CK20 Expression Enhances the Invasiveness of Tamoxifen-resistant MCF-7 Cells

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Abstract. *Cytokeratin 20 (CK20) is an intermediate filament that is known to be a prognostic marker in several types of cancer. However, little is known about CK20 expression and tumor metastasis in tamoxifen-resistant MCF-7 (TRM-7) breast cancer cells. TRM-7 cells overexpress CK20, resulting in enhanced invasiveness in vitro. CK20 silencing reduced the invasiveness of TRM-7 cells. Moreover, CK20 expression in MCF-7 cells was regulated by peroxisome proliferator-activated receptor γ (PPARγ). Our findings suggest that PPARγ-dependent CK20 expression enhances the metastatic potential of MCF-7 breast cancer cells and may be a potential therapeutic target in tamoxifen-resistant breast cancer.*

Breast cancer is the most common cancer in women and its growth is hormonally regulated. Estrogen is the main hormonal stimulant in the development and growth of breast cancer. Over the past three decades, tamoxifen, the active metabolite of which (hydroxytamoxifen) is an estrogen receptor antagonist, has been the basis of hormonal therapy and is used worldwide for women with estrogen receptor-positive breast cancer. Resistance to chemotherapy remains a major hurdle in the treatment of hormone-independent breast cancer (1, 2). Although most patients are initially responsive to tamoxifen, acquired tamoxifen resistance is a critical problem for antiestrogen therapy, and the mechanism of resistance remains elusive (3).

A member of the cytokeratin family, cytokeratin 20 (CK20), is the intermediate filament cytokeratin of epithelial cells. It has a highly conserved amino acid sequence and is classified as a type I keratin. It is mainly expressed in the cytoplasm of epithelial cells in the small and large intestine and in Merkel cells in the skin (4). Moreover, since intermediate filament proteins are retained in malignant tumors, they are suitable as histological markers of differentiation (5-7). Thus, CK20 is a prognostic marker in several cancer types (8-10).

Despite the expression of CK20 in normal tissues being restricted to the superficial epithelial layer (4), little is known about the mechanism by which the expression of CK20 is regulated in both normal tissues and tumor cells. Nonetheless, Varley *et al*. (11) showed that the peroxisome proliferator-activated receptor (PPAR) agonist troglitazone (TZ) induces the expression of CK20 in normal urothelial cells, although the *CK20* gene promoter contains no peroxisome proliferator response element (PPRE). Thus, this raises the possibility that PPARγ indirectly regulates the expression of CK20 in tumor cells. PPARγ is of particular interest since it is expressed in many types of human cancer and plays important but highly debated roles in the proliferation, differentiation, and apoptosis of cancer cells (12-15). Notably, the PPARγ isoform PPARγ1 is highly expressed in breast cancer cell lines compared to normal epithelial cells (16, 17), suggesting that it may have an important role in the development and progression of breast cancer.

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The aim of this study was to investigate whether CK20 is expressed in tamoxifen-resistant breast cancer cells (TRM-7 cells) by long-term culture of MCF-7 cells with 4-hydroxytamoxifen, and to investigate the effect of CK20 on invasive behavior using an *in vitro* model. To achieve this, we established TRM-7 cells and compared their CK20 expression and invasiveness with that of parental MCF-7 cells. We further examined whether CK20 expression and, ultimately, the invasive properties of breast cancer cells were modulated by the *in vitro* knock-down and overexpression of PPARγ.

Materials and Methods

Cell lines. An MCF-7 human breast cancer cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Tamoxifen-resistant MCF-7 (TRM-7) cells were established according to a previously described method (1). Briefly, MCF-7 cells were cultured in medium containing 1 μM tamoxifen, with the medium being replaced every 2-3 days until the cells reached confluence. The cells were continuously exposed to this treatment regimen for one week. The tamoxifen concentration was thereafter gradually increased to 3 μM over a six-month period. Although the cell growth rates were initially low, they gradually increased during exposure to tamoxifen for six months, resulting in the establishment of TRM-7 cells. To measure the effects of tamoxifen on cell viability, cells were stained with 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT; Roche, Basel, Switzerland) according to the manufacturer's instructions and absorbance at 570 nm was measured.

Transfection experiments. To knock-down *PPARγ*, and *CK20*, small interfering RNA (siRNA) was used. *PPARγ* and *CK20* siRNAs (Qiagen, Valencia, CA, USA) were transfected into cells using HiPerFect (Qiagen) according to the manufacturer's instructions.

To generate a plasmid encoding the *PPAR-γ* gene, the gene was amplified by polymerase chain reaction (PCR) and cloned into the pcDNA3.0 vector (Invitrogen, Carlsbad, CA, USA). The PPARγ construct or an empty (control) plasmid was transfected into cells using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's protocol.

Immunoblotting. Cells, including MCF-7, TRM-7, and MCF-7 transfected with PPARγ plasmid, were harvested in a lysis solution (Santa Cruz Biotechnology, Santa Cruz, CA, USA) containing a protease inhibitor cocktail (Roche) and a phosphatase inhibitor (Roche). After incubation for 30 min on ice, insoluble debris was removed by centrifugation for 10 min at 4˚C. Total protein was resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (GE Healthcare, Pittsburgh, PA, USA). The membranes were then probed with antibodies towards α-tubulin (Thermo Fisher Scientific, Fremont, CA, USA), PPARγ (Cell Signaling Technology, Danvers, MA, USA), and CK20 (Abcam, Cambridge, UK) and visualized using the SuperSignal West Femto Chemiluminescent Substrate (Thermo Fisher Scientific).

Immunofluorescence. MCF-7 and TRM-7 cells were grown on 35-mm lysine-coated, glass-bottomed culture dishes (MatTek, Ashland, MA, USA). Cells were fixed through incubation with freshly prepared 3.7% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min at room temperature. Subsequently, cells were blocked with 3% normal serum in PBS for 1 h and then incubated with anti-CK20 (Abcam) or anti-PPARγ (Cell Signaling Technology) antibodies at 4˚C overnight. They were next incubated with the appropriate secondary antibodies. Stained cells were mounted in VECTASHIELD 4', 6-diamidino-2-phenylindole (DAPI) mounting medium (Vector Laboratories, Burlingame, CA, USA) and visualized under a confocal laser scanning microscope (LSM 510; Carl Zeiss, Göttingen, Germany).

Quantitative reverse-transcription-polymerase chain reaction (PCR) assay. Total RNA was isolated from MCF-7 and TRM-7 cells and from TRM-7 cells transfected with various siRNA. The cDNA was synthesized using a QuantiTech Reverse Transcription Kit (Qiagen) and was then mixed with QuantiFast SYBR Green PCR master mix (Qiagen) and specific primers for *CK20, PPARγ,* and *GAPDH* obtained from Qiagen. Quantitative reverse-transcription PCR (qRT-PCR) was performed with an Applied Biosystems 7300 Real-Time PCR System (Life Technologies, Carlsbad, CA, USA). The raw data were analyzed by comparative Ct quantification.

Cell invasion assay. Invasiveness was assessed using a Boyden chamber system (Neuro Probe, Gaithersburg, MD, USA) with a polycarbonate membrane having a pore size of 8 μm separating the two chambers. The upper chamber was coated on ice with 100 μl of growth factor-reduced Matrigel (BD Biosciences, San Jose, CA, USA) containing 0.5 mg/ml protein and was then incubated at 37˚C for at least 4-5 h to allow gelling. Cells (1×10^5), including MCF-7, TRM-7, and MCF-7 transfected with *CK20* siRNA, in 100 μl of serum-free Dulbecco's modified Eagle's medium (DMEM) were loaded into the upper chamber. The lower chamber was filled with 600 μl of DMEM containing 10% fetal bovine serum and 5 mg/ml fibronectin. The chambers were incubated at 37˚C for 24 h in an atmosphere 95% air and 5% CO_2. The upper medium was then removed, the chamber disassembled, and the membranes were rinsed in PBS and fixed. The membranes were subsequently stained with Diff-Quik solution (Sysmex Corp. Kobe, Japan) and then washed twice. Cells attached to the bottom of the membrane were photographed at ×10 magnification.

Statistical analysis. Data are presented as the mean±standard error of the mean (SEM). *p*-Values <0.05 were considered statistically significant. Two-tailed Student's *t*-tests were conducted using GraphPad Prism ver. 5.01 (GraphPad Software, La Jolla, CA, USA).

Results

Induction of cytokeratin 20 (CK20) in tamoxifen-resistant MCF-7 breast cancer cells. To establish tamoxifen-resistant breast cancer cells (TRM-7 cells), breast cancer MCF-7 cells were cultured with tamoxifen over a six-month period. The viability of TRM-7 cells significantly increased when the tamoxifen concentration was increased to 15 μM (Figure 1A). Given the fact that TRM-7 breast cancer cells display enhanced invasive capacity *in vitro* (1), we investigated whether TRM-7 cells were more invasive than their parental MCF-7 cells in a Matrigel invasion assay. We found that the acquisition of tamoxifen resistance was accompanied by a dramatic increase in motility (Figure 1B).

Since increased expression of CK20 is well known to correlate with advanced clinical stage and lymph node metastasis in breast cancer (18), we investigated the expression of CK20 in TRM-7 cells. CK20 expression at the mRNA and protein levels was significantly higher in TRM-7 cells compared to that in parental MCF-7 cells (Figure 1C and D). Taken together, our data suggest that CK20 expression is increased in TRM-7 cells, resulting in enhanced invasiveness *in vitro* and possibly enhanced metastasis *in vivo*.

Abrogation of CK20 expression reduces the invasiveness of TRM-7 cells. To determine the functional consequence of CK20 overexpression *in vitro*, TRM-7 cells were transfected with *CK20* siRNA. *CK20* siRNA significantly reduced CK20 expression in TRM-7 cells (Figure 2A). Moreover, the invasiveness of TRM-7 cells *in vitro* was markedly inhibited by of *CK20* knock-down (Figure 2B). These data demonstrate that the induction of CK20 expression may contribute to the increased invasiveness of TRM-7 cells.

PPARγ plays a crucial role in the upregulation of CK20 expression in TRM-7 cells. The fact that CK20 expression was increased in TRM-7 cells raised the question as to which factors are involved in the regulation of CK20 expression by tamoxifen. PPARγ is expressed in many human tumors including, colon, lung, and breast cancer (16). In addition, the PPARγ agonist TZ induces the expression of CK20 in normal urothelial cells (11). We therefore investigated whether PPARγ controls CK20 expression in TRM-7 cells.

PPARγ expression at the mRNA and protein levels was higher in TRM-7 cells compared with parental MCF-7 cells (Figure 3A and B). Notably, PPARγ and CK20 were expressed at the same time in individual cells (Figure 3C), suggesting that PPARγ could be involved in the direct or indirect induction of CK20 in breast cancer cells. We next suppressed PPARγ expression through siRNA-mediated knock-down to confirm whether the expression of CK20 in TRM-7 cells was affected by PPARγ. As shown in Figure 3D, transfection of *PPARγ* siRNA significantly reduced PPARγ expression, resulting in the expression of CK20 being lowered in TRM-7 cells. Moreover, overexpression of PPARγ significantly enhanced CK20 expression in parental MCF-7 cells (Figure 3E). These data indicate that PPARγ plays an essential role in the regulation of CK20 expression in TRM-7 cells and ultimately enhances cell invasiveness.

Discussion

In this study, we demonstrated that CK20 is highly expressed *in vitro* in tamoxifen-resistant MCF-7 (TRM-7) cells, which show increased invasion (Figure 1) that is dependent on CK20 expression (Figure 2). The expression of CK20 in TRM-7 cells is regulated by the nuclear orphan receptor PPARγ, as verified by immunofluorescence and siRNA-mediated knock-down and overexpression of PPARγ (Figure 3).

In agreement with our data (Figure 1), acquired resistance to tamoxifen in breast cancer results in increased invasiveness and tumor progression (19, 20). Although autocrine epidermal growth factor receptor (EGFR) signaling *via* focal adhesion kinase (FAK) is known to be associated with increased invasive activity of TRM-7 cells *in vitro* (21, 22), little is known about the cellular/molecular mechanisms underlying acquired resistance to antiestrogens such as tamoxifen. In addition to enhanced invasiveness, we found that TRM-7 cells also have higher CK20 expression than MCF-7 cells (Figure 1). This is inconsistent with previous reports showing minimal immunostaining of CK20 in breast cancer (6, 9), although CK20 has been detected in pancreatic carcinomas, gastric carcinoma, cholangiocarcinomas, and transitional cell carcinomas, and serves as an excellent marker for metastatic pancreatic cancer (6, 23). However, Giribaldi *et al*. demonstrated, by RT-PCR, that 22% and 29% of all patients with colorectal and breast cancer, respectively, had CK20 cell equivalents in their peripheral blood, and found that the association between metastasis and CK20 cell equivalents in blood was statistically significant in patients with breast cancer (24). These data support the conclusion that TRM-7 cells, which have high invasiveness, express CK20 at higher levels than do their parental MCF-7 cells (Figure 2).

To determine whether CK20 expression directly contributes to enhanced invasiveness, we transfected TRM-7 cells with *CK20* siRNA, which led to a remarkable decrease in cell invasiveness *in vitro* (Figure 2). Urothelial cells at the leading edge (wound border) express CK20 and CK7, suggesting that CK20 may play a key role in determining the direction and rate of urothelial healing and in harmonized cell movement (25). These data and our own suggest that CK20 may be involved in modulating the motility of cells that have acquired tamoxifen resistance. Although the mechanism involved remains unclear, some evidence exist to indicate possible mechanisms by which CK20, as an intermediate filament, regulates the motility/invasiveness of breast cancer cells (26). Intermediate filaments act as signal transducers, conveying information from the extracellular matrix to the nucleus (27), thereby modulating cell function and altering gene expression, notably that of members of the integrin family of cell surface receptors (28, 29). Thus, integrin-mediated signaling may have critical consequences for cell adhesion and migration (30, 31). In agreement with this idea, co-expression of the intermediate filaments cytokeratin and vimentin in human breast cancer cells induced phenotypic interconversion (between epithelial and mesenchymal cells) and increased invasive behavior (32). Chen *et al*. (33) recently demonstrated that highly proliferative MDA-MB231 breast cancer cells have higher vimentin expression than the less proliferative MCF-7 breast

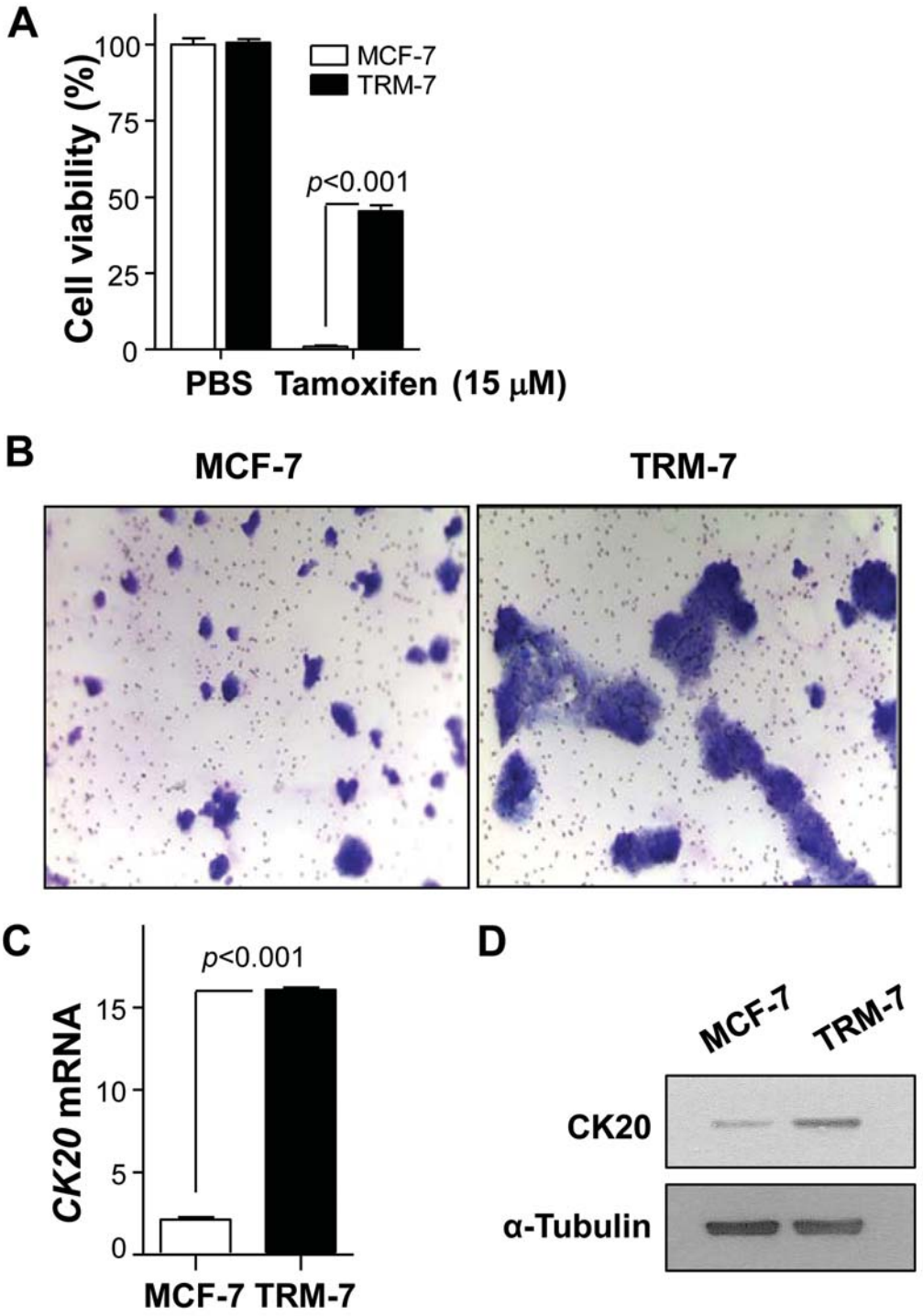

Figure 1. *Increased expression of cytokeratin 20 (CK20) in tamoxifen-resistant MCF-7 (TRM-7) cells. A: TRM-7 cells were established as described in the Materials and Methods. TRM-7 cells acquired resistance to tamoxifen, as assessed by enhanced cell viability after treatment with 15 μM tamoxifen for 24 h. The results show the mean±the standard error of the mean (SEM) of three independent experiments, each performed in triplicate. p-Values were obtained using the two-tailed Student's t-test. B: Representative Matrigel invasion assay photographs. The assay was performed in order to evaluate the invasiveness of TRM-7 cells. Cells were seeded onto a Matrigel-coated porous membrane and incubated for 24 h to allow them to migrate through the Matrigel matrix. Invading cells were fixed and subsequently stained using Diff-Quik solution. The original photographs were taken at ×10 magnification. C: The transcription level of CK20 in TRM-7 cells and in parental MCF-7 cells was analyzed by qRT-PCR. The qRT-PCR data were analyzed by comparative Ct quantification. Data are presented as the mean±SEM (n=3). p-Values were obtained using the two-tailed Student's t-test. D: CK20 protein levels in TRM-7 and MCF-7 cells were measured by immunoblotting. The results shown are representative of three independent experiments.*

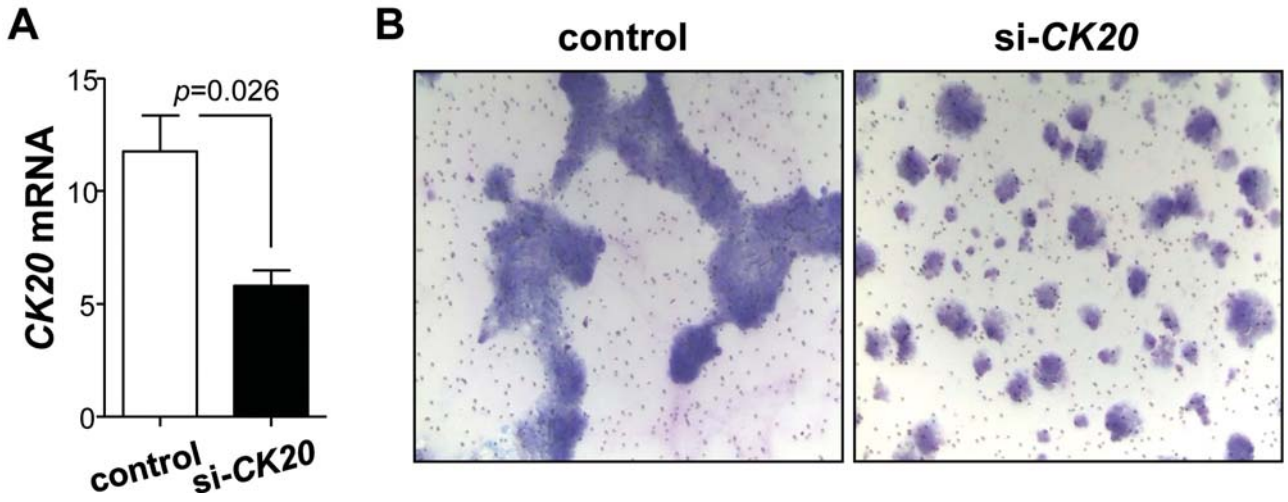

Figure 2. *CK20 silencing abrogates TRM-7 cell invasiveness in vitro. CK20 siRNA (si-CK20, 20 nM) or control siRNA (Control, 20 nM) was transfected into TRM-7 cells. A: Total RNA was isolated from cells at 48 h after transfection. The level of transcription of CK20 in the cells was analyzed by qRT-PCR. Data are presented as the mean±SEM (n=3). p-Values were obtained using the two-tailed Student's t-test. B: Representative Matrigel invasion assay photographs. The invasive behavior of TRM-7 cells in which CK20 had been silenced was assessed at 48 h after transfection. At least three independent triplicate experiments were performed.*

cancer cell line. In addition, in an experimental melanoma model, vimentin-expressing A375P human melanoma cells exhibited low invasive potential, but acquired invasive ability when CK8 and CK18 were co-expressed, suggesting possible roles for cytokeratin and vimentin in cancer metastasis (31, 34). These data suggest that intermediate filaments interact with transmembrane integrins, leading to changes in cell shape and mediating cell motility through signal transduction (35). How CK20 exerts its effect on cell motility in breast cancer cells *in vitro* and contributes to metastasis *in vivo* requires further investigation.

Given the fact that CK20 is regulated by PPARγ in normal urothelial cells during differentiation (11), we investigated whether PPARγ controls the expression of CK20 in TRM-7 cells. TRM-7 cells clearly demonstrated co-expression of CK20 and PPARγ in the nucleus (Figure 3), suggesting that PPARγ may regulate CK20 expression in breast cancer cells, as it does in urothelial cells (11). Furthermore, siRNA-mediated knock-down of PPARγ lowered the CK20 expression in the TRM-7 cells, whereas overexpression of PPARγ enhanced the expression of CK20 in MCF-7 cells (Figure 3). Since a putative PPRE with an AGGTCA motif (36) is not found in the region of the human *CK20* gene promoter, PPARγ may induce transcription factors that can bind to the *CK20* promoter to induce expression, as suggested to occur in urothelial cells (11). It was reported that a homeobox transcription factor involved in intestinal development, regulates CK20 *via* two putative binding sites in the *CK20* promoter (37). However, the expression of CDX1 did not differ between CK20-expressing TRM-7 cells and parental MCF-7 cells (*data not shown*), suggesting that CDX1 is not involved in the regulation of CK20 expression in breast cancer cells. Taken together, these data strongly suggest that PPARγ is crucial in the up-regulation of CK20 in TRM-7 cells.

In conclusion, this study provides insights into the functional significance of increased CK20 expression in MCF-7 cells following prolonged tamoxifen exposure. Knock-down of CK20 showed that expression of CK20 in TRM-7 cells is essential for invasion, suggesting that CK20 is not only a prognostic marker, but also a functional molecule in breast cancer cells. In addition, CK20 expression is dependent on the expression of the nuclear orphan receptor PPARγ. Although further study is needed to elucidate the mechanisms controlling PPARγ expression, determining whether PPARγ antagonists act on tamoxifen-resistant breast cancer would be of value.

Declaration of interest

The Authors declare that they have no competing interests.

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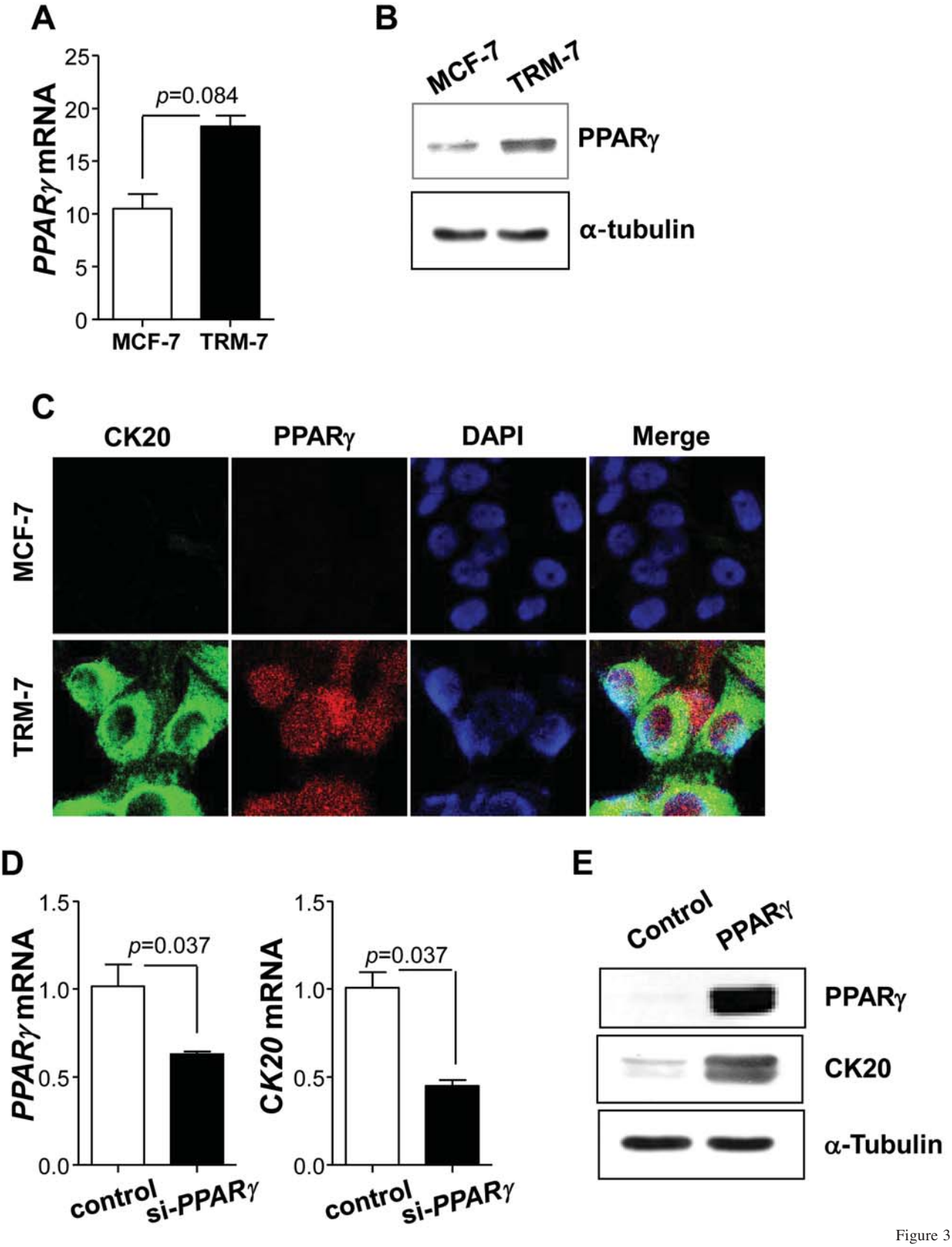
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Figure 3.

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Figure 3. *PPARγ regulates the expression of CK20. A: PPARγ mRNA expression in TRM-7 cells and parental MCF-7 cells was analyzed by qRT-PCR. Data are presented as the mean±SEM (n=3). p-Values were obtained using the two-tailed Student's t-test. B: PPARγ protein levels in TRM-7 and MCF-7 cells were measured by immunoblotting. The results shown are representative of three independent experiments. C: Cells were stained with anti-CK20 (green) and anti-PPARγ (red) antibodies, and nuclei were stained with DAPI (blue). Images were taken under a confocal microscope using a ×40 objective. At least three independent triplicate experiments were performed. D: TRM-7 cells were transfected with PPARγ siRNA (si-PPARγ, 20 nM) or control siRNA (Control, 20 nM). At 48 h after transfection, relative PPARγ (left) and CK20 (right) mRNA levels were measured by qRT-PCR. Data are presented as the mean±SEM (n=3). p-Values were obtained using the two-tailed Student's t-test. E: MCF-7 cells were transiently transfected with a plasmid encoding the PPARγ gene. Representative immunoblots show PPARγ protein levels in MCF-7 cells transfected with a plasmid encoding PPARγ gene or the empty vector pcDNA3.0 (as a control) at 48 h after transfection.*

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