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PPARγ activation by thiazolidinediones (TZDs) may modulate breast carcinoma outcome: the importance of interplay with TGFβ signalling

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Abstract

The thiazolidinediones (TZDs) are a class of synthetic antidiabetic drugs exerting its action primarily upon activation of the peroxisome proliferator-activated receptor-γ (PPARγ). Given the widespread incidence of diabetes type II and lifelong exposure of these patients to TZDs, there is a possibility that chronic treatment with TZD modifies clinical phenotypes of other common human diseases, for example breast carcinoma. There is evidence that TZDs act as breast carcinoma suppression agents, at least in the *in vitro* and animal models. Stimulation of the PPARγ by TZDs interferes with oestrogen receptor signalling, STAT5B and NF-κB signalling cascades. On the other hand, TZDs repress TGFβ signalling, a well-known suppressor of the initial stages of breast carcinoma development. Another layer of complexity arises at the later stages of tumour development, when TGFβ acts as a tumour promoter: its overexpression is associated with poor prognosis, higher degree of tumour vascularization and metastasis. Longitudinal studies of breast carcinoma development in chronic TZD users are needed. In this review, we dissect possible interplays between chronic exposure of breast tissue to TZDs and TGFβ signalling and predict influence of TZD exposure on cancer-related clinical outcome.

Keywords: thiazolidinediones (TZDs) • breast carcinoma • PPARγ • TGFβ • metastasis • proliferation • diabetes • pioglitazone • rosiglitazone

Introduction

Breast cancer is a complex disease that results from a multi-stage process involving the deregulation of a number of different signalling cascades. It is also the most frequent non-skin cancer to affect women worldwide, and remains one of the top public health burdens. There are about 212,930 new cases of

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breast cancer diagnosed every year, and 40,840 related deaths in the United States alone [1]. Despite a number of recent attempts to stratify breast carcinoma into the transcriptome-based subgroups [2–4], extreme heterogeneity of these tumours still poses a real challenge. Current therapeutic modalities for breast cancer predominantly employ cytotoxic drugs prescribed with or without adjuvant therapies. Despite the fact that many risk factors have been identified for breast carcinomas, their prognostic and predictive values remain controversial. Routine investigations often identify indolent precursors for breast cancer, including lobular and ductal carcinomas in situ (LCIS and DCIS) that are often treated by biopsy-like surgery alone [5–7]. In addition, the findings of benign breast lesions, for example ductal hyperplasia and fibroadenoma, are common [8].

Both benign and malignant changes in the breast are commonly found in patients with another widespread pathology, diabetes type II. Diabetes patients are routinely treated with thiazolidinediones (TZDs), class drugs pioglitazone (Actos, Takeda/Lilly) and rosiglitazone (Avandia, Glaxo-SmithKline) exerting glucose-lowering effects. These effects are mediated primarily by decreasing insulin resistance in the muscles and thereby increasing glucose uptake. In addition, TZDs suppress glucose production in the liver. Recently, the TZD class of medications became a mainstream diabetes therapy [9]. As TZDs are also known to suppress the proliferation and induce apoptosis of breast carcinoma cells *in vitro* [10], it is likely that the breast epithelium of diabetics exposed to TZDs will also experience those modifying effects. It might translate into the changes of breast lesion incidence or in the rates of their malignization in diabetics treated with TZDs in comparison with the general population. TZD treatment may also influence the progression of existing invasive lesions that remains yet undiagnosed.

In this review, we attempt to summarize the possible influence of TZD exposure to the molecular circuitry involved in the initiation and progression of breast carcinoma. Special attention will be paid to the epigenetic interplay of TZDs with TGFβ signalling, clearly implicated in breast carcinoma development in multiple studies.

PPARγ-dependent and -independent action of TZDs

Peroxisome proliferator-activated receptors (PPARs) belong to the superfamily of nuclear hormone receptors activated by lipophilic agonists. There are three known isoforms: PPARα, PPARβ (also known PPARδ) and PPARγ [11, 12]. There are two PPARγ isoforms derived from the alternative promoters, PPARγ1 and PPARγ2. PPARγ2 isoform is longer than PPARγ1 by additional 30 N-terminal amino acids [13]. PPARγ2 mostly expresses in adipocytes while PPARγ1 is ubiquitous. Both isoforms have the intrinsic ability to stimulate adipogenesis by induction of the similar changes in the pre-adipocyte expression profile. However, PPARγ2 could be activated by lower concentrations of the ligands and is more affine to the components of the DRIP/TRAP coactivator complex [13]. The relative expression of the PPARγ isoforms in the human tumours is still under debate [14]. The quantitative and qualitative patterns of their expression in the primary and metastatic breast carcinomas need further investigation.

The general mechanisms of gene transcription modulation by PPARs are quite similar and well understood. After PPAR binds to its specific ligand, it heterodimerizes with retinoid X receptor (RXR) and binds to specific bi-hexametric DNA sequences called PPRE elements. This binding, activation and heterodimerization processes recruit various co-activators and co-repressors that modulate expression of many human genes [15, 16]. Some of the best-known co-activator proteins for PPARγ are histone acetyltransferase p300 (CBP), SRC-1, TEF2, Drip205 (Med220) and PGC-1 [17–19]. The latter binds to PPARγ in a ligand-independent manner overlapping the 'CoR box' region required for binding of co-repressors [20], recruited by PPARγ in the absence of a ligand. Both silencing mediators of retinoid and thyroid hormone receptors (SMRT) and nuclear receptor co-repressor (NCoR) are capable of down-regulating PPARγ-dependent transcriptional activity. When a suitable ligand binds PPARγ, it is complex with the co-repressor dissociates [20], thus allowing the recruitment of co-activators.

More than 70 PPAR target genes with functional PPREs have been confirmed experimentally (see

[21] for the comprehensive list). There is no doubt that this list is skewed towards the targets involved in fatty acid metabolism and adipocyte differentiation, as these two functional pathways serve as the prime focus for recent studies. A recent genome-wide search has been conducted for the high-score PPREs in conserved elements of the 5000 base pairs upstream of all human reference genes [21]. When gene ontology (GO) annotations were retrieved for each human gene returned in the search, fatty acid metabolism appeared at ninth place in the significance table according to associated z-scores. Genes involved in cell cycle arrest, apoptosis and DNA damage response were more likely to be targeted by PPARs than those involved in fatty acids metabolism. Furthermore, five out of eight positions above fatty acid metabolism were occupied by functional categories embracing the regulation of transcription and chromatin remodelling and included genes HDAC1 and HDAC3. It should be noted that the results of the computational prediction of the transcription factor binding should be used with caution, as even the best algorithms of this kind tend to give many false-positive results. The PPRE data collected in [21] await experimental investigation.

It is of interest that most of the PPAR targets revealed by various indirect means, including microarrays, are not found in the list of the genes that contain PPREs [22]. This can be explained by the activation of the PPAR targets through its direct binding to other proteins (protein–protein interactions) that, in turn, indirectly modulate target gene's transcription. For example, Hong and colleagues showed that PPAR gamma interacts with both Sp1 and Sp4 proteins in order to activate the p21 promoter [23]. In another cellular system, PPARγ has been shown to bind Sp1 directly and to suppress transcription of the thromboxane receptor gene (TXR) through this interaction [24]. Another possible explanation of the differences between PPRE-based and microarray-based PPARγ target lists is the 'two waves' model of the PPAR-dependent transcription. The first PPAR-dependent wave involves up-regulation of the primary PPARγ targets, including the chromatin-remodelling proteins necessary for implementation of the broader changes in the cellular transcriptome; these changes are characteristic for the second PPAR-dependent wave, which covers a much larger number of secondary targets.

PPARγ is predominantly expressed in adipocytes, and is also highly expressed in the prostate, breast, colon, liver, skeletal muscles and macrophages [25]. Potential endogenous ligands for PPARγ include polyunsaturated fatty acids (PUFAs) and eicosanoids, which bind to and activate this receptor in various affinities and specificities [26–28]. An anti-inflammatory prostaglandin 15-deoxy-D12,14-PGJ2 (15d-PGJ2) that is formed from PGD2 *in vivo* is probably the most potent endogenous PPARγ ligand [26–28]. Another powerful physiological PPARγ stimulator is oxidized phosphatidylcholine [29].

A number of chemically synthesized ligands for PPARγ (thiazolidinediones, or TZDs) has been discovered and introduced in clinical practice in the late 1990s as insulin sensitizers [9]. One of them, troglitazone, was withdrawn by the FDA in 2000 due to severe hepatotoxic effects it produced in some patients [30]. Two other agents, pioglitazone and rosiglitazone, are commonly used worldwide for the treatment of the insulin resistance. In May 2000, pioglitazone passed the one million prescription mark after only 8 months in the US market. As concerns for TZDs side effects remain, particularly in patients predisposed to congestive heart disease, searches for improved synthetic PPARγ activators of non-TZD type are still ongoing [31].

In addition to PPARγ-dependent action, TZDs demonstrate a number of important PPARγ-independent effects. To name a few examples, the stimulation of the proteosomal degradation of cyclins D1 and D3 [32–34], the suppression of the NHE1 channel activity resulting in cellular acidosis [35], the block of G(1)-S transition by inhibiting translation initiation [36] and scavenging effects of some TZDs on reactive oxygen species [37] have been demonstrated.

Effects of TZDs in the breast epithelium

Many research experiments have shown that PPARγ ligands suppress the proliferation rates of many types of cancer cells, particularly those derived from liposarcoma, colon cancer, breast cancer, prostate cancer, myeloid leukemia, glioblastoma and many others [38, 39]. Moreover, various *in vitro* studies have shown that treatment of many types of cancer cells with TZD resulted in the induction of cell differentiation or apoptosis, as well as improvement in levels of

various markers for invasion and metastasis [10, 38, 39]. A body of evidence indicates that human breast carcinomas might also be responsive to TZDs [10].

PPARγ is expressed both in the normal breast tissue and in many primary breast carcinoma specimens [40, 41]. Most likely, PPARγ responsiveness is preserved in clinical breast tumours, as the mutation of the PPARγ gene is a very rare event in human malignancies [42]. Therefore, if PPARγ signalling ever undergoes alterations in the tumour cells, these alterations should be pursued at the epigenetic level. Comparative studies of PPARγ expression in breast carcinoma patients so far produced contradictory results [43–45]. Since complete loss of PPARγ signalling seems to be a rare event, it is likely that TZDs may be able to modify the phenotype of breast carcinoma cells across its histological subtypes. This makes TZDs highly promising adjuvants that could be, in theory, even more practical than hormonal ablation therapy, which largely depends on the presence of the oestrogen receptor (OR) and progesterone receptors (PR) at the tumour cell surface.

TZDs and 15d-PGJ2 inhibit the growth of both normal human mammary epithelial cells [40] and breast cancer cells [46–48]. Growth suppression by TZDs is mediated by repression of cyclin D1 by PPARγ-dependent transcriptional [49] and proteosome-dependent post-translational [32, 33] mechanisms. Levels of cyclin D3 are also suppressed by similar means in breast carcinoma calls treated with TZDs [34]. Treatment of breast carcinoma MCF7 cells with therapeutic TZD rosiglitazone has been shown to increase levels of mRNA and levels of the tumour suppressor p53 and its effector p21 (WAF1/Cip1) [50]. Interestingly, in this model the binding of PPARγ to the TP53 promoter requires its interaction with nuclear factor κB (NF-κB) binding sequence, an indication of the crosstalk between PPARγ and NF-κB pathways [50]. Another proliferation-related pathway suppressed by rosiglitazone is Akt/PTEN: TZDs increase PTEN expression in MCF7 cells and decrease Akt phosphorylation [51, 52]. In addition to the direct influence on cell proliferation, TZDs inhibit Na^+/H^+ exchanger (NHE) isoform 1 and therefore induce marked cellular acidosis in breast carcinoma cell lines; this leads to a decreased number of viable cells and suppressed cell proliferation [35]. The latter effect of TZDs is independent of PPARγ transcriptional activities [35].

The growth suppressive capabilities of TZDs are complemented by their ability to induce apoptosis. Many breast tumours are naturally resistant to the apoptotic action of the tumour necrosis factor-related apoptosis-inducing ligand (TRAIL). TZDs sensitize these cells to TRAIL at least in part by reducing levels of the anti-apoptotic protein survivin [34]. It is tempting to speculate that TZDs might also sensitize malignant cells in newly developed microscopic tumours to endogenous TRAIL, thus preventing further spread. TZDs also synergize with All-trans-retinoic acid (ATRA) in order to induce apoptosis in MCF-7 and primary breast carcinoma cells, but not in the normal breast epithelium. Interestingly, ATRA alone is unable to initiate programmed death in these cells. TZDs/ATRA combination-dependent apoptosis is associated with a dramatic decrease of BCL2 protein levels [48]. Troglitazone, but not pioglitazone or rosiglitazone, up-regulates growth arrest and DNA damage-inducible gene 45 (GADD45) in a time- and dose-dependent manner [53]. It has been shown earlier that GADD45 could be also stimulated by a sudden rise in BRCA1 levels and takes part in BRCA1-induced apoptosis [54]. In turn, expression of BRCA1 itself is enhanced in response to PPARγ activation both by natural (15dPG-J2) and synthetic (rosiglitazone) ligands [55]. Finally, troglitazone directly stimulates a promoter of gene POX that encodes proline oxidase, a redox enzyme localized in the mitochondrial inner membrane that mediates apoptosis by generating a reactive oxygen species [56].

It seems that PPARγ ligands can also influence the earliest stages of breast carcinoma development, in particular, immortalization. These data were obtained in the experiments with Li-Fraumeni Syndrome (LFS)-derived ($p53^{+/-}$, telomerase silent) breast epithelial cells that have been shown to spontaneously immortalize at a relatively high frequency of approximately 5×10^{-7} [57]. Treatment of LFS-derived breast epithelial cells just before crisis with low nontoxic doses of rosiglitazone (10 nM) reduced the frequency of spontaneous immortalization to 1.33×10^{-7}. It is of interest that treatments of the same model cells with known chemopreventive agents sulindac sulfide and celecoxib resulted in less pronounced decreases in immortalization [57].

PPARγ agonists stimulate terminal differentiation of the MCF-7 breast cancer cells *in vitro* [58, 59]. Particularly, treatment of breast carcinoma cells with

TZDs causes lipid accumulation and a profound change in breast epithelial gene expression associated with a more differentiated, less malignant state [46]. Nevertheless, expression of aP2 and adipsin, well-established markers of adipogenesis, stays unchanged in these breast carcinoma cells, thus indicating that lipid accumulation in these cells did not result from 'transdifferentiation' of carcinoma cells to adipocytes. In addition, the differentiating action of PPARγ ligands on non-malignant stromal cells may also be beneficial for the patients. It is well known that peri- and intra-tumoural fibroblasts provide structural and secretory growth promoting support to tumour tissue [60]. Moreover, malignant breast epithelial cells actively participate in the process of accumulation of stromal fibroblasts around the tumour tissue, known as desmoplastic reaction. Breast carcinoma cells secrete compounds preventing the differentiation of fibroblasts to adipocytes through down-regulation of PPARγ activity in them [61]. Chronic treatment with TZDs may counteract or delay the formation of the scirrhous component of the breast tumours and the subsequent spread of tumour cells.

PPARγ activation by its ligands also possesses anti-invasive activities in tumour cells, as it inhibits gelatinase B (MMP-9) and blocks migration of macrophages and muscle cells. Low concentration treatment of the highly aggressive human breast cancer cell line MDA-MB-231 with pioglitazone, rosiglitazone or 15d-PGJ2 inhibits the invasive capacities of this cell line in a Matrigel™ basement membrane. A mechanism of invasion inhibition in this case is probably linked to the up-regulation of tissue inhibitor of MMP-1/TIMP-1 and subsequent decrease in the gelatinolytic activities [62]. In addition to invasion suppression, PPARγ ligands demonstrate strong anti-angiogenic effects (reviewed in [39]), including direct suppression of the vascular endothelial growth factor (VEGF) expression through PPRE [63], repression of the angiopoietin-1 (Ang-1) gene transcription [64], and blocking of the production of the angiogenic ELR+CXC chemokines IL-8 (CXCL8), ENA-78 (CXCL5) and Gro-α (CXCL1) [65]. Rosiglitazone also inhibits VEGF165-induced angiogenesis through increase in NO production, followed by Maxi-K channel opening and vascular cell apoptosis [59]. On the other hand, there is accumulating evidence that in the non-cancerous settings, *e.g.* in the ischemic brain and gastric ulcers, PPARγ ligands stimulate angiogenesis [66–68]. The contradictory nature of these observations might indicate that PPARγ ligands are capable of counteracting the metastatic process by remodelling the tumour vessels, a process known as vascular normalization [69].

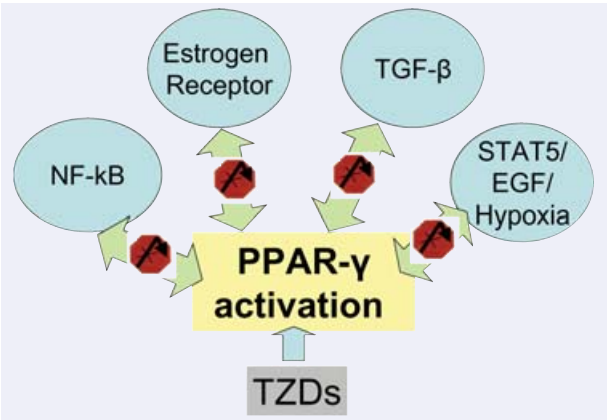

Fig. 1 TZDs activate PPARγ signalling that interferes with OR, STAT5B, TFGβ and NF-κB pathways. Most likely, this interference is mutual.

Crosstalks of the PPARγ signalling with OR, STAT5B and NF-κB pathways

PPARγ interferes with numerous cellular pathways, particularly OR, STAT5B, TFGβ and NF-κB. Most likely, the interference is mutual, as it is exerted through shared molecular components taking part in the propagation of the signal or the transcriptional regulation (Fig. 1).

A study of the immunolocalization of PPARγ in 238 human breast carcinoma samples showed that PPARγ status is significantly associated with the OR status [49]. That indicates that patients with OR-positive tumours might obtain more pronounced benefits from treatment with TZDs than the patients with OR-negative tumours. This hypothesis is further supported by findings made in the breast carcinoma cell line MCF-7 [49]. A treatment of this cell line with PPARγ ligand 15d-PGJ2 significantly inhibited OR element-dependent transcriptional activation by estradiol, which was blocked by the addition of a PPARγ antagonist GW9662 [47]. Sixteen out of 49 estradiol-dependent genes ceased to respond to that compound after treatment with the 15d-PGJ2 ligand [49]. Some important regulators of cell proliferation were

found among these genes, including intestinal trefoil factor (TFF1), cyclin D1 (CCND1) and CDK1A1 [47]. The PPARγ signalling also inhibits expression of aromatase (CYP19) that converts androgen to oestrogen, thus further reducing oestrogenic pressure on the breast epithelium. These effects of PPARγ ligands were demonstrated both in breast carcinoma cell lines [70] and in the cultured breast adipose stromal cells [71]. Most probably, an interplay between PPARγ and OR signalling involve the recently isolated scaffold attachment factor B1 (SAFB1/HET/HAP); this factor is capable of interacting with both of the above mentioned nuclear receptors and serves as a N-CoR-dependent co-repressor for ERα-dependent transcription [72, 73]. In addition to that, ciglitazone, a prototype TZD, and natural PPARγ ligand 15d-PGJ2 induce proteasome-dependent degradation of the ERα protein [33]. It is likely that binding of therapeutic TZDs to PPARγ mediate changes in the OR-mediated transcription similar to that of natural PPARγ ligands, thus mimicking the effect of oestrogen ablation.

The binding of TZDs to PPARγ suppresses the propagation of signals through the molecular pathways converging on the transcriptional factor STAT5B [74]. Phosphorylated STAT5B is a critical survival factor for normal, preneoplastic and malignant mammary epithelial cells [75]. In one study, STAT5B was found to be activated and relocated to the nucleus in 76% of breast carcinomas [76]. STAT5B becomes activated in response to epidermal growth factor (EGF) and augments expression of genes taking part in the EGF-induced DNA-synthesis [77]. Hypoxia also stimulates STAT5B-dependent transcription in mammary epithelial cells, particularly, the transcription of cyclin D1 encoding gene [78]. As PPARγ agonists suppress STATB5 activity, they might alleviate effects of EGF and hypoxia on exposed breast epitheliums.

A number of studies demonstrated the ability of PPARγ to suppress NF-αB-dependent transcription. Nuclear staining to NF-αB is a predominant finding in the OR-negative, but not in OR-positive, breast tumours [79]. Activation of NF-αB is linked to resistance to neoadjuvant chemotherapy [80]. TZDs induce a transient phosphorylation of PPARγ through the MAP kinase pathway, increasing the physical interaction of PPARγ with p65 and therefore decreasing NF-αB transcriptional activity [81]. In addition, rosiglitazone prompts SUMOylation of the ligand binding domain of the PPARγ [82]. This modification targets PPARγ to NCoR/histone deacetylase-3 (HDAC3) corepressor complexes located on the promoters of the quiescent genes that might be stimulated by NF-κB in the absence of PPARγ. Successful initiation of the transcription from these promoters requires the dismissal of the NCoR/HDAC3 complexes through their Ubc5-dependent ubiquitylation and proteosomal degradation. The recruitment of the Ubc5/19S proteosome machinery to the NCoR/HDAC3 bound promoters is prevented in the presence of the SUMOylated PPARγ. As a result, NCoR/HDAC3 complexes are not cleared from the promoter and NF-κB target genes are maintained in a repressed state [82].

PPARγ activation may interfere with TGFβ signalling

In addition to PPARγ interplays with oestrogen, EGF, NF-κB and other signalling pathways mentioned above, we would like to focus our attention on its crosstalks with transforming growth factor beta (TGFβ) signalling, an indispensable part of the molecular portrait of breast carcinoma. In the normal mammary gland, TGFβ regulates many steps of its development including branching morphogenesis, functional differentiation, cell-lineage decisions and involution [83]. It is generally accepted that TGFβ serves both as a tumour suppressor and as a tumour promoter in different tumour developmental stages and cellular contexts [84, 85]. During the initial phase of breast tumourigenesis, the TGFβ signal inhibits primary tumour development and growth by constraining cell division and possibly inducing apoptosis [85, 86]. Eventually, breast carcinoma cells cease to respond to TGFβ due to epigenetic silencing of its type I (RI) and type II (RII) receptors [87] or to aberrations in downstream SMAD signalling [88]. Both events usually cause the switch of TGFβ's role from tumour suppressor to tumour promotor; the TGFβ overproduced by tumour cells retains its ability to act on tumour stroma [89, 90] as well as on the various cellular components of the immune system [91, 92]. These effects of TGFβ promote the metastatic process by inhibiting host immune surveillance and simultaneously stimulating invasion and angiogenesis.

Accumulating evidence suggests that the activation of the PPARγ interferes with the propagation of TGFβ signalling and decreases the expression of several genes controlled by this cytokine (Fig. 2). For

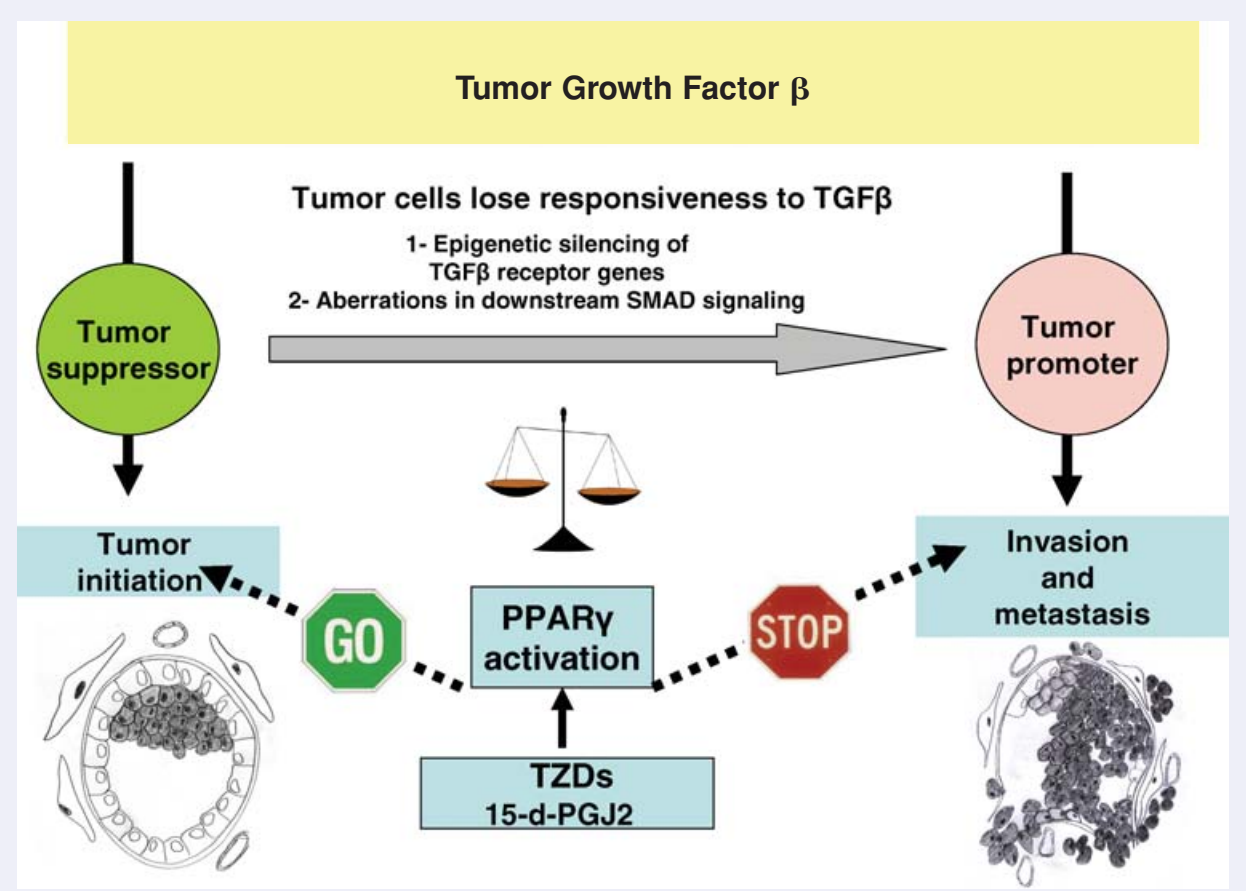

Fig. 2 During the initial phase of breast tumourigenesis, the TGFβ signal inhibits primary tumour development. Eventually, breast carcinoma cells cease to respond to TGFβ due to epigenetic silencing of its receptors or to aberrations in downstream SMAD signalling, causing the switch of TGFβ's role from tumour suppressor to tumour promoter. A crosstalk of PPARγ signalling with TGFβ pathway most likely interferes with both functions of this molecule. Most likely, the outcome of the PPARγ/TGFβ crosstalk is defined by the net effects of the TZD-related shifts in the balance of the pro-tumourigenic and tumour suppressor molecules that belong to a number of pathways affected by PPARγ.

example, pioglitazone attenuates the TGFβ driven induction of alternatively spliced mRNA and the Extra Domain A (EDA)+ protein isoform of fibronectin that are usually seen in fetal cells, tumour cells, and during wound healing, but not in normal adult cells [93]. Fibronectin molecules with EDA domains are significantly more potent in promoting local cell spread than 'classical' fibronectin [94]. EDA-containing fibronectin isoforms are present in up to 47% of breast adenocarcinoma cells and in up to 69% of adjacent stromal cells, but never found in fibroadenomas or other benign breast conditions [95]. It is possible that pioglitazone counteracts EDA^+ fibronectin-dependent invasion of breast carcinoma through the suppression of TGFβ-mediated signalling.

Both natural and synthetic PPARγ agonists suppress the conversion of fibroblasts into myofibroblasts followed by myofibroblast proliferation, the basis for desmoplastic response to invading breast carcinomas [96]. The myofibroblastic reaction is mediated by TGFβ serving as a master switch for the general fibrotic program, at least in the context of the tissue fibrosis and in some model tumours [97–99]. Myofibroblastic population is the major stromal source of extracellular matrix proteins, especially collagen, as well as of profibrogenic cytokines and

chemokines. The production of extracellular molecules and the accumulation of fibroblasts and myofibroblasts result in significant damage to the tissue architecture. PPARγ ligands inhibit both TGFβ-driven myofibroblast differentiation and type I collagen protein production by those cells without affecting their viability [96].

PPARγ activators can counteract angiogenic signals generated by connective tissue growth factor (CTGF) stimulated by TGFβ at both the transcriptional and post-transcriptional levels. Model studies performed on hepatic stellate cells revealed that TGFβ-dependent production of CTGF could be suppressed by PPARγ natural ligand 15-d-PGJ2 and by its synthetic agonist GW7845 [100]. In one study, overexpression of CTGF was registered in 55% of the primary breast tumours, where it has been associated with advanced stage of the disease, tumour size, and lymph node status [101]. Whereas the data on CTGF distribution in the breast carcinomas remain contradictory [101, 102], there is no doubt that CTGF promotes the proliferation and differentiation of the vascular endothelial cells in culture [103, 104]. Furthermore, CTGF also increases the expression of a number of metalloproteinases that play a role in vascular invasive processes and decrease the expression of tissue inhibitors of metalloproteinases (TIMPs) by vascular endothelial cells [105]. A recent study identified CTGF as a central player in the process of breast carcinoma osteolytic metastasis [106]. The treatment of mice injected with MDA231 breast carcinoma cells with CTGF-neutralizing antibody resulted in a greatly decreased number of osteolytic bone metastasis as well as in the suppression of microvasculature and osteoclastogenesis. The neutralization of CTGF also inhibits the growth of subcutaneous tumours *in vivo* and the proliferation and migration of human umbilical vein endothelial cells (HUVECs) *in vitro* [106]. It is worthy to note that expression level of CYR61, also a member of the CTGF family of growth regulators that promotes aggressive behaviour of breast carcinomas (reviewed in [107]), is also controlled by TGFβ [108]. The possibility that CYR61 production by breast carcinoma cells may be controlled by PPARγ ligands should be further studied.

PPARγ ligands are also capable of direct suppression of TGFβ production by inhibiting the expression of the TGFβ1 gene. It is interesting that both troglitazone and rosiglitazone treatments reduce TGFβ1 expression in response to high concentration of glucose, but do not change phorbol ester- and hydrogen peroxide-dependent TGFβ1 expression. According to the hypothesis proposed in two recent works [109, 110], one conceivable mechanism of TZD action is the TZD-dependent induction of diacylglycerol (DAG) kinase, which converts DAG to phosphatidic acid (thereby decreasing DAG level), and interferes with protein kinase C (PKC) activation [110]. Other studies indicate that PPARγ ligation may also block phorbol ester stimulated events, for example c-Jun-dependent AP-1 activation of the COX2 promoter in human epithelial cells [111], or ERK1/2 phosphorylation in vascular smooth muscle cells [112]. A recent study performed in mouse fibroblasts demonstrated that activation of PPARγ represses the TGFβ1 gene through dephosphorylation of transcription factor Zf9 (KLF6) [113], which also regulates expression of TGFβ receptors and collagen α (I). It is probable that active PPARγ exerts its action on Zf9 *via* induction of tumour suppressor gene PTEN that inhibits p70 ribosomal S6 Kinase-1 (S6K1) [113]. It is tempting to speculate that in addition to the suppression of the TGFβ1 promoter, inhibition of S6K1 may decrease the signal propagated by mammalian target of rapamycin (mTOR) kinase aberrantly activated in many types of human cancer, including breast carcinoma [114].

TGFβ suppressing action of activated PPARγ could be counteracted by the antagonistic action of TGFβ exerted on PPARγ signalling. Particularly, TGFβ signals decrease the expression of both C/EBPα and C/EBPβ, which are important regulators of PPARγ [115]. TGFβ also increases the level of PPARγ phosphorylation [115] and, therefore, inhibits its function as a transcription regulator. In addition to that, TGFβ suppresses activity of the PPARγ promoter through its SMAD-binding elements [116]. It is likely that the prevalence of the TGFβ or PPARγ signal in a given cell is a result of the tightly balanced molecular control. One possible candidate for the role of the molecular decision controller is Cited2 (CBP/p300-interacting transactivators with glutamic acid (E)/aspartic acid (D)-rich C-terminal domain 2), which is a CBP/p300-binding transcription co-activator without a typical DNA-binding domain. It has been demonstrated recently that Cited2 functions as a transcriptional co-activator for both TGFβ [117] and

for PPARα/PPARγ [118]. It might be possible that both TGFβ-dependent SMADs and PPARγ compete for the same pool of Cited2 molecules necessary for efficient activation of the target gene transcription by both factors.

The concept of the competitive PPARγ/ TGFβ crosstalk became more complex by observations made by VanBuskirk and coauthors [119]. It has been shown that inhibitory effects of TGFβ in accessory cells crucial for the re-stimulation of memory cytotoxic T lymphocytes are mediated by the augmented expression of PPARγ [119]. Treatment of accessory cells with ciglitazone was found to mimic the above-mentioned effects of TGFβ [119]. In monocytes, TGFβ strongly induces PPARγ2 mRNA and protein expression, with a lesser effect on PPARγ1 [120]. That means that TGFβ and PPARγ signalling cascades do not interfere with each other, but rather cooperate, at least in some cellular systems. Other studies indicate that TZDs, particularly pioglitazone, stimulate PPARγ release of TGFβ into the extracellular space and increase the nuclear recruitment of phospho-Smad2 [121]. Outcome of the TGFβ and PPARγ crosstalk may also depend on the nature of the PPARγ activation. For example, 15dPG-J2 inhibits translocation of Smad2 to the nucleus in CHO cells, while rosiglitazone enhances this process [122].

It is important to note that all observations of the crosstalk between TGFβ and PPARγ pathways were made in cultured cells. In our opinion, the outcome of this crosstalk needs to be verified in the *in vivo* carcinoma models to demonstrate which of the TGFβ effects prevail in the complex signalling milieu of the breast tissue when exposed to increased amounts of PPAR ligands.

Effects of TZDs in rodent models

The first study of the anticarcinogenic effects of TZDs in model animals was performed in 1998 [48], when immunodeficient mice were injected with breast carcinoma cells MCF-7 and simultaneously treated with troglitazone. TZD treatment alone was shown to suppress both the tumour volume and weight; observed effects were slightly more pronounced when troglitazone was used in combination with ATRA [48]. More recent model experiments involved spontaneous development of mammary tumours either in immunocompetent animals treated with various carcinogens or in animals with genetically modified PPARγ signalling. For example, in animals treated with 7,12-dimethylbenz[a]anthracene (DMBA), the experimental decrease in PPARγ signalling (caused by knocking out one of the copies of the gene PPARG) resulted in a threefold increase in the incidence of mammary adenocarcinomas ($P < 0.05$), an over threefold increase in ovarian granulosa cell carcinomas ($P < 0.05$), an over threefold increase in malignant tumours ($P < 0.02$) and a 4.6-fold increase in metastatic incidence [123]. Yin and co-authors treated FVB/N mice with medroxyprogesterone acetate followed by DMBA administration, then maintained them on either a control diet or a diet containing novel PPARγ agonist GW7845. The latter regimen conferred a chemoprotective effect as demonstrated by the 2-month delay in tumour formation for all animals [124]. These data are in concordance with the results of an earlier study that showed the ability of troglitazone, alone or in combination with RXR ligands, to prevent the induction of preneoplastic lesions by DMBA in a mouse mammary gland organ culture model [125]. In the classic rat model of mammary tumourigenesis that employs nitrosomethylurea as a carcinogen, GW7845 also significantly reduced both tumour incidence and tumour weight [126].

On the other hand, in the attempt of rosiglitazone chemoprevention of breast carcinogenesis in the MMTV-HER-2/neu transgenic mouse model, no encouraging data were produced [127]. In another study, mice that express a constitutively active form of PPARγ in the mammary gland were bred to transgenic MMTV-PyV strain prone to mammary cancer development. The development of tumours was greatly accelerated in the PPARγ/MMTV-PyV double transgenic animals, probably due to an increase in Wnt signalling [128]. One explanation for this phenomenon is that ligand-activated and Vp16-activated PPARγ may act in dissimilar ways. For example, PPARγ constitutively activated by fusion with herpes simplex virus Vp16 protein may fail to engage coactivator protein complexes and/or to displace NCoR/HDAC3 complexes in the same way as ligand-activated PPARγ. Other possible explanations include the lack of the TZD-induced SUMOylation of PPARγ in the Vp16 protein activated PPARγ mutant and the importance of the non-PPARγ mediated effects of TZDs.

Results from experiments with PPARγ chemoprevention in other tumour models were even more con-

tradictory. For example, in the ApcMin mouse model of colon carcinogenesis, troglitazone mediates an increase in the number and size of colonic tumours [129, 130] as opposed to the results obtained in similar experiments with rats. In the rat model, troglitazone alleviated azoxymethane induction of the aberrant cript foci (ACF) through stimulation of apoptosis in the colonic mucosa [131–133]. One explanation for this paradox is that the effects of PPARγ on pre-cancerous colonic mucosa depend on the state of APC protein [134]. In cells with intact APC, activated PPARγ suppresses β-catenin levels and colon carcinogenesis. In cells with mutated APC, the levels of β-catenin are constitutively elevated; so activated PPARγ can no longer serve as a colonic tumour suppressor [134]. In frame of the described model, the enhanced tumourigenesis in APCmin mice treated with PPARγ ligands observed by some authors [129, 130] could be explained by secondary, non- β-catenin mediated effects of PPARγ activation or by non-PPARγ effects of troglitazone itself. The hypothesis of APC-dependent action of PPARγ activators is supported by recent findings that demonstrated the formation of PPARγ complexes with β-catenin and Tcf-4 [135].

Another piece of information that increases the complexity of the relationship between colon carcinogenesis and PPARγ activation came from studies by Niho and co-authors. These authors demonstrated that pioglitazone inhibits formation of intestinal and colonic polyps both in Apc1309 [136] and APCmin [137] mice. Later, the same group of authors showed that polyp suppressive effects of pioglitazone in Apc-deficient mice are not dependent on its PPARγ agonistic activities, but rather rely on the non-PPARγ related boost of lipoprotein lipase activity [138]. The above-mentioned experiments clearly demonstrate that TZD effects on the tumours may be compound specific. Therefore, every potential anticancerous TZD drug needs to be studied individually, both in the chemopreventive and therapeutic settings. Precocious generalization of negative findings should be avoided.

Current and future prospects in the studies of the anticancerous effects of TZDs in human beings

The data describing the influence of the TZDs on the outcome of the pathologies of the human breast are scarce. To date, most encouraging observations were made in the recently completed PROACTIVE Study (PROspective pioglitAzone Clinical Trial In macroVascular Events) [139]. Longitudinal observations of the 5238 diabetic patients treated with pioglitazone or with placebo revealed, among other findings, a non-significant trend towards reduction of breast carcinoma incidence in the pioglitazone-treated group (3 *versus* 11 cases in equally sized pioglitazone and placebo arms of the study, respectively).

The only Phase II clinical trial of therapeutical use of the TZDs in patients with breast carcinomas was performed on the cohort of patients with advanced breast cancer refractory to at least one chemotherapy regimen. Daily oral troglitazone treatments (800 mg) of 22 patients were performed for 5 months before troglitazone withdrawal from the market. No objective responses were observed [140]. Phase I/II trial of pioglitazone in combination with tretinoin is currently underway at the Humboldt University, Germany [10], with no results of the trial made public yet. A number of case-by-case attempts of the off-label use of TZDs for the treatment of other human malignancies have been made. Some beneficial effects of TZDs were revealed in patients with resistant angiosarcomas/ hemangioendotheliomas [141], Kaposi's sarcoma [142], metastatic melanomas and soft tissue sarcomas [143]. Further research in this direction is undeniably warranted, as mentioned results were collected in non-controlled studies. A number of controlled, randomized trials for TZD therapy of the non-breast human tumours are ongoing (www.ClinicalTrials.gov).

In our opinion, the focus of the studies of TZDs in human breast carcinomas should be shifted from the 'classical' Phase II trials seeking therapeutic results to longitudinal epidemiological studies that may reveal long-term effects of exposure to TZDs. Such effects may include chemoprevention of the DCIS, LCIS, and various benign lesions of the breast, reduction in the rates of malignization for existing benign lesions, changes in the length of the non-invasive dormancy in breast tumours and modifications of its metastatic behaviour. Also, it is likely that chronic exposure to TZDs may change routes of the tumour progression; such a change might be revealed by comparative studies of the molecular portraits of breast carcinomas developed in patients treated with TZDs or in the general population.

Future longitudinal studies of breast carcinoma development in chronic TZD users need to include the collection of both epidemiological data and tissue

samples to allow the profiling of the molecular pathways. A collaborative INOVA-GMU study of this design is currently underway.

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