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Pharmacological inhibition of platelet-tumor cell cross-talk prevents platelet-induced overexpression of cyclooxygenase-2 in HT29 human colon carcinoma cells

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Running Title Page

Platelet-tumor cell cross-talk and malignancy

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Abbreviations: CRD, carbohydrate recognition domain; COX, cyclooxygenase; EMT, epithelial-mesenchymal-like-transition; EP, E-series prostanoid receptor; GP, glycoprotein; NHE, Na^+/H^+ exchanger; PDGF, platelet-derived growth factor; PDGFR, PDGF receptor; PG, prostaglandin; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; TGF, transforming growth factor; EGF, epidermal growth factor; VEGF, vascular endothelial growth factor; TX, thromboxane; TXAS, TXA_2 synthase; HuR, human antigen R.

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Abstract

Cyclooxygenase(COX)–2–derived prostanoids can influence several processes that are linked to carcinogenesis. We aimed to address the hypothesis that platelets contribute to aberrant COX-2 expression in HT29 colon carcinoma cells and to reveal the role of platelet-induced COX-2 on the expression of proteins involved in malignancy and marker genes of epithelial-mesenchymal transition(EMT). Human platelets co-cultured with HT29 cells rapidly adhered to cancer cells and induced COX-2 mRNA expression, but not protein synthesis which required the late release of platelet PDGF and COX-2 mRNA stabilization. Platelet-induced COX-2-dependent PGE_2 synthesis in HT29 cells was involved in down-regulation of $p21^{WAF1/CIP1}$ and up-regulation of cyclinB1, since these effects were prevented by rofecoxib(a selective COX-2 inhibitor) and rescued by exogenous PGE_2. Galectin-3, highly expressed in HT29 cells, is unique among galectins because it contains a collagen-like domain. Thus, we studied the role of galectin-3 and platelet collagen receptors in platelet-induced COX-2 overexpression. Inhibitors of galectin-3 function(β-lactose, a dominant-negative form of galectin-3,Gal-3C, and anti-galectin-3 antibody M3/38) or collagen receptor-mediated platelet adhesion(revacept, a dimeric collagen receptor GPVI-Fc) prevented aberrant COX-2 expression. Inhibition of platelet-cancer cell interaction by revacept was more effective than rofecoxib in preventing platelet-induced mRNA changes of EMT markers suggesting that direct cell-cell contact and aberrant COX-2 expression synergistically induced gene expression modifications associated with EMT. In conclusion, our findings provide the rationale for testing blockers of collagen binding sites, such as revacept, and galectin-3 inhibitors in the prevention of colon cancer metastasis in animal models followed by studies in patients.

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Introduction

Platelet-tumor cell interactions within the bloodstream play an important role in the metastatic dissemination of epithelial tumors. Platelets may contribute to metastasis through several mechanisms(Gay and Felding-Habermann, 2011):(i)formation of platelet aggregates surrounding tumor cells which may support tumor cell survival and protection from immune elimination, (ii)enhancement of the adhesion of tumor cells to the endothelium thus leading to tumor cell arrest and extravasation, and (iii)synthesis of lipid products[such as thromboxane(TX)A_2] and the release of proteins from α-granules[such as transforming growth factor(TGF)-β and platelet-derived growth factor(PDGF)] during platelet activation that may affect tumor vascularization and facilitate tumor cell dissemination into the bloodstream. Recently, Labelle et al.(2011) showed that the direct interaction of platelets with tumor cells synergizes with platelet released TGF-β to induce the expression of genes for epithelial-mesenchymal-like-transitions(EMT), thus increasing the invasive potential of tumor cells and their capacity to colonize the lung.

The direct interaction of platelets with tumor cells seems to be a crucial mechanism regulating the initial steps in the metastatic process. Thus, the elucidation of platelet receptors and tumor cell plasma-membrane components which participate in cell-cell interaction will facilitate progress in cancer treatment strategies. Different platelet adhesion receptors may govern platelet-tumor cell interactions depending on cell-type specific dysregulated expression of distinct membrane components induced by neoplastic transformation(Boukerche,1989;Mannori,1995). Galectin-3 is a member of a family of carbohydrate-binding proteins but uniquely consists of a C-terminal carbohydrate recognition domain(CRD), a collagen-like internal R-domain, and the N-terminal domain, is highly elevated in malignancies including colon cancer(Yang, 2008). It is localized inside the cells but also on cell surface where it mediates cell–cell and cell–matrix interactions by binding to glycoconjugates that contain β-galactosides via the CRD. Galectin-3 is unique among galectins because it has the collagen-like domain. This is suggestive of the role of galectin-3 in

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platelet-cancer cell cross-talk through the interaction with platelet collagen receptors. Several different receptors for collagen have been identified on platelets, including glycoprotein(GP)Ib, integrin α2β1 and GPVI. In humans, there is growing evidence for GPVI as the major collagen receptor for platelet activation(Nieswandt, 2003).

Another important question to unravel is the role played by other platelet-released factors, in addition to TGF-β, on tumor cell acquisition of a disseminating phenotype. PDGF, a major component of platelet α-granules, is released during platelet activation(Coppinger,2007) and it induces cell signaling pathways that stimulate EMT(Yang,2006). Moreover, the contribution of platelet TXA_2, a pro-aggregatory lipid mediator generated from arachidonic acid(AA) by the activity of COX-1(Hamberg,1975), is indirectly suggested by the finding of randomized clinical trials with aspirin showing that the drug reduced by 40-50% the likelihood that cancers would spread to distant organs(Rothwell,2012a; Rothwell,2012b; Algra,2012). The efficacy of aspirin is detected also at the low-doses, used for the prevention of atherothrombosis, which act by causing the selective inhibition of platelet COX-1 activity(Patrono, 2005). Thus, it has been proposed that the antiplatelet effect of aspirin plays a central role in its efficacy against cancer(Patrono, 2001; Dovizio, 2013).

Platelets might reprogram cancer cells to a more malignant phenotype through the induction of aberrant expression of COX-2, a hallmark of tumor invasion, metastasis and poor prognosis in colorectal cancer and other types of cancer(Prescott, 2000; Patrono, 2001). COX-2 mediates these effects by enhancing the generation of PGE_2 which triggers tumorigenic signals in target cells by coupling to four subtypes of receptors classified as EP1, EP2, EP3 and EP4(E-series prostanoid receptors) expressed on plasma membrane and/or the nuclear envelope(Regan, 2003; Cha, 2007; Bhattacharya, 1998). COX-2 overexpression, in cancer and inflammation, is associated with altered expression and/or cytoplasmic accumulation of trans-acting factors that bind to AU-rich

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elements(AREs) of COX-2 mRNA and influence its stability, such as the mRNA-stability factor HuR(Young, 2008; Dixon, 2006).

In this study, we aimed to characterize the role played by tumor and platelet surface molecules and released products on the expression of COX-2 and other proteins associated with malignancy in HT29 cells. Our findings show that platelet-tumor cell interaction, mediated by platelet collagen receptors and galectin-3, and the release of PDGF from activated platelets play a central role in the aberrant expression of COX-2 in cancer cells. The cellular interactions conferred an increased mitogenic potential to HT29 cells through downregulation of $p21^{WAF1/CIP1}$ associated with upregulation of cyclinB1. Direct platelet-tumor cell interaction and platelet-induced COX-2 overexpression, synergistically induced the expression of genes for EMT in HT29 cells. Our findings that revacept, a novel antiplatelet drug which inhibits collagen-mediated platelet adhesion(Ungerer, 2011), prevented these changes provides the basis for further investigation of agents that block collagen-induced platelet function for colon cancer chemotherapy.

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Material and Methods

Co-culture experiments with human colon carcinoma cell line HT29 and isolated human platelets

HT29 cell line, obtained from European Collection of Cell Cultures (ECC, Salisbury, UK), was cultured in McCoy's 5A medium (Invitrogen, Milan, Italy) containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 2mM L-glutamine, as previously described (Dovizio, 2012). In all experiments, $1x10^6$ cells were seeded in 6-multiwell plate containing 2ml of McCoy's 5A supplemented with FBS 0.5% and polimyxin B sulphate10μg/ml (Sigma-Aldrich, Milan, Italy). HT29 cells and platelets were cultured alone or together for different time-points (2, 4, 8, 12, 16, 20 h); then conditioned media were collected, centrifuged at 10,000xg for 2 min, to discard cell debris, and the levels of TXB_2, PGE_2, PDGF-BB, EGF and TGF-β1 were evaluated by specific immunoassays. The levels of COX-2, $p21^{WAF1/CIP1}$ and cyclin B1 were assessed in HT29 cell lysates (at 4, 8, 12, 16 and 20h). Since changes of these protein levels in HT29 cells co-cultured with platelets, occurred with different kinetics, but they were significantly modulated at 20h, the levels of other proteins, such as proliferating cell nuclear antigen (PCNA), cyclin D1, Bcl-2, ornithine decarboxylase (ODC), galectin-3 and galectin-4, and the effects of pharmacological inhibitors were evaluated at this time-point. Human platelets were freshly isolated from leukocyte concentrates obtained from Städtische Kliniken Hoechst (Frankfurt, Germany) as previously described (Dovizio, 2012). In brief, venous blood was collected from healthy adult donors and leukocyte concentrates were prepared by centrifugation (4000xg, 20min, 20°C). Leukocyte concentrate was sedimented in 5% dextran solution (Sigma-Aldrich) and the supernatant was stratified in lymphocyte separation medium (PAA Laboratories GmbH, Pasching, Austria) by centrifugation (800xg, 10min at room temperature). After centrifugation, platelet-rich plasma (PRP) was obtained. PRP was then mixed with PBS, pH 5.9 (3:2, v/v), centrifuged (2000xg, 15min, room temperature), and pelleted platelets were resuspended in PBS, pH 5.9/0.9% NaCl (1:1, v/v), washed by centrifugation (2000xg, 10min,

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room temperature), and finally resuspended in McCoy's 5A medium containing FBS 0.5% and polymixyn B sulphate10μg/ml. Fifty μl of platelet suspension containing $1x10^8$ cells was added to HT29 cells ($1x10^6$) and incubated for different times. As control conditions, HT29 cells were incubated with 50μl of culture medium and cultured alone. In some experiments, platelets were pretreated with aspirin before incubation with HT29 cells; briefly, PRP was incubated with aspirin 300μM for 30min at room temperature, then platelets were isolated, washed twice and cultured alone or co-cultured with HT29 cells. In experiments where we aimed to evaluate the effect of COX-2 inhibition, the highly selective COX-2 inhibitor rofecoxib (0.3μM, Witega Laboratorien, Berlin, Germany) was used. The drug was used to assess the contribution of HT29 COX-2 to PGE_2 released in the culture medium. Thus, in co-culture experiments performed with HT29 cells and platelets (untreated with aspirin), rofecoxib was added 20 min before the addition of platelets and the incubation continued for 20h; then conditioned media were collected and centrifuged at 10,000xg for 2 min to discard cell debris and supernatants were analyzed for PGE_2 levels. In some experiments, we aimed to verify whether platelet-induced COX-2 was catalytically active even at 20h of co-culture. Thus, HT29 cells were co-cultured with aspirin-treated platelets for 20h (under these conditions COX-2 was upregulated). Platelets were pre-treated with aspirin to eliminate the contribution of platelet COX-1 to PGE_2 levels detected in the culture medium. Then, the medium was changed with a fresh one containing rofecoxib (0.3μM) or DMSO vehicle and the cells were incubated for 1h before the addition of exogenous AA (Sigma-Aldrich) 0.5μM (to start COX product formation) and the incubation continued for 1h. At the end of the incubation, conditioned media were harvested, centrifuged at 10,000xg for 2 min to discard cell debris and supernatants were analyzed for PGE_2 levels. In other experiments, HT29 cells co-cultured with platelets for 20h in the presence of rofecoxib, were treated with exogenous PGE_2 (1.5 and 15ng/ml) (Cayman Chemical Ann Arbor, Minn, USA).

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Pharmacological Treatments

The nonselective COX inhibitor aspirin (Sigma-Aldrich), the selective COX-2 inhibitor rofecoxib (Witega Laboratorien), the inhibitor of RNA synthesis actinomycin D (Sigma-Aldrich), the PDGFR antagonist imatinib (Cayman Chemical), the P-selectin antagonist (Gallolyl-N-gaba-WVDV-OH, Calbiochem, Merck KGaA, Darmstadt, Germany), the inhibitor of PKCδ rottlerin (Calbiochem, Merck KGaA) and the inhibitor of PI3K wortmannin (Sigma-Aldrich) were dissolved in DMSO; the competitive antagonist of P2-purinoceptors suramin (Calbiochem, Merck KGaA) and the pan-galectin inhibitor β-lactose (Sigma-Aldrich) were dissolved in distilled water; the protein obtained by fusing the extracellular domain of GPVI with the human immunoglobulin Fc domain (Ungerer, 2011) (revacept, kindly provided by Dr. Münch, CorImmun GmbH, Munich, Germany) was dissolved in PBS/4% mannitol/1% sucrose; the neutralizing antibody anti-PDGF (R&D system, Minn, USA) was dissolved in PBS; the M3/38 purified anti-mouse/human galectin-3 monoclonal antibodies (Santa Cruz Biotechnology, Texas, USA) was dissolved in PBS; the truncated form of galectin-3 lacking 107 amino acids from the *N*-terminus, Gal-3C (John, 2003) (kindly provided by Dr. John, MandalMed, Inc., San Francisco, CA, USA) was dissolved in PBS, containing lactose as a stabilizer, that was removed, prior to use, by a biodialyser system with cellulose acetate membrane for 24h (Sigma Adrich); the inhibitor of Na^+/H^+ exchanger(NHE) dm-amiloride [5-(N,N-Dimethyl)amiloride hydrochloride] (Sigma-Aldrich)] was dissolved in methanol. All compounds or the appropriate vehicles were added to HT29 cells 20 min before the addition of platelets and co-cultured for 20h. Revacept or vehicle was added to HT29 cells cultured alone for 1h, then the medium was changed, replaced with platelet cell suspension and incubated up to 20h.

Biochemical Analyses

TXB_2 (the stable hydrolysis product of TXA_2) and PGE_2 levels were measured in cell culture media by previously described, validated and specific radioimmunoassay techniques (Patrono, 1980;

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Patrignani, 1994). PDGF-BB, EGF and active TGF-β1 levels released in the medium were determined by ELISA assay (R&D system)[with detection limits of 2, 0.7 and 4pg/ml, respectively]. In addition, VEGF levels were measured by ELISA assay (Pierce, Rockford, IL USA) (detection limit of 4pg/ml), according to the manufacturer's protocol.

Western blot analysis

Western Blot analysis of cell lysates from HT29 cells cultured alone or co-cultured with platelets were performed as described (Dovizio, 2012). Detailed methods are reported in Supplementary Material.

mRNA analysis

Total RNA was extracted from $1x10^6$ HT29 cells using EZNA-Total RNA KIT (Omega BIO-TEK), according to the manufacturer's protocols. Two μg of total RNA were treated with DNAse kit (Fermentas, St. Leon-Rot, Germany) and subsequently reverse transcribed into cDNA using Iscript cDNA Synthesis Kit (Bio-Rad Laboratories, CA, USA), according to the manufacturer's protocols. One hundred ng of cDNA was used for the reaction mixture and the amplification of COX-2 and GAPDH was performed using TaqMan polymerase Kit (Fermentas) and these couples of primers: COX-2- fwd: 5'GCTCAGCCATACAGCAAATCC; rev: 5'CCAAAATCCCCTTGAAGTGGG; GAPDH- fwd: 5'TCACCAGGGCTGCTTTTAAC; rev: 5' GACAAGCTTCCCGTTCTCAG, using a MyQ Single Color Real-Time PCR Detection System (Bio-Rad). COX-2 mRNA expression levels were normalized with GAPDH levels. Gene expression assays were performed by relative quantification with comparative Ct using MyQ Optical System Software 1.0 (Bio-Rad).
The stability of COX-2 mRNA was assessed in HT29 cells cultured alone or co-cultured for 4h or 20h with platelets; then the transcription was stopped by the addition of actinomycin D (2.5μg/ml) for 1 and 4h. After actinomycin D treatment, RNA was isolated and subjected to qPCR analysis.

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Regarding the expression of EMT markers, 100ng of cDNA was used for the reaction mixture and the amplification of zinc finger E-box binding homeobox 1 (ZEB1), Twist1, Vimentin and E-Cadherin was performed using TaqMan gene expression assays (Hs00232783_m1, Hs01675818_s1, Hs00185584_m1, Hs01023894_m1) (Applied Biosystem, CA, USA), per the manufacturer's instructions using a 7900HT Real-Time PCR system (Applied Biosystem). The mRNA expression levels of EMT markers were normalized with GAPDH levels (Rn99999916_s1) (Applied Biosystem). Gene expression assays were performed by relative quantification with comparative Ct using ABI Prism, SDS 2.4 software (Applied Biosystem).

Immunofluorescence

HT29 cells (0.5×10^6 cells) were cultured alone or with platelets (0.5×10^8) for 2, 4 and 20h; then, culture media were collected and cells were washed twice with PBS and fixed in cold aceton/methanol solution (40:60) for 20 min at room temperature. COX-2, COX-1, HuR and GAPDH immunostaining were detected as previously reported (Dixon, 2006) and a detailed description is reported in Supplementary Material.

Statistics

All values were reported as mean±SEM. Statistical analysis was performed using GraphPad Prism Software (version 5.00 for Windows, GraphPad, San Diego, California). Briefly, Student's t test was used to compare means of two independent groups to each other, whereas one-way ANOVA followed by Newman-Keuls post-test was used to compare the means of more than two independent groups. Linear multiple regression analysis was performed to test the relationship between PGE_2 levels and COX-2 protein levels and the coefficient of determination (r^2) was calculated using GraphPad Prism Software (version 5.00 for Windows, GraphPad). Values of $P<0.05$ were considered statistically significant.

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Results

Platelet-HT29 cell interaction triggers platelet activation and COX-2 protein overexpression in HT29 cells

In freshly isolated unstimulated platelets ($100x10^6$ cells) and HT29 cells ($1x10^6$) cultured alone or co-cultured up to 20h, we studied the time-course of TXB_2 release, the growth factors VEGF, EGF, and PDGF-BB and the pleiotropic cytokine TGF-β1(Figure 1A, B and Supplementary Figure 1A-C).

HT29 cells cultured alone did not release detectable levels of TXB_2 (Figure 1A). The incubation of platelets with HT29 cells was associated with a time-dependent increase of TXB_2 levels in the medium. TXB_2 concentrations were significantly increased 2h after the addition of platelets to HT29 cells and they continued to rise up to 4h at a fast rate; thereafter TXB_2 continued to be released in a time-dependent manner, up to 20h, albeit with a slower rate (Figure 1A). Platelets cultured alone released significant lower levels of TXB_2 than that detected in platelet-HT29 cell co-cultures, at each time-point (at 20h: 7±0.7and 116±12ng/ml, respectively, n=5-8) (Figure 1A).

HT29 cells cultured for 20h released VEGF (766±180 pg/ml, n=4) which was not affected by the co-incubation with platelets (Supplementary Figure 1A). Platelets cultured alone did not release detectable levels of VEGF (not shown).

EGF levels measured in conditioned medium of HT29 cells cultured alone were almost undetectable (roughly 5pg/ml) while platelets cultured alone released EGF in a time-dependent fashion (73±7 and 117±9 pg/ml, at 4 and 20h, respectively, n=3) (Supplementary Figure 1B). EGF concentrations were not significantly enhanced by the co-incubation of platelets with HT29 cells but instead they decreased with time (Supplementary Figure 1B).

Platelets, but not HT29 cells, released low concentrations of active TGF-β1, in a time-dependent fashion (at 20h, 116±20 pg/ml, n=3) (Supplementary Figure 1C). In co-cultures of HT29 cells and platelets, TGF-β1 levels were released at a low rate up to 4h and they were not significantly higher

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than the levels measured in the conditioned medium of platelets or HT29 cells cultured alone; at 8h of co-culture, a rapid increase of TGF-β1 release (378±80 pg/ml, n=3) was observed and then it declined in a time-dependent fashion; at 20h TGF-β1 levels detected in the medium were not significantly different from those released by platelets cultured alone (Supplementary Figure 1C).

As shown in Figure 1B, PDGF-BB levels, measured in the conditioned medium of HT29 cells cultured alone, were very low (5±2 pg/ml, n=5). Platelets, cultured alone, time-dependently released low levels of PDGF-BB (at 20h, 292±37pg/ml, n=8). In HT29 cells and platelets co-cultured for 4h, PDGF-BB levels were not significantly different from those detected in platelets cultured alone. A large increase of PDGF-BB levels was detected at 8h (818±16 pg/ml, n=5) and the levels time-dependently increased up to 20h (1450±144 pg/ml, n=5) (Figure 1B). The levels of PDGF-BB detected in the conditioned medium at 8 and 20h of platelet-HT29 cell co-cultures were roughly 2- and 8-fold higher than those of TGF-β1, respectively.

Altogether these results showed that platelets, activated by the interaction with HT29 cells, rapidly released TXB_2 (Figure 1A) while a substantial release of PDGF-BB and TGF-β1 from α-granules began after a lag-time of 4h (Figure 1B and Supplementary Figure 1C). PDGF-BB was a major protein released by platelets when co-cultured with HT29 cells, and its levels continuously accumulated up to 20h (Figure 1B). In co-cultures of HT29 cells and platelets, EGF and TGF-β1, after being released, decreased in a time-dependent manner suggesting their possible cellular re-uptake (Supplementary Figure 1B and C).

As shown in Figure 1C, in co-cultures of HT29 cells and platelets, we detected a time-dependent increase of COX-2 protein levels which occurred in parallel with the release of PDGF-BB by activated platelets. However, the onset of COX-2 synthesis was delayed compared to PDGF-BB secretion (Figure 1B).

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The temporal dynamic of platelet-HT29 cell interactions and COX-2 induction in HT29 cells was studied by performing double immunofluorescence staining of COX-1 (red) in platelets and HT29 cells and COX-2 (green) in HT29 cells followed by confocal microscopy analysis (Figure 1D). Both COX-1 and COX-2 proteins were constitutively expressed in HT29 cells (Figure 1D). At 2 and 4h of co-cultures, small platelet aggregates (expressing only COX-1) surrounded HT29 cells (Figure 1D). In contrast, at prolonged times of incubation, the majority of platelet aggregates were dissociated from HT29 cells (Figure 1D). Long-term incubation with platelets was associated with upregulation of COX-2, but not COX-1, protein expression in HT29 cells (Figure 1D).

To elucidate the importance of direct platelet-tumor cell interaction in the platelet release of TXB_2 and PDGF, we performed co-culture experiments incubating HT29 cells with platelets for 20h in the absence and in the presence of a transwell cell insert, with a pore size of 0.4μm, which avoids the direct contact between the two cell types, but permits the passage of soluble factors. As shown in Figure 1E and 1F, the increased release of TXB_2 and PDGF, respectively, was completely prevented if the two cell types were separated by the transwell.

In summary, these data suggest that HT29 cells activate platelets through a direct cell-cell interaction associated with a rapid generation of TXA_2 which may amplify the activation response and recruit additional platelets. At prolonged incubation time, platelet aggregates detach from HT29 cells and release α-granule content with PDGF-BB being more abundant than TGFβ-1.

Prolonged incubation with platelets induces COX-2 mRNA stabilization in HT29 cells

In order to clarify the mechanism involved in HT29 cell COX-2 protein overexpression induced by prolonged incubation with platelets, we compared protein and mRNA levels of COX-2 at short-term (2-4h) and long-term (20h) incubation times with platelets.

At 2-4h of incubation, COX-2 protein was not upregulated while COX-2 transcript levels were increased (Figure 2A and B). At long-term incubation (20h) both COX-2 protein and mRNA were

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increased (Figure 2A and B). Increased levels of COX-2 mRNA detected at 2, 4 and 20h of platelet-HT29 cell co-cultures were not different from each other, in a statistically significant fashion. Interestingly, COX-2 mRNA levels remained significantly elevated at 20h of incubation even when platelet aggregates dissociated from HT29 cells (Figure 2B).

A possible mechanism explaining the discrepancy between mRNA accumulation and protein synthesis of COX-2 is that platelets switched on a stabilization pathway of COX-2 mRNA (Dixon, 2006). This hypothesis was addressed by comparing COX-2 mRNA levels in HT29 cells co-cultured with platelets for 20h vs 4h or in HT29 cells cultured alone in the presence of actinomycin D to stop transcription. As seen in Figure 2C, a 4h exposure to actinomycin D resulted in a substantial decrease in the amount of mRNA present in HT29 cells that had been previously cultured alone or with platelets for only 4h. In contrast, the actinomycin D treatment led to a much smaller decrease in mRNA levels in HT29 cells previously cultured with platelets for 20h. These results show that prolonged incubation of platelets with HT29 cells induced COX-2 mRNA stabilization.

We investigated whether platelet-induced COX-2 mRNA stabilization in HT29 cells occurred by increasing the cytoplasmic accumulation of HuR. In HT29 cells co-cultured with platelets for 2-4h, HuR levels detected by Western blot in the cytoplasmic fraction were not different from those detected in cancer cells cultured alone (Supplementary Figure 2A). In contrast, enhanced HuR levels were detected in the cytoplasm of HT29 cells after prolonged incubation times with platelets by Western blot and confocal microscopy analyses (Supplementary Figure 2A and Figure 2D and E, respectively).

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Involvement of tumor galectin-3 and platelet collagen receptors, but not P-selectin, in platelet-induced COX-2 upregulation in HT29 cells

Next we aimed to characterize the cell surface constituents involved in platelet-tumor cell interactions which translated into COX-2 overexpression in cancer cells.

HT29 cells express high levels of different galectins whose involvement in cell-cell and cell-extracellular matrix adhesion is well established (Satelli, 2008). Among the galectins highly expressed in HT29 cells, there is galectin-3 (Figure 3A) which is unique among the galectin family of lectins because it contains a "collagen-like" domain (Nangia-Makker, 2008). HT29 cell galectin-3 levels were not affected by the incubation with platelets for 20h (Figure 3A). To address whether galectin-3 plays a role in platelet-induced COX-2 overexpression in HT29 cells, we used three different pharmacological tools: (i) β-lactose which competitively binds to CRD of galectins, (ii) M3/38, an antibody that inhibits galectin-3 function by binding to its *N*-terminal domain (Fukushi, 2004), and (iii) Gal-3C, a *N*-terminally truncated galectin-3 that contains the entire CRD and retains ability to bind to carbohydrate-containing ligands such as glycoproteins containing glycans but is unable to cross-link them, thus acting as a dominant-negative inhibitor of full-length galectin-3 (Markowska, 2010).

As shown in Figure 3B, β-lactose caused a significant reduction by 63% of enhanced COX-2 protein expression in HT29 cells co-cultured for 20h with platelets. M3/38 antibody and Gal-3C completely prevented platelet-induced COX-2 overexpression in HT29 cells (Figure 3C and D). Based on these results, we hypothesized that platelet collagen receptors could play a role in the direct interaction with HT29 cells. To address this question we used the novel anti-platelet drug revacept obtained by fusing the extracellular domain of the platelet collagen receptor GPVI with the human immunoglobulin Fc domain (Ungerer, 2011). Human platelet GPVI represents the major signaling receptor for collagen on platelets (Nieswandt, 2003). The *N*-terminus of revacept, constituted of the human GPVI extracellular region, binds to the ligands collagen and fibronectin in

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atherosclerotic plaques, either stable or ruptured, thus preventing platelet adhesion mediated by GPVI and possibly by other platelet receptors for collagen, i.e., α2/β1 and GPIb (Ungerer, 2011). We showed that the drug, at clinically relevant concentrations (Ungerer, 2011), completely prevented the platelet-induced upregulation of COX-2 in HT29 cells (Figure 3E). In contrast, experiments performed with a P-selectin antagonist (Appeldoorn, 2003) led us to exclude its role in platelet-HT29 cell interactions (Figure 3F).

Role of platelet-derived soluble mediators in COX-2 upregulation in HT29 cells

In a further set of experiments, the role of platelet released products, such as TXA_2, ADP/ATP and PDGF, on COX-2 overexpression in HT29 cells was assessed by a pharmacological approach. As shown in Figure 4A, platelets pretreated with aspirin (300μM) to suppress completely and persistently COX-1-dependent TXB_2 generation, retained the capacity to induce COX-2 expression in HT29 cells. Similarly, blockage of P2-purinoceptors by suramin (Hourani, 1992) did not affect platelet-induced COX-2 expression in HT29 cells (Figure 4B).

In contrast, imatinib, a non-selective PDGFR inhibitor (Buchdunger, 2002), or a PDGF neutralizing antibody completely prevented platelet-dependent induction of COX-2 protein expression (Figure 4C and D, respectively). Altogether these results suggest the involvement of the concurrent action of direct platelet/tumor cell contact and platelet-released PDGF in the aberrant expression of COX-2 in HT29 cells.

Role of downstream effectors of PDGFR signaling on platelet-dependent induction of COX-2 in HT29 cells

We studied the involvement of downstream effectors of PDGFR, i.e., PI3K, Na^+/H^+ exchanger (NHE) and PKCδ (Heldin, 1999), by using pharmacological tools affecting their activity, i.e., wortmannin, dm-amiloride and rottlerin (Doller, 2011), respectively. As shown in Figure 5A and

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5B, wortmannin and dm-amiloride reduced by 63 and 66%, respectively, platelet-dependent COX-2 protein induction. In contrast, rottlerin completely suppressed platelet-induced COX-2 upregulation (Figure 5C).

These data may place PKCδ downstream of PI3K and NHE in mediating platelet-induced COX-2 expression. PKCδ may modulate COX-2 expression by controlling intracellular HuR localization and function (Doller, 2011). As shown in Figure 5D, rottlerin, at a concentration shown to be selective for PKCδ (Doller, 2011), prevented platelet-induced cytoplasmic shuttling of HuR in HT29 cells detected at 20h of co-culture.

Platelet-HT29 cell interaction triggers PGE_2 release

We assessed the time-course of PGE_2 release by HT29 cells and platelets cultured alone or co-cultured up to 20h. As shown in Figure 6A, HT29 cells released low levels of PGE_2 (at 20h: 45±15 pg/ml, n=3). Platelets cultured alone, released higher concentrations of PGE_2. The levels of PGE_2 reached the maximum within 4h and stayed at the plateau for the remaining incubation period (Figure 6A). PGE_2 concentrations detected at each time-point, from 4 to 20h, were not significantly different from each other. In platelet-HT29 cell co-cultures, PGE_2 levels released in the medium up to 8h were comparable to those released by platelets cultured alone; at 12h, PGE_2 levels started to be higher than those released by platelets cultured alone and then they continued to raise up to 20h (Figure 6A). PGE_2 levels released from 8 to 20h, subtracted from the PGE_2 levels generated by platelets alone, linearly correlated with COX-2 protein levels detected at same time-points in cell lysates by Western blot (r^2=0.98; P=0.0097) (Figure 6B). This result indirectly suggests that COX-2 induced in HT29 cells by the interaction with platelets contributed to the enhanced levels of PGE_2 detected at late time-points. In contrast, the early generation of PGE_2 detected in the medium of platelet-HT29 cell co-cultures was mainly derived from platelets.

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To obtain a direct evidence of the contribution of COX-2 overexpression to the enhanced PGE_2 detected at 20h of HT29 cell-platelet co-cultures, we studied the effect of rofecoxib (added 20 min before platelets), at a concentration (0.3μM) which inhibits profoundly COX-2 activity of HT29 cells without affecting COX-1 activity (Dovizio, 2012). As shown in Figure 6C, PGE_2 accumulation in the medium of HT29 cells and platelets co-cultured for 20h was significantly reduced (57%) by rofecoxib. To confirm that overexpressed COX-2 was functionally active even at late time-points, we studied the effect of rofecoxib on the activity of COX-2 expressed at 20h. In this experiment, HT29 cells were incubated with platelets that were pretreated with aspirin (to completely suppress platelet COX-1 activity) and extensively washed (to eliminate the drug) before the addition to cancer cells. At 20h, the medium was changed with a fresh one containing rofecoxib or vehicle and the incubation continued for 1h; then AA (0.5 μM) (to start COX catalysis) was added for further 1h and PGE_2 levels were measured in the conditioned medium. As shown in Figure 6D, under these experimental conditions, substantial PGE_2 levels were generated and they were profoundly reduced by the treatment with rofecoxib.

Altogether, these results showed that HT29 cells had a low capacity to release PGE_2 from endogenous AA and that COX-2 overexpression induced by platelets enables the generation of increased levels of this prostanoid in these cancer cells.

Effects of platelet-HT29 cell cross-talk on the expression of malignancy-related proteins

We studied whether platelet-HT29 cell cross-talk caused changes in phenotypic biochemical markers of malignancy in HT29 cells and whether they were dependent on the activity of COX-2. As shown in Figure 7A-G, among several proteins analyzed known to be involved in proliferation, apoptosis or differentiation, $p21^{WAF1/CIP1}$(Figure 7A) was downregulated and cyclin B1(Figure 7C) was upregulated in HT29 cells co-cultured for 20h with platelets.

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We studied the kinetics of $p21^{WAF1/CIP1}$ and cyclin B1 expression changes induced in HT29 cells by the interaction with platelets. As shown in Figure 8, $p21^{WAF1/CIP1}$ was rapidly induced after the addition of platelets to HT29 cells but returned to baseline levels at 8h; later, $p21^{WAF1/CIP1}$ levels decreased in a time-dependent fashion. Incubation of HT29 cells with platelets was associated with cyclin B1 induction only at late time-points (i.e., 20h) (Figure 8).

In order to demonstrate the role of COX-2-derived PGE_2 in $p21^{WAF1/CIP1}$ and cyclin B1 changes caused by the interaction of platelets with HT29 cells, we studied the effects of rofecoxib (0.3μM). As shown in Figure 9A and B, rofecoxib reverted the reduction of $p21^{WAF1/CIP1}$ and the induction of cyclin B1 in HT29 cells co-cultured with platelets. These effects of rofecoxib were completely abrogated by exogenous addition of PGE_2 at concentrations generated in the co-cultures of HT29 cells and platelets (Figure 9C).

Altogether these results convincingly support the role of COX-2-derived PGE_2 induced by the interaction of platelets with HT29 cells in the reduction of $p21^{WAF1/CIP1}$ and the induction of cyclin B1.

Platelets induce EMT markers in HT29 cells: role of direct cell-cell interaction and COX-2 overexpression

We assessed the effects of platelets on mRNA expression of EMT-inducing transcription factors, such as ZEB1 and Twist1, the mesenchymal marker vimentin and the epithelial marker E-cadherin in HT29 cells.

First, we studied the kinetics of mRNA changes of these EMT markers in co-cultures of HT29 cells and platelets. As shown in Figure 10A, in HT29 cells, platelets induced a time-dependent increase of ZEB1 and Twist1 associated with increased expression of vimentin and a decrease in the levels of E-cadherin. A significant increase of ZEB1 and Twist1 mRNAs were detected at 4h and it persisted up to 20h. Vimentin mRNA was significantly increased at 4h and further increased at 20h.

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E-cadherin was significantly decreased (by 35%) in HT29 cells at 20h of co-incubation with platelets.

The inhibition of platelet adhesion to HT29 cells by revacept completely prevented the induction of EMT markers in HT29 cells at 20h of incubation with platelets (Figure 10B). In contrast, E-cadherin mRNA levels were significantly higher in revacept treated cells than in those treated with vehicle. These results may suggest that a direct platelet-HT29 cell interaction induces cancer cell transition to an invasive mesenchymal-like phenotype.

Platelet-induced COX-2 overexpression in HT29 cells might emanate mitogenic and survival signaling pathways to stabilize gene signature in cells undergoing EMT. Thus, we studied the effect of rofecoxib on mRNA changes of EMT markers in platelet-HT29 cell co-cultures at 20h. As shown in Figure 10B, in the presence of rofecoxib the expression of ZEB1, Twist1 and vimentin was significantly reduced while that of E-cadherin tended to increase.

The inhibitory effect of revacept on ZEB1, Twist1 and vimentin expression was higher than that caused by rofecoxib, though only ZEB1 reduction was different, in a statistically significant fashion (Figure 10B). These results may suggest that direct platelet-tumor cell interaction and platelet-induced COX-2 overexpression synergistically activate HT29 cell transition to mesenchymal-like phenotype.

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Discussion

In the present study, we show that platelet-cancer cell cross-talk led to enhanced COX-2-dependent PGE_2 generation in HT29 cells which contributed to downregulation of $p21^{WAF1/CIP1}$ and upregulation of cyclin B1 together with the induction of gene expression signatures associated with EMT.

We used a pharmacological approach to characterize the molecular partners involved in the direct platelet-HT29 cell interactions and the platelet-derived soluble factors contributing to the aberrant COX-2 overexpression in cancer cells. The role of platelet GPVI, and possibly other collagen receptors, was confirmed using revacept which is a soluble form of the platelet GPVI receptor that has been shown to bind specifically to collagen at sites of vascular damage, thus inhibiting platelet adhesion and aggregation(Ungerer, 2011).The use of three different inhibitors of galectin-3 function [β-lactose, Gal-3C(John, 2003) and anti-galectin-3 antibody M3/38(Fukushi, 2004)] showed the involvement of this galectin in platelet-induced COX-2 expression in HT29 cells. The activity of both lactose and Gal-3C are dependent on the carbohydrate-mediated functions of galectin-3. The M3/38 antibody binds to the *N*-terminal domain of galectin-3, that contains a collagen-like domain(Nangia-Makker,2008). The results suggest that galectin-3 may act as binding partner of platelet collagen receptors and, interestingly, imply that the binding is dependent on carbohydrate binding mediated by the CRD and by homologous or heterologous protein-protein binding mediated by the collagen-like domain. Our results showing that Gal-3C prevented platelet-induced COX-2 overexpression in cancer cells also suggest that this could be a mechanism of its anti-cancer activity as demonstrated in animal models(John, 2003; Mirandola, 2011). The contribution of PDGF, released by activated platelets, to the induction of COX-2 in HT29 cells was disclosed by blocking PDGFR activation with imatinib(Buchdunger, 2002) or by using a PDGF-neutralizing antibody. Herein, we provide evidence that COX-2-derived PGE_2 induced by the interaction of platelets with HT29 cells was involved in the reduction of $p21^{WAF1/CIP1}$ and the induction of cyclin B1 that are

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typical changes which allow progression through the G_2-M checkpoint(Wang, 2009). In fact, the changes of these two proteins were prevented by rofecoxib and rescued by the addition of exogenous PGE_2.

Platelet-HT29 cell co-incubation was associated with a rapid release of PGE_2 derived from platelets and a delayed release of this prostanoid dependent on COX-2 upregulation in HT29 cells. The efficacy of rofecoxib to prevent platelet-induced changes of $p21^{WAF1/CIP1}$ and cyclin B1 supports the dominant role of COX-2-dependent PGE_2 which may activate EP receptors localized, similarly to COX-2, in the nuclear compartment(Bhattacharya, 1998; Bhattacharya, 1999;Schlötzer-Schrehardt, 2002;Konger, 2005; Morita,1995). As shown in Supplementary Figure 3, EP1, EP2 and EP4 were detected by Western blot both in extra-nuclear and nuclear compartments of HT29 cells.

The kinetics of biological events induced by platelet-HT29 cell interactions, which contributed to changes of proteins associated with malignant progression, was dissected by recording(every 4h up to 20h) simultaneously the release of platelet-derived products, i.e., TXB_2, growth factors PDGF-BB and EGF and the cytokine TGF-β1, and protein levels of COX-2, $p21^{WAF1/CIP1}$ and cyclin B1 in HT29 cell lysates. In the first 4h, platelets adhered to HT29 cells and they were activated and released large amounts of TXA_2 which may recruit and activate additional platelets. In contrast, a substantial release of PDGF-BB and TGF-β1 from platelet α-granules began after a lag-time of 4h. However, PDGF-BB levels were higher than those of TGF-β1 and they continuously increased in a time-dependent fashion up to 20h. In contrast TGF-β1, after being released, decreased in a time-dependent manner suggesting its possible cellular re-uptake. Platelets cultured alone released low concentrations of EGF which were not significantly enhanced by the co-incubation with HT29 cells, but instead, they decreased over time. These results show that PDGF-BB was the dominant growth factor continuosly released by cancer cell-activated platelets.

The activation of platelet collagen receptor GPVI during the adhesion phase of platelets to HT29 cells may explain the delay in the release of α-granule proteins, such as PDGF. In fact, GPVI

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signaling is known to negatively regulates platelet granule secretion(Chari, 2009). Interestingly, in platelet-HT29 cell co-cultures, we detected a time-dependent release of soluble GPVI(Supplementary Figure 4) which seemed to parallel the detachment of platelets from HT29 cells and the release of PDGF-BB into the medium. In platelets, GPVI activation may trigger proteolytic pathways leading to GPVI ectodomain shedding resulting in the release of a soluble approx 55-kDa fragment into the platelet supernatant(Gardiner, 2004; Bender,2010).

During the adhesion phase of platelets to HT29 cells, the levels of COX-2 mRNA rapidly increased and remained stable up to 20h. In contrast, COX-2 protein synthesis began to increase after a lag-time of 8h and, then, it continued to raise, in a time-dependent manner, even when platelets were detached from cancer cells. These results show that HT29 cells can be primed for COX-2 mRNA expression by a transient interaction with platelets. However, soluble factors released by platelets at later time-points, may contribute to posttranscriptional regulation of COX-2 through the stabilization of COX-2 mRNA. In this study, we provide several lines of evidence that platelet PDGF may be involved in this phenomenon:(i) the onset of PDGF-BB secretion occurred earlier than that of COX-2 protein synthesis and COX-2-dependent PGE_2 release, (ii) the time-dependent increase of PDGF-BB levels was accompanied by a parallel upregulation of COX-2 protein, (iii) imatinib and a specific anti-PDGF antibody prevented the induction of COX-2 protein, and (iv)pharmacological inhibition of downstream effectors of PDGFR, i.e.PI3K, NHE, and PKCδ, by using wortmannin, dm-amiloride and rottlerin(Doller, 2011), respectively, reduced COX-2 protein induction.

Prolonged incubation of platelets with HT29 cells was associated with nucleo-cytoplasmic translocation of HuR which has been reported to control both mRNA decay and protein translation of COX-2(Young, 2008). The role of PKCδ in the export of HuR from the nuclear compartment was evidenced using rottlerin which has been shown to specifically interfere with PKCδ-triggered HuR phosphorylation, in a cell-free HuR phosphorylation assay(Doller, 2011).

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TGF-β1, released by cancer cell-activated platelets, seems to have a negligible, if any, role in COX-2 upregulation detected in HT29 cells. In fact, this human colon cancer cell line is resistant to TGF-β1 mediated growth inhibition and apoptosis(Winesett, 1996; Tong, 2009), due to inactivated TGF-β type II receptor and deficient expression of RUNX3 and Smad4, which are involved in the TGF-β signaling pathway. TGF-β has been shown to promote cancer cell growth through the activation of alternative signaling pathways, but at concentrations (i.e., $\geq$ 5 ng/ml)(Halder, 2005) which are more than 10-fold higher than those detected, transiently, in the medium of HT29 cell-platelet co-cultures(Supplementary Figure 1C).

The adhesion of platelets to HT29 cells was associated with a transient upregulation of $p21^{WAF1/CIP1}$ protein possibly through the activation of transcriptional and/or posttranscriptional regulatory mechanisms(Wang, 2012) which necessitate further investigation. This effect was unlikely due to released TGF-β1 because:(i) the cytokine was released later than $p21^{WAF1/CIP1}$ protein induction, and (ii) HT29 cells have been previously shown to be insensitive to $p21^{WAF1/CIP1}$ induction after TGF-β treatment(Li, 1995). In prolonged co-cultures of HT29 cells with platelets, $p21^{WAF1/CIP1}$ levels decreased in parallel to enhanced COX-2-dependent PGE_2 generation. The induction of cyclin B1 was a late response which probably required a profound downregulation of $p21^{WAF1/CIP1}$ to emerge since this protein has been previously shown to target cyclin B1 for degradation(Gillis, 2009). Platelet adhesion to HT29 cells caused an increased expression of genes involved in the EMT such as the EMT-inducing transcription factors ZEB1 and Twist1 and the mesenchymal marker vimentin(Kalluri, 2009). These changes persisted at long incubation times, when platelets detached from cancer cells, in association with a reduced expression of the epithelial marker E-cadherin. The finding that rofecoxib prevented mRNA changes of EMT markers in platelet-HT29 cell co-cultures at 20h, though less efficiently than revacept, suggests that direct platelet-tumor cell interaction and

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platelet-induced COX-2 overexpression synergistically activate the HT29 cell transition to a mesenchymal-like phenotype.

In the present study, we show that selective inhibition of platelet COX-1 activity by aspirin did not significantly affect platelet-induced upregulation of COX-2 in HT29 cells. HT29 cells may be insensitive to an enhanced release of TXA_2 by activated platelets due to the absence of TXA_2 receptor(TP) isoforms(Supplementary Figure 5). TP signaling seems to play a critical role in tumor colonization(Matsui, 2012). Our results provide the rationale for studying whether TP expression in circulating tumor cells may identify individuals who are responders to aspirin chemotherapy.

In conclusion, we have unraveled the role of platelets in inducing COX-2 upregulation in HT29 cells, considered as a key event in carcinogenesis(Prescott, 2000; Young, 2008). This program of malignancy is primed by transient platelet-cancer cell contact, involving tumor galectin-3 and platelet collagen receptors, and the release of platelet PDGF(Figure 11). These findings also reveal that inhibitors of this program such as blockers of collagen binding sites, such as revacept, and galectin-3 may represent an innovative strategies in colon cancer chemotherapy which should be tested in experimental animals followed by randomized clinical trials in colon cancer patients.

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Contributed new reagents or analytic tools: Maier, Münch, John, Suess.

Performed data analysis: Patrignani, Dovizio, Alberti.

Wrote or contributed to the writing of the manuscript: Patrignani, Steinhilber, Sgambato, Suess, Dovizio.

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Footnotes

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Figure Legends

Figure 1. Platelet-HT29 cell interaction triggers platelet activation and overexpression of COX-2 in tumor cells. HT29 cells ($1x10^6$)(HT) or isolated unstimulated platelets ($100x10^6$)(Plt) were cultured alone or co-cultured (HT+Plt) up to 20h. The release of TXB_2 (the stable hydrolysis product of TXA_2) (A) and PDGF-BB (B) were assessed in the culture medium and values are reported as mean±SEM (n=5-8, panel A and n=3-8, panel B). In panel A, **P<0.01 vs Plt alone at same time-points; in panel B, *P<0.05 and **P<0.01 vs HT at same time-points, §P<0.01 vs Plt alone at same time-points. (C) COX-2 protein expression was detected by Western blot technique in HT29 cells cultured alone (HT) or co-cultured with platelets (HT+Plt) up to 20h, and normalized to β-actin expression. Data are reported as mean of two separate experiments, as ratio between optical density (OD) values of COX-2 immunoreactive bands to that of β-actin bands. (D) Immunofluorescence analysis of COX-1 (in red) and COX-2 (in green) was performed in HT29 cells cultured alone (0h) or with platelets for 2, 4 and 20h. Arrows indicate platelet aggregates; scale bars, 10μm. (E, F) HT29 cells and platelets were co-cultured for 20h using a Transwell (pore size 0.4μm) and TXB_2 and PDGF-BB, respectively, were assessed in the medium. HT29 cells were cultured in the lower chamber of the Transwell unit while platelets were in the upper chamber of the Transwell unit. Data are reported as mean±SEM (n=3). In panel E, *P<0.05 vs Plt; §P<0.01 vs HT+Plt (Transwell); in panel F, *P<0.05 vs HT; §P<0.05 vs HT; **P<0.01 vs Plt and HT+Plt (Transwell).

Figure 2. **Prolonged incubation with platelets modulates COX-2 protein synthesis and mRNA stability in HT29 cells**. (A) Levels of COX-2 protein (normalized to β-actin levels) detected by Western blot in HT29 cells cultured alone (HT) or with platelets (HT+Plt) up to 20h. Data are reported as mean±SEM (n=6-11), as % of control, i.e., HT29 cells cultured alone. **P<0.01 vs HT. (B) In the same experimental conditions, COX-2 mRNA expression (normalized to GAPDH) in

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HT+Plt vs HT alone was assessed by qRT-PCR. Data are reported as mean±SEM (n=5-6), as % of control, i.e., HT29 cells cultured alone. *P<0.05 and ** P<0.01 vs HT. (C) Assay of COX-2 mRNA stability. Actinomycin D (2.5μg/ml) was added to HT29 cells cultured alone (HT), platelet-HT29 cells co-cultured for 4h [HT+Plt (4h)] or for 20h [HT+Plt (20h)]. COX-2 mRNA levels were assessed by qRT-PCR after 1 and 4h of treatment with actinomycin D. Data are reported as mean±SEM (n=3-4). *P<0.05 and §P<0.05 vs HT+Plt (4h) and HT. (D) HuR localization assessed by confocal microscopy analysis in HT29 cells alone (HT) or incubated with platelets (HT+Plt) for 20h. Immunostaining of HuR is shown in green, GAPDH (cytoplasmic protein) in red and DAPI (nuclear marker) in blue. Merge images are shown (merge). (E) Ratios between pixel sum of HuR staining in cytoplasm and pixel sum of HuR staining in the nucleus were calculated using LAS AF software, 2.2.1. Data are expressed as mean±SEM (n=3), **P<0.01 vs HT.

Figure 3. Tumor galectin-3 and platelet collagen receptors, but not P-selectin, regulate COX-2 upregulation in HT29 cells. Galectin-3 (A) and COX-2 protein expression (B-F) was assessed by Western blot in HT29 cells cultured alone (HT) or co-cultured for 20h with platelets (HT+Plt) and in the presence of vehicle or different pharmacological agents, i.e., (B) the pan-galectin inhibitor β-lactose (30mM), (C) the monoclonal antibody that binds the *N*-terminus of galectin-3 (M3/38)(10μg/ml), (D) a *N*-terminally truncated form of galectin-3 that acts as a dominant negative inhibitor (Gal-3C)(1μg/ml and 10μg/ml), (E) revacept (dimeric Glycoprotein VI-Fc)(4, 40 and 400μg/ml), or (F) the P-selectin antagonist (Galloyl-N-gaba-WVDV-OH peptide)(10 and 100μM). Data are expressed as mean±SEM (n=3-4). In panel B, **P<0.01 vs HT and *P<0.05 vs HT+Plt (vehicle); in panel C, *P<0.05 vs HT and §P<0.05 vs HT+Plt (vehicle); in panel D, *P<0.05 vs HT and #P<0.05 vs HT+Plt (vehicle); in panel E, *P<0.05 vs HT and §P<0.05 vs HT+Plt (vehicle); in panel F, **P<0.01 vs HT.

Figure 4. Platelet-derived PDGF, but not TXA_2 and ADP/ATP, regulates COX-2 expression in HT29 cells co-cultured with platelets. COX-2 protein levels (normalized to β-actin levels) were

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assessed by Western blot in HT29 cells cultured alone (HT) or co-cultured for 20h with platelets (HT+Plt) in the presence of vehicle or different pharmacological agents. (A) HT29 cells were incubated with aspirin-treated platelets (ASA). Moreover, HT29 cells were co-incubated with platelets in the presence or absence of (B) suramin (50μM, a P2-purinoceptor antagonist), (C) imatinib (10μM, a PDGFR antagonist) and (D) a PDGF-neutralizing antibody (anti-PDGF, 10μg/ml). The values were reported as % of control, i.e., HT29 cells cultured alone. Data are expressed as mean±SEM from 3-4 (A-C) or 2 separate experiments (D). In panel A, *P<0.05 vs HT; in panel B, **P<0.01 vs HT and *P<0.05 vs HT; in panel C, **P<0.01 vs HT and *P<0.05 vs HT+Plt (vehicle).

Figure 5. Activation of PDGFR signaling regulates platelet-dependent COX-2 expression in HT29 cells. HT29 cells were cultured alone (HT) or co-cultured for 20h with platelets (HT+Plt) in the presence of vehicle or (A) wortmannin (0.1μM), an inhibitor of PI3K, (B) dm-amiloride (10μM), an inhibitor of NHE or (C) rottlerin (10μM), a specific inhibitor of PKCδ isoform. (A-C) COX-2 protein expression was detected by Western blot analysis and quantification of optical density (OD) of different specific bands was calculated and normalized to the OD of β-actin. The values were reported as % of control, i.e., HT29 cells cultured alone. Data are expressed as mean±SEM (n=3). In panel A, *P<0.05 vs HT and §P<0.05 vs HT+Plt (vehicle); in panel B, **P<0.01 vs HT and *P<0.05 vs HT+Plt (vehicle); in panel C, **P<0.01 vs HT and #P<0.01 vs HT+Plt (vehicle). (D) Immunofluorescence localization of HuR was examined by confocal microscopy analysis in HT29 cells cultured alone (HT) or incubated with platelets (HT+Plt) for 20h in the presence of vehicle or rottlerin (10μM). Immunostaining of HuR is shown in green; GAPDH (cytoplasmic protein) is shown in red and DAPI (nuclear marker) is shown in blue. Merge images are shown (merge). Scale bars, 10μm.

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Figure 6. Platelet-HT29 cell interaction triggers PGE_2 generation. (A) HT29 cells (HT) or unstimulated platelets (Plt) were cultured alone or co-cultured (HT+Plt) up to 20h and the release of PGE_2 was assessed in the culture medium. Values are reported as mean±SEM (n=3-6). **P<0.01 vs time (0); §P<0.01 vs Plt at same time-points; #P<0.01 vs HT at the same time-point. (B) PGE_2 levels released from 8 to 20h, subtracted from the PGE_2 levels generated by platelets alone, linearly correlated with COX-2 protein levels detected at the same time-points in HT29 cell lysates by Western blot. The least-squares line and coefficient of determination r^2 were calculated by linear regression analysis using PRISM software. (C) Effect of rofecoxib (0.3μM) or vehicle on PGE_2 released by HT29 cells cultured with platelets for 20h. (D) Effect of rofecoxib on AA-induced COX activity of HT29 cells cultured alone or with aspirin-treated platelets. HT29 cells were cultured alone or with aspirin-treated platelets for 20h; the medium was changed and vehicle or rofecoxib were added for 1h and then the incubation was continued for 1h in the presence of AA (0.5μM). At the end of the incubation, the medium was collected and assayed for PGE_2. Data are expressed as mean±SEM (n=3). In panel C, **P<0.01 vs HT (vehicle); §P<0.01 vs HT+Plt (vehicle). In panel D, *P<0.05 vs HT (vehicle) and #P<0.05 vs HT+Plt (vehicle).

Figure 7. Platelet-HT29 cell cross-talk induces changes in the expression of proteins involved in malignancy. In HT29 cells cultured alone (HT) or co-cultured for 20h with platelets (HT+Plt), Western blot analysis was performed to assess the expression levels of several proteins: (A) $p21^{WAF1/CIP1}$(p21), (B) proliferating cell nuclear antigen (PCNA), (C) cyclin B1, (D) cyclin D1, (E) Bcl-2, (F) ornithine decarboxylase (ODC), (G) galectin-4 (Gal-4). The expression level of different proteins was assessed as ratio of their optical density (OD) normalized to the OD of β-actin and shown as % of control, i.e., HT29 cells cultured alone. Data are expressed as mean±SEM (n=3-6). In panel A, **P<0.01 vs HT and in panel C, *P<0.05 vs HT.

Figure 8. Time related expression of $p21^{WAF1/CIP1}$ and cyclin B1 proteins in HT29 cells co-cultured with platelets. (A) HT29 cells were cultured alone (HT) or co-cultured with platelets

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(HT+Plt) for different time-points up to 20h and p21 $^{WAF1/CIP1}$ (p21), cyclin B1and GAPDH (as loading control) protein levels were assessed by Western blot technique. (B) Quantification of optical density (OD) of different immunoreactive bands of p21$^{WAF1/CIP1}$ and cyclin B1 was calculated and normalized to the OD of GAPDH. Data are reported as mean of two separate experiments.

Figure 9. Platelet-induced COX-2 activity regulates p21$^{WAF1/CIP1}$ and cyclin B1 protein levels in HT29 cells. (A, B) The effect of a selective COX-2 inhibitor, rofecoxib (0.3μM), on protein levels of p21 $^{WAF1/CIP1}$ and cyclin B1, respectively, in HT29 cells cultured with platelets for 20h, was assessed by Western blot. Data are expressed as mean±SEM from 3 experiments, as % of control, i.e., HT29 cells cultured alone. In panel A, **P<0.01 vs HT and §P<0.05 vs HT+Plt (vehicle); in panel B, **P<0.01 vs HT and *P<0.05 vs HT+Plt (vehicle). (C) The effects of two different concentrations of exogenous PGE_2 (1.5 and 15ng/ml) on the expression levels of p21$^{WAF1/CIP1}$ (p21) and cyclin B1, in HT29 cells co-cultured with platelets for 20h in the presence of rofecoxib (0.3μM) or vehicle, were assessed by Western Blot technique.

Figure 10. Platelet adhesion to HT29 cells and upregulation of COX-2 protein expression are involved in the induction of genes involved in the epithelial-mesenchymal transition.
(A) HT29 cells were cultured alone (0h) or co-cultured with platelets for different time-points up to 20h. The mRNA levels of ZEB1, Twist1, Vimentin and E-Cadherin were assessed by qRT-PCR and normalized to GAPDH levels. Data are reported as mean±SEM (n=3) of fold change vs HT29 cells cultured alone. *P<0.05 and **P<0.01 vs HT29 cultured alone (0h). (B) The effects of revacept (400μg/ml) and rofecoxib (0.3μM) on the expression of these genes were assessed in HT29 cells co-cultured for 20h with platelets. Data are reported as mean±SEM (n=3), as % of control (vehicle); *P<0.05 and **P<0.01 vs vehicle; §P<0.05 vs rofecoxib.

Figure 11. Proposed mechanism of COX-2 overexpression in tumor cells by the interaction with platelets. Unstimulated platelets interact rapidly with tumor cells through the binding of

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platelet collagen receptors (in particular GPVI) and tumor components, such as galectin-3. This early event translates into platelet activation, as demonstrated by enhanced generation of TXA_2. Direct platelet-tumor cell interaction is associated with enhanced mRNA expression of COX-2 (but not COX-2 protein) and EMT-inducing transcription factors, such as ZEB1 and Twist1, and the mesenchymal marker vimentin. Later, platelet aggregates detach from tumor cells, possibly as a consequence of the shedding of platelet GPVI receptors and acquire the capacity to release their α-granule products, such as PDGF. The release of PDGF is associated with COX-2 mRNA stabilization via NHE-PI3K/PKCδ-dependent nucleo-cytoplasmic translocation of the mRNA-stabilizing protein HuR and COX-2 protein synthesis. In HT29 cells, overexpressed COX-2 and enhanced generation of PGE_2 emanate mitogenic and survival signaling pathways through the downregulation of $p21^{WAF1/CIP1}$ and the upregulation of cyclin B1 as well as of EMT-inducing transcription factors and mesenchymal markers, such as vimentin, in association with repression of epithelial markers, such as E-cadherin.

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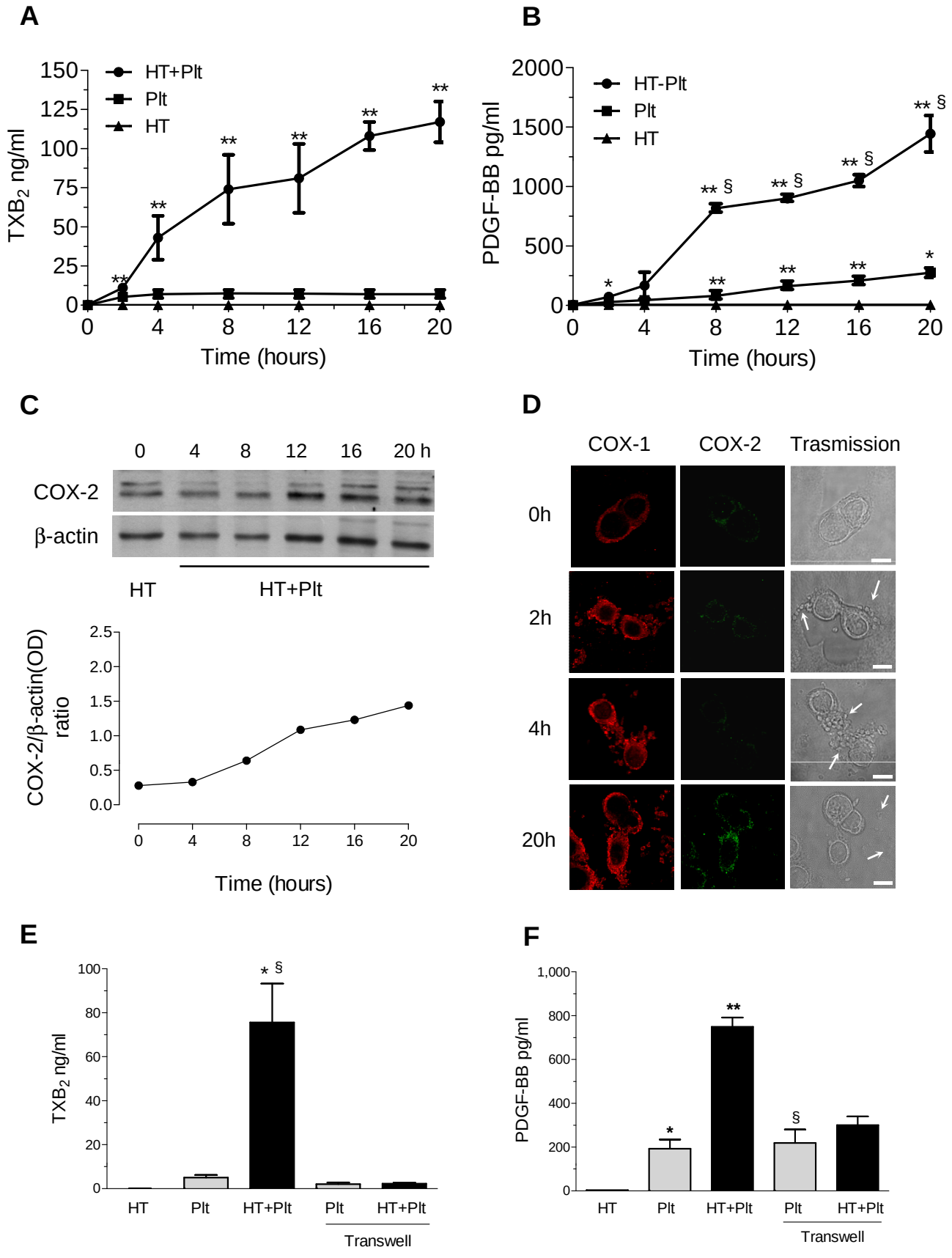

Figure 1

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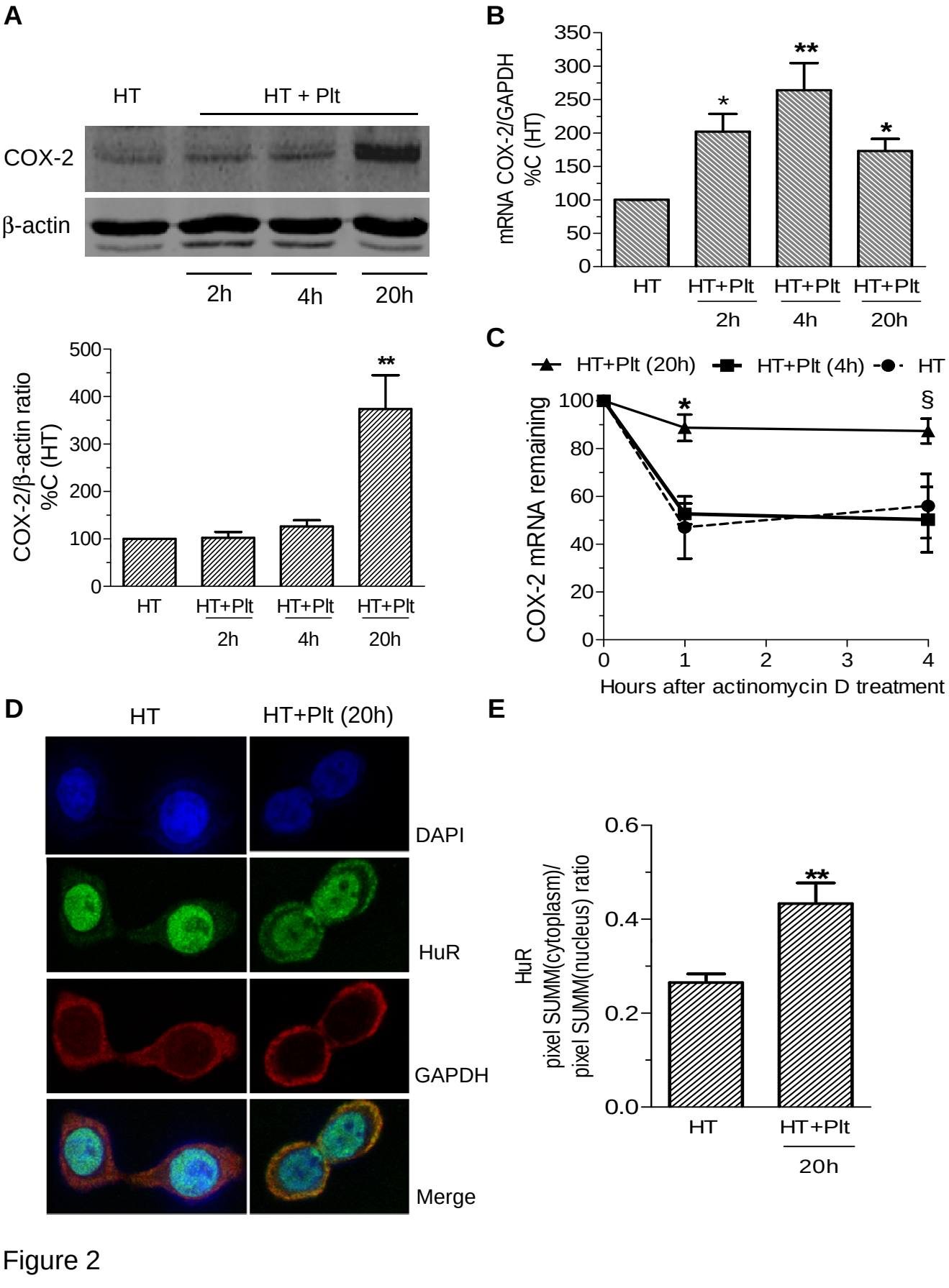

Figure 2

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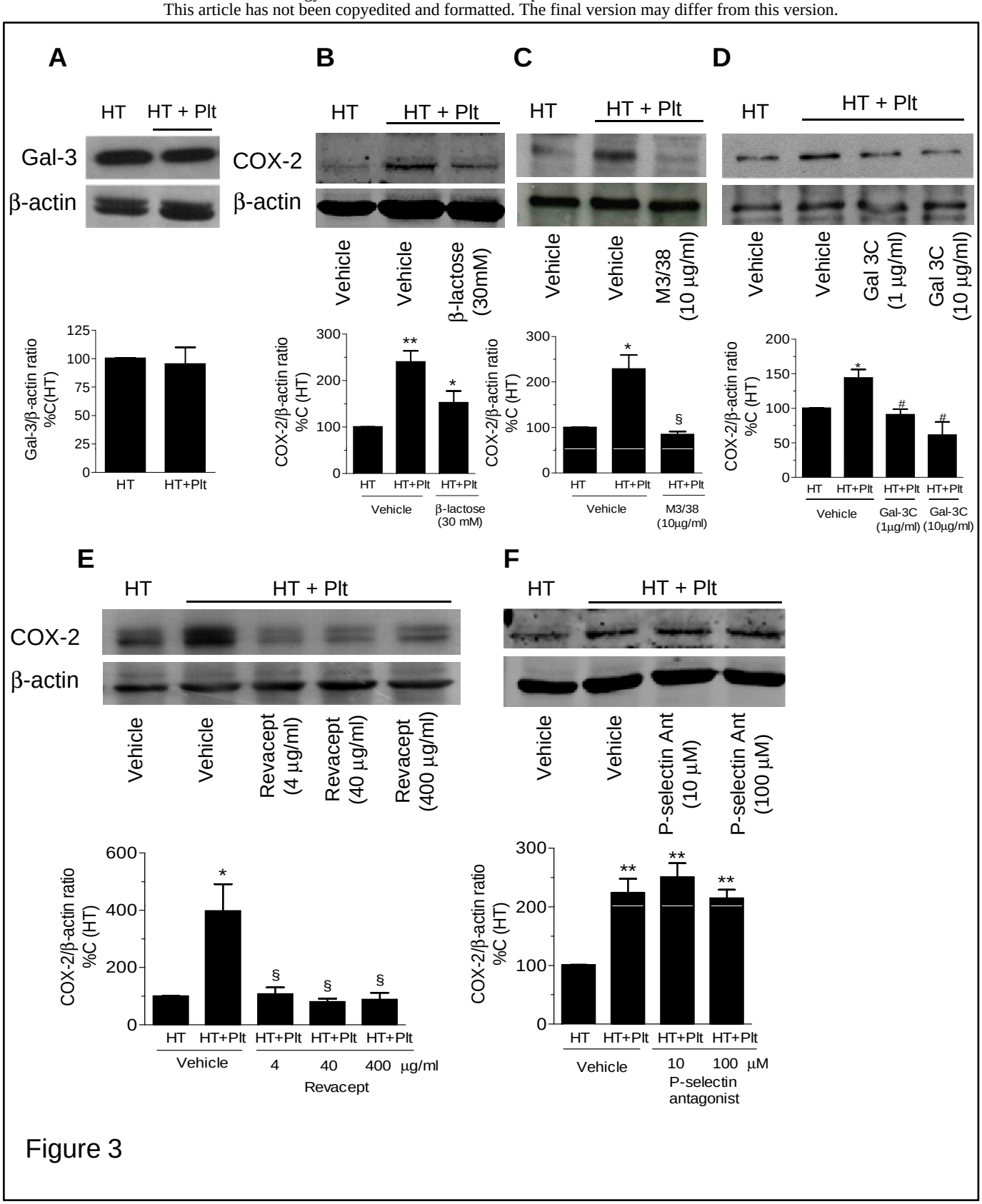

Figure 3

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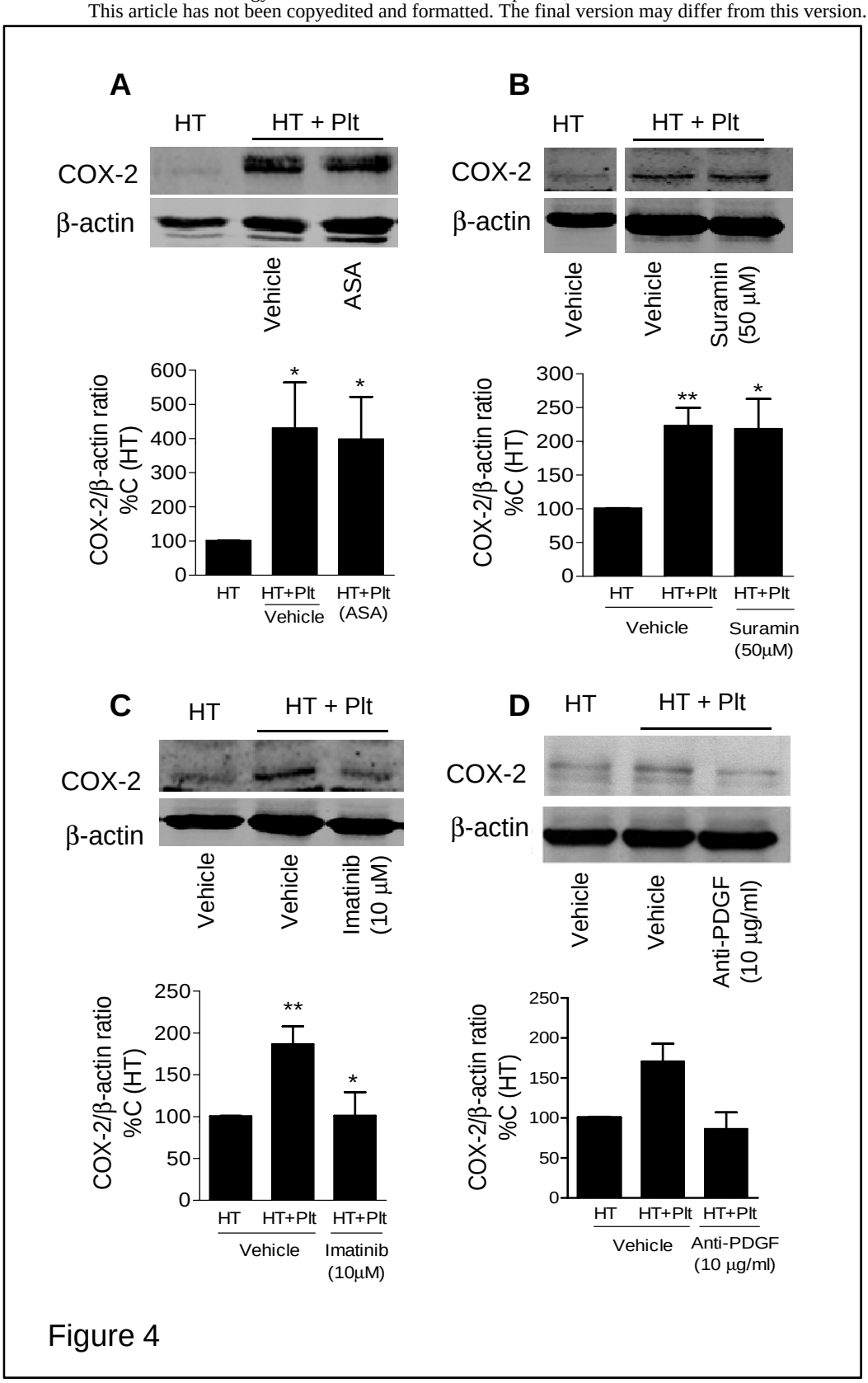

Figure 4

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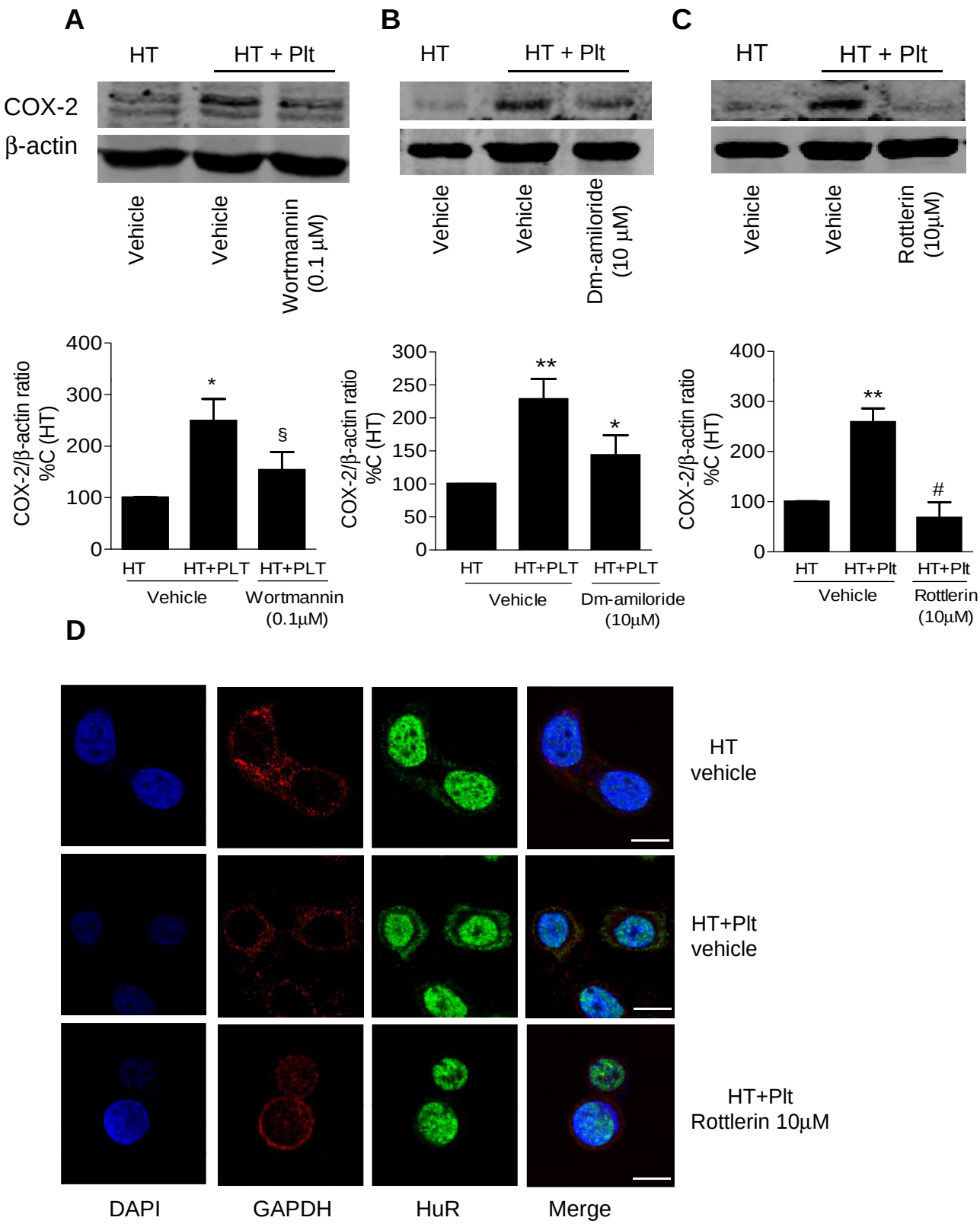

Figure 5

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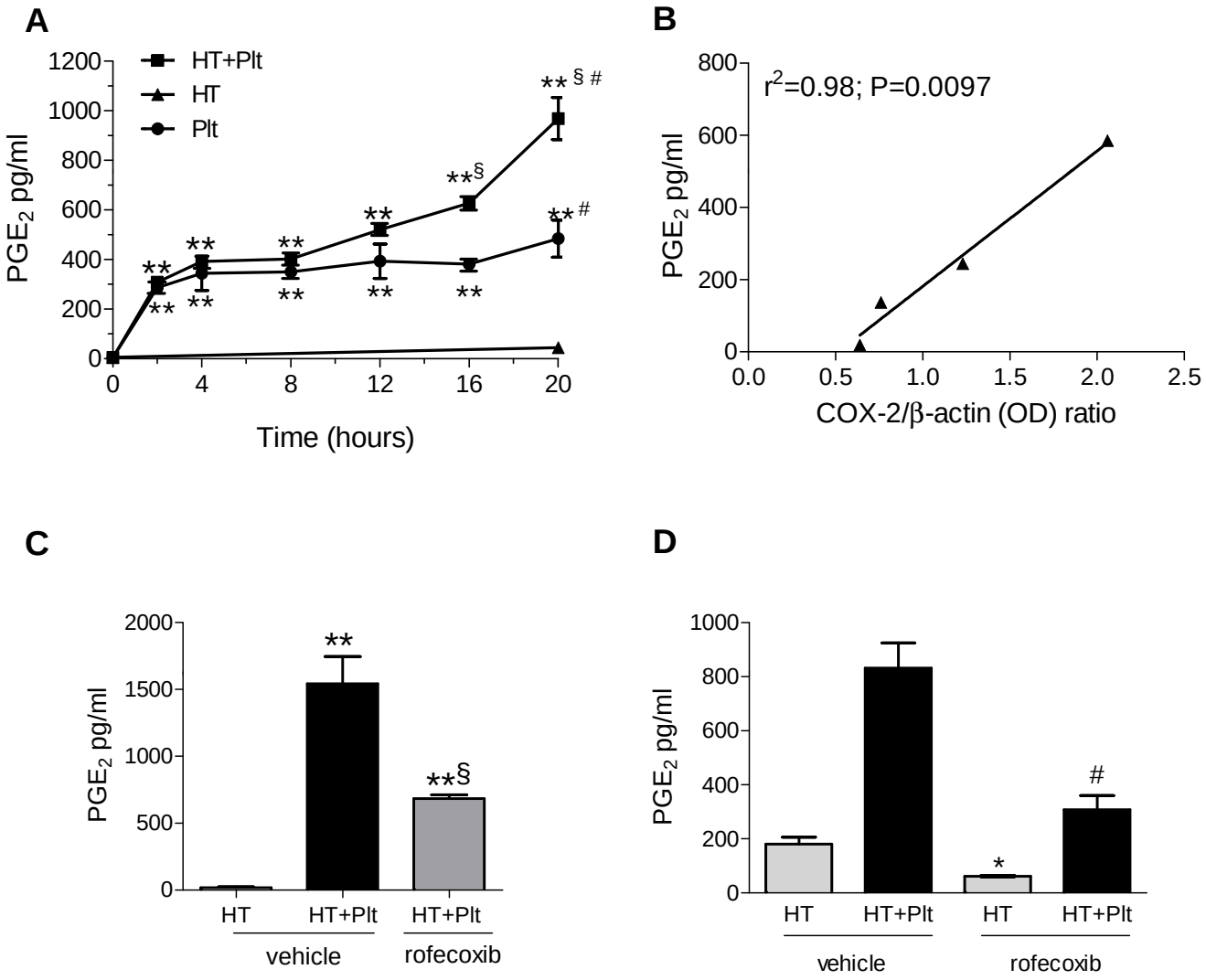

Figure 6

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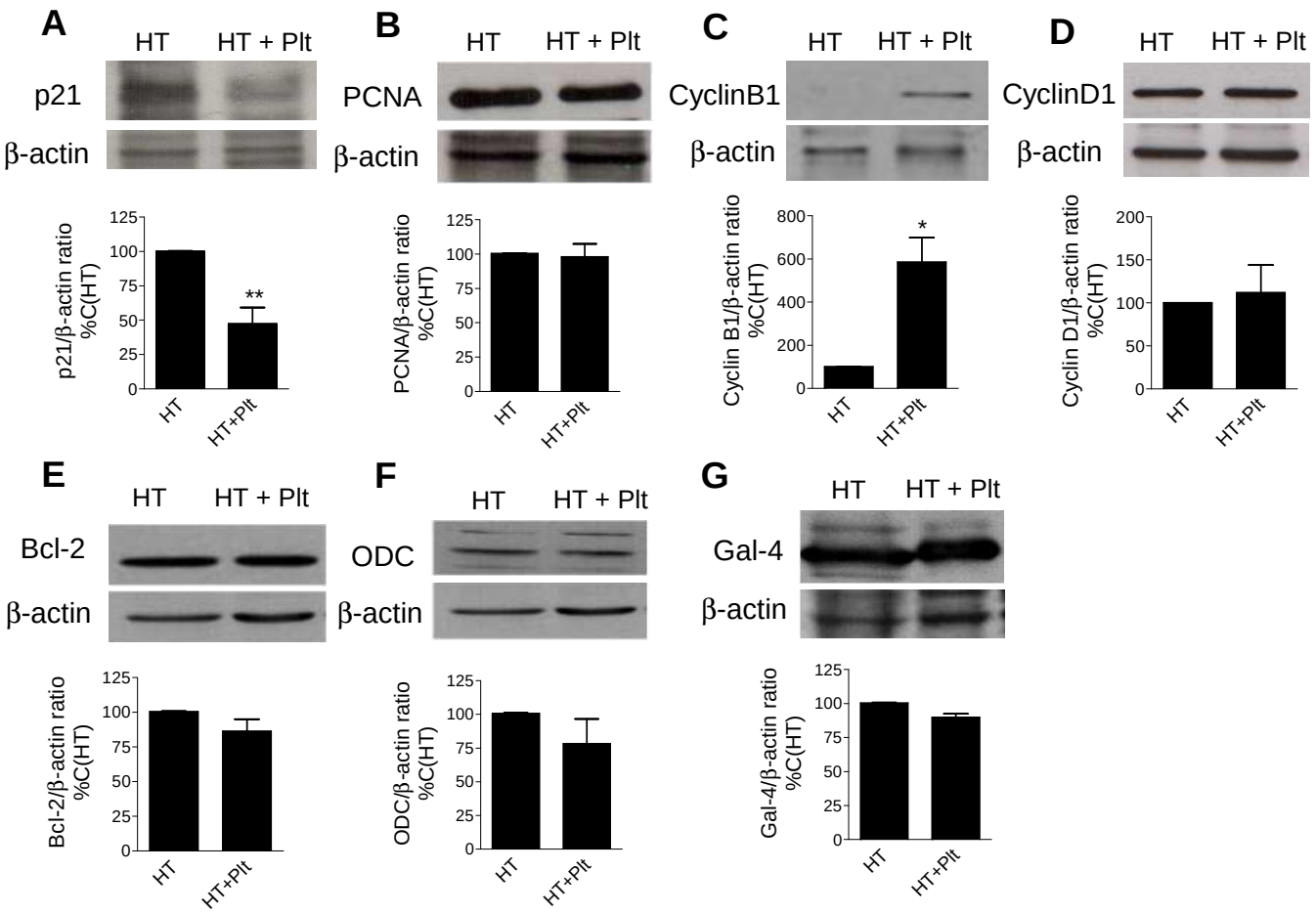

Figure 7

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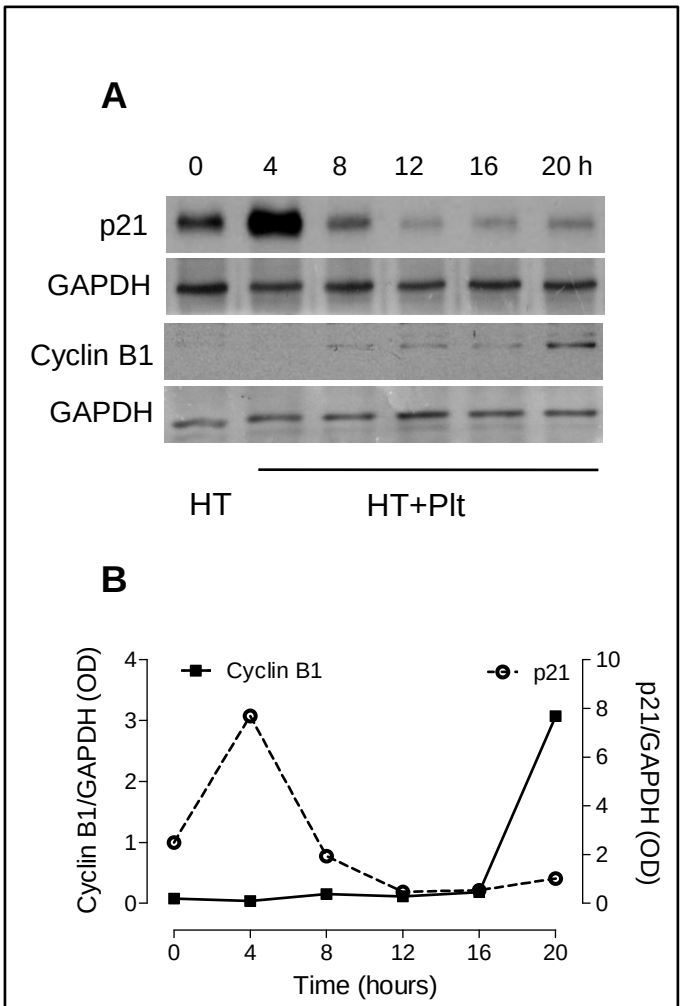

Figure 8

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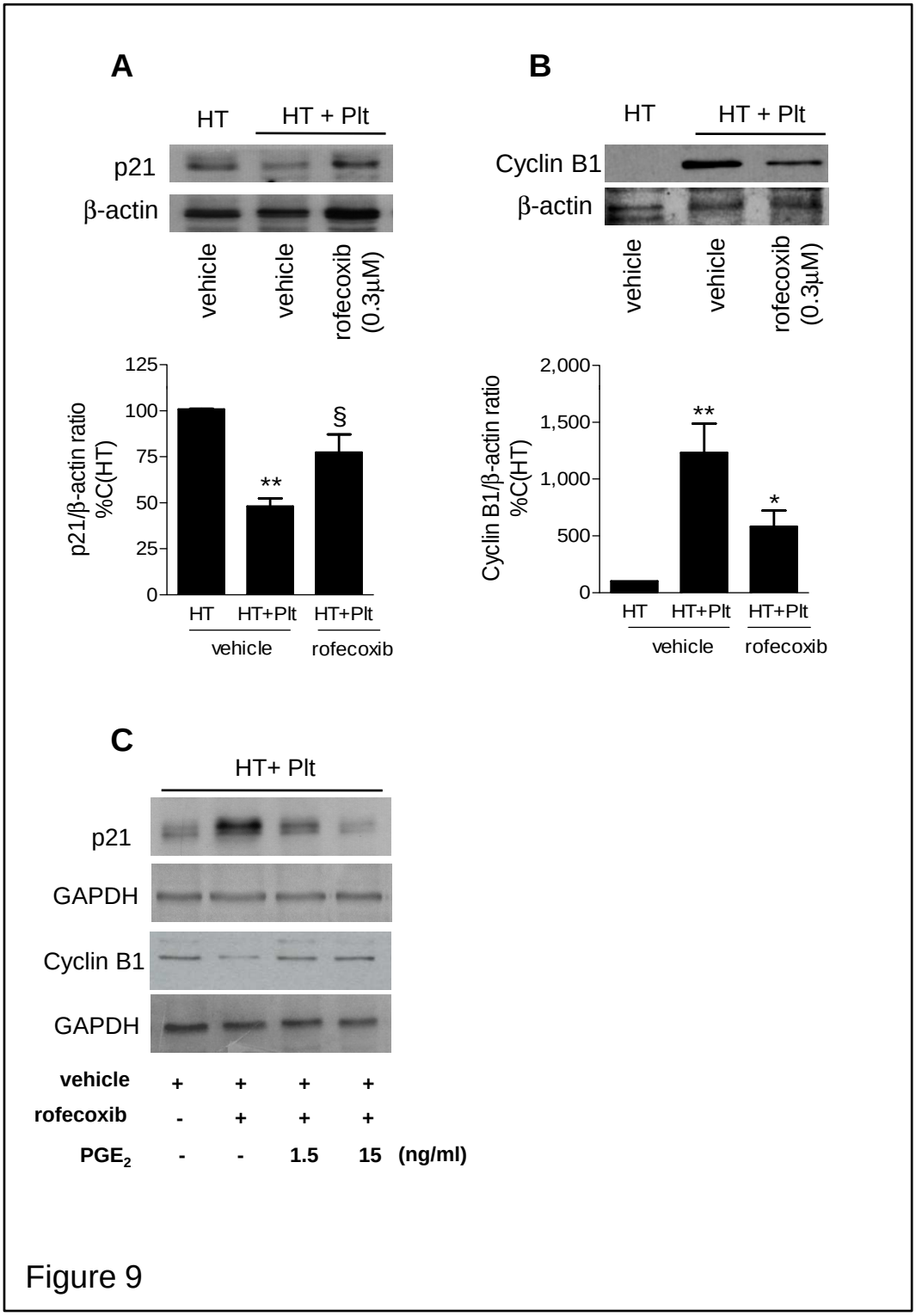

Figure 9

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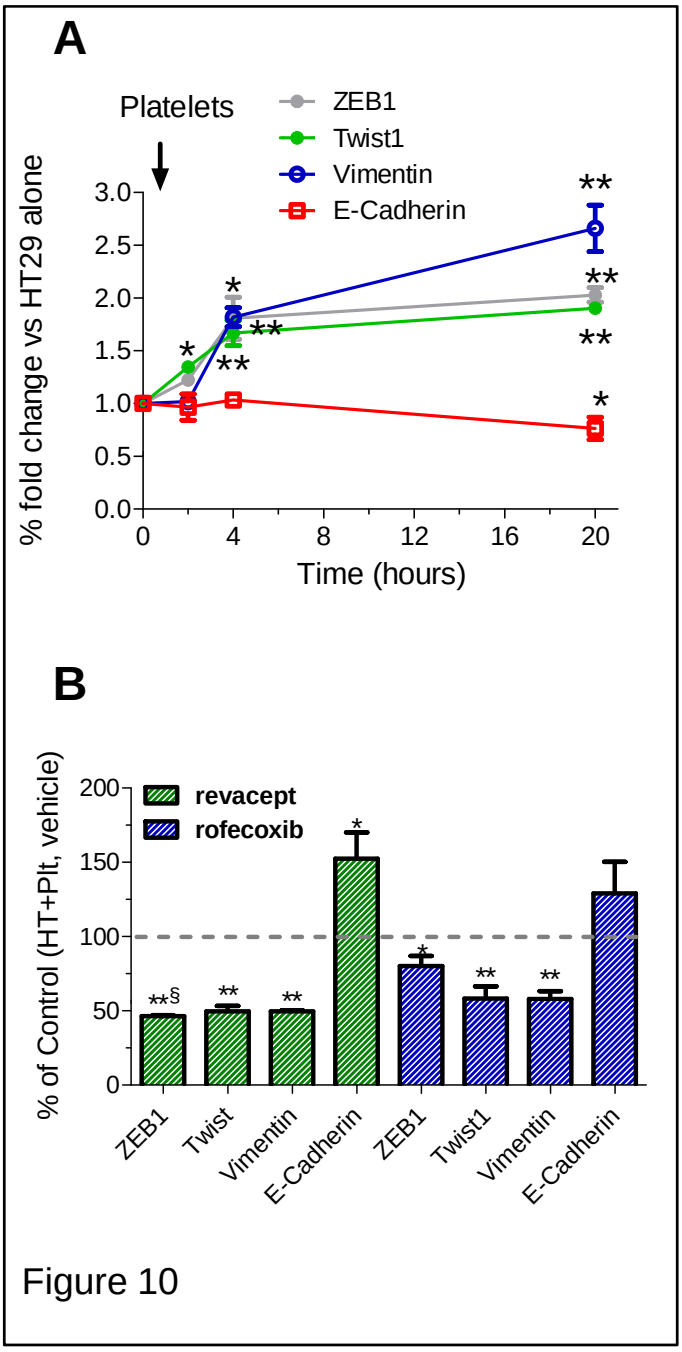

Figure 10

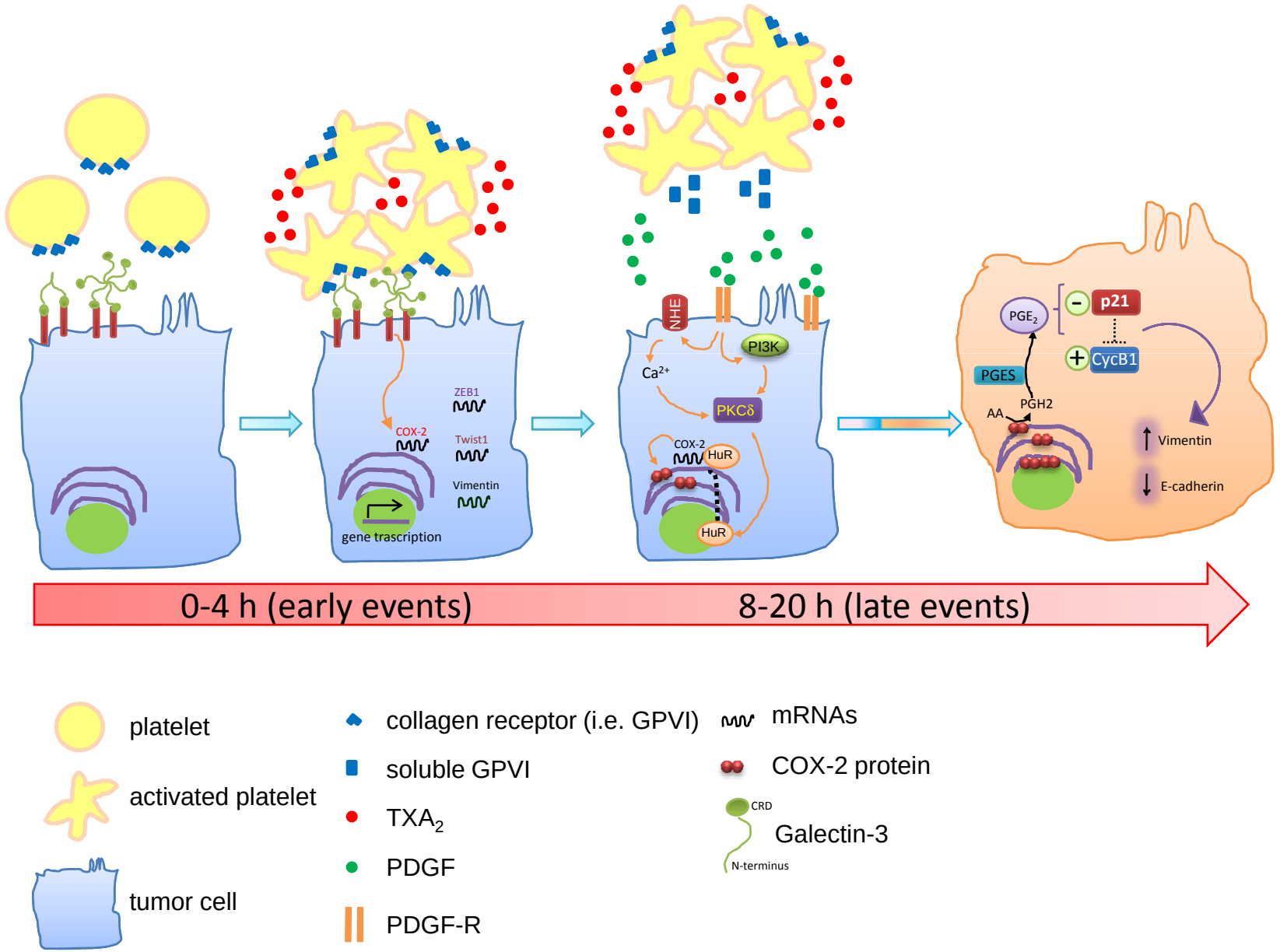

Figure 11

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