Establishment of a bovine placental trophoblast cell line and a 3-dimensional spheroid culture model: biological effects of epidermal growth factor (EGF)

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1 GENERAL REMARK

This thesis is submitted as a cumulative thesis with the main issue of establishing a permanent bovine placental trophoblast cell line. Exemplarily, the \textit{in vitro} effects of the epidermal growth factor (EGF) on the proliferation of the developed trophoblast cell line were demonstrated. The second goal of the work was the development of a 3-dimensional culture model (spheroid) for the latter cells. Therefore the thesis consists of two parts; each part being covered by one original paper published in peer-reviewed journals. The first publication deals with two major topics: (1) the isolation and characterisation of the bovine placental trophoblast cell line and (2) the influence of EGF on the proliferation of bovine trophoblast cells \textit{in vitro}. The major topic of the second publication is the development of a 3-dimensional trophoblast spheroid model in order to induce the formation of trophoblast giant cells (TGC).
2 GENERAL INTRODUCTION

2.1 THE BOVINE PLACENTA

According to its shape the bovine placenta has been classified as cotyledonary type (Placenta cotyledonaria sive multiplex), in which placentomes are formed by maternal caruncles and fetal cotyledons (Strahl 1906). The fetal and maternal tissue is in close contact to each other by interdigitation of fetal villi and maternal crypts (Mossman 1987; Strahl 1906). Due to its structure, the bovine placenta was classified as syndesmochorial (Grosser 1927). However following studies showed that the uterine epithelium persisted and therefore the bovine placenta was reclassified as epitheliochorial (Bjorkman and Bloom 1957). Wooding suggested that the term “epitheliochorial” would not be an entirely correct classification for this placenta as the uterine epithelium is indeed present at all stages of gestation but it is not left unaltered. Those alterations of the uterine epithelium are caused by particular trophoblast cells, the so called trophoblast giant cells (TGC) (Wooding 1992). In general the bovine trophoblast is composed of two populations of trophoblast cells, polarized uninucleated trophoblast cells (UTC) and trophoblast giant cells (TGC). TGC mostly have two nuclei, arise from UTC by acytokinetic mitosis (Klisch et al. 1999a) and make up 15-20% of all trophoblast cells during ruminant gestation (Wooding et al. 1997). They are non-polarized and migrate towards the maternal epithelium to fuse with single uterine epithelial cells thereby yielding feto-maternal hybrid cells which are true syncitia (Wathes and Wooding 1980; Wimsatt 1951). The hybrid cells are mostly trinuclear (Klisch et al. 1999a) and their main function is the transport of TGC specific hormones into the maternal compartment of the placenta (Wooding 1992; Wooding et al. 2005). In the end the feto-maternal hybrid cells degenerate (Wooding et al. 1997) and are most likely phagocytized by UTC (Klisch et al. 1999b). Considering on the one hand the cell fusion (“syn-“) and on the other hand that there are always two epithelial layers (“epitheliochorial“) Wooding has suggested that the bovine placenta should better be classified as synepitheliochorial
(Wooding 1992). Because the migration of TGC does not continue beyond the maternal basement membrane it is also referred to as “restricted trophoblast invasion/migration” (Pfarrer et al. 2003). TGC are not only unique because of their pattern of migration and DNA content, which can reach up to c=32n (Klisch et al. 1999b), but also due to the production of a variety of TGC specific glycoproteins and growth factors. One of those glycoproteins is bovine placental lactogen (bPL) which is expressed throughout gestation (Patel et al. 2004; Wooding and Beckers 1987) starting from day 30 of the implantation period (Yamada et al. 2002). In contrast to bPL, which is sole produced in TGC another group of trophoblast products, the pregnancy-associated glycoproteins (PAGs) are expressed in TGC as well as in UTC. However a group of PAGs remains TGC specific and is expressed from implantation to term (Green et al. 2000; Klisch et al. 2005; Wooding et al. 2005) while being the major target of lectin binding (Klisch and Leiser 2003). In addition several growth factors like vascular endothelial growth factor (VEGF) (Pfarrer et al. 2006b), platelet-activating factor (PAF) (Bucher et al. 2006) and fibroblast growth factor (FGF) (Pfarrer et al. 2006a) could be colocalized in TGC implicating that the TGC survival, migration and fusion with uterine epithelial cells is a very complex process. Taking in account all the proteins expressed in TGC it is justified to suppose that these cells are key regulators of various biological processes (angiogenesis, cell growth and differentiation and tissue remodelling) in the bovine placenta. TGC produced VEGF and FGF might have an angiogenic effect on endothelial cells in the maternal placental compartment since VEGF and FGF are known to promote the invasion (Korff et al. 2001) and differentiation and survival (Korff and Augustin 1998) of endothelial cells in vitro. It should also be mentioned that the growth factor systems listed above (VEGF, PAF, FGF) are also expressed in bovine placental cells other than TGC. An example for this is the epidermal growth factor (EGF) (Weise 2001) and components of the FGF system like FGF-1, -2 and -7 which are also found in caruncular cells during all stages of gestation (Pfarrer et al. 2006a).
2.2 BOVINE TROPHOBLAST CELLS \textit{IN VITRO}

In order to gain more knowledge about the physiology of bovine trophoblast cells primary putative trophoblast cells have been used in several previous studies (Bridger et al. 2007; Landim et al. 2007; Munson et al. 1988; Nakano et al. 2002a; Vanselow et al. 2008). However up to date only three permanent bovine trophoblast cell lines exist. Two of which have been derived from \textit{in vitro} fertilised blastocysts (Shimada et al. 2001; Talbot et al. 2000) while only one has been isolated from bovine placental tissue (Hambruch et al. 2010). A well known fact arising from those previous studies is that bovine trophoblast cells dedifferentiate when cultured \textit{in vitro} (Hambruch et al. 2010; Nakano et al. 2001; Vanselow et al. 2008). When primary TGC attach to culture dishes they adopt a flattened shape, cease to express bPL but gain cytokeratin (CK) expression. This inverse relationship between bPL and CK is a characteristic of TGC dedifferentiation and has been described before (Hambruch et al. 2010; Nakano et al. 2001). UTC also undergo dedifferentiation as they stop becoming TGC. Yet for a certain period of time TGC can still be detected in culture. In the bovine blastocystal trophoblast cell line BT-1 bPL positive TGC (on protein level) have been traced up to passage 32 (P32) (Shimada et al. 2001), while in the bovine placental trophoblast cells line F3 TGC (bPL positive, CK negative) were still observed until P2 (Hambruch et al. 2010). A reinduction of the differentiation into TGC in culture is not easy to achieve once this trait is lost. So far only one group was able to recreate the feature by culturing blastocystal BT-1 cells on collagen-I gels. After 13-17 days TGC were observed which had an increased DNA content and were bPL positive, but CK negative (Nakano et al. 2002a; Nakano et al. 2002b). Even though those studies are impressive, as they show that TGC can be induced \textit{in vitro} under certain conditions they also do have some drawbacks. The overall percentage of TGC was only 3%, which is by far to low compared to the \textit{in vivo} percentages of 15-20% (Wooding et al. 1997). Furthermore the \textit{in vivo} state of TGC differentiation, in which TGC bind the lectin DBA (Dolichos Biflorus Lectin), was never reached by BT-1 culture on collagen-I gels (Nakano et al. 2002a). \textit{In vivo} DBA specifically binds to TGC from early gestation (day 18-29) (Lehmann et al. 1992), during midpregnancy (Klisch et al. 2005; Klisch and Leiser 2003) and up to term. Yet the number of DBA
positive TGC in term placentas is considerably reduced and occasionally DBA positive TGC are completely absent (Klisch et al. 2006). In summary a major problem emerging from the \textit{in vitro} culture of bovine trophoblast cells is the complete loss of an entire cell population, namely the TGC.

\section*{2.3 EPIDERMAL GROWTH FACTOR (EGF) SIGNALLING}

\subsection*{2.3.1 EGF and the nobel prize}

EGF was discovered and isolated from the submaxillary gland of mice during a study on the nerve growth factor (NGF). In the particular study purified extracts of the murine submaxillary gland, when injected in new-born mice led to precocious opening of the eyelids and incisor eruption. In regard to the elicited biological effects EGF was firstly called the “tooth-lid factor” and was found to be a low molecular weight (~15000 kDA), heat stable protein (Cohen 1962). In directly following studies the particular growth factor was termed for the first time “Epidermal Growth Factor” (Cohen 1965) and in 1986 Stanley Cohen and Rita Levi-Montalcini were awarded the nobel prize in physiology or medicine for the discovery of EGF (Cohen 1986) and NGF (Levi-Montalcini 1987). Today it is well established that EGF is expressed in most organs of the body of mammals and body fluids (Rall et al. 1985) and in human (Moran et al. 1983) and murine milk (Grueters et al. 1985). As far as placental tissue is concerned the expression of EGF has been analyzed in the human (Maruo et al. 1995) and in the equine placenta (Lennard et al. 1998). Yet in the bovine placenta only the unphosphorylated (Weise 2001) and phosphorylated EGFR (Dilly et al. 2010) have been detected, but not EGF itself.

\subsection*{2.3.2 EGF family of growth factors and EGF receptors}

The EGF family of growth factors comprises of 13 structurally related proteins, like EGF, transforming growth factor-alpha (TNF-A), amphireticulin (AR), heparin-binding EGF (HB-EGF), betacellulin (BTC) and others (Hieda et al. 2008; Massague and Pandiella 1993; Xian 2007). All members of the family possess one or more EGF motif consisting of 6 conserved cysteins over a sequence of 35-40 amino acids (Dunbar and Goddard 2000; Massague and Pandiella 1993; Savage et al. 1972).
EGF was one of the first growth factors to be discovered and its receptor (EGFR) was also the very first receptor tyrosine kinase (RTK) to be detected (Carpenter et al. 1978). Today, four receptors of the ErbB family (ErbB1-4 or HER1-4) are known through which growth factors of the EGF family can transmit their signals (Citri and Yarden 2006 277). They all possess an extracellular ligand binding domain, a single transmembrane domain and a cytoplasmic domain, which contains a tyrosine kinase activity (Schlessinger 2002; Ullrich et al. 1984). Upon ligand binding, the receptors dimerize and the tyrosine kinase becomes activated, which in turn triggers the auto- or crossphosphorylation of tyrosine residues in the cytoplasmic receptor domain. This creates docking sites for signalling proteins (adaptor proteins and enzymes) which initiate signal transduction (Schlessinger 2002; Yarden and Sliwkowski 2001). Numerous signalling molecules that bind to the docking sites contain conserved protein domains like SH2 and SH3 (Src homology modules), pleckstrin homology domains (PH domains) or phosphotyrosine binding sites (PTB). One of the functions of those domains is to activate particular signalling pathways by causing changes in the subcellular localization of signalling components (Cohen et al. 1995; Pawson 1995).

2.3.3 Signalling pathways and biological effects of EGF

EGF activates very different signalling pathways in a cell like the phospholipase C gamma (PLC-gamma) pathway, the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, or the Ras-MAPK pathway. By doing so, EGF can influence apoptosis, migration, growth, adhesion and differentiation of cells (Moghal and Sternberg 1999; Yarden and Sliwkowski 2001). In regard to trophoblast cells, EGF has been shown to stimulate the invasion (Bass et al. 1994; Korff et al. 2004) or motility (LaMarca et al. 2008) of human cytotrophoblasts or the motility of bovine trophoblast cells (Dilly et al. 2010). Furthermore, EGF can act as a survival factor for human trophoblast cells (Perkins et al. 2002; Smith et al. 2002) and promote the secretion of matrix metalloproteinase-9 (MMP-9) and tissue inhibitor of metalloproteinase-1 (TIMP-1) in the latter cells (Qiu et al. 2004).
2.4 THREE-DIMENSIONAL (3-D) SPHEROID MODELS

2.4.1 Cell types used for spheroid formation

Spheroids are 3-dimensional cell culture models which can be formed with one or more than one cell population (co-culture spheroids). For spheroids containing only one cell population different cell types have been used like endothelial cells (EC) (Korff and Augustin 1998), stem cells (Kurosawa 2007), tumor cells (Kelm et al. 2003; Na et al. 2009), fibroblasts (Vaheri et al. 2009), granulosa cells (Hummitzsch et al. 2009) and granulosa lutein cells (Becker et al. 2007). In regard to the generation of trophoblast spheroids initially chorioncarcinoma cells have been used (Grümmer et al. 1990). In following studies other groups have also formed spheroids consisting of human cytотrophoblasts (Korff et al. 2004) and equine chorionic girdle cells (de Mestre et al. 2008). Additionally more elaborated spheroid models were developed which are made up of more than one cell population. This has been done for EC and smooth muscle cells (SMC) (Korff et al. 2001), EC and pericytes (Wirz et al. 2008), EC and fibroblasts (Wenger et al. 2005), hepatocytes and fibroblasts (Takezawa et al. 1992), tumor cells and fibroblasts (Sasaki et al. 1984; Seidl et al. 2002) and fibroblasts and uterine epithelial cells (Yamauchi et al. 2003).

2.4.2 Key benefits of 3-D spheroids compared to 2-D cell monolayers

There are several reasons for the culture of cells in 3-dimensional spheroids. One reason is that cells in spheroids can display more in vivo properties than 2-dimensional cell monolayers. This has been demonstrated for EC and tumor cells. CD34 is expressed by most EC in vivo (Baumhueter et al. 1994) but not in 2-dimensional monolayers in vitro. An induction of CD34 expression by VEGF is only possible when cells are cultured in spheroids (Korff and Augustin 1998). EMT-6 murine mammary tumor cells are highly resistant to alkylating agents in vivo but not in monolayer culture in vitro. Yet when EMT-6 sublines are cultured in spheroids they regain their resistance (Kobayashi et al. 1993). Another reason for spheroid culture is that spheroidal cells have different access to nutrients or oxygen due to culture conditions. If tumor cells are grown in a monolayer all cells are exposed to nutrients
and oxygen to the same degree. However *in vivo* in a tumor gradients are formed. Multicellular tumor spheroids (MCTS) do possess oxygen, lactic acid and glucose gradients (amongst other gradients) and therefore resemble avascular regions of tumors much better than cell monolayers (Hirschhaeuser et al. 2010).

### 2.4.3 Methods for spheroid formation

For the generation of spheroids several methods exist. One basic principle of spheroid generation is that cells are cultured under “anti-adhesive conditions”. Those “anti-adhesive conditions” prevent the binding of cells to the culture dish while at the same time promoting self-aggregation to 3-dimensional spheroids. “Anti-adhesive conditions” can be artificially created by e.g. methocel addition and upside down culture of cells (Kelm et al. 2003; Korff and Augustin 1998; Korff et al. 2004), coating of cell culture dishes with poly-HHEMA (Ivascu and Kubbies 2006; Landry et al. 1985) or by seeding of cells in rotatory bioreactors (Ingram et al. 1997). However other studies have shown that “anti-adhesive conditions” are not needed for spheroid formation as serum deprivation can lead to the aggregation of equine chorionic girdle cell into spheroids (de Mestre et al. 2008). If the formation of many spheroids of uniform size is required, the hanging drop technique should be taken into consideration. Using this technique cells are seeded in drops (20-30µl) and cultured upside down in culture dishes. Furthermore, methocel can be used to improve spheroid formation (Kelm et al. 2003; Korff and Augustin 1998; Korff et al. 2004). Some researchers also added matrigel (also referred to as reconstituted basale membrane extract) to the specimens, as certain cell lines do only form spheroids when matrigel is present (Ivascu and Kubbies 2006; Lang et al. 2001). A more complex technique has been utilized to generate bovine uterine epithelial cell/fibroblast co-culture spheroids. Firstly fibroblasts were grown to confluence in media containing vitamin C. Afterwards epithelial cells were seeded on the fibroblast monolayers. After attachment the entire cell sheet (uterine epithelial cells and fibroblasts) was transferred to agarose coated dishes. Within 14 days of culture co-culture spheroids were formed which possessed an outer layer of uterine epithelial cells underlined by fibroblasts (Yamauchi et al. 2003). Due to the variety of existing
spheroid generation techniques, a method of choice has to be determined depending on e.g. research aim and production efficiency (Kurosawa 2007; Lin and Chang 2008).

2.4.4 Light microscopical spheroid properties

Spheroids have common light microscopical properties as they are delimited to the outside (Kelm et al. 2003; Vaheri et al. 2009; Yamauchi et al. 2003) and the aggregating suspension cells become indistinguishable during ongoing spheroid formation (Korff and Augustin 1998). In addition cell spheroids are mechanically stable as they do survive pipetting after formation (de Mestre et al. 2008; Korff et al. 2004; Na et al. 2009). In contrast loose aggregates of suspension cells are not marked off to the outside (Ivascu and Kubbies 2006) and fall apart when exposed to mechanical stress (Haeger et al. 2010).

2.4.5 Examples for the application of cell spheroids

Once a cell spheroid is formed it can be used for functional studies. Such functional studies are e.g. in vitro invasion assays (human trophoblast cells) (Hohn and Denker 2002; Korff et al. 2004), or cell differentiation studies on EC (Korff and Augustin 1998), human trophoblast cells (Hohn et al. 2000) or equine chorionic girdle cells (de Mestre et al. 2008). EC/SMC co-culture spheroids have been formed to elucidate the paracrine effects of SMC on VEGF-stimulated EC. Additionally multicellular tumor spheroids of melanoma cells have been cultured with human cytotoxic T lymphocytes (CTLs) to analyze possible inhibitory effects of tumor cell-derived lactic acid on human T cells (Fischer et al. 2007). In more elaborated experimental studies cell spheroids were implanted into living animals in order to study the differentiation of equine trophoblast cells in horses (de Mestre et al. 2008), the formation of new blood vessels (angiogenesis) in mice (Laib et al. 2009) or the metastatic behaviour of tumor cells in mice under in vivo conditions (Na et al. 2009).
EGF stimulates proliferation in the bovine placental trophoblast cell line F3 via Ras and MAPK

Abstract
In the bovine placenta, multinucleate trophoblast giant cells (TGC), evolving from uninucleate trophoblast cells, are crucial for feto-maternal interaction as they show endocrine activity and the ability to migrate and fuse with caruncular epithelial cells. In contrast to caruncular epithelial cells, the isolation and culture of bovine trophoblast cells is complicated because they cease to express their specific products, like placental lactogen (PL), during prolonged culture. In the present study, we aimed to establish a bovine cotyledonary trophoblast cell line targeting our long term goal to develop an *in vitro* model for the bovine placenta. Therefore, the functional activity of important signalling pathways was tested. Primary trophoblast cells were isolated from a bovine cotyledon of a male fetus and successfully subcultured and cryopreserved. The obtained cell line, termed F3, showed epithelial morphology and characteristic binuclear giant cells in small numbers through all passages. The trophoblastic origin of F3 cells was verified by amplification of a Y-chromosome specific DNA-sequence and the presence of PL mRNA. Immunofluorescence demonstrated that F3 cells were continuously positive for zonula occludens-2 (ZO-2), cytokeratin and vimentin, whereas they expressed the TGC specific marker PL only in the first two passages. F3 cell growth was accelerated in medium supplied with epidermal growth factor (EGF). EGF-stimulated proliferation was mediated through activation of Ras and the phosphorylation of mitogen-activated protein kinase (MAPK) 42 and 44. In conclusion, the F3 cell line shows several in vivo characteristics of bovine cotyledonary trophoblast cells. The response to EGF stimulation indicates that EGF plays a role during bovine placentation, and illustrated that F3 cells may provide a valuable tool for further mechanistic studies elucidating the feto-maternal interplay.

Formation of bovine placental trophoblast spheroids

Abstract

Introduction: In this study, we aimed to form spheroids with the bovine placental trophoblast cell line F3. Spheroids are 3-dimensional culture models which can be used to conduct versatile in vitro and in vivo experiments. Materials and Methods: The spheroids were generated using the hanging drop technique, 25% methocel and matrigel. The F3 spheroids were characterized morphologically by light microscopy and transmission (TEM) and scanning electron microscopy (SEM) and immunohistochemistry (ezrin, vimentin, cytokeratin, placental lactogen). The fluorescent dyes calcein and ethidium homodimer were used to determine the viability of the spheroidal F3 cells by immunofluorescence microscopy. Results: The cell line F3 only formed spheroids by the hanging drop technique when matrigel was added. The trophoblast spheroids were delimited and fully covered by extracellular matrix (light microscopy/TEM/SEM). Cells contributing to spheroids could not be discriminated from each other (light microscopy). The outer spheroidal layer consisted of cells which possessed an apical pole with microvilli that were directed to the outside (light microscopy/TEM). All of the spheroidal F3 cells expressed ezrin, vimentin and cytokeratin, but not placental lactogen. The spheroid core contained degenerating cells whilst the F3 cells of the outer rim were viable (TEM/immunofluorescence microscopy). Discussion: We have established a 3-dimensional spheroid model for the bovine placental trophoblast cell line F3. The developed culture model might prove valuable for future in vitro studies on the differentiation of bovine trophoblast cells.

5 GENERAL DISCUSSION AND CONCLUSION

5.1 Cell isolation

The most important experimental result of this work is the establishment of a permanent bovine placental trophoblast cell line, termed F3 (Hambruch et al. 2010). Such a cell line has never been generated before. Several other studies have dealt with primary bovine placental trophoblast cells, which were more (Bridger et al. 2007; Vanselow et al. 2008; Zeiler et al. 2007) or less characterized (Landim et al. 2007). Sometimes putative bovine placental epithelial cells turned out to be of endothelial origin (Feng et al. 2000a; Feng et al. 2000b). In addition to the bovine placental trophoblast cell line F3, only two other permanent bovine trophoblast cell lines, BT-1 (Shimada et al. 2001) and CT-1 (Talbot et al. 2000), have been established from in vitro fertilized bovine blastocysts before. It is important to mention that we were only capable of producing one placental trophoblast cell line from about fifty isolations.

Very different protocols were used in these isolations in regard to the preparation of placental tissue, tissues digest (e.g. enzymes, incubation times and concentration), density gradient centrifugation and the coating of tissue culture dishes (e.g. different collagens, laminin and fibronectin). In the beginning we used collagenase for enzymatic digestion because many previous studies had used this enzyme (Bridger et al. 2007; Landim et al. 2007; Vanselow et al. 2008; Zeiler et al. 2007). But after the first unsuccessful isolations (no cell attachment) we compared the effects of collagenase and trypsin treatment on the viability of the obtained placental cells. By using FITC-annexin V (Vermes et al. 1995) and propidium iodide in flow cytometry it was clearly demonstrated that the viability of the digested cells differed greatly depending on the used enzyme. While trypsin treatment yielded about 60% of vital cells (negative for annexin V and propidium iodide) collagenase treatment only led to 10% of vital cells. Based on these results we changed the digesting enzyme from collagenase to trypsin which might have been one of the factors leading to the establishment of the bovine placental trophoblast cell line F3.
5.2 Characterization of bovine placental trophoblast cell line F3

Five overall criteria were used to characterize the established bovine placental trophoblast cell line F3: In detail those criteria were: (1) Presence of a y-chromosome, (2) Expression of CK, (3) Presence of TGC in early F3 passages, (4) exclusion of Dil-Ac-LDL uptake and (5) cell morphology.

(1) When analysing F3 we had the great advantage that the cell line was derived from a male fetus and could therefore be characterized by the amplification of a y-chromosome specific sequence by PCR. The detection of a y-chromosome has been used before to prove that putative bovine primary placental trophoblast cells were indeed trophoblast cells. In previous studies it was also shown by fluorescence in situ hybridization (FISH) that a great percentage of cells (up to ~60%) isolated from bovine cotyledons can indeed be maternal caruncular epithelial cells (Bridger et al. 2007).

(2) CK expression was used in previous studies to demonstrate that the isolated primary placental cells were actually of epithelial origin (Bridger et al. 2007; Vanselow et al. 2008; Zeiler et al. 2007). In the established cell line F3 all cells continuously express CK, proving that the cell line is an epithelial one. The fact that F3 does also possess vimentin might be irritating, as vimentin is only expressed by cells of mesenchymal origin. Yet others have shown that epithelial cells like bovine uterine epithelial cells (Bridger et al. 2007; Yamauchi et al. 2003) and primary trophoblast cells (Bridger et al. 2007) also gain vimentin expression, when cultured in vitro.

(3) Even though all F3 cells in later passages (P3-P52) do express CK we were able to observe some CK negative cells in the very early F3 passages (P1-P2). Considering the fact that the CK negative cells also express bPL it is justified to call them TGC. In the placenta TGC also express bPL but no CK (Haeger et al. 2010). In the bovine blastocystal trophoblast cell line BT-1 the presence of bPL positive TGC (on protein level) can be observed up to P32 (Shimada et al. 2001). Yet in later F3 cells passages (after P2) no more TGC can be detected as all F3 cells express CK
but no bPL. The inverse relationship between CK and bPL was also shown for BT-1 cells (Nakano et al. 2002a; Nakano et al. 2002b) and reflects the dedifferentiation of bovine trophoblast cells in vitro (Hambruch et al. 2010; Nakano et al. 2001). Furthermore we have verified the expression of bPL on mRNA level. (4) One more criterion for the characterisation of the cell line F3 was the uptake of Dil-Ac-LDL which is mainly taken up by endothelial cells (Voyta et al. 1984). Other studies have shown that it is important to exclude that the putative bovine placental epithelial cells are endothelial cells (Feng et al. 2000b). The circumstance that F3 cells do not take up Dil-Ac-LDL verifies that F3 is not an endothelial cell line. Dil-Ac-LDL has been used before to confirm the epithelial origin of bovine placental epithelial cells (Bridger et al. 2007). (5) We have also used the criterion “cell morphology” for the characterisation of the cell line F3. These cells looked very different compared to caruncular epithelial cells (BCEC) when cultured on uncoated tissue flasks. BCEC cells formed very tight curl-like colonies, which were delimited. The F3 trophoblast cells showed a cobblestone-like structure with less confined colonies. In contrast to BCEC no curls within the colonies were observed. Throughout culture of either BCEC or F3 the morphology did not change (Hambruch et al. 2010).

5.3 Effect of EGF on F3 proliferation and signalling

We hypothesized for two reasons that EGF could stimulate the proliferation of the bovine placental trophoblast cell line F3. Firstly the mitogenic effect of this growth factor is well known and secondly the positive effect of EGF on cultures of primary trophoblast cells in bovine (Munson et al. 1988) and in humans (Li and Zhuang 1997) has also been demonstrated in other studies. We found that EGF (50mg/ml) significantly stimulated the proliferation of F3 cells up to 52% compared to unstimulated probes. Interestingly the proliferation of BCEC cells was not stimulated by EGF. Our hypothesis that EGF transmits its mitogenic effect on F3 cells by the Ras-MAPK pathway was confirmed as we were able to inhibit the EGF induced proliferation of F3 cell by pre-treatment with a MEK1/2 inhibitor. Analyzing the signals responsible for F3 proliferation we observed an activation of Ras (within 2.5min) and
MAPK42/44 (maximum activation after 5 min) only upon EGF stimulation of F3 cells. Again the BCEC cells did not show Ras or MAPK42/MAPK44 activation. The presence of the EGFR in both cell lines was confirmed on mRNA and protein level (Dilly et al. 2010; Hambruch et al. 2010). We have shown that EGF stimulates the proliferation of bovine trophoblast cells \textit{in vitro} by the Ras-MAPK pathway. The fact that BCEC cells did not react to EGF stimulation needs further evaluation since EGF might utilize other signalling pathways and/or biological responses in the latter cells. Yet another possibility is that activation in BCEC cells, since they already have a high basal activity of MAPK42/44, can not be detected by western blot analysis alone.

As the F3 cells are reacting to EGF stimulation they might also be useful for the analysis of the effect of other bovine placental growth factors like VEGF (Pfarrer et al. 2006b), PAF (Bucher et al. 2006) and FGF (Pfarrer et al. 2006a).

5.4 Development of a 3-D spheroid model

In order to reinduce TGC formation in F3 cells we aimed to develop a 3-dimensional spheroid model. As a spheroid generation technique we first tried the hanging drop technique together with the addition of 20% methocoel. However this approach did only lead to loose F3 cell aggregates. This was unexpected as the addition of methocoel to suspension cells is absolutely sufficient for spheroid formation in very different cell types (Kelm et al. 2003; Korff and Augustin 1998; Korff et al. 2004). In the following, only the addition of matrigel (1%) to the specimens led to the formation of F3 spheroids in hanging drop culture. This is in agreement with the finding of another study which also has shown that matrigel sometimes can be essential for spheroid formation (Ivascu and Kubbies 2006). In regard to light microscopical appearance the F3 spheroids displayed common spheroidal characteristics like delimitation and the fact that the cells are indistinguishable from each other when participating in a spheroid. Additionally the generated F3 spheroids survived harvest thereby demonstrating stability against mechanical stress (e.g. pipetting, centrifugation). The light microscopical appearance and the mechanical rigidity are
shared by all cell spheroids (de Mestre et al. 2008; Korff and Augustin 1998; Korff et al. 2004; Na et al. 2009). But morphologically the F3 spheroid looked very different when compared to spheroids generated without matrigel. Each trophoblast spheroid was surrounded by a layer of extracellular matrix (ECM) and scanning electron microscopy (SEM) demonstrated that the spheroids itself could not be seen from the outside. Considering that bovine trophoblast cells can produce ECM (laminin and fibronectin) \textit{in vitro} (Zeiler et al. 2007) the ECM could theoretically be produced by the F3 cells themselves. However we think that the source of the ECM is matrigel for two reasons. At first SEM analysis of F3 monolayer cells did not reveal such an amount of ECM present on F3 cells (Hambruch et al. 2010). Secondly the fibroblast spheroids only possessed the same ECM when matrigel was added to the specimens. Ultrastructurally we were able to demonstrate that F3 spheroids had two compartments: an inner core of degenerating cells and an outer layer of polarized trophoblast cells which were located on a basement membrane like structure and carried microvilli at their apical poles. By the usage of Calcein AM and ethidium homodimer-1 (EthD-1) we have additionally verified that the outermost layer of F3 cells was vital contrary to nearly all cells in the core region.

5.5 Experiments concerning the reinduction of TGC

In our study we have made two attempts to reinduce TGC formation in F3 cells. The first approach was the culture of F3 cells on collagen-I gels. For the bovine blastocyst derived trophoblast BT-1 cells it was shown that culture on such collagen-I gels for 13-17 days could reinduce CK negative, bPL positive, polyploid TGC (Nakano et al. 2002a; Nakano et al. 2002b). During collagen-I gel culture BT-1 cells undergo a morphological change as the colonies appear to be more compact. The newly formed TGC were shed from the BT-1 monolayer cells and formed clusters above the BT-1 monolayer (Nakano et al. 2002b). Unfortunately the culture of F3 cells on collagen-I gels did not lead to TGC formation at all. Yet F3 cells did undergo a morphological change as they also formed much more compact and confined colonies (Hambruch et al. 2010). The second attempt we made towards TGC reinduction was the
formation of 3-dimensional trophoblast spheroids. This seemed very promising as spheroids have been used in previous studies dealing with trophoblast cell (de Mestre et al. 2008; Hohn et al. 2000) or endothelial cell (Korff and Augustin 1998) differentiation. In addition the fact that the generated F3 spheroids were surrounded by matrigel might also be beneficial since it is well established that matrigel itself promotes the differentiation of many cell types (Kleinman and Martin 2005). To analyze whether TGC differentiation of spheroidal F3 cells occurred, serial paraffin sections through one particular spheroid were stained for CK or bPL as TGC would only express bPL but no CK. Unfortunately none of the spheroidal cells showed either a downregulation of CK or an expression of bPL (Haeger et al. 2010). So after all both of the approaches to induce TGC in the cell line F3 were unsuccessful. Several reasons might be responsible for the lack of TGC formation in the spheroidal F3 cells. At first culture time of F3 cells in spheroids might have been too short. Secondly one has to take into account that sometimes the culture of cells in spheroids can alter the responsiveness of the cells to growth factors as it has been demonstrated for the VEGF responsiveness of spheroidal endothelial cells (Korff and Augustin 1998). The altered growth factor responsiveness is particularly interesting because several growth factor systems have been detected in the bovine placenta like VEGF (Pfarrer et al. 2006b), PAF (Bucher et al. 2006) and FGF (Pfarrer et al. 2006a). So another reason might be that the specimens lacked the growth factors important for TGC induction. A third reason for the absence of TGC formation could have been that the contact with, or a signal from the opposing caruncular epithelial cells might trigger this process. It is known that differentiation of epithelial cells via integrin signalling is a common feature (Gilcrease 2007). However in the end we could not explain why we did not observe TGC formation in spheroid cultures of the F3 cell line. It is possible that certain mutations in genes important for differentiation might have occurred which do inhibit TGC induction in F3 cells. The fact that the placental trophoblast cell line F3 does not form TGC while the blastocystal trophoblast cell line BT-1 does, also needs to be addressed. Firstly, this fact might reflect differences in the trophoblast programming between the early blastocystal trophoblast with no firm contact established with the caruncular epithelium and the
(differentiated?) placentomal trophoblast having established an intimate contact through interdigitating apical microvilli. Yet this assumption is highly speculative and without proof. Secondly a “defect” in the F3 cell line might be the reason for this difference (see above). Finally it has to be mentioned again that the induction of BT-1 TGC itself is impressive but the numbers of TGC are by far too low (Nakano et al. 2002b), when compared to in vivo (Wooding et al. 1997). Therefore the reinduction of TGC in the F3 cell line is subject of further, presently ongoing studies.

5.6 Concluding remark

We have established for the first time a permanent bovine placentomal trophoblast cell line. Using this cell line we have shown that EGF is a potent stimulator of the proliferation of bovine trophoblast cells in vitro and that the particular mitogenic effect is elicited by the RAS-MAPK pathway. Intriguingly our findings regarding the EGF stimulation of BCEC cells indicate that EGF regulates different functions in caruncular epithelial cells than in trophoblast cells. Furthermore we have developed the first 3-dimensional spheroid culture model for bovine trophoblast cells which might prove useful in future in vivo and in vitro studies on bovine trophoblast cells.
6 SUMMARY

Jan-Dirk Häger

Establishment of a bovine placental trophoblast cell line and a 3-dimensional spheroid culture model: biological effects of epidermal growth factor (EGF)

In this cumulative thesis the isolation of the bovine placental trophoblast cell line (F3) and the development of 3-dimensional spheroid model for the latter is reported. It is also shown that EGF stimulates proliferation in F3 cells via the Ras-MAPK pathway in trophoblast cells.

The bovine trophoblast consists of 80% uninucleate trophoblast cells (UTC) and 20% invasive trophoblast giant cells (TGC). TGC are able to fuse with single caruncular epithelial cells and synthesize unique hormones like bovine placental lactogen (bPL). In vivo they do not express cytokeratin (CK) in contrast to UTC which show cytokeratin expression. In addition several growth factor systems (VEGF, PAF, FGF) are expressed in the bovine placenta and have been colocalized in TGC. Despite several previous studies dealing with the culture of primary bovine placental trophoblast cells a permanent bovine placental trophoblast cells has never been established before. This study describes for the first time the isolation and characterization of such a cell line, termed F3. F3 was characterized by: (1) Expression of a y-chromosomal sequence, (2) CK expression, (3) Occurrence of CK negative/bPL positive TGC in early F3 passages, (4) exclusion of Dil-Ac-LDL and (5) different cell morphology than caruncular epithelial cells (BCEC). After characterization the influence of EGF (50ng/ml) on the proliferation of the bovine trophoblast cells was analyzed by MTT-Assay. To elucidate the EGF induced signalling pathways we analyzed Ras (Ras Pull-Down-Assay, western blot) and MAPK42/44 activation (western blot). We found that EGF strongly stimulated the proliferation of the trophoblast cells via the Ras-MAPK pathway while BCEC cells did not show such a reaction. A striking criterion of cell dedifferentiation in bovine
trophoblast cells *in vitro* is the complete loss of the TGC cell population. In detail the dedifferentiation is reflected by the fact that all cells express CK but no bPL. This is in contrast to the placenta where TGC are CK negative but bPL positive. In order to reinduce TGC differentiation of F3 cells different approaches were used, namely the development of a 3-dimensional spheroid model for F3 cells (1) and collagen-I gel culture of F3 cells (2). The F3 trophoblast spheroids were generated by the hanging drop technique in presence of methocoel (20%) and matrigel (1%). The addition of matrigel was shown to be the crucial factor for F3 spheroids formation within 3 of culture. The spheroids were characterized morphologically by light microscopy (LM), transmission microscopy (TEM), scanning electron microscopy (SEM) and immunohistochemistry (ezrin, vimentin, cytokeratin, placental lactogen). The fluorescent dyes Calcein AM and ethidium homodimer-1 (EthD-1) were used to determine the viability of the spheroidal F3 cells by immunofluorescence (IF). Every F3 spheroid was surrounded by ECM which most likely was matrigel itself (LM, SEM). The F3 spheroids contained an outer vital rim of trophoblast cells and an inner spheroidal core of degenerating cells (IF, TEM). The outer F3 cells were polarized and carried microvilli at their apical poles (TEM). As far as the induction of TGC in F3 cells is concerned neither the formation of trophoblast spheroids (1) nor the collagen gel culture (2) have led to TGC differentiation in F3 cells. This failure might have several physiological (e.g. lack of growth factors in culture medium) or cell line based reasons.

In conclusion, this thesis demonstrates for the first time the establishment of a permanent bovine placental trophoblast cell line. Furthermore we have shown that trophoblast and BCEC cells differ in their biological response to EGF which indicates different roles for EGF in the bovine placenta *in vivo*. Additionally a 3-dimensional, matrigel-based trophoblast spheroid model was developed which might turn out useful for future studies on the differentiation and invasion of bovine trophoblast cells *in vitro*. 
7 ZUSAMMENFASSUNG (GERMAN)

Jan-Dirk Häger
Etablierung einer bovinen plazentaren Trophoblastzelllinie und eines 3-dimensionalen Sphäroidkulturmodells: biologische Effekte des Epidermalen Wachstumsfaktors (EGF)

Die vorgelegte kumulative Arbeit beschreibt die Etablierung einer bovinen plazentaren Trophoblastzelllinie (F3) für die zusätzlich ein 3-dimensional Sphäroidkulturmodell entwickelt wurde. Es wird außerdem gezeigt, dass EGF die Proliferation der Trophoblastzelllinie über eine Aktivierung des Ras-MAPK Signalweges stimuliert.

Western Blot) oder MAPK42/44 (Western Blot) Aktivierung führt. Es konnte gezeigt werden, dass EGF seine proliferativen Effekte in der Trophoblastzelllinie über die Aktivierung des Ras-MAPK Signalweges vermittelt. Im Gegensatz dazu zeigten BCEC Zellen keine Aktivierung des Signalweges oder vermehrte Proliferation nach EGF Gabe.

Ein Hauptmerkmal der Dedifferenzierung boviner Trophoblastzellen in Kultur ist, dass die komplette Zellpopulation der TGC verloren geht. Der TGC Verlust wird dadurch gekennzeichnet, dass alle Zellen ZK besitzen, jedoch kein bPL. Im Gegensatz dazu exprimieren TGC in der Plazenta bPL, aber kein ZK. Um die Bildung von TGC aus F3 Zellen zu reinduzieren wurden zwei Ansätze verfolgt: (1) die Bildung von 3-dimensionalen F3 Trophoblastsphäroiden und (2) die Kultur von F3 Zellen auf Kollagen-I Gelen. Die F3 Sphäroiden wurden unter Zugabe von Methycellulose (20%) und Matrigel (1%) mittels Hanging drop Technik gebildet. Dabei erwies sich die Zugabe von Matrigel als ausschlaggebend für die Entstehung der Sphäroiden nach drei Tagen in Kultur. Die F3 Sphäroiden wurden morphologisch mittels Lichtmikroskopie (LM), Transmissions- (TEM) und Rasterelektronenmikroskopie (SEM) sowie Immunhistochemie (Ezrin, Vimentin, CK, bPL) untersucht. Fluoreszierendes Calcein AM und Ethidium homodimer-1 (EthD-1) wurden benutzt, um lebende und tote Zellen in F3 Sphäroiden per Immunfluoreszenzmikroskopie (IF) zu detektieren. Es zeigte sich, dass jedes F3 Sphäroid von extrazellulärer Matrix (ECM) umgeben war, welche wahrscheinlich aus Matrigel bestand (LM, SEM). Die F3 Sphäroiden wiesen eine äußere Zellschicht aus lebenden Trophoblastzellen und einen inneren Kern aus sich in Degeneration befindlichen Zellen auf (IF, TEM). Die äußeren, sphäroidalen F3 Trophoblastzellen waren polarisiert und zeigten Mikrovilli an dem apikalen Zellpol (TEM). Was die in der Studie unternommenen Versuche zur Bildung von TGC angeht (Sphäroidkultur, Kollagen-I Gele), war leider kein Erfolg zu verzeichnen. Dieser Ausgang kann mehrere „physiologische“ Gründe (z.B. Fehlen wichtiger Wachstumsfaktoren im Kulturmedium) haben, oder aber auch Ursachen, die in der Zelllinie selbst begründet sind.
Schlussfolgernd kann gesagt werden, dass erstmalig eine permanente bovine plazentare Trophoblastzelllinie etabliert wurde. Weiterhin wurde gezeigt, dass bovine Trophoblastzellen und Karunkelepithelzellen (BCEC) unterschiedlich auf eine EGF-Stimulation reagieren, was darauf hindeuten könnte, dass dem EGF in der Plazenta unterschiedliche Aufgaben in den beiden Zellpopulationen zukommen. Zusätzlich wurde ein 3-dimensionales Trophoblast Kulturmodell (Sphäroid) entwickelt, welches sich in folgenden in vitro Studien zur Differenzierung und Invasion boviner Trophoblastzellen als nützlich erweisen könnte.
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LIST OF PUBLICATIONS

9 LIST OF PUBLICATIONS

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A novel 3-dimensional cell culture model for bovine trophoblast cells
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Auguste Kekule und the discovery of the structure of Benzol in 1865. Oral presentation on the occasion of the 400th anniversary (“Cum tempore”, 20.-24.05.2007) of the Justus-Liebig University Giessen, Giessen, Germany, 23.05.2007; ([http://www.uni-giessen.de/cms/ueber-uns/400-jahre/veranstaltungen/400veranstaltungen/Vortraege/vortraege1857](http://www.uni-giessen.de/cms/ueber-uns/400-jahre/veranstaltungen/400veranstaltungen/Vortraege/vortraege1857))

How to establish a trophoblast cell line out of the bovine cotyledon and test its angiogenic potential by using a spheroid model. Seminar during a research visit at the University of Sao Paulo, Brazil, 16.11.2007
10 AWARDS AND SCHOLARSHIPS

YW Loke New Investigator Travel Award (500$) on the occasion of International Federation of Placenta Associations Meeting 2009, Adelaide, Australia, 6th – 9th October 2009 for the contribution: Bovine trophoblast cells invade collagen gels from embedded spheroids (see above)

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