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Approaches to Understanding the Importance and Clinical Implications of Peroxisome Proliferator-Activated Receptor Gamma (PPARγ) Signaling in Prostate Cancer

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Abstract The development and maintenance of the prostate are dependent upon a complex series of interactions occurring between the epithelial and stromal tissues (Hayward and Cunha [2000]: Radiol. Clin. N. Am. 38:1–14). During the process of prostatic carcinogenesis, there are progressive changes in the interactions of the nascent tumor with its surrounding stroma and extracellular matrix. These include the development of a reactive stromal phenotype and the possible promotion, by stromal cells, of epithelial proliferation and loss of differentiated function (Hayward et al. [1996]: Ann. N. Y. Acad. Sci. 784:50–62; Grossfeld et al. [1998]: Endocr. Related Cancer 5:253–270; Rowley [1998]: Cancer Metastasis Rev. 17:411–419; Tuxhorn et al. [2002]: Clin. Cancer Res. 8:2912–2923). Many molecules play an as yet poorly defined role in establishing and maintaining a growth quiescent glandular structure in the adult. Peroxisome proliferator-activated receptor gamma (PPARγ) is a candidate regulator of prostatic epithelial differentiation and may play a role in restricting epithelial proliferation. PPARγ agonists are relatively non-toxic and have been used with limited success to treat some prostate cancer patients. We would propose that a more complete understanding of PPARγ biology, particularly in the context of appropriate stromal–epithelial and host–tumor interactions would allow for the selection of patients most likely to benefit from this line of therapy. In particular, it seems reasonable to suggest that the patients most likely to benefit may be those with relatively indolent low stage disease for whom this line of therapy could be a useful additive to watchful waiting. J. Cell. Biochem. 91: 513–527, 2004. © 2004 Wiley-Liss, Inc.

Key words: prostate cancer or prostatic carcinoma; peroxisome proliferator-activated receptor gamma (γ); arachidonic acid; cyclooxygenase; lipoxygenase; signaling pathway; chemopreventative agent and chemotherapeutic agent

Prostate cancer (PCa) is unusual among commonly occurring tumors in that the disease is commonly detected as a result of a routine blood test in otherwise asymptomatic patients. As a result, the incidence of histopathologically identifiable PCa is far higher than the incidence of clinically symptomatic disease. In the United States, organ confined PCa is usually treated

Abbreviations used: PCa, prostate cancer or prostatic carcinoma; PrEC, normal prostate epithelial cell line; COX, cyclooxygenase; LOX, lipoxygenase; PPARγ, peroxisome proliferator-activated receptor gamma (γ); GLA, γ-linileic acid; AA, arachidonic acid; PUFA, polyunsaturated fatty acid; HETE, hydroxyeicosatetraenoic acid; PG, prostaglandin; TZD, thiazolidinedione; 15d-PGJ2, 15-deoxy-Δ-^{12,14}-prostaglandin J2; PSA, prostate specific antigen; PB, probasin; PSCA, prostate stem cell antigen; PIN, prostatic intraepithelial neoplasia; UGS, urogenital sinus; UGM, urogenital mesenchymal cell.

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with surgical intervention. Prostatectomy has high levels of associated morbidity.

There is a need for treatments, preferably medical in nature with few or no side effects, for patients who present with a small focus of localized disease. The option of watchful waiting, simply following the progression of the disease until a decision is taken to undergo surgery, is of course available. Given the age range of PCa patients, the ability to add treatments likely to stop or slow progression of the disease would be of enormous use. Simply delaying progression of PCa for a limited time would dramatically reduce the disease-specific death rate.

Peroxisome proliferator-activated receptors (PPAR) are nuclear hormone receptors that regulate gene transcription in response to peroxisome proliferators and fatty acids [Yu et al., 1995]. Peroxisome proliferator-activated receptor gamma (PPARγ), one of the isoforms of the PPAR family, functions as an important regulator of cell differentiation, proliferation, and apoptosis in both stromal cells (adipocyte, macrophage, endothelium, and smooth muscle) [Tontonoz et al., 1994; Barak et al., 1999; Rosen and Spiegelman, 2001] and parenchymal epithelial cells such as breast [Yee et al., 2003], colon [Saez et al., 1998; Sarraf et al., 1998b], and prostate [Shappell et al., 2001b,d]. It is also a key mediator in cellular signaling pathways such as Akt, MAPK, TGFβ, and NFκB [Karin and Ben-Neriah, 2000; Massague et al., 2000; Hsi et al., 2002].

Synthetic PPARγ agonists have minimal toxicity and moderate anti-cancer activity in patients with a number of different tumor types. They have the potential to be tested as drugs to control the growth of localized PCa. Here we review some aspects of PPARγ biology in relation to PCa. We examine applications of some of the available models for elucidating PPARγ signaling and discuss the involvement of the arachidonic acid (AA) lipid metabolism pathways in prostate development and prostatic carcinogenesis.

PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR GAMMA (PPARγ)

Nuclear hormone receptors are ligand-dependent intracellular proteins that, following activation by the appropriate ligand, stimulate transcription of specific genes by binding to restricted DNA sequences. Peroxisome proliferators are non-genotoxic carcinogens which are purported to exert their effect on cells through their interaction with members of the peroxisome proliferator-activated receptor (PPAR) family. PPARs are members of the nuclear hormone receptor superfamily of ligand-activated transcription, steroid, and thyroid hormone receptors [Sorensen et al., 1998; Desvergne and Wahli, 1999; Debril et al., 2001; Rosen and Spiegelman, 2001]. PPARs play an important role in cellular proliferation, differentiation, adipogenesis, and inflammatory signaling. PPARs interact with a number of endogenous lipids and drugs used for the treatment of human metabolic disease. There are three distinct PPAR subtypes that are the products of different genes. These are commonly designated PPARα, PPARβ(δ), and PPARγ. Each receptor shows a differential pattern of tissue expression and is activated by structurally diverse compounds. PPARs possess a highly conserved DNA binding domain (region C) and a diverse ligand-independent activation domain (region A/B), which can confer constitutive activity on the receptor. Phosphorylation within the A/B region modulates receptor activity. Upon ligand binding, PPARs regulate gene transcription by forming obligate heterodimers with the nuclear retinoid X receptor-alpha (RXRα) and binding to defined peroxisome PPAR response element (PPRE) DNA sequences, composed of direct repeats spaced by one intervening nucleotide (DR-1) [Sorensen et al., 1998; Desvergne and Wahli, 1999; Debril et al., 2001; Rosen and Spiegelman, 2001]. The different functions of the PPAR subtypes are believed to be accomplished by a combination of differential tissue expression, ligand binding, and interactions with steroid receptor co-factors and co-repressors. Human and mouse PPARγ are 95% identical at the amino acid level [Fajas et al., 1997]. In both human and mouse, there are two isoforms of PPARγ; PPARγ1 and PPARγ2, which are encoded by the same gene via alternate transcription start sites and alternate splicing, such that the proteins differ at the amino terminus [Fajas et al., 1997]. Any functional significance of the different isoforms remains to be established. Although highly conserved between species, the N-terminus does not appear to be necessary for function [Tontonoz et al., 1994], and both isoforms are activated by similar ligands [Sorensen et al.,

1998; Desvergne and Wahli, 1999; Debril et al., 2001; Rosen and Spiegelman, 2001]. In a wide variety of examined tissues, PPARγ1 is the predominant isoform expressed [Fajas et al., 1997]. In humans and mouse, PPARγ is most highly expressed in adipose tissue and colon, although it is widely expressed in a variety of other tissues. We and others have essentially uniformly detected PPARγ mRNA in benign and malignant human prostate [Mueller et al., 2000a; Shappell et al., 2001a].

PPARγ has established roles in promoting adipocyte differentiation and in glucose homeostasis [Spiegelman and Flier, 1996; Rosen and Spiegelman, 2001]. As such, PPARγ is the target for synthetic thiazolidinedione (TZD) agonists, such as rosiglitazone (Avandia), which are used to treat a subset of patients with type II diabetes [Nolan et al., 1994; Malinowski and Bolesta, 2000; Murphy and Holder, 2000]. PPARγ is also expressed in a variety of epithelial organs, such as colon, breast, and prostate [Brockman et al., 1998; Kubota et al., 1998; Sorensen et al., 1998; Mueller et al., 1998b, 2000a; Sarraf et al., 1998a; Desvergne and Wahli, 1999; Butler et al., 2000b; Debril et al., 2001; Rosen and Spiegelman, 2001; Shappell et al., 2001a]. Most data regarding the potential function of PPARγ in these organs is from experiments using immortalized cancer cell lines [Brockman et al., 1998; Kubota et al., 1998; Sarraf et al., 1998a; Mueller et al., 1998b, 2000a; Butler et al., 2000b; Shappell et al., 2001a]. It remains to be established whether PPARγ has any normal function in these organs, although it is presumed that it contributes to the regulation of epithelial cell proliferation and differentiation. Endogenous ligands for PPARγ and the genes regulated by PPARγ in non-adipose tissues remain undefined. In addition to the fact that the drugs are well tolerated in humans, the proposed use of PPARγ agonists in the treatment of PCa is supported primarily by the observed inhibition of proliferation of PCa cell lines in vitro and xenografts in vivo [Kubota et al., 1998; Mueller et al., 2000a; Butler et al., 2000b; Shappell et al., 2001a].

Recent studies found that ligand-activated PPARγ regulated differentiation and clonal growth of several types of cancer cells, including PCa, suggesting that PPARγ could be a tumor suppressor [Hisatake et al., 2000]. PPARγ may also mediate aspects of cell cycle progression, cellular proliferation, and apoptosis, and may promote differentiation or transdifferentiation of cells [Cattley, 2003].

PPARγ IN PCA CELL LINES AND TISSUES

Western blotting analysis using two different anti-human PPARγ antibodies demonstrated that constitutive expression levels for PPARγ protein were significantly higher in the ALVA, DU-145, LNCaP, and PC3 human prostate cell lines relative to normal human prostatic epithelial cells, hPrE [Nwankwo and Robbins, 2001]. Northern blot analysis showed that LNCaP cells expressed the lowest level of PPARγ mRNA whereas DU145 cells have an intermediate level. PC3 cells express PPARγ mRNA at levels that are even higher than those seen in the colon cancer cell line Moser, which was used as a positive control. The differences observed at the RNA expression level are also present at the level of the protein by using Western blotting [Mueller et al., 2000b]. PPARγ mRNA and protein were constitutively expressed in each of four primary PCa cell strains tested by real-time RT-PCR and immunoblot analysis [Xu et al., 2003]. A number of experiments have established a link between androgenic stimulation and PPARγ levels. Exposure of the PCa epithelial cell line LNCaP to the synthetic androgen mibolerone results in the down-regulation of PPARγ mRNA. Levels of PPARγ mRNA are reduced to approximately 40% of control levels in LNCaP cells exposed to 10 nM mibolerone for 96 h. PPARγ-responsive reporter plasmids derived from human ApoA-II and muscle carnitine palmityl-transferase I genes were stimulated by the PPARγ-activating ligand Wy-14,643 in LNCaP cells [Collett et al., 2000].

At one level, these experiments demonstrate a link between the androgenic and PPARγ pathways. However, at another level they raise questions about extrapolating cell line data to tumors in vivo where a host of different signaling mechanisms are interacting in a complex multi-tissue environment. These data suggest linkages into differentiation and proliferative responses of prostatic cells, responses which are linked to androgenic stimulation in complex ways which involve the surrounding stroma and extracellular matrix and which are dependent upon the nature of the specific tissues under investigation [Gao et al., 2001].

Expression of PPARs in human PCa, prostatic intraepithelial neoplasia (PIN), benign prostatic hyperplasia (BPH), and normal prostate (NP) tissue have been examined using RT-PCR and immunohistochemical staining methods. Weak immunoreactivity of PPARγ was found in BPH and NP cases. In contrast, significant expression of immunoreactive PPARγ was described in cancer cells and in PIN [Segawa et al., 2002]. The authors suggest that PPARγ is induced in PCa, and that PPARγ ligands may mediate its anti-proliferative effect against PCa cells by promoting differentiation of the cells. In contrast, another study using Northern blot analysis to examine expression mRNA levels suggested that PPARγ is expressed in both normal and malignant tissue obtained from the same patients. mRNA levels appeared to be somewhat reduced in the tumors, compared with normal tissue [Mueller et al., 2000b]. The differences between these two studies underline the need for a rigorous examination of the localization of PPARγ expression in both benign and malignant prostate.

GENERATION OF NATURAL PPARγ LIGANDS

Activation of PPARγ signaling is intimately tied to cellular AA lipid metabolism pathways. AA is an ω-6 fatty acid whose metabolism generates free radicals that can cause cellular injury. When AA is formed or ingested (e.g., from red meat or egg yolk), it undergoes metabolism by the cyclooxygenase (COX) and lipoxygenase (LOX) enzymes. Specific metabolic derivatives of AA, such as PGJ2, PGE2, PGI2, and 5-HETE, 12-HETE, 15-HETE, are created through the actions of the enzymes COX-2, 5-LOX, 12-LOX, and 15-LOX. These compounds could be natural ligands of PPARγ. These metabolites are examples of eicosanoids that have been implicated in PCa growth and metastasis [Nie et al., 2001]. Specific eicosanoids are modulators of tumor cell interactions with certain host components within the context of cancer growth, invasion, and spread. AA metabolites and PUFA are involved in tumor proliferation and angiogenesis in human PCa [Ghosh and Myers, 1998b; Augustsson et al., 2003]. This area of research is beyond the scope of the present communication, however, the AA-related metabolism pathways are shown in Figure 1 and are discussed briefly here because of their relevance to the generation of natural ligands for PPARγ signaling.

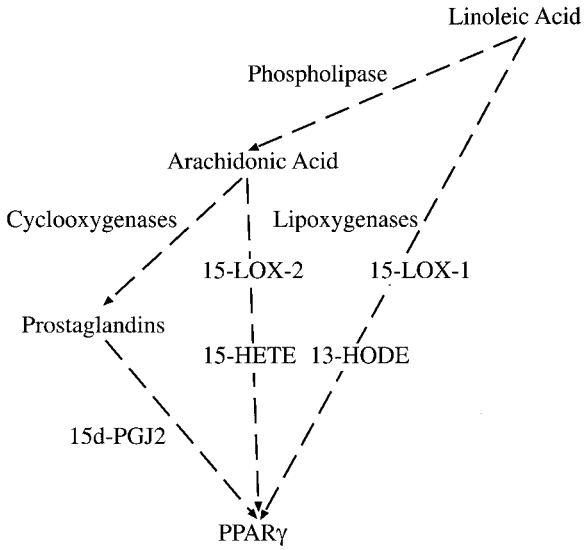

Fig. 1. Generation of PPARγ ligands by lipid metabolism. A simplified summary of the relationship between arachidonic acid metabolism and putative PPARγ ligands. Three molecules with the potential to regulate PPARγ signaling are identified. 15-deoxy-Δ-^{12,14}-prostaglandin J2 (15d-PGJ2) is formed via the cyclooxygenase pathway. 15(S)-hydroxyeicosatetraenoic acid (15-HETE) is primarily a product of the action of 15-lipoxygenase-2 on arachidonic acid. 13(S)-hydroxyoctadecadienoic acid (13-HODE) is a metabolite produced by the action of 15-lipoxygenase-1 on linoleic acid.

THE CYCLOOXYGENASE (COX) PATHWAY

COX, also known as prostaglandin (PG)-endoperoxide synthase (PTGS) or PG G/H synthase, is a key enzyme in the fatty acid metabolism pathway and the first step in the formation of PG and thromboxane (TX) from AA. COX metabolites have a wide variety of physiological and pathophysiological roles that regulate a number of homeostatic processes [Bishop-Bailey et al., 2002]. COXs and their inhibitors are the subject of intense investigation because of their potential role as anticancer agents in a number of tumors, in particular intestinal tumors [Marnett and DuBois, 2002; Saha et al., 2002; Saha and Choy, 2003; Wagenaar-Miller et al., 2003].

COX-2 expression has been reported in PCa tissue [Gupta et al., 2000; Madaan et al., 2000; Tanji et al., 2000; Yoshimura et al., 2000]. However, there is apparently no consistent overexpression of COX-2 in PCas [Shappell et al., 2001d; Zha et al., 2001]. It is still unclear to what extent and under what conditions

prostate tumors overexpress COX-2. Zha et al., described COX-2 expression in pro-inflammatory atrophic lesions in prostate, which are putative precursors of PCa. Shappell et al. also reported greater immunohistochemical staining in cancer versus benign prostate correlated with increased tumor grade (Gleason score 8 and 9 vs. 5–7) [Shappell et al., 2001d; Zha et al., 2001]. These observations suggest that COX-2 expression maybe associated with prostate carcinogenesis or PCa progression.

ARA70 is a ligand-enhanced co-activator of the androgen receptor (AR) expressed in a range of locations including adipose tissue and prostate. Heinlein et al., described a potential PGJ2-PPARγ-ARA70/AR signaling pathway in PCa. They showed that ARA70 and PPARγ specifically interact by co-immunoprecipitation. In a mammalian two-hybrid assay using DU145 PCa cells and adipocytes PPARγ and ARA70 interact in the absence of the PPARγ ligand 15-deoxy-Δ-^{12,14}-PG J2 (15d-PGJ2). The addition of 15d-PGJ2 enhanced this interaction. Co-transfection of PPARγ and ARA70 in DU145 cells induced transcription from receptor constructs driven by either three copies of an isolated PPRE or the natural promoter of the adipocyte lipid binding protein aP2, in the absence of exogenous 15-PGJ2. The PPARγ-ARA70 transactivation was enhanced by the addition of ligand [Heinlein et al., 1999].

15-d-PGJ2, is an apparently active natural ligand of PPARγ and has a strong effect on cellular S-phase arrest and non-apoptotic cell death in PC3 PCa cells [Butler et al., 2000a]. Conversely, prostaglandin E2 (PGE2) can stimulate the growth of PC-3 PCa cells [Tjandrawinata et al., 1997; Hughes-Fulford et al., 2001]. In PC3 cells, *c-fos* mRNA was found to be induced shortly after addition of AA, along with a remarkable increase in PGE2 production. This effect was blocked by the COX inhibitor, flurbiprofen. The PKA inhibitor H-89 can also abolish induction of *c-fos* expression and partially inhibit PGE2 production demonstrating that induction of *c-fos* by AA is mediated by PGE2, which activates the PKA pathway via the EP2/4 receptor [Chen and Hughes-Fulford, 2000].

THE LIPOXYGENASE (LOX) PATHWAY

LOX are a heterogeneous family of enzymes lipid peroxidizing enzymes that oxidize unsaturated fatty acids at specific positions to form active hydroperoxyl and epoxy metabolites. The LOX family is categorized with respect to their regional specificity of oxygenation and are designated as 5-, 8-, 12-, and 15-LOX isoforms, which transiently produce the end products 5(S)-, 8(S)-, 12(S)-, and 15(S)- hydroxyeicosatetraenoic acids (HETEs), respectively. Five LOX subtypes are found in humans, 5-LOX, 12-LOX, 15-LOX-1, 15-LOX-2, and 12R-LOX, the first four of which have been reported to be expressed at the mRNA, protein, and/or activity level in prostate. In a study of human PCa where 5-LOX and its metabolite 5-HETE were evaluated in malignant versus benign prostate tissue within the same patient, both 5-LOX and 5-HETE were found to be significantly overexpressed in the PCa tissue [Yang et al., 2003]. However, the function of 5-LOX in PCa development in vivo has not been determined. 5-LOX has been proposed as a potential survival factor for cultured PCa cells [Ghosh and Myers, 1998a]. The 12-LOX level was also found to correlate with the grade and stage of human prostate tumors [Gao et al., 1995]. 12-LOX was expressed in several PCa lines including DU-145 and PC-3 [Timar et al., 2000] and has been shown to regulate growth, metastasis, and angiogenesis of PCa [Pidgeon et al., 2002]. Injection of PC-3 cells over-expressing 12-LOX increased the angiogenic activity and the growth of tumors in mice [Nie et al., 1998].

The two human 15-LOXs have been shown to differ in tissue distribution and substrate preference [Hsi et al., 2001]. 15-LOX-2 has recently emerged as a potential physiologically relevant regulator of PCa development [Shappell et al., 1999, 2001d; Jack et al., 2000]. 15-LOX-2 was originally cloned from human hair rootlets [Brash et al., 1999]. The product of 15-LOX-2 activity on AA is 15S-HETE which activates PPARγ and inhibits PC3 cell proliferation in vitro [Shappell et al., 2001b]. In contrast to 15-LOX-1, 15-LOX-2 metabolized only AA to produce exclusively 15(S)-HETE. 15-LOX-2 expression is tissue-restricted being found in prostate, lung, skin, and cornea [Brash et al., 1999; Shappell et al., 2001c]. This expression pattern suggests that 15-LOX-2 may play a specific role in organs such as prostate and allows the possibility that its abnormal expression/function may contribute to tumorigenesis. This idea is given support by the observation that 15-LOX-2 expression and enzymatic

activity are decreased in PCa tissues compared to normal counterparts and that the expression levels of 15-LOX-2 are inversely correlated with the pathological grade and Gleason scores of PCa patients [Shappell et al., 1999, 2001d; Jack et al., 2000]. Reduced 15-LOX-2 expression and 15-HETE formation is the most characteristic alternation of AA metabolism in human PCa with no discernible increase in 12-HETE and 5-HETE formation.

15-LOX-1 is reported to be localized primarily in the epithelium of prostate tissue with significantly higher levels in prostate adenocarcinoma compared to normal tissue [Kelavkar et al., 2000, 2002]. In an inverse of the pattern described for 15-LOX-2, the level of 15-LOX-1 expression was strongly correlated with the degree of malignancy, as assessed by Gleason staging. Interestingly, the level of mutant p53 also correlated with the levels of 15-LOX-1 [Kelavkar et al., 2000]. In addition it was demonstrated that mutant p53 can activate the 15-LOX-1 promoter [Kelavkar and Badr, 1999]. Elevated levels of 15-LOX-1 in human PCa suggest the potential for 15-LOX-1 to act in an opposite direction to 15-LOX-2, promoting rather than suppressing tumorigenesis. It may be that there are opposing effects of 15-LOX-1 and 15-LOX-2 metabolites, respectively 13-HODE and 15-HETE. Both metabolites bind and activate PPARγ in vitro suggesting that they may function as endogenous ligands for PPARγ [Nagy et al., 1998].

The mechanisms whereby 15-LOX-2 derived 15-HETE contributes to cell functions in tissues expressing the gene or how reduced 15-LOX-2 contributes to PCa development or progression are still being elucidated. Recent data suggest that 15-HETE may be able to activate gene transcription as a ligand for the PPARγ gene [Shappell et al., 2001b; Pham et al., 2003]. Exogenous 15-HETE caused a dose-dependent inhibition of PC-3 proliferation similar to that seen in response to synthetic PPARγ agonists. Furthermore, 15-HETE activated PPARγ-dependent transcription with PPRE-containing reporter constructs and up-regulated expression of a known PPRE-containing gene. Hence, 15-HETE may constitute an endogenous ligand for PPARγ in prostate.

15-LOX-1 and 15-LOX-2 are fundamentally different enzymes with different substrate preferences, and product formation profiles [Brash et al., 1999; Kelavkar et al., 2002]. In contrast to the increasingly recognized tumor suppressive or anti-neoplastic effects of 15-LOX-2 [Tang et al., 2002; Bhatia et al., 2003], 15-LOX-1 has several described pro-neoplastic effects [Kelavkar et al., 2002]. 13-HODE produced by 15-LOX-1 potentiated EGF mediated MAPK activation and PPARγ phosphorylation in colon cancer cell lines, that was prevented by a MEK inhibitor [Hsi et al., 2001]. In PCa cell lines, the 15-LOX-2 product 15-HETE inhibited and 13-HODE increased MAPK activation and PPARγ phosphorylation [Hsi et al., 2002]. Interestingly, 15-HETE also inhibited and 13-HODE also increased Akt phosphorylation induced by IGF-1 [Hsi et al., 2002].

Activated MAP kinase (MAPK) can phosphorylate PPARγ and reduce sensitivity to PPARγ ligands thus modifying the anti-proliferative and pro-differentiating effects of PPARγ agonists in PCa cells [Hu et al., 1996]. Combination treatment in vitro with MEK inhibitors (to block phosphorylation and activation of MAPK) and PPARγ agonists restored the pro-differentiating effect of PPARγ agonists [Hu et al., 1996]. Subsequently, resistance to anti-tumor effects of PPARγ agonists in a metastasizing breast cancer cell line expressing high levels of PPARγ was shown to be due to MAPK mediated phosphorylation of PPARγ. MEK inhibitors reduced such phosphorylation and increased tumor sensitivity to PPARγ agonists [Mueller et al., 1998a]. We have made similar unpublished observations in PCa cell lines. Kelavkar et al. [2000] demonstrated that 15-LOX-1 immunostaining is increased in PCa compared to benign prostate, particularly in high grade PCas and potentially correlating with p53 mutation.

Activated MAPK phosphorylation of PPARγ has been shown to reduce sensitivity to promotion of differentiation and inhibition of proliferation by PPARγ agonists in pre-adipocytes and carcinoma cell lines [Hu et al., 1996; Mueller et al., 1998a]. By immunoblotting for phosphorylated (activated) and total MAPK variable MAPK activation was seen in primary cultures of clinically organ confined PCa [Xu et al., 2003]. Additive inhibition of proliferation of PC3 PCa cells was reported with combined treatment with a MEK inhibitor (reducing MAPK activation) and rosiglitazone [Sebolt-Leopold et al., 1999].

There are some problems associated with examining AA-metabolizing enzymes in trans-

genic mouse models in that there is not an exact correlation between the LOX enzymes found in humans and those found in mice. For example the mouse correlate of human 15-LOX-2 is 8-LOX. However, efforts have been made to examine these pathways in the LPB-Tag (LADY) model, in which a prostate epithelial-specific long probasin promoter drives expression of the SV40 large T antigen [Kasper et al., 1998]. These lines of mice (details depending upon strain) develop intensive HGPIN and invasive and metastatic carcinoma with neuroendocrine (NE) differentiation [Masumori et al., 2001; Shappell et al., 2003]. Expression of 12/15-LOX was increased in HGPIN and invasive carcinoma of the LPB-Tag transgenic mouse model similar to the increase in 15-LOX-1 seen in high-grade human PCa. The LPB-Tag model also shows increased COX-2 in HGPIN [Shappell et al., 2003]. Similar to the invasive PCa tumor in the intact LPB-Tag mouse, the NE-10 allograft tumor (derived from the 12T-10 LADY model) shows overexpression of the 12/15-LOX homologue of human 15-LOX-1 (Shappell, unpublished). Consistent with the hypothesis that 15-LOX-1 may potentiate EGF-mediated MAPK activation, Western blots for phosphorylated (activated) and total MAPK in the allograft tumor showed that MAPK is activated (Shappell, unpublished). In anticipation of possible future combination therapy strategies, treatment of nude mice with this allograft by combination therapy of an EGF receptor tyrosine kinase inhibitor and Avandia have shown a reduction in MAPK activation and PPARγ phosphorylation (Shappell, unpublished). Whether this will restore sensitivity of tumor cells to PPARγ agonists and demonstrate additive beneficial effect remains to be established.

A model linking LOX-driven AA metabolism, PPARγ signaling, and the process of carcinogenesis is presented in Figure 2. This is an attempt to rationalize the various observed effects with the role which may naturally be played by PPARγ ligands in maintaining a benign histology and growth quiescent prostate in the normal adult and the way in which these interactions may be perturbed in the process of carcinogenesis. One of the issues which presents itself in such a model is the idea that control of differentiation requires contributions from both epithelial and stromal components of the organ. Therefore therapies aimed at maintaining differentiation would probably prove most efficacious if applied early in the disease process.

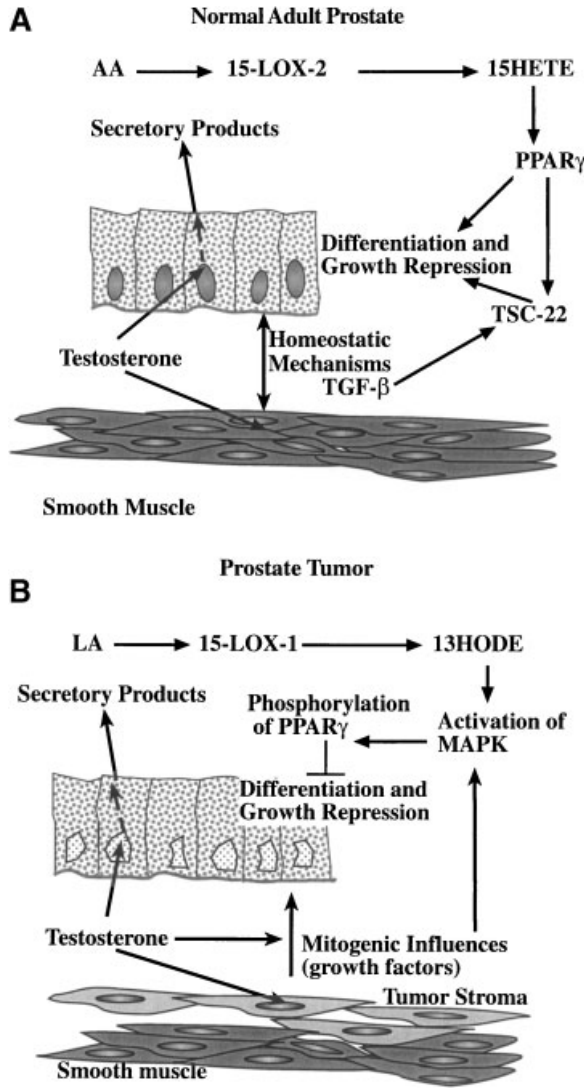

Fig. 2. A model linking lipid metabolism, PPARγ signaling, and the process of carcinogenesis. In the normal prostate (**A**) the predominant balance of lipid metabolism results in the production of 15HETE which acts, possibly in combination with stromal factors to suppress epithelial proliferation and promote differentiation, at least in part via PPARγ signaling. In contrast in prostate cancer (**B**) there is increased production of 13HODE which may act, via the activation of MAPK to phosphorylate PPARγ and suppress its antiproliferative and pro-differentiative function.

SYNTHETIC (PHARMACOLOGICAL) PPARγ LIGANDS

TZD are a class of synthetic PPARγ agonists including troglitazone, rosiglitazone, and pioglitazone that are used clinically primarily to

treat type II diabetes. These agents inhibit the proliferation of human PCa cells in vitro [Smith and Kantoff, 2002]. Pioglitazone and 15d-PGJ2 down-regulated androgen-stimulated PSA reporter gene activity in LNCaP cells using a PSA promoter/enhancer reporter assay. This inhibition of promoter activity, mediated primarily through the androgen response elements (AREs) without suppression of AR expression, may decrease expression of other androgen-responsive genes [Hisatake et al., 2000]. Rosiglitazone was used to investigate responses of primary cultures of human PCa cells to PPARγ agonists. It caused dose- and time-dependent growth inhibition that was associated with increased expression of TSC-22, and markedly increased expression of adipophilin. Other genes (aFABP, NGAL, GyK, and β-catenin) were not seen to be regulated in this study. Upregulation of adipophilin coincided with morphological changes and the appearance of cytoplasmic vacuoles with ultrastructural features of secondary lysosomes [Xu et al., 2003]. n-6 polyunsaturated fatty acids (PUFA) and γ-linolenic acid (GLA) supplementation did not affect PPARγ protein expression in malignant prostatic cells (ALVA, DU-145, LNCaP, and PC3) but caused three to sixfold increases in NP epithelial cells (PrEC) by Western blotting using two different anti-human PPARγ antibodies [Nwankwo and Robbins, 2001]. Diclofenac, a commercial antagonist of PPARγ, can be used to release DU145 PCa cell line from rosiglitazone-induced proliferation arrest [Adamson et al., 2002].

NEW EXPERIMENTAL STRATEGIES FOR ELUCIDATING PPARγ FUNCTIONS IN THE PROSTATE

As described above, there are many limitations associated with the use of cell lines to examine the biology of complex pathways. In an in vivo situation, epithelial cells are constantly interacting with the surrounding extracellular matrix and with adjacent stromal cell populations. There is also an ongoing dialog with the overall hormonal and immunological milieu of a living host. These interactions, in particular the constant cross-talk between epithelial and stromal cells are critical in determining differentiative and proliferative responses in the epithelial cells. All of these interactions are lost in cultured cell lines.

The need for model systems which accurately reflect the biological functions of PPARγ in an intact prostate has necessitated the development of new in vivo models. Approaches include the use of transgenic mice, conditional gene knockouts, and xenografting techniques. Future applications will include the specific genetic modification of epithelial and stromal components in tissue recombination models using retroviral introduction of genes and siRNA sequences under the control of constitutively active, conditional, or regulatable promoters. This approach will allow a more comprehensive examination of specific pathways in an in vivo environment.

CONDITIONAL GENE KNOCKOUT

One of the most direct approaches to determine the function of a specific gene product is simply to delete the gene. While this approach has proved successful for PPARα [Gonzalez, 1997], the deletion of the PPARγ gene is embryonic lethal due to the requirement for this gene product in the development of both placental and cardiac tissue [Barak et al., 1999]. Since direct gene deletion is impractical, conditional knockouts using the *Cre*-recombinase system to specifically target PPARγ in the luminal epithelial cells of the mouse prostate have been developed. Prostate specific probasin promoters have been used to drive expression of *Cre*-recombinase by a number of groups providing a series of new prostate-specific tools [Maddison et al., 2000; Wu et al., 2001; Jin et al., 2003].

To elucidate the biological functions of the PPARγ gene in prostate, we have crossed the ARR_2PB-*Cre* transgenic mouse line with a floxed PPARγ mouse line [Jones et al., 2002] (kindly provided by Dr. Mark Magnuson). In these animals, *Cre*-recombinase should be expressed in the luminal cells of the mouse prostate resulting in excision of the exon 2 of the PPARγ gene. Preliminary data in these animals show lesions consistent with low grade PIN in the mouse ventral prostate at three months of age and with high grade PIN with local invasion in same lobe at 7 months of age (Fig. 3).

XENOGRAFTING

An effective way to investigate the effects of biologically active compounds on human tissues in an in vivo environment is the use of

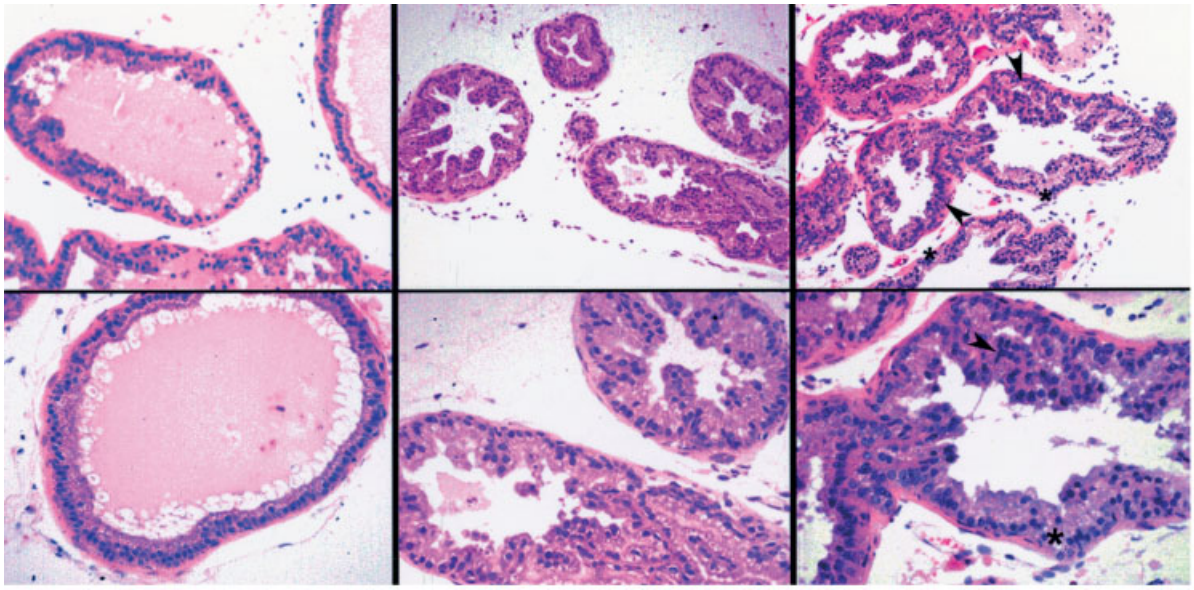

Fig. 3. Histology of prostate selective PPARγ-knockout mouse. **Top**: low mag. **Bottom**: high mag. of ventral prostate of wild type mouse (**left**), 3 month old $Cre^{tg/0}/PPAR\gamma^{flox/flox}$ (**center**), and 7 month old (**right**) $Cre^{tg/0}/PPAR\gamma^{flox/flox}$ mouse. Focal epithelial proliferation is noted at 3 months and is well developed and accompanied by cytologic atypia at 7 months (arrowheads), compatible with mouse PIN. Note residual normal epithelium (*). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

xenografting. Specifically in this context the use of cell lines as a xenograft model are ineffective as these do not recapitulate the stromal–epithelial interactions found in normal organs. In contrast, the grafting of tissue fragments containing both epithelial and stromal tissue layers allows for a more comprehensive analysis of the effects of agents on tissues in vivo. This technique has been applied to both transition zone (TZ, site of origin of BPH) and peripheral zone (PZ, site of origin of most PCas) tissues. Human TZ tissue obtained from TURPs was grafted beneath the renal capsule of intact male athymic mice [Staack et al., 2003]. An alternate application of this technique is its use to support the growth of human PCa tissue.

Patient-matched benign prostate tissues have been implanted into castrated SCID mice treated with testosterone or testosterone plus the PPARγ agonist, rosiglitazone, the results of the studies are summarized in Figure 4. Following histological analysis performed in a blinded fashion utilizing an objective scoring system, androgen deprivation of benign prostate tissue in castrated mice led to a stereotypic atrophy with loss of secretory differentiation in 100% of samples. Testosterone administration to castrated animals restored a secretory phenotype

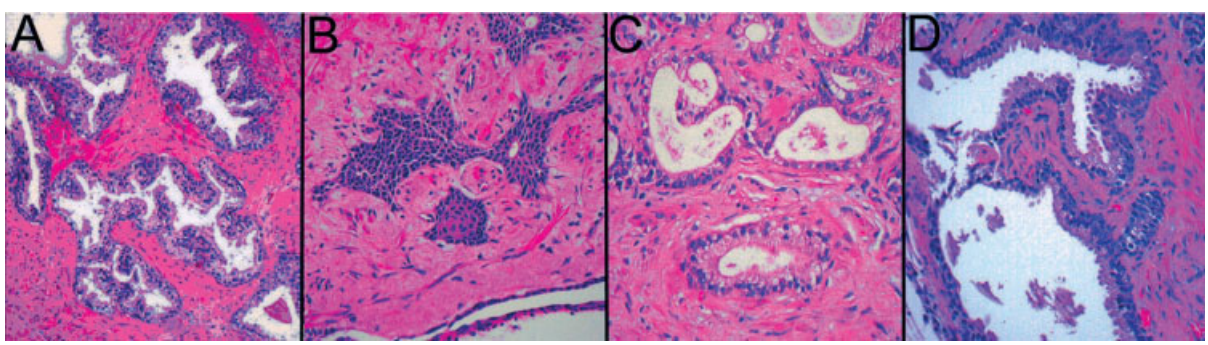

Fig. 4. Benign human prostate implanted in SCID mouse renal capsule. **A**: Prior to implantation. **B**: After 4 weeks in castrated mouse, showing marked atrophy, basal cell hyperplasia, transitional metaplasia. **C**: Same patient tissue in castrated mouse treated with testosterone (T) showing maintained secretory differentiation; **D**: same patient tissue in castrated mouse treated with T and rosiglitazone, showing greater secretory differentiation. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

to the grafted human tissue. Human benign prostate grafts in animals treated with rosiglitazone and testosterone showed more uniform and complete restoration of the secretory phenotype. Many of these grafted tissues appeared histologically identical to the tissues prior to implantation. Electron microscopy of tissues from these mice confirmed the secretory phenotype in these cells with compact apical secretory vacuoles and extracellular granules consistent with prostasomes, as seen in normal intact prostate [Carlsson et al., 2000; Kravets et al., 2000].

These studies demonstrate that in this xenograft model changes in prostatic epithelial cell secretory differentiation can be additively modulated by androgens and PPARγ agonists. They also demonstrate the development of terminal secretory differentiation that has not been achieved by stimulation of cultured prostate cells with PPARγ agonists indicating the likelihood of the identification of novel PPARγ-regulated genes compared to use of cell lines alone.

CLINICAL IMPLICATIONS AND APPLICATIONS OF PPARγ AGONISTS

PPARγ is the target for synthetic TZD agonists, such as troglitazone and rosiglitazone (Avandia), which are used to treat certain patients with type II diabetes [Nolan et al., 1994; Malinowski and Bolesta, 2000; Murphy and Holder, 2000]. Troglitazone has now been withdrawn from the market, because of rare hepatotoxicity, although clinical trial data with this drug are available. Rosiglitazone (Avandia) is more potent and appears devoid of such potential toxicity [Malinowski and Bolesta, 2000]. Such agonists are emerging as a useful candidates for therapeutic intervention in human prostate carcinoma (PCa). The practiced and proposed use of PPARγ agonists in the treatment of PCa are supported primarily by the observed inhibition of proliferation of PCa cell lines in vitro and in vivo.

Only a modest response has been seen with PPARγ agonists in patients with advanced hormone sensitive and hormone refractory PCa [Kubota et al., 1998; Butler et al., 2000b; Mueller et al., 2000a; Shappell et al., 2001a]. A small phase II clinical trial was reported with the TZD PPARγ agonist troglitazone (800 mg/day) in patients with advanced PCa [Mueller et al., 2000a]. With a median duration of treatment of 26.8 weeks, troglitazone achieved PSA reduction of >50% in 3 of 12 patients with androgen-dependent PCa. With a median duration of treatment of 14 weeks, PSA reduction of >50% was achieved in only 4 of 29 patients with androgen-insensitive PCa [Mueller et al., 2000a]. Troglitazone (600–800 mg/day) has been shown to inhibit AR mediated transcription of the PSA gene (by an effect at the ARE in the PSA promoter) [Hisatake et al., 2000]. As such, there could be concern that reduced PSA may not reflect a true clinical response. Further validation of PSA as a surrogate end-point for clinical response in PPARγ agonist trials in PCa is needed. In addition, longer time courses of PPARγ agonist treatment may be needed to see real responses in patients. Identification of molecular correlates of responsiveness in patients with more appropriate earlier PCa stages will also be important for these studies to proceed.

Recently, clinical trials with PPARγ agonists have been initiated in otherwise unselected patients at high risk for progression following definitive therapy for clinically localized PCa [Hisatake et al., 2000; Mueller et al., 2000b]. Although these trials are relatively easy to design and execute, they may not capture the patient subsets with organ confined PCa or PCa precursor lesions that are most likely to clearly benefit from treatment with synthetic PPARγ agonists. Furthermore, they do not incorporate recently described molecular alterations in PCa that may post-translationally modify PPARγ or otherwise interact with PPARγ-signaling in a manner that may alter molecular and clinical responsiveness to PPARγ agonists. Molecularly characterizing tumors in patients that respond to such treatment and developing novel strategies for prospectively identifying patients whose tumors will respond will allow optimization of targeting this promising therapeutic pathway in PCa, including in future combination therapy strategies. Collective information on PPARγ in PCa from basic experiments and clinical trials support the hypothesis that patients with organ-confined PCa or HGPIN may be candidates for future treatment with potentially pro-differentiating PPARγ agonists.

It is currently not possible to accurately predict pre-operatively which patients with biopsy proven PCa have potentially "clinically insignificant" PCa and hence may be candidates

for "watchful-waiting" or future pro-differentiating pharmacological therapy, such as with PPARγ agonists (vs. the current standards of radical prostatectomy or radiation therapy). However, strategies for preoperatively classifying PCa patients are rapidly improving, such that future pharmacological trials for patients with organ confined, low risk PCa will require rigid proof that synthetic PPARγ agonists can effectively penetrate the prostate following oral administration and modulate gene expression in the prostate in a manner translatable to a beneficial effect on organ confined tumors. We hypothesize that such a "proof of principle" trial, in which PPARγ agonists are administered to patients between the time of biopsy PCa diagnosis and radical prostatectomy (RP), will also allow for assessment of potential effects on tumor biology and correlation with genetic and molecular characteristics of the tumor that may accompany such responsiveness.

As described above, new models are starting to allow us to examine the biology of PPARγ in models that more accurately reflect the situation occurring in patients. Such approaches will allow for more rational design of clinical trials and will increase the likelihood of their success. In particular, we believe that we need an understanding and appreciation of the biomarkers which will predict and measure response to PPARγ agonists. Monitoring the modulation of gene expression makes particular biologic sense for documenting efficacy of drugs that activate transcription factors, such as PPARγ.

Even patients with clinically significant organ confined tumors may become candidates for primary pharmacological therapy as truly effective agents that can inhibit progression of PCa become available, moderating PCa progression so that it does not have the potential to cause morbidity or mortality during expected lifetime. Molecularly characterizing tumors that will respond to PPARγ agonists as monotherapy will allow future treatment of select patients with low risk organ confined PCas as an alternative to surgery or radiotherapy, reducing costs and especially treatment associated morbidity. Equally important, molecularly identifying patients that will not respond to such monotherapy will prevent future ineffective treatment in non-selective trials, and elucidating mechanisms of reduced sensitivity to PPARγ agonists will set the stage for future combination therapy incorporating strategies to restore responsiveness to PPARγ agonists.

Molecular profiling of responsive tumors will allow for possible future effective primary pharmacological treatment of defined subsets of such patients with PPARγ agonists. Approximately 30–40% of patients with clinically organ confined PCa who undergo RP will show biochemical progression within 2 years [Epstein et al., 1996] and are at risk for progression to systemic metastatic disease and PCa-related mortality. Effective pharmacological treatment of patients at risk for progression following intended definitive treatment may substantially reduce mortality from PCa. However, successful application of such treatment strategies requires protocols to identify patients who will respond to specific agents, such as PPARγ agonists.

CONCLUSIONS

The focus of the present communication is PPARγ as a regulator of prostate cell differentiation and proliferation. A number of themes have emerged. It is clear that we need a better understanding of the biology of PPARγ and its ligands and downstream effects. This will require the use of models which recapitulate the biology of tumors in vivo. It would be foolish to think of differentiation and inhibition of proliferation as being controlled by a single factor. Many factors are involved in the development and differentiation of the prostate (see Marker et al. [2003] for a recent review) and many factors are likely also involved in control of growth in the adult gland. The molecular pathways involved in growth and differentiation intersect on many levels. For example, TGFβ signaling interacts with downstream signaling pathways activated by PPARγ [Gupta et al., 2003]. This suggests that TGFβ ligands are reasonable contenders to play a cooperating role with PPARγ in maintaining prostatic differentiation. To reinforce the level of complexity it is also known that TGFβ 1, 2, and 3 mRNA levels change during postnatal development of the prostate, and may also be regulated by growth factors such as FGF-7 [Itoh et al., 1998]. It also appears that TGFβ may itself regulate expression of factors such as FGF-10 (Axel Thomson, personal communication). Thus, it is apparent that there are many levels of control of prostatic growth and differentiation.

To examine the process of cancer progression models that incorporate hormonally responsive intact epithelial–stromal interactions represent a tremendous advance over cell lines, especially in early testing of agents that promote differentiation. Inhibition of tumor cell proliferation and/or promotion of apoptosis and/or inhibition of angiogenesis in actual tissue specimens are more rational parameters to assess with PPARγ agonists for relatively short term trials in patients with clinically organ confined PCa than tumor shrinkage. Such parameters will likely correlate better with long term efficacy of cytostatic therapy in low risk patients with clinically organ confined PCa than they do for treatment responsiveness assessed as tumor shrinkage or survival with cytotoxic agents in patients with more advanced solid tumors. Use of a new models, such as xenografts and tissue recombination models, in which experimental conditions can be well controlled, and in which tumor response determined in tissue can be directly correlated with molecular and genetic alterations will be a powerful tool to identify relevant tumor progression and response markers. The intact prostate tumor tissue xenograft model also allows for combination treatments strategies, including androgen manipulation or for novel agents to be employed based on biochemical and genetic data for pathways modulating PPARγ-agonist responsiveness. Importantly, such approaches can also be applied to other clinically relevant treatments in PCa in the future, and should provide the PCa research community with a valuable tool for pre-clinical testing of new molecularly targeted agents.

PPARγ agonists may well find a place as adjuvants to watchful waiting in selected subpopulations of patients with localized PCa. The next major research challenge in this area is to identify the molecular profiles which will allow us to specifically identify the patients to be treated with these agents.

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