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CULTURE AND VITRIFICATION OF HUMAN PREEMBRYOS

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ABSTRACT

Despite improvements in stimulation protocols, culture media formulations and laboratory protocols, the success rates in human IVF remain disappointingly low. The ability to successfully cryopreserve supernumerary embryos in a given IVF cycle without losing significant embryo viability is essential to maximize the cumulative benefit of a given treatment cycle. Therefore, studies on culture, cryopreservation and gene expression of human embryos fertilized in vitro were performed.

In these studies the impact of culture media on fertilization of human oocytes *in vitro* was investigated. Furthermore, the impact of growth factor supplementation to *in vitro* culture media and embryo survival and cryodamage after vitrification were studied. Using in situ hybridization and immunohistochemistry methods, the expression of genes in the human Fallopian tube, endometrium, and pre-implantation embryos and in human embryonic stem cells (hES) cells was studied.

The findings can be summarized as follows: *in vitro* culture media has impact on normal fertilization. Supplementation of growth factors to *in vitro* culture media implicates a physiological role in regulating pre-implantation development. Vitrification of embryos is an effective way of cryopreservation. In situ hybridization, immunohistochemical and matrix assisted laser desorption/ionization time of flight mass spectrometry methods are versatile tools in reproductive medicine research.

These findings will help to identify markers for embryo development and characterisation of hESC. Furthermore, knowledge obtained will give us tools to improve formulations of culture and cryopreservation media, which in turn might increase the overall results in IVF treatment and maximise the usage of hESC.

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LIST OF PUBLICATIONS

This thesis is based on the following original publications referred to in the text by their Roman numerals.

I. **Hambiliki** F, Sandell P, Yaldir F, Stavreus-Evers A. A prospective randomized sibling-oocyte study of two media systems for culturing cleavage-stage embryos-impact on fertilization rate. J Assist Reprod Genet, 2011, 28 (4), 335-41.

II. **Hambiliki** F, Hanrieder J, Bergquist J, Hreinsson J, Stavreus-Evers , Wånggren K. Glycoprotein 130 promotes human blastocyst development in vitro. Fertil Steril. 2013 May; 99(6):1592-9.

III. Kartberg AJ, **Hambiliki** F, Arvidsson T, Stavreus-Evers A, Svalander P. Vitrification with DMSO protects embryo membrane integrity better than solutions without DMSO. Reprod Biomed Online, 2008, 17 (3), 378-84.

IV. **Hambiliki** F, Ström S, Zhang P, Stavreus-Evers A. Co-localization of NANOG and OCT4 in human pre-implantation embryos and in human embryonic stem cells. J Assist Reprod Genet. 2012 Jun 29.

V. Wånggren K, Lalitkumar PG, **Hambiliki** F, Ståbi B, Gemzell-Danielsson K, Stavreus-Evers A. Leukaemia inhibitory factor receptor and gp130 in the human Fallopian tube and endometrium before and after mifepristone treatment and in the human preimplantation embryo. Mol Hum Reprod, 2007, 13 (6) 391-7.

PUBLICATIONS NOT INCLUDED IN THIS THESIS

VI. **Hambiliki F**, Ljunger E, Karlström PO, Stavreus-Evers A. Hyaluronan-enriched transfer medium in cleavage-stage frozen-thawed embryo transfers increases implantation rate without improvement of delivery rate. Fertil Steril. 2010 Oct; 94 (5):1669-73.

VII. Zhang P, Zucchelli M, Bruce S, **Hambiliki F**, Stavreus-Evers A, Levkov L, Skottman H, Kerkelä E, Kere J, Hovatta O. Transcriptome profiling of human pre-implantation development. PLoS One. 2009 Nov 16;4(11):e7844.

VIII. Lalitkumar PG, Lalitkumar S, Meng CX, Stavreus-Evers A, **Hambiliki F**,Bentin-Ley U, Gemzell-Danielsson K. Mifepristone, but not levonorgestrel, inhibits human blastocyst attachment to an in vitro endometrial three-dimensional cell culture model. Hum Reprod. 2007 Nov; 22 (11):3031-7.

IX. Zhang P, Dixon M, Zucchelli M, **Hambiliki F**, Levkov L, Hovatta O, Kere J. Expression analysis of the NLRP gene family suggests a role in human preimplantation development. PLoS One. 2008 Jul 23; 3 (7).

X. Nordqvist S, Kårehed K, **Hambiliki F**, Wånggren K, Stavreus-Evers A, Akerud H. The presence of histidine-rich glycoprotein in the female reproductive tract and in embryos. Reprod Sci. 2010 Oct; 17(10):941-7.

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LIST OF ABBREVIATIONS

AS	Angelman Syndrome
ART	Assisted Reproduction Technology
BWS	Beckwith-Wiedemann Syndrome
DMSO	Dimethyl Sulfoxide
EG	Ethylene Glycol
FER	Frozen Embryo Replacement
FSH	Follicle Stimulation Hormone
Gp130	Glycoprotein 130
ICM	Inner Cell Mass
ICSI	Intracytoplasmic Sperm Injection
IVF	In vitro Fertilization
LH	Luteinizing Hormone
LIF	Leukemia Inhibitory Factor
LIFR	Leukemia Inhibitory Factor Receptor
TE	Trophectoderm
PrOH	Propanediol

1 INTRODUCTION

Infertility, whether male or female, is defined by the World Health Organization (WHO) as the inability of a couple to achieve conception or bring a pregnancy to term after one year or more of regular, unprotected sexual intercourse. It is estimated that around 60 - 80 million couples worldwide experience some sort of infertility. According to WHO, between 2 and 10 % of couples worldwide are unable to conceive a child and a further 10 – 25 % experience secondary infertility i.e. inability to conceive after one or more successful pregnancies (Boivin, Bunting et al. 2007). Infertility is not only a medical problem; it is regarded as psychologically stressful by most individuals and can lead to social isolation (Johansson and Berg 2005).

Assisted reproduction technology (ART) is a common name used to describe the set of various methods to help infertile couples to have a child. Conventional *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) being the two main methods used to date.

Since the birth of the first child after IVF on July 25 1978 (Steptoe and Edwards 1978), the number of ART children has increased steadily. In vitro fertilization is now acknowledged to be an effective treatment for infertility. At present, more than 2 % of all newborns are conceived with the help of IVF. Improved pregnancy and delivery rates per aspiration for IVF, ICSI and frozen embryo replacement (FER) have been reported. Recent studies show that pregnancy rates of 29.1 % and 28.6 % per aspiration for IVF and ICSI, and 20.1 % per thawing for FER can be achieved. Mean delivery rates per aspiration (per thawing for FER) are 21.1, 20.2 and 13.5 %, respectively (de Mouzon, Goossens et al. 2012). The development of human IVF was acknowledged by awarding Dr. R.G Edwards the 2010 Nobel Prize in Physiology or Medicine.

Unfortunately there are risks associated with ART, despite the fact that children born after IVF in general are as healthy as children conceived after natural conception (Ludwig, Sutcliffe et al. 2006; Basatemur and Sutcliffe 2008). Recent observations suggest a link between ART and epigenetic errors. Two imprinting disorders, Beckwith-Wiedemann Syndrome (BWS) and Angelman Syndrome (AS), have been associated with the use of IVF (Amor and Halliday 2008).

Women undergoing ART face an increased risk of multiple pregnancies due to the practice of transferring two or more embryos. The rationale to reduce multiple pregnancies is obvious: neonatal complications have been found to be more common in

children born as a result of multiple pregnancies. To address this issue of multiple pregnancies in ART, several suggestions and guidelines have been implemented. It is now well understood that single embryo transfer is the ultimate goal in ART to reduce multiple pregnancies. Sweden and other European countries have introduced mandatory or voluntarily regulatory procedures that insist on single embryo transfer (de Mouzon, Goossens et al. 2012).

To make single embryo transfer feasible, reliable methods to select the embryo with the most potential for implantation is needed. To date the most common way of selecting embryos for transfer is by morphology (Ziebe, Petersen et al. 1997; Ebner, Moser et al. 2003; Scott 2003; 2011). Recently, time-lapse imaging has emerged as a non-invasive embryo selection technique (Campbell, Fishel et al. 2013; Kirkegaard, Kesmodel et al. 2013; Montag, Toth et al. 2013). Despite improvements of stimulation protocols, culture media formulations and laboratory protocols, the success rate after human IVF treatment remain disappointingly low. Therefore, to improve implantation and thus success rates is to design culture media supplemented with growth factors and to improve cryopreservation methodology, since the ability to successfully cryopreserve supernumerary embryos in a given IVF cycle without losing significant embryo viability is essential to maximize the cumulative benefit of a given treatment cycle (Salumets, Suikkari et al. 2006).

1.1 In vivo fertilization

Women are born with a defined pool of primordial oocytes which are arrested at the dictyate stage of meiosis I. Once a month, follicle stimulating hormone (FSH) and other factors stimulate the maturation of individual follicles, generating primary and secondary follicles. Elevated levels of estradiol cause the pituitary to release a surge of luteinizing hormone (LH), which promotes maturation of the dominant follicle and meiotic resumption of the oocyte. Following this, the dominant follicle ruptures and releases the mature oocyte (MII) into the fallopian tube (oviduct) for potential fertilization.

At coitus, human sperm are deposited into the anterior vagina and moves to the upper third of the ampulla of the fallopian tube through the cervix and across the length of the uterus toward the oocyte. During travel through the uterus and oviduct, sperm undergo capacitation. The capacitated and hyper-activated sperm interact with the oocyte in the ampulla of the fallopian tube. Thermotactic and chemotactic gradients are involved

in sperm guiding towards the oocyte and motility hyperaction assist sperm in penetrating mucus in the tubes and the cumulus oophorus and zona pellucida (ZP) of the oocyte.

Upon reaching the oocyte, the sperm penetrates the corona radiata and attaches to the zona pellucida, undergoes the acrosome reaction, and binds to specific receptors (ZP1, ZP2 and ZP3) on the oocyte plasma membrane. The membranes of the oocyte and sperm fuse and the whole sperm (head and tail) enter the cytoplasma in the oocyte.

Preimplantation development is initiated as the embryo undergoes a number of cell divisions while being transported down the oviduct towards the uterus and prepares for implantation. The embryo reaches the uterus at the blastocyst stage, hatches and then implants into the endometrium (Fig. 1).

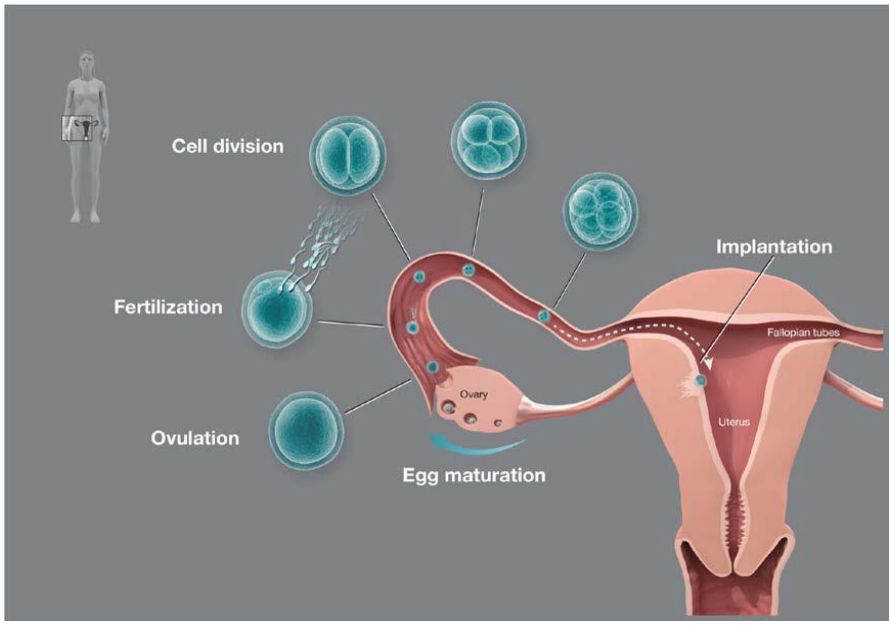

Figure 1. Illustration of in vivo fertilization (© The Nobel Committee for Physiology or Medicine, provided by the Nobel Committee for Physiology or Medicine).

1.2 In vitro fertilization

In vitro fertilization (IVF) is a process by which an oocyte is fertilized by sperm outside the body: *in vitro*. Human IVF is a major treatment for infertility after other methods of ART have failed to achieve a successful pregnancy. IVF was initially

developed for a group of patients with absence or dysfunction of fallopian tubes. The process, as developed by Robert G Edwards, involves monitoring of the woman's ovulatory process and retrieving of mature oocytes (MII) prior to ovulation from the woman's ovaries by trans-vaginal aspiration. The retrieved oocytes are placed in culture dishes with culture medium and incubated with processed spermatozoa in a laboratory. The content of the medium promotes sperm capacitation *in vitro*, which is a necessary requirement for fertilization process. The fertilised egg (zygote) undergoes a number of cell divisions *in vitro*. The embryo is then transferred to the patient's uterus with the intention of establishing a successful pregnancy (Fig. 2).

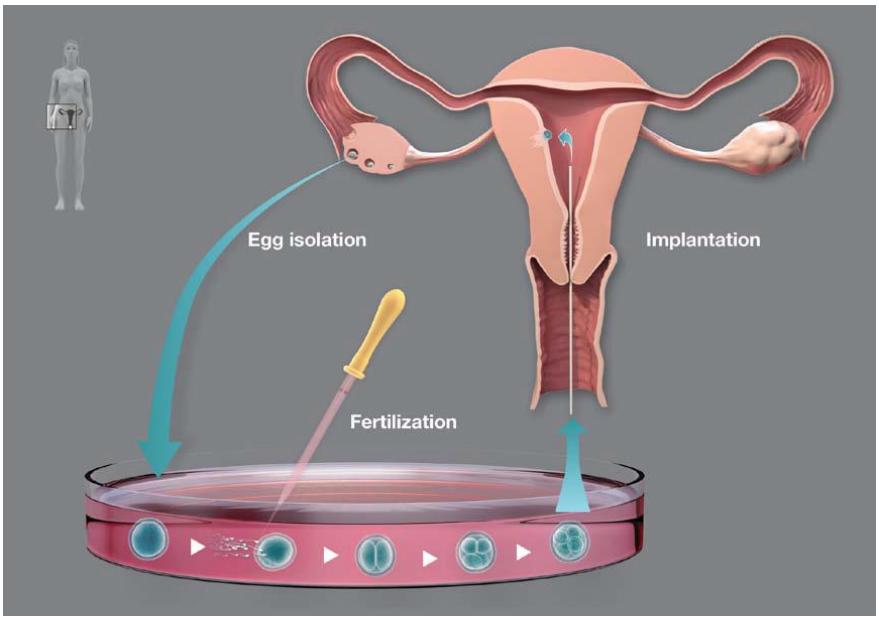

Figure 2. Illustration of in vitro fertilization (© The Nobel Committee for Physiology or Medicine, provided by the Nobel Committee for Physiology or Medicine).

1.3 Preimplantation embryo development

Early human development starts with fertilization of the oocyte by the sperm, resulting into the formation of the zygote. The zygote undergoes first mitotic division and cleaves into a two-cell embryo under the control of the maternal genome. Development continues and after 2/3 days post insemination the embryo is at four-cell stage, respective eight-cell stage. At this stage the embryonic genome is activated (Braude,

Bolton et al. 1988; Duranthon, Watson et al. 2008). Thereafter, the embryo undergoes compaction which is a major change due to the formation of the epithelium and is considered to be the first event of morphogenic and cellular differentiation. Following compaction, fluid is transported into the interior of the embryo to produce a cavity called the blastocoele and as it expands, two cell populations are formed, the trophectoderm (TE), which forms the extra-embryonic tissue and the inner cell mass (ICM), which forms the embryo lineage. Finally, the embryo hatches and implants into the endometrium.

1.3.1 **Fertilization**

Fertilization is a well-orchestrated series of cellular events triggered following sperm-oocyte interaction leading to the formation of a zygote. Fusion of the oocyte and sperm membranes initiates activation of the fertilized oocytes metabolic and biosynthetic activities and thus the commencement of early embryonic development (Ajduk, Ilozue et al. 2011)

1.3.2 **Cleavage**

The zygote cleaves into a two-cell embryo at a mean of 35.6 h post insemination (Trounson, Mohr et al. 1982; Cummins, Breen et al. 1986). Each blastomere then divides repeatedly, but not in synchrony, to yield a four-cell embryo at 44 ± 1 h post-insemination, eight-cell at 68 ± 1 post-insemination and a morula at 92 ± 2 h post-insemination. Between the four-cell and eight-cell stage, the transition from maternal to embryonic gene expression occurs; therefore, all protein synthesis up to the two-cell stage is regulated by maternal mRNA (Braude, Bolton et al. 1988; Kidder 1992).

1.3.3 **Compaction**

At the stage of 8-16 cells, the blastomeres in the morula suddenly increase in mutual adhesiveness and become packed together and form a compact sphere. The highly compact disposition of the embryo is consolidated by tight junctions that form at the

outside edges of the blastomeres, sealing off the inside of the sphere, while gap junctions form among the connecting blastomeres enabling exchange of small molecules and ions. This results into the first differentiation in mammalian development: the separation of the trophectoderm (TE) from the inner cell mass (ICM) (Tarkowski and Wroblewska 1967).

1.3.4 Blastocyst formation

The blastocyst is characterized by the inner cell mass and the trophectoderm, and a blastocyst cavity (blastocoel). The inner cell mass gives rise to the embryo proper, the amnion, yolk sac and allantois, while the trophectoderm will eventually form the placenta.

1.4 Human Embryonic Stem cells

Embryonic stem cells (ES cells) are pluripotent stem cells derived from the inner cell mass of the blastocyst stage embryo and are capable of unlimited, undifferentiated proliferation *in vitro* (Evans 1981; Martin 1981; Thomson, Itskovitz-Eldor et al. 1998; Ying, Nichols et al. 2003; Ying, Stavridis et al. 2003). ES cells differentiate into all derivates of the three primary germ layers: ectoderm, endoderm, and mesoderm.
Embryonic carcinoma (EC) cells isolated from the teratocarcinoma were previously used as *in vitro* model for early mouse development (Martin 1980). The discovery that EC cells harbor genetic aberrations led to the need to culture pluripotent cells directly from the inner cell mass of blastocysts. 1n 1981, two independent research groups managed to derivate embryonic stem cells (Es cells) from mouse embryos (Evans 1981; Martin 1981) and eventually a breakthrough was made in 1998 when researchers developed a method to isolate and culture human embryonic stem cells (Thomson, Itskovitz-Eldor et al. 1998).

Three core transcription factors, namely Oct-4, Sox2 and Nanog play a central role in the regulation of pluripotency and self-renewal in ES cells. These transcription factors are highly expressed in pluripotent cells and are considered markers of ES cells (Rosner, Vigano et al. 1990; Scholer, Ruppert et al. 1990; Avilion, Nicolis et al. 2003; Chambers, Colby et al. 2003). They are capable of inducing the expression of

each other, and are essential for maintaining the self-renewing undifferentiated state of the inner cell mass of the blastocyst, as well as in embryonic stem cells (Rodda, Chew et al. 2005).

Recently, it was shown that ES cells can be derived from single blastomeres without destroying the embryo (Klimanskaya, Chung et al. 2006). In 2006, it was reported that cells similar to ES cells can be generated by genetically reprogramming specialized adult cells (Takahashi and Yamanaka 2006).

Oct-4 together with Sox2, Klf4 and often c-Myc in mouse are the transcription factors used to create induced pluripotent stem cells, (Maherali, Sridharan et al. 2007; Okita, Ichisaka et al. 2007; Wernig, Meissner et al. 2007; Yu, Vodyanik et al. 2007). It was later shown that only two of these four factors, Oct-4 and Klf4 were sufficient to reprogram mouse adult neural stem cells (Kim, Zaehres et al. 2008). Finally, it was shown that Oct-4 alone was sufficient for this transformation (Kim, Sebastiano et al. 2009).

Human embryonic stem cells (ES) and induced pluripotent stem cells (iPSCs) have the ability to differentiate into various cell types, therefore useful for regenerative medicine.

1.4.1 **Oct-4**

The Oct-4 gene (octamer-binding transcription factor 4) is a homeodomain transcription factor of the POU family. This protein is critically involved in the self-renewal of undifferentiated embryonic stem cells. As such, it is frequently used as a maker for undifferentiated cells. Oct-4 transcription factor is initially active as a maternal factor in the oocyte but is expressed constitutively throughout the pre-implantation period (Kimber, Sneddon et al. 2008). Oct-4 is expressed in both ESCs and embryonic carcinoma cells (Okamoto, Okazawa et al. 1990; Rosner, Vigano et al. 1990; Scholer, Ruppert et al. 1990; Yeom, Fuhrmann et al. 1996) and is considered to be essential for pluripotency (Nichols, Zevnik et al. 1998; Niwa, Miyazaki et al. 2000). Deletion of the Oct-4 gene allows blastocyst formation but failure as regards pluripotency, which results in differentiation into a trophectodermal lineage (Nichols, Zevnik et al. 1998).

1.4.2 **Nanog**

Nanog is a recently described homeodomain-bearing protein. It is a transcription factor critically involved with self-renewal of undifferentiated embryonic stem cells and has been described in mouse pluripotent cells (Chambers, Colby et al. 2003; Mitsui, Tokuzawa et al. 2003). Nanog is expressed in a restricted number of cell types and only in cells that also express Oct-4, including ESCs (Chambers and Smith 2004; Zhang, Zucchelli et al. 2009; Hambiliki, Strom et al. 2012). In the mouse embryo, Nanog is localized in the centre of the morula and in the ICM of the blastocyst (Chambers, Colby et al. 2003). Nanog-induced self-renewal and nanog function, but not nanog expression, are dependent on continued Oct-4 expression. Oct-4 is required for nanog-induced self-renewal (Chambers, Colby et al. 2003; Mitsui, Tokuzawa et al. 2003; Kuroda, Tada et al. 2005). Genetic deletion of nanog in mouse ESCs resulted in the production of primitive endoderm-like cells, and nanog mutant embryos were able to produce parietal endoderm. Since embryos lacking Oct-4 are unable to develop so far this resulted in the assumption that nanog function is critical during a later developmental stage than is the case for Oct-4, and NANOG has been shown to be expressed from the 4-cell stage onwards (Kimber, Sneddon et al. 2008; Hambiliki, Strom et al. 2012). Following implantation, when the ICM is developing into epiblast and primitive endoderm, nanog is expressed in the epiblast cells (Hart, Hartley et al. 2004).

1.5 Cryopreservation

The use of ovarian stimulation of multiple dominant follicles has not only improved and simplified human IVF, but also led to the recovery of large numbers of oocytes obtained from one single oocyte retrieval (Tan, Kingsland et al. 1992). In order to limit the risk of high order multiple pregnancies and the associated obstetrical and perinatal complications and to avoid wastage of supernumerary embryos, the development of human embryo cryopreservation was initiated.

Cryopreservation is a process where cells or whole tissues are cooled and stored at temperatures where all metabolic processes are arrested. In 1972 the first mammal (rabbit) was born after the transfer of frozen-thawed morulae (Whittingham, Leibo et al. 1972). Since then, mammalian embryos of several species have been successfully frozen and stored.

The first human embryos were frozen in the late 1970s and the first clinical pregnancy derived from frozen-thawed human embryos was reported in 1983 (Trounson and Mohr 1983). The following year in 1984, the birth of Zoe Leyland was reported as the first birth following transfer of frozen-thawed embryos (Zeilmaker, Alberda et al. 1984). Since then cryopreservation of supernumerary embryos and transfer of thawed cryopreserved embryos in a subsequent cycle is now an indispensable part of human IVF.

Cryopreservation offers several important benefits in human IVF; it allows storage of good quality embryos for future use (Zeilmaker, Alberda et al. 1984), thus minimizing the risk of transfer of more embryos than currently needed. Cryopreservation protocols have been optimized and simplified by use of cryoprotectants like propanediol and sucrose for pronucleate oocytes and early cleaved embryos (Lassalle, Testart et al. 1985) and glycerol and sucrose for blastocysts (Cohen, Simons et al. 1985; Fehilly, Cohen et al. 1985; Hartshorne, Elder et al. 1991; Menezo, Nicollet et al. 1992). Currently, two basic types of cryopreservation are used irrespective of cell or tissue type: Equilibrium freezing protocols (slow freezing) and non-equilibrium protocols (vitrification/ultra-rapid freezing)

1.5.1 **Slow freezing**

Slow programmable freezing is a set of well-established techniques developed during the early 1970s which enabled the first birth from a human frozen embryo, Zoe Leyland, in 1984 (Zeilmaker, Alberda et al. 1984). Since then, most IVF programs use programmable machines to freeze human embryos before they are cryopreserved in liquid nitrogen.

Slow freezing includes dehydration of the embryo to prevent formation of intracellular ice crystals. In an isotonic medium, the formation of ice crystals takes place between -5^0C to -15^0C. The formation of ice crystals may cause mechanical damage to the embryo by disrupting and displacing organelles, or by slicing through membranes (Watson and Morris 1987). This is why slow freezing techniques use cryoprotectants and controlled ice formation at critical temperatures. The

cryoprotectants commonly used in slow freezing are dimethyl sulfoxide (DMSO), glycerol and propanediol (PrOH).

1.5.2 **Vitrification**

The purpose of vitrification is to protect the cell by avoiding ice crystal formation. This is achieved by the addition of cryoprotectants at high concentrations prior to freezing.
Vitrification was first introduced to reproductive cryopreservation in the mid-1980s (Rall and Fahy 1985). The applications and advantages of vitrification over slow freezing are increasingly clear. The method is fast and does not require expensive equipment. DMSO is the most frequently used cryoprotectant in vitrification protocols. It is a permeating cryoprotectant which protects cells from intracellular ice crystal formation when used at high concentrations. Recent publications show that vitrification of human embryos is possible with different cryoprotectant solutions, such as DMSO in combination with ethylene glycol (EG), or EG alone (Rama Raju, Haranath et al. 2005; Takahashi, Mukaida et al. 2005; Desai, Blackmon et al. 2007).

1.6 Culture media

The possibility to study mammalian preimplantation embryo *in vitro* became a reality when rabbit oocytes matured *in vitro*, reached the metaphase stage of meiosis II (Pincus and Enzmann 1935). In 1953 it was reported that eight-cell mouse embryos could development into blastocyst when cultured in a simple chemically defined medium (Whitten 1956) and that blastocyts produced in this way could develop into normal young after being transferred into the uterus of mouse surrogate mothers (Mc and Biggers 1958). Further experiments showed that *in vitro* matured rabbit oocytes could be fertilized *in vitro* and give rise to viable offspring when transferred back to adult females (Chang 1959).

Despite these advances, no progress had at that time been made regarding human *in vitro* fertilization of oocytes. A breakthrough was made when it was reported that human oocytes required 24 hours of incubation *in vitro*, before they would initiate their maturation process (Edwards 1965; Edwards 1965). Finally in 1969 it was shown that activated human spermatozoa could promote fertilization of *in vitro* matured oocytes (Edwards, Bavister et al. 1969). Edwards later reported that *in vitro* fertilization of pre-

ovulatory oocytes using *in vitro* activated spermatozoa could give rise to eight-cell (Bolton, Wren et al. 1991) stage embryos and undergo further cleavage to blastocysts *in vitro* (Edwards, Steptoe et al. 1970; Steptoe, Edwards et al. 1971). These discoveries opened the way for human IVF which culminated in the birth of Louise Joy Brown, July 25, 1978 (Steptoe and Edwards 1978).

Since then, the culture conditions for human embryos have improved considerable, increasing the viability of *in vitro* fertilized embryos. Initially, media for culture of human embryos to day 2 or day 3 post-insemination were simple media such as T6 (Trounson, Leeton et al. 1980), HTF (Quinn, Kerin et al. 1985) and EBSS (Edwards 1981). These media were composed of balanced salt solutions with added carbohydrates (glucose, pyruvate, and lactate) and a serum additive. However, they lacked important regulators of embryo development to support culture to blastocyst stage (Lopata and Hay 1989; Bolton, Wren et al. 1991).

The media used for embryo culture contain ionic components (Na^+, K^+, Ca^{2+}, Mg^{2+}, PO_4^{3-} and Cl^-) detected in the oviduct and uterine fluids (Borland, Biggers et al. 1980), in a bicarbonate buffered (HCO_3^-) isotonic atmosphere. Together with water they contribute to the osmolarity of culture media which ranges from 250 – 290 mOsmols at which embryo development is supported (Brinster 1965; McKiernan and Bavister 1990).

The main energy substrates in culture media are pyruvate, glucose and lactate. These energy substrates regulate mammalian embryo metabolism in a stage-specific manner since early human embryo metabolism differs between pre- and post- compaction stage (Gardner and Lane 1997). While, pyruvate has been shown to be essential for the early pre-implantation embryo (Conaghan, Handyside et al. 1993; Conaghan, Hardy et al. 1998), glucose in association with phosphate and in the absent of amino acids has been shown to cause developmental delay or arrest in cleavage stage embryos in non-human species (Bavister 1995). This led to omission of glucose in certain culture media. However, this omission has been shown to be non-physiological, since glucose is present in the oviduct and uterine fluids and the embryo utilizes glucose throughout the pre-implantation period via the pentose-phosphate pathway (Gardner, Lane et al. 1996). Amino acids are found in the fluids of the human reproductive tract (Borland, Biggers et al. 1980; Casslen 1987) and oocytes and embryos contain specific transport mechanisms for amino acids. They act as energy substrates, precursors for protein synthesis, osmolytes and in maintenance of intracellular pH (Gardner and Lane 1998). Previous studies have shown a biphasic requirement of amino acids during the

preimplantation period (Lane and Gardner 1997; Steeves and Gardner 1999). It has been shown that non-essential amino acids and glutamine stimulate cleavage rate and that glutamine and essential amino acids promote culture to the blastocyst stage (Lane and Gardner 1997; Lane, Hooper et al. 2001). Amino acids spontaneously break down at 37^{o}C and release ammonium which is toxic for the embryo even at low concentrations because ammonium retards embryo development, foetal growth and increases the incidence of neural tube defects (Gardner and Lane 1993). Ammonium can significantly be reduced if glutamate is replaced with the more stable alanyl-glutamine or glycyl-glutamin (Lane, Hooper et al. 2001).

Vitamins are also key components of cellular metabolism and are thus added in culture media. It has been shown that they have significant effects on embryo development of non-human species (Kane and Bavister 1988; Tsai and Gardner 1994; McKiernan and Bavister 2000). However, no benefits of vitamins on the human embryo have been shown (Tarin, de los Santos et al. 1994).

EDTA is added to culture medium for its potential as a chelating agent of metal ions and has beneficial effects on the cleavage stage mouse embryo (Gardner and Lane 1996; Gardner, Lane et al. 2000). However, EDTA should not be included in media intended for culture of post-compaction stage embryos as it inhibits the development of inner cell mass (Lane and Gardner 1997).

Protein sources in the form of serum or albumin in culture media play a role as organic osmolytes and pH buffers. In the IVF lab they prevent embryos from adhering to pipettes or plastic containers for tissue culture. There are several risks associated with addition of patient serum in culture media like potential viral transmission (Ashwood-Smith, Hollands et al. 1989), negative effects on embryo development (Lopata and Hay 1989; Dokras, Sargent et al. 1993; Ito, Fujino et al. 1996), trophectodermal (Mortell, Marmorstein et al. 1993) and mitochondrial deterioration (Thompson, Gardner et al. 1995) and metabolic perturbations (Gardner 1994). This has led to the use of chemically defined alternatives to human sera such as purified albumin (Ashwood-Smith, Hollands et al. 1989) and recombinant serum albumin (Lane, Hooper et al. 2001).

Hyaluronan is another macromolecule included in culture media. It is present in the female reproductive tract and its use has been shown to improve implantation rates (Gardner, Rodriegez-Martinez et al. 1999; Hambiliki, Ljunger et al. 2010) and increase survival after freezing, of blastocyts (Gardner, Rodriegez-Martinez et al. 1999).

Increased understanding of both the physiology of the embryo and the environment of the oviduct and uterus has led to two different approaches for design of ART specific culture media: the `back to nature´ approach (sequential culture media) and the ´let the embryo choose´ approach (monoculture). Both media reflect the change in concentrations of metabolites from the oviduct fluid to uterine fluid.

There is a need for optimization of culture conditions to enhance implantation, despite significant improvements since the inception of IVF. Co-culture systems and supplementation of growth factors in culture media are among such strategies to improve culture media.

Co-culture of human embryos with somatic cells was shown to support development of embryos to the blastocyst stage (Menezo, Guerin et al. 1990; Wiemer, Hoffman et al. 1993; Hu, Maxson et al. 1998; Wetzels, Bastiaans et al. 1998) and improve pregnancy rates (Menezo, Hazout et al. 1992; Freeman, Whitworth et al. 1995; Ben-Chetrit, Jurisicova et al. 1996), especially in patients with repeated IVF failures (Bongso, Ng et al. 1991; Olivennes, Hazout et al. 1994). Somatic cells may benefit embryo development by providing trophic factors and/or by modifying inhibitory media components (Bongso, Ng et al. 1991; Edwards, Batt et al. 1997; Liu, Chan et al. 1998).

1.6.1 Growth factors

Growth factors have been identified in the female reproductive tract fluid and receptors are present on epithelial cells of the oviduct and the uterus. Supplementation of culture media with growth factors such as leukaemia inhibitory factor (LIF) (Dunglison, Barlow et al. 1996), heparin binding-epidermal growth factor (EGF) (Martin, Barlow et al. 1998), transforming growth factor alpha (TGF-alpha) (Paria and Dey 1990), granulocyte-macrophage colony stimulating factor (GM-CSF) (Sjoblom, Wikland et al. 1999), has been shown to increase development of human embryos to blastocyst stage.

1.6.2 Leukemia inhibitory factor (LIF)

Leukaemia Inhibitory Factor (LIF) is an interleukin 6 class cytokine that affects cell growth and cell differentiation of different types of target cells in the embryo as well as in the adult (Gearing 1993), such as embryonic stem cells (Smith, Nichols et al. 1992), primordial germ cells (Matsui, Toksoz et al. 1991) and peripherial neurons (Yamamori 1992). LIF has been shown to be involved in a number of processes in reproduction

such as enhancement of sperm motility and survival (Attar, Ozsait et al. 2003) and in the physiology of ovulation (Arici, Oral et al. 1997). LIF enhances blastocyst formation and hatching (Lavranos, Rathjen et al. 1995) and has been shown to be essential for implantation in mouse (Stewart, Kaspar et al. 1992). LIF also increases implantation rates in sheep (Fry, Batt et al. 1992). It is known that addition of LIF to the culture medium significantly increases blastocyst formation in human embryos (Dunglison, Barlow et al. 1996).

The importance of LIF in human implantation is still uncertain. In the endometrium of healthy women, LIF receptor (LIFR) and glycoprotein 130 (gp130) are expressed throughout the cycle with strong increase in the midsecretory phase, coinciding with a supposed window of implantation. It has been observed that the endometrium in infertile women produces significantly less LIF during the period of receptivity than in women with proven fertility (Laird, Tuckerman et al. 1997; Hambartsoumian 1998; Lass, Weiser et al. 2001). This has led to the suggestion that recombinant human LIF might help to improve the implantation rate in women with unexplained infertility (Aghajanova, Skottman et al. 2006).

However, the LIF content in uterine secretion at the time of oocyte retrieval does not seem to be correlated to pregnancy rate (Olivennes, Ledee-Bataille et al. 2003). LIF exerts its biological effects by interaction through its specific LIF receptor (LIFR). The LIFR forms a high affinity heterodimer complex with gp130 (Robinson, Grey et al. 1994). Expression of mRNA activity for LIFR and gp130 has been demonstrated in human preimplantation embryos (Sharkey, Dellow et al. 1995; Chen, Shew et al. 1999), but the localization of LIFR and gp130 in human preimplantation embryos has to our knowledge not been reported.

LIF is produced in the human Fallopian tube. The highest levels are seen in the luminal epithelium of the ampulla, where the embryo first develops before its transport into the uterine cavity for implantation (Keltz, Attar et al. 1996; Li, Sun et al. 2004). Co-culture of mouse embryos with human oviductal cells will result in maintained mitochondrial function, decreased apoptosis in the embryo and a higher degree of blastocyst formation and hatching (Xu, Cheung et al. 2000; Xu, Cheung et al. 2001; Xu, Chan et al. 2003). Cultured bovine oviductal cells synthesize LIF that conditions the embryo for implantation (Reinhart, Dubey et al. 1998).

1.6.3 **Leukemia inhibitory factor receptor (LIFR)**

Leukemia inhibitory factor receptor (LIFR) is a subunit of a receptor for leukemia inhibitory factor. LIF action appears to be mediated through a high-affinity receptor complex composed of a low-affinity LIF binding chain (LIF receptor) and a high-affinity converter subunit, gp130. The expression of LIF and its receptor in human endometrium increases around the time of implantation and was demonstrated to appear simultaneously with pinopodes (Aghajanova, Stavreus-Evers et al. 2003). LIF is believed to be a regulatory factor essential for implantation of the blastocyst through autocrine and paracrine interaction between LIF and its receptor in the luminal epithelium (Cullinan, Abbondanzo et al. 1996; Cheng, Rodriguez et al. 2002).

1.6.4 **Glycoprotein 130**

Glycoprotein 130 (gp130) is a trans-membrane protein which is the founding member of the class of all cytokine receptors. It forms one subunit of type I cytokine cytokine receptors within the IL-6 receptor family and is important for signal transduction. The gp130 trans-membrane subunit can also, as a monomer, act as interleukin-6 (IL-6), interleukin-11 (IL-11) and ciliary neutrophic factor (CNTF) receptor (Auernhammer and Melmed 2000).

Gp130 is most abundantly expressed at the time of implantation (Classen-Linke, Muller-Newen et al. 2004), when increased secretion of soluble gp130 was seen (Sherwin, Smith et al. 2002). LIFR and gp130 mRNA is also expressed in the decidua and may play a key role during the decidualization and placentation (Ni, Ding et al. 2002). It has been shown that the secretion of gp130 was reduced in infertile women (Sherwin, Smith et al. 2002).

2 Aims of the studies

The general aim of the current thesis was to study different aspects of embryo development such as culture, cryopreservation and characterization in relation to *in vitro* fertilization.

Accordingly, the studies had the following aims:

- To compare the effects of two different sequential culture media on embryo quality.
- To investigate the effect of growth factors (LIF and gp130) on embryo development.
- To compare two vitrification protocols with and without DMSO.
- To localize transcription factors (NANOG and OCT4) in human pre-implantation embryos and in human embryonic stem cells.
- To investigate the regulation of LIF and gp130 receptors by P in the human Fallopian tube and the endometrium, and their localization in the pre-implantation embryo.

3 Materials and methods

3.1 Ethics aspects

Approval was obtained for the studies presented in this thesis from the Ethics committees at Karolinska Institutet, Uppsala University and Örebro University. The human embryos were donated for research after their 5-year storage limit has passed. The donors of the embryos did not receive any financial reimbursement. The women who donated fallopian tube and endometrial tissue gave their informed consent to participate in the study. The invasive procedures performed were not considered to have any negative effect of significance for the women.

3.2 Study materials

Human oocytes (**article I**) were collected from women undergoing IVF treatment at the Centre for Reproduction, Uppsala University Hospital, Sweden. Human embryos (**article II, III, IV and V**) were donated with informed consent by couples undergoing IVF treatment at Karolinska University Hospital/Huddinge, Uppsala University Hospital and Örebro University Hospital. Mouse embryos, (**article III**), were obtained from Embryotech (www.embryotech.com). Fallopian tube and endometrial tissue (**article V**) were obtained from healthy women undergoing sterilization at Karolinska University Hospital. Table 1 summarizes the materials used in the five articles presented.

Table I Information of samples used in the five studies

Sample	**Sample number**	**Sample source**	**Article location**
Oocytes	1206	IVF patients	I
Human embryos	465	Donated by IVF patients	II, III, VI, V
Mouse embryos	240	Embryotech	III
Biopsies	22	Healthy women undergoing sterilization	V

3.3 Methods

The details of the different methods in this thesis are described in the studies in which they are used. In this section, the basic principles for the techniques are outlined, and limitations and advantages of the methods are discussed. Table 2 summarizes the methods used in the respective articles.

Table 2 List of methods used in the different studies

Method	**Article location**
Embryo culture	I, II, III, IV
Assessment of embryos	I, II, III, IV V
Cryopreservation of embryos	I, II, III, IV V
In situ hybridization	III
Immunohistochemistry	II, III, IV V
MALDI TOF MS	II

3.4 Embryo culture

Following IVF or ICSI, oocytes were cultured individually in 20 µL droplets of culture media under mineral oil. Culture was performed at 37 °C and 6 % CO_2. In all studies, the culture conditions were kept identical as regards to temperature and pH. Embryos of good quality were either used for direct embryo transfer on day 2 or day 3, or frozen for later use by the patient (supernumerary embryos).

Considerations

First, it must be stressed that before making any comments about the impact of embryo culture medium on embryo development *in vitro*, all aspects of the culture system (laboratory and quality procedures) should be optimised.

Human pre-implantation embryos are sensitive to changes in culture milieu. In order to keep the culture conditions as constant as possible, commercial media was used throughout the studies. The embryos were cultured in the media currently used at the

clinic. The advantages of using commercial media instead of home-made media are pragmatic: they are reproducible at different times and in different laboratories, they undergo stringent quality control procedures and tests on each batch before release, and they are free of unknown activities that may interfere with the responses being studied. The main disadvantage of commercial media is that the exact composition of the solution is still kept as a company secret. It would be of great interest for culture media research if the culture media companies released the exact formulation of culture media. This would allow researchers to carry out experiments on the effect of specific components on embryo development that are reproducible by other researchers.

3.5 Assessment of embryos

On the morning of day 2 or day 3, embryo quality was assessed morphologically on the basis of the number of blastomeres (cells), fragmentation rate (volume of embryo with anucleate fragments) and multinucleation of blastomeres equivalent to the guide lines published by Alpha and ESHRE (Prados, Debrock et al. 2012). Embryos were regarded as of 'good quality' when they were at the 4-cell stage after approximately 46 h post insemination (day 2) or at the 8-cell stage, approximately 70 h post insemination (day 3) and had a score of 0–2.

Blastocyst stage embryos were scored strictly according to the guidelines published previously (Gardner, Lane et al. 2000) and by Alpha and ESHRE (Hardarson, Van Landuyt et al. 2012). In brief, the blastocyst is assessed on the basis of three parameters: blastocyst expansion and hatching status, the quality of the inner cell mass (ICM), and the quality of the trophectoderm (TE), based upon cell number and cohesion. A good quality blastocyst was classified as one with a distinct inner cell mass (ICM), a well-differentiated trophectoderm (TE), and a single large blastocoele cavity without degenerative foci on day 5/6 of development and had a score of at least 3BB.

Considerations

The evaluation of embryos was performed under light microscope. It is of interest to note that this kind of assessment of embryos only gives a score of the morphology of the embryo, and does not accurately reflect developmental potential of an embryo. New non-invasive methods for evaluating embryos could improve IVF success rates. Using

time-lapse videography, researchers have discovered characteristics that predict successful development to blastocyst stage (Wong, Loewke et al. 2010).

3.6 Cryopreservation of embryos

Cryopreservation of embryos is the process of preserving an embryo at temperatures where all metabolic processes are arrested. It involves two stages: freezing and thawing. Objectives of cryopreservation are to avoid the formation of intracellular ice crystals, which can lead to cell damage and developmental arrest (Shaw and Jones 2003). To overcome this problem two cryopreservation protocols have developed, slow freezing and vitrification. In slow freezing the growth of intracellular ice crystals is avoided by controlling the cooling rate of the embryo and its surrounding environment with the use of cryoprotectants at low concentrations. Supernumerary embryos were cryopreserved on day 2 according to a controlled slow freezing protocol including the cryoprotectants 1, 2 propanediol and sucrose in phosphate-buffered saline (PBS).

The thawing of embryos was performed using a commercial kit. The embryos were then transferred into equilibrated culture medium and carefully assessed for blastomere survival. Each embryo received a score of A (100% survival rate), B (50% ≤ survival rate < 100%), or C (<50% survival rate). Only embryos with a score of A or B were used in the studies. The embryos were then randomly allocated, with regard to embryo morphology/survival rate, into different treatment regimens and cultured up to blastocyst stage.

During vitrification, the embryo and the surrounding solution directly solidify to a glasslike state. Use of high concentrations of cryoprotectants and rapid cooling rates, eliminates both extracellular and intracellular ice crystal formation (Liebermann, Nawroth et al. 2002).

The vitrification protocol was performed at room temperature. Briefly, the embryos were rinsed and transferred to vitrification solution (with or without DMSO). One to three embryos were loaded on a cryloop and plunged into liquid nitrogen.

The warming of embryos was performed at room temperature. The cryoloop was placed directly and quickly into warming solution and the embryos were there after cultured to blastocyst stage according to standard procedures.

Considerations

Controlled ice formation during freezing is recognized as to be a key factor in determining the viability of embryos following freezing and thawing. Even if high survival rates are observed after vitrification and warming, we observed low survival rates in our study (article III). This could be due to the extended culture and definition of survival used. We defined survival as the percentage of embryos that continued to develop to the blastocyst stage *in vitro*.

3.7 In situ hybridization (ISH)

In situ hybridization was used to detect the presence of NANOG mRNA in human preimplantation embryos and human embryonic stem cells.

Considerations

In situ hybridization is a versatile tool for the examination/localization of gene expression. It detects a hybridized labeled nucleic acid probe (RNA or DNA) to a complementary sequence of mRNA. The method is extremely sensitive and can detect the amount of mRNA in a single cell. Its major advantage is that it allows the maximum use of rare tissues like human embryos. Another method we may have considered using is single cell reverse transcription (RT-PCR). It is more sensitive than ISH, but it has the disadvantage that one cannot locate the mRNA of interest.

3.8 Immunohistochemistry (IHC)

IHC was used to show the presence of specific proteins in human embryos and hESCs. In addition, immunohistochemistry was also used to detect apoptosis and damage to the cytoskeleton.

Considerations

Immunohistochemistry or IHC is a method that exploits the antibody binding to specific antigens. The advantages of IHC are that the localization of the protein is shown. However, lack of specificity, difficult of interpretation and low percentage of reproducibility are the major draw backs of IHC. We used monoclonal antibodies for consistency and standardization of experimental procedures and results.

3.9 MALDI TOF MS

Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI TOF MS) was used for direct protein profiling of intact blastocysts. It is a robust method in which a co-precipitate of an UV-light absorbing matrix and a biomolecule is irradiated by a nanosecond laser pulse. The ionized biomolecules are accelerated in an electric field and enter the flight tube. During the flight in this tube, different molecules are separated according to their mass to charge ratio and reach the detector at different times. In this way each molecule yields a distinct signal.

Considerations

The advantage of using MALDI TOF MS for detection and characterization of biomolecules, such as proteins and peptides, is that it is a relatively fast method with short measuring time and negligible sample consumption. This is important since human embryos for research are scarce.

3.10 Statistical analysis

SPSS statistical software (Chicago, II, USA) was used to calculate statistics. The Fisher's exact test (two-tailed) and Chi-square test were used to test differences between groups in articles I, III, and IV. Nonparametric statistical evaluation, ANOVA on ranks, was performed for differences in staining intensity for LIFR and gp130. Mann–Whitney Rank Sum Test was performed to compare the mRNA levels. A *P*-value of < 0.05 was considered statistically significant.

4 Results and discussion

4.1 Culture and vitrification media

The goal of embryo culture in human IVF is to improve the quality of embryos in vitro and the chances of successful delivery of a healthy baby.

In **article I**, we investigated the impact of culture media on embryo morphology and development at days 2/3. A total of 1206 oocytes from 110 women were analysed. The average age (+/-SD) of the patients was 39.9 ± 3.8 (range 24 – 40) years. Sibling oocytes were divided via alternate allocation to fertilization and culture in media system A (G-IVF ™ v5 PLUS/ G-1™ v5 PLUS) or for fertilization and culture in media system B (Universal IVF medium/EmbryoAssist ™). The main results were that normal fertilization rate and embryo utilization rate was significantly higher in group A than in group B (73.5% and 55.5% compared with 67.2% and 42.9% respectively, $p = 0.030$ and $p = 0.001$). No difference was seen regarding polyploidy and embryo quality between the two groups (Table 3).

Table 3 Outcome of 1206 sibling oocytes assigned to media system A or system B. *statistically significant ($p < 0.05$, Chi-square test)

	Media A	**Media B**	***P*-value**
Number of oocytes inseminated, *n*	622	584	0.122
Mature oocytes (MII), *n* (%)	520 (83.6)	469 (80.3)	0.137
Normal fertilized oocytes (2PN), *n* (%)	382 (73.5)	315 (67.2)	0.030*
Polyploid (>2PN), *n* (%)	29 (5.6)	34 (7.3)	0.282
Embryo quality (grade 0–2)			
with 4-cells at day 2, *n* (%)	210 (54.9)	163 (52.7)	0.396
with 8-cells at day 3, *n* (%)	23 (6.0)	11 (3.5)	0.123
Embryos cryopreserved, *n* (%)	124 (32.5)	111(35.2)	0.439
Embryo utilization, *n* (%)	212 (55.5)	135 (42.9)	0.001*

We have shown that culture media has impact on fertilization, embryo morphology and development at days 2/3. The components of the two culture media systems differ and detailed composition of the media is unknown, which makes it difficult to determine exactly which ingredients are the most optimal for embryo culture. The difference in fertilization rate between the two groups can possibly be due to the fact

that media A contains components known to be beneficial for fertilization like lactate (Kito and Ohta 2008), EDTA (Abramczuk, Solter et al. 1977; Mehta and Kiessling 1990) and taurine (Devreker and Hardy 1997). Furthermore, fertilization medium A contains a number of defined amino acids while fertilization medium B lacks amino acids. However, these differences may have been overcome if embryos were cultured to the blastocyst stage.

In summary, this study showed that today's culture media is far from optimal for supporting embryos in vitro. Our study confirms previous studies that have suggested that culture media have an impact on embryo quality in vitro (Cooke, Quinn et al. 2002). Furthermore, the effect of culture media on embryo quality has recently been reviewed by Mantikou *et al* were it was concluded that current culture media does not lead to the best success results in IVF/ICSI (Mantikou, Youssef et al. 2013).

With this in mind, we went on to conduct a study (**article II)** to see if addition of growth factors in culture media can enhance embryo development in vitro. A total number of 164 embryos which survived thawing were cultured in the presence of LIF and/or gp130 and the *in vitro* effect on human embryo development studied. The main results were that culture of embryos in media supplemented with gp130 significantly improved blastocyst development (75%) compared to control (37%), LIF (41%) or LIF + gp130 (29%), respectively (Table 4). On the other hand, LIF seemed to be detrimental for embryo development, which is contrary to what is seen in the mouse, were LIF enhanced blastocyst formation and birth rates (De Matos, Miller et al. 2008). We have in this study demonstrated the effect of gp130 on human embryo development, implicating a physiological role in regulating early embryo development. We, therefore, suggest that gp130 is beneficial for embryo development and should be included in culture media.

Table 4 Morphological embryo development assessed by light microscope

Treatment	**No of embryos**	**Blastocysts, n (%)**	**High quality blastocysts, n (%)**
Total	164	73 (44)	40 (24)
Control	43	19 (37)	10 (23)
gp130	40	29 (73)	15 (38)
LIF	46	19 (41)	9 (20)
LIF + gp130	35	10 (29)	6 (17)

Beneficial effects of growth factors in enhancing embryo development *in vitro* have been previously demonstrated. Granulocyte-macrophage colony stimulating factor (GM-CSF) (Sjoblom, Wikland et al. 1999), leukaemia inhibitory factor (LIF) (Dunglison, Barlow et al. 1996), epidermal growth factor (EGF) (Martin, Barlow et al. 1998) and insulin-like growth factor-1 (IGF-1) have been shown to increase blastocyst development in human embryos. Additionally, IGF-1 (Lighten, Moore et al. 1998) and GM-CSF (Sjoblom, Wikland et al. 1999) stimulated development of the inner cell mass. The supplementation of GM-CSF in culture media improved pregnancy rates and was shown to be beneficial in women with previous miscarriage (Ziebe, Loft et al. 2013). These results and our data demonstrate the need of optimizing human embryo culture.

Besides optimization of the media for culture, media and protocol for vitrification also need improvement. To maximize the potential for conception for IVF and prevent wastage of viable normal supernumerary embryos, cryopreservation programmes has become an essential part of IVF. Improving cryopreservation media would be of great benefit to IVF programmes. We therefore conducted a trial to evaluate different vitrification protocols.

In this study (**article III**) we set out to compare two different vitrification protocols: one with dimethyl sulphoxide DMSO, propane-2-diol, and ethylene glycol, and a second protocol with only propane-2-diol, and ethylene glycol. Viability and the importance of specific incubation times for early embryo recovery, survival and cleavage were studied. The two protocols did not differ in embryo survival rates (Table 5). However, the study showed that morphological assessment of embryos after vitrification was not a useful tool for survival assessment. Therefore, we recommend that vitrification of early embryos should be combined with extended culture before transferring to patients

Additionally, we assessed cryodamage in embryos using the Vybrant Apoptosis Assay kit and showed that DMSO-containing vitrification solutions protect embryo membrane integrity than solutions without DMSO. We have shown that DMSO-containing vitrification solution leads to less cryodamage compared to DMSO-free vitrification solution, possibly due to the fast penetrating characteristics of DMSO.

Just like this study, other researchers have previously shown that cryopreservation protocols can be improved to the better (Liebermann and Tucker 2004).

Table 5 Comparison of media with dimethyl sulphoxide DMSO) and media without DMSO in mouse and human model systems (*Survival: %, number survived/total number)*

	Mouse embryos	**Human embryos**
With DMSO	77 (43/56)	30 (16/53)
Without DMSO	80 (45/56)	35 (28/80)

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4.2 Embryo characterization

The importance of characterization of embryos cultured *in vitro* is of great importance in reproductive medicine. Using molecular and biochemical techniques key factors important for embryo development can be detected. Part of this thesis was to deploy these techniques to detect/localize some of the factors involved in embryo development.

Immunohistochemical analysis showed the presence of NANOG and OCT4 protein in human embryos and human embryonic stem cells (hESCs) (**article IV**). NANOG and OCT-4 were co-localized in human morula and blastocyst stage embryos. The time of appearance in the early morula resembled that seen in mouse embryos (Palmieri, Peter et al. 1994; Chambers, Colby et al. 2003; Hatano, Tada et al. 2005). We also observed immunostaining of OCT4 in the trophoblast cells of the blastocyst. Our results are confirmed by same observations in bovine blastocysts (van Eijk, van Rooijen et al. 1999; Kirchhof, Carnwath et al. 2000). This is also in line with the results observed in human blastocyst showing the expression of OCT-4 mRNA (Hansis, Grifo et al. 2000).

Furthermore, using whole mount *in situ* hybridization we localised NANOG mRNA in a group of cells in the morula, in cells of the ICM of blastocyst, and evenly in hESCs (**article IV)**. This method gave us the unique opportunity to visualize the expression of NANOG in both time and space in single human embryos.

Finally, we compared data from the present study with our data from gene array studies (Zhang, Zucchelli et al. 2009). The gene array data showed the presence of NANOG

mRNA in human pre-implantation embryos and hESCs (Table 6). Nanog mRNA was present from day 2 and highly expressed in hESCs. OCT4, on the other hand was weakly expressed in human pre-implantation embryos and highly expressed in hESCs (Table 6). The two transcription factors were absent in fibroblast (Table 6).

Table 6 Data from microarray analysis of human embryos and embryonic stem cells and fibroblasts.

Gene	**Signal intensity**				
	Day 2 embryos	**Day 3 embryos**	**Day 5 embryos**	**Stem cells**	**Fibroblasts**
Nanog	98.6	194.6	196.8	391.8	1.3
Oct4	32.2	67.1	46.8	310.9	5.3

In another study (**article V**), we looked at the presence of LIFR and gp130 in preimplantation embryo, the human Fallopian tube and endometrium. Using immunohistochemistry, LIFR and gp130 were localized in preimplantation embryo, Fallopian tube and endometrium.

In embryos, the presence of LIFR and gp130 was shown in all pre-implantaion embryonic stages (Fig. 4). The presence of LIFR was more evident in the Fallopian tube than in the endomentrium. The higher levels of LIFR in the Fallopian tube could be expressed by the fact that LIFR produced by the embryo acts both within the embryo and with the Fallopian tube in an auto-and paracrine manner. In the blastocyst, the presence of gp130 was mainly located in the inner cell mass, whereas LIFR was expressed in all cells.

We then went on to study the effect of mifepristone on the expression of LIFR and gp130 in the human Fallopian tube (**article V**). Our results showed that mifepristone had no effect on the expression of LIFR and gp130 in the human Fallopian tube, nor in endometrium suggesting that progesterone might not be involved in the regulation of LIFR or gp130.

Next we obtained a characteristic protein fingerprint spectra from individual blastocyst. The analysis was carried out using Matrix-assisted laser

desorption/ionization time of flight mass spectrometry (MALDI TOF MS). Protein fingerprint spectra reviewed the presence of multiple common protein species like Ubiquitin, histone H4, thymosin beta 4 and thymosin beta 10. Due to the small size and limited number of human pre-embryos, little is known about the protein patterns of early human embryo development. Here, we demonstrate that MALDI TOF MS is a valuable tool for analysis of protein patterns in pre-embryos.

Further, we confirmed some of our results from MALDI TOF MS analysis. Imunohistochemical staining showed the presence of ubiquitin, histone H4, thymosin beta 4, and thymosin beta-10 in human blastocysts. The staining reviewed that ubiquitin is present both in the ICM and in the trophectoderm of the blastocyst. Our results regarding ubiquitin are confirmed by the results in mice which showed a positive effect of ubiquitin in placentation (Bebington, Doherty et al. 2000), Histone 4 was observed only in trophectodermal cells. Thymosin beta 4 was seen in ICM and thymosin beta 10 both in ICM and trophectoderm (Figure 3).

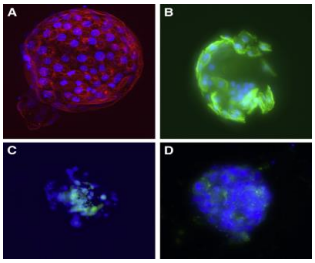

Figure 3. Immunohistochemical staining of (A) ubiquitin, (B) histone H4, (C) thymosin beta 4, and (D) thymosin beta-10 in human blastocysts. Nuclear staining with 6-diamino-2-phenylindole (DAPI) is shown in blue.

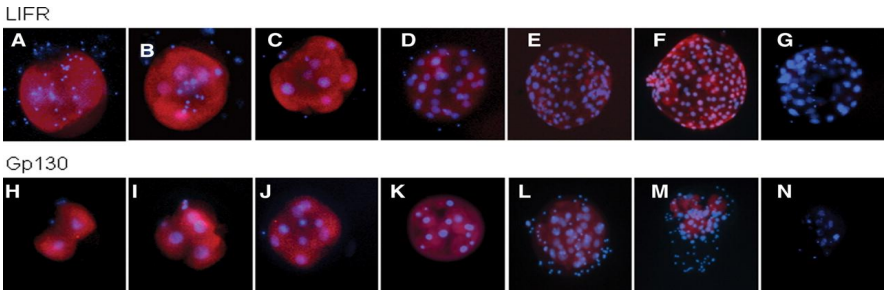

Figure 4. Immunostaining of LIFR and gp130 in human preimplantation embryos. Staining for LIFR: (**A**) 2 cell embryo (**B**) 4-cell embryo. (**C**) 8-cell embryo (**D**) Morula. (**E**) Early blastocyst. (**F**) Expanded blastocyst. (**G**) Negative control. Staining for gp130: (**H**) 2-cell embryo (**I**) 4-cell embryo. (**J**) 8-cell embryo (**K**) Morula. (**L**) Early

blastocyst. (**M**) Staining of the inner cell mass in a hatching blastocyst. (**N**) Negative control.

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5 Summary and conclusions

The current thesis gives new perspectives on culture, cryopreservation and characterization of human embryos in vitro.

The main findings of the current thesis are:

- The presence of LIFR and gp130 in the Fallopian tube and preimplantation embryo indicates a role for LIF in communication between the embryo and the Fallopian tube.

- The growth factor gp130 seems to be beneficial for preimplantation embryo development and ought to be included in culture media designed for embryo culture to the blastocyst stage.

- Culture media has impact on early embryo development and this endorses the important need for research in culture media formulation to further improve media for in vitro development of human embryos.

- Vitrification protocols consisting of DMSO as a cryoprotectant are suitable and equally efficient in both mouse and human embryo models.

- Molecular and biochemical analytical methods like whole mount in situ, immunohistochemistry and MALDI TOF MS are versatile tools in reproductive medicine research.

Studies from animal models indicate that stressful environmental conditions like in vitro culture and cryopreservation may lead to epigenetic alterations. This compelling evidence endorses the importance of basic research in optimizing culture and cryopreservation conditions in order to further advance assisted reproductive techniques (ART) and minimize epigenetic alterations.

6 Future perspectives

The findings in this thesis highlight three laboratory areas that warrant considerable discussion.

The first area for future studies is the optimization of embryo culture *in vitro*. Culture media intended for use in human IVF treatment must be developed to meet the embryo's nutrient requirements and physiology in order to reflect the in vivo environment in which the zygote forms and divides. To make these feasible, randomized trials must be performed and components of the medium disclosed. Addition of growth factors to culture medium should be undertaken with care, considering that stressful environmental conditions may lead to epigenetic alterations.

Another area of interest is non- invasive selection of viable/normal embryos for transfer. The development of time-lapse devices for the IVF laboratory has given embryologists a unique chance to observe and study human embryos in vitro. Proteomic and metabolomic profiling using microfluidics may also help in selecting embryos. These non-invasive strategies of evaluating embryos combined with the traditional qualitative morphological observation could help to improve IVF success rates and reduce multiple gestations.

The third area of interest is cryopreservation of gametes and embryos. We have in this thesis shown that there is need for more robust and safe vitrification protocols. Research areas of interest are in designing protocols with very low cryoprotectans (< 1M) and in improving devices to obtain super high cooling rates.

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EMBRYO BIOLOGY

A prospective randomized sibling-oocyte study of two media systems for culturing cleavage-stage embryos—impact on fertilization rate

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Abstract
Purpose Although several media systems have been developed, data from prospective randomised clinical studies are still lacking. In the present study we compared the effects of 2 different media systems on embryo morphology and development at days 2/3 using sibling oocytes.
Methods In this prospective sibling-split trial, 1206 oocytes from 110 women were divided via alternate allocation to fertilization and culture in media system A (G-IVF ™ v5 PLUS/ G-1™ v5 PLUS) or for fertilization and culture in media system B (Universal IVF medium/EmbryoAssist ™). *Results* The use of media system A significantly increased the normal fertilization rate (73.5% versus 67.2%; $p=0.030$) and embryo utilization rate (55.5% versus 42.9%; $p=0.001$), whereas polyploidy and embryo quality were similar in the two groups.
Conclusion The different impacts on fertilization and early embryo development between the two commercially available and commonly used media systems show the importance of evaluation of the efficacy of existing sequential culture media and the need to further improve media for in vitro development of human embryos.

Capsule Two commercially available and commonly used media systems show different impacts on fertilization and early embryo development

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Keywords Sibling oocytes · Culture media · Fertilization rate

Background

The human oocyte is fertilized in the Fallopian tube and is developing during the transport into the uterus, where it is implanted at the blastocyst stage [1–3]. The ampulla region of the Fallopian is the natural environment for fertilization and the first mitotic divisions occurs as the pre-embryo passes along the Fallopian tube to reach the uterus [3]. The in vivo environment is optimal for the developing preimplantation embryo as it moves and develops along this route. Media intended for use in IVF treatment should be optimised in order to reflect the environment in the Fallopian tube and endometrium, as the culture conditions in which the zygote forms and divides plays an important part in dictating its development and subsequent achievement of a successful pregnancy [4]. The change in environment in the different parts of the Fallopian tube has lead to development of different culture media systems.

Development of new culture media is continuously ongoing, but so far none of the media used are as complex as the tubal and endometrial fluid. Simple media has gradually been replaced with more complex culture systems; but the ability of different media to support the fertilization and culture of preimplantation embryos up to cleavage stage remains uncertain [5]. Although the use of commercial media is usually beneficial [6], these media are still under investigation and their most optimal composition remains to be determined.

The aim of the present study was to compare the effects of two different commercial culture media on early embryo

development using a prospective randomized sibling design. The primary outcome measure was normal fertilization rate. The secondary outcome measures were polyploidy rate, embryo utilization rate and embryo quality (EQ) at day 2 and 3.

Material and methods

Design of the study

This trial was designed as a prospective randomized sibling oocyte split study and included all oocytes from women undergoing IVF treatment at the Centre for Reproduction, Uppsala University Hospital, Sweden, between October 2007 and December 2007. The age limits of the patients ranged from 24 to 40 years, with a mean (± SD) of 33.9±3.8 years. The oocytes were randomly allocated into one of two groups at the time of ovum pick up. Allocation was not blinded. Oocytes in media system A (n=622) were fertilized in fertilization media A (G-IVF ^{TM} PLUS, Vitrolife AB, Kungsbacka, Sweden) and further cultured in culture media A (G-1^{TM} PLUS v5, Vitrolife AB, Kungsbacka, Sweden). Oocytes in media system B (n=584) were fertilised in fertilization media B (Universal IVF medium, MediCult A/S, Jyllinge, Danmark) and cultured in culture media B (EmbryoAssist ^{TM} MediCult A/S, Jyllinge, Danmark). When there were uneven numbers of oocytes, the additional oocyte was allocated to media system A, which was the standard system in the clinic at the time of this trial.

Ovarian stimulation and oocyte retrieval

Down-regulation for ovarian hyperstimulation was achieved by using either a gonadotropin-releasing hormone (GnRH) agonist or antagonist protocol (Suprecur Hoechst, Frankfurt, Germany, Synarela, Syntex Nordica AB, Södertälje, Sweden; Cetrotide, Serono Laboratories, Aubonne, Switzerland or Orgalutran, Organon, Oss, the Netherlands), starting on either cycle day 1 or day 21. Following down-regulation, ovarian stimulation was induced by using either recombinat FSH, (Gonal-F, Serono Laboratories, Aubonne, Switzerland, or Puregon, Organon, Oss, the Netherlands) or urine-derived Menopur® (Saint-Prex, Switzerland). The starting dose was dependent on the woman's age and/or previous response to ovarian stimulation. The ovarian response was monitored by means of serum oestradiol assays and vaginal ultrasonographic scans of follicles. Recombinant FSH or Menopur® was administered until the leading follicle had a diameter of at least 17 mm. Maturation of the oocyte was triggered by s.c injection of hCG (Pregnyl, Organon, Oss, the Netherlands or Ovitrelle, Serono Laboratories, Aubonne, Switzerland). Oocytes were retrieved by transvaginal needle aspiration under ultrasonographic guidance 34–36 h after hCG administration. The cumulus oocyte complexes were randomly allocated to fertilization medium A or fertilization medium B.

Semen preparation and fertilization

All semen preparation was carried out in Nidacon sperm preparation media. A two-density gradient (47.5/95%) (PureSperm® 100, Nidacon International, Mölndal, Sweden) was used for isolation of motile sperm. Briefly, liquefied semen was gently overlaid the 47.5% layer and centrifuged for 15 min at 300×g. The supernatant was aspirated and 0.5 ml of the pellet was transferred into a new centrifuge tube and washed twice in 5 ml equilibrated room-temperature PureSperm® Wash (Nidacon) for 10 min at 500×g. Sperm concentration was determined by pipetting an aliquot of 5 μl semen into a Makler Counting Chamber (Sefi Medical Instruments, Israel), followed by counting under a phase contrast microscope.

Conventional IVF was performed in 20 μl fertilization medium A or fertilization medium B under mineral oil and about 15,000 spermatozoa were used. The compositions of the two media used for fertilization is shown in Table 1. According to lab praxis, insemination was done at about 13.00 and fertilization check in the morning between 07.45 and 09:00 (18–20 h after insemination).

Embryo culture and grading

During the study period, the culture conditions were kept identical as regards temperature, and pH. The only difference was the culture media used and protein supplement. To ensure optimal fertilization and culture conditions in the incubator, daily manual temperature readings were performed. The pH of the media was checked once a week and the CO_2 level adjusted for optimal pH range of 7.2–7.4. Using these criteria, the pH for both media was within the optimal range (7.2–7.4) as recommended by the manufacturers.

Following fertilisation after IVF or ICSI, oocytes were evaluated and cultured in groups of 3–5 under oil in 20 μl droplets of either culture media A or culture media B at 37°C in a dual gas incubator (6% CO_2 and 94% air). Composition of the two media used for culture is shown in Table 2. On the morning of day 2 or day 3 the embryo quality was assessed morphologically on the basis of the number of blastomeres (cells), the fragmentation rate (volume of embryo with anucleate fragments) and multinucleation of blastomeres, as described previously [7, 8]. Each embryo received a score of 0 (top quality), 1 (good quality), 2 (fair quality) and 3 (poor

Table 1 Composition / Information on ingredients of the fertilization and culture media

G-IVF.v5 Plus (Vitrolife)	Universal IVF (Medicult)	GI.v5 Plus (Vitrolife)	EmbryoAssist (Medicult)
Alanyl-Glutamine	Water	Alanine	Water
Calcium chloride dihydrate	Sodium Chloride	Alanyl-glutamine	Sodium Bicarbonate
Citrate	Human Serum Albumin	Asparagine	Human Serum Albumin
EDTA	Glucose	Aspartate	Sodium Chloride
Fructose	Potassium Chloride	Calcium chloride	Amino Acids
Gentamicin	Calcium Chloride	EDTA	Hepes Acid
Glucose	Sodium Phosphate monobasic	Gentamicin	Calcium Lactate
Glycine	Magnesium Sulphate	Glucose	Potassium Sulphate
Human serum albumin	Sodium Pyruvate	Glutamate	Sodium phosphate
L-Alanine	Sodium Bicarbonate	Glycine	Vitamins
L-Asparagine H_20	Penicillin Sodium Salt	Human serum albumin	Glucose
L-Aspartate	Streptomycine Sulfate Salt	Hyaluronan	EDTA
L-Glutamate	SSR® (Synthetic Serum Replacement)[a]	Lipoic acid	Sodium Pyruvate
L-Proline		Magnesium sulphate	Magnesium Sulphate
L-Serine		Methionine	Penicillin Sodium Salt
Magnesium sulphate		Potassium chloride	Streptomycine Sulfate Salt
heptahydrate		Proline	SSR® (Synthetic Serum Replacement)[a]
Potassium chloride		Serine	
Sodium chloride		Sodium bicarbonate	
Sodium dihydrogen		Sodium chloride	
orthophosphate 1-hydrate		Sodium citrate	
Sodium hydrogen carbonate		Sodium dihydrogen phosphate	
Sodium lactate		Sodium lactate	
Sodium pyruvate		Sodium pyruvate	
Taurine		Taurine	
Water for injection (WFI)		Water for injection (WFI)	

[a] Contains Human Insulin recombinant.

quality). Embryos with a score of 0–2 were used for direct embryo transfer either on day 2 or day 3, or frozen for later use by the patient (supernumerary embryos). In this trial embryos were regarded as of 'good quality' when they were at the 4-cell stage at ~46 h post insemination (day 2) or at the 8-cell stage, ~ 70 h post insemination (day 3) and had a score of 0–2. Three embryologists graded the embryos and together decided on which embryo had the highest quality. The embryologists assessing embryo grading and selecting embryos for transfer or cryopreservation were not blinded as to which media was used. According to clinic policy one embryo was transferred in women ≤38 years old and two embryos in women >38 years old. In cases when embryo quality was not good, two embryos were transferred regardless of the woman's age.

Embryo transfer

Embryo transfers were performed using a soft transfer catheter (K-JETS-7019-SIVF, Cook, Brisbane, Australia) under transabdominal ultrasonographic guidance.

Outcome measures

Normal fertilization rate was defined as number of oocytes containing two pronuclei (2PN) divided by total number of mature oocytes (MII). *Polyploid rate* was defined as number of oocytes containing >2PN divided by total number of mature oocytes (MII). Embryo quality (EQ) was defined as number of 4-cell embryos on day 2 and number of 8-cell embryos on day 3 with a grade of 0–2, divided by number of normal fertilized embryos. Embryo

Table 2 Demographic data of patients included in the study is shown

Patients included (*n*)	110
Female age, mean±SD (years)	33.9±3.8
Infertility diagnosis, *n* (%)	
Tubal factor	8 (7.3)
Endometriosis	8 (7.3)
Unexplained	30 (27.3)
Anovulation	12 (10.9)
Male factor	37 (33.6)
Other	15 (13.6)
No. of oocytes retrieved per patient, mean±SD	10.9±4.8

utilization rate was defined as the number of embryos transferred and the number of embryos frozen in relation to the total number of embryos available. The *positive hCG rate* was determined as a ratio between the number of positive hCG tests and the total number of embryo transfers. *Clinical pregnancy rate* was defined as the number of cases with evidence of at least one gestational sacs divided by the number of transfers. *Implantation rate* was defined as the number of gestational sac/sacs verified by vaginal ultrasonography divided by the number of embryos transferred. The *Delivery rate* was defined as a ratio between deliveries and embryo transfers.

Statistical analysis

SPSS statistical software (Chicago, Il, USA) was used to calculate statistics. Power analysis showed that to detect an increase of 10% in fertilization rate between the two media systems with a power of 0.8 (alpha-level 0.05), we needed at least 269 oocytes in each group. Comparison of normal fertilisation rates, polyploid rates, embryo quality and embryo utilization rates was performed using the Chi-square test. Positive hCG rates, clinical pregnancy rates, implantation rates and delivery rates were compared by using the Fisher's exact test (two-tailed). A value of $p<0.05$ was considered statistically significant.

Results

Normal fertilization rate, polyploid rate, embryo quality and embryo utilization rate

In total, 1206 oocytes from 110 women were included in this study. A little over half of them (622; 51.6%) were fertilized and cultured in media system A (group A) and 584 were fertilized and cultured in media system B (group B). Patient demographic data including female age, infertility diagnosis and number of oocytes retrieved are given in Table 2. Regarding the causes of infertility, 7.3% were due to tubal factor, 7.3% due to endometriosis, 27.3% were unexplained, 10.9% and 33.6% were due to anovulation and male factor respectively, and 13.6% due to other factors

This was a sibling-split study and therefore differences in demographic data were eliminated. The rate of normal fertilization and the embryo utilization rate were significantly higher in group A than in group B (73, 5% and 55.5% compared with 67.2% and 42.9% respectively, $p=0.030$ and 0.001). There were no differences in polyploidy rate, embryo quality and the number of embryos cryopreserved between the groups (Table 3).

Embryo transfer

A total of 108 embryo transfers were performed. Seventeen of these were mixed transfers, were two embryos, one from each group were transferred (data not shown). Three embryologists together agreed on the best quality embryos to be used in immediate transfer. Additional embryos of high quality were frozen. A Significantly higher number of transfers were performed after culture in media system A (75.8%) compared with system B (24.2%; $p<0.0001$), as shown in Table 4.

Clinical outcomes

The positive hCG rate, the clinical pregnancy rate, the implantation rate and the delivery rate were similar in the two groups (Table 4).

Table 3 Outcome of 1206 sibling oocytes assigned to media system A or system B.
*Statistically significant ($p<0.05$, Chi-square test)

	Group A	Group B	*P*-value
Number of oocytes inseminated, *n*	622	584	0.122
Mature oocytes (MII), *n* (%)	520/622 (83.6)	469/584 (80.3)	0.137
Normal fertilized oocytes (2PN), *n* (%)	382/520 (73.5)	315/469 (67.2)	0.030*
Polyploid (>2PN), *n* (%)	29/520 (5.6)	34/469 (7.3)	0.282
Embryo quality (grade 0–2), *n* (%)			
with 4-cells at day 2	210/382 (54.9)	163/315 (52.7)	0.396
with 8-cells at day 3	23/382 (6.0)	11/315 (3.5)	0.123
Embryos cryopreserved, *n* (%)	124/382 (32.5)	111/315 (35.2)	0.439
Embryo utilization, *n* (%)	212/382 (55.5)	135/315 (42.9)	0.001*

Table 4 Clinical outcome of 91 embryo transfers, comparing the two media systems is shown. *Statistically significant ($p<0.05$, Fisher's exact test (two-tailed)

	Group A	Group B	*P*-value
Embryo transfer (*n*)	69 (75.8)	22 (24.2)	<0.0001*
Positive hCG, *n* (% per embryo transfer)	34 (49.3)	11 (50.0)	1.00
Clinical pregnancies, *n* (% per embryo transfer)	32 (46.4)	8 (36.4)	0.467
Implantation rate, *n* (%)	36/88 (40.9)	9/24 (37.5)	0.818
Deliveries, *n* (% per embryo transfer)	24 (34.8)	8 (36.4)	1.000

Discussion

In most mammalian species, the intrinsic quality of the oocyte before it is fertilized determines whether a zygote has the potential to progress through pregnancy [4]. Human embryos used for IVF are generally derived from a cohort of oocytes collected after ovarian stimulation. These oocytes differ in quality between women and the only way to study the effect of different media systems is to compare oocytes from individual women. Therefore, this study was designed as a sibling-split study, where oocytes from each woman were divided into two groups and two different culture systems were used. So far, in previous studies, randomisation procedures have been performed in relation to weekdays [9] or other randomisation procedures have been used [10], increasing the possibility that the randomisation procedure might influence the final result. By using sibling oocytes where each patient serves as her own control, we were able to control for confounding factors that are inherent to the above mentioned study designs.

The number of oocytes was higher in group A, which was a result of the fact that the media system used in this group was standard in the clinic at the time and when an uneven number of oocytes was retrieved, the additional oocyte was allocated to group A.

Detailed composition of the two media is unknown-the ingredients are to some extent known, as shown in Table 1, but their concentrations are company secrets. Therefore it is difficult to determine which ingredients are the most important for efficient embryo culture. It is noticeable that the majority of studies carried out to determine the effects of media additives on embryo development have been performed using murine or bovine embryos and the conclusions drawn from these studies might not be valid for human embryos.

The fertilization rate was significantly higher in group A than in group B. The fertilization media differ, among other things, as regards the serum component, sodium citrate, energy sources, antibiotics, EDTA and taurine. Fertilization medium A contains human serum albumin and a number of defined amino acids, while fertilization medium B contains a synthetic serum replacement component that contains serum albumin plus other ingredients. It is not known if this serum replacement component also contains amino acids—the exact composition is kept secret.

Fertilization medium A contains sodium citrate, which is not known to be included in fertilization medium B. The impact of sodium citrate on fertilization is not clearly understood. It is known that citrate does not seem to improve sperm motility after freezing [11]. Addition of citrate to bovine embryo culture appears to enhance morula and blastocyst development, and hatching [12–14], although in one study no impact on bovine blastocyst formation was found [15]. The present results indicate that it could be beneficial for early human embryo development to have citrate in the fertilization medium.

Energy is needed for fertilization. Fertilization medium A contains four sources of sugar—fructose, glucose, pyruvate and lactate—while fertilization medium B contains only glucose and pyruvate. Fructose and glucose are both energy sources and it has been shown that fructose can replace glucose, at least as regards bovine embryos [16].

Both lactate and pyruvate are favourable energy sources for spermatids [17]. For pig embryo development, lactate and pyruvate for the first 48 h resulted in significantly higher cell numbers than in those cultured with glucose only [18]. It was recently shown that lactate increases the in vitro fertilization rate in mice [19] and this could to some extent explain why the fertilization rate was higher in group A. Lactate can not be replaced by pyruvate for energy requirements, as their mechanisms of action are different. Pyruvate up-regulates ATP production in sperm and oocytes, while lactate regulates the redox process instead of increasing ATP production [20]. Lactate is present in culture medium B, but this might be too late as regards optimal early embryo development.

Insulin is used in cell culture systems to promote cell proliferation [21]. It was recently shown that insulin enhances sperm motility and the acrosome reaction, as well as nitric oxide production [22]. Only fertilization medium B contained insulin, which did not enhanced fertilization rate in comparison with medium A. However, it is important to keep in mind that these media are complex and it is difficult to identify one factor as being more important than any other.

The types of antibiotic used in culture media formulations could be of importance for embryo development. Follicle bioassay has been found to reveal a concentration-

dependent decrease in mouse oocyte nuclear maturation during continuous exposure to streptomycin and a significantly reduced rate of polar body formation. A lower fertilization rate was also seen after exposure to streptomycin. Additionally, it has been reported that exposure of hamster embryos to penicillin and streptomycin reduced the percentage of 8-cell embryos and blastocysts versus controls and in comparison with gentamycin alone [23]. This could be one explanation as to why the fertilization and cleavage rates were lower in media system B, containing both streptomycin and penicillin.

Chelating agents such as EDTA improve bovine embryo development [24], but the effect on fertilization is not known. Only fertilization medium A contains EDTA, although it is included in both culture media.

Addition of glutamine and hypotaurine has been suggested to improve embryo development by improvement of intracellular oxidative status [25]. Taurine has also been shown to improve bovine embryo culture by increasing total cell number and the rate of blastocyst formation [26]. As far as we know only fertilisation medium B contains taurine, although it is included in both culture media

Culture medium A contain hyaluronan, which is absent in culture medium B. Mouse embryos produce hyaluronan, and its receptor RHAMM is present throughout embryo development, suggesting that hyaluronan plays a role at this stage [27]. Several investigators have shown that hyaluronan-enriched medium can improve implantation and pregnancy rates [28–30], but in a recent study we found that although the implantation rate of human embryos was improved when adding hyaluronan to the transfer medium, the delivery rate was not affected, as a result of an increased number of early pregnancy losses [31].

Embryos cultured in vitro are constantly exposed to stress factors such as reactive oxygen species (ROS). These are partly formed during embryo metabolism and are associated with suboptimal composition of plastics used in embryo culture. Other sources of ROS are UV light, volatile organic compounds and oxygen in the air. Culture medium A contains lipoic acid as an antioxidant. This is a potent antioxidant which protects embryos in culture [32]. The presence of this acid in culture medium could explain the superiority of medium A over medium B.

The percentage of embryos transferred was higher after fertilization and culture in media system A. Three embryologists decided together on the embryos that were of the highest quality and thereby the best to transfer. The decision was a professional one with the aim of achieving the best possible chance of pregnancy for the individual couple. The embryologists were not blinded to the media in which the embryos had been cultured. However, the fact that a higher proportion of embryos cultured in system A reached the 4 and 8-cell stages is likely to mirror the quality of the embryos. This is supported by the significantly higher fertilization and embryo usage rates in group A.

In summary, fertilization medium A contains citrate, lactate, EDTA and taurine, all favourable for fertilization. In addition, it contains gentamycin, which is less toxic to oocytes than streptomycin, which is included in fertilization medium B. Fertilization medium B on the other hand, contains insulin, which is thought to be favourable. Taken together, the combination of nutrients in fertilization medium A enhances fertilization rate. Culture medium A is richer and contains hyaluronan, citrate which improves early embryo development, and a less toxic antibiotic, in combination with (protective) lipoic acid, which could be the reason for better early embryo development after culture in this medium.

In conclusion, the most interesting finding in the present study is that early embryonic development appears to be improved, and may even be sped up (ie. more embryos are at cleaved stage in group A), but since there is no blastocyst data, chances are that these differences may be minimised or overcome by day 5 or day 6, and that the outcomes as relates to implantation and delively are no different (non randomized outcomes in this study). The data show the importance of culture media and the fact that increased effort is needed to improve them further.

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II

Glycoprotein 130 promotes human blastocyst development in vitro

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Objective: To investigate the efficacy of leukemia inhibitory factor (LIF) and/or glycoprotein 130 (gp130) on in vitro growth of human embryos.
Design: Laboratory study.
Setting: University hospital-based IVF clinic.
Patient(s): A total of 164 frozen embryos that survived thawing were cultured in media supplemented with LIF and/or gp130 or control media.
Intervention(s): Morphological development was evaluated by light microscopy. Protein expression profiles of single blastocysts were evaluated using matrix-assisted laser desorption/ionization time of flight-based intact cell mass spectrometry.
Main Outcome Measure(s): Embryo development and protein content.
Result(s): Addition of gp130 to culture media improved blastocyst formation (73% vs. 43%). Addition of LIF to the culture media did not improve embryo development. Protein fingerprint spectra were obtained that revealed significant intensity changes for multiple molecular species including thymosin beta-10, thymosin beta-4, histone H2A, histone H2B, histone H4, ubiquitin, ubiquitin-T, and acyl-CoA binding protein.
Conclusion(s): Glycoprotein 130, but not LIF, seems to be beneficial for preimplantation embryo development, implicating a physiological role in regulating preimplantation development in humans and thus ought to be included in culture media designed for embryo culture to the blastocyst stage. Furthermore, these findings highlight the great potential of matrix-assisted laser desorption/ionization time of flight mass spectrometry and intact cell mass spectrometry as a versatile tool in reproductive medicine research. (Fertil Steril® 2013;99:1592–9. ©2013 by American Society for Reproductive Medicine.)
Key Words: Blastocyst, LIF, gp130, MALDI-TOF MS, ICMS

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Despite years of experience and many technical innovations in assisted reproduction techniques (ART), low implantation rate is still one of the major obstacles resulting in a large proportion of embryonic loss in the ART programs. One reason might be that a number of embryos fail to develop to the blastocyst stage probably due to suboptimal in vitro culture conditions with current culture media systems. A better understanding of basic needs and metabolic requirements of the early embryo at different developmental stages is needed for formulation of new sequential culture media, increased number of embryos reaching the blastocyst stage, and improvement of implantation rates.

Communication between the developing embryo and the maternal tract is of importance during the transport of the early preimplantation embryo through the fallopian tube. It is likely that the leukemia inhibitory factor (LIF) system contributes to this as it has been shown that LIF and its receptors are present in the fallopian tube and in the human preimplantation embryo (1, 2). Interestingly, glycoprotein 130 (gp130) is present in human embryos throughout embryo development; however, only

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in the inner cell mass (ICM) at the blastocyst stage (2), suggesting a need for soluble gp130 communication between the trophectodermal cells of the blastocyst to the maternal tract at later stages of embryo development, but not at earlier stages during transport and development in the fallopian tube. It has previously been shown that gp130 is needed for embryo development in mice (3), but the need for gp130 for human embryo development is not known.

Leukemia inhibitory factor is a cytokine, associated with reproductive processes such as embryo development and implantation (4). The LIF is an absolute requirement for implantation of murine blastocysts (5, 6). The action of LIF is mediated through a receptor constellation consisting of LIF receptor and gp130 subunits (7). The LIF receptor exists in both soluble and membrane-bound forms with opposite effects; the soluble form often antagonizes the actions of its ligands (8–10). gp130 can also combine with other cytokines, such as interleukin-6 (IL-6), IL-11, and ciliary neutrophic factor, to form a high affinity membrane-bound receptor complex (11, 12).

It has been shown that LIF, its receptors, and gp130, increase in the endometrium at the time of implantation (4, 7). Women with unexplained infertility have a lower LIF concentration in uterine fluid and lower protein expression of LIF receptor and gp130 in the endometrium compared with fertile women (1, 13–15), suggesting that LIF and its receptors are important for normal implantation. Soluble gp130 endometrial secretion is normally increased during the implantation window, whereas women with unexplained infertility have decreased secretion of gp130 (15).

In the present study we evaluated the influence of LIF and its receptor gp130 on embryo development using traditional morphological assessment using light microscopy. In addition to morphological assessment, protein analysis by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI TOF MS) was used for direct protein profiling of intact blastocysts followed by protein identification by using bottom-up proteomics and immunofluorescence staining.

The aim of the present study was to evaluate the effect of supplementation with LIF and/or gp130 to the embryo culture media. Furthermore, protein profiling of intact blastocysts by means of MALDI TOF MS was performed to identify potential markers of embryo development.

MATERIALS AND METHODS

Ethical Considerations

All embryos used in this study were donated by couples undergoing IVF treatment at the Centre for Reproduction, Uppsala University Hospital, Uppsala, Sweden. The embryos were thawed after 5 years, which according to the Swedish law is the limit for storage of embryos. After this time it is not possible to use the embryos for fertility treatment. All couples gave their written informed consent for the use of their frozen embryos. The study was approved by the regional research ethics review board in Uppsala.

Chemicals and Reagents

Ammonium bicarbonate, acetonitrile, 1,4-dithiothreitol (Cleland's reagent) (DTT), iodoacetamide, urea, and trifluoroacetic acid were purchased from Sigma Aldrich. Sequence grade trypsin from bovine pancreas was obtained from Roche. Sinapinic acid and protein calibration standard was purchased from Bruker Daltonics.

Embryo Scoring before Cryopreservation

On the morning of day 2, embryo quality was assessed morphologically on the basis of the number of blastomeres (cells), the rate of fragmentation (the proportion of the embryo with anucleate fragments), and the degree of multinucleation of blastomeres, as described elsewhere (16, 17). Each embryo received a score of 0 (top quality), 1 (good quality), 2 (fair quality), or 3 (poor quality). Embryos with a score of 0–2 were used for direct ET, and supernumerary embryos with scores of 0–1 were cryopreserved for later use. Three embryologists graded the embryos and together decided on which embryo(s) to transfer or cryopreserve. The embryo scoring system is validated twice a year, both internally and externally according to the International Organization for Standardization standard.

Freezing of Embryos

Before cryopreservation, embryos were cultured in microdroplets (G-1 v5 PLUS, Vitrolife AB) overlaid with mineral oil (OVOIL) at 37°C and 6% CO_2 in a humidified incubator. Supernumerary embryos were cryopreserved on day 2 (48 hours after ovum pick-up) according to a controlled rate freezing protocol involving the use of 1, 2 propanediol and sucrose solution in phosphate-buffered saline (PBS) as cryoprotectants (Vitrolife AB). This method has been described elsewhere (18, 19). All embryos used in the study had been cryostored for at least 5 years.

Thawing of Embryos

The thawing protocol was performed using a commercial kit according to the instructions from the manufacturer (Thawing Kit; Cook Medical). The embryos were then transferred into equilibrated culture medium (CCM; Vitrolife AB). Thawed embryos were then carefully assessed for blastomere survival, and each embryo received a score of A (100% survival rate), B (50%$\leq$ survival rate $<$100%), or C ($<$50% survival rate). Only embryos with a score of A or B were used for the study. The survived embryos were then randomly allocated, with regard to embryo morphology/survival rate, into four different treatment regimens and cultured up to blastocyst stage. The four groups were control, LIF, gp130, or LIF+gp130. The control embryos were cultured in the standard medium that is used in the clinic (CCM; Vitrolife AB). For treatment, LIF (Human Recombinant, 100 ng/mL; Sigma) and/or gp130 (Human Recombinant, 300 ng/mL; Sigma) was dissolved in standard medium (CCM; Vitrolife AB).

Embryo Culture

The embryos were cultured individually in 20-μL droplets and assessed daily for developmental stage. Culture was

performed at 37°C and 6% CO_2 in a humidified incubator. Each embryo received a score of 0 (top quality), 1 (good quality), 2 (fair quality), or 3 (poor quality). Fisher's exact test was used for comparison of results between the different treatment groups.

MALDI TOF MS

Matrix-assisted laser desorption/ionization (MALDI) time of flight mass spectrometry (TOF MS) is based on direct irradiation with a laser of biological samples previously coated with an crystalline chemical matrix, which facilitates ionization of peptides and proteins. The ionized biological molecules are then analyzed with a time of flight mass analyzer yielding a mass spectrum where peptides and proteins can be identified based on their molecular weight.

A total of five blastocysts from each treatment group (control, gp130, LIF, gp130+LIF) were subjected to individual MALDI TOF-based intact cell protein analysis. Each blastocyst was analyzed individually using a sandwich target preparation method with sinapinic acid, as described previously (20). Here, a polished steel target was prespotted with 1 μL of sinapinic acid matrix solution 1 (1 mg/mL; 100% acetonitrile) to give a thin layer of matrix. The individual blastocysts were washed three times with double distilled H_2O under a microscope and directly applied onto the prespotted MALDI target plate. A volume of 1 μL sinapinic acid solution II (10 mg/mL; 50% acetonitrile/0.1% trifluoroacetic acid) was spotted onto the blastocyst thin layer matrix preparation. The samples were allowed to dry and washed three times with 10 μL of ice cold 0.1% trifluoroacetic acid for salt removal.

All samples were analyzed on an Ultraflex II MALDI TOF mass spectrometer (Bruker Daltonics) equipped with a smartbeam laser. The mass range of 3–25 kDa was analyzed in linear positive mode. All spectra were baseline subtracted (Convex Hull V3), externally calibrated and further processed by means of peak picking (origin v. 8.1, originlab) and binning analysis for data reduction (pbin software, available online: www.vicc.org/biostatistics/download/MassSpec/). Bin integration (area under curve) and total ion current normalization of all raw spectra was performed using an in-house written script in R. The resulting data were evaluated in Microsoft Excel (v. 2007) by means of unbiased statistical analysis using the SAM tool (significance analysis of microarrays) available as macro for Excel (21). Here, nonparametric unpaired statistical analysis was performed for evaluating changes between the different sample groups. All SAM results were followed up by one-way analysis of variance (ANOVA) and posthoc (Tukey test) analysis (origin v. 8.1).

LC MS-based Protein Identification

A total of 30 blastocysts were washed three times in PBS followed by freezing/thawing for osmotic membrane disruption followed by 15 minutes of sonication. The sample was centrifuged at 5,000 × *g* and the supernatant collected for immediate enzymatic protein digestion. A volume of 100 μL of denaturation buffer (8 M urea, 400 mM ammonium bicarbonate) was added, followed by the addition of 10 μL of DTT (45 mM), and incubation at 55°C for 15 minutes for protein reduction. For alkylation a volume of 10 μL of iodoacetamide (100 mM) was added followed by incubation at room temperature in darkness. A total of 25 μg of trypsin was reconstituted in 250 μL of double distilled H_2O to give a final concentration of 100 ng/μL. A volume of 20 μL trypsin solution (2 μg, 1:25 wt:wt) was added to the protein solution and incubated at 37°C overnight. The samples were desalted on ZipTip C18 columns (Millipore) and dried down in a speedvac system (Thermo Scientific).

NanoLC-ESI Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS) was performed on an Agilent 1100 nanoflow system (Agilent Technologies) hyphenated to a LTQ-FT 7.0 T (Thermo Scientific) MS. A volume of 5 μL from the reconstituted ZipTip fraction was injected automatically and loaded onto a self-prepared C18 PicoFrit column (75 μm internal diameter/15 μm tip internal diameter; NewObjective) packed directly inside the electrospray needle tip using specially designed nanospray emitter tips. A water/formic acid/acetonitrile solvent system was used where solvent A was 0.1% formic acid and solvent B was 100% acetonitrile, 0.1% formic acid. Gradient elution was performed in 0 solvent B for 10 minutes, from 0–50% solvent B for 100 minutes, then from 50%–90% solvent B for 5 minutes, then in 90% solvent B for 5 minutes, and finally from 90% back to 0 solvent B for 5 minutes. Peptide elution was followed by ESI FTICR MS and tandem mass spectrometry (MS/MS) for peptide sequencing controlled by the Xcalibur software (v.2.0 SR2, Thermo Scientific). Full-scan spectra were acquired at high resolution (FWHM = 100,000) using the Fourier transform analyzer. Data-dependent acquisition was applied for MS/MS precursor selection, where the five most intense mass peaks were subjected to subsequent isolation and collision-induced fragmentation in the ion trap. Acquired raw data were subjected to database search against the Uniprot knowledgebase (www.uniprot.org) using the Mascot software (v. 2.2, Matrix Science) (22) with the following specifications: mass tolerance (±10 ppm; MS/MS, ±0.9 Da) enzyme (trypsin), fixed modifications (carbamidomethyl), variable modifications (oxidation of Met), precursor charge (1+, 2+, 3+) and instrument (ESI-TRAP). Peptide matches with a score above the confidence threshold ($P < .05$) were considered to be a significant hit. The false-positive identification rate was estimated by searching the data against a decoy database, where the false-positive identification rate threshold was set to <1%.

Embryo Staining

The blastocysts were rapidly transferred from culture and briefly washed in PBS containing 3 mg/mL polyvinyl pyrrolidone and then fixed in 2.5% paraformaldehyde in PBS for 15 minutes at room temperature. After fixation, the embryos were permeabilized in PBS/polyvinyl-pyrrolidone buffer containing 0.25% Triton X-100 for 30 minutes. Embryos were then placed in blocking buffer containing 0.1% bovine serum albumin (BSA) and 0.01% Tween 20 in PBS for 15 minutes. Primary monoclonal antibody for ubiquitin (Ubc), histone H4, and thymosin beta-10 (Tyb10; Abcam Inc.) were diluted in blocking buffer and incubated overnight at 4°C. The embryos were then washed three times

for 15 minutes each in blocking buffer to remove any unbound primary antibodies. The secondary antibody, fluorescein goat anti-rabbit IgG antibody or Texas-red horse anti-mouse IgG antibody (Vector laboratories), was diluted in blocking buffer and applied to the blastocysts for 60 minutes at room temperature in the dark. For negative controls, the primary antibody was excluded from the staining protocol. After incubation, the blastocysts were briefly washed and mounted on slides in Vectashield with 6-diamino-2-phenylindole (DAPI; Vector laboratories). The coverslips were then sealed with nail polish. Stained embryos were viewed by using an inverted microscope (Zeiss AxioVision, Carl Zeiss Microscopy GmbH) equipped with fluorescence optics and appropriate filters.

RESULTS

Embryo Culture

For the present study, a total of 164 embryos survived after thawing and were cultured for 4r days, which corresponds to day 6 after insemination. Of these, 73 embryos developed into blastocysts (44%) and 38 (24%) were of good quality (i.e., fully expanded or hatching with good quality ICM and trophectoderm) (Table 1). Culture of embryos in media supplemented with gp130 resulted in a significantly higher number of embryos that developed into blastocysts (73%) compared with controls (37%), LIF (41%), or LIF+gp130 (29%), respectively (Table 1). In addition the number of high quality blastocysts was significantly higher in the gp130-treated group compared with LIF+gp130 (Table 1). Supplementation with LIF was found not to be beneficial for embryo development. There was no difference compared with the control group (Table 1).

MALDI TOF MS

Characteristic protein fingerprint spectra were obtained from individual blastocysts from the different sample groups revealing the presence of multiple common protein species that could be preliminarily assigned according to their accurate mass value. These species included Tyb10, Tyb4, Ubc, acyl-CoA binding protein (ACBP), 10-kDa heat shock protein, histones H4, H2A, H2B, and H31, as well as hemoglobin subunits alpha and beta (Fig. 1). Further identification experiments using a LC-MS/MS-based bottom-up proteomic approach on a large control pool (n = 30) confirmed their identity based on their significant peptide fragmentation data ($P<.05$).

Intact cell mass spectrometry revealed characteristic protein patterns for the different sample groups (Fig. 2). Unbiased statistics using SAM for unpaired analysis revealed significant changes in protein expression between the different treatment groups ($P<.05$).

Comparison of gp130-treated blastocysts and controls showed significantly increased peak values for three protein species. One of the proteins that was increased is probably ACBP (peak m/z 9968), whereas the other two peaks, m/z 9772 and m/z 19938, were not possible to identify (Supplemental Fig. 1A, available online). Treatment with gp130 and LIF in combination resulted in lower protein levels of Tyb10, histones H2A, H2B, and H4 compared with controls (Supplemental Fig. 1B). Furthermore, histone H31 was found decreased in combination with gp30 and LIF treatment compared with sole treatment with gp130 (Supplemental Fig. 1C).

In contrast no difference between LIF treatment and control or between LIF and gp130 treatment was observed. A number of protein peaks were found to be elevated in LIF+gp130-treated blastocysts compared with sole LIF treatment. On the other hand, only a single unknown protein, m/z 7000.15, was decreased with combination treatment of LIF

TABLE 1

Morphological embryo development assessed by light microscopy.

Treatment	No. of embryos	Blastocysts (% of total no. of embryos)	High quality blastocysts (% of total no. of embryos)
Total	164	73 (44)	40 (24)
Control	43	19 (37)[a]	10 (23)
gp130	40	29 (73)[a,b,c]	15 (38)[d]
LIF	46	19 (41)[b]	9 (20)
LIF+gp130	35	10 (29)[c]	6 (17)[d]

Note: Statistics using Fisher's exact test.
[a] $P=.014$.
[b] $P=.005$.
[c] $P=.0002$.
[d] $P=.004$.

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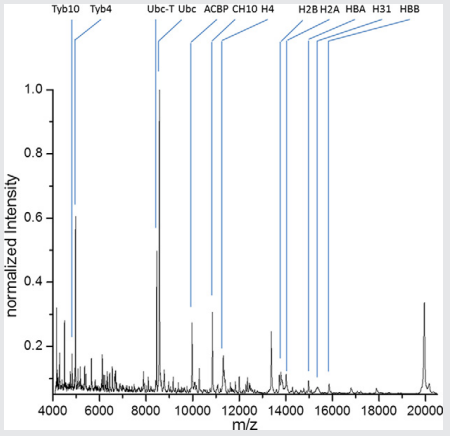
FIGURE 1



Characteristic protein fingerprint spectra of individual embryos obtained with matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI TOF MS) analysis. The peaks represent singly charged individual protein mass peaks that allow preliminary assignment of the respective protein species based on the accurate mass value. The protein annotations are based on their uniprot knowledge base entry (www.uniprot.org).

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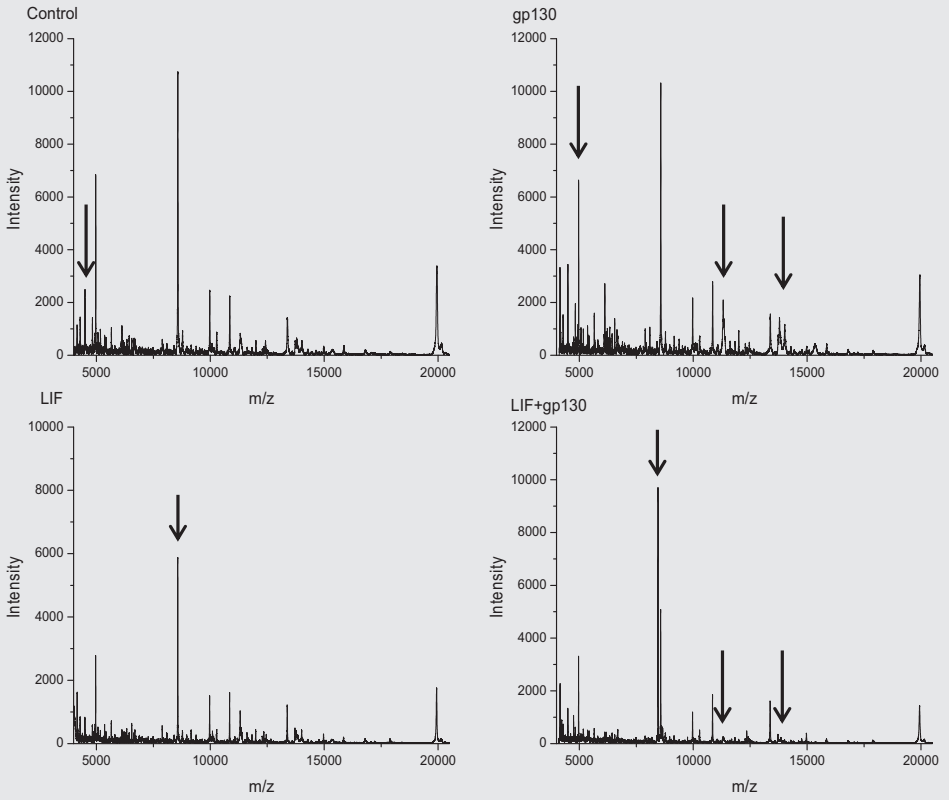
FIGURE 2



Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI TOF MS) spectra of single intact blastocysts from different treatment groups reveal characteristic changes in protein expression profiles. Different protein intensities for common species as well as exclusively present peaks were observed between the treatment groups (*arrows*). These species served as characteristic identifier masses for the respective treatment groups.

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and gp130 compared with LIF (Supplemental Fig. 1D). Interestingly, a previously reported C-terminally truncated form of Ubc1-76 (Ubc-T, Ubc1-74) (23, 24) was found to be strikingly increased in LIF+gp130 compared with LIF (Supplemental Fig. 1D, arrow).

Immunohistochemistry

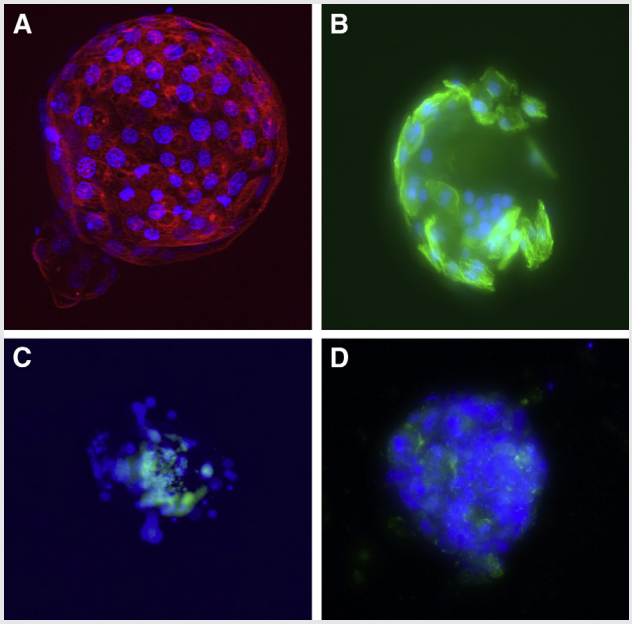
Immunohistochemical staining showed that Ubc is present both in the ICM and in the trophectoderm of the blastocysts (Fig. 3A). Histone H4 was seen in trophectodermal cells, whereas no staining appeared to be present in the ICM (Fig. 3B). Tyb4 was seen in the ICM (Fig. 3C). Tyb10 was seen mainly in the ICM and trophectoderm (Fig. 3D).

DISCUSSION

We found a significant increase in blastocyst formation after supplementation of gp130 to the embryo culture media. The natural environment for development of the human embryo is at the earlier stages in the lumen of the fallopian tube and during the later stages in the endometrium of the uterine cavity. Receptors for LIF and gp130 are present in the fallopian tube and endometrium (1, 2, 25). This is suggestive of a paracrine interaction between the embryo and the reproductive tract.

gp130 secretion is elevated in the endometrium at the time for implantation (15), regulated by the estrogen (E) and P levels (7). Altered gp130 has previously been associated with infertility (1, 15). The addition of gp130 to embryo

FIGURE 3



Immunohistochemical staining of (A) ubiquitin, (B) histone H4, (C) thymosin beta 4, and (D) thymosin beta-10 in human blastocysts. Nuclear staining with 6-diamino-2-phenylindole (DAPI) is shown in *blue*.

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culture media increased the number of embryos that developed to the blastocyst stage. This suggests that gp130 is beneficial for embryo development and could be included in culture media. One protein that was higher in the gp130 group was ACBP. Mouse homozygous ACBP-null embryos do not develop beyond the morula stage, whereas wild-type and heterozygote embryos do, which indicates that ACBP is of vital importance for embryo development (26). The influence of LIF seems to be the opposite of gp130 and LIF seem to be detrimental for embryo development. This is contrary to the mouse, where LIF enhances blastocyst formation and birth rates (27). There was no specific alteration in protein content that could explain the poor embryo development in this group. The positive effect of gp130 might be mediated through the action of other cytokines than LIF, such as IL-6 or IL-11, as gp130 also acts as a receptor for other cytokines in the IL-6 family, which might have an effect on embryo development (11, 12, 28).

To our surprise we found a negative effect of supplementation with both gp130 and LIF to the culture media. This might be a dose effect as the blastocyst itself secrets LIF (29), and that extra supplementation might result in too high levels of LIF. This could lead to disturbed development of the embryo, resulting in arrested development. The altered proteins in this group were Tyb10, histones H2A, H2B, and H4, and Ubc-T. Ubiquitin has earlier been detected in the secretome of human preimplantation embryos developing into blastocysts and has been suggested as a marker for good embryo quality (30). Ubiquitin-T, also named Tyb1 or thymosin polypeptide beta 1, is a small protein found in most tissues of eukaryotic organisms and is involved in many intracellular processes such as regulation of protein recycling. The first cell cleavage has been suggested to be regulated by a Ubc ligase (31). Studies of gene expression of human Ubc-like modifiers show their expression already in the four-cell stage embryo (32). The Ubc pathway is also important for the development of blastocysts in mice (33). Ubiquitin has been detected in the cytoplasm and the nucleus of trophoblast cells and has been suggested to be essential for placentation (34). We found staining for Ubc in both the ICM and trophectodermal cells of human blastocysts, suggesting a function for Ubc also in the human embryo development. Pre-embryos cultured with a combination of LIF and gp130 had increased Ubc-T activity compared with embryos cultured with only LIF supplementation (Supplemental Fig. 1D). This is somewhat conflicting with the above-mentioned studies as the embryos

cultured with LIF+gp130 were the least developed group. Growth factor supplementation to culture media is not necessarily an advantage. In a recent study it was demonstrated that the rate of mosaicism/aneuploidy was higher in mouse embryos cultured in media supplemented with granulocyte macrophage colony-stimulating factor (GM-CSF) compared with controls (35). Tyb10 was reduced in the LIF+gp130 group. Beta-thymosins are small proteins that regulate the actin cytoskeleton, involved in cell motility and differentiation. Tyb10 is up-regulated in bovine cumulus cells during in vitro maturation of cumulus-oocyte complexes, positively correlated with cumulus-oocyte complex expansion and P secretion and negatively correlated with apoptosis. Tyb10 expression is, however, shown to be unchanged in oocytes developing to the blastocyst stage compared with arrested preimplantation embryos (36). During embryogenesis the control of actin polymerization is essential in processes, such as cell migration, and is believed to play an important role in early embryo development in mice (37). Expression of porcine complementary DNA (cDNA) coding for Tyb10 has also been coupled to embryogenesis in swine (38). The need for Tyb10 for embryo maturation makes it a possible marker of embryo development.

Histones undergo post-transcriptional modifications that act in diverse biological processes such as gene regulation, DNA repair, chromosome condensation, and spermatogenesis (39). The oocyte is rich in histone messenger RNA, which is needed for replacement of the protamins in sperm nuclei before fertilization. This accumulated amount of histones is also needed to meet the needs of the zygote during embryonic genome activation, which normally occurs at the four- to eight-cell stage in humans. We found that H3 was up-regulated in the gp130-treated embryos, with the most favorable embryo development, compared with the least developed group treated by LIF+gp130. This might give a clue as to how gp130 exerts its biological effect on embryo development. Histone H3 could be a candidate marker, predicting blastocyst development. We found staining for histone H4 in trophoblast cells, whereas no staining appeared to be present in the ICM; therefore histone H4 could be suggested as a marker for trophoblast development. The suggested markers for embryo development need to be evaluated in further studies.

Little is known about the protein patterns of early human embryo development due to small size of the preimplantation embryo and the limited number of human embryos available for research. MALDI TOF MS is a new powerful technique used to search for both quantitative and qualitative changes in different biological processes (40). The development of this proteomic technology has made it possible to analyze the proteins of single human preimplantation embryos. The present study shows that the use of MALDI-TOF MS is a valuable tool for analysis of protein pattern in early embryo.

In conclusion, we found that supplementation of gp130 to embryo culture media improved the development of human preimplantation embryos, as seen by the formation of more blastocysts. The results need to be confirmed in further studies. Our findings also highlight the great potential of MALDI TOF MS as a versatile tool in reproductive medicine research.

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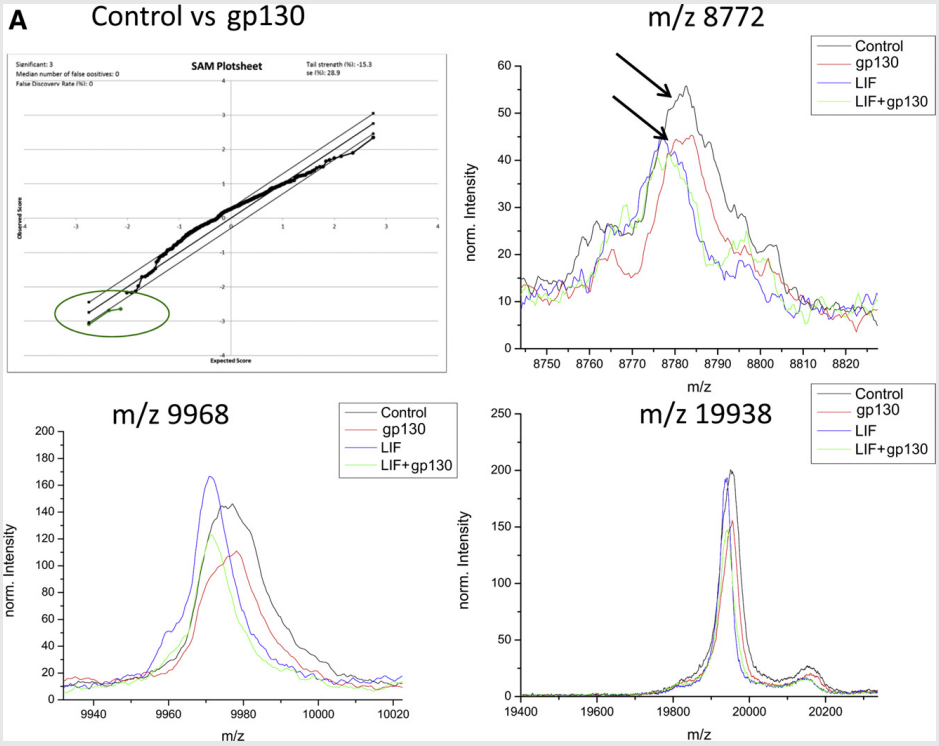
SUPPLEMENTAL FIGURE 1



Statistical investigation of matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI TOF MS) data reveals treatment-specific protein regulations. Unbiased analysis of protein peak intensities by means of multivariate, unpaired *t*-statistics was performed using the significance analysis of microarrays (SAM) software. (A) (*upper left*) Control versus gp130 shows significantly lower peak intensities (*green*) for three protein mass peaks including m/z 8772, m/z 9968, and m/z 19938. (*upper right, lower left to lower right*) Manual inspection of the MS data was performed for verification of corresponding protein intensity changes (*arrows*: black trace "control" vs. red trace "gp130") observed in SAM analysis. (B) Control versus LIF+gp130. The SAM analysis revealed significant changes for many protein peaks (*green*: lower in LIF+gp130 and *red*: higher in LIF+gp130). Here thymosin beta chains 10 (*) and 4 (*arrow*) as well as histone H4 and histone H2B were found to be significantly decreased in LIF+gp130 compared with controls. (C and D) Combinatory LIF+gp130 supplementation versus gp130 and LIF treatment, respectively. The SAM analysis revealed significant changes for many protein peaks. An up-regulation of histone H3 was observed for gp130 compared with LIF+gp130 (C). In contrast, a striking increase of ubiquitin-T was observed in LIF+gp130 compared with sole LIF treatment (D).

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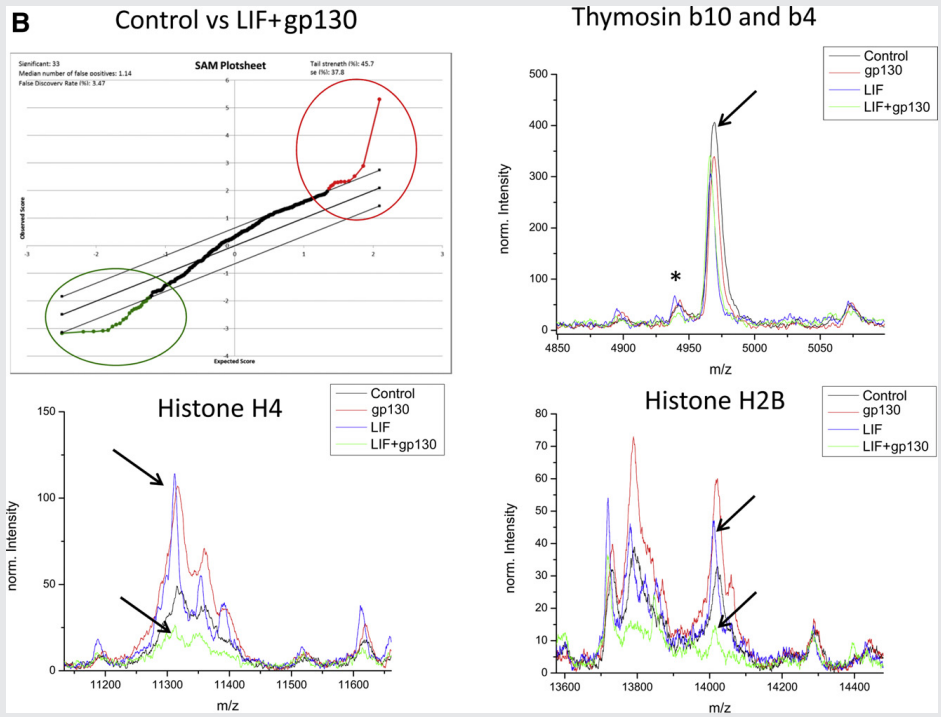
SUPPLEMENTAL FIGURE 1 Continued



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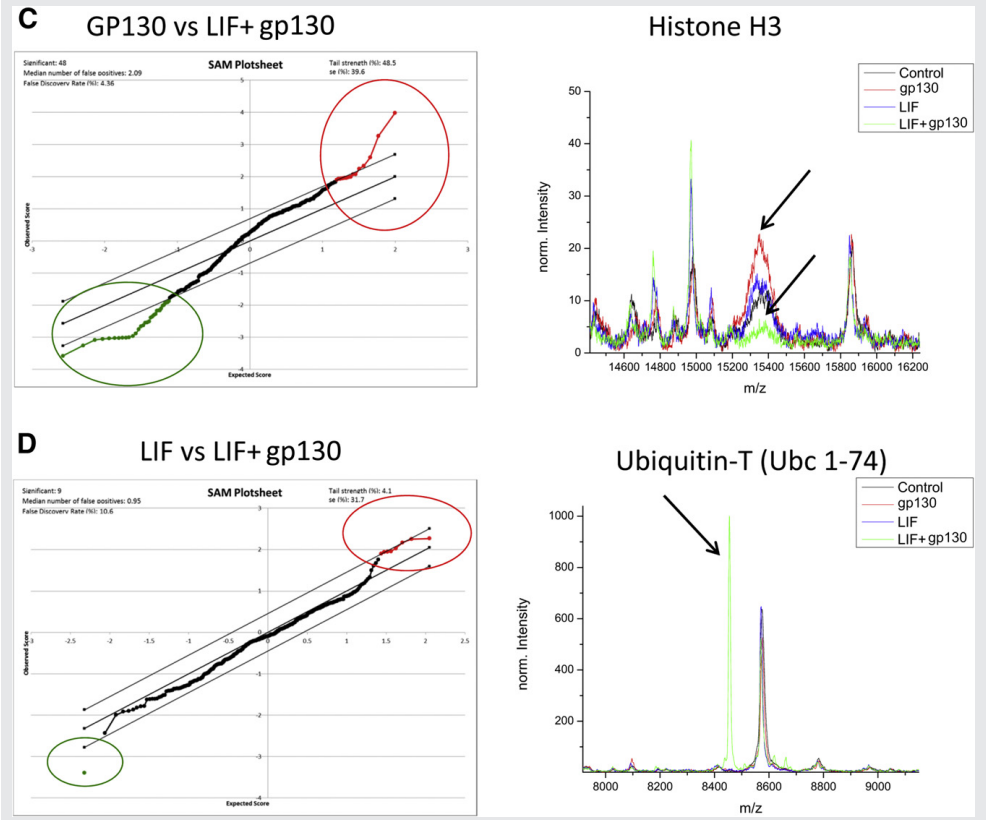
SUPPLEMENTAL FIGURE 1 Continued



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Article

Vitrification with DMSO protects embryo membrane integrity better than solutions without DMSO

Anne-Jacqueline Kartberg obtained her Bachelor's Honours degree in combined Biology and Chemistry at the University of British Columbia in Canada, and her PhD degree at Uppsala University in Sweden. Her PhD thesis was in the field of medical cell biology, focusing on asthma and allergy. As a post-doctoral fellow at the European Molecular Biology Laboratory (EMBL) in Heidelberg, Germany and at Gothenburg University in Sweden, she took interest in molecular biology. In 2005, she entered the field of reproductive technology, and started at Gothenburg IVF as the Laboratory Manager and Project Leader. Her special interests are research in embryology, vitrification, and culture media.

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Abstract

Vitrification has become common for cryopreservation of embryos. However, the most optimal protocol for vitrification is still to be found. Two vitrification protocols with similar osmolarities were compared: Protocol A, containing dimethyl sulphoxide (DMSO), propane-2-diol, and ethylene glycol, and Protocol B, containing propane-2-diol and ethylene glycol. Viability and the importance of specific incubation times for early embryo recovery, survival, and cleavage were studied. For assessment of cryodamage, embryos were labelled with Alexa Fluor 488-conjugated annexin V and propidium iodide. Vitrification studies on early mouse embryos were followed up with studies on human embryos. The two vitrification protocols did not differ in embryo survival rates and were equally efficient in both mouse and human embryo models. Morphological assessment of embryos directly after vitrification was not a useful tool for assessing survival in this study. Extended exposure of embryos with both vitrification protocols showed that the DMSO-containing vitrification solutions did not lead to cell membrane damage and death as quickly as the DMSO-free vitrification solutions. To assess embryo viability, the authors recommend that vitrification of early embryos should be combined with extended culture and assessment of normal blastocyst development before transferring to patients.

Keywords: *cryodamage, cryoloop, fluorescence imaging, post-vitrification survival, vitrification*

Introduction

Single embryo transfer decreases the risk of multiple pregnancies, which may cause preterm birth, low birth weight and perinatal death. In addition, single embryo transfer is also the most cost-effective, when combined with frozen/thawed cycles (Dare *et al*., 2004; Pinborg, 2005; Pinborg *et al*., 2005; Fiddelers *et al*., 2007). Therefore, single embryo transfer is becoming the recommended method in an increasing number of countries.

With the reduction in the number of embryos transferred per patient during IVF treatment, the need for effective cryopreservation methods has increased further. Vitrification has advantages over the traditionally used cryopreservation method of slow freezing (Rama Raju *et al*., 2005; Stehlik *et al*., 2005). Whereas slow freezing can cause intracellular ice crystal formation, which sometimes causes complete destruction of blastomeres and/or whole embryos, vitrification relies on a fast cooling rate that does not form ice crystals. Vitrified embryos seem almost unchanged morphologically after warming. Vitrification is becoming increasingly popular for cryopreserving human gametes and embryos (Rama Raju *et al*., 2005; Takahashi *et al*., 2005; Selman *et al*., 2006; Antinori, *et al*., 2007).

One perceived drawback with vitrification is the use of high concentrations of cryoprotectants leading to intense dehydration and shrinkage of the embryo. Dimethyl sulphoxide (DMSO) is a permeating cryoprotectant, which protects cells from intracellular

ice crystal formation when used at higher concentrations. This characteristic can lead to a higher toxicity compared with a cryoprotectant with lower permeation rates. Recent publications show that vitrification of human embryos is possible with different cryoprotectant solutions, such as DMSO in combination with ethylene glycol (EG), or EG alone (Rama Raju *et al.*, 2005; Takahashi *et al.*, 2005). The combination of DMSO and EG has been used successfully by Desai *et al.* (2007); however, in some IVF clinics employees are hesitant to work with DMSO. This study was undertaken to investigate the limits of these cryoprotectants. The authors wanted to consider the effects of long-term exposure of these cryoprotectants on embryo development. A functional protocol works well only if the user performs the protocol properly. With vitrification becoming more widespread, there are bound to be deviations from the instructions. Commercially available kits differ in cryoprotectant components and there is a lack of data supporting whether or not to use DMSO-containing or DMSO-free vitrification solutions. Using an in-vitro mouse embryo model, the objectives were: to compare two vitrification protocols, with and without DMSO; to assess viability of vitrified early mouse embryos compared with human embryos to recover and develop further into expanded blastocysts; to assess cryodamage through fluorescence image analysis using markers for cell membrane degeneration.

Materials and methods

Mouse embryo culture

Two-cell mouse embryos (Embryotech Laboratories, Inc, Wilmington, MA, USA) were cultured in G-1 v3 (VitroLife AB, Kungsbacka, Sweden) in IVF tissue culture dishes (Falcon 353652, Becton Dickinson, Franklin Lakes, NJ USA) at 37ºC in a humidified triple gas incubator (6% CO_2, 5% O_2, 89% N_2). Mouse embryos were cultured in groups (at least 5 µl medium/embryo) until the blastocyst stage. One to three embryos were vitrified at the same time on the cryoloop. After 17 h of culture (day 3), mouse embryos (6–8 cell) were cultured in G-2 v3 (VitroLife AB, Kungsbacka, Sweden). Blastocyst development was assessed after an additional 48 h of culture, day 5. All day 3 early embryos that developed into the 6–8-cell stage were used for the study; at least 50 embryos were used in each group, unless otherwise stated.

Collection and culture of human embryos

The use of human embryos that could not be used for fertility treatment was approved by the Ethics Committees of Örebro and Uppsala University Hospitals, in Sweden. Written informed consent was obtained from all embryo donors and none of the donors received any financial reimbursement. The embryos were obtained as donations from patients undergoing IVF at the Centre of Reproduction, Uppsala University Hospital.

Down-regulation for ovarian stimulation was achieved using a long protocol of gonadotrophin-releasing hormone agonist (GnRHa), nafarelin (Synarela; Syntex Nordica AB, Södertälje, Sweden), administered intra-nasally, starting on either cycle day 1 or 21. Following down-regulation, ovarian stimulation was induced using recombinant FSH, (rFSH; Gonal-F, Serono laboratories, Aubonne, Switzerland or, rFSH; Puregon, NY Organon, Oss, The Netherlands). The starting dose was dependent on the patient's age and/or previous response to ovarian stimulation. Ovarian response was monitored by serum oestradiol assays and vaginal ultrasound scans of follicles. GnRHa and rFSH were administered until the leading follicle had a diameter of at least 18 mm. Maturation of the oocyte was triggered by one subcutaneous injection of 10,000 IU of human chorionic gonadotrophin, (HCG; Profasi, Serono laboratories, Aubonne, Switzerland). Thirty-seven hours after HCG administration, oocytes were retrieved by transvaginal needle aspiration under ultrasound guidance.

Conventional IVF was performed in 750 µl medium (G-FERT™ PLUS, VitroLife AB, Kungsbacka, Sweden) under mineral oil (Ovoil, VitroLife AB, Kungsbacka, Sweden) containing about 250,000 spermatozoa. For intracytoplasmic sperm injection (ICSI), oocytes were stripped of cumulus cells by mechanical pipetting after brief exposure to hyaluronidase (HYASE™, VitroLife AB, Kungsbacka, Sweden). ICSI was then performed using a Nikon–Narishige micromanipulation system. Fertilization was evaluated 18–20 h after insemination. Following fertilization after IVF and ICSI, embryos were cultured in groups of three to five under oil in 20 µl droplets of G-1 v3 Plus. Embryo quality was assessed daily. On the morning of day 2 or day 3 the embryo quality was assessed morphologically on the basis of the number of blastomeres (cells), the rate of fragmentation (the volume of the embryo with anucleate fragments) and multinucleation of blastomeres, as described by Mohr *et al.* (1985) and Ziebe *et al.* (1997). Each embryo received a score of 0 (top quality), 1 (good quality), 2 (fair quality) and 3 (poor quality). Embryos with a score of 0–2 were used for direct embryo transfer either on day 2 or day 3, or frozen for later use by the patient (supernumerary embryos). Embryos surplus to treatment, all score 3, were not considered to be of acceptable quality for transfer, and were donated for the present study.

Materials and methods

Handling media used for embryo collection was G-MOPS PLUS (Vitrolife AB, Kungsbacka, Sweden). All media contained human serum albumin (HSA), concentration unspecified by manufacturer. Propane-2-diol (PrOH), DMSO and EG were all purchased from Sigma (St. Louis, MO, USA). Sucrose was purchased from Merck (Darmstadt, Germany).

Preparation of vitrification and warming solutions

The Protocol A vitrification solution 1 contained 5% PrOH, 5% DMSO and 5% EG in handling medium; vitrification solution 2 contains 10% PrOH, 10% DMSO, 10% EG and 0.5 M sucrose in handling medium.

The Protocol B vitrification solution 1 contained 7.5% PrOH and 7.5% EG in handling medium; vitrification solution 2 contained 15% PrOH, 15% EG and 0.5 M sucrose in handling medium.

The four warming solutions consisted of 1.0, 0.5, 0.25 and 0 M sucrose in handling medium. All solutions were prepared in advance, sterile filtered, stored at 4ºC and used within 6 weeks.

Experimental preparation for vitrification and warming

Solutions (500 µl) were pipetted directly into the corresponding wells of a non-toxic 4-well dish (Nunc, Roskilde, Denmark).

Handling media, vitrification solution 1, and vitrification solution 2 were aliquoted in vitrification wells 1, 2 and 3, respectively. In another 4-well dish, warming solutions 1–4 were aliquoted into warming wells 1–4. All solutions were equilibrated at room temperature in ambient atmosphere.

Vitrification of embryos

The protocol was performed at room temperature and according to media manufacturer's instructions, unless otherwise stated. Early mouse and human embryos were rinsed in G-MOPS PLUS, transferred to vitrification well 1 for 2 min with a sterile pulled glass pipette, then transferred to vitrification well 2 for 45 s. One to three embryos were immediately loaded onto a cryoloop (Hampton Research, Laguna, CA, USA) and plunged into liquid nitrogen and closed in a cryovial submerged in liquid nitrogen.

Warming of embryos

The protocol was performed at room temperature, unless otherwise stated. The cryoloop was removed from the vial at low temperature and placed directly and quickly into warming well 1. With a pulled glass pipette, embryos were moved to warming well 2 for 2 min, to warming well 3 for another 2 min and then transferred to warming well 4 for 2 min. The embryos were further cultured in G-2 v3 PLUS until blastocyst stage.

Survival assessment

After 48 h of culture in G-2 v3 PLUS, both mouse and human embryos were examined on an inverted microscope (Zeiss Axiovert 200, Germany) at ×40 magnification. Survival was assessed based on the development to the blastocyst stage and the morphological integrity of the inner cell mass and trophectoderm, according to the criteria described by Gardner *et al*. (2000).

Effect of extended exposure to cryoprotectants

In order to study the effects of an extended exposure to cryoprotectants, mouse embryos were incubated in the last vitrification solution for longer periods of time (5, 10, 20 and 30 min) before loading the cryoloop.

Cryodamage assessment

Apoptosis and necrosis were detected by using the Vybrant Apoptosis Assay Kit #2 (Molecular Probes, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, Alexa Fluor 488-conjugated annexin V was used to mark for phosphatidylserine on the plasma membrane's outer leaflet. In conjunction, the embryos were stained with propidium iodide, to detect cell membranes that were not intact. Propidium iodide only penetrates cells and stains the DNA when the membrane integrity is compromised.

Whole embryo staining using immunocytochemistry was also used for apoptosis detection and damage to the cytoskeleton. At least 10 embryos were used in each group. The embryos were fixed with 4% paraformaldehyde, stained with the anti-caspase 3 or fluorescein isothiocyanate-conjugated–anti-actin or Cy3-conjugated–anti-tubulin (Sigma-Aldrich Sweden AB, Stockholm, Sweden), nuclear stained with bisbenzamide, and mounted on glass slides for fluorescence microscopy.

Statistical analysis

Statistical comparisons of adherent cells were performed using the chi-squared test (Statistics Package for Social Sciences, Chicago, IL, USA) to compare the different groups of vitrified embryos. $P < 0.05$ was considered significant.

Results

The survival rates of mouse and human embryos after vitrification, using two different protocols were evaluated. Mouse embryos were vitrified at the 6–8-cell stage ($n > 50$ in each group) whereas human embryos were vitrified at day 2 or day 3 in each group ($n > 50$ in each group). Using Protocol A, vitrified human embryos had a survival rate of 30% (16 of 53) while for Protocol B it was 35% (28 of 80). There was no significant difference between the two protocols. Human embryos were more sensitive to the vitrification procedure than mouse embryos (**Table 1**).

Figure 1 shows the results of the effect of prolonged exposure to cryoprotectants in mouse embryos. These studies showed that Protocol A was less detrimental to the mouse embryos than Protocol B, when exposed to an extended period of time in vitrification solution 2. After 5, 10, 20 and 30 min in vitrification solution 2, the survival rate using Protocol A was 81, 84, 47, 0% ($n = 44/54$, 43/51, 24/51, 0/41) while for Protocol B it was 77, 67, 27, 2.5% ($n = 43/56$, 34/52, 15/55, 1/40). There was a significant difference between the survival rates for Protocol A and Protocol B when subjecting the mouse embryos to 10 and 20 min in vitrification solution 2 ($P \leq 0.05$). There was a significant decrease in survival rates after 20 min in vitrification solution 2 compared with the control mouse embryos ($P \leq 0.001$).

Some of the vitrified early embryos, even after prolonged exposure to the highest concentration of cryoprotectants, seemed almost unchanged morphologically after warming, but after 48 h in culture, the viability of the embryos was dramatically reduced. Despite the excellent morphology seen in most embryos exposed for 30 min to either vitrification solution 2, the embryos failed to develop further (**Figure 2**).

Whereas both protocols preserved the visible integrity of the plasma membrane even after excessive exposure to cryoprotectants, fluorescence staining showed that cell death was linked to membrane damage. Mouse and human embryos that were vitrified according to the protocol incubation times (the shortest recommended by the media manufacturer) showed no annexin V staining. Embryos exposed to longer incubation in vitrification solution 2 showed blastomeres with signs of membrane damage (**Figure 3**). The morphology in **Figure 3** depicts fixed embryos, which have fewer spherical blastomeres than seen during culture. Labelling with anti-caspase-3 (**Figure 4**), a common marker for apoptosis, showed that the previous annexin V staining was due to apoptosis in mouse embryos. There were no differences in the structures of the cytoskeletal proteins, actin and tubulin, after vitrification with either protocol.

Table 1: Comparison of Protocols A (with dimethyl sulphoxide; DMSO) and B (without DMSO) in mouse and human model systems.

	Survival (%, number survived/total number)	
	Mouse embryos	*Human embryos*
Control	83 (45/54)	32 (16/50)
Protocol A	77 (43/56)	30 (16/53)
Protocol B	80 (45/56)	35 (28/80)

There were no statistically significant differences in embryo survival compared with the control (non-vitrified) group. Survival was defined as development to the blastocyst stage *in vitro*.

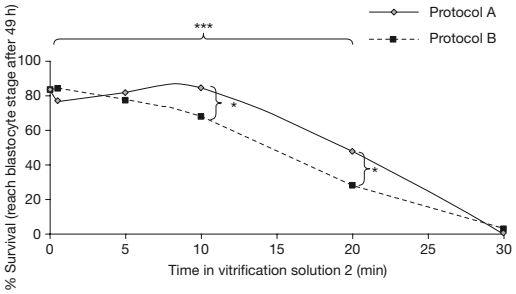

Figure 1. There was a significant difference between the survival rates of Protocol A, containing dimethyl sulphoxide (DMSO), propane-2-diol and ethylene glycol, and Protocol B, containing propane-2-diol and ethylene glycol, compared with the control (non-vitrified) mouse embryos when subjecting the mouse embryos to 10 and 20 min in vitrification solution 2, $*P \leq 0.05$. There was a significant decrease in survival rates after 20 min in vitrification solution 2 compared with the control mouse embryos, $***P \leq 0.001$ (chi-squared test).

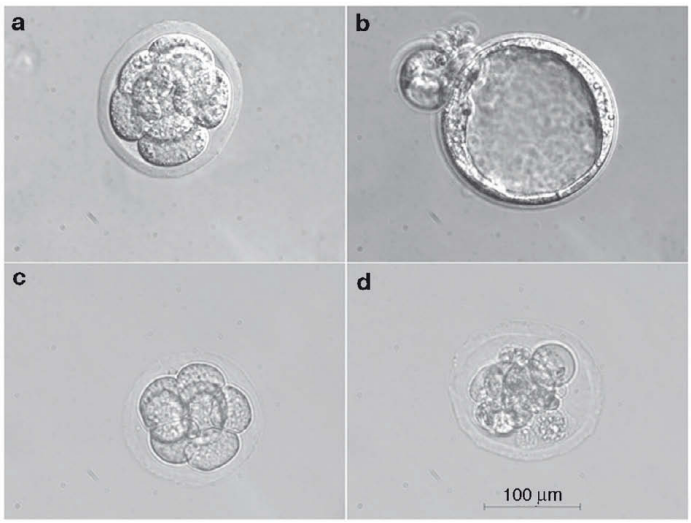

Figure 2. Embryo morphology seen directly after warming of a vitrified mouse embryo (**a**) using Protocol A according to the established protocol; after 48 h in culture, development to a blastocyst (**b**). A vitrified mouse embryo using Protocol A, exposed for 30 min in vitrification solution 2, after warming (**c**) ceased to develop further (**d**). The scale bar in panel (d) applies to all panels.

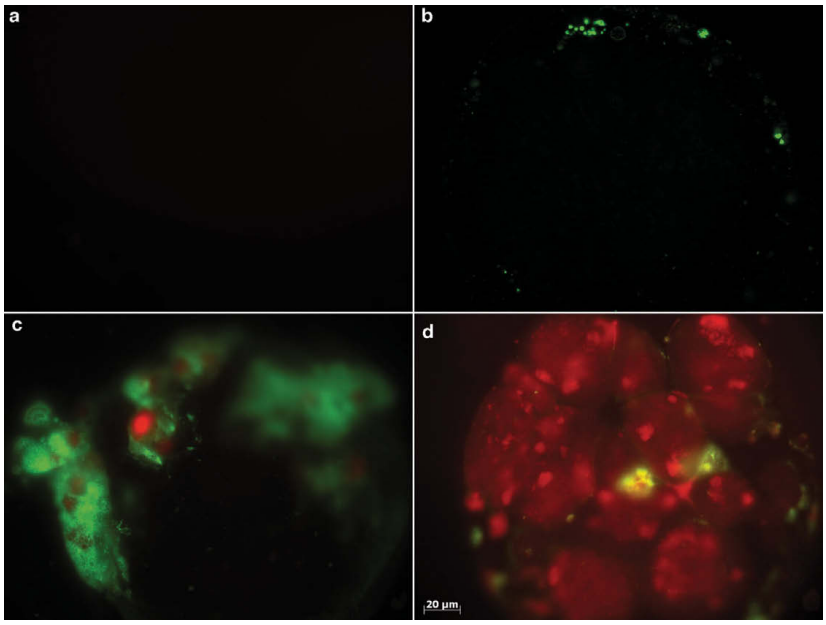

Figure 3. Staining of a control (non-vitrified) mouse embryo (**a**), a vitrified mouse embryo according to the established Protocol A (**b**) and mouse embryos exposed to vitrification solution 2 for 20 min (**c**) or 30 min (**d**) with Protocol A compared to the control (non-vitrified) mouse embryos. Green staining shows annexin V binding (early apoptosis) and red staining with propidium iodide shows necrotic cells (broken cell membranes).

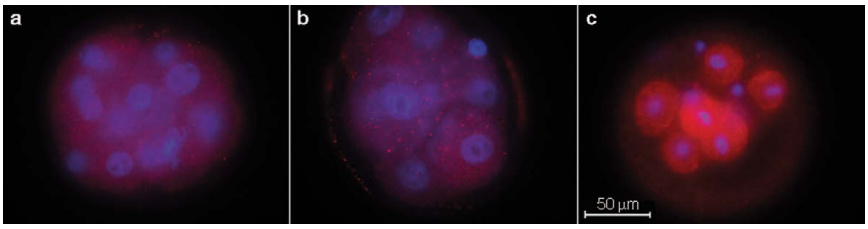

Figure 4. Immunostaining with anti-caspase-3 (red) of a control (non-vitrified) mouse embryo (**a**), a vitrified mouse embryo according to the established Protocol A (**b**) and a mouse embryo exposed to vitrification solution 2 for 30 min according to Protocol A (**c**) compared with the control (non-vitrified) mouse embryos. The blue stain is bisbenzamide, which stains all nuclei. The scale bar in panel (**c**) applies to all panels.

Discussion

The pregnancy rate is still considerably less after transfer of frozen embryos compared with fresh embryo transfer (Oktay *et al.*, 2006). This could only be partially explained by the fact that the highest quality embryos are used for fresh transfers. Therefore, it is essential that new and better vitrification techniques for embryos are developed.

Most of the successful vitrification methods published involve vitrification solutions that include DMSO and EG in combination at concentrations of up to 15% for each cryoprotectant (Matsui *et al.*, 1995; Selman *et al.*, 2006; Desai *et al.*, 2007). To minimize, but not completely abolish DMSO, Protocol A was created using DMSO, EG and PrOH. This allowed a reduced amount of each cryoprotectant; that is, 5% (vitrification solution 1) then 10% (vitrification solution 2) of each cryoprotectant. To compare this 'low-DMSO' protocol with a DMSO-free formulation, Protocol B contained EG and PrOH at 7.5% (vitrification solution 1) and then 15% (vitrification solution 2) of each cryoprotectant. Both protocols worked well, both for mouse and human embryos, when following the established procedure of 2 min exposure in vitrification solution 1 and 45 s in vitrification solution 2.

The effect of extended exposure to cryoprotectants on embryo survival was used to compare Protocol A and Protocol B. The DMSO-containing solutions used in Protocol A, were significantly less detrimental to mouse embryos after 10 and 20 min in vitrification solution 2, compared with the DMSO-free Protocol B (both $P \leq 0.05$). Low salt concentration has been used for the cryopreservation of oocytes, with acceptable pregnancy rates after frozen egg–embryo transfer (Boldt *et al.*, 2006). In the present study, the overall osmolarity for both protocols was similar so the differences in results could be due to the addition of PrOH and/or the decreased concentration of the individual cryoprotectants, namely DMSO and EG from 15% concentration to 10%. There are concerns regarding PrOH as a cryoprotectant because it has shown cytotoxicity (Fahy, 2007). However, the likelihood that the decrease in survival rates is due to the addition of PrOH is unlikely as the concentration of PrOH was only 10% and it has been reported that toxicity increased in PrOH-based vitrification solutions as the concentration increased from 30 to 40%. (Mukaida *et al.*, 1998). Cryoprotectants can be transported through the cell membrane by aquaporins (Edashige *et al.*, 2003; Yamaji *et al.*, 2006). EG diffuses into and leaves the embryos very rapidly owing to its low molecular weight, hence embryos may undergo less osmotic stress during vitrification and warming. Intracellular ice is more likely to form when the permeation of the cryoprotectants and its concentration in the cytoplasm are insufficient. DMSO is relatively polar and has a small and compact structure, which allows it to penetrate living tissues rapidly without causing significant damage.

When working with the vitrification protocol, the incubation time for the critical final step is 45 s. This step can take longer if the user is not comfortable or experienced with loading the embryos on the cryopreservation vehicle; thus leading to a prolonged time in the vitrification solution with the highest concentrations of cryoprotectants. Thus prolonged exposure of embryos during the final step was investigated.

The investigation of extended exposure to vitrification solution 2 was only performed on mouse embryos as the number of human embryos was limited. However, it is likely that the same mechanisms are involved in the cryodamage of both mouse and human embryos. Although embryos were subjected to extended exposure to the last vitrification solution, the blastomeres did not seem to be damaged upon warming. Continued culture to the blastocyst stage, however, showed otherwise. Whereas both protocols preserved the visible integrity of the plasma membrane even after excessive exposure to cryoprotectants, fluorescence staining showed that the blastomeres did not have intact cell membranes as propidium iodide could penetrate through the cell membrane and stain the DNA.

Furthermore, phosphatidyserine was shown to be on the wrong (the outer) leaflet of the cell membrane. This phospholipid asymmetry could have been a result of membrane damage due to the vitrification and warming process and/or apoptosis. To investigate apoptosis further, embryos were immunostained with anti-caspase-3. Extended exposure to high concentrations of cryoprotectants led to membrane damage characteristic of apoptosis and necrosis. These studies were performed only in mouse embryos, assuming that the same mechanism also occurs in human embryos.

The low survival rate of the human embryos used in this study could be due to the extended culture and definition of survival used. Survival, in this study, was defined as the percentage of embryos that continued to develop to the blastocyst stage *in vitro*. This extended culture was necessary as morphological assessment was not sufficient.

Vitrification of day-2 or -3 cleavage-stage embryos instead of day-5 blastocysts has several advantages. Since morphology alone of a vitrified and warmed embryo is not enough to assess viability, the possibility of culturing for a few more days before transfer can ensure that a viable embryo will be used for transfer. This is also advantageous as it was observed that embryo transfer at day 5, in comparison to day 2 or 3, gives an increased pregnancy and implantation rate (Graham *et al.*, 2000; Milki *et al.*, 2000). The reason behind this, besides natural embryo selection, may be improved embryo–uterine synchrony and decreased cervical mucus (Milki *et al.*, 2000). Another benefit to day-2 or -3 vitrification is that there is no need for artificial shrinkage at the early cleavage-stage, whereas at day 5 it is necessary to puncture the blastocoele to avoid formation of ice crystals during vitrification (Vanderzwalmen *et al.*, 2002; Hiraoka *et al.*, 2004; Mukaida *et al.*, 2006).

Based on the conclusions drawn from this study, it is obvious that morphological assessment of vitrified embryos directly after warming can be misleading. Blastomeres are almost always fully expanded and there is no morphological sign of cellular damage. Both mouse and human embryos vitrified accordingly showed very little cryodamage upon warming (less than 5%), which is comparable to other reports (Desai *et al.*, 2007). The fully expanded blastomeres can be a result of water diffusion through the cell membrane to maintain an iso-osmolar equlibrium.

Cryodamage due to vitrification can occur when the concentration of cryoprotectant is insufficient inside the cytoplasm (low concentration or low exposure or low permeation), leading to crystallization or when osmotic changes are too drastic (Storey, 1997). This is seen when the warmed embryo has a glass-like

morphology, as if the embryo was fixed. The damage is then physical and morphologically apparent. However, embryo survival cannot be evaluated solely on a morphological basis. The authors have shown that despite good morphology upon warming, developmental ability is compromised if the embryo has been exposed to cryoprotectants for too long. These embryos re-expand to their original volume upon warming but are not viable.

This study investigated the chemical injury associated with vitrification. The authors have shown that the DMSO-containing vitrification solution leads to less chemical injury upon prolonged exposure compared with the DMSO-free vitrification solution, possibly due to the fast penetrating characteristics of DMSO. DMSO can penetrate the cytoplasm quickly, thus stabilizing the osmolarity in the cells to correspond to the extracellular osmolarity.

In conclusion, the two standard vitrification protocols, DMSO-containing and DMSO-free, did not differ in embryo survival rates and were equally efficient in both mouse and human embryo models. Morphological assessment of embryos directly after vitrification is not a reliable tool for assessing survival. Extended exposure to vitrification solutions using both vitrification protocols showed that the DMSO-containing vitrification solutions were milder and did not lead to cell membrane damage and death as quickly as the DMSO-free vitrification solutions. Since it is not possible to use molecular markers on whole embryos to be used clinically, vitrification of early embryos may be combined with extended culture to the blastocyst stage with assessment of normal embryo development before transferring to patients.

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STEM CELL BIOLOGY

Co-localization of NANOG and OCT4 in human pre-implantation embryos and in human embryonic stem cells

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Abstract
Purpose NANOG and OCT4 are required for the maintenance of pluripotency in embryonic stem cells (ESCs). These proteins are also expressed in the inner cell mass (ICM) of the mouse pre-implantation embryo.
Methods Immunohistochemistry was used to show the presence of NANOG and OCT4 protein, and in situ hybridization was used to localize NANOG mRNA in human embryos from two-cell to blastocyst stage, and in human ESCs (hESCs).
Results Nanog and Oct4 were co-localized in human embryos from morula and blastocyst stages. NANOG mRNA was detected in a group of cells in the morula, in cells of the ICM of blastocysts, and evenly in hESCs. All non-differentiated hESCs expressed NANOG and OCT4 protein. Pluripotent cells expressing NANOG and Oct4 were eccentrically localized, probably in polarized cells in a human compacted morula, which appears to be different from expression in murine embryos.
Conclusion In this study, we demonstrate that whole mount in situ hybridization is amenable to localization of mRNAs in human development, as in other species.

Keywords Pluripotency · Embryo · Embryonic stem cells · NANOG · In situ hybridization

Capsule NANOG and OCT4 in human embryo and ES cells.

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Introduction

Embryo development seems to be tightly regulated during each developmental stage, although the exact mechanisms are not completely understood. The transition from morula to blastocyst involves segregation of the first two cell lineages in the pre-implantation embryo, the inner cell mass (ICM), which forms the embryo, and the trophectoderm, which gives rise to the trophoblast lineage. Two transcription factors, OCT4 and NANOG, are the two most studied factors concerning formation of the ICM in mouse embryos, and it has been shown that these two intrinsic factors are required for establishment of the ICM in an undifferentiated state [3, 18, 20]. OCT4 and NANOG are expressed in human pre-implantation embryos and human embryonic stem cells (hESCs) and these transcription factors regulate stem cell pluripotency and differentiation [6, 15, 28]. The OCT4 gene is a member of the mammalian POU family of transcription factor genes. OCT4 is expressed in both ESCs and embryonic carcinoma cells [22, 26, 27, 33] and is considered to be essential for pluripotency [20, 21]. Deletion of the OCT4 gene allows blastocyst formation but failure as regards pluripotency, which results in differentiation into a trophectodermal lineage [20]. OCT4 is expressed constitutively throughout the pre-implantation period [15].

NANOG is a more recently described homeodomain-bearing protein. It acts as a transcription factor and has been

described in mouse pluripotent cells [3, 18]. NANOG is expressed in a restricted number of cell types, and only in cells that also express OCT4, including ESCs [4]. In the mouse embryo, NANOG is localized in the centre of the morula and in the ICM of the blastocyst [3]. NANOG-induced self-renewal and NANOG function, but not NANOG expression, are dependent on continued OCT4 expression. OCT4 is required for NANOG-induced self-renewal [3, 17, 18]. Genetic deletion of *NANOG* in mouse ESCs resulted in the production of primitive endoderm-like cells, and *NANOG* mutant embryos were able to produce parietal endoderm. Since embryos lacking Oct4 are unable to develop so far this resulted in the assumption that NANOG function is critical during a later developmental stage than is the case for OCT4 [3, 18], and NANOG has been shown to be expressed from the 4-cell stage onwards [15]. Following implantation, when the ICM is developing into epiblast and primitive endoderm, NANOG is expressed in the epiblast cells [8].

The ability to visualize the expression of a gene in both time and space in human pre-embryos would be an essential tool in developmental biology. Whole-mount in situ hybridization has the advantage of showing the cellular location of specific mRNAs. However, to our knowledge, the method has not been used for studies on mRNA expression in human pre-embryos. Therefore, the aim of the present study was to optimise and use a novel whole-mount in situ hybridization method to determine the mRNA expression of NANOG in human embryos.

Materials and methods

Ovarian stimulation and in vitro fertilization

Down-regulation for ovarian hyperstimulation was achieved by using a long protocol gonadotrophin-releasing hormone agonist (GnRHa), nafarelin (Synarela; Syntex Nordica AB, Södertälje, Sweden) administered intra-nasally, starting on either cycle day 1 or 21. Following down-regulation, ovarian stimulation was induced using recombinant FSH (rFSH; Gonal-F, Serono Laboratories, Aubonne, Switzerland, or Puregon, NY Organon, Oss, the Netherlands). The starting dose was dependent on the subject's age and/or previous response to ovarian stimulation. Ovarian response was monitored by serum estradiol assays and vaginal ultrasound scans. GnRHa and rFSH were administered until the leading follicle had a diameter of at least 18 mm. Maturation of the oocyte was triggered by one s.c. injection of 10,000 IU of human chorionic gonadotrophin (hCG; Profasi, Serono laboratories, Aubonne, Switzerland). Thirty-seven hours after hCG administration, oocytes were retrieved by trans-vaginal needle aspiration under ultrasonographic guidance.

Conventional IVF was performed in 20-μl droplets of medium (IVF medium, Vitrolife AB, Gothenburg, Sweden) containing about 15,000 spermatozoa, under oil (Ovoil, Vitrolife AB, Gothenburg, Sweden).

For intracytoplasmic sperm injection (ICSI), oocytes were stripped of cumulus cells by mechanical pipetting after brief exposure to hyaluronidase (HYAS; Vitrolife AB, Gothenburg, Sweden). ICSI was then performed using a Nikon-Narishige micromanipulation system. Fertilization was evaluated 18–20 h after insemination. Following fertilization, IVF and ICSI embryos were cultured in 10-μl droplets of medium under oil (G.1.2; Vitrolife AB, Gothenburg, Sweden).

Embryo transfer was carried out either on day 2 or day 3. Excess embryos, surplus to treatment, were frozen at the 2- to 8-cell stage using a three-stage propanediol cryopreservation kit (Freeze kit 1; Vitrolife AB, Gothenburg, Sweden) according to the manufacturer's instructions. Embryos used for immunohistochemistry, $n=31$, and in situ hybridization, $n=89$, were cyopreserved and thawed after their 5-year storage limit in liquid nitrogen had passed. Frozen embryos were thawed using a thawing kit (Sydney IVF thawing kit, CooK IVF, Brisbane, Australia), and cultured to blastocysts in 10-μl droplets under oil in either BlastAssist System (Medicult, Jyllinge, Denmark) or blastocyst (Sidney IVF Blastocyst medium, CooK IVF, Brisbane, Australia) sequential media.

Human embryonic stem cell derivation and culture

The embryos used for embryonic stem cell culture had been donated on day 2 after fertilization after all embryos of good quality (minimum score of 2.0 out of 3.5 according to [19]) had been transferred or frozen. From an original score of 3.5, reductions of 0.5 at a time were made on the basis of the following features: more than 20 % cellular fragmentation, unequal size of blastomeres, multinuclear blastomeres, or the embryo did not fill the zona.

The blastocysts used for the present embryonic stem cell lines had been cultured in medium designed for blastocyst culture (MediCult, Ronnehavn, Denmark). Derivation of the present lines was carried out as described previously [13, 14]. Separation of the ICM from the trophectoderm cells was carried out by first removing the zona pellucida, using 0.5 % pronase (Sigma-Aldrich, Stockholm, Sweden). The trophectoderm was removed by immunosurgery as described earlier, using rabbit antihuman whole serum (Sigma) and guinea pig complement serum (Sigma-Aldrich, Stockholm, Sweden) [30]. The isolated ICMs were then placed on a feeder cell layer.

Human foreskin fibroblasts (CRL-2429; ATCC, Manassas, VA) were used as feeder cells. The feeder cells were mitotically inactivated using irradiation (35 Gy) and plated onto 2.84-cm^2 dishes to form a confluent monolayer to be

used as substrate cells the following day. 150,000 fibroblasts were plated for derivation of a new line, and for the passages to follow, 350,000 fibroblasts were plated. Iscove's medium (Gibco, Invitrogen, Stockholm, Sweden) supplemented (10 %) with FCS (Gibco, Invitrogen, Stockholm, Sweden) was used as culture medium.

The culture medium used for derivation and culture of hESCs consisted of Dulbecco's modified Eagle's medium (Gibco, Invitrogen, Stockholm, Sweden) supplemented (20 %) with SR medium (Knockout SR, Gibco, Invitrogen, Stockholm, Sweden), 2 mM L-glutamine (Gibco, Invitrogen, Stockholm, Sweden), 1 % penicillin-streptomycin (Gibco Invitrogen Corporation), 1 % nonessential amino acids (Gibco, Invitrogen, Stockholm, Sweden), 0.5 mM 2-mercaptoethanol, 1 % insulin-selenium-transferrin (Sigma-Aldrich, Stockholm, Sweden) and bFGF (8 ng/ml; R&H Systems, Oxon, U.K.).

After an initial growth period of 12 days, the cell aggregates were removed mechanically from the original plate and transferred to fresh feeder cells. Mechanical passage was performed by cutting the colony (approximately 2,000 μm in diameter) into eight pieces using a scalpel, under a stereomicroscope. Mechanical splitting was then carried out at 5- to 8-day intervals (mean, 7 days). Non-differentiated cells, as judged by morphology, were chosen for each further passage. The doubling time of the hESCs was approximately 24 h. In vivo pluripotency was tested in embryonic bodies and teratomas. The lines used in this study were HS181, HS235 and HS237. For cryopreservation of the cells, vitrification in pulled open straws, using ethylene glycol, dimethylsulphoxide (20 % each) and 1 M sucrose as cryoprotectants.

In situ hybridization of NANOG in human preimplantation embryos

A non-radioactive whole mount in situ hybridization method was used to detect the presence of NANOG mRNA in human preimplantation embryos. The experiment was performed on batches of ten or more embryos. The embryos were placed in culture inserts, which were thereafter transferred into wells that contained the different solutions. In the first step the embryos were washed twice in phosphate-buffered saline (PBS), and then fixed overnight at 4 °C in 4 % paraformaldehyde. After fixation the embryos were washed twice in PBS with 0.1 % Tween-20 (Sigma-Aldrich, Stockholm, Sweden) (PBT). Dehydration was performed through increasing concentrations of methanol in PBS (25 %, 50 %, 75 %) and finally twice in 100 % methanol. Rehydration was carried out through decreasing concentrations of methanol/PBS (75 %, 50 %, 25 %). The embryos were then rinsed three times with PBT followed by three washes in a detergent mix of 150 mM NaCl (Sigma-Aldrich, Stockholm, Sweden), 1 % Nonidet-P-40 (Sigma-Aldrich, Stockholm, Sweden), 0.5 % sodium deoxycholate (Sigma-Aldrich, Stockholm, Sweden), 0.1 % SDS (Sigma-Aldrich, Stockholm, Sweden), 1 mM EDTA (Sigma-Aldrich, Stockholm, Sweden) and 50 mM Tris (Sigma-Aldrich, Stockholm, Sweden), pH 8.0, at room temperature. Post-fixation was carried out in 4 % paraformaldehyde (Sigma-Aldrich, Stockholm, Sweden), 0.2 % EM-grade glutaraldehyde (Sigma-Aldrich, Stockholm, Sweden) in PBT at room temperature. Thereafter, the embryos were washed in PBT followed by a wash in a 1:1 mix of hybridization buffer (HB; 50 % ultra-pure formamide (Sigma-Aldrich, Stockholm, Sweden), 5 × SSC pH 4.5, heparin at 50 μg/ml, 0.1 % Tween 20) and PBT at room temperature. Prehybridization was then performed for 3 h at 70 °C in HB containing tRNA (100 μg/ml) and sheared denatured herring sperm DNA (Sigma-Aldrich, Stockholm, Sweden) at 100 μg/ml, followed by hybridization overnight at 70 °C in HB containing tRNA (100 μg/ml) and denatured digoxigenin-labelled riboprobe (100 μg/ml). Human NANOG ORF was subcloned from IMAGE clone 664153 using ATC TCG AGG CCG CCA CCA TGA GTG TGG ATC CAG CTT GTC C and ATG CGG CCG CTC ACA CGT CTT CAG GTT GCA TGT, subcloned into pCR2.1 and verified to be free of mutations. After linearization with *Xho* I, the probe was labelled with digoxigenin by transcription with SP6. After hybridization the embryos were washed once in a solution containing 2 × SSC, pH 4.5, 50 % formamide and 0.1 % Tween 20 at 70 °C, then twice at room temperature and finally three times at 65 °C. After cooling to room temperature and three washes in TBST (NaCl 8 g/l, KCl 0.2 g/l, 0.25 M Tris, pH 7.5, Tween-20 (1 %)), the embryos were incubated in blocking solution (10 % heat-inactivated sheep serum) for 1 h and thereafter incubated with anti-digoxigenin Fab alkaline phosphate conjugate (Roche, Stockholm, Sweden) in TBST with 1 % heat-inactivated sheep serum. This step was followed by antibody conjugate incubation overnight at 4 °C. The antibody conjugate was removed in a series of washes, first with TBST at room temperature and then with APB. The embryos were stained using Vectashield (Vectorlab Inc., Burlingame, USA) and the embryos were thereafter rinsed in PBS containing 1 M EDTA (Sigma-Aldrich, Stockholm, Sweden). Morula- and blastocyst-stage embryos incubated without the probe served as negative controls. A total of 67 embryos were used in this study.

In situ hybridization of NANOG in human embryonic stem cells

Embryonic stem cells were fixed on dishes together with feeder cells and processed in the same way as embryos. Mouse ESCs and human skin fibroblasts served as negative control cells.

Immunostaining of NANOG and OCT4 in human embryonic stem cells

Human embryonic stem cells were fixed with 4 % paraformaldehyde in PBS for 20 min at room temperature and thereafter washed with PBS and blocked with 5 % goat serum in PBS. Permeabilisation was carried out using blocking buffer consisting of 0.02 % TritonX-100 in PBS. Primary antibodies—monoclonal mouse antibodies for OCT4 (sc-5279, Santa Cruz Biotechnologies, Santa Cruz, USA), diluted 1:80, and monoclonal goat antibody for NANOG (MAB1997, R&D systems, Minneapolis, MN USA), diluted 1:200—were added in 5 % blocking buffer overnight at 4 °C and washed three times with PBS to remove any unbound primary antibodies. Secondary antibodies—FITC-conjugated goat anti-mouse IgG and FITC-conjugated bovine anti-goat IgG (both from Chemicon)—were diluted 1:200 in 5 % blocking buffer and applied to the cells for 60 min at RT in the dark. For controls, the primary antibodies were excluded from the staining protocol. Stained cells were viewed with a Zeiss Axiovert 200 M inverted microscope equipped with fluorescence optics and appropriate filters and images were acquired using Openlab 3.1.3 software. Human skin fibroblasts served as negative control cells.

Immunostaining of NANOG and OCT4 in human preimplantation embryos

Human preimplantation embryos were rapidly transferred from culture and briefly washed in PBS containing PVP (3 mg/ml) and then fixed in 2.5 % paraformaldehyde in PBS for 15 min at room temperature. Following fixation, the embryos were permeabilised in PBS/PVP buffer containing 0.25 % Triton X 100 for 30 min. The embryos were then placed in blocking buffer containing 0.1 % BSA and 0.01 % Tween 20 in PBS for 15 min. Primary antibodies—monoclonal mouse antibody for OCT4 (Santa Cruz Biotechnologies, Santa Cruz, USA), diluted 1:100, and monoclonal goat antibody for NANOG (R&D systems, Minneapolis, MN USA), diluted 1:100—were added in blocking buffer and incubated overnight at 4 °C. The embryos were then washed 3 times for 15 min each in blocking buffer to remove any unbound primary antibodies. The secondary antibodies—Alexa 568 conjugated rabbit anti-goat (1:250) and Alexa 488 conjugated rabbit anti-mouse (1:100) antibodies (Invitrogen, Stockholm, Sweden), respectively—were diluted in blocking buffer and applied to the embryos for 60 min at RT in the dark. For negative controls, the primary antibodies were excluded from the staining protocol. After incubation the embryos were briefly washed through a series of 25, 50, 75 and 100 % citifluor (with DAPI) and then mounted on slides in antifade medium under coverslips. The coverslips were then sealed with nail varnish. Stained embryos were viewed with an inverted microscope equipped with fluorescence optics and appropriate filters.

Data from gene array studies

Data from gene array studies performed at Karolinska Institutet [34] were used for comparison with data from the present study.

Ethical considerations

The pre-implantation embryos used in this study were donated with informed consent by couples undergoing in vitro fertilization treatment. Only pre-implantation embryos that could not be used in infertility treatment were used in the study. The human embryonic stem cells were derived from the inner cell masses of blastocysts that could not be used for infertility treatment. Approval was obtained from the Research Ethics Committee of Örebro University Hospital for expression studies in human pre-implantation embryos, and from the Ethics Board of Karolinska Institutet for derivation of hES cell lines, and studies regarding their properties. Both partners of the couples involved signed an informed consent form after receiving oral and written information [1]. No reimbursement was given to the couples.

Results

Presence of NANOG and OCT4 proteins in human pre-implantation embryos

Using immunohistochemistry, we observed immunostaining of NANOG and OCT4 in human pre-implantation embryos (n=31). This immunostaining was seen in an eccentrically located group of cells in the morula and in part of the ICM of blastocyst-stage embryos (Fig. 1a, b, c and d). Double staining showed that OCT4 and NANOG were present in the same cells of the ICM (Fig. 1d and e). There was also some staining of OCT4 in trophoblast cells, but no such staining as regards NANOG (Fig. 1d and e).

Expression of NANOG mRNA in human pre-implantation embryos

Expression of NANOG mRNA in human pre-implantation embryos was determined by using whole-mount in situ hybridization (n=89). NANOG mRNA was expressed in some cells of the compacted morula, with eccentric localization, and in the ICM of blastocyst-stage embryos (Fig. 2d–f). No expression was detected in 2-cell, 4-cell or 8-cell stages (Fig. 2a–c). In the blastocyst, NANOG mRNA was confined to the ICM and absent in the trophectoderm (Fig. 2f).

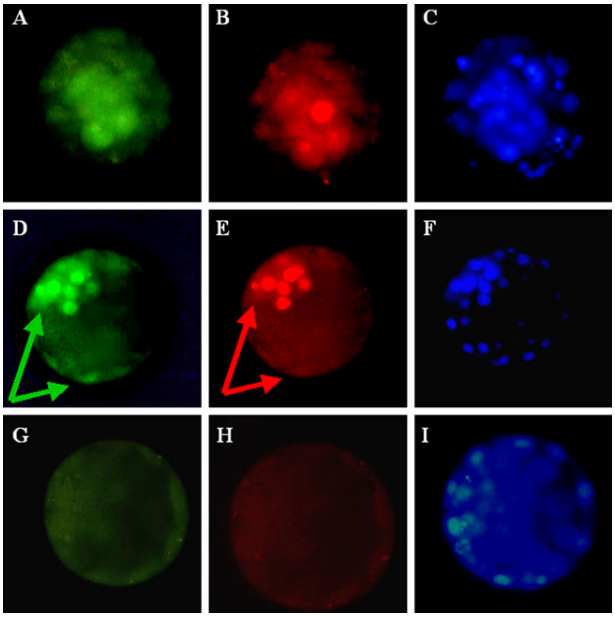

Fig. 1 Representative pictures show double staining of OCT4 and NANOG in the human morula and blastocyst. **a** Immunostaining of OCT4 in the morula. **b** Immunostaining of NANOG in the same morula. **c** DAPI staining in the morula. **d** Staining of OCT4 in the blastocyst. Immunostaining is seen both in the inner cell mass and the trophoblast; *arrows*. **e** Staining of NANOG in the same blastocyst. Staining is seen only in the inner cell mass; *arrow*. **f** DAPI staining in the same blastocyst. **g** Exclusion of OCT4 antibody. **h** Exclusion of NANOG antibody. **i** DAPI staining of the embryo without primary antibodies present

NANOG and OCT4 in human embryonic stem cells

Immunostaining showed the presence of NANOG and OCT4 proteins in all embryonic stem cells, although the staining was apparently weaker in the centre of the colony (Fig. 3b and g). Nuclear Hoechst DNA staining also showed the presence of feeder cells, which were negative for NANOG and OCT4 (Fig. 3c and g). NANOG mRNA was evenly expressed in all cells in the stem cell colonies. In situ hybridization showed NANOG mRNA in a colony of HS235 hESCs on human skin

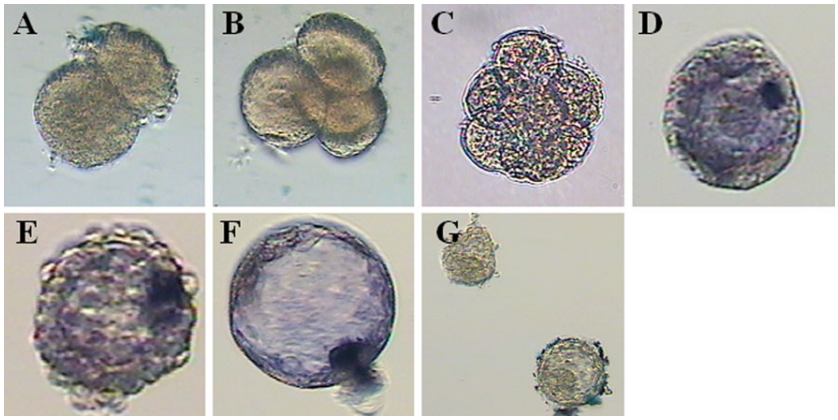

Fig. 2 In situ hybridization of NANOG in the human pre-implantation embryo. **a** 2-cell embryo, day 1. **b** 4-cell embryo, day 2. **c** 8-cell embryo, day 3. **d** morula, day 4. **e** late day 4 embryo. **f** blastocyst, day 5. **g** mouse ESCs hybridized with human NANOG primer

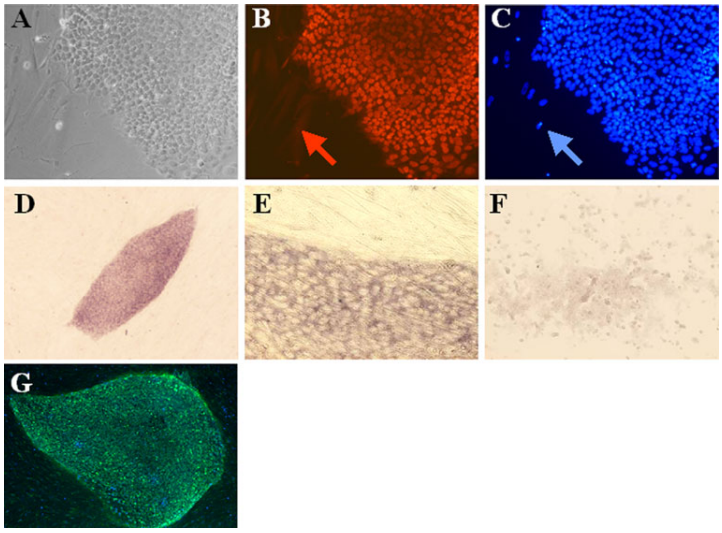

Fig. 3 Immunostaining of OCT4 and NANOG in human embryonic stem cells, and in situ hybridization of NANOG in embryonic stem cells. **a** Light microscopy of human embryonic stem cells. **b** NANOG immunostaining in embryonic stem cells. The feeder cells do not show staining for NANOG (*red arrow*). **c** Nuclear staining of embryonic stem cells and fibroblast cells. DAPI staining is seen in feeder cells (*blue arrow*). **d** & **e** In situ hybridization showing NANOG mRNA in embryonic stem cells. NANOG **f** Mouse embryonic stem cells hybridized with human NANOG primer. **g** OCT4 immunostaining of stem cell line HS426 (*green*). *Blue colour* shows nuclear DAPI staining

fibroblast feeder cells (Fig. 3d and e). Mouse stem cells did not show any staining after in situ hybridization with human NANOG probe (Fig. 1f).

Gene array data

Data from gene array studies showed that NANOG mRNA is present in pre-implantation embryos from day 2 and is highly expressed in hESCs (Table 1). OCT4 was weakly expressed in pre-implantation embryos and highly expressed in stem cells (Table 1). The two genes were absent in fibroblasts (Table 1).

Discussion

We localized the expression of NANOG and Oct4 in human pre-implantation embryos and in three human embryonic stem cell lines derived in our laboratory. The time of appearance (early morula), resembled that in mouse embryos [3, 12, 24, 25]. We found that the expression of NANOG in human compacted morula was not evenly distributed, but was strictly localized in the cells which apparently form the inner cell mass 1 day later. The expression of NANOG in human embryos seemed to be polarized earlier than in mouse embryos (see Fig. 2d and e and [3]).

The present study revealed NANOG and OCT4 protein and NANOG mRNA in the morula and blastocyst stages of human embryos. In mouse embryos, NANOG and OCT4 have also been seen in the morula to the hatched blastocyst stage [3, 12, 24, 25].

In the present study, some OCT4 immunostaining was also seen in the trophoblast cells of the blastocyst, although to a lesser extent than in the ICM. This has also been seen in bovine blastocysts [16, 32] and is in line with the results of a previous study showing the expression of mRNA for OCT4 in human trophoblast cells [9].

Additionally, we showed the expression of NANOG in human embryos during morula and blastocyst stages by using a whole-mount in situ hybridization technique. This method gave us the unique opportunity to visualize the expression of NANOG in both time and space in single human embryos. The protocol proved to be reliable and sensitive for visualization of NANOG gene expression in human embryos. This novel technique can be used to determine the precise expression

Table 1 Data from microarray analysis of human embryos and embryonic stem cells and fibroblasts. For Nanog, the average signal intensity from one probe set and for Oct4 the average number of three probe sets is shown

Gene	Signal intensity				
	Day 2 embryos	Day 3 embryos	Day 5 embryos	Stem cells	Fibroblasts
Nanog	98.6	194.6	196.8	391.8	1.3
Oct4	32.2	67.1	46.8	310.9	5.3

pattern of a gene of interest at various stages of development. Furthermore, it can be used to elucidate the genes and pathways involved in cellular processes such as differentiation, proliferation and apoptosis.

Differently from mouse embryos, the expression of NANOG was not centred at the middle of the morula. This confirms that the early human embryo is polarized at early stages, and our findings show clear polarization during the morula stage. The concept of polarization of the early embryo has been discussed in connection with the expression of hCG in early embryos [10, 11]. The distribution of mitochondria has been reported to be polarized in human oocytes and early embryos [31].

Embryonic stem cells can be derived from and reintroduced to the ICM, but this does not necessarily mean that these cells are exactly equivalent. The ICM exists only transiently and does not operate as a stem cell compartment in vivo. Stem cells might arise through selection and adaptation to the culture environment [2, 29]. Two transcription factors, Oct4 and NANOG, appear to define the potency of human and mouse ESCs. NANOG plays a fundamental role in maintaining pluripotential properties of embryonic stem cells. In a recent study [28], it was shown that ICM cells in *NANOG*-null embryos failed to develop into viable epiblast. NANOG Octamer and SOX elements, which control NANOG transcription, are able to recruit OCT4 and SOX2, respectively.

This results in upregulation of NANOG gene expression [33]. In another study it was shown that OCT4, NANOG and SOX2 were co-expressed in porcine epiblasts [5]. Human embryonic stem cells were stained positive both for NANOG and OCT4 [12]. Furthermore, Göke J et al. [7], observed that the combinatorial binding of OCT4, NANOG and SOX2 is critical for transcription in mouse and human ES. In the present study, we showed immunostaining for both NANOG and OCT4 using both immunohistochemistry and in situ hybridisation.

Whole-mount in situ hybridization has, as far as we know, not previously been used for detection of genes in human embryos. This technique permits the direct observation of the protein synthesis capacity of particular genes. Previously, the most commonly used method has been paraffin embedding and sectioning of embryos [23]. Whole-mount in situ hybridization provides a sensitive and powerful method for detection of expression patterns of genes within an individual preimplantation embryo.

Gene array data showed the presence of NANOG mRNA from D2 stage. This analysis was performed using an additional amplifying step before array analysis, showing that this method is more sensitive in detection of a gene compared with in situ hybridization. Recently it was shown that NANOG and OCT4 cDNA are present in 4- and 8-cell human embryos, expression being restricted to the ICM [6].

We showed that the expression profile of NANOG in humans resembles that in mice, suggesting that NANOG plays a physiological role in preimplantation development of the human embryo and it also provides a useful molecular marker of pluripotency. There is clear polarization of NANOG-expressing cells in human compacted morula to those cells which are probably defined to form the ICM.

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Leukaemia inhibitory factor receptor and gp130 in the human Fallopian tube and endometrium before and after mifepristone treatment and in the human preimplantation embryo

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Leukaemia inhibitory factor (LIF) is a cytokine, which is associated with reproductive processes such as embryo development and implantation. The objectives of this study were to detect the presence of LIF receptor (LIFR) and glycoprotein 130 (gp 130) in the human Fallopian tube, endometrium and preimplantation embryo and to study the effect of mifepristone on the expression of LIFR and gp130 in the Fallopian tube. Twenty-two healthy fertile women received a single dose of 200 mg mifepristone or placebo immediately after ovulation (LH+2). Biopsies were obtained from the Fallopian tubes during laparoscopic sterilization once between days LH+4 and LH+6 and from endometrium once between days LH+6 and LH+8. Preimplantation embryos were received from couples undergoing *in vitro* fertilization treatment. Immunohistochemistry was used to detect the presence of LIFR and gp130 in the Fallopian tube, endometrium and preimplantation embryo. Real-time PCR was used to study LIFR and gp130 expression in the Fallopian tube and endometrium. LIFR and gp130 were localized in the Fallopian tube, preimplantation embryo and endometrium. LIFR was more abundant in the Fallopian tube than in the endometrium. In the blastocyst, the staining of gp130 was mainly localized in the inner cell mass, whereas LIFR was expressed in all cells. The presence of LIFR and gp130 in the Fallopian tube and preimplantation embryo indicates a role for LIF in communication between the embryo and the Fallopian tube. Mifepristone did not affect the expression of LIFR and gp130 in the Fallopian tube, nor in the endometrium suggesting that progesterone might not be directly involved in the regulation of LIFR or gp130.

Keywords: embryo/endometrium/Fallopian tube/gp130/LIF receptor

Introduction

Normal microenvironment and function of the Fallopian tube are of vital importance for human reproduction. The densely ciliated fimbrial apparatus of the Fallopian tube picks up the ovum directly after ovulation. The ovum is thereafter transported into the ampulla, where it is fertilized (Pauerstein and Eddy, 1979). Following this, the embryo undergoes cleavage and development while being transported through the Fallopian tube, over a period of 3 days, after which it reaches the uterine cavity where it will hatch and implant (Croxatto *et al*., 1978).

Leukaemia Inhibitory Factor (LIF) is a cytokine, which regulates differentiation, proliferation and survival of various cells in the in the embryo as well as in the adult (Gearing, 1993), such as embryonic stem cells (Smith *et al*., 1992), primordial germ cells (Matsui *et al*., 1991) and peripheral neurons (Yamamori, 1992). LIF has been shown to be involved in a number of processes in reproduction such as enhancement of sperm motility and survival (Attar *et al*., 2003) and in the physiology of ovulation (Arici *et al*., 1997). LIF enhances blastocyst formation and hatching (Lavranos *et al*., 1995) and has been shown to be essential for implantation in mouse (Stewart *et al*., 1992). LIF also increases implantation rates in sheep (Fry *et al*., 1992). It is known that addition of LIF to the culture medium significantly increases blastocyst formation in human embryos (Dunglison *et al*., 1996).

The importance of LIF in human implantation is still uncertain. Infertile women show dysfunction in LIF production and it has been observed that infertile women have less LIF in uterine secretion than women with proven fertility (Laird *et al*., 1997; Hambartsoumian, 1998; Lass *et al*., 2001). However, the LIF content in uterine secretion at the time of oocyte retrieval does not seem to be correlated to pregnancy rate (Olivennes *et al*., 2003).

LIF exerts its biological effects by interaction through its specific LIF receptor (LIFR). The LIFR forms a high affinity heterodimer complex with glycoprotein 130 (gp130) (Robinson *et al*., 1994). The gp130 trans-membrane subunit can also, as a monomer, act as

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interleukin-6 (IL-6), interleukin-11 (IL-11) and ciliary neutrophic factor (CNTF) receptor (Auernhammer and Melmed, 2000).

Expression of mRNA activity for LIFR and gp130 has been demonstrated in human preimplantation embryos (Sharkey *et al*., 1995; Chen *et al*., 1999), but the localization of LIFR and gp130 in human preimplantation embryos has to our knowledge not been reported.

LIF is produced in the human Fallopian tube. The highest levels are seen in the luminal epithelium of the ampulla, where the embryo first develops before its transport into the uterine cavity for implantation (Keltz *et al*., 1996; Li *et al*., 2004). Co-culture of mouse embryos with human oviductal cells will result in maintained mitochondrial function, decreased apoptosis in the embryo and a higher degree of blastocyst formation and hatching (Xu *et al*., 2000, 2001, 2003). Cultured bovine oviductal cells synthesize LIF that conditions the embryo for implantation (Reinhart *et al*., 1998). LIFR and gp130 are also of great importance at later stages of embryogenesis and fetal development (Lavranos *et al*., 1995; Modric *et al*., 2000, Mitchell *et al*., 2002).

The expression of LIF and its receptor in human endometrium increases around the time of implantation and was demonstrated to appear simultaneously with pinopodes (Aghajanova *et al*., 2003). LIF is believed to be a regulatory factor essential for implantation of the blastocyst through autocrine and paracrine interaction between LIF and its receptor in the luminal epithelium (Cullinan *et al*., 1996; Cheng *et al*., 2002). Gp130 is most abundantly expressed at the time of implantation (Classen-Linke *et al*., 2004), when increased secretion of soluble gp130 was seen (Sherwin *et al*., 2002). LIFR and gp130 mRNA is also expressed in the decidua and may play a key role during the decidualization and placentation (Ni *et al*., 2002). It has been shown that the secretion of gp130 was reduced in infertile women (Sherwin *et al*., 2002).

Progesterone is essential for the development of endometrial receptivity. Treatment with a progesterone receptor antagonist, such as mifepristone, directly after ovulation reduces endometrial LIF expression at the expected time of implantation (Cameron *et al*., 1997; Danielsson *et al*., 1997). Mifepristone acts by competitive binding to the progesterone receptor. (Rauch *et al*., 1985; Teutsch *et al*., 1988). Treatment with mifepristone increases the expression of progesterone receptors (Christow *et al*., 2002; Sun *et al*., 2003) but has no effect on the expression LIF in the human Fallopian tube (Li *et al*., 2004).

Taken together LIF is an important cytokine in the female reproductive tract and in the preimplantation embryo. Still data on LIF and LIF receptor regulation is scarce and very little is known about possible communication between the embryo and the Fallopian tube. Therefore, the objective of the present study was to reveal the localization of LIFR and gp130 in the human Fallopian tube and preimplantation embryo and, in addition, to study the effect of mifepristone on these receptors in the human Fallopian tube and endometrium.

Materials and methods

Study subjects

Twenty-two healthy women (age 31–44 years) were included in the study. All women had proven fertility and had regular menstrual cycles (range 25–35 days). None of the women had taken any hormonal treatment or used an IUD for at least 3 months prior the study.

Collection of Fallopian tube tissue

Sixteen women were randomly allocated to receive treatment with a single dose of 200 mg mifepristone ($n = 8$) immediately after ovulation (LH+2) or to a control group ($n = 8$). Randomization was performed using opaque, numbered and sealed, envelopes. Laparoscopic sterilization was performed by placement of a silicone rubber ring over a segment of the isthmic part of the Fallopian tube (Yoon and King, 1975). The surgery was performed on day LH+4 to LH+6, which corresponds to the time of development and transport of the preembryo within the Fallopian tube. At surgery, biopsies were obtained from both Fallopian tubes. On one side biopsies were obtained from the isthmic part and on the other side from the ampullary part. The biopsies were immediately snap-frozen and stored in liquid nitrogen until analysed.

Collection of endometrial tissue

Endometrial biopsies were obtained from six healthy women during a control cycle on day LH+6 to LH+8, which corresponds to the assumed time of embryo implantation (Martel and Psychoyos, 1981; Nikas *et al*., 1995). An additional biopsy was obtained from the same women in the following cycle, after treatment with a single dose of 200 mg mifepristone immediately after ovulation (LH+2). The biopsies were obtained by curettage of the uterus using a Randall curette without prior dilatation of the cervix. Samples for immunohistochemistry were fixed in 4% formaldehyde for a maximum of 24 h and then stored in 70% ethanol until embedding. Samples for real-time PCR were snap-frozen and stored in liquid nitrogen until analysed.

Collection of human preimplantation embryos

Eighty human preimplantation embryos were received from patients undergoing *in vitro* fertilization (IVF) treatment. The preimplantation embryos were in all different stages from 2 cell embryos to hatching blastocysts.

Ethics

The study was approved by the local ethics committee at the Karolinska University Hospital/Karolinska Institutet. All women gave their written informed consent before entering the study. The embryos used in this study were donated by couples undergoing IVF treatment. Only embryos that could not be used for infertility treatment were used in the present study. Both partners in the couple signed an informed consent form after receiving oral and written information. No reimbursements were given to the couples.

Menstrual cycle monitoring

The day of the LH surge was estimated using a self-test detecting urinary LH (Clearplan, Searle Unipath, Bedford, UK) twice daily from cycle day 10 to the LH peak. In addition, all women collected daily urine during the cycle for analysis of estrone- and pregnanediol-glucuronide and LH using radioimmunoassay (Cekan *et al*., 1986).

Immunohistochemistry of Fallopian tube and endometrium

Biopsies from the Fallopian tube were mounted in an embedding medium (OTC Compound; Miles Inc., Elkhart, IN, USA) and serially sectioned to 9 μm using a Reichert-Jung Cryocut 1800 (Cambridge Instruments GmbH, Nussloch). The sections were mounted on glass slides and immersed in 2% paraformaldehyde in phosphate-buffered saline (PBS). The mounted sections were then wrapped in parafilm and stored in −70°C until use.

Paraffin embedded biopsies from the endometrium was sectioned to 4 μm and mounted on glass slides. The samples were thereafter deparaffinated in Bioclear (CiAB, Stockholm, Sweden) and rehydrated in decreasing concentrations of ethanol ending up in PBS.

All samples from the Fallopian tube and endometrium were hereafter treated in the same way. The samples were rinsed in PBS, incubated in darkness for 30 min in H_2O_2 (0.3 % in methanol) to block endogenous peroxidase activity and washed with PBS/BSA (Albumin, Bovine 0.05%). Hereafter the slides were blocked with 10% horse serum (in PBS/BSA) for 30 min. The sections were then incubated with the primary antibody, diluted 1:50 for LIFR and 1:25 for gp130, over night at 4°C. The primary antibody for LIFR was an affinity-purified goat polyclonal antibody (AF-249-NA, R&D Systems Inc.), raised against human LIFR. The antibody for gp130 was a goat polyclonal antibody (AF-228-NA, R&D Systems Inc.), raised against gp130 of human origin. For negative control, the primary antibody was replaced with non-immune serum of equivalent concentration from the same species. The slides were washed in PBS/BSA and thereafter incubated with the secondary antibody diluted 1:300 (horse anti-goat) for 30 min at room temperature. The slides

were then rinsed in PBS/BSA, prior to incubation with ABC complex (Vectastain Elite ABC immunoperoxidase detection system, Vector Laboratories Inc., Burlingame, CA, USA) according to the manufacturer's instructions. After washing with PBS/BSA, freshly prepared diaminobenzidine-hydrogen peroxide solution (DAB kit from Vector) was added to the slides, which were thereafter rinsed with distilled water. The slides were counterstained with 10% Mayer's Haematoxylin (VWR, Stockholm, Sweden), then washed in cold water and mounted with glycerol-gelatine.

Two persons evaluated the immunohistochemical staining independently, blinded to the identity of the samples. When the evaluation of the slides turned out differently, the average value was used. The staining was graded on a scale of 0 = no staining of cells, += faint staining, ++ = moderate staining and +++ = strong staining.

Immunohistochemistry of embryos

The embryos were rapidly transferred from culture and briefly washed in PBS containing 3 mg/ml polyvinylpyrrolidone (PVP) and then fixed with 2.5% paraformaldehyde in PBS for 15 min at room temperature. Following fixation, the embryos were permeabilized in PBS/PVP buffer containing 0.25% Triton X 100 for 30 min. Thereafter the embryos were placed in PBS blocking buffer containing 0.1% BSA and 0.01% Tween 20 for 15 min. The same primary antibodies as used for the endometrium and Fallopian tube were added in blocking buffer. For negative control, the primary antibody was replaced with non-immune serum of equivalent concentration from the same species. The embryos were incubated overnight at 4°C. The embryos were then washed three times for 15 min each in blocking buffer to remove any unbound primary antibodies. The secondary antibodies, Alexa 568 conjugated rabbit anti-goat (1:250), were diluted in blocking buffer and applied to the embryos for 60 min at RT in the dark. After incubation, the embryos were briefly washed through a series of 25, 50, 75 and 100% citifluor (with DAPI) and then mounted on slides in antifade medium under a cover slip. Stained embryos were viewed in an inverted microscope (Zeiss Axiovert 200M, Germany) equipped with fluorescence optics and appropriate filters.

RNA and cDNA preparation

Total RNA was isolated using SV Total RNA Isolation System (Promega Corp., Madison, WI, USA) according to the manufacturer's protocol. One microgram of each of the total RNA from each sample was reverse transcribed using deoxynucleotide triphosphates (10 mM each), random hexamer (250 ng/ml), ribonuclease inhibitor (40 U/μl) and Superscript reverse transcriptase (200 U/μl), using the Superscript™ II RNase H^- Reverse Transcriptase Kit (Invitrogen, Stockholm, Sweden).

Real-time PCR

Taqman real-time PCR (Applied Biosystems, Foster City, California, USA) was used to quantify the differential expression of LIFR and GP130 in the Fallopian tube ($n = 9$) and endometrium ($n = 4$).

Experiments were performed on a 96 well array format using the ABI PRISM 7300 instrument (Applied Biosystems) incorporating the target assay genes for LIFR or gp130 along with eukaryotic 18S rRNA as an internal control, which was used to normalize the expression levels of target genes in each sample. cDNA form human brain (Ambion, Austin, TX, USA) was used as positive control for the expression of both LIFR and GP130. Fluorogenic probes were 5′ labelled with 6-carboxyfluorescein (FAM) and 3′ labelled with MGB non-fluorescent quencher. Fluorescent signal detection used 'ROX' as the internal passive reference dye. The inoculum (25 μl) consists of 12.5 μl Taqman universal PCR master mix (Applied Biosystems), 1.25 μl assay probe (LIFR: Assay ID Hs00158730_m1, assay location bas 137 in the reference sequence NM_002310.3, exons 1–2. The length of the product is 63. GP130: Assay ID Hs00174360_m1, assay localization base 2016 in the reference sequence NM_175767.1, exons 13–14 and assay location base 2099 exons 14–15. The length of the product is 72, Applied Biosystems), 6.25 μl RNase/DNase free sterile water and 5 μl of diluted (1:2.5) cDNA. A standard curve was performed for both GP130 and LIFR using serial dilution of cDNA synthesized from endometrium, Fallopian tube and brain. After identifying the appropriate dilution for the respective samples, the assay was performed as follows: initial denaturation was carried out for 10 min at 95°C, followed by 40 cycles of denaturation at 95°C for 15 s and combined primer annealing/extension at 60°C for 1 min. All reactions were performed in triplicate and the mean value of the threshold cycle (Ct, the start of exponential amplification) of each sample was normalized with their respective threshold cycle of 18S, obtaining the ΔCt value. Data was analysed using SDS 1.2.3 software (Applied Biosystems). Relative expression was calculated using the following formula $100 \times 2^{-\Delta\Delta CT}$ as is described in User Bulletin number 2 from Applied Biosystems.

Statistics

Nonparametric statistical evaluation, ANOVA on ranks, was performed for differences in staining intensity for LIFR and gp130. Mann–Whitney Rank Sum Test was performed to compare the mRNA levels. A P-value of <0.05 was considered statistically significant.

Results

LIFR in the Fallopian tube

The most intense immunostaining was seen in the apical and basolateral side of the luminal epithelial cells (Fig. 1A), in vessels (Fig. 1B) and in the serosal epithelium of the Fallopian tube (Fig. 1C). There was no difference in staining intensity between the isthmic and the ampullary part of the Fallopian tube. Treatment with mifepristone did not affect the staining intensity in any compartment of the Fallopian tube (Figs. 1E–1G and 2A).

Gp130 in the Fallopian tube

Faint immunostaining for gp130 was seen in the luminal epithelium of the Fallopian tube predominately in the isthmic part (Fig. 1I). Similar immunostaining was also seen in the muscular wall, in some vessels (Fig. 1J) and in the serosal surface epithelium (Fig. 1K). Treatment with mifepristone did not affect the staining intensity in any compartment of the Fallopian tube (Figs. 1M–O and 2B).

LIFR in the endometrium

Moderate immunostaining for LIFR was seen in the luminal and glandular epithelium of the endometrium (Fig. 1Q and R). Some faint staining was also seen in the endometrial stroma and in some vessels (Fig. 1Q and S). There was no change in staining intensity after mifepristone treatment (Fig. 1S and T).

Gp130 in the endometrium

Moderate immunostaining for gp130 was seen in the luminal and glandular epithelium of the endometrium (Fig. 1V and W). There was no change in staining intensity after mifepristone treatment (Fig. 1X and Y).

Comparison of LIFR in Fallopian tube and endometrium

Taqman real-time PCR confirmed the presence of LIFR mRNA in the Fallopian tube (Fig. 3A). The relative mRNA expression of LIFR in the Fallopian tube was significantly higher than that of the endometrium, $P = 0.006$ (Fig. 3A).

Comparison of gp130 in the Fallopian tube and endometrium

Real-time PCR confirmed the presence of gp130 mRNA in the Fallopian tube (Fig. 3B). There was no significant difference in the relative expression between the mRNA levels of gp130 in the Fallopian tube compared with the endometrium, $P = 0.792$ (Fig. 3B).

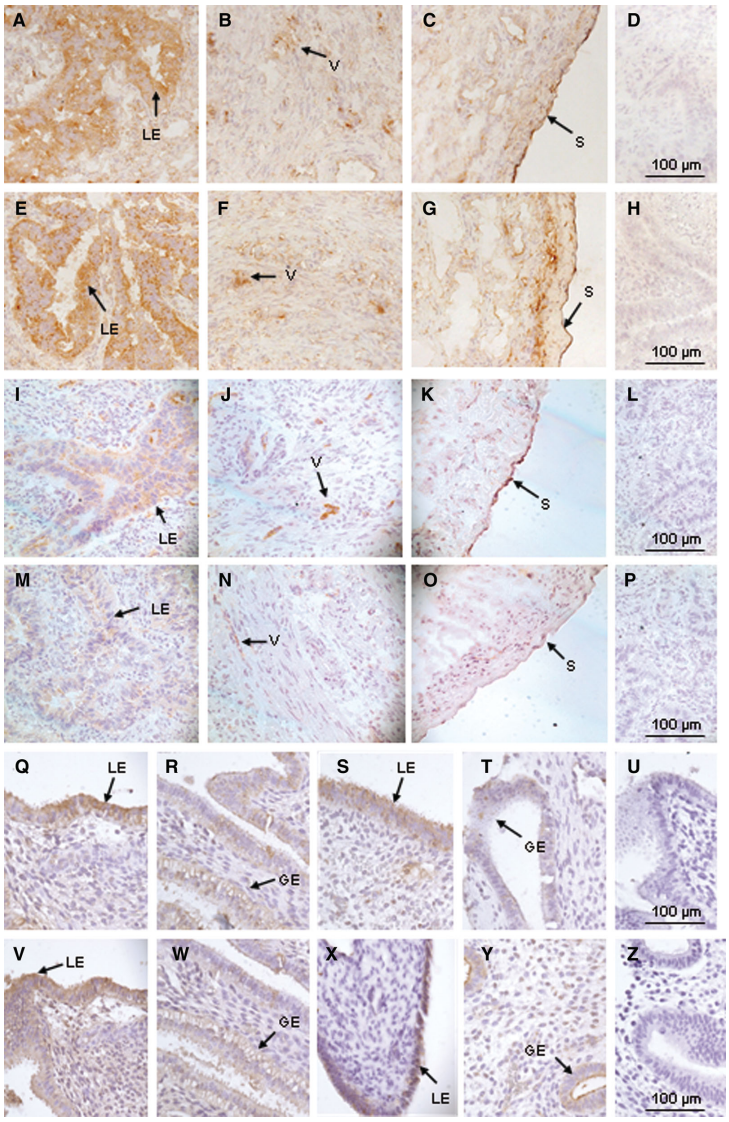

Figure 1: Representative pictures of immunostaining for LIFR and gp130 in the Fallopian tube (**A**–**P**) and endometrium (**Q**–**Z**) with and without mifepristone treatment are shown. (Arrows indicating staining in: LE, luminal epithelium; V, vessels; S, serosal epithelium; GE, glandular epithelium). (A) LIFR in luminal epithelium without treatment. (B) LIFR in muscular layer without treatment. (C) LIFR in serosal epithelium without treatment. (D) Negative control. (E) LIFR in luminal epithelium after mifepristone treatment. (F) LIFR in muscular layer after mifepristone treatment. (G) LIFR in serosal epithelium after mifepristone treatment. (H) Negative control. (I) gp130 in luminal epithelium without treatment. (J) gp130 in muscular layer without treatment. (K) gp130 in serosal epithelium without treatment. (L) Negative control. (M) gp130 in luminal epithelium after mifepristone treatment. (N) gp130 muscular layer after mifepristone treatment. (O) gp130 in serosal epithelium after mifepristone treatment. (P) Negative control. (Q) LIFR in luminal epithelium without treatment. (R) LIFR in glandular epithelium without treatment. (S) LIFR in luminal epithelium after mifepristone treatment. (T) LIFR in glandular epithelium after mifepristone treatment. (U) Negative control. (V) gp130 in luminal epithelium without treatment. (W) gp130 in glandular epithelium without treatment. (X) gp130 in luminal epithelium after mifepristone treatment. (Y) gp130 in glandular epithelium after mifepristone treatment. (Z) Negative control.

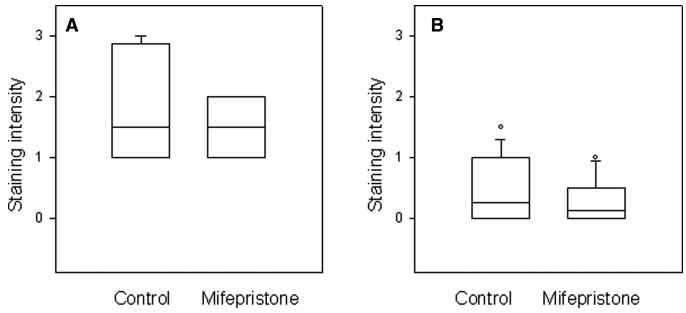

Figure 2: Staining intensity of LIFR and gp130 in the Fallopian tube. (**A**) Immunostaining of LIFR in the luminal epithelium of the Fallopian tube with and without mifepristone treatment. (**B**) Immunostaining of gp130 in the luminal epithelium of the Fallopian tube with and without mifepristone treatment.

LIFR in human preimplantation embryos

Fluorescence microscopy showed presence of LIFR in all preembryonic stages and in all cell types from the 2 cell stage to the expanded blastocysts (Fig. 4A–F).

Gp130 in human preimplantation embryos

Fluorescence microscopy showed staining of gp130 in the embryonic cells. Embryos up to the morula stage showed staining in all cells (Fig. 4H–K). In early blastocysts and in a hatching blastocyst, the gp130 staining was located to the inner cell mass (Fig. 4L and M).

Discussion

It is known that LIF is present in the human embryo (Chen *et al*., 1999; Sharkey *et al*., 1995) endometrium and Fallopian tube (Senturk and Arici, 1998). The present study shows the presence of LIFR and gp130 in the luminal epithelium of the Fallopian tube and in preimplantation embryos, suggesting paracrine interactions involving LIF between the developing embryo and the Fallopian tube in addition to autocrine interactions occurring within the developing embryo and the Fallopian tube.

Under the influence of progesterone there is a 'cross-talk' or exchange of signals between the developing embryo and the maternal tract to ensure synchronization between the blastocyst and the receptive endometrium and thus render implantation possible (Hombach-Klonisch *et al*., 2005). Progesterone is a key hormone in human embryo implantation (De Ziegler *et al*., 1994; Jabbour *et al*., 2006). In the endometrium, the highest levels of LIF and LIFR are seen during the midsecretory phase, when serum progesterone levels are high (Charnock-Jones *et al*., 1994; Kojima *et al*., 1994; Arici *et al*., 1995; Aghajanova *et al*., 2003).

Mifepristone is a potent antiprogestin that blocks the action of progesterone at the receptor level (Klein-Hitpass *et al*., 1991; Gemzell-Danielsson *et al*., 1993). Treatment with mifepristone has earlier been shown to increase the progesterone receptor level in the Fallopian tube (Sun *et al*., 2003). Administration of 200 mg mifepristone on day LH +2 has been shown to be a highly effective contraceptive method (Gemzell-Danielsson *et al*., 1993) probably acting mainly by adversely affecting endometrial receptivity (Gemzell-Danielsson and Hamberg, 1994). Although this regimen had no effect on LIF expression in the Fallopian tube on day LH +4 to +6, the same treatment resulted in significantly reduced expression of LIF in endometrial glandular epithelium on day LH +6 to +8 (Li *et al*., 2004). A daily low dose contraceptive regimen of mifepristone had similar

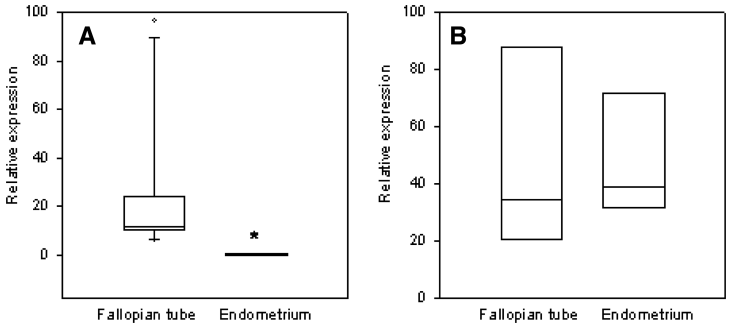

Figure 3: (**A**) Relative expression of LIFR mRNA in Fallopian tube compared with the relative expression of LIFR mRNA in the endometrium normalized to 18S rRNA expression ($P = 0.006$). (**B**) Relative expression of gp130 mRNA in Fallopian tube compared with the the relative expression of gp130 mRNA in the endometrium normalized to 18S rRNA expression (ns).

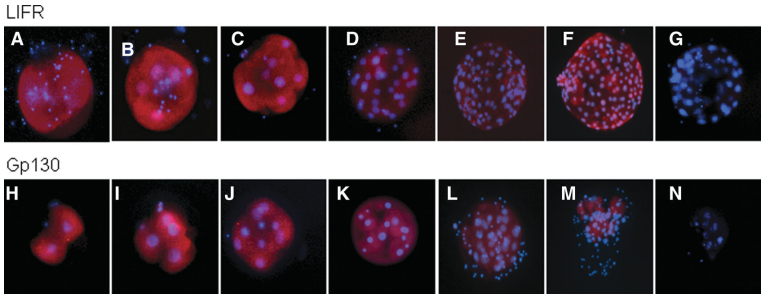

Figure 4: Immunostaining of LIFR and gp130 in human preimplantation embryos. Staining for LIFR: (**A**) 2 cell embryo (**B**) 4-cell embryo. (**C**) 8-cell embryo (**D**) Morula. (**E**) Early blastocyst. (**F**) Expanded blastocyst. (**G**) Negative control). Staining for gp130: (**H**) 2-cell embryo (**I**) 4-cell embryo. (**J**) 8-cell embryo (**K**) Morula. (**L**) Early blastocyst. (**M**) Staining of the inner cell mass in a hatching blastocyst. (**N**) Negative control.

effect on endometrial LIF expression at the expected time of receptivity (Cameron *et al*., 1997). The importance of LIF in endometrial receptivity and implantation is further supported by studies of cytokines in hydrosalpingeal fluid that show the presence of LIF in 50% of patients with hydrosalpinges (Strandell *et al*., 2004). Removal of hydrosalpinges in infertile patients resulted in increased endometrial LIF expression at the time of implantation (Seli *et al*., 2005) and improved results after IVF treatment (Strandell *et al*., 2001). Furthermore, in infertile women uterine flushings at the time of implantation demonstrated significantly lower amounts of LIF compared with fertile women (Laird *et al*., 1997). It has also been shown that the secretion of gp130 was lower in infertile women compared with fertile women (Sherwin *et al*., 2002).

In the present study, higher levels of LIFR mRNA were found in the Fallopian tube compared with the endometrium. The human embryo is known to produce LIF (Chen *et al*., 1999). The presence of LIFR in the Fallopian tube emphasise the importance of LIF for the tubal microenvironment. The present study shows that gp130 is located to the inner cell mass in the hatching blastocyst, whereas LIFR did not seem to be localized to a specific cell type. Therefore, is could be suggested that the targets of LIF produced by the blastocyst is the cells in the inner cell mass or the endometrium rather than autocrine/paracrine interactions with the cells in the trophoblast. The mechanism in the Fallopian tube might be different, where LIF produced by the embryo acts both within the embryo and with the Fallopian tube in an auto- and paracrine manner. This might explain why there are higher levels of LIFR in the Fallopian tube than in the endometrium. In contrast, gp130 expression showed no difference between The Fallopian tube and endometrium. This may be due to the fact that gp130 alone binds other cytokines such as IL6, which is known to be produced by the human embryo (Austgulen *et al*., 1995).

LIFR and gp130 did not change after mifepristone treatment. This indicates that progesterone is not the main regulator of LIFR and gp130 in the Fallopian tube and endometrium. In immortalized endometrial epithelial cells, LIF, LIFR and gp130 were unaffected after oestrogen and/or progesterone treatment (Hombach-Klonisch *et al*., 2005). This was also seen in the western spotted skunk, where prolactin, rather than progesterone, had an effect on the LIFR expression (Passavant *et al*., 2000). However, in endometrial epithelial and in endometrial decidual cells, a significant increase in secretion of soluble gp130 was observed after estradiol/progesterone treatment (Classen-Linke *et al*., 2004).

In conclusion, we found expression of LIFR and gp130 in the human Fallopian tube where it may facilitate the paracrine interaction with the embryo during its transport into the uterus. Furthermore, the staining pattern of LIFR and gp130 in the human preimplantation embryo suggests an embryonic regulation of the communication through LIFR and gp130. The communication through LIFR between the preembryo and the Fallopian tube might be important for the embryo development, and /or establishment of pregnancy. In addition, we conclude that progesterone is not likely to be a direct regulator of these receptors.

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