The Histidine-rich Glycoprotein in Reproduction

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Abstract

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Infertility affects 15% of reproductive-aged couples. The milieu surrounding the growing embryo is of outmost importance, and should be optimised during *in vitro* fertilisation (IVF). Many biological processes, such as angiogenesis, coagulation, and immune processes need to be well regulated for a pregnancy to occur and progress normally. Histidine-rich glycoprotein (HRG) is a plasma protein that regulates components of these systems by building complexes with various ligands. A single nucleotide polymorphism (SNP) in *HRG,* denoted *HRG* C633T, seem to be of importance for IVF treatment outcomes. The aim of this thesis was to further investigate the proposed human fertility effects of the *HRG* C633T SNP.

According to the findings of this thesis, the *HRG* C633T genotype is associated with primary recurrent miscarriage. Male *HRG* C633T genotype is associated with semen characteristics in infertile men, and pregnancy rates following IVF. However, the distribution of the *HRG* C633T SNP does not differ between infertile and fertile couples.

We further examined the role of the region surrounding the *HRG* C633T SNP for regulation of endometrial angiogenesis and human embryo development. The region affects primary endometrial endothelial cell migration, proliferation and tube-formation *in vitro* but does not appear to affect human embryo development. No effect of the HRG peptide was noted on the secretome of human embryos. However, early embryos secrete proteins into the surrounding culture media and the level of secretion of VEGF-A, IL-6, EMMPRIN and PlGF is greater in embryos of higher developmental stages.

In conclusion, the *HRG* C633T genotype appears to play a role only if infertility is established. The region surrounding *HRG* C633T SNP is of relevance *in vitro* for regulation of human endometrial endothelial cell angiogenesis. To predict which embryos to transfer in IVF, we have highlighted a number of proteins of interest for further investigation.

Keywords: Angiogenesis, embryo culture medium, embryogenesis, embryonic secretome, endometrium, histidine-rich glycoprotein, human embryo development, human embryo implantation, human endometrial endothelial cells, in vitro fertilisation, infertility, male infertility, proximity extension assay, recurrent miscarriage, single nucleotide polymorphism, sperm quality, time-lapse technique, vascular endothelial growth factor

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To my family

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I   Lindgren, K.E., Kårehed, K., Karypidis, H., Hosseini, F., Bremme, K., Landgren, B-M., Skjöldebrand-Sparre, L., Stavreus-Evers, A., Sundström-Poromaa, I., Åkerud, H. Histidine-rich glycoprotein gene polymorphism in patients with recurrent miscarriage. *Acta Obstet Gynecol Scand,* 2013 92(8):974–977.

II   Lindgren, K.E., Hreinsson, J., Helmestam, M., Wånggren, K., Sundström-Poromaa, I., Kårehed, K., Åkerud, H. Histidine-rich glycoprotein derived peptides affect endometrial angiogenesis in vitro but has no effect on embryo development. *Syst Biol Reprod Med,* 2016 62(3):192-200.

III   Lindgren, K.E., Nordqvist, S., Kårehed, K., Sundström-Poromaa, I., Åkerud, H. The effect of a specific histidine-rich glycoprotein polymorphism on male infertility and semen parameters. *Reprod Biomed Online,* 2016 May 16. [Epub ahead of print]

IV   Lindgren, K.E., Gülen Yaldir, F., Hreinsson., J, Holte, J., Kårehed, K., Sundström-Poromaa, I., Åkerud, H. (2016) Spent culture medium from single human embryos; differential secretomics between blastocysts and arrested embryos. *Revised manuscript submitted.*

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Contents

Abbreviations

FGF	Fibroblast growth factor
BMI	Body mass index
CASP3	Caspase 3
C/C	Homozygous for the *HRG* C633 SNP
C/T	Heterozygous for the *HRG* C633T SNP
ECM	Extracellular matrix
EMMPRIN	Extracellular matrix metalloproteinase inducer
ET	Embryo transfer
FSH	Follicle stimulating hormone
hCG	Human chorionic gonadotropin
HEE	Human endometrial endothelial
HE4	Epididymal secretory protein E4
HRG	Histidine-rich glycoprotein
HRR	Histidine-rich region
ICM	Inner cell mass
ICSI	Intracytoplasmic sperm injection
IL	Interleukin
IVF	*In vitro* fertilization
LH	Luteinizing hormone
PG	Prostaglandin
PlGF	Placental growth factor
PRR	Proline-rich region
SNP	Single nucleotide polymorphism
TSP	Thrombospondin
T/T	Homozygous for the *HRG* 633T SNP
VEGF	Vascular endothelial growth factor

Introduction

Reproduction is central to our lives as humans, and fertility is thereby of outmost importance for both men and women. Fertility is the natural capacity to give life and requires the production of a viable oocyte, its transportation through the Fallopian tube, and its fertilization by a viable spermatozoa; the resulting zygote must then enter the uterus and implant into a suitably prepared endometrial lining. Implantation initiates the development of the placenta which is essential for nutrient uptake, waste elimination, and gas exchange via the mother's blood supply.[1] A defect in any of these steps will result in infertility.

Infertility

Infertility is defined as failure to conceive after regular unprotected sexual intercourse for one year.[2] This definition reflects the knowledge that, in the general population, the conception rate (e.g. the chance of a pregnancy the first month for a couple having unprotected intercourse at ovulation) is approximately 20%.[3] More than 60% of couples are expected to conceive within the first six months, 80-90% within a year and 90-95% within two years.[4] It is estimated that infertility affects 15% of reproductive-aged couples.[5] The cause of infertility can be due to a female or male factor, a combination of both, or remain unexplained (Figure 1).[6]

Causes of infertility

Female infertility

Ovulatory dysfunction accounts for approximately17% of infertility[6] and up to 40% of female infertility. Menstrual disturbances like oligomenorrhea and amenorrhea are common features, but ovulatory dysfunction can also be less obvious. The most common reasons for ovulatory dysfunction is polycystic ovary syndrome, obesity, underweight, excessive exercise, thyroid dysfunction and hyperprolactinemia.[5]
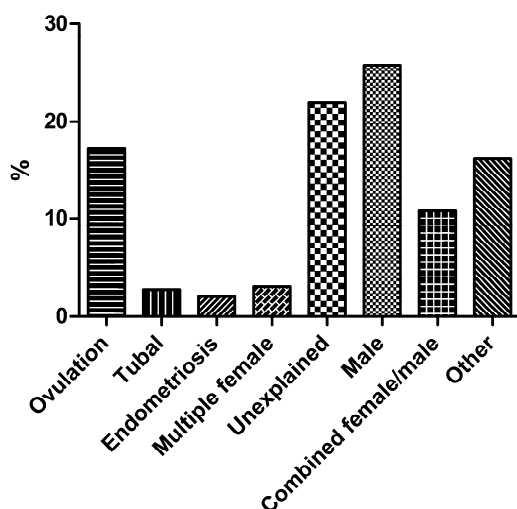
Figure 1. Causes of infertility.[6]

Tubal factors accounts for at least 25% of female infertility. Risk factors for tubal impairment include previous pelvic inflammatory disease, previous ectopic pregnancy, endometriosis and previous pelvic surgery.[7] The infertility is due to complete or partial obstruction or impaired movement through the Fallopian tubes, which may prevent oocyte pick-up and transportation of the oocyte, embryo, or spermatozoa.[1]

Endometriosis is a condition where endometrial-like tissue grows outside the uterine cavity in the ovary, oviduct or peritoneal cavity. It is considered a multifactorial disease that interferes with reproduction in several ways.[8] It is estimated that endometriosis may account for more than 17% of female infertility and the prevalence in the general population is approximately10%.[9] It is estimated that endometriosis reduces the chance of a pregnancy down to 2-10% per menstrual cycle.[10]

Male infertility

Male factors account for approximately 26% of infertility cases.[6] Male infertility is often associated with abnormal semen analysis, but can also be idiopathic with a normal semen analysis.[11] The World Health Organization (WHO) laboratory manual from 2010 established reference values for semen characteristics to guide diagnosis and treatment (Table 1).[12] Primary testicular failure is the most common cause of male infertility.[13] Other causes include systemic diseases, infections, chromosome disorders, trauma, birth defects, smoking,[14] obesity,[15] and age[16]. All of these factors can contribute to an abnormal sperm number, morphology, function and/or impaired potency.[13] The cause for an abnormal sperm sample is unknown in about 26% of infertile men.[13] It is estimated that more than 10% of spermatogenic

impairment is explained by genetic defects,[17, 18] and there is a growing literature on the genetic reasons for male infertility.[17, 19-23] For instance, it is well known that both autosomal as well as sex chromosome genes are involved in the complex regulation of spermatogenesis.[24, 25] However, also non-syndromic autosomal gene defects may result in male infertility, with mutations in the *SPATA16*, *PICK1* and *DPY192L2* genes and single nucleotide polymorphisms (SNP) in the *PRMT6*, *PEX10* and *SOX5* genes being associated with sperm defects or spermatogenesis.[19] Male autosomal chromosome variation, i.e. differences in size or staining of chromosome segments, has been negatively associated with fertilization rate[26] and clinical pregnancy rate following IVF,[24, 25] whereas such findings in women had no influence. In addition, a polymorphism in the paternal or maternal *Methylenetetrahydrofolate reductase* gene was associated with recurrent pregnancy loss.[27,28] With the rapid DNA sequencing technologies, it is anticipated that genome wide analyses might help to identify "hidden" genetic factors behind idiopathic male infertility.[18]

Table 1. World Health Organization lower reference limits (5th centiles and their 95% confidence intervals) for human semen characteristics

Parameter	Lower reference limit
Semen volume (ml)	1.5 (1.4 – 1.7)
Total sperm number (10^6 per ejaculate)	39 (33 – 46)
Sperm concentration (10^6 per ml)	15 (12 – 16)
Total motility (%)	40 (38 – 42)
Progressive motility (%)	32 (31 – 34)
Vitality (live spermatozoa, %)	58 (55 – 63)
Sperm morphology (normal forms, %)	4 (3.0 – 4.0)

Adapted from WHO.12

Unexplained infertility

In approximately 22% of infertile couples the cause remains unexplained.[6] The incidence of unexplained infertility depends on the women's age and criteria for selection in the group under study.[13] The diagnosis is given when the standard fertility evaluation is normal in both the man and the woman, and depends on the number, nature and quality of the tests used, and the interpretations made.[13] Thus, this group may include missed cases of endometriosis, premature ovarian ageing, and tubal factor infertility.[29] The underlying cause of unexplained infertility is most probably heterogeneous and the factors involved are likely to be of endocrine, immunological and genetic origin.[30] While it is quite common for these couples to conceive spontaneously, natural conception is not likely after three years without success.[13, 31] The spontaneous pregnancy rate within one year is estimated to be 13%-15%,[32, 33] but these rates rely to a great extent on the age of the women and the duration of infertility.[13]

Many couples have several diagnoses and about 11% have a combination of female and male factors. Finally, a group of infertile couples will end up having a diagnosis denoted as "other", Table 1. This group accounts for about 16% of couples and includes infertility due to cervical factor (7.6%), uterine factor (0.6%), sexual disorder (0.4%, i.e. male and female sexual problems), no diagnosis (3.2%, i.e. incomplete evaluation) and other diagnoses (4.3%).[6]

Recurrent miscarriage

Sporadic miscarriage is common, occurring in about 15% - 25%[34,35] of clinically recognised pregnancies. The number of sporadic miscarriages before being recognized clinically is even higher.[34] Recurrent miscarriage or recurrent pregnancy loss is a heterogeneous reproductive problem, with multiple aetiologies and contributing factors. Less than 5% of women experience two consecutive miscarriages, and only about 1% have three or more consecutive miscarriages.[36] A recent study demonstrated that the time to a live birth in couples with a diagnosis of unexplained recurrent miscarriage was significantly longer among those with three or more previous miscarriages than among those with only two previous miscarriages.[37] Recurrent miscarriage can be subdivided into primary recurrent miscarriage and secondary recurrent miscarriage, potentially reducing the heterogeneity of this population.[38] About 40% of women with recurrent miscarriage have delivered a child before the consecutive miscarriages and therefore have secondary recurrent miscarriage. The mechanism behind recurrent miscarriage is most often unknown[39] and there is no therapeutic strategy available to treat recurrent miscarriage.[40] After investigation, the underlying causes might be determined in about half of the cases.[41-43] Well known factors that predispose for recurrent miscarriage are maternal age, parental and foetal chromosomal aberrations, uterine abnormalities, as well as immunological, endocrine and thrombophilic disorders.[44] Unlike sporadic miscarriage, recurrent miscarriage tends to occur largely even when the foetus has a normal karyotype.[45, 46] Studies on the products of conception from couples presenting with two consecutive miscarriages revealed that 57% of embryos remain non-euploid, and among these couples, the screening is less likely to find a cause for the miscarriages.[47] Gene polymorphisms have been proposed as possible explanations to the increased risk of pregnancy loss in the group of women with recurrent miscarriage.[48-50]

Standard fertility investigation

The diagnostic evaluation is started after failure to conceive during one year or more of regular unprotected sexual intercourse. If the woman is over the age of 35 the investigation should start earlier.[5, 51] During the investigation the reproductive and medical history of both partners is obtained. This includes but is not limited to: duration of infertility and results from previous investigations and treatments, the woman's menstrual history, obstetric history, frequency of intercourse and sexual dysfunction, previous surgery, previous genital infections, systemic illnesses, family history of reproductive problems, current medications and possible exposure to environmental hazards.[5, 51] It is important to know if the female partner or a close relative suffers from thyroid disease since this can reduce fertility, increase the risk of miscarriage and affect the outcome of IVF treatment.[51] A physical examination of the female partner should include but is not limited to: body mass index (BMI), signs of androgen excess, a gynaecological examination and ultrasound scan of ovaries, Fallopian tubes and the uterus.[5, 51] Tests to assure that the woman is ovulating include either luteal phase progesterone serum concentrations or urine luteinising hormone (LH).[52] Tests on the ovarian reserve include but are not limited to: the level of anti-Müllerian hormone in blood and ultrasound-guided antral follicle count. The investigation of the male partner should include analysis of a minimum of one semen sample, according to the clinical reference ranges outlined by the WHO (Table 1), for sperm concentration, motility and morphology.[12] If the test result is abnormal a new sample should be collected around ten weeks later.[52] When the initial investigation of the male partner shows an abnormal reproductive history or detects an abnormal semen test the next step is a thorough investigation performed by a specialist in male reproduction.[5, 51]

Couples presenting with recurrent miscarriage undergo a thorough investigation including: screening of the female partner for antiphospholipid antibodies and inherited thrombophilias, pelvic ultrasound, karyotyping of both partners and hormonal tests (luteal phase progesterone, LH, thyroid hormones and androgens).[52, 53]

In vitro fertilization

In vitro fertilization (IVF) remains the treatment of choice in long-standing unresolved infertility and many infertile couples are helped by this technique. Since the first successful IVF treatment was carried out by Edwards and Steptoe in 1978 in the UK,[54] this technique is now practiced in most countries of the world. Until the end of 2013, more than five million babies were born with help of IVF.[55]

IVF and the human embryo

Controlled ovarian hyperstimulation

Controlled ovarian hyperstimulation (COH) is used to induce the ovaries to produce several mature oocytes. For this purpose, daily injections of gonadotropins are used for approximately two weeks, starting on cycle day 2-3. To prevent spontaneous ovulation a gonadotropin releasing hormone agonist or antagonist are used. A gonadotropin releasing hormone agonist is often administered as a spray begun two weeks before the start of injections and usually continued until induction of ovulation. A gonadotropin releasing hormone antagonist is given as daily injections usually starting on treatment day 5. When several oocytes have matured and the leading follicles are in correct size, monitored by vaginal ultrasound, ovulation is induced with a human chorionic gonadotropin (hCG) injection, which mimics the LH peak. Ovum pick-up (OPU) is performed 35-38 h after the hCG injection by use of vaginal ultrasound-guided needle punctures. The oocytes are placed in culture medium and transferred to an incubator.

The choice of fertilization technique depends on the male partner's semen sample analysis and the ability of sperms to fertilize, if any previous treatments have been performed. Table 1 displays the lower reference values for semen characteristics as established by the WHO. In standard IVF prepared sperm are added to the culture medium containing the oocytes and spontaneous fertilization may occur. Intracytoplasmic sperm injection (ICSI) is used when the couple has male factor infertility or if the fertility rates in previous IVF treatments were low. The principle of ICSI is injection of a single sperm directly into the oocyte. When fertilization is confirmed, the zygote(s) are cultured between two (cleavage stage embryo) to five (blastocyst stage) days in the incubator before embryo transfer (ET) into the uterus. The quality of the embryos is accessed mainly using morphological criteria for cleavage stage embryos[56] and blastocysts[56-58] and the highest quality embryo or blastocyst is chosen for ET. The remaining good quality embryos are cryopreserved. After a maximum of five years of cryopreservation the embryos have to be destroyed according to Swedish law. Couples are approached around three months prior to this, and informed that their embryos soon reach the time-limit for cryopreservation. The live birth rate today is about 40% per transfer for both fresh and frozen-thawed embryos.[59]

Oocyte maturation, embryogenesis and implantation

Successful embryo development depends upon a cascade of events. The ovaries cycle between the follicular phase, ovulation and the luteal phase and are the site for storage, maturation and release of oocytes. At the start of each menstrual cycle, about 7-10 early antral follicles in each ovary are recruited for development, mainly by FSH. Out of these, usually only one become dominant and will be ovulated. The oocyte remains arrested in meiotic

prophase until just before ovulation, when it completes the first meiotic division and enters the second. After ovulation the remaining follicle makes up the corpus luteum, a transient endocrine tissue, which produces progesterone to support a possible pregnancy. The oocyte is caught by the fimbria of the Fallopian tube and transported to the ampulla-isthmus junction where it meets up with spermatozoa to be fertilized and completes the second meiotic division.

The process of spermatogenesis that leads up to mature spermatozoa, capable of fertilizing the oocyte, takes place in the testis and epididymis. This process is dependent on gonadotrophins and several hormones produced by the Leydig and Sertoli cells of the testis. The testis is the site of production and the epididymis is the subsequent site for maturation and storage of the sperm. It is estimated that 1000 sperms are produced per heartbeat.[1]

The fusion of an oocyte and spermatozoa is initially followed by a brief period of transcriptional silence in the zygote. During this period (until day three), maternally inherited mRNA and proteins drive the first cell cycles of mitotic division.[60, 61] In the early cleavage stage the glucose consumption and metabolism in embryos are low, with pyruvate providing the main source of energy.[62] At the four- to eight-cell stage, the embryonic genome is activated and most of the maternal transcripts rapidly disappear and are replaced by novel transcripts. At this stage, a transit from pyruvate to glucose as the metabolic substrate takes place. The embryo synthesizes proteins already at the four-cell stage with a significant increase at around the eight-cell stage, which will continue throughout compaction. Already at the 6-10-cell stages the embryo starts the process of compaction and the cells (or blastomeres) get tightly connected by tight junctions to eventually form the morula. Up until this stage the embryo remains in the Fallopian tube. The next step is cavitation and expansion at the 16-32-cell stages during which water moves into the central of the embryo to form a cavity filled with fluid; the blastocoele. On day five the embryo has reached the blastocyst stage and consists of an inner cell mass (ICM) of pluripotent cells that will form the foetus, and an outer layer of trophectoderm (TE) cells that together with maternal tissues will give rise to the placenta.

During the transition from morula to blastocyst the embryo enters the uterine cavity.[63] The main function of the uterus is to provide an implantation site for the developing foetus. Pre-implantation embryos produce soluble ligands and receptors for various autocrine and paracrine interactions that support the needs of the growing embryo and render the receptive endometrium "aware" of the embryo.[64, 65] On day six of development the blastocyst is fully expanded and hatches through its glycoprotein shell; the zona pellucida.[63] In the uterine cavity the free blastocyst is surrounded by uterine secretions that provide the oxygen and nutrients needed for further development.[1] The blastocyst will now start the process of implantation into the uterine wall; the endometrium.

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The endometrium is a hormonally regulated tissue designed to provide a base for the blastocyst to implant. It cycles between the menstrual, proliferative and secretory phases. It contains a superficial luminary epithelial layer to which the blastocyst can adhere. Around the time of implantation the stromal layer underneath the epithelium is rich in high resistance and low capacity vessels (called spiral arteries) and secretory glands. The human endometrium is not receptive to blastocyst implantation at all times.

The period of receptivity is under the influence of oestrogen and progesterone and takes place in the secretory phase of the menstrual cycle[65] (6-10 days post ovulation) and lasts for approximately 4-5 days.[63, 66] During this time window a number of physiological changes take place in order to create a proper environment for the competent blastocyst. In addition to the hormonal regulation, successful implantation requires an intricate molecular cross-talk between a competent blastocyst and a receptive endometrium. The main players involved in this cross-talk are hormones, cytokines, chemokines, growth factors, adhesion molecules, extracellular matrix (ECM) proteins, and prostaglandins (PGs).[67]

When the blastocyst has hatched, the increased physical exposure of the TE to the luminary epithelium of the endometrium enables improved signalling, towards apposition and endometrial binding.[64] To facilitate close apposition of the blastocyst and the endometrium, two main structural changes occur. First the luminary epithelial lining of the endometrium is covered with pinopodes that absorb much of the fluid in the cavity,[1] secondly the anti-adhesion molecule mucin is removed from the surface.[68] During apposition, soluble mediators produced and received in a bi-directional fashion are responsible for this dialogue between the blastocyst and the endometrium. The luminary epithelium seems to secrete molecules such as interleukin-6 (IL-6) and C-X-C motif chemokine 13 (CXCL13), which attract the blastocyst. During adhesion, the interaction is mediated by factors bound to the respective membranes, and require direct contact.[69] Integrins, present on both the endometrium and blastocyst, are necessary for adhesion and invasion. Osteopontin, a protein of the ECM, interacts with integrins on both parties to facilitate connection between the implanting embryo and the endometrium. The TE of the blastocyst secrets hCG before implantation,[70] and hCG transcripts are found already in the 6-8-cell embryo.[71] This hormone has multiple roles during implantation and maintenance of pregnancy and among them is the regulation of proteins involved in blastocyst adhesion[63] and trophoblast invasion.[70]

Following adhesion, matrix metalloproteinases start to degrade the underlying compartments to allow for the embryonic trophoblasts to invade.[1] The invading embryo can take up the primary metabolic substrates that are released from the cells that are destroyed around it. It also utilizes nutrition secreted from the uterine glands situated side by side with the implantation site. These types of nutritional strategies are continued until the placenta

takes over this function after week 10. PGs are produced that promote invasion and play a major role in implantation.[1] In addition, vascular changes are necessary for the endometrium to be receptive, and during the invasion the spiral arteries are converted from their high resistance and low capacity state into low resistance and high-capacity vessels, and angiogenesis occurs. This improves the oxygen and nutrient exchange and embryo development.[72, 73] In addition to the PGs[1] that take part in these processes several members of the placental growth factor (PlGF) family are involved.[1, 73] PlGF also takes part in regulating the invasion of trophoblasts.[72] The human embryo invades deeply into the stroma and reaches the uterine vessels; the surface epithelium of the uterus is restored above it, embedding the embryo.[1] Disturbances in this cross-talk during implantation are thought to explain why many IVF treatments are unsuccessful even though high quality embryos are transferred.[65]

Choosing the best embryo for transfer

Since the first IVF baby, Louise Brown, was born in 1978[74] the technique has improved greatly. The goal with IVF today is to perform single embryo transfers and to improve success rates. To obtain these goals, there is a need to improve the tools by which the most viable embryo is chosen. Obviously, the ultimate end-point of such strategies is live-birth rate.

Morphology

Morphological evaluation of embryos has been the method for embryo selection since the beginning of IVF and is still the primary basis of embryo assessment.[75] Traditionally, embryologists evaluated embryo quality by removing them from a conventional incubator every day for a quick observation in the light microscope. Scoring systems for both cleavage[76] and blastocyst stage[57] embryos have been around for more than three decades. The system developed by Gardner and Schoolcraft in 1999[57] is a well-recognised method for grading of embryos prior to transfer. Blastocysts are given a numerical score from 1 to 6 based on their degree of expansion and hatching status (Table 2). For full blastocysts (i.e. grades 3-6) the development of the ICM and TE is assessed. A numerical interpretation of this system was later adopted.[56] The major limitations with this system are their static nature and the subjectivity of the observer.[75]

Table 2. Blastocyst score based on morphology

Scoring of 1-6 or 1-4 based on degree of expansion and hatching status

1 (1) = blastocyst cavity less than half the volume of the embryo
2 (1) = blastocyst cavity half of or greater than half the volume of the embryo
3 (2) = full blastocyst with blastocyst cavity completely filling the embryo
4 (3) = expanded blastocyst with larger blastocyst cavity, thinning zona
5 (4) = hatching blastocyst with trophectoderm starting to herniate though the zona
6 (4) = hatched blastocyst, blastocyst has completely escaped from the zona.

Scoring of A-C or 1-3 for the ICM and TE
ICM
A (1) = tightly packed, many cells
B (2) = loosely grouped, several cells
C (3) = very few cells
TE
A (1) = many cells forming a cohesive epithelium
B (2) = few cells forming a loose epithelium
C (3) = very few and large cells

ICM: inner cell mass; TE: trophectoderm. Adapted from Gardner and Schoolcraft.[57]

Time-lapse technique

In recent years, systems that incorporate a microscope and image acquisition have been developed. This allows embryologists, with or without the assistance of computer algorithms, to evaluate the quality of the embryos without removing them from the incubator. The time-lapse system takes photos of the growing embryos with 5-15 minute intervals. The benefits of time-lapse systems include the maintenance of a stable uninterrupted culture environment and the possibility to gain additional information about embryo morphology and time to different milestones in development. The rate of cell divisions is considered the most important factor of embryo viability, where too fast or too slow rates are negatively associated with implantation rates.[56] Many researchers, in their quest to access embryo viability, have focused on a combination of morphology and kinetics (morphokinetics) by the use of time-lapse evaluation.[77, 78] While the static nature of standard morphological evaluation is overcome by use of time-lapse technique,[75] the subjectivity of the observer is still a problem that remains.[79, 80]

Biomarkers

Technologies within the "omics" field such as genomics, transcriptomics, proteomics and metabolomics all have the potential to identify new biomarkers for reproductive success.[81]

The majority of biologic cell functions are carried out by proteins. Proteomics can be used to generate informative protein profiles that, when positively associated with pregnancy outcome, can help develop molecular strategies to select the best oocytes, spermatozoa and embryos to use in IVF

treatment.[82] Differences in protein expression in relation to development and viability of early embryo have been described, but also differences in protein expression between embryos of similar morphological quality.[75, 83] It is suggested that viable embryos possess a unique proteome and that some of these proteins are secreted.[84] Instead of cellular extraction for assessment of the proteome, the protein secretion into the surrounding culture media (the secretome) can be analysed.

Dominguez et al[85] studied the secretome of embryos that implanted versus those that did not. They found that granulocyte-macrophage colony stimulating factor and CXCL13 were present at lower levels in the culture medium of embryos that implanted, suggesting these proteins had been consumed. Recently the authors developed an algorithm that combined secretomics data with time-lapse technique to access embryo implantation potential.[67] They found that presence of IL-6 (secreted by the blastocyst), in combination with a certain duration of the second cell cycle, resulted in the highest implantation rates.

Whether hCG and different isoforms thereof can be used as markers of early embryo viability is debated. However, a recent report concluded that intact hCG is not released from the blastocyst until the time of hatching, and that a large proportion is hyperglycosylated hCG. Detection of hyperglycosylated hCG is indicative of invasion and may suggest a role with potential implantation.[86, 87]

Soluble human leukocyte antigen G has also been hypothesized to play a role during the initial stages of implantation, and a recent multi-centre study found a positive association between presence of soluble human leukocyte antigen G in the culture medium surrounding the embryo, and successful pregnancy outcome.[88]

The challenge with these technologies is the extremely low amount of proteins present in a medium sample, and therefore the methods used for protein quantification need to be very sensitive.[80] Advances have been made in this field with emerging assays for ultrasensitive protein analysis, that make use of nanomaterials or DNA-mediated proximity.[89] To meet the demands of proteomic analysis it is desirable that an assay can detect a whole panel of proteins.[89, 90]

The Histidine-rich glycoprotein

The Histidine-rich Glycoprotein (HRG, also denoted HRGP) was first isolated in 1972.[91] It is a multi-domain glycoprotein which is synthesized mainly by the liver[92] and the concentration in plasma is100-150 μg/ml.[93] The protein is transported as a free protein but it is also stored in the α-granules of platelets and released upon thrombin activation.[94] The concentration of HRG in plasma varies throughout life. The level of HRG in a newborn is

approximately 20% of adult levels of HRG in plasma and by the first year of age it reaches adult levels.[95] The level then continue to increase in a linear manner throughout life.[96]

Molecular structure

HRG is composed of 525 amino acids (507 amino acids without its signal peptide) that form three domains: an N-terminal domain with two cystatin-like regions (N1 and N2), a central histidine-rich region (HRR) and a C-terminal domain (C) which together forms the multi-domain structure of the protein (Figure 2).[97] The N1, N2 and C-terminal domains are highly conserved between species.[98, 99] The histidine-rich region has a proline-rich region (PRR1 and PRR2) on each side of it. HRG is highly glycosylated with several predicted glycosylation sites.[93, 100] It has five, or possibly six, disulphide bridges; two internal and three to four intra-domain bridges.[93, 99] HRG is negatively charged under physiological pH but becomes positively charged by light acidic environments and in the presence of divalent cations like Zn^{2+},[93, 101] which also induces a conformational change.[93, 102]
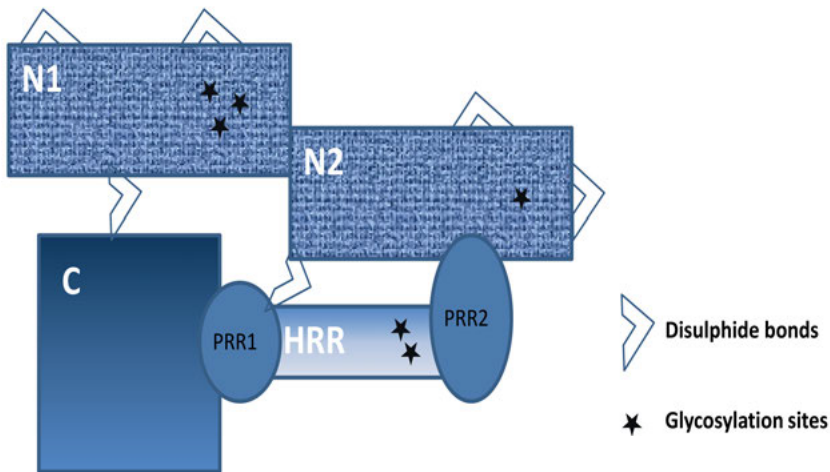
Figure 2. Adapted from Jones et al 2005.[93]

The HRG gene

The HRG gene is located on chromosome 3 and contains seven exons and six introns. There are ten naturally occurring variants within the gene,[97] corresponding to SNPs. In the *HRG* C633T SNP, located to the fifth exon, is a cytosine (C) nucleotide replaced by a thymidine (T) at position 633 in the mRNA sequence. The result is a change of amino acids from a proline to a serine at position 204 in the protein (in some publications denoted at position 186 without its 18 amino acid signal peptide). Individuals can be homozy-

gous for the *HRG* C633 SNP (C/C) and produce only the HRG protein with proline at amino acid 204; heterozygous for the *HRG* C633T SNP (C/T) and produce both HRG with proline and HRG with serine; or homozygous for the *HRG* 633T (T/T) and produce only the HRG protein with serine at amino acid position 204.

The proline containing variant of HRG has a molecular weight of 75kDa. The serine containing variant has an extra glycosylation site at amino acid position 202, which adds 2kDa in weight to this protein. Adjacent to this additional glycosylation and the polymorphic site (amino acid position 203) is one of the potential inter-domain disulfide bridges, that links over to the PRR2.[93]

The allele frequency of *HRG* C633T in a European population is 0.67 for the C-allele (proline) and 0.33 for the T-allele (serine), and the genotype frequency is 45%, 44% and 11% for the CC, C/T and TT genotypes, respectively. In other parts of the world, the T-allele is often the major allele. For instance, in populations from Africa and South Asia the reported frequencies of the T-allele are in the range of 0.5-0.7 (http://www.ensembl.org/ Homo_sapiens, rs9898, population genetics). The HRG protein that contains a proline in position 204 is unique in humans since other species, including primates, only have the HRG protein containing a serine in amino acid position 204.[103]

Function

HRG is an adaptor protein that binds to a number of different ligands.[93, 104] It is involved in regulation of many biological processes, including angiogenesis, blood coagulation, fibrinolysis and in the immune system. HRG has furthermore been shown to interact with the ability of different cell types to proliferate,[105, 106] migrate and adhere by either inducing or inhibiting these biological processes depending on the cell type and location.[93] Low pH and elevated levels of free Zn^{2+} often enhance the binding capacity of HRG to different ligands, providing a regulatory mechanism for the function of HRG.[93] These conditions are usually found during tissue injury, tumour growth, inflammation and angiogenesis.[107] A recent study, using a new technique for purifying plasmatic HRG, confirms that HRG function as an adaptor molecule involved in multiprotein complexes.[108]

Angiogenesis

Angiogenesis means formation of new blood vessels from pre-existing ones. Growth factors with promoting effects on endothelial cell angiogenesis are fibroblast growth factors (FGFs), vascular endothelial growth factors (VEGFs) and PlGF.[109, 110] Angiogenesis involve proliferation, migration and tube formation.[109] Physiological angiogenesis takes place in the formation of the corpus luteum, in the endometrium during the menstrual cycle and dur-

ing embryo implantation and placentation.[109] HRG has been reported to be both pro- and anti-angiogenic[104] owing to its multi-domain structure and the activities of a proteolytically released fragment from the HRR/PRR.[111-113]

There are a number of different ways in which HRG can act in favour of angiogenesis. Thrombospondins (TSPs) are potent inhibitors of angiogenesis that, when binding their receptor CD36,[114] inhibits the response of a number of different growth factors, including FGF-2 and VEGF.[115] HRG contains two CD36 homology domains (or CLESH-1 domains, in the N2 domain and C-terminal domain) that bind to TSP with high affinity, thereby blocking its anti-angiogenic activity.[93, 116] HRG can also reverse the inhibition of angiogenesis mediated by vasculostatin, probably mediated via its CD36 homology domain (in the C-terminal domain).[117] HRG further acts in favour of angiogenesis by tethering plasminogen or plasmin to the surface of cells, potentiating directed endothelial cell migration and invasion.[107] HRG can make FGFs available for responsive cells by displacing them from ECM or the basal membrane.[105, 118]

A proteolytic fragment from the HRR/PRR is responsible for most of the anti-angiogenic effects that HRG exert on growth factor-stimulated endothelial cells.[111] The three-dimensional structure of rabbit HRG was recently solved by Kassaar and colleagues.[98] The structure revealed a glutathione adduct at amino acid 203, close to the *HRG* C633T SNP*, that provides an explanation as to how the disulphide bridge over to the HRR/PRR fragment is reduced. When the disulphide bridge is cleaved and the fragment is released from HRG, it can rearrange focal adhesions and decrease attachment of primary endothelial cells, resulting in reduced migration.[113] A 35 amino acid peptide (HRGP330) was determined as the minimal active domain of the anti-angiogenic fragment.[112] A tumour mice-model treated with the HRR/PRR fragment displayed reduced angiogenesis and tumour volume.[113] An additional study reported reduced vascularity in a tumour mice-model after treatment with HRGP330.[112]

Heparin in complex with growth factors like VEGF, FGF2 and high mobility group box 1, induces angiogenesis. HRG can inhibit heparin-dependent angiogenesis.[118] Angiogenic growth factors are released from heparan sulphate in the ECM by heparanase cleavage. HRG can prevent this release by masking the cleavage site for heparanase on heparan sulphate.[119] HRG further competes with FGF for binding to heparan sulphate in the ECM, reducing their stimulatory effect.[105]

Immune system

The foetal-maternal immune tolerance is of great importance during implantation. An aberrant immune response and an imbalanced cytokine network may cause infertility, implantation failure after IVF, and recurrent pregnancy losses.[120] HRG is known to interact with components of the immune system in several ways. It prevents the formation of immune com-

plexes and enhances immune complex solubility and clearance by binding to IgG.[93, 121] As reviewed by Poon et al, HRG may play either a positive or a negative role in regulating the adhesion molecules used by immune cells to communicate with other cells and to migrate to inflammatory sites.[104] Further, HRG is antimicrobial under acidic conditions or when Zn^{2+} is present,[122] and HRG deficient mice are more prone to suffer from bacterial infections.[123, 124] Finally, HRG is also effective against gram positive- and negative bacteria,[125] and acts as an antifungal compound.[124]

Coagulation and fibrinolysis

HRG has been shown to interact with coagulation as well as the fibrinolytic systems.[104] HRGs anti-coagulant ability was demonstrated in a *HRG* knockout mice model, where *HRG* deficient mice had a shorter activated partial thromboplastin time and lowered thrombin generation.[126] The anti-coagulant activity is influenced by the *HRG* C633T SNP in populations of European ancestry, where the minor T-allele has an additive effect, with each T-allele decreasing the activated partial thromboplastin time.[127-130] HRG interferes with the binding of plasminogen to fibrin in clots, thereby reducing fibrinolysis.[131] On the other hand, HRG can tether plasminogen to the surface of cells,[107] thus enabling plasmin generated degradation of ECM components and directed migration and invasion of cells.

HRG in reproduction

Although HRG is known to be involved in regulation of angiogenesis, coagulation and fibrinolysis as well as in the immune system, little is known about the exact role of HRG in fertility and embryo development, implantation and placentation.

HRG is present in the female reproductive tract (Fallopian tubes, endometrium and myometrium) and in the placenta.[132] It is also present in follicular fluid surrounding the growing follicles.[132] It was quite recently discovered that human embryos, already at the 4-cell stage, produce HRG and that this production is continued up till the blastocyst stage.[132] HRG is found in almost all cells of the ICM and TE. Furthermore embryos secrete HRG into the surrounding media during culture.[132] The impact of HRG in the male reproductive system has not been studied but by using mass-spectrometry, a draft of the human proteome located HRG to the testis, prostate and seminal vesicles.[133, 134] The antibody-based Human Protein Atlas demonstrate low to moderate staining for HRG in the testis (seminiferous ducts and Leydig cells) and prostate but no expression in cells of the epididymis and seminal vesicles.[135] Furthermore, HRG is one among the more than 6000 proteins detected in the human sperm proteome.[136, 137]

Both endogenous and synthetic estrogens seem to reduce the level of HRG in plasma in a dose-dependent manner.[138-140] As the levels of estrogens

rise during pregnancy, the level of HRG starts declining in the second trimester. By the time of delivery, HRG plasma concentrations are only 50% of normal non-pregnant levels.[139] Two weeks following delivery, the HRG level has returned to normal.[141] Women with early-onset preeclampsia seem to have an imbalance between placental HRG and fibrinogen, which potentially may influence the hypercoagulability and the angiogenic imbalance seen in the pre-eclamptic placenta.[142] Furthermore, the level of HRG in plasma is lower in women who later develop preeclampsia compared to women who stay healthy throughout their pregnancy.[143]

The *HRG* C633T SNP seems to be associated with fertility. Among women undergoing IVF, the pregnancy rate was lower than expected in individuals homozygous for the T-allele.[144] Moreover, this SNP seems to be important for the ovarian reserve, where women homozygous for the T-allele obtained the lowest number of oocytes, including mature oocytes, despite a higher total amount of FSH administered. This group of women also had the lowest degree of fertilization, and their embryos were to a greater extent non-matured, meaning that they were not possible to transfer.[145] Another SNP in *HRG* (*HRG* A1042G) has been associated with recurrent miscarriage. This SNP is situated in the proteolytic fragment that is released from the HRR/PRR, known to have anti-angiogenic effects.[111, 113]

Aims

The general aim of this thesis was to investigate the proposed human fertility effects of the *HRG* C633T SNP, by achieving the following specific research objectives:

I   To investigate if the *HRG* 633T SNP is associated with primary -and secondary recurrent miscarriage.

II   To investigate the role of the region surrounding the *HRG* C633T SNP in the regulation of endometrial angiogenesis and human embryo development.

III   To investigate if male *HRG* C633T genotype is associated with semen characteristics and pregnancy rate among couples undergoing IVF. A secondary aim was to investigate if the distribution of the *HRG* C633T SNP is different between infertile and fertile couples.

IV   To investigate the secretome of human embryos. A secondary aim was to compare the secretome from embryos cultured with or without the supplementation of a HRG proline peptide.

Materials and methods

Ethical approval
The studies included in this thesis were approved by the Regional Ethics Review Board in Uppsala, Sweden and/or the Regional Ethics Review Board in Stockholm, Sweden. Written informed consent was obtained from all participants.

Paper I

Subjects and methods

Subjects
Women with a previous history of miscarriages (n = 187) were identified in the out-patient registers of four university hospitals between April 29, 2009 and June 30, 2010. The inclusion criteria were: a verified diagnosis of recurrent miscarriage between the years of 1989-2009, defined as three or more consecutive miscarriages in the first or second trimester of pregnancy (5-21 completed weeks of gestation) and ability of the patient to understand Swedish or English. The exclusion criteria for the cases were: known risk factors for recurrent miscarriage, such as systemic lupus erythematosus, diabetes mellitus type 1, severe thrombophilia and major chromosomal aberrations. Among the cases 43 had primary recurrent miscarriage and 144 had secondary recurrent miscarriage. The median follow-up time for the whole group of cases was 9 (range 3-24) years. In order to study recurrent miscarriage as a reason for sub-fertility we specifically investigated the subgroup of women with primary recurrent miscarriage. The women attended a brief health examination including measurements of weight and height and answered standardized questions on reproductive history. Data was obtained from the medical records.

The control group (n = 395) were matched for age at first planned pregnancy and were randomly chosen from the Uppsala University Hospital biobank of pregnant women. Since 31 May 2007, all women aged 18 and older attending the second trimester (16–19 weeks of gestation) routine ultrasound scan at Uppsala University Hospital have been approached for inclusion in this biobank, and the last day for inclusion to this study was June 30, 2010.

The inclusion criteria were: spontaneous pregnancy resulting in a term birth ($\geq$ 37 weeks) of a live baby, and the ability to understand Swedish or English. The exclusion criteria were: a history of miscarriage or the use of assisted reproductive techniques. Among women included as controls, 75.7% had two or more spontaneous pregnancies resulting in a live birth. The women attended a brief health examination including measurements of weight and height and answered standardized questions on reproductive history. Data was obtained from the medical records.

Cases and controls were an ethnically mixed group where 94.7% of cases and 95.4% of controls were of Caucasian origin.

Blood sampling, DNA extraction and genotyping

Blood samples were collected in EDTA-containing tubes and centrifuged at 1500 g for 10 min. Plasma and buffy coat were separated, transferred to new tubes and stored at -20°C. Genomic DNA was extracted from buffy coat using QIAamp DNA Blood Maxi kits (Qiagen, Venlo, the Netherlands).

The samples were genotyped for the *HRG* C633T SNP (rs9898), using the TaqMan SNP Genotyping Assay and run with the StepOnePlus real-time polymerase chain reaction (RT-PCR) system (Life Technologies Inc.). Briefly, PCR reactions were performed using TaqMan Universal PCR Master Mix and TaqMan primers and probes for genotyping (Applied Biosystems, US), and according to the manufacturer's protocol. For every reaction, 10ng of genomic DNA was used. Real-time fluorescence detection was performed. StepOne™ Software (Life Technologies Inc.) was used to plot fluorescence (R_n) values based on the signals from each well. The plotted fluorescence signals indicated which alleles were present in each sample.

Paper II and Paper IV

Human endometrial endothelial cells

Human endometrial endothelial (HEE) cells were isolated from endometrial tissue biopsies. Premenopausal women with regular menstrual cycles, who underwent hysterectomy for benign medical conditions at Uppsala University Hospital, Sweden, were included. No hormonal treatment had been used for at least three months prior to surgery and they were all non-smokers.

At the surgical ward, the endometrial tissue biopsies were immediately put in cold phosphate buffered saline (PBS) and transported to the laboratory. The tissues were mechanically minced, digested with enzymes and filtered. A single cell suspension was formed and this was mixed with magnetic beads coated with antibodies against the endothelial cell-surface specific antigen CD31. HEE cells were isolated from the suspension with the help of a magnetic holder, and cells not bound to the beads were discarded.

This step was repeated several times to obtain a pure fraction of HEE cells that were suspended in basal endothelial cell medium (ECM) supplemented with foetal bovine serum (FBS) and endothelial cell growth supplements (ScienCell™, US) and seeded. Sub-culturing was done through trypsination and cells in passage 2-4 were used. The magnetic beads were lost after two passages.

Expression of Thrombospondins in HEE cells

HEE cells that had been cultured in ECM (5% FBS) for 48h, were washed with PBS and harvested in lysis buffer. Total RNA was isolated according to the RNeasy Mini Kit spin protocol (Qiagen AB, Sweden). Only high quality RNA with no signs of degradation was used. Complementary DNA was synthesized from total RNA with Superscript III First Strand Synthesis (Invitrogen, US) according to the manufacturer's protocol.

Quantitative real-time PCR (qRT-PCR) analysis was performed with a Step One Plus qRT-PCR System (Applied Biosystems, US). TaqMan Gene Expression Master Mix and TaqMan primers and probes for gene expression (Applied Biosystems, US) were used according to the manufacturer's protocol. For every reaction, 2ng of complementary DNA was used. Genes of interest were *TSP1* and *TSP2*. Hypoxanthine phosphoribosyl-transferase 1 (*HPRT1*) was used to determine standard expression levels.[146] The mean efficiency was determined for each primer with the LinRegPCR software[147, 148] and was used to calculate the mean efficiency adjusted cycle threshold.[149] Each gene was analysed in triplicate and with cells from five patients.

HRG peptides

Peptides containing 35 amino-acids each, with sequences corresponding to the proline-containing polymorphism (NH2-RGGEGTGYFVDFSVRNCPRHHFPRHPNVFGFCRAD-COOH) and the serine containing-polymorphism (NH2-RGGEGTGYFVDFSVRNCSRHHFPRHPNVFGFCRAD-COOH) of HRG (amino acid position 187-221), were constructed by standard Fmoc peptide synthesis, Innovagen (Lund, Sweden). As a control, a peptide with a scrambled version of the amino-acid sequence for the HRG proline peptide was also constructed (NH2-FGRYEDHGHFGPHVFRCNDPFVRRTGPVRGFCSNA-COOH). The peptides were suspended in sterile water and stored in aliquots in -70ºC until used. The peptides were always used at a concentration of 100ng/ml.

Migration assay

The migration assay was performed in a modified Boyden chamber. HEE cells were cultured in starvation medium (ECM containing 0.2% FBS) overnight, and added to the upper compartment of the Boyden chamber. The lower chamber was filled with ECM medium supplemented with VEGF-A

(10 ng/ml, Sigma-Aldrich, US), either of the HRG proline or serine peptides, or a combination of VEGF-A and HRG proline or serine peptides. HEE cells in basal ECM were used as negative control. Between the compartments was a filter coated with collagen solution. After 5 h at 37°C the number of cells that had migrated through the filter was counted. Each condition was analysed in triplicate and with cells from three women in total.

Proliferation assay

HEE cells were seeded and after 2 h the medium was changed to starvation medium (ECM containing 0.2% FBS) containing the HRG proline or serine peptide, VEGF-A (10 ng/ml) or a combination of the HRG proline or serine peptides and VEGF-A. HEE cells in starvation media was used as negative control. Medium was changed again on day two and four. Cell numbers were counted in a Coulter counter (Beckman Coulter Inc., Sweden) after five days in culture. Each condition was analysed in triplicate and cells from five patients were used.

Tube formation assay

Tube formation assays were performed as described previously.[150] HEE cell suspension was mixed with the HRG proline or serine peptide or VEGF-A (50 ng/ml), and added on top of a gelled Geltrex™ LDEV-Free Reduced Growth Factor Basement Membrane Matrix (Gibco®, Life Technologies Inc.) and incubated for 6 h. HEE cells in basal ECM was used as negative control. The cultures were photographed in a light microscope and the images were analysed by use of a Tube Formation Image Analysis program WimTube (Wimasis GmbH, Germany) which assessed the following parameters: covered area (%), total tube length (pixels), total branching points, total loops and total tubes. Each treatment was carried out in triplicate. Cells from six patients were used. The HRG serine peptide was included in experiments with cells from the first three patients.

Human embryos

Couples that had undergone IVF treatment at the Centre for Reproduction, Uppsala University hospital or the Carl von Linné Clinic in Uppsala were asked to donate surplus cryopreserved embryos (n = 158) that, according to Swedish law, otherwise had to be destroyed after five years. Before freezing, oocytes had been fertilized with sperm and after evaluation of fertilization, only zygotes with two pro-nuclei had been selected for further culture in a humidified incubator. The two clinics used different medium for insemination of oocytes, culture after fertilization had been confirmed and for cryopreservation. Embryos with four to six blastomeres on day two, < 20% fragmentation, and without abnormalities were cryopreserved. No data on the donors were available for this study.

Human embryo culture

Rapid thawing of day-2 embryos was performed using a thawing kit (Sydney IVF Thawing kit, Cook Medical) and transferred to equilibrated culture medium used for human cleavage stage embryos (CCM, Vitrolife, Sweden). Embryos were assessed for blastomere survival.

The embryos were cultured using time-lapse monitoring in an EmbryoScope® (Unisense FertiliTech, Denmark) for four days (i.e. until day six after inseminations). In total, 150 embryos were used in seven individual experiments performed at the Centre for Reproduction. In six of the experiments embryos were treated as controls (n = 67) or had the HRG proline peptide (n = 67) added to the culture medium. In one experiment embryos were treated as controls (n = 8) or had the control peptide (n = 8) added. For immunohistochemical (IHC) staining in Paper IV, eight additional untreated embryos were used (early cleavage stage embryos, n = 4 and day-6 embryos (blastocysts), n = 4).

At the end of culture, the surrounding culture medium was collected (20µl) from 94 embryos, by careful aspiration to avoid contamination by the overlay oil (Paper IV). The samples were stored in -70°C until further analysis. Images of the embryos were recorded at 20-minute intervals.

The embryo quality was evaluated retrospectively by a person blinded for the different treatments, using standard morphological criteria according to the system developed by Gardner et al.[151, 152] Timing events related to the start of culture after thawing , were carefully monitored in terms of first cell cleavage after thawing (t_{thfl}), morula formation (t_{thm}), cavitation of the blastocysts (t_{cav}), expansion ($t_{b.xg}$) and hatching ($t_{b.hg}$).[153]

Multiplex proximity extension assay

The relative levels of 92 proteins secreted into embryo culture medium were analysed using the Proseek Multiplex Assay (Multiplex Oncology I v2^{96x96} panel, Olink Bioscience, Sweden).[154, 155] In brief, for each measured protein, a pair of oligonucleotide-labelled antibody probes binds to the targeted protein. If the two probes are in close proximity, a PCR target sequence is formed by a proximity-dependent DNA polymerization event and the resulting sequence is subsequently detected and quantified using standard qRT-PCR. The assay generates quantification cycle values that are normalised and delivered to the customer as normalised protein expression units on log2 scale. The data used for statistical analysis is in log2 normalized protein expression where a high value corresponds to high protein concentration.

The analysis included three controls (fresh culture medium, conditioned culture medium (treated similarly, but without an embryo), and conditioned oil (treated similarly, but without an embryo or medium)). The samples were run at Olink Bioscience facilities in Uppsala, Sweden, on two occasions. The first run contained 24 samples, and in the second run, the remaining 70 sam-

ples were analyzed. The two different runs were normalized to allow comparison.

Immunohistochemistry

To validate the presence of VEGF-A, caspase 3 (CASP3), epididymal secretory protein E4 (HE4) and interleukin-8 (IL-8), IHC fluorescent double staining was performed on early cleavage stage embryos (n = 4) and on day-6 embryos (blastocysts) (n = 4). Embryos were washed in PBS containing polyvinylpyrrolidon (PVP) (ORIGIO, Denmark), fixed in 2.5% paraformaldehyde in PBS and stored in PBS/PVP until later. Permeabilization was made with 0.25% Triton X-100 in PBS/PVP, blocked in blocking solution with bovine serum albumin and Tween20 in PBS. Primary antibody solutions were made up in blocking solution. Primary antibody solutions were made up in blocking solution. Antibodies specific for VEGF-A, CASP3, HE4 and IL-8 (all from Santa Cruz Biotechnology Inc., US) were used. Embryos were then incubated with secondary antibodies labelled with Texas Red® and Fluorescein (all from Vector Laboratories Inc., US). Mounting was made with VectaShield® mounting medium containing Dapi (Vector Laboratories Inc., US) on glass slides surrounded by silicone. Pictures were taken with an Axio Observer.Z1 (Carl Zeiss, Sweden).

Paper III

Subjects and methods

Heterosexual couples (n = 139) visiting Fertilitetscentrum, Stockholm between March, 2010 and February, 2012 were asked to participate in the study. The couples contributed with one blood sample per person on the day of ovum pick-up. Overall, 155 couples consented to participate but due to technical problems in handling of the blood samples, 139 couples were included in the study. Data was obtained from medical records. Semen test results were available for all but two men (n = 137). All couples had previously undergone assessment for cause of infertility and were included regardless of the cause. The infertility causes were: anovulation (10.8%), tubal (7.2%), endometriosis (5.0%), unexplained (48.9%) male factor (18.7%), multiple causes (7.2%) and other (2.2%).

The control group (n = 196) consisted of heterosexual pregnant couples, who were approached in gestational week 16-19, in conjunction with the routine ultrasound scan at Uppsala University Hospital, between February 2012 and December 2012. Inclusion criteria were: pregnancy conceived spontaneously and able to understand Swedish or English. Exclusion criteria were: previous history of infertility. Each couple contributed with one blood sample per person on the day of the ultrasound scan. The women's medical

records were reviewed for data on age, weight, length and smoking/snuff habits from their admission to the maternity centre. Age was the only data that could be retrieved on the men.

The IVF treatment type and FSH/gonadotrophin dosages were determined individually based on the anticipated response and on the results in previous treatments. Only ejaculated partner sperm was used. ICSI was used if the number of prepared sperm was ≤ 1 million or in cases with previous low fertilization (< 50 %). Conventional IVF (n = 95), ICSI (n = 38) or a combination of both (n = 5) were used. Embryos were assessed according to standardized clinical morphologic protocols,[56] and transfer was not performed if there were signs of abnormal growth.

Among infertile couples 51 (36.7 %) became pregnant after treatment, 68 (48.9 %) did not become pregnant, and 20 (14.4 %) couples had no embryo transfer for reasons mostly related to pathological development of the embryo or no oocytes being fertilized.

Blood sampling, DNA extraction and genotyping

Venous blood samples were collected in EDTA-containing tubes on the day of ovum pick-up for the infertile couples and at the ultrasound scan for the controls. All samples were handled equally. After centrifugation (*1500 g* for 10 min), buffy coat was transferred to new tubes and stored at -20°C until transferred weekly to -70°C storage. Genomic DNA was extracted from the buffy coat using QIAamp DNA Blood Mini kits (Qiagen, Venlo, Netherlands).

Genotype was determined after embryo transfer and according to the procedure described previously (Paper I).

Statistics paper I – IV

All statistical analyses were performed in the Statistical Package for the Social Sciences 18.0 or 20.0 (SPSS Inc., US) for Windows software package. Statistical tests were two-sided and *p*-values < 0.05 were considered significant. The Shapiro-Wilk test was applied to access normal distribution of continuous variables. The normally distributed data in Paper I were analysed with the Student's T-test. Odds ratios (OR) with 95% confidence intervals were calculated (Paper I) with binary- and multiple logistic regression analysis. Proliferation, migration and tube-formation data (Paper II) and the skewed data of Paper III, were analysed with the Kruskall-Wallis Test and *post hoc* Mann-Whitney U-Test. Categorical data was analysed with the Pearson Chi-Square test or Fisher's Exact Test (Paper I, II and III). Time-lapse data (Paper II) were analysed with the Student's T-test. Allele frequencies of the *HRG* C633T SNP were calculated to investigate deviation from Hardy–Weinberg equilibrium (Paper III). Normalised protein expression data (Paper IV) was analysed with the Mann-Whitney U-Test.

Summary of results

Paper I

The frequency of homozygous (C/C and T/T) and heterozygous (C/T) carriers were similar among cases and controls. The percentage of women with recurrent miscarriage was the same regardless of genotype (C/C 31.2%, C/T 32.9% and T/T 32.5%). However, thyroid disease was more prevalent among the homozygous *HRG* 633T SNP carriers (C/C 4.7%, C/T 2.8% and T/T 10.8%, p < 0.001 T/T compared with C/C or C/T).

The subgroup of 43 women with primary recurrent miscarriage was compared with controls. The frequency of homozygous *HRG* 633T SNP carriers was higher in women with primary recurrent miscarriage than in the controls (25.6% vs. 14.2%, OR: 2.49 (1.06-5.88), p < 0.05). This finding remained after adjustment for age, smoking, BMI and prevalence of thyroid diseases, Table 3.

Table 3. Factors associated with primary recurrent miscarriage

	Unadjusted OR (95% CI)	Adjusted OR (95% CI)[a]
Age		
≤35	1	1
35-40	1.22 (0.59-2.52)	1.28 (0.60-2.74)
>40	0.68 (0.09-5.35)	0.76 (0.09-6.24)
Smokers		
No	1	1
Yes	2.23 (1.00-4.96)	2.17 (0.94-4.99)
BMI		
< 25	1	1
25-30	1.52 (0.72-3.23)	1.58 (0.72-3.45)
>30	2.04 (0.83-5.04)	2.21 (0.87-5.63)
Thyroid disease		
No	1	1
Yes	5.18 (1.84-14.59) [*]	5.08 (1.74-14.88) [**]
***HRG* C633T SNP**		
C/T	1	1
T/T	2.49 (1.06-5.88)	2.44 (1.01-5.93) [*]
C/C	1.39 (0.66-2.90)	1.39 (0.65-2.95)

OR: odds ratio; CI: confidence interval; BMI: body mass index.
[a] adjusted for age, smoking, BMI, thyroid disease and SNP. [*]p < 0.05, [**]p < 0.01

Paper II and Paper IV

Migration and proliferation of HEE cells

Migration of HEE cells in response to the HRG proline peptide, HRG serine peptide and VEGF-A was investigated by using a Boyden chamber (Figure 3A). The number of cells that had migrated through a filter was counted, and ratios were calculated against migration of HEE cells in basal ECM in each experiment. Cell migration increased by 53%, 19% and 130% respectively. The VEGF-A-induced cell migration was inhibited by the HRG proline peptide.

Proliferation of HEE cells in response to the HRG proline peptide, HRG serine peptide and VEGF-A was investigated after five days of culture (Figure 3B). Ratios were calculated against proliferation of starved HEE cells in each experiment. Treatment with VEGF-A induced a 119% increase in proliferation. The HRG proline and serine peptides themselves reduced basal proliferation to 75% and 72% respectively and inhibited VEGF-A-induced proliferation.
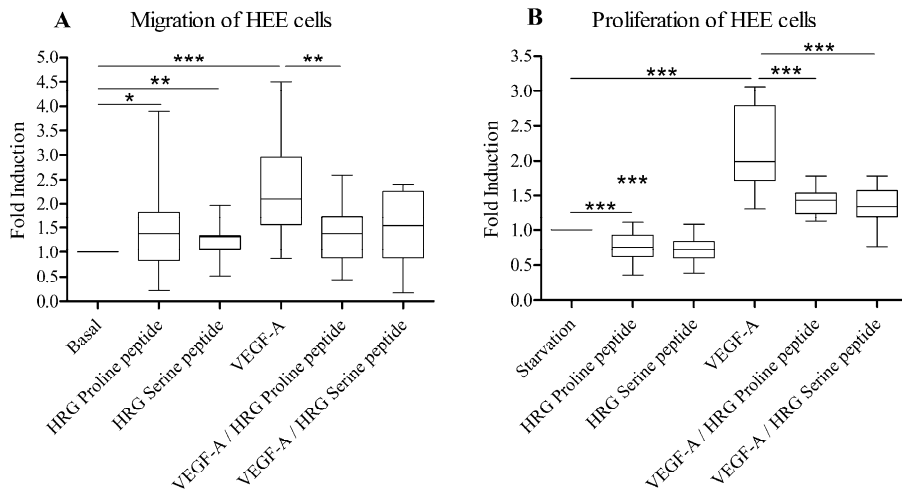
Figure 3. (A) Data are presented as fold induction in comparison with basal ECM, or basal ECM supplemented with 0.2% FBS (B).* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; Kruskall-Wallis Test and Mann-Whitney U-Test were performed.

Tube formation of HEE cells

The ability of HEE cells to differentiate and form capillary-like structures in response to VEGF-A, the HRG proline peptide and HRG serine peptide was investigated using a tube-formation assay (Table 4). The result was quantified using the WimTube software and ratios were calculated against the negative control (basal ECM). As expected, VEGF increased tube formation measured as covered area, total tube length and total tubes. Treatment with the HRG proline peptide increased the covered area and number of loops.

The HRG serine peptide was added in a total of three experiments. Treatment with this peptide increased the covered area, total tube length, branching points, loops and tubes.

Table 4. Tube formation of primary HEE cells

Tube parameters	HRG proline peptide [a]	HRG serine peptide [b]	VEGF-A [a]
Covered area, %	29.2 (14.0-44.0)**	34 (26.8-41.2)*	31.5 (16.9-37.6)*
Tube length, k pixels	30.3 (19.1-36.0)	35.2 (27.7-37.8)**	31.3 (18.8-36.8)*
Branching points, n	95 (63-131)	133 (85-149)**	114 (48-140)
Loops, n	24.0 (7.0-42.5)*	32.0 (19.5-47.0)**	18.0 (5.5-47.5)
Tubes, n	210 (173-244)	251 (188-295)*	241 (141-256)*

n; number: k; kilo. [a] Data based on six experiments. [b] Data based on three experiments.
* $p \leq 0.05$ and ** $p \leq 0.01$ compared to negative controls in basal ECM, Kruskal-Wallis Test and Mann Whitney U-Test were performed.

Quality and morphokinetics of human cryopreserved embryos treated with the HRG proline peptide

No change in embryo quality was noted following the HRG proline peptide treatment (Table 5). Except for a longer time-interval between t_{thfl} and t_{thm} in HRG proline peptide-treated embryos compared to controls (41.3 ± 8.4 (n = 53) *vs.* 37.0 ± 12.0 (n = 57) hours respectively, $p < 0.05$), no differences in morphokinetics/timing of development to different embryonic stages emerged between treatments. The control peptide resembled the HRG proline peptide in terms of embryo quality and morphokinetics.

Two recombinant full-length HRG proteins, containing a proline or serine at amino acid position 204, were also available. These proteins were tested in a small pilot study, using the same conditions as for the peptides, with 24 embryos in each group. Addition of the proline or serine variants of the HRG full-length protein did not alter embryo quality or morphokinetics compared to controls (data not shown).

Table 5. Quality and morphokinetics of day-two cryopreserved human embryos treated with the HRG proline peptide in comparison with control embryos

Embryo parameters, mean ± SD (n)	Controls n = 67	HRG proline peptide n = 67
Embryo quality, n		
Number of cells at thawing	4.3 ± 1.0 (67)	4.2 ± 0.8 (67)
Number of alive cells at thawing	3.8 ±1.1 (67)	3.5 ± 1.0 (67)
Blastocysts with a score of ≥ 3	46	44
High quality blastocysts (score of $\geq 4BB$)	33	28
Morphokinetics, h		
First cleavage after thawing (t_{thfl})	15.2 ± 9.6 (66)	13.0 ± 8.0 (66)
Morula formation after thawing (t_{thm})	50.8 ± 10.5 (57)	53.1 ± 9.1 (53)
Time between t_{thfl} and t_{thm}	37.0 ± 12.0 (57)	41.3 ± 8.4 (53)*
Time to start of cavity formation (t_{cav})	62.6 ± 11.4 (52)	64.9 ± 10.4 (49)
Time to start of expansion ($t_{b.xg}$)	62.6 ± 17.0 (48)	68.5 ± 12.6 (47)
Time to start of hatching ($t_{b.hg}$)	85.7 ± 7.0 (16)	82.1 ± 8.5 (15)

SD: standard deviation; n: number; h: hours. For continuous data the Student's t-test was used. For categorical data Chi-Square test was used. * $p < 0.05$

Proteins secreted by embryos

Ten proteins were detected in day-six culture medium from human embryos: IL-8, VEGF-A, PlGF, IL-6, extracellular matrix metalloproteinase (EMMPRIN), epithelial cell adhesion molecule (EpCAM), HE4, CASP3 and Cystatin B (CSTB), Table 6. One of the proteins detected, Adrenomedullin, was only expressed in two samples in the first run and was excluded from further analyses.

Table 6. Proteins secreted by human embryos in culture medium during days 2-6 after fertilization

Description	Symbol	UniProt-KB	n	Log2 (NPX), median (IQR)
Interleukin-8	IL-8	P10145	75	1.00 (0.80-1.40)
Vascular endothelial growth factor A	VEGF-A	P15692	91	2.30 (2.10-2.60)
Placental growth factor	PlGF	P49763	88	0.80 (0.70-1.18)
Interleukin-6	IL-6	P05231	88	2.70 (2.00-3.50)
Cystatin B	CSTB	P04080	89	1.30 (1.10-1.60)
Extracellular matrix metalloproteinase inducer	EMMPRIN	P35613	91	3.10 (2.90-3.40)
Epithelial cell adhesion molecule	EpCAM	P16422	91	3.60 (3.40-4.00)
Caspase 3	CASP3	P42574	84	1.40 (1.00-2.00)
Epididymal secretory protein E4	HE4	Q14508	87	0.70 (0.60-0.80)

n: number; NPX: normalised protein expression; IQR: interquartile range.
Data presented as median normalized protein expression (IQR) on a log 2 scale.

There were no differences in normalized protein expression between the group of embryos that had their culture medium supplemented with the HRG proline peptide and the control group. Thus, for the analyses on difference in normalized protein expression between arrested embryos and embryos that had developed into blastocysts, results were collapsed across HRG proline

peptide treatment groups. Human embryos that developed into blastocysts of high quality had significantly higher normalized protein expression levels of VEGF-A, IL-6, EMMPRIN and PlGF in comparison with arrested embryos (Figure 4).
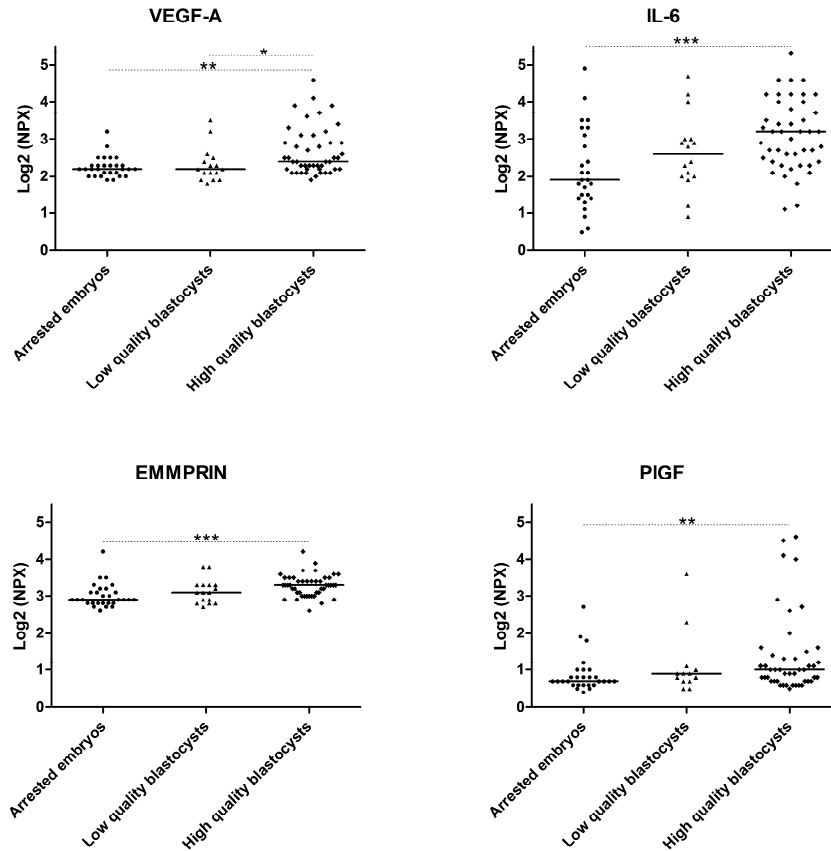
Figure 4. Normalized protein expression values (logarithmic scale, log2) in spent culture medium from embryos that arrested in development, blastocysts (<4BB) and high quality blastocysts (≥4BB). Two data points in the high quality blastocyst group are not shown; at 11.7 for IL-6 and at 9.9 for PlGF. * $p < .05$, ** $p < .01$, *** $p ≤ .001$, Mann-Whitney U-Test. VEGF-A: vascular endothelial growth factor-A; IL-6: interleukin-6; EMMPRIN: extracellular matrix metalloproteinase inducer; PlGF: placental growth factor.

Paper III

No differences in age, BMI, smoking or snuff usage were noted between the *HRG* C633T genotypes. The distribution of the *HRG* C633T SNP did not differ between infertile women and men and their respective controls.

However, men diagnosed with male factor infertility (as single factor) were more often homozygous *HRG* 633T SNP carriers than men in the pregnant control group, but this was only borderline significant (*HRG* 633T SNP; male factor infertility 19.2% versus control men 7.1%; $p = 0.05$).

Among the infertile men no differences in infertility diagnoses, treatment type, or number of embryo transfers were found across the homozygous and heterozygous carriers of the *HRG* C633T SNP. However, the male heterozygous *HRG* C633T SNP carriers had undergone fewer previous IVF/ICSI treatments than the other groups.

The *HRG* C633T SNP was not associated with pregnancy rate in the infertile women; however, in infertile men the *HRG* C633T SNP had an impact on the pregnancy rate of the current treatment cycle (Table 7). Most successful were couples with male heterozygosity where 52.0% achieved pregnancy, whereas only two couples (14.3%) became pregnant when the male partners were homozygous carriers of the *HRG* 633T SNP. These results remained even when couples with no embryo transfer were excluded from the analysis.

Figure 5 shows the pregnancy rate in relation to the frequency of T-alleles in the infertile couples. Most successful were couples with one or two T-alleles, which corresponds to one or two heterozygous *HRG* 633T SNP carriers or only one homozygous *HRG* 633T SNP carrier in the couple, where 48.1% (n = 26) and 42.1% (n = 16) became pregnant, respectively (Figure 5A). Couples carrying no or three T-alleles in the *HRG* C633T SNP had a low pregnancy rate; only 20% (n = 7) of couples with no T-alleles and 16.7% (n = 2) of couples with three T-alleles became pregnant.

Table 7. Treatment outcome according to *HRG* C633T SNP

	Men, n = 135 [a]		Women, n = 135 [a]	
	Pregnant	Not pregnant [b]	Pregnant	Not pregnant [b]
In total, n (%)	51 (37.8)	84 (62.2)	51 (37.8)	84 (62.2)
HRG C633T, n (%)				
C633	23 (32.4)	48 (67.6)	22 (33.3)	44 (66.7)
C633T	26 (52.0)	24 (48.0)	24 (42.9)	32 (57.1)
633T	2 (14.3)	12 (85.7)**	5 (38.5)	8 (61.5)

n; number.** $p \leq 0.01$, Pearson Chi-Square test. [a] Four patients are excluded due to no embryo transfer for reasons other than pathological development of the embryo or no oocytes being fertilized. [b] Patients with no embryo transfer due to pathological development of the embryo or no oocytes being fertilized are included in the non-pregnant group (n = 16).
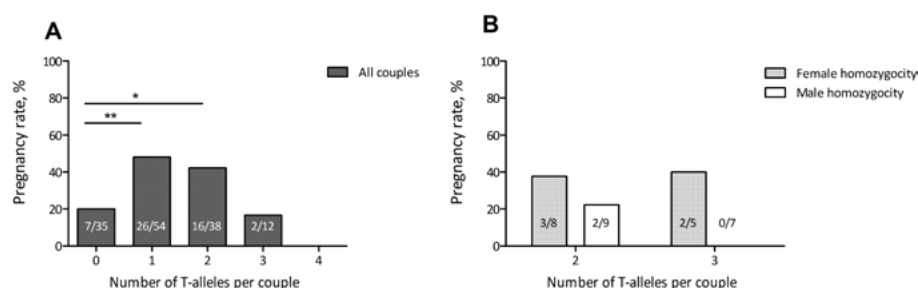
Figure 5. Pregnancy rate following IVF according to the frequency of T-alleles (*HRG* 633T SNP) in the infertile couples. Bars represent the percentage of pregnant couples according to the number of T-alleles within the couple. The total number of couples is given within each bar. (A) All pregnant couples. No couples had four T-alleles (homozygous 633T/633T), hence this bar is not shown. (B) Couples with one homozygous *HRG* 633T SNP carrier; either the woman (grey) or the man (white). Due to the small numbers no statistical analyses were performed. *$p < 0.05$, **$p < 0.01$, Pearson Chi-square test.

The semen profile, according to the male *HRG* C633T SNP, is displayed in Table 8. Male homozygous *HRG* 633T SNP carriers had lower total sperm count and motility score compared to both homozygous *HRG* C633 SNP carriers and heterozygous *HRG* C633T SNP carriers. Moreover, homozygous *HRG* 633T SNP carriers had lower sperm concentration and yield after preparation compared to homozygous *HRG* C633 SNP carriers (Table 8).

Table 8. Semen parameters of 137 infertile men, according to male *HRG* C633T

	C633, n = 72		C633T, n = 49		633T, n = 16	
	n	median (range)	n	median (range)	n	median (range)
Ejaculate volume (ml)	72	2.5 (2.0-4.0)	49	3.1 (2.6-4.0)[a]	16	2.5 (1.6-3.5)
Total sperm count (10^6)	72	131 (57-247)	49	180 (63-274)	16	81 (38-106)[a,b]
Sperm concentration ($\times 10^6$/ml)	72	52.0 (29-85)	49	52 (23-85)	16	28 (14-50)[a]
Progressive motility (%)	72	59 (45-71)	48	62 (44-70)	15	65 (40-70)
Motility score (0-3)	64	2.3 (2.2-2.4)	38	2.3 (2.2-2.4)	15	2.2 (2.0-2.3)[a,b]
Yield after preparation (%)	72	10.0 (2.5-20.0)	49	7.5 (1.2-20.5)	16	2.5 (0.6-10.1)[a]

n; number. [a] Significantly different from homozygous C633, $p < 0.05$–0.01, Kruskal–Wallis and post hoc Mann–Whitney *U*-test. [b] Significantly different from heterozygous C633T, $p < 0.05$–0.01, Kruskal–Wallis and post hoc Mann–Whitney *U*-test.

Discussion

Methodological considerations and limitations

After four years of PhD studies it is apparent that some mistakes were made along the way, that our findings are not without limitations, and that some experiments did not produce the results we had hoped for. Some of the concerns and considerations are listed below.

Sample size

The number of women diagnosed with recurrent miscarriage and the number of infertile couples is quite small for a genetic study to be performed. Further, due to the plethora of possible random associations and unreplicated findings in genetic research, there is nowadays an increasing demand for replication in independent cohorts, before genetic association studies are published. However, while this may be regarded as a limitation, it should also be acknowledged that the genetic associations we present are supported by functional biological analyses on endometrial cells and semen, strengthening the relevance of our findings. In addition, given that only women with three or more consecutive miscarriages were included, the sample size of 187 women makes this study relatively large compared to similar studies.[39] The number of embryos donated to research is limited and therefore time-consuming to collect, considering this, the more than 200 embryos used in this study is high.

It is furthermore obvious in paper III that the number of couples with male factor as the single reason for treatment was too low. A larger group with this diagnosis could have verified an association between *HRG* C633T SNP and male infertility.

Subjects

Recurrent miscarriage is a rare diagnosis, found in about 1% of the population. In order to obtain a sample that would be sufficient for genetic analysis we included women diagnosed with recurrent miscarriage during a time period of 20 years. Controls, on the other hand, were recruited at the time of the study. Thus, this work suffers from information bias, where health status over time could be obtained in the cases but not in the controls. For instance, thyroid disease was more common among women with recurrent miscarriage than controls, potentially because thyroid disorder is more common in recur-

rent miscarriage,[156] but also because these women were older when asked. In addition, the screening for thyroid disease was not conducted in the same way for cases and controls. All women diagnosed with recurrent miscarriage were tested for TSH levels according to routine clinical procedures. The controls on the other hand were subjected to targeted testing when considered to be at high risk, a method that has been shown to be unsatisfactory.[157] Even though the association between the *HRG* C633T SNP and primary recurrent miscarriage remained when excluding women with thyroid disease, future studies should exclude women with thyroid disease completely to enhance their chances of detecting genetic differences. Similarly, pre-pregnancy smoking was also higher among women with recurrent miscarriage. This could to some extent be explained by the fact that smoking among Swedish women has gradually become less common, perhaps resulting in the lower frequency of pre-pregnancy smokers in the control group. Data on smoking for the controls was collected from the medical records at inclusion to the study, and the cases were asked during the inclusion if they had been smoking during the period of their three consecutive miscarriages. Thus, both groups and especially the cases are subjected to recall-bias.

In paper III, insufficient information of demographic and clinical variables on the male controls was obtained. This is a limitation since factors such as BMI, smoking and previous surgery might affect a couple's fertility.[15, 158-161] On the other hand both partners of the infertile couple are well defined. Further, no information on the ethnicity of the couples was collected, which may diminish our chances to replicate previous findings in females, or detecting differences in relation to the controls. Ethnicity is known to affect the genetic variation of the *HRG* C633T SNP. The allele frequency of *HRG* 633T in a European population is 0.33 while in populations from Africa and South Asia the reported frequencies are in the range of 0.5-0.7.

Primary human endometrial endothelial cells

The isolation and culture of HEE cells is a well-established method in our laboratory, and the isolated cells have been characterized.[162] The endothelial cells originate from the endometrium and are therefore under the influence of cyclic hormonal changes during the menstrual cycle. In our study we did not discriminate between cells from patients of different menstrual cycle phases. However, previous work by a colleague[146] and others has shown that cultured HEE cells behave the same despite the menstrual phase they were in at the biopsy collection.[146, 163-165]

Studies on the effects of endocrine disrupting chemicals on HEE cells, in mono-cultures or in co-cultures with endometrial stromal cells, have revealed different results on gene expression and tube formation depending on the presence or absence of the stromal cells.[146, 166, 167] This is not surprising as the co-culture system allows for paracrine interactions between the cell

types, and resembles the *in vivo* situation. There are pros and cons of working with primary cells. First, the inter-individual variation, which comes with the sampling from different individuals, is greater than in established cell-lines. However, functionally they are more similar to cells *in vivo* compared to cell-lines, and cell-lines introduce other types of variations that need to be taken into account. Keeping this inter-individual variation in mind it is important to consider that the *HRG* C633T genotype of the patients donating the cells was unknown to us. Which HRG protein that was present *in vivo* could perhaps affect the response of treatment of the cells with the HRG peptides. Secondly, the process of establishing a pure colony of primary cells ready to use in an experiment is tedious and include patient selection and recruitment, a multistep isolation procedure, and culture and maintenance of the cells for several days before reaching a sufficient number. Third, the purity of the primary cells is of outmost importance. HEE cell cultures can be contaminated with endometrial fibroblasts that proliferate rapidly. A pure isolate is achieved by ensuring single cell suspensions, use of a nylon cell strainer that only let cells of $\leq 40\mu m$ pass through, and by the use of positive isolation using magnetic beads that only bind the HEE cells (via an antibody specific for endothelial cells, CD31). For these reasons, we were only able to include cells from between 3-6 patients in the different assays.

HRG peptides

Human embryos were cultured with addition of the HRG proline peptide. Since embryos donated to research are time-consuming to collect, it was decided that only one of the peptides (the HRG proline peptide) would be investigated in this thesis. Knowledge of the small difference of only one amino acid between the peptides synthesized supported this decision. In contrast, the *in vivo* full-length HRG with a serine at position 204 is glycosylated while the corresponding protein with a proline at the same position is not. The *HRG* C633T SNP seems to affect fertility in the clinic and part of our hypothesis is based on the presence or absence of this glycosylation. However, the glycosylation is not present in the HRG serine peptide. Due to this, we expected no major impact from the small difference between the two peptides, and our results with the HEE cells more or less confirmed this expectation. Given the lack of glycosylation, the HRG proline peptide was chosen since it corresponds to the predominant genotype and our hypothesis was that the full-length protein with this amino acid *in vivo* would aid fertility. To rule out that a possible positive effect of the HRG proline peptide on early human embryos would simply be due to an increase in amino acids, we synthesized a control peptide with a scrambled version of the HRG proline peptide.

The working concentration of 100ng/µl was established from literature on primary endothelial cells.[113] We performed a small pilot study on HEE cell tube formation where concentrations of 10ng/µl and 100ng/µl were tested.

We found no apparent difference between 10ng/µl and 100ng/µl and the concentration of 100ng/µl was chosen based on the previous work with primary endothelial cells.[113]

Human embryos

The embryos used in these studies were donated from two clinics. This may be considered a strength since interpretations of results based on data from one single clinic may only be representative to that clinic and not universally applicable. This is due to the variance in culture protocols and handling of embryos by different clinics and different embryologists. Results from multicentre studies may therefore apply better to the general clinical setting. This may also be considered a limitation, since the different protocols at the two clinics could not be adjusted for in the statistical analyses. The differences in medium used and culture conditions up till cryopreservation is completed could have effects on the outcome measures. Epigenetic modifications due to for example the culture medium have been suggested,[168] and an impact from the mediums used can therefore not be ruled out. In this sense it should also be kept in mind that although we did not find any differences in embryo quality, development or protein secretion after adding the HRG peptide, there might still be an impact from this that was simply not measurable with the techniques employed. As the exposure to different culture conditions (e.g. the use of different medium for fertilization and cleavage) occurred before the embryonic genome activation on day 3 of development, we believe that this difference is of minor importance for the subsequent secretion of proteins. Strengthening our studies on human embryos is the fact that only high quality embryos that were fit for cryopreservation and with a mean survival rate after thawing of more than 80% were used. Nevertheless, future studies in the search of markers for embryo selection should be based on embryos treated equally throughout the entire culture period, or the differences in culture should be adjusted for in the statistical analyses.

Since the interpretation of embryo quality is subject to intra-observer variability, part of the embryos was evaluated retrospectively by a second person blinded for the experiment. Only the embryo quality at the end of culture was evaluated by this second observer and not the time to different developmental stages. This was above all due to the fact that such observations are time consuming. Future studies including time-lapse parameters should include a second observer to correct for subjectivity. Since HRG had no effect on morphology, embryo development and normalized protein expression, we found it reasonable to include this group in the secretomics analyses, simply to increase power. We acknowledge that this is a limitation that makes a universal interpretation of these results harder. Furthermore, only four of the proteins found to be secreted by embryos were validated by IHC. This was again due to the limited number of available embryos and blastocysts. We choose to perform IHC on the four proteins that were se-

creted at differing levels in arrested embryos and blastocysts based on the first Proseek Multiplex run (24 samples). If this validation had been performed after both runs were completed then we would have chosen the proteins found to be differentially secreted between arrested embryos and high quality blastocysts. Future studies should include all proteins secreted to fully verify that the proteins are actually coming from the embryos or blastocysts.

HRG and infertility

In this thesis the proposed fertility effects of the *HRG* C633T SNP were elucidated by performing two case-control studies and two experimental studies. The experimental studies investigated the region around the HRG polymorphism, and its possible effect on the early human embryo and endometrial angiogenesis. Overall, the study findings suggest that the *HRG* C633T SNP plays a role in primary recurrent miscarriage, endometrial angiogenesis, and male infertility.

HRG and fertility

None of our studies suggested that *HRG* C633T SNP is associated with fertility *per se.* There was no difference in genotype frequencies between recurrent miscarriage cases and controls, and no difference between infertile couples and control couples. However, once infertility is established, the *HRG* C633T SNP seems to play a role. We found that women with primary recurrent miscarriage, who never had a child, were more often homozygous 633T carriers. Similarly, infertile couples with male homozygous carriers had lower chance of a successful IVF treatment, and the overall lowest semen parameter values.

HRG and recurrent miscarriage

Women with primary recurrent miscarriage were more often homozygous 633T carriers. Idiopathic recurrent miscarriage is considered to be a heterogenic disease, unlikely to be caused by one single factor.[169] Several reports have linked SNPs in different genes to idiopathic recurrent miscarriage,[39, 170] which is perhaps more likely to depend on the right combination of SNPs, and not just a single one. A meta-analysis recently suggested that recurrent miscarriage relies on a complex phenotype that consists of several SNPs, each with a minor contribution to the disease.[39]

We provide mainly three mechanistic explanations to the contribution of *HRG* C633T to primary recurrent miscarriage, and the results from our HEE cultures may be important in this aspect as well. It is well known that development of a normal well-functioning vasculature in the uterus and placenta needs cooperation between different cell types and various growth factors, in

the processes of implantation, embryo development and placentation.[171] The implanting embryo needs access to the maternal blood supply before it can create its own vascular system.[73] A properly regulated angiogenesis is vital in this process and for an adequate placentation.[73, 172] HRG is known to regulate angiogenesis both directly and indirectly, which was also indirectly confirmed in our HEE cultures. The predominant, native HRG protein contains a proline at amino acid 204 (corresponding to the *HRG* C633 SNP). Proline is, due to its unique cyclical formation with a secondary amine, important for protein structures and contributes to exceptional conformational rigidity. The variant protein that has a serine at amino acid 204 (corresponding to the *HRG* 633T SNP) could therefore have a more loose protein structure, perhaps leading to a decreased protein stability and alterations in ligand interactions. This variant also allows for a glycosylation at position 202 in the protein[93, 98] which is very close to a potential inter-domain disulphide bridge.

Secondly, the disulphide bridge connects to the region in the protein, that when released can regulate angiogenesis.[98, 112, 173, 174] This fragment in the proline-containing full-length HRG protein can inhibit both VEGF- and FGF-mediated angiogenesis of endothelial cells *in vitro*.[113] In the variant HRG protein the stability of the disulphide bridge might be altered, thereby disabling the proteolytic release of the anti-angiogenic fragment.

Finally, the tripartite interaction of HRG-TSP-CD36 is known to have an indirect pro-angiogenic effect.[175] The binding of CD36 and TSPs inhibit the response of a number of different growth factors of importance for angiogenesis, such as FGF and VEGF.[115] The substitution from proline to serine is situated in the CLESH-1 homology domain and this might lead to a disrupted interaction between CD36, TSP and HRG. The CD36 receptor is expressed in the human endometrium,[176] and we confirmed expression of TSP1 and possibly TSP2 in HEE cells. Women and embryos homozygous for HRG 633T might have an impaired regulation of angiogenesis, leading to a defect in implantation and placentation that eventually leads to expulsion of the embryo/foetus.

Our studies on how the HRG peptides, corresponding to the *HRG* C633T polymorphism, influence the angiogenic ability of HEE cells in culture may provide further insight. Both peptides had a similar effect on the angiogenic ability of HEE cells *in vitro* and we conclude that regardless of the *HRG* C633T SNP the peptides seem to regulate angiogenesis by suppressing proliferation and promoting migration and differentiation in our culture systems. We speculate that the suppressive effect of the HRG peptides on proliferation of primary HEE cells may be beneficial during implantation and placentation. Previous studies have suggested that an imbalance in endometrial angiogenesis and a too rapidly formed vascular network may result in failed implantation and first-trimester miscarriage.[177-180] TSP1 and TSP2 inhibit endothelial cell migration in response to several angiogenic factors[116] and HRG with its CD36 homology domains have been reported to inhibit the

anti-chemotactic effect of TSP1.[114] The CD36-receptor is known to be expressed on the surface of especially small vessels of the endometrium,[181] which would correspond to the primary HEE cells in this study. The HRG peptides only contained part of one of the CD36 homology domains but might still be able to block the anti-angiogenic effect of TSP1, possibly explaining the induced migration in response to the HRG proline peptide. The enhanced tube formation after treatment with the HRG peptides could be explained by the same tripartite interactions of CD36-HRG-TSP on the surface of HEE cells. However, when interpreting the results from migration and tube formation of HEE cells it should be kept in mind that no exogenous TSP was added, and the tripartite interaction is therefore dependent on endogenous expression of TSP.

HRG and IVF treatment

The *HRG* C633T SNP seems to play a role for IVF treatment, as couples with male *HRG* 633T homozygous carriers had lower chance of a successful treatment. Further, male *HRG* C633T homozygous carriers had the overall lowest semen parameter values.

However, the T-allele did prove to be needed in at least one copy in the couple, and couples with male heterozygous carriers had the highest pregnancy rates. This finding is supported by the theory of heterozygous advantage, in which heterozygous carriers present a selective advantage in viability and reproductive fitness over homozygotes in natural populations.[182] This theory has recently been described in relation to female fecundity. Laanpere et al investigated infertile women undergoing IVF treatment and the importance of several different SNPs in the folate pathway. They reported positive associations between heterozygous carriers and pregnancy rate among other outcome parameters.[183] Elenis et al found that women who were heterozygous carriers of another SNP in the *HRG* gene (A1042G) less often had recurrent miscarriage.[184] In addition, as noted in Paper I, women with primary recurrent miscarriage are less often heterozygous for the *HRG* C633T SNP. When interpreting these results, the possible mechanistic differences between the predominant and variant HRG proteins, outlined in the previous section, should be kept in mind. If the stability of the protein is altered by the pro204ser shift then the two HRG proteins created by the polymorphism could possess functions somewhat different from each other, and the heterozygous men could benefit from having both. If the stability of the disulphide bridge is altered and in turn the release of the anti-angiogenic fragment is affected, this could have implications on angiogenesis in areas where HRG is present.

Our findings highlighted a role for the *HRG* C633T SNP in the infertile male partner. In the public database ProteomicsDB[133] HRG is detected in the

testis, prostate and seminal vesicles of the human male reproductive tract,[119] and the antibody-based Human Protein Atlas (HPA) confirms a low to moderate expression of HRG in the testis and prostate.[135] Proteome analysis has also located TSP1 to the testis, prostate and seminal vesicles of men,[185] and TSP2 to testis, prostate and seminal plasma.[186] The HPA confirms a moderate to high expression of TSP1 in testis, epididymis and seminal vesicles[173] and a moderate expression of TSP2 in testis, epididymis, prostate and seminal vesicles.[187, 188] The same proteome analysis located the TSP receptor, CD36, to the testis, prostate and seminal vesicles of men but the HPA has to date not been able to confirm the presence of CD36 in these areas. The HPA did however locate CD36 to the epididymis.[189] With a possible co-localization of HRG and TSPs in these areas, the effect from an altered interaction between CD36-HRG-TSP due to the polymorphism, could have implications on the local regulation of angiogenesis. Perhaps the best balance of pro and anti-angiogenesis is achieved when the two HRG proteins (proline and serine) are present together. When only one of the proteins is present, this balance could be shifted in either direction.

HRG also influences VEGF, another key player in angiogenesis.[113] The major sources of VEGF in the male reproductive tract are the prostate and seminal vesicles, but Leydig and Sertoli cells also produce VEGF to some extent.[190] Importantly, high levels of VEGF are found in human semen[191] and VEGF receptors are present on spermatozoa.[192] A receptor for VEGF has also been suggested to be present on Leydig cells. Over-expression of VEGF results in male infertility in mice, an effect mediated through VEGF binding to Leydig cells and subsequent down-regulation of progesterone.[193] While the function of VEGF in the male reproductive tract is unclear, it appears to influence the testicular microvasculature and the composition of seminal plasma.

Male homozygous *HRG* 633T SNP carriers also had lower semen test results, which for the most part persisted after exclusion of male factor infertility. Since HRG seems to be present in the testis, this finding suggests an influence from the polymorphism on spermatogenesis. Normal spermatogenesis requires crosstalk between somatic and germ cells, relevant endocrine signalling,[24] and adequate blood flow.[194] The human testis has a highly developed microvasculature.[190] Vascular changes have been reported in testicular biopsies from infertile men,[195, 196] and potentially for the reasons given above, HRG may act as a regulator of angiogenesis during spermatogenesis and maturation of spermatozoa. HRG is also one among the more than 6000 proteins detected in the human sperm proteome.[136, 137] It is known that transcripts and proteins of importance for early embryogenesis accompany the male DNA and is introduced to the oocyte through the sperm.[197] Also, the microenvironment in which the spermatozoa are produced affects their reproductive capacity.[194] Further analyses of the sperm transcripts, pro-

teins and DNA integrity in relation to the *HRG* 633T SNP, in both fertile and infertile men, would be valuable in the future.

In addition to the many reasons for infertility, the complex nature of these types of studies is underscored by the fact that each individual of the couple contributes with intricate genetic, proteomic and metabolic differences that interact,[198] and infertility is therefore not likely to be explained by one single causative polymorphism.

HRG and the embryo

Effect on embryo development and quality

Improvement of embryo culture systems by supplementing endogenous factors to culture media in physiological concentrations is one important way to increase embryo quality and success rate in IVF. Thus, while our results are negative, this was one of the first studies where the impact of a potential novel human embryo medium supplement was evaluated by time-lapse and morphokinetics. We found no evidence that the HRG proline peptide, corresponding to the predominant genotype of this polymorphism, had any beneficial effects on embryo development. With the exception of a prolonged time between first cleavage and morula formation after thawing (Paper II), no effect on timing to other embryonic developmental events was noted. Also, pilot studies with the full-length proteins yielded similar negative results. However, since early human embryos produce HRG, it is still possible that the *in vivo* full-length HRG, in its correct three-dimensional structure, may have effects on embryo development.

Furthermore, the preimplantation period is a critical window of development.[199] Certainly, the culture environment and the manipulations of the embryo during IVF can impact its reproductive potential. If the composition of the culture media is less than perfect it may impact biochemical, metabolic, and epigenetic patterns that can affect the preimplantation embryo. There is a general hypothesis that assisted reproductive technologies increase epigenetic perturbations, with possible consequences on the long-term health outcome in the offspring.[199] Bouillon et al published a study this year comparing the longer-term effects (up till five years of infant age) of two commercially available mediums and concluded, for the first time, that the choice of embryo culture medium may impact the child development.[199] A recent animal study suggested that even the slightest changes in the nutritional milieu in early embryo development could result in for example insulin resistance and vascular system dysfunction in the offspring.[200] For these reasons, further studies on culture medium composition are needed.

Further, from our embryo culture proteome analyses we gained more knowledge about some of the factors present in medium used for embryo

culture. Thirteen proteins were detected in conditioned culture medium that had not hosted an embryo (VEGF-A, osteoprotegerin, CSTB, monocyte chemotactic protein-1, tumor necrosis factor receptor 1, interleukin 2 receptor subunit alpha, EMMPRIN, Ep-CAM, receptor tyrosine-protein kinase erbB-2**,** urokinase plasminogen activator surface receptor, fms-related tyrosine kinase 3 ligand, early activation antigen CD69 and prostasin). Some of these proteins were also secreted by embryos, see further below, but others seem to derive from the medium. The medium used for culture (CCM, Vitrolife, Sweden) from day 2-6 after fertilization is a so called defined medium, containing human serum albumin, all amino acids essential for blastocyst development, insulin, progesterone, oestradiol, and penicillin G. Most likely, these surplus proteins are the result of contamination from the human serum albumin. The influence of these contaminations on embryo development and how protein expressions differ between brands and batches is yet to be determined. A recent study comparing two different IVF culture mediums showed medium-dependent changes in gene expression of human preimplantation embryos.[201] Different albumin sources and other supplementations used in the culture mediums might have caused these changes, and the authors suggested that early adaptations of the preimplantation embryo to its milieu persist during foetal and post-natal development.[201]

Effect on the embryonic secretome

As a part of this study, the spent medium was collected at the end of culture and analysed for relative protein levels between arrested embryos and blastocysts of low- and high quality. We detected embryo secretion of nine proteins; IL-8, VEGF-A, PlGF, IL-6, EMMPRIN, EpCAM, HE4, CASP3 and CSTB.

The relative protein levels of VEGF-A, IL-6, EMMPRIN and PlGF differed between blastocysts and arrested embryos. Only a few growth factors have been identified to be expressed by the early human embryo, and one of them is VEGF. Embryos from the 3-cell stage up to the blastocyst express VEGF,[64, 202] and this early expression has been suggested to promote implantation of the embryo and its vascular development.[64] The expression of receptors for VEGF in the endometrium is well known.[203] The secretion of VEGF was higher in blastocysts than in the arrested embryos, and even higher in blastocysts of high quality compared to blastocysts of low quality. Presence of VEGF in early cleavage stage embryos and blastocysts was confirmed with IHC.

Similarly, IL-6 is produced by the growing embryo and blastocysts that secret IL-6 seem to implant to a higher extent than blastocysts that do not secret IL-6.[67] We found that IL-6 was secreted at a higher level in embryos that reached the blastocyst stage than in arrested embryos. IL-6 plays an important role in pre-implantation development of the embryo and there is growing evidence that it is of importance for blastocyst hatching,[204] implan-

tation, establishment of the placenta, and immune-tolerance of the pregnancy.[205] The IL-6 receptor is found in the blastocyst, on trophoblasts and the endometrium.[206]

We also found that EMMPRIN was secreted at a higher level in embryos that reached the blastocyst stage. EMMPRIN is a multifunctional transmembrane glycoprotein receptor with various binding partners.[207] A soluble form of EMMPRIN, which can be cleaved off from the full-length protein in the presence of matrix metalloproteinases (MMPs), has been described.[208] In line with our results, transcripts for EMMPRIN have been detected in human blastocysts,[209] and in mice it is highly up-regulated at this stage.[210] EMMPRIN is of importance in early pregnancy for adequate implantation, invasion and differentiation of trophoblasts[211] and most EMMPRIN null mice fail to implant.[212, 207]

Previous research has attempted to establish the role of PlGF for embryogenesis and implantation, but has failed to detect PlGF in spent culture medium from human embryos.[67] This is to our knowledge the first time that PlGF secretion from human embryos is reported, again, presumably due to the very high sensitivity of the proximity extension assay. A recent IVF-based study investigating the gene-expression profile of trophectoderm cells from day-5 blastocysts reported PlGF expression to be up-regulated.[213] A PlGF receptor, kinase insert domain receptor (KDR), was at the same time up-regulated in endometrial cells.[213] These findings suggest that PlGF have both an autocrine role in trophoblast function during early embryo development and a paracrine role in the subsequent angiogenesis of implantation and placentation, since trophectoderm cells at this stage were found devoid of KDR.[213, 214]

Finally, a number of proteins we expected to detect were not detected. These proteins include heparin-binding EGF-like growth factor (HB-EGF), transforming growth factor alpha (TGF-alpha), tumour necrosis factor (TNF, formerly known as TNF-alpha), and C-X-C motif chemokine 13 (CXCL13).[64] At the blastocyst stage, protein expression of HB-EGF, TGF-alpha and CXCL13 has been demonstrated. TNF has been detected at the 8-cell stage.[64] The lack of detection of these proteins could have several explanations. Potentially, embryos that are arrested in development may discharge proteases that could degrade secreted proteins, making the comparison between blastocysts and arrested embryos more complicated.

Alternatively, the previous detection of these proteins could be the result of cross-reactivity between the antibodies used in multiplex protein quantification applications. The proximity extension assay technique combines two dedicated antibodies with a qRT-PCR reaction to achieve high specificity, can be multiplexed without introducing cross-reactivity, and is thus a promising tool in the quest for biomarkers.[154, 155]

We conclude that the secretome differ between embryos that reached the blastocyst stage and those that arrested in development, and there was a dif-

ference in the level of VEGF-A between blastocysts of low- and high quality. However, this study should be considered as a pilot study that needs to be repeated not only with a prospective study design, but also with a larger sample size, limited to the nine proteins we found to be secreted by human embryos, and validated with IHC on all nine proteins.

Conclusions

The *HRG* C633T genotype appears to play a role if infertility is established. However, the *HRG* C633T SNP does not seem to associate with fertility *per se*.

The female *HRG* 633T genotype is associated with primary recurrent miscarriage.

HRG peptides corresponding to the region surrounding the *HRG* C633T SNP stimulate HEE cell migration and tube-formation. Furthermore, VEGF-A induced migration is inhibited by the HRG proline peptide. HEE cell proliferation is inhibited in presence of the HRG proline peptide as well as the serine containing peptide. Thus, the region surrounding the polymorphism appears to have an effect on different biological mechanisms *in vitro* on endothelial cells prepared from the endometrium. The HRG proline peptide has no apparent effect on human embryo development, quality, or protein secretion.

The male *HRG* C633T genotype is associated with semen characteristics among infertile men, where homozygous *HRG* 633T carriers overall have the lowest total sperm count, sperm concentration, motility score, and yield after preparation. Couples where the male partner is heterozygous for *HRG* C633T have the highest pregnancy rates. The lowest rates are achieved if the male partner is a homozygous *HRG* 633T carrier.

The early human embryo secretes proteins into culture media and the level of secretion is higher in embryos of higher developmental stages. These proteins are IL-8, VEGF-A, PlGF, IL-6, EMMPRIN, EpCAM, HE4, CASP3 and CSTB. The relative levels of four proteins, VEGF-A, IL-6, EMMPRIN and PlGF, differed between arrested embryos and blastocysts of high quality. The level of VEGF-A is further secreted to a higher degree in blastocysts of high quality than in those of low quality.

In conclusion, HRG seems to interact with pathways of importance for fertility. HRG, and mainly the region surrounding amino acid 204 in the protein, appear to be of relevance *in vitro* for regulation of HEE cell angiogenic abilities, a process that need to be well regulated for successful implantation and development of an embryo. It is furthermore shown that the male genotype

might be as important as the female genotype in fertility. For prediction, and to choose the best embryo for transfer in IVF, there are specific proteins of interest that associate with high developmental stages of the embryo. These proteins (especially the four that differed between arrested and high-quality embryos) might be biomarkers of interest for further investigation in the future.

Future perspectives

Infertility troubles millions of people worldwide and as many as one in five of these men and women never even get to know why they cannot get pregnant. Further knowledge of the aetiology and mechanisms behind this barrier to reproduction is essential for understanding and treating infertility.

Once infertility is established, there is help in the form of IVF but one third of couples remain childless after repeated treatments. In this group, the *HRG* C633T SNP might be of importance. Knowledge of a couple's *HRG* genotype could give better understanding of why some people do not succeed with their treatments, and a possibility for the medical doctor at the IVF centre to consult with the couple on their chances of becoming pregnant. The couple can then decide if they want to pursue with further IVF treatments, that can be demanding both mentally and physically, or if they want find other alternatives such as for example adoption. Similarly, in women diagnosed with recurrent miscarriage the HRG genotype could add to the diagnostic investigation. Perhaps in the future, a panel of polymorphisms may guide the clinicians and patients to the best treatment alternatives.

This was the first time that HRGs impact on male reproduction was investigated and further studies should be conducted in order to elucidate the mechanism behind the association between *HRG* C633T SNP and pregnancy success. To understand how the possible impact from *HRG* C633T SNP on sperm production and maturation is achieved, it is also important to further confirm the presence and location of HRG in the male reproductive tract.

This was also the first time that the combined genotype of the couple was investigated. The genotype of the embryo could be found out by collection of media during culture and analysis of whether it contains HRG with proline or serine. Techniques such as the proximity extension assay and antibodies for HRG with proline and HRG with serine could be used for detection. Investigating the genotype of the third part, the embryo, and its importance for implantation and a successful pregnancy, would be highly interesting. Disturbances in the molecular cross-talk between the embryo and endometrium during implantation are thought to explain why many IVF treatments are unsuccessful even though high quality embryos are transferred.[65] Since the early embryo secretes HRG prior to implantation, it might be of importance for this cross-talk whether the predominant or variant HRG protein is present.

For prediction, and to choose the best embryo for transfer in IVF, it is of great importance to add new tools to evaluate the developmental potential of embryos. However, before new techniques are implemented in daily clinical work, it is mandatory to show that such a new technology is sensitive, specific and reproducible. Analysis of proteins secreted into the culture medium surrounding the early embryo is an objective way to inform us about the physiology of the embryos. Proteins of interest are those that associate with high developmental stages of the embryo. We found four proteins that differed between arrested embryos and high quality blastocysts. One of the proteins, VEGF-A, could also distinguish between blastocysts of low- and high quality. These proteins and especially VEGF-A might be biomarkers of interest for further investigation in the future, and together with other proteins found in the literature, they could perhaps form a "reproduction panel". A complete "reproduction panel" should be able to discriminate at an early stage which embryos that have the potential to implant and subsequently develop into a healthy baby. For this to be implemented into practice large studies collecting culture medium from embryos in IVF treatment at least on a daily basis should be performed, with live birth as endpoint. Since proteins secreted by embryos exist at very low concentrations, such a panel of biomarkers should be run using a technique that is highly sensitive and specific, such as the proximity extension assay.[74, 80]

Summary in Swedish

Sammanfattning på svenska

Infertilitet definieras som utebliven graviditet efter ett år av regelbundet oskyddat samlag. Idag uppskattas det att ca 15% av alla par i reproduktiv ålder har svårigheter att bli gravida. Orsaken till varför paren inte blir gravida kan ligga antingen hos kvinnan eller hos mannen och i ungefär en tredjedel av fallen beror det på en kombination av faktorer hos båda parterna. I ungefär 20% av fallen förblir parets infertilitet oförklarad. Dessa par anses utgöra en heterogen grupp där bl.a. avvikelser i kroppens hormonsystem och immunsystem föreslagits kunna ligga bakom infertiliteten. Det kan även föreligga en genetiskt betingad obalans av de faktorer som behövs för att kunna bli gravida.

Paren kan ofta bli hjälpta med assisterad befruktning, sk *in vitro* fertilisering (IVF). Behandlingen är krävande och mer än 30% av par i Sverige får inte ens barn efter tre upprepade försök. För att öka lyckandefrekvensen behövs verktyg för att kunna välja det embryo som har störst chans att implantera och utvecklas till ett friskt barn.

Idag baseras denna selektion på hur embryot ser ut. En teknik som uppkommit är "time-lapse". Med denna teknik fotas embryot samtidigt som det växer i en inkubator på laboratoriet. Fotona sätts sedan ihop till en sekvens med vilken man bl.a. kan mäta tid till olika celldelningar och på ett bra sätt följa embryots utveckling. Många forskare är inriktade på att hitta biomarkörer hos embryot som kan hjälpa till i selektionsprocessen. Dessa markörer kan t.ex. vara proteiner som embryot syntetiserar och utsöndrar under de första dagarna efter befruktning. Mikromiljön kring könscellerna, embryot och fostret är viktig och för en lyckad graviditet och därför behöver miljön runt embryot vara optimerad även vid en IVF.

I den här avhandlingen har vi studerat plasmaproteinet HRG (histidinrikt glykoprotein) som påverkar ett flertal olika biologiska processer i kroppen genom att bilda komplex med andra proteiner och faktorer. I proteinet finns en genetisk variation, en singel nukleotid polymorfism (SNP) benämnd *HRG* C633T, som hos kvinnor visats påverka graviditetsutfallet vid IVF. I det första delarbetet studerades om denna SNP är kopplad till diagnosen upprepade missfall. Vi fann att kvinnor som aldrig fått barn i större utsträckning var homozygota för *HRG* 633T, dvs. hade fått den ovanligare T-allelen från båda föräldrarna. Vi såg även att kvinnor heterozygota för *HRG* C633T SNP

(en av vardera allel) var ovanliga i diagnosgruppen. I det andra delarbetet ville vi studera huruvida området kring SNPen i sig självt hade en påverkan på blodkärlsnybildning samt tidig embryoutveckling. Blodkärlsnybildning i livmodern är en ytterst central process för en lyckad implantation och placentation. Vi fann att regionen påverkade förmågan hos blodkärlsnybildande celler från livmodern att vandra, replikera sig samt skapa enkla blodkärl. Då HRG syntetiseras redan av det tidiga embryot var vår hypotes att denna SNP kan påverka tidig embryoutveckling. Tillsats av HRG-regionen till odlingsmediumet hade till synes ingen påverkan på det tidiga embryots kvalitet eller utveckling utvärderad med time-lapse teknik. Odlingsmedium från embryon som behandlats med HRG analyserades vart och ett med avseende på proteininnehåll och denna pilotstudie gav upphov till delarbete fyra. Av de proteiner som detekterades visades nio utsöndras av embryot själv, andra fanns i odlingsmediumet från början. Embryon som uppnått ett högre mognadsstadium (blastocyst) visade sig utsöndra högre nivåer av fyra proteiner jämfört med de som avstannat tidigt i utvecklingen. Ett av de mest centrala proteinerna för blodkärlsnybildning, VEGF, utsöndrades i högre grad av blastocyster av hög kvalitet jämfört med de av lägre kvalitet. VEGF kan därmed vara en potentiell biomarkör att undersöka vidare. HRG kunde inte visas påverka embryots tidiga utsöndring av proteiner. I det tredje delarbetet studerades frekvensen av *HRG C633T SNP* hos par som genomgick IVF behandling jämfört med fertila par. Här såg vi att mannens genotyp påverkade om paret blev gravida eller inte vid behandling. Bäst gick det för de par där männen var heterozygota för *HRG C633T* och sämst för par med män homozygota för *HRG 633T*. Vi säg även att spermiekvaliteten påverkades av vilken genotyp männen hade där homozygoti för *HRG 633T* gav sämst spermieprov. Kvinnans genotyp påverkade inte behandlingsutfallet.

Sammantaget visar delstudierna att HRG är av betydelse för chansen att bli gravid om en infertilitetsproblematik redan föreligger. En möjlig förklaring kan vara HRGs eventuella inverkan på blodkärlsnybildning. Patientnyttan är uppenbar då detta skulle kunna leda till ökad förståelse för varför vissa har lättare än andra att bli gravida med IVF behandling. Om screening av paren för *HRG C633T SNP* införs finns möjlighet för läkaren på IVF-kliniken att rådgöra och informera om sannolikheten att lyckas med IVF-försöket. Paret kan då välja om de vill investera stora pengar och mycket känslor i att försöka bli gravida eller om de väljer en annan lösning.

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