



Novel molecular markers for assessment of human embryo competence

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List of abbreviations

¹H NMR	Proton Nuclear Magnetic Resonance
aCGH	Comparative Genomic Hybridisation Array
ACN	Acetonitrile
ADO	Allele Drop Out
AmBic	Ammonium Bicarbonate
ATP	Adenosine Tri-Phosphate
BAC	Bacterial Artificial Chromosome
cDNA	cloned DeoxyriboNucleic Acid
CGH	Comparative Genomic Hybridisation
COH	Controlled Ovarian Hyperstimulation
Ct	Threshold Cycle Value
CVM	Cryologic Vitrification Method
Cy3	Cyanine 3
Cy5	Cyanine 5
dCTP	DeoxyCytidine TriPhosphate
DMSO	DiMethylSulphOxide
DNA	DeoxyriboNucleic Acid
dsDNA	Double Strand DeoxyriboNucleic Acid
dT	DeoxyThymidine
DTT	DiThioThreitol
E2	Oestradiol
EGA	Embryo Genome Activation
ELISA	Enzyme-Linked Immunoabsorbant Assay
Epi	Epiblast
eSET	Elective Single Embryo Transfer
ESHRE	European Society for Human Reproduction and Embryology
FA	Formic Acid
FC	Fold Change
FDR	False Discovery Rate
FISH	Fluorescent <i>In Situ</i> Hybridisation
FSH	Follicle Stimulating Hormone
GO	Gene Ontology
HCG	Human Corionic Gonadotropin
hESC	Human Embryonic Stem Cells
HFEA	Human Fertilisation and Embryology Authority

HLA-G	Human Leukocyte Antigen G
HPLC	High Performance Liquid Chromatography
HT	Heterozygosis
IAA	IodoAcetAmide
ICM	Inner Cell Mass
IPA	Ingenuity Pathway Analysis (Qiagen, Germany)
IRS	Interspersed Repetitive Sequences
LAMP	Loop-mediated isothermal Amplification
LH	Luteinising Hormone
LN2	Liquid Nitrogen
M	Molarity
mRNA	Messenger RiboNucleic Acid
mtDNA	Mitochondrial DNA
NGS	Next Generation Sequencing
NIRS	Near-InfraRed Spectroscopy
NMR	Nuclear Magnetic Resonance
NPB	Nucleolar Precursor Body
O2	Molecular Oxygen
OD	Optical density
OFU	Oxford Fertility Unit
PBS	Phosphate-Buffered Solution
PGD	Preimplantation Genetic Diagnosis
PGS	Preimplantation Genetic Screening
PN	ProNucleus
PrE	Primitive Endoderm
PVA	Polyvinyl alcohol
PVP	Polyvinylpyrrolidone
QC	Quality Control
qPCR	Quantitative PCR, Real-Time PCR
RCT	Randomised Controlled Trial
RI	Research Instruments Ltd
RM	Reference Mastermix
RPKM	Reads Per Kilobase per Million mapped reads
rRNA	Ribosomal RiboNucleic Acid
RT	Room Temperature
SECM	Electro Chemical Microscopy
SSC	Saline Sodium Citrate buffer
ssDNA	Single Strand DeoxyriboNucleic Acid

STR	Short Tandem Repeats
TBE	Tris/Borate/EDTA buffer solution
TE	Trophectoderm
TFE	TetraFluoroEthanol
TLS	Time-Lapse System
TM	Test Mastermix
tRNA	Transfer RiboNucleic Acid
UTP	Uridine TriPhosphate
UV	Ultra-Violet
v/v	volume on volume
WGA	Whole Genome Amplification
WTCHG	Wellcome Trust Centre for Human Genetics

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Abstract

Title Novel molecular markers for assessment of human embryo competence
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In vitro fertilization treatments are responsible for 1-5% births in industrialized countries. The safest way to generate a pregnancy is to transfer a single embryo to the mother, reducing the likelihood of multiple gestations. Hence, in order to maximize the chance of success, it is extremely important that the embryo prioritised for transfer is the most capable within the cohort of embryos generated by the patient. Along with cytogenetic components, it has been suggested that embryo protein expression patterns may correlate with its ability to implant. However, embryo proteomics strategies have not been easy to harness mainly due to the complexity of the media the embryos are cultured in, and the low concentration of the proteins that are secreted.

In this study, the use of the blastocentesis procedure, which allows the safe retrieval of embryo inner fluid (blastosol), was described. The use of the blasocoel fluid as a source of embryonic DNA for preimplantation genetic assessment was also investigated. From this highly purified embryonic sample, a comprehensive catalogue of proteins present in the human blastosol was generated using standard and custom-made mass spectrometry strategies. The embryonic origin of these proteins was validated by gene expression microarray and RNASeq analysis. These experiments also allowed the identification of differentially expressed genes in the first two cell lineages, the Inner Cell Mass and the Trophectoderm. Finally, a targeted proteomics strategy able to measure part of the previously described protein targets in single blastosol samples was employed. The correlation between the presence and abundance of proteins of interest in single blastosols and several biological characteristics of the embryo, including its chromosomal status, was assessed.

These data are of major interest for the understanding of human embryo development. The validated embryo-derived protein catalogue and blastocyst gene expression profiles generated in this study, provides access to a thorough document for consultation in human embryology proteomics-based experiment design, paving the way to next-generation proteomic-based embryo assessment.

Preface

Author contribution

I confirm that the studies described in this thesis are my original work. I have contributed to experiment design, sample collection, data acquisition and analysis in all sections discussed in this study. I have written the entirety of this thesis and performed all procedures described in it, unless stated otherwise.

Ms Jenny Spencer from the Oxford Fertility Unit and I performed Inner Cell Mass excision procedures described in chapter 4. Dr Souraya Jaroudi from Reprogenetics UK and I processed mRNA samples for microarray gene expression analysis also described in this chapter. The Wellcome Trust Centre for Human Genetics (WTCHG) performed microarray hybridization and RNASeq procedures. Dr Helen Lockstone from WTCHG performed statistical analysis also described in this chapter.

Dr Alessandro Ori from the European Molecular Biology Laboratory (EMBL) in Heidelberg and I designed experiments, acquired and analysed data described in chapter 5. Dr Alessandro Ori performed mass spectroscopy experiments also described in this chapter.

Ms Katharina Späth and I processed the samples and analysed data for array comparative genome hybridization experiments described in chapter 6. Dr Alessandro Ori, Dr Bernd Klaus from EMBL Heidelberg and I performed the statistical analysis also described in this chapter.

Prof Dagan Wells and Prof Tim Child reviewed the thesis, provided critical advice and oversaw the project.

Ethical approval

This study was integrated in an existing research study approved by the NRES Committee South Central – Oxford (Ref. 04/Q1606/44) and licensed by the Human Fertility and Embryology Authority (R0111). Patients were enrolled in this study following HFEA guidelines on surplus embryos donation to research.

1. INTRODUCTION

1.1 The in vitro fertilisation laboratory

1.2 Morphology-based embryo assessment

1.2.1 Conventional light microscopy

1.2.2 Time-lapse morphokinetics

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1.3.1 Fluorescent in situ hybridization

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1.7 Study aims

1.8 Bibliography

1.1 THE *IN VITRO* FERTILISATION LABORATORY

Since the first clinical applications of human embryology in the late '70s and early '80s, many aspects of the IVF laboratory have changed.

Firstly, in the decades following the birth of Louise Brown, the number of patients requesting IVF treatment has rapidly increased. Thousands of patients have since been referred to clinics, transforming the almost experimental practice of assisted reproduction into an affirmed healthcare service, properly recognised as a medical specialty by government authorities. National and international committees were then put in place to regulate and oversee the provision of reproductive treatments and guarantee safe, high quality service to patients. More recently, operational standardisation, protocols for quality control and monitoring of key performance indicators have been implemented and are now an essential aspect of assisted reproduction techniques.

Basic equipment such as biological safety cabinets, incubators and heated stages, has been improved to allow greater control of parameters and more stable culture conditions.

New equipment was also introduced to assist IVF professionals in executing day-to-day operations in the laboratory. Novel tools included electronic witnessing stations, incubators temperature and gas composition monitoring of incubators and electronic patient databases.

One of the most significant advances in clinical embryology in the last decades has been the introduction of sequential culture systems. Better understanding of embryo metabolic requirements (i.e. pyruvate and glucose uptake) allowed the development of culture media able to sustain prolonged in vitro culture of embryos (Gardner & Lane, 1997).

Additionally, studies on uterine physiology demonstrated that uterine transfer of blastocysts provided better synchronisation with the natural implantation process. In fact, in natural conception the human oocyte is fertilised in the Fallopian tube and it starts dividing while moving away from fimbriae, eventually reaching the uterine cavity around five days post fertilisation, at the blastocyst stage. Culture of human blastocysts for clinical purposes was first proposed at the end of 1990's (Gardner & Lane, 1997). Although it was employed only by a few clinics worldwide initially, it became widely adopted around mid 2000's. Meanwhile, modern incubators (i.e. bench tops) able to provide the required culture conditions (i.e. low O₂ concentration) were developed and new cryopreservation strategies tailored to produce high survival rates in blastocysts established. These technical achievements allowed an even larger number of IVF laboratories to adopt extended culture systems, which has now become a common procedure in IVF.

One of the main benefits introduced by blastocyst culture is the possibility to monitor embryo development for a longer period of time. Especially, it is possible to assess whether embryo genome activation (EGA) is deployed successfully and the embryo progresses to subsequent developmental stages. The first cellular divisions are controlled by proteins and mRNA inherited from the oocyte's cytoplasm. Around

Day-2/Day-3 post fertilisation, when the embryo has between 4 and 8 cells, the embryonic genome is activated through epigenetic modifications of its chromatin (Braude *et al*, 1988).

The shifting of molecular competencies from zygote to embryonic control, is a requirement for the embryo to progress to the next developmental stage.

The vast majority of normally fertilised zygotes (~95%) will divide up to the 4/8 cells stage. However, almost half of these embryos will fail to produce a blastocyst by Day-5 post-fertilisation.

Performing embryo transfer prior to EGA completion inevitably involves the transfer of some embryos with no implantation or developmental potential.

To compensate this eventuality and maintain acceptable outcomes, clinics used to transfer multiple cleavage-stage embryos. However, this approach showed major shortcomings by producing a high number of multiple pregnancies.

Multiple pregnancies are associated with several medical risks for both mother and babies. Mothers are more likely to miscarry, develop pregnancy-induced hypertension and pre-eclampsia, develop gestational diabetes and frequently require intervention during labour. Babies born from multiple pregnancies are at increased risk of premature birth, which is associated with low birth weight and often entails neonatal intensive care. Additionally, babies born from multiple pregnancies are at higher risk of developing complications that can have mid to long-term negative effects on the quality of life (i.e. respiratory distress, cerebral palsy and delay in language acquisition) (www.oneatatime.org.uk).

For these reasons, IVF clinics are under growing pressure from governing bodies to reduce the percentage of multiple pregnancies produced as a result of IVF treatment.

For example, in 2008 the UK's Human Fertilisation and Embryology Authority (HFEA) implemented a policy aimed at reducing the IVF multiple birth rate by promoting the use of elective single embryo transfer (eSET). This was as a result of the Scandinavian experience where implementation of a similar policy has resulted in a reduction of the multiple pregnancy rate following IVF to 5% (Thurin *et al*, 2004). Since 2009, every year the HFEA set a maximum multiple birth rate that is gradually reduced. IVF units were left in charge of planning their own internal multiple birth minimisation strategy aimed to reduce twin pregnancies to under 24% for 2009, under 20% for 2010 and under 15% for 2011 (Short, 2007).

To maintain acceptable pregnancy rates, many clinics implemented blastocyst culture to improve the implantation potential of the cohort of embryos they were selecting from (Papanikolaou *et al*, 2005, 2008).

Prolonging *in vitro* culture to the blastocyst stage allows monitoring of the EGA process completion and also formation of blastocysts.

Thus, embryos undergo an *in vitro* process of "self-selection", leaving available for transfer only embryos that develop after EGA, which have a high chance to implant.

Although blastocyst culture reduces the cohort of embryos to select from, it is common that more than one blastocyst would develop in a standard IVF treatment cycle. Therefore, selection of the blastocyst for transfer is usually based on embryo morphological assessment. However, the ability of morphology to predict embryo

implantation and healthy pregnancy has been challenged over the years (Kovalevsky & Patrizio, 2005) and alternative methodologies have been investigated and developed.

1.2 MORPHOLOGY-BASED EMBRYO ASSESSMENT

Embryo morphology is the most common assessment procedure used to select which embryo(s) should be transferred to the patient, cryopreserved or discarded.

Several grading systems have been proposed for the evaluation of embryos, starting from Day-1 of development up to blastocyst stage.

1.2.1 Conventional light microscopy

Zygote stage

Zygote assessment is based on the number and appearance of pronuclei (PN) and nucleolar precursor bodies (NPB). Although its predictive value is still controversial, a few studies showed that presence of PNs of similar sizes, with equally numbered, uniformly sized and centrally aligned NPBs is related to higher blastocyst formation (Balaban *et al*, 2001; Rienzi *et al*, 2002; Zollner *et al*, 2002; Nagy *et al*, 2003) and increased implantation potential (Wittemer *et al*, 2000; Montag & van der Ven, 2001). However, other studies failed to show the same relationship between zygote morphological features and clinical outcome (Skiadas & Racowsky, 2007).

Cleavage stage

Several cleavage stage assessment methodologies have been proposed. Parameters considered include number of cells, amount of fragmentation, symmetry of the blastomeres and presence of multinucleation.

All methods evaluate the number of cells within the embryo at a given time. Ideally, the embryo should possess 4 cell on Day-2 and 8 cells on Day-3, and deviations from these numbers are correlated to suboptimal development (Alikani *et al*, 2000; Giorgetti *et al*, 1995) and reduced implantation rates (Carrillo *et al*, 1998; Racowsky *et al*). Embryo fragmentation is assessed based on the percentage of intra-zonal volume occupied by fragments. This parameter was demonstrated to negatively correlate with developmental and implantation rates (Ziebe *et al*, 1997, 2007; Alikani *et al*, 1999; Racowsky *et al*). The presence of fragments was also linked to deviant cellular division possibly associated with metabolic abnormalities, apoptotic processes or aberrant chromosomal segregation (Mantikou *et al*, 2012).

Blastocyst stage

Morphology assessment schemes were also developed for blastocyst evaluation. Currently, the most used method is the one proposed by Gardner and Schoolcraft (Gardner & Schoolcraft, 1998). Evaluation criteria include the developmental stage of the embryo, assessed according to the degree of expansion of the blastocoel cavity, and the number and organization of the cells forming the Inner Cell Mass (ICM) and the Trophectoderm (TE) (Machtinger & Racowsky, 2012).

Although a correlation between blastocyst morphology and implantation rate has been shown, it is still under discussion which of the parameters analysed (expansion, ICM or TE) are the most influential on the final outcome (Thompson *et al*, 2013; Van den Abbeel *et al*, 2013).

Despite the fact that blastocyst culture results in embryos with higher implantation potential compared to cleavage stage ones, some authors have raised concerns that prolonged *in vitro* culture may disrupt genetic imprinting patterns (Market-Velker *et al*, 2010).

1.2.1.1 Considerations on conventional morphological evaluation

One important issue in evaluating the relationship of embryo morphology with clinical outcome is the intra and inter-operator variability that can affect data reproducibility. Although cell number in cleavage stage embryos is successfully identified by a majority of operators, fragmentation rate and other more qualitative parameters are not easily replicated across operators (Paternot *et al*, 2009, 2011b; Baxter Bendus *et al*, 2006). In an attempt to standardise blastocyst morphological assessment, semi-automated approaches have been proposed and tested (Santos Filho *et al*, 2012; Paternot *et al*, 2013, 2011a), however, their validity still has to be assessed in large scale studies.

Embryo morphological assessment offers the simplest and cheapest way to select embryos to prioritise for transfer and probably for this reason it is the most common method used by almost every IVF laboratory. Although blastocysts of better morphology show higher implantation rates compared to poor morphology ones

(55.1% vs 40.4% implantation rate respectively, Oxford Fertility Unit, 2013 unpublished data), this type of assessment is highly subjective and not particularly sensitive (Machtinger & Racowsky, 2012). For example, poor morphology blastocysts maintain a high implantation potential, superior to a top quality cleavage stage embryo (40.4% vs 28.3% respectively, Oxford Fertility Unit, 2013 unpublished data). Morphological assessment can also have significant consequences for embryos in cycles involving cryopreservation, since embryos considered to be of suboptimal morphology may be discarded rather than stored, due to their presumed low survival and implantation rates. However, it can be inferred from fresh treatment outcomes that, if properly cryopreserved, around 30% of poor morphology blastocysts would produce a healthy pregnancy if transferred (Gardner *et al*, 2000).

1.2.2 Time-lapse morphokinetics

Embryo morphological grading can involve single or multiple assessment sessions (i.e. Early cleavage/late Day-1, Day-2, Day-3, Day-5). Some authors reported a beneficial effect of multiple evaluations on the selection of embryos capable of implantation (Kaser & Racowsky, 2014; Nagy *et al*, 2003; Qian *et al*, 2008). However, this strategy involves the removal of embryos from culture and prolonged exposure to suboptimal conditions, including lower temperature and humidity, higher pH and light, which increase the risk of embryo development impairment.

Recently, incubators able to take pictures of the embryo without the need to remove the specimen from culture have been commercialised for clinical use (e.g. EmbryoScope, Eeva). These incubators, defined as Time-Lapse Systems (TLS), allow

monitoring of embryo development by capturing sequential pictures (every 5–20 minutes), thus revealing the dynamic changes in embryo morphology. TLS enable time measurement of morphological variation associated with cell cycle progression, including pronuclei formation, length of first cell division, time intervals between subsequent cellular cleavage, duration of compaction stage and cavitation. Through statistical and multi-variate analysis, these parameters are used to produce morphokinetic profiles of embryo development. TLS also reveals biological processes not identifiable from multiple static assessments (i.e. asynchronous appearance and disappearance of pronuclei, direct cleavage from zygote to three cells, short-time appearance of multi-nucleation).

A systematic review of three randomised clinical trials comparing clinical pregnancy, live birth and miscarriage in treatments using TLS or standard culture concluded that there is no sufficient evidence to suggest TLS can improve any of the outcomes investigated (Armstrong *et al*, 2015).

Two independent groups showed that morphology cannot be relied upon for the selection of euploid embryos due to a weak relationship between the parameters (Alfarawati *et al*, 2011; Capalbo *et al*, 2014). However, one group proposed that the use of morphokinetics could help to discriminate genetically normal from abnormal embryos (Campbell *et al*. 2013) based on the time between insemination and blastulation and insemination and full blastocyst expansion (Campbell *et al*. 2013).

1.2.2.1 Considerations on morphokinetic embryo assessment

TLS provides an excellent incubation system that avoids the exposure of embryos to suboptimal culture conditions. These systems drastically reduce the impact of the assessment procedure, allowing remote embryo monitoring.

However, studies performed so far failed to show a beneficial use of TLS in increasing pregnancy and live birth rates. These results do not change the fact that TLS helps embryologists' work by reducing manipulation and laboratory logistics. However, all TLS systems are very expensive and most laboratories cannot afford their full implementation. Also, current TLS have a limited capacity of six patients simultaneously, and 72 embryos in total, often requiring purchase of multiple devices or patient allocation to diverse incubation systems in the same laboratory. In many clinics, TLS incubation is associated with increased treatment costs paid by the patient. It can be argued that an increased cost is not justifiable until there is sufficient evidence that TLS produces better clinical outcomes.

1.3 GENETIC EMBRYO ASSESSMENT

Preimplantation genetic screening (PGS) involves the assessment of the whole chromosomal complement of one or more cells collected from the embryo. By analysing the cytogenetic status of one or a few cells, the karyotype of the whole embryo is inferred.

1.3.1 Fluorescent In Situ Hybridisation

Initially, this approach required cytogenetic techniques including cell spreading and fluorescent in situ hybridisation (FISH). One or two blastomeres were biopsied from Day-3 cleavage stage embryos and then immobilised on glass slides. Labeled DNA probes complementary to chromosome-specific sequences were hybridised and then their signal detected using fluorescence microscopy. To avoid fluorescent signal interference, the maximum number of probes that could be used per sample was limited to five (13, 18, 21, X and Y).

The main limitation of this methodology could be attributed to the lack of a comprehensive analysis of all chromosomes, which inevitably led to some embryos, aneuploid for untested chromosomes, being considered normal and therefore transferrable. Additional limitations involved reduced diagnostic rates when a single blastomere was analysed. Collecting two blastomeres from the embryo could reduce diagnosis failure, however, it was demonstrated that the removal of multiple cells

could give rise to negative consequences on embryo viability, defeating the purpose of the genetic analysis (Cohen *et al*, 2007).

1.3.2 Comprehensive genetic screening

The field of PGS was revolutionised when a new methodology was proposed for the simultaneous analysis of the whole embryonic genetic complement. Metaphase Comparative Genomic Hybridisation (CGH) consists of a competitive hybridisation of two DNA samples: one derived from one or more cells biopsied from the embryo, the second derived from a normal reference (e.g. lymphocytes of euploid karyotype). After whole genome amplification and differential fluorescent labeling, samples are co-hybridised to normal metaphase chromosomes, spread and fixed onto a glass slide. Analysis at the fluorescence microscope determines the ratio at which labeled probes hybridized to each chromosome, revealing the embryo's cytogenetic composition.

Although providing information on the whole chromosomal complement, the use of metaphase CGH analysis in a clinical context was problematic due to the length of time the method requires (Wells *et al*, 2002). Metaphase CGH in fact requires 3–4 days and analysis of data is labour intensive, precluding the analysis of large numbers of samples (Harper, 2009).

PGS expanded its clinical application when microarrays for CGH analysis were produced. The ability to construct an array, composed of bacterial artificial chromosomes (BAC) covering the whole genome, combined with analysis using high definition scanning devices and bioinformatic analysis, resulted in a comprehensive

aneuploidy screening protocol that was relatively easy to use, robust and rapid (requiring less than 24 hours).

The use of microarrays allows the identification of chromosomal aberrations such as monosomy, trisomy, Robertsonian translocations, as well as subchromosomal deletions/duplications (>5Mb).

Other approaches to comprehensive genetic screening have also been developed and clinically applied, including quantitative PCR (qPCR) and Next Generation Sequencing (NGS).

Quantitative PCR involves multiple amplification reactions targeting specific DNA sequences on each chromosome. The number of regions amplified per chromosome varies depending on the specific protocol, but 96 (four on each chromosome) is the most commonly used (Treff *et al*, 2012). Products of these reactions incorporate fluorescent dyes that allow monitoring of the amount of product generated. Based on the fluorescence detection curve, the amount of starting material derived from each chromosome, and hence the embryo's karyotype, can be inferred.

Alternatively, NGS is based on the replication of DNA fragments originating from the embryo and initially amplified using whole genome amplification. This novel methodology involves sequencing by synthesis, where the incorporation of labeled nucleotides is tracked as the DNA molecule is copied. Thousands of reactions occur in parallel, allowing the sequencing of a portion of the embryo genome and creating a catalogue of sequences analysed. Using bioinformatics tools, these sequences are aligned with a reference genome (i.e. their chromosome of origin is determined). The number of sequenced DNA fragments derived from each chromosome provides an

indication of chromosomal copy number. The use of NGS in assisted reproduction is also covered in Chapter 6.

In some cases, array-CGH has proven to be highly robust (2.9% no results) and specific (1.9% error rate) when applied to rapid (24-hour) analysis of single cells biopsied from cleavage-stage embryos (Gutiérrez-Mateo *et al*, 2011).

However, according to the ESHRE PGD Consortium, different laboratories show major variation in outcomes, suggesting that the efficiency of preimplantation genetic screening is strictly related to the experience of the embryology and genetic laboratories performing it. Studies have demonstrated that subsequent re-analysis of the embryos with a missing diagnosis can reduce the risk of inconclusive results from 7.5% to 3.1% (Colls *et al*, 2007). However, re-analysis would require an additional biopsy of the embryo.

Additionally, it has been argued that in case of mosaicism in the embryo, the cell analysed may not be representative of the rest of the embryo. Surprisingly, mosaicism in cleavage stage embryos can be as high as 25–30% and can therefore be a source of misdiagnosis in PGS cycles (Delhanty *et al*, 1997).

1.3.3 Diagnostic benefits of blastocyst culture and biopsy

In the mid 2000's, prolonged embryo culture was also extensively introduced into clinical practice and blastocyst biopsy was soon proposed as an alternative to blastomere biopsy (de Boer *et al*, 2004). This approach had some clear advantages: firstly the blastocyst is composed of more cells compared to a cleavage stage embryo and therefore is more resilient to the biopsy procedure. As the blastocyst already

shows cellular differentiation, there is no risk of removing those cells destined to produce the ICM. Also, because more cells are harvested, more DNA is available for testing, reducing the chance of failed diagnosis. Lastly, as more cells are analysed, theoretically misdiagnosis due to mosaicism should be reduced, as the test sample is believed to be more representative of the embryo's genetic status (Scott *et al*, 2013). Commercialisation of medical grade lasers to be used in micromanipulation systems also made blastocyst biopsy more practical and accessible.

1.3.4 Considerations on preimplantation genetic screening

Some studies showed improved outcomes in treatments where PGS was employed, especially in selected groups such as patients with advanced maternal age (Gianaroli *et al*, 2005) and recurrent miscarriage (Verlinsky *et al*, 2005; Munné *et al*, 2005). PGS was also shown to prevent trisomic conception by Munné and colleagues (Munné *et al*, 2006) and reduce the incidence of aneuploid miscarriages (Hodes-Wertz *et al*, 2012). However, other groups were not able to demonstrate any significant difference in implantation and pregnancy rates between untreated and PGS patients (Staessen *et al*, 2004; Platteau *et al*, 2005) or even showed a negative effect (Mastenbroek *et al*, 2007, 2011).

Since these studies were performed, genetic testing methodology has remarkably improved and now allows screening of all chromosomes with possibly smaller negative effects on the embryo viability due to the move away from cleavage stage and towards blastocyst biopsy.

Additionally, in a recent study it was demonstrated how transfer of a single euploid blastocyst delivers the same pregnancy rate as two unscreened blastocysts, whilst significantly reducing the incidence of multiple pregnancy to less than 2% (Forman *et al*, 2013). However, the lack of a randomised controlled trial (RCT) for comprehensive PGS, where inclusion criteria and protocols are set prospectively, prevents a general consensus regarding whether PGS offers increased success rates compared to unscreened treatment cycles.

However, with the extensive application of NGS technology, prices are expected to fall making PGS more accessible to patients requiring this analysis. Additionally, novel application of NGS to embryo assessment could include the simultaneous analysis of both embryo's karyotype and presence of target genes potentially involved in the implantation process, allowing an even deeper and more comprehensive assessment of embryo's viability from a genetic perspective.

1.4 GENE EXPRESSION ANALYSIS OF CUMULUS CELLS

In the ovarian cortex, granulosa cells surround the oocyte and form the inner layer of the developing follicle. Initially, granulosa cells are tightly attached to the dormant germinal vesicle. As follicle maturation progresses, they detach from the oocyte due to the formation of the follicular antrum. The antrum is a fluid filled cavity within the follicle. Some cells maintain direct contact with the oocyte during follicle maturation and are called cumulus oophorus cells. These cells are known to coordinate oocyte development and follicle maturation by regulating mRNA transcription, providing metabolic and protein synthesis substrates and exchanging molecules between the oocyte and its environment (Eppig, 2005; Gilchrist *et al*, 2008; Sutton *et al*, 2003). At the time of LH surge, cumulus cells surround the ovulated oocyte creating a protective coating.

Because of their direct connection to the oocyte, many scientists have hypothesised that the gene expression of cumulus cells may be an indicator of oocyte and embryo competence.

Typically, hundreds of cumulus cells surround the oocyte at the time of egg collection and are removed and discarded prior to ICSI or after fertilisation checks in the case of conventionally inseminated oocytes. This makes them an ideal sample for non-invasive genomic analysis.

Several groups have investigated gene expression in cumulus cells using both microarrays (Assou *et al*, 2008; van Montfoort *et al*, 2008) and quantitative PCR

(qPCR) (Hamel *et al*, 2010; Wathlet *et al*, 2011). Both methods require removal of cumulus cells from the oocyte, their collection and lysis and conversion of extracted RNA into cDNA. Microarrays allow a global analysis of tens of thousands of genes, giving a comprehensive view of the genes that are differentially expressed in one sample compared to a control (i.e. mature vs. immature oocytes, oocytes developing into implanting vs. non-implanting embryos), but they are relatively expensive and have a relatively high rate of false discovery (i.e. genes that appear to display differential expression, but are not confirmed in subsequent study). On the other hand, qPCR allows very precise quantification of transcripts at relatively low cost, but analysis must be targeted, focusing on a relatively small number of genes of interest. Given the relative merits of the two methods, it is common for researchers to perform an initial screening using a microarray and subsequently undertake confirmatory analysis of some of the candidate genes identified using qPCR. Using fluorochrome-labelled dNTPs activated by DNA polymerisation, the quantification of the original transcript using qPCR is based on the time required by the PCR reaction to generate detectable levels of fluorescence (threshold cycle value, Ct). Reference genes tested in parallel reactions provide a benchmark, enabling direct comparison of amplification curves. Relative gene expression levels can be used to identify potentially modulated (under/over-expressed) target genes.

Several groups reported the correlation of increased transcripts levels of specific genes with oocyte competence (Cillo *et al*, 2007; Feuerstein *et al*, 2007), oocyte aneuploidy (Fragouli & Wells, 2012), embryo development (McKenzie *et al*,

2004), embryo quality (Anderson *et al*, 2009) and pregnancy (Hamel *et al*, 2008, 2010; Gebhardt *et al*, 2011; Wathlet *et al*, 2012).

A number of genes have been found to be associated with better and poorer IVF outcomes. McKenzie and colleagues (McKenzie *et al*, 2004) showed that GREM1 is highly expressed by cumulus cells surrounding oocytes that developed into good morphology embryos. Wathlet and colleagues (Wathlet *et al*, 2011) showed a positive association between GREM1 and pregnancy only in patients treated with antagonist but not with agonist protocols. However, subsequent studies by other groups did not confirm these associations between GREM1 and positive outcome (Anderson *et al*, 2009; Gebhardt *et al*, 2011).

Using expression measurements of several transcripts in various ovarian stimulation conditions, Wathlet and colleagues produced statistical models to predict the achievement of pregnancy with a sensitivity of 70% and a specificity of 90% (Wathlet *et al*, 2011, 2012). Additionally, Fragouli and colleagues also investigated the relationship between cumulus cells gene expression and oocyte aneuploidy, identifying a subset of transcripts associated to abnormal karyotype (Fragouli *et al*, 2012).

1.4.1 Considerations on cumulus gene expression-based embryo assessment

Cumulus cells gene expression analysis is an interesting potential approach to embryo assessment due to its non-invasive nature. However, gene expression analysis is a relatively complex molecular approach, requiring multi-step sample preparation

and processing, which has the potential to introduce systematic or random errors, making inter-sample, inter-patient and inter-centre comparisons challenging. Furthermore, the application of microarray screening in a routine clinical context is problematic due to high processing costs, the need for expensive high-definition scanners and highly trained personnel. Conversely, qPCR offers a more direct, less labour-intense approach. While qPCR cannot deliver whole-transcriptome analysis, the lower costs of the method and its ability to measure the quantity of specific transcripts with high accuracy allows for easier inter-sample comparison and a potentially wider clinical application. However, at this moment in time there is no consensus concerning the specific genes that should be assessed in cumulus cells for the purpose of oocyte/embryo selection, which is an obstacle to the clinical application of this approach.

Gene expression alterations due to medical and technical variations such as patient age and culture environment have been reported (Adriaenssens *et al*, 2010). Therefore, the development of a comprehensive, universally applicable model may be challenging. Randomised controlled trials, taking into consideration multiple biological conditions should be undertaken before the efficacy of cumulus cells gene expression assessment in discriminating embryo competence can be determined.

1.5 MOLECULAR ANALYSIS OF CULTURE MEDIUM

During in vitro culture, the embryo interacts with its environment by the uptake of various molecules, including those that provide the energy required by cells to maintain intracellular homeostasis and support specialized functions (i.e. pyruvate and glucose), and amino acids utilised for the biosynthesis of cellular constituents and those destined for export (Leese, 2012). At the same time, the embryo releases into its surroundings by-products of its metabolism, and secretes immunogenic factors aimed at modulating the environment. Other cytoplasmic compounds may also be released by the embryo in cases where cellular damage is present.

Several groups have investigated different methodologies to identify and measure some of these molecules in the culture media where individual embryos were grown. Also, the presence of some of these molecules was found to relate to the health status of the embryo and its ability to generate a healthy pregnancy.

1.5.1 Pyruvate and glucose

A well-known metabolic modification that occurs in preimplantation embryos involves changes in the processes utilised to produce energy. In the embryo, ATP is mainly provided by oxidative metabolism (Brinster, 1973). In the first three days of development, the embryo uses pyruvate and lactate as main substrates for energy production through aerobic metabolism in the Krebs cycle. Embryo glucose consumption increases from zygote through to morula stages, when aerobic glycolysis

is fully activated. After the compaction and cavitation stages glucose becomes the main substrate for energy production in the embryo (Brison & Leese, 1994). Using enzymatic assays, studies have been performed to measure the uptake of pyruvate and glucose to monitor the energy status of the developing embryo (Gardner *et al*, 2001).

It has been shown that consumption of pyruvate declines in normally developing embryos up to three days after fertilisation, resulting in depletion of this molecule from the culture medium (Leese & Barton, 1984). Past the compaction stage, glucose levels reduce gradually in spent culture media of developing blastocysts (Leese & Barton, 1984; Gardner *et al*, 2001). Unbalanced consumption of these molecules has been hypothesised to be evidence of impaired development and reduced embryo quality (Gardner *et al*, 2001; Conaghan *et al*, 1993).

Although some authors found a correlation between glucose consumption and embryo development, other laboratories failed to demonstrate the same conclusions (Jones *et al*, 2001). It could be argued that the use of different variables in the culture systems (i.e. type of media, incubator's gas composition) affect embryo physiology and its metabolic response to the environment, making glucose consumption measurements inconclusive if not customised for the specific culture system.

1.5.2 Oxygen consumption

Compared to pre-compaction stages, a cavitating embryo requires increased amounts of energy to power its metabolic needs. Post-compaction stages are characterised by an intense oxidative phosphorylation activity aimed at producing

sufficient ATP to power the Na⁺ K⁺ ATPase activity required for the formation of the blastocoel cavity (Houghton *et al*, 2003). This energy also supports high protein synthesis rates associated with the increase of the number of nuclei and the total membrane surface of the embryo (Leese, 2012).

It has been hypothesised by several groups that oxygen consumption rates could relate to embryo quality and competence and that this could be non-invasively measured in media with minimal perturbation of the culture system.

In order to allow oxygen measurements and assess this relationship, several approaches have been investigated including micro-spectrophotometric scanning (Magnusson *et al*, 1986), electrochemical microscopy (SECM) (Shiku *et al*, 2001; Agung *et al*, 2005), loop-mediated isothermal amplification (LAMP) (Agung *et al*, 2005) and Nanorespirometer (Lopes *et al*, 2005, 2007). Although some groups showed associations between oxygen consumption and embryo quality (Shiku *et al*, 2001; Agung *et al*, 2005), these methodologies were never applied to human samples. Novel devices able to measure oxygen pressure by submersion in culture medium have recently been developed (O₂ Microsensor, Unisense, Denmark). Although this device is expected to reduce the technical challenges faced by such measurements, it has not yet been applied to human embryology and its ability to predict embryo competence requires testing.

1.5.3 Amino acid turnover

Protein synthesis requires intracellular uptake of amino acids from the culture medium and their arrangement into long peptides through the process of mRNA

translation. Also, amino acids are released into the environment as by-products of multiple metabolic pathways. It was proposed that the measurement of amino acids turnover in culture media could give information on the developmental potential of the embryo.

To perform these measurements, high pressure liquid chromatography analysis was first used by Lamb and Leese (Lamb & Leese, 1994), followed by other colleagues (Houghton *et al*, 2002; Brison *et al*, 2004). Initially, they correlated a reduced uptake of glutamine, arginine and methionine and a lower release of alanine and asparagine at Day-2/3 post-fertilisation, with an increased rate of blastocyst development (Houghton *et al*, 2002). Measurements on Day-4 at the morula stage showed instead that reduced uptake of serine and decreased release of alanine and glycine was correlated to optimal blastocyst development (Houghton *et al*, 2002). In the same study, the overall amino acids uptake and release was calculated, showing that developing embryos tend to possess a lower turnover rate than those that are non-viable. These findings are in agreement with the “quiet embryo” hypothesis (Leese, 2002; Leese *et al*, 2007, 2008), stating that developing embryos have a lower metabolic rate and consume less nutrients compared to arrested ones that instead “burn out” quickly.

Subsequent studies by the same group, showed that a reduced intake of glycine and leucine and a greater asparagine release by Day-1/2 embryos, correlates with increased clinical pregnancy and live birth rates (Brison *et al*, 2004). Interestingly, the amino acids profiles associated with better outcomes (blastocyst development, clinical pregnancy) were different in the two studies. The authors argued that embryo

metabolism changes between pre- and post- embryonic genome activation (around Day-2/3) and this is reflected in the amino acids profiles generated (Brison *et al*, 2004). Another study using the same technology showed that differences in the turnover of asparagine, glycine and valine on Day-2/3 samples and asparagine, leucine and lysin in Day-3/4 samples are significant between euploid and aneuploid embryos (Picton *et al*, 2010).

Alternatively, Seli and colleagues used proton nuclear magnetic resonance (1H NMR) to investigate amino acid composition in spent culture media collected on Day-3 post insemination (Seli *et al*, 2008). They found that glutamate levels were significantly higher in embryos that resulted in clinical pregnancy compared to those that failed to implant (Seli *et al*, 2008). Another group using a similar technology also showed a beneficial use of 1H NMR in identifying embryos leading to clinical pregnancy (Wallace *et al*, 2014). However, these tests were performed on Day-2 culture media and amino acids turnover profiles were different from Seli and colleagues. On the other hand, a recent study determined that 1H NMR amino acid profiling on Day-3 culture media could not predict implantation (Rinaudo *et al*, 2012).

1.5.5 HLA-G and leptin

The human leukocyte antigen G (HLA-G) is a protein with known immunogenic functions. HLA-G is only expressed by extra-embryonic tissues and appears to down-regulate inflammatory response in the host, allowing embryo implantation and immune tolerance during pregnancy. For these reasons it has been proposed by several authors that the secretion of HLA-G by *in vitro* developing embryos could be

used as a potential marker for embryo competence (Sher *et al*, 2004; Desai *et al*, 2006; Yie *et al*, 2005).

In assisted reproduction applications, the presence of HLA-G in culture media was assessed using chemiluminescence enzyme-linked immunoabsorbent assays (ELISA). Fuzzi and colleagues reported a necessary function of HLA-G during implantation (Fuzzi *et al*, 2002) and other authors reported a beneficial use of HLA-G detection in increasing implantation and pregnancy rates, especially if these measurements were combined with morphological assessment (Sher *et al*, 2004; Yie *et al*, 2005; Desai *et al*, 2006; Sher *et al*, 2005). However, a review from Vercammen and colleagues concluded that HLA-G measurements provided only modest diagnostic accuracy (Vercammen *et al*, 2008).

On the same line, other groups, argued that HLA-G levels were variable (Yie *et al*, 2005) and could not be reliably measured (Sargent *et al*, 2007). A multi-centre study reported that technical differences in culture systems are confounding factors leading to inconclusive results (Tabiasco *et al*, 2009). Also, Coulam and colleagues found no association between HLA-G secretion in culture media and ploidy status of the embryo (Coulam *et al*, 2009).

Although not yet proven clinically beneficial, systematic testing should be adopted to investigate its actual implantation prognostic value.

Leptin was also suggested as a potential candidate marker molecule for embryo development. This molecule is known to play a role in energy metabolism and fat storage (Friedman, 2002), however, there is growing evidence that its functions

also relate to reproduction and inflammation processes (González *et al*). Using ELISA, one group identified higher levels of leptin in the culture media of developing embryos compared to arrested embryos (González *et al*, 2000). Although leptin may have a potential role as a marker of embryo competence, present results are not sufficient to make it applicable to clinical routine and more testing is required.

1.5.6 Proteomics

Proteomics consists of the global analysis of the protein composition of a biological sample. It provides a more comprehensive assessment of protein abundance compared to single target measurements discussed above (HLA-G and Leptin). Traditional methods such as 2D-gels and Western-blot do not provide sufficient sensitivity to detect proteome composition of single embryo culture medium. This is mainly due to the low concentration of embryo-derived proteins that are released into the medium during pre-implantation development.

More recently, highly-sensitive time-of-flight mass spectrometry in combination with ProteinChip methodology has been applied (Katz-Jaffe *et al*, 2005, 2006). In these experiments, it was shown that the type of culture medium influenced the composition of embryo secretome and that protein profiles differed according to developmental stage.

Subsequent studies by the same group involved a refinement of the technique including albumin depletion from the samples and protein separation on gel prior to identification of gel band composition using tandem mass spectrometry. With these experiments they showed that abundance of Lipocalin-1 was significantly higher in

pooled culture media derived from aneuploid embryos compared to normal ones (McReynolds *et al*, 2011).

Another group investigated culture media protein composition using protein microarrays (Dominguez *et al*, 2009). This technique is similar to gene expression microarrays, however instead of oligonucleotides spotted on the array, antibodies capable of binding to specific proteins are affixed to the slide. Although they found that certain proteins (GM-CSF, CXCL13) were consumed at higher rate by implanting embryos compared to non-implanting ones, they didn't find any correlation with protein secretions (Domínguez *et al*, 2008; Dominguez *et al*, 2010).

1.5.6 Metabolomics

The metabolome is the ensemble of final and intermediate products of metabolic processes active within the cell or a biological system (i.e. the embryo). Nutrients are internalised and processed by the cell, which then excretes final products (metabolites) into the environment to avoid intracellular build up and possible toxic effects. The type and work rate of cellular metabolic activities directly affect the composition of the metabolome. Mutually, culture solution composition can modulate molecular pathways and embryo overall physiology. Diverse species of molecules including carbohydrates, amino acids, peptides, carboxylic acids, fatty acids and nucleotides form this complex mixture. Its composition involves about 3,000 metabolites, the abundance of which is highly dependent on environmental conditions (i.e. energy substrate availability, culture medium composition). Because the metabolome is formed by a limited set of molecules, it has been hypothesised that its

composition could be used as a diagnostic marker of embryo health (Hollywood *et al*, 2006).

Metabolites can be detected using different analytical approaches including mass spectrometry, nuclear magnetic resonance (NMR) and optical spectroscopy (vibrational spectroscopy).

Initial studies were performed using Near-Infrared spectroscopy (NIRS), which allows the detection and measurement of molecules in a biological fluid based on light absorbance and conformational vibrations of their key functional groups (i.e. -OH, -COOH, -NH). Initial investigations showed that the metabolic profile of implanting and non-implanting embryos differed significantly (Seli *et al*, 2007; Nagy *et al*, 2008; Vergouw *et al*, 2008). A preliminary prospective study also showed the predictive value of culture media metabolic profiling on pregnancy outcome (Scott *et al*, 2008).

Analytical algorithms to calculate embryo viability scores were developed and tested in multiple laboratories using different culture systems, showing that results were reproducible in different settings (Ahlström *et al*, 2011). Following on from these encouraging findings, a bench top device for analysis of media samples was developed for use in clinical settings, followed by commercialisation (Molecular Biometrics, Inc.). However, subsequent prospective RCTs failed to show a beneficial impact of metabolic profiling on pregnancy rates when embryos were selected based on morphology or morphology and viability score combined on Day-3 (Hardarson *et al*, 2012; Vergouw *et al*, 2012) or Day-5 post-fertilisation (Sfontouris *et al*, 2013).

The discrepancy between preliminary studies and RCTs was potentially attributed to the different procedural conditions during sample analysis that may

have affected the precision and reproducibility of readings (Hardarson *et al*, 2012), suggesting that original algorithms were not as robust as expected when applied within IVF centers (Vergouw *et al*, 2012). Researchers also identified a potential limitation of the RCT in the pre-selection of good morphology embryos, possibly biasing the study population towards a good prognosis subgroup (Vergouw *et al*, 2012). However, this inclusion criteria was required by ethical considerations, with authors not being comfortable transferring a poor morphology embryo, despite it showing the highest viability score, when a good morphology one was available (Vergouw *et al*, 2012).

These results posed the question whether inconclusive results were due to the absence of biological differences in the metabolite of implanting and non-implanting embryos or to insufficient sensitivity of the methodology used (Hardarson *et al*, 2012; Vergouw *et al*, 2012).

For these reasons, a more sensitive technology able to detail structural and dynamic information of the metabolome component was proposed (Seli *et al*, 2008). In subsequent studies, NMR was employed to investigate the relationship between an embryo's metabolic profile and its potential to implant and produce a viable pregnancy. Although two studies showed a positive correlation between NMR profile and implantation (Seli *et al*, 2008; Pudakalakatti *et al*, 2013), larger studies did not confirm these findings, concluding that no improvements in clinical outcomes were observed when NMR profiling was combined to morphological assessment in the selection of embryos for transfer (Rinaudo *et al*, 2012; Kirkegaard *et al*, 2014).

1.5.6.1 Considerations on metabolic profiling

Although studies using NIRS and NMR failed to show a correlation between metabolic profile and reproductive outcome, they demonstrated that remarkable differences can be present between embryos with the same morphological grade. This suggests that morphology does not completely reflect embryo physiology and significant biological differences may hide behind embryo's appearance (Uyar & Seli, 2014).

Also, these studies demonstrated that a model that appears robust when trialed in-house, may not deliver the same data quality when utilisation is expanded to other sites. Failure in reproducing the initial results may be due to variations in the sensitivity of the devices but more importantly in the culture system and overall laboratory conditions. Culture media composition differs across manufacturers and slight differences may also be found between batches of the same product. A recent study reported that several undisclosed (and probably uncontrolled) compounds are present in most culture media brands, including proteins involved in innate immune and inflammatory responses (Dyrlund *et al*, 2014). These proteins are potentially introduced in the solution through the supplementation of impure human serum albumin. Different culture media may therefore generate altered background signals or even trigger alternative metabolic pathways in the embryo, ultimately requiring ad hoc tuning of the predictive algorithms used for calculation of embryo viability scores.

1.6 ALTERNATIVE EMBRYO ASSESSMENT METHODS

In order to improve treatment outcomes, the field of assisted reproduction requires the development of a robust methodology able to prioritise for transfer those embryos with the highest potential to implant. In the last decade scientists in human embryology dedicated their work to investigate the translational application of several molecular techniques to this scope. However, so far no methodology has showed sufficient sensitivity in identifying those embryos that will implant to ultimately demonstrate a clear beneficial effect on treatment outcome. It is possible that a specific technique could only partially distinguish certain biological features necessary for a healthy pregnancy (e.g. euploidy) but cannot provide information regarding other aspects (i.e. metabolism or physiological functions). Nonetheless, it could be argued that the combination of complementary embryo assessment methodologies could increase overall treatment outcome.

Whilst more data is generated with the methodologies already developed and discussed above, research should also be carried out on novel potential targets with technologies that have recently emerged. Especially, these novel approaches should be used to further investigate embryo biology and to assemble detailed catalogues of molecules utilised by, produced by, or interacting with preimplantation embryos. These catalogues could then be used to select targets of interest to test with more sensitive, and likely less expensive, technologies.

With a view to eventual clinical application, novel diagnostic strategies should aim to reduce the manipulation of the embryo, ideally using specimens that would typically be discarded during the course of standard treatment, such as spent culture media. This would minimise potential iatrogenic impacts on embryo development whether direct (e.g. cell lysis following biopsy) or indirect (e.g. epigenetic modifications due to suboptimal culture conditions).

In this study, I aimed at creating a comprehensive view of embryo processes at the blastocyst stage (**Figure 1-1**).

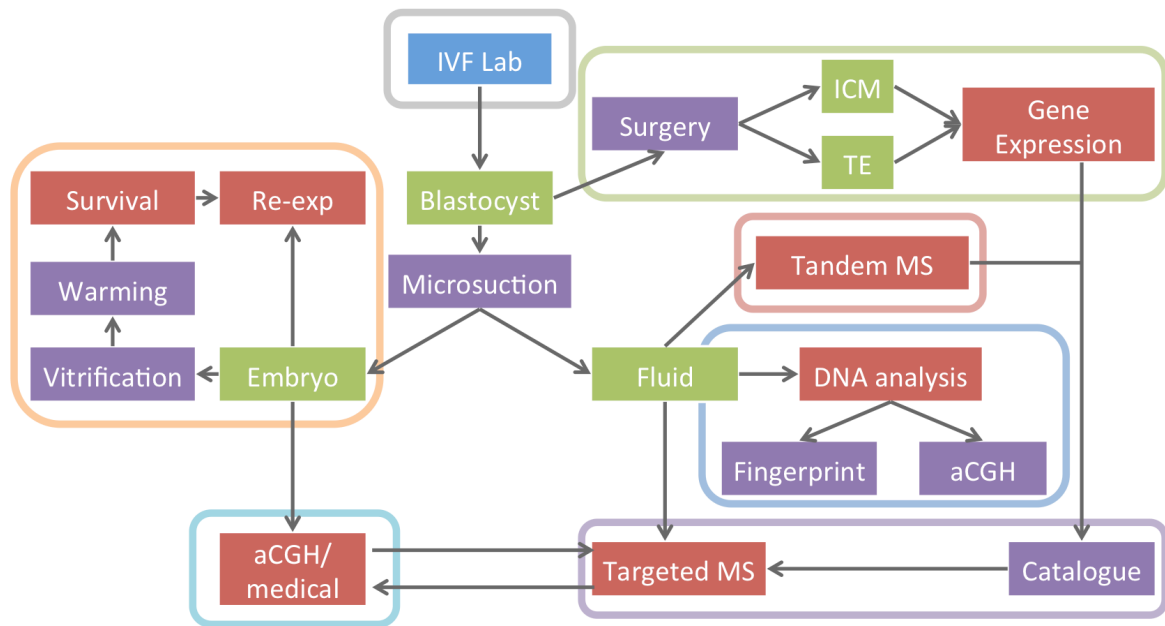


Figure 1-1 – Experimental workflow overview. Red boxes refer to molecular and biological investigative strategies employed, green boxes refer to biological samples used in the experiments and purple boxes refer to experimental interventions applied to the samples. Coloured circles refer to different investigative aims: clockwise from left, orange, embryology; green, blastocyst gene expression; red, blastocoel proteome discovery-phase, tandem mass spectrometry; blue, analysis of DNA contained in the blastocoel; purple, targeted analysis of blastocoel components, targeted mass spectrometry; light blue, assessment of embryo cytogenetic status and database retrieval of embryo and patient biological information. Abbreviations: IVF Lab, In vitro fertilization laboratory; Microsuction, blastocentesis; Surgery, Inner Cell Mass mechanical excision; ICM, Inner Cell Mass; TE, Trophectoderm; Gene Expression, Microarrays and RNASeq; Tandem MS, Tandem Mass Spectrometry; aCGH, Comparative Genomic Hybridisation array; Targeted MS, Targeted Mass Spectrometry; aCGH/medical, comprehensive cytogenetic assessment of embryo genetic complement and biological and medical characteristics of individual embryos and patients generating them; Survival, Post-vitriification survival rate; Re-exp, post-microsuction blastocoel re-expansion rate.

Here I introduce the concept of analysing a type of sample that, at the time I began our experiments, was poorly understood and investigated: the human blastocoel.

For the collection of this fluid, which I have termed blastosol, optimised micromanipulation (microsuction) techniques were developed, a process that I named Blastocentesis. Initially, the impact of the microsuction technique on embryo viability was assessed. Subsequently, blastocentesis was applied to extract and analyse both the genetic and proteomic content of the blastosol.

In parallel, gene expression analysis of the Inner Cell Mass (ICM) and Trophectoderm (TE) cells was undertaken. The aim was to define the transcriptome of the human blastocysts, identify early gene expression differences between the first two cell lineages and to validate the embryonic origin of the proteins identified in the blastosol.

Subsequently, a targeted proteomics approach was developed in order to accurately measure the amount of proteins of interest in the blastocoel. Lastly, any correlation between the levels of multiple proteins and several medical, biological and genetic features of the embryo were evaluated (i.e. patient's age, embryo morphology, karyotype).

1.7 STUDY AIMS

The aims of this project were:

- The development and assessment of a micromanipulation technique involving the collection of a highly purified embryo-derived specimen.
- The investigation of the presence and origin of DNA sourced from the blastocoel and its potential use for preimplantation genetic assessment.
- The investigation of the protein composition of the human blastocoel.
- The validation of embryonic origin of proteins detected in the human blastosol by gene expression experiments.
- The development of a targeted proteomic strategy to measure the abundance of selected proteins of interest in single blastosol samples.
- The assessment of potential correlation between the abundance of blastocoelic proteins and the cytogenetic status and other biological characteristics of the embryo.

1.8 CHAPTER 1 BIBLIOGRAPHY

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2. BLASTOCENTESIS AND ITS EFFECTS ON EMBRYO VIABILITY

2.1 Blastocentesis and embryology

2.1.1 The blastocoel

2.1.2 The blastocentesis procedure

2.1.3 Application of blastocentesis in embryo cryopreservation strategies

2.2 Effects of blastocentesis on the embryo

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2.1 BLASTOCENTESIS AND EMBRYOLOGY

2.1.1 The blastocoel

The blastocoel is a fluid filled cavity that forms within the human embryo around Day5/Day6 post insemination. The development of the blastocoel cavity results from the completion of the compaction stage that follows embryo genome activation (EGA) around Day2/Day3 post insemination. The establishment of an inner cavity is usually concomitant to the initial differentiation into the first two cellular lineages, namely the Inner Cell Mass (ICM), from which all the embryonic tissues originate, and the Trophectoderm (TE), which would later develop into extra-embryonic compartments, such as the yolk sac (**Figure 2-1**).

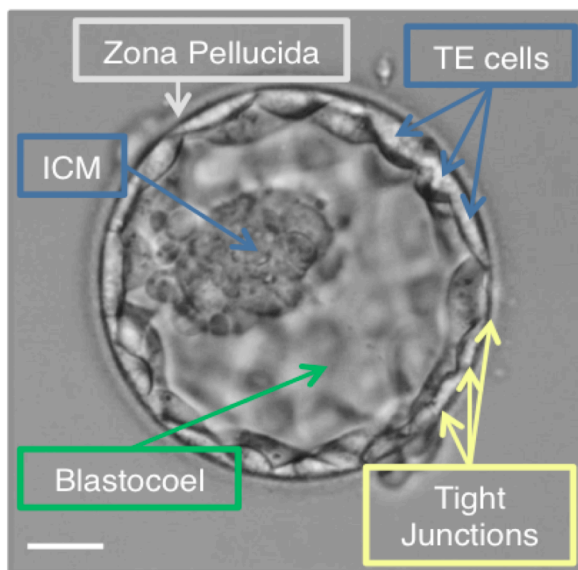


Figure 2-1 - The human blastocyst showed with phase contrast microscopy. Bar length 50 μ m

The blastocoel is surrounded by a TE cell layer that also contains the pressure increasingly building up within the cavity. In a fully expanded human blastocyst, the blastocoel cavity reaches a volume between 4 and 6 nL. Tight junctions create firm interconnections between TE cells and avoid free passage of molecules from the surroundings, maintaining a highly controlled

environment within the blastocoel. Cells forming the Inner Cell Mass (ICM) are embedded within this milieu where autocrine and paracrine signals are likely to be present.

2.1.2 The blastocentesis procedure

Blastocentesis involves the collection of the blastocoel fluid (blastosol) from a fully expanded blastocyst for subsequent genetic, metabolic or proteomic analysis. Blastocentesis involves the gentle insertion of a microneedle through the TE wall and the application of light negative pressure (**Figure 2-2** and **Video E1** in the **Supplementary Electronic Material folder**).

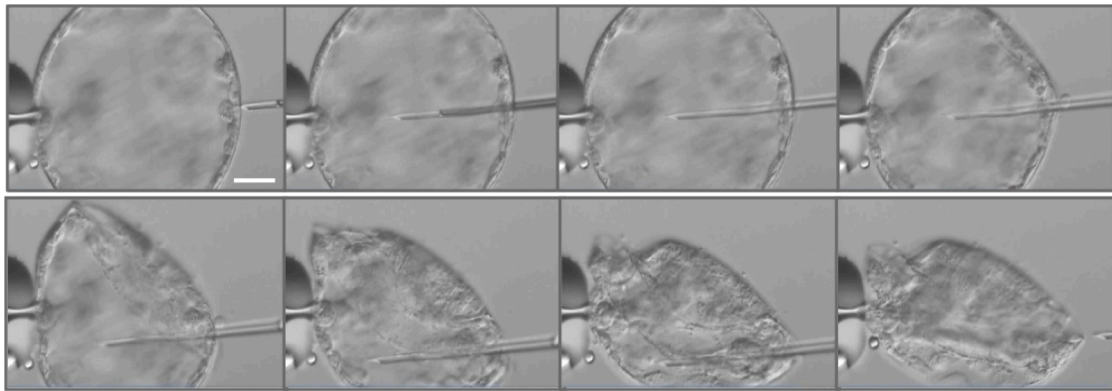


Figure 2-2 – Sequence of phase contrast microscopy showing blastocentesis procedure. Scale bar length 50 μ m.

When around 90% of the original volume is collected, the needle is removed, the content expelled in a separate drop and collected into a sterile vial or PCR tube depending on downstream applications. This operation is carried out on a micromanipulation station with heated stages at 37°C to maintain a physiological temperature during the procedure (See Materials and Methods 2.4.3). For protein

analysis, filtered phosphate buffer saline (PBS) should be used to avoid contamination from serum protein substitute present in embryo culture media.

Blastocoel aspiration was previously utilised in animal models to reduce the volume of the blastocyst cavity prior to cryopreservation (Chen *et al*, 2005).

A modified version of the technique, called blastocyst micropuncture was used in human samples, however, this method did not involve active collection of the blastocoel but was limited to puncturing the TE wall to disrupt the pressure within the cavity and allow the release of the inner fluid (Mukaida *et al*, 2006). Borland and colleagues performed the first investigation on the ionic composition of mouse blastocoel fluid in 1977 (Borland *et al*, 1977). Microsuction was later employed to determine the concentration of glucose, lactate and pyruvate in the blastocoel cavity of murine (Brison & Leese, 1994) and bovine embryos (Gopichandran & Leese, 2003).

During *in vitro* development, the human blastocyst undergoes several collapse/re-expansion cycles. These cycles appear to originate naturally and are possibly caused by pressure build-up within the cavity, leading to breaches in the TE wall eventually resulting in the release of the inner fluid in the outer compartment. It is unknown if embryo-secreted proteins are retained within the collapsing cavity during these collapsing cycles. It is possible that key solutes within the blastosol are readily replenished after collapsing. However, the dispersion of the liquid content does not appear to impair embryo development, as cryopreserved blastocysts are able to produce pregnancies although their blastocoel fluid is dispersed during the vitrification procedure. Additionally, it was demonstrated by several groups that

removal of blastocyst's inner water content significantly increases cryopreservation efficiency (Vanderzwalmen *et al*, 2002; Mukaida *et al*, 2006; Son *et al*, 2003).

2.1.3 Application of blastocentesis in embryo cryopreservation strategies

One of the factors that can detrimentally impact the freezing procedure is the formation of intra or extracellular ice crystals from free water. Ice crystals can damage cellular organelles and nuclear and plasma membranes resulting in cell lysis or induced apoptosis (Mazur, 1963; Leibo *et al*, 1978; Rall & Fahy, 1985). In the case of cleavage stage embryos, the majority of water content is contained within the cells. Using a "slow freezing" procedure, a combination of non-permeating cryoprotectant and controlled reduction of the temperature allows a gradual extraction of water from the cells. In a "vitrification" procedure, ice crystal formation is avoided by increasing the viscosity of intra and extra-cellular solutions by briefly exposing the embryo to high concentration of a permeating cryoprotectant (i.e. dimethyl sulfoxide, DMSO and ethylene glycol, EG). This type of cryoprotectant is able to lower the freezing point of water, allowing the formation of an ice-free "glassy state". Vitrification of biological material is possible only if the reduction of temperature is extremely rapid ($-10,000$ °C/sec). The ultra-rapid drop in temperature is favored by the use of a small (~ 1 μ L) loading volume. In blastocysts, although cell membranes are permeable to DMSO and EG, the cryoprotectant may not be able to diffuse properly within the blastocoel, where a relatively high volume of water is present.

This suboptimal cryoprotectant distribution can cause the formation of ice crystals and subsequent damage to cellular structures during the cryopreservation procedure,

often with lethal consequences to the embryo. It is therefore recommended to collapse the blastocyst prior to exposing it to the cryoprotective solution, in order to reduce blastocoel water content (Kader *et al*, 2009; Mukaida *et al*, 2006; Vanderzwalmen *et al*, 2002).

Several methods can be used to achieve blastocoel collapse. These strategies include micro-pipetting, where a hand pipette of a slightly smaller size than the embryo is used to "squeeze" the embryo and induce a small rupture in the TE wall (Hiraoka *et al*, 2004). Alternatively, micro-puncturing, as discussed previously, can be used to release the pressure within the cavity (Mukaida *et al*, 2006). More recent methods include hyper-dense cryopreservation solutions (Kitazato method) and the use of medical-grade laser pulse to junction points between TE cells (Iwayama *et al*, 2011; Mukaida *et al*, 2006).

According to the literature, these techniques are associated with similar successful outcomes and can allow up to >90% survival rate when performed on good morphology blastocysts. Unfortunately, a randomised controlled trial comparing these techniques has not yet been performed and laboratories, if blastocyst collapsing is carried out, use the method that suits best the equipment available and their technical or scientific preference (Edgar & Gook, 2012).

2.2 EFFECTS OF BLASTOCENTESIS ON THE EMBRYO

In order to be adopted as a clinical procedure, a novel technique has to perform no worse than the current method. Moreover, short-term effects of the new procedure should be assessed and compared to a control group. In this part of the study, I investigated the feasibility, performance and safety of using blastocentesis to achieve blastocyst collapse (**Figure 2-3**).

In the first experiment, a total of 31 fresh blastocysts were placed back in culture for 24 hours after microsuction. Using conventional phase-contrast microscopy, I evaluated the overall architecture and the presence of degenerated areas and the time required to re-expand.

In the second experiment, 50 embryos underwent microsuction prior to vitrification. Following warming, they were kept in culture for 18 hours. These embryos were morphologically assessed for the amount of degenerated cells present after warming using conventional phase-contrast microscopy. Additionally, I monitored the time each embryo required to reach full re-expansion. As control, I used a group of embryos that were vitrified for clinical purpose without prior microsuction intervention. Embryos in this group were transferred to the patient between 4 and 6 hours after warming and long-term re-expansion evaluation was not possible.

Additionally, the composition of the two groups of embryos employed in the vitrification study was also evaluated in terms of morphology following Gardner's criteria (see Materials and Methods 2.4.7).

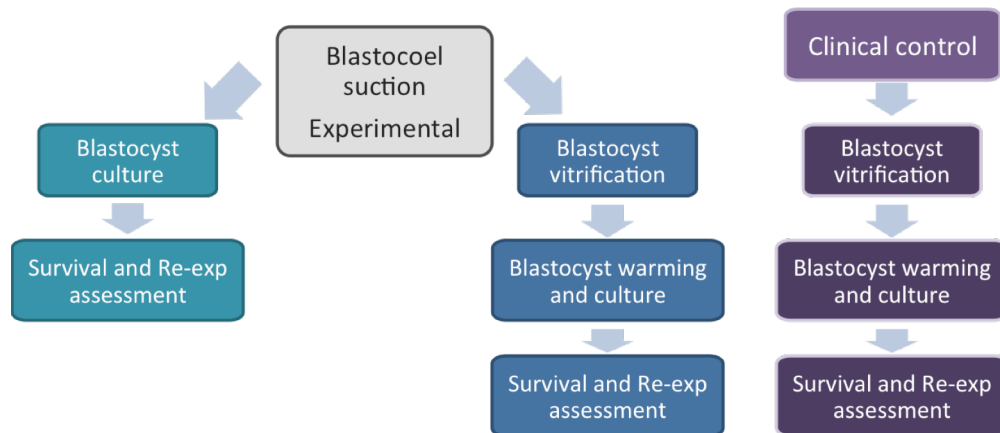


Figure 2-3 - Experimental design to test for microaspiration effect on embryo viability

Results

2.2.1 Survival and re-expansion rates of fresh embryos after microaspiration

In the first experiment, none of the cultured embryos (0/31, 0%) showed necrotic areas after blastocentesis over the time monitored (24 hours). Additionally, all the embryos (31/31, 100%) showed at least partial re-expansion within the first 5 hours. Of these 31 embryos, 28 (90.3%) were fully re-expanded at 5 hours and 30 (96.7%) at 24 hours post microaspiration (**Figure 2-4**).

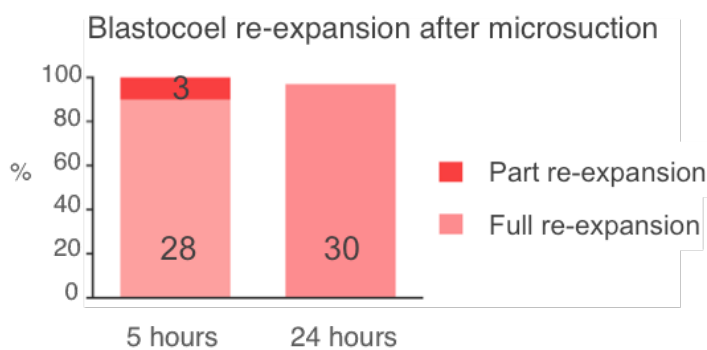


Figure 2-4 - Blastocoel re-expansion of fresh embryos. Embryos assessed at 5 and 24 hrs incubation after microaspiration

2.2.2 Survival and re-expansion rates in embryos vitrified after microsuction

The experimental group consisted of 16 *good* and 34 *poor* morphology blastocysts (32% and 68%, respectively). The control group comprised of 59 *good* and 5 *poor* morphology blastocysts (92.2% and 7.8%, respectively). Analysis of groups revealed a statistical difference in their composition (Chi-square test, C.I. 95%, $P < 0.0005$). Due to the better embryo morphology, higher survival rates were expected in the control group. On the contrary, in 48 of the 50 embryos vitrified after microsuction, more than 50% of the cells forming the embryo showed intact membranes at microscopic evaluation and were considered viable, compared to 47 out of 64 of the control group (96.0% vs 73.4% respectively, two-tail Chi-square test, C.I. 95%, $P = 0.002$) (**Figure 2-5a**). In terms of re-expansion, 44 out of 50 embryos in the research group showed their blastocoelic cavity refilled to more than 50% of its original volume at an average of 5 hours after warming, whilst the control group showed 29 out of 64 (88.0% vs 45.3% respectively, two-tail Chi-square test, C.I. 95%, $P < 0.0005$) (**Figure 2-5b**).

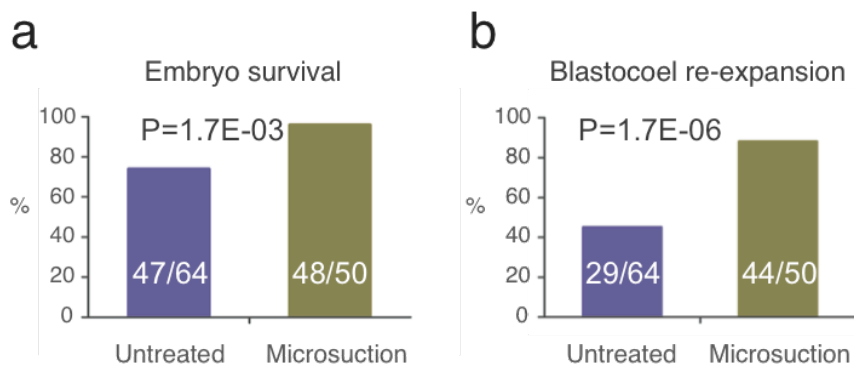


Figure 2-5 - Embryo survival and blastocoel re-expansion rates. Embryos assessed at 5 hours incubation after microsuction followed by cryopreservation.

2.3 DISCUSSION

This set of experiments was performed to investigate whether blastocoel suction had any impact on the architecture of the blastocyst. The experiment was divided into microsuction alone and microsuction plus vitrification. This set of experiments was designed to investigate if microsuction directly impacted embryo viability and if microsuction somehow affected the vitrification procedure.

In these experiments, microsuction not only appeared not to impair blastocyst viability, but it actually improved survival rates in cryopreserved blastocysts compared to a control group treated with micropipetting prior to vitrification. Firstly, fresh embryos treated with microsuction showed a rapid (>90% at 5 hours) and high (>96% at 24 hours) re-expansion rate. These data suggest that microsuction can be performed safely without damaging the embryo.

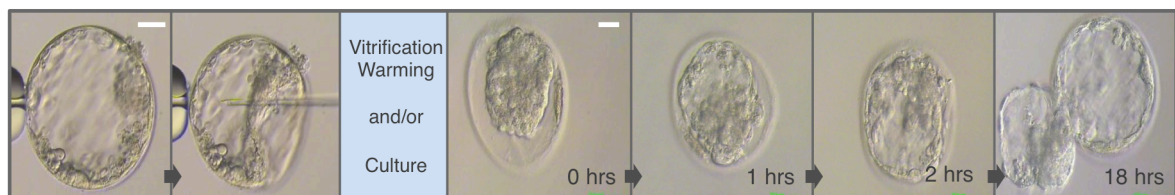


Figure 2-6 – Phase contrast microscopy of a human blastocyst collapsed using microsuction prior to cryopreservation or culture (L) and subsequent re-expansion to normal morphology (R). Scale bar $\mu\text{m}50$.

In the second set of experiments I demonstrate that microsuction can deliver a high survival rate (96%) also when coupled to the vitrification procedure. Additionally, the embryos in the experimental group had a significantly higher

survival rate compared to those in the control group (96% vs. 73.4%). This difference in survival may be due to inefficient blastocyst collapsing of micropipetting. In fact, large inner water content affects the diffusion of membrane-permeating cryoprotectants within the cavity, resulting in a higher solution freezing point and increased risks of ice crystal formation. These crystals can damage cellular organelles, nuclear and plasma membranes, resulting in cell lysis or induced apoptosis, often at lethal levels for the embryo (Vanderzwalmen *et al*, 2002; Kader *et al*, 2009).

From the literature, blastocysts collapsed by micropipetting should reach survival rates higher than 90%. However, if the pipettes used for embryo collapsing are not of the exact diameter, they can have a negative impact on the outcome. For example, if the pipette is too wide, the embryo would not collapse and the blastocyst cavity would still be filled with water, impairing the sample transition to glass state. On the other hand, if the pipette is too narrow, it is likely that mechanical damage could be produced.

In this set of experiments, I demonstrated that the mechanical procedure of blastocentesis is safe to the embryo and that the collection of the blastocoelic fluid can be performed without damaging the embryo. These findings reinforce the possibility of using blastocentesis as a clinical procedure without reducing a patient's overall chance of pregnancy.

During an IVF treatment, patients often produce multiple blastocysts. At the time of the transfer procedure, the number of embryos to transfer to the patient depends in part on her medical history. Untransferred surplus blastocysts are considered for cryopreservation only if they show top quality morphology. In this

case, morphological assessment can have significant consequences on the treatment, as embryos of suboptimal morphology may be discarded rather than stored, due to their presumed low survival and implantation rates.

However, it can be inferred from fresh treatment outcomes that around 30% of suboptimal morphology blastocysts would produce a healthy pregnancy if transferred (Gardner, 2000).

Our data suggest that high survival rates can nonetheless be achieved in poor quality embryos. This was shown by higher survival rates in the experimental group, despite the significantly lower proportion of good morphology embryos, compared to the control group.

Hence, blastocoel microsuction could increase the cohort of embryos available for subsequent frozen embryo transfer treatment cycles. In fact, although poor quality blastocysts have lower implantation rates compared to good morphology ones, they still maintain a fair implantation potential (40.4% vs 55.1%, respectively, Oxford Fertility Unit, unpublished data). In comparison, top quality cleavage stage (Day 3) embryos have even lower implantation rate than poor blastocysts (28.3% vs 40.4%, respectively, Oxford Fertility Unit, unpublished data).

These data show a significantly higher post-vitrification survival rate reached by fully collapsed embryos when compared to the control group. These data also demonstrate that blastocentesis and downstream analysis of blastosol composition could be applied clinically.

2.4 MATERIALS AND METHODS

2.4.1 Experimental groups

In this part of the study, I performed two types of experiments: the first evaluated the impact of microsuction on 31 fresh human blastocysts by re-placing the embryos back into culture after intervention and assessing the re-expansion time and any cellular degeneration. No control group was used. In the second experiment, I assessed cellular degeneration and re-expansion time after vitrification/warming. In the experimental group, 50 embryos underwent microsuction prior to cryopreservation, whilst in the control group, 64 blastocysts underwent micropipetting during cryopreservation for clinical purposes. Warming procedures were performed following the same protocol. The control group included all embryos vitrified and warmed since the introduction of vitrification at OFU. Only samples that included all data and matched developmental stage of the experimental group (Day6) were considered in the study. Data were analysed retrospectively using Chi-square statistical test.

2.4.2 Production of human embryos

Couples undergoing IVF treatment at the Oxford Fertility Unit donated embryos used in this study. All patients underwent pituitary gland down-regulation by administration of GnRH agonist spray (Nafarelin, Pfizer, USA) until occurrence of

menstruation and oestradiol (E2) levels reduced under 76 pmol/L. Patients were treated with conventional controlled ovarian hyperstimulation receiving an subcutaneous injection of FSH between 112 and 375 IU daily for a period of 9 to 14 days (Gonal-F, Merck Serono, Germany). Oocyte collection was performed following follicle triggering by 6,500 IU recombinant hCG (Ovitrelle, Merck Serono, Germany). Collected oocytes were inseminated by exposure to fixed concentration of partner's treated sperm sample or by intracytoplasmic sperm injection (ICSI). Fresh embryos were individually cultured in 50 µL microdrops of Cleavage medium (COOK, Australia) up until Day 3 post fertilization and then moved to 50 µL microdrops of Blastocyst medium (COOK, Australia) until donation.

Donated cleavage-stage frozen embryos were thawed using standard commercial kit (COOK thawing media) and then cultured to blastocyst stage using Blastocyst medium (COOK, Australia). Incubation parameters were 37 °C and 6% CO₂ and 5% O₂. Fresh embryos were donated on day 5 or 6 post insemination, whilst frozen embryos were donated at various developmental stages between 24 hours and 72 hours post insemination. Frozen embryos were thawed and cultured to day 5 or 6 until a full blastocoel cavity was present.

2.4.3 Blastocoel microsuction

Blastocysts were individually washed in 1 mL PBS to remove proteins present in the culture media prior to placing them into a microsuction set-up dish (**Figure 2-7**). Blastocoel extraction procedures were performed in 10 µL drops of sterile micro-

filtered PBS, overlaid with sterile mineral oil (COOK, Australia). The micromanipulation technique used involved anchoring the embryo to a holding pipette (Research Instruments, UK) on one side and inserting a microinjection needle (Research Instruments, UK) through the trophectoderm cell layer on the opposite side. Gentle application of negative pressure on the needle allowed fluid collection and embryo collapse (**Figure 2-2**).

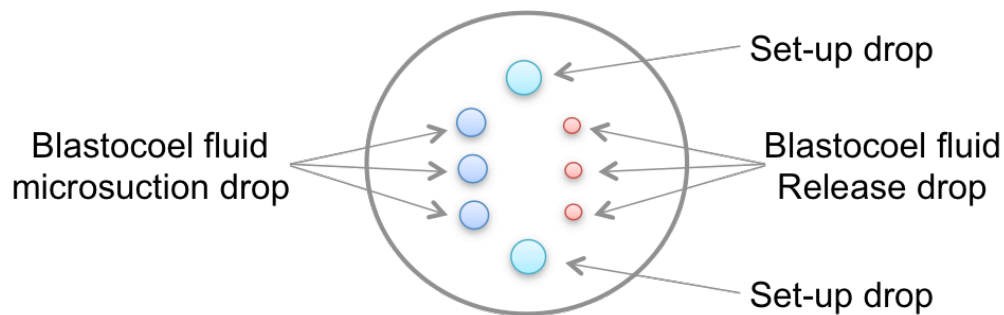


Figure 2-7. Dish set-up for blastocentesis procedure

2.4.4 Blastocyst vitrification

Blastocyst vitrification was performed using Cook Blastocyst Vitrification media kit (Cook, Australia) and CVM vitrification system (Cryologic, Australia).

The CVM system is composed of a container partially filled of liquid nitrogen (LN2) accommodating a half submerged metal block. The metal block reaches similar temperatures to liquid nitrogen (-196 °C). It is also designed to allow exposure of the sample to extremely low temperatures, avoiding direct, unprotected immersion into unsterilised LN2. The CVM sample loading device, namely Cryohook (Cryologic, Australia) is composed of a sterile plastic hook able to accommodate the 2 µL micro

drop of solution containing the embryo. Prior to vitrification, these devices were labelled with sample number, date of cryopreservation and research project name.

The media kit is composed of three solutions. The concentration of Dimethyl sulfoxide (DMSO) increases sequentially with Solution 1 at 0%, Solution 2 at 8% and Solution 3 at 16% (v/v). As recommended by the manufacturer, the embryo was washed in Solution 1 at 37 °C for 30 seconds, then moved into Solution 2 at 37 °C for 2 minutes. The embryo was then moved to Vitrification Solution 3. Within 20-30 seconds the embryo was loaded on the Cryohook. The loading device was then placed onto the vitrification block allowing the droplet containing the embryo to vitrify immediately. With a swift but controlled movement and maintaining the sample in the vitrification block, the loading device with the vitrified sample was then inserted into a straw and firmly secured by hand. After each individual embryo was vitrified, the straws were transferred to a permanent storage dewar.

2.4.5 Blastocyst warming

Blastocyst warming was performed using Cook Blastocyst Warming media kit (Cook, Australia) composed of three solutions. These solutions had sequentially decreasing concentration of Trehalose with Solution 1 at 0.33 M, Solution 2 at 0.2 M and Solution 3 at 0 M. While submerged in LN, the loading device was removed from the outer straw and the droplet containing the embryo was directly immersed into Solution 1 at 37 °C. The embryo was immediately moved to a second well containing Warming Solution 1 and incubated at 37 °C for 5 minutes. This procedure was repeated for Solutions 2 and 3. Finally, the embryo was placed in a culture dish

containing Cook Blastocyst Culture media (Cook, Australia) at standard culture conditions for a period between 3 and 18 hours prior to discarding.

2.4.6 Embryo thawing

Embryo thawing was performed using Cook Thawing media kit (Cook, Australia) composed of 4 solutions with decreasing amount of propandiol and constant concentration of sucrose.

The straw containing the embryo was removed from liquid nitrogen and thawed in air for 40 seconds. The straw was then plunged into 30 °C water for 30 seconds. The embryo was then expelled into an empty dish and moved into Solution 1 (1.0 M propandiol and 0.2 M sucrose) and subsequent wells for 5 minutes each at room temperature (Solution 2, 0.5 M propandiol and 0.2 M sucrose; Solution 3, 0.2 M sucrose, Solution 4 Cryopreservation buffer only). Finally, the embryo was placed into 50 µL of Cook Blastocyst media (Cook, Australia) if on Day 3 of development. If on Day 2 of development, they were placed in Cook Cleavage media (Cook, Australia) and moved to Cook Blastocyst media on Day 3.

2.4.7 Embryo morphological assessment

All embryos were assessed prior to vitrification or micromanipulation. Parameters evaluated included degree of expansion, ICM morphology and TE morphology, according to Gardner's blastocyst assessment criteria (Gardner & Schoolcraft, 1998) (**Table 2-1**). Embryos that scored grade A or B in ICM or TE

assessment were considered of **good** morphology unless they scored D or E for the other parameter. Embryos that showed ICM or TE of C grade were considered **good** if the other parameter was an A or a B, and **poor** if the other parameter was a C, D or E.

Table 2-1 - Blastocyst morphology assessment parameters

Expansion grade	Stage of development	ICM grade	Inner cell mass description
1	Cavitating blastocyst Blastocoel less than half the volume of the embryo	A	ICM prominent with many cells, tightly packed
2	Early blastocyst Blastocoel fills more than half the volume of the embryo	B	Several cells, loosely grouped, larger in size
3	Full blastocyst Blastocoel completely fills the embryo Zona pellucida may be starting to thin	C	Very few cells
4	Expanded blastocyst Large blastocoel volume of the size of an early embryo Zona pellucida now thinner than before	D	Cells of ICM appear degenerate or necrotic
5	Hatching blastocyst TE has started to herniate through the zona pellucida	E	No ICM discernible in any focal plane
6	Hatched blastocyst The blastocyst has completely hatched		
		TE grade	Trophectoderm description
		a	Many cells, forming a cohesive layer
		b	Few cells, forming a loose epithelium
		c	Very few large cells

2.4.8 Survival assessment

Embryo survival was assessed after warming. Similar to clinical practice procedures, embryos were evaluated using conventional phase-contrast microscopy analysis and were considered fully survived if >50% of cells were found intact. Cells were regarded as viable if plasma membranes showed a clear margin and a homogenous content. If membranes were found blurred with dark, granular content, cells were considered not viable.

2.4.9 Re-expansion assessment

After thawing, blastocysts were cultured and assessed for expansion at regular intervals. Expansion is parameter that is evaluated in clinical context to assess the ability of the blastocyst to recover from cryopreservation. Although there are no studies in the literature regarding the association between time of post-warm re-expansion and embryo implantation/clinical pregnancy rates, it is believed that a shorter re-expansion time is an indication of embryo health. Expansion was graded as ***absent*** if <20% of the original volume was recovered, ***partial*** if around 50% was recovered or ***full*** if around 100% of expansion was achieved.

2.5 CHAPTER 2 BIBLIOGRAPHY

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3. BLASTOCOEL GENOMICS

3.1 The blastocoel as a source of DNA for preimplantation genetic testing

3.2 DNA fingerprinting

3.2.1 Short Tandem Repeats

3.2.2 DNA Fingerprinting protocol development and validation

3.2.3 Discussion

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3.1 THE BLASTOCOEL AS A SOURCE OF DNA FOR PREIMPLANTATION GENETIC TESTING

The ability to safely retrieve the blastocoel fluid without harming the embryo, opened to the possibility of investigating several features of this specimen with the potential of clinical application.

As previously described in Chapter 1, a cellular sample taken from a living embryo is required for preimplantation genetic testing. Contrarily to cleavage stage biopsy, the removal of a few trophoctoderm cells from the developing blastocyst does not appear to have detrimental consequences on embryo viability (Scott *et al*, 2013). However, embryo biopsy is considered an invasive procedure and the safe outcome is highly dependent on the operator's skills and experience (Nagy *et al*, 2012). Additionally, the use of a laser beam to separate the biopsied tissue from the main embryo carries the possibility of invisible thermal damage/stress (Gardner *et al*, 2012).

The application of microsuction technique to obtain a sample of embryonic origin greatly reduces embryo micromanipulation and invasiveness. Additionally, the correct application of Blastocentesis does not require highly specialised training and can be performed without the use of laser devices, usually adopted to partially remove the zona pellucida and for detachment of the biopsied cells from the rest of the embryo. For these reasons, I investigated the use of blastocoel fluid as a source of DNA for preimplantation genetic testing.

The aim of this set of experiments was to determine whether it is possible to detect DNA within the blastocoel fluid. For this purpose, a total of 17 embryos were investigated. The study was divided into two experiments (**Figure 3-1**), both requiring blastocoel microaspiration and separate collection of the fluid and the embryo in individual PCR tubes. Results from the blastocoel samples were compared with the embryonic reference sample:

- One set of sample pairs was used to investigate DNA origin and to test the feasibility of detecting small target sequences within the fluid.
- Another set of sample pairs were used to assess the application of current BAC microarray Comparative Genome Hybridisation (CGH) technology to blastocoel DNA.

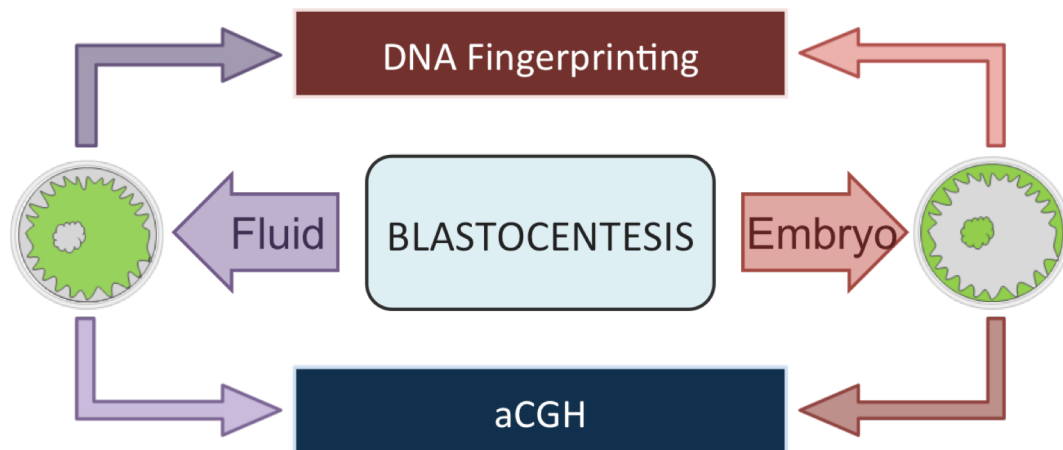


Figure 3-1 - Blastocoel genomics experimental workflow

3.2 DNA FINGERPRINTING

3.2.1 Short Tandem Repeats

DNA fingerprinting is a PCR based technique that allows the simultaneous investigation of multiple Short Tandem Repeats (STR) within the genome (Le *et al*, 1998). STRs are composed of short nucleotide sequences (2-7 bp) repeated a variable number of times in a tandem (head-to-tail) manner and located at precise genomic sites. The number of repeats in a specific STR is essentially the same in all cells of an individual. However, the length of the STR can differ within the population. Indeed, STRs loci are hypervariable in nature, having multiple alleles each comprising a different number of repeat units. STR lengths follow parental inheritance and for each STR, progeny will inherit one allele from each parent. The occurrence of inheriting different STR lengths for a specific locus is termed heterozygosity (HT). The higher the HT, the more unlikely that an individual will have two identical STR alleles, each having the same number of repeat units. However, in a population with a high rate of consanguinity, HT can be greatly reduced as the majority of the individuals inherited their STRs from a small group of ancestors with a restricted diversity of alleles. The more STRs are shared between two people, the more likely they are related. If enough loci are tested in two biological samples, matching of all alleles is a proof that both samples derived from the same individual.

3.2.2 DNA Fingerprinting protocol development and validation

To allow a reliable confirmation of DNA origin, I developed a procedure investigating 8 highly polymorphic STR regions on 7 different chromosomes. I selected the STRs regions depending on their chromosome location, their HT rate and the length of the fragment generated (**Table 3-1**).

Table 3-1 - Details of STR investigated

Marker name	Repeat type	Heterozygosity	Chromosome	Min - Max expected size	Fluorochrome	Multiplex reaction
D14S68	2	0.91	14	140 - 172	HEX	A
D15S659	4	0.88	15	166 - 210	6-FAM	
DXS8091	2	0.87	X	89 - 111	6-FAM	
D3S1581	2	0.88	3	130 - 150	6-FAM	B
D7S2847	4	0.86	7	165 - 209	HEX	
AMEL	na	na	X and Y	106 (X and Y) - 111 (Y only)	6-FAM	
D2S389	2	0.86	2	188 - 220	HEX	C
D4S2964	2	0.77	4	170 - 200	6-FAM	

For each locus of interest I designed PCR primers that anneal upstream and downstream each specific STR regions. These primers also incorporated different fluorescent dyes (HEX, green; FAM-6, blue) that help identify the final product when analysed with a capillary electrophoresis system. After DNA extraction, the amplification of the loci of interest was performed on two subsequent PCR reactions. In the first, all the loci were amplified in a single step. In the second phase, two or three loci were amplified in three multiplex reactions. The number of loci to amplify in each reaction was decided based on the expected length of the fragments and the fluorescent dye incorporated in the primer. After the two rounds of amplification, the

products of the reactions were loaded on an ABI PRISM 3100 Genetic Analyzer device (Applied Biosystems, Thermo Fisher, USA) where product length was analysed using capillary electrophoresis.

Initially, I tested the primers for first and second step PCR on buccal cells-derived from the genomic DNA of three donors. Depending on the results obtained from capillary electrophoresis, the concentration of each of the primer pairs was adjusted to normalise the signal (loci that gave a weaker signal were compensated by increasing primer concentration in the reaction). Subsequently, the performance of the DNA fingerprinting method at the single cell level (equivalent to cells biopsied from embryos) was evaluated by assessing locus amplification failure and allele drop-out (ADO) rates for each locus in 50 single buccal cells of an informative subject (see **Supplementary Table A**, in section 3.6). The locus amplification failure parameter reflects the ability of a specific primer set to successfully amplify the region of interest. A high locus amplification failure rate (>10%) may require the use of a different set of primers. The allele drop-out parameter, indicates whether a certain allele is preferentially amplified, compared to its homologous. When allele drop-out happens, the analysis of a heterozygous sample, reveals one single allele for the affected locus. This is due to the failure of amplification of one of the two alleles. The use of primers that produce high rates of ADO (>10%) increase the chance of generating false negative data, failing to detect the presence of heterozygosity. The development of single gene disorder tests for preimplantation genetic diagnosis (PGD) requires extensive assessment of locus-specific ADO rates. Linkage markers are also assessed to increase robustness of the test. Accuracy and sensitivity of tests for

PGD are fundamental to prevent the occurrence of misdiagnosis due to failed detection of the affected allele. However, due to the non-diagnostic nature of this experiment, less stringent thresholds for ADO rates were applied, allowing up to 12% risk of allele detection failure.

After validation, I performed DNA fingerprinting on 24 paired samples (11 blastocoels and 11 embryos).

Results

In the protocol validation experiments, 3 of the 50 tested single cells failed to amplify any of the loci investigated (total amplification failure 6%). However, individual locus amplification failure ranged between 4.2% and 10.6% (av. 6.1%) and ADO ranged between 2.3% and 12% (av. 7.5%), as shown in **Table 3-2**.

Table 3-2 - Locus amplification and Allele Drop Out rates

	Chr 14	Chr 15	Chr X	Chr 3	Chr 7	AMEL	Chr 2	Chr 4	Average
Locus amplification failure	4.2% (2/47)	4.2% (2/47)	4.2% (2/47)	6.4% (3/47)	4.2% (2/47)	6.4% (3/47)	10.6% (5/47)	8.5% (4/47)	6.10%
Allele Drop Out	4.4% (2/45)	8.8% (4/45)	n / a	11.4% (5/44)	11.4% (3/45)	2.3% (1/44)	12% (5/42)	2.3% (1/43)	7.50%

The majority of loci in the 11 embryo samples amplified successfully. However, only in one blastocoel sample enough alleles were detected to allow a comprehensive comparison between the embryo and blastocoel samples (**Figure 3-2**).

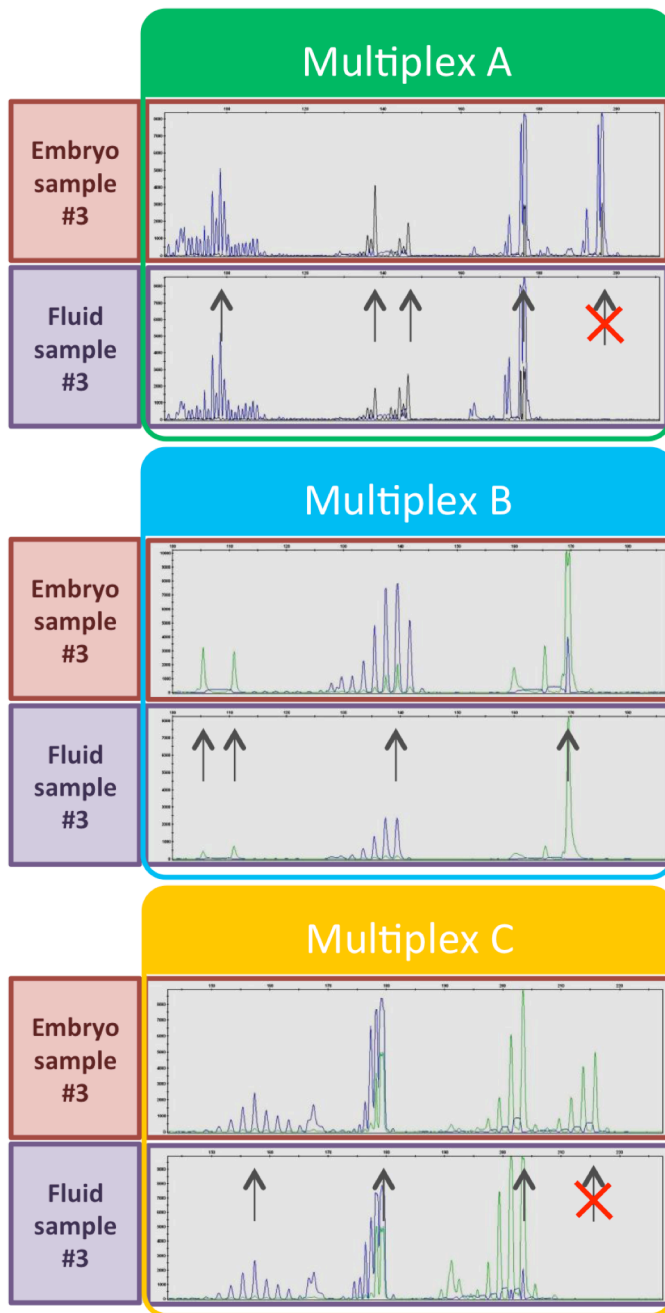


Figure 3-2 - Comparison in allele detection between embryo and blastocoel samples. Arrows show the identification of the alleles in both blastocoel and embryo samples. In where arrows are crossed, the allele was not detected in the blastocoel sample.

In this sole sample (#3), 11 out of 13 (84.6%) expected alleles were detected, confirming the embryonic origin of the DNA detected in the blastocoel. However, in the remaining 10 blastocoel pairs, only 14 out of 132 expected alleles were detected

(average 9.9%, range 0%-41.7%), prohibiting a fingerprinting analysis of the samples (Table 3-3).

Operator contamination was not detected in any of the sample. However, in pairs #4 and #11 a single, unexpected allele was detected (D7S2847 and D3S1581 respectively), suggesting the presence of fragments of contaminating DNA.

Table 3-3 - Dataset of DNA fingerprinting on blastocoels and relative embryos

	Multiplex 1			Multiplex 2			Multiplex 3		Patient
Sample N	Chr 14	Chr 15	Chr X	Chr 3	Chr 7	AMEL	Chr 2	Chr 4	
Operator	146	180 / 200	87	136 / 149	174 / 185	106 / 112	200	187 / 193	MP
Positive	146	180 / 200	87	136 / 149	174 / 185	106 / 112	200	187 / 193	
Negative	-	-	-	-	-	-	-	-	
E Negative	-	-	-	-	-	-	-	-	
B Negative	-	-	-	-	-	-	-	-	
E 1	138 / 146	176 / 196	98	140	170	106 / 112	204 / 216	157 / 179	A
B 1	138 / 146	176	98	140	170	106 / 112	204	157 / 179	A
E 2	138 / 148	176 / 196	98	140	170 / 193	106	199 / 216	157 / 179	A
B 2	-	-	-	-	-	-	-	-	A
E 3	138 / 147	180 / 196	102	140	170 / 189	106	199/204	157 / 179	A
B 3	-	-	-	-	-	106	-	-	A
E 4	146	180 / 192	-	135 / 144	185 / 189	106 / 112	197 / 216	179	B
B 4	146	-	-	142	170	106	-	179	B
E 5	146 / 151	188 / 192	98	132 / 146	174 / 185	106 / 112	210 / 216	179 / 181	C
B 5	-	-	-	-	-	-	-	-	C
E 6	146 / 151	176	-	144	178 / 189	106	210 / 216	179 / 182	C
B 6	-	-	-	-	-	-	-	-	C
E 7	146 / 151	176 / 188	98	144	178 / 189	106	197 / 216	182 / 187	C
B 7	-	-	-	-	-	-	-	-	C
E 8	135 / 137	187 / 200		142 / 151	170	106 / 112	192 / 216	176 / 187	D
B 8	-	-	-	-	-	-	-	-	D
E 9	137 / 151	180 / 184	84	140 / 148	181	106 / 112	192 / 198	176 / 187	D
B 9	151	-	-	140	-	-	-	-	D
E 10	143 / 145	172 / 180	98	140 / 152	166 / 174	106	198 / 218	176 / 180	E
B 10	-	-	-	-	166	106	198	176	E
E 11	143 / 145	180 / 200	98	136 / 140	166 / 170	106	192 / 214	176 / 182	F
B 11	-	-	-	152	-	-	-	-	F

3.2.3 Discussion

A DNA fingerprinting method, applicable to single cells, was developed in order to investigate both the origin of the DNA detected within the blastocoel and the feasibility of assessing the presence of small target sequences in the fluid. The fingerprinting protocol was thoroughly validated on a highly informative donor. All alleles were detectable with relatively low ADO rates, confirming suitability of the protocol for the investigative purpose. The sample procurement procedure and aseptic technique appear to have been acceptable as the operator's fingerprint was not detected in any of the samples assessed during the initial validation nor during the experimental runs. The injection/suction micropipette and the flexipets used were abundantly washed between sample collections, in order to minimize the risk of sample cross-contamination. Although not confirmed statistically, it seems reasonable to speculate that the DNA detected in the blastocoel is of embryonic origin.

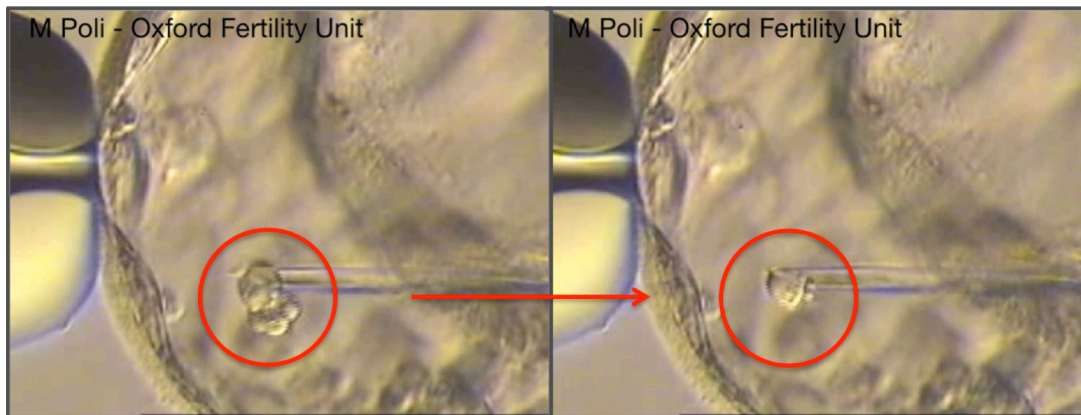


Figure 3-2 - Cellular fragments aspiration during microaspiration

This opinion derives from the fact that the vast majority of the alleles detected matched the corresponding embryonic profile. However, I experienced considerable

differences in the amplification rate of individual loci across blastocoel samples (average 17.2%, range 0% - 84.6% of loci amplified in each sample). This suggests that the amount of amplifiable DNA present in the blastocoel is highly variable. Locus amplification may have failed because the target sequences were not present in the blastocoel sample collected or the DNA was too fragmented and degraded.

The difference in results quality across blastocoel samples may have several causes. For example, some embryos may release more DNA within the cavity, possibly due to an increased apoptotic activity. If this condition involves multiple cells, more DNA templates would be available as targets for the PCR amplification, increasing the chance of producing a signal. However, if the apoptotic process is advanced, the DNA may be highly fragmented, possibly resulting in failed target amplification.

Additionally, it was noted that some blastocoel presented with large free-floating fragments that during microaspiration were aspirated together with the fluid (**Figure 3-3** and **Video E2** in the **Supplementary Electronic Material folder**). It is possible that compared to cell-free fluid, cellular fragments may contain larger amounts and/or better preserved genetic material, providing a more suitable template for PCR.

Given the fact that the great majority of loci did not amplify in blastocoel samples, it seems likely that this approach, at least using the blastocentesis methodology described here, could not be applied for preimplantation genetic diagnosis of single gene disorders.

Although most attempts at amplification of specific DNA fragments from blastocoel fluid were not successful, this does not necessarily mean that DNA present in the

blastocoel cannot provide information at the larger chromosomal level (i.e. aneuploidy detection).

3.3 COMPREHENSIVE CHROMOSOMAL SCREENING

3.3.1 Cytogenetic analysis by Comparative Genomic Hybridisation Arrays

In this part of the study I investigate whether the amount of DNA present in the blastocoel is of sufficient quality and amount to allow cytogenetic assessment for chromosomal imbalance. For this analysis I used a technology called microarray comparative genomic hybridisation (array CGH). This technology has been used for preimplantation genetic screening for several years and it is now commercially available, providing scientists with a robust and highly reproducible means of assessing embryos for aneuploidy (Wells *et al*, 2008).

To perform array CGH, one or more test cells are required. These cells are lysed and the DNA is amplified using whole genome amplification (WGA – SurePlex, Illumina, USA). Using random primers, the amplified DNA is then labelled with a cyanine-based fluorescent dye (Cy3, green). A reference normal male DNA (46,XY) is also labelled with a different dye (Cy5, red). Both test and reference DNAs are co-hybridised on a microarray slide where several thousand bacterial artificial chromosomes (BACs) have been immobilised. Each BAC clone populates a spot on the slide and covers between 150 and 350 Kb of the human genome. On average there is approximately one BAC per megabase of DNA and the microarray delivers an effective resolution (i.e. detection of losses and gains of chromosomal material) of between 10 and 20 Mb (BlueGnome, Illumina, USA). During hybridisation, the more represented of

the two samples anneals preferentially to the BACs. After allowing time for hybridisation, the microarray slide is washed of unbound DNA and read using a high definition scanner able to excite the fluorophores integrated in the sample DNA fragments. A customised software is able to quantify the amount of signal emitted by each molecule in different spots of the slide. Depending on the ratio between Cy3 and Cy5 signal, it is possible to determine whether a specific sequence is more represented in the test (gain in the test sample, trisomy) or in the reference sample (loss in the test sample, monosomy). By testing multiple sites for each chromosome, it is possible to acquire information regarding the chromosomal status of the cell(s) tested. This technique therefore allows the detection of full aneuploidies (gains and/or losses). Additionally, it allows the identification of deletions, partial chromosome gain or loss and screening of sex chromosomes.

On the other hand, array CGH does not allow detection of chromosomal abnormalities such as inversions, balanced translocations, very small gains or losses (<10 Mb) or single gene mutations (BlueGnome, Illumina, USA). In this experiment, I tested 6 paired samples (6 blastocoels and 6 embryos).

Results

As shown on **Figure 3-3**, all embryonic samples amplified successfully, giving a strong smear, composed of a diverse population of DNA fragments of differing lengths, when analysed on an agarose gel. Blastocoel samples also appeared amplified in some cases, although the smear was much weaker than the corresponding embryo. Nonetheless, all samples were processed for microarray analysis.

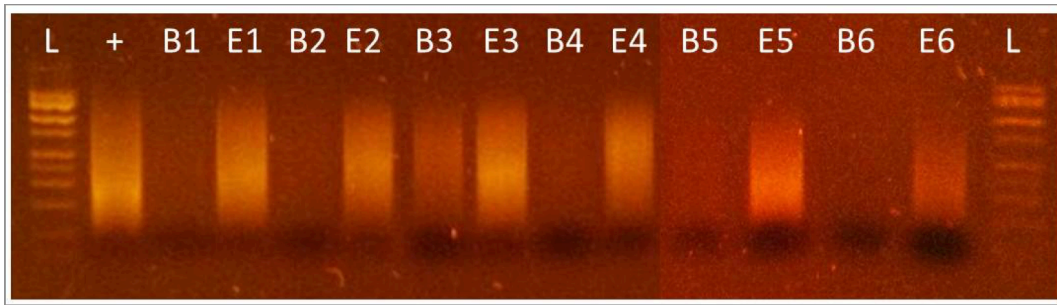


Figure 3-3 - Gel runs prior to labeling. B (Blastocoel), E (Embryo), L (Ladder), + (Positive control)

Microarray clone inclusion rate is a measure of successful microarray hybridization and is calculated based on the number of clones present on the array that provide a fluorescence signal of sufficient quality for analysis. Samples chromosomal status and clone inclusion rates are shown in **Table 3-4**.

These data show that clone inclusion rates for blastocoel samples are consistently lower than the corresponding embryonic sample suggesting a reduced DNA input or quality. In the majority of the samples, microarray hybridisation did not work, generating unreliable results. On the other hand, when sufficient DNA was collected from the blastocoel (#3), WGA protocol provided enough material for successful hybridisation and matching results between embryo and blastosol were detected.

Table 3-4 - Clone inclusion rates and karyotype details of sample pair analysed

Sample Pair	Embryo sample		Blastocoel sample		Difference in Clones inclusion rate	Matching results
	Karyotype	Clones inclusion rate (%)	Karyotype	Clones inclusion rate (%)		
1	46 XX, -15q	95.61	47 XY,+19	70.59	25.02	✗
2	46 XX	97.92	45 XO	80.86	17.06	✗
3	47 XY, +16	97.84	47 XY, +16	96.92	0.92	✓
4	47 XX, +9	98.03	48 XYY, +10	75.41	22.62	✗
5	47 XY, +1	33.76	48 XXY, +19	25.19	8.57	✗
6	46 XX	38.50	51 XX, +4, 6, 13, 14, 19	34.17	4.33	✗
Average		76.94		63.86	13.09	

3.3.2 Discussion

Although specific regions of the genome were not reliably detected after multiplex fingerprinting reactions, I could not exclude that a WGA approach could still allow the detection of cytogenetic abnormalities through microarray analysis and consequently a series of microarray experiments were undertaken.

Electrophoretic analysis of WGA products showed that blastocoel samples produced lighter smears on agarose gels, denoting suboptimal amplified DNA yields. This may be due to reduced amounts or quality of DNA collected from the blastocoel. In fact, the SurePlex protocol used for initial WGA has reduced efficiency in amplifying highly fragmented DNA.

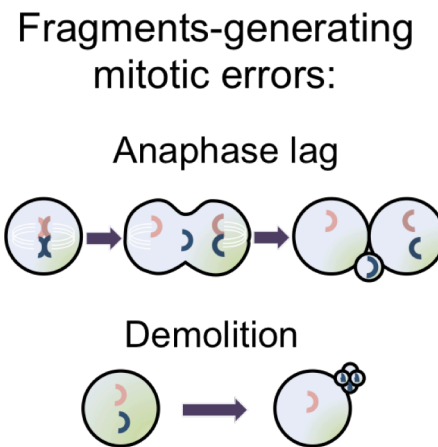
Post-hybridisation microarray clone inclusion rates were reduced for blastocoel samples, providing further evidence that certain regions of the genome were not effectively amplified from this sample type. This may be due to complete absence of parts of the genome in blastocoel samples due to high levels of DNA degradation. Qualitative differences were also noted on data plotted by the BlueFuse software, as the blastocoel samples generated more noisy profiles compared to those from embryo samples.

Two sample pairs (Samples #5 and #6) showed very low clone inclusion rates (average 32.5, range 25.2% - 38.5%) irrespective of whether they were collected from blastocoel or cellular samples. This group of specimens was processed on a different date from previous ones. The reason for these low quality results is unknown, however possible explanations could be suboptimal labeling or array hybridisation

procedures or an inconsistency between batches of microarrays and reagents kits used, as it affected both embryonic and blastocoel samples whilst collection method was unvaried.

Samples #1, #2 and #4 produced fairly high clone inclusions rates, however they failed to reproduce a cytogenetic profile similar to their corresponding embryos. Discrepancies involved both autosomes and sex chromosomes. One could therefore speculate that the cellular fragments collected via microsuction were not representative of the embryonic chromosomal status. An explanation of these outcomes can be found in biological processes called "anaphase lag" and "demolition" (Mantikou *et al*, 2012). Anaphase lag is an abnormality of mitotic division and consists of the loss of a chromosome due to failed alignment on the metaphase plate. The affected chromosome will often form its own micronucleus within one of the daughter cells and tends to be lost during subsequent cell divisions. Demolition

involves the preferential exclusion of a chromosome from the cell and incorporation and subsequent disposition into a cellular fragment. These processes have been proposed as hypothetical mechanisms of cellular self-correction of aneuploidy (Los *et al*, 1998) (**Figure 3-5**).



*Figure 3-4 - Schematic representation of mechanisms of DNA inclusion in cellular fragments (figure adapted from Mantikou *et al*, 2012)*

With these experiments it has been demonstrated that the blastocoel frequently, if not always, contains embryo-derived DNA. However, the amount of DNA detected in the cavity is variable, perhaps correlated with the amount of free-floating cellular fragments, aspirated during the procedure. According to our data, the use of DNA sourced from the blastocoel cannot be reliably used for the detection of single gene conditions using the procedures I employed. On some occasions, blastocoel DNA may be used to assess the cytogenetic status of the embryo. However, in our cohort study, the proportion of matching blastocoel/embryo chromosomal status is lower than 20%, suggesting that obtaining a clinically useful result is challenging.

Another research group has succeeded in generating embryo genetic profiles from blastocoel-extracted DNA (Gianaroli *et al*, 2014). In that study, signal from DNA collected from the blastocoel was detected in 39 out of 51 samples (76%) and blastosol and embryo karyotypes matched in 32 out of 39 samples (82%), with a final correct diagnosis efficiency of 62% (32/51).

Similarly to single gene testing, the amount of DNA available for testing may be directly influenced by the amount of fragments aspirated during microsuction. Deliberate aspiration of cellular fragments within the blastocoel may increase the amount of amplifiable DNA obtained.

However, these fragments may not carry genetic material that is representative of the rest of the embryo, potentially biasing embryo chromosomal status assessment and exposing to the risk of misdiagnosis. Therefore, more optimization to increase diagnostic rates and test procedure's accuracy should be carried out before genetic blastocentesis could be considered for clinical application. Nonetheless, despite the

current difficulties in obtaining valid data from blastosol samples, interest in this approach remains due to the multiple perceived advantages that an optimised blastocentesis protocol might deliver (e.g. minimally invasive method, not requiring an expensive laser, not requiring a biopsy-trained embryologist).

Alternative strategies to increase the DNA detection rate are being investigated by our group at the moment.

3.5 MATERIALS AND METHODS

3.5.1 Samples procurement

Thirty-four samples were enrolled in this study (11 embryo/blastocoel pairs). Embryos were created according to previously described protocol (see Chapter 2, 2.4.2) and processed fresh. Blastocoels were procured following the procedure described in Chapter 2, Blastocoel microsuction (see 2.4.3). All the microsuction procedures were video recorded and stored to allow potential retrospective analysis. After extraction of the fluid from the cavity, the sample was released from the injection pipette into a new 4 μ L drop of PBS (see **Figure 2-7**, section 2.4.3). This drop was then collected using a fine 130 μ m flexipet and expelled into a 0.2 μ L sterile PCR tube. The collapsed embryo was retrieved using a 300 μ m flexipet and collected in a separate, sterile 0.2 μ L PCR tube. All PCR tubes were kept at 4°C in a cold rack to avoid DNA degradation. All samples were then promptly frozen and maintained at -80°C until processing.

3.5.2 Genomic DNA collection and isolation

Buccal cells were used to isolate genomic DNA. Buccal cell collection was performed using a commercially available kit (SK-1S Buccal Swabs, Isohelix, UK). DNA isolation from buccal cells was performed using a commercially available kit (DDK-50, DNA Isolation kit, Isohelix, UK) and stored at 4°C until processed.

3.5.3 Buccal cells isolation

A sample of saliva was collected from the donor by rinsing the buccal cavity with PBS. A small aliquot of this sample was then moved on a dish where several drops of PBS 0.1 PVA were plated. Single cells were isolated under a dissection microscope by using a 300 μm wide flexipet. Each cell was repeatedly washed by moving it into a clean drop 4-5 times and finally transferred within 2 μL of solution into a 0.2 mL sterile PCR tube. This operation was repeated for 50 cells. Buccal cells isolation was performed in a sterile pre-amplification clean room.

3.5.4 PCR protocols

All samples (single buccal cells, whole embryos and blastocoels) were lysed by adding 1.5 μL of alkaline lysis buffer and processed at 65°C for 10 minutes before being held at 4°C. Both first and second multiplex PCR reactions were performed following standard protocols. Details of mastermixes and thermocycler programs can be found in **Table 3-5**.

Table 3-5 - PCR Mastermixes and thermocycler programs

Reaction Cocktails		Thermocycler settings		
Reagent	Volume (μL)	Temp	Time	Cycles
<u>Alkalyne Lysis Buffer</u>		<u>"Alkaline Lysis" program</u>		
H ₂ O	0.45	65°C	10'	x 1
DTT	0.75	4°C	pause	
NaOH	0.3	<u>"Qiagen Microsatellite" short program</u>		
Sample	2.0	95°C	15'	x 1
Total	3.5	94°C	30"	
<u>Multiplex PCR - 1st round</u>		59°C	90"	x 20
H ₂ O	5.82	72°C	1'	
Tricine (0.4M)	2.5	60°C	10'	x 1
Qiagen Mastermix	12.5	4°C	pause	
D2S389 F+R (10μM)	0.21	<u>"Qiagen Microsatellite" long program</u>		
D3S1581 F+R (10μM)	0.21	95°C	15'	x 1
D4S2964 F+R (10μM)	0.21	94°C	30"	
D7S2847 F+R (10μM)	0.21	59°C	90"	x 45
D14S68 F+R (10μM)	0.21	72°C	1'	
D15S659 F+R (10μM)	0.21	60°C	10'	x 1
DXS8091 F+R (10μM)	0.21	4°C	pause	
AMEL F+R (10μM)	0.21	<u>Denaturation program</u>		
Lysis Buffer + S.C DNA	2.5	95°C	3'	x 1
Total	25	4°C	pause	
<u>Multiplex PCR - 2nd round</u>				
Reaction A				
	15μl			
H ₂ O	6.31			
Qiagen Mastermix	7.5			
D14S68 F+R (10μM)	0.3			
D15S659 F+R (10μM)	0.21			
DXS8091 F+R (10μM)	0.18			
DNA	0.5			
Reaction B				
	15μl			
H ₂ O	6.4			
Qiagen Mastermix	7.5			
D3S1581 F+R (10μM)	0.21			
D7S2847 F+R (10μM)	0.21			
AMEL F+R (10μM)	0.18			
DNA	0.5			
Reaction C				
	15μl			
H ₂ O	6.58			
Qiagen Mastermix	7.5			
D2S389 F+R (10μM)	0.21			
D4S2964 F+R (10μM)	0.21			
DNA	0.5			

3.5.5 Calculation of PCR efficiencies

Total amplification failure was calculated as number of samples with failed amplification of all loci, over the number of samples processed. Locus amplification failure rate was calculated as the number of samples where the locus investigated failed to amplify, over the total amount of samples processed. Locus amplification failure rate does not account for those samples affected by Total amplification failure. Allele drop-out rate was calculated as the number of samples where one of the two expected alleles failed to amplify, over the total number of samples processed. ADO rate does not account for those samples affected by Total or Locus amplification failure. ADO rate cannot be calculated for those loci where only one allele is possible (homozygous or X-locus in males).

3.5.6 Cell lysis, DNA pre-amplification and checkpoint assessment

All samples were processed using a commercially available kit for aCGH (Sureplex, Blugnome, Illumina, USA) and following manufacturer's instructions. Both blastocoel and whole embryo samples were collected in 2 μ L of PBS and frozen at -80°C. At time of processing, they were quickly thawed and, while maintained on ice, 3 μ L of Cell Extraction Buffer were added to each individual tube. This step was performed in a dedicated pre-amplification “clean room” to avoid contamination from volatile amplified DNA.

After preparation on ice, 5 μ L of Extraction Mastermix were added to each tube, gently mixed and incubated in a thermocycler (**Table 3-6**).

Table 3-6 - DNA extraction mastermix and thermocycler program

<u>Extraction Mastermix</u>		<u>Extraction Program</u>		
	<u>Volume (μL)</u>	<u>Temp</u>	<u>Time</u>	<u>Cycles</u>
Extraction enzyme dilution buffer	4.8	75°C	10'	x1
Cell extraction enzyme	0.2	95°C	4'	x1
Total	5.0	RT	Hold	x1

A Pre-WGA Mastermix containing SurePlex pre-amplification buffer and enzyme was prepared on ice. 5 μ L of this solution were dispensed in each tube and incubated as shown in **Table 3-7**.

Table 3-7 - Pre-WGA mastermix and thermocycler program

<u>Pre-amplification Mastermix</u>		<u>SurePlex Pre-amplification Program</u>		
	<u>Volume (μL)</u>	<u>Temp</u>	<u>Time</u>	<u>Cycles</u>
SurePlex Pre-amp buffer	4.8	95°C	2'	x1
SurePlex pre-amp enzyme	0.2	95°C	15"	} x12
Total	5.0	15°C	50"	
		25°C	40"	
		35°C	30"	
		65°C	40"	
		75°C	40"	} x1
		4°C	Hold	

Similarly, the WGA Mastermix was prepared and 60 μ L of the solution were added to each sample prior to incubation (**Table 3-8**).

Table 3-8 - WGA mastermix and thermocycler program

<u>Amplification Mastermix</u>		<u>SurePlex Amplification Program</u>		
	<u>Volume (μL)</u>	<u>Temp</u>	<u>Time</u>	<u>Cycles</u>
Nuclease-free water	34.2	95°C	2'	x1
SurePlex amplification buffer	25	95°C	15"	} x14
SurePlex pre-amp enzyme	0.8	65°C	1'	
Total	60.0	75°C	1'	
		4°C	Hold	x1

This incubation was performed in a post-amplification room and the samples were never brought back to the “clean room” to prevent room contamination with amplified DNA. At the end of the DNA amplification step, 5 μ L of each sample were mixed with 2 μ L of BlueJuice Gel Loading Buffer (Invitrogen, Life Technologies, Thermo-Fisher, USA) and individually loaded on a 1% agarose 1X TBE gel containing a DNA-intercalating fluorescent dye (GelRed, Biotium, USA). The samples were run on the electrophoresis device (Bio-Rad, USA) at 200 V for 10 minutes. Assessment of DNA amplification was performed by checking the gel under a UV source. The presence of a fluorescent smear band confirmed successful amplification (see **Figure 3-4**, section 3.3.1).

3.5.7 DNA labeling and microarray hybridisation

In this step of the experiment, all procedures and master mix preparations were performed on ice unless otherwise stated. Two mastermixes were prepared according to the number of samples successfully amplified. One master mix contained Cy3 fluorescent dye for labelling of the test samples, and the other contained Cy5 dye for labelling of the reference DNA. The test sample mastermix (TM) was composed of a reaction buffer, random primers, dCTP labelling mix and Cy3 dCTPs (**Table 3-9**). Reference sample mastermix (RM) consisted of the same components as TM (apart Cy5 dCTPs instead of Cy3 dCTPs), plus an aliquot of normal male reference DNA. In a new 0.2 mL PCR tube, 16 μ L of TM were mixed with an 8 μ L aliquot of test DNA (6 produced from single blastocoel and 6 from individual blastocysts).

Table 3-9 - Test (TM) and Reference (RM) Samples Labelling Mastermixes

<u>Test Sample labelling Mastermix TM</u>		<u>Reference labelling Mastermix RM</u>	
	<u>Volume (μL)</u>		<u>Volume (μL)</u>
Reaction buffer	5	Reaction buffer	5
Primer solution	5	Primer solution	5
dCTP-labelling mix	5	dCTP-labelling mix	5
Cy3 dCTP	1	Cy5 dCTP	1
		SureRef (XY Reference DNA)	8
Total	16	Total	24

For each test sample, a 24 μL aliquot of RM was tubed (12). All samples were incubated for 5 minutes at 95°C to achieve DNA denaturation and immediately transferred to ice for 5 minutes to block any re-annealing process. An aliquot of Klenow enzyme was added to each reaction and then left to incubate for 1 hour and 30 minutes at 37°C. The Klenow enzyme is a "molecular fragment" of DNA Pol I which maintains DNA-dependant 5'-3' polymerase function that requires a 3' primer site, together with a 3'-5' exonuclease activity. During incubation, the Klenow enzyme uses the denatured DNA as a template to produce a new DNA strand incorporating labelled nucleotides. Each test sample was then combined with one reference sample. Additionally, 25 μL of COT Human DNA were added to each assembled sample, to block any non-specific cross-hybridisation of interspersed repetitive sequences (IRS) and other highly represented repetitive elements. These assembled samples were then concentrated by speed vacuuming for 43 minutes at 75°C. After centrifugation, 1-2 μL of concentrated DNA were thoroughly resuspended in 21 μL of pre-warmed DS Hybridisation Buffer (15% dextran sulfate) and then denatured at 75°C for 10 minutes. After denaturation, 19 μL of each combined sample (test and reference DNA) were loaded on a 24sure High Resolution v2.0 microarray slide (BlueGnome, Illumina,

USA). All hybridisation areas were covered by glass cover-slips and placed in a previously prepared hybridisation chamber. This chamber contained a sterile paper tissue embedded with a solution containing formamide (Sigma, USA), saline sodium citrate buffer (SSC, Fisher Scientific, USA) and water. This buffer stabilises single stranded DNA increasing stringency of the hybridisation reaction. The chamber was then sealed with Parafilm® (Bemis, USA) and placed in a 47°C closed water bath and left to incubate overnight. In the morning, slides were recovered from the chamber and placed in a coplin jar containing detergent solution (**Table 3-10**), where coverslips were carefully removed one by one. The slides were then placed in a slide holder and sequentially moved across 4 washing steps (**Table 3-10**).

Table 3-10 – Details of microarray slide washing steps

	Volume	Temp	Time	Agitation	Buffer
Cover glass removal	100 mL	RT	20"	None	2X SSC / 0.05% Tween20
Wash 1	500 mL	RT	10'	2.5 cm stir bar	2X SSC / 0.05% Tween20
Wash 2	500 mL	RT	10'	2.5 cm stir bar	1X SSC
Wash 3	500 mL	59°C	5'	None	0.1X SSC
Wash 4	500 mL	RT	1'	2.5 cm stir bar	0.1X SSC

At the end of the washing process, slides were centrifuged at 170 x *g* for 3 minutes to remove any buffer residues. While waiting to be processed with the scanner, microarray slides were placed in a dark box to avoid weakening of the fluorescent dyes.

3.5.8 Microarrays reading

Microarray slides were analysed using a InnoScan 700 scanner (Innopsys, France). To reduce background noise, this device applies specific wavelengths laser beams that excite the labelling fluorochromes (532 nm for Cy3 and 635 nm for Cy5). While exciting the cyanine dyes, the device takes detailed scans of the thousands of spots on the slide. The BlueFuse software (BlueGnome, Illumina, USA) is then able to distinguish the emission signal from each labeling dye and, quantifying the respective indicator, compare the amount of each dye present in the spot. Since each spot on the array corresponds to a specific region on a chromosome, the analysis of thousands of locations allows precise mapping of the whole genome. Calculating the Cy3/Cy5 ratio for each of these locations, the BlueFuse software is able to graphically show chromosome copy number imbalances in the whole genome (**Figure 3-6**).

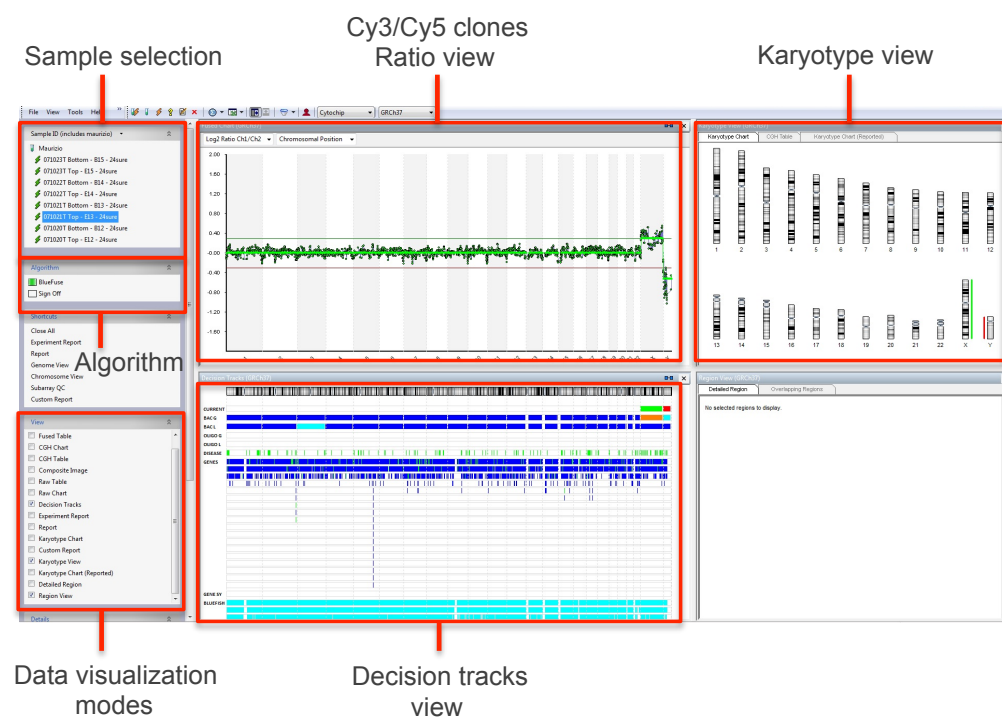


Figure 3-6 - BlueFuse Software layout. Example of a normal female (46,XX) sample

3.6 SUPPLEMENTARY MATERIAL

Supplementary Table A - Fingerprint protocol validation data

(Light red: Total amplification failure; Light blue: Locus amplification failure; Light green: Allele Drop-Out)

	Multiplex 1			Multiplex 2			Multiplex 3	
Sample N	Chr 14	Chr 15	Chr X	Chr 3	Chr 7	AMEL	Chr 2	Chr 4
Expected	145 / 147	188 / 200	87	132 / 142	170 / 185	106 / 112	197 / 212	176 / 189
Positive	✓ / ✓	✓ / ✓	✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓
Negative	X / X	X / X	X	X / X	X / X	X / X	X / X	X / X
Negative	X / X	X / X	X	X / X	X / X	X / X	X / X	X / X
1	✓ / ✓	✓ / ✓	✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓
2	X / X	X / X	X	X / X	X / X	X / X	X / X	X / X
3	✓ / ✓	✓ / ✓	✓	✓ / X	X / X	✓ / ✓	X / X	✓ / ✓
4	✓ / ✓	✓ / ✓	✓	✓ / X	✓ / ✓	X / X	✓ / X	✓ / ✓
5	✓ / ✓	✓ / X	✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓
6	X / X	✓ / X	✓	X / X	X / X	X / X	X / X	X / X
7	✓ / ✓	✓ / ✓	✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓
8	✓ / ✓	✓ / ✓	✓	✓ / X	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓
9	X / X	X / X	X	X / X	X / X	X / X	X / X	X / X
10	✓ / ✓	✓ / ✓	✓	✓ / ✓	X / X	✓ / ✓	✓ / ✓	✓ / ✓
11	✓ / ✓	✓ / ✓	✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓
12	✓ / ✓	✓ / ✓	✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓
13	✓ / ✓	✓ / ✓	✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓
14	✓ / ✓	✓ / ✓	✓	✓ / X	✓ / X	✓ / ✓	✓ / X	✓ / X
15	✓ / ✓	✓ / ✓	✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓
16	X / X	X / X	X	X / X	X / X	X / X	X / X	X / X
17	✓ / ✓	✓ / ✓	✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓
18	✓ / ✓	X / X	✓	X / X	X / X	✓ / ✓	X / X	X / X
19	✓ / ✓	✓ / ✓	✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓
20	✓ / ✓	✓ / ✓	✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓
21	✓ / ✓	✓ / X	✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓
22	✓ / ✓	✓ / ✓	✓	✓ / ✓	✓ / X	✓ / ✓	X / X	✓ / ✓
23	✓ / ✓	✓ / ✓	✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓
24	✓ / ✓	✓ / ✓	✓	✓ / X	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓
25	✓ / ✓	✓ / ✓	✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓
26	✓ / X	✓ / ✓	✓	X / X	✓ / ✓	X / X	X / X	X / X
27	✓ / X	✓ / ✓	✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓
28	✓ / ✓	✓ / ✓	✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓
29	✓ / ✓	✓ / ✓	✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓
30	✓ / ✓	✓ / ✓	✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓
31	✓ / ✓	✓ / ✓	✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓
32	✓ / ✓	✓ / X	✓	✓ / ✓	✓ / ✓	✓ / ✓	X / X	✓ / ✓
33	✓ / ✓	✓ / ✓	✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓
34	✓ / ✓	✓ / ✓	✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓
35	✓ / ✓	✓ / ✓	✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓
36	✓ / ✓	✓ / ✓	✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓
37	X / X	X / X	✓	✓ / ✓	✓ / ✓	X / X	✓ / X	X / X
38	✓ / ✓	✓ / ✓	✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓
39	✓ / ✓	✓ / ✓	✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓
40	✓ / ✓	✓ / ✓	✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓
41	✓ / ✓	✓ / ✓	✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓
42	✓ / ✓	✓ / ✓	X	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓
43	✓ / ✓	✓ / ✓	✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓
44	✓ / ✓	✓ / ✓	✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / X	✓ / ✓
45	✓ / ✓	✓ / ✓	✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓
46	✓ / ✓	✓ / ✓	✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓
47	✓ / ✓	✓ / ✓	✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓
48	✓ / ✓	✓ / ✓	✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓
49	✓ / ✓	✓ / ✓	✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓
50	✓ / ✓	✓ / ✓	✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓	✓ / ✓

3.7 CHAPTER 3 BIBLIOGRAPHY

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4. GENE EXPRESSION OF THE HUMAN BLASTOCYST

4.1 Introduction

4.2 ICM excision procedure

4.3 Gene expression analysis by microarrays

4.3.1 Samples quality control and data overview

4.3.2 Biological process and molecular function over-representation

4.4 Gene expression analysis by RNASeq

4.4.1 Samples quality control and data overview

4.4.2 Biological process and molecular function over-representation

4.4.3 Differential gene expression in ICM and TE

4.5 Discussion

4.5.1 Active biological processes in the blastocyst

4.5.2 Estrogen and blastocyst gene expression

4.5.3 Considerations on blastocyst gene expression experiments

4.6 Materials and Methods

4.7 Bibliography

4.1 INTRODUCTION

Normally fertilised oocytes possess two pronuclei, each containing a haploid set of chromosomes, one deriving from the oocyte and one from the sperm cell. Through the process of syngamy, pronuclei contents merge together, forming a final DNA complement of 46 chromosomes. The resulting zygote is able to divide and give rise to all the cell lineages that form the adult organism. For this unique characteristic, the zygote is classically defined as totipotent. During the first two divisions, cellular physiology is controlled by transcripts and proteins inherited from the oocyte cytoplasm. It was shown that at least one blastomere of a 4-cell stage embryo still maintains totipotency (Veiga *et al*, 1987). After embryonic genome activation (EGA) (Braude *et al*, 1988), as replication continues, cells lose their totipotency, beginning to show distinct localisation and function within the embryo. Thus, through epigenetically-controlled preferential activation and suppression of specific gene sets, cells are directed towards a distinct differentiation pathway.

Around 5 days after insemination, a blastocyst shows two morphologically distinct sets of cells, the Inner Cell Mass (ICM or embryoblast) and the Trophectoderm (TE or trophoblast). The TE cells arrange in a single layer forming a sphere filled with embryo secretions, the blastocoel fluid. This fluid surrounds the ICM protruding into the cavity. After implantation, these defined structures develop into embryonic and extra-embryonic tissues. The TE will contribute to the embryonic component of the placenta (cytotrophoblast).

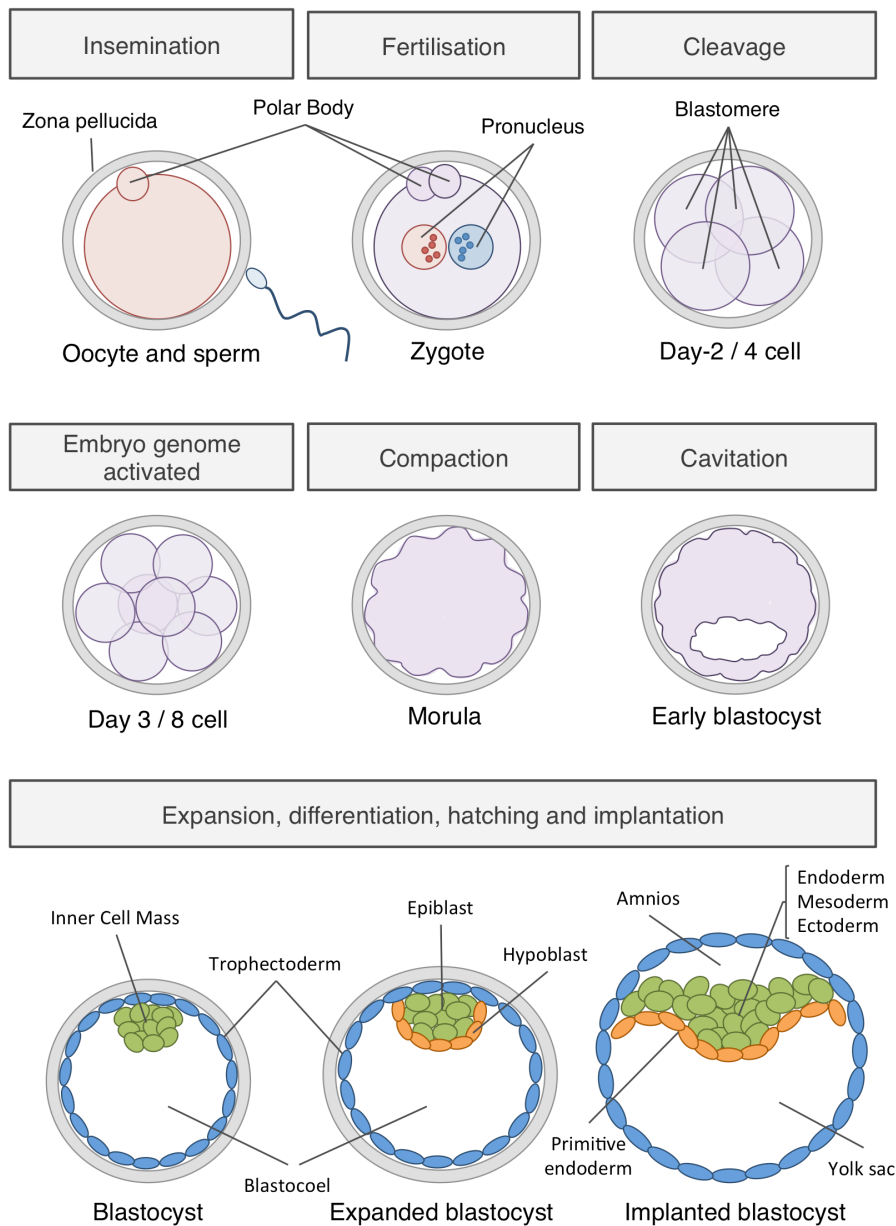


Figure 4-1 – Embryo development stages from fertilization to implantation.

Two types of cells derive from the ICM, the epiblast and the hypoblast (**Figure 4-1**). The first is located in the distal section of the ICM and it is responsible for the formation of all embryonic tissues (mesoderm, endoderm and ectoderm). For this characteristic of pluripotency, the epiblast is used to derive human embryonic stem

cell lines (hESC). The hypoblast is arranged as a layer of cells in the proximal section of the embryoblast and is in direct contact with the blastocoel fluid. These cells will form the primitive endoderm tissue, developing into the yolk sac (**Figure 4-2**).

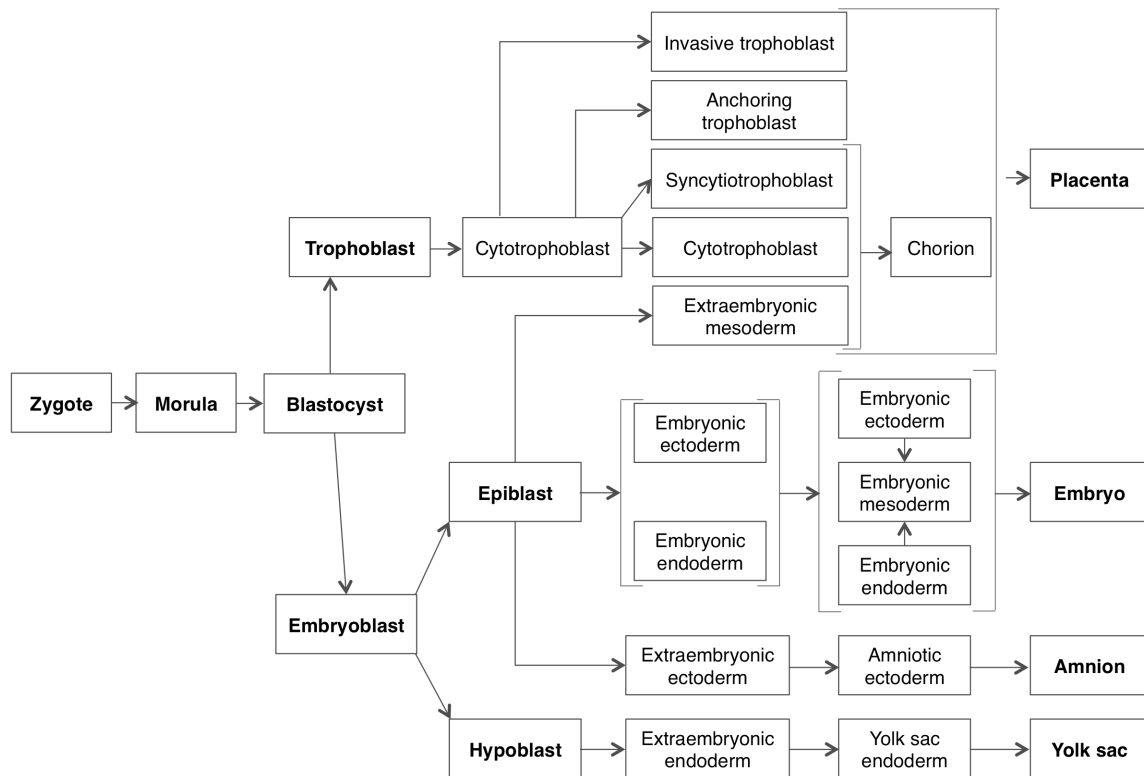


Figure 4-2 - Human differentiation patterns from zygote to fetus. Scheme adapted from Lebedev 2011 (Lebedev, 2011)

Gene expression activation and inhibition patterns are modulated by promotion and suppression of key transcription factors controlled by complex interacting molecular cascades and signalling pathways. These expression patterns modify the biological characteristics of the cells, which progressively acquire more defined and specialised functions. This gradual cellular diversification takes place across various cell replication cycles and it is controlled by epigenetic DNA modifications driving differential RNA and protein expression.

Although it is of major importance to understand the molecular basis of pluripotency, the mechanisms controlling this unique cellular state remain relatively poorly understood.

A few groups have investigated differences in gene expression across human embryos at different developmental stages. These studies were carried out using both microarray and quantitative PCR technology. In one of these studies, authors provided evidence that embryo genome activation occurs in waves of transcriptional activity in the early embryo (Vassena *et al*, 2011). Additionally, they show that degradation of maternal mRNA also occurs following stage-specific patterns (Vassena *et al*, 2011). Another study by Shaw and colleagues reported that embryos at the same developmental stage show high heterogeneity of transcripts profiles, indicating high variability in the transcriptional mechanisms controlling early embryo development (Shaw *et al*, 2013). Also, authors suggest that this high transcriptional heterogeneity could be at the basis of different implantation potential of human embryos. A study from Galán and colleagues showed that embryos developed between 5 and 8 cells show similar transcriptional profiles for genes associated to ICM and TE differentiation, suggesting that blastomeres at this developmental stage are not yet committed to a specific cell lineage (Galán *et al*, 2010).

More recently, single-cell RNASeq has been employed to assess gene expression in human and mouse embryos, as well as hESC. These studies gave the possibility to compare early differences in transcriptional profiles of ICM and TE lineages, allowing a more in depth exploration of the molecular pathways controlling

cellular pluripotency, proliferation and differentiation (Yan *et al*, 2013; Xue *et al*, 2013).

In this chapter, similarly to previous studies, I set out to define the transcriptome of the first two cell lineages in the human embryo. This set of experiments is intended to provide a catalogue of the genes expressed in the blastocyst that developed under the conditions employed in our laboratory. Also, I highlight the variations in gene expression between ICM and TE that may play a role in downstream differentiation of cell lineages. These experiments served as validation for the proteomic work discussed in Chapter 5.

4.2 ICM EXCISION PROCEDURE

In order to investigate gene expression differences between the ICM and the TE, the ICM was mechanically isolated from the rest of the embryo. Only embryos that showed good morphological features were included in these experiments, as the success of the manipulation depending on clear visualisation of the ICM. All the embryos enrolled in this study possessed a well-formed ICM and an appropriate TE wall, scoring at least B for the ICM and C for the TE (see section 2.4.7 in Chapter 2). The embryos were donated to research by patients that provided informed consents following standard HFEA procedure. Embryos were donated frozen both at cleavage and blastocyst stage. Once thawed, embryos were cultured until they reached full blastocoel expansion and were then assessed for cellular damage. Suitable samples were subjected to ICM excision procedure. For this intervention, I opted for a combination of micromanipulation and laser pulse dissection techniques. Unlike immunosurgical techniques, often used to retrieve ICMs for hESC line production, the micromanipulation approach allows collection of cells from the TE, avoiding its damage by antibody/complement complex-mediated digestion. This strategy enabled us to pair data resulting from ICM and TE cells deriving from the same embryo, strengthening statistical robustness of the experiment and reducing the number of embryos required for the study.

ICM excision was carried out using a standard Integra system kindly provided by RI (Research Instruments Ltd, United Kingdom). To create access through the zona

pellucida and detach the ICM from the TE, I used a Saturn 3 laser device also provided by RI.

Briefly, the embryo was anchored to a holding pipette with the ICM maintained at 3 o'clock and in focal plane with the mouth of the pipette (see sections 4.6.1.1 and 4.6.2.1 in Materials and Methods) (**Figure 4-3**).

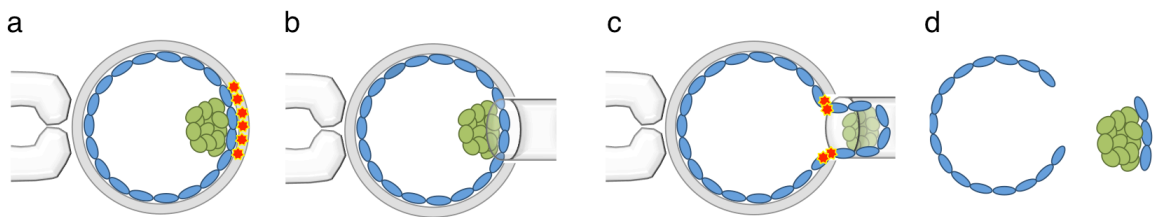


Figure 4-3 – ICM excision procedure. a) Zona pellucida removal by laser pulse (red circles indicate sites of individual pulses), b) Aspiration of ICM mass and some TE wall, c) Detachment of biopsy by lasering of connecting TE cells, d) samples tubing. ICM (green), TE (blue), laser pulse (red).

The zona pellucida was then removed from the area outside the ICM using small laser pulses (0.5 ms). Once the embryo started protruding from the incision made, a 35 μm wide biopsy pipette (Research Instruments Ltd, United Kingdom) was used to make contact with it. The ICM was then gently pulled and aspirated into the pipette, allowing to laser the surrounding TE cells and completely detach the ICM from the rest of the embryo. ICM and TE samples were then placed in different areas of the dish to avoid mismatching. ICM samples were generally composed of fewer cells (~ 10) compared to their TE counterpart (>50). In RNASeq experiments the TE samples were further divided in smaller parts, in order to collect from them a number of cell similar to the corresponding ICM (**Video E3** in the **Supplementary Electronic Material folder**).

4.3 GENE EXPRESSION ANALYSIS BY MICROARRAYS

The majority of the experiments investigating human embryo gene expression and reported in the literature involve the use of real-time PCR. This technique can provide an accurate measure of the expression levels of discrete genes through monitoring of the growing number of target sequences during the exponential phase of PCR amplification. The methodology relies on parallel quantification of the number of mRNA transcripts derived from housekeeping genes, considered to have similar levels of expression in all samples. The housekeeping genes serve as internal controls, allowing a relative quantification of the expression level of target genes. There has been some debate on the reliability of metabolic genes as references as they may also show some variation in transcription levels during preimplantation development (Radonić *et al*, 2004).

The main limitation of real-time PCR, however, is the limited number of distinct transcripts that can be simultaneously investigated. Even when multiplex real-time reactions are employed, the potential panel of targets covers only a very small fraction of the whole embryonic transcriptome.

More recently, high-throughput strategies have been developed for more comprehensive gene expression analysis. One of these is microarray technology.

As with real-time PCR, the microarray method consists of a first step involving RNA extraction and reverse-transcription to cDNA. However, the resulting material is then subjected to amplification (in cases of low input RNA) and labelling. Labelled cDNA is

then hybridised to oligonucleotide probes affixed to a solid support such as a glass slide. In some cases, the probes are directly linked to the surface of the support, forming discrete spots, while in others the probes are immobilised on microscopic beads, which are in turn spotted in defined areas of the microarray slide. After non-annealed cDNA is washed off the slide, cDNA bound to complementary probe sequences can be revealed using various staining strategies. The most common method involves detection of biotin labelled cDNA using Cy3-streptavidin complexes. Streptavidin and biotin molecules have an extraordinary affinity for each other. This characteristic allows the detection of probe-bound DNA through fluorescence. After removal of surplus streptavidin, the slide is then scanned at high resolution. This image is analysed by software able to measure the levels of fluorescence in each single spot of the array. Each spot is populated by probes of one single sequence. Typically, a total of about 48,000 sequences are represented in modern microarray platforms, covering both coding, non-coding and internal control sequences. Microarray technology allows the simultaneous detection of tens of thousands of gene products. However, its sensitivity can be reduced by small amounts of background noise caused by unbound cDNA and surplus fluorochromes. Compared to real-time PCR, microarray analyses require a larger amount of starting material. Recently, special kits have been developed to allow efficient amplification and labelling of RNA derived from small amounts of cells (one or more). It is known, that the amplification step can introduce a distortion of the quantitative relationship between genes and consequently the data produced must be interpreted with caution (Makrigiorgos *et al*, 2002). Annealing properties of different sequences may also introduce biases in the fluorescence

intensity levels observed for different genes. For these reasons, microarrays are useful for comparing the expression level of the same gene in different samples, but do not provide reliable information concerning the relative number of transcripts from different genes.

In this experiment, three to four specimens (ICM or TE), from embryos derived from different patients, were pooled together and then analysed as single sample. This strategy allowed the increase in the amount of starting material, improving the chances of generating meaningful data. However, sample batching prevented the identification of minute transcriptional differences between embryos. Nonetheless, this strategy was employed to minimise the influence of patient-specific (or embryo-specific) differences in gene expression, thus revealing as differentially expressed only those transcripts characteristic of a specific cell lineage. Using laser-assisted ICM excision, a total of 12 samples (6 ICM and 6 TE) were collected from 19 human blastocysts.

The mRNA from the samples was extracted using RNeasy-Micro Kit (Life Technologies) following manufacturer's instructions (section 4.6.1.2 in Materials and Methods). An additional step for DNA digestion was also performed to remove any potential genomic DNA contaminants.

The amount and quality of the extracted RNA was not tested prior to amplification to maximise RNA input in the downstream cDNA conversion.

Isolated RNA was amplified and biotin-labelled using TargetAmp 2-Round Biotin-aRNA Amplification Kit 3.0 (Epicentre, Illumina, USA) following manufacturer's

instructions (section 4.6.1.3 in Materials and Methods). This procedure involved two rounds of RNA-based amplification (**Figure 4-4**).

The first round started with a reverse-transcription reaction primed by a T7-Oligo(dT) primer, that produced a RNA-DNA hybrid containing a phage T7 promoter at the 5' of the DNA strand. Enzymatic digestion of the RNA strand with RNase H produced small fragments of RNA that primed the second cDNA synthesis reaction, producing double-stranded cDNA molecules.

An *in vitro* transcription step involving RNA synthesis directed by the phage T7

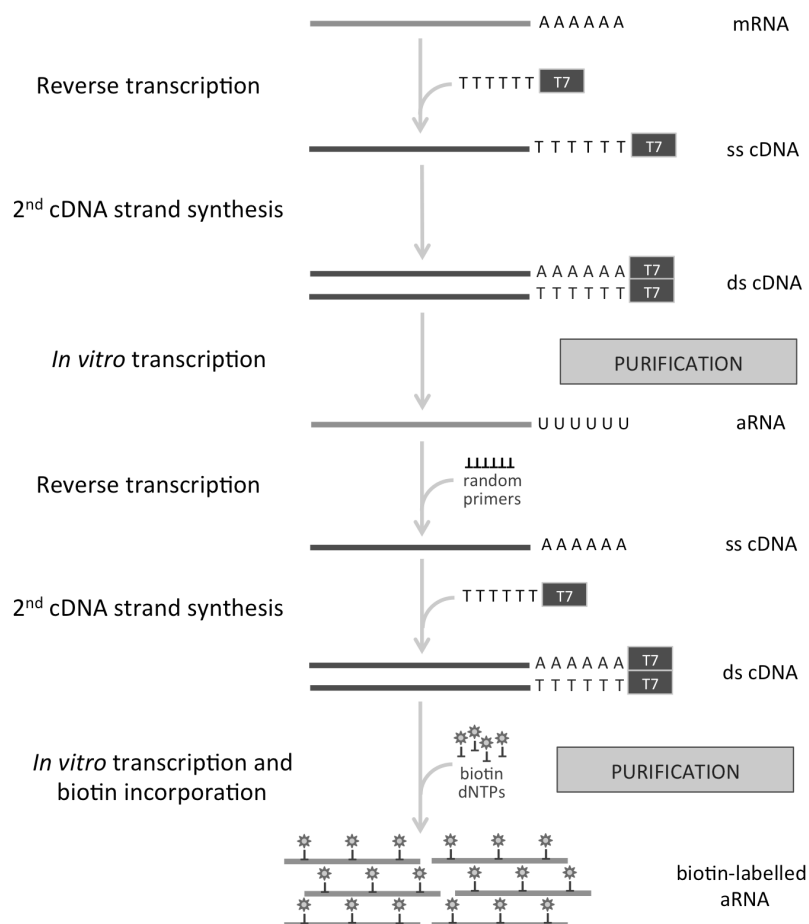


Figure 4-4 - Workflow for cDNA conversion, amplification and labeling of blastocyst mRNA for microarrays analysis

promoter produced anti-sense RNA transcripts (aRNA). In the second round of amplification, a cDNA strand complementary to the aRNA is synthesised using random primers. T7-Oligo(dT) primers are again used to produce a double strand cDNA molecule with a phage T7 promoter on both strands. A final step of *in vitro* transcription in the presence of Biotin-UTPs is performed to produce labelled aRNA. Final Biotin-aRNA purification step was performed using Qiagen MinElute Cleanup Kit as specified by the manufacturer.

Prior to hybridisation to microarrays, the amplified RNA samples were quantified using a NanoDrop device (Thermo) and normalised to 150 ng/ μ L. Samples quality control (mRNA) was performed using RNA 6000 Nano Kit and BioAnalyzer 2100 (Agilent) by the Genomic Core Facility at the Wellcome Trust Centre for Human Genetics (WTCHG), University of Oxford. Samples hybridisation to HumanHT-12 v4 Expression BeadChip microarrays (Illumina, USA) was performed according to manufacturer's protocol at the University's Genomic Core Facility (section 4.6.1.4 "Sample preparation and microarray hybridization" in Materials and Methods). It has been shown that different microarray platforms available on the market produce consistent results for what concerns gene expression data (Liu *et al*, 2012). WTCHG has great experience working with the Illumina platform and therefore it was employed for this experiment. Data analysis was performed by the Functional Data Analysis unit at the WTCHG (section 4.6.1.5 in Materials and Methods).

Functions of the expressed genes were investigated using Panther Classification System database (<http://www.pantherdb.org/>) and GeneOntology (GO) annotations (Thomas *et al*, 2006; Mi *et al*, 2013).

Results

4.3.1 Samples quality control and data overview

After amplification, final aRNA concentrations and purity were assessed using NanoDrop device (Thermo Scientific, USA). RNA concentrations and optical densities (OD) OD260/OD280 ratios per sample are shown in **Table 4-1**. All samples showed high grade of purity with OD260/OD280 ratios over 2.0 (values under 2.0 indicate the presence of salts or other contaminants in the sample). These measurements showed that ICM samples produced

Table 4-1 - Samples RNA concentrations and ODs

Sample N	NANODROP readings	
	RNA [ng/uL]	260/280
ICM 1	1926.3	2.28
TE 1	1742.7	2.12
ICM 2	154.6	2.1
TE 2	1283.5	2.17
ICM 3	675.5	2.14
TE 3	1754.1	2.15
ICM 4	1279.9	2.21
TE 4	1613.9	2.17
ICM 5	1010	2.23
TE 5	1702.3	2.15
ICM 6	791.6	2.28
TE 6	1592.9	2.19

lower yields compared to TE samples. Samples were then normalized to RNA concentrations of 150 ng/ μ L and a 3 μ L aliquot of each sample was used to assess RNA quality using a BioAnalyzer 2100 device (Agilent Technologies, USA). Electropherograms of each sample run are shown on **Figure 4-5**. Although some of them showed suboptimal quality, all 12 samples were run for microarray analysis. Initial data analysis based on average signal intensity and housekeeping genes signal suggested that all TE samples produced good expression profiles. However, some of the ICM samples failed to amplify as expected, making downstream statistical analysis inappropriate for some of the paired samples.

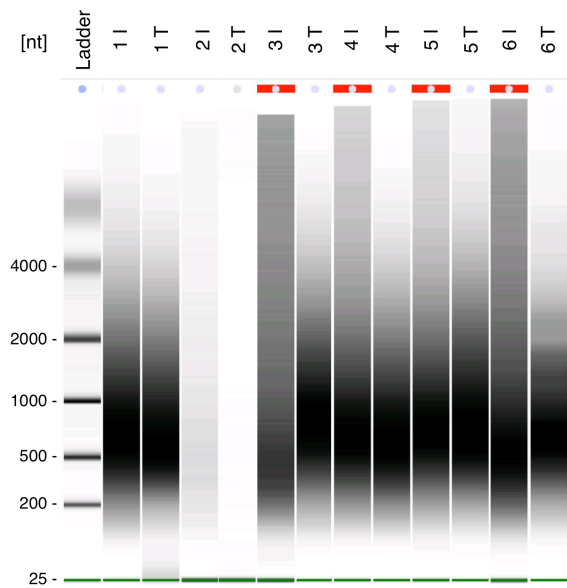


Figure 4-5 - Electropherograms of sample runs generated with Bioanalyzer. [nt] = size of RNA fragments (number of nucleotides)

As shown on **Figure 4-6**, ICM samples #2, #3 and #6 were of reduced quality compared to the other samples. After removal of these 3 paired samples from the differential gene expression analysis, the remaining samples (#1, #4 and #5), failed to provide enough statistical power to detect differences in gene expression between ICM and TE samples.

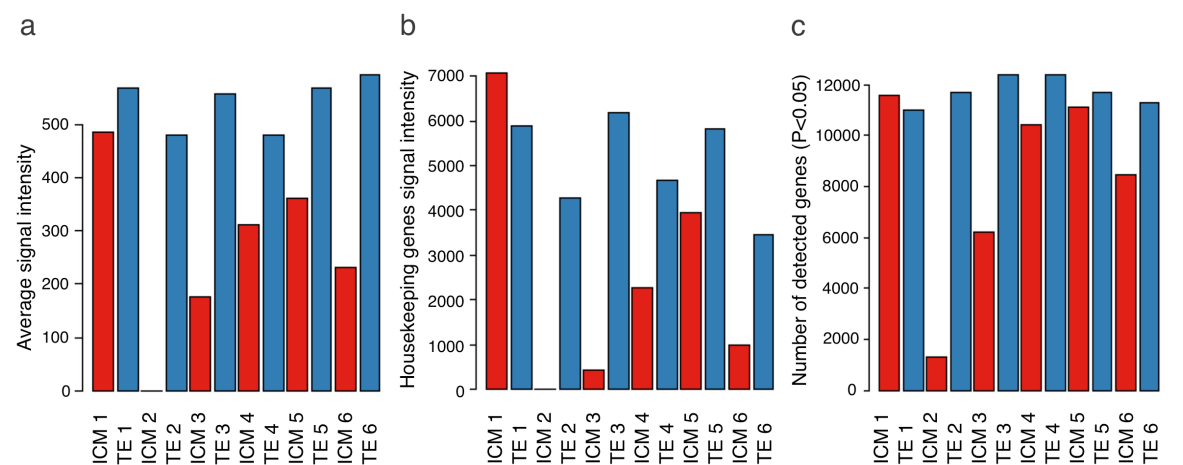


Figure 4-6 - Microarray samples quality control analysis. a) average fluorescence intensity of all probes in each sample (arbitrary units). b) average fluorescence intensity of housekeeping genes only in each sample (arbitrary units). c) number of actively transcribed genes detected in each sample.

Although comparison of relative gene expression levels was not considered reliable, the data was adequate for the construction of a catalogue of genes expressed by the human embryo at the blastocyst stage. Using only these samples of highest quality, 8,938 genes were identified which were expressed in all the ICM samples and 10,914 in all the TE samples, for a total of 11,103 actively expressed genes in the blastocyst (**Table E1** in the **Supplementary Electronic Material folder**).

4.3.2 Biological process and molecular function over-representation

To provide a functional view of blastocyst gene expression profiles, I performed gene over-representation analysis using the Panther Classification System tool, based on GO-slim biological process annotation. As shown on **Table 4-2**, the majority of the biological activities displaying >1.5 over-representation, compared to a default human cell transcriptome gene list, involve both transcription and translation processes.

PANTHER GO-Slim Biological Process	Homo sapiens REFLIST	Transcripts identified	Transcripts expected	Transcript Over/Under representation	Transcripts Fold enrichment	Bonferroni adjusted P-value	Log2 Fold enrichment
DNA repair	172	137	83.75	+	1.64	1.14E-05	0.71
mRNA processing	274	210	133.42	+	1.57	9.39E-08	0.65
mRNA splicing, via spliceosome	183	140	89.11	+	1.57	7.48E-05	0.65
natural killer cell activation	99	20	48.21	-	0.41	7.77E-04	-1.29
regulation of translation	148	118	72.07	+	1.64	8.68E-05	0.71
RNA splicing	135	101	65.74	+	1.54	6.74E-03	0.62
rRNA metabolic process	115	101	56	+	1.8	8.16E-06	0.85
sensory perception of chemical stimulus	133	12	64.76	-	0.18	2.00E-13	-2.47
translation	435	352	211.82	+	1.66	7.40E-17	0.73
tRNA metabolic process	82	77	39.93	+	1.93	2.61E-05	0.95

Table 4-2 - Panther gene statistical over-representation from microarray expression data

The increased number of active genes involved in tRNA and rRNA metabolic processes, combined with high transcription of genes required for regulation of mRNA maturation and translation, confirms an extremely active biogenesis activity in the developing blastocyst. Interestingly, genes involved in local immunological modulation (NK-cell activation and sensory perception of chemical stimulus) appear to be down regulated, which perhaps reflects an attempt to minimize maternal inflammatory response in reaction to embryonic presence. Over- and under-represented biological processes in the human blastocyst are shown in **Figure 4-7**.

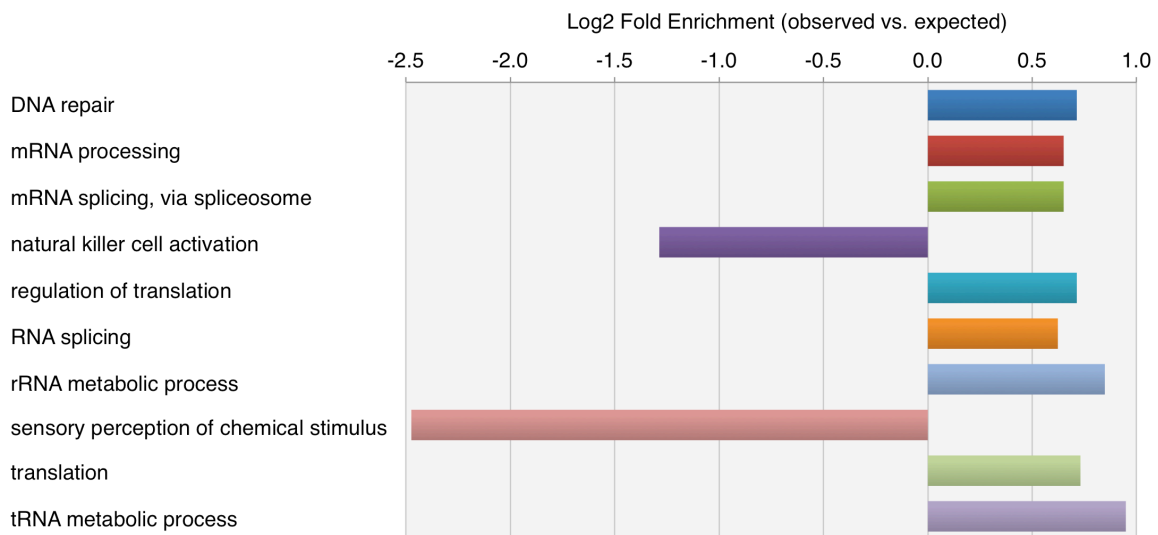


Figure 4-7 - Graphical illustration of Panther statistical gene over-representation analysis in human blastocysts samples. Only biological processes of actively transcribed genes in the human blastocyst with fold enrichment <0.5 and >1.5 and P -value <0.01 are shown. Bar lengths are displayed as Log_2 of the ratio between observed and expected number of genes for each category. Raw data and P -values for each category are shown in Table 4-2.

Microarrays data were used to verify the embryonic origin of the proteins identified in the blastocoel fluid using tandem mass spectrometry (see Chapter 5).

4.4 GENE EXPRESSION ANALYSIS BY RNASEQ

RNASeq is a relatively new methodology for the investigation of global gene expression, based on modern Next Generation Sequencing (NGS) and bioinformatic tools for sequence alignment and statistical data analysis. This investigative strategy has two main advantages compared to microarray technology. Firstly, it allows an absolute (rather than relative) quantification of the transcripts. Secondly, it permits the identification of previously uncharacterised RNA sequences. Additionally, compared to traditional Sanger sequencing, it provides higher throughput, due to its ability to perform simultaneous analysis of several samples. This is possible via the incorporation of specific sequence tags to molecules obtained from different samples. These tags, sometimes referred to as 'molecular barcodes', identify samples origin during subsequent bioinformatic sequence analysis. More recently, RNA to cDNA conversion and amplification kits have been developed for the preparation of extremely low input RNA samples (i.e single cell, 10 pg of RNA, according to SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing User Manual, Clontech, Takara Bio Inc., Japan).

In order to produce information on differential gene expression between ICM and TE, RNASeq analysis was performed on samples similar to those used for the microarray experiment. In this case, three to four specimens (ICM or TE) were pooled together per tested sample. Similarly to the previous experiment, this strategy increased the amount of starting material and minimised biological variation between

samples. Again, I performed laser-assisted ICM excision on a total of 17 specimens, subsequently divided into 10 samples (5 ICM and 5 TE) (see section 4.6.2.1 in Materials and Methods). As this protocol requires a similar amount of starting material, TE tissues were further separated in smaller pieces to reach a number of cells similar to the ICM specimens (~10 cells per specimen) (**Video E3** in the **Supplementary Electronic Material folder**). The approximate amount of cells in each sample (combining 3–4 tissue specimens) was of 30–40 cells. Cells were then moved into sterile PCR tubes and subjected to initial preparation for downstream RNASeq using SMARTer Ultra Low RNA for Illumina Sequencing kit (Clontech, Takara Bio Inc., Japan) and following manufacturer's instructions (see section 4.6.2.2 in Materials and Methods). The Clontech method allows RNA to cDNA conversion to be performed starting with cellular material rather than purified total RNA. Additionally, this kit has been tested and validated to produce reliable results starting from as little as one cell.

Samples were then frozen and maintained at -80°C until shipment to the WTCHG where RNA to cDNA conversion and library preparation was performed.

Briefly, the protocol involves an initial RNA to cDNA conversion step using a custom "*SMART CDS*" primer (**Figure 4-8**). This primer pairs to the poly-A sequence on the mRNA and initiates the reverse-transcription reaction. At the same time, a custom SMARTer oligonucleotide binds at the 5' end of the mRNA. The reverse-transcriptase synthesises the complementary sequence of the non-template oligonucleotide at the 3' of the newly formed cDNA molecule. This way, the cDNA molecule possesses custom primer sites at both ends of the fragment. By switching

template, reverse-transcription can be initiated from the 3' end of the newly synthesised cDNA. Converted double-strand cDNA is then purified using magnetic beads and then amplified. By using primers complementary to the custom sequences at both ends of the fragment, amplification rates of the flanked sequences are increased. On the other hand, the manufacturer reports that “*prematurely terminated cDNAs, contaminating genomic DNA, or cDNA transcribed from poly-A RNA, are not exponentially amplified. However, truncated RNAs with polyA tails that are present in poor quality RNA from the starting material will be amplified yielding shorter cDNA fragments*” (Clontech, Takara Bio Inc, Japan).

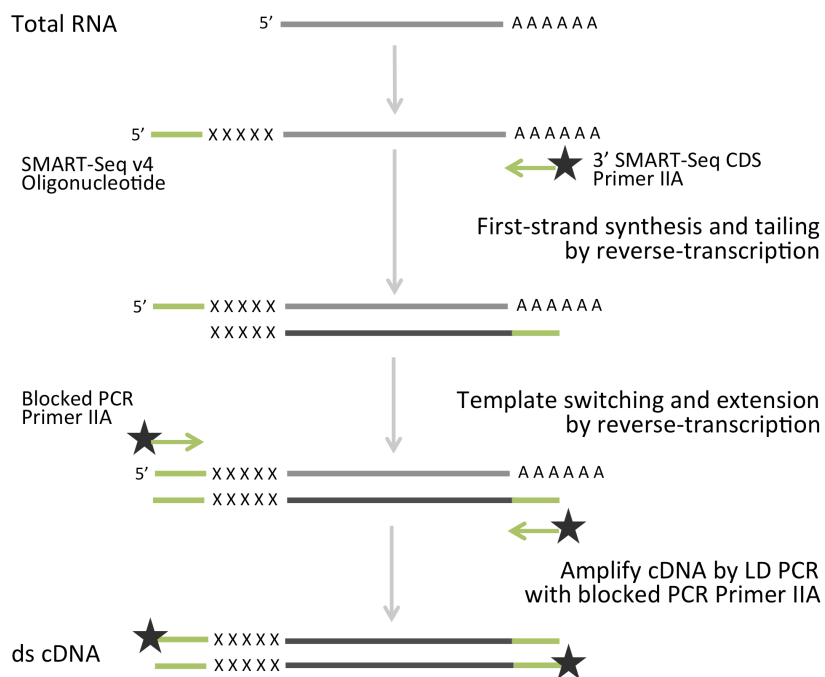


Figure 4-8 - Workflow of SMARTer Ultra Low RNA for Illumina Sequencing protocol (adapted from SMART-Seq v4 Ultra Low Input for Sequencing User Manual. Clontech, Takara Bio Inc., Japan)

The cDNA was then fragmented using acoustic shearing and subjected to NEBNext Ultra DNA Library Prep Kit for Illumina following manufacturer's recommendations (**Figure 4-9**). This protocol involved the repairing of the dsDNA, forming blunt-ended fragments. Also the 5' end was phosphorylated to enable specific adenylation of 3' ends. Overhanging dA-tails provide sites of ligation for NEBNext adaptors, creating loops at the ends of the dsDNA fragment. These loops are then cleaved by excising a U residue in the middle of the loop sequence.

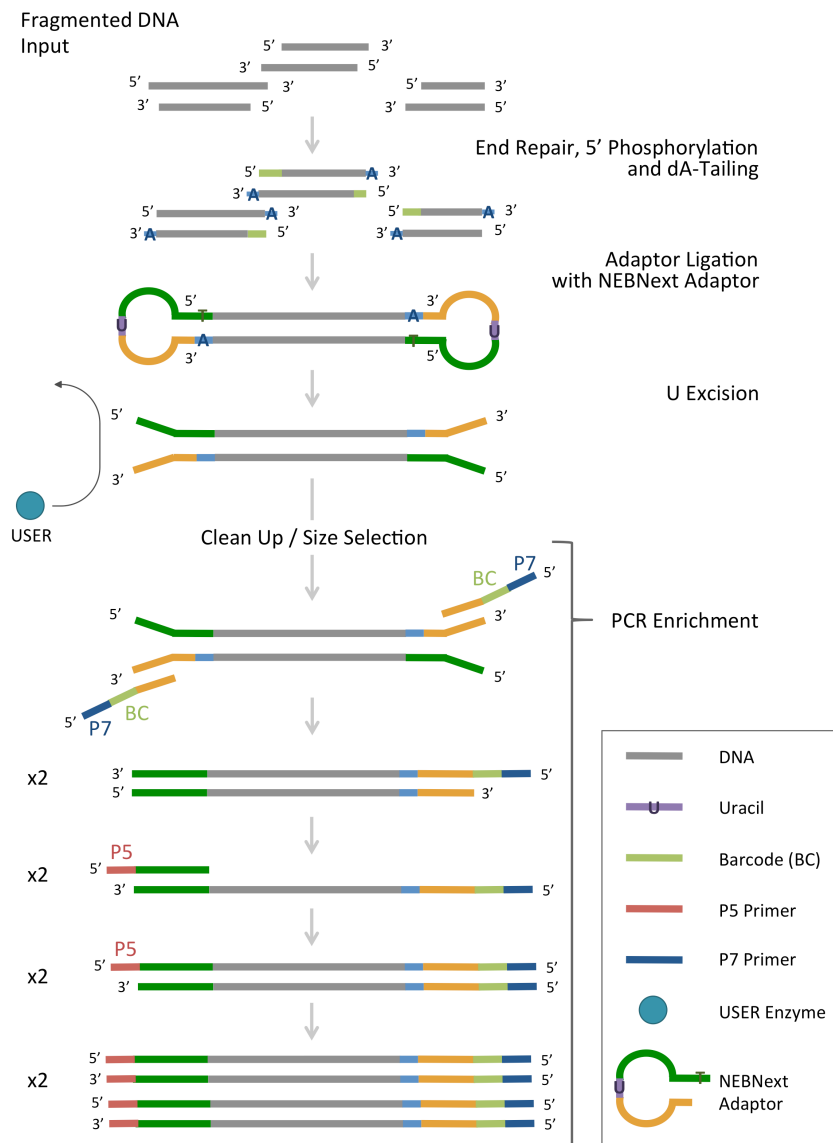


Figure 4-9 - Workflow of NEBNext Ultra DNA Library Prep for Illumina protocol (adapted from Illumina UltraDNA Workflow, Illumina, USA).

In this way the adaptors are accessible for PCR reactions. During the amplification step, fragments that have adaptors at both ends are selected and amplified. Additionally, during this step barcodes are also inserted to enable multiplexing for downstream sequencing. This step was performed using custom indexes as described by Lamble and colleagues (Lamble *et al*, 2013). Sequencing was performed using an Illumina HiSeq Sequencer at the WTCHG. Data analysis was performed by the Functional Data Analysis unit at the WTCHG. Total gene expression data was compared to microarrays data.

Additionally, pathway over-representation of differentially expressed genes was evaluated using the Panther database and Ingenuity Pathway Analysis (IPA) software (Qiagen, Germany).

Results

4.4.1 Samples quality control and data overview

Each sample analysed with RNASeq produced more than 10 million reads (**Figure 4-10**). However, after quality control checks, the number of reads of two of the samples appeared reduced (#2 and #7). These runs not only provided a smaller list of Ensemble-mapped genes, but also showed reduced similarity to the other tested samples and were therefore considered as outliers.

To allow better characterisation of differentially expressed genes, samples #2 and #7 and their pairs (#1 and #8) were removed from downstream analysis. Read counts were normalised for sequence depth of each sample using edgeR (Estimated Degree of

Gene Expression) data analysis pipeline (www.bioconductor.org). This normalisation step is based on TMM method (trimmed mean of M values) (Robinson & Oshlack, 2010).

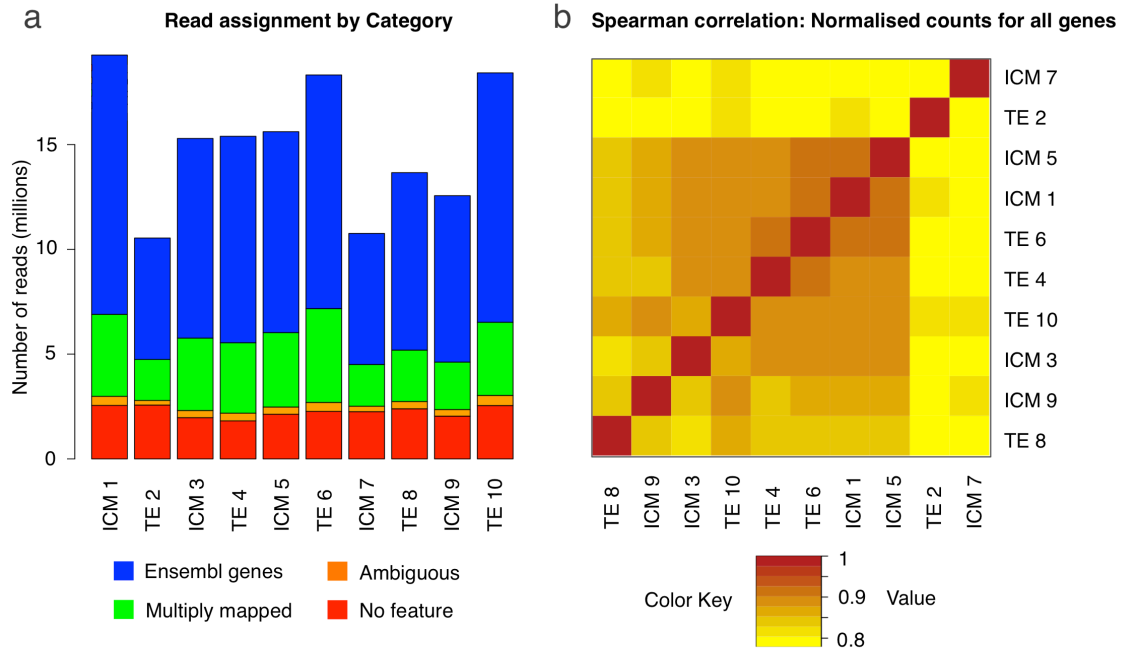


Figure 4-10 – RNASeq samples quality control analysis. a) Number of reads detected per sample. Individual reads were aligned to the Ensembl human genome database using appropriate bioinformatics tools (see section 4.6.2.3). Reads mapping precisely to the database are shown in blue. Reads mapping to multiple sites are shown in green. Reads that are not mapping precisely to the database are shown in orange. Reads not mapping to the database are shown in red. b) Correlation analysis of all gene reads per sample. The similarity between samples was tested using Spearman statistical test. Similarity values of 1 show complete match between data deriving from two compared samples (e.g. comparison of a sample with itself). The lower the similarity value, the more different are the compared samples. Samples ICM 7 and TE 2 show reduced similarity to all other samples, suggesting different biological nature of the samples. Since samples ICM 7 and TE 2 were not expected to produce different gene expression profiles compared to other samples, they were considered as outliers. This was possibly due to reduced quality of the sample, shown by a lower number of reads produced during PCR amplification step.

RNASeq data is highly complex and requires statistical adjustments before they can be used for gene expression comparison. By applying TMM statistical method, the read counts are normalised based on the depth of sequencing for each sample, taking into account the total number of reads generated in the experiment and the variety of transcript species present in the specific sample. This normalisation procedure aims

to provide a more appropriate comparison of gene expression levels across multiple samples, removing potential biases introduced during the cDNA library preparation step due to initial sample composition and different efficiency of PCR amplification.

RNASeq experiments provided a sensitive and comprehensive picture of blastocyst gene expression compared to microarray experiments. Overall, 19,724 genes were detected as expressed (with at least one read in at least one sample), significantly increasing transcript detection from previous experiments.

Comparison between the two lists of expressed genes showed that 7,899 out of 8,351 (94.6%) genes detected by microarrays were also confirmed by presence of transcripts in the RNASeq experiments. However, RNASeq provided a deeper insight into blastocyst gene expression, revealing many additional expressed genes not detected by array. In total, 7,685 of the 15,584 transcripts detected in blastocysts using RNASeq (raw gene count >5 reads, in at least one sample) were identified by this method only.

Additionally, the three paired replicates (3 ICM and 3 TE) that passed quality control checks (#3, #4, #5, #6, #9 and #10) were sufficient to provide a list of genes differentially expressed by the two cell lineages.

A complete list of expressed genes and normalised reads count is available in **Table E2** in the **Supplementary Electronic Material folder**.

4.4.2 Biological process and molecular function over-representation

In order to highlight active processes in the blastocyst, Panther gene analysis was performed using statistical over-representation test based on Gene Ontology

annotation (Thomas *et al*, 2006; Mi *et al*, 2013). For this analysis, I used a subset of genes composed of the 500 most abundant transcripts, showing the highest TMM averages across all runs (based on **Table E2**). This group of genes accounts for about 50% of the whole transcriptome composition (2,108,911 TMM). Among them, the most expressed gene was EEF1A1 with 43,621 TMM, whilst the lowest was ACP1 with 1,637 TMM. Overall, the average TMM in this subset of genes was 4,217 TMM, showing that EEF1A1, a protein involved, among other functions, in the elongation of the forming peptide chain by delivering aminoacyl-tRNAs to the ribosome complex, is 10 times more expressed than the average gene present in this subset. Also, it accounts for about 1% of all the transcripts of the 500 most highly expressed genes in the human blastocyst (43,621/sum of all average TMM 4,304,791) (**Table E3** in the **Supplementary Electronic Material folder**).

Table 4-3 - Panther statistical gene over-representation based on Molecular function Go-Slim annotation of the 500 most expressed genes

PANTHER GO-Slim Molecular Function	Homo sapiens REFLIST	Transcripts identified	Transcripts expected	Transcript Over/Under representation	Transcripts Fold enrichment	Bonferroni adjusted P-value
binding	5733	191	136.89	+	1.4	1.58E-05
DNA binding	1878	23	44.84	-	0.51	2.45E-02
hydrogen ion transmembrane transporter activity	41	10	0.98	+	10.2	1.44E-05
mRNA binding	142	17	3.39	+	5.01	1.70E-05
nucleic acid binding	2915	145	69.6	+	2.08	3.10E-16
nucleic acid binding transcription factor activity	1646	18	39.3	-	0.46	1.21E-02
oxidoreductase activity	626	33	14.95	+	2.21	4.54E-03
receptor activity	1636	12	39.06	-	0.31	3.10E-05
RNA binding	454	57	10.84	+	5.26	1.08E-21
structural constituent of ribosome	180	61	4.3	+	14.19	7.36E-47
structural molecule activity	1034	94	24.69	+	3.81	1.56E-26
translation elongation factor activity	46	7	1.1	+	6.36	2.42E-02
translation factor activity, nucleic acid binding	136	24	3.25	+	7.38	1.51E-11
translation initiation factor activity	97	17	2.32	+	7.33	6.94E-08
translation regulator activity	128	21	3.06	+	6.86	2.04E-09

Based on this subgroup of genes, significantly over- and under-represented molecular functions and biological activities are shown on **Table 4-3** and **Table 4-4**.

Panther analysis based on molecular function also highlights a reduction in receptors activity (**Table 4-4**).

Table 4-4 – Panther statistical gene over-representation based on Biological Process Go-Slim annotation of the 500 most expressed genes

PANTHER GO-Slim Biological Process	Homo sapiens REFLIST	Transcripts identified	Transcripts expected	Transcript Over/Under representation	Transcripts Fold enrichment	Bonferroni adjusted P-value
biological adhesion	606	2	14.47	-	0.14	1.17E-02
biological regulation	3918	61	93.55	-	0.65	1.26E-02
cell adhesion	579	2	13.83	-	0.14	2.09E-02
cell communication	3006	32	71.78	-	0.45	3.93E-06
cell-cell signaling	633	2	15.11	-	0.13	6.57E-03
cellular component biogenesis	310	26	7.4	+	3.51	1.30E-05
cellular process	6708	117	160.17	-	0.73	2.89E-03
DNA replication	161	14	3.84	+	3.64	1.01E-02
generation of precursor metabolites and energy	274	27	6.54	+	4.13	2.66E-07
glycolysis	25	6	0.6	+	10.00	8.15E-03
immune response	518	1	12.37	-	0.08	1.10E-02
metabolic process	8247	290	196.92	+	1.47	6.40E-15
mRNA processing	274	26	6.54	+	3.97	1.15E-06
mRNA splicing, via spliceosome	183	19	4.37	+	4.35	3.51E-05
nuclear transport	85	10	2.03	+	4.93	1.09E-02
oxidative phosphorylation	56	15	1.34	+	11.19	3.17E-09
phosphate-containing compound metabolic proc	910	6	21.73	-	0.28	1.23E-02
primary metabolic process	6825	247	162.97	+	1.52	1.11E-12
protein complex assembly	107	13	2.55	+	5.10	6.00E-04
protein complex biogenesis	108	13	2.58	+	5.04	6.63E-04
protein folding	155	17	3.7	+	4.59	7.45E-05
protein metabolic process	2692	153	64.28	+	2.38	5.80E-23
regulation of nucleobase-containing compound r	1700	16	40.59	-	0.39	1.12E-03
regulation of transcription from RNA polymerase	1319	13	31.5	-	0.41	2.53E-02
regulation of translation	148	22	3.53	+	6.23	5.59E-09
respiratory electron transport chain	217	18	5.18	+	3.47	1.64E-03
response to stimulus	2170	24	51.82	-	0.46	1.19E-03
RNA splicing	135	17	3.22	+	5.28	1.10E-05
RNA splicing, via transesterification reactions	132	17	3.15	+	5.40	7.99E-06
rRNA metabolic process	115	15	2.75	+	5.45	4.31E-05
translation	435	92	10.39	+	8.85	1.54E-54

4.4.3 Differential gene expression in ICM and TE

After Log transformation and variance stabilisation, Principal Component Analysis (PCA) of the data without outliers was performed, showing expected clustering and separation of the two types of samples (ICM and TE) (**Figure 4-9**). Of the 19,724 actively expressed genes detected, 1,322 and 1,699 transcripts were present only in ICM or TE samples respectively (**Figure 4-10**). Individual gene hits were normalised and data from ICM and TE runs were compared leading to the

identification of 278 genes significantly more expressed in ICM samples and 150 in TE samples.

Transcript levels of genes known to be differentially expressed across ICM and TE were confirmed to follow the expected transcription patterns. These included *NANOG*, *FGF4*, *SOX2* and *STAT3* for ICM and *KRT18* and *GATA3* for TE (**Table 4-5**). A complete list of genes showing a Log2 Fold Change (FC) >1 in expression levels and a false discovery rate (FDR) <0.1 is available in (**Table E4** in the **Supplementary Electronic Material folder**).

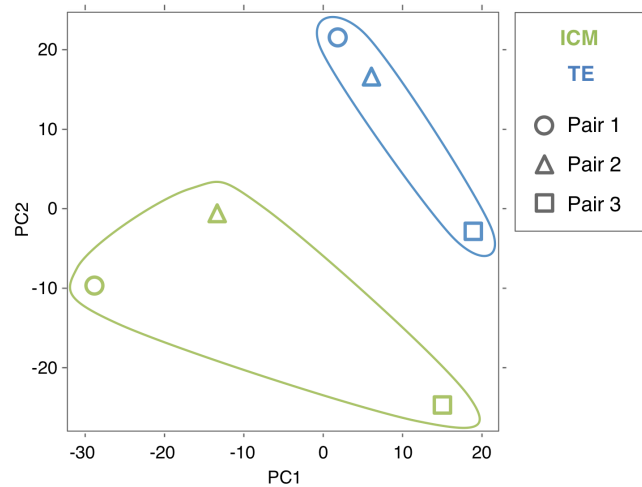


Figure 4-11 – PCA analysis showing sample clustering based on their tissue origin.

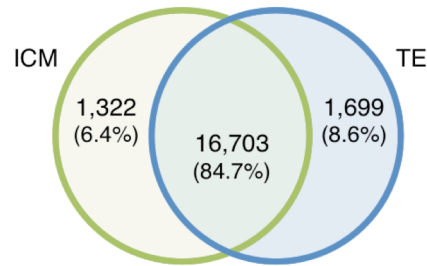


Figure 4-12 - Tissue specificity of identified gene transcripts

Table 4-5 – Tissue specific gene markers. Negative and positive fold changes refer to preferential expression by ICM and TE cells, respectively.

Log2 Fold Change	FDR	Gene ID	Gene name
-4.217	4.47E-05	NANOG	Nanog homeobox
-6.863	5.35E-03	FGF4	fibroblast growth factor 4
-5.568	1.95E-06	SOX2	SRY (sex determining region Y)-box 2
-3.059	9.67E-07	STAT3	signal transducer and activator of transcription 3
1.058	5.63E-04	KRT18	keratin 18, type I
0.842	1.48E-01	GATA3	GATA binding protein 3

Panther analysis of biological processes of genes significantly more expressed in the ICM showed a fold enrichment of 1.64 (P-value 0.046) for developmental processes.

4.4.3.1 Pathway Analysis

In this section, I use Ingenuity Pathway Analysis software (IPA, Qiagen, Germany) on the data generated by RNASeq of ICM and TE cells to identify active functional pathways within specific cell lineages. Differently from Panther analysis, which takes into consideration only the number of Gene Ontology hits for a particular genes list, without accounting for gene downstream pathway or its expression levels, IPA also accounts for gene's expression levels, allowing the identification of characteristic molecular pathways active in the tissue analysed. According to IPA, ICM samples showed a significantly higher activity in pathways involving *OCT4/POU5F1* and *NANOG* proteins (**Figure 4-11**), including their interaction with other ICM-specific growth factors and transcription regulators such as *FGF4*, *SOX2*, *SOX17*, *FZD5*, *WNT3*, *BMP2*, *BMP6* and *CD44*.

The activation of OCT4 and NANOG pathways has been shown to be related to maintenance of a pluripotent status by inhibition of multiple downstream genes (Mitsui *et al*, 2003; Loh *et al*, 2006). The interaction of OCT4 with CDX2, has been associated with deregulation of OCT4-dependent transcription inhibition, resulting in exit of the cell from pluripotent status and commitment to TE differentiation (Niwa *et al*, 2005; Chen *et al*, 2009). The data obtained during the current study confirm the activation of these cascades in *in vitro* undifferentiated cells from human ICM.

4. Gene expression of the human blastocyst

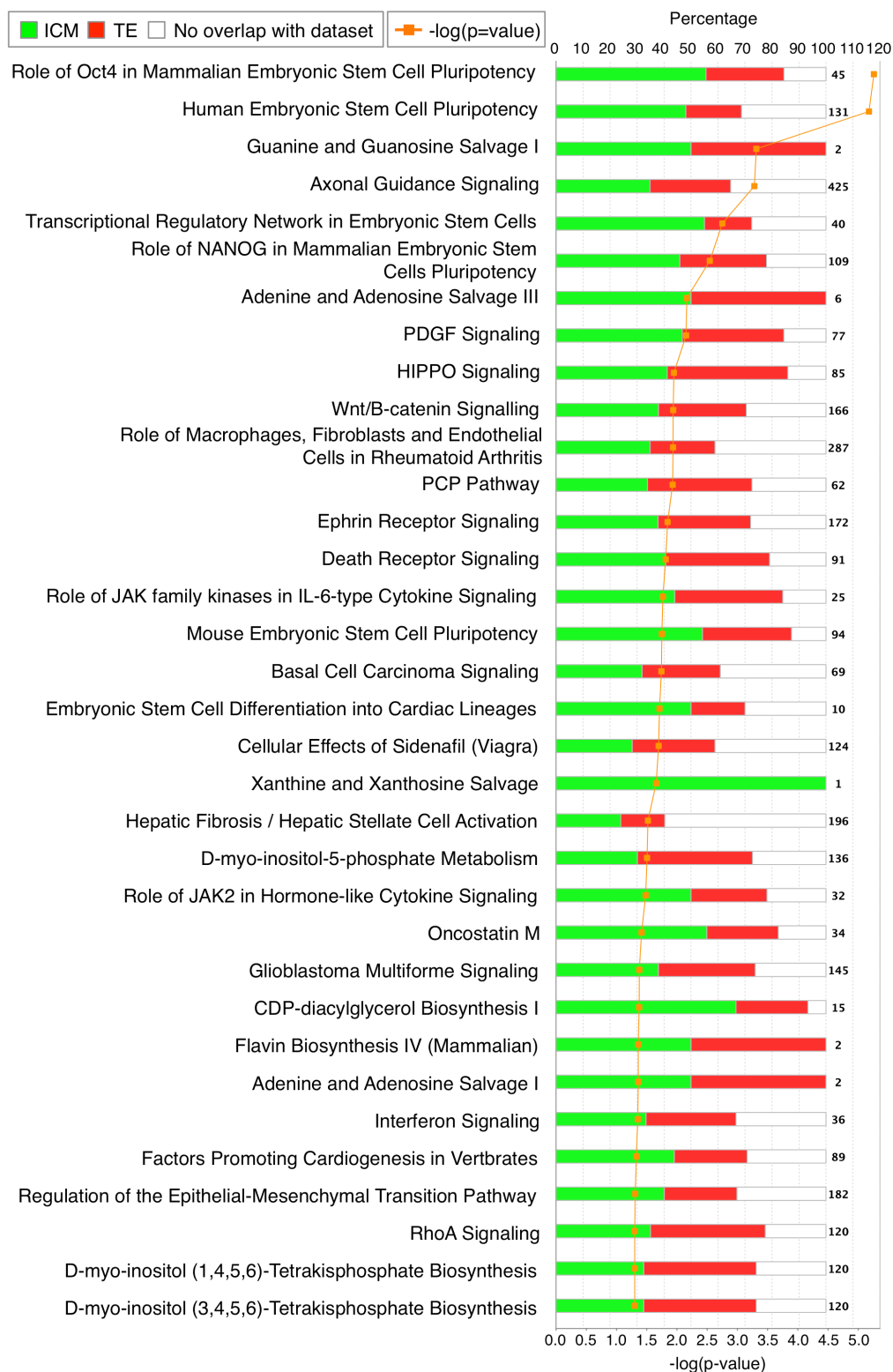


Figure 4-13 – Significantly affected biological processes following Canonical Pathway Analysis. Up-regulated genes in ICM and TE are coloured in green and red respectively. Total number of detected genes involved in the pathway are reported on the right. Orange dots indicate the likelihood of the pathway being affected expressed as $-\log(p\text{-value})$.

4.4.3.2 Autocrine and paracrine signalling across ICM and TE

IPA analysis of the RNASeq data allowed the identification of clusters of interacting proteins. A correlation between the expression of ligands and corresponding receptors in hESC and embryoid bodies has previously been shown (Dvash, 2004). Our data suggest that molecular signalling across ICM and TE is possible, indicated by differential expression of cytokines and corresponding receptors in the two lineages.

For example, the PDGF signalling pathway genes showed higher expression levels in ICM cells compared to TE samples. The activation of this pathway has been shown to induce Hypoblast (Primitive Endoderm, PrE) expansion and control Epiblast (Epi) proliferation (Artus *et al*, 2010). The data presented here shows that *PDGFRA*, *PDGFA* receptor, is highly expressed by ICM cells (Log2 FC -3.612) whilst the transcription of its ligand, *PDGFA*, is intensified in TE cells (Log2 FC 1.335), hinting at the possibility of a cross-tissue modulation of cellular proliferation operated by the TE towards the ICM. Additionally, Haouzi and colleagues suggested that *PDGFA* could act as signalling molecule directed towards the endometrium as shown by an increased transcription of its receptor *PDGFRA* in endometrial cells (Haouzi *et al*, 2011).

Similarly, Ephrin receptors *EPHA4*, *EPHA7* and *EPHBs* are relatively over-expressed in ICM cells (Log2 FC -4.406; Log2 FC -7.053, respectively), whilst mRNA transcripts for their ligands *EFNA1* and *EFNB1* are more abundant in TE cells (Log2 FC 1.870; Log2 FC 6.740, respectively). Ephrin pathway activation is associated to cell proliferation, migration, growth and differentiation and its role in embryo attachment and endometrium invasion has been previously investigated (Goldman-Wohl *et al*,

2004). However, the differential expression in ICM and TE of ligands and receptors of this protein family, could suggest a paracrine signalling pathway within the embryonic system.

Among the Fibroblast growth factors protein family (*FGFs*), *FGF4* expression was found to be restricted to embryonic tissue (Niswander & Martin, 1992). Secretion of *FGF4* was shown to be a key requirement in embryo development, promoting proliferation of undifferentiated cells and maintaining pluripotent state in hESCs, by phosphorylation of *ERK1/2* and *FGFR1*. *FGF4* transcription is activated by the OCT4 and SOX2 complex in pluripotent cells and was shown to cease following induced *in vitro* cell differentiation (Mayshar *et al*, 2008). The data in this thesis shows that the expression of both *FGF4* (ligand) and *FGFR1* (receptor) is increased ICM cells relative to TE samples (Log2 FC -6.863; Log2 FC -2.254, respectively), suggesting an autocrine molecular loop that promotes the proliferation of ICM cells. This hypothesis is also supported by increased *in vitro* proliferation patterns of hESC exposed to exogenous *FGF4* (Mayshar *et al*, 2008).

4.4.3.3 Upstream analysis / Regulator effect

IPA software also allows the identification of upstream receptor activation based on the expression patterns of specific genes. From the current dataset, IPA recognized significant activation of several molecular signalling cascades (**Table E5** in the **Supplementary Electronic Material folder**). Some of these pathways were differentially activated across ICM and TE tissues. For example, significantly activated cascades in the TE included pathways regulated by the estrogen receptor and Hdac. On

the other hand, NFkB complex, TNF cytokines and PPRC transcription regulator pathways displayed a relative up-regulation activated in ICM cells (**Table 4-6**).

Table 4-6 – IPA-inferred activated upstream regulators

Upstream regulator	Molecule type	Tissue specific Activation prediction	Activation z-score	P-value	Target molecules in dataset
estrogen receptor	group	Trophectoderm	2.138	9.43E-06	CAPG CD44 CDH11 CDH8 COL4A1 FGFR1 KRT18 LY6E MAP1B MYC PCDH19 PDGFA SAT1 SMAD6 WNT3
NFkB (complex)	complex	Inner Cell Mass	-2.168	1.28E-03	BAK1 BMP2 CD44 CFLAR CXCL12 HAS2 IL23A KIT MCL1 MMP2 MYC TWIST1
TNF	cytokine	Inner Cell Mass	-2.292	1.81E-03	BMP2 CD44 CDC42EP4 CDH11 CFLAR DUSP10 DUSP5 EFNA1 JAK1 JARID2 MCL1 MMD MMP2 MYC PLSCR1 PRDM1 PTGES SAT1 SERPINB9
Hdac	group	Trophectoderm	2.236	2.26E-03	MT1B MT1E MT1F MT1H SPP1
PPRC1	transcription regulator	Inner Cell Mass	-2.236	2.26E-03	BMP6 DUSP5 GK PHLDA1 ZFP36L1
PAF1	other	Inner Cell Mass	-2	1.47E-02	BAMBI KLF4 PNP ZC3HAV1

4.5 DISCUSSION

4.5.1 Molecular function and involvement in biological processes of genes expressed in the blastocyst

Gene statistical over-representation analysis is a bioinformatics tool based on reviewed genes GO-annotation that allows the comparison of gene expression datasets with user-provided or Panther default human genome reference. Based on the total number of identifiers in the experimental list, the number of expected expressed genes for each GO group is calculated. Differences between expected and measured number of genes are used to highlight under- and over-represented biological process and molecular function categories.

The application of this type of analysis to our experimental data shows that, at the blastocyst stage, the embryo has a high degree of biogenesis activity. In fact, biological processes displaying a relative over-representation include pathways involved in glycolysis, oxidative phosphorylation, respiratory electron transport chain and generation of precursor metabolites and energy (**Figure 4-14**).

This high energy production rate is not only required for the process of gastrulation and the production and maintenance of the blastocoelic cavity, but also for biogenetic activities and proteins synthesis. These data provide evidence for activity related to the processes of mRNA splicing and maturation, regulation of translation and

ribosomal functions, combined with assembly, folding and biogenesis of protein complexes.

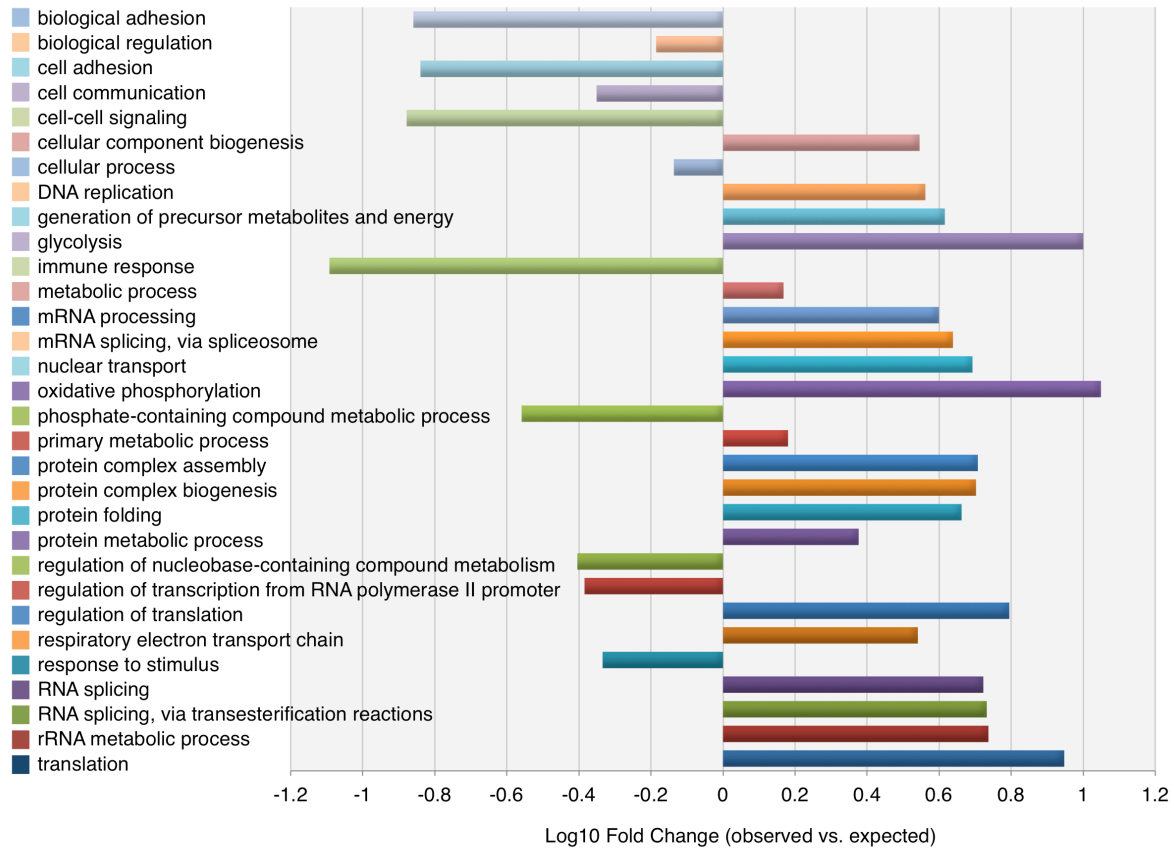


Figure 4-14 - Graphical representation of over and under represented Biological Processes in the human blastocyst according to RNASeq data. Gene statistical over-representation performed using Panther Database and Gene Ontology Annotation and displayed as Log10 Fold Change of observed/expected number of genes for each GO category. Log10 value of 1 corresponds to a 10-fold increase (or decrease in case of negative value). Log10 value of 0.4 corresponds to 2.5 and Log10 of 0.2 corresponds to 1.6 fold change respectively.

On the other hand, DNA transcription activity is relatively lower (**Figure 4-15**).

This analysis also suggests that, at the blastocyst stage (the time at which the *in vivo* fertilised embryo arrives in the uterus), the embryo may be minimising extra-cellular communications as shown by a reduction in the representation of cell adhesion, communication and signalling processes. Additionally, immunological processes in response to stimuli are also suppressed, potentially to reduce the risk of modulating

endometrium's inflammatory reaction in response to the presence of an external organism (the embryo). In previous studies, it was shown that embryo and endometrium are in communication and that inflammatory states could be modulated by molecular signalling between the two tissues, potentially affecting the implantation process (Bourdiec *et al*, 2013; Hess *et al*, 2013).

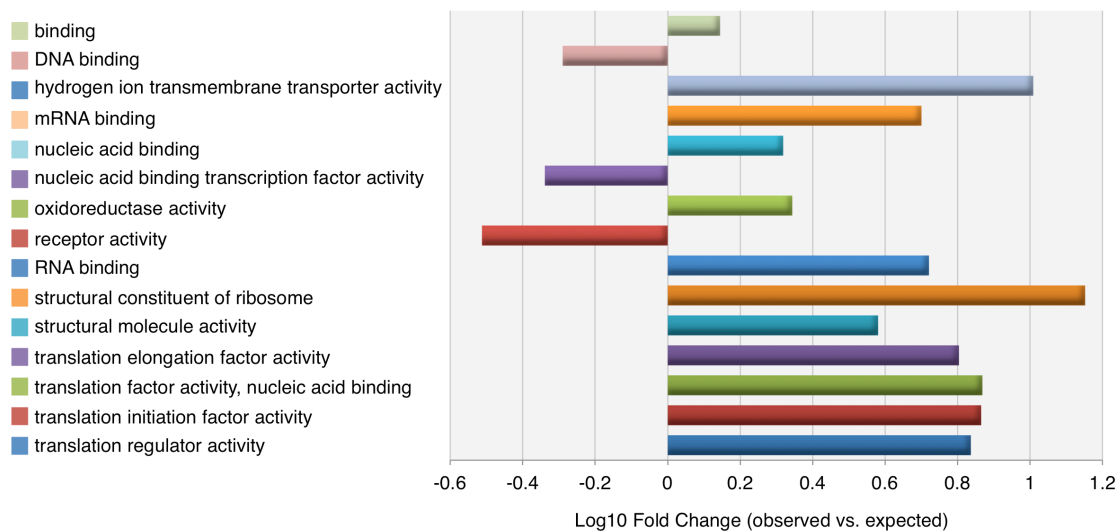


Figure 4-15 Graphical representation of over and under represented Molecular Function in the human blastocyst according to RNASeq data. Gene statistical over-representation performed using Panther Database and Gene Ontology Annotation.

On the other hand, Panther analysis of molecular functions of the expressed genes also supports data generated by microarray experiments (section 4.3.2), highlighting an increased presence of ribosome components and factors involved in RNA binding, initiation and regulation of translation and peptide elongation, and an intense ATP production manifested by an increased transmembrane hydrogen ion transport activity, reflecting an increased biosynthesis activity during blastulation and near-implantation stages (Houghton *et al*, 2003; Leese, 2012).

Additionally, evidence for enhanced oxidoreductase activity was observed, possibly to compensate the high rate of reactive oxygen species generated by the mitochondria during intense energy production (Guérin *et al*, 2001).

The maintenance of pluripotency and regulation of differentiation is a complex and yet poorly understood process. It is known that at least three protein families including FGF, WNT and TGF- β play a crucial role in modulating differentiation patterns through a cascade of signals that potentiate and inhibit their effect according to their transcription levels. This intricate network of cross-reactions and effects on downstream differentiation processes results in difficulties in the interpretation of discrete molecular pathways correlated to maintenance of pluripotency and initial commitment to distinct cellular lineages.

Additionally, *in vitro* experimental models, based upon cultured hESCs, show altered gene expression compared to ICM cells, hampering the understanding of natural cell self-renewal and differentiation processes.

Nonetheless, the current study provided evidence for potential interaction between the two cell lineages via paracrine and autocrine signalling of growth factors including Platelet-derived growth factor (*PDGF*), Ephrin (*EPH*) and Fibroblast growth factor 4 (*FGF4*).

4.5.2 Estrogen and blastocyst gene expression

Gonadotropins such as Follicle Stimulating Hormone (FSH) stimulate the growth of granulosa cells and their production of estrogen. In turn, estrogen provides negative feedback to the pituitary gland to reduce the amount of FSH secreted into the

bloodstream. Estrogen, in combination with progesterone, also stimulates the formation and organisation of the uterine lining.

During controlled ovarian hyperstimulation (COH), exogenous FSH is administered and the negative feedback provided by estrogen is inactive, allowing more follicles to develop to final maturational stages. As a consequence, more oocytes can be collected and subsequently inseminated. However, COH generates an excess of estrogen and progesterone that has been demonstrated to have negative effects on the receptivity and organization of the endometrial tissue (Toner *et al*, 1991; Meyer *et al*, 1999; Thomas *et al*, 2002).

It has been proposed by several authors that performing embryo transfer in frozen rather than fresh cycles could lead to better IVF treatment outcomes (Evans *et al*). Theoretically, by delaying the transfer of the embryo at later stages, time is given for the female patient's hormonal levels to return to more typical physiological levels.

According to the IPA upstream analysis performed with our dataset, molecular cascades downstream of the estrogen receptor are significantly activated in trophoblast cells.

Although it is not yet fully clear how these pathways operate, high concentrations of estrogen may negatively impact the outcome of IVF treatment. The gene expression data suggest the possibility that increased concentrations of estrogens could not only impair endometrial proliferation and lining organization, but also alter embryo biological activities.

4.5.3 Considerations on blastocyst gene expression experiments

Investigation of the human blastocyst gene expression is challenging. This is not only due to the precious nature of the sample itself, but also to the complicated molecular interactions that are present at this stage of development. Pluripotency equilibria and differentiation pathways that govern the initial formation of specialised tissues are finely modulated. Biological *in vivo* conditions are difficult to reproduce in *in vitro* models generating deviations from the natural state. These variables can therefore interfere with the correct interpretation of the information produced by gene expression studies.

The experiments described in this chapter produced valuable data regarding the transcription profiles of human ICM, TE and whole blastocyst. Differential expression of a subset of genes between ICM and TE cells was identified, highlighting potential molecular mechanisms active during initial cellular differentiation and peri-implantation stages. However, to validate these hypotheses, further studies will be required, using more sensitive methodologies (i.e. real-time PCR). Also, cytogenetic assessment of the tested embryos would be required to eliminate any bias due to ploidy-associated transcriptional levels. Functional studies will also be required on modified biological models (i.e. knock-out, interference RNA). Our data suggest that an intricate signalling network is present that involves paracrine and autocrine communications between ICM, TE and, as suggested by other authors, the endometrium (Herrler *et al*, 2003; Haouzi *et al*, 2011; Altmäe *et al*, 2010, 2012).

Other than providing a snapshot of the transcriptional activity in the human blastocyst, these data are fundamental for this study to validate the embryonic origin of the proteins identified in the blastocoel fluid using mass spectrometry. This approach will be discussed in Chapter 5.

4.6 MATERIALS AND METHODS

4.6.1 Microarrays

4.6.1.1 ICM excision for microarrays

Procedure set-up

Blastocyst excisions were performed using a combination of mechanical and laser-based techniques. All procedures were performed wearing sterile gloves to reduce sample contamination. Plates for micromanipulation were prepared on the day of the procedure using pre-equilibrated (non-gassed) culture media and oil. Biopsy dishes were prepared pipetting 3 x 15 μ L drops of hepes buffered media (for carrying out the excision procedure and rinsing the pipettes if necessary during the procedure), 1 x 15 μ L drop of PVP (for filling the biopsy pipette prior to intervention). Drops were completely covered with pre-equilibrated oil ($\sim$ 3.5 mL) and the lid replaced tightly. Dishes were then placed in the incubator to re-equilibrate for about 30 minutes prior to the procedure. One dish was plated per embryo to process.

Excision procedure

Holding pipette (Cook, Australia) was primed in the set-up drop in the biopsy dish and the tip was immersed (without aspiration) in PVP to reduce adhesion of the embryo to the pipette. Biopsy pipette was primed in PVP to avoid retention of cellular

material. Embryos were anchored to the holding pipette while part of the zona pellucida corresponding to the ICM was removed by laser pulse using the Saturn 3 device (Research Instruments, UK). Using a 35 μm Blastomere aspiration pipette (Cook, Australia) gentle aspiration was applied and the portion containing the ICM was extracted from the zona pellucida. As needed, small laser pulses (average pulse width of $\sim 9.0\text{-}10.0\text{ }\mu\text{m}$ and pulse time of approximately $0.500\text{-}0.510\text{ms}$) were applied to the TE to facilitate the detachment of the biopsied sample from the rest of the embryo. Three biopsied ICM samples and the corresponding TE were individually washed in serial PBS (Sigma Aldrich, USA) + 0.1% polyvinyl alcohol (Sigma Aldrich, USA) 12 μL drops previously plated and transferred to sterile PCR tubes in 2.5 μL of PBS solution and kept at 4°C to minimize cellular degradation before storing at -80°C .

4.6.1.2 RNA extraction

RNA was extracted from samples containing 3 or 4 specimen using RNeasy-Micro Kit (Life Technologies) according to manufacturer's instructions. All buffers and washing solutions described in this section were provided in the RNeasy-Micro Kit (Life Technologies) and are described with the name given by the manufacturer. Briefly, 100 μL of Lysis Buffer and 50 μL of 100% ethanol were added to each sample. Each solution was loaded onto a Micro Filter Cartridge Assembly and centrifuged for 10 seconds to allow RNA binding to the filter. 180 μL of Washing Solution 1 was added to each cartridge and centrifuged for 10 seconds. The same procedure was repeated twice with Washing Solution 2. The samples were then centrifuged at maximum speed for 1 minute. A 7 μL aliquot of Elution Solution pre-heated at 75°C was added to each filter,

stored for 1 minute at room temperature and centrifuged for 30 seconds. The elution procedure was performed twice and RNA was retrieved at the bottom of the collection tube. Subsequently, samples were also treated for DNA digestion as follows: 1 μ L of 10X DNase I Buffer and 1 μ L of DNase I (2 U/ μ L) were added to each eluted sample. The reaction was incubated for 20 minutes at 37°C. An aliquot of 2 μ L of DNase Inactivation Reagent was added and incubated for 2 minutes at room temperature. The reaction was then centrifuged for 1.5 minutes at 13,600 x *g*. The RNA was collected with the supernatant and placed in a new RNase-free PCR tube.

4.6.1.3 cDNA conversion and amplification

Purified mRNA was amplified using TargetAmp 2-Round Biotin-aRNA Amplification Kit 3.0 (Epicentre, Illumina) following manufacturer's instructions. Briefly, this kit allows two subsequent *in vitro* transcription reactions and the incorporation of biotin-labeled nucleotides in the last step, starting from purified RNA. As suggested by the manufacturer, SuperScript III and SuperScript II Reverse Transcriptase (Invitrogen, Life Technologies, USA) were used for 1st strand cDNA synthesis in the first and second amplification rounds respectively. RNA Clean & Concentrator-5 Kit (Zymo Research) was used for RNA purification after the first round of *in vitro* transcription. Final Biotin-aRNA purification was performed using RNeasy MinElute Cleanup Kit (Qiagen, Germany). No freezing step was performed during the procedure to avoid sample degradation.

Total RNA output was measured by spectrophotometry analysis using a Nanodrop device (Thermo Fisher, USA) (**Table 4-1**). Aliquots of all samples were normalized to 150 ng/ μ L to allow downstream preparation.

4.6.1.4 Sample preparation and microarray hybridisation

All buffers and washing solutions described in this section were provided in the Whole-Genome Gene Expression Direct Hybridization Assay Kit (Illumina) and are described with the name given by the manufacturer, unless otherwise stated. Individual samples were brought to a total volume of 5 μ L adding RNase-free water, mixed and left at RT for 10 minutes, then resuspended. 10 μ L of previously heated HYB solution were added to each sample. Illumina BeadChips were prepared according to manufacturer's instructions. 15 μ L of sample were added to each of the hybridisation chambers and incubated for 16 hours at 58°C with rocker speed at 5 (Illumina Hybridization Oven). On the next day, slides were collected from hybridising oven and cover slides removed in E1BC buffer. Slides were then submerged in high temperature Wash buffer for 10 minutes. Slides were immediately transferred into the staining dish containing E1BC and placed on an orbital shaker for 5 minutes at the highest speed possible. The slides were then transferred into 100% ethanol and shaken for 10 minutes in an orbital shaker and transferred to E1BC wash for 2 minutes agitating. Reactions were blocked by submerging the slides in Block E1 buffer and agitating for 10 minutes. Staining solution consisted in a 1:1,000 dilution of Cy3-Streptavidin (stock of 1 mg/mL, Fisher Scientific, USA) in Block E1 buffer. Slides were submerged in the staining solution and agitated for 10 minutes at RT and then washed in E1BC buffer for 5 minutes before

drying in a centrifuge for 4 minutes. BeadChips were then scanned using an Illumina iScan device.

4.6.1.6 Data Analysis

Raw data were imported into the R statistical software (R Development Core Team, 2013) for further processing and analysis using BioConductor packages (Gentleman *et al*, 2004). Raw signal intensities were background corrected prior to being transformed and normalised using the “*vsn*” package (Huber *et al*, 2002). After excluding one failed sample, a cautious analysis to determine which genes were expressed in each cell type was performed. The background intensity range is defined by a set of several hundred negative control probes on the array, which are designed with no match in the human genome. Probes are assigned a detection p-value, based on their signal overlap with the background distribution and I used a detection p-value <0.05 to define probes expressed above background levels. If a probe was expressed above background in all of the samples for a given cell type, it was classified as expressed in that cell type. These were annotated with gene information using the relevant BioConductor annotation package (Mark Dunning *et al*, n/a). The genomic data discussed in this chapter have been deposited in NCBI's Gene Expression Omnibus (Edgar & Lash, 2002) and are accessible through GEO SuperSeries accession number GSE72685. Human blastocyst microarray gene expression data are directly accessible through the GEO SubSeries accession number GSE71455.

4.6.2 RNASeq

4.6.2.1 ICM excision for RNASeq

ICM excision was performed on 17 fully expanded blastocysts showing good morphology ICM, as described in Chapter 2, section 2.4.7. Sample retrieval was performed as described in the section 4.6.1.1. In addition, for each sample, part of the trophectoderm was further separated to achieve a number of cells of the ICM sample. This was performed to balance the downstream sequencing analysis.

Three or four ICM and corresponding TE were placed in individual sterile 0.2 mL PCR tubes containing 0.5 µL of RNase inhibitor and 9.5 µL of 10X Lysis buffer (Clontech, Takara Bio Inc., Japan). Samples were then frozen at -80°C until shipped for downstream preparation.

4.6.2.2 Library preparation

RNA was extracted and processed at the Wellcome Trust Centre for Human Genetics in Oxford. SMARTer Ultra Low RNA Kit for Illumina Sequencing kit was used to process the samples and produce cDNA. Libraries were generated using NEBNext Ultra DNA Library Prep Kit for Illumina. Both protocols were used as per manufacturer's recommendations, with the exception of using custom indexes as described by Lamble and colleagues (Lamble *et al*, 2013).

4.6.2.3 Sequencing, mapping and gene counts

Samples were sequenced with paired-end sequencing using HiSeq2000 Sequencing system (Illumina, USA). Duplicate read pairs were removed by comparing 35bp of read pairs using filterPCRdupl.pl (<https://code.google.com/p/condetri/downloads/detail?name=filterPCRdupl.pl>).

RNAseq reads were mapped to the human genome, *Homo sapiens* (UCSC version hg19 available at <https://ccb.jhu.edu/software/tophat/igenomes.shtml>). Reads in fastq format were aligned using TopHat2 v2.0.12 (Kim *et al*, 2013) using Bowtie2 v2.2.3.0 (Langmead & Salzberg, 2012) as the core-mapping engine (--mate-inner-dist 190 --mate-std-dev 40 --report-secondary-alignments) and other default parameters. Reads mapping uniquely to annotated exon features were counted using htseq-count implemented in HTSeq, version 0.6.1p1 (Anders *et al*, 2014). Sample QC was done using statistical tools in R Core tools version 3.1.1 (R Core Team, R Foundation for Statistical Computing, Vienna, 2015). Gene counts were first VSN normalized for QC and clustering analysis using Bioconductor package DESeq2 version 1.4.5 (Love *et al*, 2014). Differential expression analysis between ICM and TE cells was done using the BIONCONDUCTOR (Gentleman *et al*, 2004) package edgeR v3.0.8 (Robinson *et al*, 2010). First low abundance genes with less than ten mapped reads in at least 3 samples were removed. Read counts were normalized for sequencing depth using edgeR's TMM normalization. Differential expression between ICM and TE samples was tested with edgeR's modified exact test and corrected for multiple testing (FDR < 0.1).

4.7 CHAPTER 4 BIBLIOGRAPHY

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5. BLASTOCOEL PROTEOMICS

5.1 Discovering the human blastocoel proteome

5.1.1 Optimisation of sample digestion and preparation for Mass Spectrometry

5.1.2 Protein characterisation of the human blastocoel

5.1.3 Validation of mass spectrometry data with gene expression analysis

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5.1 DISCOVERING THE HUMAN BLASTOCOEL PROTEOME

The steady technical improvement of mass spectrometers and bioinformatic tools over the last decade, have led to increased interest in proteomics from scientists practicing in diverse disciplines. In fact, the robustness of the techniques, the extreme sensitivity of modern devices and the reproducibility of results allowed the scientific community to gain confidence in the applicability of proteomics in basic and clinical environments. Today, like never before, scientists in genetics, oncology and regenerative medicine are looking for novel, more comprehensive methods to investigate cellular pathophysiology, sub-cellular structures and tissue systems biology. Thus far, the use of high throughput tandem mass spectrometry (MS) proteomics has allowed the identification of proteins initially separated on polyacrylamide gels, but also the characterisation of complex biological samples such as blood serum. This approach finds its main application in initial discovery phase experiments where little is known about the composition of the sample of interest.

To date, conventional proteomic approaches have been mainly developed and optimised for the processing and analysis of samples that can be sourced from large quantities of starting material such as cell lines and tissues. However, the analysis of the human blastocoel poses a difficult challenge, not only limited to the sourcing of the material which is derived from living human embryos generated *in vitro*, but also associated to an extremely small sample volume of about 4–6 nL and a minimal protein

abundance (100 ng, estimated 10x less than intracellular protein concentration based on literature (Ellis, 2001)).

Previous attempts to define the protein composition of the human blastocoel were performed by Jensen and colleagues (Jensen *et al*, 2013), managing to identify 149 proteins with a false discovery rate (FDR) of <1% from the analysis of 2 batches of 19 and 34 blastocoels. I performed the experiments described in this chapter in collaboration with Dr Alessandro Ori of the Structural and Computational Biology Unit at the European Molecular Biology Laboratory in Heidelberg, Germany. In this set of experiments I investigated the feasibility of increasing the sensitivity of protein detection in the human blastocoel proteome using tandem MS. To succeed in this objective, we employed state of the art mass spectrometry techniques, also revisiting some of the steps of sample preparation to better tailor protein digestion and peptide isolation to the minute nature of our sample. Standard and alternative procedures were then used to investigate the human blastocoel composition.

5.1.1 Optimisation of sample digestion and preparation for mass spectrometry

Prior to MS analysis, the protein content of the blastocoel fluid must be denatured and enzymatically digested (**Figure 5-1**). Different protocols have been developed to perform these preparation steps and each one may have different applicability according to the type of sample to analyse. In order to ensure complete and reproducible digestion, proteins need to be denatured. Commonly, this is achieved by

using buffers containing high concentrations of chaotropes such as urea that promotes the linear confirmation of the amino acids sequence.

In these conditions, peptide digestion is performed by proteases such as trypsin that possess high specificity.

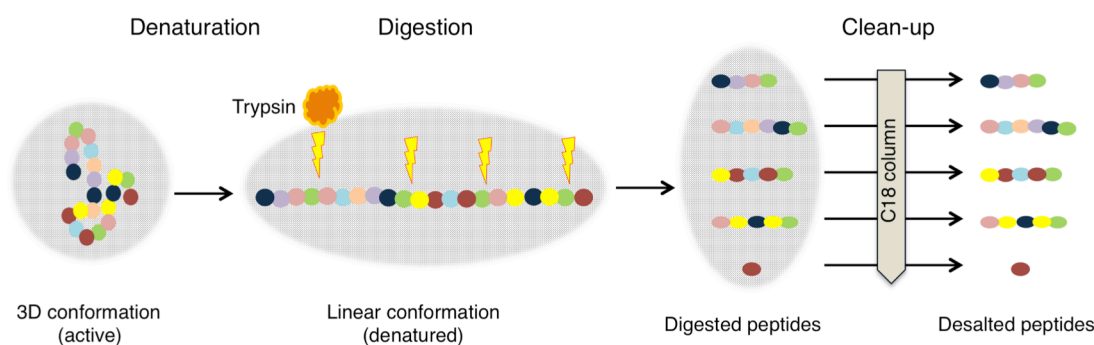


Figure 5-1 – Sample preparation workflow for conventional mass spectrometry analysis. From left, Proteins are denatured in chaotrope solution, trypsin is added to allow specific peptides cleavage. Digested peptides are submitted to C18 column. Alkyl chains in the column bind the peptides, allowing the removal of salts and other compound present in the solution by centrifugation. Peptides are finally eluted in a new tube.

This enzyme cleaves the peptide bonds exclusively at the carboxylic end of lysine and arginine residues. This mechanism is used to allow homogenous protein digestion and then exploited for peptide recognition and downstream database search.

Normally, the peptide-containing solution must then be cleaned-up from non-protein impurities and salts prior to MS analysis. This step is usually performed using reverse-phase C18 columns where the peptides are retained by the alkyl chains of the stationary phase and thus separated from other solutes. The peptides are then eluted from the C18 by solutions containing high concentration of organic solvent such as acetonitrile and thereby ready for MS analysis (sections 5.6.5 and 5.6.6).

After clean-up, between 2 and 5 μL of samples are usually injected in a liquid chromatography column where the peptides are separated based on their interaction with the stationary phase of the HPLC column. By gradually altering the composition of the mobile phase, peptides affinity for the stationary phase of the column also changes, allowing their elution. The mobile phase and the eluted peptides in the solution, are constantly sprayed through an ionizing source that charges the molecules, allowing them to fly through the magnetic field created by the mass spectrometer. Here, the most abundant peptides (top 20) are selected and their physical characteristics analysed (mass over charge ratio, m/z). These ions are then fragmented by collision-induced dissociation and further analysed in a second mass spectrometer chamber. Data on peptides physical characteristics are then collected and submitted to a proteomics database. This search allows the identification of the amino acid sequence of the peptides detected. These sequences are finally aligned to a second database to reveal the proteins they form.

These peptides will divide into:

- common to more than one protein,
- particular of a protein family (e.g. keratins)
- specific of a single protein, in which case are called proteotypic.

The peptides identified are then subjected to a target-decoy screening, to filter out those proteins that have high chance to be false positive. Acceptable false discovery rates range between 1 and 5%.

In conventional mass spectrometry experiments, sample preparation based on urea solutions allows best results, providing stable conditions for protein denaturation and digestion. However, due to the scarcity of material present within the blastocoel, and with the view of application of proteomic analysis to single blastocoel samples, we required the development of an alternative, less dispersive sample preparation protocol.

Since there is no previous literature on MS preparation of minuscule samples such as the blastocoel, we decided to compare different methods used in other applications and reported in the literature (Wang *et al*, 2005). This alternative method bypasses the clean-up steps by performing protein denaturation and digestion in an organic solvent-based solution (section 5.6.7). A major difference between these two types of preparation is the amount of sample manipulation required. The urea-based method involves more steps and requires the transfer of the sample across several tubes, thus potentially leading to both sample contamination and protein loss. On the other hand, the solvent-based method can be performed within the same tube, possibly minimizing negative consequences of sample manipulation. This latter method is from here referred to as MonoPrep.

As a test sample, we prepared a cytosolic extract from a known amount of HeLa cells (section 5.6.8) and performed three sequential dilutions of the sample to achieve protein extracts corresponding to 10,000 (10k), 1,000 (1k) and 100 cells, equivalent to an estimated protein amount of 800, 80 and 8 ng, respectively. we then divided each sample into two groups that were processed using the two alternative protocols. One group was treated using ZipTip C18 columns whilst the other group was treated with an

organic solvent (Tetrafluoroethanol, TFE). we sequentially tested the samples on the mass spectrometer, searched for detected peptides in databases and identified proteins (sections 5.6.9 and 5.6.10).

Additionally, in an attempt to optimise the solvent-based approach, we decided to test how different concentrations of two organic solvents affect protein digestion for MS analysis. Two aliquots of cytoplasmic lysates of about 1,000 HeLa cells were separately prepared using different amount of Acetonitrile (ACN) or TFE.

In the light of the results shown on **Table 5-1**, we decided to use a buffer containing 40% (v/v) ACN for the preparation of blastocoel samples.

Results

In this experiment, the efficiency of sample preparation (urea-based vs. solvent-based) was estimated on the basis of the number of peptides identified for each method.

Table 5-1 – (Left) Summary of peptide identifications using MonoPrep and Urea preparations on different concentrations of cytosolic extractions of HeLa cells

EXPERIMENT RUN	Peptide Sequences Identified	MS/MS Identified [%]
TFE 100 cells	8	0.08
TFE 1,000 cells (1k)	2129	18.81
TFE 10,000 cells (10k)	3218	28.1
ZipTip 100 cells	82	0.85
ZipTip 1,000 cells (1k)	36	0.38
ZipTip 10,000 cells (10k)	1231	13.01

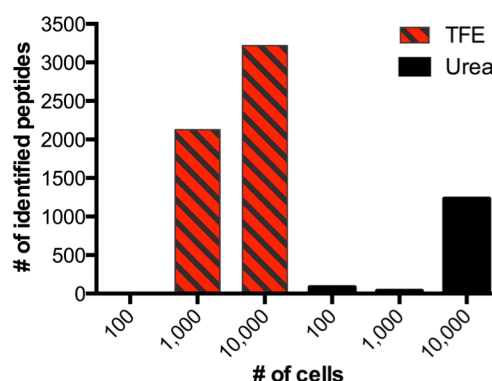


Figure 5-2 – (Right) Visual representation of peptide identifications using MonoPrep and Urea preparations on different concentrations of cytosolic lysates of HeLa cells.

As shown on **Table 5-1** and **Figure 5-2**, a few peptides were measured for both methods when a lysate of 100 cells was analysed, showing unreliable results for such low protein concentrations. However, the two preparation methods showed a significant difference when samples containing 1,000 cells lysate were analysed, with solvent-based protocol being able to identify 2129 peptides versus only 36 in the urea-based protocol (**Figure 5-3**).

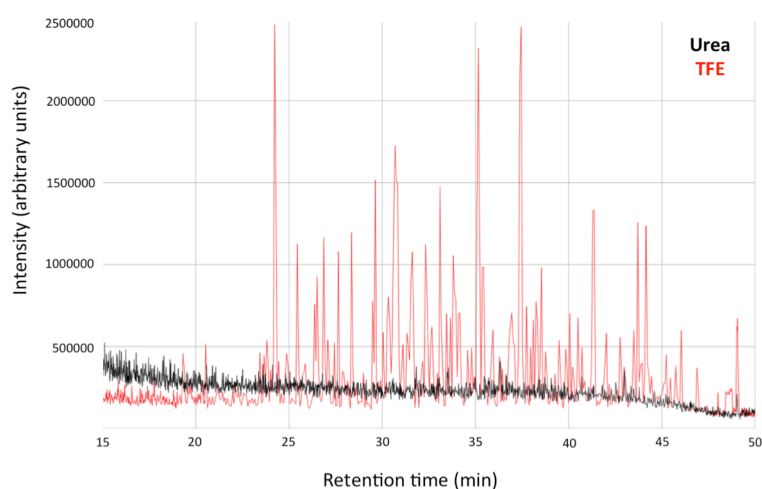


Figure 5-3 - Spectrogram showing peptide intensities using Urea and MonoPrep (TFE) preparations on cytosolic lysates from 1,000 HeLa cells

A higher performance was also noted in the analysis of 10,000 cells lysate (3218 vs. 1231 peptides, respectively), although the difference between methods performance is not as marked as in the previous experiment. These data show that the solvent-based preparation clearly outperformed the urea/C18 column method, especially for low protein amount samples (1k). In the second set of experiments, we assessed how protein digestion efficiency was affected by the type and concentration of organic

solvent in the solution. This estimate was based on the number of peptides identified in each experimental condition when a 1,000 cells lysates were analysed (**Figure 5-4**).

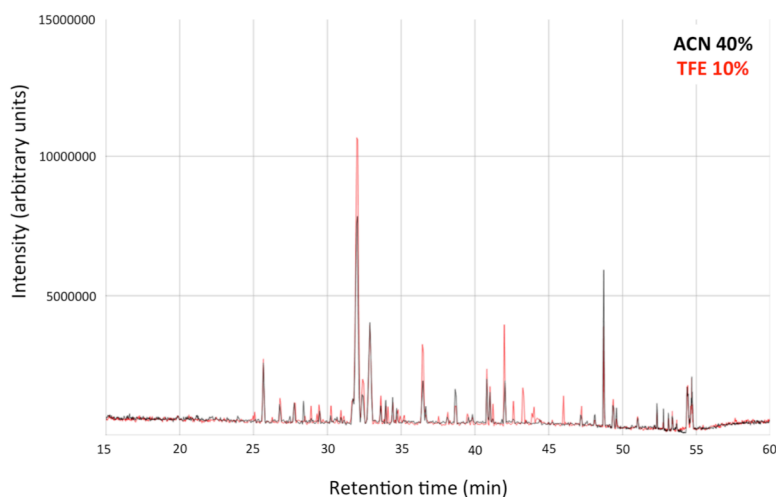


Figure 5-4 - Spectrogram showing peptide intensities using different solvent types and concentrations in MonoPrep preparations on cytosolic lysates from 1,000 HeLa cells

TFE 10%, ACN 20% and ACN 40% (v/v) delivered similar numbers of peptide identifications (1964, 1918 and 1853 peptides, respectively), whilst TFE 20% (v/v) performed sensibly less (1081 peptides) than other experimental conditions (**Figure 5-5**).

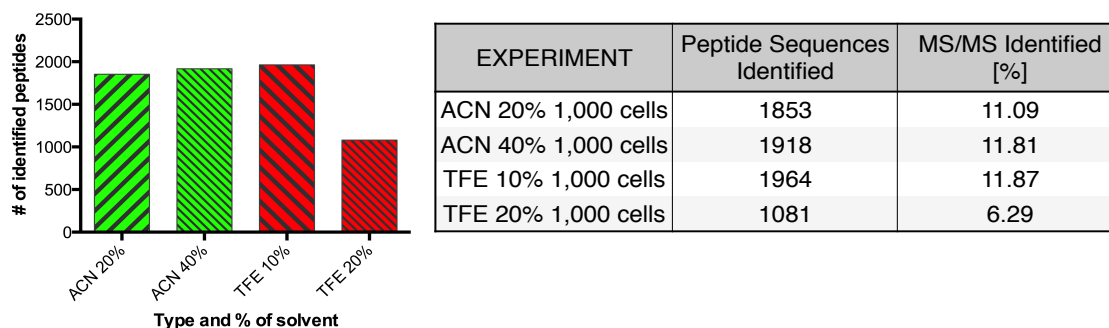


Figure 5-5 - Protein identifications employing different types and concentrations of solvent. (Left) Visual representation of peptide identifications produced using different types and concentrations of solvents on cytosolic lysates from 1,000 HeLa cells. (Right) Peptide sequences identified in cytoplasmic lysates from 1,000 HeLa cells using MonoPrep method with different solvents and concentrations

5.1.2 Protein characterisation of the human blastocoel

In order to give a full characterisation of the composition of the blastocoelic fluid, we combined the mass spectra produced by multiple MS runs. In total, we performed 4 MS runs with blastocoelic samples and 3 with blank control samples. The controls were composed of the same media used for the embryonic samples. This media was plated out and passed through a sterile injection pipette as per the experimental group. Subsequent collection of the media drops was performed without differences.

All experiments were performed on samples of 20 blastocoel fluids batched together. The first two experiments were carried out following the standard sample preparation with clean-up and desalting performed with reverse phase C18 chromatography columns (sections 5.3.3 and 5.3.5). Both these experiments involved a double sample injection of 3 μL (30% of the whole sample) in an Orbitrap Velos Mass Spectrometer (Thermo Fisher Scientific, USA) as described in Materials and Methods (sections 5.6.9).

After adjusting the sample collection procedure to be consistent with the MonoPrep protocol (40% ACN v/v), more blastocoelic samples were collected and prepared for LC-MS/MS following the method described in Materials and Methods (sections 5.3.2, 5.3.4 and 5.3.7). In these experiments we performed a single sample injection in the Mass Spectrometer of 5 μL for the first sample and 6 μL for the second (50% and 60% of the whole samples respectively). Prior to assembling a final human blastocoel protein catalogue, more stringent criteria were applied to the identification list to increase identification confidence. These involved a target/decoy search strategy

that restricted the list to proteins with a False Discovery Rate of <1%. Also, only proteins with at least 1 proteotypic peptide were considered. Additionally, peptide abundances were extrapolated and proteins with a low signal/noise ratio were also removed from the list as described in Materials and Methods (section 5.6.10). Data obtained from these experiments were used for analysis of protein sub cellular localisation and involvement in molecular processes using ProteinCenter software (Thermo Fischer, USA).

Results

In the two runs performed with urea-based method, we identified 192 and 222 proteins, whilst in similarly prepared control samples we measured 13 and 16 proteins. In solvent-based embryonic samples runs, we measured 67 and 227 proteins, whilst 27 proteins were identified in the control sample.

The total amount of peptides identified per MS injection and the relative number of proteins identified per sample is summarised in **Table 5-2**.

After removal of proteins identified in the control samples from each data set,

more stringent criteria were applied to this subset of identified targets. From the final data we derived a highly conservative list of 169 proteins from Urea-based and 150

Table 5-2 – Peptide and protein identifications using Urea or MonoPrep on batches of 20 blastocoels

Experiment	Peptides Identified	Proteins Identified
UREA control 1.1	63	13
UREA control 1.2	61	
UREA 20 samples 1.1	1321	192
UREA 20 samples 1.2	1170	
UREA control 2.1	57	16
UREA control 2.2	42	
UREA 20 samples 2.1	1368	222
UREA 20 samples 2.2	1369	
ACN 20 samples 1	88	67
ACN control 1	31	27
ACN 20 samples 2	619	227

proteins from MonoPrep embryonic samples. By combining the data from all runs, a total of 288 proteins were identified. Among these, 31 were present both in the Urea-based and the solvent-based runs (**Figure 5-6**). A complete list of identified proteins is available in **Table E6** in the **Supplementary Electronic Material folder**.

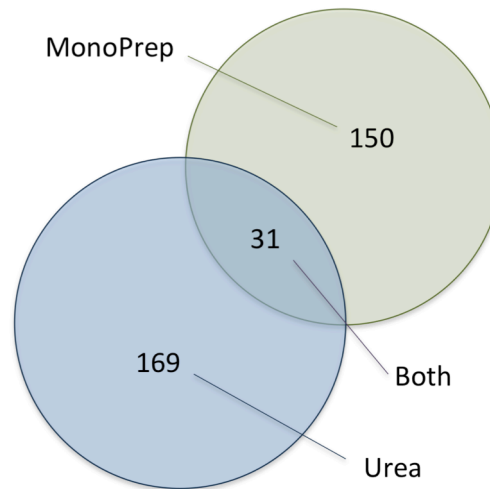


Figure 5-6 – Number of proteins identified using Urea and MonoPrep methods

5.1.3 Validation of mass spectrometry data with gene expression analysis

The highly conservative approach that we adopted for protein identification did not guarantee the embryonic origin of the targets. Although proteins identified in the blank samples were removed, contaminants could have been introduced from the media where the embryos were cultured prior to microaspiration. In fact, a recent study demonstrated that all commercial culture media shows presence of undisclosed compounds, possibly deriving from the addition of not completely purified serum albumin (Dyrlund *et al*, 2014).

In order to ensure the embryonic origin of the blastosol proteome catalogue, I cross-referenced the catalogue of proteins identified using urea-based or MonoPrep tandem mass spectrometry blastosol analysis with the catalogue of expressed transcripts in the blastocyst (described in Chapter 4).

Additionally, to validate microarray results, RNA was extracted from a batch of nine whole human blastocysts and reverse transcribed. Nested PCR was then carried out, seeking to amplify transcripts from four genes, the expression of which had been indicated by both microarrays (mRNA) and mass spectrometry analysis (protein).

The genes assessed were: Granulins (GRA); Oocyte expressed Protein (OOEP); NACHT, LRR and PYD domains-containing protein 5 (NLRP5); NACHT, LRR and PYD domains-containing protein 7 (NLRP7). I then performed gel electrophoresis of the PCR products for the identification of expected size of cDNA bands, isolated the expected cDNA gel bands and performed Sanger sequencing on inner and outer PCR products.

Results

Using Microarray data, I showed that 80 out of 169 (47.3%) proteins identified with urea-based preparations were actively transcribed in the embryo. Instead, in

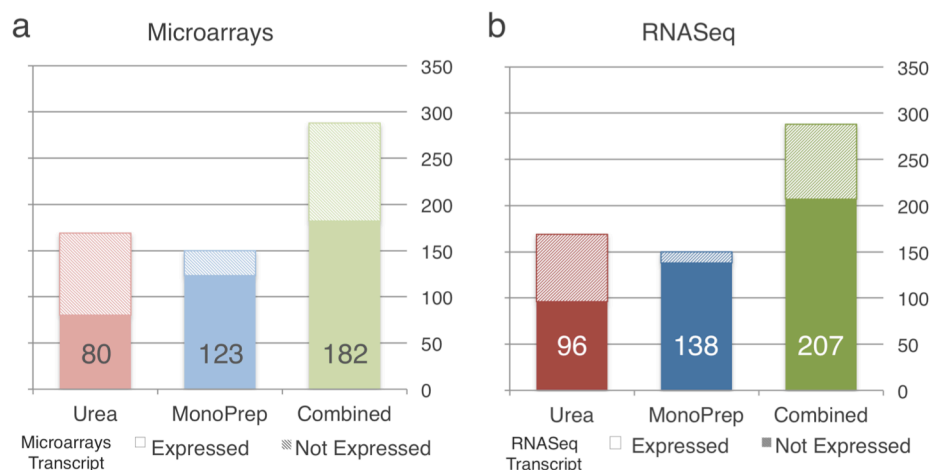


Figure 5-7 – Embryonic origin validation of proteins identified using Urea and Monoprep methods by (a) Microarrays and (b) RNASeq.

MonoPrep samples, 123 out of 150 (82.0%) identifications were confirmed (**Figure 5-**

7a). The higher sensitivity of RNASeq data increased protein validations to 96 (56.8%) for urea-based and to 138 (92%) for MonoPrep identifications (**Figure 5-7b**). Overall, 63.2% (182/288) of combined identifications from Urea and MonoPrep runs were validated by Microarray, whilst 72.2% (208/288) by RNASeq. Of the 31 proteins (10.8%) identified in both Urea and MonoPrep groups, 21 (67.7%) were confirmed by microarray analysis, whilst 10 (32.3%) were unconfirmed. A statistical analysis of the protein abundance for each group showed that shared proteins were not generally more abundant than those identified by a single method (details in section 5.3.14, Materials and Methods). Also, proteins confirmed by microarrays were not more represented in the shared group compared to the group of total identifications (Chi-square test, P-value = 0.697).

A potential explanation for the higher validation rate of the MonoPrep samples is that this procedure is less prone to the introduction of contaminant proteins such as keratins (**Table 5-3**). However, it is possible that some of the unconfirmed proteins were coded by maternal RNA prior to embryo genome activation and could have persisted to the blastocyst stage. To investigate this possibility, I cross-checked our list of identified proteins that were not confirmed by microarray with current literature on human oocyte transcriptome (Kocabas *et al*, 2006). This analysis revealed the presence of 1 protein (out of 106) of potential maternal origin (PSMA7, Proteasome subunit alpha, type 7). Although more proteins of maternal origin may persist within the cells of a blastocyst, their abundance in the blastocoelic fluid may be under the limit of detection.

MONOPREP	UniProt ID	Protein names	# peptides identified	Mw (KDa)	Abundance score (intensity/Mw)	Abundance rank	Expressed in Microarrays
10 most abundant proteins	P06454	Prothymosin alpha	5	12.191	1.24E+04	1	✓
	P62805	Histone H4	3	11.354	1.04E+04	2	✓
	P61604	10 kDa heat shock protein, mitochondrial (Hsp10)	4	10.919	6.00E+03	3	✗
	P02766	Transthyretin (ATTR) (Prealbumin) (TBPA)	2	15.869	4.02E+03	5	✗
	P09936	Ubiquitin carboxyl-terminal hydrolase isozyme L1	4	24.796	2.94E+03	6	✓
	P56279	T-cell leukemia/lymphoma protein 1A	2	13.444	2.89E+03	7	✓
	Q14764	Major vault protein (MVP)	32	99.214	2.65E+03	8	✓
	A6NGQ2	Oocyte-expressed protein homolog (OOEP)	5	17.15	2.21E+03	9	✓
	Q08257	Quinone oxidoreductase	4	35.166	1.85E+03	10	✓
	P04406	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	4	36.012	1.67E+03	11	✓
selected proteins	P06454	Prothymosin alpha	5	12.191	1.24E+04	1	✓
	A6NGQ2	Oocyte-expressed protein homolog (OOEP)	5	17.15	2.21E+03	9	✓
	P04406	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	4	36.012	1.67E+03	11	✓
	Q58738	KHDC3-like protein (ECAT1)	2	24.278	1.46E+03	13	✓
	Q99497	Protein DJ-1	1	19.868	8.06E+02	16	✓
	P00441	Superoxide dismutase [Cu-Zn]	1	15.918	7.46E+02	18	✓
	P16949	Stathmin	2	17.283	4.05E+02	27	✓
	P0DJG4	Testicular haploid expressed gene protein-like	1	52.968	3.80E+02	31	*
	Q9H808	Transducin-like enhancer protein 6 (TLE6)	4	49.771	2.19E+02	53	✓
	P14625	Endoplasmic (Heat shock protein 90 kDa beta member 1)	6	92.365	1.90E+02	56	✓
	P06576	ATP synthase subunit beta, mitochondrial	2	56.495	1.90E+02	57	✓
	P04179	Superoxide dismutase [Mn], mitochondrial	1	24.694	1.26E+02	72	✓
	P59047	NACHT, LRR and PYD domains-containing protein 5 (Mater)	5	134.187	1.11E+02	80	✓
	Q8WX94	NACHT, LRR and PYD domains-containing protein 7	4	111.678	3.86E+01	111	✓
	Q9NX02	NACHT, LRR and PYD domains-containing protein 2	1	120.376	8.94E+00	135	✓

UREA	UniProt ID	Protein names	# peptides identified	Mw (KDa)	Abundance score (intensity/Mw)	Abundance rank	Expressed in Microarrays
10 most abundant proteins	Q01469	Fatty acid-binding protein, epidermal	9	15.147	9.24E+05	5	✓
	P47929	Galectin-7	7	15.058	5.45E+05	10	✗
	P22528	Cornifin-B	3	9.876	3.44E+05	16	✓
	P10599	Thioredoxin	6	11.724	2.99E+05	18	✓
	P01040	Cystatin-A	4	10.994	2.36E+05	20	✗
	P04792	Heat shock protein beta-1	8	22.757	2.02E+05	22	✓
	P55000	Secreted Ly-6/uPAR-related protein 1 (SLURP-1)	3	11.173	1.70E+05	24	✗
	P15090	Fatty acid-binding protein, adipocyte	5	14.702	1.29E+05	26	✗
	Q06830	Peroxisomal protein, peroxisome	6	22.085	9.51E+04	28	✓
	P04406	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	11	36.012	8.89E+04	29	✓
selected proteins	Q06830	Peroxisomal protein, peroxisome	6	22.085	9.51E+04	28	✓
	P04406	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	11	36.012	8.89E+04	29	✓
	P06702	Protein S100-A9 (Calgranulin-B)	3	13.227	4.76E+04	36	✓
	P05109	Protein S100-A8 (Calgranulin-A)	3	10.822	1.57E+04	59	✓
	Q8WVV4	Protein POF1B (Premature ovarian failure protein 1B)	13	67.989	1.34E+04	61	✓
	P17931	Galectin-3	3	26.123	9.19E+03	71	✓
	P14174	Macrophage migration inhibitory factor (MIF)	1	12.462	3.53E+03	96	✓
	Q99497	Protein DJ-1	1	19.868	2.97E+03	101	✓
	Q9NZH8	Interleukin-36 gamma (IL-1 epsilon)	1	18.7	2.78E+03	104	✓
	P28799	Granulins	2	63.472	1.12E+03	134	✓

* Gene not present in Illumina HT12_v4 Microarrays platform

Table 5-3. Most abundant and selected proteins identified from samples prepared with solvent-based, MonoPrep (Blue) and urea-based, Standard (Red) methods. Proteins identified in blank samples were considered as common contaminants and removed from the catalogue of identified proteins obtained from blastosol samples. The abundance ranking column refers to the relative protein intensity levels prior to exclusion of common contaminants. The proteins detected using the urea-based method shows less continuous numbering of 'abundance rank' because a relatively large number of high abundance contaminants were detected and excluded.

To validate microarray results, RNA was extracted from a batch of nine whole human blastocysts and reverse transcribed. Nested PCR was then carried out, seeking to amplify transcripts from four genes, the expression of which had been indicated by both microarrays (mRNA) and mass spectrometry analysis (protein). The genes assessed were: Granulins (GRA); Oocyte expressed Protein (OOEP); NACHT, LRR and PYD domains-containing protein 5 (NLRP5); NACHT, LRR and PYD domains-containing protein 7 (NLRP7) (**Figure 5-8**).

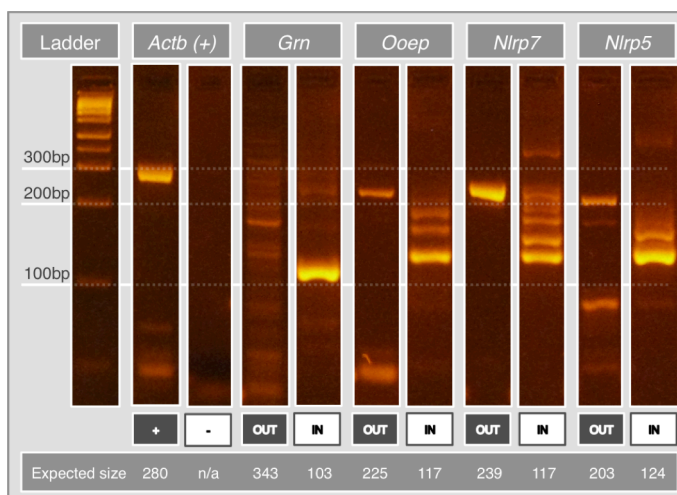


Figure 5-8 – Gel runs of target PCR amplifications of RNA transcripts of proteins of interest

Cross-check of the sequenced products with BLAST database showed high homology (>95%) to corresponding target sequences, confirming the presence of the correct transcript (**Table 5-5**).

Table 5-4 – Sequence homology of target PCR amplification products verified on BLAST database

Product Name	Homology F Fragment	pValue	Homology R Fragment	pValue
Granulin Inner	100%	8E-24	100%	8E-24
OOEP/FLOPED Outer	96%	5E-74	99%	2E-84
OOEP/FLOPED Inner	97%	2E-25	100%	5E-28
NLRP7/MATER Outer	100%	5E-91	99%	6E-91
NLRP7/MATER Inner	100%	2E-33	100%	2E-34
NLRP5 Outer	100%	8E-75	99%	8E-70
NLRP5 Inner	100%	5E-36	99%	8E-33

5.1.4 Biological characteristics of blastosol components

By applying a combination of proteomic methods, the spectrum of known blastosol components was expanded, ultimately allowing the identification of 182 proteins, which embryo-derivation was validated by microarrays. This list increases to

208 proteins when protein embryonic origin is validated with more sensitive genomic techniques like RNASeq.

Newly identified proteins include six encoded by maternal-effect genes with specific expression at the zygote-embryonic stage, prior to activation of the embryonic genome, and correlated with key developmental functions (Zheng & Dean, 2009; Akoury *et al*, 2015; Yurttas *et al*, 2010). For example, we characterized the majority of the components of the previously described SubCortical Maternal Complex (SCMC) (Li *et al*, 2008) including OOEP (also known as Floped), NLRP5 (also known as Mater), TLE6, PADI6 and ECAT1 (also known as Filia, KHDC3L or C6orf221) (**Figure 5-9**).

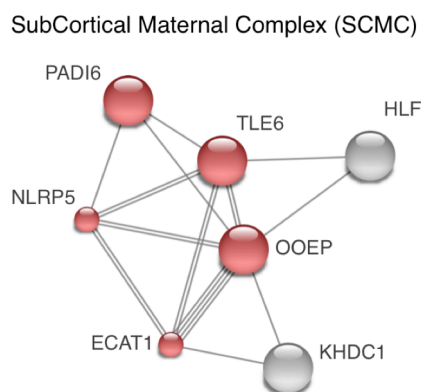


Figure 5-9 - The SubCortical Maternal Complex (SCMC) is shown as an example of embryo-specific proteins identified. Complex subunits identified with mass spectrometry (MS) are shown in red, protein interactions were derived from STRING (Jensen *et al*, 2009). Multiple lines indicate the existence of different evidences in the STRING database supporting the interaction.

Contrary to previous reports (Yurttas *et al*, 2010), we show that the components of this maternally inherited protein complex, crucial for oocyte to embryo transition, are still present both at transcript and protein level after embryonic genome activation. Additionally, recurrent hydatiform moles have been linked to the presence of mutations in maternal-effect genes encoding for two proteins also identified in our experiments, NLRP7 and KHDC3L (Murdoch *et al*, 2006). The biological function, and cellular localization of the identified proteins were explored using Gene Ontology and are shown in **Figure 5-10**.

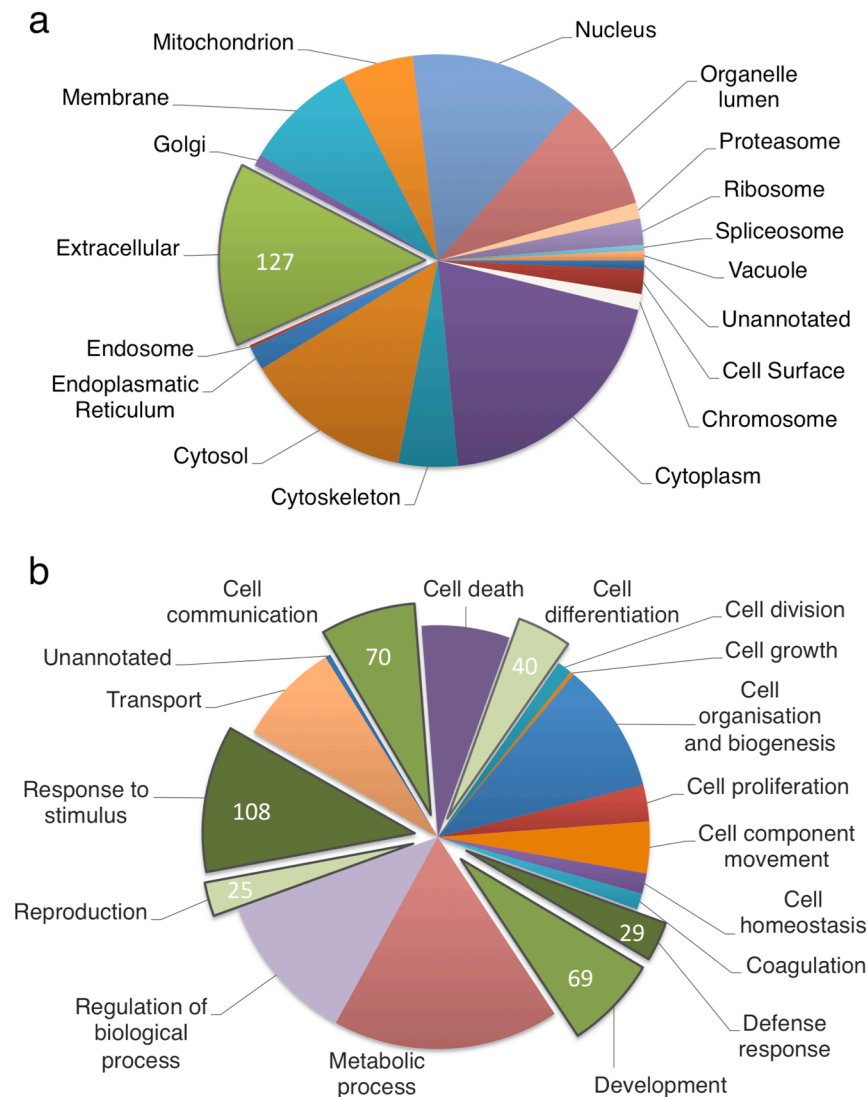


Figure 5-10 - Gene Ontology (GO) classification indicating subcellular localization (a) and function (b) of the 182 blastocoel proteins identified by MS and validated with gene expression analysis. In section C, proteins localized in the extracellular space were highlighted as potentially involved in autocrine or paracrine signaling. In section D, groups with functions associated to embryo development and implantation processes were highlighted. GO annotations were obtained using Protein Center software (Thermo Fisher, USA). Note: most proteins have multiple annotations.

In terms of localization, 127 proteins are annotated as confined and involved in functional processes in the extracellular region. On the other hand, 173 proteins have known functionality within the cytoplasm and are related to metabolic processes.

5.1.5 Discussion

The first set of experiments demonstrated that the organic solvent-based sample preparation (TFE) allows a more efficient identification of targets compared to samples prepared with ZipTip C18 technique. In fact, as shown in **Table 5-1** (section 5.1.1), in lysate samples of 10k HeLa cells, TFE allowed the identification of around 3 times more peptides than in ZipTip 10k, corresponding to an increase of about 15% in protein identification. This difference is even more remarkable if the amount of starting material is reduced to 1,000 cells lysates. In the ZipTip 1k group, almost no identifications were present, clearly showing a higher limit of detection for this methodology, compared to solvent-based protocol.

If the starting material is further lowered to a 100 cells lysate solution, there is no substantial difference in the results, with both preparations failing to provide enough material to allow protein detection, probably due to the very low protein abundance in the sample (empirically estimated around 8 ng).

Based on these results we can conclude that a ZipTip approach is not suitable for preparation of low protein abundance samples such as the blastocoel. This suboptimal performance may be explained by the permanent absorption of low concentration peptides by the reverse phase chromatography column.

The second set of results showed that sample preparations with ACN 20%, ACN 40% and TFE 10% lead to similar results. Differently, TFE 20% performs sensibly worse identifying almost 50% fewer peptides than the other preparations. This difference may

be explained by a reduced activity of trypsin when exposed to high concentrations of TFE, resulting in lower protein cleavage rates.

Despite the slightly lower amount of targets identified with ACN 40% compared to TFE 10%, we opted to adopt the first digestion condition as the protein digestion step appears to be more stable as demonstrated by minimal differences in amount of targets identified between the 20% and 40% groups.

In human blastocoel samples, similar amount of targets were identified using standard and MonoPrep preparations. A small subset of proteins (31, 10.8%) was identified in both procedures. These results were expected as proteins possess distinct affinities to different preparation solutions and therefore digestion efficiency for specific proteins may vary between two alternative conditions. Hence, some proteins showed better cleavage rates in certain conditions, improving their detection in that specific experimental group. The complementarity of two approaches enabled us to widen the spectrum of proteins identified and significantly extend the coverage of blastocoel proteome catalogue relatively to previous works (Jensen *et al*, 2013).

The characterisation of the protein contents of a minuscule sample such as the human blastocoel is an extremely challenging aim. Difficulties include both biological and technical issues related to the availability and nature of the sample. In the initial phase of this study, we wanted to evaluate if the amount of proteins present in this type of specimen was detectable using state-of-the-art mass spectrometers. By batching multiple blastocoel samples per experiment, we managed to collect detectable amounts of proteins. Additionally, in order to increase the detection of embryo-derived molecules, particular attention was paid into minimising potential sources of

contamination during sample collection. Some of these interventions included using sterile gloves throughout procedure set up and inactivated glass vials instead of common PCR tubes for sample storage. In this study, it was of critical importance to minimise sample handling to reduce dispersion of valuable material. we focused on the development of a digestion/clean-up procedure that avoided extensive manipulation of the fluid after collection as it appeared to be extremely easy to introduce contaminants during this step. The optimisation of the MonoPrep method allowed in-solution digestion and bypass the clean-up step. By avoiding the extensive sample manipulation required for standard C18 column preparations, both the risk of sample loss and of introduction of contaminants was minimised. The MonoPrep helped to address these issues and build a more robust workflow and potentially enabling analysis of single blastocoel samples. To date, we identified 288 embryo-derived proteins that constitute the blastosol. This catalogue was compiled in a conservative way, including only proteins identified with a false discovery rate lower than 1%. This approach allowed us to maintain high confidence that the proteins identified by these mass spectrometry experiments were present in the initial sample. Further validation of embryonic origin of the identified targets was achieved comparing proteomic data to blastocyst gene expression data produced both with Microarrays and RNASeq methodologies. With these data we show that protein identification by MS is extremely sensitive and that contaminations were minimised during sample collection and preparation steps, especially when MonoPrep procedure was employed. These results allowed compiling a catalogue of validated embryo-derived blastocoel proteins.

Of the total targets, around 72% (127) are classified as localised in the extra-cellular space. Many of these proteins are engaged in the modulation of local immunogenic response, and are either known or hypothesized to play roles in processes of response to stimuli, defense response and cell communication. Some of the proteins that influence immune response might conceivably play roles in embryo implantation and communication between the blastocyst and the endometrium. For example, Calgranulin A and B (also known as S100A8 and S100A9), have a pro-inflammatory function and their expression has been found to be significantly increased in products of early pregnancy loss (Nair *et al*, 2013).

Also, some molecules may be functional at an autocrine level, by coordinating communication between trophoctoderm cells and the inner cell mass and vice versa, directing cell differentiation processes.

On the other hand, 173 of the identified proteins were localised in the cytoplasmic compartment. The presence of intracellular proteins among the identifications, can be explained by the fact that during the insertion of the needle through the TE wall, some cells may rupture, releasing their content in the cavity and subsequently aspirated and processed. Additionally, we also noted that in certain circumstances, cellular fragments may be present in the blastocoel and aspirated during microsuction. These events were noted and confirmed by review of the blastocentesis video recordings (**Video E2** in the **Supplementary Electronic Material folder**). The formation of these fragments is reviewed in Chapter 3 when the presence of DNA in the blastosol was discussed.

However, intracellular proteins, such as metabolic enzymes, are expressed in high copy number within the cell, whilst regulatory proteins, such as some secretory ones, are expressed in small copy numbers. In certain conditions, this difference in abundance can reach seven orders of magnitude, resulting in easier identification by MS of intracellular components (Beck *et al*, 2011). In addition, some of the proteins known for their role in metabolic activity within the cell, may also have a role in extra-cellular processes that may yet have to be characterised.

Another large portion of the identified proteins accounts for different members of the keratin multiprotein family. The origin and presence of this protein species is unclear as they are both fundamental constituents of the cytoskeleton, which is likely to be sampled during microsuction, and also known contaminants in proteomic analysis.

The literature supports the involvement of some of the identified proteins in embryo development. Among these, Granulins (Acrogranin) appears to be necessary for blastocyst development and trophoblast outgrowth in mice (Qin *et al*, 2005; Díaz-Cueto *et al*, 2000). As we found this protein to be also secreted by the developing embryo, its presence may play a critical role on the ability of the embryo to develop and implant. Several other proteins identified in the experiments have been reported in the literature and suggested to have involvement in the implantation process. For example, Galectin-3 has been studied in mouse models and its expression by the endometrium appears to be necessary for successful implantation of mouse blastocysts (Yang *et al*, 2011). Lipocalin-1, observed in the blastocoel in the current study, has been found in high amounts in spent culture media of aneuploid embryos and has since been suggested as potential non-invasive marker of chromosome abnormality (McReynolds *et al*, 2011).

Other proteins identified include Oocyte expressed protein (OOEP), Macrophage Migration Inhibitory Factor (MIF), Neutrophil defensin 1 (DEF1), Plasminogen activator inhibitor 2 (PAI2), IL-36 and other cytokines which may play an active role in embryo development and implantation but their involvement in these processes is still under investigation.

5.2 IDENTIFICATION AND QUANTIFICATION OF PROTEIN TARGETS IN SINGLE BLASTOCOELS

Although very useful at providing a snapshot of what is most represented in a specific sample, Tandem Mass Spectrometry lacks the ability to detect less abundant and often most significant compounds. Furthermore, sample preparation for Tandem MS proteomics, generally requires a notable amount of starting material, making extremely difficult to process and analyse specimen of minute nature such as micro-dissected tissues. With the development and optimisation of targeted proteomics strategies, some of these limitations can be circumvented by focusing the sensitivity of specialised mass spectrometers to detect only a few selected peptides of interest.

In order to apply this type of analysis, a detailed knowledge of the biochemical characteristics of the targets to investigate must be previously acquired empirically, through Tandem MS experiments, or theoretically deduced using proteomics databases. Details regarding mass/charge ratio and amino acid sequence of the target peptides, combined with information of their ion fragments spectra, are necessary to develop Selected Reaction Monitoring (SRM) assays. These assays, in the form of electronic tabulates, feed information to the mass spectrometer, focusing its analysis to peptides with specific biophysical characteristics, within a complex mixture of compounds. Digestion conditions can affect the profile of peptides generated by tryptic digestion, creating specific peptide populations, otherwise not reproduced when other conditions are applied. For this reason, previous knowledge of spectral libraries from discovery

Tandem MS experiments, especially in our case where no databases are available for the ion spectra generated for our customised digestion conditions, is important information to base SRM assays on.

5.2.1 Selected Reaction Monitoring assays development

In order to enable single blastocoel analysis, I developed selected reaction monitoring (SRM) assays for 24 peptides (corresponding to 22 protein groups). These peptides were selected based on their abundance in shotgun experiments, their biological function and their biophysical characteristics. For assays development, I used spectral libraries generated from discovery phase and used a procedure for peptide selection and assay development previously described by Ori and colleagues (Ori *et al*, 2014) (section 5.3.11). Additional SRM assays aimed at identifying nuclear (H2A histone family) and cytoskeletal (ACTA) control targets were also developed. First, I validated SRM assays using isotopically labeled synthetic peptides that were spiked in pools of blastocoels obtained from 5 embryos (**Figure 5-11**). I were able to detect and validate assays for 10 peptides corresponding to 9 selected target proteins in pooled blastocoel

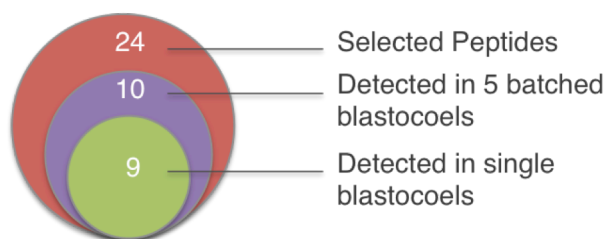


Figure 5-11 – SRM Assays validation

samples (**Figure 5-12**). I then applied these validated assays to a cohort of 21 single blastocoels, detecting 9 out of 10 targeted peptides. In order to estimate the concentration of the detected

peptides in the blastocoel, I first calibrated the signal intensity deriving from the mass

spectrometer using a pool of absolutely quantified (AQUA) peptides derived from human proteins that were injected in different known amounts (Ori *et al*, 2013).

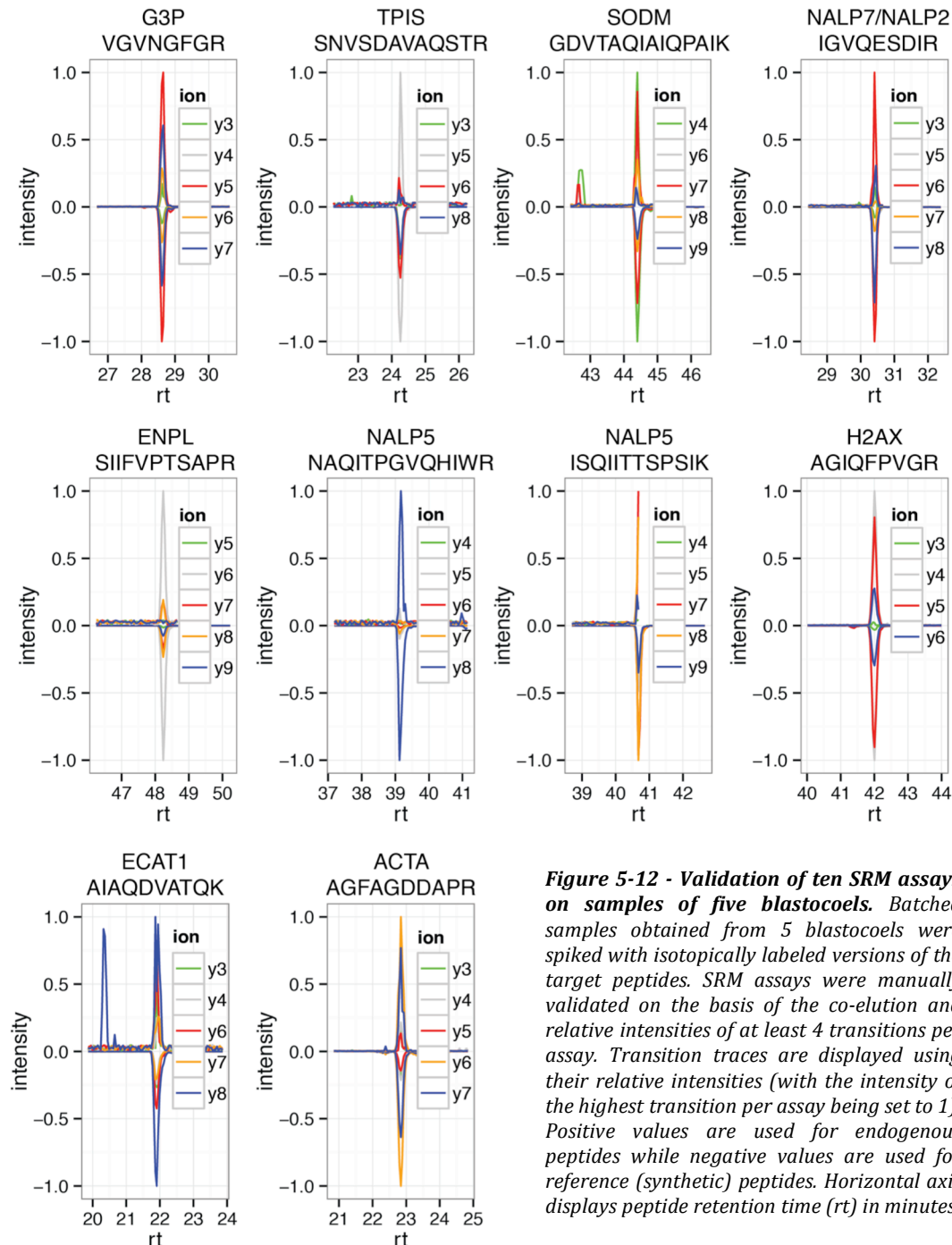


Figure 5-12 - Validation of ten SRM assays on samples of five blastocoels. Batched samples obtained from 5 blastocoels were spiked with isotopically labeled versions of the target peptides. SRM assays were manually validated on the basis of the co-elution and relative intensities of at least 4 transitions per assay. Transition traces are displayed using their relative intensities (with the intensity of the highest transition per assay being set to 1). Positive values are used for endogenous peptides while negative values are used for reference (synthetic) peptides. Horizontal axis displays peptide retention time (rt) in minutes.

We then estimated the concentration of embryo-derived peptides in the blastocoel by linear regression using the fitted equation deriving from AQUA peptides.

Results

Based on the biological importance of some proteins, SRM assays were developed for peptides selected from both urea-based and MonoPrep-based MS results. All SRM assays validated on single blastocoel samples were customized for peptides initially identified with MonoPrep sample preparation. I were not able to validate any of the SRM assays developed on peptides identified with urea-based preparations. SRM

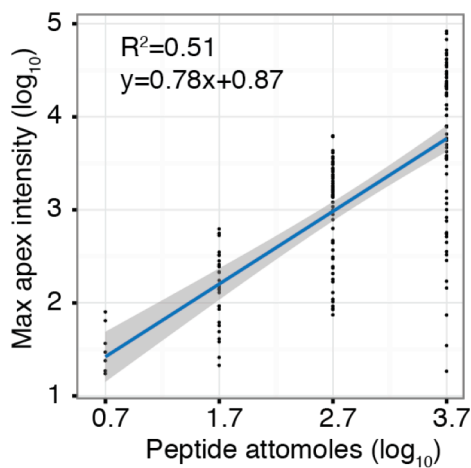


Figure 5-13 - Intensity calibration curve derived from 76 Absolutely Quantified (AQUA) peptides measured at different concentrations. The linear regression between $\log_{10}$ transformed maximum apex intensity (derived from the maximum of the most intense transition trace) and $\log_{10}$ transformed attomoles of peptide injected was used to transform SRM-derived peptides intensities for blastocoel proteins into absolute abundances.

assays were considered validated when the isotopically labeled peptides co-eluted and gave the same ion fragment spectra as the peptides from test samples (**Figure 5-12**).

We therefore used label free intensities to investigate whether the relative abundance of the detected peptides varies among samples from different embryos. Using the AQUA-generated regression formula for intensity calibration, signal intensities for each peptide were transformed into absolute protein abundances (**Figure 5-13**).

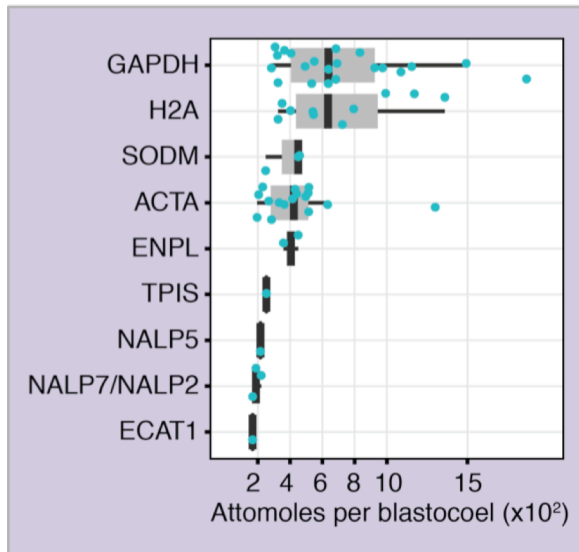


Figure 5-14 - Summary of estimated protein abundances from measurements of 9 targets in 21 single blastocoel fluids.

For 21 blastocoels, I were able to detect multiple peptides (3.3 proteins per blastocoel on average). Using an average blastocoel volume of 5 μL , the concentration of the detected target proteins spans approximately one order of magnitude, ranging from 13 $\text{ng}/\mu\text{L}$ (GAPDH) to 0.8 $\text{ng}/\mu\text{L}$ (ECAT1) (**Figure 5-14** and **Table 5-5**).

GAPDH was detected in all specimen, with an average abundance of 2.7 femtomols (3.43 Log_{10} attomols). The smallest measurement was performed for ECAT1 with 144 attomols (2.15 Log_{10} attomols).

Table 5-5 – Protein targets measurements data in single blastocoels

SAMPLES	PROTEIN ABUNDANCE (Intensity/Mw)								
	GAPDH	ACTA	H2AX	SODM	ECAT1	NALP7 NALP2	ENPL	NALP5	TPIS
S01	14665.8	6173.7	2935.6	1133.4	-	386.9	956.9	448.6	638.0
S02	9413.3	1575.0	-	-	-	-	-	-	-
S03	6455.3	1772.7	7032.4	-	-	-	-	-	-
S04	5452.5	1059.3	2904.6	-	-	453.3	-	-	-
S05	4691.9	847.4	-	-	-	-	-	-	-
S06	3861.3	570.9	4659.1	-	-	-	-	-	-
S07	3065.9	660.7	1778.2	-	-	-	-	-	-
S08	2965.7	486.6	-	-	-	-	-	-	-
S09	2815.8	1670.8	941.6	-	-	-	-	-	-
S10	2747.5	384.2	-	-	-	-	-	-	-
S11	2664.1	531.6	-	-	-	-	-	-	-
S12	2245.2	1773.8	5534.8	-	-	-	-	-	-
S13	986.7	1344.4	-	-	-	-	585.4	-	-
S14	636.8	320.2	1970.1	-	-	-	-	-	-
S15	857.4	-	-	-	-	-	-	-	-
S16	1077.1	-	-	-	-	-	-	-	-
S17	1812.6	898.4	808.9	478.1	-	-	-	-	-
S18	4111.2	2096.7	992.4	1595.4	358.8	420.9	-	-	-
S19	990.3	-	-	-	-	-	-	-	-
S20	549.4	-	-	-	-	-	-	-	-
S21	2374.9	-	-	-	-	-	-	-	-
AVERAGE	3544.8	1385.4	2955.8	1068.9	358.8	420.4	771.1	448.6	638.0

5.2.2 Discussion

A key requirement for the application of proteomics for the assessment of blastosol composition is the ability to quantify proteins in specimens derived from individual embryos. With this aim, a preparation technique that enabled the simultaneous detection of nine protein targets of interest was developed and successfully applied to 21 individual human blastocoels.

A major challenge of this methodology is the sensitivity required to detect proteins present in the minute blastosol volume. In fact, among the proteins tested, only those with higher abundance were regularly detected in the specimen (**Figure 5-14**). This limited sensitivity could be attributed both to technical and biological aspects. Firstly, of all SRM assays tested, only some of those developed based on MonoPrep sample digestion informations were validated. This was expected as specific peptide populations are generated in different digestion conditions. However, I were not able to measure each validated target in all samples tested.

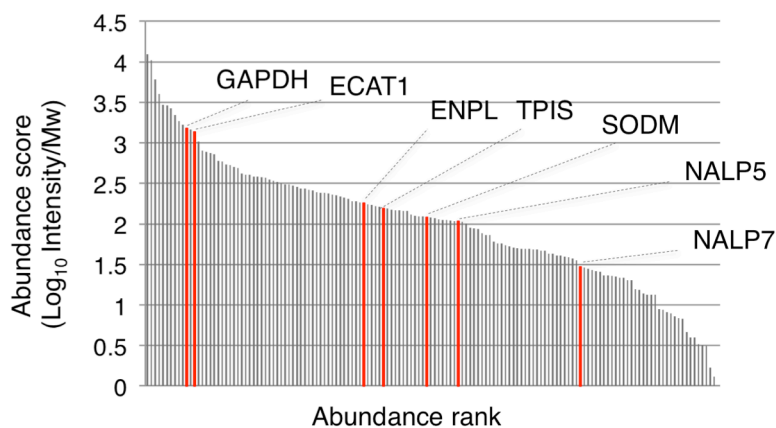


Figure 5-15 - Protein abundance profile of 20 pooled blastocoels. SRM assays were developed and validated for the proteins shown in red.

As shown in **Figure 5-15**, some of the proteins that were detected only in a subset of samples show lower than average concentrations, as judged from their intensities in shotgun proteomic experiments.

The reproducible quantification of these targets is more challenging since their concentration is close to the limit of detection of our targeted proteomic setup.

Additionally, for the majority of proteins, we developed a single SRM assay testing for a single proteotypic peptide. It is then possible that some of these target peptides were not the best candidates for MS analysis. This could be related to the peptide's biochemical characteristics that do not allow proper flyability in the mass spectrometer, leading to suboptimal detection rates.

In certain circumstances, both these unfavourable conditions may have played a part in reducing the quality and the intensity of MS measurements.

On the other hand, the inconsistent detection of some targets, as in the case of histones proteins (H2A), could be attributed to a biological variability, possibly associated to deviant physiological conditions. Increased targets detectability should be addressed in the future by targeting alternative peptides, minimizing sample loss and utilizing alternative investigative devices, including both more sensitive mass spectrometers and electronic immunoassays.

Nonetheless, we demonstrated the possibility of detecting peptides from multiple proteins deriving from single human blastocoels. Interestingly, the abundance of some of these peptides showed some correlation to embryo competence and will be discussed in Chapter 6.

5.3 MATERIALS AND METHODS

5.3.1 Embryo production and blastocoel microsuction

Human blastocysts were produced and processed as described in Chapter 2 (sections 2.4.2 and 2.4.3).

5.3.2 Blastocoel fluid collection

The aspirated fluid was released in an adjacent 3 μ L fluid collection drop of microfiltered PBS / 3 mM AmBic solution (Sigma-Aldrich Co. LLC, USA). The whole drop was then collected using a sterile 130 μ m diameter Flexipet (Research Instruments, UK) and expelled in the tube/vial where a stabilising solution was already present.

For urea-based preparations, the sample was released in a sterile 0.2 mL PCR tube containing urea 10 M and AmBic 100 mM.

For ACN/TFE based preparations, the sample was expelled into an inactivated glass vial (Agilent) containing 3 μ L of 50mM AmBic (Sigma-Aldrich Co. LLC, USA) in microfiltered PBS. The tube was tightly closed and placed on a rack off the heated stage to avoid evaporation. When all the samples were collected, they were stored at -20°C (C18 and Zip Tip preparation) or processed for protein digestion (MonoPrep).

5.3.3 Protein digestion for C18 and ZIP-TIP

The samples were thawed and spun at 13,000 $\times g$ for 5 min. The supernatant was transferred to a fresh tube and dithiothreitol (DTT) added to reach 10 mM and incubated 30 min at 37°C with mixing (800 rpm). Iodoacetamide (IAA) was added to reach 15 mM and incubated 30 min in the dark. LysC (Wako) 1:100 (w/w) was added and incubated for 4 h at 37°C. Samples were diluted to 1.5 M urea and Trypsin (Promega) 1:50 (w/w) was added and the sample incubated overnight at 37°C. Samples were acidified with 10% (v/v) Trifluoroacetic acid (TFA) and spun at 13,000 $\times g$ for 5 min. The supernatants were moved to a fresh tube and proceeded to the clean-up step.

5.3.4 Protein digestion for MonoPrep samples

ACN or TFE were added according to type of experiment (10%, 20% or 40% v/v). 1 μ L of diluted Trypsin (0.01 g/ μ L) was added to reach a protein:enzyme ratio of 1 (w/w). The outer vial (Eppendorf or glass) was capped and incubated at 37°C overnight. Samples were acidified with 10% (v/v) TFA (1:20) adding 0.55 μ L. Tubes were capped and stored at -80°C.

5.3.5 Clean-up with ultramicro-spin C18 columns

C18 columns (Harvard Apparatus, USA) were equilibrated with 50 μ L 100% MeOH spinning for 1 min at 1,000 $\times g$ and washed twice with 50 μ L 5% (v/v) ACN, 0.1% (v/v) Formic Acid (FA) spinning for 1 min at 1,000 $\times g$. The sample loaded 2x (5–200 μ L) and spun for 1 min at 1,000 $\times g$. The column was washed with 4 times with 50

μL 5% (v/v) ACN, 0.1% (v/v) FA spinning for 1 min at 1,000 $\times g$. The peptides were eluted with 3 $\times$ 25 μL 50% (v/v) ACN, 0.1% (v/v) FA spinning 1 min at 1,000 $\times g$. Samples were then placed into a speed-vac until dryness was achieved. Samples were then resuspend in 50 μL 5% (v/v) ACN, 0.1% (v/v) FA by vortexing. Samples were brought to 0.5 $\mu\text{g}/\mu\text{L}$ with 5% (v/v) ACN, 0.1% (v/v) FA, desired amount injected and the remaining stored at -20°C .

5.3.6 Clean-up procedure with C18 ZIP-TIP

Zip-Tip (Millipore, Merck, Germany) columns were equilibrated twice with 2 μL 100% MetOH and washed 5 times with 20 μL of 5% (v/v) ACN, 0.1% (v/v) FA. The sample was loaded by aspirating and transferring to a fresh tube 20 μL portions at each time (5) and then washed 5 times with 20 μL 5% (v/v) ACN, 0.1% (v/v) FA. The peptides were eluted with 5+5 μL 50% (v/v) ACN, 0.1% (v/v) FA aliquoting in a fresh tube by at least 10 time aspiration/recollection cycle. Samples were then placed into a speed-vac until dryness was achieved and then resuspended in 20 μL 5% (v/v) ACN, 0.1% (v/v) FA by vortexing. The desired amount injected and the remaining stored at -20°C .

5.3.7 Clean-up procedure with TFE or ACN

Samples were then placed into a speed-vac until dryness was achieved and then resuspended in 20 μL 5% (v/v) ACN, 0.1% (v/v) FA by vortexing. Samples were then

centrifuged for 5 min at max speed, the desired amount injected and the remaining stored at -20°C .

5.3.8 Preparation of cytoplasmic extract from HeLa cells

HeLa cells were grown in standard condition, washed twice with 13 mL of cold PBS +Ca +Mg by pipetting directly on the cell layer, and harvested by scraping with two aliquots of 4 mL PBS. The suspension was spun at $500 \times g$ for 5 min, cells were then resuspended by flicking the pellet and adding 5 mL PBS per lasagne dish. Cells were counted (1/10 dilution) and spun at $500 \times g$ for 5 min, then resuspended by flicking the pellet and 50 mM Tris pH 7.5 was added to a concentration of $2.0\text{--}4.0 \times 10^7$ cells/ml. Cells were allowed to swell on ice for 30 min and lysed by passing them 15–30 times through a 27G needle. Nuclei and cells were counted before and after lysis (1/20 dil). The preparation was spun at $1,000 \times g$ for 13 min. The supernatant was spun again at $1,000 \times g$ for 7 min and then again at $100,000 \times g$ (34,000 rpm with TLS55) for 30 min. The final supernatant was used for MS experiments.

5.3.9 High Pressure Liquid Chromatography-Tandem Mass Spectrometry (HPLC-MS/MS)

Samples were analyzed using a nanoAcquity UPLC system (Waters GmbH) connected online to a LTQ-Orbitrap Velos Pro instrument (Thermo Fisher Scientific GmbH) as described in Ori *et al* (Ori *et al*, 2014). Briefly, peptides were separated on a BEH300 C18 (75 μm x 250 mm, 1.7 μm) nanoAcquity UPLC column (Waters GmbH)

using a stepwise 145 min gradient between 3 and 85% (v/v) ACN in 0.1% (v/v) FA. Data acquisition was performed in data-dependent mode using a TOP-20 strategy where survey MS scans (m/z range 375-1,600) were acquired in the orbitrap ($R = 30,000$ FWHM) and up to 20 of the most abundant ions per full scan were fragmented by collision-induced dissociation (normalized collision energy = 35, activation $Q = 0.250$) and analyzed in the LTQ. Ion target values were 1,000,000 (or 500 ms maximum fill time) for full scans and 10,000 (or 50 ms maximum fill time) for MS/MS scans. Charge states 1 and unknown were rejected. Dynamic exclusion was enabled with repeat count = 1, exclusion duration = 60 s, list size = 500 and mass window ± 15 ppm. Raw files were analyzed using MaxQuant (version 1.2.2.5) (Cox & Mann, 2008).

5.3.10 Data processing

For urea-based procedure, samples were analyzed in technical duplicate (repeated injection of the same sample) and the results combined. MS/MS spectra were searched against the human Swiss-Prot entries of the Uniprot release 2011_12 using the Andromeda search engine (Cox *et al*, 2011). The search criteria were set as follows: full tryptic specificity was required (cleavage after lysine or arginine residues, unless followed by proline); 2 missed cleavages were allowed; carbamidomethylation (C) was set as fixed modification (only for samples from urea-based procedure); oxidation (M) and acetylation (protein N-term) were applied as variable modifications, if applicable; mass tolerance of 20 ppm (precursor) and 0.5 Da (fragments). The reversed sequences of the target database were used as decoy database. Peptide and protein hits were filtered at a false discovery rate of 1% using a target-decoy strategy (Elias & Gygi, 2007).

The “peptides.txt” output file of the MaxQuant search was used to calculate protein abundance scores from the summed intensities of proteotypic (unique) peptides normalized by the protein molecular weight, as described in Ori *et al* (Ori *et al*, 2013). Proteins were assigned as deriving from the blastocoel if they were identified by at least one proteotypic peptide and they were not detected in blank samples. Mass spectrometry proteomics data were deposited to the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) (Vizcaíno *et al*, 2014) via the PRIDE partner repository (Vizcaíno *et al*, 2013) with the dataset identifier PXD002566.

5.3.11 Targeted mass spectrometry

SRM assays development, validation and peptide quantification was performed using SpectroDive (a kind gift of Biognosys AG). Twenty-four target peptides were selected among the most intense peptides identified in the shotgun experiments (only MonoPrep samples were considered). SRM assays were developed and validated as described in Ori *et al* (Ori *et al*, 2013). Briefly, isotopically labeled version of the selected peptides were purchased from JPT Peptides Technologies GmbH, mixed and spiked into pooled samples of blastocoels derived from 5 embryos. SRM assays were validated by the detection of co-eluting transitions from the endogenous (light) and standard (heavy) peptides (**Figure 5-12** and **Table 5-6**, section 5.2.1). For the analysis of single blastocoels, we decided not to spike-in reference heavy peptides to avoid interference with the low intensity signal deriving from the endogenous peptide. However, we manually inspected that both retention time and relative order of transitions were consistent with the validated assays. For sample comparison, peptide

intensities were derived from the summed intensity of the transitions. In order to calibrate signal intensities to absolute peptide concentrations, we used a pool of 76 AQUA peptides that we previously used to determine the stoichiometry of the human nuclear pore complex. AQUA peptides were injected in four different concentrations (5, 50, 500 and 5000 attomoles) and signal intensities (max apex) were fitted by linear regression (**Figure 5-13**).

Table 5-6 – Details of peptide targets used for SRM assays development

UniProt ID	Protein description	Aminoacid Sequence	Discovery method	Confirmed in single samples
A6NGQ2	Oocyte-expressed protein homolog (OOEP)	VDDAGAAESQR	MonoPrep	✗
P04179	Superoxide dismutase, mitochondrial (SODM)	GDVTAQIAIQPAIK	MonoPrep	✓
P04406	Glyceraldehyde-3-P dehydrogenase (GAPDH)	VGVNGFGR	MonoPrep	✓
P05109	Protein S100-A8 (Calgranulin-A)	AINSIIDVYHK	Urea	✗
P06454	Prothymosin alpha	SDAAVDTSEITTK	MonoPrep	✗
P06576	ATP synthase subunit beta, mitochondrial	TVIIMEIINNVAK	MonoPrep	✗
P06702	Protein S100-A9 (Calgranulin-B)	IGHPDTINQGEFK	Urea	✗
P0DJG4	Testicular haploid expressed gene protein-like	IIQISVAK	MonoPrep	✗
P14625	Endoplasmic (ENPL)	SIIFVPTSAPR	MonoPrep	✓
P16104	Histone H2A	AGLQFPVGR	NA	✓
P16949	Stathmin 1	ASGQAFEIISPR	MonoPrep	✗
P16949	Stathmin 1	AIEENNNFSK	MonoPrep	✗
Q93045	Stathmin 2			
P17931	Galectin-3	GNDVAFHFNPR	Urea	✗
P28799	Granulins	IQSGAWGCCPFTQAVCCEDHI HCCPAGFTCDTQK	Urea	✗
P59047	NLRP5 (Mater)	NAQITPGVQHIWR ISQIITSPSIK	MonoPrep	✗ ✓
P60174	Triosephosphate isomerase	SNVSDAVAQSTR	Urea	✓
P62736	Actin A (ACTA)	AGFAGDDAPR	NA	✓
Q06830	Peroxiredoxin-1	IVQAFQFTDK	Urea	✗
Q587J8	KHDC3-like protein (ECAT1)	AIAQDVATQK	MonoPrep	✓
Q6TGC4	Protein-arginine deiminase type-6 (PADI6)	SNSQVASVYEDPNR		✗
Q86W25	NLRP13	SITHINISK	MonoPrep	✗
Q8WVV4	Premature ovarian failure protein 1B (POF1B)	NIEQENQNIR	Urea	✗
Q8WX94	NLRP7	AAIIVTTRPR	MonoPrep	✗
Q9NX02	NLRP2			
Q8WX94	NLRP7	IGVQESDIR	MonoPrep	✓
Q9NX02	NLRP2			

5.3.12 PCR of target transcripts

Embryos emptied of the inner fluid were washed in clean PBS and transferred in a sterile PCR tube in a volume of 1.5 μ L.

RNA from 9 batched blastocysts was purified and converted to cDNA using a commercially available kit “Cells to cDNA II Kit” (Ambion, Life Technologies). The protocol was employed as described by the manufacturer, apart from the cell lysis step, where a reduction of lysis buffer volume allowed an increase in overall concentration of embryo-derived material. Briefly, 9 μ L of ice-cold Cell Lysis Buffer was added into the tube containing 1 μ L of culture media and a batch of 9 human blastocysts. The solution was incubated at 75°C for 10 min and put directly on ice. A 0.2 μ L aliquot of DNase I was added to the reaction, the solution was then mixed and incubated at 37°C for 15 min and 75°C for 5 min. On ice, 4 μ L of dNTP Mix and 2 μ L of random primers to the cell lysate solution, incubated for 5 min at 75°C and transferred immediately in ice. As a final step, 2 μ L of 10X RT Buffer, 1 μ L of M-MLV RT enzyme and 1 μ L of RNase inhibitor were added to the sample, prior to incubation at 42°C for 60 min and at 95°C 10 min. The cDNA generated in this step was split in several reactions aimed at amplifying different outer products of interest.

Nested PCR amplification was used to detect products of interest with maximum sensitivity. Primers for Granulins (GRA); Oocyte-Expressed Protein (OOEP); NACHT, LRR and PYD domains-containing protein 5 and 7 (NLRP5 or MATER and NLRP7) were custom designed using PrimerBLAST, Oligocalc and Primer3 softwares. All primers were verified using GenBank BLAST Primer database for sequence specificity, primer

dimer and loop formation. Details regarding primers of each reaction can be found on **Table 5-7**.

Table 5-7 – Target PCR primers details

PROTEIN NAME	GENE NAMES	REACTION	LEFT PRIMER	RIGHT PRIMER	PRODUCT LENGTH	RefSeq Entry	Uniprot Entry
Oocyte-Expressed Protein (Floped)	OOEP C6orf156 KHD2	Outer	AGAGACCCTTTGGTGTCTACCT	ACGATGTTCTCGGTGAAACCA	225	NM_001080507.2	A6NGQ2
		Inner	GACCGAGCCATAATTCCAGAA	ATTCTGTACACGGGGCCG	117		
Granulins	GRN	Outer	TGGCAAAGAAGCTCCCTG	GACTGTAGACGGCAGCAGGT	343	NM_002087.2	P28799
		Inner	CGGTGCCCTGATGGTTCT	GGCAGCAGTGCAGGTGAT	103		
NACHT, LRR and PYD domains-containing protein 5	NLRP5 MATER NALP5	Outer	GCAGAGACAGAAGAACAAGGACA	GACTTTCGTGCAGAACAC	203	NM_153447.4	P59047
		Inner	GTGACACATGGGACTACAAGAG	TCAAAAGCACCAGCCAAC	124		
NACHT, LRR and PYD domains-containing protein 7	NLRP7 NALP7 NOD12	Outer	CTGTCTTGCTTTCATTGGGAAG	CCAGGAGCACATTGGCTG	239	NM_001127255.1	Q8WX94
		Inner	ATGCTGTGTGACCTGCTCAG	CTGGTTGGCTTTGAGGACAT	117		

Each reaction contained 1.5 μ L of 10X Buffer solution (5Prime GmbH, Germany), 0.12 μ L of each primer, 0.3 μ L of dNTP (Thermo Fisher Scientific, USA), 0.09 μ L of Perfect Taq (5Prime GmbH, Germany) enzyme solution and 1 μ L of cDNA. The volume was brought to 15 μ L with PCR grade water (Roche).

T_m for both inner and outer primers were empirically validated by amplifying genomic DNA on a gradient PCR reaction (data not shown). Elongation step times for each PCR reaction were set according to the expected product size.

The amplification conditions for all primers were as follows: denaturation for 1 min at 96°C, followed by 40 cycles of 15 s at 94°C, 60 - 150 s at 54.5°C, 45 s at 72°C, and a final extension step of 10 min at 72°C.

The T_m that produced the highest yield was subsequently used for target amplification on mRNA extracted from embryos.

Products from the outer and inner reaction were plotted on 3% agarose gel for 80 min at 80mV.

5.3.13 PCR products sequencing

Gel bands of interest were excised and DNA was purified according to Qiagen DNA gel extraction kit (Qiagen, Düsseldorf, Germany). Aliquots of the purified PCR products and primers were sent for Sanger sequencing to a commercial service provider (Source BioScience, Oxford). The origin of the mRNA sequence was then validated using BLAST database annotation homology.

5.3.14 Statistics

Protein abundance distribution per identification group (Confirmed Urea only vs. Confirmed Urea Shared; Confirmed MonoPrep only vs. Confirmed MonoPrep Shared) was assessed using SPSS software. All groups showed normal distributions. Statistical analyses of protein abundance across identification groups were performed using t-test on GraphPad software. The analysis showed no significant difference between the groups analyzed (Urea only vs. Urea Shared, t-test, P-value 0.99; MonoPrep only vs. MonoPrep Shared, t-test, P-value 0.33).

5.4 CHAPTER 5 BIBLIOGRAPHY

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6. STATISTICAL ANALYSIS OF SINGLE BLASTOCOEL PROTEOMIC DATA

6.1 Correlation between blastocoel protein levels and embryo's biological features

6.2 Discussion

6.3 Materials and Methods

6.4 Bibliography

6.1 CORRELATION BETWEEN BLASTOCOEL PROTEIN LEVELS AND EMBRYO'S BIOLOGICAL FEATURES

The identification of the embryo with the greatest developmental capacity represents a major challenge for fertility clinics. Current methods for the assessment of embryo competence are proven inefficient and the inadvertent transfer of non-viable embryos is the principal reason why most IVF treatments (approximately two-thirds) end in failure. Hence, the identification of molecular markers for human embryo competence is an important topic in reproductive medicine.

As discussed in Chapter 1, for several years, researchers have been trying to identify molecules that correlate with embryo implantation competence, including metabolites, proteins and reactive oxygen species. The methodologies used for these investigations, which ranged from Raman Spectrometry to Mass Spectrometry and Nuclear Magnetic Resonance, had different biochemical targets and varying degrees of sensitivity. So far, the vast majority of this research has focused on the analysis of the liquid in which single embryos were cultured, also described as conditioned media (Seli *et al*, 2008; Vergouw *et al*, 2008; Katz-Jaffe *et al*, 2006). One common problem of this approach is the vast excess of molecular components already present in the culture medium itself, which forms a complex background that can potentially mask key embryonic molecules present at extremely low concentrations (Dyrlund *et al*, 2014).

In this chapter, I explore the possibility of correlating the presence and abundance of a limited set of blastocoelic proteins, previously measured with targeted mass spectrometry, with several clinical and biological parameters of the embryo.

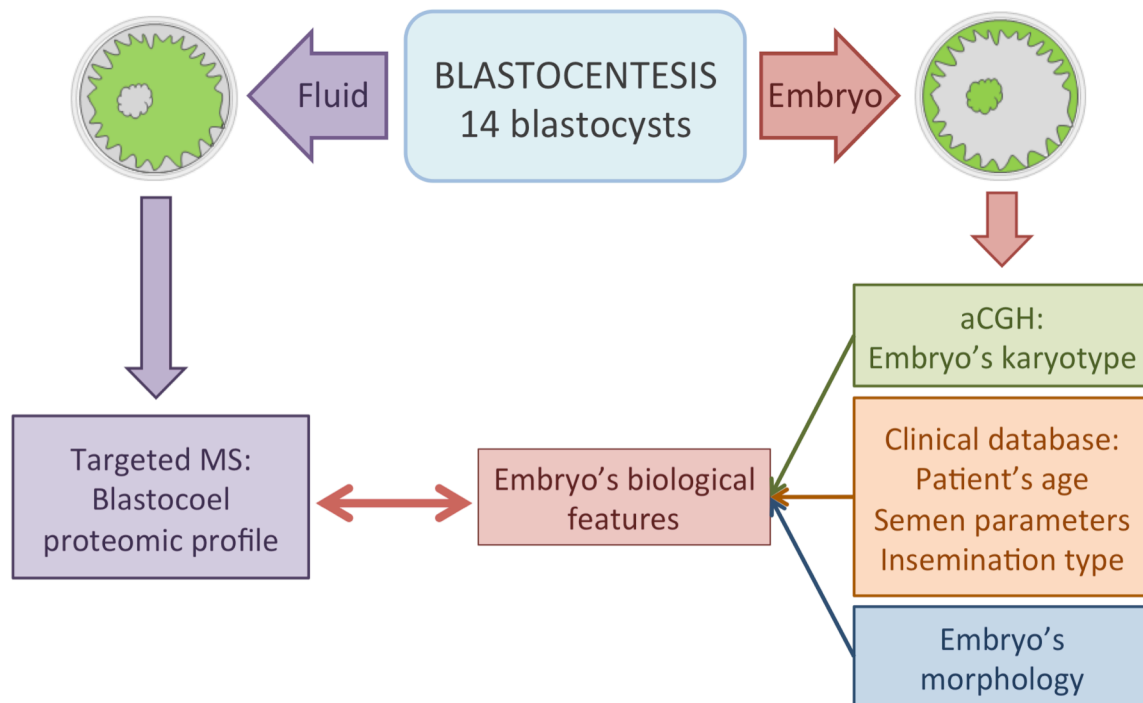


Figure 6-1 – Experimental workflow. Targeted proteomics applied to blastocoel (purple). Embryo's biological features including embryo cytogenetic status (green), clinical parameters (orange) and morphological grade (blue).

In order to accomplish this, I enrolled in the analysis 14 blastosols analysed in previous experiments. For these samples, I performed cytogenetic analysis of the embryo from which the fluids were collected and the proteins contained measured. Also, I retrieved clinical information and anonymised details of patients donating these embryos through Oxford Fertility Unit database system.

The chromosomal status of each embryo was investigated using microarray Comparative Genomic Hybridisation (array-CGH) as described by Fragouli and colleagues (Fragouli *et al*, 2011), a highly validated method that provides comprehensive detection of aneuploidy in minute samples, including single cells (described in sections 3.5.6, 3.5.7 and 3.5.8, Chapter 3). However, whole blastocysts were used to provide even more robust data on overall embryo's chromosomal status (similar to section 3.5.1, Chapter 3). This test revealed that the group of embryos analysed included seven euploid and six aneuploid embryos, seven of which were female, five male and one Turner syndrome (45,XO). Patients data retrieved from the database provided details regarding the female age at the time of oocyte retrieval, the count and motility parameters of the semen specimens used for insemination (WHO Examination and processing of human semen, 2010) and the technique used for oocyte insemination (IVF/ICSI). Embryo morphology was also assessed and recorded prior to microsuction interventions, according to Gardner's blastocyst assessment criteria (Gardner & Leese, 1999; Gardner & Schoolcraft, 1998) (section 2.4.7, Chapter 2).

Absolute protein abundance was calculated on peptides' abundance score (intensity/molecular weight) generated by mass spectrometry readings, using the intensity calibration curve produced with AQUA peptides (**Figure 5-13**, Chapter 5). To allow normal distribution of the results, I applied logarithmic transformation of absolute protein abundance data. The complete dataset is shown in **Table 6-1**.

Table 6-1 – Complete dataset

SAMPLES	Morph score	Karyotype	Sex	Patient age	Semen parameters (Count, motility)	Insemination method	GAPDH (Log10 attomoles)	ACTA (Log10 attomoles)	H2A family (Log10 attomoles)	Euploid	H2A family detected
S01	4Dc	46 XY -1q	M	35	100 M, 54%	IVF	4.226	3.744	3.330	N	Y
S02	4Cb	46 XX	F	31	4 M, 28%	ICSI	3.979	2.984	-	Y	N
S03	4Dc	45 XX	F	36	127 M, 67%	IVF	3.769	3.050	3.817	N	Y
S04	4Db	49 XY	M	31	38 M, 59%	ICSI	3.675	2.763	3.324	N	Y
S05	3Bc	46 XX	F	36	7 M, 40%	ICSI	3.591	2.639	-	Y	N
S06	4Dc	48 XX	F	36	127 M, 67%	IVF	3.483	2.419	3.588	N	Y
S07	4Db	45 XO	Turner	36	7 M, 40%	ICSI	3.355	2.500	3.051	N	Y
S08	4Dc	46 XX	F	31	4 M, 28%	ICSI	3.336	2.330	-	Y	N
S09	4Bc	46 XX	F	31	4 M, 28%	ICSI	3.307	3.017	2.697	Y	Y
S10	4Bb	46 XY	M	36	127 M, 67%	IVF	3.294	2.198	-	Y	N
S11	4Bc	46 XX	F	30	0.5 M, 11%	ICSI	3.276	2.379	-	Y	N
S12	4Db	46 XY	M	36	127 M, 67%	IVF	3.181	3.050	3.683	Y	Y
S13	4Cc	46 XY	M	33	0.5 M, 11%	ICSI	2.723	2.896	-	Y	N
S14	3Dc	46 XY	M	30	107 M, 67%	IVF	2.480	2.097	3.108	Y	Y

Taken as a whole dataset each protein showed a normal distribution, however, when subdivided in the comparison groups (i.e. euploid vs. aneuploid), normality was not confirmed. However, variance across test groups showed homogeneity. Considering these characteristics of the GAPDH and ACTA data, I performed direct comparisons using a non-parametric statistical test (Mann-Whitney U test). Since I did not produce readings of H2A protein family for all samples tested, I investigated the relationship between its detection and embryo's biological features using Fisher's Exact test.

Results

As shown in **Table 6-2** (derived from **Table 5-4**, section 5.2.1, Chapter 5), it was not possible to reproduce the measurement of all protein targets investigated across all blastosol samples tested. For this reason, the creation of a comprehensive blastocoel protein profile for each biological features compared, was not possible.

Table 6-2 – Measurement of protein targets abundance in single blastosol samples enrolled in the analysis

SAMPLES	PROTEIN ABUNDANCE (Intensity/Mw)								
	GAPDH	ACTA	H2AX	SODM	ECAT1	NALP7 NALP2	ENPL	NALP5	TPIS
S01	14665.8	6173.7	2935.6	1133.4	-	386.9	956.9	448.6	638.0
S02	9413.3	1575.0	-	-	-	-	-	-	-
S03	6455.3	1772.7	7032.4	-	-	-	-	-	-
S04	5452.5	1059.3	2904.6	-	-	453.3	-	-	-
S05	4691.9	847.4	-	-	-	-	-	-	-
S06	3861.3	570.9	4659.1	-	-	-	-	-	-
S07	3065.9	660.7	1778.2	-	-	-	-	-	-
S08	2965.7	486.6	-	-	-	-	-	-	-
S09	2815.8	1670.8	941.6	-	-	-	-	-	-
S10	2747.5	384.2	-	-	-	-	-	-	-
S11	2664.1	531.6	-	-	-	-	-	-	-
S12	2245.2	1773.8	5534.8	-	-	-	-	-	-
S13	986.7	1344.4	-	-	-	-	585.4	-	-
S14	636.8	320.2	1970.1	-	-	-	-	-	-
AVERAGE	4476.3	1369.4	3469.5	1133.4	-	420.1	771.1	448.6	638.0

However, I collected measurements of the abundance of three proteins (GAPDH, ACTA and H2A protein family) in the vast majority of samples analysed.

We therefore focused our investigation on the correlation between the presence and amount of these detected proteins and the physiological and genetic features of the embryo.

Table 6-3 - Dataset statistical analyses. Patient age was considered in years at oocyte collection. Embryo morphology assessment was based on Gardner's blastocyst assessment criteria (Gardner & Leese, 1999; Gardner & Schoolcraft, 1998). Semen classification was based on the World Health Organization criteria for semen examination (Examination and processing of human semen, 2010). Semen parameters were considered normal if count >15 Millions/mL and >40% total motility. Protein abundance statistical analyses were performed using Mann-Whitney U test (GAPDH and ACTA). MAD refers to Median Absolute Deviation. Protein detection statistical analyses were performed using Fischer's Exact test (H2AX protein family).

Protein	Median±MAD, n	Median±MAD, n	P-value
KARYOTYPE	Euploid	Aneuploid	
GAPDH	3.294 ± 0.168, n=9	3.675 ± 0.285, n=5	0.029
ACTA	2.639 ± 0.511, n=9	2.763 ± 0.426, n=5	0.317
SEX	Female	Male	
GAPDH	3.483 ± 0.261, n=7	3.2375 ± 0.706, n=6	0.295
ACTA	2.639 ± 0.458, n=7	2.829 ± 0.632, n=6	1
MORPHOLOGY	Good	Poor	
GAPDH	3.300 ± 0.023, n=4	3.419 ± 0.449, n=10	0.539
ACTA	2.509 ± 0.327, n=4	2.829 ± 0.408, n=10	0.436
PT AGE	<35	≥35	
GAPDH	3.307 ± 0.546, n=7	3.483 ± 0.280, n=7	0.318
ACTA	2.763 ± 0.377, n=7	2.639 ± 0.609, n=7	0.443
SEMEN	Normal (>15M, >40%)	Poor (<15M, <40%)	
GAPDH	3.483 ± 0.424, n=7	3.336 ± 0.089, n=7	0.805
ACTA	2.763 ± 0.510, n=7	2.639 ± 0.385, n=7	0.798
INSEMINATION	IVF	ICSI	
GAPDH	3.389 ± 0.436, n=6	3.346 ± 0.234, n=8	0.95
ACTA	2.734 ± 0.632, n=6	2.701 ± 0.359, n=8	0.846

	H2A family detected	H2A family not detected	P-value
Euploid	3	6	0.056
Aneuploid	5	0	
Female	3	4	0.59
Male	4	2	
Good Morph	1	3	0.24
Poor Morph	7	3	
Pt Age <35	3	4	0.59
Pt Age >35	5	2	
IVF	4	2	0.63
ICSI	4	4	
Normal Semen	5	2	0.59
Poor Semen	3	4	

As shown in **Table 6-3**, differences in GAPDH levels and H2A protein family detection across euploid/aneuploid groups produced P-values that respectively met and approached statistical significance following Mann-Whitney U test and Fisher's test (P-value 0.029 and 0.056, respectively) (**Figure 6-2**).

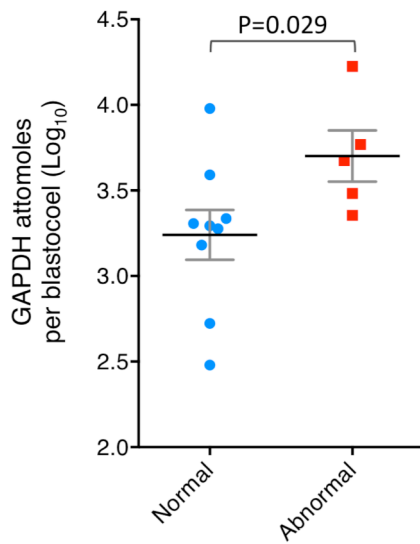


Figure 6-2 – Difference in GAPDH abundance between euploid and aneuploidy. GAPDH abundances per single blastocoeal group based on embryo chromosomal status. P-values were calculated with Mann-Whitney U test. Dark bar = Mean value; Light bars = Standard Error Mean (SEM).

In a subsequent logistic regression analysis, I tested the ability of these three proteins (GAPDH, metabolic; ACTA, cytoskeletal; H2A protein family, nuclear) to predict the policy status of the embryo. I used these three parameters as predictors and embryo's ploidy status as class label (**Figure 6-3**). The statistical model was cross validated with 3 folds and 20 repetitions. Within each cross validation step, variable selection between the three predictors was performed

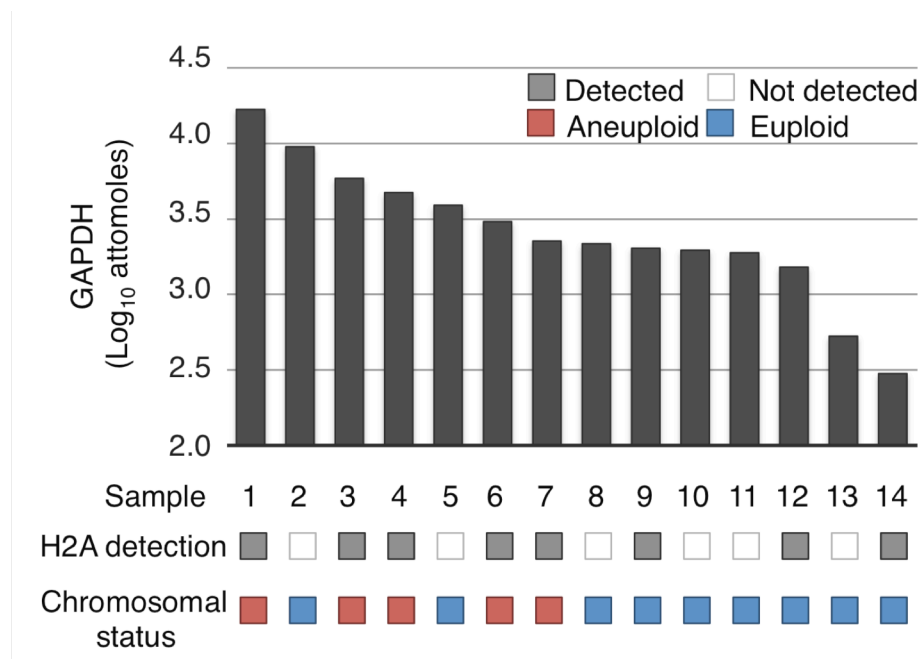


Figure 6-3 – Graphical summary of data used for regression analysis. Measurements of GAPDH and detection of H2A family proteins in samples derived from 14 single blastocoeals and chromosomal status of the embryo. The presence or absence of embryo aneuploidy was assessed by aCGH.

using best subset selection with the AIC criterion (Akaike's An Information Criterion).

ACTA was only included in 7% of the cross validation loops in the best model, while GAPDH levels and H2A protein family detection were included 92% and 97% of the time respectively. This demonstrated that, together, presence of H2A and abundance of GAPDH was able to predict whether an embryo was karyotypically normal or aneuploid. This was achieved with 100% accuracy within the cohort of samples investigated. A detailed description of the statistical analysis is reported in section 6.6.3, Materials and Methods.

It is worth noting that this accuracy estimate is likely to be overly optimistic due to the low sample size and the low number of predictors (3 in total) considered. Furthermore, validation in an independent series of samples would be necessary in order to obtain a true measure of diagnostic sensitivity and specificity.

6.2 DISCUSSION

In order to test embryo competence and ability to implant, the ideal experimental setting would involve the transfer of the tested embryo to the patient. However, due to legal and ethical limitations, embryos donated to research (and used in this project) could not be transferred back to the patient.

For this reason, I investigated the relationship between the presence of certain proteins and embryo karyotype. In our project, the cytogenetic status of the embryo acted as a surrogate marker of embryo viability, compatible with research ethical conduct.

The data obtained suggested that blastocoels of euploid embryos tend to show lower abundance of GAPDH (**Figure 6-2**). This protein is an intermediate enzyme of the glycolysis pathway, the first in a series of catabolic reactions that produce ATP. It has previously been suggested that embryos displaying increased metabolic rate and energy consumption are associated with lower developmental potential (Leese, 2002). Also, recent studies showed that increased amounts of mitochondrial DNA (mtDNA) detected in cellular biopsies are related to reduced embryo implantation rates (Fragouli *et al*, 2015). It is possible that an increased amount of mtDNA could be directly linked to higher energy production rates in the cell.

GAPDH shows both intra- and extracellular localization, therefore the precise origin of the protein detected in blastosol samples cannot be determined with certainty. However, given that the detected abundance of actin (ACTA), an entirely intracellular cytoskeletal component, was not significantly different across the samples tested, it

seems likely that the observed variation in GAPDH levels was not related to differences in the amount of cellular fragments present in the blastocoel.

Interestingly, histone H2A family members were detected more frequently in blastosol samples from aneuploid embryos, suggesting that cell-free DNA fragments may be more common in embryos with an abnormal karyotype. In this circumstance, the detection of chromatin-associated proteins may act as direct evidence of aberrant chromosome segregation. The presence of proteins, typically considered to have a nuclear localization, in the blastocoel cavity, may be indicative of release following cell lysis, possibly related to apoptotic processes, cell lysis or abnormal karyokinesis (i.e. Anaphase lag and Demolition processes discussed in Chapter 3, section 3.3.2).

The application of logistic regression analysis to the data obtained from blastosol protein profiling and aCGH suggested that it may be possible to distinguish chromosomally normal embryos from those affected by aneuploidy, based upon the levels of GAPDH and the detection of histone H2A proteins in the blastocoel fluid. In this initial cohort of samples, embryos that were aneuploid were identified with 100% accuracy. Together, the measurement of GAPDH and identification of H2A family proteins may provide a summary of the metabolic and genetic status of the embryo.

However, due to the small size of the sample population investigated, the limited amount of predictors used and the retrospective nature of the analysis, these data need to be treated cautiously. It is essential to generate additional data in larger studies in order to test the validity of this proteomic approach to preimplantation aneuploidy detection and to define the real sensitivity and specificity of this methodology.

6.3 MATERIALS AND METHODS

6.3.1 Embryo production and blastocoel microaspiration

Human blastocysts were produced and processed as described in sections 2.4.2 and 2.4.3, Chapter 2.

6.3.2 Embryo collection and processing for genetic assessment

Embryo samples were collected as described in section 3.5.1, Chapter 3. Cellular samples were processed for aCGH analysis as described in sections 3.5.6, 3.5.7 and 3.5.8, Chapter 3. The genomic data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar & Lash, 2002) and are accessible through GEO SuperSeries accession number GSE72685. Embryo cytogenetics data are directly accessible through the GEO SubSeries accession number GSE72684.

6.3.3 Statistics

Statistical analyses of protein abundance per biological feature (**Table 6-3**) were performed using Mann-Whitney U test using the R statistical environment (R Core Team, R Foundation for Statistical Computing, Vienna, 2015). Variances homogeneity was confirmed in each comparison group using SPSS software. Statistical analyses of protein detection per biological feature (**Table 6-3**) were performed using Fisher's Exact test ($\alpha = 5\%$) on GraphPad software.

Logistic regression analysis of GAPDH and ACTA levels, H2A family protein detection and embryo chromosomal status was performed using the R statistical environment (R Core Team, R Foundation for Statistical Computing, Vienna, 2015). Specifically GAPDH and ACTA levels, and the H2A family protein detection were considered for the prediction of embryo's chromosomal status. These parameters were used in the analysis, as they were the only variables measured in all the tested samples. I used logistic regression to compute the probability of the chromosomal status being euploid, given the values of the three predictors in each sample. A cutoff of 50% for this probability was used for classifying the chromosomal status of a sample.

The classifier was then evaluated using cross validation with three folds and 20 repetitions as implemented in the "crossval" R package (Korbinian, 2014). Briefly, the data was split into three parts, two of which were used to train the classifier and one was used to test the classifier (i.e. predict embryo's chromosomal status). These predictions were then compared to the true chromosomal status. This test was repeated 20 times. Within each cross validation loop, a variable selection using the three potential predictors was performed using best subset regression with the AIC criterion as implemented in the "bestglm" R package (McLeod & Xu, 2014). This analysis showed that ACTA was hardly ever part of the best model (only in 7% of the cases), while GAPDH levels and H2A protein family detection were important predictors, being part of the best model in 92% and 97% of the cross-validation runs respectively.

Note that due to the low sample size, each of the three folds contained only 4 or 5 samples. The classifier was always able to predict the chromosomal status, hence an accuracy of 100% was obtained. However, it is important to keep in mind that the prior

selection of only three features could possibly constitute an implicit a priori variable selection that could lead to biased cross-validation based accuracy estimates (Ambroise & McLachlan, 2002). Unfortunately, since I could only measure three potential predictors in all of the samples, it is not possible to elucidate this problem with the current data set.

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7. CONCLUSIONS AND FUTURE PERSPECTIVES

7.1 Summary

7.2 Development and assessment of a micromanipulation technique involving the collection of a highly purified embryo-derived specimen

7.3 Investigation of the presence and origin of DNA sourced from the blastocoel and its potential use for preimplantation genetic assessment

7.4 Investigation of the protein composition of the human blastocoel

7.5 Validation of embryonic origin of proteins detected in the human blastosol by gene expression experiments

7.6 Development of a targeted mass spectrometry strategy to measure the abundance of selected proteins of interest in single blastosol samples

7.7 Assessment of potential correlation between the abundance of blastocoelic proteins and the cytogenetic status and other biological characteristics of the embryo

7.8 Final remarks

7.7 Bibliography

7.1 SUMMARY

The field of Assisted Reproduction Techniques requires improvements in the embryo selection process. As discussed in Chapter 1, morphological scoring alone, cannot provide a sensitive enough methodology to increase pregnancy rates further. The screening of the embryonic cytogenetic complement has been proposed and presently is the most widely used and validated molecular strategy for embryo viability assessment. Although the debate is still open on whether preimplantation genetic screening improves the chances of pregnancy in an IVF cycle, it is increasingly accepted that this technique succeeds in significantly reducing risks associated to the transfer of a karyotypically abnormal embryos, such as implantation failure and miscarriage. As discussed in Chapter 1, some authors report increased pregnancy rates after the selection and transfer of a euploid embryo. However, about half of the euploid embryos transferred do not implant (Harton *et al*, 2013), suggesting that other factors may play a role in determining implantation and pregnancy outcomes.

First of all, the biopsy procedure could, in some cases, irreversibly damage the embryo, precluding its implantation even if diagnosed as euploid. While damage is believed to be rare when biopsy is carried out at the blastocyst stage, significant impairment due to cell removal is suspected to have played a role in the failure of early clinical studies in which PGS was applied at the cleavage stage (e.g. Mastenbroek *et al*, 2007). Additionally, other factors affecting pregnancy rates after embryo genetic screening include not only medical conditions of the mother (e.g. hormonal and/or

immunological imbalances, endometrial receptivity and genetic predisposition to miscarry), but also other embryo-associated biological features. For example, it has been suggested that the amount of mitochondrial DNA in embryonic cells may be inversely correlated with embryonic health (Fragouli *et al*, 2015). Therefore, even if euploid, an embryo may not implant because it lacks other critical capabilities. Some groups suggested that proteomic methodologies could increase our understanding of embryo physiology and therefore provide additional parameters for determining embryo viability and competence to produce a healthy pregnancy (Katz-Jaffe *et al*, 2006).

Based on this hypothesis experiments were carried out during the course of this DPhil research, aimed at shedding light on aspects of embryo physiology and ability to develop, using several novel cellular and molecular approaches.

7.1.1 Embryo usage

This study relied on the availability of human embryos donated to research. Appropriate approval from the local Ethical Committee (NRES Committee South Central – Oxford, Ref. 04/Q1606/44) and licensing from the Human Fertility and Embryology Authority (R0111) were put in place. Donations of embryos by consenting patients were performed following HFEA guidelines on surplus embryos donated to research. Overall, 407 embryos donated by 160 patients were accepted in this study and utilised in different experiments. In order to maximise the amount of data generated from this precious and limited resource, some embryos were used in more experiment. Embryo utilisation in the different projects is shown in **Figure 7-1**.

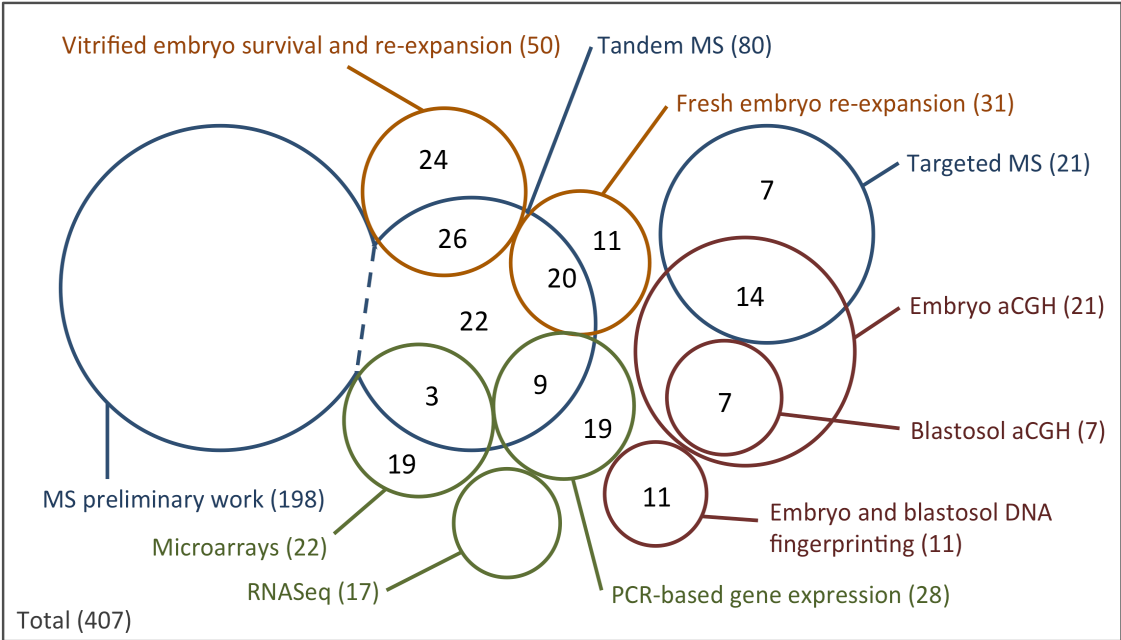


Figure 7-1 – Enrolment of embryos in the different sections of the study. Proteomics (blue), embryology (orange), gene expression (green) and genetic (red) experiments.

7.2 DEVELOPMENT AND ASSESSMENT OF A MICROMANIPULATION TECHNIQUE INVOLVING THE COLLECTION OF A HIGHLY PURIFIED EMBRYO-DERIVED SPECIMEN

This aim stemmed from the need to simplify the embryology laboratory clinical practice and reduce the operator-dependent impact on embryo viability, related to the sampling of material from the embryo. In Chapter 2, it was demonstrated that microsuction is a simple and minimally invasive procedure. Observation of treated embryos suggests that blastosol is quickly replenished following its removal, with viable embryos recovering their original volume within a few hours of the intervention. The overall architectural structure of the blastocyst, following collapse and re-expansion, is not affected by the process (Vanderzwalmen *et al*, 2002; Chen *et al*, 2005; Mukaida *et al*, 2006; Kader *et al*, 2009, 2010). Hence, blastocentesis provides a means of collecting blastosol samples for proteomic or genomic analysis, which is likely to present a minimal risk to the embryo. Indeed, survival rates of embryos subjected to blastocentesis and vitification, followed by subsequent warming, were excellent, indicating that removal of blastosol using this method may actually be beneficial for blastocyst cryopreservation.

When compared to other methods of blastocoel collapse, this study highlights certain advantages of blastocentesis. Microsuction doesn't expose the embryo to any potential thermal stress related to a laser pulse, or pipetting shear-induced stresses, allowing a low impact, cost-effective method for blastocoel collapsing prior to

cryopreservation. This technique also enables the collection of a potentially meaningful embryo-derived sample, which would otherwise be lost.

With reference to the long established amniocentesis procedure, used to test genetic defects by aspirating a sample of fetal cells suspended in the amniotic fluid, I named the collection of the blastosol fluid and its downstream analysis, blastocentesis. Downstream analysis could potentially involve a range of either genetic or proteomic investigations.

On a technical note, unlike embryo biopsy, blastocentesis does not require skills other than those routinely practiced in all IVF clinics. Furthermore, the equipment required is standard in all embryology laboratories. Since no additional equipment or training is required, it is conceivable that blastocentesis could be rapidly implemented in IVF laboratories worldwide without further investments by clinics. If future optimisations permit accurate chromosomal data to be obtained from a greater proportion of blastosol samples, it is conceivable that blastocentesis could be introduced for the purpose of PGS, with significant implications for the cost of such tests.

7.2.1 Future investigations

In order to clearly demonstrate its safety, blastocentesis should be further validated in a larger experimental population, ultimately being applied to embryos destined for transfer to the uterus (allowing implantation, miscarriage and birth rates to be calculated). All the microsuction procedures described in this study were performed by a single operator, therefore, inter-operator variability could not be assessed. Multi-

operator and/or multi-centre experiments should be performed in the future to determine how the operator's micromanipulation dexterity and laboratory settings could affect blastocentesis clinical application.

While any negative impact seems unlikely, long-term effects of this procedure on a range of clinical outcomes should be assessed in a clinical trial. Also, DNA integrity could be assessed in both treated and control human samples, similarly to the study conducted by Kader and colleagues in mice embryos (Kader *et al*, 2010).

7.3 INVESTIGATION OF THE PRESENCE AND ORIGIN OF DNA SOURCED FROM THE BLASTOCOEL AND ITS POTENTIAL USE FOR PREIMPLANTATION GENETIC ASSESSMENT

The successful application of PGS techniques is highly dependent on the skills of the embryologist performing the embryo biopsy procedure (Nagy *et al*, 2012). As discussed above, the possibility of sourcing embryonic DNA with a simple microsuction procedure, would allow widespread application of PGS in virtually every IVF laboratory without further investment in personnel training or equipment (e.g. laser). Our preliminary experiments (e.g. DNA fingerprinting) showed that embryo-derived DNA can be present in the blastocoel cavity. However, its amount is highly variable across samples. In many cases only a minority of genetic loci are detected using PCR-based methods, suggesting that only a fraction of the total genome is present. At the chromosomal level, in our small experimental population, full concordance between blastosol and embryo karyotype was achieved in a very limited proportion of samples. Hence, with current evidence, this approach shows insufficient reproducibility, precluding the applicability of blastosol DNA analysis for PGS and PGD purposes.

7.3.1 Future investigations

This reduced diagnostic efficiency may be due to the amount and quality of the DNA present in the fluid. It is likely that DNA is highly fragmented and therefore more difficult to utilise as PCR template. However, it might be possible to increase PCR

efficiency for these particular samples by using additional preparatory steps prior to amplification. For example, by applying adapter sequences at the ends of DNA molecules, the length of the fragments can be preserved. This is important if the DNA is already degraded, as any further reduction in fragment size (e.g. caused by WGA methods such as SurePlex, which routinely include a DNA fragmentation step) will reduce amplification efficiency. Additionally, a sequencing strategy could increase the sensitivity of the analysis, compared to a label and hybridise (i.e. microarray) approach. These alternatives could be investigated in future studies to identify a potential clinical use of blastosol DNA in preimplantation genetic assessment.

7.4 INVESTIGATION OF THE PROTEIN COMPOSITION OF THE HUMAN BLASTOCOEL

Most of preliminary embryo proteomics studies focused on the analysis of spent culture media composition (Katz-Jaffe & Gardner, 2007, 2008; Katz-Jaffe *et al*, 2009, 2006; Nyalwidhe *et al*, 2013; Dominguez *et al*, 2009). However, two main issues limited the sensitivity and specificity of these approaches. First, the media contains a high concentration of albumin that masks the presence of other, embryo-derived proteins. Second, it has been shown that the media contains non-declared proteins, which create uncertainty on the embryo-specificity of the compounds detected with MS. The investigation of blastocoel fluid aimed at reducing the identification of contaminants present in the media and increase the embryo-specificity of the proteins detected.

In this study, conventional tandem mass spectrometry was applied to batches of 20 blastocoel fluids and allowed the identification of 169 proteins present in these specimens. Further, the development of an alternative sample preparation method (MonoPrep), led to the expansion of this catalogue, revealing 150 proteins, 31 of which had already been detected with conventional experiments, for a combined total of 288 proteins. The development of the MonoPrep sample preparation method was necessary to enable a potential application of mass spectrometry to single blastocoel samples. MonoPrep, in fact, minimises sample handling, lowering the risk of both contamination by the operator and sample loss associated to multiple tube transfers and clean-up steps.

The creation of a catalogue of embryo-derived proteins validated by complimentary gene expression analysis is an important milestone for the investigation of blastocyst proteome. This catalogue should be of help to other research groups interested in both investigating the roles of proteins in embryonic development and generating proteomic strategies for the assessment of embryo viability.

7.4.1 Future investigations

Different protein digestion protocols can generate different peptide populations, due to modified conditions that may favour alternative protein unfolding patterns and exposure of specific amino acid residues to trypsin digestion. It is possible that the application of alternative methods for protein digestion may further enrich the catalogue of proteins identified within the blastocoel fluid. For example, a new protocol for peptide sorting and digestion using magnetic beads was recently suggested (Hughes *et al*, 2014). This method is based on the hydrophilic property of peptides that accumulate around the magnetic beads when exposed to high concentrations of solvent. This approach may bypass the need to concentrate peptides using speed-vac prior to MS analysis, eventually further reducing sample loss. There is also a possibility that this approach may be also applicable to spent culture media. However, the high amount of albumin present in the media would possibly saturate trypsin activity and limit mass spectrometer sensitivity by reducing both relative availability and detectability of less abundant cleaved peptides.

7.5 VALIDATION OF EMBRYONIC ORIGIN OF PROTEINS DETECTED IN THE HUMAN BLASTOSOL BY GENE EXPRESSION EXPERIMENTS

Initially, blastocyst gene expression was investigated to provide support to the embryonic origin of the proteins identified in tandem mass spectrometry experiments. Microarray experiments provided confirmatory results only for whole blastocyst gene expression, but were incapable of distinguishing genes specifically expressed in ICM and TE. Hence, sequencing techniques were employed to further characterise the gene expression patterns of the two cell lineages and possibly identify the tissue origin of the proteins identified. RNASeq experiments provided an exquisite sensitivity in transcript detection allowing not only to expand the transcriptomic catalogue of the human blastocyst generated with microarrays, but also to identify differentially expressed genes in the ICM and TE.

These data were analysed both from a qualitative and quantitative point of view, highlighting the potential role of certain transcripts in the regulation of human blastocyst development. Also, RNASeq made chromosomal transcriptional activity analysis possible. With these data I generated a transcriptional map at the blastocyst stage and made suggestions on how an abnormal chromosomal setting could affect transcriptional activity ultimately impacting the ability of an embryo to move on to later developmental stages. However, this preliminary analysis is mainly descriptive and provides only speculative hypotheses concerning molecular functions active in the ICM and TE.

7.5.1 Future investigations

The data generated by these experiments do not provide sufficient evidence to demonstrate how certain molecular mechanisms work at the embryo system level. They rather provide a qualitative and quantitative overview of the transcriptional activity in the blastocyst and its first differentiated cell populations. Here, different hypothesis were provided for the up- and down-regulation of specific transcript species or cellular pathways. However, to confirm their role in cellular physiology and demonstrate how gene expression modulation can affect embryonic biological processes, further experiments should be performed. These include quantitative PCR of target genes to accurately define differential expression. Also, functional studies where the specific function of a gene/protein is investigated by suppressing expression (e.g. using a knock-out biological model or by RNA interference using miRNA or siRNA) should be performed. However, this type of investigation was beyond the scope of this study and will require a specialized team focusing on the investigation of specific molecular patterns.

7.6 DEVELOPMENT OF A TARGETED MASS SPECTROMETRY STRATEGY TO MEASURE THE ABUNDANCE OF SELECTED PROTEINS OF INTEREST IN SINGLE BLASTOSOL SAMPLES

As previously discussed, the genetic status of the embryo does not correlate entirely with its ability to produce a healthy pregnancy. It is possible that the presence or abundance of certain secreted proteins may affect, or be a sign of, embryonic health. After cataloguing components of the human blastosol, I developed a technique that enabled single specimen analysis with targeted mass spectrometry.

With the application of single reaction monitoring assay, absolute measurements of the abundance of a subset of protein targets were achieved. This is a remarkable result, which highlights the potential application of this type of analysis both in research and clinical contexts. Despite the limited amount of targets measured, these data prove that measurement of proteins in extremely small samples such as the blastocoel is feasible.

These experiments pave the way to the development of more comprehensive techniques that may be able to screen a larger amount of targets and generate more in-depth blastocoel protein profiles.

7.6.1 Future investigations

As mentioned in Chapter 5, the detection of certain targets investigated did not lead to reproducible measurements. This could be due to both low levels of the target

protein in the blastocoelic fluid or to a sub-optimal SRM assay. Therefore, the development of better performing targeted proteomics panels should be researched. This could involve both further improvements in the sample preparation procedure (i.e. by using magnetic beads to separate the proteins from the solution) and the design of additional SRM assays for alternative peptides.

However, due to the specific peptides generated by different digestion conditions, the use of the magnetic beads protocol in targeted MS analysis would require new discovery and validation phases where samples would be analysed by tandem MS. Subsequently, novel SRM assays based on the ion fragments generated with the new methodology should be developed. New assays should then be evaluated using isotopically labeled synthetic peptides and peptide detection efficiency tested.

Also, targeted mass spectrometry represented the most sensitive technology that allowed us to design assays for a number of target proteins without the need for specific reagents (e.g. antibodies). In the future, alternative novel methodologies (i.e. single molecule arrays, digital ELISA) could be implemented to improve the sensitivity and comprehensiveness of human blastosol profiling in single embryos or even find new application on spent culture media. Nonetheless, these methods would require extensive optimisation and specificity/sensitivity testing.

7.7 ASSESSMENT OF POTENTIAL CORRELATION BETWEEN THE ABUNDANCE OF BLASTOCOELIC PROTEINS AND THE CYTOGENETIC STATUS AND OTHER BIOLOGICAL CHARACTERISTICS OF THE EMBRYO

Based on the measurements produced by the application of SRM assays on single blastocoel, significant differences were found in the abundance of GAPDH between blastosols deriving from euploid and aneuploid embryos (Mann-Whitney test, $P=0.029$). Although not significant, statistical testing hinted at an increased detection of H2A in blastosols deriving from aneuploid embryos compared to euploid ones (Fisher Exact test, $P=0.056$). Nonetheless, the data suggests that, employing logistic regression, using the abundance values of GAPDH and ACTA combined with the identification of H2A as predictors, the ploidy status of the embryo could be accurately estimated without the use of genetic techniques. These data derive from a small experimental population of 14 samples and require further validation in a larger prospective cohort (ideally blinded). However, if confirmed by additional evidence, these data would support the theory that an increased metabolic rate, as shown by increased presence of glycolytic enzyme (GAPDH) could be a sign of reduced embryo viability, as stated by the “quiet embryo hypothesis” (Leese, 2002) and indicated by studies on mitochondrial DNA (Fragouli *et al*, 2015). Also an increased amount of nuclear proteins associated to chromatin (H2A) in the blastocoel, could be a sign of extended cellular lysis or abnormal generation of DNA-containing cellular fragments. DNA-bearing cellular fragments have been

proposed as possible products of abnormal karyokinesis, such as “anaphase lag” and “demolition” processes (Mantikou *et al*, 2012).

7.7.1 Future investigations

For legal and ethical reasons, in the UK it is not possible to use embryos donated to research for clinical purposes. This prevented the possibility of transferring tested embryos back to the patients and monitor their subsequent development. However, it is possible that blastocentesis could be soon implemented in clinical practice to collapse embryos prior to cryopreservation. In this case, following patient’s informed consent, it could be possible to test the proteomic profile of transferred embryos. In this way, it would be possible to compare proteomic profiles of implanting and non-implanting embryos, potentially providing a helpful parameter to consider when selecting which embryo(s) to transfer to the patient.

7.8 FINAL REMARKS

In conclusion, a “next-generation” methodology able to assess embryo competence, improving IVF success rates and reducing the likelihood of multiple pregnancies, is urgently required. This set of experiments demonstrate the development of an exquisitely sensitive method of proteomic analysis, capable of detecting and quantifying specific proteins in nanoliter volumes of fluid derived from the blastocoel cavity of human preimplantation embryos. It is likely that this proteomic approach will yield novel biomarkers for the evaluation of embryo potential and could conceivably be applied in other, diverse areas of clinical research involving single cells, microscopic samples of tissue, or minute volumes of biological fluid.

Moreover, it is necessary for the field of assisted reproduction technologies to investigate novel molecular techniques to apply to clinical IVF with the aim of finding biological markers that could improve success rates in reproductive treatments. The technology is continuously being improved, illuminating new molecular targets for investigation. As seen in the reproductive genetics field, the translation of cutting-edge technology to clinical applications (starting from FISH on blastomeres, followed by arrayCGH and most recently by next generation sequencing) have resulted in a cascade of novel and robust diagnostic methodologies that a decade ago seemed unimaginable. Today, proteomics has the potential to lead the next wave of discoveries in the field of reproductive medicine.

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PUBLICATIONS, PRIZES AND ABSTRACTS

RESEARCH ARTICLES

Poli M, Ori A, Child T, Jaroudi S, Spath K, Beck M, Wells D (2015) Characterization and quantification of proteins secreted by single human embryos prior to implantation *EMBO Mol Med* In press (Impact Factor 8.665)

Filho ES, Noble JA, **Poli M**, Griffiths T, Emerson G, Wells D (2012) A method for semi-automatic grading of human blastocyst microscope images *Hum Reprod* (Impact Factor 4.569)

PRIZES

Sir Alec Turnbull Travel Scholarship, University of Oxford, April 2015

Best oral presentation, Graduate Studies Symposium, Departments of Obstetrics/Gynaecology and Paediatrics, University of Oxford, November 2012

ORAL PRESENTATIONS

“Ultra-sensitive mass spectrometry to look at the proteomic content of the blastocoel fluid with a view to embryo viability assessment” **Poli M**, CoGEN, Paris, France (invited session, September 2015)

“Association between blastocoel proteins abundance and embryo chromosomal status in humans” **Poli M**, Ori A, Child T, Jaroudi S, Spath K, Beck M, Wells D, ESHRE 2015, Lisbon, Portugal

“The blastocoel fluid as a source of DNA for preimplantation genetic diagnosis and screening” **Poli M**, Jaroudi S, Sarasa J, Spath K, Child T, Wells D, ASRM 2013, Boston, USA

“Characterisation of human blastocoel protein content by Mass Spectrometry” **Poli M**, Ori A, Child T, Turner K, Wells D, YEN 2013, London, UK

“Defining the biochemical content of the human blastocoel using Mass Spectrometry: a novel tool for identifying biomarkers of embryo competence” **Poli M**, Ori A, Child T, Turner K, Wells D, ASRM 2012, San Diego, USA

“Significant Antisperm Antibodies: ICSI is no better than IVF” **Poli M**, Keane D, Mocanu E, Fertility 2007, York, UK; IFS 2007, Cork, Ireland