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Chlorpyrifos subthreshold exposure induces epithelial-mesenchymal transition in breast cancer cells

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ABSTRACT

Chlorpyrifos (CPF) is one of the most frequently used pesticide in extensive agriculture around the world and can be incorporated by humans and animals with possible consequences on health. The effects of this pesticide on carcinogenesis are not clear and there is no consensus concerning the risks of this compound. In previous work, we demonstrated that CPF induces proliferation of breast cancer cells both *in vivo* and *in vitro*. In this work we investigate whether CPF promotes the epithelial-mesenchymal transition (EMT) in breast cancer cells. Herein, we demonstrate that 50 μM CFP induces invasion in MCF-7 and MDA-MB-231 cells. In addition, 0.05 and 50 μM CPF increases migration in both cell lines. In MCF-7 cells, 0.05 and 50 μM CPF increase the metalloprotease MMP2 expression and decrease E-Cadherin and β-Catenin expression diminishing their membrane location. Furthermore, 50 μM CPF induces Vimentin expression and Slug nuclear translocation in MCF-7 cells. 0.05 and 50 μM CPF increase MMP2 gelatinolytic activity and expression, decrease β-Catenin expression and increase Vimentin expression in MDA-MB-231 cells.

Inhibition of the oncoprotein c-Src reverses all the effects induced by CPF in MDA-MB-231 but not in MCF-7 indicating that c-Src is a kinase with a crucial role in the cells which grow in an estrogen-independent way. In MCF-7 cells both c-Src and estrogen receptor alpha must be blocked to completly inhibit the CPF-mediated effects. Our results show for the first time that the exposure to subthreshold concentrations of CPF promotes the modulation of EMT-molecular markers and pathways. These results, together with the ubiquitous distribution of the pesticide CPF, make it of utmost importance to take measures to minimize the risk of exposure to this compound.

1. Introduction

Chlorpyrifos (CPF) is an organophosphate widely used in agriculture to defend the cultured fields from plagues. It has been detected in many different surfaces or substrates that range from air or rain to solid and liquid foods (Gebremariam et al., 2012). CPF is associated with an increased risk of premenopausal breast cancer among the women who live with farmers according to a recent epidemiologic study performed in USA (Engel et al., 2017). We demonstrated that CPF induces proliferation of breast cancer cells in an estrogen receptor alpha-dependent way at pesticide concentrations that are usually found in water and soils that surround the fumigated fields (Dzul-Caamal et al., 2014; Harnpicharnchai et al., 2013; Ventura et al., 2012). In Argentinean aquatic environments, the concentrations of CPF can range from less than 0.001–0.05 μM (Alvarez et al., 2019; Bonansea et al., 2017). The limit imposed for total pesticides in drinking water in USA legislation is 0.1 μg/L (Council Directive 98/83/EC, 1998). However, an important bioconcentration of pesticides in the biota is well documented. As an example, bioconcentration factors from 100 to 5100 have been reported in fish (Racke, 1993). Furthermore, Huen et al. have reported that CPF levels in plasma from women and newborns living in an agricultural community ranged from 0 to 1726 μg/L (0–4.9 μM) (Huen et al., 2012).

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CPF detected in breast milk from nursing mothers in India ranged from 8.5 to 355 μg/L (0.02–1 μM) (Sanghi et al., 2003).

CPF is considered an endocrine disruptor (ED) at low doses (Diamanti-Kandarakis et al., 2009). It has both agonist and antagonist actions towards the aryl hydrocarbon receptor (AhR) and the estrogen receptor alpha (ERα) and is a progesterone receptor (PR) antagonist (Doan et al., 2020). ED can have effects at low doses that are not predicted by the effects at higher doses. Furthermore, sometimes the effects at low doses are more potent than those at high doses or may even have the opposite effect. We have previously studied whether CPF affects the proliferation of MCF-7 and MDA-MB-231 cell lines in a dose dependent way. We showed that CPF induces the highest estrogen dependent cell proliferation at 0.05 μM in MCF-7 estrogen-dependent cell line but this effect was not observed on MDA-MB-231 cells which is an estrogen-independent cell line. This increase was less marked at 0.5 and 5 μM. By contrast, 50 μM CPF induces inhibition of the proliferation in MCF-7 and MDA-MB-231 cell lines and this action is not dependent on the ERα signaling pathway, but it is dependent on ROS-induction and ERK phosphorylation (Ventura et al. 2012, 2015). We also showed that CPF induces mammary proliferative changes in rats (Ventura et al., 2016) and diminishes the latency period in a chemically induced mammary tumor model in rats (Ventura et al., 2019).

Bisphenol A (BPA), phthalates, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), and triclosan have been recognized as ED and they were pointed out as potential inductors of cancer metastasis by regulating epithelial mesenchymal transition markers (Lee et al., 2013). The epithelial mesenchymal transition (EMT) is defined as a process related to the latest steps of carcinogenesis associated with malignant transformation. It is also triggered in normal transformations in the first phases of the development or tissue regeneration. It is installed when epithelial cells lose their polarity and the adhesion to other cells acquiring a mesenchymal phenotype leading to an enhanced migratory capacity for metastasis (Son and Moon, 2013).

Classically, estrogen action is initiated when estrogen binds to its receptor (ER), the complex dimerizes and targets sequence-specific response elements (EREs) mediating estrogen genomic actions. The cytoplasmatic and membrane estrogen-ER complex may both interact with the oncoprotein c-Src (Frei et al., 2016; Guest et al., 2016). In turn, c-Src is followed by PI3K/AKT/GSK-3β and P38/GSK-3β downstream signaling activation (Jiang et al., 2017; Musgrove and Sutherland, 2009). In many types of tumors, the role of c-Src turns crucial in proliferation, survival, adhesion, migration, invasion and metastasis (Zhang and Yu, 2012). In EMT process, the activation of c-Src may induce the downregulation of E-Cadherin expression (Avizienyte et al., 2002; Karni and Levitzki, 2000). E-Cadherin is upregulated by the selective c-Src inhibitor, PP2 in hepatocellular and head and neck squamous carcinoma cells (Lee et al., 2008; Mandal et al., 2008). Furthermore, c-Src can phosphorylate the ERα (Arnold et al., 1995; de Leeuw et al., 2011; Lannigan, 2003) leading to the activation of PI3K/AKT pathway (Flint et al., 1993; Sun et al., 2012; Yudt et al., 1999), which is a critical event upstream GSK-3β/β-Catenin. β-Catenin is a cell adhesion protein that translocate to the nucleus to control the rate of transcription of its target genes (Shang et al., 2017) such as c-myc (Gekas et al., 2016) and cyclin D1 related to proliferation (Utsunomiya et al., 2001), and cell invasion related genes, including vascular epithelial growth factor (VEGF), cyclooxygenase 2 (COX-2) and metalloproteases expression (Bienz, 2005).

In this work we investigated the effects of CPF on the EMT process which results in a critical step in the beginning of cancer progression. In order to reach this aim, we studied whether CPF may produce the acquisition of mesenchymal phenotype of breast cancer cells and analyzed the expression of classical markers of EMT. We have also evaluated the migration and invasion and the cell signaling involved in these processes.

2. Materials and methods

2.1. Cell culture and exposure

MCF-7 and MDA-MB-231 human breast cancer cell lines were grown and exposed to CPF as we previously reported (Ventura et al., 2015).

ERα inhibitor (ICI 182,780, 1 nM) and c-Src inhibitor (PP2, 1 μM) were added to the culture 3 h before the pesticide and continued during the time of treatment when it was indicated. The duration of the treatments was specified in the corresponding legend.

2.2. Western blot analysis, immunofluorescence and hematoxylin staining

Cells were lysed, protein concentration was measured using Bradford assay (Bradford, 1976) and analyzed by SDS-PAGE and immunoassay as described (García et al., 2010). Membranes were probed overnight with primary mouse monoclonal anti-β-Catenin (1:300, Santa Cruz Biotechnology, Inc, USA), mouse monoclonal anti-E-Cadherin (1:300, Santa Cruz Biotechnology, Inc, USA), mouse monoclonal anti-Vimentin (1:500, Invitrogen, USA), mouse monoclonal anti-Slug (1:200, Invitrogen, USA), mouse monoclonal anti-MMP2 (1:200, Sigma Chemical Co., MO, USA), mouse monoclonal anti-MMP9 (1:200, Sigma Chemical Co., MO, USA) and mouse monoclonal anti-β-actin (1:1000, Sigma Chemical Co., MO, USA) as previously described (Ventura et al., 2012).

For hematoxylin staining, cells were seeded on cover slips and treated with CPF (0.05 and 50 μM) or vehicle for 72 h. Then, cells were fixed and dyed with hematoxylin or exposed to mouse monoclonal anti-β-Catenin primary antibody (1:100), mouse monoclonal anti-E-Cadherin primary antibody (1:300, Santa Cruz Biotechnology, Inc, USA), mouse monoclonal anti-Vimentin primary antibody (1:500, Invitrogen, USA), mouse monoclonal anti–Slug primary antibody (1:200, Invitrogen, USA), mouse monoclonal anti-β-actin primary antibody (1:1000, Sigma Chemical Co., MO, USA) and FITC-conjugated anti-mouse IgG (1:200, Sigma Chemical Co., MO, USA). Hoechst was used for nuclei staining. Cover slips were mounted and processed as we previously described (Ventura et al., 2012).

2.3. Invasion, migration and gelatinolytic activity

Invasion, migration and gelatinolytic activity were assayed as we described previously (Pontillo et al., 2013, 2011).

Briefly, to evaluate the invasion, cells were seeded in serum free medium into Boyden Chambers (8 μm pore size) coated with 30% Matrigel®. Then, cells were treated with CPF (0.05 μM or 50 μM) or vehicle for 24 (3×10^4 MDA-MB-231 cells) or 48 h (5×10^4 MCF-7 cells). Exposure of 48 and 24 h were chosen for MCF-7 cells and MDA-MB-231 cells, respectively, because they are shorter times than duplication rate in the absence of FBS.

RPMI with 10% FBS was used as chemoattractant. The non-invading cells were scraped from the upper chamber with a moist cotton swab. Cells on the bottom surface were fixed with ice-cold methanol and stained with hematoxylin. To evaluate the individual motion and cell's ability to squeeze through pores, we proceeded in the same way as for the invasion, but without adding Matrigel®. Five photographs of five different fields were taken for each treatment and control. The number of migrating and invasive cells were counted on the bottom side of the Boyden Chambers. Treatments were compared to Control which was considered as the 100%. For the evaluation of collective migration using wound healing assay, cells were pretreated for 3 h with a specific inhibitor of c-Src (PP2, 1 μM). The monolayer was scratched, and cells were exposed to 0.05 μM or 50 μM CPF, or vehicle in the presence or absence of PP2. The scratched area was photographed at 0 and 24 h and the distance of wound healing in each well was evaluated. Finally, the migration rate was calculated as: D t0 (distance at 0 h) − D t24 (distance at 24 h)/(D t0 × 100). Treatments were compared to Control which was considered as the 100%.

For the gelatin zymography, aliquots of 20 μl of cell-conditioned media were resuspended in Laemmli modified buffer, denatured at 95 °C for 5 min, loaded on 6 or 7.5% SDS–PAGE gels containing 1% gelatin and electrophoresed. Then, gels were washed several times with rinsing buffers and incubated for 48 h at 37 °C in developing buffer. Metalloprotease (MMP) activity was visualized by staining with 0.5% Coomassie brilliant blue R-250. Clear bands were quantified by scanning laser densitometry with the ImageJ software. β-actin was used as internal control detected by immunoblots.

2.4. Statistical analysis

Data are shown as the means ± SEM of at least three independent experiments. Statistical comparisons for significance for different treatments, with or without specific inhibitors were performed using the one-way ANOVA and Dunnett's Multiple Comparison post hoc test or two-way ANOVA and Tukey's Multiple Comparison post hoc test depending on the case. We analyzed data using the GraphPad Prism 7.0 (GraphPad Software Inc., Philadelphia, USA). p values less than 0.05 were considered as statistically significant.

3. Results

3.1. Effect of CPF on MCF-7 and MDA-MB-231 cell morphology, β-actin cytoskeleton and invasion

EMT can be induced by cytokines, growth factors, and MMPs. It is a complex process where epithelial cells acquire migratory aspect converting their cuboidal phenotype into an elongated shape. To evaluate the effects of CPF on EMT associated phenotype, MCF-7 and MDA-MB-231 cells were cultured with CPF (0.05 or 50 μM) for 72 h. MCF-7 and MDA-MB-231 treated cells lost their epithelial characteristics and were converted to a spindle-like shape with mesenchymal characteristics. CPF made the cells change into an elongated shape, with protrusive membrane structures. They were also capable of growing apart from each other as shown in the pictures of hematoxylin staining. (Fig. 1 b, b', b''; c, c', c'', h, h', h'' i, i', i''). As immunofluorescent images show, the membrane localization of β-actin in control cells was lost when MCF-7 and MDA-MB-231 cells were exposed to the pesticide. Instead, a marked increase of β-actin polymerization foci can be observed with structural changes in the cytoskeleton. Invadopodia can also be distinguished in the immunofluorescent images (Fig. 1 e, e', e'', f, f', f'' k, k', k'', l, l', l'').

As we observed that CPF induced a spindle-like shape in both MCF-7

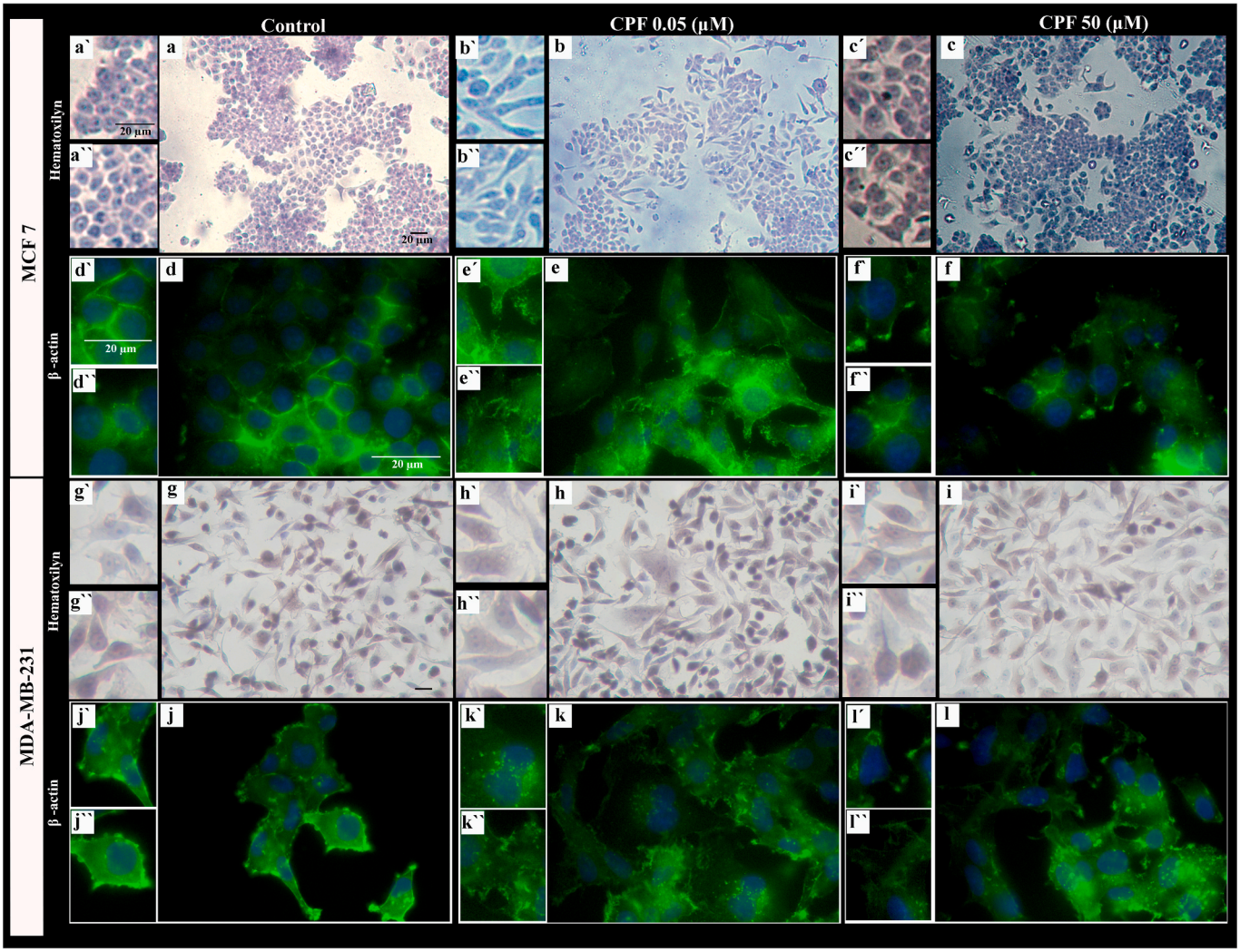

Fig. 1. ***Effect of CPF on MCF-7 and MDA-MB-231 cell morphology and β-actin cytoskeleton.*** MCF-7 and MDA-MB-231 cells were exposed to CPF (0.05 and 50 μM) or vehicle (C) for 72h. Cells stained with hematoxylin were photographed for morphology studies. Bar scale: 20 μm. Magnification: 400X. β-actin subcellular localization was determined by immunofluorescence microscopy using a specific monoclonal antibody. Bar scale: 20 μm. Magnification: 1000X.

and MDA-MB-231 cells with marked changes in β-actin cytoskeleton localization, we decided to evaluate if CPF may induce cell invasion, since these features provide a significant increase of invasive potential. For that study, we evaluated the invasion using Boyden chambers and Matrigel® to simulate the extracellular matrix. We found an increase in the number of MCF-7 cells that were able to cross the pores to invade the Matrigel® after 48 h of exposure to 50 μM CPF (110 ± 7% respect to control, ***$p < 0.001$). No significant differences were found after 0.05 μM CPF exposure in MCF-7 cells. The increase of invasion of MDA-MB-231 cells was produced in a lower gap than that for MCF-7 cells. A significant increment was observed after 24 h of exposure to 50 μM CPF in MDA-MB-231 cells (248 ± 27.5% respect to control, ***$p < 0.001$) (Fig. 2).

3.2. CPF triggers cell migration but different signaling is involved in MCF-7 and MDA-MB-231 cells, respectively

We have also analyzed the effect of CPF on migration of MCF-7 and MDA-MB-231. Experiments were performed as we described in Material and Methods Section. Wound healing assay shows the collective motion and the Boyden Chamber assay allows to evaluate the individual motion and cell's ability to squeeze through pores.

Our results show that the migration was induced in MCF-7 cells when Boyden chamber assays were performed. The increment induced by 0.05 μM CPF in MCF-7 cells was not reverted by ICI 182,780 or c-Src-inhibitor PP2 but interestingly, we observed that the effect reversed with the administration of both inhibitors together ($^{###}p < 0.001$ vs 0.05 μM CPF). We have also treated the MCF-7 cells with estradiol (E2) showing a tendency to increase the migration rates. Likewise, the increment induced by 50 μM CPF was dependent on c-Src activation ($^{###}p < 0.001$ vs 50 μM CPF) (Fig. 3).

Healing assays showed an increased migration induced by 0.05 μM CPF (130 ± 23% over control; ***$p < 0.001$) and 50 μM CPF (113 ± 17% over control; ***$p < 0.001$) after 24 h of exposure in MDA-MB-231 cells. This increment was reverted by adding c-Src inhibitor ($^{###}p < 0.001$ vs 0.05 μM CPF and $^{###}p < 0.001$ vs 50 μM CPF) (Fig. 4A). Then, we evaluated the migration using the Boyden chamber assay and we observed similar effects since 0.05 μM and 50 μM CPF induced the migration after 24 h of exposure [26 ± 6% over control (*$p < 0.05$) and 36.5 ± 11.5% over control (*$p < 0.05$), respectively]. These effects turned out to be dependent on the activation of c-Src ($^{##}p < 0.01$ vs 0.05 μM CPF and $^{#}p < 0.05$ vs 50 μM CPF, respectively) (Fig. 4B).

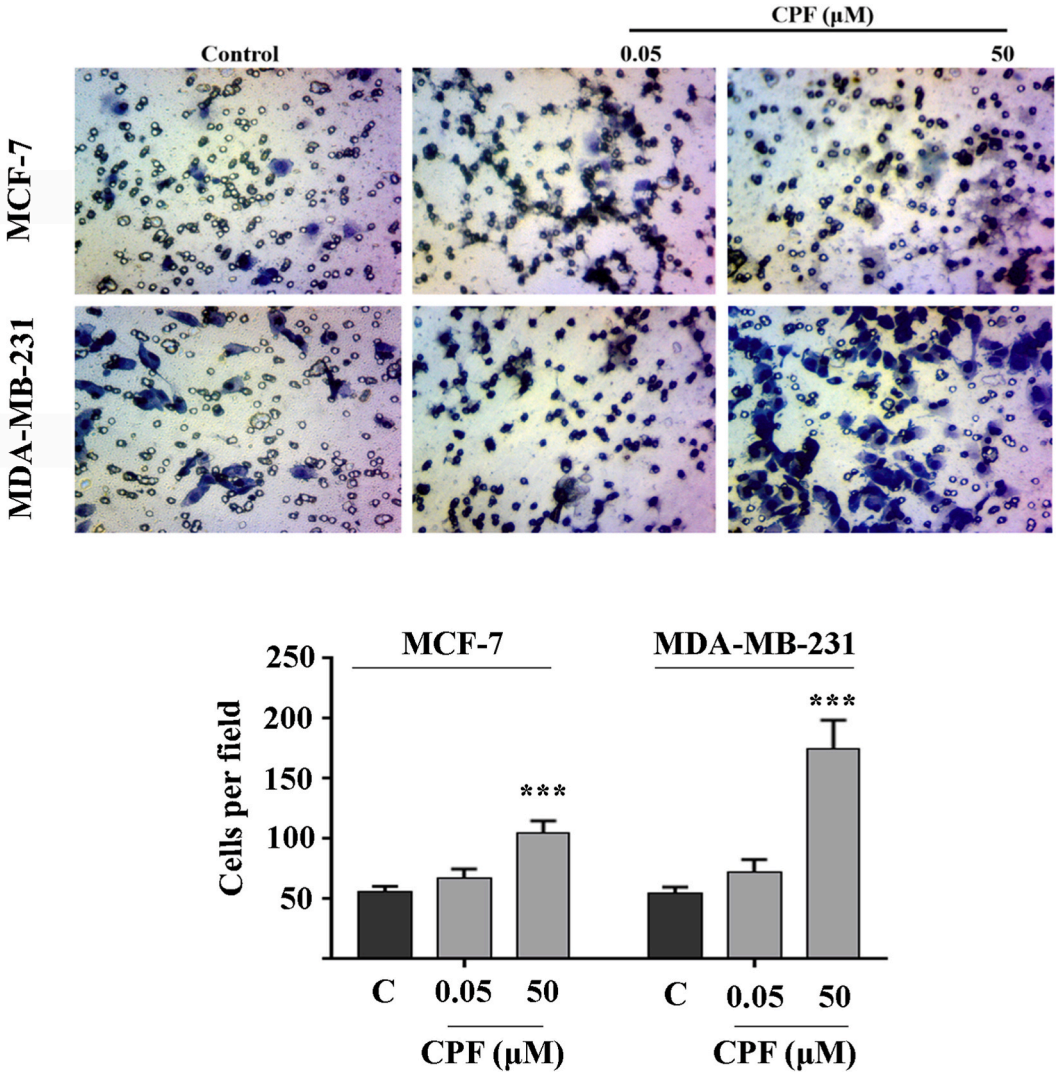

Fig. 2. ***CPF action on breast cancer cell invasion.*** MCF-7 and MDA-MB-231 cells were placed on the top of Boyden chambers covered with Matrigel®. RPMI with 10% FBS was settled in the lower chamber as chemoattractant. MCF-7 and MDA-MB-231 were exposed to vehicle (C) or CPF (0.05 or 50 μM) for 48 or 24 h, respectively. Then, cells on the lower surface of the filters were fixed, stained, and counted under an optic microscope. (***$p < 0.001$).

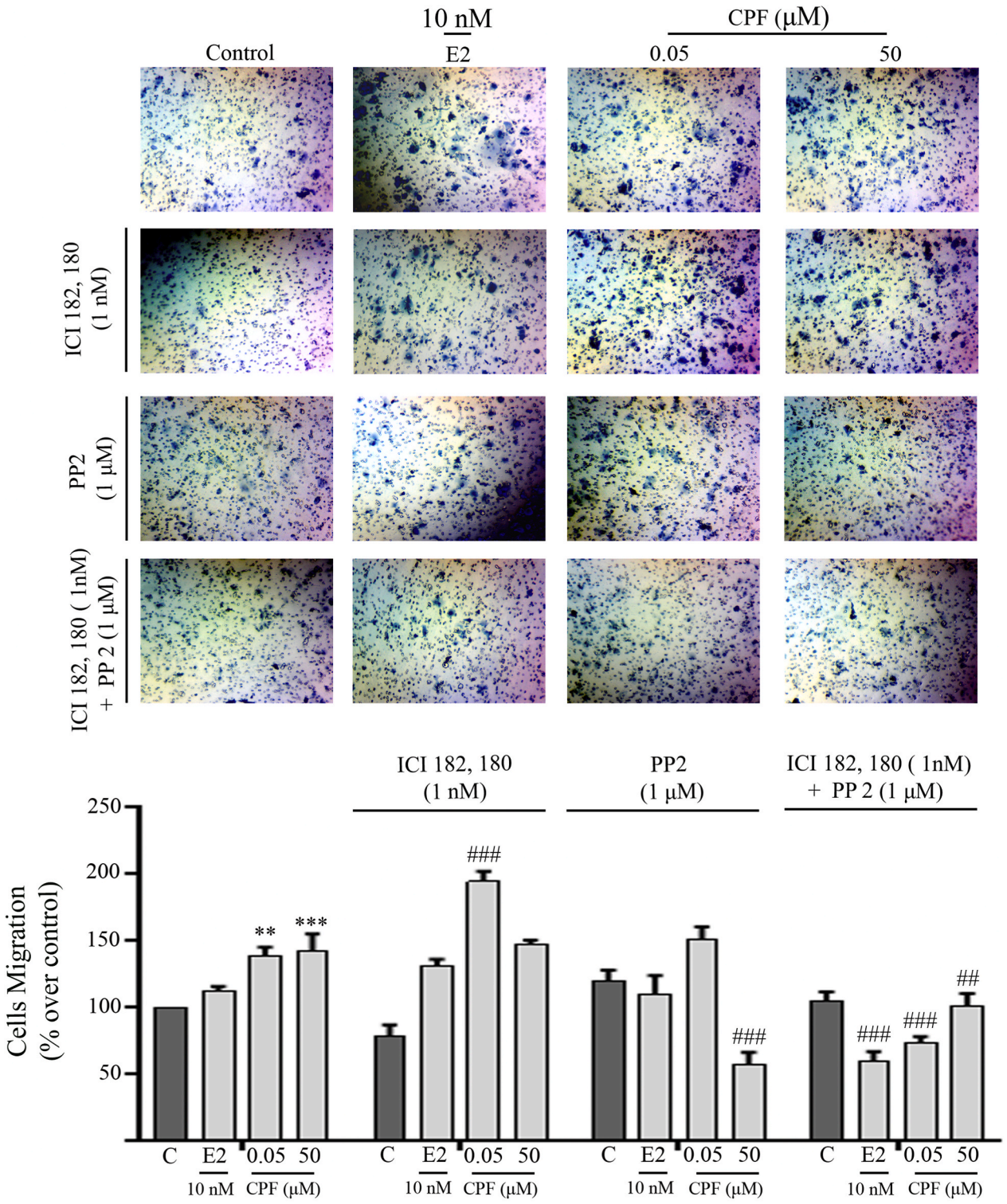

Fig. 3. ***Role of REα and c-SRC signaling pathways in CFP-induced MCF-7 cell migration.*** The migration was determined using Boyden Chambers assay. The cells were placed on the top of Boyden Chamber and pretreated for 3 h before adding the inhibitors (ICI 182,780 1 nM, PP2 1 μM or both simultaneously). Then, cells were treated with vehicle, E2 (10 nM) or CPF (0.05 or 50 μM) for 48 h (**$p < 0.01$ vs C; $^{###}p < 0.001$ vs 0.05 or 50 μM CPF).

3.3. Chlorpyrifos modulates metalloproteases activity and expression

Metalloproteases are present in invadopodia and were related to EMT processes since they are known to predominantly degrade specific components of the matrix facilitating migration and invasion. To evaluate the participation of MMPs in CPF action on EMT process, the expression and activity of MMP-2 and 9 were analyzed by Western blot and zymography assays, respectively.

CPF did not increase the gelatinolytic activity of MMP2 and MMP9 in MCF-7 cells (Fig. 5A y 5B). However, an increment of MMP2 expression was induced in the supernatant of the cultures of the MCF-7 cells that were exposed for 24 h to 0.05 μM CPF (222 ± 40% over control, ***p <

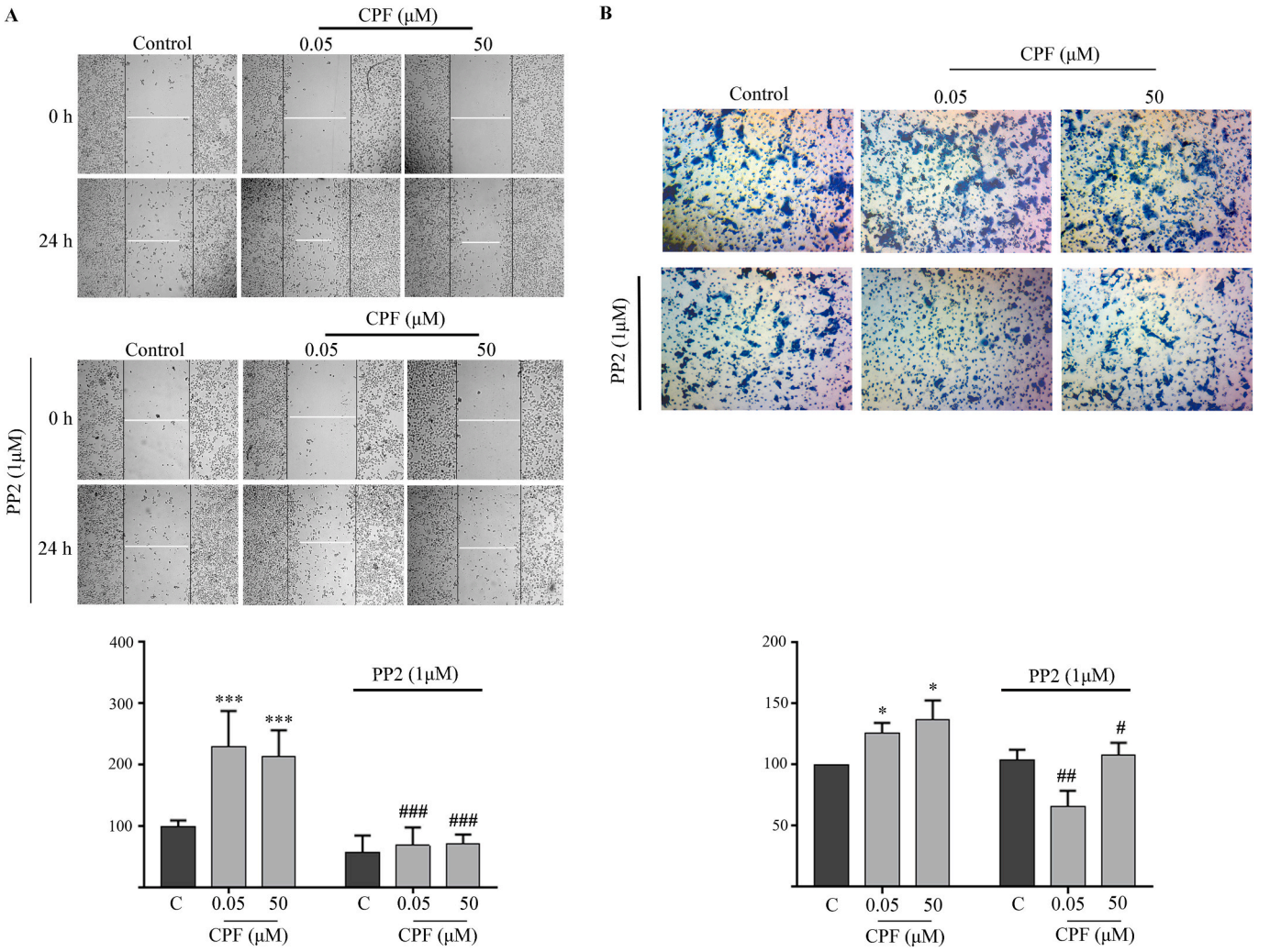

Fig. 4. ***Role of c-Src signaling pathways in CFP-induced MDA-MB-231 cell migration*. A)** Healing assay: cells were pretreated or not with PP2 1 μM. The monolayer was scratched with a pipette tip and the cells were exposed to vehicle (C) or CPF (0.05 or 50 μM) (***$p < 0.001$ vs C; $^{###} < 0.001$ vs 0.05 or 50 μM CPF). **B)** Boyden Chambers assay: cells were placed on the top of Boyden Chambers and pretreated or not for 3 h before with PP2 1 μM. Then, the cells were exposed to vehicle, or CPF (0.05 or 50 μM) for 24 h (*$p < 0.05$ vs C; $^{##} < 0.01$ vs 0.05; $^{#} < 0.05$ vs. 50 μM CPF).

0.001) or 50 μM CPF (204 ± 27% over control, ***$p < 0.001$). There is no significant increase for cells treated with E2. However, the increment induced by CPF could be reverted by ICI 182,780 as shown in Fig. 5C ($^{###}p < 0.001$ vs 0.05 μM CPF; $^{##}p < 0.01$ vs 50 μM CPF). Instead, the augment produced by the pesticide was independent of c-Src activation (Fig. 5D).

We have also analyzed the gelatinolytic activity in MDA-MB-231 cells after 24 h of exposure. Our results indicated an increment in MMP2 activity induced by 0.05 μM CPF (70 ± 19.6% over control, *$p < 0.05$) and 50 μM CPF (30% ± 5% over control, *$p < 0.05$) (Fig. 6A). We did not detect changes in the activity or expression of MMP9 in MDA-MB-231 cells (Fig. 6B). The increment of MMP2 was dependent of c-Src activation ($^{###}p < 0.001$ vs 0.05 μM CPF; $^{#}p < 0.05$ vs 50 CPF μM) (Fig. 6C). Results of MMP2 expression indicated that this gelatinase was increased by 0.05 and 50 μM CPF after 24 h (172 ± 26% over control, ***$p < 0.001$ and 385 ± 25% over control, ***$p < 0.001$, respectively), and this increase reversed with the administration of PP2 ($^{##}p < 0.01$ vs 0.05 μM CPF; $^{###}p < 0.001$ vs 50 CPFμM) (Fig. 6C). Insulin (Ins) was used as a positive control when MDA-MB-231 cells were used to perform the experiments.

3.4. *Effects of CPF on EMT proteins expression and subcellular localization*

We have also studied whether CPF modulates the expression of cancer progression markers. We analyzed E-Cadherin, β-Catenin, Vimentin and the transcription factor Slug. Immunofluorescence studies showed that E-Cadherin and β-Catenin reduced their membrane-bound expression when MCF-7 cells were exposed to 0.05 or 50 μM CPF for 72 h. In MCF-7, 50 μM CPF induced the translocation of Slug into the nucleus and the expression of Vimentin (Fig. 7A and Supplementary Image 1).

In turn, β-Catenin was found to be redistributed from the membrane to the nucleus and the perinuclear space when MCF-7 cells were exposed to 50 μM CPF. In this cell line, 0.05 μM CPF reduced E-Cadherin (38 ± 3.8% vs. control; **$p < 0.01$) and β-Catenin (34% ± 3.5% vs. control; **$p < 0.01$) after 72 h as shown in the Western blot (Fig. 7A and Supplementary image 1). Slug expression was not modified by 0.05 μM CPF. At the same timepoint, 50 μM CPF diminished the expression of E-Cadherin and β-Catenin (40% ± 4% and 43 ± 5% vs. control, respectively; **$p < 0.01$) and increased Slug expression (44 ± 6% vs. control, **$p < 0.01$). Both, E-Cadherin and β-Catenin were found diminished in an ERα-dependent way ($^{###}p < 0.001$ vs 0.05 or 50 μM CPF) (Fig. 8A). The decrease in β-Catenin and E-Cadherin expression was not dependent

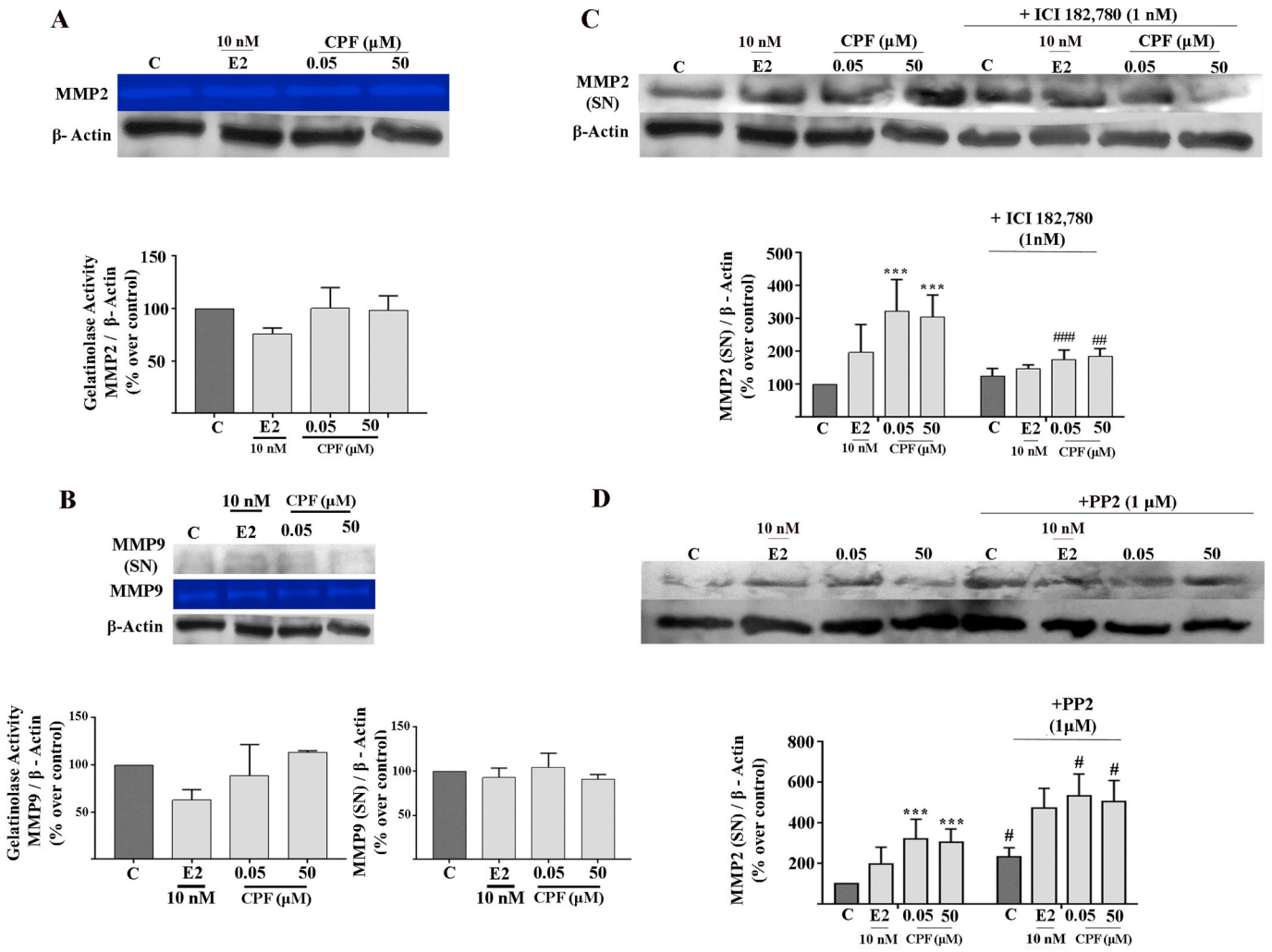

Fig. 5. ***CPF induced MMP2 secretion in MCF-7 cells.*** MCF-7 cells were treated with vehicle (C), E2 (10 nM) or CPF (0.05 and 50 μM) for 24 h. Culture supernatants were collected and a zimmography or Western Blot were performed to determine MMP2 **(A)** or MMP9 **(B)** gelatinase activities or expression, respectively **(A; B)**. **C)** Cells were treated for 3 h before adding ICI 182,780 1 nM. Then, vehicle (C), E2 (10 nM) CPF (0.05 and 50 μM) were added for 24 h. Culture supernatants were collected, and Western Blot was performed (***$p < 0.001$ vs Control; $^{###}p < 0.001$ vs 0.05; $^{##}p < 0.01$ vs 50 μM CPF). **D)** MCF-7 cells were treated 3 h before with PP2 1 μM. Then, vehicle (C), E2 (10 nM) CPF (0.05 and 50 μM) were added for 24 h. Culture supernatants were collected, and Western Blot was performed (***$p < 0.001$ vs Control; $^{###}p < 0.001$ vs 0.05; $^{##}p < 0.01$ vs 50 μM CPF).

on c-Src (Fig. 8B). PP2 markedly diminished the expression of both proteins, E-Cadherin and β-Catenin. MMP2 levels are also decreased by PP2 which suggests that c-Src is involved in the regulation of chlorpyrifos induced-EMT.

In MDA-MB-231 cells, immunofluorescence studies showed the translocation and nuclear expression of β-Catenin when the cells were exposed to 50 μM CPF for 72 h. We have also observed a reduction in expression of β-Catenin with 0.05 μM CPF (29 ±1%, ***$p < 0.01$ vs control) and 50 μM (47±7%, ***$p < 0.001$ vs control) and an increase of the expression of Vimentin (42 ±13% vs control,*$p < 0.05$ vs control; 42±7% vs control, *$p < 0.05$; 0.05 μM and 50 μM CPF, respectively) (Fig. 7B and Supplementary image 1). These effects were reversed with the administration of PP2 (Fig. 8C). We did not observe modulation of Slug expression or changes in its subcellular location (Fig. 7B and Supplementary image 1).

4. Discussion

CPF is an insecticide widely used in our country and in many others to control crops in agriculture. Some of the methods used for the application can cause CPF to reach the populations surrounding the fumigation areas with undesirable consequences on human health. We have previously reported that CPF is an ED and a breast cancer risk factor due to its ability to induce cell proliferation both *in vivo* and *in vitro* experiments (Ventura et al., 2016, 2012). We recently showed that CPF reduces the latency period and produces an augment of the number of tumors in an experimental model in rats (Ventura et al., 2019). Until now there are no reports addressing the role of CPF exposure during cancer progression. Therefore, our aim was to elucidate if CPF can also promote processes involved in the late steps of the carcinogenesis, to activate the spread of the cells from their primary sites to distant organs (Chaffer and Weinberg, 2011; Klein, 2008). Here, we specifically studied whether CPF can induce EMT process by studying morphological changes of the cells, migration and invasion promotion and the expression of different molecular markers that characterize EMT. We found that CPF induces a typical mesenchymal spindle-shaped phenotype with the appearance of protrusions compatible with invadopodia features. These characteristics have been associated with degradation of the extracellular matrix that favor cancer invasiveness and metastasis (Murphy and Courtneidge, 2011).

Several bisphenol compounds are considered as ED because they can exert estrogenic properties, induce the loss of cell to cell contacts and the

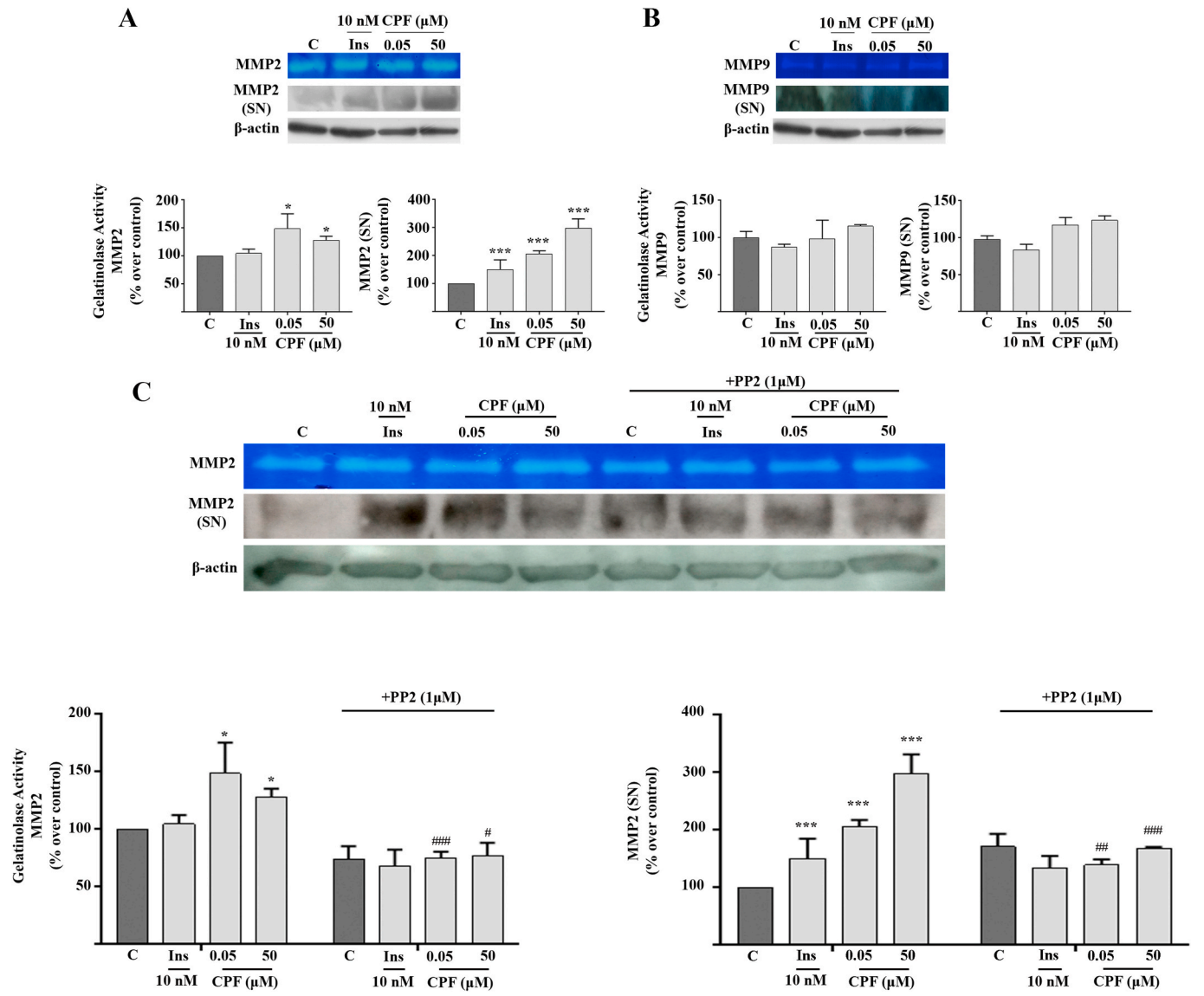

Fig. 6. ***CPF induced the activity and secretion of MMP2 in MDA-MB-231 cells in a c-Src-dependent way.*** MDA-MB-231 cells were treated with vehicle (C), Insulin (Ins) (10 nM) or CPF (0.05 and 50 μM) for 24 h. Culture supernatant were collected and zimmography or Western Blot were performed to determine MMP2 (*$p <$ 0.05 vs Control, ***$p <$ 0.001 vs Control) **(A)** or MMP9 **(B)** gelatinase activity or expression. **C)** MDA-MB-231 cells were treated for 3 h before adding PP2 1 μM. Then, cells were exposed to vehicle (C), Ins (10 nM) or CPF (0.05 and 50 μM) for 24 h. Culture supernatant were collected and zimmography and Western blot were performed (*$p <$ 0.05, ***$p <$ 0.001 vs C; $^{\#}p <$ 0.05, $^{\#\#}p <$ 0.01, $^{\#\#\#}p <$ 0.001 vs 0.05 or 50 μM CPF).

acquisition of fibroblast-like morphology (Kim et al., 2017). CPF is an endocrine disruptor that we found to induce the migration of both MCF-7 and MDA-MB-231 cell lines.

Cells can migrate collectively or as individual cells. The movement of single cells appears to be a complex mechanism since it requires the nucleus to undergo significant shape changes while cells migrate through confined spaces. In both types of migration, the cells need to acquire a motile phenotype by developing some plasma membrane protrusions through an actin cytoskeleton remodeling and formation of actin-based structures which allow the cells to adhere as well as to probe and sense different molecules of ECM. The different types of protrusions involve diverse proteins that constitute the cytoskeleton that are regulated by different pathways (Lintz et al., 2017).

In both types of movement, we observed a c-Src dependence of CPF-mediated cell migration. In MDA-MB-231 the effect is clearer than in MCF-7 because in MCF-7 cells, the ERα is also involved in CPF-mediated cell migration (Frei et al., 2016).

After silencing ERα, the intercellular adhesion mediated by the decrease of E-cadherin expression leads to morphological changes that promote the invasion of the clones that acquire the spindle cell shape (Bouris et al., 2015). According to our results, E2 slightly increased cell migration compared to the control group, but this increment was not significant. Curiously, when ERα was inhibited, E2 barely increased MCF-7 cell migration, and this increment was not significant. However, an important induction of the migration was observed at the lowest dose of CPF compared to the control cells and to the cells treated with ICI182, 180. These effects might be explained by the activation of other pathways inducing migration that could be exacerbated when ER pathway is inhibited as the c-Src signaling. Our hypothesis relies on the fact that we can only inhibit CPF-mediated or E2-mediated migration when both ER and c-Src pathways are blocked simultaneously. Recently, it was stated that the knockdown or silencing of ERα in MCF-7 breast cancer cells provokes pivotal changes in phenotype, cellular functions, mRNA and protein levels of EMT markers, and consequently, EMT status (Karamanou et al., 2019). Furthermore, it is well known that there is a cross talk between ER and c-Src pathways. c-Src can activate ER by

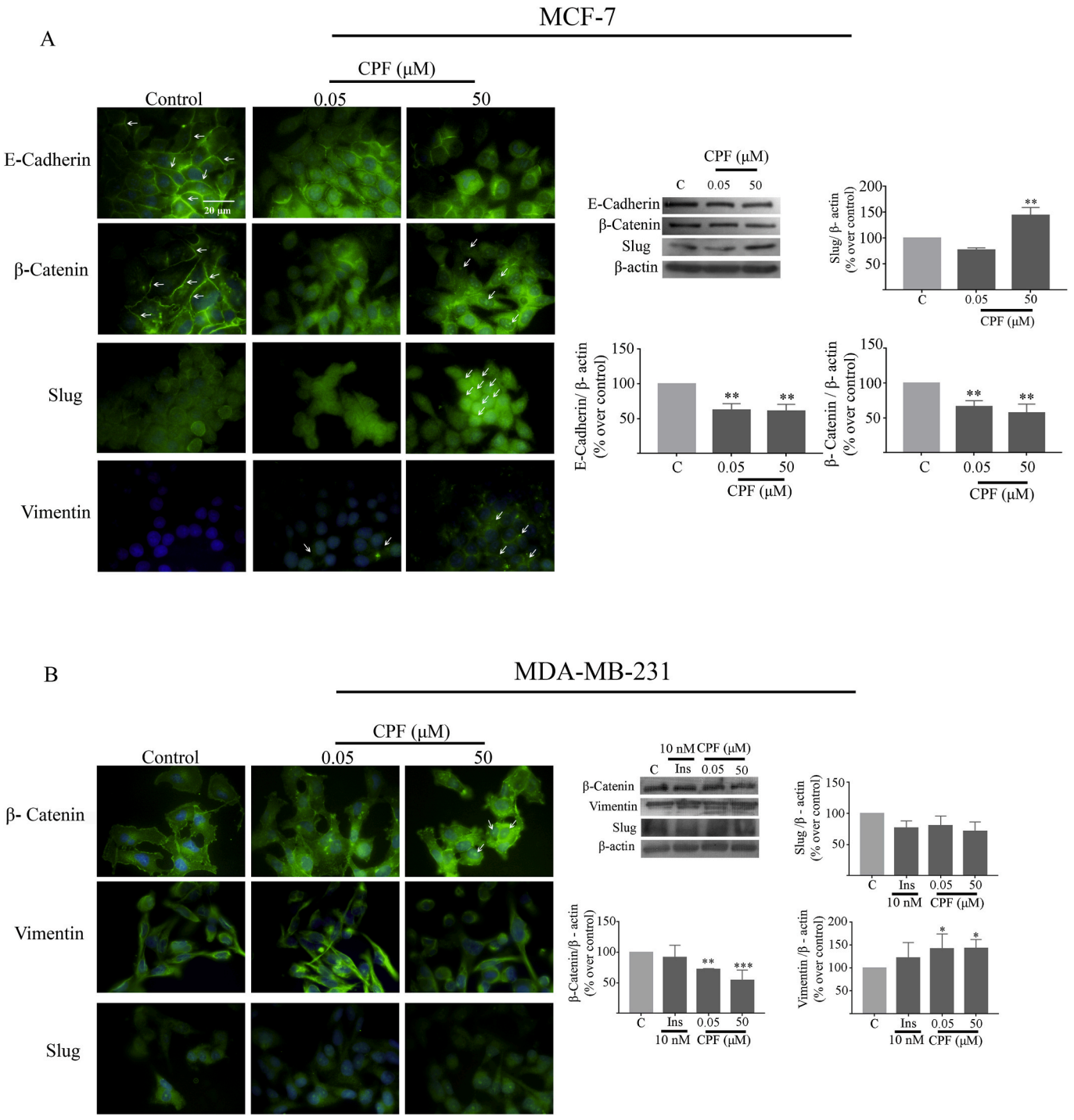

Fig. 7. ***CPF effect on* E-Cadherin, β-Catenin, *Vimentin and Slug expression in MCF-7 and MDA-MB-231 cells.*** **A)** MCF-7 cells were exposed to CPF (0.05 and 50 μM) or vehicle (C) for 72h. E− Cadherin, β-Catenin, Vimentin and Slug subcellular localization was determined by immunofluorescence microscopy using a specific monoclonal antibody. Bar scale: 20 μm. Magnification: 1000X. E− Cadherin, β-Catenin, and Slug levels were determined by Western blot (**$p < 0.01$, vs. Control). **B)** MDA-MB231 cells were exposed to CPF (0.05 and 50 μM) or vehicle (C) for 72h. β-Catenin, Vimentin and Slug subcellular localization was determined by immunofluorescence microscopy using a specific monoclonal antibody. Bar scale: 20 μm. Magnification: 1000X. β-catenin, Vimentin and Slug levels were determined by Western blot. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. Control).

phosphorylation via IGF-1R or other growth factor receptors. Our results are also supported by the work of Bouris et al. that concludes that the loss of ERα in breast cancer cells results in a potent EMT. This EMT is characterized by key changes in the profile expression of specific matrix macromolecules that highlight the role of matrix effectors in breast cancer endocrine resistance (Bouris et al., 2015). It is possible that in the estrogen-dependent MCF-7 cells, many other signals can be activated and exacerbated when ERα is inhibited and therefore blocking ERα could not be enough to revert the effect of CPF on cell migration. In this regard, it has been extensively reported the interaction between ERα and IGF system and their downstream signaling induces the EMT phenotype in epithelial tumors. We cannot rule out the participation of other growth factors such as epidermal growth factor "EGF", basic fibroblast growth factor "FGF-2", hepatocyte growth factor "HGF" or transforming

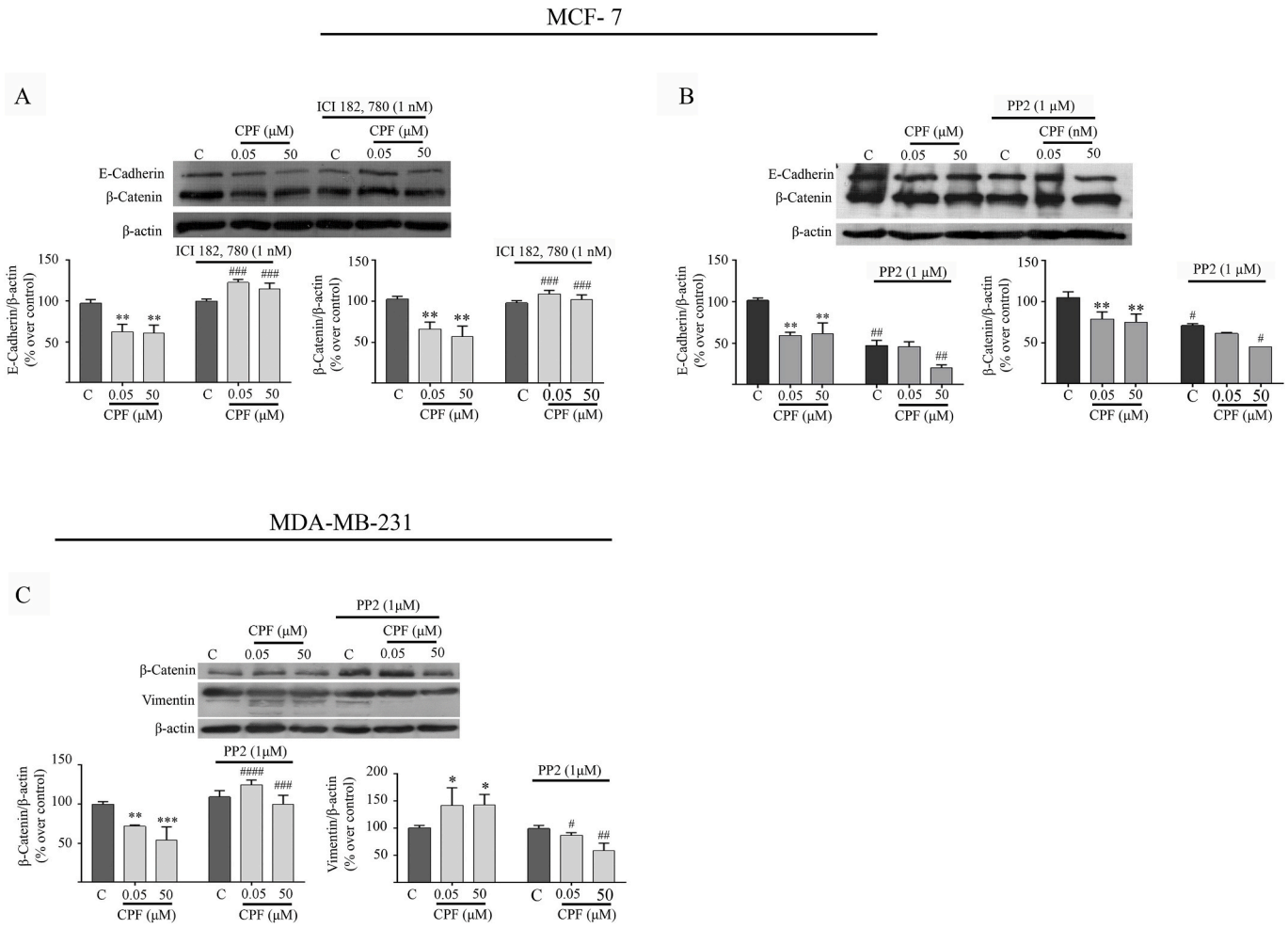

Fig. 8. ***Molecular pathways involved in the TEM induced by CPF***. E-Cadherin, β-Catenin and Vimentin levels were determined by Western blot **A)** MCF-7 cells were pretreated for 3 h before adding ICI 182,780 1 nM. Then vehicle (C), or CPF (0.05 and 50 μM) were added and incubated for 72 h. E-Cadherin, β-Catenin levels were determined by Western blot (**p < 0.01 vs Control; $^{###}$p < 0.001 vs 0.05 or 50 μM CPF. **B)** MCF-7 cells were pretreated for 3 h before adding PP2 1 μM. Then, vehicle (C), or CPF (0.05 and 50 μM) were added and incubated for 72 h. E− Cadherin, β-Catenin levels were determined by Western blot (**p < 0.01 vs Control; $^{#}$p < 0.05, $^{##}$p < 0.01 vs 0.05 or 50 μM CPF. **C)** MDA-MB-231 cells were pretreated for 3 h before adding PP2 1 μM. Then, vehicle (C), or CPF (0.05 and 50 μM) were added and incubated for 72 h. β-Catenin and Vimentin levels were determined by Western blot. (*p < 0.05, **p < 0.01, ***p < 0.001 vs Control; $^{#}$p < 0.05, $^{##}$p < 0.01, $^{##}$p < 0.01 vs 0.05 or 50 μM CPF).

growth factor β1 "TGF-β1" which have been documented to trigger or at least modulate EMT (Cevenini et al., 2018).

Consequently, in MCF-7 cells it was necessary to block both c-Src kinase and ERα to inhibit cell migration induced by 0.05 μM CPF. However, in MDA-MB-231 cells migration was prevented solely by adding PP2 to inhibit c-Src signaling.

MMPs play a key role not only in physiological ECM remodeling, but also in cancer progression. As we detected that CPF can induce invasion and migration in MCF-7 and MDA-MB-231 cells, we determined the activity and the expression of MMP2 and MMP9. We found that both 0.05 and 50 μM CPF increased the activity and expression of MMP2 in MDA-MB-231 cells. The expression was also elevated in MCF-7 cells. As MDA-MB-231 is a much more aggressive cell line than MCF-7, the increment of MMP-2 activity observed only in MDA-MB-231 cells may be related to this aspect. In this regard, it has been found that the increment of the radioresistance and invasive ability is due to a strong gelatinase activity induced in MDA-MB-231 cells after radiation that, in turn, may be traduced in a more aggressive phenotype (Artacho-Cordón et al., 2012).

The connection of MMPs with tumor progression through EMT was found in lung, ovarian, breast and prostate cancer (Scheau et al., 2019). It has been extensively studied associated with migration and invasion. MMP2 overexpression positively correlates with an aggressive malignant phenotype and poor outcome in breast cancer patients (Köhrmann et al., 2009; Radisky and Radisky, 2010) and appear to have clinical value as diagnostic and predictive factor for metastases in breast cancer (Van't Veer et al., 2002).

The traditional role of MMPs in matrix remodeling was enlarged away from those that are related to the proteolysis (Shay et al., 2015). In our experiments, MMP9 did not change when MCF-7 or MDA-MB-231 cells were exposed to CPF. It has been postulated that MMP9 inhibits angiogenesis (Deryugina and Quigley, 2006). Interestingly, when MMP9-overexpressing carcinoma cells were transplanted into wild-type recipients, the levels of expression of MMP9 and angiostatin, a strong anti-angiogenic factor, were stimulated (Pozzi et al., 2002). These results could explain why CPF did not modulate MMP9 therefore avoiding a possible antiangiogenic effect.

There are many biological markers associated with the EMT process. In this work we analyzed the expression of β-Catenin, E-Cadherin, Vimentin and Slug. Vimentin is a primary marker of EMT that stabilizes cytoskeleton interactions and responsible for maintaining the morphology of the cells and the whole integrity of the cytoplasm. Vimentin behaves like an important adaptor to maintain the assembly with proteins involved in adhesion, migration, invasion, and cell

signaling (Helfand et al., 2011; Nieminen et al., 2006). Our results indicate that 0.05 and 50 μM CPF reduced β-Catenin and increased the expression of Vimentin in MDA-MB-231 cells. Interestingly, we also detected Vimentin expression in MCF-7 cells after 50 μM CPF exposure. This change constitutes a clear signal of the transforming action of CPF since Vimentin is not normally expressed in the epithelial MCF-7 cell line because it is a distinctive intermediate filament protein that is present in mesenchymal cells.

In MCF-7 cells, 0.05 and 50 μM CPF reduced β-Catenin and E-Cadherin expression and promoted relocation of both proteins away from the plasma membrane and a decreased adhesion to facilitate the movement of the cells. β-Catenin and Slug were found in the nuclei of cells exposed to 50 μM CPF. Slug was found to be increased after 50 μM CPF exposure. As Slug represses E-Cadherin expression and promotes EMT (Adhikary et al., 2014), the reduction of E-cadherin expression could be due to Slug induction. The accumulation and nuclear translocation of these proteins could induce the expression of genes responsible for the EMT process. In the case of Cr(VI), another carcinogenic pollutant shown to accelerate EMT, Song-Ze Ding et al. described that it can induce an increment of HDAC1 with an enhanced binding of HDAC1 to the E-Cadherin promoter to repress its transcription (Ding et al., 2013). We have reported in a previous work that HDAC1 is enhanced by CPF (Ventura et al., 2019) and this effect could be due to the reduction of E-Cadherin induced by CPF. E-Cadherin repression induces a morphological change from a cobblestone-like epithelial cell phenotype to a spindle-like mesenchymal cell shape and cell migration and invasion to other sites (Ye et al., 2010). We observed the loss of cell to cell contact after CPF exposure particularly evident in MCF-7 cells. This aggressive phenotype was also demonstrated for endocrine disruptors such as aldrin, aroclor and CPF where chronic exposure induced a pro-metastatic phenotype after 50 days in prostate cancer cell line DU145 (Bedia et al., 2015).

ERα is a receptor with transcriptional activity that mediates the effects of estrogen in diverse biological processes, such as cell growth, differentiation and survival (Jiménez-Garduño et al., 2017). It was observed that MCF-7 cells acquired changes in their phenotype which was accompanied by the modulation of gene and protein expression of EMT markers (Bouris et al., 2015). We have already reported that CPF induces breast cancer cell proliferation in an estrogen receptor alpha-dependent fashion at pesticide concentrations that can commonly be found in water and soils that surround the fumigated fields (Ventura et al., 2012). Here, we investigated if REα participates in the modulation of EMT markers induced by CPF. We show that the blockage of the ERα in MCF-7 cells increased β-Catenin and E-Cadherin expression and that MMP2 in culture supernatant was reduced after CPF exposure, restoring the epithelial phenotype. Therefore, it seems that CPF generates a mesenchymal phenotype through the activation of ERα. This behavior may constitute another endocrine disrupting activity of CPF in MCF-7 cells.

In MDA-MB-231 the increment of expression and activity of MMP2, the reduction of β-Catenin and the increase of Vimentin expression after CPF exposure were reverted by adding the c-Src inhibitor, PP2. Additionally, the induction of migration was also found to be dependent on c-Src. This result agree with a large number of studies that show that estrogen independent breast cancer cells are highly susceptible to c-Src inhibition (Fan et al., 2012; Finn et al., 2007; Gilani et al., 2016; Zhang et al., 2015). In contrast, MCF-7 cells exhibit a different behavior when treated with PP2, since E-Cadherin and β-Catenin expression decreases after 72 h of CPF exposure, cell to cell adhesion is disrupted and the cells keep the mesenchymal properties.

Altogether, our results show for first time that CPF exposure modulates EMT-molecular targets, activates the invasion and migration of breast cancer cells and the pathways involved in these processes. These results together with the ubiquitous distribution of the pesticide make it of utmost importance to take measures to minimize the risk of exposure to this compound.

Credit author statement

All authors contributed to this research work. M.L. designed and carried out most of the experiments and participated in manuscript preparation. C. V. and M.S.H. designed and analyzed some experiments. M.S.H. and M.N.M. analyzed the experimental results and participated in manuscript preparation. M.L, M.S.H., G. M. and A.R. analyzed the experimental results and participated in the discussion and manuscript edition. M.N and C.C analyzed the experimental results and wrote the manuscript and C.C. supervised and was responsible for the overall progress of the project.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecoenv.2020.111312.

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