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Research Paper

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In cultured prostate cancer cells cAMP blocks proliferation and induces neuroendocrine differentiation. Pyk2 expression inversely correlates with malignancy of prostate cancer. The aim of this study was to investigate the interaction between cAMP and Pyk2 in the prostate. EPN cells, a line derived from human normal prostate expressing Pyk2, and EPN-PKM3 cells, an EPN clone bearing a Pyk2 kinase-negative mutant, were adopted as model system. cAMP inhibited cell growth in both prostate cell lines, and activated Pyk2, but not ERK1/2, in EPN cells. cAMP treatment, abolished the activation of AKT1, an important component of the pro-survival pathway, in the EPN cells but not in EPN-PKM3 cells. Finally, upon cAMP treatment, EPN and EPN-PKM3 cells exhibited different expression patterns of HOX genes, an important network controlling cell identity. These data demonstrated for the first time that Pyk2 and cAMP interact in regulating prostate cell functions and in "keeping" prostate identity.

Introduction

Unrestrained cell proliferation and progressive loss of cellular "identity" are main features of the complex sequence of events leading to cancer. Adenocarcinoma of the prostate is the most common malignancy in men and the second leading cause of cancer-related deaths.[1] In the search of new strategies to fight prostate cancer it is crucial to identify genes inducing epithelial prostate cells to transdifferentiate into a transformed phenotype.[2,3]

Proline-rich tyrosine kinase (Pyk2),[4,5] a non-receptor kinase of the focal adhesion kinase (FAK) family, regulates several cell

functions like proliferation, apoptosis, actin cytoskeleton organization and adhesion depending on the cellular context.[6-9]

Pyk2 can be activated by a variety of stimuli,[10] including stress signals, such as TNF,[11-16] UV light, changes in osmolarity,[17-23] cytoplasmic free Ca^{2+} concentration and/or activated protein kinase C (PKC).[22,24-26] In turn, Tyr-402 phosphorylated Pyk2 recruits adapter proteins Grb2 and Shc and, consequently, activates the Ras-p42/p44 extracellular signal-regulated kinase 1/2 (ERK1/2),[10,26-27] the p38 MAPK,[28,29] and Jun amino-terminal kinase (JNK) pathways.[17,20,22]

We have previously reported that Pyk2 expression inversely correlates with prostate cancer degree of malignancy[30] suggesting a role for Pyk2 as an onco-suppressor gene involved in the control of prostate identity.[31,32]

The intracellular second messenger, 3'5'-cyclic adenosine mono-phosphate (cAMP), regulates important biological processes, such as cell metabolism, growth and differentiation. The effect of cAMP on certain cellular functions has been shown to be dependent on cell-type.[33] In cultured prostate cells, agents that elevate intracellular cAMP, such as epinephrine, isoproterenol, forskolin and permeable cAMP analogues block cell proliferation and induce differentiation of prostate tumor cell lines to a neuroendocrine (NE) phenotype.[34,35] Although, areas of neuroendocrine differentiation (NED) are present, though at different extent, in all high-grade prostate cancer,[36,37] currently, the mechanisms by which prostate cancer acquires NE properties are poorly understood. Based on evidence from the literature and on our previous work, we hypothesize that the HOX genes could be important players in this complex phenomenon. HOX genes make up a network of transcription factors controlling development in embryonic life and growth and differentiation in adult life. The expression pattern of this 39 gene network is tisse-specific and may be involved in the maintenance of the differentiated phenotype. We have reported that the expression patterns of the HOX gene network varies in different human prostate cell lines, representing different components of normal prostate and different stages of prostate cancer progression.[38] Thus, by characterizing the expression

of the HOX genes in the prostate, it could possible to discriminate between different types and level of malignancy of prostate cells. Moreover, exposure of prostate epithelial cells to cAMP profoundly altered the 39 HOX genes expression pattern.[38]

The interaction between cAMP and Pyk2 has been elucidated to a very limited extent and basically in brain and neurons.[31,39,40] In prostate cancer both cAMP and Pyk2 may profoundly influence differentiation state and metastatic potential of transformed cells. We have previously reported that the ablation of Pyk2 kinase activity in the prostate cancer-derived PC3 cell line is a prerequisite for cAMP-induced NED.[31] In the present work we investigated the relation between cAMP and Pyk2 in EPN cells, a line of human epithelial non transformed prostate cells and in EPN cells bearing a dead kinase Pyk2 (EPN-PKM3 cells). Our results provided evidence that Pyk2 can influence the prostate cellular response to increased levels of cAMP. To our knowledge, these data are the first demonstration of the interaction of cAMP and Pyk2 in prostate cells.

Results

cAMP inhibits the growth of EPN and EPN-PKM3 cells in a reversible fashion and induces neuron-like morphology in EPN-PKM3 cells. Prolonged and sustained increase of intracellular levels of cAMP, achieved by treating EPN and EPN-PKM3 cells with 10 mM dBu-cAMP, in the presence of 1 mM IBMX, an inhibitor of phosphodiesterases, blocks cell proliferation in both lines (Fig. 1), and dramatically reduced cell number in EPN cells (-90%), while had a much modest effect on EPN-PKM3 cells (-35%). The inhibitory effect of dBu-cAMP is totally reversible after four days of treatment as demonstrated by the full recurrence of cell proliferation upon dBu-cAMP withdrawal. In the absence of IBMX, the decrease in cell proliferation was not observed in neither the lines tested, indicating that only prolonged and sustained levels of intracellular cAMP are able to exert an inhibitory effect (data not shown).

In the presence of sustained levels of intracellular cAMP, only the EPN-PKM3 cells acquired a neuron-like morphology developing rounded bodies and extended, long, fine processes as shown in Figure 2. The formation of the neurite-like processes, which was already visible after 24 h of treatment, continued to develop and persisted for the ten days of observation.

dBu-cAMP induces Pyk2 phosphorylation in EPN cells. Since few data are available on the possible regulation of Pyk2 by cAMP elevations, we tested whether dBu-cAMP was able to activate Pyk2 as it occurs for a variety of other extracellular signals. Treatment of EPN cells with 10 mM dBu-cAMP and 1 mM IBMX from 5–60 minutes resulted in a consistent increase of Pyk2 phosphorylation with a maximum at 15 minutes (Fig. 3).

Lysophosphatidic acid (LPA) and dBu-cAMP modulate ERK1/2 and AKT1 cascades in EPN and PKM3 cells. In other to evaluate the responsiveness of ERK1/2 and AKT1 in our experimental model we investigated the effect of LPA, the most relevant mitogen in serum. LPA-induced ERK1/2 activation was maximal after 5 min of LPA stimulation and started to decline thereafter in both EPN and EPN-PKM3 cells, as shown in Figure 4. In the same experimental conditions, AKT1 phosphorylation was maximal after 5 min of LPA stimulation and decreased slowly thereafter in EPN cells (Fig. 4). Differently, in EPN-PKM3 cells, AKT1 activation started at 60 min and remained constant at 180 min of treatment with LPA (Fig. 4).
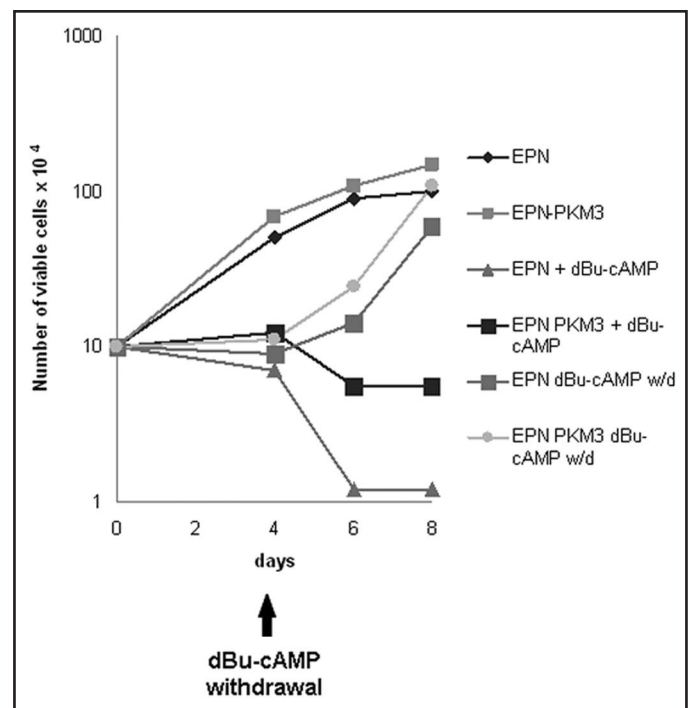

Figure 1. cAMP effect on EPN and EPN-PKM3 cell proliferation. EPN ◆—◆, EPN-PKM3 □—□, EPN + dBu-cAMP △—△, EPN-PKM3 + dBu-cAMP ■—■, EPN dBu-cAMP W/D □—□, EPN-PKM3 dBu-cAMP W/D ○—○. The arrow indicates the withdrawal (w/d) of cAMP after the fourth day of culture. Data represent the mean of triplicate measurement per each experimental point ± SD (It is to be underlined that the SD is in all cases so small to fall within the symbol).
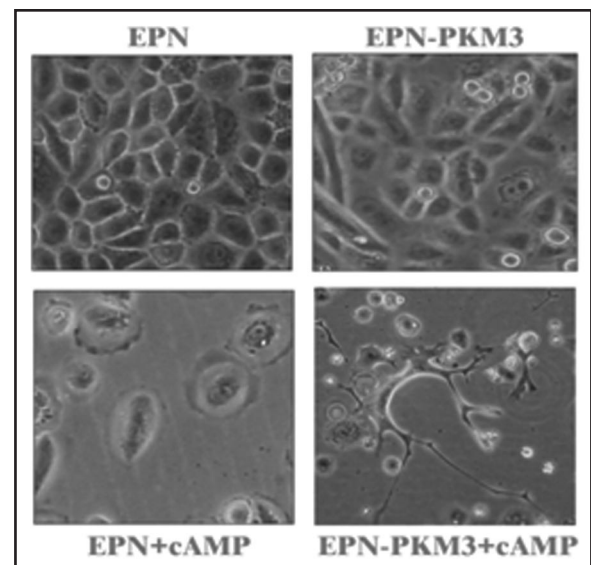
Figure 2. Effect of cAMP on the morphology of EPN and EPN-PKM3 cells. Both cell lines were incubated with 10 mM dBu-cAMP and 1 mM IBMX. By inhibiting phosphodiesterase activity, IBMX allows prolonged and sustained increase of the intracellular levels of cAMP. The figure shows a typical picture of the cultured cells after ten days of treatment. Only the EPN-PKM3 cells acquired a neuron-like morphology developing rounded bodies and extended long, fine processes. The formation of the neurite-like processes was visible already within 24 h of treatment, continued to develop and persisted for the ten days of observation. Photomicrographs: magnification x150.

Figure 3. Western blot analysis of the phosphorylation of Pyk2 on Tyr402 in EPN cells following increase of intracellular cAMP. Cells were cultured for the indicated times in the presence of 10 mM dBu-cAMP and 1 mM IBMX. (Upper) Detection of phosphorylated Pyk2 (P-Pyk2). (Lower) Detection of the total amount of Pyk2.
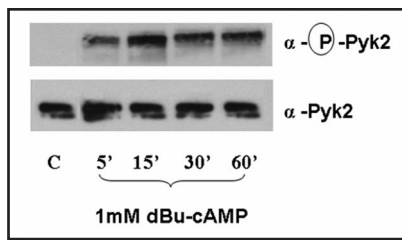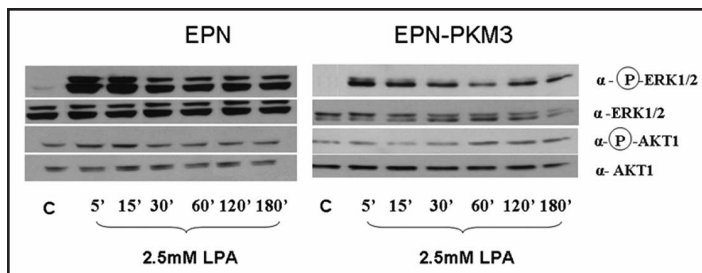

Figure 4. Western blot analysis of the phosphorylation of ERK1/2 and AKT1 in EPN and EPN-PKM3 cells following stimulation with LPA. Cells were serum-starved for 24 hours and the stimulated with 2.5 mM LPA for the indicated times. The analysis was performed for both the phosphorylated forms (pERK and pAKT1) and the total amounts (ERK and AKT1) of the kinases.

Finally, we investigated whether and how cAMP would modulate ERK1/2 and AKT1 state of activation. dBu-cAMP failed to induce ERK1/2 activation in EPN cells (Fig. 5). On the other hand, in the same experimental conditions, dBu-cAMP induced a substantial reduction (60%) of basal AKT1 activation within 30 minutes, disappearing thereafter (Fig. 6). In EPN-PKM3 cells, dBu-cAMP induced reduction of the basal level of AKT1 phosphorylation achieved within 30 minutes, was by a far smaller extent, in addition AKT1 activation remained unchanged until 24 hrs of dBu-cAMP treatment. Moreover, although a further decrease of AKT1 phosphorylation was observed at 48 hours, AKT1 was still activated up to 72 hours of treatment suggesting that cAMP and Pyk2 interact in the regulation of the AKT1 state of activation.

HOX network expression changes in EPN and EPN-PKM3 cells after dBu-cAMP treatment. We, finally, examined the effect of sustained levels of cAMP on the expression pattern of the HOX genes. In standard growth condition, EPN and EPN-PKM-3 cells display an identical pattern of HOX genes expression (Fig. 7A). Already after 24 h treatment with 10 mM of dBu-cAMP difference in HOX gene expression between EPN and EPN-PKM3 cells was observed (Fig. 7A). Five HOX genes (one cervical gene HOX D1, two thoracic genes HOX C6 ed HOX D8 and two lumbo-sacral genes HOX D9 ed HOX D11) display an altered expression in treated compared to untreated EPN cells. In contrast, a single gene, HOX D9, became silent in EPN-PKM3 cell after 24 hour exposure to dBu-cAMP, with respect to untreated cells. Exposure of the EPN and EPN-PKM3 to cAMP up to 72 h induced more dramatic effects: in EPN cells

10/39 HOX genes displayed altered expression (Fig. 7A). In contrast, EPN-PKM3 cells displayed the alteration of 8/39 HOX genes after the same dBu-cAMP treatment. Furthermore, among those genes, it is possible to detect six HOX genes whose alteration is common to both EPN and EPN-PKM3 cell lines: HOX A4, HOX A11, HOX B1, HOX D8, HOX D9 and HOX D12. In contrast, other HOX genes exhibited a specific behaviour according to the cell line: HOX A7 ed HOX A10 become silent in EPN-PKM3 cells and HOX C6 and HOX D1 become silent and HOX D11 and HOX C11 active in EPN cells (Fig. 7A and B). Moreover, the changes occurring in HOX gene expression after a 72 hour-treatment with cAMP mostly concerned lumbo-sacral HOX genes in both EPN and EPN-PKM3 cells.

Discussion

The relation between cAMP and Pyk2, is intriguing because of the variety of functions and effects that both Pyk2 and cAMP are able to generate in different cell types and in response to different stimuli. Indeed, both Pyk2 and cAMP are involved in the regulation of cell proliferation and differentiation depending on the cell type and context.[33] In prostate both Pyk2 and cAMP play pivotal role in pathophysiological conditions. The presence of Pyk2 in normal differentiated tissue, its progressive loss in adenocarcinoma of increasingly higher grade and its direct correlation with that of androgen receptor, suggest that Pyk2 could be a marker of prostate cell state of differentiation and could play the role of onco-suppressor gene controlling "prostate identity" and determining the fate of prostate cells during onset, progression and spreading of prostate cancer.[31,32,42] On the other hand, cAMP blocks cell proliferation and induces NED in prostate cancer cell lines.[31,34] At the same time, the interference of Pyk2 in such an event leads to speculate about its role in maintaining the specific prostate cellular phenotype. Thus, Pyk2 activation could be the key step linking together the pathways involved in the control of prostate cell growth and differentiation.

Here we demonstrated for the first time a relationship between cAMP and Pyk2 in the prostate. In EPN prostate cells cAMP induces Pyk2 phosphorylation, inhibits growth, and does not induce ERK 1/2 activation. Consistently, ablation of Pyk2 kinase activity caused a different growth response of EPN-PKM3 compared with EPN cells exposed to prolonged and sustained cAMP stimulation. In fact, although both cell lines cease to grow in the presence of cAMP, a substantial declining of cell number was observed in the EPN cells after eight days of treatment (-90%) while in EPN-PKM3 cells cAMP induced a by far more moderate cell decrease (-35%). This difference can be explained by the diverse effect of cAMP on the AKT1 state of activation in the two cell lines. In EPN cells cAMP treatment abolishes AKT1 phosphorylation within 30 minutes, while in EPN-PKM3 AKT1 remained still activated up to 72 hours. AKT1 is implicated in survival and apoptosis depending on its state of activation: in the phosphorylated state, AKT1 promotes cell survival by phosphorylating and inactivating the pro-apoptotic proteins BAD and caspase-9.[43] Our data are in agreement with the literature reporting that inhibition of cell proliferation correlates with reduction of AKT1 phosphorylation in several cell systems,[44] while the activation of AKT1 is significantly increased in primary prostate cancer, particularly those representing late-stage cancers.[45] On the other hand, it has been reported that AKT1 activity is influenced

by Pyk2 state of activation, vascular endothelial cells,[46] mammary epithelial cells[47] and in PC12 pheocromocitoma cells.[48] In our model system, the functional ablation of Pyk2 is associated with a smaller and slower decrease of AKT1 phosphorylation following treatment with cAMP. This observation indicates that cAMP and Pyk2 pathways interact in prostate cells and that Pyk2 influences the effect of cAMP on AKT1 activation. This suggests that in normal prostate cells Pyk2 is involved in the regulation of the apoptotic pathways activated by cAMP. On the other hand, ablation of Pyk2 kinase activity results in the longer persistence of the activated pro-survival pathway; this is consistent with the progressive disappearance of Pyk2 with increasing malignancy of prostate cancer. All the above suggest that Pyk2 might have a role in keeping the normal state of differentiation of prostate cells.

In this view, since the HOX gene network is involved in controlling differentiated specific features, we investigated cAMP and Pyk2 interaction on the 39 genes HOX network expression in EPN and EPN-PKM3 cells. Pyk2 ablation per se did not alter the pattern of HOX gene expression that was identical in EPN-PKM3 and EPN cells. However, upon cAMP stimulation HOX genes expression pattern differed between the two cell lines in a time related fashion. In that, 72 hours cAMP treatment induced variation of the expression of ten HOX genes in EPN and eight genes in EPN-PKM3, with only six HOX genes commonly modified between the two cell phenotypes. This suggests an interaction between Pyk2, cAMP and the HOX gene network as well as the involvement of the HOX gene network in cAMP-induced phenotype modifications in both epithelial cell phenotypes. One of the cAMP-altered HOX genes in EPN-PKM3 cells is HOX A7, recently described to transcriptionally interact with Pyk2 during HL-60 monocytic differentiation. We have shown that HOX D genes are involved in neuroendocrine differentiation of prostate cancer.[38] HOX C and HOX D genes are deregulated after cAMP treatment of EPN epithelial prostate cells. HOX D13 is crucial in specifying prostate identity during development and play a role in prostate ductal morphogenesis and/or cell differentiation, as results from HOX D13 knock-out mice. A potential mechanism linking the HOX gene network and Pyk2 is provided by the identification of the HOX genes as potentially interacting with the PHAS-1-eIF4E complex, which in turn is regulated by Pyk2. Thus, the expression of the HOX gene network, after exposure to cAMP, allows to discriminate between two epithelial prostate cell phenotypes that differs for a single kinase activity, but also to identify specific HOX genes influenced by Pyk2 expression, which is the kinase that makes the difference between the two cell types.

Taken together these data suggest a functional link between cAMP and Pyk2 in prostate epithelial cells that influences cell survival and possibly cell identity. One could speculate that in the prostate cAMP acts as a differentiating agent that in the presence of Pyk2 preserves prostate identity, while in the absence of Pyk2 pushes prostate cells towards NE phenotype. Since NED differentiation in prostate cancer profoundly affects the progression of the disease and is generally associated with poor prognosis, understanding the mechanisms of NED in the prostate may have important diagnostic and therapeutic implications.[49-51] A broader knowledge of the signalling pathways controlling NED might lead to identify molecular targets for novel strategies to prevent and/or antagonize the progression and spreading of prostate cancer.
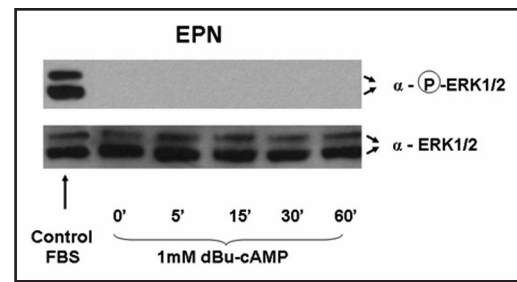

Figure 5. Western blot analysis of the phosphorylation of ERK1/2 in EPN cells following increase of intracellular cAMP. Cells were cultured for the indicated times in the presence of 10 mM db-cAMP and 1 mM IBMX. (Upper) Detection of phosphorylated Erk1/2 (pERK). (Lower) Detection of the total amount of ERK1/2.
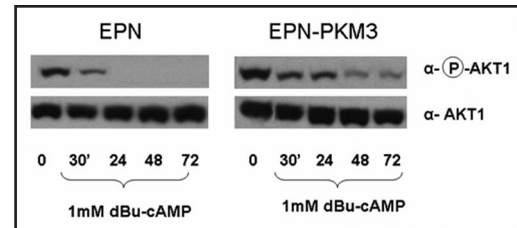

Figure 6. Western blot analysis of the phosphorylation of AKT1 in EPN and EPN-PKM3 cells following increase of intracellular cAMP. Cells were cultured for the indicated times in the presence of 10 mM db-cAMP and 1 mM IBMX. (Upper) Detection of phosphorylated AKT1 (pAKT1). (Lower) Detection of the total amount of AKT1.

Materials and Methods

Chemicals. Keratinocyte serum-free medium and Bovine Pituitary Extract were purchased from Gibco Cell Culture, Invitrogen Corporation. Penicillin, versene, streptomycin, foetal calf serum (FCS), bovine serum albumin (BSA), and phosphate-buffered saline (PBS) were purchased from Eurobio (Les Ullis Cedex, France). Aprotinin, leupeptin, phenylmethylsulfonyl fluoride (PMSF), sodium orthovanadate were purchased from Sigma (Milan, Italy). ECL System from Amersham Pharmacia (Buckinghamshire, UK), Bio-Rad assay and prestained protein standards from Bio-Rad (München, Germany). Dibutyryl cAMP (dBu-cAMP), 3-isobutyl-l-methyl-xantine (IBMX) and lysophosphatidic acid (LPA) were purchased from Sigma (Milan, Italy).

Antibodies. Antibodies were purchased from the following sources: polyclonal anti phospho-p44/42 MAP kinase (Thr202/Tyr204) antibody (#9101S), and polyclonal anti phospho-AKT1 antibody (9275s) from New England Biolab, MA; anti-actin antibody, and rabbit polyclonal anti ERK1 (#sc-94-G) from Santa Cruz Biotechnology Inc., (Santa Cruz, CA, USA); rabbit polyclonal anti-phospho-PYK2 [pY402] (# 44-618) from Biosource International Inc., (Camarillo, CA, USA); biotinylated horse-anti-goat IgG and ABC complex/HRP from Vector Laboratories (Burlingame, CA, USA).

Cell culture. EPN cells are a line of non-transformed epithelial cells derived from human normal prostate tissue spontaneously adapted to grow in culture; EPN-PKM3 cells were obtained by transfecting EPN cells with a plasmid containing a Pyk2 kinase-negative
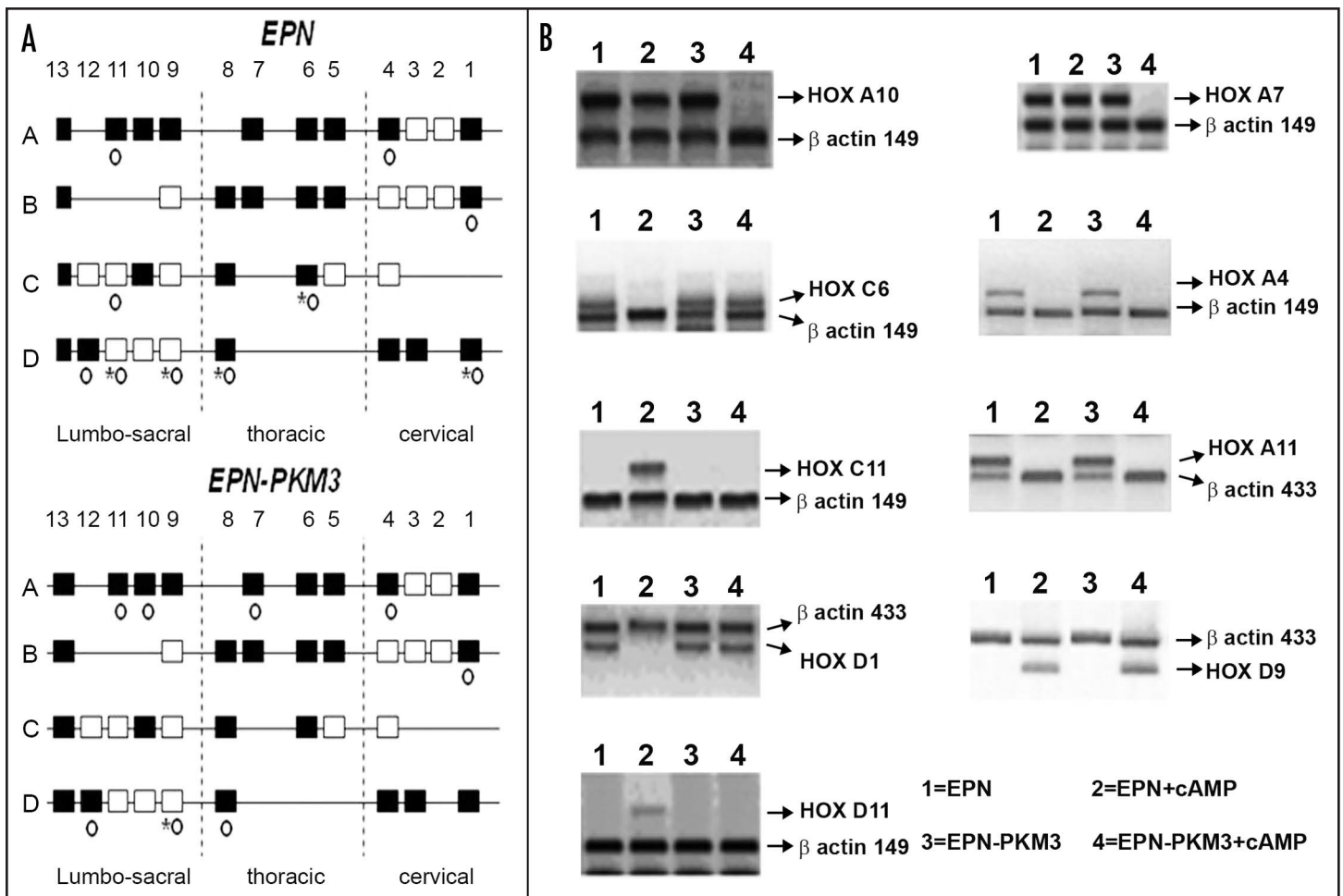
Figure 7. (A) Schematic representation of the HOX gene network (see text for details). Expression of the whole HOX gene network detected by RT-PCR in EPN and EPN-PKM3 prostate cell lines: black and white square indicate active or silent HOX genes, respectively, in basal conditions. Circles indicate HOX genes altered in EPN and EPN-PKM3 cells after 72 h cAMP exposure. Asterisks indicate HOX genes altered after 24 h cAMP exposure in EPN and EPN-PKM3 cells. Altered HOX genes are active or silent, in the same sense, after 24 and 72 h cAMP exposure. (B) RT-PCR expression of HOX A10, HOX C6, HOX C11, HOX D1, HOX D11 and HOX A7: these genes are differently expressed in EPN and EPN-PKM3 following cAMP stimulation; differently, HOX A4, HOX A11 and HOX D9 show the same response to cAMP stimulation in EPN and EPN-PKM3 cells. Control coamplification of HOX A10, HOX C6, HOX C11, HOX D11, HOX A7, HOX A4 with a 149 bp β-actin primer is reported. Control coamplification of HOX D1, HOX A11 and HOX D9 with a 433 bp β-actin primer is reported. Duplex PCR products were separated by ethidium 1.2% agarose gel electrophoresis. Lane 1 = EPN cells, lane 2 = EPN cells + 72 h cAMP; lane 3 = EPN-PKM3 cells; lane 4 = EPN-PKM3 cells + 72 h cAMP.

mutant (PKM) that was obtained by replacing Lys475 with an Ala residue as previously described.[26,32,41] Both cell lines were routinely cultured in keratinocyte serum-free medium and Bovine Pituitary Extract (KSFM) supplemented with 3% FCS.[16]

Cell proliferation assay. To study the effect of dBu-cAMP on the proliferation of EPN and EPN-PKM3, 10^5 cells were seeded in 60 mm culture dishes with medium in standard condition. After 24 hours the medium was substituted with medium supplemented with 0.5% of FBS and after three days we added 10 μM dBu-cAMP and 1 μM IBMX to inhibit phosphodiesterases activity. At appropriate intervals, triplicate dishes were trypsinized per each experimental point and cell number was determined by counting cell suspension in a Neubauer hemacytometer. The values reported represent the mean ± standard deviation (SD) of three independent samples per each experimental point.

Western blotting. To determine the effect of dBu-cAMP on EPN and EPN-PKM3, cells grown to sub-confluence in standard

medium (KSFM) were serum-starved for 48 hours. Then, medium was supplemented with 10 mM dBu-cAMP and 1 mM IBMX to inhibit phosphodiesterase activity. For LPA studies, cells were serum-starved for 24 hours and, afterward, stimulated with LPA (2.5 mM) for different periods of time (5, 15, 30 and 60 min). At appropriate time intervals cells were harvested in lysis buffer (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton-X-100, 1 mM phenylmethylsulfonyl fluoride, 1 g aprotinin, 0.5 mM sodium orthovanadate, 20 mM sodium pyrophosphate). The lysates were incubated 30 min on ice then clarified by centrifugation at 14,000 g x 10 min. Total protein concentration was estimated by modified Bradford assay.

For Western blot 25 or 50 μg/lane of total proteins were separated on SDS/10% NuNupage pre-cast gels (Invitrogen Life Technologies) and transferred to PVDF membranes (Invitrogen Life Technologies); complete transfer was assessed using pre-stained protein standards (Invitrogen Life Technologies). The membranes were blocked in

TBS/T, 25 mM Tris, pH 7.4, 200 mM NaCl, 0.5% Triton X-100, and 5% non-fat powdered milk for 1 hour at room temperature. Incubation with the primary antibody (anti ERK1/2 1:2000, anti-phospho-ERK1/2 1:2000, anti phospho-AKT1 1:1000) was carried out at room temperature for 1 hour. Finally, the membranes were incubated with the horseradish peroxidase-conjugated secondary antibody (1:3000) for 45 minutes at room temperature and the reactions detected with ECL system (Perkin Elmer).

RNA extraction and analysis. Cells were seeded in 60 mm culture dishes under standard conditions. After three days of growth, medium was supplemented with 10 mM dBu-cAMP and 1 mM IBMX to inhibit phosphodiesterase activity, and with 0.5% FBS. At appropriate time intervals (72 hours), dishes were trypsinized and total cellular RNA was extracted by the guanidinium thiocyanate technique. Four micrograms of total RNA were subjected to cDNA synthesis for 1 hour at 37°C using the "Ready to go You-Primer First-Strand Beads" kit (Amersharm Biosciences, cod. 27-9264-01) in a reaction mixture containing 0.5 μg oligo-dT (Amersharm Biosciences cod. 27-7610-01). PCR amplification of cDNA was performed in a reaction mixture (Pure Taq Ready to go PCR-beads; Amersharm Biosciences cod. 27-9558-01) containing 4 μg of cDNA sample and different primer sets (20 p/mol each). The sense/anti-sense HOX primers for PCR were designed as previously reported.[42] To prevent genomic DNA contamination, primer sense and anti-sense were designed to frame a sequence that crossed at least one intron on the genes. The co-amplification of each specific gene and human β-actin gene, as an internal control, was achieved using two primer sets in a single reaction mixture. We selected two pairs of β-actin primers to obtain amplified fragments with different length (149 and 433 bp) to be used alternatively in the co-amplification reaction. Duplex-PCR products were separated by electrophoresis on a 1.2% agarose gel containing ethidium bromide.

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