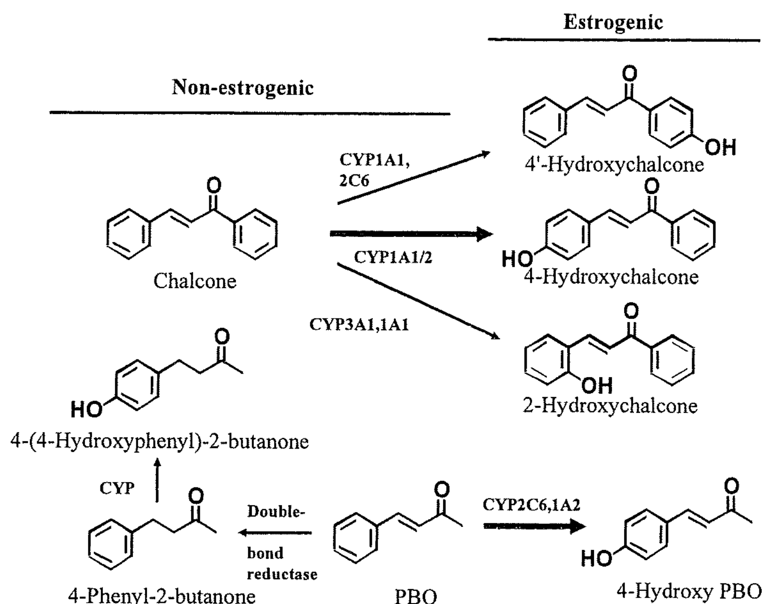

Fig. 8. Metabolic Pathways for the Activation of Chalcone and PBO to Estrogens by the Cytochrome P450 System

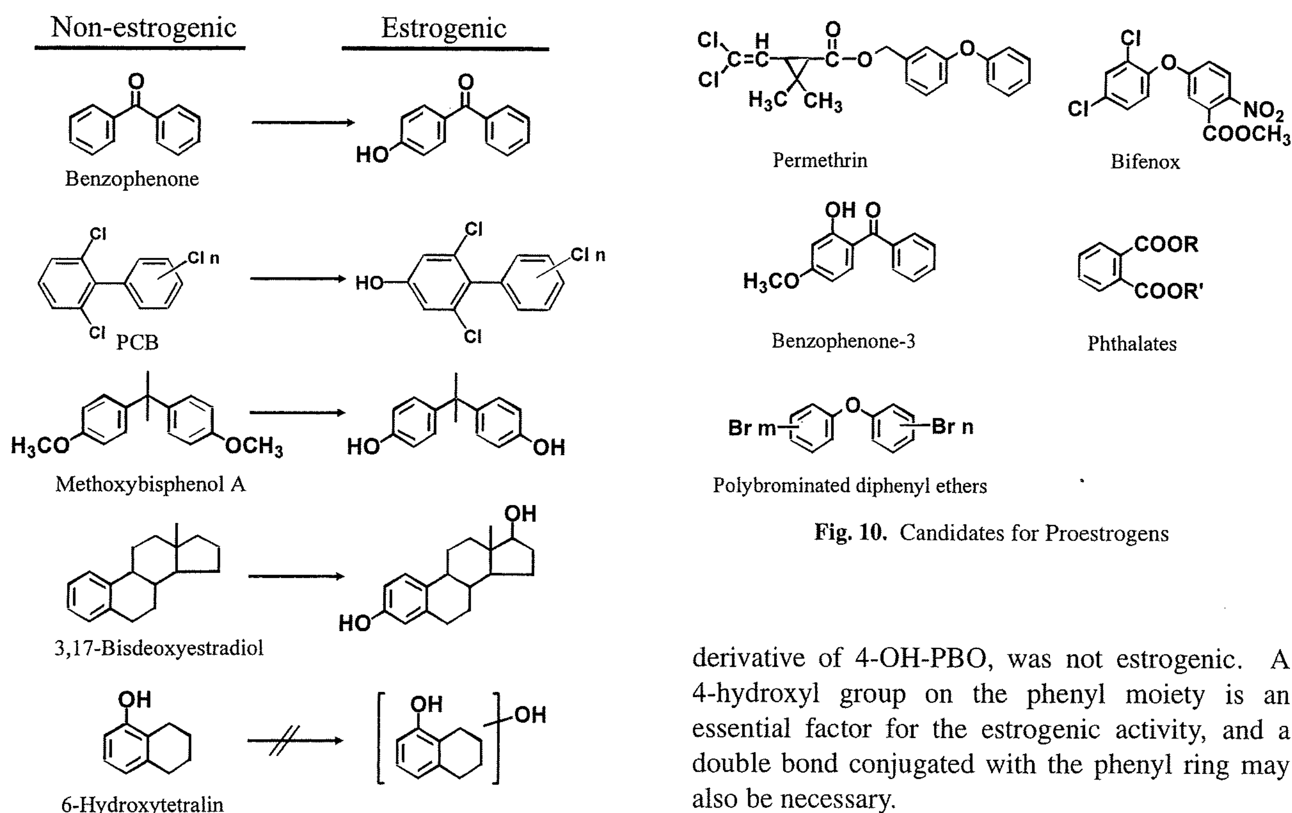

Fig. 9. Metabolic Activation of Other Proestrogens to Estrogens by the Cytochrome P450 System

Fig. 10. Candidates for Proestrogens

microsomal enzyme system, and 4-OH-PBO, which is a major metabolite in the microsomal system, exhibited estrogenic activity (Fig. 8).[16] 4-Hydroxyphenyl-2-butanone, a double-bond-reduced derivative of 4-OH-PBO, was not estrogenic. A 4-hydroxyl group on the phenyl moiety is an essential factor for the estrogenic activity, and a double bond conjugated with the phenyl ring may also be necessary.

ACTIVATION OF OTHER PROESTROGENS IN ENVIRONMENTS BY THE CYTOCHROME P450 SYSTEM

There are other examples of metabolic activation of proestrogens to estrogens, besides

the above examples. Various pesticides might be converted to active estrogens by microsomal oxidase systems, though Sumida *et al.* showed that perimethrine was not metabolically activated.[79] *p*-Hydroxybenzophenone, which is formed from benzophenone, an antifungal agent, in rat hepatocytes, is also estrogenic.[80,81] It is also reported that anethole, a flavor agent, is not estrogenic, but 4-hydroxy-1-propenylbenzene, the desmethylated metabolite of anethole, exhibited estrogenic activity.[15] Some hydroxylated polychlorinated diphenyls (PCBs), which are metabolites of PCB, show estrogenic activity.[82–84] In the case of PCB, the presence of adjacent chloride substituents decreases the estrogenic activity.[85] A catechol-type metabolite was also shown to have estrogenic activity.[32] Elsby *et al.* predicted estrogenicity by a two-stage approach, using a human liver microsome assay and a yeast estrogenicity assay.[14,86] Methoxychlor, methoxybisphenol A and 3,17-bisdesoxyestradiol were positive, but 6-hydroxytetralin, a degreasing agent, was negative in this screening system (Fig. 9).

Other environmental compounds may be proestrogenic. Candidate proestrogens are illustrated in Fig. 10. They include pyrethroids,[87] diphenyl ether herbicides (bifenox), polybrominated diphenyl ethers, a flame-retardant, and some benzophenone sunscreens. Some insecticides and medicines are also possible proestrogens. Hydroxylated derivatives of sunscreen,[88] and phthalate esters[89] show positive in estrogenicity tests. Thus, the parent compounds may be proestrogens. There may be a variety of other potentially hazardous proestrogens in our environment, too.

CONCLUSION

We have reviewed environmental proestrogens. The estrogenic activity of *trans*-stilbene in rats *in vivo* seems to be a typical example of the metabolic activation of a proestrogen.[24] It is clearly necessary to consider the activity of metabolites produced from the parent compounds for the assessment of the toxicity of environmental chemicals.

There are also pro-antiandrogen and pro-antithyroid hormonal chemicals.[85,90] It is therefore necessary, when assessing the potential *in vivo* endocrine-disrupting action of chemicals, to take into account the activities of all the metabolites produced from the parent compounds. For example, bisphenol A, a typical xenoestrogen, is further activated, when it is incubated with rat S-9 mix.[91] In this case, dimer-type metabolites, which show higher activity than bisphenol A itself, are formed. Such further activation of xenoestrogens must also also considered in the risk assessment of xenoestrogens. Much further work is needed to identify potentially hazardous proestrogens in our environment.

For the activation of proestrogen to estrogen, it is necessary to introduce a hydroxyl group, often at the 4-position of an aromatic ring, in the absence of bulky groups at the adjacent 3,5-positions. When formation of a phenolic hydroxyl group is possible after aromatic ring hydroxylation or dealkylation of chemicals, we should consider the possibility of metabolic activation to estrogens.

REFERENCES

1) Colborn, T. (1995) Environmental estrogens: Health implications for humans and wildlife. *Environ. Health Perspect.*, **103**, 135–136.
2) Andersen, H. R., Andersson, A. -M., Arnold, S. F., Autrup, H., Barfoed, M., Beresford, N. A., Bjerregaard, P., Christiansen, L. B., Gissel, B., Hummel, R., Jørgensen, E. B., Korsgaard, B., Le Guevel, R., Leffers, H., McLachlan, J., Møller, A., Nielsen, J. B., Olea, N., Oles-Karasko, A., Pakdel, F., Pedersen, K., Perez, P., Skakkeboek, N. E., Sonnenschein, C., Soto, A. M., Sumpter, J. P., Thorpe, S. M. and Grandjean, P. (1999) Comparison of short-term estrogenicity tests for identification of hormone-disrupting chemicals. *Environ. Health Perspect.*, **107**(suppl.1), 89–108.
3) Anstead, G. M., Carlson, K. E. and Katzenellenbogen, J. A. (1997) The estradiol pharmacophore: Ligand structure-estrogen receptor binding affinity relationships and a model for the receptor binding site. *Steroids*, **62**, 268–303.
4) Fang, H., Tong, W., Perkins, R., Soto, A. M., Prechtl, N. V. and Sheehan, D. M. (2000) Quantitative comparisons of *in vitro* assay for estrogenic activities. *Environ. Health Perspect.*, **108**, 723–729.
5) Shi, L. M., Fang, H., Tong, W., Wu, J., Perkins, R., Blair, R. M., Branham, W. S., Dial, S. L., Moland, C. L. and Sheehan, D. M. (2001) QSAR models using a large diverse set of estrogens. *J. Chem. Inf. Comput. Sci.*, **41**, 186–195.
6) Hong, H., Tong, W., Fang, H., Shi, L., Xie, Q.,

Wu, J., Perkins, R., Walker, J. D., Branham, W. and Sheehan, D. M. (2002) Prediction of estrogen receptor binding for 58,000 chemicals using an integrated system of a tree-based model with structural alerts. *Environ. Health Perspect.*, **110**, 29–36.
7) Nishihara, T., Nishikawa, J., Kanayama, T., Dakeyama, F., Saito, K., Imagawa, M., Takatorim, S., Kitagawam, Y., Horim, S. and Utsumi, H. (2000) Estrogenic activities of 517 chemicals by yeast two-hybrid assay. *J. Health Sci.*, **46**, 282–298.
8) Blair, R. M., Fang, H., Branham, W. S., Hass, B. S., Dial, S. L., Moland, C. L., Tong, W., Shi, L., Perkins, R. and Sheehan, D. M. (2000) The estrogen receptor relative binding affinities of 188 natural and xenochemicals: Structural diversity of ligands. *Toxicol. Sci.*, **54**, 138–153.
9) Sugihara, K., Kitamura, S., Sanoh, S., Ohta, S., Fujimoto, N., Maruyama, S. and Ito, A. (2000) Metabolic activation of the proestrogens trans-stilbene and *trans*-stilbene oxide by rat liver microsomes. *Toxicol. Appl. Pharmacol.*, **167**, 46–54.
10) Kitamura, S., Sanoh, S., Kohta, R., Suzuki, T., Sugihara, K., Fujimoto, N. and Ohta, S. (2003) Metabolic activation of proestrogenic diphenyl and related compounds by rat liver microsomes. *J. Health Sci.*, **49**, 298–310.
11) Kitamura, S., Ohmegi, M., Sanoh, S., Sugihara, K., Yoshihara, S., Fujimoto, N. and Ohta, S. (2003) Estrogenic activity of styrene oligomers after metabolic activation by rat liver microsomes. *Environ. Health Perspect.*, **111**, 329–334.
12) Fujimoto, T., Kitamura, S., Sanoh, S., Sugihara, K., Yoshihara, S., Fujimoto, N. and Ohta, S. (2003) Estrogenic activity of an environmental pollutant, 2-nitrofluorene, after metabolic activation by rat liver microsomes. *Biochem. Biophys. Res. Commun.*, **303**, 419–426.
13) Kupfer, D. and Bulger, W. H. (1987) Metabolic activation of pesticides with proestrogenic activity. *Fed. Proc.*, **46**, 1864–1869.
14) Elsby, R., Maggs, J. L., Ashby, J., Paton, D., Sumpter, J. P. and Park, B. K. (2001) Assessment of the effects of metabolism on the estrogenic activity of xenoestrogens: A two-stage approach coupling human liver microsomes and a yeast estrogenicity assay. *J. Pharmacol. Exp. Ther.*, **296**, 329–337.
15) Nakagawa, Y. and Tayama, K. (2003) Cytotoxic and xenoestrogenic effects via biotransformation of *trans*-anethole on isolated rat hepatocytes and cultured MCF-7 human breast cancer cells. *Biochem. Pharmacol.*, **66**, 63–73.
16) Kohno, Y., Kitamura, S., Sanoh, S., Sugihara, K., Fujimoto, N. and Ohta, S. (2005) Metabolism of the α,β-unsaturated ketones chalcone and *trans*-4-phenyl-3-buten-2-one, by rat liver microsomes and estrogenic activity of the metabolites. *Drug Metab. Dispos.*, **33**, 1115–1123.
17) Grundy, J. (1957) Artifical estrogens. *Chem. Rev.*, **57**, 281–356.
18) Hughes, G. M. K., Moore, P. F. and Stebbins, R. B. (1964) Some hypocholesteremic 2,3-diphenylacrylonitriles. *J. Med. Chem.*, **7**, 511–518.
19) Ali, H. A., Kondo, K. and Tsuda, Y. (1992) Synthesis and nematocidal activity of hydroxystilbenes. *Chem. Pharm. Bull.* (Tokyo), **40**, 1130–1136.
20) Metzler, M. (1984) Biochemical toxicology of diethylstilbestrol. *Rev. Biochem. Toxicol.*, **6**, 191–220.
21) Smith, O. W. and Brookline, M. (1948) Diethylstilbestrol in the prevention and treatment of complications of pregnancy. *Am. J. Obstet. Gynecol.*, **56**, 821–825.
22) Herbst, A. L., Ulfelder, H. and Poskanzer, D. C. (1971) Adenocarcinoma of the vagina: association of maternal stilbestrol therapy with tumor appearance in young women. *N. Engl. J. Med.*, **284**, 878–881.
23) Sanoh, S., Kitamura, S., Sugihara, K. and Ohta, S. (2002) Cytochrome P450 1A1/2 mediated metabolism of *trans*-stilbene in rats and humans. *Biol. Pharm. Bull.*, **25**, 397–400.
24) Sanoh, S., Kitamura, S., Sugihara, K., Kohta, R., Ohta, S. and Watanabe, H. (2006) Effect of stilbene and related compounds on reproductive organs in B6C3F1/Crj mouse. *J. Health Sci.*, **52**, 613–622.
25) Gehm, D., McAndrews, J. M., Chien, P. -Y. and Jameson, J. L. (1997) Resveratrol, a polyphenolic compound found in grapes and wine, is an agonist for the estrogen receptor. *Proc. Natl. Acad. Sci. U.S.A.*, **94**, 14138–14143.
26) Sanoh, S., Kitamura, S., Sugihara, K., Fujimoto, N. and Ohta, S. (2003) Estrogenic activity of stilbene derivatives. *J. Health Sci.*, **49**, 359–367.
27) Ambrose, A. M., Booth, A. N., DeEds, F. and Cox, A. J. Jr. (1960) A toxicological study of biphenyl, a citrus fungistat. *Food Res.*, **25**, 328–336.
28) Häkkinen, I., Hernberg, S., Karli, P. and Vikkula, E. (1973) Diphenyl poisoning in fruit paper production. *Arch. Environ. Health*, **26**, 70–74.
29) West, H. D., Lawson, J. R., Miller, I. H. and Mathura, G. R. (1956) The fate of diphenyl in the rat. *Arch. Biochem. Biophys.*, **60**, 14–20.
30) Billings, R. E. and McMahon, R. E. (1978) Microsomal biphenyl hydroxylation: the formation of 3-hydroxybiphenyl and biphenyl catechol. *Mol. Pharmacol.*, **14**, 145–154.
31) Grossman, J. (1995) Dangers of household pesti-

cides. *Environ. Health Perspect.*, **103**, 550–554.
32) Soto, A. M., Fernandez, M. F., Luizzi, M. F., Karasko, A. S. O. and Sonnenschein, C. (1997) Developing a marker of exposure to xenoestrogen mixtures in human serum. *Environ. Health Perspect.*, **105**(suppl 3), 647–654.
33) Paris, F., Balaguer, P., Terouanne, B., Servant, N., Lacoste, C., Cravedi, J. -P., Nicolas, J. -C. and Sultan, C. (2002) Phenylphenols, biphenols, bisphenol-A and 4-*tert*-octylphenol exhibit α and β estrogen activities and antiandrogen activity in reporter cell lines. *Mol. Cell. Endocrinol.*, **193**, 43–49.
34) Kawamura, Y., Sugimoto, N., Takeda, Y. and Yamada, T. (1998a) Identification of unknown substances in food contact polystyrene. *J. Food Hyg. Jpn.*, **39**, 110–119.
35) Kawamura, Y., Kawarura, M., Takeda, Y. and Yamada, T. (1998b) Determination of styrene dimers and trimers in food contact polystyrene. *J. Food Hyg. Jpn.*, **39**, 199–205.
36) Kawamura, Y., Nishi, K., Maeda, T. and Yamada, T. (1998c) Migration of styrene dimers and trimers from polystyrene containers into foods. *J. Food Hyg. Jpn.*, **39**, 390–398.
37) Sakamoto, H., Matsuzawa, A., Itoh, R. and Tohyama, Y. (2000) Quantitative analysis of styrene dimers and trimers migrated from disposable lunch boxes. *J. Food Hyg. Jpn.*, **41**, 200–205.
38) Nobuhara, Y., Hirano, S., Azuma, Y., Date, K., Ohno, K., Tanaka, K., Matsushiro, S., Sakurai, T., Shiozawa, S., Chiba, M. and Yamada, T. (1999). Biological evaluation of styrene oligomers for endocrine-disrupting effects. *J. Food Hyg. Soc. Jpn.*, **40**, 36–45.
39) Ohyama, K., Nagai, F. and Tsuchiya, Y. (2001) Certain styrene oligomers have proliferative activity on MCF-7 human breast tumor cells and binding affinity for human estrogen receptor. *Environ. Health Perspect.*, **109**, 699–703.
40) Charles, G. D., Barteles, M., Zacharewski, T. R., Gollapudi, B. B., Freshour, N. L. and Carney, E. W. (2000) Activity of benzo[*a*]pyrene and its hydroxylated metabolites in an estrogen receptor-α reporter gene assay. *Toxicol. Sci.*, **55**, 320–326.
41) Fertuck, K. C., Matthews, J. B. and Zacharewski, T. R. (2001) Hydroxylated benzo[*a*]pyrene metabolites are responsible for in vitro estrogen receptor-mediated gene expression induced by benzo[*a*]pyrene, but do not elicit uterotrophic effects *in vivo*. *Toxicol. Sci.*, **59**, 231–240.
42) Bader, A. N., van Dongen, M. M., van Lipzig, M. M., Kool, J., Meerman, J. H. N., Ariese, F. and Gooijer, C. (2005) The chemical interaction between the estrogen receptor and monohydroxybenzo[*a*]pyrene derivativer studied by fluorescence line-narrowing spectroscopy. *Chem. Res. Toxicol.*, **18**, 1405–1412.
43) De Wiele, T. V., Vanhaecke, L., Boeckaert, C., Peru, K., Headley, J., Verstraete, W. and Siciliano, S. (2005) Human colon microbiota transform polycyclic aromatic hydrocarbons to estrogenic metabolites. *Environ. Health Perspect.*, **113**, 6–10.
44) Wang, C. Y., Lee, M. S., King, C. M. and Warner, P. O. (1980) Evidence for nitroaromatics as direct-acting mutagens of airborne particulates. *Chemosphere*, **9**, 83–87.
45) Rosenkranz, H. S. and Mermelstein, R. (1983) Mutagenicity and genotoxicity of nitroarenes, All nitro-containing chemicals were not created equal. *Mutat. Res.*, **114**, 217–267.
46) Hayakawa, K., Murahashi, T., Butoh, M. and Miyazaki, M. (1995) Detemination of 1,3-, 1,6-, and 1,8-dinitropyrenes and 1-nitropyrene in urban air by high-performance liquid chromatography using chemiluminescence detection. *Environ. Sci. Technol.*, **29**, 928–932.
47) Fu, P. P. (1990) Metabolism of nitro-polycyclic aromatic hydrocarbons. *Drug Metab. Rev.*, **22**, 209–268.
48) Möller, L., Torndal, U. -B. Eriksson, L. C. and Gustafsson, J. -Å. (1989) The air pollutant 2-nitrofluorene as initiator and promoter in a liver model for chemical carcinogenesis. *Carcinogenesis*, **10**, 435–440.
49) Campbell, R. M. and Milton, L. L. (1984) Capillary column gas chromatographic determination of nitro polycyclic aromatic compounds in particulate extracts. *Anal. Chem.*, **56**, 1026–1030.
50) Beije, B. and Möller, L. (1988) 2-Nitrofluorene and related compounds: prevalence and biological effects. *Mutat. Res.*, **196**, 177–209.
51) Möller, L. (1994) *In vivo* metabolism and genotoxic effects of nitrated polycyclic aromatic hydrocarbons. *Environ. Health Perspect.*, **102**, 139–146.
52) Cui, X. -S., Eriksson, L. C. and Möller, L. (1999) Formation and persistence of DNA adducts during and after a long-term administration of 2-nitrofluorene. *Mutat. Res.*, **442**, 9–18.
53) Purohit, V. and Basu, A. K. (2000) Mutagenicity of nitroaromatic compounds. *Chem. Res. Toxicol.*, **13**, 673–692.
54) Tatsumi, K., Kitamura, S. and Narai, N. (1986) Reductive metabolism of aromatic nitro compounds including carcinogens by rabbit liver preparations. *Cancer Res.*, **46**, 1089–1093.

55) Tatsumi, K., Kitamura, S., Amano, H. and Ueda, K. (1989) Comparative study on metabolic formation of *N*-arylformamides and *N*-arylacetamides from carcinogenic arylamines in mammalian species. *Cancer Res.*, **49**, 2059–2064.
56) Möller, L., Rafter, J. and Gustafsson, J. -Å. (1987) Metabolism of the carcinogenic air pollutant 2-nitrofluorene in the rat. *Carcinogenesis*, **8**, 637–645.
57) Ueda, O., Kitamura, S., Kubo, R., Yano, Y., Kanzaki, Y., Fujimoto, T., Tatsumi, K. and Ohta, S. (2001) Metabolism of 2-nitrofluorene, 2-aminofluorene and 2-acylaminofluorenes in rat and dog and the role of intestinal bacteria. *Xenobiotica*, **31**, 33–49.
58) Stresser, D. M. and Kupfer, D. (1998) Human cytochrome P450-catalyzed conversion of the proestrogenic pesticide methoxychlor into an estrogen. Role of CYP2C19 and CYP1A2 in *O*-demethylation. *Drug Metab. Dispos.*, **26**, 868–874.
59) Schlenk, D., Stresser, D. M., McCants, J. C., Nimrod, A. C. and Benson, W. H. (1997) Influence of β-naphthoflavone and methoxychlor pretreatment on the biotransformation and estrogenic activity of methoxychlor in channel catfish (*Ictalurus punctatus*). *Toxicol. Appl. Pharmacol.*, **145**, 349–356.
60) Esaac, E. G. and Matsumura, F. (1980) Metabolism of insecticides by reductive systems. *Pharmacol. Ther.*, **9**, 1–26.
61) Kitamura, S., Yoshida, M., Sugihara, K. and Ohta, S. (1999) Reductive dechlorination of *p,p'*-DDT mediated by hemoproteins in the hepatopancreas and blood of goldfish, *Carassius auratus*. *J. Health Sci.*, **45**, 217–221.
62) Kitamura, S., Shimizu, Y., Shiraga, Y., Yoshida, M., Sugihara, K. and Ohta, S. (2002) Reductive metabolism of *p,p'*-DDT and *o,p'*-DDT by rat liver cytochrome. *Drug Metab. Dispos.*, **30**, 113–118.
63) Kelce, W. R., Stone, C. R., Laws, S. C., Gray, L. E., Kemppainen, J. A. and Wilson, E. M. (1995) Persistent DDT metabolite *p,p'*-DDE is a potent androgen receptor antagonist. *Nature*, **375**, 581–585.
64) Forster, M. S., Wilder, E. L. and Heinrichs, W. L. (1973) Estrogenic behavior of 2(*o*-chlorophenyl)-2-(*p*-chlorophenyl)-1,1,1-trichloroethane and its homologues. *Biochem. Pharmacol.*, **24**, 1777–1780.
65) Chen, C. W., Hurd, C., Vorojeikina, D. P., Arnold, S. F. and Notides, A. C. (1997) Transcriptional activation of the human estrogen receptor by DDT isomers and metabolites in yeast and MCF-7 cells. *Biochem. Pharmacol.*, **53**, 1161–1172.
66) Nelson, J. A., Struck, R. F. and James, R. (1978) Estrogenic activities of chlorinated hydrocarbons. *J. Toxicol. Environ. Health*, **4**, 325–339.
67) Nelson, J. A. (1974) Effect of dichlorodiphenyltrichloroethane (DDT) analogs and polychlorinated biphenyl (PCB) mixtures on 17β-[^{3}H]estradiol binding to rat uterine receptor. *Biochem. Pharmacol.*, **23**, 447–451.
68) Gray, L. E., Wolf, C., Lambright, C., Mann, P., Price, M., Cooper, R. and Ostby, J. (1999) Administration of potentially antiandrogenic pesticides (procymidone, linuron, iprodione, chlozolinate, *p,p'*-DDE, and ketoconazole) and toxic substances (dibutyl- and diethylhexyl phthalate, PCB 169, and ethane dimethane sulphonate) during sexual differentiation produces diverse profiles of reproductive malformations in the male rat. *Toxicol. Ind. Health*, **15**, 94–118.
69) Branham, W. S., Dial, S. L., Moland, C. L., Bruce, L. M., Hass, B. S., Blair, R. M., Fang, H., Shi, L., Tong, W., Perkins, R. G. and Sheehan, D. M. (2002) Phytoestrogens and mycoestrogens bind to the rat uterine estrogen receptor. *J. Nutr.*, **132**, 658–664.
70) Schröder, G. and Schröder, J. (1990) Stilbene and chalcone synthases: related enzymes with key functions in plant-specific pathways. *Z. Naturforsch.*, **45c**, 1–8.
71) Wadleigh, R. W. and Yu, S. J. (1987) Glutathione transferase activity of fall armyworm larvae toward α,β-unsaturated carbonyl allelochemicals and its induction by allelochemicals. *Insect Biochem.*, **17**, 759–764.
72) Opdyke, D. L. J. (1973) Monographs on fragrance raw materials. *Food Cosmet. Toxicol.*, **11**, 1011–1081.
73) Czerny, C., Eder, E. and Rünger, T. M. (1998) Genotoxicity and mutagenicity of the α,β–unsaturated carbonyl compound crotonaldehyde (butenal) on a plasmid shuttle vector. *Mutat. Res.*, **407**, 125–134.
74) Prestera, T., Holtzclaw, W. D., Zhang, Y. and Talalay, P. (1993) Chemical and molecular regulation of enzymes that detoxify carcinogens. *Proc. Natl. Acad. Sci. U.S.A.*, **90**, 2965–2969.
75) Eder, E., Scheckenbach, S., Deininger, C. and Hoffman, C. (1993) The possible role of α,β-unsaturated carbonyl compounds in mutagenesis and carcinogenesis. *Toxicol. Lett.*, **67**, 87–103.
76) Sabzevari, O., Galati, G., Moridani, M. Y., Siraki, A. and O'Brien, P. J. (2004) Molecular cytotoxic mechanisms of anticancer hydroxychalcones. *Chem. Biol. Interact.*, **148**, 57–67.
77) Motohashi, N., Ashihara, Y., Yamagami, C. and Saito, Y. (1997) Antimutagenic effects of dehydrozingerone and its analogs on UV-induced mutagenesis in *Escherichia coli*. *Mutat. Res.*, **377**, 17–25.
78) Prival, M. J., Sheldon, Jr. A. T. and Popkin, D.

(1982) Evaluation, using *Salmonella typhimurium*, of the mutagenicity of seven chemicals found in cosmetics. *Food Chem. Toxicol.*, **20**, 427–432.

79) Sumida, K., Ooe, N., Nagahori, H., Saito, K., Isobe, N., Kaneko, H. and Nakatsuka, I. (2001) An in vitro reporter gene assay method incorporating metabolic activation with human and rat S9 of liver microsomes. *Biochem. Biophys. Res. Commun.*, **280**, 85–91.

80) Nakagawa, Y. and Tayama, K. (2001) Estrogenic potency of benzophenone and its metabolites in juvenile female rats. *Arch. Toxicol.*, **75**, 74–79.

81) Nakagawa, Y. and Suzuki, T. (2002) Metabolism of 2-hydroxy-4-methoxybenzophenone in isolated rat hepatocytes and xenoestrogenic effects of its metabolites on MCF-7 human breast cancer cells. *Chem. Biol. Interact.*, **139**, 115–128.

82) Garner, C. E., Jefferson, W. N., Burka, L. T., Matthews, H. B. and Newbold, R. R. (1999) In vitro estrogenicity of the catechol metabolites of selected polychlorinated biphenyls. *Toxicol. Appl. Pharmacol.*, **154**, 188–197.

83) Korach, K. S., Sarver, P., Chae, K., McLachlan, J. A. and McKinney, J. D. (1988) Estrogen receptor-binding activity of polychlorinated hydroxybiphenyls: conformationally restricted structural probes. *Mol. Pharmacol.*, **33**, 120–126.

84) Connor, K., Ramamoorthy, M., Moore, M., Mustain, M., Chen, I., Safe, S., Zacharewski, T., Gillesby, B., Joyeux, A. and Balague, P. (1997) Hydroxylated polychlorinated biphenyls (PCBs) as estrogens and antiestrogens: structure-activity relationships. *Toxicol. Appl. Pharmacol.*, **145**, 111–123.

85) Kitamura, S., Jinno, N., Suzuki, T., Sugihara, K., Ohta, S., Kuroki, H. and Fujimoto, N. (2005) Thyroid hormone-like and estrogenic activity of hydroxylated PCBs in cell culture. *Toxicology*, **208**, 377–387.

86) Elsby, R., Ashby, J., Sumpter, J. P., Brooks, N., Pennie, W. D., Maggs, J. L., Lefevre, P. A., Odum, J., Beresford, N., Paton, D. and Park, B. K. (2000) Obstacles to the prediction of estrogenicity from chemical structure: Assay-mediated metabolic transformation and the apparent promiscuous nature of the estrogen receptor. *Biochem. Pharmacol.*, **60**, 1519–1530.

87) Nakamura, Y., Sugihara, K., Sone, T., Isobe, M., Ohta, S. and Kitamura, S. (2007) The *in vitro* metabolism of a pyrethroid insecticide, permethrin, and its hydrolysis products in rats. *Toxicology*, **235**, 176–184.

88) Suzuki, T., Kitamura, S., Khota, R., Sugihara, K., Fujimoto, N. and Ohta, S. (2005) Estrogenic and antiandrogenic activities of 17 benzophenone derivatives and sunscreens. *Toxicol. Appl. Pharmacol.*, **203**, 9–17.

89) Toda, C., Okamoto, Y., Ueda, K., Hashizume, K., Itoh, K. and Kojima, N. (2004) Unequivocal estrogen receptor-bindin affinity of phthalate esters featured with ring hydroxylation and proper alkyl chain size. *Arch. Biochem. Biophys.*, **431**, 16–21.

90) Kitamura, S., Suzuki, T., Ohta, S. and Fujimoto, N. (2003) Antiandrogenic activity and metabolism of the organophosphorus pesticide fenthion and related compounds. *Environ. Health Perspect.*, **111**, 503–508.

91) Yoshihara, S., Makishima, M., Suzuki, N. and Ohta, S. (2001) Metabolic activation of bisphenol A by rat liver S9 fraction. *Toxicol. Sci.*, **62**, 221–227.

Effects of Environmental Antiandrogenic Chemicals on Expression of Androgen-Responsive Genes in Rat Prostate Lobes

Tomoharu Suzuki,[a] Nariaki Fujimoto,[b] Shigeyuki Kitamura,[c] and Shigeru Ohta*,[a]

[a]*Graduate School of Biomedical Sciences, Hiroshima University,* [b]*Research Institute for Radiation Biology and Medicine, Hiroshima University, 1–2–3, Kasumi, Minami-ku, Hiroshima 734–8551, Japan, and* [c]*Nihon Pharmaceutical University, 10281, Komuro, Inamachi, Kitaadachi-gun, Saitama 362–0806, Japan*

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Rat prostate, which is usually used in the Hershberger assay for evaluating the antiandrogenic activity of environmental chemicals *in vivo*, has a complex structure consisting 4 lobes, *i.e.*, the ventral prostate (VP), lateral prostate (LP), dorsal prostate (DP) and anterior prostate (AP). The VP is considered to have no counterpart in primates, while the LP and DP are histologically similar to human prostate. However, the Hershberger assay focuses on the VP, not the other lobes. Moreover, there are few other methods for assessment of antiandrogenic activity *in vivo*. We therefore investigated androgen-responsive genes in the DP, as well as VP, following treatment with environmental chemicals reported to be androgen antagonists. Male castrated F344 rats were treated with testosterone ($0.5\,mg\cdot kg^{-1}\cdot day^{-1}$) alone or together with flutamide ($6\,mg\cdot kg^{-1}\cdot day^{-1}$) as a reference antiandrogen or fenthion ($25\,mg\cdot kg^{-1}\cdot day^{-1}$) or fenitrothion ($25\,mg\cdot kg^{-1}\cdot day^{-1}$) or 2,4,4′-trihydroxybenzophenone (2,4,4′-triOH-BP) ($300\,mg\cdot kg^{-1}\cdot day^{-1}$) for 7 days. Testosterone significantly increased the expression of kallikrein S3, cystatin-related protein-1 (CRP-1) and prostatein C3 mRNAs in the VP, and prostate secretory protein of 94 amino acids (PSP94) mRNA, but not stem cell growth factor (SCGF) mRNA, in the DP. Coadministration of flutamide blocked the testosterone-induced increases of all three mRNAs in the VP, but not that of PSP94 mRNA in the DP. Coadministration of fenitrothion significantly reduced the testosterone-induced increase of kallikrein S3 mRNA, while fenthion significantly increased the testosterone-induced increase of PSP94 mRNA. 2,4,4′-TriOH-BP significantly increased the testosterone-induced increases of CRP-1 and prostatein C3 mRNAs. These results indicate that the effects of environmental chemicals on the prostate are very complex. The Hershberger assay alone appears to be inadequate for risk assessment, and it may be useful to employ androgen-responsive genes as additional markers.

Key words —— antiandrogenic activity, androgen-responsive genes, rat prostate lobes, Hershberger assay, quantitative reverse transcriptase polymerase chain reaction

INTRODUCTION

Many environmental xenobiotics exert hormonal effects at the cellular and organism levels. These compounds are able to mimic the biological activity of sex hormones and thyroid hormone, and are called endocrine-disrupting chemicals. Initially, estrogenic chemicals such as alkylphenols and bisphenol A were discovered,[1,2] while more recently, several environmental pollutants were discovered to be androgen antagonists.[3,4]

*To whom correspondence should be addressed: Graduate School of Biomedical Sciences, Hiroshima University, 1–2–3, Kasumi, Minami-ku, Hiroshima 734–8551, Japan. Tel.: +81-82-257-5325; Fax: +81-82-257-5329; E-mail: sohta@hiroshima-u.ac.jp

The Hershberger assay has been used to detect chemicals with androgen receptor (AR)-mediated activity *in vivo*.[5–7] The advantages of this assay are that is straightforward, quick and relatively specific to androgenic/antiandrogenic compounds. The endpoint of this assay involves weighing the accessory sex organs of castrated male rats treated with an AR agonist and test compounds.[8,9] However, the Hershberger assay is usually focused on the rat ventral prostate (VP), not other lobes. The rat prostate has a complex structure, consisting of a VP, lateral prostate (LP), dorsal prostate (DP) and anterior prostate (AP). The rodent VP is considered to have no counterpart in primates, while the LP and DP are histologically similar to the human prostate.[10]

We recently reported the lobe-specific expres-

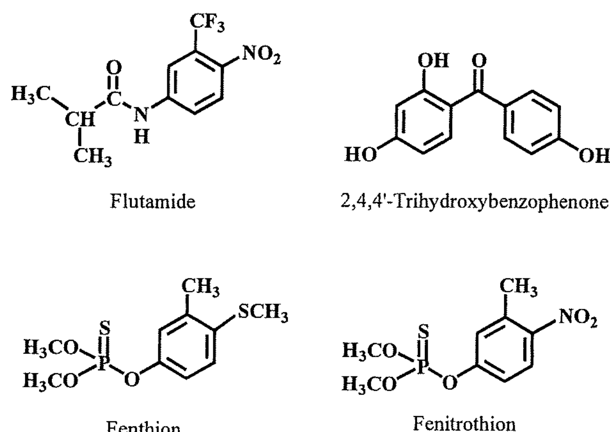

Fig. 1. Structures of Benzophenone, Fenthion and Fenitrothion

sion and lobe-specific response to androgen of several androgen-responsive genes.[11] In the VP, kallikrein S3, cystatin-related protein-1 (CRP-1) and prostatein C3 were highly responsive to androgen treatment. On the other hand, in the LP and DP, prostate secretory protein of 94 amino acids (PSP94), and stem cell growth factor (SCGF) were responsive. In the present study, we used three antiandrogenic chemicals, fenthion, fenitrothion and 2,4,4′-trihydroxybenzophenone (2,4,4′-triOH-BP), as well as the reference antiandrogen flutamide (Fig. 1), and quantitatively analyzed the changes of expression of the above genes in the DP and VP after administration of these chemicals to castrated rats using the same schedule as in the Hershberger assay.[4,12,13] Based on the results, we discuss whether androgen-responsive genes might be suitable markers for assessment of the antiandrogenic activity of environmental chemicals.

MATERIALS AND METHODS

Chemicals —— Testosterone propionate, fenthion and fenitrothion were purchased from Wako Junyaku KK, Osaka, Japan, flutamide from Sigma (St. Louis, MO, U.S.A.) and 2,4,4′-triOH-BP from Tokyo Chemical Industry Co., Ltd., Tokyo, Japan.

Animals —— Animal experiments were conducted according to "A Guide for the Care and Use of Laboratory Animals of Hiroshima University." Male F344 rats were purchased at 4 weeks of age from Charles River Japan Co. (Kanagawa, Japan) and maintained with free access to basal diet and tap water. All animals were surgically castrated at 5 weeks old. At the age of 7 weeks, they were divided into 6 groups each consisting of 6 animals. The rats were treated once a day for 7 days with subcutaneous doses of 0.3 ml of vehicle (dimethyl sulfoxide), testosterone propionate ($0.5\,mg \cdot kg^{-1} \cdot day^{-1}$), testosterone propionate plus flutamide ($6\,mg \cdot kg^{-1} \cdot day^{-1}$), testosterone propionate plus fenitrothion ($25\,mg \cdot kg^{-1} \cdot day^{-1}$), testosterone propionate plus fenthion ($25\,mg \cdot kg^{-1} \cdot day^{-1}$) or testosterone propionate plus 2,4,4′-triOH-BP ($300\,mg \cdot kg^{-1} \cdot day^{-1}$). Animals were sacrificed under anesthesia and the prostate and seminal vesicles were removed, immediately frozen in liquid nitrogen, and stored at −80 °C.

Quantification of mRNAs by Real-time RT-PCR —— RNA preparation was carried out with a Total RNA Isolation kit (Promega Co., Madison, WI, U.S.A.). Total RNA (2 µg) was reverse-transcribed with 200 U of MMLV-RT (Invitrogen Corp., Carlsbad, CA, U.S.A.) and 2.5 pmol of oligo-dT primer (Invitrogen) in 25 µl of buffer containing 1 mM dNTP, 100 mM Tris–HCl (pH 8.3), 150 mM KCl, 6 mM $MgCl_2$, 60 mM dithiothreitol and 5 U/µl RNasin with incubation at 37 °C for 60 min. A real-time PCR method with a QuantiTect Sybr Green PCR kit (Qiagen, Valencia, CA, U.S.A.) and an ABI Prism 7700 (PerkinElmer Life Sciences, Boston, MA, U.S.A.) was employed for quantitative measurement, following the supplied protocol.[14] Specific primer sets with a Tm of about 59 °C were designed for each mRNA (Table 1). The PCR conditions were 15 min of initial activation followed by 45 cycles of 20 sec at 94 °C, and 30 sec at 58 °C and 40 sec at 72 °C. Prior to quantitative analysis, PCR products were prepared separately and purified by gel-electrophoresis. Extracted fragments were used as standards for quantification. The DNA sequences were confirmed with a capillary DNA sequencer, ABI 310 (PerkinElmer Life Sciences). All mRNA contents were normalized with reference to β-actin mRNA.

Statistical Analysis —— Statistical comparisons were made using ANOVA followed by Scheffe's test.

RESULTS

Effects of Test Chemicals on mRNA Expression of Androgen-responsive Genes

In order to evaluate the effects of several environmental chemicals on the expression of androgen-

Table 1. Primers for Quantitative PCR of Rat Genes

Gene	GenBank Acc#	5′-Primer	3′-Primer
kallikrein S3	M11566	5′-AATTCCCAACCCTGGCAAGT-3′	5′-CGCTGAGCAAAGGGTTCATC-3′
CRP-1	S57980	5′-TGCTCCTACTGGCCATCTTTG-3′	5′-TGTCAGCACTGTGCGTGTTG-3′
prostatein C3	M71245	5′-CAGTGGTTCTGGCTGCAGTATT-3′	5′-CTAGAAAACACTGCTTGAATTGCTTC-3′
PSP94	U65486	5′-GATCACCTGCTGCACCAAAAC-3′	5′-TTCCTGGGTTCGTCCGTTC-3′
SCGF	XM_218611	5′-AGAGGAAACCACCACAACACCT-3′	5′-GTCCAAAACATGCAGACGGAT-3′
β-actin	X03765	5′-CTGTCCCTGTATGCCTCTGGTC-3′	5′-TGAGGTAGTCCGTCAGGTCCC-3′

Table 2. mRNA Levels Expressed by Reportedly Androgen-sensitive Genes in Castrated Rats in the Experimental Treatment Groups

Treatment group	VP			DP	
	Kallikrein S3 mRNA	CRP-1 mRNA	Prostatein C3 mRNA	PSP94 mRNA	SCGF mRNA
Vehicle Control	0.003 ± 0.0002**	0.0004 ± 0.0002**	0.02 ± 0.005**	1.3 ± 0.32**	0.018 ± 0.003
T	7.3 ± 1.1	46 ± 9.0	84 ± 4.1	11 ± 2.0	0.028 ± 0.010
T+Flu	0.060 ± 0.016**	0.30 ± 0.010**	6.4 ± 1.7**	8.7 ± 1.2	0.022 ± 0.002
T+MPP	5.5 ± 0.70	40 ± 4.8	86 ± 10	19 ± 2.2*	0.045 ± 0.011
T+MEP	4.0 ± 0.66*	23 ± 3.1	84 ± 14	15 ± 2.1	0.028 ± 0.003
T+2,4,4′-triOH-BP	7.4 ± 1.2	93 ± 10*	112 ± 9.0*	6.5 ± 1.1	0.099 ± 0.020*

Castrated male F344 rats were treated with T ($0.5\,mg\cdot kg^{-1}\cdot day^{-1}$) and/or MPP ($25\,mg\cdot kg^{-1}\cdot day^{-1}$), MEP ($25\,mg\cdot kg^{-1}\cdot day^{-1}$), BP ($300\,mg\cdot kg^{-1}\cdot day^{-1}$), Flu ($6\,mg\cdot kg^{-1}\cdot day^{-1}$) for a week. Values are mean ± S.E.M. ($n = 6$), $^{*}p < 0.05$, $^{**}p < 0.01$ *vs.* T. Abbreviations: T, testosterone propionate; Flu, flutamide; MPP, fenthion; MEP, fenitrothion; 2,4,4′-triOH-BP, 2,4,4′-trihydroxybenzophenone.

responsive genes, we carried out quantitative analysis of mRNA expression of three genes in the VP and two in the DP. All of these genes have been reported to be androgen-responsive.[11)]

In the VP, expression levels of the kallikrein S3, CRP-1 and prostatein C3 genes in castrated rats were all significantly increased by administration of testosterone (Table 2), while coadministration of flutamide essentially abrogated the effect of testosterone. Coadministration of fenthion had little effect on the action of testosterone, while coadministration of fenitrothion significantly decreased the testosterone-induced increase of kallikrein S3 mRNA. Coadministration of 2,4,4′-triOH-BP significantly enhanced the testosterone-induced increases of CRP-1 and prostatein C3 mRNAs.

In the DP, testosterone increased the expression of PSP94 mRNA, but had no effect on SCGF mRNA, while coadministration of flutamide did not significantly alter the effect of testosterone. Coadministration of fenthion further increased the testosterone-induced expression of PSP94 mRNA, while coadministration of fenitrothion had no effect. Coadministration of 2,4,4′-triOH-BP with testosterone resulted in a significant increase of SCGF mRNA compared with the testosterone-alone group.

DISCUSSION

The Hershberger assay is widely used to study the androgenic and antiandrogenic activity of environmental chemicals. Usually rat prostate is used for this assay. Rat prostate consists of four separate lobes, and although the LP and DP are considered to be homologous to the peripheral zone of human prostate and the AP is similar to the central zone, the VP has no homologous region in human prostate.[15)] However, Hershberger assays generally focus on the VP because of its high sensitivity to androgen ablation and to testosterone supplementation after castration. Moreover, there are few alternatives to the Hershberger assay to assess androgenic/antiandrogenic activity *in vivo*. In this study, we assessed the antiandrogenic activities of some known environmental antiandrogens using androgen-responsive genes expressed in the VP and DP as markers. Fenthion and fenitrothion are organophosphorus insecticides; both have been reported to have antiandrogenic activity *in vivo* in the Hershberger assay.[4, 12)] 2,4,4′-TriOH-BP, a derivative of benzophenone-3 used in sunscreen for humans, is also an antiandrogen.[13)] Kallikrein S3, CRP-1 and prostatein C3 are secreted proteins ex-

pressed abundantly in the VP and regulated by androgen.[16–18] We reported that expression of the mRNAs encoding these proteins was increased 10- to 1000-fold in the VP within 24 hr after testosterone treatment in castrated rats.[11] In this study, all the mRNA levels were confirmed to be greatly increased by testosterone and this increase was blocked by co-treatment with flutamide (Table 2). Although fenthion and fenitrothion have been reported to be antiandrogens *in vivo*, we found that they had no effect on the testosterone-induced increases of gene expression in the VP, except for a modest, but significant, decrease of the testosterone-induced increase of kallikrein S3 mRNA by fenitrothion. The reason for this may be the effect of metabolism *in vivo*. Flutamide is converted to hydroxyflutamide, with an increase of about 50-fold in antagonistic activity, while fenthion is inactivated.[12, 19] On the other hand, coadministration of 2,4,4′-triOH-BP enhanced the testosterone-induced increase in the expression of CRP-1 and prostatein C3 in the VP (Table 2).

PSP94 is one of the secreted proteins abundantly expressed in DP.[20] Expression of PSP94 was reportedly increased about 2-fold in the DP in castrated rats after testosterone treatment for 24 hr.[11] In this study, testosterone treatment increased PSP94 mRNA, but flutamide did not block this increase. Fenthion significantly enhanced the testosterone-induced increase of PSP94 mRNA, but fenitrothion and 2,4,4′-triOH-BP were ineffective. SCGF is one of the growth factors expressed in rat prostate, and is expressed highly in the DP. It is tightly regulated by androgen in the DP, being up-regulated about 5-fold within 1 hr after testosterone treatment.[11] In this study, however testosterone did not significantly increase the mRNA level of SCGF, while coadministration of 2,4,4′-triOH-BP resulted in a significant increase compared with testosterone alone (Table 2). The reason for this may be the estrogenicity of 2,4,4′-triOH-BP, which acts as an estrogen agonist in MCF-7 human breast cancer cells and ovariectomized rats.[13, 21] In the rat, estrogen receptor β (ERβ) is expressed in the prostate, and has a role in prostate growth.[22, 23] Its presence may influence the antiandrogenic activity of environmental chemicals. There are differences in response to fenthion, fenitrothion and flutamide between three genes in the VP and two genes in the DP. The reason for this may be the difference in response to testosterone; expression of three genes in the VP greatly increased, on the other hand two genes in the DP did not show great change.

In conclusion, the effects of environmental chemicals on the prostate are very complex, and the Hershberger assay alone appears to be inadequate to understand them. Androgen-responsive genes especially three genes in the VP may be good markers for assessment of androgenic/antiandrogenic activity of environmental chemicals.

REFERENCES

1) Sonnenschein, C. and Soto, A. M. (1998) An updated review of environmental estrogen and androgen mimics and antagonists. *J. Steroid Biochem. Mol. Biol.*, **65**, 143–150.
2) Kim, H. S., Han, S. Y., Yoo, S. D., Lee, B. M. and Park, K. L. (2001) Potential estrogenic effects of bisphenol-A estimated by in vitro and in vivo combination assays. *J. Toxicol. Sci.*, **26**, 111–118.
3) Kelce, W. R., Stone, C. R., Laws, S. C., Gray, L. E., Kemppainen, J. A. and Wilson, E. M. (1995) Persistent DDT metabolite p,p′-DDE is a potent androgen receptor antagonist. *Nature*, **375**, 581–585.
4) Tamura, H., Maness, S. C., Reischmann, K., Dorman, D. C., Gray, L. E. and Gaido, K. W. (2001) Androgen receptor antagonism by the organophosphate insecticide fenitrothion. *Toxicol. Sci.*, **60**, 56–62.
5) Gray, Jr. L. E. (1998) Tiered screening and testing strategy for xenoestrogens and antiandrogens. *Toxicol. Lett.*, **102–103**, 677–680.
6) O'Connor, J. C., Frame, S. R., Davis, L. G. and Cook, J. C. (1999) Detection of the environmental antiandrogen p,p-DDE in CD and long-evans rats using a tier I screening battery and a Hershberger assay. *Toxicol. Sci.*, **51**, 44–53.
7) Ashby, J. and Lefevre, P. A. (2000) The peripubertal male rat assay as an alternative to the Hershberger castrated male rat assay for the detection of anti-androgens, oestrogens and metabolic modulators. *J. Appl. Toxicol.*, **20**, 35–47.
8) Hershberger, L. G., Shipley, E. G. and Meyer, R. K. (1953) Myotrophic activity of 19-nortestosterone and other steroids determined by modified levator ani muscle method. *Proc. Soc. Exp. Biol. Med.*, **83**, 175–180.
9) EDSTAC (1998) Endocrine Disruptor Screening and Testing Adversory Committee (EDSTAC) Final Report. US Environmental Protection Agency.
10) Hayashi, N., Sugimura, Y., Kawamura, J., Donjacour, A. A. and Cunha, G. R. (1991) Morphological and functional heterogeneity in the rat prostatic

gland. *Biol. Reprod.*, **45**, 308-321.
11) Suzuki, T., Fujimoto, N., Kitamura, S. and Ohta, S. (2007) Quantitative determination of lobe specificity of mRNA expression of androgen-dependent genes in the rat prostate gland. *Endocr. J.*, **54**, 123–132.
12) Kitamura, S., Suzuki, T., Ohta, S. and Fujimoto, N. (2003) Antiandrogenic activity and metabolism of the organophosphorus pesticide fenthion and related compounds. *Environ. Health Perspect.*, **111**, 503–508.
13) Suzuki, T., Kitamura, S., Khota, R., Sugihara, K., Fujimoto, N. and Ohta, S. (2005) Estrogenic and antiandrogenic activities of 17 benzophenone derivatives used as UV stabilizers and sunscreens. *Toxicol. Appl Pharmacol.*, **203**, 9–17.
14) Woo, T. H., Patel, B. K., Cinco, M., Smythe, L. D., Symonds, M. L., Norris, M. A. and Dohnt, M. F. (1998) Real-time homogeneous assay of rapid cycle polymerase chain reaction product for identification of Leptonema illini. *Anal. Biochem.*, **259**, 112–117.
15) Cunha, G. R., Donjacour, A. A., Cooke, P. S., Mee, S., Bigsby, R. M., Higgins, S. J. and Sugimura, Y. (1987) The endocrinology and developmental biology of the prostate. *Endocr. Rev.*, **8**, 338–362.
16) Clements, J. A., Matheson, B. A., Wines, D. R., Brady, J. M., MacDonald, R. J. and Funder, J. W. (1988) Androgen dependence of specific kallikrein gene family members expressed in rat prostate. *J. Biol. Chem.*, **263**, 16132–16137.
17) Heyns, W. (1990) Androgen-regulated proteins in the rat ventral prostate. *Andrologia*, **22 Suppl 1**, 67–73.
18) Vercaeren, I., Vanaken, H., Devos, A., Peeters, B., Verhoeven, G. and Heyns, W. (1996) Androgens transcriptionally regulate the expression of cystatin-related protein and the C3 component of prostatic binding protein in rat ventral prostate and lacrimal gland. *Endocrinology*, **137**, 4713–4720.
19) Kelce, W. R., Monosson, E., Gamcsik, M. P., Laws, S. C. and Gray, L. E. (1994) Environmental hormone disruptors: evidence that vinclozolin developmental toxicity is mediated by antiandrogenic metabolites. *Toxicol. Appl. Pharmacol.*, **126**, 276–285.
20) Kwong, J., Xuan, J. W., Chan, P. S., Ho, S. M. and Chan, F. L. (2000) A comparative study of hormonal regulation of three secretory proteins (prostatic secretory protein-PSP94, probasin, and seminal vesicle secretion II) in rat lateral prostate. *Endocrinology*, **141**, 4543–4551.
21) Yamasaki, K., Takeyoshim, M., Sawaki, M., Imatanaka, N., Shinoda, K. and Takatsuki, M. (2003) Immature rat uterotrophic assay of 18 chemicals and Hershberger assay of 30 chemicals. *Toxicology*, **183**, 93–115.
22) Horvath, L. G., Henshall, S. M., Lee, C. S., Head, D. R., Quinn, D. I., Makela, S., Delprado, W., Golovsky, D., Brenner, P. C., O'Neill, G., Kooner, R., Stricker, P. D., Grygiel, J. J., Gustafsson, J. A. and Sutherland, R. L. (2001) Frequent loss of estrogen receptor-beta expression in prostate cancer. *Cancer Res.*, **61**, 5331–5335.
23) Weihua, Z., Makela, S., Andersson, L. C., Salmi, S., Saji, S., Webster, J. I., Jensen, E. V., Nilsson, S., Warner, M. and Gustafsson, J. A. (2001) A role for estrogen receptor beta in the regulation of growth of the ventral prostate. *Proc. Natl. Acad. Sci. U.S.A.*, **98**, 6330–6335.

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Quantitative Determination of Lobe Specificity of mRNA Expression of Androgen-dependent Genes in the Rat Prostate Gland

TOMOHARU SUZUKI, NARIAKI FUJIMOTO*, SHIGEYUKI KITAMURA AND SHIGERU OHTA

Department of Xenobiotic Metabolism and Molecular Toxicology, Institute of Pharmaceutical Sciences, Hiroshima University School of Medicine, Kasumi 1-2-3, Minami-ku, Hiroshima 734-8551, Japan
**Department of Developmental Biology, Research Institute for Radiation Biology and Medicine (RIRBM), Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8553, Japan*

Abstract. The rodent prostate has a complex structure, consisting of a ventral prostate (VP), lateral prostate (LP), dorsal prostate (DP) and anterior prostate (AP), and most studies so far have focused on the VP. Androgen-responsive prostatic secretory proteins, such as prostatein and kallikreins, are mainly produced in the VP, but others are abundant in the LP and DP, though little is known about differences of androgen regulation among the different lobes. Here, the mRNA expression levels of some representative androgen-responsive genes, including those encoding prostatic secreted proteins, were quantitatively determined in each of the prostatic lobes of intact rats and castrated rats treated with testosterone alone or plus flutamide. The results show that the transcriptional regulation of prostatic secretory proteins differs greatly among lobes, generally being more tightly regulated in the VP. A number of growth factor mRNAs were differentially expressed in separate lobes and were regulated by testosterone in a lobe-specific manner. Lobe-specific regulation by androgen was also found for other genes, including the DAD-1 and calreticulin genes. Thus, hormone-dependent transcriptional regulation of prostate genes differs among lobes, and there is also interlobar diversity of basal mRNA expression levels.

Key words: Rat prostate, Lobe-specific expression, Androgen regulation, Quantitative RT-PCR

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RAT prostate models have been used to study androgen-regulated gene expression of several prostatic secreted proteins, such as probasin, cystatin-related protein-1 (CRP-1), PSP94 and kallikreins [1–5], and differential display PCR and micro-array analysis have recently been applied to identify novel androgen-responsive genes. However, induction of gene expression by androgen in the prostate is not simple, since androgen may directly regulate genes, as well as indirectly influence gene expression by stimulating growth or regeneration of the gland. In the prostate, 'androgen-dependent' genes have generally been rather simply defined as those whose expression decreases following castration and recovers upon androgen replacement [6–8]. Moreover, the rodent prostate has a complex structure, consisting of a ventral prostate (VP), lateral prostate (LP), dorsal prostate (DP) and anterior prostate (AP, or coagulating gland) [9]. Most studies have focused on the VP, and the responses could be quite different in the other lobes. The best-characterized androgen-regulated gene is probably probasin in the rat [2, 5]. Although the genomic upstream regions contain consensus androgen-responsive elements (AREs) which are functional promoters in *in vitro* reporter assay [10–12], the mRNA level rapidly decreases after castration only in the VP, but not in the other lobes [4], suggesting a complexity in androgen-dependent expression *in vivo*. Other prostatic secretory proteins, including prostatein, PSP94, kallikreins,

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Correspondence to: Dr. Nariaki FUJIMOTO, Department of Developmental Biology, Research Institute for Radiation Biology and Medicine, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8553, Japan

cystatin-related protein-1 (CRP1) and SVS2, are also known to be induced by androgen. DAD-1, calreticulin, estrogen receptor β (ERβ) and a number of growth factors, such as IGF-1, EGF and SCGF, have been reported to be regulated by androgen [13, 14], but the details, such as lobe specificity and time dependency after testosterone administration, have not been examined. The rodent VP is considered to have no counterpart in primates, while the LP and the DP are histologically similar to the human prostate [12]. In experimental prostate carcinogenesis initiated with chemical carcinogens in Noble rats and other rat strains, hormone-dependent prostatic adenocarcinomas are found only in the LP, DP and AP [8, 15–17]. If the rat prostate is to be employed as a human model, more attention should be paid to androgen responses in these lobes.

We therefore examined the mRNA expression levels of androgen-responsive genes, including prostatic secreted proteins, growth factors and other recently identified genes, in the prostate of F344 rats. RNAs isolated from the different prostatic lobes, as well as the seminal vesicle (SV), were subjected to quantitative real-time RT-PCR to analyze the expression levels and to provide an outline view of the overall androgen responsiveness of genes expressed in the prostate gland.

Materials and Methods

Hormones

Testosterone propionate was purchased from Wako Junyaku KK, Osaka, Japan and flutamide from Tokyo Chemical Industry Co., Ltd., Tokyo, Japan.

Animals

Animal experiments were conducted according to 'A Guide for the Care and Use of Laboratory Animals of Hiroshima University'. Male F344 rats were purchased at 10 weeks of age from Charles River Japan Co. (Kanagawa, Japan) and maintained with free access to basal diet and tap water. All animals except those of the intact control group were surgically castrated at 11 weeks of age. To examine the effects of castration, the animals were divided into three groups (consisting of 6 animals each), *i.e.*, intact group and groups castrated 1 and 2 weeks previously. For the testosterone injection experiment, animals were castrated one week previously and divided into 5 groups (6 animals each). Testosterone and flutamide were injected ip and animals were sacrificed after 1, 4, and 24 h (T-1h, T-4h, T-24h and T plus flutamide-24h), along with intact control animals. Testosterone propionate and flutamide were dissolved in the vehicle oil, Panacete 810 (Nippon Oils and Fats Co., Ltd., Tokyo), and administered ip at 5 mg/kg body weight and 60 mg/kg body weight, respectively. After treatment, animals were sacrificed at the indicated time points under ether anesthesia and each of the prostate lobes, as well as the SV, was dissected under a microscope, weighed, immediately frozen in liquid nitrogen, and stored at –80°C.

Quantification of mRNAs by real-time RT-PCR

RNA preparation was carried out with a Total RNA Isolation kit (Promega Co., Madison, WI, USA). Total RNA (2 μg) was reverse-transcribed with 200 U of MMLV-RT (Invitrogen Corp., Carlsbad, CA, USA) and 2.5 pmol of oligo-dT primer (Invitrogen) in 25 μl of buffer containing 1 mM dNTP, 100 mM TrisHCl (pH 8.3), 150 mM KCl, 6 mM $MgCl_2$, 60 mM dithiothreitol and 5 U/μl RNasin with incubation at 37°C for 60 min.

The real-time PCR method with a QuantiTect Sybr Green PCR kit (Qiagen, Valencia, CA, USA) and an ABI Prism 7700 (PerkinElmer Life Sciences, Boston, MA, USA) was employed for quantitative measurement, following the supplied protocol [18]. Specific primer sets with a Tm of about 59°C were designed for each mRNA (Table 1). The PCR conditions were 15 min of initial activation followed by 45 cycles of 20 sec at 94°C, and 30 sec at 58°C and 40 sec at 72°C. Prior to quantitative analysis, PCR products were prepared separately and purified by gel-electrophoresis. Extracted fragments were used as standards for quantification. The DNA sequences were confirmed with a capillary DNA sequencer, ABI 310 (PerkinElmer Life Sciences). All mRNA contents were normalized with reference to β-actin mRNA.

Serum testosterone levels

Serum testosterone levels were measured with an ELISA kit, purchased from Neogen Corp. (Lexington, KY, USA).

Table 1. Quantitative PCR primers for rat genes

Gene	GenBank Acc#	5'-Primer	3'-Primer
kallikrein S3	M11566	5'-AATTCCCAACCCTGGCAAGT-3'	5'-CGCTGAGCAAAGGGTTCATC-3'
CRP-1	S57980	5'-TGCTCCTACTGGCCATCTTTG-3'	5'-TGTCAGCACTGTGCGTGTTG-3'
prostatein C3	M71245	5'-CAGTGGTTCTGGCTGCAGTATT-3'	5'-CTAGAAAACACTGCTTGAATTGCTTC-3'
probasin	NM_019125	5'-CCTCCTGCTCACACTGGATGT-3'	5'-GCGACGGAAGTAGGTCCTCA-3'
PSP94	U65486	5'-GATCACCTGCTGCACCAAAAC-3'	5'-TTCCTGGGTTCGTCCGTTC-3'
SVS2	J05443	5'-GCGCAGACAAGTGTTTCACAA-3'	5'-GGGATCCTCCAGATTTCAGCT-3'
IGF-1	M15481	5'-GACCAGAGACCCTTTGCGG-3'	5'-GCTCCGGAAGCAACACTCA-3'
EGF	NM_012842	5'-GATTATGACCCCGTGGAAAGC-3'	5'-GCAAGACCTTCTGGCGTGTC-3'
SCGF	XM_218611	5'-AGAGGAAACCACCACAACACCT-3'	5'-GTCCAAAACATGCAGACGGAT-3'
DAD-1	Y13336	5'-CGGCTACTGTCTCCTCGTGG-3'	5'-TCGCTCAGGAGAGATGCCTT-3'
calreticulin	X53363	5'-CCAAGATTGATGACCCCACAG-3'	5'-TGAATCACTGGTGGTTCCCAC-3'
FPPS	M89945	5'-CATCATGGACTCTTCCTACACTCG-3'	5'-GAACTTAAGCAGGCGGTAGATAGC-3'
ERβ	U57439	5'-TGCCAATCATCGCTCCTCTAT-3'	5'-GGCACAACTGCTCCCACTAAG-3'
AR	NM_012502	5'-CACCATGCAACTTCTTCAGCA-3'	5'-CGAATTGCCCCCTAGGTAACT-3'
β-actin	X03765	5'-CTGTCCCTGTATGCCTCTGGTC-3'	5'-TGAGGTAGTCCGTCAGGTCCC-3'

Table 2. Body and prostate lobe weights and serum testosterone levels in intact and castrated rats treated with testosterone and flutamide

	Body w. (g)	VP w. (mg)	LP w. (mg)	DP w. (mg)	AP w. (mg)	SV w. (mg)	testosterone (pg/mL)
Intact	290 ± 11.6*	216 ± 42.1**	120 ± 27.2*	129 ± 14.6**	132 ± 5.7**	701 ± 56.4**	2340 ± 448**
Castration 1W	255 ± 3.85	46 ± 2.1	37 ± 5.1	48 ± 5.7	38 ± 8.5	163 ± 7.73	70 ± 8.4
+T 1 h	242 ± 5.53	49 ± 1.1	33 ± 2.0	48 ± 3.9	31 ± 3.4	140 ± 10.1	43200 ± 7280**
+T 4 h	244 ± 4.40	58 ± 5.0	30 ± 5.0	59 ± 3.9	47 ± 6.5	143 ± 10.1	32800 ± 10000**
+T 24 h	251 ± 2.80	54 ± 5.5	51 ± 5.5	61 ± 4.1	64 ± 7.9	180 ± 10.0	1480 ± 232**
+T + Flutamide 24 h	248 ± 9.37	50 ± 1.6	43 ± 3.6	55 ± 4.8	56 ± 4.0	171 ± 3.76	1380 ± 124**

11-week-old male F344 rats were castrated for 1 week previously and testosterone (T) and flutamide were injected (i.p.) at 5 mg/kg body weight and 60 mg/kg body weight, respectively. Values are mean ± S.E.M. (n = 5)
*P<0.05, **P<0.01 vs. castration

Statistical Analysis

Statistical comparisons were made using ANOVA followed by Scheffe's test.

Results

Body and prostate lobe weights and serum T levels

One week after castration, body weights as well as the weights of the prostatic lobes, especially the VP, were significantly decreased (Table 2). The weight of each lobe showed no significant change within 24 h of testosterone administration. Serum T levels were significantly decreased in the castration group and increased within 1 h of testosterone administration, reaching 18 times the intact control level and then decreasing to below the intact control level within 24 h.

Lobe-specific mRNA expression of androgen-responsive genes and androgen receptor (AR) in control prostate

Among the prostate-secreted proteins examined, prostatein C3, kallikrein S3 and CRP1 were preferentially expressed in the VP, although substantial amounts of mRNAs for these proteins were also detected in the other lobes (*e.g.*, the prostatein C3 mRNA level was 150 mol/mol β-actin in the VP and 1.5 mol/mol in the DP) (Table 2). Prominent expression of probasin, PSP94 and SVS2 was noted in the LP and DP at 30–700 times the expression levels of β-actin. In the growth factor category, the SCGF mRNA levels were higher in the DP, while the expression of EGF was high in the AP (Table 2). Expression of DAD1 was equally higher in both the VP and LP compared with the other lobes. Calreticulin and farnesyl diphosphate synthase (FPPS) mRNA levels were 5–10 times higher in the VP than in the other lobes. The ERβ mRNA level in the

Table 3. Androgen regulation of mRNA levels

		kallikrein S3	CRP-1	prostatein C3	probasin	PSP94	SVS2	AR
VP	Intact	23 ± 5.2[a]	120 ± 19	180 ± 24	2.1 ± 0.62		0.030 ± 0.019	0.013 ± 0.0027
	Cast	0.041 ± 0.0055	0.0023 ± 0.0008	0.27 ± 0.019	0.032 ± 0.0027		0.035 ± 0.0081	0.018 ± 0.0023
	Cast + T 1 h	0.065 ± 0.014 (1.6)[b]	0.0012 ± 0.0002 (0.50)	0.26 ± 0.053 (0.96)	12 ± 2.2 (380)**	n.d.	12 ± 3.5 (340)*	0.020 ± 0.0025 (1.1)
	Cast + T 24 h	0.41 ± 0.049 (9.9)**	3.8 ± 0.56 (1700)**	11 ± 1.9 (39)**	2.8 ± 0.73 (88)**		9.8 ± 2.0 (280)**	0.0090 ± 0.0013 (0.51)*
LP	Intact				50 ± 12	34 ± 8.2	640 ± 110	0.012 ± 0.0021
	Cast				17 ± 2.3	7.6 ± 1.6	32 ± 3.4	0.025 ± 0.0020
	Cast + T 1 h	n.d.	n.d.	n.d.	32 ± 6.0 (1.8)	14 ± 2.3 (1.8)	46 ± 8.4 (1.4)	0.016 ± 0.0054 (0.63)
	Cast + T 24 h				22 ± 2.7 (1.3)	18 ± 3.8 (2.4)**	150 ± 13 (4.6)**	0.0074 ± 0.0011 (0.30)**
DP	Intact				22 ± 5.3	15 ± 2.4	24 ± 6.9	0.012 ± 0.0023
	Cast				8.9 ± 1.2	2.1 ± 0.40	2.6 ± 0.43	0.017 ± 0.0008
	Cast + T 1 h	n.d.	n.d.	n.d.	9.9 ± 1.8 (1.1)	4.1 ± 0.44 (2.0)	5.5 ± 1.4 (2.1)	0.015 ± 0.0003 (0.88)
	Cast + T 24 h				8.1 ± 2.1 (0.91)	5.5 ± 1.1 (2.6)**	7.3 ± 1.2 (2.8)*	0.0056 ± 0.0008 (0.33)**
AP	Intact				2.3 ± 0.44		12 ± 3.4	0.0065 ± 0.0014
	Cast	n.d.	n.d.	n.d.	3.2 ± 0.51	n.d.	0.33 ± 0.075	0.016 ± 0.0037
	Cast + T 1 h				2.9 ± 0.30 (0.90)		0.33 ± 0.076 (0.99)	0.017 ± 0.0036 (1.1)
	Cast + T 24 h				0.76 ± 0.15 (0.23)**		2.5 ± 0.76 (7.8)	0.0036 ± 0.0008 (0.23)*
SV	Intact						150 ± 56	0.0022 ± 0.0003
	Cast	n.d.	n.d.	n.d.	n.d.	n.d.	15 ± 3.0	0.010 ± 0.0002
	Cast + T						79 ± 8.7 (5.1)	0.0053 ± 0.0011 (0.53)**

VP was three times that in the LP or DP, while the level was 1/10 in the AP, and ERβ mRNA was not detectable in the SV. The AR mRNA showed no marked differences in expression among the prostatic lobes, although significantly lower values were obtained for the SV.

Lobe-specific changes in mRNA expression after castration

Castration reduced the mRNA expression of prostate secreted proteins, but the degree of change varied greatly among different lobes and genes (Table 3). The mRNAs of the VP-specific proteins, kallikrein S3, CRP-1 and prostatein C3, almost disappeared after castration. On the other hand, PSP94, SVS2 and probasin were moderately decreased in the LP and DP. IGF-1 and VEGF mRNAs were decreased in the VP, LP and DP, and a decrease in EGF mRNA was noted in all the lobes. SCGF mRNA was considerably reduced only in the DP and LP. DAD-1 was decreased in the VP and LP, while calreticulin and FPPS were prominently decreased only in the VP. ERβ mRNA was significantly decreased in the VP, LP and DP, but not in the AP. Castration roughly doubled the AR mRNA expression in every lobe.

Lobe-specific changes in mRNA expression after testosterone administration in castrated animals

Expression of mRNA and the fold increases over the castration group at 1 and 24 h after testosterone administration are summarized in Table 3, and graphs of time-dependent change in mRNAs for several genes are presented in Fig. 1. A VP-specific secreted protein, kallikrein S3, was increased 24 h after T treatment, but not after 1 or 4 h. Even though the expression increased by 10-fold, the absolute mRNA level reached only 1/56 of the intact control level. Similar time-dependent changes in mRNA were noted for CRP1 and prostatein C3 (data not shown). Among growth factors, IGF-1 and EGF mRNAs were elevated in a lobe-specific manner after testosterone administration. SCGF mRNA was increased in LP and DP at 1 h after testosterone administration, but reverted to the initial level within 24 h (Fig. 1). In castrated animals, T-dependent short-term induction of mRNA (in 1 or 4 h)

Table 3. Androgen regulation of mRNA levels (continued)

		IGF-1	EGF	SCGF	DAD-1	calreticulin	FPPS	ERβ
VP	Intact	0.15 ± 0.084[a]	0.088 ± 0.036	0.0020 ± 0.0005	4.6 ± 0.75	54 ± 12	53 ± 4.4	0.027 ± 0.0053
	Cast	0.055 ± 0.0062	0.016 ± 0.0029	0.0030 ± 0.0002	0.75 ± 0.15	1.6 ± 0.36	0.82 ± 0.25	0.0041 ± 0.0013
	Cast + T 1 h	0.038 ± 0.0078 (0.70)[b]	0.017 ± 0.0032 (1.1)	0.0009 ± 0.0001 (0.31)**	0.35 ± 0.075 (0.46)	4.6 ± 0.62 (2.9)**	0.83 ± 0.15 (1.0)	0.0047 ± 0.0009 (1.1)
	Cast + T 24 h	0.16 ± 0.031 (2.9)*	0.027 ± 0.015 (1.7)	0.0023 ± 0.0008 (0.77)	1.9 ± 0.29 (2.5)*	38 ± 0.59 (24)**	11 ± 0.99 (13)**	0.0079 ± 0.0017 (1.9)
LP	Intact	0.19 ± 0.022	0.15 ± 0.038	0.0065 ± 0.0022	4.4 ± 0.71	14 ± 5.9	2.5 ± 0.45	0.0086 ± 0.0014
	Cast	0.026 ± 0.0037	0.066 ± 0.014	0.0005 ± 0.0001	1.4 ± 0.15	5.0 ± 0.38	1.4 ± 0.18	0.0090 ± 0.0028
	Cast + T 1 h	0.046 ± 0.0073 (1.8)	0.065 ± 0.011 (0.98)	0.0023 ± 0.0004 (4.3)**	1.8 ± 0.23 (1.3)	11 ± 2.7 (2.2)	0.98 ± 0.20 (0.70)	0.0055 ± 0.0022 (0.61)
	Cast + T 24 h	0.10 ± 0.018 (3.9)**	0.075 ± 0.0030 (1.4)	0.0008 ± 0.0001 (1.6)	3.8 ± 0.55 (3.1)**	150 ± 33 (29)*	12 ± 1.6 (9.0)**	0.0073 ± 0.0012 (0.82)
DP	Intact	0.10 ± 0.031	0.16 ± 0.040	0.013 ± 0.0040	1.7 ± 0.15	12 ± 1.9	2.4 ± 0.36	0.0092 ± 0.0004
	Cast	0.031 ± 0.0072	0.036 ± 0.0058	0.0005 ± 0.0001	1.4 ± 0.15	4.6 ± 0.46	0.74 ± 0.13	0.0055 ± 0.0006
	Cast + T 1 h	0.032 ± 0.0045 (1.0)	0.056 ± 0.014 (1.6)	0.0019 ± 0.00037 (4.0)**	1.4 ± 0.19 (1.0)	3.3 ± 0.67 (0.70)	0.57 ± 0.24 (0.77)	0.0039 ± 0.0020 (0.71)
	Cast + T 24 h	0.051 ± 0.014 (1.6)	0.15 ± 0.018 (4.1)**	0.0004 ± 0.00004 (0.88)	1.5 ± 0.19 (1.1)	20 ± 4.6 (4.4)*	6.3 ± 1.0 (8.6)**	0.0038 ± 0.0006 (0.68)
AP	Intact	0.091 ± 0.023	0.028 ± 0.0064	0.0061 ± 0.0017	0.65 ± 0.13	7.0 ± 2.3	3.4 ± 0.77	0.0012 ± 0.0004
	Cast	0.067 ± 0.0082	0.0057 ± 0.0018	0.0061 ± 0.0016	0.39 ± 0.059	1.7 ± 0.14	0.76 ± 0.099	0.0026 ± 0.0010
	Cast + T 1 h	0.058 ± 0.0047 (0.86)	0.020 ± 0.0038 (3.5)*	0.0035 ± 0.0005 (0.58)	0.77 ± 0.040 (2.0)**	4.6 ± 0.64 (2.7)**	1.2 ± 0.44 (1.6)	0.0035 ± 0.0012 (1.4)
	Cast + T 24 h	0.14 ± 0.028 (2.1)*	0.0040 ± 0.0007 (0.70)	0.0050 ± 0.0017 (0.82)	0.42 ± 0.080 (1.1)	1.8 ± 0.38 (1.0)	1.1 ± 0.14 (1.5)	0.00065 ± 0.0002 (0.25)
SV	Intact	0.32 ± 0.21		0.0058 ± 0.0010	0.89 ± 0.33	1.5 ± 0.34		
	Cast	0.045 ± 0.010	n.d.	0.0015 ± 0.0002	0.59 ± 0.091	4.6 ± 1.2	n.d.	n.d.
	Cast + T	0.14 ± 0.017 (3.2)**		0.0021 ± 0.0006 (1.4)	0.94 ± 0.16 (1.6)	13 ± 0.29 (2.8)**		

[a] Mean ± SEM (n = 5). Values are mRNA levels divided by β-actin mRNA levels (mol/mol β-actin), n.d.: not detected
[b] Values in parentheses are fold changes in mRNA over the castrated group (Cast).
11-week-old male F344 rats were castrated and maintained for a week (Cast). They were killed 1 h (Cast + T 1 h) or 24 h (Cast + T 24 h) after testosterone (T) administration at 5 mg/kg bw, ip. Total RNA was isolated from each prostate lobe and amounts of mRNA were measured by means of real-time RT-PCR.
*$P<0.05$, **$P<0.01$ vs. Cast

was found for some genes in some lobes, *e.g.*, probasin and SVS2 in the VP, SCGF in the LP/DP and calreticulin in all the lobes. Co-administration of flutamide with T for 24 h reversed the T-induced mRNA expression in all cases.

Discussion

The rodent prostate, consisting of four or five separate lobes, is anatomically very different from the human organ [9, 19]. Developmentally, the rodent VP arises from buds in the ventral region of the urogenital sinus and there is no corresponding portion in the adult prostate in humans or other primates [4]. The LP and DP are considered to be homologous to the peripheral zone of human prostate and the AP is similar to the central zone, these lobes being most susceptible to androgen-induced carcinogenesis [16, 20, 21]. Recently, an expression profile study of the mouse prostate revealed that the gene expression patter of the dorsolateral prostate was closest to that of the human prostate peripheral zone [22]. Despite this, the rodent ventral prostate has been most intensively investigated and only limited attention has been paid to other lobes in studies on androgen regulation of genes.

AR is distributed in the target tissues, including the prostate, and mediates androgen regulation of gene expression. Our results confirm that AR mRNA expression is substantial throughout the prostatic lobes. A previous western blotting study showed that different prostatic lobes contain significantly different amounts of AR protein, *i.e.*, the level was 4–7 times higher in the VP than in the LP and DP [23], whereas the present data indicate that AR mRNA is equally expressed among the lobes. Although AR is primarily under pos-

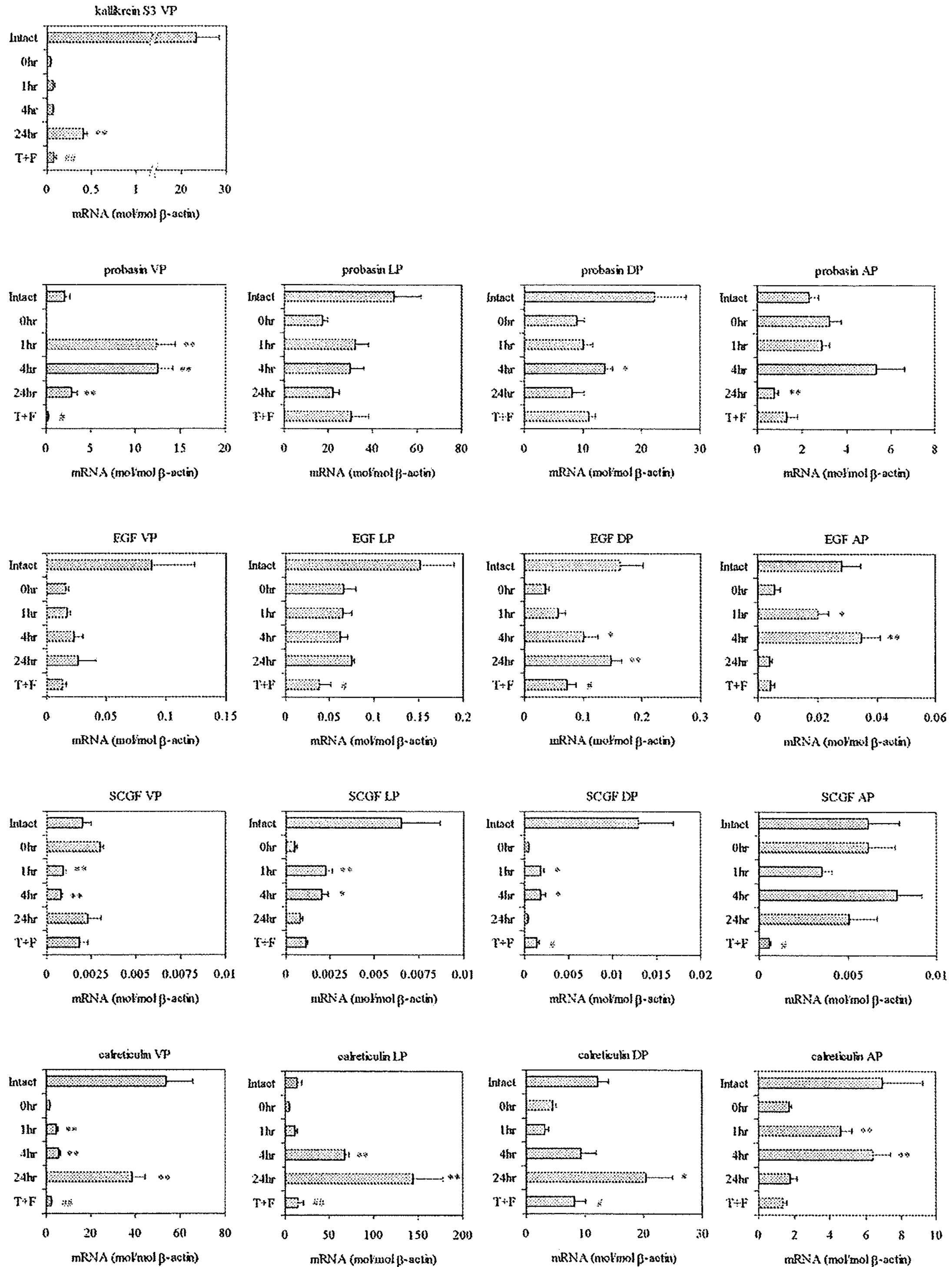

Fig. 1. The mRNA levels of prostatic proteins, growth factors and other androgen-responsive genes in different prostatic lobes at 0, 1, 4 and 24 h (0, 1, 4, 24 h) after a testosterone (T) injection (5 mg/kg body weight, ip) in rats castrated a week previously. The castrated rats were treated with T plus flutamide (F, 60 mg/kg body weight, ip) for 24 h and intact control animals were also examined. Bar indicates mean ± SEM, n = 6. *: $p<0.05$ and **: $p<0.01$ vs. 0 hr; #: $p<0.05$ and ##: $p<0.01$ vs. 24 hr.

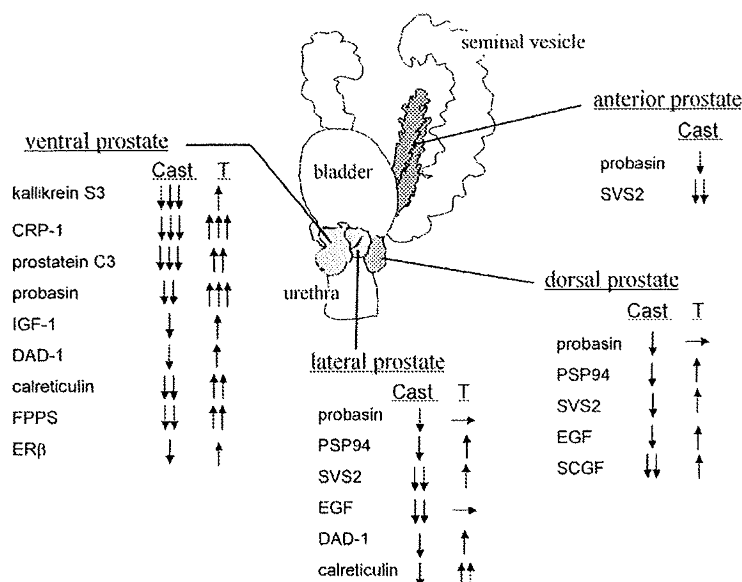

Fig. 2. A summary of differential expression of the examined mRNAs with schematic lateral view of the rat prostate. Androgen responsive genes with higher basal mRNA expression are listed in each lobe. The changes in mRNA level after castration (Cast) and the effects of testosterone (T) administration are summarized.

itive androgen control in prostatic tissues, based on ligand-binding assays [24, 25], the mRNA was down-regulated by testosterone administration according to northern blotting analysis, in agreement with our mRNA data. Discrepancy between protein and mRNA levels probably indicates that AR protein levels are partly regulated at the post-translational level in a lobe-specific manner.

Prostatein, CRP-1 and kallikreins are ventral-specific proteins. Prostatein, known as PBP or prostatic binding protein, consists of two non-identical subunits, each containing three peptides, C1, C2 and C3 [5]. Although PBP was first recognized on the basis of its low binding affinity to steroids, and accounts for 30–50% of secreted proteins in the rat VP, its *in vivo* function is still unclear. CRP-1 and -2 have been reported to make up 5–10% of the VP secretion [1]. Kallikrein S3, one of the tissue kallikreins (serine proteases), is also abundantly expressed in VP [26], and has been suggested as a counterpart for human prostatic-specific antigen or PSA [27]. Our data indeed demonstrated high levels of expression of these genes, with about 100 fold higher amounts of mRNA than that of a housekeeping gene, β-actin, used as an internal control in the present study. Similarly, mRNAs of probasin, SVS2 and PSP94 were preferentially expressed in the LP and DP, while substantial amounts of these mRNAs were also expressed in the AP.

Several growth factor families have been implicated in normal prostatic growth and differentiation, as well as in malignant changes, with interaction between stromal and epithelial cells [28]. Among them, IGF-1, EGF and SCGF have been reported to be regulated by androgen [7, 29, 30]. IGF-1 produced by stromal cells, stimulate epithelial cell growth, while TGFβ has the opposite effect. EGF is an autocrine/paracrine factor involved in epithelial cell growth. SCGF, originally found as a growth factor for primitive hematopoetic progenitor cells, was found in the DP [31], though its function is unknown. The determined values of mRNA of growth factors seem to be very low but should be significant, since the levels are normalized by abundant expression of β-actin. The present data demonstrate that mRNA levels of these growth factors greatly differ among the prostatic lobes, though they are generally lower in the VP. The other androgen dependent genes examined were calreticulin, an intercellular Ca^{2+}-binding protein that may be involved in the prevention of apoptosis of prostatic cells [32], FPPS, an essential enzyme catalyzing the synthesis of farnesyl diphosphate [33], DAD-1, a defender against apoptotic cell death [7] and ERβ [13]. Several lines of evidence suggest

that ERs are involved in regulating cell proliferation and in carcinogenesis in the prostate gland [34, 35].

To examine androgen-responsive transcription, changes in mRNA levels at one week after castration were first determined. Castration decreased most of the mRNA levels examined, although the degrees of change were most profound in the VP. For instance, probasin expression was almost shut down in the VP by castration, while it remained at one-third of the intact level in the LP and DP, and no change was noted in the AP. The different sensitivities of the lobes to castration might be related to the manner of involution in the different lobes. Androgen abbreviation by castration causes massive apoptotic death of both epithelial and stromal cells in the VP, but it only reduces epithelial cells in the other lobes [36], so that the cell population could be changed differentially among lobes. However, since the mRNA levels in the present study were normalized to β-actin, the results should be comparable.

To assess the responses more directly regulated by androgen, changes in mRNA levels at 1, 4 and 24 h after testosterone administration were determined. Induction of mRNA within 1 h was found for probasin, EGF, SCGF and calreticulin, which may suggest direct transcriptional regulation through AR. Previous studies have identified AREs in the promoter region of the genes of prostatic proteins. The probasin gene has two distinct AR-binding sites in the 5' flanking region of the genome, both of which are required for androgen responses in *in vitro* assay [37]. This promoter function probably defines the androgen response of probasin in the VP, though the gene expression could be maintained without androgen in the other lobes. Androgen-dependent induction of EGF mRNA has been reported in the salivary gland of mice [38], in addition to the prostate. The 5' flanking region contains several motifs with partial homology to ARE, which may be involved in the transcriptional regulation through AR. Calreticulin protein is expressed abundantly in epithelial cells in the VP [32]. Our results quantitatively confirmed the abundance in terms of mRNA level, which is about 50 times higher that that of β-actin. It was indeed regulated by androgen in the LP/DP/AP, as well as the VP. The regulation of SCGF mRNA seems to be rapidly and tightly regulated by testosterone, since the level was increased at 1 and 4 h and then decreased at 24 h, corresponding to the change in the serum T levels after an injection. ERβ was induced only in the VP. We previously reported that ERβ in the VP was under androgen regulation [13]. Subsequently, the promoter function was identified in the 5'flanking region of the gene and was proved to account for the androgen-dependent regulation [39].

In contrast to probasin, the VP secretory proteins prostatein C3, kallikrein S3 and CRP-1 increased only after 24 h, with the mRNA levels remaining far less than the intact control levels, while the serum testosterone level rose well above the intact control level. The prostatein C3 gene contains ARE-like motifs in the upstream region and the first intron, but only the latter apparently accounts for the androgenic responses in *in vitro* reporter assay [40]. The response may not be due to a direct interaction of AR and the ARE-like motif *in vivo*. Other androgen-responsive genes examined, including IGF-1, DAD-1 and FPPS, showed similar androgen responses. In summary, with a few exceptions such as probasin in the VP, the transcriptional induction of most of the examined genes was slow and low after a testosterone injection. Castration might cause the reduction of sensitivity to androgen, or more simply, these genes may not be under the direct transcriptional regulation of androgen. The regulation is complex, and may involve androgen-dependent regeneration of prostatic cells.

In conclusion, our present analysis has outlined the lobe and gene specificities of androgen regulation of gene expression in the prostate. Our data indicate that relatively few genes seem to be directly regulated by androgen, even though many of the prostatic proteins have been considered as androgen-dependent. The present results provide a basis for exploration of the biological regulation of individual genes in the rodent prostate, as well as improving our understanding of how rodent models can best be utilized to investigate human prostate disorders.

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Reference

1. Vercaeren I, Vanaken H, Devos A, Peeters B, Verhoeven G, Heyns W (1996) Androgens transcriptionally regulate the expression of cystatin-related protein and the C3 component of prostatic binding protein in rat ventral prostate and lacrimal gland. *Endocrinology* 137: 4713–4720.
2. Spence AM, Sheppard PC, Davie JR, Matuo Y, Nishi N, McKeehan WL, Dodd JG, Matusik RJ (1989) Regulation of a bifunctional mRNA results in synthesis of secreted and nuclear probasin. *Proc Natl Acad Sci USA* 86: 7843–7847.
3. MacDonald RJ, Southard-Smith EM, Kroon E (1996) Disparate tissue-specific expression of members of the tissue kallikrein multigene family of the rat. *J Biol Chem* 271: 13684–13690.
4. Imasato Y, Onita T, Moussa M, Sakai H, Chan FL, Koropatnick J, Chin JL, Xuan JW (2001) Rodent PSP94 gene expression is more specific to the dorsolateral prostate and less sensitive to androgen ablation than probasin. *Endocrinology* 142: 2138–2146.
5. Heyns W (1990) Androgen-regulated proteins in the rat ventral prostate. *Andrologia* 22 (Suppl 1): 67–73.
6. Desai KV, Michalowska AM, Kondaiah P, Ward JM, Shih JH, Green JE (2004) Gene expression profiling identifies a unique androgen-mediated inflammatory/immune signature and a PTEN (phosphatase and tensin homolog deleted on chromosome 10)-mediated apoptotic response specific to the rat ventral prostate. *Mol Endocrinol* 18: 2895–2907.
7. Jiang F, Wang Z (2003) Identification of androgen-responsive genes in the rat ventral prostate by complementary deoxyribonucleic acid subtraction and microarray. *Endocrinology* 144: 1257–1265.
8. Leav I, Ho SM, Ofner P, Merk FB, Kwan PW, Damassa D (1988) Biochemical alterations in sex hormone-induced hyperplasia and dysplasia of the dorsolateral prostates of Noble rats. *J Natl Cancer Inst* 80: 1045–1053.
9. Hayashi N, Sugimura Y, Kawamura J, Donjacour AA, Cunha GR (1991) Morphological and functional heterogeneity in the rat prostatic gland. *Biol Reprod* 45: 308–321.
10. Claessens F, Celis L, Peeters B, Heyns W, Verhoeven G, Rombauts W (1989) Functional characterization of an androgen response element in the first intron of the C3(1) gene of prostatic binding protein. *Biochem Biophys Res Commun* 164: 833–840.
11. Devos A, De Clercq N, Vercaeren I, Heyns W, Rombauts W, Peeters B (1993) Structure of rat genes encoding androgen-regulated cystatin-related proteins (CRPs): a new member of the cystatin superfamily. *Gene* 125: 159–167.
12. Rennie PS, Bruchovsky N, Leco KJ, Sheppard PC, McQueen SA, Cheng H, Snoek R, Hamel A, Bock ME, MacDonald BS (1993) Characterization of two cis-acting DNA elements involved in the androgen regulation of the probasin gene. *Mol Endocrinol* 7: 23–36.
13. Asano K, Maruyama S, Usui T, Fujimoto N (2003) Regulation of estrogen receptor alpha and beta expression by testosterone in the rat prostate gland. *Endocr J* 50: 281–287.
14. Liu XH, Wiley HS, Meikle AW (1993) Androgens regulate proliferation of human prostate cancer cells in culture by increasing transforming growth factor-alpha (TGF-alpha) and epidermal growth factor (EGF)/TGF-alpha receptor. *J Clin Endocrinol Metab* 77: 1472–1478.
15. Drago JR (1984) The induction of NB rat prostatic carcinomas. *Anticancer Res* 4: 255–256.
16. Noble RL (1977) The development of prostatic adenocarcinoma in Nb rats following prolonged sex hormone administration. *Cancer Res* 37: 1929–1933.
17. Powell CE, Soto AM, Sonnenschein C (2001) Identification and characterization of membrane estrogen receptor from MCF7 estrogen-target cells. *J Steroid Biochem Mol Biol* 77: 97–108.
18. Woo TH, Patel BK, Cinco M, Smythe LD, Symonds ML, Norris MA, Dohnt MF (1998) Real-time homogeneous assay of rapid cycle polymerase chain reaction product for identification of Leptonema illini. *Anal Biochem* 259: 112–117.
19. Cunha GR, Donjacour AA, Cooke PS, Mee S, Bigsby RM, Higgins SJ, Sugimura Y (1987) The endocrinology and developmental biology of the prostate. *Endocr Rev* 8: 338–362.
20. Shirai T, Tamano S, Kato T, Iwasaki S, Takahashi S, Ito N (1991) Induction of invasive carcinomas in the accessory sex organs other than the ventral prostate of rats given 3,2'-dimethyl-4-aminobiphenyl and testosterone propionate. *Cancer Res* 51: 1264–1269.
21. Shirai T, Takahashi S, Cui L, Futakuchi M, Kato K, Tamano S, Imaida K (2000) Experimental prostate carcinogenesis—rodent models. *Mutat Res* 462: 219–226.
22. Berquin IM, Min Y, Wu R, Wu H, Chen YQ (2005) Expression signature of the mouse prostate. *J Biol Chem* 280: 36442–36451.
23. Prins GS (1989) Differential regulation of androgen receptors in the separate rat prostate lobes: androgen independent expression in the lateral lobe. *J Steroid Biochem* 33: 319–326.
24. Steinsapir J, Evans AC Jr, Bryhan M, Muldoon TG (1985) Androgen receptor dynamics in the rat ventral prostate. *Biochim Biophys Acta* 842: 1–11.
25. Van Doorn E, Bruchovsky N (1978) Mechanisms of replenishment of nuclear androgen receptor in rat ventral prostate. *Biochem J* 174: 9–16.