HMGA2 Expression in the PC-3 Prostate Cancer Cell Line Is Autonomous of Growth Factor Stimulation

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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.

The gene encoding mammalian high-mobility group AT-hook 2 (HMGA2) protein is abundantly expressed during early embryonic development (1-3). In contrast, it is lacking in most differentiated cells and tissues but apparently retains the ability to be activated in some types of somatic stem cells in vivo as well as in vitro (4-7). Re-expression of HMGA2 is a frequent finding in a variety of benign tumours mostly of mesenchymal origin, as well as in subgroups of malignant neoplasms (8-10). Whereas in the former cases e.g. lipoma, uterine leiomyoma, endometrial polyp and pulmonary chondroid hamartoma, as well as in pleomorphic adenomas of the salivary glands, the transcriptional up-regulation of HMGA2 results from chromosomal rearrangements affecting its locus at the chromosomal segment 12q14-15 (9, 11, 12), malignant tumours only very rarely display these typical chromosomal translocations despite overexpression of HMGA2, and amplification of its locus also seems to be a rare finding (13, 14). However, overexpression of HMGA2 in malignant solid tumours has been found to be associated with epithelial-mesenchymal transition (15-17) thus, at least in part, explaining the worse prognosis observed for patients with malignant tumours with an abundant expression of HMGA2. Apparently, HMGA2 can enhance de-differentiation of epithelial tumour cells, leading them into a more motile mesenchymal-like state that facilitates tumour metastasis (18, 19). HMGA2 is involved in a variety of cellular processes, such as differentiation (8), stem cell renewal (5, 6), as well as cell growth and proliferation (20), but the detailed role of HMGA2 in malignant transformation has not been fully-elucidated.

In general, the main routes of HMGA2 transcriptional regulation remain obscure, as the main characteristics of its expression apparently differ between the cell types it is expressed in (11). In somatic stem cells, a temporarily very restricted expression can be stimulated by a plethora of growth factors such as fibroblast growth factor (FGF)-1 and -2, platelet-derived growth factor (PDGF)-BB and bone morphogenic protein (BMP)-4 (7, 21, 22). In benign tumours with rearrangements of 12q14-15 the expression of HMGA2 is much higher than in their malignant counterparts not exhibiting these chromosomal rearrangements (12, 23). This points to a removal of negative regulatory elements such as the 3'untranslated region (3’UTR) with microRNA let-7-binding sites (24-26) and positively acting as yet unidentified regions juxtaposed to the HMGA2 locus as a result of varying translocations (11, 27). Its expression drastically decreases, however, when these cells are put into culture (28) indicating a lack of (unknown) factors necessary for the activation of the rearranged allele in vitro. In contrast, cell lines of malignant tumours are often characterized by an overexpression of HMGA2. Nevertheless, almost nothing is known about possible mechanisms that could still force its expression.

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Herein, we have used the PC-3 cell line, derived from prostate carcinoma, in order to test the hypothesis that in malignant tumours the re-expression of *HMGA2* becomes independent of external stimuli such as foetal bovine serum (FBS), FGF1 and PDGF-BB, thus leading to the constitutional expression of the onco-embryonic gene. Additionally, we were interested in the correlation between external stimuli and proliferation, as well as its possible connection with *HMGA2* expression.

**Materials and Methods**

*Cell lines and tissue.* Cell lines MCF-7 (breast cancer; DSMZ, Braunschweig, Germany), HCT116 (colon carcinoma; DSMZ), LNCaP (prostate carcinoma; Cell Lines Service, Eppelheim, Germany), PC-3 (prostate carcinoma; Cell Lines Service), S277 (anaplastic thyroid carcinoma; Centre for Human Genetics, Bremen, Germany) and MRI-H215 (cervix carcinoma; Tumorbank DKFZ, Heidelberg, Germany) were maintained in a humidified atmosphere at 37˚C with 5% CO₂. RNA was isolated using the NucleoSpin RNA II Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer’s instructions.

As a control, RNA was isolated from a snap-frozen sample of uterine leiomyoma cytogenetically displaying a t(12;14) (12) with the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions, including the DNase I digestion.

*Cell culture.* PC-3 cells (Cell Lines Service) were cultured in RPMI-1640 medium (Life Technologies, Darmstadt, Germany) supplemented with 10% (v/v) foetal bovine serum (Life Technologies) for stimulation experiments or in Dulbecco’s modified Eagle’s medium (DMEM):Ham’s F12 (1:1) supplemented with 10% FBS (Cell Lines Service) for chromosomal analysis. When grown till confluence, cells were detached using TrypLE Express (Life Technologies) and passaged. The medium was changed twice per week.

*Chromosome preparation and Giemsa banding.* For chromosome preparation, 300 ng colcemide (Biochrom AG, Berlin, Germany) were added to the medium and cells were incubated for 1 h at 37°C in 5% CO₂. Cells were then detached with TrypLE Express and incubated with hypotonic solution (1:7) on a rocking shaker for 20 min at room temperature. The pellet was resuspended in 50 ml prewarmed banding buffer at 37°C and incubated for 8 min. Slides were incubated therein for 8 s followed by incubation in 1.5% Giemsa solution for 10 min then washing twice in water. The amount of trypsin in G-banding was reduced to 1.5 mg for subsequent fluorescence *in situ* hybridisation (FISH) analyses. Metaphases were photographed (data not shown) and then decoloured in 70% ethanol. After air drying, the slide was incubated at 60°C over night followed by FISH.

*Fluorescence in situ hybridisation.* For investigation of the *HMGA2* locus in PC-3 cells, a phage artificial chromosome covering intron 3 of the gene (29) and a probe specific for centromere 12 were used. Probe DNA was labelled using the Nick Translation Kit according to the manufacturer’s instructions (Abbott Laboratories, Abbott Park, OH, USA) with SpectrumOrange (Abbott Laboratories, *HMGA2*) or SpectrumGreen (Abbott Laboratories, Centromere 12), respectively. Probe DNA and previously prepared metaphases were co-denatured for 3 min at 80°C then hybridisation was performed at 37°C overnight in a humidified chamber. Thereafter, slides were washed for 5 min in 0.1x standard saline citrate (SSC; USB, Cleveland, IL, USA) at 61°C, rinsed three times in 1x phosphate buffered saline (PBS) solution and dehydrated in an increasing ethanol series. The slides were covered with 25 μl mounting medium with 4',6-diamidino-2-phenylindole (DAPI; Vectorlabs, Burlingame, CA, USA), BAC clones RP11-269K4 (AQ478964 and AZS16203, proximal) or RP11-293H23 (AC012264, distal) were used as break-apart probes for the detection of rearrangements of *HMGA2*. Digestion with 5 ng pepsin (Merck, Darmstadt, Germany) for 3 min at room temperature was followed by fixation with paraformaldehde (0.1% PFA/1x PBS) for 10 min and dehybridisation in an increasing ethanol series. Co-denaturation of probe and chromosomal DNA for 7 min at 77°C and hybridisation at 37°C over night were performed. Slides were washed in 0.4x SSC/0.3% NP-40 at 71°C for 2 min and briefly dipped in 2x SSC/0.1% NP-40. After air drying, the slides were covered with mounting medium with DAPI (Vectorlabs).
Spectral karyotyping. Spectral karyotyping (SKY)-FISH was performed using the Spectral Karyotyping Human Reagent and CAD-Kit (Applied Spectral Imaging, Edingen-Neckarhausen, Germany) according to the manufacturer’s protocol with modifications. Briefly, step A (trypsin digestion) was skipped. Chromosomes were denatured using the denaturation solution on a slide warmer followed by an increasing ice-cold ethanol series. Hybridisation (overnight), post-hybridisation including the treatment with blocking agent, and detection were carried out according to the original protocol.

Stimulation of HMGA2 expression with serum and growth factors. For stimulation with FBS or FGF1 and PDGF-BB, PC-3 cells were seeded in multiwell plates and allowed to attach for 24 h. Thereafter, cells were starved with RPMI-1640 supplemented with 1% FBS for another 24 h. Starvation medium was substituted with either fresh starvation medium (negative control), or medium supplemented with either 10% or 20% FBS, 1% FBS with 25 ng/ml FGF1 (Jena Bioscience, Jena, Germany) or 1% FBS with 25 ng/ml PDGF-BB (Sigma-Aldrich, Munich, Germany) and cells were incubated for 12 h or 24 h. Finally, cells were either harvested for subsequent RNA isolation or a proliferation assay was performed (see Figure 1 for workflow).

Proliferation assay. Proliferation was measured using the Cell Proliferation ELISA, BrdU (colorimetric) Kit (Roche Applied Science, Penzberg, Germany) according to the manufacturer’s instructions. Briefly, 7,500 cells per well were seeded in a 96-well plate (Greiner Bio-One, Frickenhausen, Germany) and stimulated with serum or growth factor as described above. 5-bromo-2’-deoxyuridine (BrdU) incubation was carried out for 24 h and incubation with the secondary antibody was carried out for 90 min. The incubation settings with the different stimuli were performed in octuplicates each. These were averaged and normalised to the negative control incubated with 1% FBS only for the respective time period. Outliers were removed. Absorbance was measured using a Synergy HT Multi-Mode Microplate Reader and the corresponding software KC4 (BioTek Instruments, Bad Friedrichshall, Germany). Results were analysed using Excel software (Microsoft, Unterschleißheim, Germany) and GraphPad Instat (GraphPad Software, La Jolla, CA, USA) performing a one-way analysis of variance (ANOVA).

RNA isolation from stimulated PC-3 cells. For RNA isolation from stimulated PC-3 cells, 200,000 cells per well were seeded in a 6-well plate (Nunc, Langenselbold, Germany) and treated as described above. RNA was then isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. The optional on-column DNase I digestion was included in the protocol. Each incubation setting was carried out in duplicates.

cDNA synthesis. Total RNA (250 ng) was reverse-transcribed using Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Life Technologies) with random primers (Life Technologies) according to the manufacturer’s instructions.

Real time reverse transcription (RT)-PCR. Transcripts of HMGA2 were quantified in triplicates on a 7300 Real-Time PCR System (Life Technologies) using Sequence Detection Software version 1.2.3 (Life
HMGA2 expression in response to stimulation with FBS and growth factors. Firstly, we were interested to determine if HMGA2 can be induced by FBS and growth factors. In contrast to untransformed mesenchymal cells, expression of HMGA2 did not increase in response to stimulation with FBS (Figure 4A), FGF1 nor PDGF-BB (Figure 4B) in the PC-3 prostate cancer cell line, as its expression remained stable at the same level in any incubation tested.

Proliferation of PC-3 cells stimulated with FBS, FGF1 and PDGF-BB. As proliferation of cell lines is inducible by FBS as well as growth factors, we then tested proliferation in response to the same settings tested for stimulation of HMGA2 in order to establish a possible correlation between proliferation and HMGA2 expression. The cell line did not display any significant increase of proliferation when incubated with FBS (Figure 5A), whereas significant changes in the proliferation rate induced by both growth factors were observed. An incubation period of 12 h with 25 ng/ml PDGF-BB, and with FGF1 resulted in a 1.64- (p<0.01) and 1.78-fold (p<0.001) increase in proliferation, respectively, when compared to the negative control (Figure 5B). After 24 h of incubation, the proliferation induced by FGF-1 or PDGF-BB decreased to 1.2-fold (p>0.05) that of the negative control, and significantly increased proliferation for 24 h incubation was only observed in PC-3 cells treated with FGF1 and 10% or 20% FBS, respectively (p<0.05). The differences between the remaining samples were non-significant (p>0.05).

Discussion

HMGA2 belongs to the group of oncofoetal proteins involved in a variety of cellular functions, such as differentiation, apoptosis, cellular growth and proliferation [for review see (30, 31)]. It is expressed mainly during embryogenesis and in neoplastic tissues, where its expression is re-activated (30). Nevertheless, the fundamental differences between expression of HMGA2 during embryogenesis, in benign tumours and in malignant tumours are obscure. Whereas as a rule, the expression in benign tumours is often higher than in malignant tumours, in the latter case, a strong expression of HMGA2 is actually associated with a worse prognosis (10, 17, 32, 33). However, expressional re-activation, mainly in benign mesenchymal tissues, involves chromosomal translocation fusion partners characteristic of the tumour type, such as chromosomal band 3q27~q28 in lipomas (34, 35) and 14q24 in uterine leiomyomas (36). In these translocations, the 12q14~15 breakpoint is often located in the third intron of HMGA2 or downstream but intragenic, leading to the loss of regulatory sequences in the 3’UTR that serve as binding sites for the let-7 miRNA (25, 26). Thus, the re-expression of a
truncated version containing the three AT-hooks as functional units might contribute to tumourigenesis (37, 38). Breakpoints located outside of HMGA2 also occur frequently (39), possibly separating regulatory units from the gene locus itself (40).

Generally, post-transcriptional down-regulation of HMGA2 occurs via a variety of miRNAs such as miRNA-365 in lung cancer (41) and miR-10a* and miR-21 in endothelial progenitor cells (42). The 3’UTR constitutes a negative regulatory element (43), as the binding sites for the let-7 miRNA, which is the best known miRNA for the down-regulation of HMGA2, are located herein (25). Additionally, histone deacetylases 1 and 2 suppress HMGA2 expression via up-regulation of miRNAs of the let-7 family and miR-23a, miR26a and miR-30a in human cord-blood derived multipotent stem cells (44). In contrast, miRNA-182 enhances the expression of HMGA2 in high-grade serous ovarian carcinoma probably via regulation of breast cancer 1, early onset (BRCA1) (45). Additional influence on the regulation of HMGA2 has been ascribed to a (TC)n-repeat sequence upstream of the ATG start codon, with a longer sequence being associated with increased expression (46, 47).

Little is known about the contribution of HMGA2 to tumourigenesis in malignant epithelial tumours. HMGA2 is overexpressed in non-small cell lung cancer when compared to the normal surrounding tissue (10), and increased expression has also been linked to tumours of the prostate in dogs (48). In human prostate cancer, increased amounts of HMGA2 were found in the tumour when compared to the adjacent non-tumourous tissue, and have been associated with EMT in the PC-3 cell line (49). As to possible paracrine effects of cells overexpressing HMGA2, Zong et al. recently showed that in mice, Hmga2 overexpression in prostate stromal cells is sufficient to induce prostatic intraepithelial neoplasia and hyperplasia and, in cooperation with overexpression of androgen receptor, can induce poorly-differentiated adenocarcinoma (50). On the other hand, concerning the influence on proliferation in epithelial tumour cells, the ectopic expression of Hmga2 in the Dunning rat prostate tumour cell line, which does not endogenously express Hmga2, did not reveal any significant alteration in its growth ability (51), which is supported by our findings that PC-3 cells treated with FGF1 or PDGF-BB display a
higher proliferation rate than unstimulated controls without an increase in expression of HMGA2 mRNA.

Nevertheless, the regulation of HMGA2 via growth factors is of interest in the field of tumor development and progression. Ayoubi et al. demonstrated that expression of HMGA2 is inducible via the phosphatidylinositol 3-kinase and mitogen-activated protein kinase pathway by a variety of growth factors, where FGF1 and PDGF-BB have been identified as the most potent ones in the pre-adipocyte cell line 3T3-Li (21). Interestingly, this strong inducing effect is lost in the epithelial cell line PC-3, as is indicated by our data. Accordingly, HMGA2 is neither inducible by FBS, which may be ascribed to the explanation by Ayoubi et al. stating that growth factors in the serum are responsible for the induction of delayed early response genes such as HMGA2 (21). Although there is a significant increase in the

Figure 4. Relative expression of high-mobility group AT-hook 2 (HMGA2) after stimulation with foetal bovine serum (FBS) (A) and fibroblast growth factor-1 (FGF1) or platelet-derived growth factor-BB (PDGF-BB) (B). A control taken before stimulation and after starvation referred to as 0 h was set as an expression value of 1 for calibration. Hypoxanthine phosphoribosyltransferase 1 (HPRT1) served as endogenous control. Error bars indicate standard deviation.
proliferation rate after an incubation period of 12 h with FGF1 and PDGF-BB (Figure 5), this does not correlate with a further increase of the HMGA2 mRNA level. The exact mechanism by which HMGA2 induces proliferation is not known yet, although it has been shown that HMGA2 promotes proliferation in a variety of cells such as chondrocytes (52, 53), and that silencing of HMGA2 leads to reversible reduction of proliferation in e.g. retinoblastoma cells (54), human umbilical cord blood-derived stromal cells (55) and a non-small cell lung carcinoma cell line (56). Additionally, the prostate cancer cell lines PC-3 and LNCaP were used by Peng et al. to demonstrate the influence of let-7c expression on viral transfection-induced HMGA2 expression and proliferation rate, resulting in decrease in proliferation correlating with a diminished expression of HMGA2 (26).

Figure 5. Relative proliferation rate of PC-3 cells induced by incubation with foetal bovine serum (FBS) (A), or fibroblast growth factor-1 (FGF1) and platelet-derived growth factor-BB (PDGF-BB) (B). For normalisation of the proliferation assay, a control grown in medium supplemented with 1% FBS was set as a value of 1 for the respective incubation period. Error bars indicate standard deviation.
In prostate cancer, FGF1 expression is elevated in more than 80% of the tumours investigated (57), as well as in the PC-3 cell line (58) investigated here, whereas it is barely detectable by northern blot and RT-PCR in normal prostatic tissue (59). FGF1 immunoreactivity in malignant epithelium correlates with tumour stage and Gleason score (60). Exogenous FGFs promote proliferation in normal, immortalised and fully transformed prostatic epithelial cells (57), which is confirmed by our data for FGF1.

As for PDGF-BB, its proliferation-stimulating role rather correlates with an increase in the amount of the corresponding receptor than in the cytokine itself, as stated by Nazarova et al. (61). They investigated the proliferation-inducing properties of PDGF-BB in the prostate cancer cell line LNCaP and found the cytokine did not cause any effect on proliferation due to a lack of the receptor, which is slightly more greatly expressed in PC-3 cells (61). Interestingly, both cell lines express the cytokine itself, favouring proliferation of the surrounding stroma (61). In this context, it should be noted that the expression of PDGF-BB by epithelial prostate cancer cells promotes the proliferation of mesenchymal stem cells in vitro and in vivo as shown by Cheng et al. (62). Therefore, the development of prostate cancer seems to be a complex and delicate cooperation between stroma and epithelium, in which one tissue produces or overexpresses factors that influence the other, and can, once disturbed, enhance tumour progression in the epithelial part.

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