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14. ABSTRACT PPAR δ is a member of peroxisome proliferators activated receptors which play important role in regulating cellular metabolisms. Basal-like breast cancers accounting for ~15% of all breast cancer cases tend to be very aggressive and have poor prognosis. Basal-like breast cancers are considered to be derived from mammary stem cells. Abnormal activation of Wnt signaling is often found in human basal-like breast cancers. Here we report that PPAR δ expression is induced by Wnt signaling in mammary stem cells. Activation of PPAR δ by its ligands and fatty acids promotes the proliferation of mammary stem cells. The proliferation of mammary cancer stem cells is stimulated by PPAR δ agonists. Activation of PPAR δ promotes while its inhibition suppresses the tumorigenesis of MMTV-Wnt1 transgenic mice. We also found that PPAR δ antagonist inhibits the proliferation and anchorage-independent growth of a human basal-like breast cancer cell line.					
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INTRODUCTION

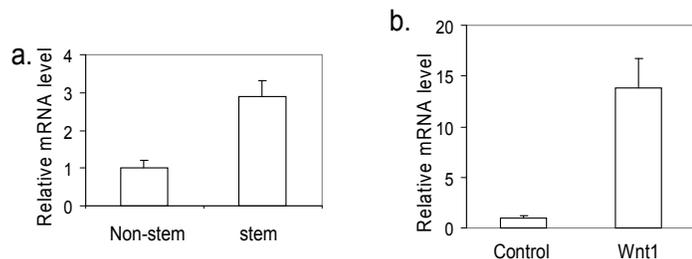
PPAR δ is a member of peroxisome proliferators activated receptors which play important role in regulating cellular metabolisms. PPAR δ can be activated by fatty acids, triglycerides, and prostacyclin. PPAR δ has been implicated in the colorectal carcinogenesis by numerous observations. PPAR δ agonist was shown to promote chemical carcinogen-induced mammary tumorigenesis. Basal-like breast cancers accounting for ~15% of all breast cancer cases tend to be very aggressive and have poor prognosis without effective treatment. Basal-like breast cancers are considered to be derived from mammary stem cells. Wnt signaling is required for the self-renewal of mammary stem cells. Overexpression of Wnt1 in mice induces the development of mammary tumors which most likely originate from stem/progenitor cells as the tumors contain both luminal and myoepithelial cells. Abnormal activation of Wnt signaling is often found in human basal-like breast cancers. The objectives of this proposal are to define the function of PPAR δ in mammary stem cells and basal-like breast cancer development.

BODY

Task 1. Determine the function of PPARD in the proliferation of cancer stem cells from triple-negative breast cancer mouse model.

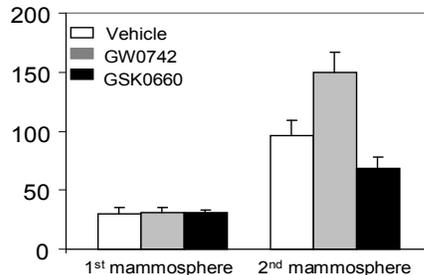
Wnt signaling induces PPAR δ expression in mammary stem cells. The mammary COMMA-1D cell line contains mammary stem cells. Our microarray analysis revealed that PPAR δ expression was dramatically increased when COMMA-1D cells expressed the activated form of β -catenin. Wnt signaling has been shown to be active in mammary stem cells. Therefore, we examined PPAR δ expression in mammary stem cells. PPAR δ expression was increased in mammary stem cell population (CD24^{med} and CD49^{high}) compared with other non-mammary stem cells (Fig. 1a). When CD24^{med} and CD49^{high} mammary epithelial cells from wild type mice were sorted out by flow cytometry. (b), Induction of PPAR δ expression by Wnt1. Real time RT-PCR was performed with RNA prepared from mammary stem cells co-cultured with NIH3T3 expressing Wnt1 or control cells.

Fig.1. (a), Increased expression of PPAR δ in mammary stem cell population compared with non-stem cells, as revealed by real-time RT-PCR. Mammary stem cell population (CD24^{med} and CD49^{high}) from wild type mice were sorted out by flow cytometry. (b), Induction of PPAR δ expression by Wnt1. Real time RT-PCR was performed with RNA prepared from mammary stem cells co-cultured with NIH3T3 expressing Wnt1 or control cells.



Activation of PPAR δ induces the proliferation of mammary stem cells. When cultured in suspension, individual mammary stem cells form mammospheres, which are composed of myoepithelial cells, luminal epithelial cells, and stem cells. We found that the number and size of primary mammospheres were not affected by the treatment of PPAR δ agonist GW0742 or antagonist GSK0660. However, the number of secondary mammospheres was increased with the treatment of PPAR δ agonist and decreased by PPAR δ antagonist (Fig. 2), suggesting that activation of PPAR δ induces the proliferation of mammary stem cells.

Fig. 2. PPAR δ agonist increased while antagonist decreased the proliferation of mammary stem cells. Mammary epithelial cells were subjected to suspension culture for mammosphere formation and treated with chemicals as indicated. The primary mammospheres were trypsinized, and equal number of cells was cultured for secondary mammosphere formation.



Arachidonic acid is more potent in inducing mammary stem cell proliferation than GW0742. As fatty acids act as agonists for PPAR δ , we examined if fatty acids could also promote proliferation of mammary stem cells. When mammary epithelial cells were treated with arachidonic acid, even more secondary mammospheres were produced compared with those treated with GW0742 (Fig. 3a). Transient transfection assay revealed that while antagonist GSK0660 completely blocks the transactivation of PPAR δ by GW0742, it only partially blocks PPAR δ activity induced by arachidonic acid (Fig. 3b). PPAR δ carries activation domain 1 (AF1) which unlike AF2 can not be inhibited by an antagonist. Therefore, we tested the effect of arachidonic acid on the activity of AF1. Arachidonic acid was found to potentiate the AF1 transcriptional activity (Fig. 3c). Saturated fatty acid palmitic acid (C16) is a weak activator of PPAR δ . Palmitic acid turns out to be a weak inducer for mammary stem cell proliferation (Fig. 3d).

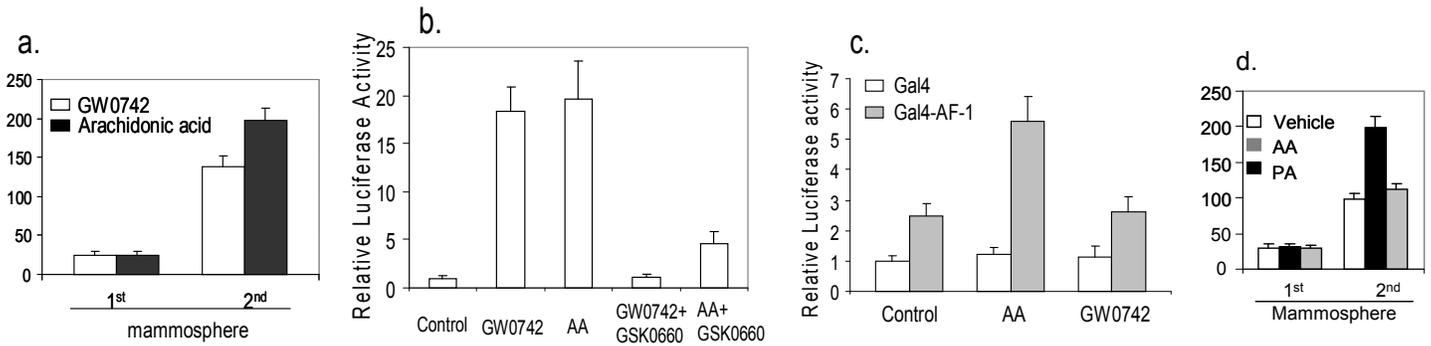
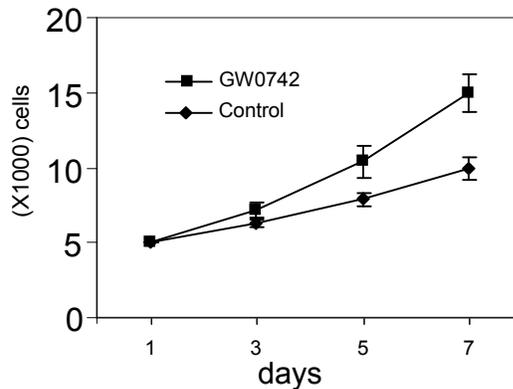


Fig. 3. (a). Arachidonic acid is more potent in inducing the proliferation of mammary stem cells than GW0472. Mammary epithelial cells were cultured for primary and secondary mammosphere formation and treated with chemicals as indicated. (b). Activation of PPAR δ by arachidonic acid (AA) can only partially be blocked by its antagonist. PPAR δ expression vector and PPARE-Luc reporter was cotransfected into CV-1 cells and treated with chemicals as indicated. (c). Arachidonic acid (AA) potentiates AF-1 activity of PPAR δ . Vectors expressing Gal4 DNA binding domain or fusion protein between Gal4 DNA binding domain and AF-1 of PPAR δ along with UAS-Luc reporter was cotransfected into CV-1 cells and treated as indicated. (d). Saturated fatty acid PA is weak in inducing mammary stem cell proliferation compared with polyunsaturated fatty acid AA. PA, Palmitic acid.

Activation of PPAR δ induces the proliferation of mammary cancer stem cells. Mammary tumors were isolated from MMTV-Wnt1 transgenic mice. The tumors were trypsinized to obtain single cells and labeled with anti-CD24/CD49f. Cancer stem cell population (CD24⁺ CD49f⁺) were sorted out by flow cytometry. Equal number of cancer stem cells was treated with control vehicle or agonist GW0742. Viable cells were counted once per two days for six days. The cells showed increased proliferation with the treatment of GW0742 (Fig. 4).

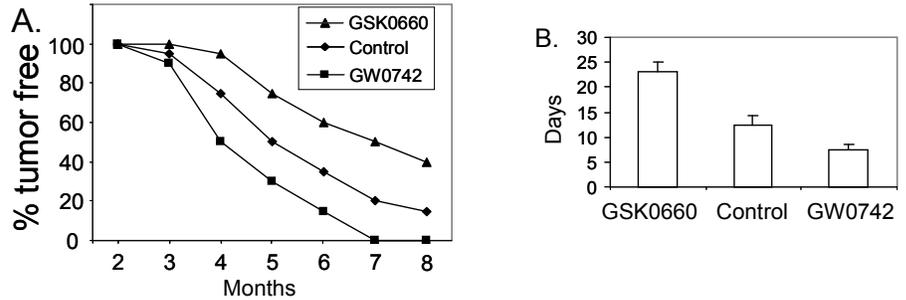
Fig. 4. PPAR δ agonist increased the proliferation of cancer stem cells. Equal number of cancer stem cells isolated through flow cytometry sorting was cultured with the treatment of GW0742 or control vehicle and counted for six days.



Task 2. Evaluate the function of PPAR δ in triple-negative breast cancer mouse model.

Activation of PPAR δ promotes while its inhibition suppresses the tumorigenesis of MMTV-Wnt1 transgenic mice. Tumors from MMTV-Wnt1 transgenic mice most likely originate from the expansion and transformation of mammary stem cells. Given that activation of PPAR δ promotes the proliferation of mammary stem cells, we treated MMTV-Wnt1 mice with PPAR δ agonist GW0742, antagonist GSK0660, or control vehicle (20 mice for each group). GW0742 promoted while GSK0660 inhibited the development of mammary tumors (Fig. 5). The histology of the tumors from GW0742 or GSK0660-treated mice and control mice was similar (data not shown). Because of the strong oncogenic effect of Wnt1, the effects of PPAR δ ligands on tumor development are relatively modest. However, for human breast cancers taking decades to develop, the impact of PPAR δ activation by fatty acids from high fat diets could be far stronger.

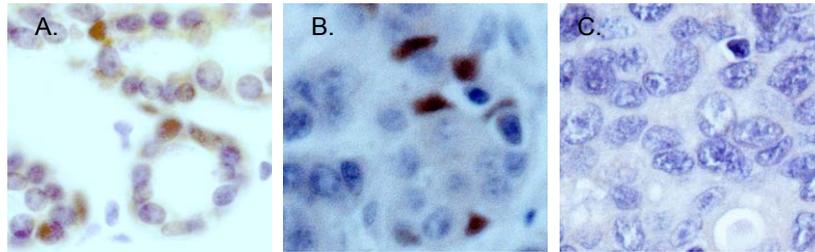
Fig. 5. PPAR δ agonist promotes while its antagonist inhibits the development of mammary tumors. (A). The onset of tumors in mice treated with GW0742 or GSK0660 compared with control mice. (B). The growth rate of tumors treated with GW0742 or GSK0660, as revealed by the time taken by tumors to reach a size of 1 cm. Differences in tumor onset and growth were statistically significant ($P < 0.05$).



Task 3. Determine the function of PPAR δ in the proliferation of cancer stem cells from human triple-negative breast cancer cell lines.

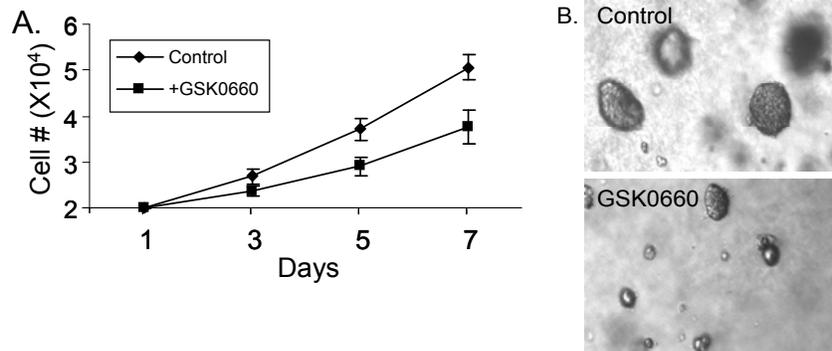
Strong expression of PPAR δ in few tumor cells in some basal-like breast cancers. Immunohistochemistry revealed that PPAR δ was expressed in both cytoplasm and nucleus in normal breast epithelial cells with strong nuclear expression in some basal cells (Fig. 6A). We examined the expression of PPAR δ in human basal-like breast cancers. High level of PPAR δ protein was detected in the nuclei of only few tumor cells in 4 of 10 tumors examined (Fig. 6B for representative picture). The few tumor cells with strong PPAR δ expression could represent cancer stem cells.

Fig. 6. Immunostaining with anti-PPAR δ . (A), normal breast tissue; (B), basal-like breast cancer expressing PPAR δ ; (C), breast cancer not expressing PPAR δ .



Suppressed proliferation of basal-like breast cancer by PPAR δ antagonist. The HCC1937 is basal-like breast cancer cell line. Treatment of HCC1937 cells with PPAR δ antagonist GSK0660 suppressed their proliferation (Fig. 7A). Furthermore, the anchorage-independent growth of HCC1937 cells was strongly inhibited by GSK0660 (Fig. 7B). Similar results were obtained from another cell line BT20.

Fig. 7. (A). PPAR δ antagonist inhibits the proliferation of HCC1937 cells. HCC1937 cells were plated at 2×10^4 cells per well and treated with $1 \mu\text{M}$ GSK0660 or control vehicle. Viable cells were counted by trypan blue staining. (B). PPAR δ antagonist suppresses the anchorage-independent growth of HCC1937 cells. HCC1937 cells were seeded in soft agar and treated with $1 \mu\text{M}$ GSK0660 or control vehicle.



KEY RESEARCH ACCOMPLISHMENTS

* We report that PPAR δ expression is induced by Wnt signaling in mammary stem/bi-progenitor cells. Activation of PPAR δ by its ligands and fatty acids promotes the proliferation of mammary stem/bi-progenitor cells. Arachidonic acid is more potent by potentiating both the N-terminal activation function AF1 and the ligand-induced activation function AF2 of PPAR δ . Saturated fatty acid palmitic acid (C16), a weak activator of PPAR δ , was found to be a weak inducer for mammary stem cell proliferation. Activation of PPAR δ induces the proliferation of mammary cancer stem cells.

* Activation of PPAR δ promotes while its inhibition suppresses the tumorigenesis of MMTV-Wnt1 transgenic mice.

* We also found that PPAR δ antagonist inhibits the proliferation and anchorage-independent growth of a human basal-like breast cancer cell line.

REPORTABLE OUTCOMES

None

CONCLUSIONS

We report that PPAR δ expression is induced by Wnt signaling in mammary stem/bi-progenitor cells. Activation of PPAR δ by its ligands and fatty acids promotes the proliferation of mammary stem/bi-progenitor cells. Arachidonic acid is more potent by potentiating both the N-terminal activation function AF1 and the ligand-induced activation function AF2 of PPAR δ . Saturated fatty acid palmitic acid (C16), a weak activator of PPAR δ , was found to be a weak inducer for mammary stem cell proliferation. Activation of PPAR δ induces the proliferation of mammary cancer stem cells. Activation of PPAR δ promotes while its inhibition suppresses the tumorigenesis of MMTV-Wnt1 transgenic mice. We also found that PPAR δ antagonist inhibits the proliferation and anchorage-independent growth of a human basal-like breast cancer cell line.

REFERENCES

None

APPENDICES

None.