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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. Activation of PPAR δ induces the proliferation of mammary cancer stem cells. PPAR δ agonist accelerates the onset of tumors and increases the growth rate of tumors in MMTV-Wnt1 transgenic. 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

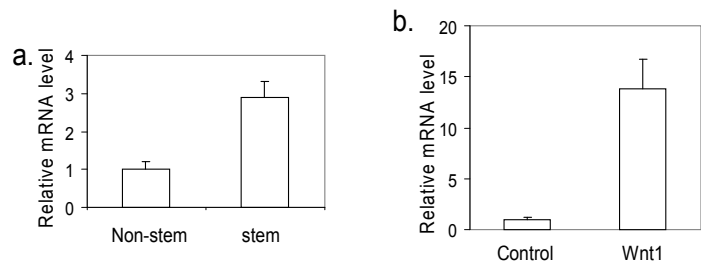
PPAR δ is a member of peroxisome proliferators activated receptors which play important role in regulating cellular metabolisms. PPAR δ can be activated by fatty acids, triglycerides, and prostacyclin. PPAR δ has been implicated in the colorectal carcinogenesis by numerous observations. PPAR δ agonist 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.

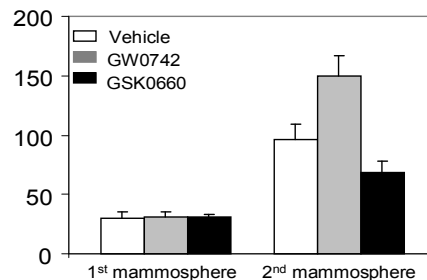
Wnt signaling induces PPARD expression in mammary stem cells. The mammary COMMA-1D cell line contains mammary stem cells. Our microarray analysis revealed that PPARD expression was dramatically increased when COMMA-1D cells expressed the activated form of β -catenin. Wnt signaling has been shown to be active in mammary stem cells. Therefore, we examined PPARD expression in mammary stem cells. PPARD expression was increased in mammary stem cell population (CD24^{med} and CD49f^{high}) compared with other non-mammary stem cells (Fig. 1a). When CD24^{med} and CD49f^{high} mammary epithelial cells from wild type mice were plated with irradiated NIH3T3 cells expressing Wnt1 or control cells as feeder cells, real time RT-PCR revealed that PPARD mRNA was markedly induced by Wnt1 (Fig. 1b). As the level of PPARD mRNA in NIH3T3 cells expressing Wnt1 was similar to that in control NIH3T3 cells (data not shown), this result indicated that PPARD expression is induced in mammary stem cells by Wnt signaling.

Fig.1. (a), Increased expression of PPARD in mammary stem cell population compared with non-stem cells, as revealed by real-time RT-PCR. Mammary stem cell population (CD24^{med} and CD49f^{high}) from wild type mice were sorted out by flow cytometry. (b), Induction of PPARD 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 PPARD 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 PPARD agonist GW0742 or antagonist GSK0660. However, the number of secondary mammospheres was increased with the treatment of PPARD agonist and decreased by PPARD antagonist (Fig. 2), suggesting that activation of PPARD induces the proliferation of mammary stem cells.

Fig. 2. PPARD 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 PPARD, 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 PPARD by GW0742, it only partially blocks PPARD activity induced by arachidonic acid (Fig. 3b). PPARD 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).

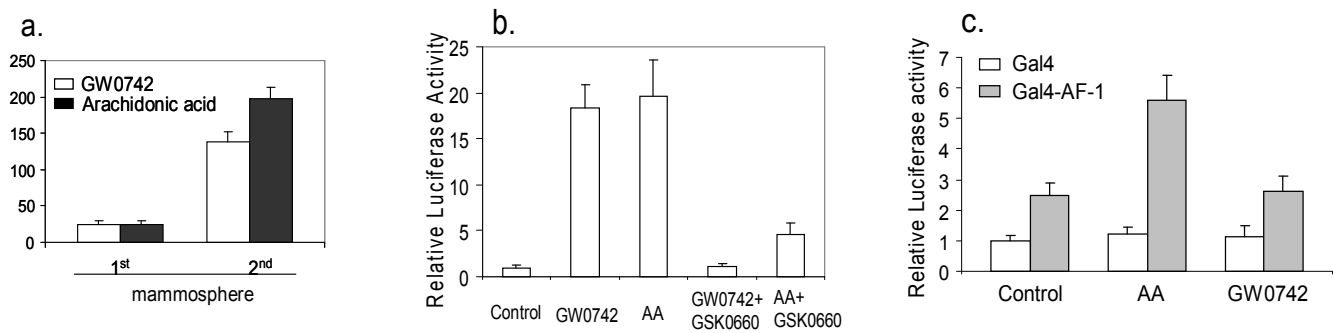
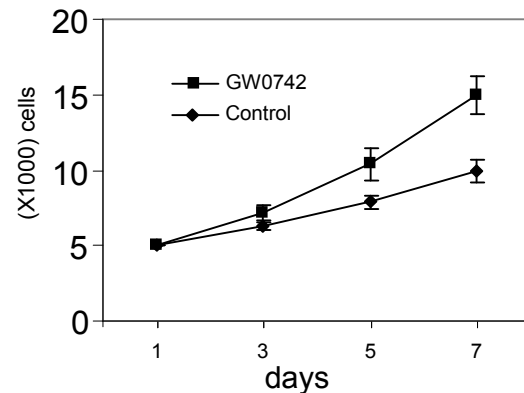


Fig. 3. (a). Arachidonic acid is more potent in inducing the proliferation of mammary stem cells than GW0472. Mammary epithelial cells were cultured for primary and secondary mammosphere formation and treated with chemicals as indicated. (b). Activation of PPAR δ by arachidonic acid (AA) can only partially 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.

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).

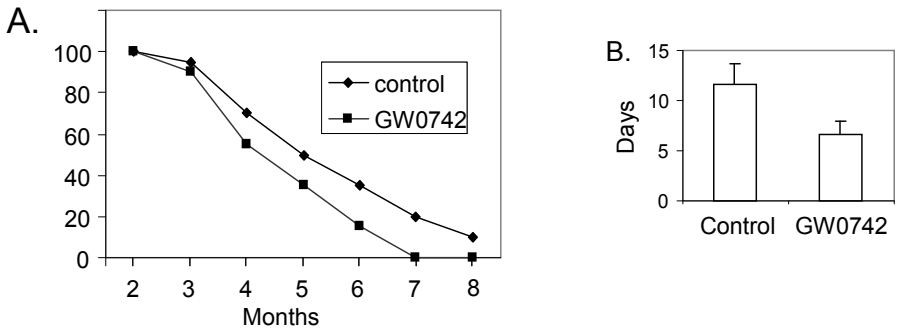
Fig. 4. PPAR δ agonist increased the proliferation of cancer stem cells. Equal number of cancer stem cells isolated through flow cytometry sorting was cultured with the treatment of GW0742 or control vehicle and counted for six days.



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

Activation of PPAR δ promotes the tumorigenesis of MMTV-Wnt1 transgenic mice. MMTV-Wnt1 transgenic mouse is a model for human triple-negative breast cancers. We treated MMTV-Wnt1 mice with PPAR δ agonist GW0742, or control vehicle (20 mice for each group). GW0742 promoted the development of mammary tumors (Fig. 5). The histology of the tumors from GW0742-treated mice and control mice was similar (data not shown). Therefore, activation of PPAR δ promotes the tumorigenesis of MMTV-Wnt1 transgenic mice. 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 the development of mammary tumors. (A). The onset of tumors in mice treated with GW0742 compared with control mice. (B). The growth rate of tumors treated with GW0742, as revealed by the time taken by tumors to reach a size of 1 cm. Differences in tumor onset and growth were statistically significant ($P < 0.05$).

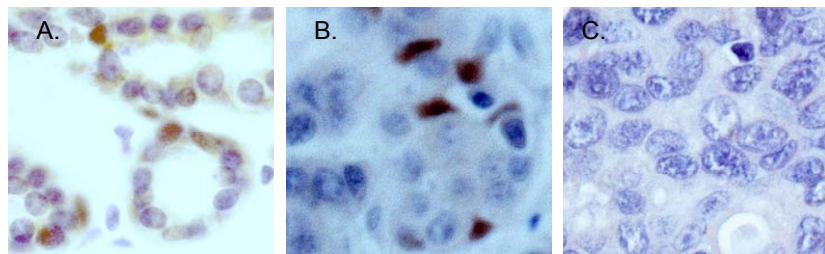


Task 3. Determine the function of PPAR δ in the proliferation of cancer stem cells from human triple-negative breast cancer cell lines.

Strong expression of PPAR δ in few tumor cells in some basal-like breast cancers.

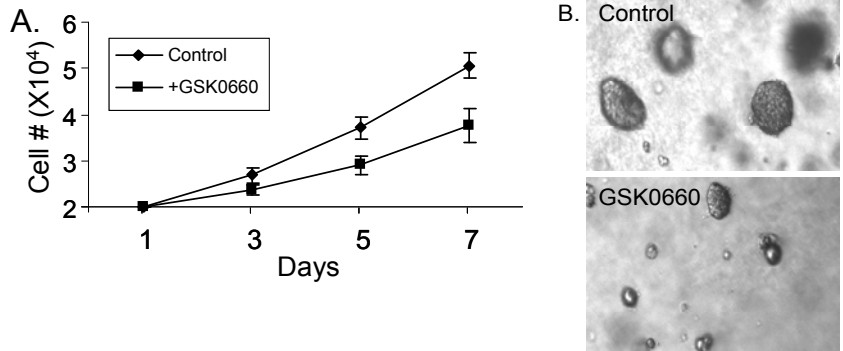
Immunohistochemistry revealed that PPAR δ was expressed in both cytoplasm and nucleus in normal breast epithelial cells with strong nuclear expression in some basal cells (Fig. 6A). We examined the expression of PPAR δ in human basal-like breast cancers. High level of PPAR δ protein was detected in the 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 and fatty acids are more potent by potentiating both the N-terminal activation function AF1 and the ligand-induced activation function AF2 of PPAR δ . Activation of PPAR δ induces the proliferation of mammary cancer stem cells.

* PPAR δ agonist accelerates the onset of tumors and increases the growth rate of tumors in MMTV-Wnt1 transgenic.

* 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 and fatty acids are more potent by potentiating both the N-terminal activation function AF1 and the ligand-induced activation function AF2 of PPAR δ . Activation of PPAR δ induces the proliferation of mammary cancer stem cells. PPAR δ agonist accelerates the onset of tumors and increases the growth rate of tumors in MMTV-Wnt1 transgenic. 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.