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The granulocyte colony-stimulating factor (G-CSF) upregulates metalloproteinase-2 and VEGF through PI3K/Akt and Erk1/2 activation in human trophoblast Swan 71 cells

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ABSTRACT

Introduction: Although the expression of the granulocyte colony-stimulating factor (G-CSF) and its receptor (G-CSFR) in placental tissues suggests that the cytokine could play a role in placental development, the relevance of G-CSF:G-CSFR interaction in trophoblast cells remains to be studied. Thus, the possible functional role of G-CSF was examined in a human trophoblast cell line (Swan 71 cells).
Methods and results: The expression of G-CSFR was detected by immunocytochemistry and Western blot assays. G-CSF treatment exerted neither a proliferative nor a protective effect on H_2O_2-mediated cell death in trophoblast cells. Gelatin zymography of supernatants collected from G-CSF-treated cells showed an increment of metalloproteinase-2 (MMP-2) activity. We also found higher MMP-2 and VEGF expression levels in conditioned medium from cells exposed to G-CSF. In addition, it was demonstrated that G-CSF induced the activation of PI3K/Akt and Erk1/2 pathways, which in turn activated NF-kB. By using selective pharmacological inhibitors, it was showed that these pathways are mediating the biological effects produced by G-CSF in Swan 71 cells.
Discussion and conclusion: We have demonstrated for the first time that G-CSF increases MMP-2 activity and VEGF secretion in Swan 71 cells through activation of PI3K/Akt and Erk signaling pathways, both contributing to the translocation of NF-kB to the nucleus. These data suggest that G-CSF is involved in the regulation of trophoblast function, and should be considered as a locally produced cytokine probably contributing to embryo implantation and the development of a functional placenta.

1. Introduction

The granulocyte colony-stimulating factor (G-CSF) is a potent hematopoietic factor that stimulates the proliferation, differentiation, survival and activation of cells of the granulocyte lineage [1]. The interaction of G-CSF with its specific receptor (G-CSFR) triggers various signaling pathways that finally lead to the induction of G-CSF biological activities [1–4]. Although G-CSFR has been originally found in different hematopoietic cells [5–7], it has also been detected in non-hematopoietic tissues, including endothelial [8,9], cardiomyocytes [10], neural stem cells [11], trophoblastic cells and placental tissue cells [12–16]. Thus, besides the essential role of G-CSF in the regulation and mobilization of hematopoietic cells, other functions have also been explored in non-hematopoietic cells [7]. In this respect, it has been demonstrated that G-CSF increases the expression of adhesion receptors and induces the proliferation and migration of endothelial cells [8,9]. G-CSF signaling also promotes survival of cardiomyocytes [10], stimulates neurogenesis [11,17] and enhances glioma cell migration [18]. In addition, G-CSF increases the migration and invasiveness of tumor cells [19,20]. Based on the expression patterns of G-CSF and G-CSFR in placental tissues [13,14], it has been suggested that this cytokine plays a role in the regulation of placental growth and trophoblast function. In particular, G-CSF was found in the stroma of fetal chorionic villi and maternal decidual tissues throughout pregnancy, whereas G-CSFR was detected in fetal first and third trimester placental tissues, but

Abbreviations: DTT, dithiothreitol; Erk, extracellular signal-regulated kinases; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; G-CSF, granulocyte colony-stimulating factor; IkBα, inhibitor of kappa B alpha; MAPKs, mitogen-activated protein kinases; MMP, metalloproteinase; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; NF-kB, nuclear factor kappa B; PI3K, phosphatidylinositol 3-kinase; PMSF, phenylmethanesulfonyl fluoride; PVDF, polyvinylidene difluoride; VEGF, vascular endothelial growth factor.

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not in maternal decidual tissues from all gestational stages. The expression of G-CSFR in first trimester placental tissues, a gestational age more related to the 7-week trophoblast cell line employed in this study, was higher in differentiated syncytiotrophoblast and extravillous cytotrophoblast [14]. In spite of these relevant findings, the significance of G-CSF signaling for placental function remains to be studied.

The matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases involved in the degradation of mostly all proteins of the extracellular matrix (ECM) [21–23]. MMPs are implicated not only in various physiological process related to embryonic development, growth, wound healing and cell migration, but also in a variety of pathological conditions, including tumor invasion, angiogenesis, inflammation, rheumatoid arthritis and osteoarthritis [21]. In particular, the invasive ability of human trophoblast cells, which is required during early pregnancy for embryo implantation and placentation, depends on the secretion of proteolytic enzymes, mostly MMP-2 (72 kDa type IV collagenase, gelatinase A) and MMP-9 (92 kDa type IV collagenase, gelatinase B) [24–26]. Both MMPs are mainly responsible for the degradation of collagen IV, the major structural collagen of the basement membrane. Although the regulation of MMPs activity by cytokines and growth factors in trophoblast and maternal decidua has been previously described [26–28], the putative role for G-CSF in placenta tissue is still unknown.

In the present study, we decided to examine whether the G-CSF exerts a functional role on human trophoblasts by using the immortalized first trimester trophoblast Swan 71 cell line, derived from a 7-week normal placenta [29]. After detecting the presence of G-CSFR in these cells, we studied the effect of G-CSF on cell proliferation, cell viability and MMP activity. In addition, taking into account that G-CSF increases the release of vascular endothelial growth factor (VEGF) from neutrophils [30], we also explored if the cytokine could behave as an angiogenic factor by stimulating VEGF secretion. The signaling pathways triggered after G-CSF:receptor interaction and the contribution of these intracellular cascades to the biological actions induced by G-CSF were further studied.

2. Materials and methods

2.1. Materials

Recombinant human G-CSF was supplied by BIO SIDUS S.A., Buenos Aires, Argentina. Rabbit polyclonal antibodies anti-MMP-2, anti-NF-kB p65 and secondary anti-mouse and anti-rabbit IgG horseradish peroxidase antibodies were obtained from Santa Cruz Biotechnology, CA, USA. Rabbit polyclonal antibodies anti-Erk1/2, anti-p-Erk1/2 (Thr202/Tyr204), anti-Akt, anti-p-Akt (Ser473), and mouse monoclonal antibody anti-IkBα were obtained from Cell Signaling Technology, Danvers, MA, USA. Mouse monoclonal antibody anti-GCSF receptor was from R&D Systems Inc., MN, USA, and rabbit anti-human VEGF antibody was from Millipore, MA, USA. Ly294002 was obtained from Santa Cruz Biotechnology, CA, USA, PD98059 was from Promega, WI, USA, and BAY11-7082 was from Sigma, St. Louis, MO, USA.

2.2. Cell line and culture conditions

The trophoblast cell line, Swan 71, derived by telomerase-mediated transformation of a 7-week cytotrophoblast isolate was kindly provided by Dr. Gil Mor (Department of Obstetrics, Gynecology and Reproductive Sciences, School of Medicine, Yale University, New Haven, USA) [29]. Swan 71 cells were maintained in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) Media (Gibco®, Life Technologies™, Argentina) supplemented with 10% FBS, 1 mM sodium pyruvate, 2 mM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin. In order to assess the effect of G-CSF on the trophoblast cell line, 1×10^5 cells/well were plated in 6-well tissue culture plates. After 24 h, cells were washed with PBS and incubated 24 h or 48 h in culture medium with no FBS in the presence or absence of different concentrations of G-CSF in a total volume of 1.5 ml. Protein concentration of cell lysates, determined with the Bradford reagent (Bio-Rad, Hercules, CA, USA), was employed to control protein loading in gelatin zymography. Supernatants were collected and stored a −20 °C for further analyses. In some experiments, Swan 71 cells were pre-treated for 1 h with 1 μM Ly294002, a selective PI3 kinase inhibitor (Santa Cruz Biotechnology, CA, USA), 1 μM PD98059, a selective MEK-1 inhibitor (Promega, WI, USA) or 0.5 μM BAY11-7082, a selective IKK complex inhibitor (Sigma, St. Louis, MO, USA).

2.3. Gelatin zymography

The presence of MMPs in supernatants obtained after incubating cells with or without G-CSF as described above was detected by gelatin zymography [31]. The harvested culture media were concentrated 8-fold in Amicon Ultra Centrifugal Filters, cut-off 10 kDa (Millipore, MA, USA). Each concentrated medium (10 μl) was diluted 1:2 with non-reducing sample buffer (0.063 M Tris/HCl, pH 6.8, 2% SDS, 10% glycerol, 0.05% bromophenol blue) and then submitted to electrophoresis using 10% acrylamide gels containing 1 mg/ml gelatin type B (Sigma, St. Louis, MO, USA). After electrophoresis, proteins were renatured by incubating gels for 1 h at room temperature with 10 mM Tris, 130 mM NaCl (TBS), pH 7.4, containing 2.5% Triton X-100 to remove SDS, then for 15 min in TBS and afterward gels were incubated for 22 h at 37 °C in 120 mM NaCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, 50 mM Tris-HCl, pH 7.5. Finally, gels were stained for 30 min with 0.25% Coomasie Brilliant Blue R-250 (Sigma, St. Louis, MO, USA) in methanol: acetic acid: water (4:1:5). Gelatinolytic activities were detected as clear bands on a uniformed blue background. For quantification of band intensities, gels were scanned using a densitometer (Gel Pro Analyzer 4.0).

2.4. Cell proliferation and survival assays

Swan 71 cells plated in 96-well microplates at a density of 1.5×10^3 cells/well were incubated for 72 h at 37 °C in the presence or absence of different concentrations of G-CSF in a total volume of 0.1 ml of culture medium without FBS. The number of viable cells was evaluated by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt) assay using the CellTiter 96®-AQueous Non-Radioactive Cell Proliferation Assay (Promega Corp, Madison, WI, USA). Absorbance at 492 nm corresponding to the conversion of MTS into a soluble formazan product was measured using a microtiter plate reader (Biotrak II, GE Healthcare, Piscataway, NY, USA). For survival experiments, 5×10^3 cells/well were incubated overnight with different concentrations of G-CSF in culture medium without FBS, and then cell viability was evaluated after treatment with 1 mM H_2O_2 for 4 h at 37 °C.

2.5. Immunocytochemistry of G-CSF receptors

Human trophoblastic Swan 71 cells grown up on coverslips were washed with PBS and fixed for 5 min at room temperature with 4% paraformaldehyde. After washing with PBS, monolayers were incubated with PBS, BSA 1% for 2 h at room temperature to block non-specific binding sites. Then, coverslips were incubated overnight at 4 °C with mouse monoclonal antibody anti-GCSF receptor (R&D Systems Inc., MN, USA) diluted in PBS, BSA 1%. In parallel, normal mouse IgG was employed as negative control. After rinsing with PBS, coverslips were incubated for 2 h at room temperature with the corresponding fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (BD Biosciences, NY, USA). Nuclear counterstaining was performed by adding the blue-fluorescent nucleic acid stain Höechst 33258 (Sigma, St. Louis, MO, USA) to the anti-fade mounting medium. Cells were examined with an Olympus BX50 epifluorescence microscope provided with a Cool-Snap digital camera.

2.6. Western blot assays

G-CSF receptors: monolayers of Swan 71 cells were detached using cell scraper, washed with cold PBS and lysed for 30 min at 4 °C in 10 μl of cold lysis buffer (1.5% Triton X-100, 50 mM NaCl, 0.5% sodium cholate, 0.2% SDS, 2% Nonidet P-40, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml trypsin inhibitor, 1 mM Na_3VO_4, 1 mM PMSF, 25 mM Tris-HCl, pH 7.5). Lysates were cleared by centrifugation for 10 min at 17,000 × g, and the supernatants diluted with sample buffer (100 μg of protein) were submitted to 8% SDS-PAGE, and then transferred onto nitrocellulose membranes. After blocking non specific binding sites, membranes were incubated with the corresponding primary antibody and then with anti-mouse IgG (horseradish peroxidase-conjugated goat IgG from Santa Cruz Biotechnology, CA, USA). Immunoreactive proteins were visualized using the Pierce® ECL Plus Western blotting substrate (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions. NFS-60 cells, derived from a murine myeloid leukemia, were used as positive control of G-CSF receptors [32].

Detection of MMP and VEGF: supernatants collected from Swan 71 cells incubated 24 h or 48 h in the presence or absence of 100 ng/ml of G-CSF were dried out in a Savant SpeedVac® Concentrator and resuspended in 20 μl of sample buffer. Samples were then submitted to 12% SDS-PAGE and transferred onto PVDF membranes. For quantification of band intensity, Western blots were scanned using a densitometer (Gel Pro Analyzer 4.0). Coomassie Blue staining of PVDF membranes was used to control protein load [33].

Akt and Erk1/2 activation: to measure the phosphorylation of Akt or Erk1/2 proteins, monolayers of Swan 71 cells, maintained 24 h in serum-free medium, were treated with or without 100 ng/ml of G-CSF for different times. Cells were then solubilized for 30 min at 4 °C in 10 μl of lysis buffer (0.5% Triton X-100, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml trypsin inhibitor, 10 mM $Na_4P_2O_7$, 10 mM NaF, 150 mM NaCl, 1 mM Na_3VO_4, 1 mM PMSF, 1 mM EDTA, 50 mM Tris, pH 7.4), and 40 μg of proteins were submitted to 10% SDS-PAGE, followed by transfer onto nitrocellulose membranes. In some experiments, cells were pre-incubated for 1 h with 1 μM

Ly294002 or 1 μM PD98059. No effect on Swan 71 cell proliferation was observed at these concentrations of inhibitors (data not shown).

2.7. Preparation of cytosolic and nuclear extracts

Monolayers of Swan 71 cells, maintained 24 h in serum-free medium, were treated with or without 100 ng/ml of G-CSF for different times. Cytosolic and nuclear cell extracts were prepared to detect IkBα and NF-kB p65 proteins, respectively. For cytosolic preparations, cells were lysed in 100 μl of lysis buffer (10 mM KCl, 2 mM $MgCl_2$, 1% Nonidet P-40, 0.5 mM DTT, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 0.2 mM EDTA, 10 mM Hepes, pH 7.9) for 10 min at 4 °C. Lysates were cleared by centrifugation for 1 min at 3000 × g, and the supernatants (cytosolic fraction) were collected and stored for further analysis. The corresponding pellets were resuspended in 15 μl of lysis buffer (0.42 M NaCl, 1.5 mM $MgCl_2$, 25% glycerol, 0.5 mM DTT, 1 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 5 μg/ml pepstatin A, 0.2 mM EDTA and 20 mM Hepes, pH 7.9) for 30 min at 4 °C. Lysates were then cleared by centrifugation for 30 min at 14,000 × g. The supernatants (nuclear extracts) were diluted 1:2 with buffer (0.05 M KCl, 20% glycerol, 0.5 mM DTT, 0.5 mM PMSF, 0.2 mM EDTA and 20 mM Hepes, pH 7.9). Both cytosolic and nuclear extracts (40 μg of proteins) were submitted to 10% SDS-PAGE and transferred onto nitrocellulose membranes (GE Healthcare, Piscataway, NY, USA). Equal protein loading was confirmed by reprobing membranes with rabbit anti-actin (Sigma–Aldrich, St. Louis, MO, USA) or mouse anti-tubulin (Abcam, Cambridge, MA, USA) antibodies. In some experiments, cells were pre-incubated for 1 h with 1 μM Ly294002, 1 μM PD98059 or 0.5 μM BAY11-7082. BAY concentration employed did not affect cell viability (data not shown).

2.8. Intracellular staining for NF-kB detection

Swan 71 cells plated on coverslips were washed twice with PBS, maintained 24 h in culture medium without FBS and then treated with or without 100 ng/ml G-CSF for 30 min. After washing with PBS, cells were fixed for 5 min at room temperature with 4% paraformaldehyde, rinsed three times with 0.2% Triton X-100 in PBS (PBS-TX) and incubated with PBS-TX containing 1% BSA for 2 h at room temperature to block non-specific binding sites. Then, coverslips were incubated overnight at 4 °C with rabbit polyclonal anti-NF-kB p65 antibody diluted in PBS-TX/1% BSA. After rinsing with PBS, coverslips were incubated for 2 h at room temperature with an indocarbocyanine (Cy3)-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Nuclear counterstaining was performed by adding Höechst 33258 to the anti-fade mounting medium. Cells were examined with an Olympus BX50 epifluorescence microscope provided with a Cool-Snap digital camera. In some experiments, cells were pre-incubated for 1 h with 1 μM Ly294002, 1 μM PD98059 or 0.5 μM BAY11-7082.

2.9. Statistical analysis

GraphPad Prism 5.00 software was used for data analysis. Results represent mean ± SEM. Comparisons were performed using one way analysis of variance (ANOVA) followed by Bonferroni post-hoc tests where appropriate. Student's *t*-test was used when the values of two groups were analyzed. Values were considered significantly different when $p < 0.05$.

3. Results

3.1. Detection of G-CSF receptors on Swan 71 cells

The presence of G-CSF receptors on Swan 71 cells was first examined by immunocytochemistry. Microscopy evaluation of cell monolayers showed that Swan 71 cells were clearly stained after

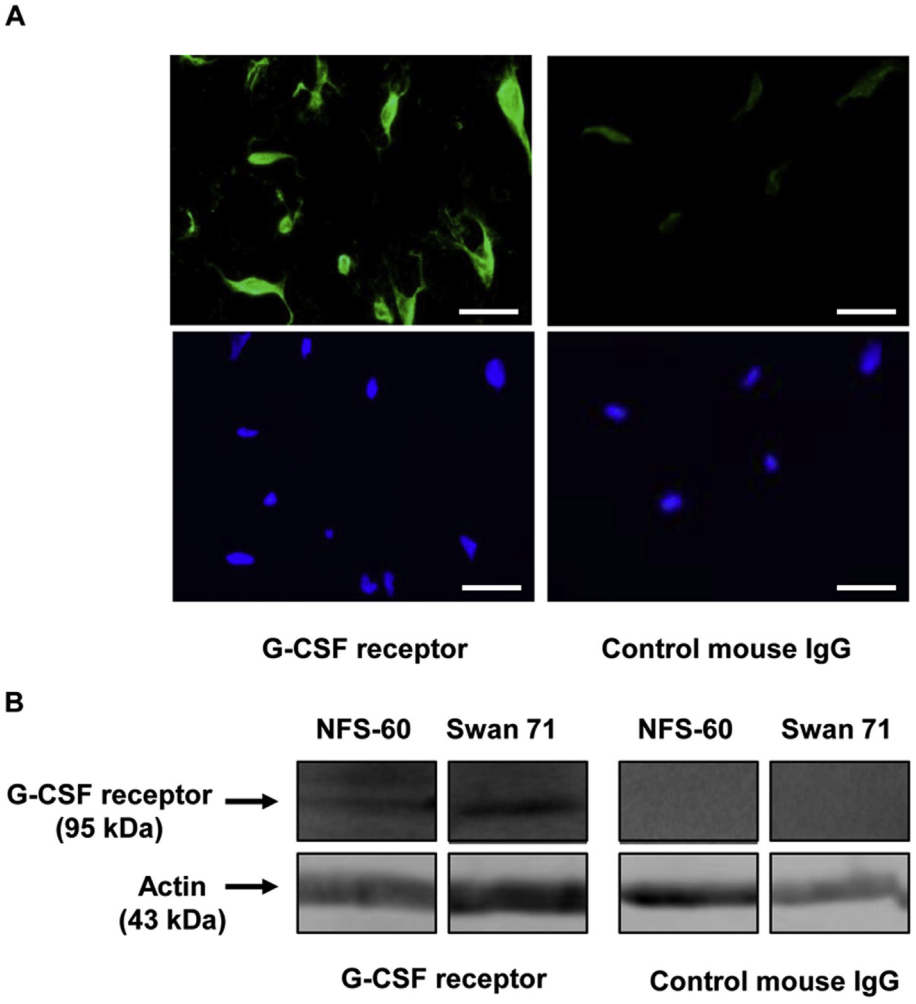

Fig. 1. Detection of G-CSF receptors. (A) Swan 71 cells plated on coverslips were fixed with 4% paraformaldehyde and stained as described in Material and methods with a monoclonal antibody anti-G-CSF receptor or normal mouse IgG (control). Nuclear counterstaining was performed with Höechst 33258. Scale bar corresponds to 50 μm. (B) Cell lysates from Swan 71 and NFS-60 cells were submitted to Western blot with anti-G-CSF receptor antibody or normal mouse IgG (control).

treatment with a specific anti-human G-CSF receptor antibody, whereas no significant fluorescence was observed when cells were incubated in the presence of normal mouse IgG used as control (Fig. 1A). G-CSF receptors were also detected after Western blot analyses of Swan 71 cell lysates (Fig. 1B). In parallel, lysates from NFS-60 cells were used as positive control of G-CSF receptors expression (Fig. 1B). In addition, although a soluble form of G-CSFR has been described [1], we could not detect this variant by Western blots assays of the conditioned media of cultured Swan 71 cells (data not shown).

3.2. Effect of G-CSF on Swan 71 cell proliferation and survival

It has been previously reported that G-CSF induces a proliferative response in NFS-60 cells [32,34]. In this sense, we formerly found that NFS-60 cell growth increased in a dose-dependent manner up to a concentration of 0.1 ng/ml of G-CSF [16]. In order to investigate the effect of G-CSF on the trophoblast Swan 71 cell line, cells were incubated in the absence of serum with different concentrations of cytokine. As shown in Fig. 2A, no proliferative effect was observed up to 1 μg/ml of G-CSF. Similar results were obtained when cells were incubated in the presence of serum (data not shown). Since it has also been found that G-CSF supports cell survival in different cell lines [10,16,17,35], we next incubated cells in culture medium deprived of FBS in the absence or presence of 1 mM H_2O_2, as an oxidizing and pro-apoptotic agent, and different concentrations of G-CSF. Whereas a similar cell proliferation was obtained with or without FBS, cell viability diminished almost 60% when cells were treated with H_2O_2 under serum deprivation (Fig. 2B). Under this experimental condition, no significant difference in the number of viable cells was found with concentrations up to 2 μg/ml of G-CSF, indicating that cytokine treatment did not exert a protective effect on Swan 71 cells (Fig. 2B).

3.3. Effect of G-CSF on metalloproteinase activity and VEGF secretion

The MMPs, a family of zinc-dependent proteolytic enzymes involved in the degradation of ECM, have been reported to play an important role in trophoblastic cells invasion [26]. Since the most studied MMPs during placental invasion are the gelatinases (gelatinase A: MMP-2; gelatinase B: MMP-9) [24,25], we decided to explore whether G-CSF modifies the activity or expression levels of MMP in Swan 71 cells. The proteolytic activity of MMPs was evaluated by gelatin zymography of supernatants collected from cells cultured with or without different concentrations of G-CSF. Although the activity of the latent form of MMP-2 (proenzyme or proMMP-2) was detected in non-stimulated cells, G-CSF treatment (100 ng/ml) increased proMMP-2 levels up to 40% or 20% after 24 or 48 h of incubation, respectively (Fig. 3A). Further studies were

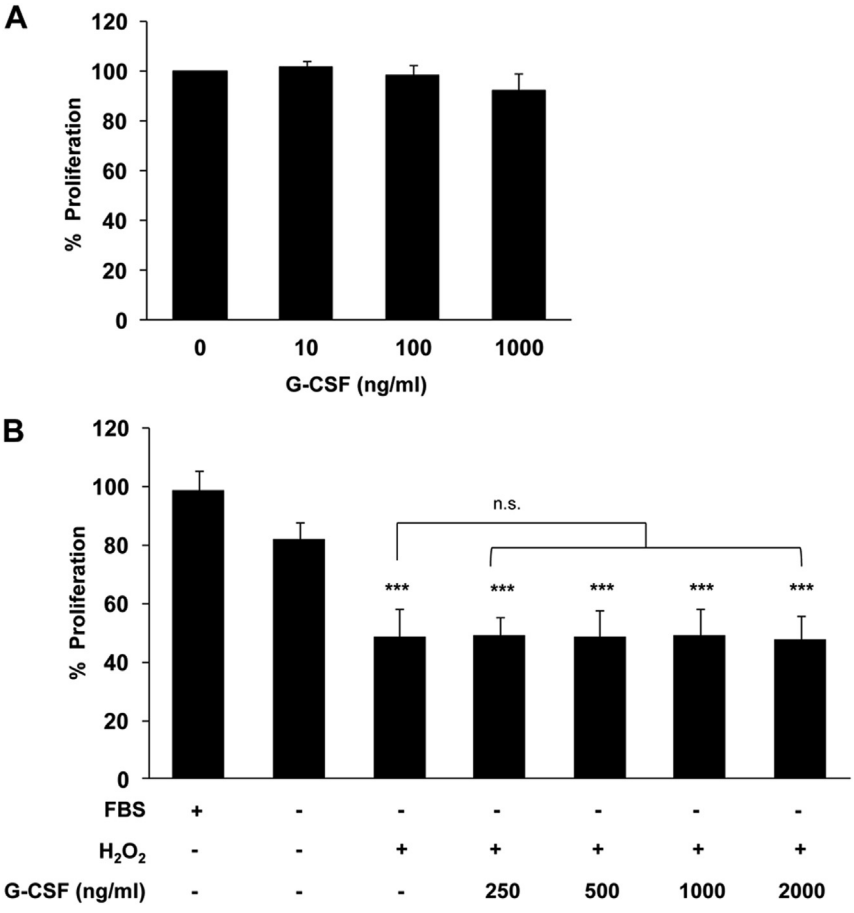

Fig. 2. G-CSF effect on proliferation or survival of trophoblastic cells. (A) Swan 71 cells were incubated for 72 h at 37 °C in the presence or absence of different concentrations of G-CSF in a total volume of 0.1 ml of culture medium without FBS. (B) Swan 71 cells were incubated 4 h at 37 °C with different concentrations of G-CSF in culture medium containing 1 mM H_2O_2 with no FBS as described in Material and methods. The number of viable cells was evaluated by MTS assay. Results are expressed as the percentage of cell viability obtained in complete culture medium and represent the mean ± SEM of three independent experiments. Statistical analyses were performed by one-way ANOVA followed by Bonferroni post-hoc tests. ***$p < 0.001$, significantly different from cells incubated in the absence of serum, n.s. non-significant with respect to cells incubated with H_2O_2 in the absence of G-CSF.

performed after 24 h of exposure to 100 ng/ml of G-CSF. Under our experimental conditions, neither the active form of MMP-2 nor the latent/active forms of MMP-9 were detected in the supernatants analyzed. The identity of MMP-2 was confirmed by Western blot analyses of supernatants arising from G-CSF-treated and non-treated cells. Under the reducing conditions employed in this assay, only the active form of MMP-2 was distinguished (Fig. 3B). A significant increase of MMP-2 was found in the conditioned medium from cells exposed for 24 h to 100 ng/ml of G-CSF (Fig. 3B). No change of MMP-2 expression level was observed at shorter incubation times (Supplemental Fig. 1A).

Embryo implantation is regulated by multiple factors, including VEGF, an homodimeric glycoprotein which is critical for placental angiogenesis [27,36,37]. In order to evaluate whether G-CSF affects VEGF secretion in Swan 71 cells, culture supernatants from stimulated and non-stimulated cells were concentrated and submitted to Western blot analyses. Results shown in Fig. 3C revealed that expression levels of VEGF were significantly up-regulated after 24 h of incubation with 100 ng/ml of G-CSF, whereas no detectable change was found after 48 h of G-CSF treatment. Furthermore, Swan 71 cells treated with 100 ng/ml of G-CSF for 8 h or 16 h show no difference in VEGF levels compared to non-treated cells (Supplemental Fig. 1B).

3.4. G-CSF stimulates Erk1/2 MAPK and PI3K/Akt pathways in Swan 71 cells

In a previous work, we demonstrated that G-CSF activates Jak/STAT and MAPK pathways in a choriocarcinoma trophoblastic cell line (JEG-3) [16]. Since the biological actions induced by G-CSF in Swan 71 cells indicated the existence of a functional G-CSFR, we decided first to examine whether G-CSF activates Erk1/2 MAPK pathway, a signaling cascade that regulates different cellular processes such as proliferation, differentiation, development and invasion [38,39]. When phosphorylation kinetics of Erk1/2 MAPK was examined in serum-deprived Swan 71 cells by Western blot analyses, a peak of phospho-Erk1/2 was observed after 15 min of stimulation with G-CSF (Fig. 4A). The addition of PD98059 (PD), a MEK-1 inhibitor, to trophoblastic cells inhibited G-CSF-induced Erk1/2 phosphorylation (Supplemental Fig. 2A). The PI3K/Akt cascade has also been related to multiple cellular functions, including cell survival, proliferation, differentiation, cellular metabolism, angiogenesis, migration and invasion [40,41]. When Akt phosphorylation was studied in Swan 71 cells, high levels of phospho-Akt were obtained after 15 min of stimulation with G-CSF (Fig. 4B). The addition of Ly294002 (Ly), a PI3K inhibitor, to trophoblastic cells strongly inhibited G-CSF-induced Akt

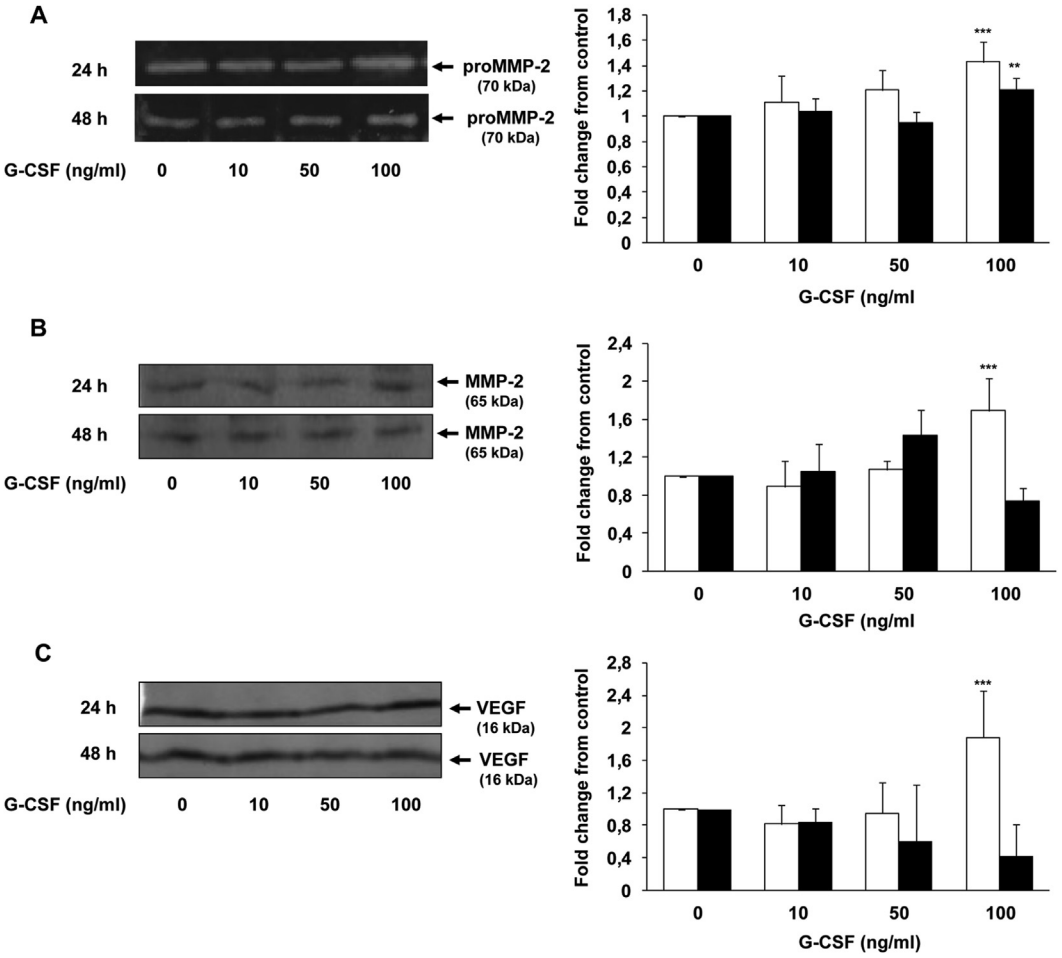

Fig. 3. G-CSF effect on MMP-2 activity and VEGF expression levels. Swan 71 cells plated in 6-well tissue plates were washed with PBS and incubated 24 h (white bars) or 48 h (black bars) with culture medium with no FBS in the presence or absence of different concentrations of G-CSF. (A) 10 μl of each concentrated medium were mixed 1:1 with sample buffer and then applied to gels for gelatin zymography. One representative zymography is shown (left panel). Quantification was performed by densitometric analysis (right panel). (B) Western blot assays of conditioned medium from G-CSF stimulated and non-stimulated cells were performed with anti-MMP-2 antibody. (C) Western blot assays of supernatants of G-CSF treated and non-treated cells were revealed with anti-VEGF antibody. Results from one representative experiment are shown (left panels). Data quantification was performed by densitometric analysis (right panels). Statistical analyses were performed by one-way ANOVA followed by Bonferroni post-hoc tests. $^{***}p < 0.001$ ($n = 4$–6), $^{**}p < 0.01$ ($n = 3$), significantly different from non-stimulated cells.

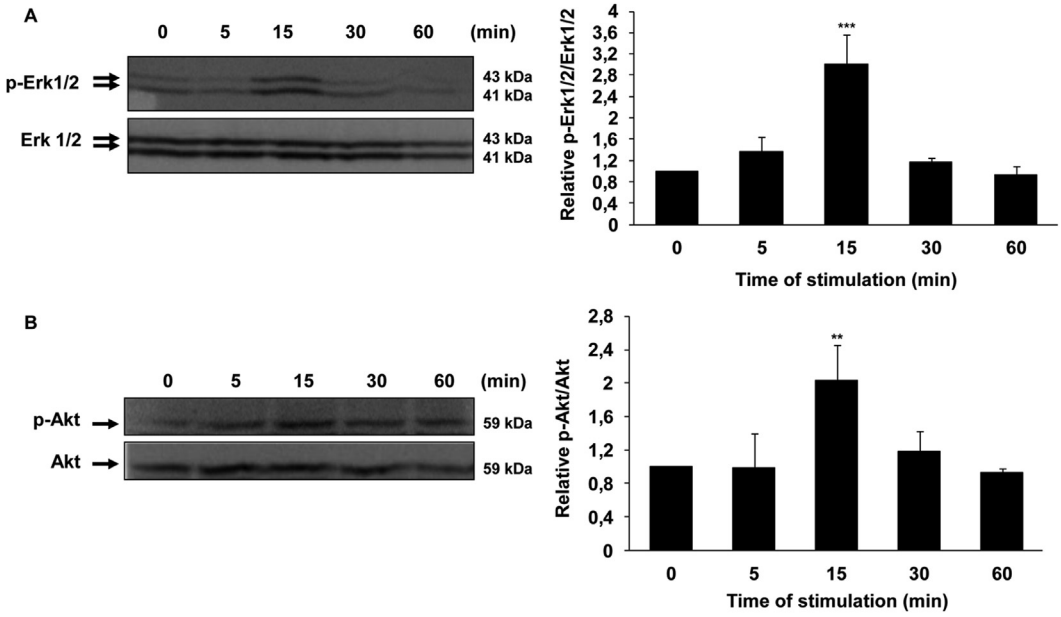

Fig. 4. G-CSF-induced Erk1/2 and Akt phosphorylation. Monolayers of Swan 71 cells maintained 24 h in medium with no FBS were incubated for different times in the presence or absence of 100 ng/ml of G-CSF. Cell lysates were subjected to SDS-PAGE under the conditions described in Material and methods. Western blot assays were performed with (A) anti-p-Erk1/2 (Thr202/Tyr204) and anti-Erk1/2, (B) anti-p-Akt (Ser473) and anti-Akt. Results from one representative experiment are shown (left panels). Data quantification was performed by densitometric analysis (right panels). Statistical analyses were performed by one-way ANOVA followed by Bonferroni post-hoc tests. $^{***}p < 0.001$, $^{**}p < 0.01$, ($n = 3$), significantly different from non-stimulated cells.

phosphorylation (Supplemental Fig. 2B). Activation of PI3K/Akt pathway results in phosphorylation of several key downstream targets, including NF-kB [41–43]. Thus, Akt activation can lead to phosphorylation of IkBα followed by its proteolytic degradation and translocation of NF-kB dimers to the nucleus [44]. In addition, it has been reported that Erk may also act as an upstream modulator of NF-kB activation [45]. In order to investigate the possible involvement of NF-kB, we then examined the kinetics of IkBα degradation and the cytosolic and nuclear expression of NF-kB in G-CSF-treated Swan 71 cells by Western blot analyses. As shown in Fig. 5A, levels of IkBα diminished after 15 and 30 min of stimulation with 100 ng/ml of G-CSF. Consequently, a significant decrease in the cytoplasmic levels of NF-kB p65 and a concomitant increase in the nuclear amount of this protein were observed after Western blot analyses of cell lysates obtained from serum-deprived Swan 71 cells stimulated 30 min with G-CSF (Fig. 5B). In addition, NF-kB p65 translocation was also evaluated by immunocytochemistry. Microscopy evaluation of Swan 71 monolayers showed a clear nuclear staining of NF-kB p65 after 30 min of exposure to G-CSF, whereas cytosolic fluorescence localization was observed when cells were incubated in the absence of cytokine (Fig. 5C). Furthermore, in order to support the participation of IkBα/NF-kB in the signaling cascades triggered by G-CSF, we demonstrated that the addition of BAY11-7082 (BAY), an inhibitor of the IkB kinase complex (IKK), to trophoblastic cells strongly inhibited G-CSF-induced IkBα degradation and NF-kB nuclear translocation (Supplemental Fig. 3).

3.5. Involvement of Erk1/2 MAPK, PI3K/Akt and NF-kB in the G-CSF-induced activation of MMP-2 and secretion of VEGF

The contribution of Erk1/2 MAPK, PI3K/Akt and NF-kB in the activation of MMP-2 and the up-regulation of VEGF induced by G-CSF in Swan 71 cells was further studied by using the corresponding selective pharmacological inhibitors. Thus, pre-treatment of Swan 71 cells with PD, Ly or BAY effectively reversed the enhancement of MMP-2 expression levels induced by G-CSF (Fig. 6A). In addition, these inhibitors also reduced the higher VEGF levels found in culture supernatants of G-CSF-exposed cells (Fig. 6B). Thus, our results suggest that the activation of Erk1/2, PI3K/Akt and NF-kB contributes to trigger the biological responses induced by the cytokine on the trophoblastic cell line.

In order to elucidate the upstream regulators responsible for NF-kB activation, G-CSF-induced degradation of IkBα was examined in Swan 71 cells pre-treated with PD or Ly. As shown in Fig. 6C, the incubation of Swan 71 cells with PD or Ly markedly reversed G-CSF effect. In addition, when NF-kB p65 translocation was evaluated by immunocytochemistry, it was observed that pre-incubation of Swan 71 cells with PD or Ly clearly diminished the nuclear localization of NF-kB p65 (Fig. 6D). Taken together, these results indicate that both PI3K/Akt and Erk pathways are required for NF-kB activation.

4. Discussion

In order to understand the functional relevance of G-CSF in placenta tissues, we previously demonstrated the presence of specific G-CSFR in human choriocarcinoma JEG-3 cells and showed that G-CSF activates some signaling proteins of the Jak/STAT and the MAPK pathways [16]. Although G-CSF stimulated the phosphorylation of several identical signaling components both in JEG-3 cells as well as in myeloid NFS-60 cells, a proliferative response was only found in the hematopoietic cell line. Thus, G-CSF might be regulating cell-type-dependent biological effects in trophoblast cells which differ, at least in part, from G-CSF-induced responses in hematopoietic tissues. In this study, we evaluated the biological actions induced by G-CSF in Swan 71 trophoblast cells. Although these cells have been reported to produce a variety of cytokines and growth factors, including G-CSF, the endogenous secretion of G-CSF is indeed not remarkable [29]. Unlike the proliferative and protective effects induced by G-CSF on myeloid cells [35,46] and even other cell types [8,10,11,16], we showed that 100 ng/ml of exogenous G-CSF neither stimulated cell growth nor exerted a protective

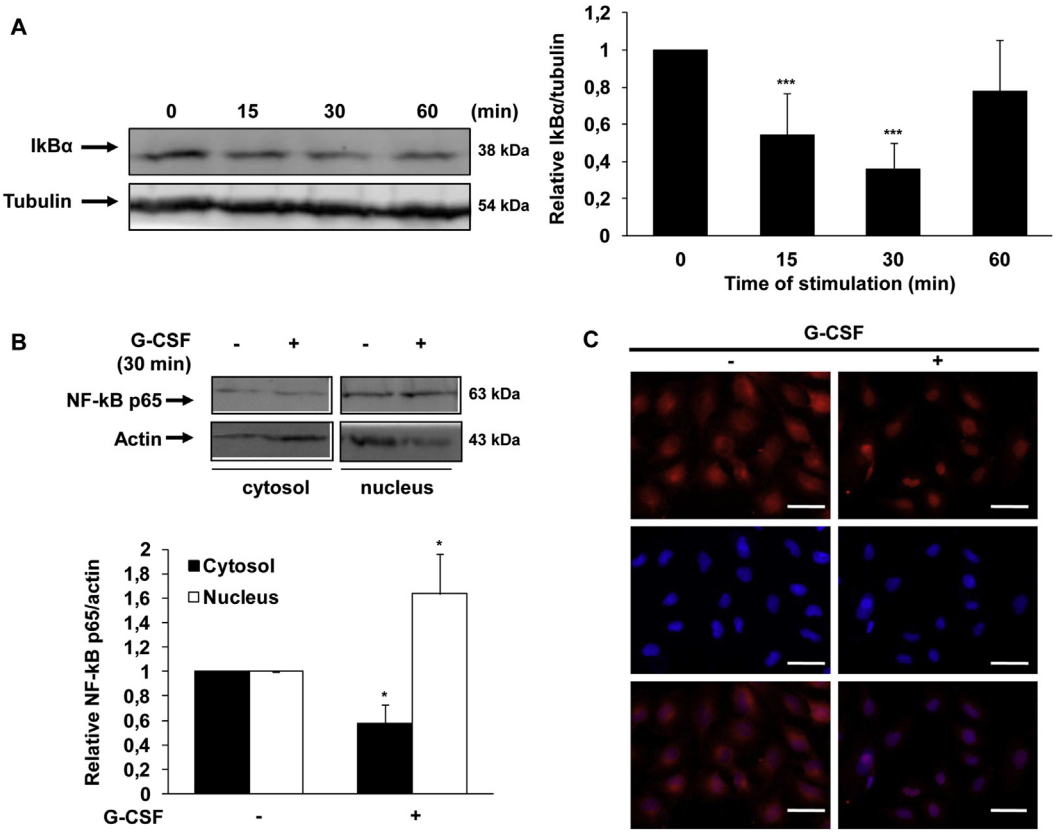

Fig. 5. G-CSF-induced IkBα degradation and NF-kB nuclear translocation. Monolayers of Swan 71 cells maintained 24 h in medium with no FBS were incubated for different times in the presence or absence of 100 ng/ml of G-CSF. (A) Cytosolic extracts were submitted to Western blot with an anti-IkBα antibody. Results from one representative experiment are shown. Data quantification was performed by densitometric analysis. Statistical analyses were performed by one-way ANOVA followed by Bonferroni post-hoc tests. $^{***}p < 0.001$ ($n = 6$), significantly different from non-stimulated cells. (B) Western blots of cytosolic and nuclear extracts obtained as indicated in Material and methods were revealed with an anti-NF-kB p65 antibody. Results from one representative experiment are shown. Data quantification was performed by densitometric analysis. Statistical analyses were performed by one-tailed Student's *t*-test. $^{*}p < 0.05$ ($n = 3$), significantly different from non-stimulated cells. (C) Swan 71 cells plated on coverslips and maintained 24 h in medium with no FBS were treated with or without 100 ng/ml of G-CSF for 30 min. After fixation, cells were incubated with an anti-NF-kB p65 antibody and then revealed with a Cy3-conjugated secondary antibody. Nuclear counterstaining was performed with Höechst 33258. Scale bar corresponds to 50 μm.

effect on Swan 71 cells. Although G-CSF concentrations herein employed are higher than G-CSF levels found in serum (range <30–163 pg/ml) [47], the local concentration of G-CSF in placenta tissues should be considered. In this regard, for comparative purposes, it is worth to mention that G-CSF levels in conditioned media from explants of first trimester chorionic villous tissue corresponding to 7 weeks of gestation, ranged from 30 to 300 ng/ml [13].

We further demonstrated that G-CSF treatment significantly increased the levels of MMP-2 in culture supernatants from G-CSF-stimulated cells. Different studies have described the contribution of both MMP-2 and MMP-9 to the process of trophoblast invasion required for embryo implantation and development of a functional placenta [24–26]. However, the secretion profile of MMPs seems to be dependent on the gestational week. Thus, MMP-2 appears to be more important in early trophoblast invasion (6–8 weeks), and afterward both MMPs participate in late first trimester trophoblast invasion (9–12 weeks) [24]. It has also been reported that MMP-2 is mainly expressed in extravillous trophoblast of first trimester placenta, whereas MMP-9 is expressed in villous cytotrophoblast [48]. In accordance with these results, we showed that the 7-week trophoblast Swan 71 cells, which exhibit a phenotype similar to extravillous trophoblasts [29,49], increased MMP-2 expression after G-CSF treatment. Thus, G-CSF stimulus contributes to enhance the levels of probably the main gelatinase involved in the early invasion of human trophoblasts. Since the molecular events leading to trophoblast invasion are similar to those related to tumor invasion, it is worthwhile to remark that G-CSF expression confers an invasive phenotype to human breast cells through up-regulation of MMP-2 [20]. Consistent with this finding, we proposed that G-CSF would play a role as a regulator of trophoblast invasion by increasing MMP-2 expression. Although decidual cells also contribute to the regulation of the invasion process by expressing a broad spectrum of MMPs [50], the role of G-CSF produced by decidual tissues remains to be studied.

We also found higher levels of VEGF in culture supernatants from Swan 71 cells incubated with G-CSF. Thus, G-CSF also promotes the secretion of an important angiogenic factor required to accomplish the invasion of uterine wall and the formation of the placenta [27,36,37]. In addition to our findings, the effectiveness of G-CSF administration in the treatment of unexplained recurrent miscarriage [51] also highlights a potential role of G-CSF on trophoblast function and early pregnancy.

We further showed that the increase of MMP-2 and VEGF expression levels induced by G-CSF in Swan 71 cells are mediated by PI3K/Akt and Erk1/2 signaling pathways. Thus, pre-treatment of Swan 71 cells with Ly294002 (PI3K inhibitor) and PD98059 (MEK inhibitor) blocked the increment of MMP-2 and VEGF expression levels induced by G-CSF. A general role of both cascades in proliferation, differentiation, survival and migration/invasion has been previously reported [38–41]. In particular, it has been showed that both PI3K and Erk signaling pathways are required to up-regulate EGF-induced MMP-9 levels and hCG-induced MMP-2 levels in

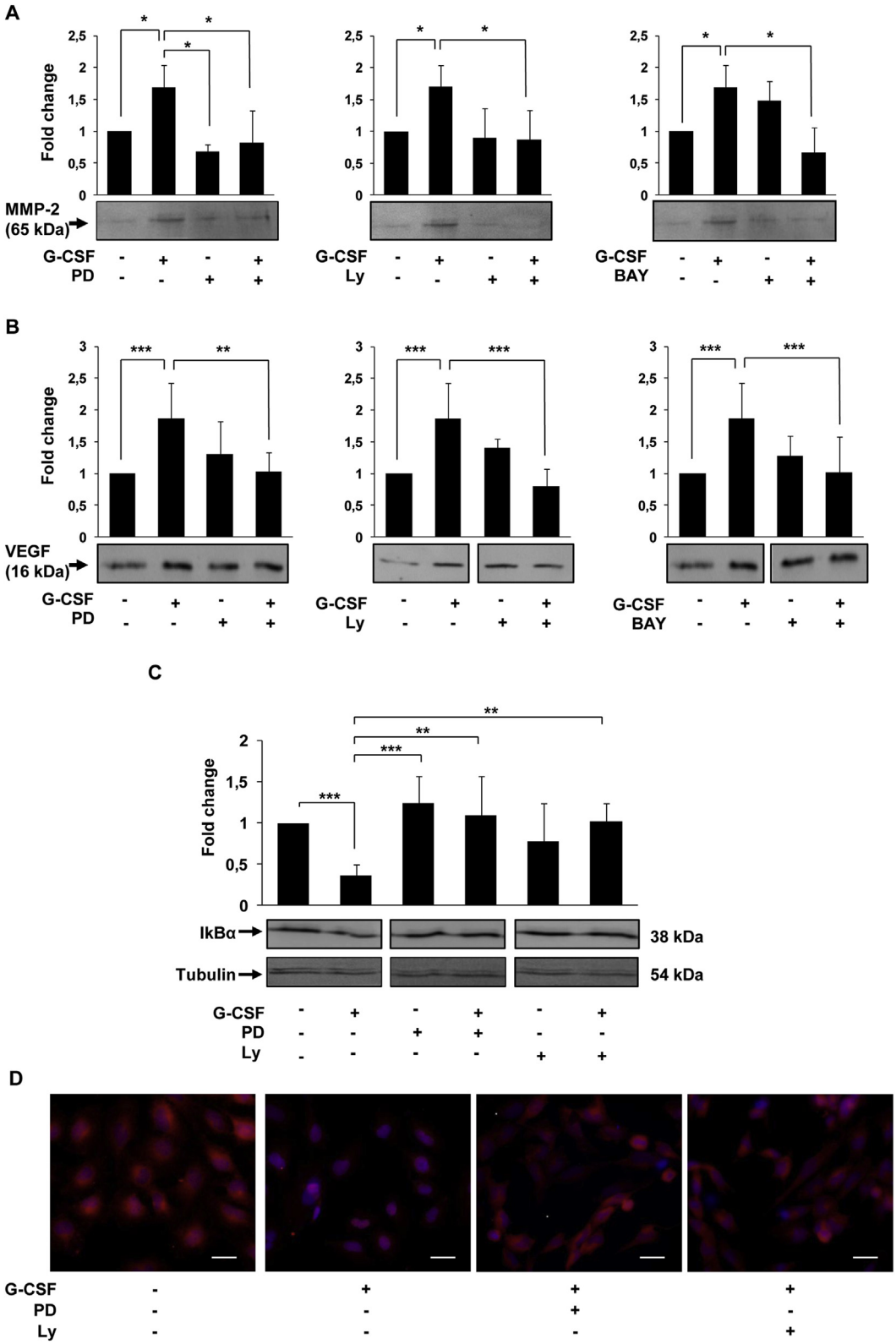

Fig. 6. Participation of Erk1/2, PI3K/Akt and NF-kB in the biological actions induced by G-CSF in Swan 71 cells. Swan 71 cells plated in 6-well tissue plates and maintained for 24 h in medium with no FBS, were pre-treated for 1 h with 1 μM PD98059, 1 μM Ly294002 or 0.5 μM BAY11-7082 and then incubated with 100 ng/ml of G-CSF for 24 h. Western blot assays of conditioned medium from G-CSF stimulated and non-stimulated cells were performed with anti-MMP-2 (A) and anti-VEGF (B) antibodies. (C) Cytosolic extracts from Swan 71 cells maintained in serum-free medium, pre-treated with PD or Ly and then exposed to 100 ng/ml of G-CSF for 30 min, were submitted to Western blot with an anti-IkBα antibody. Results from one representative experiment are shown in (A), (B) and (C). Data quantification was performed by densitometric analysis. Statistical analyses were performed by one-way ANOVA followed by Bonferroni post-hoc tests. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$, ($n = 3$–6). Comparison among the four lanes in (B) was valid, as all samples were run on a single gel (Supplemental Fig. 4). (D) Swan 71 cells plated on coverslips and maintained 24 h in medium with no FBS were pre-treated for 1 h with 1 μM PD98059 or 1 μM Ly294002 and then incubated in the presence or absence of 100 ng/ml of G-CSF for 24 h. After fixation, cells were incubated with an anti-NF-kB p65 antibody and then revealed with a Cy3-conjugated secondary antibody. Nuclear counterstaining was performed with Höechst 33258. Scale bar corresponds to 50 μm.

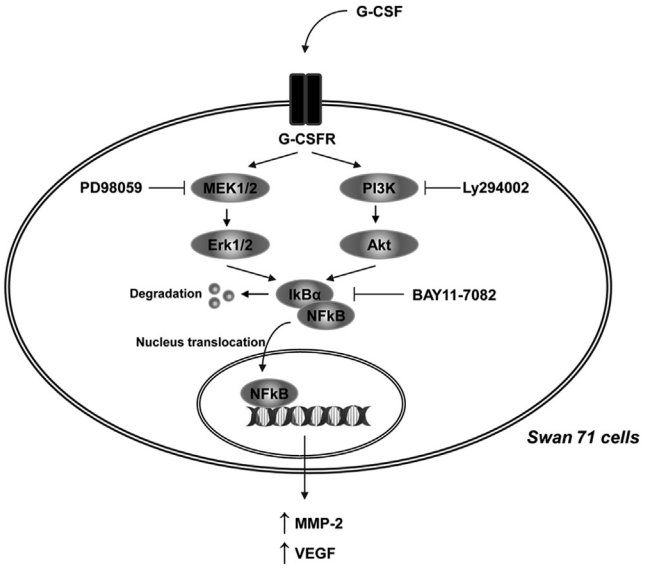

Fig. 7. Schematic representation of the signaling pathways involved in the biological actions induced by G-CSF in human trophoblast Swan 71 cells. After binding of G-CSF to G-CSF receptor, the activation of PI3K/Akt and Erk1/2 cascades leads to the translocation of NF-kB to the nucleus, inducing an increase of MMP-2 activity and VEGF secretion.

trophoblast cells [52–54], being the secretion of MMPs related to trophoblast migration/invasion processes. The effect of G-CSF in the migration/invasion of trophoblast Swan 71 cells is currently under study.

A significant decrease in MMP-2 and VEGF expression levels was also detected in the presence of BAY11-7082, an inhibitor of NF-kB signaling pathway, indicating the involvement of NF-kB in the biological responses triggered by G-CSF in Swan 71 cells. In addition, Swan 71 G-CSF-treatment resulted in an increase of IkBα degradation and NF-kB p65 translocation from the cytosol to the nucleus. We further demonstrated that NF-kB activation is mediated by both PI3K/Akt and Erk cascades. In this regard, preincubation of Swan 71 cells with Ly294002 and PD98059 clearly inhibited G-CSF-induced degradation of IkBα and diminished NF-kB p65 nuclear translocation. The contribution of both signaling pathways to NF-kB activation has been previously reported for other stimuli in different cell types [55,56].

In summary, we have demonstrated for the first time that G-CSF increases MMP-2 expression and VEGF secretion in Swan 71 cells through activation of PI3K/Akt and Erk signaling pathways, both contributing to translocation of NF-kB to the nucleus. A model illustrating the signaling pathways involved in G-CSF-induced biological actions is showed in Fig. 7. Based on our results, it is reasonable to propose that G-CSF, as it has been reported for other cytokines and growth factors, could be considered as a regulatory factor of trophoblast invasion that contributes to embryo implantation and placenta development.

Author contributions

VAF, JM, VCB performed research and analyzed data. LPR designed research, analyzed data and wrote the manuscript.

Conflicts of interest

The authors declare no potential conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.placenta.2014.09.003.

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