COMPONENT SCREENING FOR NOVEL XENO-FREE MEDIUM FOR HUMAN EMBRYONIC STEM CELLS

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1. Preface

This experimental study was performed at Regea Institute for Regenerative Medicine, University of Tampere. I want to thank my supervisors Heli Skottman and Kristiina Rajala for their help along the road. I also owe my gratitude to all the personnel of Regea, expecially to Heidi Hakala, for their help and continuous patience. I also want to thank my family and my boyfriend for supporting me through this project, this could not have been done without their help and understanding.

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Tiivistelmä:

Kantasoluja löytyy lähes kaikista aikuisen ihmisen kudoksista sekä myös kehittyvästä alkiosta. Alkiosta löytyviä kantasoluja kutsutaan ihmisen alkion kantasoluiksi (hES-solut). Nämä hES-solut ovat erilaistumattomia soluja, jotka voivat uudistua pitkiä aikoja solujakaantumisen avulla ilman, että niiden karyotyyppi muuttuu mutta ne voivat myös erilaistua kaikiksi ihmisen kehosta löytyviksi solutyypeiksi. Näin hES-solut tarjoavat erittäin lupaavia mahdollisuuksia ja työkaluja tulevaisuuden lääketieteelle, jossa voidaan vahingoittunut kudos korvata uusilla kantasoluista erilaistetulla soluilla tai kudoksilla. Perinteisesti hES-soluja on kasvatettu hiiren fibroblastien (tukisolujen) päällä ja kasvatusliuoksessa, johon tukisolut ovat erittäneet erilaisia kasvutekijöitä. Jotta hES-soluja voitaisiin käyttää lääketieteellisissä sovelluksissa, on erittäin tärkeää, että mahdollinen kasvatusliuoksessa ja tukisoluissa olevista eläinpatogeeneistä ja proteiineista johtuva immuunihyljintä tai infektio voidaan välttää. Siitä huolimatta, että tutkijat käyttävät nykyään ihmisperäisiä tukisoluja, eläinperäisiä aineksia silti vielä yleisesti käytetään hES-solujen kasvatusliuoksissa. Tutkijoiden panostuksesta huolimatta täysin toimivaa eläinperäisista ainesosista vapaata kasvatusliuosta ei ole vielä kehitetty.

Tämän tutkimuksen tarkoituksena oli parantaa itsevalmistetun kasvatusliuoksen koostumusta testaamalla erilaisia lisäaineita. Polyvinyylialkoholia testattiin kasvatuslioksessa ihmisen seerumin albumiinin sijasta. Myös transferriinin ja fifbroblastien kasvutekijän (bFGF) suurempia pitoisuuksia ja sitä kuinka ne vaikuttavat hES-solujen ominaisuuksiin ja kasvuun, testattiin. Lisäksi havainnoitiin, kuinka aktiviini A, sfingosiini 1-fosfaatti, rekombinanttinen ihmisen verihiutaleista peräisin oleva kasvutekijä AB, aminohapot, tiamiini, glutationi, insuliini ja γ-sekretaasi inhibiittori IX vaikuttavat hES-solujen erilaistumattomaan kasvuun.

Tutkimuksen aikana eri kasvatusliuoksissa kasvetettujen hES-solujen muoto-oppia, kiinnittymistä ja kasvua tarkkailtiin mikroskooppisesti aina ennen niiden jakamista. Eri kasvatusliuoksissa kasvatettujen solukolonioiden erilaistumattomuus varmistettiin immunosytokemiallisesti hyväksikäyttäen markkereita, jotka kiinnittyvät vain erilaistumattomiin hES-soluihin (SSEA-3, SSEA-4, TRA-1-60, TRA-1-81). Näiden tulosten perusteella kaikki värjätyt solu koloniat olivat erilaistumattomia. Solujen erilaistumiskyky varmistettiin erilaistamalla soluaggregaatteja eli ns. embryoid body-rakenteita, joiden geenien ilmentymistä selvitettiin RT-PCR:llä. Näin selvitettiin löytyikö kolmen eri alkion kerroksen osoittamia geenejä. Tulokset osoittivat, että solut säilyttivät täydellisen erilaistumiskyvyn hES- kasvatusliuoksessa, mutta vain yhdessä testi liuoksessa.

Tämän tutkimuksen pohjalta lupaavimpia kasvatusliuoksen lisäaineita olivat aminohapot, tiamiini ja glutationi. hES-solut, jotka kasvatettiin kasvatusliuoksissa, jotka sisälsivät erilaisen sekoituksen näitä lisäaineita, muodostivat isoja solukolonioita sekä pysyivät erilaistumattomina pisimpään verrattuna kontrolli kasvatusliuokseen. Solut, jotka kasvatettiin kasvatusliuoksessa, jossa ei ollut aminohappoja tai kasvatuslioksessa, jossa ei ollut glutationia, erilaistuivat 16 jakovälin jälkeen, kun taas tiamiinia sisältämättömän kasvatusliuksen solut pysyivät erilaistumattomina 15 jakoväliä. Näiden solujen kiinnittymis-prosenttiosuudet jaon jälkeen olivat suurimmat ja verrattuna kaikkiin kolonioihin sisälsivät eniten erilaistumattomia kolonioita. Myös kasvatusliuos, joka sisälsi aminohappoja liuotettuna sekä veteen että vetykloridiin, tuki hES-solujen erilaistumatonta kasvua hyvin (12 jakoväliä) ja näin osoitti aminohappojen lisäyksen tarpeellisuuden. Tämän tutkimuksen tulokset ovat hyvin lupaavia mutta tarvitaan silti vielä lisää perinpohjaista tutkimusta uusilla ainesosilla, jotta voidaan kehittää toimiva kasvatusliuos hES- soluille.

Avainsanat: Ihmisen alkion kantasolut**,** eläinaineksista vapaa, seerumin korvike, hES-solujen kasvatus, activiini A sfingosiini 1-fosfaatti, γ-sekretaasi inhibiittori IX, rekombinattinen ihmisen verihiutaleista peräisin oleva kasvutekijä AB, polyvinyylialkoholi

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Abstract:

Stem cells can be found in almost every tissue in adult human being and in developing embryo. Stem cells from the inner cell mass of an early embryo are called embryonic stem cells (hESC). These hESC are unspecialized cells that can renew themselves for long periods through cell division while maintaining normal karyotype but they can also differentiate into all cell types found in the human body. Therefore they offer a highly promising tool for future regenerative medicine to replace damaged tissue with new one differentiated from stem cells. Traditionally hESC have been cultured on mouse feeder cells and using a serum containing culture medium conditioned by feeder cells. But in order to use hESC in clinical applications it is crucial to avoid the possible immune rejection or infection caused by animal pathogens and proteins present in medium and in feeders. However, even though researchers nowadays use human derived feeder cells, animal derived components are still generally being used in hESC culture. But, despite of the huge efforts researchers have put in no functional xeno-free culture medium has been invented.

The aim of this study was to test different components with a house-made medium developed for hESC in order to improve the current formulation. Polyvinylalcohol was tested in place of human bovine serum albumin and the effect of increased concentration of transferrin and basic fibroblast growth factor (bFGF), into hESC characteristics and cell growth was studied. In addition, the following growth factors and components were tested: activin A, sphingosine 1-phosphate, recombinant human platelet derived growth factor AB, amino acids, thiamine, glutathione, insulin and γ-secretase Inhibitor IX, to observe how these affect the undifferentiated growth of hESC.

During the study morphology, attachment and growth of hESC colonies cultured in different test media were judged under a microscope before passaging. Human ESC cultured in different test media were characterized with immunocytochemistry using markers specific for undifferentiated cells (SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81). All stained colonies proved to be undifferentiated based on these staining results. The pluripotency of the hESC was determined with embryoid body cultivation in test culture media, following RNA isolation and RT-PCR. The results showed that the cells cultured in control hES medium but only in one test medium sustained perfect pluripotency.

According to this study, the most promising additives proved to be amino acids, thiamine and glutathione. Cells grown in media containing different mixture of them did grow well and also sustained undifferentiated growth for the longest time compared to colonies cultured in control medium. The cells cultured in media containing no amino acids or no glutathione did differentiate at passage 16 while cells grown in medium containing no thiamine sustained undifferentiated status until passage 15. The attachment rates of these cells also were the highest. And when the amount of undifferentiated cell colonies where compared to all colonies together, colonies grown in these media did have the highest amount of undifferentiated colonies. Also, the medium containing amino acids dissolved both in water and in HCl, did sustain the undifferentiated growth of hESC well (12 passages), and so proved the necessity of the addition of amino acids into culture medium. The results of this study are highly promising but it is still needed some more thorough investigation with new additives in order to create functional xeno-free culture medium for hESC.

Keywords: Human embryonic stem cells, xeno-free, Serum Replacement, Culture of hESC, Activin A, Shingosine 1-phosphate, γ-secretase Inhibitor IX, Recombinant human Platelet Derived Growth Factor AB, Polyvinylalcohol

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Abbreviations

BSA	bovine serum albumin
cDNA	complementary DNA
EB	embryoid body
ESC	embryonic stem cells
FBS	fetal bovine serum
bFGF	basic fibroblast growth factor
hESC	human embryonic stem cell
hFF	human foreskin fibroblast
HSA	human serum albumin
ICM	inner cell mass
MEF	mouse embryonic fibroblasts
mESC	mouse embryonic stem cell
PBS	phosphate buffered saline
PDGF-AB	human platelet derived growth factor AB
PVA	polyvinyl alcohol
RT-PCR	reverse transcriptase polymerase chain reaction
S1P	sphingosine 1- phosphate
SR	serum replacement
SSEA-1	stage-specific embryonic antigen-1
SSEA-3	stage-specific embryonic antigen-3
SSEA-4	stage-specific embryonic antigen-4
TGFß	tumor growth factor-ß
TRA-1-60	tumor- related antigen-1-60
TRA-1-81	tumor-related antigen -1-81
WNT	Wilms tumor gene-1
γ-sec. inh.	γ- secretase Inhibitor IX

1.1 Stem Cells

1.1.1 Types of Stem Cells

Stem cells have the ability to self-renew indefinitely and to differentiate to various kinds of cell subtypes. These unique stem cells can be derived from some adult tissues, including the pancreas, liver, nervous system, gut and epithelium of vascular system, bone marrow, adipose tissue and also from roots of tooth. These are called somatic stem cells. Stem cells are also present in fetal and in embryonic tissues and these stem cells found in human embryos are called human embryonic stem cells (hESC).

The difference between somatic and embryonic stem cells (ESC) is in their differentiation capacity. ESC are pluripotent (able to differentiate into any cell subtype in the human body) while there is no precise knowledge of that capacity in somatic stem cells which are however, thought to be multipotent (able to differentiate to different kinds of cells origin from the same embryonic germ layer). But there is some evidence that somatic stem cells also could differentiate to cell types origin of other tissue types derived from other embryonic germ layer but this has not yet been totally proven (Chiu and Rao 2003, Battula et al., 2007).

Although it is commonly known that there are many stem cell populations present in embryos and their roles in normal development have been studied well, their clinical advantages have not been widely studied because of ethical issues and also due to public opinion concerning the use of cells from embryos. In addition, in some countries there are laws prohibiting the use of hESC for research purposes (Robertson, 2001), but in Finland it is permitted with special permission. With somatic stem cells there are no such ethical issues and therefore the research around them has been and is still plentiful (De Sousa et al., 2006, Montarras et al., 2005, Salter, 2007).

Adult tissues with a high turnover rate (such as blood) are maintained by tissue-specific somatic stem cells. The role of stem cells in adults is to sustain a repertoire of cell types in essentially steady- state numbers over the whole life of an organism (Chiu and Rao 2003) and these stem cells rarely divide by themselves. Maintenance of these cells depends on infrequent divisions, where one daughter cell remains a stem cell and the other enters an amplifying population, whose rapid proliferation and ultimate differentiation maintains the

tissue in the required condition. The division may occur, for example, according to tissue injury (Zwaka, and Thomson, 2005), Figure 1.

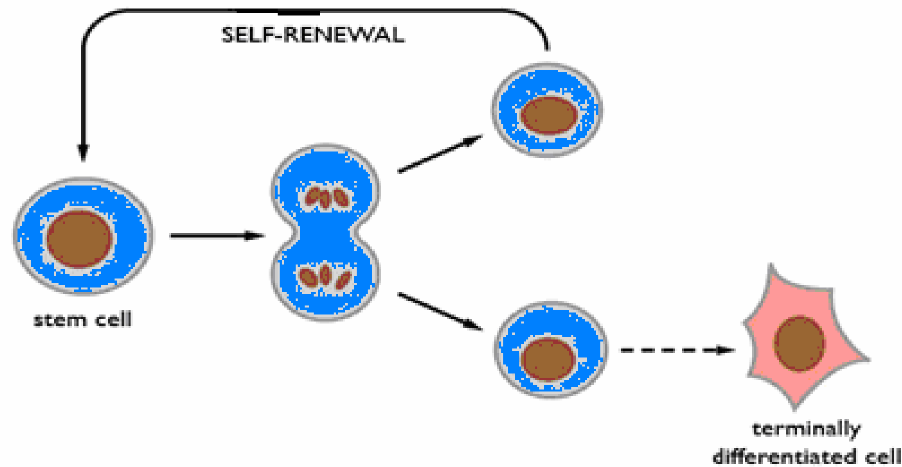

Figure 1. Self-renewal capability of a stem-cell (Figure modified from Alberts et al., 2002).

When somatic stem cells are cultured *in vitro* it is possible to get various kinds of replacement cells for future therapeutic applications. These stem cells can be used without any concerns of immunogenity issues especially when transplantable cells are from the same person as they are intended to be transplanted in (Amit et al., 2000, Cooper et al., 2000, De Sousa et al., 2006). There are no therapies with hESC available at the moment but somatic stem cells have been used for clinical approaches for many decades. For example, human adult mesenchymal stem cells from bone marrow have been used in treatments of various haematological disorders for over 40 years. The hematopoietic stem cell fraction of the bone marrow is responsible for the production of over 90% of all the body's blood cells. Hematopoietic stem cells have an extensive potential of self-renewal and also, according to recent studies, they are capable of differentiating into other lineages of cells, including cardiac phenotype (Jackson et al., 2001, Minguell et al., 2001).

Stem cells have also been isolated from human term placenta and these cells are called human placenta- derived multipotent cells. These stem cells have a variety of advantages: they are easily accessible, no invasive procedure is necessary to obtain the needed organ, since the placenta is expelled after the birth; there is no ethical concerns like with hESC because the placenta would be discarded postpartum anyway. Even though the differentiation and proliferative potentials of the placenta- derived cells are not as wide as

that with hESC, the cells are still fetal origin and may be superior compared to somatic stem cells (Yen et al., 2005). Yen and co- workers showed with immunocytochemistry that placenta- derived stem cells exhibit the same surface markers, which indicate an undifferentiated state, as the hESC: stage-specific embryonic antigen 4 (SSEA-4), tumor-related antigen-1-60 (TRA-1-60) and TRA-1-81. They also succeeded in differentiating adipogenic, osteogenic and neurogenic cells from placenta- derived stem cells. The detection of surface markers might suggest that these cells have a very primitive origin and that it may well be that the renewal and differentiation capacity of placenta- derived stem cells is better than of somatic stem cells (Chang et al., 2007, Delo et al., 2006).

There is also some evidence that somatic stem cells have two biologically different phenomena which have major public health influences: aging and cancer and maybe in the future these can be helped with stem cells (Bell, and Van Zant, 2004, Tam et al., 2007).

1.2 Human Embryonic Stem Cells

The first studies with ESC were carried out with rabbit blastocysts in the 1960s by Cole and collaborators (Cole et al., 1965, Cole et al., 1966). Although these studies were groundbreaking and paved the way for future research, later studies have been focused on mice and the first murine counterparts were derived in the 1980s (Gordon et al., 1980). The murine embryonic stem cells (mESC) are isolated from inner cell mass (ICM) of early pre-implantation embryos (Martin et al., 1987, Reubinoff et al., 2000, Tsung, and Yao, 1987). And most of the methods created for murine stem cells are also widely used for hESC even today (Thomson, and Marshall, 1998).

One reason what makes hESC so interesting for clinical research purposes is that under certain physiological or experimental conditions they can be induced to become cells with special functions. For example, cells can be induced to become beating cells of heart muscle, the insulin-producing cells of pancreas, action potential producing nervous cells or cells of liver (Jiang et al., 2007, Lee et al., 2007, Lu et al., 2007, Mummery, 2007, Zimmermann, and Eschenhagen, 2007). Many times the special diseases, such as Parkinson’s disease, in humans derive from malfunction, injury or death of just one cell or cell type in an organ or in a tissue. If these cells could be removed and changed, the disease could be cured permanently and with stem cells it is possible. Transplantable cells can be

somatic or ESC derivatives and these cells can also be gene manipulated outside the patient before transplantation if necessary (Bell, and Van Zant, 2004, Tam et al., 2007). Human ESC-derived tissue- specific cells may have therapeutic potential for the treatment of, for example, Parkinson’s disease, spinal cord injury, heart disease, diabetes and other degenerative conditions. Addition to transplantation medicine, hESC lines can also be useful in research of human developmental biology and drug discovery (Hyslop et al., 2005, Lumelsky et al., 2001, Semb, 2005).

Totipotent stem cells are produced when an egg and sperm undergo fusion in a fertilization event to form a zygote. This zygote has the necessary ability to create all the cells of the whole human body and also of the placenta. However, this capacity is limited in time and the cells in the human zygote are totipotent only during the first few divisions of a fertilized egg, which divides every 18 to 24 hours. Approximately 30 hours after fertilization the fertilized egg divides into two cells and cleavage- state begins. After sufficient mitotic divisions the cells group together and form totipotent cells in a construction called blastomere (Cowan et al., 2004, Jones, and Thomson, 2000).

On the third or fourth day after the fertilization the embryo reaches the 16- cell stage and soon after that it is called a morula. Next the cells in the morula start to differentiate and they form a fluid- filled phase called the blastocystic cavity. At that stage the embryo is called a blastocyst and it has three structures called the trophoplast, blastocoel and the ICM. The trophoplast is the outer layer of cells that surrounds the blastocyst, the blastocoel is the cavity inside and the ICM is a mass of cells at one end of the blastocoel. The cells of the ICM are now pluripotent stem cells meaning that they can give rise to any cell type in the human body except cells in the placenta or other supporting tissues of the uterus (Bradley et al., 2002). Pluripotent hESC are derived from totipotent cells of ICM of a 5-6 day old early embryo, Figure 2.

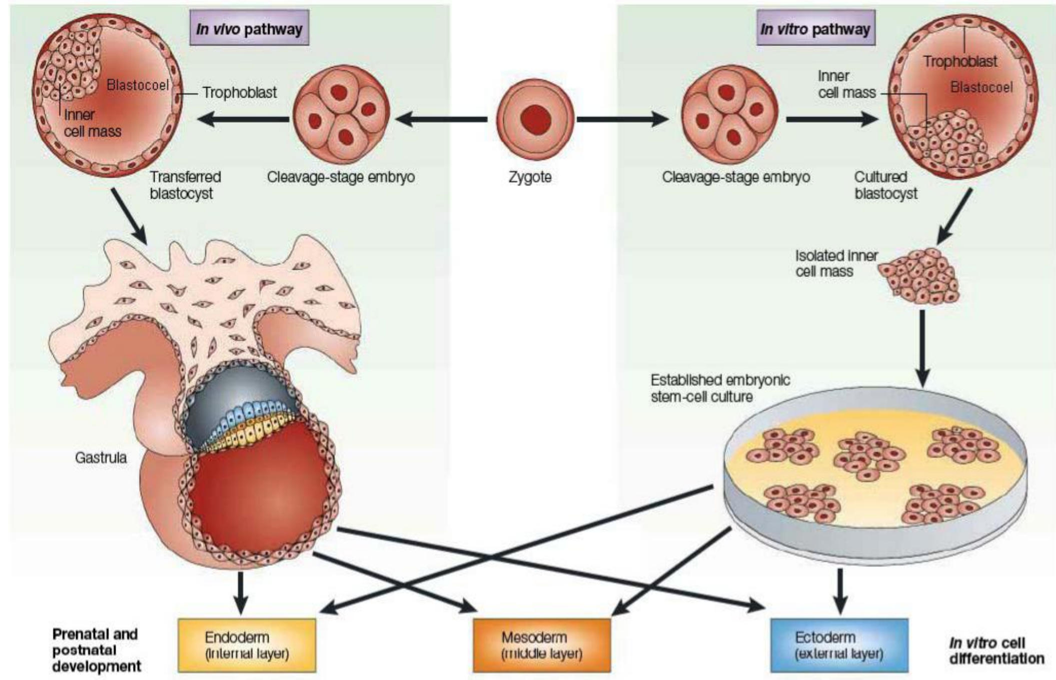

Figure 2. Two alternative fates for a fertilized zygote *in vitro* and *in vivo*. When blastocyst enters the uterus, it will become a new human baby *in vivo*, but *in vitro* it is possible to establish a continuous culture of stem cells of these ICM cells (Figure modified from Bradley et al., 2002).

Embryos used for research purposes are produced using *in vitro* fertilization for clinical purposes of couples struggling with different kinds of severe fertilization problems. The embryos donated for research can be of a quality so bad that they can not be transplanted into the uterus. But the embryos donated are usually surplus ones unnecessary for couples that have enough children.

Donated embryos can be frozen or fresh and this has no significant influence on making a stem cell line of them. The donated embryos usually are cultured until the blastocyst stage *in vitro* after which the trophoectoderm is removed and ICM is isolated. The ICM cells are cultured further in the right conditions in order to derive a stem cell line (Rodriguez et al., 2006, Thomson et al., 1998, Thomson, and Marshall, 1998).

1.2.1 Characterization of Human Embryonic Stem Cells

Human ESC have many essential characteristics: they are derived from preimplantation embryos, are capable of prolonged undifferentiated proliferation in culture and they have the ability to differentiate to trophoplast and also are capable of forming derivatives of all three embryonic germ layers. These include gut epithelium derived from endoderm; cartilage, smooth- and striated muscle, bone derived from mesoderm and also

embryonic ganglia, neural epithelium and stratified squamous epithelium which is derived from ectoderm (Amit et al., 2000, Cowan et al., 2004, Draper et al., 2004). Human ESC also have a high expression of enzyme telomerase (see review Hiyama, and Hiyama, 2007) and they also possess certain types of surface markers. Embryonic stem cells also can create teratoma when transplanted in mice proving their pluripotent capacity. Human ESC form relatively flat and compact colonies with distinct cell borders *in vitro* and grow more slowly than mESC, with a population- doubling period of ~36 hours (Cowan et al., 2004, Jones, and Thomson, 2000, Thomson et al., 1998).

Of all the approaches to define and characterize ESC, perhaps the most versatile is that based on the surface antigen phenotype of cells. Human ESC express cell surface markers characteristic for primate undifferentiated ESC. These markers include SSEA-3, SSEA-4, TRA-1-60, TRA-1-81 and alkaline phosphatase (Henderson et al., 2002). SSEA-3, SSEA-1 and SSEA-4 are glycolipids in cell surface while TRA-1-60 and TRA-1-81 are keratin sulphate proteoglycans. All these marker molecules are differently expressed in different cells: SSEA-1 in differentiated cells and SSEA-4, SSEA-3, TRA-1-81, TRA-1-60 and alkaline phosphatase in undifferentiated cells (Cooper et al., 2002, Thomson, and Marshall, 1998).

Subsequent research of hESC have pointed out that their surface antigen phenotype closely resembles that of human embryonic carcinoma cells and differs from murine counterparts. Surface markers for different differentiation stages are very useful in separation of undifferentiated cells from their spontaneously differentiated derivatives, for example, in stock cultures (Badcock et al., 1999, Mitsui et al., 2003) making the handling of these cells easier on a larger scale.

Nevertheless, the function of many of these surface antigens is still unclear, the glycolipid antigens (SSEA-1, SSEA-3, SSEA-4) present an enigma: despite of clear strong developmental regulation, studies with these antigens' inhibitors do suggest that their expression is not required for normal cell function or for normal embryonic development (Draper et al., 2004).

The transcription factors like Oct-3/4 and Nanog are also very important and useful in the characterization of hESC. They participate in maintaining the renewal of hESC and they are expressed differently in undifferentiated and differentiated pluripotent

cell populations in hESC both *in vivo* and *in vitro* (Chambers et al., 2003, Reubinoff et al., 2000).

1.3 Culturing of Human Embryonic Stem Cells

1.3.1 Immunogenic issues

For future clinical applications, hESC should be cultured under well- defined conditions, preferably in a xeno- free culture system, where exposure to animal- derived substances can be totally avoided. To be able to use hESC in human therapy applications, an animal- free culture system must be used, in order to prevent exposure to animal retroviruses and other pathogens which can cause some severe problems after transplantation (Amit et al., 2003). One very important of these pathogens is murine leukemia virus which can be found in almost all murine cells and also in mouse embryonic fifbroblasts (MEFs) which traditionally have been used in the culturing of hESC. MEFs have been used as supporting cells beneath the hESC. This endogenous murine leukemia virus is known to infect human cells and to cause diseases, including leukemias. However, even if the hESC have been in close contact with MEFs for a long time there is no evidence of infection from these viruses (Amit et al., 2005).

Another major immunogenic concern is the presence of non-human sialic acid N-glycolylneuraminic acid (see review Nasonkin, and Koliatsos, 2006). Sialic acids are a family of acidic sugars which are displayed on surface of all cell types. In mammalian cells the two most common of these sialic acids are N-glycolylneuraminic acid and N-acetylneuraminic acid, with N-acetylneuraminic acid being the metabolic precursor of N-glycolylneuraminic acid. Human ESC are genetically unable to produce N-glycolylneuraminic from N-glycolylneuraminic but they can transfer it from culture medium containing animal- derived products. Most healthy humans have circulating antibodies in their sera against this sialic acid leading to an immune response (Martin et al., 2005).

A possible cause for the rejection of hESC after transplantation may also be their major histocompability complex differences between graft and host. These problems are the same as those for tissue allografts obtained from conventional sources. But it has been proven that even if hESC do express class I antigen molecules on their surfaces, this expression is very low (see review Bradley et al., 2002). Drukker and co- workers (2006)

proved in their study with mice that although major histocompability complex I levels are sufficient for rejection by cytotoxic T cells in the human body after transplantation, the immunostimulatory capacity of hESC is very low. So, they concluded that as compared with conventional allografts or with somatic stem cells, hESC have reduced immunogenity and do not cause direct allorejection (Drukker et al., 2006). But there is many reseach results that are contradictory to Drukker`s conclusion. For example, Grinnemo and colleagues (2006) showed in their study that hESC are immunogenic in allogeneic and also xenogeneic settings (Grinnemo et al., 2006).

1.3.2 Feeder Cells

After the successful studies with mESC, Thomson's group was the first to derive a permanent hESC- line in 1998 in Wisconsin, Madison (Thomson et al., 1998). Thomson and colleagues used culture medium which contained fetal bovine serum (FBS) and mitotically inactivated MEFs as feeder layer beneath the stem cells. Fibroblasts are believed to secrete some still unknown factors into the medium and provide the necessary attachment site for the undifferentiated hESC (Amit et al., 2003). The molecular mechanism under how MEFs sustain hESC proliferation in undifferentiated state is still unclear, but it has been proposed that MEFs secrete some factors that enrich the medium and that they also form an extracellular matrix and therefore can offer a surface into which hESC can bind. They also possibly somehow detoxify the culture medium (Cooper et al., 2000, Chiu and Rao 2003).

To prevent the possible infections with animal viral or bacterial infections and with other possible causes, hESC have been cultivated on human feeder layers, such as fetal muscle, fetal skin, adult fallopian tubal epithelial cells, foreskin fibroblasts, and adult bone marrow cells (Lee et al., 2005). Additionally, also Richards and colleagues (2003) have tested many different kinds of human feeder cells derived from adult (lung, skin, muscle, endometrium), and neonatal (foreskin) tissues (Richards et al., 2003), and some of these cells are now available for commercial use (Richards et al., 2003). In 2002 they tested fetal muscle, fetal skin and human adult fallopian tubal cells and found that they are clearly superior to feeder- free matrices and that they support the undifferentiated growth of hESC (Richards et al., 2002).

An autogeneic feeder system has been successfully used for growing undifferentiated hESC over more than 44 passages. These feeders are derived from spontaneously differentiated hESC into cells with fibroblast- like morphology. Fibroblast-like cells with real morphology are separated from other cell types and cultured forward and then used as a feeder cell layer for hESC (Stojkovic et al., 2005).

1.3.3 Feeder Free Culture

Even without the immunogenicity issues with feeder cells, the problems of the laborious work load and scaling up the hESC culturing processes would still remain unsolved and should be overcome before clinical trials. Another problem with feeder layer may be that it can be an unwanted source of variability in experimental studies. So research groups worldwide have been developing different kinds of feeder- free systems to solve all these issues.

Many possible answers to these problems have been demonstrated and one of these is Matrigel. Matrigel is the manufacturer's trademark for extracellular matrix, which has been isolated from Engelbrecht-Holm-Swarm tumor cells but it still may contain mouse derived ingredients, and therefore it is not the best choice for clinical applications. Matrigel contains mostly laminin and collagen and it has been extracted in a way that avoids the lot-to-lot variations. Matrigel has been proven to support the undifferentiated growth of hESC in medium conditioned by MEFs (Xu et al., 2001). Xu and co- workers (2001) have also examined individual basement membrane components such as laminin, collagen IV and fibronectin individually but these did not prove themselves equal to Matrigel, which is a mixture of these.

Amit and co- workers (2004) have demonstrated that hESC can also grow on fibronectin coated plates in presence of transforming growth factor ß (TGFß) and basic fibroblast growth factor (bFGF). Fibronectin is a basal lamina component, which increases cell adhesion to the culture dishes and so increases the growth of hESC (Schuldiner et al., 2000). Beattie and colloquies have shown the undifferentiated growth of hESC on laminin in presence of activin A over more than 20 passages in continuous culture (Beattie et al., 2005).

Even if there are many available methods that are useful in some research group, they do not work in another study. So, more investigation is still needed in order to create universal method that supports long lasting growth of hESC.

1.3.4 Culture Medium of Human Embryonic Stem Cells

Even nowadays, animal- derived culture medium presents the possible hazard of animal- derived immunoreactive components. This should be prevented by removing or replacing these unwanted substances by human- derived derivatives in order to use hESC in clinical purposes in the future (Cabrera et al., 2006).

Traditionally, hESC have been cultured in medium containing fetal bovine serum (FBS) and conditioned by feeder cells used to supplement cell culture medium (Thomson et al., 1998). FBS can contain many beneficial and important compounds to hESC but also detrimental ones. Also, the concentrations of all the compounds can vary from batch to batch and make comparing of experimental results very difficult. The composition of conditioned medium is not exactly known which makes it difficult to replace animal- derived substances with human derived ones and to scale up the process (Skottman, and Hovatta, 2006). However, many proteins of feeder conditioned medium have been identified so far, for example Chin and her research group profiled Insulin-like growth factor binding protein 5, Pigment epithelium derived factor, Plasminogen activator inhibitor, Transcobalamin II and so forth (Chin et al., 2007). Also Lim and co- workers (Lim, and Bodnar, 2002) were able to identify a total of 136 proteins, and Prowse and collegues (Prowse et al., 2005) 102 identities present in culture medium conditioned by MEFs.

Currently FBS has been replaced with human serum in the culture medium. However, also human serum contains undefined components and is variable in quality and it may possibly contain some human derived pathogens. Therefore not being the best choice for hESC culture (Rodriguez et al., 2006). Human serum may also contain some factors that have an influence on hESC differentiation and it has been shown that the prolonged use of human serum in hESC culture medium can lead to increased differentiation rates (Koivisto et al., 2004, Richards et al., 2002, Richards et al., 2003).

Another method in replacing the FBS in media is to use Serum- Replacement (SR) which is a commercial serum replacer from Gibco. However, some supplements of

SR are still animal- derived, such as bovine serum albumin (BSA) and transferrin so some efforts are still needed to develop an optimal totally animal- free medium (Ding et al., 2006, Hoffman, and Carpenter, 2005, Li et al., 2005).

Amit and colleagues have successfully cultured hESC in feeder- and serum- free conditions (Amit et al., 2004). Their medium contained 20% SR and a combination of different growth factors, including tumor growth factor ß1 (TGFß1), leukaemia inhibitory factor and bFGF. Several research groups have reported different kinds of medium supplements in successful culture studies with hESC: bFGF (Ding et al., 2006), platelet derived growth factor (PDFG) with sphingosine-1-phosphate (S1P) (Pebay et al., 2005), Wnt, April/BAFF, insulin (vital growth factor for humans), transferrin, albumin (functions as carrier protein, antioxidant) and cholesterol (required for all mammalian cells, precursor of steroid hormones) (Lu et al., 2006). The concentration of bFGF seems to be a very important factor in supporting and maintenance of undifferentiated hESC and its effective amount has been shown to be 4ng/mL or 8ng/mL (Li et al., 2005, Inzunza et al., 2005). Pebay and co- workers (2005) showed in their study that co- incubation of both PDGF (20ng/ml) and S1P (10µM) together in culture medium successfully maintains hESC in an undifferentiated state. There were no significant results when hESC were cultured in medium containing just one of PDGF or S1P alone but together they inhibit the spontaneous differentiation of hESC in the absence of serum. The S1P receptor has been implicated in regulating smooth muscle cell migration and proliferation (Waters et al., 2003). PDGF can activate sphingosine kinases resulting in the transient increase in intracellular S1P concentration and so is responsible for cell proliferation and survival in different cell types via activating second messengers in MAPK signalling pathway (Pebay et al., 2005).

Lu and co- workers (2006) have developed a simple defined culture medium (Hesgo) which is animal component free. They showed in their study that Hesgo medium can sustain undifferentiated growth of hESC and cells cultured in it can easily be induced to differentiate into different cell lineages.

At the same time as Lu and co- workers (2006) reported their successful studies with Hesgo medium, Ludwig and colloquies (2006) reported their own defined- medium called TeSR1. TeSR1 medium is a complex mixture composed of vitamins, antioxidants, salts, trace minerals, lipids, albumin, detergent, GABA, piperolic acid, TGF, lithium

chloride and bFGF. It uses a combination of collagen, fibronectin, laminin and fibronectin as supporting matrixes. Ludwig and co- workers also reported that optimal culture conditions for cells in TeSR1 medium require pH 7.2, osmolality of 350nanoosmoles and gas atmosphere of 10% of carbon dioxide and 5% of oxygen (Ludwig et al., 2006).

Although, even if there are some culture media commercially available for hESC, none of these is totally functional and xeno- free. So, more investigation still needs to be done in order to create one.

1.3.5 Signalling Pathways

Maintenance of hESC pluripotency is still not clear but it is obvious that it confers many different signalling cascades that work interconnectally inside the cells. The better we understand these factors influencing differentiation events inside the cells the better we can keep them in the state we require. Major signalling pathways have been discovered and these include Activin/ Nodal (Parisi et al., 2003), bFGF (Greber et al., 2007) and Wilms tumor gene 1 (WNT) (Kleber, and Sommer, 2004) pathways. Activin/ Nodal signalling can block neuronal differentiation and so their action is necessary in maintaining the pluripotent status of hESC. WNT signalling is so far the only pathway reported to be active in maintaining pluripotency in stem cells but neither Nodal, Activin nor WNT is sufficient to sustain long- term undifferentiated growth of hESC *in vitro* alone but they work together with other routes (James et al., 2005). Several studies suggest that bFGF and TGFß1 could be potential regulators of these mechanisms, but the function and the interactions of these pathways still remain unclear (Amit et al., 2004, Beattie et al., 2005, Moon et al., 2002).

Activin/ Nodal/ Tumor Growth Factor-ß Signalling

Nodal and Activin are members of the TGFß superfamily, which contains about 40 ligands in humans. TGFß superfamily is important in tissue regeneration, cell differentiation, embryonic development and regulation of the immune system. Enrichment of TGFß superfamily ligands in stem cells suggest that these proteins are part of critical pathways used in these cells (Tabibzadeh, and Hemmati-Brivanlou, 2006). TGFß superfamily members signal via membrane- bound heteromeric serine- threonine kinase receptor complexes. When ligand binds type II receptor this activation leads to phosphorylation of the type I receptor and cytoplasmic protein substrates of the Smad

family. Following phosphorylation and oligomerization, the latter moves into the nucleus to act as a transcription factor to regulate the target gene expression with other factors (Gadue et al., 2006, Noggle et al., 2005, Valdimarsdottir, and Mummery, 2005).

TGFß responses are not solely the result of the activation of Smad cascade. They are highly cell- type specific and dependent of interactions of Smad signaling with a variety of other intracellular signalling mechanisms, initiated or not by TGFß itself, that may either potentiate, synergize, or antagonize, the rather linear TGFß -beta/ Smad pathway (Javelaud, and Mauviel, 2005), Figure 3.

Nodal and Activin signalling establish in normal human embryo the embryonic axes, determine left- right symmetry, induce meso- endoderm and also pattern the nervous system (Schier, 2003). Nodal and Activin act by binding to heterodimeric complexes between type I (Alk4 and Alk7) and II (ActRIIB) Activin reseptors, which in turn act through the Smad signalling pathway (Smad 2 and 3) by phosporylating. After Smad2 phosporylation and association with Smad4, it travels into the nucleus where it regulates gene expression. Activin and Nodal share type I and type II receptors and have the same Smad signalling pathway, whereas TGFß preferentially uses the TGFß receptors (Vallier et al., 2005, Xiao et al., 2006).

Vallier and co- workers (2005) showed in their study that over expression of Nodal in hESC can block neuroectodermal differentiation during embryo body formation. They also showed that Nodal is endogenously expressed in hESC and its expression fastly decreases in differentiation. Vallier's group also suggested that Activin alone can prevent differentiation for short culture period in the absence of other TGFß ligands or bFGF.

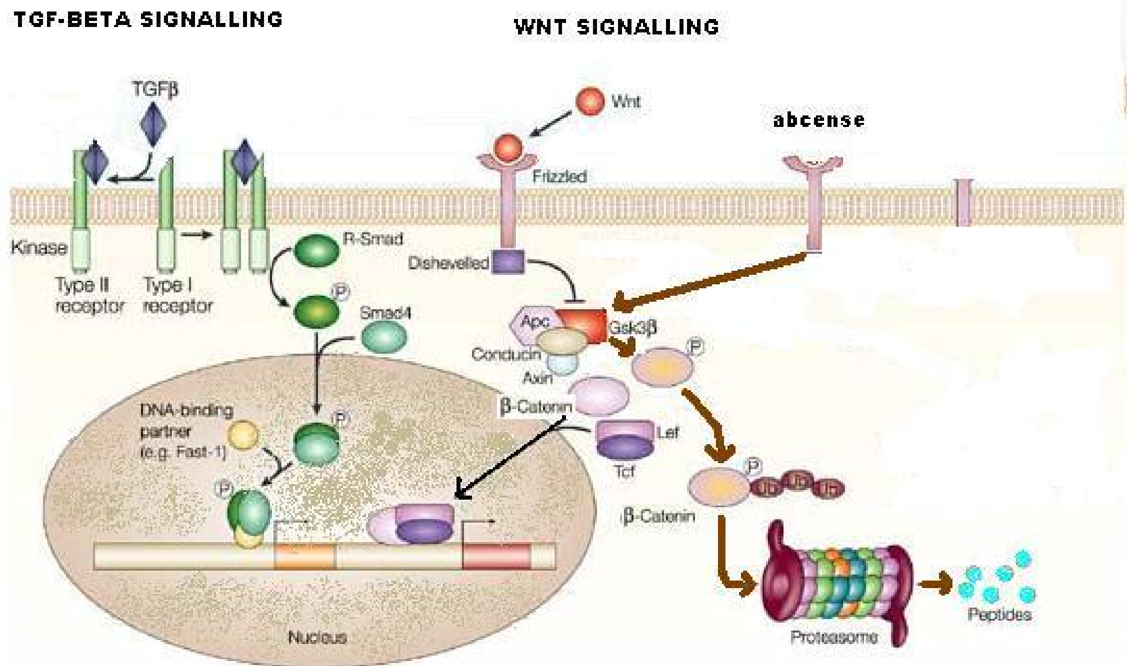

Figure 3. TGFß and WNT signalling pathways (Modified from Packard et al., 2003).

Wilms Tumor 1 Gene Signalling

Canonical WNT signalling plays a crucial role in controlling cell expansion in many stem cells and it also controls the cell lineage decisions by promoting specific fates at the expense of others. WNT together with other signalling routes mediate internal signalling during normal embryogenesis and so it widely is involved in animal development (Reya et al., 2003, Wang, and Wynshaw-Boris, 2004, Wilson et al., 2001, van Es et al., 2003).

WNT signalling proteins are cysteine- rich lipid- modified proteins which contain signal sequences and are in an important role in various processes during development including cell proliferation and differentiation, cell fate decisions, apoptosis, axial polarity and axonal patterning (Kleber, and Sommer, 2004, Wang, and Wynshaw-Boris, 2004). Recent studies have demonstrated that frizzled proteins act as receptors for WNTs. These frizzled proteins consist of seven transmembrane regions with an amino- terminal extension rich in cysteine residues that is outside the cell membrane. There are also co-receptors that work together with frizzled proteins and these include receptor- related proteins (Packard et al., 2003). WNT binding into the frizzled- receptor at the cell membrane triggers the fosforylation of sytoplasmic protein, Dishevelled. Fosforylated

Dishevelled then prevents the formation of APC (adenomatous polyposis coli)/Axin /Conducing/Gsk3ß (glycogen synthase kinase 3ß) – complex, which in absence of WNTs phosphorylates sytoplasmic ß-catenin after which ß-catenin is degraded in proteasomes. But when WNTs have been bound, ß-catenin is stabilised and it complexes with another transcription factors and initiates transcription of WNT- responsive genes in the nucleus and so makes the decision of cell's fate (Moon et al., 2002, Packard et al., 2003), Figure 3.

More results of the importance of WNT signalling for self- renewal of hESC have come from Sato's research (Sato et al., 2004). They have revealed that the activation of WNT cascade is instrumental in maintaining the molecular mechanisms for the undifferentiated growth of hESC in culture. And they also have concluded that the loss of activity of this signalling route might lead to the activation of cell's differentiation events.

Altogether, more research needs to be done in order to recognize all the events in all signalling cascades working together and influencing on hESC self- renewal and behaviour. When we know all these influencing factors in stem cell biology of 'stemness' and pluripotency maybe then we can regulate these events in hESC with some synthetic pharmacological compounds as we want. This could be a major breakthrough in research of hESC.

2. Aim of the study

The aim of this study was to test how the supplementation of house- made human embryonic stem cell (hESC) medium with activin A, recombinant human platelet derived growth factor AB (PDGF-AB), sphingosine 1- phosphate (S1P) and γ- secretase inhibitor IX affect to the undifferentiated growth, attachment and proliferation rates of hESC. In addition, polyvinylalcohol was tested to replace human serum albumin (HSA) and the effect of increased concentration of transferrin and bFGF into hESC growth and characteristics was studied. The third aim of this study was to test how amino acids, thiamine, glutathione or insulin, transferrin and HSA together influence on hESC`s behaviour in culture.

In this study, the growth and characteristics of the cultured hESC was valued by observing cell morphology, rate of cell attachment, and rate of the cell differentiation. The pluripotency of the cultured hESC was proven by embryoid body cultivation in test media, followed by RNA isolation and RT- PCR. The undifferentiated status of cultured hESC was determined by immunocytochemical analyzes.

3. Materials and methods

3.1 Human Foreskin Fibroblasts as Feeder Cells

In this study we used commercially available human foreskin fibroblasts (hFFs) (CRL-2429, ATCC) as feeder cells for hESC. HFFs are stored in liquid nitrogen and before use, they were thawed and cultured using medium containing 90% IMDM w/L glut + Hepes (Gibco Invitrogen, USA), 10% FBS (Gibco Invitrogen) and 50U/ml Penicillin/Streptomycin (Cambrex, USA). Before plating the hFFs for hESC, they were mitotically inactivated using irradiation at 40Gy. After that the hFFs were plated onto Falcon 3003 or Falcon 3653 IVF cell culturing dishes (BD Biosciences, USA) and let then to form a confluent monolayer before using under hESC. The following day after the irradiation the medium was changed to 90% IMDM w/L-glut + Hepes (Gibco Invitrogen), 10% Knock-Out Serum Replacement (KO-SR, Gibco Invitrogen) and 50U/ml Penicillin/Streptomycin (Cambrex). The feeder plates were used up to one week.

3.2 Human Embryonic Stem Cell Lines

In this study we used three different human hESC lines: HS237, HS346 and HS401. These lines have been originally derived in Sweden with the permission of the ethics committee of the Karolinska Institute. Embryos were donated for stem cell research by couples going though *in vitro* fertilization treatments.

HS181, HS237 cell lines have been derived from blastocyst stage embryos using hFFs as feeders and using 20% FBS in the culture medium but FBS was later changed for 20% KO-SR. The HS346 and HS401 lines have been derived using conventional hES medium from the beginning. The conventional hES medium contained 80% KO-DMEM (Gibco Invitrogen), 20% KO-SR (Gibco Invitrogen), 2mM GlutaMax (Gibco Invitrogen), 1% Non-essential amino acids (Cambrex), 50U/ml Penstrep (Cambrex), 0.1mM 2-mercaptoethanol (Gibco Invitrogen) and 8ng/ml human recombinant bFGF (R&D Systems).

During the experiment we found out that the cell batch of line HS237 had gained mutation in chromosome X, thus results presented in this study must be handled with some concern. The rest of the cell lines used had normal karyotypes. HS237 and HS346 lines have karyotype 46 XX and HS401 line karyotype 46 XY. The pluripotency of all these cell lines have been demonstrated by injection into immunodeficient mice where all lines

formed teratomas containing cells from all three embryonic layers. The undifferentiated cells express the normal surface markers specific for undifferentiated hESC: SSEA-4, TRA-1-60 and TRA-1-81, alkaline phosphatase and Oct3/4 (Hovatta et al., 2003; Inzunza et al., 2005; Hovatta et al., unpublished).

3.3 Human Embryonic Stem Cell Cultures

The tested media additives are summarised in Tables 1, 2 and 3 later on. The whole composition of the house- made medium is published elsewhere and is not presented in this Master of Science thesis.

The Cell Line HS237 Cells Cultured With Test Group I Media

As the control medium was used medium A, which is house- made xeno- free medium. The additives were added into the control medium A in this study. HS237 cells were adopted, before totally using of 100% test medium. The adaptation was carried in a way that during the test passage one media included 50% of conventional hES medium and after this, the cells were cultured in 100% test medium. Media components and tested passages are summarized in Table 1 below.

Used additives for media B to H were pursued from: PVA (Sigma), γ-secretase inhibitor IX (Merck), S1P (Sigma), transferrin (Sigma), PDGF-AB (R&D Systems), insulin (Gibco), HSA (Sigma) and activin A (R&D Systems). Media I to K did not contain amino acids, thiamine or glutathione, respectively.

Table 1. Summary of the test group I media compositions and passages tested with cell line HS237. S1P=sphingosine 1-phosphate, PVA= polyvinylalcohol, PDGF-AB=platelet derived growth factor-AB, HSA=human serum albumin.

MEDIUM	USED PASSAGES	HOUSE-MADE MEDIUM ADDITIVES / MODIFICATIONS
A (CONTROL)	P96-111	Xeno-free medium without additives
B	P96-97	PVA, No HSA
C	P96-97	PVA, No HSA
D	P96-106	Transferrin
E	P96-105	Activin A
F	P98-105	S1P, PDGF-AB
G	P98-109	γ-secretase inhibitor IX
H	P97-100	Insulin, Transferrin , HSA
I	P97-112	No amino acids
J	P97-111	No thiamine
K	P97-112	No glutathione

The Cell Lines HS346 and HS401 Cells Cultured With Test Group II and Test Group III Media

Media in test group II and III differed from test group I media in a way that sodium chloride was used in test groups II and III in aim to adjust osmolality to the level of 320mOsm/kg. In test group II and III conventional hES culture medium was used as a control. Based on results of the test group II the amounts of additives were changed in test group III. Commercial culture medium, Hesgro (Chemicon International) was also compared to the best performing medium SR10 during the adaptation phase. The composition of Hesgro medium is not known.

In test group II (Table 2) and in test group III (Table 3), cells were adopted per passage as follows: 80% hES medium/ 20% test medium, 50% hES medium/ 50% test medium, 20% hES medium/ 80% test medium and then 100% test medium.

Table 2. Additives used for test group II. (2X= two times the amount as in others). All the media contained house-made medium as the basal medium. γ-sec.Inh. IX= γ-secretase inhibitor IX.

Additive	SR1	SR2	SR3	SR4	SR5	SR6	SR7	SR8
AA-H2O	without additives	X	X					
AA-HCl			X					
bFGF				**2X**				
Activin A					X			
Glutathione						X		
Thiamine							X	
γ-sec.Inh. IX								X

Table 3. Additives used for test group III. (2X= two times the amount as in others, 5X= five times the amount as in other test media). All the media contained house-made medium as the basal medium. γ-sec.Inh. IX= γ-secretase inhibitor IX.

Additive	SR10	SR20	SR30	SR40	SR50	SR60	SR70	SR80	SR90
Amino acids in HCl	X		X	X	X	X	X	X	X
Amino acids in H20	X	X	X	X	X	X	X	X	X
Activin A					X				X
Glutathione						X			X
Thiamine							X		X
γ-sec.Inh. IX								X	
bFGF			**2X**	**5X**					**2X**

The summary of used cell lines and passages for test group II media is presented in Table 4 and for test group III in Table 5.

Table 4. Summary of used hESC lines and passages cultured in test group II.

TEST GROUP II		
MEDIUM		TOTAL PASSAGE
hES (CONTROL)	HS346 P59-69	11
	HS401 P69-80	12
SR1	HS346 P59-62	4
	HS401 P69-77	9
SR2	HS346 P59-62	4
	HS401 P60-77	9
SR3	HS346 P59-69	11
	HS401 P69-80	12
SR4	HS346 P60-63	4
	HS401 P69-73	5
SR5	HS346 P60-65	6
	HS401 P69-80	12
SR6	HS346 P60-63	4
	HS401 P70-75	6
SR7	HS346 P60-65	6
	HS401 P70-75	6
SR8	HS346 P70-75	6
	HS401 P70-75	6

Table 5. Summary of used hESC lines and passages cultured in test group III and in Hesgro medium.

TEST GROUP III, HESGRO		
MEDIUM		TOTAL PASSAGE
hES (CONTROL)	HS346 P59-69	11
	HS401 P69-79	11
HESGRO	HS346 P64-67	4
	HS401 P74-76	3
SR10	HS346 P64-70	7
	HS401 P74-78	5
SR20	HS346 P64-69	6
	HS401 P74-78	5
SR30	HS346 P64-70	6
	HS401 P74-78	5
SR40	HS346 P64-69	7
	HS401 P74-79	6
SR50	HS346 P64-69	6
	HS401 P74-79	6
SR60	HS346 P64-68	5
	HS401 P74-79	6
SR70	HS346 P64-68	5
	HS401 P74-78	5
SR80	HS346 P64-69	6
	HS401 P74-79	6
SR90	HS346 P64-69	6
	HS401 P74-79	6

In each tested media the cells were grown 7-9 days and then split on to a fresh hFF plate with a needle and a scalpel under the microscope (Nikon eclipse TE2000-S). Before splitting the morphology of cell colonies was judged and colonies were also photographed with EclipseNet using the microscope (Nikon eclipse TE2000-S). Medium was changed every day with pre- warmed (incubated half an hour in 37°C) medium. One medium batch prepared was used within one week and not all of it was warmed at the same time in order to avoid destroying of growth factors.

3.4 Statistics

Before passaging the cells, the morphology of the cell colonies were determined by using a microscope and the number of undifferentiated, partially differentiated and differentiated colonies were counted, Figure 4.

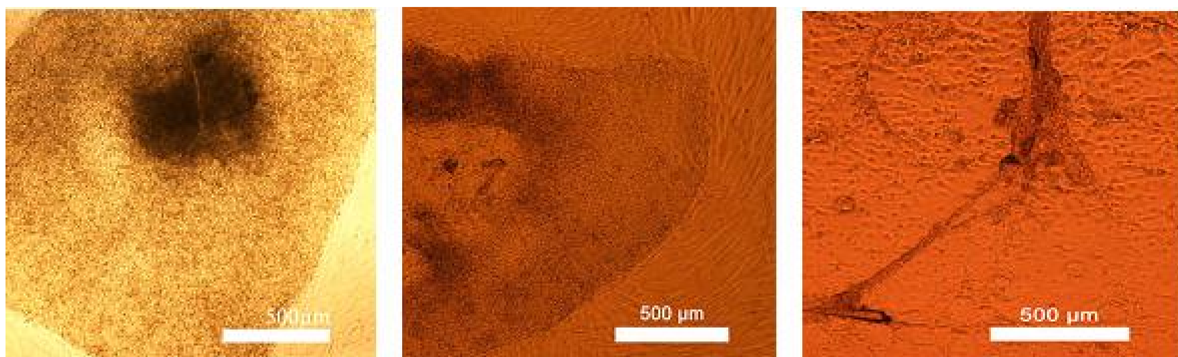

Figure 4. An example of an undifferentiated (on the left), partially differentiated (in the middle) and differentiated (on the right) hESC colony. Scale bar is 500µm and the objective 4x.

When transferred onto a fresh culture plate, the split pieces were calculated and on the following day attached pieces were calculated. So statistics are made based on these calculations and morphology judgements.

3.5 *In Vitro* Differentiation Study

3.5.1 Embryoid Body Formation

Embyoid bodies (EBs) were cultured with cell line HS346 in test media SR3, SR5, SR7 and SR8. With the cell line HS401 EBs were cultured in media SR1, SR2, SR3, SR5, SR6, SR7 and SR8. EBs were formed and cultured in order to demonstrate that hESC maintained their pluripotency by differentiating to the different cell lineages representing all three embryonic layers.

The cell colony parts were cut into small pieces and grown as suspension for at least 20 days in test medium without any growth factors. The EBs were grown in 24 well-plate which has low binding capacity to prevent EBs attachment into the bottom of the well. Medium was changed every 3-4 days with a fresh pre- warmed medium.

3.5.2 RNA Isolation

RNA isolation from cultured EBs was performed using RNAEasy Mini-kit (Qiagen, Germany). From each test media, 2-3 EB:s were taken and directly transferred in 350µl lysis buffer (was prepared 10ml RLT-buffer and 100µl ß-mercaptoethanol, Sigma-Aldrich). EBs were lysed by vigorously vortexing after which the sample was pipetted into a gDNA Eliminitor spin column with a 2ml collection tube and centrifuged 30 seconds at 10.000rpm. After this the flow- through was saved and 350µl of 70% ethanol was added and mixed by pipetting. The 700µl sample was now transferred into an RNAeasy spin colum with 2ml collection tube and centrifuged for 15 seconds at 10.000rpm (the flow-through was discarded). After centrifugation, 700µl RW1 buffer was added to the column and centrifugated for 15 seconds at 10.000rpm and the flow- through was discarded. After this 500µl Buffer RPE was added into RNAeasy spin column and centrifuged for 15 seconds at 10.000rpm after which the flow- through was discarded. The step was repeated but the centrifugation time at the second step was 2 minutes. Then, the RNAeasy column was placed in a new collection tube and centrifuged at 13.200rpm for 1 minute. This step was repeated to be sure that the column was dry. After drying, the RNAeasy spin column was moved in a new 1.5ml collection tube; 40µl pre-heated RNase- free water was added and after one minute incubation the sample was centrifuged at 10.000rpm for 1 minute. After RNA isolation, the RNA concentration and purity were measured using ND-1000 Spectrometer (NanoDrop Technologie, USA) in absorbance 260nm and 280nm.

3.5.3 Complementary DNA Synthesis and Reverse Transcriptase- PCR

Complementary DNA (cDNA) was synthesized with Sensicript Reverse Transcription kit (Qiagen, Germany). About 50ng of RNA was used in one reaction containing 1µl RNase inhibitor (10units/µl, Fermentas), 2µl 10X Reverse Transcriptase Buffer, 2µl dNTP mix (5mM), and 1µl Sensicript Reverse Transcriptase. RNase- free water was added so that the final reaction volume was 20µl. The reaction was incubated for

1 hour in 37°C. cDNA was stored in -20°C and if longer storage was needed, cDNA was stored at -70°C.

Reverse Transcriptase Polymerase chain reaction (RT-PCR) was made with a mastermix prepared for each primer. Primers are presented in Table 6 and mastermix composition in Table 7. Negative controls were made for all the analyzed genes by using sterilized water instead of cDNA. ß-actin was used as a housekeeping control.

Table 6. RT-PCR primers (Proligo,USA) used. **Ectodermal marker:** NF-68KD, (neurofilament 68 KD); **Endodermal marker:** AFP (α fetoprotein). **Mesodermal markers:** WT1(Wilms tumor gene 1) and α-CA (α-cardiac actin). ß-actin was used as a housekeeping gene, bp=basepairs.

Gene product	Forward (F) and reverse (R) primers (5´-3´)	Product size (bp)	Annealing temperature (°C)
NF-68	F:GAGTGAAATGGCACGATACCTA R:TTTCCTCTCCTTCTTCACCTTC	473	55
WT1	F:CAGGCCAGGATGTTTCCTAA R:GACACCGTGCGTGTGTATTC	459	60
α-CA	F:GGAGTTATGGTGGGTATGGGTC R:AGTGGTGACAAAGGAGTAGCCA	486	55
AFB	F:GGAGTTATGGTGGGTATGGGTC R:TCCCCTGAAGAAAATTGGTTAAAAT	216	60
ß-actin	F:GTCTTCCCCTCCATCGTG R:GGGGTGTTGAAGGTCTCAAA	302	60

Table 7. Mastermix used in RT-PCR reaction.

QUANTITY	COMPONENT
2.5 µl	10 Tag Buffer with KCL
0.250 µl	dNTP (10mM each)
0.125 µl	Tag Polymerase (0.625U)
1.5 µl	MgCl (25mM)
17.5 µl	Rnase free water
1 µl	Forward primer (5µM)
1 µl	Reverse primer (5µM)
1 µl	Sample
25 µl	**TOTAL**

RT-PCR reaction was carried out in Eppendorf Mastercycler with a program: 3 minutes at +95°C (starting denaturation), 30 seconds at +95°C (denaturation), 30 seconds at +60°C or +55°C (annealing), 1 minute at +72°C (extension) and 5 minutes at +72°C (final extension). Denaturation, annealing and extension steps were repeated 40 times.

3.5.4 Agarose Gel and Gel Run

1.5% Agarose gel was prepared for analysing the RT-PCR products. The gel was prepared with 0,5μg/ml ethidium bromide (Sigma-Aldrich). The samples were loaded with 5μl of 6X Loading buffer. The 50 base pair DNA-ladder mix (Gene-Ruler, Fermentas) was used to determine the size of the RT-PCR products. The gel was running for 1 hour and 15 minutes at 110V. Gel was photographed with Gel Doc (AppliedBiosystems).

3.6 Immunocytochemical Analyzes

The undifferentiation status of hESC colonies were examined by immunocytochemical staining. Before staining was performed, cells were washed two times (5 minutes) with DPBS (Dulbecco's Phosphate Buffered Saline, no Mg2+, no Ca+, Cambrex), after which they were fixed for 20 minutes with 4% paraformaldehyde in phosphate- buffered saline (Sigma) in room temperature and then washed three times with DPBS again. Staining was carried out using the protocol which is as follows: colonies were incubated in blocking solution (0.1% Triton X-100, 1% BSA, 10% normal donkey serum in PBS) for 45 minutes to inhibit unspecific staining, after which colonies were washed once with primary antibody solution (0.1% Triton X-100, 1% BSA, 1% normal donkey serum in PBS) without antibody. After washing, primary antibody dilutions were added onto the cells in primary antibody solution. All reagents were from Sigma- Aldrich unless stated otherwise. Used primary antibodies were: goat IgG anti- Nanog (1:200, Santa Cruz), mouse IgM anti-TRA-1-60 (1:300, Jackson Immunoresearch Laboratories, USA), mouse IgG anti- Oct3/4 (1:200, Santa Cruz).

Cells were incubated over night in +4°C. The next morning cells were washed twice (5 minutes) with secondary antibody solution (1% BSA in PBS) after which secondary antibody dilutions were added onto the cells and incubated for 1 hour light protected in room temperature. As secondary antibodies: Alexa Fluor 488 donkey anti-goat IgG (1:400, Molecular Probes Invitrogen), Rhodamine Red rat anti- mouse IgM (1:400, Jackson Immunoreseach Laboratories, USA) and Alexa Fluor 488 donkey anti-mouse (1:400, Molecular Probes Invitrogen) were used.

After incubation, the cells were washed three times (3-5 minutes) with DPBS and two times with Phosphate buffer-solutions (26.6g/l NaH2PO4, 28.6g/l Na2HPO4, deuterized H2O, Sigma). Next the cells were mounted with Vectashield Mounting Medium

for Fluorescence with Dapi (Vector Laboratories, USA) and covered with a cover slip (16mm). The stained cell colonies were viewed and photographed with a Nikon Eclipse TE2000-S phase contrast microscope with fluorescence optics and a Nikon COOLPIX 5400 camera.

Human ESC stained only with secondary antibodies were used as negative controls and cells cultured in control medium were used as positive controls. Positive controls were treated exactly the same way as the sample plates.

4. Results

4.1 Test Group I

Growth characteristics:

In test group I, different kinds of additives were used in order to see how they affect on the morphology and undifferentiated growth of hESC. As the basal medium, into additives were added, the house- made medium was used.

During the early passages, the hESC cultured in media A, D, E, F, G, I, J and K looked good but the cells cultured in media B and C, which contained PVA but no HSA, did not grow equally well. The cells cultured in B and C media grow only upward making the passaging impossible thus the cells were discarded at passage 2. The difference observed by using these two media was that the cell colonies grown in medium C tried to spread slightly but the cell colonies grown in medium B did not (see Figure 5).

During a few passage the hESC cultured in medium D, which contained addition of transferrin, looked nearly the same as the cells cultured in control medium A (see Figure 5) but later the growth rate of the cells decreased and the cells were thrown away at passage 11.

The cell colonies cultured in medium E, which included activin A, were very big in the beginning but also very thin at the same time. The thinness of cell colonies is not preferable because it makes it very difficult or even impossible to split colonies on. In the final passages the growth of the cells grown in medium E decreased and the cell colonies were the smallest ones. The cells were discarded at passage 10 (see Figure 5).

During the first few passages the cell colonies grown in test medium F, which contained S1P and PDGF-AB, looked almost the best when compared to the cell colonies grown in other test media in test group I, they were big and very thick. The cell colonies cultured in F medium started to differentiate at the centre and they were discarded at passage 8 (see Figure 5).

The cell colonies cultured in medium G, containing γ- secretase inhibitor IX, also looked very good (see Figure 5) but growth of the cells cultured in medium G decreased slowly and colonies got smaller. Cell colonies grown in test medium G lasted longer in good condition as compared to the colonies grown in medium F, until finally at passage 11

the cell colonies were very thick but also too small to be split anymore, and so the cells were discarded.

The cell colonies cultured in medium H, containing insulin, transferrin and HSA, were very small all the time and the growth rate of the cells was very slow. The cells cultured in medium H were discarded at passage 4 (see Figure 5).

Media I, J and K contained a different mixture of amino acids, thiamine and glutathione. Medium I did not contain amino acids, medium J contained no thiamine and medium K no glutathione. The cell colonies cultured in these media were thin but the growth rate of the cells was high and cells made very big colonies. Over passage ten, cell colonies grown in medium I got slightly thicker but also smaller and the cells were kept until passage 16. The cell colonies grown in medium J were big all the way but at final passages there seemed to be small holes in the cell colonies and so the cells were grown until passage 15 and then discarded.The cell colonies cultured in test medium K looked big (see Figure 5) but at the end the cell colonies cultured in this media were the thinnest as compared to all other media used and so the cells were discarded at passage 16. Summary of features of cell colonies grown in different test media is presented in Table 8. The karyotypic change in chromosome X with HS237 cell line probably had some influence on character and behaviour of these cells. And because of this the studies with the cells of cell line HS237 was terminated and the research continued with other stem cell lines.

Table 8. Summary of features of cell colonies grown in test group I. As the basal medium, into that additives were added, was house-made medium. PVA= Polyvinylalkohol, S1P= Spingosine 1-phosphate, PDGF-AB=Platelet derived growth factor-AB, HSA=Human serum albumin, γ-sec. inh IX= γ-secretase inhibitor IX.

Medium	Additive	Final passage	Good characters of the colonies	Bad characters of the colonies
A	No additives	16		Started to dif. at final passages
B	PVA	2		Cells grew onto each others
C	PVA	2	Tried to spread	Cells grew onto each others
D	Transferrin	11	Almost the same as A	Growth rate decreased
E	Activin A	10	Big colonies	Very thin, hard to split, started to differentiate at the centre and growth rate decreased,
F	S1P, PDGF-AB	8	Big, thick colonies	Growth rate decreased, finally very small
G	γ- sec. inh IX	11	Big, thick colonies	Growth rate decreased and got thinner
H	Insulin, HSA Transferrin,	4		Colonies very small, differentiated quickly
I	No amino acids	16	Big colonies, lasted longest undif.	Thin colonies
J	No thiamine	15	Big, lasted longest undif.	Thin, started to differentiate at the centre
K	No glutathione	16	Big colonies, lasted longest undif.	Thinnest at final passages

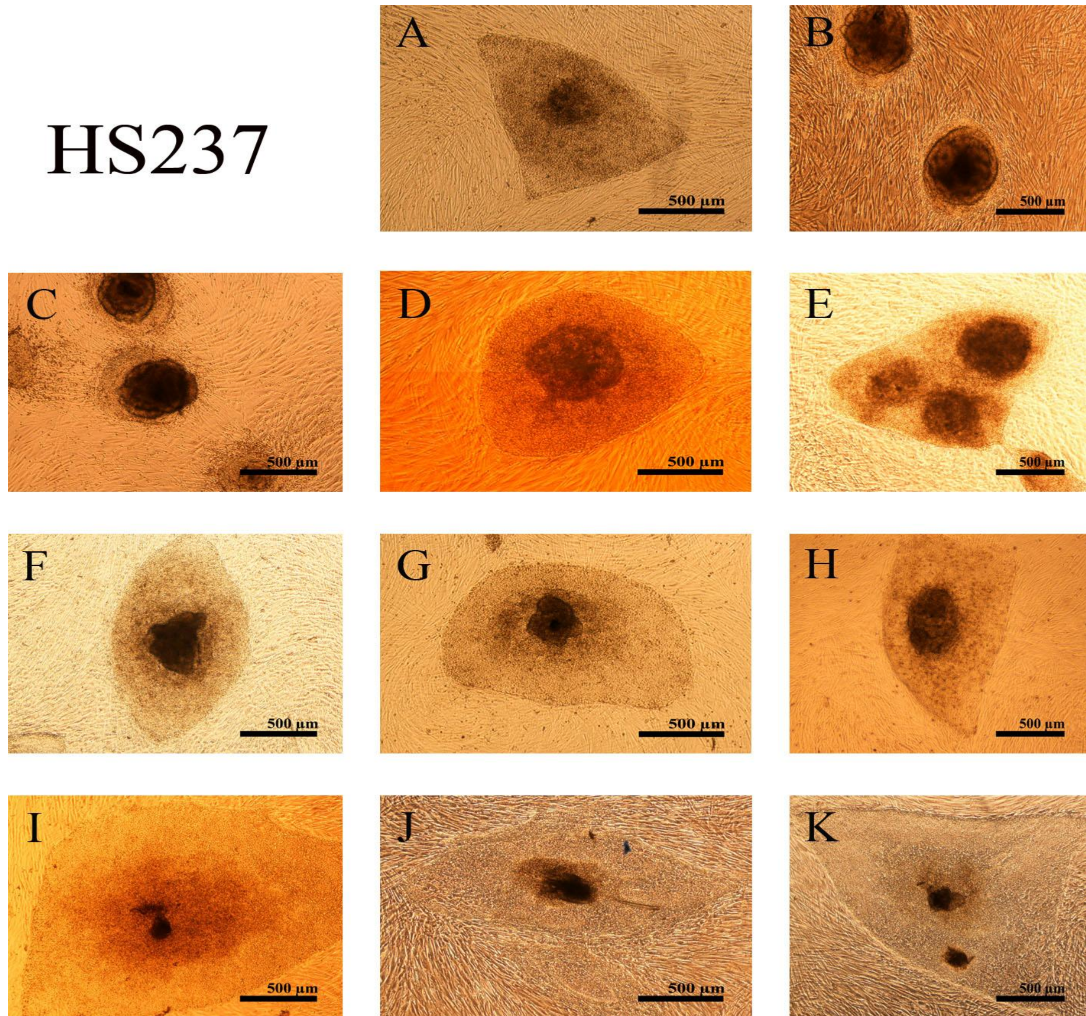

Figure 5. A representative cell colony cultured in test group I media. Letter in each picture marks for the test medium. A is control medium photographed at passage 15, B and C at passage 2, D at passage 10 and E at passage 9, F at passage 7, G at passage 11, H at passage 3, I at passage 15, J at passage 14 and K at passage 15. Scale bar is 500µm and the objective 4x.

Cell attachment:

The results in Figure 6 represent an attachment percentage of cells cultured in each test medium. The cells cultured in media B and C did survive only 2 passages so they could not have been calculated (see Figure 6). The diagram also demonstrates that all the cells grown in test media have better attachment ratio than the cells cultured in control medium A. The highest attachment ratio of the cells grown in medium H can be explained by the fact that the cells were grown just for 4 passages before differentiation and the diagram is made from passages 3 and 4, thus there is no variation between passages.

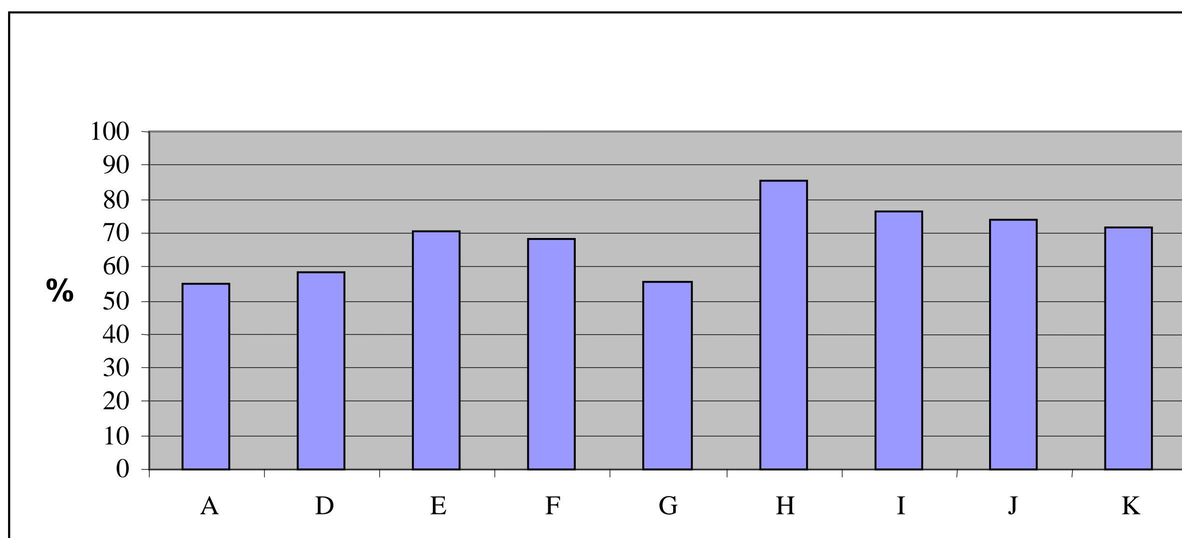

Figure 6. Attachment of HS237 cells cultured in test group I media. Media B and C did survive only 2 passages so they could not have been calculated. Total amount of all pieces is 2746. Calculated attachment from passages: A medium, P3-16 (n=294); D, P3-11 (n=257); E, P3-10 (n=286); F, P3-8 (n=232); G, P3-11 (n=282); H, P3-4 (n=28); I, P3-16 (n=439); J, P3-15 (n=353) and K, P3-16 (n=547).

Rate of differentiation:

Morphology:

From the results of Figure 7, it can be seen that the cells differentiated the most when cultured in test medium H. Differentiation levels of the cells cultured in other test media are quite similar as compared to the cells cultured in control medium A, except the fact that there are some differences as compared to the cells grown in media D and E. The cell colonies cultured in medium D differentiated almost 20% more than the cell colonies grown in the control medium. No major differences were observed on the differentiation level of other test media when compared to the test medium A.

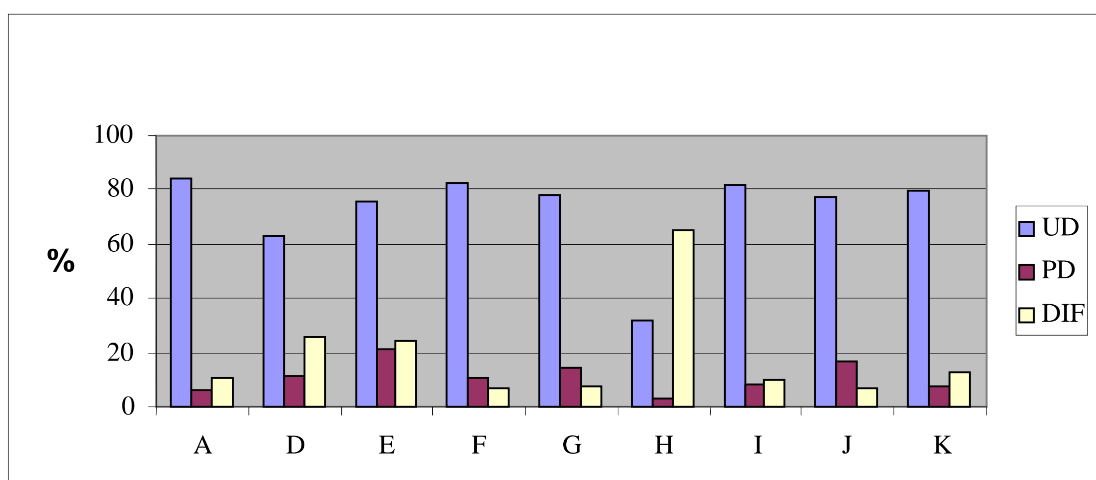

Figure 7. Differentiation of the HS237 cells cultured in test group I media. Calculated values are from passages: A-control, P2-15 (n=108); D, P2-10 (n=145); E, P2-9 (n=221); F, P2-7 (n=147); G, P2-10 (n=180); H, P2-3 (n=33); I, P2-15 (n=291); J, P2-14 (n=191); K, P2-15 (n=249). Total number of all colonies is 1565. UD=undifferentiated, PD=partly differentiated, DIF=differentiated.

Immunocytochemical Analyzes:

Representative cell colonies from tested media A, D, F, G, I, J and K stained with specific antibody markers TRA-1-60 and Nanog. These markers are specific for undifferentiated hESC. All the colonies showed specific staining for used markers, although non- equally. Figure 8 shows the staining results of the cell colonies cultured in control medium A and in medium F as an example.

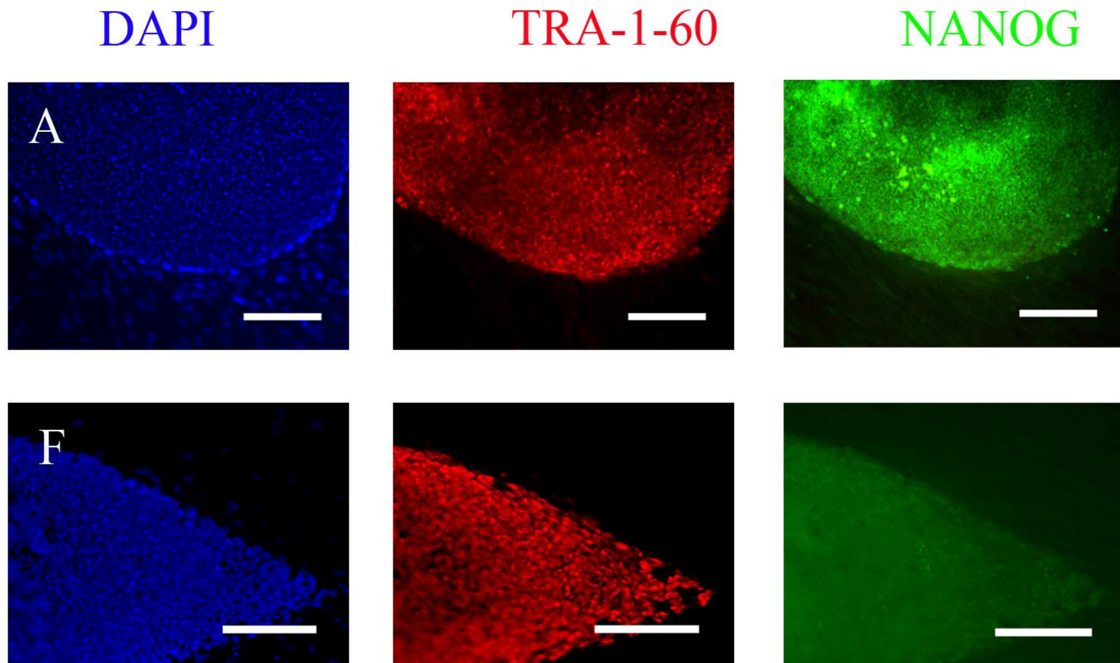

Figure 8. Immunostaining results for a representative colony of HS237 line cultured in media A (control) and F by using two markers TRA-1-60 and Nanog specific for undifferentiated hESC. The cells cultured in medium A have been at passage 16 and the cells grown in medium F at passage 8. Colonies grown in medium A has been stained with Dapi and then the same colony is shown with immunolabels TRA-1-60 (red) and with Nanog (green). Also, colony grown in test media F, containing S1P and PDGF-AB, is shown as an example. Scale bar is 200µm and the objective 10x.

4.2 Test Group II

Growth characteristics:

There were no big differences between the morphologies of the early passages of the cell colonies of cell lines HS346 and HR401 grown in the test group II media. However, after a few passages HS346 line cells cultured in medium SR3, contained amino acids both in HCl and in water, looked very promising: the cell colonies were big and thick and surveyed the best until passage 11 (see Figure 9). Also with the cell line HS401 cells, the medium SR3 proved to be the best and the cell colonies cultured in this medium did not differentiate until the passage 12. The cell colonies grown in medium SR5, which contained addition of activin A, looked good, although not as thick as cells cultured in

medium SR3, but the cell colonies differentiated spontaneously at passage 6 (HS346) and at passage 12 (HS401).

The cell colonies cultured in media SR1 and SR2 did not grow well and both cell lines had weird granular structures. The HS346 SR1 and SR2 cells also differentiated quickly at passage 4 (see Figure 9) but the HS401 cells cultured in media SR1 and SR2 survived undifferentiated until passage 9. The difference between these two media was that the medium SR1 contained no amino acids and the medium SR2 contained amino acids dissolved in water. There were no remarkable differences between the cell colony morphologies cultured in media SR4 (contained twice as much bFGF as other media), SR6 (contained addition of glutathione), SR7 (contained addition of thiamine) and SR8 (contained γ-secretase inhibitor IX) but the differentiation was observed during different passages: the HS346 cell colonies grown in media SR4 and SR6 differentiated at passage 4 and the cells grown in media SR7 and SR8 at passage 6. The cell colonies of HS401 cell line cultured in media SR4 differentiated at passage 5, the cells cultured in medium SR6 at passage 6, the cells cultured in SR7 at passage 6 and the cells grown in medium SR8 at passage 6. All test media of both cell lines were worse than control hES medium as compared by morphology and growth rate of the cells. Summary of features of cell colonies grown in different test media is presented in Table 9.

Table 9. Summary of features of cell colonies grown in test group II with HS346 line and HS401 line cells. As the basal medium was used house-made medium. HCl=hydrogen chloride, 2X= twice the amount as in others, γ-sec.Inh IX= γ-secretase inhibitor IX, diff.=differentiate.

Medium	Additive / Modification	Good characters of colonies	Bad characters of the colonies	Differentiated at passage	
				HS346	HS401
SR1	No amino acids		Small, weird stuctures	4	9
SR2	Amino acids in H2O		Small, weird stuctures	4	9
SR3	Amino acids in H2O,HCl	Big, thick colonies		11	12
SR4	2X bFGF		Small, diff quickly	4	5
SR5	Activin A	Thick colonies	Not as thick as SR3	6	12
SR6	Glutathione		Small, diff. quickly	4	6
SR7	Thiamine		Small, diff. quickly	6	6
SR8	γ-sec.Inh IX		Small, diff. quickly	6	6

One influencing factor that had an effect on the morphologies of the cell colonies was that the hFF were not confluent in the centre of plates as it usually should be. In the

beginning of this study hFF were confluent layer on all the plates but because of some unknown reason they started to grow in an unconfluent manner. Thus eventually there was a hole in the middle in most plates and because of this the cell colonies differentiated very easily and quickly. But we can say that the differentiation of the cell colonies happened despite of test media.

The conclusion on growth characteristics of culture media in test group II was that the best medium of both cell lines was the SR3 medium which contained both amino acids, diluted in water and also in HCl. The cell colonies cultured in that medium were large and thick and they also kept their undifferentiated status the longest time.

A representative colony of differentiated HS346 cell line cells cultured in test group II during final passage is shown in Figure 9.

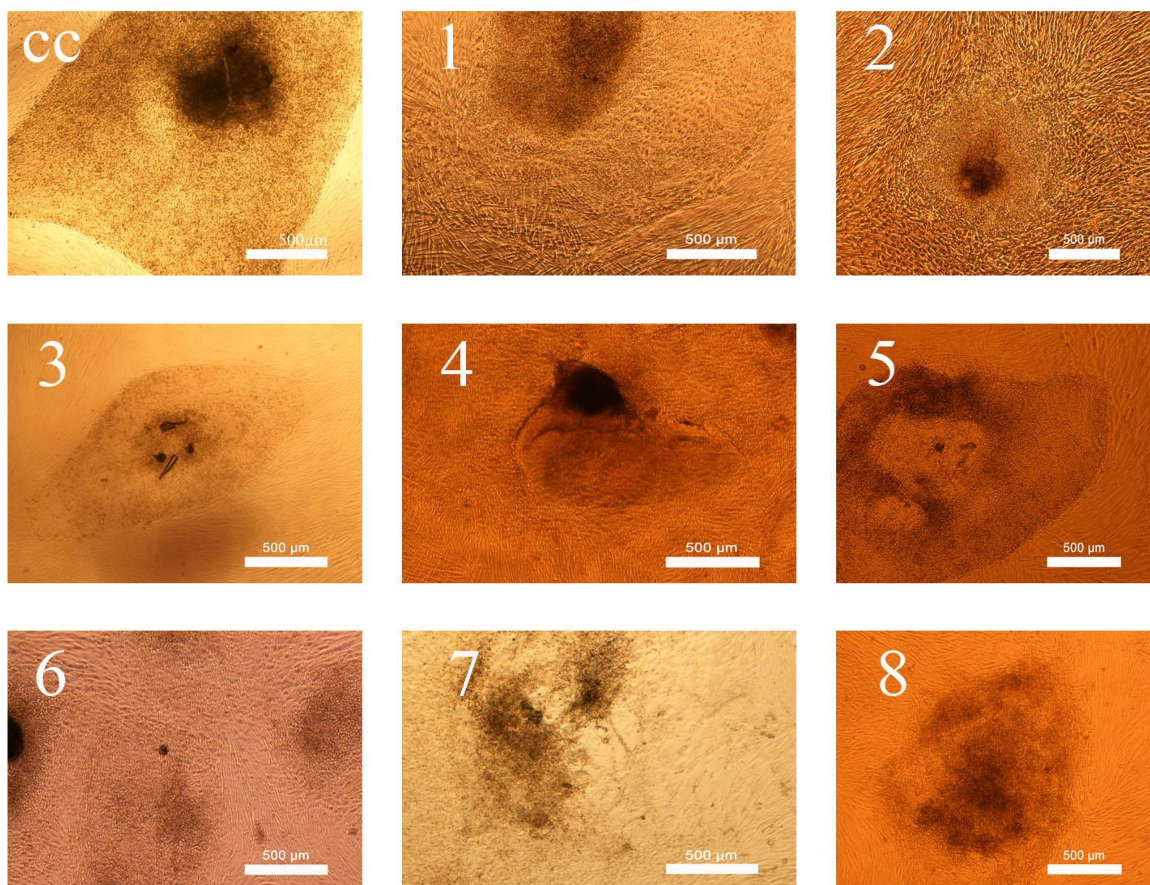

Figure 9. A representative cell colony of HS346 cells cultured in test group II media. CC represents for control. Media SR1, SR2, SR4 and SR6 have been photographed at passage 4 whereas the cell colonies of media SR3 has been photographed at passage 11 and colonies cultured in media SR5, SR7 and SR8 at passage 6. Scale bar is 500µm and the objective 4x.

Cell attachment:

There were no significant differences between attachment rates of the HS346 and HS401 cell colonies cultured in test media as compared to the cell colonies cultured in control hES medium (Figure 10). From Figure 10, it can be inferred that the HS401 cell colonies grown in medium SR3 had the lowest attachment rate and that the best attachment results for both cell lines was seen in medium SR4.

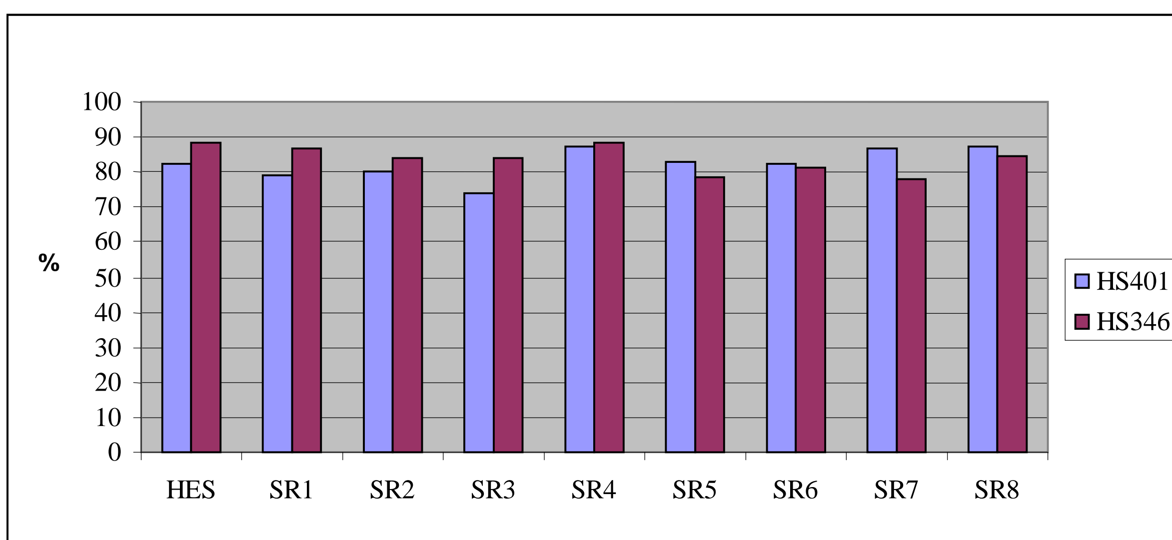

Figure 10. Attachment of HS346 and HS401 cells in test group II. Total number of all pieces is 3355. Calculated attachment rates are from passages: HS401 line cultured in hES-control medium, P1-12 (n=336); SR1 medium, P1-9 (n=173); SR2 medium, P1-9 (n=181); SR3 medium, P1-12 (n=252); SR4 medium, P1-5 (n=143); SR5 medium, P1-12 (n=227); SR6 medium, P1-6 (n=140); SR7 medium, P1-6 (n=136); SR8 medium, P1-6 (n=224); HS346 line cultured in hES medium, P1-12 (n=408); SR1 medium, P1-4 (n=212); SR2 medium, P1-4 (n=222); SR3 medium, P1-11 (n=306); SR4 medium, P1-4 (n=224); SR5 medium, P1-6 (n=116); SR6 medium, P1-4 (n=118); SR7 medium, P1-6 (n=128); SR8 medium, P1-6 (n=242).

Rate of differentiation:

Morphology:

Figure 11 shows differentiation levels for HS401 cell colonies cultured in test group II media. The highest level of undifferentiated growth was observed with cells grown in medium SR3 and that the differentiation was highest with the cells cultured in media SR6, SR7 and SR8 as compared to those cultured in control hES medium. The cells cultured in media SR6, SR7 and SR8 where much more partially differentiated and differentiated than undifferentiated. There was similar amount of undifferentiated cell colonies when media SR1 and SR2 where used and the cell colonies did not differentiate before passage 9. The cells grown in medium SR4 had clearly the highest amount of

partially differentiated cell colonies when compared to other test media in test group II. The cell colonies cultured in medium SR4 differentiated at passage 5 and the cells grown in media SR6, SR7 and SR8 differentiated at passage 6. The cells cultured in medium SR3 had almost as nice figure, in a way that there are a low amount of differentiated colonies, as the cells cultured in control hES medium. The figure of cell colonies grown in medium SR5 is very similar to the figure of media hES and SR3.

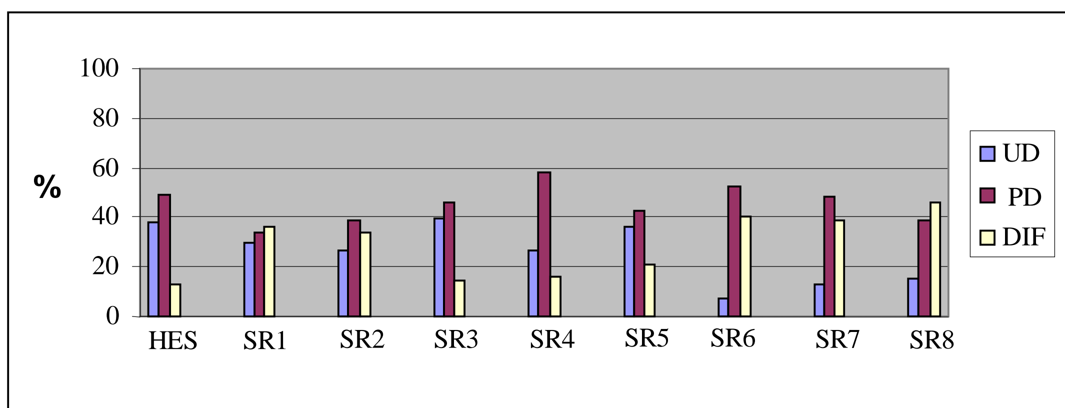

Figure 11. Differentiation of HS401 line cells cultured in test group II. Total number of all colonies is 1077. Calculated values are from passages: hES medium, P1-11 (n=242); SR1 medium, P1-8 (n=104); SR2 medium, P1-8 (n=111); SR3 medium, P1-11 (n=134); SR4 medium, P1-4 (n=76); SR5 medium, P1-11 (n=165); SR6 medium, P1-5 (n=72); SR7 medium, P1-5 (n=95) and SR8 medium, P1-5 (n=78). UD=undifferentiated, PD=partially differentiated, DIF=differentiated.

Figure 12 shows the results of HS346 differentiation levels in each tested medium. The highest level of undifferentiated growth was observed with cells cultured in medium SR3. There were even more undifferentiated colonies as compared to cells cultured with other test media but also the amount of partially differentiated colonies was very high. But cells cultured in test medium SR3 also had the lowest differentiation level. It also sustained the undifferentiated growth of the hESC the longest time, 11 passages. Cells cultured in media SR1 and SR2 had many undifferentiated cell colonies compared to other colonies but the cells have been cultured for only 4 passages in these media. The cells cultured in media SR4, SR6, SR7 and SR8 had the highest level of differentiation and also high level of partially differentiation as compared other media in test group II. What is also notable is that SR6 had exactly the same amount of partially and totally differentiated colonies and that this exceeds to 80% of all colonies cultured in this medium. Test medium SR5 had more than 50% partially differentiated colonies as compared to all colonies cultured in that medium.

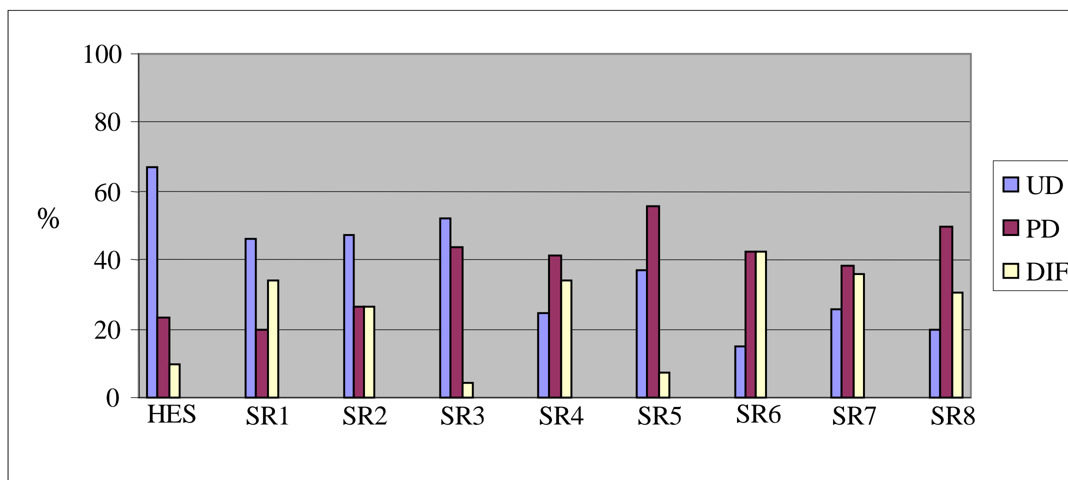

Figure 12. Differentiation of HS346 line cells cultured in test group II. Total number of all colonies is 847. Calculated values are from passages: hES medium, P1-11 (n=277); SR1medium, P1-3 (n=56); SR2 medium, P1-3 (n=61); SR3 medium, P1-10 (n=162); SR4 medium, P1-3 (n=53); SR5 medium, P1-5 (n=43); SR6 medium, P1-3 (n=61); SR7 medium, P1-5 (n=78) and SR8 medium, P1-5 (n=56). UD=undifferentiated, PD=partially differentiated, DIF=differentiated.

The cells of both cell lines (HS346 and HS401) cultured in medium SR3 had the best values; the lowest level of differentiation and a high amount of undifferentiated colonies as compared to other test media in test group II. The cell colonies had also been cultured in SR3 medium for the longest time, 11 passages with cell line HS346, and 12 passages with cell line HS401 in test group II.

Immunocytochemical Analyzes:

The cell colonies cultured in best performing test medium SR3 and in medium SR5 of both cell lines showed expression of Oct-3/4 and TRA-1-60 and so proved to be undifferentiated. The representative microscopic pictures of HS401 cell colonies cultured in media SR3 and SR5 are presented in Figure 13.

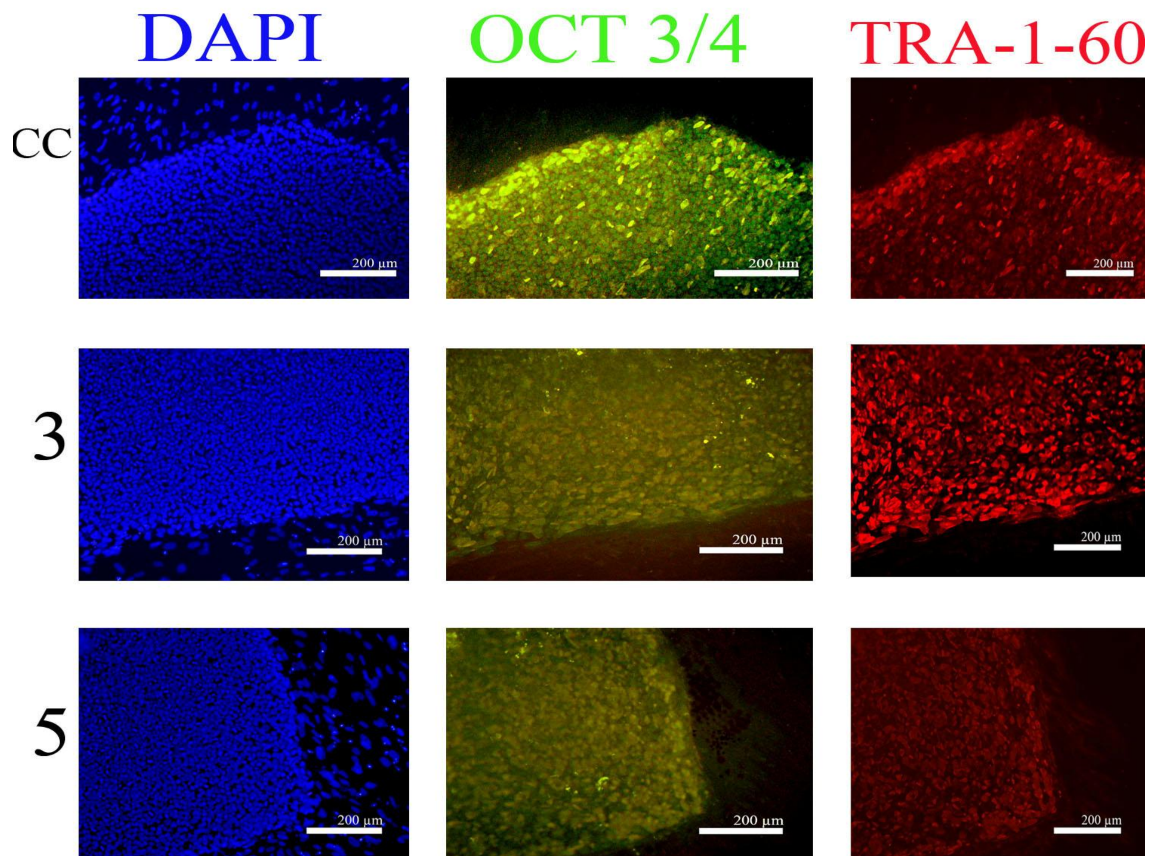

Figure 13. Fluorescence pictures for immunoassay of HS401 cells cultured in hES control medium and in SR3 and SR5 media. Microscopic pictures are taken at passages 12. Scale bar is 200µm and the objective 10x. CC=control hES medium, 3= SR3 medium, 5= SR5 medium.

Pluripotency:

Table 10 shows the results of RT-PCR analyses of EBs differentiated in test media SR7 (HS346 line) and SR3, SR5, SR6, SR7 and SR8 (HS401). There are no results of the best performing medium SR3, because the EBs did not grow. The same also happened with some other EBs cultured. The genes representing all three embryonic layers were found in from EBs of both lines cultured in control medium and from HS401 line EBs cultured in test medium SR6. On the other hand, the HS346 EBs cultured in medium SR7 expressed only genes representing two germ layers, ectoderm and mesoderm, but not endoderm. The EBs differentiated from HS401 cells cultured in media SR7 and SR8 both expressed mesoderm and cells cultured in medium SR7 also ectoderm genes, but neither showed the expression of endoderm gene. As a conclusion it can be drawn that the perfect pluripotency was sustained only in cells cultured in control media of both the cell lines and cells cultured in medium SR7 of cell line HS401.

Table 10. The results of RT-PCR analysis done for HS346 derived EBs cultured in control hES medium and SR7 medium, and also HS401 derived EBs cultured in control hES, SR5, SR6, SR7 and SR8 media. **+** = the layer was present, – =the layer was not detected.

	HS346 hES	HS346 SR7	HS401 hES	HS401 SR5	HS401 SR6	HS401 SR7	HS401 SR8
endoterm	+	-	+	+	+	-	-
ectoderm	+	+	+	-	+	+	-
mesoderm	+	+	+	+	+	+	+

As a final result from research with cells from both cell lines, HS346 and HS401, cultured in test group II media it was concluded that the best medium was SR3 which contained amino acids solvated in water and also in HCl thus these were added into all media in the next study in test group III. The conclusion was made according to the differentiation rates and also according to the morphology of the cells. SR3 medium sustained the undifferentiated growth of hESC for the longest time. But when considered the rates of cell attachment, the best medium was medium SR4 (contained two times the amount of bFGF as other media in test group II) with cells of both cell lines cultured in it. The cells cultured in medium SR3 had barely the fourth best rate of cell attachment.

4.3 Test group III

Growth Characteristics:

There were no major differences between morphologies of the cell colonies of cell lines HS346 and HS401, grown in test group III media. All the cell colonies survived in an undifferentiated state for only a few passages. One possible reason for this might be the poor condition of feeder cells on the plates. There were huge holes in the middle of the most of the feeder cell plates which may have affected the growth and survival of the hESC. This phenomenon also happened with test group II and not because of test media. The hFF should make a confluent layer onto which hESC can grow. Because of some reason, after when plated, they did not establish confluent layer but started to get loose of each others.

The cell colonies of both cell lines grown in Hesgro medium differentiated already during the adaptation stage when the amount of control hES medium was lowered

and test medium increased. The HS346 cells were cultured in Hesgro medium until the end of 80:20 adaptation phase and the HS401 cells until the end of the adaptation phase. The commercial Hesgro medium did not maintain the undifferentiated growth of hESC as compared to other test group III media.

The best of these test group III media was medium SR10 (contained amino acids in HCl and in water) in which the HS346 cells grew well in the beginning. The cell colonies had sharp edges and colonies were considerable thick and the HS346 and HS401 cells also survived 7 and 5 passages, respectively (see Figure 14). The rest of the cell colonies grown in test group III media did not grow very well but in the beginning there were some exceptions: the cell colonies grown in media SR30 (HS401, contained twice the concentration of bFGF as in other media in test group III) and SR90 (HS346, contained activin A, glutathione, thiamine and two times the amount of bFGF as in other media in test group III) looked good for a few passages but then those cells cultured in medium SR30 started to differentiate and the cells cultured in medium SR90 started to disappear. Cells of both cell lines cultured in medium SR20 (contained no amino acids in HCL) survived poorly until passage 6 (HS346) and passage 5 (HS401). HS346 and HS401 line cell colonies cultured in medium SR50 (contained activin A) were very thin and small all the time during the study and they survived until passages 6 and 5.

The HS401 cells grown in media SR60 (contained glutathione), SR70 (contained thiamine), SR80 (contained γ-secretase inhibitor IX) and SR90 also started to disappear and at the end there were almost no cells to be seen on the plate.

Representative cell colonies of the cell line HS401 during final culture passages are represented in Figure 14.

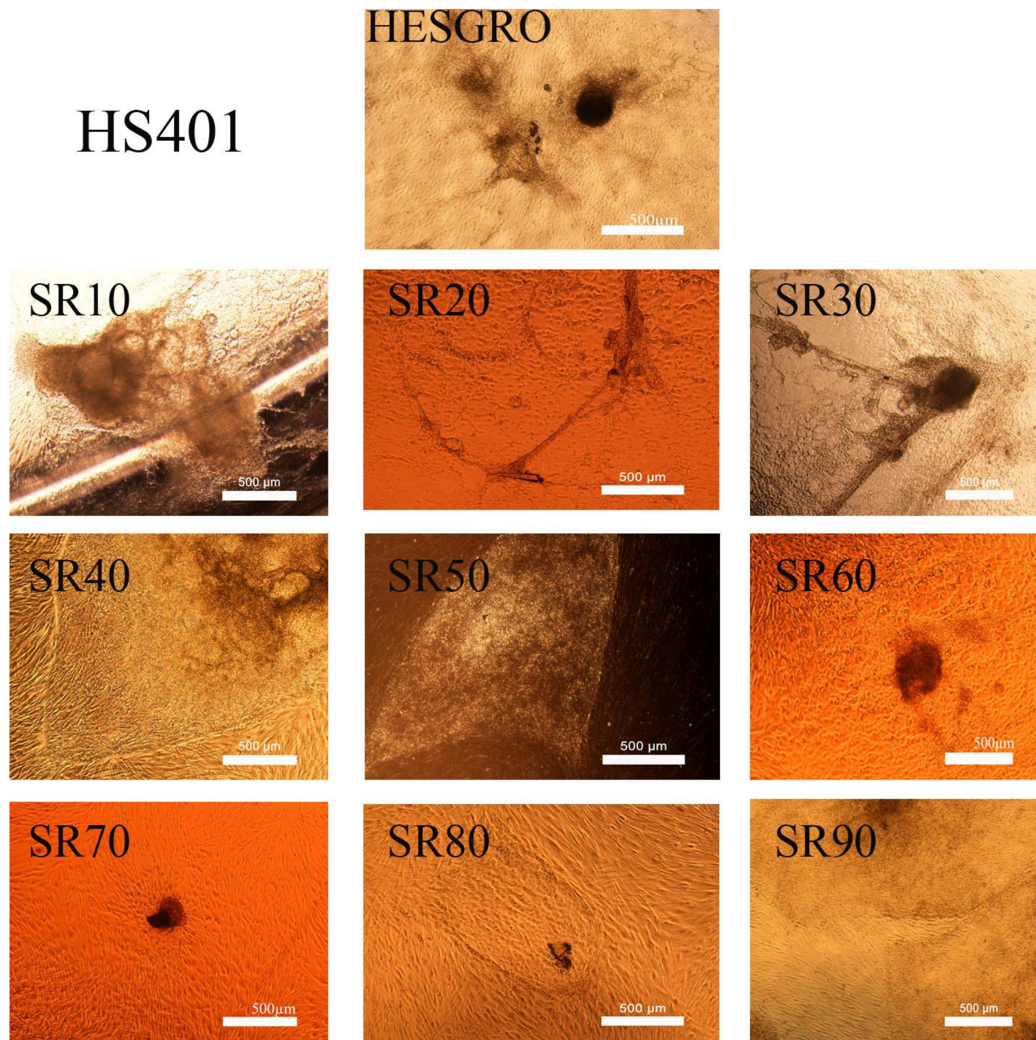

Figure 14. A representative colony of HS401 cells cultured in test group III and also in Hesgro medium during final passages. The cells in media SR10, SR20, SR30 and SR70 at passage 5, the colonies cultured in media SR40, SR50, SR60, SR80 and SR90 at passage 6 and the colonies cultured in Hesgro medium at passage 3. Scale bar is 500µm and the objective 4x.

According the results of growth characteristics in test group III, the best performing medium of both the cell lines, was medium SR10. It contained amino acids solvated in HCl and also in water.

Cell attachment:

There were no significant differences in the attachment rates between the cells cultured in test group III media, except that the attachment rate of HS346 cells cultured in medium SR70 was very low (Figure 15). The best attachment rate of the HS401 cells was observed in cells grown in media SR50 and SR80. The attachment rate of the cells cultured in medium SR50 was even higher than that of the cells cultured in control hES medium. The HS346 cells cultured in media SR80 and SR30 also had good attachment rates as

compared to other media in test group III. The HS346 cell colonies grown in media SR20, SR70 had the lowest attachment rates. The best medium based on attachment rates of the cells cultured in it, when considered both cell lines, was medium SR50. It contained activin A. Cells of the line HS346 cultured in medium SR10 had almost as high rate of attachment as cells cultured in hES medium but this was not the result with HS401 cell line cells.

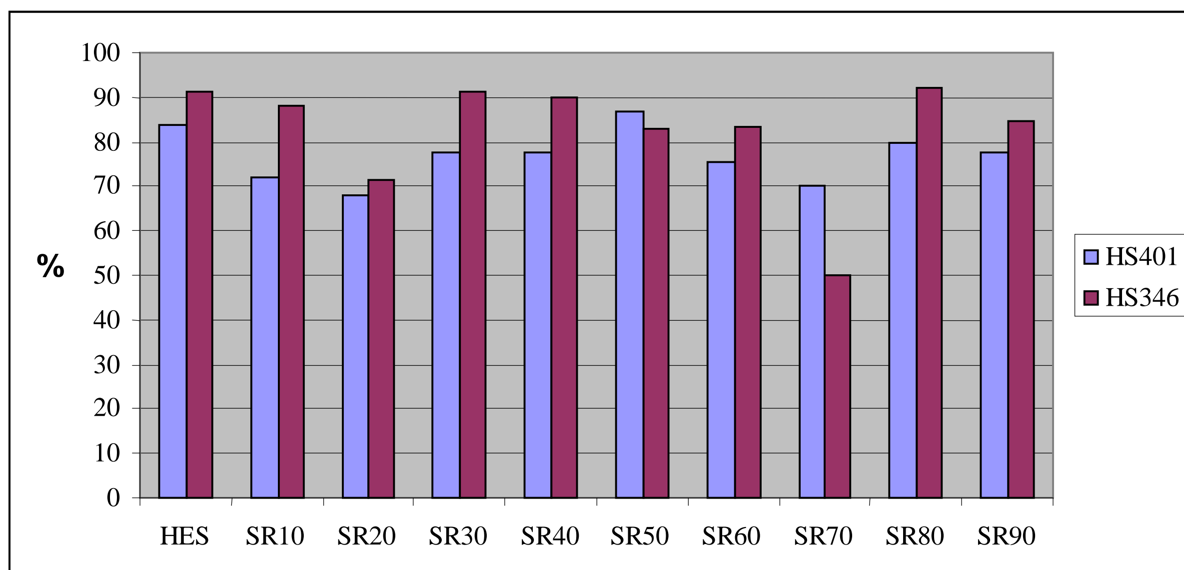

Figure 15. Attachment percentages of HS346 and HS401 cells cultured in test group III. Total number of all pieces is 807. Calculated attachment rates are from passages: HS401 cell line cultured in hES medium, P4-7 (n=105); SR10 medium, P4-5 (n=22); SR20 medium, P4-5 (n=22); SR30 medium, P4-5 (n=36); SR40 medium, P4-6 (n=54); SR50 medium, P4-6 (n=35); SR60 medium, P4-6 (n=37); SR70 medium, P4-5 (n=30); SR80 medium, P4-6 (n=20); SR90 medium P4-6 (n=63); HS346 line cultures hES medium, P4-7 (n=138); SR10 medium, P4-7 (n=25); SR20 medium, P4-6 (n=14); SR30 medium, P4-6 (n=23); SR40 medium, P4-7 (n=30); SR50 medium, P4-6 (n=47); SR60 medium, P4-5 (n=24); SR70 medium, P4-5 (n=24); SR80 medium, P4-6 (n=25) and SR90 medium, P4-6 (n=33).

Rate of differentiation:

Morphology:

The differentiation rates of the HS401 cells cultured in different culture media are presented in Figure 16. The cells cultured in medium SR10, which contained amino acids in HCl and in water, was the best medium altogether according to growth characteristics. It also had the best undifferentiation rate which was almost as high as that one of control hES medium. The cell colonies grown in SR40 which contained 5 times the amount of bFGF as in other media in test group III, SR50 (additive was activin A), SR60 (contained glutathione), SR70 (contained thiamine), SR80 (γ-secretase inhibitor IX), SR90 (contained addition of activin A, glutathione, thiamine and two times the amount of bFGF) media had

the highest differentiation (partially differentiated and totally differentiated cells) rates. Cells cultured in media SR20, SR30, SR40, SR50, SR60, SR70, SR80 and SR90 all had more partially differentiated cell colonies than undifferentiated or totally differentiated colonies and the biggest rate of these was that of cells cultured in medium SR90, more than 60% partially differentiated colonies of all colonies grown in it.

The highest amount of totally differentiated cell colonies was seen with cells cultured in media SR40, SR50, SR60 and SR70. There were not so many differences between differentiation rates of these four media.

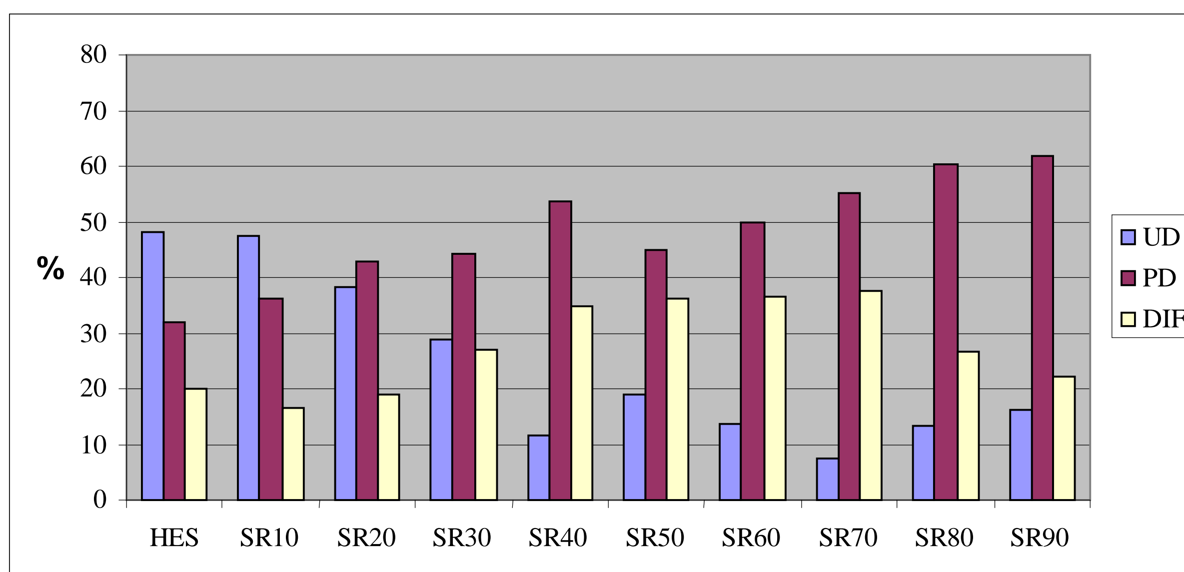

Figure 16. Differentiation of HS401cells cultured in test group III. Total number of all colonies is 739. Calculated values are from passages: hES medium, P1-5 (n=85); SR10 medium, P1-4 medium (n=61); SR20 medium, P1-4 (n=63); SR30 medium,P1-4 (n=70); SR40 medium, P1-5 (n=78); SR50 medium, P1-5 (n=69); SR60 medium, P1-5 (n=66); SR70 medium, P1-4 (n=80); SR80 medium, P1-5 (n=68) and SR90 medium, P1-5 (n=99). UD= undifferentiated, PD= partially differentiated, DIF= differentiated.

There were some differences between differentiation rates of HS346 cells cultured in test group III media (Figure 17). The cell colonies cultured in media SR90, SR70, SR60 and SR20 had the highest amount of undifferentiated cells and medium SR90 seemed to be the best of these. Over 60% of the undifferentiated colonies where observed with cells cultured in medium SR90 whereas only about 35% of cell colonies grown in that medium had partially or totally differentiated colony form. Also a very good differentiation rates were those of cells cultured in media SR70 and SR60 but when these two were compared to each other, medium SR70 had more undifferentiated cell colonies compared to partially or totally differentiated cell colonies of medium SR60.

The most differentiated (totally or partially differentiated altogether) cell colonies had been grown in media SR30, SR40 and SR50. The cells cultured in medium SR30 had the highest rate of totally differentiated colonies which amount to just over 30%. Cells grown in medium SR60 also had a very high differentiation level but as compared to medium SR30, cell colonies cultured in medium SR60 had many more undifferentiated cell colonies while medium SR30 had partially differentiated ones.

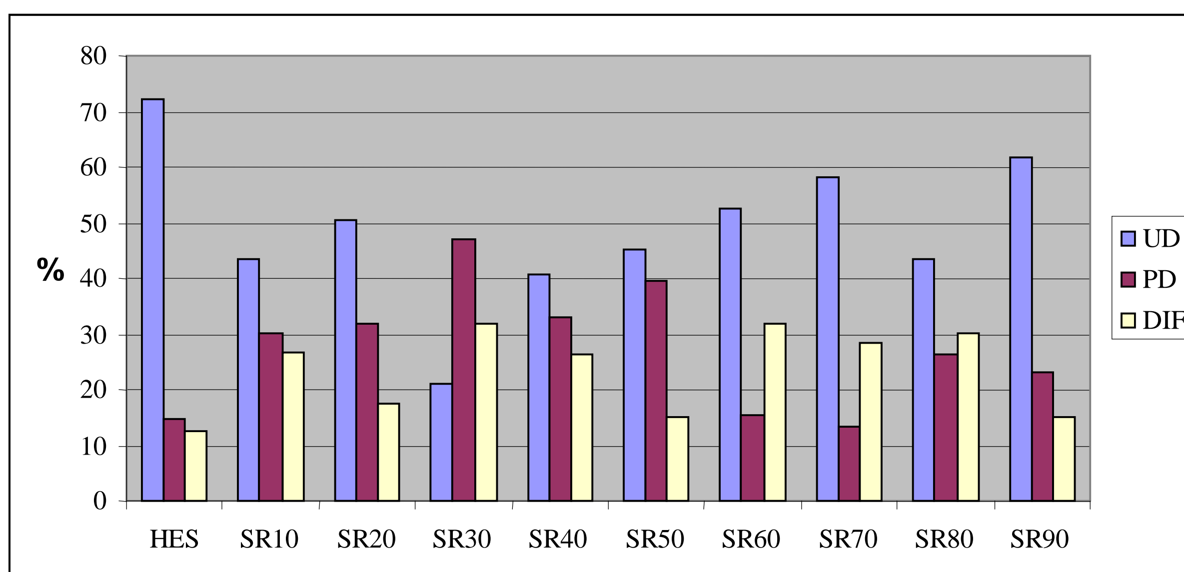

Figure 17. Differentiation of HS346 cells cultured in test group III media. Total number of all colonies is 792. Calculated values are from passages: hES medium, P1-6 (n=108); SR10 medium, P1-6 (n=83); SR20 medium, P1-5 (n=69); SR30 medium, P1-5 (n=66); SR40 medium, P1-6 (n=91); SR50 medium, P1-5 (n=93); SR60 medium, P1-4 (n=72); SR70 medium, P1-4 (n=74); SR80 medium, P1-5 (n=76); SR90 medium, P1-5 (n=60). UD= undifferentiated, PD= partially differentiated, DIF= differentiated.

According to the results of differentiation levels, the best medium with cell line HS401 cells cultured was medium SR10 while in the other hand the best medium with cell line HS346 cells was medium SR90. Medium SR10 contained amino acids in water and in HCl and medium SR90 did contain both amino acids, activin A, glutathione, thiamine and twice the amount of bFGF as in other in test group III media.

Immunocytochemical Analyzes:

The HS401 cell colonies cultured in test media SR40 and SR50 were immunostained with Oct-3/4 and TRA-1-60 antibodies. Under fluorescent microscope these hESC cell colonies showed specific staining for these antibodies. As the control was used cell colonies cultured in conventional hES medium.

As can be inferred from Figure 18, Oct-3/4 is not very bright in any of the cell colonies shown and there is a bit more background staining in picture of the cell colonies cultured in test media SR40 and SR50 than in control hES medium. However, the differences are not very big. These staining results clearly show that the stem cells have sustained the undifferentiated status.

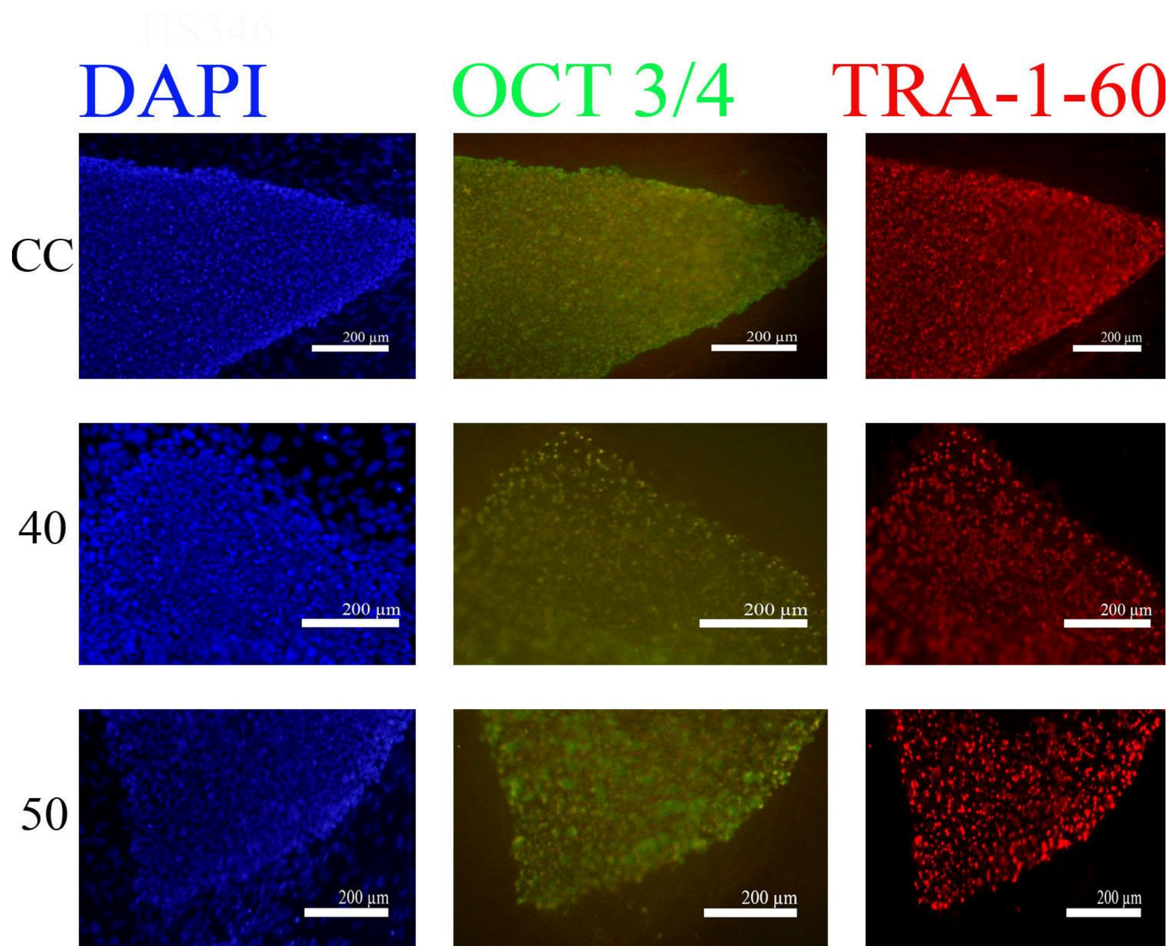

Figure 18. Fluorescence pictures for immunoassay of HS346 cell cultured in hES control medium and in SR40 and SR50 media. Microscopic pictures of cells cultured in media SR40 and SR50 have been taken at passage 6 and in hES medium at passage 11. Scale bar is 200µm and the objective 10x. CC= control hES medium, 40= SR40 medium, 50= SR50 medium.

As taken together, the best attachment rate of the HS401 line cells was observed with cells grown in SR50 and SR80 media. The attachment rate of cells cultured in medium SR50 was even higher than that of cells cultured in control hES medium. According the results of growth characteristics in test group III, the best performing medium of both of the cell lines cultured, was medium SR10. It contained amino acids solvated in HCl and also in water. HS401 line cells cultured in medium SR10 had the best undifferentiation rate which was almost as high as that one of control hES medium. But

when considering the cell line HS346 cells, the cell colonies cultured in media SR90, SR70, SR60 and SR20 had the highest amount of undifferentiated cells and of these medium SR90 seemed to be the best..

Pluripotency:

No EB culturing was made with test group III cells, because the cells did survive only poorly.

5. Discussion

The effects on PVA, transferrin, insulin, S1P, PDGF-AB, γ-secretase inhibitor IX, amino acids, thiamine, glutathione and bFGF into hESC differentiation and morphology were studied in this research with three cell lines. The aim of this study was to optimize a xeno- free culture medium which supports the undifferentiated growth of hESC in long-term culture. We also examined how HSA, insulin and transferrin together effect on hESC growth and behaviour and if HSA can be replaced with PVA in culture medium.

Test Group I

PVA in media B and C. PVA is a synthetic substance while HSA is human origin or produced with the recombinant technique in micro- organisms. We studied in this research if HSA in medium could be replaced by PVA. PVA would be safer option than human origin HSA when considered possible virus infections. Unfortunately results clearly demonstrated that media containing PVA but no HSA did produce colonies that only grew vigorously upwards, suggesting that hESC need some ingredient of HSA that influences the cell-to-cell adhesion between hESC and feeder cells in order to spread. This medium was tested many times and the conclusion was always the same the cells cultured in it still piled up on top of each other. These results indicate that the addition of PVA into hESC culture medium instead of HSA is totally unnecessary and even harmful for the culturing of hESC.

Transferrin in medium D and insulin, transferrin and HSA in medium H. Transferrin is in many research reports published as an additive (Li et al., 2005, Ludwig et al., 2006) in cell culture media but we did not find a big difference between medium containing it more as compared to one without the higher consentration. Transferrin addition had some influence on the thickness of the cell colonies but not on their size. The cell colonies grown in medium containing more transferrin looked almost like those colonies grown in the control medium, just a bit thicker. But after a few passages, the cell colonies quickly started to decrease in size and also became thinner. Comparing the cell colonies cultured in medium containing only the addition of transferrin to cell colonies grown in medium including more insulin, HSA and transferrin, the medium consisting of the addition of transferrin alone was better as a culture medium. The cell colonies grown in medium containing the addition of all three components did not support undifferentiated

growth of hESC in long- term culturing. The cells only survived a few passages before differentiating totally. Furthermore, the cell colonies were very small throughout this study but when compared to cell colonies from medium consisting only of transferrin the attachment percent of the cells was more than 20% higher so there was not as much material to lose. In this study the addition of transferrin did not approve the growth of hESC or reduce their spontaneous differentiation.

Activin A in medium E. Activin A has been identified in a wide variety of tissues as a regulator of diverse biological functions (Luisi et al., 2001). It has been reported that activin A maintains the self- renewal in hESC possibly by regulating bFGF, BMB and WNT signalling pathways. It also induces the expression of bFGF (Xiao et al., 2006). Activin A in this study had an effect on the morphology of the cell colonies grown in medium containing it. The cell colonies were very big but also very thin and so difficult to passage on. Activin A was effective only for a couple of passages in the beginning before cells got used to it and after that the cell colonies got smaller and smaller until it was impossible to split them anymore. This result is contradictory to some published reports. These reports suggest that activin A can be sufficient to promote long lasting undifferentiated growth of hESC (Brandenberger et al., 2004, Xiao et al., 2006). Xiao and co- workers used concentration of 10ng/ml of activin A and did demonstrate that it is both sufficient and necessary for the maintenance of pluripotency of hESC. But when compared to Vallier's and co- workers' research there are some similarities. Even they have also used higher amount (10ng/ml) of activin A than in this study (Vallier et al., 2005): their conclusion was that activin A is sufficient to prevent differentiation for only a short time in culture and that the effect of activin A is a dose- dependent. But Rajala's research group has tried concentration of 10ng/ml unsuccessfully (Rajala et al., unpublished) so this is contradictory to the conclusion of Vallier. The attachment was almost 70% in this study with cells cultured in medium containing activin A, so split pieces fastened on rather well compared to control cells of which only 55% did attach. When considering differentiation rates of cell colonies cultured in medium containing activin A, it must be noted that they were smaller than of the cells cultured in control medium. Based on these results more research must be done with activin A in aim of finding out the right concentration to use in order to get big as well as thick cell colonies. However, more research is required regarding the impact of activin A on hESC and on feeder cells.

S1P and PDGF-AB in medium F. S1P and PDGF-AB have an important role in maintaining undifferentiated status and inhibit the spontaneous differentiation of hESC by acting together with tyrosine kinase receptor activity (Pebay et al., 2005). They are lysophospholipids present in serum and are released by activated platelets. S1P and PDGF-AB function in many cellular functions including cell differentiation and proliferation (Pebay et al., 2005). It also has been published (Water at al., 2002) that S1P and PDGF-AB establish together a functional signalling unit that regulates MAPK signalling route in cells and so take part in cell fate decisions. In this study S1P and PDGF-AB had an influence both on the size and the thickness of the cell colonies. The cell colonies grown in media supplemented with them were the thickest and the biggest in the beginning compared to ones grown in control medium. But after a few passages the cell colonies started to differentiate partially suggesting that the confluence does not last very long. This is controversial to research of Pebay and co- workers. They used the same concentrations of S1P (10μM) and PDGF-AB (20ng/ml) as in this study but they concluded that these two additives together can prevent the spontaneous differentiation of hESC. But we did not succeed to prove this. Some cell colonies were also immunostained in this study and so their undifferentiated status was proven. But when considering the results of attachment (10% higher as with the control) and differentiation (almost the same as with control) rates these additives seem to be promising, although some more work needs to be done to define the exact concentrations to use. But altogether the results were very interesting and worthy of more research.

γ- secretase inhibitor IX in medium G. It has been published that γ- secretase inhibitors regulate NOTCH signalling pathway in mammary gland stem cells. NOTCH pathway in turn acts in cell fate decisions at in several distinct developmental stages and γ- secretase inhibitor can block this (Dontu et a., 2004). During this study medium consisting γ- secretase inhibitor IX did produce very promising cell colonies in the first few passages. The cell colonies were very thick and big and they also lasted longer in the undifferentiated state than the cell colonies cultured in medium containing S1P and PDGF-AB. However, after a while the size of the cell colonies decreased until it was impossible to split them anymore. But it is also notable that attachment rates of the cell colonies were smaller in size than the ones of other tested media. As a conclusion γ- secretase inhibitor IX possibly has some positive effect on hESC morphology as it has also been reported by Vujovic

(2007). They concluded that γ - secretases play an important role in both human mesenchymal stem cells proliferation and differentiation. According to this conclusion γ-secretase inhibitors have major implicative roles in mesenchymal stem cells self-renewal and therefore maybe also in self- renewal of hESC (Vujovic et al., 2007). Based on our study results some very promising impacts with γ- secretase inhibitor IX on hESC culture may exist.

No amino acids, thiamine or glutathione in media I, J and K. Deletion of amino acids or thiamine or glutathione in medium resulted in big but thin cell colonies. The cell colonies grown in medium containing no amino acids were the biggest cell colonies grown in this study but when compared to medium consisting also of amino acids and thiamine or glutathione there were some differences. When amino acids were added as solvated in hydrogen chloride or in water into the medium the thickness of the cell colonies altered. The cell colonies grown with amino acids were thicker than ones cultured without them. Also attachment rates of the cells were the best in this study with HS237 cell line cells (all above 70%), the difference to cells grown in control medium was more than 20%. So, the addition of amino acids, thiamine or glutathione induced undifferentiated growth of hESC the most in this study and in long term culturing (15 passages) and also enhanced attachment of the pieces. It must also be noted that cells grown in media (J and K) including no amino acids and or thiamine or glutathione all had quite good undifferentiation values; they all were almost 80% while the cells cultured in control medium had just over 80%. So, these factors have some positive influence on hESC growth and undifferentiation status and certainly need more thorough exploring.

For any serious conclusions with the cell line HS237, all these results need to be continued with some hESC line free from chromosomal mutations that could have an affect on the behaviour, morphology and differentiation of hESC in order to draw reliable conclusions and the decisions of future research activities. Because of this we continued the research with HS346 and HS401 cell lines using the best additives from test group I media (amino acids, thiamine and glutathione) again as additives.

Test Group II

Media SR1 to SR8. NaCl was added into media in test group II to adjust the osmolality into a right level of 320mOsm/kg. The best medium of these media was medium SR3 and it contained amino acids solvated in water and also in HCl. The pH of SR3 was also measured to 7.4 individually. In the beginning it seemed to be the best medium based on the appearance of the cell colonies because they were both thick and big. The cell colonies grown in SR3 also survived longest until passage 11 (cell line HS346) and passage 12 (cell line HS401) without differentiation. So, amino acids must be examined further aiming to achieve the right concentrations to use in culture medium in the future.

There were no other significant differences among colonies grown in other test media mainly because of poor condition of feeder cells so no big conclusions can not be done based on these results.

EBs were formed and cultured in media hES, SR7 (HS346), hES, SR5, SR6, SR7, SR8 (HS401) in order to demonstrate that hESC have maintained their pluripotent status in test culture media. From EBs differentiated in control hES and in test medium SR6 (HS401) all genes representing three embryonic layers were found. From HS346 EBs and also HS401 EBs differentiated and cultured in medium SR7 did express genes representing ectoderm and mesoderm but no genes of endoderm. EBs cultured in medium SR5 of HS401 cell line the ectoderm was not expressed and from EBs cultured in medium SR8 of HS401 the endoderm and ectoderm were undetected. Possible causes for these results are uncertain. One possible reason might be the handling of the RNA when extracted. There can be some nixes in RNA or helixes can be totally broken. If the RNA is broken then there are no pieces long enough for primers to attach themselves to and then the RNA`s duplication will be insufficient in PCR. After this the PCR product can not be detected in gel. But on other hand, this maybe is not the reason because the house- keeping gene did work. And this suggests that there are no big nixes at least in that gene. Undetection of the endoderm of EBs cultured in medium SR8 can be due to γ- secretase inhibitor IX activity. γ- secretase inhibitor IX can block the endodermal differentiation, but this has not been proven in this study, because the endoderm was missing also from few other EBs, not just from one grown in media containing it.

Test Group III

There were no remarkable research results with test group III media. Colonies did suffer because the feeder cells beneath them were in bad condition and so they lasted undifferentiated *in vitro* for only a couple of passages. But when considering the growth characteristics and cell differentiation rates of the cells cultured these few passages in media containing different additives, the best additives proved to be amino acids. The cell colonies cultured in medium containing amino acids did have the highest rate of undifferentiated growth when compared to cells cultured in media containing some other additive.

Exogenously added bFGF has been considered the most essential for the maintenance of hESC (Vallier et al., 2005). But when comparing medium containing twice or five times the amount of bFGF to the others, there were no remarkable differences in undifferentiation or in attachment rates. All the cell colonies lasted just a few passages but this can be due to the poor feeder cells beneath them.

Hesgro medium did not support hESC long- term culture. Colonies grown in it stayed undifferentiated only for adaptation phase passages. The cell colonies started to differentiate as soon as the concentration of conventional hES medium was lowered in the adaptation phase. So, the conclusion is that Hesgro medium as a culture medium still lacks some very important factors that have influence on the morphology and growth of hESC and more effort must be put into inventing a workable and ideal solution for culturing hESC.

6. Summary

The common aim of this study was to optimize house- made hESC culture medium towards xeno- free one that could be used instead of the conventional hESC medium. The parameters used in this study to evaluate the growth and characteristics of hESC were: observations of cell morphology, rate of cell attachment, differentiation rates, pluripotency of the cells and immunocytochemical analyzes. The results of this study indicated, that the addition of amino acids, thiamine or glutathione into a culture medium have some positive effect on undifferentiated growth, attachment and proliferation rates of hESC. We also found that HSA is not preferable to be replaced with polyvinylalcohol but is essential to undifferentiated growth of hESC. Another conclusion based on this study was that commercially available Hesgro does not support culturing of hESC in long- term basis. The optimization of house- made culture medium should be continued and the concentrations of these potential additives should also be investigated further in the future.

7. References

Amit, M., Carpenter, M.K., Inokuma, M.S., Chiu, C.P., Harris, C.P., Waknitz, M.A., Itskovitz-Eldor, J., and Thomson, J.A. 2000. Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. *Dev.Biol.* 227:271-278.

Amit, M., Margulets, V., Segev, H., Shariki, K., Laevsky, I., Coleman, R., and Itskovitz-Eldor, J. 2003. Human feeder layers for human embryonic stem cells. *Biol.Reprod.* 68:2150-2156.

Amit, M., Shariki, C., Margulets, V., and Itskovitz-Eldor, J. 2004. Feeder layer- and serum-free culture of human embryonic stem cells. *Biol.Reprod.* 70:837-845.

Amit, M., Winkler, M.E., Menke, S., Bruning, E., Buscher, K., Denner, J., Haverich, A., Itskovitz-Eldor, J., and Martin, U. 2005. No evidence for infection of human embryonic stem cells by feeder cell-derived murine leukemia viruses. *Stem Cells.* 23:761-771.

Badcock, G., Pigott, C., Goepel, J., and Andrews, P.W. 1999. The human embryonal carcinoma marker antigen TRA-1-60 is a sialylated keratan sulfate proteoglycan. *Cancer Res.* 59:4715-4719.

Battula, V.L., Bareiss, P.M., Treml, S., Conrad, S., Albert, I., Hojak, S., Abele, H., Schewe, B., Just, L., Skutella, T., and Buhring, H.J. 2007. Human placenta and bone marrow derived MSC cultured in serum-free, b-FGF-containing medium express cell surface frizzled-9 and SSEA-4 and give rise to multilineage differentiation. *Differentiation.* 75:279-291.

Beattie, G.M., Lopez, A.D., Bucay, N., Hinton, A., Firpo, M.T., King, C.C., and Hayek, A. 2005. Activin A maintains pluripotency of human embryonic stem cells in the absence of feeder layers. *Stem Cells.* 23:489-495.

Bell, D.R., and Van Zant, G. 2004. Stem cells, aging, and cancer: inevitabilities and outcomes. *Oncogene.* 23:7290-7296.

Bradley, J.A., Bolton, E.M., and Pedersen, R.A. 2002. Stem cell medicine encounters the immune system. *Nat.Rev.Immunol.* 2:859-871.

Brandenberger, R., Wei, H., Zhang, S., Lei, S., Murage, J., Fisk, G.J., Li, Y., Xu, C., Fang, R., Guegler, K., Rao, M.S., Mandalam, R., Lebkowski, J., and Stanton, L.W. 2004. Transcriptome characterization elucidates signaling networks that control human ES cell growth and differentiation. *Nat.Biotechnol.* 22:707-716.

Cabrera, C., Cobo, F., Nieto, A., and Concha, A. 2006. Strategies for preventing immunologic rejection of transplanted human embryonic stem cells. *Cytotherapy.* 8:517-518.

Chambers, I., Colby, D., Robertson, M., Nichols, J., Lee, S., Tweedie, S., and Smith, A. 2003. Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. *Cell.* 113:643-655.

Chang, C.M., Kao, C.L., Chang, Y.L., Yang, M.J., Chen, Y.C., Sung, B.L., Tsai, T.H., Chao, K.C., Chiou, S.H., and Ku, H.H. 2007. Placenta-derived multipotent stem cells induced to differentiate into insulin-positive cells. *Biochem.Biophys.Res.Commun.* 357:414-420.

Chin, A.C., Fong, W.J., Goh, L.T., Philp, R., Oh, S.K., and Choo, A.B. 2007. Identification of proteins from feeder conditioned medium that support human embryonic stem cells. *J.Biotechnol.*

Chiu, Arlene.Y. and Rao Mahendra. S. 2003. Human Embryonic Stem Cells. Humana Press inc. New Jersey.

Cole, R.J., Edwards, R.G., and Paul, J. 1966. Cytodifferentiation and embryogenesis in cell colonies and tissue cultures derived from ova and blastocysts of the rabbit. *Dev.Biol.* 13:385-407.

Cole, R.J., Edwards, R.G., and Paul, J. 1965. Cytodifferentiation in cell colonies and cell strains derived from cleaving ova and blastocysts of the rabbit. *Exp.Cell Res.* 37:501-504.

Cooper, D.K., Groth, C.G., and McKenzie, I.F. 2000. Xenotransplantation. This new form of treatment might benefit millions. *BMJ.* 320:868.

Cooper, S., Bennett, W., Andrade, J., Reubinoff, B.E., Thomson, J., and Pera, M.F. 2002. Biochemical properties of a keratan sulphate/chondroitin sulphate proteoglycan expressed in primate pluripotent stem cells. *J.Anat.* 200:259-265.

Cowan, C.A., Klimanskaya, I., McMahon, J., Atienza, J., Witmyer, J., Zucker, J.P., Wang, S., Morton, C.C., McMahon, A.P., Powers, D., and Melton, D.A. 2004. Derivation of embryonic stem-cell lines from human blastocysts. *N.Engl.J.Med.* 350:1353-1356.

De Sousa, P.A., Galea, G., and Turner, M. 2006. The road to providing human embryo stem cells for therapeutic use: the UK experience. *Reproduction.* 132:681-689.

Delo, D.M., De Coppi, P., Bartsch, G.,Jr, and Atala, A. 2006. Amniotic fluid and placental stem cells. *Methods Enzymol.* 419:426-438.

Ding, V., Choo, A.B., and Oh, S.K. 2006. Deciphering the importance of three key media components in human embryonic stem cell cultures. *Biotechnol.Lett.* 28:491-495.

Draper, J.S., Moore, H.D., Ruban, L.N., Gokhale, P.J., and Andrews, P.W. 2004. Culture and characterization of human embryonic stem cells. *Stem Cells Dev.* 13:325-336.

Drukker, M., Katchman, H., Katz, G., Even-Tov Friedman, S., Shezen, E., Hornstein, E., Mandelboim, O., Reisner, Y., and Benvenisty, N. 2006. Human embryonic stem cells and their differentiated derivatives are less susceptible to immune rejection than adult cells. *Stem Cells.* 24:221-229.

Dontu, G., Jackson, KW., McNicholas, E., Kawamura, MJ., Abdallah, WM., and Wicha, MS. 2004. Role of Notch signaling in cell-fate determination of human mammary stem/progenitor cells. *Breast Cancer Res.* 6(6):R605-R615.

Gadue, P., Huber, T.L., Paddison, P.J., and Keller, G.M. 2006. Wnt and TGF-beta signaling are required for the induction of an in vitro model of primitive streak formation using embryonic stem cells. *Proc.Natl.Acad.Sci.U.S.A.* 103:16806-16811.

Gordon, J.W., Scangos, G.A., Plotkin, D.J., Barbosa, J.A., and Ruddle, F.H. 1980. Genetic transformation of mouse embryos by microinjection of purified DNA. *Proc.Natl.Acad.Sci.U.S.A.* 77:7380-7384.

Greber, B., Lehrach, H., and Adjaye, J. 2007. Fibroblast growth factor 2 modulates transforming growth factor beta signaling in mouse embryonic fibroblasts and human ESCs (hESCs) to support hESC self-renewal. *Stem Cells.* 25:455-464.

Grinnemo, K.H., Kumagai-Braesch, M., Månsson-Broberg, A., Skottman, H., Hao, X., Siddiqui, A., Andersson, A., Strömberg, A.M., Lahesmaa, R., Hovatta, O., Sylven, C., Corbascio, M., and Dellgren, G. 2006. Human embryonic stem cells are immunogenic in allogeneic and xeno-geneic settings. *Reprod.Biomed.Online.* 13:712-724.

Henderson, J.K., Draper, J.S., Baillie, H.S., Fishel, S., Thomson, J.A., Moore, H., and Andrews, P.W. 2002. Preimplantation human embryos and embryonic stem cells show comparable expression of stage-specific embryonic antigens. *Stem Cells.* 20:329-337.

Hiyama, E., and Hiyama, K. 2007. Telomere and telomerase in stem cells. *Br.J.Cancer.* 96:1020-1024.

Hoffman, L.M., and Carpenter, M.K. 2005. Characterization and culture of human embryonic stem cells. *Nat.Biotechnol.* 23:699-708.

Hovatta, O., Mikkola, M., Gertow, K., Stromberg, A.M., Inzunza, J., Hreinsson, J., Rozell, B., Blennow, E., Andang, M., and Ahrlund-Richter, L. 2003. A culture system using human foreskin fibroblasts as feeder cells allows production of human embryonic stem cells. *Hum.Reprod.* 18(7):1404-9

Hyslop, L.A., Armstrong, L., Stojkovic, M., and Lako, M. 2005. Human embryonic stem cells: biology and clinical implications. *Expert Rev.Mol.Med.* 7:1-21.

Inzunza, J., Gertow, K., Stromberg, M.A., Matilainen, E., Blennow, E., Skottman, H., Wolbank, S., Ahrlund-Richter, L., and Hovatta, O. 2005. Derivation of human embryonic stem cel lines in serum replacement medium using postnatal human fibroblasts as feeder cells. *Stem Cells 23:544-549*

Jackson, K.A., Majka, S.M., Wang, H., Pocius, J., Hartley, C.J., Majesky, M.W., Entman, M.L., Michael, L.H., Hirschi, K.K., and Goodell, M.A. 2001. Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. *J.Clin.Invest.* 107:1395-1402.

James, D., Levine, A.J., Besser, D., and Hemmati-Brivanlou, A. 2005. TGFbeta/activin/nodal signaling is necessary for the maintenance of pluripotency in human embryonic stem cells. *Development.* 132:1273-1282.

Javelaud, D., and Mauviel, A. 2005. Crosstalk mechanisms between the mitogen-activated protein kinase pathways and Smad signaling downstream of TGF-beta: implications for carcinogenesis. *Oncogene.* 24:5742-5750.

Jiang, J., Au, M., Lu, K., Eshpeter, A., Korbutt, G., Fisk, G., and Majumdar, A.S. 2007. Generation of Insulin-producing Islet-like Clusters from Human Embryonic Stem Cells. *Stem Cells.*

Jones, J.M., and Thomson, J.A. 2000. Human embryonic stem cell technology. *Semin.Reprod.Med.* 18:219-223.

Kleber, M., and Sommer, L. 2004. Wnt signaling and the regulation of stem cell function. *Curr.Opin.Cell Biol.* 16:681-687.

Koivisto, H., Hyvarinen, M., Stromberg, A.M., Inzunza, J., Matilainen, E., Mikkola, M., Hovatta, O., and Teerijoki, H. 2004. Cultures of human embryonic stem cells: serum replacement medium or serum-containing media and the effect of basic fibroblast growth factor. *Reprod.Biomed.Online.* 9:330-337.

Lee, H., Al Shamy, G., Elkabetz, Y., Schoefield, C.M., Harrsion, N.L., Panagiotakos, G., Socci, N.D., Tabar, V., and Studer, L. 2007. Directed Differentiation And Transplantation of Human Embryonic Stem Cell Derived Motoneurons. *Stem Cells.*

Lee, J.B., Lee, J.E., Park, J.H., Kim, S.J., Kim, M.K., Roh, S.I., and Yoon, H.S. 2005. Establishment and maintenance of human embryonic stem cell lines on human feeder cells derived from uterine endometrium under serum-free condition. *Biol.Reprod.* 72:42-49.

Li, Y., Powell, S., Brunette, E., Lebkowski, J., and Mandalam, R. 2005. Expansion of human embryonic stem cells in defined serum-free medium devoid of animal-derived products. *Biotechnol.Bioeng.* 91:688-698.

Lim, J.W., and Bodnar, A. 2002. Proteome analysis of conditioned medium from mouse embryonic fibroblast feeder layers which support the growth of human embryonic stem cells. *Proteomics.* 2:1187-1203.

Lu, J., Hou, R., Booth, C.J., Yang, S.H., and Snyder, M. 2006. Defined culture conditions of human embryonic stem cells. *Proc.Natl.Acad.Sci.U.S.A.* 103:5688-5693.

Lu, S.J., Feng, Q., Caballero, S., Chen, Y., Moore, M.A., Grant, M.B., and Lanza, R. 2007. Generation of functional hemangioblasts from human embryonic stem cells. *Nat.Methods.*

Ludwig, T.E., Levenstein, M.E., Jones, J.M., Berggren, W.T., Mitchen, E.R., Frane, J.L., Crandall, L.J., Daigh, C.A., Conard, K.R., Piekarczyk, M.S., Llanas, R.A., and Thomson, J.A. 2006. Derivation of human embryonic stem cells in defined conditions. *Nat.Biotechnol.* 24:185-187.

Luisi, S., Florio, P., Reis, FM. 2001. Expression and secretion of activin a: possible physiological and clinical implications. *Eur J Endocrinol.* 142:225-236.

Lumelsky, N., Blondel, O., Laeng, P., Velasco, I., Ravin, R., and McKay, R. 2001. Differentiation of embryonic stem cells to insulin-secreting structures similar to pancreatic islets. *Science.* 292:1389-1394.

Martin, G.R., Silver, L.M., Fox, H.S., and Joyner, A.L. 1987. Establishment of embryonic stem cell lines from preimplantation mouse embryos homozygous for lethal mutations in the t-complex. *Dev.Biol.* 121:20-28.

Martin, M.J., Muotri, A., Gage, F., and Varki, A. 2005. Human embryonic stem cells express an immunogenic nonhuman sialic acid. *Nat.Med.* 11:228-232.

Minguell, J.J., Erices, A., and Conget, P. 2001. Mesenchymal stem cells. *Exp.Biol.Med.(Maywood).* 226:507-520.

Mitsui, K., Tokuzawa, Y., Itoh, H., Segawa, K., Murakami, M., Takahashi, K., Maruyama, M., Maeda, M., and Yamanaka, S. 2003. The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. *Cell.* 113:631-642.

Montarras, D., Morgan, J., Collins, C., Relaix, F., Zaffran, S., Cumano, A., Partridge, T., and Buckingham, M. 2005. Direct isolation of satellite cells for skeletal muscle regeneration. *Science.* 309:2064-2067.

Moon, R.T., Bowerman, B., Boutros, M., and Perrimon, N. 2002. The promise and perils of Wnt signaling through beta-catenin. *Science.* 296:1644-1646.

Mummery, C. 2007. Cardiomyocytes from human embryonic stem cells: more than heart repair alone. *Bioessays.* 29:572-579.

Nasonkin, I.O., and Koliatsos, V.E. 2006. Nonhuman sialic acid Neu5Gc is very low in human embryonic stem cell-derived neural precursors differentiated with B27/N2 and noggin: implications for transplantation. *Exp.Neurol.* 201:525-529.

Noggle, S.A., James, D., and Brivanlou, A.H. 2005. A molecular basis for human embryonic stem cell pluripotency. *Stem Cell.Rev.* 1:111-118.

Packard, M., Mathew, D., and Budnik, V. 2003. Wnts and TGF beta in synaptogenesis: old friends signalling at new places. *Nat.Rev.Neurosci.* 4:113-120.

Parisi, S., D'Andrea, D., Lago, C.T., Adamson, E.D., Persico, M.G., and Minchiotti, G. 2003. Nodal-dependent Cripto signaling promotes cardiomyogenesis and redirects the neural fate of embryonic stem cells. *J.Cell Biol.* 163:303-314.

Pebay, A., Wong, R.C., Pitson, S.M., Wolvetang, E.J., Peh, G.S., Filipczyk, A., Koh, K.L., Tellis, I., Nguyen, L.T., and Pera, M.F. 2005. Essential roles of sphingosine-1-phosphate and platelet-derived growth factor in the maintenance of human embryonic stem cells. *Stem Cells.* 23:1541-1548.

Prowse, A.B., McQuade, L.R., Bryant, K.J., Van Dyk, D.D., Tuch, B.E., and Gray, P.P. 2005. A proteome analysis of conditioned media from human neonatal fibroblasts used in the maintenance of human embryonic stem cells. *Proteomics.* 5:978-989.

Raikwar, S.P., Mueller, T., and Zavazava, N. 2006. Strategies for developing therapeutic application of human embryonic stem cells. *Physiology (Bethesda).* 21:19-28.

Rajala, K., Hakala, H., Panula, S., Aivio, S., Pihlajamaki, H., Suuronen, R., Hovatta, O., and Skottman, H. 2007. Testing of nine different xeno-free culture media for human embryonic stem cell cultures. *Hum.Reprod.* 22:1231-1238.

Reubinoff, B.E., Pera, M.F., Fong, C.Y., Trounson, A., and Bongso, A. 2000. Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. *Nat.Biotechnol.* 18:399-404.

Reya, T., Duncan, A.W., Ailles, L., Domen, J., Scherer, D.C., Willert, K., Hintz, L., Nusse, R., and Weissman, I.L. 2003. A role for Wnt signalling in self-renewal of haematopoietic stem cells. *Nature.* 423:409-414.

Richards, M., Fong, C.Y., Chan, W.K., Wong, P.C., and Bongso, A. 2002. Human feeders support prolonged undifferentiated growth of human inner cell masses and embryonic stem cells. *Nat.Biotechnol.* 20:933-936.

Richards, M., Tan, S., Fong, C.Y., Biswas, A., Chan, W.K., and Bongso, A. 2003. Comparative evaluation of various human feeders for prolonged undifferentiated growth of human embryonic stem cells. *Stem Cells.* 21:546-556.

Robertson, J.A. 2001. Human embryonic stem cell research: ethical and legal issues. *Nat.Rev.Genet.* 2:74-78.

Rodriguez, C.I., Galan, A., Valbuena, D., and Simon, C. 2006. Derivation of clinical-grade human embryonic stem cells. *Reprod.Biomed.Online.* 12:112-118.

Salter, B. 2007. Patenting, morality and human embryonic stem cell science: bioethics and cultural politics in Europe. *Regen.Med.* 2:301-311.

Schier, A.F. 2003. Nodal signaling in vertebrate development. *Annu.Rev.Cell Dev.Biol.* 19:589-621.

Schuldiner, M., Yanuka, O., Itskovitz-Eldor, J., Melton, D.A., and Benvenisty, N. 2000. Effects of eight growth factors on the differentiation of cells derived from human embryonic stem cells. *Proc.Natl.Acad.Sci.U.S.A.* 97:11307-11312.

Semb, H. 2005. Human embryonic stem cells: origin, properties and applications. *APMIS.* 113:743-750.

Skottman, H., and Hovatta, O. 2006. Culture conditions for human embryonic stem cells. *Reproduction.* 132:691-698.

Stojkovic, P., Lako, M., Stewart, R., Przyborski, S., Armstrong, L., Evans, J., Murdoch, A., Strachan, T., and Stojkovic, M. 2005. An autogeneic feeder cell system that efficiently supports growth of undifferentiated human embryonic stem cells. *Stem Cells.* 23:306-314.

Tabibzadeh, S., and Hemmati-Brivanlou, A. 2006. Lefty at the crossroads of "stemness" and differentiative events. *Stem Cells.* 24:1998-2006.

Tam, W.L., Ang, Y.S., and Lim, B. 2007. The molecular basis of ageing in stem cells. *Mech.Ageing Dev.* 128:137-148.

Thomson, J.A., Itskovitz-Eldor, J., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., Marshall, V.S., and Jones, J.M. 1998. Embryonic stem cell lines derived from human blastocysts. *Science.* 282:1145-1147.

Thomson, J.A., and Marshall, V.S. 1998. Primate embryonic stem cells. *Curr.Top.Dev.Biol.* 38:133-165.

Tsung, H.C., and Yao, Z. 1987. Some biological characteristics and karyotype analysis of a mouse embryonic stem cell line-ES 8501 cells. *Shi Yan Sheng Wu Xue Bao.* 20:237-251.

Valdimarsdottir, G., and Mummery, C. 2005. Functions of the TGFbeta superfamily in human embryonic stem cells. *APMIS.* 113:773-789.

Vallier, L., Alexander, M., and Pedersen, R.A. 2005. Activin/Nodal and FGF pathways cooperate to maintain pluripotency of human embryonic stem cells. *J.Cell.Sci.* 118:4495-4509.

van Es, J.H., Barker, N., and Clevers, H. 2003. You Wnt some, you lose some: oncogenes in the Wnt signaling pathway. *Curr.Opin.Genet.Dev.* 13:28-33.

Vujovic, S., Henderson, S.R., Flanagan, A.M., and Clements, M.O. 2007. Inhibition of gamma-secretases alters both proliferation and differentiation of mesenchymal stem cells. *Cell Prolif.* 40:185-195.

Wang, J., and Wynshaw-Boris, A. 2004. The canonical Wnt pathway in early mammalian embryogenesis and stem cell maintenance/differentiation. *Curr.Opin.Genet.Dev.* 14:533-539.

Waters, C., Sambi, B., Kong, K.C., Thompson, D., Pitson, S.M., Pyne, S., and Pyne, N.J. 2003. Sphingosine 1-phosphate and platelet-derived growth factor (PDGF) act via PDGF beta receptor-sphingosine 1-phosphate receptor complexes in airway smooth muscle cells. *J.Biol.Chem.* 278:6282-6290.

Wilson, S.I., Rydstrom, A., Trimborn, T., Willert, K., Nusse, R., Jessell, T.M., and Edlund, T. 2001. The status of Wnt signalling regulates neural and epidermal fates in the chick embryo. *Nature.* 411:325-330.

Xiao, L., Yuan, X., and Sharkis, S.J. 2006. Activin A maintains self-renewal and regulates fibroblast growth factor, Wnt, and bone morphogenic protein pathways in human embryonic stem cells. *Stem Cells.* 24:1476-1486.

Xu, C., Inokuma, M.S., Denham, J., Golds, K., Kundu, P., Gold, J.D., and Carpenter, M.K. 2001. Feeder-free growth of undifferentiated human embryonic stem cells. *Nat.Biotechnol.* 19:971-974.

Yen, B.L., Huang, H.I., Chien, C.C., Jui, H.Y., Ko, B.S., Yao, M., Shun, C.T., Yen, M.L., Lee, M.C., and Chen, Y.C. 2005. Isolation of multipotent cells from human term placenta. *Stem Cells.* 23:3-9.

Zimmermann, W.H., and Eschenhagen, T. 2007. Embryonic stem cells for cardiac muscle engineering. *Trends Cardiovasc.Med.* 17:134-140.

Zwaka, T.P., and Thomson, J.A. 2005. Differentiation of human embryonic stem cells occurs through symmetric cell division. *Stem Cells.* 23:146-149.