Mammakarzinom während der Schwangerschaft – Analyse des schwangerschaftsassoziierten Einflusses auf das Wachstums- und Invasionsverhalten des Tumors

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1. Abkürzungsverzeichnis

2D	zweidimensional
3D	dreidimensional
cPARP	cleaved Poly (ADP-ribose) polymerase (gespaltene Poly-ADP-Ribose-Polymerase)
CTC	circulating tumor cells (zirkulierenden Tumorzellen)
ECM	extracellular matrix (extrazelluläre Matrix)
ELISA	Enzyme-Linked Immunosorbent Assay
EGF	epidermal growth factor (epidermaler Wachstumsfaktor)
ERα	Estrogen Rezeptor α
ERß	Estrogen Rezeptor ß
FACS	fluorescence-activated cell scanning (Durchflusszytometrie)
FKS	fetales Kälberserum
FSH	follikelstimulierendes Hormon
hCG	humanes Choriongonadotropin
HE	Hämatoxylin Eosin
HER2	human epidermal growth factor receptor 2 (epidermaler Wachstumsfaktorrezeptor 2)
HLA-C	human Leukocyte Antigen-C
hPL-1	human platelet lysate (Thrombozytenlysat)
hPL-2	humanes Plazentalaktogen
HPV	humane Papillomaviren
HS	humanes Serum

ICC	Immuncytochemie
IHC	Immunhistochemie
ILGF	insulin-like growth factor (Insulinartiger Wachstumsfaktor-2)
IMUP-2	immortalization-upregulated protein-2
KIRS	killer cell immunoglobulin-like receptors
LDH	Laktatdehydrogenase
LH	Luteinisierendes Hormon
MCF-7	Michigan Cancer Foundation - 7
MCS	humane mesenchymale Stromazellen
MCTS	multicellular tumorspheroids (multizelluläre Tumorsphäroide)
MTS	[3-4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] (= Tetrazoliumsalz-Farbstoff)
MUC-1	Mucin-1
PABC	Pregnancy associated breast cancer (schwangerschaftsassoziertes Mammakarzinom)
PLAP	plazentare alkaline Phosphatase
PCR	Polymerase-Kettenreaktion
poly-HEMA	Poly(2-hydroxyethyl methacrylate)
p.c.	post conceptionem (nach der Befruchtung)
p.p.	post partum (nach der Geburt eines Kindes)
PR	Progesteron Rezeptor
siglec-6	Sialic acid-binding lg-like lectin-6 (Sialinsäure-bindende Immunglobulin-ähnliche Lektine)
SSW	Schwangerschaftswoche

TNBC	triple negative breast cancer (triple negatives Mammakarzinom)
uNK	uterine natürliche Killerzellen
WDNI	well-differentiated non-invasive

2. Zusammenfassung

Das Mammakarzinom zählt neben dem Cervixkarzinom zu einer der häufigsten malignen Erkrankung während der Schwangerschaft. Aufgrund des zunehmenden Alters schwangerer Frauen in Industrieländern, ist von einer steigenden Inzidenz dieser Tumorerkrankung auszugehen. Die Diagnose erfolgt im Vergleich zu nicht-schwangeren Frauen verspätet, welches mit einem erhöhten Risiko einhergeht die Erkrankung im fortgeschrittenen Stadium aufzufinden. Aus diesem Grund ist nach Abschluss des ersten Trimesters eine leitliniengerechte Chemotherapie indiziert, welche analog zur Behandlung von nicht-schwangeren Frauen erfolgen sollte.

Die Arbeit zielte darauf ab, die wechselseitige Beeinflussung von schwangerschaftsassoziierten physiologischen Veränderungen und Mammakarzinomerkrankung zu analysieren. Dabei sollte die Plazenta als essentielles Organ während der Schwangerschaft im Mittelpunkt der Untersuchungen stehen, welche sowohl als Einflussfaktor auf Mammakarzinomzellen untersucht als auch als Grundlage für tierversuchsfreie Modelle genutzt wurde. Weiterhin wurden andere *in vitro* Modelle etabliert, die zur Untersuchung des schwangerschaftsassoziierten Mammakarzinoms dienten.

Für das experimentelle Setup wurden drei verschiedene Mammakarzinomzelllinien (MCF-7, MDA-MB-231 und SK-BR-3) verwendet, welche die unterschiedlichen Tumorprofile, wie sie auch in der klinischen Situation vorkommen, widerspiegeln. Für eine realistische Simulation der *in vivo* Tumoren, wurden die normalerweise adhärent wachsende Tumorzellen zu Sphäroiden geformt. Insofern die Sphäroidgeneration erfolgreich verlief, erfolgte eine Co-Kultivierung mit Plazenta-Explantaten und anschließend die Analyse deren Interaktions- sowie Markerexpressionsverhalten. Außerdem dienten die Plazenta-Explantate zur Konditionierung von Zellkulturmedien, welche nachfolgend für die Inkubation der Mammakarzinomzellen genutzt wurden. Die konditionierten Tumorzellen wurden zellmorphologisch und auf Zellviabilität, -proliferation, Invasion und Rezeptorexpression untersucht.

Während des Versuches einheitlich geformte Sphäroide zu generieren, wurde deutlich, dass sich die getesteten Zelllinien in ihrer Fähigkeit kompakte, runde, sphäroidale Strukturen zu bilden, stark unterscheiden. Aus diesem Grund wurden verschiedene

Methoden und Bedingungen getestet, welche für jede Zelllinie die geeignetste Vorgehensweise erzielen sollten. Die Verwendung von hängenden Tropfen unter Zusatz von 25% Methylcellulose stellte sich als effizienteste Methode in Bezug auf Formierung gleichförmiger Sphäroide, finanzielle Ausgaben und Zeitaufwand dar. Die MCF-7 Zellen formierten unter fast allen Bedingungen gleichförmige Sphäroide. Im Fall von den MDA-MB-231 Zellen konnte nur unter Verwendung der Liquid Overlay Technique und 3,5% Matrigel eine reproduzierbare Sphäroidbildung erreicht werden. Die SK-BR-3 Zellen bildeten unter allen Bedingungen höchstens kompakte Aggregate. Anschließend wurden die MCF-7 Sphäroide mit den Plazenta-Explantaten co-kultiviert. Mikroskopische und histologische Analysen offenbarten eine Durchmischung beider Gewebetypen *in vitro*. Diese Beobachtung konnten bei den plazentaren Mammakarzinom-Metastasen *in vivo* nicht bestätigt werden, stattdessen wurde eine deutliche Abgrenzung innerhalb des intervillösen Raums zwischen den unterschiedlichen Gewebetypen detektiert. Interessanterweise zeigten die plazentaren Mammakarzinom-Metastasen strukturelle Ähnlichkeiten zu den *in vitro* generierten MCF-7 Sphäroiden. Sowohl die co-kultivierten Mammakarzinom-Sphäroide als auch Metastasen dienten zur Markersuche für zirkulierende Tumorzellen im Plazentagewebe. Das plazenta-konditionierte Medium bewirkte in allen Zelltypen unabhängig vom Rezeptorstatus morphologische Veränderungen und eine Zunahme der Invasivität. In den hormon-positiven Mammakarzinomzellen kam es zur Herunterregulierung des Estrogen Rezeptors α. Diese Beobachtung unterstützen epidemiologische Studien, welche ein kurzzeitig erhöhtes Risiko für eine Mammakarzinomerkrankung nach einer Schwangerschaft berichten. Die langfristig protektiven Faktoren, welche das Risiko in späteren Jahren reduzieren, werden unter anderem mit einer Immunisierungsreaktion gegen tumor-spezifische, glykosylierte Mucine (lat. *mucus* ‚Schleim') erklärt. Insgesamt ist der schwangerschaftsassoziierte Einfluss auf das Wachstums- und Invasionsverhalten des Mammakarzinoms bisher wenig untersucht.

Es konnte gezeigt werden, dass die Schwangerschaft als solche, aber auch die Plazenta mit ihren vielfältigen sezernierten Substanzen einen merklichen Einfluss auf die Entwicklung von Tumoren und späteren den Krankheitsverlauf ausüben, wobei molekulare Mechanismen in zukünftigen Studien näher analysiert werden sollten.

3. Einleitung

Mammakarzinom während der Schwangerschaft/ PABC

Das schwangerschaftsassoziierte Mammakarzinom (*pregnancy associated breast cancer*, PABC) wird in populationsbasierten Studien als Tumor in den *Mammae lobuli* (Drüsenläppchen) oder *ductuli* (Milchgängen) definiert, welcher während der Schwangerschaft (Ibrahim et al. 2000) oder innerhalb der Laktationsperiode bis zu 12 Monaten (Ives et al. 2005, Smith et al. 2001, Beadle et al. 2009, Halaska et al. 2009) *post partum* (p.p.) diagnostiziert wird. Studien, welche den Einfluss der Schwangerschaft auf die Prognose analysierten, erweiterten diesen Zeitraum auf zwei (Ishida et al. 1992, Johansson et al. 2011) beziehungsweise zehn Jahre (Bladstrom et al. 2003) *p.p.*. Weiterhin kann die Diagnose des Tumors bis zu 9 Monaten vor einer Schwangerschaft ebenso als schwangerschaftsassoziiert angesehen werden (Reed et al. 2003).

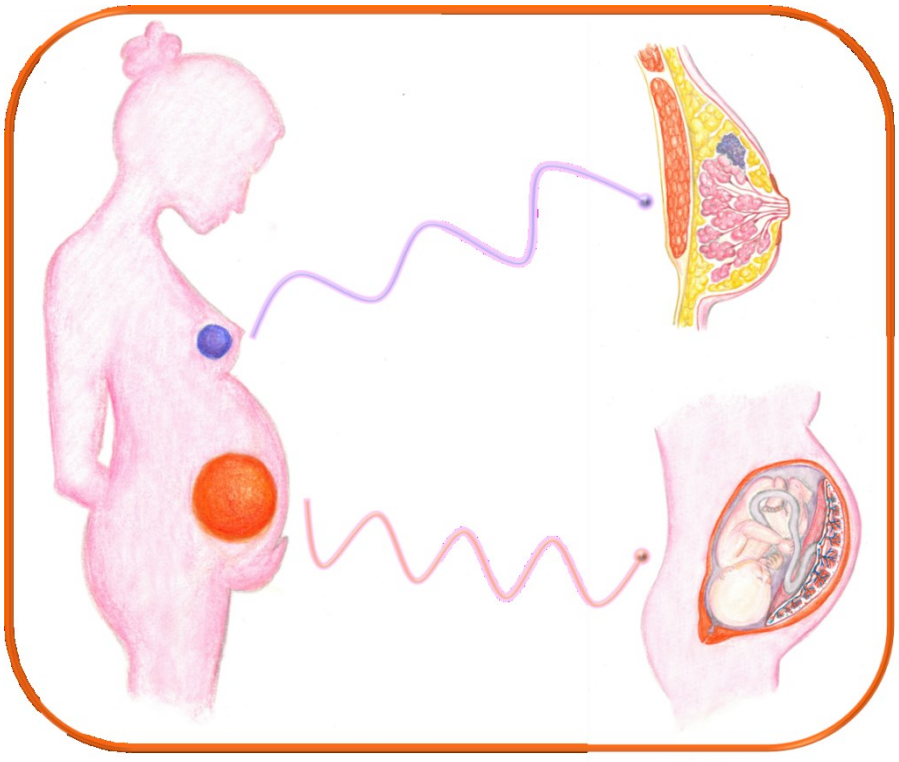


Abbildung 1: Diagnose Mammakarzinom während der Schwangerschaft: Eine seltene Koinzidenz, die sowohl in Bezug auf die Notwendigkeit einer frühzeitigen Diagnose als auch einer adäquaten Behandlung für Mutter und Fetus eine große Herausforderung für Gynäkologen und Onkologen darstellt. Zeichnungen von Edith Montag.

Bei etwa jeder tausendsten Frau wird während der Schwangerschaft eine Tumorerkrankung diagnostiziert (Nulman et al. 2001), dabei zählt das Mammakarzinom zu einer der häufigsten Entitäten. Eine maligne Erkrankung der *Cervix uteri* (Gebärmutterhals) wurde früher öfters diagnostiziert (Haas 1984), wobei sich heutzutage eine sinkende Inzidenz abzeichnet (Van Calsteren et al. 2010), Dies scheint im Zusammenhang mit den eingeführten Impfprogrammen gegen humane Papillomaviren (HPV) und umfangreichen Früherkennungsprogrammen zustehen. Neben diesen Tumoren wurden ebenso Melanome, Leukämie, Geschwüre des Ovars, Hodgkin Lymphome und non-Hodgkin Lymphome während der Schwangerschaft diagnostiziert (Haas 1984, Smith et al. 2001).

Epidemiologie

10-20% aller Mammakarzinomfälle betreffen Frauen im gebärfähigen Alter, wobei Erstgebärende mit einem derzeitigen Durchschnittsalter von 30 Jahren in Deutschland zunehmend älter sind (Kaufmann et al. 2011). Da mit fortgeschrittenem Alter auch das Risiko einer malignen Erkrankung steigt, tritt die Koinzidenz von Schwangerschaft und Krebserkrankungen zunehmend auf. Deshalb rechnet man damit, dass Gynäkologen und Onkologen in Industrieländern in Zukunft häufiger mit dieser schwierigen Situation konfrontiert werden (Loibl 2008, Voulgaris et al. 2011). Trotz alledem bleibt die maligne Erkrankung der *Mamma* während der Schwangerschaft mit einer Inzidenz von 1 zu 3,000 bis 1 zu 10,000 ein verhältnismäßig sehr seltenes Ereignis (Loibl 2009, Loibl 2008). Demgegenüber werden 0,2 – 3,8% aller Mammakarzinome während der Schwangerschaft oder innerhalb der Laktationsperiode diagnostiziert (Woo et al. 2003).

Die Diagnose erfolgt laut Studienlage um 2 bis 15 Monaten verspätet im Vergleich zu nicht schwangeren Frauen. Dies geht mit einem 2,5-fachen Risiko einher den Tumor im fortgeschrittenen Stadium aufzufinden (Woo et al. 2003, Zemlickis et al. 1992). Die Hauptursache scheint dabei zu sein, dass das während der Schwangerschaft oder der Laktationsperiode physiologisch veränderte Brustdrüsengewebe das Ertasten von Gewebeveränderungen erschwert und der behandelnde Arzt infolgedessen wahrscheinlich erst größere Geschwülste diagnostiziert (Gonzalez-Angulo et al. 2005). Darüber hinaus weisen junge Frauen generell dichtes Brustdrüsengewebe auf und werden noch nicht im Rahmen einer routinemäßigen Untersuchung mittels Mammographie erfasst (Polyak 2006).

Tumorcharakteristika

Das Tumorprofil einer schwangeren Brustkrebspatientin unterscheidet sich vom dem einer nicht schwangeren Frau. Die Karzinome weisen häufiger eine Expression des humanen epidermalen Wachstumsfaktorrezeptors (HER2) auf, andererseits wird seltener eine Expression der Hormonrezeptoren Estrogen und Progesteron (ER/ PR) nachgewiesen (Genin et al. 2012, Asztalos et al. 2015). Besonders aggressive Typen des Mammakarzinoms stellen „triple negative“ Tumore (TNBC) dar, welche keine der genannten Rezeptoren exprimieren. Diese Form scheint ebenfalls bei schwangeren Patientinnen häufiger aufzutreten (Asztalos et al. 2015). Gepaart mit der erschwerten Diagnose führt diese Tatsache dazu, dass die Karzinome oft in einem im Vergleich zu nicht schwangeren Frauen fortgeschritteneren Stadium diagnostiziert werden (Woo et al. 2003). Das veränderte Tumorprofil wird mit einem aggressiverem Wachstums- und Invasionsverhalten in Verbindung gebracht (Van den Rul et al. 2011). Jedoch bleibt zu beachten, dass ein ähnlich aggressives Tumorprofil bei nicht-schwangeren prämenopausalen Frauen charakteristisch ist. Daher scheint neben den schwangerschaftsassoziierten Einflüssen ein relativ junges Alter der Frauen einen erheblichen Anteil zur Aggressivität des Mammakarzinoms beizutragen (Puckridge et al. 2003).

Medikamentöse Behandlung der schwangeren Patientinnen

Auch wenn das PABC aufgrund der beschriebenen Charakteristika mit einem erhöhten Risiko für die Patientinnen einherzugehen scheint, wurde bisher kein verschlechtertes Outcome festgestellt, insofern das Behandlungsregime dem von nicht schwangeren Patientinnen entspricht (Cardonick et al. 2010). Die derzeitige Studienlage besagt, dass nach Abschluss der Organogenese, d.h. zu Beginn des zweiten Trimesters, eine Therapie mit Chemotherapeutika indiziert ist. Erste Leitlinien wurden von der *German Breast Group* erstellt. Diese weisen darauf hin, dass die Behandlung der schwangeren Frauen der von nicht schwangeren Patientinnen entsprechen sollen (Loibl et al. 2012). Typische Kombinationsregime sind Anthrazykline mit Cyclophosphamid und/ oder 5-Fluoruracil, wobei der Einsatz von Hormonrezeptorantagonisten sowie monoklonalen Antikörpern, wie der Anti-HER2 Antikörper Trastuzumab, wegen Fallbeschreibungen von fetalen Malformationen und Oligo-/ Anhydramnions strengstens kontraindiziert ist (Berger und Clericuzio 2008, Beale et al. 2009). Wurden diese Therapieempfehlungen eingehalten, konnte bisher kein erhöhtes Risiko für neonatale Malformationen festgestellt werden (Mir et al. 2010). Des Weiteren konnte im Rahmen einer großen

multizentrischen Fall-Kontroll-Studie, welche 129 Kinder einschloss, gezeigt werden, dass sich die Kinder trotz pränataler mütterlicher Chemotherapie unauffällig ohne kardiale oder kognitive Funktionsstörungen entwickelten (Amant et al. 2015). Weiterhin zeigten andere Studien, dass selbst eine vorzeitige Beendigung der Schwangerschaft zu keiner verbesserten maternalen Prognose führte (Cardonick 2014). Dies verdeutlicht, dass eine lebenserhaltene Therapie sowohl für die Mutter als auch für das Kind möglich ist. Insgesamt erfordert diese außergewöhnliche Situation die Zusammenarbeit eines interdisziplinären Teams, bestehend aus Gynäkologen, Perinatologen, Onkologen und Pharmakologen, welche die werdenden Eltern über Therapieentscheidungen und das Vorgehen bei Fortbestehen der Schwangerschaft kompetent beraten (Gupta et al. 2014).

Der Einfluss der Schwangerschaft auf das Mammakarzinomrisiko

Welche Auswirkung hingegen die Schwangerschaft auf die Mammakarzinomentstehung hat, wird in der Literatur konträr diskutiert. Generell fehlt ein umfassendes Verständnis für das Zusammenspiel von Schwangerschaft und der Karzinogenese in der Brust (Amant et al. 2013). P.p. haben die Frauen kurzzeitig ein erhöhtes Risiko ein Mammakarzinom zu entwickeln (Lambe et al. 1994), wobei langfristig ein schützender Effekt durch vorherige Schwangerschaften beschrieben wird (Janerich 2001). Das kurzzeitig erhöhte Risiko könnte als Folge der Immunsuppression während der Schwangerschaft zustande kommen (Polyak 2006). Dieses „kurzzeitig“ erhöhte Risiko kann bis zu 15 Jahre nach der Schwangerschaft anhalten (Lambe et al. 1994, Albrektsen et al. 2005, Chie et al. 2000). Wenn Erstgebärende älter als 30 Jahre sind, so kann diese Zeitspanne sogar auf bis zu 50 Jahre ansteigen (Chie et al. 2000, Lambe et al. 1994, Albrektsen et al. 2005, Liu et al. 2002). Im Gegensatz dazu sind frühausgetragene Schwangerschaften und Multiparität mit einem reduzierten Mammakarzinomrisiko verbunden (Agrawal et al. 1995). Ebenso eine erhöhte Dauer der Laktationsperiode und das Auftreten einer Mastitis während des Stillens haben einen positiven Einfluss auf eine Tumorsuppression (Croce et al. 2001, Cramer et al. 2013, Jerome et al. 1997). Dabei scheint eine Laktationsphase über 24 Monate hinausgehend keinen zusätzlichen Vorteil darzustellen ((Kaufmann et al. 2011) S. 61). Ein protektiver Effekt könnte in diesem Zusammenhang die Inhibition der Estrogensekretion infolge einer Prolaktinausschüttung durch die Adenohypophyse darstellen, da die weiblichen Geschlechtshormone Estrogen und Gestagen per se das Wachstum von Brustkrebszellen fördern ((Kaufmann et al. 2011) S. 37).

Tierversuchsalternativen

Die Verwendung von Tieren unterschiedlicher Spezies, sei für es den Nachweis von hormonell wirksamen Substanzen und Pyrogenen oder für die Entwicklung neuer Therapien zur Bekämpfung von Krankheiten basiert auf einer langen Tradition. Bis heute werden Tierversuche als unverzichtbar angesehen und finden in medizinisch-diagnostischen sowie wissenschaftlichen Bereichen große Anerkennung. Beispielsweise wurden noch bis zum Jahr 1960 für den Nachweis einer Schwangerschaft der Urin einer potentiell schwangeren Frau afrikanischen Krallenfröschen injiziert, die bei vorhandenem humanen Choriongonadotropin (hCG) anfingen zu laichen (S.1208 (Gressner und Arndt 2013)). Heutzutage werden hingegen standardmäßig Enzyme-Linked Immunosorbent Assays (ELISA) für den Nachweis von hCG eingesetzt. Im Allgemeinen gibt es in der heutigen Zeit Diskussionen über die Grenzen der Übertragbarkeit experimenteller Ergebnisse von Tier auf Mensch. Auch ethische und tierschutzrechtliche Debatten sind vielerorts eingekehrt, die das Ersetzen der gut etablierten altgebräuchlichen Methode fordern. Obwohl die Limitationen der Tiermodelle auch in jüngsten klinischen Studien erneut offenbart wurden (Hartung 2013), wird noch heute die Forschung an Versuchstieren als Goldstandard angesehen und ermöglicht gute Aussichten für die Publikation der Ergebnisse in hochgerankten Journalen.

Das 3R-Konzept

Diese Sichtweise bzw. Herangehensweise kann nur verändert werden, wenn gute und vielversprechende Alternativen zur Verfügung stehen. Dabei wird das von den britischen Forschern William Russell und Rex Burch 1959 entworfene 3R-Konzept, welches sie in ihrer Publikation "The Principles of Humane Experimental Techniques" formulierten, als Richtlinie für die Alternativenentwicklung angesehen (Russell et al. 1959). Das erste R steht für *Replacement* (Ersatz) und hat zum Ziel, gängige Tierversuche durch eine tierversuchsfreie Methode zu ersetzen. Ist dies nicht möglich, greifen in diesem Konzept *Reduction* (die Reduktion) und *Refinement* (die Verfeinerung), was bedeutet, dass zu einem die Zahl der Tierversuche auf ein Minimum beschränkt und zum anderen bei jedem Experiment die maximale Datenmenge ausgeschöpft und das Leiden der Tiere durch entsprechende Versuchsbedingungen möglichst gering gehalten werden sollen. Auch wenn die zwei zuletzt aufgeführten Punkte *Reduction* und *Refinement* aus ethischen Gründen

nachvollziehbar sind, verhelfen diese zu keiner verbesserten wissenschaftlichen Praxis im Sinne einer Übertragbarkeit auf humane Gegebenheiten. Die unter anderem unterschiedliche genetische Ausstattung der in Forschungslaboren häufig eingesetzten Nagern kann völlig andere Auswirkungen von den getesteten Substanzen bedingen, die in den letzten Jahren ursächlich für fatale Folgen in klinischen Studien waren.

Verfehlter Einsatz des monoklonalen Antikörpers TGN1412

Der agonistische monoklonale Antikörper TGN1412, welcher ursprünglich durch Bindung des CD28 Antigens auf T-Zellen zur Behandlung von Autoimmunerkrankungen und Leukämie vorgesehen war, führte trotz unauffälliger präklinischer Tests bei allen Probanden zu schwerwiegenden unerwünschten Ereignissen (starke Entzündungsreaktionen bis multiples Organversagen). Grund für diese Ereignisse war ein Zytokinsturm, der durch den Antikörper ausgelöst wurde und eingangs in präklinischen Studien bei Mäusen und Makaken nicht beobachtet wurde (Stebbings et al. 2007). Die getesteten Spezies exprimieren im Gegensatz zum Menschen CD28 nicht auf CD4+ T-Gedächtniszellen und somit konnten die negativen Auswirkungen von TGN1412 in den klinischen Studien nicht verhindert werden (Schraven und Kalinke 2008, Eastwood et al. 2010).

Strategien zur Ablösung von Tiermodellen

Solche gravierenden Folgen lassen sich nur durch die Umkehr gewohnter Studienpläne unter Abschaffung der traditionellen Tiermodelle vermeiden. Darüber hinaus bieten tierfreie Test- und Forschungsmethoden weitere Vorteile, wie beispielsweise eine hohe Reproduzierbarkeit nach erfolgreicher Etablierung der entsprechenden *in vitro* Methode. Der Kosten- und Zeitfaktor ist im Wesentlichen beim Arbeiten mit Gewebs- und Zellkulturen positiver zu beurteilen, die Kosten werden geringer eingeschätzt und die Ergebnisse können meist nach wenigen Stunden ausgewertet werden. Erst der Einsatz von *in vitro* Methoden ermöglicht die Anwendung von sogenannten High-Throughput-Screenings, bei denen gleichzeitig verschiedene Substanzen auf erwünschte beziehungsweise unerwünschte Wirkungen analysiert werden können (Kunz-Schughart et al. 2004). Insofern beachtet wird, dass diese Modelle nur unter Verwendung von humanen Material etabliert werden, kommt der weitaus wichtigste Vorteil zu tragen: Bei dieser Art von Experimenten werden nur human-spezifische Effekte analysiert (Fitzgerald et al. 2015). Dabei stellen

permanente Zellkulturen, die adhärent oder suspensionsartig in Zellkulturflaschen wachsen, ein viel verwendetes Modell dar, welches zur Testung verschiedener Charakteristika verwendet werden kann. Insofern diese Zellkulturen ausschließlich zweidimensional (2D) wachsen ist die Übertragbarkeit auf Geweben oder Organen eingeschränkt, da sie die tatsächlichen *in vivo* Bedingungen nicht wiederspiegeln (Schmidt et al. 2015).

Entwicklung komplexer Gewebekulturmodelle

Aus diesem Grund wurden in den letzten Jahren zahlreiche Forschungsprojekte initiiert, deren Ziel es ist, komplexe Gewebeverbände aus humanem Material unter Zuhilfenahme moderner Technologien herzustellen. Die Organ-on-a-Chip Technologie stellt dabei eine vielversprechende Methode dar, welche in der Grundlagenforschung sowie in der pharmazeutischen Industrie bereits für die Testung von Hautpflegeprodukten Anwendung findet (Alepee et al. 2014). Forscher arbeiten derzeit sogar an der Entwicklung von komplexeren Modellen, die bis zu 4 Organe auf einem Chip simulieren sollen (Maschmeyer et al. 2015). Neben den ersten Chips, die Haut, Leber, Lunge oder Niere nachahmen, wurden kürzlich die Ergebnisse von einem neuen Brain-on-a-Chip-Modell publiziert. Die Wissenschaftler brachten Amygdala, Hippocampus, präfrontalen Kortex und deren Nervenzellverbindungen auf einen solchen Chip, welcher für die Erforschung von Pathogenitätsmechanismen schizophrener oder anderen psychisch erkrankter Patienten dienen soll (Dauth et al. 2016). In Zusammenhang mit der Chip Technologie ist auch das dreidimensionale Drucken von Zellen eine oft angewandte Methode. Bei neusten Studien konnten mit diesem Verfahren neben dem neu entstehenden Gewebe zusätzlich Sensoren auf die Chips gedruckt werden. Diese Sensoren sollen zur Analyse von Arzneimittelmetabolismus und Kontraktilitätsmessung von Zellen verwendet werden (Lind et al. 2016). Neben dieser Methode gibt es andere Ansätze, die darauf abzielen die Komplexität von Organen zu simulieren. Beispielsweise gewann ein humanes dreidimensionales (3D) Hepatozyten Modell HμREL® viel Beachtung, welches für Toxizitätstests oder Biotransformationsstudien Verwendung finden soll (Novik et al. 2010). Eine ebenso erwähnenswerte Methode ist die Testung von potentiell embryotoxischen Substanzen an pluripotenten Stammzellen, welche zuvor aus Hautzellen gewonnen wurden und aufgrund embryonalähnlicher Eigenschaften für diesen Zweck verwendet werden können (Baumann et al. 2014, Baumann et al. 2016).

Serumzusätze in Zellkulturmedien

Wenn auch die beschriebenen Strategien auf Grundlage von immortalisierten oder primären Zellkulturen interessante Alternativen darstellen, verbirgt sich auch hier eine Einflussquelle jenseits humanen Ursprungs. Fetales Kälberserum (FKS) wird routinemäßig allen Kulturmedien zugesetzt und dient aufgrund seiner Zusammensetzung aus pH-stabilisierenden und wachstumsstimulierenden Faktoren wie dem epidermalen Wachstumsfaktor (EGF), Hormonen wie Parathormon, Thyroxin oder Progesteron, Vitaminen und Proteaseinhibitoren zur Zellproliferation und –differenzierung (Jayme et al. 1997, Jayme et al. 1988). Abgesehen von der tierschutzrechtlich bedenklichen Gewinnung über Herzpunktion des noch im Uterus befindlichen Kalbes wird der Einsatz von FKS hinsichtlich des Interaktionspotenzials mit humanen Zellen und potentieller Kontaminationen, bspw. mit Mykoplasmen, schon länger kontrovers diskutiert. Obwohl der Einsatz nicht mehr zeitgemäß erscheint, wird es weiterhin in der Grundlagenforschung routinemäßig verwendet (Gstraunthaler et al. 2013). Tatsächlich gibt es Bemühungen alternative Zellkulturzusätze zu finden oder ganz und gar auf chemisch definierte serumfreie Medien umzusteigen, jedoch scheinen viele Labore unter anderem aus finanziellen Gründen sich vor einem Wechsel zu scheuen. Ein weiterer Grund wird in dem hohen zeitlichen Aufwand gesehen, der notwendig ist um etablierte Zelllinien mit neuen Medien oder Zusätzen in ihren Verhaltenseigenschaften zu charakterisieren (Zähringer 2015). Dabei steigen die Preise für FKS unter anderem wegen zunehmender Monopolisierung der Serum-Industrie, was schließlich im Jahr 2013 in einem publik gewordenen Skandal um gefälschte Kälberseren mündete (Köppelle 2013). Erst 2015 kam es zu einem erneuten Vorfall, mehrere Unternehmen wurden verdächtigt über mehrere Jahre die Seren mit falschen Herkunftangaben vertrieben zu haben (Balser et al. 2015). Auch diese Vorkommnisse sollten dazu führen, dass geeignete Alternativen zu FKS in den Forschungslaboren etabliert und verwendet werden. Eine Vorreiterrolle wird hier den Stammzellforschern zugeschrieben, die beispielsweise für die Kultivierung von humanen mesenchymalen Stromazellen (MCS) Thrombozyten-Lysat (hPL-1) benutzen (Rauch et al. 2011). Nicht zuletzt stellt die Verwendung von humanen Serum (HS) ebenso eine vielsprechende Alternative dar. Zum einem gibt es die Sicherheit, dass nur human-spezifische Serum-Zell-Interaktionen auftreten und zum anderen ist dessen Gewinnung einfach praktizierbar und ohne Tierleid verbunden (Kocaoemer et al. 2007). Bei routinemäßigen Einsatz bleibt jedoch zu beachten, dass große Mengen

an HS, die nur schwer ohne Weiteres zur Verfügung gestellt werden könnten, benötigt würden.

Einsatz von multizellulären Tumorsphäroiden in der Tumorforschung

In präklinischen Studien werden Substanzen für die mögliche Anwendung als Arzneimittel häufig an den bereits erwähnten immortalisierten Zellkulturen getestet. Ziel bei diesem Vorgehen ist den geeignetsten Wirkstoff für die anschließenden *in vivo* Studien auskundig zu machen. Problematisch dabei ist, dass solche *in vitro* Untersuchungen heutzutage noch schätzungsweise um mehr als 70% an 2D Zellkulturen stattfinden (Hutmacher 2010). Diese Tatsache wird häufig als möglicher Grund für die hohe Fehlerquote in der klinischen Anwendung der zuvor im Forschungslabor erfolgreich getesteten Substanzen gesehen. Aufgrund der 2D Kultivierung der Zellen ist es nicht möglich die *in vivo* Bedingungen adäquat zu simulieren. Weder die Komplexität eines 3D Gewebsverbandes noch die zahlreichen Zell-Zell Interaktionen (Desrochers et al. 2014) und die physiologisch vorkommende Zellpolarität (Fitzgerald et al. 2015) wird durch diese *in vitro* Kultivierungsmethode erreicht. Ebenso fehlt die Ausbildung einer extrazellulären Matrix (ECM), die im *in vivo* Zellverband wichtige Funktionen, wie die Aufrechterhaltung von Zelldifferentiation, Wachstum und Zellhomöostase, erfüllt (Chitcholtan et al. 2013). Hinzu kommt, dass sich bei soliden Tumoren oder Mikrometastasen typischerweise avaskuläre Regionen ausbilden, da der unkontrollierten fortschreitenden Proliferation maligner Zellen keine ausreichende Angiogenese entgegen wirken kann. Dadurch kommt es zur Entstehung charakteristischer Nährstoff-, Sauerstoff- und pH-Gradienten (Friedrich et al. 2007) und in Folge dessen bilden sich verschiedenartig versorgte Zonen in dem Gewebe aus, welche in histologischen Analysen an proliferationsstarken und nekrotischen bzw. apoptotischen Regionen erkennbar sind (Proskuryakov und Gabai 2010, Mueller-Klieser 2000). Deshalb stehen Kliniker häufig vor dem Dilemma, über keine effiziente Chemotherapie zur Tumorbehandlung zu verfügen, da diese hypoxischen Zellen weniger stark von den proliferationshemmenden Zytostatika gehemmt werden und somit ein höheres Risiko besteht, dass es zur Resistenzentwicklung kommt (Hatok et al. 2009).

Komposition der multizellulären Tumorsphäroide

Zur Reflexion dieser komplexen Zell- und Gewebscharakteristika werden schon seit vier Jahrzehnten multizelluläre Tumorsphäroide (MCTS, im folgendem auch als

Sphäroide benannt) als geeignetes Modell in der Tumorforschung diskutiert. Der Radiobiologe Sutherland entwickelte in den frühen 70er Jahren die Kultivierung von MCTS und untersuchte dabei die Wirkung radioaktiver Strahlung auf maligne Zellen (Sutherland et al. 1971, Durand und Sutherland 1976). Weiterhin beschrieb er in seinen Publikationen noch heute geltende charakteristische Gemeinsamkeiten zwischen Tumoren *in vivo* und den MCTS *in vitro*: Es kommt in Sphäroiden zur Ausbildung heterologer Zellpopulationen in konzentrischer Anordnung; die proliferierende Randzone setzt sich nach innen fort mit einer Schicht ruhender und hypoxischer Zellen und das Zentrum des MCTS wird durch einen nekrotischen Kern charakterisiert. Der ebenso in avaskulären Tumorregionen beschriebene pathophysiologische Gradient konnte auch in diesen Zellmodellen nachgewiesen werden (Casciari et al. 1988, Mueller-Klieser 1987).

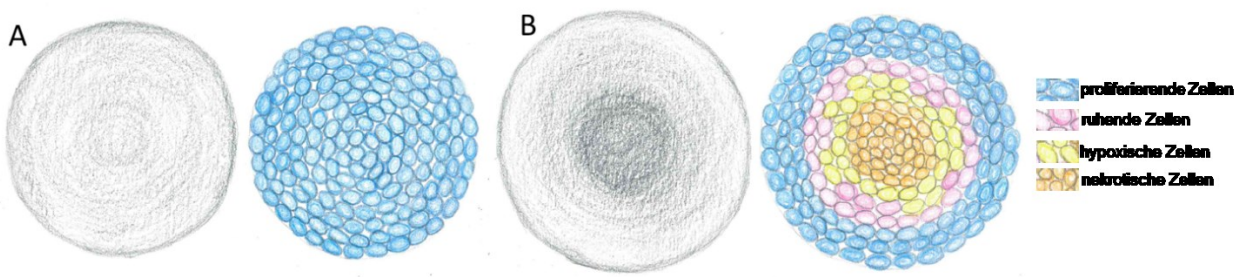


Abbildung 2: Aufbau von Sphäroiden mittels schematischer Zeichnung der Charakteristika im Lichtmikroskop (links) und nach immunhistochemischen (IHC, rechts) Analysen. A Homogene sphäroidale Struktur mit gleichmäßigem Vorkommen von proliferierenden Zellen (blau) direkt nach der Generation. B Einen ersten Hinweis auf die Stratifizierung der Sphäroide liefert die dunkle Färbung im Zentrum einer lichtmikroskopischen Aufnahme. Im Inneren weisen die MCTS eine typische Schichtung in proliferierende (blau), ruhende (rosa), hypoxische (gelb) und nekrotische (orange) Zonen auf. Hypoxische und ruhende Zellen können in allen inneren Zonen und auch in der nekrotischen Kernregion detektiert werden (Zhang et al. 2014, Zhang et al. 2015b, Ohnishi et al. 2014).

Nach diesen ersten Pionierarbeiten ging das Interesse an dem Sphäroidmodell zunächst zurück, kam es in den letzten 15 Jahren zu einem exponentiellen Anstieg Sphäroid-basierender Veröffentlichungen, weshalb mit einem Paradigmenwechsel in Richtung 3D Kultivierung zu rechnen ist (Leong und Ng 2014).

Verschiedenartige Nomenklatur der geformten Strukturen

Möglicherweise hatte dies eine uneinheitliche Nomenklatur von MCTS zur Folge, was bei der Identifikation der geformten Strukturen oder auch im wissenschaftlichen Austausch zu einigen Missverständnissen führen kann (Weiswald et al. 2015). So findet man in aktuellen Publikationen Bezeichnungen wie *spheroids, tumoroids, mixed*

spheroids, nodules, heterospheroids und *organoids* (Für eine eindeutige Terminologie erfolgt die Bezeichnung in englischer Publikationssprache). Des Weiteren fehlt trotz der hohen Anzahl an Publikationen eine exakte Beschreibung von MCTS (Nagelkerke et al. 2013). Aus diesem Grund sollten bei der Bezeichnung Sphäroid folgende Charakteristika nachgewiesen werden: 3D Wachstum, runde Form, hohe Kompaktheit, einen abgeschlossenen, glatten Rand und eine gleichmäßige Oberfläche (Froehlich et al. 2016). Für eine eindeutige Nomenklatur ist es darüber hinaus sinnvoll, wenn andere geformte Strukturen folgendermaßen nach ihrer Gestalt klassifiziert werden:

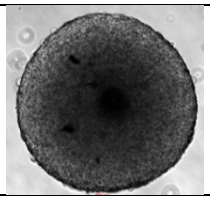
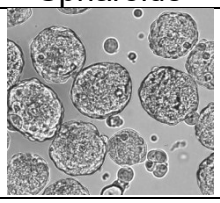
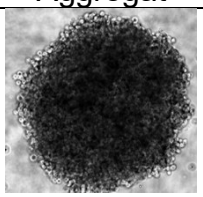
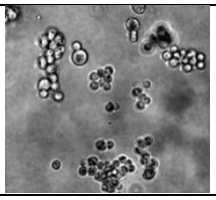
1 Sphäroid	2 kleine Sphäroide	3 kompaktes Aggregat	4 loses Aggregat	5 vereinzelte Zellen
				

Abbildung 3: Klassifikation geformter Strukturen (Froehlich et al. 2016).

Methoden zur Generierung

Die heutzutage eingesetzten Methoden zur Generierung von Sphäroiden wurden in ihren Grundzügen bereits von ihren Erfindern geprägt. Dabei werden zwei unterschiedliche Prinzipien verfolgt: Zu einem kann die Formierung durch bewegende, rotierende Systeme oder auch durch stationäre Zellkulturtechnologien initiiert werden (Friedrich et al. 2007). Während Spinnerflaschen für die Herstellung von Sphäroiden im Großmaßstab dienen, gibt es für kleinere experimentelle Ansätze vielverwendete stationäre Systeme. Zum Beispiel ist es möglich die Sphäroidbildung durch Kultivierung der Zellen im hängenden Tropfen zu induzieren. Dabei werden die 20-30 µl großen Tropfen zunächst auf einem Petrischalendeckel pipettiert und durch anschließende Inversion des Deckels für 1-3 Tage hängend kultiviert (Kelm et al. 2003). Insgesamt weist diese Art der Kultivierung viele Vorteile auf, wie geringer Kosteneinsatz, einfache Handhabung und hohe Reproduzierbarkeit, welche für toxikologische Anschlussexperimente von Bedeutung sind (Kelm et al. 2003). Die Liquid Overlay Technik, welche von Ivascu und Kubbies entwickelt wurde, fand gerade in den letzten Jahren unter vielen Wissenschaftlern große Beachtung (Ivascu und Kubbies 2006). Bei dieser Methode werden die Zellen in einer 96-Wellplatte kultiviert. Aufgrund spezieller Beschichtungen der Böden wird ein Anwachsen der Zellen verhindert und die Sphäroidbildung induziert. Mittlerweile existieren verschiedene Variationen der 96-Wellplatten, die je nach Eigenschaften und Verhalten der Zelltypen

eine verbesserte Generierung der Sphäroide anstoßen soll. Wenn zum Beispiel V-geformte (Nagelkerke et al. 2013) oder U-geformte (Ivascu und Kubbies 2006) Wells verwendet werden, kann die Zellsuspension in die unten gelegene Verengung des Wells zentrifugiert werden, was wiederum eine stärkere mechanische Aggregation der Zellen bewirken soll. Bei Verwendung von geraden F-Wells ist dies nicht möglich (Metzger et al. 2011). Für das Verhindern des Anwachsens der Zellsuspension werden auch unterschiedliche Strategien verfolgt. Entweder werden die Wellplatten mit Agarose (Charoen et al. 2014, Costa et al. 2014, Metzger et al. 2011, Friedrich et al. 2009) oder mit poly-HEMA (Poly(2-hydroxyethyl methacrylate)) (Ivascu und Kubbies 2006) vor dem Kultivieren der Zellen beschichtet, sodass eine nicht adhärente Oberfläche geschaffen werden kann. Zusätzlich bieten Firmen speziell beschichtete 96-Wellplatten für diesen Zweck an (Greiner Bio-One, Thermo Fisher Scientific). Insofern auf diese nicht-adhärierenden Wellplatten zurückgegriffen werden muss, ist jedoch mit einem wesentlich höheren Kostenaufwand im Vergleich zur Verwendung der *Hanging Drop* Methode zu rechnen. Eine andere kostengünstige Methode stellt die Suspensionskultur dar. Bei dieser werden die Zellen in herkömmlichen beziehungsweise nicht-adhärierende Petrischalen kultiviert (Froehlich et al. 2016). Um den Prozess der Formierung zu begünstigen, können die Kulturen leicht geschüttelt werden. Wenn auch hier eine Produktion im Großmaßstab möglich wäre, schließen die variable Größe und Morphologie der entstehenden Sphäroide eine Verwendung für weiterführende Versuche häufig aus (Breslin und O'Driscoll 2013). Neben unterschiedlichen Techniken ist es möglich durch Variationen von Medien, Medienzusätzen oder der Zelldichte die Sphäroidgenerationsfähigkeit der jeweiligen Zelltypen zu modulieren. Einen besonders positiven Effekt konnte dabei durch Viskositätssteigerung des Kultivierungsmediums mittels Methylcellulose (Korff und Augustin 1998) oder Matrigel® (Ivascu und Kubbies 2006) erzielt werden.

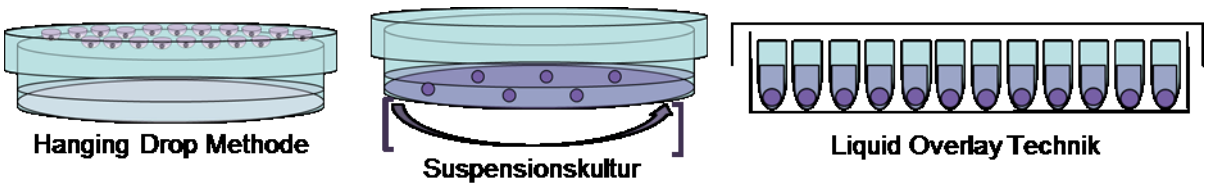


Abbildung 4: Methoden zur Generierung von Sphäroiden

Charakterisierung mit Hilfe unterschiedlicher Analysemethoden

Je nachdem, für welche Untersuchungen die geformten Sphäroide später benutzt werden, können verschiedene Kriterien berücksichtigt werden. Sphäroide mit einer

durchschnittlichen Größe von 500 µm entwickeln den bereits beschriebenen geschichteten Aufbau nach wenigen Tagen und stellen somit ein geeignetes Tumormodell dar (Timmins et al. 2004, Sutherland et al. 1986, Lin und Chang 2008). Sphäroide mit kleineren Durchmessern von 200 µm entwickeln diese unterschiedlichen Regionen nicht oder erst nach längerer Kultivierungsperiode (Kim et al. 2011, Grimes et al. 2014). Nach Bildung steht eine Vielzahl verschiedener Methoden für die Analyse der Sphäroide zur Verfügung. Des Weiteren ist es möglich, Sphäroide in unterschiedlichen Zustandsformen, wie in der Zelllebendkultur, eingefroren, in Paraffin eingebettet oder auch in dissoziierter Form, zu untersuchen (Kunz-Schughart et al. 1998). Auch molekulare Techniken wie Protein-, RNA- und DNA-Untersuchungen können ohne Weiteres auf Sphäroide angewendet werden. Die technisch einfache Analyse von Sphäroidvolumen und –durchmesser mit Hilfe von mikroskopischer Methoden wird immer noch häufig zur Analyse von proliferationsfördernden bzw. –hemmenden Effekten verwendet (Friedrich et al. 2007).

Veränderte Wirkung von Medikamenten an MCTS

Die bereits beschriebene Chemoresistenz gegenüber Zytostatika konnte auch in MCTS nachsimuliert werden (Fitzgerald et al. 2015). Neben der limitierten Diffusion der Wirkstoffe in den MCTS geht man davon aus, dass die Hochregulierung des Multidrug-Resistance-Proteins, die aufgrund hypoxischer Bedingungen im Inneren des Tumors bzw. Sphäroids stattfindet, die Wirkung der Medikamente behindert wird (Xu et al. 2014). Bisherige Forschungsergebnisse bestätigen, dass MCTS mit einer anderen Wirkstoffsensitivität – und Toxizität reagieren als 2D Kulturen. Demzufolge sehen viele Wissenschaftler die unterschiedliche Reaktion der 2D oder 3D Modelle bezogen auf die Chemosensitivität als Ursache für die hohen Fehlerraten bei Wirkstoffstudien (Fitzgerald et al. 2015). Beispielsweise entwickelten Ovarialkarzinomzellen in der Sphäroidkultur eine Paclitaxel spezifische Resistenz, während dies bei den 2D Experimenten ausblieb (Frankel et al. 1997). Die Wirkung des monoklonalen Antikörpers Trastuzumab, welcher an den HER2 bindet und damit die Proliferation von Mammakarzinomzellen inhibiert, wurde unter Verwendung des MCTS Modells sensitiver (Pickl und Ries 2009). Darüber hinaus wurden MCTS bereits in vielen anderen Sachverhalten getestet, wie Tumormetabolismus, Penetration von Adenoviren und die Wirkung mechanischen Stresses auf Tumorzellen (Review siehe (Weiswald et al. 2015)).

Perspektivischer Einsatz in der molekularbiologischen Forschung

Auch wenn die Vorteile des MCTS-Modells bei verschiedenen Sachverhalten ausreichend bekannt sind und vielfältige Applikationen eine große Zahl von Interessenten rekrutiert, scheint im Moment sein Potential nicht vollständig ausgeschöpft werden zu können. Eine der Hauptlimitationen ist dabei, dass nicht alle Zelltypen 3D Sphäroide formen und eher Aggregate wechselhafter Gestalt entstehen (Mueller-Klieser 2000). Auch wenn eine Vielzahl von Methoden bekannt sind, fehlen meist Zelltyp-bezogene technische Informationen (Rotin et al. 1986). Aus diesem Grund sind für die Generation einheitlicher geformter MCTS optimierte und standardisierte Protokolle notwendig, die dann für die jeweiligen Zelllinien spezifisch angepasst werden (Froehlich et al. 2016). Somit könnte die Vergleichbarkeit von Ergebnissen aus unterschiedlichen Forschungslaboren sichergestellt werden.

Simulation des schwangerschaftsassoziierten Mammakarzinoms

In der Tumorforschung werden zur realistischen Reflexion der *in vivo* Bedingungen Zelllinien verwendet mit dem zu untersuchenden Krankheitsbild ähnlichen Charakteristika. Zur Simulation des PABC-Tumorprofils sind folgende Mammakarzinomzelllinien für *in vitro* Untersuchungen geeignet: MCF-7, SK-BR-3 und MDA-MB-231. Die Michigan Cancer Foundation – 7 (MCF-7) Zellen wurden 1970 aus dem Brustgewebe einer kaukasischen 69-jährigen Frau isoliert und 1973 durch Soule und Kollegen als Zelllinie etabliert (Soule et al. 1973). Die Zellen sind ER/ PR positiv und HER2 negativ und werden zu den gut-differenzierten und nicht-invasiven (WDNI) Karzinomzellen gezählt. Die WDNI SK-BR-3 Zelllinie ist im Gegensatz zu der MCF-7 Zelllinie ER und PR negativ und HER2 positiv. Die Zellen wurden 1970 von dem Memorial Sloan-Kettering Cancer Center von einer 43-jährigen kaukasischen Frau isoliert (Trempe 1976). Die dreifach negative Mammakarzinomzelllinie MDA-MB-231 zeichnet sich durch ein besonders invasives Wachstum aus. Die Zellen wurden von einer 51-jährigen kaukasischen Frau im Jahr 1973 gewonnen (Cailleau et al. 1974, Brinkley et al. 1980).

Plazenta als Tierversuchsersatzmodell

Neben *in vitro* Methoden stellen auch *ex vivo* Modelle interessante tierversuchsfreie Ansätze dar, die Verwendung der Plazenta, ist ebenso aus ethischer Perspektive als sehr geeignetes Forschungsmodell anzusehen. Wie Gewebeproben, Nabelschnüre oder Material von Verstorbenen ist die Plazenta heutzutage meist ein Abfallprodukt

und kann mit Zustimmung der Patientin ohne Weiteres benutzt werden. Nur wenige Patientinnen sehen eine Eigenverwendung, beispielsweise für homöopathische Zubereitungen oder zur Plazentophagie, vor und somit sind viele bereit durch ihre Spende einen Beitrag zur Wissenschaft leisten zu können. Zustimmungen von Ethik-Komitees existieren generell weltweit (Halkoaho et al. 2010).

Entwicklung: Von der Befruchtung zur Implantation

Die während der Embryogenese angelegten Follikel reifen im Zeitraum der Menarche unter Stimulation des von der Hypophyse ausgeschütteten follikelstimulierenden Hormons (FSH), zu Graaf-Follikeln heran. Die Ovulation wird letztlich durch einen Anstieg des luteinisierenden Hormons (LH) in der Zyklusmitte ausgelöst. Nachfolgend wird die Oozyte aus dem Follikel freigegeben und gelangt über die Fimbrien in die *Tuba uterina*, wo letztendlich die Befruchtung stattfindet. Nach der Verschmelzung der beiden haploiden Vorkerne entsteht eine diploide Zygote, die sich in Folge von wiederholten mitotischen Teilungsvorgängen über 2-, 4-, 8-Zell- und Morula-Stadien zur Blastozyste entwickelt. Die Implantation der Blastozyste in den *Corpus uteri* findet am 6. Tag *post conceptionem* (p.c) statt. Die Blastozyste besteht aus Trophoblastzellen und der inneren Zellmasse, dem Embryoblasten. Die Anheftung an das Endometrium wird durch das invasive Wachstumsverhalten des Trophoblasten initiiert. Bei diesem Vorgang beginnen die Trophoblastzellen zu proliferieren und differenzieren sich in eine innere Zellschicht, den Zytotrophoblasten (Langhans-Zellen) und eine äußere synzytiale Zellschicht, den Synzytiotrophoblasten ((Moore et al. 2007) S.45). Die Zottenbildung beginnt mit der Formierung von Primärzotten zwischen dem 13. und 15. Tag p.c.. Die Primärzotten sprossen seitlich aus den Trophoblasttrabekeln in das maternale Endometrium und erodieren maternale Kapillaren, sodass Trophoblasten-Lakunen entstehen. Durch weiteres Wachstum dringen immer neue Primärzotten in das Lakunensystem ein. Die Umwandlung der Primärzotten in Sekundärzotten mit zentraler Stromachse beginnt etwa am 15.-16. Tag p.c.. Zum Zeitpunkt der Sekundärzottenentwicklung wird aus dem embryonalen Mesoderm der Haftstiel gebildet. Es erfolgt die Differenzierung der ersten Blutgefäße. Am 17.-18. Tag p.c. erreichen die Haftstielgefäße die Chorionplatte (Vogel 1996). Die Trophoblasten-Lakunen reifen durch die Aussprossung der tertiären Zotten zum intervillösen Raum aus ((Welsch 2005) S.525). Ab der 10. Schwangerschaftswoche (SSW) bilden sich die Chorionzotten am abembryonalen Pol (zum Uteruslumen gerichtet) zurück. Diesen Teil bezeichnet man als *Chorion laeve*. Am embryonalen Pol (zur Uteruswand

gerichtet) vergrößern und verzweigen sich die Zotten weiter. Diese Seite wird als *Chorion frondosum* bezeichnet und stellt den eigentlich Ursprung der Plazenta dar ((Welsch 2005) S.525). Aus dem rupturierten Follikel bildet sich das *Corpus luteum*, welches durch die Sekretion von Progesteron das Endometrium in dessen Proliferationsphase stimuliert, sodass die Implantation erfolgreich stattfinden kann. Im Anschluss beginnt die Produktion von humanen Choriongonadotropin (hCG) durch invasive Trophoblasten. Nun kommt es durch die hCG getriggerte Stimulation des *Corpus luteum menstruationes* zur Sekretion von Progesteron und Estrogen, die im Laufe der Schwangerschaft wichtig für Reifung und Entfaltung der Mammae lobuli sind. Bei Fortbestehen der Schwangerschaft geht das *Corpus luteum menstruationes* unter und die Produktion dieser Hormone wird ab der 9. SSW von der Plazenta übernommen ((Kaufmann et al. 2011) S. 33-35).

Anatomie und Funktionen der reifen Plazenta

Zwischen der 13. und 14. SSW nach der Abgrenzung des *Chorion leave* vom *Chorion frondosum* ist die vollständige Plazenta ausgebildet, die infolge von Reifungsprozessen in den nachfolgenden SSW an Größe und Gewicht zunimmt. Nach vollständiger und gesunder, normaler Entwicklung wiegt dieses scheibenförmige Organ im Durchschnitt 600g (Bruttogewicht) zur 40. SSW. Das Nettogewicht, welches ohne Nabelschnur und Eihaut bestimmt wird, beträgt ca. 500 g (Vogel 1996). Die Plazenta bildet die Grenze zwischen dem fetalen und mütterlichen Organismus und wird aus folgenden Anteilen gebildet: Eine äußere bindegewebige Chorionplatte, der das Amnionepithel anliegt und an die die Amnionhöhle angrenzt, das Plazentaparenchym, welches durch die Chorionzotten und den intervillösen Raum gebildet wird, und die überwiegende maternale, deziduale Basalplatte, die an das Myometrium des *Corpus uteri* anschließt.

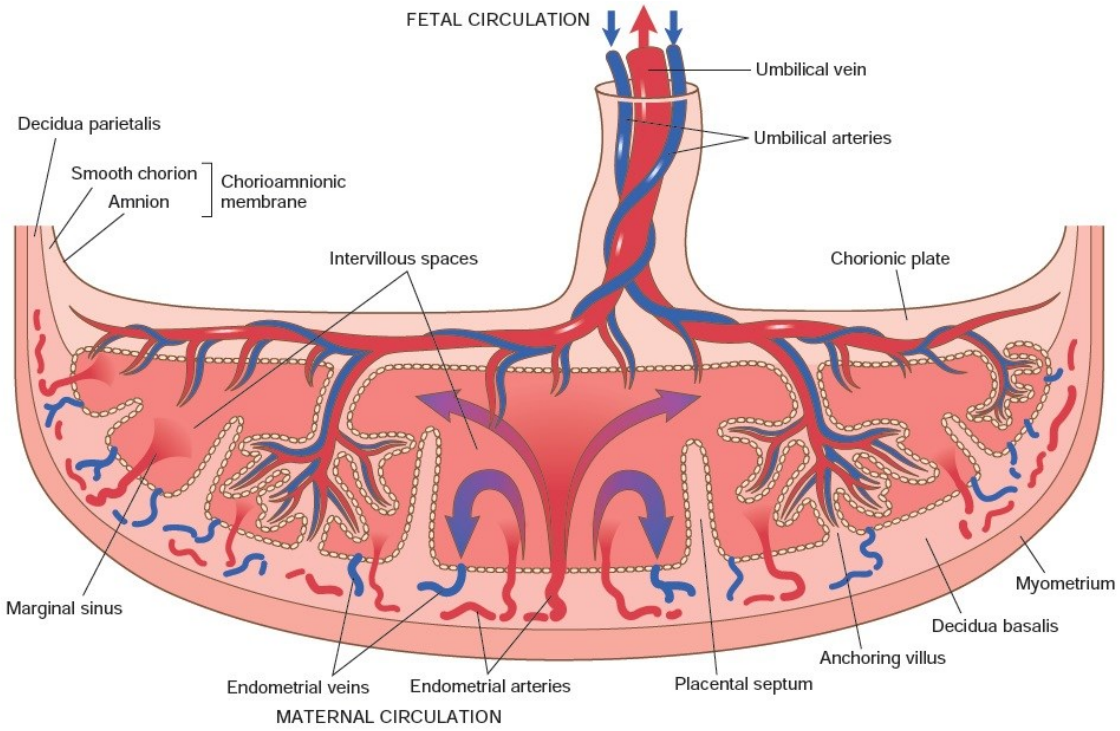


Abbildung 4: Transversalschnitt durch eine reife Plazenta. Die Chorionplatte besteht aus mehreren Epithel- und Bindegewebsschichten, an der Grenze zum Intervillosum befinden sich herdförmige Synzytiotrophoblasten, welche die Grundlage für die Zottenbildung darstellen. Von hier gehen schließlich die Stammzotten ab, welche die Allantoisgefäße der Chorionplatte aufnehmen. Die plazentare Zwischenschicht, das Plazentaparenchym, besteht aus mehreren Kotyledonen, welche durch Plazentasepten getrennt sind. Jedes Kotyledon besitzt einen Zottenstamm mit zahlreichen Verzweigungen. Das Zottenwerk ist über Zellsäulen mit der Basalplatte fest verbunden, sodass solche mit Basalplatte verbundene Zotten als Haftzotten bezeichnet werden. Direkt angrenzend an den intervillösen Raum ist mikroskopisch eine unvollständig ausgebildete Schicht von Synzytiotrophoblast- und darunter gelegenen Zytotrophoblastzellen zu erkennen. Der Nitabuch-Fibrinoidstreifen trennt den fetalen vom maternalen Anteil (in dieser Zeichnung nicht zu sehen). Es schließt sich die *Decidua basalis* an, welche hauptsächlich aus Deziduazellen besteht und regelmäßig ein- und mehrkernige extravillöse Trophoblastzellen und vielkernige extravillöse Synzytiotrophoblastzellen enthält. In der Basalplatte verlaufen die Endäste der endometrialen Spiralaterien, welche maternales Blut in den intervillösen Raum abgeben und somit zur Versorgung des Embryos dienen. Die Umbilikalarterien leiten das fetale sauerstoffarme Blut zurück zur Plazenta und die Umbilikalvene transportiert sauerstoffreiches Blut von der Plazenta zum Fetus ((Moore et al. 2007, Vogel 1996), Bild http://medicinase.com/pregnancy/ 21.09.2017).

Die Entwicklung der Plazentazotten erfolgt durch Längen- und Verzweigungswachstum sowie durch Zottenreifung. Es kommt zur Ausbildung von Intermediär- und Terminalzotten, die aus den Stammzotten hervorgehen. Das Wachstum der Zotten ist infolge der Zunahme an funktionstüchtigen Trophoblastzellen notwendig. Somit kommt es auch zur Zunahme des Volumens des Zottenparenchyms, wodurch die Vergrößerung der Zottenoberfläche auf ca. 10-14 m^2 bedingt wird ((Welsch 2005) S. 528). Die Zottenreifung dient zur Ausweitung des fetalen Gefäßsystems, der Differenzierung des Chorionepithels mit Vergrößerung der feto-

maternalen Austauschfläche und der Verkürzung der Diffusionsstrecken zwischen mütterlichem und fetalem Blut (Vogel 1996). Während bis zum Ende der 16. SSW die Plazentazotten noch von einem durchgängigen zweischichtigen Epithel bedeckt sind, welches aus basal liegenden Zytotrophoblastzellen und dem darüberliegenden vielkernigen Synzytium besteht, bilden sich ab der 17. SSW die Zytotrophoblastzellen zunehmend zurück und verschmelzen mit dem Synzytiotrophoblasten. Bei der reifen Plazenta sind nur noch vereinzelt Zytotrophoblastzellen zu finden, dafür aber eine hohe Anzahl von synzytiokapillären Membranen, die durch das Verschwinden der Zytotrophoblastzellkerne und das Auseinanderweichen der Synzytiotrophoblastzellkerne entstehen. Somit wird die feto-maternale Diffusionsstrecke signifikant von 50-100 µm in der 10. SSW auf 4-5 µm in der 40. SSW reduziert.

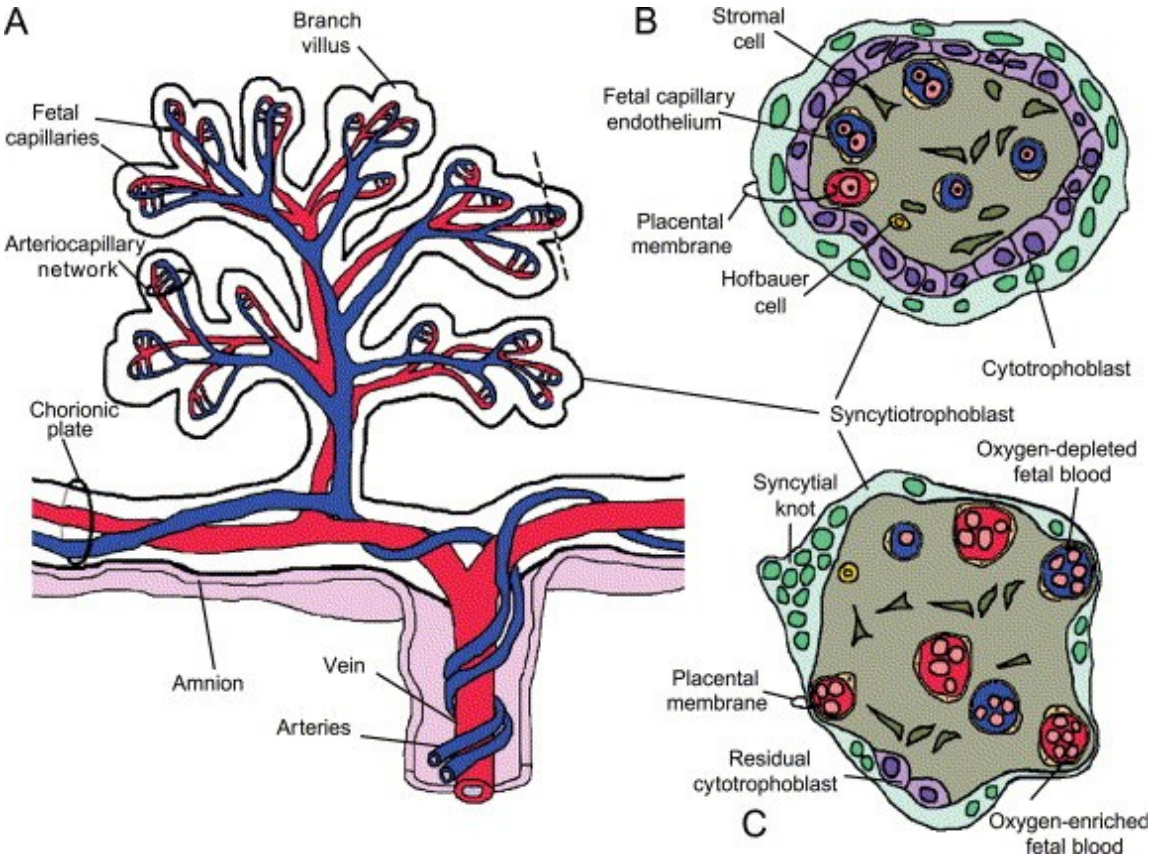


Abbildung 5: A Skizzenhafte Zeichnung einer Stammzotte mit arterio-kapillär-venösem Gefäßsystem. B-C Querschnitt durch terminale Zotten einer Plazenta aus der 10. SSW (B) bzw. einer Termplazenta (C). Während die Plazentaschranke bis zur 20. SSW noch aus vier Schichten (Synzytiotrophoblast, Zytotrophoblast, villöses Stroma, fetalen Kapillarendothel) besteht, sind diese in der 40. SSW bis auf den Synzytiotrophoblasten und dem fetales Kapillarendothel verschwunden. Die noch bestehende Barriere wird als synzytiokapilläre Membran bezeichnet und sorgt aufgrund der geringen Dicke für einen besonders effizienten Stoffaustausch. Somit kann der erhöhte Sauerstoff- und Nährstoffbedarf des Fetus gedeckt werden. Die im Stroma befindlichen Hofbauer-Zellen haben phagozytierende Eigenschaften (Moore, Bild (Gude et al. 2004)).

Neben der Versorgungs- und Schutzfunktion stellt die Plazenta ebenso ein wichtiges endokrines Organ dar. Dieses Organ bestimmt den Hauptteil des zirkulierenden Progesterons im Körper einer schwangeren Frau. Es inhibiert Uteruskontraktionen und wirkt ebenso auf den fetalen Kreislauf. Auch die Estrogene Estron, Estradiol und Estriol werden in dieser Zeit hauptsächlich von der Plazenta gebildet. Sie wirken als Wachstumshormone auf alle reproduktionsbezogenen Organe (Gude et al. 2004). Um den Fetus vor zu hohen Estrogenkonzentrationen zu schützen, sind die Hormone teilweise mit Sulfaten oder Glucuroniden konjungiert. Während hCG in den ersten 8 Wochen der Schwangerschaft in hohen Konzentrationen vom Trophoblasten sezerniert wird, kommt es ab der 12. SSW zum Abfall der hCG Konzentration im mütterlichen Blut. In der späten Schwangerschaft steigt die Konzentration von hCG wieder an, was mit einer Trophoblastdifferenzierung und für die Verschmelzung des Zytotrophoblasten mit dem Synzytium in Verbindung gebracht wird (Malassine und Cronier 2002). Andere wichtige Hormone, wie humanes Plazentalaktogen (hPL-2), viele Wachstumsfaktoren (z.B. EGF, insulin-like growth factor (ILGF) I und II), Zytokine, Chemokine, Eicosanoide, Corticotropin-releasing Hormone und Gonadotropin-releasing Hormone, werden zusätzlich von der Plazenta produziert (Gude et al. 2004).

Plazentare Modelle im experimentellem Ansatz

Obwohl die Plazenta gut verfügbar ist, gibt es nur wenige Forschungslabore, die diese in ihren experimentellen Studien integrieren. Deshalb wird sie auch als vergessenes Organ bezeichnet (Maltepe und Fisher 2015), wohingegen Plazenta-Forscher dieses Organ schon in verschiedenen Anwendungsgebieten als Modell benutzt haben, z.B. in der Reproduktionsbiologie, Immunologie, Toxikologie, Stammzell- und Krebsforschung (siehe Review (Yoshizawa 2013)). Besonders vorteilhaft erscheint hierbei, dass dieses Organ aus einer Vielzahl von verschiedenen Zelltypen, wie Trophoblastzellen, Endothelzellen, Erythrozyten, Immunzellen (besonders Hofbauer-Zellen Abb. 5), mesenchymalen Stromazellen (Fibroblasten, Myofibroblasten und glatte Muskelzellen) aufgebaut ist (Miller et al. 2005). Dieser Reichtum an Zellen ermöglicht die Untersuchung von unterschiedlichsten Fragenstellungen.

Die Plazenta-Explantate

Mit Hilfe verschiedener Präparations- und Kultivierungsmethoden können somit die jeweiligen Zellpopulationen und Gewebsverbände genauer untersucht werden. Dabei

ist die Verwendung von humanen plazentaren Explantaten (im Folgenden auch als Plazenta-Explantate benannt) eine beliebte und häufig eingesetzte Methode. Im Laufe der letzten Jahre konnten deshalb erste Untersuchungen zu Metabolismus- und Transportfunktion, der endokrinen Sekretion sowie dem Differenzierungs- und Proliferationsverhalten stattfinden (Miller et al. 2005, Orendi et al. 2011). Die Plazenta-Explantate werden entweder von Plazenten aus dem ersten oder dritten Trimester präpariert und können prinzipiell aus dem Zottengewebe, der Dezidua, den Membranen oder aus den Blutgefäßen stammen. Die Präparation erfolgt relativ einfach und schnell. Danach werden die kleinen Gewebestücke direkt kultiviert. Aufgrund dessen sind die Plazenta-Explantate relativ geringem Stress ausgesetzt und da diese auch in ihrem unter physiologischen Bedingungen vorkommenden Gewebsverband kultiviert werden, ist davon auszugehen, dass die Untersuchungen nahe der *in vivo* Bedingungen stattfinden (Gohner et al. 2014). Die Kultivierung kann auf Plastikböden mit oder ohne vorheriger Kollagen- oder Matrixbeschichtung erfolgen, verschiedene Medien wie DMEM/F12 oder RPMI und Kultivierungsperioden von bis zu 12 Tagen wurden bereits erfolgreich getestet (Miller et al. 2005). Folgend können die Kulturüberstände der Plazenta-Explantate auf die verschiedenen sekretierten Hormone, Matrix-Metalloproteinasen, Glucose, Laktat und Laktatdehydrogenase (LDH) analysiert werden (Miller et al. 2005). Zusätzlich können die Gewebestücke in Paraffin eingebettet werden und anschließend auf ihre Gewebeintegrität histologisch und immunhistochemisch untersucht werden (de Oliveira Gomes et al. 2011). Miller et. al. sieht diese Methode neben Anwendung von Elektronenmikroskopie für die Überprüfung der Gewebevitalität *in vitro* als wichtigste Untersuchung an. Der extrem sensitive Synzytiotrophoblast tendiert leicht zur Degeneration und löst sich unter Zellkulturstandardbedingung schnell ab. Dies kann wie auch die Entstehung von Fibrinoidnekrosen mit Hilfe von histologischen Untersuchungen eindeutig identifiziert werden (Miller et al. 2005). Außerdem ist es möglich, Plazenta-Explantate aus villösem Plazentaparenchym präpariert, zur Konditionierung von Medien zu benutzen, sodass diese mit Sekreten der Plazenta angereichert werden.

Co-Kultivierung mit anderen Zellkulturen

Aufgrund der bereits beschriebenen Bestrebungen, Tierversuchsmodelle durch alternative 3D Modelle abzulösen und gleichzeitig *in vivo* Bedingungen so gut wie möglich zu simulieren, gab es in den letzten Jahren viele Initiativen komplette Zellnetzwerke von Organen nachzubauen, welche die Interaktionen verschiedener

Zelltypen und der extrazellulären Matrix (ECM) wiederspiegeln. Da die Plazenta per se aus zahlreichen Zelltypen aufgebaut ist, können deren Explantate für die Co-Kultivierung mit anderen Zelltypen genutzt werden. Zur Erhaltung der 3D Bedingungen ist eine Konfrontation solcher Explantate mit anderen 3D Strukturen, wie zum Beispiel Sphäroiden, besonders erstrebenswert (Weber et al. 2013). In Hinblick auf die Tumorforschung ist eine Co-Kultivierung von Plazentagewebe und Krebszellen aus vielerlei Gründen interessant. Einerseits kann der Einfluss der plazenta-sekretierten Faktoren auf das Wachstum und die Invasivität von Tumorzellen untersucht werden (Tartakover-Matalon et al. 2010). Andererseits können solche Co-Kultur-Modelle generell für die Analyse von Metastasierungsprozessen in humanem Gewebe nützlich sein. Hierbei könnte man neben Plazentagewebe auch ähnliche Co-Kulturen mit hepatischem Gewebe herstellen, um so unterschiedliche Metastasierungsmechanismen zu analysieren. Eine weitere interessante Verwendung eines solchen Co-Kultur-Modells, bestehend aus Plazenta-Explantaten und Sphäroiden, wäre in toxikologischen Fragestellungen. Man könnte beispielsweise Zytostatika auf erwünschte, an den Sphäroiden, und unerwünschte Wirkungen, an den Plazenta-Explantaten, testen.

Isolation von plazentaren Zellen

Neben diesen vielfältigen Einsatzmöglichkeiten von Plazenta-Explantaten werden aus dem plazentarem Gewebe spezifische Zelltypen isoliert. Hauptsächlich handelt es sich hierbei um Zytotrophoblastzellen, verschiedene Immunzellen und Endothelzellen (Kammerer et al. 2004, Male et al. 2012). Zwar ist es so möglich einzelne Zelltypen getrennt voneinander zu untersuchen, jedoch ist es fraglich, ob sich diese tatsächlich noch wie *in vivo* Bedingungen verhalten. Durch aufwendige Isolationsschritte wird viel Stress induziert, der physiologische Gewebsverband aufgebrochen und deshalb ist davon auszugehen, dass sich die Zellen artifiziell verhalten (Gohner et al. 2014).

Die Plazenta-Perfusion

Die Perfusion von vollständigen plazentaren funktionellen Einheiten, den Kotyledonen, gilt als Goldstandardmethode, stellt Plazentaforscher jedoch aufgrund methodisch komplexer Vorgänge vor größere Herausforderungen. Ursprünglich wurde die Plazenta-Perfusion in den 60er bzw. 70er Jahren von den Wissenschaftlern Panigel und Schneider etabliert und noch heute werden so Substanzübertritte embryotoxischer Stoffe, z.B. Zytostatika, vom maternalen zum fetalen Kreislauf in gewöhnlich 2-6

stündigen Perfusionen getestet (Panigel 1962, Schneider et al. 1972, Berveiller et al. 2012). Anschließende Analysen auf Gewebsintegrität und –vitalität können im gleichen Ausmaß wie bei den Plazenta-Explantaten durchgeführt werden. Aufgrund der Erhaltung der Gewebestruktur (Chorion-Plazentaparenchym-Dezidua) werden die *in vivo* Bedingungen jedoch besser simuliert als dies mit der Kultivierung von Plazenta-Explantaten möglich ist. Bei der Experimentplanung muss hierbei beachtet werden, dass nach aktuellem Stand der Wissenschaft von einer Erfolgsquote meistens deutlich unter 50% ausgegangen werden kann (Myllynen und Vahakangas 2013).

Mammakarzinom-Metastasen in der Plazenta

Obwohl das schwangerschaftsassoziierte Mammakarzinom eher in einem fortgeschrittenen Stadium diagnostiziert wird und insgesamt einen aggressiveren Phänotyp aufweist, sind plazentare Metastasierung sehr selten (Epstein Shochet et al. 2012). Bisher wurden nur 17 Fälle von Brustkrebsmetastasen in der Plazenta beschrieben (Vetter et al. 2014, Alexander et al. 2003). Deswegen wird diskutiert, ob das Organ als solches einen inhibierenden Effekt auf die Tumorzellen ausübt, beziehungsweise sekretierte Faktoren stark negativ modulierende Einflüsse haben, wenn auch zu den molekularen Mechanismen wenig bekannt ist (Epstein Shochet et al. 2012). In einer experimentellen Studie konnte gezeigt werden, dass die gemeinsame Kultivierung von Plazenta-Explantaten aus dem ersten Trimester und Mammakarzinomzellen zur Elimination der Tumorzellen geführt hat, was die Hypothese der „non-supportive microenviroment for cancer cells“ unterstützt (Tartakover-Matalon et al. 2010). Wenn auch Metastasierungen sehr selten vorkommen, könnten durchaus einzelne Tumorzellen den Weg zum Plazentagewebe finden. In diesem Zusammenhang wurden zirkulierende Tumorzellen ausgehend vom Primärtumor bereits mehrfach beobachtet (Schindlbeck et al. 2016). Hinzu kommen Beobachtungen eines sogenannten Mikrochimärismus, der den Prozess eines Übertritts fetaler Zellen auf den mütterlichen Organismus aber auch maternaler Zellen auf den fetalen Organismus während der Schwangerschaft beschreibt (Boddy et al. 2015). Somit wäre es durchaus denkbar, dass nur einzelne Tumorzellen sowohl im mütterlichen als auch im fetalen Teil der Plazenta zu finden sind. Dabei stellt die spezifische Detektion dieser Mammakarzinomzellen eine besondere Herausforderung dar, da Plazenta- sowie Brustkrebszellen gleiche Proteine exprimieren. Aus diesem Grund stellt das beschriebene Co-Kultur-Modell, bestehend aus Plazenta-Explantaten

und Brustkrebssphäroiden, einen möglichen Ansatz dar, einen spezifischen Marker für die Mammakarzinomzellen zu finden.

Kooperation mit anderen Arbeitsgruppen

Während der experimentellen Arbeiten bestanden enge Kooperationen mit dem pharmakologischen Institut (Prof. Dr. Amelie Lupp) für die Durchführung von IHC-Färbungen sowie mit der experimentellen Nephrologie (Prof. Dr. Ralf Mrowka) für mikroskopische Aufnahmen am Universitätsklinikum Jena. Bei der Zusammenarbeit mit Frau Prof. Lupp erfolgte die Einbettung der Sphäroid- und Co-Kultur-Proben und die Anfertigung der Schnittpräparate sowie deren histologische und immunhistochemische Färbung selbständig ohne Hilfe Dritter. Nach intensiver Einweisung am Live Cell Imaging Mikroskop durch Herr Prof. Mrowka wurden hier ebenso alle Aufnahmen der Sphäroide selbständig ohne fremde Hilfe angefertigt. Für histologische Analysen der Plazentapräparate und die Beschreibung von plazentaren Mammakarzinom-Metastasen entstand außerdem eine Kooperation mit dem pathologischen Institut des Ullevål-Universitätsklinikums in Oslo, Norwegen (MD PhD Gitta Turowski). Dieses gemeinsame Projekt entstand im Rahmen eines 1-monatigen Forschungsaufenthaltes in Oslo und somit konnte die Aufarbeitung es Patientenfalles sowie die Beurteilung der plazentaren Mammakarzinom-Metastasen selbständig erfolgen. Diese Arbeiten wurden in diesem Zeitraum von der Pathologin Frau Dr. Turowski betreut.

4. Ziele der Arbeit

Das übergeordnete Ziel der Promotionsarbeit beinhaltete die thematische Aufarbeitung der seltenen Koinzidenz Mammakarzinom während der Schwangerschaft. Dabei sollte insbesondere das Wechselspiel zwischen den plazentaren Zellen inklusive deren sezernierten Substanzen und dem Mammakarzinom analysiert werden. Außerdem sollten während der Arbeit Modelle etabliert werden, welche das Potenzial haben, als Alternativen zu herkömmlich eingesetzten Tierversuchsmodellen verwendet werden können.

Folgende Teilziele wurden zur Realisierung des übergeordneten Ziels formuliert:

- Die Experimente sollten möglichst nah an den humanen Bedingungen durchgeführt werden. Aus diesem Grund hatte die Arbeit zum einen die Etablierung der 3D Kultivierung der drei Mammakarzinomzelllinien MCF-7, MDA-MB-231 und SK-BR-3 zum Ziel.
- Die in Plazenta-Explantat-Kulturen sezernierten Substanzen sollten zunächst isoliert und nachfolgend analysiert werden.
- Mittels eines plazenta-konditionierten Mediums sollten die Auswirkungen auf die Mammakarzinomzelllinien untersucht werden.
- Außerdem sollten einzelne Krebszellen im Plazentagewebe detektiert werden. Dafür wurden spezifische Marker sowohl für Trophoblast- als auch Mammakarzinomzellen gesucht.
- Die Interaktion zwischen MCF-7 Sphäroiden und Plazenta-Explantaten sollte in einem 3D Modell untersucht werden, um die Pathogenitätsmechanismen eines metastasierenden Mammakarzinoms in plazentares Gewebe *in vitro* analysieren zu können.
- Humanes Serum sollte als Alternative zum gebräuchlichen xenogenen fetalen Kälberserum für den standardmäßigen Einsatz als Zusatz zum Zellkulturmedium getestet werden.
- Unterschiede und Gemeinsamkeiten zwischen Mausplazenten und humanen Plazenten sollten anhand einer Literaturrecherche dargestellt werden, um den Bedarf an *ex vivo*-Studien an humaner Plazenta zu untermauern.

Diese Versuchsansätze zielten letztendlich darauf ab, den Einfluss der schwangerschaftsassoziierten Veränderungen auf das Mammakarzinom in verschiedenen tierversuchsfreien *in vitro* Modellen zu simulieren und anschließend zu analysieren. In besonderem Maße sollte in dieser Arbeit außerdem die humane Plazenta als Forschungsmodell für wissenschaftliche Fragestellungen benutzt werden, um somit auch Wissenschaftlern aus anderen Fachbereichen die vielfältigen Einsatzmöglichkeiten dieses Organes vorzustellen und Vorteile sowie Herausforderungen beim Arbeiten näher bringen zu können.

5. Publikationen

Manuskript 1: Generation of multicellular breast cancer tumor spheroids: comparison of different protocols

Artikel Typ: Original Paper

Autoren: Karolin Froehlich, Jan-Dirk Haeger, Julia Heger, Jana Pastuschek, Stella Mary Photini, Yan Yan, Amelie Lupp, Christiane Pfarrer, Ralf Mrowka, Ekkehard Schleußner, Udo R. Markert, André Schmidt

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Zusammenfassung: Drei verschiedene adhärent wachsende Brustkrebszelllinien (MCF-7, SK-BR-3, MDA-MB-231) wurden mit Hilfe von verschiedenen methodischen Ansätzen auf ihre Fähigkeit getestet 3D Kulturen zu formen. Für die Generierung von Sphäroiden erfolgte die Anwendung der Hanging Drop-Methode, Liquid Overlay Technik und Suspensionskultur. Aufgrund der Variation von verschiedenen Bedingungen (Zellzahl, Zellmedien, viskositätserhöhende Zusätze) konnten für jede Zelllinie 42 unterschiedliche Versuchsansätze getestet werden. Die MCF-7 Sphäroide wurden nach der Generierung für zwei weitere Tage kultiviert und anschließend histologisch ausgewertet (HE, Ki-67, cPARP, MUC-1).

Eigenanteil: Hauptanteil, Planung und Durchführung der Experimente, mikroskopische Aufnahmen, HE- und IHC- Färbungen, Auswertung der Daten, Verfassung des Manuskriptes.

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Generation of Multicellular Breast Cancer Tumor Spheroids: Comparison of Different Protocols

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Abstract Multicellular tumor spheroids are widely used models in tumor research. Because of their three dimensional organization they can simulate avascular tumor areas comprising proliferative and necrotic cells. Nonetheless, protocols for spheroid generation are still inconsistent. Therefore, in this study the breast cancer cell lines MCF-7, MDA-MB-231 and SK-BR-3 have been used to compare different spheroid generation models including hanging drop, liquid overlay and suspension culture techniques, each under several conditions. Experimental approaches differed in cell numbers (400–10,000), media and additives (25 % methocel, 25 % methocel plus 1 % Matrigel, 3.5 % Matrigel). In total, 42 different experimental setups have been tested. Generation of spheroids was evaluated by light microscopy and the structural composition was assessed immunohistochemically by means of Ki-67, cleaved poly (ADP-ribose) polymerase (cPARP) and mucin-1 (MUC-1) expression. Although the tested cell lines diverged widely in their capacity of forming spheroids we recommend hanging drops supplemented with 25 % methocel as the most reliable and efficient method with regard to success of generation of uniform spheroids, costs, experimental complexity and time expenditure in the different cell lines. MCF-7 cells formed spheroids under almost all analyzed conditions, and MDA-MB-231 cells under only one protocol (liquid overlay technique, 3.5 % Matrigel), while SK-BR-3 did not under neither condition. Therefore, we outline specific methods and recommend the use of adapted and standardized spheroid generation protocols for each cell line.

Keywords Breast cancer · MCF-7 · MDA-MB-231 · SK-BR-3 · Spheroids · 3D cultures · Tumor

Abbreviations
MCTS multicellular tumor spheroids

Introduction

Three-dimensional cell culture systems have been caught up with an increasing number of publications in the recent years [1]. Therefore, and due to the growing interest in applying 3D cell culture models, a shift in cell culture paradigm towards three-dimensional may be expected in the coming years [1]. For their extended use in future it is necessary to establish standardized and reproducible protocols for generation of multicellular tumor spheroids (MCTS, shortly termed as spheroids) of comparable size, structure and shape [2]. Three-dimensional cell cultures display a variety of features, which are absent in cell monolayers, such as a complex network of cell-cell contacts and

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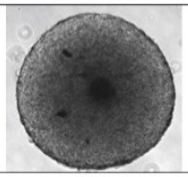
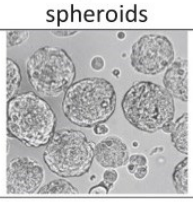
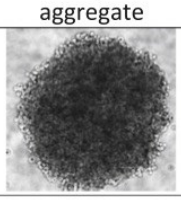
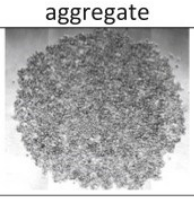
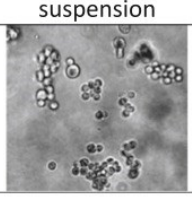
Table 1 Classification of structures formed under tested conditions. The formed structures were classified in spheroids, multiple small spheroids, compact aggregates, loose aggregates or singular cell suspension.

Spheroid	Multiple small spheroids	Compact aggregate	Loose aggregate	Singular cell suspension
				

advanced extracellular matrix. They develop pH, oxygen, metabolic and proliferative gradients causing stratification in mature spheroids, which resemble avascular stages of solid tumors [3–6] and micrometastases [7] mimicking the situation in vivo. The spheroid structure is driven by nutrient and signal gradients [6] resulting in an outer zone of proliferating cells,

Table 2 List of equipment and reagents used for generation and analysis of multicellular tumor spheroids

Plastics and equipment	Company
Petri dishes 6 cm; 10 cm	Greiner Bio-One, Frickenhausen, Germany
NuclonTM Surface dishes 6 cm	NuncTM, Roskilde, Denmark
96-well plates Cellstar® (see Table 2)	Greiner Bio-One
IKA KS 260 basic	Laborgeräte München, Munich, Germany
Cellstar® 48–well plates	Greiner Bio-One
Life-Imaging microscope with incubator Axio Observer Z1	Carl Zeiss, Jena, Germany
ZEN blue software	Carl Zeiss
MS2 Minishaker IKA®	Laborgeräte München
Heraeus Multifuge 1S Centrifuge	ThermoScientific, Waltham, MA, USA
microtome Microm	Microm, Walldorf, Germany
poly-L-lysine-coated glass slides	
Reagents	**Company**
DMEM with high/ low glucose	Sigma, St. Louis, USA
RPMI 1640	Sigma
10 % fetal calf serum	Gibco, Paisley, UK
0.05 % trypsin-EDTA	Gibco
Ham's F12	Sigma
methocel	Sigma
growth-factor reduced Matrigel	BD Biosciences, Heidelberg, Germany
2 % poly-HEMA	Polysciences, Eppelheim, Germany
pooled plasma	Institute for Transfusion Medicine, University Hospital Jena
fibrinogen	Stago, Asnières sur Seine, France
Mayer's hematoxylin and eosin Y solution	Sigma
DePex	SERVA Electrophoresis GmbH, Heidelberg, Germany
hydrogen peroxide	Sigma
Vectastatin® Elite® ABC Kit	Vector Laboratories, Burlingame, USA
primary antibodies	
Ki-67 *1:50*	Dako, Hamburg, Germany
cPARP *1:200*	New England Biolabs, Beverly, USA
MUC-1 *1:5000*	Dako
AEC substrate	BioGenex, San Ramon, USA
Mowiol	Carl Roth GmbH, Karlsruhe, Germany

followed by an inner hypoxic area with quiescent cells [8], which encloses a necrotic core [9]. Due to this in vivo-like composition, MCTS offer a great potential to study the molecular properties of tumors. However, despite this intrinsic potential for numerous applications, their advantages seem to be underestimated [10] which may be due to limited technical information available in literature [4]. Therefore, optimized and standardized protocols for spheroid formation are needed. Here, we tested 42 different experimental conditions for spheroid generation on the following breast cancer cell lines: MCF-7, a non-invasive, hormone positive and HER2 negative cell line [10], SK-BR-3, a more invasive, hormone negative and HER2 positive cell line [11], and the triple negative and invasive cell line MDA-MB-231 [12].

In contrast to the frequently missing exact description of MCTS in literature [5], we have applied a strict definition based on characteristics recognizable in light microscopy: three-dimensional growth, spherical shape, high compactness, concluded, smooth rim, and an even surface. We classified the formed structures in spheroids, multiple small spheroids, compact and loose aggregates, and single cell suspension (Table 1), based on the definition for spheroids as described in Vinci et al. [13]. In the following section we outline specific methods, which we recommend for use in this assay.

Material and Methods

Cell Culture

MCF-7 (ATCC® HTB-22TM) and SK-BR-3 (ATCC® HTB-30TM) breast cancer cells were cultured in DMEM high glucose, MDA-MB-231 (ATCC® HTB-26TM) breast cancer cells in RPMI 1640. Cells were trypsinized as soon as confluence was observed until passage 35 (MCF-7; MDA-MB-231) or 65 (SK-BR-3). All cell cultures were performed at 37 °C in a humidified atmosphere with 5 % CO_2 and all media were supplemented with 10 % fetal calf serum (for all reagents in this paper, see Table 2).

Spheroid Generation

The efficacy of three different methods for spheroid generation has been compared (Fig. 1, for protocols see Table 3): the hanging drop method (2.3.1.), liquid overlay technique (2.3.2.) and suspension culture (2.3.3.).

The Hanging Drop Method

For comparison of cell number influence on generation of spheroids, different numbers of cells (400–10,000)

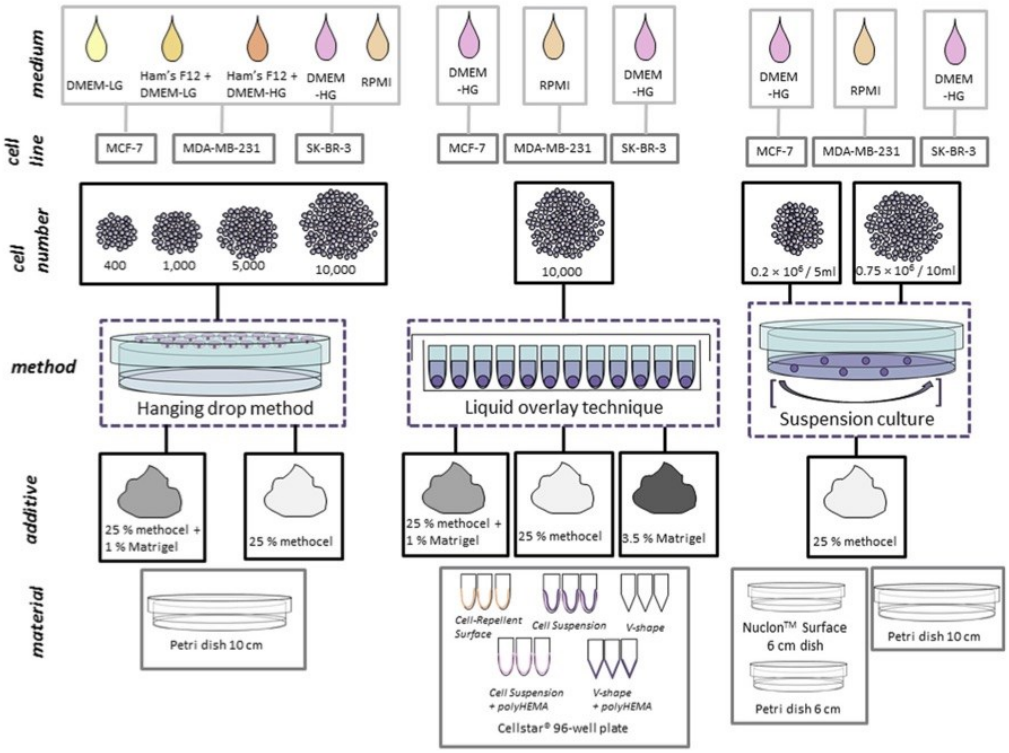


Fig. 1 Overview of the tested spheroid generation protocols. Three principally different methods have been tested and compared for their suitability for generating spheroids from 3 breast cancer cell lines. Each method has been applied under varying conditions as displayed

Table 3 Methods and simplified protocols for generation of breast cancer cell line multicellular tumor spheroids and their respective components. Recommended culture period for all is 3 days

Hanging drop method	
Place up to 45 hanging drops under the lid of a Petri dish	
Drop volume	20 μl
Cell number	400–10,000 cells/drop
Additives	25 % methocel (with or w/o 1 % Matrigel)
Media (used in this paper)	RPMI 1640, DMEM high or low glucose (each 100 % or 50:50 mixed with Ham's F12)
Liquid overlay technique	
Use 96-well plates.	
Cellstar® V-shape	coated or not with 2 % poly-HEMA
Cellstar® Cell Suspension	
Cellstar® Cell-Repellent Surface	
Volume	100 μl/well
Cell number	10,000 cells/well
Additives	25 % methocel without or with 1 % or 3.5 % Matrigel
Medium	depending on cell line
Suspension culture on non-coated plates	
Culture cells in Petri or Nunclon™ dishes under slight shaking (100 rpm, or not)	
Cell number and volume	
on a 6 cm diameter Petri dish	0.2×10^6 cells in 5 ml medium
on a 10 cm diameter Petri dish	0.75×10^6 cells in 10 ml medium
Additives	none or 25 % methocel
Medium	depending on cell line

were seeded in hanging drops (20 μl) under the lids of cell culture plates for 3 days. Experimental approaches differed in the type of media and additives in hanging drops: DMEM high glucose (medium 1, labelled as "M1"), DMEM low glucose (Sigma; "M2"), RPMI 1640 ("M3"), a mixture of Ham's F12 (Sigma) and DMEM high glucose (50 %/50 %; "M4") or a mixture of Ham's F12 and DMEM low glucose (50 %/50 %; "M5"). In addition, 25 % methocel or 25 % methocel plus 1 % growth-factor reduced Matrigel were added to the cell suspension for increasing viscosity. The methocel stock solution was prepared as described by Korff and Augustin [14]. The process of thawing and pipetting of Matrigel was done as described by Ivascu et al. [15].

Liquid Overlay Technique

Round bottom Cellstar® Cell-Repellent Surface, Cellstar® Cell Suspension and Cellstar® V-shape plates were used. Cellstar® Cell Suspension and Cellstar® V-shape 96–well plates were additionally coated with 2 % poly(2-hydroxyethyl methacrylate) (poly-HEMA). A total suspension volume of 100 μl containing 10,000 cells, cell-specific medium and additives (25 % methocel, 25 % methocel plus 1 % Matrigel or 3.5 % Matrigel) was added to wells. Cells were cultivated for 3 days.

Suspension Culture on Non-Coated Plates

Cell suspensions were seeded at defined densities in their respective medium in different cell culture dishes to compare their suitability for the generation of MCTS: 6 cm Petri dishes (0.2×10^6 cells in 5 ml), 6 cm Nuclon™ Surface dishes (0.2×10^6 cells in 5 ml) or 10 cm Petri dishes (0.75×10^6 cells in 15 ml medium). The cells were incubated in pure medium or medium supplemented with 25 % methocel for 3 days and exposed or not to slightly shaking at 100 rpm.

Microscopical Analysis of Formed Structures

Eight mm of the narrow end of 200 μl pipette tips were cut for enlarging the aperture diameter to transfer the formed structures into 400 μl cell-specific medium in Cellstar® 48–well plates. Size and morphology of the formed structures were evaluated and recorded using a Life-Imaging microscope with incubator Axio Observer Z1. Pictures were taken with an AxioCam MRm Rev.3 camera using ZEN blue software (Table 4).

Table 4 Immunohistochemistry protocol for 3D structures. Generated structures can be transferred from site of production to site of analysis by using 200 μl pipette tips after cutting eight mm of the narrow end for enlarging the aperture diameter

Embedding of formed structures
Transfer six of the formed spheroid-like structures in 200 μl pooled human plasma in a 0.5 ml tube.
Wait until sedimentation of the structures, add 20 μl fibrinogen and vortex the sample carefully for 20 s.
The sample needs 5 min for coagulation at room temperature.
Fix the coagulated structure in 4 % formalin.
Perform final paraffin embedding.
Hematoxylin and eosin (HE) staining
Cut 4 μm sections from the paraffin blocks at a microtome.
Float section onto poly-L-lysine-coated glass slides and wait until the sections are air-dried.
Deparaffinize sections in xylene and rehydrate in a graded ethanol series.
Perform HE staining according to routine protocols.
Wash sections in distilled water, dehydrate in a graded ethanol series, clear in xylene and mount in DePex.
Evaluate sections at an appropriate microscope.
Immunostaining of formed structures
After deparaffinization and rehydration in a graded ethanol series, block the endogenous peroxidase by hydrogen peroxide.
Perform antigen retrieval by boiling in 0.1 M citrate buffer (pH 6.0) for 16 min.
Wash sections in PBS and incubate them for 20 min in blocking solution.
Incubate sections with primary antibodies in a humid chamber at 4 °C overnight.
Wash sections in PBS and incubate them with biotinylated secondary antibodies, expose to the avidin-peroxidase and develop with AEC substrate.
Counterstain sections with Mayer's haematoxylin and mount in Mowiol.
Evaluate immunostained sections at an appropriate microscope.

Paraffin Embedding of Formed Structures and Cell Monolayers

Structures formed in hanging drops have been transferred into poly-HEMA coated 96-well plates and cultured conventionally. After 0 h, 6 h, or 48 h, they were placed into a 0.5 ml reaction tube filled with 200 μl pooled plasma from healthy anonymous blood donors. After sedimentation of the formed structures, 20 μl fibrinogen were added and the samples were vortexed for 20 s. After 5 min of coagulation at room temperature, samples were fixed in 4 % formalin. Cell suspensions from monolayers were centrifuged (25,200 x g, 10 min) using a Heraeus Multifuge 1S Centrifuge and the cell pellet was resuspended in 1 ml pooled plasma and treated like the formed structures. The final paraffin embedding was performed as described by Lupp et al. [16].

Hematoxylin and Eosin (HE) Staining

From the paraffin blocks, 4 μm sections were cut using a microtome, floated onto poly-L-lysine-coated glass slides and air-dried. Subsequently, sections were deparaffinized in xylene and rehydrated in a graded ethanol series. HE staining was performed according to routine protocols using Mayer's hematoxylin and eosin Y solution [16]. Thereafter, sections were washed in distilled water, dehydrated in a graded ethanol series, cleared in xylene and mounted in DePex. Sections were evaluated by use of an Axio Imager A1 microscope.

Immunostaining of Formed Structures

Sections of embedded structures and cellular monolayers were deparaffinized, rehydrated in a graded ethanol series and the endogenous peroxidase was blocked by hydrogen peroxide. Antigen retrieval was performed for the histochemical detection of all antigens by boiling in 0.1 M citrate buffer (pH 6.0) for 16 min. After washing in PBS, sections were incubated for 20 min with blocking solution followed by incubation with primary antibodies against Ki-67, cPARP and MUC-1 in a humid chamber at 4 °C overnight. After washing in PBS, the specimens were incubated with biotinylated secondary antibodies, exposed to the avidin-peroxidase complex and developed with AEC substrate. Sections were counterstained with Mayer's haematoxylin, mounted in Mowiol and evaluated at an Axio Imager A1 Microscope.

Evaluation and Nomenclature of Formed Cellular Structures

The formed structures have been classified by applying the following categories: "spheroids", "multiple small spheroids", "aggregates" (compact or loose) or "single cell suspensions"

(Table 1). Each of a total of 42 individual methodological setups has been repeated at least 3 times in 6 replicates ($n = 18$ up to $n = 200$ independent spheroid formation preparations). The most practicable setups leading to spheroids have been repeated routinely ($n > 1000$) as they have been produced for further experiments which are not part of this paper.

Statistics

Aim was to identify the most frequent outcome of each setup. The respective structures have been categorized as being generated at a frequency of >90 %, >80 % or >70 %.

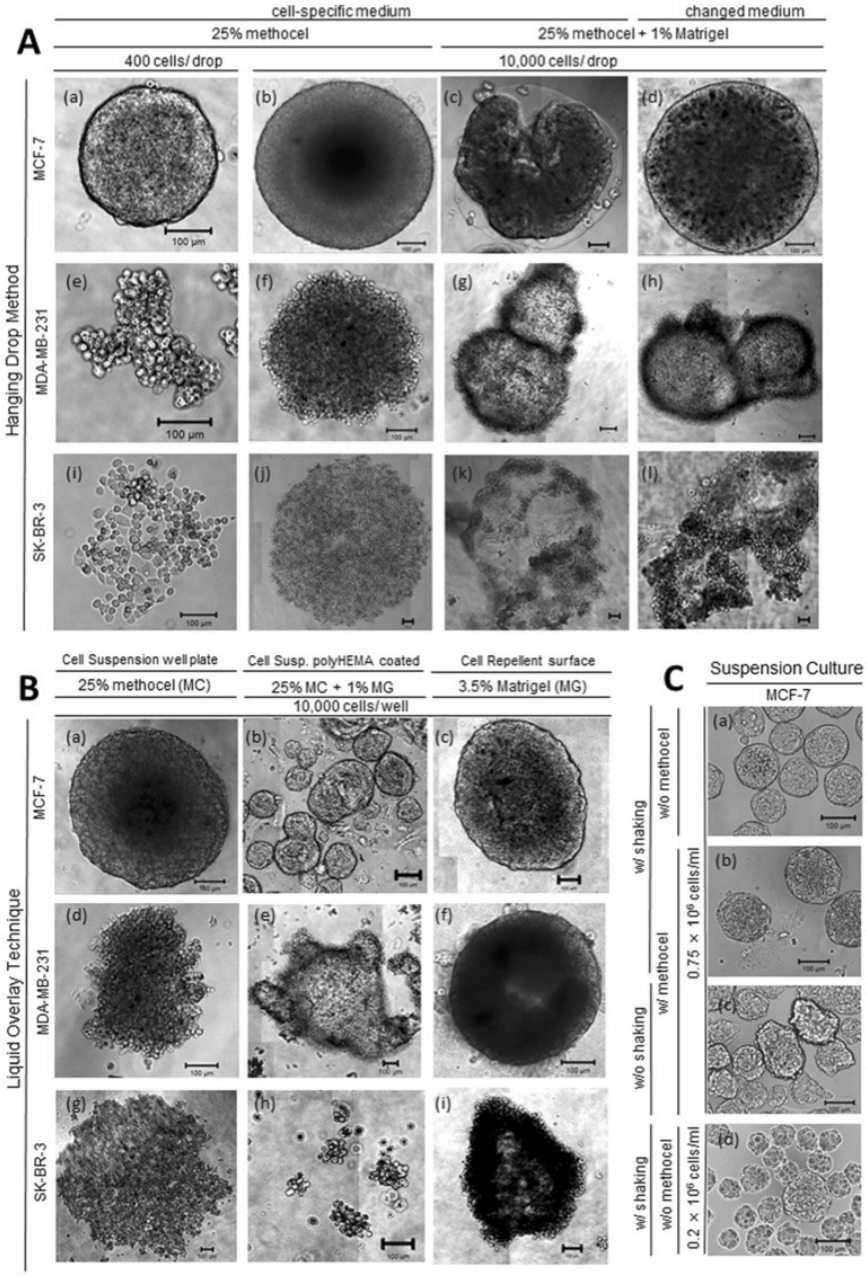


Fig. 2 Representative microphotographs of most frequently formed structures from breast cancer cell lines after 3 days culture under different conditions. MCF-7 and SK-BR-3 cells were cultured in DMEM high glucose medium, MDA-MB-231 in RPMI 1640. The generated structures have been classified as spheroids (**Aa**, **Ab**, **Ad**, **Ba**, **Bc**, **Bf**), multiple small spheroids (**Bb**, **Ca**, **Cb**), compact aggregates (**Ac**, **Af**, **Bd**, **Bi**, **Cc**, **Cd**), loose aggregates (**Ae**, **Ag**–**l**, **Be**, **Bg**) or single cell suspension (**Bh**). Each indicated setup has been repeated at least 18 times. For summary see Fig.3. Scale bars = 100 μm

Results

Breast Cancer Spheroid Formation

None of the used cell lines formed spheroids in hanging drop cultures without addition of a viscosity raiser (data not shown). The addition of 25 % methocel led to regularly shaped MCF-7 spheroids in each drop independently of the culture media. MDA-MB-231 cells generated compact aggregates at higher cell numbers and SK-BR-3 cells formed exclusively loose aggregates independently of the culture media. By applying the liquid overlay technique or suspension cultures, only very specific setups allowed the formation of spheroids. SK-BR-3 cells did not form spheroids under the tested conditions, but compact aggregates being the best observed result. The only condition for spheroid generation in MDA-MB-231cells was the liquid overlay technique on a Cellstar Cell-Repellent Surface and 3.5 % Matrigel (microphotographs in Fig. 2; summarized results in Fig. 3).

Immunostaining

After 3 days of generation in hanging drops, MCF-7 spheroids showed a homogenous inner structure (Fig. 4a-d). After additional 6 h of spheroid culture on plates, MCF-7 spheroids were morphologically altered. A minority of the spheroidal MCF-7 cells became apparently larger and had a pale cytoplasm (Fig. 4e). The

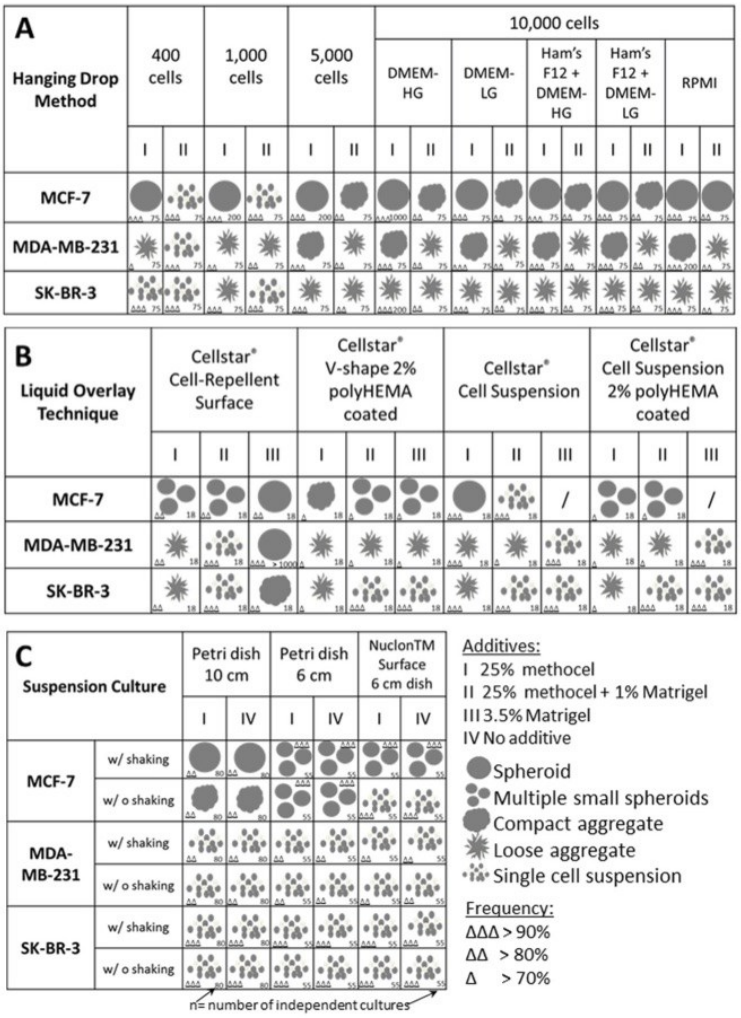


Fig. 3 Representative structures as mostly formed from breast cancer cell lines after 3 days culture under different conditions. Schematic summary of results from 42 different setups for spheroid generation in the breast cancer cell lines MCF-7, SK-BR-3 and MDA-MB-231. Representative photographs are shown and described in Fig.2. The symbols display the most frequently generated structure obtained by application of the respective setup. The triangles indicate the frequency of these structures. n varied between setups due to their different complexicity ($n = 18$ to $n > 1000$)

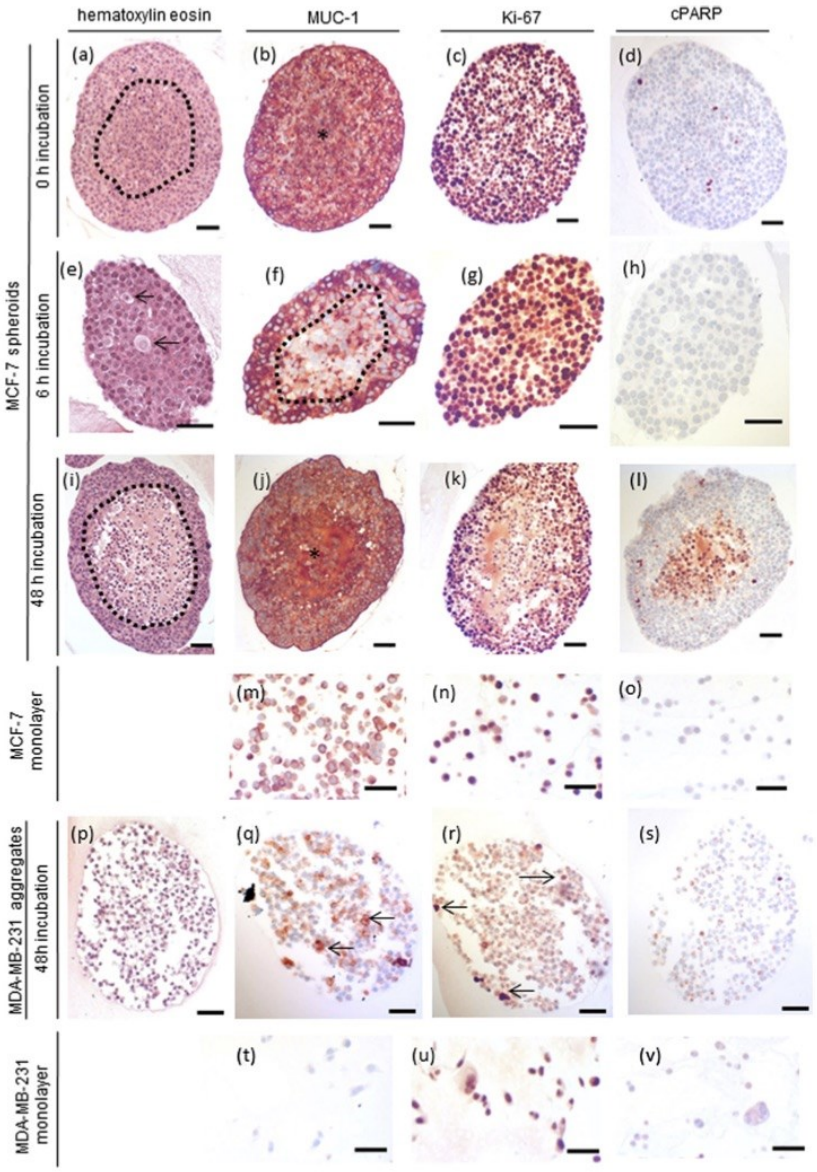


Fig. 4 HE and immunostaining of MCF-7 spheroids/ monolayers and MDA-MB-231 aggregates/ monolayers. The microphotographs show HE staining (**a**, **e**, **i**, **p**), immunlocalization of MUC-1 (**b**, **f**, **j**, **m**, **q**, **t**), Ki-67 (**c**, **g**, **k**, **n**, **r**, **u**) and cPARP (**d**, **h**, **l**, **o**, **s**, **v**) in MCF-7 spheroids (**a**–**l**), MCF-7 monolayers (**m**–**o**), MDA-MB-231 aggregates (**p**–**s**) and MDA-MB-231 monolayers (**t**–**v**). MCF-7 and MDA-MB-231 cells were cultured 3 days in hanging drops with 25 % methocel, 10,000 cells/drop. Formed MCF-7 spheroids were analyzed immediately after formation (0 h; **a**-**d**), or cultivated for further 6 h (**e**–**h**) or 48 h (**i**–**l**) in a conventional poly-HEMA coated 96 well plate. Immediately after formation, MCF-7 spheroids were compact (**a**). In some figures the spheroidal inner zone is exemplarily indicated by asterisks (**b**, **j**) or by dotted lines (**a**, **f**, **i**). Almost all cells expressed MUC-1 (**b**) and most nuclei were Ki-67 positive (**c**). In contrast, cPARP was only expressed in few cells (**d**). After 6 h, some larger MCF-7 cells with pale cytoplasm (*arrows*) appeared in the core and at the interface to the outer spheroidal rim (**e**). In the inner spheroidal cells MUC-1 expression was decreased (**f**). Most nuclei (in the spheroidal rim and core) were Ki-67 positive (**g**) and cells were cPARP negative (**h**). After 48 h, spheroids developed a disintegrated spheroidal core, whilst the outer rim was still organized (**i**). The expression of MUC-1 in the core was increased (**j**). Ki-67 positive nuclei were detectable in the outer rim but not in the disintegrated core (**k**). Almost all cells in the spheroid core were cPARP positive, but in the organized spheroidal rim cPARP negative (**l**). MUC-1 (**m**) and Ki-67 (**n**) were expressed constantly in MCF-7 monolayers which were cPARP-negative (**s**). MDA-MB-231 aggregates (**p**–**s**) were analyzed immediately after generation or after further 48 h culture in poly-HEMA coated 96 well plates. MUC-1 was expressed at different intensities in approximately half of the cells (*arrows*; **q**). Several nuclei were Ki-67 positive (*arrows*; **r**), and a few cells were cPARP positive (**s**). MDA-MB-231 monolayers were MUC-1 negative (**t**), Ki-67 positive (**u**) and cPARP negative (**v**). Scale bars = 50 μm

expression of MUC-1 was decreased, particularly in cells of the core (Fig. 3a f). After 48 h culture on a plate, the spheroidal core disorganized while the spheroidal rim remained organized (Fig. 4i) in comparison to younger MCF-7 spheroids (Fig. 4a). Simultaneously, in the core MUC-1 expression increased (Fig. 4j), Ki-67 decreased, and cells became cPARP-positive (Fig. 4a k-l). In contrast to MCF-7 spheroids, after 3 days of culture in the hanging drops, MDA-MB-231 aggregates displayed lower cell density (Fig. 4p), and consequently, did not differentiate into inner core and an outer proliferating zone (e.g., 48 h Fig. 4r-s). In two dimensional cell cultures, MCF-7 and MDA-MB-231 cells expressed Ki-67 in nuclei, and were cPARP-negative. MCF-7, but not MDA-MB-231 cells were MUC-1-positive (Fig. 4m-o; t-v).

Discussion

The **hanging drop spheroid generation** protocol established in our study differs from previous protocols which used lower concentrations of methocel (0.24 %) and did not lead to spheroid formation [5]. In general, hanging drops represent an attractive alternative for spheroid production, because they are easy to handle, inexpensive and useful for further experiments, e.g., in toxicity testing [6]. The here presented method is optimized and standardized and might therefore help to overcome previously reported problems with inconsistent spheroid size, variable cell populations and high expenses [17]. The **liquid overlay technique** established by Ivascu and Kubbies [15] is rapidly gaining popularity. Recently, several spheroid generation protocols have been established based on this method [5, 17–19]. Although the liquid overlay technique is considered to be an established method for the formation of spheroids, variations exist depending on the type of well plates. When using V-shaped [5] or U-shaped [15] wells, cells can be pelleted in the well cone by smooth centrifugation in contrast to F-shaped wells [19]. Different non-adherent substrates such as agarose [17–20] and poly-HEMA [15] prevent cell adhesion and change spheroid morphology. Further, some biotechnology companies offer 96-well plates with special non-adherent coatings as well. In order to compare the effects of different coatings on spheroid formation in breast cancer cell lines, we have used different types of 96-well plates. The best results were achieved by using the Cellstar ® Cell-Repellent Surface well plate enabling MCF-7 and MDA-MB-231 spheroid formation (Fig. 2B c,f).

In **suspension cultures**, MCF-7 cells generate spheroids which can be improved by continuous spinning [2]. Although this method displays some advantages like easy application, low costs and the possibility of scale-up production, the most critical limitation is the variability in spheroid size and morphology [21]. In our experiments suspension culture of MCF-7 cells in Petri dishes with or without methocel revealed formation of heterogeneous spheroids which complicates standardization for subsequent experiments (Fig. 2C). Compactness of inner zone of MCF-7 spheroids was proven by HE- and immunostaining. MCF-7 spheroids generated during 3 days in hanging drops were compact and almost all cells were proliferating, whereas only few cells were apoptotic. Extended spheroid culture time of up to 5 days decreased proliferation and increased apoptosis. These spheroids presented typical characteristics of central necrotic regions. The increasing depletion of oxygen and nutritive substances in growing spheroids may cause an energy and ATP deficit and an accumulation of fatty acids in the cells, detectable as lipid droplets. The process may be followed by tissue disintegration and destruction in the inner region which leads to the characteristic stratified composition of spheroids and depends on size, culture time and cell type [22]. Spheroids larger than 500 μm display the concentrically layered structure with a necrotic core surrounded by a viable layer of quiescent cells and an outer rim of proliferating cells within 3–5 days [23–26], while smaller spheroids of around 200 μm need more time (~8 days) to develop central, hypoxic core regions with necrotic areas [27, 28].

Spheroid formation required the addition of methocel and/or Matrigel to the medium in all successful approaches. Without additives no cell line generated spheroids. Previously, it has been shown that Matrigel used as viscosity-increasing additive induces MDA-MB-231 and SK-BR-3 in a single cell suspension to form cell aggregates which do not fulfill our definition of spheroids as used in the present paper [15]. In our hands, SK-BR-3 cells did not form spheroids under the tested conditions, but formed compact aggregates on Cellstar ® Cell-Repellent Surface well plates coated with 3.5 % Matrigel. Compared to previously published protocols we employed higher concentrations of Matrigel and methocel for the generation of spheroids [4, 15]. Applying Matrigel and methocel in combination had no added impact on spheroid generation in breast cancer cell lines.

Conclusions

In the present study we have analyzed the influence of 42 different experimental approaches on the capability of inducing spheroids in three commonly used breast cancer cell lines. While MCF-7 cells easily formed spheroids under different conditions, only one single protocol yielded spheroids in MDA-MB-231 cells. SK-BR-3 cells did not form spheroids under the tested setups. The different spheroid formation capabilities of different cell lines stress the need for standardization of spheroid generation protocols for better comparability of international data and for avoiding of unnecessary spending of resources for their establishment at different laboratories. Based on our results we assume hanging drops as the most appropriate method for reproducible generation of spheroids

with similar size, shape and structure. We show a comprehensive overview of the different outcomes of spheroid formation depending on the applied protocol and provide an easy-to-follow protocol as supplementary information.

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Compliance with Ethical Standards

Conflict of Interest Statement All authors declare that they have no conflict of interest.

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Manuskript 2: Human serum alters cell culture behavior and improves spheroid formation in comparison to fetal bovine serum

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Zusammenfassung: In dieser Studie wurde der Einfluss von FKS versus HS auf das Wachstumsverhalten (Proliferation, metabolische Aktivität) der Zervixkarzinomzellen HeLa und SiHa untersucht. Es erfolgte die mikroskopische Beurteilung der Zellmorphologie und die Analyse des Migrations- und Invasionsverhaltens mittels “wound healing“- und Transwell-Assays. Zusätzlich wurden die Zervixkarzinomzellen mittels der „Hanging Drop“-Methode zu Sphäroiden geformt, wobei HS die Generierung von Sphäroiden deutlich positiv beeinflusste. Weitere Kultivierung der Sphäroide in FKS bzw. HS supplementiertem Medium wurde mithilfe mikroskopischer Aufnahmen und anschließenden histologischen Analysen (HE) ausgewertet. FKS und HS stimulierten die Zellproliferation und -migration im gleichen Maße.

Eigenanteil: fachliche Beratung, Einarbeitung und Hilfestellung bei der Generierung der Sphäroide und bei der Veränderung der Kulturbedingungen zur Optimierung der Sphäroidbildung, Einarbeitung am Live Cell Imaging Mikroskop und Unterstützung bei den mikroskopischen Aufnahmen sowie Auswertungen.

Mitautorenanteil: Julia Heger (Hauptanteil, Planung, Durchführung und Auswertung der Experimente, Erstellung des Manuskripts), Jana Pastuschek (fachliche Beratung bei Verwendung von HS) Christin Baer (immunhistochemische Färbungen), Astrid Schmidt (Durchführen der MTS-Versuche mit verschiedenen Serumkonzentrationen), Ralf Mrowka (Betreuung bei mikroskopischen Aufnahmen), Ekkehard Schleußner (Betreuung), Udo R. Markert (Betreuung), André Schmidt (Betreuung).

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Human serum alters cell culture behavior and improves spheroid formation in comparison to fetal bovine serum

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ABSTRACT

Background: The use of fetal bovine serum (FBS) as growth supplement for human cell and tissue culture is widely spread in basic research as well as in clinical approaches, although several limitations must be considered, such as unstable composition and availability, biosafety and ethical aspects. Regarding interspecies differences, xenogeneic growth factors may evoke incompatibilities and non-desired interactions with human cells resulting in imprecise outcome of human-relevant data.

Methods: In this study the functionality of human serum (HS) has been investigated in comparison to FBS by assessing proliferation, migration and invasion of the human cervical cancer cell lines SiHa and HeLa. The effects of both sera on spheroid formation were analyzed microscopically.

Results: Both, FBS and HS, stimulate cell proliferation and migration similarly, whereas HS significantly enhanced cell invasion. The spheroid formation assay revealed remarkable differences between both sera, especially for SiHa cells. While in FBS supplemented medium cells only formed loose aggregates, HS induced regularly shaped spheroids under all tested conditions.

Conclusion: We were able to demonstrate that HS and FBS differently influence behavior of cells in culture which may have an impact on experimental results, especially in 3D cultures.

1. Introduction

Interspecies differences are major obstacles when translating animal studies to humans and led to the development of numerous in vitro assays based on human cell lines, primary cells, tissue explants and organ models. These in vitro approaches aim to mimic the human condition as closely as possible. While the use of human cells for in vitro models is self-explanatory regarding the numerous species- and clade-specific genes [1,2], splicing variants [3], glycosylation patterns [4] or regulatory elements [5] it is surprising that less attention is paid to the species origin of serum which is applied as growth supplement.

Traditionally, the use of FBS as universal growth supplement [6] in cell or tissue culture medium is widely accepted. Besides its function for proliferation and maintaining cellular activities [7], it provides essential compounds, such as hormones, vitamins, binding and attachment factors and is involved in additional functions, e.g. pH buffering or protease inhibition [8]. However, FBS is known to be an ill-defined medium supplement [9] and several technical disadvantages have to be taken into account. Batch to batch variations due to qualitative and quantitative geographical differences, animal age differences, biosafety aspects, e.g. contamination with endotoxins and mycoplasma and ethical perspectives in term of animal welfare [6], illustrate its weaknesses for use in cell culture and the need to consider species differences. A variety of factors, e. g. proteins and non-coding RNAs, differs between sera of different species and might contribute to species-specific modes of actions regarding cell behavior in cultures [10]. By modeling albumin-ligand interactions major differences in the ligand binding capacities of FBS and HS have been found, an important issue when results are extrapolated on humans in pharmacokinetic studies [11]. For instance, species-specific albumin binding of the antibiotic

Abbreviations: HS, Human serum; FBS, Fetal bovine serum; HPV, Human papillomavirus; DT, Doubling time; STR, Short Tandem Repeat; poly-HEMA, poly-(2-hydroxyethyl methacrylate); SD, Standard deviation; FDA, Food and drug administration; EMA, European Medicines Agency; GCCP, Good cell culture practice; MSC, Mesenchymal stromal cells; ASC, Adipose tissue-derived stem cells

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drug ceftriaxone in FBS and HS has been demonstrated [12]. Folic acid binds stronger to bovine than human serum albumin owing to an additional tryptophan residue [13]. Since folic acid is an ingredient of many cell culture media such discrepancies might influence experimental outcomes of cell cultures. Analysis of human proteome and microRNAs also remains challenging when using serum from other species. Here, foreign molecules will be evaluated in downstream analyses and generated data intermingle with those received from human cells or tissues [14]. These findings lead to the question, whether FBS really mirrors HS sufficiently and therefore, if it is useful in generating human-relevant data. It may be argued that application of sera from different species in human cell culture leads to non-desired influences on results and questionable conclusions.

In the current study, we aimed to investigate the effects of HS on growth behavior, migration and invasion of two immortalized cervical cancer cell lines in comparison to FBS: HeLa, the first established cell line, with human papillomavirus type 18 (HPV-18) genome integration and SiHa containing HPV-16. Since 3D in vitro assays are increasingly applied in cancer research and other fields, we analyzed the impact of FBS and HS on the capability of cells to form spheroids which develop different cell layers and resemble solid tumors in vivo.

2. Material and methods

The Placenta Lab strictly applies quality management and is certified after DIN EN ISO 9001

2.1. Maintenance of cell cultures

SiHa (ATCC® HTB-35TM) and HeLa (ATCC® CCL-2TM) cervical cancer cells (Table 1) were cultivated in DMEM high glucose (Gibco, Paisley, UK) as frequently published [15,16]. For maintenance of cultures, media were supplemented with 10% FBS (Sigma, Steinheim, Germany) and 50 U/ml penicillin/streptomycin (1% P/S; Gibco). Medium was replaced at least every 4 days and splitting was performed as soon as confluence was observed by incubation with 0.05% trypsin-EDTA (Gibco) until passage 65 (SiHa), respectively 75 (HeLa). Cells were maintained under standard conditions (37 °C, 5% CO_2, humidified atmosphere) and regularly screened by PCR for absence of mycoplasma. Immediately before initiating the experiments for this study, Short Tandem Repeat (STR) analyses were performed to confirm identity of cell lines.

2.2. Experimental setup of cell cultures

All experiments have been performed by using one batch each of FBS (Product No. F7524; Lot No. 072M3396; Sigma, Steinheim, Germany) and HS (Catalog No. S1–100 ml; Lot No. 1066D; Merck Millipore, Berlin, Germany), both gender mixed. All sera were aliquoted, stored at −80 °C and have been freshly thawed for each individual experiment. Upon personal information from the manufacturer, one bovine batch contains 1500 l composed by 0.5–2 l serum per animal. Each batch must be free of mycoplasma and several viruses. The manufacturer performs cell culture tests and cloning assays. The batches must fit in specified ranges of pH (6.7–8.0), osmolality (260–340 mOsm/kg) and total protein (3.0–4.5 g %). There are limits for endotoxin (< 10 EU/ml) and hemoglobin (< 25 mg %) concentrations. Osmolality and pH of HS used in our experiments were within the same ranges, but total protein (6.6 g %; manufacturer's information), albumin concentration (4.1 g % versus 1.7 g % in FBS; manufacturer's information) and estradiol (101.7 pmol/l versus 27.9 pmol/l; as assessed by an in-house immunoassay; ELECSYS, Roche Diagnostics, USA) were higher.

For comparison of variability of different sera or batches, we have compared effects of a second FBS batch (Lot No. AVC63371) and self-prepared pooled HS from 3 donors in spheroid formation assays.

Unless otherwise stated, experiments were initiated by replacement of maintenance medium with medium supplemented with 10% FBS or HS (starting point, 0 h) after washing in serum free medium.

2.3. Microscopical analyses of cell morphology

500.000 cells in 2 ml/well were seeded on 6-well plates and allowed to attach overnight in maintenance medium. For starting the experiments (0 h), culture medium was replaced with either 10% FBS or HS. 24, 48 and 72 h after medium change, cells were imaged at an Axiovert 25 CFL microscope (Carl Zeiss, Jena, Germany) using the camera AxioCam ICc1 (Carl Zeiss) to assess cell morphology.

2.4. Metabolic activity assay

In order to compare the influences of HS and FBS on cell metabolic activity a Cell Titer AQeous MTS (3-(4,5-dimethylthiazol-2-yl) − 5-(3-carboxymethoxyphenyl) − 2-(4-sulfophenyl) − 2H-tetrazolium) assay (Promega, Mannheim, Germany) has been applied accordingly to the manufacturer's instructions. Cells were seeded overnight in 96-well plates at a density of 5000 cells per 200 µl/well in maintenance medium. For initiation of experiments (0 h), culture medium was replaced with either 10% FBS or HS. After 0, 24, 48 and 72 h, metabolic activity was assessed and expressed as blank-corrected absorbance at 490 nm using a SPECTROstar Omega (BMG Labtech, Offenburg, Germany) and normalized to that of cells at 0 h. Additionally, assays were performed with different FBS or HS concentrations (0.5%, 5% and 10%) 72 h after replacing the maintenance medium. Experiments were carried out with 1000 HeLa and 2000 SiHa cells per well. Blank-corrected absorbance was normalized to number of respective cells grown in medium without serum supplement after 72 h.

2.5. Proliferation assay

Proliferation was determined using a colorimetric BrdU-incorporation ELISA (Roche Applied Science, Mannheim, Germany). 5000 cells per 200 µl/well were seeded in 96-well plates and allowed to attach overnight. Cell proliferation was assessed 0, 24, 48 and 72 h after replacing maintenance medium with respective serum supplement. After treatment, cells were incubated in fresh BrdU-containing medium for another 2 h. The colorimetric ELISA for BrdU quantification was performed following the manufacturer's instruction. Absorbance was

Table 1
Cell characteristics of cervical carcinoma cell lines examined in this study.

Cell line	Establishment	Tumor pathology	Karyotype (ATCC®)	HPV status	Copy number	E2 ORF intact?	Chromosomal location [43]	In vitro features
HeLa	1952 [44]	Adeno-carcinoma of the cervix	modal chromsome number = 82	HPV-18	10–50	No	primarily 8q24, chromosomes 5, 9, 22	24 h DT, 45% plating efficiency [45]
SiHa	1970 [46]	Squamous cell carcinoma of the cervix	modal chromsome number = 69	HPV-16	1–2	No	13q21-q31	25.9 h DT [47], homogeneous, undifferentiated

DT: Doubling time

measured at 370 and 492 nm and cell proliferation was expressed as corrected absorbance (A_{370}–A_{492}) normalized to that of cells at 0 h.

2.6. Matrigel invasion and migration assay

Cell invasion and migration were evaluated in 24-well inserts with 8 µm pore size transwell membranes (Merck Millipore). To evaluate invasion 100,000 cells in 200 µl were seeded in inserts pre-coated with growth factor reduced Matrigel (BD Biosciences, Heidelberg, Germany). Respectively, 50,000 cells were seeded in uncoated inserts to perform the migration assay. The lower chamber was filled with 500 µl respective medium. After 24 h of culture, cells remaining in the upper chamber were removed via cotton swab, while invaded or migrated cells were fixed under the insert with cooled 80% ethanol and stained with crystal violet. After washing and de-staining with 1% acetic acid, colorimetric absorbance was measured at 570 nm.

2.7. Wound healing assay

Suspensions of 600,000 cells were cultured in 2 ml maintenance medium in 6-well plates for 24 h until confluence was observed. Subsequently, cell motility was tested by scratching with a pipette tip along the axis of the plate [17]. Wells were rinsed with serum free medium to remove free floating cells and cell debris. Medium was replaced with medium supplemented with 1% FBS or HS. A low serum concentration was used to prevent proliferation. 0, 24, 48 and 72 h after medium replacement, cells were imaged as described above. Experiments were carried out in triplicates, whereby per well a total of 6 images for analysis of respective wounded areas were selected randomly (18 images of each condition for one independent experiment). The size of scratched areas was measured by using ImageJ software (version 1.49; National Institutes of Health, Bethesda, ML, USA). The measure was the percentage of repopulated surface on the total scratched area. To compare repopulation of cell free areas data were normalized to that of 0 h

2.8. Spheroid formation assay

Spheroid formation was induced by the hanging drop method as described in Froehlich et al. [18]. To study the impact of different cell numbers on generation of spheroids 1000 or 5000 were seeded in 20 µl drops on the inner side of the lid of cell culture dishes (Greiner Bio-One, Frickenhausen, Germany). Cell suspension was prepared with 10% HS or FBS. 25% methocel (M0512, Sigma) was added to the cell suspension for increasing viscosity. To sustain a humid atmosphere the bottom of the Petri dish was covered with PBS. After inversion, cells were cultured for 72 h and microscopically checked for spheroid formation. Spheroids were further cultured either in HS or FBS to analyze the effect on maintaining spheroid shapes. For this approach, spheroids were transferred into individual wells of a 96-well plate (Cellstar®, F-bottom, Greiner Bio-One) coated with 2% poly-(2-hydroxyethyl methacrylate) (poly-HEMA, Polysciences, Eppelheim, Germany) containing 260 µl medium supplemented with HS or FBS. Half of the medium was replaced every day. After 24 h, microphotographs were taken using a Life-Imaging microscope with incubator Axio Observer Z1 and an AxioCam MRm Rev.3 camera (Carl Zeiss). Images were analyzed by Zeiss ZEN lite software (Carl Zeiss).

2.9. Data analysis and statistics

Experiments were carried out in 3 replicates and each assay was repeated independently at least 3 times. Spheroid formation assays were repeated 5 times, each with 30 independent hanging drops, resulting in up to 150 spheroids. All quantitative data were presented as mean ± standard deviation (SD). Statistical analysis was done by paired two-tailed Student's *t*-test. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. HS supports metabolic activity and proliferation

Cells were cultured in medium with FBS or HS over a period of 3 days to assess metabolic activity. No differences in morphological characteristics were observed between cells cultured in FBS and HS (Fig. 1A). After 48 h, metabolic activity increased in SiHa cells, but the effect was still stronger in HeLa cells (Fig. 1B). Culturing cells in medium with HS slightly reduced the metabolic activity (Fig. 1B). In comparison to cells grown in medium without serum supplement, FBS and HS enhanced metabolic activity after 72 h which was significant for all concentrations in SiHa cells. A 0.5% concentration of FBS or HS increased their metabolic activity by 82 (FBS) or 72% (HS) while sera concentrations of 5% and 10% in culture medium led to a 138–219% higher metabolic activity. In HeLa cells, supplementation of culture medium with 10% FBS or HS increased metabolic activity by up to 405%. At 0.5% concentration FBS, compared to HS, significantly enhanced metabolic activity. In all other cases, the effects of FBS and HS were not significantly different (Fig. 1C).

During 72 h of culture, proliferation as determined by BrdU incorporation was increasing in HeLa and SiHa cells at similar levels in FBS and HS, but with a tendency to higher proliferation of HeLa cells in HS (Fig. 1D).

3.2. HS enhances cell invasion but not migration

In the transwell system, cell invasion was significantly enhanced in both cell lines by using HS compared to FBS as medium supplement. Invasiveness was increased by 23% in SiHa and 43% in HeLa cells. Migration did not differ significantly. Representative images of the transwell assays demonstrate invasion and migration characteristics (Fig. 2A, B).

Additionally, a scratch wound healing assay has been performed to analyze migration potency over a period of 3 days (Fig. 2C). Here, HS slightly enhanced migration of SiHa cells, whereas FBS increased migration in HeLa cells, but both not significantly.

3.3. HS stimulates spheroid formation

FBS supplemented medium induced spheroid formation in HeLa cells (Fig. 3A a, c), whereas only loose aggregates of SiHa cells were formed under these conditions (Fig. 3A i, k). When HS was used as medium supplement, regularly shaped spheroids of both cell lines were generated (Fig. 3A b, d, j, l). Using 25% methocel as an additional supplement improved spheroid formation and spheroid diameter and compactness increased (Fig. 3A e-h, m-p). Nevertheless, SiHa cells did never generate spheroids in FBS supplemented medium (Fig. 3A m, o), but with increasing cell numbers compact aggregates have been formed (Fig. 3A o). Results were summarized in Fig. 4A. For confirmation of these results, we have repeated all spheroid formation assays using a second FBS batch and donor HS (each used for one complete experimental series on SiHa and HeLa cells, 30 spheroids per culture condition). All results were qualitatively similar to the above described (data not shown). To check whether the change of medium composition itself affects experiments, we additionally cultured SiHa cells in HS for up to 5 passages before spheroid formation assay was performed. Here, we could confirm our previous observations showing well-shaped spheroidal structures (complete experimental series on SiHa and HeLa cells, 30 spheroids per culture condition; data not shown).

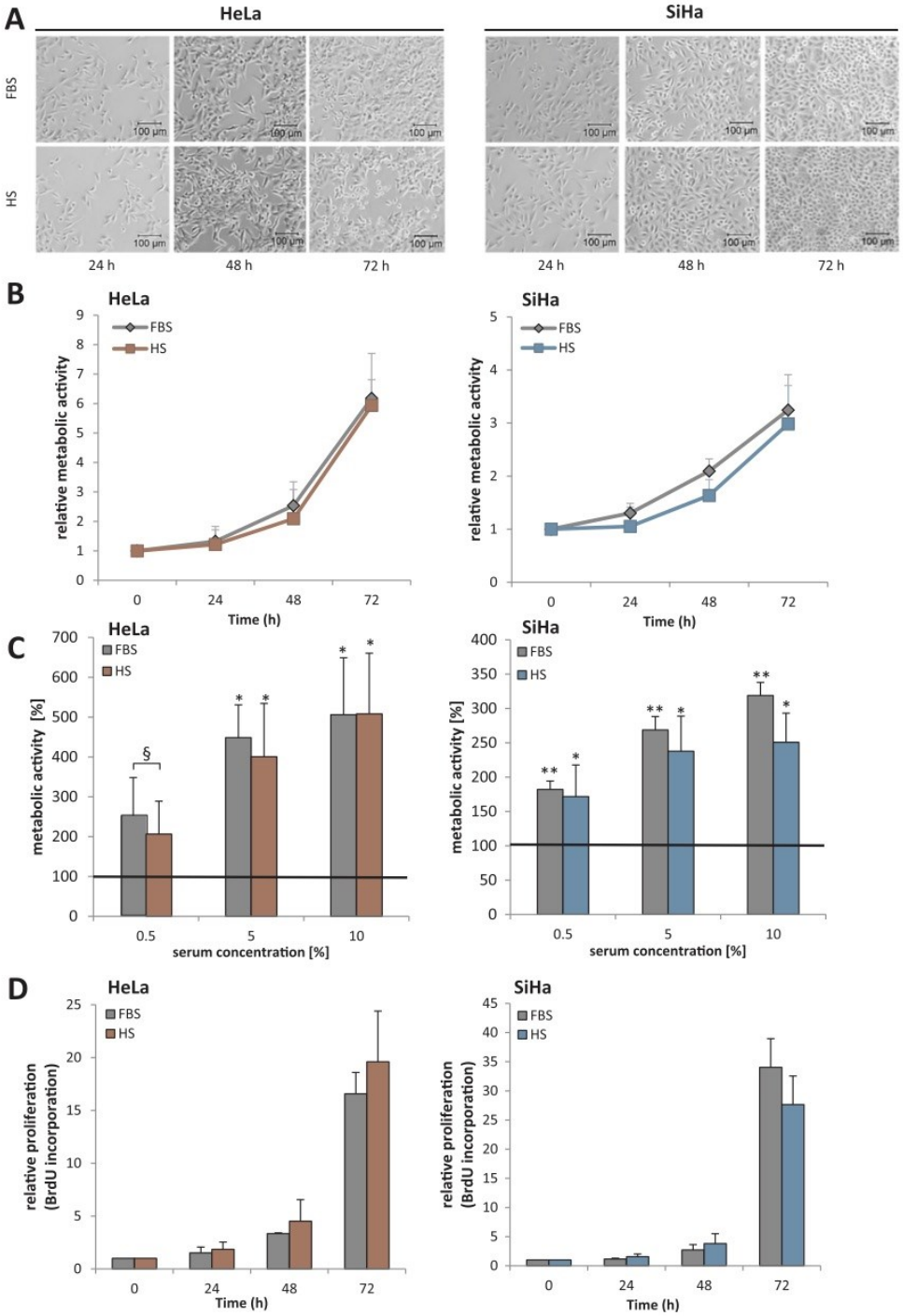

Fig. 1. Serum influence on cell morphology, metabolic activity and proliferation. HeLa and SiHa cells were seeded in 6-well plates and imaged 24, 48 and 72 h after replacing the maintenance medium with medium supplemented with FBS or HS (A). A MTS colorimetric assay was performed on cells seeded in 96-well plates to analyze cell metabolic activity. 0, 24, 48 and 72 h after replacement the maintenance medium with respective serum supplement, optical density (OD) at 490 nm was measured (B) and normalized to 0 h (defined as 1) (B). The assay was also performed with different FBS and HS concentrations (0.5%, 5% and 10%) 72 h after medium replacement. Values have been normalized to those from cultures without serum supplementation (defined as 100%, base line) (C). Respectively, cell proliferation was analyzed via BrdU time-course incorporation and normalized to 0 h (defined as 1) (D). Bars show mean values of n = 3 independent experiments, which have been performed in triplicates. Error bars indicate SD of the mean. *$p < 0.05$, **$p < 0.01$ for culture with vs without serum supplement (base line), $^{§}p < 0.05$ for FBS vs and HS; Student's *t*-test (paired, two-tailed).

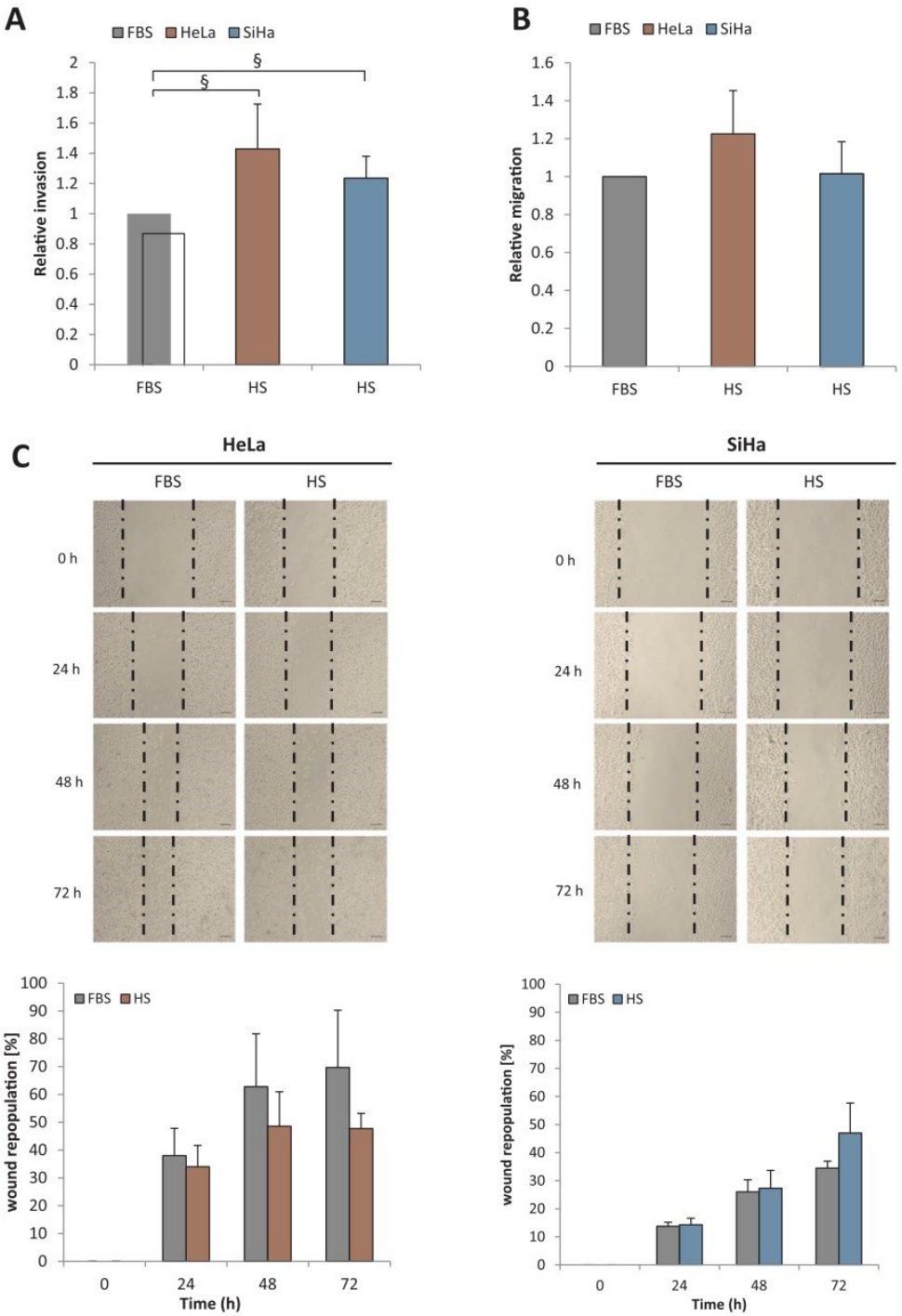

Fig. 2. Cell invasion and migration. HeLa and SiHa cells were seeded in serum free medium on transwell chambers coated with Matrigel (for analysis of invasion; A) or not (for migration; B) and incubated immediately in medium containing FBS or HS. After 24 h, invaded (A) and migrated cells (B) under the transwell membranes have been stained and photographed. Absorbance at 570 nm has been measured on a plate reader and normalized to cells cultured in FBS. Migration was further tested by a wound-healing assay (C). Each independent replicate has been calculated as mean from repopulation of 6 cell-free areas by using ImageJ software and was documented for up to 72 h. Data are expressed as mean ± SD (n = 3, each performed in triplicates). $^{\S}p < 0.05$; Student's *t*-test (paired, two-tailed). Scale bars = 100 µm.

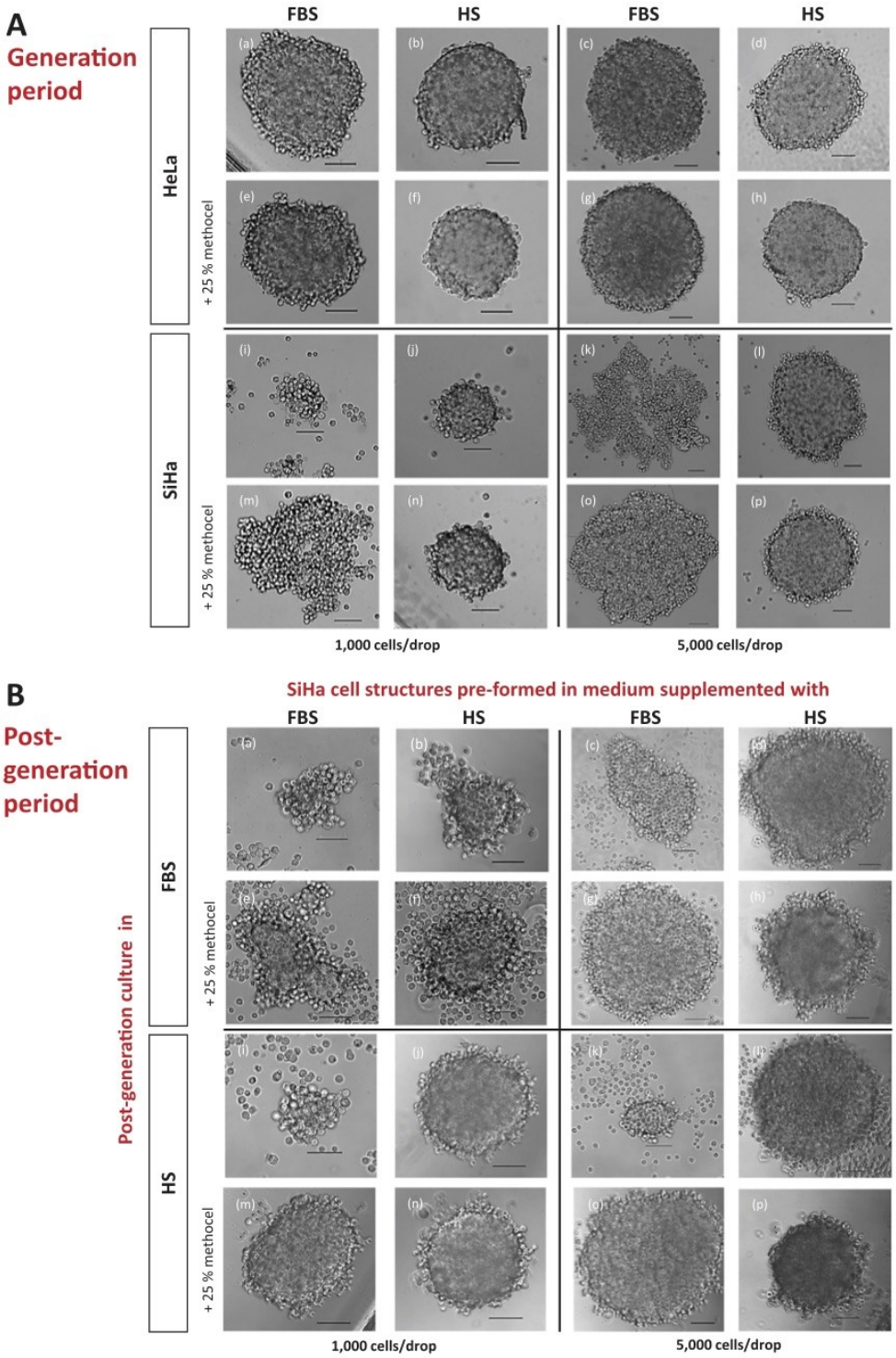

Fig. 3. Spheroid formation capacity. Spheroids were generated via hanging drops (A). HeLa (a-h) and SiHa cells (i-p) were cultured in medium supplemented with FBS or HS. Representative images of most frequently formed structures were taken after 72 h. For a higher viscosity medium was amended with 25% methocel (e-h; m-p). Assay was performed with 1000 and 5000 cells per drop. The influence of HS and FBS on pre-formed SiHa spheroids was analyzed after transfer to poly-HEMA coated 96-well plates and further 24 h culture (B). Scale bars = 100 μm.

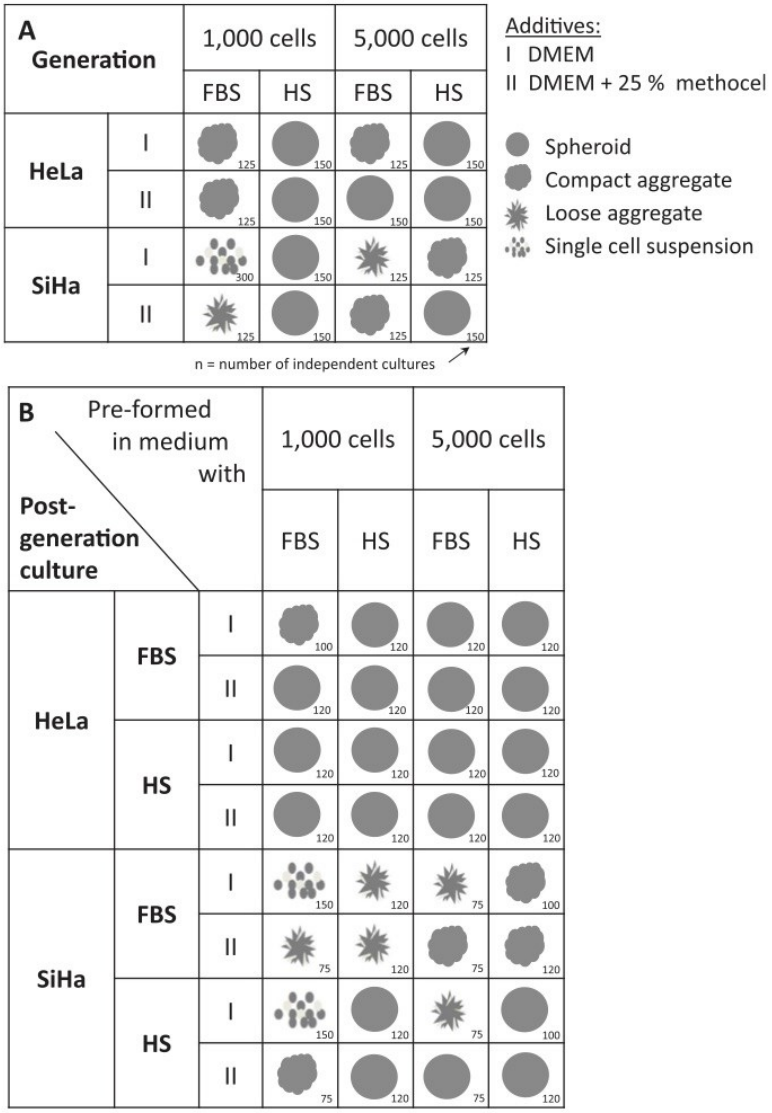

Fig. 4. Schematic summary of spheroid formation results. Representative structures as mostly formed from cervical cancer cell lines after 3 days culture under different conditions (A) and subsequent culture in 96-well plates for 24 h (B). For each culture condition the formed structures resembled > 90% of cases and are represented by respective symbols. n varied between setups due to the different compactness of generated structures (n = 75 to n = 300).

3.4. FBS and HS differently influence spheroid formation during post-generation culture

After their generation (as in Fig. 3 A + 4A), spheroids were further cultured in medium with FBS or HS which affected their structure differently: SiHa spheroids generated in HS supplemented medium disaggregated in 24 h culture in medium with FBS (Fig. 3 B b, f). This impairment was less obvious, but also observed for spheroids formed at a higher cell number (Fig. 3 B d, h). In contrast, culture medium with FBS improved the compactness of aggregates generated in FBS supplemented medium (Fig. 3 B a, c, e, g). Spheroid formation was enhanced in HS supplemented culture medium, even after generation in FBS supplemented medium (Fig. 3 B i-p). The serum influence on spheroids was lower in HeLa than in SiHa cells (Fig. S 1; schematic summary in Fig. 4 B).

4. Discussion

FBS as medium supplement for cell and tissue culture has long been discussed controversially in science. As early as Sato started to investigate those culture conditions, which enable cell growth and survival, it has been realized that media should provide an environment closely resembling in vivo situations [19,20]. Although many disadvantages of using FBS as medium supplement are obvious and serum-free alternatives are promoted by regulatory authorities, e. g. Food and Drug Administration (FDA) and European Medicines Agency (EMA), as well as by numerous research communities, many groups still hesitate with its replacement [21–23]. Since research in biomedical tissue engineering and adult stem cell therapy has become more popular, the implementation of good cell culture practice (GCCP) is required to strengthen the reliance on in vitro methods [24,25]. Indeed, numerous papers have been published investigating xeno-free medium

supplements for human mesenchymal stromal (MSC) and adipose tissue-derived stem cells (ASC) minimizing the risk of microbial contaminations, potentially inducing zoonosis and immune responses, in regard to clinical application [26,27]. Studies on effects of HS on established cell lines are still rare, although first experiments with HS as an alternative serum supplement have been performed in the 1980s. Emerman et al. determined stronger cell proliferation in primary human breast cancer cells induced by HS compared to FBS [28]. Moreover, positive growth effects induced by human AB serum have been reported in the endothelial cell lines HUVEC and HuDMEC as well as the cancer cell lines HT-29, HeLa-229 and MCF-7 [29]. The commercial bovine and human sera as used in our study at least differ in concentrations of total protein, albumin and estradiol which may contribute to their different effects on the observed cell lines. Due to the complexity of the serum composition it is not possible to extrapolate the observed effects on spheroid formation to one or more specific factors. Previous studies have shown the influence of estradiol and albumin concentrations on the growth and differentiation of mammalian cells [30,31]. The estradiol concentration in HS used in our study was approximately 4 fold higher than in FBS. Other authors have demonstrated that invasiveness of HeLa and SiHa cells increases upon stimulation with estradiol by enhancing MMP-2 and MMP-9 expression [32]. Also albumin concentration was higher in HS than in FBS which per se may influence results. Furthermore, the binding capacity of human and bovine albumin may differ as reported. For example, human serum albumin has a higher capacity of increasing cholesterol efflux from human cells than bovine [33]. Apart of concentration differences, the observed effects may also be due to absence or presence of certain factors or their species-specificity in the applied sera.

In our study no significant differences between HS and FBS were detected concerning proliferation and metabolic activity in cervical cancer cell lines. The effects strongly depended on cell line and serum concentration in culture medium. The goal is to provide a culture system resembling the human nature as closely as possible. In this regard, three-dimensional cell culture systems like spheroids present appropriate models to study tumor cell physiology or response to therapeutic agents [34]. Their stratified composition owed by nutrient and signal gradients during cultivation displays growth characteristics of avascular regions of solid tumors and micrometastases [35]. However, spheroid formation capability is cell line dependent and optimized generation protocols are limited [18]. Our investigations show that HS enables or at least improves spheroid formation of tested cell lines including spheroids with diameters > 500 µm and therefore, might contribute to an easier establishment of 3D models.

Simulating in vivo conditions it has to be kept in mind that in humans most cells are rather in contact with interstitial fluid than with blood. Nonetheless, in the course of metastasis malignant cells pass different stages [36] amongst which those of intravasation, survival in the bloodstream and extravasation might be influenced by plasma composition. Further, recent results indicated that in vivo tumor spheroids may serve as carriers for cancer stem cells in bloodstream supporting their survival under hypoxic conditions and thus promoting metastasis [37]. Consequently, in metastasis invasion during the processes of intra- and extravasation and in vivo spheroid formation appear to be central elements. In our study, we could demonstrate that these two features responded differently to FBS and HS. Under both supplements, spheroid formation was similar in HeLa cells, whereas in SiHa cells spheroid formation was possible only by supplementation with HS, even in the absence of methocel. It may be argued that these effects differ between different serum batches as the manufacturers do not guarantee exact composition but ranges of component concentrations. Therefore, we have repeated the spheroid formation assays by using a second batch of both sera leading to similar results. Nonetheless, it cannot be excluded that further batches induce other effects. SiHa spheroids preformed in HS degraded after transfer to medium supplemented with FBS indicating that HS might promote formation of extracellular matrix or adhesion relevant surface factors, e.g. ß1-integrin, potentially necessary for formation of spheroids [38]. Mazlyzam et al. have demonstrated an upregulation of extracellular matrix proteins from dermal fibroblasts by HS [39]. Discrepancies between HS and FBS have been well documented in literature [40] and are often connected with species differences [41]. These may include species-specific molecular interactions, but also variable concentrations of factors in serum from different species. This study demonstrates that quality of serum in regard to its molecular composition affects results of in vitro experiments, potentially depending on the species of origin, but also on individual serum batch composition.

5. Conclusion

In this study, we demonstrated that particular batches of HS and of FBS differently influence the behavior of two epithelial cervical cancer cell lines. Our data indicate that HS supports growth and migration comparably to FBS, but significantly enhances invasion and spheroid formation. Nevertheless, it has to be considered that different batches may induce different effects in the present, but also in other studies. Thus far, although of crucial importance, the analysis of species-specific characteristics of serum factors or ligand-receptor-interactions on cell lines appears to be widely neglected, especially when considering that human cell lines usually are cultured in FBS. Consequences on molecular level have not yet been explored deeply. It cannot be excluded that the high failure rate in preclinical research might, at least partly, result from the fact that human cells are confronted with the serum of a foreign species [42].

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Conflict of interest

The authors declare no conflict of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.yexcr.2018.02.017.

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Manuskript 3: Only humans have human placentas – molecular differences between mice and humans.

Artikel Typ: Review

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Zusammenfassung: Die humane Plazenta gehört zu den Organen, welche im Vergleich zu Plazenten von anderen Spezies eine Vielzahl von human-spezifischen Faktoren und Charakteristika aufweist. Trotz alledem wird für die Erforschung von plazenta- oder schwangerschaftsbezogener Fragenstellungen nach wie vor das Mausmodell am häufigsten verwendet. Um auf die bedeutenden Speziesunterschiede, welche beispielsweise bei den Implantationsprozessen der Trophoblastzellen von Bedeutung sind, (z.B. KIRS auf NK-Zellen, HLA-C) aufmerksam zu machen, wurden in dieser Übersichtsarbeit die typischen Merkmale von Maus- und Menschplazenten gegenübergestellt.

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Mitautorenanteil: André Schmidt (Hauptanteil, Literaturrecherche, Verfassung des Manuskriptes), Diana M. Morales-Prieto (fachliche Beratung), Jana Pastuschek (fachliche Beratung), Udo R. Markert (Betreuung)

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Only humans have human placentas: molecular differences between mice and humans

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ABSTRACT

The placenta is one of the organs with the highest evolutionary diversity among animal species. In consequence, an animal model that reflects human placentation exactly does not exist. However, the mouse is the most frequently used animal model for placenta and pregnancy research. It possesses a hemochorial placenta, which is similar, but also different from the human placenta. The question whether the similarities are sufficient for the achievement of useful results with regard to human pregnancy was debated recently at the 11th Congress of the European Society for Reproductive Immunology (Budapest, Hungary). Here, we discuss the molecular features of the human placenta that are restricted to primates or even to humans. Many of the primate-specific genetic novelties, e.g., the large microRNA cluster on chromosome 19, have been detected during the last 10–15 years and could not be referred to in earlier discussions. Now, in the light of recent findings and a better understanding of interspecies differences, we conclude that the mouse model is often overvalued. Owing to the increasing number of known human-specific factors in human placentation we consider that many aspects of human placentation can only be understood on the basis of experiments on human cells and tissues in combination with data collections from human subject studies.

1. Introduction

The mouse is the most frequently used animal model. Originally, the main reasons for this choice were not the similarity to humans, but the fact that mice are small, have a large litter size and short generation times, all features that are very practical for laboratory work. However, in recent years, the value of animal models in general and the mouse model in particular has been questioned more and more as genomic data on the one hand and experimental outcomes on the other hand have revealed marked differences between humans and all other mammals. It is considered that these differences are at least partly responsible for the slow advances in the treatment of serious diseases or the failure to predict serious adverse effects as in cases such as the disastrous clinical phase I trial of TGN1412 (Stebbings et al., 2007). Indeed, the fact that only a very small portion of the drugs that are successful in preclinical animal models enter the market (Hartung, 2013) clearly indicates that the failure of animal models (but also of the applied in vitro models) is rather systemic than restricted to single events. This is supported by a variety of publications on, e.g., inflammatory diseases (Seok et al., 2013), multiple sclerosis (Raddatz et al., 2014), diabetes (Chandrasekera and Pippin, 2014), Alzheimer's disease (Cavanaugh et al., 2014),

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Table 1
While the human placenta shows characteristics that developed in the course of primate evolution (left) the mouse has traits that are absent from primates (right).

Humans and (some) other primates	Mouse
- Chorioallantoic placenta	- Chorioallantoic placenta and yolk sac placenta
- Fetal placenta with intervillous space	- Placental labyrinth
- Monochorial	- Trichorial
- Trophoblast invasion into the inner third of the myometrium	- Trophoblast invasion restricted to the decidua
- (Hyperglycosylated) chorionic gonadotropin	-Absent in mice
- LHCGR with exon 6a	- Absent in mice
- Gene clusters for placental lactogens/growth hormones and placental galectins	- Gene clusters for prolactin-related genes and cathepsins
- Estrogen synthesis in placenta	- Absent in mice
- KIRs on natural killer cells	- Ly49 expression on natural killer cells
- HLA-C	- H2-K
- HLA-G, HLA-E	- Absence of nonclassical MHC class I molecules on trophoblast surface
- Glycodelin A	- Absent in mice
- miRNA cluster on chromosome 19	- *Sfmbt2 miRNA* cluster on chromosome 2
- Syncytin-1 and -2; suppressyn	- Syncytin-A and -B

Table 2
According to the present knowledge, the human placenta is characterized by a range of human-specific molecular novelties that cannot be found in any other mammals.

Human-specific features of the placenta
- KIR B haplotype
- Siglec-6 expression in placenta
- IMUP-2
- Several miRNAs (e.g., hsa-mir-941); miRNA targets
- Absence of Neu5Gc synthesis
- sFlt1-14

cancer (Ellis and Fidler, 2010), and atherosclerosis (Cullen et al., 2003). Furthermore, Hartung has summarized that the predictability of animal experiments in toxicology of two species is not better than 53–60%, which is close to pure chance (Hartung, 2009). Generally, it is interesting to note that problems with the translation of animal experiments are not restricted to evolutionary distant species like rodent models, but that striking differences exist even between humans and their closest relatives, the chimpanzees (Varki and Altheide, 2005; Bailey, 2011).

Bearing in mind such differences and being aware of the fact that the placenta is an organ with outstanding evolutionary diversity the question arises whether animal models of placentation are rather constructive or confusing with regard to the human biology, especially in the case of the most frequently used mouse model. Here, we aim to highlight several aspects of species differences between these two organisms, indicating that the species mouse should be regarded very critically as a model for human placentation and pregnancy (Tables 1 and 2).

2. Interspecies differences in placental anatomy

In contrast to other potential animal models such as dogs (endotheliochorial) and pigs (epitheliochorial) the mouse possesses a hemochorial placenta, which means that the trophoblast layer is in direct contact with the maternal blood and not separated by endothelium and/or epithelium. Thus, the chorioallantoic placentas of mice belong to the same group as human placentas. However, on further study it becomes obvious that the similarity is rather gross as striking differences can be found when comparing the anatomy, the cell types, and the molecular biology.

Before approaching these differences, probably the most impressive divergence between mouse and human is the fact that mice, in addition to the chorioallantoic placenta, have a choriovitelline placenta – the inverted yolk sac placenta – which becomes active early in pregnancy and persists until term. This kind of placenta, which is typical for rodents, is completely absent from humans, but plays an irreplaceable role in rodent pregnancy with failures leading to embryo malformations (Beckman et al., 1990). The existence of a second placental structure is accompanied by several problems with regard to the value of mouse experiments. The development of the inverted yolk sac placenta can be affected by chemicals and pharmaceuticals (Beckman et al., 1990; Haghighi Poodeh et al., 2012). Principally, with regard to humans, this may result in the possibility of false-positive observations of adverse effects (Holson et al., 2005), one of the central problems in toxicity testing. Further, human placental transfer cannot be mimicked well in mouse models as substances may pass the yolk sac placenta. It is interesting that plutonium is trapped effectively in the yolk sac placenta of mice and rats (Kubota et al., 1993), which may result in the maldevelopment of the yolk sac placenta (National Research Council (U.S.). Committee on the Biological Effects of Ionizing Radiations, et al., 1988). Further, plutonium is discussed as being a potential factor for an increased risk of childhood leukemia (Morgan et al., 1991), leading to the assumption that yolk sac accumulation may have a protective effect on the fetus as the access of plutonium to the fetus should be much more limited. Indeed, this is supported by data showing that whole-body fetal:maternal concentration ratios (C_F:C_M) for plutonium in a comparable state of late pregnancy is 1.3 in baboons, like humans, a species without a yolk sac placenta, while it is 0.06 for rats (Paquet et al., 1998).

Another possible implication is mentioned by Nau (2001): in rodents the chorioallantoic placenta is preceded by the functional yolk sac placenta. Thus, in comparison to rodents the chorioallantoic placenta in primates develops during an earlier period when the embryo is less developed and more sensitive to teratogenic effects. Therefore, even

comparable transfer rates between the chorioallantoic placentas of humans and mice may lead to diverging results owing to different time windows during development of the fetus.

The chorioallantoic placentas of mice and humans are structurally different (Rossant and Cross, 2001). The feto-maternal interdigitations in humans are hallmarked by a villous organization that is more open than the murine labyrinth representing a system of interconnected cavities (Georgiades et al., 2002). The placentas of mice are trichorial while those of humans develop into a monochorial phenotype after a period of two trophoblast layers in the first half of pregnancy (Malassine et al., 2008). The junctional zone in mice consists of cell types and layers that do not exist in this form in humans, e.g., the zone of trophoblast giant cells bordering the maternal decidua basalis (Georgiades et al., 2002). A species difference of central importance is the deep interstitial and endovascular invasion of trophoblast cells into the inner third of the human myometrium. In mice the invasion is restricted to the decidua basalis, a phenotype associated with placental malfunction in humans, e.g., in preeclampsia or fetal growth restriction (Moffett and Loke, 2006). The deep invasion into the inner third of the myometrium is the basis for a proper remodeling of the maternal arteries that is necessary for the optimal nutrition of the human fetus. Further, the deep invasion is accompanied by a range of primate- and human-specific factors, which are discussed below.

The chorioallantoic placentas of humans and mice feature a gross anatomical similarity, but are also hallmarked by differences with regard to feto-maternal interdigitations, trophoblast layers, and depth of extravillous trophoblast invasion. The most remarkable difference is a second placenta type in mice, the inverted yolk sac placenta, which is completely absent in humans.

3. Human and murine endocrinology

The emergence of the chorionic gonadotropin during primate evolution, representing a new hormone appearing in different isoforms with a range of biological functions, is remarkable. Amongst primates, the human chorionic gonadotropin possesses the longest half-life of up to 36 h resulting in an increased relative biopotency (Cole, 2012). Probably the most popular function is the maintenance of the corpus luteum during early pregnancy, but as discussed in detail by Cole (2012) this is just one aspect. Human chorionic gonadotropin (hCG) appears in five distinct variants with hCG and hyperglycosylated hCG being central in human pregnancy. In addition to the promotion of the corpus luteum, hCG, amongst others, supports angiogenesis of the uterine vasculature, controls cytotrophoblast differentiation, and reduces macrophage phagocytosis activity (Akoum et al., 2005). On the other hand, hyperglycosylated hCG is a central player with regard to deep trophoblast invasion into the inner myometrium and a low level of hyperglycosylated hCG is associated with an increased risk for preeclampsia (Bahado-Singh et al., 2002), a disease that does not occur naturally in rodents. HCG acts via the luteinizing hormone/choriogonadotropin receptor (LHCGR) and hyperglycosylated hCG antagonizes a transforming growth factor (TGF) beta receptor. A noteworthy novelty in the primate lineage is the emergence of a new LHCGR type that is characterized by the exon 6a. The biological role of this primate-specific receptor type has not been elucidated so far, but its potential role as a hormone scavenger is discussed (Troppmann et al., 2013).

The appearance of hCG as a new hormone in evolution, indispensable for pregnancy, is not the only endocrinological difference between humans and mice. Striking differences worth mentioning are, for example, a primate-specific gene cluster coding for growth hormones and placental lactogens (Newbern and Freemark, 2011) and 23 murine prolactin/placental-lactogen-related genes in mice compared with only one prolactin gene in humans (Simmons et al., 2008). Further, in contrast to mice, the human placenta is aromatase-positive and thus produces estrogens. This attribute is not only interesting from an endocrinological point of view as it is also suspected to determine the outcome of toxicity tests. First, the level of circulating estrogen during human pregnancy is much higher than in mice and the potential low-dose effects of weak estrogens such as bisphenol A, as reported for mice, are unlikely in humans (Witorsch, 2002). Second, in some cases the aromatase is able to metabolize xenobiotics, contributing to differences in pharmacokinetics compared with rodent models, e.g., observed for methadone (Lu et al., 2010).

Central aspects of endocrinological differences between humans and mice are the existence of different gene clusters and estrogen synthesis in the human placenta with special importance for toxicological issues. From an evolutionary point of view the emergence of hCG in primates, which exists in several isoforms, is amazing.

4. KIR/HLA-C

As mentioned above the deep trophoblast invasion is a central feature of human pregnancy. Apart from the hyperglycosylated hCG several other factors that are restricted to primates act in this process. Uterine natural killer (uNK) cells, which represent up to 70% of the leukocytes at the implantation site (Moffett and Loke, 2006) are of central importance for the regulation of extravillous trophoblast invasion. In humans these uNK cells are equipped with killer-cell immunoglobulin-like receptors (KIRs) that interact with human leukocyte antigens (HLAs). With regard to mice there are several differences. First, natural killer cells in mice do not express KIRs. Instead, they act through members of the Ly49 family, which in turn cannot be found to be functional in humans (Mestas and Hughes, 2004). Second, the HLA system shows a very taxon-specific evolution: HLA-G and HLA-C, which are expressed on the surfaces of extravillous trophoblast cells are restricted to primates, with HLA-C only occurring in humans and great apes (Older Aguilar et al., 2010). HLA-C, which is polymorphic in gorillas, bonobos, chimpanzees, and humans, may play a significant role in trophoblast invasion. There is evidence that an increased risk for preeclampsia occurs in consequence of interactions between inhibitory maternal KIRs on uNK cells and HLA-C2 on extravillous trophoblast cells (Hiby et al., 2004). It was observed that

an inhibitory maternal KIR AA genotype in combination with the expression of fetal HLA-C2, especially when inherited from the father, leads to an increased frequency of preeclampsia, which was supported by population data showing an inverse correlation between HLA-C2 and KIR-AA genotype (Hiby et al., 2004). In a recent publication, Hiby et al. (2014) presented data showing a significant positive correlation between maternal activating KIR2DS1, part of the human-specific B KIR haplotype (Parham and Moffett, 2013), and birth weight when HLA-C2 was inherited from the father. Principally, the potential role of HLA-C is supported by case reports of preeclampsia in chimpanzees and gorillas, but not for gibbons and Old World monkeys, which feature diverging invasion patterns that do not rely on MHC-C. However, because of differences in KIRs it is possible that the risk for preeclampsia in chimpanzees is lower than in humans (Carter, 2011).

The interplay of KIRs and HLA-C appears to be central to trophoblast invasion in great apes, with humans showing species-specific combinations between HLA-C1/C2 and KIR A and KIR B that might influence the risk for preeclampsia.

5. Siglec-6, IMUP-2, and galectins

The KIR/HLA interactions, in addition to the hyperglycosylated hCG are not the only invasion-relevant factors that are absent from rodents. An attribute only defining the human placenta is the expression of sialic acid-binding Ig-like lectin-6 (siglec-6) on trophoblast cells, probably due to human-specific changes in the promoter sequences (Brinkman-Van der Linden et al., 2007). Siglec-6 is upregulated in placentas from women with preeclampsia (Winn et al., 2009, Rumer et al., 2013). Indeed, Lam et al. (2011) discovered that the uterine isoform glycodelin A interacts with siglec-6, thus lowering the invasive potential of trophoblast cells (Lam et al., 2011). It is important to note that glycodelin itself is a primate-specific protein (Soni and Karande, 2010).

Jeon et al. (2010) report on a human-specific protein isoform with a potential role in the placenta, the splice variant IMUP-2 (immortalization-upregulated protein-2), which is involved in pathophysiological processes in preeclampsia. It is expressed significantly more highly in placental tissues of patients with preterm preeclampsia compared with those from women with preeclampsia who went to term. Differences between normal term patients with and without preeclampsia were not observed. Further studies based on HTR-8/SVneo trophoblast cells led to the assumption that IMUP-2 interacts with XIAP (X-linked inhibitor of apoptosis) in the nucleus under hypoxic conditions regulating human trophoblast apoptosis (Jeon et al., 2013).

Cell surface-recognizing galectins are involved in a multitude of biological processes and seem to play multiple roles at the feto-maternal interface (Than et al., 2012). The existence of galectin genes differs partly from species to species. While galectin-5 is reported for rats (Barres et al., 2010) and galectin-6 for mice (Gitt et al., 1998), both are absent from humans. On the other hand, in anthropoid primates the evolution of a galectin gene cluster is reported, which is encoded in humans on chromosome 19 (Than et al., 2009). This gene cluster is expected to play a crucial role in human placentation as three of its five members, the genes LGALS13, -14, and -16, are expressed placenta-specifically and bind preferentially N-acetyl-lactosamine, which is usually bound at the apical membrane of the syncytiotrophoblast. It is suggested that the placental galectins might be involved in the interaction between maternal immune cells and the syncytiotrophoblast by inducing apoptosis of T cells (Than et al., 2009). It is noteworthy that low serum levels of galectin-13 in the first trimester of pregnancy or mutations in LGALS13 (Huppertz et al., 2013) are associated with an increased risk for preeclampsia. In agreement with such findings, Kliman et al. (2012) discuss a galectin-13-mediated mechanism for the conversion of spiral arteries in which galectin-13 regulates maternal immune responses.

The proteins and isoforms discussed represent factors additional to KIR/HLA-C, which seem to be involved in human trophoblast invasion. Siglec-6 and IMUP-2 expression in the placenta are described as human-specific, suggesting that even in our closest relatives, the chimpanzees, trophoblast invasion is at least partly regulated in a different manner.

6. Chromosome 19 microRNA cluster

MicroRNAs (miRNAs) are small regulatory molecules that usually bind to the 3′-untranslated regions (3′UTRs) of mRNAs leading to decreased gene expression by the inhibition of translation or degradation of the mRNA (Berezikov, 2011). For the interaction between miRNA and mRNA the seed sequence at positions 2–7 of a miRNA (Lewis et al., 2005) is of special importance as the target recognition depends (in most cases) on a perfect Watson–Crick pairing between the 3′UTR and the seed region (Bartel, 2009). One single nucleotide exchange in the seed sequence is sufficient for drastic changes in the target repertoire while a mutation in the corresponding target sequence can eliminate the miRNA–mRNA interaction completely. Regulatory miRNAs can vary strongly between different species and they can occur in only one lineage or even in only one species such as the human-specific miR-941, which is also expressed by human trophoblast cells (Morales-Prieto et al., 2012).

In pregnancy, several miRNA clusters seem to play a pivotal role (Morales-Prieto et al., 2013): the miR-371-3 cluster, the chromosome 14 miRNA cluster (C14MC), and the chromosome 19 miRNA cluster (C19MC). While the small miR-371-3 cluster is well conserved in evolution, C14MC features a range of differences between mice and humans (Morales-Prieto et al., 2014). The large C19MC, which is expressed from the paternally inherited allele (Noguer-Dance et al., 2010) encodes for microRNAs that are predominantly expressed in the placenta, but also seem to act in several types of cancer (Flor and Bullerdiek, 2012). C19MC exclusively exists in primates. The role of these miRNAs still has to be elucidated further, but it is known that they are packaged in placenta-derived exosomes during pregnancy (Ouyang et al., 2014). Disorders of C19MC miRNAs have been observed in pregnancy-related pathological conditions. Their detection in peripheral maternal blood demonstrates their potential as novel biomarkers

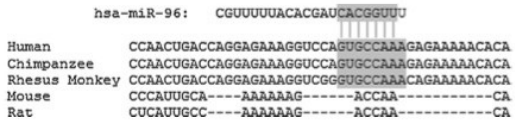

Fig. 1. The sequences of miRNAs are often strongly conserved across species, which is in contrast to the target sites. One such example of a species difference is the regulation of the progesterone receptor expression in the endometrium by miR-96. The figure shows a target site of miR-96 in the 3′UTR of the progesterone receptor, which is conserved in humans, the chimpanzee and rhesus monkey, but not in the mouse and rat. The sequences are taken from TargetScan (www.targetscan.org).

(Morales-Prieto et al., 2014). Further, some of the C19MC miRNAs are able to confer viral resistance to trophoblasts, but also via exosomes to recipient cells (Delorme-Axford et al., 2013).

Principally, it has to be considered that miRNAs with conserved seed sequences or even completely conserved sequences may differ in their target repertoire in different species. For example, computational analyses have shown that only about 10% of predicted target sites are conserved across species (Mor and Shomron, 2013). It is estimated that 30–50% of the nonconserved target sites are functional when the microRNA and the respective mRNA are co-expressed in the same cell (Chen and Rajewsky, 2006). This appears to be logical because microRNAs target numerous mRNAs, it is estimated up to hundreds, and a change in the microRNA sequence is much more dramatic from an evolutionary point of view than a step-by-step modification of the target sequences, which in each case only affects one gene. Examples of such nonconserved targets are known for the progesterone receptor (PGR) in the endometrium of the rhesus monkey (Liu et al., 2012): while miR-96 targets the mRNA of this receptor in human and rhesus monkey it does not in the mouse and rat (Fig. 1). The regulation of PGR through miR-375 is specific exclusively to the rhesus monkey and miR-219-5p regulates PGR via a primate-specific long noncoding RNA (Liu et al., 2012).

The C19MC emerged de novo in primates and is suspected of contributing to maternal immunomodulation. However, conserved microRNAs may also contribute to species differences owing to species- or lineage-specific mutations in 3′UTR target sites.

7. "Trojan Horse" Neu5Gc

It is observed that placental diseases such as preeclampsia are associated with lifestyle factors. For example, Ray et al. (2007) were able to show that immigrants to Western nations show a decreased occurrence of maternal placental syndromes, including preeclampsia, compared with original residents – "the healthy immigrant effect". The existence of lifestyle-dependent factors with regard to pregnancy-related diseases has also been described by others (Brantsaeter et al., 2009). In particular, the so-called "Western diet" appears to be inferior with regard to pregnancies while a Mediterranean diet, for example, correlates with positive effects (Englund-Ogge et al., 2014).

Discussing human-specific factors the evolutionary disappearance of N-glycolylneuraminic acid (Neu5Gc) after the human–chimpanzee split may be interesting when approaching lifestyle-factors connected to placental diseases. Usually, Neu5Gc covers mammalian cells. Because of a human-specific inactivation of the gene CMAH that catalyzes the conversion of N-acetylneuraminic acid (Neu5Ac) to Neu5Gc, human cells are not able to produce Neu5Gc (Irie et al., 1998). Instead, Neu5Gc can enter the human metabolism following the consumption of red meat and milk products. After cellular uptake, in the cytosol it is handled like an endogenous molecule because of its great similarity to Neu5Ac and transferred to the cell surface, which led to the image of Neu5Gc as a molecular "Trojan Horse." In contrast, Neu5Gc is identified as foreign by the immune system with the ultimate consequence of antibody production against it potentially contributing to chronic inflammation, as observed in atherosclerosis (Varki, 2010). As shown by Pham et al. (2009), Neu5Gc is also accumulated in the human placenta (Pham et al., 2009). Being aware of the central role of immune balance in hemochorial placentation we think that this "human-specific lifestyle factor," which is strongly connected to a "Western diet," should be analyzed with regard to potential negative influences on human placentation, especially when considering that pregnant women often increase their consumption of red meat with the aim of improving iron supply.

Neu5Gc appears to be a factor that might contribute to various diseases in humans. Human subject studies on lifestyle and Neu5Gc accumulation in placental tissue indicate its possible implication in pathological conditions such as preeclampsia.

8. Conclusion

Above we listed several aspects of interspecies differences between mice and humans, being aware of the fact that we have just scratched the surface and that there is an enormous amount of other differences among species. For instance, we did not discuss primate-specific syncytins (Dupressoir et al., 2012) and suppressyn (Sugimoto et al., 2013), HLA-G/HLA-E expression on human trophoblasts (Madeja et al., 2011), rodent-specific evolution of placental cathepsins (Mason, 2008) or mouse-specific microRNA regulation, e.g., with regard to the Sfmbt2 cluster (Zheng et al., 2011). The role of sFlt1-14, a soluble human-specific splicing variant of vascular endothelial growth factor receptor 1, in preeclampsia also remains to be elucidated (Sela et al., 2008). The last common ancestor of mice and humans is estimated to have lived about 75 million years ago (Winter et al., 2004), which allowed numerous genomic modifications over time. The existence of human-specific factors shows that even between closely related nonhuman primates and humans profound differences exist, but they are surpassed by the differences between mice and humans, leading to serious doubts as to whether mouse models can mimic human placentation in sufficiently. Principally, the use of transgenic mouse models expressing human proteins or microRNAs also appears to be of limited value, because in such cases the human molecules have to act in a molecular environment, they are evolutionarily not adapted to, thus escaping, for instance, species-specific ligand–receptor interactions, post-translational modifications or regulation of gene expression. For example, with

a high degree of probability, microRNAs with identical sequences target diverging mRNA patterns in different species owing to nonconserved target sites.

It is noteworthy that many of the factors presented are not restricted to the placenta. For example, KIRs are generally important in human killer cell functions (Campbell and Purdy, 2011), variants of hCG, members of the C19MC (Flor and Bullerdiek, 2012), and IMUP-2 (Kim et al., 2008) are associated with human cancer and Neu5Gc may act systemically, e.g., also contributing to the increased risk for cancer or atherosclerosis (Padler-Karavani et al., 2008; Pham et al., 2009).

In conclusion, the work with human cell lines, in particular human primary cells, and original placental tissues in combination with human subject studies, appears to be superior to using not only mouse models, but also other animal models, when analyzing placental diseases or toxicological issues.

Conflicts of interest

None.

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Manuskript 4: Breast cancer, placenta and pregnancy

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Zusammenfassung: Es erfolgte eine Literaturrecherche und eine anschließende Übersichtsdarstellung zum Thema Mammakarzinom während der Schwangerschaft, wobei insbesondere der Einfluss der Schwangerschaft auf das Mammakarzinomwachstum und -verhalten diskutiert wurde. Dabei wurden die plazentaren Zellbestandteile (bspw. Amnionepithelzellen) und die von der Plazenta sezernierten Hormone (Estrogene, Progesteron, hCG) als mögliche Einflussfaktoren umfassend analysiert. Außerdem erfolgte eine thematische Aufarbeitung aus immunologischer Sicht, wobei die „fetal antigen hypothesis“ als möglicher Faktor für eine spätere Immunisierung der Mutter gegen Onkoproteine nach einer Schwangerschaft diskutiert wurde.

Eigenanteil: Hauptanteil, Literaturrecherche, Erstellung der Abbildung, Verfassung des Manuskriptes.

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Breast cancer, placenta and pregnancy

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Abstract

Breast cancer is one of the most frequently diagnosed malignancies during pregnancy and tumors are often found at an advanced stage compared to non-pregnant women. Furthermore, they often present adverse pathological factors as hormone receptor negative/HER2 positive and triple-negative tumors. In general, pregnancy, including the post partum period, is associated with a transiently increased risk of developing breast cancer, but followed by a long-lasting protective period. Further, placental metastases are very rare and, until now, no breast cancer metastases in the fetal circulation have been described. In order to understand these supposedly contradictory observations more profoundly, this review discusses immunological and hormonal alterations during pregnancy potentially affecting tumor growth and behavior as well as breast cancer risk. Comparing gene expression of parous and nulliparous women, an up-regulation of breast cancer associated genes, which are involved in immunological and reproductive processes, has been observed and might be a reason for the transiently increased risk post partum. In contrast, maternal immunization against antigens found on trophoblast cells might prevent breast neoplasms development in later years. Animal models and human studies indicate that T cells may be involved in these processes. The pregnancy related increase of estrogen, progesterone and other hormones may also influence the growth and histopathology of breast cancer. Simulating pregnancy-specific conditions in human by a co-culture model comprising placental explants and hormone positive breast cancer cells, a down-regulation of estrogen α receptor was detected. Additionally, breast cancer cells proliferate less closely to the placental area, which may be an evidence for the low rate of placental breast cancer metastases. Even if the impact of hormonal milieu during pregnancy seems to be established the function of further placenta-secreted factors on cancer development is still underestimated and needs to be investigated in further studies.

Keywords: breast cancer, pregnancy, placenta, trophoblasts, fetal antigen hypothesis, MUC-1

1. Breast cancer in pregnancy

Although breast cancer and pregnancy is a rare coincidence (Stensheim et al. 2009), this disease is one of the most frequent malignancies during gestation. Having an incidence of 1 in 3,000 to 1 in 10,000 pregnancies (Loibl 2008, Loibl 2009) it seems to become the most frequent malignant disease since the decline of cervical cancer (Amant et al. 2012, Loibl et al. 2006, Van Calsteren et al. 2010). Pregnancy associated breast cancer (PABC) is defined to occur during pregnancy (Ibrahim et al. 2000) or lactation period up to 12 months post partum (Smith et al. 2001). Studies analyzing the effect of pregnancy on its prognosis extended this time period up to 2 or 10 years (Ishida et al. 1992, Bladstrom et al. 2003). Although it remains overall low, an increasing coincidence due to the tendency of delaying family planning is expected (Loibl 2008, Voulgaris et al. 2011, Cardoso et al. 2012).

Pregnant or breastfeeding cancer patients are often diagnosed with a delay of 2 to 15 months leading to a 2.5-fold higher risk for diagnosis at an advanced stage (Woo et al. 2003, Zemlickis et al. 1992, Ishida et al. 1992). Reasons may be due to several factors: At first, diagnosis itself becomes more difficult at an early stage of malignancy due to physiological changes during gestation, e.g. growing of mammary glands and milk ducts (Gonzalez-Angulo et al. 2005). Secondly, young women, pregnant or non-pregnant, present denser breast tissue and are not routinely screened by mammography (Polyak 2006). Thirdly, tumors of pregnant women present more often adverse pathological patterns, such as estrogen receptor (ER) α and progesterone receptor (PR) negative and human epidermal growth factor receptor 2 (HER2) positive expression, which are associated with higher tumor-aggressiveness (Baulies et al. 2015, Gogia et al. 2014, Genin et al. 2012). Overall, it should be considered that advanced tumor stages might also be related to the relatively young age of expectant mothers, since women below the age of 40 are generally diagnosed with more aggressive subtypes of breast cancer (Azim und Partridge 2014, Pavlidis und Pentheroudakis 2005, Ishida et al. 1992, Middleton et al. 2003, Genin et al. 2012).

Treatment of PABC

Although breast cancer in pregnancy is associated with a higher mortality risk in general, the prognosis is not principally connected with a worse outcome, if treatment schedule is identical to that in non-pregnant patients (Cardonick et al. 2010, Ring et al. 2005). Therefore, it is important to begin systemic chemotherapy immediately after completion of first trimester, when fetal organogenesis is completed. First guidelines for breast cancer treatment during pregnancy have been developed by the German Breast Group (Loibl et al. 2006) demonstrating pregnant breast cancer patients can be treated according to recommendations for non-pregnant women (Loibl et al. 2012). Anthracyclines in combination with cyclophosphamide, fluorouracil or taxanes are the most common chemotherapeutics, whereas hormonal and anti-HER2 therapies have to be avoided because delivery malformations as well as cases of abnormally low levels of amniotic fluid have been reported (Berger und Clericuzio 2008, Beale et al. 2009). Apart from this, the applied chemotherapeutic drugs seem to be well tolerated by the fetuses when applied in the second or third trimester and newborns have no higher risk for developing malformations (Berveiller und Mir 2012, Mir et al. 2008, Mir et al. 2010). This may occur due to low transport across the placental barrier because drugs such as doxorubicin, paclitaxel and vincristine are substrates for different ATP-binding cassette (ABC)–transporters (Yan und Sadee 2000, Gedeon und Koren 2006). Especially the efflux pumps ABCB1, known as phospho-glycoprotein (P-gP), as well as ABCG2, known as breast cancer resistance protein (BCRP), are involved in feto-maternal exchange processes and may support fetal protection (Iqbal et al. 2012, Aye und Keelan 2013, Evseenko et al. 2007). For instance, the anthracycline doxorubicin is a substrate for both efflux pumps, P-gP and BCRP and is known to be well tolerated during pregnancy (Gedeon und Koren 2006). Nonetheless, a reduced birth weight in newborns from PABC patients is often observed (Amant et al. 2015), which may be a consequence of disturbed placentation, impaired placental tissue and reduced nutrition transfer (Lala und Chakraborty 2003).

2. The influence of immunological factors on breast cancer risk

During the post partum period, women have transiently an increased risk of developing breast cancer, but this turns back into a long-lasting protective period (Lambe et al. 1994), which is described as dual or cross-over effect (Lyons et al. 2009, Janerich 2001). This increased risk has been supposed to persist for 10 to 15 years after pregnancy (Lambe et al. 1994, Albrektsen et al. 2005, Chie et al. 2000). If primiparous women are older than 30 years, this period extends to 30 to 50 years (Chie et al. 2000, Liu et al. 2002, Lambe et al. 1994, Albrektsen et al. 2005). Placentation is enabled by a maternal transient immunosuppressive state. Hence, a decreased immune surveillance is discussed to worsen the outcome of women diagnosed with breast cancer shortly after delivery (Polyak 2006), whereas a positive effect of immunological alterations during gestation has been considered to lead to a long-lasting protection against breast cancer (Barton et al. 2014). Moreover, multiparity seems to decrease the breast cancer risk (Agrawal et al. 1995).

Genexpression in pregnant women

In breasts of parous women regulation of several genes is changed as evidenced by an up-regulation of 238 genes as well as a down-regulation of 48 genes (Santucci-Pereira et al. 2014). Up-regulated genes are mainly related to immune response, e.g. CCL5, CD48 and IL7R, but usually decrease after delivery rapidly back to nulliparous levels. The chemokine CCL5 affects T cells and recruits leukocytes into inflammatory sites. Additionally, proliferation and activation of NK cells is induced by CCL5 in combination with cytokines (Maghazachi et al. 1996, Donlon et al. 1990). CD48, which is found on the surface of lymphocytes, dendritic and endothelial cells, induces their activation and differentiation (Mellman und Steinman 2001). The activation of lymphocytes is also triggered by IL7R, which is expressed on the surface of T cells. Its absence or functional defects leading to immunodeficiency (Noguchi et al. 1993). These activated genes may influence the short-term increased risk of breast cancer after gestation. Other up-regulated genes, which are also related to immune responses, e.g. CD38 and CXCL10, or to developmental processes, as mainly DKK3 and LAMA2, decrease their activity, but do not return to nulliparous levels after pregnancy (long-term changing genes). For instance, CD38 is expressed on the surface of several immune cells, such as CD^{4+} and CD^{8+} T cells, NK cells, B lymphocytes has activating functions (Malavasi et al. 1994). CXCL10, which is secreted by monocytes, endothelial cells and fibroblasts in response to interferon γ (IFN-γ), supports chemoattraction for different immune cells leading to anti-cancer and anti-angiogenic effects (Luster et al. 1985, Dufour et al. 2002, Angiolillo et al. 1995). To sum up, these two groups of genes may be involved in processes explaining the described cross-over effect, but experimental studies addressing this issue remain to be performed.

Glycoprotein induced immunomodulation

Moreover, pregnancy and breast feeding are assumed to induce protective anatomical and molecular changes in mammary glands. Here, the maternal immune system may be primed against pregnancy-related antigens, which are also expressed in breast cancer tumors (Bremner et al. 1981, Dell'Uomo et al. 1990, Sarcione et al. 1983). In clinical studies parous compared to nulliparous women were naturally immunized against antigens found on breast, ovarian and endometrial cancer cells (Katsanis et al. 1998, Shields et al. 1997). This observation has been described first by Janerich and termed as “fetal antigen hypothesis” (Janerich 1994). It has been suggested that a stimulation of the maternal immune system is caused by fetal antigens, which are similar to antigens on breast cancer cells and thereby, induce the protection against breast cancer (Agrawal et al. 1995). Here, the transmembrane mucin (MUC) glycoproteins, especially MUC-1, are discussed to be involved in molecular mechanisms (Beatson et al. 2010). MUC-1 comprises a membrane-bound beta-subunit and an extracellular alpha-subunit, which are subsequently characterized by a variable number of tandem repeats (VNTRs) (Vlad et al. 2004). In these VNTRs serine and threonine residues are strongly modified by O-linked glycans (Taylor-Papadimitriou et

al. 2002, Beatson et al. 2010). MUC-1 is present in normal human epithelium helping to create a protective barrier against pathogens (Kim 2012). However, MUC-1 is also one of the major cell surface mucins expressed in various malignant tissues, e.g. breast and ovaries playing a key role in cell communication in order to promote breast cancer growth (Parry et al. 1990). Interestingly, glycosylation patterns of MUC-1 are different in both cases: in healthy tissue MUC-1 is O-glycosylated with long core-2 glycans, whereas it carries truncated O-linked glycans in cancer cells (Beatson et al. 2010). Consequently, these aberrant glycosylated mucins on cancer cell surfaces are characterized by specific antigens, which are absent in healthy tissues and therefore, can be considered as neoantigens evoking immune response (Beatson et al. 2010). At the maternal fetal interface MUC-1 is secreted by uterine cells required for blastocyst growth and development. Likewise, this glycoprotein is essential for immunological processes during implantation and prevents alloreactivity (Redzovic et al. 2013, Dekel et al. 2010). Aberrant glycosylation patterns are also present in the human placenta and the lactating breast with mastitis, which exhibit similarities to those on cancer cells. Hence, the human placenta is assumed to express these normally tumor-specific epitopes (Jerome et al. 1997, Jeschke et al. 2002, Terada 2013). For example, Thomsen-Friedenreich disaccharide Galβ1–3GalNAc (TF) antigen, which is expressed in approximately 90% of cancer types and also on trophoblast cells, probably supports galectin-mediated interactions between syncytiotrophoblast and endometrium cells (Jeschke et al. 2009). In general, these aberrant glycosylated mucin epitopes might activate the maternal immune system during pregnancy helping to eliminate arising cancer cells later in life. Indeed, Croce et. al. analyzed 149 serum samples and found increased MUC1 levels in pregnant compared to non-pregnant women. Elevated values of IgM- and IgG-anti-MUC1 circulating antibodies have been detected in non-pregnant women, while lactation raises IgG-anti-MUC1 significantly (Croce et al. 2001). Here, the authors suggest that IgG antibodies are involved in the anti-carbohydrate epitope response. This hypothesis of the triggered lactation effect is further supported by two case-control studies and one case report of puerperal mastitis. Women suffering from mastitis had significant higher levels of anti-MUC1 antibodies (Cramer et al. 2013, Jerome et al. 1997). In conclusion, prior mastitis is associated with a significantly lower risk for ovarian cancer (Cramer et al. 2013). Especially activated cytotoxic CD^{3+}/CD^{8+} T cells (CTL) proliferate in response to MUC-1 and consequently recognize MUC-1 peptide sequences exposed on tumor cells. These cytotoxic T cells are able to lyse MUC-1 expressing breast tumor cells (Barnd et al. 1989, Jerome et al. 1991). In a clinical study, a higher MUC-1 expression on primary breast cancer tumors is associated with a better prognosis. The steroid hormones progesterone and estrogen, which are highly produced during human pregnancy, induce an up-regulation of MUC-1 expression on cancer cells (Brockhausen 1995).

Gender-specific aspects

Furthermore, the sex of the first child is also discussed to have an influence on breast cancer survival in young women (Olson et al. 2015), but studies reveal controversial results. It has been reported that primiparous women giving birth to a boy have an increased risk of early mortality when diagnosed with breast cancer (Janerich et al. 1994), but other studies could not observe any sex-dependent correlations (Albrektsen et al. 1995). Here, again immunological mechanisms have been suggested (Olson et al. 2015).

Animal studies

Some experimental animal models have been established to investigate the interaction between breast cancer and placenta tissue. For instance, splenocytes of parous rats develop a slight cytotoxicity against mammary tumor cells resulting in a reduced tumor aggressiveness after delivery. A re-stimualtion of these splenocytes by irradiated mammary tumor cells leads to an enhanced cytotoxic effect induced by cytotoxic T cells (Chakravarty und Sinha 2000). Due to the ability to reactivate the cytotoxic response of splenocytes, a long-term T cell memory has been suggested. In another study using mice it has been demonstrated, that CD44hi T cells with long-term memory

phenotype can survive *in vivo* for up to 70 days. Hence, it is considered that repeated pregnancies stimulate already existing mammary tumor targeting T cells. This might be an explanation for a lower breast cancer risk after multiple pregnancies (Belardelli et al. 1998). Additionally, foreign antigens isolated from rats have been detected in embryonic and placental cells, e.g. alpha fetoprotein, alpha-2 glycoprotein, beta-1 glycoprotein and placental lactogen (Sinha et al. 1988). Fetoplacental tissue and mammary tumors may produce several similar of such antigens, which further immunize the mother. This immunity remains in the mother after pregnancy.

Tumor- and pregnancy-associated proteins

In general, the progression of a malignancy is determined by multifactorial interaction between cancer cells and the host immune system. Cancerous cells are able to acquire the ability to escape from immunological surveillance, and moreover, to suppress immune responses (Saito et al. 2000). Therefore, receptor binding cancer antigen expressed on SiSo cells (RCAS1) seems to play an important role in immune-modulation of the tumor microenvironment (Nakashima et al. 1999, Sonoda et al. 2006, Sonoda et al. 2007, Dutsch-Wicherek und Wicherek 2008).It has been suggested that RCAS1 is also a determining factor in the development of immune tolerance during pregnancy. The protein has been detected in soluble as well as membrane bound forms in trophoblast, decidua and endometrium cells (Wicherek et al. 2005b, Wicherek et al. 2005a, Wicherek et al. 2006). Decreased levels of RCAS1 in blood serum have been measured during labor. Its physiological function seems to be the activation of maternal cytotoxic cells (Wicherek et al. 2008, Szekeres-Bartho et al. 1986). Recurrence of cancer is accompanied by an increase of RCAS1 levels in blood serum, whereby preeclamptic patients, who exhibit decreased RCAS1 serum levels in general, consequently suffer less frequently from neoplasms (Tskitishvili et al. 2008). RCAS1, also known as estrogen-responsive protein EBAG9 (Nakashima et al. 1999), was originally identified by monoclonal antibody (mAb) 22.1.1, which increases in mice after immunization with the human uterine cervical adenocarcinoma cell line SiSo (Sonoda et al. 1996). Subsequently, studies demonstrated that RCAS1 is not recognized by mAb 22.1.1 itself, but it recognizes the tumor-associated O-linked glycan Tn (N-acetyl-D-galactosamine, GalNAc) (Engelsberg et al. 2003). As described before, aberrant carbohydrate epitopes frequently occur on malignant cells, especially Tn and TF are often found on tumor surfaces and their impact on tumor pathology mechanism remains unknown (Springer 1984). Since early embryonic tissue also expresses these glycan epitopes it may be expected that RCAS1 associated truncated glycans may induce long term immunity against breast cancer as described for MUC-1. As RCAS1 is estrogen-inducible the high estrogen levels during pregnancy may influence the development of malignancies. RCAS1 impairs the cytotoxic activity of CTLs by formation/exocytosis of secretory lysosomes, which triggers the development of tumors (Wolf 2010).

The pregnancy-related immunomodulator placental immunoregulatory ferritin (PLIF) is involved in regulating immune responses against the embryo, but is also expressed in breast cancer manipulating the cytokine network, immune responses and tumor microenvironment. When the protein is blocked by anti-C48 treatment in a mouse model, an inhibition of placental and fetal growth as well as tumor growth can be observed (Halpern et al. 2007). In general, enhanced activity of immune suppressing factors due to the coincidence of breast cancer and pregnancy may worsen the survival of patients.

Glycodelin (GD), initially described as progesterone-associated endometrial protein, is a glycoprotein with a molecular weight of 28 kDA (Jeschke et al. 2005) consisting of 180 amino acids. The GD gene (gene name PAEP) is located on chromosome 9q34 (Kolbl et al. 2014, Schneider et al. 2015). Its glycosylation is unique and includes fucosylated LacdiNAc structures, which are uncommon in mammals (Jeschke et al. 2007). Various GD isoforms with different glycosylation and biological activity have been reported: GD-A (expressed in endometrium, decidua, amniotic fluid and maternal serum) GD-C (cumulus oophorus), GD-F (follicular fluid and oviduct) and GD-S (seminal vesicles and seminal plasma) (Lenhard et al. 2013, Seppala et al. 2009). GD-A, a member of the lipocalin protein

family and serves as a differentiation marker (Lenhard et al. 2013, Jeschke et al. 2005, Schneider et al. 2015, Amir et al. 2009). Lipocalins belong to the calycin-protein family and are available in almost all organisms from eubacteria to eukaryotic cells (Kolbl et al. 2014). As the name progesterone-associated endometrial protein suggests, the GD gene expression and GD secretion from the luteal and decidual endometrium are correlate with progesterone levels (Uysal et al. 2015, Tseng et al. 1999, Vigne et al. 2001). Also relaxin and human chorionic gonadotropin (hCG) stimulate glycodelin synthesis and secretion by endometrium cells (Vigne et al. 2001).

Increased levels of GD-A are associated with enhanced endometrial secretory functions (Mylonas et al. 2000). In an ovulatory cycle, GD-A secretion is absent during the proliferative phase (Ohta et al. 2008) and starts 4-5 days after ovulation persisting until the next menstrual cycle indicating its involvement in implantation (Tseng et al. 1999, Mylonas et al. 2000). The increased production of GD-A in early pregnancy is mainly effected by decidual cells (Tseng et al. 1999). Between the 6th and 12th week of pregnancy the GD-A concentration reaches a maximum (Vega et al. 2000). Hence, decreased expression of GD in decidual tissue indicates disturbed functions in early pregnancy (Toth et al. 2008). In contrast, in premalignant conditions like hydatidiform mole with upregulated hCG levels, GD expression is increased (Toth et al. 2008). This relationship might be caused by a feedback mechanism between hCG and GD expression (Toth et al. 2008). GD exerts immunosuppressive and immunomodulatory functions in the endometrium, especially during pregnancy, supporting implantation and maintenance of pregnancy (Tseng et al. 1999, Vega et al. 2000). These properties may also influence tumorigenesis of different malignancies (Lenhard et al. 2012). GD-A induces apoptosis of NK cells, T cells, monocytes, B cells and is involved in the suppression of cell proliferation (Schneider et al. 2015). GD expression is also associated with cell differentiation and consequently growth reduction, which might be interesting for further studies in cancer research (Seppala et al. 2002). Specific monoclonal antibodies against GD-A have been developed to further investigate its role in endometrial development, maintaining pregnancy, and tumor progression (Jeschke et al. 2006). These antibodies are able to detect GD-A in breast cancer tissue and may be useful for further studies in PABC research (Jeschke et al. 2006).

3. The effect of pregnancy associated hormones on growth and behavior of breast cancer cells

The placenta is the central organ for feto-maternal exchange, maintaining pregnancy and supporting the development and health of the growing fetus by providing nutrients and oxygen (Newbern und Freemark 2011). These functions are accompanied by the placental production of progesterone, estrogens, placental lactogen, placental growth hormone and chorionic gonadotropin (hCG) (Carter 2012) leading to a significant change of the hormonal milieu in pregnant women, which may influence growth and behaviour of breast cancer cells. Additionally, it must be considered that the main estrogen, estriol, is produced at significant amounts during pregnancy by the fetoplacental unit. However, respective published data are limited. Some authors assume that breast cancer recurrence may be stimulated by the increased levels of sex-specific hormones during pregnancy and consequently worsen the survival (Azim et al. 2011). In contrast, a significant improvement in the overall survival for women, who became pregnant following breast cancer diagnosis compared with women who were diagnosed with breast cancer but did not become pregnant afterwards has been demonstrated by an meta-analysis (Azim et al. 2011). This phenomenon is called “healthy mother effect” and was first described 1994 by Sankila et al. (Sankila et al. 1994). Combined with the rarity of breast cancer metastases in the placenta, it is discussed, whether the placenta might present a non-supportive microenvironment for cancer cells in general and especially for breast cancer cells (Epstein Shochet et al. 2012). In fact, only 87 cases of maternal tumors metastasizing to the placenta or even to the fetus have been reported from 1966 to 2002 (Alexander et al. 2003). Here, 15 cases of breast cancer metastases were described, but none with fetal involvement. Indeed, maternal-fetal transmission of tumor cells seems to be very rare. However, it has been shown experimentally in *ex vivo* one-sided perfused placentas that T cell leukaemia cells principally can cross the placental barrier (Schamberger et al. 2013). A co-culture model of first trimester

placental tissue and hormone positive breast cancer cells (MCF-7/ T47D) established by Tartakover-Matalon et al. revealed a decrease of breast cancer cells placed close to the placental explant. Additionally, the cancer cells present a decreased ERα level after co-cultivation with placental explants (Tartakover-Matalon et al. 2010). In general, ERα promotes breast cancer cell proliferation, survival and motility (Cortez et al. 2010). Likewise, an extensive cross talk between progesterone and 17beta-estradiol exists: Progesterone reduces ERα levels and exhibits some anti-estrogenic effects and vice versa (Sukocheva et al. 2009). Interestingly, most studies have demonstrated that ERß acts as negative modulator of ERα indicating a good prognosis (Li et al. 2013, Lazennec 2006). Even in triple negative breast cancer cells, which do not express ERα, PR and HER2, but ERß (Haldosen et al. 2014), ERß agonists reduce invasiveness and tumor progression (Hinsche et al. 2015). Therefore, the expression of ERß in the breast may also influence the growth of breast cancer during pregnancy. Gene expression analyses of the breast showed, that parous women have significant twofold higher ERß expression compared with nulliparous women (Asztalos et al. 2010). HCG is one of the most important pregnancy-related hormones, mainly produced in early pregnancy. Its expression decreases in the second trimester, but rises again towards delivery (Gude et al. 2004). The appearance of hCG within tumors is described to be an evidence of differentiation leading to a protective effect on breast cancer (Russo und Russo 2000). A clinical study has demonstrated that hCG given as a breast cancer treatment reduces tumor proliferation and progression (Janssens et al. 2007). This anti-proliferative effect has been confirmed on breast cancer cells in experimental studies (Bodek et al. 2003, Rahman und Rao 2009). Therefore, hCG is discussed to be a natural chemopreventive agent (Kuijper et al. 2009).

4. Anti-tumor effects of placenta derived components

Besides the effects of immunological and hormonal factors, special cell types of the human placenta and extraembryonic tissue have been suggested to be a potentially useful for inducing apoptosis in cancer cells. For instance, human amniotic epithelial cells (hAM) activate cancer cells towards apoptosis and have anti-angiogenic effects (Niknejad et al. 2014). In fact, these cells express thrombospondin-1, endostatin and heparin sulphate proteoglycan. Further, tissue inhibitors of metalloproteases (TIMP-1, -2, -3 and -4) with a potential anti-angiogenic effect have been detected in amniotic membrane (Hao et al. 2000). Therefore, application of amniotic epithelial cells has been considered for novel therapeutic anti-tumor strategies (Niknejad et al. 2014). HAM protein extracts isolated from amniotic membrane inhibit the metabolic activity of several cancer cell lines, but the commonly used breast cancer cell line MCF-7 appears to be unaffected by this treatment (Mamede et al. 2014). These currently published studies might motivate working in the PABC field to test anti- or pro-tumor effects of further placental cell types. Another study addressing the role of placental extracts revealed the inhibition of tumor growth and metastasis in cell culture and in a mouse model (Marleau et al. 2012). Furthermore, high expression of KiSS-1, a human metastasis suppressor gene, was found in placental tissue. This peptide was isolated from human placenta as an endogenous ligand of an orphan G-protein-coupled receptor, termed as well ‘metastin’. Highest concentration of metastin have been found in third trimester placenta, but 5 days after delivery plasma level of metastin returned to almost non-pregnant levels suggesting its production mainly by the placenta (Ohtaki et al. 2001). The authors concluded, that metastin can act as potential biomarker for aggressive metastasizing tumors and may offer new therapeutically approaches against metastatic cancer (Shoji et al. 2010).

5. Future directions

The pregnancy effect on breast cancer is still unsolved and contradictory. The clinical situation represents an increased risk suffering from breast cancer in early years after pregnancy, which turns into a reduced risk in later years. Since the last twenty years, this observation has been described well, even if reported time periods are highly variable and the time span for having an increased risk reaches up to 50 years (Lambe et al. 1994, Albrektsen et al. 2005, Chie et al. 2000, Liu et al. 2002). The reasons for this phenomenon have not been studied intensively, yet.

The hypothesis of immunization induced by pregnancy related antigens, described in 1994 for the first time (Janerich 1994), offers a potential explanation, but has not been investigated further. Studies on MUC-1 have led to creation of MUC-1 antigen-based vaccines for reduction of breast cancer risks (Beatson et al. 2010). Even if phase I/II clinical trials are currently ongoing (Antonilli et al. 2016), thus far, no vaccine has been launched. The liposomal vaccine Tecetomide (L-BLP25) for vaccination against non-small-cell lung cancer is currently in phase III trials (Butts et al. 2011). It may be expected that MUC-1 or other oncofetal antigens expressed during pregnancy or lactation period may have a similar potential as the absence of metastases in the placenta indicate. Current studies have shown tumor suppressive characteristics by different placenta-derived factors and placental components. Understanding their molecular mechanisms and long-term effects on development of breast cancer and its transiently increased risk during and after pregnancy may help to develop novel treatment strategies.

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Conflict of interest statement

All authors declare that they have no conflict of interest.

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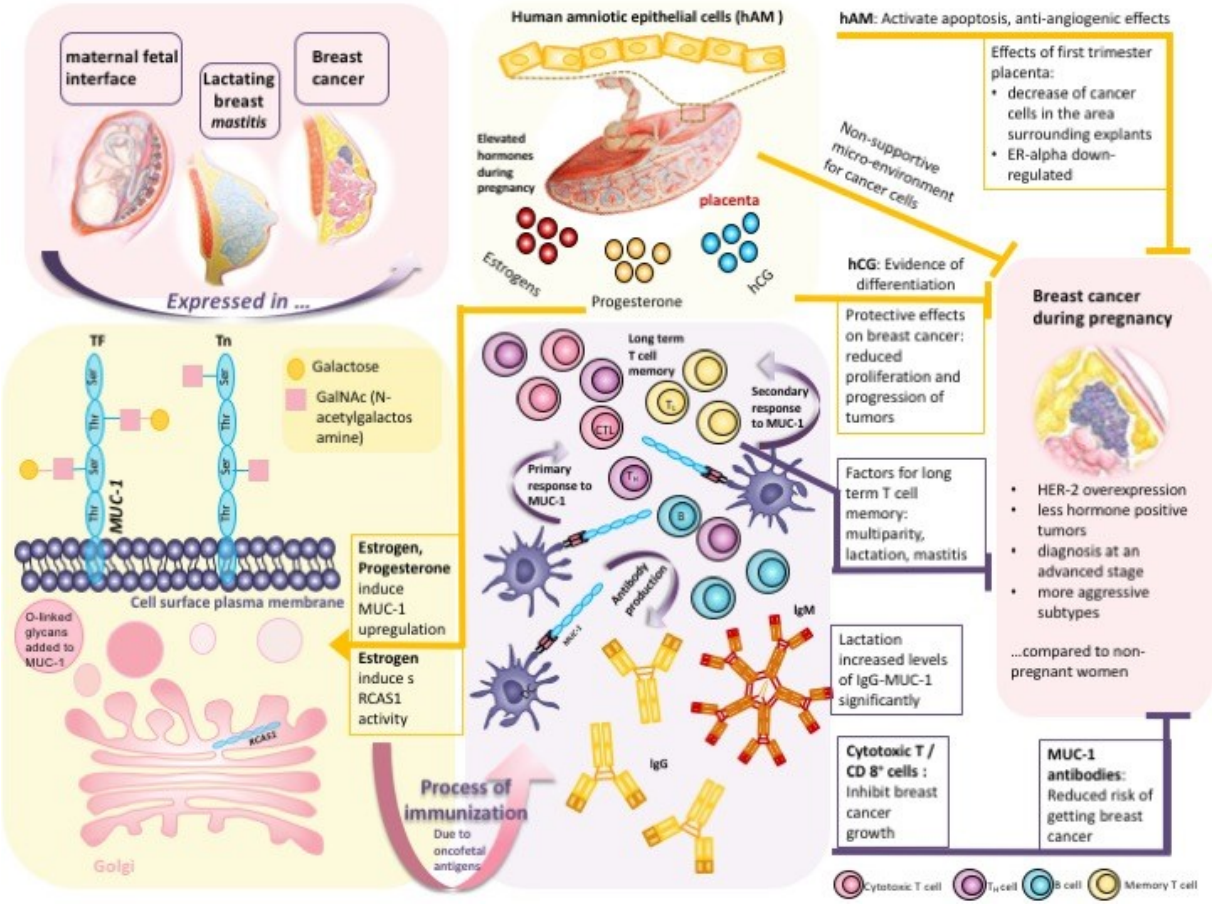
Legends to figures

Figure 1 Proposed anti-tumor effects of the placenta



During pregnancy, several breast cancer surface proteins are expressed on the cell surface membrane of trophoblast cells. The extracellular alpha-subunit of MUC-1 is characterized by alternately repeated threonine (Thr) and serine (Ser) sequences. At these positions O-linked glycosylation occurs constantly. These truncated O-linked glycans, such as Thomsen-Friedenreich disaccharide Galß1–3GalNAc (TF) antigen, are present in breast cancer, trophoblast cells and mastitis. Due to different glycosylation patterns in healthy tissue these structures may induce immunological response. The closely related tumor-associated O-linked glycan Tn (N-acetyl-D-galactosamine) is also present on the receptor binding cancer antigen expressed on SiSo cells (RCAS1) in the Golgi complex of trophoblast cells. Dendritic cells (DC) present these antigens to activate cytotoxic T cells (CTL), T_H cells and B cells leading to the secretion of IgG antibodies found in maternal serum. Memory T cells may be induced by multiparity, lactation and mastitis. All described immune regulatory effects might lead to a reduced breast cancer risk after pregnancy in later years of life. The placenta itself is described as non-supportive microenvironment for cancer cells with indirect and direct effects on breast cancer invasiveness. Estrogen and progesterone induce MUC-1 upregulation, whereby estrogen enhances RCAS1 activity. This might strengthen the process of immunization. Experiments with human chorionic gonadotropin (hCG), which is secreted in high amounts by the placenta, have shown an evidence of differentiation. Proliferation and progression of tumors were decreased by this hormone. Human amniotic epithelial cells (hAM), located at the fetal placental surface, have also anti-angiogenic effects and induce apoptosis. These direct effects of placental factors might explain the low rate of breast cancer metastases in placenta.

Manuskript 5: Breast carcinoma in pregnancy with spheroid-like placental metastases: a case report.

Artikel Typ: Case Report

Autoren: Karolin Froehlich, Hanne Stensheim, Udo R. Markert, Gitta Turowski

Journal: APMIS (Acta Pathologica, Microbiologica et Immunologica Scandinavica, Journal of Pathology, Microbiology and Immunology)

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Zusammenfassung: Ein Fall einer norwegischen Patientin, bei der während der Schwangerschaft ein Rezidiv einer Mammakarinomerkrankung diagnostiziert wurde. Es erfolgt die Aufarbeitung der früheren Patientengeschichte und der aktuellen Studiensituation hinsichtlich Behandlungsmöglichkeiten während der Schwangerschaft. Eine genaue histologische Beschreibung und Analyse der sphäroid-ähnlichen Plazentametastasen wurde vorgenommen und eine Übersicht von anderen bisher beschriebenen plazentaren Mammakarinommetastasen wurde dargestellt. Da diese Form der Metastasierung ein besonders seltenes Ereignis darstellt, wurden mögliche schwangerschaftsassoziierte Faktoren mit Hilfe von klinischen und experimentellen Studien diskutiert.

Eigenanteil: gemeinsame Aufarbeitung des Falles mit Gitta Turowski, Literaturrecherche, Erstellung der Abbildung, Verfassung des Manuskriptes.

Mitautorenanteil: Hanne Stensheim (fachliche Beratung, klinische Kontaktperson für patientenbezogene Fragen und durchgeführten Therapieregimen), Udo R. Markert (Betreuung), Gitta Turowski (mikroskopische Aufnahmen, Kontakt mit Ethik-Kommission in Oslo, Betreuung).

APMIS JOURNAL OF PATHOLOGY, MICROBIOLOGY AND IMMUNOLOGY

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CASE REPORT

Breast carcinoma in pregnancy with spheroid-like placental metastases—a case report

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Froehlich K, Stensheim H, Markert UR, Turowski G. Breast carcinoma in pregnancy with spheroid-like placental metastases—a case report. APMIS 2018; 126: 448–452.

Breast cancer is one of the most common malignancies diagnosed in pregnancy. Although the tumor is often detected at an advanced stage, placental metastases are rare. Here, we describe the case of a woman with breast cancer recurrence during pregnancy and subsequent metastases. The focus of this study is the large amount of placenta metastases, which have been analyzed immunohistochemically. Staining with trophoblast markers (placenta alkaline phosphatase, beta human chorionic gonadotropin and human placental lactogen) showed the strict localization of metastases in the intervillous space without invasion into fetal tissue. They have a large spheroidal shape and are free of blood vessels. Staining with Ki-67 revealed an outer proliferative shell and inner necrotic core. At week 28, a healthy newborn was born by elective cesarean section. A few weeks later, after surgery and FEC60 (fluorouracil, epirubicin, cyclophosphamide) cycles, the patient died. Breast cancer metastases in the placenta are rarely described. The special immunological environment in pregnancy may influence phenotype, growth, and behavior of tumor and metastases.

Key words: breast cancer; pregnancy; placenta; metastases.

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Cancer diagnosed during pregnancy ranges from 24.5 to 191.5 per 100 000 pregnancies (1–3). Breast cancer is one of the most common tumors in pregnancy with varying incidence from 2.4/100 000 to 10/100 000 (4, 5). Although pregnancy-associated breast cancer (PABC, breast cancer diagnosed in pregnancy or during the first 12 months post-partum) (6) is frequently diagnosed at a more advanced stage than in breast cancer in non-pregnant women, thus far, metastases in placenta have been described in only 17 cases (7, 8). PABC patients often present a different tumor profile at diagnosis compared with non-pregnant breast cancer patients. They have a higher frequency of hormone receptor negativity, including triple negative tumors and human epidermal growth factor receptor 2 (HER-2) positivity (9, 10). This may lead to more aggressive tumor growth (11), advanced stage, and more frequent metastases (12). However, the placenta is assumed to function as a non-supportive microenvironment for cancer cells (13). We present a patient with breast cancer, diagnosed shortly after a previous pregnancy, and initially treated with surgery and FEC60 (fluorouracil, epirubicin, cyclophosphamide) cycles. The tumor relapse was detected during a subsequent pregnancy. Delivery of a healthy child was induced in week 28 to start oncologic treatment after pregnancy. The patient died few weeks later.

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CASE PRESENTATION

A woman in her early 40s presented a self-detected nodule in the right breast approximately 2 months after pregnancy, which has been removed by surgery. The tumor was histologically classified as breast cancer (Fig. 1A, B), infiltrative ductal type,

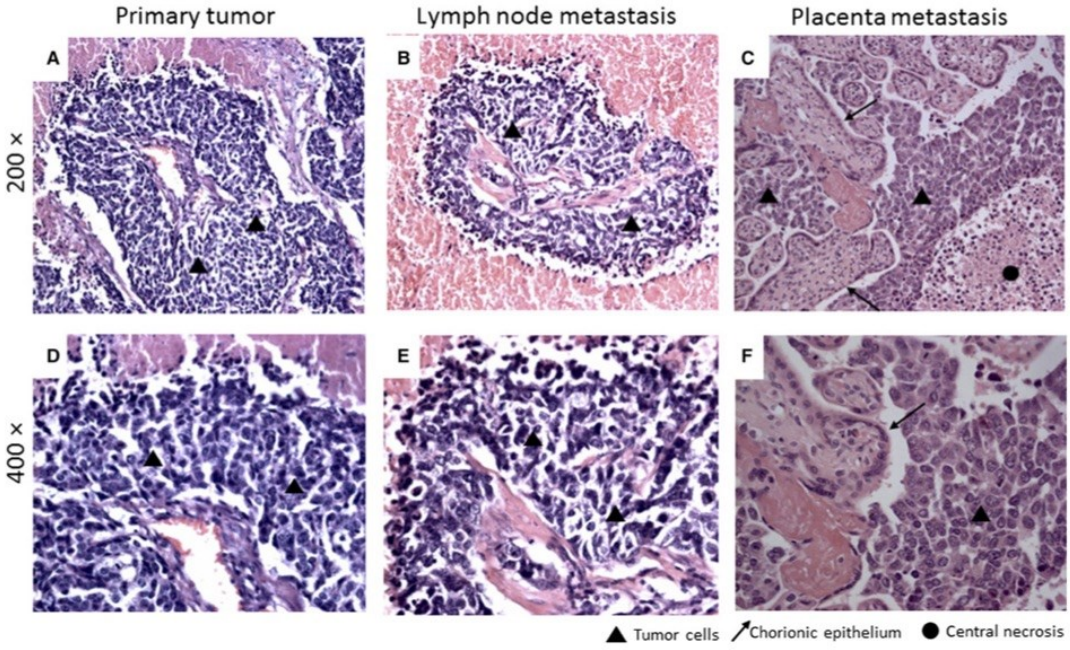

Figure 1. (A, B) Primary tumor of mammary gland; (C, D) lymph node metastasis; (E-F) placenta metastasis (hematoxylin and eosin, magnification 200 ×, 400 ×).

pT2, G3, N0 (0/2 SNB [sentinel node biopsy]), estrogen receptor (ER) negative, progesterone receptor (PR) weakly positive, and HER-2 negative. Chemotherapy was started with application of FEC60 cycles (fluorouracil 600 mg/m^2, epirubicin 60 mg/m^2, cyclophosphamide 600 mg/m^2). Due to nausea, only 5 of 6 planned cycles have been given. About 1 year after initial diagnosis, a relapse was diagnosed simultaneously with pregnancy in gestational week 11. Enlarged axillary, *supra-* and *infraclavicular* lymph nodes have been detected and extirpated. Histological analyses confirmed metastases of the primary breast cancer as infiltrating ductal mamma carcinoma (Fig. 1C, D). They were ER negative, PR positive (over 75%), and HER-2 negative in 8 of 15 lymph nodes. Chemotherapy was not applied as desired by the patient. Due to progressive cancer growth, cesarean section was performed in gestational week 28 and a healthy child was born. Large intraparenchymal metastases in the placenta have been identified by histology (Fig. 1E, F). Spheroid-like metastases presented central necrosis and multilayered epithelium with high mitotic activity, as detected by Ki-67 immunostaining (Fig. 2A, B). Nevertheless, cancer cells were observed only in the placental intervillous space, but not in the villi or other extraembryonic fetal tissues. Placental alkaline phosphatase, human chorionic gonadotropin, and human placental lactogen staining have shown a defined border between metastases and villi (Fig. 2C–H). Post-delivery, *supra-* and *infraclavicular* lymph node conglomerates, liver metastases, bilateral lung parenchyma changes, mediastinal tumors, and a thrombus in the left ovarian vein have been detected by computer tomography. For chemotherapy, the patient received weekly paclitaxel (150 mg/m^2), and because of pneumonia, the patient received antibiotic treatment. After 2 cycles and 3 weeks after delivery, she died due to respiratory failure. The publication of this case has been allowed by the Regional Ethics Committee (REK) sør-øst, Norway (see Appendix S1).

DISCUSSION

The patient has been diagnosed with breast cancer 2 months post-partum, followed by a relapse one year later during a subsequent pregnancy. As reported and discussed in several studies, pregnancy transiently increases the risk of breast cancer and may stimulate cell growth and malignant cell transformation, for example, due to hormonal changes (14). The most investigated hormones potentially influencing breast cancer risk are estrogens and

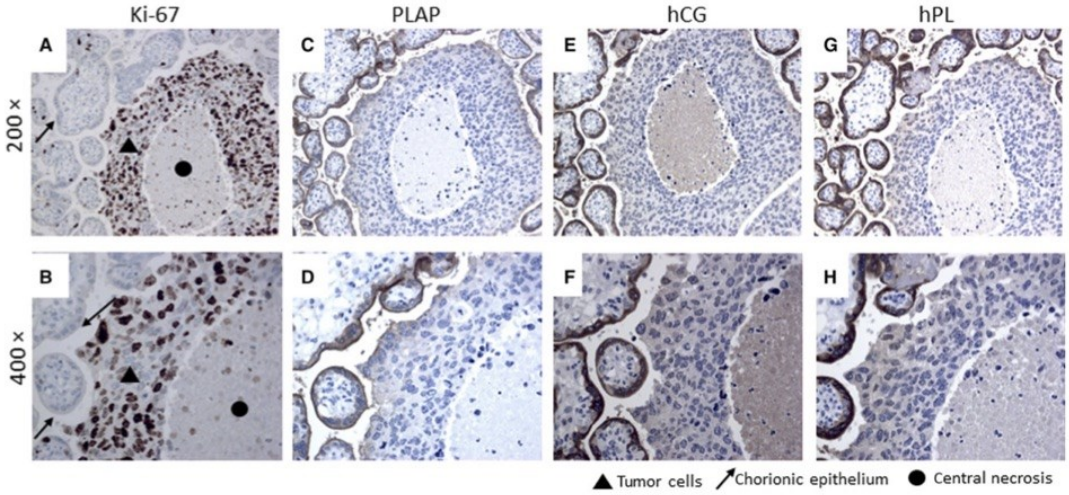

Figure 2. Immunostaining of a placenta metastasis by using the following markers: (A, B) Ki-67, (C, D) placental alkaline phosphatase, (E, F) human chorionic gonadotropin, (G, H) human placental lactogen (magnification 200 ×, 400 ×).

progesterone. However, endocrinological alterations during pregnancy are very complex and include further placental factors such as human chorionic gonadotropin, placental growth hormone, human placental lactogen, and different cytokines (15). In a xenograft model for breast cancer, cell invasion and metastases were promoted by using an involution matrix, isolated from rats with mammary glands undergoing weaning-induced involution (16), which has been described to deregulate tissue structure and activate tumor microenvironment (17). Significantly increased rates of breast cancer metastases were found in lung, liver, and kidney. In contrast, mammary matrix from nulliparous rats had no stimulating effect on invasion (16). Another study has shown that mouse mammary stem cells respond to steroid hormone signaling despite lacking ER and PR (18). Polyak et al. postulated cumulative exposure to reproductive hormones as most significant and well-characterized risk factor of breast cancer besides family history (19). In general, elevated gestational hormones during pregnancy might lead to a poor prognosis of PABC [16]. Furthermore, immunosuppression, relative insulin resistance combined with high circulating insulin levels, and altered melatonin levels during pregnancy are discussed to worsen prognosis of malignant diseases. Tissue remodeling of the mammary gland during pregnancy and after delivery (lactation and involution) is assumed to change microenvironment in breast glands and extracellular matrix, which may influence tumor genesis. Number of mammary epithelial cells, stem cells, and stromal fibroblasts are elevated during pregnancy, which may lead to a hyperplasia of stem cells with tumor-initiating genetic alterations potentially increasing breast cancer risk (19).

Although breast cancer is one of the most common malignancies during pregnancy, well-documented reports of maternal breast cancer metastases in the placenta are very rare compared with melanoma, which has a four-fold cancer risk in pregnancy with varying incidence from 10 to 280/100 000 pregnancies (20). Since the first case reported in 1866, Alexander et al. have documented a total of 87 cases of placental metastases, 15 of which from breast cancer, but none with fetal involvement. Other cancers like melanoma, hematopoietic malignancies, and lung cancer have been reported to affect the fetus, but at low frequency. To our best knowledge, after 2003, one single case of placental metastases of breast cancer has been documented (8) [PubMed Search: breast cancer + metastases + placenta]. They were located in the intervillous space without involvement of the fetal villous stroma or the fetal vascular circulation and have been diagnosed histologically. In our case, large spheroid-like metastases with central necrosis have been identified in the intervillous space without involvement of fetal villous stroma. Previously, it has been shown that cancer stem cells can form cancer spheroids with modified metastatic activity *in vivo* (21). *In vitro* generated spheroids are widely used models in tumor research because they simulate avascular tumor areas comprising proliferative and necrotic cells (22). The here

observed placental metastases present features like those in *in vitro* generated spheroids. Hence, our histological findings highlight the relevance of *in vitro* spheroids and simultaneously support the hypothesis that spheroid-like structures can also develop *in vivo*. In the here presented case, the pregnant breast cancer patient did not receive therapeutic intervention, and thus, tumor growth, progression, and metastasizing have not been inhibited. Nonetheless, metastases did not invade villous tissue, and a clear separation between chorionic villi and cancer metastases was evident. Although tumor growth was massive, invasion seemed to be hindered which may be due to placental factors acting as non-supportive microenvironment for cancer cells (13). However, in general, placental metastases may be underdiagnosed when the placenta appears macroscopically normal. In such cases, histological analyses are frequently not done (23). For this reason, we emphasize the importance of placenta examination upon pregnancy cancer by a pathologist, even when it looks macroscopically normal. Mamma carcinoma cells affect the intervillous space, but passage through the syncytiotrophoblast has not been reported, yet. It has been shown in *ex vivo* perfused placentas, that T-cell leukemia cells principally can cross the placental barrier (24), but can also be removed by the syncytiotrophoblast via phagocytosis (25). Fetal immune response affected by maternal cancer is assumed to play a role in transplacental transmission of tumor cells. The few newborns described with metastases of a maternal malignancy seem to build immunological tolerance induced by tumor antigens during development of the fetal immune system. In contrast, an intact fetal immune response should eradicate maternal cells and protect from metastases (26). In the here described case, the tumor had an infiltrative ductal type and was weakly PR positive, ER, and HER-2 negative. Therefore, no anti-hormonal and anti-HER-2 treatment, but chemotherapy was indicated. A recently published multicenter case–control study including 129 children investigated the pediatric outcome after maternal cancer diagnosed in pregnancy. They concluded that these children had normal development during testing (18–36 months), and maternal chemotherapy (anthracyclines, taxanes, platinum derivates) had no adverse effects on postnatal growth, and cognitive or cardiac functions (27).

CONCLUSIONS

Malignant diseases in pregnancy provoke a challenging situation for gynecologists, oncologists, and neonatologists. The here presented patient had a fatal outcome and died after pregnancy while her child survived. Clinical studies have shown that cancer in pregnancy can be treated by surgery, chemotherapy, or a combination of both after the first trimester (28). Preterm termination of pregnancy does not improve maternal prognosis (29). Further clinical and pathological studies are needed for better understanding of the potential impact of tumors and their therapies on the placenta, the fetus, and its later life.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

Appendix S1 Regional Ethics Committee (REK sørøst Norway).

Manuskript 6: Multicellular tumor spheroids co-cultured with human placental villous explants

Artikel Typ: Short Communication

Autoren: Karolin Froehlich, Lea Hauswald, Julia Heger, André Schmidt, Amelie Lupp, Udo R. Markert

Status: Manuskript erstellt, Prüfung und Korrektur für die Einreichung

Zusammenfassung: In diesem Artikel wurde ein Co-Kultur-Modell, bestehend aus MCF-7 Sphäroiden und Plazenta-Explantaten vorgestellt. Es erfolgte die Beschreibung der verschiedenen technischen Schritte zur Co-Kultivierung beider Komponenten beschrieben und anschaulich dargestellt. Außerdem wurden zur weiteren Charakterisierung des Co-Kultur-Modells die Überstände auf Glucose, Lactat, Progesteron, Estrogen und ß-hCG analysiert. Nachfolgend wurde die Interaktion von den MCF-7 Sphäroiden mit dem villösen Plazentagewebe nach lichtmikroskopischen Aufnahmen, HE- und IHC- (Ki-67, cPARP, MUC-1) Färbungen beurteilt. Abschließend soll das Manuskript einen Ausblick auf die vielfältigen Einsatzmöglichkeiten dieses Modells (z.B. als Metastasierungsmodell, toxikologische Anwendungen) geben.

Eigenanteil: Hauptanteil, Planung und Durchführung der Experimente in großen Teilen, Auswertung der Daten, Literaturrecherche, Erstellung der Abbildungen, Verfassung des Manuskriptes.

Mitautorenanteil: Julia Heger (fachliche Beratung bei unterschiedlichen Einsatzmöglichkeiten des Modells), Lea Hauswald (lichtmikroskopische Aufnahmen, Überstandsanalysen), André Schmidt (fachliche Beratung bei der Präparation der Plazenta-Explantate), Amelie Lupp (Betreuung bei HE- und IHC- Färbungen), Udo R. Markert (Betreuung).

Multicellular tumor spheroids co-cultured with human placental villous explants

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Abstract

Most cell line based studies on human tumors have been performed on monolayer cultures. As monolayers do not perfectly reflect the in vivo situation we have analyzed the characteristics of three-dimensional tumor spheroids.

In this study, MCF-7 multicellular tumor spheroids were co-cultured with placental villous explants. Afterwards, this three-dimensional model has been characterized by vitality assays, supernatant analysis of hormones, histology and immunohistochemistry (MUC-1, Ki-67, cPARP). Until yet, such *in vivo* – like 3D structure based on placental tissue has not create before and may open up new possibilities for diverse fields e.g. metastatic mechanisms, toxicology, metabolism and tissue engineering.

Keywords: three-dimensional co-culture models, breast cancer, multicellular tumor spheroids, placental villous explants

Introduction

Several three-dimensional (3D) tumor models have been proved to be useful for *in vitro* drug delivery and efficacy tests. They allow studying disease and resistance mechanisms due to similarities to *in vivo* situations (Elliott und Yuan 2012, Martin und Vermette 2005). Well-established 3D models are multicellular tumor spheroids (MCTS, shortly: “spheroids”) (Elliott und Yuan 2012), which have been used to study various biological characteristics of cells (e.g. proliferation, differentiation, metastasis) (Hirschhaeuser et al. 2010, Schutze et al. 2015). Currently, many studies aim to mimic or create human organs *in vitro* because of their more realistic simulation of human conditions e.g. in regard to nutrition supply and drug metabolism (Kelm und Marchan 2014, Jin und Yu 2015), which may improve transferability from experimental data to clinical hypotheses (Kelm und Marchan 2014). Also, with regard to cancer metastasis such multicellular systems may be a useful tool for investigating tumor cell growth and microenvironment, including the function of extracellular matrix (ECM) and drug diffusion kinetics (Skardal et al. 2016, Kaur et al. 2011). As breast cancer and other malignancies may occur during pregnancy, efforts have been made to investigate molecular and clinical features of such coincidences to define an optimal treatment, which does not harm the fetus (Loibl et al. 2006). Although mamma carcinoma in pregnancy has been described to be more aggressive and the tumors are occasionally diagnosed at an advanced stage, thus far, placental metastases have been described in only 18 cases (Froehlich et al. 2018, Skardal et al. 2016, Kaur et al. 2011),(Epstein Shochet et al. 2012). Indeed, all documented placental breast cancer metastases presented their localization in the intervillous space without invasion into fetal tissue (Froehlich et al. 2018). As the occurrence of placental breast cancer metastases is a very rare event, in this study we aimed to analyze cellular interactions between carcinoma and placenta tissue cells in vitro. For this approach, we co-cultured MCF-7 spheroids with placental villous explants (PVE). Moreover, we assessed the potential usefulness of this model for toxicological studies. The human placenta is described as a multitalented organ, providing the interchange between mother and fetus (Burton und Jauniaux 2015). Therefore, many studies address its role in fetal development and maternal health (Guttmacher und Spong 2015), but due to the presence of a large spectrum of cell types (e.g. trophoblast cells, fibroblasts, endothelial cells, blood cells, placental immune cells) PVE may serve also an interesting model for other issues beyond reproductive toxicology (Miller et al. 2005, Gohner et al. 2014).

Material and Methods

Cell culture and spheroid generation (1)

MCF-7 (ATCC® HTB-22TM) breast cancer cells were cultivated in DMEM high glucose (Gibco, Paisley, UK) supplemented with 10% fetal calf serum (FCS; Gibco) and 1% penicillin/ streptomycin (P/S; Gibco). When confluence was observed cells were trypsinized and counted. For initiation of hanging drop culture, 50 drops of each containing 10,000 cells in medium supplemented with 25% methocel, were pipetted onto the lid of a 10 cm Petri dish. The lids were inverted and placed on Petri dishes filled with PBS (5 ml, Gibco). Hanging drop cultures were incubated at 37°C in a humidified atmosphere with 5% CO_2 for 3 days. The methocel stock solution was prepared as described by Korff and Augustin (Korff und Augustin 1998).

Preparation of PVE (2)

Six healthy human term placentas were obtained from the Department of Obstetrics, Jena University Hospital, after spontaneous delivery or caesarian section upon written consent. An approval by the local ethical committee exists. Villi of placenta tissue were cut in small pieces, washed three times with PBS supplemented with 1% P/S and biopsy-sized PVE (2 mm diameter) were prepared by plucking. Equally sized pieces of umbilical cord explants (UCE) were used as control.

Confrontation of spheroids and PVE (3)

After 3 days generation period, MCF-7 spheroids were harvested using cut pipette tips (200 µl), which have been prepared by cutting eight mm of their narrow end for enlarging the aperture diameter. Six spheroids per group were transferred to a well containing one freshly prepared PVE or UCE (shortly termed as co-culture). All co-cultures were performed in 2% poly-2-hydroxyethyl methacrylate (poly-HEMA; no. 18894, Polysciences, Eppelheim, GER) coated 48-well plates (No. 677180, Greiner Bio-One) containing 500 µl RPMI (Gibco) per well supplemented with 10% FCS and 1% P/S for 1 h, 24 h, 48 h, 72 h or 96 h. Other experimental setups for co-culturing were tested (table 1).

Measurement of lactate dehydrogenase (LDH), glucose, lactate, human choriongonadotropin (ß-HCG), estrogen and progesterone (4)

Glucose concentration was assessed in accordance to phosphorylation and oxidation reactions and measured at 340 nm (Architect i-System, Abbott GmbH, Wiesbaden, GER). After an enzymatic transformation of lactate to pyruvate, the produced hydrogen peroxide was measured via color intensity, which is proportional to lactate and LDH concentration (Abbott GmbH). ß-HCG concentration was determined by chemiluminescence microparticle immunoassay (Abbott GmbH). Estrogen and progesterone concentration were assessed by Cobas e411 Immunoassay (Roche Diagnostics, IN, USA) according to manufacturer's instructions using biotinylated polyclonal anti-estradiol antibody (Roche Diagnostics, Mannheim, GER) or biotinylated monoclonal anti-progesterone antibody (Roche Diagnostics).

Microscopical analyses of co-cultures (5)

After the indicated time, cells were imaged by Axiovert 25 CFL microscope (Carl Zeiss, Jena, GER) using the camera Sony Cyber-shot 3.3 MegaPixels (Sony, Tokio, JPN).

Paraffin embedding of co-cultures (6)

After the indicated time, each co-culture was washed twice with 500 µl PBS and transferred into a 0.5 ml reaction tube, supplemented with 200 µl pooled plasma from healthy anonymous blood donors (Institute for Transfusion Medicine, University Hospital Jena). 20 µl fibrinogen (REF 00674, Stago, Asnières sur Seine, France) were added and the sample was vortexed for 20 seconds (MS2 Minishaker IKA®, Laborgeräte München, Munich, Germany). After 5 minutes of coagulation at room temperature, samples were fixed in 4% formalin. The final paraffin embedding was performed as described by Lupp et al. (Lupp et al. 2001).

Hematoxylin and eosin (HE) staining (7)

From the paraffin blocks, 4 µm sections were cut using a microtome (Microm HM 335 E; Microm, Walldorf, Germany), floated onto poly-L-lysine-coated glass slides and air-dried. Subsequently, sections were deparaffinized in xylene and rehydrated in a graded ethanol series. HE staining was performed according to routine protocols using Mayer's hematoxylin and eosin Y solution (Sigma) (Lupp et al. 2001). Thereafter, sections were washed in distilled water, dehydrated in a graded ethanol series, cleared in xylene and mounted in DePex (SERVA Electrophoresis GmbH, Heidelberg, GER). Sections were evaluated by use of an Axio Imager A1 microscope (Carl Zeiss).

Immunostaining (8)

Sections of co-cultures were deparaffinized, rehydrated in a graded ethanol series and the endogenous peroxidase was blocked by hydrogen peroxide (Sigma). Antigen retrieval was performed for the histochemical detection of all antigens by boiling in 0.1 M citrate buffer (pH 6.0) for 16 minutes. After washing in PBS, sections were incubated for 20 minutes with blocking solution (Vectastatin® Elite® ABC Kit, Vector Laboratories, Burlingame, USA) followed by incubation with primary antibodies against Ki-67 (No. M7240, 1:50, Dako, Hamburg, GER), cPARP (No. 5625, 1:200, New England Biolabs, Beverly, USA) and MUC-1 (No. M0613, 1:5000, Dako) in a humid chamber at 4°C overnight. After washing in PBS, the specimens were incubated with biotinylated secondary antibodies, exposed to the avidin-peroxidase complex (Vectastatin® Elite® ABC Kit) and developed with AEC substrate (BioGenex, San Ramon, USA). Sections were counterstained with Mayer's haematoxylin, mounted in Mowiol (Carl Roth GmbH, Karlsruhe, GER) and evaluated at an Axio Imager A1 Microscope.

Statistical Analyses

Paired Student`s t-test was used to analyze differences between cohorts. A p-value of < 0.05 was considered significant. All experiments were repeated independently at least three times.

Results

Supernatant analysis

Glucose levels of PVE or UCE co-cultures decreased during the incubation time (Fig. 2A). The effect was stronger in PVE than in UCE, whereby after 96 h glucose levels of PVE co-cultures turned back to UCE levels. Lactate concentrations of PVE co-cultures increased and also returned to concentration of UCE co-cultures after 96 h. ß-hCG levels of PVE co-cultures were significantly higher than in UCE co-cultures, but decreased during incubation time (Fig. 2B). High concentrations of estrogen were measured in both types of co-cultures, which slightly decreased with ongoing incubation period. Progesterone levels of PVE co-cultures were higher than in UCE co-cultures, but decreased after 48 h.

Microscopical and histological characteristics of co-cultures

At the initial state spheroids co-cultured with PVE or UCE showed a smooth rim, clearly separated from PVE or UCE (Fig. 3A a, f; dotted line implicates interface; Fig. 3B a). After 24 h PVE were already integrated in MCF-7 spheroids (Fig. 3A b; Fig. 3B b). In contrast, UCE co-cultures did not overlap each other until 72 h cultivation period (Fig. 3A g, h, I), which was started after 96 h (Fig. 3A j). Nevertheless, histology analysis of UCE co-cultures revealed no deeply interaction between both compounds (Fig. 3B f, g). After 72 h PVE-spheroid interaction became more intense, spheroids lost their round shape and cells grew in direction towards PVE (Fig. 3A d, arrows). Moreover, some villi were covered by breast cancer cells (supplementary material, Fig. S1A, Fig. S1B e). After 48 h histology analyses revealed the development of necrotic cores in spheroids (Fig. 3B c) as described in Froehlich et. al., which became more obvious after 72 h (Fig. 3B d). After 72 h 80 h placental villi were also detected in the outer rim of the spheroids (Fig. 3B d, e). Furthermore, IHC enabled a more precise differentiation between spheroids and PVE (Fig. 4). The glycoprotein MUC-1 was strongly expressed and presented a three-layered structure in spheroids (Fig. 4 a,d,g,j,m). The outer zone was strongly MUC-1 positive, followed by weakly positive cells in the intermediate zone and very strongly positive cells in the core (Fig. 4 a,d,g). Ki-67 immunolocalization enabled the identification of single breast cancer cells, which were found close to PVE (Fig. 4 n; Fig. S1B e). Apoptotic cells in the core were detected by cPARP immunostaining (Fig. 4 b,e,h,k), which became more intense after 72 h co-cultivation (Fig. 4 i). Spheroids, which interact with PVE, presented only a few cPARP positive cells (Fig. 4 l,o), while those, which did not interact with PVE developed a strongly cPARP positive core (supplementary material, Fig. S1B i,l).

Discussion

In context of economic pressure in drug development and the aim reducing animal tests *in vitro* alternatives are very popular (Wick et al. 2014). Therefore, the described co-culture model might be an interesting complement for the upcoming shift from monoculture to co-cultures resp. from 2D to 3D culture systems alone or in combination and may significantly improve the physiological function of models towards *in vivo* – like situations (Wick et al. 2014).

Glucose and estrogen are suitable markers for further experiments

We characterized the vitality and hormone production of co-cultures by supernatant analyses. Glucose levels indicated that glucose synthesis of PVE co-cultures was decreased after 72 h incubation, but enhanced again after 96 h. This indicates that trophoblast cells might regenerate after longer incubation period and revealed glucose as a suitable marker for further analysis. After 96 h incubation low concentrations of ß-hCG and progesterone were detected. Therefore, these hormones seem to be not adapted for vitality markers, whereas estrogen levels remained stable over time, suggesting a continuous estrogen production, even when a strong inter-variability has to take into account. In contrast, Sato et. al. showed in PVE mono-cultures a reduced secretion of steroidal hormones (progesterone, estrogen, 17b-estradiol) after 96 h (Sato et al. 2015). In our case, stable estrogen level might be associated with the breast cancer spheroids.

Placental villi were incorporated in MCF-7 spheroids and no evidence of breast cancer cell elimination were found

Although breast cancer during pregnancy is more aggressive than in non-pregnant women in general, placental metastases are really rare and the placenta is described to be a non-supportive microenvironment for cancer cells (Epstein Shochet et al. 2012). Due to this phenomena Tartakover-Matalon et. al. have established a co-culture model comprising PVE and breast cancer single cells (Tartakover-Matalon et al. 2010). They showed an elimination of breast cancer cells in PVE surroundings and simultaneously an increase of breast cancer cell motility. In our co-culture model elimination of MCF-7 spheroids was not remarkable. After 24 h spheroids and PVE started to interact with each other. Moreover, when no villous was found in spheroids breast cancer cells became more apoptotic suggesting a positive influence on vitality of breast cancer spheroids by PVE. After 48 h single MCF-7 cells were detected at one villous suggestion a partial dissolution of MCF-7 spheroids. As negative control UCE was also co-cultured with breast cancer spheroids. Interactions between both compounds were only found by light microscopy, but no interaction between spheroids and UCE could be revealed via histological examinations. It should be kept in mind that there exist some similarities between PVE and UCE, both contain many stem cells including mesenchymal stem cells, endothelial stem cells and epithelial stem cells (Gonzalez et al. 2010). Recently, it has been shown that UCE effects fibroblasts, keratinocytes and melanocytes similar as placental extracts (Van Pham et al. 2014).

Spheroid-PVE co-cultures can be used as toxicological model

Besides the potential investigating pathological mechanism in cancer pathways and assessing metastatic growth in placenta, this co-culture model enables a new opportunity for toxicological tests. Here, toxic agents can be incubated for up to 96 h. During culture several markers in supernatants can used to qualify the xenobiotic toxicity. Afterwards, embedded and sectioned co-cultures can be analyzed histologically, enabling further information. At first, proliferative and apoptotic staining should be performed standardized to detect general impairment. Wanted (on cancer cells) and negative side effects (on human placenta tissue) can be analyzed simultaneously. Due to the stratified structure of spheroids found also in avascular tumor regions (Froehlich et. al.), this model can be further used for studying resistance mechanism. Moreover, the breast cancer cell line can be changed, if other issues need to be examined. Features of spheroid formation should be proven before. We have also co-cultured MDA-MB-231

spheroids and PVE, based on the same protocol, but revealed another interaction between both compounds (data not shown). Our 3D model enables, after an easily paraffin embedding procedure, the analysis of various cell types, interactions between them and detection of toxic effects by agents step by step easily facilitated by sires cut. Therefore, PVE co-cultured with breast cancer spheroids may provide a new experimental approach for testing both, drug efficacy on breast cancer cells and toxicity on placenta tissue simultaneously.

Placenta can be used as tissue model and imitates other tissue types

Moreover, human placenta tissue has an expected potential to simulate other human tissues. In addition to the characteristic placental cells named cyto- and syncytiotrophoblasts, the placenta offer a riche source of other cell types: e.g. endothelial cells, fibroblasts, smooth muscle cells and many immune cell types (Gohner et al. 2014). This wide range of cell types can be used in respect of special toxicological aspects on particular cell types, e.g. human placenta was used as an *ex vivo* model for neurointerventional research (Kwok et al. 2014). Adult stem cells isolated from human placenta have been used to generate hepatocyte-like cells (Lee et al. 2011). In this context the placenta could serve as an alternative model for *in vitro* hepatotoxicity screenings. Indeed, some similarities between the placenta and the liver metabolism were found. Although placenta metabolism is not as strong as in the liver, several cytochromes P450 have been isolated, which are responsible for detoxification of xenobiotics (Yoshizawa 2013). Moreover, the syncytiotrophoblast is characterized with a variety of transporters enabling the efflux of xenobiotics (Burton und Fowden 2015). Therefore, further investigations regarding the content of common hepatic enzymes as well as efflux transports in this co-culture might to be interesting.

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Conflict of interest statement

All authors declare that they have no conflict of interest.

Fig. 1

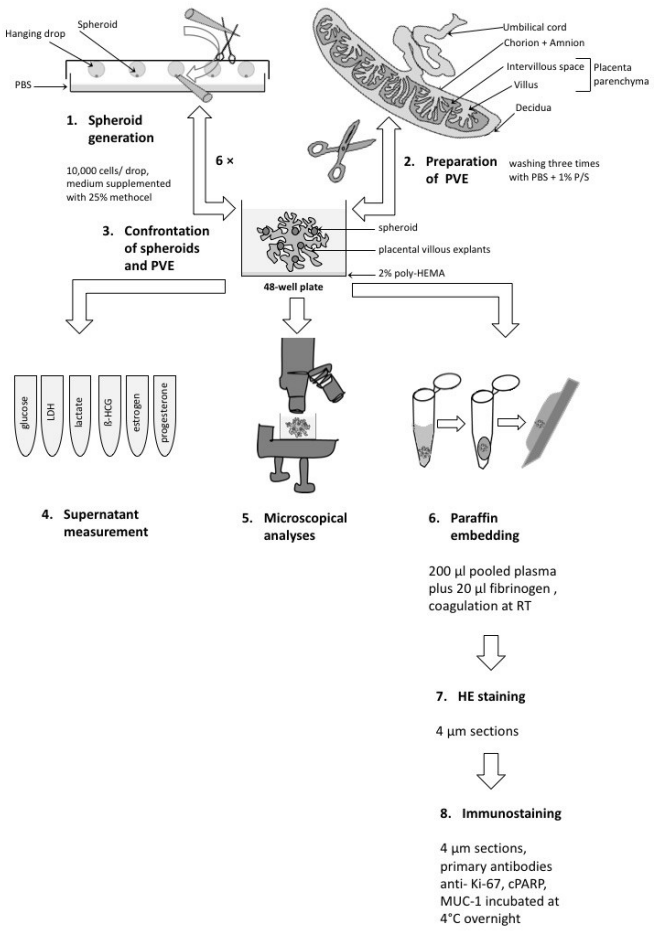


Fig. 2

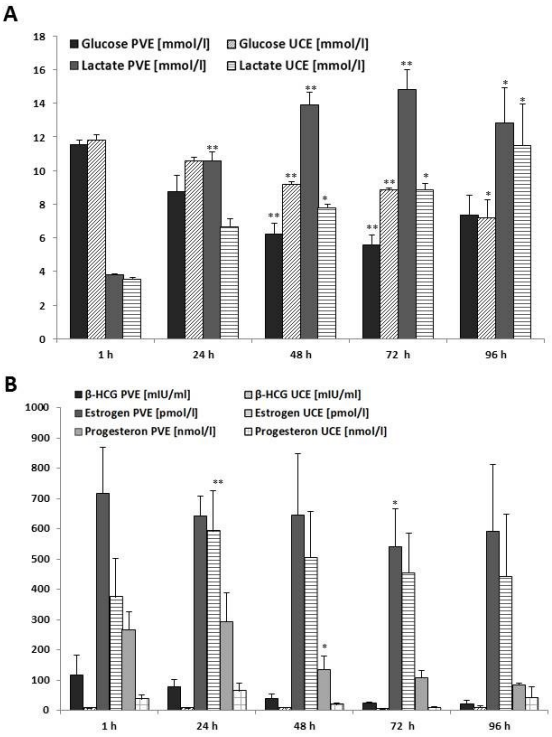


Fig. 3

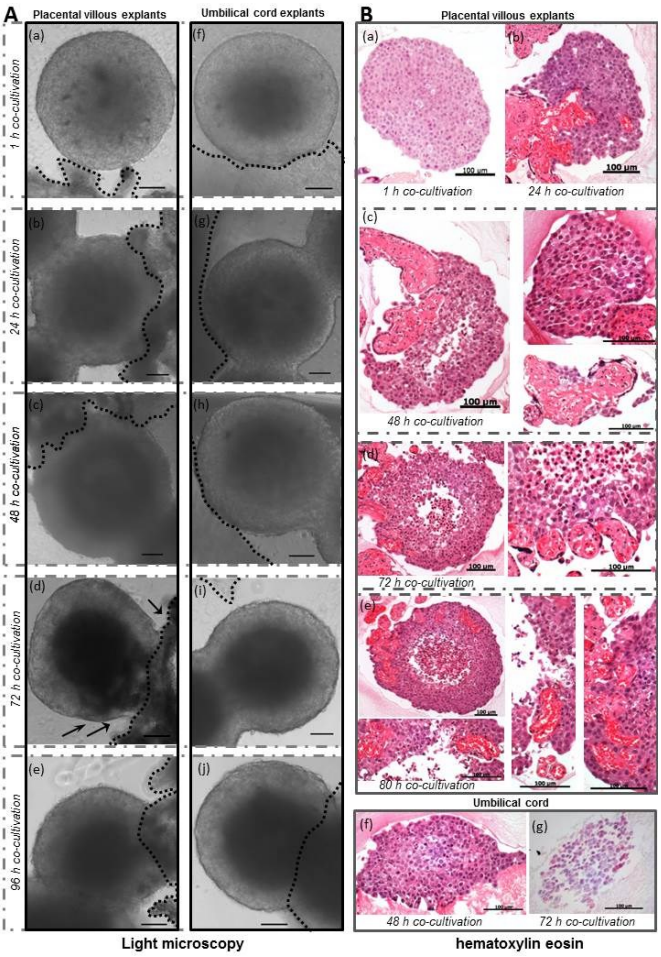
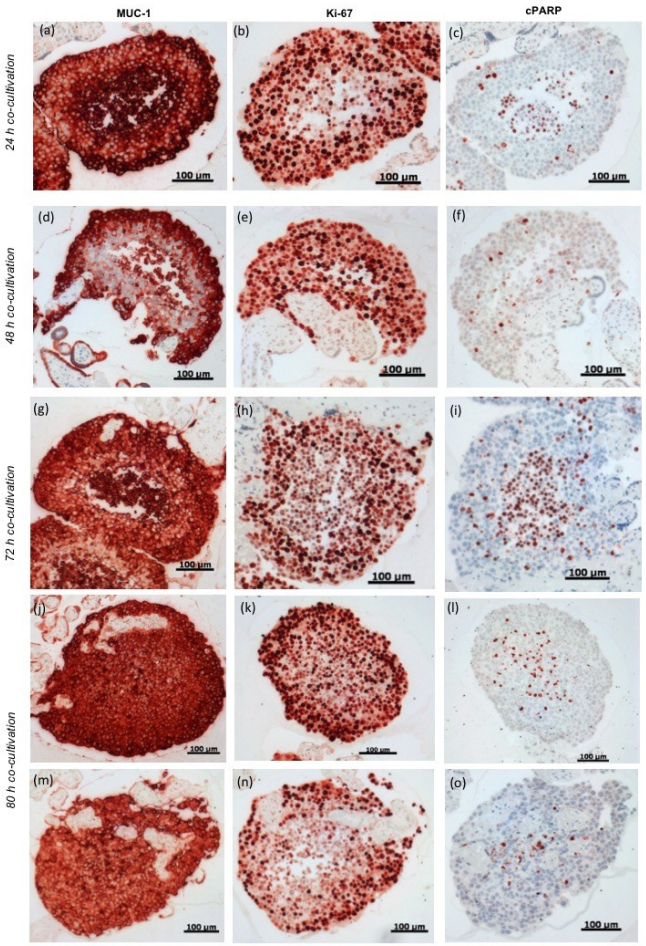


Fig. 4



Supplementary Material, Fig. S1

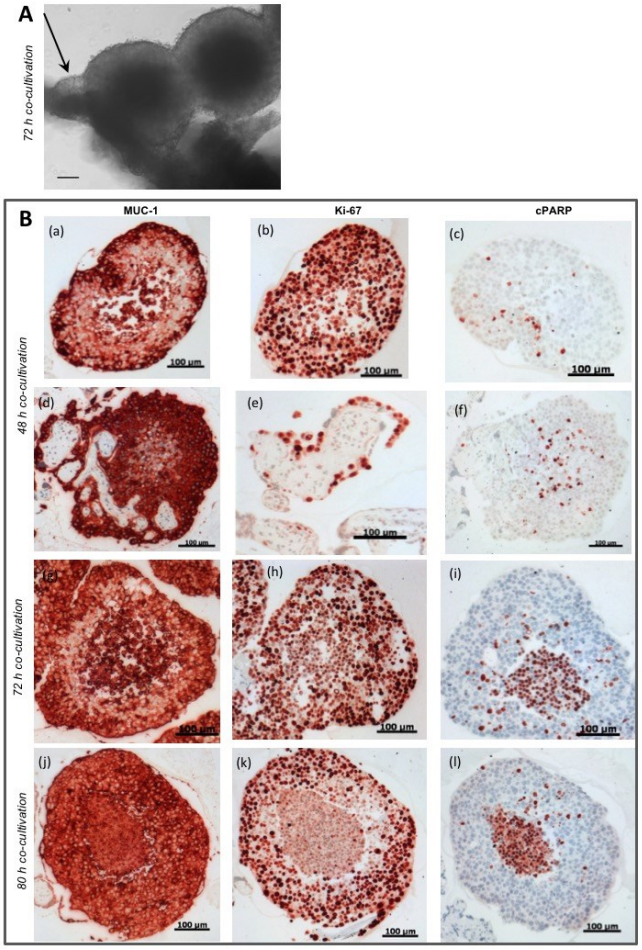


Table 1

criteria	co-culture methods		
	Hanging drops	*Reaction tube (0,5 or 1,5ml)*	*48-well plate*
adherence	no adherence	probably adherent at the reaction tube surface	after coating with 2% poly-HEMA solution no adherence
supernatant analysis	no supernatant, not possible	possible	possible
evaluation by microscopy	not possible	not possible	possible
fixation procedure	risk of structure damaging due to transfer during fixation	no transfer during fixation, lower risk of structure damaging	risk of structure damaging due to transfer during fixation

Legends to figures

Fig. 1 Experimental setup

Fig. 2 Supernatant analyses of glucose, lactate, ß-hCG, estrogen and progesterone in co-cultures

(**A**) Metabolic and (**B**) hormonal substances of PVE resp. UC co-cultures were measured for up to 96 h. Data represent the mean of n = 3 independent experiments. Bars show mean ± SE, * indicates $p < 0.05$, ** indicate $p < 0.01$, *** indicate $p < 0.001$ (Student's t- test, 1 h time point was compared with other time points, PVE and UC test was independently).

Fig. 3 Representative microphotographs and HE staining of MCF-7 spheroids confronted with PVE or UC

A MCF-7 spheroids were confronted with PVE (a-e) or UC (f-j) and have been observed for up to 96 h by light microscopy. Dotted lines show interface between spheroid and PVE resp. UC. Scale bars = 100 µm.

B The microphotographs show HE staining of PVE co-cultures cultivated 1 h (a), 24 h (b), 48 h (c), 72 h (d) or 80 h (e) and UC co-cultures cultivated 48 h (f) or 72 h (g). Scale bars = 100 µm.

Fig. 4 Immunostaining of MCF-7 spheroids confronted with PVE

The microphotographs show immunolocalization of MUC-1 (a, d, g, j, m), Ki-67 (b, e, h, k, n) and cPARP (c, f, i, l, o) in MCF-7 spheroids confronted with PVE cultivated 24 h (a-c), 48 h (d-f), 72 h (g-i) or 80 h (j-o). Scale bars = 100 µm.

Supplementary Material, Fig. S1 Microphotograph and immunostaining of MCF-7 spheroids confronted with PVE

A Microphotograph shows two MCF-7 spheroids confronted with PVE after 72 h cultivation. Scale bar = 100 µm.

B The microphotographs show immunolocalization of MUC-1 (a, d, g, j), Ki-67 (b, e, h, k) and cPARP (c, f, i, l) in MCF-7 spheroids confronted with PVE cultivated 48 h (a-f), 72 h (g-i) or 80 h (j-l). Scale bars = 100 µm.

Table 1 Different culture conditions of co-cultures. Besides the used protocol "48-wel plate" co-cultures can be incubated also in hanging drops or reaction tubes. Both alternative methods can not directly evaluate by microscopy. Supernatant analyses can be performed using the "reaction tube" method, but co-cultures may attach at the reaction tube surface. Otherwise during fixation of co-cultures no sample transfer is necessary suggesting a less damage during the processing. Due to more reliable analysis options of co-cultures we decided to use "48-wel plate" method.

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Manuskript 7: The influence of placenta-conditioned medium on breast cancer cells

Artikel Typ: Original Paper

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Status: Manuskript erstellt, Prüfung und Korrektur für die Einreichung

Zusammenfassung: Drei verschiedene Mammakarinomzelllinien (MCF-7, SK-BR-3, MDA-MB-231) wurden mit plazenta-konditioniertem Medium inkubiert, um den Einfluss schwangerschaftsassoziierter Faktoren auf das Verhalten der Mammakarinomzellen zu analysieren. Das plazenta-konditionierte Medium wurde auf den Gehalt von Glukose, Lactat, LDH, Progesteron, Estrogen und ß-hCG untersucht. Es wurde das Rezeptorexpressionsverhalten für Erα, ERß, PR, und HER2 mittels Western Blot und Immuncytochemie (ICC) analysiert. Außerdem wurde die Morphologie der Zellen lichtmikroskopisch sowie nach Anfärben mit Phalloidin-Aktin untersucht. Die Zellviabilität, Proliferation und Apoptose der Zellen wurde mittels des MTS- und FACS Assays analysiert. Weiterhin erfolgten Überstandsanalysen (Glucose, Lactat, Progesteron, Estrogen und ß-hCG) von den inkubierten Zellen. Das Rezeptorexpressionsverhalten der MCF-7 Zellen wurde zusätzlich in einem Co-Kultur-Modell bestehend aus MCF-7 Sphäroiden und Plazenta-Explantaten immunhistochemisch untersucht.

Eigenanteil: Hauptanteil, Planung und Durchführung der Experimente, Auswertung der Daten, Literaturrecherche, Erstellung der Abbildungen, Verfassung des Manuskriptes.

Mitautorenanteil: Julia Heger (Durchführung des FACS, MTS und Invasion Assays), Ann-Kathrin Fleischer (Durchführung von Western Blots), André Schmidt (fachliche Beratung), Tanja Groten (Beratung), Amelie Lupp (Betreuung bei HE- und IHC-Färbungen), Oliver Werz (Betreuung), Udo R. Markert (Betreuung).

The influence of placenta-conditioned medium on breast cancer cells

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Abstract

Purpose: Pregnancy associated breast cancer (PABC) is a rare event, but often diagnosed at an advanced stage and characterized by a hormone negative and HER2 positive expression. Therefore, we established a model for PABC with placenta-conditioned medium (PCM) and analyzed the effects on three breast cancer cell lines MCF-7, MDA-MB-231 and SK-BR-3.

Methods: In this study the influence of PCM has been investigated compared to unconditioned medium (UCM) by assessing cell viability, cell cycle phases and invasion of the breast cancer cells. Hormone levels were measured in supernatants. Morphology was studied microscopically and receptor expression of HER2, PR, ER α and ER ß was assessed by Western blot and immunofluorescence analyses. The expression of PR, ER α and ER ß was also investigated in a three-dimensional co-culture model of MCF-7 spheroids and placental villous explants (PVE) by immunostaining.

Results: Viability of MDA-MB-231 cells was slightly decreased in PCM, while MCF-7 as well as SK-BR-3 viability increased after 24 and 48 h. Invasion of conditioned MDA-MB-231 cells was significantly increased. All breast cancer cells changed their morphology towards a spindle-like shape. Receptor expression of conditioned MCF-7 cells was up-regulated; except for ER α, which was significantly reduced. Immunostaining of co-cultures revealed also a noticeable down-regulation of ER α. PCM had no influence on the receptor expression in MDA-MB-231 and SK-BR-3 cells.

Conclusions: The interactions between the different hormone receptors and HER2 are very complex and influence tumor invasiveness and proliferation. PCM altered receptor expression of the hormone positive cell line MCF-7, as ER α was down- und ER ß was upregulated suggesting reduced tumor invasiveness and progression. Nevertheless, invasion assay revealed no reduced invasion of MCF-7 cells in PCM. Moreover, invasion of conditioned MDA-MB-231 cells was increased, which indicates that other soluble factors secreted by the placenta might have an influence on breast cancer proliferation, death and invasion.

Keywords: placenta, breast cancer, MCF-7, MDA-MB-231, SK-BR-3, pregnancy, tumor

Introduction

Breast cancer and pregnancy is a rare coincidence (Stensheim et al. 2009), but since the decline of cervical cancer it has become the most common malignancy diagnosed during pregnancy (Amant et al. 2012, Loibl et al. 2006, Van Calsteren et al. 2010). The disease itself is often diagnosed at an advanced stage, which could be explained by several aspects (Woo et al. 2003, Ishida et al. 1992, Amant et al. 2013): Due to physiological changes in mammary glands of pregnant women, early diagnosis is quite rare and challenging (Amant et al. 2012, Salani et al. 2014). Also, specific histopathology and higher aggressiveness of PABC might cause an advanced stage at diagnosis. In contrast, data about placental metastasis of breast cancer cells are rare (Vetter et al. 2014): Since 1866, less than 100 cases of maternal tumours metastasizing to the placenta or foetus have been reported (Alexander et al. 2003). Tartakover-Matalon et. al. has been investigated the interaction between placenta tissue and breast cancer cells experimentally and concluded that the placenta might act as a non-supportive microenvironment for malignant cells in general and for breast cancer cells particularly (Epstein Shochet et al. 2012, Tartakover-Matalon et al. 2010). In general, the mechanisms in affecting patho- or physiological conditions are still underestimated and also the placenta, which is highly specialized in its function and presents the physiological barrier between fetus and mother as well as enables nutritional and gas exchange, need to be investigated more deeply (Newbern und Freemark 2011, Barker und Thornburg 2013, Barker et al. 2010, Borbely et al. 2014). Therefore, the Human Placenta Project has been launched in 2014 to emphasize the importance of innovative pregnancy associated studies (Guttmacher et al. 2014, Sadovsky et al. 2014). This project aims different aspects, such as improvement or development of special technologies (e.g. magnetic resonance imaging, genome analyses) for placenta assessment and further research collaborations, which also provide new approaches to areas besides classical placenta research, such as cancer pathomechanisms and treatment (Mayhew 2015). Several hormones are secreted by the placenta, whereby the hormones estrogens, progesterone and human chorionic gonadotropin (HCG) are the thoroughly researched ones (Russo et al. 1990, Rajkumar et al. 2004) and have already been analyzed in different studies. It is assumed that these hormones have an influence on the growth and behavior of breast cancer cells. Hormone positive breast cancer cells co-cultured with first trimester placental tissue decreased their cell number and receptor expression of estrogen receptor (ER) alpha near placental areas (Tartakover-Matalon et al. 2010). Moreover, Epstein Shochet et al. showed that the first trimester placental factors trigger breast cancer cells differentiation into motile cells (Epstein Shochet et al. 2015). In this context, growth and differentiation of breast cancer cells, which were exposed to altered hormonal conditions during pregnancy or postpartum might be stimulated by the women's gestational hormonal environment (Schedin 2006). Therefore, a tumor subtype-specific association of hormone-related reproductive factors on breast cancer survival has been suggested, e.g. human epidermal growth factor receptor 2 (HER2) positive tumors tend to have no better survival regarding a prolonged duration of endogenous estrogen exposure (Song et al. 2015). Hence, it should be considered that breast cancer exhibits heterogeneous clinical histopathology in case of ER, progesterone receptor (PR) and HER2 expression (Sorlie et al. 2001). Some clinical studies demonstrated a twice more often negative hormone receptor status in case of PABC. Moreover, HER2-over-expression and triple negative tumors tend to be more frequent (Genin et al. 2012, Bonnier et al. 1997).

Thus, in the current study we aim to investigate the influence of term placenta on three different breast cancer cell lines. MCF-7, a non-invasive, hormone positive and HER2 negative cell line (Levenson und Jordan 1997), SK-BR-3, a more invasive, hormone negative and HER2 positive cell line (Trempe 1976),as well as the triple negative and invasive cell line MDA-MB-231 (Cailleau et al. 1978) were incubated with PCM for at least four days. Furthermore, the impact of PVE on hormone receptor expression of MCF-7 spheroids was analyzed.

Material and Methods

The Placenta Lab strictly applies quality management and is certified after DIN EN ISO 9001

Cell culture

MCF-7 (ATCC® HTB-22TM) and SK-BR-3 (ATCC® HTB-30TM) breast cancer cells were cultivated in DMEM high glucose (Gibco, Paisley, UK). MDA-MB-231 (ATCC® HTB-26TM) breast cancer cells were cultured in RPMI 1640 (Gibco). Cells were trypsinized (0.05% trypsin-EDTA; Gibco) as soon as confluence was observed until passage 65 (MCF-7; MDA-MB-231) or 75 (SK-BR-3). Cells were cultured at 37°C in a humidified atmosphere with 5% CO_2 and all media were supplemented with 10% fetal calf serum (Gibco) and 1% penicillin/ streptomycin (P/S; Gibco). Cells regularly screened by PCR for absence of mycoplasma. Short Tandem Repeat (STR) analyses were performed to confirm identity of cell lines.

Supernatant analyses of lactate dehydrogenase (LDH), glucose, lactate, human choriongonadotropin (ß-HCG), estrogen and progesterone

Glucose concentration was assessed in accordance to phosphorylation and oxidation reactions and measuring at 340 nm (Architect i-System, Abbott GmbH, Wiesbaden, GER). After an enzymatic transformation of lactate to pyruvate, the produced hydrogen peroxide was measured via color intensity, which is proportional to lactate and LDH concentration (Abbott GmbH). ß-HCG concentration was determined by chemiluminescence microparticle immunoassay (Abbott GmbH). Estrogen and progesterone concentration were assessed by Cobas e411 Immunoassay (Roche Diagnostics, IN, USA) according to manufacturer's instructions using biotinylated polyclonal anti-estradiol antibody (Roche Diagnostics, Mannheim, GER) or biotinylated monoclonal anti-progesterone antibody (Roche Diagnostics).

Incubation of breast cancer cells with PCM

After spontaneous delivery or caesarian section, five healthy human term placentas were obtained from the Department of Obstetrics, Jena University Hospital. Villi of placenta tissue were cut in defined pieces, washed twice with PBS (Gibco) plus 1% P/S and incubated for 30 h with DMEM high glucose or RPMI (1 g villous tissue: 10 ml medium). PCM was centrifuged at 180 × g for ten minutes using Heraeus Multifuge 1S Centrifuge (Thermo Fisher Scientific, Osterode, GER). 0.25 – 0.5 × 10^6 cells in 2 ml/well were seeded on 6-well plates and allowed to attach overnight in maintenance medium. For initiation of experiments (0 h), culture medium was replaced with either PCM or UCM. All analyses were performed after 1, 24, 48 or 96 h. If cells were incubated with PCM, they are named as conditioned cells. If UCM was used, cells were designated as unconditioned/ control.

Metabolic activity assay

Metabolic activity of breast cancer cells was assessed via the Cell Titer AQeous MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay (Promega, Mannheim, GER) according to the manufacturer's instructions. Cells were seeded overnight in 96-well plates at a density of 10,000 (1, 24 h) or 5,000 (48 96 h) cells per 200µl/wel in maintenance medium. For initiation of experiments (0 h), culture medium was replaced with either PCM or UCM. The absorbance, resampling the metabolic activity, was measured in triplicates at 490 nm using SPECTROstar Omega (BMG Labtech, Offenburg, GER). Results were normalized to controls as follows: % viability = $(A_{conditioned} – A_{media})/(A_{unconditioned} – A_{media})$, A = mean absorbance.

Flow cytometry assay (FACS) for assessment of viable, apoptotic and necrotic cells

After double staining with FITC-Annexin V and propidium iodide (PI) using FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen, San Jose, CA, USA), viable, apoptotic and necrotic cells were analyzed by FACSCalibur (BD Biosciences, San Jose, CA, USA) and evaluated by the Cell Quest Pro software (BD Biosciences).

Microscopical analyses of cell morphology

After the indicated time points, cells were imaged by Axiovert 25 CFL microscope (Carl Zeiss, Jena, GER) using the camera Sony Cyber-shot 3.3 MegaPixels (Sony, Tokio, JPN). For statistical analyses spindle-like cells and total cell number was counted of each condition (n = 5).

Matrigel invasion assay

Cell invasion was evaluated in 24-well inserts with 8 μm pore size transwell membranes (Merck Millipore). 100,000 cells in 200 μl serumfree maintenance medium were seeded in inserts pre-coated with growth factor reduced Matrigel (BD Biosciences, Heidelberg, Germany). The lower chamber was filled with 500 μl PCM or UCM. After 24 h of culture, cells remaining in the upper chamber were removed via cotton swab, while invaded cells were fixed under the insert with cooled 80% ethanol and stained with crystal violet. After washing and de-staining with 1% acetic acid, colorimetric absorbance was measured at 570 nm.

Protein Isolation

After washing in PBS, cells were lysed in cell lysis buffer supplemented with protease inhibitor (SERVA Electrophoresis, Heidelberg, GER). The samples were stored at -80 °C over night to ensure the complete celllysis. After centrifugation (150 × g, 20 minutes, 4°C), supernatants were collected and protein concentrations were determined by a Pierce® BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western Blotting

30 µg of protein lysates were suspended in gel-loading buffer (62.5 mM Tris-HCl; pH 6.8; 2% SDS; 25% glycerol; 1% phenol blue; 5% β-mercaptoethanol), boiled for five minutes and resolved on 7.5% acrylamide SDS gels. After electroblotting to nitrocellulose membrane (Biotrace NT, VWR International GmbH, Dresden, GER), membranes were blocked in milk or BSA-containing buffer for 1 h (1x TBS containing 0.1% Tween-20 with 5% w/v nonfat dry milk or 5% w/v BSA (HER2 antibody)). Membranes were incubated with primary antibodies overnight at 4°C (table 1). After washing with 1x TBS containing 0.1% Tween-20 membranes were incubated with goat anti-rabbit IRDye 800CW secondary antibody (1:15,000; Li-Cor, Lincoln, NE, USA) for 40 minutes at room temperature. The blots were scanned with the Odyssey scanner (Li-Cor Biosciences, Bad Homburg, GER) according to the manufacturer's instructions.

Immunofluorescence (IF) staining

The cells were cultured on SuperFrost/Plus slides (Menzel, Germany) and fixed with 4% paraformaldehyde (PFA) solution (16 g PFA, 2 M Tris-HCl, 4 M NaCl, pH 7.6, total volume 400 ml). Afterwards, all samples were incubated with 5% goat serum (No. PK 6100, Vectastatin® Elite® ABC Kit, Vector Laboratories, Burlingame, USA) and 0.3% Triton X (AppliChem, Chicago, IL, USA) for one hour at room temperature. Samples were incubated with primary antibodies (Table 1) diluted in DAKO Antibody Diluent with Background Reducing Components (DAKO, Glostrup, DK) overnight at 4°C. After washing with PBS, all samples were incubated with AffiniPure goat anti-rabbit secondary antibody labeled with Cy3 (No. 111-165-003, Dianova, Hamburg, GER) for one hour at room temperature. Phalloidin-actin staining (1:3000; CytoPainter Phalloidin-iFluor 488 Reagent, ab176753, Abcam) was performed

and slides were mounted in Vectashield Mounting Media with DAPI (VECTOR Laboratories, Burlingame, CA, USA). Samples were evaluated using Olympus IX81 microscope (Olympus, Tokyo, JPN).

PVE co-cultured with MCF-7 spheroids

Co-cultures were prepared and cultivated as described in detail by Froehlich et al. After 1 h, 24 h, 48 h and 72 h hematoxylin and eosin (HE) staining as well as immunostaining were performed, following the protocol from Froehlich et. al. To determine hormone receptor expression of MCF-7 spheroids immunostaining was performed with PR, ER α and ER ß primary antibodies (table 1). Sections were evaluated by an Axio Imager A1 microscope (Carl Zeiss) and furthermore positive and negative cells were determined via ImageJ. With the following formula necrotic cells in the core of the spheroids were excluded: % positive cells = positive cells/ (positive+negative cells). Detailed information shown in Supplementary Material Fig. 1.

Statistical Analyses

All quantitative data were presented as mean ± standard error (SE). Paired Student`s t-test was used to analyze differences between unconditioned and conditioned cells, except for supernatant analyses of breast cancer cells. Here, PCM resp. UCM (0 h) was compared to supernatants of conditioned resp. unconditioned cells. A p-value of < 0.05 was considered significant. All experiments were repeated separately three to six times.

Results

PCM contains significant higher amounts of estrogen, ß-hCG and progesterone

After media-conditioning with human placenta tissues, conditioned DMEM medium revealed no change in LDH, but reduced glucose (0.44 ×) and increased lactate (4.5 ×), estrogen (45 ×), ß-hCG (317 ×) and progesterone (3128 ×) levels were measured compared to unconditioned DMEM (Fig. 1A). In case of conditioned RPMI glucose and LDH (0.64 ×) concentrations were reduced, whereas lactate (6.2 ×), estrogen (48 ×), ß-hCG (353 ×) and progesterone (754 ×) levels were increased.

PCM supports cell viability of MCF-7 and SK-BR-3 cells and suppresses cell viability of MDA-MB-231 cells

After 24 h and 48 h PCM incubation MTS assay revealed an increased metabolic activity of conditioned MCF-7 cells compared to unconditioned cells (Fig. 1B). The same effect was noticed in case of conditioned SK-BR-3 cells. Regarding the conditioned MDA-MB-231 cells, the metabolic activity was decreased compared to unconditioned cells. After 24 h and 48 h the percentage of viable conditioned MCF-7 cells increased, whereas a profound decrease was observed after 96 h (Fig. 1C a). Respectively, after 24 h apoptotic and necrotic conditioned MCF-7 cells levels were lowest and increased over time (Fig. 1C b, c). The percentages of viable and apoptotic conditioned MDA-MB-231 cells were lower, whereas necrotic cells were detected in higher amounts compared to unconditioned cells. (Fig. 1C) Viable and necrotic conditioned SK-BR-3 cells remained stable at same levels as unconditioned cells (Fig. 1C a, c). On the contrary, apoptotic cells decreased throughout PCM-incubation (Fig. 1C b).

PCM induces spindle-like formation of breast cancer cells

After 24 h unconditioned MCF-7 cells reached almost confluency, whereas the conditioned MCF-7 cells were confluent after 48 hours. The unconditioned MCF-7 cells remained stable in a round-square shape over the determined incubation time (Fig. 2A a-d). After 24 h the conditioned MCF-7 cells developed a spindle-like shape, which became more prominent over time (Fig. 2A f-g, 2C b, arrows indicate appendages). After 98 h the cell membranes of conditioned MCF-7 cells merged each other (Fig. 2A h). In case of unconditioned MDA-MB-231 cells

confluency was observed at 24 h cultivation time. In contrast, conditioned MDA-MB-231 did not reach confluency throughout the cultivation time and spindle-like cells was already present after one-hour culturing with PCM (Fig. 2A m-p; 2C d). After 48 h SK-BR-3 cells reached confluency in both conditions (Fig. 1A i-p). 24 h conditioned SK-BR-3 cells developed also spindle-like shape (Fig. 2A v-x, 2C f), whereas unconditioned SK-BR-3 cells remained stable with a round shape (Fig. 2A q-t; 2C e). Statistical analyses have been shown a significant higher number of spindle-like cells in all conditioned breast cancer cell cultures after 24 h (Fig. 2B).

Supernatants of SK-BR-3 cells present increased levels of estrogen and supernatants of all breast cancer cells present decreased levels of progesterone

During the cultivation of breast cancer cells, UCM of MCF-7 and MDA-MB-231 cells presented stable estrogen concentrations, while UCM of SK-BR-3 cells exhibit increased levels of estrogen after 48 h and 96 h (Fig. 3A a). PCM cultures presented the same effects (Fig. 3A b). In UCM of MCF-7 and SK-BR-3 cells no progesterone was measured, whereas progesterone was detectable in UCM of MDA-MB-231 (Supplementary Material, Fig. S3A). After 24 h PCM of all breast cancer cells presented decreased progesterone levels (Fig. 3A c).

PCM enhances cell invasion of MDA-MB-231 cells

Cell invasion was significantly enhanced in conditioned MDA-MB-231 cells, invasiveness was increased by 33%. Cell invasion of conditioned MCF-7 and SK-BR-3 did not differ significantly from unconditioned cells (Fig. 3B).

PCM up-regulates HER2, PR, ER ß and down-regulates ER α expression of MCF-7 cells

Western Blot analyses revealed a slight HER2 expression of MCF-7 cells, which was up-regulated over time (Fig. 4A a-b). This up-regulation was more dominant in the conditioned MCF-7 cells (Fig. 4A b-c). The same effect was detected in case of progesterone receptor (PR; Fig. 4A). ER α was expressed at a constant level in unconditioned MCF-7 cells (Fig. 4A a), while conditioned MCF-7 cells presented a significant down-regulated, especially after 24 h incubation (Fig. 4A b-c). Estrogen receptor beta (ER ß) was slightly expressed in MCF-7 cells and up-regulated, when cultured in PCM (Fig. 4A a-b). Western Blot analyses of MCF-7 cells were confirmed by IF (Fig. 4B). The expression of HER2 in SK-BR-3 cells was strong in both conditions (Supplementary Material, Fig. S2A b-c). After 24 h and 48 h expression of HER2 was higher in conditioned SK-BR-3 cells, but not significant (Supplementary Material, Fig. S2A a). PR and ER α were not expressed, whereby ER ß was slightly expressed in conditioned and unconditioned cells (Supplementary Material, Fig. S2A b-c). IF analyses revealed an up-regulation of HER2 and ER ß in conditioned SK-BR-3 cells (Supplementary Material, Fig. S2B). MDA-MB-231 cells expressed only ER ß and no influence of the PCM was detectable (Supplementary Material, Fig. S2C).

PVE increase PR and decrease ER α expression in MCF-7 spheroids

The expression of PR (61% positive cells (pc)), ER α (83% pc) and ER ß (93% pc) in MCF-7 spheroids (Fig. 5 A c-h) was compared to monolayer culture relatively high (Supplementary Material, Fig. S3B a-c). For calculation of positive cells, necrotic cells insight the spheroids, which were detected by HE staining, were excluded. After 48 h the expression of PR was reduced (40% pc) in MCF-7 spheroids and the expression of ER α and ER ß remained high (Fig. 5A e-h). Meanwhile, MCF-7 spheroids co-cultured with PVE showed a slight increase of PR expression, whereas a decrease of ER α expression was observed (Fig. 5B e-l; Supplementary Material, Fig. S3C). After 24 h the down-regulation of ER α was mostly affected (Fig. 5B j). In contrast, ER ß expression of co-cultured MCF-7 spheroids remained at a high level (Fig. 5B m-p; Supplementary Material, Fig. S3C).

Discussion

Three different breast cancer cells were incubated with PCM to analyze the effect of pregnancy-associated hormonal and physiological changes on breast cancer. After 24 h cells cultured in PCM changed their morphology to spindle-like formations, which imply a differentiation into motile cells (Tiezzi et al. 2007). Tartakover-Matalon's et. al. above-mentioned co-culture study with first trimester human placental explants and MCF-7/ T47D-eGFP cells revealed similar morphological alterations (Tartakover-Matalon et al. 2010). Here, a rapid disappearance from human placental explants was shown, suggesting migration process of the cells, which might be caused by high progesterone and estrogen levels (Sukocheva et al. 2009). Previous studies have been observed an influence of c-Jun N-terminal kinases via mitogen-activated protein kinases on this effect (Epstein Shochet et al. 2012). Furthermore, migration of ovarian epithelial cancer cells was also supported by medium conditioned with first trimester placenta (Amitai et al. 2015). These data suggest that soluble factors, e.g. progesterone and estrogen, promote differentiation and motility of cancer cells in general (Epstein Shochet et al. 2016). Moreover, it has been shown that human placenta-derived mesenchymal stem cells (hPMSCs) affect morphology, proliferation and differentiation of mammary epithelial cells (MCF-10F and HEMC) (Yoo et al. 2009). Interestingly, MCF-10F cells changed morphology towards spindle-like, fibroblastic shape suggesting an involvement of hPMSCs in the morphological process of change. MCF-7 cells cultured in PCM presented significantly reduced ER α expression, whereby strongest effects were observed after 24 h (Western blots, IF). Immunostaining of co-cultured MCF-7 spheroids confirmed a down-regulation of ER α. Therefore, our study has been shown the influence of PVE in a 3D model for the first time. Our results are in line with those generated in a study with PVE from first trimester placentas (Tartakover-Matalon et al. 2010). The previously mentioned transformation into spindle-like cells, resulting in a higher amount of motile cells, has been suspected to be connected with the reduced ER α levels (Platet et al. 2004). The two ER-negative cell lines, SK-BR-3 and MDA-MB-231, were both ER ß positive, which might explain their formation into spindle-like cells by an estrogen induced ezrin-dependent crosstalk between G protein-coupled receptor 30 (GPR30) and ER-ß resulting in cytoskeleton remodeling and increased migration (Li et al. 2013). A previous study has shown the expression of G protein-coupled receptor 30 (GPR30) and ER ß even in cancers, which are ER α negative (Steiman et al. 2013). The authors highlighted the importance of GPR30/ER-ß ratio in breast cancer cells, which determine the cellular response to estrogen (Li et al. 2013). Therefore, further studies need to be performed analyzing the amount of GPR30 in ER α negative cell lines, which are positive for ER ß. After 96 h cultivation supernatants of the ER-negative cell line SK-BR-3 showed increased estrogen levels, however with no effect on ER α expression. Otherwise, ER ß expression was found consistently in unconditioned and conditioned SK-BR-3 cells. Expression of ER ß in MCF-7 cells was slightly but increased significantly in PCM (detected by Western blots and ICC). In general, estriol, one of the three natural estrogens, is secreted in high amounts by the placenta and binds preferentially with ER ß, which might explain up-regulated ER ß in conditioned cells. In contrast, levels of estriol are very low in non-pregnant women (Wang et al. 2018). Immunostaining of MCF-7 spheroids revealed strong expression of ER ß in both conditions (pure medium/ cultured with PVE). MCF-7 monocultures presented a weak expression of ER ß suggesting the shift from 2D to 3D culture might lead to an up-regulation of ER ß expression (Supplementary Material, Fig. S3B c). HER2 expression of conditioned MCF-7 cells was increased compared to unconditioned MCF-7 cells. Interestingly, previous studies assumed a positive correlation between HER2 and ER ß expression in hormone-dependent breast cancer cells (Lattrich et al. 2008, Umekita et al. 2006, Choi und Pinto 2005). On the other hand, HER2 expression is known to be suppressed by ER α-dependent signaling mechanisms (Read et al. 1990). As ER α was down- and HER2 was up-regulated in MCF-7 cells, our results are in line with this fact. Moreover, interaction between ER α and ER ß is also reported, whereby ER ß can suppress ER α mediated effects e.g. fat reduction and cell proliferation in the uterus and prostate (Weihua et al. 2001, Zhou et al. 2016). Multiple studies have shown that ER ß over-expression is connected with an improved prognosis in all breast cancer subtypes (Mann et al. 2001, Honma et al. 2008). Therefore, ER ß is discussed to act as a tumor

suppressor in hormone-dependent tissue (Delaunay et al. 2000, Li et al. 2013). In correlation to our results receptor expression of conditioned MCF-7 cells has been altered in that direction suggesting a reduced invasiveness, which supports the fact that the placenta acts as non-supportive cancer microenvironment (Tartakover-Matalon et al. 2010). Therefore, an invasion assay was additionally performed. Conditioned MCF-7 cells presented the same invasion characteristics as unconditioned cells. Cell viability analyses have been shown a slight tendency towards increased viability in conditioned MCF-7 and SK-BR-3 cells, which turned back to levels of unconditioned cells. Conditioned MDA-MB-231 cells decreased viability, whereas a lower number of apoptotic cells and a higher number of necrotic cells were detectable than in unconditioned cells. Contrary, invasion of conditioned MDA-MB-231 cells was significantly increased, which might be partially facilitated by high amounts of placental growth factor in PCM (Taylor und Goldenberg 2007). To compare with the first trimester placenta co-culture study of Tartakover-Matalon et. al., reduced cell numbers of MCF-7/ T47D-eGFP near the placental area and increased amounts of apoptotic MCF-7 cells were observed. Also, ER and PR inhibitors did not change apoptosis or proliferation of breast cancer cells (Epstein Shochet et al. 2012), but the elimination of the breast cancer cells from the placenta area was mediated by estrogen and progesterone suggesting the reduced number of MCF-7 cells was mainly due to MCF-7 invasion and migration (Epstein Shochet et al. 2016). This suggests that other soluble factors secreted by the placenta may have an influence on breast cancer proliferation, death and invasion, as placenta villi in our study had no decisive impact on breast cancer cell proliferation and increased invasion of MDA-MB-231 cells.

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Conflict of interest statement

All authors declare no conflict of interest.

Fig. 1

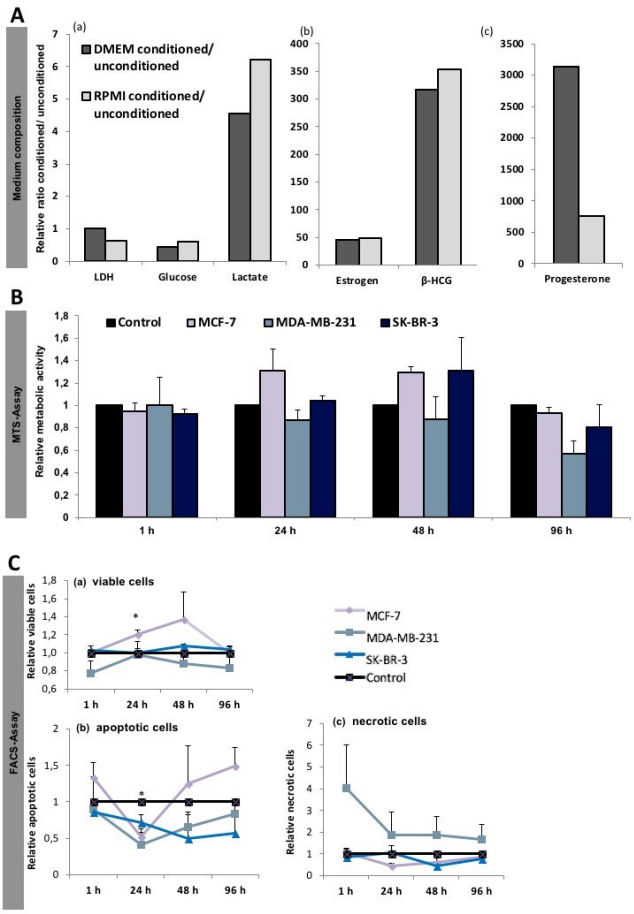


Fig. 2

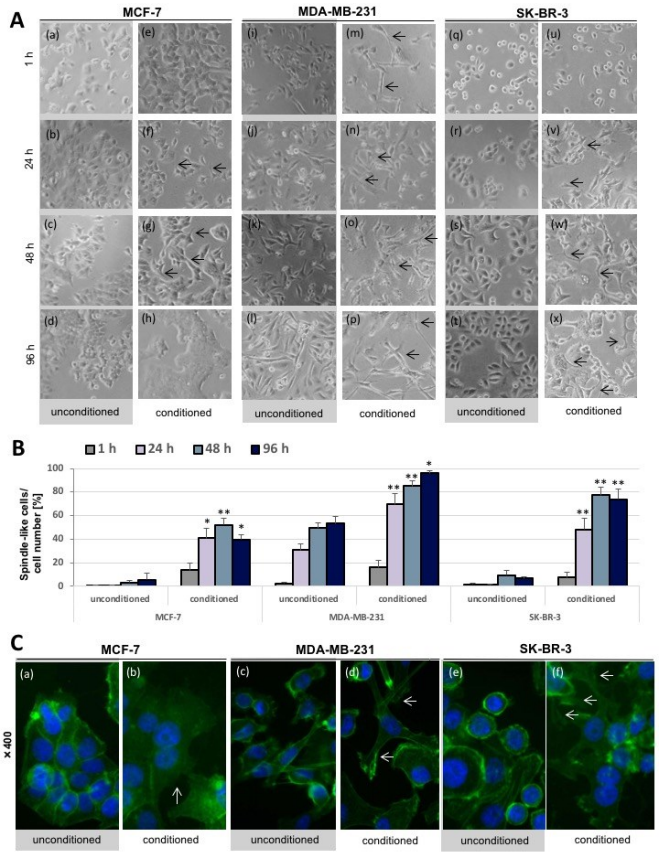


Fig. 3

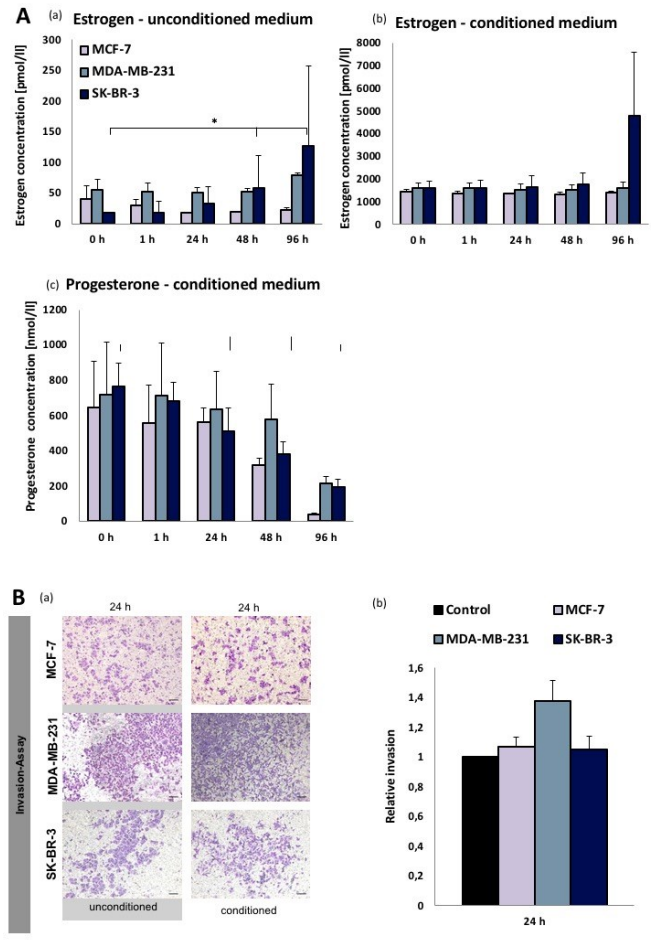


Fig. 4

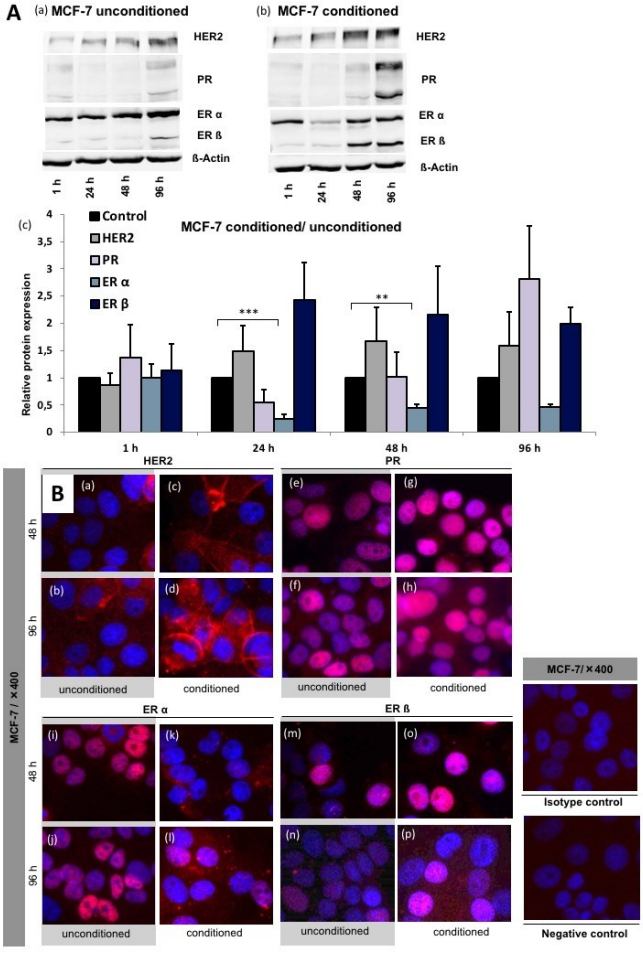
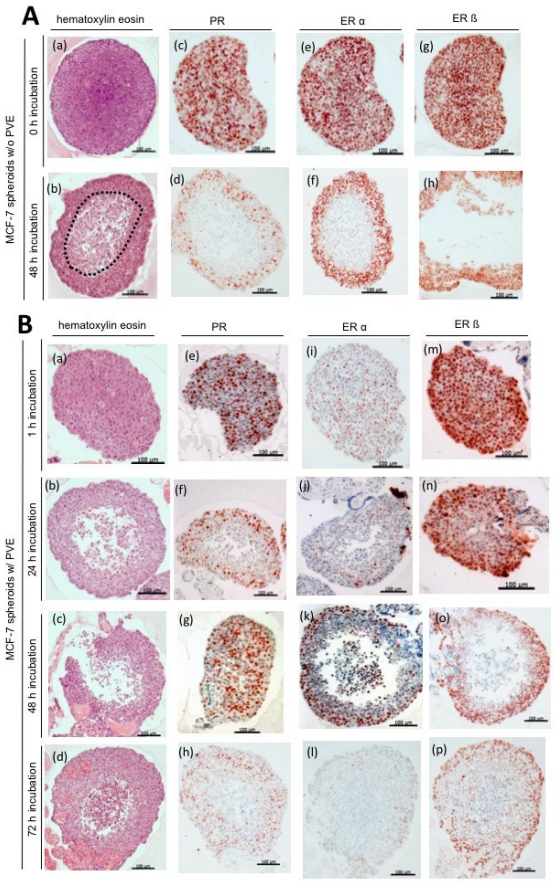
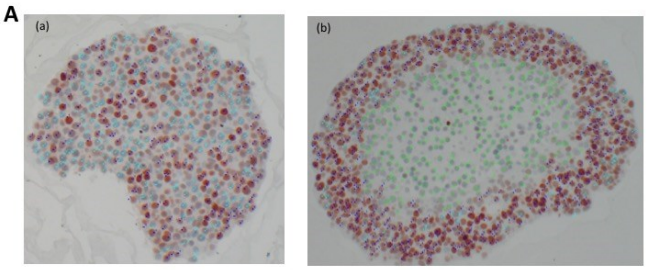


Fig. 5



Supplementary Material, Fig. S1



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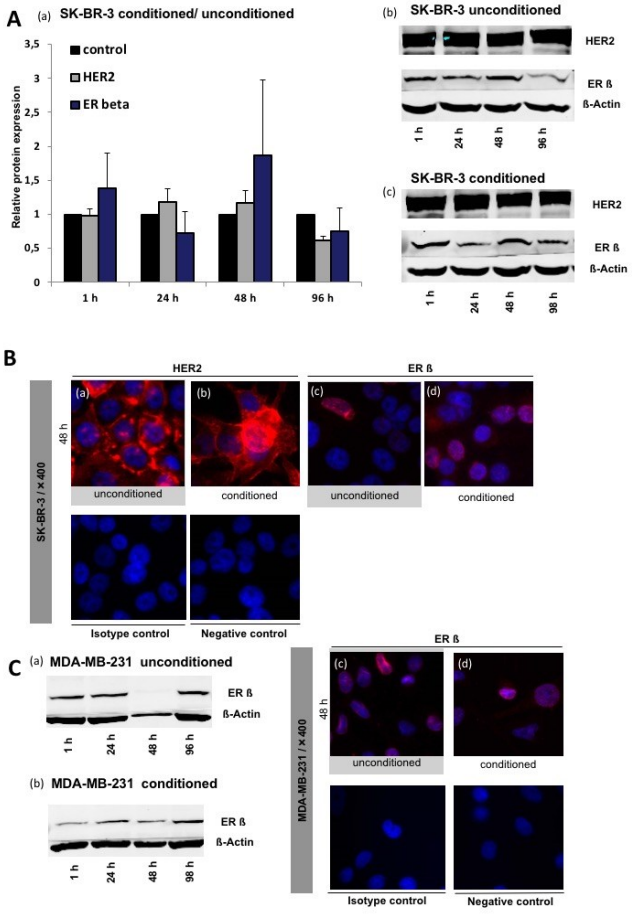

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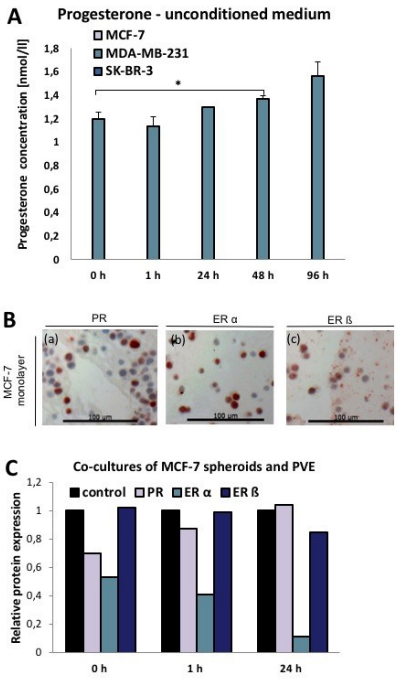

Fig. 1 Characterization of conditioned and unconditioned medium and viability of breast cancer cells

A Metabolic markers and hormone concentrations in conditioned compared to unconditioned medium. DMEM medium, used for cultivation of MCF-7 and SK-BR-3 cells, and RPMI medium, used for cultivation of MDA-MB-231 cells, were conditioned with placenta tissue (1 g villous tissue: 10 ml medium) for 30 h. After centrifugation and pooling, concentration of LDH, glucose, lactate, estrogen ß- HCG and progesterone were measured in conditioned DMEM and conditioned RPMI media and normalized to unconditioned media.

B Metabolic activity of breast cancer cells. Cells were incubated with conditioned or unconditioned medium for 1 h, 24 h, 48 h or 96 h. MTS colorimetric assays (n = 3, each performed in triplicates) were performed and optical density (OD) at 490 nm was measured to assess cell viability. Data were normalized to unconditioned cells (control). Bars show mean ± SE, * indicates $p < 0.05$, when metabolic activity of controls were compared with metabolic activity of conditioned cells; Student's t- test (paired, two-tailed).

C Flow cytometry was used for quantification of apoptosis and necrosis: annexin V–fluorescein isothiocynate (FITC) and propidium iodide (PI) stained apoptotic and necrotic cells, which were cultured before with or without (control) PCM (n = 3). Bars show mean ± SE, * indicates $p < 0.05$; Student's t- test (paired, two-tailed).

Fig. 2 Representative microphotographs of breast cancer cell lines cultured with conditioned or unconditioned medium

A Cells were seeded in 6-well plates and incubated overnight for attachment. Subsequently they were incubated with placenta conditioned medium (PCM) or unconditioned medium (n=5). After 1 h, 24 h, 48 h and 96 h, morphology was studied by light microscopy. Arrows indicate appendages of cells; these cells were defined as spindle-like cells. Magnification 100×.

B Microphotographs were used for statistical analysis. The number of spindle-like cells was counted using ImageJ software and was normalized to the total cell number in each microphotograph. Ratio of unconditioned cells was compared to conditioned cells. Bars show mean ± SE (n = 5), * indicates $p < 0.05$, ** indicate $p < 0.01$; Student's t-test (paired, two-tailed).

B Immunofluorescence analysis. Cells were seeded overnight on microscope slides until attachment and incubated with conditioned or unconditioned medium. After 1 h, 24 h, 48 h and 96 h, cells were fixed with 4% paraformaldehyde and actin filaments (Phalloidin-iFluor 488, green) and nuclei (DAPI, blue) were stained. Exemplary, 48 h incubated cells are shown. Arrows indicate appendages. Magnification 400×.

Fig. 3 Characteristics of hormone secretion and invasion of conditioned and unconditioned breast cancer cells

A Supernatant analysis of estrogen and progesterone in conditioned and unconditioned breast cancer cells. Cells were seeded in 6-well plates and incubated overnight. Subsequently, cells were incubated with conditioned or unconditioned medium. After 1 h, 24 h, 48 h and 96 h, supernatants were collected and concentration of estrogen (a-b) and progesterone (c) were measured. Data represent the mean of n = 3 independent experiments. Hormone levels of free medium (0 h) were compared to supernatants of breast cancer cells (1 – 96 h). Bars show mean ± SE, * indicates $p < 0.05$, ** indicate $p < 0.01$; Student's t- test (paired, two-tailed).

B Cells were seeded in serum free medium on transwell chambers coated with Matrigel and incubated immediately in conditioned or unconditioned medium. After 24 h, invaded cells under the transwell membranes have been

stained and photographed (a). Absorbance at 570 nm has been measured on a plate reader and normalized to unconditioned cells (b). Bars show mean ± SE (n = 5, each performed in triplicates), * indicates $p < 0.05$; Student's t- test (paired, two-tailed). Scale bars = 100 μm.

Fig. 4 Hormone receptor and HER2 analyses of MCF-7 cells

A Representative Western blots of lysates from MCF-7 cells incubated with (a) unconditioned or (b) conditioned medium for 1 h, 24 h, 48 h and 96 h. The bands of all blots (n=6) have been scanned for density analysis and the density of bands from HER2, PR, ER α and ER ß has been normalized against ß-Actin bands. Afterwards, the bands from conditioned cells were related to bands from unconditioned cells, signifying in the definition of "1" for the density of proteins in unconditioned medium (control, c). Bars show mean ± SE, * indicates $p < 0.05$, ** indicate $p < 0.01$, *** indicate $p < 0.001$; Student's t- test (paired, two-tailed).

B Immunofluorescence analysis. Cells were seeded overnight on microscope slides until attachment and incubated with PCM or unconditioned medium. After 1 h, 24 h, 48 h and 96 h, cells were fixed with 4% paraformaldehyde and stained with Cy3 labeled secondary antibody (red) for HER2, PR, ER α and ER ß. Nuclei were counterstained with DAPI (blue). Exemplary, 48 h and 96 h incubated cells are shown. Magnification 400×.

Fig. 5 HE and immunostaining of MCF-7 spheroids, co-cultures of MCF-7 spheroids and placental villous explants

A MCF-7 spheroids were harvested after formation (3 days in hanging drops with 25% methocel; 10,000 cells/drop) and optional cultivated for 48 h. Subsequently embedding in paraffin. The microphotographs show HE staining (a-b), immunlocalization of PR (c-d), ER α (e-f) and ER ß (g-h) in MCF-7 spheroids. (e) Exemplarily, the inner zone is indicated by a dotted line. Scale bars = 100 μm.

B MCF-7 spheroids co-cultured with placental villous explants. MCF-7 spheroids were incubated after formation (3 days in hanging drops with 25% methocel; 10,000 cells/drop) with biopsy-sized placental villous explants, optional cultivated for further 24 h, 48 h or 72 h, and embedded in paraffin. The microphotographs show HE staining (a-d), immunlocalization of PR (e-h), ER α (i-l) and ER ß (m-p) in MCF-7 spheroids. Scale bars=100 μm.

Supplementary Material, Fig. S1 Calculation of percentage of positive cells/ MCF-7 spheroid

A MCF-7 spheroids were harvested after formation (3 days in hanging drops with 25% methocel; 10,000 cells/drop), optional further cultivated, and subsequently embedded in paraffin. Sections were stained (brown = positive cells) for PR, ER α and ER ß and representative microphotographs were evaluated with ImageJ (1 positive, 2 negative, 3 necrotic cells). (a) MCF-7 spheroid without necrotic core. (b) MCF-7 spheroid with necrotic core.

Supplementary Material, Fig. S2 Hormone receptor and HER2 analyses of MDA-MB-231 and SK-BR-3 cells

A Representative Western blot of lysates from SK-BR-3 cells incubated with (b) unconditioned or (c) conditioned medium for 1 h, 24 h, 48 h and 96 h. The bands of all blots (n=3) have been scanned for density analysis and the density of bands from HER2 and ER ß has been normalized against ß-Actin bands. Afterwards, the bands from conditioned cells were related to bands from unconditioned cells, signifying in the definition of "1" for the density of proteins in unconditioned medium (control). Bars show mean ± SE.

B Immunofluorescence analysis. SK-BR-3 cells were seeded overnight on microscope slides until attachment and incubated with conditioned or unconditioned medium. After 1 h, 24 h, 48 h and 96 h, cells were fixed with 4%

paraformaldehyde and stained with Cy3 labeled secondary antibody (red) for HER2 (a-b) and ER ß (c-d). Nuclei were counterstained with DAPI (blue). Exemplary, 48 h incubated cells are shown. Magnification 400×.

C Receptor analyses of MDA-MB-231 cells by Western Blot and immunofluorescence. Cells were incubated with (a,c) unconditioned or (b,d) conditioned medium for 1 h, 24 h, 48 h and 96 h. Bands from MDA-MB-231 lysates as well as red stained (Cy3 labeled secondary antibody) cells via immunofluorescence indicate expression of ER ß. Magnification 400×.

Supplementary Material, Fig. S3

Table 1 List of antibodies.

Antibody	*Isotype*	*Concentration*	*Source*	*No.*
Western Blotting				
HER2	polyclonal rabbit	1-1000	Cell Signaling (Frankfurt am Main, GER)	2242
PR	monoclonal rabbit	1-1000	Cell Signaling	8757
ER alpha	monoclonal rabbit	1-1000	Cell Signaling	13258
ER beta	monoclonal rabbit	1-10,000	Abcam (Cambridge, UK)	ab133467
ß-Actin	monoclonal rabbit	1-1000	Cell Signaling	4970
Immunofluorescence				
HER2	monoclonal rabbit	1-800	Cell Signaling	2165
PR	monoclonal rabbit	1-800	Cell Signaling	8757
ER alpha	monoclonal rabbit	1-400	Cell Signaling	13258
ER beta	monoclonal rabbit	1-250	Abcam	ab92306
Immunocytochemistry				
PR	monoclonal rabbit	1-1000	Cell Signaling	8757
ER alpha	monoclonal rabbit	1-250	Abcam	ab108398
ER beta	monoclonal rabbit	1-500	Abcam	ab133467

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Manuskript 8: hCG − an endocrine, regulator of gestation and cancer

Artikel Typ: Original Paper

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Status: Manuskript erstellt, Prüfung und Korrektur für die Einreichung

Die Rolle des hCGs wird in aktuellen Studien intensiv als entscheidender Faktor während der Implantation und Schwangerschaft analysiert. In dieser Arbeit wurde die hCG Expression bzw. Konzentration in vier verschiedenen plazentaren Modellen gemessen: Die Trophoblastzelllinien JEG-3 und HTR8/Svneo wurden sowohl 2D als auch 3D kultiviert, daneben wurden Plazenta-Explantate und die einseitige Plazenta-Perfusion als komplexere Modelle herangezogen. Für Toxizitätsanalysen erfolgte die Exposition mit verschiedenen Schwermetallen. Während bei den immortalisieren Zellen sowohl in 2D als auch in 3D und der Plazenta-Perfusion stabile hCG Level zu verzeichnen waren, sank die hCG Produktion in den Plazenta-Explantaten nach dreitägiger Kultivierungszeit rapide ab. Schwermetallinkubationen supprimierten die hCG Level in den Zellkulturen. Diese Analysen zeigen, dass zum einen die hCG Produktion stark von den Kultivierungsbedingungen abhängt und zum anderen hCG als empfindlicher Indikator für Toxizitätsanalysen benutzt werden kann.

Eigenanteil: fachliche Beratung, Unterstützung bei Sphäroidbildung und mikroskopischen Aufnahmen sowie Größenbestimmung mit Hilfe des ZEN Programmes von Zeiss, Betreuung von Stephanie Morgner.

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hCG − an endocrine regulator of gestation and cancer

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Abstract

Background: The clinical relevance of hCG has been studied intensively as it functions as key modulator of placentation and maintenance of pregnancy (Cole 2012a). hCG disorders are associated with pregnancy complications like preeclampsia or trophoblastic neoplasms leading to its use in diagnostics and trials for clinical application (Cole 2014, Cole 2012b, Cole 2009b). In basic research several functions of hCG remain unclear, since contradictory results have been published (Watson et al. 1995).

Methods: We have analyzed and compared the kinetics of hCG expression in 5 different models that have been established for investigation of human placental properties: JEG-3 and HTR8/SVneo were cultured in monolayers (1) or formed to spheroid-like 3D structures (2). Placentas obtained from uncomplicated pregnancies at term were taken to prepare villous tissue explants (3), to isolate trophoblast cells (4) and for performance of a single maternal side perfusion (5). To evaluate its usefulness for toxicological assessments on trophoblast cells, hCG secretion was analyzed after exposure to heavy metals.

Results: JEG-3 cells showed a stable release of hCG, which was persistent when cells were cultured as a 3D structure. Explants, hallmarked by syncytiotrophoblast degeneration within the first days of culture, completely failed in detectable hCG secretion. In primary trophoblasts, the lowest hCG production was observed at day 1, the highest at day 3 or 4. In placenta perfusion studies, a continuous hCG secretion was maintained over the complete perfusion time. Upon exposure of JEG-3 monolayer and spheroid cultures to heavy metals hCG expression changes more sensitively in a concentration- and time-dependent manner than glucose and lactate concentrations. mercury compounds induced a significant inhibition of ß-hCG synthesis in cultures of term trophoblast cells at all applied doses.

Conclusion: hCG expression reflects trophoblast endocrine functions and cell vitality and may be used as a respective marker in cultures and toxicological testings.

Keyords:

Placenta, hCG, toxicity models, non-animal alternatives

Abbreviations

FBS	Fetal bovine serum
hCG	human chorionic gonadotropin
poly-HEMA	poly- (2-hydroxyethyl methacrylate)

Introduction

During pregnancy, the human placenta supports the fetal development by regulating the exchange of oxygen, nutrients and waste products between the maternal and fetal circulation. Its highly specific barrier is able to protect the fetus against numerous xenobiotic molecules, but enables the transfer of IgG antibodies, which thereby set up an immune protection to the fetus against infectious diseases (Gude et al. 2004). Further, it acts as endocrine organ providing a number of hormones and proteins (Honkisz und Wojtowicz 2015). In implantation and pregnancy hCG is one of the most crucial messengers, also known to be the pregnancy hormone per se. The extensive and profound research by Cole et al. during the last decades gained new findings about hCG, which is not only a promotor for progesterone production, but functions as a key modulator in physiological as well as deregulated pathways (Cole 2009b, Cole 2009c, Cole 2009a, Cole 2012a, Cole 2012b, Cole 2014). While the endocrine hormone hCG, which is produced by syncytiotrophoblast cells, maintains angiogenesis in spiral arteries and promotes differentiation of trophoblast cells, its hyperglycosylated form is released by extravillous cytotrophoblast cells and acts as an autocrine factor supporting placenta growth and implantation [2]. Hence, a permanent concentration of both types in the maternal blood serum starting from implantation is essential and levels out of the range of tolerance indicate complications during pregnancy. For instance, preeclampsia is linked with low levels of hyperglycosylated hCG at the end of the first trimester potentially due to placentation disorders (Cole 2009b). On the contrary, an excessively high production of hyperglycosylated hCG at term can be a signal for trophoblastic neoplasm or choriocarcinoma (Cole 2014). Its ß-free subunit is a potential trigger of malignancies and blocks the apoptosis pathway by antagonizing a TGF-ß receptor (Cole 2009b, Cole 2014). The ß-free subunit is secreted by nearly 55% of all cancers and may be targeted for cancer therapy (Cole 2014, Cole 2012a).

In normal pregnancies, the ß-subunit of the endocrine acting hCG type is synthesized and secreted exclusively by the syncytiotrophoblast layer of the placenta (Watson et al. 1995, Cole 2014). Its use as a viability marker for culture of placenta-derived cells has become routine as it indicates syncytial secretory capacity (Miller et al. 2005, Muoth et al. 2016, Genbacev et al. 1992, Bechi et al. 2013, Di Santo et al. 2003, Orendi et al. 2011). On the contrary, it was questioned if the hormone level in supernatants may be due to starting necrotic processes of the syncytiotrophoblast (Miller et al. 2005, Watson et al. 1995). Studies reported a degeneration of these cells even when the release of ß-hCG was rising until day 5 of culture (Watson et al. 1995). Interestingly, Simán et al. measured increasing levels of ß-hCG reaching a plateau by 5 to 6 days of culture by preparing placenta pieces with a weight of 5 mg. Moreover, it was possible to generate a newly formed syncytiotrophoblast layer, which remained viable for at least 11 days in culture (Siman et al. 2001). A constant release of ß-hCG for 10 days could also be demonstrated when first trimester explants were cultured on decidual extract supplemented collagen matrix. Here, the hormone production was connected with the differentiation of extravillous trophoblast cells (Genbacev et al. 1992). In another experimental setup, ß-hCG levels in supernatants of early and term placenta histocultures containing collagen peaked on day 1 to 4 followed by a decrease reaching a minimum at day 14, while cell viability was proved morphologically until day 10 (Morere et al. 2015, Faye et al. 2005). Hamilton et al. have cultured term placenta explants, which remained viable for up to 21 days by using the same culture protocol. Expression of hCG was consistent with constant explant viability (Hamilton et al. 2012). To exclude misinterpretations, radioactive amino acid has been added to the cultures demonstrating de novo biosynthesis by incorporation into the polypeptide (Miller et al. 2005). These findings illustrate, that the function of hCG in cell as well as tissue culture remains unclear. Since structure and secretion of hCG as well as hypergycosylated hCG are human specific and closely connected with the development of hemochorial placentation and evolution of humans, its function is hardly to investigate as no animal models can be used [1].

In this study, we focused on 4 in vitro models that have been established for human placenta derived cells and can further be used for toxicity studies (Gohner et al. 2014). These models were used to assess ß-hCG secretion. The

trophoblastic cell lines HTR8/SVneo and JEG-3 have been cultured in monolayer and formed to spheroids. With regard to the complexity of tissue structure placental explants were prepared from term placentas. This model may be useful for investigating drug-specific effects on cell-cell as well as cell-matrix interactions. We have used it to investigate the effects on ß-hCG secretion in placental cells after exposure to heavy metals to analyze its potential for toxicity assessment (Magnarelli und Guiñazú 2012). Finally, one-side placenta perfusion has been performed to study ß-hCG secretion into the maternal circulation as well as its tissue accumulation (Gohner et al. 2014).

Material and Methods

Cell culture

The human choriocarcinoma-derived placental cell line JEG-3 (DSMZ, Braunschweig, Germany) was cultured in Ham F-12 medium (Gibco, Paisley, UK). HTR-8/SVneo (kind gift from CH Graham, Kingston, Canada), an immortalized human trophoblast cell line, was cultured in RMPI-1640 medium (Gibco). Media were supplemented with 10 % fetal bovine serum (FBS) (Sigma Steinheim, Germany) and 1% penicillin/streptomycin (Gibco). Cells were maintained under standardized conditions (37 °C, 5% CO_2, humidified atmosphere) and regularly screened for absence of mycoplasma. The medium was replaced at least every three days. Cells were splitted as soon as confluence was observed by incubation with 0.05% trypsin-EDTA (Gibco). For toxicity tests JEG-3 cells were seeded in 6 well plates at a density of 4 x 10^5 cells and allowed to attach overnight before incubation was started.

Spheroid formation assay

Spheroids were generated by the hanging drop method (Fig. 1). Briefly, 30 drops containing 10,000 cells in 20 µl of their respective medium were placed on the inner side of a Petri dish lid. Lids were inverted over the PBS-filled bottom of the Petri dish and cultured for 24 h after which they were transferred into individual wells coated with 2% poly-HEMA and 260 µl culture medium. Spheroids were cultured for 48 h and fed every day by carefully aspirating 130 µl of medium and replacing it with the same volume of fresh complete medium.

Placenta explants tissue culture

Term placentas (n = 3) were obtained from the University Hospital Jena, Germany, after spontaneous delivery or caesarean section following normal pregnancies. All placentas were collected under the terms of a patient`s consent form approved by the Ethics Committee at the University Hospital Jena. The experimental approach is outlined in figure 2. Within 30 min after delivery, chorionic villi with a diameter of approximately 1 cm have been taken from central areas of the placenta after removing the decidual layer. Samples were carefully washed in PBS (Gibco) containing 1% penicillin/streptomycin to remove all blood cells and subsequently cut into pieces of 100 mg ± 5 mg. Placental villous tissue explants were cultured in 12-well plates in RMPI-1640 medium (10% FBS) for 72 h. Medium was completely changed after each 24 h.

Trophoblast isolation

Trophoblast isolation was performed using a modified method by Kliman et al. (1986). Several authors contributed significantly to the development of the following procedure (Bloxam et al., 1997, Daniels-Mc Queen et al., 1987, Fisher et al., 1989, Kaspi and Nebel, 1974, Kliman et al., 1987, Loke and Burland, 1988, Nelson et al., 1986, Schwab et al., 1984, Truman et al., 1989, Yeger et al., 1989).

Placental villous tissue samples (taken as described before) were washed in Hanks` Balanced Salt Solution (HBSS), without calcium, magnesium and phenolred, supplemented with 2% penicillin-streptomycin. Membranes and vessels were removed. The cell suspension was filtered through a tissue sieve (400 mesh) and washed twice

in washing solution. The filtrated tissue was digested during three cycles at 37°C in 100 ml cell culture bottles: 40 min incubation in 100 ml trypsin solution supplemented with 1 ml DNAse (1X-HBSS, 0,125% trypsin, 10 mg/ml DNAse, 2% AAS, 1 M HEPES), 20 min incubation in 75 ml trypsin solution supplemented with 0.5 ml DNAse, and 20 min incubation in 50 ml trypsin solution. After each cycle, the single cell suspension was removed and supplemented with 10% FBS to block trypsinization. Cells were resuspended in 10 ml DMEM / 10% FBS (37°C) after washing. The cell suspension was then passed through a second sieve (800 mesh) and washed again. Cell pellets were resuspended in 8 ml washing solution. The suspension was then separated on a 5% to 70% 40 ml discontinuous HBSS diluted Percoll (Pharmacia, Sweden) gradient. Four major layers were obtained: trophoblast cells settle in the middle layer (55% Percoll density), cell fragments in an upper layer, lymphocytes in a lower layer and erythrocytes on the bottom of the tube. The middle layer was collected, washed thrice in F-10 HAM/ 10% FCS medium and used for following cell cultures.

Trophoblast cell culture

Trophoblast cells were adjusted to $2x10^6$ cells / ml in F-10 HAM (10% FCS, 1% AAS). Every 24 hours, culture conditioned medium was collected for following analyses, stored at –80° C and substituted with fresh medium. (43, 57, 101, 111, 139, 171). 2 or 3 parallel cultures with $4x10^6$ cells each from first trimester placentae could be performed for each applied mercury concentration. Thereby, a total of 40 or 60 supernatants was obtained from each placenta. In case of term placentae, up to 6 parallel incubations were possible because of availability of larger tissue sections (table 1).

Single maternal side placenta perfusion

Term placentas (n = 5, obtained as mentioned before) were perfused as described by Göhner et al. (Schneider et al. 1972, Gohner et al. 2014), starting within 30 min after delivery (Fig. 3). In brief, an intact peripheral cotyledon was selected and placed together with its surrounding placental tissue in the 37 °C tempered perfusion chamber with the maternal side up. To initiate maternal circulation the intervillous space was perfused by using 5 cannulas at a maternal flow rate of 12 ml/min. The maternal circulation of the selected placenta cotyledon was gently perfused with DMEM low glucose (Gibco) plus Earl´s buffer medium [50:50] added with 0.8 g/l, glucose (Merck, Darmstadt, Germany), 2 g/l dextran 40 (Serva, Heidelberg, Germany), 2.5 U/mL heparin from a porcine source (ratiopharm GmbH, Ulm, Germany), 8 g/L bovine serum albumin (Roth, Karlsruhe, Germany) and aerated with technical air (21% oxygen + 78% nitrogen + 1% undefined). Adjustment of pH to 7.40 was achieved with sodium hydroxide and sodium bicarbonate throughout the experiment. The maternal circulation was perfused in an open-system for approximately 30 min for reoxygenation, to remove residual blood and to allow stabilization of circuit. Subsequently, single side perfusion was maintained for 2 h. Samples were collected every 30 min and immediately stored at -20°C for further analyses.

Cell exposure to chemicals

For toxicity tests, stock solutions were prepared for magnesium sulfate [$MgSO_4$], manganese sulfate [$MnSO_4$], nickel chloride [$NiCl_2$], mercury chloride [$HgCL_2$]. All stock solutions were prepared in distilled H_2O and stored at 8°C. The stock solutions were diluted to the final concentrations with the appropriate culture medium immediately before use (Mg, Mn and Ni: 0.005, 0.01, 0.05, 0.1, 1, 10; Hg: 0.005, 0.01, 0.05, 0.1, 1, 5). Moderate doses of Mg induce extremely low acute toxic effects on organisms and cells *in vivo* and *in vitro* and be evaluated as additional control (Toxnet). Cells, spheroids and explants were seeded on the respective plates and cultured for 24 h before they were treated for up to 48 h.

Microscopical analyses of cell morphology

Cells were imaged by an Axiovert 25 CFL microscope (Carl Zeiss, Jena, GER) using the camera AxioCam ICc1 (Carl Zeiss, Jena, GER). To evaluate spheroid growth and effects induced by heavy metals, microphotographs were taken with an AxioCam MRm Rev.3 camera and analyzed by Zeiss ZEN lite software (Zeiss, Jena, Germany).

ß-hCG concentration

ß-hCG hormone production was measured in the supernatants of trophoblastic cells, spheroids and placental tissue during culture or perfusion. Concentration was measured according to the manufacturer's instructions using electrochemiluminescent "sandwich" ELISA on magnetic microparticles (Architect Total β‑hCG assay, Abbot, Wiesbaden, Germany).

Glucose and lactate concentration

Concentrations of glucose and lactate in culture supernatants were assessed by using an analyzer type Architect Ci 16200 (Abbot).

Data analysis and statistics

Statistical analysis was performed for the toxicity tests. Experiments were carried out with 3 replicates and each assay was repeated independently at least 3 times. In spheroid assays, 8 spheroids per condition were included for statistical analysis. All quantitative data were presented as mean ± SD. Statistical analysis was done by two-tailed Student`s t-test. Values of $p < 0.05$ were considered statistically significant.

Results

hCG is a suitable marker for toxicity in 2D culture

hCG concentration has been analyzed in JEG-3 cell cultures exposed for 24 h to different concentrations of metals. The ß-hCG concentration in pure, un-conditioned medium has been assessed and was < 1mI U/ml. A MTS assay was performed to evaluate general metabolic toxicity of all applied metal concentrations, but no significant changes have been observed (data not shown). ß-hCG levels (Fig. 4.1), glucose consumption (Fig. 4.2) and lactate concentration (Fig. 4.3) decreased dose-dependently and significantly after incubation with all heavy metals. As exception, the highest applied concentration of Hg (5mM) led to a significant increase of glucose consumption.

Spheroids are able to produce hCG

Spheroids were formed via hanging drop method to check if cells keep their capability of hCG secretion in a 3D structure. After 24 h in hanging drops, spheroids were transferred to a poly-HEMA coated 96-well plate to prevent cell attachment. JEG-3 spheroids secreted ß-hCG time-dependently. After 24 and 48 h Hg and Ni exposure. ß-hCG expression decreased significantly (Fig. 5.1). Significant effects on ß-hCG secretion in spheroids could be also demonstrated after 48 h incubation with Mn (Fig. 5.1). Glucose and lactate levels were not affected (Fig. 5.2, 5.3). Further, spheroids were imaged to analyze the effect of heavy metals on morphology (Fig. S1A). While compact spheroids were detected in almost all conditions, Hg incubated spheroids exhibited a detachment of cells located at the rim and became smaller. Same effect was observed after 48 h incubation with Ni, whereas the lowest concentration led to spheroid growth. But statistical analysis of spheroid diameter revealed no significant data (Fig. S1B). In another experimental setup, heavy metals were incubated with cell suspension before spheroids were generated to test their influence on spheroid formation capacity (Fig. S2A-B).

The placenta explants model fails in releasing hCG

When culturing villous placenta tissue inter-individual variations of placenta function and metabolic activity including secretion of hormones must be taken into account (Fig. S1A). Unfortunately, ß-hCG release continuously decreased over a cultivation time of 7 days by changing half of the medium daily (Fig. S3B). After preparation of fresh derived placenta tissue high amounts of ß-hCG were detected within the first 24 h. But when a complete medium change was performed, only low concentrations of ß-hCG were detectable, which became almost zero after the medium was changed twice and PVTE were cultured for up to 72 h (Table 1). Further, HE staining revealed a degeneration of the syncytiotrophoblast layer with ongoing cultivation time (Fig. S3C), although metabolic activity until day 7 has been demonstrated (Fig. S3D). Therefore, it was not possible to evaluate the effects of heavy metals on ß-hCG secretion in placenta explants (Fig. S3E).

hCG is an approved viability marker for the placenta perfusion model

For the single side placenta perfusion samples were collected every 30 min and concentration of several markers, e.g. glucose, lactate and antipyrine, were determined in order to assess cell viability. Samples were taken each from the perfusion solution [arterial; Pl a] as well as from the perfusate [venous; Pl v]. Besides the release of hormones and metabolic substances additional systematic parameters, were checked regularly to ensure a successful perfusion due to the vulnerable equipment and a well-known high failure rate. In all included placenta perfusions a stable release of ß-hCG could be observed (Fig. 6.1), even if an accumulation during the perfusion time was induced by the methodical approach (closed, recirculating system) illustrated by increased ß-hCG level. The concentrations were slightly higher after the solution crossed the placenta [Pl v > Pl a], except for placenta 3, indicating an ongoing ß-hCG production by the placental cells. To take different sizes of the perfused cotyledons into account the concentration of ß-hCG was calculated per gram placenta tissue. Glucose consumption as well as lactate concentration of perfused medium increased continuously during the period of perfusion demonstration metabolic activity of vital cells (Fig. 6.2, 6.3).

Discussion

The placenta is a complex and highly specific organ containing a wide spectrum of cell types (Gohner et al. 2014). Its unique functions, the human origin and its availability make the placenta suitable for toxicological tests in general. As organ protecting the fetus from exposure to toxic chemicals, the placenta is an important object of investigation regarding the toxic potential of xenobiotics and pathogens on the growing fetus (Magnarelli und Guiñazú 2012). Here, we presented 4 different placenta-based models and analyzed their qualification for toxicity tests by using hCG as viability marker, as this pregnancy-related hormone is produced by trophoblast cells during the gestational period. Concerning placenta-derived immortalized cell lines, we assessed a cell-dependent capability to produce hCG, which was sustained in a 3D model. Cells formed to spheroids enhanced the release of hCG into the supernatant during cultivation time even after a medium change had been performed. Hence, it would be interesting, if the 3D-structure influences the mechanism of hormone synthesis. But our results allow no conclusion due to the different methodical approaches with various cell numbers and changes in growth behavior in spheroid culture. To compare the release of hCG in these models, numbers of vital cells need to be determined, for instance via FACS assay. Nevertheless, spheroids are a well-used model in cancer research as they develop a layered morphology with ongoing cultivation time resembling solid tumors in vivo. Regarding the secretion of hCG it has to be proofed if spheroids model the in vivo situation realistic, even if a stratified composition is avoided by a short formation time. In placenta explant cultures a continuous release of hCG was not observed. Cultivation for 72 h was characterized by a sharp decrease of hCG level in supernatants. We suggest that the hormone initially leaked out of the tissue due to the stress-causing preparation steps and then concentration was diluted by changing the culture medium daily. Same kinetics could be observed over a culture period of 7 days, even if the hCG level was much higher due to a different procedure of medium change. The high amount after 24 h might also come from residual blood in

placenta tissue. These results support previous findings on explant cultures, as the syncytiotrophoblast in term placenta tends to degenerate within the first days in culture. (Sooranna et al. 1999, Di Santo et al. 2003). A detachment of the syncytiotrophoblast was confirmed by HE-staining. Hence, our data are in line with previous findings confirming a degeneration of the old syncytiotrophoblast (Siman et al. 2001, Sooranna et al. 1999, Palmer et al. 1997). It has to be considered that in our studies explants were cultured for a short period of 72 h and therefore, a newly formed trophoblast layer after day 5 of culture as observed by Simán et al. has not been demonstrated (Siman et al. 2001). However, we cultured explants for up to 11 days, but could not observe a rising hormone release, whereas cells tend to have an ongoing metabolic activity, which was shown by a MTS assay. In addition, the experiments with placenta tissue illustrate the inter-individual variations in hormone production, which makes the correlation of data from different samples more challenging. Apart from tissue studies, research on primary cells can be a further option as these cells resemble more closely in vivo situations than immortalized cell lines. In contrast to explants, primary isolated cytotrophoblast cells seem to differentiate in culture and fuse to form functional syncytiotrophoblasts (Kliman et al. 1986). When supernatants of isolated primary human placenta trophoblasts were screened for hCG, we observed a daily increase in term as well as first trimester placenta trophoblast cells, although they are known to react very sensitive and do not longer proliferate in culture (Orendi et al. 2011). Kinetics of hormone production was different between first trimester and term placenta trophoblasts and declined at day 5 in term placenta production (Fig. S4.1). Hence, Zhang and Ni have shown maximal syncytialization of primary cells after three days in vitro and stuck to a regeneration time until experiments were started (Zhang et al. 2015, Ni et al. 2009). Concerning placenta perfusion studies, which enable new insights in drug transfer mechanisms and placenta tissue accumulation, hCG is a well-accepted and sensitive viability marker and therefore, a criterion for the success of perfusion studies. Besides volume loss for detection of leaks and antipyrine to confirm the overlap of maternal and fetal circulations, the biochemical functionality of the placental tissue is proofed by hormone synthesis and secretion (Karttunen et al. 2015, Cannell et al. 1988, Mathiesen et al. 2010). The experimental setups established by Schneider and Miller routinely measured hCG to monitor metabolic activity of the perfused placenta cotyledon (Miller und Berndt 1975, Schneider und Huch 1985, Karttunen et al. 2015). Thus, in our perfusion system, an ongoing metabolic activity was confirmed by a stable release on hCG. In addition, we measured persistent glucose consumption and subsequently an increase of lactate concentration in the perfusate of all performed perfusions, which indicate functional placental tissue (Cannell et al. 1988). But kinetics of further parameters like antipyrine, oxygen transfer, volume loss or pH must be included to assure validity of this complex and challenging methodical approach (Karttunen et al. 2015).

In a second step, we wanted to analyze effects of toxic compounds on ß-hCG to assess further its qualification function as biomarker for endocrine disruption. Hence, we performed toxicity tests by incubating the placental cells with heavy metals. Strong effects were caused by Hg in all tested conditions. With regard to the different model-dependent pattern of ß-hCG release we tested JEG-3 cells in monolayer as well as in spheroidal structure and further primary trophoblast cells. For JEG-3 cells a concentration- and time-dependent decrease of hCG for all heavy metals was observed. However, this may be a secondary effect due to the cell dying process. Therefore, we identified doses, which show no effect on cell viability in monolayer, via MTS and FACS assay (data not shown). After treatment with 0.05 mM Hg or 0.1 mM Ni and Mn cell viability was not significantly reduced compared to control, indicating that these are likely to reflect subtoxic concentrations. In monolayer, hCG level was significantly decreased after cells were incubated with 0.05 mM Hg, whereas only a slight reduction was observed after exposure to lower doses of Ni and Mn. Interestingly, spheroids reacted more sensitive to Hg shown by a significant decline of hCG in all tested conditions, whereas hCG level of cells in monolayer were not affected by low concentrations of Hg. No differences between hCG level in 2D and 3D could be observed for Ni and Mn. Additionally, spheroids were imaged via life-imaging microscope showing harming effects of Hg and Ni on morphology. The metabolic markers

glucose and lactate were not modified in spheroids after drug exposure therefore, failed revealing first harming effects of heavy metals.

Due to the loss of hCG secretion it was not possible to evaluate the effects of heavy metals on human placental villous explants. Hence, toxicity studies require improved culture conditions, which guarantee vitality of tissue including stable hCG secretion over investigation period.

Regarding primary human trophoblasts, experiments were performed with Hg in 3 different concentrations. Results demonstrated a significant dose-dependent decline of hCG production in term trophoblasts, whereas the kinetic profile seemed to be unaffected. Slighter effects were observed on first trimester trophoblasts (Fig. S4). Our findings were supported by a less glucose consumption and decreased lactate level after incubation with Hg indicating a hampered metabolic activity (Fig. S4). Perfusion studies could provide further insights into tissue accumulation and potential of placenta transfer.

Conclusion

In the presented study we analyzed secretion of hCG in several placenta derived models to evaluate their vital condition and qualification for toxicity tests. Immortalized cell lines in 2D and 3D models presented stable hCG level, while experimental work based on ex vivo models remains challenging. As the endocrine hCG is produced by syncytiotrophoblasts, a maximal syncytialization should be checked by e.g. E-cadherin or syncytin, before starting experiments on placenta explants. As it reflects physiological, regenerative as well as necrotic processes a number of assays need to be performed to prevent misinterpretations of hCG values in placenta tissue [10].We recommend to monitor further metabolic markers, e. g. LDH, to use vitality assays and check tissue integrity by morphologic examination for verifying cell condition. Regarding in vitro studies hCG level can be used for toxicity assessment. Our findings indicate harming effects of Hg even when applying low concentrations. May be due to the stratified composition hCG level in spheroids reacted more sensitive and therefore represent an useful model to reveal endocrine disruption. To corroborate these hypothesis, further research of molecular pathways remains to be performed, including placenta perfusion studies or monitoring of other placenta-derived hormones, e.g. PLAP, which indicates placenta-tissue damage (Magnarelli und Guiñazú 2012).

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Conflict of interest

The authors declare no conflict of interest.

Fig. 1

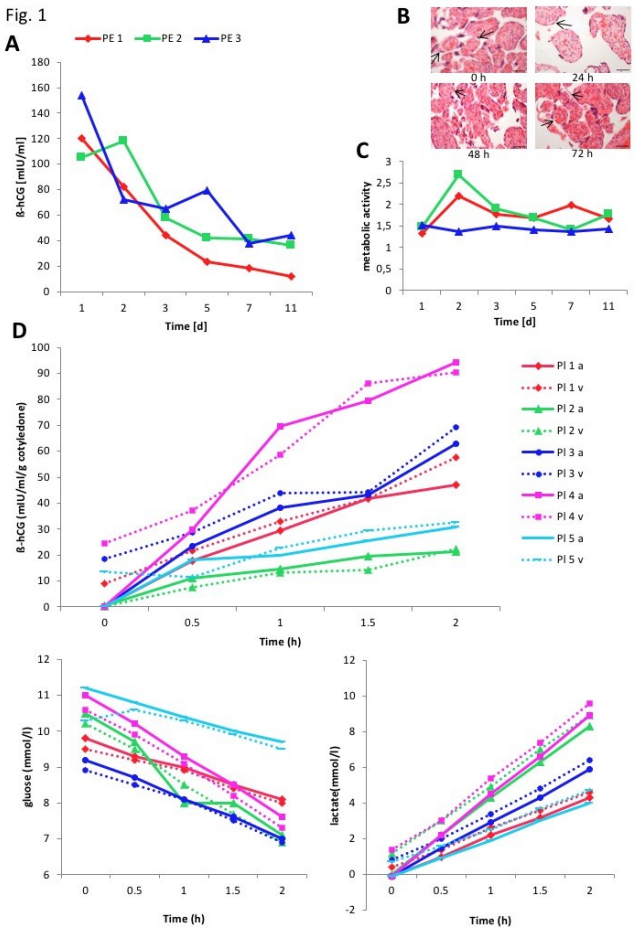

Fig. 2

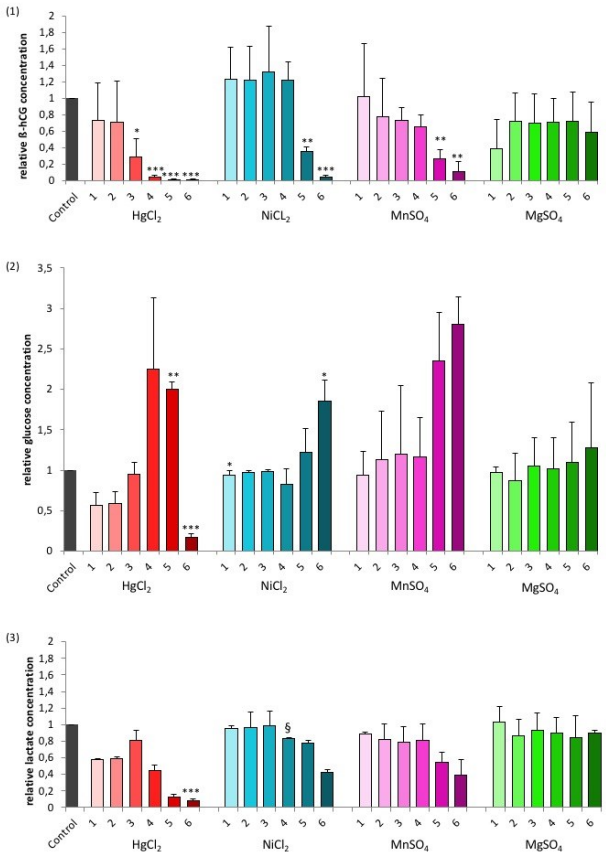

Fig. 3

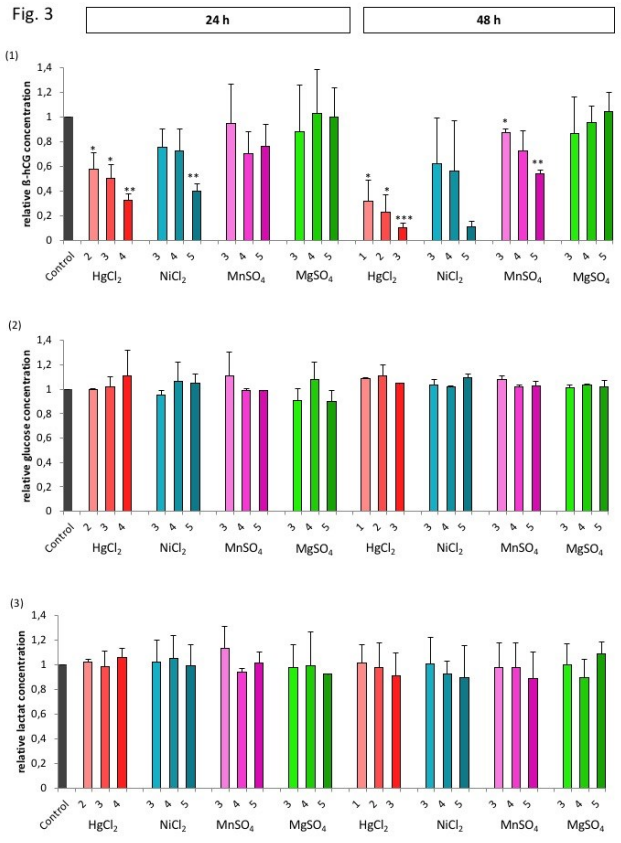

Fig. 4

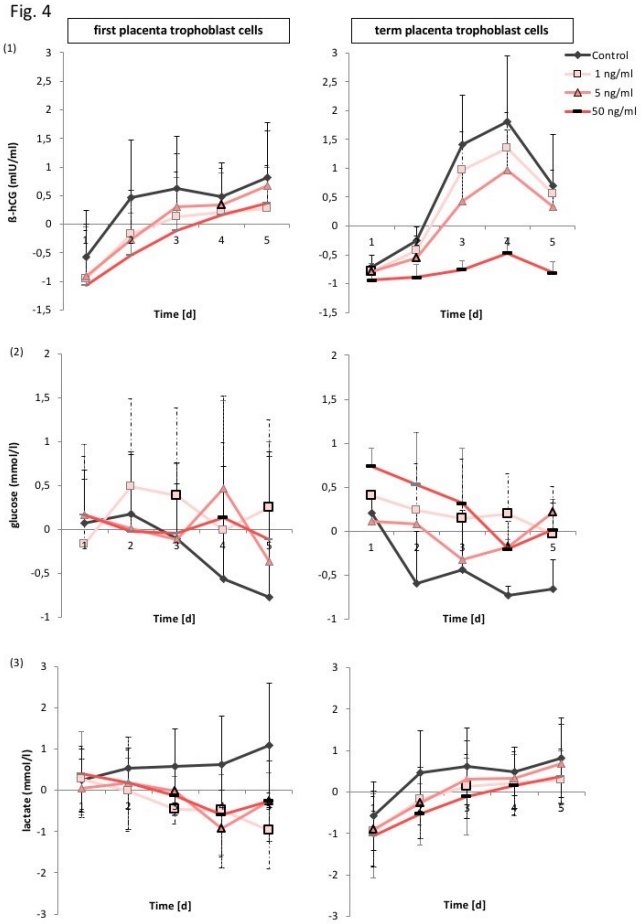

Fig. 5

***In vitro* model**	JEG-3 monolayer (n = 3)	JEG-3 Spheroids (n = 3)	placenta explants (n = 6)	primary cells 1. trim. (n = 9)	primary cells 3. trim. (n = 26)
incubation time [h]	24	48	48	120	120
$HgCl_2$	☆☆☆⇩	☆☆☆⇩	N/A	☆ ⇩	☆☆☆⇩
$NiCl_2$	☆☆ ⇩	☆ ⇩	N/A	N/A	N/A
$MnSo_4$	☆☆ ⇩	☆ ⇩	N/A	N/A	N/A
$MgSo_4$	⇨	⇨	N/A	N/A	N/A

☆ efficiency

⇩ ß-hCG concentration
(in comparison with control)

Abbreviations
N/A: not applicable or not available

Fig. S1

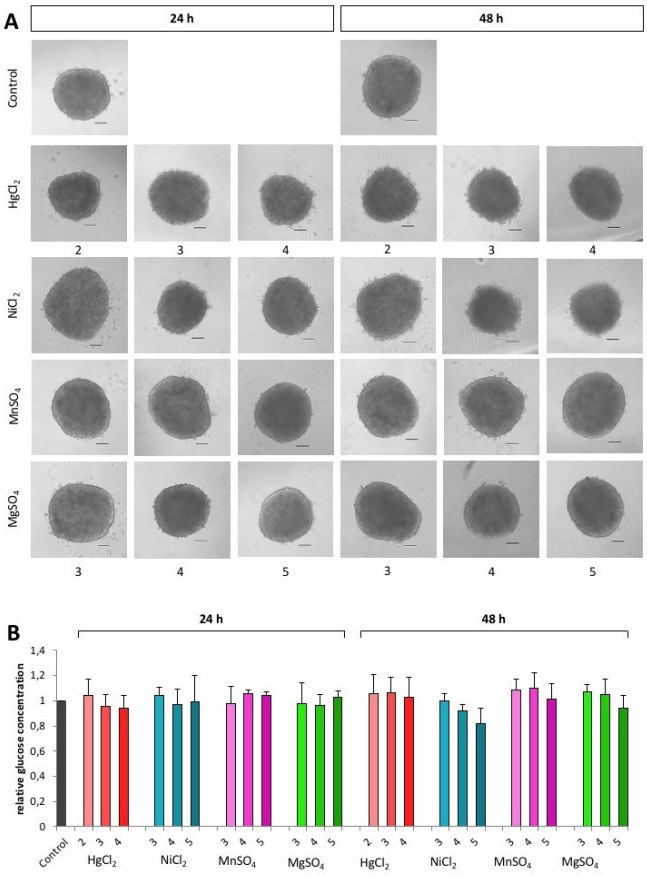

Fig. S2

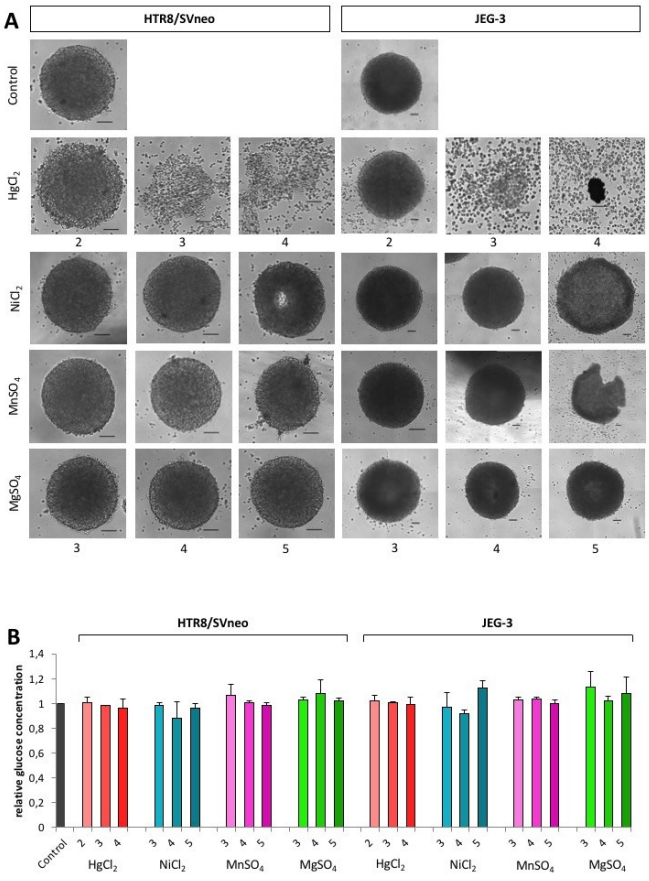

Fig. S3

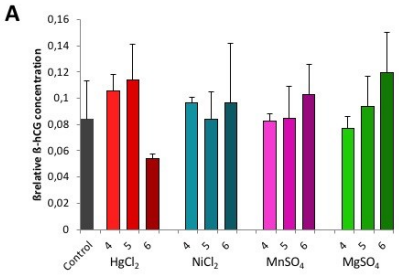

Table 1: Concentration of ß-hCG in supernatants of placental cells

Regarding experimental setups > 24 h medium change was performed daily.

experimental setup	cells/material	n	Cell number	cultivation time [h]	ß-hCG [mIU/ml]
		3	500,000	2	29
monolayer	JEG3	6	500,000	24	173.7
			10,000 / drop	24	43.6
	JEG3	6	10,000 / drop	48	100.2
			10,000 / drop	24	< 1
spheroids	HTR8/Svneo	6	10,000 / drop	48	< 1
			N/A	24	1587.7*
			N/A	48	116.7*
placenta explants	term placenta	6	N/A	72	47.5*
			N/A	0	0.46*
			N/A	0.5	1.99*
			N/A	1	3.44*
			N/A	1.5	4.19*
perfusion	term placenta	5	N/A	2	5.19*
			N/A	24	-0.58**
			N/A	48	0.46**
			N/A	74	0.62**
			N/A	96	0.49**
	1. trimester placenta	9	N/A	120	0.82**
			N/A	24	-0.71**
			N/A	48	-0.26**
			N/A	74	1.41**
			N/A	96	1.80**
primary trophoblast cells	term placenta	26	N/A	120	0.70**

* per gram cotyledone weight

** values α-standardized

Abbreviations: N/A - not applicable

Legends to figures

Fig. 1. Characterization of placenta explants in culture and perfused chorionic tissue

A: ß-hCG release over a period of 11 days in culture by replacing the culture medium daily [50/50]. B: Representative images after HE staining illustrate the morphologic changes of untreated villous tissue during culture. Scales = 100 µm. C: Metabolic activity analyzed over a period of 11 days via MTS assay D: Single maternal side placenta perfusion (n = 5) was started after a washing time of 30 min and samples were taken every other 30 min over a perfusion time of 2 h. Concentration of ß-hCG, glucose and lactate were determined. Placentas were obtained from healthy pregnancies.

Fig. 2. Effects of metals on cultured JEG-3 monolayer

JEG-3 cells were seeded in 6-well plates at a density of 500,000 cells and allowed to attach overnight before incubation with metals was started. Supernatants were collected after 24 h of incubation to measure ß-hCG concentration (1), glucose (2) and lactate level (3). Data display as mean ± SD (n = 3, each performed in triplicates). *p < 0.05; **p < 0.01; ***p < 0.001 (Student‘s t test).

Fig. 3. Effects of metals on JEG-3 spheroids

Spheroids were formed via hanging drop method using 10,000 cells each drop. After 24 h spheroids were transferred to a poly-HEMA coated 96-well plate and drug exposure was started for up to 48 h. Supernatant analyses of ß-hCG (1), glucose (2) and lactate (3) were performed after 24 and 48 h incubation time. Bars indicate mean ± SD (n = 3). For each condition, supernatants of 8 spheroids were collected for further analyses. *p < 0.05; **p < 0.01; ***p < 0.001 (Student‘s t test).

Fig. 4. Effects of $HgCl_2$ on primary trophoblast cells

Pattern of ß-hCG production (1), glucose consumption (2) and lactate release (3) over a period of 5 days with or without incubation with 3 different concentrations of $HgCl_2$. Trophoblast cells were isolated from healthy human first or term placenta. α-standardization was performed to exclude inter-individual differences [(measured value – mean) / standard deviation]. Bars show mean ± SD (1. trimester: n = 9; 3. trimester: n = 26). Significances were calculated using Student’s t test and illustrated by marked data points.

Fig. 5. Schematic summary of metal effects on ß-hCG release of placental cell

Methodical approaches were performed as described before. Incubation time with metals differs between models due to the experimental setups (24 h to 120 h). Placentas were obtained from healthy pregnancies (1. trimester: 6-11 week of pregnancy; 3 trimester 38-41 week of pregnancy). n varied between setups due to the different complexity of methods (n = 3 to n = 26).

Supplementary Material:

Fig. S1. Spheroid morphology after exposure to metals

Methodical approaches were performed as described in Fig. 3. Representative microphotographs were imaged after 24 and 48 h with a Life-Imaging microscope Axio Observer Z1. Scales = 100 µm (A). Spheroid diameters were quantified by using Zeiss ZEN lite software (B). Data are expressed as mean ± SD (n = 3) and each independent experiment has been calculated as mean from 8 spheroids.

Fig. S2. Metal effects on spheroid formation capability

For performance of spheroid formation assay drugs were added to cell suspension before 10,000 HTR8/SVneo or JEG-3 cells in 20 µl were cultured in hanging drops. Representative microphotographs were imaged after 24 h with a Life-Imaging microscope Axio Observer Z1. Scales = 100 µm (A). Spheroid diameters were quantified by using Zeiss ZEN lite software (B). Data are expressed as mean ± SD (n = 3) and each independent experiment has been calculated as mean from 8 spheroids.

Fig. S3. ß-hCG release in placenta explants after incubation with metals

ß-hCG level after exposure to metals for 24 h. Data were normalized to controls, which were collected directly after preparation. Bars indicate mean ± SD (n = 3, each performed in triplicates).

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6. Diskussion

Methoden zur Sphäroidgenerierung

Bei der Verwendung von alternativen Methoden zur Testung verschiedener Substanzen oder zur Charakterisierung molekularbiologischer Eigenschaften ist das Sphäroidmodell in den letzten Jahren zunehmend populär geworden. In dieser Arbeit wurde der Versuch vorgenommen, Sphäroide gleichmäßiger Form und Gestalt mit einer hohen Reproduzierbarkeit bei möglichst geringem Kosteneinsatz herzustellen. Dabei zeigten sich bei der Verwendung der *Hanging Drop* Methode die größten Vorteile, die aus diesem Grund als favorisierte Methode anzusehen ist. Da die Generierung von gleichmäßig geformten Sphäroiden für einige Zelllinien eine große Herausforderung darstellt, erscheint die Verwendung von Viskositätserhöhern einen enormen Vorteil zu haben. In dieser Arbeit hatte der Einsatz von 25% Methylcellulose einen stark positiven Einfluss bei der Kultivierung der Brustkrebszelllinie MCF-7. Experimentelle Studien, die trotz der Verwendung des gleichen Zelltyps und der *Hanging Drop* Methode keine Sphäroidbildung erreichen konnten, verwendeten wesentlich geringere Methylcellulose-Konzentrationen (0,24%) (Nagelkerke et al. 2013). Bezogen auf die Mammakarzinomzelllinie MDA-MB-231, welche unter allen Bedingungen im hängendem Tropfen höchstens kompakte Aggregate formte, konnten die besten Ergebnisse mit der Liquid Overlay Technik erzielt werden. Diese Sphäroidbildung war jedoch nur unter Verwendung einer speziellen Wellplatte (Cellstar Cell-Repellent Surface) und dem Einsatz von 3,5% Matrigel möglich. In dieser Arbeit wurde die Konzentration von Matrigel im Vergleich zu anderen Studien erhöht, welches die Wichtigkeit des Einsatzes und der Konzentration solcher Viskositätserhöher unterstreicht (Rotin et al. 1986, Ivascu und Kubbies 2006). Die Bedingung, die bei den MDA-MB-231 Zellen zur erfolgreichen Sphäroidgenerierung führte, stellte auch für die SK-BR-3 Zellen die beste Bedingung dar. Die oben festgelegten Kriterien für das Vorliegen von Sphäroiden wurden in diesem Fall jedoch nicht erfüllt, SK-BR-3 Zellen formten lediglich kompakte Aggregate. Dabei ist zu beachten, dass andere Autoren solche geformten Strukturen mitunter als Sphäroide definieren (Ivascu und Kubbies 2006). Der kombinierte Einsatz von Methylcellulose und Matrigel hatte bezogen auf die untersuchten Zelllinien keinen positiven Einfluss auf die Sphäroidgenerierung. Für die weitere Charakterisierung der MCF-7 Sphäroide wurden diese für histologische und immunhistochemische Untersuchungen mittels Formalin fixiert und in Paraffin

eingebettet. Die Sphäroide zeigten direkt nach der Generierung eine gleichmäßige Gestalt mit einer kompakten inneren Zone (Abbildung 6 a). Nach einer weiteren Kultivierungszeit von 2 Tagen konnte bereits in histologischen Untersuchungen die typische Stratifizierung in eine proliferierende Randzone und einen inneren nekrotischen Kern beobachtet werden (Abbildung 6 b). Der nekrotische Kernbereich zeigte sich in der HE-Färbung deutlich anhand der farblichen Differenzierung. Die Zellen in der inneren Zone sind durch zunehmende Verfettung, welche durch Sauerstoff- und Nährstoffminderversorgung bedingt ist, charakterisiert. Aufgrund der Unterversorgung kommt es zu einem ATP-Mangel, wodurch die Fettsäureoxidation eingeschränkt wird und es zur Ablagerung von Fettsäuren und Lipidtropfen kommt. Außerdem werden die Zellkerne dichter und dunkler im Vergleich zu den Äußeren und schrumpfen ein (Froehlich et al. 2016). Diese Charakteristika weisen auf eine Apoptose bzw. Nekrose im Kernbereich hin. Diese deutliche Zelldifferenzierung konnte in anschließenden immunhistochemischen Färbungen nochmals bestätigt werden (Abbildung 6 c-d). Die in der Literatur beschriebene Differenzierung von hypoxischen Arealen und ruhenden Zellen, welche zwischen den proliferierenden und apoptotischen Zellpopulationen zu finden sein sollen, konnten im Fall der MCF-7 Sphäroide hingegen nicht gefunden werden. Die Färbung mittels anti-HIF-1 alpha (Hypoxie) und anti-$p27^{Kip1}$ (Marker für ruhende Zellen) Antikörpern zeigte eine zu der Ki-67 (Proliferation) Markierung ähnliche Färbung.

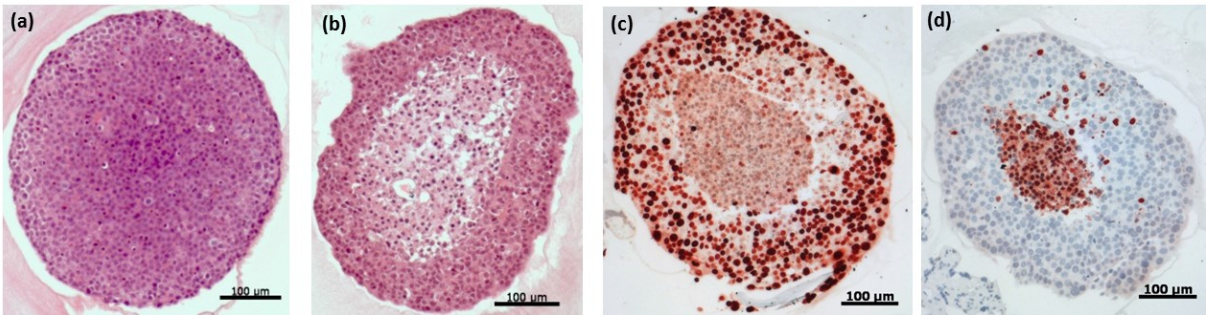

Abbildung 6: Die MCF-7 Sphäroide wurden drei Tage mittels der *Hanging Drop* Methode generiert und für zwei weitere Tage für die typische Stratifizierung kultiviert. (a) Hematoxylin/ Eosin Färbung von einem frisch generierten MCF-7 Sphäroid. (b) Hematoxylin/ Eosin Färbung von einem MCF-7 Sphäroid nach zusätzlicher 2-tägiger Kultivierungszeit. Diese Sphäroide zeigten in der immunhistochemischen Färbung die bereits beschriebene Schichtung in (c, anti-Ki-67 Färbung) proliferierende und (d, anit-cPARP Färbung) apoptotische Zone.

Die MDA-MB-231 kompakten Aggregate, welche mittels der *Hanging Drop* Methode unter Zusatz von 25% Methylcellulose geformt wurden, zeigten keine dieser Charakteristika. Vielmehr waren diese in den histologischen und

immunhistochemischen Untersuchungen durch eine lose Struktur gekennzeichnet. Die Schwierigkeit der Formierung von adäquaten Sphäroiden zeigte sich auch bei den Zervixkarzinomzelllinien HeLa und SiHa. Grundlage für die Sphäroidgenerierung bildete hierbei die zuvor favorisierte *Hanging Drop* Methode. Auch verbesserte der Zusatz von 25% Methylcellulose die Sphäroidbildung, die Sphäroide wurden kompakter und ihr Durchmesser vergrößerte sich. Während im Fall von HeLa Zellen das routinemäßig verwendete FKS als Zellkulturzusatz keinen negativen Einfluss auf die Sphäroidbildung hatte, konnten unter diesen Bedingungen bei den SiHa Zellen nur lose Aggregate gebildet werden. Der Einsatz von HS bewirkte die Bildung von SiHa Sphäroiden, welche mit zunehmender Zellzahl und durch den Einsatz von Methylcellulose ihre Kompaktheit und gleichmäßige Form steigerten. Während der weiteren Kultivierungszeit der geformten SiHa Sphäroide konnte der positive Einfluss von HS weiterhin gezeigt werden, da diese mit dem HS-Zusatz über den Kultivierungszeitraum kompakter wurden. Die verbesserte Sphäroidbildungsfähigkeit aufgrund des HS-Zusatzes weist darauf hin, dass der in den meisten Zellkulturexperimenten eingesetzte xenogene Zusatz FKS dehumanen *in vivo* Bedingungen nicht ausreichend entspricht. Bisher konnte gezeigt werden, dass bereits unterschiedliche Medien zu verschiedenen Zelleffekten führten (Emerman et al. 1987).

Einfluss von HS in der Zellkultur

Um den Einsatz von HS als alternatives Serum in der Zellkultur zu analysieren, wurden die Zervixkarzinomzelllinien zusätzlich auf typische morphologische und funktionelle Charakteristika untersucht. Es konnte gezeigt werden, dass HS sowohl die Proliferation als auch die Migration in ähnlicher Weise wie FKS stimuliert, während das Invasionsverhalten durch HS signifikant verstärkt wurde. Die Ergebnisse dieser Arbeit deuten daraufhin, dass der Einsatz von HS in Zellkulturexperimenten humane *in vivo* Bedingungen besser imitiert als FKS, so dass grundlegende Zellkultureigenschaften eher gewährleistet werden können. Zusätzlich konnte durch den Einsatz von HS eine verbesserte Sphäroidbildung erreicht werden. Bei den genannten Vorteilen sollte jedoch nicht in Vergessenheit geraten, dass die Etablierung und die Anpassung an neue Mediumsupplemente auch hier unabdingbar ist und Variationen zwischen Chargen beziehungsweise das Risiko für Infektionen genau so wie bei der Anwendung von FKS in diesem Zusammmenhang eine Herausforderung darstellen.

Speziesdifferenzen in der Plazenta

Die Plazenta zählt zu den Organen, welche sich durch die größten evolutionären Unterschiede innerhalb der verschiedenen Spezies auszeichnet. Bereits bei der makroskopischen Begutachtung der Form und der feto-maternalen Kontaktflächen gibt es erhebliche Variationen (Abbildung 7A). Die *Placenta diffusa* findet man bei Schweinen und Pferden. Die *Placenta multicotyledonaria* wird typischerweise bei Wiederkäuern während der Schwangerschaft ausgebildet. Die *Placenta zonaria*, welche den Fetus wie ein Band umgibt, wird bei Katzen oder Hunden ausgebildet. Die *Placenta discoidalis*, welche bei Primaten und Nagern vorkommt, ist aufgrund ihrer Form und somit aus anatomischer Sicht der einzige Plazentatyp welcher die Vergleichbarkeit mit humanen Bedinungen zulässt. Neben diesen Formunterschieden gibt es ebenso Variationen bei der Barriereausbildung zwischen fetalem und maternalem Blut. Primaten und Nager besitzen eine stark invasive hemochoriale Plazenta, welche die Voraussetzung für einen direkten Kontakt der Trophoblastzellen/ des Chorions mit dem maternalen Blut darstellt (Abbildung 7B c). Je nach Anzahl der Trophoblastschichten unterscheidet man zwischen hemomonochorialen (Vorkommen bei Primaten), hemodichorialen (Hasen) und hemotrichorialen (Ratten und Mäusen) Typen. Bei anderen Spezies ist dieser unmittelbare Kontakt aufgrund der Aufrechterhaltung von Endothelium und/ oder Epithelium nicht möglich. Beispielsweise findet man den epitheliochorialen Typ bei Pferden, Schweinen und Wiederkäuern (Abbildung 7B a). Bei diesem Typ ist die Trophoblastinvasion ins Endometrium sehr schwach ausgeprägt, sodass hier die spätere Plazenta nur oberflächlich auf der Gebärmutterschleimhaut liegt und keine maternalen Gefäße erodiert werden. Eine weitere Untergruppe bildet der syndesmochoriale Typ, bei dem spezifische Trophoblastzellen mit einzelnen uterinen Epithelzellen fusionieren, welcher auch bei Wiederkäuern gefunden werden kann. Der endotheliochoriale Typ wird beispielsweise bei Hunden ausgebildet (Abbildung 7B b). Bei diesem Typ wird nach der Implantation das Endometrium und maternale Gewebe verdrängt, sodass der Synzytiotrophoblast direkt mit dem maternalen Interstitium in Kontakt kommt.

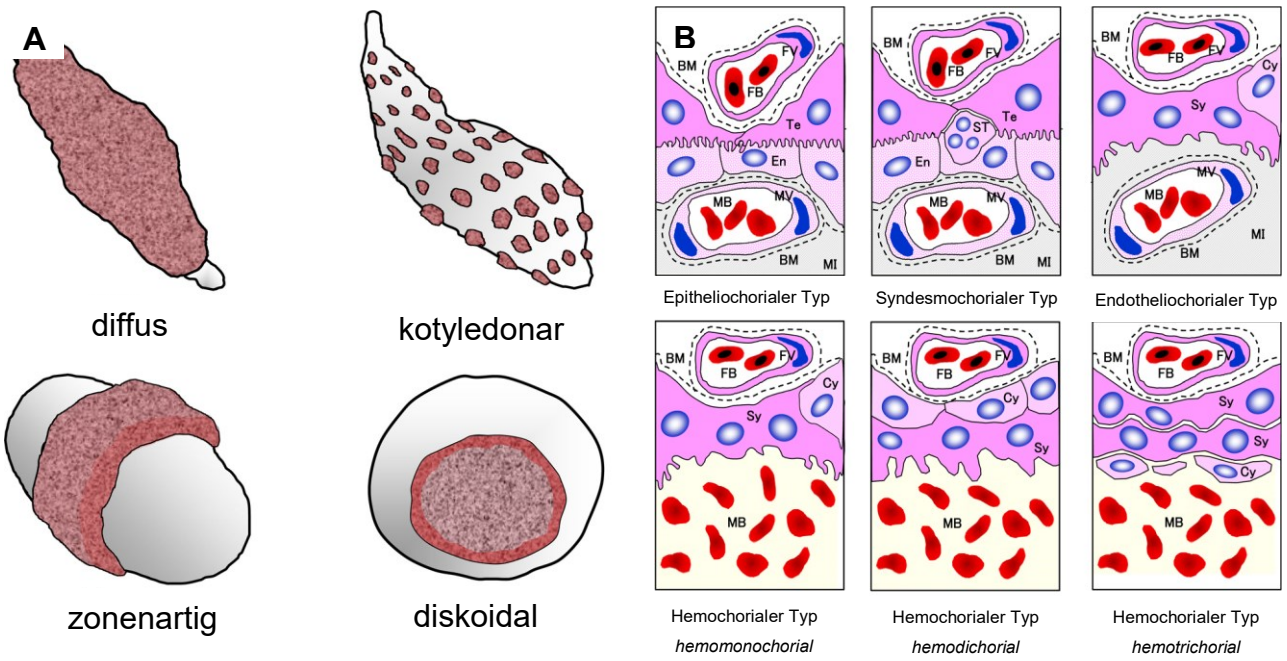

Abbildung 7: A Klassifizierung der Plazentamorphologie. **B** Verschiedene feto-maternale Kontaktflächen. BM Basalmembran Te Trophektoderm, Cy Zytotrophoblast, En Endometrium, FB fetales Blut, FV fetales Gefäß, MB maternales Blut, MI maternales Interstitium, MV maternales Gefäß, ST spezifischer Trophoblast, Sy Syzytiotrophoblast, Abbildung von (Furukawa et al. 2014).

Aufgrund dieser morphologischen Unterschiede existiert kein Tiermodell, welches die humane Plazentation exakt reflektiert. Trotz alledem wird das Mausmodell häufig zur Untersuchung von plazenta-assoziierten Fragestellungen eingesetzt. Im Gegensatz zu Menschen besitzen Mäuse eine hemotrichoriale Plazenta und außerdem zusätzlich die sogenannte *inverted yolk sac placenta*, welche in der frühen Schwangerschaft funktionsfähig wird und bis zum Ende der Schwangerschaft persistiert. Diese Art der Plazenta existiert beim Menschen nicht (Beckman et al. 1990). Zusätzlich ist die Invasion der murinen Trophoblastzellen physiologisch auf die Decidua basalis beschränkt. Diese Art der Invasion kann beim Menschen nur in pathophysiologischen Schwangerschaften, wie Präeklampsie und intrauteriner Wachstumsretardierung gefunden werden (Moffett und Loke 2006). Die in der humanen Schwangerschaft notwendige tiefe Invasion zur Erodierung der maternalen Spiralarterien ist von verschiedenen primaten- und human-spezifischen Faktoren begleitet. So scheint hyperglykosiliertes hCG, welches neben hCG am häufigsten während der Schwangerschaft vorkommt, eine entscheidende Rolle für die Invasion der Trophoblastzellen ins Myometrium zu spielen. Folglich könnten erniedrigte Level von hyperglykosiliertem hCG zu einem erhöhten Risiko von Präeklampsie beitragen (Bahado-Singh et al. 2002). Diese Erkrankung findet man bei Nagern nicht. Des

Weiteren gehören uterine natürliche Killerzellen (uNK) zu den häufigsten Leukozyten an der plazentaren Implantationsseite und spielen nach gängiger Auffassung insbesondere bei der Regulation der extravillösen interstitiellen Trophoblastzellinvasion eine zentrale Rolle. Die humanen uNK-Zellen exprimieren Killerzell-Immunglobulin-artigen Rezeptoren (KIRs), welche mit humanen Leukozyten Antigenen (HLA) interagieren. Im Gegensatz dazu exprimieren NK-Zellen in Mäusen keine KIRs und murine H2-Komplexe (vergleichbar mit humanen HLAs) zeigen im Vergleich zu humane HLAs Abweichungen. Das auf der Oberfläche von extravillösen Trophoblastzellen exprimierte HLA-C gibt es nur bei Menschen und Menschenaffen (Older Aguilar et al. 2010). Zudem wird vermutet, dass das Zusammenwirken inhibitorischer maternaler KIRs auf uNK-Zellen und HLA-C2 auf extravillösen Trophoblastzellen zu einem erhöhten Risiko von Präeklampsie führt (Hiby et al. 2004). Dieses Beispiel offenbart nochmals die eingeschränkte Eignung von Mausmodellen, speziell in der Präeklampsieforschung. Hinzu kommen weitere human-spezifische Faktoren, welche die Invasionsvorgänge der Trophoblastzellen regulieren und bei Nagern nicht vorkommen, wie bspw. die human-spezifische Expression von sialic acid-binding Ig-like lectin-6 (siglec-6) und immortalization-upregulated protein-2 (IMUP-2). Regulierende microRNAs variieren ebenso zwischen unterschiedlichen Spezies und einige werden nur in humanen Trophoblastzellen exprimiert (Morales-Prieto et al. 2014). Des Weiteren bestehen Unterschiede in dem Chromosom 14 microRNA Cluster zwischen Mäusen und Menschen (Glazov et al. 2008). Das Chromosom 19 microRNA Cluster, welches vor allem in der Placenta vorkommt, aber auch bei malignen Erkrankungen eine Rolle zu spielen scheint, ist nur auf Primaten beschränkt (Flor und Bullerdiek 2012). Zusammenfassend verdeutlichen diese Beispiele, dass zahlreiche Unterschiede zwischen Menschen und Mäusen existieren und deshalb die Übertragbarkeit von Ergebnissen aus Maus-Modellen auf humane Gegebenheiten als kritisch angesehen werden muss. Aus diesem Grund und aufgrund der Tatsache, dass die Plazenta das am wenigsten verstandene und untersuchte Organ ist, wurde in den USA das „Human Placenta Project“ initiiert, welches das Verständnis über das noch zum größten Teil unbekannte Organ revolutionieren und somit das Wohlbefinden und die Gesundheit von Mutter und Neugeborenem signifikant verbessern soll (Guttmacher et al. 2014). Ziel ist es, die plazentare Entwicklung, die Funktion und letztendlich mikro- und makroskopische Struktur in allen Stadien der Schwangerschaft zu verstehen. Dabei soll auf neue Technologien und kreative Methoden gesetzt werden, die das

Plazentagewebe und die Interaktionen mit anderen Zelltypen und –geweben in verschiedenartiger Weise analysieren können.

Spezifische Marker für Trophoblast- und Mammakarzinomzellen

In dieser Arbeit wurde humanes Plazentagewebe in unterschiedlicher Art und Weise als Versuchsmodell eingesetzt. Es wurde beispielsweise mit den bereits beschriebenen Sphäroiden co-kultiviert. Nach 48 Stunden der gemeinsamen Kultivierung präsentierten die Mammakarzinom-Sphäroide ähnlich wie bei alleiniger Kultivierung einen nekrotischen Kern. Jedoch zeigten Sphäroide, die mit dem villösen plazentaren Gewebe interagierten, nur wenige apoptotische Zellen im Vergleich zu denen, die während der Kultivierung nicht mit dem Plazentagewebe zusammenwuchsen. Einzelne Sphäroide, die nicht mit dem villösen Gewebe interagierten, hatten eine Vielzahl von apopotischen Zellen im Inneren. Mucin-1 (MUC-1), ein Glykoprotein, welches von vielen Tumoren exprimiert wird, wurde in den co-kultivierten Sphäroiden untersucht. Interessanterweise zeigten einige Sphäroide eine Dreischichtung der MUC-1 Expression. Während die äußere Zone stark positiv war, folgten eine mittlere schwächer gefärbte Zone und ein sehr stark gefärbter Kern. Diese zu Teilen starke Färbung und das charakteristische Färbeprofil ermöglichte eine gute Differenzierung von Tumor- und Plazentagewebe. Nach Markierung mit Ki-67, welche vornehmlich zur Charakterisierung des Proliferationsverhaltens der Sphäroide durchgeführt wurde, konnten ebenso einzelne Tumorzellen im Plazentagewebe identifiziert werden. Diese Beobachtungen lassen die Frage aufkommen, ob es möglich ist, spezifische Marker zu finden, die eindeutig zwischen beiden Gewebetypen unterscheiden können. Denn auch wenn eine Metastasierung vom primären Mammakarzinom ein sehr seltenes Ereignis darstellt, wurde das Phänomen von zirkulierenden Tumorzellen (*circulating tumor cells*, CTC), welche ihren Ursprung im Primärtumor haben, nach seinem Erstbeschreiber Stephen Paget 1889 als „seed and soil theory“ nun auch nachfolgend vielfach beschrieben (Ribatti et al. 2006). Die Detektion der CTC erfolgt im Blut, wobei die Erkennung nach morphologischen Kriterien aufgrund ihrer Seltenheit sowie der im Verhältnis zu Blutzellen geringen Anzahl schwierig erscheint (Schindlbeck et al. 2016). Aus diesem Grund erscheint die Separierung und Anreicherung der CTC durch bestimmte Isolations- und Zentrifugationsschritte vor einer morphologischen Charakterisierung als sinnvoll. In den meisten Studien werden die CTC mittels ICC gefärbt und durch automatisierte Mikroskopie ausgewertet. Die Suche nach spezifischen Markern für diese zirkulierenden Tumorzellen stellt eine Herausforderung dar, denn auf solchen abgelösten Mammakarzinomzellen zeigen Standardmarker wie ER/PR oder HER2

eine inhomogene und stark variierende Expression (Schindlbeck et al. 2016) und verhalten sich in ihrer Proteinexpression oft anders als der eigentliche Primärtumor. Außerdem sind sie wahrscheinlich aufgrund ihrer nicht-proliferierenden und Stammzelleigenschaften wenig sensitiv gegenüber Chemotherapeutika (Braun et al. 2000, Riethdorf et al. 2008). Aufgrund eines potenziellen Transfers mütterlicher Zellen über die Plazentaschranke ist es somit auch durchaus denkbar, dass diese zirkulierenden Tumorzellen nicht nur im intervillösen Raum, sondern auch in fetalen Gefäßen der Plazenta gefunden werden (Schamberger et al. 2013, Heinzelmann et al. 2009). Hier stellt eine weitere Herausforderung dar, einen spezifischen Marker für Tumorzellen zu finden, welcher jedoch nicht auf plazentaren Zellen exprimiert wird. Die Tabelle zeigt eine Übersicht ausgewählter Proteine und ihr Expressionsverhalten sowohl in Plazenta- als auch Mammakarzinomzellen, wodurch die Schwierigkeit, spezifische Marker zur Identifikation von zirkulierenden Mammakarzinomzellen zu finden, veranschaulicht werden soll:

Proteine	Abkürzung	Plazenta	MCF-7	MDA-MB-231	SK-BR-3	IHC Färbung
Aquaporin - 5	AQP5					×
E-cadherin	E-cadherin					×
Enhancer of zeste homolog 2	EZH2					×
Epidermal growth factor receptor	EGFR/ ErbB-1					×
Estrogen Rezeptor	ER					×
Humanes Choriongonadotropin	hCG					☆
Human epidermaler growth factor receptor 2	HER2neu					×
Humanes Plazentalaktogen	hPL					☆
4-Hydroxynonenal	4-HNE					×
Ki-67	Ki-67					×
Mammaglobin	MAM					☆
Mucin 1	MUC1/EMA					☆
Placental alkaline phosphatase	PLAP					☆
Plättchen-Endothel-Zelladhäsionsmolekül 1	PECAM-1/CD31					☆
Progesteron Rezeptor	PR					×
Prolaktin induziertes Protein	GCDFP-15					☆
Zytokeratin	CK					☆

	Expression gemäß Literatur
	Schwache Expression gemäß Literatur
	Keine Expression gemäß Literatur
	unbekannt
×	**Keine Färbung**
☆	Färbung durchgeführt

Abbildung 8: Übersicht von Proteinen und deren Expressionsverhalten im Plazentagewebe und in den Mammakarzinomzelllinien. Aufgrund des oft ähnlichen Expressionsverhaltens beider Gewebe ist es schwierig, einen eindeutig differenziellen Marker zu finden. Deshalb wurde zunächst eine Literaturrecherche für das Auffinden potenziell geeigneter Marker durchgeführt und nachfolgend ausgewählte Marker im Sphäroid-Plazenta Co-Kultur-Modell wurden immunhistochemisch gefärbt (Stern zeigt durchgeführte Färbung an).

Auch in CTC Analysen aus Blutproben sind Cytokreatine, MUC-1, HER2 oder ER/ PR gängige Marker für immunomagnetische Anreicherungen oder ICC Färbungen

(Schindlbeck et al. 2016). Bei der Literaturrecherche und bei den erfolgten IHC-Färbungen des Co-Kultur-Modells zeigte sich jedoch, dass keine dieser Marker für die besondere Fragestellung der Tumorzell-Detektion in Plazentagewebe in Frage kommt. Auch wenn MUC-1 im Brustkrebssphäroid sehr stark exprimiert war, gab es gleichzeitig eine schwache Expression dieses Proteins auf Trophoblastzellen (Abbildung 9). Es konnte kein Marker gefunden werden, der ausschließlich auf den Mammakarzinomzellen exprimiert wird. Stattdessen zeigte sich eine alleinige Expression der plazentaren alkalinen Phosphatase (PLAP) und des Endothel-Zelladhäsionsmoleküls CD31 im Plazentagewebe. Zytokeratin wird sowohl auf den Trophoblastzellen als auch auf den Mammakarzinomzellen stark exprimiert. Somit wäre ein möglicher Ansatz, dass man das Verhältnis von PLAP und Zytokeratin in einer Plazentaprobe von einer Frau mit einem Mammakarzinom bestimmt, um Rückschlüsse auf das Vorhandensein von CTC ziehen zu können. Die vielversprechendsten Erfolge sollten durch eine Kombination von ICC/ICH-Methoden und Polymerasekettenreaktion (PCR) basierender genetischer Charakterisierung erreicht werden. In dem hier präsentierten Fall eines metastasierten Mammakarzinoms zur Plazenta wurde immunhistochemisch ebenso die ausschließliche Expression von PLAP in Trophoblastzellen nachgewiesen. Das gleiche Expressionsmuster wurde bei hCG und hPL-2 festgestellt, sodass diese beiden Marker für mögliche Differenzierungsanalysen durchaus denkbar sind. Zudem zeigten die plazentaren Mammakarzinom-Metastasen ähnliche morphologische Charakteristika wie sie zuvor in den MCF-7 Sphäroiden und in dem plazentaren sphäroidalen Co-Kultur-Modell beschrieben wurden. Diese Metastasen sind durch eine sphäroidale Struktur gekennzeichnet und eine Differenzierung in proliferierende und nekrotische Zone ist genauso wie bei den *in vitro* Sphäroiden ausgeprägt. In Studien konnte bereits gezeigt werden, dass Stammzellen *in vivo* Sphäroide formen können, welche Einfluss auf das Metasierungsrisiko haben (Denes et al. 2015). Folglich erscheint es auch naheliegend, dass solche *in vivo* Sphäroide von vorherigen CTC geformt wurden. Dies offenbart das Sphäroidmodell als das für diese Fragestellung am besten geeignete Modell, da hier die veränderten Expressionseigenschaften im Vergleich zum Primärtumor weiterhin präsent sein sollten.

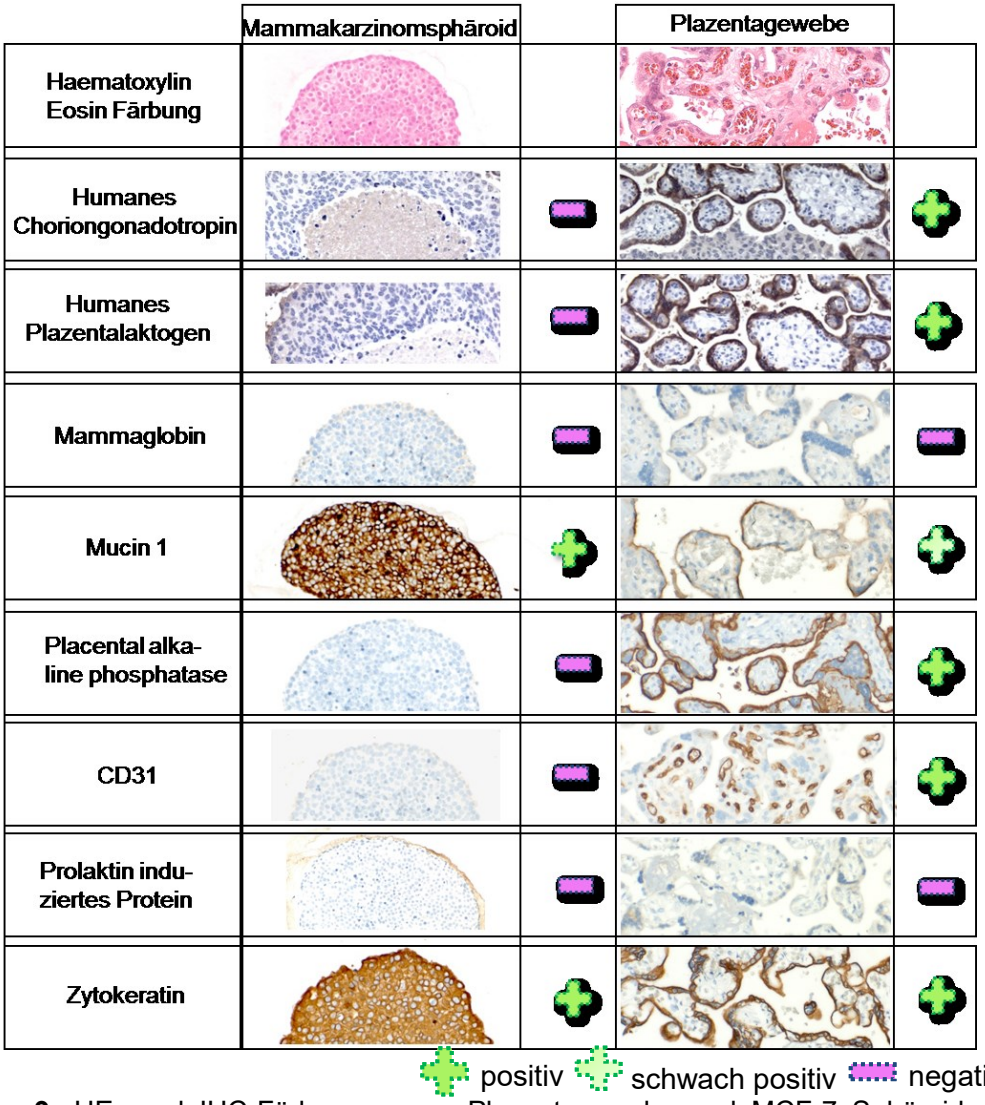

Abbildung 9: HE- und IHC-Färbungen von Plazentagewebe und MCF-7 Sphäroiden mittels ausgewählter Proteine. Die verschiedenen Zelltypen zeigten häufig das gleiche Expressionsverhalten. Ausnahmen stellen die Färbungen mit PLAP und CD31 dar. Obwohl eine Expression von den Proteinen Mammaglobin und Prolaktin induziertes Protein bei Mammakarzinomzellen in der Literatur beschrieben wurde, konnte dies nicht mittels IHC-Färbungen in MCF-7 Sphäroiden bestätigt werden.

Weiterhin wurden die plazentaren Metastasen nur im intervillösen Raum ohne fetale Beteiligung detektiert und das Tumorgewebe war klar von den Trophoblastzellen abgegrenzt. Dieses Charakteristikum konnte im Co-Kultur-Modell nicht beobachtet werden, bei dem es zur Verschmelzung von beiden Gewebetypen kam. Mögliche Ursache könnte eine Desintegration (= Freilegung der proliferierenden Cytotrophoblastzellen) des Trophoblastlayers infolge der Präparation und Kultivierung *ex vivo* sein, sodass das Verwachsen der Tumorzellen mit dem Plazentagewebe ermöglicht wurde.

7. Schlussfolgerungen

Der schwangerschaftsassoziierte Einfluss auf das Wachstums- und Invasionsverhalten des Mammakarzinoms wurde bisher nur in wenigen Studien untersucht, sodass Prognosen oder gar Empfehlungen nicht erstellt bzw. gegeben werden können. Epidemiologische Studien offenbaren ein im Anschluss an eine Schwangerschaft kurzzeitig erhöhtes Risiko, an einem Mammakarzinom zu erkranken. Dies kehrt sich jedoch in den darauffolgenden Jahren in ein reduziertes Risiko um. Fraglich ist, welchen Einfluss die Plazenta mit ihren sezernierten Hormonen und anderen Faktoren auf das Verhalten der Mammakarzinomzellen ausübt. In dieser Arbeit konnte gezeigt werden, dass plazenta-konditioniertes Medium zu morphologischen Veränderungen der Zellen unabhängig von ihrem Rezeptorstatus führte. Die Differenzierung hin zu *spindle-like* Zellen deutet auf erhöhte Motilität- und Invasionseigenschaften hin, welches im Fall der hormon-positiven MCF-7-Zellenlinie auch bestätigt werden konnte. Ähnliche Beobachtungen zeigten auch eine andere Studie an Mammakarzinomzellen, welche mit Plazenta-Explantaten aus dem ersten Trimester co-kultiviert wurden, die in gleicher Weise ihre Morphologie veränderten. Zusätzlich verschwanden die Zellen aus der Region der Plazenta-Explantate (Tartakover-Matalon et al. 2010). Weiterhin konnte gezeigt werden, dass unter dem Einfluss von plazentarem Gewebe oder plazenta-sezernierten Substanzen ERα in hormon-positiven Mammakarzinomzellen herunterreguliert wird. Diese reduzierte Expression des Rezeptors wird auch in Verbindung mit den beobachteten morphologischen Veränderungen gebracht (Platet et al. 2004). Im Fall der hormon-negativen Zellen soll eine Ezrin-abhänige Interaktion zwischen einem G Protein-gekoppelten Rezeptor und ERß einen Umbau des Zytoskeletts bewirken (Li et al. 2013). Interessanterweise zeigte sich in den eigenen experimentellen Untersuchungen, dass die Expression von ERß und HER2 in den hormon-positiven Zellen nach der Inkubation mit plazenta-konditioniertem Medium hochreguliert war. Bereits in einer anderen Studie konnte gezeigt werden, dass die Expression von HER2 und ERß in hormon-abhängigen Mammakarzinomzellen positiv miteinander korreliert (Choi und Pinto 2005). Gleichzeitig ist HER2 dafür bekannt, durch einen ERα abhängigen Signalweg herunterreguliert zu werden (Read et al. 1990). Weiterhin gibt es auch Interaktionen zwischen ERα und ERß, wobei ERß die ERα abhängigen Effekte wie Zellproliferation unterdrücken kann (Zhou et al. 2016). Neben der Zellproliferation fungiert ERα weiterhin als Promotor von Zellüberleben und Motilität (Cortez et al.

2010). Insgesamt wird ERß eine Wirkung als Tumorsuppressor in hormon-abhängigen Geweben nachgesagt (Li et al. 2013, Delaunay et al. 2000) und eine Überexpression dieses Rezeptors in Mammakarzinomsubtypen korreliert mit besseren Prognosen. Deshalb deutet die beobachtete Herunterregulierung von ERα in den MCF-7 Zellen darauf hin, dass die Tumorzellen durch das plazenta-konditionierte Medium zu weniger invasiven Subtypen transformiert werden. Jedoch konnte diese Vermutung bei Durchführung des Invasionsassays nicht bestätigt werden. Die konditionierten und unkonditionierten MCF-7 Zellen zeigten das gleiche Invasionsverhalten. Auch in dem Co-Kultur-Modell verwuchsen Brustkrebssphäroide und Plazentagewebe miteinander, welches auf tumor-supportive Eigenschaften des Plazentagewebes hindeutet. Andererseits wurde in unserem klinischen Fallbericht eine klare Abgrenzung zwischen Tumor- und Plazentagewebe beobachtet, welches mit den Ergebnissen von Tartakover-Matalon et. al. übereinstimmt (Tartakover-Matalon et al. 2010). Zudem wurden bisher keine Mammakarzinom-Metastasen in der fetalen Zirkulation beobachtet. HCG wird ebenso in Bezug auf Tumorsuppression als protektiver Faktor beschrieben, da die behandelten Zellen zur Ausdifferenzierung neigten und weniger proliferativ waren (Russo und Russo 2000, Bodek et al. 2003, Rahman und Rao 2009). Der schützende Langzeiteffekt einer Schwangerschaft kann teilweise mittels einer Immunisierung gegen tumor-spezifische, glykosylierte MUC-1 Proteine erklärt werden (Janerich 1994, Beatson et al. 2010). Dieses Protein mit den gleichen Glykosilierungsmustern wird während der Schwangerschaft auch an der feto-maternalen Grenze exprimiert (Redzovic et al. 2013, Dekel et al. 2010) und soll die Produktion von Antikörper sezernierenden B-Zellen und zytotoxischen T-Zellen im mütterlichen Organismus stimulieren, welche wiederum in späteren Jahren eventuelle Tumorzellen aufgrund der Erkennung spezifisch-glykosylierter MUC-1 Proteine eliminieren (Agrawal et al. 1995). Somit bleibt abschließend zu sagen, dass die Schwangerschaft als solche, aber auch die Plazenta mit ihren vielfältigen sezernierten Substanzen einen merklichen Einfluss auf die Tumore und den späteren Krankheitsverlauf haben. Jedoch können aufgrund der Vielzahl von beeinflussenden Faktoren und dem Mangel an Untersuchungen und Studien keine Empfehlungen gegeben werden. Der starke Einfluss des veränderten hormonellen Milieus konnte in der Arbeit gezeigt werden, wobei weitere Effekte und Mechanismen in zukünftigen Studien untersucht werden sollten.

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9. Anhang

Lebenslauf

Der Lebenslauf wird aus datenschutzrechtlichen Gründen in diesem Exemplar nicht aufgeführt.

Ehrenwörtliche Erklärung

Hiermit erkläre ich, dass mir die Promotionsordnung der Medizinischen Fakultät der Friedrich-Schiller-Universität bekannt ist,

ich die Dissertation selbst angefertigt habe und alle von mir benutzten Hilfsmittel, persönlichen Mitteilungen und Quellen in meiner Arbeit angegeben sind,

mich folgende Personen bei der Auswahl und Auswertung des Materials sowie bei der Herstellung des Manuskripts und der Publikationen unterstützt haben: Prof. Dr. med. Udo Markert, Dr. André Schmidt, Julia Heger, Jana Pastuschek, Dr. Jan-Dirk Haeger. Prof. Dr. Amelie Lupp, Dr. Gitta Turowski, Dr. Hanne Stensheim, Lea Hauswald, Ann-Kathrin Fleischer, Sarah Avemarg und Stephanie Morgner,

die Hilfe eines Promotionsberaters nicht in Anspruch genommen wurde und dass Dritte weder unmittelbar noch mittelbar geldwerte Leistungen von mir für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen,

dass ich die Dissertation noch nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht habe und

dass ich die gleiche, eine in wesentlichen Teilen ähnliche oder eine andere Abhandlung nicht bei einer anderen Hochschule als Dissertation eingereicht habe.

Ort, Datum Unterschrift des Verfassers

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