Universität Bremen

Dysregulation of the high mobility group AT-hook 2 (*HMGA2*) gene in human tumours

Dissertation to obtain the degree „Doctor rerum naturalium“

submitted by
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„Lernen ist Erfahrung,
alles andere ist einfach nur Information."

Albert Einstein

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1. Introduction

In the development of cancer, many genes and pathways that are needed in embryogenesis to successfully undergo the replication and differentiation steps essential to grow from the zygote to cells of various shape and function are reactivated. Tumour cells thus become equipped with the ability to grow in an uncontrolled fashion and to metastasize. Among these oncofoetal genes is the family coding for the high mobility group AT-hook (HMGA) proteins with its two members *HMGA1* and *HMGA2*.

The HMGA proteins belong to a group of nuclear nonhistone proteins with a size smaller than 30 kDa that were discovered in proteins extracted from calf thymus chromatin with 0.35 M NaCl. They were named in regard to their high mobility in polyacrylamide gel electrophoresis (PAGE) (Goodwin et al. 1977). HMGA2 was originally described as high-mobility group (nonhistone chromosomal) protein isoform I-C (HMGIC) and consists of three highly basic AT-hooks serving as functional domains, an acidic C-terminal domain and potential phosphorylation sites within the whole amino acid sequence indicating regulation possibilities (Manfioletti et al. 1991). It is encoded by a gene consisting of five exons mapping to chromosomal band 12q15 (Chau et al. 1995). HMGA2 proteins do not possess any secondary structure when free in solution (Cleynen and Van de Ven 2008), and specifically bind to the minor groove of AT-rich sequences of B-form DNA (Giancotti et al. 1991; Cui et al. 2005), as it was first observed for the closely related high mobility group AT-hook 1 proteins (HMGA1, formerly known as HMGIY) (Solomon et al. 1986) belonging to the same gene family. As HMGA1 and HMGA2 are highly similar proteins carrying the same characteristic functional domains and mainly differ in the distance of the three AT-hooks (Cleynen and Van de Ven 2008), a similar mode of operation for these proteins is suggested. HMGA2 is a protein with a length of 109 amino acids (aa) being translated from an mRNA with a length of 4150 bases. It is described as an architectural transcription factor that is not able to initiate transcription per se, but can enhance or suppress expression of targeted genes.

Apparently, HMGA2 generally acts by several mechanisms as a regulator of gene expression. One of the best studied mechanisms comprises interaction of the protein with DNA. In this context, the AT-hooks represent the functional domain, which specifically bind to the minor groove of AT-rich B-form DNA sequences. As a

consequence, DNA is bent thus facilitating or impairing the assembly of the transcription initiator complex and the start of transcription. This was described for the binding of HMGA2 to e.g. the promoter of the *ERCC1* gene (excision repair cross-complementing rodent repair deficiency, complementation group 1 (includes overlapping antisense sequence)) (Borrmann et al. 2003), or the region -400 base pairs (bp) to -600 bp from the transcription start site of the *ATM* (ataxia telangiectasia mutated) gene (Palmieri et al. 2011), both of which result in altered expression. HMGA2 binding to AT-rich sequences in the genomic DNA is not restricted to the promoter region alone, but can also be found in other gene-regulatory regions being located in for example introns, as seen in the regulation of *Igf2bp2* (insulin-like growth factor 2 mRNA binding protein 2) (Cleynen et al. 2007). This regulation involves also cooperation with NF-κB (nuclear factor of kappa light polypeptide gene enhancer in B-cells), which binds to a consensus binding site immediately adjacent to the AT-rich regulatory region of HMGA2, and its binding is enhanced by attachment of HMGA2 (Cleynen et al. 2007).

HMGA2 does not only bind to DNA but is also able to directly interact with other proteins to influence gene expression which thus represents a further mechanism of transcriptional regulation. Again, the AT-hooks represent the functional domains of HMGA2 that e.g. interacts with the activated, i.e. dephosphorylated form of pRB (retinoblastoma protein) by binding to the N-terminal domain or the pocket region between amino acids 703-737 of pRB involving its second AT-hook (Fedele et al. 2006). In consequence, HMGA2 replaces HDAC1 (histone deacetylase 1) from the pRB/E2F1 (E2F transcription factor 1) complex at E2F1 target promoters resulting in a more open chromatin structure by recruitment of acetylases and thus enhanced histone acetylation with initiated/increased transcription. E2F1 itself is acetylated as well and thus stabilised (Fedele et al. 2006). Protein-protein interaction of HMGA2 in DNA repair mechanisms has recently been found in the ATR (ataxia telangiectasia and Rad3 related)-CHEK1 (checkpoint kinase 1) pathway, as HMGA2 is involved in a complex with phosphorylated (p)ATR/pCHEK1, which is not altered by DNase digestion after co-immunoprecipitation suggesting a DNA independent complex (Natarajan et al. 2013).

Concerning post-translational modifications (PTMs) of the HMGA proteins impacting their function, the main focus in research up to date has been set on HMGA1 that undergoes acetylation, phosphorylation and methylation affecting its binding

properties (for review see Cleynen and Van de Ven 2008). As to HMGA2, PTMs include phosphorylation on the SQ-motif serine 102/glutamine 103 of HMGA2's carboxy-terminus by the ATM kinase that in turn results in transcriptional enhancement of this gene (Palmieri et al. 2011). Likewise, HMGA2 can be highly phosphorylated at the acidic C-terminal domain affecting its binding properties (Sgarra et al. 2009).

In its function as an architectural transcription factor, HMGA2 impacts a variety of cellular mechanisms. Among the first ones found to be influenced by HMGA2 was growth, as mice carrying mutations which led to absent *Hmga2* expression developed the so called pygmy phenotype resulting in dwarfism (Zhou et al. 1995). Vice versa, overexpression of a truncated Hmga2 lacking the acidic C-tail but including the three AT-hooks led to gigantism associated with lipomatosis in mice (Battista et al. 1999). In humans, HMGA2 also is involved in the determination of body height (Weedon et al. 2007), shown by the 12q14 microdeletion syndrome which, amongst others, is associated with low birth weight and short stature (Buysse et al. 2009; Lynch et al. 2011). Different SNP (single nucleotide polymorphism) analyses identified *HMGA2* as one of the responsible genes for the determination of adult height (Weedon et al. 2008; Yang et al. 2010) and constitutional rearrangement of *HMGA2* resulted in overgrowth associated with lipomas (Ligon et al. 2005). Further involvement of HMGA2 in cell proliferation has been revealed by its expression in preadipocytic cells as a response to incubation with growth factors or foetal bovine serum in mesenchymal cells being subjected to a mid-G_1 regulation as delayed early response genes (Ayoubi et al. 1999).

Hmga2 overexpression resulting in gigantism in combination with lipomatosis in mice (Battista et al. 1999), the pygmy phenotype displaying enormous reduction in fat tissue (Zhou et al. 1995), and *Hmga2* expression in fat depots of mice on a high fat diet (Anand and Chada 2000) led to the discovery of its involvement in the differentiation of adipocytes (Ayoubi et al. 1999). Besides, HMGA2 is involved in the differentiation of other cell types such as cardiomyocytes (Monzen et al. 2008) and chondrocytes (Kubo et al. 2006) and impacts developmental processes like the formation of myotubes (Li et al. 2012).

Furthermore, HMGA2 has been shown to influence DNA repair mechanisms, as it is involved in nucleotide excision repair (Borrmann et al. 2003), base excision repair,

(Natarajan et al. 2013) and also participates in the ATM pathway (Palmieri et al. 2011).

Finally, HMGA2 is involved in the maintenance of stemness of cancer stem cells (Dröge and Davey 2008) sustaining an undifferentiated cell state in e.g. breast cancer in linkage with the let-7 microRNAs (Yu et al. 2007) or miR-93 (Liu et al. 2012), and has impact on cell senescence as well (Nishino et al. 2008; Markowski et al. 2011; Markowski et al. 2011; Yu et al. 2012).

From this vast and broad range of cellular processes and functions that are influenced by HMGA2, it is obvious that a tight and strict regulation of its expression is required. Up to date, several regulatory mechanisms are known. Among the first ones to be discovered was the influence of the 3'UTR (3'untranslated region). Reporter assays showed an up to 12.7-fold decrease in luciferase activity upon presence of the 3'UTR pointing to a negative regulatory influence of this element, which was originally thought to be caused by AUUUA motives causing destabilization of the mRNA (Borrmann et al. 2001). It has later been shown that the *HMGA2* 3'UTR harbours seven conserved binding sites for the let-7 microRNA family which proved to be the underlying mechanism of this negative *HMGA2* regulation (Mayr et al. 2007; Lee and Dutta 2007). Consistently, expression patterns of *HMGA2* and let-7 during embryonic development are opposite to each other, i.e. *HMGA2* becomes down-regulated upon transcriptional activation and processing of *MIRLET-7* (Mayr et al. 2007). In the recent years, additional miRNA binding sites have been discovered in the *HMGA2* 3'UTR as e.g. miR-16, miR-17 (Palmieri et al. 2012), miR-23a (Lee et al. 2011), miR-26a (Lee et al. 2011; Palmieri et al. 2012), miR-30a (Lee et al. 2011), miR-33a (Rice et al. 2013), miR-93 (Liu et al. 2012), miR-98 (Hebert et al. 2007), miR-154 (Zhu et al. 2013), miR-196a2 (Palmieri et al. 2012) as well as miR-365 (Qi et al. 2012). Though *HMGA2* gene expression is regulated by a huge number of miRNAs, its expression does not only depend on this mechanism, but also has been shown to positively correlate to the length of a $TC_{(n)}$-repeat being located 500 bp upstream of the initial ATG start codon (Borrmann et al. 2003; Hodge et al. 2009). So far, there is only one study addressing epigenetic regulation by methylation of CpG (cytosine phosphate guanine)-islands and surrounding SNPs of *HMGA2* which display differential methylation in type 2 diabetes, but no association with altered splicing or gene function was found (Dayeh et al. 2013).

Generally, tumour initiation and growth is caused by one or several events that disturb the balanced gene expression profile and lead to proliferation and/or dedifferentiation of originally differentiated, resting cells. Chromosomal aberrations affecting the *HMGA2* locus on 12q14~15 resulting in re-expression of the intrinsically silenced embryonic gene are well known in a variety of benign mesenchymal tumours.

In lipomas, different translocations have been described affecting the *HMGA2* locus. Herein, the breakpoint often is located in intron 3 of the *HMGA2* locus (Ashar et al. 1995) separating the 3'UTR as regulatory unit from the open reading frame (ORF), which leads to a lost silencing effect due to the missing miRNA binding sequence and hence re-expression of *HMGA2* (Mayr et al. 2007). This is also true in the translocation t(3;12), which leads to expression of a *HMGA2/LPP* (LIM domain containing preferred translocation partner in lipoma) fusion gene (Petit et al. 1996; Bartuma et al. 2009). Interestingly, lipomas also display breakpoints 5' to *HMGA2* (Nilsson et al. 2006), which is a more common feature in uterine leiomyomas (UL) (Quade et al. 2003), but the activating mechanism is not fully elucidated yet. 50-80 % of UL are cytogenetically inconspicuous, but the subgroup of tumours carrying translocations involving 12q14~15 accounts for approximately 27 % of tumours with chromosomal aberrations (Nilbert and Heim 1990). *HMGA2* has been identified as the target of these translocations in 1995 (Schoenmakers et al. 1995). However, *HMGA2* overexpression in UL is generally found in comparison to the matching myometrial tissue (Klemke et al. 2009) and the common feature of *HMGA2* overexpression in benign mesenchymal tumours suggests a crucial role for HMGA2 in the development of these tumours.

In malignant tumours, the underlying causes for re-expression of *HMGA2* differ from those in benign cancer. *HMGA2* is frequently overexpressed in a variety of malignant epithelial tumours such as e.g. non-small cell lung cancer (Meyer et al. 2007), colorectal cancer (Huang et al. 2009), where increasing expression is associated with tumour invasiveness (Rizzi et al. 2013), ovarian serous carcinoma (McMillen et al. 2012; Hetland et al. 2012), canine prostate cancer (Winkler et al. 2007) and others. However, reports on chromosomal rearrangements as the reason for this differential expression are rare. Amplifications of the *HMGA2* locus accompanied by its overexpression have been reported in e.g. well-differentiated liposarcomas and derived cell lines (Tallini et al. 2000; Trahan et al. 2006; Persson et al. 2008;

Pedeutour et al. 2012), in uterine leiomyosarcoma (Cho et al. 2005), in high-grade serous ovarian carcinoma (Helland et al. 2011) and in carcinoma ex pleomorphic adenoma (Persson et al. 2009).

As expression of *HMGA2* is part of the mesenchymal stem cell program, its re-expression in adult epithelial cells can be critical by promoting epithelial-mesenchymal transition (EMT) thus enhancing the aggressiveness and metastatic potential of the developing tumour (Zha et al. 2013). The pathway that seems to be targeted by HMGA2 enabling EMT possibly is the WNT/CTNNB1 (wingless- type MMTV integration site family/ catenin (cadherin-associated protein), beta 1, 88 kDa) pathway (Zha et al. 2012). Silencing of *Hmga2* in KRAS (v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog)-transformed rat ovarian surface epithelial cells led to partial reversal of EMT (Schäfer and Sers 2011) and HMGA2 has been localised to the invasive front of tumours enhancing metastatic behaviour and invasiveness by supporting the canonical TGF-beta-1 (transforming growth factor beta-1) pathway via up-regulation of TGFR2 (transforming growth factor-beta type 2) (Morishita et al. 2013).

The findings that HMGA2 plays a prominent role in a variety of human and mammalian cancers make it a promising target in cancer therapeutic strategies. Therefore, it is essential to understand the main causes that underlie reactivation and expression in the distinct cancer types and in benign tumours. As to ULs, the breakpoints upstream of *HMGA2* caused by the translocation t(12;14) that do not lead to separation of the 3'UTR from the rest of the gene are not yet fully characterised. Microdeletions accompanying the microscopically balanced aberration may thus play a role in activation of the *HMGA2* gene.

Herein, the method of array based comparative genomic hybridisation (aCGH) was used to identify possible loss of genetic regulatory elements being too small for detection in conventional cytogenetics due to limited resolution in tumours displaying these aberration. ACGH provides the possibility for the detection of numerical chromosomal aberrations as small as ~10 kb depending on the resolution of the array. An array with an intermediate spacing of ~8.9 kb and ~7.4 kb for RefSeq genes was chosen for the investigation of ULs displaying a t(12;14) assessed in previous conventional cytogenetic studies (Klemke et al. 2009). The objective was to identify microdeletions and/or amplifications accompanying the breakpoints.

HMGA2 usually is expressed during embryogenesis in cells without any cytogenetic aberrations as a part of their development and can be activated in mesenchymal cells by adequate stimuli such as growth factors (GFs; Ayoubi et al. 1999). However, little is known about the behaviour of *HMGA2* in response to GFs in cancer cells, which was yet another focus of this thesis. The influence of GFs and foetal bovine serum (FBS) on *HMGA2* expression was investigated in the prostate cancer cell line PC-3 to address the question about similarities in the up-regulation of *HMGA2* in cells of mesenchymal origin and malignant epithelial cells. A possible correlation between *HMGA2* expression and proliferation was investigated by real-time RT-PCR and a proliferation assay. The PC-3 cell line displays a moderate *HMGA2* expression that allows for the detection of effects induced by GFs or FBS that might correlate to a possible increase in cell proliferation. The same issue was addressed in human umbilical vein endothelial cells (HUVECs) that play a role in the development of cancer and growth, as, with increasing size of the tumour, angiogenesis and thus the building of new vessels to ensure oxygen supply and nutrition of the growing tumourous tissue becomes necessary.

Interestingly, the levels of *HMGA2* vary between tumours of the same entity or cell lines derived thereof, ranging from complete abrogation to high expression and the mechanisms by which this is achieved might be various. The mRNA level in the prostate cancer cell line LNCaP is undetectable even by a sensitive method such as real-time RT-PCR highlighting the strict silencing of this gene in this cell line. On this account, the consequences of HMGA2 incubation on these cells were assessed by proliferation, cytotoxicity and apoptosis assays. Furthermore, different silencing mechanisms and their attenuation were investigated. In consideration for *HMGA2* silencing is its knock-down by let-7, so that DICER1, a protein involved in miRNA processing, was reduced and *HMGA2* expression was measured by real-time RT-PCR. Additionally, due to the limited data available about the methylation status of *HMGA2*, its regulation by this epigenetic mechanism is of interest in cell lines reflecting regulation *in vivo*. To identify possible influence of hypermethylation that is responsible for the non-expression of *HMGA2*, the cell line was treated with 5-Aza-2'-deoxcytidine (5-AdC), a methyltransferase inhibiting agent that allows the demethylation of proliferating cells. Trichostatin A (TSA), a histone deacetylase inhibitor, was used to supplement the experimental setup.

2. Materials and methods

2.1 Cell lines and primary cells

The human prostate cancer cell lines PC-3 and LNCaP were obtained from Cell Lines Services, Eppelheim, Germany. Pooled human umbilical vein endothelial cells (HUVECs) were purchased from Lonza, Cologne, Germany. The MCF-7 breast cancer cell line was obtained from PD Dr. G. Belge (Centre for Human Genetics, University of Bremen, Germany).

2.2 Tissues

Tissues were taken during surgery and snap-frozen in liquid nitrogen for further analysis. All patients gave their written consent.

2.3 Plasmid DNA isolation from bacterial cultures

Plasmid DNA was isolated from *Escherichia coli* JM109 cells transformed with the eukaryotic expression vector pCR3.1 (Life Technologies, Darmstadt, Germany) either containing the human *HMGA2* wild-type coding sequence (CDS) or the empty vector (Fedele et al. 1998). Briefly, a starter culture was prepared, allowed to grow for 6-7 h and used for inoculation of an overnight culture. The next morning, plasmid DNA was then isolated using the NucleoBond Xtra Plus EF Kit (Macherey-Nagel, Düren, Germany).

2.4 Cell culture

Cell lines were cultivated either in Medium 199 (Life Technologies) supplemented with 20 % foetal bovine serum (FBS; Life Technologies), RPMI 1640 (Life Technologies) supplemented with 10 % FBS, EMEM (Eagle's Minimum Essential Medium) containing 10% FBS (Cell Lines Services) or in DMEM (Dulbecco's Modified Eagle Medium)/Ham's F12 (1:1) supplemented with 10 % FBS (Cell Lines Services). LNCaP cells were cultivated in RPMI 1640 with 20 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) and 0.85 g/l $NaHCO_3$ (PAN-Biotech, Aidenbach, Germany) supplemented with L-Glutamine (Biochrom AG, Berlin, Germany) and

10 % FBS. HUVECs were maintained in endothelial growth medium (EGM)-2 (Lonza). When grown till confluence, cells were detached using TrypLE Express (Life Technologies) and passaged.

2.4.1 Stimulation with growth factors and foetal bovine serum

For stimulation with growth factors or foetal bovine serum, cells were seeded in Multidish 6 Well Plates (Nunc, Wiesbaden, Germany) for RNA isolation or in Microplates 96 Well (Greiner BioOne, Frickenhausen, Germany) for proliferation assay and allowed to attach for 24 h. Thereafter, cells were serum starved with the respective medium supplemented with 1 % FBS only for another incubation period of 24 h. Starvation medium then was replaced by medium either supplemented with 1 % FBS (control), 10 % or 20 % FBS, 1 % FBS + 25 ng/ml FGF1 (Jena Bioscience, Jena, Germany) or 1 % FBS + 25 ng/ml PDGF-BB (platelet-derived growth factor beta polypeptide; Sigma-Aldrich, Munich, Germany) followed by incubation for 12 h or 24 h. Cells were then either detached for subsequent RNA isolation or, for proliferation assay, grown for another 24 h in a final concentration of 10 µM 5-bromo-2-deoxyuridine (BrdU) added to the medium.
HUVECs were seeded in EGM-2 and serum starved with endothelial basal medium (EBM)-2 (Lonza) supplemented with 1 % FBS (Lonza). Stimulation was done with EBM-2 supplemented with 1 % FBS + 25 ng/ml FGF1, FGF2 (kindly provided by the University of Veterinary Medicine, Hanover) or VEGFA (Promokine, Heidelberg, Germany).

2.4.2 Treatment with 5-Aza-2′-Deoxycytidine and Trichostatin A

For demethylation of LNCaP cells to investigate the methylation status of *HMGA2*, 150,000 cells were seeded in Multidish 6 Well Plates (Nunc) the day before treatment and allowed to attach. 5-AdC (Merck Millipore, Darmstadt, Germany) was applied at a concentration of 5 µM, 10 µM or 50 µM diluted in RPMI/HEPES and incubated for 144 h overall. After the first 72 h, medium was refreshed followed by another 72 h incubation period. A non-treated control and a control treated with the same amount of the vehicle DMSO (dimethyl sulfoxide; Sigma-Aldrich) were included. After 144 h, cells were lysed for subsequent RNA isolation. For simultaneous incubation of LNCaP cells with 5-AdC and TSA (Merck Millipore), 50 nM TSA were added 48 h

before cell lysis. The equivalent amount of DMSO was added to the respective control.
TSA alone was added 24 h after cell seeding and incubated for 48 h followed by RNA isolation.

2.4.3 Incubation of LNCaP cells with recombinant HMGA2

The influence of recombinant HMGA2 (for production details, see Richter et al. 2009) on LNCaP cells was tested in 96 well plates. Therefore, cells were seeded (6,300 for cell viability and cytotoxicity, 12,500 for apoptosis detection) and grown over night. The next morning, growth medium was replaced by the same culture medium supplemented with HMGA2 in concentrations of 1 µg/ml; 10 µg/ml and 100 µg/ml. After 24 h and 48 h, respectively, cells were analysed following the appropriate protocol.

2.4.4 Transfection with siRNA

LNCaP cells were seeded in Multidish 6 Well Plates (Nunc) and allowed to attach for ~0.5 h. Thereafter, a cocktail of four different siRNAs (Qiagen, Hilden, Germany) targeting *DICER1* (dicer 1, ribonuclease type III) was used at a final concentration of 2.5 nM each in combination with 4 µl RNAiMAX (Life Technologies). 72 h after transfection, cells were detached and 65 % of the cell suspension was seeded out and another transfection was performed. After another 72 h, cells were lysed in Qiazol (Qiagen) for subsequent miRNA isolation.

2.4.5 Transfection with DNA

LNCaP cells were transfected with a mammalian expression vector containing the CDS for the wild-type *HMGA2* or the empty vector alone. Lipofectamine LTX Plus (Life Technologies) was used as transfection reagent. Briefly, cells were seeded and allowed to attach. Meanwhile, transfection complexes were prepared using 9.25 µl Lipofectamine and 2.5 µg vector DNA for transfections in 6 well plates, and adapted for 96 well plates for the respective viability or cytotoxicity assays. After adding the transfection complexes, the cells were grown for 24 h or 48 h and then lysed for RNA isolation or tested for vitality.

2.4.6 Proliferation assay (BrDU-ELISA)

Proliferation of stimulated living cells was assessed using the Cell Proliferation ELISA, BrdU (colorimetric) kit (Roche Diagnostics, Mannheim, Germany) following the manufacturer's instructions. Absorbance was measured using a Synergy HT Multi-Mode Microplate Reader and the corresponding KC4 software (BioTek Instruments, Bad Friedrichshall, Germany). Analysis was performed with Microsoft Excel (Microsoft, Unterschleißheim, Germany).

2.4.7 Cell viability and cytotoxicity assays

Cell viability was tested using the Cell Proliferation Reagent WST-1 (Roche Diagnostics) according to the manufacturer's instructions. Cell death was measured using the Cytotoxicity Detection Kit^{PLUS} (LDH) (Roche Diagnostics) following the original protocol.

2.4.8 Apoptosis detection assay

Apoptosis in LNCaP cells incubated with recombinant HMGA2 was detected using the Caspase-Glo 3/7 assay (Promega, Mannheim, Germany) following the manufacturer's instructions.

2.5 RNA/miRNA isolation

RNA and miRNA were isolated from tissues or cultured cells using the RNeasy Mini Kit (Qiagen) or the miRNeasy Mini Kit (Qiagen), respectively, according to the manufacturer's instructions. The optional DNase I digestion was included into the protocol.

2.6 cDNA synthesis

Total RNA (250 ng) was reverse transcribed using 150 ng random hexamers (Life Technologies), 200 u M-MLV (Moloney Murine Leukaemia Virus)-Reverse Transcriptase (Life Technologies) and 40 u RNase Out (Life Technologies) following the manufacturer's instructions.

2.7 Real time RT-PCR

Real time RT-PCR was used for the quantification of mRNA amounts relative to total RNA. PCR was run on a 7300 Real-Time PCR system (Life Technologies) using the Sequence Detection Software version 1.2.3 (Life Technologies) that also served for analysis, as well as Excel (Microsoft). The TaqMan Universal PCR Mastermix (Life Technologies) was used in combination with commercial assays for *HMGA2* (Hs00171569_m1) and *DICER1* (Hs00229023_m1). Hypoxanthine phosphoribosyl-transferase 1 (*HPRT1*; fw5'-GGC AGT ATA ATC CAA AGA TGG TCA A-3', rev5'-GTC TGG CTT ATA TCC AAC ACT TCG T-3', probe5'-6-FAM-CAA GCT TGC TGG TGA AAA GGA CCC C-TAMRA-3'; Biomers, Ulm, Germany) or 18S rRNA (fw5'-GGA TCC ATT GGA GGG CAA GT-3', rev5'-AAT ATA CGC TAT TGG AGC TGG AAT TAC-3', probe5'-6-FAM-TGC CAG CAG CCG C-MGB-3'; Life Technologies) were used as endogenous controls. When using 18S rRNA for normalisation of the mRNA amount, cDNA was diluted 1:10.

2.8 Chromosome preparation

For chromosome preparation, metaphases were arrested using 300 ng colcemid (Biochrom) and incubated for ~1 h at 37 °C. Cells were then detached using TrypLE Express (Life Technologies) and incubated in 8 ml hypotonic solution (1:7) for 20 min. Thereafter, cells were centrifuged for 10 min at 1000 rpm. Supernatant was aspirated and the pellet resuspended. Fixation was done with ice cold fixative (methanol:acetic acid 3:1) and repeated three times.

2.9 Giemsa banding

For GTG-banding, 15 mg trypsin were dissolved in prewarmed banding buffer for 8 min at 37 °C. Slides were then incubated for 8 sec followed by an incubation in 1.5 % Giemsa solution prepared with 1.5 ml Giemsa, 5 ml ethanol, 10 ml Giemsa buffer and aqua bidest ad 100 ml for 10 min at room temperature. Washing was done twice in aqua bidest. Metaphases were photographed on an Axioplan microscope (Carl Zeiss, Jena, Germany) using BandView software (Applied Spectral Imaging, Edingen-Neckarhausen, Germany).

For Giemsa banding followed by FISH analysis, the amount of trypsin was reduced to 10 % of the original amount. After photographing, metaphases were decoloured in 70 % ethanol, air dried and incubated over night at 60 °C.

2.10 Fluorescence *in situ* hybridisation

FISH analysis was performed for the detection of the *HMGA2* locus in the cell line PC-3 with a phage artificial chromosome (PAC) covering intron 3 (Hauke et al. 2002) labelled with SpectrumOrange (Abbott Molecular, Wiesbaden, Germany) and a probe targeting the centromeric region of chromosome 12 labelled with SpectrumGreen (Abbott Molecular). Probe and chromosomal DNA on a slide were co-denatured for 3 min at 80 °C on a ThermoBrite slide processing system (Abbott Molecular). Hybridisation was done over night in a humidified chamber at 37 °C. The slide was washed in 0.1 x SSC (usb, Cleveland, USA) at 61 °C for 5 min followed by an increasing ethanol series and then covered with mounting medium stained with DAPI (Vectorlabs, Burlingame, USA) after air drying.

Break-apart probes RP11-269K4 (AQ478964 and AZ516203, proximal) in combination with RP11-745O10 (AC078927, distal) or RP11-293H23 (AC012264, distal) were used for investigation of potential rearrangements of the *HMGA2* locus in PC-3 cells. Chromosomal DNA was digested with 5 ng pepsin (Merck Millipore) and fixated with 0.1 % paraformaldehyde (Merck Millipore)/1 % PBS (phosphate buffered saline; Sigma-Aldrich) for 10 min. Dehydration was done in an increasing ethanol series. Co-denaturation of probe and chromosomal DNA was carried out for 7 min at 77 °C. Hybridisation took place in a humidified chamber at 37 °C over night. The slides were washed in 0.4 x SSC/0.3 % NP-40 (Sigma-Aldrich) for 2 min at 71 °C and dipped briefly in 2 x SSC/0.1 % NP-40. After air drying, mounting medium with DAPI was applied.

Images were captured on an Axioskop 2 plus fluorescence microscope (Zeiss, Göttingen, Germany) using a high performance CCD-camera and FISHView software (Applied Spectral Imaging).

2.11 Spectral karyotyping

Spectral karyotyping was done using the Spectral Karyotyping Human Reagent and the CAD-Kit (Applied Spectral Imaging) with modifications from the original protocol.

Trypsin digestion was skipped and chromosomes were denatured with 100 µl denaturation solution on a slide warmer. Metaphases were then dehydrated in an ice cold increasing ethanol series, as indicated by the manufacturer. Hybridisation, posthybridisation including the optional blocking and detection were performed according to the manufacturer's protocol.

2.12 DNA isolation

DNA from uterine leiomyomas was isolated using the QIAamp DNA Mini Kit (Qiagen) including the optional RNase A digestion according to the manufacturer's instructions.

2.13 Array based comparative genomic hybridisation

For the detection of numerical chromosomal aberrations in uterine leiomyomas, aCGH was performed using Human Genome 244A Microarrays (Agilent, Waldbronn, Germany) according to the manufacturer's protocol. The labelling was done using the Genomic DNA Labelling Kit (Agilent).

2.14 Statistical analysis

Statistical analysis was done using Microsoft Excel (Microsoft, Unterschleißheim, Germany) and GraphPad InStat (GraphPad Software, La Jolla, CA, USA). Data were analysed in regard to Gaussian distribution and identical standard deviations by Kolmogorov and Smirnov and Bartlett tests, respectively. A One-Way ANOVA (analysis of variance) or, in case of a non-Gaussian distribution, a non-parametric Kruskal-Wallis test, were performed.

3. Results

3.1 *HMGA2* expression in response to growth factor stimulation

It is well established that in mesenchymal cells *HMGA2* belongs to the delayed early response genes whose expression can be induced upon stimulation with a variety of growth factors (Ayoubi et al. 1999). This is true for growths factors such as e.g. FGF1, FGF2, PDGF-BB as well as FBS (Ayoubi et al. 1999). In contrast, little is known about if *HMGA2* expression can be stimulated in cells of epithelial or endothelial origin as well. Therefore, the prostate cancer cell line PC-3 was chosen, as it displays a moderate *HMGA2* expression enabling the detection of significant changes in the *HMGA2* mRNA level upon incubation with growth factors. FISH analyses revealed two or three copies of the *HMGA2* locus per metaphase that mapped to a derivative chromosome t(4;12) or t(8;12). In case of three *HMGA2* copies per metaphase, the derivative t(8;12) was present twice. The derivative chromosomes were confirmed by SKY analysis. Stimulation with GFs (1 % FBS additionally supplemented either with 25 ng/ml FGF1 or PDGF-BB) as well as different concentrations of FBS illustrated that *HMGA2* expression apparently does not increase in response to these incubation settings. A BrdU ELISA to assess proliferation revealed increased proliferation caused by the incubation for a period of 12 h with FGF1 and PDGF-BB (statistically significant, as assessed by a one-way ANOVA), whereas FBS did not alter the proliferation rate of the investigated cell line. In contrast to the findings in mesenchymal cells, *HMGA2* expression did not rise in response to GF incubation.

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3.1.1 *HMGA2* expression in the prostate cancer cell line PC-3 is autonomous of growth factor stimulation

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Own contribution:

- Planning and performing of experiments including
    - Cell culture and stimulation experiments
    - *In vitro* cell proliferation assay (BrdU-ELISA)
    - RNA isolation from stimulated PC-3 cells
    - cDNA synthesis and real time RT-PCR
    - Chromosome preparation and SKY-FISH, FISH
- Data analysis (except for karyotyping and SKY-FISH analysis)
- Manuscript writing

Abstract

BACKGROUND:
High-mobility group AT-hook 2 (HMGA2) protein acts as an oncofoetal transcriptional regulator. In mesenchymal tissues, its expression can be induced by a variety of growth factors such as fibroblast growth factor-1 (FGF1) and platelet-derived growth factor-BB (PDGF-BB) as well as by foetal bovine serum (FBS), thus enhancing proliferation.

MATERIALS AND METHODS:
To examine these effects in epithelial malignancies, we used the PC-3 prostate cancer cell line for assaying proliferation and HMGA2 expression in response to incubation with growth factors and FBS. The HMGA2 locus was investigated by fluorescence in situ hybridisation (FISH) for loss, amplification or re-arrangement.

RESULTS:
PC-3 is a cell line that moderately overexpresses HMGA2. None of the growth factors nor FBS caused significantly increased expression of HMGA2. In contrast, a significantly augmented proliferation rate was observed when applying FGF1 or PDGF-BB for 12 h.

CONCLUSION:
HMGA2 is expressed independently of external stimuli, whereas proliferation stimulated by growth factors is independent of further elevated HMGA2 expression.

- unpublished data -

3.1.2 *HMGA2* expression and its correlation with proliferation in HUVECs as a consequence of growth factor stimulation

To address the question if *HMGA2* is inducible in HUVECs by GF incubation, these were grown after serum starvation in media supplemented with 1 % FBS and 25 ng/ml of different GFs each, namely FGF1, FGF2 or VEGFA. The amount of *HMGA2* was determined by real-time RT-PCR. FGF2 yielded in the highest increase of *HMGA2* mRNA after 12 h, followed by FGF1 and VEGFA (Figure 1). A proliferation assay using the same settings resulted in similar findings, with the highest proliferation increase of ~1.9-fold induced by stimulation with FGF2, and slightly less, FGF1. VEGFA induced the lowest proliferation rate when compared to the negative control treated with 1 % FBS only, as consistent with the lowest *HMGA2* expression level. Generally, the ranking of induced *HMGA2* expression and proliferation induced by the GFs corresponds to each other. This finding is supported by a Pearson product-moment correlation coefficient (R) between *HMGA2* mRNA and proliferation in HUVECs of 0.96 (Figure 2), pointing to a linear correlation between these factors. A One-Way ANOVA confirmed extremely significant differences in proliferation between the control grown in medium containing 1% FBS and the cells grown in medium supplemented with FBS and 25 ng/ml FGF2 ($p<0.001$), whereat proliferation of cells grown in medium with added VEGFA did not display a significant change. The difference in induced proliferation between the treatments with VEGFA and FGF1 was significant ($p<0.05$), and the difference between VEGFA and FGF2 is considered as highly significant ($p<0.01$) as well as the difference between 1 % FBS and FGF1. As to the differences in *HMGA2* expression, statistical analysis was done using a Kruskal-Wallis test. The mRNA increase in cells treated with FGF1 or FGF2 was about 2- and 2.5 fold referring to the 0 h control ($p<0.01$ and $p<0.001$, respectively) and the control incubated with 1% FBS ($p>0.05$ and $p<0.01$, respectively), whereat VEGFA only caused a non-significant ~1.4 fold increase. The different p-values are indicated in Figure 1 by asterisks in the respective colour (green for *HMGA2* expression and blue for proliferation, referring to the bars' colour).

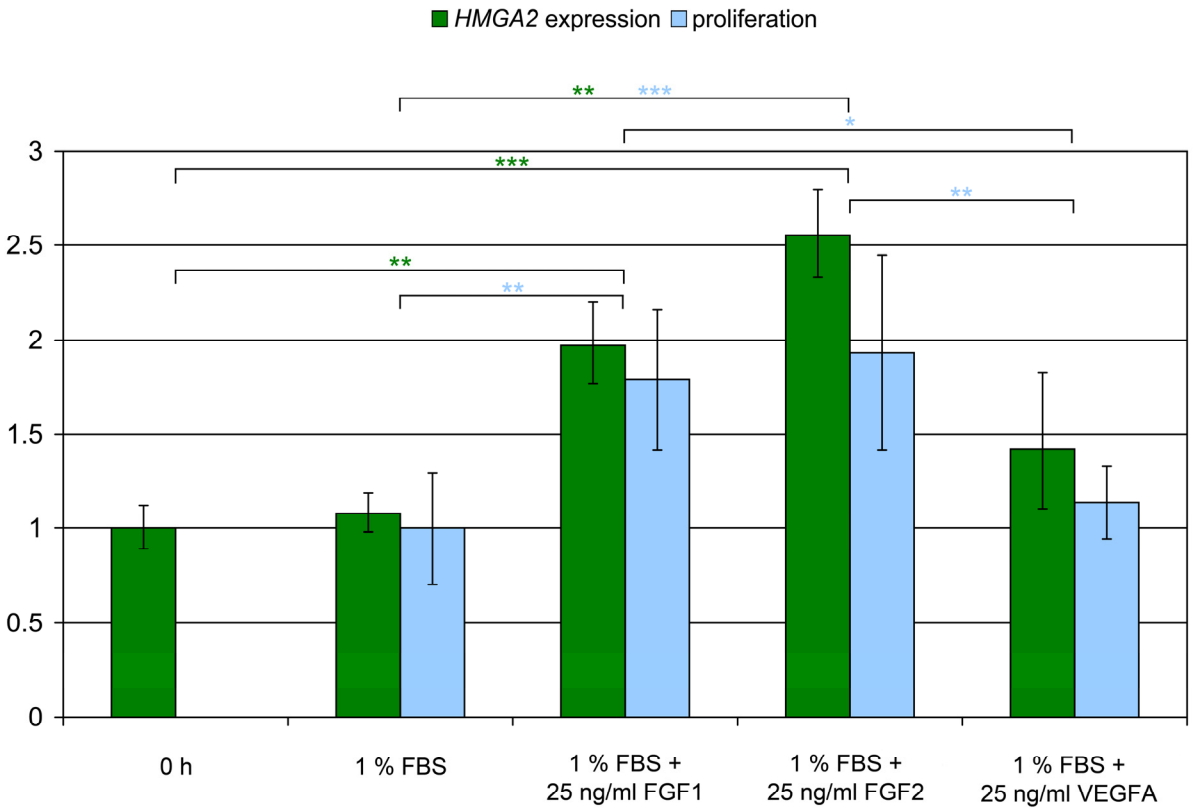

Figure 1: Relative expression of *HMGA2* in HUVECs and proliferation thereof after stimulation with GFs. As a calibrator for the mRNA level, a control taken before stimulation was used. *HPRT1* served as endogenous control. Proliferation was calibrated against a control incubated in starvation medium, i.e. EBM-2 supplemented with 1 % FBS only. Asterisks indicate significant differences in *HMGA2* expression (green) and proliferation (blue).

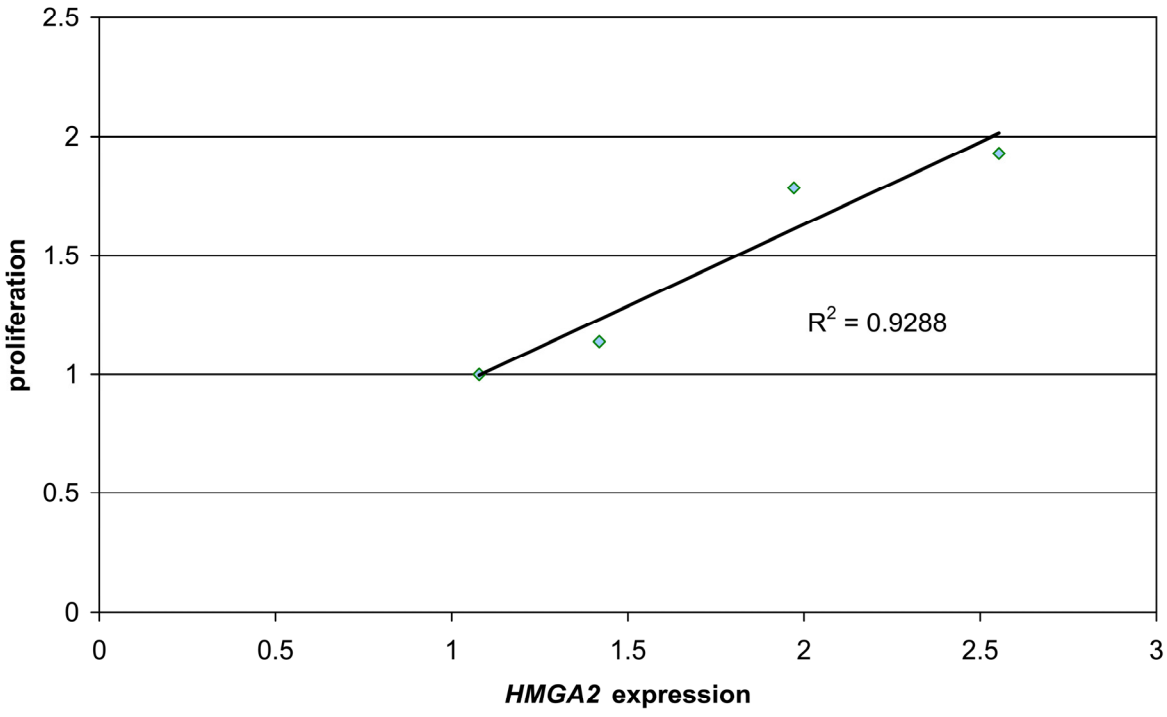

Figure 2: Correlation between proliferation and *HMGA2* expression in HUVECs after stimulation with various GFs.

- preliminary data -

3.2 Array-based comparative genomic hybridisation of uterine leiomyomas with a t(12;14)

Uterine leiomyomas frequently display a reciprocal, apparently balanced translocation involving the chromosomes 12 and 14. The *HMGA2* gene is the target for this translocation on chromosome 12 (Schoenmakers et al. 1995; Ashar et al. 1995), and *RAD51B* (RAD51 paralog B) is targeted on chromosome 14 (Schoenmakers et al. 1999). The translocation evokes very high expression of *HMGA2*, but the main mechanism has not been described yet and seems to differ from the loss of negative regulation by the let-7 miRNA family, which is described as cause for *HMGA2* overexpression in lipomas. The breakpoints on chromosome 12 in uterine leiomyomas seem to vary and are mainly located upstream of the *HMGA2* locus (Schoenberg Fejzo et al. 1996). Small deletions not visible in conventional cytogenetics due to their size can also allow the extrapolation of the breakpoint identification leading to the identification of potential regulatory sequences.

For the analysis of the aCGH experiments, a $\log_2$-ratio ranging between -0.25 and 0.25 was considered as balanced between tumour and reference probe, whereat a ratio lower than -0.25 was considered as loss in the tumour DNA, and higher than 0.25 was classified as a gain in tumour material. A series of at least 5 consecutive probes was set as minimum to be considered as potential aberration.

The experiments conducted herein led to the detection of potential deletions on chromosome 12 upstream of the *HMGA2* locus in two of the three samples investigated and are possibly caused by the translocation. In UL10, this deletion is located approximately 1.1 Mb upstream of *HMGA2* and consists of 5 probes with a log-ratio of smaller than -0.25 (green dots) distributed over a region of about 31 kb. In the other tumour, a series of 7 consecutive probes was identified ~412 kb upstream of *HMGA2* covering a region of about 62 kb (Figure 3). In the same tumour, a region downstream of *RAD51B* on chromosome 14 with a size of about 413 kb was shown to be deleted. In none of the other tumours, deletions on chromosome 14 being potentially related to the translocation were detected (Figure 4).

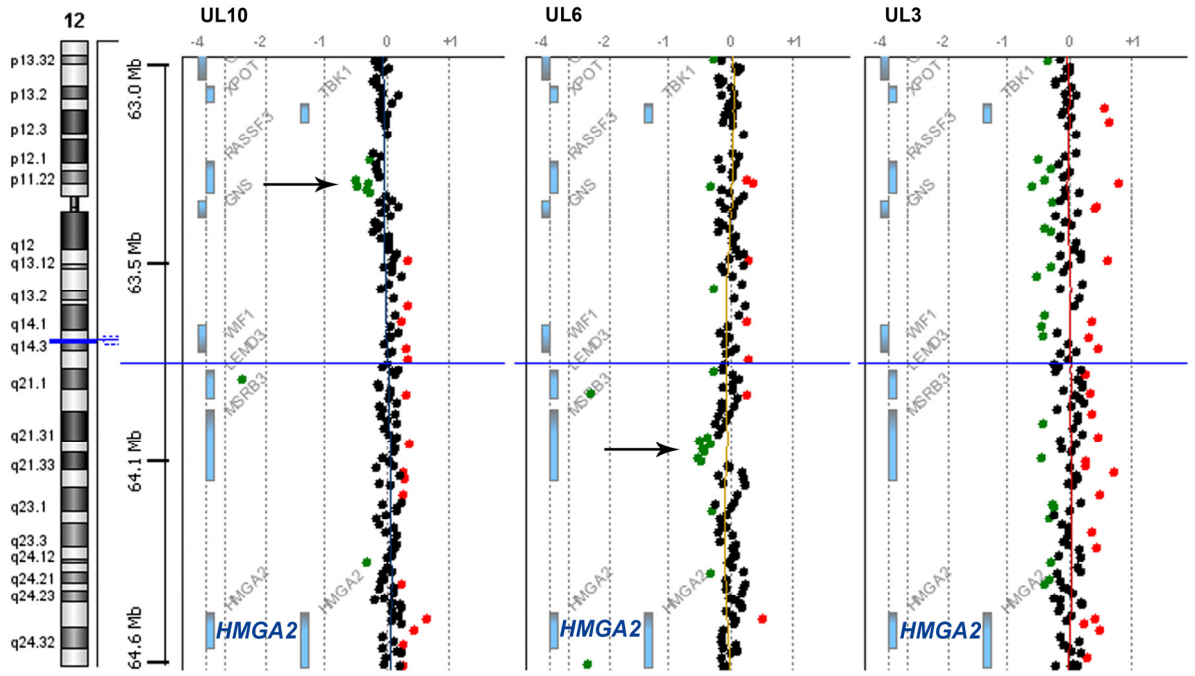

Figure 3: Depiction of the upstream region of the *HMGA2* locus on chromosome 12. Black dots indicate probes with a $\log_2$-ratio between -0.25 to 0.25. Green dots represent probes with a loss in tumour DNA, whereat red dots stand for gains in tumour DNA. Arrows indicate the deletions potentially related to the translocation t(12;14).

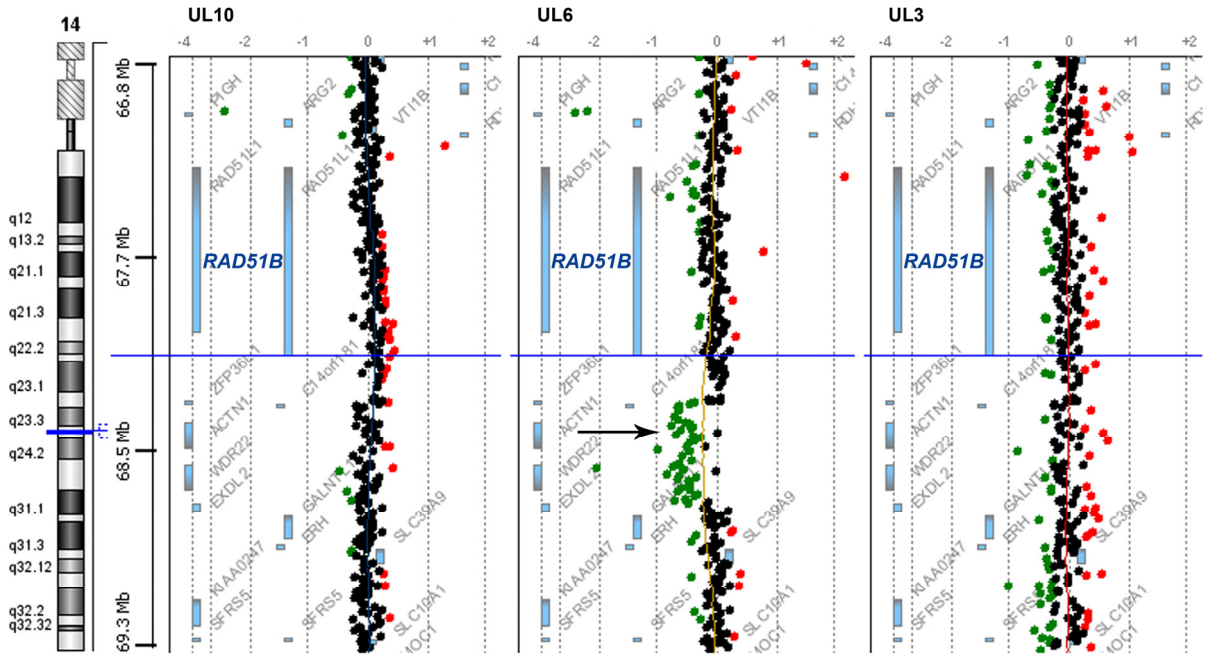

Figure 4: The chromosomal region 14q24.1 around *RAD51B*. Black dots indicate probes with a $\log_2$-ratio between -0.25 to 0.25. Green dots represent probes with a loss in tumour DNA, whereat red dots stand for gains in tumour DNA. Arrows indicate the deletions potentially related to the translocation t(12;14).

3.3 Cytotoxic effects of HMGA2 and its silencing in LNCaP cells

The cell line LNCaP, which was originally established from a lymph node metastasis of a prostate carcinoma, does not display a reliably detectable *HMGA2* expression, despite four apparently normal chromosomes 12. As *HMGA2* mainly is down-regulated by the let-7 miRNA family, miRNA processing in this cell line was decreased by *DICER1* knock-down to test for an inverse correlation of this miRNA family. Though *DICER1* expression was reduced by about 60 %, *HMGA2* expression still was undetectable by real time RT-PCR, pointing to different mechanisms in *HMGA2* knock-down. Worthy of consideration is also DNA methylation, a mechanism widely used in eukaryotes. Therefore, LNCaP cells were treated for 144 h with 5-AdC, a known methyltransferase inhibitor thus impeding methylation in proliferating cells and re-inducing gene expression. After 144 h of incubation with increasing concentrations of 5-AdC, *HMGA2* expression was reliably detected in nearly all of the replicates with the 0.31-, 0.43- and 0.38-fold expression for 5 µM, 10 µM and 50 µM 5-AdC, respectively. As non-treated LNCaP cells do not display detectable *HMGA2* expression, the cell line MCF-7 served for calibration. There was no huge difference in *HMGA2* expression between the incubation with 50 µM 5-AdC and 50 µM 5-AdC + 50 nM TSA, and incubation with 50 nM TSA alone for 48 h did not induce *HMGA2* expression. The impact of forced *HMGA2* expression was then tested by transfection of an expression vector containing the wt *HMGA2* transcript or the empty vector used as a control. Viability of cells transfected with the *HMGA2* insert as well as those that obtained the empty vector alone was reduced, so that the impact of *HMGA2* expression could not be deduced. To avoid the harmful effect of transfection with the expression vector, the cells were incubated with increasing concentrations of recombinant HMGA2. Interestingly, cell viability still was reduced by HMGA2 and resulted in a decrease to the 0.88-, 0.73- and 0.5-fold of the non-treated control for 24 h incubation with 1 µg/ml, 10 µg/ml and 100 µg/ml, respectively, as determined by WST-1 proliferation assay. Cytotoxicity assay confirmed the increasing amount of dead cells due to HMGA2, as measured by lactate dehydrogenase (LDH) release, with a proportion of dead cells of 0.05, 0.18 and 0.25. After 48 h, the amount of living cells decreased to the 0.82-, 0.62 and 0.66-fold, as revealed by WST-1 assay, when compared to the non-treated cells. LDH assay amounted to a proportion of dead cells of 0, 0.04 and 0.03, respectively. There was no huge induction of apoptosis after 24 h

HMGA2 incubation with values close to the non-treated control, as measured by caspase 3/7 activity, but an increased apoptosis rate was measured after 48 h. This effect was dose-dependent with an apoptotic rate of 1.18, 1.29, and 1.6 for 1 µg/ml, 10 µg/ml and 100 µg/ml HMGA2, respectively.

- III -

Cytotoxic effects of HMGA2 and its silencing in LNCaP cells

Marietta Henrike Müller, Norbert Drieschner,
Sabine Bartnitzke, Tim Focken, Jörn Bullerdiek

prepared for submission

Own contribution:

- Planning and performing of experiments including
    - Cell culture and demethylation
    - Chromosome preparation and karyotyping
    - DNA/siRNA transfection
    - *In vitro* viability/cytotoxicity (WST-1/LDH) assays
    - Apoptosis detection assay
    - RNA isolation
    - cDNA synthesis and real time RT-PCR
- Data analysis (except for karyotyping and SKY-FISH)
- Manuscript writing

Cytotoxic effects of HMGA2 and its silencing in LNCaP cells

Marietta Henrike Müller, Norbert Drieschner, Sabine Bartnitzke, Tim Focken, Jörn Bullerdiek

Abstract

The high mobility group AT-hook 2 (*HMGA2*) gene codes for an oncofoetal protein whose re-expression occurs in a variety of benign and malignant neoplasias. Its expression is not detectable in real-time RT-PCR in LNCaP cells derived from an early stage prostate carcinoma, making these cells a good model to determine the effects of *HMGA2* expression. To avoid the detected harmful effects of transfection of an eukaryotic expression vector, cells were incubated with recombinant HMGA2 which resulted in reduced cell viability and an increased time- and dose-dependent apoptosis rate. As underlying mechanism for the lack of *HMGA2* expression, the loss of the *HMGA2* locus was excluded. Despite their role in *HMGA2* regulation, miRNAs also seem unlikely, as *DICER1* silencing did not lead to *HMGA2* activation. Treatment with the methyltransferase inhibitor 5-aza-2'-deoxycitidine led to low *HMGA2* mRNA detection suggesting methylation to be responsible for *HMGA2* silencing.

Introduction

High mobility group AT-hook 2 (HMGA2) protein is a non-histone protein highly expressed in various types of stem cells during mammalian embryonic/foetal growth but also in adult stem cells, particularly of mesenchymal origin. In the latter case, strong expression of *HMGA2* is inducible by certain growth factors (Lanahan et al. 1992; Ayoubi et al. 1999; Markowski et al. 2011). Besides normal development and differentiation, *HMGA2* is abundantly expressed in a variety of human benign tumours mostly of mesenchymal origin due to chromosomal rearrangements targeting its locus at chromosomal region 12q14~15 (Schoenmakers et al. 1995; Ashar et al. 1995). In contrast, many malignant tumours including those from epithelial tissues display high expression of *HMGA2* due to distinct mechanisms.

These include e.g. amplifications as present in atypical lipomatous tumours (Pedeutour et al. 2012), dysregulated expression of miRNAs of the let-7 family in e.g. serous ovarian carcinoma (Helland et al. 2011), or other miRNAs targeting the *HMGA2* mRNA. High expression of *HMGA2* occurs in a large variety of malignant human neoplasias as e.g. sarcomas (Zhang et al. 2014) and carcinomas (Shell et al. 2007; Wang et al. 2011) and is associated with a poor prognosis. An increased metastatic potential has been associated with expression of HMGA2 in e.g. colorectal cancers (Wang et al. 2011), melanomas (Raskin et al. 2013), and a significant correlation between high HMGA2 expression and a poor prognosis for progression-free and overall survival of the patients was also found in ovarian cancer (Shell et al. 2007). However, the contribution of HMGA2 to malignant neoplasias is not fully elucidated yet, though different cellular processes were found to be impacted by HMGA2. *HMGA2* is mainly expressed during development (Rogalla et al. 1996; Hirning-Folz et al. 1998) and accordingly, its re-expression in some kind of malignant epithelial tumours promotes epithelial-mesenchymal transition enhancing tumour progression and aggressiveness, as observed in e.g. human hepatocellular carcinoma cells (Luo et al. 2013), gastric cancer (Zha et al. 2012; Zha et al. 2013), squamous cell carcinomas of the oral cavity (Miyazawa et al. 2004) and in prostate cancer cells (Zhu et al. 2013). HMGA2 thus is able to drive tumour metastasis, as it was found at the invasive front of human and murine tumours of epithelial origin (Morishita et al. 2013).

Accordingly, the role of HMGA2 in human malignant tumours is of interest and might offer a valuable therapeutic tool in treatment. It is thus essential to identify and elucidate the impacts of expression in epithelial tumours as well as the reasons and advantages for high and, in contrast, non-expression in other tumours of the same entity.

Herein, the impact of HMGA2 on viability of LNCaP cells which lack any detectable expression of *HMGA2* was addressed. *HMGA2* therefore was ectopically introduced into LNCaP cells using a mammalian expression vector, and cells were incubated with the recombinant protein. Cell viability and cytotoxicity was investigated using WST-1 and lactate dehydrogenase (LDH) release, as well as apoptosis was measured by caspase-3/7 activity. Additionally, several experiments have been performed to address the regulation of *HMGA2*. We tried to reactivate HMGA2 by different approaches. At first, as the let-7 miRNA family is a known down-regulator of

HMGA2, miRNA processing was abrogated by transient silencing of *DICER1* (dicer 1, ribonuclease type III), being part of the RNA induced silencing complex (RISC) and involved in the maturation of miRNAs (for review see Rana 2007). It is known from the literature, that the cell line LNCaP displays high expression levels of let-7 a-c (Nadiminty et al. 2012), indicating involvement of the let-7 family in the silencing of *HMGA2* so that *DICER1* knock-down might be followed by *HMGA2* expression. Additionally, the influence of demethylation by the methyltransferase inhibiting agent 5-aza-2'-deoxycitidine (5-AdC) and the histone deacetylase inhibitor Trichostatin A (TSA) on *HMGA2* expression was investigated, as there are few information available concerning the methylation status of the silenced *HMGA2* locus.

Materials and Methods

Cell culture

LNCaP cells were cultivated in RPMI-1640 containing 0.85 g/l $NaHCO_3$ and 20 mM HEPES (4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid) (PAN-Biotech, Aidenbach, Germany) supplemented with L-glutamine (Biochrom, Berlin, Germany) and 10% foetal bovine serum (FBS; Life Technologies, Darmstadt, Germany) or in EMEM (Eagle's Minimum Essential Medium) supplemented with 10% FBS (Cell Lines Services) in a humidified atmosphere at 37 °C/5 % CO_2. Medium was changed twice per week. When grown till confluence, cells were detached using TrypLE Express (Life Technologies) and split.

Chromosome preparation

Metaphases were arrested applying 300 ng colcemide (Biochrom) for ~1 h. After detaching of the cells with TrypLE Express (Life Technologies) and treatment with hypotonic solution (1:7), cells were fixed with methanol:acetic acid (3:1). Suspension was dropped on glass slides, air dried and incubated at 37 °C for at least 24 h.

GTG-banding

Metaphases were digested using 15 mg trypsin at 37 °C and stained in a 1.5 % Giemsa solution for 10 min. After washing the slides with distilled water and air drying, photographs were taken on an Axioplan Microscope (Carl Zeiss, Jena,

Germany) using BandView software (Applied Spectral Imaging, Edingen-Neckarhausen, Germany) that was also used for analysing the metaphases.

Spectral karyotyping

For spectral karyotyping, the protocol for the Spectral Karyotyping Human Reagent and CAD-Kit (Applied Spectral Imaging) was slightly modified. Trypsin digestion of the metaphases was skipped and chromosomal DNA was denatured using denaturation solution on a slide warmer with subsequent ice-cold increasing ethanol series. Overnight hybridisation, post-hybridisation with included slide blocking and detection of stained metaphases were performed as described in the original protocol.

Fluorescence *in situ* hybridisation

A phage artificial chromosome covering intron 3 of the *HMGA2* locus (Hauke et al. 2002) and a probe specific for centromere 12 (clone pBR12; both probes labelled using the Nick Translation Kit (Abbott Laboratories, Abbott Park, IL, USA) and SpectrumOrange or SpectrumGreen (Abbott Laboratories), respectively) were used to determine presence and number of *HMGA2* alleles. Briefly, chromosomal DNA and labelled probes were denatured for 3 min at 80 °C followed by overnight hybridisation in a humidified chamber at 37 °C. Washing of the slide was done in 0.1x SSC (standard saline citrate; USB, Cleveland, IL, USA) for 5 min at 61 °C, which was then rinsed three times briefly in 1x PBS solution and air dried. The slide was then covered with mounting medium containing DAPI (4', 6-diamidino-2-phenylindole; Vectorlabs, Burlingame, CA, USA). Photographs were taken on an Axioplan Microscope (Carl Zeiss) using a VDS CCD - 1300 camera and FISHView software (Applied Spectral Imaging) for analysis.

***HMGA2* expression vector transfection**

For transfection of the expression vector (pCR3.1; Life Technologies) encoding for the wild-type *HMGA2* (for vector construction, see Fedele et al. 1998), 175,000 cells were seeded in 6-well plates the day before transfection. Transfection complexes prepared of 9.25 µl Lipofectamine LTX, 2.5 µl PLUS and 2.5 µg vector DNA reagent (Life Technologies) were allowed to aggregate according to the manufacturer's

instruction and added to the cells. Cells were harvested for RNA isolation 24 h or 48 h after transfection.

For cell viability assays after transfection, 6,300 cells were seeded in 96-well plates (Greiner Bio-One, Frickenhausen, Germany). The amount of transfection reagent and DNA were down-scaled appropriate to the well surface.

HMGA2 incubation of LNCaP cells

LNCaP cells were seeded in 96-well plates and grown for 24 h. The next day, medium was replaced by growth medium supplemented with 1, 10 or 100 µg/ml recombinant HMGA2 (produced as described earlier, Richter et al. 2009). Cell viability, apoptosis and cell death were determined by WST-1, caspase-3/7 or LDH-assay after 24 h and 48 h.

Cell viability, cytotoxicity and apoptosis assays

Cell viability and proliferation of LNCaP cells after transfection were quantified with the Cell Proliferation Reagent WST-1 (Roche Diagnostics, Mannheim, Germany). Cell death was determined measuring the released lactate dehydrogenase (LDH) in the growth medium using the Cytotoxicity Detection Kit^{PLUS} (LDH)(Roche Diagnostics). Both kits were used according to the manufacturer's protocol. Each incubation setting was tested in quadruplicates. Apoptosis was measured using the Caspase 3/7 Glo Assay (Promega, Mannheim, Germany) according to the manufacturer's instructions. Absorbance and luminescence were measured using a Synergy HT microplate reader (BioTek, Bad Friedrichshall, Germany).

Treatment with 5-Aza-2'-deoxycytidine with and without Trichostatin A

For DNA demethylation in LNCaP cells, 150,000 cells were seeded in 6-well plates (Nunc, Wiesbaden, Germany). The next day, medium was replaced with fresh growth medium supplemented with 5-aza-2'-deoxycytidine (5-AdC, Merck Millipore, Darmstadt, Germany) at concentrations of 5 µM, 10 µM and 50 µM and cells were grown for 72 h. Thereafter, medium was replaced with growth medium again containing 5-AdC in the previous concentrations and incubated for another 72 h. Cells were then lysed in buffer RLT (Qiagen, Hilden, Germany). A non-treated control and controls grown in medium with equivalent volumes of the vehicle DMSO were included. The experiment was performed four times. For the combined incubation of

5-AdC and Trichostatin A (TSA; Merck Millipore), LNCaP cells were treated with 50 µM 5-AdC as described above. For the last 48 h before cell harvesting with buffer RLT for subsequent RNA isolation, TSA was added at a final concentration of 50 nM to the medium. TSA alone was applied 24 h after cell seeding at 50 nM and cells were incubated for 48 h. Each setting was performed in duplicates.

siRNA transfection

For transfection of LNCaP cells with siRNA (short interfering RNA), 200,000 cells were seeded in 6-well plates (Nunc) and allowed to attach for ~0.5 h. During that time, transfection complexes were prepared using 4 µl Lipofectamine RNAiMAX (Life Technologies) and a mixture of 4 different siRNAs (Qiagen) targeting *DICER1* at a final concentration of 10 nM (2.5 nM each). 72 h after transfection, cells were detached using TrypLE Express, medium was added and 65 % of the cell suspension was plated again followed by another transfection as prepared previously. After another 72 h, cells were lysed using Qiazol (Qiagen) for subsequent miRNA isolation.

RNA/miRNA isolation from cells

RNA was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Total RNA including the miRNA fraction was isolated using the miRNeasy Mini Kit (Qiagen) following the manufacturer's protocol. The optional DNase I digestion was included into both protocols as described by the manufacturer.

cDNA synthesis and real time RT-PCR

Total RNA (250 ng) was reverse transcribed with M-MLV (Moloney Murine Leukaemia Virus) Reverse Transcriptase (Life Technologies) according to the manufacturer's instructions using Random Hexamers (Life Technologies).

Quantification of mRNA transcripts was carried out on a 7300 Real-Time PCR System (Life Technologies) with the Sequence Detection Software version 1.2.3 (Life Technologies). Triplicates of each sample were run using TaqMan Universal PCR Master Mix (Life Technologies) and commercial assays for *HMGA2* (Hs00171569_m1) and *DICER1* (Hs00229023_m1; both Life Technologies). Hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) served as endogenous control

(with primers fw5'-GGC AGT ATA ATC CAA AGA TGG TCA A-3', rev5'-GTC TGG CTT ATA TCC AAC ACT TCG T-3', probe5'-6-FAM-CAA GCT TGC TGG TGA AAA GGA CCC C-TAMRA-3'; Biomers, Ulm, Germany).

Results

Transfection of mammalian expression vector results in increased cell death, and HMGA2 has cytotoxic and apoptotic effects on LNCaP cells.

The absence of *HMGA2* expression in LNCaP cells might indicate advantages for the cells not to express this gene so that consequences of re-activation are interesting. Therefore, cells were transfected using an expression vector coding for wild-type HMGA2, which caused an expression of *HMGA2* that, compared to the cell line MCF7 serving as calibrator for *HMGA2* expression, was 3,377,000- or 5,415,000-fold 24 h or 48 h post-transfection, respectively (data not shown). The transfection resulted in a decrease of living LNCaP cells of approximately 40 % and 80 % after 24 h and 48 h, respectively, as determined by WST-1 reagent (Figure 1 A+B). A decrease of cell viability was also detected in the control transfected with the empty expression vector that ranged in the same level after 48 h as the cells expressing *HMGA2*, and was about 20 % lower after 24 h. The mock control displayed slightly more living cells than the non-treated control. These data were confirmed when measuring the release of lactate dehydrogenase, as the highest amount of dead cells was detected in the cells that obtained the *HMGA2* expression vector, directly followed by the ones containing the empty vector. Equivalent to the WST-1 viability test, less dead cells were found in the mock control. To avoid the cell damaging effect of DNA transfection observed, cells were incubated with increasing amounts of recombinant HMGA2. The cell damaging effects of *HMGA2* expression were confirmed, as HMGA2 caused increased cell death and less viable cells (Figure 2 A+B). This effect was dose dependent, as cell death increased with augmented HMGA2 concentration for the incubation with 24 h, whereat, after 48 h, the amount of dead cells slowly increased from 0 to 10 µg/ml, and slightly decreased again for 100 µg/ml. There was no apoptosis inducing effect of HMGA2 after 24 h, whereat increased caspase-3/7 activity was detected after 48 h, which was dose dependent (Figure 3).

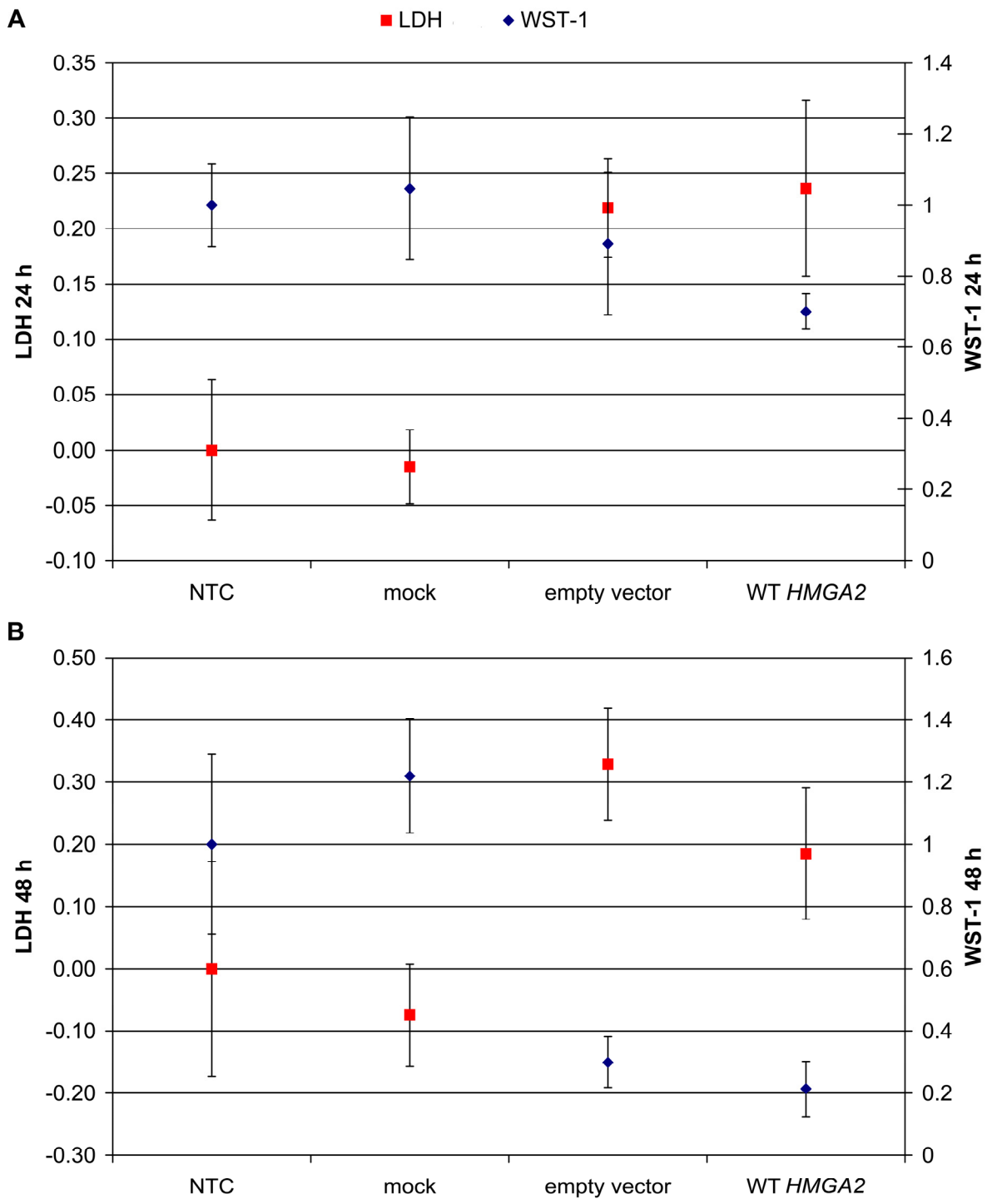

Figure 1: Cell viability using Cell Proliferation Reagent WST-1 after 24 h (A) and 48 h (B) after transfection of LNCaP cells with the expression vector pCR3.1 either containing wild-type *HMGA2* or without insert. A mock control with transfection reagent only and a non-treated control (NTC) were included into the experiment. LDH indicates the proportion of dead cells relative to the NTC set as zero. For comparison of WST-1 assays, NTC was referred to as 1.

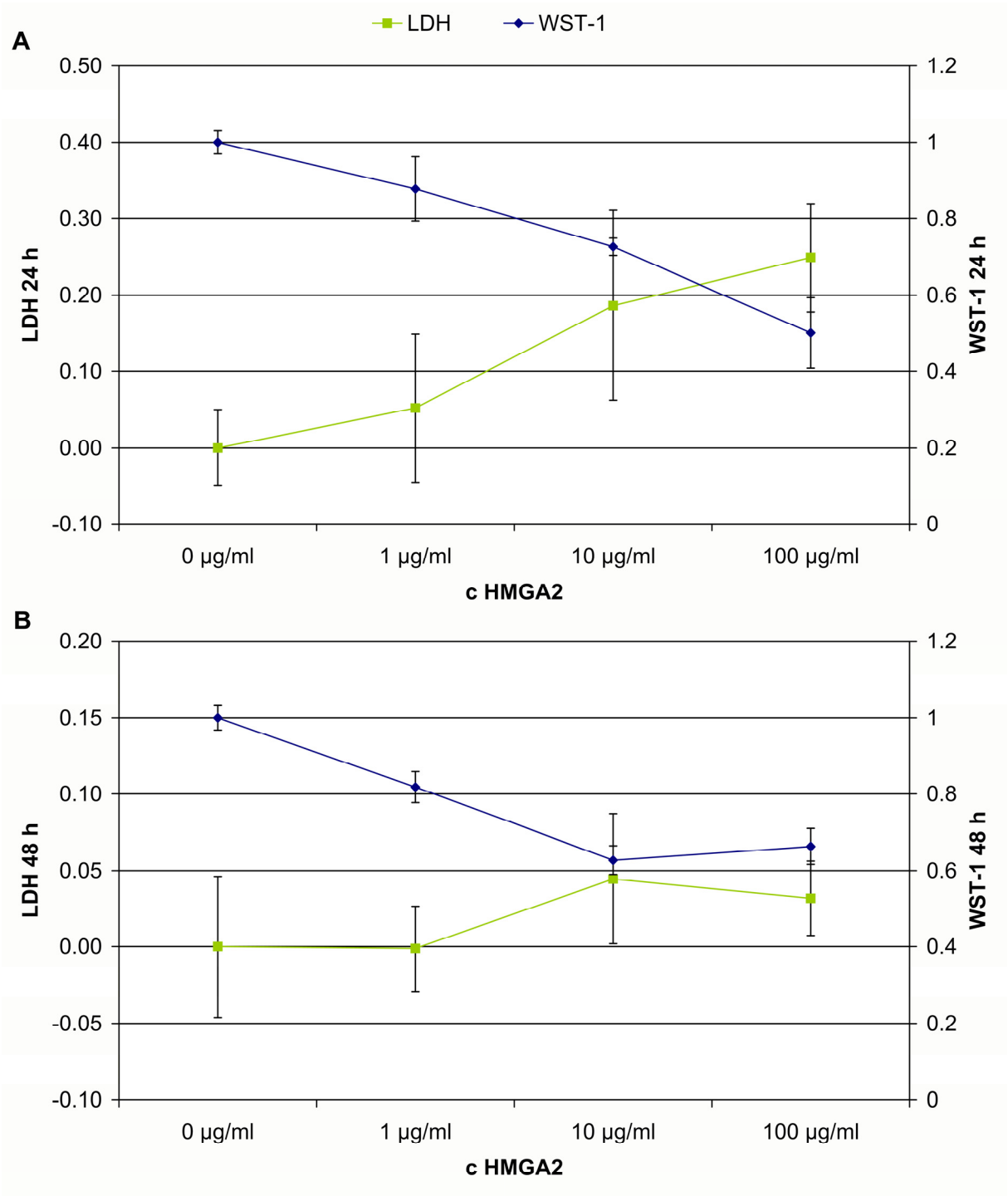

Figure 2: Cell damaging effects of HMGA2 on LNCaP cells, as determined by WST-1, LDH release and caspase-3/7 activity assay. Cells were incubated with increasing concentrations of HMGA2 (1, 10 and 100 µg/ml) for 24 h (A) or 48 h (B) followed by assay performance. Absorbance was determined on a Synergy HT microplate reader.

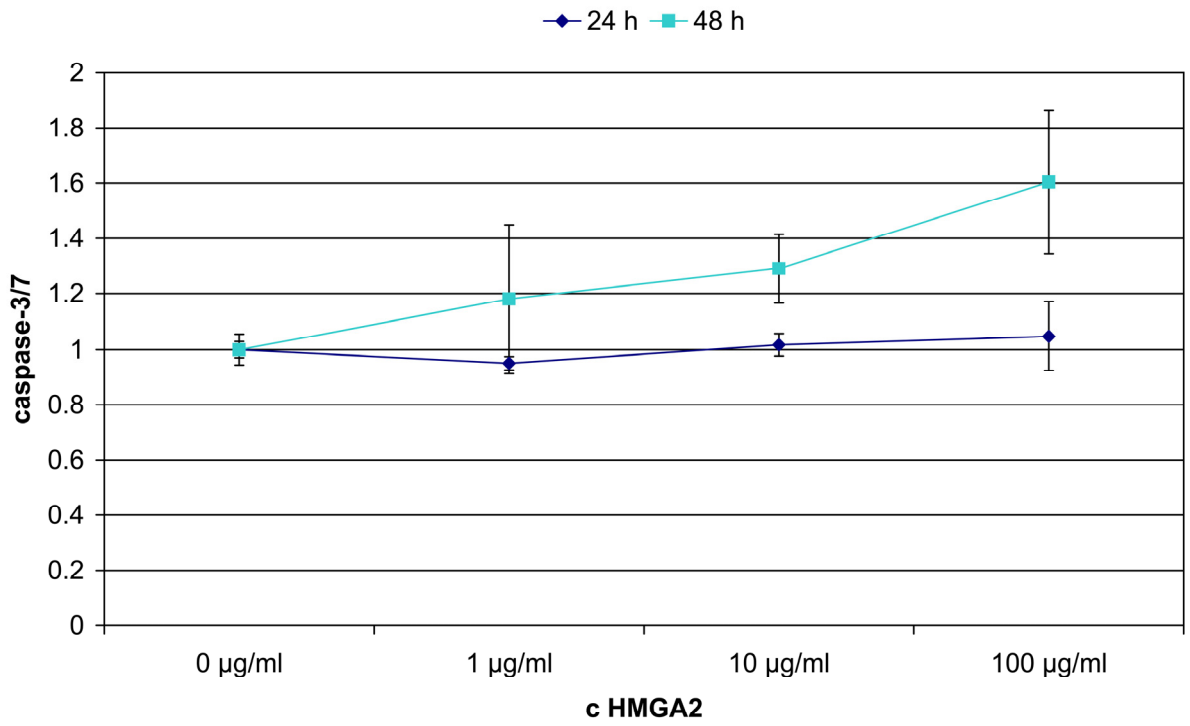

Figure 3: Detection of apoptosis by Caspase-3/7 activity after 24 and 48 h of HMGA2 incubation, respectively. Luminescence was measured on a Synergy HT microplate reader. The non-treated cells were set as 1.

Four copies of apparently normal chromosome 12 containing the *HMGA2* locus are present in the LNCaP cell line.

The cell line LNCaP does not display detectable *HMGA2* expression, as measured by real time PCR. To exclude an underlying loss of the *HMGA2* locus as a reason for this lacking expression, the cell line was analysed by conventional cytogenetics, SKY-FISH and FISH with probes specific for *HMGA2* and centromere 12. Cytogenetic analysis as well as SKY revealed four copies of apparently normal chromosomes 12 (Figure 4 A+B). Accordingly, FISH revealed four signals for centromere 12 as well as for the *HMGA2* locus, respectively (Figure 4C), with both signals co-localised on the same chromosomes.

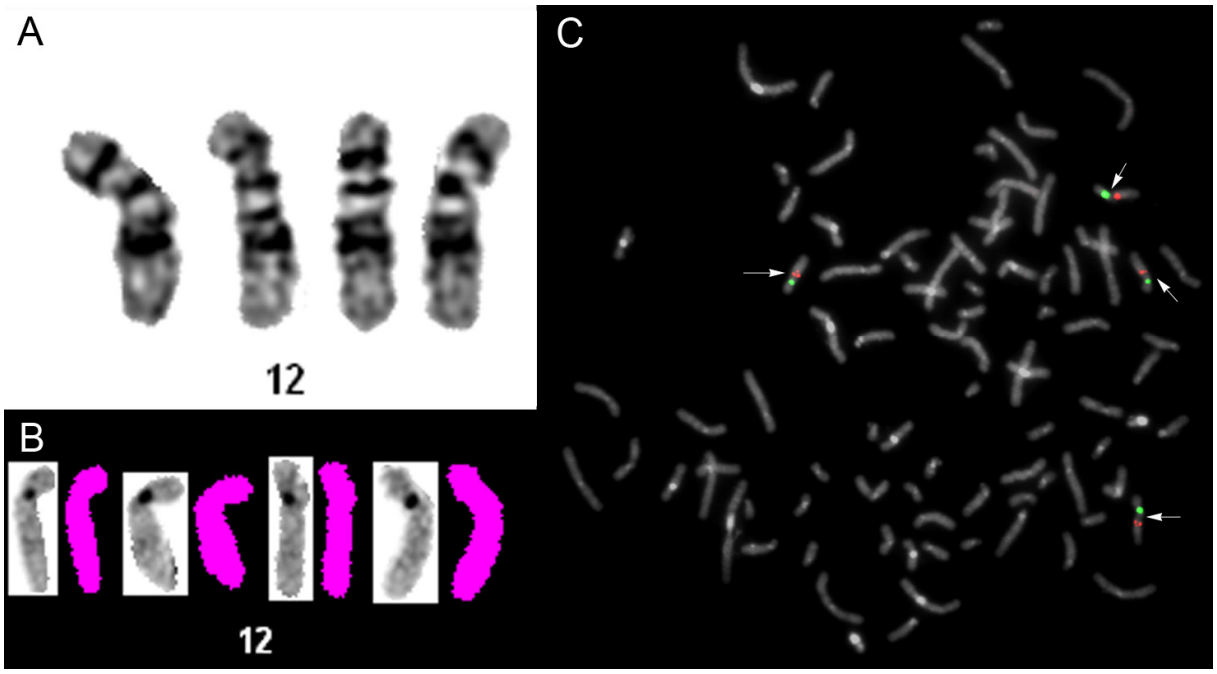

Figure 4: Cytogenetic analysis of the LNCaP cells revealed four apparently normal chromosomes 12 (A), which was confirmed by SKY-FISH analysis (B). FISH with a centromer 12 specific probe (green) and a probe covering intron 3 of the *HMGA2* locus also resulted in the detection of four signals for each probe.

The knock-down of *DICER1* to 40 % remaining expression does not lead to *HMGA2* expression.

One main actor involved in *HMGA2* regulation are miRNAs targeting the 3'UTR. Among those, the let-7 family plays a major role. The influence of miRNAs on *HMGA2* knock-out in LNCaP cells was tested by silencing of *DICER1*, encoding for a protein essential for the maturation and processing of miRNAs. Treatment with *DICER1* specific siRNAs resulted in an mRNA decrease of ~60 % for the target gene (Figure 5) compared to the non-treated control set as one, whereas the negative control siRNA and the mock transfection did not cause alterations on the mRNA level. Nevertheless, this did not lead to *HMGA2* expression, as would have been expected.

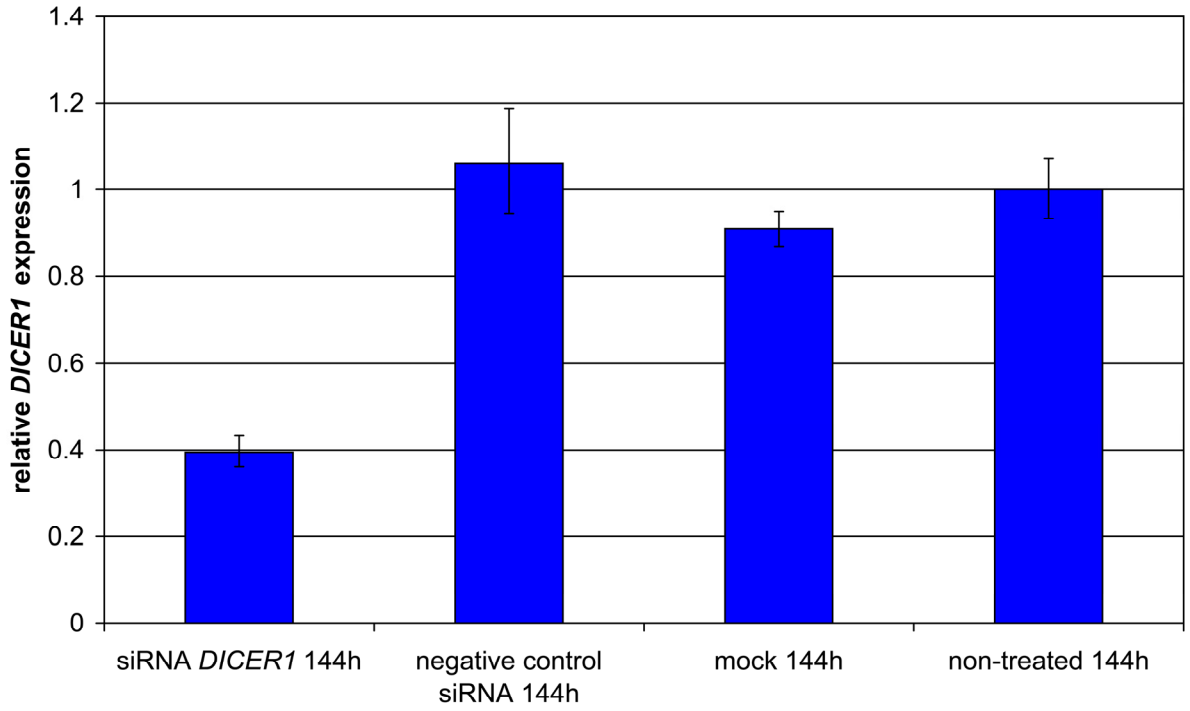

Figure 5: Relative amounts of *DICER1* mRNA after 144 h in LNCaP cells transfected twice with a cocktail of siRNAs targeting *DICER1*. *DICER1* was reduced to a level of 40 % compared to the non treated control, but *HMGA2* expression remained undetectable (data not shown). *HPRT1* served as endogenous control.

5-AdC in combination with or without TSA treatment leads to the activation of *HMGA2* expression that was not induced by TSA alone.

As disturbing of miRNA maturation did not lead to a detectable *HMGA2* expression in this cell line, methylation and histone acetylation was considered as a potential cause in HMGA2 abolishment. Therefore, the cells were treated with the demethylating agent 5-AdC, which turned out to be able to induce *HMGA2* expression. After treatment with concentrations of 5 µM, 10 µM and 50 µM, respectively, *HMGA2* expression became reliably detectable at a low level in the replicates in real-time PCR. *HMGA2* expression could not be detected in the non-treated cells (Fig. 6A). The highest expression resulted from incubation with 10 µM 5-AdC. Incubation of LNCaP cells with 5-AdC combined with the histone deacetylase inhibitor TSA also caused expression from the former silenced *HMGA2* gene (Fig. 6B). Incubation with TSA alone did not induce *HMGA2* expression (data not shown).

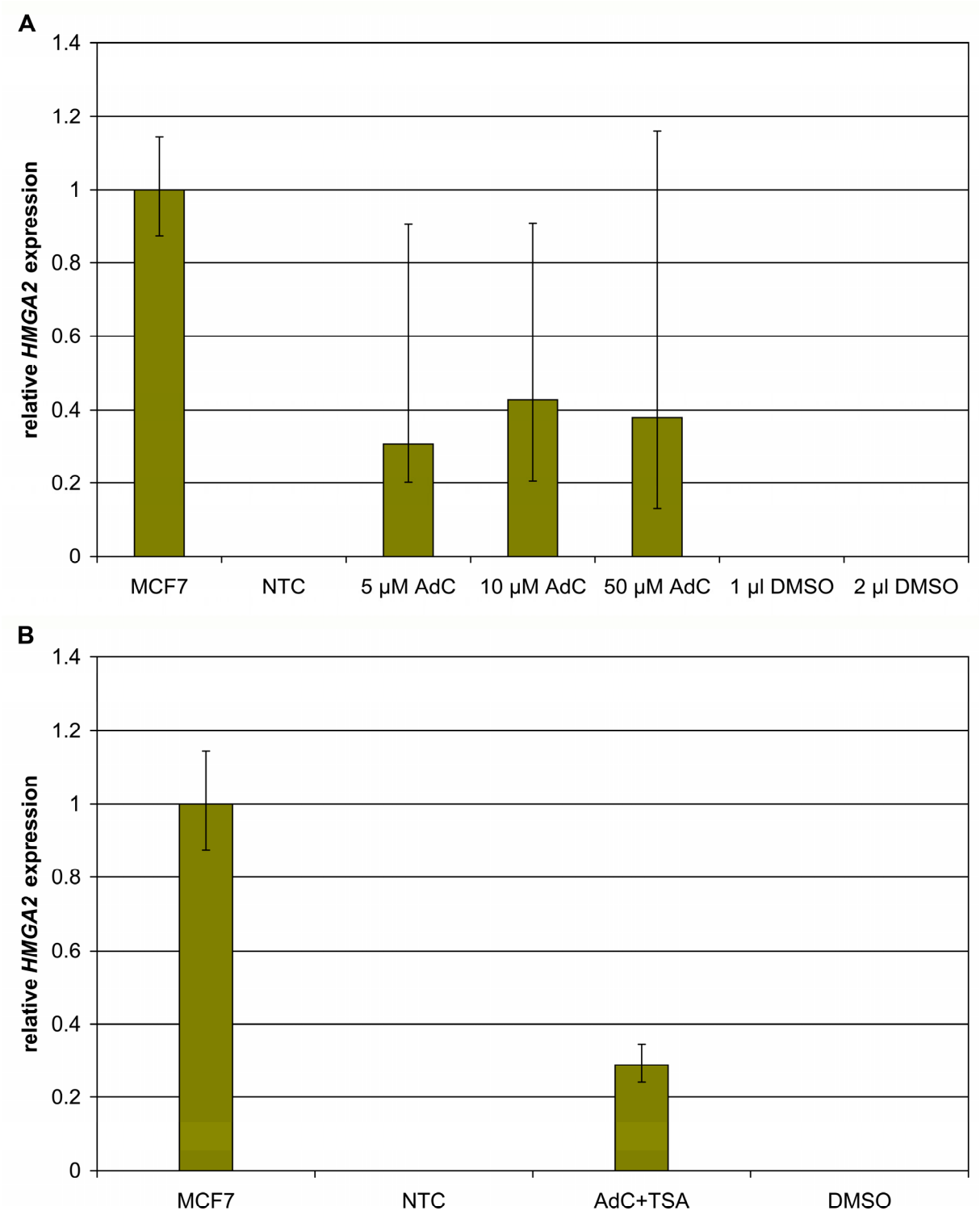

Figure 6: A) Treatment with 5-AdC in increasing concentrations caused rising expression of *HMGA2* in the cell line LNCaP. Presented are the mean values of four treatments. B) Relative expression of *HMGA2* after incubation with a combination of 50 µM 5-AdC (144 h) and 50 nM TSA (48 h), as mean value obtained by two replicates. Controls included non-treated cells (NTC) and cells incubated in medium containing the same volume of the vehicle DMSO. *HPRT1* served as endogenous control.

Discussion

The cell line LNCaP was established from a lymph node metastasis of a prostate carcinoma and, despite four apparently normal chromosomes 12, does not display any reliably detectable *HMGA2* expression. Though *HMGA2* is usually inactive in adult tissue, it becomes reactivated in certain benign as well as malignant tumours (for review see Cleynen and Van de Ven 2008), but the function of HMGA2 in malignant tumours and its contribution to tumour growth is still not fully elucidated.

One main factor that contributes to a poor prognosis in malignant epithelial tumours associated with *HMGA2* (re-) activation might be due to the protein's potential to drive EMT. As *HMGA2* expression is mainly restricted to mesenchymal development and widely down-regulated in differentiated adult tissue (Rogalla et al. 1996; Hirning-Folz et al. 1998), its re-expression in the epithelium is a critical point because HMGA2 regulates e.g. the transcription factors Twist1 (twist family bHLH transcription factor 1) and SNAI1 (snail family zinc finger 1) as regulators of EMT (Thuault et al. 2008; Tan et al. 2012). Indeed, as HMGA2 lacks in the cell line LNCaP, it displays EPCAM (epithelial cell adhesion molecule) expression, whereat staining for the mesenchymal marker VIM (Vimentin) was negative according to its immunologic profile (DSMZ datasheet, retrieved from http://www.dsmz.de/catalogues/details/culture/ACC-256.html).

Interestingly, as a consequence from incubation with HMGA2, cell viability was reduced in a dose dependent manner, as was shown by WST-1 assay and measurement of LDH release, and apoptosis was found to be slightly increased after 48 h. It is thus tempting to speculate, that *HMGA2* reactivation in malignant non-expressing cell lines might prevent tumour progression, whereat it has a favourable effect on viability and proliferation in benign tumours and in some kind of malignant neoplasias as well. In uterine leiomyomas, tumours carrying the characteristic translocation t(12;14) followed by high expression of *HMGA2* are even larger when compared to those not displaying this chromosomal aberration (Rein et al. 1998; Hennig et al. 1999), underlining the significance of HMGA2 in growth and proliferation. Despite a proliferation promoting role in other cell types, especially those of mesenchymal origin as e.g. chondrocytes and adipose-derived stem cells (Richter et al. 2009; Richter et al. 2011) but also in malignant tumour cells, such as those from retinoblastoma (Venkatesan et al. 2012), in liver cancer (Di Fazio et al. 2012) and others, this effect was not observed in LNCaP cells. Instead, HMGA2 in

the cell line LNCaP even impedes proliferation and increases cell death, which is in contrast to the finding by Peng et al. (2008) that ectopic expression of *HMGA2* promotes proliferation. Instead, cell viability decreased upon incubation with recombinant HMGA2 and the relative amount of dead cells increased. In rat Dunning G cells lacking endogenous HMGA2, ectopic *HMGA2* expression did not alter the growth ability significantly (Diana et al. 2005). The reason for the cytotoxic effect of HMGA2 in LNCaP cells described herein and its slight promotion of apoptosis after 48 h are unclear. HMGA2 has been shown to enhance genotoxic stress by consistent phosphorylation of the histone H2AX at Serine 139 (γ-H2AX), which is a hallmark of DNA double strand breaks (Boo et al. 2005). In cells with ectopic *HMGA2* expression as well as in those endogenously expressing *HMGA2*, a higher basal γ-H2AX level and delayed γ-H2AX clearance after x-ray exposure resembling the phenotype of DNA-PK deficient cells with perturbed nonhomologous end joining repair occurs (Li et al. 2009). HMGA2 might thus reinforce the apoptosis rate in LNCaP cells that, though at low rates, increases with time and concentration.

In the development of prostate cancer, the androgen receptor (AR) has a pivotal role. The AR controls and induces signalling pathways during prostate development (Meeks and Schaeffer 2011) and is also involved in the growth of prostate carcinoma due to the reactivation of androgen-induced pathways (Schaeffer et al. 2008). Interestingly, androgen deprivation leads to increased expression of the closely related *HMGA1* gene (Takeuchi et al. 2012). It might thus be interesting to investigate if a similar mechanism of increase upon androgen deprivation also exists for *HMGA2* expression. HMGA2 could then, following the androgen refraction of LNCaP cells, lead to their further dedifferentiation as they retained prostate cell specific properties and represent early stages of prostate cancer (Dozmorov et al. 2009).

As to *HMGA2*, let-7 c, a member of the let-7 miRNA family and acting as negative regulator, was found to be down-regulated in human prostate cancer, suggesting up-regulation and thus a role for HMGA2 in this tumour type as well (Nadiminty et al. 2012). As the cell line LNCaP was found to display high levels of let-7c, an inverse correlation with *HMGA2* mRNA levels can be guessed. Interestingly, *HMGA2* mRNA levels did not respond to abrogation of miRNA processing by *DICER1* silencing. The *HMGA2* suppressive mechanism in the LNCaP cell line still needs further exploration, as the let-7 family does not seem to be the main actor in the abrogation of *HMGA2*

expression therein. This is in contrast to HeLa cells that also display barely detectable *HMGA2* expression but in which increased mature mRNA could be detected after *DICER1* silencing (Lee and Dutta 2007).

Due to the four apparently normal chromosomes 12 bearing the HMGA2 locus as detected by FISH analysis, epigenetic mechanisms like methylation are also potential silencing possibilities. Treatment with the demethylating agent 5-AdC caused a low, but reliably detectable mRNA level of *HMGA2* pointing to DNA methylation being involved in its silencing. Data concerning the methylation/acetylation status of the *HMGA2* gene locus regulating its expression are found sparsely in the literature. Murine Hmga2 responds to acetylation, as the histone deacetylase inhibitor TSA represses its transcription (Ferguson et al. 2003). Introduction of CpG islands as well as differential methylation of *HMGA2*-CpG-sites and those surrounding *HMGA2* establishing an association with type II diabetes was found in human pancreatic islets, but no mRNA alterations due to differential methylation were reported (Dayeh et al. 2013).

As cell viability was strongly reduced in the empty vector control as well is in the cells transfected with the WT *HMGA2* insert, *HMGA2* expression seems to be harmful to LNCaP cells which was confirmed by incubation with recombinant HMGA2 making *HMGA2* silencing reasonable. The cell line LNCaP might hence constitute a valuable tool to explore the mechanisms responsible for *HMGA2* silencing, as it seems to deviate from the main mechanism, i.e. knock down by regulating miRNAs such as e.g. the main actor, the let-7 family. Further investigation should be assigned to the consequences of *HMGA2* expression and the influence of the protein in the cell line LNCaP as well as possible carry over to other malignant tumours without endogenous *HMGA2*.

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4. Discussion

The architectural transcription factor HMGA2 is expressed during embryonic development and impacts a variety of cellular functions such as cell differentiation, proliferation, DNA repair and others. In the adult, its reactivation is a critical point in tumourigenesis, since *HMGA2* accounts as a proto-oncogene favourable to tumour initiation and progression. Hence, comprehension of its regulation as well as the mechanisms inducing dysregulated expression in already differentiated tissue and the consequences following *HMGA2* dysregulation are of major interest, allowing the development of potential interventions to avoid *HMGA2* dysregulation as therapeutic strategies in neoplasias.

In the work presented herein, the regulation mechanisms impacting *HMGA2* expression in cells of different origin were subject of several experiments. The induction of *HMGA2* by growth factors and its correlation with proliferation were examined in HUVECs and in the prostate cancer cell line PC-3 expressing endogenous *HMGA2*. Another prostate cancer cell line lacking this expression (LNCaP) served as a model for the impact of recombinant HMGA2 on these cells and the silencing mechanism responsible for the absence of *HMGA2* expression therein. Using high-resolution aCGH, ULs with a t(12;14) were screened for common deletions associated with the translocation on chromosome 12.

Angiogenesis and vascularisation are important processes in embryonic development associated with proliferation of endothelial cells (Humar et al. 2002) building the blood vessel system in the growing embryo. Blood vessel formation is also necessary in tumourigenesis. During tumour cell proliferation and tumour growth, cell death due to insufficient oxygen occurs (Brahimi-Horn and Pouysségur 2006). The so-called hypoxia leads to the activation of HIF1A (hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)) that induces the expression of pro-angiogenic genes like *VEGFA* (Pouysségur et al. 2006) finally resulting in vessel building to ensure nutrition and oxygen supply of the tumour. The angiogenic switch thus refers to the transition from pre-vascular hyperplasia to a highly vascularised and progressively outgrowing tumour (for review see Baeriswyl and Christofori 2009). Vessel building requires the proliferation and migration of endothelial cells that finally form a lumen to enable blood flow (Bergers and Benjamin 2003), a process that is supported by a variety of other growth factors, including FGFs. FGF2 is involved in

the proliferation and migration of HUVECs (Kitamura et al. 2013), and the same is true for FGF1 (Presta et al. 2005). VEGFA possesses a high angiogenic potential (reviewed in Ylä-Herttuala et al. 2007). As in smooth muscle cells *HMGA2* was found to up-regulate pro-angiogenic factors like *FGF2* as well as to down-regulate e.g. the VEGF receptor *FLT1* (fms-related tyrosine kinase 1) (Borrmann 2003) being a negative regulator of embryonic angiogenesis (Hiratsuka et al. 2001), HMGA2 is suggested to contribute directly to tumour angiogenesis by supporting the proliferation of endothelial cells. *HMGA2* expression induced by growth factors could hence support proliferation of endothelial cells and enhance angiogenesis during embryonic development as well as in tumour growth. HUVECs are a widely used cell type to study the angiogenic processes *in vitro* and were thus chosen as experimental model. Interestingly, FGF1 and FGF2 induced the highest *HMGA2* expression with a 2- and 2.5-fold increase in expression that correlated with a rise in proliferation approximately 1.8- and 1.9-fold, respectively. The lowest expression was induced by VEGFA, which was also linked to the weakest induction of proliferation. The linear correlation between *HMGA2* and proliferation is supported by a R-value of 0.96 indicating that HMGA2 might play a role in the promotion of angiogenesis by enhancing endothelial cell proliferation. These three growth factors all are involved in endothelial cell proliferation, whereat VEGFA also contributes to vascular permeability as one of the initial step in the building of new vessels from pre-existing ones (Weis and Cheresh 2005) probably explaining the weakest proliferation induced.

The role of HMGA2 in cancer varies between tumour entities. *HMGA2* is up-regulated in prostate cancer in dogs (Winkler et al. 2007), but up to date, few data are available concerning *in vivo* deregulation of *HMGA2* in human prostate cancer. The amount of HMGA2 was found to be significantly increased in a subset of four matched tumour and normal tissues (Zhu et al. 2013), and in a mouse model, Hmga2 stromal epigenetic dysregulation was able to induce prostatic intraepithelial neoplasia (PIN) as preneoplastic lesions (Zong et al. 2012). The same study suggested a role for Hmga2 in ductal morphogenesis and regulation of epithelial differentiation, due to *Hmga2* knock-out mice displaying a smaller prostate than wt-mice and a more than six-fold decrease in cell number, whereat proliferation staining with Pcna (proliferating cell nuclear antigen) did not display any differences (Zong et al. 2012). In synergy with up-regulation of the androgen receptor (AR), Hmga2 induced poorly

differentiated adenocarcinoma, whereas AR overexpression alone did not induce hyperplastic lesions (Zong et al. 2012). Furthermore, Zong et al. (2012) were able to show that Hmga2-modified (i.e. Hmga2 overexpressing) stromal cells induce epithelial proliferation, probably mediated by ß-catenin through the Wnt pathway.

The issue of a correlation between *HMGA2* expression and proliferation of tumour cells induced by growth factors was thus addressed using the prostate cancer cell line PC-3 derived from the metastasis of a poorly differentiated adenocarcinoma. This cell line already displays constitutive expression of *HMGA2*, but at a relatively moderate level allowing the detection of increased *HMGA2* mRNA by real time RT-PCR. Similarly to HUVECs, cells were incubated with FGF1 and PDGF-BB as well as with increasing concentrations of FBS for subsequent RNA isolation or determination of the proliferation rate by means of a BrdU-ELISA. Interestingly, the stimulation of *HMGA2* expression by FGF1 previously observed in HUVECs as well as in mesenchymal cells (Ayoubi et al. 1999) did not occur as it has not been detected in quantitative real time RT-PCR. There was neither increased *HMGA2* expression due to PDGF-BB nor due to various concentrations of FBS, as detected in mesenchymal cells (Ayoubi et al. 1999). Despite the absence of a detectable increase of *HMGA2* expression, as was observed in HUVECs, FGF1 and PDGF-BB were able to increase proliferation. It thus seems that a further augmentation of *HMGA2* expression is not necessary for PC-3 to increase the proliferation rate, suggesting the amount of HMGA2 to be sufficient. A mechanism distinct from those in HUVECs that does not require further *HMGA2* increase to raise proliferation of PC-3 cells is also possible. Besides its potential role in proliferation, HMGA2 supports further mechanisms maintaining and promoting tumour growth. HMGA2 e.g. up-regulates *TERT* (telomerase reverse transcriptase) maintaining telomere length essential for tumourigenesis (Li et al. 2011).

A further pivotal process during epithelial cancer progression is the epithelial-mesenchymal transition (EMT). EMT and the reverse process, MET (mesenchymal-epithelial transition), are also fundamental processes during embryonic development and organ and tissue formation (for review see Thiery et al. 2009) that are reactivated in cancer. The transition from an epithelial to a mesenchymal cell is associated with changes in cell shape and a higher motile and invasive potential (Thiery et al. 2009). The loss of the epithelial expression marker CDH1 (cadherin 1, type 1, E-cadherin (epithelial), also known as E-cadherin) is a crucial step in EMT (Perl et al. 1998) and

associates with loosening of the tissue due to the lack of CDH1, being one of the proteins stabilising cell-cell adhesion and connection (Lamouille et al. 2014). Hallmarks of dedifferentiation of epithelial cells into a mesenchymal-like state are down-regulation of CDH1 and up-regulation of VIM (vimentin) and CDH2 (cadherin 2, type 1, N-cadherin (neuronal), also known as N-cadherin; Thiery et al. 2009). EMT is regulated by various transcriptions factors which offer possibilities for the influence of EMT by HMGA2. Likewise, repression of CDH1 is induced by transcription factors such as SNAI1 (snail family zinc finger 1) that are regulated by HMGA2 in cooperation with proteins of the Smad family (Thuault et al. 2008). The transcription factor Twist also regulating EMT is directly induced by HMGA2 through binding to its promoter (Tan et al. 2012).

Influence of HMGA2 on EMT e.g. has been described in the PC-3 cell line (Zhu et al. 2013) which was also used in this thesis. The study by Zhu et al. (2013) investigated EMT in the PC-3 as well as in the DU145 prostate cancer cell lines and revealed that miR-154 negatively regulates the expression of HMGA2, which usually down-regulates CDH1, accompanied by up-regulation of the mesenchymal marker VIM thus a critical step of EMT. Knock-down of HMGA2 by siRNA transfection or forced mir-154 expression resulted in increased CDH1 and decreased VIM expression, suggesting that miR-154 impacts expression of these two EMT-markers mediated by *HMGA2* silencing (Zhu et al. 2013). Consequently, HMGA2 reduction decreases the invasive and migratory potential of PC-3 and DU145 cells *in vitro*, as was also observed after ectopic expression of miR-154 in PC-3 cells (Zhu et al. 2013). It can thus be speculated that *HMGA2* expression is a prerequisite enabling the proliferation of epithelial cells by loosening cell-cell adhesion mediated by the suppression of the stabilising proteins like CDH1. This would allow cell growth mediated by factors such as PDGF-BB and FGF1 hence promoting tumour growth. This is in line with the finding that HMGA2 is involved in tumour cell migration and invasion which has been observed in various tumours of epithelial origin. It has e.g. been described in hepatocellular carcinoma cell lines, where it represses CDH1 and induces VIM and CDH2 (Luo et al. 2013). HMGA2 expression positively correlates with the metastatic potential of the investigated cell lines (Luo et al. 2013). In human lung cancer, *HMGA2* frequently is overexpressed (Meyer et al. 2007), and its knock-down reduces cell migration (Park et al. 2010) pointing to its involvement in EMT. In G401 cells derived from a rhabdoid tumour of the kidney, down-regulation of

overexpressed *HMGA2* almost abolished the invasive potential of the cell line and decreased proliferation (Zhang et al. 2014).

In contrast to the PC-3 cells displaying *HMGA2* expression, *HMGA2* expression was found to be absent in the cell line LNCaP that was also derived from a prostatic carcinoma, but represents an early stage with retained prostate cell specific properties (Dozmorov et al. 2009). This suggests this cell line to be a good model to study the impact of HMGA2 *in vitro*. Interestingly, application of increasing concentrations of recombinant HMGA2 protein resulted in increased cell death and a reduction of viable cells, as determined by cytotoxicity and viability assay. The apoptosis rate increased slightly after 48 h, but it remained at a relatively stable level after 24 h. The reasons for the toxic effect of HMGA2 on LNCaP cells are unclear. So far, HMGA2 mainly has been described to protect against DNA-damaging drugs (Summer et al. 2009; Palmieri et al. 2011; Yu et al. 2014). An explanation may be found in the comparison between HMGA2 and HMGA1a and HMGA1b, splice variants derived from the closely related *HMGA1* gene: in a normal rat thyroid cell line, *HMGA1b* transfected cells were unable to step through the cell cycle and instead stopped replicating at G_2-M phase and underwent apoptosis, with similar effects for the *HMGA1a* isoform (Fedele et al. 2001). Though the cell line LNCaP has been derived from a malignant tumour, the cells show strictly abrogated *HMGA2* expression, leading to a lack of detection in sensitive methods such as quantitative real time RT-PCR. This may suggest that, comparably to *HMGA1*, *HMGA2* expression would severely perturb the progression through the cell cycle leading to increased cell death and, to a lower extend, apoptosis, as was shown by incubation with the recombinant HMGA2 protein. It is unclear if the primary tumour developed with or without expression of *HMGA2*, but its expression in the derived cell line hampers survival, despite proliferation promoting and protective properties in other cell lines. It would be interesting to investigate a potential correlation between the androgen-dependence of prostate cancer cells and their expression of *HMGA2*, as LNCaP cells depending on androgens have abrogated *HMGA2* expression that is on the contrary high in the androgen-independent PC-3 cells. In prostate cancer cells, the development of androgen independence is associated with the expression of *HMGA1* maintaining cell growth of androgen-deprived or -independent cells (Takeuchi et al. 2012). A similar mechanism could also be possible for *HMGA2*.

The abrogation of *HMGA2* expression leads to the question of the silencing mechanism with which LNCaP cells achieve the complete knock-down of *HMGA2.* At the post-transcriptional level, gene regulation is impacted by miRNAs being endogenous, non-coding small RNA molecules with a final size of ~21-23 nucleotides (for detailed review see Rana 2007). Similar to classic oncogenes, particular miRNAs can enhance tumour formation by suppressing target genes functioning as tumour suppressors and vice versa, miRNAs inhibit tumour growth by the suppression of oncogenes (Esquela-Kerscher and Slack 2006).

The regulation by miRNAs plays a central role in the control of *HMGA2* expression. The *HMGA2* 3'UTR has been found to negatively regulate gene expression (Ayoubi et al. 1999; Borrmann et al. 2003) and was later shown to harbour seven let-7 binding sequences (Mayr et al. 2007; Lee and Dutta 2007). The let-7 miRNA being described for the first time in the nematode *Caenorhabditis elegans*, which was also the first organism at all in which RNA induced gene silencing was demonstrated (Fire 1999), is known to cause destabilisation and degradation of its target mRNAs (Bagga et al. 2005), including *HMGA2*. Inverse correlation between *HMGA2* mRNA and members of the let-7 family, that comprises 13 members in humans (Roush and Slack 2008), is found during development, with let-7 in the late stages of animal development (Pasquinelli et al. 2000) and *HMGA2* in the early ones (Hirning-Folz et al. 1998). During the past years, *HMGA2* has been shown to be targeted by some miRNAs. Among these are e.g. the previously mentioned let-7 family with the miRNAs let-7 (Mayr et al. 2007) and miR-98 belonging to the same family (Hebert et al. 2007), miR-10a* and miR-21 (Zhu et al. 2013), miR-23b (Leone et al. 2014), miR-26a (Zhou et al. 2014), miR-142-3p (Chiou et al. 2013), miR-154 (Zhu et al. 2013), miR-365 (Qi et al. 2012) and others.

HMGA2 overexpression often is caused by the loss of regulated gene expression of the miRNAs themselves resulting in expression or silencing of the respective miRNA genes. The let-7 miRNA family e.g. is regulated by LIN28A (lin-28 homolog A (C. elegans) and LIN28B (lin-28 homolog B (C. elegans)) *via* different regulatory mechanisms impeding let-7 maturation (Piskounova et al. 2011). Consequently, misexpression of LIN28 is found in tumours with impact on let-7 expression as well as on the let-7 target genes. LIN28A and LIN28B are overexpressed followed by down-regulated let-7 expression and thus increased *HMGA2* expression in e.g. oral

squamous cell carcinoma (Wu et al. 2013), glioblastoma (Mao et al. 2013) and head and neck cancer (Alajez et al. 2012).

In prostate cancer, the let-7 family as well as other miRNAs were found to be down-regulated in several studies (Ozen et al. 2008; Nadiminty et al. 2012; Schubert et al. 2013). The most recent one found let-7b to function as a prognostic marker that also targets *HMGA1* (Schubert et al. 2013). Dysregulation of miRNA expression might play a role in the up-regulation of *HMGA2* gene expression in the prostate carcinoma cell line PC-3, as let-7c and let-7b are down-regulated therein (Nadiminty et al. 2012; Schubert et al. 2013).

Interestingly, *HMGA2* is in competition with other let-7 targets, influencing the expression thereof independently from its activity as a transcription factor by displacing them from the Ago-complex, as described in lung cancer cells (Kumar et al. 2014). From a number of 34 putative targets, 13 were suppressed by *Hmga2* depletion (Kumar et al. 2014). Six of these targets could be rescued by wt *Hmga2* and *Hmga2* with mutated start codon, being thus identified as ceRNA (competing endogenous RNA) targets, whereat seven could be rescued by wt *Hmga2* only, thus being classified as transcriptional targets (Kumar et al. 2014). Among the ceRNA targets were genes such as, amongst others, *Tgfbr3* (transforming growth factor, beta receptor III) and, interestingly, *Hmga1* (Kumar et al. 2014). *HMGA1* just recently was found to be targeted by the let-7 miRNAs so that a synergistic effect of *HMGA1* and *HMGA2* up-regulation is imaginable. Double-knock out of *Hmga1* and *Hmga2* in mice resulted in a “superpygmy” phenotype displaying reduced vitality and an extremely small size of 75 % of wt mice (Federico et al. 2014), whereat the body size of *Hmga2* knock-out mice was reduced about 60 % only (Zhou et al. 1995).

As *HMGA2* mRNA was not detectable in the LNCaP cell line though it displays a hypotetraploid karyotype including four apparently normal chromosomes 12 bearing the *HMGA2* locus, miRNAs are suspected to be responsible for mRNA repression. In contrast to the PC-3 cell line, LNCaP cells exhibit higher levels of let-7 miRNAs that could thus be part of the *HMGA2* silencing program in this cell line (Nadiminty et al. 2012; Schubert et al. 2013). Suppression of *DICER1* which represents an essential part in miRNA biogenesis by siRNA would thus lead to increased *HMGA2* mRNA amounts due to the lack of miRNA degradation. This would thus make *HMGA2* detectable by quantitative PCR.

Interestingly, the performed *DICER1* knock-down did not lead to detectable *HMGA2* expression in LNCaP cells as expected if *HMGA2* was silenced by miRNA regulation and which was shown in HeLa cells (Lee and Dutta 2007). Therefore, alternative mechanisms being responsible for the lack of measurable *HMGA2* mRNA needed to be considered. Epigenetic mechanisms impacting gene expression at the transcriptional level as e.g. methylation of CpG island and histone acetylation are valuable candidates for gene silencing. Methylation of CpG promoter islands leads to decreased gene expression and histone acetylation changes the overall charge of the nucleosome being composed of several histone proteins from positive to neutral leading to a more relaxed DNA structure that is now accessible for transcription factors.

The treatment of LNCaP cells with increasing concentrations of the DNA methyltransferase inhibitor 5-AdC actually resulted in the detection of *HMGA2* mRNA transcripts, though the amount was still at a very low level. The level of *HMGA2* mRNA amounted to 0.31-, 0.43- and 0.38-fold of that of the calibrator MCF-7 which was used due to the lack of *HMGA2* expression in the non-treated control. A 0.29-fold expression was yielded by simultaneous treatment with the histone deacetylase inhibitor TSA, while TSA alone did not result in *HMGA2* expression.

Up to date, the methylation status of the *HMGA2* gene and its genomic vicinity is not well documented. Given the cytotoxic potential of HMGA2 in LNCaP cells, silencing of *HMGA2* still is favourable for their overall survival. The tight silencing of the *HMGA2* gene shows that cells in general are in charge of a variety of mechanisms to determine a favourable gene expression profile. Though representing the opposite situation, namely the protection of *HMGA2* mRNA from being introduced into miRNA RISC, the ability to accurately control gene expression is underlined by the recent finding that *HMGA2* mRNA is protected in IGF2BP3 (insulin-like growth factor 2 mRNA binding protein 3) RNP (ribonucleoprotein) granules (Jønson et al. 2014). Besides *HMGA2* mRNA, other let-7 target mRNAs like that coding for LIN28B, suppressing let-7 maturation, are also stored and protected in these “safe houses” thus increasing the amount of oncogenes during tumour growth (Jønson et al. 2014).

It is unclear, if the *HMGA2* promoter region itself is demethylated upon 5-AdC treatment, or other genes are demethylated, which in turn activate *HMGA2*. Nonetheless, *HMGA2* mRNA levels still were very low. Concerning *HMGA2* regulation via histone deacetylation, it is known that the murine as well as the human

HMGA2 gene become down-regulated in NIH3T3, HeLa and F9 (derived from a mouse testicular teratoma) cells upon inhibition of histone deacetylases with TSA (Ferguson et al. 2003), confirming the lacking effect of TSA alone on treated LNCaP cells. It was later shown that a negatively regulating effect of HDAC inhibition in human umbilical cord blood-derived multipotent stem cells also was mediated by transcriptional activation of miRNAs miR-23a, miR-26a and miR-30a targeting *HMGA2* (Lee et al. 2011).

In benign mesenchymal tumours, reactivation of *HMGA2* is different from that in malignant cancer, as *HMGA2* is targeted by chromosomal aberrations (Schoenmakers et al. 1995; Ashar et al. 1995). In lipomas, the negatively regulating 3'UTR (Mayr et al. 2007; Lee and Dutta 2007) is separated from the first three exons which become fused with exons obtained from *LPP* leading to a fusion protein (Petit et al. 1996). In contrast, the *HMGA2* translocation in uterine leiomyomas targets different chromosomal regions than that in lipomas and, more importantly, the breakpoints mainly are located upstream or downstream of the *HMGA2* locus (Schoenberg Fejzo et al. 1996; Quade et al. 2003). Uterine leiomyomas generally display higher *HMGA2* expression when compared to the surrounding myometrium, but the highest expression levels can be observed in tumours carrying t(12;14) (Klemke et al. 2009). Thus, re-expression of *HMGA2* not only due to the loss of the 3'UTR, but also influenced by different regulatory units probably located in the 5' region as well (Schoenberg Fejzo et al. 1996; Quade et al. 2003), can be assumed. This is underlined by the presence of the 3'UTR including the complete let-7 binding sites in eight of 13 UL with a t(12;14) (Klemke et al. 2010). The mapping of the breakpoint as well as the loss of regulatory units positively affecting *HMGA2* expression should be able to provide new insights into the mechanism leading to up-regulation by this translocation. Therefore, uterine leiomyomas bearing the t(12;14) translocation were chosen for investigation with aCGH enabling the detection of small losses associated with the translocation that otherwise seems to be balanced by means of conventional karyotyping due to the limited resolution. In two of the investigated uterine leiomyomas, a small loss that might be caused by the translocation was identified by visual inspection with DNA Analytics software (Agilent). Both potential breakpoints are located upstream of the *HMGA2* locus at a distance of ~1.14 Mb and ~412 kb, respectively, revealing that no common region was affected. FISH using probes that span those regions that were indicated as

deleted or a custom-made array with a narrower probe spacing of this region would be a useful tool for the confirmation of those potential breakpoint-associated deletions. The localisation of the potential breakpoints upstream of *HMGA2* is in line with the finding that re-expression of *HMGA2* due to loss of the 3'UTR and the resulting missing influence of let-7 can be excluded for at least 2 of the ULs investigated herein, as gene expression analysis using real-time PCR for full length *HMGA2* as well as the 3'UTR only did not reveal differential expression for these two sequences (Klemke et al. 2010). Only one of these tumours displayed higher expression for exons 1-2 than for the 3'UTR pointing to a potential further intragenic breakpoint in at least some of the cells, though there are larger differences between both assays in the tumour set investigated (Klemke et al. 2010).

The up-regulation of *HMGA2* hence is not accompanied by the deletion of a common region containing negatively regulating elements. Also in discussion is the activation of *HMGA2* through this translocation because of an enhancing region on chromosome 14 provided by the translocation partner *RAD51B* (Mehine et al. 2013). The activation of *HMGA2* can thus be compared to that of *MYC* (v-myc avian myelocytomatosis viral oncogene homolog) where *MYC* is activated through enhancing regions provided by the translocation partner *IGH* (immunoglobulin heavy locus; Hayday et al. 1984). In addition to the lost down-regulating influence of let-7 expression on *HMGA2*, Mayr et al. (2007) propose suppression of *HMGA2* fusion partners such as *RAD51B* and others that are known tumour suppressors through transfer of the *HMGA2* 3'UTR. The let-7 binding sites transferred from *HMGA2* make their mRNA susceptible to miRNA destabilization leading to degradation of their mRNA target, thus enhancing tumourigenesis. Separation of the *HMGA2* 3'UTR and the CDS is a frequent finding in lipomas (Bartuma et al. 2009) and also occurs in uterine leiomyomas, although more rarely therein (Quade et al. 2003; Klemke et al. 2010).

Nevertheless, miRNAs also seem to play a role in *HMGA2* dysregulation in uterine leiomyomas, as miR-93 and miR-106b are candidates for *HMGA2* regulation that are, besides the well-known let-7a, down-regulated in tumours overexpressing *HMGA2* (Mello et al. 2013). *MiR-93* and *miR-106b* are located in the chromosomal region 7q22 (Mello et al. 2013) that is frequently deleted in uterine leiomyomas and also harbours the transcriptional regulator *CUX1* (cut-like homeobox 1) being the target of 7q22 aberrations (Schoenmakers et al. 2013). Furthermore, a signature of miRNA

expression associated with race and tumour size was revealed (Wang et al. 2007). Conversely, the let-7 family was found to be higher expressed in the tumour than in the matching myometrial tissue (Wang et al. 2007), whereat high levels were found in small uterine leiomyomas and low levels in larger ones, matching to the report that high *HMGA2* expression is correlated with larger size (Rein et al. 1998; Hennig et al. 1999). As high proliferation indexes obtained by the proliferation marker MKI67 (marker of proliferation Ki-67) associated with higher HMGA2 expression were inversely correlated with let-7c expression, the hypothesis of other mechanisms interrupting the regulation of *HMGA2* by let-7 is supported (Peng et al. 2008). Cryptic transcripts and point mutations in the let-7 binding sites (Peng et al. 2008) may also be responsible as well as shortening of the 3'UTR by alternative cleavage and polyadenylation (Mayr and Bartel 2009), though this mechanism for *HMGA2* so far only has been described in serous ovarian carcinoma (He et al. 2014). By shortening the 3'UTR, oncogenes achieve higher mRNA stability and produce greater protein amounts by escaping miRNA regulation (Mayr and Bartel 2009). However, transcriptional activation still is a possible reason that cannot be excluded for high *HMGA2* expression in uterine leiomyomas.

Concluding, it can be said that re-activation of *HMGA2* is a major event in both benign mesenchymal and malignant epithelial tumours, further enhancing tumourigenesis. As is shown by the results presented herein, the reasons for *HMGA2* activation and its impact on the cell type as well as its silencing differ and consist of multiple and different mechanisms that need specific targeting when considering therapy.

5. Summary

In the work presented herein, mechanisms leading to the activation of the oncofoetal *HMGA2* gene in benign as well as in malignant tumours have been investigated. In mesenchymal cells, *HMGA2* expression increases upon incubation with growth factors such as FGF1, FGF2 and PDGF-BB. This effect was examined in HUVECs and in the human prostate carcinoma cell line PC-3. In HUVECs, *HMGA2* increased in response to FGF1, FGF2 and VEGFA, with the highest increase for FGF2. VEGFA induced the lowest response. The proliferation of HUVECs nearly doubled in consequence of incubation with FGF1 or FGF2, while VEGFA induced the lowest increase in proliferation. A correlation coefficient of R=0.96 indicates a linear correlation. In contrast, *HMGA2* is not inducible in the androgen-independent prostate cancer cell line PC-3 by incubation with PDGF-BB, FGF1 or FBS, also containing growth factors. Proliferation increased through incubation with FGF1 or PDGF-BB for the 1.8- or 1.6-fold, but not in response to FBS.

Another prostate carcinoma cell line, LNCaP, does dot display detectable *HMGA2* expression in real time RT-PCR, though it possesses four apparently normal chromosomes 12. It is thus a good model to study the effects of HMGA2. To avoid the cytotoxic consequences that occurred after transfecting the eukaryotic expression vector pCR3.1, these cells were incubated with increasing concentrations (1, 10 and 100 µg/ml) of a recombinant HMGA2 protein. A cytotoxic and damaging effect of HMGA2 was shown as well as a dose-dependent induction of apoptosis after 48 h. As underlying mechanism for *HMGA2* silencing in this cell line, miRNAs being huge players in the post-transcriptional regulation seem unlikely, as knock-down by siRNA targeting *DICER1*, which is essential in miRNA processing, did not lead to *HMGA2* expression. The incubation of LNCaP cells with the methyltransferase inhibitor 5-AdC resulted in the detection of a low amount of *HMGA2* mRNA by real time RT-PCR, indicating the methylation of the *HMGA2* gene or other activating genes.

In benign mesenchymal tumours, *HMGA2* often is affected by translocations. While the breakpoint in lipomas often is located in intron 3 separating the 3'UTR containing miRNA binding sites from the coding sequences for the functional AT-hooks leading to the expression of a truncated but functional protein, the breakpoints in uterine leiomyomas mainly are located upstream of *HMGA2*. This points to an activation mechanism distinct from that in lipomas. In two of three tumours, potential small

deletions upstream of *HMGA2* were identified that may be caused by the translocation and are connected to the breakpoints. A region commonly deleted was not identified in the tumours investigated, suggesting expansion of the study followed by confirmation of the potential breakpoints using FISH probes spanning the potential breakpoint regions to identify the loss of potential regulatory elements in the 5' region upstream of the *HMGA2* gene.

The functions as well as the regulation of HMGA2 are versatile and need to be considered cell-type specifically to offer a valuable and promising target in potential therapeutic applications.

6. Zusammenfassung

In der hier vorliegenden Arbeit wurden Mechanismen, die zur Reaktivierung des onkofoetalen *HMGA2*-Gens in benignen sowie in malignen Tumoren führen, untersucht. In mesenchymalen Zellen wird *HMGA2* nach Stimulation mit Wachstumsfaktoren wie FGF1 und FGF2, PDGF-BB und weiteren verstärkt exprimiert. Dieser Effekt wurde in HUVECs sowie in der humanen Prostatakarzinom-Zelllinie PC-3 untersucht. Hierbei zeigte sich, dass *HMGA2* in HUVECs durch FGF1, FGF2 und VEGFA induzierbar ist, wobei FGF2 den größten Effekt erreichte. VEGFA induzierte den geringsten, nicht-signifikanten Anstieg in der *HMGA2*-Expression. Die Proliferation der HUVECs verdoppelte sich nahezu in Folge der Inkubation mit FGF1 oder FGF2, während VEGFA hier ebenfalls die geringste Steigerung erzielte. Ein Korrelationseffizient von R=0,96 zeugt hier von einer linearen Korrelation. Im Gegensatz dazu ist *HMGA2* in der Androgen-unabhängigen PC-3 Zelllinie nicht mehr durch die Inkubation mit PDGF-BB und FGF1 sowie durch FBS, das ebenfalls Wachstumsfaktoren enthält, induzierbar. Eine erhöhte Proliferation um das 1,8- und 1,6-fache wurde durch FGF1 und PDGF-BB ausgelöst, nicht aber durch FBS.

Eine weitere Prostatakarzinom-Zelllinie, LNCaP, weist im Gegensatz zu PC-3 keine mittels Real-Time RT-PCR detektierbare *HMGA2*-Expression auf, obwohl sie über vier augenscheinlich normale Chromosomen 12 verfügt und bietet sich somit als gutes Modell zur Studie der Auswirkungen von HMGA2 an. Um die ermittelten zytotoxischen Folgen einer Transfektion mit dem eukaryotischen Expressionsvektor pCR3.1 zu vermeiden, wurden diese Zellen in Medium mit verschiedenen Konzentrationen (1, 10 und 100 µg/ml) rekombinanten HMGA2s gehalten. In Folge dessen zeigte sich, dass HMGA2 auf LNCaP-Zellen eine zytotoxische und Viabilitäts-senkende Wirkung hat und zudem nach 48 h Inkubationszeit dosisabhängig Apoptose auslöst. Als zu Grunde liegender Mechanismus des strikten Silencings in dieser Zelllinie scheint die Regulation mittels miRNAs, die eine große Rolle in der post-transkriptionellen Regulierung spielen, unwahrscheinlich, da ein Knock-Down mit Hilfe von siRNAs gegen *DICER1*, das essentiell für die miRNA-Prozessierung ist, keine Expression des *HMGA2*-Gens nach sich zog. Durch die Inkubation mit dem Methyltransferase-Inhibitor 5-AdC konnte eine, wenn auch geringe, Menge an *HMGA2*-mRNA detektiert werden, was auf eine Methylierung des *HMGA2*-Gens oder anderer, aktivierender Gene hinweist.

In benignen mesenchymalen Tumoren ist häufig das *HMGA2*-Gen von Translokationen betroffen. Während in Lipomen der 3'UTR mit den miRNA-Bindestellen durch den Bruchpunkt in Intron 3 von der kodierenden Sequenz für die funktionellen AT-Hooks getrennt wird, und ein trunkiertes, aber funktionales Protein exprimiert wird, liegen die Bruchpunkte in Uterus-Leiomyomen größtenteils stromaufwärts von *HMGA2*. Dies deutet auf einen anderen Aktivierungs-mechanismus als den in Lipomen hin. Mittels aCGH konnte hier in zwei von drei Tumoren mögliche kleine Deletionen stromaufwärts von *HMGA2* gefunden werden, die potentiell durch die Translokation verursacht wurden und den Bruchpunkt möglicherweise begleiten. Es konnte jedoch keine gemeinsame Region in den untersuchten Tumoren festgestellt werden. Hier bietet sich eine Ausweitung der Studie mit anschließender Überprüfung der potentiellen Bruchpunkte mittels FISH an, um den Verlust von eventuellen negativen Regulatoren stromaufwärts der 5'-Region von *HMGA2* zu identifizieren

Zusammenfassend kann gesagt werden, dass sowohl die Funktionen als auch die Regulationsmechanismen von HMGA2 vielseitig sind, und daher Zelltyp-spezifisch betrachtet werden sollten, um ein valides und vielversprechendes Ziel in potentiellen therapeutischen Anwendungen zu bieten.

7. Complete list of publications

7.1 Peer reviewed papers

1. Helmke BM, Markowski DN, **Müller MH**, Sommer A, Müller J, Möller C, Bullerdiek J: HMGA proteins regulate the expression of FGF2 in uterine fibroids. Molecular Human Reproduction; 2011 Feb; 17(2): 135-42

2. **Müller MH**, Reimann-Berg N, Bullerdiek J, Murua Escobar H: Genetic characterization of dogs via chromosomal analysis and array-based comparative genomic hybridization (aCGH). Tierärztliche Praxis Ausgabe K Kleintiere Heimtiere; 2012 Feb 20; 40(1): 55-8

3. **Müller MH**, Drieschner N, Focken T, Bartnitzke S, Winter N, Klemke M, Bullerdiek J: *HMGA2* expression in the PC-3 prostate cancer cell line is autonomous of growth factor stimulation. Anticancer Research; 2013 Aug; 33(8): 3069-78

4. Klemke M, **Müller MH**, Wosniok, W, Markowski DN, Nimzyk R, Helmke BM, Bullerdiek J: Correlated expression of *HMGA2* and *PLAG1* in thyroid tumors, uterine leiomyomas and experimental models. PLoS ONE; 2014 Feb; 9(2): e88126

7.2 Poster presentations

Müller MH, Drieschner N, Reimann-Berg N, Bullerdiek J: Establishment of a stable cell line with constitutionally down-regulated *HMGA2* expression. Poster presentation at the annual meeting of the German Society of Human Genetics in Regensburg, 16th-18th of March 2011

8. Abbreviations

5-AdC	5-aza-2′-deoxycytidine
aCGH	array based comparative genomic hybridisation
ATM	ataxia telangiectasia mutated
ATR	ataxia telangiectasia and Rad3 related
bp	base pair
BrdU	5-bromo-2'-deoxyuridine
CDH1	cadherin 1, type 1, E-cadherin (epithelial)
CDH2	cadherin 2, type 1, N-cadherin (neuronal)
cDNA	complementary deoxyribonucleic acid
CDS	coding sequence
ceRNA	competing endogenous ribonucleic acid
CHEK1	checkpoint kinase 1
CpG	cytosine phosphate guanine
CTNNB1	catenin (cadherin-associated protein), beta 1, 88kDa
CUX1	cut-like homeobox 1
DAPI	4',6-diamidino-2-phenyindole
DICER1	dicer 1, ribonuclease type III
DNA	deoxyribonucleic acid
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DSB	double strand break
E2F1	E2F transcription factor 1
EBM-2	endothelial basal medium-2
EGM-2	endothelial growth medium-2
ELISA	enzyme-linked immunosorbent assay
EMEM	Eagle's Minimum Essential Medium
EMT	epithelial-mesenchymal transition
ERCC1	excision repair cross-complementing rodent repair deficiency, complementation group 1 (includes over-lapping antisense sequence)
FBS	foetal bovine serum
FGF1	fibroblast growth factor 1 (acidic)

FGF2	fibroblast growth factor 2 (basic)
FLT1	fms-related tyrosine kinase 1
GF	growth factor
h	hour
HDAC1	histone deacetylase 1
HIF1A	hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)
HMGA1	high mobility group AT-hook 1
HMGA2	high mobility group AT-hook 2
HMGIC	high-mobility group (nonhistone chromosomal) protein isoform I-C
HMGIY	high-mobility group (nonhistone chromosomal) protein isoforms I and Y
HPRT1	hypoxanthine phosphoribosyltransferase 1
HUVEC	human umbilical vein endothelial cell
IGF2BP2	insulin-like growth factor 2 mRNA binding protein 2
IGF2BP3	insulin-like growth factor 2 mRNA binding protein 3
JunB	jun B proto-oncogene
kDa	kilodalton
KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
LDH	lactate dehydrogenase
let-7	microRNA let-7
LIN28A	lin-28 homolog A (C. elegans)
LIN28B	lin-28 homolog B (C. elegans)
LPP	LIM domain containing preferred translocation partner in lipoma
MET	mesenchymal-epithelial transition
min	minute
miRNA	micro ribonucleic acid
MIRLET7	microRNA let-7
M-MLV	Moloney murine leukaemia virus
mg	milligram
MKI67	marker of proliferation Ki-67
ml	millilitre

mRNA	messenger ribonucleic acid
MYC	v-myc avian myelocytomatosis viral oncogene homolog
NaCl	sodium chloride
NF-κB	nuclear factor of kappa light polypeptide gene enhancer in B-cells
ng	nanogram
nt	nucleotide
ORF	open reading frame
PBS	phosphate buffered saline
PAC	phage artificial chromosome
PAGE	polyacrylamide gel electrophoresis
Pcna	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PDGF-BB	platelet-derived growth factor beta polypeptide
pRB	retinoblastoma protein
PTM	post-translational modification
RAD51B	RAD51 paralog B
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNP	ribonucleoprotein
rpm	rotations per minute
RPMI 1640	Roswell Park Memorial Institute 1640
RT	reverse transcription
sec	second
Smad	Smad family member
SNAI1	snail family zinc finger 1
SNP	single nucleotide polymorphism
SSC	standard saline citrate
TERT	telomerase reverse transcriptase
TGF-beta-1	transforming growth factor beta-1
TGFR2	transforming growth factor-beta type 2
Tgfbr3	transforming growth factor, beta receptor III
u	unit
UL	uterine leiomyoma

UTR	untranslated region
VEGFA	vascular endothelial growth factor A
VIM	vimentim
WNT	wingless-type MMTV integration site family
ZCCHC11	zinc finger, CCHC domain containing 11

9. References

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11. Declaration/Erklärung

I herewith declare that I wrote and completed the dissertation entitled “Dysregulation of the high mobility group AT-hook 2 (*HMGA2*) gene in human tumours” on my own and used nothing but the sources as referenced.

Hiermit erkläre ich, dass ich die Doktorarbeit mit dem Titel „Dysregulation of the high mobility group AT-hook 2 (*HMGA2*) gene in human tumours“ selbstständig verfasst und geschrieben habe und außer den angegebenen Quellen keine weiteren Hilfsmittel verwendet habe.

Bremen, den 02.06.2014

Marietta Henrike Müller