Peripheral Benzodiazepine Receptor Antisense Knockout Increases Tumorigenicity of MA-10 Leydig Cells in Vivo and in Vitro †

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Peripheral Benzodiazepine Receptor Antisense Knockout Increases Tumorigenicity of MA-10 Leydig Cells in Vivo and in Vitro[†]

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ABSTRACT: Peripheral benzodiazepine receptors (PBR), first described more than 20 years ago, have been attributed with many putative functions including ones in cellular proliferation and cellular respiration. Hence, it is quite conceivable that deregulation of this receptor could lead to pathology. We and others have reported the existence of PBR overexpression in different human and nonhuman malignancies, but it has never been made clear whether this aberrant malignant PBR expression is a cause or consequence of the cancer. In the current study we induced PBR underexpression by downregulating one critical subunit of the PBR complex, the isoquinoline-binding protein (IBP), using the stable antisense knockout approach, in the MA-10 Leydig cell line. Resultant clones, showing PBR deregulation, also demonstrated increased tumorigenicity, using both in vitro (loss of contact inhibition and growth in soft agar) and in vivo (increased mortality on grafting back into isogenic mice) assays. We suggest that this type of deregulation could be a later event in natural tumor progression. Consequently, PBR deregulation should be more closely studied in human malignancy.

Peripheral benzodiazepine receptors (PBR),[1] while widely expressed throughout the body, exhibit different patterns of tissue-specific expression (*1−5*). Although the PBR were first described more than 20 years ago (*6*) and a broad spectrum of putative functions has been suggested for them (*1, 5, 7*), their primary role still has not been established. These putative PBR functions include the regulation of steroid production (*4, 7−12*), involvement in cell growth and differentiation (*11, 13−15*), regulation of cellular respiration (*16, 17*), regulation of heme biosynthesis (*18, 19*), effect on the immune and phagocytic host defense response (*20*), and modulation of voltage-dependent calcium channels (*7*). We had previously suggested that some of these putative PBR functions may be tissue-specific (*5, 12, 21*). As some studies have suggested a function for PBR in cell proliferation (*11, 13−15, 21*), we and others have found increased PBR density in different tumors and transformed cell lines (*5, 22−25*).

The PBR is an intracellular multisubunit protein receptor, located mainly on the outer mitochondrial membrane (*26*) and composed of three subunits: the isoquinoline-binding protein (IBP),[1] which is 18 kDa in mass, the voltage-

dependent anion channel (32 kDa), and the adenine nucle-otide transporter (30 kDa) (*27*). We have demonstrated that a tissue-specific relationship between these three protein subunits in the PBR complex is important (*28, 29*).

The present study was initiated to study the effect of changes in putative PBR functions in the MA-10 mouse tumor Leydig cell line by the antisense knockout of the IBP subunit of PBR. Because much of the early work on the role of PBR in steroidogenesis originated in the MA-10 Leydig cell line (*4, 7−9, 11*), we decided to extend our prior antisense knockout population studies in MA-10 cells (*12*) to specific stable clones from the original populations. To our surprise, we found that the stable antisense knockout of IBP in MA-10 cells as isolated clones indicated a direct involvement in tumor enhancement.

MATERIALS AND METHODS

Cells. The MA-10 cell line, originally cloned from the solid M5480P mouse tumor Leydig cell, was a kind gift of Dr. Mario Ascoli (Department of Pharmacology, University of Iowa College of Medicine, Iowa City, IA). MA-10 cell cultures were grown as previously described (*30*). Briefly, cells were maintained in RPMI 1640 medium with L-glutamine, containing 20 mM HEPES buffer, 15% horse serum, 300 mg/L glutamine, and 50 μg/mL gentamicin sulfate (all purchased from Biological Industries, Beit HaEmek, Israel). MA-10 transfected cells were grown in similar medium, except for the use of 7.5% horse serum and 7.5% fetal calf serum in place of 15% horse serum.

Stable Transfection of MA-10 Cells. The transfection was performed as previously described (*12, 31*). Briefly, cells

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[1] Abbreviations: IBP, isoquinoline-binding protein(s); PBR, periph-eral benzodiazepine receptors(s).

were plated a day before transfection (5×10^5 cells/90 mm^2 tissue culture dish). On the following day, 15 μg of antisense knockout plasmid [the same as that used by Kelly-Hershkovitz et al. (*12*)] was routinely cotransfected with 3 μg of the neomycin-resistant plasmid pMCI.neo.Poly(A) (Stratagene, La Jolla, CA). In control experiments, the plasmid pENKAT12 was used with pMCI.neo.Poly(A). Four hours after the transfection, the cells were subjected to glycerol shock for 90 s. The day after transfection, G418 (stable neomycin analogue) selection (300 μg/mL) was initiated and maintained for 3 weeks, at the end of which cells were subjected to [^{3}H]PK 11195 binding assays before dilution cloning.

Protein Measurements. Protein was quantitated by the method of Bradford (*32*) using BSA as a standard.

[^{3}H]PK 11195 Binding Assays. [^{3}H]PK 11195 (90 Ci/mmol; NEN Life Science Products), an isoquinoline carboxamide derivative, was used for binding studies, as described previously (*33*). PK 11195 is a specific PBR ligand. Cells were scraped from 90 mm^2 culture dishes, washed with phosphate-buffered saline (PBS), and centrifuged at 1200g for 5 min. Then the cell pellets were resuspended in 1 mL of 50 mM phosphate buffer, pH 7.4, for 10 s using a Brinkmann/Kinematica polytron (setting 6) and centrifuged at 37 000g for 30 min.

Binding assays contained 400 μL of cell membrane (0.1 mg of protein/mL) in the absence (total binding) or presence (nonspecific binding) of 1 μM unlabeled PK 11195 (Pharmuka Laboratories, Gennevilliers, France), up to a final volume of 500 μL. After incubation for 1 h at 4 °C, samples were filtered under vacuum over Whatman GF/B filters and washed three times with 3 mL of phosphate buffer. Filters were placed in vials containing 4 mL of Opti-Fluor (Packard, Groningen, The Netherlands) and counted for radioactivity in a β-scintillation counter after 12 h.

The maximal number of binding sites (B_{max}) and equilibrium dissociation constants were calculated from the saturation curve of [^{3}H]PK 11195 binding, using Scatchard analysis. For initial knockout clone selection, single-point PBR binding values were determined at 6 nM [^{3}H]PK 11195.

Dilution Cloning. Successfully transfected MA-10 populations were then subjected to two rounds of dilution cloning, so that each purified culture would represent cells derived from one transfected clone each. Twenty 100 μL aliquots of a trypsinized MA-10 population of transfected MA-10 cells were seeded in 96-well plates. From each primary seeding, six serial dilutions were prepared (1:10 dilution each time) and allowed to grow back to fill the wells. Each regrown dilution was then tested for reduced [^{3}H]PK 11195 binding, and those with about half of the control [^{3}H]PK 11195 binding value were then subjected to another round of dilution cloning, as described above. These final regrown diluted cells were expected to represent individual clones of transfected MA-10 cells.

Thymidine Incorporation. Cell cultures were incubated in 0.5 mL of medium for 4 days without change of medium. Cells were then trypsinized and plated in 24-well plates (5 $\times$ 10^4 cells/well). One hour after plating, 25 μL of [^{3}H]-methylthymidine (5 Ci/mmol; Rotem Industries, Beersheba, Israel) to a final concentration of 1 μM was added to each well of the plate. Cells were incubated for 24 h, and then each well was washed three times with ice-cold phosphate-

buffered saline, incubated with 500 μL of 10% trichloracetic acid for 30 min at 4 °C, and incubated overnight with 500 μL of 1 N NaOH. The next day, cells were counted for radioactivity in Opti-Fluor in a β-counter.

Western Blot Analysis. Whole cell protein extracts were obtained from the different MA-10 cell lines by resuspending the cells in lysis buffer B [1 mM EDTA, 250 mM sucrose, and 5 mM Tris-HCl (pH 7.5) plus Roche proteinase inhibitors] and then subjecting the suspension to light homogenization (glass–glass, 10 strokes). The homogenates were centrifuged at 1000g for 10 min. The supernatants were then centrifuged at 7500g for 20 min. The pellets were resuspended in fresh lysis buffer B and centrifuged again at 7500g for 20 min. The pellets were then suspended in lysis buffer B, and the protein in the extract was quantified. Appropriate protein amounts were then prepared in 2 $\times$ sample buffer [final concentrations 50 mM Tris-HCl (pH 6.8), 10% glycerol, 2% (w/v) SDS, 100 mM β-mercaptoethanol, and 0.1% (w/v) bromphenol blue]. The samples were boiled for 10 min and subjected to electrophoresis through 4–20% SDS–polyacrylamide gradient gels (10 μg of protein/lane). The protein extracts were then electrophoretically transferred to nitrocellulose (Hybond ECL; Amersham Life Sciences, Buckinghamshire, England) in 20 mM Tris-HCl, 150 mM glycine, and 20% methanol for 1 h at 100 V, followed by blocking of the membrane in 5% dried milk (Carnation, Glendale, CA) in TBS-T solution (20 mM Tris-HCl, pH 7.6, and 14 mM NaCl containing 0.1% Tween 20). After three washes, the membranes were incubated with polyclonal anti-18-kDa IBP antibody, prepared in our laboratory (*28*), in 1% dried milk in TBS-T for 2 h. The membranes were washed three times in TBS-T, followed by 1 h incubation with secondary antibody [anti-rabbit IgG, horseradish peroxidase-linked, from Amersham]. After three washes with TBS-T, the membranes were incubated for 1 min with ECL detection reagent (Amersham) and exposed to Hyperfilm (Amersham) for 30–60 s.

Steady-State RNA Preparation and Analysis. Total RNA was isolated using Tri Reagent (Sigma Chemical Co., St. Louis, MO). RNA (10–30 μg/lane) was quantitated by absorbance at 260 nm and fractionated by electrophoresis through 1% agarose/formaldehyde gels, and the fractionated RNA was transferred (in 20 $\times$ SSC) to 0.45 μm nylon-reinforced nitrocellulose membranes (Nytran-N; Schleicher & Schuell, Dassel, Germany) by standard procedures (*34*). These blots were probed with ^{32}P-labeled cDNA probes (rat IBP or cyclophilin): the 750-bp *Eco*RI rat IBP cDNA fragment (a kind gift from Dr. Karl E. Krueger, Georgetown University, Washington, DC) and the 700-bp *Bam*HI–*Pst*I cyclophilin cDNA (*35*). Hybridizations and washes were carried out under standard conditions (hybridization at 65 °C; two washes with 1% SDS and 2 $\times$ SSC at 65 °C for 20 min each). The blots were autoradiographed with a phosphorimaging system (model BAS-1000 MacBas; Fuji, Tokyo, Japan).

Soft Agar Analysis. Cells were grown in soft agar, as previously described (*34*). Thirty-five millimeter diameter plates had 2 mL of 0.6% (final concentration) agar in MA-10 growth medium (as described above) applied, which was allowed to solidify over 48 h. Above this first layer, 1.6 mL of 0.3% (final concentration) agar in MA-10 growth medium, with 1 $\times$ 10^4 MA-10 cells/plate, was added. Cells were added

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Rat:    295 CAGCT
            |||||
Mouse:  227 CAGCT

Rat:    301 GGCTCTGAACTGGGCATGGCCCCCCATCTTCTTTGGTGCCCGGCAGATGGGCTGGGCTTT
            |||||||||||||| |||||||||||||||||||||||||||||||||||||||||||||
Mouse:  332 GGCTCTGAACTGGGCGTGGCCCCCCATCTTCTTTGGTGCCCGGCAGATGGGCTGGGCCTT

Rat:    361 GGTGGACCTCATGCTTGTCAGTGGGGTGGCAACCGCCACTACCCTGGCTTGGCACCGAGT
            || ||| |||| ||||||||||||||||||| || ||||| |||||||||||||||||||
Mouse:  392 GGCCGATCTTCTGCTTGTCAGTGGGGTGGCGACTGCCACAACCCTGGCTTGGCACCGAGT

Rat:    421 GAGCCCACCGGCTGCCCGCTTGCTGTATCCTTACCTGGCCTGGCTGGCCTTTGCCACCAT
            |||||| || |||||||||||||||||| |||||||||||||||||||| |||||||| |
Mouse:  452 GAGCCCGCCCGCTGCCCGCTTGCTGTACCCTTACCTGGCCTGGCTGGCTTTTGCCACCGT

Rat:    481 GCTCAACTACTATGTATGGCGTGATAACTCTGGTCGGCGAGGGGGCTCCCGGCTCACAGA
            |||||||||||||||||||||||||||||||||||| |||||||||||||||||| ||||
Mouse:  512 GCTCAACTACTATGTATGGCGTGATAACTCTGGCCGGCGAGGGGGCTCCCGGCTCGCAGA

Rat:    541 GTGAGGACACCTAGCCATCAGGAATGCAGCCCTGCCAGCCAGGCATCATGGGTTGAGGTC
            |||| | ||||| |||||||||||||||||||||||||||||| ||||||| | | ||
Mouse:  572 GTGAAGGCACCCAGCCATCAGGAATGCAGCCCTGCCAGCCAGGCACCATGGGTGGCAGCC

Rat:    601 ATCCTGCTTTCATGACCATTGGGCCTGCTGGTCTACCTGGTCTTAGTCCAGGAAGCCACC
            ||| ||||||||||||||||||||||||||||||||||||||| |||||||||||||||
Mouse:  632 ATCATGCTTTCATGACCATTGGGCCTGCTGGTCTACCTGGTCTTAGCCCAGGAAGCCACC

Rat:    661 AGGTAGGTCAAGGTGGTCAGTGCTAAGTCCCATGCGGGGACAGTTGTACCTGCTTTTCTG
            ||||||||| |||||||||||| |||| | ||| | |||||| ||||||| ||||||
Mouse:  692 AGGTAGGTTAGGGTGGTCAGTGCCGAGTCTCCTGCAGACACAGTTATACCTGCCTTTCTG

Rat:    721 CACTGCTGCAGGCGTGCCCTAGGAGCATGGGGCCTTTAA
            |||||||| |||||| |||||| |||||||| | || ||
Mouse:  752 CACTGCTCCAGGCATGCCCTTAGAGCATGGTGTTTTAAA
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FIGURE 1: Sequence comparison between the rat IBP sequence, used for the current antisense knockout vector (*12*; GenBank Accession Number J05122|RATPKBSX), and the murine IBP cDNA sequence (GenBank Accession Number D21207|MUSPTBR) over the equivalent range (numbered with respect to the GenBank sequence). The 464 base pair rat IBP sequence showed 90.3% identity with the mouse sequence.

to the 0.3% agar mix at 37 °C; when the top agar layer had solidified, plates were kept at 37 °C in 5% CO_2 and 100% humidity. After 17 days, cultures were photographed.

In Vivo Tumorigenesis Assay in Isogenic C57 Black Mice. After cell trypsinization and washing suspended cells in medium with serum, cells were washed twice with PBS. Then 5×10^5 cells in 500 μL of PBS per mouse were injected interperitoneally into C57 black mice (both males and females). Control mice had PBS injected either without cells or with control cells. These mice were followed until death.

Statistical Analysis. Results are expressed as the mean ± standard error. One-way analysis of variance, Student−Newman−Keuls post hoc analysis, and nonparametric Mann−Whitney and Kruskal−Wallis tests were applied as appropriate. The nonparametric analyses were used when indicated by the Bartlett test for homogeneity of variances. A two-tailed *t* test was used to analyze the binding parameters. Statistical significance was defined as $p < 0.05$.

RESULTS

Antisense knockout stable transfections were carried out on MA-10 Leydig cells, as previously described (*12*). The antisense knockout vector contained rat IBP sequences in the antisense orientation to the orientation of transcription. As MA-10 Leydig cells are mouse cells, we compared the 464 base pair rat IBP sequence to the equivalent mouse IBP sequence (Figure 1). As this region of the IBP sequence was highly homologous (90.3%), the current vector was considered appropriate for this study in mouse cells.

Plates of MA-10 Leydig cells following stable transfection and selection were trypsinized and then subjected to dilution cloning, with the goal of isolating cell lines of knockout clones derived from single insertion events. These clones were then characterized by initial PBR-ligand binding screens to identify clones with the most reduced ability to bind [^{3}H]-PK 11195 at a single concentration of 6 nM (Table 1). Two such clones, MAAP29 and MAAP31, showed a significant drop of 61% ($p < 0.01$) and 64% ($p < 0.001$) in [^{3}H]PK 11195 binding, respectively, compared with the MA-10 pEnKAT12 (*12*) transfected (MA-Enk) control cell line (Table 1). Other clones also showed a significant drop in [^{3}H]PK 11195 binding (see Table 1, clones MAAP6 and MAAP8). For the rest of the present study, we decided to focus on the two knockout clones, MAAP29 and MAAP31, as well as on the control transfected MA-Enk. A representative Scatchard analysis on crude, whole cell membranes was then carried out for MA-Enk, MAAP29, and MAAP31 (Figure 2), resulting in B_{max} values of 8480, 3181, and 3908 fmol/mg of protein, respectively. This reduction of the knockout clones' B_{max} values is indicative of the reduced IBP abundance in these cells.

Western blot protein analysis was then carried out on the mitochondrial fraction to determine the effect of IBP antisense knockout on the IBP subunit of the PBR. IBP expression was reduced dramatically compared to the control

Table 1: PBR Binding (B) Values for Knockout Clones

P value[b]	mean B value ± standard error[a]	clone
	7267 ± 621	MA-Enk
<0.01	2975 ± 805	MAAP6
<0.01	2739 ± 537	MAAP8
NS[c]	4695 ± 549	MAAP9
NS	4704 ± 337	MAAP10
NS	4297 ± 344	MAAP11
NS	6506 ± 2733	MAAP12
NS	6621 ± 439	MAAP13
NS	6109 ± 646	MAAP21
NS	5241 ± 384	MAAP22
NS	4313 ± 184	MAAP24
NS	7015 ± 133	MAAP27
<0.01	2847 ± 142	MAAP29
<0.001	2630 ± 297	MAAP31
NS	4875 ± 226	MAAP34

[a] Each B value results from at least three separate experiments. [b] P value for knockout clone with respect to P value for MA-Enk control. [c] NS = no significant differences were detected.
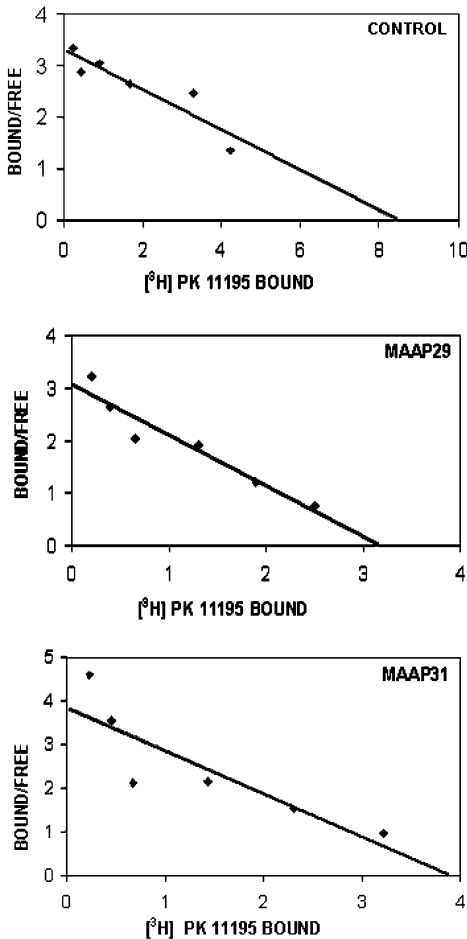

FIGURE 2: Scatchard plot analysis for control and PBR antisense knockout MA-10 clones.

in MAAP29 (97%) and also greatly reduced in MAAP31 (76%) (Figure 3). As an equal amount of protein was added to each lane, these differences in IBP protein expression give clear evidence that stable antisense knockout was effective. Furthermore, with Northern blot analysis we also saw a 68% decrease in IBP total RNA expression in MAAP31 cells compared to control MA-Enk cells after normalization to cyclophilin expression ($p < 0.05$; $n = 4$) (data not shown).

On cell cultures it was apparent to us that, as opposed to control MA-Enk cells, these two knockout clones grew
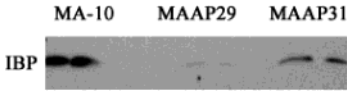

FIGURE 3: Western blot analysis of IBP expression in MAAP29 (two lanes) and MAAP31 (two lanes) IBP antisense knockout clones compared to MA-10 control cells (two lanes). IBP expression is strongly reduced in both IBP antisense knockout clones.
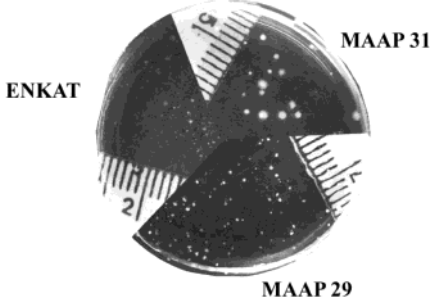

FIGURE 4: Montage of PBR antisense knockout clones and control MA-Enk MA-10 cells grown in soft agar. Here we see that both knockout clones grow with larger colony size and number than control MA-Enk cells.
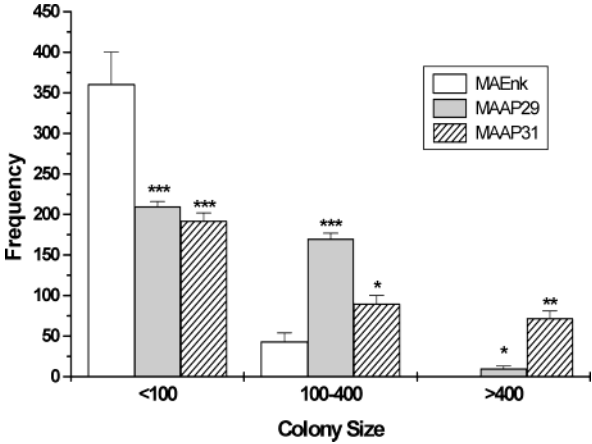

FIGURE 5: Variation in colony size (cells per colony) of PBR antisense knockout clones compared to MA-Enk control cells grown in soft agar: (open column) MA-Enk; (shaded column) MAAP29; (striped column) MAAP31. Error bars represent standard error of the mean (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

without contact inhibition, as they were seen as growing on top of each other as well as on the plate. This suggested that at least these two independent PBR knockout clones may have become more tumorigenic than their parent Leydig cell line. To further confirm and clarify this observation, we chose to study the ability of these cells to grow in soft agar, which is a measure of in vitro tumorigenicity. Results of MA-10 clone growth in soft agar are shown in Figures 4 and 5. Untransformed cells typically do not grow in this environment, while transformed cells become increasingly able to do so (*36*). In general, MA-10 cells, which are a low-level transformed cell line, grew as small clusters, mostly under 100 cells per colony (Figure 4). In Figure 4, we can also see the stronger growth of both of the MA-10 knockout clones (MAAP29 and MAAP31) compared with MA-Enk control cells. These differences are quantified in Figure 5 and are particularly clear for clone MAAP31 ($p < 0.01$ in comparison to control), where a large number of large colonies were typically seen (>400 cells/colony), which were not seen at all in parent MA-10 or control MA-Enk cells. A small but significant ($p < 0.05$) number of large colonies were also
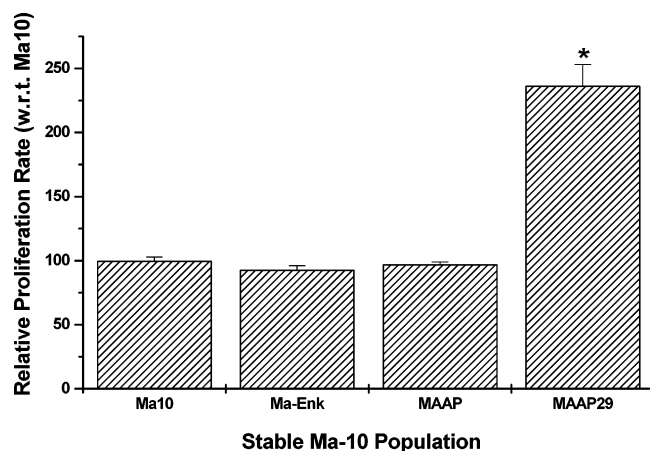
FIGURE 6: Effect of IBP knockout on cell proliferation. Thymidine incorporation, indicative of cell multiplication in mixed cultures of IBP antisense knockout clones (MAAP) and MA-Enk control cells, was measured and found not to differ ($n \geq 16$ for each group), except for antisense knockout clone MAAP29. Error bars represent the standard error of the mean (*, $p < 0.05$).
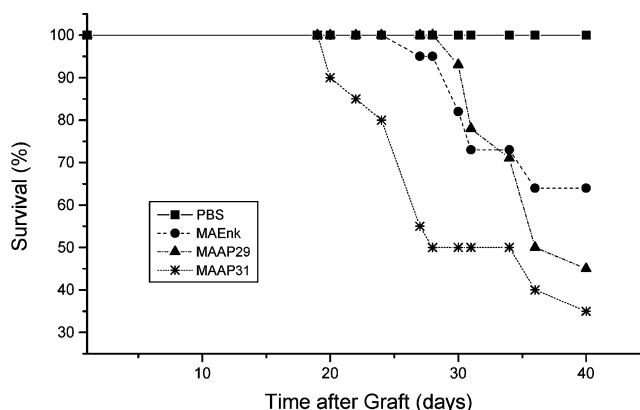

FIGURE 7: Survival curve for isogenic mice grafted intraperitoneally with either saline (solid line), control MA-Enk (dashed line), MAAP29 (dotted-dashed line), or MAAP31 cells (dotted line).

generated by MAAP29 IBP antisense knockout cells (Figure 5). Medium-sized colonies (100−400 cells/colony) were also generated in greater number by both antisense knockout clones compared to control cells ($p < 0.001$ for MAAP29 and $p < 0.05$ for MAAP31). Control MA-Enk cells usually grew as small colonies (<100 cells/colony) in soft agar (Figures 4 and 5). It must be remembered here that this assay is not 100% efficient, and hence direct comparison of the number of large and small colonies may not be valid. These data indicate that the antisense knockout clones became more tumorigenic than their parental controls.

To test the effect of IBP antisense knockout on MA-10 cell proliferation, we measured [³H]methylthymidine incorporation in exponentially growing cell cultures of MA-Enk control cells and IBP antisense knockout cell lines. Interestingly, upon comparison of the population average of knockout clones (MAAP) compared to control data (Ma-Enk or Ma10), shown in Figure 6, we do not see an effect. This was also the case for antisense knockout clone MAAP31 (data not shown). Ordinarily this would suggest that this genetic manipulation did not lead to a functional change in cell proliferation. As we also have found that the antisense knockout clone MAAP29 did have significantly elevated cellular proliferation compared to the controls ($p < 0.05$, Figure 6), we know that the population effect we saw was the average of many clones that were no different to the controls (with respect to cell proliferation) and a few that were. Significantly, this clone (MAAP29) was also very tumorigenic (in vitro) in the current study (Figure 4, 5). This is reminiscent of data presented in an earlier study (*12*).

To investigate whether this in vitro transformation assay translated into increased in vivo tumorigenicity, we started a series of experiments to measure the effect of intraperitoneal injections of these clones into isogenic C57 mice. Cells were washed in PBS alone and were injected interperitoneally into groups of 20 mice. The survival of these mice was then monitored (Figure 7). MA-Enk control cells did kill C57 mice, as was expected, as the MA-10 parent cell line is an established transformed cell line. Furthermore, it is clear from data presented in Figure 7 that the antisense knockout clone MAAP31 killed mice more quickly than any of the other

clones or controls tested. The difference between MAAP31 and MA-Enk was also clear in the soft agar in vitro tumorigenicity assay (Figures 4 and 5) and was consistent with this in vivo result. MAAP29 cells were less aggressive killers in this in vivo assay (Figure 7) compared with MAAP31, which was consistent with their ability to grow fewer large colonies in soft agar than MAAP31 (in Figures 4 and 5). MAAP29 cells still killed more mice in less time than control MA-Enk cells and hence appear to be more tumorigenic than control cells.

DISCUSSION

Although numerous molecular studies have implicated many putative functions for the PBR, such as in steroidogenesis, cellular respiration, and cell proliferation, its primary role in the cell is still an enigma (*5*). Hence, this study was originally designed to study putative functions of PBR in MA-10 Leydig cells after having purified PBR antisense knockout clones and controls. We chose the antisense knockout approach after applying it in other studies (*12, 31*). The antisense knockout we describe in this study was focused on the IBP, one of the PBR protein subunits. We chose this subunit because there are many proteins involved in the mitochondrial PBR complex, some of which are not unique to this complex, and because the main PBR-specific ligands require the IBP subunit for binding (see review, ref *5*).

The B_{max} values determined in the present study are all at the same level of magnitude, indicating the consistency of the applied method. These B_{max} values for the MA-10 Leydig cells, however, are 5−8-fold lower than for crude cell membrane homogenates of MA-10 Leydig cells reported elsewhere (*8, 12*). We have chosen to assay binding in MA-10 stationary cultures to improve the reproducibility and reduce the variability of the resulting values. Cells grown in exponential cultures can show much greater B_{max} values. However, this can be at the cost of substantial replicate variability. Such manifold differences also are apparent when comparing reports of other cell lines, even coming from the same laboratory, where each report presents a different B_{max} value, ranging from 1000 fmol/mg, over 16000 fmol/mg, to 23000 fmol/mg in near confluent C6 cells (e.g., refs *37−39*).

Two of the most studied functions attributed to the PBR are its roles in steroid biosynthesis and regulation of cell proliferation. In recent years more attention has focused on

the latter function, as a growing number of neoplastic tissues are being reported to express higher levels of PBR densities than in untransformed control tissues (refs *21*, *23*, and *24* and studies reviewed in ref *5*). Moreover, several investigators have found that PBR-specific ligand treatments can also slow cell proliferation in some of these cells (*5*, *13*, *21*). None of these earlier investigators could discover whether changes in PBR density were an indirect result of cellular transformation or, in fact, were associated with the cause of this pathology. Hence, following our genetic manipulation of these Leydig cell cultures, when we first noticed evidence that suggested a change in the transformation level of the cells, we set out to further characterize and confirm our observations.

Loss of contact inhibition is a common attribute associated with cellular transformation. It can be seen in established cell cultures but is not a requirement for the continuous culture of cells in vitro. We are unaware of any reports suggesting that MA-10 Leydig cells do not show contact inhibition. Thus, on observing evidence of loss of contact inhibition in our genetically manipulated cells, but not in control cultures, the need for further transformation assays became apparent. We had previously reported that PBR subunit ratios show tissue and treatment specificity (*28*, *29*). We hypothesized that, by changing the expression of one such subunit (IBP) within the PBR complex, we could possibly induce a dysfunction in these cells. The antisense knockout approach we used proved to be an appropriate tool to perturb PBR subunit ratios in MA-10 cell cultures, as we not only changed the PBR binding densities in knockout clones but also reduced IBP protein expression and RNA levels (Figures 2 and 3).

The ability of transformed cells to grow in a soft agar matrix was also useful in the current study to demonstrate that some of the clones became more transformed upon IBP antisense knockout (Figures 4 and 5), without affecting the cells' ability to proliferate. This was extended to the in vivo survivability assay (Figure 7). It is interesting that we were not measuring primary transformation of these Leydig cells, as the MA-10 cell line is already an established cell line capable of weak growth in soft agar. It could be argued that a decrease of IBP expression may lead to dedifferentiation and tumorigenesis with a concurrent change in steroidogenesis. In MA Leydig cells, the data in the current study do not support a direct effect of IBP antisense on an increase in cell proliferation, in general, with the exception of clone MAAP29. This clone may be exceptional, as other clones, while tumorigenic (e.g., MAAP31), did not show a significant change in cell proliferation. In fact, the present data support a separation of cell proliferation and tumorigenesis for Leydig cells. Moreover, this apparent uncoupling of MA-10 cell proliferation from the tumorigenic state may represent the different embryonic origin to other tissues, where this uncoupling does not occur. It should be remembered that here there is a secondary genetic event (due to the transfection and expression of antisense IBP RNA in cells), which changes IBP expression. A parallel, more natural variant of this scenario could cause tumor progression in Leydig cell cancers in vivo (Figure 8) and would be interesting to discover. Additionally, "natural" PBR overexpression in tumors may be due to an uncoupling to a natural PBR-associated antitumor pathways within the cell. Hence, the
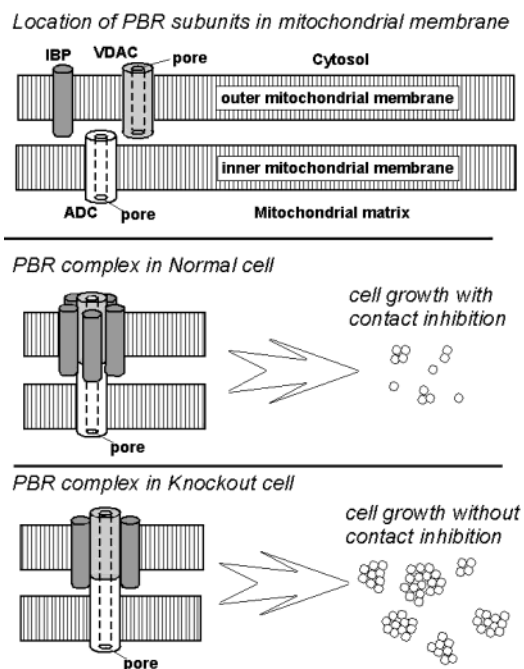

FIGURE 8: Schematic illustration of the effect of antisense knockout of the IBP subunit (short gray cylinders) within the PBR complex (all cylinders combined) on colony growth of MA-10 Leydig cells in soft agar (a measure of tumorigenesis). The reduced number of IBP in the complex may result in the more malignant growth of MA-10 cells.

downregulation of cell proliferation due to PBR ligands that we and others have found (*12*, *13*) may represent a remnant of the coupling just mentioned. Further studies are necessary to determine whether IBP expression changes can be a secondary event in different human cancers as they progress in vivo. A useful tool for this study would be cell lines containing IBP mitochondrially directed overexpression vectors.

In a more global perspective, PBR traffic could affect the function of the mitochondrial transition pore (MTP) either directly (by being apart of it) or indirectly by changing or otherwise affecting the ion gradient across the mitochondrial membrane. Hence, a homeostatic response to molecules passing through the PBR in the mitochondrial membrane would require the MTP to correct the change. If further studies substantiate this model, far-reaching consequences in health and disease could result. To explain the overexpression we and others see in PBR in different tumors and cancer cell lines, an uncoupling between the PBR and the ion gradient sensing apparatus necessary for maintaining the mitochondrial ion gradient could have occurred, the result of which could be the cell attempting to make more PBR to compensate for the nonfunctioning sensor. Furthermore, this uncoupling could result in extra ATP synthesis (through increased oxidative phosphorylation), which in turn would allow increased cell proliferation, steroid biosynthesis, and tumorigenesis to result. On antisense knockout of IBP, in MA-10 cells, a pool of less active PBR could also cause imbalance in the mitochondrial ion gradient, causing further synthesis of ATP, which in turn causes more tumorigenesis, less apoptosis, and so on.

In conclusion, we have presented evidence suggesting that IBP deregulation, or at least the perturbation of IBP expression, can lead to progression in the severity of a murine

model for testicular cancer (Leydig cell type). This event is probably not the only type of change that leads to cancer progression. Future studies are necessary to dissect and understand this type of event in human cancer.

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