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Sildenafil stimulates human trophoblast invasion through nitric oxide and guanosine 3',5'-cyclic monophosphate signaling

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Abstract

Objective: To determine the effect of sildenafil, a phosphodiesterase type 5 inhibitor, on trophoblast invasiveness.

Design: Laboratory investigation.

Setting: Academic medical center.

Patient(s): Placental tissues discarded after first-trimester terminations were obtained from patients with informed consent.

Intervention(s): A cell line, HTR-8/SVneo, established from first-trimester cytotrophoblast, and villous explants, was treated with or without sildenafil, guanosine 3',5'-cyclic monophosphate (cGMP) analog, cGMP inhibitor, or L-NAME (N^G-nitro-L-arginine methyl ester hydrochloride) and cultured on fibronectin or Matrigel. Integrins $\alpha 6\beta 4$ and $\alpha 1\beta 1$ were detected by immunocytochemistry.

Main Outcome Measure(s): Trophoblast outgrowth from villous tips, cytotrophoblast cell invasion, and integrin immunostaining were assessed in cytotrophoblast and explant cultures.

Result(s): Integrin expression in trophoblast cells ex vivo switched from $\alpha 6$ to $\alpha 1$, and invasiveness increased, when exposed to sildenafil or cGMP agonist. Either cGMP antagonist

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or L-NAME blocked integrin switching and invasion induced by sildenafil. Elevation of nitric oxide pharmacologically induced invasion, but not when cGMP antagonist was present.

Conclusion(s): Sildenafil altered trophoblast phenotype through a process dependent on nitric oxide availability and cGMP accumulation. In addition to its vasoactivity, sildenafil directly stimulates trophoblast extravillous differentiation, which would be favorable for implantation and reduce risk for adverse pregnancy outcomes. (*Fertil Steril*® 2015;103: 1587–95. ©2015 by American Society for Reproductive Medicine.)

Keywords

Trophoblast invasion; phosphodiesterase-5 inhibitor; nitric oxide; guanosine 3',5'-cyclic monophosphate; integrins

Conversion of the endometrium to a receptive state for embryo implantation requires increased vascular permeability, edema, altered membrane fluidity, and programmed epithelial cell death in response to blastocyst adhesion (1). Production of nitric oxide (NO) and guanosine 3',5'-cyclic monophosphate (cGMP) at the blastocyst implantation site prepares the endometrium for receptivity (2) by inducing vasodilation, immune function, and inflammation (3, 4). Nitric oxide increases the production of cGMP, which activates vascular permeability, edema, and uterine receptivity (5). When NO production is prevented in the rat, cGMP synthesis is suppressed, reducing receptivity of the endometrium and inhibiting embryonic development (2). In pregnant rats, inhibition of NO can reduce the cGMP concentration and generate characteristics of pre-eclampsia (6, 7), suggesting an additional role in placentation. The experimental evidence supports a role for NO signaling in mouse and human trophoblast differentiation (8–10) and survival (4).

Sildenafil is a phosphodiesterase type-5 (PDE5) inhibitor that is in a class of drugs used since 1989 for erectile dysfunction (11). By inhibiting PDE5, cGMP accumulates to elevate blood flow through smooth muscle relaxation. Downstream of cGMP, sildenafil activates the protein kinase G pathway (12). Phosphodiesterase type-5 inhibitors can correct endothelial dysfunction, reduce risk for cardiovascular complications, prevent adverse pregnancy outcomes, and restore adequate numbers of circulating progenitor cells (13). Phosphodiesterase type-5 messenger RNA is expressed in the uterus and placenta (14). Interestingly, recent studies have shown that sildenafil can be used successfully to correct a thin endometrium and reduce recurrent implantation failure (15, 16), and therapeutically for intrauterine growth restriction (IUGR) and pre-eclampsia (17, 18). Others have refuted the suggested benefits of sildenafil as a treatment for pre-eclampsia (19, 20), but the intervention might have been applied too late to reverse endothelial damage and placental dysfunction. Recent studies have examined therapeutic interventions for the treatment of pre-eclampsia in a high-risk population, based on dietary supplementation with L-arginine in food bars, and antioxidant vitamins (21). Endothelial dysfunction, which is due largely to overproduction of soluble fms-like tyrosine kinase (sFLT1) and soluble endoglin (sENG) (22), and vasoconstriction, were prevented with L-arginine, correcting the dysregulation of nitric oxide synthase known to occur in pre-eclampsia. L-Arginine, a precursor of NO, can reduce blood pressure to prevent pre-eclampsia by increasing NO bioavailability and activating the cGMP pathway (23). Nitric oxide donors with or without antioxidants could

also suppress other sequelae associated with the disease, including IUGR and preterm delivery (23).

Migrating first-trimester cytotrophoblast cells switch their expression of integrin isoforms as they differentiate in the decidua from a stationary ($\alpha6\beta4$) phenotype to a more invasive ($\alpha1\beta1$) state (24). Integrin switching initiates normal endovascular invasion during placentation (24). Aberrant trophoblast invasion and failed integrin switching are associated with adverse pregnancy outcomes, including pre-eclampsia and IUGR. As first trimester trophoblast cells differentiate to the invasive extravillous phenotype, an extravillous trophoblast-specific major histocompatibility complex class Ib protein, HLA-G, also accumulates (25).

The potential therapeutic benefits of sildenafil include alleviation of perinatal disorders, such as pre-eclampsia, IUGR, abruptio placenta, miscarriage, and implantation failure, which are all linked to poor trophoblast invasion and failure to remodel the uterine spiral arteries (26). Pre-eclampsia and IUGR are associated with disruption of NO and cGMP production (27, 28). Therefore, we hypothesize a link between positive pregnancy outcomes in infertile patients and the direct effect of sildenafil on trophoblast function. The aims of this study were to [1] investigate the ability of sildenafil to induce invasiveness of a human trophoblast cell line and first-trimester villous explant cultures, [2] determine the effect of sildenafil on integrin switching and extravillous differentiation of trophoblast cells, and [3] investigate signaling pathways that mediate the effects of sildenafil on trophoblast function.

MATERIALS AND METHODS

Cell Culture

The HTR-8/SVneo cytotrophoblast cell line was cultured on plastic in T-75 tissue culture flasks (Corning) in Dulbecco's Modified Eagle Medium (DMEM) and Ham's F12 (1:1; DMEM/F12) media (Invitrogen) containing 10% fetal bovine serum in a humidified incubator at 5% CO₂. Culture medium was replaced with serum-free medium 24 hours before all experiments, as previously described (25).

Villous Explant Culture

Placental tissues were obtained with Wayne State University institutional review board approval and patient informed consent. Specimens were isolated from first-trimester terminations at a Michigan Family Planning Facility. Fresh tissue was placed on ice in phosphate-buffered saline (PBS) and immediately transported to the laboratory. The chorionic villi were dissected into individual villi or villous clusters and cultured on Millicell-CM inserts (12-mm diameter, 0.4- μ m pores; Millipore) precoated with 0.2 mL of undiluted Matrigel in a 24-well culture plate for a total of 72 hours (29, 30). The bottom chamber contained 500 μ L DMEM/F12 medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin, and the upper chamber contained approximately 25 μ L of medium. Chorionic villi were added with 150 μ L of medium to the upper chamber, with or without supplementation with 350 ng/mL sildenafil citrate (Sigma), and the culture was continued for 72 hours. The attached villi were fixed in 4% paraformaldehyde, rinsed

three times with PBS, and assessed for outgrowth. Extravillous trophoblast migration was determined for each villous tip from digital images captured using a Leica DM IRB epifluorescence microscope, and a Hamamatsu Orca digital camera. Trophoblast outgrowth length from the villous tip at the point of initial attachment to the distal point of migration was measured using Simple PCI (Hamamatsu) imaging software.

Treatments

Cell or villous explant cultures were treated by supplementing culture medium with 35–3,500 ng/mL (52.5 nM–5.25 μ M) sildenafil citrate with or without 10 μ M N²,2'-O-dibutyl-guanosine 3':5'-cyclic monophosphate (cGMP analog), 10 μ M 8-(4-chlorophenylthio)-guanosine 3',5'-cyclic monophosphorothioate, Rp-Isomer (cGMP antagonist), 10 μ M (\pm)-S-nitroso-N-acetylpenicillamine (SNAP; NO donor), 10 μ M N^G-nitro-L-arginine methyl ester, hydrochloride (L-NAME, NO inhibitor), or the control compound D-NAME (all from Sigma).

Immunocytochemistry

Cytotrophoblasts grown in 96-well plates were fixed in PBS containing 4% paraformaldehyde for 30 minutes and washed three times with PBS. Cells were permeabilized for 10 minutes by incubation in PBS containing 0.1% Triton-X100. Permeabilized cells were labeled with 1 μ g/mL of mouse monoclonal antibodies against Ki67 (DAKO), the integrin subunit α 1, or α 6 (Upstate Biotechnology), and primary antibody controls were 10 μ g/mL nonimmune mouse IgG (Jackson ImmunoResearch), all prepared in antibody diluent (DAKO). After incubation with primary antibodies, cells were rinsed three times with PBS. To visualize and quantify antigen, an Envision System peroxidase anti-mouse kit (DAKO) was used. Staining was imaged using a Leica DM IRB epifluorescence microscope, and images were captured using a Hamamatsu Orca digital camera. Images were processed to determine gray level using Simple PCI (Hamamatsu) imaging software, as previously described (31, 32). Values obtained with IgG controls were subtracted from each sample. We have found, using matched cell samples, that measurement of antibody stain intensity by image analysis increases linearly with measurement of the corresponding antigen by ELISA (32). Moreover, quantitative immunocytochemistry is comparable to Western blotting for demonstration of integrin switching when using highly specific antibodies (Supplemental Fig. 1, available online).

Invasion Assay

Cytotrophoblast cells (100,000 per well) were cultured for 72 hours on Matrigel (Collaborative Research) in 6.5-mm transwell inserts (Corning), as previously reported (25). Cells that penetrated the Matrigel and populated the lower chamber were detached using trypsin–ethylenediaminetetraacetic acid solution, fixed, and counted.

Statistical Analysis

All assays were conducted in triplicate, and all experiments were repeated at least three times. Data were analyzed statistically using SPSS version 21.0 (IBM) and are reported as mean \pm SEM. Independent-sample *t* tests were used to determine significance in explant

outgrowth and integrin switching studies. One-way ANOVA was used to examine the differences among sildenafil concentrations and the efficacy of inhibitors for cGMP and NO pathways. Means were separated using Tukey's method following significant ANOVA ($P < .05$).

RESULTS

Sildenafil Stimulates Trophoblast Outgrowth from Villous Explants

To evaluate the direct effect of sildenafil on human trophoblast cells in first-trimester villous explants, culture medium was supplemented with 0 or 350 ng/mL sildenafil, and trophoblast outgrowth on Matrigel was quantified. All villi exposed to sildenafil produced large outgrowths from their tips, whereas control villi had very small, thinly populated areas of trophoblast outgrowth (Fig. 1A and B). The length of villous tips increased significantly after 72 hours of culture in explants treated with sildenafil (97.2 ± 17.9 vs. 443.2 ± 42.9 ; $P < .05$; Fig. 1G). To determine whether the increase was due to proliferation or migration, we labeled nuclei with 6-diamino-2-phenylindole in trophoblast outgrowths and determined cell number (Fig. 1C and D). Cell number was not significantly different between outgrowth areas of nontreated and sildenafil-treated villous explants (385 ± 50 vs. 444 ± 47 ; $P = .46$). Expression of the nuclear proliferation protein Ki67 was also unaffected by sildenafil treatment (Fig. 1E and F). When the percentage of Ki67-expressing cells in sildenafil-treated and control cells in the explant outgrowths was compared (Fig. 1H), no significant differences were found (14.7 ± 1.6 vs. 15.2 ± 1.8 ; $P = .82$). Trophoblast outgrowth stimulation by sildenafil was apparently due to cell migration away from the villous tip and not proliferation of the migrating cells.

Sildenafil Increases Cell Invasion in Vitro

We examined the sildenafil dose dependency of trophoblast invasion using a Matrigel invasion assay with the human first-trimester cytotrophoblast cell line, HTR-8/SVneo. Sildenafil significantly stimulated ($P < .05$) trophoblast invasion at or above 350 ng/mL (Fig. 2A). Cytotrophoblast cells cultured with 350 or 3,500 ng/mL sildenafil showed a four- to fivefold increase in invasion, as assessed by penetration through Matrigel.

Sildenafil Alters Trophoblast Phenotype

We previously found that HLA-G expression is induced, and integrin expression switches from predominantly $\alpha 6 \beta 4$ to $\alpha 1 \beta 1$, in HTR-8/SVneo cytotrophoblast cells cultured on Matrigel (25). This is similar to cultures of primary cytotrophoblasts as they become highly invasive, and extravillous trophoblast cells at the base of the anchoring villi in utero, which demonstrate a critical switch in integrin expression from $\alpha 6 \beta 4$ to $\alpha 1 \beta 1$ (33, 34). Because sildenafil increased trophoblast outgrowth of first-trimester villous explants and invasion of HTR-8/SVneo cells, we determined whether it also induced integrin switching in the cytotrophoblast cell line, comparable to induction by Matrigel (Supplemental Fig. 1). Cytotrophoblast cells cultured without sildenafil expressed high levels of integrin $\alpha 6$ (29.6 ± 2.38), and very low levels of integrin $\alpha 1$ (3.5 ± 0.75), as shown for vehicle treatment in Figure 2B. Cells treated with 35 ng/mL of sildenafil expressed similar levels of both integrins (17.1 ± 3.32 and 16.46 ± 1.67), indicating partial phenotypic change, whereas cells

treated with 350 (10.4 ± 1.47 and 28.7 ± 2.59) or 3,500 ng/mL (10.9 ± 2.71 and 30.2 ± 6.84) exhibited a clear switch from integrin $\alpha 6$ to integrin $\alpha 1$ (Fig. 2B and C–F).

Sildenafil Operates through the NO and cGMP Signaling Pathways

Sildenafil inhibits PDE5 to block cGMP turnover; however, the accumulation of cGMP depends on the NO activation of guanylyl cyclase for its synthesis. To examine the involvement of these signaling pathways in sildenafil induction of integrin switching, we evaluated the effects of specific inhibitors and agonists. Both the cGMP analog (N2,2'-O-dibutyrylguanosine 3':5'-cyclic monophosphate), and the NO donor SNAP induced integrin switching from $\alpha 6$ to $\alpha 1$, similarly to 350 ng/mL sildenafil (Fig. 3A). Moreover, the specific cGMP inhibitor [8-(4-chlorophenylthio)-guanosine 3',5'-cyclic monophosphorothioate, Rp-Isomer], and the NO antagonist L-NAME each blocked the ability of sildenafil to induce integrin switching in cytotrophoblast cells. As a control for L-NAME, D-NAME did not block the effect of sildenafil. The cGMP inhibitor also blocked integrin switching induced by SNAP, demonstrating that cGMP was the required downstream mediator of NO activity (Fig. 3A). Examples of immunostaining of the $\alpha 6$ and $\alpha 1$ integrin subunits in cells cultured under these various conditions are provided in Supplemental Figure 2.

The NO and cGMP signaling pathways were examined with respect to trophoblast invasion using the Matrigel invasion assay. The approximately fourfold increase in invasion induced by sildenafil ($P < .05$, compared with vehicle) was completely blocked in the presence of either L-NAME or cGMP inhibitor (Fig. 3B). Again, the NO donor SNAP increased invasion above vehicle control, and cGMP inhibitor blocked this effect (Fig. 3B). These findings suggest that sildenafil alters the NO–cGMP signaling axis to convert cytotrophoblasts from a nonmotile, proliferative phenotype to invasive cells required for implantation and proper placentation.

DISCUSSION

This is the first study to demonstrate that sildenafil directly induces trophoblast phenotypic differentiation at the molecular level, indicated by cells switching from $\alpha 6\beta 4$ to $\alpha 1\beta 1$ integrin expression. These changes increased cellular invasive activity in both villous explants and first-trimester cytotrophoblasts. In vitro experiments using the trophoblast cell line demonstrated that both NO and cGMP signaling pathways are critical for integrin switching and stimulation of invasion. The findings strongly support a mechanism (Fig. 3C) in which NO stimulation of guanylyl cyclase activity induces extravillous trophoblast differentiation specifically through downstream cGMP signaling. The potential therapeutic effects of sildenafil arise from its inhibition of PDE5 to block cGMP turnover and produce intracellular cyclic nucleotide accumulation (11). However, NO activation of guanylyl cyclase activity is essential for sildenafil-mediated elevation of cGMP (Fig. 3C). Whereas NO would be the expected initiator of invasive activation of trophoblast cells, cGMP appears to be a requisite downstream mediator of the signal. Potential targets of cGMP that might stimulate trophoblast invasion include protein kinase G, cGMP-gated cation channels, and PDEs (12).

By examining trophoblast cells in the absence of endothelial cells during in vitro culture, evidence was provided that autologous NO production, enhanced by sildenafil, can drive extravillous differentiation. In placental villi, only the syncytiotrophoblast expresses endothelial NO synthase (NOS3), whereas in the decidua, extravillous trophoblast cells also express the enzyme (35). The cellular distribution of NOS3 in cytoplasm is consistent with production of NO for signaling, whereas NOS2 localizes at the front of actively migrating cells, possibly as part of the invasive mechanism (36). Thus, an endogenous NO signaling pathway that regulates extravillous trophoblast differentiation is feasible.

Pre-eclampsia is a complex disorder of pregnancy affecting multiple organ systems that can precipitate serious and deadly complications, including neurologic disorders, liver failure and rupture, pulmonary fluid overload, hemorrhage, stroke, renal complications, and maternal or fetal death (23). It is a major health issue, affecting 2%–8% of all pregnancies, with the ability to cause not only significant maternal morbidity and mortality, but also adverse outcomes in the newborn, including prematurity, IUGR, and fetal death (37). Placentation anomalies are common and central to the manifestations of pre-eclampsia (38). There is a significant paucity of medical interventions for pre-eclampsia and IUGR. Although the exact pathophysiologies are not known, evidence suggests a role for placental abnormalities, including chronic ischemia, vascular endothelial dysfunction, and fibroid necrosis (39). Endothelial dysfunction is found in women with pre-eclampsia and ultimately diminishes NO, as well as cGMP. A detrimental cascade of events can ensue that further decreases trophoblast invasion, leading to inadequate uteroplacental blood flow and growth restriction. At the molecular level, errors in integrin switching accompany a shallow pattern of trophoblast invasion (34) and up to 50% of the invading trophoblast subpopulation in pre-eclamptic placentas is associated with significant programmed cell death (40, 41). Pre-eclamptic patients exhibit high circulating concentrations of sFLT1 and sENG, with a reduction of vascular endothelial growth factor (VEGF) (42, 43). Rapid resolution of clinical symptoms is associated with a reduction in sFLT1 and increase in VEGF.

Sildenafil is a PDE5 inhibitor that potentiates NO downstream signaling by enhancing the accumulation of cGMP through its reduced turnover (23). Nitric oxide is generated in the endothelium, and its production is up-regulated in normal pregnancies. Both NO and cGMP are required for proper placental vasodilation and vascular compliance (44, 45). Improvement in endothelial function has been observed after sildenafil treatment by increasing endothelial progenitor cells, cGMP, growth factors such as VEGF, and matrix metalloproteinase (13, 46, 47). Endothelial progenitor cells develop into mature progenitor cells during repair of endothelial damage and differentiation (48). These cells are involved in the growth, differentiation, remodeling, and repair of vascular endothelial cells throughout the body (49, 50).

Sildenafil has been shown in animal studies to significantly reduce elevated sFLT1 and sENG, increase free VEGF, and reverse oxidative stress (51–53). Sildenafil treatment in pre-eclamptic rat studies has lowered blood pressure, reduced proteinuria, and enhanced placental perfusion (52). Sildenafil has proven to be a palliative remedy in a knockout mouse model of IUGR through a decrease in vascular resistance of the fetal placental unit and increased blood flow in placental ischemia-induced hypertension (54). The use of sildenafil

therapeutically for severe IUGR in the human is under investigation (17). In related studies, we found that sildenafil treatment prevents trophoblast apoptosis during oxidative stress (55). Taken together with the present findings, we suggest that sildenafil can improve trophoblast survival and invasion, which are both deficient in uteroplacental insufficiency.

This study suggests potentially useful strategies for intervening in several major categories of pregnancy complications. Adverse pregnancy outcomes, including pre-eclampsia and IUGR, involve trophoblast dysfunction and vascular endothelial anomalies (56). Hypoxic insults arising from trophoblast and endothelial dysfunction can affect placental growth and blood flow to the fetus, resulting in impairment of fetal well-being (38, 56). With an emerging understanding of the mechanism of sildenafil action in the placenta, enthusiasm for its use in treating obstetric disease can be envisioned. The findings of this study demonstrate an important role for NO activation of cGMP in extravillous differentiation of human trophoblast cells. Clinical investigations to assess the risks and benefits of using this PDE5 inhibitor to impede uteroplacental insufficiency are certainly warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgment:

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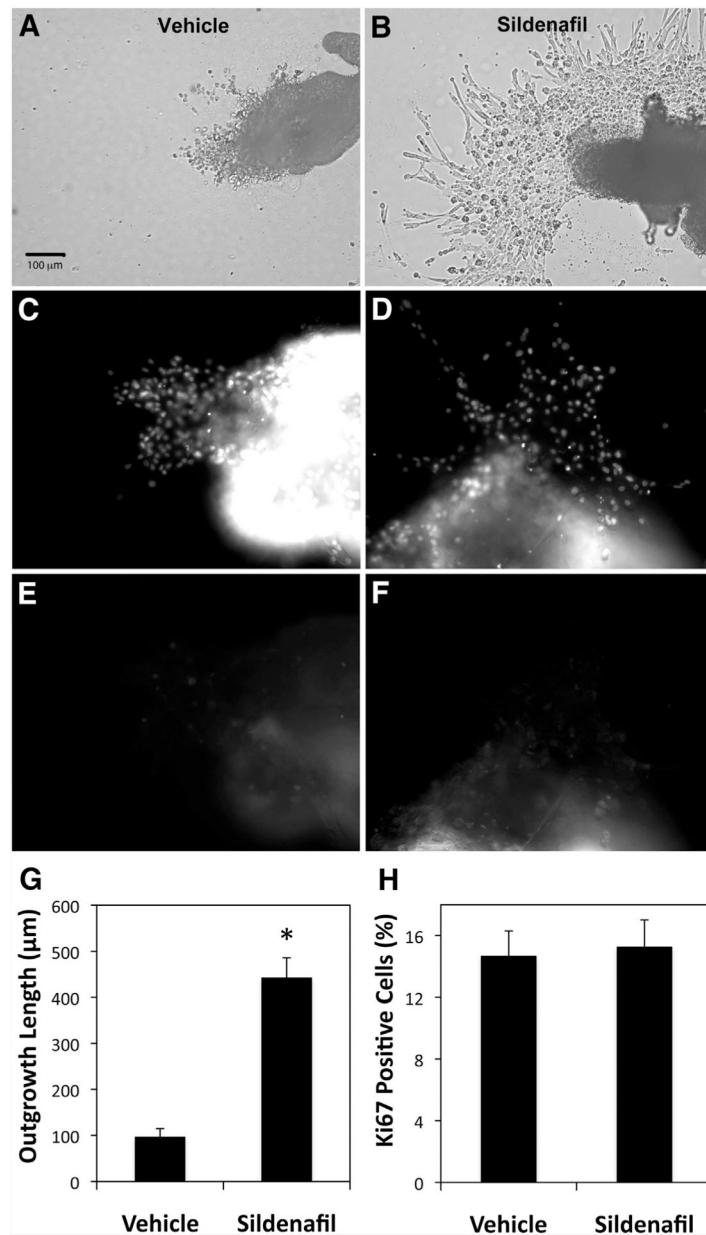
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**FIGURE 1.**

Trophoblast invasion from first-trimester chorionic villous explants. First-trimester villous explants were cultured on Matrigel-coated transwell inserts and treated with vehicle or 350 ng/mL sildenafil for 72 hours before fixation. Examples of outgrowths from villous explants cultured with vehicle (**A**, **C**, **E**) or 350 ng/mL sildenafil (**B**, **E**, **F**) are shown. Bright-field images (**A**, **B**) and paired fields labeled with 6-diamino-2-phenylindole (**C**, **D**) or anti-Ki67 (**E**, **F**) are included. Size bar in (**A**) applies to all image panels. Outgrowth length and cell proliferation were measured using Simple PCI image analysis software (**G**, **H**). The graph in (**G**) shows outgrowth lengths for triplicate experiments in which 10 villous explants were measured for each treatment. * $P < .05$ vs. vehicle. The graph in (**H**) shows the percentage of cell nuclei in the outgrowth regions that were labeled with anti-Ki67 antibody. The

differences between vehicle- and sildenafil-treated explants were not significant ($n = 8$).
Error bars represent SEM.

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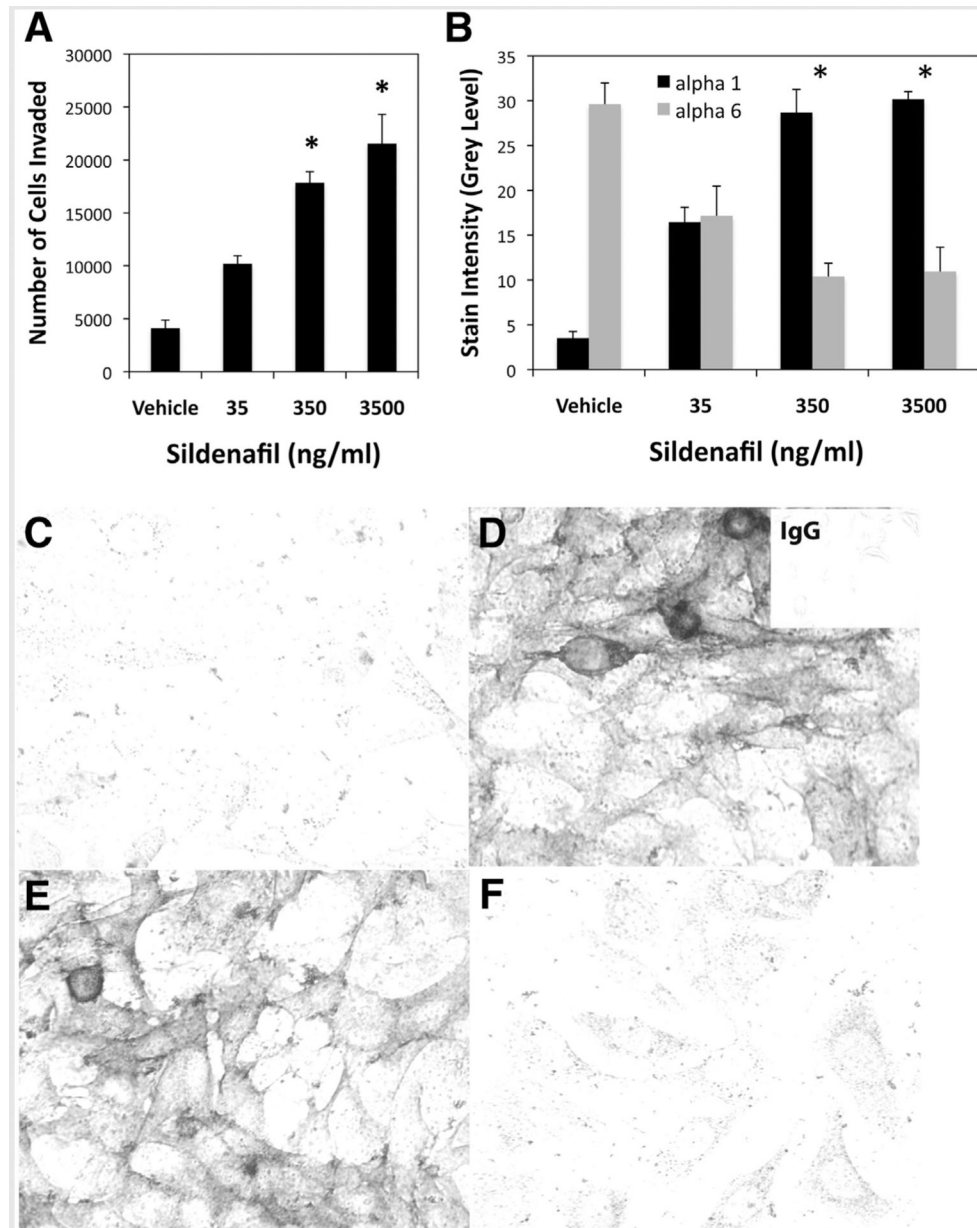


FIGURE 2. Extravillous trophoblast differentiation and invasion. (A) HTR-8/SVneo cytotrophoblast cells were treated with vehicle or 35–3,500 ng/mL sildenafil for 72 hours during culture on Matrigel-coated transwell inserts. Cells that invaded through the Matrigel were trypsinized from the bottom of the insert, fixed, and counted. (B) Cytotrophoblast cells treated for 24 hours with vehicle or 35–3,500 ng/mL sildenafil were assessed for $\alpha 1$ or $\alpha 6$ integrin subunits by immunocytochemistry and quantitative image analysis. Untreated cells expressed predominantly $\alpha 6$, whereas cells exposed to 35 ng/mL sildenafil showed equivalent $\alpha 1$ and $\alpha 6$ expression. Cells exposed to 350 and 3,500 ng/mL sildenafil demonstrated integrin switching from $\alpha 6$ to $\alpha 1$. * $P < .05$ vs. vehicle. (C–F) Examples of immunocytochemical staining of $\alpha 1$ (C, E) and $\alpha 6$ (D, F) in confluent cells cultured

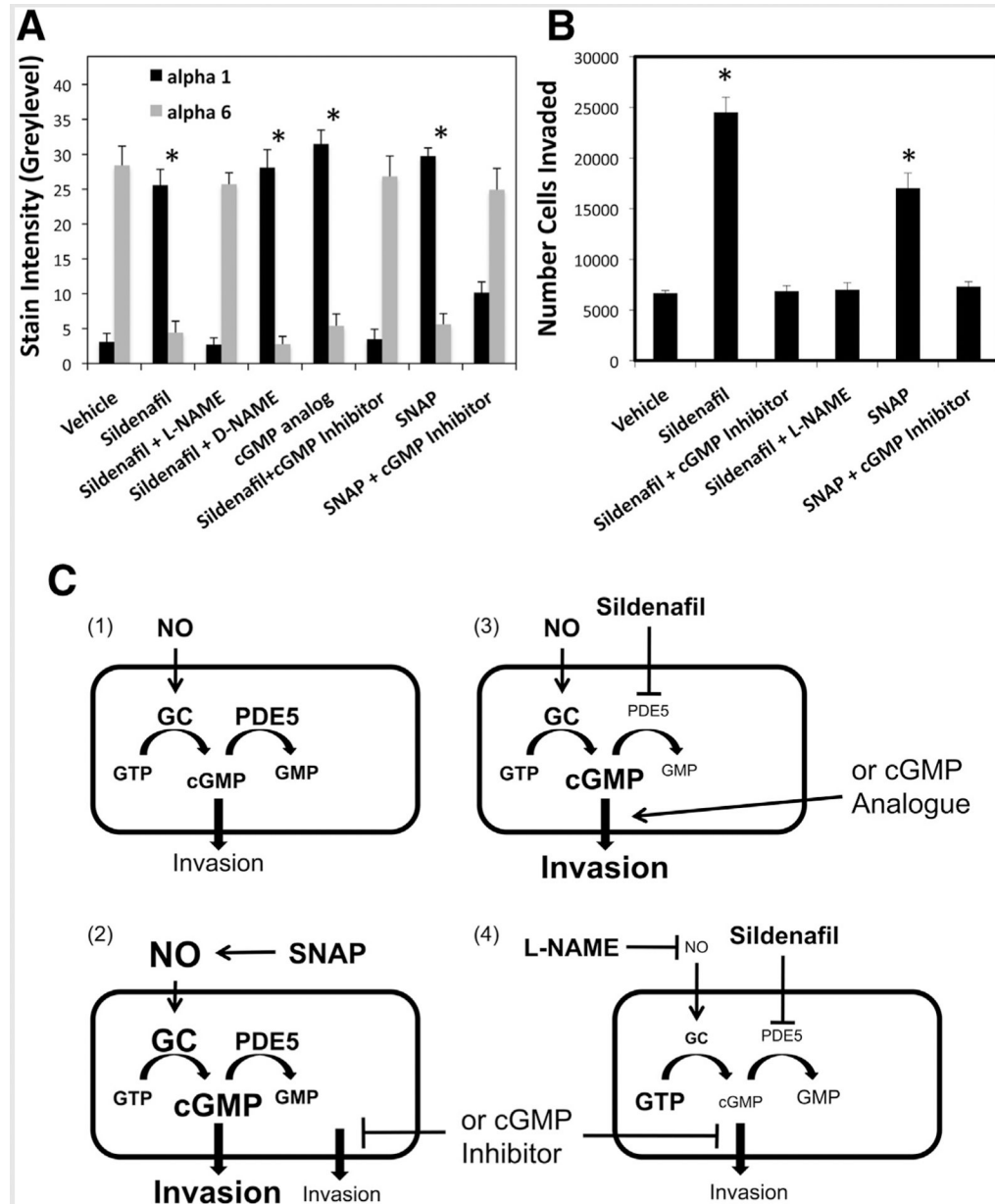
with vehicle (**C, D**) or 350 ng/mL sildenafil (**E, F**). Negative control cells labeled with non-immune IgG are shown in the inset in the upper right corner of (**D**).

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**FIGURE 3.**

Sildenafil induces trophoblast differentiation through the cGMP and NO signaling pathways.

(A) Cytotrophoblast cells were treated during culture for 24 hours with vehicle, 350 ng/mL sildenafil, SNAP, cGMP analogue, or the indicated combinations of sildenafil with L-NAME, D-NAME, or cGMP inhibitor. Expression of the $\alpha 1$ and $\alpha 6$ integrin subunits was determined as in Figure 2B. (B) Invasion was determined as in Figure 2A, for cytotrophoblast cells treated with vehicle, 350 ng/mL sildenafil, or the indicated combinations of sildenafil with cGMP inhibitor or L-NAME. * $P < .05$ vs. vehicle.

(C) Summary of trophoblast invasion mechanisms suggested by the results, assuming the following. [1] Nitric oxide activates guanylyl cyclase (GC) to induce extravillous differentiation, and that PDE5 modulates cGMP levels through conversion to GMP. [2]

production of NO by SNAP increases invasion, owing to resulting GC activation and production of cGMP. Inhibition of SNAP-induced invasion by cGMP inhibitor confirms that NO increases invasion specifically through cGMP. [3] Sildenafil inhibition of PGE5 prevents degradation of cGMP, which accumulates to increase invasion, similarly to direct elevation of cGMP signaling by the cGMP analogue. [4] Sildenafil-mediated elevation of cGMP requires continuous NO stimulation of GC and invasion is, thus, repressed by L-NAME inhibition of NO, similarly to inhibition of cGMP signaling by the cGMP inhibitor. *Arrows* indicate activation, and *bars* indicate inhibition.

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