Loss of the Peroxisome Proliferation-activated Receptor gamma (PPARγ) Does Not Affect Mammary Development and Propensity for Tumor Formation but Leads to Reduced Fertility*

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The peroxisome proliferation-activated receptor gamma (PPARγ) is expressed in many cell types including mammary epithelium, ovary, macrophages, and B- and T-cells. PPARγ has an anti-proliferative effect in pre-adipocytes and mammary epithelial cells, and treatment with its ligands reduced the progression of carcinogen-induced mammary tumors in mice. Because PPARγ-null mice die in utero it has not been possible to study its role in development and tumorigenesis in vivo. To investigate whether PPARγ is required for the establishment and physiology of different cell types, a cell-specific deletion of the gene was carried out in mice using the Cre-loxP recombination system. We deleted the PPARγ gene in mammary epithelium using WAP-Cre transgenic mice and in epithelial cells, B- and T-cells, and ovary cells using MMTV-Cre mice. The presence of PPARγ was not required for functional development of the mammary gland during pregnancy and for the establishment of B- and T-cells. In addition, no increase in impaired fertility was observed. However, loss of the PPARγ gene in oocytes and granulosa cells resulted in impaired fertility. These mice have normal populations of follicles, they ovulate and develop corpora lutea. Although progesterone levels are decreased and implantation rates are reduced, the exact cause of the impaired fertility remains to be determined.

The peroxisome proliferation-activated receptor gamma (PPARγ) is a member of the nuclear receptor superfamily. It is expressed in many cell types, including adipocytes, epithelial cells, B- and T-cells, macrophages, endothelial cells, neutrophils, and smooth muscle cells (1-3). PPARγ regulates gene expression by binding as a heterodimer with retinoid X receptors (RXRs) to specific response elements (PPREs) in the promoter regions of target genes (4, 5). PPARγ ligands mediate a diversity of cellular effects, such as the regulation of adipocytes differentiation, lipid metabolism, and glucose homeostasis (6-8). A versatile array of ligands for PPARγ includes naturally occurring compounds such as fatty acids and the prostaglandin D2 metabolite 15-deoxy-A12,14-prostaglandin J2 (9). They also include synthetic compounds such as the thiazolidinedione (TZD) class of insulin-sensitizing agents that are used to treat type II diabetes (10). The extensive use of agonists in vitro has resulted in some understanding of PPARγ function in adipogenesis. However, the function of PPARγ is not restricted to adipogenesis and insulin sensitization (11, 12). In peripheral monocytes and macrophages, PPARγ agonists are reported to inhibit the production of inflammatory cytokines (13) and to stimulate lipid metabolism and transport (11). Furthermore PPARγ ligands can induce differentiation and apoptosis in breast (14-17), prostate cancer cells (18), and choriocarcinoma cells (19).

Using a traditional gene-targeting approach, PPARγ-deficient null embryos have been generated, which die at around embryonic day 10 because of defects in placental vascularization that lead to extensive myocardial thinning (20). A single PPARγ-null embryo that was rescued at term exhibited a lethal combination of pathologies, including lipodystrophy and multiple hemorrhages. Because PPARγ is found in a broad spectrum of cell types, tissue-specific gene targeting of the PPARγ gene is necessary to expand our knowledge of its physiological role. Conditional disruption of the PPARγ gene in macrophages resulted in lowered expression of ABCA1, ABCG1, and apoE and reduced cholesterol efflux (21).

In this report, the role of PPARγ was investigated by deletion of the gene in mammary epithelium, ovary, and B- and T-cells using Cre-loxP-mediated recombination. Mice were generated that carried loxP sites in the first and second intron of the PPARγ gene (21) and Cre transgenes under control of the whey acidic protein (WAP) gene promoter and the mouse mammary tumor virus long terminal repeat (MMTV-LTR) (22, 23). Through the generation of mice that carry two targeted PPARγ alleles and a Cre transgene, we were able to investigate the roles of PPARγ in mammary gland development and tumorigenesis, in the ovary and the hematopoietic system.

MATERIALS AND METHODS

Transgenic Mice—Conditional PPARγ-null mice were previously generated by floxing exon 2 of the PPARγ gene (21). These mice were...
mated with MMTV-Cre and WAP-Cre transgenic mice (22) and with ROSA26 reporter mice (24). The genotypes of the mice were determined by PCR analysis. Primers for the PPARγ gene were F (5'-ctc caa tgt ctc cca act tac-3'), R1 (5'-gat gac tca tgg aag ttg acc-3'), and R2 (5'-gta ttc tgt gct cag tgc-3'), which yielded a 225-bp band from the wild type allele and a 197-bp band from the floxed allele, or a 200-bp band from the null allele (95 °C, 30 s; 60 °C, 30 s; 72 °C, 90 s; 35 cycles). Primers for the Cre transgenes were 5'-tag agc tgt gcc agc ctc ttc c-3' (which binds in the WAP gene promoter), 5'-ggt tgc cat gat cgc gct gc-3' (which binds in the MMTV-LTR), and 5'-cat cac tct gcg cga ccg g-3' (which binds in the Cre sequence). The WAP-Cre transgene produced a 240-bp fragment and MMTV-Cre transgene yielded a 280-bp fragment (95 °C, 30 s; 65 °C, 30 s; 72 °C, 1 min; 30 cycles). The ROSA26 transgene produced a 425-bp product with primers 5'-gat ccg cgc tgg cta ccg gc-3' and 5'-gga tac tga cga aac gcc tgc c-3' (95 °C, 30 s; 65 °C, 30 s; 72 °C, 1 min; 30 cycles). All products were separated in 2% agarose Tris acetate/EDTA gels. In the study, all the control mice were PPARγ fl/fl littermates.

Northern Blot—Total RNA from mammary gland samples was isolated at different time points by TRIzol reagent (Invitrogen). Northern blots were prepared with 20 μg of total RNA per lane. The hybridization probe was an approximate 1-kb BamHI/SpeI fragment from the 3'-part of the PPARγ cDNA. The identity of the probe was confirmed by sequencing.

Histological Evaluation of Mammary Glands and Ovaries—The inguinal mammary gland was biopsied at the indicated times of development and spread on a glass slide. After fixation for 4 h in Carnoy's solution, the glands were hydrated and stained with carminalum and -cat cac tcg ttg cat cga ccg g-3

mm; 72 °C, 1 min; 30 cycles). All products were separated in 2% agarose Tris acetate/EDTA gels. In the study, all the control mice were PPARγ fl/fl littermates.

Flow Cytometry—Single cell suspensions from spleen were depleted of erythrocytes and 10⁶ cells were incubated with different combinations of antibodies for two-color fluorescence surface staining. Data were collected in a FACs calibr flow cytometer (BD PharMingen) and analyzed using CELLquest software (BD PharMingen). The following monoclonal antibodies were used: anti-B220 (clone RA3-6B2), anti-CD11b (clone MacI), anti-CD4 (clone GK1.5), anti-CD8 (clone 53-6.7) and anti-CD19 (clone B 30). All the antibodies were purchase from BD PharMingen.

RESULTS

Conditional Deletion of the PPARγ Gene in Mouse Tissues—Because PPARγ-null mice die by day E10 (20), it has not been possible to investigate the function of PPARγ in tissue development and physiology. To overcome this obstacle, we generated mice in which the PPARγ gene can be deleted in specific cell types using the Cre-loxP recombination system. Exon 2 of the PPARγ gene was flanked by loxP sites to generate PPARγ-null mice (primers F/R2). The flox band (275 bp) is from the primers F/R1 with one loxP insertion.

Flow Cytometry—Single cell suspensions from spleen were depleted of erythrocytes and 10⁶ cells were incubated with different combinations of antibodies for two-color fluorescence surface staining. Data were collected in a FACS calibr flow cytometer (BD PharMingen) and analyzed using CELLquest software (BD PharMingen). The following monoclonal antibodies were used: anti-B220 (clone RA3-6B2), anti-CD11b (clone MacI), anti-CD4 (clone GK1.5), anti-CD8 (clone 53-6.7) and anti-CD19 (clone B 30). All the antibodies were purchase from BD PharMingen.

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by PCR analysis of fl/fl; MC(F) mice. Extensive excision was observed in mammary tissue, B- and T-cells, and to a lesser extent in granulosa cells (Fig. 1B). Isolated B- and T-cells from the spleen of the conditional knockout mice exhibited a high recombination efficiency of the *PPARγ*/*H9253* gene. There was no excision of the *PPARγ*/*H9253* gene in the uterus, which demonstrated the absence of Cre recombinase expression.

*PPARγ* Is Not Required for Functional Mammary Gland Development—It has been shown that the *PPARγ* gene is expressed in both normal mammary epithelial cells and stromal cells (15). We further established the profile of *PPARγ* during mammary development by using northern blot analyses (Fig. 2A). *PPARγ* mRNA levels were high in virgin tissue and during pregnancy, decreased during lactation, and were reestablished at day 4 of involution. Highest levels of *PPARγ* mRNA were detected in cleared fat pad, which demonstrates that *PPARγ* is more abundant in stromal cells than in epithelial cells.

To investigate the role of *PPARγ* in mammmopoiesis, we monitored ductal and alveolar development as well as mammary function in fl/fl; MC and fl/fl; WC mice. Ductal elongation and branching during puberty were normal upon inactivation with both the fl/fl; MC(D) and (F) line (Fig. 2B). Similarly, the formation and differentiation of the alveolar compartment appeared normal in fl/fl; WC and fl/fl; MC(D) mice (Fig. 3), and the dams could support their litters. However, pregnancy-mediated alveolar development in fl/fl; MC(F) mice was impaired, and the fat pad was rarely filled with lobules (Fig. 3). Those dams that had only a sparsely developed lobular compartment could not nurse their pups.

To investigate whether the mammary gland phenotype in fl/fl; MC(F) mice was autonomous to the epithelium or caused by systemic defects, we performed mammary epithelial transplants. Epithelium from fl/fl; MC(F) mice was transplanted into the cleared fat pad of athymic nude mice, and mammary development was evaluated at parturition. At parturition, fl/fl; MC(F) mammary epithelium into wild type mice at day 1 lactation. Original magnification: ×200.

*PPARγ Ablation from the Epithelial Compartment Did Not Induce Mammary Tumors*—*PPARγ* is highly expressed in...
breast cancer cell lines and infiltrating ductal breast adenocarcinomas (15), and the activation of PPARγ has been known to inhibit growth and induce apoptosis and terminal differentiation of breast cancer cells \textit{in vitro} and \textit{in vivo} (15, 17). Based on these observations, we hypothesized that the loss of PPARγ would sensitize mice to tumor formation. To investigate this possibility, we observed more than 30 PPARγ fl/fl; MC mice, 20 fl/fl; WC mice, and an equal number of control mice over a period of 12 months. These mice were bred \textit{ad libidum}. None of these mice developed tumors over the 12 months. After 15 months 2 fl/fl; MC mouse and 1 control mouse developed breast tumors. These results suggest PPARγ is not a strong and dominant tumor suppressor.

Loss of PPARγ in the Ovary Results in Reduced Fertility

PPARγ has been found in bovine (27–29), rat (30, 31), and human (32) ovaries. Reverse transcription-PCR results confirmed that the PPARγ gene is also expressed in the mouse ovary (data not shown). One-third (11/35) of fl/fl; MC(F) females were infertile, and the remainder exhibited impaired fertility. On the average fl/fl; MC(F) conceived after 22 days of mating, while it took control mice only 8 days ($p < 0.01$) (Fig. 4). In addition, litter sizes of fl/fl; MC(F) dams were small (3 ± 2 pups), while there were 6 ± 3 pups in the fl/fl mice ($p < 0.01$) (Fig. 4).

To investigate the cause of the impaired fertility, we performed morphometric analyses on ovaries from 3-month-old fl/fl; MC(F) ($n = 4$) and fl/fl females ($n = 4$). There was no significant difference in the numbers of primordial (9720 ± 3595 \textit{versus} 9460 ± 5006 in fl/fl; MC(F) \textit{versus} fl/fl ovaries, $p = 0.935$), primary (2940 ± 1253 \textit{versus} 2760 ± 847, $p = 0.819$) and preantral/antral (5240 ± 1201 \textit{versus} 6620 ± 2145, $p = 0.304$) follicles. We also treated fl/fl; MC(F) ($n = 2$) and control ($n = 2$) mice with PMSG (5 IU/mouse) and hCG (5 IU/mouse) to induce superovulation. There was no difference in the number of oocytes (25 \textit{versus} 20) released in response to PMSG.

Reduced fertility of PPARγ fl/fl; MC(F) mice could also be the result of decreased levels of progesterone. We therefore measured progesterone levels in virgin mice at the estrus day ($n = 4$ in each group). Although the progesterone level in fl/fl; MC(F) mice (6.3 ± 3.1 ng/ml) was lower than that in the control (12.5 ± 7.5 ng/ml) mice, the difference was not significant ($p = 0.204$). We also measured the progesterone levels in mice in-

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\caption{Normal B- and T-cell population in spleen of PPARγ fl/fl; MMTV-Cre and control mice. Two-color FACS analysis of spleen from 6–8-week-old mice. \textit{Upper panels} show staining with anti-B220 and anti-MacI antibodies (B-cell and monocyte surface marker, respectively) and \textit{lower panels} show anti-CD8/anti-CD4 staining (T-cell marker).}
\end{figure}
jected with 5 IU of PMSG followed 48 h later by 5 IU of hCG injection. Progesterone levels were 43.6 ± 21.2 ng/ml in fl/fl; MC(F) mice and 51.5 ± 19.6 in fl/fl mice (n = 6 in each group). There was no significant difference. We collected and fixed the ovaries from these virgin and pseudopregnant mice, and measured the size of the corpus luteum. There were no differences in morphology or size of the corpus luteum between the fl/fl; MC(F) and control mice ovaries.

We also examined the number of implantations in uterus of PPARγ fl/fl; MC(F) and control mice. Implantation occurs between days 3.5 and 4 (33), and we counted implantation sites at day 6.5 postcoitus. We found six implantation sites in one of the three PPARγ fl/fl; MC(F) mice but none in the other two. In the two control mice we found five and seven implantation sites, respectively. As described earlier (Fig. 1), Cre was not expressed in the uterus, and the PPARγ gene had remained intact.

**B- and T-cells Develop in the Absence of PPARγ—**A possible role for PPARγ in the differentiation of B- and T-cells has been reported (3, 34). To address whether the development of B- and T-cells requires the presence of PPARγ, we analyzed B- and T-cell populations from spleen using FACS cytometry (Fig. 5). In control mice, B-cells constitute ~50%, T-cells 35%, and macrophages 5% of the total cells in spleen. The same ratio was observed in spleens from PPARγ fl/fl; MC(F) mice. These results suggest that PPARγ is not required for the generation of B- or T-cells.

**DISCUSSION**

A variety of functions have been attributed to PPARγ. Activation of PPARγ promotes differentiation and induces apoptosis in a broad range of human malignant cell lines, including breast cancer (15, 17), prostate cancer (18), non-small cell lung cancer (35), and liposarcomas (36). Furthermore, activation of PPARγ reduces tumor progression in xenograft models of prostate (18) and colon (37) cancers, and it induces regression or stasis of DMBA (9,10-c12-methyl-1,2-benzanthracene)-induced tumors (14, 16). In contrast with this, other studies show that activation of PPARγ promotes the development of colon tumors in C57BL/6-APC–/– mice (38, 39). The use of mice in which the PPARγ gene is inactivated should shed light on the role of PPARγ on normal development, physiology, and tumorigenesis. Because traditional PPARγ-null mice are embryonic lethal (20), we have now investigated the role of PPARγ through the deletion of the gene in several cell types using Cre-loxP-mediated recombination. Inactivation of the PPARγ gene in mammary epithelium with WAP-Cre or MMTV-Cre (D) mice did not interfere with normal development during pregnancy, and lactation was not impaired. Thus unlike other members of the steroid receptor family (40), PPARγ is not essential for ductal and lobulo-alveolar development. Furthermore, we did not observe an increased incidence of mammary tumors. This suggests that PPARγ by itself is not vital for development and is not a dominant tumor suppressor. It is possible that other members of this family, such as PPARα and PPARβ, compensate for the loss of PPARγ, similar to the pRb family (41). The expression of an active oncogene in mammary epithelium devoid of PPARγ (possibly through a transgene) will eventually establish whether PPARγ has any tumor suppressor function in the breast.

Inactivation of the PPARγ gene with an MMTV-Cre(F) transgene resulted in impaired fertility and abrogated mammary development. However, lack of functional mammary development is probably a consequence to the ovarian dysfunction for several reasons. Results of mammary epithelial transplants demonstrated the PPARγ-null epithelium could develop into a differentiated mammary gland. In situ hybridization has shown that PPARγ mRNA is present in the ovary and primarily in the granulosa cells of developing follicles, but not in the oocytes (31). Following the luteinizing hormone surge, levels of PPARγ mRNA decline suggesting a role in ovarian function (31). Furthermore, the MMTV-Cre(F) line of transgenic mice expresses Cre in oocytes, granulosa cells, and the corpora lutea, and the impaired fertility could be the result of subfunctional physiology of these cell types.

PPARγ fl/fl; MC(F) mice appeared to ovulate normally but exhibited impaired implantation. Because Cre was not expressed in uterine tissue and the PPARγ gene was intact, uterine dysfunction can be ruled out. In the mouse, secretion of progesterone from newly formed corpora lutea, accompanied by preimplantation ovarian estrogen secretion on day 4 of pregnancy, is critical for the establishment of uterine receptivity for implantation (42). The activation of PPARγ has been shown to affect progesterone production. PPARγ ligands inhibited progesterone production in cultured human and porcine granulosa cells (43); however, they stimulated the secretion of both progesterone and E2 in cultured rat granulosa cells (31). In our in vivo study, progesterone levels were reduced in virgin mice upon inactivation of the PPARγ gene in granulosa cells and the corpora lutea. These mice had normal follicle numbers and normal estrous cycles. When stimulated by PMSG/hCG injection the progesterone levels increased to the normal range, and the morphology and size of the corpora lutea were normal, suggesting the ovary was functional upon exogenous hormone challenge. Under physiological conditions, the ovarian function might not be sufficient to induce implantation, which could explain the reduced fertility of PPARγ fl/fl; MC(F) mice.

**REFERENCES**

PPARγ Loss Leads to Reduced Fertility