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

MARIETTA HENRIKE MÜLLER, NORBERT DRIESCHNER, TIM FOCKEN, SABINE BARTNITZKE, NINA WINTER, MARKUS KLEMKE and JÖRN BULLERDIEK

Center for Human Genetics, University of Bremen, Bremen, Germany

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.

Correspondence to: Jörn Bullerdiek, Centre for Human Genetics, University of Bremen, Leobener Str. ZHG, 28359 Bremen, Germany. Tel: +49 42121861500, Fax: +49 42121851505, e-mail: bullerd@uni-bremen.de

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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% CO_2. 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% CO_2. Cells were then detached with TrypLE Express and incubated with hypotonic solution (1:7) on a rocking shaker for 20 min at room temperature. Thereafter, cells were centrifuged for 10 min at 1000 ×*g*. The pellet was resuspended in the remaining fluid and fixative (methanol:acetic acid 3:1) added. Centrifugation followed by fixation was repeated twice. The cell suspension was dropped onto a glass slide, air dried, and incubated at 37˚C for at least 24 h.

For Giemsa banding, 15 mg trypsin were dissolved 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 0.1× standard saline citrate (SSC; USB, Cleveland, IL, USA) at 61˚C, rinsed three times in 1× 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 AZ516203, proximal) in combination with RP11-745O10 (AC078927, distal) 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 paraformaldehyde (0.1% PFA/1× PBS) for 10 min and dehydration 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 0.4× SSC/0.3% NP-40 at 71˚C for 2 min and briefly dipped in 2× SSC/0.1% NP-40. After air drying, the slides were covered with mounting medium with DAPI (Vectorlabs).

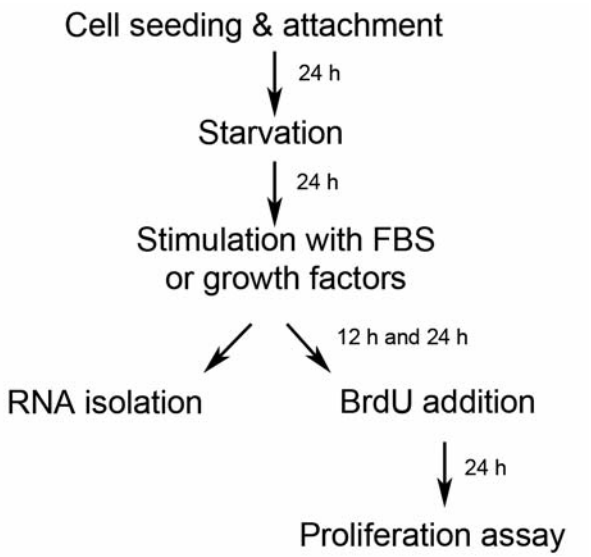

Figure 1. *Schematic workflow of the cell culture experiments presented herein. PC-3 cells were seeded in the respective multiwell-plates and allowed to attach for 24 h. Thereafter, the medium was replaced with starvation medium supplemented with 1% foetal bovine serum (FBS). After 24 h, the medium was replaced with either fresh starvation medium (serving as control) or stimulation medium supplemented with either 10% or 20% FBS, 1% FBS with 25 ng/ml fibroblast growth factor 1 (FGF1) or 1% FBS with 25 ng/ml platelet-derived growth factor-BB (PDGF-BB). After 12 h or 24 h, respectively, either RNA was isolated or 5-bromo-2'-deoxyuridine (BrdU) was added for another 24 h incubation to assess proliferation.*

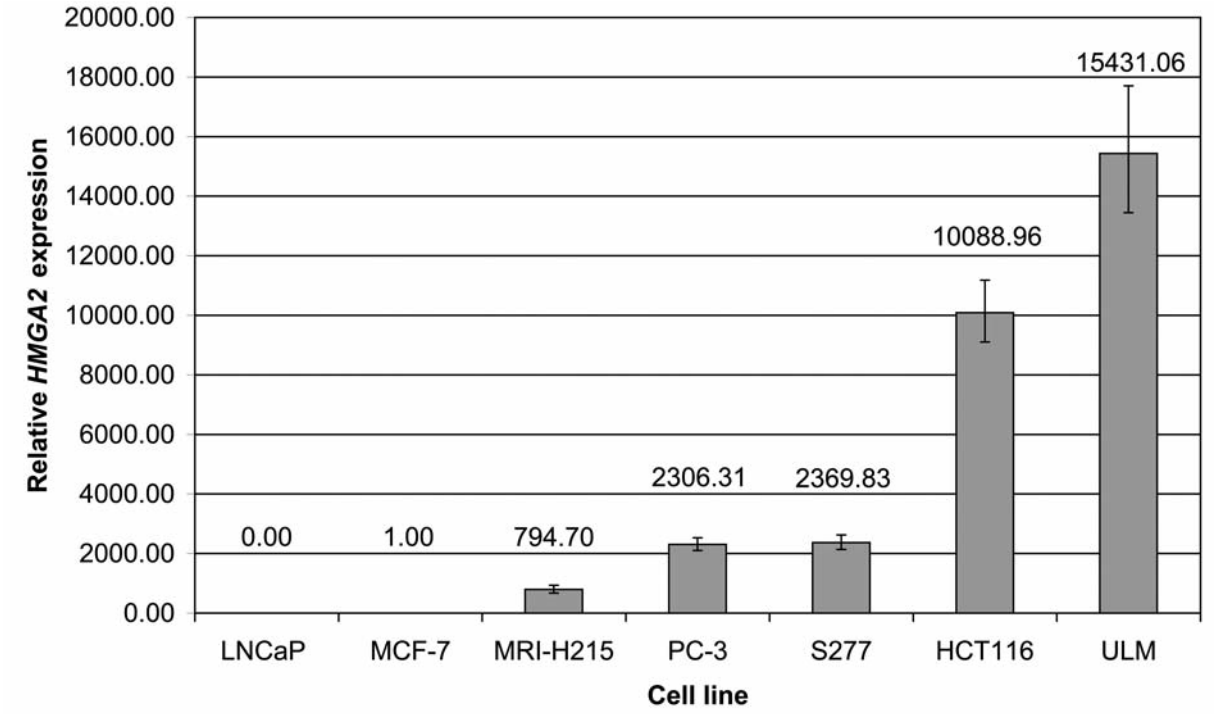

Figure 2. *Relative expression of high-mobility group AT-hook 2 (HMGA2) in the cell lines LNCaP, MCF-7, MRI-H215, PC-3, S277, HCT116, and in a uterine leiomyoma (ULM) with de-regulated HMGA2 expression. 18S rRNA was used for normalisation of the amount of mRNA. Error bars indicate standard deviation.*

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

Technologies) and a commercial assay (Hs00171569_m1, Life Technologies). Either *hypoxanthine phosphoribosyltransferase 1* (*HPRT1*) (with the 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) 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) served as endogenous control to normalise the RNA amount used for reverse transcription. Results were analysed using Sequence Detection Software (Life Technologies) and Excel (Microsoft).

Results

Choice of an appropriate cell line. In order to choose an appropriate epithelial cell line with a moderate *HMGA2* expression, we compared the expression level of various cell lines. A sample of uterine leiomyoma with de-regulated *HMGA2* gene due to chromosomal rearrangement was included as a control for high expression. The PC-3 cell line was found to express *HMGA2* at an intermediate level (Figure 2), thus allowing further increase of *HMGA2* mRNA as a response to stimulation with growth factors and FBS. Therefore it was chosen for the stimulation and proliferation experiments.

Cytogenetics and FISH analysis of the HMGA2 locus. Although chromosomal rearrangements of the *HMGA2* locus have been very rarely reported in malignant solid tumours, amplifications may be more frequent. Classical cytogenetics revealed a hyperdiploid karyoytpe with a variety of structurally rearranged chromosomes. In the absence of normal chromosome 12, three derivative chromosome 12s were detected (Figure 3A). By multicolour FISH, these derivatives were found to be two apparently identical t(8;12) translocation chromosomes and one other t(4;12) translocation chromosome (Figure 3B). In neither of these chromosomes was evidence for rearrangement of the *HMGA2* locus obtained. Nevertheless, to exclude amplifications or rearrangements that escaped identification by classical cytogenetics, we used FISH with appropriate locus-specific probes. In line with the presence of a hyperdiploid karyotype, as a rule, two or three copies of the *HMGA2* locus/metaphase were detected by FISH: From a total of 24 metaphases scored, five (20.83%) and 19 (79.17%) showed two or three copies, respectively, of the *HMGA2* locus (Figure 3C). However, the use of break-apart probes detecting most of the chromosomal rearrangements of the *HMGA2* locus as seen in benign solid tumours did not reveal any evidence for the existence of hidden rearrangements of this locus in the cell line (data not shown). Thus, there is no evidence that the high expression of *HMGA2* is explained by a rearrangement of the *HMGA2* locus.

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 3q27~28 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

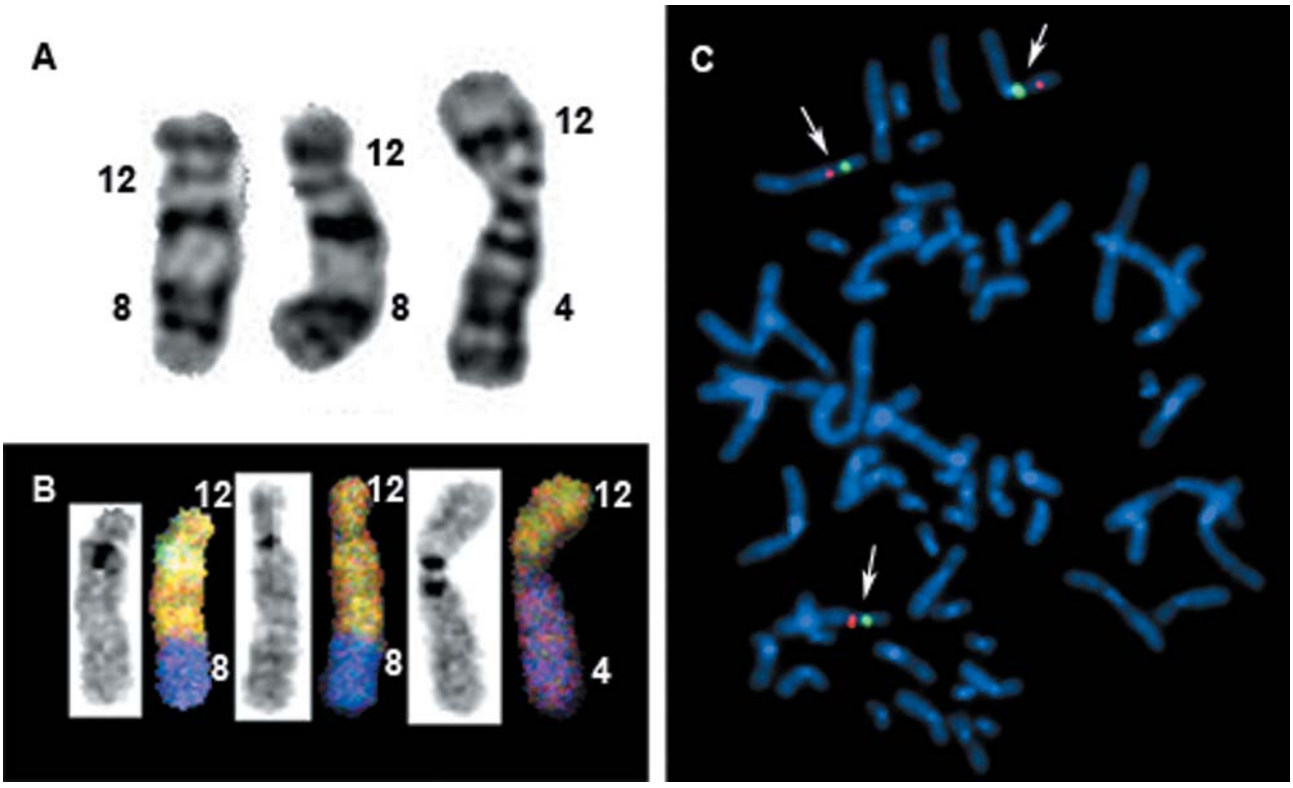

Figure 3. *Conventional and molecular cytogenetics did not reveal evidence for rearrangements or amplification of the high-mobility group AT-hook 2 (HMGA2) locus: Giemsa-banded derivative chromosomes 12 (A) and spectral karyotyping (SKY-FISH) (B) of both of the derivative chromosomes t(8;12) and t(4;12). FISH analysis revealed the HMGA2 locus (red) on the q-arm of both of the derivate chromosomes t(8;12) and on the p-arm of the derivative dicentric t(4;12). Arrows indicate derivative chromosomes 12. Centromere 12 (green) was present on all chromosomes with HMGA2 locus (C).*

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 deacatelysases 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

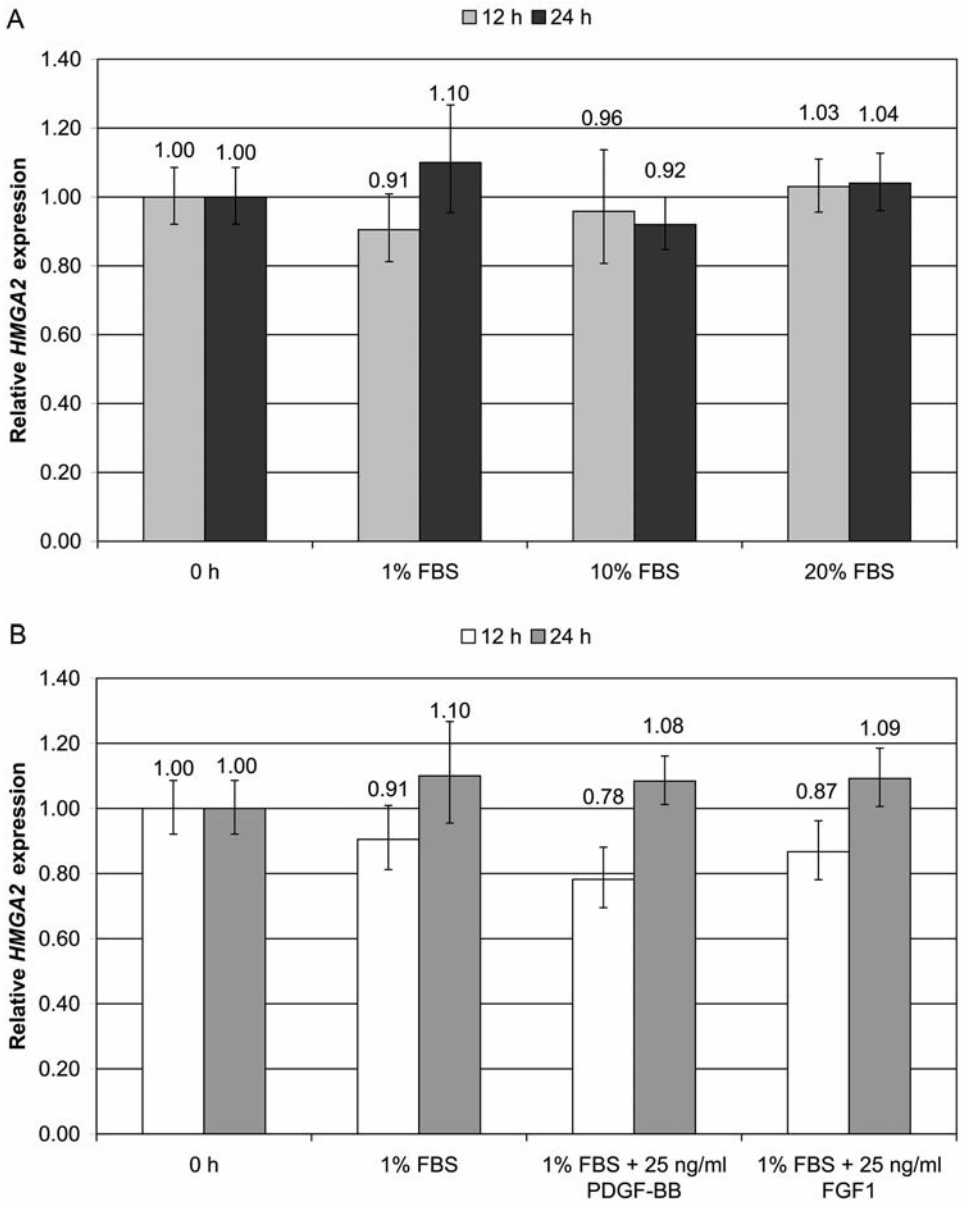

Figure 4. *Relative expression of high-mobility group AT-hook 2 (HMGA2) after stimulation with foetal bovine serum (FBS) (A) and fibroblast growth factor-1 (FGF1) or platelet-derived growth factor-BB (PDGF-BB) (B). A control taken before stimulation and after starvation referred to as 0 h was set as an expression value of 1 for calibration. Hypoxanthine phosphoribosyltransferase 1 (HPRT1) served as endogenous control. Error bars indicate standard deviation.*

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 tumour 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

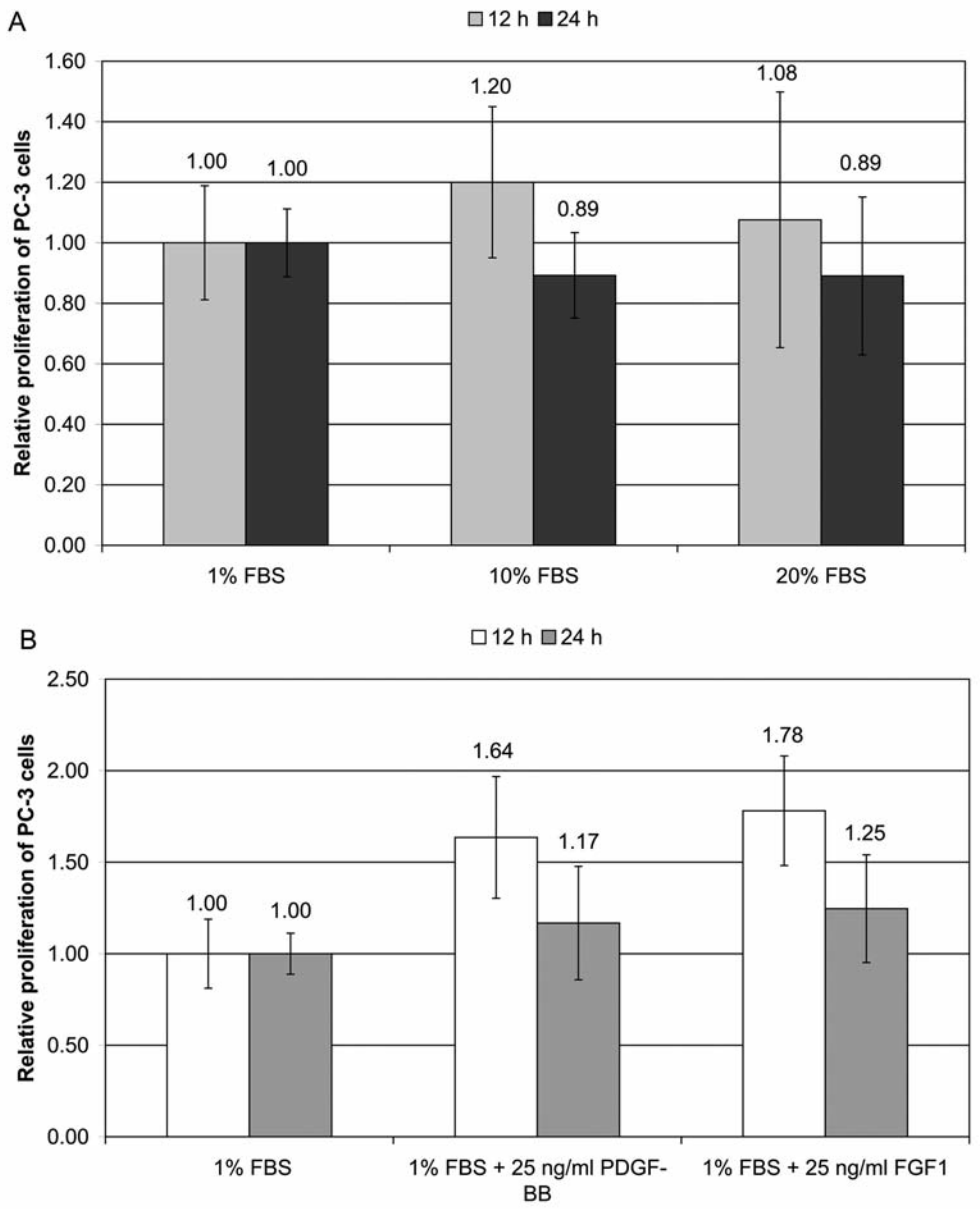

Figure 5. *Relative proliferation rate of PC-3 cells induced by incubation with foetal bovine serum (FBS) (A), or fibroblast growth factor-1 (FGF1) and platelet-derived growth factor-BB (PDGF-BB) (B). For normalisation of the proliferation assay, a control grown in medium supplemented with 1% FBS was set as a value of 1 for the respective incubation period. Error bars indicate standard deviation.*

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).

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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