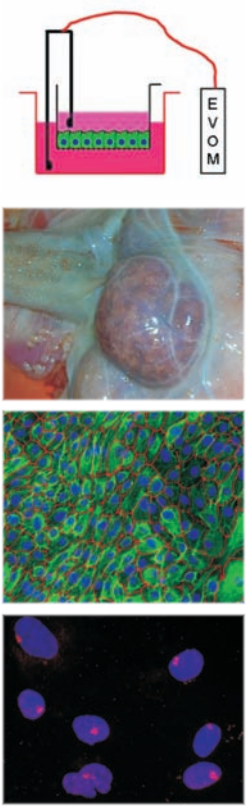
VALIDATION AND ESTABLISHMENT OF CELL CULTURE MODELS TO STUDY INVASION AND FETO-MATERNAL INTERACTION IN THE BOVINE PLACENTOME

PHILIP SIMON BRIDGER



INAUGURAL DISSERTATION
(Cumulative Thesis)
in partial fulfillment of the requirements
for the PhD-Degree of the Faculties of
Veterinary Medicine and Medicine
of the Justus-Liebig-University Giessen

édition scientifique
VVB LAUFERSWEILER VERLAG

1. Auflage 2008

1st Edition 2008

VVB LAUFERSWEILER VERLAG
édition scientifique

STAUFENBERGRING 15, D-35396 GIESSEN
Tel: 0641-5599888 Fax: 0641-5599890
email: redaktion@doktorverlag.de

www.doktorverlag.de

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Giessen 2008

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Date of Doctoral Defense: 3rd April 2008

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„This dissertation is submitted as a cumulative thesis according to the charter of the PhD-Program of the Faculty of Veterinary Medicine and Medicine of the Justus-Liebig-University. The thesis includes an interconnection of three original papers, two of which describe and discuss the majority of my experimental work during the course of the program. The experimental data from the third paper was produced during the same time period in collaboration with a colleague (Susanne Haupt) who submitted part of the work as doctoral dissertation."

Philip Bridger

To Nicole

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1 Abbreviations

Ac-LDL	Acetylated low-density-lipoprotein
BEND	Bovine Endometrial cell line
BVDV	Bovine Viral Diarrhea Virus
cp-virus	Cytopathogenic virus
DMEM	Dulbecco's Modified Eagle's Medium
ECM	Extracellular Matrix
EMT	Epithelial-mesenchymal Transition
FAK	Focal Adhesion Kinase
FCS	Fetal Cow Serum
FISH	Fluorescence In-Situ Hybridization
ncp-virus	Non-cytopathogenic virus
PI	Persistently infected
TGC	Trophoblast Giant Cell(s)
ZO-1	Zonula occludens-1

2 List of papers

I. P. S. Bridger, S. Haupt, K. Klisch, R. Leiser, H.-R. Tinneberg, C. Pfarrer

Validation of primary epitheloid cell cultures isolated from bovine placental caruncles and cotyledons

Theriogenology, 2007; 68 (4):592-603

II. P. S. Bridger, C. Menge, R. Leiser, H.-R. Tinneberg, C. D. Pfarrer

Bovine Caruncular Epithelial Cell line (BCEC-1) isolated from the placenta forms a functional epithelial barrier in a polarized cell culture model

Placenta, 2007; 28 (11-12):1110-1117

III. P. S. Bridger, S. Haupt, R. Leiser, G. A. Johnson, R. C. Burghardt, H.-R. Tinneberg, C. Pfarrer

Integrin activation in bovine placentomes and in caruncular epithelial cells isolated from pregnant cows

Biology of Reproduction, 2008; in press

3 Introduction

The placenta is an ephemeral organ within the female body of placental mammals (*Placentalia*) responsible for fetal nutrition and regulating embryonic development. During pregnancy, the placenta undergoes various and impressive processes of growth, remodeling and degradation. Gaining access to the organ as such without interfering with other functional tissues, i.e. uterus or fetal membranes, is difficult. It is therefore important to be aware of which structures and what functional properties are related to the placenta.

The most comprehensive definition of the placenta was given by Mossman (1937) as "an apposition of parental (usually maternal) and fetal tissue for the purposes of physiological exchange". The maternal part consists of a derivative of the endometrium (e.g. uterine epithelium and connective tissue) which faces the fetal part consisting of the chorionic epithelium derived from the ectoderm also named trophoblast. Under a more functional point of view Steven (1975) characterized the placenta as an "arrangement of one or more transporting epithelia between fetal and maternal circulations" (cited in Wooding and Flint, 1994). This includes an efficient exchange of various metabolites and hormones as well as O_2, CO_2 and particular nutrients (e.g. iron) via different types of transport pathways such as active transport, pinocytosis, phagocytosis and passive diffusion. Taken together, the placenta acts as a surrogate fetal lung, gut and kidney protecting the usually allogeneic fetus from the mother's immune system (Wooding and Flint, 1994).

3.1 The bovine placenta

The bovine chorioallantoic placenta is classified according to its shape as a cotyledonary type (placenta cotyledonaria sive multiplex; Strahl, 1906). This term describes 70-150 circumscribed areas of feto-maternal contact named placentomes (Figure 1) protruding into the amnion/allantoic cavity (Wooding and Flint, 1994). These placentomes evolve in those regions where the trophoblast comes into contact with preformed areas (caruncles) of the uterine epithelium (Atkinson et al., 1984). During pregnancy both maternal caruncular and fetal chorionic (now termed cotyledon, components grow extensively. This process is characterized by the formation of branching cotyledonary villi which interdigitate with the caruncular tissue thus creating complementary septa and crypts (villous placenta; Leiser and Kaufmann, 1994). This leads to a firm anchorage of fetal membranes to the endometrium (Leiser et al., 1998) and to a significant increase in feto-maternal exchange area. Prior to birth this exchange area is calculated by means of stereology to be approximately 120 m^2 (Baur, 1981).

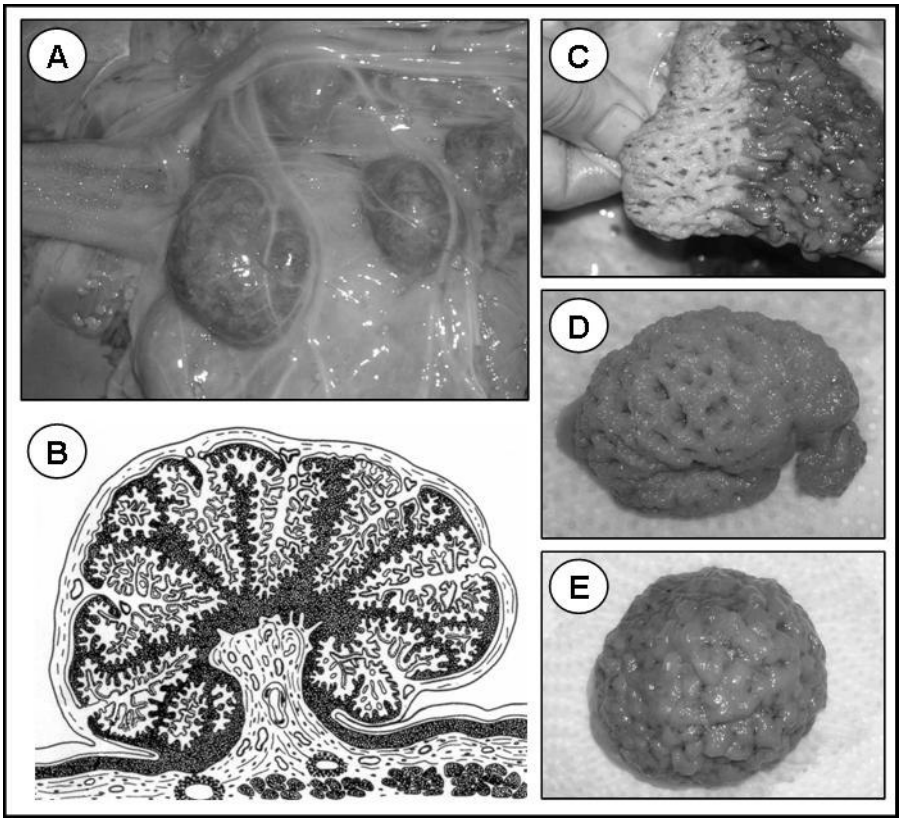

Figure 1: The bovine placentome

[A] Multiple placentomes protruding into the amnion/allantoic cavity. [B] Schematic cross sectional view (from Leiser and Kaufmann, 1994) showing caruncular septa (dark) interdigitating with cotyledonary villi (white) [C] Manual separation of a placentome into its maternal caruncular (left) and fetal cotyledonary (right) components. [D] Feto-maternal interface of a caruncle characterized by crypts formed by septa. [E] Feto-maternal interface of a cotyledon characterized by villi.

3.1.1 The feto-maternal interface

From a histological point of view, the ruminant placenta was originally classified as syndesmochorial by Grosser (1909). However, the term "synepitheliochorial" introduced by Wooding (1992) was found to be more appropriate. This latter classification comprises two features: the cell layers separating the fetal and maternal blood vasculature as well as the functional relationship of both epithelia. The chorionic epithelium (trophoblast) faces an intact maternal caruncular (endometrial) epithelium within the placentomes. In addition, trophoblast giant cells (TGC) migrate towards and fuse with maternal epithelial cells to form feto-maternal syncytia (Figure 2).

The formation of feto-maternal syncytial cells is a major process in the bovine feto-maternal interaction and is also called “restricted” trophoblast invasion (Pfarrer et al., 2003) (Figure 2C). TGC, which are in most cases binucleate and located within the chorionic epithelium (Wimsatt, 1951), emerge from mononuclear trophoblast cells via acytokinetic mitosis (Klisch et al., 1999a). Thereby they lose their epithelial-specific properties, e.g. loss of tight junctions, gap junctions, apical microvilli and contact to the basal membrane (Wooding and Flint, 1994). During the “maturation” process (Wooding, 1992), the TGC produce a large amount of periodic acid-Schiff (PAS)-positive cytoplasmic granules (Wimsatt, 1951) containing a variety of signaling molecules such as bovine placental lactogen (Wooding and Beckers, 1987) and pregnancy-associated glycoproteins (Zoli et al., 1992). In addition, TGC participate in the synthesis of steroids (Ullmann and Reimers, 1989; Matamoros et al., 1994) and prostaglandins (Reimers et al., 1985). Mature TGC then migrate towards the maternal epithelium, fuse with single epithelial cells (feto-maternal syncytium) and release, amongst others, the metabolites mentioned above into the maternal circulation (Wooding, 1992). Finally, the feto-maternal hybrid cells degenerate and are phagocytized by mononuclear trophoblast cells (Klisch et al., 1999b). In comparison to placentation in other ruminants, the fusion of binucleate trophoblast cells with only one endometrial epithelial cell is a unique feature of the bovine placenta. In sheep for example, trophoblast giant cell migration and fusion leads to the formation of multinuclear syncytial plaques (Wooding, 1984).

An epithelium-specific polarized morphology of both layers displaying apical microvilli and junctional complexes (Figure 2B) are characteristic for the feto-maternal interface (Björkman, 1973; Leiser, 1975). The passive exchange of fetal and maternal metabolites is limited to small molecules only. Maternal antibodies, for example, cannot enter the fetal circulation resulting in the necessity of the calf to receive colostrum within the first few hours after birth.

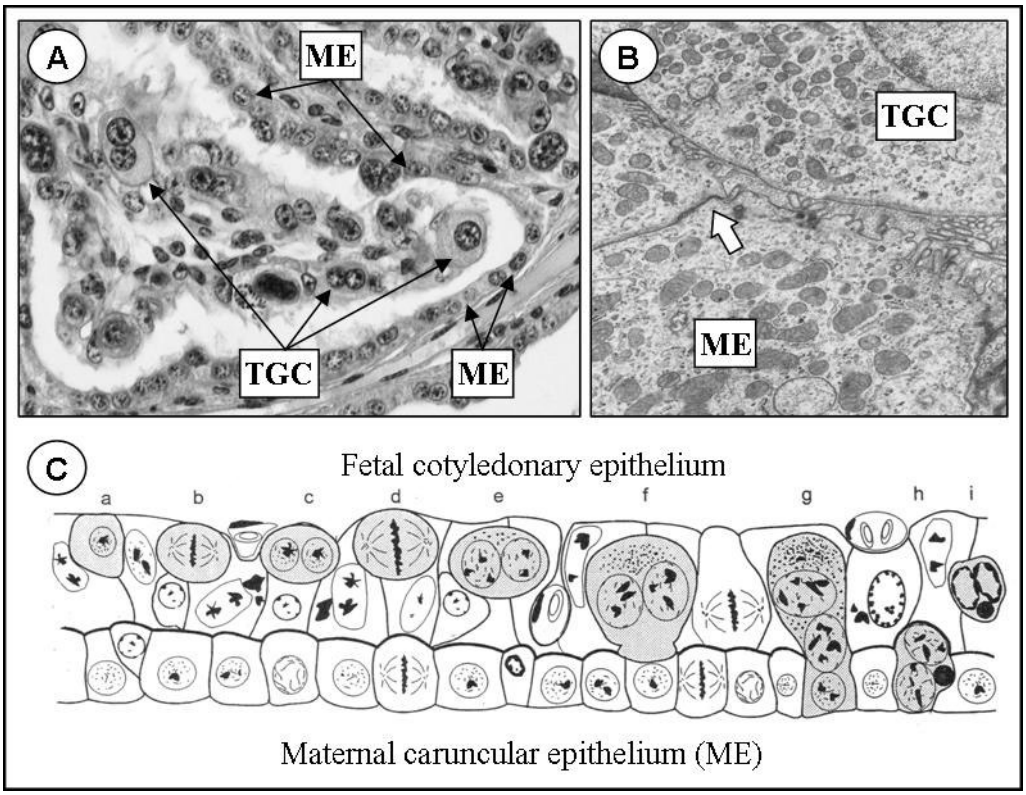

Figure 2: The feto-maternal interface

[A] Haematoxylin-eosin stained cryosection of the bovine placentome showing a caruncular crypt delineated by maternal epithelial cells (ME) facing the fetal chorionic epithelium consisting of mononuclear trophoblast and binucleate trophoblast giant cells (TGC). [B] Transmission electron micrograph of the feto-maternal contact interface showing maternal epithelial cells (ME) with apical microvilli and junctional complexes (arrow) facing a TGC. [C] Schematic view of the feto-maternal interface displaying the phases (a-i) of restricted trophoblast invasion. Thereby TGC emerge through acytokinetic mitosis of mononuclear trophoblast cells, migrate towards and fuse with a maternal epithelial cell. Finally they degenerate and become phagocytized (from Klisch et al., 1999b).

3.1.2 Comparative placentation

When comparing the bovine feto-maternal interface with other species distinct differences are observed (Leiser and Kaufmann, 1994). As in the bovine, both equine and porcine placentae are epitheliochorial. While in the horse trophoblast cell invasion and disruption of the endometrial epithelium is observed exclusively in defined regions (endometrial cups) between day 36 to 38 of gestation (Allen et al., 1973), no trophoblast invasion occurs in the sow (Dantzer, 1985). In the endotheliochorial placenta of carnivores, the endometrial epithelium degenerates at the attachment sites (zonary placenta) allowing the grossly parallel fetal lamellae to invade the maternal tissue in close contact to intact maternal

blood vessels (Leiser and Koob, 1993). The most "destructive" type of placentation is the haemochorial placenta present in rodents, rabbit, ape and man (Enders, 1965). Feto-maternal contact in this placenta is characterized by a highly invasive trophoblast resulting in degeneration and partial loss of endometrial tissue. This includes the blood vessel walls and consequently permits the fetal villi to be irrigated by the maternal blood. However, there are considerable differences between these species. In humans the feto-maternal interface is haemomonochorial (single trophoblast layer) and of villous architecture (Jones and Fox, 1991), whereas in mice and rats a haemotrichorial (triple trophoblast layer) arrangement and a labyrinthine architecture is present (Enders, 1965; Enders and Welsh, 1993). Nonetheless, the latter species is commonly used as a model to study placental disorders in man (Carter, 2007).

3.2 Placenta and invasion

Studying cellular processes of invasion, tumor invasion in particular, is a common topic in research. The majority of these studies aim to find targets for new therapeutic approaches capable of curing cancer in man. When looking at the cell behavior of invasive trophoblast cells, similarities to tumor cells are obvious. In the human placenta for example, extravillous cytotrophoblast cells invade the maternal tissue massively, remodel the spiral arteries (Jones and Fox, 1991) and express a variety of molecules also present in cancer cells (Soundararajan and Rao, 2004; Ferretti et al., 2007). However, in contrast to tumor cells trophoblast-mediated cellular invasion in the placenta is limited to a certain extent and time period. Thus, specific regulatory mechanisms controlling trophoblast behavior, not only within the invading trophoblast cells themselves but also in the surrounding tissue, are very likely to be present. In human decidua cells for example, the secretion of transforming growth factor beta (TGF-β) inhibits trophoblast proliferation and invasion (Tse et al., 2002). In the bovine placenta, TGC migration and invasion takes place between two cell layers only, namely fetal chorionic and maternal endometrial epithelium (Wooding and Flint, 1994), a phenomenon also termed restricted trophoblast invasion (Pfarrer et al., 2003). This small and well defined region suggests the bovine placenta to be an ideal model to study invasive processes.

Important factors for tumor and trophoblast cell migration and invasion are extracellular matrix (ECM) proteins (such as fibronectin, collagen and laminin) and their receptors (such as the integrins) as well as enzymes degrading this matrix (such as matrix-metalloproteinases) (Soundararajan and Rao, 2004). The ECM is an extracellular network of secreted proteins and glycoproteins playing a vital role in cellular function. Interaction with the neighboring cells predominantly occurs via integrin receptors participating in "inside-out" and "outside-in"

signaling pathways (Hynes, 2002). These receptors are heterodimers and consist of an "α" and a "β" subunit which are divided into further subclasses (Hynes, 2002; Reddy and Mangale, 2003). Integrin receptors not only mediate cell-ECM but also cell-cell adhesion and interaction. Damsky et al. (1994) demonstrated that antibody perturbing of collagen type IV and laminin as well as their receptor-associated subunits α_1-integrin and α_6-integrin inhibited human cytotrophoblast invasion. In contrast, disruption of fibronectin binding and its receptor-associated subunit α_5-integrin enhanced cytotrophoblast invasion. In the bovine, the expression patterns of the ECM proteins fibronectin, collagen I, collagen IV and laminin as well as the integrin subunits α_1, α_2, α_3, α_4, α_5, α_6, α_v, β_1, β_3 and β_4 have been demonstrated during implantation and in the placenta throughout pregnancy (MacIntyre et al., 2002; Pfarrer et al., 2003). The results suggested that TGC migration and invasion employ mechanisms used by tumor cells such as migration along laminin matrices mediated by $\beta_1\alpha_6$-integrin receptors (Pfarrer et al., 2003). Furthermore, TGC fusion with the maternal epithelium may induce integrin and ECM changes in the subepithelial stroma such as α_1-integrin downregulation as well as laminin and collagen IV upregulation (MacIntyre et al., 2002).

3.3 Bovine placental disorders

Studying feto-maternal interaction in the bovine placenta is not only of relevance as a model for human diseases. In fact, reproduction is the major issue in herd management of cattle and subsequently in veterinary medicine and thus has a major impact on the economical success of farms, dairy farms in particular. Therefore, the physiology and pathology of bovine pregnancy including disorders and infectious diseases is an important topic in veterinary research.

3.3.1 The retained placenta

One of the major disorders in bovine pregnancy is the retention of the fetal membranes post partum (Laven and Peters, 1996). In contrast to other ruminants this disease is a routine problem in cattle and water buffalos, which may be related to the firm anchorage of fetal cotyledons in maternal caruncles being very susceptible to pathological conditions (Leiser et al., 1998).

Physiologically, in the cow the fetal membranes are released within the first few hours after birth. In the case of a retained placenta, an incomplete loosening of the membranes from the maternal caruncles occurs resulting in the retention of fetal tissue in the uterine cavity. If not treated in time secondary infection and inflammation can result in severe reproductive disorders. Many studies have identified a variety of risk factors such as genetical background,

nutrition, husbandry and dystocia. These induce either mechanical obstruction (< 2 %), myometrial dysfunction (< 10 %) or maintenance of the feto-maternal union which result in retained placenta (Laven and Peters, 1996). It is a generally accepted hypothesis that the placenta undergoes a "maturation" process ante partum (Woicke et al., 1986) as a prerequisite for successful detachment of the fetal compartment post partum. That is why the induction of parturition with corticosteroids, for example, prior to placental maturation, results in the development of a retention of the fetal membranes in nearly every case. From a cellular point of view, the maturation process is characterized by a flattening of the caruncular epithelium (Björkman and Sollen, 1960) and a decrease in the number of TGC (Gross et al., 1991). In retained placentae, for example, a decrease in the number of TGC was not observed (Gross et al., 1991). Considering the fact that the placenta is an endocrine organ, the involvement of hormones in the regulation process of maturation and subsequent pathogenesis of retained placenta, is likely to be present (Grunert et al., 1989). However, the relation between impaired hormonal values and incomplete placental maturation is still unclear. Studies comparing pre- and postpartal conditions are often contradictory and inconclusive due to further parameters which influence hormone concentration levels (Laven and Peters, 1996).

Taken together, the maturation process of the bovine placenta is far too complex to be studied by in-vivo methods. The underlying mechanisms, i.e. the factors that regulate feto-maternal detachment are still unknown. Therefore, new methods are necessary to gain insight in this process.

3.3.2 Bovine viral diarrhea

As described above, the synepitheliochorial placenta serves as a barrier between maternal and fetal tissue thus protecting the fetus from the majority of toxic and infectious agents circulating in the mother's body and vice versa. However, some microbial pathogens are capable of bypassing this barrier which may have consequences for the outcome of pregnancy (e.g. abortion, abnormalities, infertility).

Infection with the bovine viral diarrhea virus (BVDV-1 and BVDV-2) is considered one of the most important endemic pathogens in cattle in the developed world leading to substantial economic losses (Houe, 2003). The effects on health and production include increased mortality (young animals), increased occurrence of other diseases, growth retardation, reduced milk production and reproductive performance, unthriftiness and early culling. Infections during pregnancy can also result in fetal death, abortion, mummification, fetal malformation and retained placenta (Larsson et al., 1994). In Europe BVDV-1 dominates

with a prevalence of more than 90 % and approximately 50 % of the herds are considered to harbor persistently infected (PI) animals (Lindberg et al., 2006).

Apart from the economical consequences, the pathogenesis of infection and in particular the pathogenesis leading to PI animals, makes the BVDV so interesting for research. The basis for the development of a PI bovine is the infection of the dam with a non-cytopathogenic (ncp)-virus during pregnancy (Thiel et al., 1996). If infection and viremia occur and the virus is transmitted to the fetus within the first 120 days of pregnancy, the developing fetal immune system recognizes the virus as self. The resulting immunotolerance allows the virus to persist lifelong and though the animal is clinically healthy, it sheds the pathogen almost continuously in high concentrations in saliva, milk, urine and feces. Endogenous mutation of the ncp-virus to a cytopathogenic (cp)-virus or an infection with the exogenous but closely related cp-virus, both termed superinfection, precede the development of the lethal mucosal disease (MD).

The distribution pattern of BVDV antigen in the placenta of PI-animals in comparison to acutely infected cattle via immunohistology revealed interesting findings (Fredriksen et al., 1999a; Fredriksen et al., 1999b). In pregnant PI-cows (n=3) virus antigen was found predominantly in the maternal epithelium of both caruncular and intercaruncular regions whereas in the trophoblast BVDV-antigen staining was found in selected TGC only (Fredriksen et al., 1999a). In the subepithelial region of both compartments, cells surrounding blood vessels, arteries in particular, and selected cells of mesenchymal origin were positive for BVDV. Furthermore, viral antigen was detected in fetal intestine, liver, lung and spleen as well as in the uterus and ovary of the dam. In acutely infected cattle (n=7), which were inoculated with BVDV on day 85/86 of pregnancy and culled 7-22 days post infection, virus antigen was detected in a time dependent manner but somewhat different from the PI-animals. Interestingly, virus antigen was initially detected in the fetal organs from day 14 post oculation onwards whilst BVDV positive cells were observed in the mesenchyme of the fetal intercotyledonary and cotyledonary tissue on day 18 and 22, respectively. The maternal compartment as well as the uterine and ovarial tissue remained negative. However, in all cases virus isolation and antibody detection from maternal blood samples were successfully performed from day 5/6 and 13/16 post infection, respectively. The authors concluded that in acutely infected animals passage of BVDV occurs primarily via the vasculature rather than local cell-cell pathways prior to manifestation in endometrial epithelium. But the question how the virus transfers the placental barrier has not yet been answered.

The design of both studies, especially the small number of animals, demonstrates the difficulties and limitations when performing in-vivo studies in cattle. Even though in-vivo studies with BVDV are well under way, the majority focuses on the properties of the virus using cell lines (MDBK - Madin Darby Bovine Kidney cell line, in particular) derived from tissues which are not involved in the vertical transmission of BVDV (Lackner et al., 2004; Lee et al., 2005; Schweizer et al., 2006).

3.4 Cell cultures of the placentome

For the interpretation of data obtained from uterine/placental primary cell cultures, it is important to determine the exact origin and the properties of the cells. Regarding the maternal compartment, the differentiation between caruncular and intercaruncular regions, which are both already present in the fetal uterus (Atkinson et al., 1984), is essential. In in-vivo studies, the morphological differences are clearly associated with functional differences, both in pregnant as well as non-pregnant cyclic tissue (Del Vecchio et al., 1991; Kimmins and MacLaren, 1999; Kizaki et al., 2001; Banu et al., 2005; Pfarrer et al., 2006b). Furthermore, it is evident that the differentiation of the cell type, i.e. epithelial cells or stroma-derived fibroblasts, has a major impact on the outcome of experiments due to the structural organization, location and function of the cell.

3.4.1 Epithelial cell cultures from the caruncle

Primary bovine endometrial cell cultures derived from endometrial tissue of non-pregnant animals are a common finding in in-vitro studies dealing with the physiology and pathology of reproduction (Soto-Belloso et al., 1976; Fortier et al., 1988; Munson et al., 1988a; Lindenberg et al., 1989; Horn et al., 1998; Asselin et al., 1998; Xiao and Goff, 1998; Skarzynski et al., 2000; Takahashi et al., 2001). However, in the majority of these studies no differentiation between the cell types (epithelial or stromal) and the regions (caruncular or intercaruncular) was performed. That this differentiation should be considered was demonstrated by Asselin et al. (1998) showing that both stromal and epithelial cells originating from caruncular or intercaruncular regions exhibited specific responses to oxytocin or interferon-tau treatment. Furthermore, significant differences in the prostaglandin production to stimulating agents between epithelial and stromal cells was demonstrated not only in this study but also in various previous studies of the same group (Kim and Fortier, 1995; Asselin and Fortier, 1996; Asselin et al., 1996; Asselin et al., 1997b).

Soto-Belloso et al. (1976) were the first authors to describe a method to isolate primary endometrial cells from fetuses of 5-7 months of gestation and to successfully infect them with

cp-BVDV. However, no differentiation between endometrial region and cell type was performed, a fact discussed by the authors who suggested that more work is necessary. Munson et al. (1988a) addressed this matter by demonstrating a method to isolate and cultivate primary epithelial cells from caruncular regions of uteri derived from fetuses of mid to late stages of pregnancy. The cells demonstrated epitheloid morphology and expressed epithelial-specific cytoskeletal cytokeratin. These cells were kept in culture for at least 10 passages. In the same study epithelial cells derived from intercaruncular regions of non-pregnant and pregnant adult cows were also cultured but were less viable (four passages). Therefore the following studies based on this method included caruncular epithelial cells derived from the uterus of a fetus (Munson et al., 1990; Munson et al., 1991; Munson et al., 1996).

In January 1998 a bovine endometrial epithelial cell line (BEND) was deposited and characterized by T.R. Hansen, K.J. Austin and G.A. Johnson (Hansen et al., 1997; Staggs et al., 1998) at the American Type Culture Collection (ATCC number CRL-2398). The cells were isolated in 1997 via explant outgrowth and cultured on matrigel in 40 % Modified Eagle Medium (MEM, D-valin modified), 40 % Ham's F12 medium, 10 % heat inactivated FCS, 10 % heat inactivated horse serum, 1 % antibiotic-antimycotic solution and 0.2 U/ml insulin. Subculturing was performed with a trypsin/EDTA solution. After four passages the cells were seeded without matrigel. Immortalization of the cell line was spontaneous. The ATCC received the cell culture after passage 6 and tested it positive for BVDV. Nonetheless, the BEND cell line has been used successfully in various studies (Austin et al., 1999; Perry et al., 1999; Johnson et al., 1999a; Binelli et al., 2000; Binelli et al., 2001; Pru et al., 2001; Thatcher et al., 2001; Badinga et al., 2002; Flint et al., 2002; Mattos et al., 2003; McDonnel et al., 2003; Guzeloglu et al., 2004a; Guzeloglu et al., 2004b; Guzeloglu et al., 2004c; Parent and Fortier, 2005; Rempel et al., 2005; Tekin et al., 2005; MacLaren et al., 2006). The majority of these studies focused on prostaglandin and interferon-tau synthesis and signaling pathways. According to Binelli et al. (2000), the BEND cell line can be used for at least 25 passages without morphological alterations although the authors failed to present data to confirm this statement. In addition, Binelli et al. (2001) characterized the cells by immunostaining and immunoblotting and confirmed their epithelial origin by demonstrating their capability to express cytokeratin.

In contrast to studies using endometrial-derived cells of non-pregnant cows, primary cultures isolated from pregnant animals, caruncular cells in particular, are a rare finding. Shemesh et al. (1983) were the first to publish a short description of a method to isolate and

cultivate cells from caruncular and cotyledonary tissue of day 145 to 265 of gestation. However, these cells were not further characterized in terms of their morphological appearance and cell type. Following studies from the same group failed to address this matter, although significant differences in prostanoid production dependent on the origin of the cells and gestational age were demonstrated (Shemesh et al., 1984a; Shemesh et al., 1984b; Shemesh et al., 1984c; Izhar et al., 1992; Shemesh et al., 1994). A similar isolation and cultivation procedure of caruncular and cotyledonary cells was performed by Fecteau and Eiler (2001). However, no differentiation and characterization of the cell types was performed. The authors demonstrated a mitogenic effect of serotonin (5-hydroxytryptamine) on these cells which was similar to the effect observed in trophoblast cells donated by L. Munson (Munson et al., 1988b). In conclusion, there is no study available that describe a method to isolate primary caruncular epithelial cells from pregnant cows including a clear identification and characterization of the cell type in culture.

3.4.2 Trophoblast cell cultures from the cotyledon

Similar to the situation for caruncular epithelial cells, primary trophoblast (not TGC) cultures isolated from the cotyledon are rare. Again the lack of characterization and identification questions the interpretation of the results when culturing cells from the cotyledon as performed by Shemesh et al. (Shemesh et al., 1983; Shemesh et al., 1984a; Shemesh et al., 1984b; Shemesh et al., 1984c; Shemesh et al., 1988; Shalem et al., 1988; Shemesh et al., 1994).

Despite extensive search in the literature, Munson et al. (1988b) appear to be the only group that successfully isolated trophoblast from cotyledons of cows at early to mid pregnancy and identified them as such. The cells displayed a polygonal morphology and expressed epithelial-specific cytokeratin but not Factor VIII-related antigen specific for endothelial cells. Six cell lines were established, three of which could be kept in culture up to 48 passages. Feng et al. (2000a) also generated cultures from the cotyledon (3-4 months of gestation) and differentiated three cell types: polygonal, fan-shaped and epitheloid. Apart from the morphological assessment no further characterization was performed. In the following publication the authors determined the polygonal cells to be of endothelial origin, due to the uptake of acetylated low-density lipoproteins (Ac-LDL; specific for endothelial cells) and the expression of vimentin and angiotensin converting enzyme, ACE (Feng et al., 2000b). Furthermore, the cells were stained negative for cytokeratin as well as desmin and α-smooth muscle actin which are both markers for cells of smooth muscle-derived cells. The differences in the identification of the polygonal shaped cells between Munson et al. (1988b)

and Feng et al. (2000b) may be related to the differing isolation procedures and culture conditions: Munson et al. digested fragments of cotyledonary chorion in collagenase type II and seeded the resulting cell suspension after filtration in Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F12 Medium (1:1) supplemented with epidermal growth factor (EGF), an insulin, transferring, selenium solution, 10 % fetal calf serum (FCS) and antibiotics. Feng et al. digested minced cotyledonary villi in trypsin, followed by a Percoll gradient centrifugation step and finally seeded the resulting cell suspension in M199 medium supplemented with 10 % FCS and antibiotics. M199 is a medium commonly used in cell culture models of endothelial cells, whereas DMEM/Ham's F12 is considered to be a "basic" medium for multiple applications.

When reviewing the data of all research groups that isolated different cell populations from either the caruncular or cotyledonary compartment, it appeared that none of the groups presented proof that their cells were truly of maternal or fetal origin.

The difficulties in the isolation of pure trophoblastic cell cultures from the cotyledon due to the heterogeneity of the tissue can be avoided when using non-implanted blastocysts as a source for trophoblast cells (Stringfellow et al., 1987; Shimada et al., 2001). Shimada et al. (2001) established a cell line (BT-1) via explant outgrowth of in-vitro fertilized in maturated blastocysts displaying cubical morphology and expressing cytokeratin. Furthermore, the expression of interferon-tau mRNA in the cells, a signaling molecule predominantly secreted by the trophoblast during implantation (Spencer et al., 2007), and the secretion of the protein into the medium was shown. In culture the formation of binucleate cells was observed and identified as evolving TGC via the detection of bovine placenta lactogen in these cells only (Nakano et al., 2002). Even though trophoblast cultures derived from preimplantation blastocysts may be an alternative to cotyledonary-derived cells, it should be kept in mind that blastocyst-derived cells are less differentiated and may not possess all properties characteristic for the cotyledonary trophoblast.

3.5 Aim of the study

In summary, the process of restricted trophoblast invasion within only two cell layers is a characteristic and unique feature of the bovine placenta and suggests the presence of specific regulatory mechanisms that control this event. Understanding this process may not only help prevent and treat reproductive disorders in the cow, but may also have an impact on tumor research. Furthermore, the protective, transportive and communicative properties of the feto-maternal interface are far too complex to be studied in-vivo especially considering the

difficulties when performing animal experiments, not only due to the size of the animal but also under the aspect of animal welfare. The establishment of an in-vitro model is overdue.

In a preceding study of our group the basis for an in-vitro model of bovine placentation was established (Zeiler et al., 2007). In that study manually separated caruncles and cotyledons were tied together thus positioning the epithelial layer outside, disaggregated in collagenase type I, and the resulting cell suspension was cultured in DMEM/Ham's F12 (caruncle) or Quantum 286 (cotyledon) medium. Apart from other cell types, epitheloid shaped cells forming monolayers were identified as caruncular epithelial cells if derived from caruncular tissue and trophoblast cells if derived from cotyledonary tissue. A modified trypsination protocol according to Munson et al. (1988a) allowed the reduction of contaminating fibroblasts so that after the second passage the cultures contained predominantly epitheloid cells. Both cultures demonstrated expression of cytokeratin as well as extracellular matrix proteins and integrin receptors (Zeiler et al., 2007) corresponding to in-vivo findings (Pfarrer et al., 2003). Epithelial cells from the caruncle were isolated and cultured successfully whereas gaining sufficient amounts of trophoblast cells was difficult.

Based on these results the objective of the PhD-thesis was to validate and improve the method to isolate and culture primary placentomal cells of the feto-maternal interface of pregnant cattle. This included an extensive characterization of the phenotypic and functional properties of the cells (caruncular epithelial cells in particular) participating in feto-maternal communication and restricted trophoblast invasion. Furthermore, the first bovine caruncular epithelial cell line (BCEC-1) derived from a pregnant animal was established, introducing new methods and possible applications for future research.

4 Material and Methods

The details of the material and methods applied in the different parts of the study are given in each of the publications which are part of this thesis. Nevertheless, this chapter briefly summarizes the applications to give the reader an overview.

- Primary cell culture: Fresh placentomes were excised from uteri of pregnant cows obtained at the local abattoir (Schlachthof Giessen) during routine slaughtering. Individual primary cell cultures of the caruncle and the cotyledon were generated and different isolation and culture conditions to improve the method were assessed. Within the heterogeneous culture, three predominant cell populations, namely epitheloid, fibroblastoid and polygonal (during the course of the study) shaped cells were identified (inverse light microscopy). A two-step trypsination protocol enabled the propagation of cultures predominantly consisting of epitheloid shaped cells. All cell types were characterized with the methods described below (Paper I, II and III).
- Immunofluorescence: The detection of selected cytoskeletal proteins (cytokeratin, vimentin, α-smooth muscle actin and desmin) and zonula-occludens 1 protein (Paper I, II and III) via immunofluorescence was performed on all cell types. Double-labeling of cytokeratin with the remaining proteins was done to clearly identify the properties of the epitheloid, fibroblastoid and polygonal shaped cells. To assess whether functional integrin binding was present, primary caruncular epithelial cells were stained for β_1-integrin and integrin-associated signaling proteins: α-actinin, focal adhesion kinase, phosphotyrosine and talin (Paper III). If antibodies were derived from different hosts double-labeling was performed to illustrate co-localization of the respective proteins. All antibodies were additionally applied to cryosections of shock frozen placentomes in order to determine whether the observations in-vitro were comparable to the situation in-vivo.
- Western blot: The specificity of the antibodies applied and the purity of the primary caruncular epithelial as well as fibroblastoid cell cultures were confirmed via western blot (Paper I, II and III). Protein isolated from shock frozen placentomes served as a control.
- Fluorescence in-situ hybridization (FISH): Provided the fetus was male, the origin (fetal or maternal) of the cells was determined by detecting Y-chromosome positive cells directly after isolation from either the caruncle or the cotyledon and in cultured epitheloid and polygonal shaped cells isolated from the same sources (Paper I). Bull-derived leucocytes served as positive controls.

- Acetylated-low density lipoprotein (Ac-LDL) labeling: In order to exclude the presence of endothelial cells in the cultures Ac-LDL-labeling was performed (Paper I). Bovine umbilical vein cells served as positive controls.
- Cell line: A cell line termed bovine caruncular epithelial cell line-1 (BCEC-1) was established by spontaneous immortalization during continuous subculture of a primary caruncular epithelial culture isolated from a pregnant cow (Paper II).
- Transmission and scanning electron microscopy (TEM and SEM): TEM and SEM were performed to confirm the polarized phenotype of primary caruncular epithelial cell cultures and BCEC-1 cells, and to identify characteristic ultrastructural features of an epithelial barrier (Paper II). In these experiments primary caruncular epithelial and BCEC-1 cells were cultured in Transwell® - clear inserts.
- Transepithelial electrical resistance (TEER) measurements: In order to confirm epithelial barrier functionality, the TEER of BCEC-1 cells cultured in Transwell® inserts was measured (Paper II). Primary fibroblasts served as negative controls.
- Bovine viral diarrhea virus (BVDV) detection and infection: Detection of viral antigen was performed via immunofluorescence to assess whether the BCEC-1 cultures were positive for BVDV (Paper II). If cells were shown to be negative, susceptibility to infection was investigated. BVDV infected and non-infected Madin Darby bovine kidney (MDBK) cells served as positive and negative controls, respectively.
- Influence of extracellular matrix proteins (ECM): In order to assess whether specific matrices had an impact on cellular properties (proliferative activity and integrin signaling), primary caruncular epithelial cells were cultured on fibronectin, laminin, collagen A (predominantly containing collagen type I) and collagen type IV-coated dishes (Paper II, III). The influence on cell proliferation was determined via TEER measurements (Paper II) and cell counting (Paper III). The effect on integrin signaling was qualitatively assessed via immunofluorescence thereby detecting β_1-integrin, phosphotyrosine and talin (Paper III).

5 Results and Discussion

The thesis presented firstly focuses on two major aspects of culturing primary bovine placental epithelial cells: methodology and functionality.

The comparison of FISH with the generally accepted description of the phenotype together with the detection of cytoskeletal filaments has shown that cell cultures isolated from caruncles and cotyledons of manually separated placentomes each were “contaminated” with cells from the neighboring compartment. This fact highlights the importance of appropriate characterization of cell types as well as the determination of origin when using primary cell cultures derived from placentomal tissue, and leaves doubts concerning the reliability of any result obtained in past and future studies where this distinction has not been done. Functional experiments conducted with primary caruncular epithelial cells have shown that integrin-mediated signaling occurs and can be enhanced by specific matrices, resulting in an increased cell proliferation and expression of signaling proteins.

The second part of the thesis concentrates on the establishment of a spontaneously immortalized bovine caruncular epithelial cell line (BCEC-1) from a pregnant animal maintaining major characteristics of differentiated epithelial cells. In addition, when grown on Transwell® inserts, BCEC-1 develops a functionally intact epithelial barrier. As BCEC-1 cells were shown to be negative for BVDV, but susceptible to infection, new perspectives emerged for the development of an in-vitro model for infectious diseases.

The results presented confirm that both caruncular epithelial cell models (primary cells and BCEC-1) may serve as a basis for future research on cellular invasion strategies, pathways of transport and feto-maternal communication as well as pathogenesis of pregnancy-associated diseases.

5.1 Primary caruncular epithelial cells

5.1.1 Characterization of caruncle-derived epitheloid shaped cells

The first aim of this thesis was to validate and, if necessary, improve the method of culturing primary epithelial cells from the placentome. Taken together, 88 % of all attempts to isolate primary caruncular epithelial cells resulted in the culture of a sufficient number of cells for further experiments (Paper I). The method to isolate and culture primary caruncular epithelial cells from pregnant cows was further modified enabling work under standardized conditions when subjecting cultures to experiments. This included the usage of commercially available ready-to-use medium, culture on fibronectin-coated dishes and the establishment of

a protocol to successfully cryopreserve the cells (Paper I, II and III). All results confirmed that the morphologically identified epitheloid cells are indeed maternal caruncular epithelial cells.

Similar to the findings of Zeiler et al. (2007), epitheloid cells grew as a monolayer and expressed epithelial cytokeratin but not desmin or α-smooth muscle actin (Paper I). Only vimentin, an intermediate filament protein of mesenchymal origin (Evans, 1998), was present in both epitheloid and fibroblastoid cell types. The fibroblastoid cells were positive for desmin and α-smooth muscle actin. Vimentin expression in epithelial cells indicates the presence of initial steps towards dedifferentiation also termed epithelial mesenchymal transition (EMT). Nonetheless, vimentin has been detected in endometrial epithelial cell cultures derived from pigs, rats, humans, sheep and dogs (Zhang et al., 1991; Arslan et al., 1995; Classen-Linke et al., 1997; Johnson et al., 1999b; Galabova-Kovacs et al., 2004). Bovine primary epithelial cultures derived from non-pregnant endometrial tissue as well as the BEND cell line were shown to be positive for vimentin (Munson et al., 1988a; Betts and Hansen, 1992; Binelli et al., 2001; Yamauchi et al., 2003). All these studies concluded to have successfully cultured functional epithelial cells suggesting that the caruncular cells propagated in the present study maintained their epithelial phenotype.

EMT is a phenomenon observed in idiopathic pulmonary fibrosis and epithelial tissue-derived cancer as well as during embryonic development (Shook and Keller, 2003; Willis et al., 2005; Kokkinos et al., 2007). The major features of EMT include the loss or reduction of epithelial cell adhesion and apical-basally oriented cytoskeleton, de novo synthesis of smooth muscle actin and actin re-organization, and enhanced cell migration and invasion (Kokkinos et al., 2007). In order to rule out EMT and to assess additional features of the caruncular epithelial cells further experiments were performed.

The presence of junctional complexes and apical microvilli via ultrastructural analysis (Paper II) as observed in vivo (Leiser, 1975) as well as the expression of zonula occludens-1 protein (ZO-1; Paper I) at the lateral borders of the epithelial cells confirmed the formation of a structural intact polarized epithelial monolayer. ZO-1 as a member of the tight junctional-associated protein family (Denker and Nigam, 1998) is frequently used for the demonstration of cell polarization (Buse et al., 1995; Bowen et al., 1996; Gath et al., 1997; Weng et al., 2005; Shi and Zheng, 2005; Wakabayashi et al., 2007). Buse et al. (1995) demonstrated a disruption of ZO-1 expression after adding transforming growth factor-α (TGF-α) to the basolateral compartment of cultured mammary epithelial tumor cells. This observation was accompanied by the loss of transepithelial electrical resistance (TEER) between apical and basolateral sides of the epithelial monolayer. TEER measurement is a well known procedure

to demonstrate epithelial barrier functionality (Buse et al., 1995; Gath et al., 1997; Weng et al., 2005; Wakabayashi et al., 2007) frequently used in toxicological studies (Brown et al., 2002; Bouhet et al., 2004; Shi and Zheng, 2005). Increased TEER values correlated with the degree of confluence and were also present in cultured caruncular epithelial cells confirming the functionality of the epithelial barrier (Paper II). However, Zo-1 expression is also observed in endothelial cells as these are classified as simple squamous epithelium. Therefore, it was ruled out that the cultured cells were mistaken for endothelial cells by showing that the cells did not take up acetylated low-density-lipoprotein (Ac-LDL, Paper I) based on a well established method originally described by Voyta et al. (1984). LDL is normally metabolized by macrophages and monocytes only, but when acetylated, bovine aortic endothelial cells are capable of metabolizing LDL at an accelerated rate (Stein and Stein, 1980).

The final proof for the definite maternal origin of the cultured epithelial cells was the absence of the Y-chromosome via fluorescence in-situ hybridization (FISH) in pure epithelial cultures derived from the caruncle provided the fetus was male (Paper I). Detection of the Y-chromosome is an elegant method to discern cells derived from fetal or maternal origin and has been used to assess the frequency of polyploidization in bovine TGC (Klisch et al., 1999a; Klisch et al., 2004).

5.1.2 Integrin signaling in caruncular epithelial cells

An important feature of the bovine placenta is the occurrence of restricted trophoblast invasion (see chapter 3.1.1). This process requires complex interactions of integrin receptors on TGC, mononuclear trophoblast cells and caruncular epithelial cells with various extracellular matrix (ECM) proteins in the surrounding tissue (MacLaren and Wildeman, 1995; Johnson et al., 2001; MacIntyre et al., 2002; Pfarrer et al., 2003; Lang et al., 2004; Spencer et al., 2004). Zeiler et al. (2007) demonstrated that cultured primary caruncular epithelial cells produced laminin and fibronectin, and expressed the integrin receptors $\alpha_6\beta_1$ and $\alpha_v\beta_1$ as observed in vivo. β_1-integrin is the most common integrin subunit present in heterodimeric integrin receptors in addition to a variable and ligand-specific α-subunit (Hynes, 2002). The cytoplasmic tail of β_1-integrin interacts with the actin cytoskeleton via a variety of linking molecules such as α-actinin, focal adhesion kinase (FAK), phosphotyrosine and talin (Zamir and Geiger, 2001). In culture, activation of integrin receptors is characterized by an assembly of integrins and integrin-associated molecules as well as clustering at sites named “focal adhesions” (Wozniak et al., 2004). Thus, a further aim of this thesis was to determine whether integrin receptors within these cultured cells are activated to form “focal adhesions” and participate in “outside-in” and “inside-out” signaling pathways (Paper III). In

addition, the effect of coating cell culture flasks with ECM proteins on cell proliferation and expression of integrin subunit β_1 and focal adhesion molecules was assessed (Paper II+III).

In the bovine placentome β_1-integrin and the integrin signaling molecules FAK, phosphotyrosine, and talin were found to be expressed in specific subcellular localizations in the majority of the cells (caruncular epithelial cells in particular, Paper III). In cultured primary caruncular epithelial cells β_1-integrin was co-localized with FAK and talin, respectively, which were also co-localized with each other (Paper III). These findings clearly demonstrated that integrin-mediated signaling is functional in-vitro. In addition, the expression pattern observed at the basal compartment of the cells was typical for focal adhesions. However, phosphotyrosine co-localized with FAK, but not with β_1-integrin. In fact, both molecules were expressed side by side. Tyrosine phosphorylation is one of the key events during integrin signaling and is necessary for FAK-mediated integrin activation (Wozniak et al., 2004; Ginsberg et al., 2005). However, tyrosine phosphorylation of the cytoplasmic tail of β_1-integrin mediated by Src-kinase can inhibit talin-mediated integrin activation (Ling et al., 2003). In fact, the binding site for β_1-integrin to talin is a phosphotyrosine-binding domain (Garcia-Alvarez et al., 2003). Thus, tyrosine phosphorylation of β_1-integrin inhibits talin binding resulting in the disassembly of focal adhesions as was shown by Johansson et al. (1994). The authors developed an antibody ($PY\beta_1$) to detect tyrosine phosphorylated β_1-integrin only. In fibroblasts transfected with a transforming protein ($P60^{v\text{-}src}$) of the Rous sarcoma virus, high tyrosine phosphorylation of cellular proteins were induced and diffuse staining of β_1-integrin as well as of $PY\beta_1$ was observed. In non-transfected fibroblasts the staining pattern for β_1-integrin was characteristic for focal adhesions indicating integrin activation, whereas no $PY\beta_1$ signals were detected. This finding is consistent with various studies showing that focal adhesion disassembly via tyrosine phosphorylation and mediated by Src-family kinases is an important process in cells participating in hemostasis, migration and transformation (Ratnikov et al., 2005). These features are not characteristic for caruncular epithelial cells. Hence, the co-localization of phosphotyrosine with FAK but not with β_1-integrin in addition to co-localization of FAK with β_1-integrin suggests integrin activation mediated by FAK to be present. On the other hand, the absence of phosphotyrosine-β_1 co-localization, observed in the caruncular epithelial cells, enables talin-mediated integrin activation which is considered indispensible and plays an important role in “inside-out” integrin signaling (Ginsberg et al., 2005).

The co-localization of α-actinin with β_1-integrin at the cell membranes of neighboring caruncular epithelial cells showed once more that integrin signaling is functional in caruncular

epithelial cells in-vitro (Paper III). In contrast to the integrin signaling described above, this result suggests that the primary function of α-actinin-mediated integrin signaling in the bovine placentome is to participate in feto-maternal interaction via apical cytoplasmic membranes rather than basement membrane-ECM adhesion and signaling. α-actinin is a further actin-integrin linking protein and crucial for integrin activation (Greenwood et al., 2000; Wozniak et al., 2004). As indicated, α-actinin expression was not observed either at sites of focal adhesions towards the basal compartment of the cell or at the outer borders of the cell colonies. A similar expression pattern was found in-vivo where α-actinin was predominantly detected in the apical compartment of the caruncular epithelial cells (Paper III; Lang et al., 2004). Apical α-actinin linking to β-integrin subunits as a response to osteopontin-mediated trophoblast-endometrial signaling in the ovine and porcine endometrial epithelium as well as cultured epithelial and trophectodermal cells has been demonstrated (Johnson et al., 2001; Garlow et al., 2002). The authors concluded that osteopontin binding to integrin heterodimers induce focal adhesion sites that promote and stabilize attachment of trophoblast to the endometrial epithelium thereby influencing conceptus development, adhesion and placentation.

ECM-proteins, fibronectin, laminin and collagen in particular, are major ligands of β_1-integrin receptors (Hynes, 2002). Thus, coating cell culture flasks with these matrices may influence integrin expression and activation and subsequent cell behavior. Indeed, coating dishes with fibronectin derived from bovine plasma, laminin derived from human placenta and collagen type IV derived from bovine placental villi significantly increased proliferation in comparison to uncoated dishes (Paper III). Fibronectin-induced proliferation was even significantly higher than proliferation induced by laminin and collagen IV. In addition, the amount of focal adhesions increased markedly in comparison to the uncoated control when detecting β_1-integrin, phosphotyrosine and talin. Thus, increased proliferation and expression of focal adhesions as a response to fibronectin coating, is likely to be mediated by integrin signaling. Fibronectin as a major component of the extracellular matrix is, amongst other locations, expressed in the basement membrane of the caruncular epithelium (Pfarrer et al., 2003). Furthermore, Zeiler et al. (2007) demonstrated that primary caruncular epithelial cells produce fibronectin and laminin in culture. In addition, the expression of the integrin subunits α_6 and β_1 (capable of forming the laminin receptor) as well as α_v and β_1 (capable of forming the fibronectin receptor) was shown. The potency of fibronectin to enhance culture growth was confirmed when comparing TEER measurements performed with cells cultured in uncoated and inserts coated with fibronectin or collagen (Paper II). Fibronectin coating led to

an earlier confluence and subsequently induced higher TEER values at an earlier stage. The involvement of integrin-mediated signaling in the process of proliferation is well known (Yelian et al., 1995; Guan, 1997; Oktay et al., 1999). On the one hand, integrins cooperate with growth receptors producing synergistic stimulation of one or more mitogenic pathways. On the other hand, integrin-mediated induction of cell proliferation (progression through the G1 phase of the cell cycle) can be induced independent of the growth receptor-associated pathways (Oktay et al., 1999). Furthermore, these authors demonstrated that this induction is mediated by FAK and in the presence of fibronectin. High proliferation rates are a unique property of caruncular epithelial cells in-vivo (Schuler et al., 2000) which may be necessary for tissue remodeling and compensation of the destructive activity of the invading TGC. Furthermore, FAK may play an important role in this event due to the fact that phosphotyrosine linked focal adhesions (crucial for FAK-mediated integrin signaling) was increased in caruncular epithelial cells cultured on fibronectin-coated dishes (Paper III).

5.2 Primary cotyledonary cells

5.2.1 Characterization of cotyledon-derived epitheloid shaped cells

Establishing and maintaining cultures of primary trophoblast cells derived from cotyledonary tissue was found to be a very difficult task (Paper I). In only approximately half of the attempts to isolate this cell population, epitheloid colonies could be identified. However, most of these colonies were outnumbered by fibroblastoid cells resulting in further losses of cultures when trying to diminish the contaminating cells. Culturing the cotyledon-derived epitheloid shaped cells in Quantum 286 medium (Q286) in comparison to the standard used DMEM/Ham's F12 medium had no positive effect on the outcome of the procedure (Paper I). This observation may be related to the fact that Q286 is a DMEM based medium (according to the manufacturer). DMEM alone or in combination with Ham's F12 is a commonly used basic medium applicable for the cultivation of almost all cell types (Freshney, 2005). This medium has been successfully used to culture primary bovine endometrial epithelial and trophoblast cells (Munson et al., 1988a; Munson et al., 1988b).

Since a long term goal of our research group was to develop a co-culture model including both caruncular epithelial and trophoblast cells, the prerequisite for this model was the capability to distinguish between both populations. Until then both epitheloid cultures derived from the caruncle and cotyledon could neither be differentiated on a morphological basis nor based on the expression pattern of proteins. In addition to the expression of cytokeratin, zonula-occludens-1, vimentin and selected integrin subunits, both epitheloid cell

cultures expressed the estrogen receptor-α (ERα) and were negative for connexin (cx)43 and cx32 (unpublished data). In-vivo, ERα was present in the caruncular epithelium, but not in the trophoblast (Schuler et al., 2002). Gap junctional cx43 was expressed in the trophoblast but not in the caruncular epithelium (Bridger et al., 2005; Pfarrer et al., 2006a), whereas cx32 was solely found in caruncular epithelial cells at the tips of the maternal septa (Pfarrer et al., 2006a). The reason for the inability to discern the potentially fetal (trophoblast) and maternal (caruncular) epithelial cells became evident when fluorescence in-situ hybridization (FISH) demonstrated that all cells with epitheloid morphology were of maternal origin independent of the placental compartment from where they were isolated (Paper I). Thus, manual separation of the caruncle and cotyledon cannot be performed without leaving bits of neighboring tissue in the individual compartment. This is the first report to describe the contamination of cotyledonary samples with maternal cells. This fact should always be considered when performing experiments with samples either from maternal or fetal placentomal tissue. Depending on the sensitivity of the method applied, false positive results may occur not only when culturing cells but also when using tissue homogenates.

Considering the fact that in-vivo both maternal and fetal compartments interdigitate deeply (Leiser et al., 1998), one should assume that manual separation is likely to result in remaining bits of neighboring tissue in the individual compartment. Such a “contamination” has been described for the presence of fetal tissue in caruncular samples but not vice versa (Shemesh et al., 1984c; Reimers et al., 1985; Shemesh et al., 1994). Reimers et al. (1985) considered the contamination to be so severe that it may lead to false positive results. In their study the authors isolated primary TGC, which can easily by differentiated from the other cell types based on their size and binucleate morphology. In the thesis presented here, FISH analysis was also performed on cells directly after enzymatic disaggregation prior to seeding (Paper I), revealing that both suspensions (caruncle and cotyledon) contained cells of the neighboring compartment. When analyzing the attempts to isolate trophoblast cells from the cotyledon in a retrospective manner, half of the primary cotyledonary cell cultures included at least one (maternal) epitheloid cell colony independent of the gestational age. These maternal epithelial cells may be less demanding concerning culture conditions than trophoblast cells allowing a minor contamination in cotyledonary-derived cell suspensions to result in outgrowth of maternal cells in cell culture dishes. This hypothesis is supported by the fact that Fortier et al. (1988) and all the following studies based on the method of this group cultured primary endometrial epithelial cells in RPMI 1640, a medium which is usually used for lymphoid cultures (Freshney, 2005). Betts and Hansen (1992) cultured primary endometrial

cells in medium consisting of 40 % Eagle's minimal essential medium (MEM), 40 % Ham's F12, 10 % FCS, 10 % defined horse serum and 0.1 IU/ml insulin. A similar recipe was used to culture the BEND cell line (Hansen et al., 1997; Staggs et al., 1998). On the other hand Munson et al. (1988b) compared various culture conditions in order to enhance trophoblast growth resulting in the conclusion that the addition of certain supplements is required to significantly increase trophoblast proliferation (see next chapter).

5.2.2 Characterization of polygonal shaped cells

During the course of the thesis a third polygonal shaped cell population was identified in cultures derived from the cotyledon (Paper I). Extensive characterization confirmed the polygonal shaped cells to be trophoblast cells, as they expressed cytokeratin, zonula occludens-1 and vimentin but not α-smooth muscle actin and desmin. In addition, the cells showed no uptake of Ac-LDL. FISH analysis demonstrated that the nuclei possessed the Y-chromosome confirming the fetal origin of these cells. A preliminary experiment (immunofluorescence) demonstrated the polygonal cells to be positive for cx43 in contrast to the epitheloid shaped cells (unpublished observations) suggesting cx43 to be a potential marker for the identification of trophoblast cells. Munson et al. (1988b) described a similar polygonal phenotype of cultured trophoblast cells. However, these cells were only characterized based on their morphology and their capability to generate large binucleate cells (supposedly TGC) after some days in culture. In addition, all cells expressed both cytokeratin and vimentin. Therefore, Munson et al. (1988b) seem to be the only authors describing a method to isolate and cultivate mononuclear trophoblast cells from the cotyledon.

During the course of this thesis, culturing polygonal cells still remained difficult, because the isolation procedure resulted in highly heterogeneous primary cell cultures. The subculturing procedure (two-step trypsination) which successfully removes contaminating cells from the caruncular epithelial cultures was not as efficient due to the less adhesive properties of the trophoblast cell colonies in comparison to the caruncular cells. Thus, further modifications of the isolation and cultivation protocol are necessary.

A more "aggressive" digestion procedure of the tissue may result in a higher number of isolated primary trophoblast cells. Collagenase type I, as used in the current protocol, is considered to be a moderate digesting enzyme whereas collagenase type II used by Munson et al. (1988b) is ranked one step higher on the enzyme digestion scale (Worthington, 2007). Trypsin is ranked the highest and is a commonly applied enzyme in primary cell culture (Freshney, 2005), although it should be kept in mind that "aggressive" digestion may lead to alterations in the properties of the cell membrane and subsequent functional aberrations

(Wolffe and Tata, 1984). Trypsin was used by Feng et al. (2000a) who isolated polygonal shaped cells from the cotyledon which were finally identified as cells of endothelial origin (2000b). Munson et al. (1988b) compared trypsin and collagenase II digestion of cotyledonary tissue and conclude that the viability was significantly greater with collagenase than with trypsin (85 vs 50 %).

Munson et al. (1988b) compared different culture conditions (DMEM/Ham's F12 with or without supplements) whilst generating trophoblast cell lines. Adding epidermal growth factor (EGF), insulin, transferrin and selenium increased proliferation significantly in comparison to culture conditions without or only single use of these additives. The absence of these supplements may explain the low cell yield in the present study. Shimada et al. (2001) cultured their bovine trophoblast cell line (BT-1) in fibroblast conditioned medium based on DMEM/Ham's F12. Attempts to reproduce these culture conditions had no positive effect on the yield of polygonal cells isolated from cotyledons (unpublished observations). However, it should be kept in mind that BT-1 was isolated from a blastocyst which is present long before the placenta is established and therefore BT-1 cells are likely not to be as differentiated as cotyledonary trophoblast cells.

Overall, the low number of publications reporting on experiments with cotyledonary (mononuclear) trophoblast cells reflects the difficulty of this procedure.

The isolation of TGC has been performed more successfully (Reimers et al., 1985; Gross and Williams, 1988; Nakano et al., 2001; Bainbridge et al., 2001; MacIntyre et al., 2002; Landim, Jr. et al., 2007). This is probably due to the large size of TGC, which allows the application of gradient centrifugation and sedimentation methods helping to purify the cell suspensions prior to seeding. One way to gain a higher percentage of mononuclear cells in the primary cell suspension may be to adapt this approach.

5.3 Bovine caruncular epithelial cell line (BCEC-1)

After validating and improving the method to successfully isolate and culture primary caruncular cells, a continuous cell line was generated which allowed the application of standardized experimental conditions when subjecting the cells to further research on trophoblast invasion, feto-maternal communication, transport and infection in the bovine placenta (Paper II). To date, the only available endometrial cell line derived from a comparable region, namely the BEND cell line (Johnson et al., 1999a), had limitations regarding its use as a model to study physiology and pathology of pregnancy, because the cells were isolated from a non-pregnant endometrium without the differentiation between

caruncular and intercaruncular regions. However, Asselin et al. (1998) demonstrated this differentiation is indeed necessary. The authors showed that amongst other results, significant differences in the prostaglandin-$F_{2\alpha}$ ($PGF_{2\alpha}$) response to oxytocin stimulation in endometrial epithelial cells derived from caruncular vs. intercaruncular areas.

5.3.1 Establishment of BCEC-1

The prerequisite for the development of a continuous cell line is that the cells become "immortal" and maintain their cell-specific properties throughout their life span. This transformation process can be initiated either actively (using mutagens or oncogens) or passively (spontaneous immortalization) (Freshney, 2005). The latter requires careful subculturing of a primary cell culture hoping that some cells survive the normally genetically terminated senescence. This was indeed the case when primary caruncular epithelial cells were subcultured 32 times over a period of approximately nine months (Paper II). However, a marked decrease of cell viability and proliferation was observed between passage 7 and 12 (Paper II). Beyond this point viability and proliferation increased significantly resulting in a relatively constant growth pattern until passage 30. Similar observations were made when the the human breast epithelial cell line MCF-10 was established. MCF-10 transformed (spontaneously immortalize) between passages 2-7 and 12 (Soule et al., 1990). In addition, the BEND cell line (Binelli et al., 2000) and the trophoblastic cell lines established by Munson et al. (1988b) were kept in culture for at least 25 and 46-48 passages, respectively, without active immortalization procedures. Despite the fact that Binelli et al. did not show evidence for their findings, both cell lines were reported to stain positive for cytokeratin and vimentin (Binelli et al., 2001). BCEC-1 was characterized every 5 (±1) passages and cytokeratin, ZO-1 and vimentin but not desmin and α-smooth muscle actin were detected in each experiment (Paper II) corresponding to the findings in the primary cultures (see chapter 5.1.1). After the successful adaption to the Transwell® insert model system further properties of the cells were assessed (Paper II). Transmission and scanning electron microscopy confirmed that the cells form an intact polarized epithelial monolayer. Corresponding to the findings in vivo (Leiser, 1975), apical microvilli and junctional complexes were expressed. Furthermore, TEER values were significantly increased after confluence was reached confirming epithelial barrier functionality. The formation of an intact and functional barrier is a characteristic feature of epithelial cell lines and prerequisite for studies focusing on properties and integrity of the epithelium (Buse et al., 1995; Brown et al., 2002; Wakabayashi et al., 2007). Culturing BCEC-1 on fibronectin or collagen A-coated inserts resulted in an enhanced proliferation and subsequent earlier increase of TEER in comparison to uncoated

inserts (Paper II). Although not statistically analyzed, fibronectin coating seemed superior in comparison to collagen A. As described and discussed above (see chapter 5.1.2), fibronectin-coated dishes lead to an enhancement of cell proliferation in primary caruncular epithelial cells (Paper III). Coating dishes and inserts with fibronectin is an additional step to improve culture conditions and is now a standard procedure when working with primary caruncular epithelial cells or the BCEC-1 cell line.

5.3.2 Future applications of BCEC-1 (Outlook)

BCEC-1 is the first caruncular epithelial cell line derived from a pregnant cow and has successfully passed the first and major steps of characterization. However, more work is necessary including the comparison of cultures from different passages by karyotype and DNA fingerprint. Continuous cell lines are usually aneuploid including heteroploidy within the population and due to their "tumorigenic" character, gene alterations may occur (Freshney, 2005). Furthermore the cells should be assessed for the maintenance of further properties specific for the tissue in-vivo. In the case of caruncular epithelial cells this could include their prostaglandin response to oxytocin, sex steroids and/or interferon-tau as described for primary cultures of endometrial cells (Asselin et al., 1996; Asselin et al., 1997a; Asselin et al., 1997c; Staggs et al., 1998; Asselin et al., 1998).

The culture in Transwell® inserts that mimicks an intact epithelial barrier, introduces the BCEC-1 in-vitro model for various applications. This includes (1) co-culture invasion assays with TGC or tumor cells, (2) studies on pathways of transport, communication and metabolism during pregnancy and (3) pathways of pregnancy-associated diseases.

(1) The isolation of primary TGC at a high percentage of the whole cell suspension has been performed successfully as described above (Reimers et al., 1985; Gross and Williams, 1988; Nakano et al., 2001; MacIntyre et al., 2002; Landim, Jr. et al., 2007). However, culturing TGC over a longer time period as described by Landim et al. was only possible because mononuclear trophoblast cells continuously differentiating into TGC were obviously present in those cultures. This would not have been possible with TGC alone, because TGC have a finite life span and do not proliferate (Wooding and Flint, 1994). Until now the process of restricted trophoblast invasion has not been studied in-vitro. Using a confluent monolayer of BCEC-1 and applying primary TGC to the apical surface may make the observation of this process possible on a local cellular basis. Helpful tools could be live cell imaging and ultrastructural analysis as described above. Furthermore, factors supposedly involved in this process can be assessed, i.e. integrins and extracellular matrix proteins (Pfarrer et al., 2003; Zeiler et al., 2007), growth factors such as fibroblast growth factors and vascular endothelial

growth factors (Pfarrer et al., 2005; Pfarrer et al., 2006b) and hormones, in particular bovine placental lactogen (Wooding, 1992). Munson et al. (1991) demonstrated the attachment of 14 day old blastocysts to primary cultures of polarized bovine endometrial epithelial cells in-vitro using ultrastructural analysis and immunohistochemistry. In addition, Ludwig et al. (2002) demonstrated a TEER breakdown when tumor cells and their secreted proteinases were added to a monolayer of Madin Darby canine kidney (MDCK) cells. Both publications suggest the BCEC-1 model to be a useful tool for further investigations in this field. The identification of factors involved in the regulation of TGC invasion may not only increase the understanding of this process in bovine pregnancy but may also be the basis for new approaches in cancer research and treatment.

(2) The feto-maternal interface, especially within the placentomes, is the center of feto-maternal communication and nutritional exchange. Pathways and metabolism of these processes are difficult to assess in-vivo due to the heterogeneity of the surrounding tissue. The insert model system can be used to assess these pathways from the basal to the apical compartment and vice versa. This could be of interest, especially for the study of transport mechanisms used by steroids, as they have a major impact on the outcome of pregnancy (Hoffmann and Schuler, 2002; Spencer and Bazer, 2004). This not only includes endogenous metabolites but also exogenous (environmental) substances, e.g. phytoestrogens (Woclawek-Potocka et al., 2005) and pharmacological agents (Hornof et al., 2005). Protein transport in an insert model system was investigated by Wyrwoll et al. (2005), who showed directional transport of lectins in human choriocarcinoma BeWo cells. Identifying transport mechanisms may help find new targets for the treatment and prevention of pregnancy-associated disorders in the bovine which are related to dysfunction in the feto-maternal interface. Furthermore, BCEC-1 could serve as part of a screening assay for toxicological studies.

(3) As described above, the bovine placenta serves as a barrier which generally protects the fetus from infectious and (feto-) toxic agents. Nonetheless, some agents are capable of bypassing this barrier which can lead to severe pregnancy-associated disorders (for both mother and fetus) including abortion. In order to introduce BCEC-1 as a model to study pregnancy-associated infectious diseases the cells were tested for the presence of BVDV, a virus of great importance in bovine medicine (see chapter 2.3.2.). All experiments showed that the cells were negative for the virus but susceptible to infection (Paper II). To date, the majority of all in-vitro studies with BVDV have been performed using the Madin Darby bovine kidney cell line (MDBK). However, the kidney is not affected by BVDV infection and vertical transmission. Thus, BCEC-1 may be an alternative as it is a cell line derived from a

tissue involved in the pathogenesis of the disease. Viral and cellular mechanisms which participate in the vertical transmission can be studied and in the future will possibly lead to the development of new prevention (e.g. vaccines) and treatment strategies. In a comparable insert culture model of human bronchial epithelial cells (Calu-3), Tseng et al. (2005) demonstrated the entry and release of the severe acute respiratory syndrome corona virus (SARS-CoV) almost exclusively from the apical border. BCEC-1 may also be the basis for future studies on infectious diseases that can lead to severe reproductive disorders such as bovine rhinotracheitis (IBR), brucellosis, listeriosis, aspergillosis and neosporosis (Anderson, 2007). Many mechanisms involved in placental bypass and/or infection are still unknown. In-vitro models, cell cultures in particular, may not replace experiments in-vivo but may be more efficient in generating preliminary results and thus replace animal experiments to a certain degree.

5.4 Conclusion

This thesis verified and modified a method to isolate primary caruncular epithelial and trophoblast cells isolated from a bovine placentome. The importance of appropriate cell type identification and characterization to generate reliable results was highlighted. The fact that primary caruncular epithelial cells maintain a variety of epithelial-specific features in culture and participate actively in integrin-mediated “outside-in” and “inside-out” signaling pathways confirms that this model can be applied for studying the involvement of integrin signaling pathways in bovine placentation (physiology and pathology). The BCEC-1 cell line exhibits properties of differentiated epithelial cells and is the first caruncular epithelial cell line derived from a pregnant cow, thus offering unique possibilities to study the role of the caruncular epithelium in feto-maternal interaction. Using the culture in Transwell® insert membranes, more properties of the maternal part of the placental barrier can be assessed and also introduces the model for various new applications, e.g. transport of pharmacologically active substances and models to study the pathogenesis of pregnancy-associated diseases. Therefore the BCEC-1 line has been patented for commercial use (PCT/DE 2007 001274).

6 Summary

A unique feature of bovine synepitheliochorial placenta is the occurrence of “restricted” trophoblast invasion characterized by the migration and fusion of trophoblast giant cells (TGC) with single maternal epithelial cells. Unlike tumor invasion, this process is limited to the depth of the caruncular epithelium suggesting the bovine placenta to be an ideal model to study mechanisms regulating cellular invasion. In-vitro models of the reproductive tissues involved are lacking though these models would also allow the assessment of pathogenesis of pregnancy-associated diseases und thus reduce the number of animal experiments.

The thesis presented discusses an interconnection of three articles published in peer reviewed journals ([1] Theriogenology, 2007; 68 (4):592-603; [2] Placenta, 2007; 28 (11-12):1110-1117; [3] Biology of Reproduction, 2008; in press) focusing on the characterization and functional assessment of primary caruncular epithelial cells as well as the development of a cell line.

Primary caruncular epithelial cells were successfully isolated and cultured from manually separated caruncles from the third month of gestation onwards. Fluorescence in-situ hybridization (FISH) not only confirmed the maternal origin of these cells but also demonstrated a contamination of cotyledonary-derived cultures with maternal epithelial cells. An extensive phenotypic and functional characterization defined these cells to be of epithelial origin forming an intact epithelial monolayer in culture. The cells grew as a monolayer, and via transmission and scanning electron microscopy a polarized morphology with apical microvilli and junctional complexes was demonstrated. Expression of epithelial-specific cytokeratin and Zonula occludens-1 protein as well as vimentin but not α-smooth muscle actin and desmin was shown by immunofluorescence. Furthermore, transepithelial electrical resistance measurements (TEER) of cultures grown on Transwell® inserts confirmed the presence of an intact epithelial barrier. The expression and co-localization of integrin subunit β_1 and associated signaling molecules (α-actinin, focal adhesion kinase, phosphotyrosine and talin) showed that caruncular epithelial cells actively participate in integrin-mediated “inside-out” and “outside-in” signaling pathways. Culture on dishes coated with proteins of the extracellular matrix, fibronectin in particular, resulted in increased proliferation and enhanced the expression of integrin and integrin-associated signaling molecules. By continuous subculture and spontaneous immortalization of the first caruncular epithelial cell line (BCEC-1) derived from a pregnant cow was established. The cells maintained their epithelial properties as shown for primary cells for up to 32 passages. In

addition, the cells were tested negative for the BVDV but remained susceptible to infection. In order to introduce this model for commercial use (i.e. screening essays), BCEC-1 has been patented (PCT/DE 2007 001274).

In conclusion, the thesis demonstrates the importance of appropriate cell characterization and identification especially when cells are isolated from epitheliochorial placentae. The phenotypic and functional properties of primary caruncular epithelial cells and BCEC-1 suggest that both models provide excellent potential for further functional studies, i.e. co-culture invasion assays as well as the physiology and pathology of feto-maternal interaction and pregnancy-associated diseases. In-vitro models, cell cultures in particular cannot completely replace experiments in-vivo however may be more efficient in generating preliminary results, and subsequently less animal experiments will be required.

Supported by a grant of the German Research Foundation (DFG).

7 Zusammenfassung (German)

Eine Besonderheit der bovinen synepitheliochorialen Plazenta ist die „eingeschränkte“ Trophoblastinvasion, die durch migrierende Trophoblastriesenzellen (TGC) charakterisiert ist, welche jeweils mit einer einzelnen maternalen Epithelzellen fusionieren. Im Gegensatz zur Tumorinvasion ist die Trophoblastinvasion gerichtet und ausschließlich auf das karunkuläre Epithel beschränkt. Daher ist es naheliegend die bovine Plazenta als Modell zur Untersuchung von zellulären Invasionsmechanismen zu verwenden. Bislang gibt es jedoch keine geeigneten in-vitro Modelle aus den Geweben, die an der Trophoblastinvasion beteiligt sind. Derartige Modelle würden auch Studien zur Pathogenese Trächtigkeits-assoziierter Erkrankungen ermöglichen und somit auch zur Reduktion von Tierversuchen beitragen.

Diese kumulative PhD-Arbeit diskutiert drei begutachtete und publizierte Manuskripte ([1] Theriogenology, 2007; 68 (4):592-603; [2] Placenta, 2007; 28 (11-12):1110-1117; [3] Biology of Reproduction, 2008: in press), die sich schwerpunktmäßig mit der Charakterisierung und funktionellen Untersuchung von primären bovinen Karunkelepithelzellen sowie der Etablierung einer Zelllinie befassen.

Primäre Karunkelepithelzellen wurden erfolgreich aus manuell separierten Karunkeln (3.-9. Trächtigkeitsmonat) isoliert und kultiviert. Die maternale Herkunft dieser Zellen sowie die Kontamination primärer kotyledonärer Kulturen mit maternalen Zellen wurden mittels Fluoreszenz in-situ Hybridisierung gezeigt. Eine umfangreiche phänotypische und funktionelle Charakterisierung bestätigte die epitheliale Herkunft der Zellen sowie die Ausbildung einer intakten epithelialen Barriere in-vitro. Mittels Transmissions- und Rasterelektronenmikroskopie konnte die epithelspezifische polarisierte Morphologie mit apikalen Mikrovilli und Schlussleistenkomplexen des zellulären einschichtigen Zellrasens gezeigte werden. Die Immunfluoreszenz ergab, dass die Zellen epitheliales Zytokeratin und Zonula occludens-1 Protein aber auch Vimentin exprimierten. Dagegen bilden die Zellen kein α-smooth muscle Aktin und Desmin aus. Messungen des transepithelialen Widerstandes konfluent wachsender Zellen, die in Transwell® inserts kultiviert wurden, zeigten das Vorhandensein einer intakten epithelialen Barriere. Die Expression und Kolokalisation der Integrin Untereinheit β_1 und Integrin-assoziierten Signalmolekülen (α-Actinin, Focal Adhesion Kinase, Phosphotyrosin und Talin) zeigte, dass Karunkelepithelzellen in-vivo und in-vitro aktiv an Integrin-vermittelten „outside-in“ und „inside-out“ Signalwegen beteiligt sind. Das Kultivieren der Zellen in mit Proteinen der extrazellulären Matrix (Fibronectin im Besonderen) beschichteten Gefäßen, beschleunigte die Zellproliferation und erhöhte die

Expression von Integrin β_1 und dessen assoziierten Signalmolekülen. Durch fortwährende Subkultivierung und der daraus resultierenden spontanen Immortalisierung konnte die erste bovine Karunkelepithelzelllinie (BCEC-1) aus einem trächtigen Tier generiert werden. Die Zellen behielten ihre epithelialen Eigenschaften bis zur 32. Passage bei. Weiterhin wurden die BCEC-1-Zellen negativ für das Vorhandensein des BVD Virus getestet. Andererseits konnten die Zellen mit dem Virus infiziert werden. Um BCEC-1 dem kommerziellen Gebrauch anzubieten, wurden verschiedene Anwendungsmöglichkeiten patentiert (PCT/DE 2007 001274).

Schlussfolgernd zeigt diese Arbeit die Bedeutung einer adäquaten Charakterisierung und Identifizierung von Zellen, vor allem wenn diese aus epitheliochorialen Plazenten isoliert werden. Die phänotypischen und funktionellen Eigenschaften von primären Karunkel-epithelzellen und BCEC-1 stellen ein hervorragendes Potential für zahlreiche weitere Studien dar. Dazu könnten Kokultur-Invasionsassays und Modelle zur Untersuchung der Physiologie und Pathologie Trächtigkeits-assoziierter Erkrankungen zählen. In-vitro Modelle, Zellkulturen im Besonderen, können Tierversuche nicht vollständig ersetzten. Dennoch sind sie in der Generierung vorläufiger Ergebnissen sehr effizient. Diese wiederum könnten unnötige Tierversuche vermeiden.

Diese Arbeit wurde durch die Deutsche Forschungsgemeinschaft (DFG) gefördert.

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9 Acknowledgments

First of all I would like to thank my supervisor and mentor Prof. Dr. Christiane Pfarrer for her exemplary help and support during the whole period of the thesis. She always had time and an open mind for fruitful discussions, the review of abstracts, posters, presentations and manuscripts as well as personal concerns. Her encouragement and support to attend both national and international scientific meetings introduced me to the fascinating field of research, a field which I hope to remain working in as long as I can. Her personality and character taught me a lot about dealing with challenging situations and communication with others. “Dear Christiane, you made my career!”

Secondly, I would like to thank Prof. Dr. Leiser, who was head of the Department of Veterinary Anatomy when I started my thesis. His door was always open for scientific and personal discussions including the revision of paper work named above. His dedication to the field of placentology surely had impact on the great interest in my work.

I would not have managed to have performed the experiments and analysis without the help and guidance of my colleagues and co-workers in the Institute of Veterinary Anatomy. In particular, I would like to thank K. Wolf-Hofmann, S. Schubert-Porth, S. Kettner and M. Fink for the excellent technical advice and support. Furthermore, special thanks go to Dr. M. Zeiler, Dr. K. Bücher, Dr. S. Hartmann and Dr. R. Brehm for the introduction into the methods including various discussions about the theoretical background and interpretation of the data. I will not forget V. Kowalewski, our good spirit in the office who keeps everything organized and everyone together. “To you all: I had a great time and I will surely miss you!”

Special thanks go to my colleague and friend Dr. S. Haupt. We shared the same office and laboratory, had to cope with the same or similar problems and could rely on each other. “Dear Sanne, without your support and discussions I would not have managed to get this far.”

During the course of this thesis and due to the great collegiality of the people involved, I had the opportunity to gain access to methods, equipment and fruitful discussions in other facilities of the Faculty of Veterinary Medicine and Medicine of the Justus-Liebig University. Therefore, I would like to thank H. Greven and Dr. G. Schuler (Clinic of Obstetrics, Gynecology and Andrology for Large and Small Animals), Dr. C. Rummel and Prof. Dr. R. Gerstberger (Institute of Veterinary Physiology), Dr. P. König and Prof. Dr. W. Kummer (Institute of Anatomy and Cell Biology), S. Engel and Dr. W. Hecht (Institute of Veterinary Pathology), Dr. A. Taubert (Institute of Veterinary Parasitology), M. Mohr and Dr. C. Menge (Institute for Hygiene and Infectious Diseases of Animals), E. Lattwein and Prof. Dr. N. Tautz

(Institute of Veterinary Virology), A. Hild (Laboratory of Experimental Trauma Surgery) and Dr. M. Hardt (Central Biotechnical Unit).

In addition to this, I would like to thank Dr. K. Klisch (Neuroanatomy, Medical School Hannover, Germany) for the introduction of methods performed in his laboratory, the very helpful and frequent communications and in particular his and his family's hospitality. "Dear Karl, I wish you all the best for your new challenge in Nottingham."

The foundation of this thesis was a reliable scholarship and the membership in the graduate seminar "Molecular Veterinary Medicine, GK455" supported by the German Research Foundation (DFG). The GK455 can and should serve as an example for others and I hope that the faculty will manage to establish a comparable program in the near future. In particular, I would like to thank both chairmen (and my mentors) Prof. Dr. E. Petzinger and Prof. Dr. R. Bauerfeind whose dedication to organize a superior program of lectures, seminars and practical training gave us the opportunity to obtain an additional qualification in the field of science. Special thanks go to J. Heber, our "mother" and the manager behind the scenes and of course to all my colleagues. "Dear all: we had a great time together, during and after the courses!"

I am also grateful to the PhD-Program of the faculties of Medicine and Veterinary Medicine. Special thanks go to Prof. Dr. H. M. Pieper and L. Stamer to have established an internationally accepted degree to the Justus-Liebig University.

I would also like to thank my current Supervisors Prof. Dr. G. Baljer and Dr. C. Menge (Institute for Hygiene and Infectious Diseases of Animals) for giving me the time and patience to complete my thesis in the Institute.

My gratitude goes to my family, my parents in particular, who supported me during the whole course of my study of veterinary medicine and thesis. Though they would have liked to see me counting elephants in Africa, I can say that I am counting cells, which may be a little smaller but much more interesting!

Last but not least my thanks go to my beloved wife Nicole. She has been the main reason for me to return to Giessen and perform my thesis here, a decision I am very grateful for, have never regretted and never will regret. The past years have been full of exciting events of which our marriage was the most extraordinary. "Dear Nicole, I am looking forward to a joint and happy future with you, I love you!"

"Und am allermeisten danke ich M.U.T.I.G. '99!"

10 Curriculum vitae

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11 Annexes

Paper I

Paper II

Paper III

Available online at www.sciencedirect.com
ScienceDirect
Theriogenology 68 (2007) 592–603
Theriogenology
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Validation of primary epitheloid cell cultures isolated from bovine placental caruncles and cotyledons

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Received 22 January 2007; received in revised form 4 April 2007; accepted 5 May 2007

Abstract

In order to study feto-maternal interactions in the bovine synepitheliochorial placenta primary cell cultures of both placentomal components throughout pregnancy, namely caruncular epithelial cells and trophoblast cells were developed. The aim of this study was to validate and improve a method to culture caruncular epithelial cells and fetal trophoblast from manually separated placentomes. Prior to seeding the presence of fetal cells in caruncular samples and vice-versa could be demonstrated by the detection of the Y-chromosome via fluorescence in situ hybridization (FISH) provided the fetus was male. Epitheloid shaped cells present in both cultures (cotyledon and caruncle) were characterized on a morphological basis as well as by immunofluorescence and Western blot thereby detecting cytokeratin, zonula occludens-1 and vimentin but not α-smooth muscle actin and desmin. The absence of the Y-chromosome demonstrated the caruncular origin of epitheloid cells. In addition, a population of polygonally shaped cells derived from the cotyledon was propagated and displayed the same cytoskeletal characteristics as described above. The presence of the Y-chromosome confirmed the fetal origin of these cells and the lacking uptake of fluorescence conjugated low density lipoprotein, specific for endothelial cells, identified polygonally shaped cells as fetal trophoblast cells. In conclusion, the cross-contamination of maternal and fetal cells in manually separated placentomes should be considered in future experiments as it may lead to false positive results dependent on the sensitivity of the method applied. This study highlights the importance of an appropriate cell characterization and identification, especially when isolating primary cells.

Keywords: Cow; Placentome; Cell culture; Fluorescence in situ hybridization; Cytoskeleton

1. Introduction

In the bovine placenta, the key sites of feto-maternal interaction are placentomes protruding into the amnion/allantoic cavity. These placentomes consist of two components: the fetal cotyledon and the maternal caruncle, which interdigitate and create an extensive branching network of fetal villi and maternal septa [1]. This leads to a firm anchorage between both compartments [2]. Both tissues are delimited by epithelial layers, trophoblast and maternal epithelium, respectively, defining the bovine placenta as synepitheliochorial. A special feature of this placenta is the occurrence of a ‘‘restricted’’ trophoblast invasion [3]. Thereby trophoblast giant cells (TGC), which evolve from mononuclear trophoblast cells by acytokinetic mitosis, migrate towards the maternal epithelium, fuse with an epithelial cell and degenerate having released a variety

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doi:10.1016/j.theriogenology.2007.05.046

of substances [4,5]. This process indicates the presence of specific regulatory mechanisms in TGC and its surrounding tissues controlling migration and invasion. To study this process on a local basis, cell cultures of the cells involved, namely TGC and mononuclear trophoblast as well as maternal epithelial cells, are of great interest.

A variety of studies describe the isolation and cultivation of primary epithelial cells from the endometrium of non-pregnant cows [6–9]. In most cases no differentiation between caruncular and intercaruncular tissue was performed. That this kind of differentiation is indeed necessary can be concluded from in vivo and in vitro experiments showing that both areas have different properties. For example significant differences in the production of prostaglandins were observed between isolated primary caruncular and intercaruncular epithelial cells [10]. These findings correlate with studies describing differences in protein and gene expression of the caruncular and intercaruncular tissue, especially during pregnancy [11–13]. When cells from caruncles of pregnant animals were cultured no differentiation was performed between the cell types [14].

One of the main difficulties in isolating cells from the caruncle is the fact that the manual separation of both fetal and maternal components leaves bits of cotyledonary tissue in the caruncular crypts [14–16]. However, the contamination of manually separated cotyledons with maternal tissue has not been observed so far [14,17].

In contrast to endometrial cell cultures originating from pregnant and non-pregnant animals, trophoblast cell cultures (not TGC) isolated from cotyledonary tissue are a rare finding. Munson et al. [18] described a method of isolation and long term cultivation of trophoblast cells and characterized the cells as polygonal uninucleated cells expressing cytokeratin and their ability of developing into TGC. Feng et al. [19] isolated and cultured three cell types from the cotyledon (epitheloid, polygonal and fan-shaped cells) without correlating them to a cell type in vivo.

We recently developed a method to isolate and cultivate primary epitheloid cells from the caruncle and trophoblast from the cotyledon [20]. Thereby, manually separated caruncles and cotyledons were disaggregated in collagenase I and cultured in Dulbecco's Modified Eagle Medium (DMEM)/Ham's F12 medium (caruncle) or Quantum 286 medium (cotyledon), a cell type specific medium for epithelial cells based on DMEM. After the second passage the cultures were analyzed for the expression of extracellular matrix proteins and integrin receptors, revealing that both caruncular epithelial cells and trophoblast cells coexpressed integrin subunits α_6 and β_1 in addition to cytokeratin corresponding to in vivo findings in the peri-implantation period [21] and throughout gestation [3].

An important issue for the suitability of cell cultures as models is the determination of origin as well as the characterization of the cells regarding their specific properties, as they are observed in vivo. Classical epithelium-specific markers are the cytoskeletal protein cytokeratin and the tight junctional protein zonula occludens-1 [22]. Besides, cultured epithelial cells are characterized by the absence of α-smooth muscle (sm) actin, desmin and/or vimentin [23,24]. Nonetheless, vimentin is commonly expressed in cultured epithelial cells [23,25,26]. In the bovine placentome, cytokeratin is expressed in the maternal epithelial and fetal trophoblast cells. Vimentin is localized in the maternal stroma and fetal mesenchyme whereas α-sm actin and desmin are expressed in the smooth muscle cells of blood vessels and pericytes of both compartments [27]. Due to the fact that cultured endothelial cells are of similar morphology to epithelial cells we wanted to exclude the presence of this cell type. In the course of the study a further cell population with polygonal shaped cells morphologically similar to cultured endothelial cells was identified. In order to exclude that these cells were of endothelial origin, the determination of low-density lipoprotein (LDL) uptake was performed. This is a well-established method as endothelial cells only are capable of internalizing LDL [28].

Up to date, no cellular markers have been reported to discern maternal caruncular epithelial cells from trophoblast in vitro. In order to confirm the fetal or maternal origin of the isolated and cultured cells fluorescence in situ hybridization was performed detecting the Y-chromosome in presumed fetal cells provided that the fetus was male. Furthermore, we aimed to validate and, if necessary, improve the previously developed method resulting in a standardized method. This included the development of a protocol for the cryopreservation of cultured epitheloid cells from bovine caruncles and cotyledons.

2. Materials and methods

2.1. Tissue collection

Tissues were collected as described before [20]. Briefly, uteri from pregnant cows (*Bos taurus*) were retrieved from the local slaughterhouse 20–30 min after killing and brought to the laboratory for subsequent

processing. Due to the fact that placentomes with a minimum size of 1–2 cm^3 were needed for experiments, uteri with a gestational age of at least 3 months and up to term were chosen.

Prior to incision of the uterine wall the site was cleaned and disinfected. Placentomes were collected in a sterile manner as possible (using sterile glows and scalpel blades) by opening the uterus along the large curvature and cutting off the placentomes at the attachment site to the uterine wall. Placentomes collected for histology as well as protein extraction were snap-frozen in liquid nitrogen and stored at −80 °C. Placentomes collected for cell culture were put in a sterile beaker which was immediately placed under the laminar flow hood for subsequent processing.

The crown-rump length of the fetus was measured to estimate the day of gestation according to the formula by Keller cited in [29]. In addition, the gender of the fetus was documented.

2.2. Primary cell culture

Primary cell cultures were generated as described previously [20] including minor modifications. Briefly, prior to tissue collection 50 ml tubes containing 200 U/ml collagenase I, 10% Hank's Salt Solution with Ca^{2+} and Mg^{2+} (Biochrom, Berlin, Germany) and 90% medium in a 10 ml solution were prepared for disaggregation. The medium was composed of either Dulbecco's Modified Eagle Medium, DMEM/Ham's F12 or Quantum 286 (Q286, PAA, Cölbe, Germany) containing 10% fetal calf serum (FCS) and 1% Penicillin/Streptomycin, 10,000 IU/10,000 μg/ml (Biochrom, Berlin, Germany).

The placentome was manually separated into fetal cotyledon and maternal caruncle. Depending on the size of each tissue a 1–2 cm^3 piece of cotyledon and caruncle was dissected and attached to a sterile cotton thread and immersed in the above mentioned disaggregation solution with the epithelial surface facing the solution. After a 60 min incubation period (37 °C) the tissue was discarded. The solution was centrifuged for 5 min (160 × *g*) and the pellet resuspended in medium, seeded in four 24 cm^2 cell culture flasks (7 ml/Flask) and incubated in 5% CO_2/95% air at 37 °C. Pellets derived from caruncular tissue were cultured in DMEM/Ham's F12 and pellets originating from cotyledonary tissue were cultured in Q286 or DMEM/Ham's F12. After 4–6 days 5 ml medium/flask was exchanged for fresh medium. In general, two cell populations could be identified: epitheloid and fibroblastoid cells. Ten to fourteen days post isolation the first passage was performed by a wash in Hank's Salt Solution without Ca^{2+} and Mg^{2+} and a 3–5 min incubation period (37 °C) in a 0.05% Trypsin/0.02% EDTA solution (Biochrom, Berlin, Germany) leading to a detachment of predominantly fibroblastoid cells. The solution was then discarded and a second 2 min incubation period in 1 ml Trypsin/EDTA followed leading to the detachment of the epitheloid cells, which was supported by using a cell scraper. The resulting cell suspension was resuspended in medium and subsequently seeded into two new cell culture flasks. After another 5–10 days in culture the cells reached confluence and where then further processed for the application needed.

In order to assess the success rates of the cultures they were divided into three groups: early, mid and late stage of gestation (Table 1). Before the first passage the

Table 1
Number of isolations performed and tissue samples (caruncle or cotyledon) taken from placenta early (3–4 months), mid (5–6 months) and late (7–9 months) of gestation

Gestational age	Isolations	Caruncles	Success rate before the first passage (%)	Success rate before the second passage (%)
Epitheloid cultures derived from caruncle (cultured in DMEM)				
3–4 months	6	13	88	89
5–6 months	11	22	86	89
7–9 months	5	9	83	84
Epitheloid cultures derived from cotyledon (cultured in Q286)				
3–4 months	4	11	42	39
5–6 months	8	19	33	44
7–8 months	2	4	6	100
Epitheloid cultures derived from cotyledon (cultured in DMEM)				
4–9 months	5	9	33	60

The success rates, assessed prior to subculturing (first and second passage), were defined as the percentage of cell culture flasks containing an estimated amount of more than 50% epitheloid cells and free of fungal or bacterial contamination. Please note that in some cases flasks containing less than 50% epitheloid cultures derived from the cotyledon were further processed.

Table 2
Antibodies used for immunofluorescence (IF) and Western blot (WB) analyses

	Cell type specificity	Dilution IF	Dilution WB	Manufacturer
Primary antibodies				
Rabbit anti-cow cytokeratin (polyclonal)	Epithelial cells	1:300	1:2000	Dako Cytomation, Hamburg, Germany, Cat. No.: Z0622
Rat anti-Zo-1 tight junction associated polypeptide (monoclonal)	Epithelial cells	1:50	–	Chemicon Int., Temecula, U.S.A., Cat. No.: MAB1420
Mouse anti-vimentin (monoclonal)	Cells of mesenchmal origin	1:100	1:500	Dako Cytomation, Cat. No.: M7020
Mouse anti-human α-smooth muscle actin (monoclonal)	Smooth muscle and myoepithelial cells and myofibroblasts	1:50	1:500	Dako Cytomation, Cat. No.: M0851
Mouse anti-human desmin (monoclonal)	Smooth and striated muscle and mesothelial cells	1:100	1:500	Dako Cytomation, Cat. No.: M0760
Secondary antibodies				
Donkey anti-rabbit IgG, Fluorescein conjugated		1:200	–	Chemicon Int., Temecula, U.S.A., Cat. No.: AP182F
Donkey anti-rat IgG, Cy3 conjugated		1:300	–	Chemicon Int., Cat. No.: AP189C
Donkey anti-mouse IgG, Cy3 conjugated		1:300	–	Chemicon Int., Cat. No.: AP192C
Biotinylated anti-mouse/anti-rabbit IgG		–	1:500	Vector Lab., Burlingame, U.S.A., Cat. No.: BA-1400

fraction of epitheloid cells was estimated by judging the culture under an inverse microscope. Cultures containing a fraction of epitheloid cells less than 50% and cultures showing signs of fungal or bacterial contamination were discarded. Due to the difficulties encountered when isolating epitheloid cells from the cotyledon, a higher rate of non-epitheloid cells was tolerated in these cultures. A second evaluation of success thereby discarding cultures containing a fraction of epitheloid cells less than 50% was performed prior to the second passage (Table 1).

2.3. Cryopreservation

Confluent cultures containing a high fraction of epitheloid cells (>90%) were dissociated with Trypsin/EDTA as described above, resuspended in medium, and centrifuged for 5 min (160 × *g*, Room Temperature, RT). The pellet was resuspended in fresh medium and centrifugation was repeated twice. The pellet was then resuspended in 1 ml cryosolution composed of 10% dimethylsulphoxide (DMSO) (Sigma–Aldrich, Taufkirchen, Germany), 30% FCS and 60% DMEM/Ham's F12 and stored at −80 °C. Prior to experiments, the cells were thawed rapidly, resuspended in medium and centrifuged three times as described above and transferred to the culture flasks required for the subsequent experiment.

Validation of the method was performed by comparing cryopreserved caruncular epithelial cell cultures with corresponding non-cryopreserved cultures using immunofluorescence detection of cytoskeletal proteins listed in Table 2. The comparison and detection was repeated at least five times for all proteins listed.

2.4. Immunofluorescence

Cultures used for immunofluorescence were passaged into 6-well cell culture plates containing cover slips. After another 5–10 day period confluence was reached and the cells were fixed in 100% methanol for 10 min by −20 °C and air dried for approximately 30 min. All washing steps were performed in 0.02 M phosphate buffered saline (PBS, pH 7.3) containing 0.3% Tween 20. Sera and antibodies were diluted in a PBS/0.3% Tween containing 0.1 g/ml bovine serum albumin and glycerol at a ratio of 2:1 (pH 8.0). After blocking non-specific binding sites (60 min, RT) in 1:10 normal donkey serum (Chemicon, Temecula, U.S.A.), incubation over night at 4 °C or for 60 min at 37 °C with primary antibodies (listed in Table 2) was performed. A 3 × 10 min wash was followed by a 60 min incubation period at RT with fluorescence conjugated secondary antibodies (listed in Table 2). Finally, following a 3 × 10 min wash the cover slips were removed from the wells and placed face down on slides in Vectashield Hard + Set mounting medium with DAPI (Vector, Burlingame, U.S.A.) and viewed under a Olympus BX50 fluorescence microscope (Hamburg, Germany). Immunofluorescence performed on 12 μm frozen sections of placentomes retrieved during cell isolation served as positive controls. Incubations with buffer

instead of primary antibodies were used as negative controls. If primary antibodies were derived from different hosts, double labeling was performed.

2.5. Western blot analysis

Primary cell cultures of pure epitheloid cell populations were chosen for Western blot analysis. Protein samples from shock frozen placentomes and pure fibroblastoid cell populations served as controls. Protein extraction was performed according to the manufacturer's instructions using 1 ml/cm^2 cell layer TRIzol-Reagent (Invitrogen, Karlsruhe, Germany). Protein pellets were resuspended in a 1% sodiumdodecylsulphate (SDS) solution, diluted to a final concentration of 1 mg/ml protein (±10%), measured by photometry using bovine serum albumin as a standard and stored at −20 °C until further processing. Gel electrophoresis and Western blotting were performed with the NuPAGE Gel System (Invitrogen) according to the manufacturer's protocol using 10% and 12% NuPAGE Novex Bis–Tris gels. Blotting on a nitrocellulose membrane (0.2 μm pore size) was followed by immunodetection of all cytoskeletal proteins with the primary antibodies listed in Table 2. Firstly, blocking was performed in 0.1 M phosphate buffered saline (PBS, pH 7.4) containing 5% skimmed milk powder and 5% bovine serum albumin (BSA) for 30 min. After washing, the membrane was incubated over night in PBS containing 1% BSA, 0.1% Tween 20 and the primary antibody which was diluted as described in Table 2. After washing in buffer, an additional block was performed in PBS containing 5% horse serum (Vector) for 30 min followed by a 60 min incubation period in PBS containing 1% BSA, 0.1% Tween 20 and the biotinylated secondary antibody which was diluted as described in Table 2. After washing in buffer, incubation in an avidin and biotinylated horseradish peroxidase solution (Vectastain ABC Kit, Vector) was performed for 60 min. After washing in buffer, the colorimetric reaction was induced using TrueBlue peroxidase substrate (KPL, Gaithersburg, U.S.A.) and stopped by placing the membrane in distilled water.

2.6. Preparation of nuclei for FISH

Cells were retrieved directly after isolation, before the first passage and then again before the second passage. They were resuspended in medium and centrifuged for 10 min (1000 × *g*, RT). The pellet was resuspended in a 50 mM potassium chloride solution and incubated for 10 min at 37 °C and centrifuged for 10 min (800 × *g*, RT). The pellet was then resuspended in Carnoy's solution (3:1 absolute methanol:glacial acetic acid) and washed 3 times via centrifugation (160 × *g*, 10 min, RT). Cells could be stored in the solution at −20 °C. For the FISH procedure a drop of nuclei-solution was placed on an ice-cold slide, shortly held over 70 °C steam and air-dried.

2.7. Fluorescence in situ hybridization (FISH)

FISH was performed by the method described by Klisch et al. [30]. Briefly, a DNA probe was used which detected the long arm of the Y-chromosome labeled with biotin-14-dUTP (BAC clone Texas28 from the BAC library described by Cai et al. [31], a gift from D. S. Gallagher Jr., Dept. of Animal Science, Texas A&M University, USA, using a standard nick translation reaction. Hybridization sites were detected with streptavidin-Cy3 conjugated secondary antibody (Jackson Immuno Research, West Grove, U.S.A.) using one round of amplification with biotinylated anti-avidin (Vector). Preparations were mounted in Vectashield Hard + Set mounting medium with DAPI (Vector) counterstaining the nuclei. Cell cultures derived from uteri containing a male fetus were assessed to determine either fetal (cotyledonary) or maternal (caruncular) origin. Chromosomal preparations from leucocytes derived from a bull (gift from Dr. W. Hecht, Dept of Veterinary Pathology, Justus-Liebig-University, Giessen) and cultures derived from uteri containing a female fetus hybridized simultaneously in every experiment served as positive and negative controls, respectively. The slides were viewed under the Olympus BX50 fluorescence microscope (Hamburg, Germany) and at least 200 nuclei per slide were assessed counting the number of positive hybridization signals.

2.8. LDL-labeling

Cultures used for LDL-labeling were passed into 6-well cell culture plates containing cover slips. After confluence was reached, the cells were incubated for 4 h (37 °C) in a medium containing 10 μg/ml human LDL acetylated and labeled with the fluorescent probe, 1,1′-dioctaldecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (Dil-Ac-LDL; Harbor Bio-Products, Norwood, U.S.A.). After washing in PBS the cultures were fixed for 10 min in a 2% paraformaldehyde solution and the cover slips were mounted as described above. Bovine umbilical vein endothelial cells (BUVEC, a kind gift from Dr. A. Tauber, Dept. of Parasitology, Justus-Liebig-University Giessen, Germany) served as positive controls.

3. Results

3.1. Cells isolated from the caruncle

Cultivating the cells directly after disaggregation without the usage of any filtering method resulted in relatively high amounts of cell detritus and tissue in the medium. Nonetheless after the change of medium after 5–7 days the majority of it was removed. Two distinct cell populations, namely epitheloid and fibroblastoid cell colonies, could be identified (Fig. 1a). Due to their adhesive properties the majority of the fibroblastoid cells could be removed by passaging. This resulted in a high fraction epitheloid cell cultures (83–89%). Success rates between isolations from different gestational ages did not differ (Table 1). However, the outcome of a culture was strongly related to individual tissue samples, i.e. cells isolated from two caruncles taken from the same animal sometimes grew differently. Eighty four percent of the discarded cultures contained a too high a fraction of fibroblastoid cells, whereas 16% were contaminated with fungi.

FISH performed on samples taken directly after disaggregation revealed the presence of fetal tissue in the caruncle (Table 3; Fig. 2). This contamination with fetal cells could not be quantified exactly due to the small number of samples taken. However, after the second passage, all samples containing epitheloid cells only were negative, confirming the maternal origin of the cells.

Characterization of the epitheloid cells revealed the presence of cytokeratin, zonula occludens-1 and vimentin while they remained negative for α-sm actin and desmin (Fig. 3). The specificity of the antibodies was confirmed by Western blot analysis thereby detecting protein bands at molecular weight levels characteristic for the detected proteins (Fig. 4). The cells showed no uptake of LDL (Fig. 5). No differences in protein expression could be observed when using cells which were previously cryopreserved. The cultures could be kept at −80 °C for about 1 year without a substantial loss of viability. Beyond this point viability was severely decreased.

3.2. Cells isolated from the cotyledon

Based on the assumption that epitheloid cells isolated from the cotyledon had the same phenotype like caruncular epitheloid cells, we firstly focused on gaining cultures containing predominantly the cell type described above. On a morphological point of view these cells could not be differentiated from epitheloid cultures derived from the caruncle (Fig. 1a). The success rate for obtaining viable epitheloid cultures was very low (Table 1). Altogether 43 cotyledonary samples were disaggregated leading to only 56% of cultures containing epitheloid cells, which were frequently outnumbered by fibroblastoid cells. Therefore, a higher amount of fibroblastoid cells was tolerated in primary cultures retrieved from the cotyledon in comparison to those isolated from the caruncle. Over 92% of the discarded cultures contained too high percentage of fibroblasts or fibroblasts only. The usage of Quantum 286 medium which apparently enhances epithelial cell viability and growth had no positive effect. Cultivating the cells in DMEM/Ham's F12 medium showed the same or even slightly better results (Table 1). Characterization via immunofluorescence demonstrated the presence of cytokeratin, zonula occludens-1 and vimentin and the absence of smooth muscle actin and

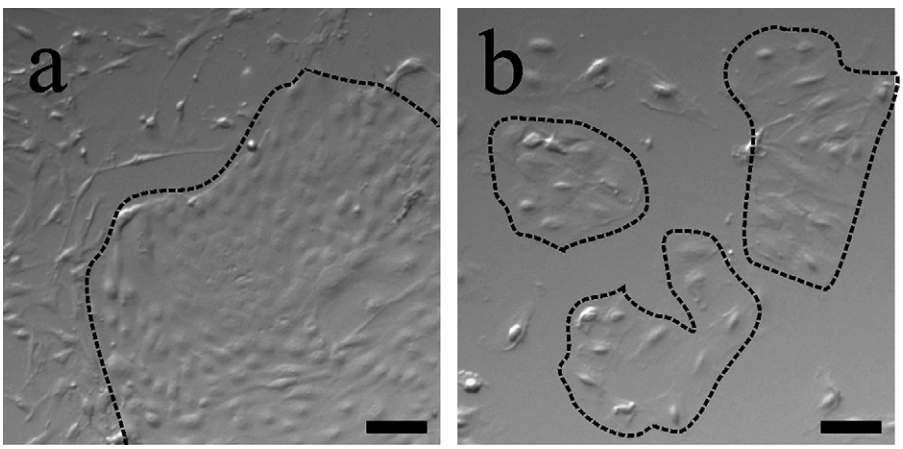

Fig. 1. Morphological identification of epitheloid shaped cells in a caruncular epithelial cell colony (a: lower right) surrounded by fibroblastoid shaped stromal cells, and polygonal shaped cells in trophoblast cell colonies (b: outlined by stippled lines). Bar: 100 μm.

Table 3
Percentages of Y-chromosome positive cells (FISH) in cells isolated directly after enzymatic disaggregation (ED), after seeding (P0) and after the first and second passage, respectively (P1; P2)

Animal Nr.	Gestational age (months)	Isolation after	Y-chromosome positive cells (%)	Remarks
Cells isolated from the caruncle				
14	3	ED	6	
16	7	ED	51	
17	5	P0	3	
18	7	P0	0	
17	5	P1	0	>95% epitheloid cells
10	6	P1	0	>95% epitheloid cells
18	7	P1	0	>95% epitheloid cells
Cells isolated from the cotyledon				
14	3	ED	43	
16	7	ED	63	
10	6	P1	63	>50% fibroblastoid cells
21a	4	P2	0	>95% epitheloid cells
21b	4	P2	3	>95% epitheloid cells
21c	4	P2	97	>95% polygonal cells

Leukocytes from a bull (positive control): 98%.

desmin in cotyledonary epitheloid cells as observed in cultures derived from the caruncle.

FISH performed on samples taken directly after disaggregation revealed the existence of maternal tissue in the cotyledon (Table 3, Fig. 2) independent of the gestational age. A quantification of the contamination with maternal cells was not possible due to the small sample size. However, all samples taken from cell cultures containing epitheloid cells only were negative, confirming the maternal origin of the cells.

In the course of the study a third polygonal shaped cell type forming epitheloid like colonies could be identified growing in cultures isolated from the cotyledon, and in few primary cultures derived from the caruncle (Fig. 1b). Since the adhesive properties of the polygonal shaped cells were similar to those of the fibroblastoid cells selective trypsination could not be used to gain cultures containing predominantly polygonal shaped cells. Here a combination of mechanical detachment with a cell scraper together with focal trypsination and gentle pipetting resulted in subcultures containing >90% polygonal shaped cells. These were maintained in culture in DMEM/Ham's F12 medium for up to 10 passages. Performance of FISH confirmed the cells to be of fetal origin (Table 3).

Characterization via immunofluorescence revealed the presence of epithelial cytokeratin, zonula occludens-1 as well as vimentin, and the absence of α-sm actin and desmin (Fig. 3). LDL-uptake was negative for polygonal shaped cells confirming that these cells were

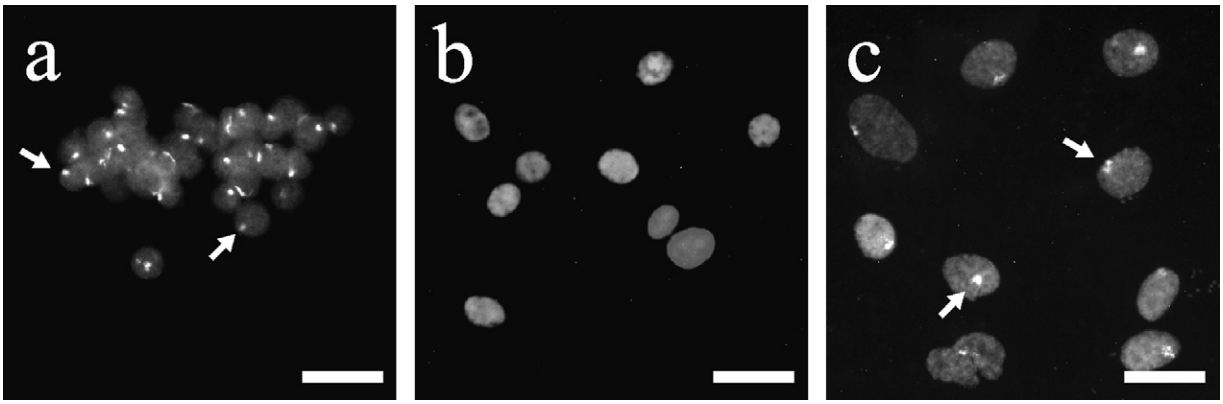

Fig. 2. Fluorescence in situ hybridization (FISH) performed on bull leukocytes (a, positive control), maternal caruncular epithelial cells (b), and fetal trophoblast cells (c, male fetus) detecting the Y-chromosome. Positive hybridization signals (arrows) in DAPI stained nuclei occur only in bull leukocytes and cultured trophoblast cells. Bar: 25 μm.

not of endothelial origin (Fig. 5). Taken together, our results are suggesting that polygonal shaped cells isolated from the cotyledon are indeed mononuclear trophoblast cells.

4. Discussion

The present study demonstrates the importance of cell characterization and proper identification when using primary cell cultures from epitheliochorial placentae for experiments. In addition, we describe a successful and reliable method to isolate, culture and cryopreserve primary caruncular epithelial cells from pregnant cows. Furthermore, a polygonal shaped cell population derived from the cotyledon was propagated and identified as mononuclear trophoblast cells.

4.1. Caruncular epithelial cells

We were able to identify contaminations of caruncular epithelial cell cultures with fetal cell fractions by FISH. The bovine placentome is an organ containing heterogeneous tissue of two individuals. Due to the firm anchorage of the cotyledon and caruncle to each other, the manual separation of both compartments cannot guarantee not having bits of the neighboring tissue in each sample. The presence of cotyledonary tissue in a caruncular sample has already been described to be present from day 140 of pregnancy onwards [14]. Ten years later the same authors reported that only caruncular samples from earlier than day 100 of pregnancy were free of fetal tissue [15]. Reimers et al. [16] concluded that the contamination of caruncular samples with fetal tissue is so severe that it leads to false positive results. In our hands every cell solution of caruncular tissue from early and late gestational periods gained by enzymatic disaggregation contained fetal cells in varying amounts (6–51%). When interpreting these high amounts it should be considered that in our method the caruncle is not minced prior to disaggregation. Only the outer tissue layer of the sample is digested leading to a concentration of epithelial cells from the outer area of the caruncle. The percentage of contamination with fetal tissue in comparison to the whole caruncular sample is more likely to be much lower.

The epitheloid cells gained from caruncles of pregnant cows are of maternal origin and express characteristic epithelial properties such as epithelial cytokeratin and tight junctional zonula occludens-1 protein, justifying the identification as primary caruncular epithelial cells. In contrast to the conditions in vivo the primary epithelial cells from bovine caruncles express the intermediate filament (IF) vimentin, which is present in many cell types of mesenchymal origin including fibroblasts, endothelial cells, macrophages, neutrophils and lymphocytes. Interestingly, vimentin IFs can be associated with other organelles, components of the cytoskeleton and membrane adhesions but they are not required for the viability of cells [32]. Mice having a null mutation of the vimentin gene show no obvious differences in development, breeding, structural or physiological properties of various tissues and organs [33]. Since then however there is growing evidence that vimentin IFs play an important mechanical role in living cells [34]. The expression of vimentin in primary epithelial cell cultures and epithelial cell lines is a well known phenomenon independent of the tissue or organ they were isolated from. It has been described in endometrial cell cultures derived from pigs, rats, humans, sheep and dogs [23,25,26,35,36]. In addition primary epithelial cells isolated from the endometrium of non pregnant cows were positive for vimentin [8,9,37,38]. Other authors could not show vimentin in primary cultured epithelial cells [8,24], but it should be considered that the experiments were performed on cells shortly after isolation and with no subculturing performed. In all studies the cells retained their specific epithelial properties resulting in successful experiments. So far, in our cell culture we have no evidence to believe otherwise.

4.2. Trophoblast cells

The present study proves that manual separation of cotyledon and caruncle may also result in the contamination of cotyledonary cells with maternal tissue. Prior to this study, epitheloid cells isolated from the cotyledon were thought to be of mononuclear trophoblast origin [20]. Morphologically, these cells could not be differentiated from epitheloid cells isolated from the caruncle. FISH analysis, however, clearly demonstrated the maternal origin of epitheloid cell colonies. Interestingly, also a polygonally shaped cell type forming epitheloid like colonies could be identified in cotyledonary cell cultures. These cells expressed epithelial properties and were shown to be of fetal origin by FISH analysis, thus concluding them to be trophoblast cells. The morphology of these cells corresponds to the one described by Munson et al. [18] and Feng et al. [19] both reporting the isolation of mononuclear cells from the cotyledon. Munson et al. [37] established six trophoblastic cell lines using a collagenase-II disaggregation procedure with subsequent filtering through a sterile cheesecloth and culture in DMEM/Ham's F12 medium. The resulting polygonal

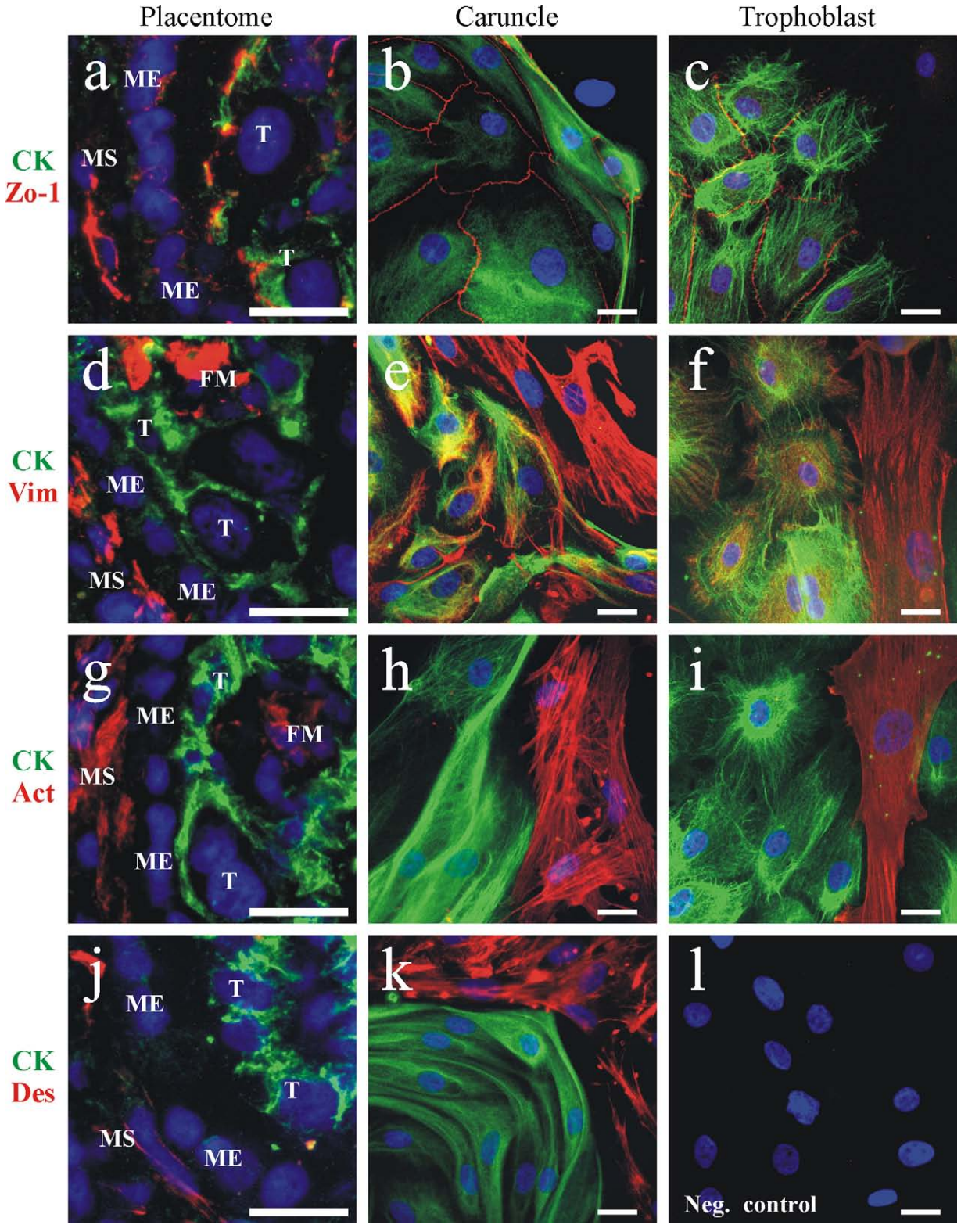

Fig. 3. Immunofluorescence performed on cryosections of placentomes (a, d, g, j: in vivo control) as well as primary maternal caruncular epithelial (b, e, h, k) and primary fetal mononuclear trophoblast cell cultures (c, f, i) containing fibroblastoid cells originating from maternal stroma (MS) or fetal mesenchyme (FM). Double labeling of epithelial specific cytokeratin (a–k: green) and tight junctional zonula occludens-1, ZO-1 (a–c: red) is

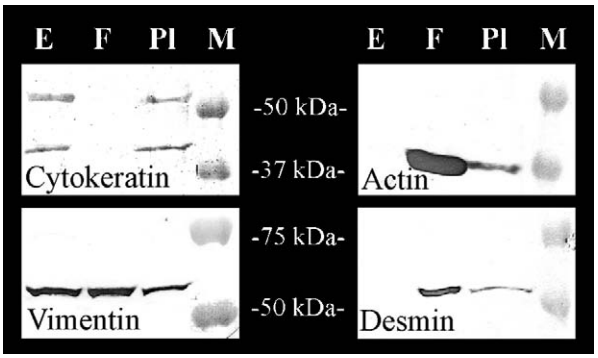

Fig. 4. Western blot of protein samples derived from caruncular cell cultures containing more than 95% epitheloid cells (E), caruncular cell cultures containing more than 95% fibroblastoid cells (F) and placentomal tissue (Pl; positive control) detecting the cytoskeletal proteins cytokeratin (wide spectrum screening, 40–54 kDa) and vimentin (57 kDa) in all fractions, while α-smooth muscle actin (42 kDa), and desmin (53 kDa) are not expressed in caruncular epithelial cells. Molecular weight marker (M).

cell population expressed cytokeratin and vimentin corresponding to our findings. Feng et al. [19] morphologically identified polygonal cells with migratory properties as well as epitheloid and fan-shaped cells, however, without correlating them to a cell type in vivo. In a further study, however, the polygonal shaped cells isolated from the cotyledon were identified as endothelial cells according to the uptake of low-density lipoprotein (LDL), and by detecting the angiotensin-converting enzyme (ACE) [39]. In our case the isolated cultures demonstrated no uptake of LDL and considering the expression of cytokeratin and tight junctional zonula occludens-1 together with the presence of the Y-chromosome the polygonal shaped cells were proven to be fetal mononuclear trophoblast cells. Taken together these findings indicate that one can morphologically distinguish trophoblast from caruncular epithelial cells concluding that if cells exhibiting the classical epitheloid phenotype are present in cultures derived from the cotyledon, these samples contained maternal tissue.

The usage of different media had no effect on the outcome of the cultures. Caruncular epithelial cells could be isolated and cultured in Quantum 286 (DMEM based medium) nearly as efficient as in DMEM/Ham's. In fact DMEM alone or in combination with Hams's F12 is a frequently used medium in endometrial epithelial cell cultures [8,9,37,38].

To determine the percentage of contaminating tissue in the whole sample was not the aim of this study, but over half of the enzymatically disaggregated cotyledonary samples contained maternal epithelial cells. This only reflects isolations resulting in epithelial cell growth and not other cell types such as fibroblasts. This was independent of the gestational age. Other factors, which may have influence on the amount of left over caruncular tissue such as size of the placentome and area from which the sample is taken were not assessed.

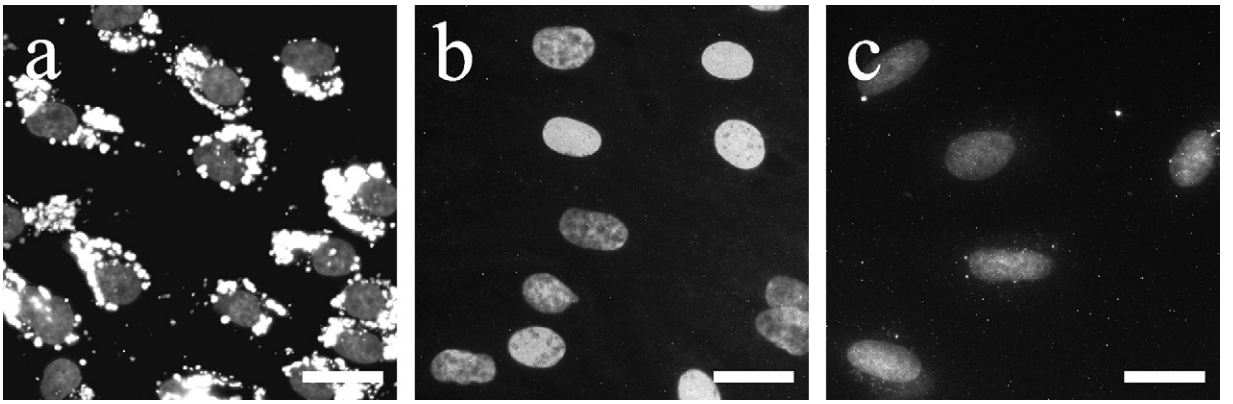

Fig. 5. Low density lipoprotein (LDL) labeling of bovine umbilical vein endothelial cells, BUVEC (a, positive control), maternal caruncular epithelial cells (b), and fetal trophoblast cells (c). Both caruncular epithelial and trophoblast cultures are negative ruling out the presence of endothelial cells. DAPI stained nuclei (blue). Bar: 25 μm.

observed in maternal caruncular epithelial cells (a and b: ME) and fetal trophoblast cells (a and c: T). In addition, ZO-1 fluorescence is located in the endothelial cells in the maternal stroma (a: MS). In vivo, vimentin, is expressed in the maternal stroma and fetal mesenchyme (d: red), whereas in culture, vimentin staining is not only located in fibroblastoid cells but also in the caruncular epithelial and trophoblast cells (g and j: red). α-Smooth actin (g–i: red) and desmin (j and k: red) both present in cells of the maternal stroma and fetal mesenchyme are located in the corresponding fibroblastoid cells in culture. Nuclei are counterstained with DAPI (blue). Desmin staining on trophoblast cells is not shown. Representative negative control on primary caruncular epithelial cells (l). Bar: 25 μm.

In our hands, retrieving and culturing mononuclear trophoblast cells from cotyledonary tissue was difficult. The heterogeneity of the sample resulted in a primary culture of various cell types as was also seen in primary cultures derived from the caruncle. Selective passaging with trypsin was unsuccessful, not only due to the high amount of non-trophoblastic cells but also because the adhesive properties of polygonal shaped cells were similar to those from fibroblastoid cells. Eliminating undesired cell types can be performed by initial filtration methods after disaggregation in a more or less successful way [18,19]. In the end, however, careful subculturing is the key to success [18,37] and corresponds to our experience. Nonetheless, we encountered great difficulties in subculturing sufficient amounts of trophoblast cells. This may be the reason that Munson et al. [18] were the only researchers who isolated, cultured and identified mononuclear trophoblast cells from the cotyledon. In contrast isolating binuclear trophoblast giant cells (TGC) via gradient centrifugation and sedimentation methods has been described more often [16,21,40,41,42,43]. Another successful approach to gain bovine trophoblast cells utilized in vitro matured and fertilized blastocysts, which were plated on coated dishes. The outgrowth of the trophectoderm was then collected and subcultured for many passages thereby creating the bovine trophoblastic cell line BT-1 [44]. Modifying culture conditions led to the appearance of binucleated cells which were stained positive for bovine placental lactogen (bPL), a marker for TGC.

In summary, we have established a method to successfully isolate and culture primary caruncular epithelial cells which have been characterized by confirming their origin and their cell type specific cytoskeletal properties. Morphologically, they can be clearly differentiated from mononuclear trophoblast cells which have been characterized in the same manner.

In conclusion, we have shown that cotyledon and caruncle from the third month of pregnancy onwards cannot be separated manually without leaving bits of the neighboring tissue in the individual compartment. This should always be considered when performing experiments based not only when working with isolated cells but also when analyzing the separated placentomal tissue (e.g. homogenates). Highly sensitive methods such as gene expression analysis or Radio Immuno Assay (RIA) for steroid hormone analysis may lead to false-positive results. Thus the characterization of cultured cells according to the expression of cytoskeletal filaments is not sufficient when working with primary cell cultures derived from epitheliochorial placentae. The fact that cell culture models are often the only mean to study complex regulatory mechanisms highlights the importance of an appropriate cell characterization and identification.

Acknowledgements

Special thanks to S. Kettner, S. Schubert-Porth and K. Wolf (Dept. of Veterinary Anatomy, Justus-Liebig-University (JLU), Giessen, Germany) for their technical assistance. Furthermore, the authors want to thank Dr. W. Hecht (Dept. of Veterinary Pathology, JLU), and Dr. A. Taubert (Dept. of Veterinary Parasitology, JLU) for the donation of material as well as their technical advice, and Prof. Dr. R. Gerstberger (Dept. of Veterinary Physiology, JLU) for providing the fluorescence microscope. This study was supported by a grant from the German Research Foundation (DFG).

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Placenta 28 (2007) 1110–1117

Bovine Caruncular Epithelial Cell Line (BCEC-1) Isolated from the Placenta Forms a Functional Epithelial Barrier in a Polarised Cell Culture Model

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Accepted 5 July 2007

Abstract

In the bovine synepitheliochorial placenta key sites of fetal–maternal interaction are placentomes consisting of maternal caruncles interdigitating with fetal cotyledons. The aim of this study was to establish an epithelial cell line from caruncles of pregnant cows and to develop a model to study restricted trophoblast invasion, pathogenesis of pregnancy associated diseases and pathways of infection and transport. Primary epithelial cells were isolated, successfully subcultured for 32 passages and cryopreserved at various stages. The cultures were termed bovine caruncular epithelial cell line-1 (BCEC-1). Cytokeratin, zonula occludens-1 protein and vimentin but neither α-smooth muscle actin nor desmin were detected by immunofluorescence performed every 5 (±1) passages. These results were confirmed by Western blotting. BCEC-1 were then cultured either without matrix or on fibronectin or collagen coated Transwell® polyester membrane inserts, respectively, enabling separate access to the basal or apical epithelial compartments. Transmission and scanning electron microscopy of BCEC-1 revealed ultrastructural features also observed in vivo, such as apical microvilli and junctional complexes. Transepithelial electrical resistance (TEER) was measured regularly and revealed an increase with advancing confluence in all cultures. Cultures on coated inserts reached confluence and corresponding TEER-levels at an earlier stage. In addition, the cells were tested negative for bovine virus diarrhoea (BVD) virus, but were permissive for the virus. In conclusion, the BCEC-1 cell line retained characteristics of maternal caruncular epithelial cells as observed in vivo and in primary cell cultures and thus will be a highly useful tool for future studies of pathways of invasion, fetal–maternal communication, transport and infection.

Keywords: Bovine placenta; Cell culture; Polarised epithelial cell line; Transepithelial electrical resistance; Bovine virus diarrhoea virus

1. Introduction

In contrast to the haemochorial placenta of humans and rodents, the synepitheliochorial placenta of cows resembles a polarised uterine epithelial barrier facing the fetal chorionic (trophoblast) epithelium. This barrier protects the fetus from a variety of infectious and/or toxic agents. Even maternal immunoglobulins cannot pass this barrier and therefore newborn calves rely on receiving colostrum within the first hours after birth.

Key sites of fetal–maternal interaction are placentomes consisting of maternal caruncles interdigitating with fetal cotyledons. A special feature is the occurrence of ''restricted'' trophoblast invasion performed by trophoblast giant cells migrating towards and fusing with maternal epithelial cells [1,2]. Trophoblast invasion may be compared with tumour invasion but in contrast to tumour cells, the trophoblast can regulate its tumour-like attributes and the depth of invasion is limited in a species-specific manner [3]. In the bovine, this migration takes place between only two cell layers

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0143-4004/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.placenta.2007.07.002

suggesting the bovine placenta to be an ideal model to study invasive processes. So far, the expression of potential regulating factors has been studied on in vivo material only [2,4,5].

From a clinical point of view, one of the major disorders in bovine reproduction is the retention of the fetal membranes post partum due to an incomplete loosening of the feto-maternal attachment [6]. If not treated in time, it can result in severe reproductive disorders and subsequent economic loss. Surprisingly and different from other ruminants this disease frequently occurs in the cow and the water buffalo. Although many studies have been conducted there is no solution to this problem yet, because the underlying mechanisms are far too complex to be elucidated by in vivo studies.

Taken together, the establishment of an in vitro model to study bovine placental mechanisms is overdue. However, when looking for suitable cell cultures it appears that the majority of studies dealt with primary epithelial cells isolated from the endometrial tissue of non-pregnant cows and seldom differentiated between caruncular and intercaruncular tissues [7,8]. Such a differentiation, however, is essential because caruncular and intercaruncular (interplacentomal) epithelia differ from each other functionally, e.g. according to the expression pattern of mRNA and proteins [9–11].

Cell cultures derived from the caruncle of pregnant animals have rarely been established. Furthermore, the few studies undertaken failed to differentiate between the cell types, epithelial cells in particular [12]. So far only one bovine endometrial (epithelial) cell line (BEND) originating from a non-pregnant animal has been characterised and deposited at the American Type Culture Collection (ATCC# CRL-2398) [13]. The BEND cell line has been used successfully in many studies; the majority of which have dealt with prostaglandin synthesis and regulation pathways [14,15]. However, since it was derived from a non-pregnant animal without differentiating between caruncular and intercaruncular regions, it raises the question as whether it is suitable to study processes occurring during pregnancy. Moreover, the ATCC tested the cells positive for the virus of bovine viral diarrhoea (BVD), a disease of great importance in bovine medicine and management, since it can lead to a reduced milk yield, decreased fertility and abortion. If transmitted intrauterinely to the fetus within the first 120 days of pregnancy, the calf develops an immune tolerance resulting in persistent infection. Superinfection or mutations within the virus in the first few months after birth result in the development of the lethal mucosal disease [16].

In the attempt to create a cell culture model to study fetal–maternal interactions, our group recently established and validated a method to isolate and cultivate primary epithelial cells from the caruncle of pregnant cows from day 100 of pregnancy onwards [17,18]. The cells were proven to be of maternal origin by fluorescence in situ hybridisation (FISH). It was demonstrated that these cells expressed the epithelial specific proteins cytokeratin and tight junctional zonula occludens-1 as well as integrin subunits and proteins of the extracellular matrix. Endothelial origin could be excluded by the absence of low density lipoprotein (LDL) labelling. Furthermore, it was shown that cryopreservation of the cells is possible. To standardise future experimental conditions, the aim of this study was to create a cell line, representing a functional, differentiated caruncular epithelium from pregnant cows including the assessment of the BVD virus status. We thereby provide the basis for future research on trophoblast invasion, feto-maternal communication, transport and infection in the bovine placenta.

2. Materials and methods

2.1. Tissue collection and primary caruncular epithelial cell culture

A uterus from a pregnant cow (*Bos taurus*) with a gestational age of 4 months estimated by measurement of the crown-crump length of the fetus (formula by Keller [19]) was retrieved from the local abattoir 20–30 min after slaughter. A primary cell culture of caruncular epithelial cells was created as described by Zeiler et al. [18]. Briefly, a placentome was excised and manually separated into fetal cotyledon and maternal caruncle. The maternal tissue was disaggregated in 200 U/ml collagenase I (Biochrom AG seromed, Berlin, Germany) and the cells were cultured on plastic in flasks supplied with Dulbecco's Modified Eagle Medium (DMEM)/Ham's F12 (PAA Laboratories GmbH, Coelbe, Germany) containing 10% fetal calf serum (FCS, Biochrom) and 1% penicillin/streptomycin (10,000 IU/10,000 μg/ml; Biochrom) in an incubator (5% CO_2/95% air at 37 °C). Morphologically, two cell populations could be differentiated: fibroblastoid cells and epithelioid cells previously identified as caruncular epithelial cells [17].

2.2. BCEC-1 cell line

Contaminating fibroblastoid cells were removed during the first 3–4 passages by a two step trypsin incubation protocol (0.05% trypsin/0.02% EDTA solution; Biochrom). In the first step, fibroblastoid cells detaching prior to the epithelial cells were discarded. Then, the remaining epithelial cells were retrieved and seeded in two new cell culture flasks. Subculturing was performed as soon as confluence was reached or after being 14 days in culture. Confluent cultures were either continuously subcultured until passage 32 or cryopreserved in a solution containing 10% dimethylsulphoxide (DMSO, Sigma–Aldrich GmbH, Taufkirchen, Germany), 30% FCS and 60% DMEM/Ham's F12. For immunofluorescence and BVD virus experiments confluent and/or thawed cultures were seeded in 6-well cell culture plates with cover slips. For transmission (TEM) and scanning (SEM) electron microscopy and measurements of the transepithelial electrical resistance (TEER) the cells were seeded in uncoated and coated (fibronectin or collagen A) polyester membrane Transwell®-Clear inserts (12 μm diameter, 0.4 μm pore size, Corning Inc., Acton, USA). Coating with 10 μg/ml fibronectin from the bovine placenta (Sigma–Aldrich) and 0.5 mg/ml collagen A (Biochrom) was performed according to the manufacturer's instructions. A preliminary experiment also included inserts with biopore and polycarbonate membranes (Millicell, Millipore, Billerica, USA) with a pore size of 0.4 μm.

2.3. Immunofluorescence

Immunofluorescence was performed with cultures from passages 4, 15, 18, 21, 25 and 31 using primary antibodies detecting cytokeratin, zonula occludens-1, vimentin, desmin and α-smooth muscle (sm) actin (Table 1) as previously described [17]. In this study, all antibodies were applied to sections of placentomal tissue (in vivo control) and primary cultures demonstrating cell type specific binding. Briefly, cultures were washed in 0.02 M phosphate buffered saline (PBS, pH 7.3) containing 0.3% Tween-20 (Sigma–Aldrich) three times between each incubation step. Sera and antibodies were diluted in a PBS/0.3% Tween containing 0.1 g/ml bovine serum albumin and glycerol at a ratio of 2:1 (pH 8.0). The cells were fixed in 100% methanol for 10 min and then blocked for 60 min in a 10% normal donkey serum solution (Chemicon Int., Temecula, USA). Incubation with primary antibodies (Table 1)

Table 1
Antibodies used for immunofluorescence and Western blot analysis

		Dilution IF (μg/ml)	Dilution Western blot (μg/ml)	Manufacturer
Primary antibodies				
Rabbit anti-cow cytokeratin (wide spectrum screening)	Polyclonal	35.7[a]	5.4[a]	DakoCytomation, Carpinteria, USA, Cat. No.: Z0622
Rat anti-Zo-1 tight junction associated polypeptide	Monoclonal	20 μl/ml	–	Chemicon Int., Temecula, USA, Cat. No.: MAB1420
Mouse anti-vimentin	Monoclonal	0.69[b]	0.14[b]	DakoCytomation, Carpinteria, USA, Cat. No.: M7020
Mouse anti-human α-smooth muscle actin	Monoclonal	1.4[b]	0.14[b]	DakoCytomation, Carpinteria, USA, Cat. No.: M0851
Mouse anti-human desmin	Monoclonal	2.4[b]	–	DakoCytomation, Carpinteria, USA, Cat. No.: M0760
Secondary antibodies				
Donkey anti-rabbit IgG, Fluorescein conjugated		5.0[a]	–	Chemicon Int., Temecula, USA, Cat. No.: AP182F
Donkey anti-rat IgG, Cy3 conjugated		3.33[a]	–	Chemicon Int., Temecula, USA, Cat. No.: AP189C
Donkey anti-mouse IgG, Cy3 conjugated		3.33[a]	–	Chemicon Int., Temecula, USA, Cat. No.: AP192C
Biotinylated anti-mouse/anti-rabbit IgG		–	1.05[b]	Vector Lab., Burlingame, USA, Cat. No.: BA-1400

[a] Total protein concentration.
[b] Total IgG concentration.

was done overnight at 4 °C. After being rinsed in PBS/Tween, the cultures were incubated for another 60 min with fluorescence conjugated secondary antibodies (Table 1). Finally, the cover slips were removed from the wells and placed face down on slides in Vectashield Hard+Set mounting medium with DAPI (Vector Lab., Burlingame, USA) and viewed under an Olympus BX50 fluorescence microscope (Hamburg, Germany). Incubation in buffer without primary antibodies was used as negative control for unspecific binding of secondary antibodies. If the primary antibodies were derived from different hosts, double labelling was performed.

2.4. Western blot analysis

BCEC-1 cultures (passages 18 and 28) were chosen for Western blot analysis. Protein samples from a shock frozen placentome and a primary culture containing a pure epithelioid cell population isolated from a caruncle served as controls. Protein extraction, electrophoresis, Western blot and immunodetection with the antibodies listed in Table 1 was performed as previously described [17]. In this study protein derived from primary fibroblasts isolated from the caruncle (negative for cytokeratin) was included as well as the detection of a desmin protein specific band in placentome derived and fibroblast derived protein only. Briefly, protein was extracted with TRIzol-Reagent (Invitrogen, Karlsruhe, Germany), resuspended in a 1% sodium dodecyl sulphate (SDS) solution, diluted to a final concentration of 1000 μg/ml protein (±10%) and stored at −20 °C until further processing. Gel electrophoresis and Western blotting were performed with the NuPAGE Gel System (Invitrogen) according to the manufacturer's protocol using 10% and 12% NuPAGE Novex Bis-Tris gels. Blotting on a nitrocellulose membrane (0.2 μm pore size) was followed by immunodetection (Vectastain ABC Kit, Vector Labs). The colorimetric reaction was induced using TrueBlue peroxidase substrate (KPL, Gaithersburg, USA).

2.5. Transmission- and scanning electron microscopy (TEM and SEM)

TEM was performed on sections of confluent cultures (passages 11, 18 and 28 as well as a primary cell culture). The monolayer was fixed during a first step in 0.1 M cacodylate buffer (pH 7.2) containing 2% paraformaldehyde, 2% glutaraldehyde and 0.02% picric acid and in a second step in 1% osmium tetroxide in 0.1 M cacodylate buffer. After dehydration in ethanol followed by xylene, the membrane was embedded in Epon (Serva Electrophoresis GmbH, Heidelberg, Germany). Prior to the preparation of ultra thin sections for TEM, semithin sections were cut with a Reichert Ultracut S (Leica GmbH, Wetzlar, Germany), stained with Richardson's stain and assessed via light microscopy to obtain an overview of the sample. Finally, ultra thin sections (70–100 nm) were cut, counterstained with uranyl acetate and lead citrate with a Reichert Ultrastainer (Leica) and viewed under a transmission electron microscope (LEO 912AB, Zeiss, Oberkochen, Germany). Sections were assessed for the presence of epithelium specific structures, i.e. tight junctions, adherent junctions and apical microvilli.

SEM was performed with confluent cultures (passages 12 and 28). The monolayer was fixed in Soerensen buffer containing 1% glutaraldehyde. After dehydration in ethanol followed by isoamylacetate, the cells were dried in a Critical Point Dryer CPD 030 (Bal-Tec GmbH, Schalksmuehle, Germany) and sputter-coated with gold in a Sputtering Device (SCD 020, Balzer Union). The membrane was mounted to the sample holder and viewed using a scanning electron microscope (LEO 1430 Gemini, Zeiss). Samples were assessed for the presence of epithelial specific structures, i.e. the presence of a regular monolayer and apical microvilli.

2.6. Transepithelial electrical resistance (TEER) measurements

TEER measurements were performed on cultures (passages 6, 12 and 19) grown in duplicates on uncoated or fibronectin or collagen A coated polyester membrane Transwell-Clear inserts as described above. TEER measurements with the Epithelial Voltohmmeter (EVOM) and a STX2 Electrode (WPI Inc., Berlin, Germany) were performed regularly until confluence was reached according to the manufacturer's instructions. The cultures were considered confluent as soon as an estimated >95% of the insert membrane was covered with a monolayer observed via light microscopy. TEER levels of cultures from the same origin grown on coated and uncoated inserts were compared. Primary cultures of fibroblastoid cells isolated from the caruncle and seeded in duplicates on coated and uncoated inserts served as a negative control. As soon as TEER values exceeded 200 Ω/cm^2 the monolayer was considered to be intact and polarised.

2.7. Infection with the bovine virus diarrhoea (BVD) virus

The BVD virus experiments were performed on cultures of passages 6, 12, 17 and 28 as well as Madin Darby bovine kidney (MDBK) cells (positive control, ATCC Number CCL-22, kindly provided by N. Tautz, Department of Virology, Justus-Liebig-University, Giessen, Germany) which were seeded in triplicate in 6-well cell culture plates. Prior to confluence one well of each cell culture was infected with a non-cytopathogenic BVD virus (NCP8-strain, kindly provided by E.J. Dubovi, New York State College of Veterinary

Medicine, Cornell University Ithaca, NY, USA [20]) with a multiplicity of infection (MOI) of 10 and incubated for 48 h. Finally, virus protein was detected via immunofluorescence as described by Lackner et al. [21]. Briefly, the cells were fixed in 2% paraformaldehyde and pestivirus specific NS2/3 protein was detected by the primary antibody code 4 (mAk 8.12.7, kindly provided by N. Tautz [22]) followed by a fluorescence cy3-conjugated secondary antibody (Jackson ImmunoResearch, Suffolk, UK). The cultures were viewed under an inverse fluorescence microscope (Axiovert 35, Zeiss) and the presence of virus was assessed in the non-infected wells and in the infected wells to confirm that the cultures were not contaminated with virus prior to infection and to determine whether infection was possible, respectively.

3. Results

By continuously subculturing, a primary cell culture of bovine caruncular epithelial cells the removal of contaminating fibroblastoid cells was possible within the first four passages, and resulted in a population of cells displaying epithelioid morphology only. During the first 8 passages the cells reached confluence after 11 days (±2). The following three passages were characterised by a low viability of the cells (Fig. 1) and confluence was hardly reached. By day 14 in culture no further proliferation was observed. Subculturing at this time point stimulated cell proliferation and from passage 12 onwards the proliferation rate accelerated markedly resulting in confluence within 7 days (±1) in culture and remained constant for the subsequent 18 passages. From passage 30 onwards the proliferation rate decreased and because the amount of cryopreserved cultures was deemed sufficient, it was decided to stop further subculturing after the 32nd passage. During the process of subculturing cultures of various passages were successfully cryopreserved for later experiments. The cell line was termed BCEC-1 (Bovine Caruncular Epithelial Cell line 1).

Characterisation of BCEC-1 cells at various time points during subculturing revealed the presence of cytokeratin, zonula occludens-1 and vimentin while the cells remained negative for both α-smooth muscle actin and desmin (Fig. 2). This result was confirmed by Western blot analysis (Fig. 3). In addition, TEM and SEM analysis confirmed the presence of epithelium specific characteristics as they show a polarised morphology, the presence of apical microvilli and junctional complexes (zonula occludens, zonula adherens and desmosomes) as well as the growth pattern as a homogenous monolayer of cells (Fig. 4).

All BCEC-1 cultures used for TEER experiments displayed marked increases in TEER values with progressing confluence independent of culture conditions in comparison to confluent fibroblasts which never developed TEER $> 200\ \Omega/cm^2$. Culturing cells on coated membranes resulted in an earlier attainment of confluence and subsequent earlier increase of TEER values (Fig. 5).

All cultures tested negative for the BVD virus (Fig. 6). After 48 h of incubation with non-cytopathogenic BVD virus, virus protein could be detected in the cytoplasm of the BCEC-1 cells. Corresponding to the controls (MDBK cells) no cytopathogenic effect was observed.

4. Discussion

This is the first study to successfully establish a cell line of bovine caruncular epithelial cells (BCEC-1) from pregnant animals with a morphologically and functionally intact epithelial barrier.

The first issue was to create a population of epithelial cells. The primary culture consisted of several cell types and though the majority of cells was epithelioid, fibroblastoid cells originating from the stroma were also present. Different adherent properties of the cells allowed purification of the culture using a two step trypsination procedure which was carried out on every passage even though no fibroblastoid cells were identified beyond the fourth passage. As has been previously shown in primary cultures of caruncular epithelial cells [17], the BCEC-1 cell line also consistently expressed epithelial cytokeratin and tight junctional zonula occludens-1 as well as vimentin, an intermediate filament protein of mesenchymal origin [23]. The expression of vimentin in primary epithelial cell cultures and epithelial cell lines is a well known phenomenon irrespective of which tissue or organ they were isolated from. It has been described in endometrial cell cultures derived from sheep, pigs, dogs, rats and humans [24–28]. Furthermore, primary epithelial cells isolated from the

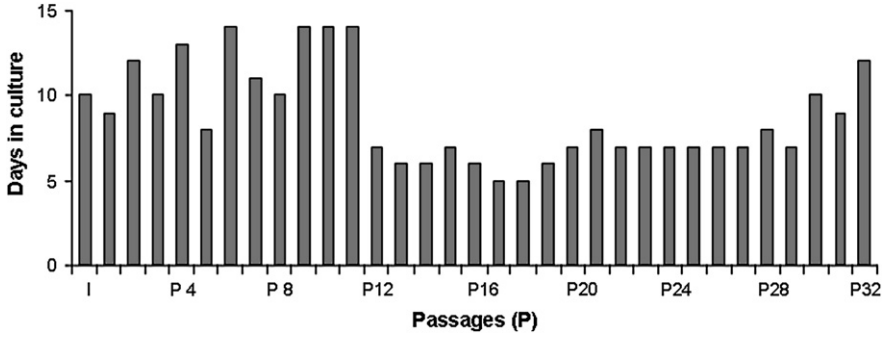

Fig. 1. Proliferation characteristics of BCEC-1 during establishment of the cell line. The number of days in culture until confluence was reached (*y*-axis) is shown for primary cells (I) and each subsequent passage (P). During the first 8 passages confluence was reached after 11 days (±2) followed by a period of crisis (low viability and proliferation) until passage 11. From passage 12 onwards viability and proliferation increased markedly resulting in constant time periods (7 days ± 1) in which confluence was reached.

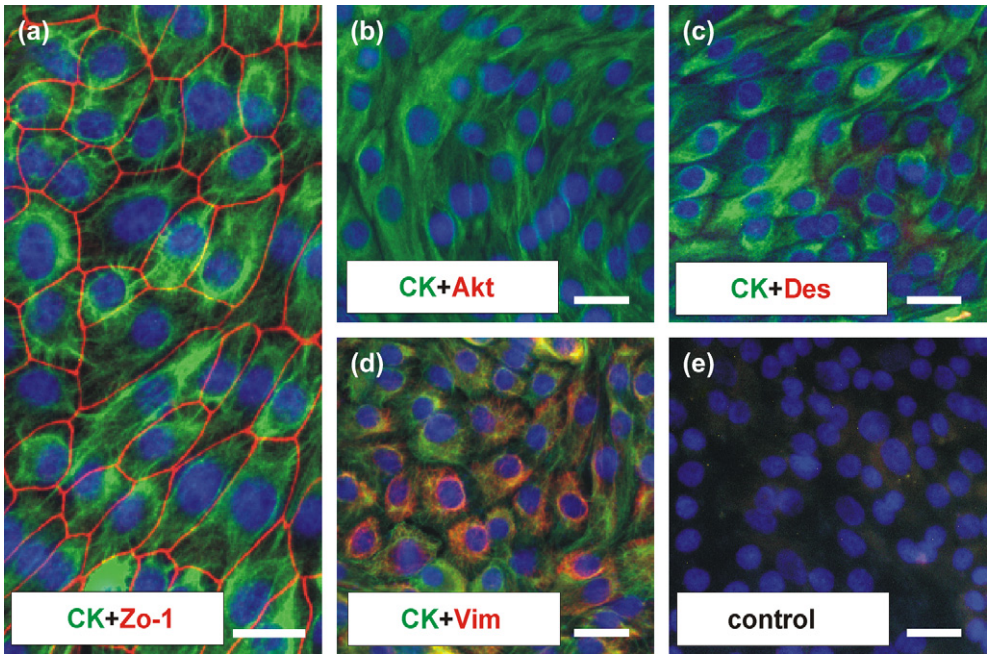

Fig. 2. Representative pictures demonstrating the characterisation of BCEC-1 via immunofluorescence at regular intervals in culture. The cells of epithelial origin constantly expressed epithelial cytokeratin (a–d, green) and tight junctional zonula occludens-1 (Zo-1) protein (a, red; passage (P) 25). Neither α-smooth muscle actin (Akt) protein (b; no red colour: P15) nor desmin (Des) protein (c; no red colour: P21) was detected. Only vimentin (d, red; P11) a protein of mesenchymal origin was present, a well-known phenomenon in epithelial cell cultures. Nuclei were counterstained with DAPI. Representative negative control (e; P18). Bar: 25 μm.

endometrium of non-pregnant cows as well as the BEND cell line were shown to be positive for vimentin [29–31]. It was reported that the cells preserved their epithelial specific properties resulting in successful experiments.

In BCEC-1 cultures the cell morphology, protein expression and the ability to form an intact epithelial barrier was maintained for at least 32 passages. In comparison, the BEND cell line was successfully subcultured for at least 25 passages [14]. In BCEC-1 the variable and prolonged cultivation periods until confluence during the initial passages are partly referable to the process of spontaneous immortalisation. A similar growth pattern was observed during the establishment of the human breast epithelial cell line MCF-10 [32]. The number of viable and proliferating cells decreased within the first 7 passages and spontaneous immortalisation occurred beyond passage 12. Nonetheless, it should be taken into account that the BCEC-1 cells were not counted prior to each seeding and therefore the number of cells transferred to the new flasks was not identical on each occasion. Thus, the differing periods until confluence was reached could possibly be related to different numbers of cells per passage. Nevertheless, the number of days between two passages remained relatively constant between passages 12 and 30. Later experiments with cryopreserved BCEC-1 cells enabled the defining of an optimal seeding density of 7500 cells/cm^2, resulting in a subcultivation ratio of 1:4 and a cultivation period of 6 days until confluence on fibronectin coated flasks. These values corresponded to those suggested by the ATCC for the BEND cell line.

The BCEC-1 cells grew successfully on Transwell polyester (PET) membranes allowing further assessment of morphological and functional characteristics. A preliminary experiment revealed that culture was not successful on Millicell inserts with biopore or polycarbonate membranes. Ultrastructural analysis via electron microscopy demonstrated growth of polarised epithelial monolayers with apical microvilli and junctional complexes as described in vivo [33]. The localisation of the tight junctional protein zonula occludens-1 supported the hypothesis that this cell culture model forms an intact epithelial barrier. This assumption was unequivocally confirmed by the detection of a significant transepithelial electrical resistance a soon as confluence was reached.

Coating the membrane with extracellular matrix proteins enhanced cell growth and optimised culture conditions. In most cases fibronectin resulted in higher TEER values than coating with collagen A (personal observations). The positive effect of fibronectin on growth properties of the monolayer was confirmed by the results obtained from cell counting of primary caruncular epithelial cells cultured on different extracellular matrices [34]. Fibronectin is a soluble multiadhesive matrix protein and a major component of the extracellular matrix. Amongst other locations in the bovine placenta, fibronectin is expressed in the basement membrane of the caruncular epithelium [2]. In addition, in primary cultures of caruncular epithelial cells fibronectin secretion was shown to be associated with the expression of specific integrin receptors [18]. Fibronectin secretion is a frequently observed feature of epithelial cell cultures and

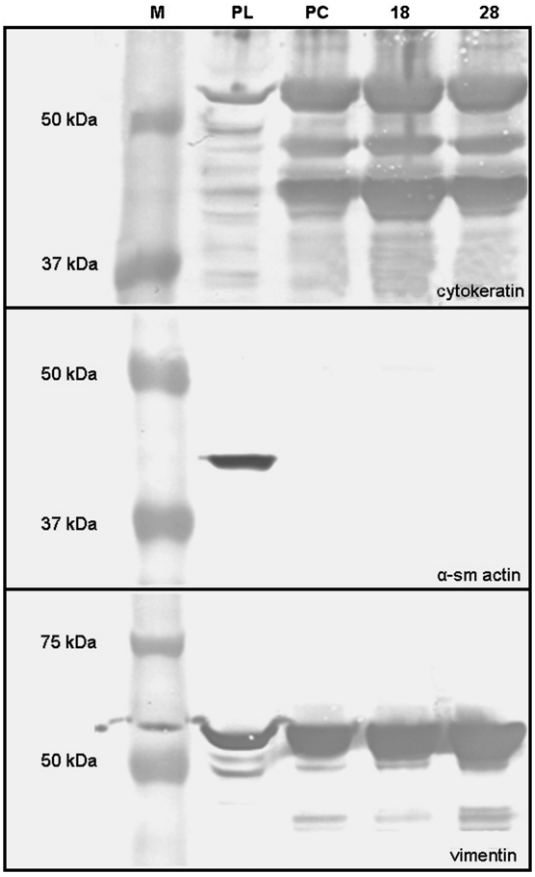

Fig. 3. Western blot analysis of protein samples derived from the placentome (PL; positive control), primary epithelial cells (PC) and BCEC-1 cells from passage (P)18 and 28 confirming the specificity of the antibodies and purity of the cultures. Specific bands corresponding to epithelial cytokeratin (wide spectrum screening, 40–54 kDa) and vimentin (57 kDa), but not α-smooth muscle (sm) actin (42 kDa: negative control for cell culture derived protein) were detected in all epithelial cell cultures. M, Molecular weight marker.

occurs particularly at the basolateral surface [35,36]. Taken together these results provide evidence that fibronectin may have a positive effect on the culture conditions of BCEC-1, a procedure which is now used routinely when working with this cell line.

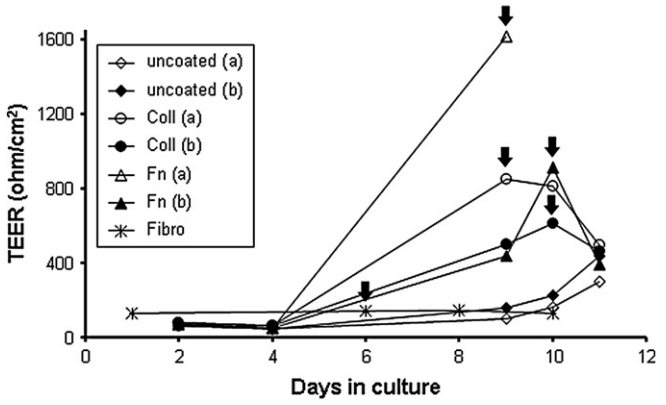

Fig. 5. Representative graph of one out of three independently and in duplicates performed transepithelial electrical resistance (TEER) experiments showing the TEER development of a BCEC-1 passage 12 culture seeded in duplicates (a + b) on uncoated as well as collagen A (Coll) and fibronectin (Fn) coated Transwell insert membranes. Culture on coated inserts lead to an increase in proliferation and an earlier attainment of confluence as demonstrated by an increase of TEER values at an earlier stage when compared to uncoated inserts. This was the case in all experiments. In the experiment shown confluence (arrows) was reached in fibronectin and collagen A coated inserts within day 9 and 10, respectively, whereas the cultures grown on uncoated inserts were close to confluence by the end of the measurements at day 11. Cultures of primary caruncular fibroblasts (Fibro) reaching confluence by day 6 served as negative controls.

In contrast to the BEND cell line, the BCEC-1 cultures were negative for the BVD-virus antigen but were found to be permissive for the virus suggesting a possible application of the BCEC-1 cell line in future studies on the pathogenesis of pregnancy-related diseases in cattle. Bovine virus diarrhoea (BVD) is a disease of great importance in bovine medicine and has a worldwide distribution [16]. If transmitted transplacentally to the fetus within the first 120 days of pregnancy, the calf develops an immune tolerance resulting in persistent infection with a non-cytopathogenic (ncp) BVD virus. In the first few months after birth superinfection with ncp-virus or mutations within the virus leading to a transformation into a cytopathogenic (cp) virus, result in the development of the lethal mucosal disease. Immunohistological

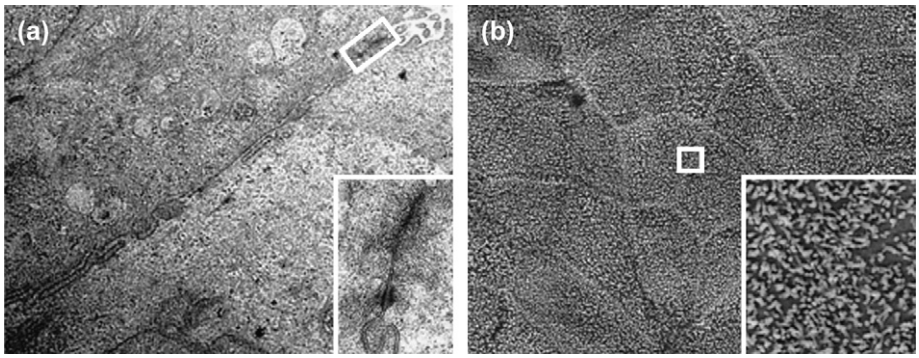

Fig. 4. Transmission (TEM) and scanning (SEM) electron microscopy of BCEC-1 cultures of passage 28 (a) and 11 (b) grown on Transwell inserts. The TEM image (a) shows the lateral contact area of two neighbouring epithelial cells. Higher magnification of the apicolateral region (inset) demonstrates the presence of an epithelial specific junctional complex (zonula occludens, zonula adherens and desmosome). The SEM image (b) shows the apical side of the epithelial monolayer. Higher magnification (inset) demonstrates the presence of caruncular epithelium specific apical microvilli as shown in vivo [33].

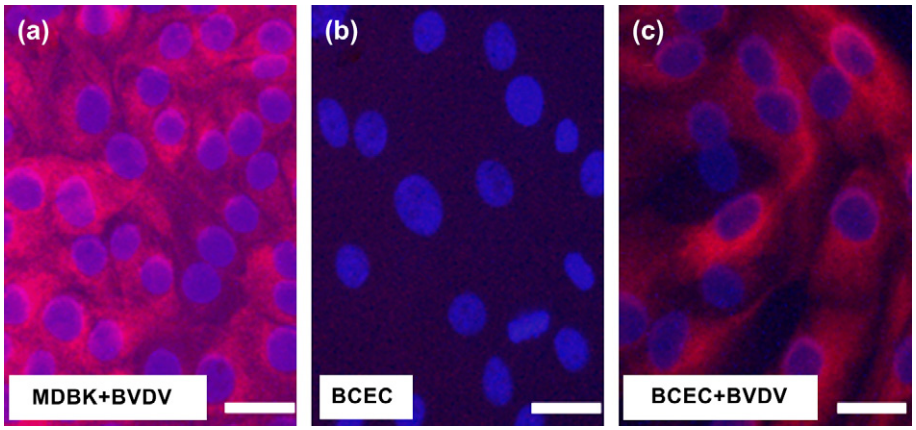

Fig. 6. Detection of bovine viral diarrhoea (BVD) virus antigen. Cultures of Madin Darby bovine kidney (MDBK) cells (a, positive control) and BCEC-1 cells (b, c) were incubated with (a, c) and without (b) BVD virus for 48 h and virus antigen was detected in the cytoplasm of infected cells demonstrating that BCEC-1 cells are negative for BVD virus and that an infection is possible. The nuclei were counterstained with DAPI. Bar: 20 μm.

studies demonstrated strong staining of BVD virus antigen in the caruncular epithelial cells of pregnant persistently infected animals [37]. To date, the pathways of infection in a polarised cell culture model derived from the tissue actually participating in vertical transmission have not yet been studied. In a comparable polarised cell culture model of human bronchial epithelial cells (Calu-3) the entry and release of the severe acute respiratory syndrome corona virus (SARS-CoV) almost exclusively from the apical border was demonstrated [38].

In conclusion, BCEC-1 is the first cell line derived from caruncular epithelial cells of a pregnant cow displaying characteristic epithelial features on a morphological and functional basis for at least 32 passages. Culture of BCEC-1 cells in an insert culture system makes a variety of future applications possible, i.e. co-culture invasion assays with trophoblast or tumour cells [39]; studies on pathways of transport, communication and modification of metabolic substances (e.g. hormones, pharmaceuticals) [40–42] and pathways of infection of pregnancy associated diseases caused by parasites (e.g. *Neospora caninum*), bacteria (e.g. *Bacillus* spp.) and viruses (e.g. BDV virus); as well as studies on environmental toxins (e.g. plants, mycotoxins) [38,43–45].

Acknowledgements

The authors are grateful to M. Fink, A. Hild, S. Kettner, S. Schubert-Porth and K. Wolf (Department of Veterinary Anatomy, Justus-Liebig-University (JLU), Giessen, Germany) for their expert technical assistance. Furthermore, the authors would like to thank Dr. N. Tautz (Department of Veterinary Virology, JLU), Dr. M. Hardt (Centre of Biotechnology, JLU) and Prof. Dr. R. Gerstberger (Department of Veterinary Physiology) for the donation of material as well as the usage of their facilities as well as M. Mohr (Institute for Hygiene and Infectious Diseases of Animals, JLU) and E. Lattwein (Department of Veterinary Virology, JLU) for introduction in TEER measurements and BVD virus detection. This study was supported by a grant from the German Research Foundation (DFG).

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BOR Papers in Press. Published on April 16, 2008 as DOI:10.1095/biolreprod.108.067637

Integrin activation in bovine placentomes and in caruncular epithelial cells isolated from pregnant cows

Running title: Functional integrin binding in bovine placental cells

Summary sentence: Integrin binding is functional in bovine low passage caruncular epithelial cells from pregnant cows according to the co-localization of signaling proteins with integrin β_1, and the enhancement of signaling molecule expression and cell proliferation by extracellular matrix proteins.

Keywords: bovine placentome, caruncular epithelium, integrin, α-actinin, FAK, fibronectin phosphotyrosine, talin

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Abstract

In the bovine synepitheliochorial placenta, restricted trophoblast invasion requires complex interactions of integrin receptors with proteins of the extracellular matrix (ECM) and integrin receptors of neighboring cells. Activated integrins assemble to focal adhesions and are linked to the actin cytoskeleton via signaling molecules including alpha-actinin (ACTN), focal adhesion kinase (PTK2 or FAK), phosphotyrosine and talin (TLN1). Aims of this study were to assess integrin activation and focal adhesion assembly within epithelial cells of bovine placentomes and low passage (not transformed) placentomal caruncular epithelial cells cultured on dishes coated with ECM proteins. Immunofluorescence analysis was performed to co-localize the signaling molecules ACTN, PTK2, phosphotyrosine and TLN1 with each other and with β_1-integrin (ITGB1) in placentomal cryosections throughout pregnancy and in caruncular epithelial cells in vitro. Antibody specificity was confirmed by Western blot. Cells were cultured on uncoated dishes and dishes coated with fibronectin (FN), laminin (LAMA) and collagen IV (COL4) thereby statistically assessing cell number and qualitatively assessing the expression pattern of ITGB1, phosphotyrosine and TLN1. Results demonstrated integrin activation and focal adhesion assembly in the placentome and that low passage caruncular epithelial cells maintain integrin associated properties observed in vivo. Expression and/or co-localization of signaling molecules with ITGB1 confirmed, for the first time, integrin activation and participation in “outside-in” and “inside-out” signaling pathways. The prominent role of ECM, and FN in particular, in integrin signaling is supported by the in vitro enhancement of proliferation and focal adhesion expression. Thus, this in vitro model provides excellent potential for further mechanistic studies designed to elucidate feto-maternal interactions in the bovine placentome.

Introduction

The synepitheliochorial placenta is the key site of feto-maternal communication regulating embryonic development and maintenance of pregnancy in ruminants. In bovine placentomes, trophoblast invasion is restricted to two cell types, the chorionic and maternal epithelia [1]. Trophoblast giant cells (TGC) develop through acytokinetic mitosis of mononuclear trophoblast cells, migrate towards the intact maternal epithelium and fuse with single epithelial cells forming feto-maternal hybrid cells which, upon degeneration, deliver a variety of metabolic substances to the endometrium [1,2]. This process requires complex interactions between integrin receptors on TGC and various extracellular matrix (ECM) proteins in the surrounding tissue. Therefore, the bovine placentome provides an excellent model to study the mechanistic involvement of integrins and ECM to initiate, support and limit cell migration and invasion.

Integrins are α and β heterodimeric cell surface receptors belonging to a family of transmembrane adhesive glycoproteins. They are major ligands for ECM proteins and counter receptors on adjacent cells, and in vertebrates, play an important role in cell-cell adhesion and mechanotransduction [3,4]. Integrin mediated cell-ECM and cell-cell adhesion involves rearrangement of cytoskeletal proteins and activation of intracellular signaling cascades that profoundly effect cell functions critical for tissue development, the immune response, hemostasis, angiogenesis, tumorigenesis, embryo implantation and placentation during pregnancy [5,6]. Activation of integrins can result from initial low affinity ligand binding that converts to high affinity binding and this causes ligand-induced clustering within the plasma membrane. This “outside-in” activation process is followed by the assembly of complex “focal adhesions” within the cell through which mechanical forces are transmitted and signaling cascades are activated. Transmission of mechanical forces from integrin bound to ECM involves structural components

68 such as TLN1 (talin), ACTN (α-actinin) and FLNA (filamin) link the integrin cytoplasmic tails to
69 the actin cytoskeleton.

70 In contrast to the “outside in” activation pathway, the affinity state of integrins for their
71 ligands can be increased through an “inside-out” pathway where cytoplasmic signals lead to
72 integrin activation and focal adhesion assembly. “Inside-out” signaling involves the binding of
73 TLN1 to the β integrin tail as a final common step in integrin activation leading to integrin
74 clustering, activation of PTK2 (also known as focal adhesion kinase or FAK) and tyrosine
75 phosphorylation of a number of focal adhesion constituents [7].

76 Focal adhesions are therefore dynamic structures in which protein-protein interactions
77 between more than 150 different components can occur to include cytoskeletal and actin-binding
78 proteins, adaptor proteins, serine/threonine and tyrosine kinases and phosphatases, GTPases and
79 numerous other classes of proteins [3,8-10]. The size of focal adhesions, their composition and
80 signaling activity is dependent upon forces generated between the matrix and cytoskeleton, as is
81 the extent of tyrosine-phosphorylated protein found within focal adhesions [4].

82 The temporal and spatial expression patterns of the ECM proteins COL1 (collagen type I),
83 COL4 (collagen type IV) FN (fibronectin) and LAMA (laminin) as well as the integrin subunits
84 ITGA1, ITGA2, ITGA3, ITGA4, ITGA5, ITGA6, ITGAV, ITGB1, ITGB3 and ITGB4 (α_1, α_2, α_3,
85 α_4, α_5, α_6, α_v, β_1, β_3 and β_4, respectively) have been described for the bovine placenta throughout
86 pregnancy [11,12]. We recently established and validated a cell culture model of caruncular
87 epithelial cells derived from pregnant cows [13,14]. The cells were subcultured twice prior to
88 experiments and were therefore defined as low passage non transformed cell lines [15]. These
89 epithelial cells produced LAMA and FN, and expressed the integrin receptors $\alpha_6\beta_1$ and $\alpha_v\beta_1$ [13].
90 The aim of the present study was to determine whether integrin receptors of epithelial cells within
91 bovine placentomes are activated to form focal adhesions and to determine whether integrin

activation in caruncular epithelial cells in vitro occurs in a manner similar to what occurs in vivo. Aggregation of the ITGB1 integrin, actin-binding proteins TLN1 and ACTN, and a signal generator PTK2 involved in “inside-out and “outside-in” signaling as well as tyrosine phosphorylated proteins were used as classic markers for focal adhesion assembly [16,17]. In addition, we assessed whether coating of cell culture flasks with COL4, LAMA or FN affected cell proliferation and expression of integrin subunit ITGB1 and focal adhesion molecules. These results highlight the critical utility of well-characterized in vitro cell culture systems to dissect the mechanistic events that are essential for embryo survival during pregnancy.

Material and Methods

Tissue collection

Tissues were collected as described previously [13]. Briefly, uteri from pregnant cows (*Bos taurus*) with a gestational age of 3 months and up to term were retrieved from the local slaughterhouse 20-30 min after killing and brought to the laboratory for subsequent processing. The crown-rump length of the fetus was measured to estimate the day of gestation according to the formula by Keller [18].

Placentomes were collected in a sterile manner by opening the uterus along the large curvature and cutting off the placentomes at the attachment site to the uterine wall. Placentomes collected for histology as well as protein extraction were snap-frozen in liquid nitrogen and stored at -80°C. The samples were divided into three groups (n=6 per group): early (3-4 months), mid (5-6 months) and late (7-9) stages of pregnancy. Placentomes collected for cell culture were put in a sterile beaker which was immediately placed under the laminar flow hood for subsequent processing.

Cell culture

Low passage (non transformed) cell cultures were generated and cryopreserved as described previously [13,14]. Briefly, prior to tissue collection 50 ml tubes containing 200 U/ml collagenase I, 10% Hank's Salt Solution with Ca^{2+} and Mg^{2+} (Biochrom, Berlin, Germany) and 90% medium composed of DMEM/Ham's F12 (PAA, Cölbe, Germany) containing 10% fetal calf serum, FCS and 1% Penicillin/Streptomycin, 10000 IU/10000 µg/ml (Biochrom) in a 10 ml solution were prepared for disaggregation.

The placentome was manually separated into fetal cotyledon and maternal caruncle. A 1-2 cm^3 piece of caruncle was dissected and attached to a sterile cotton thread and immersed in the disaggregation solution with the epithelial surface facing the solution. After a 60 min incubation period (37°C) the tissue was discarded. The solution was centrifuged for 5 min (160 x g) and the pellet resuspended in medium, seeded in four 24 cm^2 cell culture flasks (7 ml/flask) and incubated in 5% CO_2/95% air at 37°C. Ten to fourteen days post isolation the first passage was performed by a wash in Hank's Salt Solution without Ca^{2+} and Mg^{2+} and a 3-5 min incubation (37°C) in a 0.05% Trypsin/0.02% EDTA solution (Biochrom) leading to detachment of predominantly fibroblast cells. The solution was then discarded and a second 2 min incubation period in Trypsin/EDTA allowed for detachment of the epithelial cells using a cell scraper. The resulting cell suspension was resuspended in medium and subsequently seeded into two new cell culture flasks. After another 5-10 days in culture the cells reached confluence and were passaged again as described above. Cultures containing at least 90% caruncular epithelial cells, which was determined by qualitative assessment of the confluent culture dishes by the same observer, were cryopreserved by resuspension in 10% dimethylsulphoxide (DMSO) (Sigma-Aldrich, Taufkirchen, Germany), 30% FCS and 60% DMEM/Ham's F12 and storage at -80°C. Thawing

was performed by resuspension in culture medium and centrifugation for 5 min (160 x g) three times prior to seeding.

Western blot analysis

Primary antibodies used to detect integrin subunit ITGB1, phosphotyrosine, PTK2, TLN1, and ACTN are listed in Table 1. Protein samples from low passage caruncular epithelial cells (>95 %; no fibroblastoid cells identified by qualitative assessment) were chosen. Protein samples from snap-frozen placentomes obtained during the procedure of gaining primary cells served as positive controls. Incubation without primary antibodies served as negative controls for unspecific secondary antibody binding. Total-protein extraction was performed by incubating the cells or tissue in 250 µl Laemmli sample buffer (Sigma-Aldrich) containing 20 µl/ml Benzonase (10,000 U/vial; Merck, Darmstadt, Germany) per 10 cm^2 cell monolayer and for 30 min at room temperature followed by 7 min denaturation at 65°C. Gel electrophoresis and Western blot were performed with the NuPAGE Gel System (Invitrogen, Karlsruhe) according to the manufacturer's protocol using Novex Bis-TrisGels 4-12% and Novex Tris-Acetate Gels 3-8%, respectively and nitrocellulose membranes (0.2 µm pore size). The APAAP-method firstly described by Cordell at al. [19] was chosen to detect specific bands predicted for the antibodies applied. Briefly, membranes were incubated in blocking solution, 0.1 M TRIS buffer containing 5 % skimmed dry milk, for 60 min. Over night blocking was necessary when detecting integrin subunit ITGB1. Blots were then washed in 0.1 M TRIS, followed by overnight incubation at room temperature with primary antibodies (ITGB1, PTK2, TLN1, and ACTN; details listed in Table 1) diluted in 0.1 M TRIS [19]. Blots were again washed, and incubated for 45 min with a polyclonal rabbit anti-mouse bridging antibody (Dakocytomation, Hamburg, Germany), diluted 1:500. Finally, after a further washing step the blots were incubated for 45 min in APAAP-complex (Dakocytomation), diluted 1:500. In order to detect primary antibodies derived from rabbits

(ITGB1 and PTK2) a further incubation step (45 min with a monoclonal mouse anti-rabbit antibody Clone 4G10, diluted 1:500; DakoCytomation,) prior to the bridging antibody was performed. The colorimetric reaction was induced using BCIP/NBT substrate (KPL, Gaithersburg, U.S.A.) until bands were clearly visible. Detection was stopped by washing the membrane in distilled water.

Detection of protein specific bands for each of focal adhesion-specific antibodies at appropriate molecular weights for ITGB1-integrin (~130 kDa), ACTN (~100 kDa), PTK2 (~125 kDa) and TLN1 (~270 and 220 kDa) validated the use of these reagents (Figure 1) for the temporal spatial analysis of focal adhesions within the placentome and caruncular epithelial cell cultures by immunofluorescence microscopy.

Immunofluorescence microscopy

Immunofluorescence was performed with cultures seeded in uncoated or coated (see below) 6-well cell culture plates containing cover slips. Incubation in buffer without primary antibodies was used as negative controls to exclude nonspecific binding of the secondary antibodies. If primary antibodies were derived from different hosts, double labeling was performed. Epithelial cells were characterized using antibodies directed against cytokeratin, tight junctional zonula occludens-1 (TJP1), vimentin (VIM), desmin (DES) and smooth muscle actin (ACTA2) as described previously [14]. Specificity of primary antibody binding was determined via western blot analysis as described above. Cryosections of placentomes served as positive controls. In addition, cell membrane associated signals both in vivo and in vitro as well as co-localization with ITGB1-integrin and the formation of focal adhesions strongly suggest primary antibody binding to be specific. Cultures and sections were fixed in 100 % methanol (-20°C) and blocked in a 10 % normal donkey serum solution (Chemicon Int., Temecula, U.S.A.). Incubation with primary antibodies (for details see Table 1) was done overnight at 4°C or for 60 min at 37°C.

After each incubation period the cells were washed with 0.02 M phosphate buffered saline (PBS, pH 7.3) containing 0.3% Tween 20 (Sigma-Aldrich). The cultures were incubated for another 60 min with appropriate fluorescein- (FITC) or Cy3-conjugated secondary antibodies (for details see Table 1). Sera and antibodies were diluted in a PBS/0.3% Tween containing 0.1 g/ml bovine serum albumin and glycerol at a ratio of 2:1 (pH 8.0). For colocalization studies, combinations of mouse and rabbit primary antibodies were applied sequentially with appropriate fluorochrome-conjugated secondary antibodies. Finally, the cover slips were removed from the wells and placed face down on slides in Vectashield Hard+Set mounting medium with DAPI (Vector Lab., Burlingame, U.S.A.). Cryosections were mounted with cover slips in the same manner. All samples were viewed under an Olympus BX50 fluorescence microscope (Hamburg, Germany) equipped with DAPI, FITC and Cy3 filter sets and images were captured using Spot Insight Software (Diagnostic Instruments, Michigan, USA). The resulting black-and-white images were dyed and overlaid using Metamorph (Visitron Systems GmbH, D-Puchheim) software.

Cell culture on ECM proteins

Caruncular epithelial cells were seeded in triplicate (minimum) in 6-well dishes containing cover slips coated with FN, LAMA, or COL4. Uncoated dishes served as controls. Experiments were repeated at least 5 times. Coating was performed by incubating the wells with 150 µl/cm^2 10 µg/ml FN from bovine plasma (Sigma-Aldrich, Taufkirchen, Germany), 10 µg/ml LAMA from human placenta (Sigma-Aldrich) and 10 µg/ml COL4 from bovine placental villi (Chemicon Int., Temecula, U.S.A.), respectively, for 30 min at room temperature. The remaining solution was discarded and low passage caruncular epithelial cell cultures were seeded into the wells as described above. After 5 days in culture, the cover slips were prepared for immunofluorescence as described above.

Cell proliferation was determined by counting the number of DAPI-stained nuclei. Samples containing epithelial cells only were analyzed. Briefly, digital images were taken of 10 randomly chosen visual fields at the same magnification (200x). All nuclei identified in each visual field were counted and summed up using the AnalySIS software program (Olympus Soft Imaging Solutions, Münster, Germany). The mean value of the experiments was used to compare number of cells cultured on uncoated and coated (as described above) dishes. Statistical analysis was performed with the Statistical Analysis System (SAS, Institute Inc. Cary, U.S.A., Release 8.2) using a two-way ANOVA with the fixed factors: matrix and cell culture. Differences in the numbers of samples per factor were equalized on the basis of the Least Square Means. P-values less than 0.05 were considered significant.

ITGB1, phosphotyrosine and TLN1 expression were assessed by image capture of five selected visual fields per sample at the same magnification using an identical exposure time for all cultures. Three pictures per sample were randomly chosen, and signal intensity and distribution as they related to focal adhesions of cells cultured on coated dishes were qualitatively assessed as to whether there was increase in comparison to corresponding signals of cells cultured on uncoated dishes. No statistical analyses were performed for the expression of these genes.

Results

Localization of ITGB1-integrin and associated focal adhesion proteins in placentomes

Over the course of gestation, no differences in the spatial distribution of the integrin subunit ITGB1, ACTN, and phosphotyrosine were observed (Table 2, Figure 2). ITGB1 occurred basally in both maternal and fetal epithelia and endothelia within the maternal stroma and the fetal mesenchyme, while TGC staining was in the plasma membrane. ACTN clearly demonstrated

apicolateral staining in the maternal epithelium as well as moderate expression in both trophoblast cell types. Weak ACTN staining was present in the maternal stroma and fetal mesenchyme. Phosphotyrosine showed strong expression in TGC, while maternal epithelium and stroma were weakly stained. TLN1 was predominantly present in the maternal epithelium and the TGC, displaying differences in a temporal manner. As pregnancy progressed, TLN1 expression increased in the TGC and the surrounding caruncular epithelial cells and decreased in the basal compartment of the caruncular epithelial cells. Weak TLN1 staining was also observed in the maternal stroma and the fetal mesenchyme, respectively. In the early stages of pregnancy, PTK2 protein was present in TGC, demonstrating focal cytoplasmic staining. As pregnancy progressed, this staining in TGC increased and additional PTK2 expression was observed basally in maternal epithelial cells.

Localization of ITGB1-integrin and associated focal adhesion proteins in low passage cultures of bovine caruncular epithelial cells

In vitro, the integrin subunit ITGB1 and associated focal adhesion proteins were detected in all epithelial cells but displayed distinct differences in intracellular localization and staining patterns (Figure 3). ITGB1 was strongly expressed along the lateral cytoplasmic membrane and accumulations of staining characteristic of focal adhesions were observed at the basal surface of the cells attached to the coverslip. Co-localization of ITGB1 with PTK2 and ACTN along the lateral cell borders as well as ITGB1 with PTK2 at the sites of focal adhesions was observed. ACTN was present at the apicolateral borders between neighboring cells, whereas TLN1 was only present at focal adhesions. Phosphotyrosine was present along the cytoplasmic membrane and at focal adhesions. Co-localization of phosphotyrosine with PTK2 was observed at focal adhesions. Phosphotyrosine did not perfectly co-localize with ITGB1, however, these proteins were situated side-by-side.

Effect of coating with different matrix proteins on the proliferation rate of low passage caruncular epithelial cells and the expression pattern of ITGB1-integrin, phosphotyrosine and TLN1

The proliferation of cultured low passage caruncular epithelial cells increased significantly on all matrices over controls. FN in particular supported significantly greater cell proliferation than all other treatments (Figure 4). The average number (± standard deviation) of nuclei counted on FN (1618.71 ± 329.87; p=0.0001), LAMA (592.58 ± 213.79; p=0.0013) and COL4 (691.35 ± 297.67; p=0.04) coated dishes was significantly higher than on uncoated (436.06 ± 199.48) dishes. FN coating was also significantly superior to coating with LAMA (p=0.0001) and COL4 (p=0.0001).

Coating with LAMA and COL4 did not effect the distribution of ITGB1 and phosphotyrosine in low passage caruncular epithelial cells, whereas TLN1 staining at focal adhesions was markedly increased on all coating treatments (Figure 5). In contrast, FN coating markedly increased the number of focal adhesions that formed at the basal surface of cells. In addition, immunostaining for ACTN at the cell membrane markedly increased in cells cultured on FN in comparison to the other culture conditions.

Discussion

Focal Adhesions assemble between caruncular epithelial cells of the bovine placentome.

The bovine placentome is a complex placental structure that provides restricted trophoblast invasion and maximal vascular exposure for the transport of molecules across the epitheliochorial feto-maternal interface [20]. By co-localizing aggregates of ITGB1, phosphorylated tyrosine, PTK2, TLN1 and ACTN, we have, for the first time conclusively shown the activation of ITGB1-

integrin to form focal adhesions in caruncular epithelial cells that inhabit the feto-maternal interface.

In the present study, PTK2, phosphotyrosine and TLN1 were all localized to the majority of the cells in the bovine placentome in vivo also expressing aggregates of the ITGB1-integrin subunit. The ability of focal adhesions to be maintained as stable structures requires continuous application of local force either exerted from the contractile machinery within the cell, or from outside of the cell by application of external forces [21]. Therefore the increase in TLN1-dependent focal adhesions in TGC suggests a cell type-specific and temporal increase in physical forces between these cells, the neighboring cells and the ECM as pregnancy advances. TGC are highly migratory and displayed strong staining for phosphotyrosine keeping in mind the tyrosine phosphorylation is not a specific feature of integrin signaling alone. The increased TLN1 expression in the TGC as pregnancy progressed suggests that both “inside-out” signaling mediated by TLN1 and “outside in” signaling may occur simultaneously, at different sites, or alternately at the same sites. In both sheep and pigs, apical accumulation of TLN1 in uterine luminal epithelial cells and trophoblast cells has previously been used a sensitive functional index to confirm functional integrin activation and outside-in signalling [22,23].

The distribution of PTK2 in the bovine placentome exhibited a remarkable temporal and spatial pattern of expression. During the initial stages of pregnancy, only TGC demonstrated focal cytoplasmic staining for PTK2 while caruncular epithelial cell expression of PTK2 began at mid-gestation and continued to the end of pregnancy. It may be significant that studies performed on human placental tissue demonstrated similar findings. PTK2 could not be detected in the vasculature and its surrounding tissue of human placental stem villi [24], but was strongly expressed in cytotrophoblast in vivo and in vitro. Indeed, dependent on the site of phosphorylation, PTK2 is considered to be a marker for cytotrophoblast invasion [25].

Furthermore, PTK2 is hypothesized to have important roles in cell migration and invasion, especially in tumor cells [26]. Therefore, aggregation of PTK2 in TGC correlates nicely with the invasive phenotype of TGC. The exquisite temporal and cell-type-specific expression of PTK2 in the caruncular epithelium indicates that mechanical forces at this interface increase with fetal growth as pregnancy progresses as an adaptation to increased tension, compression and/or shear load at this interface and as part of the maturation of the placentome and placenta, respectively. On the other hand, the increasing nutritional demand of the fetus requires additional uptake of glucose at the feto-maternal interface. The localization of glucose transporters type 1 and 3 in the bovine placentome has recently been demonstrated by Wooding et al. [27]. In skeletal myotubes, it was demonstrated that PTK2 regulates insulin mediated cytoskeletal rearrangement essential for normal glucose transport and glycogen synthesis [28].

The in vivo data were strongly supported by results from cultured caruncular epithelial cells grown on rigid ECM. Co-localization of ITGB1 with PTK2 and TLN1, respectively, followed by co-localization of PTK2 with TLN1 in these cells confirmed functional integrin activation in vitro. Interestingly, phosphotyrosine co-localized perfectly with PTK2, but phosphotyrosine and ITGB1 were expressed side by side, lacking perfect co-localization as would be expected of a plasma membrane integrin receptor and underlying tyrosine-phosphorylated proteins distributed within focal adhesions. These results agree with previous studies that suggest integrin phosphorylation in some cases can actually inhibit integrin activation [7]. Although certainly phosphorylation of FAK is necessary for FAK mediated integrin activation [29], the β_1-integrin binding site for TLN1 is also a phosphotyrosine binding domain (PTB). Tyrosine phosphorylation of β_1-integrin mediated by src-kinase inhibits TLN1 binding [30] resulting in the disassembly of focal adhesions [8,31]. This process is important for cells participating in migration, hemostasis and

transformation [7], all properties which are not observed for caruncular epithelial and mononuclear trophoblast cells.

Additional confirmation of the functional activation of integrins in low passage caruncular epithelial cells was the co-localization of ACTN with ITGB1. In our study, ACTN was located only at the cell borders between neighboring cells in association with ITGB1 but not at the outer borders of the cell colonies and not within the focal adhesion sites at the basal compartment of the cell. This in vitro observation correlated with observations in vivo where ACTN expression was localized to the apicolateral compartment of the caruncular epithelial cells. Similar localization of ACTN was previously reported for the bovine placentome via electron microscopy with the immunoperoxidase/DAB staining [32]. Further, apical ACTN linking to β-integrin subunits as a response to SPP1 (osteopontin) mediated trophoblast-endometrial signaling in the ovine and porcine endometrial epithelium as well as cultured epithelial and trophectodermal cells has been demonstrated [22,23]. Taken together, these results suggest that the primary function of ACTN mediated integrin signaling in the bovine placentome is to participate in feto-maternal membrane communication rather than cell-basement membrane adhesion. However, ACTN also plays an additional role in mechanotransduction as an actin-binding component of the zonula adherens of epithelial cells in association with CDH1 (E-cadherin) and therefore, the presence of ACTN at the borders between neighboring cells is likely to include a contribution from zonula adherens-associated ACTN as well [33].

Focal adhesion assembly in bovine caruncular epithelium is dependent on the composition of the ECM.

Coating cell culture dishes with ECM proteins is a common and well accepted method to enhance cell adhesion and subsequent proliferation. Depending on the cell type cultured, the effect of coating varies significantly [34]. The results of the present study clearly demonstrate

that low passage caruncular epithelial cells isolated from placentomes of pregnant cows express functionally active ITGB1-integrin and multiple associated focal adhesion molecules that participate in “outside-in” and “inside-out” signaling that is critical for communication between cells and ECM at the feto-maternal interface. Further, the formation of these focal adhesions is affected by the composition of the surrounding ECM. Coating culture dishes with LAMA, COL4 and FN lead to a significant increase of proliferation in comparison to the cells grown on uncoated dishes. Enhancement of growth using LAMA and FN matrices is supported by recent results showing that low passage caruncular epithelial cells produce both proteins in culture [13]. Furthermore, this previous study demonstrated the presence of the integrin subunits IGTA6 and ITGB1 as well as ITGAV and ITGB1, which may form receptors for LAMA and FN, respectively. FN secretion in various cultures of epithelial cells is well described, especially at the basolateral surface [35,36] and additional coating enhances cell proliferation [37]. In the present study, FN coating lead to a significantly higher cell proliferation than coating with other ECM proteins. Continuously high cell proliferation, especially in the caruncular epithelium and independent of total caruncular growth has been shown to be present in placentomes throughout pregnancy [38]. Thus, high proliferation, especially in this area of the placentomes, is necessary for tissue remodeling and the compensation of the destructive activity of the invading TGC. FN and FN-associated integrin signalling may play a crucial role in this event.

These quantitative results were supported by a qualitative increase of the focal adhesions in cells growing on a FN matrix when detecting ITGB1, TLN1 and phosphotyrosine. The importance of FN for integrin assembly in murine primary trophoblast cell cultures was demonstrated by Yelian et al., who coated dishes with fragments of the FN molecule which clearly modulated trophoblast adhesion [39]. Furthermore, it is well established that FN induces ITGB1-integrin activation mediated by tyrosine phosphorylated PTK2 [40] lending strong

support to our findings. As described above, TLN1 binding to β-integrins promotes focal adhesion assembly and clustering followed by the activation of PTK2 [8,22]. We therefore conclude that FN coating induced additional ITGB1-integrin aggregation and assembly of focal adhesions in bovine low passage caruncular epithelial cells mediated by TLN1 and PTK2-dependent tyrosine phosphorylation. Thus FN not only mediates cell proliferation as mentioned above but may also play an important role in cell-cell communication within the caruncular epithelium in vivo.

In conclusion, this is the first report describing integrin activation, focal adhesion assembly, and the distribution pattern of phosphotyrosine, ACTN, TLN1 and PTK2 in the bovine placentome from the 3rd month of pregnancy to near term. Significantly, this study also demonstrates that low passage caruncular epithelial cells isolated from pregnant cows maintain integrin associated properties observed in vivo. In addition, the integrin-mediated response to FN coating not only resulted in enhanced cell proliferation but also increased the formation of functional focal adhesions. We suggest that this in vitro model provides excellent potential for further mechanistic studies designed to elucidate the complex interactions that develop between fetal and uterine cells at the feto-maternal interface required for fetal survival and pregnancy maintenance. These studies support continued and intensified interest in the characterization of in vitro methods that incorporate those cells that participate in the process of feto-maternal interaction such as TGC and mononuclear trophoblast cells.

Acknowledgments

The authors are grateful to S. Kettner, S. Schubert-Porth and K. Wolf (Dept. of Veterinary Anatomy, Justus-Liebig-University (JLU), Giessen, Germany) for their expert technical assistance. We also wish to thank Dr. M. Zeiler (Dept. of Veterinary Anatomy, JLU Giessen,

Germany) for sharing her expertise on integrin signalling and Prof. Dr. D. Brockmeier (Dept. of Pharmacology, JLU Giessen, Germany) for helping with statistical analysis. Furthermore, the authors would like to thank Dr. C. Rummel and Prof. Dr. R. Gerstberger (Dept. of Veterinary Physiology, JLU Giessen, Germany) for the assistance with image capturing and the usage of their facilities.

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Figure legends

Figure 1: Western blot and immunodetection on protein samples derived from the whole placentome (PL) and low passage caruncular epithelial cells (EC). Protein bands were detected at molecular weight ranges predicted for ITGB1 (130 kDa), ACTN (100 kDa), PTK2 (125 kDa) and TLN1 (270 KDa and 220 KDa fragment). Identical incubation conditions without the primary antibodies served as a control.

Figure 2: Schematic overview of the synepitheliochorial interface in the bovine placentome [A]; maternal stroma (MS); blood vessels (BV); maternal caruncular epithelium (ME); mononuclear trophoblast cells (MTC, chorionic epithelium), trophoblast giant cells (TGC); fetal mesenchyme (FM). Immunofluorescence performed on cryosections of snap frozen placentomes detecting integrin subunit ITGB1 [B], phosphotyrosine (PT) [C], ACTN [D], TLN1 [E & F] and PTK2 [G

& H]. All experiments were performed with control sections incubated without primary antibodies displaying no nonspecific binding of the secondary antibodies (not shown). Bar = 25 µm

Figure 3: Double labeling immunofluorescence performed on low passage caruncular epithelial cells. Co-localization is present where the overlay of green and red fluorescent signals is yellow/orange. Pictures were taken at the basal focal plane towards the ground of the cover slip (right column) to visualize the characteristic protein aggregations identified as focal adhesions. Pictures taken at the apicolateral focal plane (left column) visualize the cell membranes. IGTB1 (green) and PTK2 (red) [A & B]; Phosphotyrosine (PT, red) and PTK2 (green) [C]; PT (red) IGTB1 (green) [D]; TLN1 (red) and IGTB1 (green) [E]; ACTN (red) and ITGB1 (green) [F]; PTK2 (red) and TLN1 (green) [G]; control without primary antibody [H]. TLN1 was not present along the cell membrane whereas staining for ACTN at the FA was not observed (not shown). [G] Co-expression of PTK2 (red) with TLN1 (green) was present at the FA. [H]. Bar = 25 µm

Figure 4: Influence of coating with COL4 (n=5), LAMA (n=7) and FN (n=7) on the proliferation (cell number) of cultured low passage caruncular epithelial cells. The graph summarizes the data displaying the mean of counted nuclei for each culture condition (±standard deviation). Different superscripts indicate significant differences ($p < 0.05$) between treatment groups.

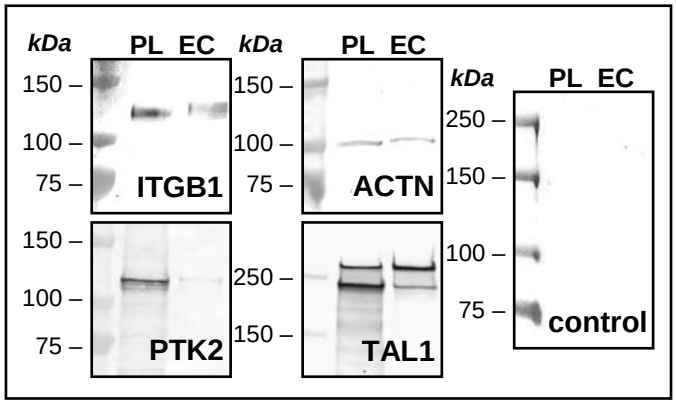
Figure 5: According to the immunofluorescence detection of IGTB1-integrin, phosphotyrosine and TLN1, the number of focal adhesions (FA) at the basal surface of cells and the signal intensity was increased when cells were cultured on COL4, LAMA or FN. Bar = 25 µm

Table 1: Antibodies used for immunofluorescence (IF) and Western blot (WB) analyses

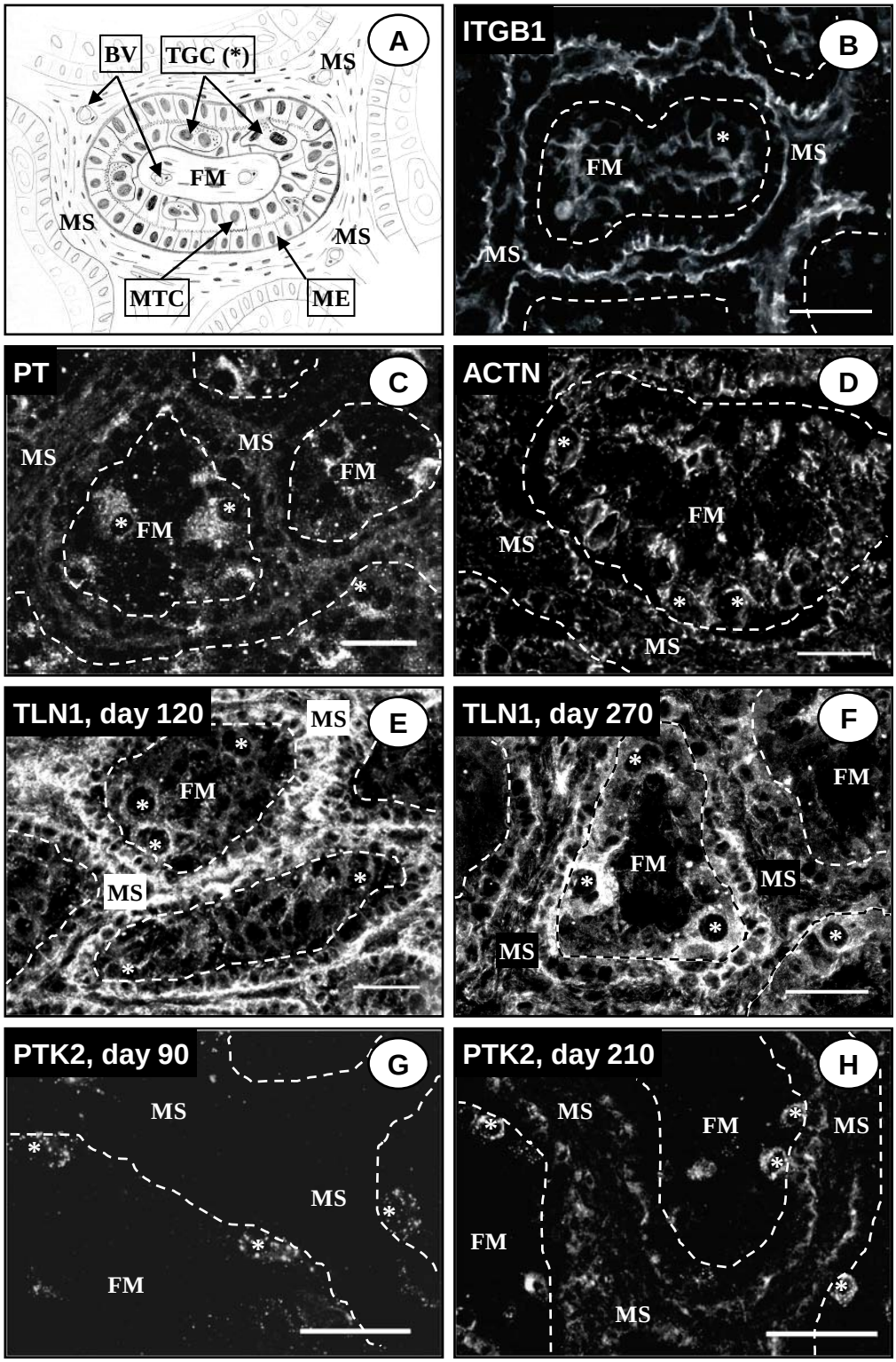
Primary antibodies		**Dilution IF**	**Dilution WB**	**Manufacturer**
Rabbit anti-Integrin β_1 (ITGB1)	poly-clonal	1:100	1:600	Chemicon Int., Temecula, U.S.A., Cat. No.: AB1952
Mouse anti-Integrin β_1 (ITGB1)	mono-clonal	1:100	-	Natu Tec, Frankfurt, Germany Cat. No.: FW$-101
Mouse anti-α actinin (ACTN)	mono-clonal	1:200	1:2000	Sigma-Aldrich, Taufkirchen, Germany Cat. No.: A5044
Rabbit anti-FAK (PTK2)	poly-clonal	1:100	1:200	Santa Cruz, Heidelberg, Germany Cat.No.: sc-558
Mouse anti-Phosphotyrosine	mono-clonal	1:50	-	Upstate / Biomol, Lake Placid, U.S.A. Cat. No.: 05-321
Mouse anti-Talin (TLN1)	mono-clonal	1:400	1:2000	Sigma-Aldrich, Cat. No.: A5044
Secondary Antibodies		**Dilution IF**	**Dilution WB**	**Manufacturer**
Donkey anti-rabbit IgG, Fluorescein (FITC) conjugated		1:200	-	Chemicon Int., Cat. No.: AP182F
Donkey anti-mouse IgG, Fluorescein (FITC) conjugated		1:200	-	Chemicon Int., Cat. No.: AP192F
Donkey anti-rabbit IgG, Cy3 conjugated		1:300	-	Chemicon Int., Cat. No.: AP182C
Donkey anti-mouse IgG, Cy3 conjugated		1:300	-	Chemicon Int., Cat. No.: AP192C

Table 2: Distribution pattern of integrin subunit ITGB1 and its associated focal adhesion proteins in vivo throughout pregnancy. Immunofluorescence performed on cryosections of placentomes of 3-4 months (early), 5-6 months (mid) and 7-9 months (late) of pregnancy were assessed thereby differing between location and intensity of the signals (+++ strong, ++ moderate, + weak, - none).

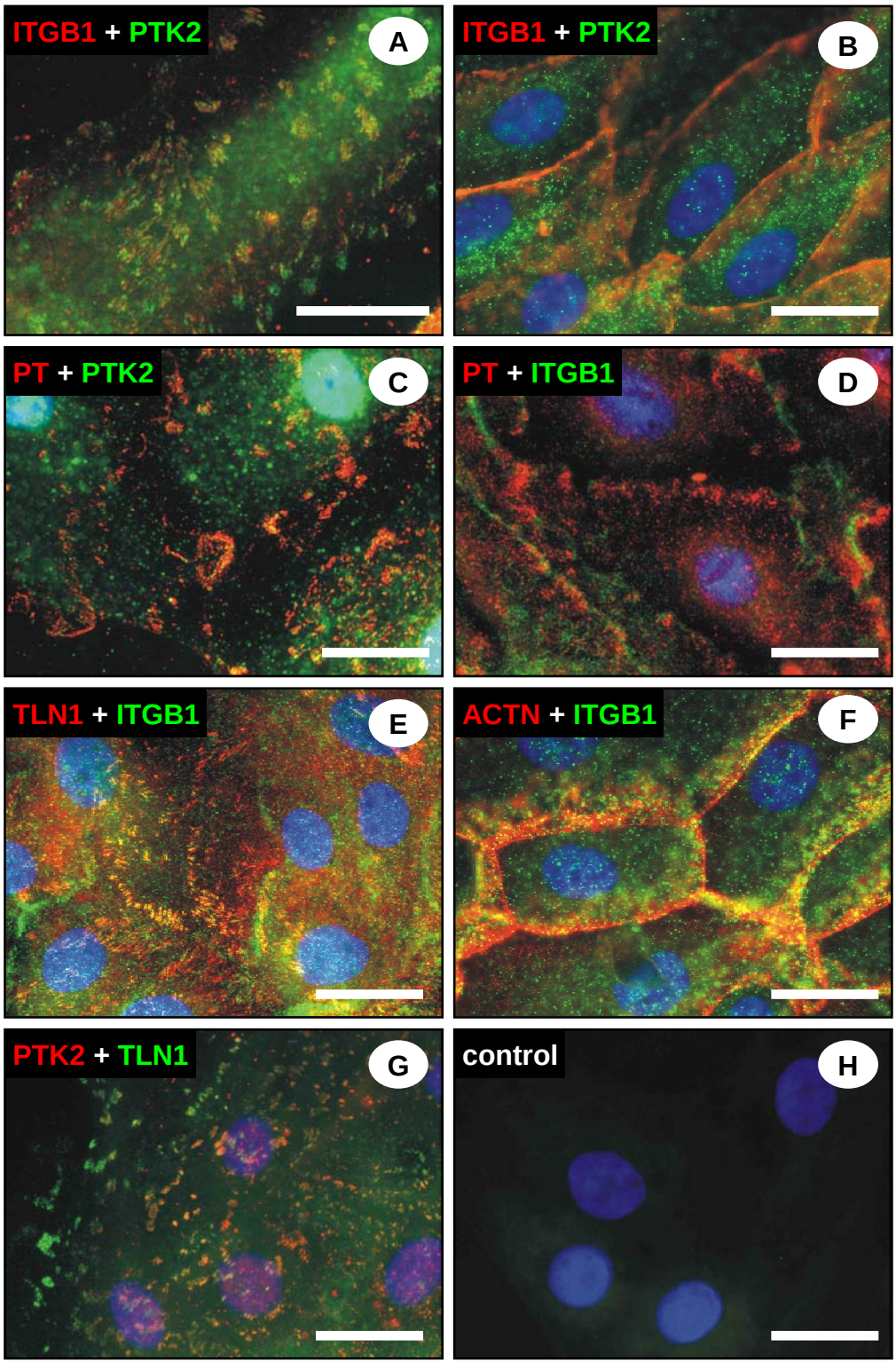
Gestational stage	**Early (n=6)**	**Mid (n=6)**	**Late (n=6)**
ITGB1-integrin			
mat. Stroma	+	+	+
mat. epithelium basal	+++	+++	+++
mat. epithelium cytoplasm	+	+	+
mat. epithelium apical	-	-	-
fetal mononuclear trophoblast	++	++	++
fetal TGC	++	++	++
fetal mesenchyme	+	+	+
Phosphotyrosine			
mat. Stroma	+	+	+
mat. epithelium basal	+	+	+
mat. epithelium cytoplasm	-	-	-
mat. epithelium apical	-	-	-
fetal mononuclear trophoblast	-	-	-
fetal TGC	+++	+++	+++
fetal mesenchyme	-	-	-
PTK2			
mat. Stroma	-/+	+	+
mat. epithelium basal	-/+	++	++
mat. epithelium cytoplasm	-	+	+
mat. epithelium apical	-	+	+
fetal mononuclear trophoblast	-	-	-
fetal TGC	++	+++	+++
fetal mesenchyme	-	-	-
TLN1			
mat. Stroma	**++**	**++**	**++**
mat. epithelium basal	+++	+++	+
mat. epithelium cytoplasm	++	++	++
mat. epithelium apical	++	++	++
fetal mononuclear trophoblast	+	+	+
fetal TGC	-/+	+/++	++/+++
fetal mesenchyme	+	+	+
ACTN			
mat. Stroma	+	+	+
mat. epithelium basal	-	-	-
mat. epithelium cytoplasm	++	++	++
mat. epithelium apical	+	+	+
fetal mononuclear trophoblast	++	++	++
fetal TGC	++	++	++
fetal mesenchyme	+	+	+



Bridger et al., Figure 1



Bridger et al., Figure 2



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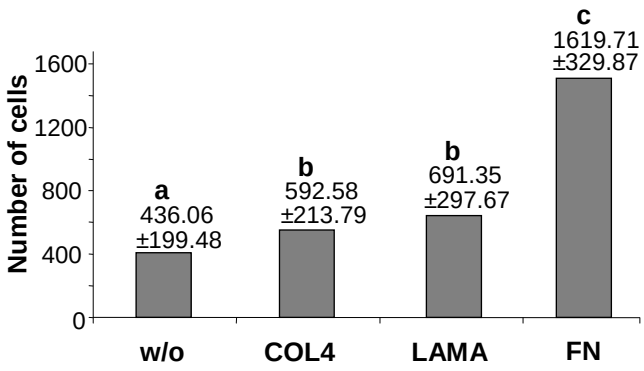

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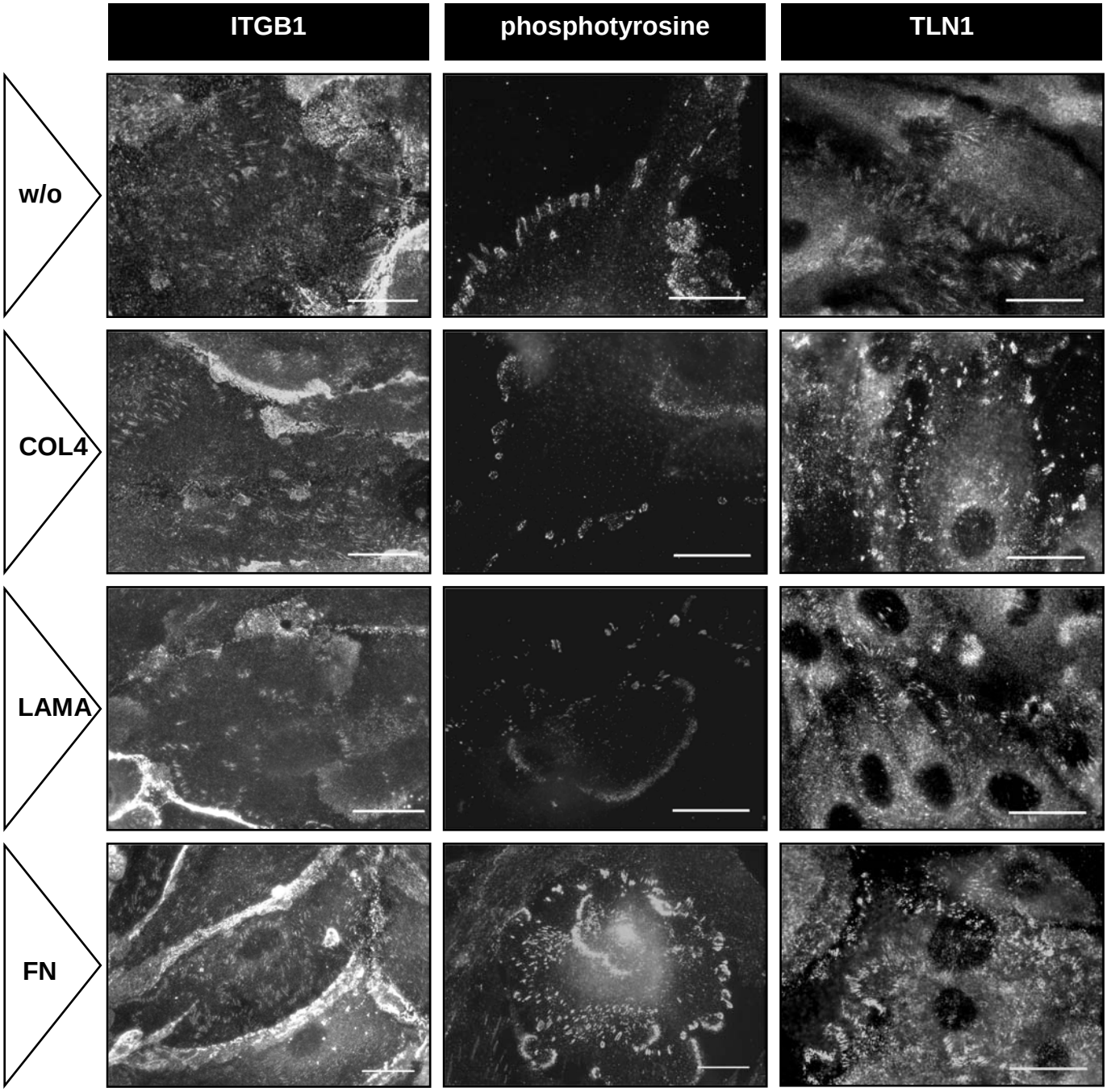

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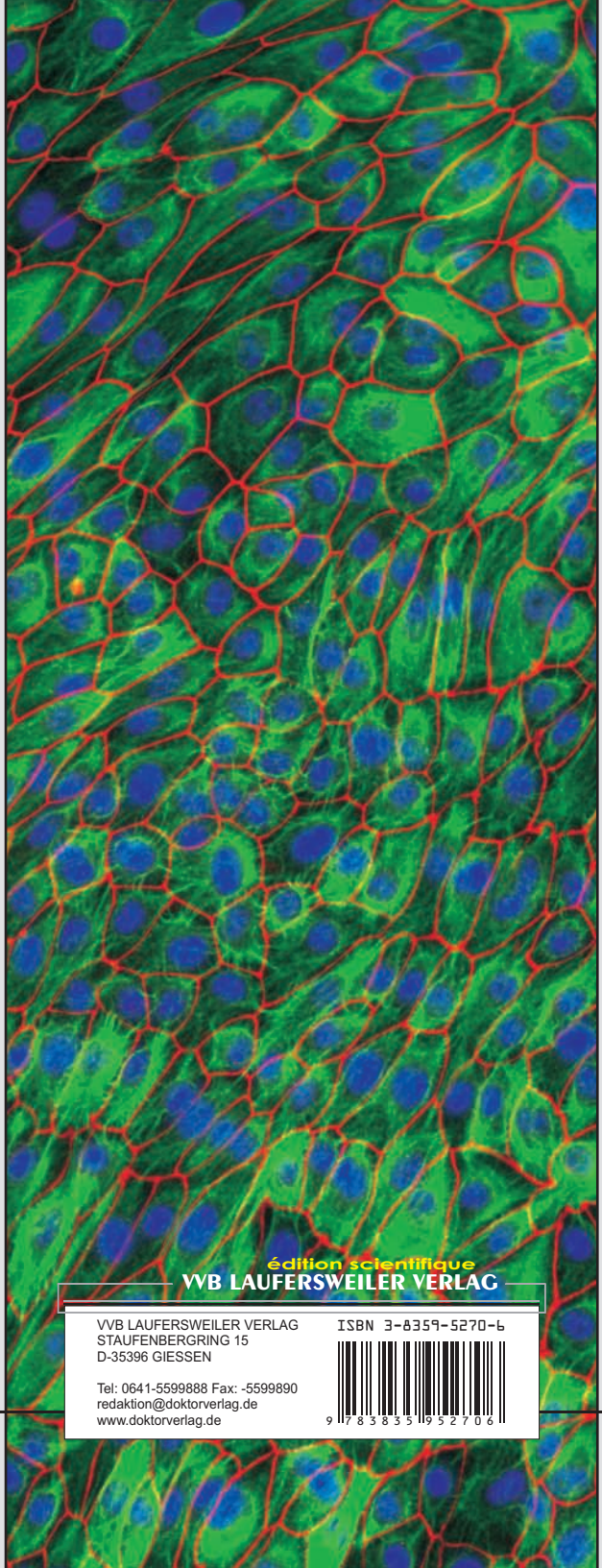

édition scientifique
VVB LAUFERSWEILER VERLAG

VVB LAUFERSWEILER VERLAG
STAUFENBERGRING 15
D-35396 GIESSEN

Tel: 0641-5599888 Fax: -5599890
redaktion@doktorverlag.de
www.doktorverlag.de

ISBN 3-8359-5270-6