Universität Bremen

Untersuchungen zur Expression ausgewählter Stammzellgene an embryonalen Geweben und Tumoren

Dissertation

zur Erlangung des Grades eines Doktors der Naturwissenschaften

- Dr. rer. nat –

dem Promotionsausschuss Dr. rer. nat. im Fachbereich Biologie/Chemie

der Universität Bremen

vorgelegt von

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Bremen, im März 2017

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Datum der Disputation:
30.05.2017

So eine Arbeit wird eigentlich nie fertig, man muss sie für fertig erklären, wenn man nach Zeit und Umständen das Möglichste getan hat.

Johann Wolfgang von Goethe

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Abkürzungsverzeichnis

°C	Grad Celsius
µl	Mikroliter
µm	Mikrometer
AWMF	Arbeitsgemeinschaft der Wissenschaftlichen Medizinischen Fach-gesellschaften e.V.
BAC	Bacterial artificial chromosome
bp	Basenpaare
C19MC	Chromosome 19 micro-RNA cluster
CDK2	Cyclin-dependent kinase 2
cDNA	Copy DNA
CpG	Cytosin-phosphatidyl-Guanin
Ct	Cycle threshold
ddCt	Delta delta Ct
DMRT1	Doublesex and mab-3 related transcription factor 1
DNA	Desoxyribonukleinsäure
dNTP	Desoxy-Nukleosid-5`-Triphosphat
EMT	Epithelial-mesenchymale Transition
EVT	Extravillöser Trophoblast
FFPE	Formalin fixed paraffin embedded
HE	Hämatoxilin-Eosin
HMGA1	High Mobility Group AT-Hook Protein 1
HMGA2	High Mobility Group AT-Hook Protein 2
HMGA2-P3	High Mobility Group AT-Hook Protein 2-Peptide 3
HPRT	Hypoxanthin-Guanin-Phosphoribosyl-Transferase
HTR-8/SVneo	First trimester Human trophoblast cells
IGF2BP1	Insulin-like growth factor 2 mRNA-binding protein 1
ING1	Inhibitor of growth family member 1
ITGCNU	Intratubular germ cell neoplasia undifferentiated
kbp	Kilobasenpaare
min	Minute
miRNA	microRNA
M-MLV	Moloney-Murines-Leukemia-Virus

mRNA	Messenger RNA
ng	Nanogramm
NK-Zellen	Natürliche Killerzellen
O_2	Sauerstoff
OCT3/4	Octamer-binding-transcription factor 4
PCR	Polymerase-chain-reaction
PRKG1	Protein kinase, cGMP-dependent, type I
qRT-PCR	Quantitative Real-time PCR
RNA	Ribonukleinsäure
RNU6B	RNA, U6 small nuclear 6, pseudogene
ROC	Receiver-Operating-Characteristic
RQ	Relative quantification
siRNA	Small interfering RNA,
snoRNA	Small nucleolar ribonucleic acid
ß-HCG	Humanes Choriongonadotropin ß
SSW	Schwangerschaftswoche
STAT3	Signal transducer and activator of transcription 3
TBPC	Trophoblast progenitor cells
TGCT	Testicular germ cell tumours
U	Enzymeinheit

1 Einleitung

Die embryonale Entwicklung des Menschen ist eine Phase hoher Genaktivität. Da der im Entstehen begriffene Mensch schnell wächst, findet ein permanenter Umbau statt. Gewebe entstehen, differenzieren sich und werden zum Teil durch apoptotische Prozesse wieder abgebaut. Daher sind in der Entstehungsphase des Menschen sehr viel mehr Gene aktiv, als sie in adulten Geweben zur Selbsterneuerung und Aufrechterhaltung der Funktion gebraucht werden. Einige dieser Gene werden in späteren Phasen wieder reaktiviert und werden mit Tumorentstehung in Zusammenhang gebracht. In der vorliegenden Arbeit wurden als Vertreter embryonal exprimierter und in Tumorzellen wieder reaktivierter Gene microRNAs (miRNAs) der Cluster C19MC und mir-371-3 (hier beispielhaft miR-517a-3p, miR-520c-3p, miR-371a-3p, miR-372-3p und miR-373-3p) sowie das High Mobility Group ATHook Protein 2-Gen (*HMGA2)* untersucht.

miRNAs sind etwa 22 Nucleotide lange, nicht codierende, einzelsträngige RNA-Moleküle, deren Funktion es ist, regulierend auf die Expression von mRNAs einzuwirken. Derzeit sind beim Menschen fast 1900 Gene identifiziert worden, die für etwa 2600 miRNAs codieren (miRBase 2014). Vermutlich werden mehr als 60% aller mRNAs von miRNAs reguliert (Friedman et al. 2009). Jede miRNA kann verschiedene Targets regulieren, umgekehrt kann eine mRNA von vielen verschiedenen miRNAs reguliert werden (Selbach et al. 2008). Viele miRNAs liegen benachbart auf einem DNA-Abschnitt in Clustern, deren Mitglieder durch einen gemeinsamen Promotor reguliert werden können. Die Transkription erfolgt dann als polycistronisches Transkript (Lee et al. 2002). Etliche miRNAs sind in ihrer Sequenz konserviert, was die Hypothese mitbegründet, dass sie während der evolutionären Entwicklung durch Amplifikation entstanden sind (Tanzer, Stadler 2004). In diesen amplifizierten Sequenzen wiederum sind Modifikationen vorhanden, so dass neue miRNAs entstanden sind, die die erwähnten Cluster bilden (Zhang, Wang & Su 2008).

Die in dieser Arbeit untersuchten miRNAs des C19MC-Clusters sind vermutlich aus dem hier ebenfalls untersuchten miR-371-3-Cluster hervorgegangen. Ähnliche Seed-Sequenzen sowie die Tatsache, dass C19MC nur in Primaten vorkommt, während von miR-371-3 Orthologe bei Hund, Maus, Ratte, Pferd und Rind gefunden wurden,

führen zu diesem Schluss (miRBase 2014, Zhang, Wang & Su 2008, Bentwich et al. 2005, Morales-Prieto et al. 2013).

Beide Cluster befinden sich etwa 25 kbp voneinander entfernt in der chromosomalen Bande 19q13.4. C19MC ist das größte bekannte miRNA-Cluster im menschlichen Genom (Bentwich et al. 2005). Es umfasst 46 Gene, die für 62 reife miRNAs codieren (miRBase 2014, Bentwich et al. 2005, Bortolin-Cavaille et al. 2009). C19MC wird fast ausschließlich in der Plazenta exprimiert (Bentwich et al. 2005, Liang et al. 2007, Luo et al. 2009).

Eine Besonderheit der miRNAs des Clusters ist, dass sie nur vom paternalen Allel exprimiert werden, während das maternale Allel methyliert ist (Noguer-Dance et al. 2010). Die Methylierung der DNA ist einer der wichtigsten Mechanismen zur Kontrolle der Genexpression in unterschiedlichen Zelltypen oder verschiedenen Entwicklungsstadien von Zellen (Tsai et al. 2009). Im C19MC-Cluster setzt die Methylierung an einer CpG-Insel 17,6 kb upstream des Clusters an. Sind beide Allele hypermethyliert, ist das Cluster inaktiv (Tsai et al. 2009).

Das miR-371-3-Cluster ist mit drei Genen, die für sechs reife miRNAs codieren, wesentlich kleiner. Ein viertes Gen, das für weitere zwei miRNAs des Clusters codiert, wird auf dem Minusstrang vermutet (miRBase 2014). Physiologisch wird miR-371-3 in embryonalen Stammzellen exprimiert (Cao, Yang & Rana 2008, Laurent et al. 2008, Morin et al. 2008).

In verschiedenen Tumoren wurde eine Reexpression der miRNAs beider Cluster gefunden. He et al. (2014) fanden ein durch miR-371-5p-Überexpression deutlich gesteigertes Tumorwachstum in Pankreastumoren. In in-vitro Versuchen konnten die Autoren zeigen, dass miR-371-5p den Tumorsuppressor ING1 (inhibitor of growth 1) herunter reguliert und damit die Migration von Tumorzellen fördert. In testikulären Keimzelltumoren konnte eine onkogene Funktion von miR-372 und miR-373 gezeigt werden (Voorhoeve et al. 2006). Hohe Expression von miRNAs des C19MC bzw. des miR-371-3-Clusters wurden mit Metastasierung und verringerter Überlebenszeit in Colorektalkarzinomen (Yamashita et al. 2012) und oralen Plattenepithelkarzinomen assoziiert (Tu et al. 2015). Rippe et al. (2010) zeigten in Schilddrüsenadenomen eine Korrelation zwischen Rearrangierungen der chromosomalen Bande 19q13.4 und der Überexpression von miRNAs beider Cluster, die zur Proliferation von Adenomen bei-

trägt. Eine Rolle in der Entstehung von Schilddrüsenkarzinomen konnte für keines der beiden Cluster nachgewiesen werden (Rippe et al. 2012).

Auf der anderen Seite zeigten verschiedene Untersuchungen tumorsuppressive Effekte von miRNAs beider Cluster. In Zervixkarzinomen (Tian et al. 2011) und Nierenzellkarzinomen (Huang et al. 2015) wurde eine im Vergleich zum Normalgewebe deutliche Unterexpression von miR-372 beobachtet. Für Zervixkarzinome konnte gezeigt werden, dass CDK2 (cyclin-dependent kinase 2) und Cyclin A Targets von miR-372 waren. Hohe Aktivität beider Gene und entsprechende starke Proteinbildung führen zu einem forcierten Zellzyklus. Im Falle der Nierenzellkarzinome wurde die mRNA des IGF2BP1 (insulin-like growth factor 2 mRNA-binding protein 1) als Target identifiziert und gezeigt, dass eine niedrige miR-372-Expression die Proliferations- und Invasionsfähigkeit der Tumorzellen steigert. Tumorsuppressive Eigenschaften einer miRNA des C19MC konnten Hong et al. (2016) in Gewebeproben und Zellkulturen von Gliomen nachweisen. Die Autoren zeigten, dass niedrige Expression von miR-519a mit der Progression der Krankheit invers korreliert. Als Target von miR-519a wurde STAT3 (signal transducer and activator of transcription 3) identifiziert, ein Transkriptionsfaktor, der in vielen Tumoren überexprimiert wird. Eine Übersicht über miRNAs des C19MC in der Funktion als Tumorsuppressoren publizierten Flor et al (2012).

Darüber hinaus wurde in dieser Arbeit *HMGA2*, ein ebenfalls in der frühen Entwicklung hoch exprimiertes Gen, untersucht. HMGA-Proteine sind kleine, chromatinassoziierte nicht-histon-Proteine. Sie haben drei DNA-bindende Domänen, die AT-Hooks, und einem sauren carboxy-terminalen Bereich. HMGA-Proteine haben sehr viele Funktionen. Der von Cleynen & Van de Ven (2008) publizierte Artikel „The HMGA proteins: a myriad of functions" bietet hierzu eine sehr gute Übersicht. Eines der Funktionsprinzipien ist die Bindung an Chromatin oder DNA in AT-reichen Abschnitten der kleinen Furche der DNA-Doppelhelix (Bustin, Reeves 1996). Durch die so entstehenden Konformationsänderungen wird die Transkription verstärkt oder abgeschwächt (Bustin 1999). Das *HMGA2*-Gen befindet sich in der chromosomalen Bande 12q14~15 (Ashar et al. 1995, Schoenmakers et al. 1995). Es ist etwa 160 kbp lang und besteht aus fünf Exons. Die mRNA ist 4150 bp lang. Das HMGA2-Protein

der häufigsten Transkriptionsvariante 1 besteht aus 109 Aminosäuren (Cleynen, Van de Ven 2008).

Hohe *HMGA2*-Expression wurde in embryonalen und fötalen Geweben nachgewiesen (Zhou et al. 1995, Rogalla et al. 1996, Hirning-Folz et al. 1998). In ausdifferenzierten gesunden Geweben ist nur eine geringe (Kloth et al. 2015, Smeti et al. 2014) bis keine Aktivität messbar (Rogalla et al. 1996).

HMGA2 gilt unter anderem als Stammzellfaktor, seine Rolle liegt vermutlich darin, Impulse zur Selbsterneuerung von Stammzellen zu geben (Nishino et al. 2008, Markowski et al. 2011). Daher ist es nicht überraschend, dass in sich entwickelnden embryonalen und fötalen Geweben hohe *HMGA2*-Expressionen detektierbar sind. Die *HMGA2*-Expression kann sowohl in benignen als auch malignen Tumoren reaktiviert sein. So wurden in benignen mesenchymalen Tumoren mit 12q15-Rearrangierungen, wie sie häufig bei Lipomen (Ashar et al. 1995), Leiomyomen (Hennig et al. 1997) und Lungenhamartomen (Dal Cin et al. 1993, Kazmierczak et al. 1995) vorkommen, *HMGA2*-Expressionen nachgewiesen.

Eine Reexpression von *HMGA2* wird auch in vielen malignen Tumoren beobachtet. Dies konnte beispielsweise in Schilddrüsentumoren (Belge et al. 2008, Klemke et al. 2014), Mammakarzinomen (Ahmed, Tsai & Lee 2010, Fabjani et al. 2005), Leukämien (Meyer et al. 2007, Nyquist et al. 2012, Tan et al. 2016) und Colonkarzinomen (Helmke et al. 2012) gezeigt werden.

In der vorliegenden Arbeit wurden exemplarisch für die physiologische Expression in einem embryonalen Gewebe Plazenten unterschiedlicher Schwangerschaftswochen hinsichtlich ihrer miRNA- und *HMGA2*- -Expression untersucht. Im Folgenden werden Aufbau und Funktion der Plazenta beschrieben.

Die Plazenta ist ein spezifisches und temporäres Organ der Embryonal- und Fetalentwicklung. Sie bildet die Schnittstelle von Mutter zu Kind und dient der Ernährung des wachsenden Menschen. Verschiedene Untersuchungen deuten darauf hin, dass die Plazenta außerdem eine Rolle in der feto-maternalen Kommunikation spielt und dazu beiträgt, dass der semiallogene Embryo und Fötus vom mütterlichen Körper toleriert wird (Kshirsagar et al. 2012, Kambe et al. 2014, Ishida et al. 2015, Burton, Jauniaux 2015, Costa 2016).

Die Plazenta besteht aus einem kindlichen, vom extraembryonalen Teil der Blasto-zyste gebildeten, und einem mütterlichen Teil (Hinrichsen 1990). Der kindliche Teil der Plazenta besteht aus sich im Laufe der Schwangerschaft fortlaufend verzweigen-den Zotten. Das Zottenepithel der frühen Plazenta ist aus zwei Zellschichten, dem innenliegenden Zytotrophoblasten und dem außen liegenden Synzytiotrophoblasten aufgebaut. Bis etwa zur 12. Schwangerschaftswoche (SSW) ist dieser zweischichtige Aufbau die dominante Form. Mit fortschreitender Entwicklung verschwindet die Zytotrophoblastenschicht, so dass das Zottenepithel ab der 20. SSW fast ausschließ-lich aus Synzytiotrophoblastzellen besteht (Vogel 1996). Das Innere der Zotten be-steht aus lockerem mesenchymalen Bindegewebe, dem Stroma. Es wird von kindli-chen Blutgefäßen durchzogen, die die aus dem mütterlichen Blut aufgenommenen Nährstoffe zum Embryo transportieren. Außerdem enthält es eine hohe Anzahl an Immunzellen, die Hofbauerzellen (Hinrichsen 1990). Der mütterliche Teil der Pla-zenta wird durch Deziduazellen und Spiralarterien gebildet. Deziduazellen versorgen den Embryo mit Nährstoffen, bevor die Plazenta ihre Funktion voll erfüllt (Oreshkova, Dimitrov & Mourdjeva 2012). Spiralarterien weiten und entspiralisieren sich während der Schwangerschaft unter dem Einfluss einwandernder Trophoblastzellen und ge-währleisten damit eine gute Blutversorgung. Im ersten Schwangerschaftstrimester wächst der Embryo jedoch zunächst in einer sauerstoffarmen Umgebung auf (Jauniaux et al. 2000). Durch massenhafte Einwanderung extravillöser Trophoblast-zellen (EVT) werden die mütterlichen Spiralarterien vorübergehend wie ein Pfropf verschlossen (Salomon et al. 2014). Der Embryo wird in dieser Phase vom Plasmafil-trat der Mutter sowie Exkreten der Deziduazellen und der uterinen Drüsen ernährt (Burton et al. 2002, Huppertz et al. 2009, Moser et al. 2010). Durch Auflösung des Trophoblastpfropes und dadurch steigende O_2-Konzentration wird etwa in der 12. SSW ein Höhepunkt der Invasion von extravillösen Trophoblastzellen erreicht (Silva, Serakides 2016). Hierdurch verwächst die Plazenta zunehmend fest mit dem mütterlichen Endometrium (Schneider, Raio & Knöfler 2011)

Die Fähigkeit invasiv zu wachsen ist ein Hauptkennzeichen maligner Tumoren und so blickt die Tumorforschung seit jeher mit Interesse auf die Regulation des invasiven Wachstums in der Plazenta. Die Plazenta kann deshalb als eine Art „kontrollierter Tumor" betrachtet werden. Erkenntnisse über die kontrollierenden Regelmechanis-

men könnten helfen, Tumorwachstum besser zu verstehen und damit zu beeinflussen.

Als Beispiel für einen aus embryonalen Zellen hervorgehenden Tumor wurden in der vorliegenden Arbeit testikuläre Keimzelltumoren erwachsener Männer hinsichtlich ihrer *HMGA2*-Expression untersucht. Diese Tumoren haben ein sehr diverses Erscheinungsbild. Allen Subgruppen ist gemeinsam, dass sie die Zellmorphologie der embryonalen Entwicklung imitieren (Looijenga et al. 2007). Keimzelltumoren werden histologisch in Seminome (bei Frauen Germinome genannt), Embryonalkarzinome, Dottersacktumoren, Teratome und Chorionkarzinome unterschieden. Aus therapeutischen und prognostischen Gründen wird eine Unterscheidung in Seminome und Nicht-Seminome vorgenommen (Eble et al. 2004, Ulbright 2005, Krege et al. 2008). Nicht-Seminome präsentieren verschiedene Grade der Gewebedifferenzierung von sehr ursprünglichen Embryonalkarzinomen bis hin zu reifen Teratomen. Sie zeigen sowohl embryonale und als auch extraembryonale Differenzierungen (Looijenga 2009, van de Geijn, Hersmus & Looijenga 2009).

Testikuläre Keimzelltumoren sind mit einer Inzidenz von etwa 10/100.000 Männern eine seltene Tumorerkrankung (Chia et al. 2010, Bray et al. 2006). Sie treten, im Gegensatz zu den meisten anderen Tumoren, überwiegend bei jungen Männern auf. In den letzten Jahrzehnten wurde weltweit ein Anstieg der Inzidenz beobachtet, für den bislang hinreichend schlüssige Gründe fehlen (Chia et al. 2010, Huyghe, Matsuda & Thonneau 2003, Purdue et al. 2005). Die Differentialdiagnose der Keimzelltumor-Subgruppen ist anspruchsvoll. Insbesondere vor dem Hintergrund, dass 10-54 % der Keimzelltumoren eine gemischte Histologie aus mehreren Nicht-Seminomgruppen bzw. Seminom und Nicht-Seminom präsentieren (Horwich, Shipley & Huddart 2006, Sesterhenn, Davis 2004), kann die Diagnostik sehr schwierig sein. Auf Grund dieser Komplexität und der steigenden Inzidenz ist eine sichere Differentialdiagnose daher umso wichtiger. Hierfür steht ein breites Panel an immunhistochemischen Markern zur Verfügung. Nach wie vor gibt es aber schwierig zu diagnostizierende Fälle, die einen zusätzlichen Marker wünschenswert machen.

Der Fokus dieser Arbeit lag auf embryonalen Geweben und embryonale Gewebe imitierenden Tumoren. Hierfür wurde die Expression der Cluster C19MC und miRNA-371-3 und die *HMGA2*-Expression in Plazenten sowie die *HMGA2*-Expression in testikulären Keimzelltumoren untersucht. Im Vordergrund der Untersuchungen standen

Expressionsänderungen im Schwangerschaftsverlauf sowie die Frage nach differenzierter *HMGA2*-Expression in verschiedenen Subgruppen von Keimzelltumoren. Ein besonderes Augenmerk lag in allen drei Publikationen auf der Frage, ob die Expression der untersuchten Gene als diagnostische Marker für die Früherkennung von drohendem Abort bzw. zur Differenzierung der Subgruppen testikulärerer Keimzelltumoren geeignet sein könnte.

2 Material und Methoden

2.1 Gewebe- und Serumproben

Die Plazenten wurden vom Pathologischen Institut des Elbe-Klinikums Stade als formalin-fixierte Paraffingewebeblöcke (FFPE) zur Verfügung gestellt. Weitere postpartal gewonnene Plazentaproben wurden im Helios-Klinikum Cuxhaven sowie im Ev. Diakonie-Krankenhaus Bremen gesammelt. Die Gewebeproben wurden unmittelbar nach der Entbindung entnommen und in gepuffertem 4%igem Formalin gelagert. Die weitere Verarbeitung zu FFPE erfolgte durch das Pathologische Institut des Elbe-Klinikums Stade (Publikation I u. II).

Serumproben wurden von Schwangeren des Helios-Klinikums Cuxhaven zur Verfügung gestellt (Publikation I). Die Proben wurden präpartal gewonnen.

Hodentumoren sowie Hoden-Normalgewebe wurden von der Pathologie des Albertinen-Krankenhauses in Hamburg, vom Pathologischen Institut des Elbe-Klinikums Stade-, vom Pathologischen Institut des Klinikums Bremen Mitte sowie vom Leibniz-Instituts für Präventionsforschung und Epidemiologie (BIPS) als FFPE zur Verfügung gestellt. Zusätzlich stellte das Pathologische Institut des Klinikums Bremen Mitte schockgefrorene Frischgewebeproben von normalem Hodengewebe bereit (Publikation III).

Die Verwendung aller Proben erfolgte entsprechend der Regeln der Helsinki-Deklaration (World Medical Association 2013) und wurde durch die Ethikkommission der Ärztekammer Bremen genehmigt. Im Falle der postpartal gewonnenen Plazenten sowie der Serumproben wurde das schriftliche Einverständnis der Mütter eingeholt.

2.2 Expressionsanalyse

2.2.1 RNA-Isolierung

Für die RNA-Isolierung aus Plazenta-FFPEs (Publikation I u. II) wurden je nach Größe des eingebetteten Gewebes 5 bis 10 Schnitte á 5 μm angefertigt. Im Falle der Hodengewebe-FFPEs (Publikation III) wurden je nach Größe des eingebetteten Gewebes 6 bis 8 Schnitte á 5 μm angefertigt. Die Isolierung erfolgte jeweils mit Hilfe des innuPREP MicroRNA Kits (Analytik-Jena, Jena) nach Herstellerangaben mit folgen-

der Modifikation: Die der eigentlichen RNA-Isolierung vorangehende Lysis der Paraffinschnitte wurde mit Hilfe der TLS-Lysis solution sowie Proteinase K aus dem innuPREP DNA Mikro Kit (Analytik Jena) durchgeführt.

Für die RNA-Isolierung aus schockgefrorenem Frischgewebe (Publikation III) wurde das Gewebe zunächst mit Hilfe eines TissueLysers (Qiagen, Hilden) homogenisiert. Die RNA-Isolierung erfolgte mit Hilfe des RNeasy Mini Kits (Qiagen, Hilden).

Für die RNA-Isolierung aus Serum (Publikation I) wurden jeweils 200 µl Serum vorsichtig aufgetaut. Mit Hilfe des RNeasy Mini Kits (Qiagen, Hilden) wurde entsprechend des Herstellerprotokolls die RNA isoliert.

Die RNA-Konzentration und –qualität wurde mit einem Eppendorf Photometer (Bio-Photometer, Eppendorf, Hamburg) ermittelt.

2.2.2 cDNA-Synthese

Um die miRNA-Expression in Plazenten und Serum zu messen (Publikation I), wurde die RNA mit Hilfe des TaqMan MicroRNA Reverse Transcription Kits (Applied Biosystems, Darmstadt) in cDNA transkribiert. Hierbei wurden spezifische stem-loop Primer benutzt, die in den jeweiligen TaqMan microRNA-Assays enthalten sind, (Applied Biosystems).

Plazenten: Je Probe wurden 5 µl RNA (entspricht 200 ng), 4,16 µl nukleasefreies Aqua bidest, 1,5 µl Puffer, 0,15 µl dNTPs, 3 µl stem-loop Primer, 0,19 µl RNAse Inhibitor sowie 1 µl Reverse Transkriptase in die Reaktion eingesetzt. Für jede Probe wurde außerdem eine Negativkontrolle (ohne Reverse Transkriptase) sowie für jedes gemessene Gen eine weitere Negativkontrolle (ohne RNA) angesetzt. Als endogene Kontrolle diente die snoRNA RNU6B.

Serum: Je Serumprobe wurden 55 ng RNA und ein Primerpool mit je 0,75 µl der miRNAs 371a-3p und 20a eingesetzt. Die restlichen Komponenten des cDNA-Ansatzes wurden in den gleichen Mengen wie für die Plazentaproben verwendet, der Ansatz wurde mit nukleasefreiem Aqua bidest auf 15 µl aufgefüllt. Als endogene Kontrolle diente miRNA-20a.

Die cDNA-Synthese der miRNAs wurde in Placenta- und Serumproben unter folgenden Reaktionsbedingungen durchgeführt: 30 min bei 16 °C, 30 min bei 42 °C, 5 min bei 85 °C gefolgt von Kühlung bei 4 °C.

RNAs für die Analysen von *HMGA2*-Expression in Plazenten (Publikation II) und Hodentumoren (Publikation III) wurden mit Hilfe des M-MLV Reverse Transcriptase (Invitrogen, Karlsruhe) nach Herstellerprotokoll in cDNA umgeschrieben. Es wurden jeweils 250 ng RNA, 200 U Enzym sowie 150 ng Random Hexamer Primer (Invitrogen) eingesetzt. Das Reaktionsvolumen betrug 20 µl. Als endogene Kontrolle der *HMGA2*-Analysen diente für Plazentaproben (Publikation II) und Hodentumoren (Publikation III) *HPRT*. Die cDNA-Synthese erfolgte unter folgenden Reaktionsbedingungen: 10 min bei 25 °C, 50 min bei 37 °C und 15 min bei 70 °C.

Sämtliche Proben wurden entweder sofort in der qRT-PCR weiterverarbeitet oder zunächst bei -20 °C eingefroren.

2.2.3 Präamplifikation

Um im Serum die Signalstärke der relativ schwach konzentrierten miRNAs zu verstärken (Publikation I), wurde vor der quantitative Real-time PCR eine Präamplifikation durchgeführt. 0,75 µl Assay je Probe und miRNA wurde mit 13,5 µl nukleasefreiem Aqua bidest versetzt. 12,5 µl dieser Verdünnung wurden mit 12,5 µl cDNA sowie 25 µl TaqMan Universal PCR Master Mix (Applied Biosystems) versetzt. Die PCR wurde unter folgenden Bedingungen durchgeführt: 10 min bei 95 °C, gefolgt von 14 Zyklen á 15 s bei 95 °C und 4 min bei 60 °C im GeneAmp PCR System (Applied Biosystems). Das Präamplifikationsprodukt wurde für die qRT-PCR 1:5 in nukleasefreiem Aqua bidest verdünnt.

2.2.4 Quantitative Real-time PCR (qRT-PCR)

Die qRT-PCR wurde mit Hilfe des Applied Biosystems Sequence Detection Systems 7300 entsprechend der TaqMan Genexpressionsassay-Protokolle in 96-well-Platten (Applied Biosystems) durchgeführt.

Die Expressionsmessungen der plazentaren miRNAs (Publikation I) wurden nach folgendem Pipettierschema angesetzt: 1,33 µl cDNA mit 1 µl TaqMan Assay (Applied Biosystems), 10 µl TaqMan Universal Mastermix (Applied Biosystems) sowie 7,67 µl nukleasefreiem Aqua bidest. Für die miRNA-Messungen im Serum (Publikation I) wurden je Reaktion 9 µl der präamplifizierten cDNA, 1 µl TaqMan microRNA Assay sowie 10 µl TaqMan Universal PCR Master Mix eingesetzt. Für die *HMGA2*-Expres-

sionsmessungen (Publikation II u. III) (Assay Nummer Hs00171569, Applied Biosys-tems) wurden je Reaktion 2 µl cDNA (entspricht 50 ng), 1 µl TaqMan Assay, 10 µl TaqMan Universal Mastermix (Applied Biosystems) sowie 7 µl nukleasefreies Aqua bidest pipettiert. Für die *HPRT*-Expressionsmessungen (Publikation II u. III) wurden 2 µl cDNA (entspricht 50 ng), 1,2 µl (600 nM) forward und 0,2 µl (200 nM) reverse Primer, 10 µl TaqMan Universal Mastermix (Applied Biosystems) sowie 5,4 µl nukle-asefreies Aqua bidest pipettiert.

Alle Proben wurden als Dreifachbestimmung sowie jeweils mit einer Negativkontrolle (cDNA ohne Reverse Transkriptase) gemessen. Je untersuchtem Gen und 96-well-Platte wurden außerdem zwei Negativkontrollen (cDNA: ohne Reverse Transkrip-tase; PCR: ohne cDNA) mitgeführt. Alle qRT-PCR wurden im ABI-7300-Cycler (Applied Biosystems) nach dem folgenden Protokoll durchgeführt: 2 min bei 50 °C gefolgt von 10 min bei 95 °C, 50 Zyklen á 15 s bei 95 °C gefolgt von 1 min. bei 60 °C. Die Auswertung erfolgte mit Hilfe der Software „7300 System Sequence Detection Software" (Version 1.2.3) (Applied Biosystems). Die relative Genexpression wurde nach der ddCt-Methode (Livak, Schmittgen 2001) ermittelt.

2.2.5 Immunhistochemie

Die immunhistochemische Detektion des HMGA2-Proteins (polyklonales Kaninchen-anti-HMGA2-P3, Biocheck Incorporation, Foster City, USA) wurde mit Hilfe des DAKO-Detektions-Kits (DAKO Chem-Mate; DAKO, Glostrup, Dänemark) und einem semiautomatischen Färbegerät (DAKO TechMate) nach Herstellerangaben durch-geführt. Die Antigen-Demaskierung der Schnitte erfolgte in einem PT Link modul (DAKO) mit Hilfe der EnVision FLEX Target Retrieval Solution, low pH (DAKO). Der Antikörper wurde für die immunhistochemischen Untersuchung der Plazenten (Publi-kation II) 1:500 verdünnt, während für die Hodentumoruntersuchungen (Publikation III) ein 1:1000 verdünnter Antikörper eingesetzt wurde.

In Publikation II wurde die Färbeintensität in fünf Stufen beurteilt: ungefärbt, sehr schwache, schwache, mittlere und starke Färbung. Für die Auswertung in Publikation III wurde ein Score vergeben, der sich aus folgenden Parametern zusammen-setzte:1. Prozentualer Anteil der positiven Zellen und 2. Färbeintensität: ungefärbt (0), schwache (1), schwache bis mittlere (1,5), mittlere (2) mittlere bis starke (2,5)

und starke (3) Färbung. Beide Werte wurden miteinander multipliziert, wodurch ein maximaler Score von 3 (100% gefärbte Zellen x Färbeintensität 3) erreicht werden konnte.

2.2.6 Statistik

Die statistischen Analysen wurden mit Hilfe der Software R (R Core Team) durchgeführt.

Der Wilcoxon Rangsummentest (= Mann-Whitney U-Test) wurde für alle Analysen zweier unabhängiger Gruppen durchgeführt (Publikation I, II u. III). Waren mehr als zwei unabhängige Gruppen zu vergleichen, wurde der Kruskal-Wallis-Test gewählt (Publikation I). Vergleiche zwischen erwarteten und beobachteten Werten sowie zum Zusammenhang zwischen zwei Variablen wurden mit Hilfe der linearen Regression (Publikation I, II u. III) bzw. der linearen und der nicht-parametrischen Regression angestellt (Publikation II). In Publikation II wurden beide Modelle schließlich durch einen likelihood ratio Test verglichen und das besser passende Modell wurde angewandt. Um die Eignung einer beobachteten Expressionshöhe für die Diskriminierung zwischen verschiedenen Tumorsubtypen zu untersuchen wurde eine Receiver-Operating-Characteristic (ROC)-Analyse durchgeführt (Publikation III).

Ein Signifikanzwert (p-Wert) von ≤0,05 wurde als signifikant, ein p-Wert von ≤0,001 als hochsignifikant angesehen.

Die statistischen Tests wurden in Zusammenarbeit mit Dr. Rolf Nimzyk (Zentrum für Humangenetik, Universität Bremen) (Publikation I) und mit Dr. Werner Wosniok (Institut für Statistik, Universität Bremen) (Publikation II u. III) durchgeführt.

3 Ergebnisse

Die im Rahmen der Dissertation erhobenen Daten werden im Folgenden präsentiert. Beginnend mit der physiologischen Expression von miRNAs und *HMGA2* in Plazenten wird danach die pathologische *HMGA2*-Expression in testikulären Keimzelltumoren beschrieben.

3.1 Publikation I:
Quantitative Analyse von miRNAs der Cluster C19MC und 371-3 in Plazenten

miRNAs sind Marker der Schwangerschaftsentwicklung bzw. für Störungen derselben (Morales-Prieto et al. 2012, Hromadnikova et al. 2013, Doridot et al. 2014, Svobodova et al. 2016). Eine Publikation unserer Arbeitsgruppe deutete bereits auf eine Rolle von miRNAs des plazentaspezifischen Clusters C19MC für den Schwangerschaftsverlauf hin (Flor et al. 2012). Deshalb wurden darauf aufbauend diese miRNAs im Schwangerschaftsverlauf untersucht. Das Cluster miR-371-3 wird genau wie C19MC in der Plazenta exprimiert, deshalb wurde das Spektrum um drei miRNAs dieses Clusters erweitert. miR-371-3 wird physiologisch auch in embryonalen Stammzellen exprimiert und gilt als Stammzell- und Proliferationsmarker (Laurent et al. 2008, Voorhoeve et al. 2006). Diese Eigenschaften teilt das miR-371-3 Cluster mit *HMGA2*, bislang wurden aber keine gemeinsamen pathways identifiziert.

Im Fokus der Untersuchungen für Publikation I standen grundlegende Fragen. Einerseits sollte geprüft werden, wie sich die Expressionswerte der ausgewählten miRNAs unterschieden und wie sie sich im zeitlichen Verlauf darstellten. Die Überlegung, dass miRNAs des C19MC eine Rolle in der feto-maternalen Kommunikation bzw. der Immunmodulation haben können, war bereits von verschiedenen Autoren geäußert worden (Kambe et al. 2014, Ishida et al. 2015, Donker et al. 2012, Bullerdiek, Flor 2012). Daher sollte als zweites untersucht werden, ob das Abortgeschehen auch anhand aberranter miRNA-Expression erkannt werden könnte.

Für die Untersuchung der Expression von miRNAs des C19MC-clusters sowie des 371-3-clusters wurden 85 Plazenten aus drei Subgruppen untersucht und miteinander verglichen: induzierte Aborte (7. - 13 SSW), Spontanaborte (7. – 33. SSW) und Plazenten aus termingerechten Entbindungen (38. - 42. SSW). Zunächst wurden

Termplazenten untersucht. Die Expressionsstärke der fünf untersuchten miRNAs unterschied sich sehr deutlich. Sehr hohe Aktivität wurde für miR-517a-3p (Median RQ = 133200) gemessen, während miRNA 520c-3p (Median RQ = 9182), 371a-3p (Median RQ = 2300) und 372-3p (Median RQ = 2697) eine hohe Aktivität zeigten. Eine geringe Expression war für miR-373-3p messbar (Median RQ = 52.5). Überraschenderweise waren die Expressionen aller fünf untersuchten miRNAs individuell sehr unterschiedlich hoch. Die Expression der miRNAs des miR-371-3- Clusters schwankte bis Faktor143, während die miRNA-Expressionen des C19MC individuelle Schwankungen in geringerer Bandbreite (bis Faktor 20) zeigten. Da die Gewebeproben an zufällig ausgewählten Stellen der Plazenta genommen wurden, wurde in nächsten Schritt überprüft, ob sich systematische Expressionsunterschiede an definierten Probeentnahmestellen nachweisen lassen. Dies konnte nicht bestätigt werden, so dass die Expression tatsächlich individuell unterschiedlich zu sein scheint. Um zu überprüfen, ob sich die starken individuellen Schwankungen auch im peripheren Blut zeigen, wurden außerdem die miRNA mit den stärksten individuellen Schwankungen, miRNA-371a-3p, in vier präpartalen Serumproben gemessen. Hier zeigten sich ebenfalls individuell unterschiedliche Expressionsstärken, die Bandbreite lag bei Faktor 77. Verglichen mit Faktor 143 von miR-371a-3p in Termplazenten ist dies eine geringere, aber noch hohe Schwankung.

Mit Fortschreiten der Schwangerschaft stieg die Expression der untersuchten miRNAs des C19MC-Cluster signifikant an, während bei miRNA-371a-3p und 372-3p eine leichte, aber nicht signifikante Expressionssteigerung beobachtet werden konnte.

Die oben skizzierte Hypothese, dass sich Ursachen für das Abortgeschehen auch anhand aberranter miRNA-Expression in Plazentageweben aus Spontanaborten sichtbar machen lassen würden, ließ sich nicht bestätigen. Die Expression von induzierten- und Spontanaborten unterschied sich nicht signifikant, wenn auch der Median der Spontanaborte niedriger war als bei den induzierten Aborten. Wie bei den Termplazenten wurden auch bei Spontan- und induzierten Aborten deutliche individuell unterschiedliche Expressionsstärken gemessen.

I

The Expression of miRNA Encoded by C19MC and miR-371-3 Strongly Varies Among Individual Placentas but Does Not Differ between Spontaneous and Induced Abortions

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Eigenanteil

- Studiendesign mit Inga Flor und Jörn Bullerdiek
- Durchführung der RNA-Isolierung mit Inga Flor und Lars Kloth
- Durchführung der cDNA-Synthese, der qRT-PCR sowie deren Auswertung mit Inga Flor
- Statistische Auswertung mit Rolf Nimzyk
- Verfassen des Manuskriptes mit Jörn Bullerdiek

The Expression of miRNA Encoded by C19MC and miR-371-3 Strongly Varies Among Individual Placentas but Does Not Differ between Spontaneous and Induced Abortions

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Abstract

Most miRNAs of the largest human miRNA gene cluster at all i.e. C19MC are almost exclusively expressed in the placenta. Nevertheless, only little is known about the interindividual variation of their expression and even about possible influence of gestational age conflicting data is reported. Basically, the same holds true for miRNAs of the much smaller miR-371-3 cluster. Therefore, our present study aims at analyses of the expression of miRNAs from both clusters at different times of pregnancy, possible differences between placenta samples obtained from spontaneous or induced abortions in the first trimester, and the possible variation of miRNA expression at different sites within the same placentas. miR-371a-3p, miR-372-3p, miR-373-3p, miR-517a-3p, and miR-520c-3p were quantified in 85 placental samples and in addition miR-371a-3p was quantified in maternal serum samples taken immediately before delivery. While for miRNA-517a-3p and miR-520c-3p the expression increased significantly with increasing gestational age, the present study also revealed strong interindividual differences in the expression of miR-371-3 cluster in full-term placenta tissue ranging between 111and 15,890 in miR-371a-3p, between 113 and 11,890 in miR-372-3p and between 6 and 713 in miR-373-3p. Interindividual differences were also seen for miRNAs of the C19MC cluster tested, but the levels differed to a much lesser extent than for the former microRNA. Also, strong interindividual differences were noted between the serum samples (range 1 – 77.) As a straightforward explanation, differences related to

the site of the placenta where the sample has been taken from were excluded. For neither of the data from placental tissue the study revealed differences between the spontaneous and induced abortion group. Thus, from our data the interindiviudal differences do not in general seem to be related to first trimester abortion but it remains to be elucidated whether or not they affect other fundamental processes during prenatal life.

Introduction

Chromosome 19 microRNA cluster (C19MC) is primate specific and encodes more than 50 mature miRNAs [1,2]. As to its presumed evolution this large cluster has been emerged in a relatively short period of time from a pre-existing much smaller cluster (miR-371-3) orthologous of which can be detected in all mammalian species (e.g. mmu-mir-290-295 in mice and rno-mir-290-295 in rat) [3,4]. Compared to the size of C19MC little is known about its function and even the functions of the smaller predecessor cluster miR-371-3 encoding only six mature miRNAs have been elucidated not fully so far. The expression of both clusters is limited to only a few tissues mainly of the embryonic and foetal period of life and C19MC, with few exceptions, is almost exclusively expressed in extraembryonic tissues as in particular the placenta [5]. Formerly, it was thought that its expression is even restricted to the trophoblast but then C19MC-derived miRNAs were also detected in the mesenchymal core of chorionic villi and in the mesenchyme of the amniotic membrane [6]. miRNAs of this cluster constitute the major part of the miRNA cargo of placental exosomes suggesting that they serve important functions not only in their cells of origin but also in recipient cells of the released exosomes as in particular maternal NK cells [7-9]. One important mechanism seems to be influencing the immune response against viral infections as demonstrated by Delorme-Axford et al. [10] in a series of experiments. The authors found out that placental trophoblast cells are highly resistant to several virus infections and additionally confer their resistance to other non-placental cells via exosomes containing, among others, specific miRNAs of C19MC. Furthermore, immunomodulatory functions related to the establishment and maintenance of embryo-maternal tolerance have also been assumed to be influenced by these miRNAs [8,9,11]. In addition, there is increasing evidence that contribution to proper implantation and placenta development is another group of functions addressed by the miRNAs of both clusters [12]. Nevertheless, given the variety of miRNAs encoded by C19MC alone as well as the differences of the seed sequences it is tempting to assume that the majority of functions of these miRNAs still remain to be detected. Furthermore, conflicting data exists as for their expression at different time points during pregnancy [13,14]. On the other hand, the question if this variation may be due to different sampling procedures and the position of the sample within the placenta has not been addressed in detail. Therefore, our present study aims at the analyses of the expression of representative miRNAs from both clusters at different times of pregnancy, possible differences between placenta samples obtained from spontaneous or induced abortions in the first trimester, and the possible variation of miRNA expression at different sites within the same placentas. We feel that such study is still missing and its results may help to understand the functions of these miRNAs.

Methods

Tissue samples

Formalin-fixed paraffin-embedded (FFPE) placenta samples of induced and spontaneous abortions (gestational age (GA) week 7-33) were retrieved from the archive and comprised cases from 2010 to 2014. Pathological examinations were performed after haematoxylin and eosin staining of the samples for diagnostic purposes. Full-term placental tissue was collected after timely delivery (estimated date ± two weeks). Placental samples of women suffering from preeclampsia were excluded. In case of full-term placentas a randomly sampled piece of placenta was transferred to 4% buffered formalin immediately after delivery followed by paraffin embedding according to standard techniques.

In addition, six samples of three full-term placentas each were obtained from defined sample sites in two depths (A,B,C: chorion plate; D,E,F about two cm closer to basal plate) to prove if collection site has an influence on expression levels. Amniotic membrane was removed before sampling. Sampling points were: A and D: near umbilical cord, B and E: middle distance between umbilical cord and marginal sinus, C and F: near marginal sinus. Samples were transferred to 4% buffered formalin immediately and further processed to FFPE samples.

In total, FFPE tissue samples from 85 placentas were examined. These comprised of 23 induced abortions (AR), 39 spontaneous abortions (MSA, including missed abortions) and 23 full-term placentas (TE). Three additional full-term placentas were investigated for analysis of possible influences of the collection site.

Serum samples
Serum was collected from women shortly before timely delivery (estimated date ± two weeks). Blood was centrifuged at 2700 x g for 10 min within 30 min after collection. Serum aliquots were transferred into cryotubes and immediately stored at -34 °C for 5 to 30 days. Subsequently, the serum samples were frozen at -80 °C before further processing.

RNA isolation
For RNA isolation, six to twelve tissue sections from FFPE samples of 5 µm each were used. Total RNA isolation was performed using the innuPREP Micro RNA Kit (Analytik Jena AG, Jena, Germany) according to the manufacturer's instructions with the following modifications: Lysis of the paraffin sections preceding RNA isolation was conducted using TLS-Lysis Solution and Proteinase K from the innuPREP DNA Micro Kit (Analytik Jena AG) without prior deparaffinisation. Sections were incubated for 1 h at 60 °C and 15 min at 80 °C.
For RNA isolation from serum samples 200 µl of serum was thawed on ice. Total RNA was extracted using the miRNeasy Mini Kit (Quiagen, Hilden, Germany) according to the manufacturer's instructions with one minor modification: 400 µl of the aqueous phase were mixed with 600 µl of ethanol.

Reverse transcription
To quantify the expression of miR-371a-3p, miR-372-3p, miR-373-3p, miR-517-3p, and miR-520c-3p in FFPE samples and of miR-371a-3p in serum samples, miRNAs were reverse-transcribed into cDNA using TaqMan microRNA RT kit (Applied Biosystems, Darmstadt, Germany). 200 ng of total RNA of each FFPE sample were used for reverse transcription, whereas 55 ng of total serum RNA were used. Specific miRNA stem loop primers (Applied Biosystems, Darmstadt, Germany) were used (assay numbers: 371a-3p: 002124; 372-3p:000560; 373-3p: 000561; 517a-3p: 002402; 520c-3p: 002402; RNU6B: 001093, 20a: 000580). For serum samples, a primer pool consisting of 0.75 µl each of stem loop primer miR-371a-3p and miR-20a-3p (as an endogenous control) was used. For each sample a negative control (-RT) was measured and for each microRNA a non-template control was included. The reactions with a final volume of 15 µl were incubated in a thermal cycler (Biometra TGradient or Biometra Trio-Thermoblock, or, for serum samples, GeneAmp PCR-System 2700 (Applied Biosystems) for 30 min at 16C, 30 min at 42 °C and 5 min at 85 °C, respectively. Samples were stored at 4 °C for immediate qRT-PCR or at -20 °C for later processing.

Pre-amplification of RT-products in serum samples
0.75 µl of miRNA-371a-3p and miRNA-20a-3p assay were diluted in 13.5 µl nuclease-free water. The PCR with a final volume of 50 µl (12.5 µl of this solution, 12.5 µl of RT product, 25 µl TaqMan Universal PCR Master Mix (Applied Biosystems) was performed at 95 °C for 10 min, followed by 14 cycles of 95 °C for 15 s and 60 °C for 4 min using the GeneAmp PCR-System 2700 (Applied Biosystems). The pre-amplification product was diluted 1:5 in nuclease-free water.

Real-time PCR
Real-time PCR was performed using the Applied Biosystems sequence detection system 7300 (Applied Biosystems, Darmstadt, Germany) in 96-well microtiter plates with a total volume of 20 µl. Each reaction of FFPE sample cDNA

consisted of 7.67 µl nuclease-free water, 10 µl TaqMan Universal Mastermix, 1 µl TaqMan microRNA assay and 1.33 µl probe. Each reaction of serum cDNA consisted of 9 µl of the pre-amplification product, 10 µl TaqMan Universal PCR Master Mix and 1 µl TaqMan microRNA assay. For each sample, PCR was performed in triplicate and one negative control was run. Non-template controls of cDNA and PCR of each miRNA were run on every plate. PCR conditions were 50 °C for 2 min, followed by 95 °C for 10 min and 40 (serum) or 50 (FFPE) cycles of 15 s at 95 °C and 1 min at 60 °C.

Relative quantification was performed using the ddCT method [15]. For FFPE samples, a thyroid tumour with 19q13 rearrangement known to overexpress C19MC and miR-371-3 (S958) served as a positive control. Another thyroid tumour without 19q13 rearrangement and therefore with low expression in both miRNA clusters (S925) was used as a calibrator as described in Flor al. [6]. In FFPE samples, RNU6B served as endogenous control for normalization according to Luo et al. [16], Donker et al. [7], Flor et al. [6], Gu et al. [13] and others. In serum samples, miR-20a served as an endogenous control as suggested by Gillis et al. [17].

Statistical analysis

The two-sided Wilcoxon signed rank test was used to compare averages from two groups. The Kruskal-Wallis test was used when more than two groups were compared. Relationships between two observed or measured amounts were quantified by linear regression. A p-value of less than 0.05 was considered being significant, a p-value of less than 0.001 was termed "highly significant". Statistical calculations were done using the R package, version 3.2.3 [18].

Ethics Statement

All samples of induced and spontaneous abortions investigated were initially taken for diagnostic purposes. Samples were de-identified before their use following the rules of the Helsinki declaration. All full-term placentas and serum samples were collected after written informed consent was given by the mother. The study was approved by the local ethics committee (Ärztekammer Bremen, reference number 330).

Results

miRNA expression levels differ within a broad range

In eighty-five placentas, the concentration of representative miRNAs of the two neighbouring clusters C19MC and miR-371-3 was measured. These were: miRNA 371a-3p, 372-3p, 373-3p, 517a-3p, and 520c-3p. The samples belonged to either of three subgroups: 23 were placentas collected after timely delivery (TE) (GA 38-42), 23 were induced abortions (AR) (GA 7-13), and 39 were spontaneous abortions (MSA) (GA 7-33) (S1 Table).

First, we analysed expression levels in full-term placentas, presenting with only a small variety of their GA. Expression was largely different in the investigated miRNAs with low expression of miR-373-3p (median RQ of full-term placentas = 52.5), middle expression of miR-371a-3p (median RQ of full-term placentas: 2,300), miR-372-3p (median RQ of full-term placentas: 2,697), and miR-520c-3p (median RQ of full-term placentas: 9,182), and extremely high expression of miR-517a-3p (median RQ of full-term placentas: 133,200). All five miRNAs analysed showed a wide expression range with the largest amplitude from lowest to highest expression in miR-371-3 and smaller amplitudes in miR-517a-3p and miR-520c-3p, respectively (Table 1). As the next step we analysed the expression levels in induced and spontaneous abortions. The wide expression range observed in full-term placentas was measured in both subgroups of abortions also (Table 1).

Table 1: Relative quantification of miRNA expression (fold change) in subsets of placenta samples

miRNA	Type of abortion or delivery			
	All	AR	MSA	TE
371a-3p	n=85	n=23	n=39	n=23
median	1521	1774	1193	2300
mean	2436	2413	1636	3814
SD	3002	3006	1832	4056
range	52.9-15980	140.7-14900	52.9-10720	111.7-15980
n fold max/min	302	106	203	143
372-3p	n=85	n=23	n=39	n=23
median	1334	1156	984	2697
mean	2013	1631	1448	3354
SD	2404	2022	1762	3164
range	45.2-11890	92.9-10020	45.2-10450	113.4-11890
n fold max/min	263	108	231	105
373-3p	n=84	n=22	n=39	n=23
median	33.28	37.2	29.8	52.5
mean	64.6	51.4	35.6	126.4
SD	104	60	35	173
range	1.8-713.9	3.2-299.7	1.8-218.1	6.3-713.9
n fold max/min	397	93	124	113
517a-3p	n=65	n=23	n=19	n=23
median	52080	36800	44850	133200
mean	94800	39330	54910	183200
SD	115809	27281	42704	154979
range	1940-481300	2250-119400	1940-160400	25790-481300
n fold max/min	248	53	83	19
520c-3p	n=84	n=22	n=39	n=23
median	4540	4472	3224	9182
mean	7387	5149	4111	15080
SD	9260	3045	2596	14734
range	285.6-49960	1664-13840	285.6-13060	2453-49960
n fold max/min	175	8	46	20

AR: induced abortion; MSA: spontaneous abortion; TE: delivery at term +-2 weeks; SD: standard deviation; n fold max/min: ratio of highest expression divided by lowest expression

Expression levels are independent of sampling site

To check how expression varies between different sampling sites and therefore the broad range in expression might be determined by sampling site, in three additional full-term placentas six samples each were taken at defined sites. Measurement of all five miRNAs did not show an influence of the collection site but individual differences in expression between the three placentas (Fig 1, S1 Fig). No differences in expression between means of the six sampling sites and 23 randomly sampled full-term placentas were detected by Wilcoxon signed rank test (miR-371a-3p, p=0.7046; miR-372-3p, p=1; miR-373-3p, p=0.7046; miR-517a-3p, p=0.4415; miR-520c-3p, p=0.0785).
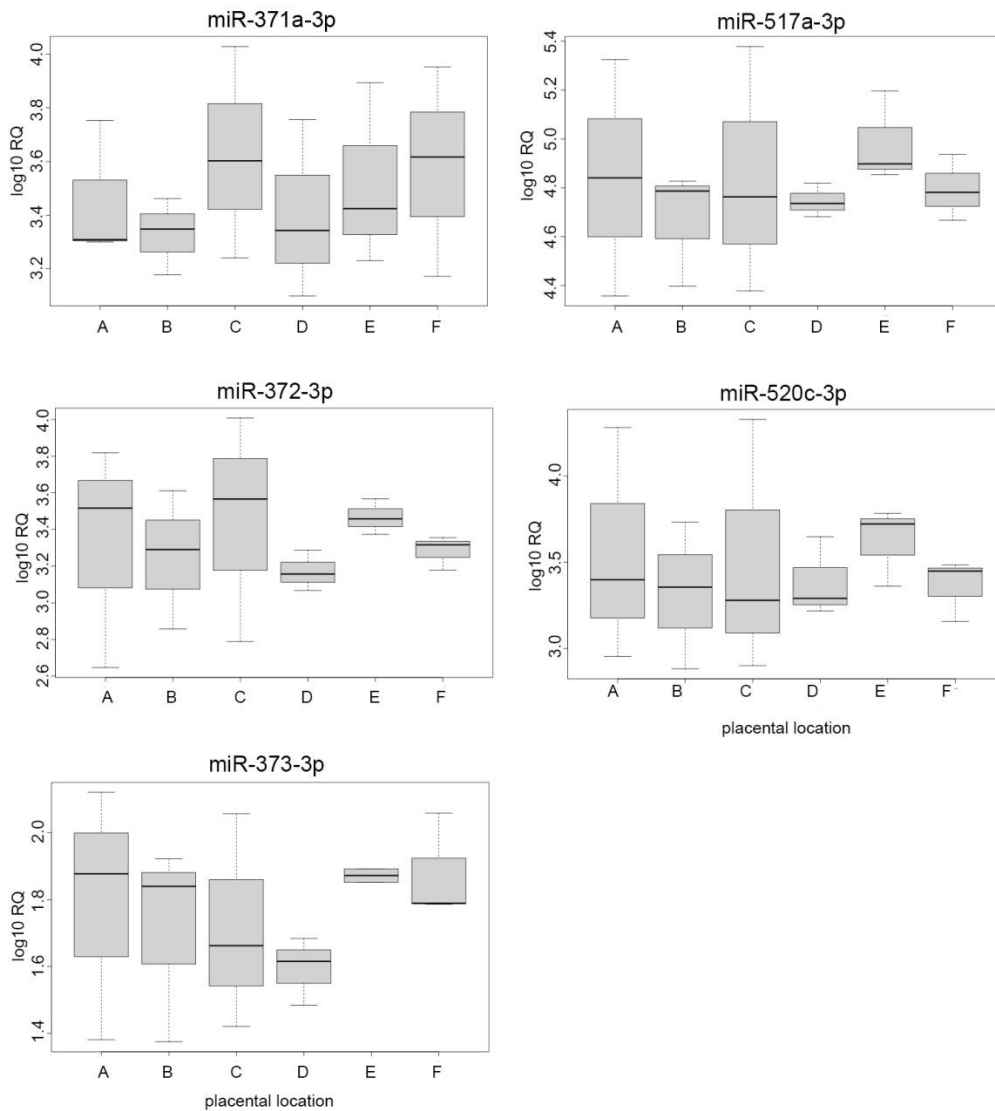
Fig 1: Boxplots showing miRNA expression in six sampling sites of three placentas
A= chorion plate; near umbilical cord, B= chorion plate; middle distance between umbilical cord and marginal sinus, C= chorion plate; near marginal sinus, D= 2 cm closer to basal plate, near umbilical cord, E= 2 cm closer to basal plate, middle distance between umbilical cord and marginal sinus, F= 2 cm closer to basal plate, near marginal sinus. Kruskal-Wallis analysis of variance showed no significant differences in expression between six sampling sites (miR-371a-3p: $p= 0.9369$, miR-372-3p: $p=0.6265$, miR-373-3p: $p=0.6788$, miR-517a-3p: $p=0.6159$, miR-520c-3p: $p=0.8767$). Boxes contain the central 50% of values, lines inside boxes denote the median, whiskers extend to the extreme values or to 1.5 * box height, whatever is smaller.

Expression of miR-517a-3p and miR-520c-3p increases significantly with the progression of pregnancy

Concerning the change in expression from first to third trimester, conflicting results have been published. Morales-Prieto et al. [14] found increasing expression in miRNAs of the C19MC cluster in first and third trimester trophoblast cells whereas no significant changes were observed in miR-371-3. On the other hand, Gu et al. [13] found decreasing expression of miR-371-3 and miR-520c and unchanged expression of miR-

517a in first compared to third trimester placental tissue. To check if and how expression in placental tissue changes with progression of pregnancy we next analysed expression patterns of the above mentioned miRNAs as a function of GA. Analysis focused on gestational week without stratification in induced and spontaneous abortion.

Due to multiple testing, a Bonferroni correction was made resulting in α = 0.01. Linear regression analysis revealed a highly significant increase from first to third trimester of miR-517a-3p (p=0.000003, fold change: 3.9) and miR-520c-3p (p=0.000001, fold change: 3.4), whereas no significant differences in expression could be observed in different stages of pregnancy in miR-371a-3p (p=0.3905, fold change: 2.0); miR-372-3p (p=0.1474, fold change: 2.2) and miR-373-3p (p=0.018, fold change:3.0) (Fig 2).
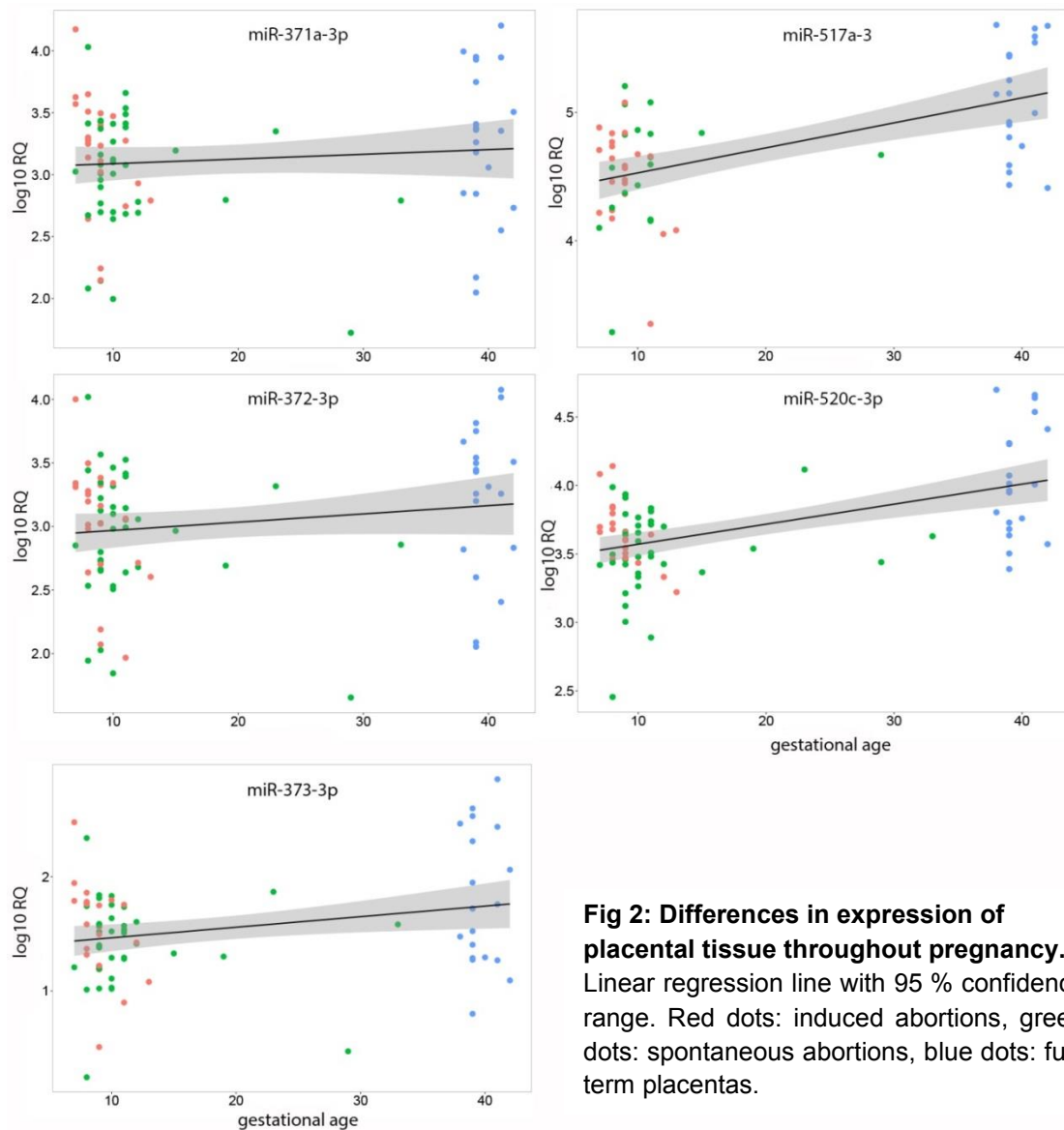

Fig 2: Differences in expression of placental tissue throughout pregnancy. Linear regression line with 95 % confidence range. Red dots: induced abortions, green dots: spontaneous abortions, blue dots: full-term placentas.

No differences in expression of induced and spontaneous first trimester abortions were observed

Since miRNAs of the C19MC cluster play a role in viral resistance [10] and are also suspected to have immunomodulatory functions [11] and since the miR-371-3 cluster is suspected to be involved in foeto-maternal crosstalk as well [19,20] we were interested to see if variations in expression of these miRNAs might partly explain miscarriage events. We therefore analysed expression levels of induced and spontaneous first trimester abortions separately. Wilcoxon signed-rank test showed no significant differences in expression of induced compared to spontaneous first trimester abortions in each of the investigated miRNAs (Table 2).

Table 2: Expression levels of miRNAs in placenta tissue of induced and first trimester spontaneous abortions.

miRNA	AR		MSA (first trimester)		p-value
	n	Median RQ ± SD	n	Median RQ ± SD	
371a-3p	22	1827 ± 2981	33	1193 ± 1925	0.1734
372-3p	22	1305 ± 2000	33	1060 ± 1855	0.7015
373-3p	22	37 ± 59	33	32 ± 37	0.1963
517a-3p	22	37610 ± 26530	19	38240 ± 44192	0.7814
520c-3p	21	4567 ± 3008	33	3131 ± 2266	0.1108

Differences were analysed by the Wilcoxon signed-rank test. A p-value of <0.05 was considered as significant, a p-value of <0.001 was considered as highly significant. AR= induced abortion, MSA=spontaneous abortion, RQ= relative quantification, SD= standard deviation

miRNA concentration in serum samples differs interindividually

The unexpected finding of the wide expression range in placentas prompted us to see, if this could be observed in serum as well. Therefore, concentration of miR-371a-3p, the miRNA showing the widest expression range in full-term placental FFPEs, was measured in four serum samples taken shortly prior to timely delivery. Also in these samples, concentration differed individually, though in a range as half as wide compared to FFPE. The sample displaying the highest CT served as a calibrator. The other three samples showed RQ-levels of 14.6, 21.8 and 77.3, respectively.

Discussion

C19MC is nearly exclusively expressed in the placenta [1,16], suggesting an important role in placental development and function. Specific function and regulation of C19MC in the placenta is still not completely clear (reviewed by [21]), but three functional complexes are likely to be influenced by C19MC. These are: viral resistance [10], implantation and placenta development [12,22,23] and immunomodulation in terms of foetal-maternal crosstalk [8,9,11].

This is, to our knowledge, the first report investigating expression of C19MC miRNAs and miR-371-3 cluster in placental tissue comparing three groups (induced and spontaneous abortions and full-term placentas) in a large cohort. MiR-517a-3p and miR-520c-3p represented the placenta specific C19MC. The miR-371-3 cluster was chosen because of its suspected role in stem cell maintenance [24] and cell differentiation [25], pointing at its possible role in trophoblast differentiation.

Overall, individual expression levels differed remarkably within each group (induced and spontaneous abortions and full-term placentas) in each of the five miRNAs investigated. In contrast,

Gu et al. [13] found relatively homogenous expression in six first and six third trimester placenta tissue using microarray analysis. Our finding prompted us to examine if expression levels are dependent on sampling site. Analysis revealed that though expression differs within one placenta, it does not show relation to sampling site. Expression levels therefore seem to vary individually. This finding is partly in line with Wyatt (2005) who found no association between placental sampling site and expression of some hypoxia-related genes (NDRG1, adipophilin and human placental lactogen) whereas others were differently expressed. By investigating the placental transcriptome Sood et al. [26] found interindividual differences in expression patterns of diverse genes as well.

The expression of C19MC, C14MC and the miR-371-3 cluster was examined by Morales-Prieto et al. [14] in first and third trimester trophoblast cells. The authors found tenfold change in miR-520c and about 20 fold increase in miR-517a. Our data confirm this finding, but increase was lower. Since we investigated placental tissue instead of cell-lines our findings might mirror the in vivo situation more accurately. In concordance with Morales-Prieto et al. [14], we did not find significant change in expression of miR-371-3 cluster throughout pregnancy. Increase of C19MC expression was also observed in peripheral natural killer cells (NK) of pregnant women in third compared to first trimester [9] with a rapid decline post-delivery [8,9]. In contrast, Gu et al. [13] observed a downregulation of miR-371-3 and miR-520c in placental tissue of third compared to first trimester. We have no straightforward explanation but this conflicting data might partly be due to small sample size.

Xie et al. [12] found higher expression of C19MC in villous trophoblasts than in extravillous trophoblast suggesting a role in migration of extravillous trophoblast. Increasing expression in extravillous trophoblast results in decreasing invasion. Since trophoblast invasion usually takes place during early pregnancy, but can be observed in second trimester pregnancies as well [27], increasing levels of C19MC might restrict invasion as pregnancy progresses and might therefore serve as protection against deep invasion of the trophoblast leading to placenta accreta. According to Umemura et al. [28], trophoblastic cells of placenta accreta and especially of placenta increta and percreta show aggressive invasion into the myometrium. The authors found miR-34a, a miRNA inversely associated with invasiveness and metastasis, downregulated in placenta accreta. Regulation of trophoblast invasion is a complex process which is tightly regulated by an interaction of diverse factors (reviewed in [29]). For example, the suppression of trophoblast invasion by β-1,4-galactosyltransferase III (B4GALT3) was investigated by Liao et al. [30]. The authors showed increased expression of B4GALT3 in third trimester extravillous trophoblasts implicating a role in invasion control. Nevertheless, the potential role of miRNAs of the C19MC cluster in formation of placenta accreta remains to be elucidated.

A recent study revealed different expression of miRNAs of the miR-371-3 cluster and C19MC cluster in women suffering from recurrent miscarriage (RM) [20]. Our hypothesis when starting the study was that miR-371-3, and C19MC might play a role in miscarriage events, but we did not find significant differences in expression of any of the miRNAs investigated between induced and spontaneous abortions. Wang et al. [20] focused on women with RM and compared expression in decidua and in villous trophoblasts. The authors found miR-517a-3p upregulated in decidua, whereas miR-371a-5p and miR-372 were downregulated in villi of RM patients compared to induced abortions of normal pregnancies. While the authors did exclude parental chromosomal alterations as a cause of RM, one might speculate if aberrant expression of these miRNAs e.g. due to paternal deregulation can indicate a general problem of proper placenta development in these couples. From our data, this question cannot be answered because we do not know in which cases of the abortions had occurred more than once.

Moreover, since we investigated whole FFPE without microdissection and therefore cannot differentiate between the expression of decidua and villi, results might not be comparable. Furthermore, information about RM in our spontaneous abortion group was not available.

Nevertheless, taken together, the miRNAs investigated in our study are not likely to play an important role in miscarriage events in general since we did not find significant differences in expression of induced and spontaneous abortions.

Luo et al. [16] first found miRNAs of C19MC in exosomes released from trophoblast cells. Donker et al [7] showed that C19MC miRNAs are even predominantly present in these exosomes. These findings suggested a possible use in monitoring of pregnancy. So far, studies have found that circulating miRNAs of C19MC can indeed serve as biomarkers in preeclampsia, foetal growth restriction, and hypertension [31-35]. Compared to the broad range in expression of all miRNAs investigated in placental FFPE, individual variation in maternal serum was still high, but nevertheless half as broad. There are diverse results concerning the correlation of miRNA levels in placenta and serum. Higashijma et al. [33] found different expression of C19MC miRNAs in placentas of foetal growth restriction (FGR) compared to normal pregnancies but no differences in serum levels between these groups. On the other hand, Mouillet et al. [31] found inverse correlation of placental and serum miRNA levels in FGR pregnancies. Williams et al [36] investigated placental tissue and plasma probes of mother-child pairs. Some miRNAs of C19MC were more abundant in maternal and foetal plasma than in corresponding placenta, some showed reverse values and others, e.g. miR-517a were highly expressed in placenta and plasma of both mother and foetus. However, heterogeneity of serum concentration correlated with corresponding placenta samples in Williams´ data.

While for the miRNAs of C19MC tested the expression increased with increasing gestational age, the present study revealed strong interindividual differences in the expression of the miR-371-3 cluster in placenta tissue. Differences were also seen for the miRNAs of the C19MC cluster tested, but the levels differed to a much lesser extent than for the former microRNA. As a straightforward explanation, differences related to the site of the placenta where the sample has been taken from were excluded. From our data these differences do not in general seem to be related to first trimester abortion but it remains to be elucidated whether or not they affect other fundamental processes during prenatal life.

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Supporting Information

S1 Tab.: Overview of all placenta samples used for expression studies

| Case	Type of delivery/ abortion	GA	Trim	RQ miR-371a-3p	RQ miR-372-3p	RQ miR-373-3p	RQ miR-517a-3p	RQ miR-520c-3p	maternal age	new born sex	new born weight	c section
PT002	TE	39	3	1511,467	1810,874	19,396	38803,43	4317,67	30	m	3155	no
PT003	TE	42	3	539,296	680,204	12,353	25786,5	3725,896	30	m	4250	yes
PT004	TE	39	3	111,696	122,93	18,744	27221,826	3186,471	41	m	3435	yes
PT007	TE	40	3	1141,731	2060,242	19,663	54704,406	5747,842	32	f	3060	yes
PT008	TE	39	3	147,883	113,4	6,298	34128,742	2452,904	26	m	3760	yes
PT010	TE	39	3	2557,233	3477,939	88,837	280705,469	20038,373	37	m	3430	yes
PT011	TE	38	3	708,449	660,254	29,907	138829,297	6369,422	31	f	2855	yes
PT018	TE	41	3	354,823	255,263	18,526	98839,766	10152,48	30	m	3310	yes
PT020	TE	41	3	8846,765	10388,873	273,348	350839,625	43584,906	35	f	3970	no
PT027	TE	39	3	700,622	398,75	25,369	80128,172	9182,019	20	f	3110	no
PT034	TE	41	3	2258,726	1810,311	57,315	391268,344	45813,23	32	f	3880	yes
PT036	TE	38	3	9872,229	4646,313	291,615	481344,719	49961,301	30	m	3220	yes
PT043	TE	39	3	2299,906	3146,983	52,742	178134,078	11808,915	26	m	3190	yes
PT045	TE	39	3	5594,139	2696,786	204,729	84244,406	4801,711	25	m	3300	no
PT046	TE	39	3	8925,931	5624,016	339,818	273056,094	10298,127	31	f	3330	no
PT047	TE	39	3	8492,016	6513,779	396,427	280698,063	20369,65	30	m	3090	no
PT048	TE	41	3	15982,176	11890,08	713,925	451713,781	34398,973	35	f	3510	no
PT050	TE	42	3	3206,956	3225,013	115,105	473529,281	25771,98	32	m	3510	no
PT051	TE	39	3	2394,598	2789,313	52,484	140909,016	8887,091	24	m	3435	yes
PT052	TE	39	3	1826,228	1585,193	33,371	63970,762	5354,211	29	m	3440	no
PT053	TE	NA	3	1911,014	1756,505	28,164	52083,992	3982,805	32	m	3370	yes
PT054	TE	NA	3	5301,91	6989,457	66,881	133186,156	10308,977	30	f	2685	yes
PT055	TE	NA	3	3047,378	4492,803	42,483	79634,242	6355,147	30	m	2700	yes
P002	MSA	8	1	120,404	88,186	1,752	1939,673	285,573	NA	-	-	-
P004	MSA	10	1	498,369	321,769	10,419	NA	2149,997	NA	-	-	-
P005	MSA	11	1	4564,57	3349,706	54,396	119847,815	6820,025	NA	-	-	-
P006	MSA	9	1	793,235	449,221	16,395	115832,922	2935,668	NA	-	-	-
P007	MSA	9	1	907,353	531,295	10,494	160431,234	4601,139	NA	-	-	-
P008	AR	9	1	3130,573	2120,485	24,506	119406,227	4569,093	NA	-	-	-
P009	MSA	9	1	2667,709	2220,745	25,142	67133,719	2884,4	NA	-	-	-
P010	MSA	9	1	1022,165	544,788	15,457	NA	2649,643	NA	-	-	-
P011	MSA	11	1	2413,809	1397,377	34,479	NA	3027,16	NA	-	-	-
P012	MSA	10	1	2570,865	1422,844	33,191	72997,406	2279,053	NA	-	-	-
P013	AR	7	1	3717,387	2193,634	61,404	76407,469	12106,528	NA	-	-	-
P014	MSA	10	1	438,205	339,933	12,826	NA	3011,283	NA	-	-	-
P015	MSA	15	2	1563,602	924,276	21,288	69190,078	2324,708	NA	-	-	-
P016	MSA	11	1	3073,325	2604,838	33,711	14622,547	5184,022	NA	-	-	-
P017	AR	8	1	4456,751	1898,603	60,274	68830,789	5280,65	NA	-	-	-

Case	Type of delivery/ abortion	GA	Trim	RQ miR-371a-3p	RQ miR-372-3p	RQ miR-373-3p	RQ miR-517a-3p	RQ miR-520c-3p	maternal age	new born sex	new born weight	C section
P018	MSA	11	1	2588,752	2478,752	32,214	39349,148	6464,33	NA	-	-	-
P021	MSA	29	3	52,859	45,209	2,957	46662,977	2753,703	NA	-	-	-
P022	MSA	NA	NA	1521,226	1813,32	29,771	89609,055	4347,249	NA	-	-	-
P023	MSA	8	1	2588,296	2766,138	55,267	37131,012	3131,252	NA	-	-	-
P024	AR	9	1	2498,31	2414,888	56,077	69362,266	8502,312	NA	-	-	-
P025	AR	8	1	3223,683	3141,247	72,408	43846,836	7008,713	NA	-	-	-
P026	MSA	11	1	482,114	435,841	19,088	14276,58	776,273	NA	-	-	-
P027	MSA	12	1	602,62	1140,819	25,927	NA	2666,176	NA	-	-	-
P028	MSA	9	1	1201,687	1060,493	32,898	NA	4383,012	NA	-	-	-
P029	AR	8	1	1773,681	1788,088	59,094	58817,27	6244,989	NA	-	-	-
P030	AR	9	1	1288,466	1052,003	36,122	36795,434	3197,762	NA	-	-	-
P031	MSA	9	1	2730,038	3682,483	68,947	NA	8622,758	NA	-	-	-
P032	MSA	8	1	10718,678	10445,96	218,066	NA	9723,083	NA	-	-	-
P033	MSA	19	2	624,26	492,353	19,99	NA	3455,765	NA	-	-	-
P034	MSA	23	2	2236,838	2073,991	73,678	NA	13064,995	NA	-	-	-
P035	AR	9	1	174,756	155,142	15,466	38425,449	3974,972	NA	-	-	-
P036	MSA	9	1	138,704	106,487	15,655	NA	1010,242	NA	-	-	-
P037	MSA	11	1	3442,6	983,752	37,142	68082,688	5473,248	NA	-	-	-
P038	MSA	12	1	492,317	479,778	40,174	NA	5008,378	NA	-	-	-
P039	MSA	10	1	1330,544	1256,059	56,709	NA	5089,508	NA	-	-	-
P041	MSA	33	3	617,354	719,583	38,232	NA	4258,204	NA	-	-	-
P042	MSA	10	1	99,074	70,061	10,671	NA	1833,185	NA	-	-	-
P043	MSA	9	1	497,814	461,263	37,778	NA	1319,172	NA	-	-	-
P044	AR	8	1	439,618	435,412	23,374	54410,086	6857,538	NA	-	-	-
P045	MSA	9	1	585,635	629,713	23,924	NA	8129,214	NA	-	-	-
P046	MSA	10	1	1259,319	2905,706	43,277	27061,875	3922,322	NA	-	-	-
P047	MSA	10	1	1846,303	2082,067	67,839	NA	5838,539	NA	-	-	-
P048	MSA	10	1	1016,201	958,594	19,509	NA	4513,697	NA	-	-	-
P050	MSA	9	1	1448,631	1333,603	38,649	NA	6177,616	NA	-	-	-
P054	AR	NA	NA	629,618	258,074	NA	10178,404	3044,674	NA	-	-	-
P066	AR	7	1	4227,068	2046,045	87,95	16519,908	4979,657	NA	-	-	-
P068	AR	9	1	1052,175	503,907	16,654	29882,613	2954,046	NA	-	-	-
P072	AR	8	1	1374,147	955,776	20,733	14918,381	2904,694	NA	-	-	-
P095	AR	9	1	1711,788	1453,697	31,28	23147,205	4057,633	NA	-	-	-
P096	AR	9	1	140,654	118,193	3,224	28147,623	3490,873	NA	-	-	-
P097	AR	13	1	617,384	401,853	11,988	12074,559	1663,995	NA	-	-	-
P098	AR	12	1	850,4	517,938	26,573	11281,497	2148,42	NA	-	-	-
P099	AR	11	1	555,307	92,927	7,911	2249,735	NA	NA	-	-	-
P100	AR	8	1	1894,015	1032,432	57,642	17300,973	4779,673	NA	-	-	-
P101	AR	7	1	14901,793	10017,551	299,676	50985,059	4567,033	NA	-	-	-
P105	MSA	9	1	2345,833	1670,094	64,917	23641,512	1630,648	NA	-	-	-
P106	AR	10	1	2966,031	2190,918	62,35	47287,43	2718,263	NA	-	-	-

Case	Type of delivery/ abortion	GA	Trim	RQ miR-371a-3p	RQ miR-372-3p	RQ miR-373-3p	RQ miR-517a-3p	RQ miR-520c-3p	maternal age	new born sex	new born weight	C section
P108	MSA	11	1	1193,056	1132,888	19,584	44848,383	3224,046	NA	-	-	-
P109	AR	8	1	1996,592	1573,007	38,287	28853,424	13839,526	NA	-	-	-
P110	MSA	8	1	469,841	341,736	10,262	18114,381	2736,13	NA	-	-	-
P111	AR	11	1	1880,475	1156,222	56,918	45542,535	4377,629	NA	-	-	-
P112	MSA	7	1	1057,443	709,388	16,093	12605,536	2626,771	NA	-	-	-

AR: induced abortion; MSA:spontaneous abortion; TE: delivery at term +-2 weeks; GA:calendar gestastional age in weeks; NA:information not available; Trim:Trimester of pregnancy; RQ:relative quantification; -: not applicable; C section: Caesarean section
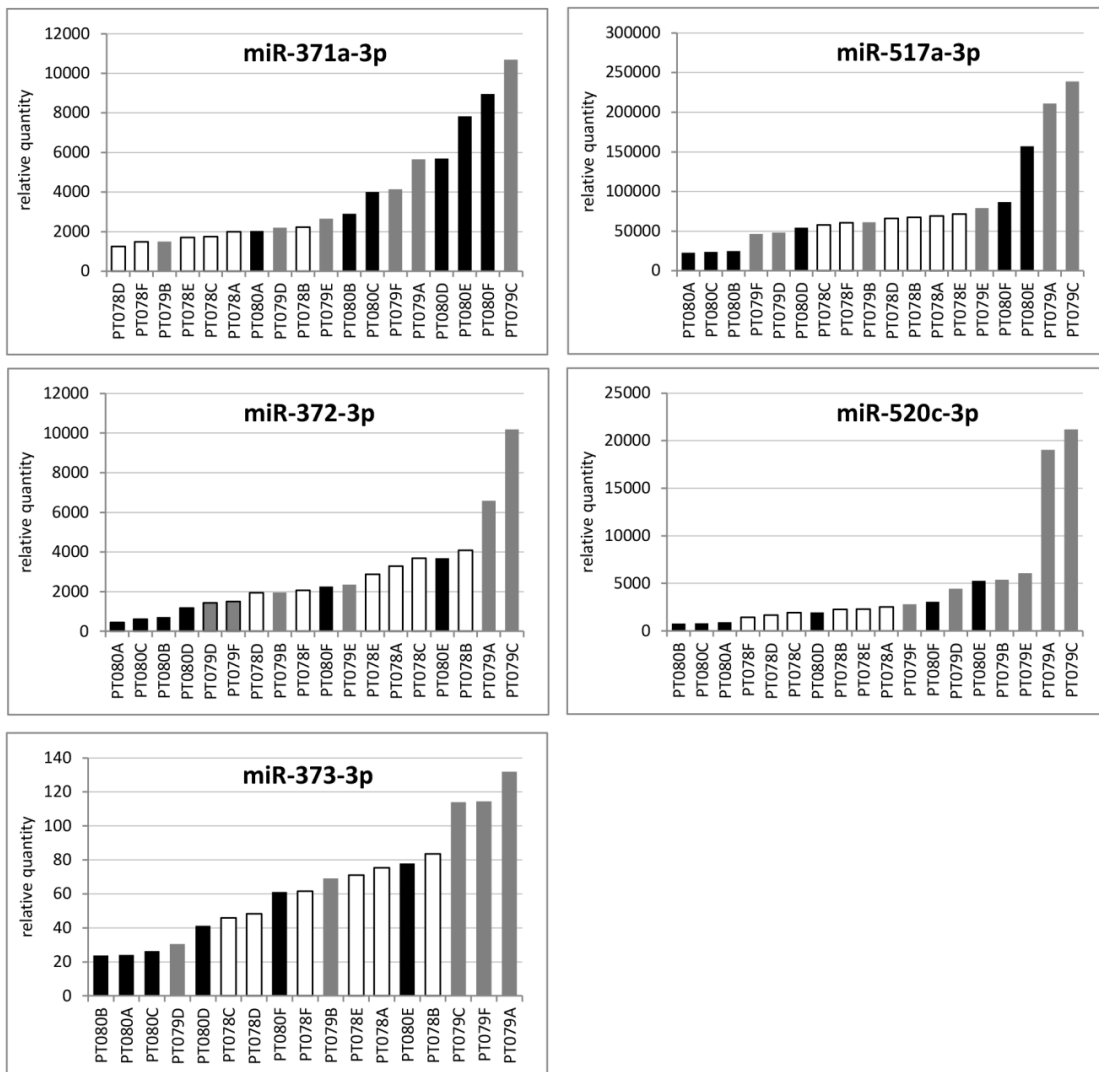
S1 Fig: miRNA expression in six sampling sites of three placentas.
White columns: samples PT078A-F, grey columns: samples PT079A-F, black columns: samples PT080A-F. A= chorion plate; near umbilical cord, B= chorion plate; middle distance between umbilical cord and marginal sinus, C= chorion plate; near marginal sinus, D= 2 cm closer to basal plate, near umbilical cord, E= 2 cm closer to basal plate, middle distance between umbilical cord and marginal sinus, F= 2 cm closer to basal plate, near marginal sinus.

3.2 Publikation II:
Quantitative und qualitative Analyse von *HMGA2*-Expression in Plazenten

Neben den untersuchten miRNAs ist auch *HMGA2* ein während der embryonalen Entwicklung hoch exprimiertes Gen. Über seine Expression in Plazenten gibt es nur wenige Studien (Rogalla et al. 1996, Gattas et al. 1999, Genbacev et al. 2016). Zum Expressionsverlauf über die gesamte Schwangerschaft in Gewebeproben gibt es bislang keine Daten. Daher war das Ziel der Untersuchung die genauere Beschreibung des räumlich-zeitlichen Expressionsmusters in Plazenten unterschiedlicher Schwangerschaftswochen. Darüber hinaus wurden auch für Publikation II Plazenten induzierter und spontaner Aborte im Hinblick auf Unterschiede in der *HMGA2*-Expression untersucht. Hierfür wurden 89 Plazenten aus drei Subgruppen mit Hilfe der qRT-PCR untersucht. Die Subgruppen umfassten 19 induzierte Aborte (5. – 20. SSW), 63 Spontanaborte (5. – 32. SSW) und 4 Plazenten, die nach Lebendgeburten gesammelt wurden (32. – 41. SSW). Bei drei Abortproben konnte nicht ermittelt werden, ob sie spontan oder induziert waren. Es zeigte sich, wie vorab als Hypothese formuliert, dass die *HMGA2*-Expression mit steigender Schwangerschaftswoche abnahm. Im ersten Schwangerschaftstrimester waren signifikant höhere *HMGA2*-Expressionen zu messen als im zweiten und dritten Trimester. Ab Mitte des zweiten Trimesters war nur noch eine bis zur Geburt zu beobachtende Basisexpression messbar. Dies galt gleichermaßen für induzierte- wie für Spontanaborte. Signifikante Expressionsunterschiede zwischen induzierten und Spontanaborten wurden für *HMGA2* nicht gefunden.

Ein weiteres Ergebnis dieser Untersuchung war, dass im mütterlichen Teil der Plazenta kein *HMGA2* exprimiert wird. Dies zeigte sich an Proben, die einen hohen Anteil an mütterlichem Gewebe enthielten, bereits in der qRT-PCR. In diesen Fällen war keine bzw. eine für die Schwangerschaftswoche ungewöhnlich geringe Expression detektierbar. Die in der qRT-PCR gewonnenen Ergebnisse wurden mittels Immunhistochemie überprüft und bestätigt. In der Immunhistochemie zeigte sich außerdem, dass HMGA2 im Wesentlichen in den Zellkernen des Zottenstromas detektierbar ist. In den Trophoblastzellen war HMGA2, in etwas niedrigerer Intensität, nur im Zytoplasma nachweisbar.

II

Expression of HMGA2 in fetal placenta correlates with gestational age

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Eigenanteil:

- Histologische Beurteilung der Plazenten mit Burkhard Helmke
- Beurteilung der immunhistochemischen Färbung mit Burkhard Helmke
- Überarbeitung des Manuskriptes mit Jörn Bullerdiek

Expression of *HMGA2* in fetal placenta correlates with gestational age

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Keywords: placenta, HMGA2, gestational age, development, abortion

Abstract

Background

High-mobility group AT-hook 2 (HMGA2) expression can be detected in many embryonic and fetal tissues but becomes down-regulated during postnatal life except for many benign and malignant tumors. In the latter case, its expression has been correlated with epithelial-mesenchymal transition and invasive growth. The placenta contributes essentially to proper development of the embryo and the fetus. In a tumor-like manner it shows rapid invasive growth during the first weeks of gestation. To address the possible role of HMGA2 during placental development, we have measured its expression throughout the prenatal period from week five to 41 by mRNA quantification as well as by immunohistochemistry.

Methods

Expression of *HMGA2* and *HPRT* was measured on 89 embryonal, fetal and full-term placentas, encompassing calendar gestational age of five to 41 weeks, using quantitative

real time-PCR. In eleven cases, in addition immunohistochemistry was used to determine the localization of HMGA2 and to check data obtained by quantitative real time-PCR.

Results

The expression of *HMGA2* was found to be inversely correlated with gestational age (*p* < 0.001). For the better part of the first trimester the level of *HMGA2* is high, after that the expression shows a decline down to a baseline level, where it remains until the birth. HMGA2 protein was mainly detected in the nuclei of the stromal cells in the placental villi.

Conclusions

During pregnancy, the expression of *HMGA2* follows a non-linear pattern of decrease. In the first trimester, from two to three weeks after the implantation of the conceptus until the blood supply is established, the expression is high, indicating a critical role in the early development of this organ and in the control of its invasive behavior.

Background

High-mobility group AT-hook protein 2 belongs to a family of non-histone chromatin proteins, encoded by two genes, *HMGA1* and *HMGA2*. Due to alternative splicing, there are four known proteins (HMGA1a, HMGA1b, HMGA1c, and HMGA2) (reviewed in [1]). All HMGA-proteins are architectural transcription factors and contain three DNA-binding domains, called AT-hooks, and an acidic carboxy-terminal tail. As such, they do not have an intrinsic transcription factor capacity, but rather enhance or silence transcription through a change in chromatin structure and interaction with nuclear proteins (reviewed in [2, 3]). They play a key role in stem cell renewal, growth and development of tissues and the differentiation of cells [4-6]. As to *HMGA2*, its expression is not detectable in most adult tissues and cells [7-10], with few exceptions as e.g. some types of mesenchymal stem cells [11], spermatocytes and spermatids in testis [12]. *HMGA* reactivation in adult tissue was first reported in HeLa cells [13]. Since then, the overexpression of *HMGA1* and *HMGA2* was detected in numerous types of cancers [reviewed in [1, 14, 15] as well as in a variety of benign tumors [16, 17].

The placenta is the only normal human organ infiltrating surrounding tissue, albeit, as a rule, in a tightly restricted manner. It serves as the connection between the mother and the embryo or fetus, providing nutrients and oxygen for the developing child. Additionally, it removes waste products from the fetus and forms a maternal-fetal barrier, protecting the fetus from infectious diseases. About six days after fertilization, the blastocyst starts implantation into the maternal uterus and placentation. Five weeks after conception the basic structure of the placenta has formed. To support the developing embryo and fetus, it continues to grow throughout gestation.

The detection of *HMGA2* expression in the placenta dates back to 1996 [8]. To detect *HMGA2* mRNA, several embryonal/fetal tissues of a gestational age between eight to twelve weeks and samples of the maternal and the fetal part of a placenta (36th weeks of pregnancy) were analyzed by RT-PCR. The experiments yielded positive results for the embryo, but negative findings for the placenta. In an investigation by Hirning-Folz *et al*. [10], RNA in situ hybridization was used on mouse embryo sections for detection of *HMGA2* mRNA. Fetal placenta showed reduced expression compared to nearly all parts of the embryo at a developmental stage of 9.5. *HMGA2* mRNA was also detected by RT-PCR, but not by northern blot analysis in three human fetuses of 19 to 22 weeks estimated gestational age by Gattas *et al*. [18]. Genbacev *et al*. [19] identified the chorionic mesoderm as a niche for human trophoblastic progenitor cells that support placental growth. *HMGA2* was found to be one of the factors associated with the self-renewal or differentiation of these cells.

The aim of the present paper was to determine if there is a temporally and spatial expression pattern of *HMGA2* in the human placenta. In case of a correlation between the pattern and the invasive growth of the placenta, this would provide insights into the role of *HMGA2* in the development of this organ.

Methods

Tissue specimens and RNA isolation

Formalin-fixed paraffin-embedded (FFPE) tissue samples were collected at the Institute for Pathology, Elbe Clinic Stade-Buxtehude, Germany. Pathological examinations were performed after haematoxylin and eosin staining of the samples. Six to eight sections of 5 µm for each sample were used to for isolation of total RNA. Isolations were performed using the innuPREP Micro RNA Kit (Analytik Jena AG, Jena, Germany) according to the manufacturer's instructions with the following modifications: Lysis of the paraffin sections preceding RNA isolation was conducted using TLS-Lysis Solution and Proteinase K from the innuPREP DNA Micro Kit (Analytik Jena AG) without prior deparaffinization. Sections were incubated for 1 h at 60°C and 15 min at 80°C.

cDNA-synthesis and quantitative real-time RT-PCR

RNAs were reverse-transcribed into cDNA by M-MLV Reverse Transcriptase (Invitrogen, Karlsruhe, Germany). Quantitative real-time PCR (qRT-PCR) was performed using the Applied Biosystems 7300 sequence detection system according to Taq-Man Gene Expression Assay Protocol (Applied Biosystems, Darmstadt, Germany) in 96-well microtiter plates with a total volume of 20 µl. In the case of the TaqMan gene expression assay for *HMGA2* (assay number Hs00171569, Applied Biosystems, Foster City, USA), each reaction consisted of 2 µl of cDNA reverse transcribed from 25 ng of total RNA, 10 µl of TaqMan Universal PCR Master Mix (Applied Biosystems), 1 µl of TaqMan assay, and 7 µl of ddH$_2$O. For the *HPRT* assay, using HPRT FP and HPRT RP primers [20], each reaction consisted of 2 µl of cDNA reverse transcribed from 25 ng of total RNA, 10 µl of TaqMan Universal PCR Master Mix, 600 nM (1.2 µl) of forward and reverse primers, and 200 nM (0.2 µl) of probe [20] and 5.4 µl of ddH$_2$O. Thermal cycling conditions were 2 min at 50°C followed by 10 min at 95°C, 50 cycles at 95°C for 15 s and 60°C for 1 min. For each sample, a negative control of the previous cDNA synthesis (missing reverse transcriptase) and for each plate a non-template control of amplification and a non-template control of previous cDNA synthesis were included in each run. Sequence Detection Software 1.2.3 (Applied Biosystems) was used to analyze data. All testing reactions were performed in triplicate. *HPRT* was used as endogenous control, as it has previously been shown to be stably expressed in human fetal placenta [21-23]. As recommended for FFPE samples [24] the fragment sizes amplified by both assays were small, ranging between 65 and 80 bp. A validation of these values was performed via gel electrophoresis of the PCR product (data not shown). When applying the comparative C$_T$ method, the sample with the lowest value was used as calibrator.

HMGA2 immunohistochemical analysis

Slides utilized for the immunohistochemical analysis were produced using consecutive sections directly adjacent to those for the qRT-PCR investigation. Immunohistochemical staining for HMGA2 (rabbit polyclonal anti-HMGA2-P3, Biocheck, Inc., Forster City, USA) was performed using a detection kit (DAKO ChemMate; DAKO, Glostrup, Denmark) and a semi-automated stainer (DAKO; TechMate) according to the specifications of the manufacturer. For antigen retrieval the slides were treated in a PT Link module (DAKO) using the EnVision™ FLEX Target Retrieval Solution, low pH (DAKO). The antibody dilution used was 1:500.

A FFPE sample from a uterine leiomyoma carrying a HMGA2 rearrangement leading to its overexpression served as positive control, whereas negative control was performed by omission of the primary antibody. Staining extent was classified as negative, very weak, weak, moderate and strongly positive, respectively.

Statistical analysis

The two-sided Wilcoxon rank sum test to compare averages from two independent groups was used. Relationships between two observed or measured amounts were quantified by linear regression or by a nonparametric spline model, if the latter turned out to have a significantly better fit. Models were compared by the likelihood ratio test. A *p*-value of less than 0.05 was considered significant, a *p*-value below 0.001 was deemed highly significant. Statistical calculations were done using the R package, version 2.3.2 [25].

Ethics Statement

All samples investigated were initially taken for diagnostic purposes and only secondarily used for the present study. Samples were deidentified before their use in this study, in line with the rules of the Helsinki declaration. The study was approved by the local ethics committee (Ärztekammer Bremen: reference number 371).

Results

qRT-PCR analysis

Eighty-nine samples of human fetal placenta were tested for the expression of *HMGA2* mRNA (table 1A and 1B). Eighty-five were collected after termination of the pregnancy or abortion, respectively (63 were spontaneous, 19 were induced, (one with medical indication). In three cases, no information was available about the type of abortion. In addition, four specimens collected after birth were examined.

The placenta samples showed a relative expression ranging from 1 to 498 (fig. 1). Overall, there was a strong correlation between gestational age and the level of *HMGA2* expression. For all placenta samples, the correlation coefficient was $0.653(p = 2.37*10^{-12})$ (fig. 2). When the analysis was restricted to the specimens gathered after induced abortion (IA) and after delivery (AD), the *r*-value was 0.889 $(p = 3.88*10^{-9})$ (fig. 3). The samples collected after a spontaneous abortion (SA) showed a correlation coefficient of 0.431 $(p = 1.30*10^{-4})$ (fig. 4). The relation between gestational age and *HMGA2* expression did not significantly differ between these groups ($p = 0.095$, likelihood ratio test).

HMGA2 levels from samples taken during the first trimester of pregnancy differed from those of the second and third trimester highly significantly ($p = 3.82*10^{-7}$), Wilcoxon signed rank test). Using the same test, significant differences were also found within the SA subgroup ($p = 0.00257$) and within the IA and AD joint subgroups ($p = 1.98 * 10^{-5}$).

Taking all samples into consideration, a relatively wide ranging but overall high level of expression was observed up to the calendar gestational age (CGA) of 13 weeks. After that, the level of expression showed a decline up to the CGA of 28 weeks and finally leveled out to a steady value

for the rest of the pregnancy. In specimens of non-spontaneous abortions, the observed drop was more pronounced and happened earlier, at around nine weeks of gestation.

Pathological examinations of the specimens after hematoxylin and eosin staining revealed a considerable percentage of maternal decidua in several samples (see also table 1). After the immunohistochemical analysis (see also below), the decidua was found to be HMGA2 negative (data not shown). Therefore, it seems conceivable that the presence of this tissue is the reason for the strong differences in *HMGA2* expression observed among the samples.

Immunohistological analysis

Eleven samples were investigated for the presence and localization of HMGA2 via immunohistochemical analysis. Overall, the results from the immunostaining are in concordance with those from the qRT-PCR, except for two relatively mild outliers. Samples from early gestation (eight to ten weeks CGA) showed intense signals (fig. 5A). The strongest staining was visible in the nuclei of the stromal cells, slightly less intense signals were detectable in the cytoplasm of the trophoblast. Samples from CGA between 19 and 41 weeks showed only weak to very weak signals (fig. 5B). For case number 34 (eight weeks CGA) the qRT-PCR suggested a relatively low expression in comparison to other samples of the same developmental stage, but the signals for the protein were strong (fig. 5C). For case no. 52, the measured mRNA level was above the average value for the developmental stage, but after the immunostaining only a weak signal was visible on the slide (fig. 5D). This might have been due to protein degradation in this case.

Discussion

To quantify the amount of *HMGA2* mRNA in human fetal placentas throughout the pregnancy, 89 samples encompassing CGA from five to 41 weeks were investigated. A highly significant correlation was found between the level of expression of *HMGA2* and the CGA. The level of *HMGA2* is high in the early CGA samples and declines towards the end of the first trimester, followed by an apparently stable level until birth. In the samples from induced abortions, the

HMGA2 level dropped around the ninth week of CGA. Concerning induced and spontaneous abortions as one group, the decrease is less steep and takes place several weeks later. For the specimens collected after spontaneous abortion, it is conceivable that in some cases the underlying cause of the abortion affected the development of the placenta and the expression of *HMGA2*. In addition, the CGA is less precise, because the embryo or fetus might have died days or even weeks before abiosis of the placenta [26]. Therefore, despite a smaller sample size, more reliable results may be obtained from the group of specimens gathered after induced abortions and after birth. Differences in *HMGA2* expression between the samples at an identical specified CGA might be explained by the uncertainty to exactly determine the age of the embryo or fetus. In a group of 29 pregnant women, Blaas *et al*. [27] found virtually the same growth velocities for the embryo or fetus, respectively, between seven and twelve weeks of gestation, but considerable differences between the individuals. The authors discussed differences in development before week seven of the pregnancy as possible explanations. Transferring this argumentation to the development of the fetal placenta, differences in *HMGA2* expression might also be related to the uniqueness of each placenta's development in the early course (before week nine CGA) of pregnancy.

The stable level of *HMGA2*-expression detected in samples from the third trimester differs from the analysis by Rogalla *et al*. [8] revealing an absence of *HMGA2*-expression in term placentae. A possible explanation is the higher sensitivity of qRT-PCR compared to conventional RT-PCR.

HMGA2 is known to participate in the proliferation of tissues by upregulation of genes involved in cell proliferation and invasion (reviewed in [1, 28]). Therefore, the results presented in this study are in accordance with those published by Sitras *et al.* [29], who tested more than 29,000 genes in human placentas. Applying RNA microarray analysis, the authors found that genes involved in cell proliferation, differentiation, and angiogenesis are upregulated in human first trimester placentas. In this latter study, the expression of HMGA2 was not tested.

As demonstrated here, high *HMGA2* expression correlates with the uterine low oxygen environment in early pregnancy. As a result of trophoblast invasion into the maternal decidua, spiral arteries are plugged during the first 7 to 8 weeks of pregnancy (reviewed in [30]). Filtrated plasma enriched with secretions from the endometrial glands can be found in the placental intervillous space, providing histiotrophic nutrition [31]. A low oxygen environment has been shown to be necessary for the proliferation of cytotrophoblast cells [32, 33]. Around week 7 to 8 of gestation (week 9 to 10 CGA), maternal uterine circulation to the placenta begins [34], resulting from disintegration of spiral arterial plugs [35] indicating the starting of haemotrophic nutrition of the fetus which is reaching its full function from week 12 of gestation on. The increasing oxygen level coincides with the decrease of *HMGA2* expression.

Spatially and temporally accurate proliferation and invasion of trophoblast cells are crucial for an undisturbed pregnancy. Superficial implantation of the placenta leading to poor placental and uterine perfusion is characteristic for preeclampsia [36], reviewed in [37]. Preeclampsia affects 2 % to 8 % of all pregnancies [38] and is the cause of direct maternal death in 16 % [39] to 39 % [40] of cases. The only treatment of this serious medical condition is planned preterm delivery. Preeclampsia is not yet fully understood, and there are few markers for diagnosis [41]. In combination with oxidative stress of the placenta (reviewed in [26]), several transcription factors involved in the proliferation and differentiation of the trophoblast have been detected to contribute to an elevated risk of preeclampsia [42-45]. For some proteins, a significantly higher expression has been shown in early gestation [43, 44, 46], similar to the results for *HMGA2*. While symptoms of preeclampsia do not appear before the 20th week of gestation, it seems to result from earlier changes of proliferation and differentiation of the trophoblast that play a key role in the implantation of the embryo during low placental oxygenation (reviewed in [47]). In this investigation, case number 89 was diagnosed as a severe form of preeclampsia, which led to a premature delivery at the gestational age of 32 weeks (see also table 1). The measured value for *HMGA2* was within the normal range of probes of

this late stage of pregnancy. In case of a correlation between *HMGA2* and preeclampsia, a deviation might be restricted to first trimester samples, since in that period of time the proliferation and invasion of the trophoblast determines whether an elevated risk for preeclampsia will exist. A similar situation has been proposed by Jeon *et al*. [41] for IMUP-2. They suggest an association of this protein with preeclampsia, but in term placentas their findings do not reveal differences for patients with or without preeclampsia.

Whereas shallow infiltration of the trophoblast is a sign for preeclampsia, overly deep infiltration indicates another obstetric complication: placenta accreta (including the closely related forms of increta and percreta). This severe complication during pregnancy has been associated with decidual deficiency and an overinvasive trophoblast (reviewed in [48]). Placenta accreta accounts for about 1 % of maternal mortality in the USA [39], and approximately 5 % of the women with this complication die [49-51]. In addition, fetal deaths occur in almost 26 % of the cases [51]. Since the 1970s, the incidence has risen from one in approximately 4,000 deliveries to one in 333 [49, 50, 52, 53]. The reasons are unknown, making further investigations necessary. Since there is no definitive method to detect this complication before birth [54], a possible test would be of high interest. However, a relation with deviant *HMGA2* expression is not easy to investigate because due to miscarriage risk, the invasive sampling of chorionic villi is not considered being an appropriate screening method. Further investigation is needed to prove if HMGA2 or its mRNA in maternal serum might help in identifying these patients.

By immunostaining it became apparent that HMGA2 is strongly expressed in the stromal cells of the placental villi. Positive staining mainly was noted in the nucleus, but in a lesser concentration also observed in the cytoplasm of the trophoblast. This pattern is significantly different from that of HMGA1 [55] found exclusively in the trophoblast cells. This suggests different roles for the two members of the HMGA family. The results of this study are in concordance with those from Genbacev *et al*. [56] who also found HMGA2 in the villi, as well as a change from nuclear to cytoplasmatic location. In general, HMGA proteins are considered

nuclear proteins [1], even though cytoplasmatic expression of HMGA1 has been reported before [55]. In the majority of papers, only nuclear immunoreactivity for HMGA2 was noted [57, 58] with only few exceptions [12, 59].

Conclusions
High expression of *HMGA2* was noted in the early fetal placenta, with a rapid decline around the end of the first trimester. Thus, *HMGA2* may perform essential functions in early placental development. Overall, these findings could serve as the basis for further studies examining the role of *HMGA2* in gestation and its possible correlation with the develoment of placenta accreta and/or preeclampsia or other placental diseases.

Authors' contributions
LK conceived and designed the study, carried out the molecular genetic studies, took part in the immunohistochemical and the statistical analysis and wrote the manuscript. AG took part in the pathological determination of the samples, in immunohistochemical analysis and revised the manuscript. BMH provided the study material, carried out the pathological determination of the samples, the immunohistochemical analysis, and the clinical workout. WW carried out the statistical analysis. JB conceived the study and participated in its design and coordination, helped to draft, and also revised the manuscript. All authors have read and approved the final manuscript.

Declaration of competing interests
The authors declare that they have no competing interests.

Tables

Table 1 - Summary of all samples investigated for the expression of *HMGA2*.

1A early termination samples

case no.	RQ	GA	type of abortion	Immunostaining	note
1	140,523	7	spontaneous		
2	9,902	7	spontaneous		percentage of decidua
3	3,12	15	induced		
4	95,469	9	spontaneous		
5	46,382	10	spontaneous		
6	68,581	8	spontaneous		
7	36,058	8	spontaneous		
8	32,201	8	induced		
9	29,015	8	spontaneous		
10	497,846	8	spontaneous	+++	
11	19,692	10	spontaneous		
12	89,261	9	spontaneous		
13	67,173	6	induced		
14	99,902	9	spontaneous		
15	19,433	14	spontaneous		
16	45,969	10	spontaneous		
17	49,632	7	n.a.		
18	141,657	10	spontaneous		
19	4,725	11	spontaneous		percentage of decidua
20	13,69	9	spontaneous		percentage of decidua
21	3,507	28	spontaneous		
22	130,567	7	spontaneous		
23	109,029	8	induced	++	
24	188,477	7	induced	+++	
25	9,02	10	spontaneous		
26	85,272	11	spontaneous		
27	55,483	8	spontaneous		
28	136,494	7	induced		
29	32,358	8	induced		
30	96,846	8	spontaneous		
31	64,117	7	spontaneous		
32	1,027	18	spontaneous		
33	23,595	22	spontaneous		Part of umbilical cord
34	68,913	8	induced	+++	
35	244,977	8	spontaneous	+++	
36	23,64	10	spontaneous		
37	39,999	11	spontaneous		
38	24,045	9	spontaneous		
39	2,22	20	induced		
40	1,057	32	spontaneous	+-	
41	198,117	9	spontaneous	+++	
42	87,735	7	induced	++	
43	26,091	8	spontaneous		

case no.	RQ	GA	type of abortion	Immunostaining	note
44	8,117	9	spontaneous		
45	54,529	9	spontaneous		
46	30,706	9	spontaneous		
47	9,355	7	spontaneous		percentage of decidua
48	80,126	8	spontaneous		
49	21,238	10	spontaneous		
50	44,867	9	spontaneous		
51	1	7	spontaneous		percentage of decidua
52	152,952	19	spontaneous	+	
53	63,482	7	spontaneous		
54	250,892	11	spontaneous		
55	15,773	7	induced		percentage of decidua
56	160,201	7	spontaneous		
57	247,132	11	spontaneous		
58	92,824	7	spontaneous		
59	74,893	9	spontaneous		
60	162,155	7	spontaneous		
61	126,681	6	induced		
62	186,271	9	spontaneous		
63	28,572	8	induced		
64	47,861	7	induced		
65	31,963	9	spontaneous		
66	154,653	7	spontaneous		
67	95,824	7	induced		
68	31,415	9	induced		
69	72,805	7	spontaneous		
70	56,65	10	spontaneous		
71	6,058	7	spontaneous		percentage of decidua
72	22,586	14	spontaneous		
73	123,656	7	induced		
74	28,452	9	spontaneous		
75	18,37	5	induced		percentage of decidua
76	20,153	7	induced		percentage of decidua
77	87,926	8	spontaneous		
78	109,971	12	spontaneous		
79	152,111	5	spontaneous		
80	128,672	7	spontaneous		
81	47,139	9	spontaneous		
82	3,306	28	n.a.		
83	4,502	30	n.a.		
84	23,446	27	spontaneous		
85	6,276	24	spontaneous		

RQ: relative quantification; CGA: calendar gestational age in weeks; n.a.: information not available; percentage of decidua: sample consisted of at least 50 % decidua; umbilical cord: sample contained a section of the umbilical cord; immunostaining: sample was used for immunostaining with an HMGA2-specific antibody. Staining extent: +- very weak, + weak, ++ moderate, +++ strong

1B samples collected after birth

case no.	RQ	GA	age	sex	weight	C section
86	1,557	38	30	m	3155	no
87	1,711	41	30	m	4250	yes
88	1,525	38	41	m	3435	yes
89	1,698	32	28	m	1750	yes

RQ: relative quantification; CGA: calendar gestational age in weeks; age: age of the mother at delivery; sex: sex of the neonate; weight: weight of the neonate in grams; C section: Caesarean section

Figures
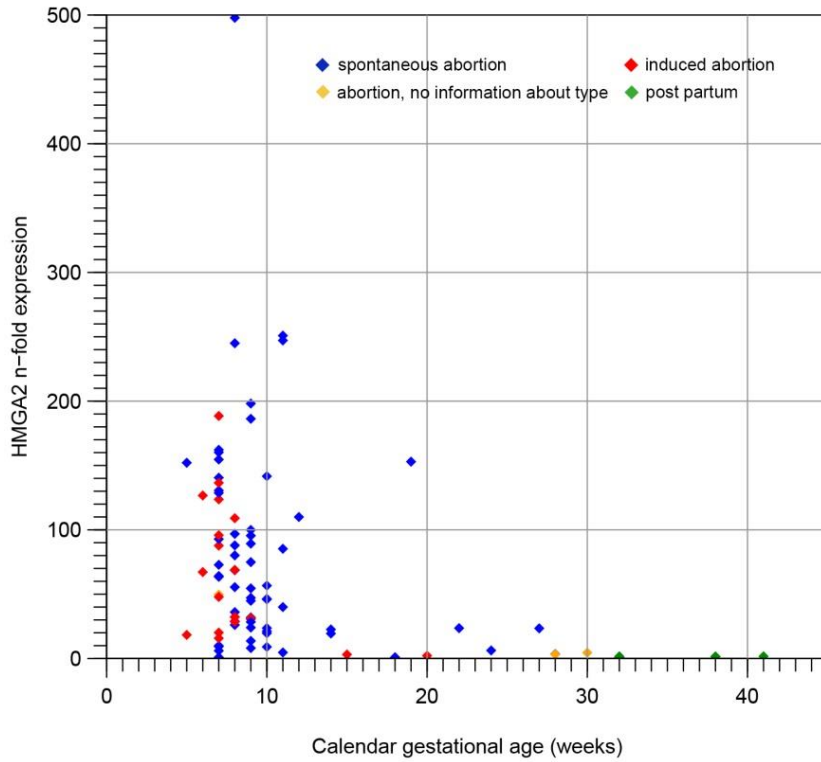
Figure 1 - *HMGA2* expression in relation to the gestational age. Linear display for *HMGA2* expression, all samples.
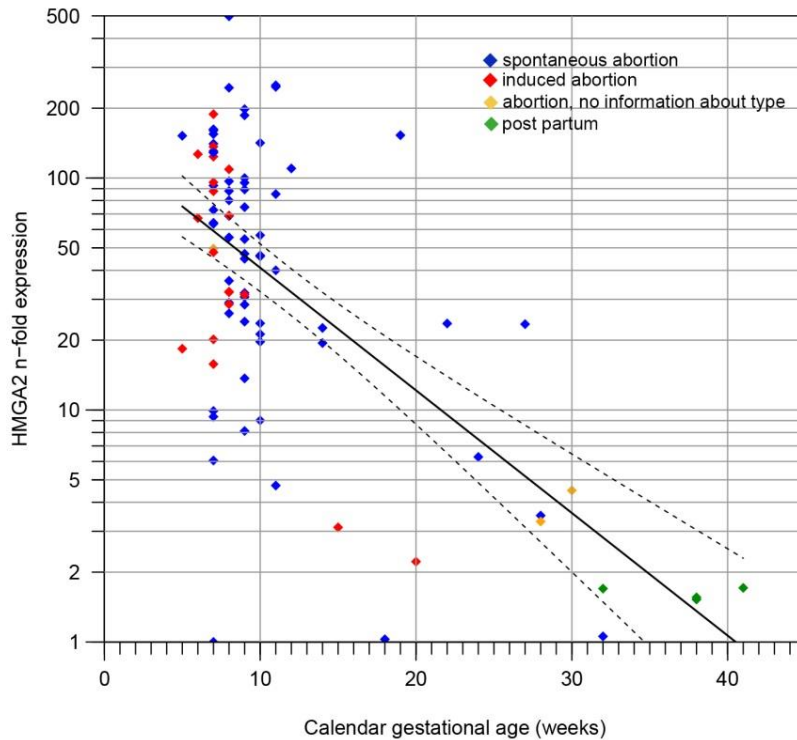
Figure 2 - *HMGA2* expression in relation to the gestational age including the linear regression line with 95% confidence range. Logarithmic display for *HMGA2* expression, all samples.
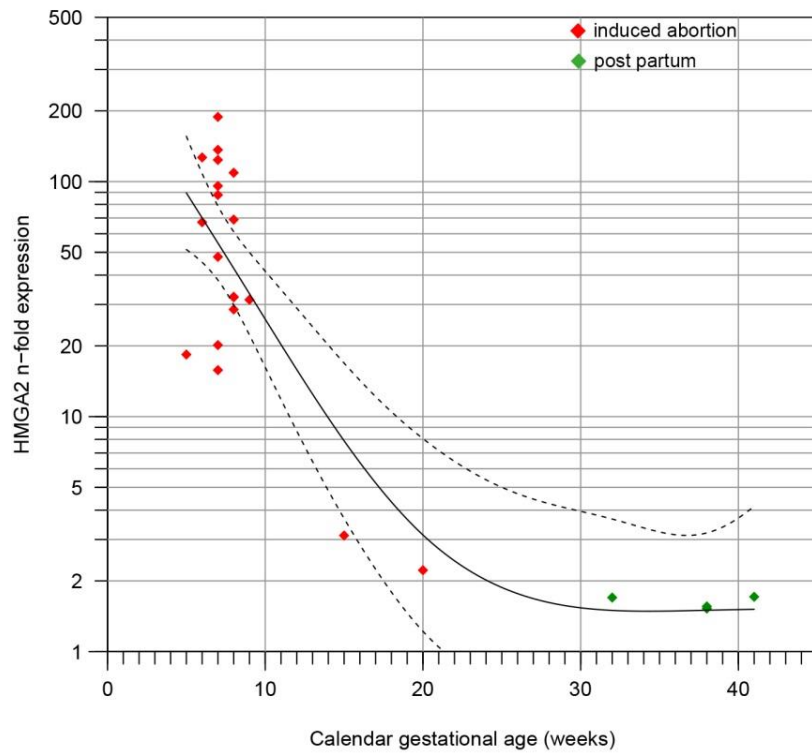
Figure 3 - *HMGA2* expression in relation to the gestational age including the spline fit with 95% confidence range. Logarithmic display for *HMGA2* expression, samples collected after induced abortion and after birth.
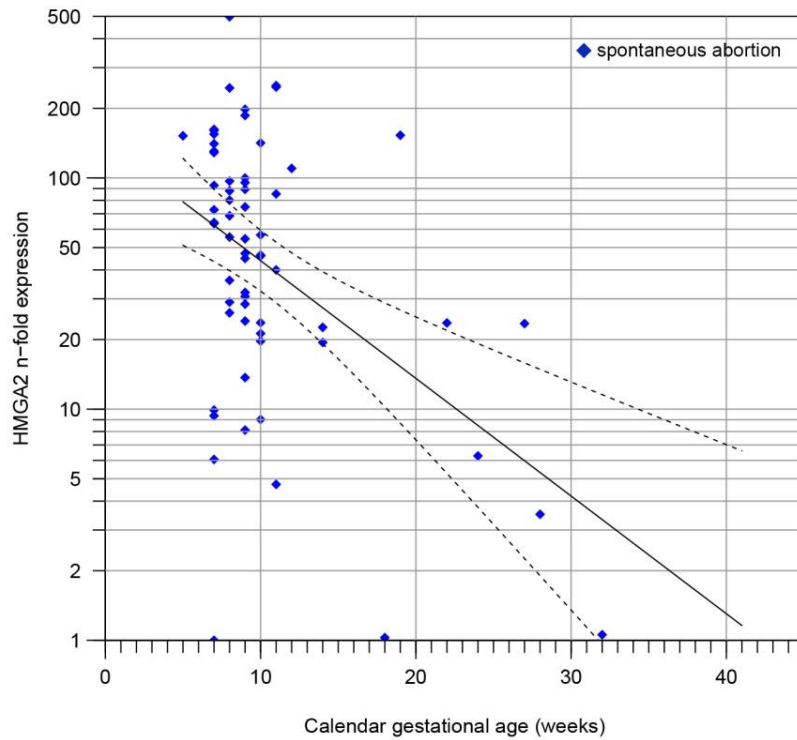
Figure 4 - *HMGA2* expression in relation to the gestational age including the linear regression line with 95% confidence range. Logarithmic display for *HMGA2* expression, spontaneous abortion samples.
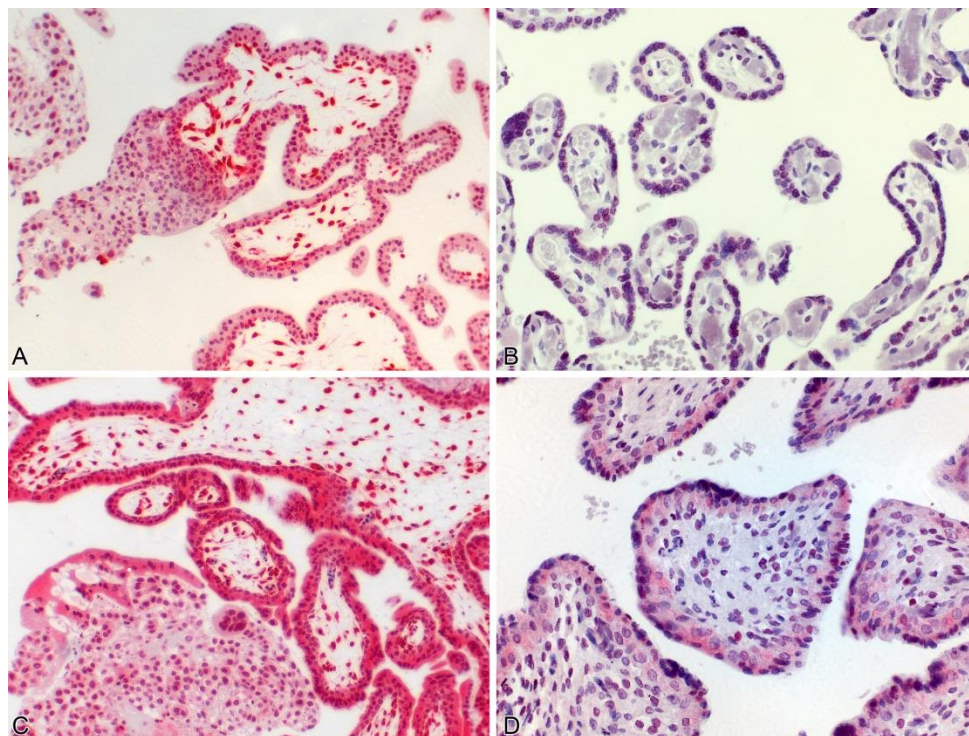
Figure 5 - Immunoreactivity for HMGA2.
As mentioned in the text, in all but two cases the qRT-PCR data was in concordance with the interpretation of the HMGA staining. For the outliers, see (C) and (D). (A) (seven weeks calendar gestational age) shows an intensive signal for HMGA2 and a high expression as measured in the qRT-PCR. (B) in case no. 88 (38 weeks calendar gestational age) the signal is barely visible, the qRT-PCR showed a very low expression. (C) case number 34 (eight weeks calendar gestational age) shows a strong signal, the measured expression was average, (D) in case no. 52 (19 weeks calendar gestational age) the signal is weak, the qRT-PCR data suggested a relatively high expression (see also table 1A and 1B). In all samples with a level high enough for visual detection, HMGA2 was found in the nuclei of the stroma cells of the villi and in the cytoplasm of the trophoblast.

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3.3 Publikation III:
Quantitative und qualitative Analyse von *HMGA2*-Expression in testikulären Keimzelltumoren

In Publikation II wurde die physiologische *HMGA2*-Expression in Plazenten im Schwangerschaftsverlauf untersucht, während im nächsten Schritt nun dessen mögliche Reexpression in testikulären Keimzelltumoren (TGCTs) erwachsener Männer betrachtet werden sollte. Keimzelltumoren imitieren unterschiedliche Stufen embryonaler Entwicklung (Looijenga et al. 2007), was sich schon in der Bezeichnung histologischer Subgruppen andeutet, nach der sie in Seminom, Embryonalkarzinom, Chorionkarzinom, Dottersacktumor und Teratom unterteilt sind (Chaganti, Houldsworth 2000, Nogales, Preda & Nicolae 2012). *HMGA2* wird im frühen Embryo stark, mit fortschreitender Embryonalentwicklung aber schwächer exprimiert (Rogalla et al. 1996, Hirning-Folz et al. 1998). Daher stand im Zentrum dieser Publikation die Frage, ob diese Imitation unterschiedlicher Stufen der Embryonalentwicklung sich auch in der *HMGA2*-Expression verschiedener TGCT-Subgruppen widerspiegelt.

Es wurde mit Hilfe der qRT-PCR gezeigt, dass Seminome praktisch kein *HMGA2* exprimieren, während Embryonalkarzinome schwach exprimieren. Teratome zeigten ein heterogenes Expressionsmuster, abhängig vom Reifegrad des Teratoms. Reife Teratome exprimieren *HMGA2* schwächer bis gar nicht im Vergleich zu unreifen. Die nur in wenigen untersuchten Proben enthaltenen Chorionkarzinome zeigten ebenfalls ein heterogenes Bild, aber eine Tendenz zu eher starker Expression. Durchgehend hohe *HMGA2*-Expression wurde in Dottersacktumoren gemessen. Bei reinen Formen war mit der qRT-PCR eine Unterscheidung in die verschiedenen Entitäten möglich, schwieriger war dies in Mischtumoren. Aber auch hier zeigte eine Modellierung der anhand der Zusammensetzung zu erwartenden Expression eine gute Übereinstimmung mit den tatsächlich gemessenen Werten. Etwa 50% der untersuchten TGCTs waren Mischtumoren. Hier war eine Unterscheidung der Subgruppen mit Hilfe der qRT-PCR zwar möglich, für diagnostische Zwecke ist diese Methode aufgrund des recht hohen Aufwandes und einer nur statistischen Aussage aber nur bedingt geeignet. Um die qRT-PCR-Ergebnisse zu überprüfen und vor allem auch zu analysieren, welche Subgruppen in welcher Intensität HMGA2-Protein exprimieren und wie sich Expressionsmuster in Mischtumoren präsentieren, wurden an ausgewählten TGCTs immunhistochemische HMGA2-Färbungen durchgeführt. Hierbei konnten die bereits

in der qRT-PCR gewonnenen Ergebnisse bestätigt werden. Darüber hinaus wurde deutlich, dass die Subgruppen mit Hilfe der Immunhistochemie relativ genau differenziert werden konnten. Insbesondere die Dottersacktumoranteile in den Mischtumoren konnten verlässlich detektiert werden. Zur Unterscheidung der teilweise im histologischen Bild sehr ähnlichen Seminome und Embryonalkarzinome war die HMGA2-Immunhistochemie nicht verlässlich geeignet, weil beide Subgruppen wenig bis kein HMGA2-Protein exprimieren. Ebenso war die Identifizierung von Teratomanteilen nicht verlässlich möglich. Über die Eignung von HMGA2-Immunhistochemie zur Detektion von Chorionkarzinomanteilen konnte anhand der untersuchten Proben keine belastbare Aussage gemacht werden, da die Probenanzahl mit nur drei Proben zu klein war. Eine positive Tendenz ließ sich aber aus drei Proben bereits ablesen.

Interessanterweise war sowohl in der qRT-PCR als auch in der Immunhistochemie die Expression in Seminomen nahe oder unter der Nachweisgrenze, während Hoden-Normalgewebe eine schwache Expression erkennen ließ. Dies war, wie sich im immunhistologischen Bild zeigte, auf Spermien-Vorläuferzellen (Spermatozyten und Spermatiden) im Normalgewebe zurückzuführen.

III

HMGA2 expression distinguishes between different types of postpubertal testicular germ cell tumour

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* diese Autoren sind gleichberechtigte Erstautoren des Artikels

Eigenanteil

- Studiendesign mit Jörn Bullerdiek und Lars Kloth
- Beurteilung der immunhistochemischen Färbungen mit Thomas Löning, Burkhard Helmke und Käte Burchardt
- Statistische Auswertung mit Werner Wosniok und Lars Kloth
- Verfassen des Manuskriptes mit Lars Kloth

Original Article

HMGA2 expression distinguishes between different types of postpubertal testicular germ cell tumour

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Abstract

The group of postpubertal testicular germ cell tumours encompasses lesions with highly diverse differentia-tion – seminomas, embryonal carcinomas, yolk sac tumours, teratomas and choriocarcinomas. Heterogeneous differentiation is often present within individual tumours and the correct identification of the components is of clinical relevance. *HMGA2* re-expression has been reported in many tumours, including testicular germ cell tumours. This is the first study investigating *HMGA2* expression in a representative group of testicular germ cell tumours with the highly sensitive method of quantitative real-time PCR as well as with immunohisto-chemistry. The expression of *HMGA2* and *HPRT* was measured using quantitative real-time PCR in 59 postpu-bertal testicular germ cell tumours. Thirty specimens contained only one type of tumour and 29 were mixed neoplasms. With the exception of choriocarcinomas, at least two pure specimens from each subgroup of tes-ticular germ cell tumour were included. In order to validate the quantitative real-time PCR data and gather information about the localisation of the protein, additional immunohistochemical analysis with an antibody specific for HMGA2 was performed in 23 cases. Expression of *HMGA2* in testicular germ cell tumours depended on the histological differentiation. Seminomas and embryonal carcinomas showed no or very little expression, whereas yolk sac tumours strongly expressed HMGA2 at the transcriptome as well as the protein level. In teratomas, the expression varied and in choriocarcinomas the expression was moderate. In part, these results contradict data from previous studies but HMGA2 seems to represent a novel marker to assist patho-logical subtyping of testicular germ cell tumours. The results indicate a critical role in yolk sac tumours and some forms of teratoma.

Keywords: testicular germ cell tumour; HMGA2; seminoma; embryonal carcinoma; yolk sac tumour; teratoma; choriocarcinoma; qRT-PCR; immunohistochemistry; biomarker

Introduction

Testicular germ cell tumours (TGCTs) are relatively rare, but in many countries they represent the most prevalent cancer in men between 15 and 40 years of age [1]. For unknown reasons its incidence has increased significantly over recent decades in many populations globally [1,2]. Nevertheless, the world-wide frequency varies considerably between different races and countries, with several European countries showing the highest incidences [1,3,4]. In these regions, up to one in 200 men is affected [3]. The

assumed originating cells are primordial germ cells, which undergo malignant transformation thus becoming an intratubular germ cell neoplasia undifferentiated (ITGCNU), formerly referred to as testicular intraepithelial neoplasia or carcinoma *in situ* [reviewed in [5,6]]. In almost all cases of ITGCNU, a TGCT with invasive growth eventually develops [reviewed in [7,8]]. TGCTs are divided into pure seminoma (∼50–54%) and nonseminoma (ca. 46–50%) subgroups. The latter also contains mixed tumours [9,10]. This subtyping is of clinical and prognostic relevance [10–12]. Nonseminomas display different degrees of differentiation from embryonal carcinoma (EC) to mature teratoma and display embryonic and extraembryonic differentiation [8,13]. Mixed forms of two or more nonseminomas, or seminoma and nonseminoma, are common: between 13 and 54% of tumours have been reported to contain mixed histology [14,15]. Although a panel of immunohistochemical biomarkers helps to differentiate tumour subgroups, diagnosis can still be a challenge [16].

High-mobility group AT-hook (HMGA) proteins are small and highly charged, consisting of three DNA-binding domains and an acidic carboxy-terminal tail. As architectural transcription factors they lack intrinsic transcription factor capacity but interact with nuclear proteins and enhance or silence transcription through changes in chromatin structure [reviewed in [17,18]]. There are four known HMGA proteins in humans (HMGA1a, HMGA1b, HMGA1c and HMGA2), encoded by two genes [reviewed in [19,20]]. High *HMGA* expression has been detected at embryonic and foetal stages in mammalian tissues. Conversely, no or only very low *HMGA2* expression has been identified in adult tissue; slightly higher levels have been reported for *HMGA1* in some tissues [21–25]. The reactivation of *HMGA* expression has been reported for a multitude of tumours [reviewed in [19,26,27]]. Chieffi et al. [28] found *HMGA2* to be critically involved in spermatogenesis in mice. Furthermore, Di Agostino *et al*. [29] found that *HMGA2* interacts with Nek2 in a MAPK-dependent manner in mouse spermatogenesis. In addition to the participation in normal testicular processes, *HMGA2* has also been suggested as a marker for testicular cancer [30 and reviewed in [5,31,32]]. Franco *et al*. [30] showed moderate to high expression of *HMGA2* in ECs and yolk sac tumours (YSTs).

The aim of this study was to investigate the role of *HMGA2* in postpubertal germ cell tumours of the testis. For the first time, highly sensitive quantitative real-time PCR (qRT-PCR) has been applied in combination with immunodetection, to allow more distinctive differentiation of expression levels of *HMGA2* in the subgroups. Another key aspect was determining whether protein level could serve as a diagnostic marker for clinical application.

Methods

Tissue samples

Formalin-fixed paraffin-embedded (FFPE) tumour tissue and snap-frozen samples of normal testis were collected at the Department of Pathology, Albertinen Hospital, Hamburg, Germany, the Department of Pathology, Clinical Centre Bremen-Mitte, Bremen, Germany, and the Institute of Pathology, Elbe Clinic Stade-Buxtehude, Germany. Additional FFPE samples were collected under the supervision of the Leibniz Institute for Prevention Research and Epidemiology, Bremen, Germany. Pathological examinations were performed after haematoxylin and eosin staining of the samples for diagnostic purposes. In complex cases, additional immunostaining with antibodies specific for PLAP, OCT4, CD30, CD117, glypican 3, AFP and β-HCG was conducted according to the relevant pathology department's protocol. FFPE tissue samples from 59 postpubertal patients with TGCT and three snap-frozen normal testis tissues were examined in the study. Histology was re-evaluated by three of the authors (B.H., T.L. and K.B.) according to the WHO classification. Histological subgroups were: 12 pure seminomas, three mixed tumours with a predominant component of seminoma, 10 pure ECs, 13 mixed tumours with a predominant component of EC, three mixed tumours with two equally predominant components of EC/teratoma or EC/YST, two YSTs, three mixed tumours with a predominant component of YST, six pure teratomas, seven mixed tumours with a predominant component of teratoma (see also Table 1). All samples investigated were initially taken for diagnostic purposes and secondarily used for the present study. Samples were deidentified before their use in this study, in line with the rules of the Helsinki declaration. The study was approved by the local ethics committee (Ärztekammer Bremen, reference number 371).

RNA isolation

Depending on the size of the embedded tissue, FFPE blocks were cut into six to eight sections of 5 μm for each sample using a microtome. Total RNA isolations were performed using the innuPREP Micro RNA Kit (Analytik Jena AG, Jena, Germany) for

Table 1. Overview of all testicular cancer samples

Case	RQ	Seminoma (%)	EC (%)	YST (%)	Teratoma (%)	CC (%)	Undetermined (%)	Normal tissue, absolute value (%)	Immunohistochemistry	Patient's age	Tumour size (cm)
HT01	7,998	0	4	4	92	0	0	75	−	32	2,8
HT02	3,617	100	0	0	0	0	0	11	−	46	6,5
HT03	9,182	0	50	10	40	0	0	70	−	18	2,7
HT04	0.763	100	0	0	0	0	0	70	−	60	1,6
HT05	0.507	100	0	0	0	0	0	90	−	35	1,3
HT06	0.901	0	100	0	0	0	0	80	−	21	2.6
HT07	3.689	0	100	0	0	0	0	70	+	29	1.9
HT08	0.312	100	0	0	0	0	0	15	−	31	4.5
HT09	130.584	0	40	40	0	0	20	25	+	39	3.5
HT10	46.510	0	0	0	70	30	0	50	+	23	6
HT11	0.865	100	0	0	0	0	0	30	−	55	1.5
HT12V	1.808	0	0	0	100	0	0	20	−	23	1.8
HT13	0.234	100	0	0	0	0	0	80	−	39	1.5
HT14	0.438	60	40	0	0	0	0	30	−	18	2
HT15	1.624	0	100	0	0	0	0	85	−	27	2
HT16	0.733	100	0	0	0	0	0	20	−	41	5.5
HT17	111.724	0	40	40	0	20	0	17	+	28	2.5
HT19	0.1*	100	0	0	0	0	0	70	+	38	2.8
HT20	1.765	100	0	0	0	0	0	90	+	50	1.5
HT22	6.922	0	100	0	0	0	0	64	+	53	1.5
HT23	1.880	0	100	0	0	0	0	90	−	35	3.5
HT24	10.833	0	4	0	96	0	0	75	−	28	0.9
HT25	1.041	75	20	5	0	0	0	56	+	20	2.8
HT26	0.681	100	0	0	0	0	0	10	−	26	3.3
HT27	0.143	100	0	0	0	0	0	30	+	39	6.5
HT28	0.322	100	0	0	0	0	0	85	−	46	2.4
HT29	0.873	0	0	0	100	0	0	15	+	21	2
HT30	74.481	0	0	20	80	0	0	38	+	66	4
HT31	17.595	0	90	5	0	5	0	43	−	37	6.5
HT32	31.621	0	60	30	0	5	5	40	−	24	8
HT33	32.310	0	80	10	0	10	0	25	−	23	4
HT34	6.474	0	96	4	0	0	0	33	−	33	1.8
HT35	0.379	0	0	75	25	0	0	38	+	35	4
HT36	50.418	0	0	100	0	0	0	26	−	36	1.5
HT37	61.414	0	80	20	0	0	0	47	−	22	3.4
HT38	1.594	0	0	5	95	0	0	20	−	35	2.6
HT39	7.565	0	90	10	0	0	0	50	−	47	3
HT40	1.403	0	100	0	0	0	0	11	+	30	n.a.
HT41	14.691	0	59	5	35	1	0	11	−	40	1.2
HT42	109.424	0	0	100	0	0	0	25	+	38	6
HT43	99.796	0	5	10	85	0	0	15	+	31	3.5
HT44	147.842	0	40	18	40	2	0	30	+	24	3.2
HT45	6.866	0	0	0	100	0	0	70	+	43	5.5
HT46	18.707	20	60	20	0	0	0	40	−	19	2.7
HT47	15.294	0	95	5	0	0	0	50	−	48	2
HT48	2.960	0	100	0	0	0	0	80	−	23	1
HT49	0.897	0	0	0	100	0	0	50	−	24	1.8
HT50	0.853	0	0	0	100	0	0	80	−	21	1.5
HT51	626.427	0	10	20	70	0	0	20	+	40	4.5
HT52	230.972	75	5	20	0	0	0	30	+	19	3
HT53	28.455	0	90	10	0	0	0	30	+	18	n.a.
HT54	130.314	0	0	95	5	0	0	41	−	43	3
HT55	13.557	0	95	5	0	0	0	22	+	23	3
HT56	6.136	0	100	0	0	0	0	29	−	29	2.1
HT57	2.301	0	0	0	100	0	0	20	−	n.a.	3.5
HT58	3.549	0	100	0	0	0	0	33	−	43	2
HT59	6.824	0	100	0	0	0	0	38	−	30	4.5
HT62	3.733	0	81	10	9	0	0	70	+	27	n.a.
HT63	119.312	0	50	50	0	0	0	26	+	38	2.5

RQ, relative quantification; *HMGA2* expression, EC, embryonal carcinoma; YST, yolk sac tumour; CC, choriocarcinoma; Immunhistochemistry, sample was used in HMGA2-specific immunohistochemistry investigation; *: set value, expression below detection level (see text for further explanation), n.a., not available.

RNA isolation according to the manufacturer's instructions, with the following modifications: Lysis of the paraffin sections preceding RNA isolation was conducted using TLS-Lysis Solution and Proteinase K from the innuPREP DNA Micro Kit (Analytik Jena AG, Jena, Germany) without prior deparaffinisation. Sections were incubated for 1 h at 60°C and 15 min at 80°C.

cDNA-synthesis and quantitative real-time RT-PCR

RNAs were reverse-transcribed into cDNA by M-MLV Reverse Transcriptase (Invitrogen, Karlsruhe, Germany). Real-time PCR was performed using the Applied Biosystems 7300 sequence detection system, software 1.2.3, according to the Taq-Man Gene Expression Assay Protocol (Applied Biosystems, Darmstadt, Germany) in 96-well microtitre plates with a total volume of 20 μl. For the TaqMan gene expression assay for *HMGA2* (assay number Hs00171569, Applied Biosystems, Foster City, USA), each reaction consisted of 2 μl of cDNA reverse transcribed from 25 ng of total RNA, 10 μl of TaqMan Universal PCR Master Mix (Applied Biosystems), 1 μl of TaqMan assay and 7 μl of ddH$_2$O. For the *HPRT* assay, using HPRT FP and HPRT RP primers [33], each reaction consisted of 2 μl of cDNA reverse transcribed from 25 ng of total RNA, 10 μl of TaqMan Universal PCR Master Mix, 600 nM (1.2 μl) of forward and reverse primers, 200 nM (0.2 μl) of probe [33] and 5.4 μl of ddH$_2$O. Thermal cycling conditions were 2 min at 50°C followed by 10 min at 95°C, 50 cycles at 95°C for 15 s and 60°C for 1 min. In each run, a negative control of previous cDNA synthesis (missing reverse transcriptase) was included for each sample and a nontemplate control of amplification and a nontemplate control of previous cDNA synthesis were included for each plate.

All testing reactions were performed in triplicate. Considering the expression range of *HMGA2*, *HPRT* was chosen as the endogenous control as generally suggested by de Kok *et al*. [34], and as used for testicular samples by McIntyre *et al*. [35], Looijenga *et al*. [36] and Wermann *et al*. [37]. The C_T values of both genes were in concordance (*HMGA2*: 21,914–36,006; *HPRT*: 23,421–37,459). As recommended for FFPE samples [38] the fragment sizes amplified by both assays were small, ranging between 65 and 80 bp; validation of these values was performed via gel electrophoresis of the PCR amplicons (data not shown). Relative quantity (RQ) was calculated using the ddCT method [39]. Snap frozen tissue of normal testis was tested against FFPE from the same sample giving highly comparable results.

Because of disposability of snap frozen normal testis tissue, the average of three such tissues was used as calibrator.

Immunohistochemical analysis

Slides utilized for the immunohistochemical analysis were produced using cuts directly adjacent to those used for the qRT-PCR investigation. Immunohistochemical staining for HMGA2 (rabbit polyclonal anti-HMGA2-P3, Biocheck, Inc., Forster City, USA) was performed using a detection kit (DAKO Chem-Mate; DAKO, Glostrup, Denmark) and a semiautomated stainer (DAKO; TechMate) according to the specifications of the manufacturer. For antigen retrieval, the slides were treated in a PT Link module (DAKO) using the EnVision^{TM} FLEX Target Retrieval Solution, low pH (DAKO). The antibody dilution used was 1:1000. Term placenta was used as a positive control whereas negative control was performed by omission of the primary antibody.

Interpretation of HMGA2 staining was done using a Zeiss Axioplan (Carl Zeiss Microscopy GmbH, Göttingen, Germany) microscope. Immunoreactivity in the nucleus was considered positive (although perinuclear granulation in cytoplasm was observed occasionally). In each slide, three to five high-power fields were rated. Staining extent was scored by multiplying intensity of staining (0: no staining, 0.5: very weak, 1: weak, 1.5: weak-moderate, 2: moderate, 2.5: moderate-strong and 3: strong) by percentage of stained tumour cells. Lack of available tissue was one of the reasons why we did not perform IHC in all cases analysed by qRT-PCR.

Statistical analysis

RQ values and immunohistochemical scores were described by number of values, arithmetic means, standard deviations and minimum and maximum. Boxplots were used to summarize the distribution of data values. Averages of immunohistochemical scores were compared by the Wilcoxon two-sample rank sum test. The ability of the RQ value to discriminate between tumour subgroups was quantified for all pairs of tumour subgroups by sensitivity and specificity, obtained from Receiver-Operator-Characteristics (ROC) analysis, thereby using a normal approximation of the empirical data. This analysis was performed for all samples containing only a single type of tumour. The relationship between lg(RQ) and the proportion of tumour components – these expressed as proportion of the total section area – was analysed by linear regression. An intercept was omitted from the

Table 2. *HMGA2* expression in pure tumours

Case	RQ	Type of tumour
HT02	3.617	seminoma
HT04	0.763	seminoma
HT05	0.507	seminoma
HT08	0.312	seminoma
HT11	0.865	seminoma
HT13	0.234	seminoma
HT16	0.733	seminoma
HT19	0.1*	seminoma
HT20	1.765	seminoma
HT26	0.681	seminoma
HT27	0.143	seminoma
HT28	0.322	seminoma
HT06	0.901	EC
HT07	3.689	EC
HT15	1.624	EC
HT22	6.922	EC
HT23	1.880	EC
HT40	1.403	EC
HT48	2.960	EC
HT56	6.136	EC
HT58	3.549	EC
HT59	6.824	EC
HT36	50.418	YST
HT42	109.424	YST
HT12V	1.808	teratoma
HT29	0.873	teratoma
HT45	6.866	teratoma
HT49	0.897	teratoma
HT50	0.853	teratoma
HT57	2.301	teratoma

RQ, relative quantification; EC, embryonal carcinoma; YST, yolk sac tumour; *, set value; expression below detection level (see text for further explanation).

Table 3. *HMGA2* expression in pure tumours by group

Type of tumour	n	Average	St dev
Seminoma	12	0.904	1.004
EC	10	3.589	2.292
YST	2	79.921	41.724
Teratoma	6	2.266	2.332

n, number of cases; St dev, standard deviation; EC, embryonal carcinoma; YST, yolk sac tumour.

normal testicular samples used for normalisation. Overall, the samples showed an *HMGA2* expression level between 0.143 and 626.427: this relates to a range of about 1–4381. For one sample, the expression was 0, ie the expression was below the detection limit.

Focussing on the samples with only one tumour subgroup, there was a clear classification between the groups (Tables 2 and 3, Figure 1). Seminomas showed the lowest values; with two exceptions, all measured data were below the expression in normal tissue. ECs and teratomas showed slightly elevated levels, while the levels expressed by YSTs were by far the highest. This clustering could be visualized by aligning the samples by level of expression (Figure 2, including the mixed tumours).

To statistically validate the visual impression on discriminatory ability, ROC analyses were performed (Table 4). Comparisons involving YST showed a sensitivity of at least 0.988 and a specificity of 0.997, indicating a clear distinction from the other tumour subgroups. These numbers, however, must be treated with caution, since the YST group consisted of only two samples. In addition to the comparison of individual groups, seminomas were tested against all other subgroups. This analysis indicated that seminomas and nonseminomas were separated moderately well by real-time *HMGA2* expression data alone with a sensitivity of 0.912 and a specificity of 0.680.

To analyse qRT-PCR data from samples with two or more tumour components and to accommodate for varying percentages of normal tissue content, a linear regression was calculated between the logarithmic RQ and the tumour components (Table 5, Figure 3). Comparison of observed and predicted RQ values showed good agreement; in particular, no indication of systematic deviation was identified. The model achieved an adjusted coefficient of determination of 0.6625 with a *p* value of $1.112*10^{-12}$, indicating *HMGA2* expression is dependent on the tumour subgroup. With the exception of seminomas, each subgroup's contribution turned out to be significant or highly significant (see also Table 5). There are four values with large differences between observed and
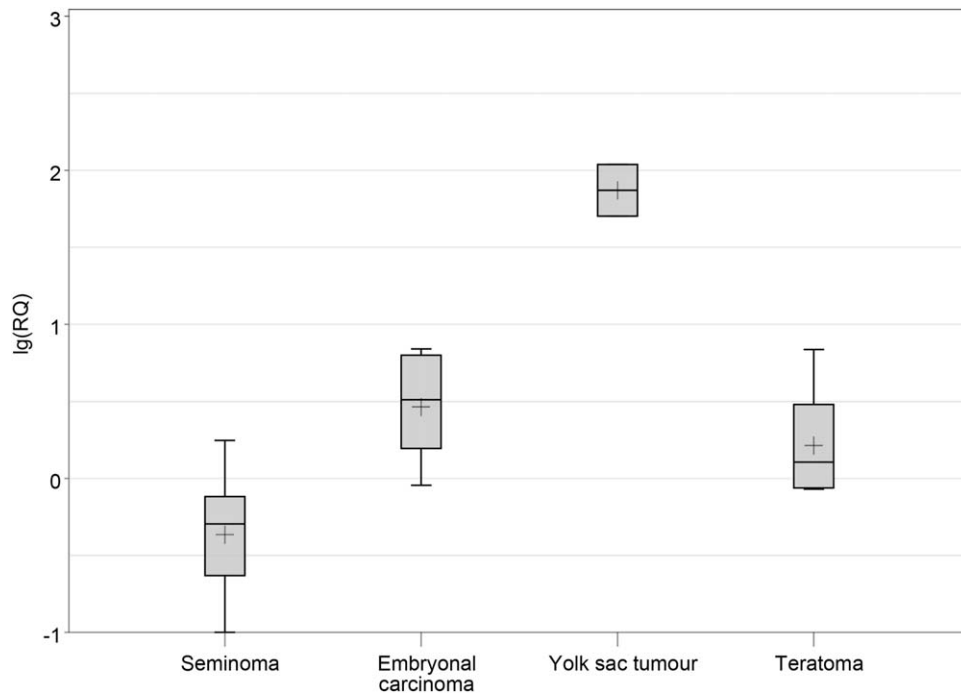
regression equation, because a tumour proportion of zero is by definition associated with $\lg(RQ) = 0$. To allow for the logarithmic transformation of all values, the RQ of zero observed in one case was replaced by $RQ = 0.1$. This value still lies below the smallest observed RQ value. All cases were included in this analysis. A *p* value of less than 0.05 was considered significant, a *p* value of less than 0.001 highly significant. Statistical analyses were undertaken using the SAS/STAT and SAS/GRAPH software (version 9.2 for Windows, copyright 2002–2008 SAS Institute Inc.), and the R software [40].

Results

qRT–PCR analysis

Fifty-nine FFPE samples of human TGCTs were tested for the expression of *HMGA2* (Table 1). Of these tumours, 30 were pure tumours (12 seminomas, 10 ECs, two YSTs and six teratomas), 29 were mixed GCTs. These were accompanied by three snap-frozen

Figure 1. *HMGA2* expression in pure tumours. Boxplots for the relative quantification of *HMGA2* expression in TGCTs. Tumour type at *x*-axis, logarithmic RQ at *y*-axis. Boxes contain the central 50% of all values and a bar at the position of the median, whiskers extend to the extreme values or to 1.5 * box height, whichever is smaller. The plus sign shows the arithmetic mean.
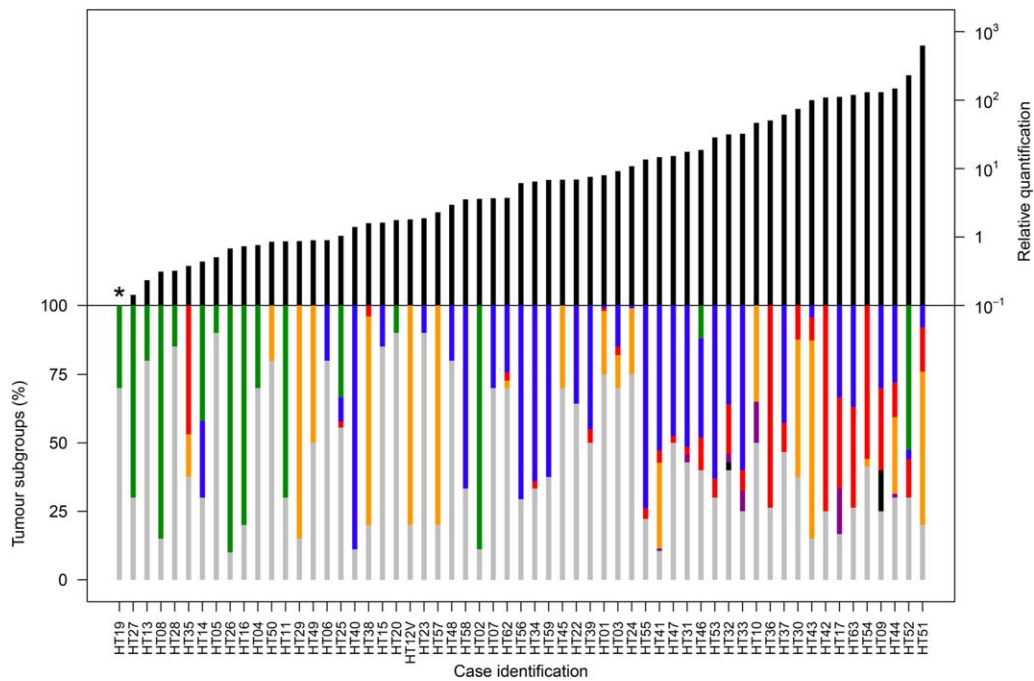

Figure 2. Overview of all testicular cancer samples. Bar plot with RQ in the upper part, tumour composition in the lower part. Samples are arranged by level of *HMGA2* expression. *: set value, expression below detection level (see text for further explanation). Colour code for the tumour composition: green: seminoma, blue: embryonal carcinoma, red: yolk sac tumour, orange: teratoma, purple: choriocarcinoma, black: undetermined, grey: normal tissue.

Table 4. ROC analysis of pure tumours

Type of tumour	n		Seminoma	EC	YST	Teratoma
Seminoma	12	sens.		0.868	0.998	0.581
		spec.		0.815	0.999	0.828
EC	10	sens.			0.988	0.883
		spec.			0.997	0.371
YST	2	sens.				0.996
		spec.				0.998
Teratoma	6	sens.				
		spec.				

n, number of cases; sens., sensitivity; spec., specificity; EC, embryonal carcinoma; YST, yolk sac tumour.

Table 5. Linear regression analysis of the relationship between lg(RQ) and the proportion of tumour components

Tumour component	Estimate	Standard error	t value	p value
Seminoma	−0.001496	0.003134	−0.477	0.63502
EC	0.014816	0.002875	5.153	$3.72*10^{-6}$
YST	0.030189	0.004875	6.192	$8.38*10^{-8}$
Teratoma	0.01078	0.00327	3.297	0.00173
CC	0.06092	0.029689	2.052	0.04504

Estimate, estimate of the regression coefficient; positive values indicate an RQ value increasing with tumour proportion, t value, test statistic for the hypothesis 'Coefficient is zero'; p value, level of significance; EC, embryonal carcinoma; YST, yolk sac tumour; CC, choriocarcinoma. The model contains no intercept, as a proportion of zero is by definition associated with lg(RQ) = 0; data: all samples.

predicted expression. HT35 is a clear outlier, with measured expression far below the expected value. As the sample was composed of YST and teratoma, taking the overall results into consideration, much higher expression of *HMGA2* had been expected. HT51 and HT52 showed values higher than expected considering their composition. HT19 is the sample with the set value. In all cases, qRT-PCR data were confirmed by immunohistochemical analysis (see below).

Immunhistological analysis

In 23 cases, the section of the FFPE block following those used for qRT-PCR analysis was investigated for HMGA2 protein expression patterns. Since 14 samples contained two or more histologically different areas, 45 immunohistochemical scores were gathered (Figure 4). Concordant with results from qRT-PCR, the HMGA2 scores in seminomas were nearly zero (mean: 0.0375). One seminoma (HT20) showed focally strong immunoreactivity; all the others were negative. No or very weak staining was observed in EC components, whereas a wide range was observed in teratoma components. There was a tendency for immature structures to be positive, whereas mature teratomas were negative. Exceptions were observed, eg mature glandular structures as goblet cells were often, but not always, strongly positive. On the other hand, primitive neuroepithelium showed weak staining; muscular structures were negative. YST components were strongly positive. Two exceptions were found: one YST was negative (HT25: 5% YST, 75% seminoma, 20% EC) and one showed weak to moderate staining (HT35: 75% YST, 25% teratoma). Both also showed unexpectedly low HMGA2 expression by qRT-PCR. HT51 and HT52, both displaying very high qRT-PCR values, showed equally strong immunostaining. In choriocarcinomas (CCs) syncytiotrophoblasts as well as cytotrophoblasts showed weak to moderate staining (Figure 5, Supplemental Figure 1). HMGA2 expression was

seen in 80–100% of syncytiotrophoblastic cells and in 60% of cytotrophoblast components. As we did not find syncytiotrophoblastic cells in our seminoma cases we can neither confirm nor exclude HMGA2 expression in this situation. In normal tissue, HMGA2 was detected in the cytoplasm of the spermatogonial cells. Nuclear expression was weak in spermatocytes and strong in spermatids. Spermatozoa were negative for the protein (Supplemental Figure 1).

A Wilcoxon two-sample rank sum test (Table 6) was conducted to evaluate the separation of tumour entities. Due to multiple testing, a corrected α = 0.005 was used. Despite this restriction, significant differences were detected when comparing scores from YSTs with those from ECs and teratomas. Testing seminoma scores against nonseminoma scores resulted in a significant difference in protein level ($p = 0.0154$). Performing the same test with YST values against all other scores revealed a highly significant difference ($p = 3.821*10^{-6}$). Table 7 gives the results from both real-time PCR and immunostaining analysis.

Discussion

An investigation using real-time PCR and immunohistology was performed to study the expression of *HMGA2* in all subgroups of TGCT. Overall, in comparison to normal tissue, seminomas showed a marginal decrease and ECs a slight upregulation. In teratomas, the expression level was variable and appeared to depend on cellular differentiation. CCs (syncytiotrophblastic giant cells and to a lesser extent cytotrophoblasts) and especially YSTs showed considerably increased expression. In normal testicular tissue, low *HMGA2* expression was detected by real-time PCR. This is most likely caused by temporarily high expression in cells involved in spermatogenesis.
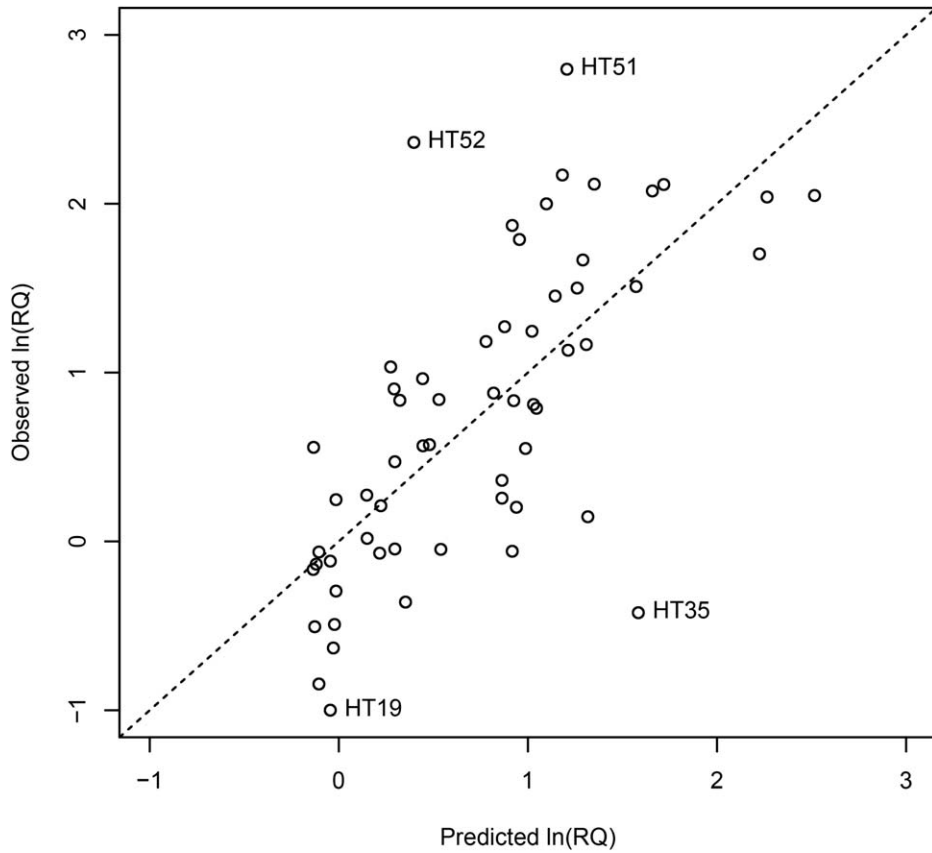
Figure 3. Linear regression analysis of the relationship between lg(RQ) and the proportion of tumour components. Predicted RQ at *x*-axis, observed RQ at *y*-axis, logarithmic scale. Each circle represents one sample. Outliers are marked by case identification (see text for details).
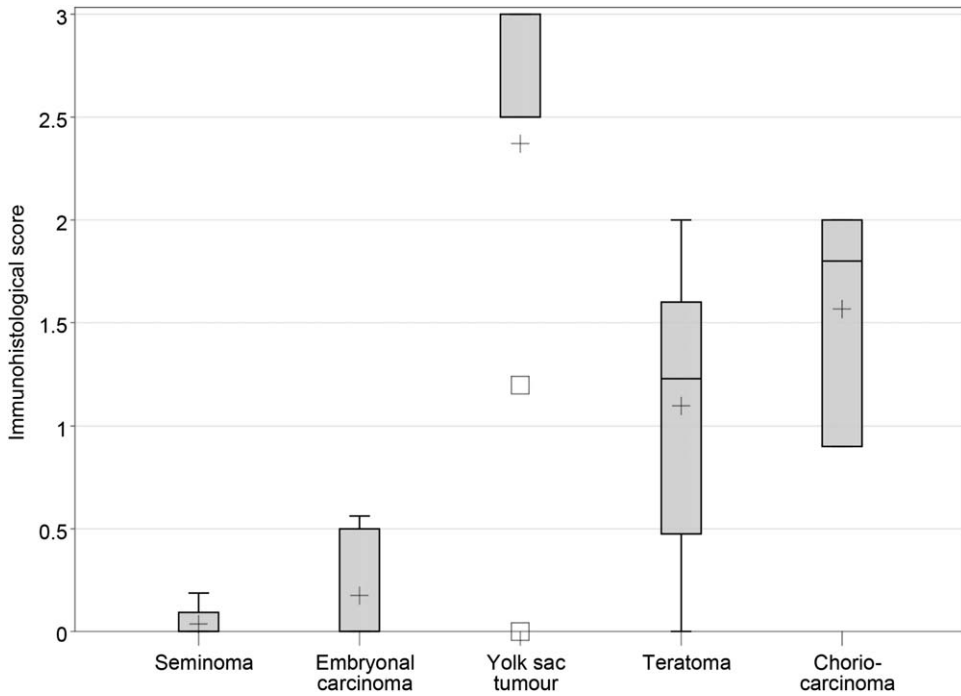

Figure 4. Immunostaining score by type of tumour. Boxes contain the central 50% of all values and a bar at the position of the median, whiskers extend to the extreme values or to 1.5* box height, whichever is smaller. The plus sign shows the arithmetic mean, the rectangles denote outliers.
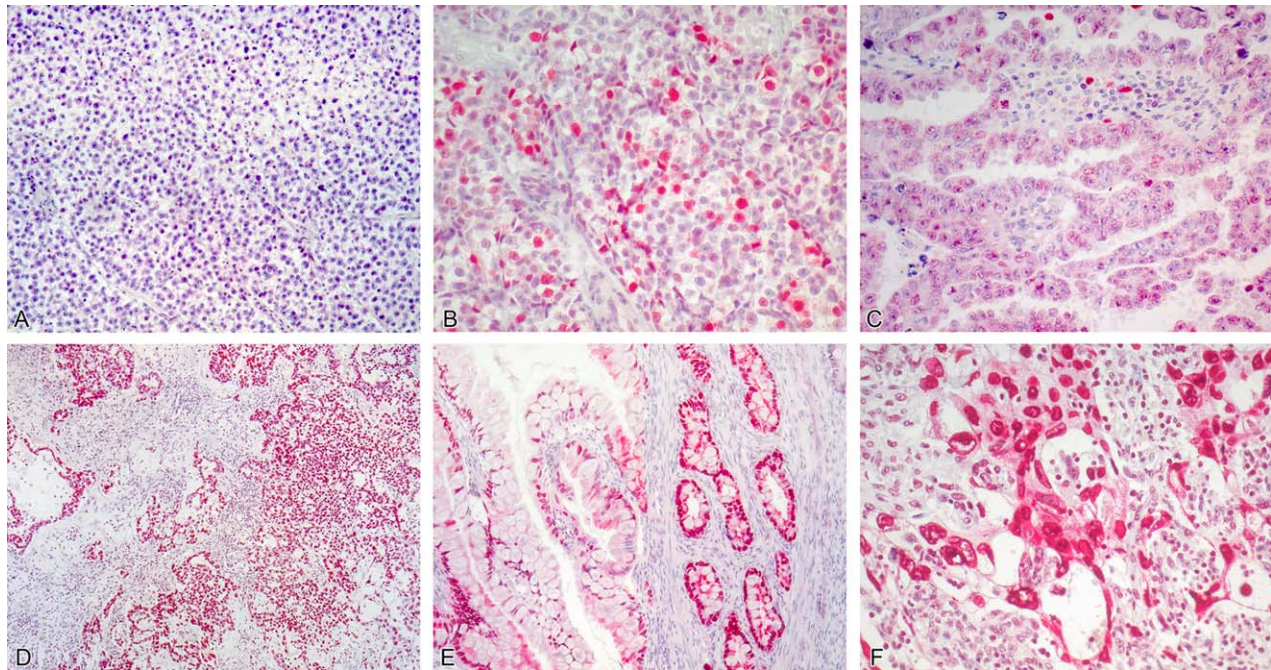
Figure 5. Immunoreactivity for HMGA2 in different tumour subgroups. A: seminoma; B: seminoma with focal HMGA2 reactivity; C: EC with weak granulation; D: YST, microcystic pattern; E: mature teratoma, heterogeneous staining intensity; F: CC intense staining in syncytiotrophoblasts. Original magnifications are given.

Contrary to normal tissue, no such pattern could be detected in most seminomas and immunostaining also showed no HMGA2 expression. Since all pure seminomas contained a percentage of normal tissue, it is plausible that the presence of *HMGA2* mRNA results from that portion. It is also possible that the very low expression detected by real-time PCR is below the threshold of immunohistochemical analysis. One seminoma with relatively high expression (>1) was also investigated using immunohistochemistry. Signals were restricted to one area where single HMGA2 positive cells were scattered in between negative seminoma cells (see Figure 5). One might hypothesize that further transformation of seminoma cells has taken place here. It is known that seminomas can progress into nonseminomas via EC [reviewed in [6]]. As HMGA2 staining in EC was in

the range between not detectable and very weak, another type of transformation seems more likely. Nettersheim *et al*. [41] found that the seminoma cell line TCam-2 differentiates into mixed nonseminoma-like cell types without an intermediate step of EC after stimulation with growth-factors TGF-β1, EGF and FGF4. Gopalan *et al*. [42] also suggested a model in which teratoma, YST and CC develop directly from seminoma. Honecker *et al*. [43] found formations of germ cells developing in nonseminomas, but explicitly not in seminomas. This interesting finding remains to be investigated further. At this stage it can just be stated that we detected cells with enhanced self-renewing capacity within one seminoma.

Table 6. Wilcoxon two-sample test comparing the immunohistochemical score by group

Type of tumour	n	Seminoma	EC	YST	Teratoma	CC
Seminoma	5		0.3219	0.0065	0.0223	0.0616
EC	14			0.0003	0.0054	0.0147
YST	14				0.0045	0.0544
Teratoma	9					0.3294
CC	3					

n, number of values, EC, embryonal carcinoma; YST, yolk sac tumour; CC, choriocarcinoma. In each cell, the *p* value is stated (2-sided *t* approximation with continuity correction); α with Bonferroni correction: 0.005.

Table 7. Summary of qRT-PCR and immunohistochemical results

Tumour component	n (qRT − PCR)	n (immunohisto)	HMGA2 expression
Seminoma	12	5	0
EC	10	14	0–+
YST	2	14	+++
Teratoma	6	9	0–++
CC	–	3	++*

n (qRT − PCR), number of values from real-time PCR; *n* (immunohisto), number of immunohistochemical scores; 0, no expression; (+), very weak expression; ++, moderate expression; +++, strong expression; *, preliminary deduction. Results obtained by linear regression analysis (see Table 5) were used in addition to qRT-PCR data and immunohistochemical scores for this summary.

ECs showed some variation, but always at a low to very low level. Tumours with YST components had a strong tendency towards high *HMGA2* expression, which was clearly confirmed by the immunohistochemical analysis. Teratomas showed a heterogeneous pattern of expression. It seemed that positivity depended on the type of teratoma structure. CC is the most uncommon type of TGCT [10]. Due to the limited availability of CCs, no qRT-PCR-data from homogeneously differentiated tumours could be gathered, and results from the immunohistochemical analysis were limited. Even though the samples present in this study showed a clear tendency, no definitive statement can be made for this subgroup at this point. These data partly confirm the results presented in Franco *et al*. [30]. Besides immunohistology, the authors used western blot analysis and RT-PCR. They also detected high expression of *HMGA2* in YSTs. For seminomas, no expression was reported, which coincides with our results from the immunohistochemical analysis. The residual presence of *HMGA2* expression is likely due to either the high sensitivity of this method, or to the presence of normal cells. In three of six pure teratomas in our study, qRT-PCR values were below one, the other three were slightly elevated. Taking the immunohistochemical analysis into account, and including mixed tumours with a teratoma component, teratomas turned out to be mostly positive, with only one exception of a pure mature teratoma without any positive staining. This is in contrast to the findings presented by Franco *et al*. [30] who did not find expression of *HMGA2* in teratomas. As they did not discriminate between immature and mature teratoma components, direct comparison cannot be performed. Even though it seems unlikely, it is not impossible that all of the 15 samples investigated by Franco *et al*. [30] were mature forms. Results for EC could not be reproduced: Franco *et al*. [30] reported one EC with moderate expression while 14 others showed high expression. This is in clear contrast to our results, for which we can not offer a straightforward explanation. Murray *et al*. [44] also reported positive results for EC. The authors investigated the LIN28/let-7 pathway in malignant germ cell tumours, and found a strongly negative correlation between LIN28 and let-7. As a minor aspect, the study also encompassed the analysis of the expression of *HMGA2*, but, due to the limited sample size of ECs ($n = 3$: one postpubertal, two paediatric), the results might not be representative for this particular group of neoplasms.

As HMGA2 is a nuclear protein, expression analysis generally focuses on nuclear staining [45–49]. Nevertheless, in the present study cytoplasmic positivity was observed occasionally in teratomas and to a lesser extent also in EC (Supplemental Figure 1). Other researchers have made similar observations in different tissues [47,50,51]. Taking into account these data and using a highly specific antibody, it seems less plausible that the cytoplasmic signal was artefactual. The task of developing an approach to clarify these findings remains.

Bearing a high mortality until the mid-1970s, today patients with a TGCT have a 5-year survival rate of 90–95% [52–54]. The remaining deaths are mostly due to chemoresistance of certain subgroups of TGCT: teratomas are benign-appearing, but metastases can form in 29% [15]. Mature teratomas have lost their embryonic features and are therefore completely resistant to cisplatin-based chemotherapy and other clinical treatment strategies [55]. After initial chemotherapy in patients with mixed TGCT with a portion of teratoma, teratoma can be found in the residual mass in 82% of cases [56]. CC metastasises early, therefore a high percentage of mixed tumours show a poor prognosis [15,57]. For several years, different studies found an amount of >50% EC to confer a higher risk for relapses [58]. Recently a follow-up study showed that the any presence of EC, independently of the amount, increases the relapse risk [59]. This illustrates the importance of determining the composition of the particular tumour. A proven set of antibodies for determination of the subgroups exists. Nevertheless, identification can pose a challenge for the pathologist [16,30], and a false diagnosis rate of 4–32% has been reported [60–62].

HMGA2 expression in YST turned out to be different from other types of TGCTs. To a somewhat lesser degree, immunohistochemical staining was also positive for (immature) teratoma components and CC. YST has a wide variety of growth patterns; it can be difficult to differentiate from seminomas, which is of therapeutic relevance [63]. AFP, the only immunohistochemical marker of YST for a long time, often shows only focal staining [15,64]. In recent years glypican-3, SALL4 and LIN28 have been established as diagnostic markers [15,64–68]. Glypican-3 has a higher sensitivity than AFP, but also shows focal staining [64,69]. This was confirmed in the present study. In most glandular growth patterns glypican-3 and HMGA2 showed identical expression. Yet much more HMGA2 positivity was observed in primitive reticular components with non-cohesive cells (Supplemental Figure 1G, 1I). Therefore HMGA2 staining seems to be more sensitive than glypican-3. HMGA2 shows expression (to a somewhat lesser degree) also in (immature) teratomas and CCs. Ota *et al*. [64] also found glypican-3

positivity in teratoma and CC. The specificity of HMGA2 and glypican-3 is therefore comparable for germ cell tumours. SALL4 and LIN28 are both sensitive markers. SALL4 is positive in all germ cell tumour subgroups including ITGCNU [65]. Therefore, it cannot be used to distinguish between different subgroups. LIN28 is sensitive for ITGCNU, seminomas, ECs and YSTs [66]. β-HCG is an established marker for CC, but as Lempiäinen *et al.* [70] showed recently, it can also be positive in ECs. Furthermore the authors found no expression in one of three pure CCs and two mixed TGCTs containing a CC component.

Our data suggest that the use of a HMGA2-specific antibody could be a sensible addition to existing markers and potentially help to decrease the rate of false diagnoses. A study composed of a larger number of FFPE and fresh-frozen samples, including a representative number of CCs, could bring this method even closer to clinical application. In addition, investigation of the expression of *HMGA2* in ovarian and extragonadal germ cell tumours would be of particular interest.

Acknowledgements

Contract/grant details

Authors' contributions

L.K. and J.B. conceived study. L.K., A.G. and J.B. designed study. L.K. carried out molecular genetic studies. A.G., L.K., B.H., T.L. and K.B. carried out immunohistological studies. B.H., T.L. and K.B. performed pathological analysis. B.H., T.L., K.B., G.B. and K.G. provided study material. W.W., L.K. and A.G. carried out statistical analysis. L.K. and A.G. wrote the manuscript. All authors revised the manuscript and had final approval of the submitted and published versions.

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SUPPLEMENTARY MATERIAL ON THE INTERNET

Additional Supporting Information may be found in the online version of this article.

Supplemental Figure 1. Examples of HMGA2 immunoreactivity in normal testis tissue and different tumour subgroups. A: normal seminiferous tubules showing nuclear staining in spermatocytes and spermatids and weak cytoplasmic staining in spermatogonial cells, B: EC surrounded by YST, C: mature teratoma, glandular structure, D: immature teratoma, mesenchymal appearance, E: same as D, negative glypican-3 staining shows that no YST components are present, F: mature teratoma, glandular structures positive, muscular structures negative, G: glypican-3 staining in YST components restricted to glandular growth patterns, H: same as G, HMGA2 staining in YST is also strongly positive in primitive reticular components with noncohesive cells. Original magnifications are given.

Supplemental Information
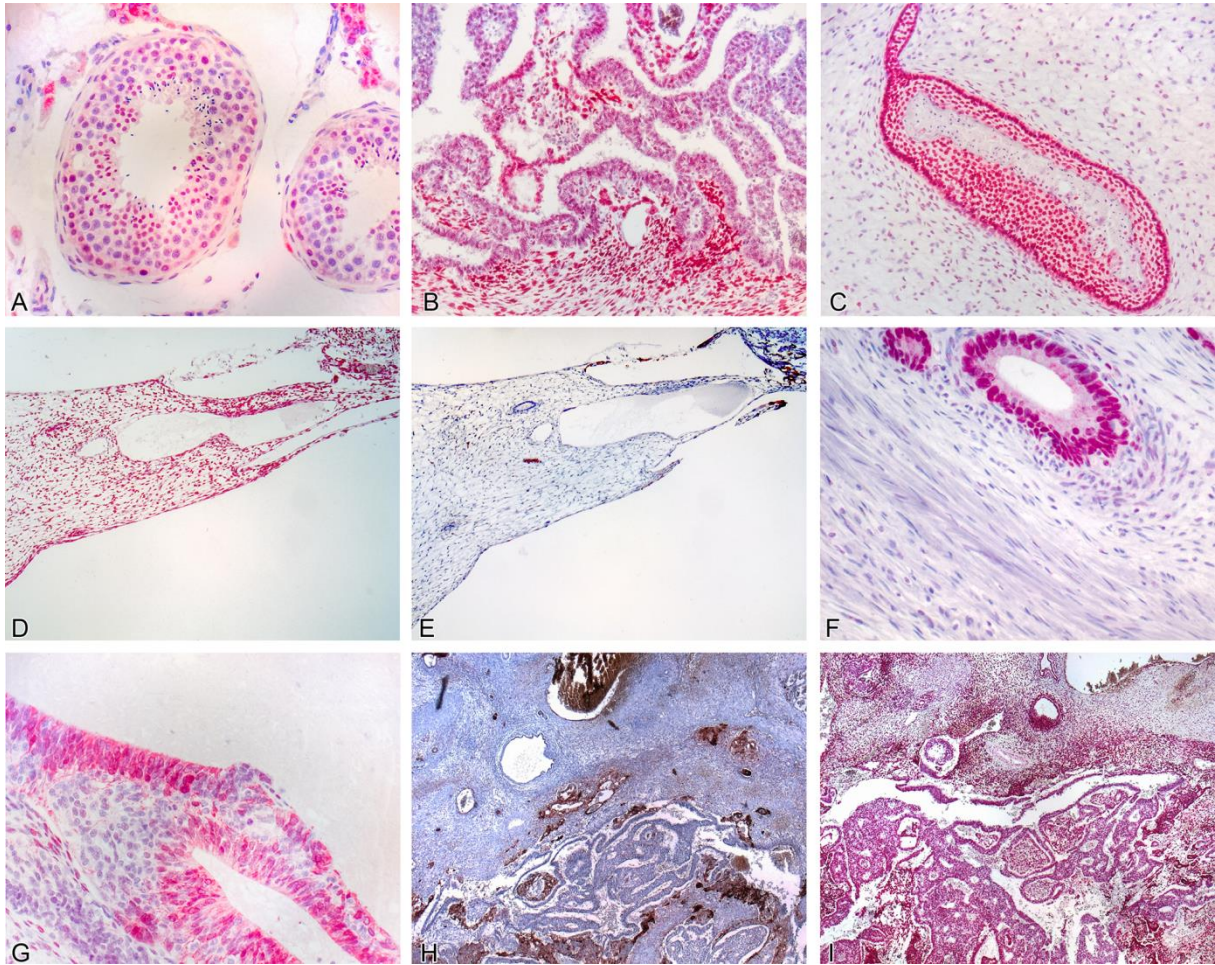
Supplemental Figure 1: Examples for HMGA2 immunoreactivity in normal testis tissue and different tumour subgroups. A: normal seminiferous tubules showing nuclear staining in spermatocytes and spermatids and weak cytoplasmatic staining in spermatogonial cells, 200x; B: EC surrounded by YST, 100x; C: mature teratoma, glandular structure, 100x; D: immature teratoma, mesenchymal appearance, 50x; E: same as D, negative glypican-3 staining proves that no YST components are enclosed, 50x; F: mature teratoma, glandular structures positive, muscular structures negative, 200x; G: glypican-3 staining in YST components restricted to glandular growth patterns, 25x; H: same as G, HMGA2 staining in YST is strongly positive also in primitive reticular components with noncohesive cells.

4 Diskussion

Während der Tumorentstehung erwerben maligne Zellen embryonale Eigenschaften, deshalb teilen sie etliche Charakteristika mit embryonalen Zellen. So sind z.B. Trophoblast- und Tumorzellen invasiv, entgehen der Immunabwehr, beeinflussen angiogenetische Prozesse und exprimieren (häufig) humanes Choriongonadotropin (Simpson et al. 2005).

Eine essentielle Eigenschaft von Tumorzellen ist die Fähigkeit zu migrieren und dadurch Metastasen zu bilden. Trophoblasten und Keimzellen migrieren ebenfalls, werden dabei aber über eine Vielzahl von Wachstumsregulations- und -Kontrollgenen eng kontrolliert. In Tumorzellen werden einige dieser Gene wieder reaktiviert. Im Falle des in dieser Arbeit untersuchten Stammzellfaktors *HMGA2* führt die Reexpression unter anderem dazu, dass eine höhere Invasivität und damit häufig auch schlechtere Prognose zu beobachten ist (Morishita et al. 2013, Rizzi et al. 2012). Als ein Grund für höhere Invasivität konnte eine Rolle vom *HMGA2* bei der epithelial-mesenchymalen Transition (EMT) gezeigt werden (Morishita et al. 2013, Thuault et al. 2008, Ding et al. 2014).

Neben *HMGA2* wurden in dieser Arbeit exemplarisch miRNAs der Cluster C19MC und miR-371-3 untersucht. Die spezifische Rolle von miRNAs der Cluster C19MC und miR-371-3 in der embryonalen Entwicklung wird weiterhin entschlüsselt. miRNAs des C19MC werden physiologisch fast ausschließlich in der Plazenta exprimiert (Bentwich et al. 2005, Liang et al. 2007, Luo et al. 2009). Sie finden sich in hoher Konzentration in Exosomen im peripheren Blut der Mutter (Luo et al. 2009, Donker et al. 2012) und spielen außerdem eine Rolle bei der Immunreaktion in Zuge von Virusinfektionen (Delorme-Axford et al. 2013). Daher liegt es nahe, anzunehmen, dass die miRNAs des C19MC eine entscheidende Rolle in der feto-maternalen Kommunikation spielen und dazu beitragen, den Fetus vor dem mütterlichen Immunsystem zu schützen. Die miRNAs des miR-371-3-Clusters haben eine Funktion in der Erhaltung von Stammzelleigenschaften (Langroudi et al. 2015) sowie in der Zelldifferenzierung (Kim et al. 2011), sie werden vor allem in embryonalen Stammzellen exprimiert. Bentwich et al. (2005) fanden beim Vergleich von Plazenta, Hoden, Thymus, Gehirn und Prostata die mit Abstand höchste Expression des miR-371-3-Clusters in der Plazenta.

In den folgenden Abschnitten werden die Untersuchungen zur Expression von miRNAs der Cluster C19MC und miR-371-3 sowie die*HMGA2*-Expression in physiologischem (Plazenta) und pathologischem (testikuläre Keimzelltumoren) Zusammenhang diskutiert.

4.1 Plazentare Expression von miRNAs der Cluster C19MC und miR-371-3 sowie von *HMGA2* im Schwangerschaftsverlauf

Die Plazenta ist das am wenigsten verstandene Organ des Menschen (Guttmacher, Maddox & Spong 2014). Sie hat großen Einfluss auf die Gesundheit von Mutter und Kind, nicht nur während der Schwangerschaft, sondern lebenslang (Barker, Thornburg 2013). In der vorliegenden Arbeit sollte anhand der Genexpression von miRNAs der Cluster C19MC und miR-371-3 sowie von *HMGA2* ein Beitrag zum besseren Verständnis der plazentaren Entwicklung geleistet werden. Im Fokus standen hierbei generell die Expressionsentwicklungen im Schwangerschaftsverlauf sowie die Frage nach der Rolle der untersuchten Gene im Abortgeschehen.

In Publikation I wurde gezeigt, dass die untersuchten miRNAs des C19MC mit zunehmender Schwangerschaftsdauer stärker exprimiert werden, während das miR-371-3-Cluster eine nur leichte, nicht signifikante Expressionssteigerung vom ersten zum dritten Trimester aufwies. Eine Arbeit von Xie et al. (2014) zur Funktion von miRNAs des C19MC in Plazenten im Schwangerschaftsverlauf zeigte, dass miRNAs des C19MC im villösen Trophoblasten stärker exprimiert werden als im extravillösen. Durch Transfektion von HTR-8/SVneo-Zellen (immortalisierte extravillöse Trophoblast-Zellen (EVT)), die nur wenig C19MC exprimieren) mit dem C19MC-Transkript wurden die Invasivität dieser Zellen deutlich reduziert, so dass angenommen werden kann, dass C19MC die Invasivität des EVT beeinflusst. Interessanterweise fanden Xie et al. (2014) keinen Expressionsunterschied zwischen Erst- und Dritttrimester-Proben. Das steht im Gegensatz zu den hier dargestellten Ergebnissen und zu Ergebnissen von Morales-Prieto et al (2012) und Gu et al. (2013). Während die für die vorliegende Dissertation durchgeführten Untersuchungen sowie Morales-Prieto et al. (2012) einen signifikanten Anstieg der Expression von miRNAs des C19MC vom ersten zum dritten Trimester zeigten, fanden Gu et al. (2013) einen Expressionsabfall im Verlaufe der Schwangerschaft.

Geht man davon aus, dass EVT in der frühen Schwangerschaft und bis in das zweite Trimester in die Spiralarterien und das Endometrium einwandern (Pijnenborg et al. 1980) und eine gleichbleibend starke Invasion zu einem noch tieferen Einwachsen führt, würde man erwarten, dass die Expression von C19MC mit zunehmender Schwangerschaftsdauer ansteigt und damit zur Invasionskontrolle beiträgt. Befunde von Ding et al. (2015) stützen diese These. Die Autoren zeigten, dass miR-519d-3p bei Präeklampsiepatientinnen stärker exprimiert wird als bei gesunden Schwangeren und vermuten einen Zusammenhang mit der bei Präeklampsien zu beobachtenden zu schwachen Invasion des EVT.

Ishida et al. (2015) fanden bei Untersuchungen der natürlichen Killerzellen (NK) aus peripherem mütterlichem Blut einen Anstieg der exosomalen miRNAs des C19MC vom ersten zum dritten Trimester. Die miRNAs haben in diesem Zusammenhang eine andere Funktion. Im mütterlichen Blut ist ihre Rolle nicht die Steuerung der Invasivität, sie scheinen stattdessen an der Immunmodulation beteiligt zu sein.

miRNAs des C19MC werden von der Plazenta in Exosomen sezerniert und von den mütterlichen NK-Zellen aufgenommen (Kambe et al. 2014). Im Kulturversuch mit einer Chorionkarzinom-Zelllinie (BeWo) zeigte sich, dass die von diesen Zellen ausgeschleusten C19MC enthaltenden Exosomen von Jurkat-Zellen, einer humanen T-Lymphozyten-Zelllinie, aufgenommen werden. Kambe et al. (2014) zeigten für miR-517a-3p, dass sie die *PRKG1* mRNA (protein kinase, cGMP-dependent, type I) herunterreguliert. PRKG1 reguliert in Immunzellen die T-Zell-Aktivierung und -Proliferation. Eine Steuerung der mütterlichen Immunantwort über einen miR-517a-3p-PRKG1-Signalweg scheint damit denkbar und könnte ein weiteres Mosaiksteinchen im Verständnis der Rolle von C19MC in der feto-maternalen Toleranz sein.

miRNAs des C19MC sind im Zusammenhang mit Resistenz gegenüber viralen Infektionen relevant. Delorme-Axford et al. (Delorme-Axford et al. 2013) wiesen nach, dass primäre Trophoblastzellen immun gegen eine Reihe von Virusinfektionen sind, weil die in ihnen exprimierten miRNAs des C19MC an der Induktion von Autophagie nach Viruskontakt beteiligt sind. Diese Immunität wiederum konnte durch den Transfer Exosomen-haltigen Mediums auf andere Zelltypen übertragen werden. Autophagie wurde ebenfalls ausgelöst, nachdem Zellen, die kein C19MC exprimieren mit einem C19MC BAC-Vektor oder mit mimics von miR-517a/b-3p, -516b-5p

oder -512-3p transfiziert wurden. Damit zeigt sich eine zunehmende Evidenz dafür, dass die Sezernierung von plazentaren miRNAs in das mütterliche Blut im Zusammenhang mit feto-maternaler Toleranz steht (Ouyang et al. 2014, Mouillet et al. 2015, Sadovsky et al. 2015).

Demgegenüber ist das miR-371-3-Cluster im Kontext der Schwangerschaft bislang sehr wenig untersucht. Das in der vorliegenden Arbeit gewonnene Ergebnis, dass sich die Expression im Schwangerschaftsverlauf nicht signifikant ändert, stimmt mit dem von Morales-Prieto et al. (2012) überein. Im Gegensatz dazu fanden Gu et al. (2013) eine verringerte Expression vom ersten zum dritten Trimester. Weitere Evidenz zur Rolle von miR-371-3 im Schwangerschaftsverlauf lieferten Wang et al. (2016) bei Untersuchungen wiederholter Spontanaborte, wie weiter unten diskutiert wird.

Zur *HMGA2*-Expression in Plazenten gibt es ebenfalls nur wenige Daten. Frühere Untersuchungen von Gattas et al. (1999) konzentrierten sich auf einen eingeschränkten Zeitraum (19. – 22. SSW). In einer neuen Arbeit zur *HMGA2*-Expression in Plazenten untersuchten Genbacev et al. (2016) Trophoblastprogenitorzellen (TBPC) in Zellkulturen aus Gewebeproben aller drei Trimester. Im Verlaufe der Schwangerschaft blieb die Expression in den TBPC, wie für einen Stammzellfaktor zu erwarten, hoch. Für Publikation II wurde erstmals die *HMGA2*-Expression über den gesamten Schwangerschaftsverlauf in vivo untersucht. Erstmals wurden auch Plazenten von induzierten Schwangerschaftsunterbrechungen mit denen von Spontanaborten verglichen. Die hohe *HMGA2*-Expression im ersten Trimester fiel mit Beginn des zweiten Trimesters ab um sich dann bis zum Ende der Schwangerschaft auf einem niedrigen Niveau (im Mittel Faktor 30 niedriger als im ersten Trimester) einzupendeln. Die Phase der hohen *HMGA2*-Expression geht einher mit einer niedrigen Sauerstoffkonzentration in der Plazenta. Nach Huppertz et al. (2009) proliferieren die Trophoblastzellen in der sauerstoffarmen Umgebung stark, während sie mit steigender O_2-Konzentration stärker invasiv wachsen und damit den kindlichen Teil der Plazenta fest mit dem mütterlichen verzahnen. *HMGA2* fungiert hier also möglicherweise als Proliferationsfaktor.

Einen Beleg für diese These fanden kürzlich Genbacev et al. (2016). Die Autoren untersuchten die Rolle von *HMGA2* in TBPC, indem sie durch siRNA die *HMGA2*-

Expression reduzierten. Parallel dazu ging auch das Zellwachstum deutlich zurück, so dass gefolgert wurde, dass *HMGA2* in TBPC die Proliferation undifferenzierter Zellstadien fördert.

In einer vorher publizierten Arbeit untersuchten Genbacev et al. (2011) Trophoblastzellkulturen und stellten fest, dass *HMGA2* in TBPC besonders stark, mit zunehmender Differenzierung aber schwächer exprimiert wird. In differenzierten Zytotrophoblastzellen fanden Genbacev et al. (2011) eine deutlich niedrigere Expression.

Weitere plazentare pluripotente Stammzellen sind nach Genbacev et al. (2016) wahrscheinlich im Zottenstroma lokalisiert. Diese Annahme wird von unseren Daten gestützt: HMGA2-Protein wurde vor allem im Zottenstroma detektiert. Der mesenchymale Gewebeanteil im Zottenstroma wird im Laufe der Schwangerschaft geringer, schon in der 13. SSW besteht es hauptsächlich aus retikulärem Bindegewebe, obwohl der Zottenbaum bis spät in die Schwangerschaft hinein reift (Hinrichsen 1990). Daher erscheint der Expressionsabfall ab dem zweiten Trimester plausibel. In Synzytiotrophoblasten- und Zytotrophoblastenkernen war in Publiktation II nur geringe HMGA2-Aktivität detektierbar, stattdessen zeigte das Zytoplasma beider Zelltypen deutlich positive Reaktionen. Genbacev et al. (2011) beschrieben dieses Phänomen ebenfalls für sich differenzierende Trophoblastzellen. Die zytoplasmatische Lokalisation des HMGA2-Proteins wurde bislang nicht oft beschrieben (Kloth et al. 2015, Ding et al. 2014, Genbacev et al. 2011, Wang et al. 2011). Vermutlich wird sie häufiger beobachtet, aber als Artefakt angesehen und deshalb nicht diskutiert. Ob es sich um ein echtes Signal handelt, ist bislang nicht systematisch untersucht. Die hohe Spezifität des eingesetzten HMGA2-P3-Antikörpers (Wang et al. 2011) spricht allerdings dafür, dass es sich nicht um einen Artefakt handelt. Obwohl HMGA generell als rein nukleäre Proteine gelten (Cattaruzzi et al. 2007), ist von HMGA1 bekannt, dass sich seine Expression von nukleär zu zytoplasmatisch wandelt, wenn Zytotrophoblasten sich zu extravillösen, also migrierenden, Trophoblasten differenzieren (Bamberger et al. 2003). Ob HMGA2 eine ähnliche Funktion hat, bleibt zu untersuchen.

Welche Rolle haben miRNAs der Cluster C19MC und miR-371-3 sowie *HMGA2* im Abortgeschehen? Dies war die zweite Frage, der sich diese Arbeit widmete.

Etwa 20 % der bereits diagnostizierten Schwangerschaften führen zum Spontanabort (Wilcox et al. 1988), etwa die Hälfte davon ist auf chromosomale Aberrationen zurückzuführen (Guerneri et al. 1987). Die Ursachen der anderen 50 % der Spontanaborte sind Gegenstand vieler Untersuchungen. Implantationsstörungen, Plazentabildungsstörungen, Abstoßungsreaktionen aufgrund eingeschränkter maternaler Immuntoleranz, Infektionen, Hormonstörungen und weitere Störungen können Gründe sein. Entsprechend vielfältig fallen die Befunde bei der Ursachensuche aus. Zunehmend werden hierfür miRNAs in den Fokus genommen. Beispielsweise fanden Ventura et al. (2013) miR-17 und miR-19b bei Spontanaborten signifikant niedriger exprimiert. Diese beiden miRNAs fördern den Zellzyklus sowie Proliferation, Invasion und Migration von Zellen. Sie werden außerdem in verschiedenen Tumoren wie z.B. Lungenkarzinomen (Hayashita et al. 2005), und Colonkarzinomen (Monzo et al. 2008) überexprimiert. Nach Ergebnissen von Wang et al. (2016) gehen wiederholte Spontanaborte mit etwa vierfach erniedrigter Expression von miR-371a-5p und miR-372 im villösen Trophoblasten sowie mit 5-20 fach erhöhter Expression diverser miRNAs des C19MC in der mütterlichen Dezidua einher. Targetgenanalysen ergaben für die Dezidua hauptsächlich Targetgene, die mit Zelltod, Apoptose, Proliferation und Hormonrezeptivität im Zusammenhang stehen. Demgegenüber wurden im villösen Trophoblasten vor allem Targets identifiziert, die mit der Embryonalentwicklung im Zusammenhang stehen, wie Proliferation, Anti-Apoptose und Angiogenese. Daraus folgerten die Autoren, dass die im Trophoblasten aberrant exprimierten miRNAs die Invasion und damit die Plazentabildung beeinflussen, während einige miRNAs des C19MC in der Dezidua unter anderem eine gesteigerte Apoptoserate bewirken. In der hier vorgelegten Untersuchung (Publikation I) wurden keine signifikanten Expressionsunterschiede zwischen induzierten- und Spontanaborten gefunden. Möglicherweise deuten die Ergebnisse von Wang et al. (2016) auf väterliche Deregulation der miRNA-Expession hin, die zu einem systematischen Problem der Plazentaentwicklung bei betroffenen Paaren und damit wiederholten Aborten führt. In der hier vorgelegten Arbeit standen keine Informationen über die Anzahl der vorher bereits stattgefundenen Aborte zur Verfügung, so dass die Ergebnisse nicht ohne weiteres vergleichbar sind.

Es ist trotz negativer Ergebnisse nicht ausgeschlossen, dass die untersuchten miRNAs am komplexen Abortgeschehen beteiligt sind. Ihre potentielle Rolle dürfte aber eher klein sein.

Die *HMGA2*-Expression von induzierten- und Spontanaborten unterschied sich ebenfalls nicht signifikant (Publikation II). Der Expressionsabfall verlief in beiden Gruppen ähnlich. Daraus kann vorsichtig gefolgert werden, dass auch *HMGA2* keine offensichtliche Rolle im Spontanabortgeschehen hat. Für ein Monitoring der frühen Schwangerschaft bezüglich drohenden Abortes bieten sich die untersuchten miRNAs und *HMGA2* daher nicht an.

Ob sie im Rahmen von Präeklampsie-Früherkennung als Biomarker genutzt werden können, war keine ursprüngliche Fragestellung, deshalb wurde das Untersuchungs-kollektiv nicht mit Präeklampsieplazenten angereichert.

Diese Frage sollte weiter untersucht werden, denn Präeklampsien sind mit 2-8% aller Schwangerschaften eine der häufigsten Komplikationen (Duley 2009). Sie entstehen auf dem Boden gestörter Plazentaentwicklung durch zu geringe Invasion des EVT bereits in der frühen Schwangerschaft. Vermutlich durch Entzündungsreaktionen der Mutter wird eine Kaskade in Gang gesetzt, die in Proteinurie und Bluthochdruck mündet (LaMarca et al. 2016) und in schweren Verlaufsformen lebensgefährlich für Mutter und Kind sein kann. Klinische Symptome der Präeklampsie treten in der Regel erst nach der 20. SSW auf (AWMF 2014), die plazentaren Dysfunktionen beeinträchtigen Mutter und Kind aber schon deutlich vorher. Eine Identifizierung von Risikopatientinnen bereits vor der 16. SSW könnte es ermöglichen, durch niedrig dosierte Aspiringabe die Präeklampsieprävalenz zu halbieren (Bujold et al. 2010).

Montagnana et al. (2016) kommen in einem kürzlich erschienenen Review zu dem Schluss, dass miRNAs vielversprechende Kandidaten für ein frühes Monitoring sein könnten. Nachdem viele der in den letzten Jahren publizierten Arbeiten zu miRNA-Expression bei Präeklampsien (Hromadnikova et al. 2013, Noack et al. 2011, Anton et al. 2015, Miura et al. 2015) und die häufig damit einhergehende fetale Wachstumsretardierung (Hromadnikova et al. 2013, Higashijima et al. 2013, Hromadnikova et al. 2015) spätere Phasen der Schwangerschaft untersuchten (> 27. SSW), wird seit Neuestem die Eignung von miRNAs als Risikoindikator in der Früh-schwangerschaft untersucht. Hromadnikova et al. (2017) zeigten kürzlich, dass im

Serum von Schwangeren, die später Präeklampsie entwickeln, bereits in der 10. - 13. SSW signifikant erhöhte miR-517a-5p-Konzentrationen messbar waren.

4.2 *HMGA2* als Marker für postpubertäre testikuäre Keimzelltumoren

Die nächste in dieser Arbeit behandelte Frage war die nach der Eignung von *HMGA2* als diagnostischer Marker für testikuläre Keimzelltumoren. Postpubertär auftretende testikuläre Keimzelltumoren sind histologisch eine äußerst vielfältige Gruppe, dementsprechend zeigen sie ein sehr heterogenes Erscheinungsbild. Ihre präzise Diagnose ist nach wie vor eine Herausforderung.

Zur *HMGA2*-Reexpression in Keimzelltumoren sind nur wenige Daten publiziert. Franco et al. (2008) untersuchten die HMGA1- und HMGA2-Expression in testikulären Keimzelltumoren und kamen zu dem Ergebnis, dass HMGA1 und HMGA2 insbesondere in Kombination eine hilfreiche Ergänzung zu bereits etablierten Biomarkern sind und die Diagnostik damit verbessern könnten. Die wenigen später erschienenen Artikel zur HMGA2-Expression in Keimzelltumoren waren Reviews (Favilla et al. 2010, Chieffi 2011, Chieffi, Chieffi 2013, Chieffi, Chieffi 2014), die die Erkenntnisse von Franco et al. (2008) lediglich zitierten.

Die in der vorliegenden Arbeit gewonnenen Ergebnisse waren in einigen Punkten konträr zu denen von Franco et al. (2008). So war in der hier beschriebenen Untersuchung in 58 von 59 Proben eine Basisexpression von *HMGA2* messbar. Wie von Chieffi et al. (2002) festgestellt, exprimieren unreife Spermienstadien HMGA2, so dass die Basisexpression vermutlich auf die in jeder der von uns untersuchten Proben vorhandenen Anteile von normalem Hodengewebe zurückzuführen war. Vermutlich war sie aufgrund der höheren Empfindlichkeit der von uns eingesetzten qRT-PCR detektierbar.

Deutlich unterschieden sich die in Publikation III gewonnenen Ergebnisse allerdings hinsichtlich der *HMGA2*-Expression in Embryonalkarzinomen und Teratomen von denen Francos und Kollegen (2008). Während Franco et al. (2008) starke Expression in Embryonalkarzinomen fanden, konnten in der vorliegenden Arbeit weder in der qRT-PCR noch immunhistochemisch nennenswerte Expressionen in dieser Subgruppe detektiert werden. Demgegenüber exprimierten Teratome in unserer Untersuchung - anders als von Franco et al. (2008) beschrieben - meistens *HMGA2*. Abhängig vom Reifegrad des Teratoms waren mittlere bis hohe Expressionen bei

unreifen und niedrige Expressionen bei reifen Teratomen zu beobachten. Publikation III zeigte, dass anhand der qRT-PCR-Ergebnisse Tumorsubgruppen abgegrenzt werden können. Reine Dottersacktumoren ließen sich von reinen Seminomen, Embryonalkarzinomen und Teratomen anhand der *HMGA2*-Expression verlässlich unterscheiden.

Für die Unterscheidung in Mischtumoren (d.h. mit mehr als einer Subgruppe) wurde ein Modell entwickelt, das es erlaubte, mit einem Bestimmtheitsmaß von 0,66 und einer Irrtumswahrscheinlichkeit von $1,1*10^{-12}$ anhand der Zusammensetzung des Mischtumors auf die Expression zu schließen. Für die Routinediagnostik ist die qRT-PCR recht aufwendig und teuer und sie bietet bislang auch keine Vorteile gegenüber der Immunhistochemie. Das berechnete lineare Regressionsmodell lässt außerdem nur die statistische Aussage zu, dass mit Y% Wahrscheinlichkeit bei einer gegebenen Expression ein Anteil von Tumorsubtyp X in der Probe enthalten ist. Die lineare Regression war zudem für Seminomanteile nicht signifikant. Für diagnostische Zwecke ist diese Aussage daher nicht präzise genug. Deshalb wurde exemplarisch an 23 der 59 Keimzelltumoren das Expressionsmuster des HMGA2-Proteins untersucht.

Ergänzend zur Basisdiagnostik an HE-gefärbten Schnitten wird in vielen Fällen bereits seit langem ein umfangreiches Panel an immunhistochemischen Markern eingesetzt, um die histologische Zusammensetzung von Keimzelltumoren sicherer zu ermitteln. Die präzise Diagnose ist sowohl von therapeutischer als auch von prognostischer Relevanz (Eble et al. 2004, Ulbright 2005, Cheng, Lyu & Roth 2017). Das bereits etablierte und breit genutzte Panel lässt aber nach wie vor in Zweifelsfällen keine sichere Differenzierung zu. Eine Ergänzung der Diagnostik durch zusätzliche Marker ist daher von hohem Nutzen (Ota et al. 2006, Stoop et al. 2008, Cao et al. 2009, Gopalan et al. 2009, Klumper et al. 2015).

In Publikation III zeigte sich, dass von wenigen Ausnahmen abgesehen die in der qRT-PCR gewonnenen Ergebnisse sehr gut mit den immunhistochemischen Untersuchungen übereinstimmten. Dottersackanteile konnten mit Hilfe der HMGA2-Immunhistochemie verlässlich entdeckt werden. Dies kann für die Routinediagnostik eine hilfreiche Ergänzung sein, denn Dottersacktumoren präsentieren sich in einer großen morphologischen Vielfalt und sind deshalb teilweise schwierig zu erkennen. Entsprechend werden sie am häufigsten übersehen (Cheng, Lyu & Roth 2017).

Ermutigende Ergebnisse für die Eignung von HMGA2 als diagnostischer Marker deuteten sich auch bei Chorionkarzinomen an. Je größer der Chorionkarzinomanteil im Tumor, desto schlechter die Prognose, deshalb ist ein möglichst genaues Bestimmen der jeweiligen Tumoranteile wichtig (Wojno, Bégin 2010). Die hier untersuchte Stichprobe war mit nur drei Chorionkarzinomanteilen sehr klein, so dass sich aus den Ergebnissen nur eine Tendenz ablesen lässt. Da der bislang in der Diagnostik eingesetzte Chorionkarzinommarker ß-HCG relativ spezifisch aber nicht sehr sensitiv ist (Lempiäinen et al. 2014), wäre ein zusätzlicher Biomarker für Chorionkarzinome deshalb wünschenswert.

HMGA2 hat sich in der vorliegenden Arbeit als vielversprechender Kandidat für eine Ergänzung des diagnostischen Panels gezeigt. Um HMGA2 in die diagnostische Praxis zu integrieren, sollte eine größere Stichprobe untersucht werden. Insbesondere müssten deutlich mehr Chorionkarzinome einbezogen werden und die gesamte Vielfalt der morphologisch unterschiedlichen Dottersacktumoren sollte abgebildet werden.

Interessant wäre es außerdem, Teratome genauer zu untersuchen, um über die Tendenz „je reifer desto geringere HMGA2-Expression" verlässlichere Aussagen machen zu können. Therapeutisch und prognostisch spielt es keine Rolle, ob ein Teratom aus reifen oder unreifen Zellen besteht oder wie das Verhältnis beider zueinander ist (Sesterhenn, Davis 2004, Cheng, Lyu & Roth 2017, Howitt, Berney 2015). Die weitere Untersuchung der Rolle von HMGA2 in testikulären Keimzelltumoren könnte aber neue Impulse für Vorstellungen zur Pathogenese dieser Tumoren geben. Dabei ist nicht notwendigerweise damit zu rechnen, dass die HMGA2-Expression komplett mit den bisher etablierten histologischen Differenzierungsmustern korreliert. Denkbar ist auch, dass die HMGA2 Expression Informationen z.B. zu Reifegrad, Differenzierungspotenz und Ausbreitung geben kann, die nur teilweise durch das histologische Bild der jeweiligen Tumoren reflektiert werden und eventuell mit dem hier nicht berücksichtigten genetischen Typ des Tumors (häufigste Aberration: Isochromosom 12p) korrelieren.

Zur Pathogenese testikulärer Keimzelltumoren schlugen Oosterhuis und Looijenga (2005) ein inzwischen weitgehend akzeptiertes Schema vor. Die Tumoren entwickeln

sich demnach aus malignen Vorläuferzellen, den intratubulären undifferenzierten Keimzellneoplasien (intratubular germ cell neoplasia undifferentiated, ITGCNU). Etwa 70% dieser Läsionen präsentieren sich innerhalb von sieben Jahren schließlich als Keimzelltumor (Looijenga, Stoop & Biermann 2014). Aus ITGCNU entwickeln sich entweder Seminome, die frühen Keimzellen ähneln oder Embryonalkarzinome, die embryonalen Stammzellen der inneren Zellmasse der frühen Blastozyste imitieren (Honecker et al. 2006). Durch Reprogrammierung können sich Seminome in Embryonalkarzinome umwandeln (Oosterhuis, Looijenga 2005). Differenzieren sich Embryonalkarzinome weiter, entwickeln sich die somatischen Zellen ähnelnden Teratome oder die extraembryonale Strukturen imitierenden Dottersacktumoren und Chorionkarzinome (Looijenga 2009, Wittekind, Neid 2003).

Interessanterweise bilden die in dieser Arbeit erstmals beschriebenen HMGA2-Expressionsmuster das von Osterhuis und Looijenga (2005) vorgeschlagene Schema gut ab: In ITGCNU wird HMGA2 differenziert exprimiert, denn nur in einem Teil der malignen Zellen ist HMGA2 detektierbar. Im Gegensatz dazu detektiert der routinemäßig in der Diagnostik eingesetzte Pluripotenzmarker OCT3/4 sehr sensitiv alle ITGCNU. Hier wären genauere Untersuchungen nötig, um die Ursache für die differenzierte Expression zu finden. Möglicherweise zeigt eine HMGA2 exprimierende ITGCNU-Zelle ein frühes Stadium der Entwicklung an, während die negativen ITGCNUs bereits weiter in Richtung invasiver Verläufe transformiert sind, denn die weitere Entwicklung in Seminom oder Embryonalkarzinom resultiert in einer Deaktivierung von HMGA2. Jorgensen et al. (2013) fanden differenziert exprimiertes DMRT1, ein im Mitose-Meiose-Übergang involviertes Protein, in ITGCNU-Zellen und äußerten die Vermutung, dass die Expression einen frühen oder „schlafenden" Zustand der ITGCNU anzeigen könnte.

Im Rahmen der Differenzierung in Dottersacktumor, Teratom und Chorionkarzinom ist schließlich eine HMGA2-Reaktivierung zu beobachten (Abb. 1).
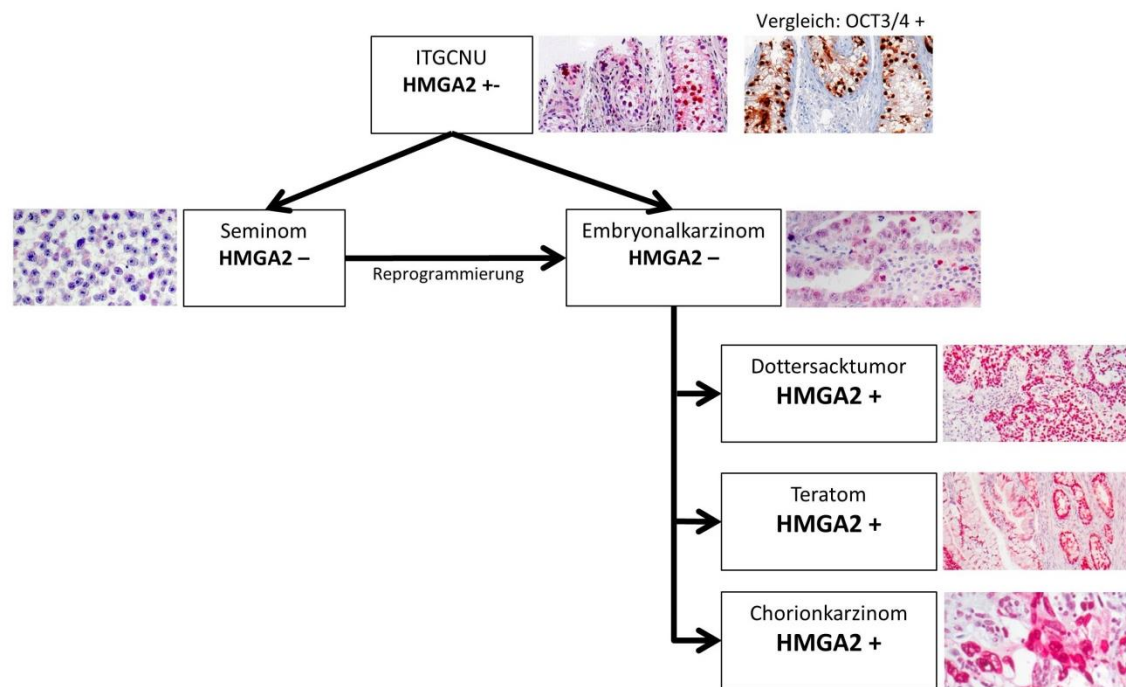
Abb. 1: Die immunhistochemische HMGA2-Detektion in Hodentumorvorstufen (ITGCNU) zeigt nur teilweise positive ITGCNU-Zellen. Im Gegensatz dazu detektiert der routinemäßig eingesetzte Marker OCT3/4 ITGCNU-Zellen verlässlich. Mit fortschreitender Tumorentwicklung in die invasiven Formen Seminom und Embryonalkarzinom wird kein HMGA2 mehr exprimiert. Auch die Reprogrammierung eines Seminoms in ein Embryonalkarzinom führt nicht zur Reexpression. Die Differenzierung vom Embryonalkarzinom in jedes andere Nicht-Seminom geht mit einer Reaktivierung von HMGA2 einher.

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6 Zusammenfassung

HMGA2 und die miRNAs der Cluster C19MC und miR-371-3 werden physiologisch hauptsächlich in embryonalen Geweben exprimiert und in etlichen Tumoren wieder reaktiviert. Um den Einfluss dieser Gene auf das Wachstum im embryonalen Gewebe und in Tumoren näher zu charakterisieren, wurde die Expression dieser Gene in Plazenten und in den embryonale Gewebe imitierenden testikulären Keimzelltumoren bestimmt. Plazenten zeigen ähnlich wie Tumoren ein invasives Wachstum. Das Entschlüsseln der Regulationsmechanismen plazentarer Wachstumssteuerung könnte Rückschlüsse auf die Wachstumsinduzierung von Tumoren liefern.

In den dieser Arbeit zu Grunde liegenden Untersuchungen an postpartalen Plazenten sowie Plazenten spontaner und induzierter Aborte wurde gezeigt, dass die miRNAs des C19MC Clusters mit zunehmender Schwangerschaftsdauer signifikant stärker exprimiert werden. Dieser Anstieg scheint nach Literaturdaten mit der Regulierung der Invasivität von Trophoblastzellen im Zusammenhang zu stehen. Die Expressionsstärke insbesondere der miRNAs des miR-371-3-Clusters unterschied sich individuell stark. Ein Zusammenhang zwischen Expressionsstärke und Probenentnahmeort ließ sich nicht herstellen, so dass es sich tatsächlich um individuelle Unterschiede handelt. In Serumproben konnte die individuell unterschiedliche Expressionsstärke exemplarisch an miR-371a-3p bestätigt werden. Der Einfluss dieser individuell unterschiedlichen Expressionsstärken auf die pränatale Entwicklung ist noch ungeklärt. Die Hypothese, dass die untersuchten miRNAs beider Cluster am Abortgeschehen beteiligt sind konnte nicht bestätigt werden, ihre Expression unterschied sich zwischen induzierten und spontanen Aborten nicht.

Die für diese Arbeit durchgeführten Untersuchungen zeigten, dass *HMGA2* in Plazenten im ersten Trimester hoch exprimiert wird um danach deutlich abzufallen und bis zum Ende der Schwangerschaft nur noch als Basisexpression messbar ist. Die Phase hoher *HMGA2*-Expression fällt mit der Phase niedriger O_2-Konzentration, in der Trophoblastzellen stark proliferieren, zusammen. Möglichweise fungiert *HMGA2* hier also als Proliferationsfaktor. Ein Expressionsunterschied zwischen spontanen und induzierten Aborten wurde nicht gefunden, so dass eine Rolle von HMGA2 im Abortgeschehen nicht angenommen werden kann. Der Stammzellfaktor HMGA2 wird vor allem in den Stromazellen des Zottenbaumes exprimiert. Dieses Ergebnis bestä-

tigt Literaturdaten, nach denen im Zottenstroma Stammzellen lokalisiert sind. Das HMGA2-Protein, das normalerweise im Zellkern zu finden ist, wurde demgegenüber im Trophoblasten im Wesentlichen auf das Zytoplasma beschränkt detektiert. Möglicherweise ist dieses Phänomen, wie schon für HMGA1 beschrieben, im Zusammenhang mit der Differenzierung von Zytotrophoblasten zu extravillösen, d.h. migrierenden, Trophoblasten zu sehen.

Neben der physiologischen Expression in embryonalen Geweben und Stammzellen wird *HMGA2* in einer Reihe von benignen und malignen Tumoren reexprimiert. Als Beispiel für einen embryonale Gewebe imitierenden Tumor wurde die Expression von *HMGA2* in verschiedenen Subgruppen von testikulären Keimzelltumoren erwachsener Männer untersucht. Anhand der Genexpression ließ sich für reine Seminome, Embryonalkarzinome, Teratome und Dottersacktumore eine klare Abgrenzung der Subgruppen feststellen. In den ebenso häufig vorkommenden Mischtumoren ist dies schwieriger, aber mit Hilfe der immunhistochemisch nachgewiesenen HMGA2-Expression konnten die Subgruppen gut voneinander abgegrenzt werden. Um HMGA2 als Biomarker für die Routinediagnostik zu etablieren ist die Untersuchung einer größeren Stichprobe notwendig. Für die Identifizierung von Chorionkarzinomanteile zeigten sich bereits ermutigende Hinweise auf eine Eignung als Marker. Für die Detektion von Dottersackkomponenten hat sich HMGA2 auf Grund der vorliegenden Untersuchung bereits als geeigneter Marker erwiesen.

7   Summary

HMGA2 and miRNAs of C19MC and miR-371-3 are mainly expressed in embryonic tissues. Reexpression of these genes can be found in different types of tumours. The influence of the above mentioned genes on development and growth was investigated in placentas and testicular germ cell tumours, which mimic embryonic development. Invasive growth is a characteristic of placentas and tumours. Therefore, analysis of the underlying regulation processes in placental development might help to understand the induction of invasive behavior in tumours. In this thesis, investigation of spontaneous and induced abortions and of full-term placentas revealed increasing expression of miRNAs of C19MC with the progression of pregnancy. According to literature, this seems to be related to the regulation of invasive growth of trophoblast cells. Particularly the expression level of miRNAs of the miR-371-3 cluster showed strong interindividual differences. This was not caused by the site of the placenta where the sample has been taken from. Additionally, interindividual differences in expression of miR-371a-3p were also observed in serum samples. It remains to be elucidated whether or not they affect processes during prenatal life. Since there were no differences in expression of spontaneous compared to induced abortions, the miRNAs examined do not play an obvious role in miscarriage events.

Investigations conducted in this thesis revealed high *HMGA2* expression in first trimester placentas followed by a decline to a baseline level which can be observed until birth.

High *HMGA2* expression correlated with the low oxygen environment in the uterus in early pregnancy which is known to be a requirement for the proliferation of trophoblast cells. Hence, *HMGA2* might function as a proliferation factor in this context. No difference in expression of spontaneous compared to induced abortions could be detected. This indicates that *HMGA2* has no obvious role in spontaneous abortions. The stem cell factor HMGA2 was mainly detected in stromal cells of placental villi. This is in concordance with the previous finding that stem cell are located in the stroma of placental villi. Another interesting result of this thesis was the finding that in trophoblast cells HMGA2 is located mostly in the cytoplasma.

Cytoplasmatic expression might be related to extravillous differentiation of cytotrophoblast as shown for HMGA1.

While physiological expression of *HMGA2* is mainly restricted to embryonic tissues and stem cells, reexpression can be observed in different types of tumours. Due to the fact that testicular germ cell tumours mimic embryonic tissues, *HMGA2* expression was measured in different types of postpubertal testicular germ cell tumours. Tumours comprising of only one subgroup could be distinguished by qRT-PCR expression levels. In frequently found mixed tumours, classification via *HMGA2* expression is less distinct. By using HMGA2 immunohistochemistry subgroups could be distinguished. To establish HMGA2 in clinical application, the investigation should be extended on a larger sample. HMGA2 staining showed promising results in detecting choriocarcinoma components. Convincing evidence was found for HMGA2 as an appropriate marker for the detection of yolk sac components.

8 Publikationsübersicht

1. Markowski DN, Thies HW, **Gottlieb A,** Wenk H, Wischnewsky M, Bullerdiek J: HMGA2 expression in white adipose tissue linking cellular senescence with diabetes. Genes Nutr. 2013 Sep;8(5):449-56. doi: 10.1007/s12263-013-0354-6.

2. Kloth L***, Gottlieb A*,** Helmke B, Wosniok W, Löning T, Burchardt K, Belge G, Günther K, Bullerdiek J: HMGA2 expression distinguishes between different types of postpubertal testicular germ cell tumour. J Pathol Clin Res. 2015 Sep 12;1(4):239-51. doi: 10.1002/cjp2.26.

3. Kloth L, **Gottlieb A,** Helmke B, Wosniok W, Bullerdiek J: Expression of HMGA2 in fetal placenta correlates with gestational age.

   Submitted to *BMC Women´s Health*

4. **Gottlieb A*,** Flor I***,** Nimzyk R, Kloth L, Helmke B, Langenbuch M, Spiekermann M, Feidicker S, Bullerdiek J: The Expression of miRNA Encoded by C19MC and miR-371-3 Strongly Varies Among Individual Placentas but Does Not Differ between Spontaneous and Induced Abortions.

   Submitted to *PLOS ONE*

* gleichberechtigte Autorenschaft der Erst- und Zweitautoren in Publikation 2 und 4.

9 Danksagung

Ich danke

- Prof. Dr. Jörn Bullerdiek für die Überlassung des interessanten Themas, die Betreuung des Promotionsvorhabens und die Übernahme des Referats.

- Prof. Dr. Andreas Dotzauer für die Übernahme des Ko-Referats.

- Prof. Dr. Ursula Dicke, Manuela Grund und Finja Hennig für die Teilnahme am Prüfungsausschuss.

- Dr. Rolf Nimzyk für seine jederzeit zur Verfügung gestellte Zeit und Geduld, die statistische Beratung und die Teilnahme am Prüfungsausschuss.

- Prof. Dr. Burkhard Helmke, Prof. Dr. Thomas Löning, Prof. Dr. Klaus Junker, Prof. Dr. Ulrich Deichert, Marc Langenbuch, Prof. Dr. Susanne Feidicker, Prof. Dr. Wolfgang Ahrens sowie den Schwangeren der Frauenkliniken Cuxhaven und DIAKO in Bremen für die Überlassung von Probenmaterial.

- Prof. Dr. Thomas Löning, Prof. Dr. Burkhard Helmke und Käte Burchardt für die Beurteilung histologischer Präparate.

- Dr. Werner Wosniok für die Unterstützung bei statistischen Auswertungen.

- allen noch nicht genannten Kolleg*innen der Humangenetik, die mich jederzeit mit Rat, Tat, Konstruktivität, Aufmunterung, Diskussionsbereitschaft und Bonbons unterstützt haben: Dr. Lars Kloth, Dr. Inga Flor, Dr. Volkhard Rippe, Meike Spiekermann, Dr. Nina Winter, PD Dr. Gazanfer Belge, Dr. Arlo Radtke, Dr. Yvonne Kiefer und Angelika Schneider-Uhlhorn.

- meinen Freund*innen Prof. Dr. Julia Lademann, Dr. Jenny Peplies, Dr. Kathrin Günther, Dr. Petra Seling-Biehusen und Dr. Wolfgang Biehusen für die moralische Unterstützung; einigen von ihnen auch für das Drängeln, damit dieses Projekt ein gutes Ende finden konnte.

- aus tiefstem Herzen Norbert und Leon dafür, dass sie da sind und mich nie vergessen lassen, was noch wichtiger ist als ein wissenschaftliches Vorhaben.

10 Erklärung

Hiermit erkläre ich, dass ich die Doktorarbeit mit dem Titel:

„Untersuchungen zur Expression ausgewählter Stammzellgene an embryonalen Geweben und Tumoren"

selbständig verfasst und geschrieben habe und außer den angegebenen Quellen keine weiteren Hilfsmittel verwendet habe.

Ebenfalls erkläre ich hiermit, dass es sich bei den von mir abgegebenen Arbeiten um drei identische Exemplare handelt.

Bremen, den 28.03.2017

Andrea Gottlieb