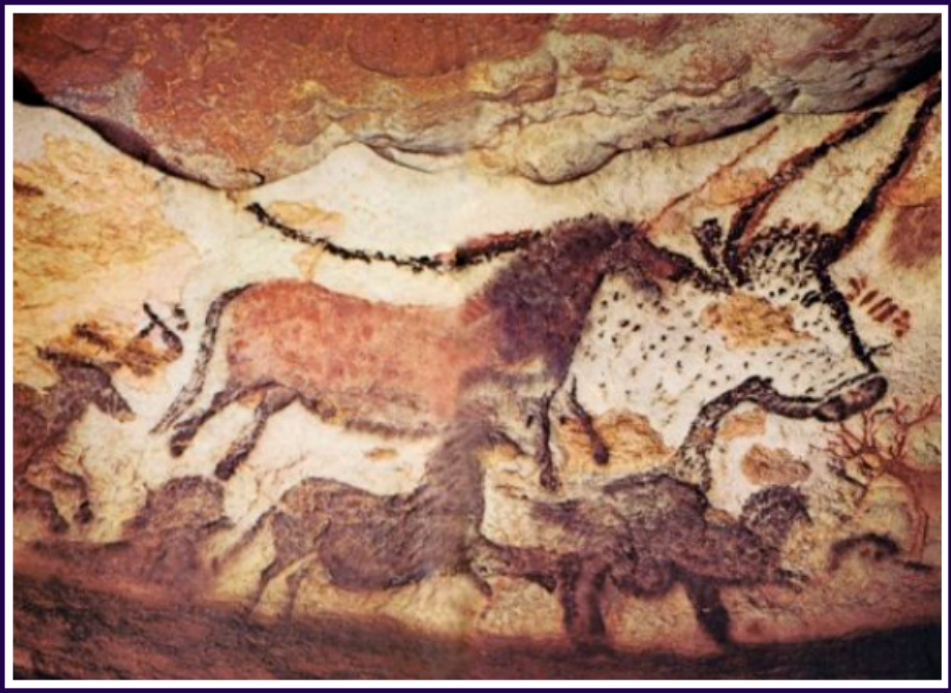
Aus dem Institut für Veterinär-Anatomie, -Histologie und -Embryologie
der Justus-Liebig-Universität Giessen

Charakterisierung der Entwicklung der Wiederkäuerplazentome



Habilitationsschrift
vorgelegt dem Fachbereich Veterinärmedizin
der Justus-Liebig-Universität Giessen

von
Dr. med. vet. Christiane Pfarrer, geb. Krebs
aus Oldenburg in Holstein

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Meinen Eltern

1. Abkürzungen

AP-I	Protein 1 (activator protein-1)
BM	Basalmembran
Cx	Connexin
ECM	Extrazelluläre Matrix (extracellular matrix)
ERK	Extrazellulär-regulierte Kinase (extracellular-regulated kinase)
FAK	Fokale Adhäsionskinase (focal adhesion kinase)
FGF	Fibroblastenwachstumsfaktor (fibroblast growth factor)
FGFR	FGF-Rezeptor
GLUT 1	Glukosetransporter 1
HA	Hochgebirgsschaf (high altitude sheep)
IHC	Immunhistochemie
ISH	In situ Hybridisierung
JNK	c-Jun N-terminale Kinase
MAPK	mitogen-aktivierte Proteinkinase (mitogen-activated protein kinase)
MEKK	mitogen-aktivierte Proteinkinase/ERK Kinasekinase
OPN	Osteopontin
p.c.	post conceptionem
p.i.	post inseminationem
PL-I	plazentäres Laktogen I
pMAPK	phosphorylierte (= aktivierte) MAPK
p.p.	post partum
RT-PCR	Reverse Transkriptase Polymerasekettenreaktion (reverse transcriptase polymerase chain reaction)
SL	Meereshöhe Schaf (sea level sheep)
SSL	Scheitel-Steiss-Länge
TGC	Trophoblastriesenzelle (trophoblast giant cell)
UTMP	Protein der Uterinmilch (uterine milk protein)
VEGF	Gefäßendothel-Wachstumsfaktor (vascular endothelial growth factor)
VEGFR	VEGF-Rezeptor

2. Vorbemerkung

Die vorgelegte Habilitationsschrift setzt sich aus acht thematisch zusammenhängenden Originalveröffentlichungen in referierten wissenschaftlichen Zeitschriften und zwei eingereichten Arbeiten zusammen, die jeweils mit relevanten Arbeiten der internationalen Literatur diskutiert werden.

Die vorliegende Schrift umfasst zwei Abschnitte, da die Wiederkäuerplazenta aus zwei Blickwinkeln beleuchtet wird. Die Arbeiten des ersten Abschnitts befassen sich mit verschiedenen Signalwegen in der Wiederkäuerplazenta, die über zelluläre Strukturen, Rezeptoren und extrazelluläre Proteine charakterisiert werden. Im zweiten Abschnitt wird die Gefäßarchitektur und Angiogenese der Wiederkäuerplazenta analysiert.

Illustrationen und Abbildungen finden sich nur in den vorgelegten Veröffentlichungen. Die eigenen Ergebnisse wurden bewusst nicht mit Selbstzitaten versehen, da die entsprechenden Originalveröffentlichungen Teil dieser Arbeit sind.

3. Einleitung

3.1 Funktionelle Morphologie der Ruminantenplazenta

3.1.1 Klassifizierung

Die Ruminanten besitzen eine kotyledonäre Plazenta (Placenta cotyledonaria sive multiplex), in der sich fetale Kotyledonen und maternale Karunkeln zu Plazentomen zusammensetzen (Strahl, 1906). Fetales und maternales Gewebe interdigitiert in Form von Zotten und Krypten miteinander (Strahl, 1906; Mossmann, 1987). Ursprünglich wurde die Wiederkäuerplazenta auf Grund der Zahl und Art der Schichten, die fetales und maternales Blut trennen, als syndesmochorial klassifiziert (Grosser, 1927). Detailierte, elektronenmikroskopische Untersuchungen zeigten allerdings, dass das uterine Epithel bei Wiederkäuern persistiert, und es sich daher um einen epitheliochorialen Plazentatyp handelt (Björkman, 1954; Ludwig, 1962).

Im Chorionepithel differenzieren sich zwei Populationen von Trophoblastzellen, erstens mononukleäre, polarisierte Zellen und zweitens meist binukleäre Trophoblastriesenzellen (TGC) (Wooding und Wathes, 1980), die nicht polarisiert sind und mit maternalen Epithelzellen zu feto-maternalen Hybridzellen fusionieren. Da es sich bei diesen genaugenommen um Synzytien handelt, wurde von Wooding (1992) der Begriff synepitheliochorial geprägt. Die TGC entwickeln sich aus mononukleären Vorläufern durch azytogenetische Mitosen (Wimsatt, 1951; Klisch et al., 1999b) und sind zur Migration befähigt. Im Verlaufe dieser lösen sich die TGC aus dem Trophoblastzellverband und wandern auf das uterine oder Karunkelepithel zu, um dort mit einzelnen Epithelzellen meist dreikernige, feto-maternale Hybridzellen zu bilden, die das Endprodukt beim Rind darstellen. Dagegen entstehen beim Schaf durch weitere Fusionen von TGC mit Hybridzellen regelmäßig größere Synzytien (Wooding, 1984). Durch Migration und Fusion der TGC werden hormonelle Produkte, wie plazentäres Laktogen und schwangerschaftsspezifische (Glyko)Proteine, an das maternale Kompartiment übertragen (Wooding und Beckers, 1987). Die Hybridzellen degenerieren in der Folge (Wimsatt, 1951), wahrscheinlich durch Apoptose, und werden in Richtung des fetalen Kompartiments abgestoßen und dort möglicherweise phagozytiert (nicht publizierte eigene Ergebnisse).

3.1.2 Die Trophoblastinvasion

Epitheliochoriale Plazenten zeichnen sich im Allgemeinen durch einen nicht invasiven Trophoblasten aus und gehören daher zu den Placentae adeciduata. Das heißt, das uterine Epithel bleibt während der Implantation und Plazentation bis hin zum Geburtszeitpunkt mehr oder weniger unversehrt (Strahl, 1906). Dieses ist bei den endotheliochorialen Plazenten der Fleischfresser und den hämochorialen Plazenten der Primaten und Rodentia nicht der Fall. Diese werden als deziduate Plazenten klassifiziert, da der Trophoblast in das Endometrium einwandert und zur Degeneration des Endometriums führt, so dass nach der Geburt bei der Ablösung der Nachgeburt zum Teil erhebliche Gewebsverluste auf der mütterlichen Seite zu verzeichnen sind (Strahl, 1906). Die synepitheliochoriale Plazenta der Wiederkäuer stellt möglicherweise ein Intermediärstadium zwischen den echten epitheliochorialen adeziduaten und den endothelio- und hämochorialen deziduaten Plazenten dar, da eine eingeschränkte Invasion von TGC in das maternale Epithel stattfindet (Pfarrer et al., 2003). Die Trophoblastinvasion ist ein extrem empfindlicher Prozess, der einer Reihe von Faktoren unterworfen ist (Fisher und Damsky, 1993; Damsky et al., 1994; Aplin, 1997). Bei Störungen der Regulation kommt es zu tiefgreifenden pathologischen Veränderungen, die sich nicht nur auf funktioneller Ebene sondern auch auf morphologischer Basis niederschlagen. Zum Beispiel ist die Präeklampsie des Menschen (Schwangerschafts-Bluthochdruck) durch eine mangelnde Invasionstiefe gekennzeichnet (Zhou et al., 1997), die von einer veränderten Integrinexpression begleitet wird (Zhou et al., 1993) und auf hypoxische Verhältnisse im Bereich der invasiven Zytotrophoblastzellen zurückzuführen ist (Zhou et al., 1998).

Der Sauerstoffpartialdruck ist einer der Schlüsselregulatoren des plazentären Wachstums (Kingdom und Kaufmann, 1997). Der frühe Embryo mit seinen Hüllen entwickelt sich in einer relativen Hypoxie, die eine essentielle Rolle für die embryonale Frühentwicklung spielt (Cross et al., 1994). Diese Tatsache wurde auch durch in vitro Studien bestätigt, in denen sich Sauerstoff als kritische Determinante für die Tiefe der Trophoblastinvasion herauskristallisierte (Genbacev et al., 1996). Während der normalen menschlichen Schwangerschaft differenziert sich eine Subpopulation fetaler Zytotrophoblastzellen weiter und wandert in das Endometrium und seine Arteriolen ein. Bei der Präeklampsie ist diese Differenzierung nur unvollständig und flach, was zu einer relativen Hypoxie der Plazenta führt. In vitro reagieren menschliche Zytotrophoblastzellen auf einen verringerten Sauerstoffpartialdruck mit der Inkorporation von [3H]Thymidin und 5-Bromo-2'-Deoxyuridin und dem Verlust der Migrationsfähigkeit (Genbacev et al., 1996).

Die Fähigkeit zur Migration von Zellen ist eng mit einer Interaktion von Integrinen mit Proteinen der extrazellulären Matrix (ECM) verknüpft. Integrine sind Glykoproteine, die als Heterodimere aus einer α- und einer β-Untereinheit transmembran gelegene Rezeptoren an der Zelloberfläche bilden. Diese interagieren mit einer Vielzahl von Liganden, wie der ECM und Oberflächenmolekülen, und vermitteln so Signale in die Zelle (outside-in signaling) wie auch aus ihr hinaus (inside-out signaling) (Hynes, 1987; Ruoslahti, 1991; Bosman, 1993). Eine regulatorische Funktion der Interaktion von Integrinen mit der ECM wurde für die Implantation und Trophoblastinvasion der hämochorialen Plazentation des Menschen festgestellt (Fisher und Damsky, 1993; Aplin, 1997), wo ein Umschalten (Integrin-switch) von Integrinen auf einen Wechsel der zellulären Differenzierung zwischen Proliferation und Migration hinweist (Damsky et al., 1994). Auch für die epitheliochoriale Implantation des Schweines gibt es eine spezifische Adhäsionskaskade, die mit einer Veränderung der Integrin- und ECM-Expression einhergeht (Bowen et al., 1996; Burghardt et al., 1997) und auch in vitro nachzuweisen ist (Bowen et al., 1997). Weiterhin wurde die Beteiligung des β_1 Integrins für die Migration der TGC in der frühen Implantation des Rindes vermutet (MacLaren und Wildeman, 1995) und Veränderungen im Expressionsmuster von weiteren Integrinen während der Implantation und in isolierten TGC beobachtet (MacIntyre et al., 2002).

Eine Integrinbindung führt über die Anlagerung von Zytoskelett-assoziierten Proteinen, wie α-Aktinin, Talin und Vinculin, die fokale Adhäsionskomplexe bilden, zu Konformationsänderungen des Zytoskelettes, die in der Folge zu Transkriptionsänderungen im Zellkern führen (Miyamoto et al., 1995). Das Auftreten von fokalen Adhäsionskomplexen gilt als Nachweis einer funktionellen Integrinbindung (Pavalko und Otey, 1994). Parallel zu der Signalübertragung über das Zytoskelett werden durch die Integrinbindung auch Phosphorylierungskaskaden verschiedener Kinasen in Gang gesetzt, die zu einer Translokation der phosphorylierten und somit aktivierten mitogen-activated protein kinase (MAPK) in den Zellkern führt und dort wiederum Transkriptionsänderungen bewirkt (Chen et al., 1994a; Morino et al., 1995; Aplin et al., 2002). Derartige Integrin-induzierte Signalkaskaden können auch mit Wachstumsfaktoren synergistisch wirken (Miyamoto et al., 1996). Die Verlagerung der aktivierten Form der MAPK (auch extracellular-regulated Kinase, ERK) in den Zellkern steht auch in engem Zusammenhang mit der Steuerung der Zellmigration (Cho und Klemke, 2000; Glading et al., 2001; Howe et al., 2002).

Die Migration, die bei Invasionsvorgängen in Tumoren wie auch der Trophoblastinvasion auftritt, wird weiterhin durch interzelluläre Kommunikation über Gap junctions beeinflusst.

Funktionelle Gap junctions setzen sich aus jeweils zwei Halbkanälen (Connexonen) benachbarter Zellen zusammen, die wiederum aus hexamerisch arrangierten Proteinen, den Connexinen (Cx), bestehen (Yeager und Gilula, 1992). Durch diese Kanäle können kleine Moleküle von einer Größe von 1-2 kDa (anorganische Ionen und Second Messenger, z.B. cAMP) direkt von einer Zelle in die andere übertreten (Murray und Fletcher, 1984; Sandberg et al., 1992). Daraus ergeben sich auch die Hauptfunktionen der Gap junctions, die schnelle Übertragung von Regulations- und Informationsmolekülen in einem Zellverband und das Abkoppeln von geschädigten Zellen von intakten Nachbarzellen (Bruzzone et al., 1996). Auf diese Weise sind Cx an der Kontrolle von Wachstums- und Proliferationsprozessen beteiligt (Yamasaki, 1990; Loewenstein und Rose, 1992; Hotz-Wagenblatt und Shalloway, 1993), und so auch für die physiologische Differenzierung von Zellen in Richtung Invasivität und Zellfusion (Firth et al., 1980; de Virgiliis et al., 1982; Cronier et al., 1994) sowie für das Tumorwachstum verantwortlich (Yamasaki und Naus, 1996).

In hämochorialen Plazentationstypen mit hochinvasivem Trophoblasten zeigt sich eine speziesabhängige räumliche und zeitliche Veränderung der Cx-Expression während der kritischen Periode der Implantation, die mit dem funktionellen Status der Trophoblastzellen korreliert (Cronier et al., 2001). In der frühen Schwangerschaft des Menschen exprimieren proliferative, extravillöse Trophoblastzellen Cx40, welches während der Migration verloren geht, aber in den Trophoblastzellinseln wieder auftaucht. Gleichzeitig findet sich in den Zytotrophoblastzellen Cx43, wohingegen TGC Cx32 und Cx43 exprimieren (Winterhager et al., 1999). Im Spongiotrophoblast von Nagern wird eine Verschiebung der Expression von Cx31 zu Cx43 beobachtet, die eine Schalterfunktion zwischen Invasion/Proliferation und Differenzierung vermuten lässt (Winterhager et al., 1996). Vergleichbare Regulationen wurden bei Spezies mit nicht invasiven epitheliochorialen Plazenten nicht festgestellt (Day et al., 1998).

3.1.3 Die Angiogenese

Die Gefäßarchitektur bestimmt die Form der fetalen Plazentazotten maßgeblich, da die Kapillaren sich direkt an die Basalmembran der Oberflächenepithelien des Trophoblasten und des Endometriums anschließen (Leiser und Koob, 1992). Die Entwicklung eines funktionierenden Blutgefäßsystems ist daher die entscheidende Determinante für die spätere Form der interdigitierenden fetalen und maternalen Anteile der Plazenta (Leiser und Koob, 1992). Dieser Zusammenhang wird auch bei aus somatischen Zellen geklonten Rinderfeten beobachtet, die schon in der frühen Trächtigkeit eine erhöhte Mortalität aufweisen, da die

reduzierte Entwicklung von vaskulären Strukturen im fetalen Kompartiment Plazentome verminderter Größe entstehen lässt (Hill et al., 2000).

Das Wachstum von Gefäßen wird in die de novo Formation von Blutgefäßen im sich entwickelnden Embryo, die Vaskulogenese, und das sich daran anschließende Wachstum von Gefäßen, die Angiogenese, eingeteilt (Risau, 1997). Die Vaskulogenese, also die Bildung von Angioblasten und hämatopoetischen Vorläuferzellen, wird von Mitgliedern der Fibroblastenwachstumsfaktoren (FGFs) induziert (Risau, 1997). Allerdings ist auch der vascular endothelial growth factor receptor (VEGFR)-2 (flk-1) von Bedeutung, da in flk-1 defizienten Mäusen die Bildung von Blutinseln und die Vaskulogenese unterbleiben (Shalaby et al., 1995). Dagegen spielt der VEGFR-1 später bei der Formation von Blutgefäßen aus einzelnen Angioblasten eine Rolle (Fong et al., 1995). Auch vascular endothelial growth factor (VEGF) selbst ist für die Differenzierung der Angioblasten wichtig, da Mäuse, denen eine Kopie des VEGF Genes fehlt, auf Grund von fehlgeleiteter Gefäßentwicklung im Dottersack und im Embryo in utero sterben (Carmeliet et al., 1996).

Die Angiogenese wird durch die Migration, Proliferation und Invasion von Endothelzellen charakterisiert (Folkman und Klagsbrun, 1987), wobei jeder dieser Schritte einer eigenen Regulation unterworfen ist. Auch bei der Angiogenese sind Integrine involviert, da Untersuchungen gezeigt haben, dass die Migration von Endothelzellen durch Integrinbindung vermittelt wird (Friedlander et al., 1995; Senger et al., 1997; Kim et al., 2000). Die Migration glatter Muskelzellen der Gefäße wird ebenso durch Interaktion von Integrinen mit der ECM, besonders Osteopontin (OPN), stimuliert (Yue et al., 1994; Liaw et al., 1995; Pickering et al., 1997).

Eine Reihe von weiteren Faktoren, wie VEGF, placenta growth factor (PlGF), FGFs und Zytokine, wurden als Angiogenesefaktoren identifiziert (Norrby, 1997; Powers et al., 2000; Ferrara, 2001; Poole et al., 2001). Häufig wirken mehrere Faktoren gleichzeitig oder regulieren einander. Eine solche autokrine Induktion von VEGF wird spezifisch in Endothelzellen von Kapillaren beobachtet, nicht aber in FGF2-stimulierten Fibrozyten, und ist durch Antikörper gegen VEGF blockierbar (Seghezzi et al., 1998). Die Mitglieder der Familie der FGFs vermitteln ihre angiogene Wirkung über ihre Mitogenität und die Stimulation der Migration über Chemotaxis (Powers et al., 2000). Auch die Kommunikation von Zellen wird durch FGF beeinflusst, da sich die Expression von Cx43 in Endothelien von Gefäßen durch FGF2 erhöht (Pepper und Meda, 1992).

In vitro wird die VEGF-Expression durch hypoxische Bedingungen stimuliert (Shweiki et al., 1992; Wheeler et al., 1995), was auch für das menschliche Endometrium in/ex vivo berichtet wird (Sharkey et al., 2000). Die Stimulation der Angiogenese in der Plazenta durch Hypoxie wurde für den Menschen und das Meerschweinchen bestätigt (Bacon et al., 1984; Scheffen et al., 1990; Jauniaux et al., 1992, 1994; Reshetnikova et al., 1994). Bei beiden Spezies erfolgt die Adaptation über die Vergrößerung des Kapillarbettes und damit über die Ausweitung der feto-maternalen Austauschfläche, die entweder im fetalen und/oder im maternalen Kompartiment beobachtet wird.

Weiterhin kann eine Modulation der angiogenen Wirkung über die ECM (wie Glykosaminoglykane und Syndecane), Integrine, Zelladhäsionsmoleküle (CAMs) und SPARC (secreted protein, acid and rich in cysteine) erfolgen (Polverini, 1996; Madri, 1997; Norrby, 1997; Sage, 1997; Kim et al., 2000; Ornitz, 2000). Ein Teil der Stimulatoren ist zellulären Ursprungs, das heißt, diese werden von Makrophagen und Mastzellen produziert (Norrby, 1997). Neben ihrer mitogenen Aktivität vermitteln einige der genannten Faktoren ihre Wirkung über die Stimulation der Migration von Gefäßendothelien und glatten Muskelzellen (Yoshida et al., 1996; Rousseau et al., 1997; Bernatchez et al., 1999; Kim et al., 2000). Hierfür sind wiederum Veränderungen des Zytoskelettes und Aktivierung von Phosphorylierungskaskaden eine Voraussetzung (Rousseau et al., 1997, 2000).

4. Zielsetzungen

In folgendem Konzept laufen die Fäden der Studie zusammen: Faktoren, die einerseits die Angiogenese stimulieren, werden andererseits durch Proteine moduliert, die im Zusammenhang mit Zellmigration und -invasion stehen, wie dem Zytoskelett, den Integrinrezeptoren und der ECM als auch mit den Gap junctions bildenden Connexinen.

Um dieser Vernetzung Rechnung zu tragen, und den Modellcharakter der eingeschränkten Trophoblastinvasion in der Plazenta des Rindes für andere invasive Prozesse zu prüfen, wurden nachstehende Untersuchungen durchgeführt:

4.1 Charakterisierung von Signalwegen in der Wiederkäuerplazenta

1. Das ***Zytoskelett*** und seine ***assoziierten Proteine*** sind als intrazelluläre Einheit möglicherweise in der Lage, über die Expression von Integrinrezeptoren und einer spezifischen extrazellulären Matrix, den feto-maternalen Kontakt, sowie das Verhalten und die Aktivität der TGC zu beeinflussen. Aus diesem Grund wurden im ***Rinderplazentom*** das Aktinzytoskelett sowie die assoziierten Proteine α-Aktinin und Vinculin mittels Immunelektronenmikroskopie und RT-PCR dargestellt. Da eine Integrinbindung auch stets eine intrazelluläre Phosphorylierungskaskade in Gang setzt, wurde mit der gleichen Methode parallel auch das "Schlüsselenzym" ***mitogen-activated protein kinase (MAPK)*** lokalisiert, welches nach Phosphorylierung, sprich Aktivierung, in den Kern verlagert wird (Veröffentlichung Nr. 1: Lang et al., 2004).

2. Bei dem nahe verwandten ***Schaf*** wurde die Expression von ***Integrinrezeptoren*** und korrespondierenden Proteinen der ***extrazellulären Matrix*** während der Implantationskaskade mittels Immunfluoreszenz in vivo und vitro untersucht (Veröffentlichung Nr. 2: Johnson et al., 2001).

3. Weiterhin wurde im ***Schaf Osteopontin***, ein spezielles Protein der extrazellulären Matrix, über den gesamten Verlauf der Trächtigkeit im Uterus und der Plazenta lokalisiert. Für diese und die vorhergehende Studie (Nr. 2.) wurde der Proteinnachweis über Immunfluoreszenz mittels molekularbiologischer Methoden zum mRNA Nachweis ergänzt (Veröffentlichung Nr. 3: Johnson et al., 2003).

4. Im gleichen Zuge wurde die Expression von ***Integrinrezeptoren*** und der ***extrazellulären Matrix*** im ***Rinderplazentom*** vom 90. bis zum 270. Tag der Gravidität mit immunhistochemischen Methoden untersucht (Veröffentlichung Nr. 4: Pfarrer et al., 2003).

5. Die Modulation der ***Connexinexpression*** im Endometrium des ***Schafes*** als Antwort auf die Trächtigkeit wurde mittels Immunfluoreszenz untersucht, da auch die Kommunikation jeweils zwischen zwei Zellen über Gap junctions spezifische Signalwege der Reproduktion in Gang setzen kann (Veröffentlichung Nr. 5: Gabriel et al., 2003).

6. Die spezifische Fähigkeit der Trophoblastriesenzellen des ***Rindes*** zur Migration und Fusion lässt auf eine spezielle ***Connexin***ausstattung schließen, die sich von der der echten epitheliochorialen Plazentatypen unterscheidet. Diese Hypothese wurde mittels Proteinnachweis über Immunhistochemie und mRNA Nachweis über RT-PCR und in situ Hybridisierung überprüft (Veröffentlichung Nr. 6: Pfarrer et al., 2004).

4.2 Gefäßarchitektur und Angiogenese der Wiederkäuerplazenta

1. Als Grundlage für die Analyse der ***Gefäßentwicklung*** und ***-architektur*** diente die rasterelektronenmikroskopische Untersuchung von Gefäßausgüssen der ***Rinderplazentome*** aus dem gesamten Verlauf der Trächtigkeit (Veröffentlichungen Nr. 7 und 8: Leiser et al., 1997; Pfarrer et al., 2001).

2. Die Hypothese, dass ***Hypoxie*** die ***Angiogenese*** in der ***Plazenta des Schafes*** stimuliert, wurde rasterelektronenmikroskopisch an Gefäßausgüssen und mittels computergestützter Bildanalyse an histologischem Plazentamaterial von Tieren untersucht, die während der Trächtigkeit im Hochgebirge (hypobare Hypoxie) gehalten wurden (Veröffentlichung Nr. 9: Krebs et al., 1997).

3. Da FGF als einer der wichtigsten Angiogenesefaktoren auch in Migrations- und Differenzierungsprozesse eingreift, wurde die Expression von verschiedenen Mitgliedern des ***FGF-Systems*** (Faktoren und korrespondierende Rezeptoren) in der ***Rinderplazenta*** während der Trächtigkeit und unmittelbar vor der Geburt mittels Immunhistochemie auf Proteinebene und über RT-PCR sowie in situ Hybridisierung auf mRNA-Ebene untersucht (Veröffentlichung Nr. 10: Pfarrer et al., 2004).

5. Ergebnisse und Diskussion

5.1 Charakterisierung von Signalwegen in der Wiederkäuerplazenta

5.1.1 Zytoskelett, assoziierte Proteine und mitogen-activated protein kinase (MAPK)

Aktin wurde im Rinderplazentom apikal und lateral in maternalen Epithelzellen sowie in fetalen mononukleären Trophoblastzellen gezeigt. Dagegen war Aktin in TGC membranassoziiert, mit deutlichen Ansammlungen im Bereich der Pseudopodien. Weiterhin exprimierten Endothelzellen beider Kompartimente Aktin. Die immunzytochemischen Ergebnisse wurden mit dem mRNA Nachweis für Aktin per RT-PCR bestätigt. Die hauptsächlich in den Pseudopodien der TGC befindlichen Aktinfilamente haben sich als wichtige Grundlage der Zellmigration erwiesen, denn Veränderungen der Zellform, Verankerung und Zellmotilität schließen eine dynamische Reorganisation des Aktinzytoskelettes ein (Pavalko und Otey, 1994; Small et al., 1999b), welches sich durch Injektion von α-Aktinin Fragmenten unterbrechen lässt (Pavalko und Burridge, 1991). Weiterhin ist α-Aktinin zu β_1-Integrin kolokalisiert, was eine direkte Verbindung zwischen Integrinen und Aktin vermuten lässt (Pavalko und Burridge, 1991). Aktin steht mit Integrinen über Talin direkt oder über Vinculin in Kontakt, wobei Integrine auch an α-Aktinin binden können, welches gleichzeitig mit Aktin verbunden ist. Diese Bindung kann oder kann nicht Vinculin einschließen (Geiger et al., 1995). Eine zytoskelettale Antwort auf die Ligation von Integrinen an Fibronectin-beschichtete Kügelchen ist die Induktion von fokalen Adhäsionskomplexen aus α-Aktinin, Talin und Vinculin, die dem Zytoskelett assoziiert sind (Miyamoto et al., 1995). In vitro führt die transmembrane Akkumulation von α-Aktinin und Talin an der apikalen Zelloberfläche von luminalen Uterusepithelzellen und Trophoblastzellen des Schafes auf den Kontakt mit OPN-beschichteten Kügelchen zu funktioneller Integrinaktivierung und Reorganisation des Zytoskelettes, die wahrscheinlich auch während der Implantation des Schafembryos stattfindet (Johnson et al., 2001). Das Vorhandensein von Aktin in fetalen und maternalen Endothelien ist nicht überraschend, da die Migration von Endothelzellen ein allgemeines Phänomen der Angiogenese ist (Risau, 1997), welche im fetalen und maternalen Kompartiment der Rinderplazenta während der gesamten Trächtigkeit zu beobachten ist (Leiser et al., 1997b; Pfarrer et al., 2001).

α-Aktinin findet sich kolokalisiert zu Aktin apikal in dem maternalen und fetalen (Trophoblast-) Epithel boviner Plazentome, in denen die Expression von α-Aktinin mRNA mittels RT-PCR bestätigt wurde. Daher ist eine direkte Signalübertragung über Integrine

während der Migration und Fusion boviner TGC mit einzelnen uterinen Epithelzellen zu feto-maternalen Hybridzellen (Wooding, 1992) sehr wahrscheinlich, denn auch die entsprechenden Integrine werden in den TGC beobachtet (Pfarrer et al., 2003). Die Bedeutung von α-Aktinin für die Migration der TGC wird dadurch betont, dass α-Aktinin als Ligand für die β-Untereinheit von Integrinen dient und so als Verankerungsprotein wirkt sowie nach einer Bindung Konformationsänderungen von Aktin induziert (Alberts et al., 1999). Die Modulation von Adhäsionsmolekülen ist unter anderem auch für die Invasivität des Trophoblasten mitverantwortlich (Damsky et al., 1992). Als besonderer Kandidat für die Invasion von Zytotrophoblastzellen hat sich das $\alpha_1\beta_1$ Integrin erwiesen, da eine Behandlung mit Antikörpern, die die Interaktion von Integrin $\alpha_1\beta_1$ mit Laminin oder Kollagen Typ IV blockieren, die Invasion des Zytotrophoblasten unterbinden konnte (Damsky et al., 1994).

Die nicht polarisierte Aktinrinde einer menschlichen endometrialen Zelllinie (RL95-2) ist möglicherweise für die Adhäsion des Trophoblasten wichtig (Thie et al., 1997) und könnte somit auch für die Anheftung von mononukleären Trophoblastzellen an bovine Uterusepithelzellen oder die Fusion mit TGC zur Verfügung stehen. Die nicht polarisierte Aktinrinde in bovinen TGC könnte dagegen an der Migration beteiligt sein, da auch die Zell-Zellkontakte über das Integrin $\alpha_2\beta_1$ während der Wanderung der TGC einen transienten Charakter haben (Pfarrer et al., 2003).

Veränderungen der äußeren Form von Zellen während der Entwicklung werden von einer Reorganisation des Zytoskelettes begleitet, die der Bildung von Verankerungstrukturen ähnelt. Aktin und Intermediärfilamente sind mit Zell-Zell- und Zell-Matrixverbindungen assoziiert (Small et al., 1999a). Dabei sind die Verbindung von Aktin zur Zellmembran und die Integrität des Aktin-Zytoskelettes eine Voraussetzung für Zell-Zell- und Zell-Matrix-Interaktionen, Zellmotilität, Rezeptor-Liganden Interaktionen (Pavalko und Otey, 1994) sowie die Tyrosinphosphorylierung von MAPK (Morino et al., 1995). Zum Beispiel führt die Behandlung von Fibroblasten mit Zytochalasin D zu einer selektiven Unterbrechung des Netzwerkes von Aktinfilamenten und verhindert so die adhäsionsvermittelte Aktivierung von MAPK (Morino et al., 1995).

Das Vorhandensein von Vinculin mRNA in bovinen Plazentomen und der immunzytochemische Nachweis von Vinculinprotein apikal, lateral und membranassoziiert in Trophoblastzellen sowie in der Nähe der Kontaktstelle von mononukleären Trophoblastzellen mit TGC lässt vermuten, dass Vinculin ein Verankerungspunkt für Trophoblastzellen ist und

möglicherweise als Fixpunkt für die Migration der TGC dient. An motilen Fischkeratozyten wurde gezeigt, dass neu entstandene fokale Adhäsionen Vinculin enthalten, was eine Voraussetzung für die Adhäsion ist und von einem diagonalen Aktinnetzwerk unterlagert wird (Small et al., 1999a). Auf diese Weise fungiert Vinculin während der Signalübertragung in fokalen Adhäsionen als Kupplung zwischen Talin, α-Aktinin, Aktin und der Integrin β-Untereinheit (Clark und Brugge, 1995; Garratt und Humphries, 1995).

Die spezifische Lokalisation von α-Aktinin und Vinculin in den TGC boviner Plazentome deutet auch auf eine Beteiligung an der Fusion von TGC mit einzelnen uterinen Epithelzellen hin, denn nicht polarisierte TGC (Wimsatt, 1951; Davies und Wimsatt, 1966) sind vorübergehend, bis zur Fusion mit dem uterinen Epithel, in die Tight junctions des "Trophektoderms" eingebaut (Wooding und Wathes, 1980; Morgan und Wooding, 1983). In vitro werden Vinculin und α-Aktinin nach Injektion in Madin-Darby Nierenzellen des Rindes (MDBK) sofort in Adhärenz- und Tight junctions inkorporiert, worauf sich die fokalen Adhäsionen nicht verändern, aber eine Lösung der Zell-Zellkontakte erfolgt (Palovuori und Eskelinen, 2000). Stattdessen werden Polykaryonten aus kurzen Zellmembranfragmenten aufgebaut, die die injizierten Proteine (sowie Aktin, beta-Catenin, Claudin, Occludin und Zonula occludens 1) enthalten, ein Vorgang, der die Desintegration von Adhärenz- und Tight junctions indiziert. Im Gegensatz führt die Mikroinjektion von Fluoreszein-Isothiocyanat (FITC)-markierten Kopfdomänen von Vinculin zur Ablösung der Zellen von ihrem Substrat, was vermutlich auf der hohen Affinität des Vinculinkopfes zu Talin beruht (Palovuori und Eskelinen, 2000). Die gleichen Autoren berichten auch, dass ein Überschuss an α-Aktinin and Vinculin die Zell-Zell Adhäsionen von dem intrazellulären Zytoskelett abkoppelt, was zur Fragmentierung von Zellverbindungskomplexen und Zellfusion führt.

Im Rinderplazentom findet sich phosphorylierte MAPK (pMAPK) in den Kernen von fast allen Trophoblastzellen und in einzelnen Kernen des uterinen Epithels. Die erfolgreiche Amplifikation von spezifischer MAPK mRNA bestätigt die potentielle Fähigkeit von bovinen Plazentomzellen, MAPK-Signalwege zu nutzen. Transmissionselektronenmikroskopisch ist die pMAPK, neben ihrer Anwesenheit im Kern, auch schwach im zellmembrannahen Zytoplasma beider Populationen von Trophoblastzellen des Rindes zu finden. Allerdings ist eine schwache Reaktion im Zytoplasma nicht überraschend, da die ERK oder auch MAPK im Zytoplasma phosphoryliert (aktiviert) und als aktivierte Form in den Kern überführt wird; ein Prozess, der für die Signalübertragung essentiell ist (Glading et al., 2001; Howe et al., 2002). Eine nukleäre Lokalisation von pMAPK in bovinen Plazentomzellen spricht für einen

aktivierten Zustand und somit für eine funktionelle Rolle der MAPK. Die Migration der TGC wird möglicherweise durch diese Aktivierung von Proteinkinasen beeinflusst, da ERK in vitro Migrationsprozesse vermitteln kann (Cho und Klemke, 2000; Glading et al., 2001; Howe et al., 2002). Weiterhin sind MAPK als Regulatoren von Zellwachstum, Differenzierung und Genaktivierung bekannt (Chen et al., 1994b; Miyamoto et al., 1996). Beispielsweise ist die Aktivierung des Genes für das plazentäre Laktogen-I (PL-I) sensitiv gegenüber der Unterbrechung des MAPK Signalweges und der Aktivierung der Protein-1 (AP-1) Signalkette (Peters et al., 2000). In differenzierenden Trophoblastzellen der Ratte spielen auto- und/oder parakrine Reaktionswege, unter Beteiligung von epidermal growth factor receptor (EGFR), FGFR1, MAPK und AP-1, bei der Regulation des PL-I Genes eine Rolle. Aus diesem Grund ist auch eine Aktivierung des Genes für plazentäres Laktogen über pMAPK in den TGC der Rinderplazentome denkbar. Die Anwesenheit von plazentärem Laktogen in den TGC der bovinen Plazenta ist seit langem bekannt (Wooding und Beckers, 1987) und ist, zusammen mit der Invasivität, ein den TGC verschiedener Spezies gemeinsames Phänomen (Hoffman und Wooding, 1993).

Die Anwesenheit von Aktin in bovinen plazentomären TGC, zusammen mit der Präsenz von pMAPK im Kern, lässt auf ein intaktes Aktinzytoskelett schließen, welches eine Voraussetzung für die Induktion der Migration der TGC über ERK-1 und ERK-2 ist (Morino et al., 1995) und gleichzeitig die Verlagerung der ERK vom Zytoplasma in den Kern von einem intakten Aktinzytoskelett abhängig ist (Aplin et al., 2001). Weiterhin wurde α-Aktinin als Bindungsprotein für MEKK1 (Aktivator für JNK, ERK und p38 MAPK) identifiziert, was bedeutet, dass aktivierte ERK andere Zielproteine (wie Talin) beeinflussen kann und so eine Rolle in der Regulation von Adhäsion und Konformation des Zytoskelettes spielt (Fincham et al., 2000).

Die zytoplasmatische pMAPK in Zellen des bovinen Plazentoms ist spezifisch in der Nähe der Zellmembranen lokalisiert. Die Assoziation von pMAPK mit Zellmembranen von NR6 Zellen wurde mit Wachstumsfaktor induzierter Zellmotilität in Zusammenhang gebracht (Glading et al., 2001). Die zusätzliche Nachbarschaft von ERK zu integrinspezifischen Gebieten wird als potentiell wichtig für die Ausbreitung und Bewegung von Zellen erachtet (Howe et al., 2002). Daraus ergibt sich die Hypothese, dass die membranassoziierten ERK-1 und ERK-2 Faktoren für die Induktion der Migration der TGC sind. Unterstützt wird diese Annahme durch die Tatsache, dass die Tyrosinphosphorylierung und die enzymatische Aktivität durch die Bindung des β_1 Integrins vermittelt wird (Morino et al., 1995), welches

während der Implantation (MacLaren und Wildeman, 1995) und der gesamten Gravidität (Pfarrer et al., 2003) in bovinen TGC lokalisiert ist.

Es lässt sich schlussfolgern, dass die Lokalisation von Aktin und seinen assoziierten Proteinen sowie der pMAPK für eine Beteiligung an der Migration der TGC in bovinen Plazentomen spricht. Damit könnte die eingeschränkte Trophoblastinvasion in der Plazenta des Rindes auch Modellcharakter für andere invasive Prozesse besitzen.

5.1.2 Integrinrezeptoren und extrazelluläre Matrix bei Schaf und Rind

Die ovine Implantationskaskade

Es ist bekannt, dass von der Implantation bis hin zur Formation einer definitiven Plazenta eine Adhäsionskaskade abläuft, welche mit einer engen Anlagerung des Embryos an das Endometrium beginnt. Adhäsive, normalerweise von Muzinen maskierte Liganden des luminalen Epithels werden während der rezeptiven Phase des Endometriums, die auch als Implantationsfenster bezeichnet wird, freigelegt und ermöglichen so die Aktivität verschiedener adhäsiver Moleküle, entweder in einer Sequenz oder nebeneinander, um eine stabile Verbindung des Trophoblasten zu dem luminalen Epithel zu etablieren (Kimber, 2000). Die zeitlichen und räumlichen Veränderungen der Expression von Muzinen, Proteinen der ECM und ihren Integrinrezeptoren beeinflussen das Erreichen der endometrialen Rezeptivität bei Nagern, Primaten, Rind, Ziege und Schwein (Lessey et al., 1994; Surveyor et al., 1995; Aplin et al., 1996; Bowen et al., 1996; Fazleabas et al., 1997; McNeer et al., 1998; Aplin, 1999; Johnson et al., 1999a, 2000). In der vorliegenden Studie wurden folgende strukturellen und funktionellen Schlüsselmoleküle der Implantationskaskade des Schafes identifiziert: 1. Das antiadhäsive, an der gesamten luminalen Oberfläche ausgeprägt vorhandene Muc-1 nimmt direkt vor der Adhäsionsphase ab; 2. Die Integrinuntereinheiten α_v, α_4, α_5, β_1, β_3 und β_5, welche die bekannten Osteopontinrezeptoren $\alpha_v\beta_3$, $\alpha_v\beta_1$, $\alpha_v\beta_5$ und $\alpha_4\beta_1$ bilden können, werden gleichbleibend in hohem Maße an der apikalen Oberfläche des luminalen Uterusepithels und des Trophoblasten exprimiert; 3. Osteopontin (OPN) wird vor und während der Anheftung des Embryos am Uterusepithel aus den uterinen Drüsen in das Uteruslumen sezerniert (Johnson et al., 1999a, b); 4. OPN Protein ist an der apikalen Oberfläche des uterinen Epithels und des Trophoblasten vorhanden (Johnson et al., 1999a); und 5. in vitro bindet OPN die auf dem luminalen Epithel und Trophoblast exprimierten Integrine und aktiviert so das outside-in signaling über die Integrine.

Die luminale Oberfläche des Uterus ist mit stark glykosylierten Muzinen bedeckt, die über den apikalen Zellpol herausragen und auch menschliche Blastozysten exprimieren Muc-1 (Meseguer et al., 2001). Die Membranmuzine Muc-1 und Muc-4 befeuchten und schützen den Uterus gegen mikrobielle Infektionen; allerdings stellen sie auch ein sterisches Hindernis für die Zell-Zell- und Zell-Matrix-Adhäsion dar (Wesseling et al., 1995; Komatsu et al., 1997), was auch die Anheftung des Embryos an das Endometrium behindern könnte (DeSouza et al., 1999). Generell steigt die Muc-1 Expression in der rezeptiven Phase bei Kaninchen und Menschen, ist aber lokal an den Orten der Embryoanheftung reduziert (Hoffman et al., 1998; Aplin, 1999). Eine Annäherung des Embryos wird erst möglich, wenn ein Keratinsulfatverlust bei Muc-1 im Endometrium des Menschen eine Senkung der abstoßenden Ladungsdichte erzeugt. Ergebnisse aus in vitro Studien deuten darauf hin, dass parakrine Signale der Blastozyste für die Abregulierung von Muc-1 an den Implantationsorten verantwortlich sind (Aplin, 1999; Meseguer et al., 2001). Beim Schaf wird vor der Implantation eine Abnahme der Muc-1 Expression an der apikalen Oberfläche des luminalen Epithels beobachtet, was mit den bei Nagern und dem Schwein erhobenen Befunden übereinstimmt (Surveyor et al., 1995; Aplin et al., 1996; Bowen et al., 1996). Diese Abregulation könnte möglicherweise durch Progesteron gesteuert sein, da die Implantation und die Abregulation von Muc-1 durch Gaben eines Progesteronantagonisten (RU-486) blockiert werden (Surveyor et al., 1995; Aplin et al., 1996) und auch Progesterongaben bei ovariektomierten Sauen die Muc-1 Expression senken (Bowen et al., 1996). Außerdem werden in der Trächtigkeit beim Schaf Muc-1 zwischen Tag 7 und 17 und der Progesteronrezeptor zwischen Tag 13 und 19 herunterreguliert (Spencer und Bazer, 1995). Interessant ist in diesem Zusammenhang, dass die Adhäsion des Schafembryos zwischen dem Tag 16 und 18 der Trächtigkeit erfolgt und damit genau den Zeitpunkt der niedrigsten Muc-1 Expression (Tag 17) trifft (Guillomot et al., 1981). Der Verlust von Muc-1 vor der Implantation beim Schaf könnte also eine Barriere entfernen, die ein sterisches Hindernis für die Interaktion von Integrinen mit der extrazellulären Matrix darstellen.

Die Integrinrezeptoren der Zelloberfläche verbinden die extrazelluläre Matrix mit dem intrazellulären Zytoskelett und vermitteln so Signale durch die Zellmembran. Damit stellen sie die wichtigsten Mediatoren für Signale aus der Matrix dar, die Funktionen wie Zell-Zell- und Zell-Matrix-Adhäsion, Zellform und Regulation der Genexpression beeinflussen (Choquet et al., 1997; Giancotti und Ruoslahti, 1999). Die hormonabhängige zeitliche und räumliche Verteilung von Integrinen im menschlichen Uterus und die entwicklungsabhängige

Regulation auf der invadierenden Mausblastozyste liefern schlagkräftige Beweise für die Beteiligung von Integrinen bei der Implantation (Lessey et al., 1992; Sutherland et al., 1993). In der aktuellen Studie werden die Integrinuntereinheiten α_v, α_4, α_5, β_1, β_3 und β_5 im Endometrium während des Zyklus und der Trächtigkeit sowie im Trophoblasten des Schafes exprimiert. Das Vorkommen aller Untereinheiten wurde mittels RT-PCR am Tag 15 des Zyklus und der Trächtigkeit sowie in einer Trophoblastzelllinie bestätigt. Die mRNA der Integrinuntereinheiten α_v, α_5, β_1 und β_3 wurde weiterhin auch in einer immortalisierten Zelllinie aus dem luminalen Epithel nachgewiesen. In Immunfärbungen an in vivo Material des Schafes wurde die apikale Lokalisation von α_v, α_4, α_5, β_1, β_3 und β_5 in glandulären und luminalen Epithelzellen und im Trophoblasten gezeigt. Diese apikale Expression war konstitutiv und ist generell in der peri-implantativen Phase nicht angestiegen. Obwohl das Protein von α_4 und β_5 auf den endometrialen Epithelien vorhanden war, konnte nur sehr wenig korrespondierende mRNA amplifiziert werden, beziehungsweise war in der Trophoblast- und luminalen Epithelzelllinie überhaupt nicht vorhanden. Da die Primerauswahl auf einer cDNA Sequenz für menschliches α_4 und β_5 basierte, konnte die mRNA hier möglicherweise nicht amplifiziert werden. Daher scheint beim Schaf die Empfänglichkeit für die Implantation nicht auf einer veränderten Integrinexpression zu beruhen, da die Untereinheiten apikal und konstitutiv exprimiert werden. Dieses spezifische Muster steht im Gegensatz zu Studien an anderen Spezies, die einen Zusammenhang zwischen Veränderungen in der Integrinausstattung und dem Implantationsfenster festgestellt haben. Beim Menschen werden unmittelbar vor der Anheftung des Embryos vermehrt $\alpha_v\beta_3$ und $\alpha_4\beta_1$ Integrine im luminalen Epithel beobachtet (Lessey et al., 1994, 1996). Während $\alpha_v\beta_3$ und $\alpha_v\beta_5$ an der apikalen Zelloberfläche des luminalen Epithels zu finden sind (Aplin, 1996; Lessey et al., 1996), liegen $\alpha_6\beta_4$, $\alpha_2\beta_1$ und $\alpha_3\beta_1$ vorwiegend an den lateralen und basalen Zelloberflächen (Aplin, 1997). Beim Schwein sind die Untereinheiten α_v, α_3, α_4, α_5, β_1 und β_3 an der luminalen Oberfläche zu finden, wovon α_v und β_3 hoch und konstitutiv exprimiert werden. Dagegen sind α_4, α_5, und β_1 während des Zyklus und der frühen Trächtigkeit räumlich und zeitlich reguliert und zeigen in der frühen Implantation die höchste Expression (Bowen et al., 1996). Der Pavian, als Vertreter der hämochorialen Plazentatypen, exprimiert das $\alpha_v\beta_3$ Integrin im Drüsenepithel und den dezidualisierenden Stromazellen des graviden Endometriums (Fazleabas et al., 1997), während die Expression um die Implantation der Maus auf endometriale Stromazellen beschränkt ist (Illera et al., 2000). Die Ausbildung von Integrinrezeptoren beim Schaf scheint sich auch von der anderer Ruminanten zu unterscheiden. Beim Rind werden $\alpha_v\beta_3$, α_3, α_6 und β_1 basolateral im luminalen Epithel, oberflächlichen Drüsen und im subluminalen Stroma

beobachtet, wobei die $\alpha_v\beta_3$ Expression im Verlauf des Zyklus variiert (Kimmins und MacLaren, 1999). Auch bei der Ziege wurde die β_1 Untereinheit an den basolateralen Zellmembranen von Drüsen- und Oberflächenepithel lokalisiert, wobei sich im luminalen Epithel eine starke Reduktion am Ort der Implantation zeigte (Guillomot, 1999). Diese tierartlichen Differenzen reflektieren möglicherweise Unterschiede in der Art der Apposition (Anlagerung), Anheftung und Invasion während der Implantation. Auf jeden Fall aber lässt die Lokalisation an der Oberfläche des Uteruslumens und des Embryos vermuten, dass Integrine bei der Vermittlung der Anheftung des Embryos beim Schaf beteiligt sind.

Die integrinvermittelte Anheftung schließt wahrscheinlich auch Brückenmoleküle ein, die mit den Rezeptoren von Uterusepithel und Trophoblastzellen Kontakt aufnehmen. Ein solches Brückenmolekül ist das OPN, dessen 45 kDa Fragment in der peri-implantativen Phase in großen Mengen im Uteruslumen vorkommt. Dabei liegt das OPN-Protein direkt an den apikalen Zelloberflächen dieser Epithelien, die beide selber keine OPN mRNA exprimieren und legt so die Vermutung nahe, dass die Ansammlung von OPN in dieser Lokalisation durch Bindung an Integrinrezeptoren hervorgerufen wird (Johnson et al., 1999a, 2000). Das ovine OPN ist ein saures 70 kDa Glykoprotein der ECM, welches eine RGD-Bindungssequenz enthält, die Integrinrezeptoren bindet. OPN lässt sich durch Einfrieren und Auftauen oder entsprechende Proteasebehandlung in 24- und 45 kDa Fragmente spalten (Fresno et al., 1981). Das 45 kDa Fragment hat eine höhere Bindungsaffinität für Integrine als die native Form, was möglicherweise durch ein Freilegen der RGD-Sequenz verursacht wird (Senger und Perruzzi, 1996). Grundsätzlich führt die Bindung von Integrinen an Proteine der ECM zu einer weiteren Aggregation von Integrinen und initiiert eine Kaskade, die zu einer Anlagerung von Proteinen des Zytoskelettes und über 20 Signaltransduktionsmolekülen an die zytoplasmatische Domäne der β_1 Untereinheit führt (Miyamoto et al., 1995). Das Ergebnis ist die Bildung eines Komplexes aus Proteinen der ECM, Integrinen und Proteinen des Zytoskelettes, die als fokale Adhäsionskomplexe bekannt sind (Miyamoto et al., 1995; Defilippi et al., 1999; Giancotti und Ruoslahti, 1999). Die Anlagerung von c-src Substraten, Tensin und focal adhesion kinase (FAK) kann durch Integrinaggregation alleine erfolgen, dagegen erfordert die Zusammenlagerung der zytoskelettassoziierten Proteine, wie Talin, α-Aktinin, Vinculin und F-Aktin, neben der Integrinaggregation auch eine Bindung des Liganden (Miyamoto et al., 1995). Aus diesem Grund bietet die Immundetektion der angesammelten Integrine, Talin oder α-Aktinin ein sensitives System zum Nachweis der funktionellen Integrinaktivierung und des outside-in signalings. Die präsentierten Ergebnisse der in vitro Versuche zeigen klar, dass

eine Bindung von Integrinen an die ECM die Bildung fokaler Adhäsionskomplexe nach sich zieht und demonstrieren so in Zelllinien aus ovinem Uterusepithel und Trophoblast erstmalig eine funktionelle Integrinaktivierung mit korrespondierender Reorganisation des Zytoskelettes als Antwort auf eine Bindung von OPN. Die Akkumulation von Talin und α-Aktinin wurde exakt an der Kontaktfläche von OPN-beschichteten Polystyrenkügelchen und den apikalen Zellmembranen von luminalen Epithelzellen und Trophoblastzellen gefunden. Diese fokalen Adhäsionskomplexe sind das Ergebnis einer RGD-Integrin Interaktion, da sich ihre Bildung mit Mutationen der RGD-Sequenz blockieren ließ. Die Identität der an der Bindung beteiligten Integrine ist bislang unbekannt. Erstaunlicherweise finden sich die Integrinuntereinheiten α_v und β_3, die zusammen den $\alpha_v\beta_3$ Rezeptor (bindet verschiedene Matrixproteine wie OPN, Vitronectin und Fibronectin) bilden (Burghardt et al., 1997), an den Orten der Verankerung der luminalen Epithel- und Trophoblastzellen an das Substrat und würden somit auch für die Bildung von fokalen Adhäsionskomplexen bereitstehen. In Trophoblastzellen induzierten die OPN beschichteten Kügelchen auch eine Integrinaktivierung, wobei die Zahl der angelagerten Kügelchen mindestens doppelt so hoch war wie in luminalen Epithelzellen, was dem hochadhäsiven Status des Embryos entspricht. Korrespondierend wurde in Trophoblastzellen auch eine größere Zahl der basal angesammelten α_v und β_3 Integrinuntereinheiten beobachtet. Daraus lässt sich schließen, dass die Bindung von OPN an Integrine im trächtigen Uterus des Schafes die Bildung ähnlicher fokaler Adhäsionskomplexe induziert, die die Förderung und Stabilisierung der Anheftung des Trophoblasten an das luminale Epithel während der Implantation unterstützen.

Es lässt sich schlussfolgern, dass die Verminderung von Muc-1 auf dem uterinen Epithel in der Implantationsperiode, die apikal angeordneten Integrinuntereinheiten α_v, α_4, α_5, β_1, β_3 und β_5 freilegt, die daraufhin für die Interaktion mit OPN zur Verfügung stehen. Die gleichzeitig exprimierten Untereinheiten machen die Bildung der OPN-Rezeptoren $\alpha_v\beta_3$, $\alpha_v\beta_1$, $\alpha_v\beta_5$ und $\alpha_4\beta_1$ auf dem uterinen Epithel und Trophoblasten möglich, was beim Schaf zusammen mit dem Nachweis der funktionellen Interaktion mit OPN für eine Funktion von OPN als Brückenmolekül zwischen den Integrinenrezeptoren beider Epithelien im physiologischen Ablauf der Implantation spricht.

Osteopontin während der Trächtigkeit beim Schaf

Es werden die ersten Ergebnisse zur Lokalisation von OPN im gesamten Kontaktbereich von Embryo und Uterus zwischen dem Tag 30 und 120 der Trächtigkeit beim Schaf präsentiert, wobei die Befunde vermuten lassen, dass OPN, als Teil der Histotrophe von uterinen Drüsen produziert, eine spezifische feto-maternale Bindung unterstützt. Diese Befunde sind interessant, weil sie mit OPN definitiv ein sekretorisches Produkt der uterinen Drüsen entlang der feto-maternalen Kontaktlinie des Schafes lokalisieren, wo OPN möglicherweise die Kommunikation zwischen fetalem und maternalem Kompartiment vermittelt und so Einfluss auf die Entwicklung und das Wachstum von Fetus und Plazenta nimmt.

Zwischen dem Tag 40 und 80 der Trächtigkeit beim Schaf steigen die Gehalte an totaler OPN mRNA im Endometrium und der Plazenta ungefähr um den Faktor 30, wobei der größte Anteil mit den uterinen Drüsen assoziiert ist, aber von einem Anstieg im Stratum compactum des subepithelialen Stromas begleitet wird, der zwischen dem Tag 20 und 25 beginnt. Dieser Unterschied zwischen Stroma und Drüsenepithel wird bei dem Vergleich der Stärke der Hybridisierung am Tag 80 und 100 der Trächtigkeit besonders deutlich. Die Veränderung der mRNA Expression korreliert mit dem Wachstum der uterinen Drüsen in den letzten zwei Dritteln der Gravidität, wo sich der Uterus an die Entwicklung und das Wachstum des Feten anpasst (Wimsatt, 1950). Während der Trächtigkeit des Schafes erlebt das Drüsenepithel bis zum Tag 50 eine Hyperplasie, die von Hypertrophie gefolgt wird und eine maximale Produktion von Histotrophe ab dem Tag 60 bewirkt (Stewart et al., 2000). Ein ähnlicher Zusammenhang zwischen Entwicklung der uterinen Drüsen und Genexpression während der Trächtigkeit besteht auch für ein weiteres sekretorisches Produkt der uterinen Drüsen, das uterine milk protein (UTMP) (Stewart et al., 2000). Die Morphogenese der uterinen Drüsen, wie auch die Genprodukte der Drüsenepithelien (OPN, UTMP), stehen wahrscheinlich unter der Kontrolle von Östrogenen, Progesteron, Interferon Tau, plazentärem Laktogen und Wachstumshormon (Spencer et al., 1999; Noel et al., 2003). Dieses wird am Modell des ovariektomierten Schafes demonstriert, in dem Progesterongaben eine erhöhte Expression von OPN und UTMP bewirken (Wimsatt, 1950; Spencer et al., 1995, 1999; Fisher et al., 1995; Stewart et al., 2000; Johnson et al., 2000, 2002a) und die zusätzliche Injektion von Antagonisten des Progesteronrezeptors diesen Effekt auf die OPN mRNA blockiert (Johnson et al., 2000). Außerdem lassen sich die OPN und UTMP Spiegel bei progesteronbehandelten, ovariektomierten Schafen durch die intrauterine Applikation von plazentärem Laktogen

erhöhen (Spencer et al., 1999; Noel et al., 2003). Auffallend ist, dass sich Gaben von Wachstumshormon im gleichen Modell unterschiedlich auf das OPN und UTMP Gen auswirken. Während sich im Drüsenepithel die mRNA Spiegel von OPN nicht verändern, wird ein Anstieg der mRNA von UTMP beobachtet (Spencer et al., 1999; Noel et al., 2003). Grundsätzlich kann in ovariektomierten Schafen mit Gaben von Progesteron, Interferon Tau, plazentärem Laktogen und Wachstumshormon eine vergleichbare Drüsenentwicklung provoziert werden wie unter dem Einfluss von plazentärem Laktogen aus der Plazenta während der Trächtigkeit (Noel et al., 2003).

Eine Aussage über den möglichen Wirkungsmechanismus von OPN während der Trächtigkeit des Schafes wird durch eine Immunfärbung mit Antikörpern ermöglicht, die entweder gegen das aminoterminale (LF-124) oder das karboxyterminale Ende (LF-123) von dem Spaltprodukt des rekombinanten menschlichen OPN (70 kDa) gerichtet waren, welches mit Thrombin an der RS-Sequenz gespalten wurde (Fisher et al., 1995). Beide Immunglobuline detektieren das native 70 kDa OPN, wobei nur der LF-124 Antikörper das die RGD-Sequenz enthaltende 45 kDa Fragment erkennt, welches die Zellanheftung und -ausbreitung durch eine bessere Zugänglichkeit für Integrinrezeptoren steigert (Senger und Perruzzi, 1996). Nur das 45 kDa OPN unterstützt die $\alpha_9\beta_1$ und $\alpha_4\beta_1$ vermittelte Zellmigration und Adhäsion (Bayless et al., 1998; Smith und Giachelli, 1998) sowie eine Anheftung durch weitere Bindungsmoleküle ohne RGD-Sequenz (Katagiri et al., 1996). Das 45 kDa Fragment ist die in uterinen Spülungen am häufigsten vorkommende Form (Johnson et al., 1999a, b) und wurde in der aktuellen Studie vom Tag 14 an der apikalen Zelloberfläche des uterinen Epithels, vom Tag 20 auf dem Trophoblasten und ab hier bis zum Tag 120 der Trächtigkeit entlang der gesamten feto-maternalen Kontaktfläche (plazentomär und interplazentomär) des Schafes lokalisiert. Da die OPN mRNA vom Epithel der Drüsen, nicht aber vom luminalen Epithel, produziert wird, bestätigt die Immunreaktion für das 45 kDa Fragment erstens die Sekretion von OPN in den Drüsen und zweitens die Bindung an allen Orten, wo fetale und maternale Gewebe aneinander geheftet sind. Die Quelle des an der feto-maternalen Kontaktzone lokalisierten OPN Proteins ist sehr wahrscheinlich das Sekret der benachbarten uterinen Drüsen im interplazentomären Bereich. Außerdem exprimieren Stroma- und Immunzellen der Plazentome OPN, welches auch an die Kontaktfläche zwischen Trophoblast und uterinem Epithel transportiert werden könnte (Johnson et al., 2002a, b).

Die Implantation und Plazentation ist ein fortschreitender Prozess, der einen adhäsionsmolekülabhängigen Gewebeumbau des Endometriums und des Embryos

einschließt. Bei der Frau ist die vorübergehende endometriale Expression von $\alpha_v\beta_3$ und $\alpha_4\beta_1$ Integrinen zyklusabhängig und definiert das Implantationsfenster. Ein verändertes Auftreten gerade dieser Integrine ist mit mehreren Phänomenen gekoppelt, die zur Infertilität führen (Lessey et al., 1995). Nullmutationen der α_v, α_5, β_1 und β_5 Integrin-Gene der Maus führen durch Fehler bei der Implantation zu frühembryonalem Tod und fehlender Fusion von Chorion und Allantois (Hynes, 1996) und die funktionelle Blockade von α_v und β_3 bei Mäusen bewirkt eine Verminderung der Anzahl der Implantationsorte (Illera et al., 2000). Eine endometriale Integrinexpression wurde auch für Spezies mit nicht invasiver Implantation, wie Schaf, Schwein, Ziege und Rind bestätigt (Bowen et al., 1996; Guillomot, 1999; Johnson et al., 1999a, 2001; MacIntyre et al., 2002). Uterines, plazentäres und/oder embryonales Auftreten von OPN wurde bei Rind, Schwein, Maus, Pavian, Mensch und Schaf gezeigt. Eine erste Studie für das Rind lokalisiert OPN Protein an der apikalen Zelloberfläche des uterinen Drüsenepithels, im interkotyledonären und karunkulären Stroma und wahrscheinlich auch auf Trophoblastzellen vom Tag 80 bis 270 der Gravidität (Pfarrer et al., 2002). Beim Schwein erscheint die OPN mRNA ab dem Tag 12 bis 15 der Trächtigkeit in diskreten Regionen des luminalen Epithels und breitet sich danach über das ganze Epithel aus, wobei die ersten Signale im Drüsenepithel ab Tag 30 auftreten (Garlow et al., 2002). In der Maus finden sich OPN Transkripte im Trophoblasten, der Dezidua (metrial gland cells) und der Plazenta (Nomura et al., 1988), während das OPN Protein des Pavians in Drüsenepithelien und dem dezidualisierten Stroma lokalisiert ist (Fazleabas et al., 1997). Bei Frauen wurde OPN mRNA zuerst in der Dezidua und in den Drüsen des sekretorischen Uterus identifiziert (Young et al., 1990), später aber auch in den hypersekretorischen Drüsen schwangerer Frauen gefunden (Brown et al., 1992; Coutifaris et al., 1997). Progesteron scheint regulativ auf die OPN Expression zu wirken, da OPN Protein spezifisch nur in Zytotrophoblastzellen der Chorionzotten erhöht wurde, nicht dagegen im Synzytiotrophoblasten, was darauf schließen lässt, dass OPN gerade in Zytotrophoblastzellen Adhäsion und/oder Signalketten über α_v Integrine vermittelt (Omigbodun et al., 1997). Die endometriale Expression von OPN wird ab Tag 16/17 des Zyklus durch Progesterongaben erhöht, wobei die Quelle des an der apikalen Oberfläche des luminalen Epithels lokalisierten Proteins das Drüsenepithel zu sein scheint (Coutifaris et al., 1997; Apparao et al., 2001).

Beim Schaf ist OPN ein Bestandteil der Histotrophe, der in Spülungen von tragenden Tieren vom Tag 11 bis 17 ansteigt (Johnson et al., 1999a). Progesteron induziert OPN mRNA nur im Drüsenepithel, wohingegen das Protein an der apikalen Zelloberfläche des luminalen

Epithels und des Trophoblasten erscheint (Johnson et al., 1999a, b, 2000). Die Funktion von OPN als Brücke zwischen den Integrinrezeptoren als essentieller Bestandteil der Implantationskaskade wurde für Schaf und Mensch vorgeschlagen (Coutifaris et al., 1997; Johnson et al., 1999a, 2001; Kaartinen et al., 1999). Der OPN Anstieg im menschlichen Endometrium der rezeptiven Phase wird mit der high density microarray Methode bestätigt. Ein Vergleich der Genexpression vom Tag 8-10 des Zyklus mit den entsprechenden Tagen der Schwangerschaft zeigt einen 8,1-fachen Anstieg der OPN mRNA (Kao et al., 2002). Weiterhin lässt sich ein 12,3-facher Anstieg der Expression beim Vergleich der Tage 2-4 mit den Tagen 7-9 der Schwangerschaft nachweisen (Carson et al., 2002). Die Ergebnisse der aktuellen Studie korrelieren mit den Befunden am Schwein (Garlow et al., 2002) und Rind (Pfarrer et al., 2002) und bestätigen so, dass OPN auch über die Implantation hinaus eine Rolle bei der feto-maternalen Interaktion spielt. Ein Beweis für die spezifische Funktion steht noch aus, allerdings hat dieses Matrixmolekül das Potential Adhäsion, Gewebeumbau und Zell-Zell- sowie Zell-Matrix-Kommunikation an den Orten des feto-maternalen Kontaktes zu vermitteln. Die Anwesenheit des hochadhäsiven (Senger und Perruzzi, 1996) und flexiblen (Fisher et al., 2001) 45 kDa OPN Spaltproduktes, welches diverse Bindungssequenzen für verschiedene Proteine besitzt (Davis und Bayless, 2002) und in der Lage ist, Multimere zu bilden (Kaartinen et al., 1999), unterstützt die Hypothese, dass OPN während der gesamten Trächtigkeit als adhäsiver Ligand zwischen Placenta fetalis und materna wirkt. Aber auch andere Funktionen sind denkbar. Zum Beispiel können OPN Fragmente Zellproliferation und -migration induzieren (Agnihotri et al., 2001) und so auf die Reorganisation des Zytoskelettes bei der Plazentation Einfluss nehmen. Weiterhin kann das Überleben von Zellen durch das Unterbinden der Apoptose durch Bindung von OPN an Integrine unterstützt werden (Stupack und Cheresh, 2002).

Die beschriebene Lokalisation von OPN mRNA und Protein während der Trächtigkeit des Schafes bestätigt, dass OPN eine Komponente der Histotrophe ist, die an die feto-maternale Kontaktfläche bindet und dort möglicherweise wichtige Funktionen ausübt, die allerdings erst durch weitergehende Untersuchungen bestimmt werden können.

Expression von Integrinen und extrazellulärer Matrix in der Rinderplazenta

Laminin ist in den Basalmembranen (BM) fetaler und maternaler Epithel- und Endothelzellen in bovinen Plazentomen im gesamten Verlauf der Gravidität zu beobachten. Dazu kolokalisiert finden sich die α_6 und β_1 Integrinuntereinheiten an der basalen Seite von fetalen und maternalen Epithelien und Endothelien, was das Vorhandensein eines funktionellen $\alpha_6\beta_1$ Lamininrezeptors vermuten lässt, der Trophoblast und maternale Epithelzellen wie auch Endothelien an der BM verankert (Ruoslahti, 1991; Bosman, 1993). Dieses gemeinsame Auftreten ist in der frühen Trächtigkeit (um den Tag 80 post inseminationem, p.i.) besonders auffallend, wird aber für die α_6 and β_1 Integrinuntereinheiten auch im Endometrium des Rindes während des Zyklus (Kimmins und MacLaren, 1999) und im Zeitraum der Implantation (MacIntyre et al., 2002) beschrieben. Außerdem kann die Integrin α_6 Untereinheit auch mit der β_4 Untereinheit Heterodimere bilden, die dann Epithelzellen über Hemidesmosomen an der BM verankern (Jones et al., 1998). Diese Art der Verankerung wird für menschliche Zytotrophoblastzellen in vivo und in vitro beschrieben (Damsky et al., 1992, 1994), erscheint aber in bovinen Plazentomen nur für uterine Epithelzellen möglich, da die β_4 Untereinheit ausschließlich in Zellen des maternalen Stromas und assoziiert mit BM detektiert wurde.

Obwohl sich die TGC in einem nicht polarisierten, migratorischen Zustand befinden, exprimieren sie die α_6 und β_1 Integrinuntereinheiten während der gesamten Trächtigkeit, wiederum kolokalisiert mit Laminin. In der frühen Trächtigkeit (Tag 24) wird das Auftreten der β_1 Integrinuntereinheit in binukleären Trophoblastzellen mit der Wanderung dieser Zellen assoziiert (MacLaren und Wildeman, 1995). Ein faszinierender Zusammenhang von Laminin und Migration ergibt sich für bestimmte Tumorzellen, die während der Extravasation entlang einer Lamininmatrix wandern (Schuppan et al., 1994). Die enge Verwandtschaft der Invasivität von Trophoblast und wandernden Tumorzellen lässt ähnliche Prozesse vermuten, da Tumorzellen und/oder Metastasen nicht nur entlang von Lamininmatrices wandern, sondern sogar ihre eigene Lamininmatrix produzieren (Tani et al., 1997; Scarpa et al., 1999; Lohi et al., 2000; Schaumburg-Lever et al., 2000). In Keratinozyten kann die Integrinuntereinheit α_2 auch mit der β_1 Untereinheit Heterodimere bilden, die entweder als Lamininrezeptoren fungieren (Decline und Rousselle, 2001) oder direkte Zell-Zellkontakte vermitteln (Symington et al., 1993). Daraus ergibt sich die Hypothese, dass die α_2 Untereinheit, die in der basalen Hälfte der TGC lokalisiert ist, für beide Möglichkeiten zur

Verfügung steht, da Laminin vorhanden ist und eine Kommunikation mit benachbarten mononukleären Trophoblastzellen während der Migration sehr wahrscheinlich ist. Kollagen Typ IV ist in fetalen BM von Trophoblast und Endothelzellen in bovinen Plazentomen während der gesamten Trächtigkeit reichlich vorhanden, und auch die Expression im maternalen Stroma und BM ist während der frühen Trächtigkeit (< Tag 150 p.i.) von ähnlicher Intensität, dagegen nimmt sie bis zur späten Trächtigkeit (Tag 270 p.i.) insofern stark ab, als dass nur noch das maternale Stroma und darin enthaltene Blutgefäße eine schwache Immunreaktion zeigen. Im Gegensatz findet sich die Majorität von Kollagen Typ I im maternalen Bindegewebe, während das Mesenchym der fetalen Zotten nur eine schwache Reaktion aufweist. Dagegen beobachten Boos und Stelljes (2000) keine Veränderung der Kollagen Typ IV Expression im Verlauf der Trächtigkeit des Rindes und lokalisieren auch Kollagen Typ I und III, wobei das Auftreten von Kollagen Typ I mit der mechanischen Stabilität der Uteruswand in Verbindung gebracht wird. Der Verlust der Kollagen Typ IV Expression in den maternalen BM während der Implantation der Ziege wird eher als Modifikation der BM denn als ihre Zerstörung interpretiert (Guillomot, 1999). Potentielle Liganden für die Bindung von Kollagenen sind Heterodimere aus den Untereinheiten α_1, α_2 und α_3 mit β_1, die in der aktuellen Studie alle in fetalem und maternalem Stroma und/oder BM im Rinderplazentom identifiziert werden.

Fibronectin wird in der vorliegenden Studie im maternalen und fetalen Stroma sowie in BM der Epithelien und Endothelien der bovinen Plazenta vom Tag 80 bis 270 p.i. detektiert, ist aber in präimplantativen Stadien (Tag 14-24 p.i.) nicht nachzuweisen (MacLaren und Wildeman, 1995). Grundsätzlich werden zwei Typen von Fibronectin beschrieben, die mit der Anheftung des Konzeptus des Menschen (Aplin et al., 1999) und des Schweines (Burghardt et al., 1997) in Verbindung gebracht werden. Der Antikörper in der vorliegenden Studie erlaubt eine Unterscheidung von beiden Typen nicht, aber die Lokalisation ausschließlich in fetalem Zottenmesenchym und Bindegewebe der maternalen Septen lässt eine Beteiligung bei der feto-maternalen Adhäsion sehr unwahrscheinlich erscheinen. Allerdings macht die Fähigkeit zur Bindung von anderen Matrixproteinen (wie Kollagen, Heparin und Rezeptoren der Zelloberfläche) Fibronectin zu einem interessanten Kandidaten für die Stabilisierung der Matrix und die Kontrolle der Zellmotilität (Alberts et al., 1995). Das Integrin $\alpha_5\beta_1$ ist der "klassische" Fibronectinrezeptor (Hynes, 1992), daneben sind aber auch die Integrinheterodimere $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_v\beta_1$ und $\alpha_v\beta_3$ in der Lage, Fibronectin zu binden (Ruoslahti, 1991; Bosman, 1993). Für isolierte menschliche Zytotrophoblastzellen wird

Fibronectin als Mediator für die Anheftung und die Ausbreitung des Trophoblasten sowie die Bildung des Synzytiotrophoblasten präsentiert (Bischof et al., 1995). Fibronectin kann ferner die Ausbildung spezifischer Integrinrezeptoren regulieren. Zum Beispiel wird eine Umschaltung ("switch") vom $\alpha_6\beta_4$ Integrin zu dem Heterodimer $\alpha_5\beta_1$ an dem Zeitpunkt beobachtet, wenn menschliche extravillöse Zytotrophoblastzellen ihre Wanderung durch das fibronectinreiche maternale Stroma beginnen und markiert so den Übergang von einem proliferativen zu einem invasiven Zellphänotyp (Damsky et al., 1994; Kaufmann und Castellucci, 1997). Im Endstadium werden diese Zellen positiv für das Integrin $\alpha_1\beta_1$, was das Erreichen ihrer vollen Invasivität kennzeichnet (Damsky et al., 1992).

Im Rinderplazentom werden die Integrinuntereinheiten α_1, α_2, α_3, α_5 und β_4 wie auch α_v und β_3 im maternalen Stroma deutlich exprimiert. Eine Modulation der Expression (die Mehrheit von β_3 in den Stammsepten in der frühen Trächtigkeit und eine langsame Ausweitung bis in die Septenspitzen steht im Gegensatz zum Auftreten von α_1, α_2, α_5, α_v, und β_4 in den Septenspitzen während der gesamten Trächtigkeit mit einer gleichzeitigen Reduktion in Stamm- und Intermediärsepten in der späten Gravidität) unterstützt die Idee, dass die unterschiedlichen Integrine verschiedene Funktionen ausüben, wie die Regulation der Proliferation der Stammsepten durch β_3 sowie die Entwicklung und Differenzierung einer funktionellen tertiären Austauscheinheit in den Septenspitzen durch einen Pool von Integrinen (α_1, α_2, α_5, α_v, und β_4). Die gleichzeitige Anwesenheit von mehreren Integrinuntereinheiten im Zusammenhang mit Proteinen der ECM wird mit der Proliferation und Differenzierung von maternalen Stromazellen assoziiert (Fazleabas et al., 1997) und erlaubt die Hypothese, dass unterschiedliche Gebiete des maternalen Stromas auch unterschiedliche Stadien der Entwicklung und Differenzierung von Stammsepten bis in die tertiären Septenspitzen zeigen können.

Das $\alpha_v\beta_3$ Integrin ist der Rezeptor für Vitronectin, kann aber auch an Fibronectin binden (Ruoslahti und Pierschbacher, 1987). Beide, die α_v und β_3 Untereinheit, sind beim Rind besonders stark in den Septenspitzen der späten Gravidität exprimiert. Da das $\alpha_v\beta_3$ Integrin in engem Zusammenhang mit Tumorprogression und Invasivität steht (Gladson und Cheresh, 1991), lässt das lokal in den Septenspitzen konzentrierte Signal vermuten, dass diese Stromazellen eine erhöhte Differenzierungs- und Proliferationsaktivität besitzen. Ein Nachweis von Vitronectin im bovinen Plazentom steht noch aus. Ein weiterer Ligand diverser Integrine (wie $\alpha_v\beta_3$, $\alpha_v\beta_1$, $\alpha_v\beta_5$, $\alpha_4\beta_1$, $\alpha_5\beta_1$) ist Osteopontin, ein Glykoprotein, welches von

uterinen Drüsen sezerniert wird und anschließend zwischen dem luminalen Epithel des Uterus und dem Trophoblasten zu liegen kommt und auf diese Weise als Brücke der Embryoanheftung bei Primaten (Fazleabas et al., 1997), dem Schwein (Garlow et al., 2002) und dem Schaf (Johnson et al., 2001) fungiert.

Neben dem Kollagen Typ IV und Laminin der BM ist Fibronectin das überwiegende Protein der ECM im fetalen Kompartiment des Rinderplazentoms. Als Bindungspartner für Kollagen werden die Integrinuntereinheiten α_1, α_2 und α_3 identifiziert, die allerdings nur schwach exprimiert werden, sowie β_1, während α_4 und α_5, die mit β_1 Fibronectinrezeptoren bilden, weitaus stärkere Signale liefern. Dagegen wird α_4 während des Zyklus im Stroma des bovinen Endometriums lokalisiert (Kimmins und MacLaren, 1999).

Es lässt sich schlussfolgern, dass die Migration der TGC oder die eingeschränkte Trophoblastinvasion des Rindes Modellcharakter für invasive Prozesse hat, da ähnliche Mechanismen genutzt werden wie bei der Tumorinvasion, wie zum Beispiel die Produktion einer Lamininmatrix und die Expression des $\alpha_6\beta_1$ Integrins, welches normalerweise für die basale Anheftung von Epithelzellen an die Basalmembran dient. Weiterhin scheint auch im Rinderplazentom, in der Zeit nach der Implantation bis hin zur Geburt, ein Zusammenwirken vieler Integrine mit ihren Liganden der ECM wichtig zu sein.

5.1.3 Connexine bei Schaf und Rind

Connexinexpression im zyklischen Endometrium des Schafes während der Gravidität und um die Geburt

Implantation

In der frühen Trächtigkeit des Schafes scheint die räumliche und zeitliche Verteilung der Connexine Cx26 und Cx43 direkt mit der Aufnahme des feto-maternalen Kontaktes im Zusammenhang zu stehen. Wie bei Nagern sind Cx26 und Cx43 die Hauptvertreter der Kanalproteine im Endometrium und Cx32 spielt bei den trächtigkeitsabhängigen Differenzierungsprozessen im Endometrium keine Rolle. Während der gesamten Trächtigkeit stellte Cx26 die epitheliale Isoform dar und Cx43 war immer auf das Stroma beschränkt. Beim Schaf scheinen beide Connexine in Abhängigkeit von der Reproduktionsphase reguliert zu werden. Am Beginn der Implantation (Tag 15 post conceptionem, p.c.) ist die Expression von Cx43 in den endometrialen Stromazellen ähnlich wie im Östrus und Cx26 fehlt im

Epithel, was sich von den Befunden bei der Ratte unterscheidet (Winterhager et al., 1993; Risek et al., 1995), aber natürlich das Vorkommen einer anderen, bislang nicht identifizierten Isoform nicht ausschließt. Mit Beginn der definitiven Anheftung der Blastozyste am Tag 16-18 p.c. (Guillomot et al., 1981) ist die Cx Expression im Stroma erhöht und steigt weiter bis zum Tag 21 p.c. Die starke Aufregulierung des Cx43 Proteins im karunkulären und interkarunkulären Stroma deutet auf eine trächtigkeitsspezifische Rolle der Kommunikation über Gap junctions beim Schaf hin.

Der enge Zusammenhang von Trophoblastinvasion und Cx43 (und Cx26) Induktion, der die regulative Funktion von Connexinen für die Implantation betont, wurde in der Dezidua von Ratten demonstriert (Winterhager et al., 1991). Den Connexinen wird eine synchronisierende Rolle für den Stoffwechsel, die Sekretion, Differenzierung und Abbauvorgänge in den Deziduazellen zugeschrieben (Ono et al., 1989; Burghardt und Fletcher, 1990). Allerdings fehlt hierfür bisher der funktionelle Beweis, da Cx43 defiziente Mäuse kurz nach der Geburt auf Grund eines Truncus pulmonalis Defektes sterben (Reaume et al., 1995). Cx26 wurde weder im nicht trächtigen, noch im präimplantativen Endometrium des Schafes detektiert, wird aber in den Epithelien der oberflächlichen uterinen Drüsen ab dem Tag 21 p.c. induziert. Dieses Phänomen scheint eine spezifische Reaktion auf das Eindringen des Trophoblasten zu sein, welches zwischen den Tagen 15 und 21 beobachtet wird (Guillomot et al., 1981; Wooding et al., 1982). Vorwölbungen (Papillen) des Trophoblasten ragen dabei in die Öffnungen der uterinen Drüsen und beginnen so die Verankerung des Embryos, ein Prozess, der mit großer Wahrscheinlichkeit durch Gap junctions unterstützt wird. Im Kaninchen wird eine ähnliche Antwort des maternalen Gewebes auf die Erkennung des Embryos beobachtet. Hier wird Cx32 vor dem Eindringen des Trophoblasten durch Fusion spontan im Epithel der Implantationskammer induziert (Winterhager et al., 1988; Antoskiewicz et al., 1996). Die Ergebnisse der vorliegenden Studie weisen darauf hin, dass beim Schaf in allen untersuchten Stadien, tragend oder nicht tragend, Cx26 das entscheidende epithelspezifische Gap junction Protein ist. Das trifft auch für die Ratte zu, in der Cx26 vor der Trophoblastinvasion lokal im Epithel der Implantationskammer induziert wird und später in der den eindringenden Trophoblasten begleitenden Deziduazone beobachtet wird (Winterhager et al., 1993). Es wird vermutet, dass die auf das Epithel der Implantationskammer beschränkte Expression von Cx26 den kontrollierten Zelltod des uterinen Epithels reguliert, welcher den Implantationsprozess bei der Ratte begleitet (Welsh und Enders, 1991). Dagegen hat der Trophoblast des Schafes nur eine geringe Tendenz zur

Invasion. In der synepitheliochorialen Plazenta bleibt das uterine Epithel weitgehend intakt, wird aber durch das Einwandern von Trophoblastzellen zu einem Synzytium aus feto-maternalen Hybridzellen (Wooding, 1992; Leiser und Kaufmann, 1994). Interessanterweise fehlen die Cx26, Cx32 und Cx43 im luminalen Epithel von Spezies mit nicht invasiver Implantation, wie Pferd und Schwein (Day et al., 1998). Cx43 wird hier nur im Stroma beobachtet, allerdings exprimiert die Stute, die eine geringgradig höhere Invasivität zeigt als das Schwein, Cx43 auch im Epithel der uterinen Drüsen. Diese Befunde lassen darauf schließen, dass das Schaf eher einen invasiven Implantationstyp repräsentiert.

Im Uterus des Schafes wird die Cx Expression hauptsächlich durch Steroidhormone moduliert, wie für das Myometrium berichtet wurde (Garfield et al., 1979; Verhoeff et al., 1985). Die spärliche Cx43 Expression während der Progesterondominanz im Zyklus (Diöstrus) deutet auf einen hemmenden Effekt hin, während Östrogene für die Stimulation der Gap junction Connexine im Östrus verantwortlich sind, wie es auch für das Endometrium von Ratte und Mensch vorgeschlagen wurde (Grümmer et al., 1994; Jahn et al., 1995). Wie bei der Ratte wird die epitheliale Cx Expression beim Schaf in der präimplantativen Phase unterdrückt, ein Phänomen, das als zellbiologischer Indikator der Rezeptivität angesehen wird und progesteronabhängig ist (Grümmer und Winterhager, 1998). Vergleichbar mit den Verhältnissen bei Kaninchen und Ratte (Winterhager et al., 1988, 1993) scheinen embryonale Signale (Grümmer et al., 1999), wie Prostaglandine, fetale Östrogene und andere (parakrine) Proteine, die dominierenden Faktoren für die Bildung von Gap junctions bei der Schafimplantation zu sein. Die Induktion von Connexinen mit einem künstlichen Stimulus (aus einer Kombination von Östrogenen und Prostaglandinen mit einem mechanischen Stimulus) wird im uterinen Epithel des Kaninchens demonstriert (Antoskiewicz et al., 1996). Fetale Östrogene werden während der frühen Trächtigkeit beim Schaf detektiert (Carnegie und Robertson, 1978; Findlay et al., 1981), und ein vom Trophoblasten produziertes ovines embryonales Interferon (OTP-1) wird als Kandidat diskutiert, der die maternale Erkennung der Blastozyste und Veränderungen im Endometrium initiiert (Godkin et al., 1982; Hansen et al., 1989; Wooding et al., 1991). Allerdings füllen die fetalen Embryonalhüllen beim Schaf beide Hörner des Uterus ab dem Tag 17 p.c. vollständig aus (Davies und Wimsatt, 1966), so dass als Folge denkbar ist, dass ein früher embryonaler Stimulus synchron im gesamten Uterus zelluläre Veränderungen, wie die Bildung von Gap junctions, bewirkt. Dieses würde erklären, warum in den untersuchten Stadien keine Unterschiede in der Cx Expression des tragenden und nicht tragenden Uterushornes gefunden wurden.

Späte Trächtigkeit und Geburt

Um den Tag 131 der Trächtigkeit hat die Cx43 Expression im endometrialen Stroma des Schafes abgenommen, außerdem wurde eine Kolokalisation zu Cx26, allerdings in schwächerem Ausmaß, beobachtet. Am Tag 145 p.c. war die Expression beider Connexine im Stroma reduziert. Dagegen deutet die starke Erhöhung von Cx26 Protein im Epithel bei fortschreitender Trächtigkeit beim Schaf auf spezifische Funktionen dieser Gewebe hin, die mit einer erhöhten Stoffwechsel- und Proliferationsrate einhergehen und bis in die tiefen Bereiche der uterinen Drüsen reichen (Wrobel und Kühnel, 1966; Hoyes, 1972). Ein präpartaler Anstieg von Cx26 im endometrialen Epithel wird auch für die Ratte bestätigt (Risek et al., 1990) und scheint direkt mit der hormonabhängigen Sekretion und Proliferation der Epithelzellen gekoppelt zu sein (Risek et al., 1995). Wie in der Implantation werden auch während der späten Trächtigkeit keine Unterschiede in der Cx Expression zwischen dem tragenden und nicht tragenden Uterushorn beobachtet, was auf eine synchrone hormonelle Steuerung des gesamten Uterus hinweist. Bei der Ratte existiert ein zellspezifischer Kontrollmechanismus für endometriale Connexine (Risek und Gilula, 1991; Grümmer et al., 1994, 1999; Risek et al., 1995). In diesem stimuliert Östrogen die Expression von Cx43 im Stroma, was wiederum durch Progesteron gehemmt wird. wohingegen Cx26 und Cx43 im Epithel der uterinen Drüsen und im luminalen Epithel unterschiedlich reguliert werden. Ein ähnlicher Regulationsmechanismus über Steroidhormone würde die Abregulation von Connexinen im Drüsenepithel und den oberflächlichen Anteilen des Stromas kurz nach der Geburt (20 min post partum, p.p.) beim Schaf erklären, während gleichzeitig das luminale Epithel und das Stratum reticulare des interplazentomären Stromas eine erstaunlich hohe Proteinexpression aufweisen. Die Konzentration unkonjugierter Östrogene im maternalen Blutplasma, die während der gesamten Trächtigkeit des Schafes relativ niedrig sind, steigen in den letzten 24 Stunden vor der spontanen Geburt signifikant an (Challis, 1971; Challis und Patrick, 1981). Im gleichen Zeitraum werden auch im Endo- und Myometrium erhöhte Östrogenwerte festgestellt (Challis und Olson, 1988). Ein Sinken der maternalen Progesteronkonzentration im Blutplasma wird in den letzten 5-15 Tagen der Trächtigkeit beim Schaf beobachtet, welches mit niedrigen Gewebekonzentrationen einhergeht (Elsner et al., 1980; Power und Challis, 1987). Der Anstieg der maternalen Plasmaöstrogenspiegel, der mindestens teilweise aus der plazentären Östrogenproduktion entstammt (Challis et al., 2000), korreliert mit einem Anstieg von Cx43 Transkripten im Myometrium des Schafes (McNutt et al., 1994). Die Bedeutung der Modulationsunterschiede zwischen invasiven und nicht

invasiven Implantationstypen ist noch unklar, besonders im Hinblick auf die anscheinend nicht oder kaum vorhandene interzelluläre Kommunikation über Gap junctions während der Trächtigkeit bei Pferd und Schwein (Day et al., 1998).

Aus den Ergebnissen der Studie lässt sich schließen, dass die verschiedenen uterinen Gewebe programmiert sind, zu bestimmten Zeiten Gap junctions zu bilden. Dieses geschieht mit einer hohen Plastizität unter spezifischen hormonellen und embryonalen Einflüssen. Die Befunde deuten auf eine essentielle Funktion der endometrialen Gap junctions bei der Aufnahme feto-maternalen Kontaktes und der Erhaltung der Trächtigkeit hin.

Connexinexpression in der Rinderplazenta

Die vorliegende Studie ist der erste Bericht über Zell-Zell-Kommunikation über Gap junctions im Modell der eingeschränkten Trophoblastinvasion der synepitheliochorialen Rinderplazenta. Im Vergleich zu anderen epitheliochorialen Plazentatypen bei Nicht-Ruminanten zeichnet sich das Rinderplazentom, neben spezifischen Mustern von Wachstum und Differenzierung, durch die Anwesenheit von Migrations-, Invasions- und Fusionsprozessen aus, die zumindestens teilweise durch gap junctionale Connexine beeinflusst werden können.

Cx32 ist in bovinen Plazentomen im endometrialen Epithel exklusiv an den Septenspitzen zu finden, die bereits als Wachstumszonen beschrieben wurden (Pfarrer et al., 2003). Im Gegensatz dazu findet sich Cx43 im Bindegewebe der maternalen Karunkel und im fetalen Mesenchym. Die Architektur und das Wachstum der maternalen Septen und fetalen Zotten der Rinderplazenta könnten durch die Anwesenheit von Cx43 Gap junctions in den bindegewebigen Anteilen des Plazentoms beeinflusst werden. Eine solche Beteiligung von Cx43 in strukturgebenden Geweben wird in Cx43 defizienten Mäusen besonders deutlich, da diese an Herzmissbildungen leiden, die auf fehlende Zell-Zell-Kommunikation via Cx43 Gap junction Kanäle zurückzuführen sind (Reaume et al., 1995; Nicholson und Bruzzone, 1997). Allerdings wird in präimplantativen Cx43 defizienten Mausembryonen auch gezeigt, dass eine Cx Expression in dieser Periode nicht essentiell ist, aber die Etablierung eines Kommunikationsnetzwerkes für die postimplantative Entwicklung fördert (Houghton et al., 2002). Missbildungen werden auch in den Plazentomen geklonter Kälber beobachtet, die schon im ersten Trimester der Trächtigkeit auf Grund von Anomalien der Plazenta, wie kleinen Kotyledonen und Mangelernährung, sterben (Hill et al., 2000). Eine Verbindung zu

einem veränderten Expressionsmuster von Connexinen ist hier aber noch nicht beschrieben.

Die Migration der TGC in bovinen Plazentomen wird möglicherweise durch Cx26 und Cx43 beeinflusst, da die spezifische mRNA und das Protein beider Cx in TGC exprimiert werden. Von Cx32 ist nur die mRNA vorhanden, was vermuten lässt, dass Cx32 mRNA im Plazentom des Rindes nicht translatiert wird. Die Migration ist ein Differenzierungsweg, der es dem Trophoblasten erlaubt, für eine gewisse Zeit invasive Fähigkeiten zu besitzen (Cross et al., 1994). Ein temporärer Verlust oder die Expression von spezifischen Cx ist daher wichtig für den Grad der Invasivität des Trophoblasten (Winterhager et al., 1999). In der Plazenta von Maus und Ratte verändert sich die Cx-Expression gerade zu der Zeit, wenn der physiologische Zustand von einem invasiven Typ zu einem "funktionellen" Typ umschaltet (Reuss et al., 1996). Menschliche Zytotrophoblastzellen exprimieren in der Proliferationsphase Cx40, aber die Ablösung der Zellen aus den Zellsäulen und die Migration in das Plazentabett werden von einer sinkenden Cx40 Expression begleitet (Winterhager et al., 1999). Diese Befunde lassen vermuten, dass die Fähigkeit zur Migration der bovinen TGC von der Expression spezifischer Cx abhängig ist.

Die TGC im bovinen Plazentom synthetisieren mRNA von allen drei untersuchten Cx (26, 32, 43) und exprimieren Protein von Cx26 und Cx43, während das gegenüberliegende maternale Karunkelepithel Cx26 besitzt. Auf diese Weise könnte eine Fusion von TGC mit maternalen Epithelzellen von Cx26 und Cx43 unterstützt werden. In der menschlichen Plazenta zeigt sich, dass eine interzelluläre Kommunikation über Cx43 Gap junctions essentiell für die Fusion von Zytotrophoblastzellen zum Synzytiotrophoblasten ist (Frendo et al., 2003). Weiterhin gibt es Hinweise, dass Gap junctions zwischen Zytotrophoblast und Synzytiotrophoblast verantwortlich für Fusionsprozesse in der Plazenta des Meerschweinchens sind (Firth et al., 1980). In vitro Versuche mit menschlichen Zytotrophoblastzellen haben gezeigt, dass Zellfusionen durch das Vorhandensein von Cx43 gefördert werden, welches seinerseits durch die Anwesenheit von hCG und cAMP stimuliert wird (Cronier et al., 1994, 1997, 2001).

Im bovinen Plazentom ist der prädisponierte Ort für die Invasion die Chorionplatte, von der aus fetale Kotyledonen ihre Interdigitation mit maternalen Karunkeln beginnen. Cx32 wird beim Rind spezifisch in dieser Zone exprimiert, nämlich ausschließlich im uterinen Epithel an der Spitze der maternalen Septen, und ist damit der interessanteste Connexin-Kandidat für eine Kontrolle der Invasion und damit für die Beeinflussung der Zahl der Primärzotten. Der Stimulus für eine Cx32 Proteinexpression könnte von dem wachsenden

Trophoblasten ausgehen (Winterhager et al., 1988; Antoskiewicz et al., 1996) und/oder von Steroidhormonrezeptoren (Orsino et al., 1996). Im Endometrium von Kaninchen ist nämlich ein mechanischer Stimulus, der die Blastozyste ersetzt, in Kombination mit einer Applikation von 17-beta-Östradiol nötig, um eine Cx32 Expression auszulösen, die der des tragenden Uterus entspricht (Antoskiewicz et al., 1996). Untersuchungen an tragenden, nicht tragenden und pseudograviden Uteri von Kaninchen haben gezeigt, dass die Anwesenheit der Blastozyste für die Induktion der Bildung von Gap junctions unbedingt erforderlich ist (Winterhager et al., 1988). Die Art der feto-maternalen Verbindung wird durch den Implantationstyp und die Tiefe der Trophoblastinvasion bestimmt (Leiser und Kaufmann, 1994). Der erste Kontakt zwischen fetalen und maternalen Geweben führt zu Veränderungen von Zellen im Endometrium, die als Dezidualisierung bezeichnet werden (Christian et al., 2002). Im Gegensatz zur hämo- und endotheliochorialen Plazentation, bei der der Trophoblast in das maternale Stroma invadiert, ist die bovine epitheliochoriale Plazenta durch Trophoblastzellen charakterisiert, die nicht in das maternale Stroma eindringen (Grosser, 1927). Die Anordnung von Cx26 im maternalen Epithel und Cx43 in maternalen Stromazellen könnte einen Teil der Regulationsmechanismen darstellen, die die Invasionstiefe bei der Kuh begrenzen. Ein ähnliches Verteilungsmuster findet sich auch im Endometrium der Ratte während der Implantation (Winterhager et al., 1993). In der Umgebung der Blastozyste ist Cx26 im maternalen Epithel und Cx43 in der Dezidua exprimiert. Sobald das maternale Epithel zerstört ist, übernehmen die den Trophoblast begleitenden Zellen der Dezidua die Cx26 Expression, während Cx43 in den tiefergelegenen Zellen der Dezidua beobachtet wird. Die Abwesenheit von Connexinen in maternalen Zellen des luminalen Epithels und in Trophoblastzellen der epitheliochorialen Plazenta des Pferdes und Schweines könnte ein Grund für die totale Abwesenheit einer Trophoblastinvasion bei diesen Tierarten sein (Day et al., 1998). Dagegen lässt die spezifische Lokalisation von Cx26 und Cx43 im Endometrium des Schafes während der Implantation und der Trächtigkeit vermuten, dass das Schaf einen etwas invasiveren Implantationstyp darstellt (Gabriel et al., 2003).

Die Anwesenheit von Cx26 Protein an der feto-maternalen Kontaktfläche, zusammen mit dem Auftreten der entsprechenden mRNA im maternalen Epithel boviner Plazentome, deutet auf das Vorhandensein eines interzellulären Austausches zwischen fetalem und maternalem Kompartiment hin. Eine Art "Ernährungsfunktion" von Cx26 Gap junctions wird auch in den Plazenten anderer Spezies vermutet, da sich das Cx26 in Wildtyp Mäusen in den Zytotrophoblastzellen des plazentären Labyrinths befindet (Pauken und Lo, 1995), und ein

Glukosetransport durch Cx26 Gap junctions in Cx26 Knockout Mäusen bestätigt wurde (Gabriel et al., 1998). In der Plazenta der Ratte sind die Lagen des Synzytiotrophoblasten I und II auch über Cx26 verbunden (Risek und Gilula, 1991). Die an das mütterliche Blut angrenzenden Lagen des Synzytiotrophoblasten exprimieren GLUT I (Takata et al., 1994), ein Protein, welches die Diffusion von Glukose unterstützt (Baldwin, 1993). Auch in der hämomonochorialen Plazenta des Menschen ist GLUT I auf der apikalen und basalen Oberfläche des Synzytiotrophoblasten zu finden (Takata et al., 1992). Diese Anordnung fördert möglicherweise den Transport von Glukose aus dem maternalen in den fetalen Blutkreislauf (Shin et al., 1996). Obwohl der immunhistochemische Nachweis von Connexinen in der menschlichen Plazenta bislang nicht möglich war (Winterhager et al., 1999), wurden Gap junctions zwischen Synzytiotrophoblast und Zytotrophoblast im ersten Trimester der Schwangerschaft identifiziert (de Virgiliis et al., 1982). Auch die Verbindung von zwei verschiedenen Zelltypen ist möglich, wie zwischen Oozyten und Granulosazellen der Ratte demonstriert wird (Gilula et al., 1978). Die Tatsache, dass Cx26 mRNA nur im maternalen Epithel boviner Plazentome gefunden wird, lässt die Ausbildung heterotypischer Kanäle vermuten, die mindestens ein bisher nicht identifiziertes Connexin enthalten. Eine weitere Erklärung für das Auftreten von Connexinen ohne Gegenstück einen interzellulären Kanal zu bilden, ist das Konzept der ungepaarten Connexone, die in Teilen der Zellmembran lokalisiert sind, in denen kein Zell-Zell-Kontakt auftritt. Diese Connexone sind möglicherweise an der Abgabe von Signalmolekülen, der Aktivierung von Kinasekaskaden und am Überleben der Zelle beteiligt (Goodenough und Paul, 2003). Indirekt könnte auch Cx43 am feto-maternalen Austausch involviert sein, da das Cx43 endothelialer Zellen eine Rolle bei der Bildung und/oder der Wirkung von Stickoxid spielt (Liao et al., 2001), welches als potenter Vasodilatator die Blutflussgeschwindigkeit senkt und so mehr Zeit für den feto-maternalen Austausch erlaubt.

Es lässt sich schlussfolgern, dass Cx26, Cx32 und Cx43 während der gesamten Trächtigkeit spezifische Funktionen in bovinen, nicht hämochorialen Plazentomen haben. Diese erstrecken sich auf die Kontrolle des plazentomären Wachstums und der Differenzierung (Gewebearchitektur), die Regulation der Trophoblastinvasion (Migration und Fusion der TGC mit uterinen Epithelzellen) und den materno-fetalen Austausch (Ernährung durch Glukosetransfer).

5.2 Gefäßarchitektur und Angiogenese der Wiederkäuerplazenta

5.2.1 Das Gefäßsystem der Rinderplazenta

Frühe Trächtigkeit

In der frühen Trächtigkeit des Rindes (unter 16 cm Scheitel-Steiss-Länge, SSL, etwa 4. Monat) war das Ausgießen des fetalen kotyledonären Gefäßsystems mit flüssigem Plastik auf Grund der Zartheit der Gefäße und des umgebenden Mesenchyms nicht möglich, da Extravasation des Ausgussmediums regelmäßig auftrat und häufig zu einem kompletten Einschluss der ausgegossenen Gefäße führte. Auch Versuche mit unterschiedlichen Spülmedien, die Fixative und/oder Vasodilatatoren enthielten, konnten die Extravasationen nicht verhindern. Dieser Mangel ließ sich zum Teil durch mit Indischer Tinte ausgegossene histologische Präparate ausgleichen, durch welche ein Einblick in das Verhältnis fetaler und maternaler Blutgefäße möglich wurde, da in den ungefärbten dicken Schnitten mehrere Ebenen sichtbar waren. In den Gefäßausgüssen späterer Trächtigkeitsstadien war eine solche Beurteilung auf Grund der hohen Kapillardichte oft nicht möglich.

Maternale Gefäßarchitektur

Die Spiralarterien des bovinen Karunkelstiels sind erheblich stärker gewunden als die dazugehörigen Venen, wobei dieses Phänomen in der frühen Trächtigkeit deutlicher als in den späten Stadien ist (Leiser et al., 1997a). Diese Tatsache weist darauf hin, dass eine Spiralisierung zumindestens während der frühen Plazentation des Rindes eine essentielle Rolle spielt und den Blutstrom in das Plazentom abfedert, um so eine Kompression der zarten fetalen Chorionzotten zu verhindern, wie auch für die menschliche Plazenta hypothetisiert wird (Carter, 1975; Benirschke und Kaufmann, 1995). Im gleichen Sinne könnten sich die Spiralgefäße ähnlich wie "Bettfedern" verändern, wenn der Karunkelstiel durch Bewegungen des Fetus und/oder der Mutter bewegt wird (Leiser et al., 1997a).

Das initiale Gefäßsystem der Karunkel besteht aus einer zentral gelegenen leitenden Einheit, welche sich aus Stamm- und Intermediärgefäßen zusammensetzt, und einer kapillären Austauscheinheit in der Peripherie, über die der feto-maternale Austausch läuft (Leiser et al., 1997b). In der frühen Trächtigkeit dominiert die Leitung, die das Blut von der Basis der Karunkel durch die Stammsepten bis an die Spitze der Intermediärsepten transportiert (Leiser et al., 1997b). Die Tatsache, dass die Stammarterien und -venen der Leitungseinheit in der frühen Trächtigkeit relativ kurz sind und die intermediären Arteriolen und Venulen nahezu bis an die fetale Oberfläche des Plazentoms reichen, deutet auf das Vorhandensein eines

Gefäßrahmens hin, der in der weiteren Entwicklung des Plazentoms eine Umwandlung der Arteriolen und Venulen in weitere Äste der Stammgefäße erlaubt und so bestehende Ressourcen ausnutzt. In der frühen Trächtigkeit sind diese verzweigten Stammgefäße der Ausgangspunkt vieler neu entstehender Intermediärarteriolen und -venulen, die das sauerstoffreiche Blut auf geradem (kürzestem) Wege an die fetale Oberfläche des Plazentoms transportieren und sich dort in Form von "Trauerweiden" in das rückläufige, das heißt maternal gerichtete, Kapillarsystem verzweigen. Obwohl das Plazentom durchschnittlich um den Faktor drei wächst (Tsutsumi, 1962) bleibt diese generelle Gefäßarchitektur auch in der zweiten Trächtigkeitshälfte erhalten, da sich nur die Länge und die Verzweigungshäufigkeit der Gefäße verändern (Leiser et al., 1997a),. Die im frühen Plazentom nur spärlich vorhandenen Kapillaren der Austauscheinheit bilden eine dünne Schicht auf der Septenoberfläche, die nur flache Krypten erkennen lässt. Im Bereich der terminalen Septen liegen die maternalen Kapillaren in direkter Nachbarschaft der fetalen Zotten, woraus sich ein kurzer interhämaler Abstand der beiden Gefäßsysteme ergibt, der den feto-maternalen Austausch fördert. In diesem Sinne positiv wirkt sich auch die Vergrößerung der Kapillaroberfläche durch einen gewundenen Verlauf und sinusoidale Erweiterungen aus. Sinusoide in der Nähe der Septenspitzen unterstützen den feto-maternalen Austausch, da der Blutfluss durch lokale Erweiterungen in einem Röhrensystem verlangsamt wird und so mehr Zeit für den Stofftransfer zur Verfügung steht (Arts, 1961). Dieser Vorgang wird zusätzlich durch die relativ dünnen Wände der Venulen gefördert (Björkman, 1954; Tsutsumi, 1962), was die Diffusions- und Transportvorgänge erleichtert. Bis zur Hälfte der Trächtigkeit erhöht sich die Kapazität für den feto-maternalen Austausch deutlich (Björkman, 1954), was möglicherweise auf die progressive Entwicklung neuer Tertiärkrypten zurückzuführen ist, welche sich dicht an dicht in die Sekundärkrypten einsenken. Die Tertiärkrypten werden wiederum von interdigitierenden Tertiärzotten der Kotyledone eingestülpt und führen so zu einer festen Verankerung der fetalen Plazenta im Uterus (Leiser et al., 1998). Alle beschriebenen Phänomene steigern sich fortschreitend bis zum Ende der Trächtigkeit (Leiser et al., 1997a, b).

Fetale Gefäßarchitektur

Das fetale Gefäßsystem der Rinderplazenta zeigt schon in der ersten Hälfte der Plazentation die gleichen Hauptcharakteristika wie in der späten Trächtigkeit (Leiser et al., 1997a, b). Entsprechend zur Organisation der maternalen Krypten entwickeln sich primäre, sekundäre und tertiäre fetale Zotten, die mit den Krypten interdigitieren. Im Unterschied zu

dem Gefäßsystem der Karunkel bleiben die Gefäße der Primärzotten deutlich voneinander getrennt, wenn sie sich von den großen Allantochoriongefäßen abzweigen. Von dort wachsen die Stammgefäße und ihre Äste (Arteriolen und Venulen) schon von Beginn der Entwicklung an durch die gesamte Kotyledone gerade auf die Karunkel zu. Dabei verlaufen die arteriellen Gefäße zentral in der Stammzotte, während die venösen Gefäße eher peripher in der Zotte liegen. Dieser Verlauf garantiert die kürzeste Distanz von den Leitungsgefäßen zu den Kapillaren der Austauscheinheit (Leiser et al., 1997b), wo der Hauptteil des plazentären Austausches stattfindet, und führt zu einem Treffen der fetalen und maternalen Gefäße nach dem Gegenstromprinzip (siehe unten). Zur Mitte der Trächtigkeit ist das fetale Gefäßsystem durch lange, parallel angeordnete, anastomosierende Kapillarschlingen gekennzeichnet, in welchen das vaskuläre Grundgerüst für die fächerartige Ausbildung von in Serie geschalteten Kapillarkomplexen der späten Trächtigkeit erkennbar ist (Leiser et al., 1997b). Der erhöhte Bedarf des Fetus manifestiert sich also einerseits in einer Verlängerung der Kapillarstrecke und andererseits in der regelmäßigen Ausbildung von sinusoidalen Erweiterungen im Kapillarbett (Leiser et al., 1997a). Dadurch erfährt der Blutfluss eine Verlangsamung (Faber und Thornburg, 1983), welche die Bedingungen für eine Diffusion von Sauerstoff und Kohlendioxid wie auch den aktiven langsamen Transport löslicher Stoffe erleichtert (Alberts et al., 1983). Der Austausch von Stoffen wird zusätzlich durch die Anordnung der Gefäße im Zottenbaum gefördert, da die Terminalgefäße ganz außen, also direkt benachbart zu den Kapillaren der Krypten, lokalisiert sind.

Blutstromverhältnis fetaler und maternaler Gefäße

Das Blutstromverhältnis zwischen fetalem und maternalem Gefäßsystem der Rinderplazenta verläuft in der frühen Trächtigkeit (3./4. Monat) nach dem Gegenstromprinzip, da der Hauptteil der Tertiärstruktur, also der Kapillaren, noch nicht ausgebildet ist und somit die Stamm- und Intermediärgefäße von Karunkel und Kotyledone parallel zueinander verlaufen. Auf diese Weise entsteht ein Gegenstromsystem, das theoretisch effektivste Austauschsystem (Faber und Thornburg, 1983), in welchem das Fehlen eines ausgedehnten Kapillarnetzes (normalerweise Grundvoraussetzung für einen Austausch) sehr effizient kompensiert wird. Dieser Verlauf der Stammgefäße bleibt bis zum Ende der Trächtigkeit erhalten (Tsutsumi, 1962), verliert allerdings an Bedeutung, da sich die Austauscheinheit tertiärer Zotten und Krypten fortschreitend entwickelt und so die fetalen und maternalen Stamm- und Intermediärgefäße in den meisten Bereichen räumlich voneinander trennt. Im Gegensatz zu den Stammgefäßen treffen sich die Kapillaren der fetalen Zotten und

maternalen Krypten nach dem Querstromprinzip (Leiser et al., 1997b), welches als nicht so effizient angesehen wird (Faber und Thornburg, 1983). Trotzdem vergrößert sich durch die Kombination beider Systeme die Austauschoberfläche, einerseits durch progressives Kapillarwachstum (Baur, 1981) und andererseits durch die spezifische Gefäßarchitektur mit kurzer Zuleitungseinheit, welche eine Plazenta mit relativ hoher Austauschkapazität entstehen lassen. Dieses wird besonders deutlich, wenn man das Verhältnis der Masse von Fetus zu Plazenta betrachtet, welches beim Rind etwa 13:1 beträgt (Dantzer et al., 1988). Weiterhin muss natürlich auch die Anwesenheit verschiedener von der Morphologie unabhängiger, austauschfördernder Faktoren, wie VEGF und FGF (Norrby, 1997; Torry und Torry, 1997), für die hohe Effizienz der Rinderplazenta am Ende der Trächtigkeit in Betracht gezogen werden.

Späte Trächtigkeit

Auswahl des Materials

Um die Plazentome verschiedener Rinder vergleichbar zu machen, wurden diese nur aus der Nähe des Embryos aus dem mittleren Bereich des tragenden Uterushornes entnommen. In dieser Zone beginnt die Implantation und in der Folge zeigen die Plazentome den höchsten Entwicklungstand (Leiser, 1975), da sich die Blutgefäße vom Beginn der Plazentation ausgehend optimal entwickeln (Leiser et al., 1997a). Plazentome aus der Spitze des Uterushornes und aus dem nicht tragenden Horn (Leiser, 1975), wie auch akzessorische Plazentome, die im Allgemeinen kleiner als implantationsnahe waren (Andresen, 1927; Björkman, 1954), wurden nicht berücksichtigt.

Wachstum des Plazentoms und der fetalen Zottenbäume

Die Rinderplazenta wird vom 170. Tag der Trächtigkeit als voll entwickelt angesehen (Björkman, 1954) und nach dieser Zeit verlangsamt sich das Wachstum (Björkman, 1969). Die Plazentome vergrößern sich weiterhin, wobei das Wachstum hauptsächlich in deren Peripherie beobachtet wird, wodurch die Pilzform zustande kommt (Ebert, 1993; Leiser et al., 1997a). Die aktuelle Studie zeigt aber auch, dass neue Zottenbäume im Zentrum entstehen. Ein Wachstum der Plazentome über den 170. Tag hinaus erscheint sinnvoll, weil der Fetus gerade zu dieser Zeit exponentiell wächst und somit auch ein erhöhter Bedarf an Stoffaustausch besteht. Dieser Zusammenhang wird von der Sauerstoffkonzentration der Nabelvene (Reynolds et al., 1986) und der gleichzeitig zunehmenden Masse des Fetus reflektiert (Reynolds et al., 1990).

Die fetale Austauschoberfläche

Der transplazentäre Austausch ist morphologisch von den Ausmaßen der austauschaktiven Oberflächen im fetalen Zottenbaum abhängig (Björkman, 1968), die von der äußeren Oberfläche des Trophoblasten und den daruntergelegenen Oberflächen der Gefäßlumina repräsentiert werden. Somit kann die Effizienz des Stoffaustausches durch eine Vergrößerung dieser beiden Oberflächen pro Volumeneinheit des Plazentoms gesteigert werden (Faber und Thornburg, 1983). In der zweiten Hälfte der Gravidität vergrößert sich die Zottenoberfläche nicht nur durch das allgemeine Plazentomwachstum, sondern auch durch ein verändertes Arrangement der fetalen Zottenbäume, welches im Vergleich mit früheren Stadien deutlich wird (Leiser et al., 1997a) und sich besonders auf das Verzweigungsmuster der Stammgefäße bezieht. Die Zottenbäume werden schlank und konisch, mit relativ kurzen Ästen, die kaum eine Variation der Länge erkennen lassen und regelmäßig mit Terminalzotten besetzt sind. Auf diese Weise ist eine dichtere Anordnung dieser Bäume möglich als bei den breit-konischen der frühen Trächtigkeit (Leiser et al., 1997a), was durch eine größere Anzahl von Bäumen pro Einheit des Plazentoms dokumentiert wird. Weiterhin vertieft sich auch die feto-maternale Interdigitation und somit auch die Ausnutzung des gegebenen Raumes, da die Äste der Stammzotten im rechten Winkel abzweigen und so tote Winkel vermieden werden, in denen fetale und maternale Gefäße zu weit voneinander entfernt sind. Die Gefäßoberfläche lässt sich in die zuleitenden Arterien/Arteriolen und Venen/Venulen sowie die Kapillaren der Austauscheinheit einteilen (Leiser et al., 1997a), wo der Hauptanteil des transplazentären Stoffaustausches stattfindet (Benirschke und Kaufmann, 1995). Zur Optimierung des Austausches sollte der blutleitende Anteil so klein wie möglich sein, das heißt, die Stammgefäße müssen theoretisch gerade von der Chorionplatte bis an die Spitzen der Terminalzotten verlaufen. Diese Theorie wird durch die aktuellen Befunde bestätigt, die den geraden Verlauf einer einzelnen Stammarterie im Zentrum eines Zottenbaumes, umgeben von zwei bis drei Stammvenen, zeigen. Venen und Venulen folgen diesem Schema nicht so streng. Ein Grund hierfür könnte die extrem dünne Wand der venösen Gefäße sein, durch die, im Gegensatz zu den arteriellen Gefäßen, ein feto-maternaler Austausch auch möglich ist. Die Austauscheinheit, die aus einer Vielzahl von nur mit Endothelien begrenzten Kapillarschlingen besteht, stellt die am weitesten peripher liegende Einheit des Zottenbaumes dar. Diese wird nur vom Trophoblasten bedeckt und grenzt direkt an das maternale Epithel. In der zweiten Hälfte der Gravidität vergrößert sich die Kapillaroberfläche signifikant durch das Wachstum der Kapillaren, welches sich in einer

starken Windungsaktivität und Anastomosierung dieser Komplexe äußert (Leiser et al., 1997a).

Fetale Gefäßarchitektur

Das arterielle und venöse Gefäßsystem der fetalen Zotten der Rinderplazentome wird durch eine Vielzahl von Kapillarkomplexen von der Basis bis zur Zottenspitze verbunden. Jeweils zwei bis fünf dieser Komplexe sind zusätzlich in Serie geschaltet, so dass eine Gesamtlänge von bis zu 1000 µm erreicht wird (Leiser et al., 1997a). Diese Verbindungen gewinnen zum Ende der Trächtigkeit, wenn der Zottenbaum bis zu 4 cm lang geworden ist, zunehmend an Bedeutung (Ebert, 1993). Dabei stellt sich die Frage, wie die Blutzirkulation in einem derartig langen Kapillarsystem erhalten bleibt. Die sinusoidalen Erweiterungen in der Nähe der Zottenspitzen bieten eine physikalische Erklärung nach dem Gesetz von Hagen-Poiseuille, da der Gefäßwiderstand sinkt, wenn sich das Lumen erweitert und ein regelmäßiger Wechsel von eng- und weitlumigen Abschnitten zu wechselnden Flussgeschwindigkeiten führt, die ein Sistieren des Blutflusses verhindern. Ein gleicher Zusammenhang wird auch für die menschliche Plazenta beschrieben (Kaufmann et al., 1985; Leiser et al., 1991). Denn Anastomosen zwischen dem Kapillarbett und dem venösem System haben hier eine ähnliche blutzirkulationserhaltende Wirkung und optimieren zudem den gleichmäßigen Abfluss des venösen Blutes. Im Bereich der sinusoidalen Erweiterungen wird durch die Verlangsamung des Blutflusses und eine vergrößerte luminale Fläche die Austauschkapazität noch erhöht. Dieses beeinflusst den schnellen Gasaustausch durch Diffusion kaum (Faber und Thornburg, 1983), erleichtert aber die Bedingungen für den langsamen aktiven Transport von löslichen Stoffen (Alberts et al., 1983). Alle Gefäße des venösen Schenkels eines Zottenbaumes im Rinderplazentom sind häufiger vertreten als die des arteriellen Schenkels (Verhältnis Arterien zu Venen = 1 : 3) und besonders dünnwandig. Gleichartige Befunde für den Menschen lassen vermuten, dass in diesem Bereich ein aktiver Transport möglich ist (Arts, 1961). Der enge parallele Verlauf von fetalen und maternalen Stammgefäßen deutet darauf hin, dass in diesem Gegenstromsystem (Leiser et al., 1997a) eine Rückdiffusion von Substanzen, wie plazentären Hormonen (Reimers et al., 1985; Wooding, 1992), aus dem venösen in das arterielle System erfolgt. Ein solches Prinzip wird auch für die Plazenta des Schweines vorgeschlagen (Reynolds et al., 1985; Dantzer und Leiser, 1993).

Die an Gefäßausgüssen rasterelektronenmikroskopisch beschriebene Gefäßarchitektur und -morphologie lässt zwischen Rind, Schaf und Ziege keine messbaren Unterschiede im Verhältnis fetaler und maternaler Gefäße zueinander erkennen, welches in der zweiten

Trächtigkeitshälfte eine Kombination aus Gegenstrom- und Querstromprinzip darstellt (Leiser 1987, 1997a; Krebs et al., 1997). Allerdings wird beim Rind mit 13:1 ein günstigeres Gewichtsverhältnis des Fetus zur Plazenta beobachtet, als bei Schaf und Ziege (10:1) oder beim Menschen (6:1), welches die plazentäre Effizienz zu einem gewissen Grad erkennen lässt (Dantzer et al., 1988; Kaufmann, 1990; Leiser et al., 1997a). Das erfolgreichere Gewichtsverhältnis beim Rind muss also auf Unterschiede in der Plazentaphysiologie zurückzuführen sein, die in der Rinderplazenta einen höheren Austausch über feto-maternale Diffusion erlauben (Faber und Thornburg, 1983) als bei Schaf und Ziege. Diese Theorie wird durch Messungen der Differenz der Sauerstoffgehalte zwischen der Arteria uterina und der Vena umbilicalis bestätigt, die beim Rind höher ist als beim Schaf (Reynolds et al., 1986; Wilkening und Meschia, 1992). Mit 6:1 ist das Gewichtsverhältnis des Neonaten zur Plazenta beim Menschen ungünstiger als bei den Wiederkäuern, obwohl sich beide durch eine ähnliche fetale Blutgefäßarchitektur der Zotten auszeichnen. Ein Grund hierfür könnte in der Ausbildung des maternalen Gefäßsystems liegen, welches bei den Wiederkäuern einen geordneten Blutfluss innerhalb regulärer, endothelbegrenzter Gefäße besitzt, aber in der hämochorialen Plazenta des Menschen in Form eines offenen Blutpools (lakunären Blutsystems) vorliegt, in dem das Blut nicht so streng geordnet verläuft wie in echten Gefäßen (Leiser et al., 1997a).

Zusammenfassend lässt sich sagen, dass das Volumen des Rinderplazentoms in der zweiten Trächtigkeitshälfte nur wenig zunimmt (Reynolds et al., 1990; Reynolds und Redmer, 1995). Allerdings wird die Architektur des Zottenbaumes adaptiert, um den Austausch an die wachsenden Bedürfnisse des Fetus anzupassen. Diese Verbesserung erfolgt über zwei grundsätzliche Mechanismen: 1. die spezifische Veränderung des Verzweigungsmusters, 2. die weitere räumliche Annäherung der zwei absorptiven Oberflächen, Trophoblast und Endothelien der Zottenbäume, welche beide die einfache feto-maternale Diffusion wie auch aktive Transportmechanismen fördern (Faber und Thornburg, 1983; Leiser und Koob, 1992; Leiser und Kaufmann, 1994; Benirschke und Kaufmann, 1995). Allerdings steht eine nähere Untersuchung der aktiven plazentären Transportvorgänge beim Rind noch aus (Wooding 1992; Wooding und Flint, 1994), die das Verständnis der bovinen feto-maternalen Physiologie erweitern würde (Reynolds und Redmer, 1995).

5.2.2 Adaptation der Schafplazenta an hypobare Hypoxie

Nomenklatur der plazentomären Blutgefäße

Der Verlauf der großen Blutgefäße im Schafplazentom wurde schon früh durch Instillation von Mischungen aus Gelatine und Farbe oder Latex und Farbe und anschließende Verdauung mit Kalilauge dargestellt (Barcroft und Barron, 1946). Eine Weiterentwicklung mit Latex (Turox oder Neopren) oder Indischer Tinte erlaubte die Beschreibung der Primärstruktur der plazentomären Blutversorgung (Tsutsumi, 1962; Makowski, 1968). In der aktuellen Studie wurde das Gefäßsystem komplett mit einem flüssigen Plastik ausgegossen und so nicht nur die Primärstruktur sondern auch die Sekundär- und Tertiärstruktur (Kapillaren) des Schafplazentoms sichtbar gemacht. Nach der Einführung in die Plazentaforschung (Leiser und Kohler, 1983; Leiser, 1985) wurde diese Technik für Wiederkäuer (Ziege und Rind) angepasst (Leiser, 1987; Ebert, 1993), wobei sich ein fetaler Gefäßbaum entsprechend der menschlichen Plazenta zeigte (Kaufmann et al., 1985; Leiser et al., 1985, 1997a). Aus diesem Grund wird die Nomenklatur vom Menschen übernommen, die die Anteile des fetalen Zottenbaumes auf Grund der enthaltenen Gefäßen unterscheidet. Danach finden sich Stammarterie und -vene in der Stammzotte, Arteriolen und Venulen in den Intermediärzotten sowie Kapillaren in den Terminalzotten; entsprechend wird auch das maternale oder septale System benannt, welches beim Schaf und anderen Ruminanten geschlossen verläuft (Leiser et al., 1997a) und sich dadurch vom offenen Blutpool des Menschen unterscheidet.

Morphologische Grundlagen für die Beurteilung von Gefäßausgüssen

Das Schafplazentom ist "napfförmig" (Barcroft und Barron, 1946; Tsutsumi, 1962; Björkman, 1965; Leiser et al., 1997a) wie das der Ziege (Leiser, 1987), wohingegen beim Rind eine Pilzform vorliegt (Björkman, 1954; Ebert, 1993; Leiser et al., 1997a). Grundsätzlich passt sich die Form der Zottenbäume in ihrer Gesamtheit der äußeren Form der Plazentome an. Daher wird bei Schaf und Ziege ein zylindrisch-schlanker, "pappelähnlicher" Zottenbaum beobachtet (Leiser, 1987; Leiser et al., 1997a) während beim Rind eine konische, "tannenbaumähnliche" Form beschrieben wird (Ebert, 1993; Leiser et al., 1997a). In Plazentomen von Schaf und Ziege in der späten Trächtigkeit erlaubt die Form dieser Zottenbäume die Entwicklung von vier bis sechs Abzweigungsebenen von den Stammgefäßen innerhalb eines Baumes (Leiser et al., 1997a), dagegen werden acht bis zehn Abzweigungsebenen in einem bovinen Zottenbaum beobachtet (Ebert, 1993; Leiser et al.,

1997a). Außerdem liegen im Kapillarbett des Schafplazentoms in Serie geschaltete Kapillarschlingen, die den Blutfluss aufrecht erhalten, wie dies auch für die menschliche Plazenta beschrieben wird (Kaufmann et al., 1985). Bei Schaf und Ziege sind bis zu drei Kapillarkomplexe aneinander gereiht (Leiser, 1987), wogegen es beim Rind bis zu fünf sein können (Ebert, 1993; Leiser et al., 1997a). Obwohl die allgemeine Architektur der Kapillarendkomplexe, bestehend aus einer zuleitenden Arteriole in der Terminalzottenachse, umgeben mit einem dichten Netz von Kapillaren, bei Ziege und Rind gleich ist, lassen die geringere Zahl von präkapillären Verzweigungen und die kürzeren Serien von Schlingen eine relativ kleinere feto-maternale Austauschfläche vermuten. Der parallele Verlauf fetaler Venulen auf die Stammvenen zu wird auch bei Ziege und Rind beschrieben (Leiser, 1987; Ebert, 1993; Leiser et al., 1997a), wodurch eine funktionelle Rückdiffusion von in diesen Gefäßen enthaltenden Substanzen in die Arteriolen über ein Gegenstromsystem vermutet werden kann (Reynolds et al., 1985).

Histomorphometrischer Vergleich der Plazentomgefäße zwischen Schafhaltung auf Meereshöhe und im Hochgebirge

Es ist bekannt, dass Hypoxie über einen langen Zeitraum das Wachstum plazentärer Gefäße, besonders das der Kapillaren fördert (Bacon et al., 1984; Kaufmann et al., 1985; Jackson et al., 1987a, b; Geisen et al., 1990; Scheffen et al., 1990). Die histomorphometrischen Ergebnisse der aktuellen Studie zeigen, dass die fetalen Blutgefäße der Schafplazenta aus dem Hochgebirge (high altitude sheep, HA) sich an die hypobarische Hypoxie durch eine Vegrößerung anpassen. Das heißt, die Anzahl von Gefäßanschnitten im histologischen Schnitt ist zwar reduziert, aber dafür nimmt die Fläche der Gefäßlumina signifikant zu. Im Vergleich zu den Plazentomen von auf Meereshöhe gehaltenen Schafen (sea level sheep, SL) ist das ein leichter (7,5 %) Anstieg der Gefäßlumina im gesamten Schnitt. Diese Veränderung konnte durch die rasterelektronenmikroskopische Untersuchung nicht quantifiziert werden, deutete sich aber durch ein starkes Windungs- und Verzweigungsmuster der Kapillaren von HA Tieren an. Diese Tendenz spiegelt sich auch in den Befunden von Reshetnikova et al. (1994) und Burton et al. (1996) am menschlichen Gefäßsystem der Plazenta in großer Höhe wider, da das Volumen der fetalen Gefäße dort insgesamt erhöht ist und erweiterte Sinusoide in den Kapillaren auftreten. Das heißt, die Ausweitung der fetalen Gefäßoberfläche trägt bei Mensch und Schaf zur Verbesserung der fetalen Versorgung unter Sauerstoffmangelverhältnissen bei. Nach dem Gesetz von Hagen-Poiseuille wird die Flussgeschwindigkeit des Blutes durch Sinusoide zwar lokal verlangsamt

und so die zur Verfügung stehende Austauschzeit erhöht, grundsätzlich wird aber ein Stocken des Blutstromes auf Grund der überlangen Kapillarstrecke verhindert (Kaufmann et al., 1985). Im Gegensatz dazu stehen die Befunde von Jackson et al. (1987a, b), die in der Plazenta des Menschen im Hochgebirge ein verringertes Volumen fetaler Zotten und Gefäße bei gleichzeitig vergrößertem maternalen Blut- beziehungsweise Zwischenzottenraum feststellen. Die beobachtete Diskrepanz beruht vermutlich auf einer Unterbewertung des fetalen Gewebes, da die Plazenten nicht perfusionsfixiert wurden und so nach der Ausstoßung kollabierten. Die Vergrößerung des maternalen Blutraumes wird als Kompensation des Minderwachstums der fetalen Zotten in großer Höhe interpretiert (Mayhew et al., 1990). Auch bei Meerschweinchen, die in Versuchskammern mit Sauerstoff unterversorgt wurden, zeigt sich eine erhöhte Zahl von fetalen Kapillaranschnitten der Plazenta, allerdings bei gleichzeitig reduziertem Kapillardurchmesser, was zu der Vermutung führte, dass die fetalen Kapillaren unter hypoxischen Bedingungen länger wachsen (Bacon et al., 1984). Diese Theorie wird durch die Untersuchung von Scheffen et al. (1990) an Gefäßausgüssen der Plazenta von unter ähnlichen Bedingungen gehaltenen Meerschweinchen bestätigt, die eindeutig zeigte, dass die fetalen Kapillaren mehr gewunden und verzweigt sind. Bei der Interpretation der Befunde in der vorliegenden Studie, also an plazentären Gefäßen aus großer Höhe, können nur eingeschränkt identische Ergebnisse erwartet werden, da die Schafplazenta epitheliochorial ist und jeweils ein geschlossenes fetales und maternales Gefäßsystem besitzt und sich damit deutlich von den hämochorialen Plazenten von Mensch und Meerschweinchen unterscheidet. Das heißt, beim Schaf verläuft auch das maternale Blut der Krypten in regulären Blutgefäßen, die komplementär zu den fetalen Gefäßen in den Zotten verlaufen. Dagegen fließt das maternale Blut der hämochorialen Plazenten von Mensch und Meerschweinchen frei durch die labyrinthartigen Lakunen zwischen den fetalen Zotten, und obwohl eine Art Gegenstromprinzip realisiert wird, ist der Blutfluss nicht so gleichförmig geführt wie in einem geschlossenen System. Aus diesem Grund können beim Schaf beide, das fetale und maternale Gefäßsystem, aktiv im Sinne der Angiogenese und Gefäßarchitektur in ihrer Funktion beeinflusst werden, um eine Adaptation an Hochgebirgsbedingungen zu erreichen. Dass diese Anpassung in der Tat stattfindet, zeigt sich bei der Betrachtung der maternalen Gefäße in HA-Plazentomen, die sogar stärkeren Veränderungen ausgesetzt sind als die fetalen Gefäße (HA versus SL maternale Gefäße: +3,4 %; HA versus SL fetale Gefäße: +0,4 %). Die großen Veränderungen in der Kapazität der maternalen Plazentagefäße beim Schaf könnten auch erklären, warum die Adaptation des fetalen Gefäßsystems weniger deutlich ausfällt als beim Menschen und dem Meerschweinchen. Die Adaptationen der

maternalen Plazentagefäße an große Höhe sind durch ein generell vergrößertes Gefäßsystem gekennzeichnet, wobei die Zahl der Gefäßanschnitte gleich blieb, dafür aber die Größe und der prozentuale Anteil der Gefäßlumina in HA Schafen signifikant höher war als in SL Tieren. Ein weiterer Grund für die starken Veränderungen der maternalen Gefäße könnte auch in der Tatsache begründet sein, dass der Aufbau der Septengefäße durch die schon im Uterus vorhandenen Karunkeln erleichtert wird, da diese natürlich schon bei Beginn der Gravidität ein Gefäßsystem mit sofortiger reaktiver Fähigkeit zu gesteigerter Angiogenese in Folge eines verminderten Sauerstoffpartialdruckes im maternalen Blut besitzen (Andresen, 1927; Boshier, 1969; Kaufmann et al., 1993). Die variablen histomorphometrischen Ergebnisse verschiedener Gebiete im Plazentom (Chorionplatte, Intermediärzone, Basalplatte) deuten auch darauf hin, dass bestimmte Zonen bevorzugt perfundiert werden, was aber die Existenz von Shunts voraussetzt, die die Blutversorgung innerhalb der einzelnen Plazentome regulieren (Steven, 1966; Wilkening und Meschia, 1992).

Erstaunlicherweise ist die gemessene Strecke zwischen fetalen und maternalen Kapillaren in der aktuellen Studie bei HA Tieren 9,7 % größer als bei SL Schafen. Diese Veränderung ist nicht signifikant, was an der kleinen Tierzahl liegen kann, unterscheidet sich aber deutlich von den Befunden an der menschlichen Plazenta im Hochgebirge, bei denen eine reduzierte interhämale Distanz beobachtet wird (Jackson et al., 1988a, b; Reshetnikova et al., 1994). Gründe für die Reduktion in der Menschenplazenta liegen in einer Verdünnung des Trophoblasten, dem Phänomen der Peripherisierung der fetalen Kapillaren an den Zottenspitzen und in einer Vergrößerung des Kapillardurchmessers (Burton et al., 1996). Die Verkürzung des feto-maternalen Gefäßabstands beim Menschen wird von Jackson et al. (1987a, b) in Verbindung mit einer Reduzierung der Zottenoberfläche beobachtet, und demnach als Kompensationsmechanismus hierfür interpretiert (Mayhew et al., 1990).

In den HA Schafen könnte die relativ weite interhämale Strecke im Zusammenhang mit einer verbesserten Stabilität der Gefäßwände stehen, die auf Grund der sinusoidalen Ausweitung fetaler und maternaler Gefäße erforderlich ist. Obwohl das klassische Schema nach Grosser (1909, 1927) davon ausgeht, dass eine Verkürzung der Strecke zwischen fetalen und maternalen Gefäßen Austauschvorgänge unterstützt, weisen physiologische Untersuchungen darauf hin, dass diese Sichtweise nicht immer korrekt ist (Faber und Thornburg, 1983; Longo, 1987; Leiser und Kaufmann, 1994). Offensichtlich sind in der Schafplazenta bestimmte Transportmechanismen distanzunabhängig, wie aus Studien zum Sauerstofftransfer bekannt ist (Longo et al., 1967, 1972; Longo und Ching, 1977; Longo

1987; Wilkening und Meschia, 1992). Auch das geometrische Arrangement der fetalen und maternalen Kapillaren im Austauschgebiet lässt bei Schafen, was die Diffusion von Substanzen betrifft, auf eine relativ effiziente Plazenta schließen (Faber und Thornburg, 1983; Leiser und Kaufmann, 1994). Frühe morphologische Studien haben in der Schafplazenta ein Gegen- (Barcroft und Barron, 1946; Tsutsumi, 1962) oder Querstromprinzip (Steven, 1966, 1975; Makowski, 1968) vermutet. In den SL Schafen und der Ziege (Leiser, 1987) scheint eine Mischung aus beiden Systemen vorzuliegen (Leiser et al., 1997a). Allerdings lässt sich diese Beobachtung auf Grund der erschwerten morphologischen Erfassbarkeit der starken Windungs- und Verzweigungsmuster in den HA Tieren nicht eindeutig bestätigen. Trotzdem sind die an SL Schafen erhobenen Befunde im Einklang mit der Hypothese, dass der Blutfluss keinen großen Einfluss auf die Effektivität hat (Wilkening und Meschia, 1992), da nur der Konzentrationsunterschied zwischen Arteria uterina und Vena umbilicalis entscheidend für die Sauerstoffaustauschkapazität ist (Longo, 1987).

Zusammenfassend lässt sich sagen, dass der Grad der Verzweigungen und Windungen der fetalen Blutgefäße in der Plazenta von Schafen aus großer Höhe erhöht ist. Dieses wird durch histologische Befunde bestätigt, die einen schwach erhöhten prozentualen Anteil fetaler Gefäße pro Gesichtsfeld und eine deutliche Erhöhung des prozentualen Anteils maternaler Gefäße zeigen. Diese Ergebnisse lassen eindeutig erkennen, dass Änderungen in der Morphologie und Architektur des Gefäßsystems in der Schafplazenta und die damit erzielte Vergrößerung der feto-maternalen Austauschfläche ausreichend sind, um die Minderversorgung mit Sauerstoff unter hypobarischer Hypoxie auszugleichen.

5.2.3 Fibroblastenwachstumsfaktoren (FGFs) in der Rinderplazenta

Es handelt sich hier um die ersten Ergebnisse zur räumlichen und zeitlichen Verteilung von mRNA und Protein einiger Mitglieder der Familie der FGFs in der synepitheliochorialen Rinderplazenta.

Im gesamten Verlauf der Trächtigkeit zeigen alle untersuchten FGFs im Rinderplazentom in der Immunhistochemie (IHC) und der in situ Hybridisierung (ISH) ähnliche Verteilungsmuster. In unreifen TGC und Zellen des Gefäßsystems wird die höchste Expression beobachtet. Diese Befunde deuten auf eine wichtige Rolle des FGF Systems bei der Regulation der Differenzierung der Trophoblastzellen, der Angiogenese sowie weiterer gefäßassoziierter Funktionen hin und lassen vermuten, dass teilweise von verschiedenen

FGFs gleiche Aufgaben erfüllt werden. Die Tatsache, dass im bovinen Plazentom FGFs und die entsprechenden Rezeptoren in denselben Zelltypen lokalisiert sind, spricht für eine Vermittlung der biologischen Effekte über autokrine Mechanismen, die unter der Kontrolle von übergeordneten Hormonen, wie Progesteron und plazentärem Östrogen, stehen (Rider und Psychoyos, 1994; Siegfried et al., 1995; Ka et al., 2001).

Bedeutung der FGFs für die plazentomäre Angiogenese

Da FGF1 und FGF2 ausgeprägt im Gefäßsystem des Rinderplazentoms exprimiert werden, könnten beide Faktoren an der Regulation vaskulärer Funktionen, wie der Angiogenese und der Gefäßpermeabilität, beteiligt sein. Dieses wird für Zell-Zell-Verbindungen zwischen menschlichen Endothelzellen aus plazentären Blutgefäßen demonstriert, da nur die Proliferation und Durchlässigkeit für Makromoleküle von Endothelien aus Mikrogefäßen, nicht aber aus großen Gefäßen, durch FGF1 über eine Aktivierung von Tyrosinkinasen stimuliert wird (Dye et al., 2001). Auch FGF2, welches von menschlichen Trophoblastzellen aus der frühen Schwangerschaft freigesetzt wurde, zeigt einen angiogenesestimulierenden Effekt auf vaskuläre Endothelzelllinien (Hamai et al., 1998). In vitro stimuliert FGF2 die Proliferation von Endothelzellen aus der menschlichen Nabelvene, und induziert gleichzeitig die Migration von Gefäßendothelzellen (Yoshida et al., 1996) sowie glatten Muskelzellen der Gefäße durch eine Veränderung der Interaktion von Integrinen mit der extrazellulären Matrix (Pickering et al., 1997). Auch in Plazentomen des Schafes wurde FGF2 als Angiogenesefaktor für Gefäßendothelien identifiziert (Zheng et al., 1997). Die Stimulation der Proliferation von Gefäßendothelien in der Zellkultur durch FGF2 erfolgt durch die Aufregulation der VEGF Expression (Seghezzi et al., 1998). Weiterhin konnte in vitro gezeigt werden, dass FGF2 über den MAPK-Signalweg die endotheliale Stickoxidsynthase (eNOS) in Endothelzellen aus kotyledonären Arterien des Schafs signifikant erhöht (Zheng et al., 1999). Auf diese Weise wird eine Erweiterung der Gefäße induziert, die eine Verlangsamung des Blutstromes im fetalen und/oder maternalen Kompartiment der Plazenta bewirkt und demnach die für den feto-maternalen Austausch zur Verfügung stehende Zeit verlängert und so die Austauschkapazität erhöht. In vitro konnte auch FGF7 das Wachstum von glatten Muskelzellen der Gefäße stimulieren (Onda et al., 2003), was die Angiogenese in den Septenspitzen des bovinen Plazentoms anregen könnte, da in dieser Lokalisation große Mengen spezifischer FGF7 mRNA in der ISH beobachtet werden.

Zelluläre Lokalisation der FGF Isoformen

FGF2 wurde immunhistochemisch parallel mit zwei Antikörpern dargestellt, erstens mit einem spezifischen monoklonalen Antikörper (bFM-2) und zweitens mit einem polyklonalen anti-Rind FGF2 Antiserum (rαbFGF2). Mit beiden Methoden werden in Plazentomen des Rindes nahezu identische Färbemuster erzielt, mit Ausnahme der Färbung in reifen TGC, in denen mit bFM-2 kein Signal erzeugt werden kann, während rαbFGF2 eine deutliche Kernfärbung in reifen TGC produziert. Diese Differenz im Färbemuster lässt sich durch die unterschiedliche Erkennung der verschiedenen FGF2 Isoformen erklären. FGF1, -2 und FGFR werden nicht nur im Zytoplasma sondern auch in den Kernen fetaler und maternaler Zellen im Rinderplazentom beobachtet. Diese nukleäre Expression von Liganden und Rezeptoren eines Peptidwachstumsfaktorsystems lässt sich wie folgt erklären: Das Auftreten von FGF2 im Kern kann durch die Aufnahme niedermolekularer Isoformen während eines autokrinen Mechanismus sowie durch eine direkte nukleäre Translokation von hochmolekularen Isoformen bedingt sein und erfüllt offensichtlich intrakrine Funktionen. Die spezifische Lokalisation von FGFs in Kern oder Zytoplasma einer Zelle wird mit verschiedenen biologischen Aufgaben in Verbindung gebracht (Delrieu, 2000). Beispielsweise wird in mit FGF2 behandelten Fibroblasten (3T3-Zellen) ein zeit- und dosisabhängiger Anstieg einer FGFR1 Immunreaktion im Kern beobachtet, die eine Beteiligung von FGFR1 an der Regulation von Genaktivität vermuten lässt (Maher, 1996).

Differenzierung und Migration von Trophoblastzellen

Unreife TGC, die naturgemäß auch den geringsten Differenzierungsgrad aufweisen, zeigen die höchste Signalintensität für alle FGFs und FGFRs im Rinderplazentom. Diese Koexpression lässt vermuten, dass das FGF System an der auto- und parakrinen Kontrolle der Differenzierung boviner TGC beteiligt ist und so auch den gleichzeitig ablaufenden Migrationsprozess beeinflusst. In Trophoblastzellen der Maus wird die in vitro Migration des Trophoblasten durch den Zusatz von FGF2 in das Kulturmedium stimuliert, wie signifikant erhöhte Raten von Trophoblastausbreitung und -wachstum belegen (Taniguchi et al., 1998). Weiterhin induziert FGF2 die Migration von menschlichen Gefäßendothelien und glatten Muskelzellen (Yoshida et al., 1996; Pickering et al., 1997). In Herzmuskelzellen der Ratte erhöhen hochmolekulare Isoformen von FGF2 die Zweikernigkeit (Pasumarthi et al., 1996), ein charakteristischer Prozess, der auch während der Differenzierung der TGC im Rinderplazentom beobachtet wird (Klisch et al., 1999a). Auch die Produktion der hormonellen Produkte der TGC, wie plazentäres Laktogen, könnte durch hochmolekulare

Isoformen von FGF2 beeinflusst werden, da in vitro Experimente zeigen, dass der endokrine Phänotyp von PC12 Zellen durch FGF2 stabilisiert werden kann (Grothe et al., 1998).

FGF7 System

Im Allgemeinen wird FGF7 (auch keratinocyte growth factor, KGF) als ein Wachstumsfaktor angesehen, der in mesenchymalen Zellen gebildet wird, aber das Wachstum benachbarter Epithelzellen fördert (Powers et al., 2000). In bovinen Plazentomen werden mittels ISH geringe Mengen spezifischer mRNA von FGF7 und seinem spezifischen Rezeptor (FGFR2IIIb) in allen Karunkelepithelzellen detektiert, mit der Ausnahme der vergrößerten Spitzen der maternalen Septen, wo das Karunkelepithel mit den direkt darunter liegenden Stromazellen eine starke Hybridisierung zeigen. Die erhöhte Expression von FGF7 in den Spitzen der maternalen Septen korreliert mit der Ansicht, dass diese die Wachstumszonen des Plazentoms darstellen (Pfarrer et al., 2003) und wird durch das Auftreten einer erhöhten proliferativen Aktivität unterstützt (Schuler et al., 2000). Große Mengen von FGF7 und seinem Rezeptor werden außerdem in unreifen TGC beobachtet. Daraus lässt sich schließen, dass bovine TGC als Intermediärtypen zwischen echten polarisierten Epithelzellen und nicht polarisierten mesenchymalen Zellen beide Systeme exprimieren, epitheliale und mesenchymale FGF:FGFR Paare.

Veränderungen im FGF System kurz vor der Geburt

Die ISH zeigt grundlegende präpartale Veränderungen aller untersuchten Anteile des FGF Systems im Rinderplazentom, da eine Verschiebung des mRNA Signals von den unreifen TGC hin in das Stroma der maternalen Septen auftritt. Das heißt, die Zahl der positiven unreifen TGC ist stark reduziert und karunkuläre Stromazellen, insbesondere die der Gefäße, zeigen eine deutliche Hybridisierung für alle FGF(R). Der signifikante Abfall der FGF(R) positiven TGC etwa 24 Stunden vor dem Beginn der Wehentätigkeit lässt vermuten, dass der Grund für die stark verminderte Zahl von TGC kurz vor der Geburt eher ein Stopp der TGC Differenzierung als ein erhöhter Zell Turn-over oder die Zerstörung von reifen TGC ist, und dass die FGFs und ihre Rezeptoren wirklich an der Regulierung der Trophoblastzelldifferenzierung beteiligt sind. Die beobachtete Aufregulierung der FGF(R) Expression in den Stromazellen der bovinen Karunkel ist konsistent mit der Verbreiterung der maternalen Septenspitzen in der späten Trächtigkeit und der parallel dazu festgestellten erhöhten proliferativen Aktivität in dieser Lokalisation (Schuler et al., 2000). Weiterhin könnte der präpartale Anstieg von FGF(R) im karunkulären Stroma die Regression des Uterus

postpartum unterstützen, die mit umfangreichen Gewebeumbildungen und Angiogenese einhergeht (Reynolds und Redmer, 1995).

Schlussfolgernd lässt sich sagen, dass das breitgefächerte Auftreten von Mitgliedern des FGF Systems und die charakteristischen präpartalen Veränderungen für eine wichtige Rolle des FGF Systems bei der lokalen Regulation des Wachstums der bovinen Plazenta, sowie deren Differenzierung und Funktion sprechen.

6. Zusammenfassung

Die vorliegende Arbeit charakterisiert die der Plazentation der Wiederkäuer, insbesondere Rind und Schaf, zu Grunde liegenden Mechanismen unter besonderer Berücksichtigung der Migration der Trophoblastriesenzellen (TGC) und der plazentären Angiogenese.

Im Plazentom des Rindes wird das zelluläre Zytoskelett und ein Schlüsselenzym der intrazellulären Phosphorylierungskaskade (mitogen-activated protein kinase, MAPK) mit immunelektronenmikroskopischen Methoden analysiert und über einen mRNA Nachweis bestätigt. Weiterhin wird im bovinen Plazentom die Expression von Integrinen und extrazellulärer Matrix (ECM) immunhistochemisch sowie von Connexinen auf Protein- und mRNA-Ebene untersucht. Beim Schaf werden Integrine und ausgewählte Proteine der ECM während der Implantation in vivo und in vitro evaluiert sowie Osteopontin, als Produkt der uterinen Drüsen, im Verlauf der Trächtigkeit mit Immunfluoreszenz und molekularbiologischen Methoden untersucht. Connexine werden beim Schaf im Zyklus und während der Gravidität mittels Immunfluoreszenz dargestellt.

Die Entwicklung des Gefäßsystems, als formgebende Determinante der feto-maternalen Interdigitation, wird während der Plazentation des Rindes rasterelektronenmikroskopisch mit Hilfe von Gefäßkorrosionspräparaten untersucht. Der Einfluss von Hypoxie als Angiogenesefaktor wird in Gefäßausgüssen der reifen Schafplazenta rasterelektronenmikroskopisch ermittelt und mittels Morphometrie an histologischen Schnitten bestätigt. Weiterhin wird mit verschiedenen Mitgliedern der Fibroblastenwachstumsfaktoren (FGFs) ein wichtiges Regulationssystem, welches in die Angiogenese sowie die Zelldifferenzierung eingreift, in der Rinderplazenta auf Protein- und mRNA-Ebene untersucht.

Aktin, α-Aktinin, Vinculin und die MAPK zeigen eine zellspezifische Lokalisation im Rinderplazentom, die für eine Beteiligung an der eingeschränkten Trophoblastinvasion spricht. Die konstitutive Expression von Integrinen und die spezifische Modulation von Proteinen der ECM während der Implantation des Schafes sowie die in vitro Akkumulation von mit dem Zytoskelett assoziierten Proteinen als Reaktion auf Osteopontinbindung, deuten auf eine Funktion von Osteopontin im Verlauf der Implanationskaskade sowie während der Gravidität des Schafes hin. Im Plazentom des Rindes zeichnen sich die TGC durch eine besondere Ausstattung an Integrinrezeptoren und ECM aus, die mit der von Tumorzellen vergleichbar ist, und daher möglicherweise bei der Migration der TGC eine Rolle spielt. Beim

Schaf spricht die spezifische Modulation der Connexinexpression als Antwort auf eine Trächtigkeit und um die Geburt für einen invasiveren Status, als bei nicht invasiven epitheliochorialen Plazentationstypen. Dagegen zeichnen sich die TGC im Rinderplazentom durch die gleichzeitige Expression von "epithelialen" und "mesenchymalen" Connexinen aus, was auch beim Rind einen Status zwischen invasiver und nicht invasiver Plazentation vermuten lässt.

Die Analyse des Gefäßsystems der Rinderplazenta zeigt im sterischen Zusammentreffen der fetalen Zottenbäume und maternalen Krypten die Etablierung einer effektiven feto-maternalen Austauscheinheit. Zu Beginn der Trächtigkeit treffen sich fetale und maternale Gefäße nach dem Gegenstromprinzip, wohingegen sich mit fortschreitender Entwicklung der Kapillaren, das heißt ab der Mitte der Gravidität, ein Querstrommuster etabliert. Der Vergleich des Gefäßsystems geburtsnaher Plazentome von auf Meereshöhe und im Hochgebirge gehaltenen Schafen demonstriert die Adaptation an hypobare Hypoxie über eine Vergrößerung der fetalen und maternalen Austauschfläche über Angiogenese.

Die spezifische Lokalisation von FGFs nicht nur in gefäßassoziierten Zellen sondern auch in unreifen TGC im Rinderplazentom deutet auf Funktionen im Rahmen der plazentären Angiogenese sowie für die Differenzierung und Migration der TGC. Der präpartale Wechsel der Expression aus dem Epithel in das maternale Stroma des Plazentomes deutet auf eine Umstellung auf die bevorstehende Regression des Uterus nach der Geburt hin. Der schon bei der Connexinexpression beobachtete Intermediärstatus der bovinen TGC bestätigt sich auch in der parallelen Ausbildung von mesenchymalem FGF1 und FGF2 sowie epithelialem FGF7. Die Expression von FGFs und FGF Rezeptoren im Zytoplasma sowie in Kernen plazentärer Zellen des Rindes lässt autokrine und/oder intrakrine Regulationsmechanismen vermuten.

Zusammengenommen unterstützen die Ergebnisse der vorgelegten Schrift die Hypothese, dass die eingeschränkte Trophoblastinvasion in der Plazenta des Rindes auch als Modell für andere invasive Prozesse gelten kann. Allerdings sind weitere Analysen, besonders die Etablierung eines in vitro Zellkultursystems für eine abschliessende Beurteilung sowie die anschliessende Anwendung dieses überschaubaren Invasionsmodells unabdingbar.

7. Summary

The basic mechanisms of ruminant placentation are characterized with special reference to trophoblast giant cell (TGC) migration and placental angiogenesis.

In bovine placentomes the actin cytoskeleton and its associated proteins as well as one key-enzyme of intracellular phosphorylation cascades (mitogen-activated protein kinase, MAPK) are evaluated by immuno transmission electron microscopy and RT-PCR. The expression of integrins and proteins of the extracellular matrix (ECM) in placentomes of the cow are examined by immunohistochemistry. Furthermore, protein and mRNA of gap junctional connexins are shown by immunohistochemistry and RT-PCR. During sheep implantation integrins and selected components of the ECM are evaluated in vivo and in vitro, and osteopontin, a uterine gland product is studied in the course of gestation by immunofluorescence and molecular biological methods. Connexins are detected by immunofluorescence in cyclic and pregnant sheep.

The development of the bovine placentomal vasculature as shaping power of the feto-maternal interdigitating structures is studied by scanning electron microscopy of vascular corrosion casts throughout gestation. The angiogenic properties of hypoxia in near-term sheep placentomes are tested by scanning electron microscopy of vascular casts and morphometry of histological sections. Since fibroblast growth factors (FGFs) are involved in both, angiogenesis and differentiation of cells, various members of the FGF system are studied in placentomes of cattle on protein and mRNA level.

In bovine placentomes actin, α-actinin, vinculin, and MAPK show a cell-specific localization indicating a role during restricted trophoblast invasion. The constitutive expression of integrins and the specific modulation of ECM proteins as well as the in vitro accumulation of cytoskeleton-associated proteins following adhesion of osteopontin suggest a functional role for osteopontin during implantation and placentation of sheep. Since bovine placentomal TGC are characterized by a specific expression of integrin receptors and ECM components, which is similar to the one of tumor cells, we may hypothesize that these proteins are relevant for TGC migration. The specific modulation of sheep connexins in response to pregnancy and around parturition indicates that the trophoblast in sheep is more invasive than in non-invasive epitheliochorial placental types. Bovine placentomal TGC coexpress epithelial and mesenchymal connexins, a fact supporting the idea that cows are representing an intermediate status between invasive and non-invasive placentation.

The bovine placental vasculature is characterized by the establishment of an efficient feto-maternal exchange unit, embedded in fetal villous trees and maternal crypts. In early pregnancy, fetal and maternal vessels meet each other in a countercurrent fashion, whilst in the second half of gestation, a crosscurrent system develops, due to the progressing growth of capillaries. The adaptation of fetal and maternal placental blood vessels to hypobaric hypoxia by angiogenesis resulting in an increased exchange surface is shown in near term sheep placentomes.

In bovine placentomes, the specific localization of FGFs in cells of the vasculature and immature TGC suggests their involvement in placental angiogenesis and TGC differentiation and migration. The prepartal switch from FGF expression in the epithelia to maternal stroma indicates programming for the regression of the uterus after parturition. The intermediate status of bovine TGC, which is also proposed due to connexin expression, is corroborated by the parallel occurrence of mesenchymal FGF1 and FGF2 and epithelial FGF7. The presence of FGFs and their receptors not only the cytoplasm but also in nuclei of bovine placental cells suggests auto- and/or intracrine functions.

Taken together the presented results support the hypothesis that the restricted trophoblast invasion in the bovine placenta may serve as a model for other invasive processes. However, further studies, including the establishment of an in vitro cell culture system, are needed for a final judgement and the subsequent application of this model.

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9. Vorgelegte Veröffentlichungen

zu: Charakterisierung von Signalwegen in der Wiederkäuerplazenta

1. Lang C, Hallack S, Leiser R, **Pfarrer C** (2004) Cytoskeletal filaments and associated proteins during restricted trophoblast invasion in bovine placentomes: light- and transmission electron microscopy and RT-PCR. Cell Tissue Res 315, 339-348. Online Publikation am 15.01.2004 (doi: 10.1007/s00441-003-0842-x).

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REGULAR ARTICLE

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Cytoskeletal filaments and associated proteins during restricted trophoblast invasion in bovine placentomes: light and transmission electron microscopy and RT-PCR

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Abstract Trophoblast cell migration is unusual in epitheliochorial placentae but occurs in placentomes of cows as "restricted" trophoblast invasion of binucleated trophoblast giant cells (TGC). Migration may be induced by integrin binding to the extracellular matrix initiating two pathways: (1) conformational changes of the actin cytoskeleton induced by an accumulation of its associated proteins and (2) integrin-dependent phosphorylation of various protein kinases. In cow placentomes, actin, its associated proteins (α-actinin, vinculin) and a key protein kinase of the signal transduction cascade (phosphorylated mitogen-activated protein kinase, pMAPK) were localized by immunogold-silver enhancement and immunoperoxidase staining at the light- and transmission electron-microscopical levels. Findings were confirmed by amplification of specific mRNA transcripts by reverse transcriptase/polymerase chain reaction. Actin and α-actinin were co-localized apically in mononuclear trophoblast cells, along the cytoplasmic membrane of TGC and apically in maternal crypt cells. The actin and α-actinin immunoreaction occurred as a band of electron-dense particles beneath the cytoplasmic membrane. Vinculin labelling was membrane-associated in TGC and in fetal and maternal endothelial cells. MAPK was observed as nuclear clusters in both kinds of trophoblast cells and was less dense in single uterine epithelial cells. Most MAPK immunoreactivity was detected in the nuclei of the trophoblast epithelium but was also sometimes membrane-associated in the cytoplasm. Thus, actin, α-actinin, MAPK and vinculin may be involved in the regulation of TGC migration. "Restricted" trophoblast invasion could serve as a model for invasive processes.

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Introduction

Epitheliochorial bovine placentomes are composed of fetal cotyledons and maternal caruncles (for a review, see Leiser and Kaufmann 1994). Trophoblast giant cells (TGC) within the fetal chorionic epithelium originate from mononuclear trophoblast cells by acytokinetic mitoses (Wooding 1992; Klisch et al. 1999). TGC are present from about day 16 of gestation (Greenstein et al. 1958; Leiser 1975) until term, migrate towards the uterine epithelium (Wimsatt 1951; Davies and Wimsatt 1966) and finally fuse with single uterine epithelial cells, forming trinucleated feto-maternal hybrid cells during the whole gestational period (Wooding 1992). As a result of this fusion, hormonal products of TGC, placental lactogen and pregnancy-specific (glyco)proteins can be delivered to the maternal compartment (Wooding 1992; Klisch et al. 1999). The migration of TGC through chorionic tight junctions (Wooding 1992) leads to apical fusion with the uterine epithelium via the formation of pseudopodia (Wathes and Wooding 1980) and ends with the degeneration of the hybrid cells (Wooding 1992; Hoffman and Wooding 1993) and/or their resorption by the trophoblast (Schuler 2000). This migration (invasive phenomenon) may be interpreted as "restricted" trophoblast invasion (Pfarrer et al. 2003).

Trophoblast invasion during haemochorial placentation of humans and rodents and invasive processes during tumour growth are characterized by the expression of specific integrin receptors binding to proteins of the extracellular matrix (Damsky et al. 1994, Lohi et al. 2000). These heterodimeric transmembrane glycoprotein receptors consist of an α- and β-subunit (Hynes 1987) and are intimately associated with the cytoskeleton and signalling proteins (Giancotti and Ruoslahti 1999) partic-

ipating in bidirectional signalling involving both "outside-in" and "inside-out" pathways (Hynes 1987).

In the bovine placenta, the integrin β_1 subunit occurs basolaterally on fetal mononuclear trophoblast cells and TGC at day 24 post-insemination, a finding that has been interpreted by MacLaren and Wildemann (1995) as being the functional relevance of β_1 integrin in trophoblast migration and cell development. Pfarrer et al. (2003) have suggested that TGC migrate along their own laminin matrix and maintain cell-cell contacts with neighbouring cells via $\alpha_2\beta_1$ integrin.

Within the repertoire of signalling molecules exists a family of protein kinases leading to phosphorylation cascades. These are known as mitogen-activated protein kinase (MAPK) modules (for a review, see Pearson et al. 2001). MAPK are major components of pathways controlling embryogenesis, cell differentiation, cell proliferation and cell death (for a review, see Pearson et al. 2001) and are also regulators of cell growth and gene activation (Miyamoto et al. 1996; Chen et al. 1994a).

Because of the importance of the cytoskeleton in cell-contact-dependent signal transduction pathways and the involved cytoskeletal changes, we investigated the localization of actin and its associated proteins α-actinin and vinculin in the epitheliochorial bovine placenta with special reference to TGC migration. To provide evidence of whether a signal cascade via integrin-mediated phosphorylation of protein kinases is possible in bovine placentomes, we decided to detect the possible presence of phosphorylated MAPK.

Materials and methods

Collection and preparation of placentomal samples

Placentomes of 12 pregnant cows, including three cows for the isolation of mRNA for reverse transcription/polymerase chain reaction (RT-PCR) from around days 60, 210 and 280 of gestation, were taken directly after evisceration during the slaughtering process. Gestational age was assessed according to fetal crown-rump length (Schnorr and Kressin 2001).

Three placentomes per animal were immediately shock frozen in liquid N_2 for immunogold labelling at the light-microscopical level and stored at −80°C. Three other placentomes were cut into pieces of about 0.5×1×0.3 cm, which were immersed in fixatives containing (1) 3% buffered paraformaldehyde (pH 7.4) and (2) 3% buffered paraformaldehyde (pH 7.4) plus 0.1% glutaraldehyde, for 2 h at room temperature, for transmission electron-microscopical investigations. Subsequently, the samples were rinsed (3×45 min) in phosphate-buffered saline (PBS; pH 7.3), dehydrated in a graded series of ethanol at room temperature, finally infiltrated with Epon resin (for pre-embedding immunoreaction) or LR-White resin (for post-embedding labelling) and polymerized at 60°C for 12–16 h or under UV light (360 nm) at 4°C for 48–72 h. Ultrathin sections were deposited on collodium-coated nickel grids.

For the peroxidase/diaminobenzidine (DAB) pre-embedding method, placentome samples were fixed in 3% buffered paraformaldehyde (pH 7.4) for 2 h at room temperature, washed (3×45 min) in PBS (pH 7.3) at room temperature and stored in 18% saccharose-PBS solution (pH 7.3) overnight at 4°C. Then they were shock-frozen in liquid N_2 and stored at −80°C until use.

Light- and electron-microscopical procedures

The immunogold-silver-enhancement technique was performed according to protocols of the manufacturer (Aurion, Wageningen, Netherlands) as follows.

Light microscopy

Cryosections (12 µm thick) were fixed in 0.1% glutaraldehyde, 3% paraformaldehyde (pH 7.4) for 1 h and washed (3×5 min) in PBS (pH 7.4). Free aldehyde groups were blocked in 0.1% $NaBH_4$ for 10 min. This was followed by a protein-blocking step in Aurion blocking solution for gold conjugates raised in goat for 30 min and a washing step (3×10 min) in a buffer containing bovine serum albumin (BSA) viz. PBS plus 0.1% BSA-c (Aurion), pH 7.4. Monoclonal primary antibodies against actin (Chemicon, Hofheim, Germany; 1:800 in BSA-c buffer), α-actinin (Sigma, Taufkirchen, Germany; 1:800 in BSA-c buffer), vinculin (Sigma; 1:800 in BSA-c buffer), phosphorylated MAPK (pMAPK; Sigma; 1:800 in BSA-c buffer) were applied to sections overnight at 4°C. Negative controls were incubated with BSA-c buffer only. After being washed in BSA-c buffer (3×10 min), sections were incubated in goat-anti-mouse ultra-small gold particle (GAM Fab'2, Aurion) secondary antibody, diluted 1:100 in BSA-c, for 4 h at room temperature or overnight at 4°C. Several rinsing steps in BSA-c buffer (6×5 min) and PBS (3×5 min) were followed by post-fixation in 2% glutaraldehyde in PBS for 8–10 min at room temperature and several washing steps (1×5 min) in PBS and (5×2 min) in distilled water at room temperature. Silver enhancement was performed for 20–30 min in Aurion R-Gent solution, followed by washes in distilled water (5×2 min). Specimens for light microscopy were counterstained with haematoxylin and, after dehydration in a graded series of ethanol/xylene, were mounted in Vitro Clud (Langenbrinck, Emmendingen, Germany).

Electron microscopy (pre-embedding)

Cryosections (60 µm thick) were fixed in 0.1% glutaraldehyde, 3% buffered paraformaldehyde (pH 7.4) for 1 h and washed (3×5 min) in PBS (pH 7.4). Free aldehyde groups were blocked in 0.1% $NaBH_4$ for 10 min, followed by a protein-blocking step for 30–60 min with Aurion blocking solution at room temperature. The sections were washed (3×5 min) in BSA-c buffer at room temperature before primary antibody incubation (same dilutions as above) overnight at 4°C and subsequent washing steps in BSA-c buffer (6×10 min). The ultra-small gold particles were diluted in the same buffer by 1:50 and incubation was performed overnight at 4°C, followed by washing steps in BSA-c buffer (6×10 min) and PBS (6×5 min). Post-fixation in 2% glutaraldehyde in PBS (pH 7.4) was performed for 30 min, with subsequent rinsing steps in PBS (1×5 min) and distilled water (5×2 min). Silver enhancement lasted for up to 45 min and was followed by washes in distilled water (5×2 min). The samples were then dehydrated in a graded series of pure ethanol (each step for 5 min), infiltrated with a mixture of ethanol and xylene (100% ethanol plus xylene 1:1 for 5 min), pure xylene (2×5 min), xylene and Epon (Serva, Heidelberg, Germany; 1:1) for 30 min and pure Epon resin overnight. Polymerization was performed at 60°C for 16 h.

Electron microscopy (post-embedding)

For post-embedding, immersion-fixed samples were embedded in LR-White resin (Agar Scientific, Essex, UK) because of its suitability for immunohistochemistry. Ultrathin sections were picked up on collodium-coated nickel grids. The immunogold-silver-enhancement technique was performed according to protocols of the manufacturer (Aurion) as below.

Free aldehyde groups were blocked in 0.05 M glycine (Sigma-Aldrich, Steinheim, Germany) in PBS for 15 min. Aurion blocking

solution for goat gold conjugates was applied for 30 min and was followed by washing steps in BSA-c buffer (3×10 min) and then incubation with monoclonal primary antibodies as described for light microscopy. Negative controls remained in BSA-c buffer or were incubated with mouse IgG. After being washed in BSA-c buffer (6×5 min), sections were incubated with goat-anti-mouse ultra-small gold particle secondary antibody diluted 1:50 in BSA-c for 4 h or overnight at room temperature. Several rinsing steps in BSA-c buffer (6×5 min) and PBS (3×5 min) were followed by post-fixation in 2% glutaraldehyde in PBS for 5–10 min at room temperature, and washing steps in PBS (1×5 min) and distilled water (5×2 min) at room temperature. Silver enhancement in Aurion R-Gent solution was applied for approximately 25 min and followed by washes in distilled water (5×2 min).

A second pre-embedding procedure was performed with immunoperoxidase/DAB (Sigma D8001) on 50-µm-thick cryosections, which were finally embedded in Epon as described above. Protein blocking was accomplished by incubation in 10% normal pig serum in 0.0005 M PBS-NaCl (0.45%) buffer (pH 7.4), followed by incubation with primary antibodies (see above) overnight at room temperature and washes in PBS-NaCl buffer (3×5 min). Incubation with directly bound secondary antibody, viz. Anti-POX, 1:100 (sh-α-ms-Ig, POX, Amersham-Pharmacia, Freiburg, Germany) in PBS-NaCl (pH 7.4) buffer, was carried out for 1 h at room temperature. Sections were rinsed in PBS-NaCl buffer (1×5 min) and TRIS-HCl buffer (2×5 min, 0.05 M TRIS-HCl, pH 8.6), before being treated for the DAB reaction. For nickel enhancement, a nickel solution (25 mM) was prepared by solving 0.2 g nickel ammonium sulfate in 20 ml TRIS-HCl plus 250 µl DAB solution (10 µg/ml); 4 ml of this solution was taken for a 10-min pre-incubation without H_2O_2 and then the rest of the solution plus 1.2 µl H_2O_2 (30%) was used for a 45-min incubation, followed by a washing step (3×5 min) with TRIS-HCl buffer. The specimens were then treated with 1% OsO_4 in distilled water for 20 min at room temperature. After being rinsed (3×5 min) in 0.05 M maleate buffer (pH 5.2), the sections were counterstained with 1% uranyl acetate in 0.05 M maleate buffer (pH 6.0) for 1 h in darkness at room temperature, followed by a rinsing step (3×5 min) in maleate buffer (pH 5.2) and dehydration in graded ethanol, an ethanol:propylene oxide mixture (1:1, 5 min) and then pure propylene oxide (2×5 min). Finally, the samples were infiltrated with propylene oxide plus Epon (1:1, 30 min) and then pure Epon overnight at room temperature followed by polymerization in fresh Epon at 60°C for 16 h.

RT-PCR technique

mRNA was extracted from whole bovine placentomes with Trizol reagent (Gibco BRL, Md., USA) according to the manufacturer's protocol. The presence of transcripts for β-actin, α-actinin, vinculin and pMAPK was shown by RT-PCR. First strand cDNA synthesis was performed by using the Superscript II Kit (Invitrogen Life Technologies, Karlsruhe, Germany), according to the manufacturer's protocol. Primers were designed from sequences published in the data base of the National Center for Biotechnology Information (α-actinin: BU787657, vinculin BC008520 and pMAPK [ERK-2]: M84489) or from the literature (β-actin; White et al. 2002), giving an expected PCR product of about 661 bp for β-actin, 446 bp for α-actinin, 373 bp for vinculin and 339 bp for pMAPK [ERK-2]. The PCR master mix for β-actin and pMAPK contained 36.5 µl distilled water, 5 µl 10× PCR buffer (Promega, Mannheim, Germany), 4 µl 25 mM $MgCl_2$ (Promega), 1 µl 10 mM dNTP-Mix (Promega), 1 µl forward primer (β-actin: 5'-tgacggggtcacccacactgtgcccatcta-3', and pMAPK [ERK-2]: 5'-tacggcatggtgtgctctgcttat-3') and reverse primer (β-actin: 5'-ctagaagcattgcggtggacgatggaggg-3', and pMAPK [ERK-2]: 5'-acggtgcagaacgttagctgaatg-3'; all primers from MWG, Ebersberg, Germany), 1 µl cDNA and 0.5 µl *Taq* DNA polymerase (Promega).

The PCR master mix for α-actinin and vinculin contained 36.5 µl distilled water, 5 µl 10× PCR buffer, 4 µl 25 mM $MgCl_2$ (both from AmpliTaq Gold, Applied Biosystems, Roche Molecular Biochemicals, Mannheim, Germany), 1 µl 10 mM dNTP-Mix (Promega), 1 µl forward primer (α-actinin: 5'-aactgtcacttg-gcgggcaggg-3', and vinculin: 5'-atgccggtgtttcacacgcgta-3') and reverse primer (α-actinin: 5'-aagggcatcagccaggagcagat-3', and vinculin: 5'-taagcagtaggtcagatgtgc-3'; all primers from MWG), 1 µl cDNA and 0.5 µl *Taq* DNA polymerase (AmpliTaq Gold, Applied Biosystems, Roche Molecular Biochemicals).

PCR for α-actinin and vinculin was carried out with an initial denaturation at 95°C for 10 min and continued for 55 amplification cycles (each started at 94°C for 45 s) with an annealing temperature for α-actinin of 62°C and vinculin 63°C for 45 s, whereas PCR for β-actin and ERK-2 was performed with an initial denaturation at 95°C for 1 min and continued for 40 amplification cycles with an annealing temperature for β-actin of 65°C and for ERK-2 of 63°C for 1 min, followed by 72°C for 2 min and afterwards a final step at 72°C for 10 min, in a T_3 thermocycler (Whatman Biometra, Göttingen, Germany). RT-PCR products were analyzed by gel electrophoresis on a gel containing 2% agarose, 1× TRIS acetate EDTA and 10 µl ethidium bromide (Sigma) on an electrophoresis system from Whatman Biometra.

Controls were performed by applying the whole PCR master mix without cDNA. Basepair length was determined by using a 100-bp marker (New England Biolabs, Herfordshire, UK).

Results

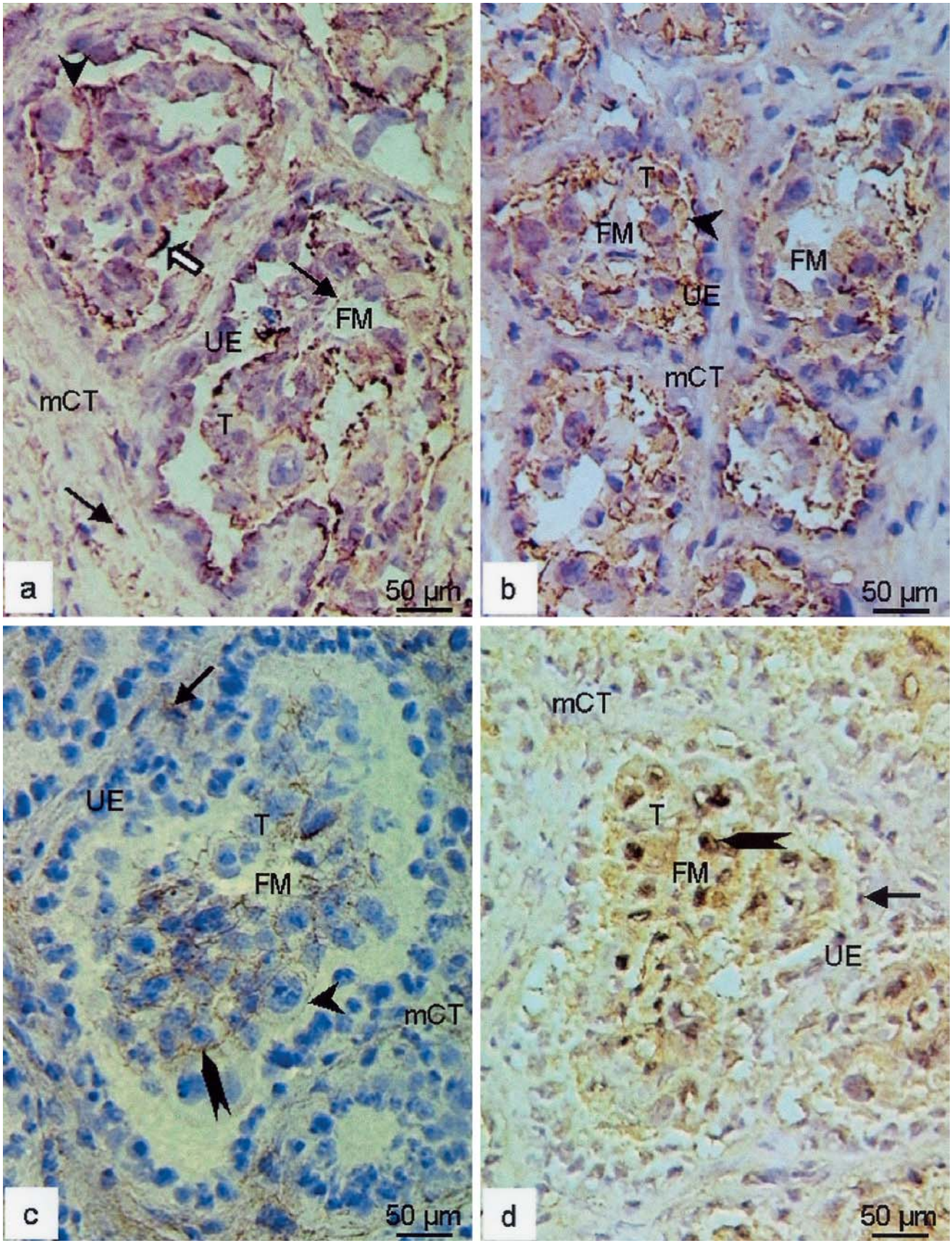
No differences in the signal expression were observed among the animals at different gestational ages. Negative controls showed no electron-dense silver accumulations, no dark cloudy peroxidase precipitations and no brown colour attributable to DAB staining. Background phenomena were negligible.

Localization of actin

Uterine epithelial cells showed staining for actin in apical and lateral cellular locations (Figs. 1a, 2a, b). Maternal endothelial cells of all kinds of blood vessels were also positive for actin (Figs. 1a, 2a) as were smooth muscle cells of the vessel walls (not shown). In the fetal compartment, actin was detected apically in mononuclear trophoblast cells, occasionally with apical accumulations (Figs. 1a, 2c, d). In the TGC, a circular membrane-associated localization of actin was visible, plus an accumulation in the area of the pseudopodium (Fig. 2a, c). Endothelial cells in the fetal mesenchyme were also positive (Figs. 1a, 2d).

Localization of α-actinin

Like actin, α-actinin was localized apically in uterine epithelial and mononuclear trophoblast cells. In TGC, electron-dense silver particles accumulated adjacent to the cytoplasmic membrane, thus showing a co-localization with actin (Figs. 1b, 3a, b). In contrast to actin, neither maternal nor fetal endothelial cells were stained (Figs. 1b, 2b, 3a, b).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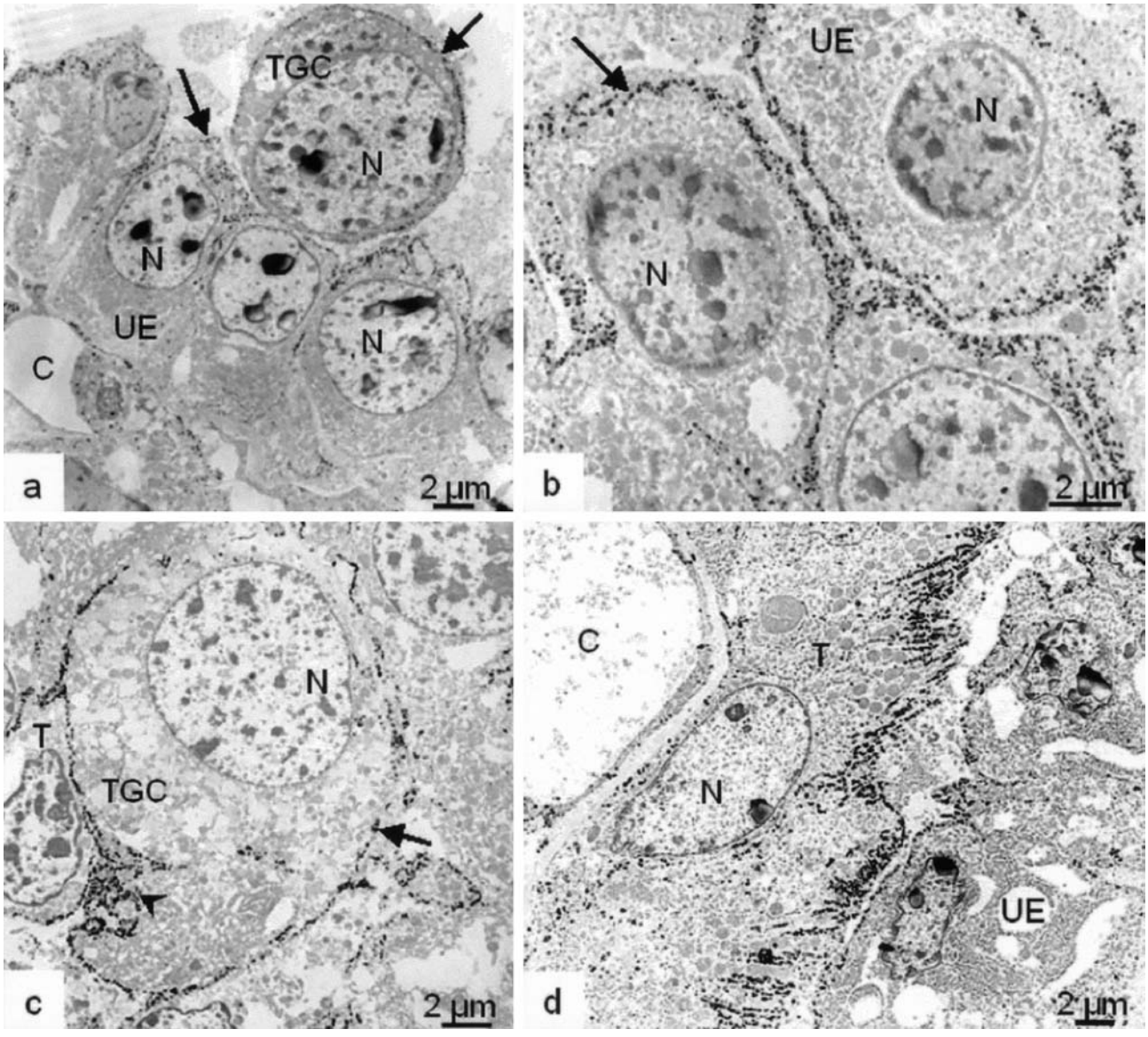

Fig. 2a–d Transmission electron-microscopical demonstration of actin in bovine placentomes from approximately day 130 of gestation by immunogold-silver-enhancement technique. **a** Uterine epithelium (*UE*) and an adjacent trophoblast giant cell (*TGC*); electron-dense silver granules occur apically and laterally in *UE* and are membrane-associated in *TGC* (*arrows*). Endothelial cells of capillaries (*C*) are also labelled by silver granules (*N* nucleus). **b** Detail of **a**, sectioned across *UE*, showing a continuous arrangement of black silver-enhanced gold particles (*arrow*) adjacent to apical parts of the cytoplasmic membrane. **c** TGC with pseudopodium displaying an agglomeration of silver-enhanced gold particles near the pseudopodium area (*arrowhead*) oriented in the direction of migration and dispersed directly beyond the membrane (*arrow*). *T* Mononuclear trophoblast cell. **d** Overview of *T*, in contact with *UE*, showing a dense arrangement of silver-enhanced gold particles in the apical microvillous membrane (*C* fetal capillary)

Fig. 1a–d Light microscopy of the immunogold-silver-enhancement technique in bovine placentomes from approximately day 180 of gestation. Note that the empty space between trophoblast (*T*) and uterine epithelium (*UE*) is an artefact of preparation. **a** Actin. Deposition of brown to black silver granules is observed apically in uterine epithelial cells (*UE*) and mononuclear trophoblast cells (*T*, *white arrow*), in contrast to TGC (*arrowhead*) where silver granules are located along the cytoplasmic membrane. Fetal and maternal (*black arrows*) endothelial cells are also labelled (*mCT* maternal connective tissue, *FM* fetal mesenchyme). **b** α-Actinin co-localization to actin (compare with **a**). Brown to black silver granules are found apically in *UE* and *T* and are associated with the cytoplasmic membrane in TGC (*arrowhead*). **c** Vinculin immunoreaction is distinctly associated with the cytoplasmic membrane in TGC (*arrowhead*) and peripherally seen in *T* (*black block arrow*). It is also observed in fetal and maternal (*black arrow*) endothelial cells. **d** Phosphorylated (activated) MAPK can be observed as black nuclear clusters in both kinds of trophoblast cells (*black block arrow*) and to a much lesser extent in *UE* (*arrow*)

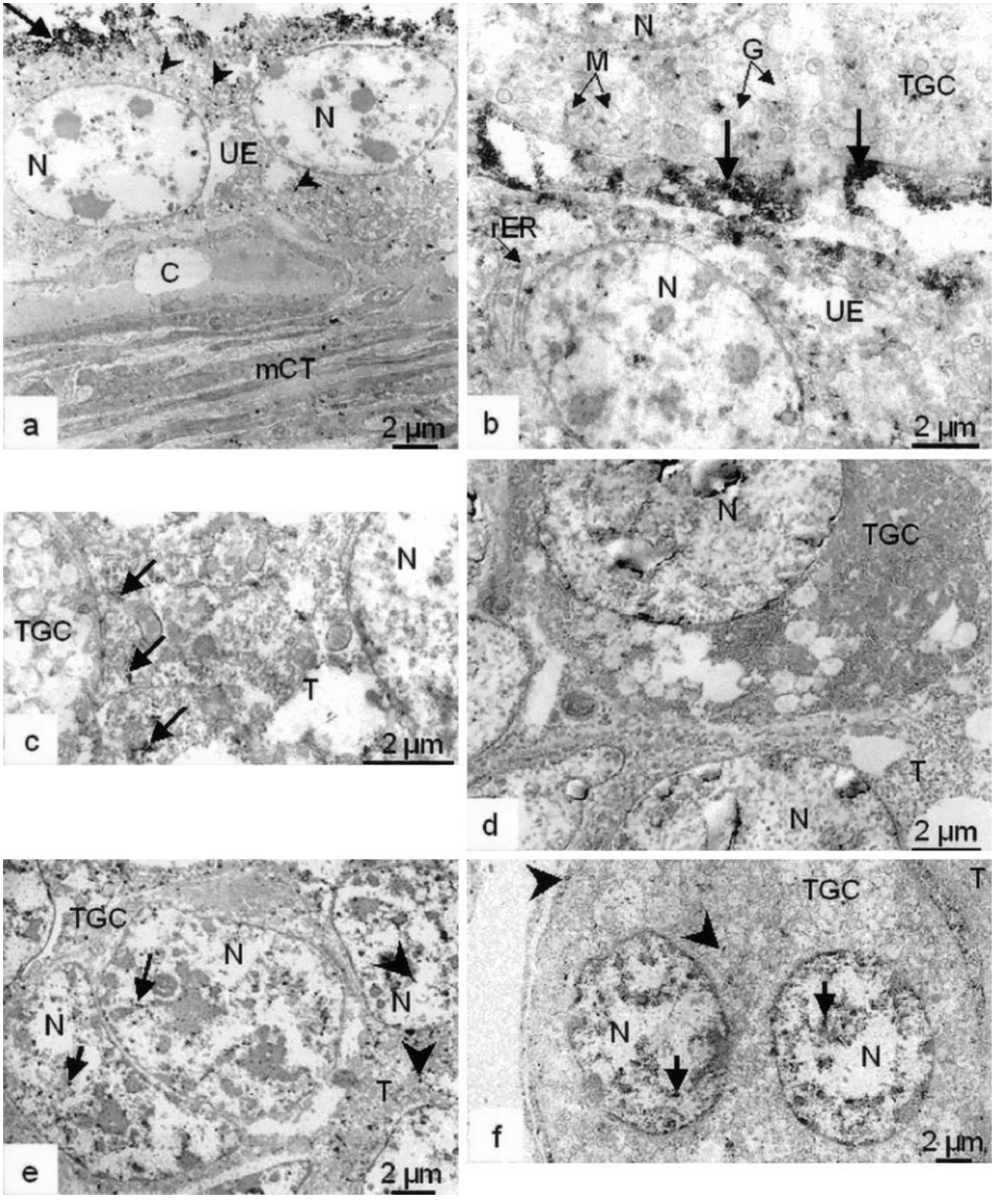

Fig. 3a–f Transmission electron-microscopical demonstration of α-actinin, vinculin and pMAPK in bovine placentomes by immunoperoxidase/DAB staining, which appears as black electron-dense precipitations. **a–c, e, f** Approximately day 180 of gestation. **d** Approximately day 130 of gestation. **a**, **b** α-Actinin. **a** Overview of the uterine epithelium (*UE*) and subepithelial stroma showing anti-α-actinin immunoperoxidase reaction at the feto-maternal contact zone (*arrow*), which is torn by preparative separation from the trophoblast, and to a lesser extent in the cytoplasm of *UE* (*arrowheads*). *C* Capillary, *mCT* maternal connective tissue, *N* nucleus. **b** Detail of the feto-maternal interface revealing a distinct black immunoperoxidase/DAB reaction product at the borderline (*arrows*) between *UE* and a trophoblast giant cell (*TGC*). *G* Granules, *M* mitochondria, *rER* rough endoplasmic reticulum. **c**

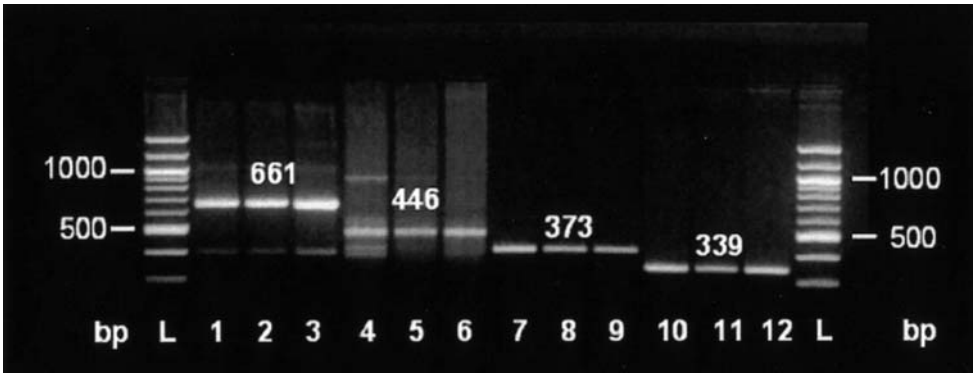

Fig. 4 RT-PCR for β-actin, α-actinin, vinculin and pMAPK. Expression of β-actin, α-actinin, vinculin and pMAPK (ERK-2) mRNA in cow placentome samples of various gestational stages: *lanes 1, 4, 7, 10* day 60, *lanes 2, 5, 8, 11* day 210, *lanes 3, 6, 9, 12* day 280. Specific RT-PCR products were detected by gel electrophoresis in all three gestational stages: β-actin (661 bp, *lanes 1–3*), α-actinin (446 bp, *lanes 4–6*), vinculin (373 bp, *lanes 7–9*), pMAPK (339 bp, *lanes 10–12*). *Lane L* 100-bp DNA ladder

Localization of vinculin

Light microscopically, vinculin produced a very sparse staining of the apical cytoplasmic membrane of uterine epithelial cells but was distinctly localized adjacent to the cytoplasmic membrane in the TGC (Fig. 1c). Maternal and fetal endothelial cells were also positive. Transmission electron microscopically, vinculin was localized in the cytoplasm closely to the cytoplasmic membrane as was apparent following the immunoperoxidase/DAB method (Fig. 3c).

Localization of pMAPK

Light microscopy demonstrated pMAPK immunoreaction as brown to black silver particles precipitated as dense nuclear clusters in both kinds of trophoblast cells and, to a much weaker extent, in uterine epithelial cells (Fig. 1d). Transmission electron microscopy revealed that the majority of immunoreactive material was deposited in the nuclei of trophoblast cells; small amounts (mostly associated with the cell membrane) were present in the cytoplasm of the cells (Fig. 3e, f).

Vinculin immunoperoxidase/DAB staining (*arrows*) in the cytoplasm of a mononuclear trophoblast cell (*T*) adjacent to a *TGC*. **d** Representative negative control section showing a mononuclear trophoblast cell neighbouring a *TGC* with no electron-dense silver particles or DAB precipitations. **e**, **f** pMAPK. **e** Detection of pMAPK in nuclei (*arrows*) and of a membrane-associated form in the cytoplasm of a *TGC*. In *T*, immunoreaction for pMAPK occurs in the cytoplasm and nucleus (*arrowheads*). **f** An immature *TGC* (roundish nuclei) showing strong immunoperoxidase/DAB reaction in nuclei (*arrows*) and weak reaction in the cytoplasm (*arrowheads*). Cytoplasm of *T* shows no reaction

RT-PCR technique

The expression of mRNAs for β-actin, α-actinin, vinculin and pMAPK [ERK-2] in the three different gestational stages (approximately days 60, 210 and 280) appeared as specific RT-PCR products (β-actin: 661 bp, α-actinin: 446 bp, vinculin: 373 bp and ERK-2:339 bp) on agarose gels (Fig. 4). There was no distinct difference in the expression of each protein within the three gestational stages tested. The identity of the bands was confirmed by sequence analysis (Qiagen, Hilden, Germany).

Discussion

Actin in the studied bovine placentomes has been detected by immunocytochemistry apically and laterally in maternal epithelial and in fetal trophoblast cells and apically in mononuclear trophoblast cells, in a membrane-associated form in TGC, with distinct accumulations in the area of the pseudopodium, and in the endothelial cells of both compartments. The potential ability of the tissues examined to form actin has been demonstrated at the mRNA level. The mechanical properties of the actin cytoskeleton may play a significant role in migration, particularly in the pseudopodia of TGC, because changes in cell shape, anchorage and motility include the dynamic reorganization of the actin-cytoskeleton (for a review, see Pavalko and Otey 1994; for overviews, see Small et al. 1999; Steven 1975). Pavalko and Burridge (1991) have reported a disruption of actin organization after microinjection of a 53-kDa α-actinin fragment into living cells. Furthermore, α-actinin is co-localized to integrins (β_1), suggesting a direct link between integrins and actin within cells (Pavalko and Burridge 1991). Several modes of F-actin-membrane interactions have been observed, e.g. the interaction of integrins with actin via talin, either directly or through vinculin (for a review, see Geiger et al. 1995). In addition, integrins may bind to α-actinin, which is also linked to actin, and this interaction again may include

vinculin (see Geiger et al. 1995). The cytoskeletal response to integrin binding to fibronectin-coated beads is the induction of a focal accumulation of a variety of cytoskeleton-associated molecules, including vinculin, talin, α-actinin and F-actin (for a review, see Miyamoto et al. 1995). In vitro, the transmembrane accumulation of talin and α-actinin at the apical surface of ovine luminal epithelial and conceptus trophectoderm cells upon contact with osteopontin-coated beads has revealed that functional integrin activation and cytoskeletal reorganization might occur during embryo implantation in sheep (Johnson et al. 2001). The presence of actin in fetal and maternal endothelia is not surprising, since endothelial cell migration is a common feature during angiogenesis (Risau 1997), which takes place in the fetal and maternal compartments of the bovine placenta throughout gestation (Leiser et al. 1997; Pfarrer et al. 2001).

Bovine TGC migrate towards the uterine epithelium and finally fuse to give hybrid symplasms with single uterine epithelial cells (Wooding 1992). During this migration, a direct signal transduction via integrins to the cytoskeleton is likely, since we have found that actin and α-actinin are co-localized apically in the maternal and fetal epithelium (trophoblast) of bovine placentomes, and α-actinin mRNA expression has also been confirmed by RT-PCR. The relevance of α-actinin for TGC migration is emphasized because α-actinin as a β-subunit ligand of integrins acts as an anchorage protein and induces conformational changes of actin (Alberts et al. 1999). Damsky et al. (1992) have suggested that the modulation of adhesion receptors is responsible for the invasiveness of the trophoblast. They furthermore assume that $\alpha_1\beta_1$ integrin is important for cytotrophoblast invasion because antibody perturbation of interactions involving laminin or collagen type IV and their integrin $\alpha_1\beta_1$ receptor inhibits invasion of the cytotrophoblast (Damsky et al. 1994).

Our findings also correlate with the results of Thie et al. (1997), who have shown that a human endometrial cell line (RL95-2) possesses a non-polarized peripheral actin cortex, which is probably necessary for trophoblast adhesion and which may also be present in the bovine uterine epithelial cells, either by attaching to mononuclear trophoblast cells or by fusing with TGC. However, we suggest that the non-polarized actin cortex in bovine TGC is involved in trophoblast migration, since cell-cell contact via integrin $\alpha_2\beta_1$ during TGC migration (Pfarrer et al. 2003) must be of a transient character because of the migratory character of these cells. Furthermore, cell shape during development is characterized by cytoskeletal reorganization, which is closely related to the formation of anchorage structures. Thereby, both actin and intermediate filaments are associated with cell-cell and cell-matrix connections (for an overview, see Small et al. 1999). Furthermore, the connection of actin to the cytoplasmic membrane and the integrity of the actin-cytoskeleton seem to be of great significance for cell-cell and cell-extracellular matrix interactions, cell motility, receptor-ligand interactions (for a review, see Pavalko and Otey 1994) and tyrosine phosphorylation of MAPKs (Morino et al. 1995). Treatment of human skin fibroblasts with cytochalasin D leads to a selective disruption of the network of actin filaments and consequently inhibits adhesion-mediated MAPK activation (Morino et al. 1995).

The presence of vinculin mRNA in bovine placentomes and the immunocytochemical localization of vinculin protein, apically and laterally and in a membrane-associated form in trophoblast cells and near the contact area between mononuclear and TGC, allows, first, the assumption that vinculin serves as an anchorage point for trophoblast cells and, second, that it functions as a fixation point during the migration and fusion of the TGC. On motile fish keratocytes, newly built focal adhesions have been shown to be vinculin-containing figures, which are essential for adhesion; they are underlayed with a diagonal actin network (Small et al. 1995). In this way, vinculin is capable of acting as a juncture between talin, α-actinin, actin and the integrin β-subunit during signal transduction in focal adhesions (Clark and Brugge 1995; Garrat and Humphries 1995).

When injected into Madin-Darby bovine kidney cells, vinculin and α-actinin are immediately incorporated into adherent and tight junctions (Palovuori and Eskelinen 2000). Focal adhesions do not change; however, cell-cell contacts disappear within 1 h after injection and 5–13 nucleated polycaryonts containing short cell membrane fragments with injected proteins (such as actin or beta-catenin) are built, indicating the disintegration of adherent and tight junctions. In contrast, microinjected fluorescein-isothiocyanate-labelled head domains of vinculin lead to the ablation of the cells from their substrate, probably because of the high affinity of the vinculin head to talin (Palovuori and Eskelinen 2000). Palovuori and Eskelinen (2000) suggest that an excess of α-actinin and vinculin uncouples cell-cell adhesion junctions from the intracellular cytoskeleton, followed by fragmentation of junctional complexes and cell fusion. The localization of α-actinin and vinculin in TGC of bovine placentomes in the present study indicates that this mechanism is also involved in the fusion of TGC with single uterine epithelial cells. Unpolarized TGC (Wimsatt 1951; Davies and Wimsatt 1966) form a transient component of "trophectodermal" tight junctions until the cells fuse with the uterine epithelium (Wooding 1980; Morgan and Wooding 1983). Moreover, an agglomeration of cytoplasmic proteins (e.g. ZO-1, 2 and 3, α-catenin, AF-6) beyond tight junctions is supposedly responsible for the networking of transmembrane proteins (such as occludin, claudin), which form a connection to the actin-cytoskeleton around the tight junctions and transmit regulatory signals as a control for paracellular barriers (for an overview, see Fanning et al. 1999).

pMAPK is present in the nuclei of nearly all trophoblast cells and in single nuclei of the uterine epithelium. Successful amplification of MAPK mRNA establishes the ability of bovine placentomal cells to utilize signal transduction pathways involving MAPKs. Transmission electron microscopically, pMAPK, in addition to its clear

presence in nuclei, is weakly recognizable in the cytoplasm adjacent to the plasma membrane of mononuclear trophoblast cells and TGC. A weak cytoplasmic reaction is not surprising, since ERK or MAPK is phosphorylated and is thus activated in the cytoplasm; it is translocated into the nucleus upon activation, a process that is essential for signal transduction (Glading et al. 2001; Howe et al. 2001). Nuclear localization of pMAPK in bovine placentomal cells suggests an activated status and thus a functional role for MAPK. This phosphorylation and thus the activation of protein kinases may influence TGC migration, since migratory processes are also mediated by ERK (Cho and Klemke 2000; Glading et al. 2001; Howe et al. 2001). MAPK also serves as regulator of cell growth, differentiation and gene activation (Chen et al. 1994b; Miyamoto et al. 1996).

Placental lactogen-I-gene (PL-I) activation is sensitive to the disruption of MAPK and the activation of protein-1 (AP-1) signaling pathways (Peters et al. 2000). Peters et al. (2000) conclude that autocrine and/or paracrine pathways, involving epidermal growth factor receptor and fibroblast growth factor receptor-1, MAPK and AP-1, participate in the regulation of the PL-I gene in differentiating trophoblast cells of the rat. Therefore, pMAPK may also play a role in the activation of this gene in bovine placental TGC. The presence of placental lactogen in TGC of bovine placentomes has been demonstrated (Wooding and Becker 1987) and is, together with their invasive properties, a common feature of TGC in various species (Hoffman and Wooding 1993). However, TGC of rodents are mononuclear, do not fuse with maternal epithelial cells and differ from bovine mostly binucleate TGC in their DNA content (Hoffman and Wooding 1993).

In bovine placentomal cells, cytoplasmic pMAPK shows a specific association with cytoplasmic membranes. The occurrence of pMAPK in the vicinity of the membrane of NR6 cells (Glading et al. 2001) has previously been reported and is associated with growth-factor-induced cell motility. Therefore, we hypothesize that ERK-1 and ERK-2 (pMAPK) localization in the vicinity of the cytoplasmic membrane may be a stimulus for the induction of trophoblast cell migration, because tyrosine phosphorylation and enzymatic activity seems to be mediated by ligation of the β_1 integrin (Morino et al. 1995). The presence of the β_1 integrin subunit in bovine TGC (MacLaren and Wildemann 1995; Pfarrer et al. 2003) supports the hypothesis that TGC migration is initiated by integrin ligation. This theory is corroborated by the finding that ERK (pMAPK) is localized near cytoplasmic membranes and integrin-specific areas and thus may be important for cell spreading and cell movement (Howe et al. 2001).

The presence of actin in bovine placentomal TGC confirms the integrity of the actin cytoskeleton and allows the induction of trophoblast cell migration via ERK-1 and ERK-2 (pMAPK; Morino et al. 1995), since the nucleocytoplasmic exchange of ERK is also associated with the presence of an intact actin cytoskeleton (Aplin et al. 2001). Furthermore, α-actinin is detected as a binding protein for MEKK1 (upstream activator for JNK, ERK and p38 MAPK), meaning that activated ERK can influence other target proteins (such as talin) and may thus play a role in the regulation of adhesion and cytoskeletal conformation (Fincham et al. 2000).

In conclusion, the localization of actin, its associated proteins and pMAPK suggests their involvement in TGC migration in bovine placentomes. The "restricted" trophoblast invasion in bovine placentomes may therefore serve as a model for other migratory processes, such as tumour invasion.

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Muc-1, Integrin, and Osteopontin Expression During the Implantation Cascade in Sheep[1]

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ABSTRACT

The extracellular matrix protein osteopontin (OPN) is a component of histotroph that increases in uterine flushings from pregnant ewes during the peri-implantation period and is localized on the apical surfaces of the uterine luminal epithelium (LE) and conceptus trophectoderm (Tr). The potential involvement of OPN in the implantation adhesion cascade in sheep was investigated by examining temporal, spatial, and potential functional relationships between OPN, Muc-1, and integrin subunits during the estrous cycle and early pregnancy. Immunoreactive Muc-1 was highly expressed at the apical surfaces of uterine luminal (LE) and glandular epithelium (GE) in both cycling and pregnant ewes but was decreased dramatically on LE by Day 9 and was nearly undetectable by Day 17 of pregnancy when intimate contact between LE and Tr begins. In contrast, integrin subunits α_v, α_4, α_5, β_1, β_3, and β_5 were constitutively expressed on conceptus Tr and at the apical surface of uterine LE and GE in both cyclic and early pregnant ewes. The apical expression of these subunits could contribute to the apical assembly of several OPN receptors including the $\alpha_v\beta_3$, $\alpha_v\beta_1$, $\alpha_v\beta_5$, $\alpha_4\beta_1$, and $\alpha_5\beta_1$ heterodimers on endometrial LE and GE, and conceptus Tr in sheep. Functional analysis of potential OPN interactions with conceptus and endometrial integrins was performed on LE and Tr cells in vitro using beads coated with OPN, poly-L-lysine, or recombinant OPN in which the Arg-Gly-Asp sequence was replaced with RGE or RAD. Transmembrane accumulation of talin or α-actinin at the apical surface of uterine LE and conceptus Tr cells in contact with OPN-coated beads revealed functional integrin activation and cytoskeletal reorganization in response to OPN binding. These results provide a physiological framework for the role of OPN, a potential mediator of implantation in sheep, as a bridge between integrin heterodimers expressed by Tr and uterine LE responsible for adhesion for initial conceptus attachment.

implantation, pregnancy, signal transduction, trophoblast, uterus

INTRODUCTION

Implantation in mammals is a highly coordinated process that begins with apposition, attachment, and adhesion of uterine lumenal epithelium (LE) and conceptus trophectoderm (Tr). Remodeling of the glycoprotein adhesion molecules of the apical surfaces of these cells precedes implantation [1]. In ruminants implantation is also influenced by secretions from both the embryo that signals its presence through interferon (IFN)τ [2] and the endometrial glands that produce histotroph to nourish the conceptus [3]. In contrast to primates and rodents with invasive implantation, sheep have superficial/central implantation in which a prolonged preattachment period (~15 days) is followed by incremental apposition and attachment until zones of Tr invade and fuse with uterine LE (~40 days) [4].

The LE is a simple, polarized cell layer that mediates cell-cell and cell-extracellular matrix (ECM) interactions. The apical LE domain is normally nonadhesive; however, this character is lost during development of receptivity [5, 6] when apical adhesion between LE and Tr defines the onset of implantation. The nonadhesive properties of LE are partially due to apical expression of mucins with extensive glycosylation and extended structure that sterically inhibits cell-cell and cell-ECM adhesion [7, 8]. These properties of the mucin Muc-1 presumably impair interactions between adhesive glycoproteins at the surfaces of LE and Tr [9]. The relationship between the mode of implantation and the spatial and temporal expression of Muc-1 is not obvious. In rodents and pigs, Muc-1 expression on uterine LE decreases just prior to implantation [10, 11], whereas it increases during the receptive phase in rabbits and humans but may be reduced locally at sites of conceptus apposition [12, 13]. Muc-1 expression in sheep uterus has not been reported.

Integrins are dominant glycoproteins in adhesion cascades. They comprise a ubiquitous family of cation-dependent, heterodimeric intrinsic transmembrane glycoprotein receptors that mediate cellular differentiation, motility, and adhesion [14–16]. The central role of integrins in the implantation adhesion cascade stems from their ability to bind ECM ligand(s) to mediate adhesion, cause cytoskeletal reorganization to stabilize adhesion, and transduce cellular signals through numerous signaling intermediates [17–19]. Lessey and coworkers have established that transient endometrial expression of $\alpha_v\beta_3$ and $\alpha_4\beta_1$ integrins is cycle-dependent and defines the implantation window in women [20, 21]. Altered expression of these integrins is correlated with several causes of infertility [22, 23]. Null mutations of α_v, α_5, β_1, or β_5 integrin genes in mice lead to peri-implantation lethality, and failure of chorion-allantois fusion [24], while functional blockade of α_v and β_3 integrins reduces the number of implantation sites [25]. Endometrial

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TABLE 1. Summary of PCR primer sequences, annealing temperature and product size.

Primer	Sequence of forward and reverse primers (5′ → 3′)	Annealing temperature (°C)	No. of cycles	Product size (bp)	GenBank accession number
IFNτ	GGAAACTCATGCTGGATGC AAGGTGGTTGATGAAGTGAGG	55	35	460	AF158823
Integrin α_4	AGCACCATCAGAGAGGAAGG GCAGAATCAGACCGAAAAGC	59	35	383	AF349458
Integrin α_5	GAGCCTGTGGAGTACAAGTCC CCTTGCCAGAAATAGCTTCC	59	35	299	AF349459
Integrin α_v	CTGGTCTTCGTTTCAGTGTGC GCCTTGCTGAATGAACTTGG	59	30	295	AF349464
Integrin β_1	GACCTGCCTTGGTGTCTGTGC AGCAACCACACCAGCTACAAT	55	30	313	AF349461
Integrin β_3	AGATTGGAGACACGGTGAGC GTACTTGCCCGTGATCTTGC	59	30	392	AF349462
Integrin β_5	GTCTGAAGATTGGGGACACG GGTACACGCTCTGGTTCTCC	59	30	285	AF349463

integrin expression of several species that exhibit noninvasive implantation has also been reported including pig, sheep, goat, and cow [11, 26–28]. In the pig, integrin subunits expressed by uterine LE and conceptus Tr potentially form $\alpha_v\beta_3$, $\alpha_4\beta_1$, $\alpha_v\beta_1$, and $\alpha_5\beta_1$ heterodimers at implantation sites [11].

The $\alpha_v\beta_3$, $\alpha_4\beta_1$, and $\alpha_v\beta_1$ heterodimers present during the implantation window in humans and pigs bind the Arg-Gly-Asp (RGD) amino acid sequence found in osteopontin (OPN), an ECM ligand that binds integrins to promote cell-cell attachment and spreading [29–31]. Osteopontin is expressed at high levels by epithelium and decidualizing stroma of human uterus [32–34] and cytotrophoblast of the chorionic villus [35, 36]. It is also expressed in invading cytotrophoblast, glandular epithelium (GE), and decidualizing stromal cells in the baboon [37]. Osteopontin is produced by mouse trophoblast and metrial gland cells of decidua and placenta [38], while mRNA and protein are present in LE and GE of pregnant pigs during the peri-implantation period [39]. In sheep, OPN is a component of histotroph that increases in uterine flushings from pregnant ewes between Days 11 and 17 [26]. Although exposure to progesterone induces OPN mRNA expression only in the endometrial GE of pregnant ewes [40, 41], OPN protein is localized on the apical aspect of the endometrial LE, GE, and conceptus Tr [26, 40]. Therefore, it is hypothesized that OPN binds integrin heterodimers expressed by Tr and LE to 1) stimulate changes in morphology of conceptus extraembryonic membranes; and 2) induce adhesion between LE and Tr essential for implantation and placentation [26, 41]. Objectives of this study were to 1) examine relationships between Muc-1, selected integrins, and OPN in the implantation cascade in sheep; and, 2) perform functional analysis of potential OPN interactions with conceptus and endometrial integrins.

MATERIALS AND METHODS

Animals

Mature western-range ewes of primarily Rambouillet breeding were observed daily for estrous behavior in the presence of vasectomized rams. After experiencing at least two estrous cycles of normal duration (16–18 days), ewes were assigned randomly on Day 0 (estrus/mating) to cyclic or pregnant status. Ewes assigned to pregnant status were mated to intact rams three times at 12-h intervals beginning at estrus. Fifty-two ewes were hysterectomized (n = 4 ewes/day) on Day 1, 3, 5, 7, 9, 11, 13, or 15 of the estrous cycle or Day 11, 13, 15, 17, or 19 of gestation. At hysterectomy uteri were flushed with 0.9% NaCl, and pregnancy was verified by recovery of an apparently normal conceptus in uterine flushes. Several sections (1–1.5 cm) of uterine wall from the middle of each uterine horn were snap frozen in Tissue-Tek OCT compound (Miles, Oneonta, NY). The remaining endometrium was dissected from myometrium, frozen in liquid nitrogen, and stored at −80°C. All experimental and surgical procedures involving animals were approved by the Agricultural Animal Care and Use Committee of Texas A&M University (Animal Use Protocol AG-239AG).

Immunocytochemical Analyses

Antibodies used for immunocytochemistry included rabbit anti-Muc-1 (generously provided by Dr. Dan Carson, University of Delaware); rabbit anti-α_v (AB1930), α_4 (AB1924), α_5 (AB1928), β_1 (AB1952), β_3 (AB1932), and β_5 (AB1926) from Chemicon (Temecula, CA); mouse anti-talin clone 8d4 (7-3287), rabbit anti-α-actinin (A-2543), normal rabbit IgG (15006), and normal mouse IgG (15381) from Sigma (St. Louis, MO); and, fluorescein-conjugated goat anti-rabbit IgG (65-611) and fluorescein-conjugated goat anti-mouse IgG (65-6411) from Zymed (San Francisco, CA).

Proteins were localized in frozen uterine tissue sections (8–10 μm) and in cell lines grown on LabTek four-well chamber slides (Nunc, Naperville, IL) by immunofluorescence staining as previously described [26, 42]. Frozen sections or monolayer cell cultures were fixed in −20°C methanol, permeabilized with 0.3% Tween 20 in 0.02 M PBS, blocked in 5% normal goat serum, and incubated overnight at 4°C with 2 μg/ml primary antibody. Immunoreactive protein was then detected using a fluorescein-conjugated secondary antibody for 1 h at room temperature. Slides were overlaid with a coverglass and Prolong antifade mounting reagent (Molecular Probes, Eugene, OR). For each antibody, representative fluorescence images of cross sections for each day were recorded using a Zeiss Axioplan microscope (Carl Zeiss, Thornwood, NY) equipped with a Hamamatsu chilled 3CCD color camera (Hamamatsu, Japan) using Photoshop 5.0 (Adobe Systems, Seattle, WA) image capture software.

Reverse Transcription-Polymerase Chain Reaction

The presence of mRNAs for IFNτ and the integrin receptors α_v, α_4, α_5, β_1, β_3, and β_5 in cell lines (Tr and LE) and endometrium (Day 15 of the estrous cycle and pregnancy) was examined by reverse transcription-polymerase chain reaction (RT-PCR). Total cellular RNA was extracted using Trizol reagent (Gibco BRL, Grand Island, NY). Total RNA (5 μg) was reverse transcribed to obtain cDNAs using Superscript II reverse transcriptase (Gibco BRL), acid-ethanol precipitated, resuspended in 20 μl water, and stored at −20°C. The cDNA templates were then diluted (1:10) with sterile water and amplified by PCR using AmpliTaq DNA polymerase (Perkin Elmer, Foster City, CA) and the specific primers listed in Table 1. The PCR products were separated on 2% agarose gels, visualized by ethidium bromide staining, and analyzed using an Alpha Innotech (San Leandro, CA) imaging system. The identity of each amplified PCR product was verified by sequence analysis after cloning into the pCRII vector (In Vitrogen, San Diego, CA).

Functional Analysis of OPN-Induced Integrin Activation

A ligand-coated bead assay, described by Miyamoto et al. [43] and utilized to identify the transmembrane aggregation of cytoskeletal and sig-

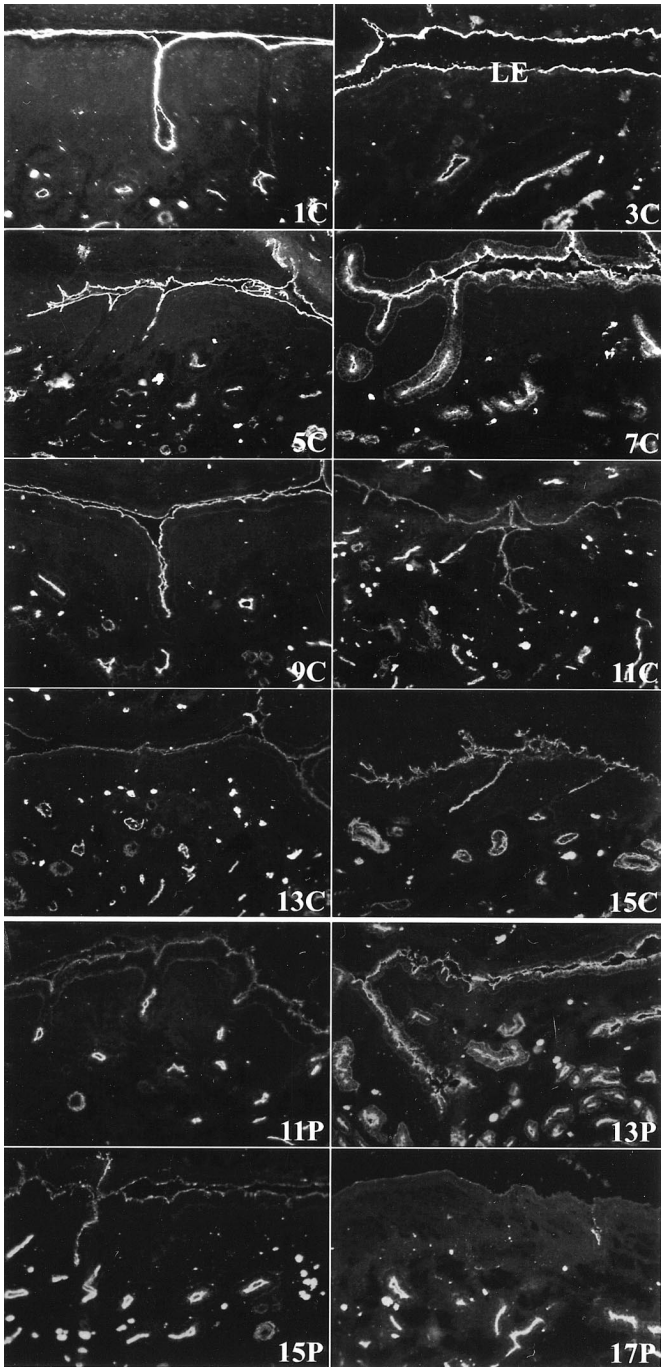

FIG. 1. Muc-1 immunofluorescence in the endometrium of cycling and pregnant ewes. Days 1, 3, 5, 7, 9, 11, 13, and 15 of the estrous cycle (C), and Days 11, 13, 15, and 17 of early pregnancy (P) are shown. Muc-1 was expressed at the apical aspect of all uterine epithelia but decreased on LE after Day 7 of the estrous cycle or early pregnancy. Refer to Figure 3 for an example of representative background staining for the rabbit IgG (IgG) control. ×80.

naling molecules induced by integrin activation, was employed to detect functional activation of integrins by OPN in immortalized LE and primary cultures of Tr from ewes. The differentiated properties of the immortalized ovine LE cells that include estrogen and progesterone receptors, STAT proteins, and several IFNτ-inducible genes expressed by ovine LE in vivo have been described [42]. Ovine LE cells were maintained in Dulbecco modified Eagle medium with F-12 salts (DMEM-F12; Sigma) supplemented with 10% fetal bovine serum and antibiotics. Primary ovine Tr cells were isolated using mechanical dispersion from conceptuses harvested on Day 15 of gestation [44–46] and were maintained in DMEM-F12 supplemented with 5% fetal bovine serum, antibiotics and 0.1 U/ml bovine insulin (Sigma). Both cell types were seeded onto two-well Lab-Tek coverglass chambered slides (Nunc) and cultured for 48 h prior to addition of ligand-coated beads.

Polystyrene beads (6.0 μm; Polysciences Inc., Warrington, PA) were washed in sterile high phosphate PBS (hPBS; 0.1 M NaCl, 2.7 mM KCl, 5 mM Na_2PO_4, 0.85 mM KH_2PO_4, 50 mM NaH_2PO_4), pH 7.4, and centrifuged at 7000 × *g* for 5 min five times. Beads were coated with poly-L-lysine, wild-type OPN or with mutated OPN in which the RGD sequence was replaced with RGE or RAD (100 μg/ml in hPBS; [47]) and incubated at room temperature with constant agitation for 24 h. The beads were washed five times in hPBS as described above and then incubated overnight with sterile BSA (1 mg/ml in hPBS) at room temperature with constant agitation and then stored at 4°C until used [48].

Cells were washed once in DMEM-F12 + 1 mg/ml BSA (810013, crystalline bovine albumin; ICN Pharmaceuticals) prior to initiation of the assay. Beads were diluted in DMEM-F12 + 1 mg/ml BSA, to a final concentration of 2×10^6 beads/2-ml/chamber, and incubated with cells at 37°C for 1 h in a humidified, 5% CO_2/air incubator. Cells were then fixed with 4% paraformaldehyde in PBS (10.14 mM Na_2PO_4, 1.76 mM KH_2PO_4, 136.9 mM NaCl, 2.68 mM KCl) for 10 min at room temperature, rinsed twice with PBS, and once with PBS containing 2% BSA. Cells were incubated at room temperature for 30 min in 10% normal serum followed by overnight incubation at 4°C with anti-talin or anti-α-actinin primary antibody. Following removal of primary antibody, cells were rinsed once in PBS/BSA and three times in PBS for 5 min each. Secondary antibody conjugated to fluorescein isothiocyanate was diluted 1:200 and incubated with cells at room temperature for 1 h in the dark. Cells were rinsed twice with PBS and twice with Hepes buffer (10 mM Hepes, 150 mM NaCl, 0.08% NaN_3). After removing the last rinse, 400 μl mounting medium (0.2 M PBS, 2 mg/ml *p*-phenylenediamine, 90% glycerol) was added, and slides were stored at 4°C in the dark prior to viewing.

Fluorescence imaging of ligand-coated beads was performed with a digital fluorescence imaging system consisting of a charge-coupled device camera and image-capturing software (CELLscan; Scanalytics Inc., Bedford, MA) integrated with a Zeiss Axiovert inverted fluorescence microscope. Sequential optical slices from the basal-to-apical surface of the cells were collected with a high numerical aperture objective lens. Image collection was initiated approximately 1 μm below the basal surface of the cell, and optical slices were collected at 0.5-μm steps up through the apical cell surface and attached beads.

The percentage of ligand-coated beads with apical surface-induced focal adhesions was determined by combined phase-contrast/fluorescence imaging. Phase-contrast imaging was used to locate cells and count adherent beads. Fluorescence microscopy was then used to identify transmembrane accumulation of immunoreactive talin below the ligand-coated beads. For each treatment group, the percentage of ligand-coated beads in contact with cells that exhibited apical focal adhesions was obtained by multiplying the ratio of focal adhesion-positive beads to the total number of beads in contact with the cell by 100. Three to five separate experiments on different days were performed for each of the ligands tested. Data collected from each experiment were subjected to arcsine transformation. General linear models analysis was conducted to test for significance followed by Tukey multiple comparison test to identify significance between groups. A P value of <0.05 was considered significant. Data are presented as mean percentage of beads ± SD inducing apical focal adhesions.

RESULTS

Localization of Muc-1 in Ovine Endometrium

Immunoreactive Muc-1 was restricted to the apical surface of uterine LE and GE (Fig. 1). Expression of Muc-1 on uterine GE remained elevated and constant throughout the estrous cycle and early pregnancy. In contrast, intense apical staining of Muc-1 on uterine LE was observed from Days 1 to 7 followed by a continuous reduction of immunostaining through Day 13 of the estrous cycle. Staining remained low for the remainder of the cycle and early pregnancy and was barely detectable on LE by Day 17 of pregnancy, when intimate adhesion between Tr and LE begins in the ewe [4]. No immunoreactive Muc-1 was detected on Day 15 conceptus flushed from the uterus or on Day 15–17 conceptus tissues examined in situ (data not shown).

Uterine/Conceptus Expression of Integrin Subunits

Primers were designed to amplify mRNAs for the integrin subunits α_v, α_4, α_5, β_1, β_3, and β_5 from Day 15 cyclic and Day 15 pregnant total ovine endometrial RNA (Table 1). Reverse transcription-PCR confirmed the presence of mRNAs for α_v, α_4, α_5, β_1, β_3, and β_5 integrins in endometrium from both cyclic and pregnant ewes (Fig. 2A). The identity of each specific PCR product was confirmed by sequence analyses (data not shown).

Immunoreactive α_v, α_4, α_5, β_1, β_3, and β_5 integrin subunits were detected at the apical surface of uterine LE and GE, and on conceptus Tr (Fig. 3). The α_5 integrin subunit was also detected in uterine stroma. Each of these integrins was expressed by endometrium of both cyclic and pregnant ewes, and the apical expression patterns of each integrin subunit did not change during pregnancy. The apical expression of these subunits could potentially contribute to the apical assembly of several OPN receptors including the $\alpha_v\beta_3$, $\alpha_v\beta_1$, $\alpha_v\beta_5$, and $\alpha_5\beta_1$ heterodimers on endometrial LE and GE, and conceptus Tr in sheep. Attempts to identify other known OPN receptors including $\alpha_9\beta_1$ and CD44 using commercially available antibodies were unsuccessful.

Characterization of LE and Tr Cell Lines

Immortalized uterine LE [42] and primary Tr cells were developed to investigate integrin-mediated signaling at the apical surfaces of these cells. The Tr cells exhibited an epithelial morphology and were evaluated for the expression of IFNτ mRNA, the most definitive marker of this cell type. A specific IFNτ PCR product was detected in total RNA from Tr cells but was not present in total RNA from immortalized LE cells (Fig. 2B). Sequence analyses identified the PCR product as ovine IFNτ (data not shown).

Using RT-PCR (primers described in Table 1), mRNAs for the integrin subunits α_v, α_5, β_1, and β_3 were detected in total RNA samples from immortalized ovine LE and primary conceptus Tr cells (Fig. 2A). Although α_4 and β_5 integrin mRNAs were present in Tr cells, they were not detected in the immortalized LE cell line.

The α_v and β_3 integrin subunits were abundantly expressed at the basal surface of immortalized ovine LE and primary Tr cells (Fig. 4). The similar punctate subunit staining patterns at sites of cell anchorage to the substrate suggest both the presence of $\alpha_v\beta_3$ heterodimers aggregated at focal adhesion sites as well as the abundance of this OPN receptor in both cell types. Although immunoreactive β_1, β_5, and α_5 integrin subunits were also detected in LE and Tr cell lines, the aggregation of these proteins at focal adhesions was not as well defined as those shown for α_v and β_3 integrins.

Functional Activation of LE and Tr Integrins by OPN

Figure 5 illustrates the cytoskeletal reorganization (outside-in signaling) observed at the cytoplasmic side of the interface between OPN-coated beads and the apical cell surface of immortalized ovine LE using immunocytochemical localization of the cytoskeletal protein talin to detect the response. Talin was selected as the response variable for this assay because of its central role in binding cytoplasmic domains of β_1 and β_3 integrins, cytoskeletal proteins, and focal adhesion kinase [49]. Within 1 h of adding OPN-coated beads to cultured ovine LE, approximately 20% of the beads in contact with cells exhibited intense immunostained talin aggregates. Similar aggregates were

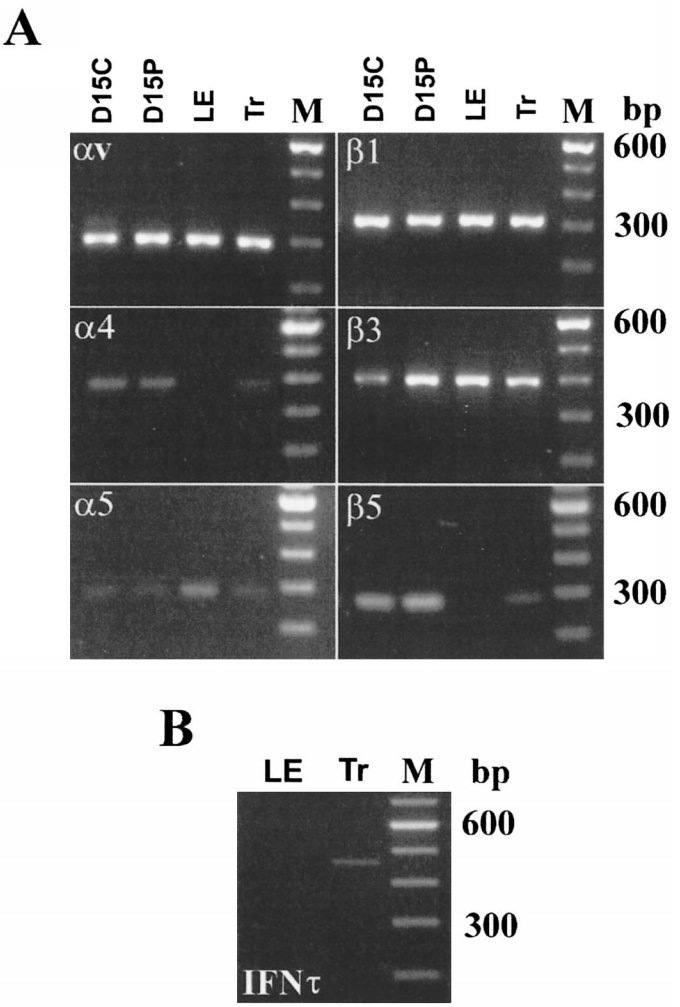

FIG. 2. The RT-PCR analysis of integrin and IFNτ mRNAs. **A**) Analyses of α_v, α_4, α_5, β_1, β_3, and β_5 mRNAs in endometrial total RNA from Day 15 cyclic (D15C) and pregnant (D15P) ewes, and immortalized ovine LE (LE) and primary ovine Tr (Tr) cell lines. Messenger RNA for each of these integrins was present in endometrium from both cyclic and pregnant ewes. **B**) Reverse transcription-PCR analysis of IFNτ mRNA in total RNA from immortalized ovine uterine LE and primary ovine conceptus Tr cells. Note the presence of a single PCR product in RNA from Tr cells only. The identity of each specific PCR product was confirmed by sequence analyses. The molecular weight markers (M) are noted to indicate base pairs (bp) in the PCR product.

detected at the surface of conceptus Tr although about 50% of the cells associated with beads were talin positive. Similar staining patterns were detected in cells stained with antibody directed against α-actinin, but the results were not quantified. Table 2 summarizes the talin results from multiple in vitro microbead-cell adhesion assays.

DISCUSSION

It is generally accepted that the process of implantation that begins with intimate association of the conceptus and uterine endometrium and ends with the formation of a placenta involves an adhesion cascade. Adhesive LE ligands, normally masked by mucins, become exposed during the receptive period, and various adhesion molecules then function sequentially, or in parallel, to stabilize adhesion of Tr to LE [50]. Temporal and/or spatial changes in expression of mucins, ECMs, and their integrin receptors influence the establishment of endometrial receptivity in rodents, pri-

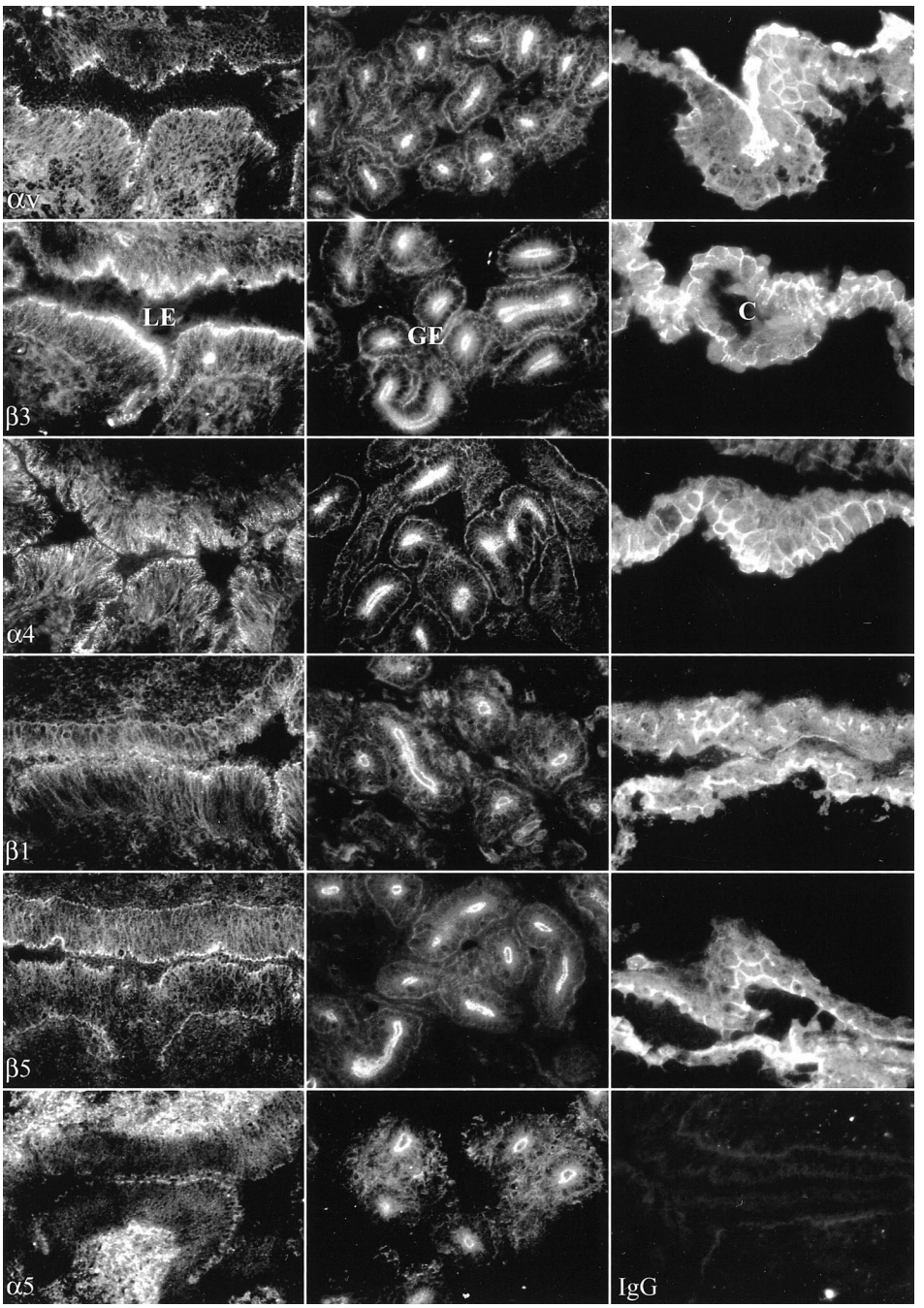

FIG. 3. Integrin subunit expression in ovine endometrium and conceptus tissue. Integrin subunits α_v, α_4, α_5, β_1, β_3, and β_5 in ovine endometrial LE (left column), GE (middle column), and conceptuses (C, right column) from Day 16 of pregnancy were detected using immunofluorescence staining of frozen sections. The uterine cross-sections shown represent both the estrous cycle and pregnancy because the staining pattern did not differ due to day or pregnancy status. Compare the absence of antibody staining in endometrium when rabbit IgG (IgG) was used to detect immunoreactive proteins. ×100 (columns 1 and 2) and ×260 (column 3).

mates, cattle, goats, and pigs [10, 11, 13, 20, 26, 37, 40, 51, 52]. The present and previous studies identified key structural and functional elements of the implantation adhesion cascade in sheep that include 1) elevated expression of antiadhesive Muc-1 at the apical aspect of uterine LE that declines prior to the attachment phase of implantation; 2) the integrin subunits α_v, α_4, α_5, β_1, β_3, and β_5 that could form known OPN receptors including $\alpha_v\beta_3$, $\alpha_v\beta_1$, $\alpha_v\beta_5$, and $\alpha_4\beta_1$, and are constitutively and highly expressed on the apical surface of uterine LE and on conceptus Tr; 3) OPN is secreted from the endometrial GE into the uterine lumen immediately before and during conceptus attachment [26, 41]; 4) OPN protein is present at the apical surfaces of both uterine LE and conceptus Tr [26]; and 5) OPN binds integrins expressed on LE and Tr cells in vitro to initiate integrin activation and outside-in signaling.

The lumen of the uterus is covered by mucins that are heavily glycosylated and project above the apical surface of LE cells. Human blastocysts also express Muc-1 [53]. Membrane mucins such as Muc-1 and Muc-4 serve to lubricate and protect the uterus against microbial infection; however, these proteins also sterically inhibit cell-cell and cell-ECM adhesion [7, 8], and presumably impair Tr access to the uterine LE [9]. Overall Muc-1 expression increases during the receptive phase in rabbits and humans but is locally reduced at sites of conceptus attachment [12, 13]. It is hypothesized that Muc-1 in human endometrium undergoes a reduction of repulsive charge density through the loss of keratin sulfate to allow conceptus apposition. Recent in vitro evidence suggests that paracrine signals from the blastocyst lead to down-regulation of Muc-1 from both LE and trophectoderm at sites of implantation [13, 53]. In con-

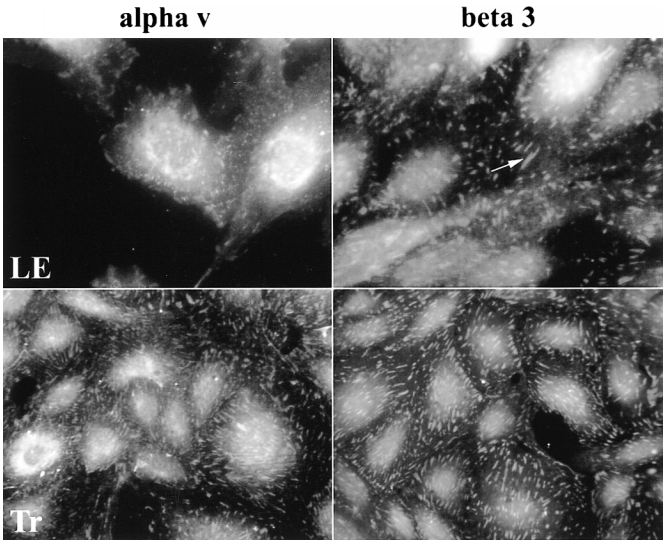

FIG. 4. Immunofluorescence analyses of α_v and β_3 integrin subunit expression in cultured immortalized ovine LE (LE) and primary conceptus Tr (Tr) cell lines. Note that both α_v and β_3 integrins are detected at focal adhesions (arrow) along the basal aspect of cells, representing sites of cell anchorage to the substrate. ×240.

trast, Muc-1 expression at the apical LE surface decreases prior to implantation in rodents and pigs [10, 11, 52].

The present studies clearly show that in sheep, similar to rodents and pigs, the ovine adhesion cascade initiates through down-regulation of Muc-1. Although speculative, it is reasonable to infer that loss of Muc-1 on ovine LE is influenced by progesterone. Both implantation and Muc-1 down-regulation are blocked by administration of RU-486 in rodents [10, 52], and injection of ovariectomized gilts with progesterone decreases Muc-1 expression [11]. Further, Muc-1 decreases dramatically between Days 7 and 17 of pregnancy in sheep (see Fig. 1). This pattern of Muc-1 expression is temporally similar to that of progesterone receptor gene expression in pregnant ovine endometrium. Progesterone receptors are expressed in LE on Days 9 and 11 of pregnancy but are progressively lost between Days 13 and 19 [54]. It is noteworthy that expression of Muc-1 on LE declines to barely detectable levels by Day 17 of pregnancy because adhesion of conceptus Tr to a still-intact uterine LE occurs between Days 16 and 18 in sheep [4]. Loss of Muc-1 prior to ovine implantation may remove a barrier on LE that sterically hinders the ability of ECMs to interact with apically expressed integrins.

TABLE 2. Percentage of beads inducing apical focal adhesions.*

	OPN-RGD	OPN-RGE	OPN-RAD	Poly-L
LE	21.7 ± 8.4 B	<1 A	<1 A	<1 A
n	4	4	4	5
Tr	48.3 ± 9.4 C	<1 A	<1 A	<1 A
n	3	3	3	3

* Polystyrene beads (6 μm) were coated with either recombinant rat OPN (OPN-RGD), rat OPN in which the RGD sequence was replaced with RGE (OPN-RGE), RAD (OPN-RAD) or poly-L-lysine as a negative control. The number of separate experiments (n) conducted on different days is indicated. Values followed by different letters are significantly different ($P < 0.05$).

Cell surface integrin receptors connect the ECM to the intracellular cytoskeleton and transmit biochemical signals across the plasma membrane. Integrins are the primary mediators of matrix effects including cell-cell and cell-ECM adhesion, influence on cell shape, and regulation of gene expression [19, 55]. Hormone-dependent temporal and spatial distribution of integrins in the human uterus and developmental regulation on the invading mouse blastocyst provide strong circumstantial evidence for integrin involvement in the events of implantation [22, 56]. In sheep, α_v, α_4, α_5, β_1, β_3, and β_5 integrin subunits are expressed by endometrium of both cyclic and pregnant ewes and by conceptus Tr. All subunits were identified by RT-PCR in Day 15 cyclic and pregnant endometrium and in a conceptus Tr cell line (see Fig. 2). Expression of α_v, α_5, β_1, and β_3 integrin subunits in LE was confirmed by detection of mRNAs in an immortal LE cell line (Fig. 2). Immunostaining showed that α_v, α_4, α_5, β_1, β_3, and β_5 are apically expressed on LE and GE and on conceptus Tr in vivo (Fig. 3). Apical expression of these integrins was constitutive and, in general, did not increase during the peri-implanta-

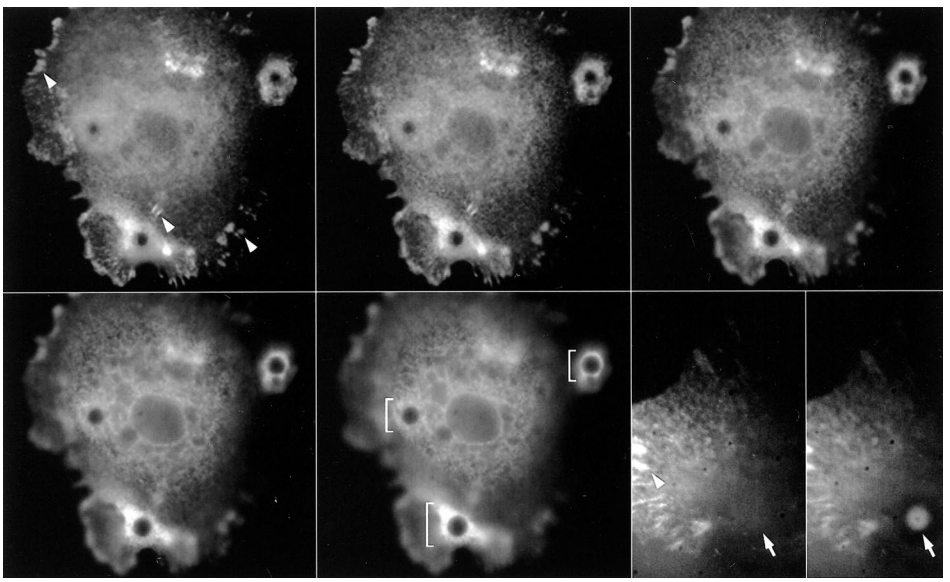

FIG. 5. Integrin receptor binding to OPN RGD sequences leads to cytoplasmic reorganization and induction of focal adhesions. Because cytoplasmic aggregation of cytoskeletal proteins requires ligand occupancy and integrin aggregation, immunodetection of talin shown here at focal adhesions provides a sensitive functional index of integrin activation and outside-in signaling. A series of five optical slices recorded at 4 μm apart (moving in the basal-to-apical direction) in an LE cell exposed for 1 h to OPN-coated beads is represented. Focal adhesions that function to anchor cells to the substrate (arrowheads) provide a reference point for the basal aspect of cells. Brackets indicate the talin aggregation reaction induced by three different beads attached to the apical surface. No focal adhesions were induced by polylysine-coated beads or beads coated with mutant recombinant rat OPN in which the RGD sequence was replaced with RAD or RGE. This is illustrated in the lower right two panels that represent a nonreactive bead (arrow) at different focal planes. ×400.

tion period. Although α_4 and β_5 subunit proteins were present on endometrial epithelia, mRNA was minimal in endometrium and Tr cells and was not detected in LE cells. The primers used to amplify ovine α_4 and β_5 were designed using the cDNA sequence for human α_4 and β_5. Perhaps detection of these integrin subunits was limited due to an RT-PCR reaction that was less efficient than those for the other integrins.

The ewe does not appear to limit receptivity to implantation by modifying temporal and spatial patterns of integrin expression as integrins are expressed apically and constitutively. The constitutive apical expression of integrins on uterine epithelia in sheep contrasts with studies from other species which suggest that alterations in integrin expression may frame the putative window of implantation. In women, $\alpha_v\beta_3$ and $\alpha_4\beta_1$ increase on LE prior to embryo attachment [20, 21]. While $\alpha_v\beta_3$ and $\alpha_v\beta_5$ are found on the apical surface of LE [21, 57], $\alpha_6\beta_4$, $\alpha_2\beta_1$, and $\alpha_3\beta_1$ are distributed primarily along the lateral and basal cell surfaces [58]. In pigs, subunits α_v, α_3, α_4, α_5, β_1, and β_3 are present on the apical LE surface, with α_v and β_3 integrins showing intense and constitutive expression. However α_4, α_5, and β_1 are spatially and temporally regulated throughout the estrous cycle and early pregnancy, reaching maximal levels during early implantation [11]. Baboons express $\alpha_v\beta_3$ integrin in both GE and decidualizing stromal cells of pregnant endometrium [37], whereas in mice, endometrial $\alpha_v\beta_3$ integrin is limited to stromal cells during implantation [25]. Integrin expression in the LE of sheep also appears to contrast with other ruminants. In cows, $\alpha_v\beta_3$, α_3, α_6, and β_1 are localized to the basolateral surfaces of LE, shallow GE, and within the sublumenal stroma. The pattern of expression for $\alpha_v\beta_3$ changes over the estrous cycle [28]. Similarly, β_1 integrin has been detected along the basolateral membranes of LE and GE in goats but is decreased in LE at sites of conceptus adhesion [27]. These variations of integrin expression among species may reflect differences in types of apposition, attachment, and invasion during implantation. However, the presence of integrin receptors on endometrial and conceptus surfaces suggests their involvement as mediators of conceptus attachment to uterine LE common to all mammalian species.

Integrin-mediated attachment may involve bifunctional bridging ligands that interact with receptors expressed on these apposing surfaces to adhere fetal and maternal membranes. OPN is a likely candidate integrin bridging ligand in sheep because the 45-kDa fragment is abundant within the uterine lumen during the peri-implantation period. Moreover, OPN protein is present at apical surfaces of LE and Tr cells that do not express OPN mRNA, providing circumstantial evidence that OPN accumulates at these surfaces through interaction with integrin receptors [26, 40]. Ovine uterine OPN is an acidic 70-kDa matrix glycoprotein that contains an RGD integrin receptor binding sequence [59]. Upon freezing and thawing or treatment with proteases, the 70-kDa protein gives rise to 24- and 45-kDa fragments [60]. The 45-kDa fragment has greater binding affinity for integrins than the native 70-kDa form, possibly because protease cleavage makes the adjacent RGD sequence more accessible for interaction with specific integrins [61].

Binding of integrins to ECM proteins promotes the aggregation of integrins and triggers a hierarchical response leading to transmembrane accumulation of cytoskeletal proteins and over 20 signal transduction molecules to the β-integrin subunit cytoplasmic domain [43]. The result is assembly of well-developed aggregates composed of ECM proteins, integrins, and cytoskeletal proteins that are known as focal adhesions [19, 43, 62]. Attachment of the c-Src substrates, tensin, and focal adhesion kinase can result from integrin aggregation alone, but aggregation of cytoskeletal proteins including talin, α-actinin, vinculin, and F-actin requires ligand occupancy and integrin aggregation [43]. Therefore, immunodetection of aggregated integrins, talin, or α-actinin at focal adhesions can provide a sensitive functional index of integrin activation and outside-in signaling. The in vitro studies reported here exploit the ability to induce focal adhesions by integrin-ECM interactions to show, for the first time, functional integrin activation and cytoskeletal reorganization in uterine LE and conceptus Tr cells in response to OPN binding. Accumulation of talin and α-actinin was detected at the interface between OPN-coated polystyrene beads and the apical membranes of LE and Tr cells. The focal adhesions are the result of RGD-integrin interactions because mutation of the OPN RGD sequence eliminated cytoskeletal aggregation (Table 2), although the identity of activated integrins remains unknown. Interestingly, α_v and β_3 integrin subunits that form the $\alpha_v\beta_3$ receptor, which is capable of binding multiple matrix proteins including OPN, vitronectin, and fibronectin [18], aggregate at sites of cell anchorage to the substrate in both LE and Tr cells, suggesting the presence of this versatile receptor at focal adhesion sites (see Fig. 4). OPN-coated beads also induce integrin activation in Tr cells. The number of beads associated with Tr cells was at least double the number formed at the apical surface of immortalized LE, and this was consistent with the greater abundance of α_v and β_3 containing aggregates at the basal surface of cultured Tr cells. This is not unexpected in light of the highly adhesive nature of the conceptus. Therefore, it is reasonable to predict that in the pregnant ovine uterus, OPN binding to integrin heterodimers induces similar focal adhesion sites that promote and stabilize attachment of Tr to LE for implantation.

The present study suggests that a decline in Muc-1 on uterine LE exposes apically oriented α_v, α_4, α_5, β_1, β_3, and β_5 integrins to interaction with OPN during the peri-implantation period. The coincident presence of these integrins that could form known OPN receptors including $\alpha_v\beta_3$, $\alpha_v\beta_1$, $\alpha_v\beta_5$, and $\alpha_4\beta_1$, on uterine LE and conceptus Tr, along with evidence that OPN can functionally interact with integrins expressed on LE and Tr cells, provide a physiological framework for OPN to act as a mediator of implantation in sheep that bridges integrin receptors expressed by Tr and LE to induce adhesion essential for initial conceptus attachment.

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Osteopontin Is Synthesized by Uterine Glands and a 45-kDa Cleavage Fragment Is Localized at the Uterine-Placental Interface Throughout Ovine Pregnancy[1]

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ABSTRACT

Osteopontin (OPN) is a phosphorylated and glycosylated, secreted protein that is present in various epithelial cells and biological fluids. On freezing and thawing or treatment with proteases, the native 70-kDa protein gives rise to 45- and 24-kDa fragments. Secreted OPN functions as an extracellular matrix (ECM) protein that binds cell surface receptors to mediate cell-cell adhesion, cell-ECM communication, and cell migration. In sheep and humans, OPN is proposed to be a secretory product of uterine glandular epithelium (GE) that binds to uterine luminal epithelium (LE) and conceptus trophectoderm to mediate conceptus attachment, which is essential to maintain pregnancy through the peri-implantation period. Cell-cell adhesion, communication, and migration likely are important at the interface between uterus and placenta throughout pregnancy, but to our knowledge, endometrial and/or placental expression of OPN beyond the peri-implantation period has not been documented in sheep. Therefore, the present study determined temporal and spatial alterations in OPN mRNA and protein expression in the ovine uterus between Days 25 and 120 of pregnancy. The OPN mRNA in total ovine endometrium increased 30-fold between Days 40 and 80 of gestation. In situ hybridization and immunofluorescence analyses revealed that the predominant source of OPN mRNA and protein throughout pregnancy was the uterine GE. Interestingly, the 45-kDa form of OPN was detected exclusively, continuously, and abundantly along the apical surface of LE, on conceptus trophectoderm, and along the uterine-placental interface of both interplacentomal and placentomal regions through Day 120 of pregnancy. The 45-kDa OPN is a proteolytic cleavage fragment of the native 70-kDa OPN, and it is the most abundant form in uterine flushes during early pregnancy. The 45-kDa OPN is more stimulatory to cell attachment and cell migration than the native 70-kDa protein. Collectively, the present results support the hypothesis that ovine OPN is a component of histotroph secreted by the uterine GE that accumulates at the uterine-placental interface to influence maternal-fetal interactions throughout gestation in sheep.

placenta, pregnancy, uterus

INTRODUCTION

All mammalian uteri contain endometrial glands that secrete ''histotroph.'' Histotroph is a complex mixture of a variety of enzymes, growth factors, cytokines, lymphokines, hormones, transport proteins, and other substances [1] that plays a role in conceptus nourishment, attachment, implantation/placentation, and immunoprotection [2, 3]. Studies of ewes in which uterine glands have been epigenetically ablated by neonatal progestin exposure confirm that histotroph is required to maintain pregnancy through the peri-implantation period, when conceptus trophectoderm must adhere to the luminal epithelium (LE) [4].

Osteopontin (OPN) is a component of histotroph [5, 6] that is hypothesized to be involved in ovine implantation [7, 8]. This highly phosphorylated and glycosylated, secreted protein is present in various biological fluids [9]. It also has been found on epithelial cells of the gallbladder, pancreas, lung, sweat ducts, placenta, gastrointestinal, urinary, mammary, and reproductive tracts [10–14]. On freezing and thawing or treatment with proteases, the native 70-kDa protein gives rise to 45- and 24-kDa fragments [15]. Secreted OPN functions as an extracellular matrix (ECM) protein that regulates the activity of a variety of cells by signaling through integrin or CD44 receptors [16, 17]. Reported effects of OPN include mediation of cell-cell adhesion, cell-ECM communication, and cell migration [16, 18].

Uterine OPN and its integrin receptors have become the focus of studies concerning conceptus adhesion for implantation in a number of species, including humans, in which OPN expression is increased by progesterone during the menstrual cycle, starting on Days 16–17, and secretory glandular epithelium (GE) appears to be a source of OPN localized on the apical LE surface [19]. In pregnant sheep, OPN protein is a component of histotroph that binds to the apical surface of LE and GE where several integrins are apically expressed and could form known OPN receptors, including $\alpha_v\beta_3$, $\alpha_v\beta_1$, $\alpha_4\beta_5$, $\alpha_4\beta_1$, and $\alpha_5\beta_1$ [6, 7]. The form of OPN protein that increases in uterine flushings from pregnant ewes between Days 11 and 17 is predominantly a 45-kDa cleavage fragment that binds with higher affinity to integrins and has greater biological activity than the native 70-kDa OPN [20]. The OPN protein is also present on trophectoderm of Day 14–19 ovine conceptuses despite the absence of conceptus OPN mRNA [6]. The OPN mRNA is expressed in the GE of pregnant ewes and is induced when ovariectomized ewes are given exogenous progesterone [5, 21]. Indeed, uterine gland knockout ewes lack uterine glands and exhibit a peri-implantation defect that is associated with the absence of OPN in uterine secretions [4,

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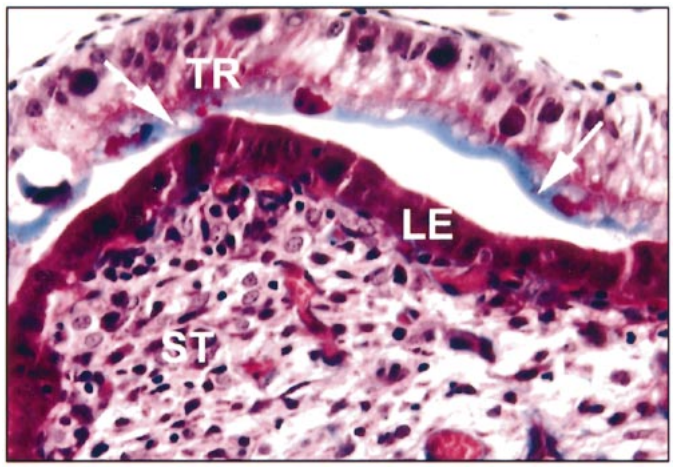

FIG. 1. Representative photomicrograph of endometrium and placenta from a Day 35 pregnant ewe subjected to Masson trichrome staining. This procedure stains nuclei black, cytoplasm and muscle fibers red, and ECM blue. Note the presence of ECM along the interface between endometrial LE and placental tissue (white arrows). ST, Uterine stroma; TR, conceptus trophectoderm. Width of each field is 250 μm.

8]. Therefore, OPN is proposed to be a progesterone-induced secretory product of the GE that binds to uterine LE and conceptus trophectoderm to mediate conceptus elongation and attachment, which is essential to maintain pregnancy through the peri-implantation period in ewes.

Although cell-cell adhesion, cell-ECM communication, and cell migration undoubtedly continue to be critical at the interface between uterus and placenta throughout pregnancy, to our knowledge endometrial and/or placental expression of OPN beyond the peri-implantation period has not been documented in ewes. Our working hypothesis is that OPN is a component of histotroph that is secreted by the endometrial GE throughout pregnancy in the ewe and contributes to an ECM that supports maternal-conceptus adhesion and communication. Therefore, the present study determined temporal and spatial alterations in OPN mRNA and protein expression in the ovine uterus and placenta between Days 25 and 120 of pregnancy.

MATERIALS AND METHODS

Animals and Tissue Collection

Experimental and surgical procedures complied with the Guide for the Care and Use of Agricultural Animals and were approved by the Texas A&M University Institutional Agricultural Animal Care and Use Committee.

Mature, western-range ewes were observed daily for estrous behavior. After experiencing at least two estrous cycles of normal duration (16–18 days), ewes were assigned randomly on Day 0 (estrus/mating) to cyclic or pregnant status. Fifty-two ewes were ovariohysterectomized (n = 4 ewes/day) on Days 14, 20, 25, 30, 35, 40, 45, 50, 55, 60, 80, 100, or 120 of pregnancy. At hysterectomy, uteri from Day 14 pregnant ewes were flushed with 0.9% NaCl to verify pregnancy by recovery of an apparently normal conceptus. Pregnancy was verified on all later days by visual observation of conceptus tissues undergoing attachment and placentation. Several sections (thickness, ~1–1.5 cm) from the middle of each uterine horn were fixed in fresh 4% paraformaldehyde in PBS (pH 7.2) for 24 h and then embedded in Paraplast Plus (Oxford Labware, St. Louis, MO). Several sections were also embedded in Tissue-Tek Optimal Cutting Temperature Compound (Miles, Oneonta, NY), snap-frozen in liquid nitrogen, and stored at −80°C. The remaining endometrium was dissected from the myometrium, frozen in liquid nitrogen, and stored at −80°C for RNA extraction.

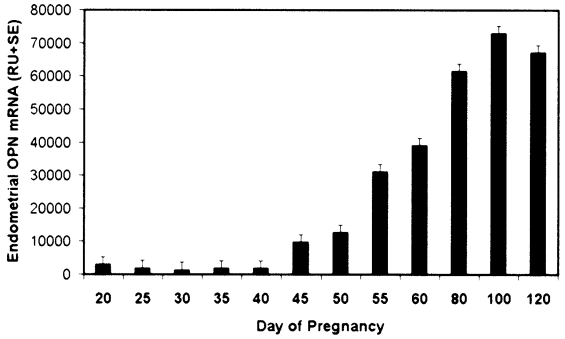

FIG. 2. Slot-blot hybridization analysis of steady-state levels of OPN mRNA in ovine total endometrium from Day 20 through Day 120 of pregnancy. The OPN mRNA levels are expressed as relative units (RU), normalized for differences in sample loading using the 18S rRNA, in 20 μg of total endometrial mRNA. Data are presented as least-square-means RU with overall SEM.

Histological Analysis

Ovine uteri were sectioned (thickness, 5 μm), deparaffinized in CitraSolv (Fisher Scientific; Pittsburgh, PA), and rehydrated through a graded alcohol series to distilled water. Tissues were then exposed to a Masson trichrome staining procedure as previously described [8]. This staining procedure results in black nuclei, red cytoplasm and muscle fibers, and blue ECM components.

RNA Isolation and Analyses

RNA isolation. Total cellular RNA was isolated from endometrial tissue samples using TRIzol reagent (Invitrogen, Carlesbad, CA) according to the manufacturer's recommendations.

Slot-blot analysis. Steady-state levels of OPN mRNA were assessed in endometrial total RNA samples by slot-blot hybridization analysis as described previously [22] using 20 μg of endometrial total RNA and ^{32}P-labeled antisense ovine OPN cRNA probe [5]. To correct for variation in total RNA loading, a duplicate RNA slot membrane was hybridized with ^{32}P-labeled antisense 18S rRNA cDNA (pT718S; Ambion, Austin, TX). The radioactivity in each slot was quantified by a Typhoon 8600 Imager (Molecular Dynamics, Piscataway, NJ) and expressed as total counts.

In situ hybridization analysis. Osteopontin mRNA expression in ovine uterine tissues was localized by in situ hybridization as previously described [5]. Deparaffinized, rehydrated, and deproteinated uterine cross-sections (thickness, 5 μm) were hybridized with ^{35}S-radiolabeled antisense or sense cRNA probes for ovine OPN [5]. Following washes and RNase A digestion, slides were dipped in Kodak NTB-2 liquid photographic emulsion (Kodak, Rochester, NY), stored at 4°C for 5 days, developed in Kodak D-19 developer, counterstained with Harris modified hematoxylin (Fisher Scientific), dehydrated, and protected with coverslips.

Immunofluorescence Analyses

Antibodies used for immunofluorescence staining included rabbit anti-OPN amino terminal (LF-124) and carboxyl terminal (LF-123) [23] and a fluorescein-conjugated goat antibody against rabbit immunoglobulin G (Chemicon International, Temecula, CA). Proteins were localized in frozen ovine uterine cross-sections as previously described [6]. Briefly, tissues were fixed in −20°C methanol, permeabilized with 0.3% Tween 20 in PBS, blocked in 10% normal goat serum, incubated overnight at 4°C with 2 μg/ml of primary antibody, and detected with fluorescein-conjugated secondary antibody. Slides were overlaid with a coverglass and Prolong antifade mounting reagent (Molecular Probes, Eugene, OR).

Photomicrography

Photomicrographs of representative fields of in situ hybridization, Masson trichrome staining, and immunofluorescence staining were evaluated with a Zeiss Axioplan2 microscope (Carl Zeiss, Thornwood, NY) fitted with a Hamamatsu chilled 3CCD color camera (Hamamatsu Corporation, Bridgewater, NJ). Digital images were captured using Adobe Photoshop 4.0 (Adobe Systems, Seattle, WA) and an Apple PowerMac G3 computer (Apple Computer, Cupertino, CA).

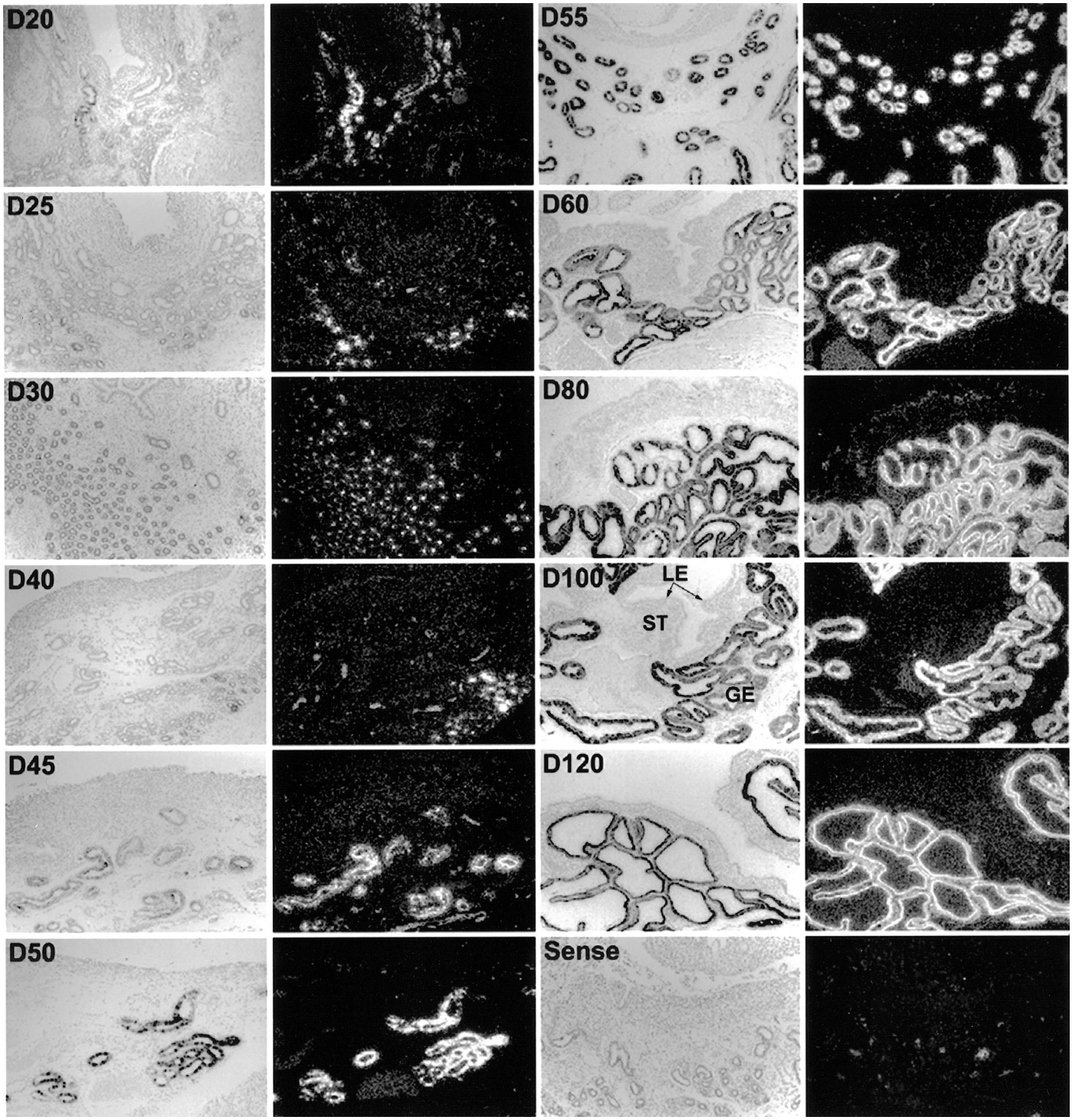

FIG. 3. In situ hybridization analysis of OPN mRNA in endometrial glandular epithelium (GE) of pregnant ewes. Corresponding bright- and dark-field images of endometrium from different days of pregnancy are shown. A representative section from Day (D) 40 hybridized with radiolabeled sense cRNA probe (Sense) serves as a negative control. LE, luminal epithelium; ST, Uterine stroma. Width of each field is 940 μm.

Statistical Analysis

Data from slot-blot hybridization analyses were subjected to least-squares ANOVA using the general linear models procedures of the Statistical Analysis System (SAS Institute, Cary, NC). Effects of day on steady-state levels of endometrial OPN mRNA were examined by regression analysis. Hybridization data (relative units) were normalized for differences in sample loading using the 18S rRNA data as a covariate in ANOVA. Data are presented as least-square-means relative units with overall SEM.

RESULTS

Localization of ECM at the Uterine-Placental Interface

In support of our working hypothesis that OPN is a component of histotroph contributing to an ECM that supports maternal-conceptus adhesion and communication throughout pregnancy, Masson trichrome staining of paraffin-embedded, Day 35 cross-sections revealed the deposition of

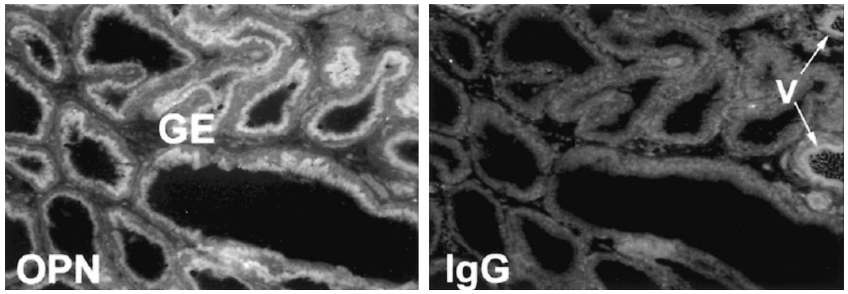

FIG. 4. OPN protein expression in ovine endometrial GE from a Day 100 pregnant ewe. The uterine cross-section shown represents Days 20 through 120 of pregnancy, because the staining pattern did not differ as a result of the day of pregnancy. The OPN was detected using immunofluorescence staining of frozen sections. Compare the absence of antibody staining in Day 100 endometrium when rabbit immunoglobulin G (IgG) was used to detect immunoreactive proteins. V, Vasculature. Width of each field is 940 μm.

ECM (blue staining) along the interface between uterine LE and placental tissue (Fig. 1). The following results indicate that OPN is an important constituent of this ECM.

Expression of OPN mRNA in Total Endometrium

Temporal changes in steady-state levels of OPN mRNA in total ovine endometrium from Days 25 through 120 of pregnancy were quantified by slot-blot hybridization (Fig. 2). The OPN mRNA remained constant through Day 40. Steady-state levels of endometrial OPN mRNA were affected ($P < 0.001$) by day of pregnancy. Overall, OPN mRNA levels were lowest on Days 20–40, increased 39-fold from Days 40 to 100, and remained maximal thereafter (cubic effect of day, $P < 0.0001$, $r^2 = 0.94$) (Fig. 2).

Localization of OPN mRNA Within Endometrial Glands

In situ hybridization analysis of endometrial cross-sections revealed expression of OPN mRNA in the middle to deep GE from Days 20 through 120 of pregnancy (Fig. 3). An increase in total hybridization was evident concomitant with the hypertrophy in endometrial glands that occurs between Days 50 and 60 of pregnancy (Fig. 3). As previously reported, OPN mRNA was also present in endometrial stratum compactum stroma between Days 25 and 120 of pregnancy [24]. However, hybridization was much less in these cells than in GE and is not readily apparent in Figure 3 because of the relative intensity of the GE signal.

Localization of OPN Protein in the Uterus and Placenta

The location of OPN protein in pregnant ovine uterus and placenta was assessed by immunofluorescence staining of frozen cross-sections using the LF-123 rabbit anti-OPN antibody that detects both native and the carboxyl half of OPN starting at the Arg-Ser (RS) thrombin-cleavage site or the LF-124 antibody that detects both native and the amino half of OPN ending at the RS site (see Fig. 5A).

OPN protein in endometrial glands. In agreement with in situ hybridization results, immunoreactive OPN protein (LF-123 antibody) was present in the endometrial GE of all ewes between Days 20 and 120 of pregnancy (Fig. 4).

OPN protein at the uterine-placental interface. Immunolocalization of OPN protein by immunofluorescence staining of serial cross-sections from Days 14 through 120 of pregnancy with LF-123 and LF-124 antibodies is illustrated in Figure 5B. Previous studies have established that LF-124 antibody detects a 45-kDa OPN cleavage product that contains the Arg-Gly-Asp (RGD) integrin-binding sequence and is secreted into the uterine lumen of ewes during early pregnancy, whereas LF-123 antibody does not detect 45-kDa OPN [6]. Both antibodies immunoreact with native 70-kDa OPN in immunofluorescence staining. Importantly, however, clear differences exist in the endometrial distribution of native 70-kDa OPN and the secreted 45-kDa fragment, which is exclusively, continuously, and abundantly present along the apical surface of LE, on trophectoderm, and along the entire uterine-placental interface of both interplacentomal and placentomal regions through Day 120 of pregnancy (Fig. 5B). As previously reported [24], OPN protein is also expressed in uterine stroma between Days 30 and 120 of pregnancy (Fig. 5B). Stromal OPN is detectable with either the LF-123 or LF-124 antibody, indicating expression of native 70-kDa OPN.

DISCUSSION

Results of the present study are the first, to our knowledge, to demonstrate that OPN is present at all sites of intimate contact between conceptus and maternal tissues between Days 30 and 120 of pregnancy in sheep. Furthermore, the results strongly suggest that OPN protein is synthesized and secreted from the GE as a constituent of histotroph that specifically binds to the uterine-placental interface throughout gestation in sheep. These findings are significant, because they definitively localize a secretory product of the GE to regions of contact between conceptus and uterus where OPN can potentially influence fetal/placental development and growth and mediate communication between placental and uterine tissues to support pregnancy.

Steady-state levels of total endometrial OPN mRNA increased approximately 30-fold between Days 40 and 80 of gestation. Although increases in total OPN mRNA in the sheep are confounded by a complex temporal and spatial pattern of expression that includes both an increase in OPN gene expression in stratum compactum stroma that begins between Days 20 and 25 of pregnancy [24] as well as constitutive expression within the GE, even a perfunctory examination of mRNA localization (Fig. 3) clearly indicates that the major source of OPN is GE. This is readily seen by comparing the relative magnitude of hybridization between stroma and GE on Days 80 and 100 of pregnancy.

Changes in OPN mRNA correlate with growth of endometrial glands that occurs as the uterus remodels to accommodate rapid conceptus development and growth during the last two-thirds of pregnancy [25]. During ovine gestation, GE undergoes hyperplasia through Day 50, followed by hypertrophy and maximal production of histotroph after Day 60 [26]. A similar correlation between GE development and gene expression during pregnancy has been shown for the well-defined secretory product of GE, uterine milk protein (UTMP) [26]. Endometrial gland morphogenesis and, by inference, increases in GE gene products, such as OPN and UTMP, likely are influenced by sequential uterine exposure to estrogen, progesterone, interferon-tau, pla-

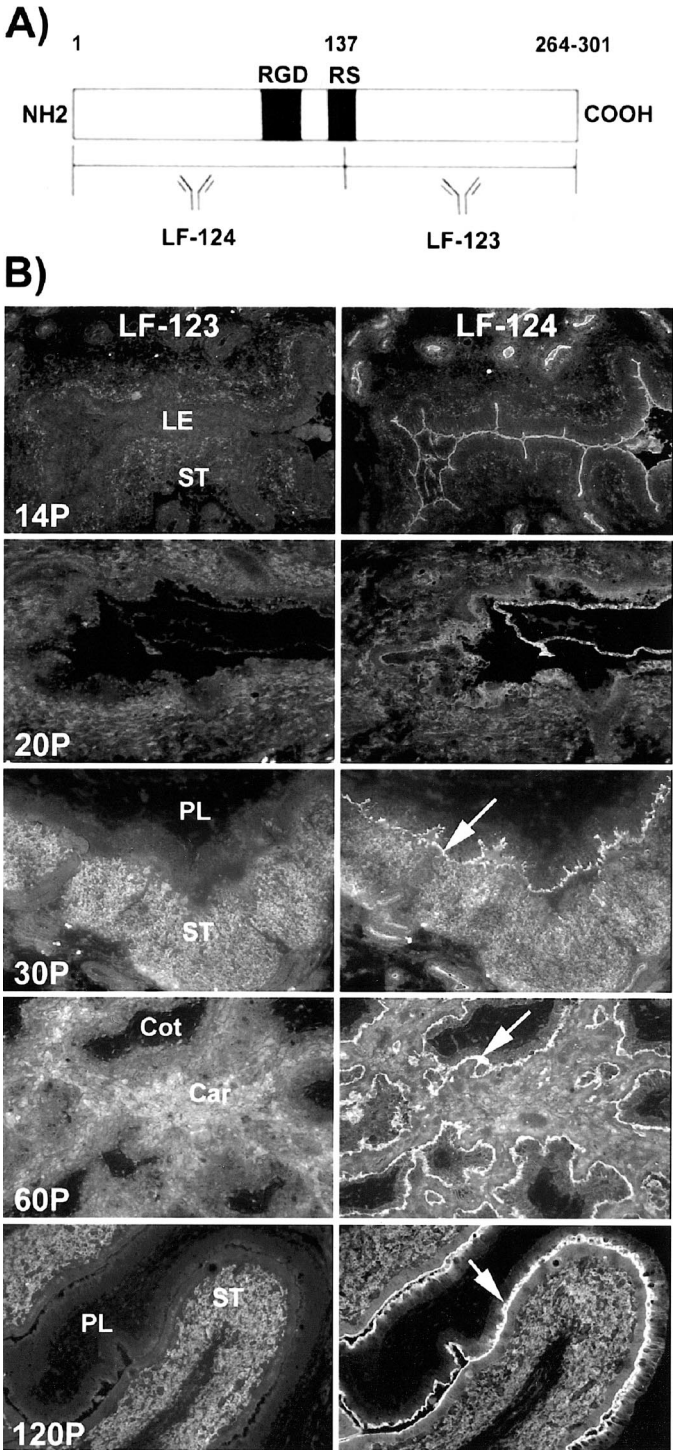

FIG. 5. Detection of OPN protein in ovine uterus and placenta using immunofluorescence staining of frozen sections. **A**) Immunoreactive OPN proteins were detected using immunoglobulin (Ig) G (LF-123; [24]) against the carboxyl half of human recombinant OPN (rhOPN) starting at the thrombin (RS) cleavage site and using IgG (LF-124; [23]) against the amino half of rhOPN ending at the RS site. **B**) White arrows denote the uterine-placental interface. Refer to Figure 4 for an example of representative background staining for the rabbit IgG control. Car, Caruncle; Cot, cotyledon; PL, placenta; ST, uterine stroma. Width of each field is 940 μm.

cental lactogen, and placental growth hormone [27, 28]. Using an ovariectomized ewe model, administration of progesterone increased GE expression of OPN and UTMP [21–27], and injection of a progesterone-receptor antagonist prevented progesterone effects on OPN mRNA [21]. Furthermore, intrauterine infusion of placental lactogen to progesterone-treated ovariectomized ewes increased OPN and UTMP levels over those with progesterone alone [27, 28]. Interestingly, regulation of OPN and UTMP genes diverge in regard to exposure to growth hormone. Whereas infusion of growth hormone into progesterone-treated ovariectomized ewes increased GE expression of UTMP mRNA, OPN mRNA was not affected [27, 28]. It is important to note that sequential treatment of ovariectomized ewes with progesterone, interferon-tau, placental lactogen, and growth hormone resulted in GE development similar to that observed under the influence of placental lactogen from the placenta during pregnancy[28].

The differential staining observed when serial cross-sections were immunolabeled with LF-123 and LF-124 antibodies provides significant insights regarding the mechanistic role of OPN during pregnancy. These antibodies were generated against the amino (LF-124) and carboxyl (LF-123) terminal fragments of recombinant human OPN that form when the native 70-kDa protein is cleaved by thrombin at its RS sequence [23]. Both immunoglobulins detect the native 70-kDa OPN, but only the LF-124 antibody recognizes the 45-kDa cleavage fragment that contains the integrin-binding RGD sequence and has increased cell attachment and spreading properties through increased accessibility of the RGD to integrin receptors [20]. Only the 45-kDa OPN supports $\alpha_9\beta_1$- and $\alpha_4\beta_1$-mediated cell migration and adhesion [29, 30] as well as attachment through several other non-RGD sites [31]. This fragment is the predominant form of OPN in uterine flushes from pregnant ewes [5, 6]. The 45-kDa OPN fragment is clearly present at the apical surface of uterine LE on Day 14 of pregnancy, on Day 20 conceptus trophectoderm, and along the entire uterine-placental interface of both placentomal and interplacentomal regions through Day 120 of gestation. Because OPN mRNA is synthesized by GE, but not by LE or conceptus, immunodetection of 45-kDa OPN protein at these locations confirms that OPN is secreted by GE and binds to all sites of direct attachment between conceptus and uterus. The source of OPN protein that localizes at the interface between caruncular epithelium and chorion likely is OPN secreted from neighboring GE in the intercaruncular endometrium. However, stromal and immune cells of placentomes express OPN, which may be transported to the interface between caruncular epithelium and chorion [24, 32].

Implantation/placentation is a progressive process involving adhesion molecule-dependent remodeling of endometrium and conceptus trophectoderm. Recently, OPN and its integrin receptors have become the focus of studies concerning adhesion for implantation. Lessey et al. [33] have established that transient endometrial expression of $\alpha_v\beta_3$ and $\alpha_4\beta_1$ integrins is cycle-dependent and defines the implantation window in women. Altered expression of these integrins is correlated with several causes of infertility [34]. Null mutations of α_v, α_5, β_1, or β_5 integrin genes in mice lead to peri-implantation lethality and failure of chorion-allantois fusion [35], whereas functional blockade of α_v and β_3 integrins in mice reduces the number of implantation sites [36]. Endometrial integrin expression has also been reported in several species that exhibit noninvasive implantation, including the sheep, pig, goat, and cow [6, 7, 37–39]. Uterine, placental, and/or conceptus expression of OPN has been demonstrated in cows, pigs, mice, baboons, humans, and sheep. Preliminary studies in cows suggest that OPN protein is localized to the apical surface of GE,

to stroma, and on trophectoderm cells of midpregnancy cows [40]. In pigs, OPN mRNA is synthesized in discrete regions of the uterine LE between Days 12 and 15 of pregnancy, is present throughout the entire LE thereafter, and appears in GE by Day 30 [41]. In the mouse, OPN is transcribed by trophectoderm, metrial gland cells of decidua, and placenta [42], whereas OPN protein is found in GE and decidualized stroma of baboons [43]. In women, high levels of OPN mRNA were first localized by Young et al. [10] to decidual stromal cells as well as endometrial glands of nonpregnant secretory uterus. The OPN transcript was later detected in hypersecretory endometrial GE of pregnant women [11, 44]. Omigbodun et al. [45] subsequently determined that progesterone increased OPN protein on cytotrophoblasts of the chorionic villus, but not syncytial trophoblasts, and concluded that OPN mediates adhesive and/or signaling events between these trophoblast cell types via α_v integrins. Importantly, endometrial expression of OPN is increased by progesterone during the menstrual cycle, starting on Days 16–17, and secretory GE appears to be the source of OPN protein that is localized on the apical LE [19, 44].

In sheep, OPN is a component of histotroph that increases in uterine flushings from pregnant ewes between Days 11 and 17 [6]. Exposure to progesterone induces OPN mRNA expression only in endometrial GE, but OPN protein is present on the apical aspect of the endometrial LE and trophectoderm [5, 6, 21]. Indeed, OPN has been proposed to bridge integrin receptors expressed by trophectoderm and uterus to induce adhesion between LE and trophectoderm essential for initial conceptus attachment in sheep and humans [6, 7, 44, 46]. Two recent reports using high-density microarray screening of human endometrium during the receptive phase for implantation have confirmed that OPN increases markedly during the window of implantation. A comparison of gene expression between endometrium from Days 8 to 10 of the cycle with Days 8–10 postmidluteal LH surge showed an 8.1-fold increase in OPN mRNA [47]. Similarly, microarray comparison of endometrium from Days 2 to 4 with Days 7 to 9 post-LH surge showed a 12.3-fold up-regulation [48]. Results from the present study support the results from studies of pigs [41] and cows [40] and firmly establish that glandular OPN continues to have a role in maternal-fetal interactions well beyond the receptive phase for implantation. The role of OPN at the uterine-placental interface is not clear, but this ECM protein has potential to mediate adhesion, remodeling, and cell-cell/cell-ECM communication at sites of contact between maternal- and fetal-derived tissues. The presence of the highly adhesive [20] and flexible [49] 45-kDa OPN cleavage fragment containing multiple binding sequences for interaction with different proteins [50] and capable of multimer formation [46] supports the hypothesis that OPN continues to serve as an adhesive ligand that bridges receptors on uterus and placenta throughout gestation. However, other roles for OPN are possible. For example, OPN fragments are known to initiate cell proliferation and migration [51] and may be involved in cytoskeletal reorganization for placentation. In addition, ECM proteins, such as OPN, can bind integrins to promote survival in cells that would otherwise undergo apoptosis [52], and OPN sequesters Factor-H, a plasma protein that binds and inactivates complement pathway components, to the cell surface and inhibits complement-mediated cell lysis [53]. Interestingly, both OPN and another endometrial secretory protein, decay-accelerating factor, which functions in a similar way to inhibit complement-mediated cell lysis, exhibit comparable temporal and spatial increases of expression in the peri-implantation human endometrium [54]. Therefore, OPN may serve to stabilize cells at the uterine-placental interface.

Collectively, OPN mRNA and protein localization in the present study confirm that OPN is a component of ovine histotroph that binds to the uterine-placental interface, where it could have profound influence throughout gestation. Experiments that address physiological, cellular, and molecular mechanisms involved in OPN-mediated attachment, proliferation, migration, and signal transduction using in vitro, ex vivo, and in vivo models are warranted to define the role of OPN during pregnancy.

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Interaction of Integrin Receptors with Extracellular Matrix is Involved in Trophoblast Giant Cell Migration in Bovine Placentomes

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Integrins are heterodimeric glycoproteins involved in cell–cell and cell–extracellular matrix adhesion and signal transduction. We evaluated the distribution and the putative role of integrin receptors and extracellular matrix (ECM) proteins during trophoblast giant cell (TGC) migration and fusion with uterine epithelial cells in the cow. Placentomes from 24 cows, covering day 80 to day 270 of gestation, were used for indirect immunohistochemistry against integrin subunits α_1, α_2, α_3, α_4, α_5, α_6, α_v, β_1, β_3, β_4 and ECM proteins collagen type I and IV, fibronectin, laminin.

The basement membranes of fetal and maternal epithelia and endothelia were immunoreactive for laminin, fibronectin and collagen IV. Collagens I and IV were found in maternal stroma, while fibronectin was present in fetal and maternal stroma. The integrin subunits α_2, α_6 and β_1 were observed in basal aspects of fetal and maternal epithelial and endothelial cells. Additionally, the α_6 and β_1 integrin subunits were colocalized with laminin on TGC. The integrin α_2 subunit was also found on TGC, but localized with a strong gradient to the basal side. Cells of the maternal connective tissue, including endothelium, expressed α_1, α_2, α_3, α_5, α_6, α_v, β_3 and β_4. The expression of α_2, α_5, α_v, β_3 and β_4 occured mainly in the septal tips. Cells of the fetal mesenchyme were positive for integrin subunits α_1, α_2, α_3, α_4, α_5, α_6, and β_1.

Our results indicate that $\alpha_2\beta_1$ collagen and $\alpha_6\beta_1$ laminin receptors anchor epi- and endothelial cells to basement membranes. We suggest that TGC migrate along a matrix of laminin and maintain cell–cell contact with mononuclear trophoblast cells via $\alpha_2\beta_1$ heterodimers. Integrins in maternal stroma and fetal mesenchyme may be involved in the regulation of proliferation and differentiation of maternal septa and fetal villi.

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INTRODUCTION

Integrins are cell surface adhesion receptors belonging to a family of transmembrane glycoproteins which display a heterodimeric structure of an α- and β-chain (Hynes, 1987). They interact with a variety of ligands including extracellular matrix (ECM) glycoproteins and cell surface molecules (Ruoslahti, 1991; Bosman, 1993) . The binding of integrins to their ECM proteins with regulatory functions during implantation and trophoblast invasion has been confirmed for the haemochorial human placenta (Fisher and Damsky, 1993). Large amounts of ECM proteins, like collagen type IV, fibronectin and laminin were shown during all stages of gestation in the placenta of macaques (Blankenship, Enders and King, 1992; Blankenship and King, 1993). Additionally, a diversity of integrin subunits is expressed throughout gestation in the human placenta and the macaque (Korhonen et al., 1991; Damsky, Fitzgerald and Fisher, 1992; Aplin, 1993; Burrows, King and Loke, 1993; Malak and Bell, 1994; Ruck et al., 1994; Douglas, Thirkill and Blankenship, 1999).

In contrast to haemochorial conditions found in primates and rodents, placentation in the cow is characterized by a 'restricted' invasion, since trophoblast giant cells (TGC) migrate towards the endometrium and fuse with uterine epithelial cells (Wooding, 1992; Klisch et al., 1999). That means the migration does not continue beyond the maternal basement membrane. Knowledge of the cell–cell interactions in the bovine placenta is limited and confined either to the secreted products of the embryo (Roberts et al., 1990) or to the time around implantation (Bazer, Ott and Spencer, 1994; MacLaren and Wildeman, 1995; MacIntyre et al., 2002). Larson, Ignotz and Currie (1992) suggested that the ECM, specifically fibronectin, plays a role in the early development of bovine embryos. During implantation of the closely related goat, Guillomot (1999) showed both the expression of the β_1 integrin subunit in uterine epithelial cells and collagen type IV

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and laminin in the basal lamina of the luminal and glandular epithelia and vascular endothelium, and their loss in uterine compartments adherent to the trophoblast. In cyclic and pregnant sheep integrin subunits α_v, α_4, α_5, β_1, β_3, and β_5 were constitutively expressed on conceptus trophectoderm and at the apical surface of uterine luminal and glandular epithelium during the implantation window thus being available for the assembly of osteopontin receptors (Johnson et al. 2001). Kimmins and MacLaren (1999) investigated the bovine endometrium during the estrous cycle and found the integrin subunits α_3, α_6, β_1, and the integrin receptor $\alpha_v\beta_3$ in luminal epithelium; from these α_6, α_v and β_3 showed a cycle dependent expression. In addition, the distribution of the α_6 and β_1 subunits in basement membranes of glandular epithelium and blood vessels suggests the presence of the $\alpha_6\beta_1$ laminin receptor (Kimmins and MacLaren, 1999). Day 24 TGC showed intense staining with the β_1 antibody, suggesting that β_1-integrin is involved in TGC migration (MacLaren and Wildeman, 1995). Recently, implantation-associated changes of integrin subunits a1, a3, and a6 as well as extracellular matrix proteins collagen IV and laminin in bovine endometrium and isolated binucleate trophoblast cells have been observed (MacIntyre et al., 2002). Also, changes in the localization of collagens I, III, IV and VI in the bovine uterine wall during the estrous cycle were reported (Boos, 2000). In contrast, there are no detectable structural changes in the basement membranes during epitheliochorial implantation in the porcine uterine epithelium (Bowen and Hunt, 1999) and the implantation cascade of the pig which lacks TGC is well defined (Burghardt et al., 1997).

In vitro studies with invasive trophoblast and TGC of rodents and the human, where the outgrowth of trophoblast cultured on different substrates was investigated, serve as models for implantation (Sutherland, Calarco and Damsky, 1988, 1993; Stephens et al., 1995; Yelian et al., 1995; Kilburn et al., 2000). Fibronectin-mediated trophoblast cell outgrowth was inhibited by antibodies against either the β_1 or the β_3 subunit suggesting that RGD-binding integrins, $\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ could mediate murine trophoblast adhesion in vitro and may play an important role during implantation (Yelian et al., 1995). The activity of trophoblast cells cultured on laminin was inhibited by antibodies against the β_1 integrin subunit (Stephens et al., 1995). The integrin subunits α_4, α_v, β_1 and β_3 were detected by immunofluorescence in TGC and in different areas of the murine placenta (Bowen and Hunt, 1999). The variable patterns of expression of certain integrin subunits in the murine pregnancy suggest that these proteins are directly involved in the process of implantation and normal placental formation and function (Sutherland, Calarco and Damsky, 1993; Aplin, 1997; Burghardt, Bowen and Bazer, 1997).

In this study, immunohistochemical techniques were used to evaluate the distribution and thus the possible role of integrin-ECM interactions during TGC migration and invasion in placentomes of the cow.

MATERIAL AND METHODS

Tissue preparation

Placentomes of 24 cows were collected at the local abattoir and assigned to six groups of gestation (days 80, 120, 150, 220, 240, 270 p.i.; *n* per group=4) according to fetal crown–rump-length (Schnorr, 1996). Tissue samples of about 1 cm^3 from at least three placentomes per animal were taken, snap-frozen in liquid nitrogen and stored at –80°C until use. Tissue blocks were mounted on specimen holders with Tissue Tec® (Sakura, USA) and cryostat sections (10–12 μm) were cut with a Reichert–Jung cryostat (Heidelberg, Germany) at −22°C and subsequently mounted on chrome–alum-coated glass slides (Pappas, 1971). In parallel, at least three other placentomes were perfusion-fixed with 4 per cent buffered formaldehyde, rinsed in phosphate-buffer (PB) at pH 7.4 and routinely embedded in paraffin. Sections of 3 μm were cut, mounted on Superfrost glass slides, deparaffinized in xylene, and rehydrated in a series of graded alcohol.

Immunohistochemistry

Cryostat sections were air dried for 1 h at room temperature and fixed with acetone/methanol (70 : 30) for 10 sec at room temperature. In the following procedure, all incubation steps were performed in a moist chamber at room temperature and all dilutions were carried out with phosphate-buffered saline (PBS, pH 7.2). After quenching the activity of endogenous peroxidase with 1 per cent H_2O_2 in PBS for 10 min, the sections were rinsed in PBS (3 × 5 min) and incubated in 0.02 per cent bovine serum albumin (BSA) for 20 min to reduce nonspecific binding. This was followed by incubation with primary antibodies for 1 h. The details of the antibodies used are shown in Table 1. After rinsing in PBS (3 × 5 min), the sections were incubated with a biotinylated horse anti-mouse/anti-rabbit secondary antibody for 20 min and rinsed again in PBS (3 × 5 min). Subsequently, incubation with an avidin/biotinylated horseradish–peroxidase complex (ABC-Method; Vectastain-Universal-ELITE-ABC-Kit®, Vector Laboratories, Germany) was used for signal amplification (45 min) according to the protocol of the manufacturer. After rinsing in PBS (3 × 5min), specific immunostaining was visualized with the chromogen 3-amino-9-ethylcarbazole (AEC; Vector Laboratories), allowing 10 min for staining. Finally, the sections were rinsed in distilled water (3 × 5min) and then counterstained with haematoxylin (30 sec) and rinsed with tap water (10 min) before they were mounted with Kaisers Gelatine. Control reactions were carried out without specific antibodies and normal mouse immunoglobulins (for monoclonal antibodies) or normal rabbit sera (for polyclonal antibodies) instead. A second control section was incubated with PBS. Slides were viewed with a Zeiss Axiophot photomicroscope (Oberkochen, Germany) and photographed with Ektachrome 64T film. A subjective scoring of the staining intensity was based on a five-point scale as strong, moderate, weak, variable and no immunoreaction.

Table 1. Details of the antibodies used

Antibodies	Clone (isotype)	Host	Dilution	Supplier (order no.)
Fibronectin	IST-3 (IgG1)	mouse	1 : 250	Sigma (F0791)
Laminin	Polyclonal	rabbit	ready-to-use	BioGenex (AR078)
Collagen IV	CIV 22 (IgG1)	mouse	1 : 100	Dako (M 785)
Integrin α_1	Polyclonal	rabbit	1 : 800	Chemicon (AB1934)
Integrin α_2	Polyclonal	rabbit	1 : 400	Chemicon (AB1936)
Integrin α_3	Polyclonal	rabbit	1 : 75	Chemicon (AB1920)
Integrin α_4	Polyclonal	rabbit	1 : 25	Chemicon (AB1924)
Integrin α_5	Polyclonal	rabbit	1 : 400	Chemicon (AB1928)
Integrin α_6	BQ 16 (IgG1)	mouse	1 : 75	Dako (M3511)
Integrin α_v	Polyclonal	rabbit	1 : 800	Chemicon (AB1930)
Integrin β_1	JB 1a (IgG)	mouse	ready-to-use	BioGenex (298M)
Integrin β_3	PM 6/13 (IgG1)	mouse	1 : 100	Chemicon (MAB1381)
Integrin β_4	Polyclonal	rabbit	1 : 1600	Chemicon (AB1922)
Secondary Antibody:				
Anti mouse/anti rabbit (biotinylated)	IgG (H+L)	horse	10–20 µl/ml	Vector (BA-1400)

Immunofluorescence

After rinsing in PB paraffin sections were pretreated with 20 µg/ml Proteinase K (Boehringer Mannheim, Germany) for 20 min at 37°C and rinsed again in PB (3 × 5 min). Normal goat serum (10 per cent) was used to block nonspecific binding IgG binding (1 h) before primary antibodies were applied to sections and incubated at 4°C overnight in a moist chamber. This was followed by rinses in washing buffer (PB with 0.2 per cent BSA, 3 × 10 min), incubation with secondary fluorescein-conjugated antibodies (1 h) at room temperature and final rinsing in washing buffer (3 × 5 min). Negative controls were incubated with either normal rabbit serum or mouse immunoglobulins and PB instead of primary antibodies. Counterstaining of nuclei was accomplished by the addition of 2 mg/ml Hoechst's dye (Sigma) to the first wash. The sections were mounted in Mowiol (Hoechst)-propyl gallate mounting medium and examined with a Reichert and/or Zeiss Axioplan photomicroscope equipped with epifluorescence illuminating systems using Ilford Delta 400 ASA film.

RESULTS

Staining patterns for all results presented were consistent among the animals of the same gestational age, but showed slight variation of the staining intensity at times. Negative controls showed no red staining or flourescent reaction. One representative negative control for each method is shown [Figure 2 (F), Figure 3 (C)].

ECM distribution in the bovine placentome

Potential ligands are shown in Table 2, and the distribution patterns for collagen type I (C–I) [Figure 3 (A)], collagen type IV (C–IV) [Figure 1 (A)], fibronectin (FN) [Figure 2 (D)] and laminin (Lam) [Figure 1 (E)] as well as for the different integrin subunits in the bovine placentome are summarized in Table 3.

Maternal stroma was strongly stained for FN and C–I throughout gestation, whereas C–IV and Lam immunostaining was weaker and Lam expression was confined to early gestation (days 80–150). FN was abundant in fetal stroma, whereas C–IV displayed weak immunoreactivity, and C–I and Lam were absent in fetal stroma throughout gestation.

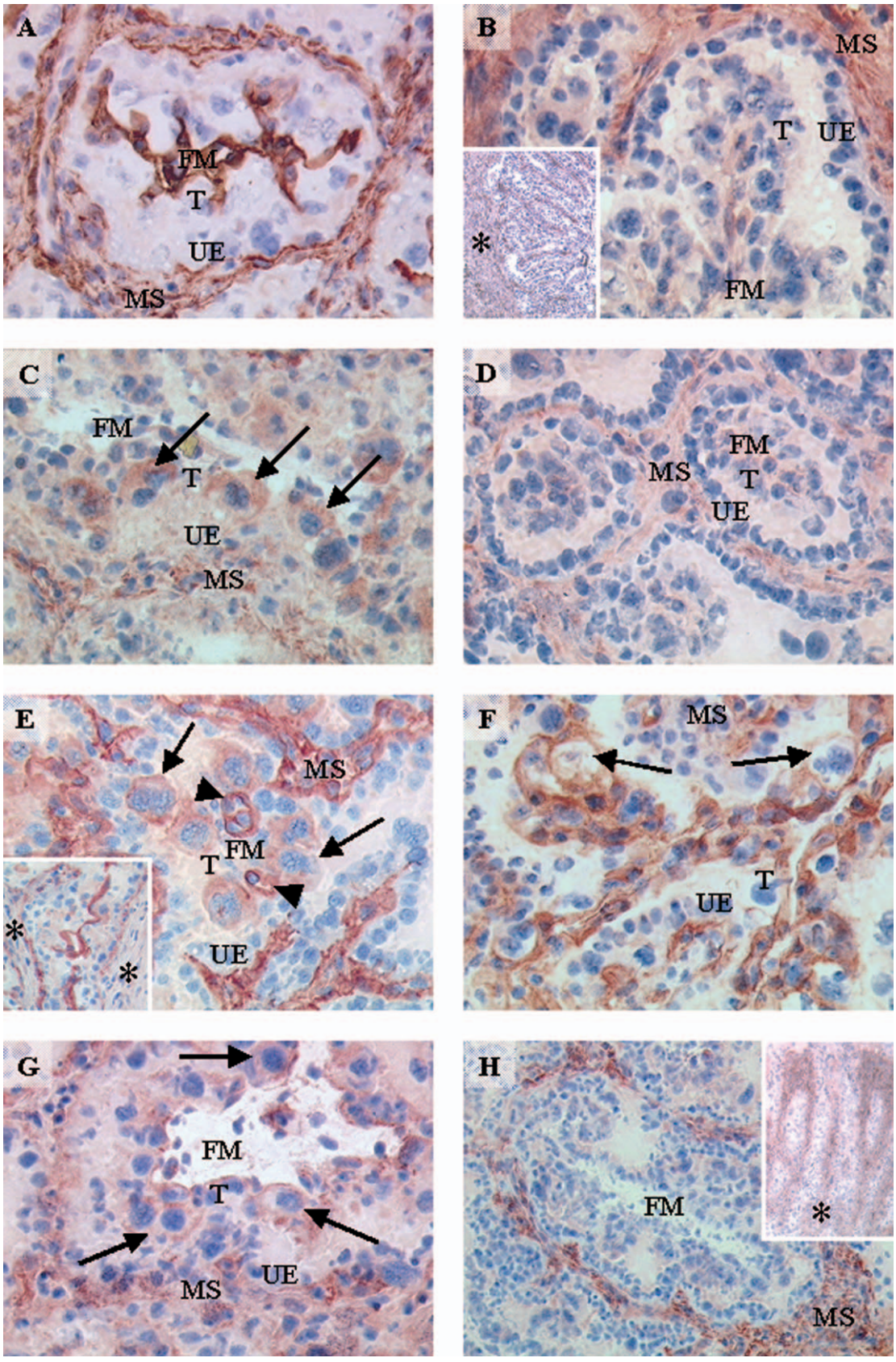
Walls of large maternal and fetal blood vessels continuously expressed FN, C–I, C–IV and Lam predominantly in the tunica media allowing a first distinction between thicker-walled arteries and thin-walled veins. In maternal blood vessels the signal of C–IV and Lam decreased late in gestation and only weak staining remained at day 270.

Maternal basement membranes (BM) strongly stained for FN throughout gestation. In the first half of gestation, Lam and C–IV were abundant in maternal BM, whereas later in gestation, the expression of Lam and C–IV diminished and at day 270 local loss of Lam was found, and C–IV was nearly undetectable in BM. Fetal BM showed intense staining for C–IV, FN, and Lam throughout gestation.

In addition, trophoblast giant cells (TGC) showed homogeneous cytoplasmic staining for Lam, especially early in gestation [Figure 1 (E)]. This signal shifted to staining along the cytoplasmic membrane later in gestation (day 220) where it remained until near term (day 270).

Integrin distribution in maternal septa

The α_1 [Figure 1 (B)], α_2 [Figure 1 (C)], α_3 [Figure 1 (D)], α_5 [Figure 2 (B)], α_v [Figure 2 (E)], and β_4 [Figure 1 (H)] integrin subunits were primarily expressed in cells of the maternal stroma, with highest intensity in the septal tips. The β_3 subunit was also found in maternal stroma, but its expression was confined to stroma cells of the stem septa in early gestation [Figure 2 (G)] and only reached the stromal cells of the tips of septa in late stages of pregnancy [Figure 2 (H)]. In contrast, expression of the α_1, α_2, α_5, α_v, and β_4 subunit remained the same in the septal tips, but decreased in stem and intermediate septa in late gestation [see insets to Figure 1 (B and H), Figure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2 (B and E)]. Integrin α_6 [Figure 1 (F)] and β_1 [Figure 1 (G), Figure 2 (C), Figure 3 (B)] immunoreactivity was observed at the basal aspect (adjacent to BM) of maternal epithelial and endothelial cells throughout gestation. Cells of the maternal stroma showed a weak immunoreactivity for the β_1 subunit also. In addition, the α_1 and α_2 subunits were associated to maternal blood vessels throughout gestation. The α_4 [Figure 2 (A)] integrin subunit immunostained maternal endothelial cells with a low intensity.

Integrin distribution in the fetal villi

Fetal mesenchymal cells showed intense immunoreactivity for the α_4 [Figure 2 (A)] and a weaker reaction for the α_3 [Figure 1 (D)] and β_1 [Figure 1 (G), Figure 2 (C), Figure 3 (B)] integrin subunit throughout gestation. Embedded endothelial cells strongly expressed the α_4 and β_1 subunit in all stages of gestation, whereas subunit α_2 [Figure 1 (C)] showed intense staining in late gestation (>day 220). Only a weak signal for the α_1 [Figure 1 (B)], α_5 [Figure 2 (B)] and α_6 [Figure 1 (F)] integrin subunits was detected in fetal vessels throughout gestation. Basal aspects of mononuclear trophoblast and fetal endothelium were positive for the α_6 [Figure 1 (F)] and β_1 [Figure 1 (G), Figure 2 (C), Figure 3 (B)] subunits. Unpolarized TGC highly expressed the α_6 [Figure 1 (F)] subunit along the cytoplasmic membrane at all stages of gestation and furthermore displayed a weaker, cytoplasmic staining for the integrin α_2 [Figure 1 (C)] in the basal half as well as a membrane-associated immunoreaction for the β_1 [Figure 1 (G), Figure 2 (C), Figure 3 (B)] subunit which both were most pronounced in early stages of gestation.

DISCUSSION

This is the first study presenting distribution patterns of ECM and potential integrin receptors in bovine placentomes throughout gestation, with special regard to TGC migration.

Laminin is found in BM of fetal and maternal epithelial and endothelial cells in bovine placentomes throughout gestation. In addition, the α_6 and β_1 integrin subunits are colocalized at the basal aspect of fetal and maternal epithelial and endothelial cells, thus neighbouring BM, suggesting the presence of a functional $\alpha_6\beta_1$ laminin receptor which anchors trophoblast and maternal epithelial cells as well as endothelium to the underlying BM (Ruoslahti, 1991; Bosman, 1993). This characteristic is most pronounced in early placentation (around day 80) and has also been proposed for α_6 and β_1 integrin subunits occurring in bovine cyclic endometrium (Kimmins and MacLaren, 1999) and during implantation (MacIntyre et al., 2002).

Alternately, the integrin subunit α_6 can heterodimerize with β_4 integrin in order to link epithelial cells to BM via hemidesmosomes (Jones, Hopkinson and Goldfinger, 1998). This has been shown for human cytotrophoblast cells in vivo and in vitro (Damsky, Fitzgerald and Fisher, 1992; Damsky et al., 1994). In bovine placentomes this seems to be possible only for uterine epithelial cells, since we detected the β_4 subunit only in cells of the maternal stroma and associated to BM.

Despite their unpolarized, migrating state, TGC also express the α_6 and β_1 integrin subunits colocalized with laminin throughout gestation. The presence of the β_1 integrin subunit in binucleated trophoblast cells in very early stages of the developing bovine placenta (day 24) has been associated with binucleate cell migration (MacLaren and Wildeman, 1995). Intriguing evidence for the involvement of laminin in migratory events has been presented for tumour cells, which migrate along a laminin matrix during extravasation (Schuppan et al., 1994). The close relation of invasiveness or migration of trophoblast and invading tumour cells suggests similar processes, since tumour cells and/or metastases not only migrate along laminin matrices, but can also produce their own laminin (Tani et al., 1997; Scarpa et al., 1999; Lohi et al., 2000; Schaumburg-Lever et al., 2000). In keratinocytes integrin α_2 may also heterodimerize with the β_1 integrin subunit forming either laminin receptors (Decline and Rousselle, 2001) or direct cell–cell contacts (Symington, Takada and Carter, 1993). We hypothesize that the α_2 subunit found in basal halves of TGC may be involved in both, since laminin is present and communication with neighbouring mononuclear trophoblast cells during migration is very likely. Collagen IV is abundant in fetal BM of trophoblast and endothelial cells in the bovine placentome throughout gestation, whereas the expression in maternal stroma and BM was of similar intensity

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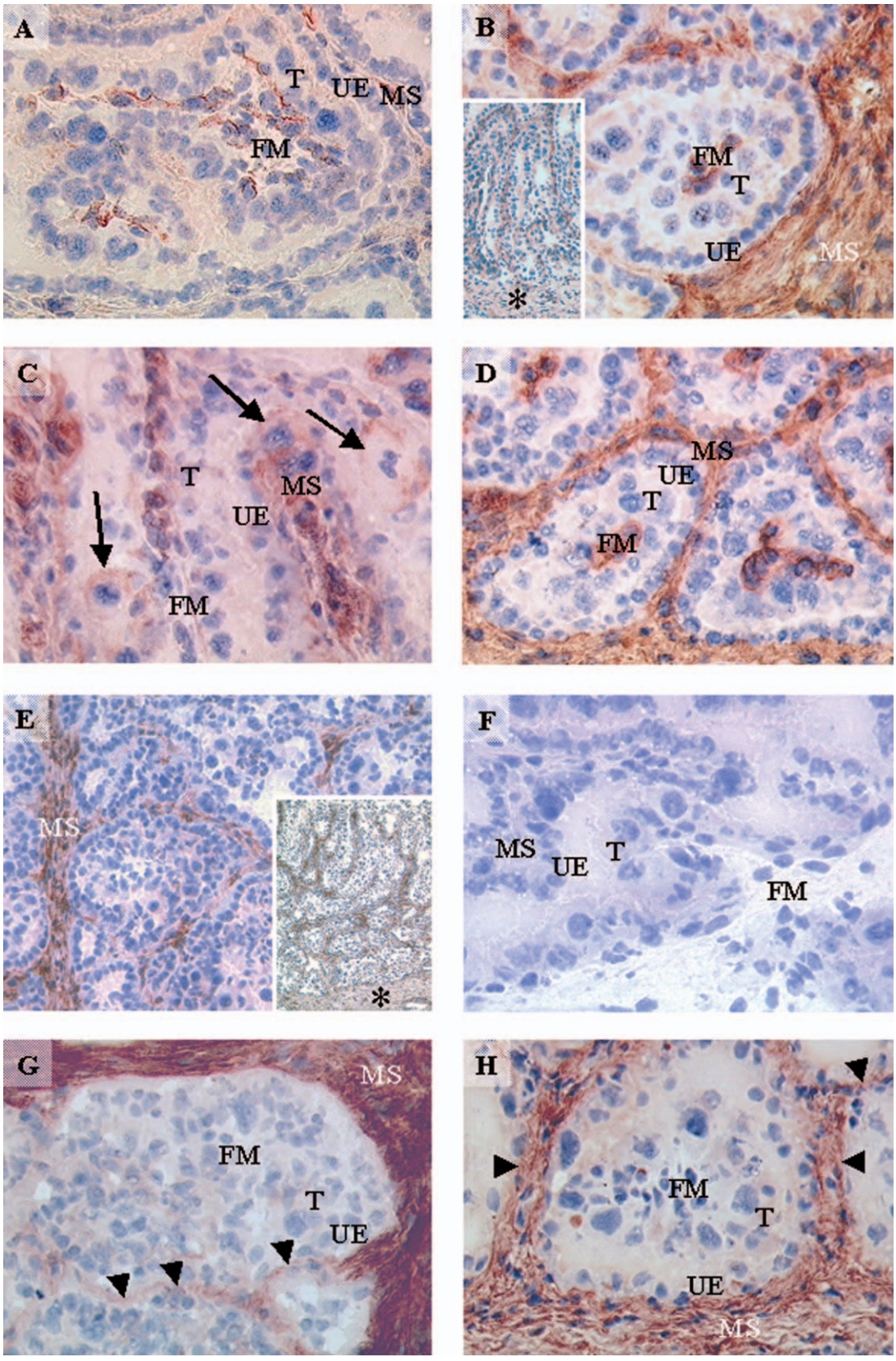
Figure 1. Immunohistochemistry for collagen IV (C–IV), laminin (Lam) and their integrin receptor subunits. **A**: day 270; C–IV is localized in maternal stroma with the majority found in basement membranes as well as in fetal basement membranes. B–D: alpha subunits of collagen receptors. **B**: day 80; in early gestation α_1 immunoreactivity is detected in maternal stroma of all parts of the villous tree and fetal mesenchyme. Inset: day 270 overview shows a distinct reduction of reactivity in maternal stem septa (asterisk) when compared to tips of septa (right side of inset). **C**: day 270; α_2 immunolabelling is observed in maternal stroma and associated to blood vessels in fetal mesenchyme. Trophoblast giant cells show a infranuclear cytoplasmic staining (arrows). **D**: day 115; α_3 is also detected in maternal stroma and fetal mesenchyme. **E**: day 85; Lam immunoreaction in fetal (arrowheads) and maternal basement membranes as well as in trophoblast giant cells (arrows) and maternal stroma. Inset: day 270; Lam is now confined to maternal and fetal basement membranes and trophoblast giant cells, while expression in maternal stroma (asterisks) is greatly reduced. **F**: day 270; Integrin α_6 (alpha subunit of laminin receptor) staining appears at the basal aspect of maternal and fetal epithelial and endothelial cells. Trophoblast giant cells (arrows) are labelled all along the cytoplasmic membrane. G–H: beta subunits of collagen (β_1 integrin) and laminin (β_1 and β_4 integrins) receptors. **G**: day 220; β_1 immunostains basal aspects of fetal and maternal epithelium and endothelium as well as trophoblast giant cells (arrows) along the cytoplasmic membrane. **H**: day 160; β_4 immunoreaction is observed in basal aspects of maternal epithelial and endothelial cells and in maternal stroma of all parts of the villous tree. Inset: day 270 overview distinctly shows that only the tips of septa (top of inset) remain to express the β_4 integrin subunit when compared to intermediate septa (asterisk). FM, fetal mesenchyme; MS, maternal stroma; T, trophoblast; UE, uterine epithelium. Original magnifications: A–G × 125; H × 62.5; insets: B, H × 31.25; E × 62.5.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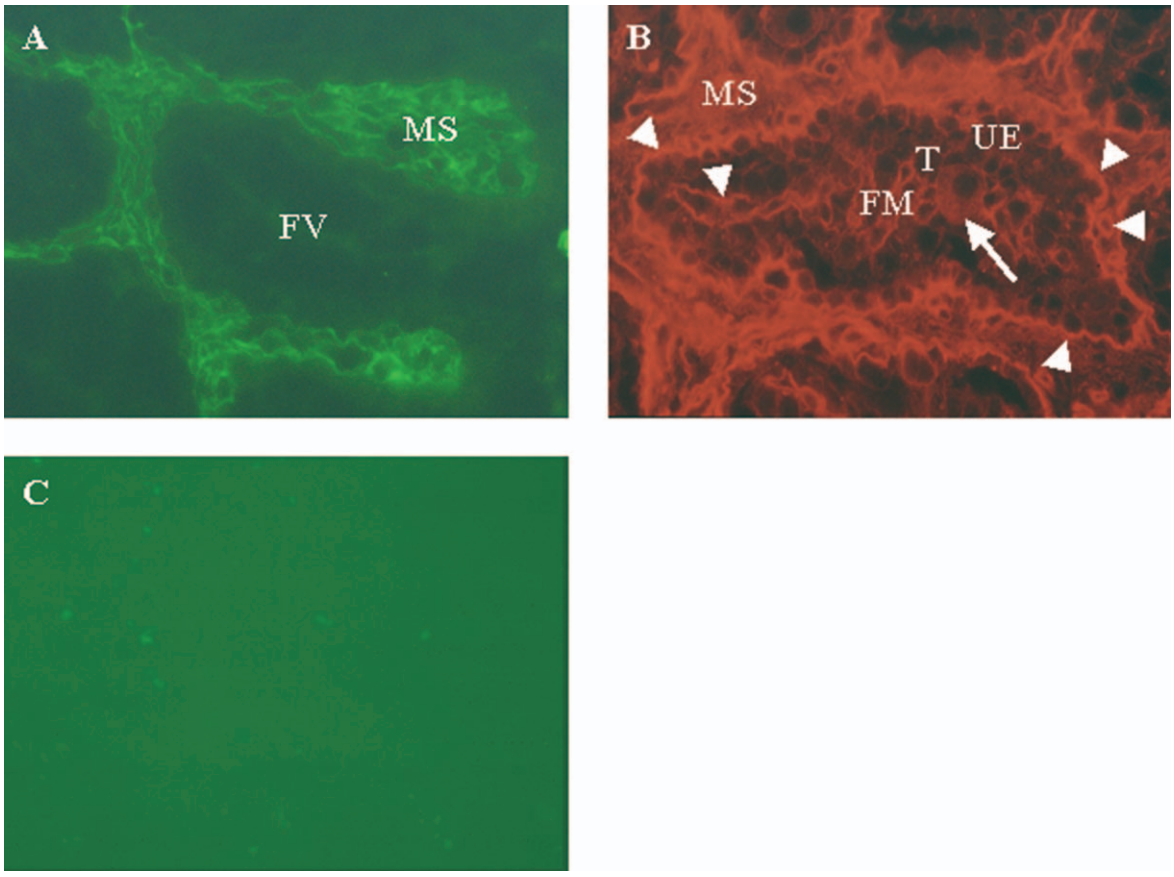

Figure 3. Immunofluorescence for collagen type I (C–I) and integrin β_1 subunit. **A**: day 220, tips of maternal septa; C–I immunofluorescence is confined to connective tissue of maternal septa. Fetal villus (FV) shows no immunofluorescence. **B**: day 150; β_1 immunofluorescence reveals that the labelling of the basal aspects of fetal and maternal epithelial and endothelial cells (arrowheads) is stronger than the stroma reaction. Trophoblast giant cells are also positive (arrow). **C**: day 150; negative control shows no immunofluorescence. FM, fetal mesenchyme; MS, maternal stroma; T, trophoblast; UE, uterine epithelium. Original magnification: × 125.

in early stages of gestation (<day 150), but decreased until late gestation (day 270), when only maternal stroma and embedded vessels showed a weak immunoreactivity. In contrast, the majority of C–I was found in maternal connective tissue, whereas the fetal villous mesenchyme showed only a slight reaction. Boos and Stelljes (2000) found no changes in the Collagen IV expression in the course of gestation, and were able to localize C–I and C–III also, associating C–I with the mechanical stability of the uterine wall. The loss of collagen IV in maternal BM during goat implantation was interpreted as a modification of the BM composition rather than its destruction (Guillomot, 1999). Potential ligands for collagens are heterodimers of α_1, α_2 and α_3 with β_1 which have all been detected in fetal and maternal stroma and/or BM of bovine placentomes.

Fibronectin was observed in maternal and fetal stroma as well as in BM of epithelia and endothelia in the bovine placentome throughout gestation, but was not detectable in preimplantational stages (d 14–24) (MacLaren and Wildeman, 1995). Two types of fibronectin have been characterized, maternal fibronectin and trophoblast-derived oncofetal fibronectin, which have been associated with the mediation of conceptus attachment in humans (Aplin et al., 1999) and pigs

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Figure 2. Immunohistochemistry for fibronectin (FN) and its integrin receptor subunits. A–B: alpha subunits of fibronectin receptors $\alpha_4\beta_1$ and $\alpha_5\beta_1$. **A**: day 120; Integrin α_4 was localized in fetal mesenchyme and maternal endothelium. **B**: day 80; α_5 immunoreactivity in maternal stroma of stem and terminal septa and fetal mesenchyme. Inset: Around day 270 the immunoreaction in stem septa (asterisk) is markedly reduced when compared to tips of septa (top of inset). **C**: day 240; β_1 immunolabelling is observed in maternal stroma and fetal mesenchyme with the majority found in endothelium. Trophoblast giant cells (arrows) are stained along the cytoplasmic membrane. **D**: day 90; FN immunoreaction was abundant in maternal stroma and fetal mesenchyme. E–F: Integrin subunits of fibronectin receptor $\alpha_v\beta_3$. **E**: day 105; α_v immunoreaction is found in maternal stroma of stem and terminal villi. Inset: day 220; a distinct reduction of the stem septal staining (asterisk) can be observed. **F**: day 150; negative control section shows blue counterstaining exclusively. **G**: day 150; in early gestation β_3 integrin stains only connective tissue of maternal intermediate and stem septa. In contrast, terminal septa (arrowheads) show only a faint β_3 immunoreaction. **H**: day 270; during late gestation, integrin β_3 stains maternal stroma of stem (bottom) and terminal septa (arrowheads). FM, fetal mesenchyme; MS, maternal stroma; T, trophoblast; UE, uterine epithelium. Original magnifications: A–D, F–H × 125; E × 62.5; insets: B × 62.5; E × 31.5.

Table 2. Ligand-specificity of integrin receptors

Collagen	Laminin	Fibronectin	Vitronectin	Tenascin
$\alpha_1\beta_1$	$(\alpha_1\beta_1)$	$(\alpha_3\beta_1)$	$\alpha_8\beta_1$	$\alpha_v\beta_1$
$\alpha_2\beta_1$	$(\alpha_2\beta_1)$	$\alpha_4\beta_1$	$\alpha_9\beta_1$	
$\alpha_3\beta_1$	$(\alpha_3\beta_1)$	$\alpha_5\beta_1$	$\alpha_v\beta_3$	
	$\alpha_6\beta_1$	$\alpha_8\beta_1$	$\alpha_v\beta_5$	
		$(\alpha_v\beta_3)$		
		$\alpha_v\beta_6$		

Brackets indicate weak binding affinity, $\alpha_6\beta_4$ associated to hemidesmosomes

Table 3. Distribution pattern of ECM components and integrins in the bovine placentome

	Fetal compartment					Maternal compartment			
	FS	FV	BM	T	TGC	UE	BM	MV	MS
C–I	–	–	–	–	–	–	+	+	++
C–IV	+	++	+++	–	–	–	+++	++	++
FN	+++	+++	+++	–	–	–	+++	+++	+++
Lam	–	++	+++	–	++*	–	++	++	+
α_1	+	++	–	–	–	–	–	+++	++
α_2	+	+++	–	–	++*	–	–	++	+
α_3	+	–	–	–	–	–	–	+	++
α_4	++	+++	–		–	–	–	+	+
α_5	++	++	–	–	–	–	–	+++	+++
α_6	+	++	–	+++	+++†	+++	–	++	++
α_v	–	–	–	–	–	–	–	++	+++
β_1	+	+++	–	+	++†	++	–	++	+
β_3	–	–	–	–	–	–	–	++	+++
β_4	–	–	–	–	–	–	–	++	+++

Abbreviations: FS, fetal stroma; FV, fetal vessels; BM, basement membrane; T, mononuclear trophoblast; TGC, trophoblast giant cells; UE, uterine epithelium; MV, maternal vessels; MS, maternal stroma.Staining intensity: [+++], strong immunoreactivity; [++], moderate immunoreactivity; [+], weak immunoreactivity; [+/−], variable signal; [−] no staining.

* Homogeneous stained cytoplasm.

† Staining along cytoplasmic membrane; d, days.

(Burghardt et al., 1997). The antibody used in this study did not discern between maternal and oncofetal fibronectin, but its localization exclusively in fetal mesenchyme and maternal connective tissue makes an involvement in feto-maternal adhesion not very likely. However, the ability of fibronectin to bind other matrix proteins, like collagen and heparin as well as cell surface receptors of different cell types, makes it a perfect candidate for the stabilization of the matrix and presumably also for the promotion and control of the motility of cells (Alberts et al., 1995). The $\alpha_5\beta_1$ integrin is the 'classic' fibronectin receptor and has been extensively investigated (Hynes, 1992), but the integrin heterodimers $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_v\beta_1$ and $\alpha_v\beta_3$ are likewise capable of binding cells to fibronectin (Ruoslahti, 1991; Bosman, 1993). In isolated human cytotrophoblasts fibronectin has been suggested to be a mediator of attachment, trophoblast spreading and syncytial formation (Bischof, Haenggeli and Campana, 1995). Also, a switch from integrin $\alpha_6\beta_4$ to $\alpha_5\beta_1$, marking the transition from proliferative to invasive phenotype, has been observed in the human extravillous cytotrophoblast when migrating through the fibronectin-rich maternal stroma (Damsky, Fitzgerald and Fisher, 1992; Damsky et al., 1994; Kaufmann and Castellucci, 1997). Finally these cells became $\alpha_1\beta_1$ positive, indicating their fully invasive state.

In the bovine placentome subunits α_1, α_2, α_3, α_5, and β_4 as well as α_v and β_3 were strongly expressed in the maternal stroma. Modulation of the expression (majority of β_3 in stem septa in early gestation and slow extension into septal tips contrasting the occurrence of α_1, α_2, α_5, α_v, and β_4 in tips of septa throughout gestation, together with a reduction in stem and intermediate septa in late gestation) supports the idea of differing functions, like regulation of stem septal proliferation by β_3 and development and differentiation of a functional tertiary exchange unit in the tips of the septa by a pool of integrins (α_1, α_2, α_5, α_v, and β_4). The presence of several integrin subunits in connection with the proteins of the extracellular matrix has been associated with proliferation and differentiation of the cells in the maternal stroma (Fazleabas et al., 1997) and gives rise to the hypothesis that different areas of the maternal stroma show different stages of development and differentiation from stem septa to the septal tips.

The $\alpha_v\beta_3$ integrin is a receptor for vitronectin, but can also bind to fibronectin (Ruoslahti and Pierschbacher, 1987). Both α_v and β_3 subunits are strongly expressed particularly in the septal tips in late gestation. Because of the locally concentrated signal within the septal tips we suggest that these stromal cells have an increased ability to differentiate and proliferate, since $\alpha_v\beta_3$ integrin heterodimer has been associated with tumour progression and invasiveness (Gladson and Cheresh, 1991). Vitronectin has not yet been reported in the bovine placentome. Another ligand for several integrin heterodimers, including $\alpha_v\beta_3$, $\alpha_v\beta_1$, $\alpha_v\beta_5$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, is osteopontin, a glycoprotein secreted by uterine glandular epithelium and deposited between uterine luminal epithelium and trophoblast, thus serving as a bridging ligand during attachment of the conceptus in primates (Fazleabas et al., 1997), pig (Garlow et al., 2002), and sheep (Johnson et al., 2001).

Besides collagen IV and laminin in basement membranes, the predominant extracellular matrix protein in the fetal compartment was fibronectin. Integrin subunits available for collagen binding were α_1, α_2, α_3, which were weakly expressed and β_1, while α_4 and α_5, both heavily expressed, dimerizing with β_1, constitute fibronectin receptors. In cyclic bovine endometrium α_4 was expressed in the endometrial stroma (Kimmins and MacLaren, 1999).

We conclude that the trophoblast giant cell migration or 'restricted' trophoblast invasion employs mechanisms used by tumour cells namely, the aquisition of laminin and the utilization of integrin subunits α_6 and β_1 for the basal attachment of epithelial cells to the basement membrane which can serve as model for invasive processes. Moreover, even after implantation and during placentation until term an orchestrated interaction of a variety of integrin receptors with extracellular matrix proteins occurs in the placentome of the cow.

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Modulation of Connexin Expression in Sheep Endometrium in Response to Pregnancy

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The expression pattern of two typical gap junction channel proteins, connexin 43 and connexin 26 (Cx43 and Cx26), was identified in the endometrium of sheep, a species with epitheliochorial type of implantation, by indirect immunohistochemistry during the cyclic phases, early and late pregnancy, and immediately after birth. The extent of Cx43 immunoreaction bound to endometrial stromal cells of the early implantation stage (day 15 p.c.) was comparable to the situation observed in oestrus. The subsequent intensification of feto–maternal contact correlated with a striking increase of stromal Cx43 in the intercaruncular and caruncular regions of the uterus (days 18 and 21 p.c.) and the induction of Cx26 in the glandular epithelium of late implantation (day 21 p.c.). In contrast, both gap junction proteins, coexpressed in the stroma of placentomes and interplacentomal sections on days 131 and 145 p.c., decreased during late pregnancy, while an intense and augmenting staining for Cx26 was detected at the cell borders of the glandular and luminal epithelium. The spatial and temporal distribution of both connexins suggests that, under embryonal and hormonal influences, gap junctional communication is involved in the implantation process and the regulation of endometrial tissue functions during sheep pregnancy and indicates further, that this connexin expression path resembles more the invasive type of implantation.

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INTRODUCTION

Cell–cell-coupling via gap junctions has been shown to play an important role in tissues of the reproductive tract during the last two decades. Representing specialized contact areas between adjacent cells, gap junctions provide pathways for direct intercellular communication, that could be associated with essential biological functions such as cell growth and differentiation, proliferation of tissues and embryonic development. Gap junctions contain aggregates of channels, each formed by the hemi-channels of two neighbouring cells, the connexons, which are composed of the hexamere arrangement of integral proteins, the connexins [1,2]. Being members of a multigene family with now 20 members in the human genome [3] the cell-typic expression of these proteins could be identified immunologically in many tissues. The exchange of ions and small molecules such as second messengers via gap junctions is known to permit both the electric and metabolic coupling and coordination of cells [4–6]. In hormonal target organs connexins have been shown to be regulated by steroid hormones and mediators of the inflammatory cascade. In the myometrium, a strong rise of gap junction expression at term could be related to the development of synchronous, effective labour contractions in several species including the sheep [7–10].

In mammalian species, pregnancy requires above all an appropriate differentiation of the endometrium which permits taking-up and maintenance of the feto–maternal contact to establish a nutrition route. As revealed by indirect immunocytochemistry, the decisive gap junction proteins in rat endometrium are Cx43 in the stromal compartment and Cx26 in the uterine epithelium, expressed in a characteristic spatial and temporal manner, beside the much less abundant connexins, Cx32 and Cx37 [11–13]. Studies on both connexin transcripts have demonstrated that gap junction formation in endometrial tissues of rodents is completely suppressed during receptivity by maternal progesterone. At implantation, however, connexins are locally induced in response to the implanting blastocyst by unknown embryonic signals [12,14–16]. The structural and functional coupling of uterine cells could be interpreted there as important precondition for successfull invasive implantation. Very little is known about endometrial gap junctions in animals with non-invasive modus of implantation. The sheep with its placenta cotyledonaria s. multiplex [17,18] represents a type not only advantageous but also very interesting for investigation because of its high rate of embryonic death (30 per cent) and complications during parturition [19,20]. In

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contrast to animals with invasive type of implantation, in the ruminants all endometrial layers remain intact and consequently nutrition is transported from maternal vessels via the stromal compartment and uterine epithelium to the embryonic compartment. In order to clarify, if a specific development of endometrial connexins characterizes this kind of feto–maternal junction, we studied the Cx43 and 26 expression pattern in endometrial tissues of the sheep during early and late pregnancy and compared it to the cyclic phases and the situation after birth. As a result we can report here on a connexin induction during the progression of implantation which is species-specific, but resembles the invasive type of implantation.

MATERIAL AND METHODS

Animals

Ten merino ewes were supervised and covered in the Clinic for Veterinary Obstetrics, Gynaecology and Andrology of the Justus-Liebig-University Giessen, Germany. Ewes were killed during oestrus and dioestrus, on days 15, 18, 21, 131 and 145 of pregnancy and 20 min after delivery. Blood was drained from the animals after they had been shot by bolt and the reproductive tract was removed immediately. At day 131 of pregnancy euthanasia of the fetuses was necessary, while lambs of the 145th day were well and viable. Two animals were sacrificed for each phase of late pregnancy, one pregnant with one fetus and the other with twins. For the states of early pregnancy, the pregnant uterus horn had been determined before by ultrasonics. Experimental and surgical procedures as well as the slaughtering of the ewes were approved by the Ethical Committee of the Justus-Liebig-University Giessen.

Immunocytochemistry

Specimens were taken from different localizations of both uterus horns and, after a quick plunge bath in Isopentane, were immediately frozen in liquid nitrogen. Cryostat sections (4.5 μm) were postfixed in ice-cold absolute ethanol for 5 min, then rinsed twice in PBS (phosphate buffered saline) for 5 min and kept overnight in a 0.5 per cent BSA (bovine serum albumin) in PBS solution at 4°C. Indirect immunocytochemistry was received using affinity-purified, polyclonal rabbit antibodies against the C-terminus of rat Cx43 [21] and Cx26 as well as Cx32 from mouse liver gap junctions [22] at a dilution of 1 : 25 with PBS/BSA. Tissue sections were incubated for 90 min at cool temperature (4°C). After rinsings three times in PBS, they were incubated with the secondary antibody, FITC (fluorescein isothiocyanate)-labelled rabbit immunglobulins, conjugated from swine (Dako, Hamburg), at a dilution of 1 : 40 with PBS/BSA. After three rinses in PBS/BSA sections were left for a short moment in aqua bidest, then covered with ice-cold p-phenylendiamin-glycerin (Sigma) to avoid fading of the fluorescence. To test the cross reactivitiy and specifity of anti-Cx26, 32 and 43 in sheep tissues immunohistochemistry was performed on mouse as well as rat heart and liver sections and compared to liver and skin of the sheep. Controls were performed by omitting the primary antibody and using preimmune sera instead. Photographs were taken with an Axiophot microscope (Zeiss) equipped for epifluorescence. All photographs were taken with the same camera setting, films were developed according to the same standard protocol and prints were produced using the same shutter settings. The intensity of immunofluorescence on the prints was assessed by the same investigator (S. G.) and scored from none to strong immunofluorescence: −, (+), +, ++, +++. Generally, a whole population of cells within a certain tissue (for example: luminal epithelium) showed immunofluorescence, therefore the scoring index refers to the intensity observed.

RESULTS

Cross reactivity of the Cx-antibodies with sheep connexins

Cxs26, 32 and 43 antibodies showed a clear cross reactivity with sheep connexins. Specific punctate staining for Cx26 and for Cx32 in the sheep liver is shown in Figure 1 [A, C]. Similarly to the situation in mouse liver tissues Cx26 immunoreactivity is less intensive in sheep liver compared to Cx32 staining (Figure 1 [A–D]). Cx43 showed a specific staining in keratinocytes of sheep skin and in sebaceous glands (data not shown).

Cx expression in sheep endometrium

In all stages investigated no specific staining for Cx32 could be detected in any endometrial compartment. Results for Cx26 and Cx43 are summarized in Table 1.

Oestrous cycle. In the progesterone-dominated cyclic phase (dioestrus) an only weak immunolocalization of the Cx43 antigen was noticed in the upper endometrial stromal compartment (stratum compactum) underlying the uterine epithelium (Figure 2 [A]). In contrast, the oestrous phase was characterized by a significant elevated expression of Cx43 spreading out into the whole stroma in a typical punctate manner (Figure 2 [B]). The epithelia lacked any specific reaction using the Cx43 antibodies. The Cx26 protein was missing in all tissues of the cyclic phases (Table 1).

Pregnancy.

Implantation. At day 15 of pregnancy the caruncular sites had become more prominent and folded, while approximation of trophoblast and maternal surface was characterized by a still lose contact of both tissues. At this stage of apposition, distribution and intensity of Cx43 immunofluorescence bound

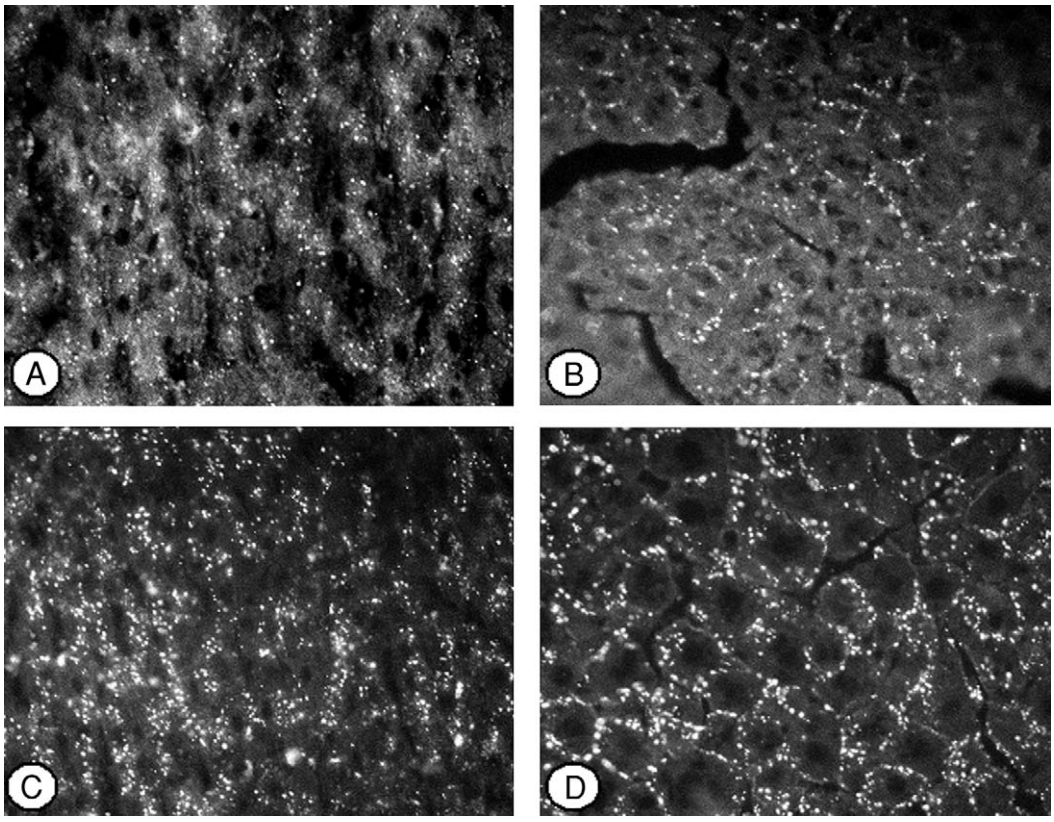

Figure 1. Immunofluorescence for Cx26 (A, B) and Cx32 (C, D) in sheep (A, C) and mouse (B, D) liver. A typical punctate staining at the liver cell membranes is seen for both connexins in sheep as well as in mouse liver. In both species Cx26 staining is less intensive compared to Cx32 immunoreactivity. × 525.

Table 1. Quantitative synopsis of results

Non-pregnant/ pregnant state	Intercaruncular/interplacentomal stroma stratum compactum/ stratum reticulare		Stroma of caruncles/placental stroma		Luminal epithelium	Glandular epithelium
	Cx43	Cx26	Cx43	Cx26	Cx26	Cx26
Dioestrus	(+)	−	(+)	−	−	−
Oestrus	++	−	++	−	−	−
15 days p.c.	++	−	++	−	−	−
18 days p.c.	+++	−	+++	−	−	−
21 days p.c.	+++	−	+++	−	−	+
131 days p.c.	++/(+)	(+)/−	++	+	+	++
145 days p.c.	++/(+)	(+)/−	++	+	++	+++
20 min p.p.	+/++	−	+	−	+++	++

Range of immunofluorescence intensities: −, none; (+), weak and only occasional; +, weak; ++, intermediate; +++, strong. /, distinguishes between stratum compactum/stratum reticulare.

to the endometrial stromal cells was comparable to that observed in oestrus (Figure 2 [C]). The progression of implantation and definitive attachment of the blastocyst correlated with a dramatic increase in number and size of Cx43 plaques in all intercaruncular (Figure 2 [D, E], and Figure 3 [A, B]) and caruncular (Figure 3 [D]) stromal compartments of both the pregnant and non-pregnant horn (days 18 and 21 p.c.). A remarkably high density of Cx43 protein was noticed in the periphery of big maternal blood vessels in all implantation phases (Figure 2 [D]). At day 21 p.c. induction of small Cx26-specific spots was detected at the lateral cell borders of the upper glandular epithelium in the intercaruncular region, but not in the luminal epithelium (Figure 3 [C], Figure 4 [A]). No Cx26 could be recognized within the caruncles which were denuded from luminal epithelium at this time.

Late pregnancy. A striking change in connexin expression followed in the stages of late pregnancy, harmonizing partially with the morphologic alterations from implantation onwards. Thickness and density of the maternal stroma had diminished,

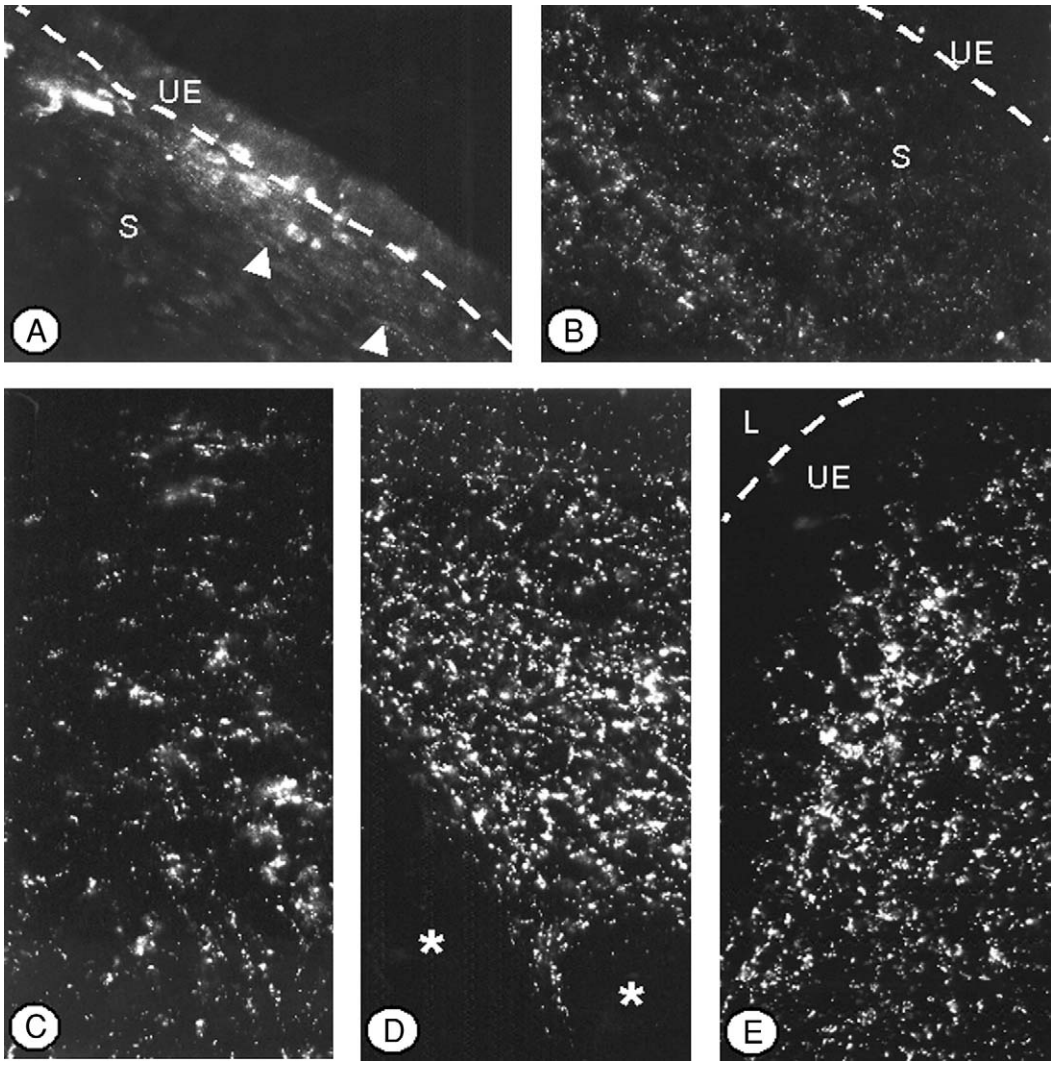

Figure 2. Immunofluorescence for Cx43 in uteri of sheep during estrous cycle and around implantation. A: Dioestrus; Cx43 presence in the stroma (S) is sparse and restricted to the upper part of the stratum compactum (arrowheads). No specific staining can be observed in the uterine epithelium (UE). The dotted line points out the location of the basement membrane. B: Oestrus; abundant typical punctate spots indicating the Cx43 protein is distributed in the stroma (S). The uterine epithelium (UE) is free of fluorescence. Dotted line, position of basement membrane. C: Day 15 p.c.; the expression pattern of stromal Cx43 antigen at this early stage of pregnancy is comparable to the situation found in the oestrous phase (compare to Figure 2 [B]). D: Day 18 p.c.; the density of Cx43 immunolocalization in the stroma has increased. Compare higher protein concentration in the vicinity of big maternal blood vessels (asterisks in non-fluorescent vascular lumen) to area in top of micrograph. E: Day 21 p.c.; Cx43 plaques in the stroma show maximal size and quantity, whereas the uterine epithelium (UE) lacks any specific reaction. The dotted line marks off lumen (L) and underlying uterine epithelium, where Cx43 fluorescence is missing. × 525.

correlating especially in the interplacentomal region with a reduced and smaller Cx43 reaction at the stromal cells of the stratum compactum. Colocalization of Cx26 (only stratum compactum) was seen occasionally at a lower and also decreasing level towards birth. Cx26-specific plaques were always smaller than those indicating the Cx43 protein. Cx43 detection in the deep interplacentomal stroma which enclosed the glands (stratum reticulare) was extremely weak (Table 1).

The stromal components of the late placentome were represented by a cell-rich stromal base and the interdigitating septa of the placentomal labyrinth containing maternal vessels and connective tissue cells. Cx43-specific immunoreaction was more abundant in the placentomal than in the neighbouring interplacentomal stroma, but also declined towards delivery (Figure 5 [A]). Interestingly, Cx26 coexpression was noticed in all sections of the placentomal stroma (Figure 5 [B]), in contrast to the interplacentomal regions. The maternal septa were marked by less punctate but more diffuse staining (Figure 5 [E]). These observations were compatible in the four animals of the late pregnant stages. No difference in Cx26 and Cx43 fluorescence was found between the pregnant and non-pregnant horn in the uteri containing only one fetus!

In the interplacentomal sites of late pregnancy, an intense and increasing Cx26 reaction was bound to the lateral and basal cell borders of the luminal and glandular epithelium (Figure 4 [B, D]). The latest one (day 145 p.c.) exhibited the strongest Cx26 presence found in all tissues and stages investigated. In both epithelia precise punctate spots were arranged along

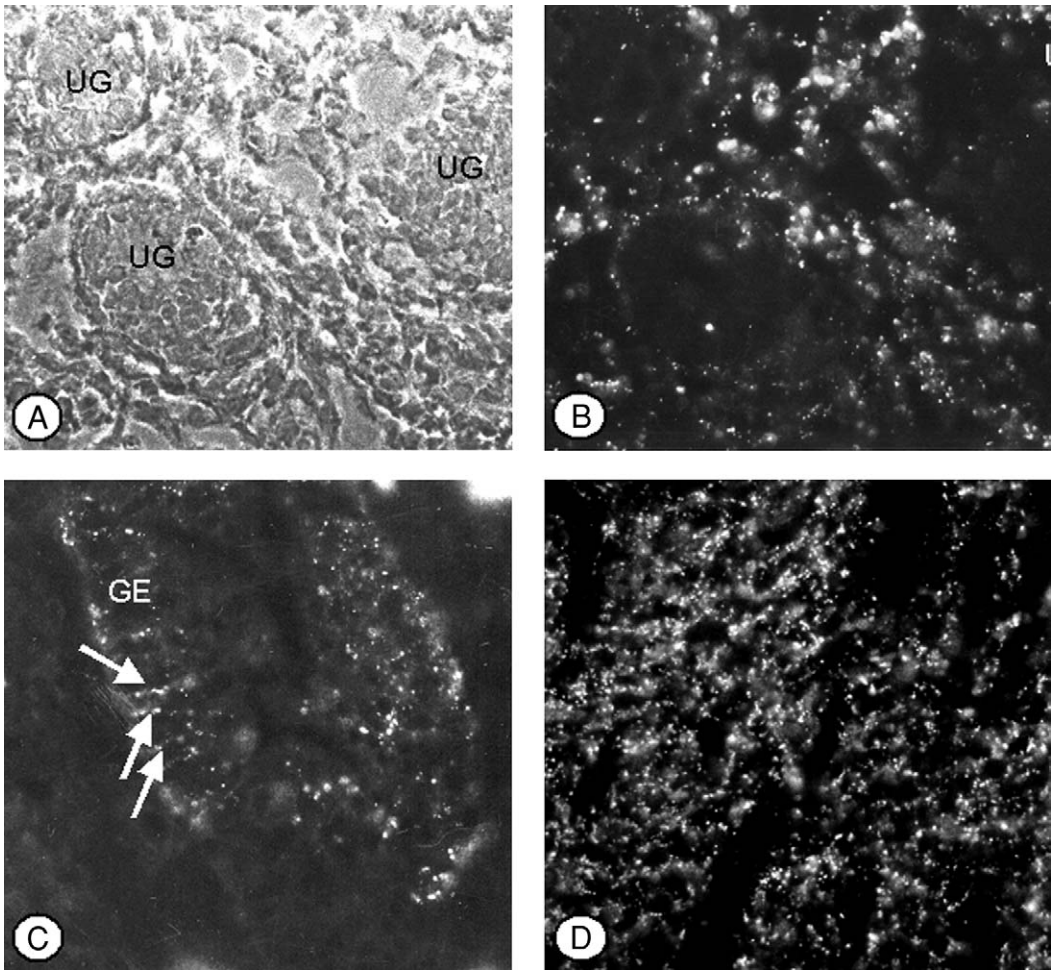

Figure 3. Endometrial localization of Cx26 and Cx43 around implantation. A, B: Day 18 p.c. A: Phase-contrast micrograph shows sections of uterine glands (UG), enclosed by stromal cells of the stratum reticulare. B: Immunofluorescence of the same section showing that Cx43 antigen is restricted to the stroma, but missing in the glandular epithelium. C: Day 21 p.c.; immunohistochemical staining with anti-Cx26 antibody shows induction of Cx26 protein at the cell borders (arrows) of the glandular epithelium (GE). D: Day 21 p.c.; extremely high amounts of Cx43 antigen are bound to the stromal cells of the caruncular regions. × 525.

the lateral cell borders whereas bright and fuzzy staining characterized the basal lining of the cells towards birth.

Postpartum. Twenty min after delivery levels of Cx43 in the interplacentomal stratum compactum and the placentomal stroma and of Cx26 in the glands had been reduced significantly (Figure 4 [C], Figure 5 [C]). Stromal coexpression of Cx26 had disappeared (Figure 5 [D]). In contrast, Cx26 abundance in the luminal epithelium was even enhanced, restricted now to the lateral cell borders (Figure 4 [E]), while a surprising rise of Cx43-specific staining was found at the stromal cells of the reticular layer (Table 1).

DISCUSSION

Specificity of the connexin antibodies

The polyclonal rabbit antibodies raised against the carboxyterminus of rodent connexins showed a cell specific expression of Cx26 and Cx32 in sheep liver as well as Cx26 and Cx43 in sheep skin. Furthermore, a typical punctate reaction indicating gap junction plaques at the cellular borders were demonstrated without any unspecific background staining. Cross reactivity of rabbit polyclonal antibodies against rodent Cx26, Cx32 and Cx43 has already been demonstrated by Grazul-Bilska et al. [23] for sheep ovarian follicles and corpora lutea. The specificity of each connexin isoform can be explained by the differences of the amino acid sequences of the carboxyterminus. Thus, the cross reactivity with the specific connexin in sheep tissues is due to the highly conserved amino acid sequence of the carboxyterminus among numerous species [3].

Implantation

In early pregnant sheep endometrium the spatial and temporal pattern of the two gap junction connexins Cx26 and Cx43 seems to be directly related to the taking up of feto-maternal contact. Like in rodents, Cx26 and Cx43 are the major channel

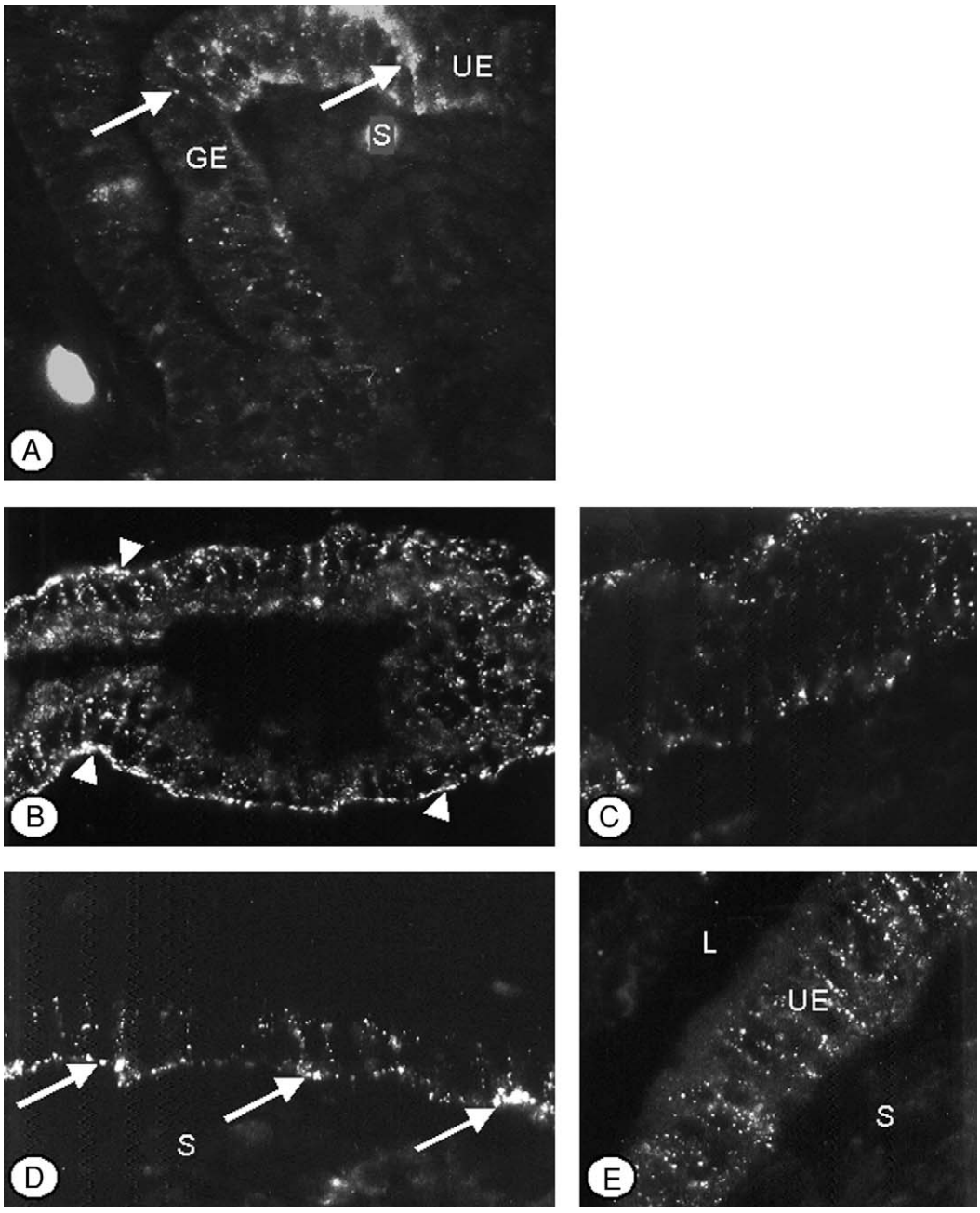

Figure 4. Cx26 immunofluorescence during early and late pregnancy and post partum. A: Day 21 p.c.; induction of small Cx26-specific punctate reaction is restricted to the cell borders (arrows) of the glandular epithelium (GE). Note that no immunoreaction is found in the adjacent luminal epithelium (UE) and stroma (S). B: Day 145 p.c.; high levels of Cx26-antigen are arranged in typical spots at lateral cell borders of the glandular epithelium, whereas a bright and more fuzzy staining characterizes its basal lining (arrowheads). C: 20 min p.p.; Cx26 immunofluorescence in the glandular epithelium has been reduced significantly. D: Day 145 p.c.; the luminal epithelium exhibits a similar but weaker Cx26 expression during late pregnancy. Cx26 plaques at the basal cell borders are marked by arrows. The underlying stroma (S) lacks any specific reaction. E: 20 min p.p. Postpartal presence of Cx26 in the luminal epithelium (UE) is enhanced at the lateral cell borders. S, Stroma. L, Lumen. × 525.

proteins characterizing the endometrium and Cx32 seems not to play a role in the pregnancy-related differentiation processes of the endometrium. Throughout gestation Cx26 represents the connexin isoform for the epithelium and Cx43 was always restricted to the stromal compartment.

In the sheep, both connexins seem to be regulated in correlation to the reproductive phase. At the very beginning of implantation (day 15 p.c.) the expression of Cx43 bound to the endometrial stromal cells is still similar to that observed in oestrus and Cx26 in the epithelium is missing. Since cross-reactivity of the antibody with sheep Cx26 showed that these findings are different from the findings in rats [15,24] however, it cannot be excluded that other isoforms not tested yet are present instead. At the definitive attachment period of the

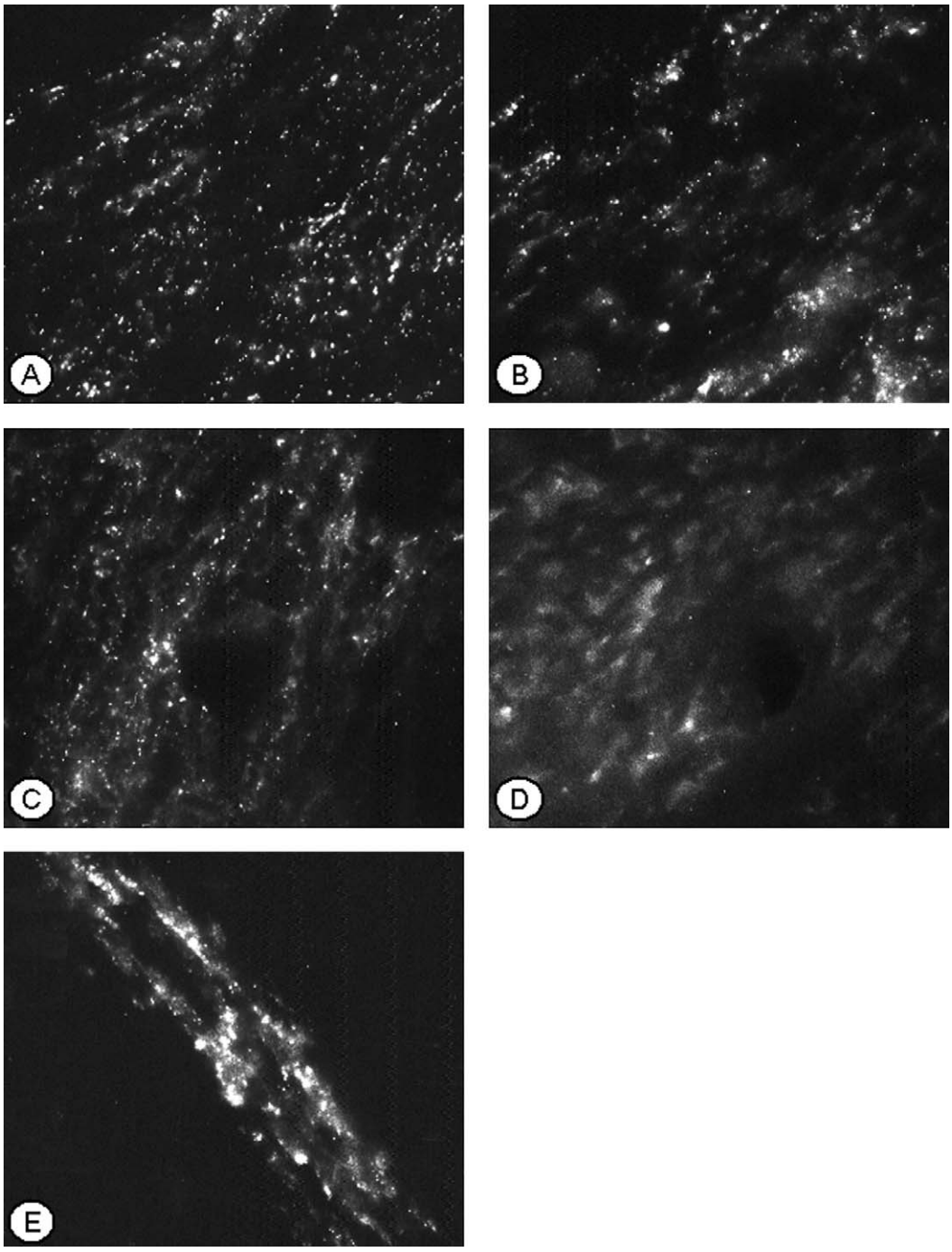

Figure 5. Cx26 and Cx43 immunofluorescence in endometrial stroma at late gestation and post partum. A: Day 131 p.c.; intensity of Cx43 immunoreaction in the placentomal stroma has been reduced compared to the state of early implantation (Figure 2 [C]). B: Day 131 p.c.; coexpression of Cx26 at the stromal cells is characterized by smaller size and quantity of the punctate spots. C: 20 min p.p.; Cx43-specific staining is only sparse in the postpartal placentomal stroma. D: 20 min p.p.; stromal copresence of the Cx26 antigen has disappeared after delivery. E: Day 145 p.p.; precise punctate reaction and diffuse staining indicate Cx43 in the stroma of a maternal sept. No antigen can be noted in the surrounding epithelial components of the placentomal labyrinth. × 525.

blastocyst between days 16 and 18 p.c. [25] however, Cx43 presence is enhanced in the stromal compartment and increases up to day 21 p.c. The strong build-up of Cx43 protein in the intercaruncular and caruncular stroma points to a pregnancy-specific role of gap junctional intercellular communication (GJIC). In previous investigations the close correlation between trophoblast invasion and Cx43 (and Cx26) induction in the developing decidua of rats has demonstrated, that gap junctions have regulatory functions in the implantation process [12]. They are thought to synchronize the decidual cells for metabolic, secretory, differentiation and degradation operations [26,27]. However, the functional

evidence is still missing, since Cx43 gene deficient mice die directly after birth due to a truncus pulmonaris defect [28].

Cx26, neither detectable in non-pregnant, nor in the early implantative sheep endometrium, is locally induced in the upper glandular epithelium by day 21 p.c. This phenomenon seems to be a specific reaction provoked by interacting trophoblastic tissue. The invasion of trophoblastic protuberances (papillae) into the openings of the uterine glands between days 15 and 21 of sheep pregnancy has been documented by Wooding et al. [29] and Guillomot et al. [25]. They concluded an informatory role for the establishment and anchoring of the embryo region, a process, that could be supported by gap junctions. In rabbits, maternal tissue exhibits a similiar response to embryo recognition: Cx32 is induced spontaneously in the epithelium of the implantation chamber before trophoblast penetration by fusion [14,30]. The data obtained in our studies indicate, that, during all pregnant and non-pregnant stages investigated, Cx26 seems to be the important epithelium-specific gap junction protein, comparable to the situation in rats. In this species, Cx26 is locally induced in the epithelium of the implantation chamber prior to trophoblast invasion, then detected in the decidual zone lining the invading trophoblast [15]. It has been proposed, that the restricted expression of Cx26 in the epithelium of the implantation chamber regulates the controlled cell death of the uterine epithelium accompanying the implantation process in the rat [31]. However, at sheep implantation, the contact of the trophoblast with the maternal stroma is performed only by a weak tendency for invasion. Characterizing the placenta syndepitheliochorialis of the sheep, the maternal epithelium stays largely intact, but is transformed into a syncytial layer by feto-maternal hybrid-cells [32,33]. Interestingly, in other non-invasive implantation models like mare and pig, Cx26, Cx32 or Cx43 isoforms were not present in the uterine luminal epithelium throughout oestrous cycle and pregnancy, except for Cx43 in the stromal compartment [34]. Only in the mare, where a small degree more invasivity can be found, the glandular epithelium revealed Cx43 (and not Cx26) staining with some modulations during oestrus and pregnancy. Thus we conclude from our observations, that the spatiotemporal expression of endometrial connexins in sheep resembles more the invasive type of implantation.

In all uterine tissues of the ewe, connexin expression seems to be modulated mainly by steroidhormonal factors, as already documented for the myometrium [9,35]. The sparse endometrial Cx43 presence during the progesterone-dominated phase of cyclus (dioestrus) can be explained by the suppressive effect of this hormone, whereas oestrogens are responsible for gap junction stimulation in oestrus, a phenomen also described for the endometrium of rats and humans [16,36,37]. Like in rats, epithelial connexin expession in sheep is suppressed in the preimplantation period, a phenomenon, which is interpreted as cell biological indication for receptivity and caused by progesterone [13,36]. Comparable to the situation in rabbits and rats [14,15] embryonic signals seem to be the dominating factors influencing gap junction formation in sheep implantation. Prostaglandins, fetal oestrogens and other (paracrine) proteins have been discussed as stimulating embryonal substances [36]. Connexin induction in the rabbit uterine epithelium by an artificial stimulus (combination of oestrogens and prostaglandins such as oestrogens and a mechanic stimulus) has been described by Antoskiewicz et al. [30]. Several authors demonstrated the existence of fetal oestrogens in early sheep pregnancy [38–40] and an ovine embryonal interferone (OTP-1), produced by trophoblastic tissue, has been considered to initiate maternal blastocyst-recognition and changes of the endometrium [41–43]. However, in sheep the fetal membranes are known to fill out completely both uterus horns (concerning also the horn containing no fetus) by day 17 p.c. of pregnancy [44]. As a consequence, an early trophoblastic stimulus could be able to provoke the synchronous realization of specific cellular programs including the formation of gap junctions at all sites of the implantative ovine endometrium. This would explain, why we failed to find any difference in connexin expression between the 'empty' and fetus-containing uterine horn in the three implantation stages investigated here.

Late pregnancy and delivery

At day 131 of pregnancy stromal Cx43 expression has decreased, but was now colocalized to Cx26 at lower levels. Immunolabelling of both connexins was even more reduced at day 145 p.c. The strong epithelial increasing amount of Cx26 protein during the progression of sheep pregnancy points to specialized functions of these tissues correlating with an active epithelial metabolism and proliferation tendency that reaches also the deeper parts of glandular epithelium [45,46]. Prepartal elevation of Cx26 in the endometrial epithelium has also been documented for the rat [11]. Epithelial connexin expression in this species seems to be associated directly with the hormone-modulated secretory and proliferative properties of the epithelial cells [24].

Like during implantation, we were not able to find any differences in connexin immunoreaction between the empty and fetus containing contralateral uterine horn of a pregnant sheep during late pregnancy. This attributes to an obviously synchronous hormonal regulation of all uterine compartments. A cell-specific steroidhormonal control mechanism for endometrial connexins in rats has been documented by Risek and Gilula [47], Risek et al. [24], and Grümmer et al. [16,36]: Oestrogen stimulated stromal Cx43 expression, which was down-regulated by progesterone. These investigators pointed out also, that Cx43 and Cx26 are differently regulated in the glandular and luminal epithelium of the rat uterus. A similiar regulatory mechanism would explain why in sheep, due to the steroidhormonal changes around parturition, connexin presence in the glandular epithelium and the upper stromal compartments has decreased until 20 min after delivery, while the postpartal luminal epithelium and the reticular layer of the interplacentomal stroma exhibit a surprisingly high protein expression. Maternal plasma concentrations of unconjugated

oestrogens, remaining low during most of sheep pregnancy, rise significantly during the final 24 h before spontaneous parturition [48,49] and elevated local oestrogen levels have been registrated in the endo- and myometrium at this time [50]. A decline of the maternal plasma progesterone niveau is recognized during the last 5–15 days of ovine gestation, correlating with low tissue progesterone concentrations [51,52]. Furthermore this increase in the maternal plasma oestrogen level derived at least from oestrogen production from the placenta [53] is correlated to an increase of Cx43 transcripts in sheep myometrium [54]. The significance of differences in the modulation of connexin expression in invasive versus non-invasive implantation animal models is still not clear, especially when considering the study of Day et al. [34], who demonstrated non or only moderate regulation of the intercellular communication channels during pregnancy in the equine and pig endometrium. However, we can conclude from the data obtained in this study, that different uterine tissues are programmed at different times to produce gap junctions with high plasticity under distinct hormonal and embryonal influences. From these observations we propose that endometrial gap junctions in the sheep have essential functions in the taking-up of feto-maternal contact and maintenance of pregnancy.

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Expression of Gap Junctional Connexins 26, 32 and 43 in Bovine Placentomes During Pregnancy

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Gap junctional connexins (Cx) are induced in the endometrium during implantation in rodents, the human receptive window, and in the decidua Cx26 and Cx43 expression increases in response to trophoblast invasion. In contrast, this gap junctional response and decidualization is absent in non-invasive epitheliochorial placentae of pigs and horses. Bovine (syn)epitheliochorial placentation represents an intermediate type of trophoblast invasion, since it is characterized by the continuous migration and fusion of trophoblast giant cells (TGC) with uterine epithelial cells. Therefore the objective of the present study was to investigate the expression of Cx26, Cx32, and Cx43 in placental tissues during bovine pregnancy, to determine if Cx expression patterns correlate with the depth of trophoblast invasion. Cx26, Cx32, and Cx43 proteins were detected by immunohistochemistry and corresponding specific mRNAs were shown by RT-PCR and localized in tissue sections by in situ hybridization. Cx26 protein was detected at the feto-maternal contact interface and as cytoplasmic staining in TGC. Cx26 mRNA was located in maternal epithelium and in TGC. Cx32 protein expression was observed in the maternal epithelium exclusively on the tips of maternal septa, whereas Cx32 mRNA was detected in all maternal epithelial cells and single TGC. Cx43 protein and mRNA were coexpressed in TGC. Cx43 protein was present in maternal septal stroma and to a lesser extent in chorionic villous mesenchyme, while Cx43 mRNA was associated with the vasculature. In the course of gestation, expression of Cx26, Cx32, and Cx43 did not change. In conclusion, the intermediate invasive status of bovine trophoblast is supported by the fact that TGC coexpress Cx26, Cx32, and Cx43, which may be important for trophoblast migration (invasion), and fusion with maternal epithelial cells. Cx32 could be involved in the control of invasion.

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INTRODUCTION

Gap junctions are intercellular channels which allow small molecules with a molecular weight up to 1–2 kDa, like inorganic ions (Na^+, K^+, Ca^{2+}, etc) and second messengers (cAMP, inositol 1,4,5-triphosphate) [1,2] to pass directly from one cell to another. Their protein subunits, connexins, belong to a multigene family of related proteins characterized by their molecular weight. Six connexins (Cx) assemble to form a hemichannel called a connexon, and paired connexons of neighbouring cells align to form a complete dodecameric gap junction channel [3,4]. The main function of gap junctions is the rapid transmission of regulatory and informational molecules across an interconnected network of cells, and the uncoupling of damaged cells from healthy neighbours [5]. These properties form the basis for Cx involvement in cell growth, proliferation, differentiation, and fusion [6–11]. Indeed, Cx play important roles in cellular remodeling along tissue invasive pathways including implantation and tumorigenesis [9–12]. Additional functions of connexins, including the regulation of cell growth, are not related to the classical gap junction channel function [13,14].

The expression of Cx has been examined in a variety of species during the critical period of implantation and early placentation. Hemochorial placental types with highly invasive trophoblast showed spatiotemporal alterations of their Cx expression pattern which correlated to the functional state of the trophoblast cells [15]. In humans, Cx40 is expressed in proliferative extravillous trophoblast, is lost during migration, but reappears in multinucleated trophoblastic cells. Human cytotrophoblast expresses Cx43, while multinucleated trophoblastic cells show Cx32 and 43 [16]. In spongiotrophoblast of rodents a change from Cx31 to Cx43 can be observed indicating a switch from invasion and proliferation to differentiation [17]. In contrast, no regulation of Cx expression

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in the trophoblast of non-invasive epitheliochorial placentae is observed [18]. This leads us to our working hypothesis that TGC migration in the bovine placenta, which is considered as an intermediate between invasive and non-invasive placental types, may be correlated to a specific connexin expression pattern.

The bovine synepitheliochorial placenta is characterized by placentomes which are formed by interdigitating fetal cotyledons and maternal caruncles [19,20]. Fetal trophoblast cells accompanied by fetal mesenchyme are linked to maternal epithelium by microvilli [21–23]. An extraordinary feature of the cow placenta are migrating trophoblast giant cells (TGC) [24,25]. These multinuclear cells are able to loosen from the trophoblast and migrate along tight-junctions into the maternal epithelium where they fuse with single maternal epithelial cells, release hormonal products, and finally degenerate [25–30]. This process is characteristic for the cow and may be interpreted as a restricted type of invasion [31], thus contrasting with the pig which has true non-invasive epitheliochorial placentation [32].

Placentation is similar to tumorigenesis in that it is a highly invasive and proliferative process. However, unlike tumors, trophoblast invasion during placentation is exquisitely controlled. In this study, the expression of Cx26, Cx32 and Cx43 in this process was elucidated in cattle to gain insight into the control of invasiveness in proliferation zones, migratory TGC, and at the feto-maternal interface.

MATERIAL AND METHODS

Animals

Placentomes from 40 healthy cows were collected at the local slaughterhouse and perfusion-fixed with either Bouin's solution or 4% buffered formalin and embedded in paraffin. In parallel, placentomes were snap frozen in liquid nitrogen and stored at −80 °C. Tissues were allocated to 4 groups of gestational age (Days 60–90; Days 120–150; Days 150–220; Day 220 to parturition; n per group = 10) according to fetal crown-rump-length [33]. Three different tissue samples per animal were examined from 10 animals per stage.

Immunohistochemistry

Cx26 and Cx43 immunostaining had to be performed on frozen sections (4–8 μm thickness) due to the properties of the antibodies, while the Cx32 antibody was suitable for immunoreaction on paraffin-embedded sections (3–5 μm). Slices were mounted on 3-aminopropyl-triethoxysilane-coated slides. Paraffin sections were pretreated in boiling citrate-buffer for 20 min. Both frozen and paraffin-embedded sections were blocked with 1% H_2O_2 in phosphate buffered saline (PBS) for 10 min at room temperature (RT), and subsequently incubated with 1 g bovine serum albumin and 21 μl Triton-X-100 in 70 ml PBS for 60 min at RT prior incubation with primary antibodies (AB) overnight at 4 °C. Monoclonal mouse anti-connexin-26 (1:100) and mouse anti-connexin-43 (1:100) and a polyclonal rabbit anti-connexin-32 (1:50) (all from Zymed, San Francisco, CA) were used. Specificity of the polyclonal AB for bovine Cx has been confirmed previously [34]. Detection of primary AB was accomplished by incubation with a biotinylated secondary AB (horse anti-mouse/-rabbit; Vector Laboratories, Burlingame, CA) for 20 min at RT and the ABC system (Vector Laboratories, Burlingame, CA) for 45 min at RT. For visualization slides were incubated with a peroxidase-substrate AEC solution (AEC Substrate Kit for Peroxidase, Vector Laboratories) for 5 min at RT. Control sections were treated with the same dilution of normal rabbit serum (for polyclonal AB) or mouse IgG instead of the primary AB as well as with PBS.

RNA extraction and RT-PCR amplification

RNA was extracted from placental frozen tissue with Trizol reagent (Life Technologies, Karlsruhe, Germany) and first strand cDNA synthesis was performed using the Superscript II Kit (Life Technologies, Karlsruhe, Germany). Both procedures were carried out according to the manufacturer's protocol. The cDNA sequences were generated using polymerase chain reaction (PCR). The PCR master mix contained 38.5 μl diethylpyrocarbonate-water (DEPC), 5 μl 10× PCR-buffer (Perkin Elmer, CA, USA), 4 μl 25 mM $MgCl_2$, 1 μl mM dNTP (Perkin Elmer), 1 μl forward primer (Cx26: 5′CTGCCTTCATGTATGTCTTCTACGT3′ [NCBI Accession: AJ293886]; Cx32: 5′ATCAGCGTGGTCTTCCGGCTGT3′ [NCBI Accession: X95311]; Cx43: 5′CCATCTCTAACTCTCATGCACAGC3′ [NCBI Accession: AF 151980]), 1 μl reverse primer (Cx26: 5′AATCAGCAAGTAACACAGCTCAGTGA3′; Cx32: 5′TCCTGCTCACTCAGCAGCTTGTT3′; Cx43: 5′TGGCACGACTGCTGGCTCTGCTT3′), 1 μl cDNA and 0.5 μl Taq DNA-Polymerase (Perkin Elmer). All primers were purchased from MWG, Ebersberg, Germany. Probes were run on a thermocycler (T3, Biometra, Göttingen, Germany) with following temperatures: Cx26: 95 °C 1 min, 67 °C 1 min and 72 °C 2 min for 40 cycles; Cx32: 95 °C 1 min, 65 °C 1 min and 70 °C 2 min for 40 cycles; Cx43: 95 °C 1 min, 66 °C 1 min, 72 °C 2 min for 10 cycles, 95 °C 1 min, 62 °C 1 min and 72 °C 10 min for 25 cycles.

The PCR master mix for the housekeeping gene β-actin contained 40.5 μl aqua bidest., 5 μl 10× PCR-buffer complete (Perkin Elmer), 1 μl 10 mM dNTP-Mix (Perkin Elmer), 1 μl forward primer (5′TGACGGGGTCACCCACACTGTGCCCATCTA3′ [35]) and reverse primer (5′CTAGAAGCATTGCGGTGGACGATGGAGGG3′ [35]) (both MWG), 1 μl cDNA and 0.5 μl Taq DNA-Polymerase (Perkin Elmer). PCR for β-actin was run at 95 °C 3 min; followed by 95 °C 1 min, 65 °C 1 min, 72 °C 2 min for 40 cycles, and 72 °C 10 min resulting in a product of 661 base pairs (bp).

Amplicons were separated on a 2% agarose gel and visualized with ethidium bromide. All experiments included controls lacking the RT enzyme to exclude contamination with genomic DNA or were run with water instead of RNA to

exclude contamination from buffers and tubes. Both controls were negative.

Digoxigenin-labelled mRNA probes

The PCR products of Cx26, Cx32 and Cx43 were subcloned in pGEM-T (Promega, Heidelberg, Germany), before being transformed into an XL1-Blue *Escherichia coli* strain (Stratagene, Heidelberg, Germany) and extracted by column purification, according to manufacturer's instructions (Qiagen, Hilden, Germany). Identity was confirmed by sequencing (Qiagen Sequence Service, Hilden, Germany).

The vectors containing the Cx-inserts were digested with *Nco*I or *Not*I (New England Biolabs, Schwalbach, Germany) for the production of sense and antisense cRNA. Heat inactivation was performed at 65 °C for 20 min. In vitro transcription of digoxigenin (DIG)-labelled cRNA of Cx26, Cx32 and Cx43 was carried out using the RNA-DIG labelling Kit (Promega, Mannheim, Germany), DIG RNA labelling mix (Roche, Mannheim, Germany), RNA-polymerase T7 (Promega), and SP6 (Promega).

In situ hybridization

To localize Cx26, Cx32 and Cx43 mRNA, in situ hybridization was performed on Bouin-fixed, paraffin-embedded sections of 3−5 μm thickness. The sections were deparaffinized, rehydrated and subsequently handled using RNAase-free conditions. They were placed in 0.2 N HCl and transferred into 2× standard saline citrate (SSC) for 20 min at 70 °C. Incubation with Proteinase K (20 μg/ml, 1× PBS) for 30 min at 37 °C to achieve partial digestion was followed by treatment with 0.2% glycine in PBS and 20% acetic acid in diethylpyrocarbonate (DEPC). Sections were postfixed in 4% paraformaldehyde for 10 min and prehybridized in 20% glycerol for 30 min. Hybridization with the DIG-labelled sense and antisense cRNA probes was performed overnight at 37 °C in a humid chamber containing 50% formamide in 2× SSC. The cRNA probes were used at a dilution of 1:50 in a hybridizing-buffer containing 50% deionized formamide, 10% dextran sulphate, 2× SSC, 1× Denhardt's solution, 10 μg/ml salmon sperm DNA and 10 μg yeast tRNA. Post-hybridization was carried out by following steps: 4× SSC (3 × 10 min), incubation with RNAse (30 min, 37 °C), 4× SSC (4 × 5 min, 37 °C), 2× SSC (15 min, 60 °C), 0.2× SSC (15 min, 42 °C), 0.1× SSC (5 min at RT), 2× SSC (5 min at RT), 1× Tris−HCl-, NaCl-, $MgCl_2$-, Triton-X-100 buffer (TNMT; 10 min at RT), 3% BSA in TNMT for 60 min at RT. Tissue sections were incubated with anti-DIG Fab-antibody conjugated to alkaline phosphatase overnight at 4 °C. RNA was visualized by developing sections with nitroblue-tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate in a humid and dark chamber. Finally sections were mounted in Glycergel (Kaisers Glycergelatine, Merck, Darmstadt, Germany). For each test, control incubations were performed using DIG-labelled cRNA sense probes as well as incubation with hybridization buffer instead of sense or antisense probes. These sections were completely negative.

RESULTS

Positive immunohistochemical staining was intense red (Figure 1, A1, B1, C1), and positive in situ hybridization reaction was brown to black (Figure 1, A2, B2, C2). Representative negative controls are shown (Figure 1, D1 [immunohistochemistry], D2 [in situ hybridization]). RT-PCR (Figure 2) showed specific bands in the equivalent kDa/bp axis. No obvious changes in the expression pattern during pregnancy were observed with the different techniques applied.

Cx26

Cx26 was localized at the feto-maternal border throughout pregnancy (Figure 1, A). TGC expressed both, Cx26 mRNA and protein, which appeared either associated with cytoplasmic membranes or as diffuse cytoplasmic staining. Fetal mononuclear trophoblast cells showed immunostaining for Cx26 protein in apical cytoplasmic membranes (Figure 1, A1), while maternal epithelial cells only showed a positive reaction with in situ hybridization (Figure 1, A2). The existence of Cx26 mRNA in the bovine placentome was confirmed by RT-PCR (Figure 2) showing a positive band at 222 bp.

Cx32

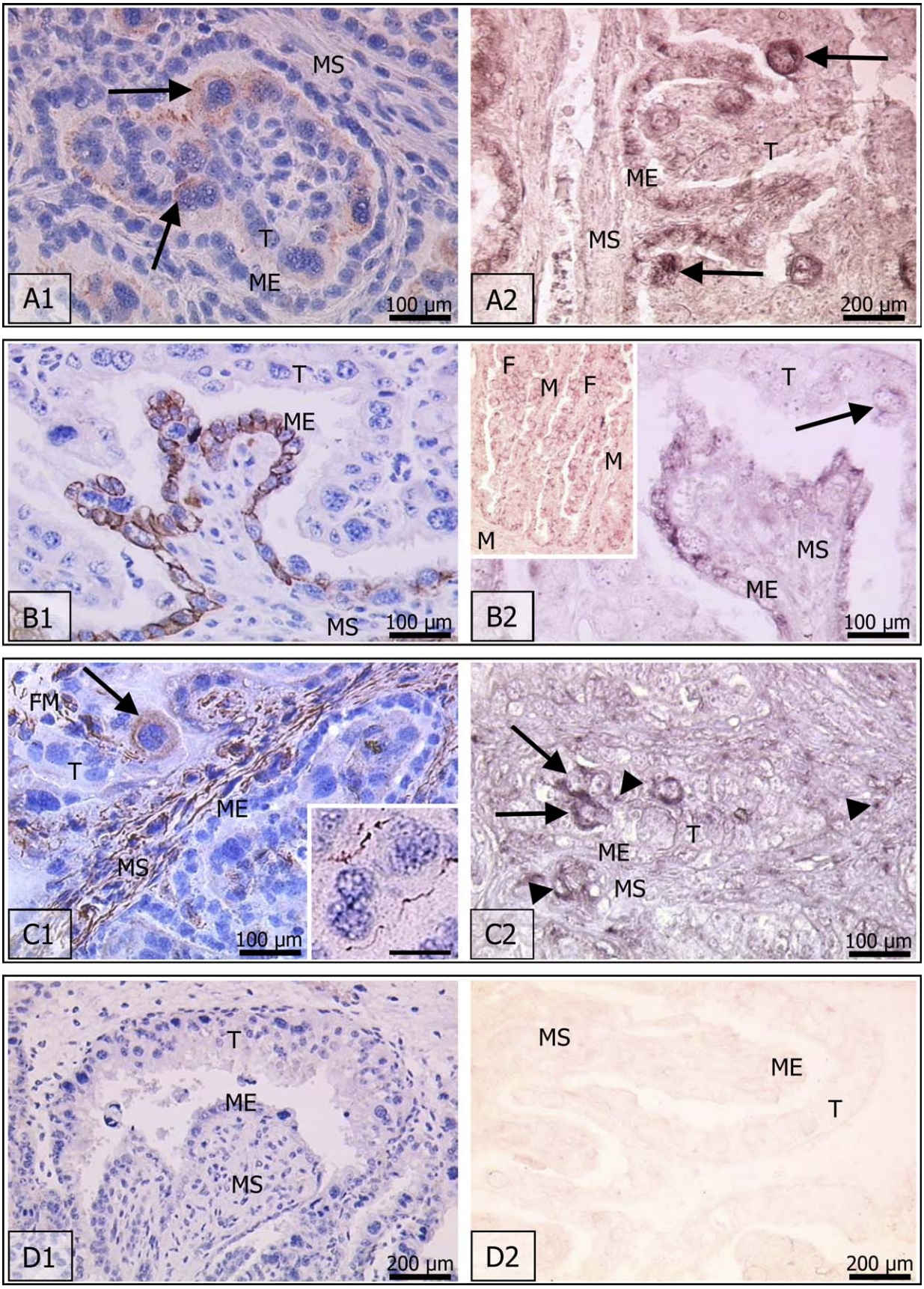
Immunohistochemical staining for Cx32 was found along the lateral cell borders of caruncular epithelium at the tips of maternal septa, facing the fetal primary or stem villi (Figure 1, B1) throughout gestation. Protein expression was strictly confined to this area, whereas mRNA occurred in maternal epithelial cells of the entire placentome as is shown by in situ hybridization (Figure 1, B2 inset). Cx32 mRNA, but not protein was localized in TGC (Figure 1, B2). RT-PCR analysis for Cx32 showed a specific band of 362 bp (Figure 2).

Cx43

Cx43 protein was detected in the stroma of maternal crypts and to a lesser amount in fetal mesenchyme at all gestational stages (Figure 1, C1). Migrating TGC were also immunostained, but with qualitative differences: TGC in the vicinity of stem villi showed intracytoplasmic staining (Figure 1, C1), whereas TGC in secondary and tertiary villi exhibited staining in the cytoplasmic membrane (Figure 1, C1 inset). Cx43 mRNA was observed in TGC and in the vasculature of maternal crypts and fetal villous mesenchyme (Figure 1, C2). The presence of Cx43 mRNA in the bovine placentome was confirmed by RT-PCR resulting in a specific band of 137 bp (Figure 2).

DISCUSSION

This is the first report documenting gap junction expression during the restricted trophoblast invasion of synepitheliochorial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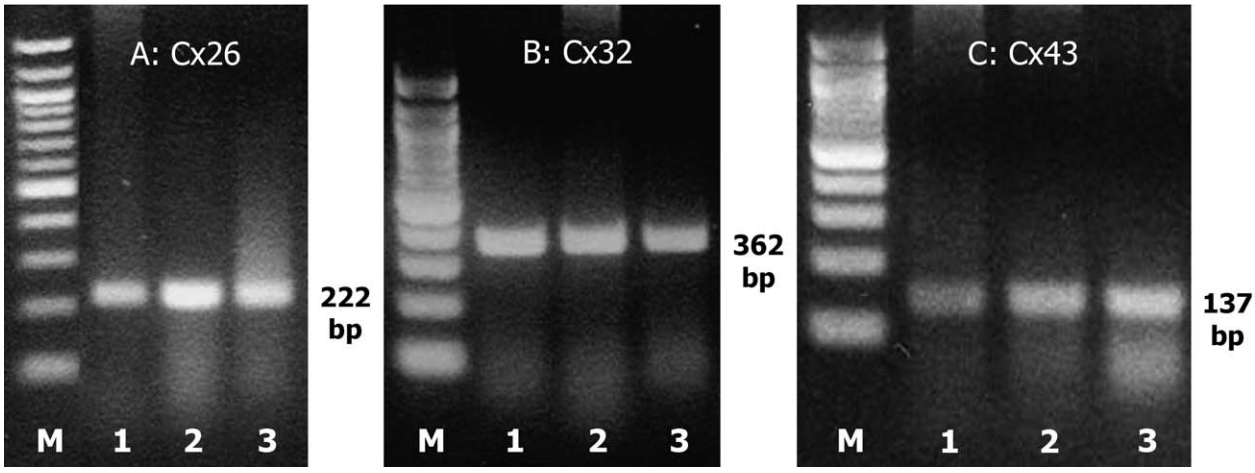

Figure 2. Expression of Cx26, Cx32 and Cx43 mRNA in bovine placentomes. A) Transcripts for Cx26 (222 bp) are shown for Day 120 (lane 1) and Day 180 (lanes 2, 3) of gestation. B) Cx32 (362 bp) amplificates are demonstrated for Day 120 (lane 1) and Day 180 (lanes 2, 3). C) Cx43 (137 bp) transcripts are shown for Day 120 (lanes 1, 2) and Day 220 (lane 3). M, 100 bp DNA ladder.

cow placentation. In comparison to other non-ruminant epitheliochorial placental types bovine placentomes, besides showing specific patterns of growth and differentiation, are characterized by migration, invasion, and fusion processes, which could at least partly be influenced by gap junctional connexins.

Connexins and placentomal growth and differentiation

In bovine placentomes Cx32 is localized in the endometrial epithelium exclusively in the tips of maternal septa, which have been described as growth zones [31]. In contrast, Cx43 occurs in caruncular connective tissue and fetal mesenchyme. Architecture and growth of maternal septa and fetal villi may be influenced by the presence of Cx43 gap junctions in stromal components of placentomes. The involvement of Cx43 in structuring tissues has been confirmed in Cx43 deficient mice which have a malformed heart outflow tract due to a lack of cell–cell communication via Cx43 gap junction channels [36,37]. However, Cx expression does not appear to be essential for embryo survival during preimplantation development in Cx43 deficient mice, but facilitates the establishment of a communication network for postimplantative developmental stages [38]. Maldevelopment is also observed in cloned bovine calves which die within the first trimester due to starvation and placental anomalies including small cotyledons [39], but a relation to altered Cx expression patterns has not yet been established [40]. Additionally, and independently from gap junction formation, Cx43 may control cell growth [13,14].

Connexins and trophoblast migration

TGC migration in bovine placentomes may be influenced by Cx26 and 43, since both mRNA and protein are expressed in TGC. Although mRNA for Cx32 is present in TGC, protein is not detected suggesting that Cx32 is not translated. Trophoblasts have invasive properties for only a defined period of placental development [41]. Temporary loss or expression of certain Cx is important for the degree of invasiveness of the trophoblast [16], because in the mouse and rat placenta Cx expression changes when the physiological status switches from an invasive type to a "functional" type [42]. Human cytotrophoblast cells express Cx40 in the proliferation phase, but loosening from the cell columns and migration into the placental bed is accompanied by loss of Cx40 expression [16]. These findings suggest that the migratory properties of bovine TGC may be dependent on the expression of specific Cx.

Connexins and trophoblast giant cell fusion

In the bovine placentome, fetal TGC synthesize mRNA for Cx26, 32 and 43 and express Cx26 and 43 proteins, while the

Figure 1. A) Cx26 expression. A1) Immunohistochemistry from around Day 240 reveals positive red staining along the feto-maternal barrier, which can be localized to mononuclear trophoblast cells (T). TGC (arrows) show an annular red staining. A2) In situ hybridization from around Day 80 of gestation. Cx26 mRNA is located in maternal epithelial cells (ME) and in higher degree in some TGC (arrows). B) Colocalization of Cx32 protein (B1) and mRNA (B2) in exclusively those maternal epithelial cells which border the tip of a maternal septum. B1) Immunohistochemistry from around Day 120. Maternal epithelial cells display a distinct basolateral staining in septal tips exclusively. B2) In situ hybridization from around Day 220 illustrates colocalization of Cx32 mRNA in those maternal epithelial cells which border the tip of a maternal septum, but the inset shows that Cx32 mRNA is also located in maternal epithelial cells throughout the placentome and in TGC of fetal villi (F). M, maternal caruncular tissue. Bar inset = 1000 μm. C) Cx43 expression. C1) Immunohistochemistry from about Day 240. Cx43 protein is expressed in maternal stroma (MS) and endothelial cells of the fetal mesenchyme (FM). TGC close to the chorionic plate exhibit a cytoplasmic, annular staining (arrow), whereas TGC in the center of the placentome show a typical macular staining (inset). Bar inset = 40 μm. C2) In situ hybridization from around Day 270. Cx43 mRNA is expressed in blood vessels (arrowheads) of fetal mesenchyme and maternal stroma, as well as in TGC (arrows). D) Representative control sections for immunohistochemistry (D1) and in situ hybridization (D2).

maternal caruncular epithelium expresses Cx26. Thus, fusion of TGC with maternal epithelial cells could be supported by Cx26 and Cx43. However, the presence of gap junctional channels in this specific location has not yet been demonstrated. In the human placenta, where syncytiotrophoblast arises from the fusion of cytotrophoblast cells, intercellular communication via Cx43 gap junctions is essential for these cell fusions [43]. Similarly, gap junctions between cytotrophoblast and syncytiotrophoblast are believed to be responsible for fusion processes in the placenta of guinea pigs [9]. In vitro studies with human cytotrophoblast cells have shown that cell fusion is stimulated by the expression of Cx43 which is promoted by the presence of hCG and cAMP [11,15,44].

Connexins and trophoblast invasion

In the bovine placentome the most exposed place for invasion is the chorionic plate where fetal cotyledons begin to interdigitate with maternal caruncles. Cx32 is specifically expressed in uterine epithelium at the tips of maternal septa within this zone, and is therefore a likely candidate for the control of invasion or determination of the quantity of primary or stem villi. The stimulus for Cx32 protein expression could be the growing trophoblast [45,46] and/or steroid hormone receptors [47], since in the endometrium of rabbits a mechanical stimulus, replacing the blastocyst, in combination with administration of 17-beta-estradiol is necessary to provoke Cx32 expression corresponding to that found in pregnant uteri [45]. Investigations with pregnant, nonpregnant and pseudopregnant rabbit uteri emphasize the necessity of the presence of the blastocyst for the induction of gap junction formation [46]. The feto-maternal junction is determined by the type of implantation and trophoblast invasion [32]. The first contact between maternal and fetal tissues leads to cellular changes, summarized as decidualization [48]. In contrast to the hemo- and endotheliochorial placentation, where the trophoblast penetrates into the maternal stroma, the bovine epitheliochorial placenta is characterized by trophoblast cells, which do not invade the maternal stroma [49]. The arrangement of Cx26 in maternal epithelium and Cx43 in maternal stromal cells may be involved in regulating the depth of trophoblast invasion in the cow. A similar distribution pattern has been reported for the rat endometrium during implantation [50]. In rats, Cx26 is expressed in maternal epithelium in the vicinity of the blastocyst, while Cx43 is present in the decidua. Once the maternal epithelium is destroyed the decidual cells accompanying the trophoblast begin to express Cx26 while Cx43 is expressed in deeper layers of the decidua. The absence of connexins in maternal luminal epithelial cells and trophoblast in the epitheliochorial placenta of horses and pigs might be a reason for the absence of trophoblast invasion in these species [18], while the specific localization of Cx26 and 43 in the sheep endometrium during implantation and gestation suggests that the sheep has a more invasive type of implantation [51].

Significance of connexins for materno-fetal exchange

The presence of Cx26 protein at the feto-maternal interface together with the synthesis of the corresponding mRNA in the maternal epithelium of bovine placentomes indicates intercellular exchange between fetal and maternal compartments. A nutritional function has been proposed for Cx26 in placentae of other species. In wild type mice Cx26 is located in cytotrophoblast cells of the placental labyrinth [52], whereas in Cx26 knockout mice the transport of glucose through Cx26 gap junction has been verified [53]. The syncytiotrophoblast layers I and II in the placenta of the rat are also connected by Cx26 [54]. These layers facing fetal and maternal blood express GLUT I [55], a protein that supports diffusion of glucose [56]. In the hemomonochorial human placenta GLUT I is localized in both apical and basal aspects of the syncytiotrophoblast [57]. This arrangement likely promotes the transport of glucose from maternal into fetal blood circulation [58]. Initially, the presence of gap junctions was shown electron microscopically in first trimester human placenta between syncytiotrophoblast and cytotrophoblast [10]. However, recently Cx43 was localized "in situ" between cytotrophoblast cells and cytotrophoblast cells and syncytiotrophoblast in human first trimester villous trophoblast [59]. Certainly, different cell types can be connected by gap junctions, as is seen between in rat oocytes and granulosa cells [60]. The fact that Cx26 mRNA is only seen in maternal epithelium of bovine placentomes suggests the presence of heterotypic channels, including at least one yet unidentified connexin. However, unpaired connexons without counterparts to form intercellular channels have been detected in non-junctional membranes of cells, but are associated with exchange-independent functions, e.g. release of signaling molecules, activation of kinase cascades, and cell survival [61]. Indirectly, Cx43 may also contribute to materno-fetal exchange in bovine placentomes, because endothelial cell Cx43 plays a role in the action or formation of nitric oxide [62], which dilates blood vessels to slow down blood flow and thus allows more time for exchange.

We conclude that Cx26, Cx32, and Cx43 have specific functions in bovine, non-hemochorial placentomes throughout gestation. Placentomal growth and differentiation (architecture), trophoblast invasion (TGC migration and fusion with uterine epithelial cells), and materno-fetal exchange (nutrition through transfer of glucose) may be influenced by these connexins.

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The three-dimensional feto-maternal vascular interrelationship during early bovine placental development: a scanning electron microscopical study

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ABSTRACT

Both the fetal and maternal microvasculature of bovine placentomes was examined by scanning electron microscopy of vascular casts. So far the development of the vascular architecture of the bovine placentome in early gestation has only been studied 2-dimensionally due to technical difficulties arising from the fragility of the early placental blood vessels. Repeated experiments led to the selection of the microvascular corrosion casts presented here. The vasculature of the maternal compartment is supplied by large caruncular stalk or spiral arteries, which release short maternal stem arteries. In the 3rd month of gestation, these arteries branch into several arterioles at their base, thus providing the vascular framework for the lower part of the septal walls of the primary crypts. In the 4th month, due to progressive longitudinal growth of the stem arteries, branching into arterioles occurs not only at the base, but over the whole length of the stem arteries. These arterioles supply the capillary complexes of the septa which resemble the major part of the septal vasculature and face the secondary crypts. Further indentation results in the formation of tertiary crypt capillary complexes, encircling the earlier secondary unit. From the 6th month of gestation the architecture resembles the fully developed maternal placenta with stem arteries running directly to the fetal side to branch into 4 to 6 arterioles, which turn back to enter secondary and tertiary septa. Maternal venules, collecting the blood from the capillary bed of secondary and tertiary septa, converge onto stem veins leaving the caruncle via branches of the uterine vein. The fetal part of the placentome is supplied by the cotyledonary arteries, which branch into fetal stem arteries that are the tributary to single villous trees. Over their whole course towards the maternal side, these give off arterioles entering secondary villi. The tertiary or terminal villous vasculature consists of capillaries, which are organised in serial capillary loops. This system is progressively elaborated in the course of gestation. In the 4th month there are only finger-like loops, whereas from the 6th month large fan-like structures can be observed. In early gestation the maternal and fetal blood vessels meet predominantly in a countercurrent fashion, changing to the less efficient crosscurrent exchange when the tertiary unit develops. These results indicate the development of a highly elaborated fetomaternal villous-crypt exchange system, already established in the 1st half of gestation, thus meeting the increasing needs of the fetus.

Key words: Bovine placentation; early gestation; corrosion casts; vasculature.

INTRODUCTION

Implantation in the bovine species starts around d 18 and 19 post insemination (p.i.) (overview by Leiser, 1975) and is characterised by the formation of a synepitheliochorial (Wooding, 1992), caruncular-cotyledonary or crypt-villous interrelationship (Mossmann, 1987) starting about d 30 p.i. (Melton et al. 1951; Greenstein et al. 1958). Dependent on the development of the underlying vasculature, the degree

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of placentomal elaboration increases until parturition (around d 280 p.i.). This has been shown for the 2nd half of gestation where the degree of fetomaternal indentation and, hence, the anchorage of the chorion in the uterus (Leiser et al. 1998) is distinctly elaborated (Leiser et al. 1997*a*, *b*). Information regarding the origin and development of the microvessels and their architecture during early placentome formation is so far lacking. We therefore attempted to elucidate the development of both the maternal and fetal vascular systems in the early bovine placenta.

MATERIALS AND METHODS

Placentomes from 9 cows (5 per animal) were selected immediately after slaughtering and excision of the uterus. The crown-rump-length (CRL) of the fetuses was recorded and used to allocate them to 3 different gestational ages, 3 (8–12 cm CRL), 4 (16–23 cm CRL), and 6 (35–38 cm CRL) mo (according to Schnorr, 1996). The placentomal vasculature was rinsed by perfusion with phosphate buffer (30 s, 0.1 M, pH 7.3; anticoagulant: 1000 IU/l heparin; vasodilator: 1% procaine) either through fetal (branches of the umbilical artery) or maternal vessels (branches of the uterine artery). Subsequently liquid plastic compounds (Batson no. 17 compound [Polysciences, Eppelheim, Germany] or Mercox CL-2R [Nordwald, Hamburg, Germany]) were freshly prepared and instilled. The procedure of casting and specimen preparation has been described in detail (Leiser & Kohler, 1983; Krebs et al. 1997; Leiser et al. 1997*b*). Finally, the casts were examined with a scanning electron microscope (Zeiss DSM 940, Oberkochen, Germany). Measurements of vessel diameter were made using on-screen callipers and the data were described by mean and range.

For comparison with histology, the fetal and maternal vasculature of additionally buffer perfused placentomes was injected with Indian ink (green [fetal blood vessels] and red [maternal vasculature] drawing ink, Rotring, Hamburg, with bovine serum: 1:1). The placentomes were then immersion fixed in 4% buffered formaldehyde (pH 7.3) for 3 d. After dehydration in a graded series of ethanols and embedding in Epon, 150 μm sections were obtained with a Polycut microtome (Leica) and examined without staining.

Rationale for the use of vascular corrosion casts

The basic principle is that the blood vessels intimately adjoin the single layered epithelium of the caruncle and the cotyledon thus following every contour of the tissue surface (Leiser & Koob, 1992). Therefore, a maternal crypt is formed by a vascular crypt, the surrounding stroma and the uterine epithelium (Wooding & Flint, 1994). Correspondingly, a fetal villus consists of its vascular villous tree, stroma components and the covering trophoblast (Leiser et al. 1998). The fetal villous and maternal septal or cryptal, microvasculature, respectively was classified according to established criteria (Leiser et al. 1997*a*, *b*). The different vessel types were distinguished by the shape of the impressions of protruding endothelial cell nuclei on the casts (Leiser & Kohler, 1983; Leiser et al. 1989). These impressions were deep and spindle-shaped in arteries, shallow and spindle-shaped in arterioles, long and oval in veins, just oval in venules and round in capillaries.

RESULTS

Relation of general maternal and fetal vascular components to the whole placentome

The typical mushroom-like form (Fig. 1*a*) with a caruncular stalk (Fig. 1*b*) of the bovine placentome was already present in the 3rd mo of gestation. The vasculature of the maternal caruncle consisted of a base with projecting blood vessels of fetally-oriented septa which formed vascular crypts (Figs 1*a*, 2*a*, *b*). These crypts were indented by chorionic villi, resulting in a villous-crypt fetomaternal relationship of the placentome. The vessels in the core of maternal septa and fetal villi were stem arteries and veins, or the primary structure, which ramified into arterioles and venules of intermediate septa and villi. These were the secondary structures, which supplied blood to or from the tertiary structures, which were the terminal capillaries of septal and villous placentomal parts (Figs 1*a*, 2*a*, 5*e*, 7*b*). The relation of tissues and corresponding microvasculature is summarised in Table 1. The measurements of vessel diameters from the 3rd to 6th mo of pregnancy are summarised in Table 2.

Maternal vasculature

In the early gestation placentomes (8–12 cm CRL, 3rd mo of gestation) the caruncular stalk arteries were showing a more spiral course than the corresponding veins (Figs 1*a*, *b*, 4). In the basal plate of the placentome or caruncle base (Fig. 1*a*) each ramified at right angles to maternal stem arteries and veins, respectively, which entered the main septa as a pair, and followed them up to about one third of the height

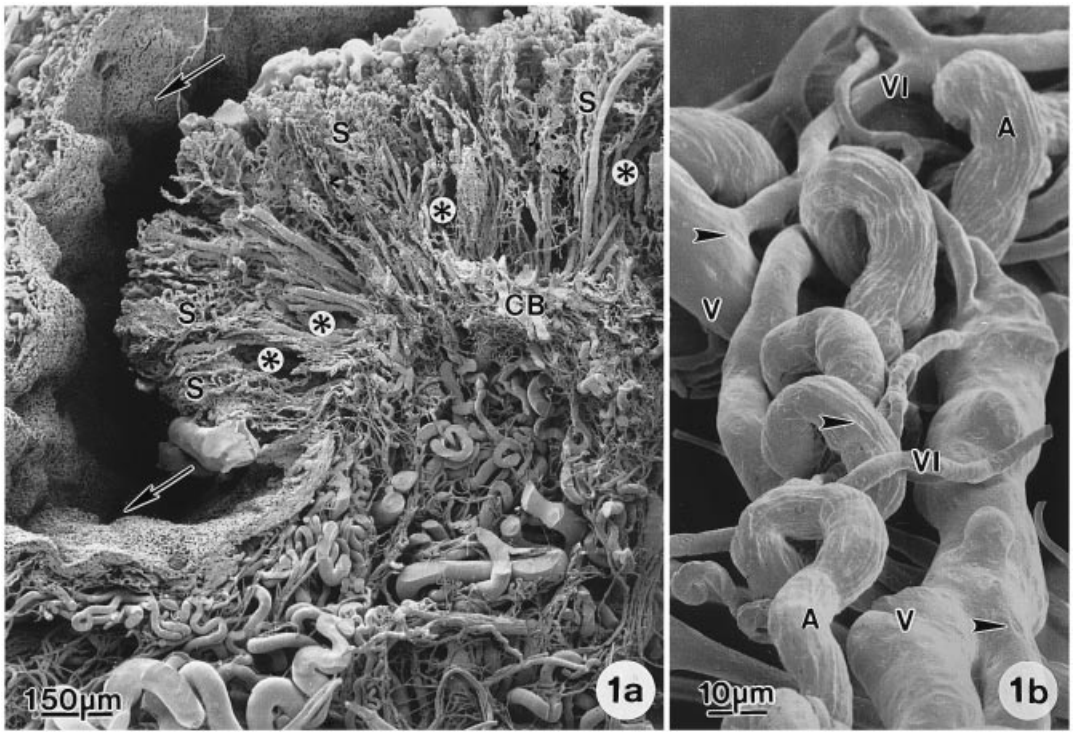

Fig. 1. Maternal vasculature. (*a*) Scanning electron micrograph of a cracked, laterally viewed maternal vessel cast of bovine endometrium; fetus of 8 cm CRL. Overview of the intercaruncular vasculature (arrows) which extends from the stalk (below right) to the body of caruncular vasculature of placentome (top right). Note spiral arteries running from the caruncle stalk into the base of the caruncle (CB). Blood vessels of main septa (S) and primary crypts (asterisks) are indicated. (*b*) Detail of a caruncle stalk, fetus of 23 cm CRL. The coiling of the spiral artery (A) contrasts with the veins (V) and venules (Vl) which are straighter and have a relatively larger diameter. Impressions of endothelial perikarya can be observed (arrowheads).

of the caruncle (Figs 1*a*, 2*a*). Therefore, these stem vessels built up a sector of the main septum which was the wall of a primary crypt (Figs 2*a*, 4). With the ramification of the stem vessels into arterioles and venules (Fig. 2*b*) intermediate septa were formed, which as walls of secondary crypts could reach almost to the top of the caruncle (Figs 1*a*, 2*a*, *c*, 3, 4). Several venules (3–4), sometimes anastomosing with each other, accompanied a single arteriole (Figs 2*a*, *c*, 4). The arterioles ran in the centre of intermediate septa, whereas the venules were located in the periphery (Fig. 2*c*). The average venule diameter of 35 µm was exceeded in branching points of the crypts (35–65 µm) (Fig. 2*b*). Along intermediate vessels there were many ramifications leading to a terminal capillary complex (Fig. 2*a*, *b*) which bordered the secondary crypts as a vascular tuft (Figs 2*a*, 3). With higher magnification, this tuft at the rim of the septal system showed serially linked, anastomosing and winding capillary loops with distinct dilations (Figs 2*d*, 3, 4), which were seen to be related to rather primitive tertiary crypts when viewed from the fetal side of the placentome (Fig. 3).

Up to midgestation (35–38 cm CRL or 6th mo of pregnancy) the maternal vascular system of bovine placentomes developed until similar appearances to those at near term were achieved (see Leiser et al. 1997*a*, *b*). The stem vessels which ran to the convex side of the placentomal surface also branched, the average diameter increased (artery: 146 µm; vein: 276 µm), and the length was distinctly elongated (Figs 5*a*, 6). Along their way, the stem arteries and veins, which were usually centred as a pair in the main septae (Figs 5*b*, 6), increasingly gave off arterioles (Fig. 5*a*) and venules (Fig. 5*e*). Most of these vessels could be seen close to the fetal surface, together forming 'weeping willow'-like structures (Figs 5*c*, 6). Hence, depending on the number of arterioles and venules (which were the core of intermediate septa), there were only a few and indistinctly formed secondary crypts close to the caruncular base as seen on a cross-section of the cast (Fig. 5*b*) whereas, on the opposite side of placentome, secondary crypts were numerous (with 4–6 surface arterioles) and thus identical with the deep indentations seen in the weeping willow (Fig. 5*c*). Secondary crypts appeared as marginal niches from primary crypts (Fig. 5*a*, *c*). The border of this cryptal system, a dense tuft of capillaries in columnar form in a maternofetal orientation (Fig. 5*a*) and as large as the width of primary crypts (Fig. 5*a*, *b*), was developed from the 3rd to the 6th mo of gestation. Viewed at higher magnification, these capillaries represented complexes linking the ramifications of the inter-

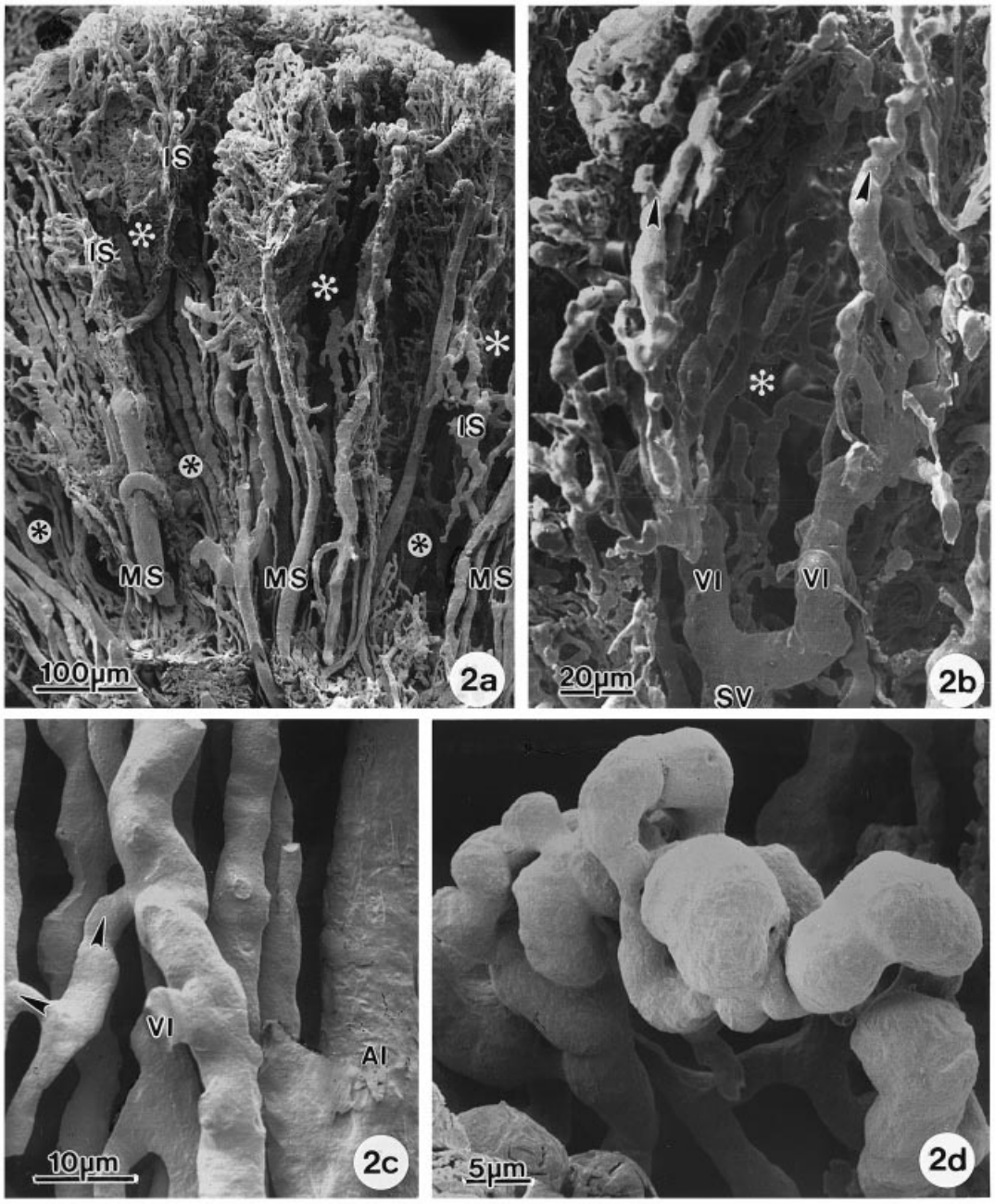

Fig. 2. Caruncular vasculature of 8 cm CRL fetus. (*a*) Main vascular septa (MS), mostly consisting of arterial and venous stem vessels which reach from the caruncle base up to about one third of the height of caruncle, form the walls of primary vascular crypts (small asterisks). Intermediate septa (IS), containing arterioles and venules, extend almost to the top of caruncle, thus providing the vascular framework for the secondary crypts (large asterisks), which are bordered by relatively small superficial complexes of terminal capillaries. (*b*) Higher magnification of the central area of *a*. A stem vein (SV), branching into 2 venules (Vl) gives rise to a secondary vascular crypt (asterisk) which is formed by the secondary septa and their adjacent terminal capillary complexes (arrowheads). (*c*) Detail of the microvasculature of a secondary septum showing one arteriole (Al) which is accompanied by several, parallel partly anastomosed (arrowheads) venules (Vl). (*d*) Terminal capillary loops clearly displaying sinusoidal dilations.

mediate septal arterioles and venules (Fig. 5*a*) and were arranged in a centrifugal way around the axis of the stem vessel pair (Figs 5*b*, 6). At the surface of these complexes, distinct tertiary crypts were formed, together appearing as a honeycomb-like system (Fig. 5*c*, *d*). Sinusoidal dilations on terminal capillaries were still a frequent phenomenon.

Fetal vasculature

The fetal vasculature of the bovine placentome could not be prepared by the method of vessel casting in very early placentation in this study (see Discussion) and therefore only stages from 16 to 38 cm CRL or 4th to 6th mo of gestation are shown.

Table 1. *Terminology of bovine placentomal microvasculature*

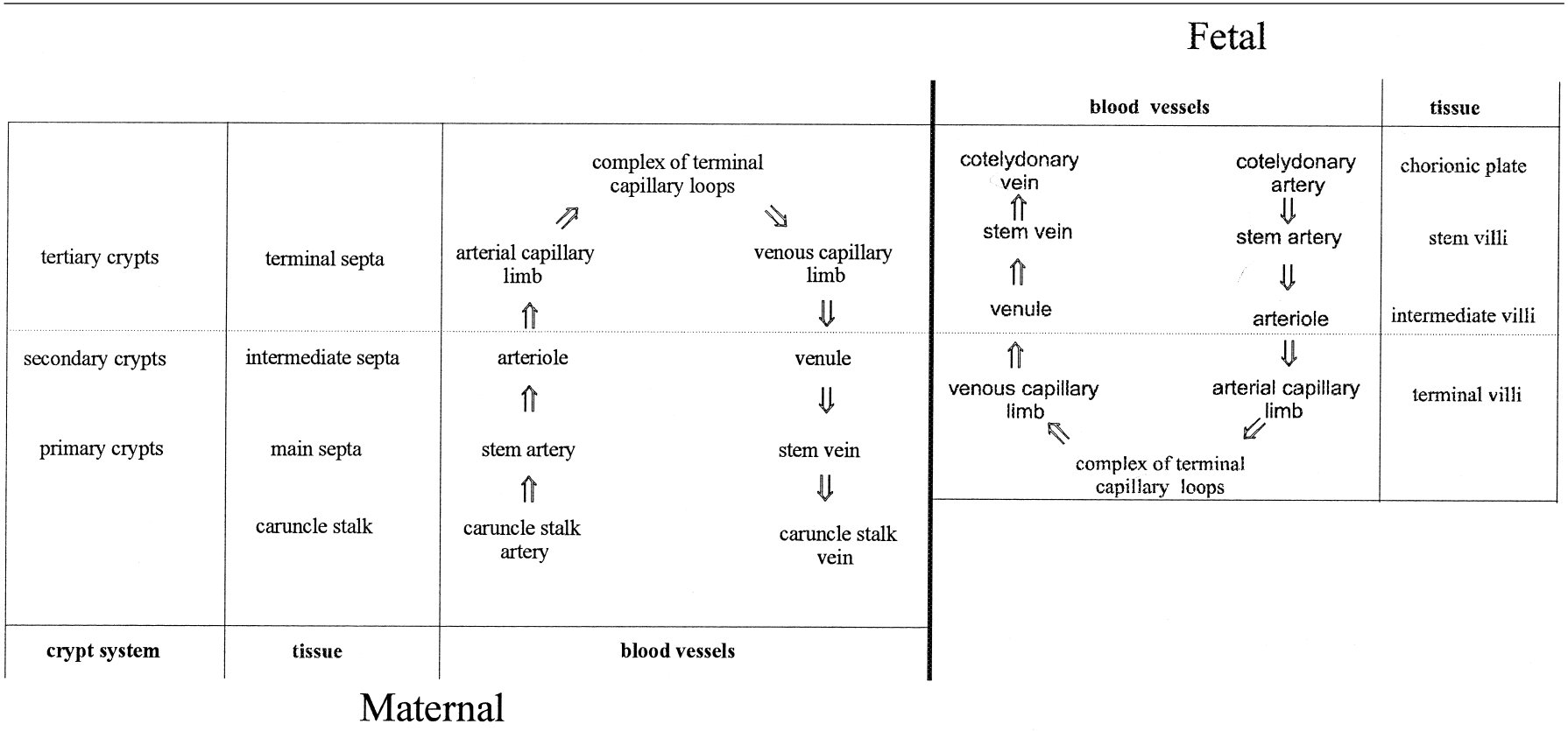

Table 2. *Vessel diameters*

Placentome	Maternal vasculature (gestational age)			Fetal vasculature (gestational age)	
Vessel type	~ 3rd month (8 cm CRL)	~ 4th month (23 cm CRL)	~ 6th month (36 cm CRL)	~ 4th month (23 cm CRL)	~ 6th month (38 cm CRL)
Stem arteries μm (mean)	88–105 (90)	108–300 (146)	143–263 (192)	108–145 (122)	107–214 (154)
Arterioles μm (mean)	40–60 (50)	57–101 (83)	90–130 (112)	25–63 (38)	32–50 (40)
Capillaries μm (mean)	18–23 (19)	13–23 (17)	14–28 (19)	8–14 (11)	7–12 (10)
Venules μm (mean)	15–58 (35)	40–83 (62)	38–120 (82)	30–90 (56)	35–45 (40)
Stem veins μm (mean)	98–125 (114)	260–300 (276)	168–270 (198)	104–160 (136)	108–276 (190)

n = 15 (per vessel type, compartment and gestational age).

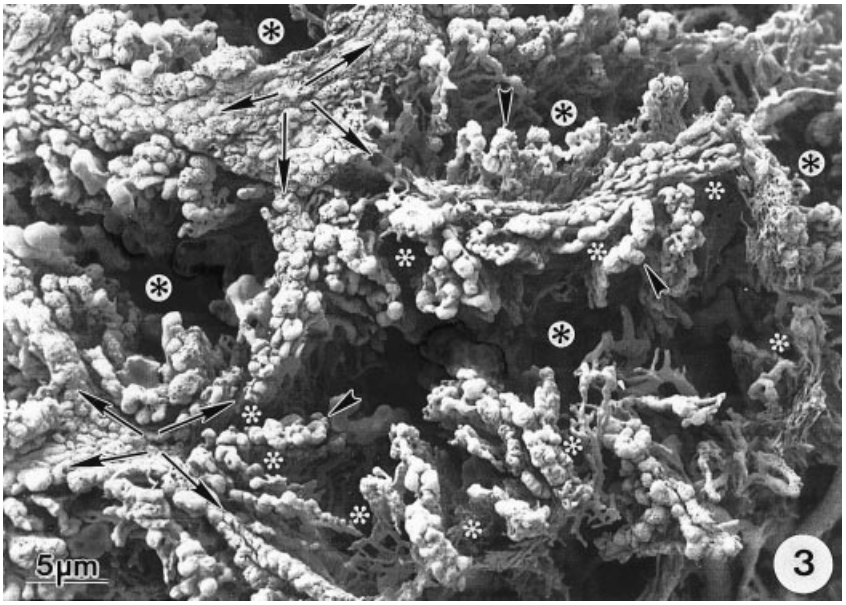

Fig. 3. Caruncular vascular cast viewed from the fetal side (8 cm CRL fetus). The top of caruncle which is covered with terminal capillary complexes allows insight into a vascular system of ramified primary (arrows) and secondary septa (arrowheads), enclosing primary (black asterisks) and secondary crypts (white asterisks).

Cotyledonary arteries of the chorionic plate, which were represented by 1–3 arteries with a straight course and branch at an acute angle supplied 1 placentome, whereas the 1–3 cotyledonary veins were thicker in diameter than the arteries and showed a rather winding course and variable-angled branchings. Many arteries and veins originated from these ramifying allantochorionic vessels and branched frequently before entering the vascular villous trees. From the base of the villous trees they were called stem vessels (Figs 7*a*, *b*). Villous trees on casts were easy to recognise not only by scanning electron microscopy (Fig. 7*b*) but also macroscopically since their length varied from 4 to 8 mm. Their orientation was fetomaternal.

The arterial and venous systems inside the villous tree ran in parallel thus being different from the allantochorionic vasculature outside the trees. The pairs of stem arteries and veins were oriented parallely in the centre of the stem villi (Fig. 7*c*) and gave off arterioles and venules in an irregular manner (Fig. 9). With progressing gestation, more than one stem vein developed to accompany a single stem artery.

As ramifications from the stem vessels, a single arteriole accompanied by several venules represented the core of an intermediate villus, which was short in relation to a stem villus (Fig. 7*c*). These arterioles branched into capillaries (arterial capillary limb) which supplied the terminal villi from their centre (Figs 5*e*, 7*c*, 8, 9). One terminal villus was composed of several capillary loops (2–4: Fig. 8) each about 400–700 μm long. These terminal villous capillaries were frequently arranged in parallel or in a slightly fan-like shape with many anastomoses. With progressing gestation, the loops were increasingly linked in a serial manner (Fig. 8). The terminal capillary loops converged to venous capillary limbs, which had a more peripheral position in the terminal villi when

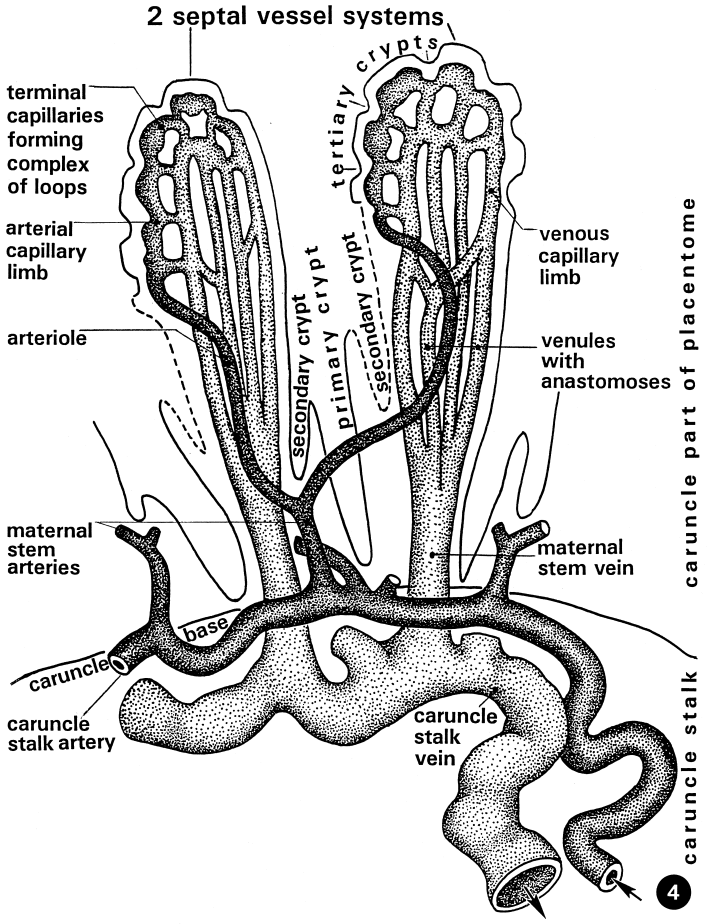

Fig. 4. Schematic drawing of the bovine maternal (caruncular) microvasculature of the placentome in the 1st trimester.

compared with the arterial capillary limbs (Figs 8, 9). Sinusoidal dilations of capillaries were rare, but occurred at sites of 'U turns' (Figs 8, 9). From the 4th to 6th mo of pregnancy, an increasing number of capillary 'vasa vasorum' were observed along the stem vessels.

DISCUSSION

Technical remarks

In early gestation (before 16 cm CRL, ~ 4th mo), casting of the fetal, cotyledonary vasculature was impossible due to the fragility of the blood vessels. The fetal mesenchymal tissues were very delicate at the beginning of gestation, so that extravasation of the casting resin occurred frequently, completely covering most of the remaining vessels. Even rinsing procedures with various fixatives and/or vasodilators did not improve the results. Indian ink histology was necessary to gain insight into the relation of fetal and maternal blood vessels, due to the transparency of the unstained thick sections. The dense microvasculature prevented this assessment in corrosion casts.

Implications of architectural changes

In the caruncle stalk vasculature of the bovine placentome 'spiral' arteries show a more winding course than the corresponding veins, but both are more distinct in early gestation than in later stages of pregnancy (Leiser et al. 1997*b*). Spiralling, therefore, seems to be essential at least in early placentation for curbing the blood pressure and flow in cattle in order to avoid compression of the delicate fetal villi as has also been discussed in man (Carter, 1975; Benirschke & Kaufmann, 1995). Of less importance may be the fact that this arrangement might also deform, as do 'bed springs', when the caruncle stalk becomes flexed by movements of the fetus and the mother (Leiser et al. 1997*b*).

The early *caruncular vasculature*, with its supply represented by stem and intermediate vessels (Leiser et al. 1997*a*), exceeds the working part by volume, the former guiding the blood from the caruncular base through the stem septa to the top of the intermediate septa (Leiser et al. 1997*a*). The latter includes the capillaries being active for exchange at the caruncular periphery (see also Fig. 1*a*).

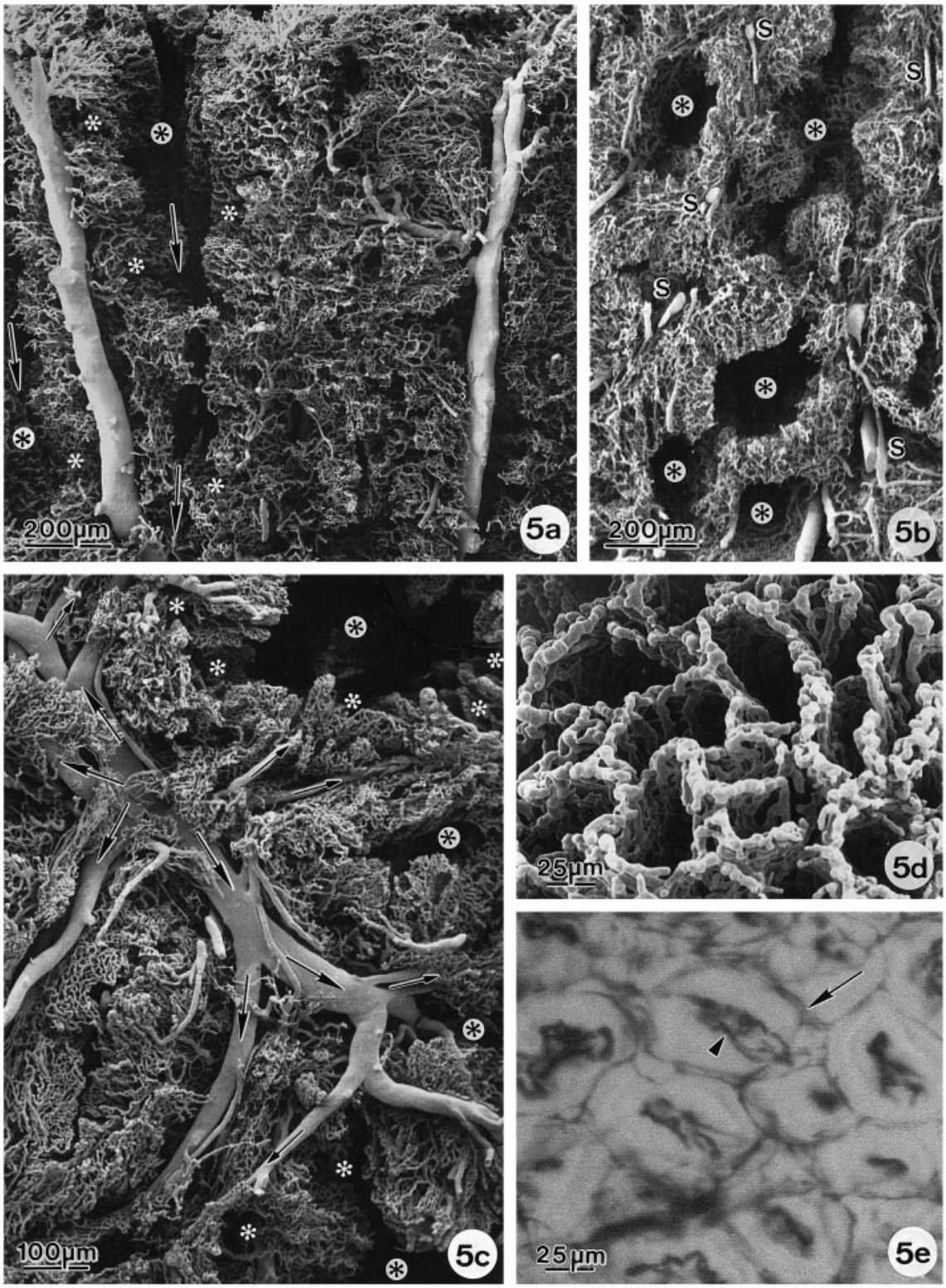

Fig. 5. Maternal vasculature of 36 cm CRL fetus. (*a*) Lateral view of a cracked arterial cast of the caruncle. Two maternal stem arteries, each running in the centre of a main/primary septum from the base (below) to the top of a placentome (above), ramify into the arterioles (most of them broken) of the secondary vascular crypts (white asterisks; arrows indicate orientation of fetal stem villi) which supply the large terminal capillary complex. Primary vascular crypts are indicated by black asterisks. (*b*) Horizontally cracked caruncular cast from the same animal. Primary crypts (black asterisks) are surrounded by the rough reticulum of the capillary complex. Inside this complex, conspicuous stem vessels (S) mark the centres of main (primary) septa. (*c*) Caruncle vasculature with view of the fetally oriented surface. A stem artery branches into centrifugally oriented ramifying arterioles (large arrows) which with a bend of about 90° reach into intermediate septa (small arrows) and ultimately supply the terminal capillary complex. This resembles the branching pattern of a 'weeping willow'. Note the primary (black asterisks) and secondary crypts (white asterisks). (*d*) Higher magnification of the caruncular microvasculature. The terminal capillary

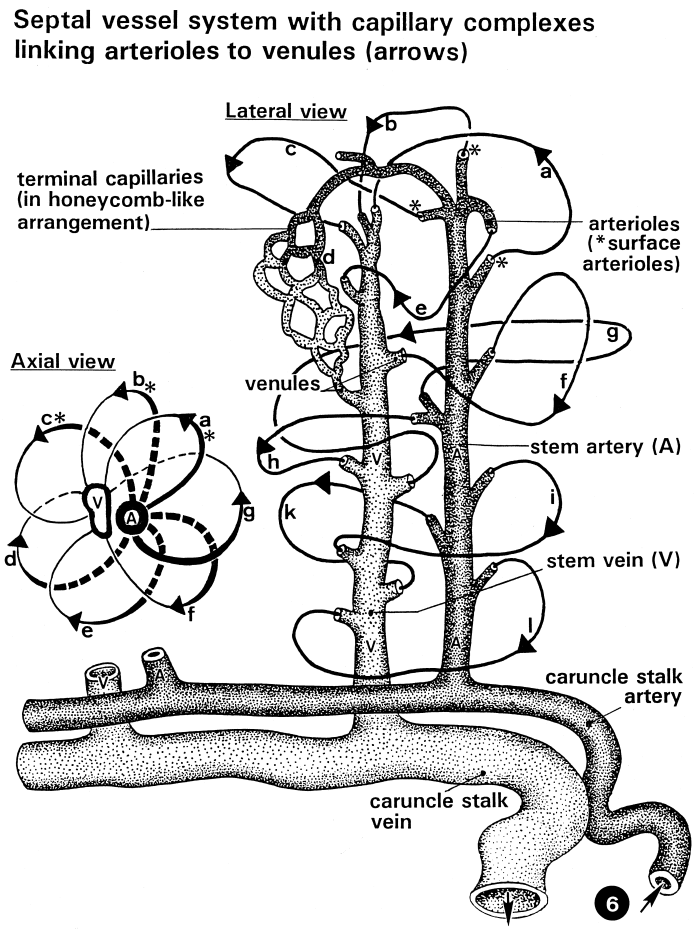

Fig. 6. Schematic drawing of the bovine maternal microvasculature of the placentome in the 2nd trimester. Arrangement of capillary complexes (*a–l*) in fetomaternally directed (left) and lateral (right) views with respect to the axis of a main septum.

The fact that stem arteries and veins of the supplying part are relatively short in the lower third of the caruncle and intermediate arterioles and venules reach up almost to its tip, a 'vascular frame' is given in early pregnancy which allows these arterioles and venules to turn into branches of stem vessels in the middle of gestation (Fig. 6). In early gestation, these branched stem vessels are the basis of many newly growing intermediate arterioles and venules which transport the blood to the fetal side of the placentome and distribute it in a 'weeping willow' pattern, thus providing a relatively short link from the stem vasculature to the capillaries (Fig. 6). This vascular system continues as a basic ramification principle for the second half of pregnancy, since only length and ramification density of vessels will change when compared with the increasing size ($\times 3$) of the placentomes (Tsutsumi, 1962; Leiser et al. 1997*a*).

The capillaries, or working part of the early caruncular vasculature, are conspicuously poorly developed, enclosing a small layer on top of the septal system with as yet little formation of tertiary crypts (Fig. 4). The capillaries inside the terminal septa are directly adjacent to the extremely short fetal villosity, which guarantees a short interhaemal distance for transplacental exchange. The large surface, due to the winding course and sinusoidal dilations of these capillaries, favours substance transfer. Sinusoids located near the septal tips may slow the blood flow, providing a specific opportunity for maternofetal exchange (Arts, 1961). This is also sustained by the fact that venules are especially thin-walled in early placentation (Björkman, 1954; Tsutsumi, 1962) and therefore easy to penetrate.

Up to the middle of pregnancy, the capacity for substance transfer is distinctly increased (Björkman,

loops of the tertiary septa are arranged in a honeycomb-like fashion, thus enclosing the tertiary crypts. (*e*) Micrograph of a combined fetomaternal Indian ink perfused histological section, 35 cm CRL fetus. Blood vessels of maternal tertiary crypts (arrow) surround fetal terminal villous capillaries (arrowhead) in a honeycomb-like fashion.

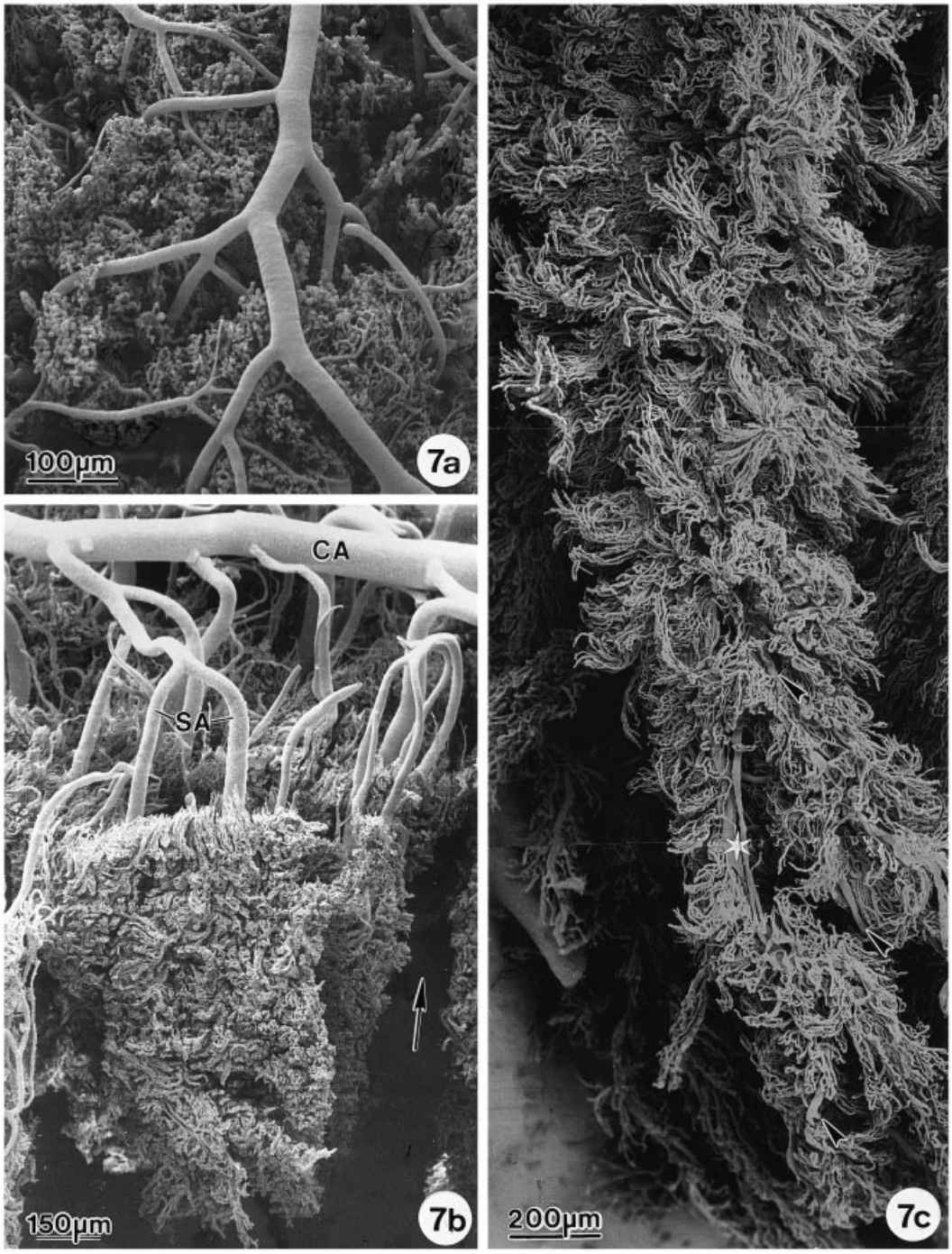

Fig. 7. Fetal vasculature. (*a*) Scanning electron micrograph of vessel cast showing the allantochorionic surface of a cotyledon or fetal part of a bovine placentome (16 cm CRL fetus). Stem arteries, ramifying from a cotyledonary artery of the chorionic plate, disappear in several capillary complexes which are not organised in the shape of villous trees at this time. (*b*) Scanning electron micrograph illustrating the branching pattern of a large cotyledonary artery (CA) into many smaller ones which are followed by stem arteries (SA); fetus of 38 cm CRL. The cotyledonary arteries bend from the chorionic plate into a straight uterine direction before reaching the centres of their conical villous trees. The intervillous space (arrow) corresponds to a caruncular septum. Note that the surface of these vascular trees consists of the terminal capillary complexes. (*c*) Micrograph of the tip of a villous tree (50 cm CRL fetus). The innumerable capillary loops of the terminal villi are missing in some places and therefore allow sight onto the stem vessels (star) as well as arterioles and venules (arrowheads).

1954) probably by the newly developing tertiary crypts, which are embedded in the large and dense capillary tuft located along the secondary crypts. Tertiary crypts are indented by tertiary villi of the cotyledon, a phenomenon which, besides substance transfer, results in anchoring of the bovine fetal placenta in the uterus (Leiser et al. 1998). All this increases progressively until the end of gestation (Leiser et al. 1997*a*,*b*).

The fetal vasculature of the bovine placentome

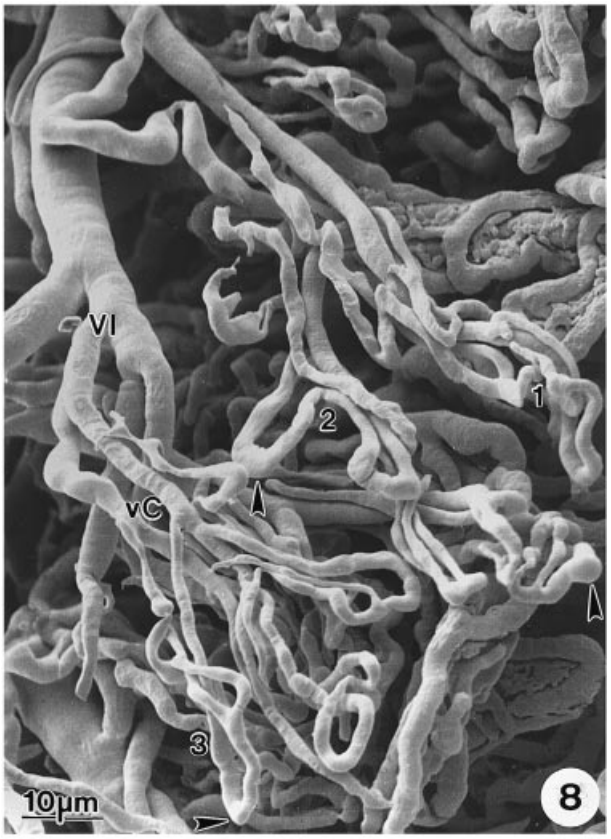

Fig. 8. Detail of a complex of serially-linked fetal capillary loops (38 cm CRL fetus). The capillary loops (1, 2, 3, …) each consisting of 2–4 terminal capillaries, converge to venous capillary limbs (vC), venules (Vl), and a stem vein (top left). Note some sinusoidal dilations of the capillaries (arrowheads).

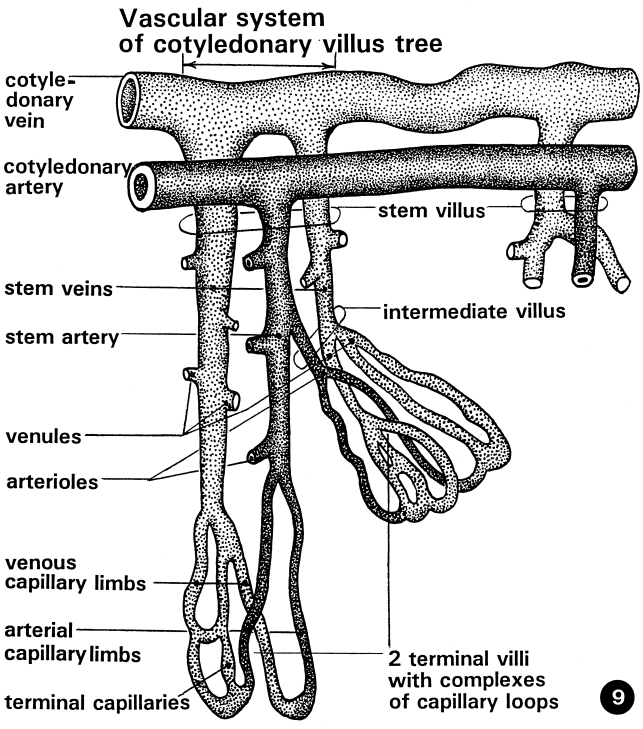

Fig. 9. Schematic drawing of the bovine fetal microvasculature of the placentome in the second trimester.

shows the same main characteristics as in later placentae by the first half of pregnancy (compare Fig. 9 with Leiser et al. 1997*a*, *b*). Conforming to the cryptal organisation of the caruncle primary, secondary, and tertiary villi develop, indenting these crypts. Differing from the caruncular vasculature, the vessels of the primary villous vasculature are distinctly isolated from each other when branching from the cotyledonary vessels. Hence, the stem vessels and their ramifications—arterioles and venules—from the beginning of their development almost completely follow the whole height of the placentome with a more central location for the arterial vessels and a peripheral one for stem and intermediate villous vessels. This provides the shortest distance from supplying vessels to the capillaries of the working part (Leiser et al. 1997*a*) where more of the transplacental exchange takes place, and results in a countercurrent blood flow interrelationship of the fetal and maternal vasculatures (see below).

The fetal capillary system of midgestation is characterised by its parallel anastomosed loops being relatively long in comparison with the size of the whole placentome (Fig. 9). They are already representing a rough 'framework' for the vasculature seen at term (see fig. 5*d* in Leiser et al. 1997*b*). Hence, in fulfilling the increasing need for placental exchange until term, the capillaries will shape into a typical fan-like, serially-linked arrangement of loops or complexes which are distinctly convoluted and show an abundance of dilations to aid blood flow (Leiser et al. 1997*a*, *b*). In general, slow blood flow should occur in the relatively wide-calibre venous capillary limbs and venules of terminal and intermediate villi and therefore could ameliorate conditions not only for diffusional O_2 or CO_2 exchange (Faber & Thornburg, 1983), but also for slow-transported solutes of the active placental exchange (Alberts et al. 1983). Substance transfer might also be facilitated by the vicinity of terminal vessels which are located peripherally along the villous axes.

Development of maternofetal blood flow interrelationship

The bloodflow interrelationship is mainly countercurrent in the relatively early placentation of the 3rd and 4th mo of gestation. The lack of most of the capillaries of the tertiary structure allows the stem and intermediate vessels both of the caruncular and cotyledonary parts of the placentome to meet in a countercurrent fashion (compare Figs 4 and 9). Thus one of the most efficient transplacental exchange

systems is established (Faber & Thornburg, 1983; Leiser & Kaufmann, 1994), substituting for the lack of capillaries very efficiently.

From the middle third up to the end of gestation, the mainly countercurrent (Tsutsumi, 1962) bloodflow interrelationship of the stem vessels is maintained, but its significance reduces with the development of tertiary crypts and interdigitating terminal villi. The fetal and maternal capillary complexes of the tertiary unit, where the majority of the exchange takes place are in a crosscurrent blood flow relation with each other (compare fig. 6 with fig. 12 in Leiser et al. 1997*a*). The crosscurrent exchange is considered to be not as efficient (see Faber & Thornburg, 1983), but (1) the combination of both systems, (2) the multiplication of the exchange surface (Baur, 1981) due to progressive capillary growth and (3) the vascular architecture displaying a short supplying component of the vasculature results in a placenta of relatively high substance transfer, especially when considering the ratio of fetal and placental weight, which is about 12:1 in cattle (Dantzer et al. 1988). Additionally, the presence of morphology independent exchange-promoting factors, such as vascular endothelial growth factor (Torry & Torry, 1997), may compensate for the reduced efficiency of the crosscurrent exchange condition.

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Fetal villosity and microvasculature of the bovine placentome in the second half of gestation

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ABSTRACT

The architecture of the fetal villous tree and its vasculature in the bovine placentome were studied in the second half of gestation using both conventional histology and histology of ink-filled blood vessels. These were compared with corrosion casts of plastic fillings of the vasculature, prepared for scanning electron microscopy. This combination of morphological methods allows perception of the villous tree throughout gestation from broad-conical to tall-conical form where branch ramification occurs mainly at right angles to the stem. The stem villus typically contains a single central artery and several peripheral veins arranged in parallel. The proximal branches to the stem, the intermediate villi, contain a central arteriole and accompanying venules. The distal branches, the terminal villi, enclose capillary convolutions which consist of an afferent arterial capillary limb, capillary loops and efferent venous capillary limbs. Vascular interconnections exist within the terminal villi, as capillaries or venules between the capillary convolutions, serially bridging them in up to 5 places, and as capillary anastomoses between the capillary loops. Coiling and sinusoidal dilatations of these loops develop near the end of gestation. The intraplacentomal rearrangement of villous trees with progressive gestation and their morphological vascular adaptations are discussed in relation to placental function, including the ever increasing need for transplacental substance exchange. This adaptation allows the blood to traverse the shortest possible arterioarteriolar route to the periphery of the trees where exchange takes place. The need for an increasing blood flow stimulates capillary growth and at the same time optimises the blood flow reaching the placental barrier represented by the vessel cast surface. The capillaries also carry the blood back into the very voluminous system of venules and veins where back diffusion may occur. The total volume of terminal villi of bovine placentome, the 'working part' of villous trees, hence distinctly increases with respect to the stem and intermediate villi, the 'supplying part' of the villous tree. In morphological terms the efficiency of the bovine transplacental diffusional exchange is higher than in the closely related 'co-ruminants' sheep and goats and distinctly higher when compared with the human placenta.

Key words: Placenta; microvasculature; corrosion casts.

INTRODUCTION

The bovine placenta is classified by multiple separate cotyledons which display a villous interdigitation of fetal villi with maternal crypts of the complementary caruncles (for review, see Mossman, 1987; Leiser & Kaufmann, 1994; Wooding & Flint, 1994). The interhaemal barrier is epitheliochorial (Björkman, 1954, 1968) or synepitheliochorial (Wooding, 1992) because binucleated cells of the syncytial trophoblast fuse with the syncytium of the uterine epithelium. The blood flow interrelationship between the maternal and fetal vessel systems is a mixture of crosscurrent to countercurrent in the cow (Leiser et al. 1997) which is regarded as an efficient type for transplacental diffusional exchange, e.g. oxygen, when compared

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with other species (reviews by Faber & Thornburg, 1983; Dantzer et al. 1988).

These classifying characteristics guarantee functions such as anchoring, substance exchange, and hormonal control; however, for a better understanding the exact 3-dimensional structural arrangement of both fetal and maternal compartments of the placenta needs to be determined (reviewed by Faber & Thornburg, 1983; Mossman, 1987).

The placenta is a highly vascularised organ. Therefore, its 3-dimensional structure can be demonstrated by microvascular casts which reflect the shape of the whole or part of the organ, and include specific details such as the capillary architectural structure (Leiser et al. 1989). Filling of the placental vasculature by liquid plastic and, after polymerisation of the plastic and tissue corrosion, its visualisation by scanning electron microscopy can technically be advanced in 3 ways: treatment of maternal and fetal circulatory systems in common or each of the 2 systems separately (Leiser, 1985; Leiser et al. 1989).

In the placentomes of ruminants, e.g. goat (Leiser, 1987), sheep and cow (Leiser et al. 1997), the technique of separate casting is technically easy and allows an impressive demonstration both of the fetal villosity of the cotyledon and the maternal septal/cryptal structure of the caruncle. The systems complement each other (Tsutsumi, 1962; Ebert, 1993). In this study we aimed to elucidate the 3-dimensional structure of the cotyledonary vasculature of the bovine placentome in the second half of pregnancy, which serves as a core for the shape of the whole fetal villosity. Methods used are histology of perfusion fixed tissue as well as immersion fixed vascular India ink-injected tissue, and vessel corrosion casting with subsequent analysis by scanning electron microscopy.

MATERIALS AND METHODS

Fifteen bovine placentae from uncomplicated, second half gestations were excised from slaughtered animals with postinsemination (p.i.) age as follows: 3 from day (d) 150, 1 d 180, 2 d 200, 3 d 220, 2 d 240, 4 d 270. Suitable placentomes (4 per placenta) were immediately perfused for 30 s with phosphate buffer (0.1 M, pH 7.3) which contained 1000 IU/l heparin as anticoagulant and 1% procaine for vasodilation.

For histology without special treatment, perfusion of buffer was followed by perfusion fixation with 'yellow fix' (2% glutaraldehyde, 2% paraformaldehyde, 0.02% picric acid in 0.1 M phosphate buffer, pH 7.3; Ito & Karnowsky, 1968). After excision of the placentomes smaller blocks ($\sim 0.5 \times 1$ cm) were cut and this tissue was postfixed in the same fixation solution for 3 h.

For specialised histology, the fetal vasculature of additional buffer perfused placentomes was injected with India ink (green drawing ink, Rotring, Hamburg, mixed 1:1 with bovine serum). Before subsequent sectioning (see above) the placentomes were immersion fixed in buffered (pH 7.3) 4% formaldehyde for 3 d.

Tissue specimens from both histological preparations were dehydrated through a series of graded ethanols, embedded in Epon, and sectioned with a Polycut microtome (Leica). Sections of tissue (3 μm) without ink treatment were deplastinised by a saturated ethanolic solution of NaOH. The sections were stained by a silver impregnation technique (Movat) and counterstained with Mayer's haematoxylin (Böck, 1989). Sections (150 μm) of ink-injected material were examined 'unstained'.

For corrosion casting, after an additional perfusion rinse with buffer at 4 °C (see above), the fetal vascular system was instilled with a mixture of liquid plastic, Batson no. 17 compound (Polysciences) and Sevriton (De Trey Dentsply, D-Konstanz) 33:12 (see Leiser & Kohler, 1983; Leiser, 1985). The plastic mixture was freshly prepared and cooled prior to instillation, thus delaying polymerisation for approximately 10 min. The plastic was instilled via a syringe under manual pressure at a flow rate of approximately 5 ml/min. Chorionic arteries and veins were clamped after instillation to prevent efflux of plastic and to maintain the instillation pressure within the system. The placentomes were excised and placed in a water bath at 20 °C for 30 min, followed by immersion in a water bath at 80 °C for several hours to allow hardening of the plastic.

Corrosion was performed over several days by alternating immersion of the plastic-instilled tissue in 40% KOH and distilled water, both at 60 °C. Suitable material for scanning electron microscopy was obtained as follows. The casts were embedded in warm 20% gelatine (50 °C), cooled to -5 °C, and cut with a knife. After thawing, the gelatine was removed by a second corrosion procedure, followed by very thorough and repeated washes at room temperature in distilled water, then in 5% Extran (Merck) and finally in distilled water. After final drying the pieces were mounted onto stubs by a conductive carbon cement, sputter-coated with gold (3 nm), and examined by scanning electron microscopy. For further technical details, see Leiser (1985) and Leiser et al. (1997).

RESULTS

Relation of general fetal components to the whole placentome

The shape of the bovine placentome was generally mushroom-like and consisted of 2 components (Fig. 1): maternal tissue of the caruncular stalk sustaining the placentomal basal plate and septae, and the fetal tissue which forms the convex fetally oriented cotyledon with the chorionic plate of placentome covering the maternal tissue like a cap. Because of this convexity the shape of a single villous tree was conical, with a large base thinning out to its top like a Christmas tree (Fig. 1; see also Leiser et al. 1997). These trees radiated strictly from the chorionic plate in a maternal direction, eventually ramifying to form several tree-tops.

Formation and shape of villous trees

The development of young villous trees was primarily characterised by the budding of particularly large branches from the lowest part of stem villus (Fig. 1). Secondly, these branches may have separated from their original villous tree and became new villous trees arising from the fetal side or chorionic plate. This process caused a rapid extension of the convexity of the cotyledonary part of the placentome (Figs 1, 12). As the number of villous trees increased (Andresen, 1927; Björkman, 1954) the conical shape became slim and tall. At d 220 of pregnancy (Fig. 2*a*, *b*), the histological picture revealed a triangular cross-section of a villous tree (Fig. 2*a*). This histological picture was corroborated by the structure of extravasated vessel casts, which represent a filling of almost the whole stromal compartment, producing a solid 3-dimensional aspect of the fetal villosity (Fig. 2*b*, *c*).

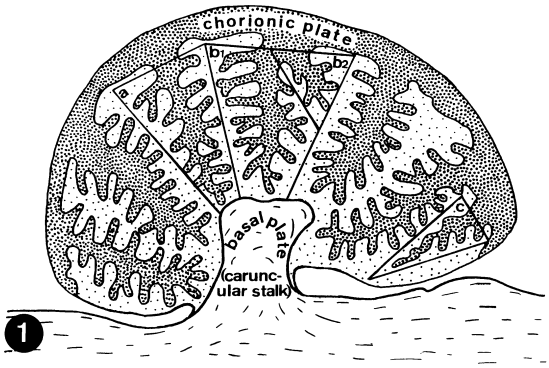


Fig. 1. Schematic drawing of the bovine mushroom-shaped placentome. The villous trees (densely stippled) extend from the fetal chorionic plate (convex) to the maternal basal plate (concave). Three different types of villous tree are schematised: tall-conical of mainly late gestation (a), Christmas tree-like of midgestation in full-grown (b_1) and in budding (b_2) stage, new-grown on the periphery (c). The branches ramify mainly at right angles to the stem of the villous tree.

Multiple branches projected from the stem villus in these casts (Fig. 2*b*), generally oriented at right angles to the middle third and at acute angles to the upper third of the stem villus (also compare Figs 1, 12). They ramified by several orders, decreasing in number from the base to the top of the villous tree. An obliquely cracked vascular cast (Fig. 3) and an ink-vascular-filled histological section 150 μm in thickness (Fig. 4) allowed insight into the interior of the villous tree, revealing both the angles and the ramification pattern. Only well-chosen sections of conventional histology were also able to demonstrate these phenomena (Fig. 5).

Vasculature of villous trees

The vasculature of the stem of villous trees was marked by one main stem artery which arose from the allantochorionic arterial system. The stem artery ran a straight fetomaternal course, and at several locations ramified at an acute angle, forming new main stem arteries as well as subordinated arteries according to the principle of the villous tree. Both types of stem arteries were strictly located centrally in the stem (Fig. 3). Several stem veins formed a tube-like system, which ran parallel to and enclosed the stem artery. These veins were anastomosed to each other by intervenous bridges (Figs 3, 4, 6, 12). In contrast to the stem artery, the stem veins were slightly convoluted and had a variable diameter, at times forming pouch-like structures (Figs 6, 12).

The vasculature of the branches ramifying from the stem villus was classified according to the character of their main vessels (Leiser, 1987; Leiser et al. 1997). Arterioles and venules, which were recognisable histologically by a relatively thin muscle layer (Fig. 9), marked the intermediate villus, whereas capillaries, histologically devoid of muscle cells (Fig. 9), carried blood to the terminal villi. Intermediate villi were variable in ramification and length, e.g. simpler in early pregnancy and on tree tops and complex in advanced gestation and in the basal part of the villous tree (Fig. 2). The terminal villi were more constant in length with respect both to gestation and location.

The vasculature of intermediate villi generally consisted of one arteriole which formed the central axis of the villi and venules located in the axial periphery (Figs 7, 8). Thus the spatial orientation of these vessels was determined by the angle of ramification of the intermediate villus from the stem villus

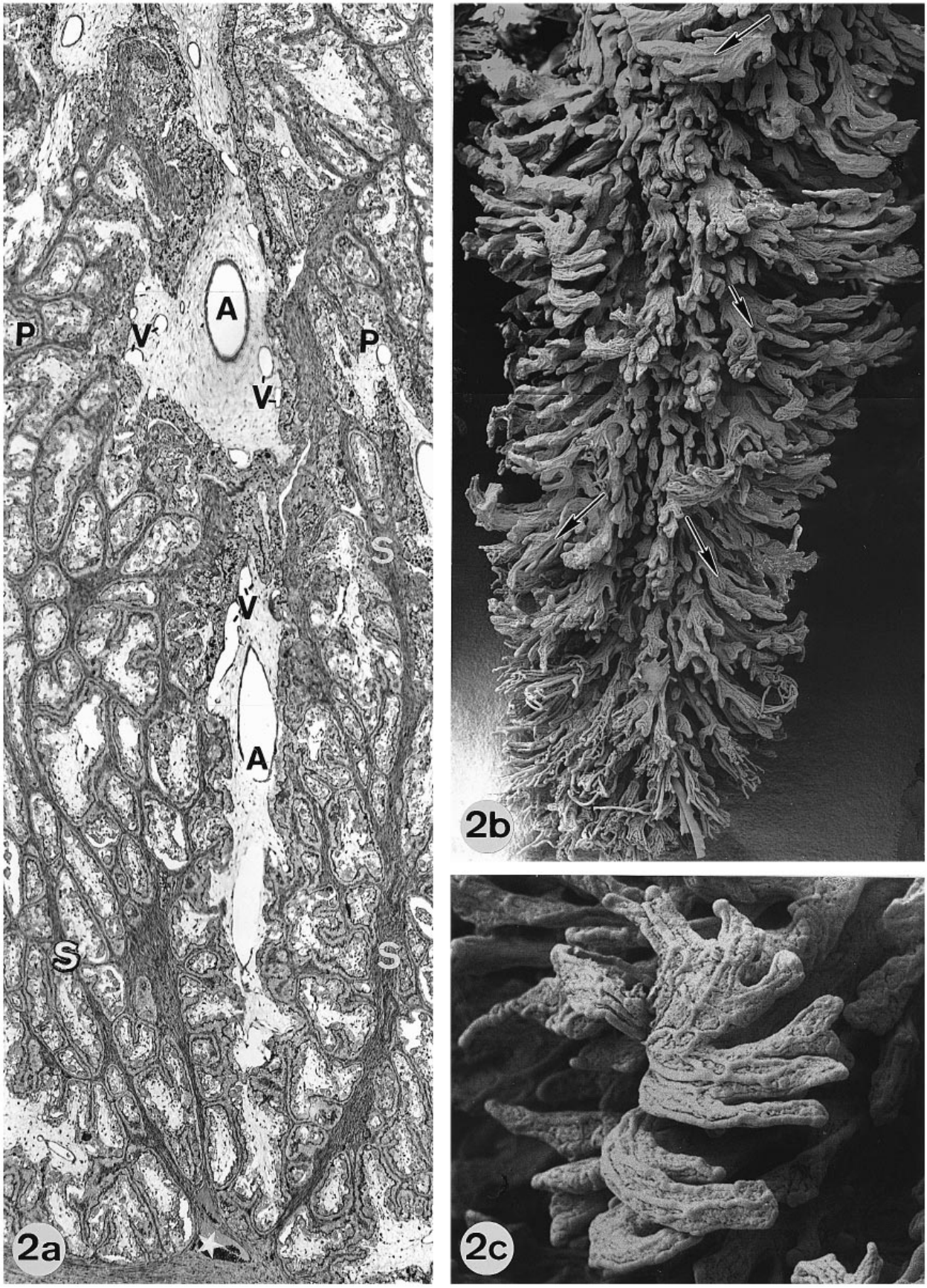


Fig. 2. Tall-conically formed, full-grown villous trees, d 220 p.i. (*a*) Histological preparation demonstrating the connective tissue of a stem villus with a centrally located artery (A), whereas several thin-walled veins (V) are found at the periphery of the placental parenchyma (P) which consists of intermediate and terminal villi (bright) indenting maternal septal tissue (dark). Thick septae (S) mark the peripheral border

(see above and Figs 4, 7). The venules converged (Fig. 8) into the tube-like venous system of the stem villi (see above and Fig. 4). They lay in a similar plane, forming a fan-like anastomosing system (Fig. 6). Venules were more numerous in the complex intermediate villi (Fig. 6) than in the simpler ones (Fig. 7). They demonstrated a typical wavy course and variable diameter, whereas the arterioles were straight and had a smaller constant diameter, resulting in a rather smooth surface on casts (Figs 8, 12).

The vasculature of terminal villi consisted of capillaries which, initially tightly arranged, formed the short 'necks' of terminal villi (Ebert, 1993) and then convolutions formed the several outmost-reaching loops. This system was outlined by conventional histology (Fig. 9), but demonstrated more clearly by the histology of ink-filled blood vessels (Fig. 7) and vessel casts (Figs 8, 10). The angle of terminal villi to the main axis of intermediate villi was generally about 45° (Fig. 10) but varied from no angle, e.g. at the top of the tree (Figs 2*b*, 10) to obtuse angles (Figs 2*c*, 9).

The neck of terminal villi contained one arterial capillary limb situated centrally and a few venous capillary limbs running in parallel (Figs 7, 10). The capillary convolutions of terminal villi projected from the neck region (Fig. 10), forming up to 6 capillary loops (Figs 10, 12). The degree of coiling and the number of anastomoses at the bases of these loops (Fig. 11) increased during gestation (compare Figs 4, 7 with 6, 10), as did sinusoidal dilatations at the extremities of loops (Figs 9, 10). From the neck area to the convolutions the terminal villi were linked by capillary or venular bridges, serially connecting up to 5 terminal villi (Figs 4, 7, 10, 12).

DISCUSSION

Selection of placentomes for study

In this study, the specific method of placentome selection allowed comparable material to be obtained from different cows. With respect to gestation, the most developmentally advanced placentomes are located near the conceptus in the midsection of the pregnant uterine horn, where implantation of the embryo first occurs (Leiser, 1975), and thus where the blood vessels develop optimally from the beginning of placentation (Leiser et al. 1997). Therefore, placentomes located in the tips of gravid and in nongravid uterus horns (Leiser, 1975), as well as accessory placentomes (Andresen, 1927; Björkman, 1954) which are generally smaller than circumconceptional ones, were excluded.

General placentomal and fetal villosity growth in second half of gestation

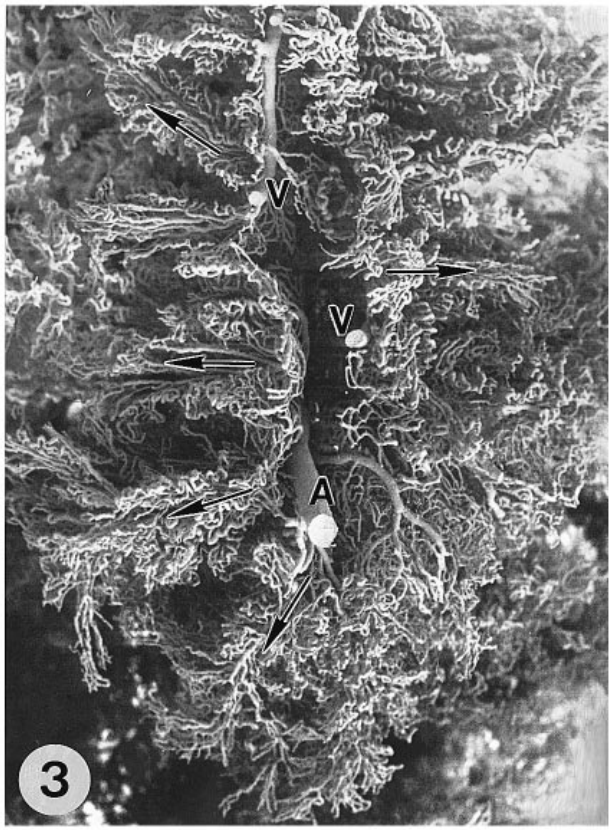
Björkman (1954, 1969) described the bovine placenta as fully developed after d 170 of gestation. After this time growth slows. The placentomes still enlarge but growth is observed mainly at the placentomal periphery, thus forming the mushroom-like shape of the placentomes (Ebert, 1993; Leiser et al. 1997). Our study reveals that, in addition, new fetal villous trees also develop in the centre of the placentomes. This growth is necessitated by the increasing demand for maternofetal substance transfer during the second half of pregnancy. This is reflected by the concentration of oxygen measured in the umbilical vein (Reynolds et al. 1986) and the increasing fetal weight (Reynolds et al. 1990).

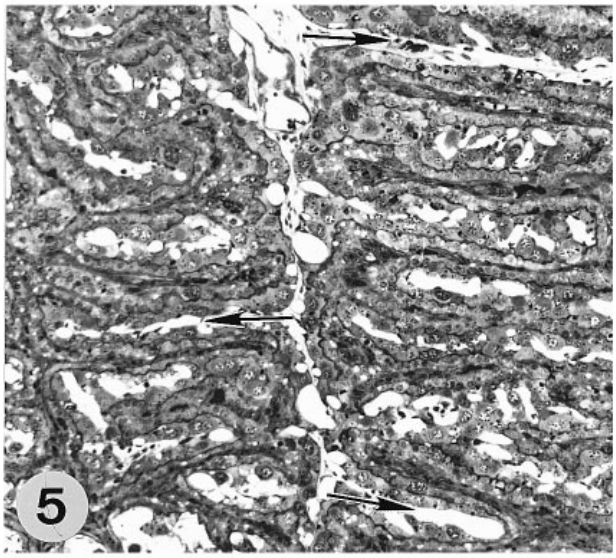
Fetal absorptive surfaces favouring placental substance exchange

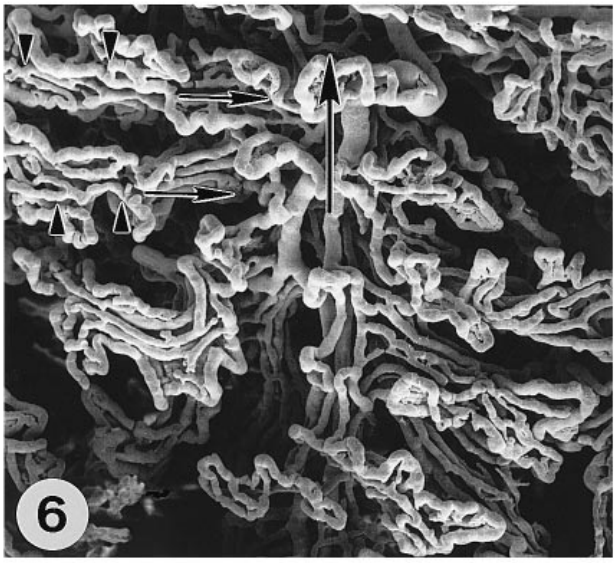
Transplacental exchange depends upon 2 substance-absorbing surfaces of the placentomal villosity (Björkman, 1968), the outer villous trophoblast surface and the inner vascular endothelial surface. The efficiency of substance exchange is improved by the increasing area of both these surfaces per volume of placentome (Faber & Thornburg, 1983).

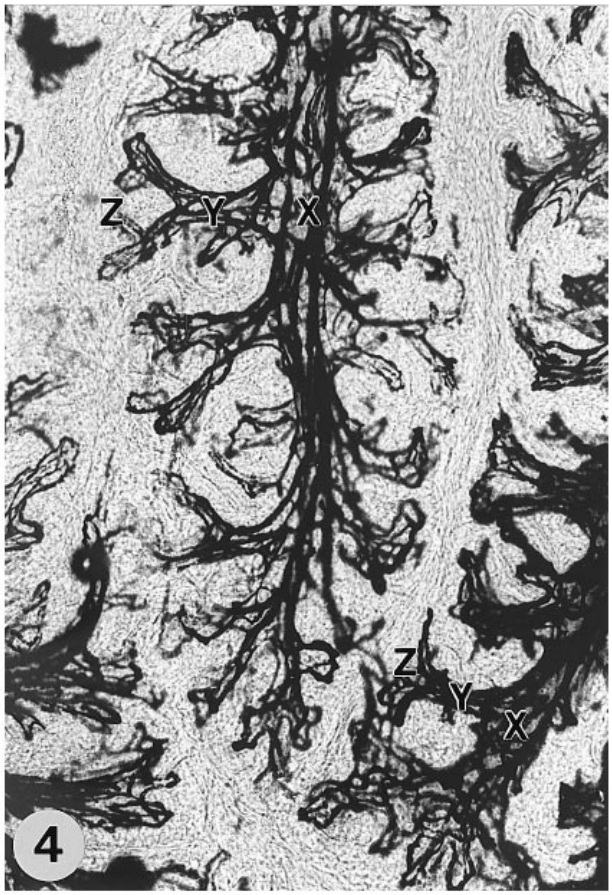
In the second half of bovine gestation, the villous surface increases not only by general growth of the placentomes (see above) but also, as compared with earlier stages (Leiser et al. 1997), by an altered arrangement of villous trees, which occurs by a change in the form of the villous trees, specifically the pattern of branches ramifying from the stem of villous tree. The villous trees become tall and conical with relatively short branches which show little variation in length and are well equipped with terminal villi (see below). This allows a denser arrangement of trees

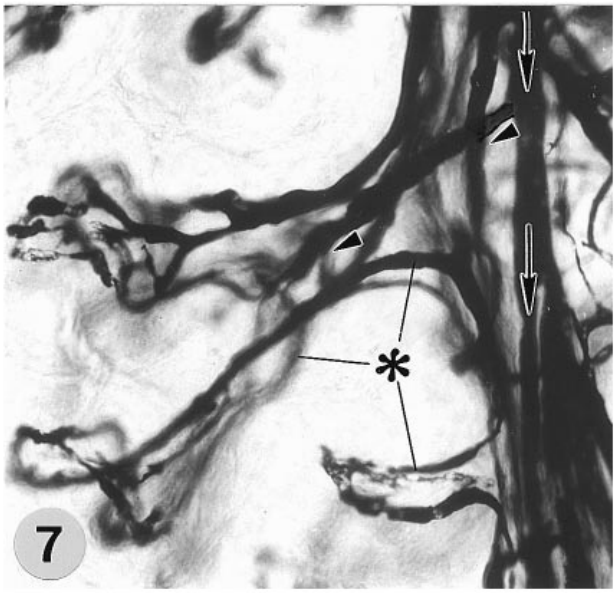
of the centrally located villous tree. Endometrial tissue of the caruncular stalk containing a vein (star) is located at the bottom. ×60. (*b*) Following casting, the stroma of the villous tree is almost completely filled with plastic, except for the top of the tree (bottom). The complex ramification of intermediate villi into terminal villi is clearly visible (arrows). ×185. (*c*) Higher magnification of above illustration displaying a fan-like ramification of a terminal villus (appearing from left) into capillary loops, which are partly bent backwards. The capillary filling can be distinguished from the stromal filling. ×600.











Figs 3–7. For legend see opposite.

than in the young broad-conical ones observed earlier in gestation (Leiser et al. 1997). This is documented by a higher number of tree-units per placentome. Additionally, the indentation of fetal with septal tissue is almost complete, because the villous branches mainly ramify at right angles to the stems of the villous trees (Figs 4, 5), therefore 'blind corners' of maternofetal contact are excluded to achieve the maximal functional surface area of villous tissue in a given space.

The vascular surface of villous trees can be classified, first, according to the vessel groups of arteries/arterioles and venules/veins providing the 'supplying part' of the placentome (Leiser et al. 1997) and secondly, by the blood vessels of the capillary bed or 'working part', where most of the transplacental substance exchange takes place (Benirschke & Kaufmann, 1995).

In order to favour the working part, the supplying part has to be as small as possible. Therefore the vessels of the villous tree have to run as straight and directly as possible from the chorionic plate to the capillaries of terminal villi. This study supports this hypothesis, showing the straight, central course of the single artery in the stem and the arterioles in the branches or intermediate section of the villous tree (Fig. 12). Veins and venules follow this scheme less strictly, because their peripheral location in stem and branches (Fig. 12) and their thin wall allows them to function in substance transfer, as well as the specific blood flow characteristics described below.

The working part of the villous tree consists of thin-walled vessels, mainly capillaries, which are located in the periphery or terminal villi, close to the maternal tissue. Numerous terminal villi in the periphery provide a large vascular surface for substance exchange. This effect is progressively enhanced during the second half of bovine gestation by distinct coiling and anastomosing of the capillary convolutions, as observed in this study and by Leiser et al. (1997).

Fetal vascular architecture related to blood flow characteristics and placental substance exchange

Numerous capillary convolutions serve as interconnections between the arterial and venous villous tree vasculature from the base to the top of the tree. The capillary convolutions are also serially linked to each other between 2 and 5 times (Fig. 12); thus the total length of this capillary system reaches up to 1000 μm (Leiser et al. 1997). The interconnections become more developed in late gestation when the villous tree has grown extremely long (up to 4 cm; Ebert, 1993). How can this large interconnecting system allow full and even circulation of blood flow? Kaufmann et al. (1985) and Leiser et al. (1991) suggested that in the human placenta capillary sinusoids or dilatations—also abundant near the tips of terminal villi in the bovine placenta—encourage blood flow according to the law of Hagen–Poiseuille. Reduced vascular resistance occurs whenever the vascular lumen widens, therefore blood flow through a very extended capillary system such as terminal villi in the cow placenta is guaranteed. The same effect is attributed to anastomoses of the capillary bed and the venous system (Fig. 12). In addition, the latter may support the drainage of blood from the capillary system into the particularly voluminous system of venules and veins in the intermediate and stem parts of the villous tree (Fig. 12).

Sinusoidal dilatations offer an extended absorptively active endothelial surface and may slow blood flow locally. This should not influence the rapidly equilibrating diffusional transplacental exchange with, e.g. O_2 and CO_2 (Faber & Thornburg, 1983) but may ameliorate conditions for slow-transported solutes of the active placental exchange (Alberts et al. 1983). Venous capillary limbs, venules and veins are abundant and specifically thin-walled in the bovine fetal placenta and therefore, after Arts's (1961) conclusion

Fig. 3. Low magnification of obliquely cracked corrosion cast of middle part of the villous tree, d 270 p.i. One relatively large stem artery (A) is distinctly separated from stem veins (V) which are located close to the capillary complex. Branches of the villous tree ramify at right angles to the stem (arrows). ×150.

Fig. 4. Thick histological section of the maternally oriented tops of villous trees (bottom) visualised by ink-filled vasculature, d 150 p.i. The stem (X) and intermediate (Y) vessels of villous trees (1 artery/arteriole and several venules/veins) are oriented in parallel bundles. The branching angle to the stem is generally 90°, but may become acute at the top of the villous trees. Capillary convolutions of terminal villi are clearly demonstrated (Z). ×80.

Fig. 5. Histological section showing the middle part of a villous tree, d 200 p.i. Intermediate villous branches occur at right angles (arrows) to a small stem villus oriented vertically. ×140.

Fig. 6. Corrosion cast of the venous system and capillaries comparable to Figure 5, d 270 p.i. Note the convergence of numerous terminal capillary convolutions (vertical arrowheads) into the venules of intermediate villi (horizontal arrows), which subsequently meet the stem venules (vertical large arrow) at right angles. ×200.

Fig. 7. Ink-filled vasculature magnified from a histological section corresponding to Figure 4, d 150 p.i. One arteriole (arrowheads) branching from the stem artery (arrows) can be identified in the centre of an intermediate villus. Venous capillary limbs or venules (asterisk) serially interconnect by curves peripherally located capillary convolutes of terminal villi. ×220.

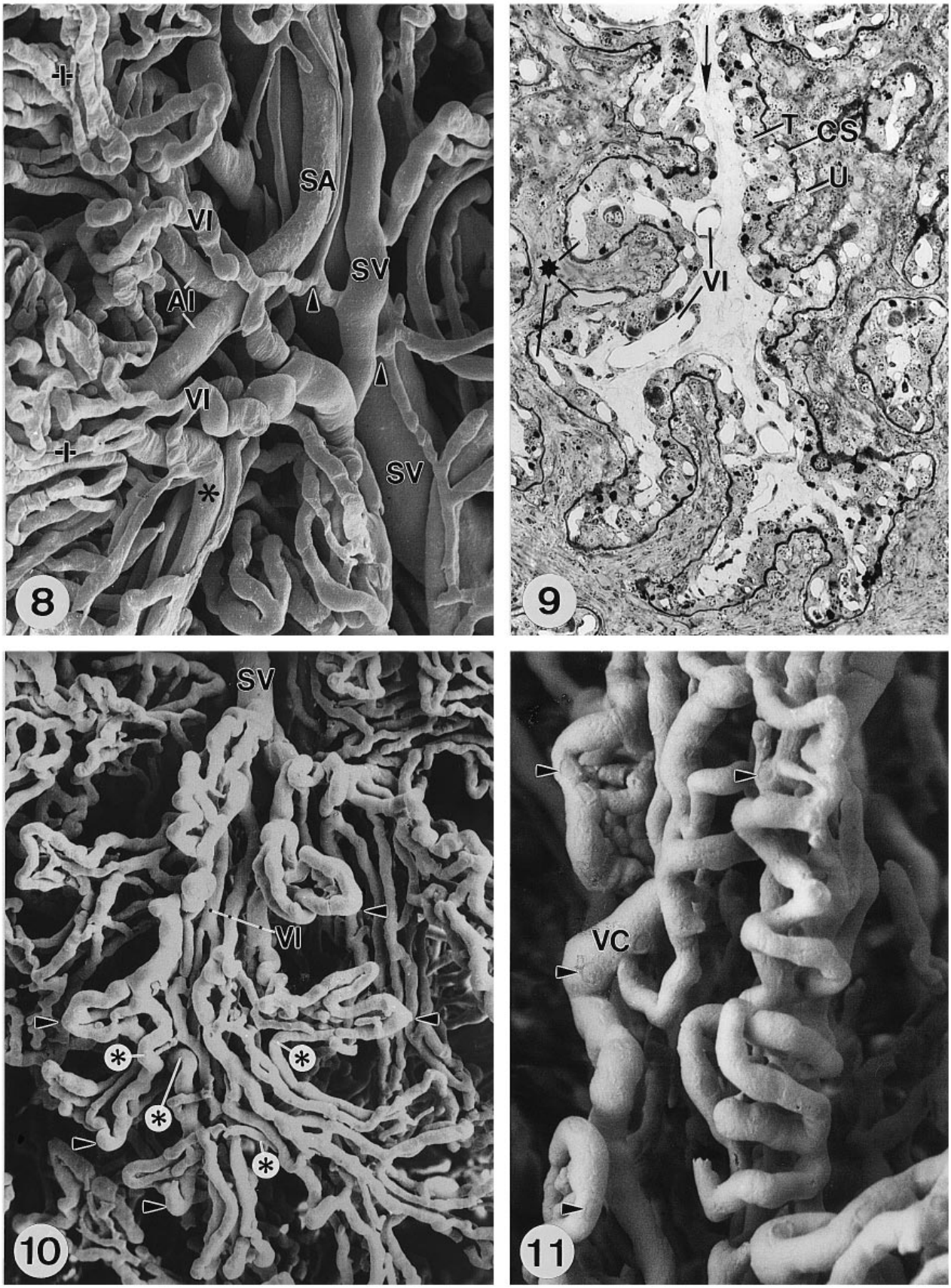


Fig. 8. Corrosion cast of a parastem vascular area, d 180 p.i. The stem villus, in the background (right), consists of 2 veins (SV) and an artery (SA). This artery branches into 2 short arterioles (Al) which while visible are each situated in the centre of an intermediate villus. They are surrounded by venules (Vl) and capillaries in the neck region of 2 terminal villi (crosses). Asterisk, venule connecting terminal villi. Vasa vasorum of stem vessels join capillaries of terminal villi (arrowheads). $\times 700$.

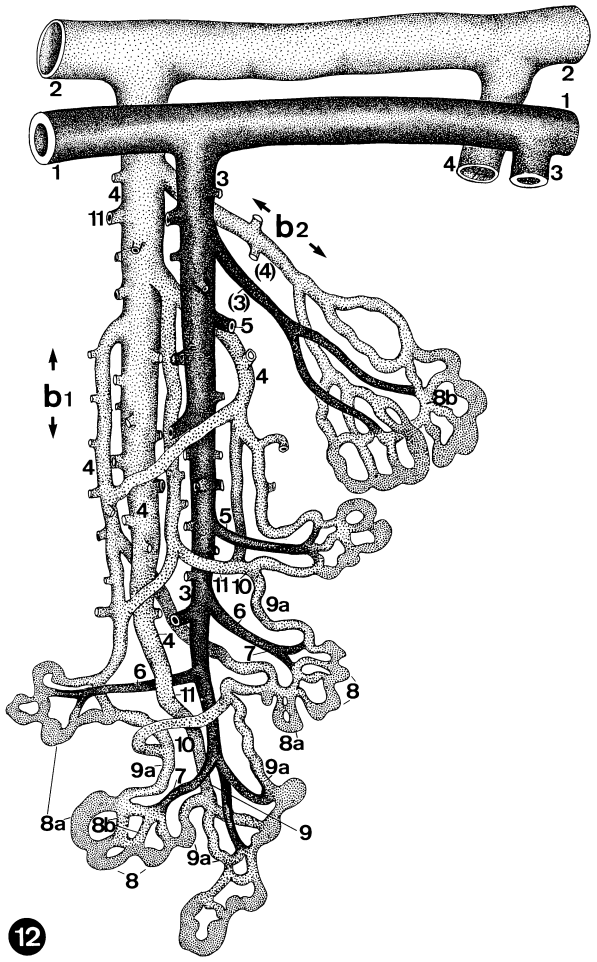


Fig. 12. Schematic drawing of the microvasculature of villous trees in full-grown (b_1) and budding (b_2) stages (compare with Fig. 1) illustrating a cotyledonary artery (1) and cotyledonary vein (2) of the chorionic plate; stem arteries (3) and stem veins (4) of a stem villus; branch arteries/arterioles (5); arterioles of intermediate villi (6); the capillary complex of terminal villi with capillary convolutions consisting of arterial capillary limbs (7), capillary loops (8) with dilatations (8a) and anastomoses (8b), and venous capillary limbs (9); capillaries or venules connecting capillary convolutions (9a); venules of intermediate villi (10); and branch venules/veins (11).

for the human, may also support active substance transfer. The voluminous venous system because of its very close association to the corresponding segments of the arterial system, being oriented in the opposite way, however, provides a condition suitable for countercurrent exchange (Leiser et al. 1997). It would allow back-diffusion of substances from the venous into the arterial system (Fig. 12) such as hormones produced by the bovine placenta (Reimers et al. 1985; Wooding, 1992), a principle also discussed for the pig by Reynolds et al. (1985) and Dantzer & Leiser (1993).

The vascular architecture and morphology shown by scanning electron microscopy of vessel casts in this study does not reveal any measurable differences in the blood flow interrelationship of maternal and fetal vessel systems between the cow and the sheep or goat, a relationship which is a mixture of crosscurrent and countercurrent (Leiser, 1987; Krebs et al. 1997; Leiser et al. 1997). However, the weight ratio of neonate to placenta (showing how many grams of placenta are produced per 1 g of fetus at term) is 13:1 in cattle and 10:1 in sheep and goats (or 6:1 in the human) and thus reflects the efficiency of the placenta to some extent (Dantzer et al. 1988; Kaufmann, 1990; Leiser et al. 1997). The better weight ratio of the cow, compared with that of the closely related 'co-ruminants', obviously expresses physiological conditions, and thus correlates with the amount of substance transferred with the maternofetal diffusional exchange (compare Faber & Thornburg, 1983). In fact, this amount, measured as the difference of O_2-content between the uterine artery and umbilical vein, is higher in cattle (Reynolds et al. 1986) than in sheep (Wilkening & Meschia, 1992). The neonate/placenta ratio of the human (6:1) is distinctly less favourable than that in ruminants (see above), even though both the human and the ruminants show a similar villous fetal blood vessel system. As thoroughly discussed in a comparative study by Leiser et al. (1997), the main cause for a better ratio in the ruminants may be the fact that ruminants have intimately associated maternal and fetal vascular systems both showing distinctly guided ways of blood flow, whereas in the human, the maternal placental blood space is open, preventing a strict and smooth course of the blood to the fetal villi enclosing the fetal vasculature.

Fig. 9. Tissue section showing an intermediate villus (arrow) with several terminal villi, d 270 p.i. Impregnation with silver clearly stains the contact surface (CS) between the trophoblast (T) and the barely visible uterine epithelium (U). Many capillaries have an 'intraepithelial' position in the trophoblast (star), thus reducing the placental barrier. Venules (Vl). ×220.

Fig. 10. Corrosion cast of the vasculature of an intermediate villus and of terminal villi comparable to the histological appearances shown in Figure 9, d 270 p.i. Capillary convolutions of terminal villi are composed of a central arterial capillary limb (not visible), capillary loops with sinusoidal dilatations (arrowheads), and of venous capillary limbs accompanying the arterial capillary limb or typically bridging parts of the convolutions (asterisks). Several postcapillary venules (Vl), arranged in parallel, run along the axis (bottom to top) of the intermediate villus and join the stem vein (SV). ×350.

Fig. 11. Detailed corrosion cast of a near-term capillary convolution, d 270 p.i. The lateral view illustrates impressively the capillary convolution with vigorous coiling. VC, venous capillary limb. Arrowheads mark impressions of endothelial perikarya. ×1200.

Conclusions

There is little increase in placentome volume during the second half of bovine gestation (Reynolds et al. 1990; Reynolds & Redmer, 1995). However, the structure of the placentome villous trees is transformed to facilitate transplacental substance exchange. This transformation occurs by 2 principal mechanisms: the specifically altered branching pattern of the villous trees, and the improved spatial relationship between the 2 absorptive surfaces, endothelium and trophoblast, of the villous trees. These adaptations clearly promote simple diffusional transplacental exchange (Leiser & Koob, 1992; Leiser & Kaufmann, 1994) and by their very nature will also benefit active transport exchange mechanisms (overview by Faber & Thornburg, 1983; Benirschke & Kaufmann, 1995). Research into bovine placental active transport mechanisms is poorly advanced (overviews by Wooding, 1992, 1994). Such research is beyond the scope of our morphological studies but is now needed to further complete our understanding of the bovine fetomaternal relationship in utero (compare Reynolds & Redmer, 1995).

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Term Ovine Placental Vasculature: Comparison of Sea Level and High Altitude Conditions by Corrosion Cast and Histomorphometry

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The placental vascular architecture differs significantly at high altitude from that at sea level in the human and guinea-pig. Four sheep between 137 and 140 days of gestation, kept near sea level throughout gestation, were used as a normoxic control group for comparison of the placental vasculature with 10 other ewes, kept at high altitude (3820 m above sea level; Barcroft Laboratory, White Mountain Research Station, CA, USA). Placentomes from both groups were prepared for histology and scanning electron microscopy of vascular corrosion casts. Singular perfusion of fetal placentae, as well as combined maternal/fetal injection was performed. The influence of long-term hypoxaemia was determined by qualitative and semi-quantitative evaluation of corrosion casts and histological sections. The fetal vessel casts show a distinct difference in the arrangement of vessels of all sizes in response to long-term hypoxaemia. In the control group, stem arteries and veins are straight and parallel. In contrast, this is much less evident in the hypoxaemic group because arterioles and venules branch off the stem vessels more frequently and in an irregular manner. This leads to a capillary bed that is much more dense due to increased branching and capillary coiling. These observations are confirmed by histomorphometry. In the fetal vessels of high altitude sheep placentomes, we observed a decreased number of vascular cross sections (21.6 ± 4.7 SEM versus 27.7 ± 4.0 SEM; $P=0.02$). However, the average luminal size per cross section (77.9 ± 10.5 μm^2 SEM versus 59.4 ± 7.4 μm^2 SEM; $P=0.004$) was increased at high altitude and the percentage of lumina of the total area (5.7 ± 0.5 SEM versus 5.3 ± 0.3 SEM; $P=0.09$) indicated a trend towards an increase. In maternal vessels of high altitude placentomes, the number of vessel cross sections (6.5 ± 0.7 SEM versus 6.0 ± 0.5 SEM; $P=0.2$) remained unchanged, whereas the average luminal size (1108 ± 122 μm^2 SEM versus 844 ± 77 μm^2 SEM; $P<0.001$) and the percentage of lumina out of the total area (20.9 ± 1.8 SEM versus 17.5 ± 1.7 SEM; $P<0.001$) were increased. The interhaemal distance appeared to be slightly but not significantly increased at high altitude. These findings indicate that at high altitude the sheep placenta develops an increased materno–fetal absorptive surface to help guarantee substance exchange. © 1997 W. B. Saunders Company Ltd
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INTRODUCTION

According to recognized schemes of placental classification (for review see Mossmann, 1987; Leiser and Kaufmann, 1994; Wooding and Flint, 1994) the ovine placenta at term is described as chorioallantoic and chorioamniotic with multiple separate cotyledons in which there is a villous interdigitation (Strahl, 1906; Andresen, 1927; Wimsatt, 1962) with the maternal crypts of the complementary caruncles. The interhaemal barrier, which separates fetal and maternal blood of the sheep placenta, is epitheliochorial (Ludwig, 1962; Björkmann, 1965; Davis and Wimsatt, 1966; Lawn, Chiquoine and Amoroso, 1969) or synepitheliochorial, because binucleated cells of the syncytial trophoblast fuse with the syncytium of the uterine epithelium (Wooding, 1992).

The efficiency of the transplacental oxygen exchange may be influenced by the way fetal and maternal blood vessels are arranged in the placental barrier (for review see Faber and Thornburg, 1983; Dantzer et al., 1988; Benirschke and Kaufmann, 1990; Leiser and Kaufmann, 1994). In the ovine placenta there are different opinions because countercurrent (Barcroft and Barron, 1946; Tsutsumi, 1962) or cross-current (Steven, 1966, 1975; Makowski, 1968) arrangements have been proposed.

It is well known that capillary growth is stimulated by reduced oxygen supply, both in the human at high altitude (Jackson, Mayhew and Haas, 1988a,b; Soma, Watanabe and Hata, 1995, Reshetnikova et al., 1996) and the guinea-pig in hypobaric oxygen chambers (Bacon et al., 1984; Scheffen et al., 1990). In sheep, prolonged placental hypoxia has been achieved by both breathing a low oxygen concentration (Koos et al., 1988; Alonso et al., 1989; Kitanaka, Gilbert and Longo,

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1989; Kitanaka et al., 1989) and a reduction (occlusion) of utero–placental blood flow (Challis et al., 1989; Wilkening and Meschia, 1992).

The hypobaric hypoxia of high altitude offers natural conditions to study the mechanisms of placenta adaptation. Because many humans (~2–3 million) live at high altitude permanently, the haemochorial human placenta has been subject to intensive stereological studies, revealing major morphological changes (Jackson et al., 1985; Jackson, Mayhew and Haas, 1986, 1987a,b, 1988a,b; Mayhew, Jackson and Haas, 1990; Reshetnikova, Burton and Milovanov, 1994; Burton et al., 1996). Sheep represent domesticated animals that have adapted to various climates and altitudes, and are the focus of interest in numerous physiological studies on placental oxygen transfer (Longo, Power and Forster, 1967; Longo, Hill and Power, 1972; Longo and Ching, 1977; Longo, 1987; Wilkening and Meschia, 1992).

This study focuses on the vasculature of the ovine placenta to determine the influence of high altitude (hypobaric long-term hypoxia) on the structural changes/adaptations of the placental vasculature compared with sea level (SL), using scanning electron microscopy of vascular casts and morphometry of tissue sections.

MATERIALS AND METHODS

Materials

After mating of 14 Western breed sheep, four were kept near SL throughout gestation while 10 were taken to high altitude (HA) (White Mountain Research Station, Barcroft Laboratory, University of California, 3820 m, 480 Torr) after confirmation of pregnancy (day 41–49 of gestation). Before the experiment, the latter were taken down to Loma Linda University, CA, USA, for study.

We performed a hysterectomy under general anaesthesia at a gestational age of 140–142 days in SL ewes, or 137–140 days in HA ewes. The uterine horns were opened along the curvatura major to remove the fetus(es) whose weight(s) was (were) recorded (50 per cent singleton gestations). A minimum of six placentomes per placenta, originating from different areas, were chosen, and the corresponding afferent arteries were identified and prepared for perfusion for either histology or scanning electron microscopy. In order to achieve optimal perfusion or filling, only placentomes with one afferent artery were picked out.

Methods

Procedure of casting and treatment of vascular casts. The selected placentomes and their adjacent main vessels were clamped to guarantee a permanent perfusion pressure and to avoid loss of the casting resin during the process of instillation. Preliminary perfusion with physiological buffered saline (PBS) was required for casting with Mercox CL-2R® resin (Vilene, Tokyo, Japan). The buffer perfusion was continued until the venous outflow was clear and the placentomes became distinctly pale. Subsequently, the placentomes were perfused with liquid plastic compounds [Mercox® or modified Batson® (Polysciences, Warrington, USA)], see also Leiser, Dantzer and Kaufmann, 1989).

After polymerization of the plastic, the placentomes were excised and stored in warm water overnight (40°C). During the casting procedure with the modified Batson®, the placentomes were cut in a semi-polymerized state to allow viewing of the central parts of the cast thereby avoiding damage to the specimen by cutting the fully polymerized casts. The placental tissue was removed through corrosion, by alternating immersion in 20 per cent potassium hydroxide (KOH) and distilled water for a few days (40°C). The corrosion casts were washed repeatedly in distilled water and 5 per cent neutral Extran® (Merck, Darmstadt, Germany), air-dried, and split into smaller pieces from which suitable specimens were selected by stereomicroscopy. These specimens were fixed on mounts with a conductive carbon cement (Leit C, Göcke, Münster, Germany), sputter coated with 3 nm gold and examined qualitatively by scanning electron microscopy (Zeiss DSM 940). For more detailed instructions for casting see Leiser, Dantzer and Kaufman (1989).

Treatment of histological material. Quickly after opening of the uterus each placentome was perfused (50 mmHg) with PBS at pH 7.3 and 4°C for 10–20 sec with a maximum of 1–2 ml. This was followed by perfusion fixation (50 mmHg) with 2.5 per cent glutaraldehyde (GA) in 0.1 M cacodylate buffered solution at pH 7.2 and 4°C. When the placentomes were firm and pale (after ~10 min), they were cut into 2-mm slices (three pieces per placentome), post-fixed in the above mentioned GA-solution for 3 h, and stored in cacodylate buffer at 4°C. Subsequently, they were dehydrated and embedded in hard plastic (Medim®) by routine methods. Sections (three per block) of 3 μm (Polycut, Leica) were stained after Richardson, and silver-impregnated after Movat (Romeis, 1989). The sections were studied by light microscopy, and examined morphometrically (two-dimensional) using an image analysis system (SIS, Münster, Germany).

In three different zones of the placentome (chorionic plate side, intermediate area, basal plate side), we measured the following parameters, using a minimum of 10 microscopic fields of view per zone at a magnification of × 200 (Olympus microscope BH-2), (see Figure 8): (1) the number of fetal and maternal vessel cross sections, (2) their average luminal size, (3) the calculated percentage of fetal and maternal lumina out of the total area, and (4) the interhaemal distance. The data were described by mean ± standard error of the mean (SEM). The groups were tested for equal distribution and an independent-samples *t*-test (SPSS for Windows™ Release 6.0) was carried out to test whether or not the changes were significant.

Placental and fetal weights were not taken in consideration because interindividual variations caused by a varying number of fetuses and the variations of the gestational age did not allow a statistical calculation.

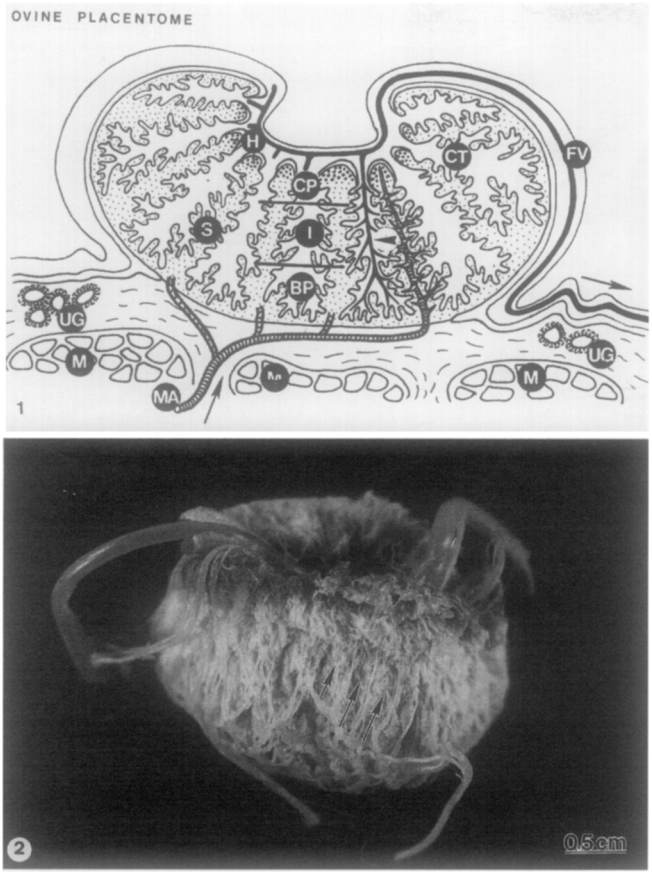

Figure 1. Schematic representation of a vertical section of a near-term ovine placentome. This illustrates fetal (clear) and maternal (stippled) compartments. Their supplying blood vessels are indicated as follows: MA, maternal artery (dashed lines) and FV, fetal vein (solid black). The arrows show the blood flow of the arterial (or oxygenated) blood, and the arrowhead marks the direction the transplacental oxygen transport. The areas of morphometric measurements are marked in the chorionic plate zone (CP), intermediate zone (I), basal plate zone (BP), Additionally marked are connective tissue of fetal primary villus or villous tree, respectively (CT), maternal primary septum (S), with haematoma (H) on its top, uterine glands (UG), and myometrium (M).

Figure 2. Vessel cast of an near-term ovine placentome of a normoxic (SL) ewe, combined feto-maternal modus of plastic instillation. The fetal portion of the placentome (top) receives only one to three major arteries, while the maternal part of the placentome (bottom) is supplied by numerous arteries originating from minor (smaller) branches of the uterine artery. The stem vessels (arrows) are oriented in a strictly parallel manner.

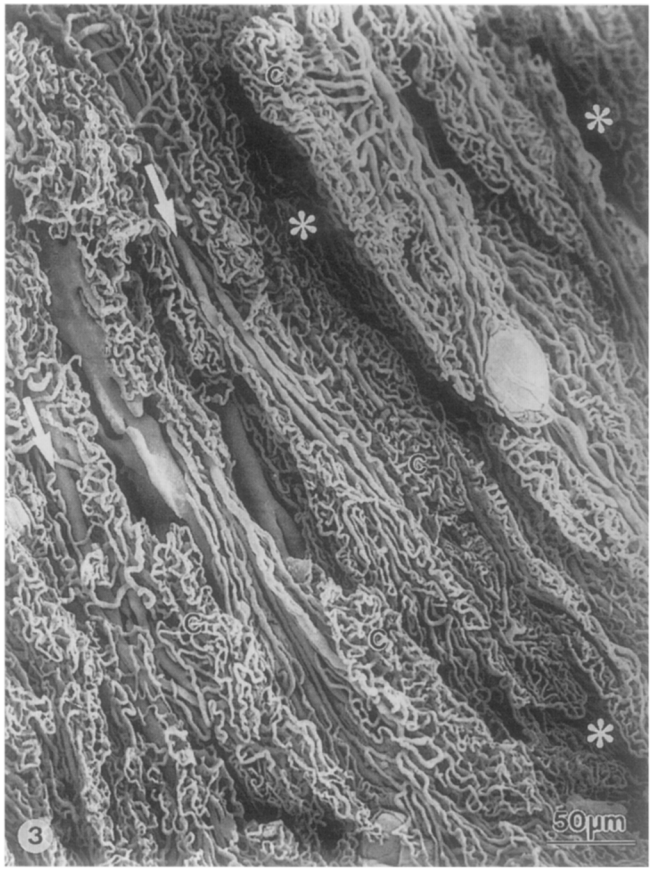

Figure 3. Overview of a fetal cast of a SL ewe. Transition of intermediate zone to basal plate zone (top area of fetal villous tree is oriented towards bottom right). Each 'vascular tree' of a villus is clearly separated by empty space (white asterisks) from the other 'trees', which mainly corresponds to corroded maternal septal tissue. The stem vessels are oriented in a parallel manner (white arrows), and supply units of capillaries (C) distinctly separated from each other.

RESULTS

Scanning electron microscopy of placentomal vascular casts)

Arrangement of large fetal placental vessels results in shape and primary structure of the placentome.

A placentome consists of a complementary system of fetal villi indenting deeply into the maternal crypts separated by septa (Figure 1). Correspondingly, a cast represents those blood vessels that were injected with the liquid plastic: both fetal and maternal vessels (Figure 2), or only fetal (Figures 3–7) or maternal vessels (not specifically shown in this study).

The combined cast reflects the cup-like shape of the whole placentome due to the extremely dense vascularization (Figure 2). Its large fetal vessels consist of cotyledonary arteries, resembling branches of the two umbilical arteries, and of cotyledonary veins (Figures 1 and 2), which converge to the single umbilical vein. Both vessel types run over the fetal side of the placentomes to descend into the central concavity of the cup-like placentomes (Figures 1 and 2), where they turn off at an angle of 90° to run in feto–maternal direction (Figure 1). Within the placentome, they branch repeatedly into numerous stem arteries, each supplying one primary villus. Maternal caruncular arteries and veins, originating from radial branches of the uterine artery adjoin the placentome from its endometrial side (Figures 1 and 2) and ramify into stem vessels of the numerous septa. Both fetal and maternal stem vessels are oriented in a strictly materno–fetal direction (Figure 2), each with the tributary arterioles, venules, and the capillary complexes. These are clearly visible on the fetal cast in Figure 3. The stem arteries mark the centre of a single cylindrically-shaped fetal vascular (villous) tree (Figures 3 and 4) or of a maternal cryptal or septal system, respectively (Figure 3).

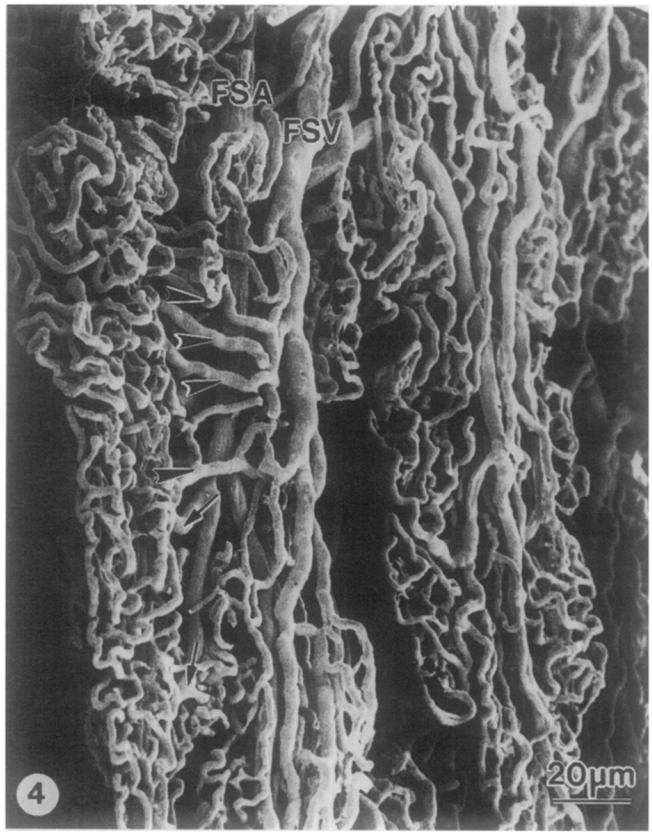

Figure 4. Fetal cast of a SL ewe. The stem vessels [fetal stem artery (FSA), fetal stem vein (FSV)], are usually found within the centre of a vascular tree. Few arterioles branch off the stem arteries (arrows) to enter the capillary system, where it is easy to follow single vessels until they enter numerous venules (arrowheads) which run towards the stem of the vascular tree in a parallel manner.

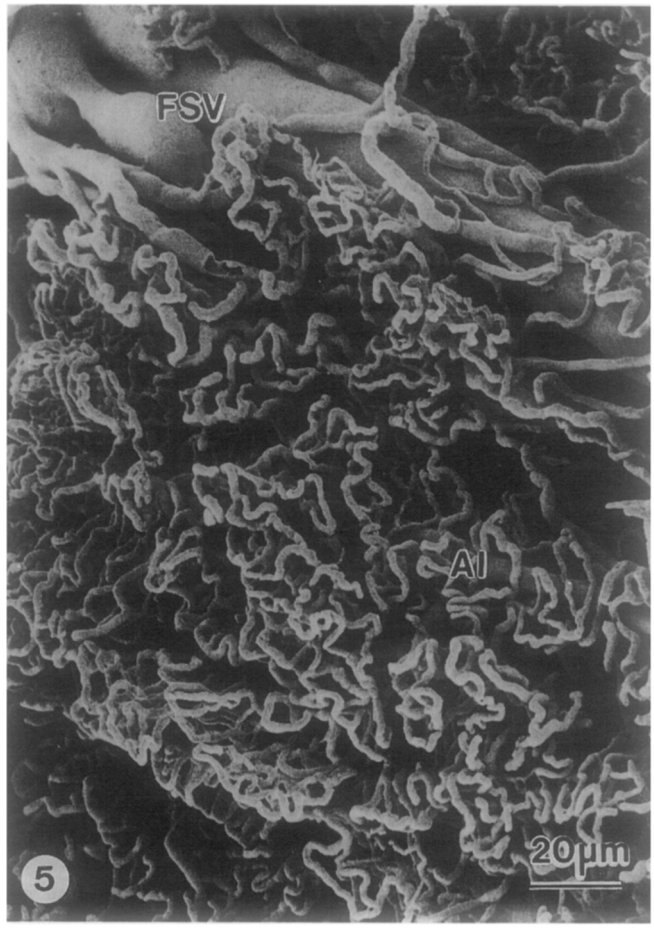

Figure 5. Fetal vessel cast of a HA ewe. Note the fairly coiled impression of the entire vessel architecture. The stem vessels [only the fetal stem vein (FSV) is visible] are moderately coiled. Arterioles (Al) and venules are usually hidden among numerous strongly coiled capillaries.

In casts, the different types of vessels are identified by the impressions of endothelial cells or their perinuclear parts, respectively. Arteries show deep spindle-like impressions, which are longitudinally oriented, whereas veins exhibit polygonal cells with ovally shaped imprints. Arterioles and venules show basically the same features, but on a smaller scale.

Architecture of small fetal placental vessels (resulting in vascular phenomena such as orientation and course, branching and coiling, occurrence of sinusoidal dilatations) represents the secondary structure of the placentome

The fetal stem arteries and stem veins are oriented materno–fetally, particularly straight and in a parallel manner in placentomes of SL ewes (Figure 3), whereas they are moderately coiled in the placentomes of HA animals (Figures 5 and 7).

Small arterioles branch off the stem arteries to enter the terminal villi capillary system. The frequency of branching into arterioles is distinctly increased in placentae of HA animals, in which the modus of branching is conspicuously irregular. The degree of fetal vascular ramification from the stem vessels into the capillary complex is four to six orders in organs of SL, and higher (up to eight orders) in placentomes of HA. In both, SL (Figures 4 and 6) and HA ewes, numerous venules converge to stem veins in a parallel manner, outweighing arterioles (ratio 1 : 8), however this parallelity is lost in HA placentae (Figures 5 and 7).

The terminal villi capillary system of a single villous tree consists of serially linked capillary convolutions or loops (compare Leiser et al., 1996). The proximal parts of these loops, arterial and venous limbs, are arranged in a parallel manner, whereas the distal parts show a more tortuous course (Figures 4 and 5). In placentomes of SL ewes, it is easy to follow the course of a single capillary loop, due to a moderate degree of anastomosing and coiling (Figure 4). On the other hand in HA ewes, the capillary loops exhibit a highly conspicuous amount of coiling, branching and anastomosing, and parallelism of arterial and venous capillary limbs is not observed (Figure 5). This results in a dense capillary bed, preventing distinction of single capillary loops from each other. Consequently arterioles and venules are most often hidden among the highly coiled capillaries. Sinusoidal dilatations on the extremes of capillary loops are found in both groups in moderate number and quality (Figures 6 and 7).

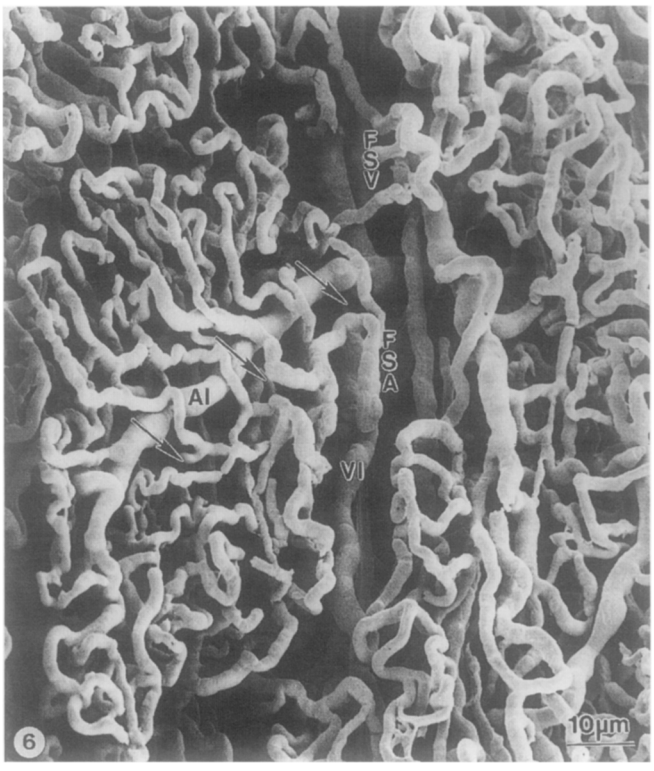

Figure 6. Capillary loop in a fetal vessel cast of a normoxic ewe. Stem artery (FSA) and stem vein (FSV) are located in the background (impressions of endothelial nuclei and the muscular vessel wall are not clearly visible here). The supplying arteriole (AI) and the efferent venule (VI) of a single capillary loop are indicated. The distal part of the vessel loop is coiled in a convoluted manner. Note the distinct parallel orientation of the efferent capillaries before they enter the venule (arrows show direction of blood flow).

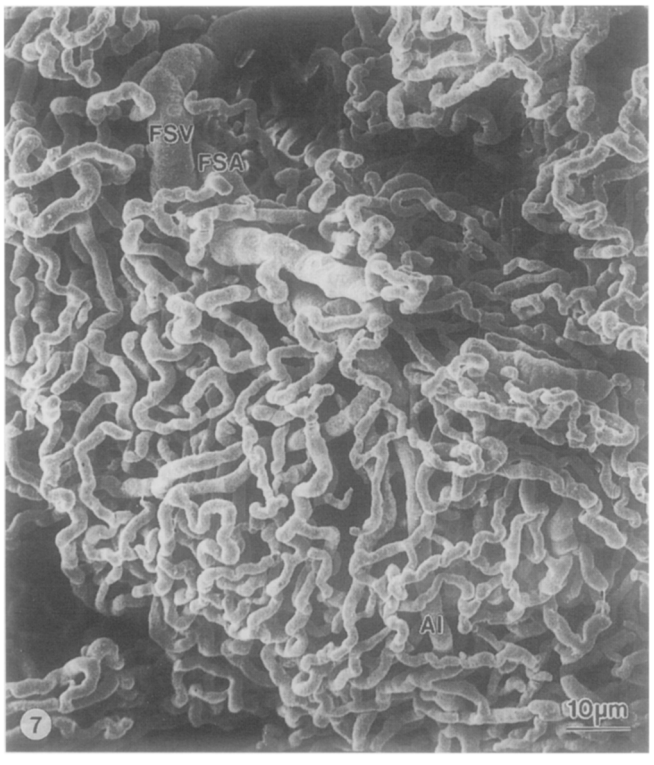

Figure 7. Capillaries in a fetal cast of a HA ewe. Note the distinct coiling of a stem artery (FSA) and a stem vein (FSV). The density of the capillary bed does not allow distinguishing single capillary loops from each other. Most portions of arterioles (AI) and venules are hidden by the highly coiled capillaries. Sinusoidal dilatations are remarkable.

Histomorphometry of placentomes

We examined the different areas of the placentome to evaluate whether there was a different adaptation in the parts of the placentome namely in the chorionic plate, the intermedial zone, and the basal plate zone. The variable results revealed that this is not the case; therefore, only pooled data were used.

Fetal vessels. The means and SEM for number of fetal vessel cross sections per microscopic field of view, average luminal size per vessel cross-section, percentage of luminal surface out of the total histological area and *P*-values for significance are given in Table 1. The mean number of cross sections was 27.7 for SL placentae and 21.6 for HA placentae, a significant decrease of 22 per cent (P=0.022). The mean luminal size of the fetal vessels was 59.4 μm^2 in placentae of SL ewes and 77.9 μm^2 in the ones of HA ewes, a significant increase of 31 per cent (P=0.004). The mean percentage of fetal lumina out of the total area showed a trend (7.5 per cent) to be higher in placentomes of HA ewes (5.7 per cent) versus SL ewes (5.3 per cent), but the difference failed to reach significance (P=0.097).

Maternal vessels. The results are given in Table 1 as well, summarizing the means, SEM and *P*-values for the number of maternal vessel cross-sections per microscopic field of view, the average luminal size per cross-section of a vessel, and the percentage of luminal surface out of the total histological area. The number of luminal cross-sections was 6.0 ± 0.5 for SL placentomes and 6.5 ± 0.7 for the placentae of HA ewes. This increase of 9 per cent lacked statistical significance (P=0.219). The luminal size was 844 ± 77 μm^2 in placentae of SL ewes, whilst that for HA placentae was 1108 ± 122 μm^2, an increase of 31 per cent (P <0.001). Corresponding values for the mean percentage of maternal lumina out of the total area of a microscopic field of view were 17.5 ± 1.7 per cent for SL organs and 20.9 ± 1.8 per cent for HA placentomes, an increase of 19 per cent (P<0.001).

Interhaemal distance. Table 1 presents also the results for the mean interhaemal distance. The interhaemal distance was 8.2 ± 1.0 µm for SL placentomes and 9.0 ± 1.0 µm for the organs of HA ewes, representing a nonsignificant increase of 10 per cent (P=0.1).

DISCUSSION

Placentome vessel nomenclature

To describe the general course of the main fetal and maternal placentome vessels in sheep, Barcroft and Barron (1946)

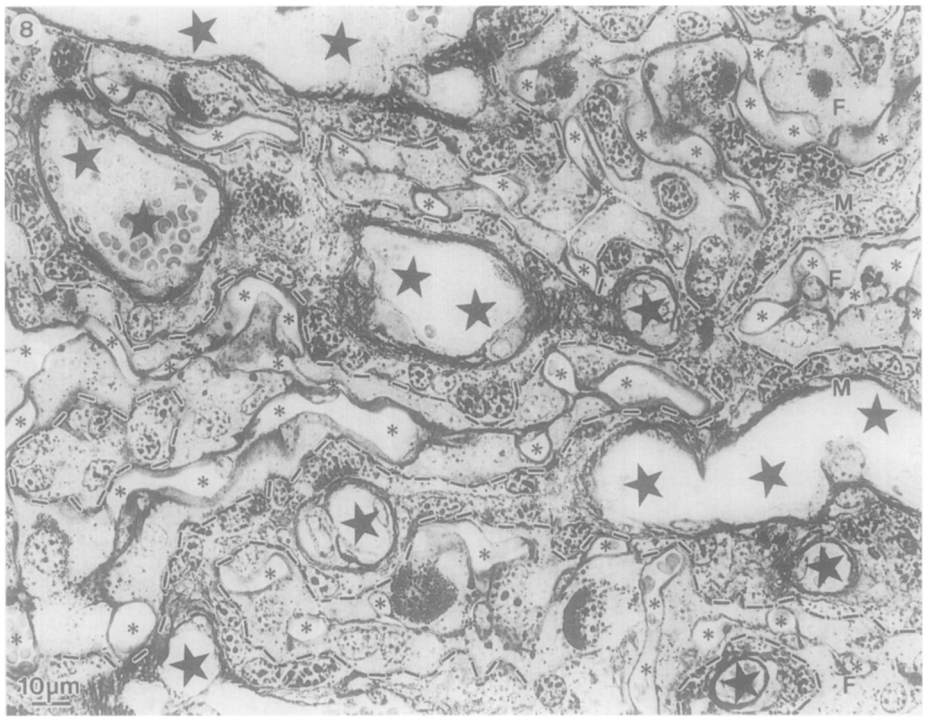

Figure 8. Histological section of an ovine placentome (SL ewe). Maternal vessel lumina (stars), fetal vessel lumina (asterisks), and contact line (dotted line) between fetal (F) and maternal (M) tissues.

Table 1. Morphometrical results

	Sea level group (n=4) ± SEM		High altitude group (n=10) ± SEM		Significance
Fetal vessels					
1	27.7	± 4.0	21.6	± 4.7	P=0.02
2	59.4 µm^2	± 7.4	77.9 µm^2	± 10.5	P=0.004
3	5.3%	± 0.3	5.7%	± 0.5	P=0.09
Maternal vessels					
1	6.0	± 0.5	6.5	± 0.7	P=0.2
2	844 µm^2	± 77	1108 µm^2	± 122	P<0.001
3	17.5%	± 1.7	20.9%	± 1.8	P<0.001
Interhaemal distance					
	8.2 µm	± 1.0	9.0 µm	± 1.0	P=0.1

Mean values for number of luminal cross-sections per microscopic field of view. (1), Luminal size per vessel cross-section (2), and percentage of vessel lumina out of the total area (3) are given for fetal and maternal vessels; and mean values for interhaemal distance are shown.

instilled gelatine-colour or latex-colour mixtures into the vasculature and subsequently digested the tissue with 'KOH'. Their casting technique was further developed by Tsutsumi (1962) and Makowski (1968) who used latex (turox or neoprene) or India ink, and roughly described the primary structure of the placentomal vasculature, which is also presented here. In the present study, the total filling and the subsequent full description of the capillary system, or secondary structure of the placentome, was made possible using liquid plastic as casting resin, followed by corrosion, and using scanning electron microscopy. After being introduced into placental vascular research (Leiser and Kohler, 1983; Leiser, 1985) this new technique was adapted for ruminants (goat: Leiser, 1987; cow: Ebert, 1993), revealing a fetal vessel tree similar to that of the human (Kaufmann et al., 1985; Leiser et al., 1985, 1997). Therefore, in both human and sheep, this similarity enables distinction between the different parts of the fetal villous tree according to the type of vessels they contain: stem artery in the stem villus, arterioles in intermediate villi, and capillaries in terminal villi. Likewise, the same nomenclature can be used for the maternal or septal system in the sheep (or other ruminants), which is 'closed' (see below and Leiser et al., 1997), and thus different from the human with an 'open' blood pool (no septal system).

Morphological implications for the interpretation of corrosion casts in sea level ewes and comparison with other ruminants

The placentome shape, or its primary structure, is 'cup-like' in sheep (Barcroft and Barron, 1946; Tsutsumi, 1962; Björkman, 1965; Leiser et al., 1997) and goat (Leiser, 1987), whereas it is 'mushroom-like' in the cow (Björkmann, 1954; Ebert, 1993; Leiser et al., 1997). Obviously, the form of the fetal villous tree (secondary structure) is dependent on the primary structure of the placentome (macroscopically visible outer shape of the cast). In the sheep and goat, each villous tree is cylindrically and slender like a 'poplar tree' (Leiser, 1987; Leiser et al.,

1997; see also Figure 1), whereas in the bovine, Ebert (1993) and Leiser et al. (1997) described its shape as conal like a 'Christmas tree'. The form of these villous trees allows development of vascular ramifications from the stem vessels to the capillary complex of only four to six branching orders in the near term ovine and caprine placentome (Leiser et al., 1997) while eight to 10 branching orders can be observed in the bovine (Ebert, 1993; Leiser et al., 1997). Moreover, the capillary bed, consisting of a system of serially connected convolutions, guarantees a smooth blood flow through the elongated capillary system, as also described in the human by Kaufmann et al. (1985). These convolutions number up to three in the sheep and goat (Leiser, 1987) compared with up to five in the cow (Ebert, 1993; Leiser et al., 1997). Despite the fact that the vessel architecture of a single ovine capillary convolution is in general agreement with that of the goat and cow (consisting of a supplying arteriole, being located in the centre of the terminal villus, and highly coiled and anastomosed capillaries, which surround the arteriole), the fewer vascular pre-capillary ramifications and shorter series of convolutions in the sheep must reflect a relatively smaller materno–fetal contact surface for placental exchange.

Fetal venules of SL ewes display a parallel arrangement during their course towards the stem veins. This is also characteristic in the goat (Leiser, 1987) and cow (Ebert, 1993; Leiser et al., 1997), where it may function as a countercurrent system for diffusion back to the fetal arterioles (Reynolds, Ford and Ferrell, 1985).

Interpretation of histomorphometrical findings comparing SL and HL placental vasculature of sheep

Long-term hypoxia stimulates placental vessel growth, particularly that of capillaries (Hölzl, Lüthje and Seck-Ebersbach, 1974; Bacon et al., 1984; Kaufmann et al., 1985, 1988; Jackson, Mayhew and Haas, 1987a,b; Geisen et al., 1990; Scheffen et al., 1990; Olivo et al., 1992; Reynolds, Killilea and Redmer, 1992). The histomorphometrical results in this study indicate that fetal vessels of sheep HA placentae adapt to hypobaric hypoxia by becoming larger, e.g. the number of vessel cross sections is decreased, while the luminal size per vessel cross section is significantly increased. Compared with the SL sheep, this is a slight (7.5 per cent) trend towards an increase of vessel lumina out of the total area. This was not evident on mere visual inspection of the casts with the scanning electron microscope; however, conspicuous coiling and branching of the capillaries (see above) suggested such a result in HA animals.

The findings of Reshetnikova, Burton and Milovanov (1994) and Burton et al. (1996) in the human placental vasculature at high altitude (e.g. increased fetal vascularity, and the occurrence of dilated capillary sinusoids) were consistent with our findings in sheep. Therefore, the increased vascular surface area creates a more efficient materno–fetal exchange at high altitude in both sheep and human. In accordance with the law of Hagen/Poiseuille, the blood-flow rate would be slower providing more time for the exchange process. Capillary sinusoids prevent blood stagnation, which could be suspected due to the long distance of the capillary systems (compare Kaufmann et al., 1985).

In contrast to these findings, Jackson, Mayhew and Haas (1987a,b) reported a reduced volume of fetal villi or vessels, and an increased maternal intervillous blood space in human. This discrepancy might be due to an underestimation of the amount of fetal tissue, because the placentae were not perfusion fixed, and therefore, collapsed following delivery. Based on these findings Mayhew, Jackson and Haas (1990) suggested that the enlargement of the lacunal blood space could be a compensation of the 'impoverished growth of villi' at high altitude. In guinea-pigs, which inspired reduced oxygen content, Bacon et al. (1984) showed an increased number of fetal placental capillary transsections, with a reduced mean diameter. These workers suggested that, under hypoxic conditions, fetal capillaries might grow longer. In agreement with this, Scheffen et al. (1990), using casts to illustrate their histological results, revealed that the increased number of capillary cross sections was due to the increased fetal capillary coiling and branching.

The findings and explanations of HA placental vessel adaptations in different species should not be expected to be the same. They must be understood in view of the sheep placenta representing the epitheliochorial type with a closed materno–fetal blood-flow interrelationship, in contrast to the haemochorial type of the human and guinea-pig. In other words, the sheep has a maternal cryptal or septal vascular system with a strictly guided blood flow, being complementary to the fetal vessel system of the villi. In contrast, humans and guinea-pigs have a labyrinthine or lacunal intervillous system, where the maternal blood flow, although being countercurrent, is not as uniform and structured. Therefore, in the sheep, both fetal and maternal vascular systems may be active for the regulation of vessel growth, architecture, and function to optimize adaptation to HA hypoxia. That this is true becomes evident when viewing the maternal vessels in HA sheep placentomes, which are subject to alterations more distinct than fetal vessels (see above), e.g. the mean percentage of vessels lumina out of the total area in SL ewes versus HA sheep rises 0.4 in the fetal vasculature, whereas it rises 3.4 in the maternal part. The distinctly changed capacity in the ovine maternal system could also explain why morphological adaptations at HA in fetal placental vessels are less pronounced or needed than in the human and guinea-pig. Maternal adaptations to HA is reflected by an overall increased vascularity. The number of vessel cross-sections remained the same, but the size and the percentage of the vessel lumina (per cross-section) is significantly larger than in SL animals. The establishment of the maternal septal vasculature is facilitated through pre-formed caruncles of the uterus, which already contain a vascular system (Andresen, 1927; Boshier, 1969) able to regulate angiogenesis due to decreased oxygen partial

pressure of the maternal blood (Kaufmann, Kohnen and Kosanke, 1993).

Wilkening and Meschia (1992) suggest preferential perfusion of some areas over others due to a supposed existence of shunts which regulate a varying blood supply for single placentomes (compare also Steven, 1966). These theories, based on physiological studies give a possible explanation for the variable histomorphometrical results found in the different areas (chorionic plate, intermedial zone, basal plate zone) of the placentome.

Surprisingly, the interhaemal distance measured in this study was 9.7 per cent larger in HA than SL ewes. For this parameter significance is lacking, but this might be due to the small sample size. In humans, however, this distance is reduced (Jackson, Mayhew and Haas, 1988a,b; Reshetnikova, Burton and Milovanov, 1994), due to a thinning of the trophoblast and the peripheralization of fetal capillaries in the tips of the villi. An increase of the capillary diameter may contribute to the thinning of the villous membrane (Burton et al., 1996). Mayhew, Jackson and Haas (1990) concluded that the reduced villous surface area for exchange (Jackson, Mayhew and Haas, 1987a,b) was compensated for by a decrease in the harmonic mean thickness of the villous membranes. In the HA sheep, the relatively thick interhaemal membrane could be related to enhanced stability of vessel wall, which could be a need in the enlarged vessels of both the fetal and maternal vascular systems. Even though it was assumed in the classical Grosser scheme (Grosser, 1909, 1927) that decreasing thickness and tissue layers of the interhaemal membrane facilitate transplacental exchange, physiological studies (for review see Faber and Thornburg, 1983 and Longo, 1987) have shown that this view is not necessarily correct (Leiser and Kaufmann, 1994). Obviously in sheep, particular transplacental transport mechanisms are more independent from the thickness of the placental barrier, as extensively studied and hypothesized in respect to oxygen transfer (Longo, Power and Forster, 1967; Longo, Hill and Power, 1972; Longo and Ching, 1977; Longo, 1987; Wilkening and Meschia, 1992). The geometric arrangement of maternal and fetal capillaries and the blood-flow interrelationship in the exchange area suggests placental efficiency in respect to the diffusion of substances (for review see Faber and Thornburg, 1983; Leiser and Kaufmann, 1994). Early morphological studies suggest that the blood flow is of a countercurrent (Barcroft and Barron, 1946; Tsutsumi, 1962) or a cross-current type (Makowski, 1968; Steven, 1966, 1975). In the SL ewes, and the goat (Leiser, 1987), it appears to be a mixture of cross-current and countercurrent flow (Leiser et al., 1997). Because of the conspicuous vascular coiling and branching, in HA animals, this statement can not be made with the same certainty. Nonetheless, our findings in SL ewes are consistent with the hypotheses of Wilkening and Meschia (1992), who conclude that the flow might be ineffective due to the high gradient of oxygen partial pressure from the uterine artery to the umbilical vein suggesting that the mean gradient between maternal and fetal blood in the placental exchange area is responsible for the efficiency (Longo, 1987).

CONCLUSION

In summary, branching and the grade of coiling is increased in fetal blood vessels of the placentomes of HA ewes. Histologically, the percentage of fetal vessels per section showed a tendency to be slightly higher, whereas the percentage of maternal vessels was distinctly increased. This suggests that morphological alterations of the ovine placental vasculature (increase of the materno–fetal absorptive surface) may improve the exchange sufficiently to compensate in cases of hypobaric hypoxaemia.

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Fibroblast growth factor (FGF)-1, FGF2, FGF7 and FGF receptors are uniformly expressed in trophoblast giant cells during restricted trophoblast invasion in cows[★]

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ABSTRACT

The bovine placenta is characterized by a limited invasion of trophoblast giant cells (TGC). In contrast to mononuclear trophoblast cells (MTC), TGC are non polarized cells, which migrate and fuse with single uterine epithelial cells throughout gestation. Fibroblast growth factors (FGF) were shown to be associated with the migratory activity of cells, cell differentiation and angiogenesis, and due to its localization in trophoblast cells were proposed as important regulating factors in hemochorial placentae of rodents and humans, and the (syn)epitheliochorial placenta of pig and sheep. Since migrating bovine TGC are of epithelial origin, but exhibit similarities to mesenchymal cells we hypothesize that the restricted trophoblast invasion in cattle is characterized by a specific FGF expression pattern. Therefore, the spatiotemporal expression of specific FGF factor:receptor pairs, either acting on cells of mesenchymal origin or on epithelial cells was examined in bovine placental tissues throughout gestation and prepartum by immunohistochemistry, semiquantitative RT-PCR and in situ hybridization. FGF1 protein was found in trophoblast, caruncular epithelium (CE) and stroma (CS), stroma of chorionic villi (SCV), and in fetal and maternal blood vessels. FGF2 signals dominated in maternal vascular endothelia (VE), immature TGC, and MTC, whereas staining in other cell types was clearly weaker. FGF7 protein was detected in fetal and maternal blood vessel as well as in immature TGC and MTC predominantly at the chorionic plate. FGFR immunoreaction was localized in immature TGC, MTC, and to a clearly lesser extent in CS, CE and fetal and maternal blood vessels. Mature TGC stained negatively for all examined factors and FGFR. The corresponding mRNAs specific for FGF1, -2, -7, total FGFR, and FGFR2 isoforms IIIb and IIIc were colocalized in immature TGC, whereas hybridization was substantially lower in CE and absent in CS, SCV and mature TGC throughout gestation, but switched to CS and VE immediately prepartum. Semiquantitative RT-PCR revealed higher mRNA levels for FGF1, FGFR, and FGFR2IIIc in cotyledons

compared to caruncles ($p<0.05$), whereas it was the opposite with FGF2 ($p<0.001$). FGF7 and FGFR2IIIb mRNA levels did not differ between caruncles and cotyledons. Significant changes ($p<0.05$) of mRNA levels related to gestational age were found for FGF1 and FGFR2IIIc, but not for FGF2, -7, total FGFR, and FGFR2IIIb. The specific localization of all examined FGF family members in TGC suggests that TGC, apart from their classical function as producers of hormonal products, play other important roles in the regulation of bovine placentomal growth, differentiation and angiogenesis.

INTRODUCTION

Throughout its life span the placenta has to adapt itself to steadily changing demands of the growing fetus by permanent growth and differentiation. A specific phenomenon in synepitheliochorial placentomes of the cow is the continuous migration of trophoblast giant cells (TGC) into the maternal epithelium and the subsequent fusion with single caruncular epithelial cells [1]. This migration does not proceed beyond the maternal basement membrane, and, in contrast to placentation in the sheep, generally results in the formation of trinucleated feto-maternal hybrid cells; therefore it is considered as restricted trophoblast invasion [2]. Up to date TGC have been associated with the production of hormones, like progesterone, prostacyclins, prostaglandins, and placental lactogen and pregnancy-specific (glyco)proteins [3-5]. However, the fact that TGC are of epithelial origin, but are not polarized and thus programmed differently led us to suspect that they may indeed have additional functions. The control of the limited invasion of TGC is still poorly understood, however, likely candidates to be involved may be FGF7 and FGF10, which are expressed by cells of mesenchymal origin [6] but specifically promote the proliferation of epithelial cells [7]. In sheep uterus and placenta, FGF7 and FGF10 mRNAs are localized in different tissue compartments of mesodermal origin, while the common receptor is found exclusively in epithelial cells, suggesting independent roles for both factors [8].

The pregnancy-related expression of FGFs in the uteroplacental unit has been suggested to be species specific and was associated with the modulation of uterine-conceptus interactions [9]. However, the presence of a conceptus may be responsible for the differential control of FGF expression [9]. Proliferative activity and angiogenesis in the placenta and/or endometrium are attributed to the presence of FGF1 and/or FGF2 in the human, rat, mouse, pig and sheep, representing hemochorial and (syn)epitheliochorial placental types. Embryo implantation in mice seems to be supported by the action of FGF1 and/or FGF2 [10]. In pigs,

pregnancy-specific expression of FGF1 and FGF2 in early gestation indicates roles for both factors during non-invasive embryo implantation of this species [9]. However, the localization of FGF1 and FGF2 in different tissue layers suggests that different ways of action are utilized [9]. In the synepitheliochorial placenta of the sheep, which resembles a slightly more invasive type than the pig, the expression of FGF2 is associated with angiogenesis and stimulation of proliferation and differentiation of trophoblast cells [11].

FGFs predominantly exert their actions through binding of low molecular weight isoforms to specific membrane receptors (FGFRs) which are subject to alternative splicing [7, 12-14]. Binding studies revealed that FGF1 can activate all FGF receptor splice variants, while FGF2 preferentially activates the c splice forms, and FGF7 almost exclusively activates the b splice form of FGFR2 [12]. However, high molecular weight isoforms may be responsible for auto/intracrine signaling pathways [15].

In order to gain more information on the control of the restricted trophoblast invasion of the bovine placenta, and the function and programming of TGC, and consequently the impact on placentomal growth and angiogenesis we evaluated the expression of specific factor:receptor pairs (FGF1 and total FGFR, FGF2 and FGFR2IIIc, and FGF7 and FGFR2IIIb) in bovine placentomes from different stages of gestation and immediately prepartum on protein and mRNA level.

MATERIALS AND METHODS

Sample Collection and Fixation

For immunohistochemistry, placentomes from 150, 220, 240 and 270 days of pregnancy and from parturient cows forming five observational groups each consisting of three animals were collected, perfusion-fixed in 10% neutral phosphate buffered formalin and paraffin embedded. The three prepartal cows were defined by declining serum progesterone levels

(<1.5 ng/ml) detected by automated chemiluminescence system (ACS180) using a PGRE kit (Bayer Vital GmbH, Fernwald, Germany). Serum levels were tested in eight hour intervals during the last two weeks of gestation and caesarean sections were done immediately after progesterone had declined when no signs of active labor were visible. The collection of samples from living animals was approved by the local authority (Regierungspräsidium Giessen, protocol II25.3-19c20/15cGI18/14).

In order to extend the study to mRNA level, additional placentomes from 52 generally healthy cows were collected at a slaughterhouse, perfusion-fixed with Bouins solution and paraffin embedded for in situ hybridization. In parallel, placentomes from the same cows were snap-frozen in liquid nitrogen for RT-PCR. The animals were assigned to the following groups: early gestation (≤ 3 months, n = 6 and 4th month, n = 9) and late gestation (5th month, n = 5; 6th month, n = 6; 7-8th month, n = 5, and > 8 months, n = 5). Additionally, placentomes from 8 cows were separated into maternal caruncle and fetal cotyledon in order to detect differences in the expression between fetal and maternal components of the placentome in early gestation (≤ 4 months, n = 4) versus late gestation (≥ 5 months, n = 4). The gestational age was assessed according to fetal crown-rump-length [16].

Immunohistochemistry

Antibodies (ABs) against FGF1 (rabbit-anti-bovine-FGF1 antiserum, rαbFGF1, catalog Nr. 06-101, UBI/Biomol, Hamburg, Germany), FGF2 (1. murine monoclonal anti-bovine-FGF2, clone bFM-2, catalog Nr. 05-118, UBI/Biomol, 2. rabbit-anti-bovine-FGF2, rαbFGF2 [17], FGF7 (rabbit anti-FGF7, Acris Antibodies, Hiddenhausen, Germany) and FGFR (monoclonal murine IgM, clone VBS1, recognizing FGFR1 [*flg* gene] and to a lesser extent FGFR2 [*bek* gene], Chemicon International, Hofheim, Germany) were used for indirect immunohistochemistry (ABC method, Vectastain-Universal-ELITE-ABC-Kit®, Vector

Laboratories, Burlingame, CA, USA). As secondary ABs biotinylated goat-anti-rabbit-IgG (BA-1000), horse-anti-mouse-IgG (BA-2000), and goat-anti-mouse-IgM (BA-2020, all Vector Laboratories) were used. Negative controls were set up by replacing rαbFGF1, –2 and –7 with heat inactivated normal rabbit serum adjusted to a corresponding protein concentration and with isotype-specific irrelevant monoclonal antibodies replacing monoclonal antibodies (C48-6: murine IgM against trinitrophenol, BD PharMingen, Hamburg, Germany; murine IgG1 anti-clostridium-perfringens-toxin provided by Dr. L. Wieler, Institute for Hygiene and Infectious Diseases of Animals, Justus-Liebig-University Giessen). Negative control sections from pregnant animals were negative except for a non-specific staining of the lumina of blood vessels. In parturient animals, diffuse background staining was observed due to labor-related hemorrhage or extravasation.

The proportion of positive cells and staining intensity of individual cell types were analyzed semiquantitatively in three sections of a randomly chosen placentome per cow by the same person (S. W.) who was blinded for the animals. According to morphological criteria and localization, trophoblast cells were assigned to two types, mononuclear trophoblast cells (MTC) and trophoblast giant cells (TGC). The latter group comprised immature TGC, characterized by intermediate cell and nuclear size, and mature TGC, which were distinctly larger and tended to lay in direct association to the caruncular epithelium.

Semiquantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The presence of mRNAs for FGF1, FGF2, FGF7, FGFR, FGFR2IIIb and FGFR2IIIc was evaluated by reverse transcription-polymerase chain reaction (RT-PCR). Total cellular RNA was extracted from whole bovine placentomes or from manually separated maternal caruncles and fetal cotyledons using TRIzol reagent (Gibco BRL, MD, U.S.A.). RNA was dissolved in water and spectroscopically quantified at 260 nm. Aliquots were subjected to 1% (w/v)

denaturing agarose gel electrophoresis and visualized by ethidium bromide staining to verify quantity and quality of RNA. Two micrograms of total RNA were used to generate single-strand cDNA in a 60 µl reaction mixture as described previously [18]. The optimal amount of total RNA for reverse transcription was evaluated by testing different RNA concentrations. Specific primers, designed according to bovine sequences listed in the GenBank database (Table 1) were commercially synthesized (Amersham-Pharmacia, Freiburg, Germany). Only the FGFR primers were deduced from consensus cDNA portions of highly conserved regions within the cytoplasmic tyrosine kinase domains of all four FGFR types [19]. The number of amplification cycles, primer sequences and size of resulting fragments for all examined factors are shown in Table 1. RT-PCR was run on a gradient cycler (Eppendorf, Hamburg, Germany). The PCR for all examined factors contained 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$ (FGF2; 1.0 mM $MgCl_2$), 0.1% (v/v) Triton X-100, 0.6 µM of each primer and 0.5 units of thermostable polymerase PrimeZyme (Biometra, Göttingen, Germany) to 3 µl cDNA (final volume 25 µl). Ubiquitin PCR was performed under the same conditions, but a higher concentration of primer (1.0 µM) was used. All amplifications were done in the following sequence: initial denaturation step 94°C for 2 min, each cycle 94°C for 1 min, 60°C for 1 min (FGF2 and FGFR2IIIc, 61°C) and one final elongation step 72°C for 2 min. Samples for the house-keeping gene ubiquitin were amplified for 20 cycles: a single denaturation step 94°C for 2 min, each cycle 94°C for 45 sec, 55°C for 45 sec and afterwards one additional elongation step 72°C for 2 min. To determine the optimal quantity of reverse transcript needed for PCR and to verify that the cDNA product was dependent on the input of transcript, varying quantities of transcript were used in the PCR reaction. The RT product in 3 µl was in the linear range for these amounts and produced a visible band. To exclude contamination with genomic DNA, all experiments included controls lacking the RT enzyme. RT-PCR contamination from buffers and tubes was excluded using a negative control with

water instead of RNA. Aliquots of the PCR reaction products (5 µl) were separated by agarose gel (2.0% [w/v]) electrophoresis in a constant 70 V field containing ethidium bromide and analyzed by a video documentation system (Amersham-Pharmacia). To determine the length of the products, a 100-bp marker (Gibco BRL) was used. All gels being compared were run and stained at the same time to ensure that apparent differences detected were indeed due to treatments and not the result of between-run variability. The ethidium bromide stained gels were evaluated by a video documentation system (Image Master VDS, Pharmacia, Freiburg, Germany), and band intensities were analyzed by computerized densitometry using the Image Master-1D-software (Pharmacia) for relative quantification. The expression data of the investigated factors were normalized by the housekeeping gene ubiquitin (ratio: optical density [OD] of the target gene/OD of the reference gene). Thus, RT-PCR and the evaluation technique used are relative and not a strictly quantitative method. PCR product identity was verified by subcloning of the cDNA into a transcription vector (pCR-Script, Stratagene, La Jolla, CA), followed by commercial DNA sequencing (TopLab, Munich, Germany).

The statistical significance of differences in mRNA expression of all examined factors was assessed by ANOVA, followed by Fisher's LSD as a multiple comparison test. All experimental data are shown as the mean ± S.E.M.

Generation of Probes for In Situ Hybridization

The PCR products of the bovine FGF1, -2, -7, total FGFR, FGFR2IIIb, and FGFR2IIIc genes were subcloned in pGEM-T (Promega, Heidelberg, Germany). The plasmids were transformed in the XL1-Blue Escherichia coli strain (Stratagene, Heidelberg, Germany) and extracted by column purification, according to the manufacturer’s instruction (Qiagen, Hilden, Germany). In vitro transcription of digoxigenin (DIG)-labeled cRNA was performed using the RNA-DIG Labeling Kit (Roche Molecular Biochemicals, Mannheim, Germany) and RNA-

polymerases T7 and SP6. The vectors containing the respective inserts were linearized with NcoI or NotI (New England Biolabs, Schwalbach, Germany) for the production of sense (NcoI) or antisense cRNA (NotI). Finally, enzymes were heat inactivated (20 min 65°C). Identity of the probes was verified by sequence analysis in a commercial laboratory (Qiagen).

In Situ Hybridization

For detection of FGF1, -2, -7, total FGFR, FGFR2IIIb and FGFR2IIIc mRNA, 3 µm tissue sections were deparaffinized, rehydrated, placed into 0.2 N HCl for 20 min and transferred into 2x standard saline citrate (SSC) for 20 min at 70°C. Following partial digestion with Proteinase K (20 µg/ml 1x PBS) for 30 min at 37°C, sections were treated with 0.2% (w/v) glycin in PBS and 20% (v/v) acetic acid in diethylpyrocarbonate (DEPC) treated H_2O. Sections were postfixed in 4% (v/v) paraformaldehyde for 10 min and prehybridized in 20% (v/v) glycerol for 30 min. Sections were then covered with the DIG-labeled sense and antisense cRNA probes. All cRNA were used at a dilution of 1:25 in a hybridizing-buffer containing 50% (v/v) deionized formamide, 10% (v/v) dextran sulphate, 2x SSC, 1x Denhardt's solution, 10 µg/ml salmon sperm DNA (Sigma), and 10 µg/ml yeast tRNA (Sigma). Hybridization was performed overnight at 37°C in a humid chamber containing 50% (v/v) formamide in 2x SSC. Post-hybridization washes were performed as follows: 4x SSC for 4x10 min at 37°C, 2x SSC for 15 min at 60°C, 0.2x SSC for 15 min at 42°C, 0.1x SSC for 5 min at room temperature, 2x SSC for 5 min at room temperature, and 3% (w/v) BSA in Tris-HCl-, NaCl-, $MgCl_2$-, Triton-X-100 buffer (TNMT) for 1 h. Tissue sections were incubated with the anti-DIG Fab-antibody conjugated to alkaline phosphatase (Roche Molecular Biochemicals) overnight at 4°C. Staining was visualized by developing sections with nitroblue-tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (Sigma) in a humid chamber protected from light. Finally, sections were mounted in Glycergel (Dako, Hamburg,

Germany). For each test control incubations were performed using DIG-labeled cRNA sense probes as well as incubation with hybridization buffer instead of sense or antisense probes. These sections were completely negative.

RESULTS

Immunohistochemistry for FGF1, FGF2, FGF7 and FGFR in Bovine Placentomes

FGF1, FGF2, FGF7 and FGFR were detected in bovine placentomes throughout gestation, however immediately prepartum, the immunoreaction differed. The spatiotemporal distribution and subcellular localization as well as staining intensities are shown in Table 2, and representative areas are demonstrated in Figure 1.

Specificity and Validation of RT-PCR Data

The presence of mRNA specific for all examined factors in bovine cotyledons and caruncles was verified by conventional RT-PCR (Fig. 2). Each PCR product showed 100 % homology to the known bovine genes after sequencing. Ubiquitin was examined in all samples to confirm the integrity of the mRNA templates and RT-PCR protocol, and also served as housekeeping gene for the quantification of mRNA-levels. The results were expressed as the ratio of densitometric readings for examined factors to ubiquitin, therefore indicating relative changes in RNA levels.

mRNA Levels of FGF System Members in Whole Placentomes in the Course of Gestation

Using semiquantitative RT-PCR analysis of complete placentomes (Fig. 3), significant changes of mRNA levels as a function of gestational age were found for FGF1 (Fig. 3A; $p<0.05$) and FGFR2IIIc (Fig. 3F; $p<0.05$) with an increase from around Day 80 until Day 200, followed by a decrease until term. FGF2, FGFR and FGFR2IIIb exhibited similar mRNA levels and expression patterns (Fig. 3B, D, E). However, the observed changes in the course

of gestation were statistically not significant. FGF7 mRNA levels varied insignificantly throughout gestation (Fig. 3C).

mRNA levels of FGF System Members in Cotyledons and Caruncles at Early and Late Gestation

As investigated by semiquantitative RT-PCR, FGF1 specific mRNA levels were higher in the cotyledon compared to the caruncle (Fig. 4A). However, this difference was statistically different only at early gestation ($p<0.05$) due to a significant decrease ($p<0.05$) in the cotyledon at late gestation. FGF2 mRNA expression was significantly higher ($p<0.001$) in the caruncle compared to the cotyledon (Fig. 4B). FGF7 mRNA expression between caruncles and cotyledons and during early and late gestation was not significantly different (Fig. 4C). FGFR mRNA was higher ($p<0.05$) in the fetal compared to the maternal compartment in early gestation. In late gestation this discrepancy was reduced, because cotyledonary expression decreased, while caruncular levels were maintained. FGFR2IIIb mRNA tended to be higher in cotyledons than in caruncles (Fig. 4E), but remained the same when comparing early and late gestation. FGFR2IIIc mRNA expression was different between cotyledons and caruncles (Fig. 4F) with significantly higher levels in the fetal part of the placentome at late gestation ($p<0.05$).

In Situ Hybridization for FGF1, -2, -7, total FGFR, FGFR2IIIb, and FGFR2IIIc

During gestation, large amounts of FGF1 mRNA were detected in the cytoplasm of immature TGC situated in close vicinity to basement membranes (Fig. 5A), whereas no signals were found in mature TGC. In prepartal animals, only few immature TGC expressed FGF1 mRNA (Fig. 5B). Hybridization in CEC was weak and occurred in the infranuclear cytoplasm; it increased on a low level in the course of gestation, but was again almost absent

in prepartal animals. During gestation, no FGF1 mRNA was detected in fetal and maternal stroma (Fig. 5A). However, in placentomes from prepartal cows, distinct hybridization associated with caruncular and cotyledonary blood vessels was observed (Fig. 5B), indicating a switch of the FGF1 mRNA expression from epithelial to stromal components of the placentome.

FGF2 mRNA was also predominantly localized in immature TGC and to much lesser extent in CEC, whereas mature TGC, and both fetal and maternal stroma were nearly devoid of FGF2 mRNA (Fig. 5C). In the course of gestation the FGF2 expression did not change. However, immediately prior to parturition, a similar switch from epithelial to stromal cells, predominantly vasculature occurred as with FGF1 mRNA (Fig. 5D). CEC remained to express FGF2 mRNA only in the tips of maternal septa, and the number of immature TGC expressing FGF2 mRNA was distinctly reduced.

FGF7 transcripts were detected in immature TGC of fetal villi in the course of gestation (Fig. 5E), however in intervillous areas of the basal plate only few immature TGC were labeled. CEC showed weak FGF7 mRNA expression in an irregular pattern, only CEC at the tips of maternal septa displayed a stronger hybridization. In this specific location, an expression of FGF7 mRNA was also detected in the maternal connective tissue underlying FGF7 positive CEC. Endothelial cells of allantochorionic blood vessels located within the chorionic plate also expressed large amounts of FGF7 mRNA, whereas within the placentome only few fetal and maternal endothelial cells showed hybridization. Immediately preceding parturition, FGF7 mRNA expression also switched from immature TGC and CEC to the maternal stroma, leaving only few TGC expressing FGF7 transcripts (Fig. 5F).

Total FGFR (Fig. 5G), FGFR2IIIb (Fig. 5I) and FGFR2IIIc (Fig. 5L) mRNAs were colocalized to mRNA of FGF1, -2, and -7 in high amounts in immature TGC, and to a clearly

lower extent in CEC. Immediately prior to parturition, parallel changes from TGC to caruncular stroma and CEC at the tips of maternal septa were observed (Figs. 5H, K, M).

DISCUSSION

This is the first report on the spatiotemporal expression of protein and mRNA of specific FGF factor:receptor pairs in the synepitheliochorial bovine placenta, a placental type which is characterized by the limited invasion of migrating, mostly binucleated trophoblast giant cells into the maternal (caruncular) epithelium.

Differentiation, Migration and Functions of Trophoblast Giant Cells

Immature bovine placentomal TGC expressed the highest amounts of all FGF factor:receptor pairs examined in the present study, while mature TGC during their migration and feto-maternal hybrid cells were completely negative. These findings indicate 1) the examined FGF system components may be involved in the auto- and/or paracrine control of bovine TGC differentiation, migration, and limitation of trophoblast invasion, implicating that TGC have other functions during pregnancy than just the production of hormones; 2) not polarized TGC are intermediates between epithelial and mesenchymal cells.

The migratory activity of trophoblast cells is essential for a successful embryo implantation in vitro in hemochorial placental types [20]. In the mouse, this in vitro trophoblast migratory activity is stimulated by FGF2 as its inclusion into the culture medium significantly promotes the rates of trophoblast spreading and outgrowth via paracrine action [10]. Moreover, as mentioned above, FGF2 induced migration in human vascular endothelial and smooth muscle cells [21, 22]. Additionally, cell binucleation, a characteristic process occurring in the course of bovine TGC differentiation may be promoted by high molecular weight isoforms of FGF2 which are mainly located in the nucleus, a phenomenon that has

also been observed in rat cardiac myocytes [23]. TGC production of hormonal products, like placental lactogen, may also be affected by high molecular weight FGF2, since in vitro experiments have shown that these high molecular weight isoforms were able to stabilize an endocrine phenotype of PC12 cells [24].

Immature bovine TGC expressed also high amounts of FGF7 and its receptor. Generally, FGF7 is considered a growth factor produced in mesenchymal cells promoting the growth of adjacent epithelial cells [7]. However, moderately invasive TGC as intermediates between real polarized epithelial cells and non polarized mesenchymal cells seem to express both systems, epithelial and mesenchymal FGF:FGFR pairs. An evolutionary diversification was suggested for rodents, where FGFR2IIIb, receptor for FGF7 is localized to surface ectoderm, while FGFR2IIIc, receptor for FGF2 is expressed in the adjacent mesoderm [25]. Bovine placentomal CEC also expressed FGF7 and its receptor (FGFR2IIIb) in all placentomal locations with the exception of the broadened superficial tips of caruncular septa, where distinct signals were found in the caruncular epithelium and in the underlying maternal stroma. The pronounced expression of FGF7 at the tips of maternal septa is in accordance with the concept that these areas are the predominant zones of placentomal growth [2], and is corroborated by the occurrence of increased proliferative activity [26]. In the pig, a species with true epitheliochorial placentation, FGF7 mRNA is expressed by endometrial epithelia particularly between Days 12 and 15 of the estrous cycle and pregnancy, while FGFR2IIIb is expressed in both endometrial epithelia and conceptus trophectoderm, suggesting that FGF7 may act on the uterine endometrial epithelium in an autocrine manner and on the conceptus trophectoderm in a paracrine manner [27]. In vivo experiments revealed also that estrogen, the pregnancy recognition signal from the conceptus in pigs, increases uterine epithelial FGF7 expression, which, in turn, stimulates the proliferation and differentiation of conceptus trophectoderm [28]. In contrast, invasive hemochorial human placentation is characterized by

a progesterone dependent expression of FGF7 and its receptor [29]. The localization of FGF7 in the decidua and pseudopregnant endometrium, and FGFR2IIIb in chorionic villi in early pregnancy suggests that FGF7 plays a role in the interaction between human decidua and chorion [29] and speaks for a paracrine modus of action. In vitro FGF7:receptor interaction stimulates hCG secretion indicating an important role in the embryo-endometrial/decidual interaction; however, in the same time cell proliferation was not affected [30]. The proliferative activity of trophoblast cells was associated with high levels of FGF7 and FGFR2IIIb and most binding to each other in hemochorial placentae of rhesus monkeys in early gestation [31]. A similar involvement in embryo implantation of FGF7 in other species with invasive implantation can be hypothesized, since the surface area of the trophoblast of mice can be significantly increased by FGF7 treatment [10].

Impact of FGFs for Placentomal Angiogenesis

FGF1, FGF2 and FGFR were extensively expressed in the bovine placentomal vascular system and in the surrounding stroma. Thus, both factors may play important roles in the regulation of angiogenesis and vascular functions such as capillary permeability.

In vitro, FGF1 stimulates proliferation and increases macromolecular permeability through activation of tyrosine kinases between the cell-cell junctions of human placental microvascular endothelial cells, but not of large vessel cells [32]. FGF2 released by human trophoblast cells from early gestation exerted an angiogenesis promoting effect on vascular endothelial cell lines [33]. In vitro, FGF2 is a potent stimulator of proliferation of human umbilical vein endothelium, but also induces migration of vascular endothelial cells [21] and promotes migration of vascular smooth muscle cells through alteration of the interaction of integrin-mediated contact to the ECM [22]. In peri-implantative pigs, characterized by non invasive trophoblast, different roles in uterine function and conceptus development are

proposed, since FGF1 expression changes from a diffuse stromal staining during estrous cycle to a concentration around uterine glands and luminal epithelium, whereas FGF2 was localized in luminal and glandular epithelium as well as in concepti [9]. In synepitheliochorial placentomes of sheep, displaying a slightly higher degree of trophoblast invasiveness, FGF2 has also been identified as an angiogenic agent for vascular endothelial cells [11]. The mitogenic response of 3T3 cells can be enhanced by interaction of FGF2 with HSPG [34]. This specific interaction has been reported for the human placenta where colocalization of FGF2 and HSPG in the growth zones indicates an important role in placental angiogenesis and morphogenesis [35]. FGF2 action includes also the involvement of VEGF, since the stimulatory effect on cultures of vascular endothelial cells has been shown to be mediated by the up-regulation of VEGF expression [36]. Moreover, FGF2 may promote the feto-maternal exchange by the induction of vasodilation, since FGF2 treatment significantly increased eNOS levels via the MAPK cascade in ovine fetoplacental artery endothelial cells [37]. Vasodilation results in a reduced flow velocity of fetal and/or maternal blood in the placenta, thus allowing more time for feto-maternal exchange. In vitro, also FGF7 enhanced the growth of vascular smooth muscle cells co-expressing the corresponding FGFR2IIIb [38], and thus may stimulate angiogenesis in bovine septal tips where high levels of specific mRNA were observed using ISH.

Prepartal Changes of FGF Factor:Receptor Pairs

All FGF factor:receptor pairs investigated in this study showed the same pronounced prepartal switch. Expression of FGF1, -2, -7 and their receptors in immature TGC decreased while the expression in maternal stroma cells, predominantly endothelial cells increased.

The significant reduction of FGFs and their receptors in immature TGC prior to the onset of active labor suggests that the process underlying the dramatic decrease of TGC at

parturition [39] is a stop in TGC differentiation rather than an increased turn-over or destruction of mature TGC. That means, via FGFs and their receptors, TGC may actually be involved in the autocrine regulation of their own differentiation. The observed up-regulation of the FGF(R) expression in CEC is consistent with the broadening of maternal caruncular septa in late gestation together with an enhanced proliferative activity in this cell type [26]. Furthermore, the prepartal up-regulation of FGF factor:receptor pairs in the caruncular stroma may support the post partum regression of the uterus, which is associated with tissue remodeling and angiogenesis [40].

Subcellular localization of FGF family members

FGF1, -2 and FGFR have not only been observed in the cytoplasm, but also in nuclei of fetal and maternal cell types of the bovine placentome.

The nuclear expression of ligands and receptors of a peptide growth factor system is at first view unexpected. However, it has been shown that nuclear localization of FGF2 can result from internalization of low molecular weight isoforms during an intracrine mechanism, and via direct nuclear translocation of high molecular weight isoforms, which obviously exert intracrine functions [15]. The specific localization of FGFs in either cytoplasm or nucleus of a cell has been associated with specific and different biological functions [15]. The nuclear localization of FGF2 in dividing but not in non-dividing human placental cells speaks for a role in cytotrophoblast proliferation in vivo, a concept which is supported by the fact that the proliferation of JEG-3 cells is stimulated by FGF2 [41]. In FGF2 treated 3T3 fibroblasts, a time- and dose-dependent increase in the association of FGFR1 immunoreactivity with the nucleus was observed suggesting participation in the regulation of gene activity [42].

In conclusion, the specific expression of mesenchymal and epithelial FGF factor:receptor pairs in bovine placentomal TGC suggests 1) TGC are in fact intermediates between real epithelial cells and cells of mesenchymal origin, 2) TGC have multiple functions, like paracrine influence on angiogenesis and placentomal growth besides auto- and/or intracrine impact on their own function (production of hormones, differentiation and migration). The characteristic changes in the expression of all examined FGF family members implies that they play important roles in the maintenance and local regulation of bovine placental growth and angiogenesis as well as TGC differentiation and function.

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Figure Legends

Figure 1. Immunolocalization of FGF1 (**A, B**), FGF2 (**C, D**) FGF7 (**E, F**) and FGF receptors (**G, H**) in bovine placentomes. **A**) FGF1 is detected almost ubiquitously in bovine placentomes with pronounced cytoplasmic staining in immature TGC (arrows, day 150). **B**) Predominantly nuclear staining for FGF1 is observed in endothelium (arrowhead) and wall of a small cross-sectioned caruncular artery (CA) and in surrounding caruncular stroma cells (CS). Staining in residual blood plasma components is non-specific (day 270). **C**) Immunostaining for FGF2 in a cross-sectioned tertiary chorionic villus and surrounding caruncular septa with pronounced cytoplasmic staining in immature TGC (arrows), while mature TGC (asterisks) are negative (day 240). **D**) Caruncular vascular endothelial cells (arrowhead) of a large CA display an intense nuclear staining for FGF2 (day 150). **E**) FGF7 protein is localized in immature TGC and MTC as well as in fetal and maternal endothelial cells throughout gestation (day 150). **F**) Immediately prepartum, strong FGF7 immunoreactivity is found in vascular endothelial cells and CS, while staining of the trophoblast has almost disappeared. **G**) The signal intensity for FGFR in the trophoblast (T) gradually decreases from the origin of a chorionic stem villus (left side of figure) at the chorionic plate towards secondary and tertiary villi (to the right of figure, day 220). **H**) A corresponding detail of a cross-sectioned chorionic villus shows that immature TGC and MTC as well as fetal and maternal capillaries are distinctly immunostained for FGFR. Mature TGC (asterisks) are negative (day 270). C, capillary; CA, caruncular artery, CV caruncular vein, CE, caruncular epithelium; CS, caruncular stroma; SCV, stroma of chorionic villi; asterisks, mature TGC; arrows, immature TGC; arrowheads, endothelial cells. Black bars = 25 μm; white bars = 100 μm.

Figure 2. Representative gels showing specific PCR products for a) Ubiquitin (189+417 bp) and b) FGF family members in bovine placentomes separated by agarose gel electrophoresis.

1) FGF1 (317 bp); 2) FGF2 (288 bp); 3) FGF7 (304 bp); 4) FGFR (417 bp); 5) FGFR2IIIb (178 bp); 6) FGFR2IIIc (139 bp). M represents 100 bp DNA ladder.

Figure 3. Semiquantitative analysis of total placentomal mRNA of FGF system members (OD of specific mRNA/OD of UBQ mRNA; arbitrary units) in the course of gestation. **A**) FGF1 (28 cycles, C). **B**) FGF2 (32 C). **C**) FGF7 (38 C). **D**) Total FGFR (28 C). **E**) FGFR2IIIb (28 C). **F**) FGFR2IIIc (28 C). n per Month of gestation = 5-9. Data are expressed as means+SEM. Different superscripts denote statistically different values ($p<0.05$).

Figure 4. Semiquantitative analysis of differences in the mRNA expression of the FGF system (OD of specific mRNA/OD of UBQ mRNA; arbitrary units) in fetal cotyledons and maternal caruncles compared in early and late gestation. **A**) FGF1 (28 cycles, C). **B**) FGF2 (32 C). **C**) FGF7 (38 C). **D**) Total FGFR (28 C). **E**) FGFR2IIIb (28 C). **F**) FGFR2IIIc (28 C). F-/M-, fetal/maternal compartment; Early gestation, Month 2-4; Late gestation, Month 5-9; n per group = 4. Data are expressed as means+SEM. Different superscripts denote statistically different values ($p<0.05$).

Figure 5. Representative micrographs of in situ hybridization for FGF family members in bovine placentomes during gestation (**A, C, E, G, I, L**) and immediately prepartum (**B, D, F, H, K, M**). During gestation the majority of mRNA for FGF1, -2, -7 and total FGFR, FGFR2IIIb, FGFR2IIIc is localized in immature trophoblast giant cells (arrows) and caruncular epithelium (arrowheads). In contrast, immediately prepartum the mRNA expression of all FGFs and receptors investigated has changed to the vasculature of maternal and fetal stroma (asterisks) as well as connective tissue cells of the caruncular stroma (CS). SCV, stroma of chorionic villi. bars = 100 µm.

Table 1. Gene transcript, primer sequences, number of cycles used, and resulting fragment size.

Target	Sequence of forward and reverse primers (5´ → 3´)	No. of cycles	Annealing temperature	Fragment size (bp)	Accession number
FGF-1	GCTGAAGGAGAAACCACGAC GTTTTCCTCCAACCTTTCCA	28	60	317	X13221
FGF-2	GAACGGGGGCTTCTTCCT CCCAGTTCGTTTCAGTGCC	32	61	288	M13440
FGF-7	CTGCCAAGTTTGCTCTACAG TCCAACTGCCAGGGTCCTGAT	38	60	294	S72475
FGFR	GARATGGAGRTGATGAAGMTGATYGG CCCRAARGACCASACRTCACTCTG	28	60	471	Z68150
FGFR-2IIIb	AGCAAATGCCTCCACTGTG TGCATTGGAACTATTTATCCCC	28	60	178	AJ419173
FGFR-2IIIc	GGTGTTAACACCACGGACAA CTGGCAGAACTGTCAACCAT	28	61	139	AJ413268
Ubiquitin	ATGCAGATCTTTGTGAAGAC CTTCTGGATGTTGTAGTC	20	55	189	Z49056

R: A or G; M: A or C; Y: C or T; S: G or C.

Table 2.
Results for the semiquantitative evaluation of immunohistochemical staining for FGF1 (**A**), FGF2 (**B**), FGF7 (**C**) and FGF-receptors (**D**) in cytoplasm (cyt), nuclei (nuc) and cell membranes (mem) of different cell types in bovine placentomes. Signal intensity is presented by + = weak staining, ++ = distinct staining, +++ = intense staining. The proportion of positive cells is indicated for each cell type by arabic numbers in brackets: 0 = no positive cells to 5 = ubiquitous staining. TGC = trophoblast giant cells; imTGC = immature TGC; maTGC = mature TGC; MTC = mononuclear trophoblast cells.

A) FGF1

Group		caruncular stroma	caruncular epithelium	cotyledonary stroma	trophoblast (MTC + TGC)
Day 150	cyt	[0]	++/+++ [3]	[0]	+/+++ [3]
(n=3)	nuc	+/+++ [3]	+/+++ [4]	+/+++ [3]	+/+++ [4]
Day 220	cyt	[0]	+/++ [4]	[0]	+/+++ [4]
(n=3)	nuc	+/+++ [2]	+/+++ [4]	+/+++ [2]	+/+++ [4]
Day 240	cyt	[0]	++/+++ [5]	[0]	+/+++ [5]
(n=3)	nuc	+/+++ [4]	++/+++ [5]	+/+++ [4]	++/+++ [5]
Day 270	cyt	[0]	+/+++ [5]	[0]	+/+++ [5]
(n=3)	nuc	+/+++ [4]	+/+++ [5]	+/+++ [3]	++/+++ [5]
Term	cyt	[0]	– [1]	[0]	+/++ [4]
(n=3)	nuc	+/+++ [4]	– [1]	+/+++ [4]	+/+++ [2]

B) FGF2

Group		caruncular stroma	caruncular epithelium	cotyledonary stroma	trophoblast MTC	trophoblast imTGC	trophoblast maTGC
Day 150	cyt	[0]	+/++ [5]	[0]	+/+++ [4]	++/+++ [5]	[0]
(n=3)	nuc	+/+++ [2]	[0]	+/++ [2]	[0]	[0]	[0]
Day 220	cyt	+ [2]	+ [1]	[0]	+/+++ [2]	+/+++ [2]	[0]
(n=3)	nuc	+/+++ [2]	[0]	+/++ [1]	[0]	[0]	[0]
Day 240	cyt	[0]	+/++ [5]	[0]	+/++ [3]	+++ [3]	[0]
(n=3)	nuc	+/+++ [2]	[0]	+++ [1]	[0]	[0]	[0]
Day 270	cyt	[0]	+/++ [3]	+/++ [1]	+/++ [2]	++/+++ [2]	[0]
(n=3)	nuc	+/+++ [2]	[0]	+/+++ [1]	[0]	[0]	[0]
Term	cyt	[0]	– [1]	[0]	[0]	[0]	[0]
(n=3)	nuc	+/+++ [3]	– [1]	+/+++ [1]	[0]	[0]	[0]

C) FGF7

Group		caruncular vasculature	caruncular epithelium	cotyledonary vasculature	trophoblast MTC + imTGC	maTGC
Day 150	cyt	++ [2]	+ [1]	+++ [4]	+/++ [3]	[0]
(n=3)	nuc	[0]	[0]	[0]	[0]	[0]
Day 220	cyt	++ [2]	+ [1]	+++ [4]	+/+++ [3]	[0]
(n=3)	nuc	[0]	[0]	[0]	[0]	[0]
Day 240	cyt	++ [2]	+ [1]	+++ [4]	++/+++ [4]	[0]
(n=3)	nuc	[0]	[0]	[0]	[0]	[0]
Day 270	cyt	++ [2]	+ [1]	+++ [4]	+/+++ [3]	[0]
(n=3)	nuc	[0]	[0]	[0]	[0]	[0]
Term	cyt	+++ [4]	– [1]	+++ [4]	+/++ [4]	[0]
(n=3)	nuc	[0]	– [1]	[0]	[0]	[0]

D) FGF-receptor

Group		caruncular stroma	caruncular epithelium	cotyledonary stroma	trophoblast MTC + imTGC	maTGC
Day 150	cyt/mem	+/++ [2]	[0]	[0]	+/++ [3]	[0]
(n=3)	nuc	+ [2]	[0]	+ [1][2]	[0]	[0]
Day 220	cyt/mem	+/+++[3]	[0]	[0]	+/+++ [3]	[0]
(n=3)	nuc	[0]	[0]	+/+++[1][2]	+ [1]	[0]
Day 240	cyt/mem	+/++ [3]	+ [3]	[0]	++/+++[4]	[0]
(n=3)	nuc	[0]	+ [3]	+/++[1][2]	[0]	[0]
Day 270	cyt/mem	+/+++[2]	+ [1]	[0]	+/+++ [3]	[0]
(n=3)	nuc	[0]	+ [1]	+/++[2][2]	[0]	[0]
Term	cyt/mem	+ [4]	– [1]	[0]	+/++ [4]	[0]
(n=3)	nuc	[0]	– [1]	+ [1][2]	[0]	[0]

1 No evaluation was performed because caruncular epithelial cells partly disappear prior to parturition and thus the quantification of the remaining flat cells is doubtful.

2 weak nuclear signals associated with more pronounced perinuclear staining; mainly associated with major blood vessels.

Figure 1

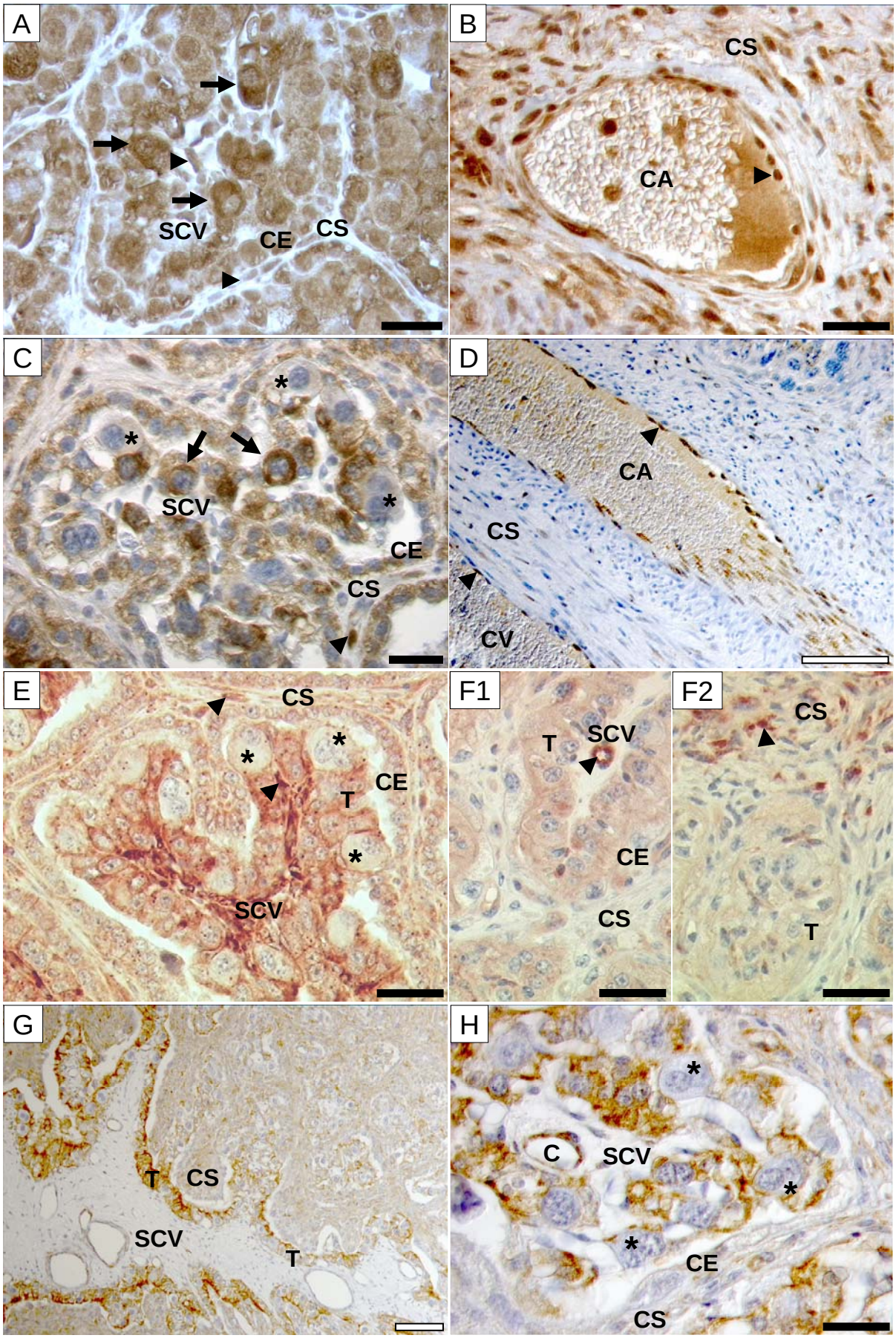

A
SCV
CE
CS
B
CS
CA
C
SCV
CE
CS
D
CA
CS
CV
E
CS
T
CE
SCV
F1
T
SCV
CE
CS
F2
CS
T
G
T
CS
SCV
T
H
C
SCV
CE
CS

Figure 2

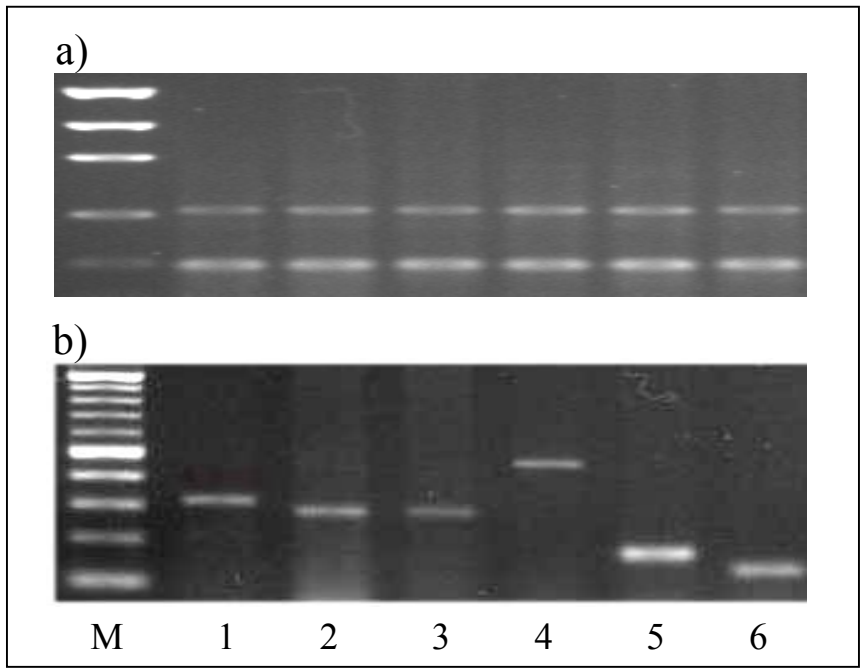


Figure 3

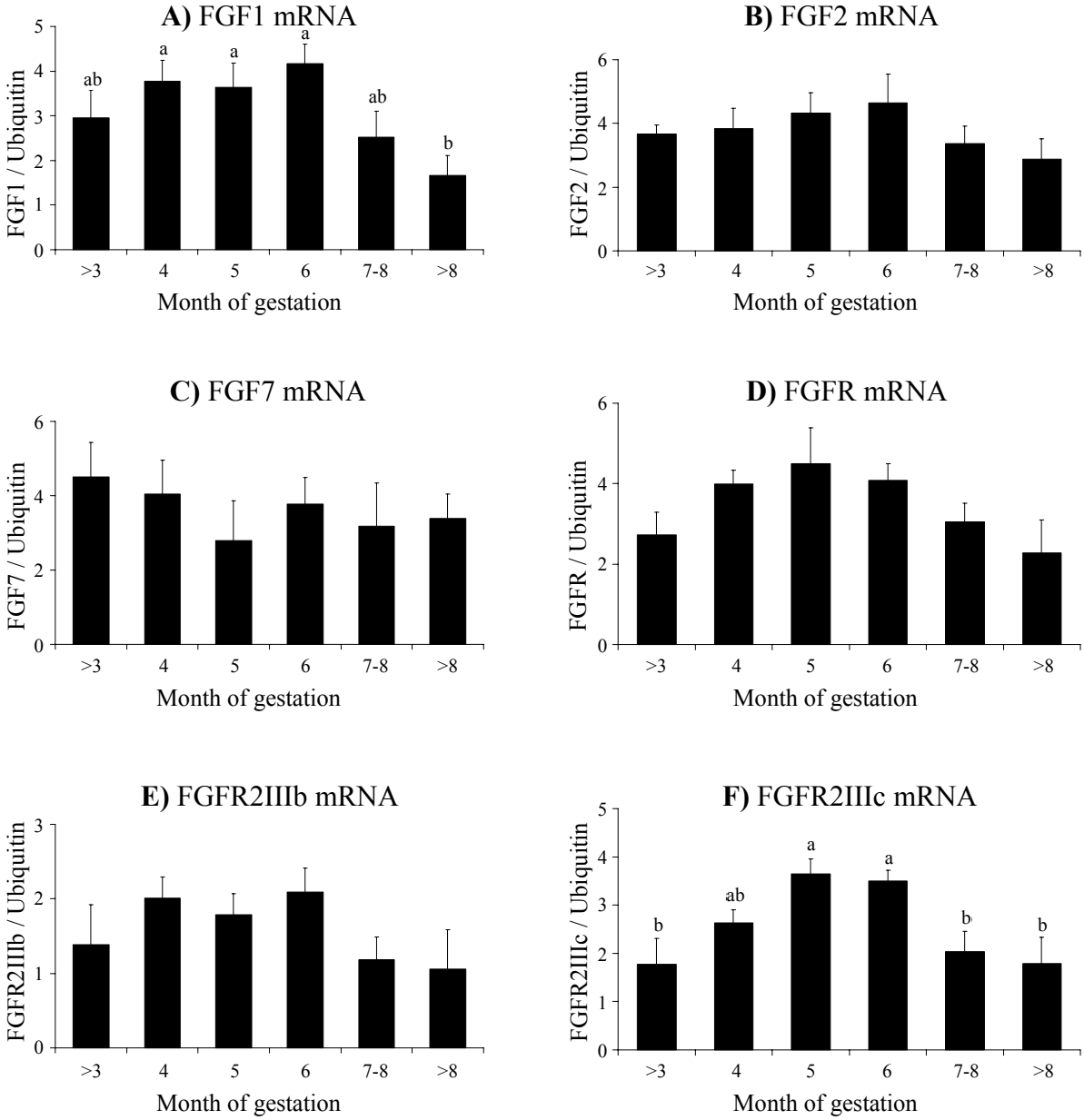

A) FGF1 mRNA
FGF1 / Ubiquitin
ab
a
a
a
ab
b
B) FGF2 mRNA
FGF2 / Ubiquitin
C) FGF7 mRNA
FGF7 / Ubiquitin
D) FGFR mRNA
FGFR / Ubiquitin
E) FGFR2IIIb mRNA
FGFR2IIIb / Ubiquitin
F) FGFR2IIIc mRNA
FGFR2IIIc / Ubiquitin
b
ab
a
a
b
b
>3
4
5
6
7-8
>8
Month of gestation

Figure 4

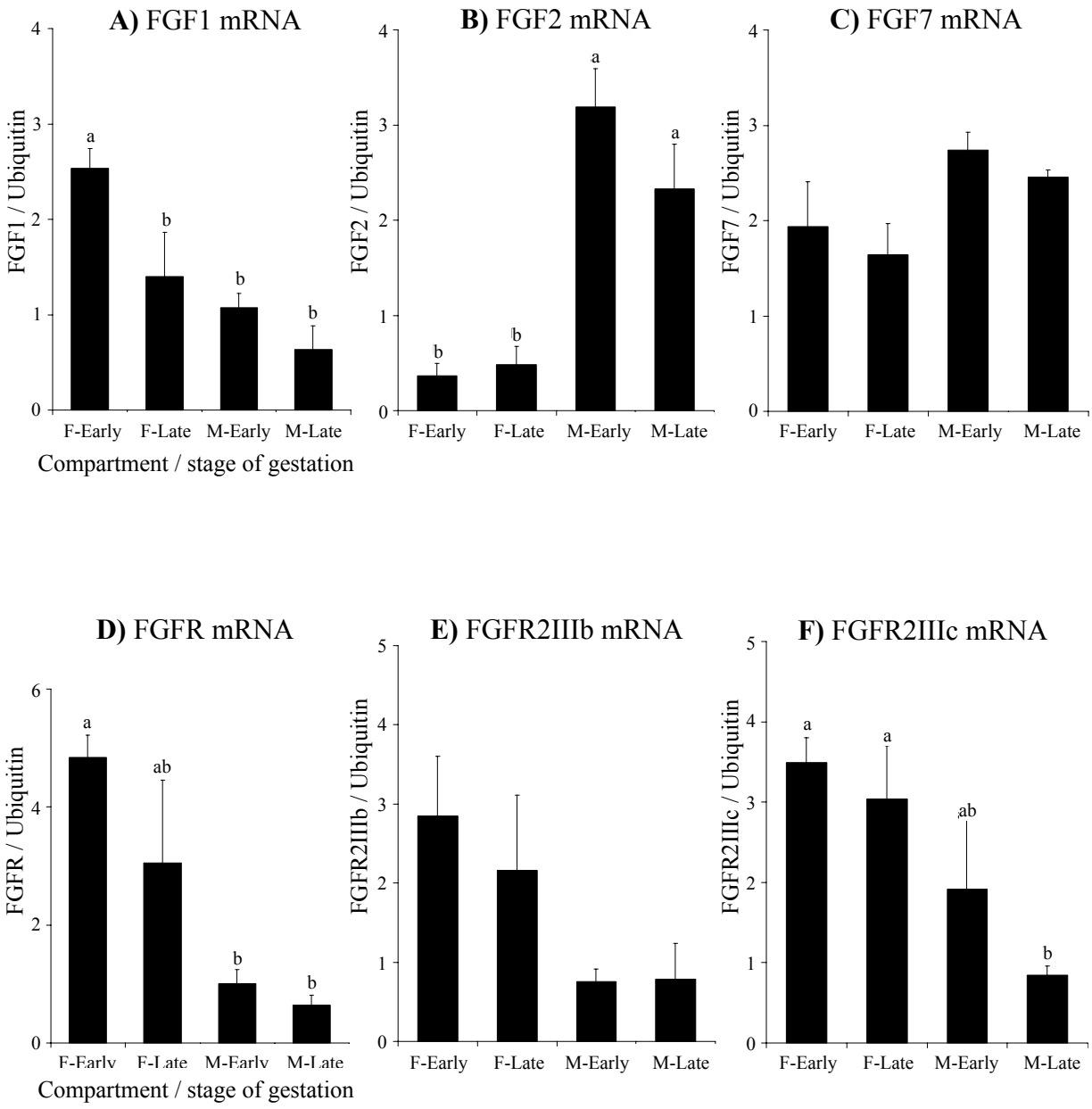
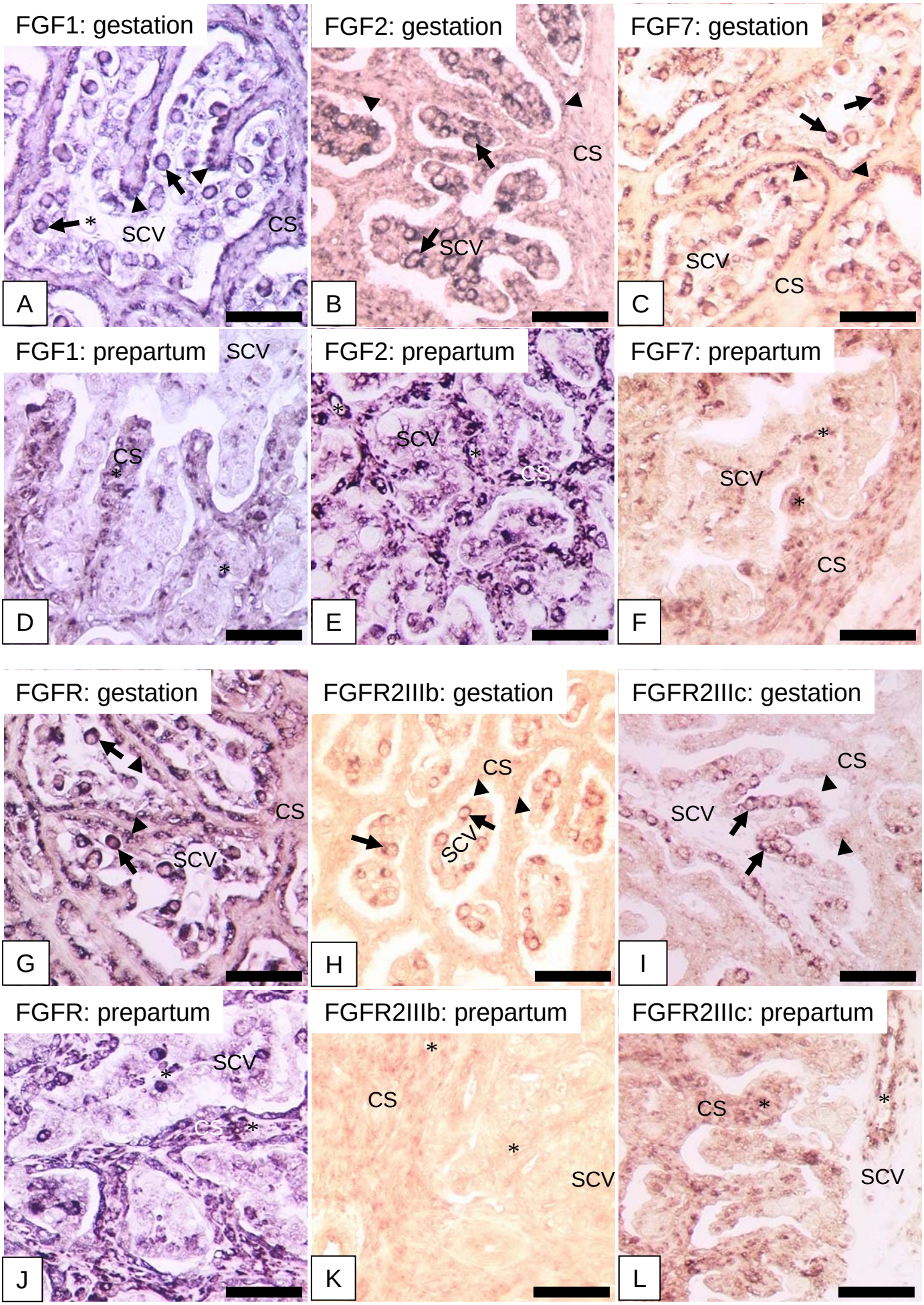

A) FGF1 mRNA
FGF1 / Ubiquitin
B) FGF2 mRNA
FGF2 / Ubiquitin
C) FGF7 mRNA
FGF7 / Ubiquitin
F-Early
F-Late
M-Early
M-Late
Compartment / stage of gestation
D) FGFR mRNA
FGFR / Ubiquitin
E) FGFR2IIIb mRNA
FGFR2IIIb / Ubiquitin
F) FGFR2IIIc mRNA
FGFR2IIIc / Ubiquitin

Figure 5


FGF1: gestation
SCV
CS
A
FGF2: gestation
CS
SCV
B
FGF7: gestation
SCV
CS
C
FGF1: prepartum
SCV
CS
D
FGF2: prepartum
SCV
CS
E
FGF7: prepartum
SCV
CS
F
FGFR: gestation
CS
SCV
G
FGFR2IIIb: gestation
CS
SCV
H
FGFR2IIIc: gestation
CS
SCV
I
FGFR: prepartum
SCV
CS
J
FGFR2IIIb: prepartum
CS
SCV
K
FGFR2IIIc: prepartum
CS
SCV
L

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