HUMAN AMNIOTIC EPITHELIAL CELLS: ISOLATION AND CHARACTERISATION

RUTH GOMEZ DOMINGUEZ

INAUGURALDISSERTATION
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vorgelegt von

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1 INTRODUCTION

1.1 Human placenta and membranes

The placenta, an organ characteristic for mammals, mediates the contact between mother and fetus, providing endocrine secretion and selective exchange of soluble substances through an apposition of uterine and trophoblastic vascularized parts. At the end of the pregnancy the oval shaped placenta is about 15-25 cm in diameter and 2-3 cm thick. It weighs about 500-600 g. During labour, the placenta separates from the uterus wall and will be expulsed around 30 min following birth of the baby.

1.1.1 Function of the placenta

Hormone production

The placenta has an important endocrine function. Hormones are synthetised mostly in the syncytium. The implantation acts as a trigger for hormone production. The placenta synthesises as well not only pregnancy-associated proteins, but also protein hormones and steroid hormones. In these cases, the trophoblast needs maternal and/or fetal precursors to produce them.

Human chorionic gonadotrophin (hCG) is synthesised by the placental syncytiotrophoblast, as opposed to the modulatory Human chorionic gonadotrophin releasing hormone (hCGRH) which is synthesized by the cytotrophoblast. Reaching the mothers´ blood stream, hCG induces progesterone synthesis in the ovary. Eliminated from the mother through the urine, hCG is used as an early pregnancy marker. The most important functions of hCG at early pregnancy are the maintance of the corpus luteum, the prevention of menstruation and the stimulation of steroid production from the placenta. At fetal compartments hCG affects the fetal gonades and estimulates the fetal adrenal cortex. Furthermore hCG promotes cellular motility and can be considered as an angiogenic factor, inducing neovascularization from preexisting blood vessels during the uterine adaptation to early pregnancy (Zygmunt et al., 2002; Zygmunt et al., 2003). Another hormone produced by the syncytium is human placental lactogen (HPL) which has an anabolic, lipolitic and insulinogenic effect, controlling glucose levels. HPL is responsible for the increasing exogen and endogen insulin resistance during the pregnancy.

The most important steroid hormones are estrogens (estradiol, estrone and estriol) and progesterone. Estrogens have an anabolic effect and are responsible for the

uterine muscle fiber excitement. They are produced in increasing levels by the placenta, with a maximum almost at the end of the pregnancy and their precursors include dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEAS). Progesteron synthesis is mostly done (over 90%) by maternal cholesterin precursors and by a few pregnenolon precursor from maternal adrenal cortex. Fetal contribution is therefore very small. At the end of the fourth month of pregnancy, the placenta becomes the primary tissue of progesterone synthesis, maintaining the ongoing pregnancy. Together with the estrogens, progesterone is responsible for the uterine volume and vascularisation increasement as well as breast development during the pregnancy. Progesterone in the fetus works as a precursor for the gluco- and mineralocorticosteroid synthesis.

Exchange of gases and nutrients

The major function of the placenta is to mediate an efficient oxygen exchange between mother and fetus, without allowing the two circulations to mix. Gas exchange in blood operates by simple diffusion. At the end of the pregnancy the fetus takes 20-30 ml oxygen per minute from the maternal circulation.

Glucose and milk acids operate by facilitated diffusion, erythrocytes and lymphocytes by diapedesis and water molecules by filtration. The placenta is permeable as well to nutrients such as aminoacids, free fatty acids, electrolytes and vitamins which are transported in an active way (requiring cell energy) from mother to fetus. Not only nutrients, but most drugs and infectious agents also pass easily through the placenta.

Passive immunity

Different mechanisms are being discussed to explain why the fetus and placenta, containing 50% of paternal HLA are not rejected by the mother. Most probably, maternal protective antibodies previously sensibilised would prevent the destructive effects that an immune response could induce. Moreover, the transport of maternal antibodies from the fetus allows the fetus to acquire immunity against diphteria, smallpox, measles and other infectious diseases.

Warm exchanger

The fetus is 0.6-1.0°C warmer than the mother. Around 85% of the warm production flows through the placenta and the rest through the fetal skin, amniotic fluid, membranes and the uterus.

1.1.2 Early placental development

The fertilized oocyte experiences several mitotic cycles growing up to 32 cells (morula) during its way through the Fallopian tube. At nearly day 5 post conceptionem (p.c.) it comprises 58 cells and is called a blastocyst (Figure 1.I).

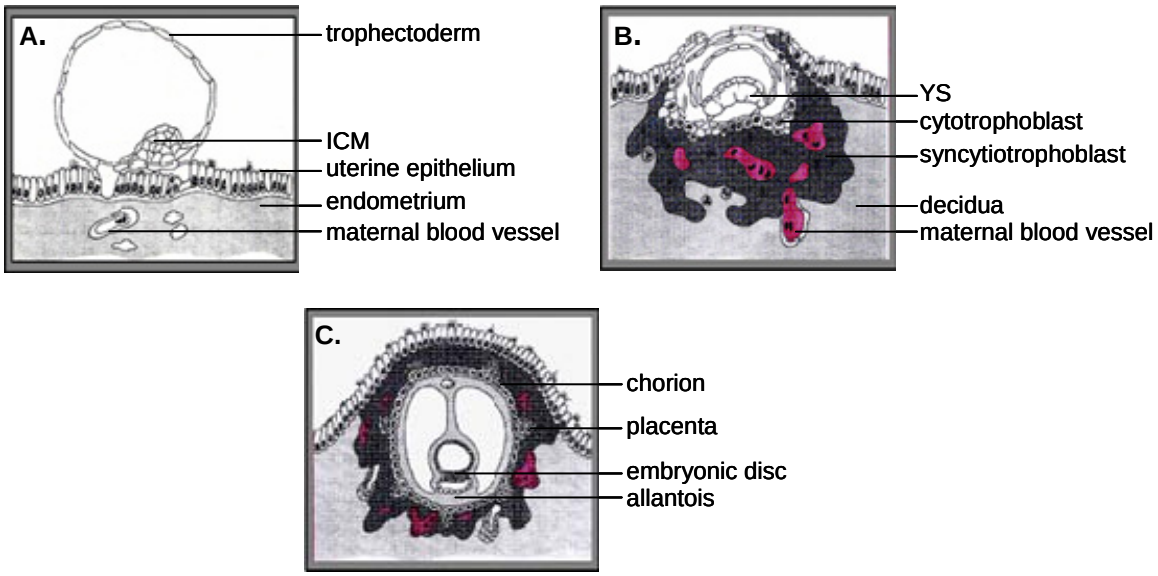

Figure 1.I – Human blastocyst implantation and early placentation. Implantation involves stable adhesion of blastocyst to uterine epithelium (**A**). As the blastocyst transgresses the epithelial layer, trophectodermal cells proliferate and invade the uterine deciduas (**B**). The process of embryogenesis results in formation of the embryonic sac. Extra-embryonic yolk sac (YS) also forms during this time. Further invasion into deciduas results in total blastocyst encapsulation (**C**). The chorion arises trough differentiation of trophectodermal cells and will eventually consist of cytotrophoblast and syncytiotrophoblast. In humans, fusion of the chorion and allantois results in formation of a chorioallantoic placenta. ICM: inner cell mass. Figure modified from Moore, 1988

At implantation the blastocyst is composed of two cell layers; the blastocyst enters the uterus and, during implantation, attaches to the uterine mucosa and penetrates the decidua, which is the maternal endometrium modified by the pregnancy.
Approximately 53 cells build up the outer layer, called trophoblast, (troph, Greek = to feed, to nourish; blast, Greek = sprout, shoot) because it supplies the inner layer, called embryoblast, with nutrients and oxygen.

Proliferation of the trophoblast

While the blastocyst penetrates the decidua, trophoblasts proliferate. The outer trophoblastic cells, situated right beneath the maternal tissue in utero, merge losing their individual cell membrane and forming a syncytium.

Cytotrophoblast and Syncytotrophoblast

Already at day 8 p.c. the trophoblast is divided into an external part called syncytiotrophoblast and an internal part called cytotrophoblast.
Syncytiotrophoblast develops where trophoblast cells come in contact with maternal tissue. The growing syncyciotrophoblast leads to cavities in the maternal decidua, which is the beginning of the trabecular stage (day 8 p.c.). Parts of the syncytiotrophoblast sprout into the cavities building primary villi (13 to 14 days p.c.). Therefore, the chorionic villi are initially composed of a central mass of cytotrophoblast surrounded by a thick layer of syncytiotrophoblast.
At the same time maternal vessels are erroded and maternal blood flows into the cavities.
The primary villi develop to secondary villi by the formation of a mesodermal core. Capillaries sprout into these secondary villi generating tertiary villi (Figure 1.II). This takes place in the 3rd week p.c..The villi are situated in the intervillous space that is filled with maternal blood provided by the uteroplacental arteries. Fetal nutrient exchange occurs at the interface between the intervillous space and the chorionic villi. Mature human placenta is composed by a fetal and maternal side (Fig. 1.III).
The part of the placenta facing the mother is composed of 15-20 cotyledons, the functional subunits of the placenta. Maternal blood flows from the spiral arteries into the cotyledons and is drained back to the maternal blood circulation by the endometrial veins.

The part of the placenta facing the fetus comprises the chorion, the amnion and the umbilical cord and is developed from the chorionic plate. The chorionic plate forms the ´roof´ of the developing placenta and consists of the amnion and the trophoblast.

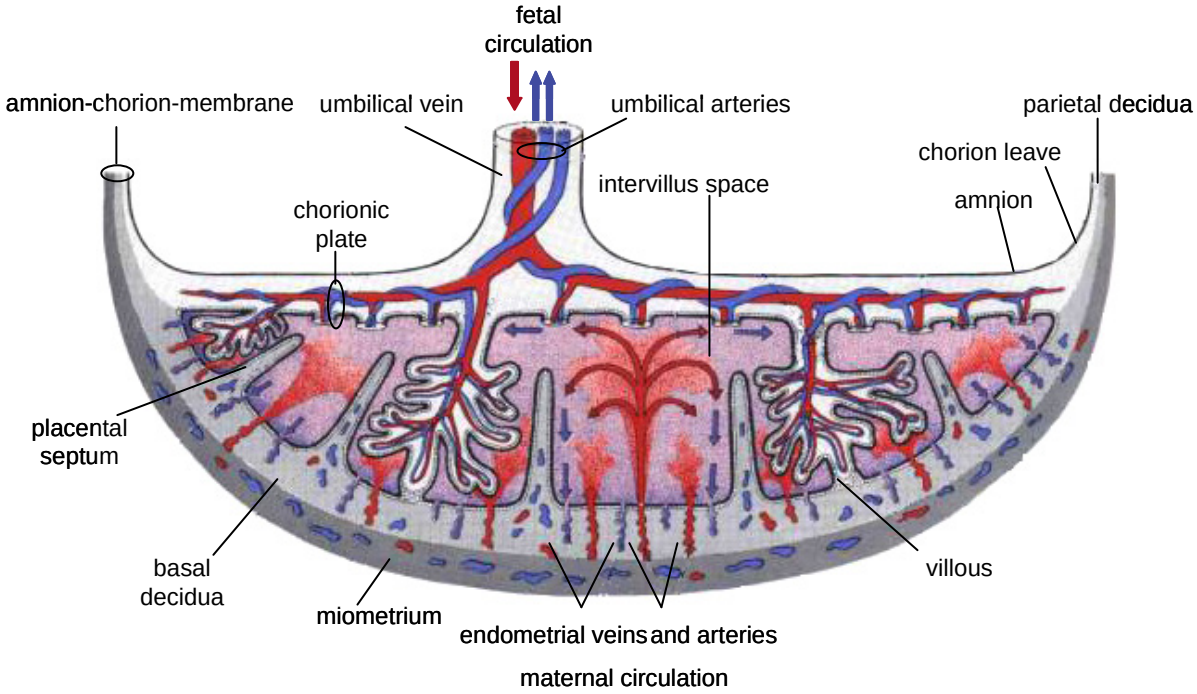

Figure 1.II – Schematic representation of a mature placenta. A mature placenta consists of a fetal and maternal side. The fetal side is built from the chorionic plate with the villous, the cytotrophoblast and the intervillous space. The cotyledons are separated through the decidual septi from the maternal placenta. Every cotyledon is formed by two or more villi and their ramifications. Source: (Moore, 1998).

The basal plate is covered by syncytiotrophoblast on the maternal side and consists of a basal decidua, which is the portion of the decidua where the implantation site is located. The more or less excentrically inserted umbilical cord is covered with amnion, a thin avascular layer that continues past the edges of the placenta to line the entire hollow sphere of chorion except where it is reflected to cover the umbilical cord.

The amnionic and the chorionic membrane represent the bag that encloses the fetus. The membranes normally insert at the edge of the placenta and contain the amnionic fluid and the fetus. The amnion covers embryo from the inner side, while the chorion does it from the outer side.

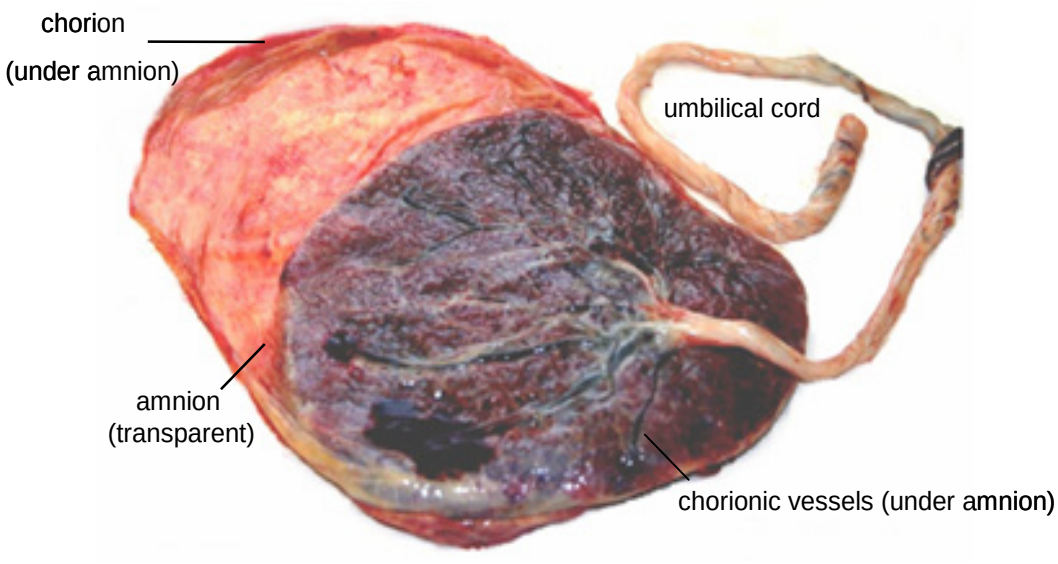

Figure 1.III – Picture of the placenta, the fetal membranes and the umbilical cord

The chorionic membrane is derived from the trophoblast layer and is considered an extraembryonic tissue. It is comprised by a mesenchyma and a stratified epithelial layer passing over into a trophoblastic layer (cytotrophoblast and syncytiotrophoblast).

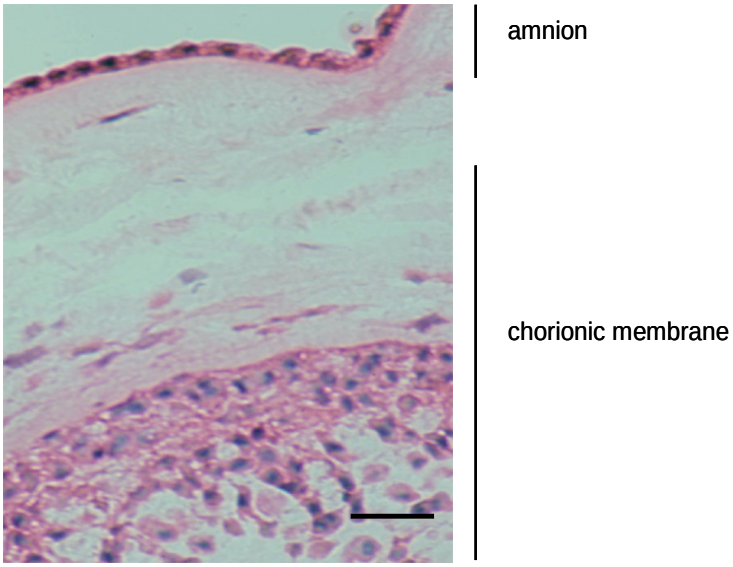

Figure 1.IV – Composition of the fetal membranes: amnion and chorionic membrane. Bar represents 50 µM

The amnion is formed from the epiblast from the inner cell mass at day eight after fertilization. As we can see in Figure 1.V the epiblast gives rise to the amnion as well as all three embryonic germ layers (ectoderm, endoderm and mesoderm).

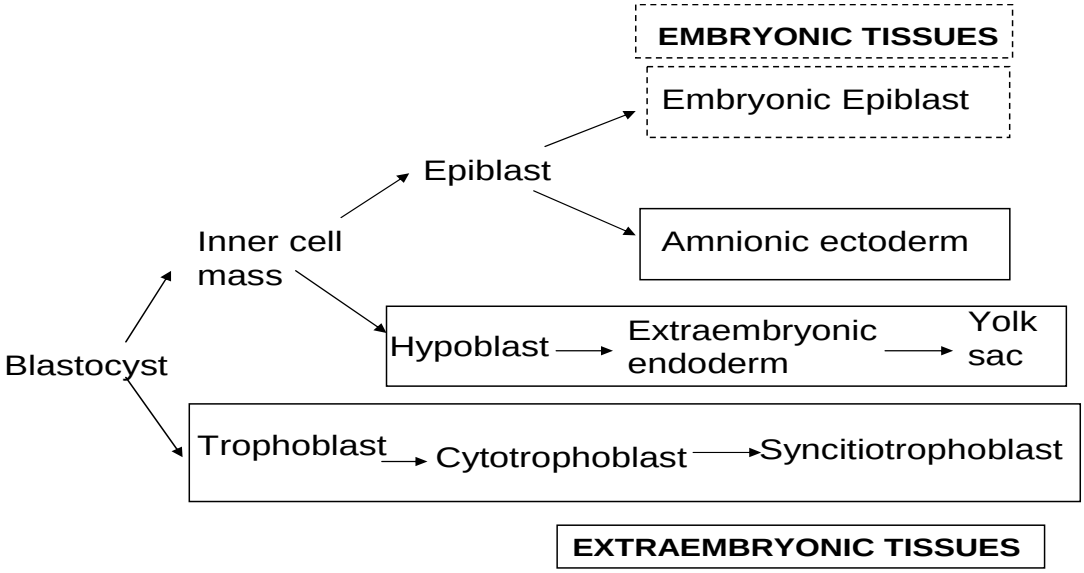

Figure 1.V – Origin of the embryonic and extraembryonic tissues. Diagrammatic Illustration

During the 4th week of gestation, small spaces appear between the inner cell embryonic mass and the trophoblast ring. By the 22nd day after the last menstruation, these spaces have coalesced to form the amniotic cavity.

Around the 5th week of gestation, amniotic fluid begins to accumulate within the cavity. This fluid increases in quantity and causes the amnion to expand.

Towards the end of the 12th week the amnion adheres with the mesenchymal inner surface of the chorionic plate, a “fusion” of amnion and chorion and the secondary yolk sac disapears.

From a histological point of view, the amniotic membrane is composed of a single stratum of slightly flattened epithelial cells, the human amniotic epithelial cells (HAE cells), resting on a basement membrane. Under it lays the amniotic mesenchyma which is composed of connective tissue synthesized by fibroblast-like cells (see Table 1.I).

AMNIOTIC MEMBRANE	Bilayer	amniotic epithelium + amniotic mesenchyma
CHORIONIC MEMBRANE	Trilayer	Chorionic mesenchyma + chorionic epithelium chorionic trophoblast

Table 1.I - Composition of the Fetal Membranes: overview

1.1.3 Role of the early amniotic membrane

The amnion mediates the exchange of water and possibly nutrients and hormones between the maternal blood vessels in the decidua and the amniotic fluid. The intact amnion sac provides a buoyant environment that protects the fetus from trauma, and allows freedom of fetal movements.

The functions of the early amniotic membrane and the amniotic epithelium are as follows:

Nutrient supply

The source of nutrients and oxygen may be supplied by diffusion from the deeper layers of the uterine wall or via the amnionic fluid from the fetus.

The presence of glucose transporter proteins 1 and 3 predominantly at the apical surface of amniotic epithelial cells has been described, whereas glucose transporter protein 4 and insulin receptor could not be detected (Wolf and Desoye, 1993; Desoye et al., 1997). Thus it is likely that these cells cover their glucose requirements from the amnionic fluid with an insulin-independent mechanism.

Intraamniotic lipid synthesis

Ultrastructural findings are thought to favour intraamniotic lipid synthesis, rather than a degenerative origin of the lipid droplets found in the amniotic membrane. The lipids are derived from the maternal circulation and are secreted into the amniotic fluid after chemical modifications. Throughout pregnancy the amount of lipid droplets increases (Yoshimura et al., 1980).

Glycogen storage as an energy source

The rich intracellular stores of glycogen present in the amniotic epithelium in early pregnancy may provide an energy source that is necessary in view of the poor complement of organelles such as mitochondria and endoplasmic reticulum and

limited pinocytotic activity of HAE cells. It is not known if the amniotic cells manage those glycogen stores themselves (Robb and Hytten, 1976).

Role of the amniotic membrane in prostaglandin biosynthesis

The amniotic membrane is a nearly exclusive source for prostaglandin E2 (PGE2) (Okazaki et al. 1981a; Gibb and Sun, 1996; Surendran, 2001; Kumar et al., 2004; McCoshen et al., 1989), which play a significant role in the initiation and maintenance of uterine contractions. The amnion plays an important role during parturition, because of the strategic anatomical location, with its close relation to amniotic fluid, myometrium and uterine cervix.

Enzymes involved in prostaglandin biosynthesis have been found in human amnion and include phospholipases, prostaglandin synthase, and prostaglandin endoperoxidase synthase (or cyclooxygenase) (Okazaki et al., 1981; Bryant-Greenwood et al., 1987; Toth et al., 1996a; Smieja et al., 1993; Olson et al., 1991).

hCG receptors are found on the amniotic epithelium as well suggesting a role for hCG in regulating prostaglandin biosynthesis (Reshef et al., 1990; Toth et al., 1996b).

Furthermore, corticotropin-releasing hormone (CRH) (Jones and Challis, 1989b) and glucocorticoids (Gibb and Lavoie, 1990; Whittle et al., 2000b) modulate prostaglandin production (Whittle et al., 2000a). CRH mRNA was demonstrated in amniotic membrane of term pregnancies (Okamoto et al., 1990); its production is modulated by glucocorticoids and progesterone (Jones and Challis, 1989a; Jones and Challis, 1990b; Jones and Challis, 1990a).

Interleukins are well known to regulate prostaglandin biosynthesis and to participate in trophoblast invasion into the uterus. Normal amniotic and chorionic cells of the membranes produce interleukin- 1ß in vitro, so that intraamnionic infections could induce the production of interleukins, which increase prostaglandin production (Menon et al., 1995).

Oxitocine receptors, which act as the myometrial contraction stimulator only when combined with increased levels of PGF2α, have been demonstrated in the amnion as well as in the chorion (Benedetto et al., 1990).

Leukotriene synthesis

Leukotrienes, a cytokine group derived from arachidonic acid, are produced by the amnion (Rees et al., 1988). These cytokines may affect uterine contractility in addition to their function as mediators of immune reaction and vasodilatation.

Regulation of the pH of the amniotic fluid

Amnion epithelium has been shown to exhibit carbonic anhydrase activity which is involved in bicarbonate/carbon dioxide exchange (Benirschke, 1998; Bernischke K, 2000), suggesting that the amnion epithelium may also be partly responsible for regulating the pH of amniotic fluid (Gilbert and Brace, 1989).

Steroid transformation

17ß-hydroxysteroid dehydrogenase activity has also been demonstrated in human amnion epithelium from 7 to 20 weeks of pregnancy, suggesting that these cells have the ability to transform steroids (Cheung et al., 1992). Comparing early amniotic membranes with third trimester membranes, there is an increase in the amniotic epithelium complexity: the lateral intercellular connections within the amniotic epithelium become more complex, with many interdigitations between the desomosomal contacts and the number of microvilli increases as well, allowing more water exchange between the mother and the amniotic fluid (Wang et al., 2003).

1.1.4 Characterisation

The amnion starts to differentiate early in development, at the 8th day after fertilization. That is prior to the start of organogenesis, which occurs approximately 3 weeks after fertilisation. Therefore, amniotic cells have been hypothesised to retain pluripotent properties of early epiblast cells. In spite of this interesting “privileged” early embryological origin, HAE cells have been poorly characterised.

The amniotic epithelium arises out of the ectoderm. HAE cells express epithelial specific markers such as cytokeratin (Regauer et al., 1985a) and CA125 which is a differentiation antigen of fetal coelomic epithelium derived from the mesoderm of germ cells (Nanbu et al., 1989a).

Additionally, non-epithelial but ectodermal markers have been identified as indicators for the pluripotent status of HAE cells. This comprises neural markers such as: acetylcholine, norephinephrine, dopamine, brain-derived neurotrophic factor (BDNF),

neurotrophin 3 (NT-3), activin A and noggin (Sakuragawa et al., 1996b; Elwan et al., 1998a; Elwan et al., 2003f; Elwan and Sakuragawa, 1997b; Kakishita et al., 2000a; Koyano et al., 2002c; Okawa et al., 2001; Sakuragawa et al., 1997b; Kakishita et al., 2003; Sakuragawa et al., 2001; Uchida et al., 2000e; Uchida et al., 2003; Sakuragawa et al., 2004d; Elwan et al., 2003d; Elwan et al., 1999e; Ishii et al., 1999a).

Furthermore, it has been shown that HAE cells express endoderm specific proteins like hepatocyte nuclear factor-3ß, αFP, albumin and GLUT-2, characteristic for hepatic cells (Sakuragawa et al., 2000b; Takahashi et al., 2002; Elwan et al., 2003c; Nakajima et al., 2001). The expression of endodermal markers has been of functional relevance, because HAE cells can secrete insulin under specific cultivation conditions (Wei et al., 2003).

Finally, vimentin as an intermediary filament of mesenchymal cells arising from mesoderm has been also detected in HAE cells (Ko et al., 2004b; Virtanen et al., 1981; Cremer et al., 1981).

Like many progenitor stem cells, HAE cells are known to inhibit cellular immune function, a property which makes them an attractive candidate for universal donor tissues in clinical transplantation. (Akle et al., 1981c; Bailo et al., 2004; Enosawa et al., 2003; Heeger, 2004; Kaviani et al., 2002; Mitka, 2001; Miyamoto et al., 2004; Terada et al., 2000a). Flow cytometric analyses have revealed that these cells do not express any class II antigen on their surfaces. Conversely, Class I antigen is slightly expressed (Sakuragawa et al., 1995c; Kubo et al., 2001a; Hunt and Fishback, 1989).

Various antiangiogenic and antiinflammatory proteins are expressed in HAE cells (Hao et al., 2000; Djian et al., 1996; Talmi et al., 1991a) which explains as well the antiangiogenic and antinflammatory effects of amniotic membrane transplantation. Such properties have already led to the use of amnion tissue as adjuvant therapy for a number of disease states, most notably, conjunctiva and cornea surface reconstruction (Tseng et al., 1997b; Grueterich et al., 2003a; Solomon et al., 2003b; Ueta et al., 2002b; Koizumi et al., 2000; Kubo et al., 2001b; Nakamura et al., 2004b), tracheal epithelium reconstruction (Noguchi et al., 1995; Goto et al., 1999) and in metabolic diseases (Kosuga et al., 2001; Kosuga et al., 2000; Sakuragawa et al., 1995b; Takahashi et al., 2001c). This makes HAE cells very interesting also for further application because they are easily available during cesarean section.

2 AIM OF THE STUDY

Since no standarised protocols for HAE cells are available, the aim of the study was:

1. to optimize and standarise the isolation and cultivation method for HAE cells

2. to characterize HAE cells ex vivo

3. to examine the expansion and pluripotency markers on HAE cells

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Equipment

Canon Eos 300 Digital, Canon Germany GmbH, Krefeld

Centrifuges: Labofuge GL, Heraeus Sepatech, Hanau

ELISA-Reader, Dynatech MR 5000, Dynatech, Guyanocourt, France

FACScalibur, Becton Dickinson Labware, Heidelberg

Incubator for Cell Culture: Heraeus 6000, Heraeus, Hanau

Laboratory Vacuum pump, Vacusafe, Integra bioscience, Fernwald

Leica Microscope MS-5, Light source: KL 1500 LCD, Wetzlar

Leica DM-LB microscope, Wetzlar

Micropipettes, Eppendorf, Hamburg

Multipipettes plus, Eppendorf, Hamburg

Pipettboy acu, Integra bioscience, Fernwald

PowerPac 200, Bio-Rad Laboratories GmbH, Munich

Spectophotometer SmartSpec 3000, Bio-Rad Laboratories GmbH, Munich

Sterile Flow Bank: Gelaire, Flow Laboratories

Thermal cycler, Mastercycler gradient, Eppendorf, Hamburg

Vortex, Heidolph Instruments GmbH&Co. KG, Schwabach

Waterbath, Memmert GmbH&Co. KG, Schwäbach

WesternBlot, Mini Trans-Blot Electrophoretic Transfer Cells, Bio-Rad Laboratories GmbH, Munich

3.1.2 Materials

Cell Scraper, TPP AG, Trasadingen, Switzerland

Cell Strainer, Becton Dickinson Labware, Heidelberg

Cryotubes TM Vials, Nunc TM Brand Products, Roskilde, Denmark

Electrophoretic Paper, Pharmacia GmbH, Karlsruhe

FACS tubes, Becton Dickinson Labware, Heidelberg

Falcon tubes, Greiner, Frickenhausen

Hemocytometer, Neubauer, Roth, Karlsruhe

OTC-compound "Tissue-Tek", Sakura Finetek Europe, Zoeterwoude, Holland

Pasteur pipettes, Brand GmbH, Wertheim

Pipettes (serological), Becton Dickinson Labware, Heidelberg

Pipettes tips, Sarstedt, Nümbrecht

SuperFrost Ultra Plus microscope slides, Menzel, Braunschweig

Scalpel, PFM, Cologne

Cell culture flasks, Biochrom, Berlin

3.1.3 Chemical products

Agarose Neeo, Ultra-Qualität, Roth, Karlsruhe

Ammoniumpersulfate (APS), Sigma-Aldrich, Taufkirchen

Bisbenzimide Höchst 33342, Merck Biosciences, Darmstadt

Bromphenolblue, Sigma-Aldrich, Taufkirchen

Bisacrylamide, Roth, Karlsruhe

Collagenase, Sigma-Aldrich, Taufkirchen

Dil-Ac-LDL, Biomedical Technologies Inc, Stoughton, MA, USA

Dimethylsulfoxide (DMSO), Roth, Karlsruhe

dNTP Mix, Eurogentec, Seraing, Belgium

Donkey serum, Jackson ImmunoResearch, West Grove, Pennsylvania, USA

Dithiotreitol (DTT), Invitrogen, Karlsruhe

ECL WesternBlotting Analysis System, Amersham Bioscience, Freiburg

ECL Hyperfilm, Amersham Bioscience, Freiburg

ECL horseradish peroxidase (HRP) labelled anti-mouse, Amersham Bioscience, Freiburg

Ethylenediamine Tetraacetic Acid (EDTA), Sigma-Aldrich, Taufkirchen

Endothelial Cell Basal Medium-2 (EBM-2), Cambrex Bioscience, Walkersville, USA

Endothelial Basal Cell Medium MV2, Promocell, Heidelberg

Eosin, Merck, Darmstadt

Ethanol, Fischer, Saarbrücken

Ethidium Bromide, Sigma Aldrich, Taufkirchen

Fetal Calf Serum (FCS), Biochrom, Berlin

Gene Ruler, DNA ladder 100bp, Fermentas GmbH, St. Leon-Rot

Glycine Hydrochloride, Sigma-Aldrich, Taufkirchen

Glycerol, Sigma-Aldrich, Taufkirchen

Hämatoxylin, Waldeck, Münster

Hank´s balanced salt solution (HBSS-buffer) 10X, Invitrogen, Karlsruhe

Loading Dye Solution 6x, Fermentas GmbH, St.Leon-Rot

Mammary Epithelial Cell Growth Medium plus Supplement Mix, Promocell, Heidelberg

MEM Medium, Invitrogen, Karlsruhe

Methanol (MetOH), Riedel-de Häen, Seelze

MiniProtean-3 kit , Bio-Rad Laboratories GmbH, Munich

NP-40, Sigma-Aldrich, Taufkirchen

Sodiumhydroxide (NaOH), Merck Biosciences, Darmstadt

Ammonium Cloride (NH4 Cl), Fluka Chemie, Buchs

Ammonium Bicarbonat (NH4 HCO3), Fluka Chemie, Buchs

Paraformaldehide, Sigma-Aldrich, Taufkirchen

PBS tablets 1x, Invitrogen, Karlsruhe

Penicilline/Streptomycine, Sigma-Aldrich, Taufkirchen

Picric Acid solution, Fluka Chemie, Buchs

Bio-Rad Dc Protein Assay Kit, BioRad Laboratories GmbH, Munich

Polyvinylidenedifluoride (PVDF) membrane, Amersham Bioscience, Freiburg

Retinoic Acid, Sigma-Aldrich, Taufkirchen

RNA extraction kit, RNeasy Mini kit, Qiagen, Hilden

RNase Reverse Transcriptase, SuperScript II, Invitrogen, Karlsruhe

Dodecylsulfate sodium salt (SDS), Roth, Karlsruhe

Slow Fade Light Antifade, Molecular Probes, Eugene, Oregon, USA

Sucrose, Serva, Heidelberg

Temed, Roth, Karlsruhe

Taq DNA Polymerase, Invitrogen, Karlsruhe

Tris (2-Amino-2-(hydroxymethyl)-1,3-propanediol), Roth, Karlsruhe

Triton X-100, Sigma-Aldrich, Taufkirchen

Tryphan Blue, Flow Laboratories, Irrine, Scotland

Trypsin-EDTA (1X) Invitrogen, Karlsruhe

Tween 20, Roth, Karlsruhe

3.1.4 Antibodies

Following antibodies were used in this study:

AFP-FITC, Santa Cruz, Heidelberg

CD14 PerCP, Becton Dickinson, Heidelberg

CD45 PerCP, Becton Dickinson, Heidelberg

CD34 FITC, Becton Dickinson, Heidelberg

CD140b PE, Becton Dickinson, Heidelberg

CD141 PE, Becton Dickinson, Heidelberg

CD62e APC, Becton Dickinson, Heidelberg

CD31 FITC, Becton Dickinson, Heidelberg

CD83 APC, Becton Dickinson, Heidelberg

CD144 unlabeled, Becton Dickinson, Heidelberg

CD133 PE, Milteny Biotech, Bergisch Gladbach

CD105 unlabeled, Serotec, Düsseldorf

Cytokeratin 14, 15, 16, 19 FITC, Dako Cytomation, Hamburg

Goat IgG- APC, Becton Dickinson, Heidelberg

Mouse IgG1 –FITC, Becton Dickinson, Heidelberg

Mouse IgG2a –FITC, Becton Dickinson, Heidelberg

Mouse IgG1 –PE, Becton Dickinson, Heidelberg

Vimentin-RhodRedX, Santa Cruz, Heidelberg

Vimentin PE, SantaCruz, Heidelberg

vWF unlabeled, Becton Dickinson, Heidelberg

Further details are presented in Chart 2.III

3.2 Methods

3.2.1 Isolation and cultivation of human amniotic epithelial (HAE) cells

Human placentas were obtained from uncomplicated term pregnancies delivered by elective Cesarean Sections (CS) from healthy women between the ages of 19 and 31. Written consent for the use of these tissues was obtained. The study was approved by the local Ethics Comitee at the Medical Faculty, University of Giessen, Germany.

There were no evidences of congenital malformation, genetical aberration, severe diseases (fetal or maternal) or signs of infection. 3 males and 1 female infant were born from 3 different women (one of the placentas was monoamniotic bichorial).

HAE cells were isolated immediately following the CS using a modified method based on previously published approaches (Casey and MacDonald, 1996b; Wei et al., 2003; Bilic et al., 2004b). All manipulations were carried out under sterile conditions. Human amniotic membrane tissue was mechanically peeled free from the chorion (Figure 2.I) and was cut with a scalpel about 2 cm from the placental disc to avoid the "zone of altered morphology" (McLaren et al., 1999; El Khwad et al., 2004).

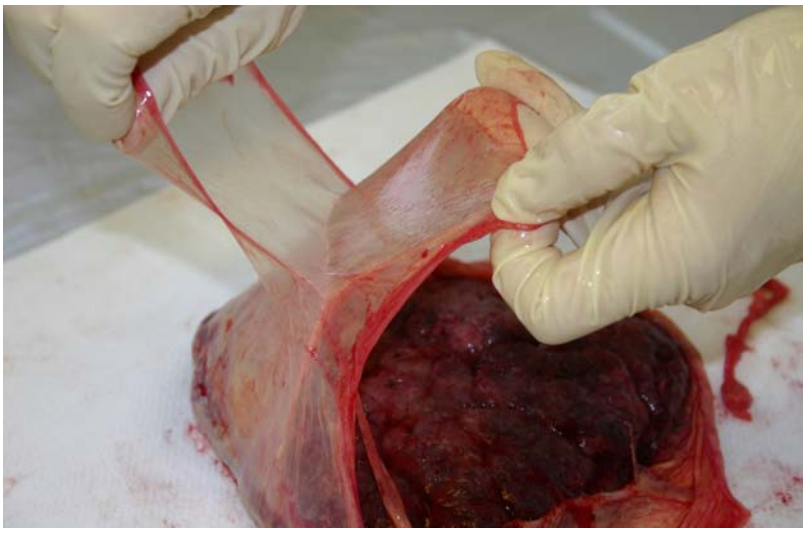

Figure 2.I - Separation of the amniotic membrane (left) from the chorionic membrane (right) by blunt dissection.

Amnion was washed several times with ice-cold, calcium-free, phosphate buffered saline pH 7.2 (PBS) (Invitrogen, Karlsruhe) to remove blood and cellular debris. Blood-free amnion was divided in 3-4 pieces, (1-3 g each). Each piece was then

transferred into a Falcon tube (Greiner, Frickenhausen; Germany) containing 20 ml of 0.05% Trypsin-EDTA (Invitrogen) and was incubated at 37°C for 20 min in a shaking waterbath. The digest was poured over gauze through a stainless steel mesh (Becton Dickinson Labware, Heidelberg, Germany) in order to separate the dispersed amniotic epithelial cells from undigested tissue. Cells were then suspended in 60 ml HBSS (Invitrogen) containing 10% heated-inactivated fetal calf serum (FCS, Biochrom, Berlin) and pelleted by a 5 min centrifugation at 1200 rpm. Pelleted epithelial cells from the 1st trypsination were resuspended in Mammary Epithelial Cell Growth Medium (MECGM), containing Supplement Mix (Promocell, Heidelberg) and 10% of FCS. The suspended cells were kept on ice.

The remaining tissue was subjected to 2nd and 3rd step of trypsination for additional 20 min. The dispersed epithelial cells of the 2nd and 3rd trypsination were collected and combined with the first cell fraction and poured over a cell strainer.

Cells were cultured in two 75 cm^2 tissue culture flasks, in MECGM plus Supplement Mix with 10% FCS in humidified atmosphere of 5% CO2 at 37°C.

In order to allow for a high quality RNA extraction at day 0, 1/3 of the final supernate was incubated in Erythrocyte Lysis Buffer for 2 min, centrifuged and shock frozen in liquid nitrogen. Freshly isolated cells were analysed with flow cytometry at day 0.

On the 1st day after isolation, cells were washed two times with PBS and the medium was replaced.

To lift adherent HAE cells, cells were washed twice in ice-cold PBS buffer, treated with 20 ml of 0.05% Trypsin-EDTA, incubated for 5 min at 37°C and exposed to the same amount of MEM medium with 10% FCS. Afterwards, cells were centrifuged for 5 min at 1500 rpm and the supernatant was discarded. The cell pellet obtained was resuspended in MECGM and transferred to a 75 cm^2 tissue culture flasks. At a 1:2 split ratio, cells reached the 5th-passage after 5 weeks. HAE cells were used to passage 7. The cultivation methods were optimised by testing different media: Epithelial Cell Growth Media as well as Endothelial Basal Medium (EBM-2) were also used. The optimised method resulted to be Epithelial Cell Growth Medium, because upon visual inspection, the majority of HAE cells cultured in Epithelial Cell Growth Media survived passaging as opposed to HAE cells cultured in EBM-2.

Chart 2.I - Buffer solutions

HBSS-Buffer	Erythrocyte Lysis Buffer
HBSS	4.1 g NH_4Cl
10% FCS	0.4 g NH_4HCO_3
2.5 µg/ml Amphotericin B	8.5 mg EDTA
200 mM L-Glutamine	Distilled water added to total volume of 0.5 l

PBS
PBS (pH 7.2)
104 U/ml Penicillin/Streptomycin (10 mg/ml)
2.5 µg/ml Amphotericin B

Mammary Epithelial Cell Growth Medium
500 ml Mammary Epithelial Cell Growth Medium
10 ml Supplement Mix®: BPE (0.4%), Epidermal Growth Factor (10 ng/ml), Insulin (5 µg/ml), Hydrocortisone (0.5 µg/ml), Amphotericin B (50 µg/ml) Gentamicin (50 µg/ml)
50 ml FCS

3.2.2 Cell counting

Determination of cell number was performed with a hemocytometer (Neubauer; Roth, Karlsruhe, Depth: 0.1 mm, 0.0025 mm2). Cells were suspended in 50 ml of PBS and 10 µl of this cell suspension was mixed with 90 µl of Trypan Blue (Flow Laboratories, Irrine, Scotland). Finally 10 µl of the mixture was transferred to a capillary chamber. Cells were counted in 4 quadrants and the total cell number was determined using the following formulation:

$$n = x \cdot 10^4 \cdot V \cdot DF$$

n - Cell number (mean of the 4 quadrants)

V - Suspension Volume (50)

DF - Dilution Factor (10)

3.2.3 Freezing and thawing HAE Cells

Cells were trypsinated and centrifuged as previously described. Supernatant was discarded and the pellet was mixed with freezing medium. Cells from a confluent 75 cm2 culture flask were resuspended in 2 ml cold freezing medium and separated into 2 cryotubes. The tubes were stored at in isopropanol for 24 h at -80°C before transferred into liquid nitrogen long-term storage tanks.

Freezing medium

60%	FCS
30%	Mammary Epithelial Cell Growth Medium Buffer
10%	DMSO

To thaw HAE cells, cells were removed from liquid nitrogen to a 37°C waterbath for 3-5 min and transferred to a tube containing 9 ml of Mammary Epithelial Cell Growth Medium. After a centrifugation at 4°C, the cell pellet was resuspended in Mammary Epithelial Cell Growth Medium and cultured in a culture flask.

3.2.4 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Characterisation of HAE cells began with analysis of RNA expression of pluripotency markers using RT-PCR.

RNA Extraction

Total RNA from HAE cells at passages 0,1 and 5 was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer´s instructions. Cells were lysed by adding an appropiate volume of Buffer RLT. To homogenize the sample, the lysate was directly pipeted onto a QIA shredder spin column (Qiagen) placed in a 2 ml collection tube, which was centrifuged for 2 min at 1300 rpm. One volume (usually 350 µl or 600 µl) of 70% ethanol was added to the homogenized lysate, carefully mixed, applied to a RNeasy mini column placed in a 2 ml collection tube and centrifuged for 15 s at 1300 rpm. The flow-through was discarded, 700 µl Buffer RW1 were added to the RNeasy column and centrifuged for 15 s at 1300 rpm. Flow-through and tube were discarded again and transferred into a 2 ml collection tube. 500 µl Buffer RPE was pipetted onto the RNeasy column and centrifuged for 15 s at maximum speed. Flow-through was discarded, 500 µl Buffer RPE was added to

the column and centrifuged for 2 min at 1300 rpm to dry the RNeasy silica-gel membrane. To elute, the column was transferred to a new 1.5 ml collection tube, 30-50 µl RNase free water was directly pipetted onto the silica-gel membrane and the column was centrifuged for 1 min at 1300 rpm.
RNA concentration was measured using a Spectophotometer (SmartSpec 3000, Bio-Rad Laboratories GmbH, Munich).

cDNA synthesis

During the following step, total cellular mRNA will be transcribed into a complementary DNA (cDNA).To transcribe mRNA into cDNA, Superscript II Reverse Transcriptase (Invitrogen) was used according to manufacturer´s instruction. In detail, 5 µg total RNA was added to 1 µl of random Primers (Promega, Madison, Wisconsin, USA) with distilled water to a volume of 12 µl. The mixture was heated to 70°C and chilled 10 min on ice. Afterwards, 4 µl 5x First-Strand Buffer (Invitrogen), 2 µl 0,1 M DTT (Invitrogen) and 1 mM dNTP (Eurogentec, Seraing, Belgium) were added and incubated at 42°C for 2 min. 1 µl SuperScript II Reverse Transcriptase was added and incubated again at 42°C for 50 min. The reaction was completed after 15 min at 70°C.

PCR Reaction

During PCR, high temperature separates the DNA molecules into single strands. Synthetic sequences of single-stranded DNA (18-25 nucleotides) serve as primers. 2 different primer sequences are used to bracket the target region; a 2nd primer is complementary to a sequence in the opposite DNA strand at the end of the target region. To perform a PCR reaction, a small quantity of the target cDNA is added to a test tube with a buffered solution containing DNA polymerase (see Figure 2.II), the cofactor MgCl2, oligonucleotide primers and the 4 deoxynucleotides. The PCR mixture is taken through replication cycles consisting of:

1. One to several minutes at 94-96°C, this mediates denaturation of the cDNA into single strands;
2. 30 sec at 50-65°C, to allow the primer annealing to their complementary sequences on either side of the target sequence;

3. 30 sec at 72°C, where the polymerase binds and extends a complementary DNA strand from each primer.

As amplification proceeds, DNA sequence doubles after each cycle. Following 30 cycles, a theoretical amplification factor of 1 billion is attained.

All components were gently mixed and added to a thermal Cycler (Mastercycler gradient, Eppendorf, Hamburg). Following conditions were used (see Chart 2.II B.):

Chart 2.II - PCR-Reaction

A. Reaction

41.5 µl	H2O
5 µl	10 x Buffer
1 µl	cDNA Product
1 µl	Primer 1 (10 µM)
1 µl	Primer 2 (10 µM)
0.5 µl	DNA-Taq-Polymerase

B. PCR-running conditions

1x	4 °C	5 min
1x	80 °C	Pause
30x	94 °C	45 sec
	Annealing temperature	30 sec
	72 °C	45 sec
1x	72 °C	10 min

C. Primers

Sox-1	848 bp fragment, annealing temperature 58°C
Primer 1	5´- CTC ACT TTC CTC CGC GTT GCT TCC -3´
Primer 2	5´-TGC CCT GGT CTT TGT CCT TCA TCC-3´
Sox-2	448 bp fragment, annealing temperature 60°C
Primer 1	5´-CCC CCG GCG GCA ATA GCA-3´
Primer 2	5´-TCG GCG CCG GGG AGA TAC AT-3´
FGF-4	370 bp fragment, annealing temperture 55°C
Primer 1	5´-CTA CAA CGC CTA CGA GTC CTA CA-3´
Primer 2	5´-GTT GCA CCA GAA AAG TCA GAG TTG-3´
Rex-1	306 bp fragment, annealing temperature 56°C
Primer 1	5´-GCG TAC GCA AAT TAA AGT CCA GA-3´
Primer 2	5´-CAG CAT CCT AAA CAG CTC GCA GAA T-3´
Oct-4	182 bp fragment, annealing temperature 56°C
Primer 1	5´ GGG CTC GAG AAG GAT GTG GT-3´
Primer 2	3´ GGG CTC CCA TAG CCT GGG-5´

Electrophoresis

PCR fragments were separated by their length using Agarose gel electrophoresis. 20 µl of the PCR reaction was mixed in 6x Loading Dye Solution (Fermentas GmbH, St.Leon-Rot) and loaded onto a 1% agarose gel (Roth).

The agarose was dissolved in 1x TAE- Buffer and 6*104% Ethidium Bromide (Sigma Aldrich, Taufkirchen) was added. cDNA bands were visualised, photographed and evaluated with the Gel-Doc 2000 and Quantity One Software (Bio-Rad Laboratories).

1x TAE Buffer pH 8

40 mM	Tris-Acetate
1 mM	EDTA

3.2.5 Flow Cytometry

To analyse HAE cell phenotype at the protein level, flow cytometry was performed. Antibodies marked with fluorochromes bind specifically to different epitopes. The fluorochromes can be excited by lasers and the emitted fluorescence will be detected and analysed using FACScalibur (Becton Dickinson). Flow Cytometer has 3 components: the fluidics, the optics and the electronics.

The fluidics are based on the hydrodinamic focusing principle, where the sample is introduced as a single cell suspension in the FACScalibur and focused for interrogation (Figure 2.II).

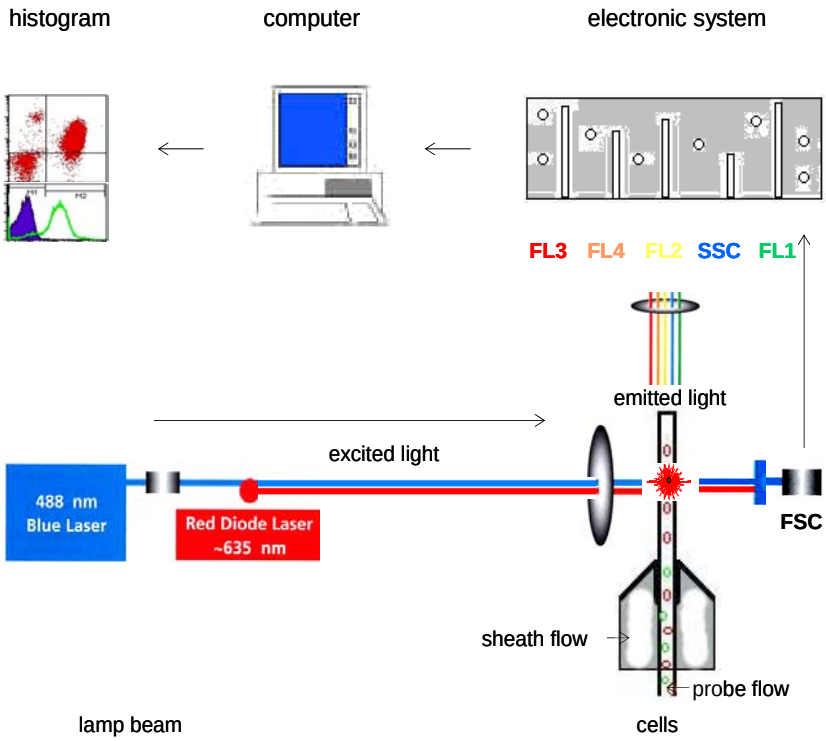

Figure 2.II - Diagram of FACS. Fluorescence marked cells are focused for interrogation in the FACS. The sheath fluid accelerates the cell suspension and brings them one in a row to the center of the measuring capillary (hydrodynamic focusing). When the cell passes the laser beam, a fluorochrome will be excited and will be sent as emitted light to the photomultiplier. The light signal will be enforced and converted to an electronical signal. The computer analyses the signals and presents it digitally (histogram).

In general, the optics generate focused light and collect the light signals deviated by the cells.There is 2 types of light sources: Forward Scatter and Side Scatter Light (Figure 2.III).

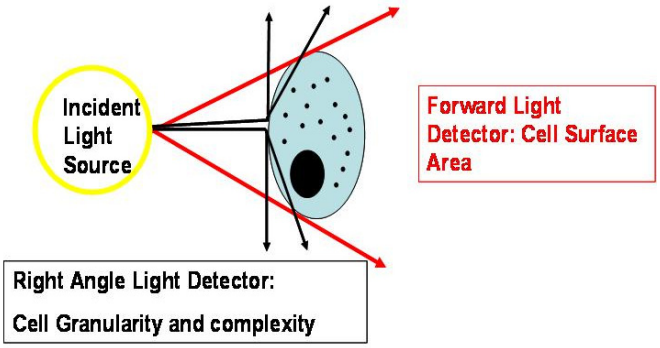

Figure 2.III - Diagram showing the light sources and detector in a FACS machine.

Forward Scatter, or Diffracted Light, does not enter the cell. Its detector, which converts the light to electrical pulses, is located along the axis of the incident light in the forward direction and gives information about the cell surface area.

Side Scatter, or Refracted Light, enters the cell and is deflected by it. Therefore, the detector which is located at 90° to the laser beam gives information about cell granularity and complexity.

Furthermore, the laser can excite fluorochromes, which are coupled to specific antibodies binding to cell epitopes. As the sample intercepts the laser beam, the electrons of the fluorochromes are excited to a higher energy state. When the electrons fall to their starting energy level, energy is released as a photon with specific emission spectra unique to different fluorochromes. Therefore a parallel analysis of one sample with several antibodies which are conjugated with different fluorochromes can be performed.

The electronic system converts the optical signals to proportional electronic signals and digitises them for computer analysis. Data in this work were presented as the so called Two Parameter Dot Plots. At the x- and y-axes, the results for every fluorescences (FITC: fluorescein isothiocyanat and PE: R-phycoerythrin, respectively in the Figure 2.IV) are shown. Every dot represents a counted cell (event).

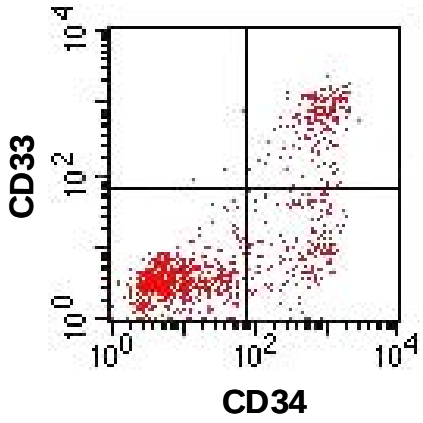

Figure 2.IV - Two parameter Dot Plot displaying CD34 on the x axis and CD33 on the y axis.

To perform flow cytometry analyses of HAE cells of passages 0, 1 and 5, the cells were used directly after isolation or were subjected to the trypsination protocol to obtain a single cell suspension. Depending if the antibodies recognised an extra- or intracellular epitope, the cells were subjected to different protocols.

Extracellular staining

Detection of extracellular epitopes was performed with antibodies shown in Chart 2.III. 200.000 HAE cells (per tube/probe) were resuspended in 100 µl of PBS. To minimise unspecific binding, cells were blocked with 10 µl human serum for 15 min on ice. Primary antibodies were added (see Chart 2.III) and the cells were incubated on ice for 45 min. Cells were washed with 1 ml PBS and centrifuged for 5 min at 1200 rpm. The supernatant was discarded and the pellet vortexed.

Afterwards, cells marked with directly coupled antibodies were resuspended in 0.5 ml PBS and kept in dark. Probes with uncoupled primary antibodies were incubated with secondary antibodies coupled with APC (1 µl, Becton Dickinson) and kept on ice in the dark for 30 min. Samples were finally washed with 1 ml PBS, centrifuged for 5 min at 1200 rpm. The pellet was resuspended in 0.5 ml PBS and measured with the FACScalibur (Becton Dickinson).

Chart 2.III - Antibodies used for Extracellular Stainining

Epitope	Source	Final Concentration	Staining Concentration	Vol/200.000 HAE cells	Fluorochrome
CD14	Mouse	5 µg/ml	0,25µg/ml	5 µl	FITC
CD45	Mouse	25 µg/ml	1,25µg/ml	5 µl	PerCP
CD34	Mouse	55 µg/ml	2,75µg/ml	10 µl	FITC
CD140b	Mouse		20 µl/10^6cells	5 µl	PE
CD141	Mouse		20 µl/10^6cells	5 µl	PE
CD62e	Mouse		20 µl/10^6cells	5 µl	APC
CD31	Mouse		20 µl/10^6cells	5 µl	FITC
CD83	Mouse		20 µl/10^6cells	5 µl	APC
CD144	Mouse	0.1 mg/ml	5µg/ml	5 µl	-
CD133	Mouse	55 µg/ml	2,75µg/ml	10 µl	PE
CD105	Mouse	0.2 mg/0.2ml		3 µl	-
mIgG	Goat		1 µg/10^6cells	1 µl	APC

Intracellular staining

Following epitopes were analysed by an intracellular staining (see Chart 2.IV):

Chart 2.IV - Antibodies used for intracellular staining

Epitope	Source	Final Concentration	Staining Concentration	Vol/500.000 HAE cells	Fluorochrome
vWF	Mouse	0.1 mg/ml	5 µg/ml	5 µl	-
Cytokeratin 14, 15, 16, 19	Mouse	50 µg/2ml		5 µl	FITC
Vimentin	Mouse	30 µl/10^6cells		7 µl	PE

500.000 HAE cells per probe were used. Cells were incubated for 20 min on ice with Cytofix/Cytoperm (Becton Dickinson) (0.5 ml Cytofix/Cytoperm per 1*106 cells) in order to permeabilise cell membranes and fix cell structures. After 5 min centrifugations at 1200 rpm, cell pellets were washed two times with Permwash Buffer (Becton Dickinson). Cells were then resuspended in Permwash, 10 µl of Human Serum was added and the samples incubated 10 min on ice. Primary

antibodies were added and kept on ice for 45 min. Afterwards, cells were washed with 1 ml Permwash, centrifuged, and the remaining pellet was vortexed. Cells marked with directly coupled antibodies were resuspended in 0.5 ml Permwash and kept in dark. Probes with uncoupled primary antibodies were incubated with APC-conjugated secondary antibodies (1 µl, Becton Dickinson) and kept on ice in the dark for 30 min. Samples were then washed with 1 ml Permwash and centrifuged for 5 min at 1200 rpm. The pellet was resuspended in 0.5 ml Permwash. Samples were subjected to flow cytometric analysis using FACScalibur and Cell Quest Pro software program (Becton Dickinson)

As isotype controls, the following antibodies for both intracellular and extracellular staining were used (Chart 2.V):

Chart 2.V – Antibodies used for isotype control

Epitope	Source	Final Concentration	Vol/500.000 HAE cells	Fluorochrome
mIgG1	mIgG1	20 µl/10^6cells	5 µl	FITC
mIgG2a	mIgG2a	20 µl/10^6cells	5 µl	FITC
mIgG1	mIg1κ	20 µl/10^6cells	5 µl	PE

3.2.6 Immunohistochemistry and Immunocytochemistry

Protein expression of HAE cells was additionally analysed with immunohistochemistry. Amniotic and chorionic tissues were fixed for 30 minutes in Zamboni solution (Jahr and Bretzel, 2003), washed 4 times with PBS first at room temperature and then overnight at 4°C, and finally incubated for 24 h in sucrose solution (0.18 g/ml PBS). Fixed tissues were embedded in OTC-compound ("Tissue-Tek", Sakura Finetek Europe, Zoeterwoude, Netherlands) and frozen in liquid nitrogen. Afterwards, fixed tissues were cut into 8 µm slices and put on SuperFrost Ultra Plus microscope slides (Menzel, Braunschweig). A single cell suspension of HAE cells was fixed for 10 min in Zamboni solution, washed overnight with PBS, and resuspended in PBS containing 5% native FCS. Small drops (about 5 µl each) of the cell suspension were placed on SuperFrost Ultra Plus microscope slides, dried at 37°C for 20 min, and were then stored at 4°C up to 2 weeks, or at -80°C for longer. Tissues or cells were blocked for 10 min with 10% donkey serum (Jackson ImmunoResearch, West Grove, Pennsylvania). Primary antibodies, diluted in 1%

donkey serum/0.1% Triton X-100 (Sigma-Aldrich, Taufkirchen) were incubated on the tissue slices or cells overnight at 4°C.
The following primary antibodies were used:

Chart 2.VI – Antibodies used for immunohistochemistry

Epitope	Source	Secondary Antibody	Fluorochrome	Final Concentration
Cytokeratin 19	Mouse	Donkey	FITC	1:100
Vimentin	Goat	Donkey	RhodRedX	1:200
AFP	Goat	Donkey	FITC	1:100

Binding of primary antibodies was then visualized following incubation (1 h, room temperature) with a mixture of donkey anti-mouse-FITC and donkey anti-goat RhodRedX (both from Jackson ImmunoResearch) diluted 1:400 in PBS containing 5% human serum. Concerning AFP staining, incubation was performed overnight with a mixture of polyclonal goat anti-AFP antibody (Santa Cruz) diluted 1:100 in PBS containing 1% donkey serum and 0.1% Triton X-100. Nuclei were counterstained (blue fluorescence) with bisbenzimide (Höchst 33342, Merck Biosciences, Bad Soden; final concentration 5 µg/ml) for 5 min at room temperature. After a final washing step with PBS 3 times, slides were embedded into Slow Fade Light Antifade (Molecular Probes, Eugene, Oregon). Unspecific binding of primary antibodies was estimated using isotype controls. Fluorescence was observed with a Leica DM-LB microscope (Wetzlar, Germany) equipped with a Leica DC-200 camera and Leica IM 1000 software.

Zamboni Solution

20 g	Paraformaldehyde
150 ml	C6H3N307 (Picrin Acid)
2.5%	NaOH
	Phosphate Buffered Saline (PBS) pH 7.3 up to a final volume of 1 l

3.2.7 Protein isolation and Western blot analysis

In order to investigate the protein expression of the transcription factor Oct-4, Western Blot analysis was performed.

Protein Extraction

HAE cells were grown to ~80% confluency, and subjected to trypsination as previously described. The cell pellet was washed twice in ice cold PBS and centrifuged for 5 min at 1500 rpm.

To obtain cytoplasmic protein extracts, the cell pellet was washed quickly in Buffer A, and centrifuged at 1300 rpm for a few seconds. The pellet was resuspended in a restricted volume of Buffer A plus NP-40 (~120 µl per 10*106 cells), incubated for 10 min on ice and centrifuged at 3500 rpm for 5 min at 4°C.

Supernatants, containing the cytoplasmic protein fraction, were collected and stored at -80oC. To obtain the nuclear protein extract, the pellet was resuspended in a restricted volume of Buffer C (~120 µl per 10*106 cells) and incubated for 30 min on ice. After centrifuging at 1400 rpm for 10 min at 4°C, supernatants containing the nuclear protein fraction were stored at -80°C.

Chart 2.VII – Protein extraction

Buffer A	
10 mM	Hepes pH 7.9
1.5 mM	MgCl2
10 mM	KCL
0.1%	NP-40

Buffer C	
20 mM	Hepes pH 7.9
1.5 mM	MgCl2
420 mM	NaCl
0.2 mM	EDTA pH 8.0
25%	Glycerin

Proteinase inhibitors	
2 µg/ml	Antipain
2.2 µg/ml	Aprotinin
1 mM	DTT
2 µg/ml	Leupeptin
1 mM	PMSF
1 µg/ml	Pepstatin A

Determination of protein concentration

The protein concentration of the cellular protein fractions was quantified utilising the BioRad DC Protein Assay Kit (BioRad Laboratories). It is a colometric determination method where Cystine, Cysteine, Histidine, Tryptophane and Tyrosine molecules react with cuprictartrate that allows the statistical determination of the whole protein concentration. Afterwards, the Cu+ protein complex reduces the following folin-reagents by using 1, 2 or 3 carbon-dioxide atoms, during a 15 min incubation at room temperature. Those reduced molecules have a characteristic blue colour, detected at

650 nm by an ELISA-Reader (Dynatech MR 5000, Dynatech, Guyanocourt, France). In parallel, a standard curve was also measured. The protein probes were related to the standard curve and protein concentrations determined.

Protein separation using SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The protein samples were separated under denaturating conditions utilising the Laemmli method. With the use of DTT, the disulfate bridges in the native proteins are reduced; SDS maintains the protein denaturation and mediates a negative charge on the protein complex. This allows for protein separation depending on molecular weight. The proteins (20-50 μg/ well) first passed a 6 % stacking gel at 80 V and were separated in a 12% SDS-PAGE. The MiniProtean-3 kit (Bio-Rad Laboratories) was used for the protein separation.

Chart 2.VIII – Reagents for SDS-PAGE

SDS-PAGE Buffer (5x Laemmli-Buffer pH 8.3)

25 mM	Tris-HCl
250 mM	Glycine
0.1 %	SDS

Protein Loading Buffer (3x Sample Buffer)

187.5 Mm	Tris-HCl (pH 6.8)
6%	SDS
150 mM	DTT
30%	Glycerol
0.3%	Bromophenolblue

	Separating gel (12%)	**Stacking gel (6%)**
Distilled water	5.25 ml	6.1 ml
Tris-HCl	3.75 ml (pH 8.8)	2.5 ml (pH 6.8)
Bisacryl	6.0 ml	1.3 ml
APS	75 µl	75 µl
Temed	10 µl	10 µl

Western Blot analysis

The separated proteins were transfered to a polyvinyllidendifluoride (PVDF) membrane (Amersham, Bioscience, Freiburg). Before transfer, PVDF membranes were activated with Methanol for 15 s, then washed for 2 min with destilled water and kept in Transfer Buffer for 5 min. The transfer was carried out in Towbin Buffer with the electrophoresis Mini Trans-Blot Electrophoretic Transfer Cells (Bio-Rad

Laboratories) according to manufacturer´s instruction. The transfer was performed at 50 V for 45 min.

Immunoprobing

The immobilised proteins were analysed by the specific antibodies. While the primary antibody bound to Oct-4 protein, the horseradishperoxidase (HRP) (Amersham Bioscience, Freiburg) conjugated secondary antibody recognised a fragment of the primary antibody. After the addition of ECL (Amersham Bioscience), the substrate-peroxidase reaction emitted photons which were detected on a Hyperfilm ECL (Amersham Bioscience). In detail, the PVDF membrane was first treated for 2 h at room temperature with Blocking Buffer. Following blocking, the membrane was incubated with 1:1000 monoclonal Oct-4 (Chemicon, Temecula, California, USA) antibody solutions in Blocking Buffer at 4°C overnight.

The blots were thoroughly washed in Tris Buffered Saline plus Tween 20 (TBST, Roth, Karlsruhe) (3 x 5 min) and subsequently incubated for 60 min with HRP labeled secondary antibody solution (Amersham Bioscience; 1:2000 dilution in Blocking Buffer). The detection of the protein bands was done after 3 x 10 min washing with TBST by an ECL WesternBlotting Analysis System (Amersham Bioscience). Finally the membranes were exposed to an ECL Hyperfilm (Amersham Bioscience) and the film was processed.

Chart 2.IX – Reagents for Immunoprobing

TBST

1x	Tris Buffered Saline
0.1%	Tween-20

Blocking Buffer

150ml	1x TBS
7.5 g	BSA
0.1%	Tween-20

4 RESULTS

4.1 HAE cells characterization ex vivo

The fetal membranes comprise both amnion and chorion. To provide an overview of the lysed tissue, fetal membranes were stained with Hematoxilyn/Eosin (H/E) (Figure 3.I A). In parallel, fetal membranes were stained with cytokeratin and vimentin unconjugated primary antibodies and fluorochrome-conjugated secondary antibodies, to differentiate between epithelial and mesenchymal layers (Figure 3.I B)

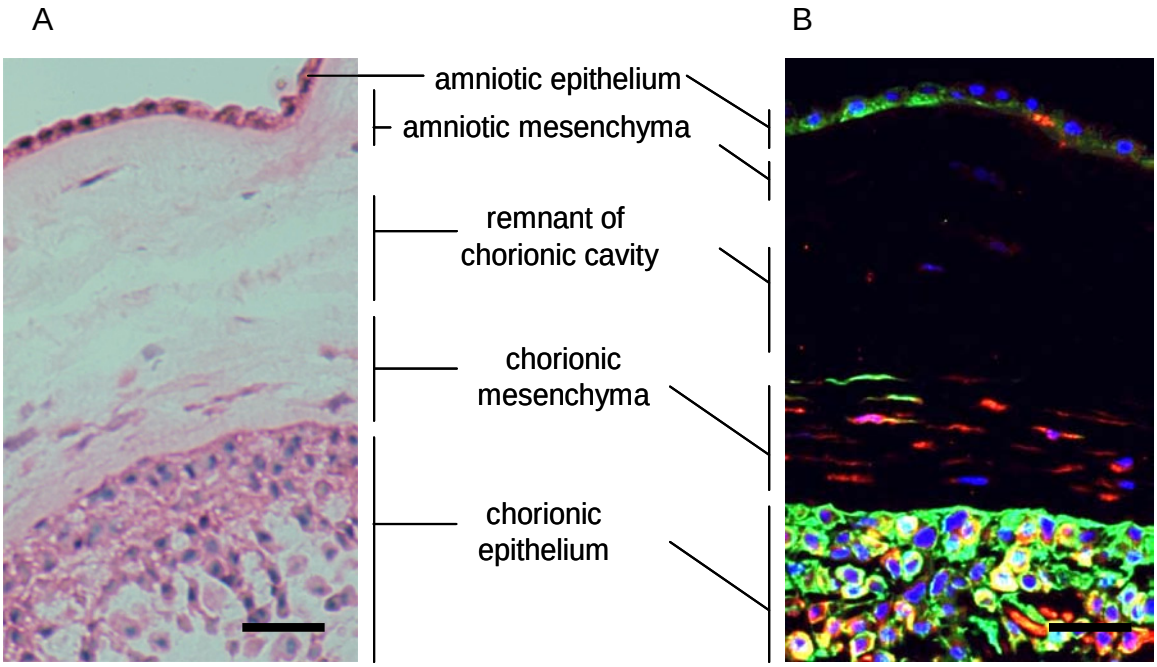

Figure 3.I - HE staining (A) and Immunofluorescence (B) of fetal membranes. Immunofluorescence detecting positive Vimentin (red) and Cytokeratin (green). Höchst staining is shown in blue. Scale bar: 50 µM

The first layer of the fetal membranes to be found under the amniotic cavity is the amniotic epithelium (Figure 3.I), where HAE cells were isolated from. It includes a single monolayer of flattened, cuboidal cells resting on a basement membrane. Underlying the amniotic epithelium basement membrane is a compact stromal layer representing the amniotic mesenchym.

The following spongy intermediate zone resulting from incomplete fusion of amniotic and chorionic mesenchyma embryologically represents a remnant of the chorionic cavity. This zone has a highly variable thickness.

The remnant of the chorionic cavity continues into the next connective tissue layer, the chorionic mesenchyma. Passing over the chorionic mesenchyma, a stratified epithelial layer, the chorionic epithelium, is found. In the HE staining the remnant of the chorionic cavity can easily be seen. Due to this remnant cavity, both amniotic and chorionic membranes can be easily separated. To verify that only amniotic cells were isolated, the amniotic membrane was stained after being dettached from the chorion (Figure 3.II). This figure confirms that only the amniotic membrane and not the chorion was used for further experiments.

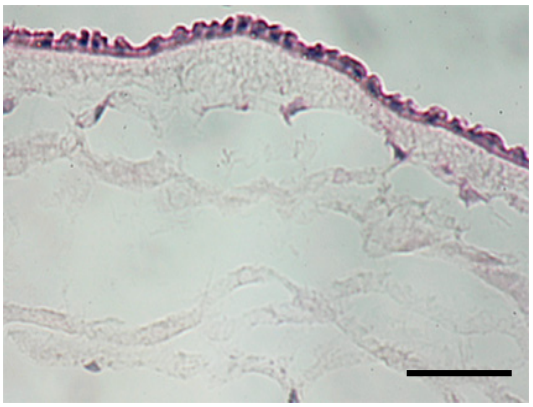

Figure 3.II – H/E staining of the amniotic membrane after dissection from the chorionic membrane. Scale Bar: 50 µM

4.2 Isolation of HAE cells for in vitro culture

In order to investigate which HAE cell isolation method was more adequate, different published protocols were analysed and modified. Most of these isolation methods involved longer or shorter trypsination steps. As most cells were obtained after the first and second trypsination, more than three trypsination did not alter the final amount of HAE cells (approximately 12 million cells).

Freshly isolated and attached HAE cells (Passage 0) were flat, cuboidal to columnar cells as described by Bernischka and Kaufmann (2000). During cultivation, HAE cells acquired a spindle-shaped morphology.

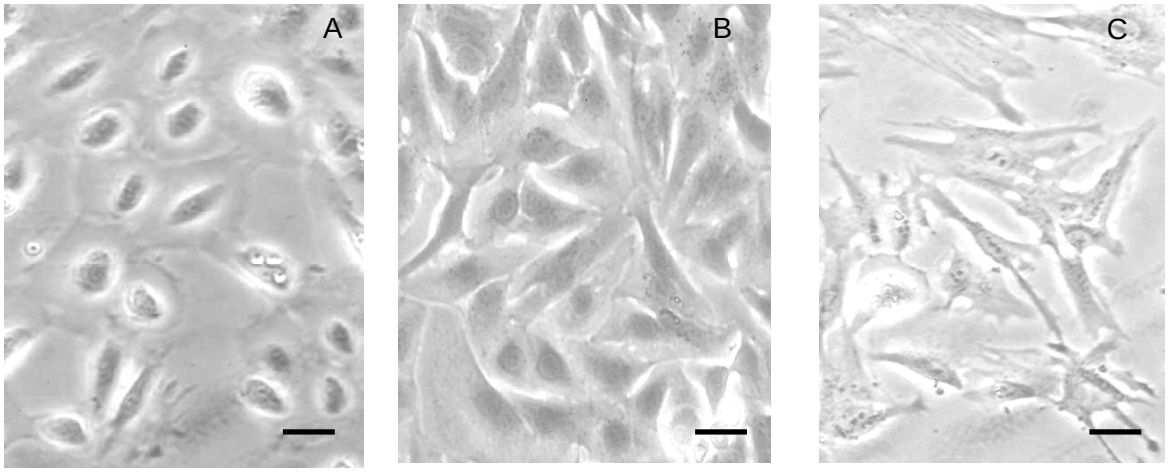

Figure 3.III - Appearance of HAE cells culture at passages 0 (A), 1 (B) and 5 (C). Scale Bar: 66 µM

Following in situ characterisation of HAE cells, cells were also analysed directly after isolation and during cultivation (passage 6) by flow cytometry. The results are average values from 3 different experiments. Representative data is shown in Figure 3.IV. Following epitopes characteristic for different cell types and/or germ layers were used:

Within epithelial markers, cytokeratin 19 was strongly positive at passage 0 and 1 and decreased to 0% at passage 6. Mesenchymal markers like vimentin remained strongly positive at passage 0, 1 and 6. Another mesenchymal marker is CD140b (syn. PDGF Receptor ß) which is expressed in stromal cells and some endothelial cells. Its expression was negative at passage 0 and 1, but it was upregulated at passage 6, with 90% positive CD140b cells. Following some endothelial markers, it is known that CD31 (syn. PECAM-1, EndoCam) is a transmembrane glycoprotein strongly expressed by all endothelial cells. It is supposed to be an adhesion molecule for platelets and belongs to the immunoglobulin superfamily. Regarding our results, this marker was negative throughout the passages. Another endothelial marker is CD62e (syn. E-Selectin) which is an adhesion molecule expressed in the endothelium. Its expression in HAE cells was negative throughout the passages. Same results were obtained with vWF. Furthermore, CD144 (syn. VE Cadherin) is a marker expressed in cadherins and endothelial cells. 20% positive CD144 cells were detected at passage 0. CD144 expression was downregulated at passage 6. Another marker expressed in vascular endothelial cells is CD141 (syn. Thrombomodulin). CD141 expression in HAE cells remained strongly positive at passage 0, 1 and 6.

CD105 (syn. Endoglin) is a marker not only expressed in endothelial cells but also in activated monocytes, macrophages and bone marrow cell subsets. Around 30% positive cells were detected in HAE cells at passage 0. At passage 6 CD105 expression was upregulated, with 80% positive cells. CD83 (syn. HB 15), which is expressed in dendritic cells, B cells and Langerhans´ cells was also expressed in HAE cells. Approximately 50% positive cells were detected at passage 0. CD83 expression was downregulated at passage 1 and 6, with 90% negative CD83 cells. Following more endothelial markers, CD34 (syn. gp105-120) is expressed in hematopoetic stem cells and in the capillary endothelium. Around 40-50% positive CD34 cells were detected at passage 0. CD34 expression decreased to 10% at passage 6. CD133 (syn. AC133, Prominent One) is a pluripotency marker expressed in stem cells (Baal et al., 2004a). Approximately 40% CD133 positive cells were detected at passage 0. CD133 expression dropped to 10-20% at passage 6. Furthermore, CD45 (syn. LCA, T200, Non lineage) which is expressed in hematopoetic cells, was not expressed in HAE cells, neither at passage 0,1 nor 6. Finally, CD14 (syn. LPS), expressed in myeloid cells, was not expressed neither at passage 0, 1 nor 6.

In summary, HAE cells upregulated mesenchymal markers throughout cultivation, in contrast to the downregulation of the epithelial marker cytokeratin. Furthermore, the cells did not show strongly any endothelial marker profile. Interesting to remark is the expression of CD141, CD105 and more specifically the expression of CD140b, so called PDGF receptor ß, which was positive in all isolations at passage 6 (see Table 3.I)

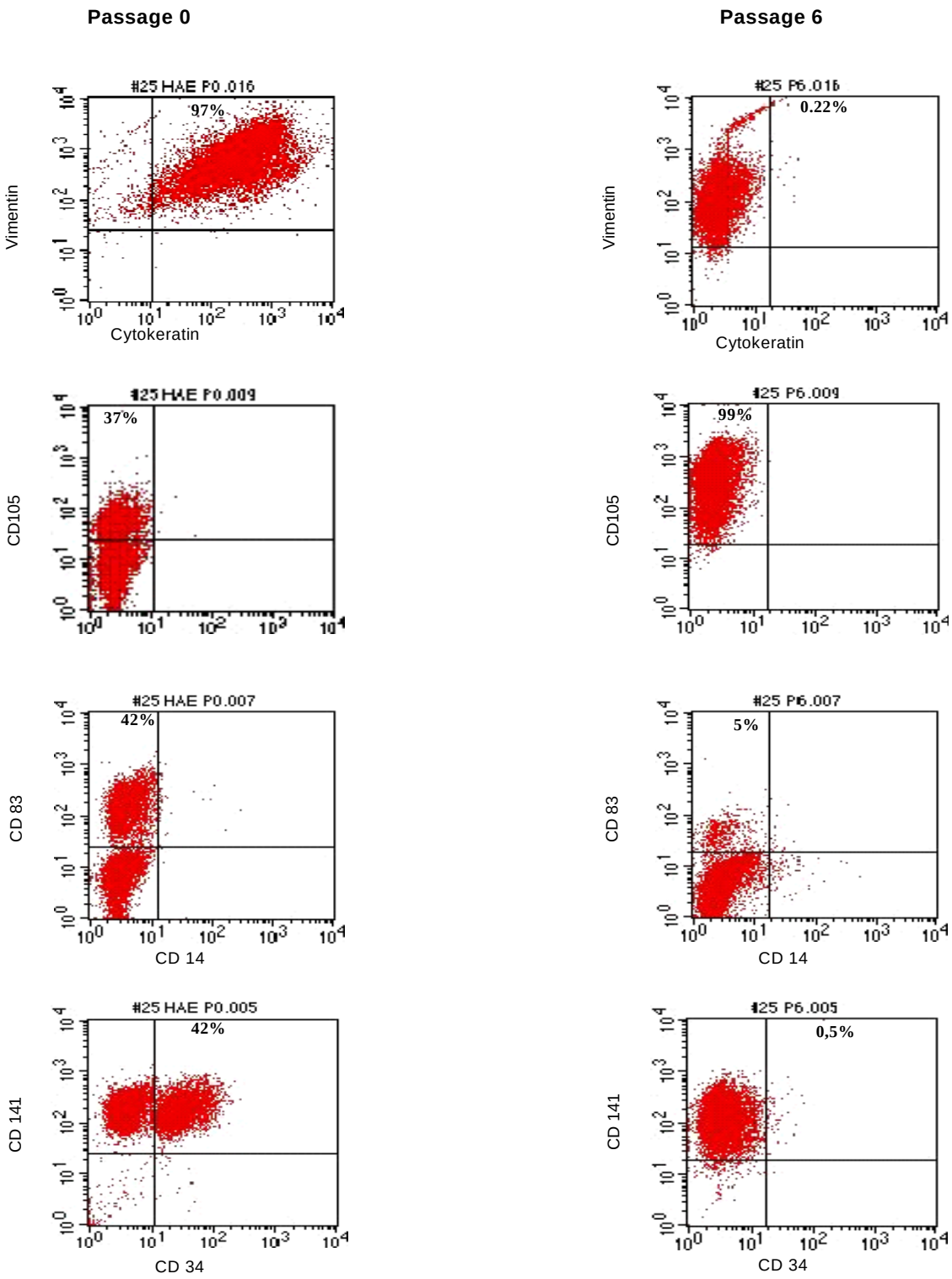

Figure 3.IV – Representative Flow Cytometry Analysis of HAE cells.

Directly after isolation (Passage 0) Cytokeratin+/Vimentin+ population was 96.72%, CD105+ population was 37.49%, CD83+/CD14- population was 42.12% and CD141+/CD34+

population was 41.61%. At passage 6 (day 28) these populations were 0.22%, 99.38%, 4.73% and 0.51% respectively (Figure 3.IV).

Chart 3.I – HAE cell characterization during in vitro culture.
Overview (+++: 75-100% cells, ++: 25-75% cells; -: 0-25% cells)

	Passage 0	**Passage 6**
Cytokeratin 19	+++	-
Vimentin	+++	+++
CD140b	-	+++
CD31	-	-
CD62e	-	-
vWF	-	-
CD144	+	-
CD141	+++	+++
CD105	++	+++
CD83	++	-
CD34	++	+
CD133	++	+
CD45	-	-
CD14	-	-

Flow cytometric analysis showed the expression of different markers and different germ layers. As previously mentioned, HAE cells are formed from amnioblasts separated from the epiblast at about the 8th day after fertilisation. Due to this early embryological origin, they are presumably able to retain some pluripotency capacity.

4.2.1 HAE cells express pluripotency markers

The expression of different germ layer specific proteins may indicate a pluripotent status of the HAE cells. Therefore, expression of pluripotency marker mRNA like Oct-4, Rex-1, Sox-1, Sox-2, FGF-4 and Nanog was investigated by RT-PCR. As a positive control CD133 positive cells from the umbilical cord blood as a model for endothelial progenitor cells were used (Baal et al., 2004b).

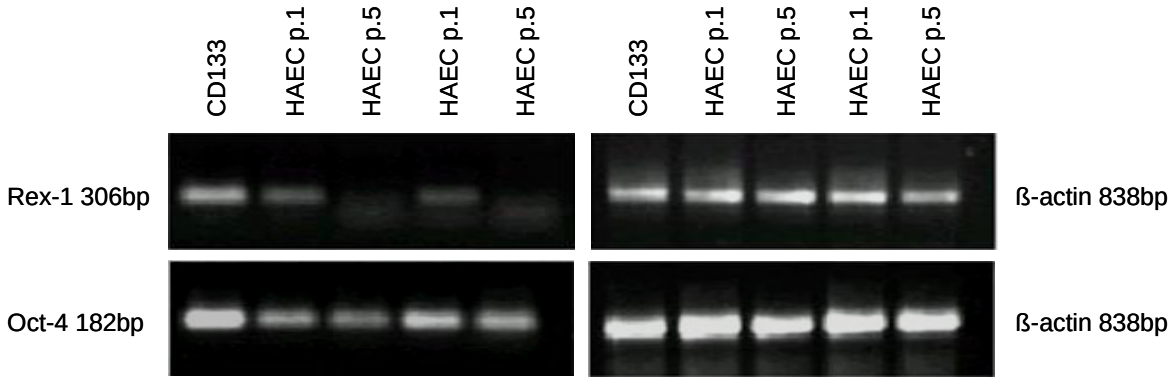

Figure 3.VI – Representative RT-PCR analysis of HAE cells

In Figure 3.VI, the expression of Oct-4 was detected in both passages 1 and 5 from 2 different HAE cell cultures derived from separate isolations. The expression of Rex-1 was shown only in passage 1 from 2 different HAE cell cultures. The expression of Sox-2 and Nanog mRNA was weak at passage 1 and negative at passage 5 (data not shown). Sox-1 and FGF-4 expression was negative. Oct-4 mRNA has a functional relevance because the protein was also expressed in HAE cells (Figure 3.VII). As no commercial Rex-1 antibody was available, the analysis concentrated on Oct-4 protein expression.

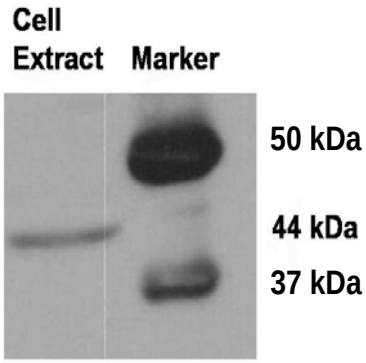

Figure 3.VII – Representative Western Blot analysis of HAE cells. Expression of Oct-4 in HAE cells directly after isolation.

The detection of Oct-4 Protein in HAE cells confirms that HAE cells have the potential to not only to express Oct-4 mRNA but also to synthesize it. This stem cell marker shows that directly after isolation HAE cells maintain this pluripotency capacity. As a remark, no Western Blot analysis was performed in passage 1 or 5.

4.2.2 Chorionic epithelial cells express AFP

Alpha-Fetoprotein (AFP) is known as an oncodevelopmental 70-kDa protein with a growth-regulative and immunosuppressive activity (Dudich et al., 1998).

AFP is secreted by the embryonic liver and yolk sac during perinatal development. After 12 week of gestation, the yolk sac degenerates and the fetal liver becomes the main site of AFP synthesis. The protein expression level in the circulation of the fetus is very high (1-10 mg/ml), but it decreases abruptly soon after birth. By the end of the second month postpartum, only trace amounts of AFP can be detected in the circulation, and it is almost completely substituted by human serum albumin (Dudich et al., 1998). The important role of AFP as proangiogenic factor during vascular morphogenesis at the fetomaternal unit has been demonstrated by our group in a previous publication (Liang et al., 2004).

AFP is expressed by some types of malignant tumors, and appears to play a role in the suppression of the mother´s immune response to the developing embryo as well as in that of a patient to a developing tumor.

Fetal membranes were stained with AFP unconjugated primary antibodies and fluorochrome-conjugated secondary antibodies (Figure 3.VIII). In the immunofluorescence, AFP expression is shown at the chorionic membrane, specially in the chorionic epithelium bordering the trophoblast. No AFP expression was shown at the amniotic membrane.

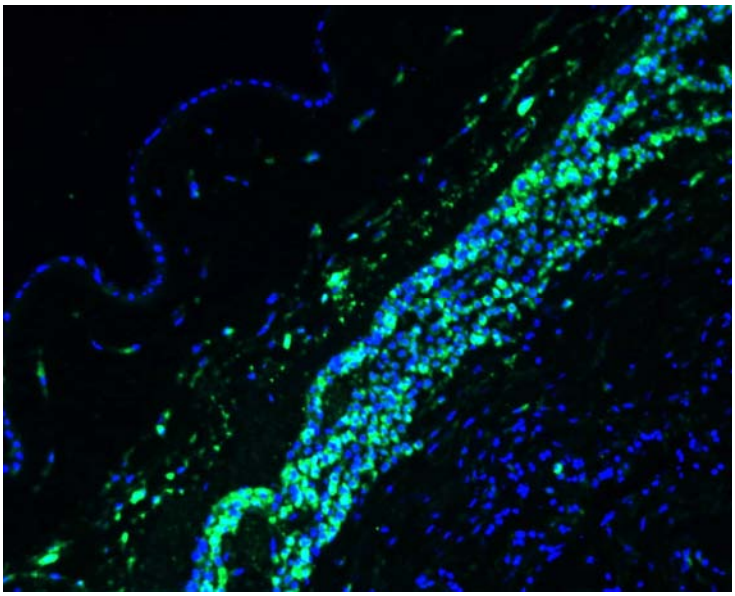

Figure 3.VIII – Immunofluorescence of fetal membranes. Immunofluorescence detecting positive AFP (green). Stratification matches Figure 3-I. Scale bar: 50 µM

5 DISCUSSION

The interesting properties described in HAE cells arise out of the following considerations. Taking into account that human placental tissues are discarded after the delivery of the newborn, HAE cells are fetal epithelial cells that usually exist less than 10 months in nature. Like stem cells and some immature cells, HAE cells can inhibite cellular immune response making them interesting for allogenic transplantation. The uncomplicated obtainment of HAE cells is another appealing feature. Placenta is abundantly available as a discarded tissue following normal delivery. Large number of cells can be derived from this source, without obvious ethical problems, as opposed to embryonic stem cell research, which requires the destruction of the blastocyst. All of the previous described issues make HAE cells a highly interesting source of pluripotent cells.

Analysing already published approaches, HAE cells express neural (Sakuragawa et al., 1996), hepatic (Sakuragawa et al., 2000a) and pancreatic (Wei et al., 2003) markers. HAE cell development before the start of the organogenesis would lead these cells to have similar properties like early epiblast cells and thus to differentiate and contribute to cells from all three germ layers.

To test whether HAE cells have putative multipotentiality, several studies investigated HAE cell expression of neuron, astrocyte or oligodendrocyte markers (Sakuragawa et al., 1996; Sakuragawa et al., 2004a). Furthermore, it has been demonstrated that HAE cells can synthethise and release acetylcholine in a time-dependent manner (Sakuragawa et al., 2000a; Ishii et al., 1999; Sakuragawa et al., 1996; Sakuragawa et al., 2004c). HAE cells can also synthesise and release catecholamines such as norepinephrine and dopamine (Elwan and Sakuragawa, 1997c; Elwan et al., 2003a; Elwan et al., 2003h; Elwan et al., 2003f; Elwan et al., 1999d; Elwan et al., 1999b; Elwan et al., 1998a). Furthermore, extensive analyses with various techniques have demonstrated that HAE cells and immortalised HAE cells synthesise and release brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and nerve growth factor (NGF) (Uchida et al., 2000e). Finally, the synthesis and release of activin A and noggin has been found in HAE cells (Koyano et al., 2002b).

Continuing with the hepatic system, interesting markers relating the liver are expressed in HAE cells. The fetal part of human placenta is composed only from ectodermal and mesenchymal tissue (Moore and Persaud 1998, Benirschke and

Kaufmann 2000). In remarkable contrast to this view, definitive endodermal markers GATA-4 and hepatocyte nuclear factor-3ß have been detected in amnion-derived cells by RT-PCR (Wei et al., 2003), and the expression of the endodermal/hepatic markers AFP and albumin was shown both at the mRNA and at the protein level (Sakuragawa et al., 2000b; Takahashi et al., 2002; Takahashi et al., 2001).

Cultured amniotic cells strongly expressed mRNA for GLUT-2, a glucose-sensing molecule characteristic for beta cells and hepatocytes (Wei et al., 2003). Finally, the evidence of albumin synthesis and excretion by HAE cells by immunostaining and enzyme-linked immunoassay has been shown (Sakuragawa et al., 2000a).

Concerning the pancreatic system, markers of pancreatic differentiation, like insulin and glucagon, have been found in HAE cells. More concretely, the Wei group, (Wei et al., 2003) demonstrated the normalisation of blood glucose in diabetic mice transplanted with HAE cells induced to differentiate to insulin expressing cells after culture with 10 mM nicotinamide for up 4 weeks.

Relating the heart, a recent publication showed cardiomyocyte-related gene expression of myosin light chain 2 (MLC-2A, MLC-2V) and the transcription factors GATA-4 and Nkx 2.5 in induced HAE cells cultured over 14 days in ascorbic acid supplemented media (Miki et al., 2005).

All aforementioned published approaches shed light into the theory that HAE cells can express markers belonging to all three germ layers (endoderm, mesoderm and ectoderm), apparently due to their epiblastic origin.

HAE cells lack the expression of MHC class II, and express very low MHC class I antigens (Akle et al., 1981; Sakuragawa et al., 1995; Hunt et al., 1988). They are not tumorigenic because there was no evidence of tumorigenicity in humans when isolated amniotic cells were transplanted into human volunteers to examine their immunogenicity or into patients in attempt to correct lysosomal storage diseases (Akle et al., 1981). In fact, antimicrobial properties have been shown as well in human amniotic membranes (Talmi et al., 1991) when closely situated to a wound surface, adhering to it. Therefore its use in conjunctiva or corneal surface reconstruction.(Tseng et al., 1997; Grueterich et al., 2003a; Solomon et al., 2003; Ueta et al., 2002a; Nakamura et al., 2004b).

An optimal isolation and cultivation of the cells is needed in order to obtain a maximum number of isolated cells in an optimal state. Finally it is not only necessary to find an optimised method of isolation and cultivation but also a precise

characterisation, in order to demonstrate the HAE cell marker profile. Therefore this doctoral thesis can be considered as an attempt to reach this aims. It is important to remark that the results shown in this study are derived from HAE cells isolated from different human placentas.

When incubated with collagenase A, cells were collected following centrifugation after 2 h. Concerning the number of trypsin incubations performed, different strategies are described:

Some authors prefer to discard the supernatant resulting from the first trypsination in order to eliminate red blood cells; supernatant obtained from the 2nd (Wei et al., 2003), 3 rd and 4 th trypsinations (Casey and MacDonald, 1996b; Bilic et al., 2004b; Sakuragawa et al., 1997) is then combined. Other authors combined all supernatants from 6 (Terada et al., 2000b) or 3 (Kakishita et al., 2000a; Sakuragawa et al., 1996) trypsination steps, including the 1st trypsination.

According to Moore et al. (2003) protocol, cells were submitted to a single 1.5 h incubation and, following centrifugation, the supernatant collected.

The isolation of HAE cells described in this doctoral thesis was likewise performed with trypsin. Regarding HAE cell location on the most external fetal membrane layer, amnion epithelium is considered a single monolayer which does not establish strong cell-cell adhesion at their lateral sides like other epithelial cells. Consequently trypsin is able to to dettach HAE cells from the amniotic basement membrane.

Collagenase could digest further internal tissues, increasing the risk of mesenchymal cell contamination. Therefore, collagenase was not used in this investigation during HAE cell isolation.

Due to the high number of cells obtained after the first trypsination compared to further trypsinations, it was decided not to discard any supernatant. Approximately 70% of HAE cells were obtained after the 1st trypsination, ~ 15% after the 2nd trypsination and ~ 10% after the 3rd trypsination, as measured by hemocytometer cell counting method after every trypsination. After the 3rd trypsination very few cells were obtained. Consequently the here described method optimises previously methods by achieving similar amounts of HAE cells using less trypsination steps and therefore less time and reagent volume.

Almost all red blood cells and cellular debris were eliminated from the tissue after rinsing the amniotic membrane with PBS. Hence, approximately 5% of the cell

population at the end of the isolation consisted of red cells. This population of non-adherent erythrocytes was discarded after replacing the medium on the 2nd day.
After dissecting the amnion from the chorion, HE staining was performed in order to verify that only amniotic cells were trypsinated.
Within the amnion, both epithelial and mesenchymal cells are found. HAE cell detachment to the basement membrane can be performed by single trypsination, whereas longer trypsination periods are needed to obtain amniotic mesenchymal cells, because of stronger cell-matrix adhesion. Still, mesenchymal cell contamination of the HAE cell culture can unfortunately not be excluded.

5.1 HAE cell characterisation

Prior to this study a precise characterisation of HAE cells was lacking. Some publications had shown HAE cells protein expression focusing on the nerve system, the hepatic system, the pancreatic system or the cardiac tissue.
To test whether HAE cells have putative multipotentiality, several studies investigated HAE cell expression of neuron, astrocyte or oligodendrocyte markers (Sakuragawa et al., 1996; Sakuragawa et al., 2004b).
Furthermore, it was demonstrated that HAE cells can synthethise and release acetylcholine in a time-dependent manner (Sakuragawa et al., 2000a; Ishii et al., 1999; Sakuragawa et al., 1996; Sakuragawa et al., 2004c). HAE cells can also synthesise and release catecholamines such as norepinephrine and dopamine (Elwan and Sakuragawa, 1997a; Elwan et al., 2003d; Elwan et al., 2003i; Elwan et al., 2003g; Elwan et al., 1999e; Elwan et al., 1999a; Elwan et al., 1998c). Furthermore, extensive analyses with various techniques demonstrated that HAE cells and immortalised HAE cells synthesise and release brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and nerve growth factor (NGF) (Uchida et al., 2000d). Finally, the synthesis and release of activin A and noggin was demonstrated in HAE cells (Koyano et al., 2002a).
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amnion-derived cells by RT-PCR (Wei et al., 2003), and the expression of the endodermal/hepatic markers AFP and albumin was shown both at the mRNA and at the protein level (Sakuragawa et al., 2000b; Takahashi et al., 2002; Takahashi et al., 2001).

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All aforementioned published approaches shed light into the theory that HAE cells can express markers belonging to all three germ layers (endoderm, mesoderm and ectoderm), apparently due to their epiblastic origin.

In order to close the existing gap in HAE cell characterisations, the present study demonstrated a precise systematic characterisation of HAE cells in vitro.

It is necessary to remark that the amniotic epithelium is composed of unique amniotic epithelial cells exhibiting quite characteristic morphological features. They have a relatively small number of intracytoplasmic organelles, microvilli on the apical surface, abundant cytoplasmic processes to the lateral and basal sides, and loose intercellular connections (Benirschke and Kaufmann, Pathology of the human placenta, Springer Verlag, p 268-314)(Matsubara and Sato, 2000).

This single cell layer appearing as a uniform cell type (King, 1980) is illustrated in Figure 3.I. Furthermore, Figure 3.I illustrates that fetal membranes are composed of both amnion and chorion, each with an epithelial and mesenchymal component and separated by an intermediate zone.

Cytokeratin is a group of intermediate filaments that is almost exclusively expressed in epithelia and represents one of the most important parts of the cytoskeleton. It

consists of at least 20 different proteins varying in molecular weight (from 40 to 70 kD) and showing some epithelium-, organ-, or tumor-specificity (Moll et al., 1982; Santini et al., 1996). It is a marker for epithelial cells with an epidermal origin. Cytokeratins are relatively stable in vivo, as they remain expressed in most high-grade carcinomas and traces of it can even be detected in the sarcomatous component of a mixed tumor.

Vimentin is a cytoskeleton protein from mesenchymal cells, this means, fibroblasts, condrocytes, osteocytes, endothelial cells and leucocytes.

Interestingly enough, immunohistochemical data (Figure 3.I B) demonstrates that HAE cells in situ express two types of intermediate filaments within the same cell, cytokeratin and vimentin. This reforces previously published approaches where HAE cells, despite their clearly ectodermal-epidermal origin not only expressed general epithelial markers such as cytokeratins (Beham et al., 1988) or epidermal markers such as CA 125 (Nanbu et al., 1989b), but also expressed a marker for mesenchymally derived cells, vimentin (Wolf et al., 1991; Sakuragawa et al., 1996; Uchida et al., 2000c; Regauer et al., 1985b).

Intermediate filaments play an important role in the differentiation and proliferation of cells. During differentiation, transdifferentiation and neoplastic formation, dramatic changes occur in the intermediate filament protein expression and organization (Bouwens et al., 1997). This expression has been specifically studied in the pancreatic system. Vimentin positive cells have been observed in epithelial ducts during embryogenesis (Rosenberg, 1995), in the dedifferentiation from adult to progenitor ß cells (Zulewski et al., 2001), and during ductal cell proliferation during carcinogenesis (Petersen et al., 2003). Conversely, cytokeratin expression alone is shown in mature pancreatic epithelial cells. Consequently, vimentin in pancreatic duct cells can be considered a useful marker for pancreatic precursor cells (Ko et al., 2004). Taking into account that vimentin is expressed in cultured HAE cells, their ability to differentiate into pancreatic precursor cells could be especulated. This hypothesis is enforced by the Wei et al. study, where HAE cells were differentiated to secrete insulin.

Alpha-fetoprotein (AFP) is a protein expressed during development of primitive endoderm and reflects therefore endodermal differentiation. It is normally produced by the liver and yolk sac of a fetus and it decreases soon after birth. Fetal spina bifida or other defects of the fetus' neural tube are associated with elevated levels of AFP.

AFP measurement can be performed along with the amniocentesis during pregnancy. In contrast to the Sakuragawa group (Sakuragawa et al., 2000b; Takahashi et al., 2002; Takahashi et al., 2001), no expression of AFP was shown by HAE cells in situ. Positive staining for AFP though, could be demonstrated at the epithelial layer of the chorion, specially in the cells bordering the trophoblast. This is the same location where most of the double-positive cytokeratin/vimentin chorionic cells are found. Because AFP is present in fetal serum, it cannot completely be excluded that AFP expression results from a passive absorption/uptake from circulating AFP. However, the nuclei of AFP-positive cells does not show any signs of apoptosis and the strong and strictly cellular localization of AFP suggests an endogenous cellular AFP synthesis.

Regarding the cultivation media, we tested HAE cell cultivation not only in epidermal cell culture media (Epidermal Cell Growth Medium and Accutase)(Accutase PAA, Austria) but in media indicated for endothelial cell culture (EBM-2) and for amniotic fluid cells (Amniomax and AmnioGrow plus, Invitrogen).The most adequate results in terms of number of cells were obtained using Epidermal Cell Growth Medium.

After culturing HAE cells in Epidermal Cell Growth Media, cells were disposing more elongated shape throughout the passages, losing their originally rounder aspect, reminding a typical mesenchymal cell morphology (Figure 3.III). Again, as mesenchymal cell contamination in HAE cell cultures remains possible, an epithelial to mesenchymal transition of HAE cells can be neither confirmed nor excluded.

HAE cells were characterized using flow cytometry at passage 0, 1 and 6 in order to test differences between freshly isolated and cultured cells. Both shorter (1st passage) and longer (6th passage) cultured periods were tested. To reduce the possibility of mesenchymal cell contamination, trypsinated cells resulting from two 20 min incubation periods were submitted to flow cytometry. Results illustrate a homogenic pattern of cells (Fig. 3.IV), guaranteeing at single cell population of cells and excluding the possibility of 2 different cell types.

After speculating about HAE cell plasticity, the following question arises: can HAE cells express differentiation markers? Thus, the expression of epithelial, mesenchymal and endothelial markers was investigated.

Decreased expression of cytokeratin is, in some epithelial cell types like breast epithelium, accompanied by the emergence of vimentin (Osborn et al., 1984; Seshadri et al., 1996). In vitro studies indicated a link between vimentin and

increased motility or invasion in breast (Sommers et al., 1994; Zajchowski et al., 2001; Gilles et al., 1994b; Ebert et al., 2000), prostate (Ramaekers et al., 1989) and cervical (Ebert et al., 2000; Gilles et al., 1994a) cancer lines. Vimentin/Cytokeratin co-expression is found in some carcinoma types like renal cell carcinoma, synovial sarcoma, endometrium carcinoma or epitheloid sarcoma without evidence of dedifferentiation, and even in normal epithelium. This is particularly true for epithelia that have evolved from mesenchymal precursors like the urogenital organs (Auersperg et al., 2001; Holthofer, 1990), as if these epithelial cells recalled their mesenchymal roots. Ovarian surface epithelial cells (Auersperg et al., 1994) and normal endometrial glands (Dabbs et al., 1986) mainly in their proliferative phase express vimentin.

HAE cells had showed a cytokeratin-vimentin co-expression pattern immediately after isolation (day 0). At passage 6 the expression of cytokeratin decreased and the expression of vimentin remained positive.

A combination of markers were used in conjunction with flow cytometry to explore the pluripotency of HAE cells in vitro (Figures 3.I and 3.IV).

CD105 (syn. endoglin) binds members of the TGF-ß superfamily. It has been found that this endothelial cell marker may play a role during early hematopoetic development, and so it could be considered as a marker of differentiation (Cho et al., 2001). The strongly positive expression of CD105 at passage 6 supports our hypothesis on HAE cell pluripotency.

Likewise, CD141 (thrombomodulin) is strongly expressed in HAE cells. CD141 has a receptor that binds thrombin and that results in the activation of protein C, which degrades clotting factors Va and VIIIa. Mutations in this gene are a cause of thromboembolic disease, also known as inherited thrombophilia. It is a useful molecular marker for controlling vascular complications in human diabetic neuropathy, Behcet´s disease or in hemorrhagic stroke.

CD140b (platelet-derived growth factor receptor beta) is a member of the platelet-derived growth factor family that are mitogens from cells of mesenchymal origin. A translocation in two chromosomes results in chronic myelomonocytic leukemia. The expression of CD140b in HAE cells up to Passage 6 shows a further hint of HAE cell differentiation in mesenchymal cell type (Figure 3. IV).

Epithelial, mesenchymal and endothelial markers are expressed in HAE cells at day 0. After HAE cell cultivation in Epidermal Cell Growth Medium, the epithelial marker

cytokeratin decreased expression throughout the passages. In contrast, mesenchymal markers like Vimentin and CD140b were upregulated. With the exception of CD141 and CD105, endothelial markers CD31 and vWF were not strongly expressed in HAE cells at day 0.

After guaranteeing a homogenic population of cells at Passage 0, 1 and 6, it is interesting to notice how epithelial origin cells lose their epithelial marker expression and acquire a mesenchymal-like marker profile. In spite of HAE cell cultivation in an epidermal cell medium, epithelial characteristics of these cells decreases throughout the passages. A similar induction of mesenchymal cell markers has been observed during the process of epithelial-mesenchymal transition in the pancreas, where vimentin filaments follow pre-existing cytokeratin networks (Casaroli-Marano et al., 1999; Pagan et al., 1996). This epithelial-to-mesenchymal transition has been shown to generate proliferative human islet precursor cells (Gershengorn et al., 2004) and is related to the insulin expression by differentiated HAE cells (Wei et al., 2003).

Apparently this evidence could lead us to a false euphory regarding the possibility of HAE cells differentiating into insulin secreting cells.

In the Wei article, a much lower insuline level expressed by the nicotinamide induced HAE cells was described, compared to therapeutical levels needed in diabetes treatment. Finally, it is not clear if HAE cells demonstrated an authentic epithelial to mesenchymal transition or if this induction of mesenchymal cell markers was induced by other undescribed factors.

5.2 HAE cell pluripotency

Regarding flow cytometric characterisation, HAE cells demonstrated a potential to develop into different cell types. The pluripotency of those cells was confirmed as well by the expression of Oct-4 mRNA and protein and Rex-1mRNA at passage 1 in HAE cells.

Oct-4 is a transcription factor unique to totipotent stem cells and is essential for the establishment and maintenance of undifferentiated pluripotent stem cells (Pesce and Scholer, 2001). When embryonic stem cells are differentiate, Oct-4 is downregulated. In vivo mutagenesis has shown that loss of Oct-4 at the blastocyst stage causes the cells of the inner cell mass to differentiate into trophectodermal cells (Pesce and Scholer, 2001).

Rex-1 is expressed in embryonic stem cells and F9 teratocarcinoma cells. Rex-1 promoter promotes the activity in undifferentiated F9 cells and is involved in the decrease of retinoic acid expression (Ben Shushan et al., 1998). When embryonic stem cell begin to differentiate into a neuronal cell type, this marker is downregulated. The negative expression of Rex-1 mRNA at passage 5 suggest a decrease in pluripotency throughout the passages.

CD 133 is a cell surface glycoprotein localized in membrane protrusions or microvilli and is expressed by stem and progenitor cells of hematopoetic and endothelial lineages (Yin et al., 1997; Baal et al., 2004a), neurons and glial cells (Uchida et al., 2000a), and by some epithelial cells (Corbeil et al., 2000).

40% HAE cells expressed CD 133 protein at the time of isolation as shown by flow cytometry analysis, but not at passage 6, suggesting a possible ability of these cells to differentiate into hematopoetic cells.

The importance of these findings leads as well in the possibility of these genes to be expressed at day of isolation, even before cells are placed into culture.

Recently published study described the stem cell characteristics of HAE cells(Miki et al., 2005). The authors focused on the capacity of these cells in vivo to differentiate into three germinal layers (endoderm –liver, pancreas-, mesoderm –cardiomyocyte- and ectoderm –neural cells).

Similarly, this article describes a HAE cell isolation based on 3 trypsination steps for 30 min. The culture medium used was containing EGF (Epidermal Growth Factor), which induced a “robust proliferation” of HAE cells, with five cell doublings over 8 days, in contrast to the lower proliferation rate observed with Mammary Epithelial cell Growth Medium.

Immunohistochemistry results showed not only cytokeratin expression but also Oct-4 in HAE cells.

Likewise, freshly isolated HAE cells expressed not only Oct-4 and Rex-1 by RT-PCR but also FGF-4, Sox-2 and Nanog, as opposed to the RT-PCR data shown in this work, with negative FGF-4, Sox-1, Nanog and Sox-2 mRNA expression.

More specifically, the expression of nanog and Oct-4 in the Miki et al. article was higher in middle layer fraction of the cultures than in the adherent HAE cell fraction, suggesting a role of autologous feeder layer serving as a substrate for attachment or providing secreted factors which can help induce or maintain undifferentiated HAE cells.

The article shows no expression of CD34, in contrast to the flow cytometry data shown in our work, with 30-40% positive CD34 cells at Passage 0 decreasing to 10% at Passage 6.

HAE cells in the Miki article were submitted as well to flow cytometry, showing the presence of the SSEA 3 and 4, TRA1-60 and 1-81 markers, which are not tested in this work.

Furthermore, it demonstrates the expression of pancreas duodenum homeobox 1 (PDX-1), pair box homeotic gene 6 (Pax-6), NKX2 transcription factor related, locus 2 (Nkx 2.2), Insulin and Glucagon mRNA after stimulating HAE cells with Nicotinamide, atrial and ventricular myosin light chain 2 (MLC-2A, MLC-2V) GATA-4 and Nkx 2.5 transcription factors after ascorbic acid stimulation and glial fibrilary acidic protein (GFAP) and cyclic nucleotide phosphodiesterase (CNP) expression after all-trans retinoic acid and FGF-4 stimulation.

Albumin and alpha-1-antitrypsin (A1AT) expression was shown by real-time quantitative PCR when cells were cultured in EGF and dexamethasone.

In conclusion, the results of this publication confirm some of the data presented in our work. Furthermore, other aspects like HAE cell differentiation, which was initally shown by Weit et al., were throroughly investigated in the article as well, confirming HAE cell capacity to differentiate into multiple cell types.

6 SUMMARY

The amniotic membrane is part of the fetal membrane and is composed of the amniotic epithelial (HAE) and mesenchymal (HAM) cells that are derived from the inner cell mass in the blastocyst. Thus, HAE and HAM cells may be multipotent. It has been reported that some differentiation markers for neuronal and hepatic parenchymal cells were expressed in HAE cells, suggesting that HAE cells may be a good source of stem cells for various cellular therapies. In addition, amniotic cells do not express the MHC class II, which may help prevent immune rejection, and can be obtained after delivery.

We established a new method for isolation and cultivation of HAE cells and tested the pluripotency in different passages. HAE cells were cultured in different enriched mediums (with different growth factors) for expansion.
We have found that HAE cells express Oct-4 mRNA as well as Rex-1 mRNAs (pluripotency markers) at passage 0.

Phenotypic characterisation of HAE cells was carried out by a flow cytometer. HAE cells from passages 0, 1 and 5 demonstrated the transitive expression of epithelial, mesenchymal and endothelial markers.
In summary, amnion from discarded placenta can be an interesting source of cells for regenerative medicine

7 ZUSAMMENFASSUNG

Das Amnion ist ein Teil der fetalen Membran, die aus amniotischen Epithelzellen (HAE) sowie amniotischen Mesenchymzellen besteht. HAE entwickeln sich aus der inneren Zellmasse und zeigen eine vielseitige Expression von neuronalen, pankreatischen und hepatischen Differenzierungsmarkern. Anderseits exprimieren die Zellen keine MHC-2 Moleküle. Aufgrund dieser Eigenschaften konnten die Zellen für verschiedene Zelltherapien angesetzt werden.

Für die nähere Untersuchung von HAE-Zellen in vivo wurde im Rahmen der vorliegenden Arbeit eine neue Aufreinigungsmethode und die Kultivierungsbedingungen etabliert. Es konnte gezeigt werden, dass HAE-Zellen direkt nach der Aufreinigung wichtige Pluripotenzmarker wie *Oct-4 und REX-1* exprimieren.

Um das Phänotyp von HAE-Zellen zu charakterisieren, wurden FACS-Analysen mit unterschiedlichen Antikörpern durchgeführt. Die Zellen zeigten eine gleichzeitige Expression von epithelialen, mesenchymalen und endothelialen Markern. In Laufe der Kultivierung der Zellen in vivo stiegt Expression von mesenchymalen Markern (Vimentin und CD140b) auf während die Expression von Zytokeratinen und endothelialen Markern sich zurück bildete.

Die Ergebnisse dieser Arbeit zeigen, dass die HAE-Zellen ein Pluripotenzpotenzial besitzen und könnten in Zukunft in der regenerativen Medizin verwendet werden.

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10 ABBREVIATIONS

HAE: human amniotic epithelial cells

HAM: human amniotic mesenchymal cells

HCG: human chorionic gonadotrophin

hCGRH: human chorionic gonadotrophin releasing hormone

HPL: human placental lactogen

DHEA: dehydroepiandrosterone

DHEAS: dehydroepiandrosterone sulfate

HLA: human leukocyte antigen

CS: cesarean section

MECGM: mammary epithelial cell growth medium

FCS: fetal calf serum

EBM-2: endothelial Basal Medium

cDNA: complementary DNA

PVDF: polyvinyllidendifluoride

HRP: horseradishperoxidase

FITC: fluorescein isothiocyanat

PE: R-phycoerythrin

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