Untersuchungen zur Funktion der HMGA- und HMGB-Subfamilien bei der Tumorentstehung

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„Hiermit erkläre ich, Lars Borrmann, geboren am 03. September 1972 in Bremen, dass ich die vorliegende Dissertation „Untersuchungen zur Funktion der HMGA- und HMGB-Subfamilien bei der Tumorentstehung“ selbstständig verfasst und keine anderen, als die angegebenen Quellen und Hilfsmittel verwendet habe.“

Bremen, 26. August 2003

(Lars Borrmann)

INHALTSVERZEICHNIS

ABKÜRZUNGSVERZEICHNIS

As	Aminosäure
bp	Basenpaare
cDNA	komplementäre DNA
CIP	Calf Intestinal alkaline Phosphatase
cpm	counts per minute
C-terminal	Carboxyterminal
dATP	Desoxyadenosin-5'-Triphosphat
dCTP	Desoxycytidin-5'-Triphosphat
DEAD	Aminosäure Sequenzmotif (Asp-Glu-Ala-Asp)
DEAH	Aminosäure Sequenzmotif (Asp-Glu-Ala-His)
dGTP	Desoxyguanosin-5'-Triphosphat
DNA	Desoxyribonukleinsäure
DNase	Desoxyribonuklease
dNTP	Desoxynukleosid-5'-Triphosphat
ΔHMGA1a	C-terminal trunkiertes HMGA1a
ΔHMGA2	C-terminal trunkiertes HMGA2
DTT	Dithiotreithol
dTTP	Desoxythymidin-5'-Triphosphat
EDTA	Ethylendiamintetraacetat
EP	Endometriumpolyp
ER	Östrogen-Rezeptor (Estrogen-Receptor)
ERE	Bindungsstelle für den Östrogen-Rezeptor (Estrogen Responsive Element)
EST	Expressed Sequence Tagged
FISH	Fluoreszenz *in situ* Hybridisierung
g	Erdbeschleunigung (g = 981 cm x s^{-2})
gb	GenBank Datenbank
h	Stunde
HMG	High Mobility Group
HMGA1	*HMGA1*-Gen (ehemals *HMGIY*)
HMGA1	Proteine des *HMGA1*-Gens (HMGA1a und HMGA1b)
HMGA1a	Splicevariante: HMGA1a Protein des *HMGA1*-Gens (ehemals HMGI)
HMGA1b	Splicevariante: HMGA1b Protein des *HMGA1*-Gens (ehemals HMGY)
HMGA2	HMGA2-Gen (ehemals HMGIC)
HMGA2	Protein des *HMGA2*-Gens (ehemals HMGIC)
HMGA2/LPP	Fusionsprotein aus HMGA2 und LPP
HMGB1,2,3	*HMGB1,2,3*-Gen (ehemals *HMG-1,2,4*)
HMGB1,2,3	Protein des *HMGB1,2,3*-Gens (ehemals HMG-1,2,4)
kb	Kilo Basenpaare
kDa	Kilo Dalton
LBH	Genbezeichnung: Located Behind HMGIY
M	Molar
min	Minute
ml	Milliliter
mM	Millimolar
MMLV	Moloney-Mäuse-Leukämie-Virus
µl	Mikroliter
µm	Mikrometer
nm	Nanometer
OD	optische Dichte
ORF	Open Reading Frame
PAC	P1 abgeleitetes, artifizielles Chromosomen
PCH	chondroides Lungenhamartom
PCR	polymerase chain reaction
pM	Pikomolar
RACE	Rapid Amplification of cDNA Ends
RAGE	Receptor for Advanced Glycation Endproducts
RNA	Ribonukleinsäure
RNase	Ribonuklease

rpm	rotation per minute
RT	Raumtemperatur (ca.23 °C)
SDS	Sodiumdodecylsulfat
sec	Sekunde
SEM	Simple and Efficient Method
T4	Phage T4
U	Unit
UL	Uterus Leiomyom
UTR	Untranslatierte Region
UV	ultraviolett
V	Volt
Vol	Volumen

HERSTELLERANGABEN

Amersham Biosciences	Amersham Biosciences, Buckinghamshire, England
BD Biosciences Clontech	BD Biosciences Clontech, Palo Alto, USA
Biometra	Biometra, Göttigen, Deutschland
Bio-Rad	Bio-Rad, Hercules, USA
Eppendorf	Eppendorf, Hamburg, Deutschland
Incyte	Incyte, Palo Alto, USA
Invitrogen	Invitrogen, Carlsbad, USA
Lumac BV	Lumac BV, Landgraaf, Niederlande
Promega	Promega, Madison, USA
PromoCell	PromoCell, Heidelberg, Deutschland
QIAGEN	QIAGEN, Hilden, Deutschland

EINLEITUNG

Die Familie der „High Mobility Group (HMG) Proteine“ umfasst Chromatin-assoziierte Nichthiston-Proteine, die als sogenannte architektonische Transkriptionsfaktoren an AT-reiche Regionen der DNA binden (Bustin and Reeves, 1996). Das Binden der HMG-Proteine an diese AT-reichen Regionen erfolgt dabei primär strukturspezifisch und geht mit einer Änderung der Chromatin-Konformation einher (Bustin, 1999). Funktionell lässt sich die Familie der HMG-Proteine in die Subfamilien A, B und N unterteilen.

Die HMGN-Subfamilie umfasst dabei ubiquitär exprimierte Proteine, die an die Innenseite nukleosomaler DNA binden und damit die Interaktion zwischen DNA und dem Histon Oktamere beeinflussen (Shick *et al*., 1985; Bustin and Reeves, 1996). Durch Interaktion mit den Histonen H1 und H3 können sie die Chromatinstruktur modifizieren, um dadurch die Transkription zu erleichtern, ohne dabei selbst ein Bestandteil des Transkriptionskomplexes zu sein (Trieschmann *et al*., 1995, Bustin 1999).

Die Proteine HMGB1, HMGB2 und HMGB3 werden unter der HMGB-Subfamilie zusammengefasst. Als gemeinsames Charakteristikum besitzen sie am N-Terminus jeweils zwei, etwa 80 Aminosäuren umfassende HMG-Boxen, deren drei α-Helices in einer charakteristischen L-Form angeordnet sind (Bustin, 1999). Mit Hilfe dieser HMG-Boxen erfolgt das strukturspezifische Binden der DNA. Für das in der chromosomalen Bande 13q12 codierte HMGB1 wurden als Targets sekundäre DNA-Strukturen wie beispielsweise supercoiled DNA (Stros und Reich, 1998), kruziforme DNA (Bianchi *et al*., 1989), four-way-junctions (Hill und Reeves 1997; Hill *et al*., 1999) und Cisplatin-geschädigte DNA (Locker *et al*., 1995) bestimmt. Das Binden von HMGB1 an seine Targets bewirkt dabei ein Biegen der DNA, sodass Transkriptionsfaktoren sich besser an ihre Zielsequenzen anlagern können (Zappavigna *et al*., 1996). Aufgrund dieser Beobachtungen wird für HMGB1 eine Rolle bei Replikation und Rekombination sowie bei DNA-Reparatur und Transkriptionsaktivierung diskutiert (Bustin, 1999).
Die Lokalisation von HMGB1 ist nicht allein auf den Nukleus beschränkt, vielmehr kann HMGB1 zelltypspezifisch auch frei im Zytoplasma, membrangebunden oder auch extrazellulär auftreten (Merenmies *et al*., 1991; Rauvala *et al*., 2000). Als extranukleäres Protein kann HMGB1 als Ligand für den RAGE-Rezeptor sowohl den NF-κB als auch den MAPK Pathway aktivieren (Riewald *et al*., 2002). Extrazellulär kann HMGB1 die Zellmobilität und die Tumor-Metastasierung stimulieren und Zytokin Signalwege aktivieren (Wang *et al*., 1999; Andersson *et al*., 2000).
Im Rahmen der Entstehung benigner mesenchymaler Tumore zeigte sich, dass die Bruchpunkte der chromosomalen Aberrationen der Region 13q12 bei acht Lipomen weder

intragenisch, noch in der unmittelbaren Umgebung des *HMGB1* lokalisiert sind (Kazmierczak *et al*., 1999b). Allerdings kann eine Überexpression von HMGB1 zum Nutzen therapeutischer Tumorbehandlungen, durch das Binden des Proteins an Cisplatin-geschädigte DNA-Addukte, die Schadenserkennung durch die zellulären Reparaturmechanismen verhindern und somit Tumorzellen sensitiver für chemotherapeutische Behandlungen mit Cisplatin oder Carboplatin machen (McA'Nulty *et al*., 1996; He *et al*., 2000).
Die Erfolgsaussichten einer Hormontherapie bei der Behandlung von Mamma-Karzinomen stehen zum Einen in Abhängigkeit zu dem Status der Östrogen- (ER) und Progensteronrezeptoren (PR) des jeweiligen Tumors (Molina *et al*., 1999), können aber andererseits durch Co-Faktoren, die die Funktion der Steroidrezeptoren modulieren, beeinträchtigt werden. HMGB1 ist als ein solcher Co-Faktor bekannt, der zu einer Intensivierung der Bindung zwischen Steroidrezeptor, insbesondere der Östrogen- und Progesteronrezeptoren und deren Zielsequenzen im Genom führt (Onate *et al*., 1994; Verrier *et al*., 1997; Boonyaratanakornkit *et al*., 1998). Da die Expression von *HMGB1* seinerseits durch Östrogene verstärkt wird (He *et al*., 2000), wird HMGB1 ein besonderes Augenmerk im Rahmen hormonell bedingtem Tumorwachstum oder der Hormontherapie zuteil.

Das Hauptinteresse dieser Arbeit galt neben dem *HMGB1* primär der Subgruppe der HMGA-Proteine, die aus dem in der chromosomalen Region 12q14-15 gelegenem *HMGA2*-Gen und *HMGA1* in 6p21.3 mit seinen sich um 33bp unterscheidenden Splicevarianten HMGA1a und HMGA1b gebildet wird (Chau *et al*., 1995; Johnson *et al*., 1989).
Die Proteine der HMGA-Subfamilie binden in die schmale Furche AT-reicher DNA (Disney *et al*., 1989; Elton *et al*., 1987), können mit anderen Proteinen interagieren (Thanos und Maniatis, 1992) und bewirken im Gegensatz zu HMG-Box Proteinen, keine scharfen Knicke an der DNA, sondern biegen intrinsisch gebogene DNA wieder leicht zurück (Falvo *et al*., 1995). Dabei binden die HMGA-Proteine nicht nur mit hoher Affinität in die schmale Furche AT-reicher B-Form-DNA (Solomon *et al*., 1986), sondern sind auch in der Lage an four-way junction DNA (Hill und Reeves, 1997), an supercoiled Plasmide (Nissen und Reeves, 1997) und an nukleosomale Kern-Partikel (Reeves und Nissen, 1993) zu binden und DNA zu biegen, zu strecken, zu entwickeln und supercoils in sie einzufügen (Bustin und Reeves, 1996). Durch die Induktion der Konformationsveränderungen kann die Bildung stereospezifischer Transkriptionskomplexe oder Enhanceosomenkomplexe beeinflusst werden, wodurch es zu einer Aktivierung oder Inhibierung der Genexpression kommen kann (Thanos und Maniatis, 1995; John *et al*., 1995). Eine positive genregulatorische Wirkung ist im Falle von HMGA1 unter anderem für den Tumor Nekrose-Faktor-β (Fashena *et al*., 1992) und *E-Selektin* (Lewis *et al*., 1994) beschrieben worden, während HMGA1 beim *IgE*-Gen der Maus (Kim *et al*., 1995), bei *Interleukin-4* (Chuvpilo *et al*., 1993) und *GP91-PHOX* (Skalnik und Neufeld, 1992) einen inhibitorischen Einfluss auf die Expression ausübt. Protein:Protein-

Interaktionen mit anderen Transkriptionsfaktoren sind für HMGA1 beispielsweise mit den Transkriptionsfaktoren NF-κB (Thanos und Maniatis, 1992), ATF-2 (Du und Maniatis, 1992), IRF, c-Jun und Elf-1 (John *et al*., 1995) bekannt.
Das *HMGA2*-Gen mit einer Transkriptionsorientierung in Richtung Telomer besteht aus 5 Exons und erstreckt sich über eine Länge von 142kp mit einem Intron 3, dass einen Bereich von 112kb abdeckt (Schoenmakers *et al*, 1995; Chau *et al*., 1995; Hauke *et al*., 2002) wohingegen das humane *HMGA1* insgesamt lediglich einen Bereich von 9349bp einnimmt, aber über 8 Exons und vier verschiedene Promotoren verfügt (Friedmann *et al*., 1993). Als besondere Auffälligkeiten codieren sowohl *HMGA2*, als auch *HMGA1* für außergewöhnlich große 3' untranslatierte Regionen von 2927bp und 1345bp Länge (Friedmann *et al*., 1993; Chau *et al*., 1995).
Alle drei Proteine der HMGA-Subfamilie verfügen über je drei hochkonservierte DNA-bindende Domänen (AT-hooks) sowie eine saure C-terminale Domäne, die im Falle von HMGA2 durch einen 11As langen Spacer von den AT-hooks getrennt ist (Chau *et al*., 1995). Darüber hinaus sind über die Proteine verteilt, diverse Positionen zur posttranskriptionellen Modifikation identifiziert worden, an denen durch Phosphorylierung, Acetylierung, Methylierung, Glycosylierung oder poly(ADP)-Ribosylierung die Wirkungsweise der Proteine moduliert werden kann (Elton und Reeves, 1986; Lund und Laland, 1990; Nissen *et al*., 1991; Sgarra *et al*., 2003). So wird je nach Position der Phosphorylierung entweder die Degradation der Proteine durch Proteinasen oder die Bindungsaffinität zur DNA beeinflusst (Wisniewski und Schwanbeck, 2000).

Hauptsächlich aktiv sind die HMGA-Proteine bei Prozessen zellulärer Differenzierung. Dementsprechend ist eine starke Expression dieser Gene während der Embryonalentwicklung und in undifferenzierten Zellen zu finden. Im adulten, differenzierten Gewebe ist lediglich in der Retina *HMGA1* stark exprimiert, während in den übrigen Geweben HMGA2 gar nicht und HMGA1 nur noch in sehr geringen Konzentrationen detektierbar ist (Bussemakers *et al*., 1991; Chiappetta *et al*., 1996; Rogalla *et al*., 1996; Zhou *et al*., 1995; Chau *et al*., 2000). Eine reaktivierte Expression von HMGA-Proteinen in differenziertem Normalgewebe wird dabei mit dem Wachstum und der Differenzierung von Adipozyten (Zhou *et al*., 1995; Anand und Chada, 2000; Melillo *et al*., 2001), der Proliferation glatter Muskelzellen in den Blutgefäßen nach Gefäßverletzungen (Chin *et al*., 1999; Foster *et al*., 1999), der Immunantwort bei entzündlichen Reaktionen (Pellacani *et al*., 1999; Foster *et al*., 1999) sowie mit apoptotischen Prozessen (Diana *et al*., 2001; Sgarra *et al*., 2003) assoziiert. Die Menge an HMGA1 variiert dabei in Abhängigkeit von der Proliferationsrate der Zellen (Johnson *et al*., 1990).
Untersuchungen zur embryonalen Expression von HMGA in verschiedenen Entwicklungsstadien der Maus zeigten, dass in frühen embryonalen Phasen sowohl *HMGA1*

als auch *HMGA2* abundant in nahezu allen Geweben exprimiert wird. In der zweiten Hälfte der Embryonalentwicklung ist HMGA2 beschränkt auf mesenchymale Gewebe, die sich zu Knorpel und Muskel differenzieren, sowie auf einige epitheliale Zellschichten und Teile des zentralen Nervensystems (Hirning-Folz *et al*., 1998). Dagegen wird *HMGA1* zwar noch ubiquitär exprimiert, ist aber nur in einigen Geweben noch abundant zu finden (Chiappetta *et al*., 1996). Dabei bewirken HMGA-Proteine vermutlich bereits im frühen Zygoten-Stadium eine Änderung der Chromatinkonformation in einen transkriptionskompetenten Status, sodass regulatorische Faktoren einen besseren Zugang zu ihren Zielsequenzen bekommen (Beaujean *et al*., 2000).
Im Rahmen der Charakterisierung maligner Tumore konnte für Karzinome der Prostata (Bussemaker *et al*., 1991; Tamimi *et al*., 1993), der Schilddrüse (Chiapetta *et al*., 1995) des Colons (Fedele *et al*., 1996; Abe *et al*., 1999), der Brust (Ram *et al*., 1993), der Cervix (Bandiera *et al*., 1998), der Bauchspeicheldrüse (Abe *et al*., 2000) sowie bei Lymphomen (Wood *et al*., 2000) eine stark reaktivierte HMGA1-Expression bestimmt werden. Die Stärke der HMGA1-Expression des jeweiligen Tumors ist dabei als prognostischer Marker mit dessen Metastasierungspotential korreliert und stellt ein charakteristisches Merkmal für eine maligne transformierte Zelle dar (Giancotti *et al*., 1987). Dagegen ist die in Brust- (Rogalla *et al*., 1997) und Lungenkarzinomen (Rogalla *et al*., 1998) beobachtete Expression von HMGA2 mit dem Tumorgrading zu korrelieren.

Mit Hilfe zytogenetischer Untersuchungen konnten in den letzten Jahren für eine ganze Reihe von benignen Tumoren mesenchymalen Ursprungs Veränderungen der chromosomalen Region 12q14-15 das *HMGA2* Gen betreffend gefunden werden. Bei benignen mesenchymalen Tumoren handelt es sich um die größte Gruppe gutartiger Neoplasien des Menschen, die neben normalen Karyotypen eine Vielzahl mikroskopisch sichtbarer Veränderungen aufweisen. Die häufigste dieser Veränderungen ist dabei die strukturelle Aberration der chromosomalen Region 12q14-15. Derartige Aberrationen wurden bereits für Leiomyome des Uterus (Heim *et al*., 1988; Turc-Carel *et al*., 1988; Vanni und Lecca 1988) und Lipome (Heim *et al*., 1986; Turc-Carel *et al*., 1986; Mandahl *et al*., 1987; Sreekantaiah *et al*., 1991; Belge *et al*., 1992) beschrieben. Die Leiomyome des Uterus, mesenchymale Muskeltumoren, gehören mit einer Inzidenz von etwa 30% bei Frauen über 30 Jahren zu den häufigsten Tumoren der Frau überhaupt (Norris und Zaloudek, 1982). Weitere Tumorentitäten mit Veränderungen der Region 12q14-15 sind Endometriumpolypen (Walter *et al*., 1989; Vanni *et al*., 1993; Dal Cin *et al*., 1995), chondroide Hamartome der Lunge (Fletcher *et al*., 1991; 1995; Dal Cin *et al*., 1993), pleomorphe Adenome der Kopfspeicheldrüsen (Mark *et al*., 1980; Mark und Dahlenfors, 1986; Bullerdiek *et al*., 1987), Hämangiopericytome (Mandahl *et al*., 1993), chondromatöse Tumore (Mandahl *et al*., 1989; Bridge *et al*., 1992), benigne Tumore der Brust (Birdsal *et al*., 1992; Rohen *et al*., 1995; Staats

et al., 1996), aggressive Angiomyxome (Kazmierczak *et al*., 1995a), diffusen Astrocytome (Jenkins *et al*., 1989) und Osteoclastome (Noguera *et al*., 1989).
Durch genauere Analyse der Rearrangierungen der Region 12q14-15 mit Hilfe von FISH-Experimenten, konnten die chromosomalen Bruchpunkte genauer eingegrenzt werden. Dabei wurden für Lipome (Schoenmakers *et al*., 1995; Ashar *et al*., 1995), Uterus Leiomyome (Schoenmakers *et al*., 1995), pleomorphe Adenome der Kopfspeicheldrüsen (Schoenmakers *et al*., 1995), chondroide Hamartome der Lunge (Schoenmakers *et al*., 1995; Kazmierczak *et al*., 1995a, 1996a), Fibroadenome (Schoenmakers *et al*., 1995) sowie für Angiomyxome (Schoenmakers *et al*., 1995) und Endometriumpolypen (Schoenmakers *et al*., 1995; Bol *et al*., 1996) primär Bruchpunkte im Intron 3 oder 4 von *HMGA2* gefunden. Zusätzlich zu diesen intragenischen Bruchpunkten sind für Lungenhamartome (Wanschura *et al*., 1996) sowie Leiomyome des Uterus (Fejzo *et al*., 1996; Quade *et al*., 2003) extragenische Bruchpunkte beschrieben worden, die sowohl 5' oberhalb von *HMGA2* oder in der 3' flankierenden Region lokalisiert sein können. Unveröffentlichte Daten über die Bruchpunkteingrenzung bei 87 chondroiden Lungenhamartomen mit 12q14-15 Aberration stützen diese Daten und zeigen, dass bei etwa 58% der Hamartome ein chromosomaler Bruchpunkt im Intron 3 oder 4 vorliegt, während 36% der Rearrangierungen die Region 5' vor *HMGA2* betreffen und nur 6% der Brüche in den 3' flankierenden Regionen lokalisiert sind (unpublizierte Ergebnisse).
Als Folge der chromosomalen Bruchereignisse kommt es in den verschiedenen Tumorentitäten zu einer Überexpression von nativem HMGA2 oder zur Expression von trunkiertem oder chimärem HMGA2. Die Entstehung von vielen der trunkierten Transkripte ist dabei das Resultat von aberrantem Splicen mit Sequenzen aus dem Intron 3 oder Intron 4 von *HMGA2* (Hauke *et al*., 2001; 2002). Betrachtet man die chimären Formen von HMGA2, stellt die Fusion zwischen den AT-hooks von *HMGA2* und den LIM-Domänen der Exons 9-11 des *LPP*-Gens das am häufigsten zu beobachtende Fusionprodukt dar, das bisher sowohl in Lipomen als auch in Lungenhamartomen mit einer t(3;12)(q27;q14-q15) gefunden wurde (Petit *et al*., 1996; Rogalla *et al*., 1998b). Weitere Fusionspartner für das *HMGA2* sind beispielsweise *ALDH2* (Kazmierczak *et al*., 1995b), *FHIT* (Geurts *et al*., 1997), *RAD51L1* (Schoenmakers *et al*., 1999) und *LHFP* (Petit *et al*., 1999) sowie einige andere.
Trotz dieser Vielzahl an Fusionspartnern und der ebenso zahlreichen Formen von trunkiertem HMGA2 (Schoenmakers *et al*., 1995; Kottickal *et al*., 1998; Klotzbücher *et al*., 1999), haben die aberranten Formen doch immer eine Gemeinsamkeit, sie beinhalten alle die drei DNA-bindenden Domänen, verlieren aber sowohl die saure C-terminale Domäne als auch auf Ebene der RNA die Informationen des 3'UTRs.

Eine weitere große Subgruppe benigner mesenchymaler Tumore ist charakterisiert durch chromosomale Veränderungen in der Region 6p21.3. Derartige Aberrationen wurden bislang unter anderem für Uterus Leiomyome (Mark *et al*., 1988; Ozisik *et al*., 1993), Lipome

(Sreekantaiah *et al*., 1990), sowie Endometriumpolypen (Fletcher *et al*., 1992; Dal Cin *et al*., 1995) und chondroide Hamartome der Lunge (Fletcher *et al*., 1991; Johansson *et al*., 1992; 1993) beschrieben. Durch die genauere Eingrenzung der Bruchpunkte mittels FISH-Analysen, konnte gezeigt werden, dass sowohl in Uterus Leiomyomen (Kazmierczak *et al*., 1996b; Williams *et al*., 1997) als auch in chondroiden Lungenhamartomen (Kazmierczak *et al*., 1996c) die chromosomalen Bruchpunkte in einem ca. 120kb großen Bereich um das *HMGA1*-Gen lokalisiert sein müssen.

Ziel dieser Arbeit war es, den Zusammenhang zwischen der Entstehung benigner mesenchymaler Tumore und den Veränderungen von HMGA1 und HMGA2 genauer zu analysieren. Dazu wurden die für die Tumoren charakteristischen Situationen sowohl auf genomischer, funktioneller als auch auf Expressionsebene untersucht, um für die zytogenetischen Subgruppen benigner mesenchymaler Tumore mit Veränderungen der Regionen 12q14-15 und 6p21.3 Aussagen für die HMGA-Familie treffen zu können. Untersuchungen zur Funktion von nativem und aberrantem HMGA sollten einen tieferen Einblick in die Mechanismen der HMGA-Expressionsregulation ermöglichen und die Funktion der aberranten Proteinformen in bezug auf die Tumorentstehung genauer beschreiben.
Für *HMGB1* war das Ziel, die bis dato nur partiell bekannte genomische Struktur des Genes zu komplettieren um darauf aufbauend mehr über die Mechanismen der *HMGB1*-Expressionsregulation zu erfahren.

MATERIAL UND METHODEN

ZELLMATERIALIEN

PAC-DNA

Die PAC-Klone 8603, 8604 und 8605 wurden mit Hilfe *HMGA1*-spezifischer Primer gescreent (Kazmierczak *et al*., 1996a).

Die Isolierung von PAC1363, der das komplette humane *HMGB1*-Gen umfasst, erfolgte wie beschrieben (Kazmierczak *et al*., 1999b).

Primäres Zellmaterial

Folgendes Tumormaterial und Normalgewebe wurden verwendet (Tab.1):

Tabelle 1: Karyotypen der verwendeten Tumore (PCH: chondroides Lungenhamartom; EP: Endometriumpolyp; UL: Uterus Leiomyom; F: weiblich; M: männlich; -/-: keine Angaben; [a]Kazmierczak *et al*., 1996c; [b]Kazmierczak *et al*., 1996b; [c]Kazmierczak *et al*., 1998).

Tumor	Bezeichnung in [c]Kazmierczak *et al*., 1998	Alter/ Geschlecht	Karyotyp
PCH1[a,c]	PCH1	60/F	46,XX,t(6;14)(p21.3;q24)[13]
PCH28	-/-	71/M	46,XY,t(6;14)(p21.3;q24)[20]
PCH31[a,c]	PCH4	56/F	46,XX,der(6)t(6;8)(p21.3;q12 or q13),der(14)t(6;14)(p21.3;q24)[13]
PCH38[a,c]	PCH5	64/F	46,XX,t(6;14)(p21.3;q24)[19]
PCH41[a,c]	PCH6	53/M	46,XY,t(6;10)(p21.3;q22.3)[21]
PCH47[c]	PCH12	54/F	46,XX,del(6)(p21.3),der(14)t(6;14)(p21.3;q24)[19]
PCH50[a,c]	PCH7	59/F	46,XX,t(6;14)(p21.3;q24)[7] / 46,XX[11]
PCH56	-/-	66/M	46,XY,del(2),ins(6;2)(q15,p21-p25)
PCH63[a,c]	PCH9	57/M	46,XY,der(1)t(1;14)(p34.1;q24),der(6)t(6;17)(p21.3;q23),der(14) t(6;14)(p21.3;q24),der(17)t(1;17)(p34.1;q23)[13]
PCH66[a,c]	PCH10	64/F	46,XX,der(6)(6qter->6q26::6p21.3->6q26::9p13->9pter), der(9)t(9;14)(p13;q24),der(14)t(6;14)(p21.3;q24)[23]
PCH72	-/-	68/F	46,XX,t(6;10)(p21.1;q26.1)[16]
PCH76	-/-	36/F	46,XX,der(6)t(6;?)(p21.3;?)t(6;15)(q27;q24),der(14)t(6;14)(p21.3;q24), der(15)t(14;15)(q24;q24)[20]
PCH88[c]	PCH13	62/F	46,XX,t(6;12)(p21.3;q22)[9]
PCH94	-/-	53/M	46,XY,der(14)[8]
PCH99	-/-	49/M	46,XY,der(4)(6pter->6p21.3::14q13->14q24::4p14->4qter),add(6)(p21.3),del(14)(q13q24), der(18)t(4;18)(p14;p11.3)[24]
PCH119[c]	PCH15	59/M	46,XY,der(1)t(1;6)(p32,p21.3),del(4)(q12),der(6)t(4;1;6)(q12;p36p21.3;p21.3)[18]
PCH123[c]	PCH16	47/M	46,XY,t(6;14)(p21.3;q24)[12]
PCH125[c]	PCH17	68/W	47,XX,t(5;6;14)(q22;p21.3;q24),+8[12]
PCH130	-/-	60/M	46,XY,der(1)del(1)(p32)del(1)(q32),add(5)(p15.1),add(7)(q22),add(10)(q24),add(16)(q24)[10]
PCH137	-/-	56/M	46,XY,t(6;10)((p21.3;q11.2 or q21)[19]
PCH138	-/-	67/M	46,XY,t(1;6)(p33;p21.3)[18]

PCH161	-/-	48/M	46,XY,der(6)t(6;20)(p21.1;q11.2),t(14;19)(q24;q13.3),add(17)(p13),del(20)(q11.2) [9]
PCH168	-/-	50/M	46,XY,der(6)del(6)(p22)der(6)t(6;13)(q21;q14),der(13)t(6;13)(q21;q14)[11]
PCH182	-/-	58/M	46,XY,t(6;14;8)(p21.3;q24;q24.1),tas(15;21)(p13;p13)[11]
PCH191	-/-	40/M	46,XY,t(6;10)(p21.3;q22)[7]
PCH216	-/-	59/W	46,XX,t(2;6)(p21;p21.3)[10]
PCH231	-/-	42/M	46,XY,der(2)t(2;7)(q23 or q33;q22),der(6),del(7)(q22),der(14)t(2;14)(q32 or q33;q24) [11] / 46,XY [9]
PCH234	-/-	78/M	46,XY,t(X;6)(p22.1;p21.3)[15]
PCH251	-/-	65/M	46,XY,t(6;9;14)(p22;q22;q23)[16]
PCH253	-/-	65/M	46,XY,t(6;10)(p21.3 ;q22)[15]
PCH255	-/-	64/M	46,XY,t(2;6)(p21;p21.3)[17]
PCH256	-/-	63/M	46,XY,t(2;6)(p21;p21.3)[16]
PCH262	-/-	69/M	46,XY,der(1)t(1;8),der(1),der(6),der(7)t(1;7),der(8),der(9),der(13),der(18)[17]
PCH266	-/-	66/W	46,XX,t(6;8)(p21.3;q24)[15]
PCH278	-/-	49/W	46,XX,t(6;14)(p21.3;q24)[20]
PCH280	-/-	56/M	46,XY,der(6),der(14),der(19)[36]
PCH287	-/-	57/M	46,XY,t(6;14)(p21.3;q24),t(9;10)(q22;p15)[12]/46,XY[9]
PCH289	-/-	58/W	46,XX,der(4)t(4;14)(q35;q24),del(6)(p21.3),inv(9)(p11q12)c,der(14)t(6;14)(p21.3;q24)[21]
PCH290	-/-	52/M	46,XY,inv(6)(p21.3;q24)t(6;14)(q24;q24),der(14)t(6;14)(p21.3;q24)[23]
PCH301	-/-	33/M	46,XY,der(3)t(3;14)(p25;q24)ins(3;6)(p25;p21.1p25),der(6)t(3;6)(p25;p21.1),der(14) del(14)?[18]/46,XY[3]
PCH304	-/-	54/M	46,XY,t(1;6)(p32;p21.3)[4] + 5 andere Klone mit t(1;6) Translokationen
PCH308	-/-	53/M	46,XY,t(6;10)(p23,q22)[17]
PCH310	-/-	49/M	46,XY,t(3;8)(p24;q12),t(6;14)(p22;q24),ins(14;?)(q24;q31q37)[19]
PCH316	-/-	66/M	46,XY,t(1;6)(p32;p21.3)[17]
PCH342	-/-	66/M	46,XY,t(6;15)(p21.3 or p22;q25)[23]/46,XY[6]
PCH347	-/-	69/M	46,XY,t(6;17)(p21.3;q24),inv(9)(p11q13)c,-13,+mar[23]
PCH356	-/-	50/W	46,XX,der(2),der(6),der(14)[15]/46,XX[19]
PCH375	-/-	73/M	46,XY,t(6;14)(p21.3;q24),t(20;21)(q13.1;q11.2)[14]/46,XY[4]
PCH376	-/-	67/M	46,XY,t(6;17)(p21.3;q23)[24]/46,XY,[3]
PCH377	-/-	76/W	46,XX,t(6;17)(p21.3;q21)(24)
PCH380	-/-	57/M	46,XY,ins(1;?)(q22),der(6)t(6;14)(p21.3;q24),add(3)(p13),der(14)t(6;14)(p21.3;q11.2 or q12)[14]
PCH391	-/-	58/M	46,XY,t(6;14)(p21.3;q24)[16]
PCH398	-/-	54/W	46,XY,t(2;6)(p21;p21.3)[23]

EP1[c]	EP1	-/-	46,XX,t(6;20)(p21;q13),der(8q)
EP2[c]	EP2	-/-	46,XX,del(6)(p21.3p23)
EP3[c]	EP3	-/-	46,XX,t(2;6;7)(q35;p23;q22)
EP4[c]	EP4	-/-	46,XX,t(2;6)(q35;p21)
EP5[c]	EP5	-/-	46,XX,inv(6)(p21q22)
EP6[c]	EP6	-/-	46,XX,t(6;10)(p21;q22)

Lipom[c]	lipoma	-/-	46,XX,t(3;6)(q28;p21)
UL1[b,c]	UL1	-/-	Komplexe 6p21.3 Aberration
UL2[c]	UL2	-/-	46,XX,t(2;14;6)(p13;q24;p21.3)

MyMeL03	-/-	43/W	46,XX
MyMeL06	-/-	43/W	46,XX

Zelllinien

Folgende Tumor-Zelllinien benignen, mesenchymalen Ursprungs wurden vom Zentrum für Humangenetik der Universität Bremen zur Verfügung gestellt (Tab.2):

Tabelle 2: Karyotypen der verwendeten Zelllinien vor SV40 Transformation (PCH: condroides Lungenhamartom; UL: Uterus Leiomyom)

Zelllinie	Bezeichnung in Kazmierczak *et al*., 1998	Karyotyp vor SV40-Transformation
PCH4[c]	PCH11	46,XY,t(3;6)(p25 or p26;p21.1 or p21.2),der(9)t(9;12)(q34;q14 or q15), der(12) t(12;16)(q14 or q15;q21),del(16)(q21)[14]
PCH44	-/-	46,XX,der(4)t(4;6;14)(q35;p21.3p25;q24),der(6)t(4;6)(q35;p11.2),der(14)t(6;14) (14pter->14q24::6p21.2->6p12:)[16]
PCH51 [a,c]	PCH8	46,XX,der(6)t(6;14)(p21;q24),der(14)t(6;14)(p21.2;q24),der(16) (16pter->16q24::6p21.2->6p12:)[20]
UL 242.1	-/-	Komplexer Karyotyp mit 6p21.3 Aberration

Weitere verwendete Zelllinien:

HeLa	immortale Cervix-Karzinomzelllinie
MCF-7	immortale Mamma-Karzinomzelllinie
L14^{TSV40}	SV40 immortalisierte Zellline des Lipoms L14

METHODEN

Isolierung von Nukleinsäuren

Die Isolierung von **Plasmid-DNA** erfolgte je nach Volumen der eingesetzten Bakterienkultur mittels des „QIAprep Spin Kits“ oder der „QIAGEN Plasmid Kits“ (QIAGEN) jeweils strikt nach den Angaben des Herstellers. Dabei wurden für 5ml Übernachtkulturen in LB-Medium QIAprep-Spin-Säulen verwendet, während für größere Volumina die DNA-Isolierung mit den „QIAGEN Plasmid Kits“ erfolgte.
Die Elution der gereinigten Plasmid-DNA erfolgte in einem adäquaten Volumen 10mM Tris-HCl, pH 8,5.

PAC-DNA wurde strikt nach der Methode des „Plasmid Preparation # 1, P1 DNA preparation from 10 ml culture for sequencing or mapping“, des Vertreibers der PAC-Klone (Incyte) oder nach einem modifizierten Protokoll des „QIAGEN Plasmid Kit“ (QIAGEN) isoliert
Für die Isolierung mit dem „QIAGEN Plasmid Kit“ wurde eine 5ml Übernacht-Vorkultur mit einem einzelnen Klon einer zuvor beimpften Petrischale angeimpft. 250µl der Übernachtkultur wurden zum Animpfen einer 250ml Kultur genutzt, die dann nach 7,5h bei 37°C im Schüttelschrank 40min bei 3700xg, 4°C pelletiert wurde. Die Aufreinigung des Bakterienpellets erfolgte mit dem „QIAGEN Plasmid Maxi Kit“, wobei jeweils 9ml der Puffer

P1 - P3 verwendet und die Säulen 4 mal mit Quffer-QC gespült wurden. Die gereinigten Plasmid-DNA wurde in einem adäquaten Volumen 10mM Tris-HCl, pH 8,5 eluiert.

Genomische DNA wurde aus konfluent bewachsenen 25cm^2 Kulturflaschen mit dem Puregene DNA Isolation Kit nach dem „Cultured Cell and Body Fluid Protocol for 3-5 Million Cell Sample Microfuge Prep“ isoliert.
Die Zellen einer Kulturflasche wurden mit 600µ Cell-Lysis-Solution und mehrmaligem Auf- und Abpipettieren lysiert und in ein 1,5ml Reaktionsgefäß überführt. Nach Zugabe von 3µl RnaseA-Lösung und 25 maligem Invertieren wurden die Proben für 15 - 60min bei 37°C im Wasserbad inkubiert. Nach Abkühlen auf Raumtemperatur und Zugabe von 200µl Protein-Precipitation-Solution wurde das Cup für 20sec kräftig gevortext und anschließend 3min bei 14000xg zentrifugiert. Dem Überstand wurden 600µl reines Isopropanol zugegeben und die Probe ca. 50 Mal invertiert, bis die genomische DNA ausgefallen ist. Anschließend wurden die Proben 1min bei 14000xg pelletiert, mit 600µl 70% Ethanol gewaschen und das DNA-Pellet in TE-Puffer aufgenommen.

RNA wurde mit dem Trizol-LS-Reagenz (Invitrogen) oder dem „RNeasy Mini Kit“ (QIAGEN) entsprechend der Herstellerangaben isoliert. Qualität und Quantität isolierter RNA wurde durch UV-Spektrometrie oder durch denaturierende Formaldehyd Gelelektrophorese überprüft (Sambrook *et al*.1989).

Für die RNA-Isolierung mit Trizol wurden folgende Modifikationen eingefügt: 100mg Gewebe wurden mit einem Skalpell klein geschnitten und dann mit 1ml Trizol lysiert, bzw. Zellkulturen direkt in der Kulturflasche mit 1ml Trizol lysiert; RNA-Pellets wurden zweimal mit 1ml 75%igem, eiskaltem Ethanol gewaschen und getrocknete RNA in 60 - 100µl RNase freiem dH_2O resuspendiert.

Für die RNA-Isolierung mit dem „RNeasy Mini Kit“ wurden die Zellkulturen mit PBS-Lösung gespült, mit 500µl Trypsin-Lösung (0.05% Trypsin, 0.02% EDTA) abgelöst und in 1ml Medium aufgenommen. Abgelöste Zellen wurden in ein 2ml Reaktionsgefäß überführt, bei 300xg, 4°C, 5min pelletiert und einmal mit 1ml PBS gewaschen. Das Zellpellet wurde in 350µl RLT-Lysepuffer (QIAGEN) aufgenommen und mit Hilfe des QIAshredder (QIAGEN) homogenisiert. Die RNA-Isolierung erfolgte entsprechend der Angaben des Herstellers mit einem optionalen Verdau für 15min bei 30°C mit RNase-freier DNaseI (QIAGEN) direkt auf der Säule. Die RNA-Elution erfolgte zweimal mit 50µl RNase freiem dH_2O.

Verdau von DNA mit Restriktionsendonukleasen

Für den enzymatischen Verdau von DNA mit Restriktionsendonukleasen wurden die spezifischen 10x Restriktionspuffer des Herstellers verwendet und auf 1x Konzentration verdünnt. Die Aufreinigung geschnittener DNA erfolgte wahlweise über die Ethanol-Fällung

oder mit Hilfe des „QIAquick PCR Purification Kits“ (QIAGEN) entsprechend den Anweisungen des Herstellers.

Plasmid-DNA wurde geschnitten, indem 0,5 - 1,5µg DNA mit 5U Restriktionsendonuklease für 1 - 2h bei der enzymspezifischen Reaktionstemperatur im Wasserbad inkubiert wurde.

PAC-DNA wurde geschnitten, indem 0,5 - 1,5µg DNA mit 30U Restriktionsenzym für 2 - 3h bei der enzymspezifischen Reaktionstemperatur im Wasserbad inkubiert wurde.

Genomische DNA wurde mit 100U Restriktionsenzym, bei 20µg eingesetzter DNA über Nacht bei der enzymspezifischen Reaktionstemperatur im Wasserbad geschnitten. Vor Zugabe der Restriktionsendonuklease erfolgte eine Inkubation von 8h in einem 37°C Wasserbad zum Aufquellen der DNA.

Partielle Restriktionen wurden durchgeführt nach Sambrooks *et al*. (1989). Im Detail wurden dazu in Vorversuchen parallele Ansätze mit unterschiedlichen Enzymkonzentrationen für eine Stunde bei der enzymspezifischen Reaktionstemperatur im Wasserbad inkubiert und anschließend, sofern für das jeweilige Enzym möglich, die Reaktion durch Inkubation im Wasserbad für 15min bei 70°C abgestoppt. Nach Überprüfung der Restriktionsmuster mittels Gelelektrophorese wurde die Enzymkonzentration, bei der das gewünschte Bandenmuster auftrat, auf den experimentellen Ansatz entsprechend angewendet.

Gelelektrophorese

Die gelelektrophoretische Auftrennung von DNA wurde je nach Fragmentgröße in 0,7 - 1,2 %igen (w/v) Agarosegelen in 1x TAE-Puffer (0.04M Tris-Base; 1mM EDTA (pH 8,0); 20mM Eisessig) durchgeführt. Die Proben und passende Molekulargewichtsmarker wurden mit 1/5 Volumen 6x Gelbeladepuffer gemischt, in die Taschen des Agarosegels pipettiert und bei konstanter Spannung von 2 - 6 V/cm für 1 – 20h aufgetrennt.
Der Nachweis aufgetrennter Nukleinsäuren erfolgte durch Zugabe von Ethidiumbromid im UV-Durchlicht bei 254nm Wellenlänge. Für die Dokumentation wurden die Gele mit einer Polaroid MP-4 Land-Kamera bei einer Blende von 5,6 und einer Belichtungszeit von 120 - 150 sec auf einen Polaroid-Film Typ 665 fotografiert.
Für die Isolierung von DNA aus Agarosegelen wurde entweder QIAEX II (QIAGEN) oder ein Elektroeluator entsprechend der Herstellerangaben verwendet.

Denaturierende Formaldehyd-Gelelektrophoresen für RNA-Proben wurden in einem Agarosegel mit 1% Agarose in 1x MOPS (20mM MOPS (pH 7,0); 5mM Natriumacetat: 1mM EDTA) und 6% Formaldehyd, bei konstanter Spannung von 6V/cm für 1 - 2h in 1x MOPS-

Puffer durchgeführt. Zur Vorbereitung der RNA-Proben wurden diese mit RNA-Gelbeladungspuffer (50% Formamid; 2,2M Formaldehyd; 1/10Vol 10x MOPS; 1% Ficoll; 0,02% Bromphenolblau) gemischt, 10min bei 65°C inkubiert und anschließend für 5 - 10min auf Eis gelagert.

PCR

Die Amplifikation von DNA-Fragmenten mittels PCR erfolgte nach Standardmethoden. Dazu wurden je nach Ansatz Standard-*Taq*-Polymerase, proofreading *Pfu*-Polymerase oder das „TripleMaster PCR System“ (Eppendorf) mit dem jeweils zugehörigen 10xPuffer verwendet. Zusammen mit 25pM der jeweiligen Primer, 0,2mM eines jeden dNTPs und geeignetem Template erfolge die Amplifikation in 0,2 oder 0,5ml PCR-Reaktionsgefäßen im Mastercycler Gradient (Eppendorf). Spezifische Angaben zu Primern, eingesetzten Templates und Amplifikationsprofilen sind in den Publikationen beschrieben.
Die direkte Aufreinigung von RCP-Ansätzen erfolgte mit Hilfe des „QIAquick PCR Purification Kits“ (QIAGEN) entsprechend der Herstellerangaben.

3’RACE (cDNA-Synthese + 3’RACE-PCR)

cDNA-Synthese erfolgte unter Verwendung der M-MMLV-Reversen-Transkriptase und des zugehörigen 5x Erst-Strang-Puffers (Invitrogen) entsprechend der Herstellerangaben. Dazu wurden in 20µl Reaktionsvolumen 1µM Poly(A)-Adapter-Primer (AP2) 5´-AAG GAT CCG TCG ACA TC $(T)_{17}$-3´, 5 - 7.5µg RNA, 4µl Erst-Strang-Puffer, 10mM DTT und 500µM von jedem dNTP mit 200U MMLV-RT für 30min bei 42°C inkubiert. Das Abstoppen der Reaktion erfolgte für 5min in einem 55°C Wasserbad, anschließend wurde die cDNA bei -20°C bis zur weiteren Verwendung gelagert.

3´-RACE-PCRs wurde semi-nested durchgeführt. Für die erste und zweite PCR-Reaktion wurden als Antisense-Primer der Universal-Adapter-Primer (UAP2) 5´-CUA CUA CUA CUA CUA AAG GAT CCG TGG ACA TC-3´ und als Sense-Primer genspezifische nested-Primer eingesetzt. Die PCR-Reaktionen erfolgten nach Standard PCR-Methoden, bei denen 5µl der cDNA-Synthese als Template für die erste PCR und 1µl der ersten PCR als Template für die zweite PCR eingesetzt wurden. Spezifische Angaben zu Sense-Primern und Amplifikationsprofilen sind in den jeweiligen Publikationen beschrieben.

Herstellung von Klonierungsvektoren

Zur Herstellung von Klonierungsvektoren wurde Vektor-DNA mit singulär schneidenden Restriktionsendonukleasen über Nacht enzymatisch verdaut und anschließend mit dem „QIAquick PCR Purification Kit“ (QIAGEN) entsprechend der Herstellerangaben

aufgereiningt. Die Entfernung überstehender Phosphatreste an den Restriktionsschnittstellen erfolgte nach der in Sambrook *et al*. (1989) beschriebenen Methode. Dabei wurden je 100pmol 5'-überstehende Enden mit 1U CIP-Enzym in einem Ansatz mit 1/10 Vol 10x CIP-Reaktionspuffer für 30min bei 37°C dephosphoryliert. Die Reaktion wurde durch Zugabe von 1/9Vol 10x CIP-Stop-Puffer und Inkubation für 30min bei 56°C gestoppt und mit dem „QIAquick PCR Purification Kit" aufgereiningt.

Ligationen

Die Ligation von Restriktionsfragmenten in Klonierungsvektoren erfolgte in 20µl Reaktionsvolumen mit Insert-DNA und geschnittenem, dephosphoryliertern Vektor in einem molaren Verhältnis von etwa 3 : 1 mit 0,5U T4 DNA-Ligase (LifeTechnologies) in 1x T4 DNA-Ligase-Puffer für 1h bei RT oder über Nacht bei 4°C.

TA-Cloning von PCR-Fragmenten wurde mit Hilfe des „pGEM-T Vektor System I" (Promega) durchgeführt. In 10µl Gesamtvolumen wurden PCR-Fragmente und pGEM-T-Vektor-DNA im molaren Verhältnis 1 : 1 mit 1µl Ligationspuffer und 3U T_4-DNA-Ligase über Nacht bei 4°C legiert.

3'-RACE PCR-Produkte wurden über PCR-Primer modifizierte Fragmentenden in den pAMP1-Vektor mit Hilfe des „CloneAmp® pAMP1 System for Rapid Cloning of Amplification Products" (Invitrogen) entsprechend der Herstellerangaben legiert. Dazu wurden auf Eis 2µl des PCR-Produkts (10 - 50ng), 2µl pAMP1-Vektor-DNA, 2µl Uracil-DNA-Glycosylase und 15µl Annealing-Puffer (1x) gemischt und für 30min bei 37°C im Wasserbad inkubiert.

Transformation von *E.coli*

Herstellung transformationskompetenter Bakterien

Die Herstellung transformationskompetenter Bakterien aus *Escherichia coli* K12-Sicherheitsstämmen erfolgte nach dem „Standard Protokoll für die SEM Präparation kompetenter Zellen" (Inoue *et al*., 1990).

250ml SOB-Medium wurden mit 10 - 12 vereinzelten Kolonien der jeweiligen Bakterienstämme angeimpft und bei 18,5°C bis zu einer OD_{600} von 0,6 wachsen gelassen. Die Bakterienlösung wurde bei 500xg, 4°C für 30min pelletiert, einmal mit eiskaltem TB-Transformationspuffer gewaschen, in 20ml TB-Puffer resuspendiert und nach Zugabe von DMSO bis zu einer Endkonzentration von 7% (v/v) und einer Inkubation für 10min im Eisbad aliquotiert, in flüssigem Stickstoff eingefroren und bis zum weiteren Gebrauch bei -80°C gelagert.

Transformation von *E.coli*

Die Transformation von *E.coli* mit Plasmiden erfolgte nach einer modifizierten Methode nach Inoue *et al*. (1990).

Pro Transformationsanzsatz wurden 100µl SEM-kompetente-Bakterien auf Eis aufgetaut und in eiskalte 5ml Polycarbonat-Röhrchen überführt. Nach Zugabe von 1µl Ligationsansatz wurde die Kultur vorsichtig geschwenkt und für 30min auf Eis inkubiert. Die Aufnahme der Plasmide in die Zelle erfolgte per Hitzeschock für 30sec bei 42°C im Wasserbad. Die Proben wurden anschließend auf Eis gestellt und nach Zugabe von 800µl SOC-Medium 1h bei 37°C im Schüttelinkubator wachsen gelassen. Die Selektion transformierter Bakterien erfolgte auf Agarplatten mit Selektionsmedium.

Zellkultur

Anlegen von Primärkulturen

Gewebeproben wurden direkt nach der Entnahme in ein Röhrchen mit Hanks-Lösung (200IU/ml Penicillin, 200µg/ml Streptomycin) gegeben. Nach einer mechanischen Zerkleinerung der Gewebestücke wurden diese mit 0.3% Collagenase-Lösung (200U/ml) bis zum Gewebeaufschluss bei 37°C (5% CO_2, 95% Luftfeuchtigkeit) inkubiert. Die Zellsuspension wurde anschließend für 5min bei 800xg zentrifugiert, das Zellpellet in geeignetem Wachstumsmedium resuspendiert und in gleichen Teilen auf 2 - 4 Kulturflaschen mit je 5ml Wachstumsmedium verteilt. Bei Adhäsion der Zellen erfolgte nach 1 bis 2 Tagen der erste Mediumwechsel mit gleichzeitigem Spülen der Zellen mit 5ml PBS.

Kultivierung von Monolayerkulturen

Zellkulturen wurden entweder in Medium TC199 (20% fetales Kälberserum; 200IU/ml Penicillin; 200µg/ml Streptomycin) oder in smooth muscle cell growth medium 2 (PromoCell; Ergänzt mit 200IU/ml Penicillin; 200µg/ml Streptomycin) bei 37°C (5% CO_2; 95% Luftfeuchtigkeit) kultiviert. Ein Mediumwechsel erfolgte alle 2 bis 3 Tage. Konfluent bewachsene Kulturflaschen wurden mit PBS gespült, trypsiniert (0.05% Trypsin; 0.02% EDTA) und auf neue Kulturflaschen mit je 5ml Medium passagiert.

Kryokonservierung von Zellkulturen

Zur Konservierung von Zellkulturen wurden die Zellen in Stickstoff eingefroren. Dazu wurde eine konfluent bewachsene Kulturflasche mit PBS gespült, trypsiniert, in 2ml eiskaltem Medium mit 10% DMSO aufgenommen und in ein Kryoröhrchen überführt. Der Einfriervorgang der Zellen erfolgte einem Einfriergerät (CTE 880, Cryotechnik Erlangen) stufenweise in folgenden Temperaturschritten: 0.7°C/min auf -13°C, 0.3°C/min auf -15°C und 1°C/min auf -120°C. Nach Beendigung des Programms wurden die Zellen in flüssigem Stickstoff gelagert.

Auftauen eingefrorener Zellen

Eingefrorene Zellen wurden direkt nach Entnahme aus dem flüssigen Stickstoff im Wasserbad bei 37°C aufgetaut. Aufgetaute Zellsuspension wurde in ein Sarstedt-Röhrchen mit 8ml Medium überführt und 10min bei 120xg zentrifugiert. Der Überstand wurde verworfen, das Pellet in Medium resuspendiert, in eine Zellkulturflasche mit 5ml Wachstumsmedium überführt und bei 37°C (5% CO_2; 95% Luftfeuchtigkeit) inkubiert. Nach einem Tag erfolgt ein Mediumwechsel.

Chromosomenpräparation

Chromosomenanalysen wurden am Zentrum für Humangenetik nach der Methode von Bullerdiek *et al*., (1987) durchgeführt.

FISH-Analysen

Für die FISH-Analysen, die am Zentrum für Humangenetik durchgeführt wurden, wurde eine Modifikation der Methode nach Kievits *et al*. (1992) benutzt (Kazmierczak *et al*., 1998).

Transformation eukaryontischer Zellen

Für die Transfektion eukaryontischer Zellen wurden die Transfektionsreagenzien Superfect (QIAGEN) und Effectene (QIAGEN) verwendet.

Die Transfektion der Zelllinien MCF-7 und HeLa erfolgte mit dem **Superfect**-Transfektionsreagenz (QIAGEN). Dazu wurden Zellen so passagiert, dass sie zum Zeitpunkt der Transfektion etwa 60% konfluent gewachsen waren und sich in einer aktiven Teilungsphase befanden. Standardmäßig wurde für den Maßstab eines Wells einer 6-Well Platte folgendes Protokoll verwendet: Komplettes entfernen des Wachstumsmediums, spülen der Zellen mit 3ml PBS und Zugabe der „Transfektionskomplexe“, die vorher mit 800µl Wachstumsmedium gemischt wurden. Die optimale Zusammensetzung der Transfektionskomplexe wurde für jedes Experiment in Vorversuchen bestimmt und ist in den entsprechenden Publikationen beschrieben. In der Regel wurden 1µg Experimentelle-DNA und ggfl. 250 – 500ng Referenzvektor mit 7 – 10µl Superfect gemischt, mit Medium (ohne Zusätze) auf ein Gesamtvolumen von 100µl gebracht und für 10min bei RT inkubiert. Nach Zugabe der Transfektionskomplexe in 800µl Wachstumsmedium wurden die Zellkulturen für 3h bei 37°C (5% CO_2; 95% Luftfeuchtigkeit) inkubiert, anschließend der Mediumüberstand komplett entfernt, die Zellen 1x mit 3ml PBS gespült und nach Zugabe von 3ml frischen Wachstumsmedium bei 37°C inkubiert.

Transfektionen mit **Effectene** wurde für Primärkulturen aus Uterus Myometrium-Zellen optimiert. Dazu wurden Zellkulturen so auf 25cm^2 Kulturflaschen passagiert, dass sie nach 38h zu 60-70% konfluent gewachsen waren. Für die Transfektionskomplexe wurden 2.14µg Plasmid-DNA, 535µl Puffer-EC (QIAGEN), 17.14µl Enhancer-Lösung (QIAGEN) und 42.9µl Effectene-Reagenz entsprechend der Angaben des Herstellers gemischt und mit 1ml Wachstumsmedium auf die Zellen gegeben. Zuvor wurde das alte Medium entfernt und die Zellen einmal mit 5ml PBS gespült. Jeweils nach 24h erfolgte ein kompletter Austausch des Mediums.

Luziferase Assays

Zur Durchführung von Luziferase Assays wurden Reportergen- und entsprechende Normalisierungsvektoren transient in eukaryontische Zellen, die in 6-Well Platten kultiviert wurden, transfiziert. Die Durchführung der Luziferasemessung erfolgte 48h nach der Transfektion mit Hilfe des „Dual-Luciferase Reporter Assay System“ (Promega), entsprechend der Angaben des Herstellers in einem Luminometer (Biocounter M2010, Lumac BV). Jedes Experiment wurde in zwei parallelen Ansätzen bestimmt und mehrere Male wiederholt.

Hybridisierungen

Transfer auf Nylonmembranen

Der **Southern Blot** erfolgte nach einem modifizierten Protokoll des „Hybond-N$^+$; Handbuch für Positiv geladener Nylon-Membranen, Version 2.0" (Amersham Biosciences) mit Hilfe eines Vakuumblotters (Vakuumblotter 785, BIO-RAD).
Vor dem Transfer der DNA wurden die Gele 15min in 0.125N HCl depuriniert (bei DNA-Fragmenten über 5kb), 2x 15min in 0.5M NaOH; 1.5M NaCl denaturiert und 2x 15min in 1,5M NaCl; 0.5M Tris-HCl pH 7.2; 1mM EDTA neutralisiert.

Northern Blot Transfers erfolgten im Anschluss an denaturierende Formaldehyd-Gelelektrophorese von RNA-Proben. Dazu wurden die Gele mehrmals für 10min in RNase-freiem dH_2O geschwenkt und bei Gelen mit mehr als 1% Agarose, mehr als 0,5 cm Dicke, oder bei RNA mit über 2,5kb Länge zusätzlich 20min in 0,05M NaOH partiell hydrolysiert. Des weiteren wurden die Gele 2x 15min in 1,8M NaCl; 0,5M Tris-HCl pH7,5 neutralisiert und 2x 15min in 10x SSC (1,5M NaCl; 0.15M Tri-Natrium-Citrat) equilibriert. Der Transfer der RNA erfolgte auf Hybond-N$^+$-Membranen für 1 - 2h bei 3mm Hg mit 10x SSC als Blottingpuffer. Geblottete Membranen wurden bis zur weiteren Verwendung bei 4°C gelagert.

Hybridisierungsprotokolle

Als **Sonden** für die Hybridisierungen wurden PCR-Fragmente verwendet, die mit dem „QIAquick PCR Purification Kit“ (QIAGEN) entsprechend der Herstellerangaben aufgereiningt und in 10mM Tris-HCl pH 8,5 gelöst wurden. Radioaktives [α^{32}P]dCTP (Redivue, Amersham Biosciences) Labeling der Sonden erfolgte mit dem „Random Primed Labeling Kit“ (Roche) entsprechend der Herstellerangaben. Sonden-DNA wurde im kochenden Wasserbad für 10min denaturiert und anschließend bis zur weiteren Verwendung im Eis inkubiert. Das Labeling von 50ng DNA erfolgte in einem Volumen von 20µl mit je 1µ dATP, dTTP, dGTP, 5µl [α^{32}P]dCTP und 5U Klenow-Enzym für 30min bei 37°C. Direkt vor Verwendung der gelabelten Sonden bei der Hybridisierung wurden die Sonden nochmals 5min im kochenden Wasserbad denaturiert. Alle Hybridisierungs- und Waschschritte erfolgten in Schraubdeckelflaschen im Rotationsinkubator. Im Anschluss an die Waschschritte wurden die Membranen in Folie eingewickelt und zur Exponierung mit einem Phosphorimaging Screen inkubiert. Die Entwicklung des Phosphorimaging Screens erfolgte mit dem „STORM Phosphorimager“ (Amersham Biosciences).

Für **Southern Blot Hybridisierungen** wurden die Nylonmembranen 30min bei 60°C mit 5 - 10ml vorgewärmter ExpressHyb-Lösung (BD Biosciences Clontech) prähybridisiert und 1h bei 60°C in 5ml ExpressHyb mit denaturierter Sonde hybridisiert. Das Waschen der hybridisierten Membranen erfolgte 4x 10min in 2x SSC; 0,05% SDS bei Raumtemperatur und 2x 20min mit 0,1x SSC; 0,1% SDS bei 50°C.

Northern Blot Hybridisierungen erfolgten nach dem gleichen Protokoll wie die Southern Blot Hybridisierung, allerdings mit der Änderung, dass Prähybridisierung und Hybridisierung bei 68°C durchgeführt wurden.

cDNA-Expressionsarray Hybridisierungen

Zur Durchführung der cDNA-Expressionsarray Hybridisierungen wurde das „Atlas Human Cancer 1.2 cDNA-Expression Arrays System“ (BD Biosciences Clontech) entsprechend des Handbuchs PT3140-1 (Version PR89832) des Herstellers verwendet.

Die **Sondenherstellung** erfolgte eine cDNA Synthese an insgesamt 5µg Gesamt-RNA (pro Array), die mittels des „RNeasy Mini Kits“ (QIAGEN) isoliert und sowohl qualitativ als auch quantitativ überprüft worden war. Dazu wurden 5µg Gesamt-RNA und 1µl CDS-Primer-Mix (BD Biosciences Clontech) in einem Volumen von 8µl im Thermocycler für 5min bei 65°C denaturiert und anschließend langsam auf 42°C abgekühlt. Danach wurden 5mM DTT, 1,5mM dNTP-Mix, 1x First-Strand-Buffer, 5µl [α^{32}P]dATP (Amersham Biosciences) und 50U Superscript (Invitrogen) hinzugegeben und der Ansatz bei 42°C für 50min inkubiert. Durch

Inkubation für 15min bei 70°C wurde die Reaktion abgestoppt. Die Aufreinigung der Sonden von uninkorporierten Nukliden erfolgte mittels Chroma-Spin-Säulen (BD Biosciences Clontech) entsprechend der Herstellerangaben. Die Scintillation der verschiedenen Reinigungsstufen wurde gemessen um Sonden mit vergleichbaren cpm für die Hybridisierung zu vergleichender Arrays einzusetzen. Die Sonden wurden mit 1/10 des Gesamtvolumens 10x Denaturierungspuffer (1M NaOH; 10mM EDTA) gemischt, der Ansatz für 20min bei 68°C inkubiert, anschließend 5µg C_ot-1-DNA und 1Vol 2x Neutralisierungslösung (1M NaH_2PO_4 pH 7,0) hinzugegeben, für weitere 10min bei 68°C inkubiert und dann für die Hybridisierung verwendet.

Für die **Hybridisierung der Arrays** wurden die Nylonmembranen in Schraubdeckelflaschen für 30min bei 68°C mit 10ml vorgewärmtem ExpressHyb (versetzt mit 1mg Salmon-Testis-DNA) prähybridisiert und über Nacht bei 68°C in 5ml ExpressHyb (versetzt mit 0,5mg Salmon-Testis-DNA) und denaturierter Sonde hybridisiert. Das Waschen der hybridisierten Membranen erfolgte 4x 30min in 2x SSC; 1% SDS bei 68°C und 2x 30min mit 0,1x SSC; 0,5% SDS bei 68°C. Zu vergleichende Arrays wurden parallel behandelt. Im Anschluss an die Waschschritte wurden die Membranen in Folie eingewickelt und zur Exponierung mit einem Phosphorimaging Screen inkubiert. Die Entwicklung des Phosphorimaging Screens erfolgte mit dem „STORM Phosphorimager“ (Amersham Biosciences).

Auswertung und Vergleich der Arrays wurde mit der AtlasImage 1.01 Software (BD Biosciences Clontech) entsprechend der Angaben im Handbuch durchgeführt. Dabei wurden die Signalintensitäten der Gene auf den Arrays gegen die Gesamtintensität aller Gene eines Arrays normalisiert.

Statistische Auswertungen

Für die statistische Auswertung von Messdaten wurden der t-Test für ungekoppelte Proben und die Bravais-Pearson-Korrelationsanalyse mit Hilfe des Programms MS-Excel (Microsoft) durchgeführt. Eine Hauptkomponentenanalyse, die nach Strukturen innerhalb von Korrelationsmatrizen sucht, wurde am Institut für Statistik der Universität Bremen, Deutschland berechnet.

ERGEBNISSE

1. ANALYSEN AUF GENOMISCHER EBENE

1.1 Bestimmung der *HMGA1*-Transkriptionsorientierung

VIII: Kazmierczak et al., *Genes Chromosomes Cancer*, 23: 1998

Um die chromosomalen Bruchpunkte der Region 6p21.3 mit Hilfe der Fluoreszenz *in situ* Hybridisierung (FISH) in Bezug zum *HMGA1* charakterisieren zu können, war es notwendig, in einem vorherigen Versuch die Orientierung von *HMGA1* auf dem Chromosomen 6 zu bestimmen. Dazu wurden mit Hilfe zweier PAC-Subklone, deren Orientierung relativ zum *HMGA1* bekannt war, eine Doppel-FISH durchgeführt. Es ergab sich eine Transkriptionsorientierung, die vom Telomer in Richtung Centromer verläuft, wodurch die Arbeit von Xiao *et al*. (1997) in dem Punkt bestätigt werden konnte.

1.2 Eingrenzung der chromosomalen Bruchpunkte der Region 6p21.3 in benignen mesenchymalen Tumoren

VIII: Kazmierczak et al., *Genes Chromosomes Cancer*, 23: 1998
unpublizierte Ergebnisse

Nachdem erste Versuche gezeigt hatten, dass die chromosomalen Bruchpunkte der Region 6p21.3 bei 10 chondroiden Lungenhamartomen (Kazmierczak *et al*., 1996c) sowie einem Uterus Leiomyom (Kazmierczak *et al*., 1996b) in einem Bereich von 120kb um *HMGA1* lokalisiert sind, sollte im Rahmen dieser Arbeit die Bruchpunktregion genauer eingegrenzt werden.

Dazu war es notwendig, die PAC-Klone, die später für die FISH-Analysen verwendet wurden, genauer zu charakterisieren. Es konnte festgestellt werden, dass die drei PAC-Klone 8603, 8604 und 8605 jeweils das komplette *HMGA1* überspannen. Durch die Subklonierung von Restriktionsfragmenten, Sequenzanalysen und Datenbankabgleiche konnten für PAC8603 und PAC8605 sowohl deren Größe als auch der genomische Bereich identifiziert werden, der von ihnen überspannt wird. PAC8603 hat ein Insert von etwa 84kb Länge, das auf genomischer Ebene etwa 68kb vor (Telomer-seitig) *HMGA1* beginnt und 6,2kb dahinter endet. PAC8605 beginnt 45kb vor *HMGA1* und endet entsprechend einer Insertlänge von 158kb 103kb Centromer-seitig 5' hinter *HMGA1*. Die Ergebnisse für PAC8604 waren hingegen nicht eindeutig. Wahrscheinlich beginnt PAC8604 65kb vor *HMGA1*, hat entsprechend der Restriktionsfragment-Analyse ein Insert von etwa 128kb Länge und müsste somit etwa 53kb hinter *HMGA1* enden.

Southern Blot Hybridisierungen an verdauter genomischer DNA der Hamartome PCH1, PCH4 und PCH51 sowie der Endometriumpolypen EP1-5 mit verschiedenen Sonden aus dem Bereich des *HMGA1* konnten keine intragenischen Veränderungen von *HMGA1* detektieren.

Mit Hilfe von FISH-Experimenten, die die PACs 8603, 8604 und 8605, als auch klonierte Restriktionsfragmente dieser PACs als Sonden nutzten, konnten auf Grund der genomischen Zuordnung der PAC-Klone, die chromosomalen Bruchpunkte der Region 6p21.3 genauer eingegrenzt werden (Tab. 3). Da PAC8603 6,2kb hinter *HMGA1* endet, wurden bei Bruchpunkten, die 3' hinter *HMGA1* lokalisiert sind entsprechend der Transkriptionsorientierung von *HMGA1* (vom Telomer in Richtung Centromer) FISH-Signale auf dem normalen Chromosomen 6 und dem Derivatchromosomen des Translokationspartners detektiert. Im Gegensatz dazu zeigten intragenische, oder 5' gelegene Bruchpunkte Signale auf Chromosomen 6, der(6) und eventuell dem Derivatchromosomen des Translokationspartners.

Tabelle 3: Ergebnisse der Untersuchung von Tumoren mit 6p21.3 Veränderungen mittels FISH. Als Sonden wurden die PAC-Klone 8603 – 8605 und die Klone EH2 und EH7 verwendet. Dargestellt sind bisher unpublizierte Daten sowie die in Kazmierczak *et al*. (1998) beschriebenen Ergebnisse. Die Lage der chromosomalen Bruchpunkte in Relation zum *HMGA1* ist beschrieben. PCH: chondroides Lungenhamartom; EP: Endometriumpolyp; UL: Uterus Leiomyom

Tumor	**Karyotyp**	Lokalisation des Bruchpunktes relativ zu *HMGA1*
PCH1	46,XX,t(6;14)(p21.3;q24)[13]	3'
PCH4	46,XY,t(3;6)(p25 or p26;p21.1 or p21.2),der(9)t(9;12)(q34;q14 or q15),der(12) t(12;16)(q14 or q15;q21),del(16)(q21)	3'
PCH28	46,XY,t(6;14)(p21.3;q24)[20]	Intragenisch
PCH31	46,XX,der(6)t(6;8)(p21.3;q12 or q13),der(14)t(6;14)(p21.3;q24)[13]	3'
PCH38	46,XX,t(6;14)(p21.3;q24)[19]	3'
PCH41	46,XY,t(6;10)(p21.3;q22.3)[21]	5'
PCH44	46,XX,der(4)t(4;6;14)(q35;p21.3p25;q24),der(6)t(4;6)(q35;p11.2), der(14)t(6;14) (14pter->14q24::6p21.2->6p12:)[16]	Intragenisch
PCH47	46,XX,del(6)(p21.3),der(14)t(6;14)(p21.3;q24)[19]	3'
PCH50	46,XX,t(6;14)(p21.3;q24)[7] / 46,XX[11]	kein Ergebnis
PCH51	46,XX,der(6)t(6;14)(p21;q24),der(14)t(6;14)(p21.2;q24),der(16)(16pter->16q24::6p21.2->6p12:)[20]	3'
PCH56	46,XY,del(2),ins(6;2)(q15,p21-p25)	kein Ergebnis
PCH63	46,XY,der(1)t(1;14)(p34.1;q24),der(6)t(6;17)(p21.3;q23),der(14) t(6;14)(p21.3;q24),der(17)t(1;17)(p34.1;q23)[13]	3'
PCH66	46,XX,der(6)(6qter->6q26::6p21.3->6q26::9p13->9pter), der(9)t(9;14)(p13;q24),der(14)t(6;14)(p21.3;q24)[23]	3'
PCH72	46,XX,t(6;10)(p21.1;q26.1)[16]	5' oder intragenisch
PCH76	46,XX,der(6)t(6;?)(p21.3;?)t(6;15)(q27;q24),der(14)t(6;14)(p21.3; q24), der(15)t(14;15)(q24;q24)[20]	3'

PCH88	46,XX,t(6;12)(p21.3;q22)[9]	3’

PCH94	46,XY,der(14)[8]	3’
PCH99	46,XY,der(4)(6pter->6p21.3::14q13->14q24::4p14->4qter),add(6)(p21.3),del(14)(q13q24), der(18)t(4;18)(p14;p11.3)[24]	3’
PCH119	46,XY,der(1)t(1;6)(p32,p21.3),del(4)(q12),der(6)t(4;1;6)(q12;p36p21.3;p21.3)[18]	5’
PCH123	46,XY,t(6;14)(p21.3;q24)[12]	3’
PCH125	47,XX,t(5;6;14)(q22;p21.3;q24),+8[12]	3’
PCH130	46,XY,der(1)del(1)(p32)del(1)(q32),add(5)(p15.1),add(7)(q22),add(10)(q24),add(16)(q24)[10]	3’
PCH137	46,XY,t(6;10)((p21.3;q11.2 or q21)[19]	3’
PCH138	46,XY,t(1;6)(p33;p21.3)[18]	3’
PCH161	46,XY,der(6)t(6;20)(p21.1;q11.2),t(14;19)(q24;q13.3),add(17)(p13),del(20)(q11.2) [9]	3’
PCH168	46,XY,der(6)del(6)(p22)der(6)t(6;13)(q21;q14),der(13)t(6;13)(q21;q14)[11]	5’
PCH182	46,XY,t(6;14;8)(p21.3;q24;q24.1),tas(15;21)(p13;p13)[11]	3’
PCH191	46,XY,t(6;10)(p21.3;q22)[7]	5’
PCH216	46,XX,t(2;6)(p21;p21.3)[10]	3’
PCH231	46,XY,der(2)t(2;7)(q23 or q33;q22),der(6),del(7)(q22),der(14)t(2;14)(q32 or q33;q24) [11] / 46,XY [9]	kein Ergebnis
PCH234	46,XY,t(X;6)(p22.1;p21.3)[15]	3’
PCH251	46,XY,t(6;9;14)(p22;q22;q23)[16]	5’ oder intragenisch
PCH253	46,XY,t(6;10)(p21.3 ;q22)[15]	3’
PCH255	46,XY,t(2;6)(p21;p21.3)[17]	5’
PCH256	46,XY,t(2;6)(p21;p21.3)[16]	5’ oder intragenisch
PCH262	46,XY,der(1)t(1;8),der(1),der(6),der(7)t(1;7),der(8),der(9),der(13),der(18)[17]	3’
PCH266	46,XX,t(6;8)(p21.3;q24)[15]	3’
PCH278	46,XX,t(6;14)(p21.3;q24)[20]	3’
PCH280	46,XY,der(6),der(14),der(19)[36]	3’
PCH287	46,XY,t(6;14)(p21.3;q24),t(9;10)(q22;p15)[12]/46,XY[9]	3’
PCH289	46,XX,der(4)t(4;14)(q35;q24),del(6)(p21.3),inv(9)(p11q12)c,der(14)t(6;14)(p21.3;q24)[21]	3’
PCH290	46,XY,inv(6)(p21.3;q24)t(6;14)(q24;q24),der(14)t(6;14)(p21.3;q24)[23]	3’
PCH301	46,XY,der(3)t(3;14)(p25;q24)ins(3;6)(p25;p21.1p25),der(6)t(3;6)(p25;p21.1),der(14) del(14)?[18]/46,XY[3]	3’
PCH304	46,XY,t(1;6)(p32;p21.3)[4] + 5 andere Klone mit t(1;6) Translokationen	3’
PCH308	46,XY,t(6;10)(p23,q22)[17]	5’
PCH310	46,XY,t(3;8)(p24;q12),t(6;14)(p22;q24),ins(14;?)(q24;q31q37)[19]	3’
PCH316	46,XY,t(1;6)(p32;p21.3)[17]	5’
PCH342	46,XY,t(6;15)(p21.3 or p22;q25)[23]/46,XY[6]	3’
PCH347	46,XY,t(6;17)(p21.3;q24),inv(9)(p11q13)c,-13,+mar[23]	3’
PCH356	46,XX,der(2),der(6),der(14)[15]/46,XX[19]	kein Ergebnis
PCH375	46,XY,t(6;14)(p21.3;q24),t(20;21)(q13.1;q11.2)[14]/46,XY[4]	3’
PCH376	46,XY,t(6;17)(p21.3;q23)[24]/46,XY,[3]	3’
PCH377	46,XX,t(6;17)(p21.3;q21)(24)	5’
PCH380	46,XY,ins(1;?)(q22),der(6)t(6;14)(p21.3;q24),add(3)(p13),der(14)t(6;14)(p21.3;q11.2 or q12)[14]	3’
PCH391	46,XY,t(6;14)(p21.3;q24)[16]	3’
PCH398	46,XY,t(2;6)(p21;p21.3)[23]	kein Ergebnis

EP1	46,XX,t(6;20)(p21;q13),der(8q)	extragenisch
EP2	46,XX,del(6)(p21.3p23)	extragenisch
EP3	46,XX,t(2;6;7)(q35;p23;q22)	extragenisch
EP4	46,XX,t(2;6)(q35;p21)	extragenisch
EP5	46,XX,inv(6)(p21q22)	extragenisch
UL1	Komplexe 6p21.3 Aberration	3'

Durch die hier präsentierten Ergebnisse konnte erstmalig gezeigt werden, dass die chromosomalen Bruchpunkte der Region 6p21.3 auch bei Endometriumpolypen zu Veränderungen im Bereich des *HMGA1* Gens führen.

Im Rahmen der genaueren Eingrenzung der Bruchpunkte bei chondroiden Lungenhamartomen, zeigten die in Tabelle 3 beschriebenen FISH-Ergebnisse bei 75% der untersuchten Tumore einen Bruchpunkt in der Region 3' hinter *HMGA1*, während nur etwa 15% der Rearrangierungen 5' vor dem Gen lokalisiert sind. Bei 3 Tumoren liegt der Bruchpunkt voraussichtlich im 5' flankierenden Bereich, allerdings konnte auf Grund der gewählten Sondenkombinationen ein intragenischer Bruchpunkt nicht ausgeschlossen werden. Zwei bereits in der Literatur beschriebene Lungenhamartome zeigten einen intragenischen Bruchpunkt (Kazmierczak *et al*., 1996c).

1.3 Lokalisierung eines neuen Gens in der Region 6p21.3

VII: Kazmierczak et al., *Genomics*, 56: 1999

Im Rahmen der molekulargenetischen Charakterisierung der chromosomalen Bruchpunktregion 6p21.3 wurde in der 3' flankierenden Region von *HMGA1* ein neues Gen identifiziert, dessen Polyadenylierungs-Stelle 1bp hinter der für *HMGA1* beschriebenen Sequenz (gb: L17131) liegt. Dieses Gen, dass auf Grund seiner Lage als *LBH*-Gen (located behind *HMGA1*) bezeichnet wurde (GeneBank-Bezeichnung C6orf1; Accession Number: AY062936), ist auf dem zu *HMGA1* komplementären Strang codiert und in tail-to-tail Richtung orientiert:

Es verfügt bisher über 5 identifizierte Exons, deren Splice-Site-Sequenzen sowohl der Donor-Akzeptor-„GT-AG"-Regel (Breathnach *et al*., 1978) als auch der Consensussequenz für Donor- und Akzeptor-Splice-Sites (Mount 1982) entsprechen. Sequenzanalysen zeigten, dass der codierende Bereich mit 91% Homologie zwischen den *LBH*-Transkripten vom Menschen und denen der Maus evolutionär stark konserviert blieb.

Auf Expressionsebene zeigten Northern Blot Hybridisierungen in allen untersuchten Normalgeweben sowohl 1kb als auch 3kb lange Transkripte sowie in den Geweben von Milz und Schilddrüse ein drittes, etwa 1,8kb langes Transkript.

Um zu untersuchen, ob die Expression von *LBH* durch die chromosomalen Aberrationen der Region 6p21.3 beeinflusst wird, wurden Northern Blot Hybridisierungen an RNA der chondroiden Lungenhamartome PCH4, PCH44 und PCH51, des Uterus Leiomyoms UL1, sowie eines Schilddrüsen-Normalgewebes durchgeführt. Die Ergebnisse dieser Hybridisierung waren identisch mit der oben beschriebenen Untersuchung des Normalgewebes und zeigten keine signifikanten Aktivitätsunterschiede zwischen Tumorgewebe und Nomalgewebe. Lediglich für das PCH44 konnte eine leicht erhöhte *LBH*-Expression festgestellt werden.

1.4 Funktionelle Untersuchung des *HMGA2*-Promoters

III: Borrmann et al., *Oncogene*, 22: 2003

Basierend auf den in der Literatur beschriebenen Daten (Ashar *et al*., 1996; Ayoubi *et al*., 1999; Chau *et al*., 1999; Rustighi *et al*., 1999) wurden zwei Regionen bestimmt, in denen die *HMGA2*-Transkription hauptsächlich initiiert wird. Entsprechend der genomischen Lage relativ zum ATG-Codon der Translationsinitiation wurden diese Regionen als proximale (pRTI: nt -161 bis -201) und distale Region (dRTI: nt -790 bis -893) der Transkriptionsinitiation bezeichnet. Ausgehend von diesen Regionen wurden verschiedene Deletionsmutanten des *HMGA2*-Promoters erzeugt und mittels des Luciferase Promoter Assays in den Zelllinien HeLa, MCF-7 und L14$_{TSV40}$ auf ihre Funktionen bei der Expression untersucht.
Es zeigte sich, dass sowohl pRTI, als auch dRTI jeweils über einen funktionell unabhängigen Promotor verfügen, wodurch die Vermutung von Chau *et al*. (1999) bestätigt werden konnte, dass die Expression von *HMGA2* von zwei, von einander unabhängigen Transkriptions-Initiationsstellen erfolgen kann. Ferner wurden negativ regulatorische Elemente für die Region –1194 bis –1717, eine zelltypspezifische Regulation für die Region –1719 bis –2097 sowie eine positiv regulatorische Region -2098 bis –2240 bestimmt. In Übereinstimmung mit den Daten von Ayoubi *et al*. (1999) wurden aktive *HMGA2*-Promotoren sowohl in HMGA2-negativen als auch in HMGA2-positiven Zelllinien gefunden.

Für ein in der Region –500 bis –600 lokalisiertes TCTCT$(TC)_{36}$-Repeat zeigte sich, dass es zelltypspezifisch die Aktivität des Promoters (im Verhältnis zu einem Fragment ohne das Repeat) nahezu verdoppelt. Da dieses Repeat als polymorph beschrieben wird (Ishwad *et al*., 1997), wurden die Repeatvarianten TC_{36}, TC_{22} und TC_1 genauer analysiert, um zu untersuchen, ob es durch interindividuelle Unterschiede in den Repeatlängen zu einer genetisch bedingten Disposition für unterschiedlich aktive *HMGA2*-Promotoren kommen könnte. Die Analyse der Repeatvarianten die mit Hilfe des Promoterfragmentes –1418 bis –1

erfolgte, zeigte für die TC_1-Variante eine signifikante (p<0,05), um 45% verminderte Promoteraktivität im Verhältnis zum TC_{36} Repeat. Ferner wurde zwischen den sich um 14 Dinukleotide unterscheidenden Varianten TC_{36} und TC_{22} ein 16%iger Aktivitätsunterschied festgestellt.

2. ANALYSEN AUF EXPRESSIONSEBENE

2.1 Untersuchung der *HMGA1*-Expression in benignen mesenchymalen Tumoren mit 6p21.3 Aberration

VIII: Kazmierczak et al., *Genes Chromosomes Cancer*, 23: 1998

Da für Tumore mit 12q14-15 Veränderungen eine charakteristische Überexpression von nativem HMGA2 oder die Expression von chimärem oder trunkiertem HMGA2 bekannt war, wurden zum Vergleich an der Subgruppe der Tumore mit 6p21.3 Veränderung Expressionsstudien von *HMGA1* durchgeführt. Dazu erfolgten 3'RACE Analysen an vier chondroiden Lundenhamartomen (PCH4, PCH31, PCH41, PCH63; entsprechend der Nomenklatur in Tab.1), einem Endometriumpolypen (EP6) sowie an einem Uterus Leiomyom (UL1). Bis auf das Hamartom PCH41, dessen Bruchpunkt auf die Region 5' vor *HMGA1* eingegrenzt wurde, hatten alle weiteren untersuchten Tumore Bruchpunkte in der 3' flankierenden Region.

Gegenüber den normalen *HMGA1*-Transkripten der Positivkontrolle (HeLa) zeigten sämtliche Tumore aberrante Transkripte mit Verkürzungen um 1238bp bis 1449bp, wobei keines der Transkripte ein intaktes 3'UTR aufweisen konnte. Während in zwei Fällen (UL1, PCH41) der ORF intakt blieb, lediglich Bereiche des 3'UTR betroffen waren, sind in den übrigen Tumoren sowohl die saure C-terminale Domäne, als auch Teile des dritten AT-hooks (EP6, PCH4) oder zusätzlich der komplette dritte AT-hook (PCH31, PCH63) deletiert. Die Veränderungen des 3'UTR umfassen dabei zwei Bereiche, für die in der Literatur eine starke Sequenzkonservativität beschrieben wurde (Eckner und Birnstiel, 1989).
Im Falle des Tumors PCH41 mit einer t(6;10)(p21;q22), dessen chromosomaler Bruchpunkt per FISH auf die Region 5' vor *HMGA1* eingegrenzt wurde, konnten im Anschluss an die Deletion im 3' UTR eine 87bp ektopische Sequenz gefunden werden. Datenbankabgleiche assoziierten diese ektopische Sequenz zu 100% mit dem 3'UTR der von Nagase *et al*. (2000) beschriebenen hypothetischen DEAD/DEAH-Box-Helikase DDX37.

Die hier beschriebenen Fälle zeigen in Verbindung mit publizierten Daten (Tkachenko *et al*., 1997; Xiao *et al*., 1997; Klotzbücher *et al*., 1999), dass die Tumore mit 6p21.3 Aberration, ebenso wie die Tumore mit Veränderungen der *HMGA2*-Region, durch eine Überexpression

des nativen Proteins, oder die Expression trunkierter und chimärer HMGA1-Formen charakterisierbar sind.

2.2 Analyse des Einflusses der 3'untranslatierten Regionen auf die Expression von *HMGA1* und *HMGA2*

V: Borrmann et al., *Oncogene*, 20: 2001

Die Beobachtung, dass in Tumoren ein erhöhter HMGA-Level nicht immer mit einer Erhöhung auf mRNA-Ebene korreliert ist, wurden von Klotzbücher *et al*. (1999) mit einer Regulation der Expression of posttranskriptioneller Ebene erklärt. Da alle in Tumoren gefundenen aberranten Transkripte, sowohl vom *HMGA2* (Ashar *et al*., 1995; Kazmierczak *et al*., 1995b; 1996b; Petit *et al*., 1996; Geurts *et al*., 1997) als auch von *HMGA1* (Xiao *et al*., 1997; Tkachenko *et al*., 1997; Kazmierczak *et al*., 1998) Veränderungen im Bereich des 3'UTR aufweisen und regulatorische Effekte für im 3'UTR lokalisierte Elemente zum Beispiel die RNA-Stabilität (Jain *et al*., 1997; Wolford und Signs, 1995) oder die Translationseffizienz (Fajardo *et al*., 1997; Izquierdo und Cuezva, 1997) betreffend bereits beschrieben worden waren, war ein weiteres Ziel dieser Arbeit, die Analyse der 3'UTR der *HMGA*-Gene hinsichtlich ihrer Effekte auf die Expression.

In silico Analysen zwischen *HMGA1*-3'UTR-Sequenzen von Mensch, Maus und Ratte zeigten zusätzlich zu den zwei von Eckner und Birnstiel (1989) beschriebenen konservierten Regionen zwei weitere Bereiche, die mit 83% bis 94% Homologie evolutionär sehr stark konserviert geblieben sind. Für *HMGA2* konnten neben einer 39bp G/C-reichen Sequenz am Anfang des 3'UTR noch zehn AUUUA-Motive detektiert werden, von denen acht bereits bekannt waren (Geurts *et al*., 1997). Derartige AUUUA-Elemente, die bei „delayed early response"-Genen für kurze Halbwertszeiten der mRNA verantwortlich sind (Malter *et al*., 1989), beschleunigen die Deadenylierung und die damit verbundene Degradation der mRNA (Decker und Parker, 1995). Für eine 58bp lange, hoch konservierte A/U-reiche Sequenz, die 13bp vor dem ersten AUUUA-Cluster lokalisiert werden konnte, zeigte die Literatur, dass sie bei untersuchten RNAs die destabilisierenden Effekte der AUUUA-Motive noch weiter verstärken kann (Xu *et al*., 1997).

Ausgehend von den vier konservierten Regionen im 3'UTR von *HMGA1* und den verschiedenen Sequenzmotiven bei *HMGA2* wurden Deletionsmutanten der 3'UTR hinter ein Reportergen kloniert und mittels Luziferase Assays funktionell genauer analysiert.

Für das native des 3'UTR von *HMGA1* konnte im untersuchten Zellsystem (HeLa) keinerlei Auswirkungen auf die Expression festgestellt werden. Deletionsmutanten, die vom 3' Ende her mindestens um die vier konservierten Regionen verkürzt waren, zeigten im Vergleich zur Positivkontrolle eine um 20% erhöhte Expressionaktivität. Zwei der konservierten Regionen

(Bereiche 2 und 4; Borrmann *et al*., 2001) bewirkten durch ihre Deletion eine um 10% bzw. 20% verminderte Expression, weshalb für diese Bereiche eine positiv-regulatorische Wirkung bei der Expression von *HMGA1* zu vermuten ist. Im Unterschied dazu, sind die Bereiche 1 und 3 durch eine, die Expression inhibierende Wirkung zu charakterisieren.
Deletionsmutanten, die den trunkierten *HMGA1*-3'UTR in den Tumore PCH41 und UL1 entsprechen (Kazmierczak *et al*., 1998), führten bei diesen Analysen zu einer 1,2-fach erhöhten Expression.

Die Analysen der verschiedenen *HMGA2*-3'UTR-Deletionsmutanten zeigten, dass die native Form *per se* zu einer 12,7-fachen Verminderung der Expressionsaktivität führt. Hierdurch konnte das von Ayoubi *et al*. (1999) beschriebene Ergebnis, dass das 3'UTR von *HMGA2* im Rahmen von Luciferase-Assays zu einer um 7 - 15-fach verringerten Expression führt, bestätigt werden.
Die Deletionsmutante des 3'UTR ohne 593bp des 3'-Endes zeigte im Vergleich zum nativen Fragment nur noch die Hälfte der Expressionsaktivität. Insgesamt kann durch die Anwesenheit dieses 3'UTR-Fragmentes die Effizienz der Expression eines Transkriptes um das 22,7-fache vermindert werden. Alle Deletionen des 3'UTR, die mehr als diese 593bp umfassen, führten zu einem kontinuierlichen Anstieg in der Expressionsaktivität. Dabei hatte das mit 485bp kürzeste hier analysierte 3'UTR-Fragment, dem sowohl die 10 AUUUA-Motive als auch die vorgelagerte A/U-reiche Sequenz deletiert waren, eine um das 2,6-fach stärkere Expressionsaktivität als das *native* 3'UTR.

3. ANALYSEN AUF FUNKTIONELLER EBENE

3.1 Funktionelle Analyse von HMGA2 und seiner aberranten Formen am Beispiel des *ERCC1*-Promoters

II: Borrmann et al., *Nucleic Acids Research*, eingereicht

Obwohl bekannt war, dass sich die Tumorentitäten mit 12q14-15 Aberration durch die Expression von Wildtyp HMGA2, trunkiertem oder chimärem HMGA2 charakterisieren lassen, waren die exakten Mechanismen, durch die diese Proteine an der Tumorprogression beteiligt sind weiterhin ungeklärt. Um die Eigenschaften dieser Tumor-charakteristischen Proteine im Bezug auf DNA-Bindungsaffinität, DNA-Bindungsmuster und Genregulierung genauer zu untersuchen, wurden für wildtyp und C-terminal trunkiertes HMGA2 (ΔHMGA2) Bindungsstudien am Beispiel einer Promoterregion exemplarisch durchgeführt. Dazu wurde die Promoterregion des DNA-Reparaturproteins ERCC1 verwendet, da sich in initialen Microarray-Experimenten gezeigt hatte, dass HMGA2 in Uterus Leiomyomen mit normalem

Karyotyp eine positive Wirkung auf die Expression dieses Gens ausübt (unveröffentlichte Daten). Des weiteren wurde im Rahmen der vorliegenden Arbeit der Einfluss von HMGA2, ΔHMGA2 sowie des HMGA2/LPP-Fusionsproteins auf die Expressionsaktivität des *ERCC1* Promoters mittels Luziferase Promoter Assays untersucht.

Durch DNA-Bindungsstudien konnte in einer AT-reichen Region –323bp bis -298bp vor dem *ERCC1*-Transkriptionsstartpunkt eine hoch affine HMGA2-Bindungsstelle lokalisiert werden, die von HMGA2 mit einer 10-fach höheren Affinität gebunden wird, als es die Proteine des *HMGA1*-Gens tun. Analysen des DNA-Bindungsmuster zeigten, dass innerhalb der Bindungsstelle ein Molekül HMGA2 stark an zwei Regionen (–323 bis –318; –312 bis –304) im Top-Strang mit Maxima bei -321 und -309, und zwei Regionen (–314 bis –305; –303 bis -298) im Bottom-Strang mit Maxima bei –310 und –301 bindet.

Vergleichende Studien mit C-terminal trunkiertem ΔHMGA2 wiesen ein leicht verändertes Bindungsmuster auf, bei dem die von ΔHMGA2 im Bereich des Top- und des Bottom-Strang gebundenen Regionen vergrößert sind und gleichzeitig stärker vor dem Verdau mit Hydroxyl-Radikalen geschützt werden, als es bei HMGA2 der Fall ist. Ebenso konnten klare Unterschiede in der Stöchiometrie der DNA-Bindung festgestellt werden. Während bei HMGA2 nur 1:1 Komplexe mit der DNA entstehen, werden für ΔHMGA2 sowohl diese 1:1-Komplexe als auch 2:1-Protein:DNA-Komplexe gefunden, wobei die Verkürzung von HMGA2 zu einer Transition von einem nicht-kooperativen zu einem kooperativen DNA-Bindungsmechanismus führt. Die Charakteristika der 1:1-Komplexe von HMGA2 und ΔHMGA2 sind dabei ähnliche, wohingegen in den 2:1-Komplexen der durch zwei ΔHMGA2-Moleküle gebundene Bereich im *ERCC1*-Promoter stark vergrößert ist und im Gegensatz zu HMGA2 und HMGA2/LPP zu signifikanten Auswirkungen auf die DNA-Konformation führt.
Allerdings konnte gezeigt werden, dass die DNA-Bindungsaffinität unabhängig von der sauren C-terminalen Domäne bei HMGA2 und ΔHMGA2 gleich groß ist.

Mit Hilfe des Luziferase Promoter Assays wurden zwei verschiedene Regionen (-3900 bis +1; -425 bis +1) des *ERCC1*-Promoters auf deren Beeinflussbarkeit durch HMGA2, HMGA2/LPP und ΔHMGA2 untersucht. Es zeigte sich, dass während HMGA2/LPP nur eine leichte Verminderung der Promoteraktivität bewirkt, beide Promoterfragmente durch HMGA2 signifikant um etwa 15% in ihrer Aktivität reduziert werden, wohingegen ΔHMGA2 eine hoch signifikane Aktivitätsminderung um 63% verursacht. Diese unterschiedlich starken Auswirkungen von HMGA2 und ΔHMGA2 auf die Aktivität des *ERCC1*-Promoters bestärken eine Funktionalität der für diese beiden Proteinformen gefundenen Unterschiede bezüglich Bindungsmuster, Stöchiometrie der DNA-Bindung und Auswirkungen auf die DNA-Konformation.

3.2 Funktionelle Analyse des Einflusses von HMGA-Proteinen und deren aberranter Formen auf die zelluläre Genexpression mittels cDNA-Expressionsarrays

I: Borrmann et al., *Oncogene*, in Vorbereitung

Um die am Beispiel des *ERCC1*-Promoters exemplarisch gezeigten funktionellen Unterschiede zwischen HMGA2, ΔHMGA2 und HMGA2/LPP genauer zu untersuchen und um herauszufinden, wieso die Expression unterschiedlicher HMGA-Proteine und deren aberrante Formen als Folge chromosomaler Aberrationen jeweils charakteristisch für die gleichen Tumorentitäten sein können, wurden auf Ebene der Regulation zellulärer Genexpression cDNA-Expressionsarray-Studien durchgeführt, um für verschiedene HMGA-Varianten spezifische Target-Gene zu identifizieren und um vergleichbare Expressionsprofile zu bekommen. Dazu wurden Myometriumzellen des Uterus mit HMGA2, ΔHMGA2 und HMGA2/LPP sowie HMGA1a, HMGA1b und trunkiertem ΔHMGA1a induziert und die Veränderungen der Gen-Expressionsprofile mittels cDNA-Expressionsarray Analysen dokumentiert.

Es zeigte sich, dass von den 1176 Genen, die auf jedem Array repräsentiert waren, im Durchschnitt lediglich 4,5% durch HMGA-Induktion differenziell exprimiert wurden. Davon wurden etwa 1,5% in ihrer Expressionsintensität hochreguliert, während 3,5% der Gene in der Expression reprimiert wurden. Darunter befanden sich Gene wie *FGF2*, *COL1A2*, *TRAM* und *AKAP12*, die von allen untersuchten HMGA-Varianten hochreguliert, bzw. im Falle von *NOTCH4* runterreguliert wurden. Hingegen zeigten sich die Gene *CDC10*, *CXCL12* und *SEMA3A* in dem untersuchten Zellsystem als HMGA1-spezifische Targets, während *MT3* und *LIG1* nur von den Varianten des HMGA2 reguliert wurde.

Auf Basis statistischer Auswertungen der Expressionsprofile konnte gezeigt werden, dass die verschiedenen HMGA-Varianten miteinander funktionell verwandt sind und ihnen ein gemeinsamer Mechanismus der Expressionsregulation zu Grunde liegt, wobei lediglich für ΔHMGA1a eine leichte Tendenz zum unabhängigen Verhalten aufweist. Da die DNA-bindenden Domänen das einzige Charakteristikum sind, dass allen hier untersuchten HMGA-Varianten gemein ist, wird der gemeinsame Mechanismus, der der Expressionsregulation zugrunde liegt, vermutlich über die AT-hooks vermittelt.

Auf Ebene der Expressionsprofile wurde diese Verwandtschaft dadurch sichtbar, dass zwischen verschiedenen HMGA-Varianten bei jeweils nur 7-30% der regulierten Gene klare Unterschiede in Form von Verlust oder Neugewinn von Funktion, oder eine um mehr als das 2-fache abweichende Fähigkeit zur Genregulation auftraten, während etwa jeweils die Hälfte der betroffenen Gene keinen Unterschied zeigten. Für die Gruppe der HMGA1-Varianten

wurden dabei 58% der differentiell exprimierten Gene von HMGA1a, HMGA1b sowie ΔHMGA1a auf annähernd identische Weise reguliert. Die Deletion der sauren C-terminalen Domäne führte bei HMGA1a lediglich in nur 7% der betroffenen Gene zu klaren Unterschieden im Regulationsverhalten.
Zwischen HMGA2 und seiner trunkierten und chimären Form wurden 49, bzw. 42% der Gene annähernd identisch reguliert. Die Deletion des C-Terminus führte in 20% der betroffenen Gene zu klaren Unterschieden im Regulationsmuster, während der Zugewinn der ektopischen LPP-Sequenz nur bei 12% der Gene klare Auswirkungen hatte.

Bei der Betrachtung der durch die verschiedenen HMGA-Varianten regulierten Gene zeigte sich insbesondere bei den HMGA1-Varianten, dass es in Myometriumzellen zu einer Herabregulierung der Gene kommt, die funktionell an Proliferation und Differenzierung beteiligt sind, wobei der stärkste Effekt bei der Gruppe der Zell-Zyklus-Regulatoren beobachtet wurde.

Entsprechend der Funktion von HMGA bei der Proliferation von glatten Muskelzellen der Blutgefäße (Chin *et al*., 1999; Foster *et al*., 2000) konnten durch die Array Experimente Target-Gene identifiziert werden, die funktionell mit Angiogenese und Myogenese oder deren pathogenen Formen verknüpft sind. Zu diesen Genen zählen für den Bereich Angiogenese *FLT1*, *NOTCH4*, *VEGFC*, *SERPINE1*, *FGF2* und durch ihre Wirkung auf die Insulin-ähnlichen Wachstumsfaktoren auch *IGFBP2*, *IGFBP4* und *IGFBP5*, während *SLC2A3*, *MAPK12*, *CDH2* und *DES* in Prozesse der Myogenese involviert sind. Für die Funktion im Bereich der Proliferation und Differenzierung von Fettgewebe (Zhou *et al*., 1995; Battista *et al*., 1999; Arlotta *et al*., 2000; Anand and Chada, 2000; Melillo *et al*., 2001) konnten *COL1A2* ebenso wie die bereits für die Angiogenese beschriebenen Gene *SERPINE1*, *IGFBP2*, *IGFBP4* und *IGFBP5* als potentielle Zielgene identifiziert werden.

4. ANALYSE DES *HMGB1*

Nachdem in den letzten Jahren immer mehr über die Funktionen von HMGB1 bekannt geworden ist und man weiß, dass es nicht nur an nukleären Prozessen wie Replikation, Rekombination, DNA-Reparatur und Transkriptionsaktivierung beteiligt ist, sondern auch im Zytoplasma, membrangebunden oder extrazellulär an der Aktivierung von Signalkaskaden oder der Tumor-Metastasierung beteiligt sein kann, ist HMGB1 immer mehr in den Blickpunkt der Forschungsinteressen gerückt.
Da zu Beginn dieser Arbeit allerdings die komplette genomische Sequenz des humanen *HMGB1* noch nicht bekannt war, ging es im Rahmen der Grundlagenarbeit zuerst darum, die

genomische Struktur komplett zu entschlüsseln und den *HMGB1*-Promoter funktionell genauer zu charakterisieren.

4.1 Komplettierung der genomischen Struktur des humanen *HMGB1*

VI: Borrmann et al., *Anticancer Research*, 21: 2001

Da für das humane *HMGB1* nur die Sequenz zwischen dem Start des ORF in Exon 2 und einem Teil des terminalen Exon 5 bekannt war, sollte die genomische Struktur dieses Genes komplett entschlüsselt werden. Dazu wurden, ausgehend vom PAC1363 zwei Restriktionsfragmente, die zusammen das *HMGB1* überspannen, subkloniert und anschließend mittels Sequenzierungen genauer analysiert.

1440bp der 5' flankierenden Region, ein 170bp langes Exon 1, das 1888bp Intron 1 und die restlichen Basen des Exon 2 (zusammen gb-Accession Number AF281043) sowie 1580bp neuer Sequenz des terminalen Exon 5 (AF169650) konnten dadurch erstmalig bestimmt werden.

Durch *in silico* Analysen dieser neu etablierten Sequenzen zeigte sich, dass die *HMGB1*-Promoterregion keine TATA-Box besitzt, dafür aber drei CCAAT-Box Bindungsstellen für die RNA-Polymerase II enthält. Zusätzlich sind mehrere Bindungsstellen für den Transkriptionsfaktor VBP vorhanden, der an der Östrogen-abhängigen Expression des Vitellogenin des Huhns beteiligt ist (Iyer *et al*., 1991). Im Bereich des Intron 1 konnte die Existenz einer 1,1kb großen CpG-Insel entsprechend der Kriterien von Gardiner-Garden und Frommer (1987) sowie zwei Bindungsstellen für den Östrogen-Rezeptor (ERE) nachgewiesen werden. Da ERE funktionell nicht auf die 5' flankierende Promoterregion limitiert sind (Hyder *et al*., 1992), könnten diese ERE, eventuell in Verbindung mit VBP, an der Östrogen-induzierten *HMGB1*-Expression (He *et al*., 2000) beteiligt sein. Im neu etablierten Bereich des Exon 5 wurden drei Poly(A)-Signalsequenzen gefunden, durch die die Poly(A)-Stellen der meisten bisher bekannten ESTs erklärt werden können.

Durch Sequenzvergleiche mit öffentlichen Datenbanken wurden darüber hinaus zwei cDNA-Klone (AA295279, AA294975) identifiziert, die anstatt mit Exon 1 mit Sequenz aus dem Intron 1 beginnen und somit auf einen alternativen Transkriptionsstartpunkt in Intron 1 direkt hinter der CpG-Insel schließen lassen.

4.2 Funktionelle Analyse der humanen *HMGB1*-Promoterregion

VI: Borrmann et al., *Anticancer Research*, 21: 2001

Aufbauend auf den *in silico* Analysen der Promoterregion wurde der Bereich –1332 bis +51 (relativ zum in GeneBank beschriebenen Transkriptionsstartpunkt) des *HMGB1*-Promoters mittels Luziferase Promoter Assays genauer analysiert, um die Mechanismen der *HMGB1*-

Regulation besser verstehen zu können. Das Promoterfragment –1332 bis –7 hatte dabei eine 1,4-fach höhere Aktivität als Positivkontrolle unter Einfluss des SV40-Promoters. Luciferase-Assays mit verschiedenen Deletionsmutanten dieser Promoterregion konnten für die Region -553 bis +58 einen starken Promoter definieren, der durch die drei CCAAT-Box RNA-Polymerase II Bindungsstellen charakterisiert ist. Die Häufung von Bindungsstellen für den als Repressor der Transkription bekannten Transkriptionsfaktor GFI1 (Zweidler-McKay *et al*., 1996) könnte ursächlich an der für die Region -1057 bis –554 beobachteten, reprimierten Expression beteiligt sein. Dagegen zeigte der Bereich –1332 bis –1058 einen positiven Einfluss auf die Transkriptionsrate.

4.3 Expressionsstudien von *HMGB1* in Mamma-Tumoren

IV: Flohr et al., *Anticancer Research*, 21: 2001

Um Variationen im Level der *HMGB1*-Expression in Mamma-Karzinomen zu untersuchen, wurden 13 Brust-Tumore unterschiedlichen Subtyps und unterschiedlichem histologischen Gradings per Northern Blot Hybridisierung genauer untersucht. Dabei konnte für allen Proben die Expression der in der Literatur beschriebenen (Wen *et al*., 1989; Xiang *et al*., 1997; Chau *et al*., 1998) spezifischen 1,4kb und 2,4kb Transkripte nachgewiesen werden. Für den quantitativen Vergleich der *HMGB1*-Expressionslevel der verschiedenen Tumoren, erfolgte eine Normalisierung gegen *GAPDH*. Der Vergleich der Tumore untereinander zeigte, dass die Stärke der Expression von *HMGB1* je nach Tumor verschieden ist, wobei für das 1,4kb Transkript eine intertumorale Variation um den Faktor 8,5 festgestellt wurde, während die Expression des 2,4kb Transkriptes in den verschiedenen Tumoren sich um das 14,5-fache unterschied.

DISKUSSION

Die im Rahmen dieser Arbeit präsentierten Ergebnisse liefern einen Beitrag zum Verständnis der Funktionen von HMGA-Proteinen bei der Tumorentstehung. Dabei verknüpfen sie die zytogenetischen Subgruppen benigner mesenchymaler Tumore mit Veränderungen der Regionen 12q14-15 und 6p21.3 und zeigen deren gemeinsame Charakteristika. Die funktionellen Untersuchungen verschiedener HMGA-Varianten liefen darüber hinaus einen tieferen Einblick in die Wirkungsweisen dieser architektonischen Transkriptionsfaktoren.

Benigne mesenchymale Tumoren, als die größte Gruppe gutartiger Neoplasien des Menschen bilden, lassen sich zytogenetisch in Subgruppen mit normalem Karyotyp, mit Veränderungen der chromosomalen Regionen 12q14-15 und 6p21.3 sowie mit Deletionen im langen Arm von Chromosomen 7 u.a. unterteilen. Da fast die gleichen Tumorentitäten, die durch Rearrangierungen des in 12q14-15 gelegenen *HMGA2*-Gens charakterisiert sind, ebenfalls Veränderungen der Region 6p21.3 aufweisen, lag der Schluss nahe, dass das in dieser Region lokalisierte *HMGA1*-Gen von den Rearrangierungen betroffen sein könnte. Erste Versuche an Uterus Leiomyome (Kazmierczak *et al*., 1996b; Williams *et al*., 1997) sowie chondroiden Lungenhamartomen (Kazmierczak *et al*., 1996c) bestätigten diese Vermutung und zeigten, dass die chromosomalen Bruchpunkte in einer Region von ca. 120kb um das *HMGA1*-Gen lokalisiert sind. Zur genaueren Eingrenzung der chromosomalen Bruchpunkte der Region 6p21.3 wurden im Rahmen dieser Arbeit in einem ersten Schritt die genomische Region des *HMGA1* an Hand von PAC-Klonen genauer analysiert. Ausgehend von diesen PAC-Klonen, die den Bereich von 68kb 5' vor, bis 103kb 3' hinter *HMGA1* überspannen, konnte für *HMGA1* eine Transkriptionsorientierung bestimmt werden, die vom Telomer in Richtung Zentromer verläuft (Kazmierczak *et al*., 1998), und in diesem Punkt die Arbeit von Xiao *et al*. (1997) bestätigt. Die Bestimmung der Orientierung von *HMGA1* stellte einen entscheidenden Schritt für die genauere Charakterisierung der chromosomalen Bruchpunktregion dar, denn durch sie wurde es möglich, die Ergebnisse von FISH-Analysen an Tumorzellen zu interpretieren, um genauere Aussagen über die Lokalisation der Bruchpunkte treffen zu können.
Durch die im Anschluss durchgeführten FISH-Analysen und Southern Blot Hybridisierungen konnte für Tumore mit 6p21.3 Veränderung die Lage der chromosomalen Bruchpunkte in Relation zum *HMGA1* genauer bestimmt werden (Kazmierczak *et al*., 1998 und unpublizierte Ergebnisse). Für 51 untersuchte chondroide Lungenhamartome und ein Uterus Leiomyom zeigte sich in 75% der Fälle ein Bruchpunkt im Bereich 3' hinter *HMGA1*, durch den, unter Berücksichtigung der in Richtung Zentromer verlaufenden Transkriptionsorientierung, das komplette *HMGA1* auf das Chromosomen des Translokationspartners verschoben wurde.

Weitere 15% der Tumore hatten Rearrangierungen 5' vor *HMGA1* und in den übrigen Fällen wurden Bruchpunkte 5' vor HMGA1 oder intragenisch beobachtet.

In Verbindung mit den in der Literatur beschriebenen Daten zeigen sich, im Gegensatz zu den Daten für *HMGA2*, bei dem die Bruchpunkte primär intragenisch lokalisiert sind, bei *HMGA1* nur sehr wenige intragenische Bruchereignisse. Stattdessen scheint die 3' Region hinter *HMGA1* das bevorzugte Ziel chromosomaler Veränderungen zu sein. Beispielhaft für intragenische Bruchereignisse in *HMGA1* sind zwei Lipome mit intragenischen Deletionen (Tkachenko *et al*., 1997) sowie ein Lungenhamartom, bei dem ein Bruch im Intron 7 zu einer Fusion mit dem *LAMA4**-Gen führt (Xiao *et al*., 1997).

Auffallend bei der Betrachtung der Karyotypen der Tumore sind die häufig auftretenden, identischen Translokationsereignisse. Bei denen in dieser Arbeit analysierten 56 Lungenharmatomen mit 6p21.3 Aberrationen war die am häufigsten auftretende zytogenetische Veränderung der t(6;14)(p21.3;q24), bei 22 Tumoren zu finden, wobei für alle diese Tumore ein chromosomaler Bruchpunkt in der 3' flankierenden Region von *HMGA1* nachgewiesen werden konnte (Kazmierczak *et al*., 1998 und unpublizierte Ergebnisse). Für vergleichbare Tumore mit einer t(12;14)(q14;q24) konnte *RAD51L1* auf Chromosomen 14 als das Fusionsgen von *HMGA2* identifiziert werden (Schoenmakers *et al*., 1999). Daher ist es denkbar, dass innerhalb der Gruppe der Tumoren mit 6p21.3 Aberration, die Subgruppe der t(6;14)(p21.3;q24)-Veränderungen durch Rearrangierungen mit dem *RAD51L1*-Gen oder dessen regulatorischen Regionen charakterisiert sein könnte. Für *HMGA1* und seinen primär 3' gelegenen Bruchpunkten könnte es durch die Rearrangierung des Chromosomenmaterials, wie es beispielhaft für den T-Zell-Rezeptor-alpha (*TCR*α) beschrieben wurde (Ho und Leiden 1990), zu einem Verlust reprimierender Elemente und dadurch zu einer Reaktivierung der *HMGA1*-Expression kommt. Für die wenigen 5' gelegenen Bruchpunkten ist es denkbar, dass *HMGA1* in transkriptionsaktivere Regionen oder in die Nähe von Enhancer-Elementen transloziert wird, die dann zu einer verstärkten Aktivität des *HMGA1*-Promoters führen. Ein derartiger Mechanismus ist zum Beispiel für das Burkitt Lymphom dargestellt (Davis *et al*., 1984; Joos *et al*., 1992). Hierbei wird das *MYC*-Gen durch chromosomale Rearrangierungen mehrere 100kb oberhalb des Gens, mit dem Intron-Enhancer des Gens für die schweren Ketten der Immunglobuline fusioniert, in dessen Folge es zu einer verstärkten Expression von *MYC* und der damit verbundenen Tumorentstehung kommt. Die Betrachtung der wenigen Tumore mit Bruchpunkten 5' von *HMGA1* lässt eine leichte Überrepräsentation von Tumoren mit einer t(6;10)(p21.3;q22) oder t(2;6)(p21;p21.3)-Aberration erkennen.

Um darüber hinaus zu untersuchen, welche Auswirkungen die chromosomalen Aberrationen der Region 6p21.3 auf die Expression von *HMGA1* haben, wurden *HMGA1*-Expressionsstudien an der Subgruppe der Tumore mit 6p21.3 Veränderung durchgeführt. Vier untersuchte Lungenhamartome, ein Endometriumpolyp sowie ein Uterus Leiomyom die bis auf ein Hamartom mit 5' lokalisiertem Bruchpunkt, Veränderungen in der 3' flankierenden Region aufwiesen, zeigten alle ausschließlich trunkierte Transkripte, wobei von den Verkürzungen immer auch Teile des 3'UTR betroffen waren (Kazmierczak *et al*., 1998). Dabei waren bei zwei Tumoren die ORF intakt, während in den übrigen Tumoren sowohl die saure C-terminale Domäne, als auch Teile des dritten AT-hooks oder zusätzlich der komplette dritte AT-hook deletiert waren.
Für das Hamartom mit dem 5' lokalisiertem Bruchpunkt und der t(6;10)(p21;q22) konnte darüber hinaus eine Fusion mit einem 87bp langen Teil des 3'UTR der auf 12q24.32 lokalisierten hypothetischen DEAD/DEAH-Box-Helikase DDX37 nachgewiesen werden. Die Ursache für die Entstehung dieses Fusionsgens, trotz eines 5' von *HMGA1* gelegenen Bruchpunktes, ist unklar. Allerdings wurden ähnliche Ergebnisse auch für Uterus Leiomyome beschrieben, bei denen Tumore HMGA2/RAD51L1 Fusionstranskripte zeigten, obwohl sie jeweils Bruchpunkte in der Region 5' oder 3' von *HMGA2* aufwiesen (Quade *et al*., 2003). Eventuell lässt sich für solche Tumore ein komplexerer Karyotyp mit Mikroinsertionen vermuten, die mit Hilfe der Standardmethoden der zytogenetischen Chromosomenanalyse nicht mehr wahrnehmbar sind.
Expressionsstudien für das neu identifizierte *LBH*-Gen, dass direkt 3' hinter *HMGA1* in tail-to-tail Orientierung lokalisiert ist, zeigten dagegen bei keinem der untersuchten Tumoren eine signifikante Änderung der Expression (Kazmierczak *et al*., 1999).

Durch die in dieser Arbeit präsentierten Ergebnisse in Verbindung mit weiteren Daten aus der Literatur (Ashar *et al*., 1995; Schoenmakers *et al*., 1995; Kazmierczak *et al*., 1995b; 1996b; Petit *et al*., 1996; Geurts *et al*., 1997; Tkachenko *et al*., 1997; Xiao *et al*., 1997; Kottickal *et al*., 1998; Klotzbücher *et al*., 1999) konnte eindeutig gezeigt werden, dass es bei verschiedenen Tumorentitäten sowohl mit Veränderungen der *HMGA1*-Region als auch 12q14-15 Aberrationen durch die chromosomalen Veränderungen zu einer Reaktivierung der *HMGA*-Expression kommt, in deren Folge für beide zytogenetischen Subgruppen charakteristisch natives Protein überexprimiert wird, oder trunkierte und chimäre HMGA-Formen entstehen. Dabei wurde gezeigt, das die chromosomalen Bruchpunkte sowohl intragenisch als auch extragenisch lokalisiert sein können.
Während die Entstehung trunkierter Transkripte zum Teil über intragenische Deletionen (Tkachenko *et al*., 1997), oder über aberrantes Splicen (Hauke *et al*., 2001; 2002) erklärt werden kann, sind die chimären Formen fast ausschließlich das Resultat intragenischer Rearrangierungen (Schoenmakers *et al*., 1995; Kazmierczak *et al*., 1995b). Die ursächlichen

Mechanismen für die Entstehung aberranter Transkripte als Folge extragenischer Bruchpunkte bleiben jedoch weiterhin ungeklärt.
Da für chimäres HMGA2 eine Vielzahl verschiedener Fusionspartner bekannt sind, die zum Teil nur wenige Aminosäuren zum Protein beisteuern, liegt die Vermutung nahe, dass nicht der Zugewinn ektopischer Sequenz, sondern eher der Verlust der sauren C-terminalen Domäne das entscheidende Ereignis für die Tumorgenese darstellt. Unterstützt wird diese Theorie durch die Feststellung, dass sowohl trunkiertes ΔHMGA2, als auch chimäres HMGA2/LPP in der Lage sind, ohne nennenswerte Unterschiede eine neoplastische Transformation von NIH-3T3 Zellen zu bewirken (Fedele *et al*., 1998). Durch den Verlust der C-terminalen Domäne verlieren die Proteine posttranslationale Modifikationsstellen für die Casein Kinase 2 (CK2), die mit einer reduzierten DNA-Bindungsaffinität und der Degradation durch Proteinasen assoziiert sind (Wang *et al*., 1995; Wisniewski und Schwanbeck, 2000).

Auf Ebene der Transkription zeichnet sich aberrante Formen zusätzlich durch den Verlust im 3'UTR codierter Informationen aus. Im Falle von *HMGA1* sind dabei häufig Regionen betroffen, deren Sequenzen evolutionär stark konserviert geblieben sind (Eckner und Birnstiel, 1989; Borrmann *et al*., 2001). Funktionale Untersuchungen dieser Regionen zeigten, dass sie sowohl positiv-regulatorische Einflüsse, als auch inhibierende Wirkungen auf die Expression ausüben können und deren Deletion zu einer Erhöhung der Expressionsaktivität um 20% im Vergleich zum nativen 3'UTR führt (Borrmann *et al*., 2001). Darüber hinaus zeigten Deletionsmutanten, die den trunkierten Transkripten der Tumore PCH41 und UL1 vergleichbar waren (Kazmierczak *et al*., 1998), eine im Vergleich zum nativen 3'UTR um 1,2-fach erhöhte Expression (Borrmann *et al*., 2001). Funktionell könnte es sich bei den konservierten Regionen um Zielsequenzen für RNA-bindende Proteine handeln, deren Interaktion mit einer Erhöhung (Jain *et al*., 1997) oder Verringerung (Wolford und Signs, 1995) der Expressionsaktivität einher geht.
Vergleichende Studien mit dem 3'UTR von *HMGA2* zeigten, dass dessen Wildtyp 3'UTR *per se* eine Verminderung der Expressionsaktivität um das 12,7-fache bewirkt, wodurch die Daten von Ayoubi *et al*. (1999) bestätigt werden konnten. Die expressionssteigernden Effekte, die durch Verkürzungen des 3'UTR hervorgerufen werden konnten, lassen sich vermutlich auf die im 3'UTR lokalisierten AUUUA-Elemente sowie der vorgelagerten, hoch konservierten A/U-reichen Region zurückführen, die bei Untersuchungen anderer Gene eine destabilisierende Wirkung auf die RNA ausübten (Malter *et al*., 1989; Xu *et al*., 1997). Eine sukzessive Deletion dieser Elemente führte dabei zu einem kontinuierlichen Ansteigen der Expression, bis auf den 2,6-fachen Wert des nativen 3'UTR (Borrmann *et al*., 2001).
Der hier beschriebene Mechanismus der vermutlich posttranskriptionellen Regulation der *HMGA*-Expression durch funktionelle Element im 3'UTR, könnte zu dem von Klotzbücher *et*

al. (1999) beschriebenen Phänomen beitragen, dass eine Erhöhung des HMGA-Proteinlevels nicht immer mit einer Erhöhung der mRNA-Konzentration korreliert ist.

Ferner wurde im Rahmen dieser Arbeit untersucht, welche Veränderungen der Proteinfunktion in Bezug auf die Expressionsregulation mit den aberranten HMGA-Formen verbunden sind. Dazu wurde in einem ersten Schritt das Bindungsverhalten von HMGA2 und ΔHMGA2 exemplarisch am Beispiel des *ERCC1*-Promoters untersucht. Bei ERCC1 handelt es sich um ein DNA-Reparatur-Protein, dass unter anderem an der Nukleotid-Exzisionsreparatur-Reparatur beteiligt ist.
Nachdem eine hoch-affine HMGA2 Bindungsstelle im ERCC1-Promoter identifiziert werden konnte, zeigten vergleichende Studien zwischen HMGA2 und ΔHMGA2, dass die Bindungsaffinität für den *ERCC1*-Promoter unabhängig von der C-terminalen Domäne ist (Borrmann *et al*., eingereicht). Allerdings konnten Unterschiede im Bindungsmuster verdeutlicht werden, bei denen die von C-terminal trunkiertem ΔHMGA2 im Top- und Bottom-Strang gebundenen Bereiche sowohl vergrößert, als auch stärker vor dem Verdau mit Hydroxyl-Radikalen geschützt sind, als es für natives HMGA2 der Fall ist. Außerdem wurden für ΔHMGA2 zusätzlich Protein:DNA-Komplexe mit einer 2:1-Stöchiometrie gefunden, während hingegen normales HMGA2 nur als 1:1-Komplex mit der Promoterregion interagiert. Dabei ist auffällig, dass die C-terminale-Verkürzung zu einer Transition von einem nicht-kooperativen zu einem kooperativen DNA-Bindungsmechanismus führt. Genauere Analysen der 2:1-Komplexe konnten zeigen, dass die von zwei ΔHMGA2-Moleküle gebundene Region im *ERCC1*-Promoter stark vergrößert ist. Auf funktionaler Ebene sind diese Unterschiede im Bindungsverhalten mit signifikanten Änderungen der DNA-Konformation sowie einer um das 4,2-fach stärkeren Inhibition der *ERCC1*-Promoteraktivität durch ΔHMGA2 korreliert.
Die hier beschriebenen Ergebnisse bestätigen zum Teil die kürzlich von Noro *et al*. (2003) veröffentlichten Daten, die unter anderem ebenfalls zeigen konnten, dass die C-terminale Domäne keinen Einfluss auf die Spezifität der HMGA2-Bindung hat. Das Bindungsverhalten von HMGA2, ΔHMGA2 und HMGA2/MAD (Mitochondriale Aldehyd Dehydrogenease) zeigte bei hoch affinen Bindungsstellen wie beispielsweise im *HLA-DRA*-Promoter ebenfalls kaum Unterschiede. Bei höheren Proteinkonzentrationen konnten wie in dieser Arbeit beschrieben, für ΔHMGA2 ebenfalls Protein:DNA-Komplexe über eine 1:1-Stöchiometrie hinaus beschrieben werden. Allerdings zeigten sich gravierende Unterschiede im Bindungsverhalten bei Promotoren mit geringer HMGA2-Bindungsaffinität.
Diese Daten beschreiben erstmalig detailliert die Auswirkungen der C-terminalen Domäne auf das Bindungsverhalten von HMGA2. Während der Verlust der C-terminalen Domäne von HMGA2 zu signifikanten DNA-Konformationsänderungen und einer 4,2-fach stärkeren Inhibition des *ERCC1*-Promoters führen, zeigten Studien am *IFNβ*-Promoter, dass die

sauren C-terminalen Domänen von HMGA1 keinerlei Auswirkung auf die Expressionsaktivität ausübt (Yie *et al*., 1997; Thanos und Maniatis, 1992). Ebenso belegen die Daten für HMGA2, dass dessen C-terminale Domäne keinen Einfluss auf die DNA-Bindungsaffinität von HMGA2 an hochaffine Promotoren hat (Noro et al., 2003; Borrmann et al., eingereicht) wohingegen die saure C-terminale Domäne von HMGA1 die Bindung an Nukleosomen zwar nicht beeinträchtigt (Reeves und Nissen, 1993), aber sowohl die Bindungsaffinität für supercoiled Plasmide als auch die Effizienz zur Induktion negativer Supercoils erhöht (Nissen und Reeves, 1995). Darüber hinaus ist für die C-terminalen Domäne bekannt, dass sie selbst nicht in der Lage sind, die Aktivität von Transkriptionsfaktoren oder deren Binden an die DNA zu beeinflussen (Chin *et al*., 1998; Noro *et al*., 2003) und

In einem zweiten Schritt zur Untersuchung der funktionellen Unterschiede zwischen nativem und aberrantem HMGA wurden cDNA-Expressionsarray Analysen mit HMGA2, HMGA1a, HMGA1b, ΔHMGA2, ΔHMGA1a und HMGA2/LPP induzierten uterinen Myometriumzellen durchgeführt (Borrmann *et al*., in Vorbereitung). Dabei zeigte sich, dass etwa 4,5% der insgesamt untersuchten Gene differenziell exprimiert wurden, von denen etwa 1/3 hochreguliert und 2/3 in der Expression reprimiert wurden. Bei einer geschätzten Zahl von 35.000 menschlichen Genen, gäbe es somit etwa 1600 potentielle Zielgene für die Familie der HMGA-Proteine. Unter diesen Genen befinden sich beispielsweise *FGF2*, *COL1A2*, *TRAM* und *AKAP12*, die sowohl von den nativen, als auch von den aberranten HMGA-Formen gleichermaßen hochreguliert, bzw. im Falle von *NOTCH4* runterreguliert wurden. Dagegen sind *CDC10*, *CXCL12* und *SEMA3A* in Myometriumzellen HMGA1-spezifisch und *MT3* und *LIG1* nur spezifisch von den Varianten des HMGA2 reguliert.

Darüber hinaus konnte durch die Expressionsarray Analysen gezeigt werden, dass die funktionellen Unterschiede zwischen den nativen und aberranten HMGA-Formen nicht sehr groß sind und nur relativ wenige Gene unterschiedlich reguliert werden. Für die C-terminale Verkürzung von HMGA1a konnte beispielsweise bei nur 7% der Gene ein klar verändertes Regulationsmusters festgestellt werden. Die Verkürzung von HMGA2 zeigte bei 20% der Gene eine klare Veränderung der Expressionsaktivität. Diese relativ wenigen, durch die C-terminalen Verkürzungen unterschiedlich regulierten Gene, könnte dadurch erklärt werden, dass lediglich bei Promotoren mit niedriger Bindungsaffinität drastische Unterschiede zwischen HMGA2 und ΔHMGA2 zu finden sind, während deren Bindungscharakteristika bei hoch affinen Bindungsstellen annähernd identisch sind (Noro *et al*., 2003, Borrmann *et al*., eingereicht).
Unter den wenigen Genen, die im Vergleich zu den nativen Formen durch die trunkierten Formen klar unterschiedlich reguliert werden, sind für ΔHMGA1a die Gene *CDC10*, *CDC37*,

CDK3, deren Proteine an der Regulation des Zellzyklusses beteiligt sind, sowie das in die Progression von Lungen- und Mamma-Tumoren involvierte *Basigin*. ΔHMGA2-spezifisch werden einige in die Zellproliferation involvierte Gene wie *CLK3*, *IGFBP2* und *IGFBP4*, der *Oncostatin M Rezeptor*, *EGR1* oder *Stratifin* stark unterschiedlich reguliert. Inwieweit diese Gene an der Entstehung benigner mesenchymaler Tumoren beteiligt sind, bedarf jedoch weiterer Analysen.

Zusätzlich zeigen die Expressionsstudien an den Myometriumzellen, dass die verschiedenen HMGA-Varianten, insbesondere die HMGA1-Formen in den Zellen des Uterus-Normalgewebes, eine inhibitorische Wirkung auf Gene ausüben, deren Proteine funktionell an Proliferation und Differenzierung beteiligt sind. Dabei wird der stärkste Effekt bei der Gruppe der Zellzyklus-Regulatoren beobachtet. Durch diese Ergebnisse könnte erklärt werden, wieso eine Suppression der HMGA1-Expression in normalen Fibroblasten zu einer stark erhöhten Wachstumsrate (Melillo *et al*., 2001) und die Überexpression von HMGA1b in normalen Schilddrüsenzellen zu einer Deregulation des Zellzyklusses führt (Fedele *et al*., 2001).

Der Vergleich, der im Rahmen dieser Arbeit präsentierten Daten (Borrmann *et al*., in Vorbereitung) mit Daten einer vergleichbaren Studie bei denen Myomzellen normalen Karyotyps mit HMGA2-induziert wurden (unveröffentlichte Daten) zeigt, dass nur vier Gene (DCC, ARHA, XRCC1 und Alpha-Catenin) durch HMGA2 sowohl in den Zellen des Uterus-Normalgewebes als auch in den Zellen des Uterus-Tumorgewebes gleichermaßen reguliert werden.
Ein ähnliches Bild ergibt sich bei dem Vergleich mit dem Expressionsprofil von HMGA1b-induzierten Zellen der Mamma-Karzinomzelllinie MCF-7 (Reeves *et al*., 2001). Während bei Tumor-Zelllinie MCF-7 83% der Gene durch HMGA1b aktiviert wurden, sind in den Zellen des Uterus-Normalgewebes nur 28% der Gene hochreguliert. Darüber hinaus werden in den beiden Zelltypen ganz unterschiedliche Genprofile reguliert. Von den 106 Genen, die durch HMGA1b in MCF-7 differentiell exprimiert werden, sind lediglich drei Gene in den Myometriumzellen vergleichbar reguliert. Gene, deren Proteine an Proliferation und Differenzierung beteiligt sind, werden durch HMGA1b in den Myometriumzellen primär runterreguliert, während sie in den Mamma-Karzinomzellen stark aktiviert werden.
Auf Grund dieser unterschiedlichen HMGA-induzierten Expressionsprofile in den Zellen des Normalgewebes und in Zellen benigner und maligner Tumoren, lässt sich für die HMGA-Proteine ein Wirkungsmechanismus vermuten, der abhängig ist vom zellulären Kontext des betrachteten Systems. So kommt es durch die verschiedenen HMGA-Varianten im Kontext normaler Myometriumzellen zu einer Regulation eines ganz anderen Genprofils als im Kontext einer bereits neoplastischen Zellen. Die Ursache für diesen Zellkontext-spezifischen

Wirkungsmechanismus liegt wahrscheinlich darin begründet, dass HMGA-Proteine als architektonische Transkriptionsfaktoren nur ein Bestandteil des Transkriptionsapparates sind, in dem sie die DNA-Konformation modulieren, aber selbst nicht in der Lage sind Transkription zu initiieren (Thanos und Maniatis, 1992). Die Wirkung der HMGA-Proteine ist somit abhängig von den zellulären Gegebenheiten wie beispielsweise des Transkriptionsfaktor-Milieus, der übergeordneten Chromatinstruktur oder der jeweiligen Aktivität von Proteinen, die durch posttranslationale Modifikationen die Wirkung von HMGA beeinflussen.
Durch die Abhängigkeit der HMGA Wirkungen vom zellulären Kontext ließe sich auch erklären, weshalb die Überexpression von *HMGA1* einerseits ein charakteristisches Merkmal für maligne Neoplasien ist (Giancotti *et al*., 1987) und dessen Blockade die Apoptose von Karzinomzelllen induziert (Scala *et al*., 2000), während andererseits sowohl HMGA1 als auch HMGA2 nicht in der Lage sind, im Kontext normaler Schilddrüsenzellen, eine neoplastische Transformation hervorzurufen (Fedele *et al*., 2001; Scala *et al*., 2001). Ebenso wird im Kontext normaler Zellen eine durch HMGA1 hervorgerufene Verminderung der Proliferationsrate (Melillo *et al*., 2001) und verzögerte G2-M-Transition (Fedele *et al*., 2001) beobachtet, während im Kontext maligner Tumorzellen HMGA1 ein Anstieg der Proliferationsrate bewirkt (Reeves *et al*., 2001). Diese Zellkontext-spezifische Wirkungsweise der HMGA-Proteine erklärt auch, weshalb Expressionsstudien an HMGA2-induzierten Myomzellen einen aktivierenden Einfluss von HMGA2 auf den *ERCC1*-Promoter zeigten (Unveröffentlichte Daten), während in der Cervix-Karzinomzelllinie HeLa eine durch HMGA2 inhibierte *ERCC1*-Promoteraktivität beobachtet wird (Borrmann *et al*., eingereicht).

Vergleicht man die durch HMGA regulierten Gene in Zellen des Uterus Normalgewebes (Borrmann *et al*., in Vorbereitung) mit den für Uterus Leiomyomen charakteristisch exprimierten Genen (Tsibris *et al*., 2002) zeigt sich, dass die unterschiedlichen HMGA-Varianten lediglich in der Lage sind drei (*IGFBP5*, *CDH2*, *IGKC*) von 105 Tumor-spezifischen Genen in den Zellen des Uterus-Normalgewebes gleichermaßen zu regulieren, somit die untersuchten HMGA-Formen nicht in der Lage sind, in den Zellen des Uterus-Normalgewebes ein Tumor-spezifisches Expressionsprofil zu induzieren. In Verbindung mit der Zellkontext-spezifischen Wirkungsweisen von HMGA wird dadurch die Theorie der HMGA-Veränderung als ein sekundäres Ereignis in der Tumorgenese weiter bestärkt.
Diese Theorie basiert auf den Erkenntnissen, dass es für eine Reihe von benignen mesenchymalen Tumorentitäten neben den Tumoren mit chromosomalen Aberrationen auch Tumore gibt, die zytogenetisch einen normalen Karyotyp aufweisen (Mark *et al*., 1990; Sreekantaiah *et al*., 1991; Stern *et al*., 1992), wobei für Uterus Leiomyome gezeigt werden konnte, dass die Tumore mit klonalen Aberrationen in 12q14-15 signifikant größer sind, als die Tumore mit normalem Karyotyp (Hennig *et al*., 1999). Ebenso sind die gefundenen

Tumore mit einem Mosaik aus zytogenetisch normalen und aberranten Karyotypen signifikant kleiner als vergleichbare Tumore, die ausschließlich Zellen mit einem aberranten Karyotyp aufweisen (Rein *et al*., 1998), wobei beide Zellpopulationen des mosaiken Karyotyps einem gemeinsamen, monoklonalen Ursprung entstammen (Mashal *et al*., 1994). Obwohl die Untersuchung von 61 chondroiden Lungenhamartomen normalen Karyotyps eher die Theorie der chromosomalen Veränderung als primäres Ereignis befürworten (Lemke *et al*., 2002), lassen die in dieser Arbeit präsentierten, funktionellen Daten doch eher auf einen Mechanismus schließen, bei dem die Tumorgenese, insbesondere die Genese von Uterus Leiomyomen sich auf Grund eines bisher unbekannten primären Ereignisses, beispielsweise einer Virusinfektion (Bullerdiek, 1999) vollzieht. Durch den veränderten zellulären Kontextes im Anschluss an das Primärereignis, könnten die Veränderungen von HMGA1 oder HMGA2 als sekundäre Ereignisse dann das Tumorwachstum begünstigen (Hennig *et al*., 1999).

Als ein weiteres Ergebnis der Expressionsarray Analysen HMGA-induzierter Myometriumzellen (Borrmann *et al*., in Vorbereitung) wurden im Rahmen dieser Arbeit HMGA-Zielgene identifiziert, die die Bedeutung von HMGA bei der Proliferation glatter Muskelzellen der Blutgefäße und der pathologischen Situation der Arteriosklerose (Chin *et al*., 1999; Foster *et al*., 2000) erklären könnten. Derartige Zielgene sind beispielsweise der Wachstumsfaktor VEGFC sowie FLT1 als Rezeptor für VEGF, die von entscheidender Bedeutung für die Regulation der Angiogenese im Rahmen embryonaler Gefäßentwicklung sind und bereits mit pathologischen Situationen wie Arteriosklerose und Tumorwachstum assoziiert wurden (Shibuya *et al*., 1994; Fong *et al*., 1995; Cao *et al*., 1998; Salven *et al*., 1998). Ebenso sind der durch alle HMGA-Varianten in Myometriumzellen runterregulierte Rezeptor NOTCH4, der während der Embryonalentwicklung entscheidend für die Verzweigung des Gefäßsystems verantwortlich ist (Krebs *et al*., 2000) sowie die Gene FGF2, IGFBP2, 4 und 5 Zielgene für HMGA. Im Gegensatz zum Wachstumsfaktor FGF2, der von allen HMGA-Proteinen in seiner Expression hochreguliert wurde und sowohl in Angiogenese und Wundheilung involviert, als auch mit Arteriosklerose und der Gefäßversorgung von Tumoren assoziiert ist (Chen und Henry, 1997; Schmidt *et al*., 1999), ist eine mögliche Wirkung von IGFBP2, 4 und 5 auf die Proliferation von Gefäßzellen indirekt, über deren inhibierende Wirkung auf die Insulin-ähnlichen-Wachstumsfaktoren IGF1 und IGF2 begründet, die ihrerseits an normalem Gefäßwachstum und Arteriosklerose beteiligt sind (Hellstrom *et al*., 2001; Zaina *et al*., 2002). Während die von HMGA in normalem Myometrium regulierten Gene *SLC2A3*, *MAPK12*, *CDH2* und *DES* alle in Prozesse der Myogenese involviert sind (Hahn *et al*., 1998; Lechner *et al*., 1996; Cifuentes-Diaz *et al*., 1993; Munoz-Marmol *et al*., 1998) ist für SERPINE1 bekannt, dass es sowohl in arteriosklerotischen Arterien als auch in Adipozyten bei Fettleibigkeit überexprimiert wird (Yamamoto und Saito,

1998; Samad *et al*., 1997). Somit stellt SERPINE1 ein potentielles Zielgen sowohl für die Funktion von HMGA bei der Gefäßzellenproliferation als auch bei der Proliferation und Differenzierung von Fettgewebe dar (Zhou *et al*., 1995; Battista *et al*., 1999; Arlotta *et al*., 2000; Anand and Chada, 2000). Ebenso könnten wieder IGFBP2, 4 und 5 über deren Wirkung auf IGF1 und IGF2 und damit verbundenen Auswirkungen auf die Fettleibigkeit (Woods *et al*., 2000; Jones *et al*., 2001) sowie COL1A2, dessen Verkürzung phänotypisch unter anderem mit einer Reduzierung des Fettgewebes einher geht (Nathanson *et al*., 1997), HMGA-Zielgene für die Entwicklung von Fettgewebe, oder im pathologischen Sinn für Adipositas sein.

Betrachtet man den Einfluss der HMGA-Expression bei der Proliferation und Differenzierung von Adipozyten sowie glatten Muskelzellen der Blutgefäße oder im pathologischen Sinne bei Adipositas und Arteriosklerose, ist das Verständnis der Mechanismen der HMGA-Expressionsregulation von einscheidender Bedeutung. Interessanterweise konnte durch die hier präsentierten Ergebnisse gezeigt werden, dass ein polymorphes Dinukleotid-Repeat im HMGA2-Promoter einen entscheidenden Einfluss auf dessen Expressionsaktivität ausübt, wobei die Promoteraktivität von der Länge des Repeats abhängig ist (Borrmann *et al*., 2003). Aufgrund des polymorphen Charakters dieses (TC)-Repeats (Ishwad *et al*., 1997), machen diese Ergebnisse eine individuelle Disposition für eine HMGA2-Promoteraktivität und damit für Erkrankungen wie Adipositas und Arteriosklerose denkbar. Inwieweit allerdings eine genetische Disposition tatsächlich gegeben ist und ob erkranktes Gewebe durch spezifische Repeatlängen zu charakterisieren ist, bedarf weiterer Analysen.
Der regulatorische Effekt, den das (TC)-Repeat auf den Promoter hat, wird dabei vermutlich über die Ausbildung von Nicht-B-Form-DNA-Konformationen wie beispielsweise triple-helikaler H-DNA bewirkt, zu deren Bildung (TC)-Repeats in der Lage sind (Htun und Dahlberg, 1988) und mit denen eine transkriptionsaktivierende Wirkung assoziiert ist (Michel *et al*., 1992; Firulli *et al*., 1994; Kim *et al*., 1998). Ein derartiger Mechanismus wurde bereits für einen konservierten Polypyrimidin/Polypurin Abschnitt im HMGA2-Promoter beschrieben, der ebenso wie das hier beschriebene (TC)-Repeat einen positiven Einfluss auf die Promoteraktivität ausübt (Rustighi *et al*., 2002).

Die Progression hormonabhängiger Tumoren, beispielsweise der Mamma-Karzinome steht in einem klaren Zusammenhang mit der transkriptionsaktivierenden Wirkung des Östrogen-Rezeptors (ER) auf Steroidhormon-induzierbare Gene (Jiang und Jordan, 1992). Die Bindung der Östrogen-stimulierten ER an die Zielsequenzen (ERE) betroffener Gene und deren transkriptionsaktivierende Wirkung wird dabei stark durch zusätzliche Co-Regulatoren sowie akzessorische Proteine beeinflusst (Smith und Toft, 1993; Landel *et al*., 1997). Als

solche akzessorischen Faktoren konnten sowohl HMGA1 als auch HMGB1 identifiziert werden, deren Protein:Protein-Interaktionen mit dem ER zu einer verstärkten Bindung des Rezeptors an das ERE führt (Verrier *et al*., 1997; Massaad-Massade *et al*., 2003).

Durch die in der vorliegenden Arbeit präsentierten Ergebnisse zur Untersuchung von *HMGB1* konnte nicht nur die komplette genomische Struktur des humanen *HMGB1* erstmalig beschrieben werden, sondern es wurden zusätzlich in der Promoterregion regulatorische Elemente analysiert, die entscheidend an der Expression von *HMGB1* beteiligt sind. Darunter sind Bindungsstellen für Transkriptionsfaktoren, inklusive des Östrogen-Rezeptors, mit deren Hilfe zum Beispiel der Mechanismus Östrogen-induzierter *HMGB1* Expression (He *et al*., 2000) erklärt werden könnte.

Im Gegensatz zu HMGA1, dessen Expression in Mamma-Karzinomen als Marker für das Metastasierungspotential des Tumors verwendet werden kann (Ram *et al*., 1993) und bei Expression im Blut mit der durchschnittlichen Lebenserwartung bei metastasierendem Brustkrebs korreliert ist (Langelotz *et al*., 2003), war über die Expression von HMGB1 in Mamma-Tumoren nichts bekannt. Die hier vorgestellten Ergebnisse belegen erstmalig intertumorale Variationen in der Stärke der HMGB1-Expression, wobei für die 1,4kb und 2,4kb Transkripte Variationen gezeigt werden konnten, die sich um den Faktor 8,5 bzw. 14,5 unterschieden (Flohr *et al*., 2001).

In Anbetracht des steigenden Interesses für die Erforschung von HMGB1 eröffnen diese Daten die Möglichkeit das Expressionsverhalten von *HMGB1* auf Gen-Ebene genauer zu untersuchen. Dieses könnte insbesondere im Rahmen der Funktion von HMGB1 bei der Sensitivierung von Tumorzellen für chemotherapeutische Agenzien (McA'Nulty *et al*., 1996; He *et al*., 2000) und der Hormontherapie zur Kontrolle des Tumorwachstum (Molina *et al*., 1999) für therapeutische Tumorbehandlungen von großem Interesse sein.

ZUSAMMENFASSUNG

Die Proteinen der Gene *HMGA1*, *HMGA2* und *HMGB1* zählen zu den architektonischen Transkriptionsfaktoren, deren Binden an AT-reiche Regionen der DNA zur Änderung der DNA-Konformation führt, wodurch es zur Beeinflussung der Bildung funktioneller Transkriptionskomplexe und damit verbunden zur Regulation der Genexpression kommt.
In zytogenetischen Subgruppen verschiedener benigner mesenchymaler Tumorentitäten, die durch Rearrangierungen in den chromosomalen Regionen 12q14-15 oder 6p21.3 charakterisiert sind, kommt es zu Veränderungen in den Bereichen des *HMGA2* bzw. *HMGA1*-Gens. Um die Funktion der HMGA-Subfamilie bei der Tumorentstehung genauer zu analysieren, wurden im Rahmen dieser Arbeit die für die Tumor charakteristischen Situation auf genomischer, funktioneller und Expressionsebene genauer untersucht.

- Untersuchungen zur genauen Lage der chromosomalen Bruchpunkte der Region 6p21.3 zeigten, dass bei etwa 75% der Tumore der Bruch 3' hinter *HMGA1* lokalisiert ist.
- Obwohl die chromosomalen Bruchpunkte sowohl intragenisch, als auch in den 3' und 5' flankierenden Regionen lokalisiert sein können, sind die verschiedenen Tumorentitäten durch die Überexpression von nativem HMGA oder der Expression C-terminal trunkiertem und chimärem HMGA charakterisierbar, wobei für die aberranten Formen nicht der Zugewinn ektopischer Sequenz anderer Gene, sondern eher der Verlust der sauren C-terminalen Domäne von entscheidender Bedeutung ist.
- Das *LBH*-Gen, dass erstmalig beschrieben werden konnte, ist direkt 3' hinter *HMGA1* in tail-to-tail Orientierung lokalisiert, zeigte aber keine Tumor-spezifischen Änderungen im Expressionsprofil.
- Ferner konnten für *HMGA2* und *HMGA1* Mechanismen der Expressionsregulation mit funktionellen Elementen in den Regionen der 3'UTR verknüpft werden, deren Beeinträchtigung in trunkierten Transkripten mit einer erhöhten Expressionsrate assoziiert ist.
- Für *HMGA2* wurde die Existenz von zwei unabhängigen Promotoren nachgewiesen, von denen zumindest die Aktivität einer der beiden Promotoren stark in Abhängigkeit von der Länge eines polymorphen (TC)-Repeats variiert und somit eine individuelle Disposition für die *HMGA2* Aktivität und Erkrankungen wie Adipositas und Arteriosklerose möglich macht.
- Funktionelle Untersuchungen zu nativem HMGA2 und ΔHMGA2 zeigte am Beispiel des *ERCC1*-Promoters, dass die saure C-terminale Domäne keinen Einfluss auf die Bindungsaffinität hat. Allerdings wurden Unterschiede sowohl im Bindungsmuster, als auch bei der Stöchiometrie und dem Mechanismus der DNA-Bindung beobachtet. Die

Unterschiede im Bindungsverhalten von ΔHMGA2 sind mit signifikanten Änderungen der DNA-Konformation und einer 4,2-fach stärkeren Inhibition der *ERCC1* Promoteraktivität assoziiert.

- Expressionsarray Analysen mit HMGA2, HMGA1a, HMGA1b, ΔHMGA2, ΔHMGA1a und HMGA2/LPP induzierten Myometriumzellen des Uterus-Normalgewebes zeigen, dass diese HMGA-Varianten trotz der strukturellen Unterschiede funktionell miteinander verwandt sind und lediglich bei 7 - 30% der betroffenen Gene klare Unterschiede im Regulationsverhalten gefunden werden.
- Die ganz klaren Unterschiede im Profil der regulierten Gene zwischen den Expressionsstudien dieser Arbeit und vergleichbaren Studien an Zellen benignen und malignen Ursprungsgewebes lassen für die HMGA-Proteine einen Wirkungsmechanismus vermuten, der abhängig ist vom zellulären Kontext. Dabei werden in Myometriumzellen Gene, deren Proteine funktionell an der Proliferation und Differenzierung beteiligt, sind primär runterreguliert.
- Diese Abhängigkeit der HMGA-Wirkung vom zellulären Kontext in Verbindung mit der Feststellung, dass die HMGA-Varianten in Uterus Myometriumzellen lediglich in der Lage sind 3 von 105 Uterus Leimyom-spezifischen Gene zu regulieren, bestärkt die Theorie der HMGA-Veränderungen als sekundäre Ereignisse in der Tumorgenese.
- Durch die Expressionsarray Analysen konnten Zielgene für HMGA-Proteine identifiziert werden, die in Prozesse der Angiogenese, Myogenese und Fettgewebsentwicklung involviert sind und so den Einfluss der HMGA-Proteine bei der Proliferation und Differenzierung von Adipozyten sowie glatten Muskelzellen der Blutgefäße oder im pathologischen Sinne bei Adipositas und Arteriosklerose erklären hilft.
- Für das humane *HMGB1* konnte durch diese Arbeit nicht nur die komplette genomische Struktur erstmalig beschrieben werden, sondern es wurden zusätzlich in der Promoterregion regulatorische Elemente analysiert, die entscheidend an der Expression von *HMGB1* beteiligt sind und erste Ansätze zum Verständnis Östrogen-induzierter *HMGB1*-Expression liefern.
- Für eine Reihe von Mamma-Karzinomen wurden erstmalig intertumorale Variationen in der Stärke der *HMGB1*-Expression nachgewiesen, die sich je nach betrachtetem *HMGB1*-Transkript um den Faktor 8,5 bzw. 14,5 unterschieden.

SUMMARY

Proteins encoded by the genes *HMGA1*, *HMGA2* und *HMGB1* are architectural transcription factors whose interaction with the narrow minor groove of AT-rich DNA sequences lead to conformational changes in the DNA. Due to this chromatin remodeling, these proteins can affect the assembly of transcriptional complexes thus influencing gene expression.

Cytogenetic subgroups of different benign mesenchymal tumor entities being characterized by chromosomal aberrations within 12q14-15 or 6p21.3 show rearrangements within the region of *HMGA2* or *HMGA1*, respectively. To give a more detailed view on the functional aspects of HMGA proteins in terms of tumorigenesis, this work was designated to analyze the characteristics for these types of benign mesenchymal tumors on genomic, expressional, and functional level.

- Fine mapping of the breakpoints affecting the chromosomal region 6p21.3 showed that in about 75% of the tumors analyzed the breakpoints were located within the region 3' of *HMGA1*.
- Although chromosomal breakpoints were located either intragenic or in the 3' or 5' flanking regions of *HMGA* genes, different tumor entities can be characterized by a dysregulated expression of native HMGA or an expression of C-terminally truncated or chimeric HMGA. For the aberrant forms of HMGA the removal of the acidic C-terminal domain, rather than the gain of ectopic sequences from other genes, seem to be of functional relevance.
- The *LBH* gene being located in tail-to-tail orientation directly behind HMGA1 did not show any significant alteration in expression level within tumors having a 6p21.3 aberration.
- It was shown that the expression of *HMGA1* and *HMGA2* is under control of functional elements located within their 3'UTR. Truncation of these elements as found within the aberrant tumor-specific transcripts is associated with an increased expression.
- Promoter analyses for *HMGA2* revealed two functionally independent promoter and a (TC)-repeat whose repeat length is crucial for expressional activity. Due to the polymorphic character of this (TC)-repeat, an individual predisposition for HMGA2 promoter activity and thus for certain disorders such as arteriosclerosis and obesity can be postulated.
- DNA-binding studies mapped a high affinity HMGA2 binding site to an AT-rich region within the *ERCC1* promoter. Comparison of DNA-binding between HMGA2 and C-terminally truncated ΔHMGA2 demonstrated that the acidic C-tail did not influence the affinity of DNA-binding but revealed differences within the binding patterns and

the stoichiometry and the mechanisms of DNA-binding. These differences led to a significant change in DNA-conformation and a 4.2-fold stronger inhibition of *ERCC1* promoter activity for ΔHMGA2.

- Expression array analyses using HMGA2, HMGA1a, HMGA1b, ΔHMGA2, ΔHMGA1a und HMGA2/LPP induced myometrial cells showed that besides their structural differences these HMGA variants are functionally related to each other. Only 7-30% of the affected genes were regulated completely different between the HMGA variants.
- Clear differences in the pattern of regulated genes, as observed between the data presented herein and studies performed within cell of benign and malignant origin, indicates that the function of the HMGA proteins depends on the cellular context in which they are expressed. Genes functionally involved in proliferation and differentiation were predominantly down-regulated in normal myometrial cells by HMGA, especially by HMGA1 proteins.
- As HMGA proteins are only capable to regulate 3 out of 105 uterine leiomyomata-specific genes. These data in consideration with the HMGA function depending on the cellular context further strengthen the idea of HMGA dysregulation as a secondary event in tumorigenesis.
- Due to the expression array analyses, several HMGA target genes were identified that might be involved in HMGA-dependent proliferation of adipocytes and vascular smooth muscle cells or in pathological disorders as obesity and arteriosclerosis.
- The genomic sequence of human *HMGB1* was completed by the experiments presented herein. Furthermore, functional analyses of the *HMGB1* promoter revealed functional elements and putative transcription factor binding sites that can give a better insight into expressional control of human *HMGB1* and its estrogen-induced expression.
- Results presented herein demonstrated a strong intertumoral variation of *HMGB1* expression in breast cancer by factor 8.5 or 14.5 for the 1.4kb and 2.4kb transcript, respectively.

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PUBLIKATIONSÜBERSICHT

In der folgenden Übersicht sind die der vorliegenden Arbeit zugrunde liegenden Publikationen chronologisch geordnet und fortlaufend nummeriert.

I. **BORRMANN L, BECKER K, FLÖTHE L, MURUA ESCOBAR H, BELGE G, RIPPE V, GOHLA G, BONK U, BULLERDIEK J.** (2003). Differential gene expression in normal human Myometriumcells induced by HMGA1a, HMGA1b, HMGA2, and aberrant HMGA proteins as displayed by micro array experiments. *Oncogene*, in Vorbereitung.

II. **BORRMANN L, SCHWANBECK R, SEEBECK B, ROGALLA P, BULLERDIEK J, WISNIEWSKI JR.** (2003). Carboxy-Terminally truncated High Mobility Group A2 protein inhibits activity of DNA repair gene *ERCC1. Nucleic Acids Research*, eingereicht.

III. **BORRMANN L, SEEBECK B, ROGALLA P, BULLERDIEK J.** (2003). Human *HMGA2* promoter is coregulated by a polymorphic dinucleotide (TC)-repeat. *Oncogene*, 22: 756-760.

IV. **FLOHR AM, ROGALLA P, MEIBOOM M, BORRMANN L, KROHN M, THODE-HALLE B, BULLERDIEK J.** (2001). Variation of *HMGB1* expression in breast cancer. *Anticancer Research*, 21: 3881-3886.

V. **BORRMANN L, WILKENING S, BULLERDIEK J.** (2001b). The expression of *HMGIY* genes is regulated by their 3'UTR. *Oncogene*, 20: 4537-4541.

VI. **BORRMANN L, KIM I, SCHULTHEISS D, ROGALLA P, BULLERDIEK J.** (2001a). Regulation of the expression of HMG1, a co-activator of the estrogen receptor. *Anticancer Research*, 21: 301-305.

VII. **KAZMIERCZAK B, BORRMANN L, BULLERDIEK J.** (1999). Assignment of a new gene (*LBH*) located downstream of *HMGIY*. *Genomics*, 56: 136-137.

VIII. **KAZMIERCZAK B, DAL CIN P, WANSCHURA S, BORRMANN L, FUSCO A, VAN DEN BERGHE H, BULLERDIEK J.** (1998). *HMGIY* is the target of 6p21.3 rearrangements in various benign mesenchymal tumors. *Genes Chromosomes Cancer*, 23: 279-285.

I.

BORRMANN L, BECKER K, FLÖTHE L, MURUA ESCOBAR H, BELGE G, RIPPE V, GOHLA G, BONK U, BULLERDIEK J. (2003). Differential gene expression in normal human Myometriumcells induced by HMGA1a, HMGA1b, HMGA2, and aberrant HMGA proteins as displayed by micro array experiments. *Oncogene*, in Vorbereitung.

Eigenanteil an dieser Publikation:

- Planung aller Arbeiten
- Sammeln des Zellmaterials (in Zusammenarbeit mit dem Pathologischen Institut des ZKH Bremen-Nord)
- Bis auf Chromosomenanalyse und statistische Auswertung Durchführung alle Arbeiten
    - Die HMGA2 Expressionsvektoren wurden von Herrn V. Rippe zur Verfügung gestellt
    - Die Durchführung der radioaktiven Arrayhybridisierungen wurden unterstützt von Herrn H. Murua Escobar
- Verfassen der Publikation

Differential gene expression in normal human myometrium induced by HMGA1a, HMGA1b, HMGA2, and aberrant HMGA proteins as displayed by array experiments

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Abstract

Uterine leiomyomata are the most frequent tumors in woman. Within a subset of these tumors showing rearrangements of chromosomal regions 6p21.3 and 12q14-15 *HMGA* genes and their truncated and chimeric forms are similarly associated to molecular pathogenesis. To analyze the hypothesis that retaining of the DNA-binding domains and not the loss of the acidic C-tail or the gain of ectopic sequence is essential in terms of tumor development the gene regulation patterns for HMGA1a, HMGA1b, HMGA2, C-terminally truncated ΔHMGA1a and ΔHMGA2, and chimeric HMGA2/LPP within normal human uterine myometrial cells were monitored by cDNA expression array experiments.

Genes like FGF2, COL1A2, TRAM, and AKAP12 were up-regulated and NOTCH4 down-regulated by all six HMGA variants whereas genes CDC10, CXCL12, and SEMA3A were specific HMGA1 target genes and MT3 and LIG1 were only regulated by HMGA2 variants. HMGA-target genes for angiogenesis, myogenesis or development of adipose tissue were detected. Statistical analyses revealed that the HMGA proteins and their derivatives are related to each other in terms of their gene regulatory function using a similar mechanism for gene regulation. As the DNA-binding domains are in common to all HMGA derivatives the results presented herein further strengthening the hypothesis that overexpression of the AT-hooks is essential for tumor development.

Introduction

Uterine leiomyomata are the most frequent female genital tumors found in up to 70% of women in reproductive age (Cramer and Patel, 1990). As a leading cause of hysterectomy in premenopausal women uterine leiomyomata are a major public health problem. Although the pathogenesis of these frequent tumors remains still unclear until now, recurrent chromosomal abnormalities have been described. Upon these, rearrangements of chromosomal regions 6p21.3 and 12q14-15 are the most frequent structural aberrations affecting both members of the high mobility group family A of non-histone chromatin proteins (HMGA1, HMGA2) (Kazmierczak et al., 1996a; Schoenmakers et al., 1995). These proteins consist of three DNA-binding domains (AT-hooks) enabling their binding to the minor groove of AT-rich DNA and an acidic C-tail being responsible for protein-protein interactions (for review see Wiśniewski and Schwanbeck, 2000). Changes in DNA-conformation induced by DNA-binding of the so called “architectural transcription factors” HMGA1a, HMGA1b, and HMGA2 modulate the chromatin environment for the assembly and function of transcriptional complexes thus influencing gene expression.

Whereas both *HMGA* genes are highly expressed during embryonic development only HMGA1 can be detected at very low levels in differentiated cells (Chiappetta et al., 1996;

Zhou et al., 1995; Abe et al., 2000). A reactivation of *HMGA* expression due to aberrations in chromosomal regions 6p21.3 or 12q14-15 is characteristic for benign tumors including uterine leiomyomata, pulmonary chondroid hamartomas, and endometrial polyps (Schoenmakers et al., 1995; Kazmierczak et al., 1996b). Each of these tumor entities can be characterized by the overexpression of either wild type HMGA1 or HMGA2 or chimeric and truncated forms of these proteins. These derivatives retain the three DNA-binding domains of HMGA but lack the acidic C-tail (Schoenmakers et al., 1995; Geurts et al., 1997; Tkachenko et al., 1997; Kazmierczak et al., 1998; Klotzbucher et al., 1999). For the chimeric forms of *HMGA2* several fusion partner genes such as *LPP* (Petit et al., 1996), *RAD51L1* (Schoenmakers et al., 1999), and *ALDH2* (Kazmierczak et al., 1995) have been described whereas only few fusion genes such as *LAMA4* (Xiao et al., 1997) are known for *HMGA1*.
However, the exact mechanism by which HMGA contributes to pathogenesis of these frequent benign tumors is not yet understood and until now it even remains unclear why wild type proteins of the HMGA family and their derivatives are similarly associated with the same tumor entities. As to the huge variety of different HMGA derivatives we favor the hypothesis that relevant in terms of tumor development is the retaining of the DNA-binding domains and not the loss of the acidic C-tail or the gain of ectopic sequence. To find an experimental approach aimed at these questions and to identify target genes for HMGA1a, HMGA1b, and HMGA2 these proteins and their derivatives were overexpressed in normal uterine myometrium and differential expression patterns were monitored by cDNA expression array experiments.

Material & Methods

Cell culture

Myometrium tissue samples being removed directly after surgery were washed in Hank's solution supplemented with 20% FCS, penicillin (200 IU/ml) and streptomycin (200 µg/ml). Samples were minced into small pieces and treated with 0.5% collagenase (Serva, Heidelberg, Germany) solution for 2-3 hr at 37°C. Suspensions containing small fragments and single cells were centrifuged for 5min at 800xg and resuspended in smooth muscle cell growth medium 2 (PromoCell, Heidelberg, Germany) supplemented with penicillin (200 IU/ml) and streptomycin (200 µg/ml). Dissolved cells transferred to 25cm2 cell culture flasks were incubated at 37°C in an atmosphere of 5% CO_2 in air. Chromosome preparation was followed routine methods as described earlier (Bullerdiek et al., 1987). Cells were PCR-tested negative for mycoplasm contamination.

Cloning of HMGA expression vectors

Vectors expressing either wild-type HMGA2 (HMGA2), truncated HMGA2 lacking exons 4 and 5 corresponding to the spacer and acidic domain (ΔHMGA2), or a fusion protein consisting of the three DNA-binding domains of HMGA2 (exons 1-3) and three LIM-domains of LPP (exons 9-11) (HMGA2/LPP) were generated as described elsewhere (Fedele et al., 1998).

Wild type HMGA1a, HMGA1b, and truncated HMGA1a lacking the C-terminal acidic domain (ΔHMGA1a) were PCR amplified. Upper primer 5'-GCCGCCACCATGAGTGAGTCGAGCTCG-3' was used in combination with lower primer 5'-TCACTGCTCCTCCTCCGA-3' for normal HMGA1a and HMGA1b or primer 5'-TTACAGTTTTTTGGGTCTGCCC-3' for ΔHMGA1a. Standard hot start PCR were performed with *Pfu* DNA polymerase (Promega, Madison, USA) in a Mastercycler gradient (Eppendorf, Hamburg, Germany) using the following protocol: 5min 94°C, (30sec 94°C, 30sec 56°C, 45sec 72°C) 25x, 10min 72°C. PCR fragments were purified from agarose gel using QIAEX II (Qiagen, Hilden, Germany) were A-tailed using *Taq* polymerase and were cloned into eukaryotic expression vectors pCR3.1 (Invitrogen, Karlsruhe, Germany). Positively screened clones were verified by sequencing.

Plasmid DNA was purified using Plasmid Isolation Kit (Qiagen) following the instructions of the manufacturer.

Transient transfections

Transient transfection of 1st passage myometrial smooth muscle cells were performed 38h post-seeding at about 60-70% confluence in 25cm^2 cell culture flasks using 2.14µg plasmid DNA, 535µl buffer EC, 17.14µl enhancer solution, and 42.9µl of Effectene transfection reagent following the instructions of the manufacturer (Qiagen). 24hr post-transfection cells were washed once with PBS solution and were grown for further 24hr in smooth muscle cell growth medium 2. Myometrial cells from two independent donors (MyMeL03 and MyMeL06) were used for transfection experiments. Plasmids used for transfection expresses either wild-type HMGA2, truncated HMGA2 (ΔHMGA2), a HMGA2/LPP fusion protein (HMGA2/LPP), normal HMGA1a, normal HMGA1b, truncated HMGA1a (ΔHMGA1a),or no HMGA (pCR3.1, negative control).

To determine transfection efficiencies vector pEGFP-CI (BD Biosciences Clontech Palo Alto, USA) expressing a green fluorescence protein (GFP) was transfected in a matching sample. Monitoring of transfection efficiency was performed 48h post-transfection by fluorescence microscopy.

RNA isolation

48h post-transfection cells were washed once in PBS solution and were then harvested using trypsin (0.05% trypsin, 0.02% EDTA) Detached cells were spun down at 300xg for 5 min at 4°C and were washed once with 1ml PBS. RNA isolation was performed using RNeasy Mini Kit (Qiagen), following the instructions of the manufacturer. Additionally, cells were homogenized using QIAshredder (Qiagen) and genomic DNA contaminations were eliminated by DNaseI digestion for 15min on RNeasy column using RNase-free DNase (Qiagen) following instructions of the manufacturer. Quality and quantity of isolated total RNA were checked by UV-spectrometry and denaturating formaldehyde gel electrophoresis as described elsewhere (Sambrook et al.1989).

RT-PCR to test expression of HMGA-constructs

Level of construct-specific HMGA expression was tested by RT-PCR. In detail, cDNA synthesis was performed with tailed poly(A) primer AP2 5'-AAGGATCCGTCGACATCTTTTTTTTTTTTTTTT-3' using Superscript reverse transcriptase (Invitrogen, Karlsruhe, Germany) following the instruction of the manufacturer. PCR was performed using primer UAP2 complementary to the tail of primer AP2 in combination with a vector specific primer. PCR reactions were performed with Triple Master polymerase (Eppendorf) in a Mastercycler gradient (Eppendorf) with the high fidelity buffer using the following amplification protocol: 5 min 94°C, 1 min 55°C, 10 min 72°C, (30 sec 94°C, 20 sec 55°C, 2 min 72°C) x10, (30 sec 94°C, 20 sec 55°C, 2 min 72°C + 1 sec for each cycle) x30, 10 min 72°C, 10 sec 20°C. RT-PCR products were separated by electrophoresis in a native agarose gel.

Array hybridization

Array hybridizations were performed using Atlas Human Cancer 1.2 cDNA expression array (BD Biosciences Clontech).

RNA samples were isolated from 1st passage myometrial cells transfected with an plasmid expressing either wild-type HMGA2, truncated HMGA2 (ΔHMGA2), a HMGA2/LPP fusion protein (HMGA2/LPP), normal HMGA1a, normal HMGA1b, truncated HMGA1a (ΔHMGA1a), or no HMGA (pCR3.1). For each array 5µg of total RNA were used for preparation of cDNA probes and array hybridizations following the protocols supplied by the manufacturer (manual PT3140-1, version PR89832). Hybridized membranes were exposed to a phosphorimaging screen that was afterwards developed using STORM phosphorimager (Amersham Biosciences Buckinghamshire, England). Qualitative and quantitative assessments were performed using AtlasImage 1.01 software (BD Biosciences Clontech) following the

instruction of the manufacturer. Signal intensities were normalized against the overall value of all genes on the array with a normalization coefficient being calculated by the sum method. Array experiments were performed twice using total RNA of transfection experiments with myometrial cells from two independent donors (MyMeL03 and MyMeL06). Differential gene expression was analyzed by comparing the signals on the experimental arrays with the signals on the control array. Only genes being differentially expressed by factor 2 were taken into account. Of these latter genes, for higher specificy only those genes were considered to be differential expressed whose activity was either increased or was decreased in both cell lines (MyMeL03 and MyMeL06).

Statistical analysis

For statistical analyses a Bravais-Pearson-correlation test was performed. Afterwards the coefficient of determination was calculated explaining proportion of variance explained by a linear relationship between the two variables. To find out if different HMGA proteins mediates gene-regulation by similar mechanism or do behave totally independent of each other, a principle components analysis that searches for structures within a correlation matrix and combines variables which behave in a parallel manner into one principle component while variables with different behavior are assigned to different principle components was performed.

To compare the functional effects between two HMGA variants on a single gene the quotient of the differential expression values was calculated. HMGA variants were determined to act different when the quotient was 1.3 or more and act almost equally when the quotient was less then 1.3. Clear differences in HMGA functions were set to have a quotient of 2 or more.

Results

To identify genes that are regulated by HMGA1, HMGA2 and their derivatives differential gene expression induced by either wild-type HMGA2, HMGA1a or HMGA1b, C-terminally truncated HMGA2 (ΔHMGA2) or HMGA1a (ΔHMGA1a), a HMGA2/LPP fusion protein, or no protein (control) within 1st passage myometrial cells was measured by cDNA expression arrays. Experiments were performed in duplicate for independent myometrial cells MyMeL03 and MyMeL06.

The efficiencies of myometrial cell transfection as measured by fluorescence microscopy were roughly 25-30%. Expression of HMGA constructs was verified by construct-specific RT-PCR. (data not shown)

Table 1 summarizes the genes being differentially expressed in normal human myometrial cells in response to overexpression of HMGA1a, HMGA1b, HMGA2, and their derivatives. Of

the 1176 genes presented on the array only 4.5% were differentially expressed with 1.5% being up-regulated due to HMGA overexpression and 3% of the genes being decreased in their transcriptional activity (differential expression ≥ 1.4-fold).

Of the genes being regulated, five genes including fibroblast growth factor 2 (FGF2) (no.22, table 1), collagen COL1A2 (no.59), translocating chain-association membrane protein (TRAM) (no.73) being involved in the translocation of secretory proteins, topoisomerase I (no.41), and cell architectural protein AKAP12 (no.56) were transcriptionally up-regulated by more than 2-fold by almost all HMGA variants tested whereas only oncogene NOTCH4 (no.28) is decreased more than 2-fold by HMGA proteins and their derivatives. In contrast to that, genes like CDC10 (no.3), growth factor CXCL12 (no.26), and SEMA3A (no.66) that is involved in cell outgrowth were strongly decreased by proteins of the HMGA1-subgroup but were almost unaffected by HMGA2 variants. Complementarily, cell cycle regulator MT3 (no.11) and ligase LIG1 (no.47) were down-regulated by all HMGA2 variants but not by HMGA1 proteins.

To determine the degree of relationship between the different HMGA protein variants in terms of their gene regulatory function a Bravais-Pearson correlation analysis was performed calculating highly significant values for the correlation coefficients (r) within a range from 0.26 to 0.74 (fig. 1). In combination with the coefficients of determination (r^2) these correlation coefficients revealed the highest degree of relationship between HMGA1a and HMGA1b as more than 54 % ($r^2 = 0.5431$) of variance of one of these proteins can be explained by the variance of the other protein. The weakest relationship with the r-values between 0.2648 and 0.4660 was calculated for truncated ΔHMGA1a. Additionally, a principle components analysis showed only one Eigenvalue > 1.0 that is represented by the 1st principle component (Eigenvalue of principle component 1 = 3.76909505) and explains a proportion of 63% of all possible variances (fig. 2). These statistical data revealed that the different HMGA variants are related to each other and uses a similar mechanism to regulate transcription. Of the protein variants analyzed herein only truncated ΔHMGA1a showed a tendency of independent behavior a finding further confirmed by the principle components analysis as a proportion of 14% Eigenvalue of principle component 2 is observed for this protein variant.

Gene expression mediated by HMGA1 protein variants

Results from array experiments showed that genes functionally involved in proliferation, differentiation, and apoptosis were predominantly down-regulated by HMGA1a, HMGA1b, and ΔHMGA1a. Within functional category “proliferation” genes involved in cell cycle control (genes number 1-14, table 1) were strongly decreased in their activity in all three samples with the exception of CDC37 (no.4) that was down-regulated by HMGA1a and HMGA1b but 10.7-fold increased in signal intensity due to ΔHMGA1a.

A comparison of the regulation pattern of the three different HMGA1 proteins showed that in case of the HMGA1 splice variants HMGA1a and HMGA1b 58% of genes were almost identical expressed (quotient of differential expression values < 1.3). Of the remaining genes, 29% differ in the intensity of transcriptional regulatory (e.g. TNFRSF10B (no.38)), 11% are affected by one of the splice variants whereas almost unaffected by the other (e.g. CDC34 (no.2); cadherin CDH2 (no.49)), and 2% are regulated by both splice variants but in opposite directions (SP100 (no.45); ribosomal protein kinase RPS6KA1 (no.96)).

Deletion of the C-terminally domain of HMGA1a as presented by ΔHMGA1a had almost no effect on protein function in about 58% of the affected genes. Of the remaining 42% only 7% showed clear differences in HMGA function (quotient of differential expression values ≥ 2.0) between wild type HMGA1a and its truncated form. As to that, cell cycle regulating genes CDC10 (no.3) and CDK3 (no.7) were strongly increase in gene activity due to the C-terminally truncation. In case of CDC37 (no.4) being 1.4-fold decreased by HMGA1a the truncation led to a 10.7-fold increase in gene expression. For transcription factor JUNB (no.85) and apoptosis-related protein PIG7 (no.36) truncation of HMGA1a resulted in the complete loss of the ability to regulate these genes. In contrast, expression of cell architectural protein BSG (no.68) is only regulated by the truncated form of HMGA1a.

Gene expression mediated by HMGA2 protein variants

Comparing expression profiles for HMGA2 and HMGA1a regulated genes revealed that 72% of the genes were regulated in a different manner. For instance, several genes were affected by either HMGA1 or by HMGA2, e.g. IGFBP5 (no.17), CXLC12 (no.26), and CASP9 (no.35), and LIG1 (no.47), respectively. A regulation in opposite directions by these two HMGA variant is clearly observed for transmembrane trafficking protein 21 (no.74), being down-regulated by HMGA1a but increased in expression by HMGA2. Furthermore, among the genes involved in cell cycle regulation fewer genes were affected by HMGA2 than regulated by HMGA1a.

Truncated and chimeric forms of HMGA2 restored the function of wild type HMGA2 in 49%, respectively 42% of the affected genes. Among the genes being differentially expressed approximately 16% were affected in a clearly different manner. Addition of LPP to truncated HMGA2 changed the protein function in about 49% of the affected genes whereas only 12% were regulated clearly different. Changes in gene regulation as a consequence of HMGA2 truncation and/or LPP fusion are were observed as a gain or a loss of function as well as the regulation in opposite directions and restoration of lost function. For example, truncated HMGA2 had the ability to down-regulate MX1 (no.72) by 3-fold whereas normal and chimeric protein did not influence MX1 expression. Similarly, desmin (no.57) is 1.7-fold activated by wild type HMGA2 and HMGA2/LPP but 3.3-fold decreased by ΔHMGA2. For tumor susceptibility gene 101 (TSG101) (no.78) normal and truncated HMGA2 are almost equally

capable to increase transcription whereas chimeric protein HMGA2/LPP down-regulates TSG101 by 2-fold. In cases of SFN (no.32) truncated and chimeric HMGA2 completely lost the ability to inhibit gene expression relatively to wild type HMGA2 whereas in case of CASP9 (no.35) only a partial loss of inhibitory function was observed for the derivatives.

Discussion

Although HMGA1 and HMGA2 as well as their aberrant forms are thought to be implicated in the pathogenesis of the subsets of uterine leiomyomata showing rearrangements of chromosomal regions 6p21.3 or 12q14-15 (Kazmierczak et al., 1996a; Schoenmakers et al., 1995) the exact mechanisms by which these proteins contribute to tumorigenesis are still unknown. It remains even unclear why wild type proteins of the HMGA family as well as their derivatives are similarly associated with the same tumor entities. To identify HMGA target genes and to analyze differences between wild type HMGA proteins and their derivatives we have over-expressed different HMGA variants in normal 1st passage uterine myometrial cells and analyzed differential expression profiles by cDNA expression array experiments.

Results of these experiments showed that wild type HMGA1a, HMGA1b, and HMGA2 as well as C-terminally truncated ΔHMGA1a and ΔHMGA2 and chimeric HMGA2/LPP regulate about 4.5% of the genes analyzed with most of them, about 67% being decreased in their transcriptional activity. Major changes presented as differences in gene regulation greater than 2-fold, as a complete loss or a gain of function, or as gene regulation in opposite directions were observed in about 7-20% of the affected genes. The effect of a truncation of the acidic C-tail or the fusion of LPP-domains led to gene-specific effects but did not completely change the function of the protein. These gene-specific effects may be caused by alterations in stoichiometry of DNA-binding as well as in DNA-bending as for example observed upon binding of HMGA2 and its derivatives to the *ERCC1* promoter region (Borrmann et al., submitted).

Statistical analyses of the array data revealed that the different HMGA variants are besides their relationship on DNA level also related to each other in terms of their gene regulatory function and uses a similar mechanisms for gene regulation. Of the protein variant analyzed herein, only truncated ΔHMGA1a showed a tendency of independent behavior. The results presented herein strengthen our hypothesis that in terms of tumor development the expression of the DNA-binding domains is essential explaining why such a huge variety of different HMGA aberrations as well as their wild type proteins are similarly associated with the same tumor entities as they all share the DNA-binding domains but differ in their C-terminal regions.

Furthermore, the results do also show that HMGA1-mediated gene regulation and at least HMGA-regulation may depends on the cellular context in which these proteins are expressed. Differential expression profiles of HMGA1b-regulated genes were found to differ between normal uterine smooth muscle cells analyzed herein and breast cancer cell line MCF-7 presented by Reeves et al., 2001. In cell line MCF-7 83% of the genes were increased in transcription by HMGA1b whereas only 28% were up-regulated in normal myometrial cells. Comparing the 106 genes described to be HMGA1b-regulated in epithelial breast cancer cell line MCF-7 (Reeves et al. 2001) with the data presented herein, only 14 of these genes were specifically regulated in human uterine smooth muscle cells (fig. 3). Of these 14 genes, only 3 genes were regulated in the same direction although with much lower intensities whereas the remaining 11 genes were regulated in the opposite direction by HMGA1b in normal myometrial cells. The higher intensities of transcriptional regulation as described by Reeves et al. 2001 may be explained by the use of a stably transfection strategy relatively to a transient transfection strategy with only 30% transfection efficiency performed herein. Furthermore, functional categorization of HMGA1b-affected genes showed that genes functionally involved in proliferation and differentiation were predominantly down-regulated in normal myometrial cells whereas been up-regulated in cancer cell line MCF-7. Similar regulation pattern were only observed for functional categories of apoptosis and cell-adhesion. Additionally, comparison the data presented herein with micro array analysis performed for uterine leiomyomata and matching myometrium of 9 different patients (Tsibris et al., 2002) shows that only three out of 105 uterine leiomyomata characteristic genes, namely IGF binding protein 5, N-cadherin, and Ig rearranged k chain were also regulated by HMGA-proteins in normal myometrium.

The pattern of predominantly down-regulated genes functionally involved in proliferation and differentiation by HMGA1 and at least HMGA2 variants within normal myometrial cells may possibly explain why a suppression of HMGA1 expression in normal fibroblasts is capable to increase growth rate and impair adipocytic differentiation (Melillo et al., 2001) and why HMGA1 and HMGA2 are not capable of leading to a neoplastic transformation in normal thyroid cells (Fedele et al., 2001; Scala et al., 2001). In opposite to that, when the cellular context is already a tumor cell HMGA-proteins can increase tumor cell proliferation and can lead to the formation of primary and metastatic tumors in nude mice and an anchorage independent soft agar growth (Reeves et al., 2001). These findings further strengthen the hypothesis presented by Henning et al., 1999 of HMGA aberration being a secondary event in tumor development where leiomyomata develop due to an as yet unknown primary event e.g. transformation by a virus with oncogenic potential (Bullerdiek, 1999), whereas HMGA expression as a secondary event in tumor progression increases the growth potential of the corresponding tumor cells.

Besides gene regulatory effects described above some HMGA-target genes were found that are associated with angiogenesis or myogenesis. These genes, FLT1 (no.27), NOTCH4 (no.28), VEGFC (no.23), SERPINE1 (no.90), FGF2 (no.22) and IGFBP2 (no.15), IGFBP4 (no.15), and IGFBP5 (no.17) for angiogenesis and SLC2A3 (no.102), MAPK12 (no.14), CDH2 (no.49), and DES (no.57) in case of myogenesis might contribute to the formation of arteriosclerotic plaques and aortic restenosis, diseases in which HMGA could be involved (Chin et al., 1999). Similarly, genes SERPINE1 (no.90), COL1A2 (no.90) and IGFBP2 (no.15), IGFBP4 (no.15), and IGFBP5 (no.17) due to their inhibitory function on IGF were found that might be involved in HMGA dependent development of adipose tissue (Zhou et al., 1995; Battista et al., 1999; Arlotta et al., 2000; Anand and Chada, 2000). But as results presented herein showed that HMGA-dependent gene regulation depends on the cellular context in which these genes are expressed HMGA target genes relevant for the cellular processes described above have to be analyzed in the specific tissues.

Whether the gene regulatory effects exerted by the HMGA proteins are due to direct interaction with the different promoters or are indirect mediated by intermediary genes is still unknown. Moreover, differential expression analysis performed in leiomyomata cells comparable to the studies presented herein have to be performed to give more insights into aspects of HMGA-mediated gene regulation in terms of the cellular context.

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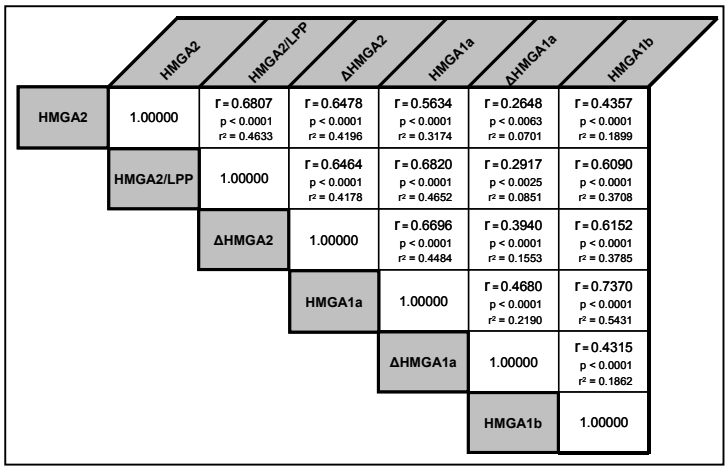
Figures and Figure legends



	HMGA2	HMGA2/LPP	ΔHMGA2	HMGA1a	ΔHMGA1a	HMGA1b
HMGA2	1.00000	r = 0.6807 p < 0.0001 r² = 0.4633	r = 0.6478 p < 0.0001 r² = 0.4196	r = 0.5634 p < 0.0001 r² = 0.3174	r = 0.2648 p < 0.0063 r² = 0.0701	r = 0.4357 p < 0.0001 r² = 0.1899
HMGA2/LPP		1.00000	r = 0.6464 p < 0.0001 r² = 0.4178	r = 0.6820 p < 0.0001 r² = 0.4652	r = 0.2917 p < 0.0025 r² = 0.0851	r = 0.6090 p < 0.0001 r² = 0.3708
ΔHMGA2			1.00000	r = 0.6696 p < 0.0001 r² = 0.4484	r = 0.3940 p < 0.0001 r² = 0.1553	r = 0.6152 p < 0.0001 r² = 0.3785
HMGA1a				1.00000	r = 0.4680 p < 0.0001 r² = 0.2190	r = 0.7370 p < 0.0001 r² = 0.5431
ΔHMGA1a					1.00000	r = 0.4315 p < 0.0001 r² = 0.1862
HMGA1b						1.00000

Fig. 1: Bravais-Pearson correlation matrix of different HMGA variants determined by using micro array data. Bravais-Pearson correlation coefficient (r) (with r = 1 as an absolute correlation and r = 0 as no correlation), coefficient of determination (r^2) and p-values for statistical significance (p<0.01 = highly significant) are shown.

Eigenvalues of the Correlation Matrix

	Eigenvalue	Difference	Proportion	Cumulative
1	3.76909505	2.92176199	0.6282	0.6282
2	0.84733306	0.30708144	0.1412	0.7694
3	0.54025163	0.19374115	0.0900	0.8594
4	0.34651048	0.09029528	0.0578	0.9172
5	0.25621520	0.01562063	0.0427	0.9599
6	0.24059457		0.0401	1.0000

Eigenvectors

	Prin1	Prin2
HMGA2	0.394775	-0.436452
HMGA2/LPP	0.433522	-0.310597
ΔHMGA2	0.437456	-0.113926
HMGA1a	0.453335	0.096629
ΔHMGA1a	0.287774	0.808873
HMGA1b	0.420140	0.190912

Fig. 2: Tables presenting the results of a Principle components analysis using correlation matrix of the different HMGA variants. Data for principle components 1-6 and Eigenvectors Prin1 and Prin2 for the six HMGA proteins are shown.

data corresponding to table 1				Reeves et al., 2001	
number in table 1	Position on Human Cancer	Gene Name (Gene Symbol) [Sequence Accession IDs] HUGO database update: March 2003	HMGA1b (normal myometrial cells)	HMG-Y (MCF-7 breast cancer cell line)	Gene Name Reeves et al. 2001
1	A12l	cell division cycle 25C (CDC25C) [NM_022809]	-7.5	+9.1	cdc25C
3	A10 m	CDC10 cell division cycle 10 homolog (S.cerevisiae) (CDC10) [S72008]	-2.6	+5.2	CDC10 protein homolog
5	A06i	cyclin D1 (PRAD1: parathyroid adenomatosis 1) (CCND1) [Z23022; NM_053056]	-2.9	+3.1	Cyclin D
22	E11d	fibroblast growth factor 2 (basic) (FGF2) [NM_002006]	+2.3	-29.3	FGF2b
28	A06h	Notch homolog 4 (Drosophila) (NOTCH4) [NM_004557]	-3.5	+4.1	Notch-4
29	A12d	epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian) (EGFR) [NM_005228]	-1.1	-3.5	EGF receptor
46	C12f	ataxia telangiectasia mutated (includes complementation groups A, C and D) (ATM) [NM_138293]	+2.1	-3.9	ATM
59	E06n	collagen, type I, alpha 2 (COL1A2) [Z74616; NM_000089]	+2.5	+85.9	Collagen type I
60	E11n	collagen, type VI, alpha 1 (COL6A1) [NM_001848]	+1.5	+24.4	Collagen type VI alpha-1
63	B14b	mitogen-activated protein kinase 14 (MAPK14) [L35263; NM_001315]	+2.6	+5.7	p38 MAPK
65	B04j	ras homolog gene family, member C (ARHC) [NM_005167]	-2.3	+3.9	RhoC
80	F02l	interferon, alpha-inducible protein (clone IFI-6-16) (G1P3) [NM_002038]	-1.4	+6.5	Leukocyte IFN-inducible peptide
89	E04j	a disintegrin and metalloprotease domain 9 (meltin gamma) (ADAM9) [U41766; NM_003816]	-2.3	+5.8	MDC9
90	E08j	serine (or cysteine) proteinase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1 (SERPINE1) [M16006; NM_000602]	-1.8	+5.4	PAI-1

Fig. 3: Comparison of HMGA1b-mediated gene regulation as a result of array experiments performed with normal human myometrial cells and genetically engineered human breast cancer cell line MCF-7. Data on the left side are extracts of table 1 with the corresponding results presented in Reeves et al., 2001 displayed on the right side.

Table 1. Comparative list of genes being transcriptionally regulated by different HMGA proteins as determined by cDNA expression array experiments. Genes are named following HUGO gene nomenclature. Genes not jet approved by HUGO are written in italics. Values of differential expression are given as the gene activity on the experimental array relatively to the control array and are mean values of two independent experiments. Genes differentially expressed above threshold level are boxed in gray. Differential-expression-values for all 6 HMGA protein variants are given for a comparative overview although not always above threshold level. n.c.r. = no clear result. According to their functional information genes were classified into functional categories.

number	Gene Name (Gene Symbol) [Sequence Accession IDs] HUGO database update: March 2003	Position on Human Cancer 1.2 Array	HMGA2	ΔHMGA2	HMGA2/LPP	HMGA1a	ΔHMGA1a	HMGA1b	Functional Category (according to BD Biosciences Clontech)	chromosomal location
	Proliferation									
1	cell division cycle 25C (CDC25C) [NM_022809]	A12l	-1.3	n.c.r.	-1.6	-5.4	-7.1	-7.5	other cell cycle regulators	5q13
2	cell division cycle 34 (CDC34) [L22005; NM_004359]	E08l	+1.1	+1.1	n.c.r.	-1.2	-2.3	-2.0	protein turnover	19p13.3
3	CDC10 cell division cycle 10 homolog (S.cerevisiae) (CDC10) [S72008]	A10m	+1.1	-1.1	-1.6	-2.1	-5.5	-2.6	other cell cycle regulators	7q14.3-14.1
4	CDC37 cell division cycle 37 homolog (S. cerevisiae) (CDC37) [U63131]	A05n	-1.3	+1.2	-2.2	-1.4	+10.7	-1.9	other cell cycle regulators	19p13.2
5	cyclin D1 (PRAD1: parathyroid adenomatosis 1) (CCND1) [Z23022; NM_053056]	A06i	-1.4	-1.5	-1.9	-3.3	-2.3	-2.9	cyclins	11q13.3
6	cyclin D3 (CCND3) [NM_001760]	A08i	-1.8	-2.0	n.c.r.	n.c.r.	n.c.r.	-1.4	cyclins	6p21
7	cyclin-dependent kinase 3 (CDK3) [NM_001258]	A01l	-2.1	-1.6	-1.4	-2.6	-5.2	-2.8	cell cycle regulating kinases	17q22-ter
8	cyclin-dependent kinase 8 (CDK8) [X85753]	A04l	-1.3	-1.2	n.c.r.	-2.1	-1.9	-3.0	cell cycle regulating kinases	11q13-14
9	CDC-like kinase 3 (CLK3) [L29220]	A09k	-2.4	+1.2	-1.5	-1.5	-1.4	-1.7	cell cycle regulating kinases	15q24
10	proliferation-associated 2G4, 38kDa (PA2G4) [NM_006191]	A08n	n.c.r.	-1.2	-1.4	-1.6	-2.7	-2.5	other cell cycle regulators	12q13
11	metallothionein 3 (growth inhibitory factor (neurotrophic)) (MT3) [NM_005954]	A02n	-3.8	-2.1	-2.1	n.c.r.	n.c.r.	n.c.r.	other cell cycle regulators	16q13
12	mitogen-activated protein kinase kinase 2 (MAP2K2) [L11285; NM_030662]	B02c	-1.6	-1.3	-1.8	-1.5	-1.1	-1.2	intracellular kinase network members	-
13	mitogen-activated protein kinase 6 (MAPK6) [L77964; NM_002748]	A09j	n.c.r.	n.c.r.	n.c.r.	-2.2	-1.2	-1.6	cell cycle regulating kinases	15q21
14	mitogen-activated protein kinase 12 (MAPK12) [NM_002969]	A02l	-2.1	-1.7	-2.0	-1.6	n.c.r.	-1.7	cell cycle regulating kinases	22q13.3
15	insulin-like growth factor binding protein 2, 36kDa (IGFBP2) [NM_000597]	A08f	-1.1	-2.6	n.c.r.	-1.1	n.c.r.	-1.1	other oncogenes	2q33-34
16	insulin-like growth factor binding protein 4 (IGFBP4) [M38177; NM_001552]	B05a	n.c.r.	+2.1	+1.2	+2.0	+2.7	+2.7	extracellular transporter	17q12-21.1
17	insulin-like growth factor binding protein 5 (IGFBP5) [NM_000599]	B06a	1.0	n.c.r.	1.0	+1.7	+1.9	+2.1	extracellular transporter	2q33-36
18	v-myc myelocytomatosis viral oncogene homolog (avian) (MYC) [NM_002467]	A03c	-1.4	-1.4	-2.0	-2.3	-1.9	-1.4	transcription factor-related oncogene	8q24
19	protein kinase, cAMP-dependent, regulatory, type I, alpha (tissue-specific extinguisher 1) (PRKAR1A) [NM_002734]	B08c	n.c.r.	-1.3	-2.3	-1.2	-1.1	-1.2	intracellular kinase network members	17q23-24
20	oncostatin M receptor (OSMR) [U60805; NM_003999]	D13j	+1.7	-1.5	-2.0	-1.1	-1.4	-1.3	interleukin & interferone receptors	-
21	non-metastatic cells 4, protein expressed in (NME4) [Y07604; NM_005009]	F09b	-1.1	-1.1	+1.4	+1.3	+1.8	+1.3	metabolic pathways	16p13.3
22	fibroblast growth factor 2 (basic) (FGF2) [NM_002006]	E11d	+3.6	+2.7	+3.3	+2.3	+2.2	+2.3	growth factors, cytokines & chemokines	4q25-27
23	vascular endothelial growth factor C (VEGFC) [NM_005429]	E06c	-2.0	n.c.r.	n.c.r.	-1.4	n.c.r.	-1.3	growth factors, cytokines & chemokines	4q33-34
24	IK cytokine, down-regulator of HLA II (IK) [NM_006083]	E04e	n.c.r.	+1.1	n.c.r.	-2.7	-1.6	-1.3	growth factors, cytokines & chemokines	20q11.1-11.3
25	stem cell growth factor; lymphocyte secreted C-type lectin (SCGF) [AF087658; NM_002975]	E11e	n.c.r.	+3.3	n.c.r.	n.c.r.	-1.1	n.c.r.	growth factors, cytokines & chemokines	16p13.3
26	chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1) (CXCL12) [L36033; NM_000609]	E06e	-1.1	-1.6	-1.2	-4.1	-2.7	-3.5	growth factors, cytokines & chemokines	17q25
27	fms-related tyrosine kinase 1 (vascular endothelial growth factor receptor/vascular permeability factor receptor) (FLT1) [AF063657]	A03e	-2.9	-1.5	-2.3	-2.3	-1.8	-1.3	tyrosine kinase receptors	13q12
	Proliferation / Differentiation									
28	Notch homolog 4 (Drosophila) (NOTCH4) [NM_004557]	A06h	-2.1	-2.2	-2.8	-2.9	-2.4	-3.5	other oncogenes	6p21.3
29	epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian) (EGFR) [NM_005228]	A12d	-1.3	-1.3	-1.4	-1.7	1.0	-1.1	tyrosine kinase receptors	7p12
30	early growth response 1 (EGR1) [NM_001964]	C12j	-2.1	n.c.r.	1.0	n.c.r.	n.c.r.	n.c.r.	transcription factors	5q23-31
31	alanyl (membrane) aminopeptidase (aminopeptidase N, aminopeptidase M, microsomal aminopeptidase, CD13, p150) (ANPEP) [M22324; NM_001150]	E02j	+1.8	+1.4	1.0	+2.0	+1.2	n.c.r.	protein turnover	15q25-26
32	stratifin (SFN) [NM_006142]	B03m	-2.5	1.0	-1.1	-3.4	-3.6	-2.0	kinase substrates & inhibitors	-
33	prostate differentiation factor *(PLAB)* [AF019770]	E09e	+1.1	-1.1	-1.1	-1.8	-1.5	n.c.r.	growth factors, cytokines & chemokines	
34	plexin A3 (PLXNA3) [X74609]	D01m	+2.0	+1.6	+2.0	n.c.r.	+1.4	n.c.r.	other receptors	Xq28

Apoptosis

35	caspase 9, apoptosis-related cysteine protease (CASP9) [U56390; NM_001229]	C11b	-3.3	-1.5	-1.9	-1.2	n.c.r.	n.c.r.	caspases	1p36.3-36.1
36	LPS-induced TNF-alpha factor *(PIG7)* [AF010312]	A11g	-1.2	-1.5	-1.7	-2.6	-1.3	-1.6	other oncogenes	16p13.13
37	tumor necrosis factor receptor superfamily, member 6 (TNFRSF6) [M67454; NM_152877]	C04a	-3.1	-1.4	-3.0	-2.4	-2.5	-1.2	death receptors	10q23
38	tumor necrosis factor receptor superfamily, member 10b (TNFRSF10B) [AF012628; NM_147187]	C05a	-1.8	-1.1	-1.9	-4.3	-2.5	-1.9	death receptors	8p22-21
39	2',5'-oligoadenylate synthetase 1, 40/46 kD (OAS1) [X04371; NM_002534]	D02n	n.c.r.	-1.8	-1.1	-1.6	-1.3	-1.9	stress response proteins	12q24.2

Chromatin structure / Genome integrity

40	MCM4 minichromosome maintenance deficient 4 (S. cerevisiae) (MCM4) [X74794]	C06f	+1.1	+1.1	n.c.r.	-2.2	-2.5	-1.5	DNA polymerases, replication factors & recombination protein	8q12-13
41	topoisomerase (DNA) I (TOP1) [NM_003286]	C11e	+4.5	+3.9	n.c.r.	+3.2	+3.7	+4.5	DNA polymerases, replication factors & recombination protein	20q12-13.1
42	chromatin assembly factor 1, subunit A (p150) (CHAF1A) [U20979; NM_005483]	D13a	-1.8	-1.7	-3.0	-1.8	-1.6	-2.4	DNA-binding & chromatin proteins	19p13.3
43	histone 1, H4b (HIST1H4B) [X67081; NM_003544]	D08b	-2.1	n.c.r.	-2.0	+1.1	-1.3	-1.2	DNA-binding & chromatin proteins	6p21.3
44	high-mobility group box 2 (HMGB2) [NM_002129]	D07b	-2.2	-1.3	-1.2	+1.2	-1.2	+1.3	DNA-binding & chromatin proteins	4q31
45	nuclear antigen SP100 (SP100) [AF056322; NM_003113]	D10b	+1.5	+1.1	-1.1	+2.2	+1.3	-2.0	DNA-binding & chromatin proteins	2q37.1
46	ataxia telangiectasia mutated (includes complementation groups A, C and D) (ATM) [NM_138293]	C12f	+1.9	+1.9	+1.6	+1.3	+1.2	+2.1	DNA damage repair proteins, ligases & helicases	11q22-23
47	ligase I, DNA, ATP-dependent (LIG1) [NM_000234]	C01g	-3.5	-2.3	-2.1	1.0	+1.1	+1.1	DNA damage repair proteins, ligases &	Chr19
48	X-ray repair complementing defective repair in Chinese hamster cells 1 (XRCC1) [NM_006297]	C01h	-2.7	-2.1	n.c.r.	-2.0	-1.3	-1.4	DNA damage repair proteins, ligases & helicases	19q13.2

Cell architecture

49	cadherin 2, type 1, N-cadherin (neuronal) (CDH2) [S42303; NM_001792]	D10d	n.c.r.	+2.1	+1.4	+2.9	+1.9	+1.1	cell adhesion receptors & proteins	19p13.3
50	cadherin 11, type 2, OB-cadherin (osteoblast) (CDH11) [L34056; NM_033664]	D03d	-1.9	1.0	-1.1	-1.3	-1.2	-1.8	cell adhesion receptors & proteins	6p21.3-21.2
51	catenin (cadherin-associated protein), alpha 1, 102kDa (CTNNA1) [NM_001903]	D09d	-2.9	-2.6	-1.3	+1.4	+1.2	-1.1	cell adhesion receptors & proteins	18q12.1
52	integrin, alpha 5 (fibronectin receptor, alpha polypeptide) (ITGA5) [NM_002205]	D04e	+1.2	+1.2	+1.2	+2.0	+1.8	+1.3	cell adhesion receptors & proteins	16q21-22.1
53	integrin, beta 5 (ITGB5) [NM_002213]	D14d	-2.6	-1.6	-1.4	-1.5	1.0	+1.1	cell adhesion receptors & proteins	5q31
54	ninjurin 1 (NINJ1) [NM_004148]	D11f	-1.8	n.c.r.	-1.6	-1.7	-1.7	-1.7	cell adhesion receptors & proteins	7q22.1
55	polycystic kidney disease 1 (autosomal dominant) (PKD1) [NM_000296]	D11e	-1.9	-1.2	-1.1	-1.2	-1.2	-1.8	cell adhesion receptors & proteins	21q22.3
56	A kinase (PRKA) anchor protein (gravin) 12 (AKAP12) [U81607; NM_144497]	F09g	+2.9	+3.2	+1.7	+2.4	+2.0	+2.2	cytoskeleton & motility proteins	2q37
57	desmin (DES) [NM_001927]	F05g	+1.7	-3.3	+1.7	+2.4	+1.8	+2.2	cytoskeleton & motility proteins	12q11-13
58	microtubule-associated protein 1B (MAP1B) [L06237]	F14g	-1.6	-1.8	-1.2	-1.3	1.0	-1.2	cytoskeleton & motility proteins	Chr3
59	collagen, type I, alpha 2 (COL1A2) [Z74616; NM_000089]	E06n	+3.4	+2.5	+2.1	+2.4	+2.0	+2.5	immune system proteins	9q22
60	collagen, type VI, alpha 1 (COL6A1) [NM_001848]	E11n	n.c.r.	+1.2	+1.1	+1.9	+1.8	+1.5	immune system proteins	16p13.3
61	collagen, type VI, alpha 3 (COL6A3) [NM_057167]	E13n	-2.0	+1.2	-1.1	+1.2	1.0	+1.1	immune system proteins	2q37-ter
62	nidogen (entactin) (NID) [NM_002508]	F10a	+1.4	-1.3	+1.3	+2.1	+2.2	+1.7	immune system proteins	Xp11.3-11.23
63	mitogen-activated protein kinase 14 (MAPK14) [L35263; NM_001315]	B14b	+1.7	+1.8	n.c.r.	+1.7	+2.1	+2.6	intracellular kinase network members	6q24-25
64	ras homolog gene family, member A (ARHA) [NM_001664]	A13e	+1.3	-1.5	-1.7	-1.8	1.0	-1.5	intracellular signal transduction-related oncogenes	2q35
65	ras homolog gene family, member C (ARHC) [NM_005167]	B04j	+1.2	-1.4	n.c.r.	-2.2	-1.6	-2.3	G proteins	1q43
66	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3A (SEMA3A) [L26081; NM_006080]	D13n	n.c.r.	+1.1	-1.4	-7.8	-6.0	-6.1	neuromediators	22q13
67	zyxin (ZYX) [X95735; NM_003461]	B07m	-1.8	+1.3	1.0	+1.6	+1.3	+1.4	other intracellular signal transduction modulators & effectors	3p21
68	basigin (OK blood group) (BSG) [L10240; NM_001728]	E09l	+1.4	-1.1	+1.8	1.0	-2.3	-1.2	protein turnover	1p21-13
69	cathepsin D (lysosomal aspartyl protease) (CTSD) [NM_001909]	E03j	n.c.r.	1.0	-1.2	+1.9	+1.7	n.c.r.	protein turnover	6q22-27
70	tissue inhibitor of metalloproteinase 1 (erythroid potentiating activity, collagenase inhibitor) (TIMP1) [NM_003254]	E10j	+1.4	+2.0	+1.4	+2.6	+1.6	+1.8	protein turnover	7q34-35
71	villin 2 (ezrin) (VIL2) [NM_003379]	A03b	-2.5	-1.2	-2.5	-2.2	-1.4	n.c.r.	tumor suppressors & related proteins	11p15.5

Vesicular transport

72	myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse) (MX1) [NM_002462]	F05h	1.0	-3.0	1.0	n.c.r.	n.c.r.	n.c.r.	trafficking proteins (endocytosis & exocytosis)	21q22.3
73	translocating chain-association membrane protein *(TRAM)* [X63679]	F09h	+3.3	+4.1	+2.3	+3.3	+3.4	+3.2	trafficking proteins (endocytosis & exocytosis)	8q13.3
74	transmembrane trafficking protein 21 *(TMP21)* [X97442]	F05i	+2.7	n.c.r.	n.c.r.	-2.8	-2.4	n.c.r.	trafficking proteins (endocytosis & exocytosis)	14q24.3
75	pleckstrin homology, Sec7 and coiled/coil domains 2 (cytohesin-2) (PSCD2) [X99753; NM_017457]	B01k	-1.1	-1.3	-1.5	-2.0	-1.7	-2.0	GDP/GTP exchangers & GTPase stimulators/inhibitors	19q13.3

Others

76	deleted in colorectal carcinomas (DCC) [NM_005215]	A03a	-3.0	n.c.r.	n.c.r.	n.c.r.	n.c.r.	n.c.r.	tumor suppressors & related proteins	20q11.2
77	microtubule-associated protein, RP/EB family, member 1 (MAPRE1) [U24166]	A02b	-2.4	-1.7	-2.2	-2.0	-1.2	-1.2	tumor suppressors & related proteins	10q25.1
78	tumor susceptibility gene 101 (TSG101) [U82130]	A11b	+1.2	+1.5	-2.0	-1.2	-1.3	+1.1	tumor suppressors & related proteins	11q23.3-qter
79	Finkel-Biskis-Reilly murine sarcoma virus (FBR-MuSV) ubiquitously expressed (fox derived); ribosomal protein S30 (FAU) [NM_001997]	F12k	-2.8	+1.1	+1.3	1.0	+1.3	+1.3	translation	Chr3
80	interferon, alpha-inducible protein (clone IFI-6-16) (G1P3) [NM_002038]	F02l	-2.5	1.0	n.c.r.	-1.2	-1.5	-1.4	translation	6q27
81	interferon, alpha-inducible protein 27 (IFI27) [X67325; NM_005532]	F10m	-1.3	+2.8	+1.2	+1.6	+1.9	+1.4	translation	6q23
82	serine/arginine repetitive matrix 2 (SRRM2) [AF201422; NM_016333]	F04l	-4.1	-3.2	n.c.r.	-1.6	-1.5	-1.2	translation	7q21.3-22
83	ELK3, ETS-domain protein (SRF accessory protein 2) (ELK3) [NM_005230]	C01j	-1.1	+1.1	n.c.r.	-1.4	-1.5	-1.8	transcription factors	12p13.3
84	FOS-like antigen 1 (FOSL1) [X16707; NM_005438]	A13c	-1.5	-1.3	-1.2	-2.0	-1.4	-1.7	transcription factor-related oncogene	17p13
85	jun-B proto-oncogene (JUNB) [NM_002229]	A01d	-2.2	n.c.r.	-1.9	-2.5	-1.1	-1.4	transcription factor-related oncogene	16q13
86	v-jun avian sarcoma virus 17 oncogene homolog (JUN) [NM_002228]	A01c	n.c.r.	-1.4	n.c.r.	-1.9	-1.3	-1.5	transcription factor-related oncogene	12q23
87	glutathione synthetase (GSS) [NM_000178]	D07m	-1.1	-2.0	-1.8	-1.5	-1.1	-1.2	stress response proteins	11q13
88	glutathione-S-transferase like *(GSTTLP28)* [U90313; NM_004832]	D09m	+3.0	n.c.r.	n.c.r.	-1.3	-1.4	-1.7	stress response proteins	19p13.2
89	a disintegrin and metalloprotease domain 9 (meltin gamma) (ADAM9) [U41766; NM_003816]	E04j	n.c.r.	+1.6	n.c.r.	-2.5	-2.3	-2.3	protein turnover	1p32-31
90	serine (or cysteine) proteinase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1 (SERPINE1) [M16006; NM_000602]	E08j	+1.9	+1.9	+1.4	+1.2	-1.6	-1.8	protein turnover	18q21.1
91	tissue inhibitor of metalloproteinase 2 (TIMP2) [NM_003255]	E11j	-2.6	+1.6	+1.2	+1.3	+1.6	+1.6	protein turnover	11p15
92	suppression of tumorigenicity 13 (colon carcinoma) (Hsp70 interacting protein) (ST13) [NM_003932]	F08j	-1.5	-1.6	-3.3	-2.1	-1.7	-1.9	post-translational modification & folding	-
93	active BCR-related gene (ABR) [NM_021962]	A13h	+1.1	-1.1	-1.7	-2.3	-1.6	-2.2	other oncogenes	11q13
94	linker for activation of T-cells (LAT) [AF036905]	B01m	-1.6	-2.3	-3.1	-2.1	-2.2	-1.9	kinase substrates & inhibitors	10q11.1
95	Cas-Br-M (murine) ecotropic retroviral transforming sequence (CBL) [NM_005188]	A03f	-1.5	-1.6	-2.9	-2.5	-1.9	-1.4	intracellular signal transduction-related oncogenes	2p15-14
96	ribosomal protein S6 kinase, 90kD, polypeptide 1 (RPS6KA1) [NM_002953]	B13d	-2.5	-1.8	-2.6	-1.9	n.c.r.	+1.5	intracellular kinase network members	2p11.2
97	ribosomal protein S6 kinase, 90kD, polypeptide 2 (RPS6KA2) [NM_021135]	B14d	-2.7	n.c.r.	-1.8	-2.8	-1.6	-1.3	intracellular kinase network members	1p35
98	serum/glucocorticoid regulated kinase (SGK) [AJ000512]	B03g	+1.3	+1.2	1.0	-2.3	-1.5	-2.2	intracellular kinase network members	14q32
99	interleukin 15 receptor, alpha (IL15RA) [U31628; NM_172200]	D11j	-1.9	-1.8	-2.1	-1.3	-1.6	-1.2	interleukin & interferone receptors	10q15-14
100	interleukin 17 receptor (IL17R) [U58917; NM_014339]	D10j	-1.3	-1.8	-2.3	-1.3	-2.2	1.0	interleukin & interferone receptors	Chr22
101	immunoglobulin kappa constant (IGKC) [J00241]	E05m	-2.4	-1.3	-1.7	-1.8	-1.9	n.c.r.	immune system proteins	5q13
102	solute carrier family 2 (facilitated glucose transporter), member 3 (SLC2A3) [M20681]	A14n	n.c.r.	-1.6	-1.6	-1.7	-1.2	-2.3	facilitated fusion proteins	19q13.3

II.

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Eigenanteil an dieser Publikation:

- Vorversuche zu dieser Arbeit
- Klonierung der für die Bindungsstudien benötigten ERCC1 Promoterfragmente
- Durchführung der funktionellen ERCC1 Luziferase Promoter Assays
- Verfassen der Publikation in Zusammenarbeit mit Herrn R. Schwanbeck

Carboxy-Terminally Truncated High Mobility Group A2 Protein Inhibits Activity of DNA Repair Gene *ERCC1**

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Running title: **Truncated HMGA2 affects activity of *ERCC1* gene**

Abstract

High mobility group A2 (HMGA2) chromosomal nonhistone protein and its derivatives play an important role in development of benign tumors, obesity and arteriosclerosis although the underlying mechanisms of these conditions are poorly understood. Therefore, we tried to identify target genes for this transcriptional regulator and to provide insights in the mechanism of interaction to its target. Multiple genes have been identified by microarray experiments as being transcriptionally regulated by HMGA2. Among these we chose the *ERCC1* gene, encoding a DNA repair protein, for this study. To obtain more functional data on *ERCC1* gene regulation DNA-binding studies were performed using HMGA2, chimeric HMGA2/LPP, and C-terminally truncated ΔHMGA2. A high affinity HMGA2 binding site was mapped to an AT-rich region located –323 to –298 upstream of the *ERCC1* transcription start site. The observed 1:1 stoichiometry for the binding of wild-type HMGA2 to this region was altered to 1:2 upon binding of truncated ΔHMGA2 causing a DNA-bending. Furthermore, the regulatory effect of HMGA2 was confirmed by luciferase promoter assays showing that *ERCC1* promoter activity is down-regulated by all three HMGA2 forms with the most striking effect exerted by ΔHMGA2. Our results provide the first insights into how HMGA2 and its aberrant forms bind and regulate the *ERCC1* promoter.

Introduction

Nucleotide excision repair (NER)[1] is the main pathway by which mammalian cells protect themselves from helix-distorting DNA lesions induced by UV-light and chemical mutagens (reviewed in 1). ERCC1 (excision repair cross-complementing rodent repair deficiency, complementation group 1) is one of the proteins being essential for the NER pathway and is considered as a marker for NER activity (2). The ERCC1 gene is located at 19q13.2-q13.3 and encodes a 32 kDa protein that is highly conserved with homologs in mouse, *mErcc1* (3), *Saccharomyces cerevisiae*, *RAD10* (4), and *Schizosaccharomyces pombe*, *swi10* (5). *In vivo,* ERCC1 forms a tight heterodimer with XPF (*syn.:* ERCC4) (6,7) that acts as a structure-specific endonuclease cutting single-strand DNA near the junction between single and double-stranded DNA. Within the NER pathway the ERCC1/XPF complex is responsible for the cleavage of the damaged DNA strand 16-25 nt upstream of the lesion (8–10).

The fact that ERCC1 and XPF-deficient mammalian cells, unlike other NER-deficient cells, are sensitive to DNA crosslinking agents indicated a role for ERCC1/XPF in NER-independent interstrand crosslink repair (11). For example, the repair of intrastrand and/or interstrand cross-links induced by chemotherapeutic agent cisplatin, are primarily repaired by the NER pathway (12–14). Furthermore, in the last few years it was shown that the ERCC1/XPF complex is also involved in recombination events such as targeted homologous recombination (15) and targeted gene replacement (16).

Interestingly, an overexpression of *ERCC1* during cisplatin-based chemotherapy is associated with tumors being clinically resistant to therapy (17). Understanding the mechanisms by which ERCC1 expression is regulated could thus be an important tool in developing strategies that interfere with ERCC1 expression and might improve the effectiveness of cisplatin-based chemotherapy.

HMGA2 belongs to the high mobility group (HMG) family of non-histone chromatin proteins (for review see 18) and its gene is located on chromosome 12q14-15 in humans. HMGA2 consists of three DNA-binding domains (AT-hooks) enabling their binding to the minor groove of AT-rich DNA and an acidic C-tail responsible for protein-protein interactions (for review see 19). The DNA binding of the so called “architectural transcription factors” HMGA were shown to alter DNA conformation and bend the DNA in some cases, so that the assembly and function of transcriptional complexes is modulated resulting in a regulation of gene expression. However, the available information about gene regulatory effects on a molecular level is mostly restricted to HMGA1, a related protein family (consisting of HMGA1a and HMGA1b) that is encoded from another gene and with different expression patterns and functions.

HMGA2 is expressed at very high levels during embryonic development whereas it is almost undetectable in differentiated cells (20,21). Reactivation of expression in differentiated cells is characteristic for malignant (22–25) and benign tumors (26,27) and is implicated in the formation of

arteriosclerotic plaques, aortic restenosis (28) and adipogenesis (29,30). Aberrations of the chromosomal region 12q14-15 affecting the *HMGA2* gene, a frequent event in a variety of human benign tumors, is the main cause for reactivated *HMGA2* expression or the expression of chimeric or truncated forms of *HMGA2* (26,31,32). These chimeric and truncated transcripts consist predominantly of the three DNA-binding domains of HMGA2 but lack the acidic C-tail and its 3'UTR. For the chimeric forms of HMGA2 several fusion partner genes such as *LPP* (33), *RAD51L1* (34), and *ALDH2* (35) have been described.

Expression of both normal and truncated HMGA2 is capable of inducing neoplastic transformation *in vitro* (36). But in contrast to full length HMGA2, transgenic mice carrying a truncated HMGA2 develop a giant phenotype along with adiposity and show an abnormally high prevalence of lipomas (37,38)

Data of cDNA expression array experiments that were performed within primary cells of three independent human myomata with normal karyotype (unpublished data) had revealed that an overexpression of HMGA2 leads to an increase in *ERCC1* transcription. As the expression of this gene is involved in the resistance of tumors to chemotherapeutic treatment we sought for mechanisms by which HMGA2 exerts its regulatory role on the expression of *ERCC1*. To address this question, electrophoretic mobility assays, DNA-footprinting, and methylation interference assays were performed to map binding sites for HMGA2, chimeric HMGA2/LPP, and C-terminally truncated ΔHMGA2 within the *ERCC1* promoter. Luminescence resonance energy transfer (LRET) measurements were used to monitor changes in DNA-bending induced by these proteins. To give insights into the functional role of these HMGA2 protein variants in terms of *ERCC1* gene regulation *ERCC1* promoter regions were used to perform transcription assays using a luciferase promoter system.

Materials and Methods

Preparation of the HMGA2 proteins **–** The proteins were expressed in *E. coli* and purified as described previously (39,40). The purified products were quantified on Coomassie Blue R - stained SDS polyacrylamide gels using a spectrophotometric determined tryptophane-containing mutant of *Chironomus* HMGA protein as a standard (41).

Preparation of ERCC1 DNA **–** Different fragments of the *ERCC1* promoter were cut out from the appropriate plasmids using EcoRI and overhanging 5' end was filled up with Klenow fragment using [α-^{32}P]dATP. For end-labeling purpose DNA was cut asymmetrically with SpeI resulting in a 3'-labeled top strand. All labeled inserts were purified on a 2% agarose/TBE gel. The 44 bp fragment of the *ERCC1* promoter was prepared from synthetic oligonucleotides (MWG-Biotech, Germany) comprising promoter region –330 to –287 relative to the transcription start. For DNA footprinting experiments and mobility shift assays the single strands were 5' end-labeled by T4 polynucleotide kinase and complementary strands were annealed by a temperature gradient from 90 ºC to 20 ºC over 2 h. The double stranded DNA fragments were purified by ionic exchange chromatography using a Gen-Pak FAX column (Waters, 4.6 × 100 mm) with a linear gradient of 40–55% Eluent B (1 M NaCl, 1 mM EDTA, 25 mM Tris/HCl, pH 7.9) in 30 min (Eluent A: 1 mM EDTA, 25 mM Tris/HCl, pH 7.9).

Mobility shift assay **–** Electrophoretic mobility shift assays were carried out as described previously (42). Briefly, purified proteins were incubated with less than 1 nM of labeled DNA in 180 mM NaCl, 1 mM $MgCl_2$, 0.01% BSA, 8% glycerol, 10 mM Tris/HCl, pH 7.9 at 20 °C for 10 min. The DNA and DNA·protein complexes were run on 6% or 8% polyacrylamide gels in a circulating electrophoresis buffer containing 6.6 mM Tris, 3.3 mM acetate, pH 7.9.

Hydroxyl-radical DNA footprinting **–** For footprints of the long DNA fragments 16,000 cpm of the *ERCC1* DNA (–426 to –257) labeled at the 3' end of the top strand was partially digested with hydroxyl-radicals in 10 µl reaction volume in presence or absence of 100 nM HMGA2 protein in 180 mM NaCl, 20 ng/µl BSA and 10 mM MOPS buffer, pH 7.2 at room temperature for 20 min as described previously (43). The reaction products were separated on 8% polyacrylamide sequencing gels containing 7 M urea/TBE. For the footprints of the short DNA fragments 10–15 kcpm of the 5'-

labeled *ERCC1* fragment –330 to –287 was partially digested as described above. The reaction products were separated on 18% polyacrylamide sequencing gels containing 7 M urea/TBE. All gels were scanned by PhosphorImager (Molecular Dynamics) and the data analyzed as described previously (43).

Methylation Interference Assay – The top strand 5'-labeled –330 to –287 fragment was methylated with dimethyl sulfate (44). 500 nM modified DNA was incubated with 1 μM HMGA2 or ΔHMGA2, and the protein·DNA complexes were separated from unbound DNA by gel electrophoresis. The DNAs out of the complexes were eluted from the gels and cleaved at methylated purines with piperidine. Finally, equal amounts of radioactivity (~5000 cpm) of the cleavage products were analyzed on 18% acrylamide sequencing gels. G+A standard was generated according to Maxam and Gilbert (45). The gels were scanned and the data analyzed as described previously (43). Briefly, the peaks of the intensity plots were aligned using the program ALIGN (Dr. T. Heyduk, St. Louis, MO) and gel-loading efficiency was normalized. The intensities of the modified bands were integrated, and binding interference expressed as normalized difference using following formula: $\Delta_{norm} = (I_{bound} - I_{unbound})/I_{unbound}$, where Δ_{norm} is the normalized difference, I_{bound} and $I_{unbound}$ is the integration of the normalized intensities at a single nucleotide position bound or unbound to HMGA2 (or ΔHMGA2) protein, respectively.

Luminescence resonance energy transfer (LRET) measurements – 23 nt oligonucleotides corresponding to *ERCC-1 promoter region from –316 to –296* were synthesized on an Applied Biosystems model 394 DNA synthesizer (Foster City, CA) using standard phosphoramidite chemistry. Oligonucleotides were purified and labeled with luminescence donor [(Eu^{3+})DTPA-AMCA-maleimide; prepared as described in (46) and fluorescence acceptor (Cy5; from Amersham Pharmacia Biotech, Piscataway, NJ) as described previously (47). The Amine-VN Phosphoramidite (Clontech, Palo Alto, CA) was used to incorporate the reactive amine into the internal positions within the oligonucleotides. LRET (48) measurements were performed at 25° C in a 120 μl cuvette on a laboratory-built two-channel instrument described earlier (49). Reaction mixtures contained 15 nM labeled DNA duplex in 10 mM Tris/HCl (pH 7.9) buffer containing 180 mM NaCl, 1 mM $MgCl_2$ and various concentrations of HMGA2 proteins. The donor emission was collected using a 620 nm interference filter (Oriel, Stratford, CT) whereas sensitized acceptor signal was detected using a 668 nm interference filter (Oriel, Stratford, CT). Sensitized acceptor (49) decay curves were analyzed by nonlinear regression using SCIENTIST (Micromath Scientific Software, Salt Lake City, UT) according to:

$$I = \Sigma I_i * \exp(-t/\tau_i) + B$$

where I_i and t_i are the amplitude and the lifetime of the ith component and B is the background noise. Energy transfer (50, 51) was calculated using:

$$E = 1 - \tau_{DA}/\tau_D$$

where τ_{DA} and τ_D are luminescence lifetimes of the donor in the presence and absence of the acceptor, respectively. The distances between donor and acceptor were calculated using procedures outlined in (49) according to:

$$R^6 = R^6_o(1 - E)/E$$

where R is a distance between a donor and an acceptor, and R_o is a distance at which the energy transfer is 0.5. The R_o for (Eu^{3+})DTPA-AMCA and Cy5 donor-acceptor pair (55 Å) was calculated as described previously (52).

Construction of ERCC1 promoter plasmids – *ERCC1* promoter fragments were PCR amplified using XhoI-linker primer 5'-CCCTCGAGCTCCCCAACACTTCCAATCCTCT-3' (nt 26426-26404; M63796) for the 3.9kb promoter fragment (nt –3900 to +1 relatively to the transcriptional start site) or BglII-linker primer 5'-AGATCTAACCGTAAGCTCCGGGAGGACAAC-3' (nt 22952-22929) for the 426bp promoter region (nt –425 to +1) in combination with HindIII-linker primer 5'-AAGCTTTCCGGCCTCTCTGGCCCCGC-3' (nt 22527-22546).
Standard hotstart PCRs were performed with Pfu DNA polymerase (Promega, Madison, USA) in a Mastercycler gradient (Eppendorf, Hamburg, Germany) using following protocols: 5 min 95 °C, (45

sec 94 °C, 45 sec 65 °C, 4 min 72 °C) 30×, 10 min 72 °C. Amplification of the 3.9 kb *ERCC1* fragment PCR was performed with the TripleMaster PCR system (Eppendorf) under high-fidelity-PCR conditions according to the instructions of the manufacturer. PCR profile was: 3 min 94 °C (20 sec 94 °C, 15 sec 69 °C, 2 min 45 sec 72 °C) 30×, 10 min 72°C. 150 ng of genomic DNA were used as templates. PCR fragments were cloned into the BglII-HindIII, respectively the XhoI-HindIII sites of reporter-gene vector pGL3-Basic (Promega).

Luciferase promoter assays – *ERCC1* promoter constructs -3900 to +1 and -425 to +1 as well as empty vector pGL3-Basic were each transiently co-transfected with a vector expressing either no HMGA2 (vector pCR3.1 (Invitrogen, Groningen, Netherlands) as reference sample), wild-type HMGA2, C-terminally truncated HMGA2 lacking its last two exons corresponding to the spacer and acidic domain (ΔHMGA2), or chimeric HMGA2/LPP consisting of the three DNA-binding domains of HMGA2 (exons 1–3) and three LIM-domains of LPP (exons 9–11) (vectors as described elsewhere (36)). To provide a standard for normalized vector pRL-Tk (Promega) was also co-transfected with each sample.

Transient transfections were carried out in HMGA2-negative HeLa cells being cultured in medium TC199 supplemented with 20% fetal calf serum (FCS) and antibiotics (200 IU/ml penicillin, 200 µg/ml streptomycin). Prior to transfection cells were seeded on 6-well plates and were grown to about 60% confluence. Growth medium was completely removed, cells were washed with PBS and "transfection complexes" mixed with 800 µl culture medium were added. Transfection complexes containing 1 µg of promoter construct DNA, 1 µg of HMGA2 expression plasmid, 250 ng pRL-TK, and 10 µl SuperFect transfection reagent (Qiagen, Hilden, Germany) were formed in a total volume of 100 µl in TC199 medium (without supplements) by incubating the sample for 10 min at room temperature according to the instructions of the manufacturer. After an incubation for 3 h cells were washed with PBS, 2.5 ml of fresh 20% culture medium were added and cells were than grown for further 48 h with renewal of the growth medium after 24 h.

Luciferase activities were measured in a luminometer, (Biocounter M2010, Lumac BV, Netherlands) using the Dual-Luciferase Reporter Assay System (Promega) following the instructions of the manufacturer. Experiments for each sample were performed in duplicate and were repeated several times. Data normalization and adjusting was performed as suggested by the manufacturer (Promega).

For statistical analysis mean values of the independent experiments as well as standard deviations were calculated. To test for statistical significance one sample t-test were performed with $p \leq 0.05$ for significant and $p \leq 0.01$ for highly significant differences.

Results

High affinity binding sites within the ERCC1 promoter – DNA fragments spanning three regions of the basal *ERCC1* gene promoter (Fig. 1A, *top panel*) were ^{32}P-labeled and assayed for binding to HMGA2 protein. Mobility shift experiments revealed that HMGA2 binds tightly to the –426 to –257 fragment of the promoter whereas the other fragments showed just unspecific DNA binding at higher protein concentrations without any complex formation (Fig. 1A). Digestion of this DNA fragment with a restriction enzyme into two smaller fragments showed that the –350 to –257 fragment was shifted in the presence of the protein whereas the –426 to –351 fragment did not (Fig. 1B). Note that the restriction enzyme Eam1104I does not digest its substrate completely and that the HMGA2·[–350 to –257] complex co-migrates with the full length DNA (–426 to +1) so that the mobility shift pattern is more complex. However, the experiment clearly indicates that the –350 to –257 region contains a HMGA2 binding site.

To obtain more quantitative binding data, the mobility shift assays were repeated in a more narrow range of protein concentrations (Fig. 1C). Quantification of HMGA2 affinity to the –426 to –257 fragment revealed a $K_{d(app)}$ of 1.75 ± 0.305 nM (Fig. 1D). Hence, HMGA2 posses a fairly high affinity to this promoter region, whereas HMGA1b and HMG1b (formerly HMGY and HMGI) revealed a much lower affinity (Fig. 1C). The HMGA2 concentration needed for shifting of 50% of the *ERCC1* promoter was at least one order of magnitude lower than the concentrations of HMGA1b and

HMGA1a necessary to achieve the same effect. Thus, these results indicate that HMGA2 exhibits a clear specificity for a site located on this fragment.

Mapping of the HMGA2 binding site on the ERCC1 promoter – In order to obtain detailed information on the position of the HMGA2 binding site DNA footprinting analyses were performed. In an initial experiment the large –426 to –257 fragment labeled on the 3' end of the top strand was analyzed. Even if the individual bands produced by the hydroxyl-radical digestion are difficult to distinguish on the plain PhosphorImage (Fig. 2A) the quantitative analysis of the digestion patterns of the free and protein bound DNA revealed two strongly protected regions that were much less cut than the average 100% (Fig. 2B). The maxima of these two sites were mapped to –321 and –310. More detailed quantitative DNA footprinting analysis using a 44 bp fragment comprising the nucleotides –330 to –287 showed that binding of the protein results in the protection of regions –323 to –318 and –312 to –304 on the top strand with maxima at –321 and –309, respectively. On the bottom strand the regions –314 to –305 and –303 to –298 with maxima at –310 and –301, respectively, were protected (Fig. 3A and B, *black bars*). The results indicate that HMGA2 binds tightly within the AT-rich minor grove spanning the region from –312 to –305. It contacts both strands in this region whereas the binding at two other sites appears to be weaker and involves just one strand.

Binding of the truncated HMGA2 to the ERCC1 promoter – DNA footprinting experiments using the C-terminally truncated ΔHMGA2 revealed altered DNA binding properties when compared to the wild-type protein (Fig. 3A, B, *gray bars*). When using the C-terminally truncated ΔHMGA2 the top strand central binding region around –309 was extended to the 3' end to –301 with a second peak at –304. The bottom strand region between the protection maxima –310 and –301 was much stronger protected than by the wild-type HMGA2. Furthermore, the truncation resulted in an additional protected region with a maximum at –295 (Fig. 3B, *gray bars*). In agreement with these results mobility shift experiments using the –330 to –287 fragment with HMGA2 and ΔHMGA2 revealed clear differences in the stoichiometry of binding to the *ERCC1* promoter. Whereas the wild-type protein formed only 1:1 complexes (Fig. 4A, *HMGA2*) the truncated protein formed complexes with both a 1:1 and 2:1 protein to DNA ratio (Fig. 4A, *ΔHMGA2*). Furthermore, the slope of the binding curves with a corresponding Hill coefficient of 1.4 ± 0.2 for HMGA2 compared to 4.5 ± 0.9 for ΔHMGA2 indicates a transition from a non-cooperative to a cooperative binding upon truncation of the protein (Fig. 4B). However, the binding affinity is independent of the presence of the acidic tail. ~10 nM of both proteins were necessary for shifting 50% of the 44 bp *ERCC1* DNA (Fig. 4B). For methylation interference assays both the 1:1 and the 2:1 complex visible in the mobility shift experiments (Fig. 4A) were isolated from preparative gels and compared to the single complex occurring with HMGA2. The experiments clearly demonstrated that the nature of the complexes with the 1:1 stoichiometry for the wild-type and the truncated proteins was similar (Fig. 4C, D, *black* and *gray bars*). The second molecule of ΔHMGA2 in the 2:1 complex however bound in a different manner 5' from the central binding region (–309) thereby covering a much larger region than the wild-type protein (Fig. 4D, *crisscrossed bars*).

Binding of the truncated HMGA2 induce strong conformational perturbation of the ERCC1 promoter – To analyze conformational changes of the *ERCC1* promoter upon binding of HMGA2 a series of duplex DNA spanning the promoter region from –316 to –294 were prepared. The duplexes were labeled at the 3' end of the top or bottom strand with (Eu^{3+}) chelate as a donor and with Cy5 as an acceptor at 3 different positions within backbone of the complementary strand. The distances between the different combinations of these fluorophores were measured by LRET for various protein concentrations (Fig. 5). The analyses revealed that ΔHMGA2 affects the DNA conformation significantly, whereas the effect of wild type protein and the LPP fused proteins is negligible low. The effect is not a simple bending because the changes are asymmetric, e.g. the distance from one end are decreasing (Fig. 5. *panels A – C*) whereas the distances from the other end (Fig 5. *panels D – F*) do not change *(E*) or increase (*D* and *F*). This explains why the total length between both ends remains constant in the presence of each of the proteins (data not shown). The model in Fig. 6 summarizes the events that take place upon ΔHMG2A binding.

ERCC1 promoter activity is down-regulated by different HMGA2 proteins – To investigate the effects that HMGA2 exerts on *ERCC1* transcriptional activity a basal promoter fragment of *ERCC1* spanning region nt –425 to +1 and a 3.9 kb promoter fragment (spanning nt –3900 to +1) were cloned into reporter vector pGL3-Basic. Luciferase reporter gene assays were used to measure promoter activity of these constructs using HMGA2-negative HeLa cells for transient transfection. Both promoter constructs were co-transfected with constructs expressing either normal HMGA2, C-terminally truncated ΔHMGA2, a HMGA2/LPP fusion protein, or no protein (vector pCR3.1) (Fig. 7).

Co-transfection experiments of the two ERCC1 promoter fragments with different HMGA2 protein variants showed decreased ERCC1 promoter activity due to HMGA2 proteins in all samples tested relatively to promoter constructs co-transfected with empty vector pCR3.1 expressing no HMGA2 (Fig. 7). Whereas, the decrease in *ERCC1* promoter activity induced by wild-type HMGA2 was approximately 15% for both promoter constructs the co-expression of truncated ΔHMGA2 decreased promoter activity to approximately 63%. In contrast to that, only minor changes in *ERCC1* promoter activities were observed for co-transfections with HMGA2/LPP. These promoter data are statistically significant ($p=0.039$ 3.9 kb promoter; $p=0.010$ basal promoter) for co-transfection experiments with wild-type HMGA2 and highly significant ($p=0.003039$; $p=2.9\times10^{-8}$) for C-terminally truncated ΔHMGA2.

As revealed by these experiments, the basal 426 bp *ERCC1* fragment had a 1.3-fold higher promoter activity with respect to the 3.9 kb fragment (data not shown) suggesting negative regulatory elements within the 3.5 kb 5' of the basal *ERCC1* promoter fragment.

Discussion

Although HMGA2 as well as its aberrant forms are thought to be implicated in the pathogenesis of benign mesenchymal tumors showing rearrangements of chromosomal region 12q14-15 (26) the exact mechanisms by which these proteins contribute to tumorigenesis are still unknown. It remains even unclear why wild type proteins of the HMGA family as well as their derivatives are similarly associated with the same tumor entities. Based on the results of cDNA expression array experiments we selected the *ERCC1* gene to analyze the mechanism of interaction of wild type HMGA2 and its derivatives to target DNA.

We were able to map a high affinity HMGA2 binding site to an AT-rich region located −323 to −298bp upstream of the *ERCC1* transcription start site. Despite their structural similarities results presented herein demonstrated clearly that HMGA2 protein has at least one order of magnitude higher affinity to this *ERCC1* promoter region than both of the HMGA1 proteins (HMGA1a and HMGA1b). These data are consistent with studies comparing DNA binding properties of various HMGA proteins showing that HMGA1 and HMGA2 might interact differently with the same DNA fragment (40). For example, HMGA1 contacts the *IFNβ* promoter using three AT-hooks, whereas binding of HMGA2 protein involves only two AT-hooks resulting in an approximately 8-fold lower affinity than HMGA1a and a ~2-fold lower affinity than HMGA1b to the *IFNβ* promoter (40, 43). Moreover, comparison of the binding pattern of HMGA2 with the *IFNβ* promoter (43) and the *ERCC1* promoter (this work) reveals that the same protein interacts differently with distinct DNA templates. Thus, our biochemical data strongly suggest that the *ERCC1* promoter harbors an HMGA2-specific binding site.

Despite to the opinion that the HMGA proteins bind unspecifically to stretches of AT-rich DNA we showed that this property alone is not sufficient. Even AT-stretches that are spaced in the appropriate distance (10-11bp from center to center) and would represent potential HMGA binding sites might be in fact not bound by the protein (this work and Schwanbeck, unpublished results). Unlike the sequence-specific binding of transcription factors the actual binding of architectural transcription factors like the HMGA proteins to certain DNA sequences is difficult to predict, and a generally valid algorithm has to be found for HMGA2, HMGA1a, and HMGA1b DNA binding with its gene regulating effects.

Binding studies for truncated ΔHMGA2 revealed that the derivative form of HMGA2 covers an extended region on the *ERCC1* promoter with the bottom strand region being much stronger protected than by wild-type HMGA2. Clear differences were also observed for the stoichiometry of binding to the *ERCC1* promoter. Whereas the wild-type protein formed only 1:1 complexes truncated protein

formed complexes with both a 1:1 and 2:1 protein to DNA ratio with a transition from a non-cooperative to a cooperative binding upon truncation of the protein. Although the nature of the complexes with the 1:1 stoichiometry for the wild-type and the truncated proteins were similar the second ΔHMGA2 molecule binds in a different manner covering a much larger region than the wild-type protein having a significant effect on DNA conformation. However, the affinity of binding was found to be independent of the presence of the acidic tail. The results presented herein confirmed the data described by Noro et al. (53) showing that the acidic C-tail is not involved in determining the specificity of HMGA2 DNA-binding in case of a high affinity HMGA binding sites. In contrast, differences in protein-DNA complexes were observed for low affinity binding sites upon C-terminal truncation of HMGA2 resulting in high molecular weight protein-DNA complexes similar to the 2:1 ΔHMGA2-DNA complexes described herein.

These differences in behavior of truncated ΔHMGA2 upon binding to the *ERCC1* promoter relatively to wild type protein were also seen for the luciferase promoter assays. Whereas luciferase assays showed that the activity of the *ERCC1* promoter is down-regulated by various HMGA2 proteins the most striking effect was exerted by the truncated ΔHMGA2. The differences in DNA-binding stoichiometry between normal and truncated HMGA2 correlate well with their different capabilities of repressing *ERCC1* promoter activity as measured by luciferase promoter assays. At first sight these results seem to be in disagreement with initial microarray experiments that predicted an up-regulation by HMGA2. However, taking into account that the cell lines used for microarray were different from those used in luciferase assays thereby containing a different set of transcription factors, it seems reasonable that by different interactions to the various transcriptions factors the regulation can be negative or positive depending on the environment, respectively. Furthermore, higher-order chromatin structure may also play a role in the gene-regulatory effect as well as the posttranslational modifications of HMGA2 that may control its activity and can be different in various cell lines.

In contrast to HMG box proteins that introduce sharp kinks into the DNA the alterations induced by HMGA proteins are more subtle (54) employing a reversal of intrinsically bend DNA. These slight changes that can be crucial to form a stereospecific 3-dimensional multiprotein complex are difficult to monitor. FRET or LRET analysis can be a very sensitive tool used to observe these slight changes in DNA bending as we demonstrated previously (40,55). We show in this work that the ΔHMGA2 protein is also able to alter the DNA conformation within the *ERCC1* promoter probably by the additional DNA contacts in the region –310 and –301 compared to the wild-type protein. Thus, it can be anticipated that binding of more than one ΔHMGA2 protein changes DNA conformation within the element to an extent affecting binding of transcription factors constituting potential *ERCC1* enhanceosomes.

The *ERCC1* gene that does not contain classical promoter elements like TATA or GC boxes (56) can possibly been down-regulated by HMGA2 and its aberrant forms by modulating the chromatin structure thus making the assembly and function of transcriptional complexes more difficult. A target of this environmental change could be the AP1 site that is located 48 bp upstream of the HMGA2 binding site. Furthermore, a comparative displacement of transcription factors by HMGA2 or their interactions can also be relevant in terms of gene expression. Sequence analysis of the HMGA2 binding site revealed putative binding sites for the transcription factors Pit-1a, TEC1, Elf-1, C/EBP, ICSBP, and ISGF-3. For the proteins Elf-1 and C/EBP physical interactions with HMGA1, another member of the HMGA family, have already been described (57,58). In further studies composition and assembly of the *ERCC1* enhanceosome upon intact and mutated HMGA2 proteins needs to be studied.

In terms of the mechanisms by which HMGA proteins contribute to tumorigenesis, a reactivated expression of either HMGA2 or its derivative forms as observed within several benign mesenchymal tumor entities (26) can decrease the expression of DNA-repair gene *ERCC1* leading to an altered genomic stability. Furthermore, as decreased ERCC1 level are responsible for a higher sensitivity of tumor cells to cisplatin-based chemotherapy (17) results presented herein showed that HMGA2 or at least ΔHMGA2 might be considered as therapeutic agent that could improve the effectiveness of cisplatin-based chemotherapy.

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Figure legends

FIG. 1. ***Identification of the high affinity HMGA2 binding site on the ERCC1 promoter.*** *A*, ≫l nM 32P-end-labeled fragments –426 to –257, –307 to –137, and –191 to –12 of the *ERCC1* promoter (top of this panel) were incubated with increasing concentrations of HMGA2 and electrophoresed on 6% polyacrylamide gels in low ionic strength buffer. The gels were dried, and radioactivity was scanned by a PhosphorImager. *B*, The end-labeled –426 to –257 fragment was partially digested with the Eam1104I restriction nuclease cutting the fragment between –351 and –350.

The digestion mixture was incubated with increasing concentrations of HMGA2 and analyzed as described in *A*. *C*, Comparison of the binding affinities of HMGA2, HMGA1b and HMG1a to the –426 to –257 *ERCC1* fragment using a more narrow protein concentration range as described in *A*. *D*, Quantification of the HMGA2 binding data of *C*. 100% – free DNA was plotted against the protein concentration on a logarithmic scale. The *line* represents the theoretical curve calculated from the relationship K_d = [100% – % free DNA] × [free protein]/[complexes] using SigmaPlot Hill regression. $K_{d(\text{app})}$ was 1.75 ± 0.31 nM for the –426 to –257 fragment.

FIG. 2. ***Footprinting of the HMGA2 on the end-labeled –426 to –257 fragment of the ERCC1 promoter.*** The *ERCC1* promoter DNA that was ^{32}P end-labeled at the 3' of the top strand was digested with hydroxyl-radicals in the absence (–) or presence (+) of 100 μM HMGA2. *A*, reaction products were separated on 8% acrylamide sequencing gels and dried gels were scanned by PhosphorImager. A *G+A* standard according to Maxam and Gilbert (57) is shown in the right lane. *B*, quantification of the DNA footprinting data shown in *A*. 100% cutting frequency corresponds to digestion of the DNA fragment in the absence of protein so that lower values mean protection upon HMGA2 binding. *Arrows* label the maxima of protection. The presented results are mean values from 3 independent experiments.

FIG. 3. ***Fine mapping of HMGA2 (black bars) and ΔHMGA2 (gray bars) binding to the –330 to –287 region of ERCC1 promoter fragment.*** *A*, either the top or the bottom strand was 5' end-labeled with T4 polynucleotide kinase and the double stranded DNA was digested with hydroxyl-radicals in the absence or presence of 100 nM of the proteins. Reaction products were separated on 18% acrylamide sequencing gels and dried gels were scanned by PhosphorImager. *B*, quantification of the DNA footprinting of the top strand (*top panel*) or bottom strand (*bottom panel*). Each bar shows relative cutting frequency at a single base. 100% cutting frequency corresponds to digestion of the DNA fragment in the absence of protein so that lower values mean protection upon protein binding. The presented results are mean values from 4 independent experiments. The sequence of the fragment is shown between the two panels. *Arrows* label the maxima of protection of the wild-type protein with the corresponding nucleotide number relative to the transcription start.

FIG. 4. ***Truncation of the HMGA2 affects protein binding to ERCC1 promoter.*** *A*, electrophoretic mobility shift assay. ≫1nM of the 32P-end-labeled –330 to –287 *ERCC1* fragment was incubated with increasing concentrations of HMGA2 and ΔHMGA2 and electrophoresed on 8% polyacrylamide gels in low ionic strength buffer. The gels were dried, and the radioactivity was scanned by PhosphorImager. In addition to the 1:1 complex (*complex #1*) a second complex is visible with ΔHMGA2 (*complex #2*) probably reflecting two protein molecules binding to the DNA fragment. *B*, quantification of the EMSA data of *A* using ImageQuant software and SigmaPlot Hill regression (see *Fig. 1D*). *C*, Methylation interference assay. The –330 to –287 fragment labeled at the 5' of the top strand was methylated with dimethyl sulfate. 500 nM modified double stranded DNA was incubated with 1 μM HMGA2 or ΔHMGA2, and the protein-DNA complexes were separated from unbound DNA by gel electrophoresis as shown in *A*, *ΔHMGA2*. The DNAs out of the complexes were eluted from the gels and cleaved at methylated purines with piperidine. Finally, equal amounts of radioactivity (~5000 cpm) of the cleavage products were analyzed on 18% acrylamide sequencing gels. *D*, quantitative analysis of the methylation interference experiment of *C*. The gels were scanned and the data analyzed as described in *Material and Methods*. Negative values indicate binding interference upon the methylation of the corresponding nucleotide. *Black*, *grey*, and *crisscrossed bars* refer to protein to DNA complexes 1:1 of HMGA2, 1:1 of ΔHMG2, and 2:1 of ΔHMGA, respectively. *Arrow* indicates the maximum of interference of the central binding site and the corresponding *number* represents the distance relative to the transcription start.

FIG. 5. ***Perturbation of DNA ERCC1 promoter conformation by HMGA2 and its mutants.*** A series of DNA constructs comprising promoter region –316 to –294 were produced either with inserted luminescence *donor* [(Eu3+)DTPA-AMCA-maleimide at the 3' of the top strand (*A* – *C*) or at the bottom strand (*D* – *F*) and the fluorescence acceptor (*X*) Cy5 at different positions within the complementary strand, respectively. The LRET measurements were performed in the absence or

presence of 12.5 nM, 37 nM, and 100nM of HMGA2 (triangles), ΔHMGA2 (circles), and HMG2/LPP (squares). The concentration of labeled duplex was 15 nM.

FIG. 6. ***Model of the ΔHMGA2 induced conformational changes on the ERCC1 promoter according to the EMSA and LRET experiments in Fig. 4 and 5.*** The promoter region contains a prebent element (left side). Upon binding of two molecules ΔHMGA2 this prebending is reversed whereas on the other end of the promoter DNA a bending is introduced.

FIG. 7. ***Activity of the ERCC1 promoter is affected by HMGA2, HMGA2/LPP, and ΔHMGA2.*** *ERCC1* promoter fragments –3900 to +1 and –425 to +1 relatively to the transcriptional start site were cloned in luciferase reporter-gene vector pGL3-Basic. These promoter constructs were transient co-transfected with a vector expressing either no HMGA2 (reference vector), wild-type HMGA2, chimeric HMGA2/LPP, or C-terminally truncated ΔHMGA2 and vector pRL-Tk used for normalization. Results are the mean value of several independent experiments performed within HeLa cells. The change in promoter activity of the 3.9 kb and 426bp *ERCC1* promoter fragments in dependence of different HMGA2 protein variants are shown relatively to the data obtained for the corresponding ERCC1 construct co-transfected with the reference vector. *Black*, *light grey*, and *dark grey bars* refer to co-transfection with wild-type HMGA2, chimeric HMGA2/LPP, and truncated ΔHMG2, respectively.

Footnotes

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1 Abbreviations used: NER, nucleotide excision repair; ERCC1, excision repair cross-complementing rodent repair deficiency, complementation group 1; HMGA2, high mobility group A2 protein; ΔHMGA, C-terminally truncated HMGA2; HMGA2/LPP, fusion of HMGA2 and lipoma preferred partner (LPP)

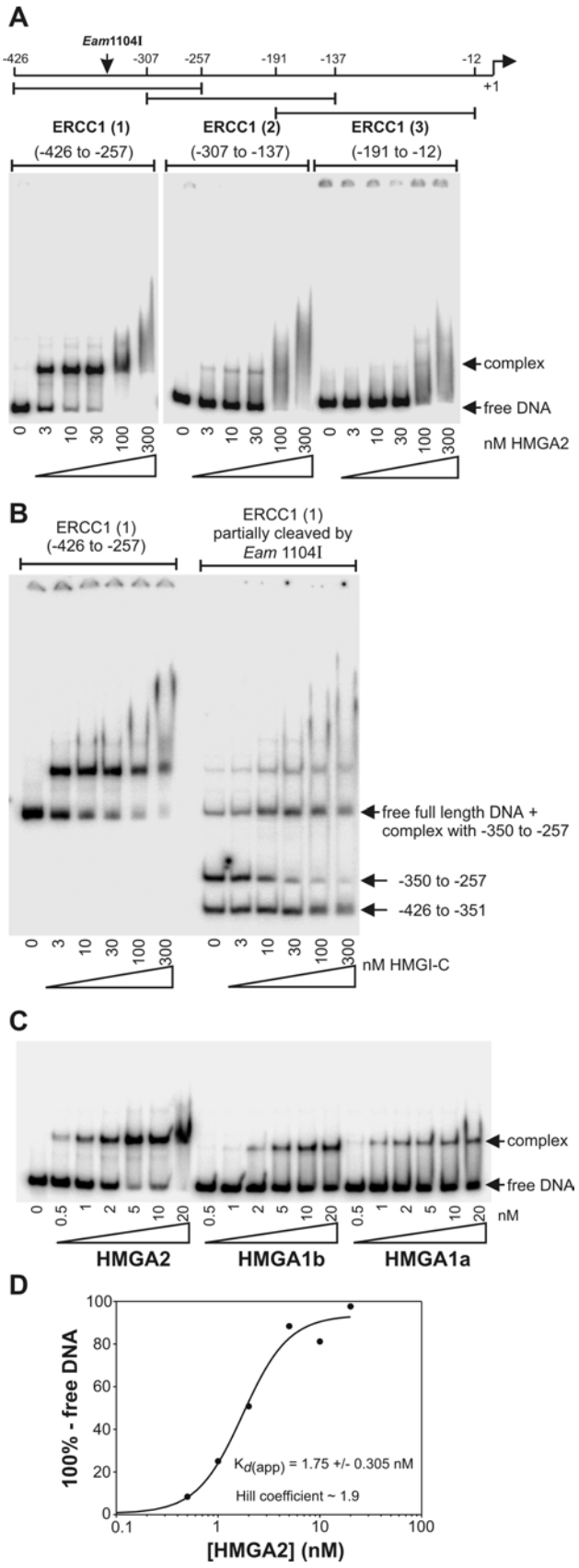
A
Eam1104I
-426
-307
-257
-191
-137
-12
+1
ERCC1 (1)
(-426 to -257)
ERCC1 (2)
(-307 to -137)
ERCC1 (3)
(-191 to -12)
complex
free DNA
0 3 10 30 100 300
nM HMGA2
B
ERCC1 (1)
(-426 to -257)
ERCC1 (1)
partially cleaved by
Eam 1104I
free full length DNA +
complex with -350 to -257
-350 to -257
-426 to -351
0 3 10 30 100 300
nM HMGI-C
C
complex
free DNA
0 0.5 1 2 5 10 20
nM
HMGA2
HMGA1b
HMGA1a
D
100% - free DNA
100
80
60
40
20
0
0.1
1
10
100
Kd(app) = 1.75 +/- 0.305 nM
Hill coefficient ~ 1.9
[HMGA2] (nM)

Fig. 1

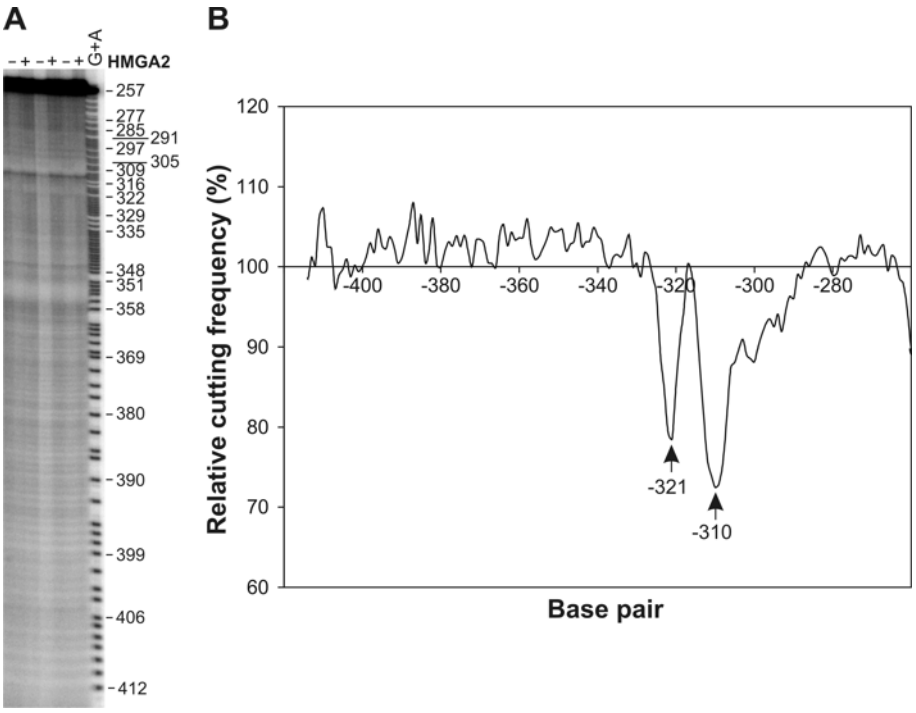
A
B
HMGA2
G+A
257
277
285
291
297
305
309
316
322
329
335
348
351
358
369
380
390
399
406
412
120
110
100
90
80
70
60
Relative cutting frequency (%)
-400
-380
-360
-340
-320
-300
-280
-321
-310
Base pair

Fig. 2

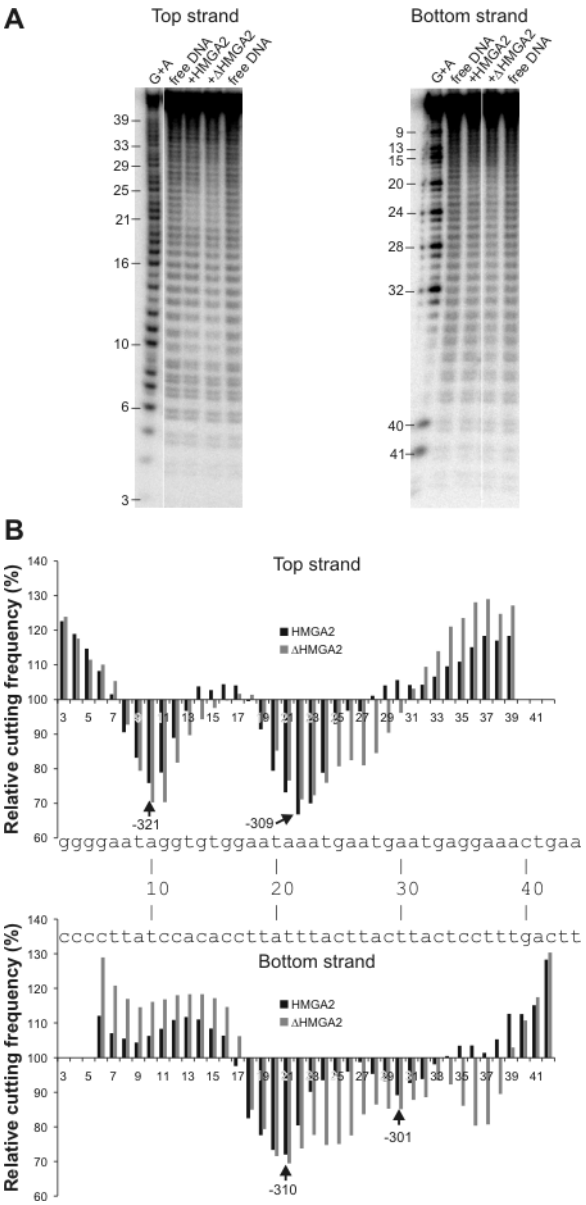
A
Top strand
Bottom strand
G+A
free DNA
+HMGA2
+ΔHMGA2
free DNA
39
33
29
25
21
16
10
6
3
9
13
15
20
24
28
32
40
41
B
Top strand
Relative cutting frequency (%)
HMGA2
ΔHMGA2
-321
-309
ggggaataggtgtggaataaatgaatgaatgaggaaactgaa
10
20
30
40
ccccttatccacaccttatttacttacttactcctttgactt
Bottom strand
-301
-310

Fig. 3

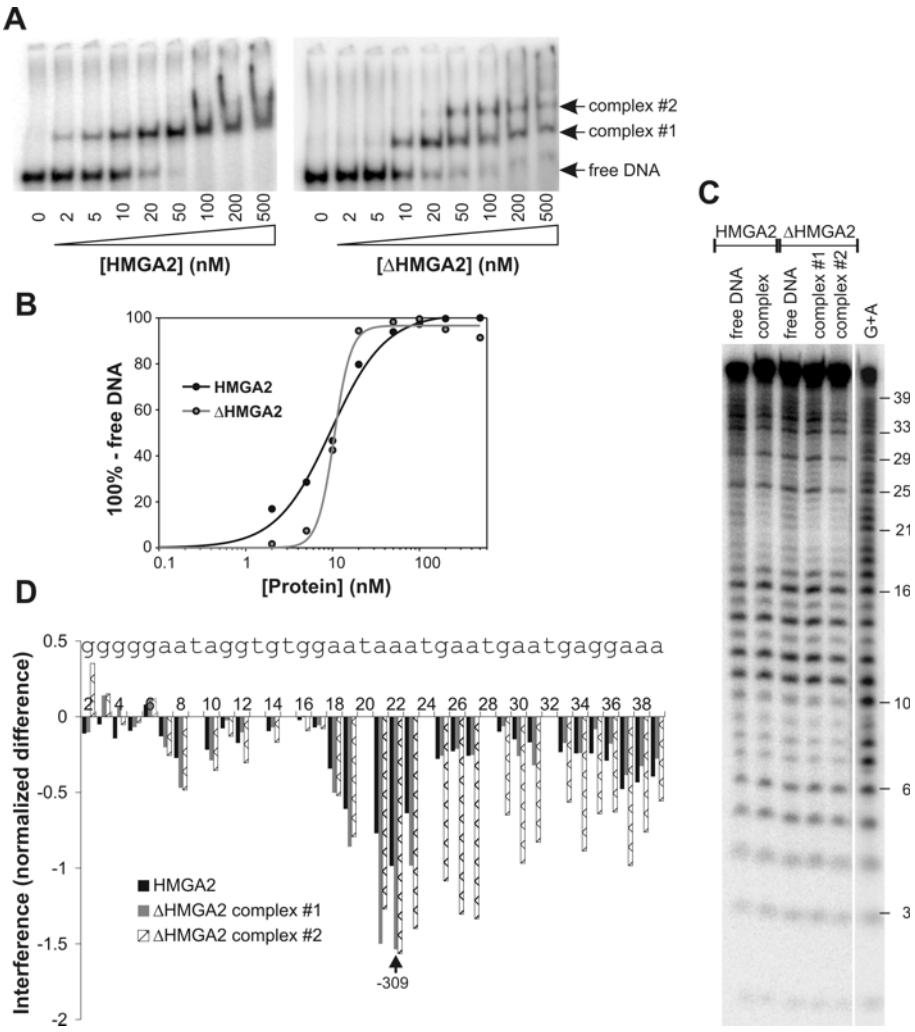
A
complex #2
complex #1
free DNA
0 2 5 10 20 50 100 200 500
[HMGA2] (nM)
0 2 5 10 20 50 100 200 500
[ΔHMGA2] (nM)
B
100% - free DNA
HMGA2
ΔHMGA2
[Protein] (nM)
C
HMGA2 ΔHMGA2
free DNA
complex
free DNA
complex #1
complex #2
G+A
39
33
29
25
21
16
10
6
3
D
gggggaataggtgtggaataaatgaatgaatgaggaaa
Interference (normalized difference)
HMGA2
ΔHMGA2 complex #1
ΔHMGA2 complex #2
-309

Fig. 4

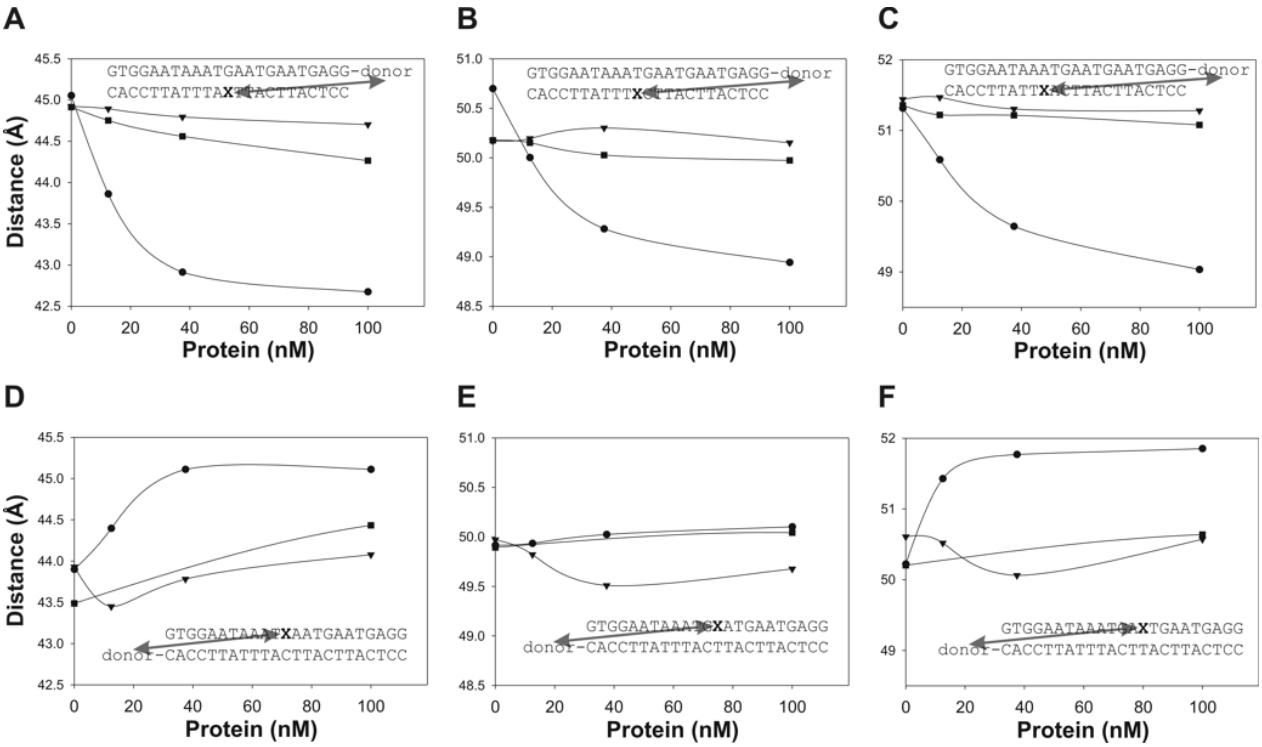
A
B
C
D
E
F
Distance (Å)
Protein (nM)
GTGGAATAAATGAATGAATGAGG-donor
CACCTTATTTAXTTACTTACTCC
donor-CACCTTATTTACTTACTTACTCC

Fig.5

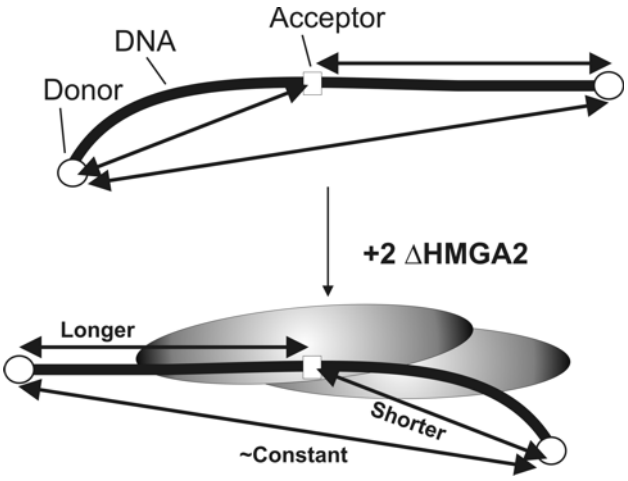
DNA
Acceptor
Donor
+2 ΔHMGA2
Longer
Shorter
~Constant

Fig. 6

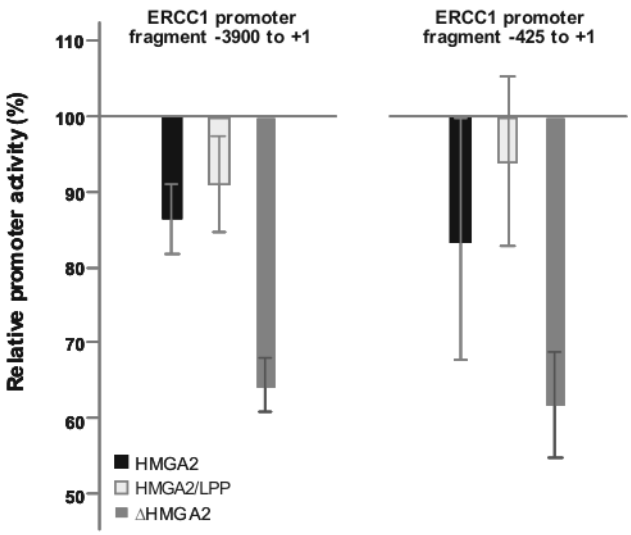
ERCC1 promoter fragment -3900 to +1
ERCC1 promoter fragment -425 to +1
Relative promoter activity (%)
110
100
90
80
70
60
50
HMGA2
HMGA2/LPP
ΔHMGA2

Fig. 7

III.

BORRMANN L, SEEBECK B, ROGALLA P, BULLERDIEK J. (2003). Human *HMGA2* promoter is coregulated by a polymorphic dinucleotide (TC)-repeat. *Oncogene*, 22: 756-760.

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- Betreuung der wissenschaftlichen Arbeit bei der Planung, Durchführung und Auswertung
- Verfassen der Publikation in Zusammenarbeit mit von Frau B. Seebeck

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Human *HMGA2* promoter is coregulated by a polymorphic dinucleotide (TC)-repeat

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HMGA proteins are thought to be causally involved in the progression of different diseases, including benign and malignant tumors, obesity, arteriosclerosis, and restenosis. As HMGA proteins are architectural transcription factors, their binding to DNA leads to changes in DNA-conformation modulating the environment for the assembly and function of transcriptional complexes, thus influencing the expression of a huge variety of genes. Despite the emerging role of HMGA proteins for important diseases, only limited information is available about mechanisms regulating the expression of the *HMGA2* gene. In this report, 2240 bp of the 5′ flanking region of the *HMGA2* gene were functionally analyzed by luciferase assay experiments. Besides the identification of novel positive and negative regulatory elements, it was shown that transcription is initiated from two independent promoter regions within cell lines HeLa, MCF7, and L14TSV40. Furthermore, a functional polymorphic dinucleotide repeat (TCTCT(TC)$_n$) 500 bp upstream of the ATG translational start codon was found to regulate strongly the human *HMGA2* promoter with an activation pattern that correlates to its TC-repeat length.
Oncogene (2003) **22,** 756–760. doi:10.1038/sj.onc.1206073

Keywords: high-mobility group proteins; HMGA; promoter analysis; cancer; obesity; arteriosclerosis

Introduction

The HMGA2 (syn.: HMGIC) protein belongs to the high-mobility group (HMG) family of nonhistone chromatin proteins that act as architectural transcription factors (for a review, see Bustin and Reeves, 1996). HMGA2, whose gene maps to 12q14–15, consists of three DNA-binding domains designated as AT-hooks, enabling its binding to the minor groove of AT-rich DNA, and an acidic C-tail responsible for protein–protein interactions (Solomon *et al*., 1986; Chau *et al*., 1995). The interaction of HMGA2 with DNA leads to changes in DNA-conformation modulating the environment for the assembly and function of transcriptional complexes, thus influencing the expression of a huge variety of genes.

Whereas HMGA2 is expressed at very high levels during embryonic development, it is almost undetectable in differentiated cells (Chiappetta *et al*., 1996; Rogalla *et al*., 1996). The embryonic expression of HMGA2 has been studied in detail in mice (Hirning-Folz *et al*., 1998). At an early embryonic stage, HMGA2 mRNA is found in all tissues, whereas the pattern of expression is restricted particularly to mesenchymal derivatives, some epithelial cell layers, and parts of the central nervous system in the second half of embryonic development. Furthermore, the role of HMGA2 in adipogenesis has emerged based on the observations that *HMGA2* knockout mice show a pygmy phenotype with a characteristic reduction of body weight, mainly affecting fat tissue (Zhou *et al*., 1995), and that transgenic mice carrying a truncated *HMGA2* gene lacking the acidic C-tail develop a giant phenotype and predominantly abdominal and pelvic lipomatosis (Battista *et al*., 1999). Moreover, knockout mice lacking *HMGA2* resisted diet-induced obesity (Anand and Chada, 2000). A reactivation of *HMGA* correlates with the formation of arteriosclerotic plaques and aortic restenosis (Chin *et al*., 1999) as well as the development of malignant (Tamimi *et al*., 1996) and benign tumors (e.g. uterine leiomyomas) (Ashar *et al*., 1995; Schoenmakers *et al*., 1995; Kazmierczak *et al*., 1996; Scala *et al*., 2000).

So far, a restricted number of investigations have aimed at the analysis of the *HMGA2* promoter in humans and mice (Ayoubi *et al*., 1999; Chau *et al*., 1999; Rustighi *et al*., 1999, 2002), showing multiple transcription initiation sites as observed in many TATA-less promoters. Herein, we performed luciferase reporter assays in both HMGA2 positive and negative tumor cell lines to identify novel sequences influencing *HMGA2* promoter activity.

Based on previously published data (Ashar *et al*., 1996; Ayoubi *et al*., 1999; Chau *et al*., 1999; Rustighi *et al*., 1999) and sequence alignments (e.g., gb-accession-no.: NM_003483; Z31595; U28749), two regions of main transcription initiation (RTI) could be determined ranging from nucleotide (nt) −161 to −201 (proximal RTI (pRTI)) and nt −790 to −893 (distal RTI (dRTI)) relative to the ATG translational start codon of human

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HMGA2. Based on these analyses, several overlapping promoter fragments spanning the region nt −1 to −2240 were cloned upstream of a luciferase reporter gene and were analyzed for promoter activity in three different human cell lines (HeLa, MCF7, and L14TSV40). Of these clones, construct P5 (region −1418 to −1) spanning the distal and proximal region of transcription initiation (pRTI and dRTI) showed the highest promoter activity in all cell lines tested and was set at 100% relative promoter activity for comparative analyses (Figure 1a). Clones P2 and P7 spanning pRTI and dRTI, respectively, showed promoter activity in all three cell lines tested, thus revealing the presence of at least two independent functional gene promoters that are capable of inducing *HMGA2* transcription on their own. These data are consistent with the sequence alignments described above showing that *HMGA2* gene transcription is initiated at two different regions (pRTI and dRTI), and previous experiments in the hepatoma cell line PLC/PRF/5 indicating that *HMGA2* gene transcription might be initiated from two independent transcription initiation sites (Chau *et al*., 1999).

Furthermore, we performed luciferase experiments with deletion mutants (clones P7–P10) of the distal promoter (Figure 1a). Although only minor changes were measured by these experiments, the significant differences between clones P7–P10 led to the identification of negative regulatory elements (region −1194 to −1717), elements that are regulated in a cell-type-specific manner (region −1718 to −2097), and a positive regulatory element 2098–2240 bp upstream of the *HMGA2* ATG start codon. Furthermore, these experiments showed that the distal promoter element (dRTI, clone P7) showed a remarkably high promoter activity in cell line L14TSV40 relative to HeLa and MCF7, suggesting that the variations in promoter activity

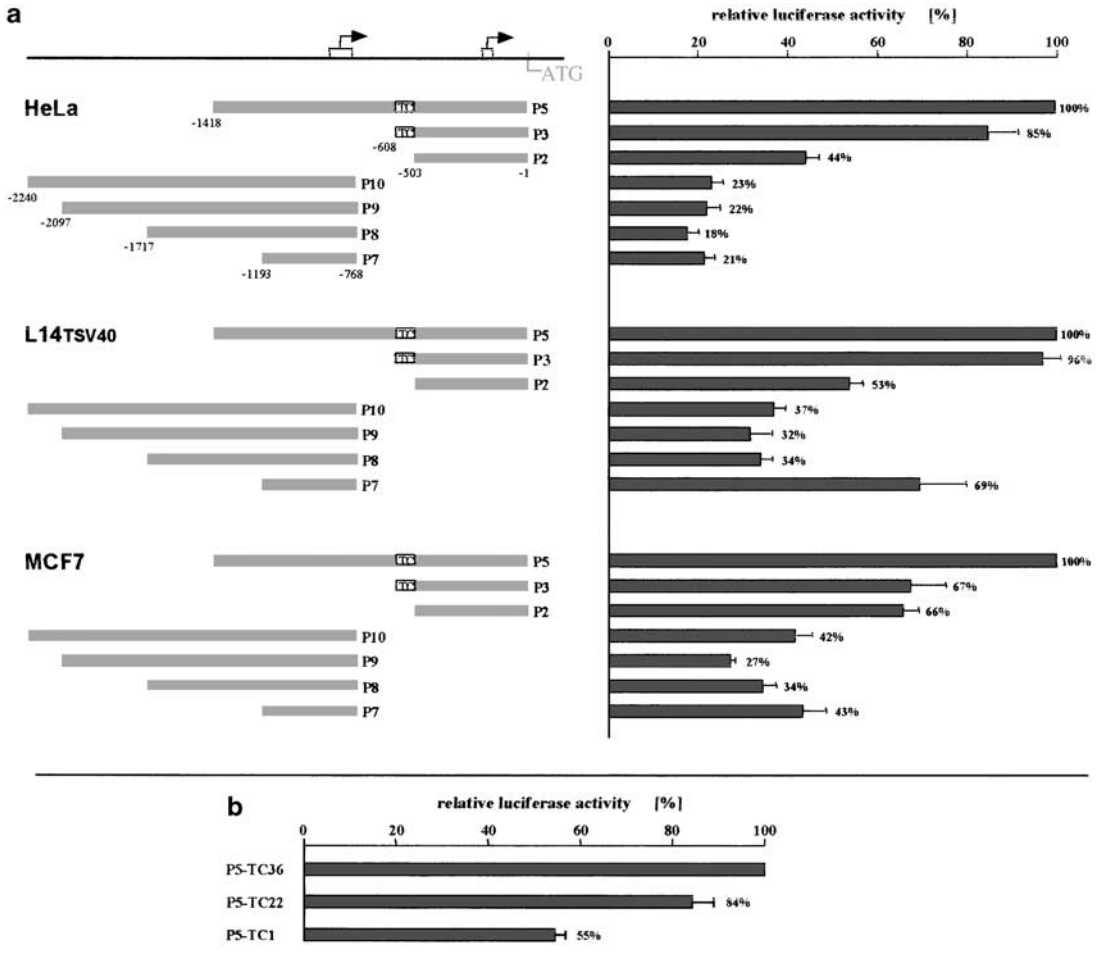

Figure 1 Characterization of the different *HMGA2* promoter fragments and their relative luciferase activity as revealed by luciferase assays. (**a**) *Left side*: schematic presentation of the region 5′ of the ATG translational start codon of human *HMGA2*. Proximal and distal regions of transcription initiation are indicated by arrows. Different promoter fragments used for luciferase experiments are shown corresponding to the experiments performed in cell lines HeLa, L14TSV40, and MCF7. Positions of the fragments relative to ATG are displayed for experiments performed in HeLa. The location of the TC-repeat ($(TC)_{36}$) is highlighted by the rectangles lettered with TC. *Right side*: diagram of relative luciferase activities obtained by the promoter fragments presented on the left side. Assays were performed in cotransfection with vector pRL-Tk as internal control for data normalization. All data are related to the activity obtained by promoter clone P5. Results are a summary of at least three independent luciferase assays per cell line. (**b**) Functional analysis of the polymorphic TC-repeat upstream of *HMGA2* within cell line L14TSV40. Relative luciferase activities of different clones of fragment P5 containing TC-repeats of 36, 22, and one TC-dinucleotides in length. All data are related to the activity obtained by promoter clone P5

underline cell-type-specific activation mechanisms because of different transcription factor settings in the analyzed cell lines.

As the most interesting result, luciferase experiments with promoter construct P3 containing a TCTCT(TC)$_{36}$-repeat at its 5′ end almost doubled the promoter activity in comparison to the construct P2 lacking the TCTCT(TC)$_{36}$-repeat and 40 bp upstream of it in a highly significant manner for HeLa ($P<0.01$) and L14TSV40 cells ($P<0.01$). In contrast, only slight activity difference between the two constructs was detected in MCF7 cells. To finally prove that the TC-repeat coregulates the promoter activity, we generated two clones of fragment P5 containing one (TC$_1$) and 22 (TC$_{22}$) TC-dinucleotides, respectively. Luciferase experiments performed within cell line L14TSV40 showed a significant ($P<0.05$) reduction in activity of about 45% of clone P5-TC$_1$ relative to clone P5 containing a TC$_{36}$-repeat (Figure 1b). These data are consistent with the results obtained for clone P3 in relation to P2 within L14TSV40 (Figure 1a). Furthermore, although clone P5-TC$_{22}$ had only 84% promoter activity compared with clone P5-TC$_{36}$, this clone showed a significant ($P=0.05$) 1.5-fold higher activity when compared to clone P5-TC$_1$. These data indicate that the TC-repeat contributes to the transcriptional activation of *HMGA2*. Moreover, by performing sequence analysis of this TC-repeat from 20 unrelated, apparently normal individuals, we could identify a length polymorphism ranging between 22 and 36 dinucleotide copies. These findings are consistent with previously described studies revealing dinucleotide repeat variations between 18 and 37 copies (Ishwad *et al*., 1997). In contrast to previous publications (Patel *et al*., 1994) and sequence databases (L41044; U28750) showing a TCTCT(TC)$_n$TG(TC)$_n$-repeat, our sequencing approach of several individuals did not verify the intermediate TG dinucleotide, and accordingly the repeat motif was described as TCTCT(TC)$_n$.

Interestingly, we determined promoter activity in both *HMGA2* mRNA positive (MCF7, L14TSV40) and negative (HeLa) tumor cell lines consistently with previously published data (Ayoubi *et al*., 1999), also showing an active promoter in *HMGA2* mRNA positive and negative cell lines. These results suggest that beyond a constitutive active promoter, there have to be negative regulatory elements that were not present in the 5′ flanking regions analyzed so far controlling the *HMGA2* expression. One of those regulatory elements could be located within the 3′UTR of *HMGA2* because it was shown that *HMGA2* expression depends on the length of the 3′UTR (Borrmann *et al*., 2001), suggesting expressional control at the post-transcriptional level.

To find out more about regulatory mechanisms of the *HMGA2* gene in this report, a series of functional promoter assays with different fragments of its promoter region were performed. Besides the confirmation of at least two independent promoter regions explaining the main cluster of transcription initiation, the existence of novel regulatory elements was identified and the polymorphic dinucleotide repeat (TCTCT(TC)$_n$) 502 bp upstream of the ATG was found to be a strong positive regulator of the *HMGA2* promoter. Furthermore, this is the first paper reporting on TCTCT(TC)$_n$ as the only sequence motif in all analyzed cases, in contrast to previous published sequences showing an internal TG dinucleotide element. The effect of this repeat element on promoter activity could probably be explained by the formation of non-B-form-DNA-conformations that are involved in transcription. For example, as described in the literature, (dT-dC)$_n$ repeats are able to adopt a hinged DNA structure (H-DNA), which is composed of triple-stranded and single-stranded regions (Htun and Dahlberg, 1988, 1989). Moreover, those polypyrimidine repetitive sequences capable of forming H-DNA are known to occur with high frequency in eucaryotic genomes, particularly around promoter regions, and have been shown to be involved in chromatin structure formation (Lu *et al*., 1993; Espinás *et al*., 1996) and transcriptional activation (Michel *et al*., 1992; Firulli *et al*., 1994; Kim *et al*., 1998). In addition to the results presented herein, an evolutionary conserved polypyrimidine/polypurine tract (nt −235 to −197; L41044) that is also a positive regulatory element on the human (Chau *et al*., 1999) and murine (Rustighi *et al*., 1999) *HMGA2* promoter was recently described to adopt non-B-DNA-conformations, such as triple-helical H-DNA *in vitro* (Rustighi *et al*., 2002).

As the most interesting result, we were able to show not only that the polymorphic dinucleotide repeat (TCTCT(TC)$_n$) is a strongly positive regulator of the *HMGA2* promoter but also that different repeat lengths contribute to clear differences in *HMGA2* promoter activity. These results offer strong evidence that the length of the polymorphic TCTCT(TC)$_n$ element is a crucial factor for *HMGA2* transcriptional activity, thus implicating an individual or ethnical predisposition for certain disorders such as arteriosclerosis, restenosis, and obesity in which HMGA2 dysregulation is involved. Furthermore, these data may give a first explanation for the much higher incidence of uterine leiomyomas in African Americans compared to Caucasians (Meilahn *et al*., 1989), as there are clear ethnical differences in allelic frequency of different TCTCT(TC)$_n$-repeat lengths between African Americans and Caucasians (Ishwad *et al*., 1997).

Nevertheless, further experiments need to be done to give a more detailed view on the mechanisms of transcriptional activation exerted by the TCTCT(TC)$_n$-repeat. Promoter clones of different TCTCT(TC)$_n$-repeat lengths are under construction and will be analyzed.

Material and methods

PCR amplification of HMGA2 promoter elements

For amplification of *HMGA2* promoter fragments, HindIII-primer 5′-CCCAAGCTTCTGCCCGGGCTG-GAAGTTTTC-3′ was used in combination with *Bgl*II-primer 5′-GGAAGATCTAGGACAACTGCCCAGG-GGTGAC-3′ (clone P7, nt 1749–2172, L41044),

5′-GGAAGATCTCCTTTACCCTGGGAACATCGG-ATTC-3′ (P8, nt 1224–2172), 5′-GGAAGATCT-ACTCCTGCTTAGGGCTGCAAAGTAGG-3′, (P9, nt 844–2172), and 5′-GGAAGATCTCAACGTGCAG-CAAAGTGTCCCA-3′ (P10, nt 701–2172). *Bgl*II-primer 5′-GAAGATCTTCCCTGCCTCCCGCCGCCG-CTACC-3′ was used in combination with *KpnI*-primer 5′-GGGGTACCCCTCGCAGGGTGGGGGGAAGA-GGA-3′ (P2, nt 2438–2940), 5′-GGGGTACCCCAT-CCTCCTTTGCTTTCCGACTGC-3[1] (P3, nt 2333–2940), and 5′-GGGGTACCCCCGCCCGCCCGCAG-GCTCTGTGG-3′, (P5, nt 1523–2940). For clone P5-TC_1 two fragments were amplified using pGL3-vector primer 5′-CTAGCAAAATAGGCTGTCCC-3′ and 5′-CTTTATGTTTTTGGCGTCTTCC-3′ in combination with primer 5′-AGAGATTGAGATTGAAAGTGC-3′ and 5′-TCGCAGGGTGGGGGGAAGAGGA-3′, respectively. PCR reactions were performed with *pfu* polymerase in a Mastercycler gradient (Eppendorf, Hamburg, Germany) using the following protocol: 5 min 94°C, (1 min 94°C, 1 min 60–64°C, 1 min 72°C) $\times$ 35, 10 min 72°C. As template, a cosmid spanning the *HMGA2* promoter region was used. Clone P5 was used as a template for the amplification of P5-TC_1 fragments. For P5-TC_{22}, genomic DNA of a person carrying a TC_{22} allele was used as a template for PCR.

Plasmid constructions, cell culture, and promoter assay

The amplified promoter fragments were cloned into the *Hind*III-*Bgl*II or *Kpn*I-*Bgl*II sites of pGL3-Basic vector (Promega, Madison, USA). P5-TC_1 PCR fragments were cut with *Kpn*I and *Bgl*II, blunt end ligated, and afterwards cloned into the *Kpn*I-*Bgl*II site of pGL3-Basic vector. Plasmid DNA was isolated using the Plasmid Isolation Kit (Qiagen, Hilden, Germany) following the instructions of the manufacturer. Clones were verified by sequencing.

Cell culture of cell lines HeLa (cervix carcinoma), MCF7 (breast cancer), and L14TSV40 (lipoma, having a *t*(3; 12)(q27; q14) translocation involving *HMGA2*) as well as promoter assay experiments were performed as described elsewhere, with some minor modifications (Borrmann *et al*., 2001). Briefly, luciferase activities were measured after 48 h, and the experiments for each construct were performed in duplicate and repeated three times. For statistical analysis, a one-sample *t*-test was performed, with $P \leq 0.05$ for significant and $P \leq 0.01$ for highly significant differences.

Detection of polymorphic CT-repeat

DNA of 20 unrelated, normal individuals was isolated by the salt extraction method. A PCR spanning the CT repeat was performed with primer 5′-ATCCTCCTTTGCTTTCCGACTGC-3′ and 5′-AC-CTCATCTCCCGAAAGGTGCTG-3′ using Hot Star Taq (Qiagen, Hilden, Germany) in a Mastercycler gradient (Eppendorf, Hamburg, Germany) by the following protocol: 15 min 95°C, (1 min 94°C, 1 min 66°C, 1 min 72°C) $\times$ 35, 10 min 72°C. For analysis of polymorphic variants, PCRs were separated on 4% small DNA agarose gel (Biozym, Hessisch Oldendorf, Germany), fragments were isolated from gel using QIAEX II (Qiagen, Hilden, Germany), cloned into pGEM T-easy (Promega, Madison, USA), and analyzed by sequencing with a special protocol for repetitive sequences (Seqlab, Göttingen, Germany).

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IV.

FLOHR AM, ROGALLA P, MEIBOOM M, BORRMANN L, KROHN M, THODE-HALLE B, BULLERDIEK J. (2001). Variation of *HMGB1* expression in breast cancer. *Anticancer Research*, 21: 3881-3886.

Eigenanteil an dieser Publikation:

- Durchführung der radioaktiven Northern Blot Hybridisierungen

Variation of *HMGB1* Expression in Breast Cancer

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Abstract. *The amount of steroid hormone receptor proteins does not always correlate with the response of breast cancers to endocrine therapy. This may partly be due to the fact that binding of the estrogen receptor (ER) to estrogen responsive elements (ERE) of its target genes is mediated by additional cellular proteins. One of these is the high mobility group protein HMGB1, known to interact with ER thus dramatically increasing its binding to ERE. This is the first report analysing the expression patterns of HMGB1 in breast cancer cells. Northern blot analyses of the 1.4 kb and the 2.4 kb transcripts of HMGB1 in 13 breast cancer samples revealed a strong intertumoural variation by a factor of 8.5 and 14.5, respectively. This variation may contribute to the different response of estrogen receptor- positive breast tumours to endocrine therapy, making HMGB1 a marker of considerable clinical interest.*

Breast cancer remains one of the main causes of death for women in Western countries, with a lifetime risk of developing this tumour of 12.2% and a lifetime risk of death of 3.6% (1). Accordingly, considerable effort has been put into establishing and improving prognostic and predictive molecular markers and therapeutical concepts for breast cancer.

The main prognostic and predictive markers determined in breast cancer tissue are the steroid receptor levels, mutations of *p53*, amplification of *HER2,* proportion of S-phase cells and ploidy. However, at present steroid receptors are the only parameters widely accepted in standard practice (1). The determination of steroid receptors has both prognostic and predictive impact in breast cancer. Untreated patients with newly-diagnosed estrogen receptor (ER)-negative breast cancer have a higher risk of relapse than ER-positive patients presenting a similar stage (1). In adjuvant therapy, the anti-estrogen tamoxifen reduces the rate of both recurrence and mortality in women with early breast cancer (2).

However, despite the clinical significance of ER there is no complete correlation of the hormone receptor status with the response of breast cancers to endocrine therapy. Thus the molecular characterization of co-factors influencing the binding of steroid hormone receptors to hormone responsive elements of their target genes is of high potential interest.

The high mobility group protein HMGB1 is one of the proteins which have been shown to interact with steroid hormone receptors, thus dramatically enhancing their binding to estrogen and progesterone responsive elements (3-5). Nevertheless, nothing is yet known about a possible variation of *HMGB1* expression in hormone dependent tumours, *e.g.* breast cancer samples. To address the possible role of the HMGB1 protein in hormone-dependent growth, this paper is aimed at the quantitation of *HMGB1* expression in a series of 13 breast cancer samples.

Materials and Methods

Tissue samples. Breast cancer samples taken directly after surgery were (after pathological examination) immediately frozen in liquid nitrogen and stored at –80°C. Histological subtypes were determined in accordance with Rosen and Oberman (6), TNM and grading in accordance with Sobin and Wittekind (7), while steroid hormone receptor status was determined as described by Remmele and Stegner (8). Adjacent nonmalignant tissue was not analysed because it was either predominantly composed of adipocytes or showed the typical admixture of stromal and epithelial cells. For each tumour sample the percentage of the stromal component was roughly estimated by microscopical analysis of the tissue section.

Northern blot hybridisation. Total RNA extraction of the breast cancer samples was performed with the acid guanidine isothiocyanate-chloroform method using the Trizol reagent (Life Technologies, Eggenstein, Germany), following the manufacturer's instruction. For Northern blot hybridisation 20 µg total RNA of each sample was separated on a 1.2% denaturing agarose gel containing 0.65% formaldehyde. RNAs were transferred onto Hybond-N+ -positive nylon membrane (Amersham Pharmacia Biotech, Buckinghamshire, England) by vacuum blot. A 550 bp cDNA fragment derived from the 3´UTR of *HMGB1* served as a molecular probe for hybridisation and was generated by PCR with a primer set (*H1up* and *L1do*) as described

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Key Words: HMGB1 expression, high mobility group protein B1, breast cancer, estrogen receptor, endocrine therapy.

Table I. *Breast cancer samples analysed in this study.*

Breast cancer sample	Age	Histological type	Grading	TNM	ER status	PR status	Stromal component
BrCr 1	76	i.d.	3	pT2/pN0	6	9	50%
BrCr 2	52	i.d.	2	pT2/pN0	12	8	30%
BrCr 3	74	i.d.	3	pT2/pN1bii	0	0	50%
BrCr 4	84	i.d.	3	pT1c/pN1b	6	12	80%
BrCr 5	76	i.d.	2	pT1c/pN0	6	0	40%
BrCr 6	41	i.l.	2	pT1c/pN1a	8	8	15%
BrCr 7	59	i.l.	2	pT1c/pN0	6	0	30%
BrCr 8	74	i.d.	2	pT2/pN1bi	9	9	30%
BrCr 9	71	i.d.	2	pT2/pN0	12	0	40%
BrCr 10	78	i.d.	3	pT4c/pN2	0	0	10%
BrCr 11	76	i.l.	2	pT1c/pN0	6	0	80%
BrCr 12	60	i.l.	2	pT2/pN0	4	0	40%
BrCr 13	39	i.d.	3	pT2/pN0	4	1	20%

Histological type (i.d. = invasive ductal; i.l. = invasive lobular), histological grading (according to Rosen and Oberman (6)), TNM (in accordance to Sobin and Wittekind (7)), hormone receptor status (ER = estrogen receptor; PR = progesterone receptor; IRS score according to Remmele and Stegner (8)) and stromal component of breast cancer samples analysed in this study. The content of stromal cells was roughly estimated by microscopical analysis.

previously (9). A 299 bp cDNA probe detecting the 1.3 kb transcript of *GAPDH* was generated with a primer set (*GAPDH2* and *GAPDH3*) as described previously (10). The probes were labeled with ^{32}P using a random primer extension protocol (11). Employing the ExpressHyb hybridisation solution (Clontech Laboratories, Palo Alto, USA), prehybridisation was carried out for 30 minutes and hybridisation for 1 hour at 68°C. The membranes were washed twice for 20 min at room temperature in 2 x SSC / 0.05% SDS, and twice for 20 minutes at 68°C in 0.1 x SSC / 0.1% SDS. Signals were visualized by using a STORM imager (Molecular Dynamics, Sunnyvale, USA). Quantitation of the 1.4 and 2.4 kb band of *HMGB1* and the 1.3 kb band of *GAPDH* was performed using the software programme ImageQuant (Molecular Dynamics, Sunnyvale, USA). The relative *HMGB1* expression of the 1.4 and 2.4 kb transcript, respectively, was determined by the ratio of *HMGB1*-RNA to *GAPDH*-RNA.

Results

We performed Northern blot hybridisation on a subset of 13 breast cancer samples of different histological subtypes and grading (Table I) using a cDNA probe derived from the 3'UTR of *HMGB1*. In all breast cancer samples examined, we were able to detect two *HMGB1* mRNA species of approximately 1.4 and 2.4 kb (Figure 1a) which have been described previously (12-14). In order to quantify the expression of *HMGB1*, membranes were co-hybridised with a *GAPDH* specific cDNA probe (Figure 1b) and the ratios of *HMGB1*-RNA / *GAPDH*-RNA were calculated.

The 13 breast cancer samples analysed revealed a strong intertumoural variation in the expression of the 1.4 kb and 2.4 kb transcripts of *HMGB1*, respectively (Figure 2). Whereas expression of the 1.4 kb transcript varied by a factor of 8.5, the expression of the 2.4 kb transcript showed a 14.5-fold variation (Figure 2). Statistical analysis revealed a quantitative correlation between both transcripts ($k=0.94$; $p>0.001$) as would be expected because both transcripts are under the control of the same promotor (15). On average, *HMGB1* expression signals of the 1.4 kb transcript were 1.6- fold stronger than those of the 2.4 kb transcript ($S_x=0.45$).

In order to analyse whether the intertumoural variation of *HMGB1* expression was due to an intertumoural variation of stromal cells, the percentage of the stromal component of each tumour was estimated. There was no correlation between *HMGB1* expression and stromal part content (Table I and Figure 2).

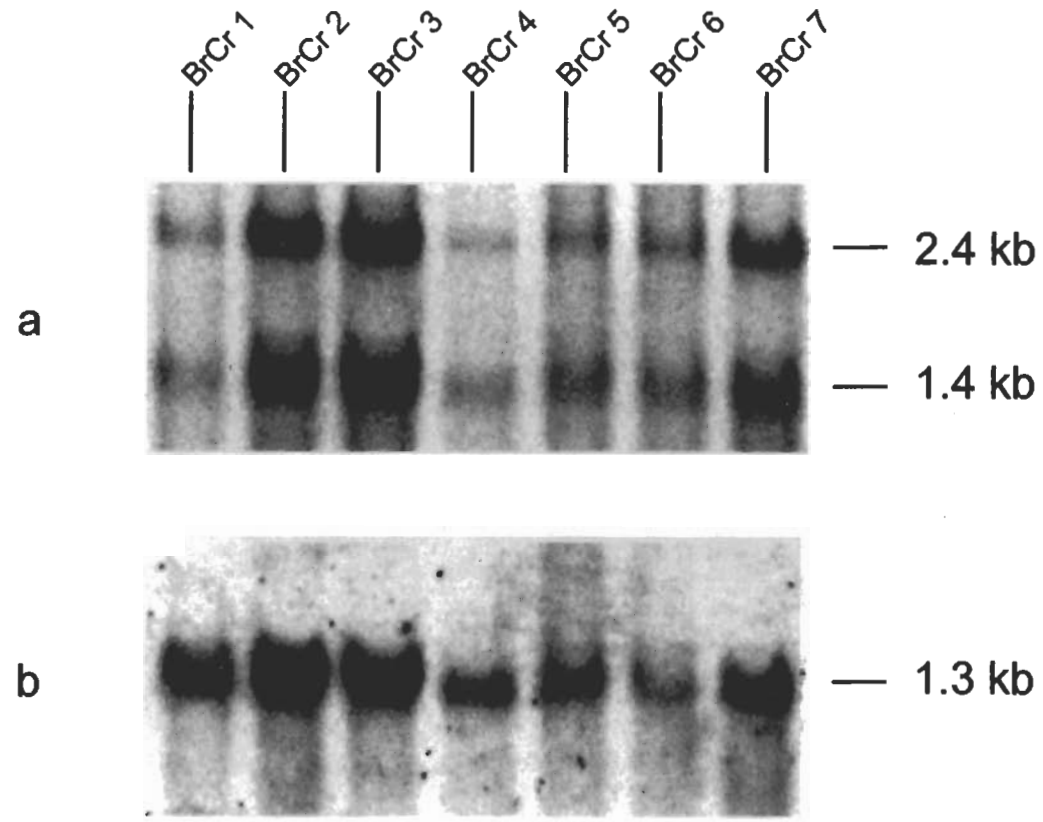

Figure 1. *a) Northern blot analyses of seven breast cancer samples using a 550 bp cDNA probe derived from the 3'UTR of HMGB1 revealed the two HMGB1 mRNA species of approximately 1.4 and 2.4 kb. b) Co-hybridisation of the same membrane with a GAPDH specific cDNA probe detecting a 1.3 kb transcript.*

Discussion

The results of the present study clearly showed that *HMGB1* expression among breast cancer samples varies. The expression of *HMGB1* in malignant human tumours deserves particular attention for two reasons. First, HMGB1 has a strong co-regulatory role in steroid receptor-mediated gene expression (3-5). Secondly, HMGB1 sensitizes cancer cells to the anticancer drug cisplatin by shielding cisplatin-DNA adducts from nucleotide excision repair (16).

In this study we analysed the expression of the 1.4 and 2.4 kb transcript of *HMGB1* in 13 breast cancer samples resulting in an intertumoural variation of *HMGB1* expression by factors of 8.5 and 14.5, respectively. This strong intertumoural variation obviously was not caused by a different stromal content of the tumour samples. As for its expression patterns in other malignant tumours, in hepatocellular, gastric, and colorectal cancer a higher expression of *HMGB1* than in the corresponding non-malignant tissues has been observed (13,17).

At present, only approximately 60% of ER- positive and 75% of ER- and PR- positive breast cancers respond to hormone therapy in contrast to only 15% of ER- negative carcinomas (1). An intertumoural variation of co-activators of ER may also contribute to the different response of estrogen receptor-positive breast tumours to endocrine therapy.

The question arises as to how the strong variation of *HMGB1* expression in breast cancer samples can be explained at the molecular level. It is possible that the observed differences are due either to a different regulation of the gene or, akin to the *HER2* gene, to an amplification (18). Certainly, comparative genomic hybridisation experiments have revealed both gains and losses of the chromosomal region 13q harbouring *HMGB1* (19-22). In addition, genetic alterations of the long arm of chromosome 13 have been shown by loss of heterozygosity (23-26). Thus, *HMGB1* may be strongly affected by these chromosomal alterations resulting in the observed variation of *HMGB1* expression in breast cancer. Accordingly, it may well be possible that allelic loss of the gene mediates loss of estrogen responsiveness. For these reasons *HMGB1* can be considered an interesting gene in the development and progression of breast cancer.

In summary, this is the first report showing a marked intertumoural variation of *HMGB1* expression in breast

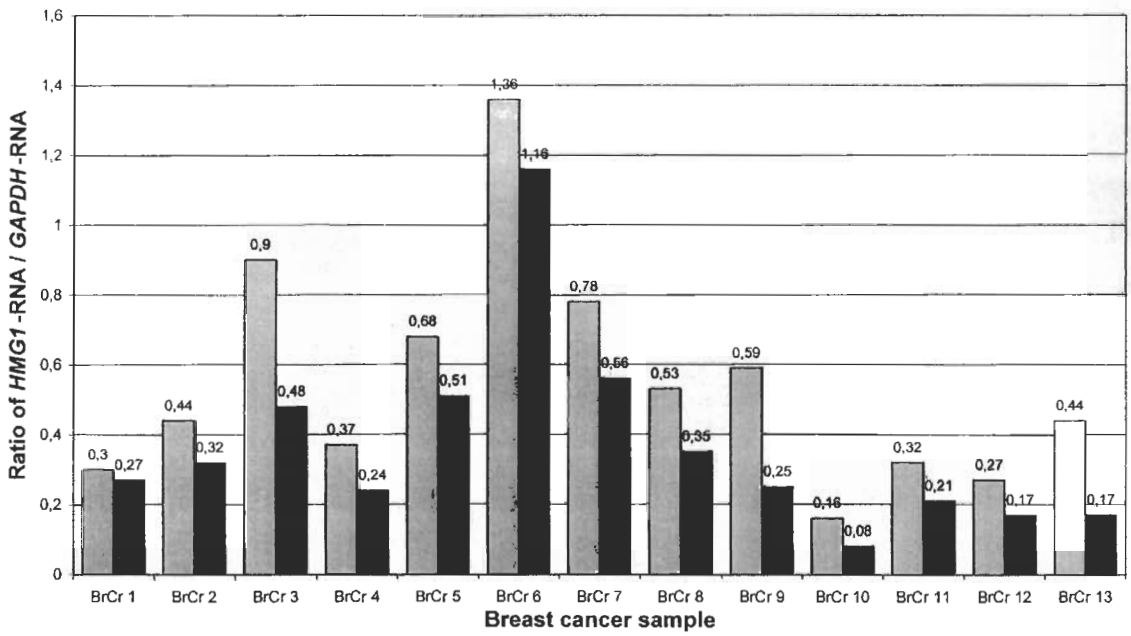

Figure 2. *Variation of relative HMGB1 expression of the 1.4 kb (light grey bars) and 2.4 kb transcript (dark grey bars), respectively, in 13 breast cancer samples. The data were obtained by Northern blot analyses using a 550 bp cDNA probe derived from the 3'UTR of HMGB1. The relative HMGB1 expression of both transcripts was determined by the ratio of HMGB1-RNA / GAPDH-RNA.*

cancers. For this reason and because of its role in steroid receptor-mediated gene expression, *HMGB1* can be considered to be one of the major players involved in the mechanisms of steroid hormone-dependent growth of breast cancer cells.

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Eigenanteil an dieser Publikation:

- Planung der Arbeiten
- Herstellung des modifizierten Klonierungsvektors
- Durchführung aller Arbeiten für das 3'UTR von *HMGA1*
- Betreuung der Arbeiten für das 3'UTR von *HMGA2*
- Verfassen der Publikation

Oncogene (2001) 20, 4537–4541

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The expression of *HMGA* genes is regulated by their 3′UTR

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Many benign mesenchymal tumors are characterized by chromosomal abnormalities of the regions 12q15 or 6p21.3 leading to aberrant expression of either HMGA2 (formerly HMGIC) or HMGA1 (formerly HMGIY). The proteins of both genes belong to the HMGA (formerly HMGI(Y)) family of architectural transcription factors. As a rule, aberrant *HMGA* transcripts found in a variety of benign tumors have intact coding regions at least for the DNA binding domains with a truncation of their 3′ untranslated regions. Adding this to the finding that an altered HMGA protein level is not always correlated with an increased amount of corresponding mRNA indicates a posttranscriptional expression control mediated by regulatory elements within the 3′UTR. To check if HMGA expression is under control of such elements we performed luciferase assays with several *HMGA2* and *HMGA1* 3′UTRs of different length. Experiments showed that an up to 12-fold increase in luciferase activity is obtained by the truncation of the 3′UTRs suggesting that the expression of HMGA2 and HMGA1 is controlled by negatively acting regulatory elements within their 3′UTR. Chromosomal aberrations affecting the *HMGA* genes may therefore influence their expression by an altered stability of the truncated transcripts as a result of the cytogenetic aberrations. *Oncogene* (2001) **20,** 4537–4541.

Keywords: HMGA; high mobility group proteins; 3′UTR

Introduction

During the last 5 years three members of the high mobility group proteins i.e. HMGA1a, HMGA1b, and HMGA2 were gathering increasing interest of molecular oncologists because of their oncogenic potential. As to their genes, *HMGA1* is coding for two alternative splice products HMGA1a and HMGA1b (Friedmann *et al*., 1993) whereas the related *HMGA2* encodes a different protein (Manfioletti *et al*., 1991). All HMGA proteins contain three DNA-binding domains designated as AT-hooks enabling their binding to the minor groove of AT-rich DNA (for review see Bustin and Reeves, 1996). This DNA-binding by HMGA proteins leads to a conformational change in DNA modulating the environment for the binding of transcription factors.

Whereas HMGA2 is almost exclusively expressed in proliferating undifferentiated cells during embryogenesis and in benign and malignant tumors, HMGA1 can also be detected at very low levels in differentiated cells (Chiappetta *et al*., 1996; Zhou *et al*., 1995; Bussemakers *et al*., 1991; Abe *et al*., 2000). An increased HMGA1 gene expression has been considered as a general diagnostic marker for neoplastic transformation (Goodwin *et al*., 1985; Giancotti *et al*., 1987, 1989) and the metastatic potential of malignant neoplasms (Bussemakers *et al*., 1991; Tamimi *et al*., 1993; Chiappetta *et al*., 1995).

Aberration of chromosomal regions 12q14-15 and 6p21.3 occurring in a variety of human benign tumors of mesenchymal origin including lipomas, uterine leiomyomas, pulmonary chondroid hamartomas, and endometrial polyps are known to affect the two genes of the high mobility group protein family i.e. *HMGA1* and *HMGA2* (Schoenmakers *et al*., 1995; Ashar *et al*., 1995; Kazmierczak *et al*., 1996a, 1998; Xiao *et al*., 1997). In contrast to *HMGA2* where numerous fusion genes have been reported (Schoenmakers *et al*., 1995; Ashar *et al*., 1995; Kazmierczak *et al*., 1996b) gene fusion of *HMGA1* seems to be a very rare event because most of the chromosomal breaks affecting that gene are mapping to a 150 kb region 3′ of the gene (Kazmierczak *et al*., 1998; unpublished results).

Although the chromosomal breakpoints affecting *HMGA1* are located predominantly in the 3′ flanking region of the gene numerous *HMGA1* transcripts with a truncated 3′ untranslated region (3′UTR) have been reported (Tkachenko *et al*., 1997; Kazmierczak *et al*., 1998). These transcripts were supposed to originate from so far unknown mechanisms as a result of chromosomal rearrangements extragenic of *HMGA1*. As to *HMGA2*, truncated transcripts have also been reported (Geurts *et al*., 1997; Klotzbücher *et al*., 1999). However, the occurrence of transcripts of both genes with truncated 3′UTR in tumors raises the question if there are elements in the 3′UTR of either *HMGA1* or *HMGA2* that are responsible for a post-transcriptional regulation of their expression and if a deletion of these elements as a result of chromosomal aberrations is of functional relevance in terms of tumorigenesis.

There are two findings suggesting post-transcriptional processes regulating the expression of either *HMGA1* or *HMGA2*: Firstly, Ayoubi *et al*. (1999)

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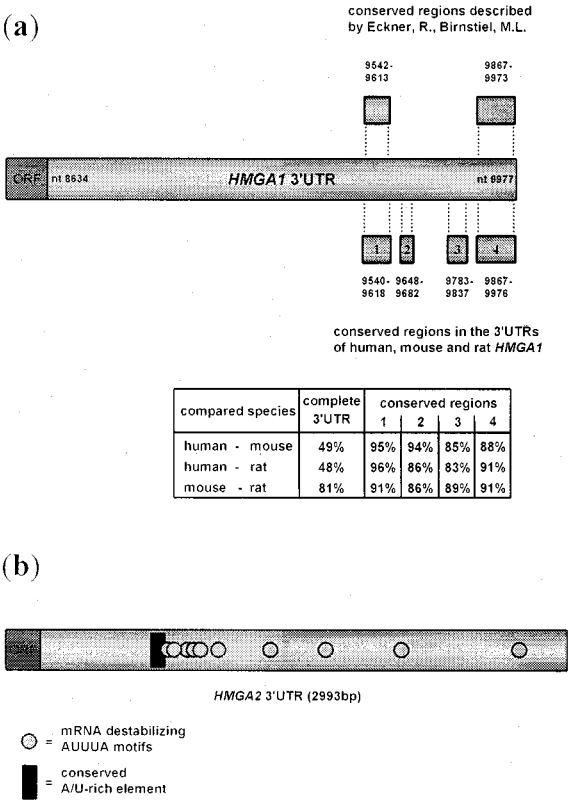
found an active *HMGA2* promoter in cells that do not express *HMGA2*, at Northern blot detection level, and secondly, an altered HMGA protein level is not always correlated with an increased amount of corresponding mRNA (Klotzbücher *et al*., 1999).

To investigate whether or not the HMGA 3′ untranslated regions are implicated in expressional control we first performed sequence comparison between the HMGA 3′UTRs of different species to identify possible target sequences for RNA-binding proteins. This sequence analysis of the 3′UTRs of human, mouse and rat *HMGA1* revealed an overall sequence homology of about 49%. Within these, four highly conserved regions exist with a sequence homology between 83–96% (Figure 1a) with two of them (conserved regions 1 and 4) described earlier by Eckner and Birnstiel (1989). Database searches with these four regions showed only a weak homology of conserved region number 4 (nt 9867–9976) to a pyrimidine-rich element within the 3′UTR of alpha-globin. This element protects the alpha-globin mRNA from degradation through an unknown mechanism, by protecting it against erythroid cell specific destabilization (Russell *et al*., 1997).



compared species	complete 3'UTR	conserved regions 1	2	3	4
human - mouse	49%	95%	94%	85%	88%
human - rat	48%	96%	86%	83%	91%
mouse - rat	81%	91%	86%	89%	91%

Figure 1 (**a**) Schematic presentation of the different conserved regions within the 3′UTR of *HMGA1*. The localization and the position of the four regions (Arabic numbers 1–4) being conserved between human, mouse and rat is given below the grey bar representing the 3′untranslated region of *HMGA1*. Protein coding region is designated as ORF. The conserved regions described by Eckner and Birnstiel with their position in the *HMGA1* gene are indicated by the boxes above the bar. Nucleotide numbering is corresponding to human genomic *HMGA1* (gb-accession L17131). The homologies of the four regions or the entire 3′UTR are given in the table below. (**b**) Schematic presentation of the relative position of the mRNA destabilizing AUUUA motifs (dots) and the conserved A/U-rich element (black box) within the 2993 bp long 3′UTR of *HMGA2*. Protein coding region is designated as ORF

To find out if the different conserved regions and sequence motifs located within the 3′UTRs of *HMGA1* and *HMGA2* are involved in post-transcriptional processes influencing expression, several deletion mutants of both 3′UTRs were cloned into 3′ position of a luciferase reporter gene. The different constructs were then analysed in luciferase assays for their expressional activity. Analysis of the different *HMGA1* 3′UTR fragments showed that the 1343 bp wild type (*wt*) 3′UTR, when positioned 3′ behind the reporter gene does not influence post-transcriptional processes in a positive or negative way indicated by a 100% relative luciferase activity equal to that of the positive control (Figure 2a). Deletion of parts of or entire conserved region 4 leads to a drop in luciferase activity of about 30% indicating that this region has a positive influence on expression. The same holds true for the other conserved region (number 2) as its deletion also leads to a decrease of luciferase activity. In contrast to that, the regions number 1 and 3 are putative negatively regulating elements as its deletion leads to a 1.5-fold, respectively 19% increase in activity. Truncation of larger parts, extending region 1, leads to an increased expression exceeding that of the positive control as indicated by up to about 120% relative luciferase activity.

In contrast to *HMGA1* the wild type 3′UTR of *HMGA2* leads *per se* to a 12.7-fold decrease in luciferase activity relatively to that of the positive control (Figure 2b). Deletion of about 593 bp of the 3′ end of the 3′UTR further reduces the activity to 4.4% indicating weak positive regulatory elements within this region. From that point on further truncation of the 3′ end lead to an increase in luciferase activity. The activity of the smallest fragment analysed in this series showed a relative luciferase activity of about 20.5% that is 2.6-fold higher than that of the *wt HMGA2* 3′UTR but still five times lower than the positive control.

As demonstrated herein the results for *HMGA1* indicate that its wild type 3′UTR does not influence expression in a positive or negative way, while the presence of *wt HMGA2* 3′UTR leads *per se* to a 12.7-fold decrease in expression. This finding fits with the data from Ayoubi *et al*. (1999), who described that the

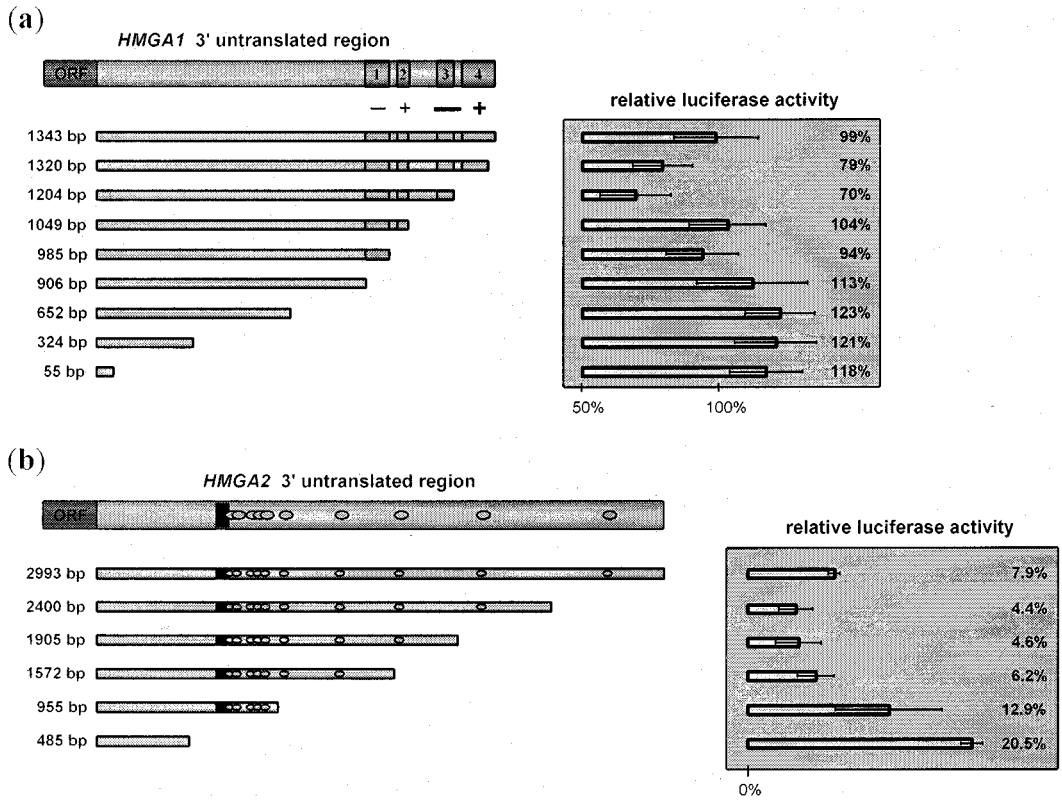


Figure 2 Diagram of the different HMGA 3′UTR deletion mutants and their relative luciferase activity obtained by luciferase assay experiments. *Left side*: schematic presentation of the 3′UTRs and the deletion mutants of different length according to Figure 1. *Right side*: diagram of the relative luciferase activity obtained by the different 3′UTR elements presented on the left side. The activity measured by the positive control was set 100% relative activity. (**a**) Experiments performed for HMGA1; the conserved regions are highlighted as darker grey boxes; 'plus' and 'minus' indicates positive and negative role on expression as measured by luciferase assays. (**b**) experiments performed for HMGA2; localization of the sequence motives according to Figure 1b

complete *HMGA2* 3′UTR is responsible for a 7–15-fold lower luciferase activity. As HMGA2 belongs to the group of 'delayed early response' genes that are known to be expressed at the beginning of embryogenesis (Lanahan *et al*., 1992), most of these mRNAs have a very short half-life (Caput *et al*., 1986; Chen and Shyu, 1994). Studies have shown that the short half-life of many 'delayed early response' genes is linked to a number of AUUUA elements found in their 3′UTR (Malter, 1989) promoting rapid deadenylation and subsequent decay of mRNA (Decker and Parker, 1995). This findings may explain the results obtained for the *HMGA2* 3′UTR deletion mutants that a truncation of the 3′UTR coincident with a deletion of different AUUUA motives leads to an increase in luciferase activity, presumably due to an increase in mRNA stability. Furthermore, an insertion of an A/U-rich element 20–30 bp in front of a AUUUA cluster as we found for the 3′UTR of *HMGA2*, can further increase the destabilizing effect of the AUUUA motif (Xu *et al*., 1997) explaining the increased luciferase activity when deleting this element. Although, when deleting all the destabilizing AUUUA elements and the A/U-rich element the remaining fragment (485 bp) still has an activity that is fivefold lower than the positive control. This effect could probably be linked to the conserved 39 bp G/C rich sequence of unknown function located at the beginning of the 3′UTR of *HMGA2*.

In contrast to *HMGA2*, the 3′UTR of *HMGA1* did not show any known functional elements, but we were able to detect regions of positive (nt 1–906, conserved regions 2 and 4) and negative (conserved regions 1 and 3) effect on expression. So far only little is known about regulatory elements within the 3′ untranslated region of genes. But putatively the different *HMGA1* 3′UTR regions may be involved in post-transcriptional processes regulating the expression of *HMGA1*. Conceivably they can promote degradation similar to the elements within the 3′UTR of *HMGA2* or they can provide target sites for RNA-binding proteins that activate (Fajardo *et al*., 1997) or inhibit (Izquierdo and Cuezva, 1997) translation. But further experiments will have to be done to find out more about the function of the putative regulatory elements within the 3′UTR of both *HMGA* genes.

Summarizing we can say that the expression of *HMGA2* is post-transcriptionally regulated by negative regulatory elements being located within the 3′UTR and that truncations of this 3′UTR as it is found in all

aberrant transcripts, are leading to an increased activity of the gene. And as to *HMGA2* the truncation of the 3′ untranslated region of *HMGA1* also lead to an increased activity of the gene.

All studies so far indicate that the normal effects of HMGA proteins and their pathological function are dose-dependent. For example, the onset of transcription in one-cell mouse embryos depends on the amount of HMGA1a, probably due to a competitive displacement of histone H1 or other general repressors, making chromatin regions accessible to the transcription machinery (Beaujean *et al*., 2000). In addition, only transgenic mice expressing high levels of truncated HMGA2 lacking the acidic C-tail showed an increased adiposity and a giant phenotype (Battista *et al*., 1999). Thus, aberrations of *HMGA* genes affecting the 3′UTR as found in all aberrant transcripts and analysed in this work, are sufficient to induce an increase in expression beyond normal activity. Furthermore, tumors that showed in Northern blot transcripts of apparently normal length can have basepair deletions or exchanges at sensitive sites leading to increased expression patterns.

Nevertheless, this is the first experiment ever that showed that a truncation of the 3′UTR of either *HMGA1* or *HMGA2* could indeed be responsible for their aberrant expression probably due to altered post-transcriptional processes, leading to the conclusion that the 3′UTR truncations are of more pathological relevance than expected so far.

Material and methods

PCR amplification of 3′UTR elements

For the amplification of *HMGA1* 3′UTR fragments the upper *Eco*RI linker-primer 5′-GGAATTCCCCATGCGTGCCGCCTGC-3′ (nt 8635–8652 of *HMGA1*) was used in combination with the following lower *Xba*I linker-primer to establish fragments of different length: 5′-GCTCTAGACAGAAAAGGATATTTTTTTTATTCAAG-3′ (nt 9977–9951), 5′-GCTCTAGACAAGTAACTGCAAATAGGAAACC-3′ (nt 9954–9932), 5′-GCTCTAGAGGGCCCCCTTGCTCACTC-3′ (nt 9838–9821), 5′-GCTCTAGAGAGGATGAACATTTGGCGCTG-3′ (nt 9683–9663), 5′-GCTCTAGAAAAAAACAACACAACTGTCCAGAG-3′ (nt 9619–9596), 5′-GCTCTAGAAGGAGGGGGGTTGGGAGCG-3′ (nt 9540–9522), 5′-GCTCTAGACCCCTCCTGCCTTCCTGTAG-3′ (nt 9286–9268), 5′-GCTCTAGAAGTGGGGACGCAGCAGGTG-3′ (nt 8960–8941), 5′-GCTCTAGAGTCCAGTCCCAGAAGGAAGC-3′ (nt 8689–8670).

Different *HMGA2* 3′UTR fragments were amplified by using the *Eco*RI linker-primer 5′-GGAATTCGGGGCGCCAACGTTCGATTTC-3′ (nt 1–22 of *HMGA2* 3′UTR) in combination with the *Xba*I linker-primer 5′-GCTCTAGATTGATCTATTTTGACCAAACTTTATTAC-3′ (2993 bp fragment, entire *HMGA2* 3′UTR). 5′-GCTCTAGAATGAGCTGGCCAATGAGGTTTC-3′ (2400 bp), 5′-GCTCTAG-AAAAGCAGGGCAAGAAGCATC-3′ (1905 bp), 5′-GCTCTAGAGTGATGTGTAGTGTGATTGTGG-3′ (1572 bp), 5′-GCTCTAGATTTCGCTCCTCCCACCTCATA-3′ (955 bp), 5′-GCTCTAGACAGGGAGT-GGGTTGGGGTGGTA-3′ (485 bp).

PCR reactions for both 3′UTRs were performed in a Mastercycler gradient (Eppendorf, Hamburg, Germany) using the following protocol: 1 min 94°C, 1 min 57–62°C, 2 min 72°C × 35, 10 min 72°C. As template different plasmids and PACs spanning the desired 3′UTR regions of *HMGA1* or *HMGA2* were used.

Construction of plasmids

For directed cloning of 3′UTR elements to the 3′ end of the luciferase reporter-gene of pGL3-Control (Promega. Madison, WI, USA) the vector was modified by inserting a CTAGTGAATTCCGGCT element into the *Xba*I restriction site downstream of the luciferase reporter-gene. Isolated PCR fragments were then cloned into the new *Eco*RI–*Xba*I site of the modified pGL3-Control vector. Plasmid DNA was isolated using the Plasmid Isolation Kit (QIAGEN, Hilden, Germany) following the instructions of the manufacturer.

Cell culture and luciferase assay

HeLa cells were cultured in medium TC199 supplemented with 20% fetal calf serum (FCS) and antibiotics (200 IU/ml penicillin, 200 μg/ml streptomycin). For transient transfections HeLa cells were seeded on six-well plates. DNA of the different constructs, pGL3-Control (positive control), and pGL3-Enhancer (negative control) were each co-transfected with pRL-TK (Promega, Madison, WI, USA) expressing a *Renilla* luciferase serving as an internal control value to which the experimental data were normalized. Transfection was performed using SuperFect transfection reagent (Qiagen, Hilden, Germany) following the instructions of the manufacturer. After 24 h Luciferase activities was measured in a luminometer, (Biocounter M2010, Lumac BV, Netherlands) using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) following the instructions of the manufacturer.

Experiments for each construct were performed in triplicate and were repeated several times.

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VI.

BORRMANN L, KIM I, SCHULTHEISS D, ROGALLA P, BULLERDIEK J. (2001a). Regulation of the expression of HMG1, a co-activator of the estrogen receptor. *Anticancer Research*, 21: 301-305.

Eigenanteil an dieser Publikation:

- Betreuung der Arbeiten zur Komplettierung der genomischen Sequenz des 5' Bereiches von *HMGB1*
- Durchführung der Sequenzanalysen in Zusammenarbeit mit Frau I. Kim
- Durchführung aller wissenschaftlichen Arbeiten zur funktionellen Analyse des *HMGB1* Promoters inklusive der *in silico* Analysen für die Promoterregion
- Verfassen der Publikation

ANTICANCER RESEARCH *21:* 301-306 (2001)

Regulation of the Expression of HMG1, a Co-activator of the Estrogen Receptor

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Abstract. *HMG1 is a protein of high clinical significance. Besides shielding of DNA adducts against repair enzymes making cells more sensitive to cisplatin therapy, it is also a co-modulator of the activity of steroid hormone-regulated genes. Although HMG1 is regulated by various factors, including steroid hormone estrogen, nothing was known about regulatory sequences. Also the sequence of parts of HMG1 including its promoter remains still unknown. We have completed the genomic organization of human HMG1 and characterized its regulatory region by luciferase assay for promoter activity. Insights into the regulation of HMG1 expression are given by the promoter analysis showing a strong functional promoter, enhancing and negative regulatory regions together with a CpG island in intron 1 and several transcription factor binding sites. Furthermore, the finding of two estrogen responsive elements within intron 1 is relevant as they indicate a direct mechanism of HMG1 up-regulation by estrogen making the presence of an estrogen receptor a significant marker for a combined treatment of special tumor types with estrogen and the anticancer drugs cisplatin or carboplatin.*

HMG1 is an evolutionary highly conserved and ubiquitously expressed non-histone chromosomal protein implicated in transcription, replication and recombination as well as in chromatin organization (for review: 1). HMG1 contains two highly basic DNA-binding domains (HMG boxes A and B) and a negatively charged C-terminal domain involved in interactions with other proteins *e.g.* histones, p53, Oct, RAG1, TBP, HOX and some components of the basal transcriptional machinery (2-6). Recently, HMG1 has gathered considerable interest in molecular oncology. First, it sensitizes cancer cells to the anticancer drugs cisplatin and carboplatin by binding to distorted DNA and protecting it from proteins of the DNA-repair machinery (7,8). Secondly, it is a known co-activator of the estrogen-receptor (ER), promoting the binding of ER to the estrogen response element (ERE) leading to the formation of a more stable receptor-DNA complex (9).

However, relatively little is known about the regulation of *HMG*1 itself. Although estrogen leads to a rapid increase in HMG1 expression in the breast cancer cell line MCF-7 (10), there is so far no evidence that *HMG*1 itself is a target gene of the estrogen receptor because the sequence of parts of *HMG1* including its promoter remain unknown. Therefore, we have completed the genomic sequence of *HMG1* and characterized its promoter region.

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Key Words: Estrogen receptor, estrogen, HMG1, high mobility group protein.

Materials and Methods

Isolation and analysis of HMG1 genomic subclones. 4.3kb and 2.1kb HindIII restriction fragments of PAC1363 (11) spanning *HMG*1 were purified from an agarose gel using QIAEX II (Qiagen, Hilden, Germany) and were cloned into pGEM-11Zf(+) vector (Promega, Madison, USA) for sequencing. Plasmid DNA was isolated using the Plasmid Isolation Kit (Qiagen, Hilden, Germany). Clones were sequenced by primer walking with an ABI PRISM 377 DNA Sequencer (Perkin Elmer Langen, Germany) using the dideoxy chain termination method.

Cloning of promoter constructs. For cloning the *HMG1* promoter elements different 5'-flanking regions of the *HMG1* gene were PCR amplified using the following BglII/*Hin*dIII linker primer combinations: 5'-GAAGATCTTGAATAGGGGAGTGGTCTG-3'/5'-CCCAAGCTT-GTCTGGCTCCCGCTCTCA-3'(-1332 to -7); 5'-GAAGATCTGGTG-TGATGSGAGGGAAA G-3'/5'-CCCAAGCTTGTCTGGCTCCCGCT-CTCA-3' (-1058 to +58); 5'-GAAGATCT GCAGAGACCCCAGT-TTTCAG-3'/5'-CCCAAGCTTGTCTGGCTCCCGCTCTCA-3' (-553 to +58); 5'-GAAGATCTTCAGGACAATGGGAGGTATC-3'/ 5'-CCC-AAGCTTGTCTGGCTCCCGCTCTCA-3' (-147 to +58). PCR reactions for amplification of the promoter elements were run on a gradient cycler (Mastercycler gradient, Eppendorf, Hamburg, Germany) using the following protocol: (45 seconds 94°C, 30 seconds 60-62°C, 2

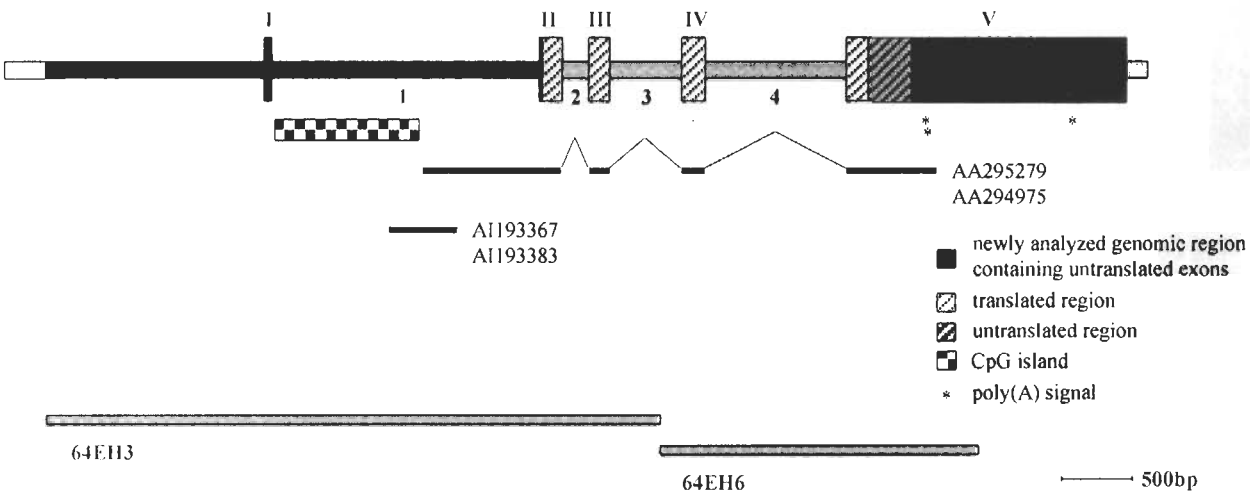

Figure 1. *Diagram of the human HMG1 gene showing regions of newly analyzed genomic sequences and so far known regions. Human HMG1 is 5.3 kb in length and contains five exons (Roman numeral I-V) and four introns (Arabic numbers 1-4). Shading key is shown in the lower right corner of the diagram. Bold lines indicate sequences of cDNA clones starting in intron 1 with small lines representing the lack of intron sequence. The relative position of the genomic clones 64EH3 and 64EH6 is given in the lower part of the diagram.*

minutes 72°C) x 30, 10 minutes 72°C. As template genomic clone 64EH3 was used. Isolated PCR fragments were cloned into the *Bg*II/*Hin*dIII sites of the luciferase reporter vector pGL3-Enhancer (Promega, Madison, USA). Plasmid DNA was isolated as previously described.

Cell culture and transient transfection assay. HeLa cells were cultured in medium TC199 supplemented with 20% fetal calf serum (FCS) and antibiotics (200 IU/ml penicillin, 200 µg/ml streptomycin). For transient transfections HeLa cells were seeded on 6-well plates. After 18 hours the growth medium was completely removed and transfection complexes were added. Transfection complexes containing 1 µg of plasmid DNA, 1 µg of pUC18 carrier DNA and 10 µl transfection reagent (SuperFect Transfection Reagent, Qiagen, Hilden, Germany) were formed in a total volume of 100 µl in TC199 medium (without supplements) by incubating the sample for 10 minutes at room temperature. Complexes were mixed with 800 µl culture medium and immediately transfered to the cells of one well. After overnight transfection 2.5 ml 20 % culture medium were added to each well and the cells were grown for further 24 hours.

Measurement of the promoter activity. Promoter activities were measured using the Luciferase Assay Kit (Stratagene, La Jolla, USA). After removing the growth medium from the cells, 500 µl cell lysis buffer were added to each well and the plates were incubated for 15 minutes on a shaker. 20 µl of the cell lysate were gently mixed with 100 µl of luciferase substrate-assay buffer and luciferase activities were measured immediately in a luminometer, (Biocounter M2010, Lumac BV, Netherlands). Experiments for each construct were performed in triplicate in five independent transfection assays.

Results

Isolation and sequencing of HMG1 genomic clones. For completion of the yet unknown genomic regions 5' of exon 2 and of the 3' end of human *HMG1* gene two *Hin*dIII fragment (64EH3 and 64EH6) from PAC1363 were subcloned (Figure 1). By sequencing the clone 64EH3 we were able to recieve sequence for 1534 bp of 5'-flanking region, 76 bp of exon 1 (corresponding to mouse exon 1 (X80458)) and a 1888 bp long intron 1 (gb-accession no. AF281043) (Figure 2). Sequence comparison between the newly established sequence of human *HMG1*, cDNA clones from public databases and pseudogene sequences (unpublished data) show a 99% homology of exon 1 extending up to nt -94 indicating a transcription start site located further upstream than previously described (X80458). Homology searches of intron 1 revealed homology to cDNA clones lacking exon 1 sequence but starting at position nt 918 (*e.g.* AI193367, AI193383) and nt 1134 (AA295279 and M294975), respectively within intron 1 suggesting an unknown alternative splice variant of *HMG1* (Figure 1, 2).

Sequencing of PAC1363 and clone 64EH6 spanning the 3' end of *HMG1* leads to the establishment of 1699 bp (gb-accession no. AF169650) containing 1580 bp of new sequence 3' of incomplete human *HMG1* exon 5 (U51677). This sequence includes a consensus polyadenylation signal at position 1346 to 1351 and two non-consensus AGTAAA and AATACA poly(A) signals at positions 308 to 313 and 312 to 317, respectively. Blast research in the NCBI database (National Center for Biotechnology, http://www.ncbi.nlm.nih.gov/BLAST/) confirmed that these poly(A) signals explain most of the EST sequences established so far.

Functional analysis of the promoter region. To identify specific elements in the 5'-flanking region of *HMG1* required for promoter activity several deletion mutants spanning bases -1332 to +51 were cloned into the promoterless luciferase reporter vector pGL3-Enhancer (Figure 3). The promoter

Figure 2. *The 3662 bp of newly analyzed genomic sequence spanning the 5'-flanking region to exon 2 of the human HMG1 (gb accession no AF281043). Nucleotide numbering is due to the postulated transcriptional start site in mouse HMG1 (16). Exons are shaded. Putative transcription factor binding sites in the promoter region (-1534 to -1) and in intron 1 (78 to 1964) are underlined and named below the sequence. The translation start site (ATG) in exon 2 is indicated in boldface type. The 5'-end of the fragments used in luciferase assay are given by bold arrows corresponding to the elements described in the brackets. Small arrows within intron 1 indicate the start of cDNA clones containing intron 1 sequence.*

activity of each construct was determined by measuring luciferase activity in HeLa cells that had been transiently transfected with these promoter constructs. Compared with the positive control driven by a SV40 promoter, the largest fragment tested (-1332 to -7) had a 1.4-fold higher luciferase activity indicating a strong *HMG1* promoter (Figure 3). Deletion of 275 bp of the 5' end resulted in a 2-fold decrease in luciferase activity (fragment -1058 to +51) suggesting the presence of positive regulatory elements within the region -1332 to -1058. Further deletion of 505 bp reincreased the

luciferase activity about 1.4-fold (fragment -553 to +51) to a level equal to the positive control indicating negatively regulating element(s) within the region -1058 to -554 suppressing HMG1 expression. The low level luciferase activity achieved by the smallest fragment -147 to +51 suggests the existence of a strong functional *HMG1* promoter within the region -553 to -1 and a core promoter for basal low level expression in the region -147 to -1.

Characterization of the 5'-flanking region. Analysis of the 5' sequence of *HMG1* by the luciferase assay revealed several potential binding sites for transcription factors (as revealed by the program MatInspector (12)). Whereas no consensus TATA box was found in the promoter region of *HMG1*, three different CCAAT boxes are localized in the region -173 to -86 upstream of exon 1 (Figure 2).

Although HMG1 expression is up-regulated by steroid hormones like estrogen or progesterone neither estrogen responsive element nor binding sites for other members of steroid hormone receptors, like AR, COUP, PPAR, PR, or VDR, were found in the 5'- flanking region. Nevertheless, two estrogen responsive elements (ERE) at positions 1516 ($ATGTT_{TTC}TGACC$) and 1571 ($ATGTT_{AAG}TGACC$) within intron 1 were predicted by MatInspector (Figure 2). In addition, two other GGTCA sequences identical to the first half-site of the consensus ERE (GGTCAnnnTGACC) were found at positions -149 and 168.

Among the other binding sites found by MatInspector in the 5'-flanking region of *HMG1* there are six potential binding sites for USF, four sites for C/EBPß, five sites for VBP and six sequence motives for GFI1 located upstream of exon 1 (Figure 2). Furthermore a 1.1 kb long CpG island matching the criteria described by Gardiner-Garden and Frommer (13) was found at the beginning of intron 1 (Figures 1, 2).

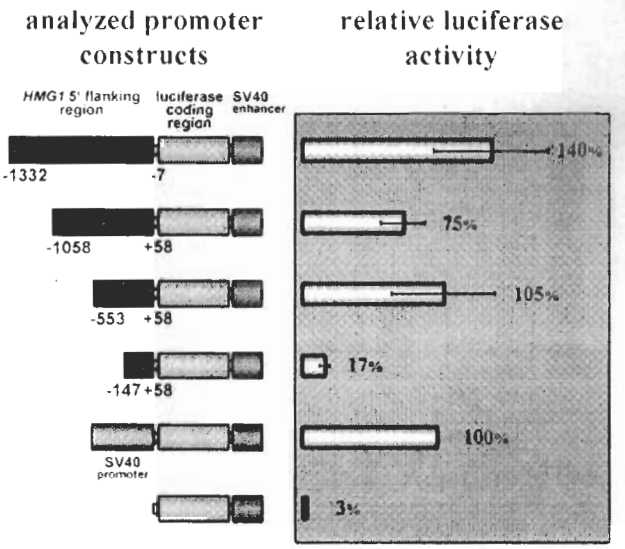

Figure 3. *Diagram of different reporter gene constructs and their corresponding relative promoter activity as revealed by luciferase assay. The localization of the tested promoter fragments are shown on the left with numbers indicating their relative position with respect to Figure 2. Both lower constructs illustrate the promoter features of pGL3-Promoter and pGL3-Enhancer that were used as positive or negative control, respectively. The promoter activity of the positive control was set 100%*

Discussion

HMG1 is an architectural transcription factor that binds and mediates bending of DNA to provide a more favorable environment for the assembly of a functional transcription complex. An example is the binding of estrogen receptor homodimers to the estrogen responsive element. Aimed at a better understanding of the regulation of *HMG1* the present study completed the genomic structure of *HMG1* and elucidated the regulation of *HMG1* expression. Sequencing of two genomic subclones derived from *HMG1* has lead to the establishment of 3662 bp (AF281043) 5' and 1699 bp (AF169650) 3' new sequences thus completing the genomic organization of *HMG1* (Figure 1).

The data presented herein indicates two potential estrogen responsive elements in intron 1 of *HMG1* and several potential binding sites for VBP, a protein involved in the estrogen-dependent transcription of the chicken vitellogenin gene (14). As EREs are not limited to the 5'-flanking region as revealed by the finding of EREs in the 3'-flanking region of the murine c-fos protooncogene (15) the two EREs described herein are likely targets for an estrogen-mediated transcriptional activation of *HMG1*. Recently, it was shown that estrogen treatment leads to HMG1 overexpression and accordingly sensitizes the breast cancer cell line MCF-7 to the anticancer drug cisplatin (8). Because the pathways by which estrogen lead to an up-regulation of HMG1 remained unknown the criteria making cancer patients suitable for this combined steroid hormone/anticancer drug treatment were not reliable. The data presented herein indicated a direct up-regulation of HMG1 induced by the binding of a estrogen/ER compelex to the ERE found within the gene.

Further insights into expressional control were obtained by characterizing the 5'- flanking region of *HMG1*. This region contains a strong eukaryotic promoter and regions responsible for enhanced and repressed expression. The functional promoter up to position -553 has a promoting activity similar to the SV40 promoter and is characterized by three RNA polymerase II binding sites (CCAAT boxes). The negative regulatory effect of the region -1058 to -554, as revealed by luciferase assay, may be due to the predominant localization of GFI1 binding sites within this region since GFI1 is known to act as a transcriptional repressor (16). Upstream of this repressor region there is a further region

with a positive regulatory effect (-1332 to -7058). Binding sites for C/EBPß, USF and VBP might be relevant for the enhancer element detected in the region -1332 to -1058. Furthermore C/EBPß and USF two proteins being implicated in differentiation and cell growth (17-19) may be of functional relevance for the regulation of *HMG1* during development.

In summary, the localization of binding sites for different transcription factors in combination with the results of the luciferase assay and the finding of two EREs and a CpG island in the newly established sequence of intron 1 give insights into the control of *HMG1* expression. In the future, the molecular mechanisms of the regulation of *HMG1*, a strong co-activator of the estrogen receptor, may help to influence hormone-dependent growth.

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VII.

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Eigenanteil an dieser Publikation:

- Bis auf die radioaktiven Northern Blot Hybridisierungen die Durchführung aller wissenschaftlichen Arbeiten
- Mithilfe beim Verfassen der Publikation

Assignment of a New Gene (*LBH*)[1] Located Downstream of *HMGIY*

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The genomic region surrounding high-mobility group protein isoforms I and Y (*HMGIY;* MIM 600701) assigned to 6p21.3 is a frequent target of chromosomal aberrations in subgroups of a huge variety of human benign mesenchymal tumors, e.g., uterine leiomyomas, lipomas, or pulmonary chondroid hamartomas (PCH) (1–5, 7, 8, 10, 11).

Because it has been shown that most of the breakpoints map 3′ of *HMGIY* (6, 12), we have cloned and sequenced the 3′ flanking region of the *HMGIY* gene. Sequencing data were obtained by using an ABI Prism 377 DNA sequencer (Perkin–Elmer, Langen, Germany). By a positional cloning strategy (6), we were able to detect a yet unknown gene 3′ of *HMGIY,* which we have preliminarily named *LBH* (located behind *HMGIY*). It is located in a tail-to-tail orientation to *HMGIY* (Fig. 1A), and its transcriptional orientation is thus directed toward the telomere. A homology search revealed 100% homology of its exons to different *Homo sapiens* cDNA clones (GenBank Accession Nos. R07003, R95998, and AA043491) and also a high homology to several fetal mouse expressed sequence tagged sites (GenBank Accession Nos. W18017, W30375, and W56987). The length of the *H. sapiens* cDNA clones ranged between 772 and 849 bp, comprising the complete sequence of all five exons detected thus far. Alignment of the cDNA sequences to the genomic sequence 3′ of *HMGIY* allows for the conclusion that *LBH* contains at least five exons with exon/intron junctions corresponding to the consensus sequences of donor/acceptor splice sites (9). Because of the absence of exon X-III′ (Fig. 1A) in one of the cDNA clones (AA043491), it was supposed that this exon leads to a splice variant. The polyadenylation site of the unknown gene lies 1 bp behind the 3′ end of the *HMGIY* gene. The homology between the *H. sapiens* and the mouse cDNA clones was about 91% within the coding sequences and decreases in the untranslated region to 22%. For analysis of the expression patterns of the *LBH* gene, Northern blotting experiments were performed on a "Multiple Tissue Northern Blot Human IV" (Clontech Laboratories Inc., Palo Alto, CA) containing mRNAs from several different normal tissues. According to the manufacturer's manual, each of the lanes is loaded with 2 μg mRNA. As a molecular probe, we used the cDNA clone AA043491 containing exons X, X-I, X-II, and X-III of *LBH*. The probe was labeled with ^{32}P using a random primer extension protocol. For prehybridization and hybridization, the ExpressHyb hybridization solution (Clontech Laboratories Inc.) was used. Prehybridization was carried out for 30 min, and hybridization was performed for 1 h at 68°C. Blots were washed twice for 20 min at room temperature in 2 × SSC/0.05% SDS and twice for 20 min at 68°C in 0.1× SSC/0.1% SDS. Northern analysis revealed two transcripts of 1 and 3 kb, in spleen, thymus, prostate, testis, uterus, small intestine, colon (mucosal lining), and peripheral blood leukocytes. In spleen and thymus, an additional band of 1.8 kb was detected (Fig. 1B). The size of the transcripts indicates that we have not yet determined the complete genomic structure of *LBH*.

As the genomic region 3′ of *HMGIY* is frequently involved in chromosomal rearrangements found in benign mesenchymal tumors, we tested additionally RNAs from three PCH and one uterine leiomyoma with 6p21.3 aberrations in a Northern blot for aberrant expression of *LBH*. For the Northern blot, 30 μg total RNA of each tumor was separated by gel electrophoresis in a denaturing gel (1% agarose in Mops buffer and 6% formaldehyde) and blotted onto a Hybond-N$^+$ nylon membrane (Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany). The amount of RNA loaded to each lane was equal in lanes 1 and 3, slightly higher in lane 2, and slightly lower in lane 4, as estimated after ethidium bromide staining. Integrity of RNA was proven by the presence of the 18S and 28S bands. The hybridizations were performed as described above. As in normal tissue, all tumors showed transcripts of 1 and 3 kb in length. In none of the tumors was an aberrant transcript detected, but one PCH showed an enhanced transcription level (Fig. 1C). The differences observed after hybridization cannot be explained by the rather small differences in RNA loading. Nothing is yet known about the function of this new gene, and a homology search of the coding region with different databases did not reveal any similarity to known proteins or protein domains. Cloning of the complete cDNA sequence will presumably help to elucidate the function of the *LBH* gene. Furthermore, we cannot exclude a coordinate regulation, either positive or negative, between *LBH* and *HMGIY*. A suppression of *LBH* due to *HMGIY* expression would be possible, but must be determined in a functional *in vitro* assay.

[1] The gene has been submitted to public databases as C6orf1.

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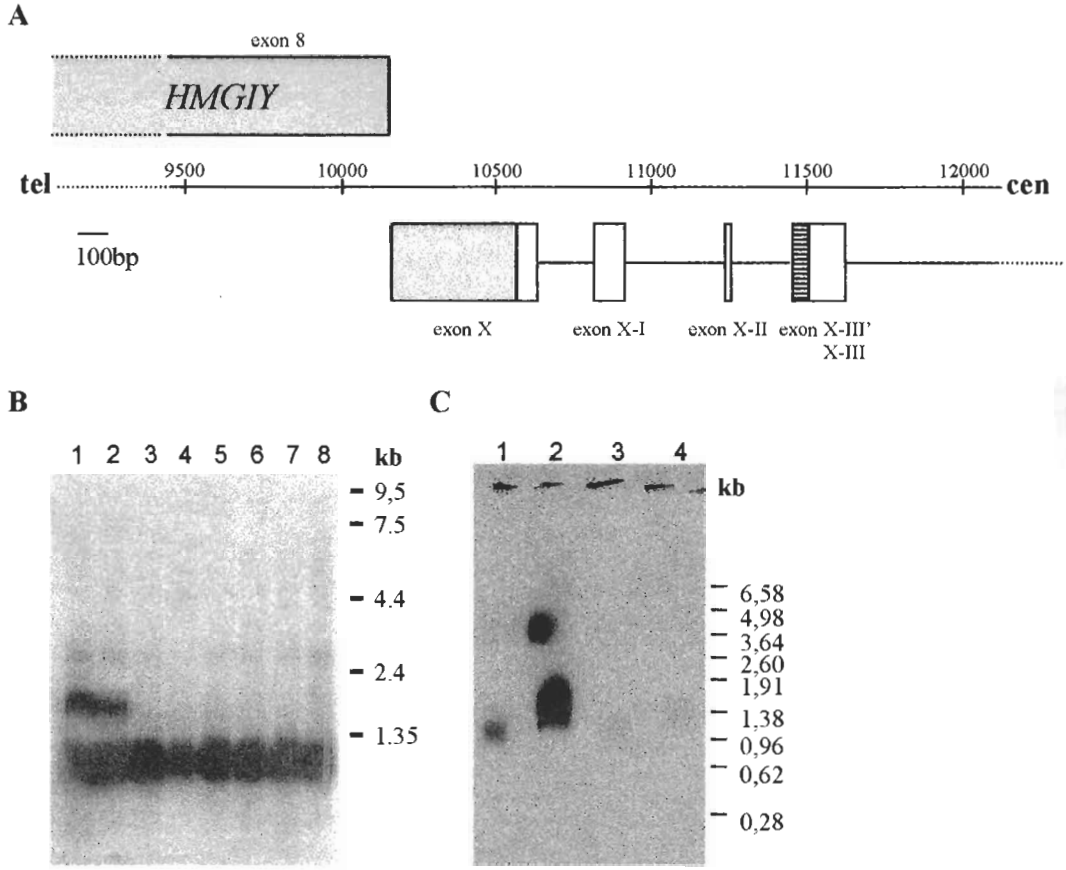

FIG. 1. (**A**) Schematic illustration of the genomic structure of the *LBH* gene in relation to *HMGIY*. The boxes indicate the known exons of *LBH* and exon 8 of *HMGIY*. Scale between boxes shows the numbered nucleotides, whereas the first basepair of *HMGIY* was defined as zero. White boxes: coding region; gray boxes: 3′ UTR of *HMGIY* and *LBH;* tel:telomere, cen:centromere. (**B**) Expression pattern of the LBH gene in various normal human tissues. Analyses were performed using the Multiple Tissue Northern Blot Human IV (Clontech Laboratories Inc.) containing mRNAs from eight different normal tissues (lanes **1–8:** spleen, thymus prostate, testis, uterus, small intestine, colon (mucosal lining), peripheral blood leukocytes). As molecular probe, the cDNA clone AA043491 containing exons X, X-I, X-II, and X-III of *LBH* was used. (**C**) Northern analysis of the *LBH* gene in three different chondroid hamartomas (lanes **1–3**), and one uterine leiomyoma (lane **4**) with 6p21.3 aberrations. As molecular probe, the cDNA clone AA043491 containing exons X, X-I, X-II, and X-III of *LBH* was used.

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VIII.

KAZMIERCZAK B, DAL CIN P, WANSCHURA S, BORRMANN L, FUSCO A, VAN DEN BERGHE H, BULLERDIEK J. (1998). *HMGIY* is the target of 6p21.3 rearrangements in various benign mesenchymal tumors. *Genes Chromosomes Cancer*, 23: 279-285.

Eigenanteil an dieser Publikation:

- Subklonierung der Region des *HMGA1* und Charakterisierung der PAC-Klone
- Herstellung der Southern Blots und dafür verwendeten Sonden
- Herstellung und Charakterisierung der in der FISH verwendeten Sonden
- Durchführung der 3'RACE Expreimente inklusive deren Auswertung
- Verfassen der Publikation in Zusammenarbeit mit einigen Co-Autoren

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RESEARCH ARTICLES

HMGIY Is the Target of 6p21.3 Rearrangements in Various Benign Mesenchymal Tumors

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Specific chromosomal abnormalities of chromosomal region 6p21.3 have been described in subsets of many benign mesenchymal tumors. In the presented study, we investigated a series of 36 such cases by FISH, and Southern blot analyses for *HMGIY* rearrangements. FISH results revealed that the chromosomal breakpoints of 11 pulmonary chondroid hamartomas (PCHs), 12 endometrial polyps (EPs), one lipoma, and two uterine leiomyomas (ULs) were located within a 80 kb region surrounding the *HMGIY* gene. In 11 PCHs and one UL the breakpoints were located 3' of *HMGIY,* and one PCH showed a breakpoint 5' of *HMGIY*. Southern blot analyses with intra- and extragenic probes were performed of primary tumor material or cell lines from one UL, three PCHs, and five EPs. In none of these cases was an intragenic rearrangement found. Finally, we were able to detect expression of truncated *HMGIY* transcripts by 3'-RACE PCR. Our data clearly show the role of a further member of the *HMGI* family in the development of benign mesenchymal tumors. Although most of the breakpoints of the chromosomal translocations involving *HMGIY* are located outside the gene, aberrant transcripts resembling the structure of those observed in the case of *HMGIC* have been found. Our molecular investigations thus led to the identification of the molecular mechanism by which rearrangements of either of two closely related genes lead to the development of frequent benign mesenchymal tumors in humans. *Genes Chromosomes Cancer 23:279–285, 1998.*

INTRODUCTION

Cytogenetic analysis of more than 2,000 benign solid tumors has revealed a variety of specific chromosomal aberrations. Among these, rearrangements of either 12q14–15 or 6p21 are particularly frequent (Mitelman, 1994). Chromosomal aberrations of the segment 12q14–15 leading to rearrangements of the *HMGIC* gene have been shown in pleomorphic adenomas of the salivary glands, uterine leiomyomas, lipomas (Ashar et al., 1995; Schoenmakers et al., 1995), pulmonary chondroid hamartomas (Kazmierczak et al., 1996c), and endometrial polyps (Bol et al., 1996), The breakpoints were either located within the third or fourth intron of the gene, or mapped upstream or downstream of it (Wanschura et al., 1996). Recently, Fejzo et al. (1996) reported seven uterine leiomyomas, all with 12q14–15 translocations, the breakpoints of which were mapped 10 to >100 kb upstream of *HMGIC* by fluorescence in situ hybridization (FISH). The existence of breakpoints outside the gene has led to the assumption that transcriptional activation per se is sufficient to induce tumor development by mechanism(s) not yet known in detail.

Interestingly, almost all tumor entities with subgroups showing *HMGIC* rearrangements also have cytogenetically defined subgroups characterized by 6p21 abnormalities, that is, of the region where *HMGIY* has been mapped (Friedmann et al., 1993). Because of the close similarity of the *HMGIY* gene to *HMGIC*, it is tempting to propose that the 6p21 aberrations are affecting *HMGIY*. This hypothesis is supported by our previous studies indicating that the 6p21 breakpoints of one uterine leiomyoma cell line (Kazmierczak et al., 1996a) and of 10 pulmonary chondroid hamartomas (PCHs) (Kazmierczak et al., 1996d) all mapped within a region of about 120 kb, including the *HMGIY* gene. These results were confirmed by two recent reports determining the breakpoints of four PCHs and one uterine leiomyoma. These studies showed one breakpoint within *HMGIY* in a PCH and extragenic breakpoints in the remaining four cases (Williams et al., 1997; Xiao et al., 1997). Detailed studies of a larger series also including lipomas and endometrial polyps are lacking so far, and molecular studies at

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the transcriptional level are still rare for *HMGIY* aberrations (Xiao et al., 1997).

The exact molecular mechanisms by which *HMGIY* contributes to the initiation of benign mesenchymal tumors is still unknown. Characterizing molecularly the 6p21 aberrations leading to *HMGIY* rearrangements in benign mesenchymal tumors could be an important step toward the elucidation of these mechanisms, and was the aim of the present study.

MATERIALS AND METHODS

Tumors

The material at our disposal for this study consisted of 36 tumors: 17 PCHs, 16 endometrial polyps, one lipoma, and two uterine leiomyomas, all showing aberrations of chromosomal region 6p21.3 (Table 1).

Southern Blot Analysis

High-molecular-weight DNAs were extracted from primary cultures or cell lines of five endometrial polyps, one leiomyoma, and three PCHs. In parallel to the DNA extractions, the karyotypes of the cell lines were rechecked for the presence of the translocation chromosomes, which were found in all of them. DNA extracted from lymphocyte cultures of healthy donors with normal karyotypes was used as a control. DNAs were digested with restriction enzymes *Bam*HI, *Eco*RI, *Hin*dIII, and *Pst*I, fractionated by agarose gel electrophoresis, and blotted onto Hybond N^+ nylon membranes. As molecular probes for *HMGIY* we used gel-purified PCR products from different positions within the gene. Probe 1 was derived from intron 3 (nt 2422–2738), probe 2 from intron 5 (nt 5668–6333), and probe 3 from the 3' UTR (nt 8600–8805 and 8805–9451). Furthermore, we cloned the flanking regions of *HMGIY* and used them as probes to detect rearrangements outside the gene. For subcloning the vector pGem-11Zf+ (Promega, Mannheim, Germany) was used. The probes were labeled with ^{32}P using a random primer extension protocol. Prehybridization and hybridization buffer consisted of 5 × SSPE/0.5% SDS, 5 × Denhardt's solution, and denatured salmon sperm DNA (0.5 mg/ml). Prehybridization was carried out for 5 hours and hybridizations for 16 hours at 65°C. Blots were washed twice for 5 minutes at room temperature in 2 × SSC/0.1% SDS, once for 30 minutes at 65°C in 1 × SSC/0.1% SDS, and for a further 30 minutes in 0.1 × SSC/0.1% SDS.

Rapid Amplification of cDNA Ends (RACE)

Amplification of 3' cDNA ends was performed using the GIBCO/BRL protocol with minor modifications. For cDNA synthesis, the adapter primer 5'-AAG GAT CCG TCG ACA TC $T_{(17)}$-3' was used. For first and secondary rounds of PCR, the universal amplification primer 5'-CUA CUA CUA CUA AAG GAT CCG TGG ACA TC-3' was used as a 'reversed primer.' The specific forward primer used in the first PCR was: 5'-TCGAGCTC-GAAGTCCAGC-3' (exon 5). In the second round of PCR, the following nested primer was used: 5'-CAU CAU CAU CAU AGG ACG GCA CTG AGA AGC-3' (exon 5). The nested specific primers and the universal amplification primer were CUA/CAU-tailed because cloning of the gel-extracted fragments was performed using the CloneAmp cloning system (GIBCO/BRL, Eggenstein, Germany). Sequence data were obtained by using an ABI PRISM 377 DNA Sequencer (Perkin–Elmer, Langen, Germany).

Assignment of the Ectopic Sequence

Chromosomal origin of the ectopic sequence was established by CASH (chromosome assignment using somatic cell hybrids) using the human/rodent somatic hybrid mapping panel 2 (Coriell Cell Repositories, Coriell Institute for Medical Research, Camden, NJ): With a primer set derived from the ectopic sequence, PCR was performed on genomic DNAs of somatic cell hybrids containing only single human chromosomes.

Fluorescence In Situ Hybridization (FISH)

To identify the chromosomes unambiguously, FISH analysis was performed after GTG-banding of the same metaphase spreads. Treatment of metaphase spreads, subsequent FISH experiments, and probe DNA labeling (with biotin-14-dATP) were performed using the protocol of Kievits et al. (1990). Chromosomes were counterstained with antifade solution consisting of DABCO (2 g/100 ml), propidium iodide (0.5 µg/ml), 0.1 M Tris-HCl, pH 8.0, 0.02% NaN_3, and glycerol (90%). As molecular probes, three different PACs and three different subclones of these PACs were used. All three PACs contained the complete sequence of *HMGIY*.

For each slide, 150 ng of labeled DNA was used. After hybridization overnight at 37°C slides were washed according to Kievits et al. (1990) and incubated for 20 minutes with avidin-FITC. Subsequently, washing steps in buffer (0.1 M Tris-HCl,

TABLE 1. FISH Data of Tumors With Rearrangements of *HMGIY* or Its Immediate Surroundings

Tumor[a]	Karyotype	*HMGIY* rearrangement (R) tested by PACs no.	Breakpoint assignment—FISH: EH2 and EH7, or PAC 8603 as molecular probes
PCH 1[b]	46,XX,t(6;14)(p21.3;q24)	R: pool 8604 + 8605	EH2, EH7: moved to der(14), Southern blot extragenic 3′ of *MHGIY*
PCH 2[b]	46,XY,t(6;14)(p21.3;q24)	R: pool 8604 + 8605	Intragenic
PCH 3[b]	46,XX,der(4)t(4;6;14)(q35;p21.3p25;q24),der(6)t(4;6)(q35;p11.2),der(14)t(6;14)(p12p21.2;q24)	R: pool 8604 + 8605	Intragenic
PCH 4[b]	46,XX,der(6)t(6;8)(p21.3;q12 or q13),der(14)t(6;14)(p21.3;q24)	R: pool 8604 + 8605	n.t.[c]; truncated transcript
PCH 5[b]	46,XX,t(6;14)(p21.3;q24)	R: pool 8604 + 8605	8603 moved to der(14), 3′ of *HMGIY*
PCH 6[b]	46,XY,t(6;10)(q21.3;q22.3)	R: pool 8604 + 8605	n.t., truncated transcript
PCH 7[b]	46,XX,t(6;14)(p21.3;q24)/46,XX	R: pool 8604 + 8605	n.t.
PCH 8[b]	46,XX,der(6)t(6;14)(p12;q24),der(14)t(6;14)(p21.2;q24),der(16)t(6;16)(p21.2p12;q24)	R: pool 8604 + 8605	EH2, EH7: moved to der(14), Southern blot extragenic, 3′ of *HMGIY*
PCH 9[b]	46,XY,der(1)t(1;14)(p34.1;q24),der(6)t(6;17)(p21.3;q23),der(14)t(6;14)(p21.3;q24),der(17)t(1;17)(p34.1;q23)	R: pool 8604 + 8605	8603 moved to der(14), 3′ of *HMGIY*, truncated transcript
PCH 10[b]	46,XX,der(6)inv(6)(p21.3q26)t(6;9)(q26;p13),der(9)t(9;14)(p13;q24),der(14)t(6;14)(p21.3;q24)	R: pool 8604 + 8605	8603 moved to der(14), 3′ of *HMGIY*
PCH 11	46,XY,t(3;6)(p25 or p26;p21.1 or p21.2),der(9)t(9;12)(q34;q15),der(12)t(12;16)(q15;q21),del(16)(q21)	R: 8603, 8605: moved to der(3)	EH2, EH7: moved to der(3), Southern blot extragenic, 3′ of *HMGIY*, truncated transcript
PCH 12	46,XX,del(6)(p21.3),der(14)(p21.3;q24)	pool 8603 + 8605: moved to der(14)	8603 moved to der(14), 3′ of *HMGIY*
PCH 13	46,XX,t(6;12)(p21.3;q22)	R: pool 8603 + 8605	8603 moved to der(12), 3′ of *HMGIY*
PCH 14	46,XY,der(14)ins(14;6)(q13;p21.3p21.3)del(14)(q22q24)	pool 8603 + 8605: moved to der(14)	8603 moved to der(14), 3′ of *HMGIY*
PCH 15	46,XY,der(1)t(1;6)(p32;p21.3),del(4)(q12), der(6)t(4;1;6)(q12;p36p21.3;p21.3)	pool 8603 + 8605: remains on der(6)	8603 remains on der(6), 5′ of *HMGIY*
PCH 16	46,XY,t(6;14)(p21.3;q24)	pool 8603 + 8605: moved to der(4)	8603 moved to der(14), 3′ of *HMGIY*
PCH 17	47,XX,t(5;6;14)(q22;p21.3;q24),+8	pool 8603 + 8605: moved to der(14)	8603 moved to der(14), 3′ of *HMGIY*
EP 1	46,XX,t(6;20)(p21;q13),der(8q)	R: pool 8603 + 8605	Southern blot, extragenic
EP 2	46,XX,del(6)(p21.3p23)	n.t.	Southern blot, extragenic
EP 3	46,XX,t(2;6;7)(q35;p23;q22)	n.t.	Southern blot, extragenic
EP 4	46,XX,t(2;6)(q35;p21)	R: pool 8603 + 8605	Southern blot, extragenic
EP 5	46,XX,inv(6)(p21q22)	n.t.	Southern blot, extragenic
EP 6	46,XX,t(6;10)(p21;q22)	n.t.	n.t., truncated transcript
EP 7	46,XX,inv(6)(p21–22q21)/46,XX	R: pool 8603 + 8605	n.t.
EP 8	46,XX,t(6;8)(p21;q12)/46,XX	R: pool 8603 + 8605	n.t.
EP 9	46,XX,add(6)(p21)/46,XX	R: pool 8603 + 8605	n.t.
EP 10	46,XX,add(6)(p25)/46,XX	R: pool 8603 + 8605	n.t.
EP 11	46,XX,t(5;6;14)(q35;p21;q?)/46,XX	R: pool 8603 + 8605	n.t.
EP 12	46,XX,t(1;6)(p32;p21)/46,XX	R: pool 8603 + 8605	n.t.
EP 13	46,XX,t(6;7)(p21;p15)/46,XX	R: pool 8603 + 8605	n.t.
EP 14	46,XX,t(6;15)(p21;q21)	R: pool 8603 + 8605	n.t.
EP 15	46,XX,t(2;6)(p22;p21)	R: pool 8603 + 8605	n.t.
EP 16	45,XX,inv(6)(p21q23)	R: pool 8603 + 8605	n.t.
lipoma	46,XX,t(3;6)(q28;p21)	R: pool 8603 + 8604	n.t.
UL 1[d]	Complex 6p21.3 aberration	R: pool 8604 + 8605	FISH/Southern blot, extragenic, 3′ of *HMGIY*
UL 2	46,XX,t(2;14;6)(p13;q24;p21.3)	R: pool 8603 + 8605	n.t.

[a]PCH, pulmonary chondroid hamartoma; EP, endometrial polyp; UL, uterine leiomyoma.
[b]J Natl Cancer Inst 88:1234–1236 (1996). Kazmierczak et al., 1996d.
[c]n.t., not tested.
[d]Genes Chromosomes Cancer 17:191–193 (1996). Kazmierczak et al., 1996a.

pH 7.5, 0.15 M NaCl, 0.05% Tween 20) followed and a second round of incubation with goat anti-avidin-biotin was applied. After a third incubation for 20 minutes at 37°C using avidin-FITC and dehydration through an ethanol series, the slides were analyzed on a Zeiss Axioplan fluorescence microscope (Oberkochen, Germany). Results of GTG-banding and FISH were processed and recorded with a Power Gene Karyotyping System (PSI, Halladale, GB).

RESULTS AND DISCUSSION

The *HMGIY* gene is 10.144 kb in length and contains eight exons and seven introns. Whereas exons 1–4 are not transcribed, exons 5–7 encode three DNA binding domains, and exon 8 encodes the acidic carboxy-terminal end. *HMGIY* is a structural transcription factor and binds to the minor groove of AT-rich DNA, thus altering DNA confirmation (e.g., by introducing bends and supercoils). Numerous examples documenting the in vivo involvement of *HMGIY* in the positive induction of gene transcription have been published. These include the genes for β-interferon, the α-subunit of the interleukin-2 receptor, and E-selectin. In addition, by binding to promoter regions of other genes including the genes for interleukin-4, and GP91-PHOX, *HMGIY* seems also to be involved in their negative regulation (for review, see Bustin and Reeves, 1996). As for its tumorigenic potential, there is some evidence now that *HMGIY* is the target of 6p21 changes observed in many benign mesenchymal tumors. This study was aimed at a more detailed molecular characterization of 6p21 aberrations in four different tumor entities. We previously reported the isolation of three different PAC clones (PAC 8603–8605) from a PAC/BAC library by PCR screening with a primer set specific for intron 3 of *HMGIY* (nt 2422–2738) (Kazmierczak et al., 1996a,d). In a first step of this study, 25 benign mesenchymal tumors with 6p21 aberrations were investigated by FISH with the PACs as molecular probes. The 6p21 breakpoints of 10 PCHs (Kazmierczak et al., 1996d), two uterine leiomyomas, one lipoma, and 12 endometrial polyps were found to map within the genomic region covered by these PACs, as revealed by split signals on both derivative chromosomes resulting from the chromosomal abnormalities (Table 1, Fig. 1). In PCH 3, the signals were moved to chromosomes 4 and 14, respectively—as reported previously (Kazmierczak et al., 1996d). For PCH 11, 12, 14, 15, 16, and 17, PACs 8603 and 8605 did not show split signals, whereas in PCH 15, the signals remained on the der(6) in cases 11, 12, 14, 16, and 17 and the signals were moved to the translocation partners of the der(6). By cloning various *Bam*HI fragments downstream of *HMGIY* (EH7, EB19, and EB13), we were able to show that the genomic sequence of PAC 8603 ends 6.2 kb after the 3' end of *HMGIY* (Fig. 2). By subcloning the flanking regions of the *HMGIY* gene we established new probes for mapping the breakpoints. We established two different *Bam*HI fragments: one containing nt 1–6313 of *HMGIY* and about 4.3 kb sequences 5' outside the gene (EH2), and one containing nt 6588–10144 of *HMGIY* and about 1.5 kb 3' outside the gene (EH7). By two-color FISH with EH7 (3' end of *HMGIY*) and a 8.5 kb *Bam*HI fragment (B9) 5' upstream of *HMGIY*, we were able to show that the transcriptional orientation was directed toward the centromere. As the PAC 8603 sequence ends about 6.2 kb 3' of *HMGIY*, we performed FISH studies using this PAC alone as a probe on PCH 9, 10, 12, 13, 14, 15, 16, and 17. In PCH 15 the signals remained on the der(6), confirming the results obtained with PAC 8603 and 8605 as a pool and showing that in this tumor the breakpoint is located 5' of *HMGIY*. In all other PCH tested with PAC 8603 as a molecular probe, the signals moved to the translocation partners of der(6) indicating that in these tumors the breakpoints are located 3' of *HMGIY*. By FISH on metaphase spreads of the uterine leiomyoma UL1 and the pulmonary hamartomas PCH 1, 8, and 11 (Table 1) with EH2 and EH7 as separate probes, the signals for both probes were assigned to the translocation partners of chromosome 6 denoting that the breakpoints were extragenic, and that the complete *HMGIY* gene was moved to the translocation partners. The transcriptional orientation of the gene along with these results allows for the conclusion that the breakpoints of 11/12 PCH tested so far were located 3' of *HMGIY*, and only one was located 5' of *HMGIY*.

Subsequently, Southern blot analyses of DNAs from one uterine leiomyoma cell line (UL1), three pulmonary hamartomas (PCH 1,8,11), and five primary endometrial polyps (EP1–5), all with 6p21.3 aberrations (Table 1) were performed for detection of intragenic *HMGIY* rearrangements. In none of the cases was a rearrangement detected by using the PCR products from intron 3, intron 5, and 3'UTR as probes (Fig. 3). In addition, we used the two *Bam*HI fragments EH2 and EH7 as probes for Southern blot analysis. No detectable rearrangement was found with any of the probes. We then isolated another 4.7 kb fragment (B41) upstream of *HMGIY* and EH2 as a molecular probe for Southern

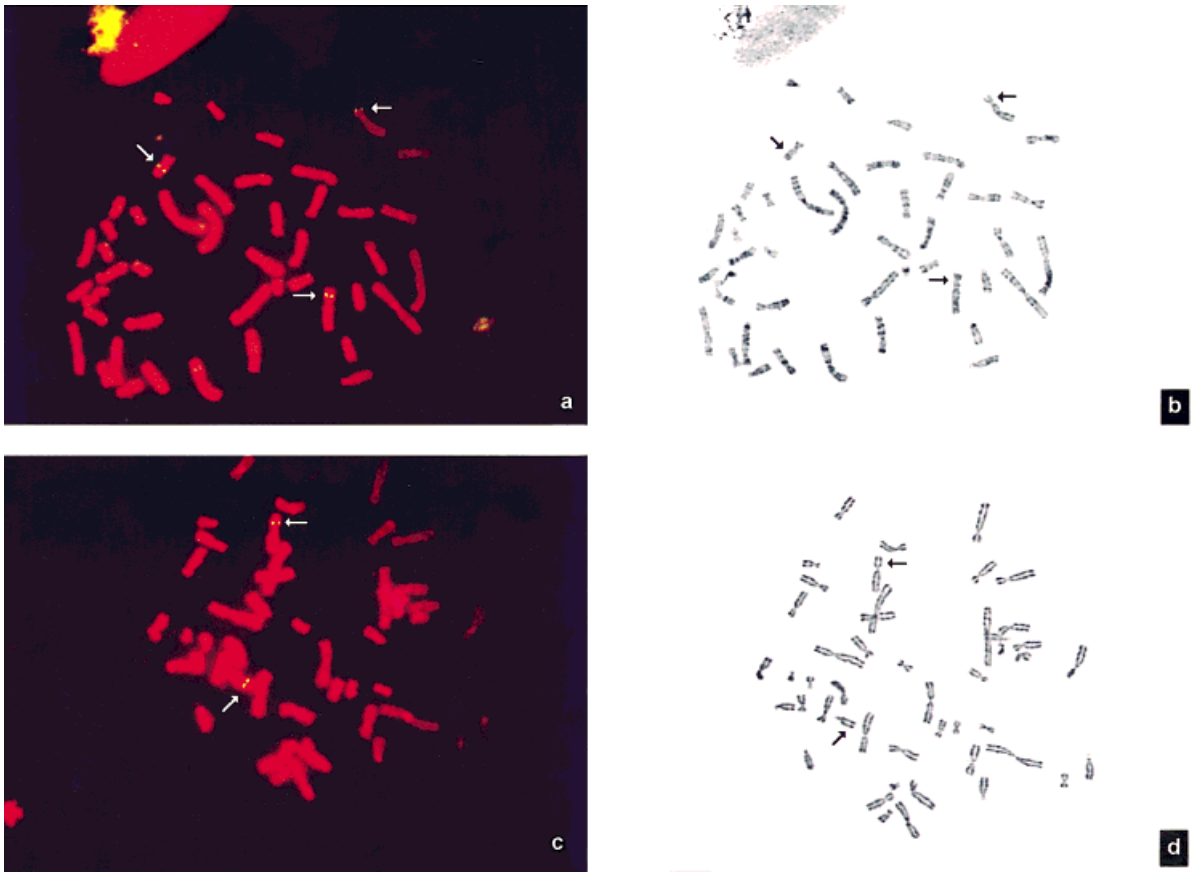

Figure 1. Localization of the PAC clones spanning the breakpoint region 6p21.3 in metaphases of one endometrial polyp and one chondroid hamartoma. **a:** Metaphase spread of the endometrial polyp EP1 with a t(6;20). PACs 8603 and 8605 were used as a pool, and signals are visible on the normal chromosome 6 and on the derivative chromosomes 6 and 20 due to the translocation (signals are indicated by arrows). **b:** Same metaphase spread of EP1 GTG-banded prior to FISH (normal and aberrant chromosomes 6 and der(20)) are indicated by arrows. **c:** Metaphase spread of PCH 17 using PACs 8603 and 8605 as probes. Signals are visible on normal chromosome 6 and on the derivative chromosome 14 (signals are indicated by arrows). **d:** Same metaphase spread of PCH 17 GTG-banded prior to FISH (normal chromosome 6 and der(14) are indicated by arrows). Chromosomes were stained with propidium iodide, and PACs were labeled with FITC.

blot hybridization. These fragments again did not detect a rearrangement. Previously, we reported on two pulmonary hamartomas with intragenic *HMGIY* rearrangements (PCH 2 and 3) (Kazmierczak et al., 1996d). An intragenic rearrangement of *HMGIY* has also been detected by Xiao et al. (1997). However, this analysis of 36 benign mesenchymal tumors indicates that the breakpoints of most tumors with 6p21.3 aberrations are located 3' of *HMGIY*.

In addition to Southern blot hybridization, 3'-RACE PCR experiments were performed. HeLa cells served as controls revealing 3'-RACE clones representing perfect partial cDNA copies of 3' *HMGIY* mRNA sequences after sequence analysis. In contrast, truncated transcripts were detected in 4 PCHs (PCH 4, 6, 9, 11), one endometrial polyp (EP 6), and one uterine leiomyoma (UL 1) (Table 1). A schematic presentation of the aberrant transcripts is given in Figure 4. All aberrant RACE clones were lacking a part of 1285–1447 bp, mostly from the 3'UTR in contrast to the normal *HMGIY* transcript found in HeLa cells. None of the clones had an intact 3'UTR region. PCH 6 with a t(6;10)(p21;q22) showed a deletion in the 3'UTR at nt 8692, then 87 bp of an ectopic sequence preceded the poly A tail. A homology search of that ectopic sequence showed 100% homology to the 3' end of two Homo sapiens cDNA clones derived from a cDNA library of the WashU-Merck EST project. With a primer set derived from the ectopic sequence and CASH we were able to map this sequence back to chromosome 6. Therefore, it can be assumed that this t(6;10) at the molecular level is not a simple reciprocal translocation, but a more complicated translocation with an additional small inversion of 6p not visible at the cytogenetic level.

For benign mesenchymal tumors with aberrations of *HMGIC*, a frequent event is the loss or

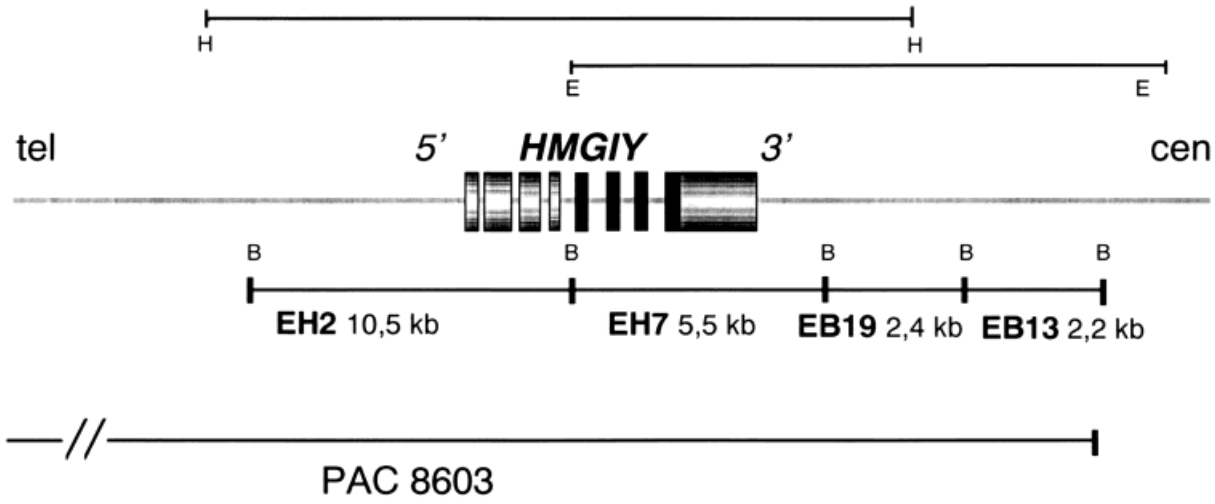

Figure 2. Partial restriction map of PAC 8603. The *HMGIY* gene is presented schematically with its genomic orientation. Black solid lines represent different cloned fragments used for FISH analysis. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII.

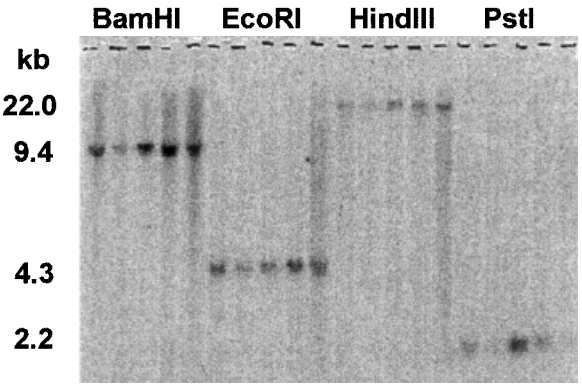

Figure 3. Southern blot analysis of genomic DNA from PCH 1 (lanes 1, 6, 11, and 16), PCH 8 (lanes 2, 7, 12, and 17), PCH 11 (lanes 3, 8, 13, 18), the uterine leiomyoma (lanes 4, 9, 14, and 19), and the control (lanes 5, 10, 15, and 20). DNA was digested with *Bam*HI, *Eco*RI, *Hin*dIII, and *Pst*I. Molecular probe: PCR product located in intron 3 (nt 2422–2738). Molecular weight markers are indicated.

replacement of the part encoding its carboxy-terminal end (exons 4–5). Losses of the carboxy-terminal end have been described as enhancing the ability of mutant *HMGIY* proteins to introduce negative supercoils 8–10 times more effectively than native proteins (Nissen and Reeves, 1995). For *HMGIY*, we also found transcripts missing exons 7 and 8, although the genomic structure of the translocations is not directly comparable to the situation described for *HMGIC*. For *HMGIC*, the breakpoints are frequently located within intron 3 (Wanschura et al., 1996), probably due to a cluster of RTVLH-related sequences located within intron 3 (Kazmierczak et al., 1996b). Nevertheless, breaks 5' upstream of *HMGIC* have been described in cases with t(12;14)(q15;q24) as well, and 14q24 is also one of the main translocation partners for 6p21 aberrations in PCH. For those cases, a reactivated or enhanced transcription has been proposed as a mechanism of tumor initiation (Fejzo et al., 1996; Wanschura et al., 1996). In 11/12 PCH and one uterine leiomyoma with aberrations of the chromosomal band 6p21 tested in this study (by the two probes EH2 and EH7, and PAC 8603 alone), the complete *HMGIY* gene was moved to the translocation partners. As a consequence, negative regulators located on chromosome 6 in the proximity of *HMGIY* may have been replaced by enhancers from the translocation partners. Disruption of regulatory sequences has also been found (e.g., in Burkitt lymphoma), where breakpoints more than 100 kb upstream of *MYC* result in aberrant expression and tumorigenesis. Because the translocations were not always accompanied by altered transcripts detectable by 3'-RACE PCR experiments, our data presented here support the idea that transcriptional up-regulation of the *HMGIY* gene per se may be sufficient to induce the development of frequent benign mesenchymal tumors. Aberrant transcripts like those detected in the present study resulting from breakpoints outside the gene may have additional effects.

In summary, as for the genesis of frequent mesenchymal tumors, the results of this study strictly underline the functional similarity between particular mutations of *HMGIC* and *HMGIY*. Furthermore, this is the first report comparing the involvement of the same region 6p21.3 in four

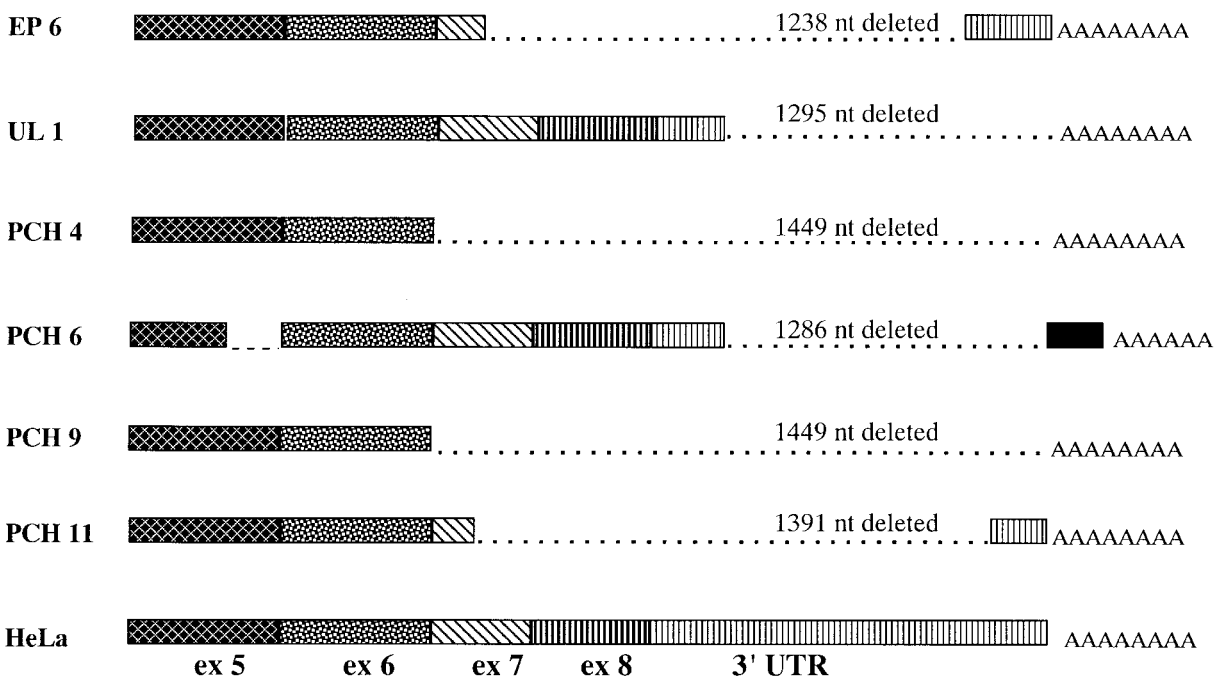

Figure 4. Schematic representation of truncated transcripts found by 3'-RACE analyses on four hamartomas, one endometrium polyp, and one uterine leiomyoma. The dotted line indicates the deleted sequences. The dashed line in PCH 6 indicates the 33-bp HMGIY-specific deletion. Black box before poly A tail in PCH 6: ectopic sequence fused to *HMGIY* transcript.

different tumor entities of benign mesenchymal tumors.

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