Journal of Steroid Biochemistry & Molecular Biology 121 (2010) 368–371

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Tumor progression in the LPB-Tag transgenic model of prostate cancer is altered by vitamin D receptor and serum testosterone status☆

Sarah Mordan-McCombs [a,b], Theodore Brown [b], Wei-Lin Winnie Wang [a], Ann-Christin Gaupel [a], JoEllen Welsh [a], Martin Tenniswood [a,*]

[a] Cancer Research Center, Department of Biomedical Sciences, School of Public Health, State University of New York at Albany, Rensselaer, NY 12144, United States
[b] Department of Biological Sciences University of Notre Dame, Notre Dame, IN 46556, United States

A R T I C L E I N F O

Article history:
Received 28 October 2009
Received in revised form 19 March 2010
Accepted 22 March 2010

Keywords:
Vitamin D
Prostrate cancer
Androgen

A B S T R A C T

Previous studies have suggested that 1,25 dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) induces cell cycle arrest and/or apoptosis in prostate cancer cells *in vitro*, suggesting that vitamin D may be a useful adjuvant therapy for prostate cancer and a chemopreventive agent. Most epidemiological data however shows a weak link between serum $25(OH)D_3$ and risk of prostate cancer. To explore this dichotomy we have compared tumor progression in the LPB-Tag model of prostate in VDR knock out (VDRKO) and wild type (VDRWT) mice. On the C57BL/6 background LPB-Tag tumors progress significantly more rapidly in the VDRKO mice. VDRKO tumors show significantly higher levels of cell proliferation than VDRWT tumors. In mice supplemented with testosterone to restore the serum levels to the normal range, these differences in tumor progression, and proliferation are abrogated, suggesting that there is considerable cross-talk between the androgen receptor (AR) and the vitamin D axis which is reflected in significant changes in steady state mRNA levels of the AR, PCNA, cdk2 survivin and IGFR1 and 2 genes. These alterations may explain the differences between the *in vitro* data and the epidemiological studies.

© 2010 Elsevier Ltd. All rights reserved.

1. Introduction

Previous studies have shown that $1,25(OH)_2D_3$ induces cell cycle arrest [1,2] and/or apoptosis [3] in prostate cancer cells *in vitro*. These effects appear to be mediated through the effects of vitamin D on the expression of genes such as p21, cyclin A [4] and p27 [5]. *In vivo* studies with LNCaP xenograft tumors have shown that treatment with the $1,25(OH)_2D_3$ analog, EB1089, results in decreased tumor growth [6] suggesting that vitamin D axis is a potential target for chemotherapeutic intervention for early stage prostate cancer. However, unlike breast and colon cancer, epidemiological studies have generally shown a weak correlation between low circulating $25(OH)D_3$ and increased risk of prostate cancer [7,8], suggesting that vitamin D may not play a significant role in chemoprevention of prostate cancer. There have been very few studies in autochthonous models of prostate cancer examining the impact of the vitamin D axis on the initiation and progression of the disease. Since both stromal and epithelial prostate cells express the androgen receptor (AR) (NR3C4) and the vitamin D receptor (VDR) (NR1I1) [9], respond to $1,25(OH)_2D_3$ and express transcriptionally active VDR [10], these *in vivo* models may better recapitulate the initiation and progression of human prostate cancer than xenograft or *in vitro* studies. In this study, we have utilized the LPB-Tag mouse model for prostate cancer [11] crossed with a VDR knockout (VDRKO) mouse [12], to determine whether the VDR plays a significant role in tumor initiation and progression.

2. Materials and methods

2.1. Animals

Animals were bred at the Freimann Life Sciences Center at the University of Notre Dame. Males were weaned onto a high calcium rescue diet containing 20% lactose (Harlan TEKLAD TD 96348) at 3 weeks of age, and were given food and water *ad libitum*. Tumor progression was evaluated at 7, 9, 12, 15, and 18 weeks of age, essentially as previously described [13]. Where indicated, slow-release testosterone pellets (1.5 mg; Innovative Research, Sarasota, FL) were implanted subcutaneously at 5–6 weeks of age. Animals were genotyped by PCR for the LPB-Tag transgene and males were also genotyped for presence of the wild type and knockout VDR alleles Animals that were hemizygous for the LPB-Tag transgene, and homozygous for either the wild type VDR or knockout VDR were used for these studies, with non-transgenic (LPB-Tag), age-matched littermates as controls.

☆ Special issue selected article from the 14th Vitamin D Workshop held at Brugge, Belgium on October 4–8, 2009.

* Corresponding author. Tel.: +1 518 591 7231; fax: +1 518 591 7201.
E-mail address: mtenniswood@albany.edu (M. Tenniswood).

0960-0760/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jsbmb.2010.03.062

2.2. Tissue collection, processing and histological analysis

Based on preliminary studies of tumor growth rates in the C57BL/6 VDRWT LPB-Tag mice it was determined that for 80% confidence with a mean growth rate of 0.5 g per week, a minimum of 10 animals per group was necessary (GraphPad Instat), therefore eleven animals per genotype were necropsied at each time point and the urogenital complex, including bladder, seminal vesicles, and all prostatic lobes, were removed and weighed. Tissues were fixed in 4% phosphate buffered formalin for 12–16 h, paraffin-embedded, and sectioned to 5 μm. Sections were stained with hematoxylin and photographed with a Leica DM IRB light microscope and Optronics LCD camera. Two sections each from three animals per genotype per time point were rated using the mouse-modified Gleason Scale (MGS) for tumor progression (scored by two observers blinded to the genotype) as previously described [13]. Five samples per group per time point were flash frozen in liquid nitrogen for RNA isolation and analysis. Average ratings per time point were compared by ANOVA followed by appropriate post-tests (Tukey or Bonferroni) using GraphPad Prism software, $p < 0.05$.

The expression of SV40 large T-antigen and PCNA was evaluated by immunohistochemistry using PAb416 (Calbiochem, San Diego, CA) and ab29 (Abcam Inc.), respectively. TUNEL analysis was performed using the *In Situ* Cell Death Detection Kit, POD (Roche Diagnostics, Indianapolis, IN). Slides were pretreated for antigen retrieval using 10 mM sodium citrate buffer, pH 6.0 at 95 °C (PCNA, SV40 large T-antigen), counterstained with Harris hematoxylin and eosin, and developed using the Vectastain Elite ABC system with mouse IgG (Vector Labs, Burlingame, CA). TUNEL-positive and PCNA positive cells were counted and divided by the total number of cells in representative sections to determine the percent of positively stained cells. Cellularity was determined by counting the total number of cells per unit area in representative sections. Data is expressed as mean ± SE, and differences were assessed by ANOVA and determined to be significant if $p < 0.05$.

2.3. Serum testosterone

Blood samples were obtained at time of necropsy by heart puncture and testosterone levels were analyzed by ELISA (Cayman Chemical, Ann Arbor, MI). Results are expressed as mean ± SE and were analyzed by ANOVA ($p < 0.05$).

2.4. RNA isolation and real-time PCR

RNA was isolated from frozen tissues from 15-week-old animals using TRIzol Reagent (Invitrogen, Carlsbad, CA). RNA quality was assessed using an Agilent Bioanalyzer 2100. cDNA synthesis was performed using 1 μg of total RNA per 100 μL reaction. RT-PCR was run on an ABI 7900 using SYBR green (Applied Biosystems, Foster City, CA). Gene expression was normalized to 18S rRNA and fold change was analyzed using the $2^{-\Delta\Delta Ct}$ method [14]. Gene expression was assessed in five tumors from each genotype in triplicate. Due to variations in the epithelial–stromal ratio in the tumors we have used a conservative cutoff for the fold change of >3 (VDRKO vs. VDRWT) for effects to be considered significant.

3. Results

3.1. The VDR confers a protective effect against LPB-Tag tumor progression in the absence of testosterone supplementation

LPB-Tag animals that differentially express the VDR develop autochthonous prostate tumors in the dorsolateral lobes of the prostate beginning at 7 weeks of age (Fig. 1A). Based on the MGS, the LPB-Tag driven initiation and progression of the prostate tumors on

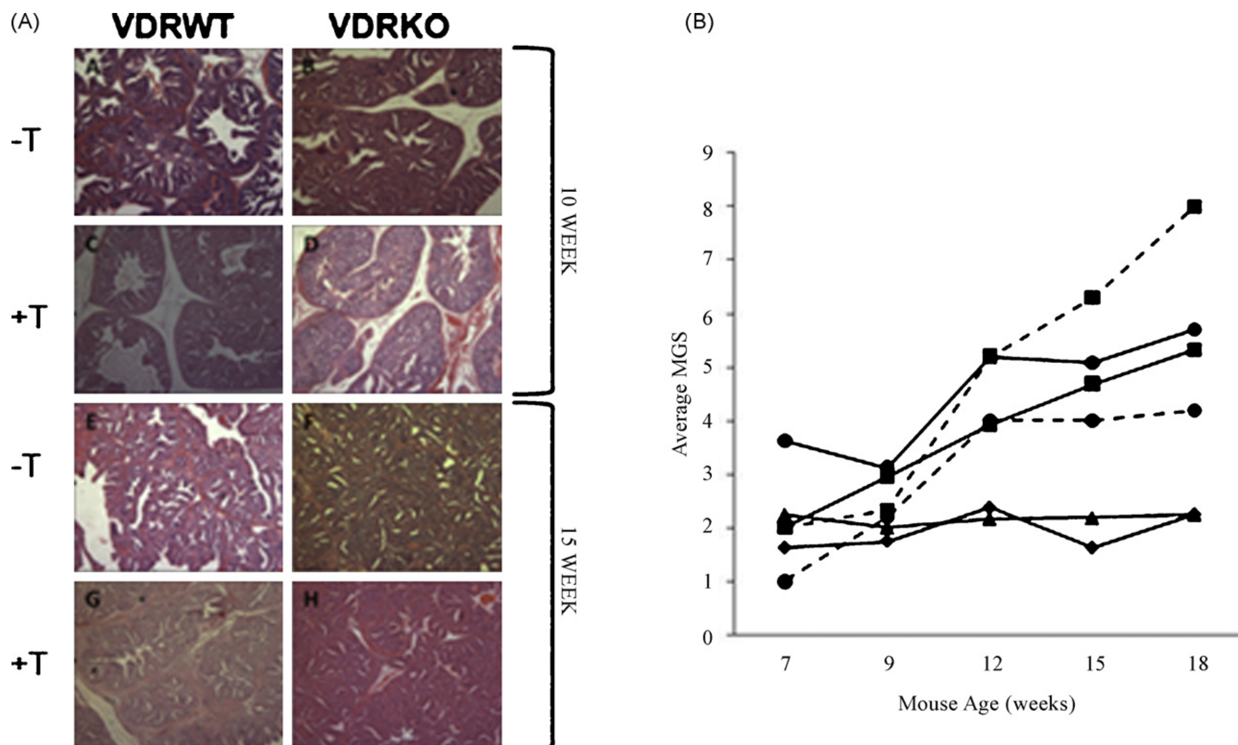

Fig. 1. Tumor progression of LPB-Tag prostate tumors in VDRWT and VDRKO animals unsupplemented and supplemented with exogenous testosterone. Sections were stained with hematoxylin and eosin (A) and progression was scored by mouse-modified Gleason Score (MGS) as described in materials and methods. Tumors initiate earlier in animals with testosterone supplementation (B). Animals expressing the VDR show inhibition of tumor progression in the absence of testosterone, but this protection is lost in the presence of exogenous testosterone [●–● VDRWT/Tag +T; ■–■ VDRKO/Tag +T; ●– · –● VDRWT/Tag −T; ■– · –■ VDRKO/Tag −T; ▲–▲VDRKO/NT +T, ♦–♦ VDRKO/NT −T].

the VDRWT and VDRKO background is not significantly different prior to 10 weeks of age (Fig. 1B). However, beginning at the 10-week time point, tumor progression diverges. VDRWT tumors reach an average MGS of 3.8, indicative of midrange tumor severity, with epithelial cell layers folding into the glandular lumen, eliminating luminal space. While tumors continue to grow, as indicated by an increasing tumor weight to body weight ratio (data not shown), they do not progress in VDRWT animals, reaching a maximal average MGS of 4.2. The stroma is abundant and remains well organized in the VDRWT tumors. In contrast, at the 10-week time point, the average MGS in VDRKO animals is already 5.0, and tumors continue to progress to a maximal average MGS of 8 at 18 weeks of age (Fig. 1) at which point tumors show little to no luminal space, and a high degree of epithelial infolding, and a smaller stromal compartment.

Due to the highly variable and lower than normal serum testosterone levels in the C57Bl/6 mice previous studies [13], serum testosterone levels were assessed. Both LPB-Tag and non-transgenic VDRWT or VDRKO animals on the C57BL/6 background showed widely variable serum testosterone levels (3.3 ± 1.8 ng/mL, n = 10 per genotype), the majority of which were in the castrate range, below the normal range of 5–15 ng/mL, although these levels were sufficient to maintain continuous expression of the SV40 large T-antigen transgene (data not shown).

3.2. In the presence of testosterone the VDR does not influence prostate tumor progression in LPB-Tag transgenic mice

To model prostate cancer progression in normal males, both VDRWT and VDRKO LPB-Tag transgenic animals were supplemented with exogenous testosterone at 5–6 weeks of age (prior to the time at which tumor initiation in unsupplemented animals), which restores the serum testosterone levels to approximately 9.9 ± 3.4 ng/mL (n = 10 per genotype), within the normal range of serum testosterone in C57BL/6 mice. These animals predictably develop autochthonous tumors of the dorsolateral prostate beginning at 7 weeks, at which time the tumors in the VDRWT animals have an average MGS of 2.0, indicative of low grade PIN while tumors in the VDRKO animals have an average MGS of 3.6, more indicative of high-grade PIN (Fig. 1B). VDRWT tumors progress steadily to an average MGS of 5.3 by 18 weeks of age; VDRKO tumors progress more rapidly reaching an average MGS of 5.2 by 12 weeks of age, however they show no further progression between 12 and 18 weeks, although the tumors continue to grow. At no point during the time course do the differences in MGS between the LPB-Tag driven tumors in VDRWT and VDRKO cells in animals supplemented with testosterone, reach statistical significance (Fig. 1B). In contrast the MGS in unsupplemented animals, studied in parallel are considerably more divergent, showing continued tumor progression in VDRKO mice compared to VDRWT.

3.3. VDR modulates cell proliferation in prostate tumors generated by SV40 large T-antigen in testosterone supplemented animals

Assessment of cell proliferation (using PCNA staining) and cell death (using TUNEL staining) has established that ablation of the VDR leads to increased proliferation in LPB-Tag prostate tumors, early in tumor progression (between 7 and 12 weeks), but not at later stages of tumor progression. PCNA labeling is almost exclusively in the epithelial compartment of the dorsolateral prostate in both VDRWT and VDRKO tumors, with minimal labeling in the stromal cells (data not shown). TUNEL labeling is also predominantly in the epithelial compartment of both VDRWT and VDRKO tumors, with negligible labeling in the stromal compartment. Quantitation of TUNEL-positive cells shows a higher percentage of TUNEL-positive cells in VDRWT tumors over VDRKO tumors, especially at later time points (Fig. 2), however the difference between these genotypes never reaches statistical significance. These data indicate that loss of the VDR results in an overall increase in cell proliferation and slight decrease in apoptosis within developing VDRKO tumors. The morphological outcome of these differences in proliferation and apoptosis is that the epithelial/stromal ratio is significantly higher in LPB-Tag driven prostate tumors in VDRKO mice than in the VDRWT mice. While this leads to increase infolding and reduced luminal volume, the increased cellularity does not significantly affect the MGS even at later time points.

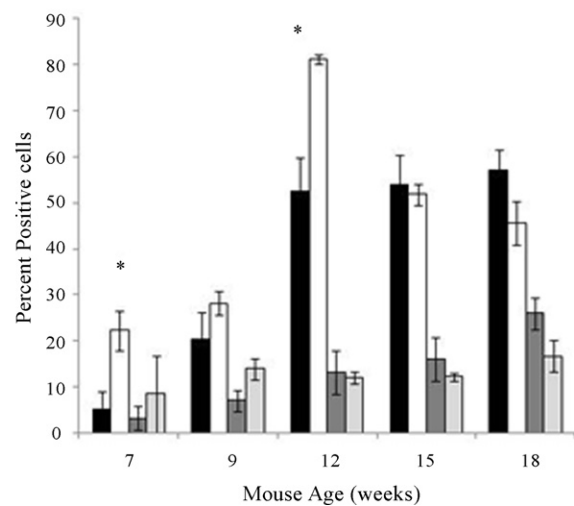

Fig. 2. Quantitation of TUNEL and PCNA staining in VDRWT and VDRKO tumors after exogenous testosterone supplementation. Sections were stained with TUNEL or PCNA and percent of positive-stained cells was quantified as described in materials and methods. Values shown are mean ± SE (n = 3, * indicates $p < 0.05$) [■, VDRWT PCNA; □, VDRKO PCNA; ■ VDRWT TUNEL; ■ VDRKO TUNEL].

3.4. VDR modulates expression of AR mRNA and expression of genes associated with cell cycle, cell death and stromal–epithelial interactions

Ablation of the VDR results in significant alterations in the expression of several genes associated with proliferation, apoptosis and stromal–epithelial interactions (Table 1). The steady state level of AR mRNA is substantially elevated in the VDRKO mice, suggesting that the VDR plays a role in the regulation of AR mRNA transcription or stability. PCNA mRNA levels are also higher in VDRKO tumors than VDRWT tumors, in agreement with the immunohistochemistry, while neither of the cell cycle inhibitors previously implicated in the regulation of proliferation of cancer cells *in vitro*, p21 and p27, showed significant change in expression level between VDRWT and VDRKO tumors. The expression of the anti-apoptotic gene survivin is 5-fold higher in VDRKO tumors over VDRWT tumors. This corre-

Table 1
Relative expression of genes regulating cell cycle, apoptosis, and stromal–epithelial signaling in VDRKO vs. VDRWT LPB-Tag prostate tumors.

Gene	Relative expression (VDRKO/VDRWT)
PCNA	7.41
Cdk2	6.35
p21	NS
p27	NS
AR	6.30
Survivin	4.86
IGF1	NS
IGF2	NS
IGFR1	4.53
IGFR2	4.72

lates well with the immunohistochemical data which shows lower rates of cell death in VDRKO tumors. Furthermore, while there is no significant difference in IGF1 or IGF2 mRNA levels between VDRKO and VDRWT tumors, the steady mRNA levels of their cognate receptors (IGFR1 and IGFR2) are substantially higher in LPB-Tag driven tumors in VDRKO mice compared to VDRWT. This may explain the increased number of epithelial cells within the VDRKO tumors, due to an enhanced ability of the epithelial cells to respond to growth signals from the surrounding stroma.

4. Discussion

4.1. The impact of the VDR on tumor morphology in LPB-Tag prostate tumors

Based on considerable *in vitro* data, it has been postulated that there is potential signaling cross-talk between the AR and VDR which may affect cellular responses to $1,25(OH)_2D_3$ [3,15,16]. The data presented here demonstrate that in animals with low serum testosterone levels, the VDR confers a protective effect and considerably slows the rate of tumor progression, resulting in a plateau in tumor progression at 12 weeks of age at an average MGS of 4.0 in VDRWT animals, while tumors in the VDRKO mice progress to an average MGS of 8.0 at 18 weeks of age. However, in testosterone supplemented animals, this protective effect is lost, and from 9 weeks of age onward the difference in average MGS between the LPB-Tag driven tumors in the VDRWT and VDRKO mice is not statistically significant. The changes in tumor progression correlate well with the changes in cell proliferation and apoptosis. The observation that ablation of the VDR results in increased proliferation and decreased apoptosis, suggests that it negatively regulates cell cycle progression and protects cells from cell death.

4.2. Loss of the VDR alters gene expression of the LPB-Tag prostate tumor in testosterone supplemented mice

In testosterone supplemented animals, ablation of the VDR results in a substantial increase in the steady state levels of several genes including survivin and cdk2 which correlates with increased cell proliferation and decreased cell death leading to tumor progression in the VDRKO LPB-Tag tumors. These genes are most likely expressed in the epithelial compartment of the tumor. The increase in the steady state levels of IGFR1 and IGFR2 in the VDRKO tumors indicates that it is probable that the enhanced survival and proliferation seen in VDRKO tumors is due to growth factor signaling from the stroma to the epithelium through the IGFR family of receptors. It has been suggested that alterations in epithelial–stromal signaling during carcinogenesis may be due to altered expression of various transcriptional coregulators [17]. VDR-mediated transcriptional activity has been shown to be altered in stromal cells isolated from human prostate tumors, and this difference has been attributed to differences in recruitment of the coregulators SRC-1, CBP, and SMRT to VDREs in carcinoma-associated fibroblasts [18], suggesting that nuclear receptor coregulators may play a mechanistic role in stromal–epithelial signaling in the VDRWT and VDRKO LPB-Tag tumors.

The majority of *in vitro* studies which have been performed to determine the efficacy of $1,25(OH)_2D_3$ as an antiproliferative agent against prostate cancer have been done under conditions where physiological concentrations of testosterone were not added to the culture media [3]. Recent studies have shown that treatment of LNCaP human prostate cancer cells with $1,25(OH)_2D_3$ results in cell cycle arrest and increased expression of the AR. The antiproliferative effects of $1,25(OH)_2D_3$ appear to be most profound in the absence of testosterone, indicating that the AR signaling pathway may interfere with VDR signaling. The studies presented here suggest that there is significant cross-talk between androgen signaling and vitamin D signaling, and that this cross-talk may have important implications in prostate cancer treatment, particularly for the use of vitamin D_3 or its analogs as adjuvant therapy. These data also demonstrate that in the presence of normal levels of testosterone (as would be the case in a chemopreventive setting), signaling through the VDR may not play a critical role in slowing tumor initiation and/or progression.

Acknowledgement

This worked was funded by an operating grant from the USPHS, National Cancer Institute (RO1 CA101114-01).

References

[1] S.H. Zhuang, K.L. Burnstein, Antiproliferative, effects of 1α,25-dihydroxyvitamin D_3 in human prostate cancer cell line LNCaP involves reduction of cyclin dependent kinase-2 activity and persistent G_1 accumulation, Endocrinology 139 (1998) 1197–1207.
[2] D.M. Peehl, R.G. Sellers, Antiproliferative effects of 1,25-dihydroxyvitamin D_3 on primary cultures of human prostatic cells, Cancer Res. 54 (1994) 805–810.
[3] S. Murthy, I.U. Agoulnik, N.L. Weigel, Androgen receptor signaling and vitamin D receptor action in prostate cancer cells, Prostate 64 (2005) 362–372.
[4] L.P. L.P.Freedman, Transcriptional targets of the vitamin D3 receptor-mediating cell cycle arrest and differentiation, J. Nutr. 129 (1999) (1999) 581S–586S.
[5] E.S. Yang, K.L. Burnstein, Vitamin D inhibits G1 to S progression in LNCaP prostate cancer cells through p27Kip1 stabilization and cdk2 mislocalization to the cytoplasm, J. Biol. Chem. 278 (2003) 46864–46868.
[6] S.E. Blutt, T.C. Polek, L.V. Stewart, M.W. Kattan, N.L. Weigel, A calcitriol analogue, EB1089, inhibits the growth of LNCaP tumors in nude mice, Cancer Res. 60 (2000) (2000) 779–782.
[7] E.A. Platz, M.F. Leitzmann, B.W. Hollis, W.C. Willett, E. Giovannucci, Plasma 1,25-dihydroxy-and 25-hydroxyvitamin D and subsequent risk of prostate cancer, Cancer Causes Control 15 (2004) 255–265.
[8] J.-E. Damber, G. Aus, Prostate cancer, Lancet 371 (2008) 1710–1721.
[9] R. Lou, I. Laaksi, H. Syvala, M. Blauer, T.L.J. Tammela, T. Ylikomi, P. Tuohimaa, 25-Hydroxyvitamin D_3 is an active hormone in human primary prostatic stromal cells, FASEB J. 18 (2004) 332–334.
[10] M. Kivineva, M. Blauer, H. Syvala, T. Tammela, P. Tuohimaa, Localization of 1,25-dihydroxyvitamin D_3 receptor (VDR) expression in human prostate, J. Steroid Biochem. Mol. Biol. 66 (1998) (1998) 121–127.
[11] S. Kasper, P.C. Sheppard, Y. Yan, N. Pettigrew, A.D. Borowsky, G.S. Prins, J.G. Dodd, M.L. Duckworth, R.J. Matusik, Development, progression and androgen-dependence of prostate tumors in probasin-large T-antigen transgenic mice: a model for prostate cancer, Lab. Invest. 78 (1998) 319–333.
[12] Y.C. Li, A.E. Pirro, M. Amling, G. Delling, R. Baron, R. Bronson, M.B. Demay, Targeted ablation of the vitamin D receptor: an animal model of vitamin D-dependent rickets type II with alopecia, Proc. Natl. Acad. Sci. U.S.A. 94 (1997) 9831–9835.
[13] S. Mordan-McCombs, T. Brown, J. Welsh, M. Tenniswood, Dietary calcium does not affect prostate tumor progression in LPB-Tag transgenic mice, J. Steroid Biochem. Mol. Biol. 103 (2007) 747–751.
[14] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) method, Methods 25 (2001) 402–408.
[15] E.S. Leman, J.A. Arlotti, R. Dhir, R. Getzenberg, Vitamin D and androgen regulation of prostatic growth, J. Cell. Biochem. 90 (2003) 138–147.
[16] B.-Y. Bao, Y.-C. Hu, H.-J. Ting, Y.-F. Lee, Androgen signaling is required for the vitamin D-mediated growth inhibition in human prostate cancer cells, Oncogene 23 (2004) 3350–3360.
[17] P. Cano, A. Godoy, R. Escamilla, R. Dhir, S.A. Onate, Stromal–epithelial cell interactions and androgen receptor-coregulator recruitment is altered in the tissue microenvironment of prostate cancer, Cancer Res. (2007) 511–519.
[18] A.A. Hidalgo, R. Paredes, V.M. Garcia, G. Flynn, C.S. Johnson, D.L. Trump, S.A. Onate, Altered VDR-mediated transcriptional activity in prostate cancer stroma, J. Steroid Biochem. Mol. Biol. 103 (2007) 731–736.