and the combination of these two fluorochromes (FDA+PI), to distinguish between live and dead blastomeres, the staining of blastomeres exposed to lethal treatment (the blastomeres were incubated for 10 min in 99% of DMSO) was compared with that of control blastomeres.

Cell staining by FDA+PI: A stock solution of FDA was prepared by dissolving 5mg/ml FDA in acetone. The FDA working solution was freshly prepared before use by adding 0.04 ml of stock to 10 ml of PBS. The propidium iodide stock solution was made by dissolving 1 mg PI in 50 ml PBS. To stain with FDA+PI, 0.1ml (2 μg) of FDA working solution and 0.03 ml (0.6μg) of PI stock solution were added directly to the resuspended cells. The blastomeres were stained in the dark for 3 min. The evaluation of viability was conducted by fluorescent microscopy (cells fluorescing bright green were considered to be viable, while nonviable cells stained bright red). FDA+PI stained blastomeres were examined with an inverted microscope with additional incident light fluorescence unit (LEICA DM IL), equipped with band pass 450-490 nm (blue) excitation filter, 510 nm dichromatic mirror, long pass 515 suppression filter and with band pass 515-560 nm (green) excitation filter, 580 nm dichromatic mirror, long pass 590 suppression filter. These filters did not permit both green and red fluorescing blastomeres to be seen simultaneously (Zampolla et al., 2008).

2.3.5 Statistical analysis

For statistical analysis one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test were used. ANOVA (confidence level < 0.05) was used in order to test for significant differences between the mean values from experimental treatments and Tukey's post-hoc test was used in order to determine which groups specifically differ. Inscription 'N = ….' in graph legends indicates the total number of

embryos used for the corresponding experiment. The results of the study are presented in ‘mean value ± standard error’.

2.4 Identification of suitable housekeeping genes (HKGs) for use in gene expression studies of zebrafish embryos and blastomeres following cryopreservation

2.4.1 Embryo chilling treatment

Embryos from each developmental stage (50% epiboly, 75% epiboly, 100% epiboly, 6 somite and 20 somite) were placed in a cryoprotectant solution of 2M methanol and 0.2M sucrose in PBS and then incubated at 1ºC for 24 hours. Chilled embryos were then returned to room temperature and were washed three times in fresh PBS over a 30 minute period in order to remove the cryoprotectants and allow recovery.

2.4.2 Blastomere cryopreservation

The optimal protocol for cryopreservation of zebrafish blastomeres from 50% epiboly embryos used a cryoprotectant mixture of 1.5M DMSO and 0.1M sucrose in PBS with 10% foetal bovine serum (incubation time of 30 min), and a cooling protocol of 5°C/min from 22°C to a seeding temperature of -6°C, held for 15 min; then cooled at 0.3^{o}C/min to -40°C; followed by cooling at 2°C/min to -80°C, held for 10min, and then direct plunge into liquid nitrogen (see section 2.3.2).

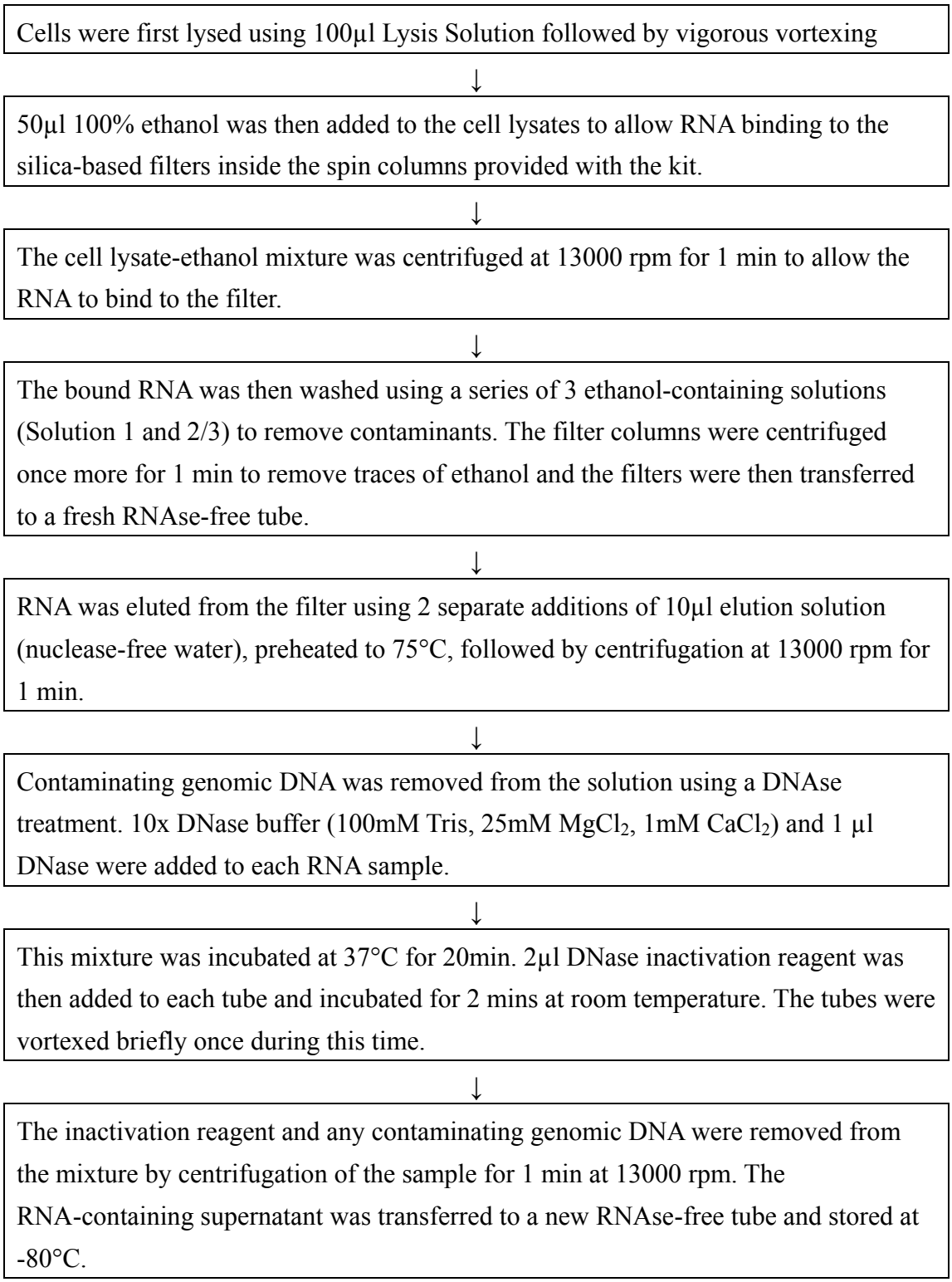
2.4.3 Culture of blastomeres

Groups of both fresh and frozen-thawed blastomeres were cultured at 28°C for either 2, 5, 7 or 15 hours and samples were harvested into 1.5ml tubes for initial storage at -80°C before RNA extraction (culture condition see session 2.2.1.5).

2.4.4 RNA extraction and DNase treatment

RNA was collected from groups of 15 fresh or chilled embryos and from fresh and frozen-thawed blastomere samples using the RNAqueous-Micro RNA Isolation Kit (Ambion, Huntingdon, UK), according to the manufacturer's instructions (Figure 2.3). This included a DNase I treatment and DNase inactivation step to remove any contaminating DNA. The RNA was then stored at -80°C before further processing.

Figure 2.3 The protocol of RNA extraction.



2.4.5 Reverse transcription (cDNA synthesis)

A 5µl (for embryo samples) or 200ηg (for blastomere samples) volume was reverse transcribed with oligo(dT) $_{18}$ primers using SensiMix^{TM}Two-Step Kit (Quantace, London, UK) following the manufacturer's protocol. The first step of reactions took place in 10µl volumes consisting of oligo $(dT)_{18}$ (2µl), 10mM dNTP (1µl), and DEPC-treated water (5µl) and incubation at 65°C for 10 min. The second step of reaction took place in 20µl volumes consisting of a sample from the first step (10µl), 5x SensiMix reverse transcriptase buffer (4µl), RNase inhibitor (1µl), SensiMix reverse transcriptase (0.25µl), and diethylpyrocarbonate (DEPC)-treated water (4.75µl) and incubated at 45°C for 50 min followed by incubation at 70°C for 15 min to terminate the reaction. The reverse transcription reaction was run in a PCR machine (Tech gene, Techne, Luton, UK). The cDNA was then diluted 1:100 in molecular biology grade water and stored at -80°C before analysis by real-time PCR. For each sample, a duplicate reaction was set up containing no reverse transcriptase enzyme. Subsequent PCR amplification would, therefore, allow confirmation of successful elimination of genomic DNA. A reaction containing no RNA was also set up, allowing for the detection of any contaminated reagents.

2.4.6 Generation of standards for real time PCR

The external standard of the appropriate size for each potential housekeeping gene was generated by conventional PCR using primers for the specific genes (see Table 2.2). PCR reactions were performed in 20µl volumes using 1x PCR buffer (Bioline, London, UK), 1.5mM $MgCl_2$ (Bioline), 0.5µM each primer (see Table 2.2), 200µM dNTP mix (Bioline), and 2U BioTaq polymerase (Bioline). PCR products were run on 2% agarose gels, and DNA was extracted from the excised bands using the EZNA Gel extraction kit (Omega Bio-tek) according to the manufacturer's instructions (Figure 2.4). The DNA concentration of the PCR products was quantified using spectrophotometry at 260ηm and extractions were diluted initially to 2ηg/µl followed by 10-fold serial dilutions to generate standards for real-time PCR.

Figure 2.4 The protocol of gel extraction.

400µl of the Binding Buffer was added to 1.5 ml tube contain agarose of DNA fragment and incubated with the agarose at 55-60°C for 7 min.

↓

The solution was applied to the column assembled in a 2 ml collection tube and then the liquid was discarded after centrifugation at 13000rpm for 1 min.

↓

The column was washed using a series of Binding buffer (300µl) and Wash buffer (700µl), followed by centrifugation of the empty column for 2 min at max speed to dry.

↓

DNA was eluted from the column with 30 µl elution buffer.

2.4.7 Real time PCR

Real-time PCR was performed on a RotorGene 6000 cycler (Corbett Research, UK) using a 72 well rotor. Reactions tubes contained 7.5µl SensiMix dT 2x mix and 0.3µl SYBR green (both from Quantace), 333ηM each primer (see Table 2.2) and 2µl of each sample or standard, made up to 15µl with molecular biology grade water. The reaction conditions were 1 cycle at 95ºC for 10 min, followed by 50 cycles of 95ºC for 10 sec, 59ºC for 15 sec and 72ºC for 15 sec. Data were acquired on the FAM/SYBR channel at the end of each extension step. Melt curves were analysed to check for the absence of mispriming and amplification efficiency from a standard curve was also determined (efficiency and R^2 were close to 1, see Appendix B). The possibility of a genomic DNA influence on the results was eliminated by the use of primers that crossed introns. A total of 45 embryos per developmental stage and blastomeres isolated from a total of 150 embryos per time point were used to obtain three biological replicates, each of which was analysed three times. Mean ± Standard error of the mean (SEM) relative expression levels for each gene were calculated using the RotorGene software (Version 1.7, Corbett Research) and Microsoft Excel.

2.4.8 Interpretation of results of real time PCR

The target products of PCR reactions were detected using the DNA binding dye, SYBR Green. As described previously, this dye fluoresces when it is bound to double-strand DNA. Fluorescence measurements collected at the end of each cycle are on a graph (Figure 2.5a). Early PCR cycles show undetectable levels of fluorescence, but once levels became detectable an exponential graph is generated until a plateau is reached. DNA standards in a series of 10-fold dilutions are analysed and show equally-spaced curves on the fluorescence graphs (Figure 2.5b). As the sample is diluted, it takes more cycles for a threshold level of fluorescence to be reached. The cycle number at which the threshold level is reached can be used to create a standard curve from which sample data can be generated (Figure 2.5c). The melting point of the product is also determined at the end of the amplification (Figure 2.5d). All PCR products should have the same melting temperature (80 to 82°C) unless the samples are contaminated, mispriming, occurs or primer-dimers are formed. Contaminating DNA or primer dimers would show as an extra peak separate from the target amplicon peak (Figure 2.5d).

2.4.9 Statistical analysis

Statistical analysis was carried out on mean relative expression data for each sample. Data were analysed using: 1) ANOVA with Games-Howell post hoc tests following logarithmic transformation of the data where necessary (SPSS and Microsoft Excel); 2) GeNorm software (Vandesompele, 2002) which selects the most stable pair of genes from those analysed and 3) NormFinder software (Andersen, 2004), which analyses the stability of each gene individually. GeNorm compares the variation between one gene against all other genes within the group being screened as possible HKG, and does this for each of the genes. It then eliminates the gene with the most variation compared to the others. To investigate the optimal number of reference genes, it was determined whether the stepwise inclusion of less stable genes significantly affected the normalisation factors according to the value V. A score of 0.15 is taken as a cut-off value, below which the inclusion of an additional reference

gene is not required. The process is repeated and the most variable gene is eliminated until only two genes are left. They are the best two HKGs. NormFinder analyses the inter (between groups) and intra (within groups) group variation for each gene separately. The gene with the least variation is considered the most stable.

Each potential HKG was analysed for stability of expression across 1) fresh embryos of different stages; 2) fresh and chilled embryos of different stages; 3) fresh blastomeres during culture and 4) fresh and cryopreserved blastomeres during culture. Stability of gene expression at individual stages of development was also investigated using GeNorm and NormFinder.

Table 2.2 Primer sequences, amplicon size and PCR efficiency of zebrafish candidate housekeeping genes.

	Orientation	Primer 5'-3' sequence	Bases	Melting temperature (MT)	Amplicon size
Beta actin (β-actin)	For	CCAGCTGTCTTCCCATCCA	19	59	86
	Rev	TCACCAACGTAGCTGTCTTTCTG	23		
Beta 2 microglobulin (B2M)	For	AAACGCCAGCTGTCTGGCTAAA	22	59	190
	Rev	TTCGAAGGCCAGATCTGTCTGTGT	24		
Elongation factor 1 alpha	For	CTGGAGGCCAGCTCAAACAT	20	59	87
(EF1α)	Rev	ATCAAGAAGAGTAGTACCGCTAGCATTAC	29		
Glyceraldehyde-3-phosphate	For	CGCTGGCATCTCCCTCAA	18	59	83
dehydrogenase (GAPDH)	Rev	TCAGCAACACGATGGCTGTAG	21		
RNA polymerase subunit D	For	TTGCTAGTGTGCGCAGTTTGCT	22	59	184
(RNAP)	Rev	TGGAAGCTGCGTTTGGTCTGAA	22		
Ribosomal protein L13a	For	TCTGGAGGACTGTAAGAGGTATGC	24	59	148
(Rpl13a)	Rev	AGACGCACAATCTTGAGAGAGCAG	24		
Succinate dehydrogenase	For	ACACTGATCTGGTGGAAACGCT	22	59	189
complex subunit A,	Rev	AGTGCTGCTCGAAAGGCTTCTT	22		
flavoprotein (SDHA)					

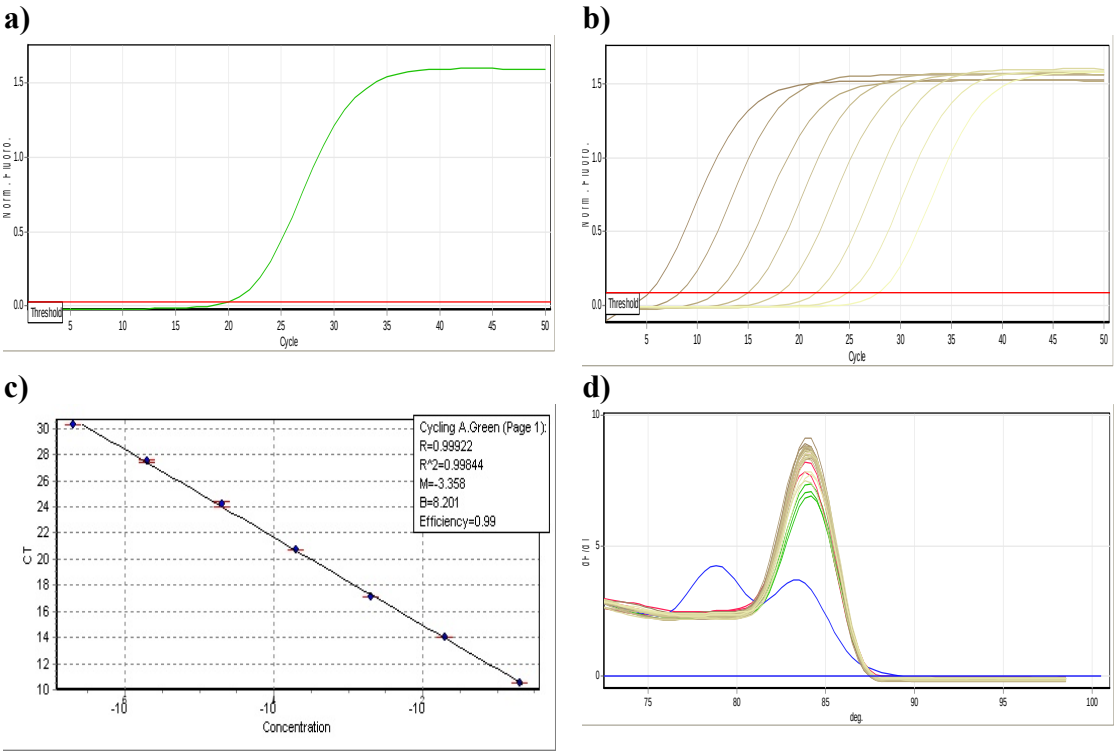


Figure 2.5 Graphs produced by real time PCR. a) Fluorescence measurements at each cycle. b) Standards of decreasing concentration crossing the threshold at increasing cycle numbers c) Standard curve from 10-fold dilutions due to an equal number of cycles separating standards of 10-fold concentration difference. d) Derivative melting curve with a primer-dimer using real time PCR.

2.5 Effect of chilling and cryopreservation on expression of Pax genes in zebrafish embryos and blastomeres

2.5.1 Preliminary experiment to determine timing and level of Pax2a and Pax2b genes in fresh blastomeres at different developmental stages using a One-step RT-PCR method

The following primers were used:

Pax 2a
(RT) primer: 5'-TACTGAGCCAGTCGTTCGGTC-3'
Reverse primer: 5'-ATCTCTCGACCTGTCACTACTG-3'
Forward primer: 5'-ACGTGATGATGATGGTTCAGATG-3'

Pax 2b
RT primer: 5'-TACGGTAACTTGATCATTGCG-3'
Reverse primer: 5'-ACTGGGAATTATAGTGTGTCCTG-3'
Forward primer: 5'-GTATTCACTCGCCTGCTCTGG-3'
Forward primer: 5'-AACGCACGGTCATGCCTGAC-3'

RNA extraction used Trisure RNA Isolation Reagent (Bioline), at the targeted stages of development. The RT reaction was carried out using a mix of RT primers and reverse transcriptase enzyme at 45°C for 30 min to prime the RT reaction and to produce cDNA. The PCR conditions were an initial denaturation step of 95°C for 10min followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 61 and 63°C for 30s, and extension at 72°C for 40s. The PCR products were analysed and confirmed by ethidium-bromide-stained 2% agarose gel electrophoresis. When a single band of the correct size was visualised on the gel, the primers were considered specific.

Information on the embryo chilling treatment, blastomere cryopreservation, culture of blastomeres, RNA extraction, reverse transcription and generation of standards for real time PCR are given in session 2.4.1 to 2.4.5.

2.5.2 Real-time PCR

In order to measure Pax2a, Pax2b, Pax5 and Pax8 gene expression. The amplification protocol used was as follows: Initial 10min denaturation and enzyme activation at 95°C, followed by 50 amplification cycles of denaturation at 95°C for 10 sec, annealing for 15 sec at the appropriate temperature (see Table 2.3), elongation at 72°C for 15 sec. Data were acquired on the FAM/SYBR channel at the end of each extension step. Melt curves were analysed to check for the absence of mispriming, and amplification efficiency from a standard curve were also determined (Appendix C).

2.5.3 Reference genes

B-actin and EF1α were chosen as the reference genes for the experiment. The housekeeping gene primer sequences and annealing temperatures are shown in Table 2.2.

2.5.4 Data analysis and statistics

Data were analysed using SPSS and Microsoft Excel. The one-sample Kolmogorov-Smirnov test was performed to determine whether the data were normally distributed and logarithmic transformation of the data was carried out where necessary. One-way ANOVA with least significant difference post-hoc tests were used to compare fresh and chilled embryos at different stages as well as fresh and frozen-thawed blastomeres following different periods of culture. Where variances were not homogeneous, Welch tests with Games-Howell post hoc analysis were carried out instead.

Table 2.3 Name, primer sequences, accession numbers, TM and amplicon size of zebrafish Pax and reference genes.

Name	Primer 5'-3' sequence	Chromosome location	Accession no.	TM	Amplicon size
Pax2a	F: CCGCGTTATTAAGTTCCCCT R: TGGCGTATCCATCTTCAATCC	13	ENSDARG00000028148	57	173
Pax2b	F: TACCTGGATATCCACCTCAC R: GAGCCTCTGGATGCACTATA	12	ENSDARG00000032578	55	199
Pax 5	F: GTGGCAGTGACGCAGGTTTCT R: GCTGTTCTTCATCTCCTCCAA	1	ENSDARG00000037383	55	279
Pax 8	F: GCTCCGCCGTCACTCCTCCTG R: GCTGTCGCTGCTGTAGTGTCT	5	ENSDARG00000015879	63	254

CHAPTER 3: CRYOPRESERVATION OF ZEBRAFISH (*DANIO RERIO*) BLASTOMERES BY CONTROLLED SLOW COOLING

3.1 Introduction

Sperm has been successfully cryopreserved in many fish species (Cabrita, 1998; Ritar and Campet., 2000; Tsvetkova et al.,1996) and cryopreserved sperm has been used in both aquaculture and for conservation purposes. Androgenesis using cryopreserved sperm has been successful with several teleost species, but this technique does not overcome the loss of mitochondrial DNA, which is inherited maternally. Successful cryopreservation of fish oocytes and embryos has not yet been achieved. Whilst relatively little work has been done on cryopreservation of oocytes, embryos have been well studied and their large size, high yolk content, thick chorion, poorly permeable membranes and complex structure in development, have all been cited as factors limiting successful cryopreservation (Hagedorn et al., 2002; Zhang and Rawson, 1995, 1996). One approach for maintaining the genetic diversity of both the nuclear genome and mitochondrial DNA is cryopreservation of the blastomeres (Harvey, 1983; Nilsson and Cloud, 1992), which can also be transplanted into recipient blastulae after thawing. Different methods have been successfully used to produce fish chimeras by blastomere transplantation into blastula embryos (Satoshi et al., 2004; Hong et al., 1998; Yamaha et al., 1997). Although cultured somatic-cells from fish have also been cryopreserved successfully, their value is limited because of loss of developmental potential. Since blastomeres from the early embryos of fish still retain pluripotency (Ho and Kimmel, 1993), their cryopreservation may be a promising approach not only to preserve the genotypes of zygotes but also to enable reconstitution of the organism.

The process of cryopreservation involves many potentially damaging procedures relating to both the chemical (e.g. toxicity of cryoprotectants) and physical (e.g. intracellular ice formation) insult of the material treated (Jun et al., 2000). There is no

information on cryoprotectant toxicity to zebrafish blastomeres. As the first step of establishing a successful cryopreservation protocol for zebrafish blastomeres, toxicity of cryoprotectants to 50% epiboly stage zebrafish blastomeres was studied.

The cryopreservation strategy typically employed involves the use of a controlled slow cooling procedure, which is now a common procedure to cryopreserve many different cell and tissue types (Mazur, 1990). In general, this procedure consists of an initial slow cooling (room temperature to freezing point) and slow freezing period (approximately -5 to -50°C) followed by rapid freezing as the samples are plunged into liquid nitrogen for final storage. In the initial slow cooling step, most procedures for cryopreservation utilize an initial slow cooling rate between body temperature or the temperature at which the sample is collected (often ambient temperature) (Foote 1975; Sherman 1973). When cells are cooled rapidly from this temperature to -5°C or lower, without freezing, they undergo apparently irreversible loss of viability. This phenomenon has been termed "cold shock" (Watson 1995). Cold shock occurs in bacteria, protozoa, algae, fungi, higher plants, fish and mammalian somatic cells and spermatozoa (Morris and Watson, 1984). In the second freezing step, extracellular ice is induced at a temperature just below the solution freezing point and slow freezing is continued in the presence of this growing ice phase, which raises the extracellular solute concentration in the unfrozen fraction and results in water exiting the cell via exosmosis. As this freezing procedure continues, the slow freezing step is terminated at an intermediate temperature at which samples were plunged into LN_2. If the second freezing step is conducted in a way that allows formation of a critical concentration of intracellular solute, then the CPA will interact with the remaining water in the cell, which results in the formation of a glass-like structure by the intracellular solution and prevention of damaging intracellular ice formation (Jun et al., 2000).

Successful cryopreservation of blastomeres has been reported for several fish species. Harvey (1983) reported a survival rate of 84.8% after cryopreservation of 50% epiboly zebrafish blastomeres using a two-step freezing procedure, with ice-seeding at -6°C, and cooling to -25°C, followed by immersion in liquid nitrogen. However, freezing rates were not controlled, rather tubes were allowed to equilibrate in cooled alcohol baths with very small sample size. Vitrification of zebrafish blastomeres has been studied recently and showed a high survival rate (93.4%) with

the cryoprotectant DMSO (Cardona-Costa and García-Ximénez, 2007). Calvi and Maisse (1998) reported a rainbow trout blastomere controlled slow cooling procedure with a 95% survival. They also reported that the controlled slow cooling protocol adopted for rainbow trout was successfully applied to carp blastomere with survivals of 94% and 96% at the early blastula stage and the late blastula stage (Calvi and Maisse, 1999). Lower survival rates of cryopreserved blastomeres have been reported for other fish species (Kududa et al., 2004, 2002; Strussmann et al., 1999). Although controlled slow cooling of zebrafish blastomeres has been reported (Kopeika et al., 2005) the survival rate was low (25%). There is a need for a defined controlled cooling protocol that gaves a high and reproducible level of viability. The aim of the present study was to develop a successful cryopreservation protocol for zebrafish blastomeres using controlled slow cooling.

3.2 Experimental design

The first step in the freezing protocol design was a study of the impact of cryoprotectants on blastomeres. After CPA toxicity studies, investigations of optimal cooling and freezing rates were carried out using controlled slow cooling over three different temperature ranges 1) from 20°C to seeding temperature 2) from seeding temperature to -40°C. 3) from -40°C to -80°C. The effect of cryoprotective supplements and the comparison of viability staining methods, TB and FDA+PI were also examined.

3.3 Results

3.3.1 Toxicity of cryoprotectants to zebrafish blastomeres

NOECs for METH, PG, DMSO and EG for 50% epiboly stage blastomeres are given in Table 3.1, and the toxicity of the cryoprotectants to 50% epiboly blastomeres are given in Fig 3.1. The results showed that the toxicity of cryoprotectants generally increased with concentration. DMSO was the most toxic cryoprotectant to zebrafish

blastomeres, and there are no differences between toxicity of METH, PG and EG. The survivals assessed with TB staining for 50% epiboly blastomeres after exposure to NOEC concentrations of cryoprotectants 2 M METH, PG, EG and 1.5 M DMSO for 30 min at room temperature were 93.4 ± 2.7%, 95.9 ± 2.0%, 96.1 ± 2.1% and 91.7 ± 3.6% respectively.

Table 3.1. NOEC of cryoprotectants to 50% epiboly blastomeres at 22°C obtained from different viability tests. The highest concentration of cryoprotectants used in the experiments was 5 M for 30 min.

Cryoprotectants	50% epiboly blastomeres
METH	2 M
PG	2 M
DMSO	1.5 M
EG	2 M

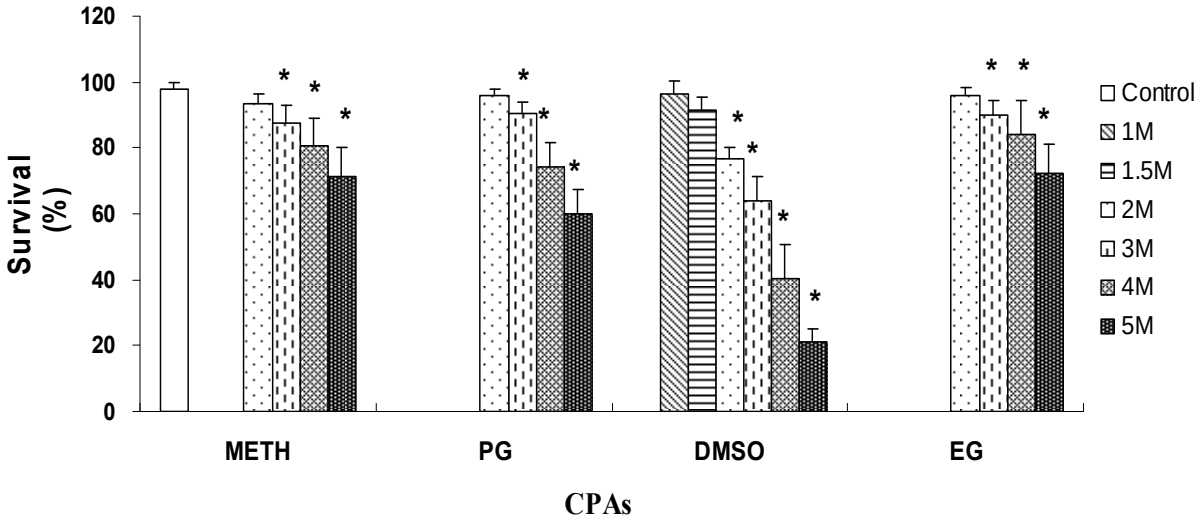
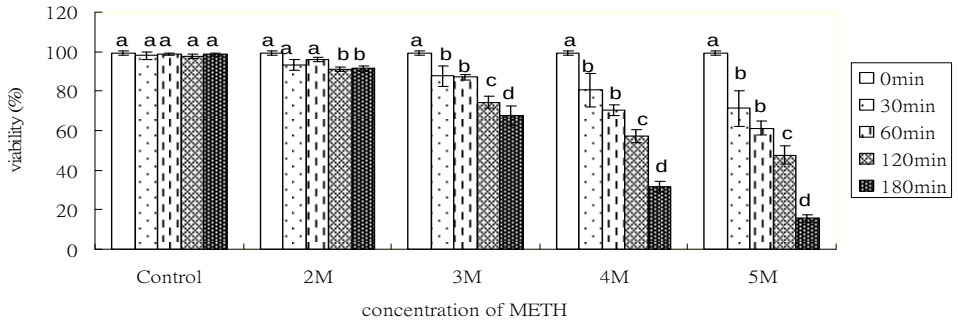


Figure 3.1 Toxic effect of cryoprotectants to 50% epiboly zebrafish blastomeres assessed with TB test. Blastomeres were exposed to cryoprotectants at concentration of 2 to 5 M for 30 min at room temperature. In addition, concentrations of 1 and 1.5 M DMSO were also used in the experiments. Error bars represent standard errors. Stars represent significant difference from control ($P<0.05$). (n=270 embryos per treatment).

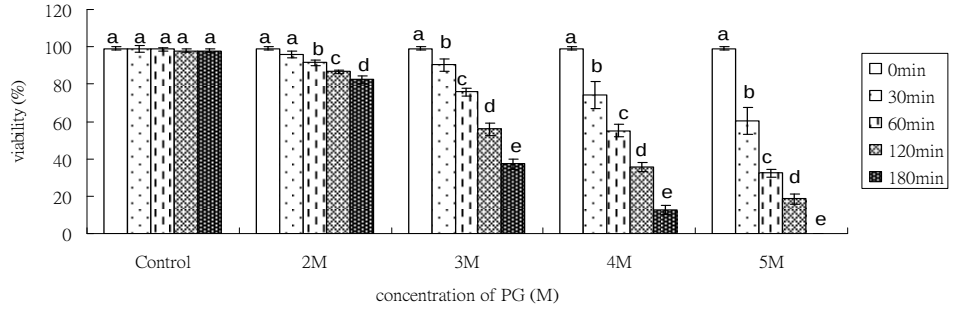
3.3.2 Effect of concentration and exposure time

The comparisons of blastomere survival rates using different concentrations of cryoprotectants are shown in Figure 3.2. Blastomere survival rates after treatment in 3, 4 and 5M of all cryoprotectants for 30 min were found to be significantly different to the control. However, no significant differences were found in blastomere survival rates when blastomeres were treated in 2M Meth, PG or EG. There is no survival after treatment with 4M and 5M DMSO for 180 min and 120 min. The toxic effect of these cryoprotectants on blastomeres increased with exposure time.

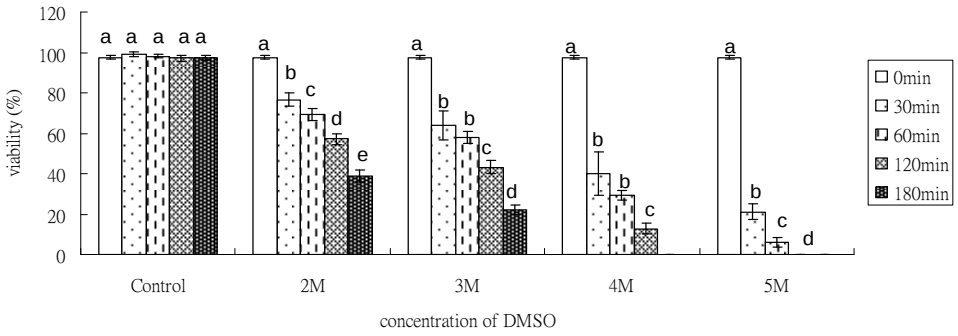
a)



b)



c)



d)

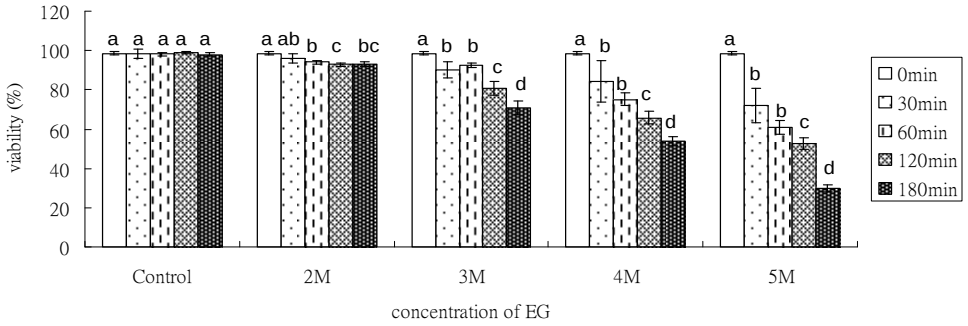


Figure 3.2 Comparisons of toxic effect of concentration and exposure time on 50% epiboly blastomeres assessed with TB test. Blastomeres were exposed to 2M, 3M, 4M and 5M METH, PG, DMSO and EG for different time periods. Error bars represent standard error. Different letters represent significant differences between times at the same concentration ($P<0.05$). (n=270 embryos per treatment)

3.3.3 Effect of freezing rate

As shown in Figure 3.3, the highest survival rate from 3 different freezing rates over the range of seeding temperature to -40°C was 50%, which was obtained with 0.3°C/min and 1.5 M DMSO. In the presence of DMSO, this freezing rate gave significantly higher survival values than both 1°C/min (37%) and 2°C/min (32%) freezing rates. No significant differences were observed between the freezing rates of 1 and 2 °C/min. The slower freezing rates appear to be more advantageous with DMSO than with the other three cryoprotectants. Cryopreservation with METH produced a survival rate of more than 20% but less than 40% at all the freezing rates examined. Cryopreservation with EG gave 35% survival with freezing rates of 2°C/min and gave significantly higher survival values (35.2 ± 8.6) than both 0.3°C/min (12.6 ± 2.6) and 1°C/min (21.3 ± 3.8). The faster freezing rates appear to be more advantageous compared to slower rate with this cryoprotectant. On the contrary, the maximum survival rates of PG were lower than those of the other three cryoprotectants mentioned above.

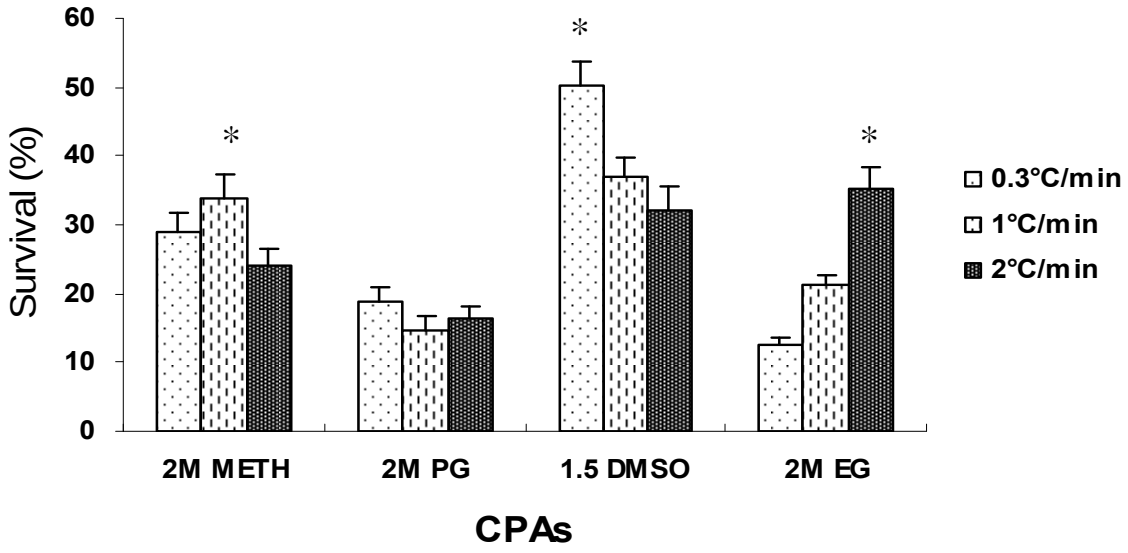


Figure 3.3 Effect of different freezing rates (0.3, 1 and 2°C/min) on survival of zebrafish blastomeres in 2 M METH, EG and PG and 1.5 M DMSO using the following protocol: 1°C/min to -7.5°C (except DMSO which was -6°C seeding temperature) hold for 15min followed by freezing rate 0.3, 1 or 2°C/min to -40°C; 2°C/min to -80°C, hold for 10 min. Samples were then directly plunged into liquid nitrogen. Error bars represent standard error. Asterisks represent freezing rates with significantly higher survival rates than other freezing rates for the same CPA ($P<0.05$). (n=450 embryos per treatment)

Cooling rate can be an important contributor to the survival of frozen cells. However, there is no information on blastomeres regarding the effect of cooling rates from room temperature to seeding point. The effects of three different cooling rates (1, 2 and 5°C/min) from 20°C to seeding temperature were studied using 1.5 M DMSO (Figure 3.4). The results showed that there were no significant differences in blastomere survival rates between the three cooling rates. The survival rates of the blastomeres after freeze-thawing were 54.5 ± 2.4%, 54.0 ± 2.0% and 56.7 ± 2.5% for 1, 2 and 5°C/min respectively. Although, the statistics did not reveal any significant differences in mean survival rates due to the different cooling rates (1,2 and 5°C/min), a rate of 5°C/min was chosen for the subsequent experiments.

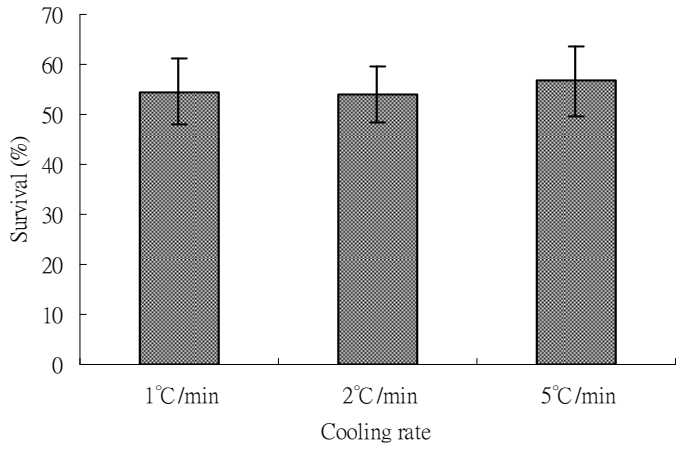


Figure 3.4 Effect of different cooling rates (1, 2 and 5°C/min) with 1.5M DMSO on survival of zebrafish blastomeres assessed by TB. The freezing and thawing procedures were performed from 20°C, at 1, 2 and 5°C/min to -6°C, seeding at -6°C, held for 15min, 0.3°C/min to -40°C, 2°C/min to -80°C, held for 10 min, then directly plunged into liquid nitrogen. Error bars represent standard error. (n=450 embryos per treatment)

The results of the present study identified 0.3°C/min from seeding temperature to -40°C and 5°C /min from 20°C to seeding temperature as the best freezing rates for zebrafish blastomeres (Figure 3.3 and 3.4). A further study on the effect of three different freezing rates (2, 5, and 10°C/min) from -40 to -80°C on blastomere survival was also carried out and the results showed that the highest survival rates were obtained when 2°C/min was used (Figure 3.5). There was no significant difference in blastomere survival rates between 5 and 10°C/min. The survival of blastomere using the three freezing rates of 2, 5 and 10°C/min were 56.7 ± 2.5%, 51.5 ± 4.8% and 47.2 ± 5.5%. The slower freezing rates appear to be more effective for zebrafish blastomeres.

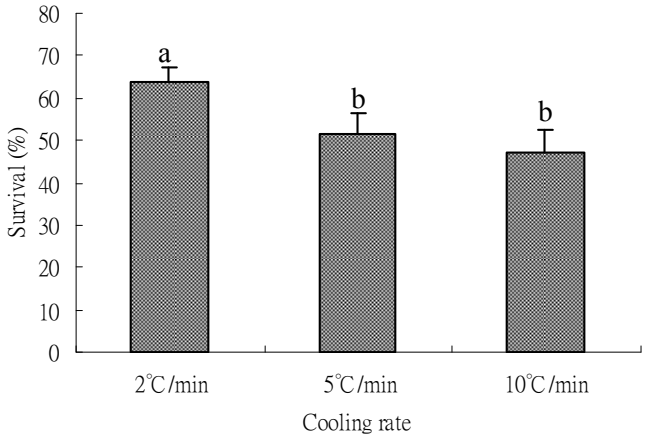


Figure 3.5 Effect of different freezing rates (2, 5 and 10°C/min) from -40°C to -80°C with 1.5M DMSO on survival of zebrafish blastomeres assessed by TB. The cooling, freezing and thawing procedures were performed from 20°C, 5°C/min to -6°C, seeding at -6°C, hold for 15min, 0.3°C/min to -40°C, 2, 5 and 10°C/min to -80°C, hold for 10 min, then directly plunged into liquid nitrogen. Error bars represent standard error. Different letters represent significant difference between groups ($P<0.05$). (n=450 embryos per treatment)

3.3.4 Effect of liquid nitrogen plunging temperature

Figure 3.6 shows the effect of different liquid nitrogen plunge temperatures on cryo-survival of blastomeres. With the exception of PG and EG, -80°C plunge temperature resulted in significantly higher survival rate than -40°C plunge. The highest survival rate (56.6 ± 2.9%) was obtained using 1.5 M DMSO.

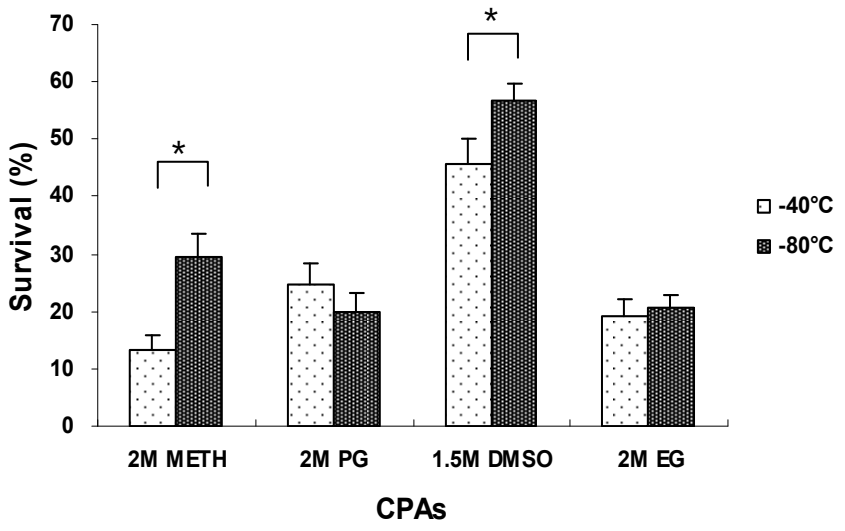


Figure 3.6 The effect of different liquid nitrogen plunge temperatures on TB-assessed cryo-survival of 50% epiboly stage zebrafish blastomeres using four cryoprotectants. Cooling from 20°C to -7.5°C (DMSO -6°C) at 5°C/min; seeding and hold for 15 min. Samples were then frozen at 0.3°C/min to -40°C and plunged into LN_2 after holding for 10 min. Other samples were further cooled from -40°C to -80°C at 2°C/min and then plunged into LN_2 after holding for 10 min. Error bars represent standard error. Asterisks represent significant differences between groups ($P<0.05$). (n=450 embryos per treatment)

In the light of the results from these studies, the following optimal procedure was adopted for further study: cooling at 5°C/min from 22 to -6°C, held for 15 min; 0.3°C/min from -6 to -40°C; 2°C/min from -40 to -80°C, held for 10 min; and then directly plunged into liquid nitrogen.

3.3.5 Effect of cryoprotective supplements

The effect of combining the penetrating cryoprotectant DMSO (1.5 M) with sugar supplements was investigated and the results are shown in Figure 3.7. The highest post-thaw survival (70.2 ± 3.2%) was achieved using 0.1 M sucrose supplement, which differed significantly ($P<0.05$) from the control (1.5 M DMSO). In the case of trehalose, maximum survival rates (60.6 ± 3.1%) were also obtained at a concentration of 0.1 M. A concentration of 0.2 M resulted in significantly lower survival for all the three sugars compared to controls ($P<0.05$).

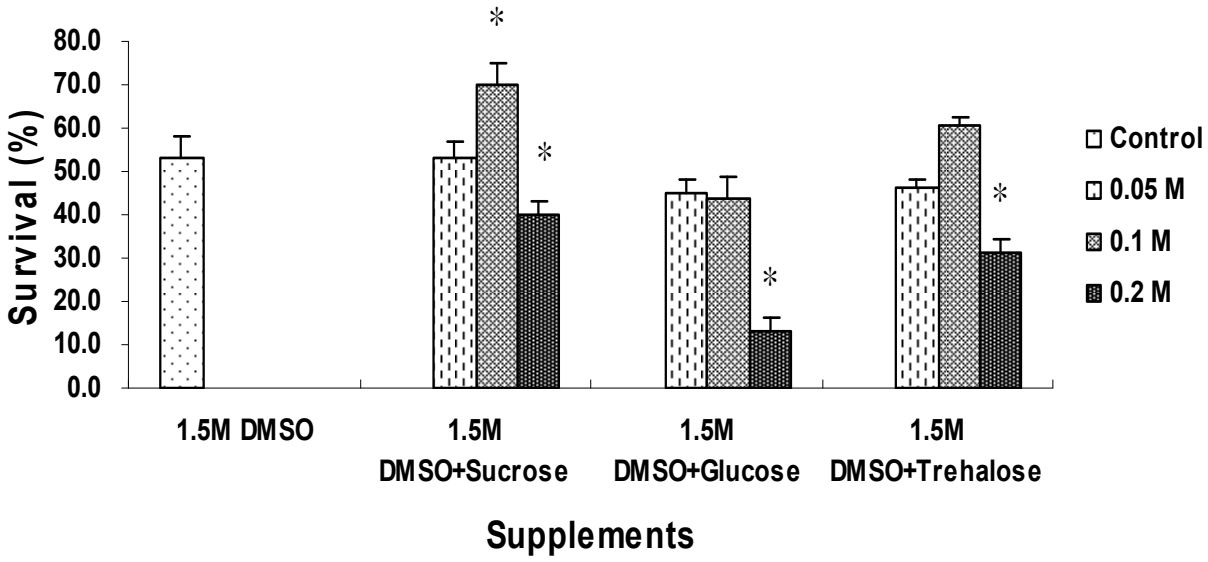
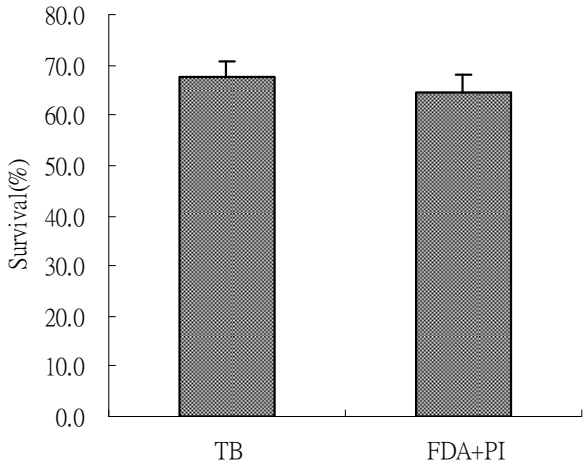


Figure 3.7 Post-thawed survival of blastomeres frozen to -196°C in 1.5 M DMSO PBS+10% FBS solutions with supplements. The freezing procedure was cooling from 20°C to -6°C at 5°C/min hold for 15min; 0.3°C/min to -40°C; 2°C/min to -80°C, hold for 10 min. Samples were then directly plunged into liquid nitrogen. Error bars represent standard error. Asterisks represent significant difference from control ($P<0.05$). (n=450 embryos per treatment)

3.3.6 Effect of different viability assessment methods

The comparisons of the two viability assessment methods are shown in Figure 3.8. No significant difference was found between survival rate assessed by TB and FDA+PI. Blastomere viability after cryopreservation at room temperature with TB and FDA+PI was 67.5±9.5 and 64.6±10.3 respectively. In general terms, these two viability assessment methods produced similar results and both were considered to be suitable for 50% epiboly zebrafish blastomeres viability assessment.



Fugure 3.8 TB-assessed survival of blastomeres frozen to -196°C in 1.5M DMSO PBS+10% FBS solutions with supplements after cryopreservation and thawing. The freezing and thawing procedures were performed from 20°C, 5°C/min to -6°C hold for 15min, 0.3°C/min to -40°C, 2°C/min to -80°C, hold for 10 min. Samples were then directly plunged into liquid nitrogen. (n=450 embryos per treatment).

3.4 Discussion

3.4.1 Toxicity of cryoprotectants to zebrafish blastomeres

In the present study, DMSO treated blastomeres showed higher survival percentages after freezing than those treated with METH, PG or EG. Interestingly, DMSO was the most toxic of the cryoprotectants to zebrafish blastomeres when exposed at room temperature. These results agree with several previous findings where DMSO were used successfully as a cryoprotectant for the cryopreservation of many species of fish sperm (Tsvetkova et al., 1996; Ritar, et al., 2000; Cabrita et al., 1998) and fish blastomeres (Kusuda et al., 2002, 2004; Strussmann et al., 1999; Harvey, 1983; Cardona-Costa and García-Ximénez, 2007). DMSO has been reported to be a membrane modifier and plastifier (Orvar et al., 2000; Yamamoto, 1989) and fluidises plasma membranes during freezing, so that the membrane becomes less brittle and less likely to be disrupted (Zeron et al., 2002). Cells with more fluid plasmalemmae have been shown to survive freeze-thawing better than cells with rigid membranes.

3.4.2 Effect of freezing rate and liquid nitrogen plunging temperature

During the process of cooling and freezing, cells are subjected to a series of drastic changes in their physical and chemical environment (Watson, 2000). The first change that the cell has to cope with is cooling from body temperature to near the freezing point of water (Watson, 2000; Medeiros et al., 2002), which causes phase transitions of the membranes lipids (commonly refered as cold shock) (Woelders, 1997). In the past, the effects of cold shock on survival, metabolism and the mode of action of protective substances have been recognised. Previous studies showed that cold shock greatly reduced the respiratory activity of bull semen (Hancock, 1952; Blackshaw and Salisbury, 1957). Cold shock is related to phase transitions in the sperm plasma membrane which correlate with changes in the membrane permeability to ions such as K+ and Ca^{2+} (Hammerstedt et al., 1990; Parks and Lynch, 1992; Drobnis et al., 1993; White, 1993). The results of the present study demonstrated that

there were no significant differences in blastomeres among three different cooling rates (1, 2 and 5°C/min). This demonstrated that the three different cooling rates of cold shock did not influence zebrafish blastomere damage in terms of survival.

A second change in the cells environment takes place when liquid water is converted into ice (ice crystal formation). Of considerable importance for the freezing regime is the freezing rate in the critical temperature range (approximately -5 to -50°C) that determines whether the cell will remain in equilibrium with their extracellular environment or become progressively supercooled with the increasing possibility of intracellular ice formation (Kumar et al., 2003). Therefore, the ability to survive cooling in the critical temperature range from ice seeding to liquid nitrogen plunge, depends upon the optimum-freezing rate (Andrabi, 2007). This needs to be slow enough to allow a sufficient degree of cell dehydration during freezing which, in combination with cryoprotectant, prevents intracellular ice formation and allows the dehydration of the blastomere to the point of osmotic equilibrium between the intracellular and extra-cellular space (Muldrew and McGann, 1990). It has been suggested that the successful cryopreservation of fish blastomeres would require a freezing rate of 1 ^{o}C/min or slower (Kusuda et al., 2004; Strussmann et al., 1999; Harvey, 1983). The present study found that a freezing rate of 0.3^{o}C/min from a seeding temperature of -7.5 ^{o}C to -40 ^{o}C was optimal for 50% epiboly zebrafish blastomeres. Several workers have shown that slow freezing rates result in better survival of fish blastomeres after freezing (Calvi and Maisse, 1999 and 1998; Harvey, 1983).

In most cases cells, including fish gametes, are cooled to -80°C and not to -40°C before liquid nitrogen plunge, and the effect of different freezing rates from -40°C to -80 ^{o}C on survival is important (Trounson and Mohr, 1983). Human embryos are generally frozen at a rate of 0.3 ^{o}C/min to -30 ^{o}C or -40 ^{o}C, and then at an increased freezing rate of 10-15 ^{o}C /min to -80 ^{o}C before plunging into LN_2 (Zeilmaker et al., 1984; Kattera et al., 1999; Hartshorne et al., 1991; Testart et al., 1986; Cohen et al., 1988; Gardner et al., 2003; Kaufmann et al., 1995). Surprisingly, the result of the present study demonstrated that a relatively slow freezing rate of 2 ^{o}C /min gave significantly higher survival than a freezing rate of 10 ^{o}C /min. This may suggest that on reaching -40°C blastomeres still have not reached the eutectic point, and further

removal of residual water was dependant on the rate of cooling. The fast freezing rates increased the risk of intra-cellular ice formation. The results are also consistent with the results obtained for the different LN_2 plunge temperatures, with the plunge temperature of -80°C giving significantly higher mean survival rates than a plunge temperature of -40°C.

3.4.3 Effect of cryoprotective supplements

Various cryoprotective supplements are capable of assisting the preservation of cell function during freezing and thawing and the success of sugars as extenders in sperm cryopreservation can be explained by their role as membrane stabilizers (Watson, 2000). Sugars also provide an osmotic buffer that prevents excessive cell volume excursion during dilution of cryoprotectant (Mazur, 1990). The results of the present study showed that the addition of a low concentration (0.1 M) of sucrose to the freezing medium provides the best protection with regard to post-thaw survival rates, and that sucrose was a more effective supplement for zebrafish blastomeres than trehalose or glucose. This result was in agreement with reports which proposed that the sucrose supplementation were more effective than glucose in reducing cryoprotectant toxicity to the embryos and spermatozoa by reducing the extracellular concentration of the cryoprotectant (Fuku et al, 1995; Kasai et al, 1990., Molinia et al., 1994). However, not all studies have given the same results. For example, use of trehalose was shown better than sucrose for improving the viability of ultrarapidly frozen mouse morulae (Kim et al., 1986).

3.4.4 Effect of different viability assessment methods

Reliable fish cell quality assessment methods are essential in developing cryopreservation protocols (Jones and Senft, 1985). Two different viability assessment methods were used in the present study, TB and FDA+PI staining. FDA and PI which can be used singly or in combination have primarily been used to determine viability of mammalian cells (Jones and Senft, 1985; Rotman and Papermaster, 1966; Widholm, 1972). TB is a vital dye, its reactivity is based on the fact that the chromophore is

negatively charged and does not interact with the cell unless the membrane is damaged (Plachinta et al., 2004; Isayeva et al., 2004). The TB test assesses only the membrane integrity; cells with an intact membrane are able to exclude the dye while cells without an intact membrane take up the colouring agent (Tsai et al., 2008). FDA molecules enter the cell, are hydrolyzed by cell esterases, and fluorescein is produced and if the membrane is intact, is retained within the cell. PI, an intercalating dye, is known to pass only through the membranes of dead or dying cells and intercalates with DNA and RNA to form bright red fluorescence complex seen in the nuclei of dead cells. Jones and Senft (1985) considered the combination of these two dyes to be a reliable method for assessing cell viability. The study also found TB and FDA+PI to be suitable for assessment of blastomeres and FDA+PI was used here for the first time to investigate zebrafish blastomeres. Examination of the effect of different assessment methods on the survival rates of frozen blastomeres yielded some interesting results. The results obtained in this study do not fit the pattern of the results for fish oocytes which show FDA+PI has lower survival rate than these obtained with TB in this laboratory (Tsai et al., 2008; Zampolla et al., 2006). It is not clear why the results for zebrafish blastomere stained differ from the zebrafish oocyte.

3.5 Summary

The optimal protocol for cryopreservation of zebrafish blastomeres from 50% epiboly embryos was found to be the use of the cryoprotectant mixture of 1.5 M DMSO and 0.1M sucrose, and a cooling protocol of 5°C/min from 22°C to a seeding temperature of -6°C, held for 15 min; then cooled at 0.3^{o}C/min to -40°C; followed by cooling at 2°C/min to -80°C, hold for 10min, and then direct plunge into liquid nitrogen. This protocol gave a survival of 70.1 ± 3.2 %. Although Harvey (1983) reported a survival rate of 84.8% after cryopreservation of 50% epiboly zebrafish blastomeres using a two-step freezing procedure, the results were obtained from a very small sample size of 3 embryos and 270 cells. In the present study 450 embryos were used for each treatment condition. Vitrification of zebrafish blastomeres has been studied recently and a high survival rate (93.4%) for blastula stage embryos was obtained (Cardona-Costa and García-Ximénez, 2007). This stage is different from our

study using gastrula stage. Although the highest survival rate obtained from the present study (70.1 ± 3.2 %) is lower than that obtained by Calvi and Maisse (1998, 1999) for rainbow trout and carp blastomeres using controlled freezing, it is higher than that achieved with gold fish, whiting, pejerrey, medaka and chum salmon blastomeres (Kududa et al., 2002, 2004; Strussmann et al., 1999). However any comparisons between these studies must take into consideration the differences between the species examined and the methodology. Optimum freezing protocols identified here were used in studies on the effect of cryopreservation at the molecular level including gene expression in the next chapter.

This work has been accepted for publication by Cryoletters.

CHAPTER 4: IDENTIFICATION OF SUITABLE HOUSEKEEPING GENES FOR USE IN GENE EXPRESSION STUDIES OF ZEBRAFISH EMBRYOS AND BLASTOMERES FOLLOWING CRYOPRESERVATION

4.1 Introduction

The study of gene expression is becoming increasingly important for the determination of regulatory and disease pathways within cells and for validation of the effects of various insults or treatments. Conventional PCR and microarrays have been widely used for qualitative and semi-quantitative methods of analysing gene expression. Real time PCR is a more sensitive and accurate quantitative technique, it has great potential to detect smaller differences in gene expression between samples. However, variation between samples can influence the data obtained by real time PCR. This is very important when studying embryonic development, as embryos are known to develop at different rates (Lonergan et al., 2000) and also because transcriptional activity can vary between different developmental stages (Mathavan et al., 2005). An internal reference factor is therefore required to ensure that the data collected has an acceptable level of accuracy (Sturzenbaum and Kille., 2001; Bustin 2002).

It is generally accepted that there are a number of genes that contribute to basic cellular functions and are therefore expressed at relatively similar levels across many types of cells. These genes are known as housekeeping genes and are commonly used for normalisation of real time PCR data. The use of some of these housekeeping genes quickly became widespread, with limited validation of their expression stability over the treatment to be analysed. Later studies showed that many common housekeeping genes were not actually stably-expressed and that their stability varies significantly among different groups of samples and embryo stages (Lonergan et al., 2000; Filby and Tyler, 2007). Suitable reference genes for each type of sample group should be validated as part of experimental design processes. This process has been simplified by the development of software such as ‘GeNorm’ (Vandesompele et al., 2002) and

'NormFinder' (Andersen et al., 2004) that analyse the stability and the most suitable number of genes from a selection of potential housekeeping genes across development stages in embryos and periods of culture in blastomeres.

There are a number of studies on the suitability of various housekeeping genes in different tissues. For example, eukaryotic translation elongation factor 2 was found to have high expression stability across 15 different tissues in mice (Kouadjo et al., 2007) and a study of 17 pig tissues indicated that ACTB, RPL4 and HPRT1 were all suitable housekeeping genes in this species (Nygard et al., 2007). Some work has also been carried out to investigate suitable housekeeping genes for studies involving early embryos in cow (Goossens et al., 2005), pig (Kuijk et al., 2007) and mouse (Mamo et al., 2007) and for studies involving human and mouse embryonic stem cells (Willems et al., 2006). In the zebrafish, suitable housekeeping genes have been identified in both developmental time-course studies and different tissues (Tang et al., 2007). However, despite some studies in other species (Spinsanti et al., 2006; Filby and Tyler, 2007; Mamo et al., 2007), there are currently no data available on the effect of cryopreservation of embryos and embryonic cells on expression of housekeeping genes in zebrafish. This information is essential for the selection of suitable housekeeping genes for the accurate study of the effect of cryopreservation on the gene expression in these cell types.

The aim of the present study is to validate reference genes for use in cryopreservation studies of zebrafish embryos. However, cryopreservation of zebrafish embryos has not been successful so intact embryos were cooled to 1 ºC, whilst isolated blastomeres were subjected to cryopreservation at -196 ºC in LN_2.

4.2 Experimental design

Seven potential housekeeping genes were analysed across 1) fresh embryos during development; 2) fresh and chilled embryos during development; 3) fresh blastomere samples following different periods of culture and 4) fresh and freeze-thawed blastomere samples following different periods of culture. Real-time PCR was

performed to analyse cycle threshold (CT) values (cycle number at which threshold fluorescence level was reached), PCR efficiency and relative expression levels. The data were analysed using descriptive statistics and mean comparisons. The stability of expression of each of the genes was also investigated using both GeNorm and NormFinder. As GeNorm also suggests the number of HKGs necessary for an accurate normalization, GeNorm program is able to determine the minimum number of HKGs for fresh and chilled intact embryos and for fresh and frozen-thawed cultured blastomeres at multiple or individual stages of development.

4.3 Results

4.3.1 Analysis of CT values and PCR efficiency

As similar quantities of RNA (equivalent to 3.75 embryos for embryo samples and 200ηg for blastomere samples) were included in each reverse transcription reaction, the CT values obtained from the real time PCR reactions can provide rough estimation of the abundance of transcripts for each gene. EF1α was the most abundantly expressed potential housekeeping gene, as indicated by the low CT values (16.32±0.28 – 22.00±0.41), followed by β-actin and Rpl13a (see Table 4.1). GAPDH was the least abundantly expressed gene (CT values ranging from 26.42±1.00 to 31.71±2.43), followed by RNAP and B2M (see Table 4.1). The expression level of SDHA fell between the two groups, but was relatively more highly expressed in intact embryos (CT= 20.58±0.20 – 22.30±0.22) than in isolated blastomeres (CT= 27.07±1.03 – 29.43±0.46; see Table 4.1). In general, embryo samples contained more transcripts for most genes than the equivalent blastomere samples, as indicated by their lower CT values (see Table 4.1). However, the opposite trend was noted with GAPDH and B2M. Interestingly, all genes produced lower CT values for chilled embryos than for fresh embryos. However, this was not always the case for frozen blastomere samples compared to their fresh equivalents (see Table 4.1). For standard curve evaluation of housekeeping genes in zebrafish embryos and blastomeres, the amplification efficiency and a correlation coefficient from a standard curve of cDNA were determined for each gene utilizing real time PCR (Figure 4.1, 4.2 and 4.3). All of the target genes had acceptable efficiencies and correlation coefficients from 0.7 to 1.1 (Pfaffle, 2003).

Table 4.1 CT values for each group of samples

Gene	Fresh embryos CT (Mean ± S.E.M.)	Chilled embryos CT (Mean ± S.E.M.)	Fresh blastomeres CT (Mean ± S.E.M.)	Frozen blastomeres CT (Mean ± S.E.M.)
β-actin	21.52±0.32	19.27±0.40	21.06±0.72	24.49±0.28
EF1α	18.37±0.21	16.32±0.28	19.49±0.60	22.00±0.41
GAPDH	31.71±2.43	29.68±0.30	26.42±1.00	28.42±0.46
Rpl13a	21.79±0.42	20.32±0.45	21.46±0.92	26.16±0.33
RNAP	27.40±0.33	25.05±0.24	28.16±0.77	32.14±0.38
SDHA	22.30±0.22	20.58±0.20	27.07±1.03	29.43±0.46
B2M	31.17±0.40	28.95±0.40	24.84±0.42	26.62±0.27

CT: Cycle number at which threshold fluorescence level was reached

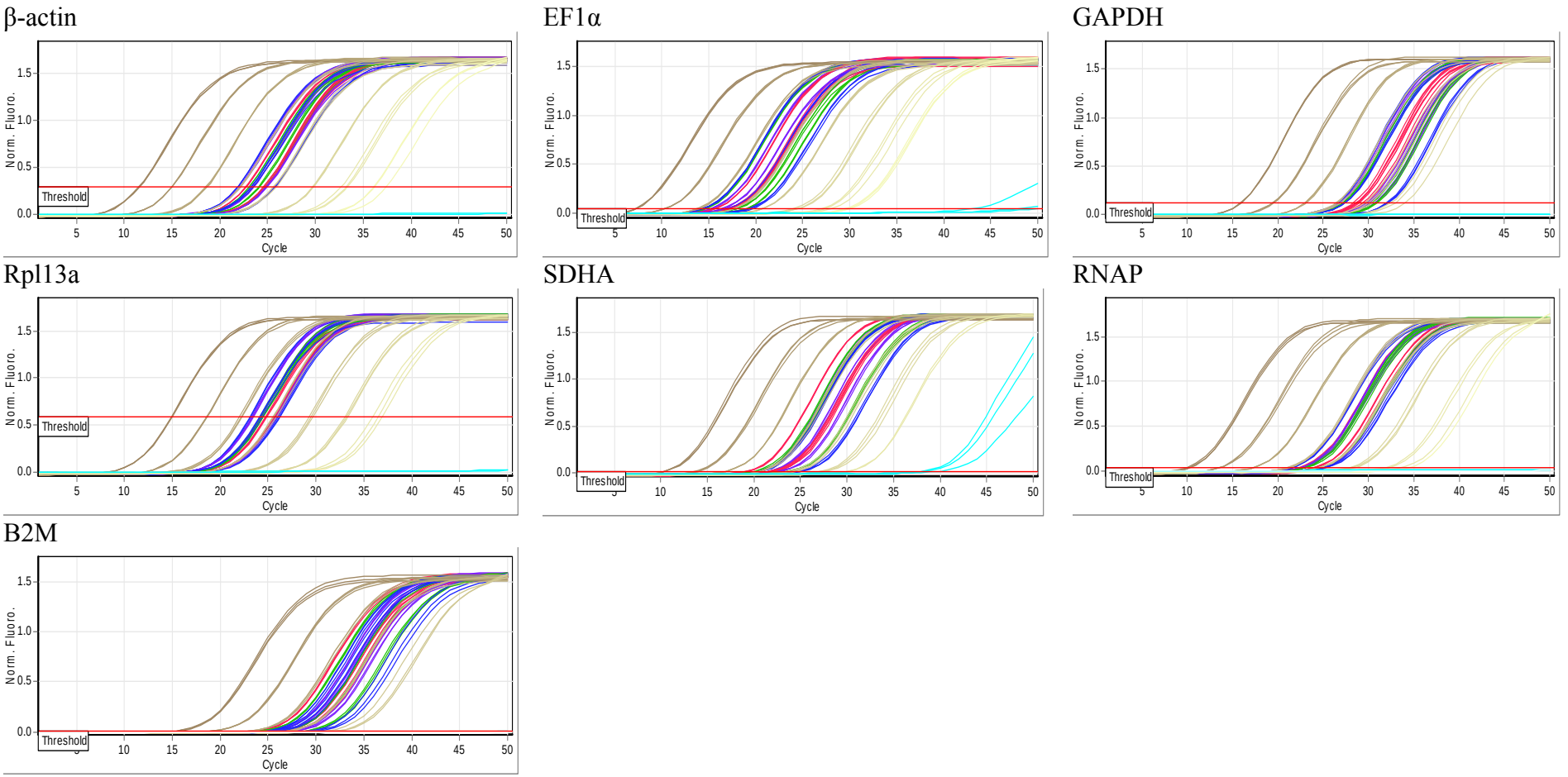
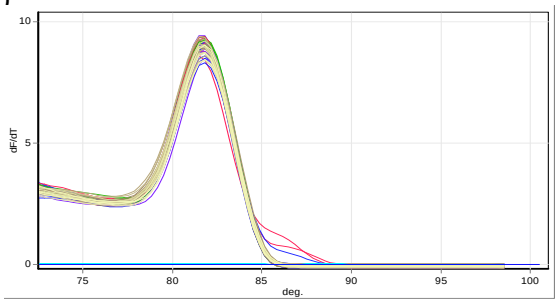
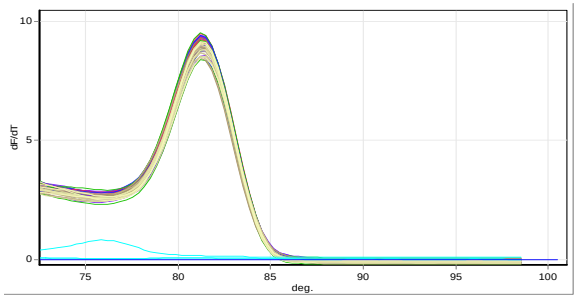


Figure 4.1 Example of fluorescence measurements for seven housekeeping genes. Fluorescence graphs show fluorescence measurements obtained for standards (brown) of 10-fold dilutions, negative control (blue) and other colors relate to embryo and blastomere samples.

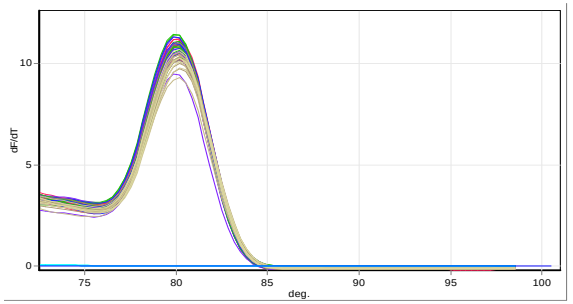
β-actin



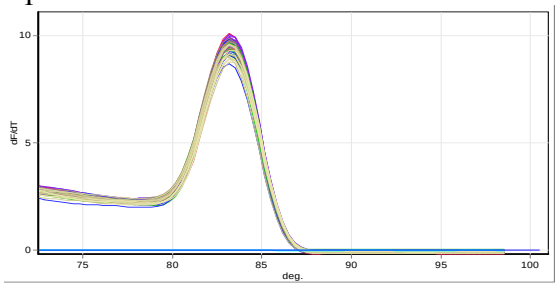
EF1α



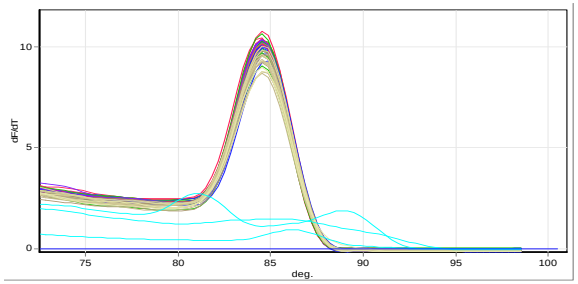
GAPDH



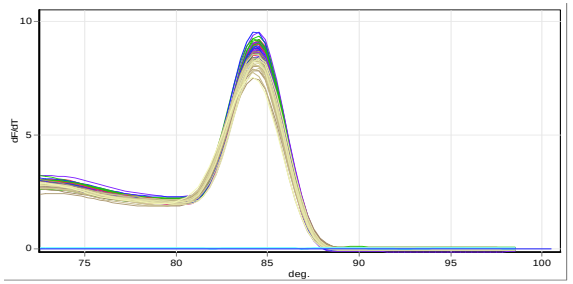
Rpl13a



SDHA



RNAP



B2M

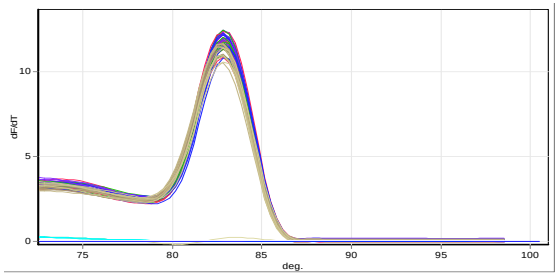
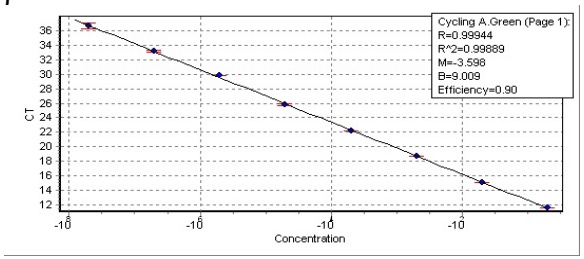
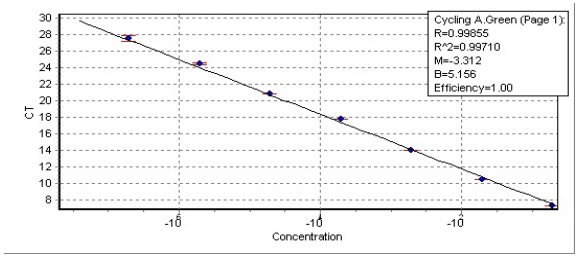


Figure 4.2 Example of melting curve profile of seven housekeeping genes. Standard (brown), negative control (blue) and samples (other colors) melt curves from different stages and time periods. All samples produced the same peak. The samples are therefore not contaminated, have no misprming and no primer-dimers.

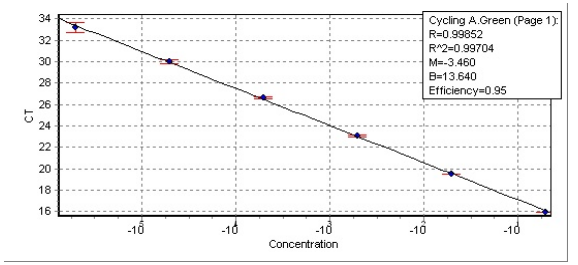
β-actin



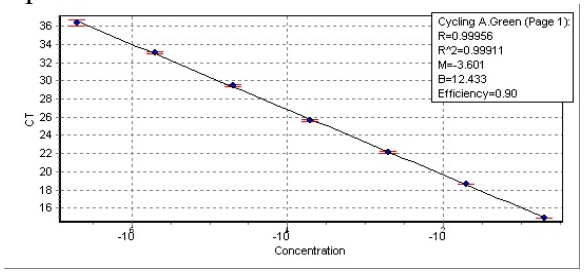
EF1α



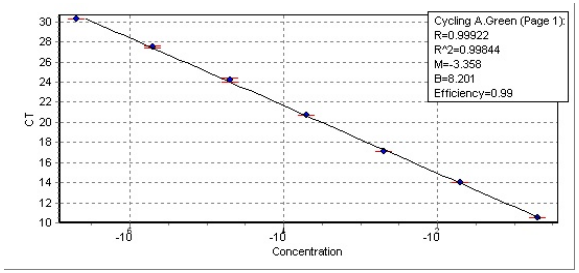
GAPDH



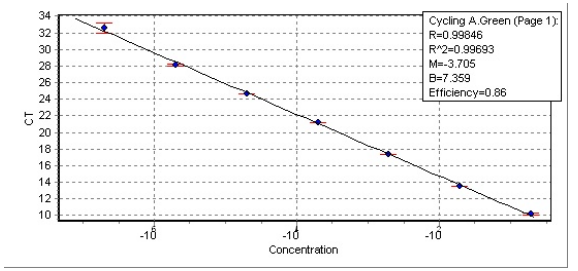
Rpl13a



SDHA



RNAP



B2M

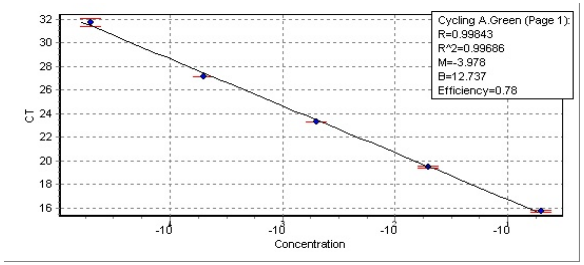


Figure 4.3 Example of standard curves for seven housekeeping genes. Mean ± SEM CT values are plotted against the log concentration of the standards of 10-fold dilutions. Each curve has an R^2 value > 0.99, due to an equal number of cycles separating standards of 10-fold concentration difference. Each curve has an efficiency value in with the acceptable range of between 0.7 and 1.1 (Pfaffle, 2003).

4.3.2 Expression stability in intact embryos during development

The expression level of seven potential housekeeping genes was compared over six stages of development of fresh embryos, covering the period between the late-blastula stage and early segmentation (see Figure 4.4). There were no significant differences in expression across the developmental stages for the majority of the genes, although there was a slight increase in GAPDH expression (4.1-fold, $P>0.05$, Figure 4.4) and a slight decrease in expression of SDHA (10-fold, $P= 0.008$) as development progressed (see Figure 4.4). There was also a slight peak in expression at the 75% epiboly stage for β-actin, EF1α and Rpl13a (2.7-fold, 3.2-fold, and 3.3-fold, respectively, compared to the oblong stage; see Figure 4.4), although this increase was only statistically significant for EF1α.

4.3.3 Expression stability in intact embryos following chilling treatment

In general, the gene expression in chilled embryos followed a similar pattern throughout development to that observed in the fresh controls. However, expression levels were consistently higher in chilled embryos compared to fresh embryos at the same stage (see Figure 4.4). Very few of these differences were statistically significant, however, significant increases were present at the oblong stage for β-actin ($P=0.009$), GAPDH ($P=0.03$), and SDHA ($P=0.02$). A peak in expression was observed at the 100% epiboly stage for GAPDH ($P=0.009$ compared to the oblong stage).

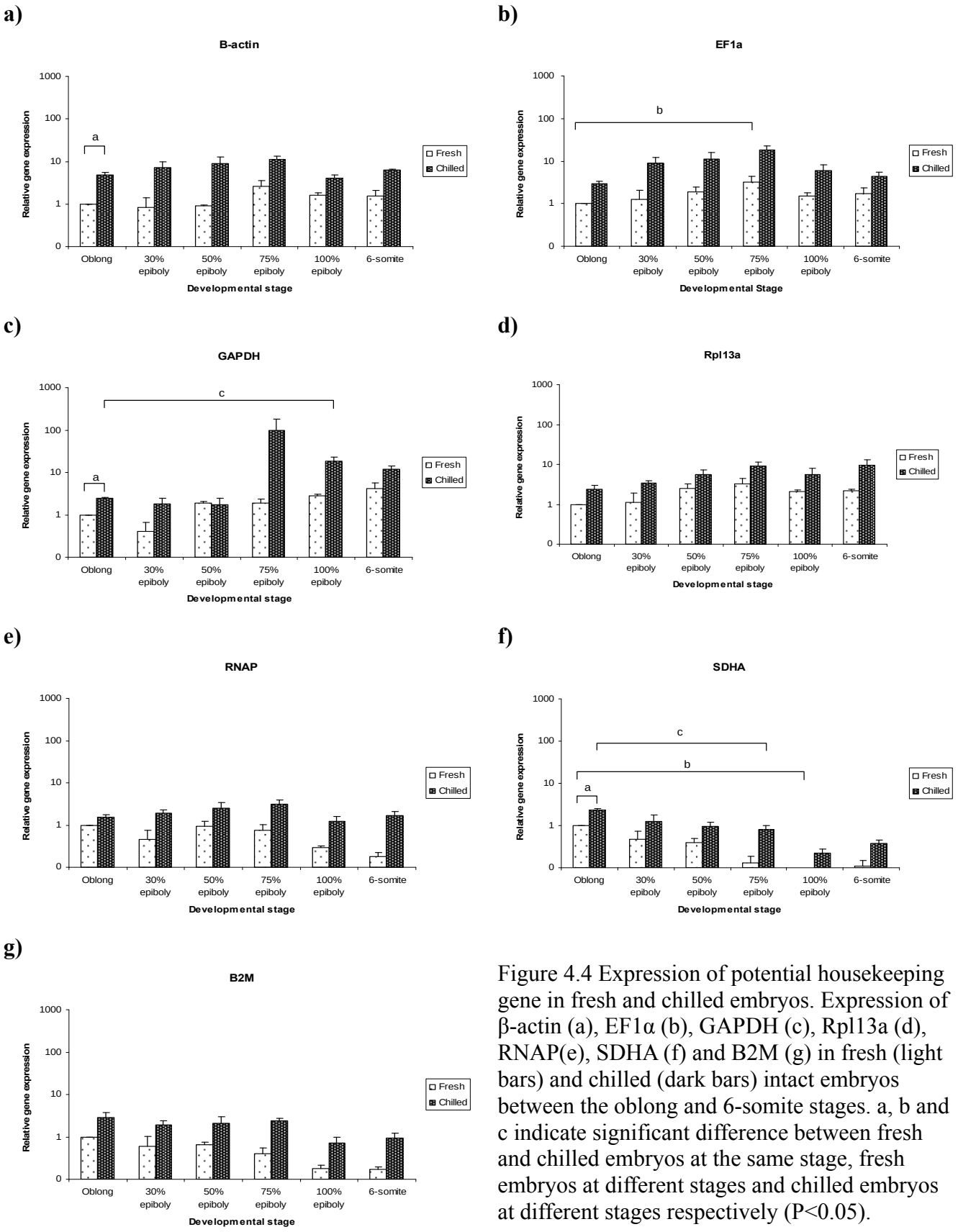


Figure 4.4 Expression of potential housekeeping gene in fresh and chilled embryos. Expression of β-actin (a), EF1α (b), GAPDH (c), Rpl13a (d), RNAP(e), SDHA (f) and B2M (g) in fresh (light bars) and chilled (dark bars) intact embryos between the oblong and 6-somite stages. a, b and c indicate significant difference between fresh and chilled embryos at the same stage, fresh embryos at different stages and chilled embryos at different stages respectively ($P<0.05$).

4.3.4 Expression stability in isolated blastomeres during development

The expression level of seven potential housekeeping genes was compared at five time points over 15 hours of culture of fresh blastomeres isolated from embryos at the 50% epiboly stage. The first four time points were equivalent (in hours) to the latest 4 stages of intact embryos (50% epiboly, 75% epiboly, 100% epiboly and 6 somite stage), although it was not determined whether embryo development was comparable to blastomere development. There were no significant differences in expression of any of the genes analysed over the 15 hours of culture. However, with the exception of B2M (see Figure 4.5), all genes demonstrated a similar tendency to increase expression during the culture period by at least 10-fold (see Figure 4.5).

4.3.5 Expression stability in isolated blastomeres following cryopreservation

As for the fresh blastomeres, there were no significant differences in expression of any of the genes across the culture period following cryopreservation. However, in contrast to the fresh blastomeres, cryopreserved blastomeres demonstrated a tendency to decrease expression of all genes except B2M (see Figure 4.5) between 7 hours and 15 hours of culture by approximately 10-fold, whilst expression remained relatively constant during the initial stages of culture (see Figure 4.5). As for the chilled embryos, cryopreserved blastomeres up to 7 hours in culture were consistently expressing the potential housekeeping genes at a higher level than the equivalent fresh blastomeres, although these increases were not statistically significant. This situation was reversed following 15 hours in culture due to the opposing trends in the fresh and cryopreserved blastomeres (see Figure 4.5).

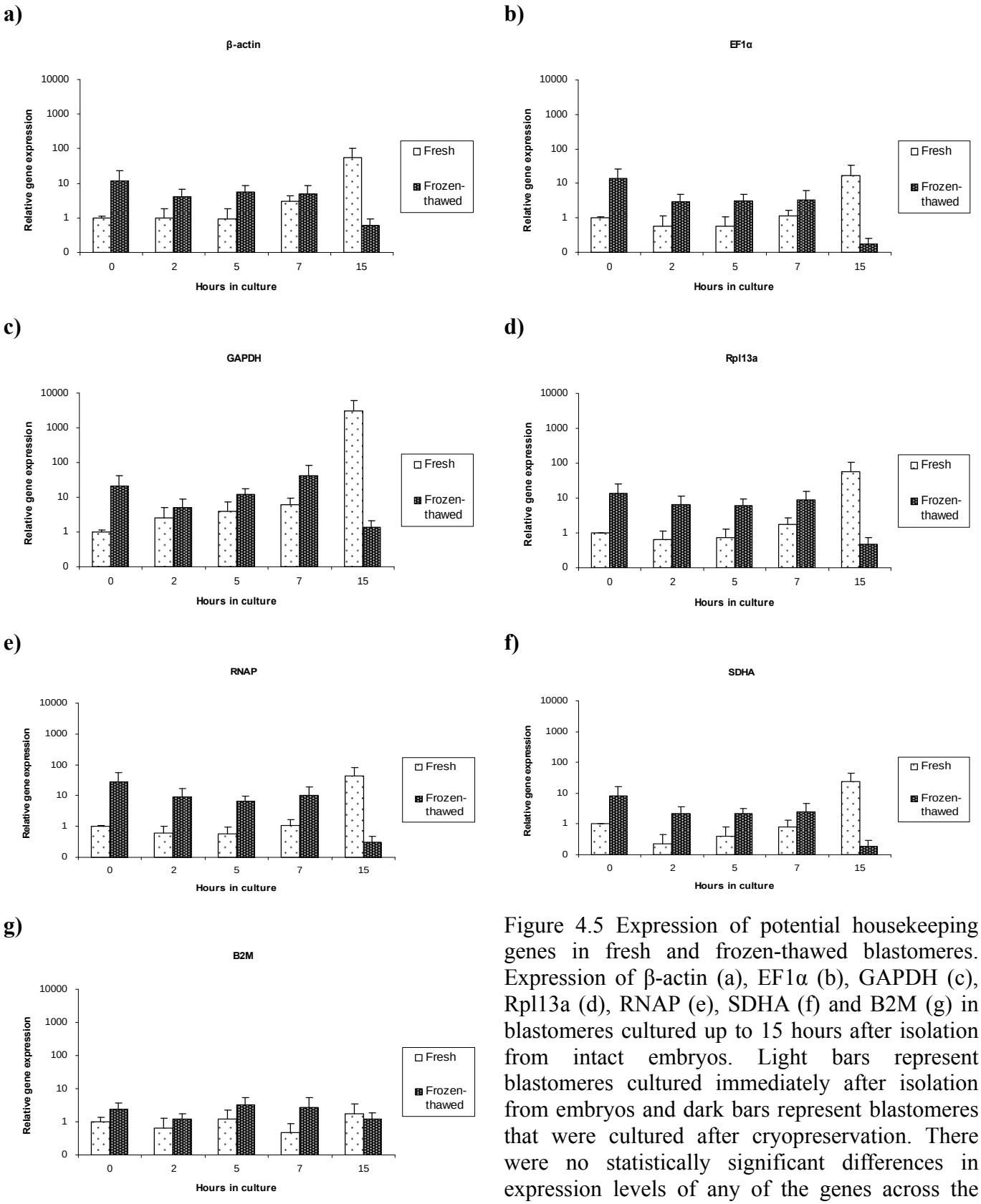


Figure 4.5 Expression of potential housekeeping genes in fresh and frozen-thawed blastomeres. Expression of β-actin (a), EF1α (b), GAPDH (c), Rpl13a (d), RNAP (e), SDHA (f) and B2M (g) in blastomeres cultured up to 15 hours after isolation from intact embryos. Light bars represent blastomeres cultured immediately after isolation from embryos and dark bars represent blastomeres that were cultured after cryopreservation. There were no statistically significant differences in expression levels of any of the genes across the culture period or between fresh and frozen-thawed samples after the same length of time in culture.

4.3.6 Stability of housekeeping genes in embryos as determined by GeNorm and NormFinder software.

Both GeNorm and Normfinder software packages were used to determine which genes were most stable during the developmental period being studied in fresh embryos. EF1α was the most stable and β-actin was the second most stable gene according to the Normfinder software (see Table 4.2a). These genes were also the two most stable genes according to the GeNorm software (which does not differentiate between the best two genes; see Figure 4.6a). Both programs also put Rpl13a in third place, B2M in fourth place, RNAP in fifth place and SDHA and GAPDH as the two least stable genes. When the results for both fresh and chilled embryos were considered together, despite these differences in β-actin expression, β-actin and EF1α remained as the most stable genes according to the GeNorm software (see Figure 4.6b). NormFinder software also confirmed EF1α as the most stable gene but placed β-actin in third place, after RNAP (see Table 4.2b). Both packages ranked the remaining genes in the same order and the results were similar, although not identical, to those obtained using only fresh embryos.

4.3.7 Stability of housekeeping genes in blastomeres as determined by GeNorm and NormFinder software.

In fresh blastomeres, β-actin and Rpl13a were ranked as the genes with the highest stability across the culture period according to both GeNorm and Normfinder software packages (see Figure 4.6c and Table 4.2c). Both programs also indicated the least stable genes as B2M, GAPDH and SDHA and placed EF1α and RNAP in third and fourth place respectively (see Figure 4.6c and Table 4.2c). When the results for both fresh and frozen-thaw blastomeres were considered together, the ranking of expression stability was identical using both the GeNorm and Normfinder software packages, with β-actin and EF1α being classified as the most stable genes (see Figure 4.6d and Table 4.2d). These were followed by Rpl13a and RNAP, with B2M, GAPDH and SDHA being the least stable, as observed for fresh blastomeres (see Figure 4.6d and Table 4.2d).

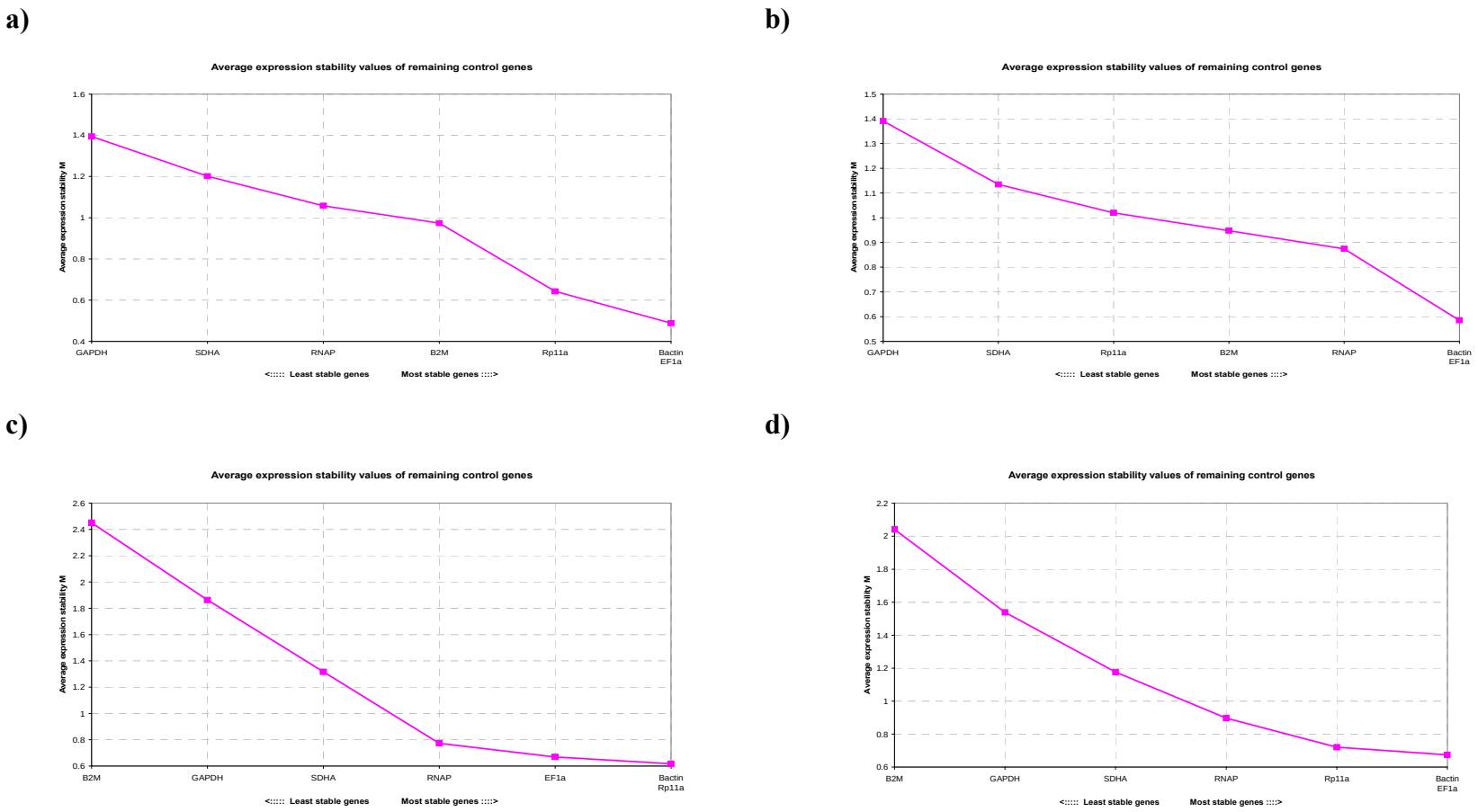


Figure 4.6 Stability of housekeeping genes as determined by GeNorm software. Graphs represent GeNorm output for fresh embryos (a), fresh and chilled embryos combined (b), fresh cultured blastomeres (c) and fresh cultured blastomeres combined with those cultured after cryopreservation (d). The most stable genes are at the right hand side of each graph.

a)

Gene name	Stability value	Standard error
EF1α	0.336	0.110
β-actin	0.476	0.117
Rp113a	0.543	0.123
B2M	0.555	0.125
RNAP	0.560	0.125
SDHA	1.040	0.191
GAPDH	1.182	0.212

b)

Gene name	Stability value	Standard error
EF1α	0.376	0.076
RNAP	0.378	0.076
β-actin	0.384	0.077
B2M	0.512	0.083
Rp113a	0.622	0.092
SDHA	0.962	0.125
GAPDH	1.308	0.161

c)

Gene name	Stability value	Standard error
β-actin	0.214	0.362
Rp113a	0.214	0.362
EF1α	0.241	0.331
RNAP	0.243	0.330
SDHA	1.455	0.317
GAPDH	2.182	0.434
B2M	2.603	0.506

d)

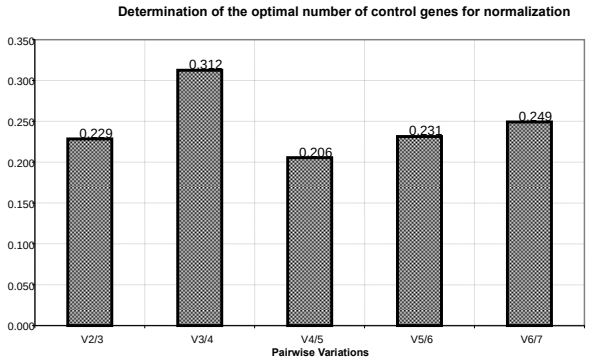
Gene name	Stability value	Standard error
β-actin	0.227	0.166
EF1α	0.234	0.163
Rp113a	0.236	0.162
RNAP	0.631	0.131
SDHA	1.030	0.163
GAPDH	1.611	0.226
B2M	2.173	0.292

Table 4.2 Stability of housekeeping genes as determined by NormFinder software. Values represent NormFinder output for fresh embryos (a), fresh and chilled embryos combined (b), fresh cultured blastomeres (c) and fresh cultured blastomeres combined with those cultured after cryopreservation (d). The most stable gene being at the top of each table followed by remaining genes with decreasing stability.

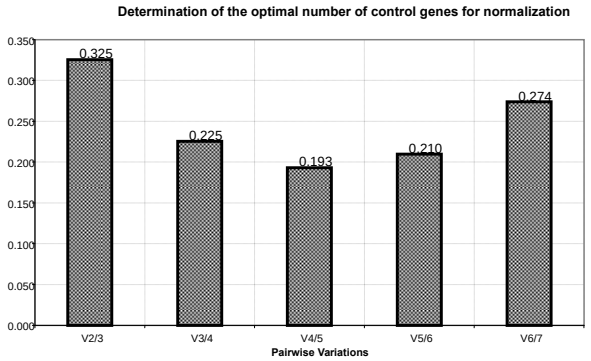
4.3.8 Determination of the number of HKGs necessary for accurate normalisation across development stages in embryos and periods of culture in blastomeres

To determine the minimum number of housekeeping genes necessary for an accurate normalization, a pairwise variation Vn/n+1 analysis was performed (Vaerman et al., 2004). Geometric averaging of 2 genes in zebrafish embryos required for accurate normalization (Figure 4.7a and 4.7b) and 4 genes would be required for accurate normalization of zebrafish blastomeres (Figure 4.7c and 4.7d).

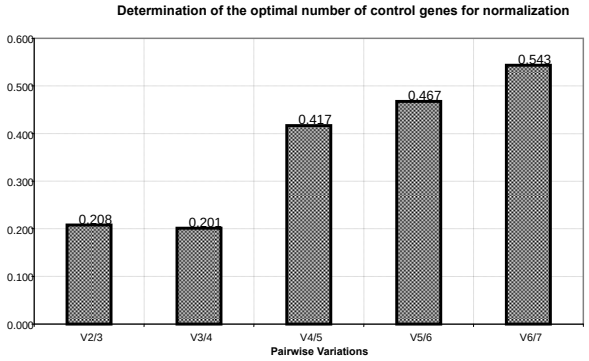
a)



b)



c)



d)

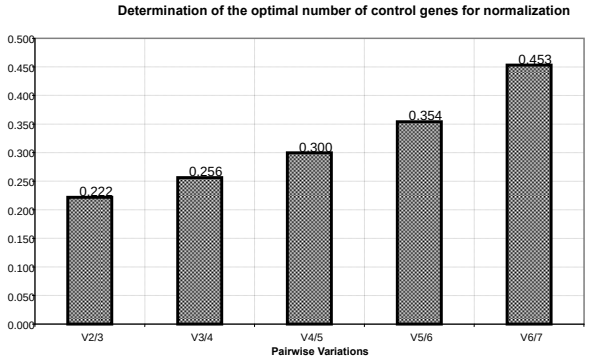


Figure 4.7 Determination of the optimal number of HKGs in fresh embryos (a), chilled embryos (b), fresh blastomeres (c) and frozen-thawed blastomeres (d) for normalization calculated on the basis of the pair-wise variation by GeNorm software

4.3.9 Stability rankings of housekeeping genes in fresh and chilled embryos at individual stages of development

Expression stability following chilling treatment was also investigated at individual stages of development. Interestingly, SDHA, B2M and GAPDH tended to rank higher by GeNorm and Normfinder software at individual developmental stages than they did across multiple stages whilst EF1α, β-actin and also RNAP ranked lower (see Table 4.3). The two programs used presented different orders of the four most stably expressed genes which were EF1α, Rpl13a, SDHA and B2M in fresh and chilled embryos of oblong and 100% epiboly stage. At the stages of 30% epiboly, 75% epiboly and 6 somite, β-actin, EF1α, SDHA and B2M emerged as the four most stable genes. The Rpl13a, RNAP, SDHA and B2M were ranked among the best genes at 50% epiboly stage.

4.3.10 Stability rankings of housekeeping genes in fresh and frozen-thawed blastomeres after individual periods of time in culture

The rankings of these genes at individual developmental stages was very similar to those observed across multiple periods of time in culture, with the exception of SDHA, which tended to be more stable across the cryopreservation procedure at individual periods of time in culture and β-actin, which tended to be less stable (see Table 4.4). GAPDH and B2M are the least stable HKGs in all periods of time in culture recognized by the two algorithms used. The rankings of the HKGs in fresh blastomeres following 0, 2, 5, 7 and 15hr periods of culture were similar to fresh and frozen/thawed blastomeres. Although the order at each developmental stage is different from fresh and frozen/thawed blastomeres, the four most stable genes still maintained similar patterns except for RNAP which was placed at third place in fresh blastomeres instead fifth place in fresh and frozen/thawed blastomere after 7hrs of culture.

Table 4.3 Stability rankings of housekeeping genes in fresh and chilled embryos at individual stages of development

Gene	Oblong			30% epiboly			50% epiboly			75% epiboly			100% epiboly			6-somite		
	G	N	C	G	N	G	N	C	C	G	N	C	G	N	C	G	N	C
β-actin	7	7	**7**	1	3	**2**	6	6	**6**	4	3	**4**	6	6	**6**	3	2	**1**
EF1α	4	4	**4**	1	2	**1**	5	5	**5**	1	3	**2**	1	1	**1**	1	5	**3**
GAPDH	5	5	**5**	6	6	**6**	7	7	**7**	7	7	**7**	7	7	**7**	1	4	**1**
Rpl13α	1	2	**1**	7	7	**7**	1	4	**2**	6	6	**6**	3	5	**4**	7	7	**7**
RNAP	6	6	**6**	5	4	**4**	1	3	**1**	5	5	**5**	5	4	**5**	6	6	**6**
SDHA	3	1	**2**	4	5	**4**	3	2	**2**	1	2	**1**	4	3	**3**	4	2	**3**
B2M	1	3	**2**	3	1	**2**	4	1	**2**	3	1	**2**	1	2	**2**	5	1	**3**

G: GeNorm

N: NormFinder

C: Combined rankings from GeNorm and NormFinder

Table 4.4 Stability rankings of housekeeping genes in fresh and frozen-thawed blastomeres after individual periods of time in culture

Gene	0hr			2hr			5hr			7hr			15hr		
	G	**N**	**C**	**G**	**N**	**C**	**G**	**N**	**C**	**G**	**N**	**C**	**G**	**N**	**C**
β-actin	1	1	**1**	1	1	**1**	3	5	**4**	1	4	**2**	4	2	**3**
EF1α	1	2	**2**	1	1	**1**	1	1	**1**	1	1	**1**	3	2	**2**
GAPDH	5	5	**5**	5	5	**5**	7	7	**7**	6	6	**6**	6	6	**6**
Rpl13α	4	3	**3**	3	3	**3**	4	3	**3**	4	2	**3**	1	1	**1**
RNAP	6	6	**6**	4	4	**4**	5	3	**4**	5	5	**5**	5	4	**5**
SDHA	3	4	**3**	6	6	**6**	1	1	**1**	3	3	**3**	1	5	**3**
B2M	7	7	**7**	7	7	**7**	6	6	**6**	7	7	**7**	7	7	**7**

G: GeNorm

N: NormFinder

C: Combined rankings from GeNorm and NormFinder

4.3.11 Determination of the number of HKGs necessary for an accurate normalization in embryos at individual stages of development

To determine the minimum number of housekeeping genes necessary for an accurate normalization, a pairwise variation Vn/n+1 analysis was performed. Geometric averaging of 2 genes was required in zebrafish oblong, 50% epiboly, 75% epiboly, 100% epiboly. The addition of further HKGs did not significantly affect the reliability of the determined normalisation factor, yielding a V2/3 value pair-wise variation between two sequential normalization factors of 0.089, 0.139, 0.103, 0.117 and 0.145. However, the 6 somite stage would require 3 genes for accurate normalization and 30% epiboly stage would require 4 genes (see Figure 4.8).

4.3.12. Determination of the number of HKGs necessary for an accurate normalization in blastomeres after individual periods of time in culture

The minimum number of housekeeping genes necessary in cultured blastomeres for 2 to 5 hrs culture would be 2 genes required (V2/3 value of 0.189, 0.187 and 0.101, Figure 4.9). However, 7 and 15 hrs of culture would require 3 genes (V3/4 value of 0.169 and 0.133) for accurate normalization (see Figure 4.9).

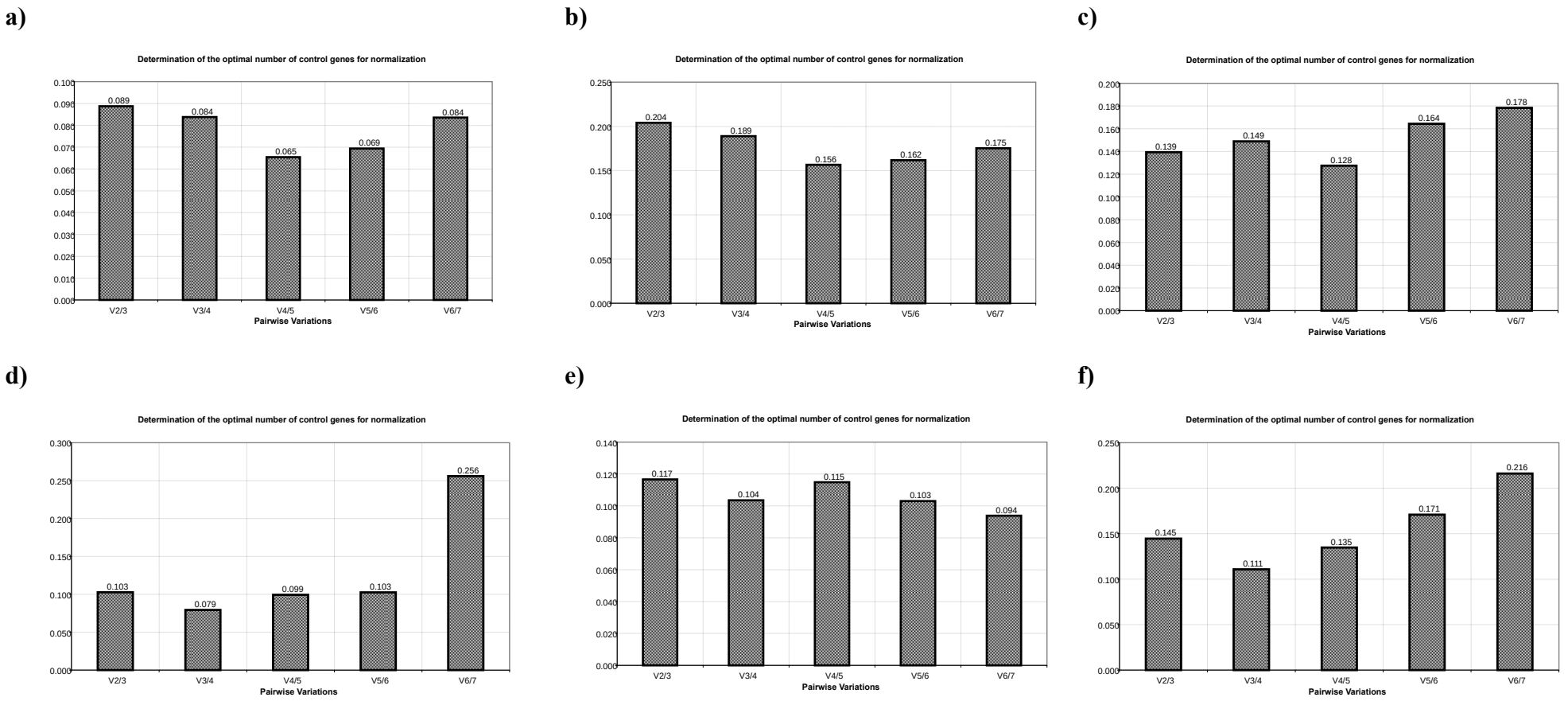


Figure 4.8 Determination of the optimal number of HKGs in fresh and chilled embryos at oblong (a), 30% epiboly (b), 50% epiboly (c), 75% epiboly (d), 100% epiboly (e) and 6 somite (f) stage for normalization calculated on the basis of the pair-wise variation by GeNorm software.

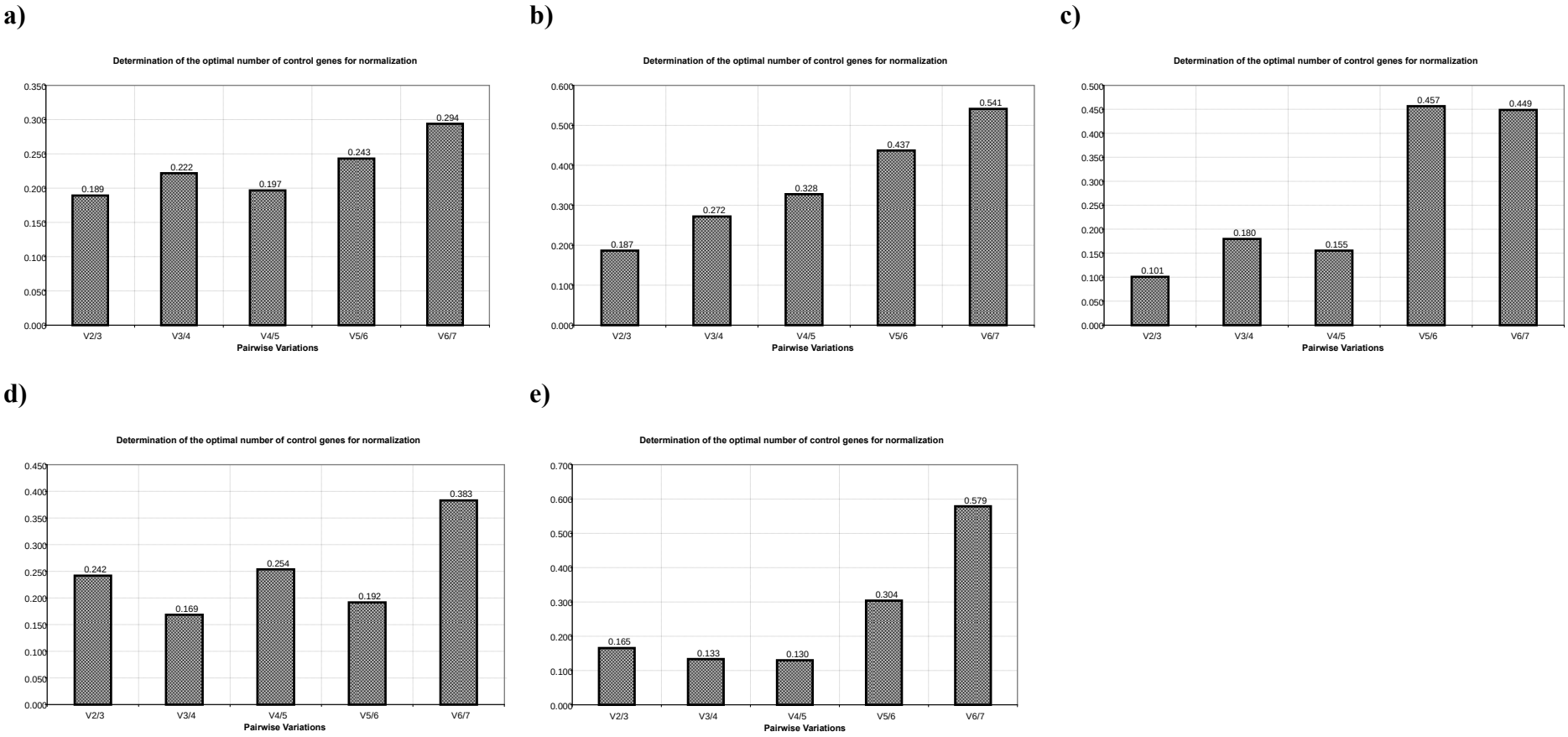


Figure 4.9 Determination of the optimal number of HKGs in fresh and frozen/thawed blastomeres at 0 (a), 2 (b), 5 (c), 7 (d) and 15 (e) hours of culture for normalization calculated on the basis of the pair-wise variation by GeNorm software.

4.4 Discussion

4.4.1 Expression stability in intact embryos during development and following chilling treatment

This study aimed to validate reference genes for use in cryopreservation studies of zebrafish embryos. Firstly, the expression levels of seven potential housekeeping genes were determined during embryo development. There were no significant differences in expression from many of these genes. However, β-actin, EF1α and Rpl13a showed a similar trend of a peak in expression at the 75% epiboly stage. It is possible that it has some biological significance, despite the lack of statistical significance. A potential explanation is a general increase in transcription at this stage of development in preparation for organogenesis. 50% to 75% epiboly stages were targeted as it is the start of midblastula transition (MBT) in zebrafish (Kane and Kimmel, 1993), which is characterized by activation of transcription and appearance of cell motility (Newport and Kirschner, 1982), both characteristics essential for the ensuing process of gastrulation. However, this has not been reported previously and cannot be concluded from this study alone. Interestingly, a similar trend with reducing expression in the later stages were noted in SDHA, RNAP or B2M. It is possible that the products of these genes are not required at this relatively early stage in development and that the existing transcripts are not replaced until they are required at a later stage.

As intact zebrafish embryos are not yet able to be cryopreserved successfully, the effect of chilling was investigated at each stage of development. Expression levels of all seven housekeeping genes in chilled embryos were consistently higher compared to fresh embryos at the same stage (see Figure 4.4). It is possible that these housekeeping genes were upregulated during or following the chilling treatment in order to ensure basic function was maintained. The increased expression in chilled embryos compared to fresh embryos was particularly large at the oblong stage, where the differences reached statistical significance for three of the seven genes analysed. As a result, this provides the information that early staged embryos are less able to withstand chilling treatments (Zhang et al., 2005; Zhang

and Rawson, 1993). However, these increases could also have occurred due to a reduction in the degradation of existing transcripts at the lower temperature.

4.4.2 Expression stability in isolated blastomeres during development and following cryopreservation

As isolated blastomeres can now be cryopreserved relatively successfully (as described earlier), the expression of housekeeping genes in isolated blastomeres was also investigated. Fresh blastomeres were initially analysed during culture. A gradual increase in expression was observed for all genes analysed, especially at 15 hours of culture. The increase in expression of housekeeping genes at the later stages of culture might be a 'stress' response to the non-physiological conditions, similar to that observed in non-physiological cancer cells compared to healthy controls (Waxman et al., 2007). This is not the same gene expression pattern as intact embryos, although the time points analysed corresponded broadly to the later developmental stages analysed in intact embryos. The results suggest that development of blastomeres did not proceed at the same rate as embryo development, although this was not investigated directly. It is also possible that an increase in expression of all genes was detected towards the end of the culture period analysed, which could be a delayed equivalent to the peak in expression detected at the 75% epiboly stage in intact embryos.

The gene expression was consistently higher in frozen-thawed blastomeres cultured up to seven hours than in cultured fresh blastomeres. Then, there was a general tendency for frozen-thawed blastomeres to decrease gene expression during the culture period at 15 hours of culture. This might be due to a negative feedback response to the increased expression observed in the earlier stages of culture. Ultimately, this could also be due to a failure to increase expression at a vital stage of development.

4.4.3 Stability of housekeeping genes in embryos and blastomeres as determined by GeNorm and NormFinder software.

The seven housekeeping genes analysed in this study are commonly used in many species including the zebrafish. Tang et al. (2007) used embryos from the sphere stage (4 hpf) to the protruding mouth stage (~72 hpf) to study expression of nine potential housekeeping genes. To our knowledge, this is the only study of housekeeping genes in zebrafish. The study investigated expression stability during embryo development but did not assess the effect of chilling or cryopreservation. The study demonstrated that β-actin, EF1α and Rpl13a were found to be the three most stable genes and GAPDH was the least stable, according to the GeNorm software. Although these results are not directly comparable to those presented here due to the analysis of different developmental stages, the study was in agreement with this report. This conclusion was also reached following analysis of our data using NormFinder. In fact, with one minor exception, all data generated identical results using the two software programs. Moreover, Olsvik et al. (2005) and Infante et al. (2008) give support and highlight the most stable gene is EF1α and the least stable gene is GAPDH for gene expression based on GeNorm calculations in Atlantic salmon and flatfish.

In contrast to the results obtained for zebrafish, studies in mice (Mamo et al., 2007) and fathead minnows (Filby and Tyler, 2007) selected β-actin as an unsuitable housekeeping gene, whilst a study of bovine embryos identified GAPDH as one of the most stable genes (Goossens et al., 2005), as did a study of skin biopsies in striped dolphin (Spinsanti et al., 2006). It might be expected that gene expression patterns are not identical across different species, however, changes in housekeeping gene expression stability have also been noted in response to different tissue and environmental conditions. For example, human airway epithelial cells taken from patients with different respiratory diseases expressed a variety of housekeeping genes with different levels of stability (He et al., 2008) and the expression of housekeeping genes has also been reported to be disrupted in tumour cells (Waxman et al., 2007; Kagedal et al., 2007). It is therefore important to validate potential housekeeping genes before use in gene expression studies with new types of samples.

There are some similarities in gene expression stability between cultured fresh blastomeres and fresh intact embryos during development. For example, β-actin, EF1α and Rpl13a were classified as the three most stable genes for both sets of samples. Similarly, a comparison of *in vivo* and *in vitro* derived mouse embryos also demonstrated very similar results between the two groups of samples (Mamo et al., 2007). However, the ranking of B2M was reduced in the fresh blastomeres compared to the fresh intact embryos. It is possible that the stability of this gene is affected more than others by the isolation and *in vitro* culture of the cells. As B2M is known to play a role in cell adhesion, it is not surprising that its stability is reduced during the period shortly after isolation and separation of adjacent cells when adhesion requirements alter significantly (Jaradat et al., 2001). However, this effect was not observed with any of the genes in the mouse study (Mamo et al., 2007), probably because *in vitro* culture of mouse embryos does not involve isolation of the cells. It is therefore less disruptive to the cells and will have less of an effect on gene expression.

On the other hand, cryopreservation of isolated blastomeres did not significantly alter the ranking of housekeeping gene stability and stability values from sample groups that included and excluded frozen-thawed samples were fairly similar. This indicated that the process of cryopreservation did not significantly affect the stability of housekeeping genes. However, some genes were still more stably expressed than others, with β-actin being considered the most stable housekeeping gene, followed by EF1α and Rpl13a.

4.4.4 Stability rankings of housekeeping genes at individual stages of development in fresh and chilled embryos and after individual periods of time in culture in fresh and frozen-thawed blastomeres

When fresh and chilled embryos or frozen-thawed blastomere samples were analysed at individual stages of development rather than across multiple stages, EF1α was the most stably expressed gene at 30% and 100% epiboly stages in fresh and chilled embryos and 2hr, 5hr and 7hr periods of culture in fresh and

frozen-thawed blastomeres. The results obtained in this study are in agreement with previous studies in salmon which showed EF1α is the most stable HKG for transcript studies (Olsvik et al., 2005; Jorgensen et al., 2006; Ingerslev et al., 2006). Although β-actin was the most stably-expressed gene across 6 developmental stages in fresh and chilled embryos (see Figure 4.4 and Table 4.3), the results from the individual stages show β-actin in particular to be less stable. β-actin has been the most commonly used HKG for normalizing real-time PCR data in zebrafish (Rongying et al., 2007). However, β-actin gene expression was found to vary greatly between tissue samples (Olsvik et al., 2005; Jorgensen et al., 2006; Ingerslev et al., 2006). Therefore, caution must be exercised when using β-actin as a HKG at individual stage in zebrafish experiments.

Interestingly, when fresh and chilled embryos or frozen-thawed blastomere samples were analysed at individual stages of development rather than across multiple stages, SDHA and B2M in particular ranked more highly. SDHA and B2M have low transcript abundance and have mean CT values above 25 cycles. These data might suggest that the expression of genes such as SDHA is affected little by the cryopreservation process but more significantly by embryonic development, whilst the reverse is true for genes such as β-actin. However, Giacomo (2006) suggested that the use of target genes with low levels of expression should always be avoided, because of the considerable variance due to experimental fluctuations. The exclusion of SDHA and B2M from the control genes is recommended for this reason and because they ranked low according to GeNorm and NomFinder.

4.4.5 Determine the number of HKGs necessary for accurate normalization across development stages in embryos and periods of culture in blastomeres

Recently reported studies quantifying cellular mRNA levels reveal that many continue to use single housekeeping genes without demonstrating their appropriateness. The obvious alternative is to use multiple housekeeping genes (Bustin et al., 2005). GeNorm program determines the minimum number of HKGs necessary for an accurate normalization by a pairwise variation Vn/n+1 analysis. GeNorm ranks reference genes according to the similarity of their expression profile

using a pair-wise comparison and uses their geometric mean as a normalization factor (Vandesompele 2002). The reliability of the normalization factor increases together with the number of stably expressed HKGs included in its calculation. This study examined that various combinations and numbers of housekeeping genes were optimal for different individual experiments. Two to four genes were found to be suitable for all embryos and blastomeres work including across development stages in embryos, across periods of culture in blastomeres, individual stages of development in embryos and individual periods of time in culture in blastomeres (see figure 4.6, 4.8 and 4.9). However, using up to four housekeeping genes were considered costly, time-consuming and unnecessary for some combinations. In order to make all experiments comparable, it would be ideal to use the same combination of housekeeping gene. EF1α and β-actin were consistently among the top three stable genes and these genes were therefore chosen as HKGs to normalize Pax gene in the next chapter.

4.5 Summary

It has been shown that of the genes investigated the most ideal housekeeping genes vary according to the type of cell and treatment used, which strengthens the requirement for validation of housekeeping genes before use with new types of samples. Although validation of housekeeping genes has now been carried out for many applications, this is the first analysis of the stability of housekeeping genes in chilled and cryopreserved embryonic cells of the zebrafish. The combined use of β-actin and EF1α as reference genes would be suitable for work on both zebrafish embryos and blastomeres across the developmental stages analysed and also on studies involving chilling and/or cryopreservation of those cells.

This work has been accepted for publication by Theriogenology.

CHAPTER 5: EFFECT OF CHILLING AND CRYOPRESERVATION ON EXPRESSION OF PAX GENES IN ZEBRAFISH EMBRYOS AND BLASTOMERES

5.1 Introduction

In chapter three, the optimal freezing protocol was found for the cryopreservation of blastomeres using controlled slow cooling and the most suitable housekeeping genes were identified in chapter four. The results from these investigations were used to study the effect of chilling and cryopreservation on expression of Pax genes in zebrafish embryos and blastomeres.

Two methods of cryopreservation have become well established, controlled slow cooling and vitrification. The effect of the freeze-thaw process on cells is most often assessed in terms of survival (Zilli et al, 2003). However, this parameter provides no information on more subtle potential effects of cryopreservation, such as the level of gene expression. The analysis of differences in the level of gene expression after cryopreservation may help us to understand developmental stage dependent cryotolerance and make major advances in knowing the adaptations needed for freezing survival. Therefore, quantitative analysis of mRNA abundance of certain genes would give a more complete picture of the consequences of chilling and cryopreservation treatments.

Although only a limited number of studies have investigated the effect of cryopreservation on gene expression, some data is available. For example, Tachataki et al (2003) demonstrated that cryopreservation does alter the normal pattern of gene expression during preimplantation development of human embryos. Rodriguez-Vargas et al (2002) used mRNA differential display to assess yeast gene expression and found both up- and down-regulation of genes with repression being more common than increased expression after chilling and freezing. Furthermore, previous experiments (chapter four) showed that even some housekeeping genes were not totally stable

throughout the chilling and freezing conditions analysed. Cryopreservation has also been shown to affect DNA stability of rainbow trout sperm (Labbe et al 2001) and chromatin structure in human sperm (Spano et al 1999) and Zilli et al (2005) showed that there is also an effect at the protein level in sea bass sperm. However, little or no effect on gene expression was observed in boar spermatozoa (Hamamah et al., 1990; Evenson et al., 1994).

The genes studied, Pax2a, Pax2b, Pax5 and Pax8, belong to the same group of Pax genes which are transcription factors and play important roles in embryogenesis (Noll, 1993; Mansouri et al., 1996; Chi and Epstein, 2002; Dahl et al., 1997; Pfeffer et al., 1998). Pax genes have been classified into four groups based on their sequence similarity, expression pattern and protein structures (Noll, 1993; Mansouri et al., 1996; Chi and Epstein, 2002; Dahl et al., 1997; see section 1.5.1). Evidence indicates that pax genes play important roles in embryonic development and organogenesis in a variety of species. For example, Bouchard et al (2000) demonstrate that Pax2 and Pax5 have maintained equivalent biochemical functions since their divergence early in vertebrate evolution. Terzic et al (1998) demonstrate that the differentiation of neural tube, eye, ear and kidneys were mediated by the Pax2 gene at defined stages of human development. Lack of Pax2 expression results in major defects in the brain, ear, eye and kidneys (Torres et al., 1995, 1996; Favor et al., 1996). Whilst Pax8 deficient mice develop thyroid glands devoid of follicular cells and Pax5 deficient mice fail to develop B-lymphocytes.

This study aimed to discover if chilling and cryopreservation has an impact on Pax2a, Paxe2b, Pax5 and Pax8 gene expression in zebrafish embryos and isolated blastomeres

5.2 Experimental design

The one-step RT-PCR method was used in a preliminary experiment to confirm that the genes of interest could be detected. Real time RT-PCR was used in subsequent experiments to compare levels of gene expression between fresh

embryos at various stages of development and embryos of equivalent stages chilled at 1°C in the presence of cryoprotectants. The effect of cryopreservation in liquid nitrogen was then analysed using isolated blastomeres which are able to withstand the lower temperature.

5.3 Results

5.3.1 Preliminary experiment to determine timing of Pax2a and Pax2b genes in fresh blastomeres at different developmental stages using a one-step RT-PCR method

Pax2a expression was visible at the 75% epiboly stage, but strongly increased until 10 hours of embryogenesis giving a large band at 100% epiboly and the intensity of Pax2a gene expression generally increased with embryonic development (Figure 5.1). There does not appear to be any product for either of the pax2b PCRs at 50 and 75% epiboly, but product is seen at 100% epiboly, implying that this gene is not expressed until 100% epiboly (Figure 5.2). Therefore, Pax2a appears to be expressed earlier than Pax2b.

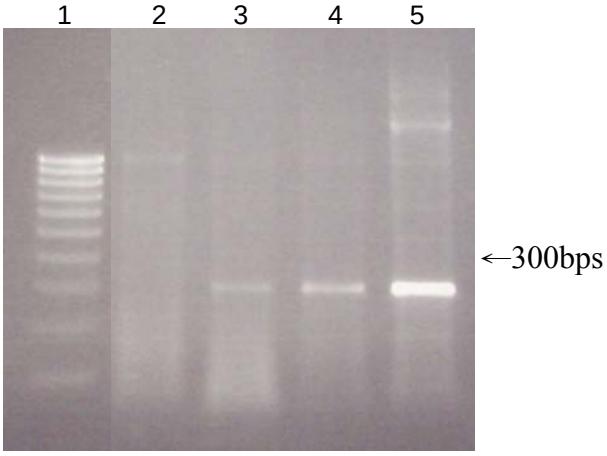


Figure 5.1 Determination of zebrafish Pax2a (300bp) gene expression by a reverse transcription-PCR analysis. Pax2a gene expression was analysed in different stages of zebrafish embryogenesis by RT-PCR. Lane 1: Hyperladder (1Kb). The blastomeres assayed were from embryos at 50% (lane 2), 75% (lane 3), 90% (lane 4) and 100% (lane 5) epiboly.

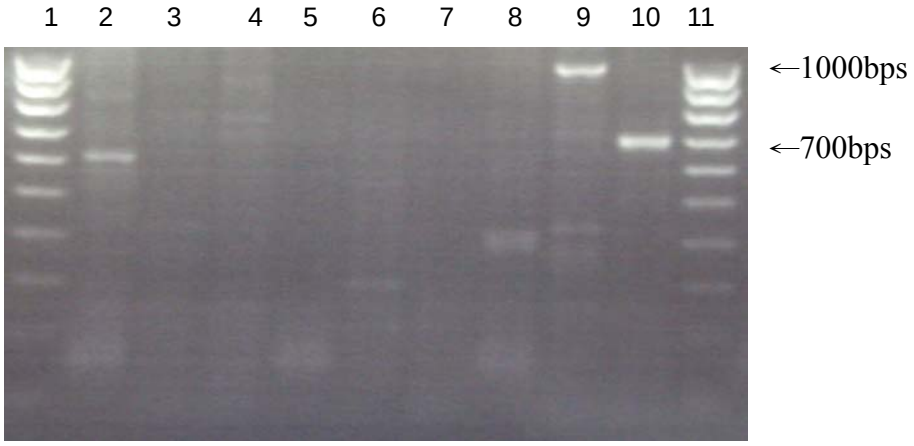


Figure 5.2 Determination of zebrafish Pax2b (360, 960 and 700bp depending on primer combination) gene expression by a reverse transcription-PCR analysis. Pax2b gene expression was analysed at different stage of zebrafish embryogenesis by RT-PCR. Lanes 1 and 11: Hyperladder (1Kb). The blastomeres assayed were from embryos at 50% (lanes 2, 3 and 4), 75% (lanes 5, 6 and 7), and 100% (lanes 8, 9 and 10) epiboly.

5.3.2 Fluorescence measurements, melt curves and standard curve evaluation of Pax genes

After the initial experiment, studies were carried out using real time PCR. Fluorescence levels within each tube were measured at the end of every cycle (Figure 5.3). Amplification efficiency and a correlation coefficient from a standard curve of cDNA at different 10-fold dilutions were also determined for each Pax gene utilizing real time PCR (Figure 5.4). All of the target genes had acceptable efficiencies and correlation coefficients from 0.7 to 1.1 (Pfaffl, 2003). Melting curves were carried out to ensure that the primers amplified a single product. All of the target genes had a single peak for melting curve (Figure 5.5). Therefore, there is no contamination in these reactions.

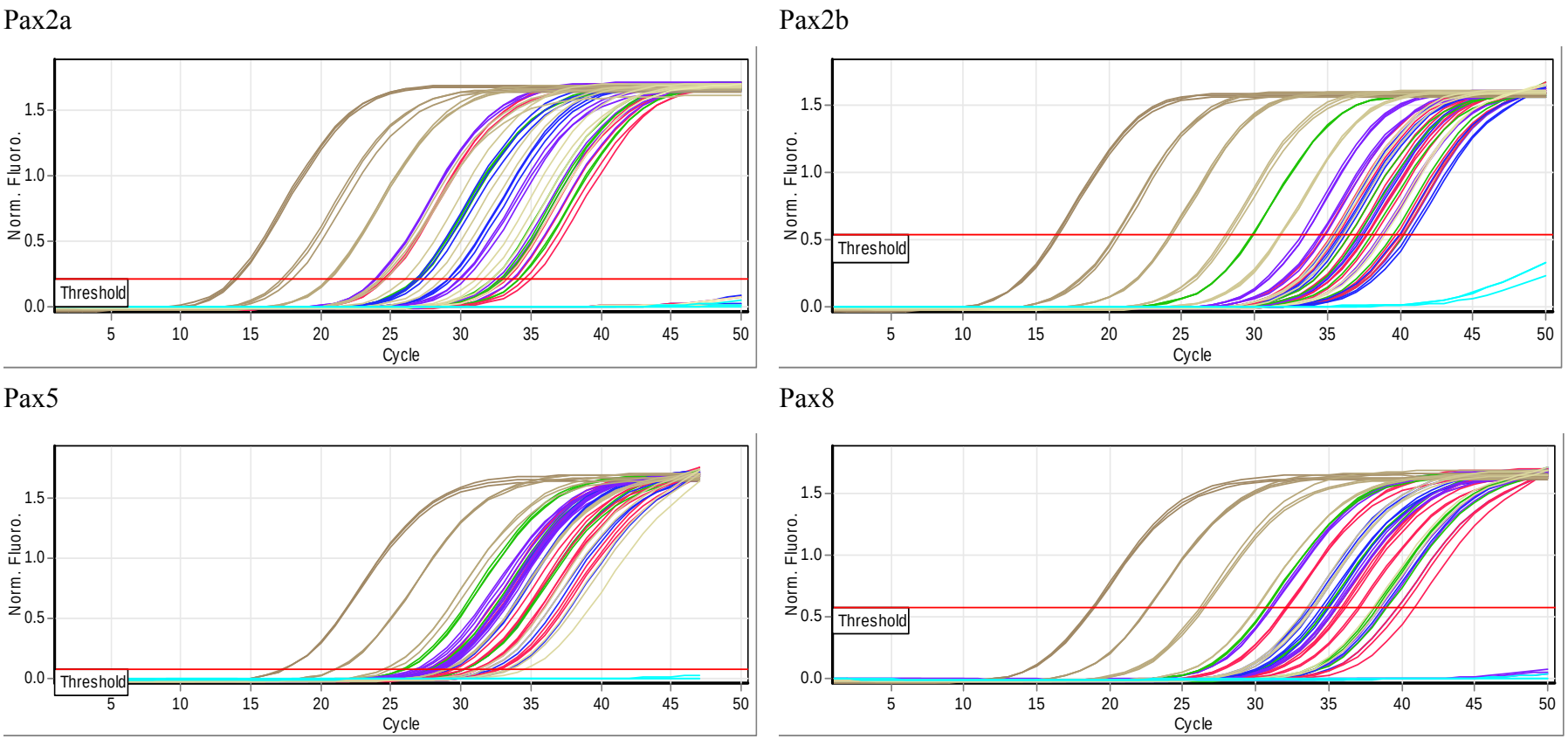
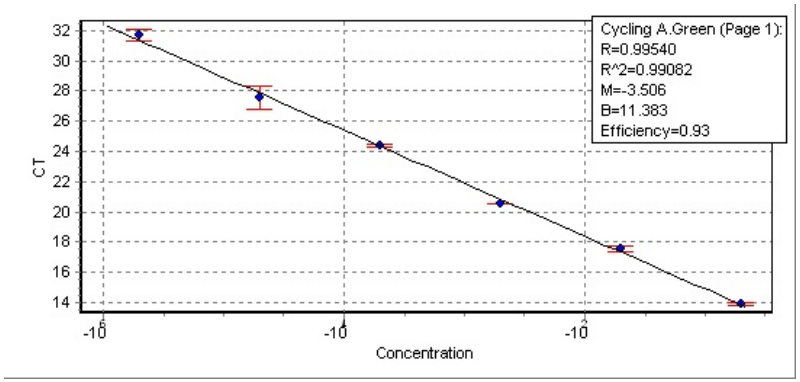
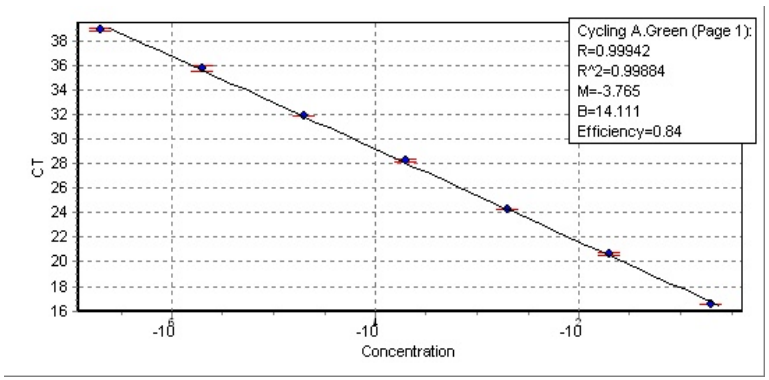


Figure 5.3 Example of fluorescence measurements for Pax2a, Pax2b, Pax5 and Pax8 genes. Fluorescence graphs show fluorescence measurements obtained for standards (brown) of 10-fold dilutions, negative control (blue) and other colours relate to embryo and blastomere samples.

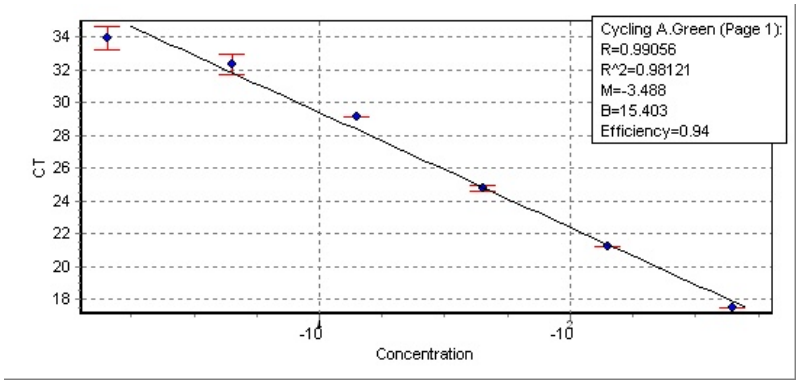
Pax2a



Pax2b



Pax5



Pax8

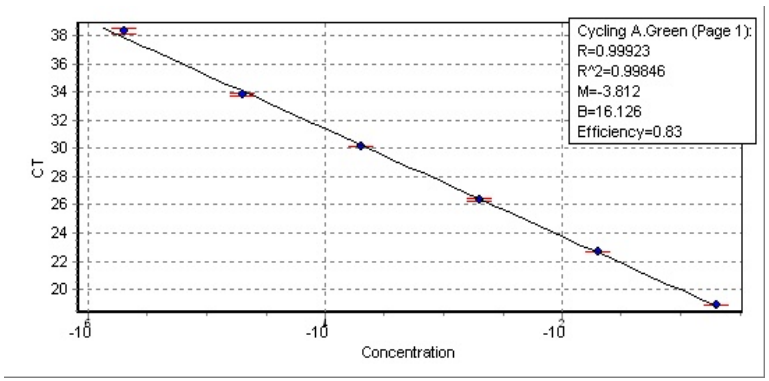
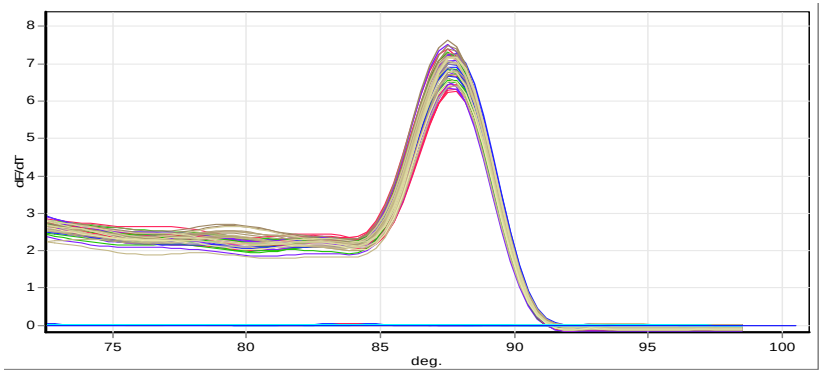
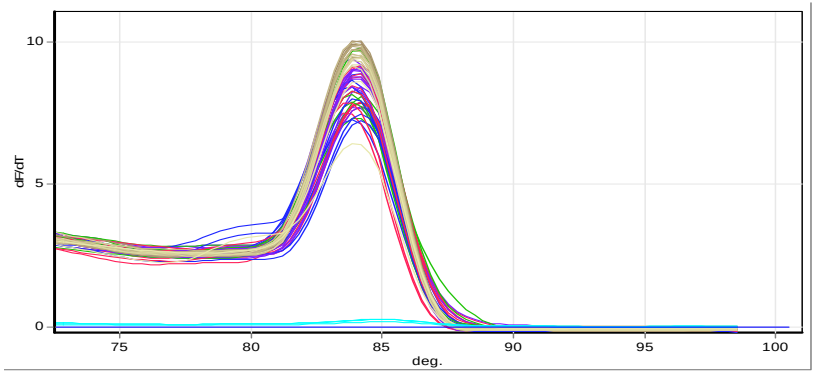


Figure 5.4 Example of standard cures for Pax2a, Pax2b, Pax5 and Pax8 genes. Mean ± SEM CT values are plotted against the log concentration of the standards of 10-fold dilutions. Each curve has an R^2 value > 0.98, due to an equal number of cycles separating standards of 10-fold concentration difference. Each curve has an efficiency value with in the acceptable range of between 0.7 and 1.1 (Pfaffle, 2003).

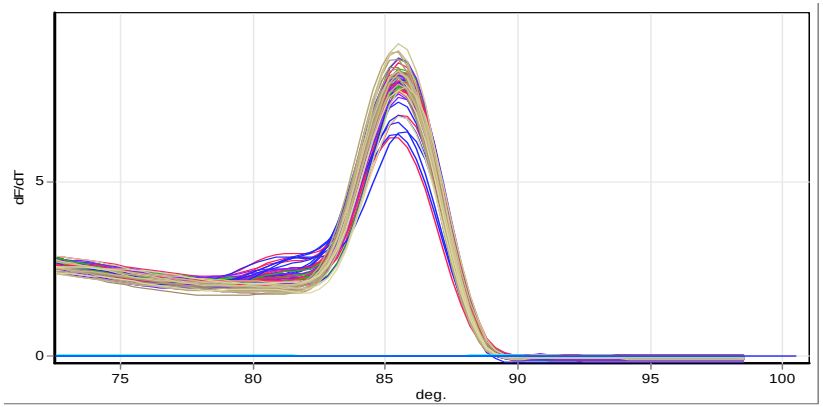
Pax2a



Pax2b



Pax5



Pax8

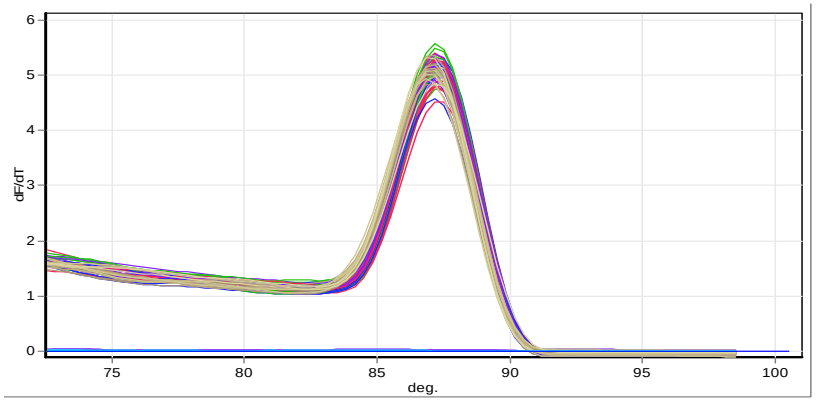


Figure 5.5 Example of a melting curve profile of Pax2a, Pax2b, Pax5 and Pax8 genes. Showing standard (brown), negative control (blue) and sample (other colours) melt curves from different stages and time periods. All samples produced the same peak. The samples are therefore not contaminated, have no misprming and no primer-dimers.

5.3.3 Expression levels of Pax genes in zebrafish fresh and chilled embryos of different developmental stages

Gene expression profiles normalised to the internal reference genes β-actin and EF1α at different stages of embryo development are shown in Figure 5.6. The comparisons between fresh and chilled embryos demonstrated no significant differences statistically. The expression levels of Pax2a, Pax2b and Pax8 were gradually increasing with embryonic development in both fresh and chilled embryos, although there were some differences between the 2 groups. For example, the expression of Pax2a and Pax8 were significantly increased between the 50% and the 75% epiboly stage in fresh embryos, and further increased with continued development (Figure 5.6a and 5.6d). These increases in chilled embryos were delayed until the 100% epiboly stage. A gradual rise in Pax2b was observed throughout development of fresh embryos becoming significantly higher than the 50% epiboly stage by the 20 somite stage. This was also the case for chilled embryos, although levels were lower than fresh embryos at all stages beyond 75% epiboly (Figure 5.6b). Furthermore, Pax5 expression was significantly increased between the 100% epiboly and the 6-somite stage in fresh embryos, following an initial decrease. However, chilled embryos maintained relatively constant levels of Pax5 until they significantly increased at the 20 somite stage (Figure 5.6c). Smaller increases were detected at intermediate stages in all Pax genes.

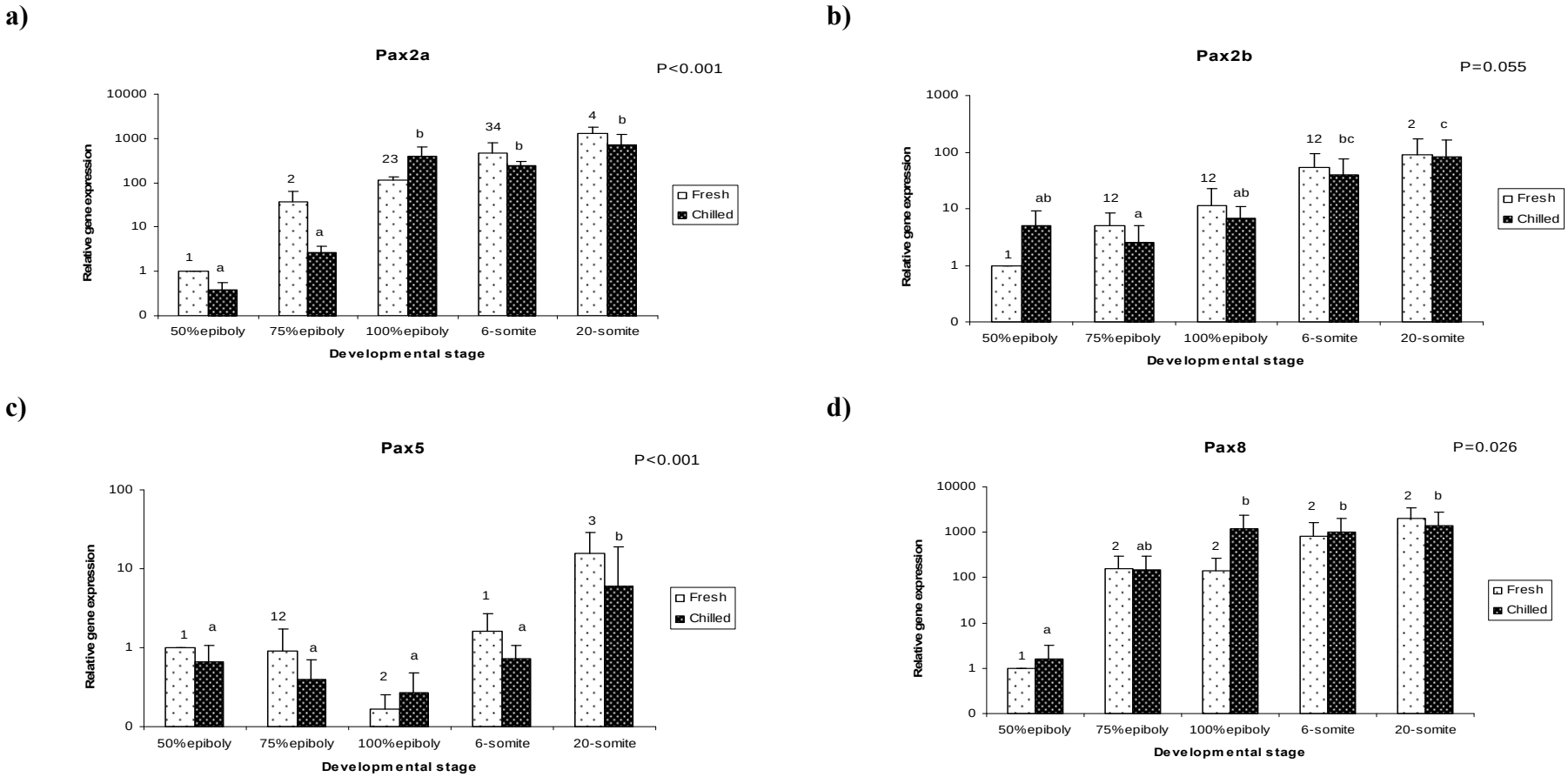


Figure 5.6 Relative expression of Pax2a (a), Pax2b (b), Pax5 (c) and Pax8 (d) in fresh and chilled embryos at different developmental stages, assessed by real-time RT-PCR. Error bars represent the standard error of the mean (SEM). Fresh bars with different numbers are significantly different ($p<0.05$). Chilled bars with different letters are significantly different ($p<0.05$). Statistical difference was determined using Welch tests.

5.3.4 Expression levels of Pax genes in fresh and frozen-thawed blastomeres following different periods of culture

The trypan blue staining of fresh blastomeres after culture for 0, 2, 5, 7 and 15hr demonstrated that viability levels were maintained higher than 98% throughout the culture period with no significant differences between time points. The survival rate of blastomeres following cryopreservation was significantly reduced to between 55 and 68% (Figure 5.7). The viability of blastomeres that survived was further evidenced by their ability to form morulae in culture, albeit slightly more slowly than their fresh equivalents (Appendix D). Analysis of Pax gene expression was subsequently carried out in order to determine whether these apparently unaffected cells had any more subtle abnormalities.

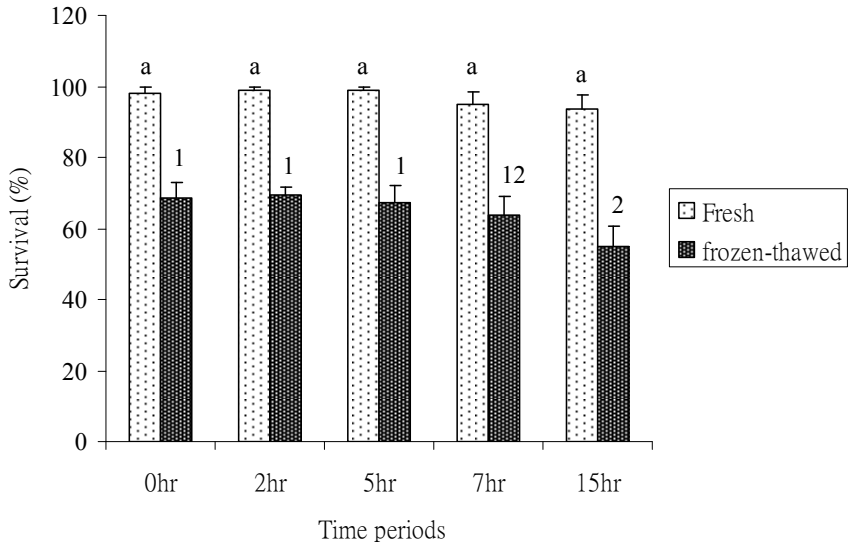


Figure 5.7 The trypan blue staining of fresh and frozen-thawed blastomeres after culture for different time periods. Error bars represent the standard error of the means (SEM). Fresh bars with common letters are not significantly different ($p>0.05$), chilled bars with different numbers are significantly different ($p<0.05$).

Gene expression profiles normalised to the internal reference genes β-actin and EF1α after different periods of blastomere culture are shown in Figure 5.8. The expression of Pax2a increased (30-fold compared to 0 hour of culture) and peaked at hour 2 of culture in fresh blastomeres (Figure 5.8a). Similarly, this peak also appeared in frozen-thawed blastomeres after the same time in culture, but was smaller (2-fold compared to 0 hour of culture).

The expression of Pax2b increased at 5 hours of culture in fresh blastomeres, a decrease in expression was then observed. Frozen-thawed blastomeres appeared to follow this trend, however, it was slightly delayed as the peak did not occur until 7 hours of culture (Figure 5.8b).

Expression of Pax5 in fresh blastomeres decreased significantly during the 15 hours of culture. However, the decrease was not observed in frozen-thawed blastomeres which therefore did not demonstrate the same trend as fresh blastomeres (Figure 5.8c).

Interestingly, the expression of Pax8 revealed a slight increasing trend with time in culture until hour 15 in fresh blastomeres (11-fold, $P>0.05$) but larger increases were detected in frozen-thawed blastomeres (25-fold, $P>0.05$, Figure 5.8d) which contained higher levels than fresh blastomeres at all time points.

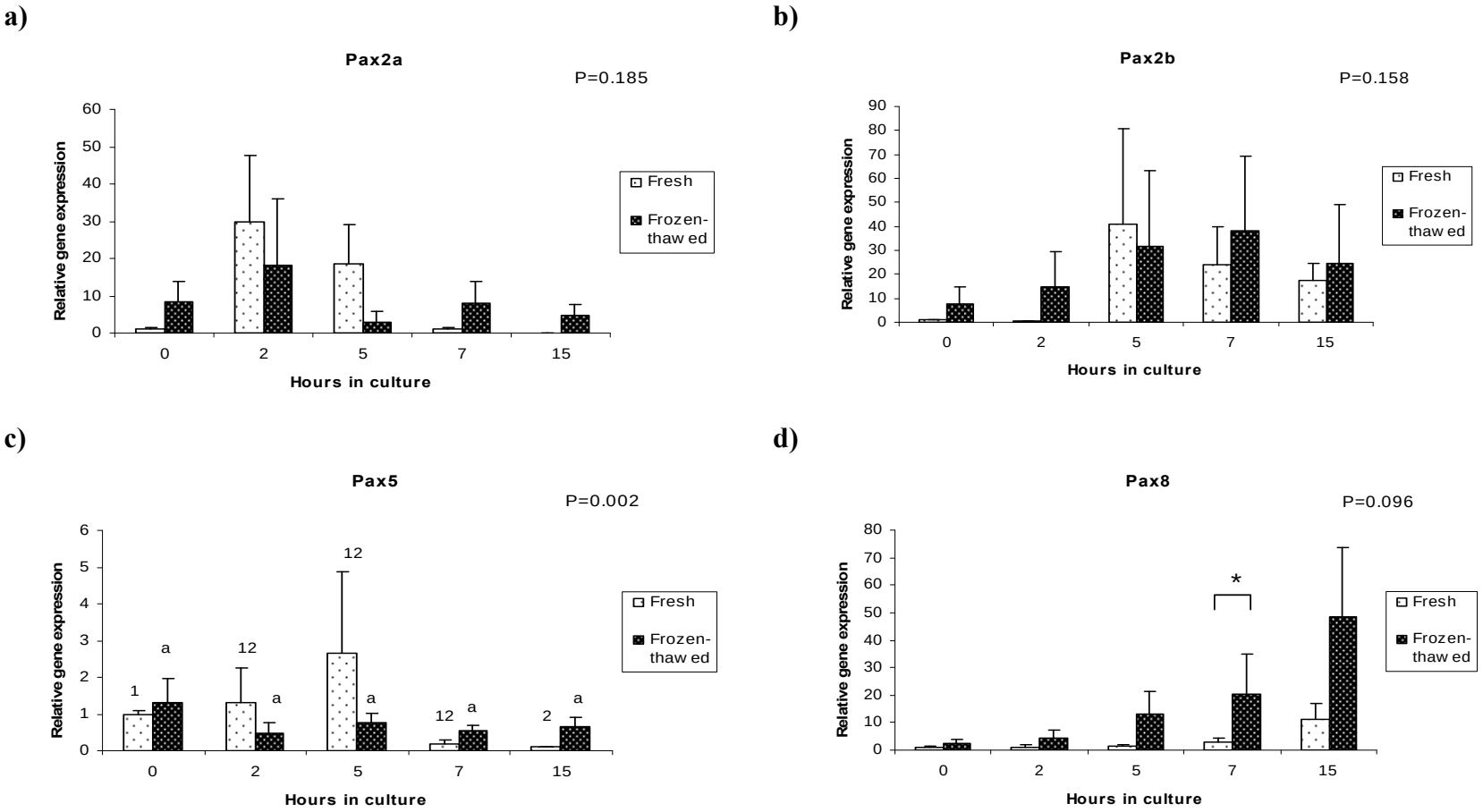


Figure 5.8 Relative expression of Pax2a (a), Pax2b (b), Pax5 (c) and Pax8 (d) in fresh and frozen-thawed blastomeres after different periods of time of culture, assessed by real-time RT-PCR. Error bars represent the standard error of the mean (SEM). Asterisks indicate significant differences between fresh and frozen-thawed blastomeres ($P<0.05$). Fresh bars with different numbers are significantly different ($p<0.05$). Frozen-thawed bars with different letters are significantly different ($p<0.05$). Statistical difference was determined using Welch tests.

5.4 Discussion

5.4.1 Expression levels of Pax genes in zebrafish fresh and chilled embryos of different stages

The fresh embryos were firstly assessed at different developmental stages for the level of Pax gene expression. The study demonstrated that significantly higher levels of Pax2a and Pax8 were present at the 75% epiboly stage compared to 50% epiboly stage. In addition, significantly higher levels of Pax2b and Pax5 were detected at the 20 somite stage compared to the 50% epiboly stage. These results are similar but have some differences to a previous report by Pfeffer et al. (1998), who demonstrated that the Pax2a, Pax2b, Pax5 and Pax8 were first expressed and detected at 100% epiboly, 5 somite, 5 somite and 75% epiboly stage, respectively, using conventional RT-PCR in zebrafish embryos. However, the study was able to detect relatively low levels of each Pax gene as early as 50% epiboly using real time PCR. The more sensitive real time technique used here accounts for our detection of low levels at an earlier stage than previously reported and also allowed us to quantify any effects of the chilling and freezing treatments.

The ability of fish embryos to withstand chilling has been widely used in the past as a useful indicator of quality (Zhang and Rawson 1995; Zhang et al. 2005; Valdez et al 2004; Dinnyes et al 1998). However, there are only a few examples of measuring effects at the level of mRNA expression (Hamamah et al., 1990; Evenson et al., 1994; Tachataki et al., 2003; Rodriguez-Vargas et al., 2002). Chilling is the first part of most cryopreservation protocols and chilling injury is a factor in failure to survive cryopreservation (Watson, 1995). The comparisons between fresh and chilled embryos demonstrated no significant differences statistically. However, the trends in Pax2a, Pax2b and Pax5 expression observed throughout development of fresh embryos were slightly reduced in chilled embryos or slightly delayed. As these embryo samples were saved for analysis shortly after chilling, it is possible that any larger effects of the chilling process had not had time to manifest themselves or that the chilling temperature was not low enough, therefore, only slight differences were observed.

5.4.2 Expression levels of Pax genes in zebrafish fresh and frozen-thawed blastomeres following different periods of culture

Although intact embryos have not yet been cryopreserved successfully, there are several reports of cryopreservation of isolated blastomeres in fish species (Harvey, 1983; Cardona-costa and Garcia-Ximenez, 2007; Strüssmann et al., 1999; Kududa et al., 2002, 2004; Calvi and Maisse 1998, 1999). However, each of these studies used only survival rates as a measure of success. To further investigate those trends in gene expression observed in chilled embryos, the studies have been carried out on isolated blastomeres which are able to withstand the full cryopreservation procedure. Fresh and cryopreserved blastomeres were also maintained in culture for a short period to allow further analysis at time points beyond the initial treatment period. The trends (profile changes) of gene expression of Pax2a and Pax5 were smaller in frozen-thawed blastomeres than in fresh blastomeres whilst Pax8 was greater. These data demonstrated that the cryopreservation procedure may affect the pattern of gene expression. This is in agreement with some previous publications which reported alteration of gene expression in mammalian oocytes and embryos following cryopreservation. Succu et al. (2008) reported that freezing procedures lower the ovine oocyte mRNA levels of developmental genes. Similarly, a decrease in CD9 expression was reported in frozen-thawed mouse oocytes (Wen et al., 2007). In addition, Tachataki et al. (2003) demonstrated the reduced expression pattern of the tuberous sclerosis TSC2 gene across preimplantation development of human embryos after cryopreservation. The gene expression results obtained in these studies are in agreement with the results reported for the mouse β-actin, Hsp 70, HnSOD and CuSOD, CirpB, Rbm3, Trp53 (Boonkusol et al., 2006), Bcl2 and p53 (Dhali et al., 2007) and GLUT1 (Uechi et al., 1997). These studies concluded that cryopreservation does affect the normal pattern of gene expression during embryonic development.

The trends in expression of Pax2b were delayed in frozen-thawed blastomeres compared to fresh blastomeres. Indeed, cryopreservation has been reported to delay other processes such as cell division and blastocyst formation in mouse embryos (Balakier et al., 1991) and proliferation of granulosa cells in mouse prenatal follicles (Choi et al., 2008). As zebrafish Pax2a, Pax2b, Pax5 and Pax8 genes play important

roles in embryonic development and organogenesis (Majumdar et al., 2000; Kwak 2006), even slightly altered expression patterns may cause subtle abnormalities in those tissues specifically regulated by these genes (Pfeffer et al., 1998; Mansouri et al., 1996). For example, insufficient expression of Pax2a, Pax2b, Pax5 and Pax8 has resulted in major defects in the ears, eyes, kidneys, brain or thyroid gland and could affect processes such as hematopoiesis (Torres et al., 1995, 1996; Favor et al., 1996; Rolink et al., 1999; Schaniel et al., 2002). Any disruption of the normal gene expression pattern could therefore result in subtle defects in these tissues during development.

In some cases, any resulting defects might be compensated by other genes. Indeed, the existence of genetic compensation is well accepted (Nowak et al 1997; Winzeler et al 1999; Wagner 2000; Gu et al 2003) and there is some evidence of that occurring from these data. For example, Pax8 is largely increased in cryopreserved blastomeres compared to fresh blastomeres, which might compensate for the reduced or delayed expression of Pax2a and Pax2b in these cells. Pfeffer et al (1998) reported that different members of the Pax family may potentially compensate for the loss of Pax2a function and that Pax2a and Pax2b appear to partially overlap in function with Pax8. Indeed, disruption of both Pax2a and Pax2b function has been demonstrated to be compensated by Pax8 (Hans et al 2004; Kwak 2006). Although compensation mechanisms may restore normal function in those cryopreserved cells with slightly abnormal gene expression, it is also possible that many genes do not overlap in function with others. It is therefore not guaranteed that normal function will be possible. As gene expression is highly regulated, any disruption should be considered important and should be avoided.

Although not one of the direct aims, the results have demonstrated interesting differences in expression patterns of Pax genes between fresh intact embryos and isolated blastomeres. For example, expression of all of the Pax genes gradually increased during development of fresh intact embryos. However, whilst Pax8 expression also increased during the culture of fresh isolated blastomeres, Pax2a, Pax2b and Pax5 expression levels actually decreased between and 5 hours and 15 hours of culture. There are three possibilities to explain these results. Firstly, there is considerable room for improvement in the culture conditions used if cultured

blastomeres are to be used as models for intact embryos. Although successful cultures of blastomeres have been made that produced holoblastic morulae from the reaggregation of blastomeres, the different patterns of gene expression existing between fresh embryos and fresh blastomeres may be due to suboptimal culture conditions in this study. Indeed, culture conditions have previously been demonstrated to alter gene expression in mouse (Khosla et al 2001; Lee et al 2001) and bovine (Massip et al 1995) and even cause incorrect and late embryonic genome activation (Natale et al 2000). Sub-optimal culture conditions could also cause phenotypic alterations as demonstrated in bovine embryos and blastocysts (Farin et al 2006; Lonergan et al 2006). There is another possibility which is related to cell-to-cell communication (Beyer et al., 1990; Sa´ez et al., 1993). Intact embryos and cultured blastomeres have very different physical structures and individual cells are therefore exposed to different external environments in terms of links with other cells. Differences in cell-cell signalling could result in significantly different patterns of gene expression which may explain some of the changes observed here. The third possibility is that although the time points analysed corresponded broadly to the developmental stages analysed in intact embryos, the developmental rates were not the same. This may be due to the disruption of the individual cells during isolation and/or due to differences in cell-cell signaling. The increase in expression of Pax8 detected towards the end of the culture period could be a delayed equivalent to the significant increase in expression at the 75% epiboly stage in intact embryos. More investigations are needed to determine which, if any of these hypotheses actually caused the effects observed.

5.5 Summary

It is evident from this study that different members of the zebrafish Pax gene family are expressed at different stages of development and that their expression patterns can be subtly disrupted following chilling and cryopreservation. Although the changes in gene expression observed were very subtle, it is important to be cautious in making judgements of the safety of cryopreservation techniques in reproductive biology such as blastomere transplantation into blastula embryos in light of these and

other results. It is important that further studies are carried out to investigate these effects on a larger scale and over a larger period of time in order to understand these very subtle effects of cryopreservation that may not be evident immediately post-thaw.

This work has been submitted for publication to Cryobiology.

CHAPTER 6: CONCLUSIONS

6.1 Reiteration of aims

Immediate post-thaw survival is most often used to assess the effect of the freeze-thaw process on cells (Satoshi et al., 2004; Hong et al., 1998; Nilsson and Cloud, 1992; Varadi and Horvath, 1997; Yamaha et al., 1997). However, this parameter provides no information about the more subtle effects of cryopreservation that may not be evident immediately post thaw. This includes changes at the molecular level such as the level of gene expression. The aim of the present investigation was to determine whether cryopreservation has an impact on gene expression in zebrafish blastomeres.

Although cryopreservation of blastomeres has been studied in many fish species (Harvey, 1983; Cardona-Costa and García-Ximénez, 2007; Calvi and Maisse 1998; Kududa et al., 2004, 2002; Strussmann et al., 1999), there has been only one report of controlled slow cooling of zebrafish blastomeres (Kopeika et al., 2005). However, the survival rate was low (25%). The present study assessed the toxicity of different cryoprotectants and the success of various cooling rates which allowed optimisation of the cryopreservation protocol. The optimal freezing protocol was then used in studies investigating the effect of cryopreservation on gene expression.

Quantitative analysis of gene expression using real-time PCR typically requires the use of stably expressed housekeeping genes as an internal control for normalisation. Using housekeeping genes as an internal control allows for the correction of experimental variations caused by pipetting errors, inhibitory compounds, RNA extraction efficiency and reverse transcription efficiency. Many studies either do not use housekeeping genes or use housekeeping genes that are later found to be influenced by the treatment being studied (Lonergan et al., 2000; Filby and Tyler, 2007). Therefore, experiments were carried out to validate housekeeping genes for use in gene expression studies of zebrafish embryos and blastomeres following cryopreservation.

Among many transcription factors, Pax genes have been demonstrated to play important roles in embryonic development and organogenesis (Noll, 1993; Mansouri et al., 1996; Chi and Epstein, 2002; Dahl et al., 1997; Pfeffer et al., 1998). The analysis of changes in gene expression, in response to cryopreservation may explain some of the observed differences in viability. Therefore, this study aimed to determine the effect of freezing on the expression of four Pax genes using real time PCR in order to understand the subtle effects of cryopreservation which may impact on further development of the embryos.

6.2 Review of the main findings

6.2.1 Cryopreservation of zebrafish blastomeres by controlled slow cooling

The objectives of this work were to study the effect of the toxicity of cryoprotectants, cooling and freezing rates and the use of cryoprotective supplements on zebrafish blastomeres suvival, using the controlled slow cooling method. The results showed that DMSO was the most toxic of the cryoprotectants to zebrafish blastomeres and there were no differences in toxicity between METH, PG and EG., in terms of the NOEC. Whilst DMSO was found to be the most toxic of the cryoprotectants to zebrafish blastomeres, 1.5M DMSO treated blastomeres showed the highest survival percentages after freezing compared to those treated with METH, PG and EG.. Studies with controlled slow cooling of zebrafish blastomeres indicated that 0.3°C/min (from seeding temperature to -40°C), 5°C/min (from 20°C to seeding temperature) and 2°C/min (from -40°C to -80°C) were the optimal cooling rates for cryopreservation of 50% epiboly blastomeres. The addition of 0.1M sucrose as a cryoprotective supplement improved the cryo-survivals. The study also compared different methods of viability assessment. There was no statistical difference between two different viability assessment methods: TB and FDA+PI staining.

The subsequent study used this optimal freezing protocol in order to find the most stable housekeeping genes for use in cryopreservation and embryochilling studies.

The protocol was subsequently used to study the effect of cryopreservation at the molecular level of Pax gene expression.

6.2.2 Identification of suitable housekeeping genes for use in gene expression studies of zebrafish embryos and blastomeres following cryopreservation

In this study potential housekeeping genes for quantitative gene expression analysis in zebrafish embryos and blastomeres were investigated. EF1α was the most abundantly expressed potential housekeeping gene and GAPDH was the least abundantly expressed gene. The stability of housekeeping genes in embryos and blastomeres was assessed using GeNorm and NormFinder software. EF1α and β-actin were most stable during the developmental period being studied in embryos across all stages and GAPDH and B2M were least stable genes. Similarly, when the results for blastomeres were considered, β-actin, EF1α and Rpl13a were found to be the most stable genes and B2M and GAPDH still remained the least stable.

The stability rankings of housekeeping genes at individual stages of development in embryos and after individual periods of time in culture in blastomeres were also studied. SDHA, B2M and GAPDH were ranked higher at individual developmental stages than across multiple stages in embryos whilst EF1α, β-actin and RNAP ranked lower.

This study also determined that varying combinations and numbers (2-4) of housekeeping genes were optimal for different individual experiments. In order to make all experiments comparable, genes were selected that were good for all embryo and blastomere work. Whereas, up to four housekeeping genes would be optimal for some experiments (see figures 4.6, 4.8 and 4.9), this number was considered too costly and time-consuming and was unnecessary for some combinations. Therefore, two housekeeping genes that were among the top three for each group of samples were selected for use in the subsequent Pax gene study – EF1α and β-actin.

6.2.3 Effect of chilling and cryopreservation on expression of Pax genes in zebrafish embryos and blastomeres.

The aim of this study was to discover whether chilling and cryopreservation had an impact on Pax2a, Pax2b, Pax5 and Pax8 gene expression in zebrafish embryo and blastomeres. It was considered that cryopreservation might damage cell DNA, and if true it might affect gene expression. Although all the Pax genes were expressed from the 50% epiboly stage, the first significant increase in expression of Pax genes was different from gene to gene in embryos, for example Pax2a and Pax8 expression were significantly increased at 75% epiboly in fresh embryo while Pax2b and Pax5 expression were significantly increased at the 20 somite stage. However, the gene expression of Pax2a and Pax8 were delayed in chilled embryos until 100% epiboly.

The expression patterns of Pax2a, Pax2b, Pax5 and Pax8 were different between fresh and frozen-thawed blastomeres. The trends of gene expression of Pax2a and Pax2b were smaller and delayed respectively in frozen-thawed blastomeres compared to fresh blastomeres. Pax5 expression was significantly decreased over the period of culture in fresh blastomeres, whilst this trend was not seen with cryopreserved blastomeres. Whilst fresh blastomeres maintained relatively constant levels of Pax8 expression throughout the culture period, cryopreserved blastomeres increased expression of Pax8. It was hypothesised that this might compensate for the reduced and delayed expression of Pax2a and Pax2b.

Although not one of the study's direct aims, differences in expression patterns of Pax genes were noted between fresh intact embryos and isolated blastomeres. Expression patterns of Pax2a, Pax2b and Pax5 in blastomeres were discordant from the expression patterns in intact embryos which always remained high and increased with embryonic development.

More investigations are needed to determine whether optimisation of cryopreservation is effective in reducing the adverse effect; whether such abnormal gene expression affects the overall cell functioning and has any consequences for future development and whether similar changes occur at the protein level. Although the changes observed were subtle, it is important to be cautious in making judgements

of the safety of cryopreservation techniques in reproductive biology such as blastomere transplantation into blastula embryos in light of these results.

6.3 Conclusions

There are three key areas where progress has been made in developing a method for investigating the effect of cryopreservation on gene expression: (1) development of an optimal controlled slow cooling method for zebrafish blastomeres; (2) identifying suitable housekeeping genes for use in gene expression studies with zebrafish embryos and blastomeres following cryopreservation; (3) identifying the effect of chilling and cryopreservation on expression of Pax genes in zebrafish embryos and blastomeres.

6.3.1 Cryopreservation of zebrafish blastomeres by controlled slow cooling

Blastomeres are capable of dividing and renewing themselves for long periods as so called embryonic stem cells which are different from muscle cells, blood cells or nerve cells. Blastomeres can also give rise to specialised cells because of their pluripotency. Blastomere cryopreservation therefore, is an important approach to enable reconstitution of an organism. Indeed, there are several reports of germ-line chimeras created using the transplantation of blastomeres into zebrafish (Lin et al. 1992), medaka (Wakamatsu et al., 2001) and rainbow trout (Takeuchi et al., 2001) embryos. Kusuda et al. (2004) transplanted frozen-thaw blastomeres into goldfish embryos and the blastomeres differentiated into primordial germ cells. Although only in one of the 32 chimeric fish examined, this report demonstrated that germ-line cells from cryopreserved blastomeres may develop into mature gametes of chimeric fish if blastomeres are not damaged by cryopresevation. Therefore, development of successful cryopreservation techniques is very important.

The effect of cryoprotectants on zebrafish blastomeres has not been studied before. In this present investigation, four widely used cryoprotectants were investigated for

their toxicity following different exposure times. It was shown that DMSO was the most toxic to zebrafish blastomeres. However, DMSO is also the best cryoprotectant in terms of survival of zebrafish blastomeres. Therefore, it is possible that the cryoprotective effect of DMSO may be greater than its toxicity effect.

The protocol for controlled slow cooling of blastomeres needed to be improved and the present study has progressed the post-thaw survival rate from 25% (Kopeika et al., 2005) to 70%. Although this survival rate is lower than that obtained by using two step (Harvey, 1983) and vitrification (Cardona-Costa and García-Ximénez, 2007) methods, the comparisons between these studies must take into consideration the different embryonic stages and methodology. Kuleshova and Lopata (2002) compared controlled slow cooling with vitrification in human embryos and oocytes. Although the controlled slow cooling of oocytes gave very low survival rate, both methods have resulted in successful cryopreservation and healthy birth. Moreover, Uechi et al. (1999) showed vitrification caused a more harmful effect than controlled slow cooling in terms of morphology and further development in mouse 2-cell and blastocyst embryos. It has also been reported that controlled slow cooling is more efficient than rapid cooling in human embryos (Van den Abbeel et al., 1997). Therefore, further investigations are needed to compare these methods and determine whether biological materials are phenotypically and genetically normal post-thaw before they are used routinely.

The optimal protocol presented here can also be applied to other stages of zebrafish blastomeres and give cryobiologists a template protocol to modify in research of blastomeres and embryonic stem cells in other fish species.

6.3.2 Identification of suitable housekeeping genes for use in gene expression studies of zebrafish embryos and blastomeres following cryopreservation

The optimal controlled slow cooling protocol was used in order to find the most stable housekeeping genes for use in cryopreservation and chilling embryos studies. Although validation of housekeeping genes has now been carried out for many applications, this is the first study of housekeeping genes in chilled and cryopreserved

embryonic cells of the zebrafish.

There are three popular methods (GeNorm, NormFinder and Bestkeeper) to analyse the stability of housekeeping genes. The present study did not use Bestkeeper to analyse data as the data did not meet the standard deviation requirements for this algorithm. Similar results were obtained from Hibbeler et al. (2008) who measured total RNA from whole organs and the results of standard deviation were too high to use the BestKeeper's index. Therefore, this method is only suitable under limited conditions and experimental setups.

Based on detailed quantification, pattern analyses and using the GeNorm and Normfinder applications, the most suitable housekeeping gene combination is β-actin and EF1α. These are suitable as reference genes for both zebrafish embryos and blastomeres across the developmental stages analysed and also on studies involving chilling and/or cryopreservation of those cells. Other genes may be equally or more suitable such as SDHA if individual developmental stages are to be investigated. The least stable genes were GAPDH and B2M in both chilling and cryopreservation experiments, which indicates their inappropriateness as housekeeping genes in zebrafish embryo cryo-work, although GAPDH was found to be a suitable housekeeping gene for bovine embryo work (Goossens et al., 2005).

This study has confirmed that ideal housekeeping genes vary according to the type of cell and treatment used, which strengthens the requirement for validation of housekeeping genes before use with new types of samples. Use of the wrong housekeeping genes for normalization can result in serious errors in the study of gene expression. Therefore, this work will significantly enhance future studies investigating the impact of cryopreservation on gene expression in zebrafish embryos.

6.3.3 Effect of chilling and cryopreservation on expression of Pax genes in zebrafish embryos and blastomeres.

Because of the importance of embryonic development and cryopreservation on growth and deformities, they have been the subjects of gene expression studies using

conventional PCR-based methods (Cao et al., 2003; Huang et al., 1999; Succu et al. 2008; Tachataki et al. 2003; Zilli et al., 2005). As adequate housekeeping genes (β-actin and EF1α) for accurate quantification have been found, the analysis of differences in the level of gene expression after cryopreservation may give a further picture of the consequences of chilling and cryopreservation treatments.

Transcription factor proteins can bind to the DNA and either activate or repress transcription of specific genes (Chau et al., 2002). Therefore, transcription factors are crucial regulators of gene expression in cells. The genes that have been studied, Pax2a, Pax2b, Pax5 and Pax8 are transcription factors and important in embryogenesis. This study demonstrated that different zebrafish Pax genes are regulated differently (Pax2a, Pax2b, Pax5 and Pax8) under chilling and freezing conditions. These results might be due to abnormal expression of other transcription factors (Memili and First 2000), epigenetic effects such as DNA methylation (Cezar et al., 2003) and chromatin remodelling (Kim et al., 2002). However, the mechanisms of these effects are still not clear.

Pax8 may potentially compensate for Pax2a, Pax2b and Pax5 after the cryopreservation insult. The existence of genetic compensation is well accepted (Nowak et al 1997; Wagner 2000; Tong et al 2001). Pfeffer et al (1998) reported the different members of the Pax2a, Pax2b Pax5 and Pax8 may potentially compensate for the loss of Pax2a function. This interaction relies on genes or pathways that can compensate for the loss of one another (Tong et al 2001, 2004). However, the mechanism of gene compensation is still unclear (Wong and Roth, 2005), for example, are compensatory genes well expressed to protect gene loss? How does the cell find loss of a gene and react by up-regulating compensatory genes? Although the results from the present study can not prove the consequences of compensation of Pax8, any genetic abnormality caused by cryopreservation should be considered important and avoided.

The effect of chilling and cryopreservation on expression of transcription factor genes is studied here for the first time. The level of gene expression in zebrafish blastomeres can be disturbed by cryopreservation, but it varies between different species and individuals (Succu et al., 2008; Uechi et al., 1997; Tachataki et al., 2003;

Hamamah et al., 1990; Stachowiak et al., 2007). Although the present study did not investigate whether disrupted gene expression has any long term impact on cell function, progress has been made in providing additional information on the genetic impact of cryopreservation.

Long-term studies should now be carried out to further assess the effect of cryopreservation techniques, not only during the period of embryo development, but even longer including post-embryonic development up to late adulthood. In cancer research, Pax genes have been already found to relate with cancer growth and Pax2 was expressed in a high proportion of primary tumors in breast, ovarian, lung, colon, prostate and lymphoma (Muratovska et al. 2003). Moreover, influence on DNA stability by cryopreservation may relate to the incidence and risk of some cancers (Duthie et al., 2002; Auroux et al., 2004). These studies may tell us whether cryopreservation is really safe.

6.4 Suggestions for the future work

A broad spectrum of questions are raised by the present study, related both to problems of cryopreservation and molecular biology.

6.4.1 Microarrays to study a larger range of genes

The microarray is a recently developed technology which allows scientists to detect genome-wide expression patterns. The advantage of the microarray assay is that a large number of genes can be analysed together at one time. The microarray assay has been applied to a wide range of different fields such as biology, medicine, disease and toxicology research (Fathallah-Shaykh HM, 2005; Timmons et al., 2005; Nuwaysir et al., 1999; Marx, 2000). A study can be carried out to survey a large number of zebrafish genes expressed in blastomeres that might be affected by cryopreservation. Whilst microarray analysis has a reduced ability to achieve quantitative results compared to real time PCR, it allows identification of genes

worthy of further study which can be analysed in more detail using this more sensitive and accurate technique.

6.4.2 Effect of cryopreservation on proteins in zebrafish blastomeres

Most of the changes in gene expression during development are associated with measurable changes in the level of cellular RNAs. The data presented here demonstrate the possibility of changes in mRNA levels of Pax genes. However, the presence of the mRNA does not necessarily indicate the presence of the protein (Rizos et al., 2003). The efficacy of translation is often affected by transcriptional modulation of regulatory genes. Moreover, it has been discovered that small non-protein-coding RNAs (small nucleolar RNAs, microRNAs, short interfering RNAs and small double-stranded RNAs) also regulate gene expression, including translation in developmental processes (Mattick and Makunin, 2005). There are many reports on the effect of cryopreservation on protein (Cao et al., 2003; Huang et al., 1999; Zilli et al., 2005; Succu et al., 2008; Uechi et al., 1997; Dhali et al., 2007). However, there is no information on the effect of cryopreservation on blastomere protein levels or function. As Pax proteins are important in regulation of developmental processes including differentiation, growth, survival and morphogenesis (Chi and Epstein 2002; Dahl et al 1997; Mansouri et al 1996), studies of Pax proteins under the chilling and freezing condition could be carried out.

6.4.3 Explore the effect of changes in protocol on gene expression, in particular comparison of vitrification and controlled slow cooling.

Research comparing the effect of different freezing methods has been carried out and the results are very controversial. Some reports demonstrated no difference between controlled slow freezing and vitrification in mouse (Rall and Wood, 1994, Graves-Herring and Boone, 2007) and bovine (Van Wagtendonk-De Leeuw et al., 1995) while other reports suggested that one method is more beneficial than the other (Uechi et al., 1999; Van den Abbeel et al., 1997; Kuleshova and Lopata, 2002). However, little work has been devoted to functional or genetic analysis of the cells

following freezing. As the optimal controlled slow cooling protocol affects gene expression as found in the present study, it is important to compare this method with the rapid cooling method known as vitrification. Vitrification involves using high concentrations of cryoprotectants to produce extreme viscosity to transform from a liquid stage into a glassy solid without ice formation during freezing (Fahy et al., 1984). Vitrification of zebrafish blastomeres has been studied recently and showed a high survival rate (93.4%) for blastula stage using DMSO as a cryoprotectant (Cardona-Costa and García-Ximénez, 2007). The comparison of gene expression after controlled slow freezing and vitrification may give a more complete picture of the consequences of the different cryopreservation treatments.

6.4.4 Long-term study of the effect of cryopreservation

Changes in gene expression profiles were found in zebrafish blastomeres after cryopreservation. A further study could be carried out on the long-term effects of cryopreservation. Indeed, Dulioust et al. (1995) demonstrated significant differences in body weight, pre-weaning development and learning capacity in mice derived from cryopreserved embryos. Although several studies reported that children born from cryopreserved embryos presented no major pathological features (Olivennes et al., 1996; Wennerholm et al., 1997; Aytoz et al., 1999), Wennerholm et al. (1998) mentioned that minor handicaps, behavioural disturbances, learning difficulties, and dysfunction of attention and perception can not be excluded. Studies on the long term effects can be quite sensitive to detect some genetic related effects, although such effects are more difficult to explain. Moreover, experimentation on human and other mammalian species are more difficult than fish in terms of the relatively small number of available samples. Studies on the effect of cryopreservation on embryonic development have reported larvae deformation following use of cryopreserved sperm in several fish species such as turbot (Suquet et al., 1998), African catfish (Otémé et al., 1996), hirame (Tabata and Mizuta, 1997), tilapias (Rana and McAndrew, 1989) and channel catfish (Tiersch et al., 1994). More detailed examinations and long-term studies are therefore necessary to assess the consequences of cryopreservation in depth.

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APPENDIX A

The chemicals used in the present study

Chemical	Source	Product No.
10x PCR buffer	Bioline	BTK5-1020
Agarose	Bioline	41025
amphotericine B	Sigma	127K4001
BioTaq polymerase kit	Bioline	BTK5-1020
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	154938
EDTA	BDH	16079
Ethylene glycol (EG)	BDH	10324
EZNA Gel extraction kit	Omega Bio-tek	A112907W
Fluorescein diacetate (FDA)	Sigma	F7378
Gentamicin	Sigma	G1272
Glucose	Sigma	G767
HEPES	Sigma	H3375
L-15 medium (Leibovitz)	Sigma	L4386
L-glutamine	Sigma	G5792
Methanol (METH)	Sigma	M3641
$MgCl_2$	Aldrich	208337
NaCl	Aldrich	43,320-9
$NaHCO_3$	BDH	10247
Penicillin	Sigma	095k0625
Phosphate buffered saline PBS	Fluka	79382
Propidium iodide (PI)	Sigma-Aldrich	P4170
Propylene glycol (PG)	BDH	29673
RNAqueous-Micro kit	Ambion	0712016
Sea salt	ZM Ltd	N/A
SensiMix kit	Quantace	QT 505-05
Sensimix Two-Step kit	Ambion	0712016
Sucrose	Sigma-Aldrich	55016
Trehalose	Fluka	90208
Trypan blue (TB)	Sigma	T8154
Ultroser	Life Sciences	292050

APPENDIX B

Standard curve evaluation of seven housekeeping genes in zebrafish embryos and blastomeres

	Embryos R^2	Embryos PCR Efficiency	Blastomeres R^2	Blastomeres PCR Efficiency
B-actin	0.99889	0.9	0.98210	0.91
	0.99823	0.93	0.99898	0.94
	0.99640	1		
B2mic	0.99875	0.82	0.99640	0.84
	0.99686	0.78	0.99540	1.00
	0.99135	0.81		
EF1a	0.99962	0.96	0.99959	0.91
	0.99710	1.0	0.99900	0.92
	0.99872	1.03		
GAPDH	0.99704	0.95	0.97785	1.26
	0.99789	0.84	0.99585	1.00
	0.97172	1.11		
RNAP	0.99591	0.88	0.99523	0.90
	0.99862	0.86	0.99481	0.85
	0.99913	0.87		
Rpl13a	0.99911	0.9	0.97490	0.78
	0.99855	0.99	0.98685	0.85
	0.99889	0.88		
SDHA	0.98641	1.11	0.99548	0.92
	0.99844	0.99	0.99658	1.03
	0.99472	1.08		

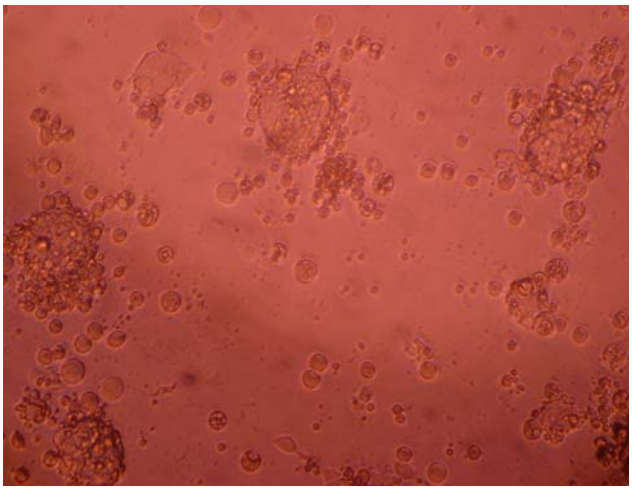
APPENDIX C

Standard curve evaluation of Pax genes in zebrafish embryos and blastomeres

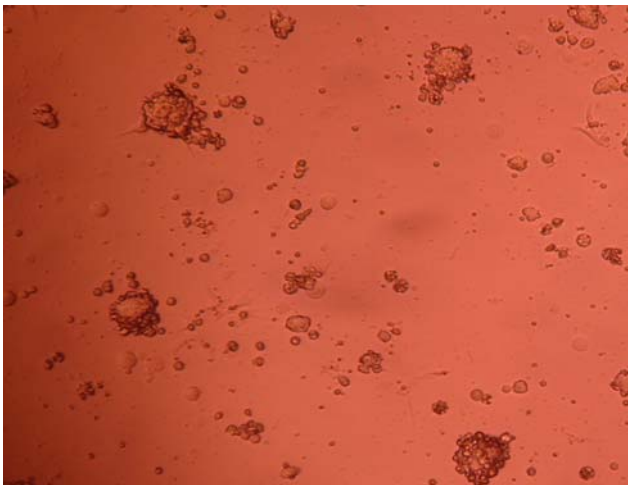
	Embryos R2	Embryos PCR Efficiency	Blastomeres R2	Blastomeres PCR Efficiency
Pax2a	0.97309	0.89	0.98597	0.83
	0.99683	0.91	0.99851	0.89
	0.98894	0.96		
Pax2b	0.99889	0.90	0.99765	0.85
	0.99884	0.84	0.99432	0.80
	0.99916	0.92		
Pax5	0.99304	0.81	0.99136	0.82
	0.99770	0.81	0.98242	0.83
	0.98121	0.94		
Pax8	0.99317	0.9	0.97171	0.84
	0.99846	0.83	0.98799	0.99
	0.99615	0.94		

APPENDIX D

(a)



(b)



Holoblastic morula form from the reaggregation of the fresh (a) and the frozen-thawed (b) blastomeres in L15 culture medium for 24 hr.

APPENDIX E: Publications

Lin C., Zhang T., Rawson D M. Cryopreservation of zebrafish (*Danio rerio*) blastomeres by controlled slow cooling. Cryoletters, in press (2008)

Lin C., Spikings E., Zhang T., Rawson D M. Identification of suitable housekeeping genes for use in gene expression studies of zebrafish embryos and blastomeres following cryopreservation. Theriogenology, in press (2008)

Lin C., Spikings E., Zhang T., Rawson D M. Effect of chilling and cryopreservation on expression of Pax genes in zebrafish (*Danio rerio*) embryo and blastomeres. Cryobiology, in submission (2009)

Lin C., Zhang T., Elgar G., Rawson D M. Initial studies on cryopreservation and gene expression in isolated blastomeres of zebrafish (*Danio rerio*). Cryobiology 55: 365. (2007). Abstract

Lin C., Zhang T., Elgar G., Rawson D M. cryopreservation and gene expression in zebrafish blastomere. Cryoletters 29(1): 85. (2008) Abstract