

# Nuclear receptors agonists exert opposing effects on the inflammation dependent survival of breast cancer stem cells

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Recent literature highlights the importance of pro-inflammatory cytokines in the biology of breast cancer stem cells (CSCs), unraveling differences with respect to their normal counterparts. Expansion of mammospheres (MS) is a valuable tool for the *in vitro* study of normal and cancer mammary gland stem cells. Here, we expanded MSs from human breast cancer and normal mammary gland tissues, as well from tumorigenic (MCF7) and non-tumorigenic (MCF10) breast cell lines. We observed that agonists for the retinoid X receptor (6-OH-11-O-hydroxyphenanthrene), retinoic acid receptor (all-*trans* retinoic acid (RA)) and peroxisome proliferator-activated receptor (PPAR)- $\gamma$  (pioglitazone (PGZ)), reduce the survival of MS generated from breast cancer tissues and MCF7 cells, but not from normal mammary gland or MCF10 cells. This phenomenon is paralleled by the hampering of pro-inflammatory Nuclear Factor- $\kappa$ B (NF- $\kappa$ B)/Interleukin-6 (IL6) axis that is hyperactive in breast cancer-derived MS. The hindrance of such pathway associates with the downregulation of MS regulatory genes (*SLUG*, *Notch3*, *Jagged1*) and with the upregulation of the differentiation markers estrogen receptor- $\alpha$  and keratin18. At variance, the PPAR $\alpha$  agonist Wy14643 promotes MS formation, upregulating NF- $\kappa$ B/IL6 axis and MS regulatory genes. These data reveal that nuclear receptors agonists (6-OH-11-O-hydroxyphenanthrene, RA, PGZ) reduce the inflammation dependent survival of breast CSCs and that PPAR $\alpha$  agonist Wy14643 exerts opposite effects on this phenotype.

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## Introduction

Breast cancer is a heterogeneous set of diseases, which constitutes the first cause of cancer mortality in women in western countries.<sup>1</sup> The existence of a minor sub-population of cancer cells, capable of initiating tumor growth in xenografts upon serial passage and to recapitulate primary tumor heterogeneity, led to hypothesize the existence of breast cancer stem cells (CSCs).<sup>2,3</sup> CSCs are likely to sustain the growth of the primary tumor mass, as well as to be responsible for disease relapse and metastatic spreading.<sup>4,5</sup> Consequently, CSCs represent the most significant target for new anti-cancer drugs.<sup>3</sup>

Cells disclosing a CSCs phenotype can be expanded *in vitro* as multicellular spheroids, named mammospheres (MS), obtained from breast cancer surgical specimens and cell lines.<sup>6–11</sup> The activity of nuclear factor- $\kappa$ B (NF- $\kappa$ B) pathway has been recognized to be of pivotal importance in MS survival.<sup>8–11</sup> In MS from aggressive breast cancer the

over-expression of interleukin-6 (IL6), an NF- $\kappa$ B controlled gene, has been reported.<sup>7</sup> In MS, IL6 upregulates survival by promoting the Notch3/Jagged1 ligand/receptor system.<sup>6,7</sup> Besides IL6, other pro-inflammatory cytokines, such as interleukin-8 (IL8) were found to contribute to breast CSCs activity and survival.<sup>5,12</sup> According to these findings, tumor necrosis factor- $\alpha$  (TNF $\alpha$ ), a major NF- $\kappa$ B inducer, was shown to enhance MS formation,<sup>8,9</sup> by upregulating SLUG, an NF- $\kappa$ B controlled regulator of the breast CSC phenotype.<sup>9,10</sup> In hematopoietic and prostate CSCs, NF- $\kappa$ B upregulation has been reported.<sup>13,14</sup> A kind of 'NF- $\kappa$ B activity addiction' has been therefore proposed to likely render CSCs more susceptible to NF- $\kappa$ B inhibitors than their normal counterparts.<sup>13–15</sup>

Retinoids are among the most effective pro-differentiating molecules and have been proposed as anticancer agents.<sup>16</sup> Interestingly, it has been shown that all-*trans* retinoic acid (RA), by inhibiting the activator protein-1, reduces MS

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**Abbreviations:** MS, mammospheres; CSCs, cancer stem cells; IIF, 6-OH-11-O-hydroxyphenanthrene; RA, all-*trans* retinoic acid; PGZ, pioglitazone; WY, Wy14643; TNF $\alpha$ , tumor necrosis factor  $\alpha$ ; NF- $\kappa$ B, nuclear factor- $\kappa$ B; IL6, interleukin-6; IL8, interleukin-8; NRs, nuclear receptors; RAR, retinoic acid receptor; RXR, retinoid X receptor; PPAR, peroxisome proliferator-activated receptor; RXRE, retinoid X response element, RARE, retinoic acid response element; PPRE, peroxisome proliferator response element; ER $\alpha$ , estrogen receptor- $\alpha$ ; KRT18, keratin-18; ApoE, apolipoprotein E

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formation and the expression of IL6.<sup>17</sup> In inflamed human chondrocytes, retinoids hamper the expression of nitric oxide synthase, cyclooxygenase-2 and of various chemokines,<sup>18</sup> thus leading to argue in favor of their anti-inflammatory activity.<sup>19</sup> The effects of retinoids are mediated by two nuclear receptors (NRs): the retinoic acid receptor (RAR) and retinoid X receptor (RXR), that both exist in three different isoforms, encoded by different genes ( $\alpha$ ,  $\beta$  and  $\gamma$ ).<sup>20</sup> 6-OH-11-O-hydroxyphenanthrene (IIF) is a recently developed RXR ligand that activates the retinoid X response element (RXRE) and exerts higher antitumor effects compared with RA without measurable side effects.<sup>21–23</sup> The RAR ligand RA activates the retinoic acid response element (RARE), which function is mediated by RAR homodimers, as well as by RAR–RXR heterodimers.<sup>20</sup> RXR receptors regulate gene expression by forming heterodimers with peroxisome proliferator activated receptors (PPAR  $\alpha$ ,  $\beta$  and  $\gamma$ ).<sup>24</sup> Activated PPAR $\gamma$  and PPAR $\alpha$  bind peroxisome proliferator DNA response element (PPRE).<sup>25</sup> Pioglitazone (PGZ), a drug of the Thiazolidinedione family, is a specific PPAR $\gamma$  agonist that inhibits inflammation and reduces breast cancer cell proliferation.<sup>26</sup> Intriguingly, PPAR $\alpha$ , which is activated by the chemical agonist Wy14643 (WY), induces both pro-inflammatory and anti-inflammatory response in target cells.<sup>27–29</sup>

Here, MS from normal and tumor breast tissues, as well as from tumorigenic MCF7 and non-tumorigenic MCF10 human breast cell lines, were exposed to RARs, RXRs and PPARs agonists. Our data point out that NRs agonists (IIF, RA and PGZ) impair MS formation. This phenomenon is paralleled by the hampering of pro-inflammatory NF- $\kappa$ B/IL6 axis and associates with the downregulation of MS regulatory genes (*SLUG*, *Notch3*, *Jagged1*).<sup>6,7,9,10</sup> We also report that, at variance with the other NRs agonists, the PPAR $\alpha$  specific agonist WY exerts a promoting role on MS formation and on the accompanying pro-inflammatory phenotype.

## Results

**NRs agonists reduce the formation of breast cancer MS, but not of their normal counterparts.** We started this investigation observing that the NRs agonists PGZ, IIF and RA induce their cognate response elements (RXRE-Luc, RARE-Luc and PPRE-Luc) in the breast tumorigenic cell line (MCF7) and in MCF7-derived MS (MCF7-MS) at higher extent than in non-tumorigenic MCF10 and MCF10-MS (Figure 1a). Then, we observed that the NRs agonists, when administered both at 24 and 72h, exert anti-proliferative effects on MCF7, but not on MCF10 cells (Figure 1b). These data were confirmed by the reduced viability in MCF7, but not in MCF10 cells, when exposed to the same treatments (Supplementary Figure 1a). Also MDA-MB-231, an estrogen receptor- $\alpha$  (ER $\alpha$ ) negative, highly tumorigenic cell line, show a reduction of viability in the same experimental setting (Supplementary Figure 1b). Similar effects on cell viability were observed under hypoxic conditions (1% pO<sub>2</sub>), which represent the environment where cancer cells are harbored *in vivo*<sup>30</sup> (Supplementary Figure 1c and Supplementary Figure 1d). Notably, in the above experiments, the RXR agonist IIF was the most active

molecule in reducing MCF7 and MDA-MB-231 cells viability, in a dose dependent manner. Upon both normoxia and hypoxia, the administration of NRs agonists to MCF7 cells was followed by an upregulation of the respective NRs receptors (*e.g.*, PPAR $\gamma$  for PGZ, RXR $\gamma$  for IIF and RAR $\alpha$  for RA), which promoters contain NRs consensus sequences<sup>31–32</sup> (Supplementary Figure 1e).

Then, we tested the impact of the three NRs agonists on MS formation capability, which is currently considered the gold standard assay to assess the breast CSC phenotype *in vitro*.<sup>6–11,33</sup> We found that all the three NRs agonists reduce the number of MCF7-MS, but not of MCF10-MS with respect to control, and that IIF is the most active molecule (Figure 1c and Supplementary Figure 2a). Moreover, we compared the activity of NRs agonists on MS from tumor mammary gland (T-MS) and normal tissue samples (N-MS) (Table 1). We observed that NRs agonists (being IIF the most active molecule) reduce T-MS but not N-MS formation (Figure 1d, Supplementary Figure 2b and Supplementary Figure 2c).

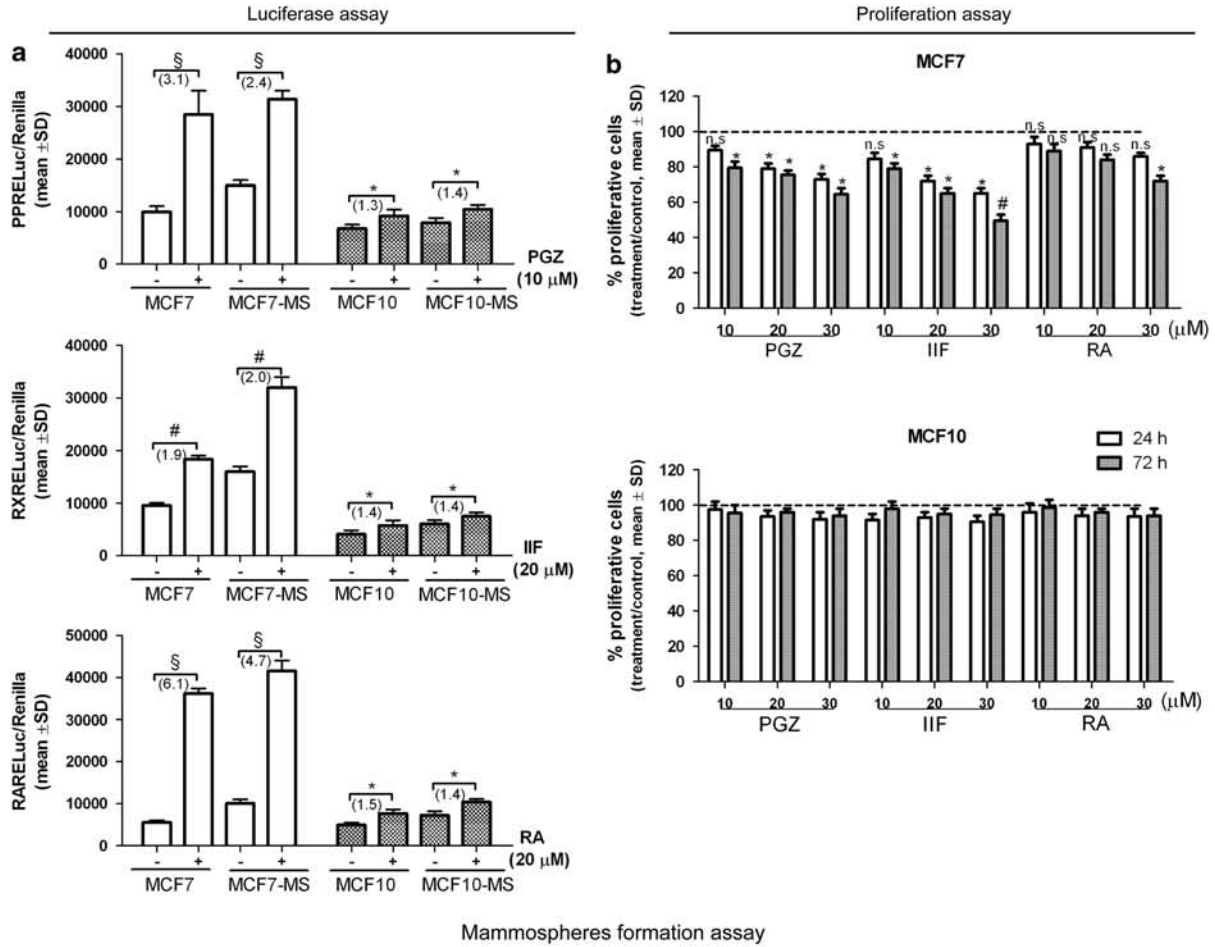
We finally proved that both in normoxia and hypoxia, NRs agonists induce cytotoxic effects in MS (Supplementary Figure 3a) and upregulate their cognate receptors (Supplementary Figure 3b). We therefore reasoned that such data underpin an higher activity of NRs agonists on CSCs compared with normal counterparts, both upon in normoxic and in hypoxic environment.

**NRs agonists reduce NF- $\kappa$ B/IL6 axis activity, downregulate MS regulatory genes and elicit differentiation markers.** MS formation has been recently found to be controlled by the activation of the pro-inflammatory NF- $\kappa$ B pathway<sup>9,11</sup> and to be sustained by NF- $\kappa$ B regulated genes, such as *SLUG*<sup>9,10</sup> and the pro-inflammatory cytokine IL6.<sup>5–7,11,12</sup> In line with these data, when compared with adherent MCF7 cells, MCF7-MS show increased levels of the NF- $\kappa$ B responsive reporter (NF- $\kappa$ BLuc), the SLUG promoter driven reporter (SLUGLuc), the IL6 promoter driven reporter (IL6Luc), but not of the IL6Luc carrying an inactive NF- $\kappa$ B-binding site, (IL6MLuc, Figure 2a). Upon exposure of MCF7 and MCF7-MS to NRs

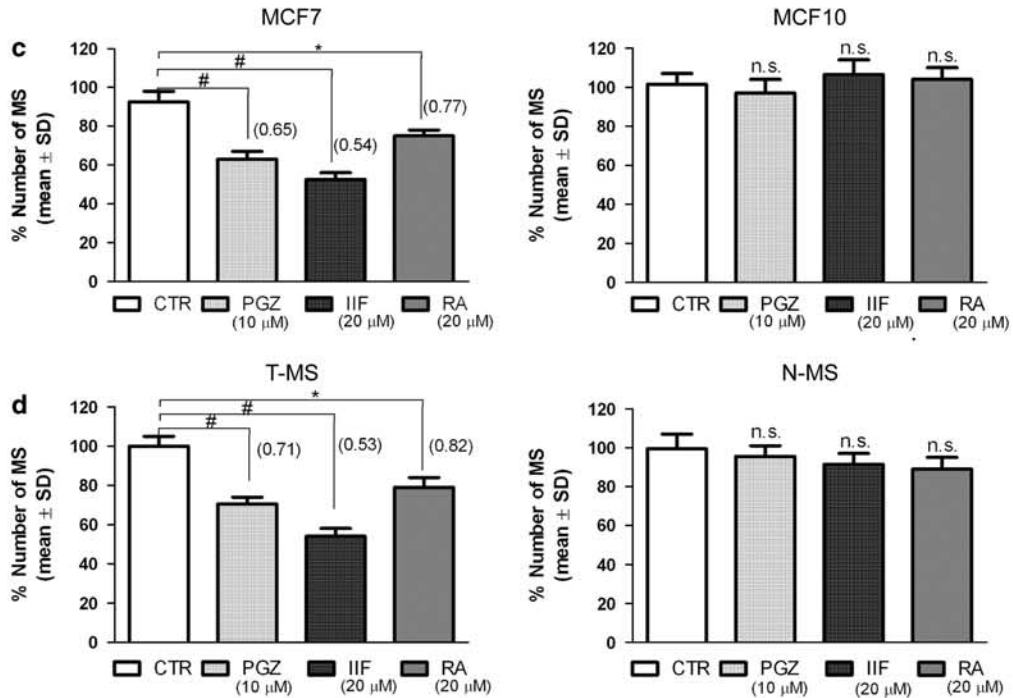
**Table 1** Clinical–pathological parameters of 14 breast carcinomas used for T-MS, N-MS and fibroblasts isolation

Sample	Age (year)	pT	pN	G	NG	ER	HER-2	EGFR
1	60	T2	N3	G1	NG1	100	0	0
2	80	T2	N0	G3	NG3	0	0	2
3	70	T2	N2	G3	NG3	0	0	2
4	67	T1	N0	G2	NG2	100	0	0
5	88	T2	N1	G1	NG2	100	0	0
6	76	T1	N0	G2	NG3	100	1	0
7	50	T1	N3	G3	NG3	0	2	0
8	47	T1	N0	G1	NG1	100	1	0
9	78	T1	N1	G2	NG3	100	0	2
10	45	T1	N3	G3	NG3	0	3	0
11	64	T2	N0	G1	NG2	100	1	0
12	41	T1	N3	G1	NG2	100	0	0
13	66	T3	N0	G3	NG3	32	0	2
14	47	T1	N0	G2	NG3	100	2	0

Abbreviations: G, grade; NG, nuclear grade; pN, nodal involvement; pT, tumor size; HER-2, ERB2 kinase receptor; EGFR, epidermal growth factor receptor.



## Mammospheres formation assay



agonists (PGZ, IIF and RA), we observed a reduction of NF- $\kappa$ BLuc, SLUGLuc and IL6Luc (but not IL6MLuc), a phenomenon that is particularly evident after IIF treatment (Figure 2b). Interestingly, ER $\alpha$ Luc activity is downregulated in MCF7-MS with respect to MCF7 adherent cells and is enhanced by the administration of IIF and RA (Figure 2c). Concomitantly, quantitative real-time PCR (qPCR) analysis in MCF7-MS exposed to NRs agonists revealed a downregulation of SLUG, IL6 and of two IL6-dependent MS regulatory genes (i.e., *Notch-3* and *Jagged-1*<sup>6,7,34</sup>), as well as an upregulation of ER $\alpha$  mRNA expression (Figure 2d). These data indicate that the NRs agonists-induced reduction in MCF7-MS formation is accompanied by a downregulation of the NF- $\kappa$ B controlled stem cell regulatory genes.

**The over-activation of NF- $\kappa$ B/IL6 axis in T-MS is hampered by NRs agonists.** As far as tissue-derived MS, we observed that IL6Luc and NF- $\kappa$ BLuc are more active in T-MS than in N-MS (Figure 3a). Accordingly, higher IL6 mRNA expression was found in T-MS with respect to N-MS (Figure 3b). Interestingly, we found that RXRE-Luc, RARE-Luc, but not PPRE-Luc activity is higher in T-MS exposed to NRs agonists compared with N-MS (Figure 3c). In line with these findings, RXR $\alpha$  and RXR $\gamma$  (targets of IIF), RAR $\alpha$  (target of RA) are expressed at substantial lower level in N-MS compared with T-MS, whereas the difference between N- and T-MS in PPAR $\alpha$  and PPAR $\gamma$  levels (targets of PGZ) are negligible (Figure 3d).

We thus exposed T-MS and N-MS to IIF, the most active molecule in the MCF7 cells model, and we found that IIF strongly downregulates IL6Luc and NF- $\kappa$ BLuc in T-MS, as well as slightly reduces NF- $\kappa$ BLuc in N-MS (Figure 3e). Accordingly, qPCR analysis revealed that IIF downregulates the NF- $\kappa$ B targets SLUG and IL6 in T-MS, but not in N-MS, in which IL6 is barely detectable (Figure 3f). In T-MS, and at a lesser extent in N-MS, IIF, as well as the other NRs agonists, strongly downregulate the IL6-dependent MS regulatory genes,<sup>6,7</sup> *Jagged1* and *Notch3*, and upregulate the expression of differentiation markers ER $\alpha$  and Keratin18 mRNA (Figure 3f).

**NRs agonists inhibit the expression of inflammatory cytokines in breast cancer-associated fibroblasts (CAF).** Stromal cells, a crucial component of the tumors milieu, are a major source of inflammatory mediators.<sup>5,12,34</sup> Here, we found that in breast CAF and in normal mammary gland fibroblasts (NF), NRs agonists downregulate NF- $\kappa$ BLuc and IL6Luc promoter activity (Figure 4a). In CAF, NRs agonists reduce the expression of a variety of inflammatory cytokines that promote MS formation,<sup>5-12</sup> namely IL6, IL8 and TNF $\alpha$  (Figure 4b). We then observed

that supernatants of CAF treated with NRs agonists disclose a reduced capability to sustain MCF7-MS formation compared with controls (Figure 4c). These data indicate that NRs agonists exert a reduction of breast CSC phenotype via the downregulation of inflammatory mediators in tumor microenvironment.

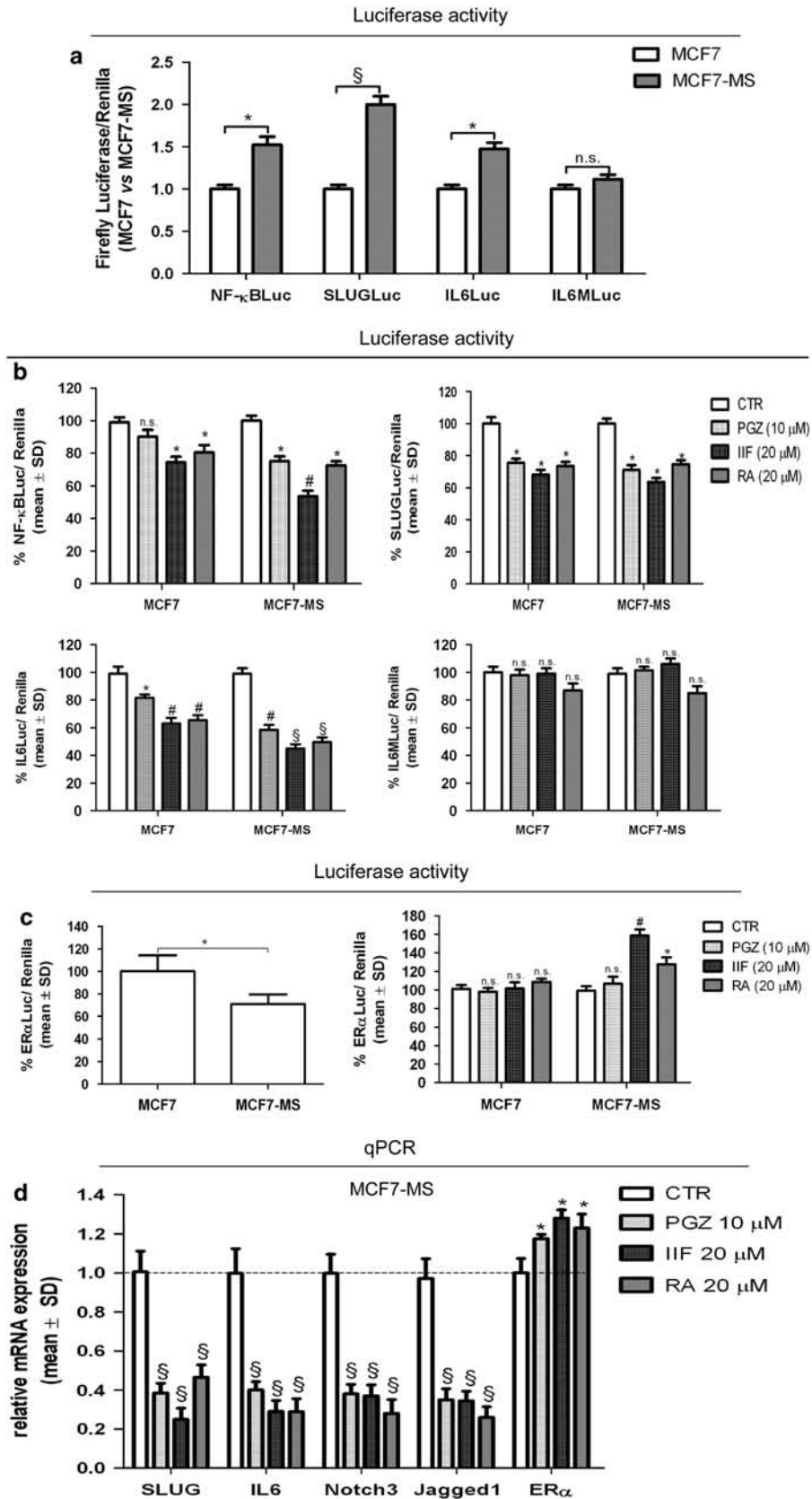
**PPAR $\alpha$  promotes MS formation and upregulates NF- $\kappa$ B/IL6 axis.**

As far as NRs expression is concerned, we observed lower expression of RXRs, RARs and PPARs levels in MCF7-MS than in adherent MCF7 cells, with the only exception of PPAR $\alpha$  level, which was higher in MCF7-MS (Figure 5a). Similar gene expression regulation was observed upon hypoxic conditions (Supplementary Figure 4). To investigate the function of PPAR $\alpha$  in MCF7-MS, we administered the PPAR $\alpha$ -specific agonist Wy14643 (WY), as well as a specific PPAR $\alpha$  siRNA. We found an increased number of MCF7-MS after WY treatment (Figure 5b). Conversely, a decreased MS formation upon PPAR $\alpha$  siRNA administration was found (Figure 5c). Indeed, in MCF7-MS cells, WY-administration triggers the activity of NF- $\kappa$ B-Luc, IL6Luc (but not IL6MLuc) and SLUGLuc reporters (Figure 6a). qPCR analysis revealed that WY also upregulates IL6, Notch3, Jagged1 and SLUG mRNA, as well as decreases ER $\alpha$  mRNA expression (Figure 6b). Consistently, PPAR $\alpha$ -siRNA transfection, which hampers p65NF- $\kappa$ B subunit expression (Supplementary Figure 5), elicits a reduction in NF- $\kappa$ BLuc, IL6Luc (but not IL6MLuc) and SLUGLuc activity (Figure 6c). qPCR analysis showed that PPAR $\alpha$ -siRNA transfection hinders IL6, Notch3, Jagged1 and SLUG mRNA, as well as upregulates ER $\alpha$  mRNA expression (Figure 6d). Interestingly, both in T-MS and N-MS WY treatment induces IL6Luc, as well as PPAR $\alpha$  siRNA downregulates IL6Luc activity (Figure 6e). These data indicate that PPAR $\alpha$ , in contrast to other NRs, promotes the inflammatory stem cell phenotype in CSCs.

**Discussion**

CSCs are believed to sustain primary growth of tumors, as well as to be responsible for metastatic spreading and growth at secondary sites.<sup>3-5,14</sup> Owing to such a pivotal role in cancer biology, CSCs are considered one of the most important targets in cancer therapy.<sup>3,13</sup> In this study, we exposed MS from normal and tumor tissues, as well as from tumorigenic MCF7 and non-tumorigenic MCF10 human breast cell lines to NRs agonists, namely the rexinoid IIF,<sup>21-23</sup> the classic retinoid receptor agonist RA and the ligand of PPAR $\gamma$  PGZ. Our first observation is that the breast CSCs (represented by tumor tissues and MCF7 derived MS) are more responsive to the NRs agonists than those obtained from normal tissues and

**Figure 1** NRs agonists reduce the formation of breast cancer derived MS, but not of their non-tumorigenic counterparts. (a) PPRE-Luc, RXRE-Luc and RARE-Luc luciferase reporters assay in MCF7, MCF7-MS, MCF10 and MCF10-MS cells in presence/absence of NRs agonists (PGZ, 10  $\mu$ M; IIF, 20  $\mu$ M; and RA, 20  $\mu$ M), respectively; (b) Cell proliferation assay (MTT) of MCF7 and MCF10 cells in presence/absence of 10 to 30  $\mu$ M of each NRs agonist for 24 and 72 h; (c) MS formation assay of MCF7 and MCF10 cells in presence/absence of NRs agonists for 24 h; (d) MS formation assay in tumor (sample no. 5) and normal (sample no. 6) human mammary gland specimen, in presence/absence of NRs agonists for 24 h. Bar: 100  $\mu$ m. Fold increase are in brackets. Data are expressed as mean  $\pm$  S.D.,  $n = 3$ , \* $P < 0.05$ , # $P < 0.01$ , § $P < 0.005$ , ANOVA test. n.s.: not significant



non-tumorigenic MCF10 cells. In particular, we show that MS forming capacity, the *in vitro* hallmark of the stem cell phenotype,<sup>6–11,33</sup> is decreased by such molecules.

We then provide evidence that NRs agonists interfere with the over-activation of the NF- $\kappa$ B/IL6 pro-inflammatory axis in CSCs, thus furnishing support to the notion that NF- $\kappa$ B activation is a hallmark of CSCs<sup>13,14</sup> and that such cells are therefore more sensitive to NF- $\kappa$ B inhibiting drugs than their normal counterparts.<sup>15</sup> Here, we also confirm previous data that tumor MS express higher levels of IL6 compared with normal ones<sup>7</sup> and that the expression of the cytokine correlates with that of the Notch3/Jagged1 stem cell regulatory axis.<sup>6,7,34</sup> SLUG, an NF- $\kappa$ B controlled pivot of the stem cell phenotype,<sup>9,10</sup> also takes part to such a regulatory activity of NRs agonists. Interestingly, SLUG is a repressor of differentiation markers,<sup>35,36</sup> and its regulation helps to explain the upregulation of KRT18 and ER $\alpha$  expression upon NRs agonists administration.

The importance of the pro-inflammatory cytokine network in breast CSC biology gained growing importance in the recent past.<sup>5–7,9–12,34</sup> Stromal cells are crucial components of the tumors milieu and are a major source of inflammatory mediators.<sup>5,12,34</sup> Here, we are able to show an NRs dependent downregulation of pro-inflammatory cytokines expression in CAF, and a concomitant reduction of their capability to sustain MS formation. Our data therefore suggest that the *in vivo* action of NRs on CSCs may involve a co-operative effect of their anti-inflammatory activity on tumor stroma. As far as the potential *in vivo* action of NRs, we verified that NR activity is maintained also in the hypoxic microenvironment, a condition which frequently occurs in tumor tissues.<sup>30</sup> This finding is of particular importance, as both normal and tumor stem cells are *in vivo* harbored in an oxygen poor environment.<sup>37</sup>

Previous data reported that RA reduces breast CSC population, while inducing differentiation.<sup>17</sup> Our data show that, among the NRs agonists tested, the RXR agonist IIF is the most active molecule in inhibiting the pro-inflammatory NF- $\kappa$ B/IL6 pathway and in inducing the expression of differentiation markers (KRT18 and ER $\alpha$ ) in breast CSCs. This finding recalls recent data showing that RXR ligands are endowed with higher antitumor efficacy compared with RAR ligands.<sup>23</sup> Such molecules selectively target tumor cells without inducing the typical *in vivo* side effects of retinoids.<sup>21,38</sup> Moreover, we found that PGZ, a ligand of PPAR $\gamma$ , is the least active molecule among the NR agonists tested, thus showing that NRs agonists are not interchangeable in their potential biological activity on breast CSCs. In this regard, we found that PPAR $\alpha$  is endowed with a significantly different biological activity compared with the other NRs, being PPAR $\alpha$  expression the only one that undergoes upregulation in MS. We show that the PPAR $\alpha$  agonist WY promotes, while PPAR $\alpha$  knock-down abolishes MS formation,

thus suggesting that PPAR $\alpha$  expression positively correlates with the CSCs phenotype. Accordingly, we found that WY upregulates the NF- $\kappa$ B/IL6 pro-inflammatory axis as well as MS regulatory genes (*SLUG*, *Jagged1*, *Notch3*).<sup>6–11</sup> These data are in agreement with a recent report that PPAR $\alpha$  agonists exert pro-inflammatory activities, and with previous findings stating that certain PPAR $\alpha$  agonists promote breast cancer cells proliferation.<sup>28,29</sup>

PPAR $\alpha$  also regulates the expression of lipoprotein receptors and cholesterol transporters, as apolipoprotein E (ApoE), that contains a PPRE sequence on its promoter.<sup>39</sup> Intriguingly, ApoE over-expression has been documented in normal MS<sup>33</sup> and in prostate CSCs.<sup>14</sup> We here observed that ApoE gene is over-expressed in T-MS compared with N-MS, and that ApoE expression is regulated by PPAR $\alpha$  expression in MCF7-MS (Supplementary Figure 6). Owing to the role of ApoE in a variety of inflammatory age-related diseases, including cancer,<sup>40</sup> these data lead to speculate that the PPAR $\alpha$ /ApoE crosstalk may take part to the pro-inflammatory CSCs phenotype, encouraging to study lipid metabolism in the context of CSCs biology.

Overall, the data here presented sustain the notion that the NF- $\kappa$ B inhibition<sup>8,13–15</sup> may constitute a suitable strategy to target CSCs. Our findings suggest to harness the capability of NRs to control such a pro-inflammatory axis to design new molecules aimed at selectively hitting CSCs (Figure 7).

## Materials and Methods

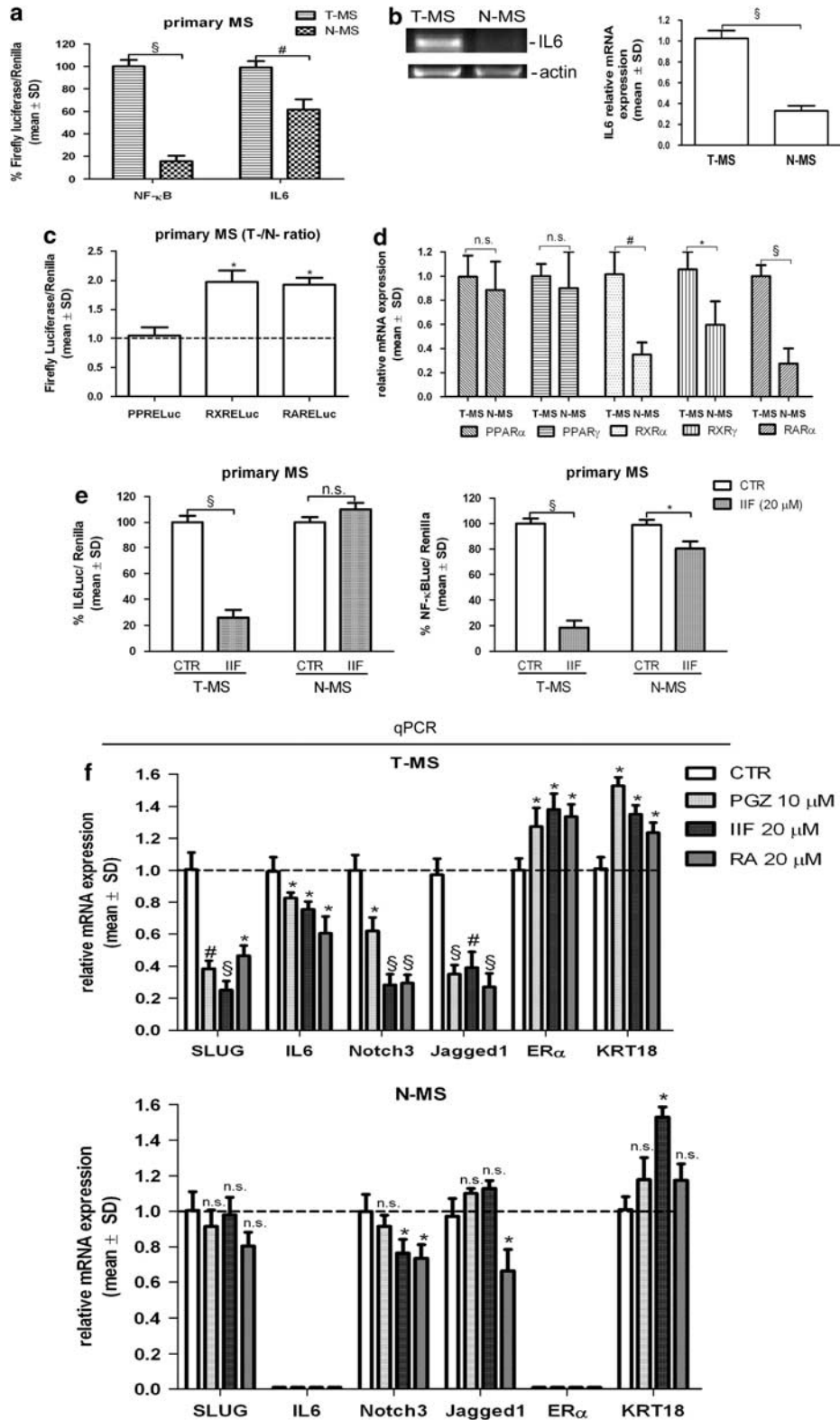
**Reagents.** 6-OH-11-O-hydroxyphenanthrene (IIF) (pat. WIPO W0 00/17143 of Dr. K Ammar, Bologna, Italy) was dissolved in propylene glycol (stock solution 0.0078 M). *All-trans-retinoic acid* (RA) (Sigma, St-Louis, MO, USA) was dissolved in ethanol (stock solution 0.01 M). PGZ (Alexis, Lausen, Switzerland) and PPAR $\alpha$  agonist Wy14643 (WY) (Sigma) were dissolved in dimethylsulfoxide (stock solution: 0.1 M). All reagents were diluted to their final concentrations (10–30  $\mu$ M) using cell culture medium. The concentration of the solvent in the highest dose of drugs did not affect cell proliferation and vitality of the cell lines.

**Generation of MS from normal and ductal breast carcinoma human tissue specimens.** Tumor samples (ductal breast carcinoma) were separated from the surrounding normal tissue, under sterile conditions, and were diagnosed as normal or neoplastic, following standard diagnostic procedures.<sup>6,7</sup> Normal and tumor tissues were processed to generate MS as previously described.<sup>6,7,33</sup> Primary MS started forming after 4–6 days and processed for secondary MS on which experiments were performed. The procedure was approved by the local ethical committee and by the patients' written informed consent.

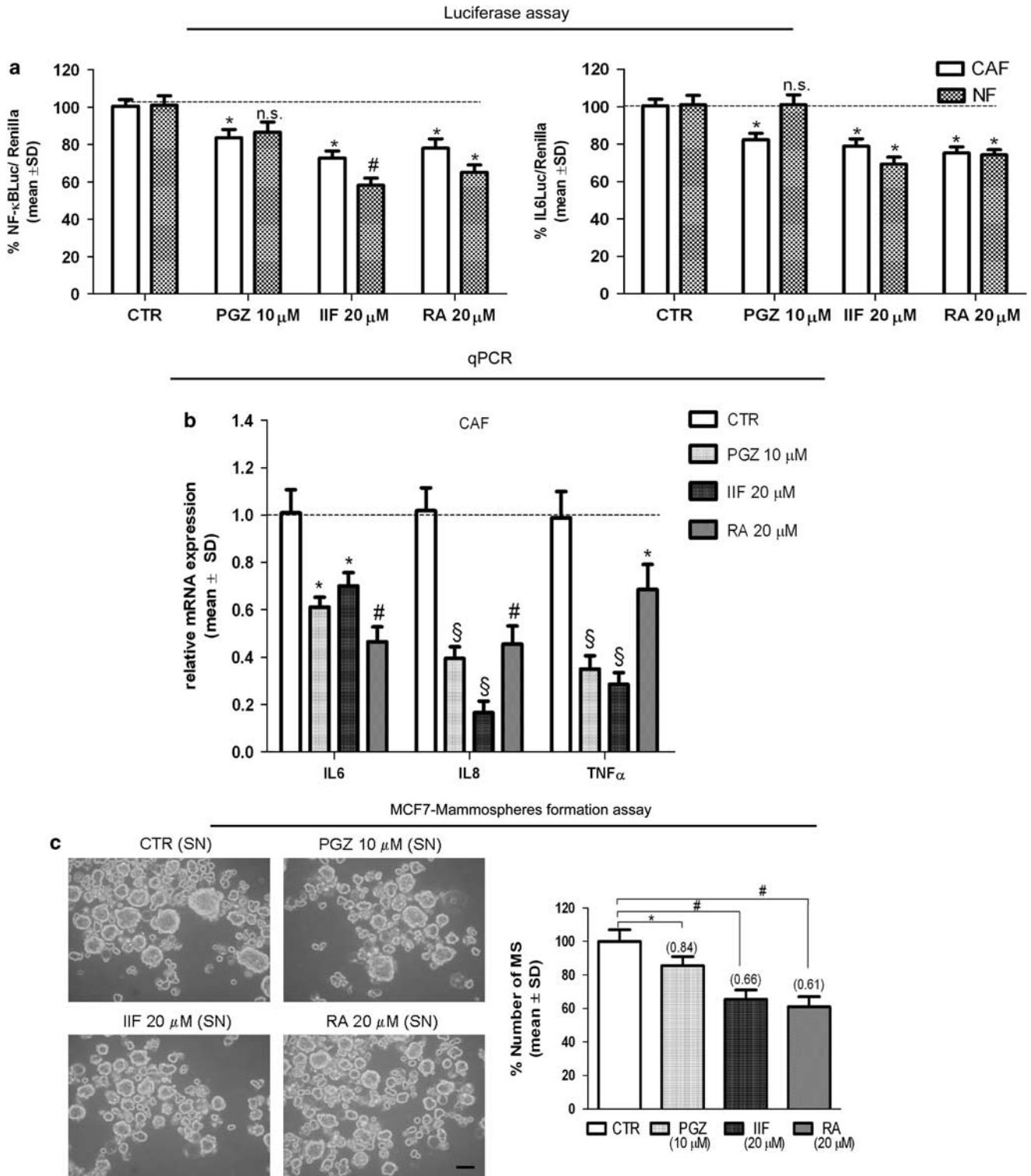
**Fibroblasts were obtained from normal and carcinoma breast human tissue specimens.** Fibroblasts were obtained from tissues processed to generate MS: after collagenase/hyaluronidase digestion, cells were centrifuged at 500 g for 5 min and the pellet containing fibroblasts was suspended in DMEM medium supplemented with 20% fetal bovine serum (FBS) (Euroclone, Milan, Italy), penicillin–streptomycin and glutamine (Sigma), seeded in six wells plates and cultured for 1 month.

**Cell cultures.** MCF7, MCF7-MS and MDA-MB-231 cells were grown in RPMI 1640 medium with 10% FBS, penicillin–streptomycin and glutamine. MCF10 cells, kindly provided by Dr. Stephan Reshkin (University of Bari, Italy), were grown in

**Figure 2** NRs agonists reduce NF- $\kappa$ B/IL6 axis activity, downregulate MS regulatory genes, and elicit differentiation markers in MCF7 cells. SLUGLuc, NF- $\kappa$ BLuc, IL6Luc and IL6MLuc assay (a) in MCF7 cells compared with MCF7-MS; (b) MCF7 cells and MCF7-MS exposed to NRs agonists for 24 h, compared with untreated cells (CTR); (c) ER $\alpha$ Luc assay in MCF7 compared with MCF7-MS and in MCF7 and MCF7-MS exposed to NRs agonists for 24 h; (d) Quantitative Real-time PCR (qPCR) analysis of SLUG, IL6, Notch3, Jagged1 and ER $\alpha$  mRNA level in MCF7-MS exposed to NRs agonists for 24 h. Data are expressed as mean  $\pm$  S.D.,  $n=3$ , \* $P<0.05$ , # $P<0.01$ ,  $^{\S}P<0.005$ , ANOVA test. n.s.: not significant



**Figure 3** The over-activation of NF-κB/IL6 axis in T-MS is hampered by NRs agonists. (a) NF-κBLuc and IL6Luc activity in T-MS versus N-MS (sample no. 12); (b) RT-PCR (sample no. 7) and qPCR (sample no. 13) analysis of IL6 mRNA in T-MS and N-MS; (c) PPRE-Luc, RXRE-Luc and RARE-Luc assay in T-MS and N-MS (samples no. 8, no. 10, no. 11), exposed to IIF 20 μM, RA 20 μM and PGZ 10 μM, respectively and expressed as T/N-MS ratio; (d) qPCR analysis of PPARα,γ, RXRα,γ and RARα mRNA level (samples no. 13 and no. 14) in T- and N-MS; (e) NF-κB and IL6 Luc assay in T-MS and N-MS exposed to IIF 20 μM for 24 h (sample no. 12); (f) qPCR analysis of SLUG, IL6, Notch3, Jagged1, ERα and KRT18 mRNA level in T-MS (sample no. 5) and N-MS (sample no. 6) exposed to NRs agonists. Data are expressed as mean ± S.D.,  $n = 3$ , \* $P < 0.05$ , # $P < 0.01$ , § $P < 0.005$ , ANOVA test. n.s.: not significant

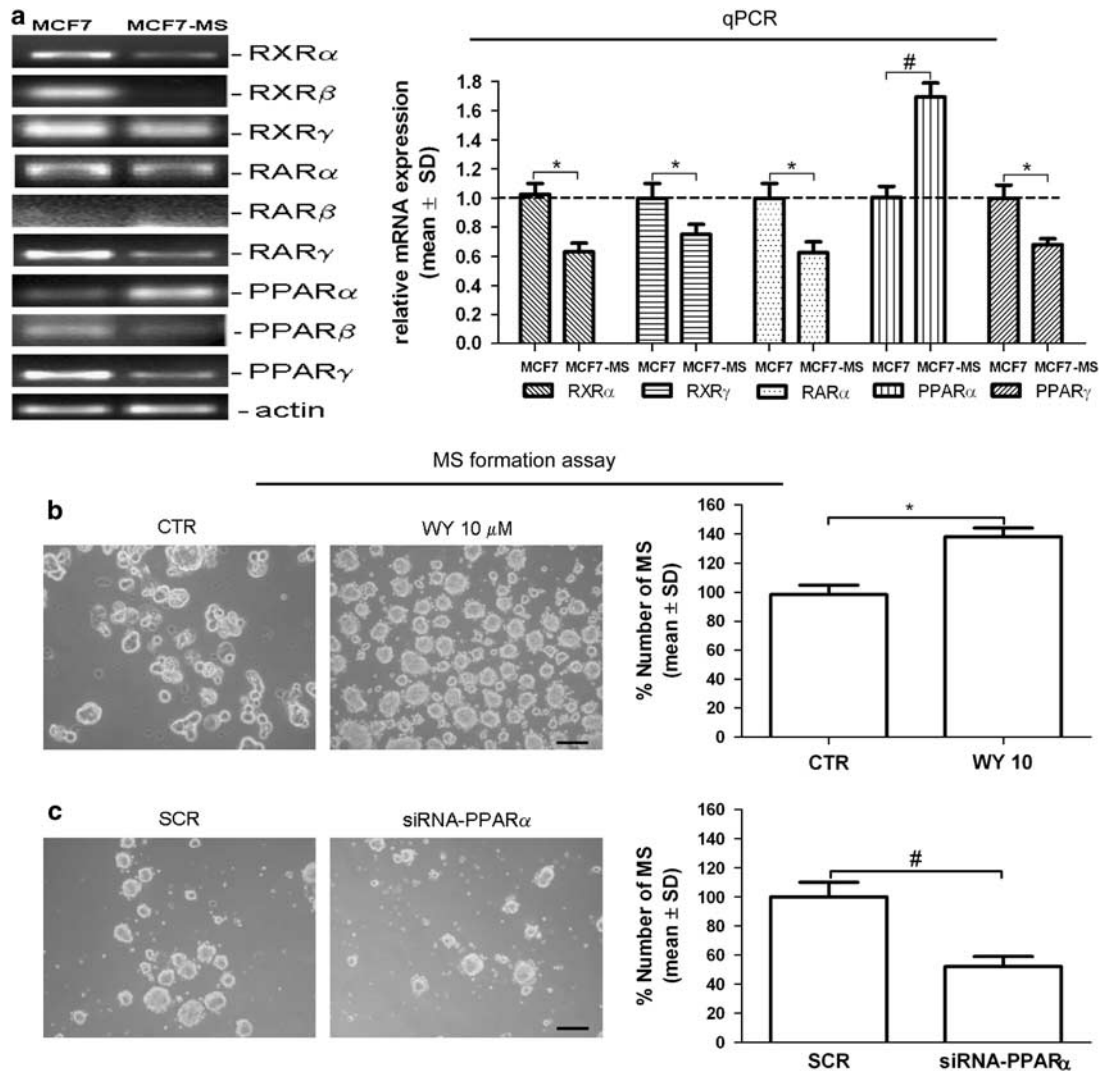


**Figure 4** NRs agonists inhibit the expression of pro-inflammatory cytokines in breast CAF. (a) NF-κB and IL6 Luc assay in breast CAFs and breast NF (sample no. 1 and 2) exposed to NRs agonists for 24 h; (b) qPCR analysis of IL6, IL8 and TNFα mRNA level in CAF (sample no. 3) exposed to NRs agonists for 24 h; (c) MCF7-MS formation assay in presence of supernatants (SN) of CAF exposed to NRs agonists for 24 h. Fold increase are in brackets. Data are expressed as mean ± S.D.,  $n = 3$ , \* $P < 0.05$ , <sup>#</sup> $P < 0.01$ , <sup>§</sup> $P < 0.005$ , ANOVA test. n.s.: not significant

DMEM medium with 20% FBS, supplemented with 10 μg/ml insulin, 10 μM hydrocortisone and 10 μg/ml epidermal growth factors. MCF7-MS and MCF10-MS were generated in to 3-cm<sup>2</sup> low-attachment wells in MS medium after 48–72 h.

Hypoxia (1% O<sub>2</sub>, 5%CO<sub>2</sub>, 94% N<sub>2</sub>) was generated in a *in vivo* 2 300 hypoxia cabinet (Ruskin Technologies, Dublin, Ireland). Experiments were performed after 24 h of hypoxic incubation.





**Figure 5** PPAR $\alpha$  promotes MS formation in MCF7 cells. (a) RT-PCR analysis of RXR $\alpha,\beta,\gamma$ , RAR $\alpha,\beta,\gamma$  and PPAR $\alpha,\beta,\gamma$  and qPCR analysis of RXR $\alpha,\gamma$ , RAR $\alpha$  and PPAR $\alpha,\gamma$  mRNA level in MCF7 cells and MCF7-MS; MCF7-MS formation assay upon (b) administration of the PPAR $\alpha$  agonist Wy14643 (WY) 10  $\mu$ M for 24 h or control (CTR); (c) transfection of scramble (SCR) or PPAR $\alpha$ -specific siRNA for 72 h. Data are expressed as mean  $\pm$  S.D.,  $n=3$ , \* $P<0.05$ , # $P<0.01$ , ANOVA test. n.s.: not significant

**Cell viability.** Cell death was measured by Trypan blue dye exclusion, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, Sulforhodamine B assay as previously described.<sup>22</sup> CellTiter-Glo assay was performed following manufacturer's instructions (Promega, Madison, WI, USA).

**RNA extraction and RT-PCR (reverse transcription-PCR).** Total RNA was extracted from cultured cells, MS and fibroblasts, frozen in liquid nitrogen using TRIzol (Invitrogen, Carlsbad, CA, USA).  $\beta$ -actin was used as an internal control. Sequences primer and parameters of PCR are in Supplementary Table 1.

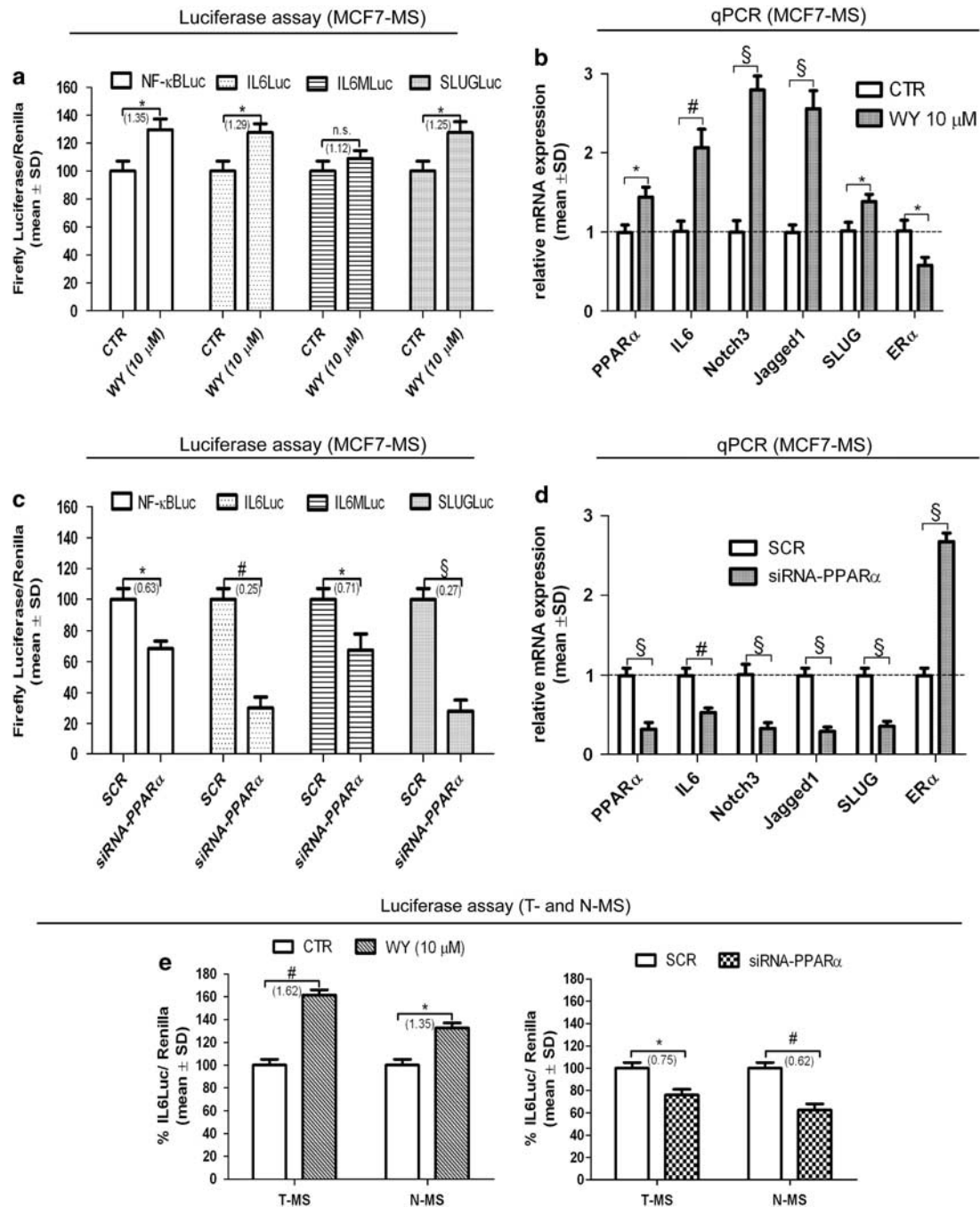
**qPCR (Real-time quantitative RT-PCR).** Real-time PCR analysis was performed by TaqMan approach in a Gene Amp 7000 Sequence Detection System (Applied Biosystems, Carlsbad, CA, USA). Each sample was analyzed in replicates. Sets of primers and fluorogenic probes specific for the target genes (Supplementary Table 2) were purchased from Applied Biosystems. The relative amount of the target genes was calculated using the expression of human  $\beta$ -glucuronidase (Applied Biosystems) as an endogenous control. The final results were determined as follows:  $N \text{ target} = 2^{-(\Delta C_t \text{ sample} - \Delta C_t \text{ calibrator})}$ , where  $\Delta C_t$  values of the

sample and calibrator were determined by subtracting the  $C_t$  value of the endogenous control gene from the  $C_t$  value of each target gene.

**Transient Transfection and RNA interference.** Double-strand RNA oligonucleotides (siRNA) directed against PPAR $\alpha$  mRNA (5'-AGAACUACGUAAU AGCUAUUUCUU-3'), and appropriate control scrambled (5'-GUACAAUUCUUAU UCUAUUUACUA-3') siRNA were purchased from Origene (Rockville, USA). siRNA was transfected to adherent MCF7 cells ( $10^5$  cells in a 3-cm<sup>2</sup> well) at a concentration of 1  $\mu$ g/well using Lipofectamine 2000 (Invitrogen). siRNA transfection in MS was performed by mixing 1  $\mu$ g siRNA with *in vitro* JET-PEI reagent (Poly plus transfection, Illkirch, France) for 72 h.

**Western-blot analysis.** Cell lysates were prepared, run and blotted using standard methodologies<sup>22</sup> and probed with specific antibodies: rabbit polyclonal anti-PPAR $\alpha$  (Cell Signaling, Danvers, MA, USA), anti-p65, (Assay Designs, Ann Arbor, MI, USA) and anti-actin (Sigma) as control.

**Luciferase assay.** PPRE-Luc, RXRE-Luc and RARE-Luc were kindly provided by Dr. Ronald Evans (Salk Institute, La Jolla, CA, USA) and Dr. D Mangelsdorf

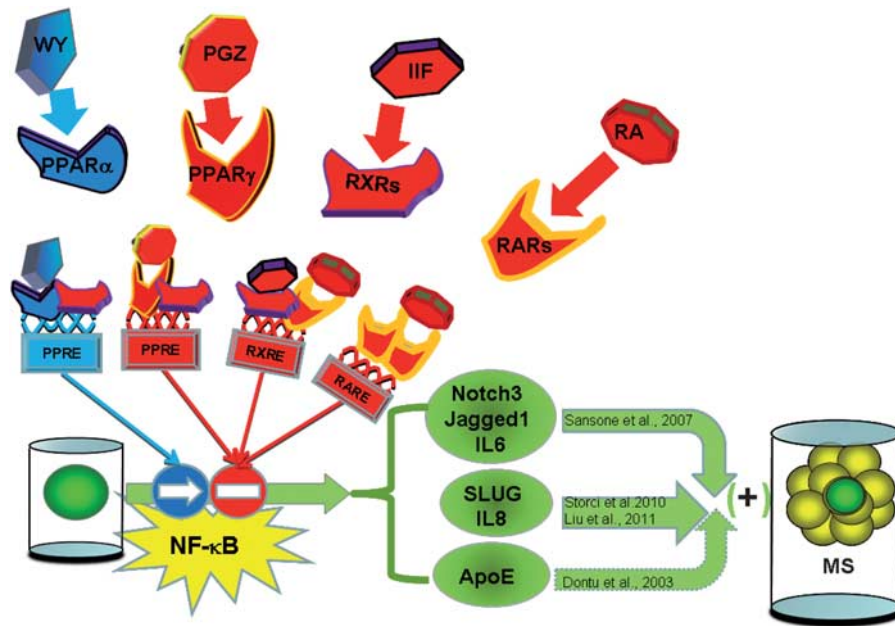


**Figure 6** PPAR $\alpha$  induces NF- $\kappa$ B/IL6 axis and MS regulatory genes. MCF7-MS in presence of WY (10  $\mu$ M for 24 h): (a) NF- $\kappa$ BLuc, IL6Luc, IL6MLuc, SLUGLuc and ER $\alpha$ Luc assay and (b) qPCR analysis of PPAR $\alpha$ , IL6, Notch3, Jagged1, SLUG and ER $\alpha$  mRNA level; SCR/PPAR $\alpha$  siRNA-transfected MCF7-MS: (c) NF- $\kappa$ BLuc, IL6Luc, IL6MLuc, SLUGLuc and ER $\alpha$ Luc assay and (d) qPCR analysis of PPAR $\alpha$ , IL6, Notch3, Jagged1, SLUG and ER $\alpha$  mRNA level; (e) IL6Luc assay in T-MS and N-MS (sample no. 9) in presence of WY (10  $\mu$ M for 24 h) or SCR/PPAR $\alpha$ -siRNA. Fold increase are in brackets. Data are expressed as mean  $\pm$  S.D.,  $n = 3$ , \* $P < 0.05$ , # $P < 0.01$ , § $P < 0.005$ , ANOVA test. n.s.: not significant

(Howard Hughes Medical Institute, Dallas, TX, USA); SLUGLuc and Estrogen Response Element (ER $\alpha$ Luc) and NF- $\kappa$ BLuc plasmids were kindly provided by Dr. Togo Ikuta (Saitama Cancer Centre, Saitama, Japan) and Dr. Rakesh Kumar (Department of Molecular and Cellular Oncology, MD Anderson Cancer Center, Houston, TX, USA) and Dr. KB Marcu, Stony Brook University, Stony Brook, NY, USA, respectively. IL6Luc and NF- $\kappa$ B-unresponsive IL6Luc (IL6MLuc) were kindly provided by Dr. WL Farrar, (NCI-Frederick Cancer Research and Development Center, Frederick, MD, USA). Each of the above plasmids (1  $\mu$ g) were transfected with Lipofectamine 2000 (Invitrogen) in co-transfection with a thymidine kinase

promoter driven Renilla luciferase (40 ng) plasmid as a reference control (Promega). Luciferase activity was assayed after 24 h using the Dual-Luciferase Reporter assay system (Promega), according to the manufacturer's instructions. Luciferase activity was normalized over Renilla activity and all reported experiments were performed in triplicate.

**Statistical analysis.** Statistical significance was assessed by ANOVA followed by Bonferroni's multiple comparison test or two-tail Student's  $t$ -test, as



**Figure 7** Schematic representation of the data. Antagonistic and agonistic effects of NRs in MS. Agonist of PPAR $\gamma$  (PGZ), of RXRs (IIF) and of RAR $\alpha$  (RA) hamper MS formation inhibit NF- $\kappa$ B/IL6 axis and switch-off MS regulatory genes (*SLUG*, *Notch3*, *Jagged1*). At variance the agonist of PPAR $\alpha$  (WY) promotes formation of MS, inducing inflammation by NF- $\kappa$ B pathway

appropriate, using PRISM 5.1. (Graphpad Software, La Jolla, CA, USA). The level for accepted statistical significance was  $P < 0.05$ .

### Conflict of Interest

The authors declare no conflict of interest.

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- Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T et al. Cancer statistics. *A Cancer J Clin* 2008; **58**: 71–96.
- Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci USA* 2003; **100**: 3983–3988.
- Liu S, Wicha MS. Targeting breast cancer stem cells. *J Clin Oncol* 2011; **28**: 4006–4012.
- Bartkowiak K, Wiczorek M, Buck F, Harder S, Moldenhauer J, Effenberger KE et al. Two-dimensional differential gel electrophoresis of a cell line derived from a breast cancer micrometastasis revealed a stem/progenitor cell protein profile. *J Proteome Res* 2009; **8**: 2004–2014.
- Korkaya H, Liu S, Wicha MS. Breast cancer stem cells, cytokine networks, and the tumor microenvironment. *J Clin Invest* 2011; **3**: 3804–3809.
- Sansone P, Storci G, Giovannini C, Pandolfi S, Pianetti S, Taffurelli M et al. p66Shc/Notch-3 interplay controls self-renewal and hypoxia survival in human stem/progenitor cells of the mammary gland expanded *in vitro* as mammospheres. *Stem Cells* 2007; **25**: 807–815.
- Sansone P, Storci G, Tavolari S, Guarneri T, Giovannini C, Taffurelli M et al. IL6 triggers malignant features in mammospheres from human ductal breast carcinoma and normal mammary gland. *J Clin Invest* 2007; **12**: 3988–4002.
- Zhou J, Zhang H, Gu P, Bai J, Margolick JB, Zhang Y. NF- $\kappa$ B pathway inhibitors preferentially inhibit breast cancer stem-like cells. *Breast Cancer Res Treat* 2008; **111**: 419–427.
- Storci G, Sansone P, Mari S, D'Uva G, Tavolari S, Guarneri T et al. TNF $\alpha$  up-regulates SLUG via the NF- $\kappa$ B/HIF1 $\alpha$  axis, which imparts breast cancer cells with a stem cell-like phenotype. *Cell Physiol* 2010; **225**: 682–691.
- Bhat-Nakshatri P, Appiah H, Ballas C, Pick-Franke P, Goulet Jr R, Badve S et al. SLUG/SNAI2 and tumor necrosis factor generate breast cells with CD44+/CD24-phenotype. *BMC Cancer* 2010; **10**: 411–420.
- Iliopoulos D, Hirsch HA, Struhl K. An epigenetic switch involving in NF $\kappa$ B, Lin28, Let-7 micro-RNA and IL6 link to inflammation to cell transformation. *Cell* 2009; **139**: 693–706.
- Liu S, Ginestier C, Ou SJ, Clouthier SG, Patel SH, Monville F et al. Breast cancer stem cells are regulated by mesenchymal stem cells through cytokine networks. *Cancer Res* 2011; **71**: 614–624.
- Konopleva MY, Jordan CT. Leukemia stem cells and microenvironment: biology and therapeutic targeting. *J Clin Oncol* 2011; **29**: 591–599.
- Rajasekhar V, Studer L, Gerald W, Socci ND, Scher HI. Tumor-initiating stem-like cells in human prostate cancer exhibit increased NF $\kappa$ B signaling. *Nat Commun* 2011; **12**: 162.
- Chaturvedi MM, Sung B, Yadav VR, Kannappan R, Aggarwal BB. NF- $\kappa$ B addiction and its role in cancer: 'one size does not fit all'. *Oncogene* 2011; **30**: 1615–1630.
- Tang XH, Gudas LJ. Retinoids, retinoic acid receptors, and cancer. *Annu Rev Pathol* 2011; **28**: 345–364.
- Ginestier C, Wicinski J, Cervera N, Monville F, Finetti P, Bertucci F et al. Retinoid signaling regulates breast cancer stem cell differentiation. *Cell Cycle* 2009; **8**: 3297–3302.
- Ho LJ, Lin LC, Hung LF, Wang SJ, Lee CH, Chang DM et al. Retinoic acid blocks proinflammatory cytokine-induced matrix metalloproteinase production by down-regulating JNK-AP-1 signaling in human chondrocytes. *Biochem Pharmacol* 2005; **70**: 200–208.
- Hung L-F, Lai J-H, Lin L-C, Wang S-J, Hou T-Y, Chang D-M et al. Retinoic acid inhibits IL-1-induced iNOS, COX-2 and chemokine production in human chondrocytes. *Immunol Invest* 2008; **37**: 675–693.
- Germain P, Chambon P, Eichele G, Evans RM, Lazar MA et al. Retinoid X receptors. *Pharmacol Rev* 2006; **58**: 760–772.
- Papi A, Tatenhorst L, Terwel D, Hermes M, Kummer MP, Orlandi M et al. PPAR $\gamma$  and RXR ligands act synergistically as potent antineoplastic agents *in vitro* and *in vivo* glioma models. *J Neurochem* 2009; **109**: 1779–1790.
- Papi A, Rocchi P, Ferreri AM, Orlandi M. RXR $\gamma$  and PPAR $\gamma$  Ligands in Combination to Inhibit Proliferation and Invasiveness in Colon Cancer Cells. *Cancer Lett* 2010; **297**: 65–72.
- Papi A, Bartolini G, Ammar K, Guerra F, Ferreri AM, Rocchi P et al. Inhibitory effects of retinoic acid and IIF on growth, migration and invasiveness in the U87 MG human glioblastoma cell line. *Oncol Rep* 2007; **18**: 1015–1021.
- Moraes LA, Piqueras L, Bishop-Bailey D. PPAR and inflammation. *Pharmacol Ther* 2005; **110**: 371–385.

25. Bonofiglio D, Cione E, Qi H, Pingitore A, Perri M, Catalano S *et al*. Combined low doses of PPAR $\gamma$  and RXR ligands trigger an intrinsic apoptotic pathway in human breast cancer cells. *Am J Pathol* 2009; **175**: 1270–1280.
26. Szanto A, Nagy L. The many faces of PPAR $\gamma$ : anti-inflammatory by any means? *Immunobiology* 2008; **213**: 789–803.
27. Zhang JZ, Ward KW. WY-14643, a selective PPAR $\alpha$  agonist, induces proinflammation. *Int J Toxicol* 2010; **29**: 496–504.
28. Suchanek KM, May FJ, Robinson JA, Lee WJ, Holman NA, Monteith GR *et al*. Peroxisome Proliferator-Activated Receptor  $\alpha$  in the Human Breast Cancer Cell Lines MCF7 and MDA-MB-231. *Mol Carcinog* 2002; **34**: 165–171.
29. Conzen S. Nuclear receptors and breast cancer. *Mol Endocrinol* 2008; **22**: 2215–2228.
30. Vaupel P, Höckel M, Mayer A. Detection and characterization of tumor hypoxia using pO<sub>2</sub> histography. *Antioxid Redox Signal* 2007; **9**: 1221–1235.
31. Balmer J, Blumhoff R. Gene expression regulation by retinoic acid. *J Lipid Res* 2003; **43**: 1773–1809.
32. Mandard S, Muller K, Kersten S. Peroxisome Proliferator activated receptor alpha target genes. *Cell Mol Life Sci* 2004; **61**: 394–416.
33. Dontu G, Abdallah WM, Foley JM, Jackson KW, Clarke MF, Kawamura MJ *et al*. *In vitro* propagation and transcriptional profiling of human mammary stem/progenitor cells. *Genes Dev* 2003; **17**: 1253–1270.
34. Studebaker AW, Storci G, Werbeck JL, Sansone P, Sasser AK, Tavorali S *et al*. Fibroblasts isolated from common sites of breast cancer metastasis enhance cancer cell growth rates and invasiveness in an interleukin-6-dependent manner. *Cancer Res* 2008; **68**: 9087–9095.
35. Dhasarathy A, Kajita M, Wade PA. The transcription factor snail mediates epithelial to mesenchymal transitions by repression of estrogen receptor- $\alpha$ . *Mol Endocrinol* 2007; **21**: 2907–2918.
36. Tripathi MK, Misra S, Chaudhuri G. Negative regulation of the expressions of cytokeratins 8 and 19 by SLUG repressor protein in human breast cells. *Biochem Biophys Res Commun* 2005; **329**: 508–515.
37. Mazumdar J, Dondeti V, Simon MC. Hypoxia-inducible factors in stem cells and cancer. *J Cell Mol Med* 2009; **13**: 4319–4328.
38. Szanto A, Narkar V, Shen Q, Uray IP, Davies P, Nagy L. Retinoid X receptors: X-ploring their (patho)physiological functions. *Cell Death Differ* 2004; **11**: S126–S143.
39. Yue L, Rasouli N, Ranganathan G, Kern PA, Mazzone T. Divergent effects of peroxisome proliferator-activated receptor gamma agonists and tumor necrosis factor alpha on adipocyte ApoE expression. *J Biol Chem* 2004; **279**: 47626–47632.
40. Chen YC, Pohl G, Wang T, Morin PJ, Risberg B, Kristensen GB *et al*. Apolipoprotein E is required for cell proliferation and survival in ovarian cancer. *Cancer Res* 2005; **65**: 1331–1338.

Supplementary Information accompanies the paper on Cell Death and Differentiation website (<http://www.nature.com/cdd>)