Lamin A/C protein is overexpressed in tissue-invasive prostate cancer and promotes prostate cancer cell growth, migration and invasion through the PI3K/AKT/PTEN pathway

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Prostate cancer (PC) remains the second most common cause of cancer-related death in Western countries. A previous proteomics study suggested that the nuclear membrane protein lamin A/C to be a maker to discriminate low- and high-Gleason score tumors and to identify high-risk cancers. To characterize its function in PC cells, we performed a detailed expression analysis in PC tissue and explored the consequences of down or upregulation of lamin A/C in PC cells. Our results confirm an increased lamin A/C protein expression in high-risk cancers and show association of expression with tumor cell formations at the invasion fronts of tumors and in invasion ‘spearheading’ tumor cell clusters. In the prostate tumor cell lines, LNCaP, DU145, and PC3 small hairpin RNA knockdown or overexpression of lamin A/C resulted in inhibition or stimulation, respectively, of cell growth, colony formation, migration and invasion. Further mechanism studies suggested that the lamin A/C-related malignant behavior is regulated through modulation of the phosphoinositide 3-kinase (PI3K)/AKT/PTEN signaling pathway. Western blot results indicated that knockdown or overexpression of lamin A/C decreased or increased, respectively, protein levels of the PI3K subunits p110 and p85 in all three cell lines; phosphor-AKT in the PTEN-negative cell lines LNCaP and DU145, and PC3 small hairpin RNA knockdown or overexpression of lamin A/C resulted in inhibition or stimulation, respectively, of cell growth, colony formation, migration and invasion. Further mechanism studies suggested that the lamin A/C-related malignant behavior is regulated through modulation of the phosphoinositide 3-kinase (PI3K)/AKT/PTEN signaling pathway. Western blot results indicated that knockdown or overexpression of lamin A/C decreased or increased, respectively, protein levels of the PI3K subunits p110 and p85 in all three cell lines; phosphor-AKT in the PTEN-negative cell lines LNCaP and PC3, and, increased or decreased, respectively, PTEN protein levels in PTEN-positive DU145 cells. Together, our data suggest that lamin A/C proteins are positively involved in malignant behavior of PC cells through the PI3K/AKT/PTEN pathway. Lamin A/C may represent a new oncogenic factor and a novel therapeutic target for PC.

Introduction

In the Western industrialized world, prostate cancer (PC) is the most common malignancy and the second leading cause of cancer-related death (1–3). Despite the use of prostate-specific antigen (PSA) screening for early detection and curative treatment, still many PC patients develop life-threatening metastatic disease. For this tumor stage, only palliative treatment is possible. Development of resistance to androgen ablation and chemotherapy finally leads to the death of patients and hampers patient care and quality of life of patients suffering from end-stage disease (4,5). Despite numerous attempts on development of new molecular therapy drugs and ongoing clinical trials, more effort for the identification of new potential targets and pathways involved in PC dissemination and progression is needed to understand the crucial drivers of progression and improve the diagnostic and therapeutic armamentarium for PC patients.

In a proteomics profiling approach using two-dimensional differential gel electrophoresis in combination with laser capture microdissection and MALDI-TOF/TOF mass spectrometry for the identification of proteins, we retrieved 19 proteins differing in abundance (6). When comparing well to moderately differentiated low Gleason score (GS) 6 tumors to dedifferentiated high GS 8–10 tumors, the protein with the highest difference was lamin A/C (6). In the current study, we thoroughly investigated the expression pattern of lamin A/C protein in malignant prostate tissue and analyzed the functional effects of this protein in PC cells in order to uncover its implications for tumor progression and explore its usability as a therapeutic target.

Humans express seven different laminas, encoded by three distinct genes, LMNA, LMNB1 and LMNB2. The LMNA gene, located at chromosome 1q21.1–21.3, encodes for lamin A and lamin C proteins (7). These two lamin subtypes are both derived from LMNA through alternative splicing. Both are expressed in most differentiated cells. Lamin C is identical to lamin A up to codon 566 but lacks a part of exon 10 and exons 11 and 12. At the COOH terminus, it contains five unique basic amino acids (7). Lamins, including lamin A, lamin C and also lamin B (encoded by the genes LMNB1 and LMNB2), are major components of the nuclear membrane (8,9). Lamins interact with membrane-associated proteins to form the nuclear lamin layer on the interior of the nuclear envelope. They are involved in the breakdown and reformation of the nuclear envelope during mitosis, as well as the positioning of nuclear pores. As central structural components of nuclei they influence nuclear shape, stability and mechanics and are involved in chromatin dynamics, gene regulation and transcription, cell division and organization of mitosis (10). Mutations in the LMNA gene or defects of lamin A posttranslational modifications are associated with several diseases including Emery-Dreifuss muscular dystrophy, familial partial lipodystrophy, limb girdle muscular dystrophy, dilated cardiomyopathy, Charcot–Marie-Tooth disease and Hutchinson–Gilford progeria syndrome (11–13).

A-type laminas influence the activity of the retinoblastoma protein (pRb) and oncoproteins such as b-catenin (14,15). Consequently, it has been speculated that their expression level may also influence carcinogenesis and tumor progression. Immunohistochemical studies revealed downregulation of lamin A/C in several tumor types, for example gastrointestinal cancers (16). Reduced expression correlated with poor histological differentiation and poor prognosis in primary gastric carcinomas (17), tumor recurrence and aberrant nuclear morphology and aneuploidy in ovarian and breast cancer (18,19). Somehow contradicting these results, Willis et al. (20) described lamin A as a putative colonic epithelial stem cell biomarker, which identifies colorectal tumors with a more aggressive phenotype and found a worse prognosis for colorectal cancer patients with tumors expressing high levels of lamin A. The putative role of lamin A/C in PC progression and risk of worse outcome has not yet been investigated. As lamin A/C is higher expressed in dedifferentiated prostate tumors, we wondered if it enhances the malignant behavior of PC cells and what is the molecular mechanism behind its role in PC cells.

Cell migration and invasion are critical events during the progression to metastasis. Without motile function, cancer cells are unable to leave the primary tumor site, invade through the basement membrane and form secondary tumors. In line with this, prostate tumor cells derived from distant metastases sites are much more invasive than cells obtained from lymph node carcinoma of the prostate (22). In this study, we performed a detailed analysis of the expression pattern
in high-grade tumors and investigated the role of lamin A/C using knockdown and overexpression in several tumor cell lines. Our results indicate that indeed lamin A/C is involved in cell proliferation, migration and invasion of PC cells.

**Materials and methods**

**Immunohistochemistry**

The expression of lamin A/C was analyzed by immunohistochemistry (IHC) in PC specimens derived from previously untreated patients who were diagnosed in a PSA-based screening program performed in Tyrol by the Department of Urology, Innsbruck Medical University and underwent radical prostatectomy (23,24). The use of archive tumor tissue samples was approved by the Ethics Committee of the Innsbruck Medical University. Twenty-nine cases were analyzed using paraffin tissue slides covering the whole specimen. In addition, three tumor-infested regional lymph nodes and six seminal vesicles removed together with the prostate were scrutinized by IHC. A tissue microarray containing tissue cores of 94 cancer cases (40 low GS ≤6 and 54 high GS >7 tumors) was used for statistical evaluation of expression. The tissue microarray was assembled using a manual tissue arrayer (Beecher Instruments/Etigen, Tarfù, Estonia) with three cancer and one benign tissue cores of each case. Cores of cultured PC, bladder cancer and prostate stromal cell lines were included as specificity controls. For analysis of lamin A/C expression by IHC, 4 μm sections of formalin-fixed and hematoxylin/eosin-stained as well as pS6/AKT/MAPK IHC double staining were performed to control the correct histological diagnosis and tumor boundaries. Lamin A/C immunostaining was performed on a Ventana Discovery—XT staining automat (Ventana, Roche) using a rabbit polyclonal antibody (Sigma, St. Louis, MO, HPA066660) at a dilution of 1:10 after antigen retrieval according to Ventana protocol CC2. Immunoreactivity was scored by an experienced uropathologist (G.S.) according to an intensity scale from 0 to 3 (no staining, weak, moderate, strong, staining), which considers the number of positive cells and the intensity of immunostaining (25), and stratified according to tissue Gleason patterns (GPs).

**Cell culture and experimental reagents**

Human PC cell lines PC3 and DU145 were grown in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, 100 U/ml penicillin and 2.0 mM glutamine. LNCaP cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, 100 U/ml penicillin, 10 mM N2-hydroxyethylylperazine-N2-ethanesulfonic acid, 2.5 g/l D-glucose, 1.0 mM Na-pyruvat and 2 mM glutamine. Cells were grown in a humidified incubator comprising 94 tumor cases for statistical evaluation. Immunoreactivity was stratified according to tissue Gleason patterns (GPs).

**Lamin A/C overexpression or knockdown**

The LMNA complementary DNA (cDNA) plasmids encoding LMNA transcript variant 1 (lamin A), transcript variant 2 (lamin C) or the corresponding empty control plasmids were purchased from Beijing OriGene Technologies (Beijing, China). For lamin A/C overexpression, cells were transfected with cDNA expression or control plasmids, respectively, using lipofectamine 2000 (Invitrogen) according to the protocol of the manufacturer. After transfection, transiently cDNA overexpressing prostate cells were selected for 1 week in the presence of G418 antibiotic. Efficiency of transfection and selection was controlled by inspection of cell cultures for coexpressed green fluorescence protein (GFP) using a fluorescence microscope. After 1 week of selection, 80% or more of the cells were GFP positive. The G418 selection concentration (300 μg/ml for PC3, 200 μg/ml for Du145 and 500 μg/ml for LNCaP cells) was determined as the lowest dose of antibiotic that killed 100% of non-transfected cells in 5–7 days. After 1 week of selection, the G418 concentration was reduced to a maintenance concentration of 30, 20 and 50 μg/ml, respectively, for PC3, Du145 and LNCaP cells. For proliferation, colony formation, invasion, migration assay and scratch assays, transfected cells selected in G418-supplemented medium for 1 week were used.

**Lamin A/C knockdown small hairpin RNA (shRNA) plasmids**

PPU6/GFP/NeO-shRNA1 and PPU6/GFP/NeO-shRNA2 and PPG6/NeP/GFP/NeO-shRNAC were purchased from Shanghai Gene Pharma siRNA Company (Shanghai, China). For lamin A/C knockdown, cells were transfected with the shRNA or control vectors, respectively and afterward selected in G418-supplemented medium and controlled for transfection efficiency as described above for overexpression vectors. After 1 week of selection, 60% or more of the cells were positive for coexpressed GFP and this percentage further increased when selection was continued.

**Western blot analysis**

Primary polygonal rabbit antibodies for lamin A/C (ab 58529), phospho-lamin A/C (phospho S392, ab 58528) were purchased from Abcam Biotechnology (Cambridge, UK). AKT1 (#2938), Phospho-Akt (Ser473) antibody (#9271), anti-PI3K-p110α (#4255), anti-PI3K-p85α (#4292) and anti-Pten (#8552) were purchased from Cell Signaling Technology (Beverly, MA). The mouse monoclonal antibody to GAPDH (14C10, #2118) was from Millipore (Billerica, MA). Whole-cell lysate of the cells was prepared by ultrasonication in a mixture of sodium dodecyl sulfate lysis buffer (BioRad, Berkley, CA). Total proteins were fractionated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane for western blotting. The membrane was incubated in primary antibodies (1:1000 dilution) in 2 ml of blocking buffer with 0.1% (2 μl) Tween 20, washed, incubated with secondary fluorescence-labeled antibody (Millipore) in 4 ml phosphate-buffered saline with 0.2% Tween 20 or in 4 ml blocking buffer with 0.2% Tween 20. Finally, membranes were scanned on an Odyssey Infrared Imaging System (LI-COR, Lincoln, NE) and analyzed with the Odyssey software.

**Proliferation assay**

The transfected and selected PC cells (5 × 10⁴) were grown in a 96-well culture plate wells and subsequently incubated with 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide (MTT) reagent (0.5 mg/ml) at 37°C for 1 h and the absorption measured at 595 nm as described (26). Results were plotted as mean ± SD of three separate experiments with five replicate samples per experiment for each experimental condition.

**Scratch cell migration assay**

A total of 2.5 × 10⁵ cells were seeded in six-well plates and transfected with lamin A/C shRNA or cDNA. After G418 selection for 1 week, scratch wounds were created in the cell monolayer using a sterile 20–200 μl pipette tip. After washing away suspended cells, the open area of the scratch was captured at different time points using a microscope (ULWCD Olympus) equipped with a digital camera, and the widths of the gaps were quantified using the MATLAB TScratch assay software 10.4 (27). In a typical experiment, each group consisted of three different plates. Five measurements of wound width were made for each wound at randomly chosen points. All measurements were made by an observer unaware of the treatments.

**Colony formation assay**

Colony formation assay is an in vitro cell survival assay based on the ability of a single cell to grow into a colony. After transfection, PC cells were selected for 1 week in the presence of G418 antibiotic. To test the effect on colony formation, 1000 transfected or control PC3 and DU145 cells were grown in 75cm² flasks to assess the effects of transfection on clonogenic survival. The cells were then incubated for 7–10 days at 37°C in a 5% CO₂ incubator. The colonies were fixed in 100% methanol and stained with 0.5% crystal violet in 20% methanol and counted with CoCount (Oxford Optronix, Oxford, UK). The surviving fraction was normalized to untreated control cells with respect to clonogenic efficiency. Each colony formation assay was carried out in triplicate and repeated at least three times.

**Transwell migration and invasion assays**

Cell migration and invasion were assessed using 24-well BD FalconTM Cell Culture inserts (BD Biosciences, Franklin, NJ) with 8 μm pores according to the manufacturer’s protocol. For invasion assays, the insert membranes were coated with 50 μl per well of diluted matrigel (BD Biosciences) (1:3). After getting at 37°C, transfected PC3 cells, DU145 (1 × 10⁵) and LNCaP (2 × 10⁴) cells in 0.4 ml of medium supplemented with 1% serum were seeded into the upper chamber and the bottom wells were filled with complete medium (10% fetal bovine serum) as chemoattractant. After 24 h of incubation, the cells on the upper membrane surface were removed by using a cotton swab and washing in phosphate-buffered saline for three to four times to make sure no cells were left on the surface. The cells were fixed using 100% methanol for 10 min at room temperature. After washing with phosphate-buffered saline, the membranes were cut out and mounted on an object slide with 4 ,6-diamidino-2-phenylindole-containing medium. The fluorescent cells were photographed using a Zeiss Axiovert 200 equipped with a LMS510 META detector (Zeiss, Jena, Germany) and counted using TissueFAX software (TissueCinometrics, Vienna, Austria).

**Results**

**Expression pattern of lamin A/C in benign and malignant prostate tissue suggests association with dedifferentiation and invasion**

Lamin A/C expression in prostate tissue and prostate tumors was assessed by IHC using classical tissue sections and a tissue microarray comprising 94 tumor cases for statistical evaluation. Immunoreactivity was stratified according to tissue type. The Gleason grading system is used for the histological grading of prostate tumors (28,29). The
GPs indicate the extent of dediffererentiation and are defined by morphological criteria. Since prostate tumors are often multifocal and heterogeneous, the GS, which is the sum of the two most prevalent GPs found in the tumor, is used for tumor characterization (e.g. GP4 + GP3 = GS7). GPs range from 1 as the most differentiated pattern with highly structured glands to 5 as the most dedifferentiated pattern that displays a complete loss of prostatic gland pattern. If there is only one predominating tumor GP, this pattern is doubled to get the corresponding GS (e.g. GP3 + GP3 = GS6). Thus the range of GSs is 2 (highly differentiated tumor) to 10 (dedifferentiated tumor). High GPs and scores represent one of the most reliable—although not perfect—risk factors for progression and worse prognosis of PC.

Lamin A/C was highly expressed in the functionally differentiated epithelial cell layers of benign glands with the strongest expression in the p63-positive basal cell layer (mean staining score 1.3) (Table I; Figure 1A and B). The cells in the surrounding benign stroma also showed strong immunostaining with an intensity score of 1.4 (Table I; Figure 1B). In contrast, lamin A/C expression was weak or absent in the majority of the tumor cells of low-grade GPs (Figure 1A and B). In these well to moderately differentiated tumors, lamin A/C promotes PC cell growth, migration and invasion.

Table I. Statistic evaluation of lamin A/C expression in prostate tumors using immunohistochemical analysis

<table>
<thead>
<tr>
<th>Tissue class (tumor GP)</th>
<th>Number of evaluable tissue cores</th>
<th>Mean staining score ± SD</th>
</tr>
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<tbody>
<tr>
<td>Benign epithelium</td>
<td>186</td>
<td>1.3 ± 0.38</td>
</tr>
<tr>
<td>Stromal cells</td>
<td>320</td>
<td>1.4 ± 0.30</td>
</tr>
<tr>
<td>Low-grade GP 3</td>
<td>175</td>
<td>0.7 ± 0.28</td>
</tr>
<tr>
<td>High-grade GP 4 or 5</td>
<td>84</td>
<td>1.2 ± 0.27</td>
</tr>
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</table>

A PC tissue array containing 376 tissue cores of 94 PC cases, 3 tumor and 1 benign cores of each case were employed to assess expression of lamin A/C protein by IHC. Immunostaining was scored by an uropathologist according to a four-point intensity scale: 0, no; 1, weak; 2, moderate and 3, strong immunoreactivity and statistically evaluated according to the GPs found in the tumor cores. In case of more than one GP in a tumor core, each GP tumor region was scored and assigned to the low- or high-GP group individually. Immunoreactivity decreased in low-grade tumor tissue (GP3) compared with benign epithelial cells and increased in high-grade tumor tissue (GP4 and GP5). Stromal cells also were immunopositive displaying a immunoreactivity similar to benign epithelial cells.
cells that are still capable of forming glandular structures, the mean staining intensity was 0.7 (Table I). A contrasting picture was seen in the dedifferentiated tumor cells of GP 4 and 5 tumor regions (Figure 1A and C). There, significantly, more cells positive for lamin A/C were found and the mean staining intensity score was 1.2 (Table I).

Characteristic for high-grade tumors was also a heterogeneous distribution of positive tumor cells (overview in Figure 1D). We observed a significant increase of the number of positive cells in subcapsular or capsule-infiltrating cancer formations (Figure 1E) and in tumor areas surrounding nerve tracts (Figure 1F), whereas the number of positive cells and staining intensity decreased toward the central part of the tumor masses (Figure 1G). In the majority of 14 cases with prostate capsule invasion, such cell formation was found. This pattern of lamin A/C upregulation suggested an association with the invasion front of the tumors. This link was further demonstrated in tumor cases with extracapsular extension, e.g., in seminal vesicle infiltrations and in lymph node metastases. Figure 2A shows a tumor invading seminal vesicle tissue. It is a representative case of altogether six cases with seminal vessel infiltration. The tumor cell spearheads leading the front of invasion into the healthy tissue (Figure 2A, B and C) show lamin A/C immunoreactivity, whereas tumor cells in the tumor center are almost negative (Figure 2A and D). A very similar pattern of expression was found in regional lymph node metastases. An example of three analyzed cases with positive lymph nodes is presented in Supplementary Figure 1, available at Carcinogenesis Online. This lymph node is almost full of PSA-positive cancer cells. The expression of lamin A/C is heterogeneous with low immunostaining of central tumor areas and higher intensity staining at tumor boundary areas especially in subcapsular extensions and in a region of tumor growth into the adjacent adipose tissue.

In summary, the expression pattern of lamin A/C in PC radical prostatectomy specimens, local tumor extensions and lymph node metastases revealed a decrease of expression in low-grade tumors and a distinct pattern of lamin A/C reexpressing cells in high-grade tumors that is reminiscent of tumor cells at sites of infiltrative and invasive tumor growth. This prompted us to investigate the functional impact of lamin A/C expression in prostate tumor cells.

Lamin A/C promotes PC cell growth and colony formation

The effects of lamin A/C protein levels on cell growth and survival were assessed by employing shRNA knockdown and overexpression of lamin A/C cDNA and measuring cell viability and colony formation ability. We transfected PC3, DU145 and LNCaP PC cell lines with human PGPU6/GFP/NeO-shRNA1, PGPU6/GFP/NeO-shRNA2 or negative control shRNA plasmids or with cDNA expression plasmids or empty control vectors and maintained the cells under G418 selection for 1 week before experiments were performed. Efficiency of transfection was confirmed by western blot (Figure 3A and B, Figure 3E and F) and immunofluorescence of coexpressed GFP (data not shown). Whereas lamin A/C knockdown caused significant cell growth inhibition at different time points (Figure 3C), overexpression significantly promoted cell growths in all three PC cell lines tested (Figure 3G). Accordingly, colony formation in PC3 and DU145 cells compared with control was significantly reduced ($P = 0.03$ and $P = 0.0008$, respectively, Figure 3D and Supplementary Figure 2 is available at Carcinogenesis Online) or enhanced in case of lamin A/C overexpression ($P = 0.007$ and $P = 0.02$, respectively, Figure 3H and Supplementary Figure 2 is available at Carcinogenesis Online). Due to the unique growth characteristic of LNCaP cells, which do not form colonies from single cells, colony formation analyses were restricted to PC3 and DU145 cells. It was no problem to establish stable overexpression clones but all attempts to establish long-term stably expressing lamin A/C shRNA subcell lines failed. All colonies died after some time of selection and passaging of cells and thus underscored a key role of lamin A/C in PC cell survival.

Lamin A/C enhances PC cells migration and invasion

To further evaluate effects of lamin A/C on PC progression, we tested the influence on PC cell migration and invasion. Migration was assessed by scratch and transwell migration assays, whereas invasion was assessed using a Matrigel invasion test. Downregulation of lamin A/C significantly delayed wound healing and decreased migration of LNCaP, PC3 and DU145 compared with control-transfected cells through the pores of a transwell ($P = 0.039$, 0.042 and 0.030, respectively, Figure 4A and B and Supplementary Figure 3 is available at Carcinogenesis Online). The effect on invasion properties changed similarly. In all three cell lines, the number of cells invading through the matrigel layer decreased significantly after knockdown of lamin A/C in LNCaP, DU145 and PC3 cells ($P = 0.037$, 0.048, 0.041, respectively, Figure 4C). On the other hand, upregulation of lamin A/C significantly accelerated wound healing and increased the number of migrating cells in all three cancer cell lines compared with control ($P = 0.003$, 0.014, 0.036, respectively, Figure 4D and E and Supplementary Figure 3 is available at Carcinogenesis Online). Overexpression also increased the number of invading cells ($P = 0.035$, 0.009 and 0.02, respectively, Figure 5F).
Fig. 3. Lamin A/C knockdown decreases and overexpression enhances cell growth and colony formation of PC cells. LNCaP, PC3 and DU145 cells were transiently cotransfected with shRNA or control constructs. After transfection, PC cells were selected for 1 week in the presence of G418 antibiotic before assays were performed. Transfection efficiency was controlled by fluorescence microscopy of coexpressed green GFP protein and was 60% or higher after 1 week of selection. (A) Lamin A/C protein levels were downregulated by shRNA in all three PC cells. A representative western blot is shown. (B) Histogram representing densities of lamin A and lamin C western blot bands normalized with GAPDH. Lamin A/C protein levels were downregulated up to 2-folds in LNCaP cells, 1.5-folds in DU145 cells and 3-folds in PC3 cells, respectively. (C) Downregulation of lamin A/C inhibited cell proliferation. (D) Transfected PC3 and DU145 cells were examined for their ability to form colonies in vitro in G418-supplemented selective medium. The histogram shows the number of colonies formed in
In summary, both enhancement of proliferation and colony formation and stimulation of migration and invasion properties point to an association with malignant behavior of PC cells through increased levels of lamin A/C.

Lamin A/C regulates the PI3K-PTEN-Akt signaling pathway in PC cell lines

Recent data suggest that nuclear lamins interact with the PI3K-PTEN-Akt survival pathway (32,33). This pathway is frequently aberrant in prostate tumors (34–36). Therefore, we explored the effect of different levels of lamin A/C on activation of protein kinase Akt (protein kinase B, PKB) and pathway components upstream of Akt (Figure 5A). DU145 tumor cells have a functional PTEN with one wild-type PTEN allele and a second variant allele (M134L). They show little basal activation of Akt whereas in PC3 cells with a homozygous deletion of PTEN and in LNCaP cells with a deletion of one PTEN allele and mutation of the other allele this pathway is constitutively activated (37) (see Figure 5A for a schematic pathway overview).

Overexpression and knockdown of lamin A/C modulated lamin A/C protein levels and in parallel also levels of the phosphorylated form phospho-Akt (pAkt) as revealed by western blot analysis (Figure 5B). Downregulation by shRNA decreased the levels of the two subunits of PI3K, p110α and p85α in all three cell lines and of the PTEN protein in DU145 cells. The opposite regulation of protein levels was observed after overexpression of lamin A/C (Figure 5B). In LNCaP cells, in addition also Akt protein decreased but remained unchanged in PC3 and DU145 cells. Activity of Akt kinase, measured as phosphorylated Akt protein, was affected in both cell lines with constitutive Akt activity (LNCaP and PC3). It decreased after knockdown and increased after overexpression of lamin A/C, respectively, (Figure 5B and C). No regulation of phospho-Akt levels was found in DU145 cells or changes were below the sensitivity of detection. These data suggest a stimulation of the PI3K-PTEN-Akt survival pathway when lamin A/C protein levels are increased, especially in PC cells that lost PTEN function and harbor a constitutively activated protein kinase Akt.

Discussion

Nuclear lamins, also known as class V intermediate filaments, are fibrous proteins providing structural function and transcriptional regulation in the cell nucleus. Their phosphorylation is a key event in the mitotic breakdown of the nuclear membrane (38). Lamins are the only intermediate filament proteins to possess a nuclear localization signal sequence (39) and a C-terminal CaaX motif that undergoes posttranslational modification (40). Lamin A and C are the most common A-type nuclear lamin proteins and are splice variants of the LMNA gene found at 1q21. Recent reports have shown that lamin A/C expression is lost or decreased in some tumor types, such as ovarian, breast or colon cancers (18,19,41). Our previous study revealed upregulation in high-grade PC (6). In this study, we show that cell proliferation, colony formation, survival, migration and invasion of PC cells are modulated by lamin A/C levels. This function is in agreement with the distinct expression pattern in aggressive invasive prostate tumors. Thus, our results provide in vitro and in vivo evidence for an oncogenic role of lamin A/C and a potential driver of prostate tumor progression.

Decreased or aberrant expression of nuclear lamins was associated with worse prognosis in gastrointestinal tract cancers, colorectal and breast cancers (16,17,19,41). Furthermore, lamin A/C was also described as a marker for colorectal cancer and for colorectal cancer displaying a more aggressive phenotype (20,21). Our immunohistochemical analysis of prostate tumors showed a decrease of protein levels in low-grade tumors compared with the benign epithelium and increased levels comparable with those of the benign cells in high-grade tumors, which possess an inherent higher probability for aggressive behavior and progression. Noteworthy, in the benign epithelium, the basal cells lining the base of prostate glands were uniformly positive for lamin A/C, whereas secretory cells showed low immunoreactivity. In contrast to the uniform staining pattern of the benign basal epithelium, the pattern of expression in high-grade tumors was more heterogeneous. Tumors extending into the prostate capsule or into tissue outside the prostate showed a very distinct immunoreactivity with cell formations at the forefront of invasive growth showing the highest immunoreactivity and the lowest immunostaining in the cells of the central parts of tumor masses. Tumor cells at the tumor boundaries and tumor clusters that invaded deeply into the surrounding tissue as well showed high lamin A/C immunoreactivity in comparison with the bulk of the tumor suggesting a specific role in spearheading of tissue invasion. The observation that this boundary effect was also evident in extraprostatic tumor masses that did not display different GPs and stained quite uniformly for PSA suggests that this is induced by the invasion process.

The mechanism of Lamin A/C upregulation at the sites of invasive growths remains to be established in detail. At the boundaries of tumors infiltrating into healthy tissue, tumor cells release proteases such as metalloproteinases and other enzymes to degrade extracellular matrix and pave the path for invasive tumor extension (42,43). Extracellular matrix degradation releases growth factors, chemokines and angiogenesis regulators bound there, which retroact on the tumor cells and—among many other effects—this could enhance the levels of Lamin A/C.

To further explore the molecular mechanism by which lamin A/C upregulation affects the oncogenic properties of PC cells, we investigated its effects on cell proliferation, migration and invasion and examined a possible link to the PI3K-PKB survival pathway. Lamin A/C was recently identified as a substrate for phosphorylation by PKB/Akt1 in Emery–Dreifuss muscular dystrophy and this modification was involved in the regulation of lamin A/C expression levels (33). In PC, the PI3K/Akt pathway is often altered through loss or mutation of the tumor suppressor gene PTEN and this is crucially involved in aberrant regulation of cell proliferation, transformation, tumor growth and angiogenesis (34,35,44,45).

We investigated the oncogenic functions of lamin A/C in the androgen-dependent PC cell line LNCaP and the androgen-insensitive tumor cell lines DU145 and PC3. In all three cell lines, downregulation of lamin A/C by shRNA transfection decreased and upregulation by cDNA overexpression increased proliferation, migration and invasion through matrigel. Furthermore, colony formation was decreased or enhanced, respectively, in DU145 and PC3 cells. LNCaP cells did not form colonies under the condition of our test system and this property was not changed by manipulation of lamin A/C levels. Taken together, our in vitro results show that lamin A/C enhances basal oncogenic properties of PC cells. This finding is in agreement with increased protein levels in tumors with a high risk of invasive growth and metastatic progression, and the specific expression at the invasion front observed in tumor specimens.

In vitro and in vivo effect of lamin A/C on cell proliferation and colony formation. Representative images are presented in Supplementary Figure 2, available at Carcinogenesis Online. For overexpression of lamin A/C, LNCaP, PC3 and DU145 cells were stably transfected with LMNA transcript variant 1 or 2 expression plasmids or with a control plasmid, respectively, and selected in G418-supplemented medium for 1 week. Transfection efficiency was controlled by fluorescence microscopy of coexpressed green GFP protein and was 80% or higher after 1 week of selection. (B) Lamin A/C protein levels were upregulated in all three cell lines transfected with lamin A/C cDNA, as controlled by western blotting. (F) Statistics analyses shown as histograms revealed that lamin A/C protein levels were upregulated up to 2-folds in lamin A/C cDNA-transfected LNCaP, DU145 and PC3 cells compared with control vector-transfected cells. (G) Upregulation of lamin A/C promoted proliferation detected by MTT assay in all three PC cell lines. (H) Overexpression of lamin A/C promoted colony formation of PC3 and DU145 cells. For representative images see Supplementary Figure 3, available at Carcinogenesis Online. LNCaP do not form colonies under these conditions. Data are expressed as average value ± standard deviation of three independent experiments. *P < 0.05, **P < 0.01 compared with controls.
Fig. 4. Effect of downregulation and overexpression of lamin A/C on PC cell migration and invasion. LNCaP, PC3 and DU145 cells were transiently cotransfected with plasmids expressing shRNAs or cDNA expression vectors, selected in the presence of G418 antibiotic and controlled for transfection efficiency as described in Figure 3. To evaluate migration of shRNA-transfected cells, a wound-healing scratch assay was performed and quantified by measurement of the open area of the scratch at different time points using the MATLAB TScratch assay software 10.4 (A). Data show that downregulation of lamin A/C retarded wound healing in all three transfected cell lines. Representative images are shown in Supplementary Figure 3, available at Carcinogenesis Online. Downregulation of lamin A/C significantly decreased cell migration through the membranes of a transwell in all three tumor cell lines (B) and cell invasion through a matrigel-coated transwell membrane in all three tumor cell lines (C). The wound-healing scratch assay performed with prostate tumor cells overexpressing lamin A/C revealed accelerated cell migration and wound closure (D). Representative images are shown in Supplementary Figure 3, available at Carcinogenesis Online. Upregulation of lamin A/C also significantly increased the number of cells that migrated through the pores of the transwell membrane (E) or through the matrigel-coated transwell membrane (F). The histograms in B, C, E and F show the mean average number of cells of 10 microscope fields that migrated. Data are expressed as average value ± standard deviation of three independent experiments. *P < 0.05, **P < 0.01 compared with controls.
Activation of the PI3K/AKT pathway by mutation or inactivation of PTEN function is a prominent feature of human PC. While previous studies indicated that lamin A/C is regulated by the PI3K/AKT/PTEN pathway in laminopathies (32), in the current study, we found that lamin A/C also modulated the PI3K/AKT/PTEN pathway. Our results indicate that there exists a lamin A/C-PI3K feedback loop in PC cells. Downregulation of lamin A/C decreased PI3 kinase subunits p110α and p85α and diminished protein levels of activated Akt protein (phosphor-Akt), especially in PTEN-negative PC3 and LNCaP cells. On the other hand, overexpression resulted in the opposite effect. The level of phosphorylated lamin A/C changed pretty much in parallel to total lamin A/C under the conditions investigated here indicating that the level of lamin A/C protein and not hypo- or hyperphosphorylation was the trigger for the effects observed.

The tumor suppressor gene PTEN mapping to chromosome 10q23 and encoding a dual specificity phosphatase, which attenuates PI3K activity, is a negative regulator of cell migration and cell survival. Mutated or deleted PTEN is correlated with pathological markers in both PC cell lines and primary PCs and with poor prognosis of PC patients (46,47). We conclude from our results that lamin A/C over-expression enhances activation of the PI3K/Akt pathway and that it cooperates with this pathway to stimulate migration, invasion and oncogenic properties of PC cells.

In summary, our data indicate that lamin A/C is involved in cell proliferation, migration and invasion of PC cells. The PI3K/Akt1 cell survival pathway seems to be enhanced by lamin A/C. Lamin A/C could be a molecular marker in progression of PC and is a potentially novel target for prostate cancer therapeutics.

Fig. 5. Lamin A/C enhances the PI3K/AKT/PTEN signaling pathway in PC cells. (A) Summary diagram illustrating the PI3K/AKT/PTEN signaling pathway. A ligand engaged receptor tyrosine kinase binds PI3K, either directly or indirectly, removing the inhibitory action of its p85 subunit on the catalytic p110 subunit. PIP3 formed by the activated PI3K stimulates the phosphorylation and activation of Akt/PKB. Akt transmits the signal to downstream targets, thus orchestrating a variety of key cellular functions, including growth, apoptosis, proliferation and survival. (B) and (C) show representative western blot images of proteins of the PI3K pathway detected in whole-cell lysates with antibodies indicated. Tumor cells transfected with lamin A/C shRNA (B) or lamin A/C cDNA (C) and selected in G418 and controlled as described in Figure 3 were compared with respective controls. In the PTEN-positive DU145 cells, the level of phospho-lamin A/C, p110α, p85α and PTEN were decreased by downregulating lamin A/C with shRNA and increased by overexpression of lamin A/C. In the PTEN-negative PC3 and LNCaP cell lines, the level of phospho-lamin A/C, p110α, p85α and phosphorylated AKT was decreased by downregulation of lamin A/C with shRNA and increased by overexpression of lamin A/C.
Supplementary material
Supplementary Materials, Figures S1–S3 can be found at http://carcin.oxfordjournals.org/

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