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Patrick Fafet, Cosette Rebouissou, Thierry Maudelonde, Marie-Luce Vignais. Opposite Effects of Transforming Growth Factor- β Activation and Rho-Associated Kinase Inhibition on Human Trophoblast Migration in a Reconstituted Placental-Endometrial Coculture System. *Endocrinology*, 2008, 149 (9), pp.4475 - 4485. 10.1210/en.2008-0253 . inserm-01855902

HAL Id: inserm-01855902

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Submitted on 8 Aug 2018

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Opposite Effects of Transforming Growth Factor- β Activation and Rho-Associated Kinase Inhibition on Human Trophoblast Migration in a Reconstituted Placental-Endometrial Coculture System

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Placental implantation involves highly regulated trophoblast invasion of the endometrial stroma. TGF β is a known regulator of this process. This study examines the effect of TGF β on extravillous cytotrophoblastic cell (EVCT) migration in cocultures of first-trimester human chorionic villus explants and primary human endometrial fibroblasts. Migration of EVCTs was followed by phase-contrast time-lapse microscopy and was shown to highly depend on the endometrial fibroblast matrix. Interstitial EVCT invasion was also analyzed by confocal microscopy of fluorescently prelabeled trophoblasts and endometrial fibroblasts. As expected, addition of TGF β led to inhibition of EVCT invasion of the endometrial cell layer. This inhibition was characterized by

formation of compact EVCT stacks at migration fronts and displacement of endometrial fibroblasts. We tested the role of the RhoA/Rho-associated kinase (ROCK) pathway, a TGF β -dependent pathway known to regulate cell migration. Interestingly, blocking ROCK with the chemical inhibitor Y27632 had an effect opposite to TGF β activation because it promoted superficial EVCT migration on the endometrial cell layer. These data suggest a role for ROCK in the TGF β -dependent control of trophoblast migration. Furthermore, they indicate that even though ROCK signaling plays a role in human trophoblast cell invasion, EVCT migration can still occur in the absence of ROCK activity. (*Endocrinology* 149: 4475–4485, 2008)

PLACENTAL IMPLANTATION during pregnancy gives rise to naturally occurring cell invasion. In humans, trophoblast cells produced from the trophoblast of the blastocyst are responsible for the invasion of the entire endometrium and a fraction of the myometrium in the so-called interstitial invasion (1). During placental implantation a fraction of the chorionic villi, called the anchoring villi, develop and produce multilayered columns of nonpolarized extravillous cytotrophoblastic cells (EVCTs). These cells further differentiate into highly invasive EVCTs that migrate as individual cells and are involved in the interstitial invasion of the endometrial decidua and myometrium (2).

Trophoblast and cancer cells share striking similarities in their migratory and invasive capacities, which suggests the use of common molecular mechanisms. Cancer cells are known to actively interact with the surrounding stroma that can either act as a powerful tumor suppressor or, alternatively, help promote tumor growth and invasion (3, 4). Similarly, successful trophoblast invasion and blastocyst implantation depend on widespread metabolic and endocrine changes of the endometrium, a process that is known as

endometrial decidualization and that is under the control of the trophoblast itself. Decidualized endometrial fibroblasts express a wide array of cytokines and growth factors such as prolactin and IGF binding protein (IGFBP)-1 that are established markers of decidualization and can regulate EVCT migration (5, 6). *In vitro*, decidualization of endometrial fibroblasts can be achieved by the combined action of progesterone and cAMP (7). It can also be induced *ex vivo* by the interaction with first-trimester trophoblast explants as shown recently by Popovici *et al.* (8).

Apart from their marked similarities, migration and tissue invasion by trophoblast and cancer cells also bear major differences. Most notably, contrary to malignant cells, extravillous cytotrophoblastic cells display a controlled invasive capacity that is limited in both space and time. Loss of this control can lead to pathological conditions such as development of hydatidiform moles or choriocarcinomas. This indicates that, despite comparable invasive characteristics, trophoblast and cancer cell behaviors rely on clearly distinct regulatory circuits.

TGF β is a pluripotent cytokine that plays key roles in numerous cellular processes (9). Functions such as proliferation or differentiation can be regulated positively or negatively, depending on cell type and context (10, 11). Because of its negative effect on epithelial cell proliferation, TGF β signaling was proposed as a tumor suppressor pathway. However, TGF β overexpression by cancer cells, loss of TGF β -dependent control of proliferation in these cells and

First Published Online May 22, 2008

Abbreviations: EVCT, Extravillous cytotrophoblastic cell; FBS, fetal bovine serum; HLA, human leukocyte antigen; IGFBP, IGF binding protein; PRL, prolactin; ROCK, Rho-associated kinase.

Endocrinology is published monthly by The Endocrine Society (<http://www.endo-society.org>), the foremost professional society serving the endocrine community.

otherwise TGF β -induced metastatic invasiveness now make TGF β signaling a target for cancer therapy (12).

TGF β is closely associated with tissue remodeling in the physiological processes of embryonic development and placental implantation. It also regulates invasion of the endometrial decidua by trophoblast (5, 13–18). TGF β and its receptors are expressed by both the placenta and endometrium (14, 19–22). After degradation of the endometrial epithelium and in sharp contrast to its effect on cancer cells, TGF β acts as a major repressor of cytotrophoblast invasion and migration (5, 13, 18). Interestingly, this TGF β -dependent inhibition of trophoblast invasion is lost in malignant choriocarcinomas, such as JARs or JEGs (23).

Various signaling pathways mediate the biological effects of TGF β . Among these, the pathway involving the small GTPase RhoA and its downstream effector Rho-associated kinase (ROCK) has been associated with cell migration regulation (24, 25). ROCK is reported to increase the invasive capacity of tumor cells, a capacity blocked by the specific Y27632 inhibitor (26, 27). Likewise, a function for the RhoA-ROCK signaling cascade was also proposed for the regulation of extravillous cytotrophoblast migration (28–30).

Several model systems, which rely on different sources of trophoblast and matrix, have been used to study trophoblast migration and invasion *ex vivo*. A number of trophoblast cell lines derived from choriocarcinomas or artificially immortalized from normal placentas have been described and proven powerful research tools (31). However, because of the diverse origins and means of immortalization of these cell lines, their characteristics can widely vary as recently shown by comparative microarray analysis of the BeWo and JEG3 cell lines (32). Alternatively, trophoblast cells or chorionic explants freshly isolated from first-trimester or term placentas can be used, the explants offering the valuable advantage of maintaining an intact villus structure most likely capable of secreting diffusible regulatory factors (17, 33–35). Artificial matrices like collagen or Matrigel have been used to support trophoblast migration (17, 35, 36). However, a coculture of the tissues that interact *in vivo* is more likely to reproduce naturally occurring interactions as it was recently shown for the characterization of trophoblastic endovascular invasion (37). To study trophoblastic interstitial invasion, cocultures between endometrial tissues and placental villous explants or between cells isolated from these tissues have been used (33, 34). Recently gene expression profiling of human endometrial stromal cells grown in coculture with first-trimester trophoblast explants showed the impact of trophoblast on endometrial gene expression therefore validating this experimental approach (8).

Whereas there is strong evidence that TGF β regulates trophoblast invasion, little is known about the signaling pathways involved. It is also unclear how this regulation takes place and what mechanisms are involved. The aim of the present investigation was to characterize the effect of TGF β on the regulation of interstitial migration/invasion of EVCTs and investigate the role of the RhoA/ROCK pathway. To address these questions, we set up an *in vitro* coculture system between human trophoblastic villi and primary endometrial fibroblasts. This system enables trophoblast-dependent decidualization of the endometrium and therefore

recapitulates some of the interactions taking place *in vivo* between these two tissues. We characterized the migration of extravillous trophoblast cells and confirmed the inhibitory effect of TGF β on trophoblast migration. Furthermore, we observed that inhibition of ROCK by Y27632 treatment induced an effect opposite to that of TGF β and unexpectedly promoted two-dimensional migration of trophoblast cells.

Materials and Methods

Patients and samples

Endometrial tissues were obtained from women undergoing cesarean sections at the Hospital Arnaud de Villeneuve (Montpellier, France). They were at term for a normal pregnancy. Trophoblast villi were isolated from placentas (n = 20) obtained from legal early pregnancy terminations of uncomplicated, unwanted pregnancies. Abortions were induced by mifepristone. All samples were obtained at 4–7 wk gestational age. Pregnancy was dated by echography and measurement of both the crown-rump length and mean gestational sac diameter. Average mother age was 27 \pm 8 yr. All samples were obtained after informed written consent of the patients and the protocol was approved by the local ethical committee.

Endometrial fibroblast isolation and *in vitro* decidualization

Endometrial samples were obtained from normal pregnant women (n = 4) undergoing cesarean section at term. Two-millimeter-long explants were mechanically chopped from fresh endometrial tissue and seeded on 6-cm dishes in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS). These endometrial fibroblasts had a doubling time of roughly 24 h and entered senescence after about 50 d in culture. They were passaged twice a week and used before passage 6. For *in vitro* decidualization, endometrial fibroblasts were plated in FBS 10% at a concentration of 10⁵ cells per 4-cm dish. When confluent, they were cultured in the presence of medroxyprogesterone acetate (1 μ M) and 8-bromoadenosine-cAMP (0.5 mM; Sigma, Saint-Quentin Fallavier, France) for 4 d.

Establishment of cocultures

After uterine evacuation, placentas were immediately collected in DMEM supplemented with heat-inactivated FBS 10%, penicillin (200 U/ml), streptomycin (200 μ g/ml), fluconazole (4 μ g/ml; Pfizer, Sandwich, UK), and amphotericin B (20 μ g/ml; Bristol-Myers Squibb, Princeton, NJ; DMEM). They were processed for dissection within the next 2 h. Meanwhile, they were kept at room temperature and protected from light. Before dissection, placentas were gently washed with warm PBS three times or more to eliminate blood. Placentas were then transferred for 20–30 min in the above-mentioned DMEM. Placental villousities were dissected with sterile tweezers and scissors to get tissue pieces about 1 mm long. Dissociation of villousities in small pieces was done by pipetting them up and down with a 10-ml pipette. Villousities were then transferred to a 50-ml Falcon tube and let to decant. They were rinsed once and resuspended in a medium composed of 50% of the above-mentioned DMEM and 50% of Ham F12 medium supplemented with CaCl₂ (2 mM), MgSO₄ (2 mM), and NaHCO₃ (0.5 mM; DMEM/Ham medium).

Endometrial fibroblasts were seeded on glass coverslips (10⁵ cells in 4 cm diameter dishes) in DMEM supplemented with FBS 10% 3 d before the coculture so that fibroblasts were confluent by the time coculture was started. Before setting up the coculture, the fibroblast medium was changed to DMEM/Ham medium. Approximately 10 trophoblast explants were seeded per 4-cm dish and let to adhere for 16 h at 37 C. At that point, the medium was aspirated, and plates were rinsed once with the DMEM/Ham medium to eliminate all unattached villousities and further incubated in the same medium. This was considered to be time zero for all measures of trophoblast migration and for further treatment.

Treatments

TGF β 1 (R&D Systems, Minneapolis, MN) was used at a concentration of 10 ng/ml. The pharmacological inhibitor Y-27632 (Alexis Biochemi-

als, Lausen, Switzerland) was used at a final concentration of 5–10 μ M. The pan anti-TGF β antibody is a monoclonal murine antibody (clone 1D11; R&D Systems) used at a concentration of 4 μ g/ml. Treatments were done at 37 C from 8 to 24 h as indicated.

Immunofluorescence

Cells were fixed with paraformaldehyde (4%) for 10 min and permeabilized with Triton (0.1%, 2 min). Alternatively, for IGFBP1 and prolactin (PRL) labeling, cells were permeabilized with ethanol (EtOH 70%, 30 min at 4 C). After three PBS washes, saturation was performed with FBS 10% for 30 min at room temperature. Cells were incubated for 1 h at room temperature with the primary antibodies in 150 μ l PBS/BSA 1%. The antibodies used were the following: mouse antihuman cytokeratin 7 (1:400, clone OV-TL 12/30; DakoCytomation, Glostrup, Denmark), mouse antivimentin cy3 conjugate (clone V9, 1:2000; Sigma), mouse anti-human leukocyte antigen (HLA)-G (clone 4H84, 1:100, Exbio, Vestec, Czech Republic), rabbit anti-PRL (1:400) and anti-IGFBP1 (1:200; Upstate Biotechnology, Lake Placid, NY). Immunofluorescence was monitored by incubation at room temperature for 1 h with either Cy-3 or fluorescein isothiocyanate-conjugated antirat, antimouse, or antirabbit IgG. Hoechst 33342 (Molecular Probes, Leiden, The Netherlands) was used for nuclei staining. After three additional PBS washes, slides were mounted with ProLong Gold antifade reagent (Molecular Probes). For vital dye labeling, the green 5-chloromethyl-fluorescein diacetate and red CMTPX CellTracker probes (Molecular Probes) were used. Briefly, confluent endometrial fibroblasts were incubated for 30 min at room temperature in DMEM supplemented with FBS 10% and the green 5-chloromethyl-fluorescein diacetate (4 μ M). The 1-mm-long trophoblastic villi were incubated for 45 min at room temperature in DMEM supplemented with FBS 10% and the red CMTPX (10 μ M). Cells and villousities were then independently rinsed twice with FBS 10% and further incubated with FBS 10% at 37 C for 30 min. They were rinsed twice more with FBS 10% and put in the DMEM/Ham medium (see above). Cocultures were then set up between green-stained fibroblasts and red-stained villi. The reverse combination of CellTracker probes for, respectively, the endometrial fibroblasts and the trophoblastic villi was also used.

Imaging

Trophoblast cell invasion was followed by time lapse with a phase-contrast DMIR microscope (Leica, Heidelberg, Germany) in an incubation chamber providing controlled temperature, CO₂ concentration, and hygrometry. Pictures were taken every 2 min during 24–48 h using a ORCA 100 and the HPDCPx32 program (Hamamatsu, Bridgewater, NJ). After imaging, all time points were compiled and exported as a Quick-time (avi) file using the MetaMorph software (Universal Imaging Corp., West Chester, PA). Alternatively, images were taken with a Leica DMIRE 2 2002 microscope and a MicroMax 1300 Y/HS (1999) camera (RS-Princeton Instruments, Trenton, NJ) with the MetaMorph 7 acquisition program. Image analysis and measurement of migration velocities was performed with the MetaMorph and Excel (Microsoft Corp., Redmond, WA) softwares. Individual cell tracking was done with the Flash MX 2004 (Macromedia, San Francisco, CA) software. Immunofluorescence and CellTracker stainings were visualized with a LSM 510 Meta confocal laser system (Carl Zeiss, Jena, Germany). Images were converted with MetaMorph and processed with the Adobe Photoshop software (Adobe Systems Inc., San Jose, CA).

Statistical analysis of phase-contrast images

For analysis of EVCT migration, two-way ANOVA was used to compare the effects of the culture treatments (TGF β 1, Y27632, or control) and the different placental origins. Unpaired Student's *t* tests with Welch correction were performed to compare villi distributions for the different experimental conditions. Data on lacune formation after TGF β 1, Y27632, or control treatments were compared as nonnormal distributions using the Mann-Whitney rank sum test. Statistical analysis was done with the Prism software (GraphPad Inc., San Diego, CA) (*, *P* < 0.05; **, *P* < 0.01; ns, not significant).

Results

Reconstitution of the trophoblastic invasion of the endometrium

For setting up our *ex vivo* coculture system involving placental explants and endometrial fibroblasts, we used placentas from early elective pregnancy terminations (4–7 wk gestation). Within a couple of hours of placenta expulsion, trophoblast villi were trimmed off the placentas, cut into 1- to 2-mm pieces and seeded on top of confluent layers of primary endometrial fibroblasts. The human primary endometrial fibroblasts were isolated independently from endometrial tissues removed during cesarian section. They doubled every 24 h and entered senescence after about 50 generations (data not shown). They were used before passage 6. For setting up cocultures of placental explants and fibroblasts, trophoblast villi were let to adhere to the fibroblast layer for 12–16 h at 37 C. At that time, culture dishes were gently washed with culture medium to get rid of any unbound placental material and cultures were submitted to various treatments (see following text) or left untreated (Figs. 1 and 2). Production of trophoblast cells by the villi and migration of these EVCTs away from the villi, onto the fibroblast layer, were followed by phase-contrast time-lapse analysis (Fig. 1). Important changes in trophoblast cell shapes were observed, suggesting an adaptation of the EVCTs to the fibroblast extracellular matrix and their ability to squeeze through narrow spaces. Trajectories of isolated trophoblastic cells were tracked and confirmed that: 1) cells migrate away from the villosity, 2) EVCT trajectories are not strictly straight and show a strong dependence on fibroblast orientation patterns, and 3) consequently, EVCT migration velocities for a given trophoblastic cell also depend on the fibroblast cell layer pattern, ranging in this case from 10 to 40 μ m/h (see trajectories I, II, and III in Fig. 1B).

To follow EVCT migration over longer distances, we performed phase-contrast time-lapse acquisition of contiguous fields followed by image reconstruction over a 35-h period (see reconstituted 5 \times 5 contiguous fields in Fig. 2). Velocities of trophoblast cell migration fronts were determined (see migration directions I, II, and III in Fig. 2B). As for individual trophoblast cells, the measured velocities showed a clear dependence on fibroblast orientation patterns with a faster EVCT migration rate along the longer fibroblast axis. From a total number of six placentas analyzed, and 30 EVCT speed values determined, we found that EVCT migration velocities in our coculture system ranged from 11 to 46 μ m/h, with an average EVCT migration velocity of 23 μ m/h (data not shown).

Trophoblast-induced decidualization of the endometrial fibroblasts

The endometrial fibroblasts were characterized by vimentin expression and their cAMP and progesterone-induced decidualization, as shown by the expression of specific markers of decidualization. Indeed, expression of PRL and IGFBP1 was detected under these decidualizing conditions but not without treatment (control conditions) (Fig. 3A).

To assess how representative of *in vivo* conditions our model culture system was, we investigated whether endo-

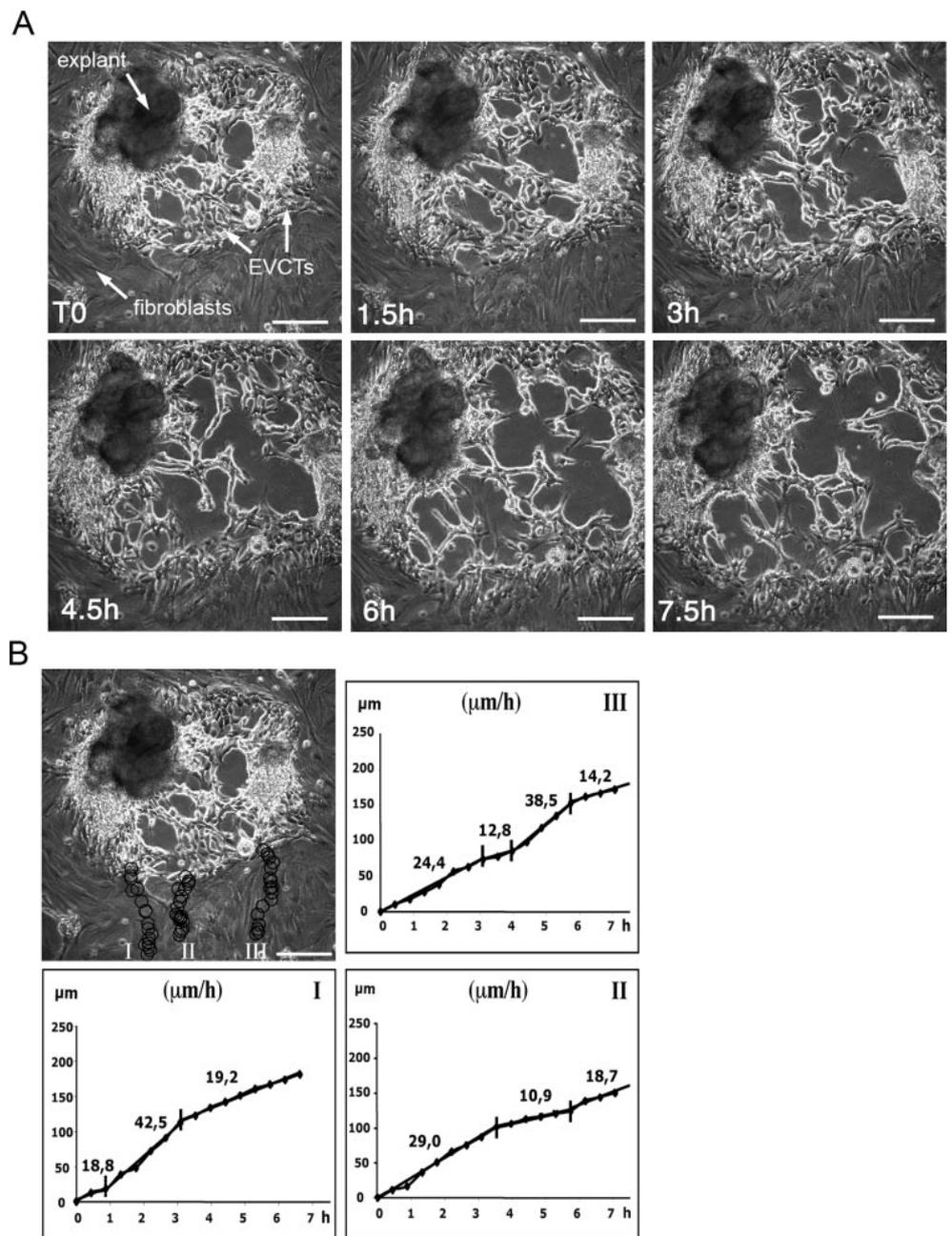


FIG. 1. Invasion of endometrial fibroblasts by trophoblastic cells produced by placental explants. **A**, Time-lapse analysis of the migration of cytotrophoblast cells on confluent layers of endometrial fibroblasts. Frames corresponding to time-lapse phase-contrast images show EVCT migration at the indicated times. *Scale bar*, 100 μm . **B**, Trajectories and kinetics of three independent EVCTs within the endometrial fibroblast layer. Kinetics curves shown in panels I, II, and III refer to the corresponding trajectories in the phase-contrast micrograph.

metrial decidualization, known to take place *in vivo* in response to trophoblast invasion, was also taking place in our coculture system. We detected expression of PRL in endometrial fibroblasts cocultured with trophoblastic villi (Fig. 3B). Similar data were obtained in independent experiments using two distinct placentas. This indicated that endometrial fibroblasts in culture with trophoblastic villi could undergo decidualization, without addition of decidualization-inducing reagents such as cAMP or progesterone (see above). These data showed that an effective interaction between the two cell types, respectively, the trophoblastic epithelial cells and the endometrial fibroblasts, was taking place in these coculture experimental settings, at least partly reproducing interactions occurring *in vivo* between the placenta and the endometrium.

Analysis of the EVCT invasion of the endometrial stromal cells

To track the two cell types interacting in the coculture system, the trophoblastic villi and endometrial fibroblasts were labeled, respectively, with red and green CellTracker dyes before setting up the coculture. Attachment of the trophoblastic villi to the endometrial fibroblast layer was let to occur for 16 h at which time phase-contrast pictures of the coculture were taken. Another set of phase-contrast pictures was taken 24 h later (Fig. 4, A–C). Cultures were fixed, labeled with Hoechst, and analyzed by confocal laser-scanning microscopy. Trophoblastic villi as well as the EVCTs produced by the stained trophoblast explants showed incorporation of the red CellTracker. This allowed

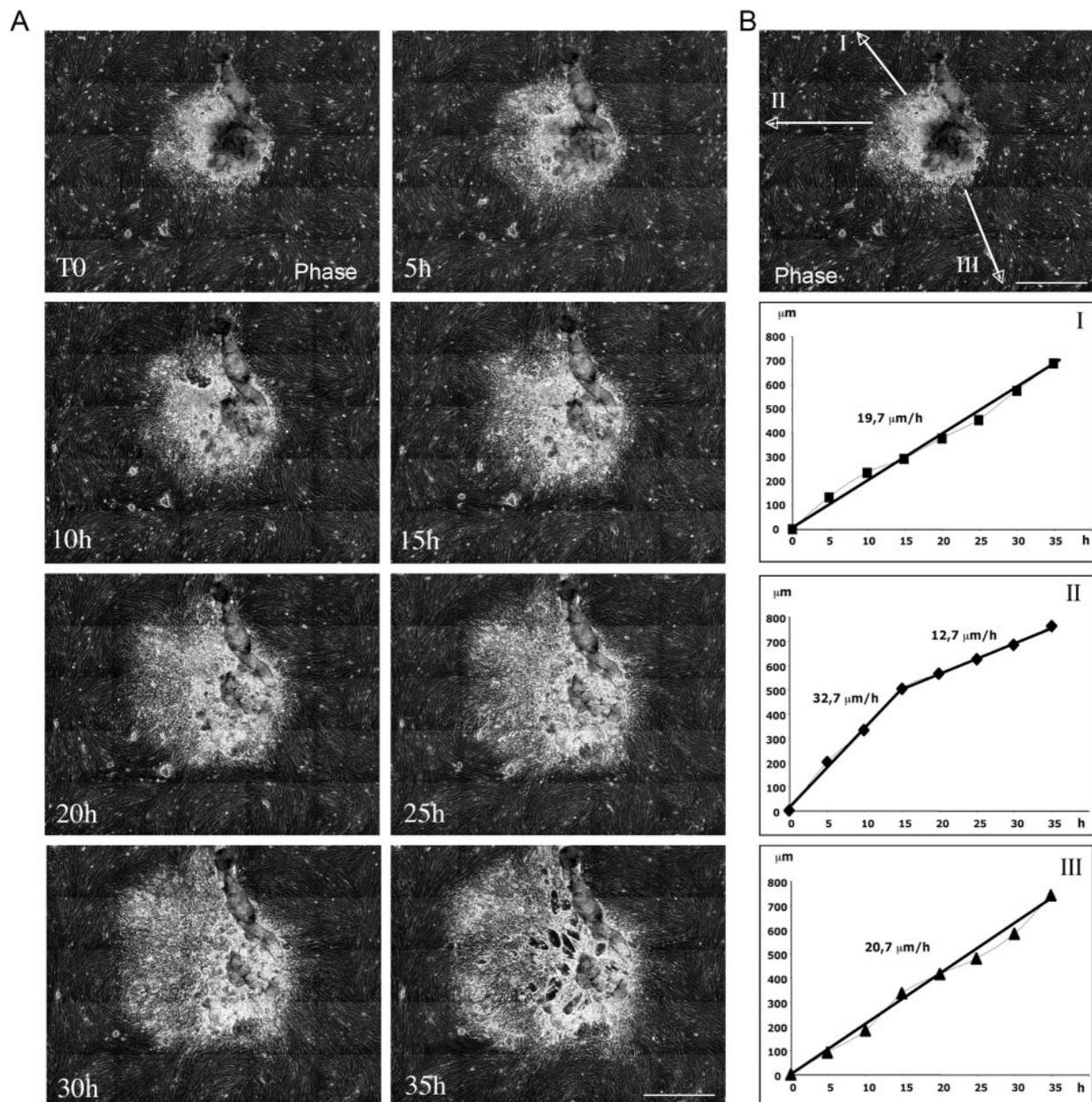


FIG. 2. Invasion of EVCTs on endometrial stromal cells. Kinetics of migration are shown. A, The production and migration of extravillous trophoblastic cells was followed by time-lapse phase-contrast microscopy with camera-scanned fields. The phase-contrast micrographs show image reconstitution from the 5×5 contiguous fields (total of 25 fields) from the time-lapse images taken at the indicated times. Scale bar, 1 mm. B, The migrating kinetics (shown in panels I, II, and III) were determined for the three corresponding EVCT migrating fronts whose trajectories are indicated in the phase-contrast micrograph. Scale bar, 1 mm.

their clear distinction from the green CellTracker-stained endometrial fibroblasts and enabled the visualization of the trophoblastic interstitial invasion of endometrial fibroblasts (Fig. 4, D–H). The reverse CellTracker incorporation combination (*i.e.* green CellTracker-stained villi and red CellTracker-stained endometrial fibroblasts) was also tested showing similar EVCT invasion patterns (data not shown). To confirm the specificity of the CellTrackers, we labeled the trophoblastic villi with the green CellTracker and immunostained the cocultures for either cytokeratin 7, HLA-G, or vimentin. As expected, the cytokeratin 7 and HLA-G labeling was found to fully match with the vital

dye stainings (Fig. S1, A and B, published as supplemental data on The Endocrine Society's Journals Online web site at <http://endo.endojournals.org>), whereas, on the other hand, vimentin and CellTracker stainings were exclusive of one another (Fig. S1, C). These observations showed the specificity of the CellTracker dye and its suitability for identifying trophoblastic cells amid fibroblasts. The reverse experiment, involving prestained endometrial fibroblasts, was also performed and likewise showed optimal correlation with fibroblast markers (data not shown). Altogether these observations fully validated the use of CellTrackers for identifying, respectively, extravillous cytotrophoblasts and endometrial fibroblasts.

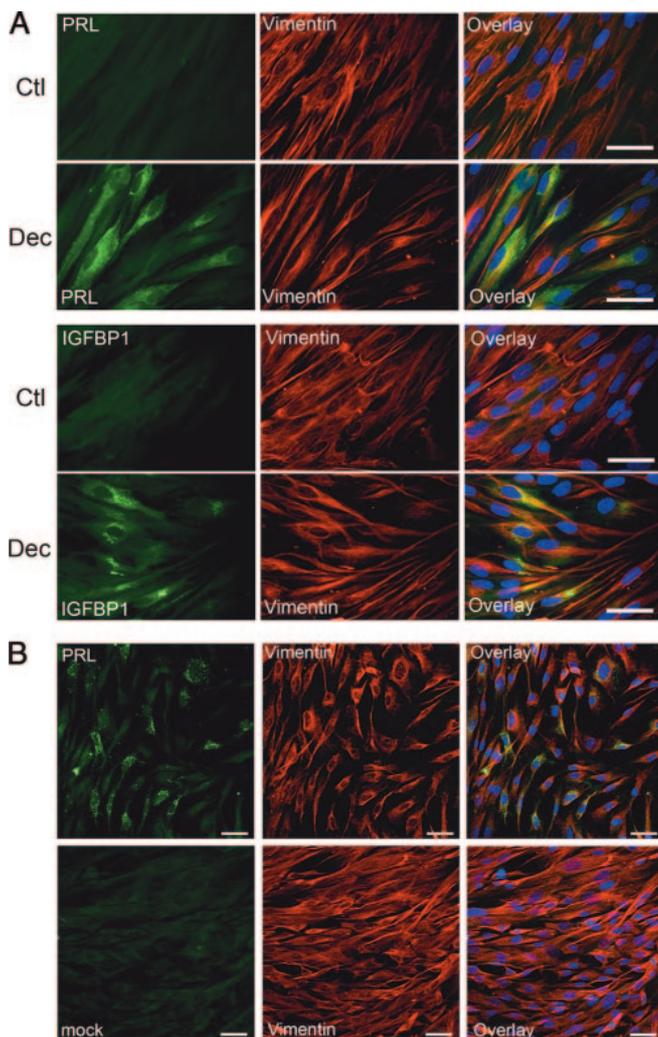


FIG. 3. Decidualization of endometrial fibroblasts. **A**, *In vitro*-induced decidualization of endometrial primary fibroblasts. Endometrial fibroblasts were treated with medroxyprogesterone acetate (1 μ M) and 8-bromoadenosine-cAMP (0.5 mM) for 4 d. Cells were fixed and stained for PRL and IGFBP1. Fibroblasts were counterstained with vimentin and Hoechst (triple staining in the *overlay panels*). Scale bar, 50 μ m. Ctl, Control; Dec, decidualization conditions. **B**, Trophoblast-induced decidualization of endometrial fibroblasts. Endometrial fibroblasts were cocultured with placental explants for 48 h. Cell cultures were then fixed and stained for PRL, vimentin, and Hoechst (triple staining in the *overlay panels*). The mock labeling was done in the absence of the PRL antibody. Scale bar, 50 μ m.

Regulation of trophoblastic migration/invasion by TGF β : role of the RhoA-ROCK pathway

A role for TGF β in controlling trophoblastic invasion has been documented in various experimental settings. We first investigated whether our *in vitro* coculture system was amenable to reproduce this effect. The trophoblastic explants and endometrial fibroblasts were respectively labeled with the red and green CellTrackers and trophoblastic invasion was let to take place for about 16 h. At that time, TGF β 1 was added for 24 h, and effects of this cytokine were analyzed by both phase-contrast and confocal immunofluorescence microscopy (Fig. 4, *bottom panel*). Two major TGF β 1-related effects were observed: 1) blockade of EVCT migration within

the fibroblast layer, resulting in the formation of stacks of EVCTs and 2) formation of large lacunes devoid of both EVCTs and endometrial fibroblasts. Similar effects were observed for the TGF β 2 and TGF β 3 isoforms (data not shown). Because both EVCTs and endometrial fibroblasts constitutively express TGF β (see above and our own unpublished RT-PCR data), we tested the effect on EVCT migration of anti-TGF β antibodies that prevent binding of all three TGF β isoforms to the receptor.

We also tested the effect of an inhibition of the Rho-ROCK signaling pathway, using the Y27632 inhibitor. Quite interestingly, both treatments induced an enhanced superficial trophoblastic migration that appeared to occur at the expense of interstitial invasion of the fibroblast layer. Indeed, extravillous cytotrophoblast migration exhibited a lesser dependence on the fibroblast orientation patterns as EVCTs were also able to migrate across transversally oriented endometrial fibroblasts (Fig. 5A, *bottom panels*). Y27632-dependent inhibition of ROCK was linked to an elongated EVCT cell morphology, as shown by cytokeratin 7 staining (Fig. 5B). In addition, confocal analysis of cocultures of prestained EVCTs and endometrial fibroblasts showed that in presence of Y27632, migration of EVCTs had a lesser impact on the endometrial fibroblast layer integrity, compared with control conditions or TGF β 1-treated cultures (Fig. 5C). Whereas TGF β 1 treatment led to the disappearance of endometrial fibroblasts after passage of EVCTs, in Y27632 conditions, the cell morphology of the endometrial fibroblasts apparently remained unchanged. Because of the variability between villi, inherent to the use of human specimens, we extended our survey of the effects of TGF β 1 and Y27632 treatments to a total of five different placentas, analyzing about 40 villi for each of the three conditions (TGF β 1, Y27632, or control) (Fig. 6). For each placenta considered, we evaluated the effect of the above-mentioned treatments on two main characteristics of the cocultures: 1) EVCT migration capacity (Fig. 6, A–C) and 2) size of lacunes made by EVCTs within the endometrial fibroblast layer (Fig. 6D). As previously observed (see Figs. 1, 2, and 4), EVCT migration in the control conditions took place preferentially along the fibroblast longer orientation axis (migration dependent on the fibroblast orientation pattern). Treatment with TGF β appeared to frequently block EVCT migration, whereas, on the contrary, Y27632 treatment seemed to induce EVCT migration, whatever the fibroblast orientation pattern (migration independent of the fibroblast orientation pattern).

The statistical significance of these data were determined first by two-way ANOVA tests. They showed that for each type of migration, dependent on the fibroblast orientation pattern (Fig. 6A), blocked (Fig. 6B) or independent of the fibroblast pattern (Fig. 6C), the effects of the treatments were significant ($P = 0.032, 0.0001, \text{ and } 0.003$, respectively). On the other hand, the effects of the placentas (variations in the distributions due to the placental origin) were not considered significant (P values ranging from 0.52 to 0.98). The villi distributions in the various culture conditions were then compared using unpaired Student's t tests, which confirmed our initial observations. Indeed, in control conditions 70% of the trophoblastic villi were found to produce EVCTs that migrated along the fibroblast longer axis (Fig. 6A). Both

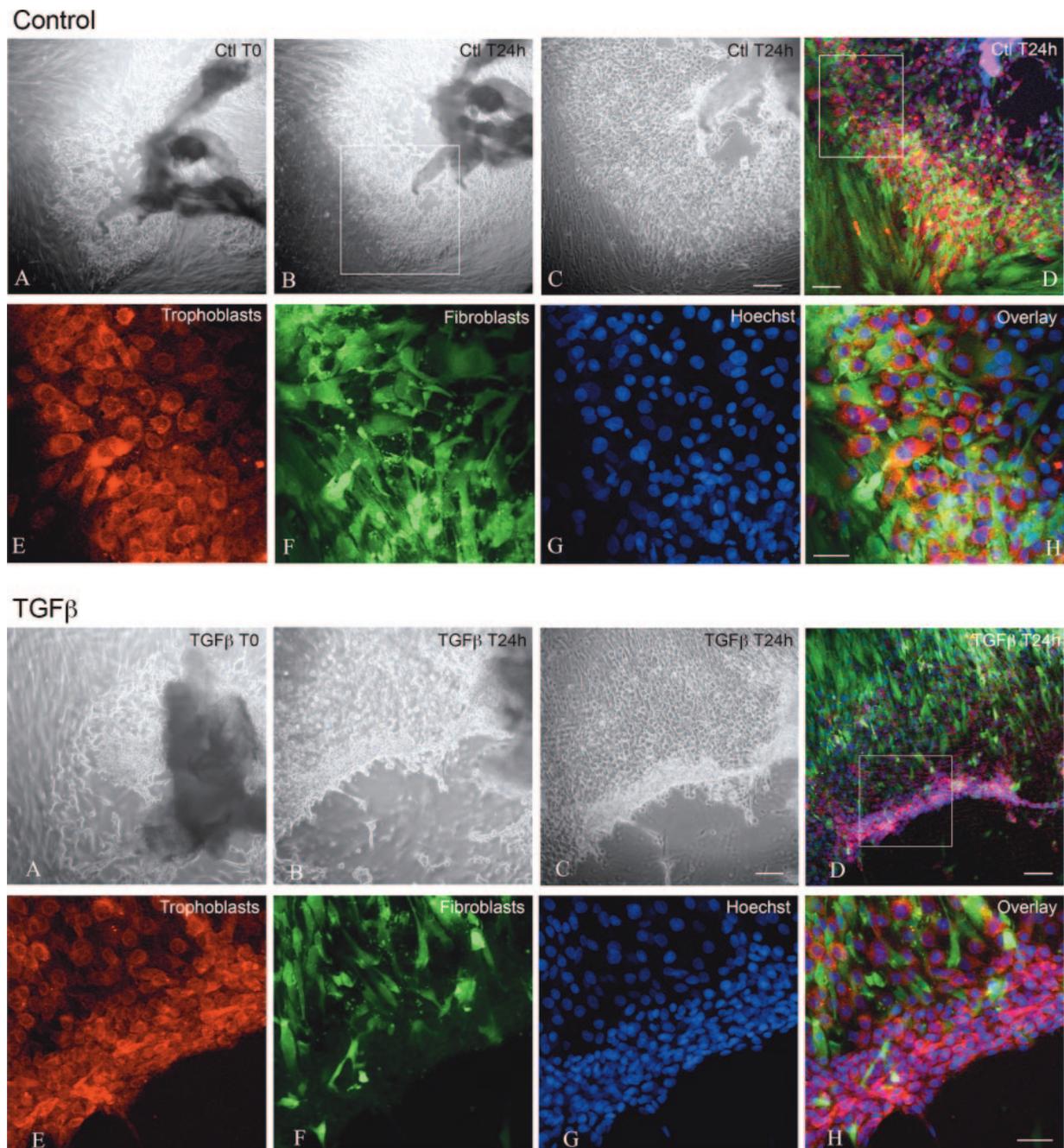


FIG. 4. Interstitial trophoblastic invasion of endometrial fibroblasts. Inhibitory effect of TGF β on trophoblastic cell migration. Placental explants and endometrial fibroblasts were labeled beforehand with the red and green CellTrackers, respectively. They were subsequently cocultured for about 16 h. Phase-contrast pictures were taken (T0). Cultures were further incubated for 24 h at which time phase-contrast pictures were taken again (T24 h). Cultures were fixed and nuclei were stained with Hoechst. Phase-contrast micrographs are shown at times T0 and T24 h (A and B). C, High magnification of B; scale bar, 100 μ m. Confocal microscopy sections of the fields shown in C are shown in low magnification (D, scale bar, 100 μ m) and high magnification (E–H, scale bar, 50 μ m). Top panels, Control conditions. Bottom panels, TGF β treatment. At time T0, cultures were treated with TGF β 1 (10 ng/ml) and further incubated for 24 h at which time phase-contrast pictures were again taken (TGF β T24 h). Cultures were fixed and analyzed as for control conditions.

TGF β 1 and Y27632 treatments gave rise to significantly different EVCT migration preferences ($P = 0.008$ and 0.015 , respectively) because this dependent migration was followed by only 20% of the villi. On the other hand, blockade of EVCT migration was observed in roughly 80% of the villi of the TGF β 1-treated cultures, a value significantly different from that of the control ($P = 0.001$ in Fig. 6B). Finally, EVCT

migration independently of the fibroblast orientation pattern was a feature that was observed mostly in Y27632-treated cultures (75% of the villi) and only 15% of the control cultures ($P = 0.004$ in Fig. 6C). TGF β 1 rarely induced this type of EVCT migration ($P = 0.078$). The size of lacunes formed within the fibroblast layer after EVCT migration was also found to change in response to the coculture treatment. La-

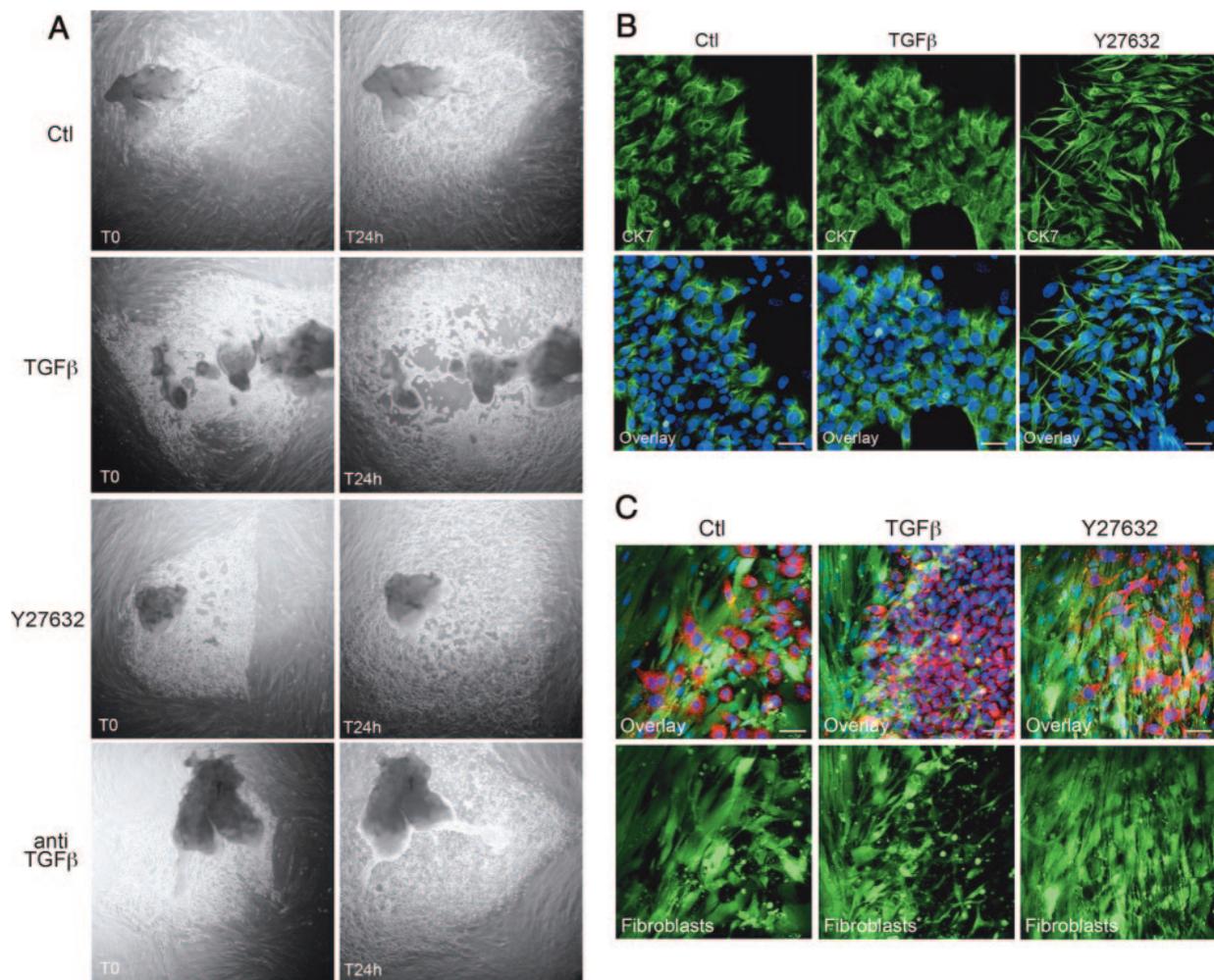


FIG. 5. Effect of exogenous TGF β , ROCK inhibitor Y27632, and anti-TGF β antibodies on extravillous trophoblast cell invasion. Placental explants and endometrial fibroblasts were cocultured for about 16 h. Phase-contrast pictures were taken of fields showing a beginning of trophoblastic invasion (T0). Cultures were treated with TGF β 1 (10 ng/ml), the ROCK inhibitor Y27632 (5 μ M), or anti-TGF β antibodies (4 μ g/ml) or left untreated (Ctl) and further incubated for 24 h, at which point phase-contrast pictures of the same fields were taken (T24 h). A, Phase-contrast micrographs of a representative experiment performed with trophoblastic villi obtained from the same placenta. B, At time T24 h, cultures were fixed and stained for cytokeratin 7 (CK7) and Hoechst (shown in the *overlays*). C, Before the coculture, the placental explants and endometrial fibroblasts were labeled with CellTrackers. They were otherwise treated as in A. EVCTs and endometrial fibroblasts are identified as *red* and *green* cells, respectively. The Hoechst staining is shown in the *overlays*. B and C, Confocal microscopy sections of the cultures are shown (scale bar, 50 μ m).

cunes were found to slightly increase with TGF β 1 treatment, even though the difference with the control was not considered significant by Mann-Whitney evaluation (Fig. 6D, $P = 0.322$). On the other hand, Y27632 treatment considerably limited the size of lacunes compared with either control or TGF β 1 conditions ($P = 0.003$ and 0.002 , respectively), an observation consistent with the more superficial migration of EVCTs in these culture conditions.

Discussion

Cocultures of human trophoblastic villi and primary endometrial fibroblasts were used in this work to characterize trophoblast migration. Extravillous cytotrophoblast migration was followed by time-lapse phase-contrast microscopy. Trophoblast explants, as opposed to isolated cytotrophoblast cells or immortalized EVCT cell lines, were used, and therefore, we analyzed the migration of EVCTs produced con-

temporaneously during the coculture. The data shown are based on experiments performed on a total of 20 different placentas obtained at very early pregnancy (4–7 wk). The young age of these placentas enabled us to observe migration phenomena such as formation of cytotrophoblast cell columns within very short time frames (16–48 h after seeding the explants), compared with other reports in which the use of older placentas (8–12 wk) led to delayed trophoblast migration (of the order of 76 h) (33, 35). Boyden chambers are often used for three-dimensional migration assays that measure motility as the number of cells that migrate across a filter separating two culture compartments. They have provided valuable information on the migration of a number of strains, including transformed cell lines. However, this technique mostly relies on artificial matrices and accordingly does not take into account interactions with the normal cellular microenvironment. This limitation led us to set up a system

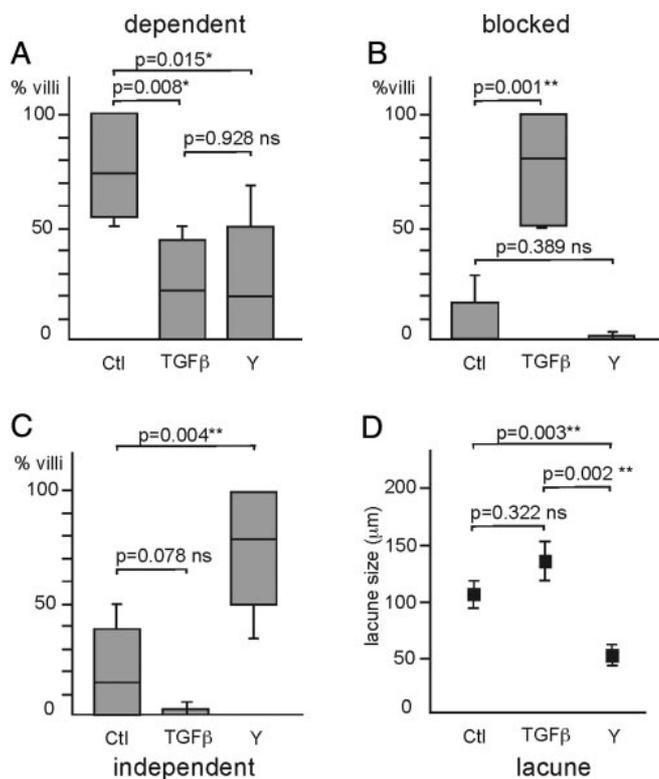


FIG. 6. Statistical analysis of the effects of TGF β and Y27632 on trophoblast migration. Two main trophoblast invasion criteria were analyzed: migration (A–C) and formation of lacunes (D). A, Distribution of trophoblastic villi producing EVCTs that migrate preferentially along the longer fibroblast axis (migration dependent on the fibroblast orientation pattern). B, Distribution of villi showing a blockade in EVCT migration upon culture treatment. C, Distribution of villi producing EVCTs whose migration is independent on the fibroblast orientation pattern. In the box and whisker diagrams, *whiskers* mark 10th and 90th percentile, *boxes* mark 25th and 75th percentile. *P* values were determined with unpaired Student's *t* tests. D, Quantification of lacune length in the different culture conditions, shown are the median values of the distributions \pm SD. Data were obtained from experiments performed on five different placentas and at different times. These placentas provided material to analyze a total of 16 independent culture plates with a total of 48 trophoblastic villi for the control conditions, 12 plates, and 36 villi for the TGF β conditions and 10 plates and 37 villi for the Y27632-treated cultures.

whereby stromal endometrial fibroblasts are used as a matrix for trophoblastic cell migration. The observed trophoblast-induced decidualization of the endometrial fibroblasts indicated that our coculture system could recapitulate interactions that occur *in vivo* between the placenta and the endometrial stroma, giving further support to the use of human primary endometrial fibroblasts for studying trophoblast invasion.

Extravillous cytotrophoblast migration was followed by phase-contrast time-lapse microscopy and fluorescence microscopy of vitally stained cells. EVCTs were found to migrate in nearly straight paths away from the villi. However, individual EVCT migration trajectories were clearly influenced by the underlying endometrial fibroblast orientation pattern and showed a strong preference for a migration along the fibroblast longer axis. Interestingly, EVCT migration characteristics differed, depending on the tissue origin of the fibroblasts. When derm instead of endometrium primary

fibroblasts (isolated from the same donor) were used in the coculture, EVCT migration was found to occur more superficially on the fibroblast layer (our unpublished data). These observations further emphasized the role of the fibroblast matrix for EVCT migration and could be related to recent data showing that different repertoires of genes are expressed in fibroblasts, depending on the tissue they originate from (38). EVCT velocities in our coculture system ranged from 10 to 40 $\mu\text{m}/\text{h}$ with an average measured velocity of 23 $\mu\text{m}/\text{h}$. These values are close to velocities found for carcinomas in three-dimensional collagen matrices, ranging from 8 to 14 $\mu\text{m}/\text{h}$ (39, 40), or for keratinocytes (42 $\mu\text{m}/\text{h}$) (41). They are much lower, however, than velocities found for lymphocytes, of the order of 400 $\mu\text{m}/\text{h}$ (42, 43).

We analyzed the effect of TGF β on EVCT migration by time-lapse phase-contrast microscopy and confocal microscopy of fluorescently labeled trophoblasts and endometrial fibroblasts. TGF β was previously reported to block EVCT migration (5, 13–18). We found a similar effect of TGF β in our coculture system, and all three TGF β isoforms, TGF- β 1, TGF- β 2, and TGF- β 3, were found to inhibit EVCT invasion of the endometrial fibroblasts. Besides, TGF β treatment induced the formation of compact EVCT stacks up to 20 μm in height, as observed by confocal microscopy. Formation of these stacks was correlated with increased E-cadherin expression at the EVCT plasma membrane (data not shown). TGF β treatment also induced EVCTs to form large lacunes, devoid of cells, within the endometrial fibroblast layer by a mechanism that still needs to be determined. Because murine trophoblast cells were reported to phagocytose cells from the endometrium (44), we looked for such a phenomenon using vitally stained cells in our coculture system. However, we were unable to provide evidence that such an engulfment mechanism was taking place between human extravillous trophoblastic cells and endometrial fibroblasts. Alternatively, metalloproteinases secreted by the trophoblast might also be involved (45, 46). Determining their putative role in the formation of the lacunes observed will need further investigation.

Previous reports showed a role for the RhoA/ROCK pathway in supporting trophoblast migration for both primary EVCTs and immortalized trophoblastic cell lines (28, 30, 47). This was tested in Boyden chamber-type assays in which migration efficiency is scored as the number of cells crossing a synthetic matrix. These assays are obviously very different, in their biochemical and biophysical properties, from the system we used that involved an endometrial fibroblast-based matrix. Nevertheless, we observed, as they did, that addition of the chemical ROCK inhibitor Y27632 in the coculture disrupted the actin stress fibers found in untreated EVCTs, leading to cortical relocalization of F-actin (data not shown), and induced an elongated EVCT morphology. However, in contrast to their observations, we found that EVCT migration was not abolished in Y27632-treated cocultures. Unexpectedly, inhibition of the RhoA-ROCK pathway promoted efficient EVCT two-dimensional migration. This increased migration capacity was apparently linked to weakened interactions between EVCTs and the endometrial fibroblasts because EVCT migration showed a lesser dependence on the fibroblast orientation pattern and because, conversely, the fibroblast layer integrity was maintained after

passage of the EVCTs. ROCK activity has been shown to be important for the migration of tumor cells, in particular in the context of amoeboid cell motility (26, 48). However, different modes of cell migration with distinct requirements for Rho/ROCK signaling could be found in tumor cells (49). Whereas ROCK activation was required for bleb-associated motility, elongated protrusive movements were found to occur independently of ROCK. Recent reports also established that ROCK activation could be required in the stromal fibroblasts supporting invasion but not in the actual invasive tumor cells, as shown for squamous cell carcinoma cells (50). Whether such phenomena occur in our coculture system and could explain why treatment with the Y27632 inhibitor induces a switch between three-dimensional and two-dimensional trophoblast migration will require further investigation.

Acknowledgments

We thank the Montpellier RIO imaging facility (MRI) for providing adequate environment for time-lapse analysis and confocal microscopy. The authors also thank the staff of the Obstetrics Department of Hospital Arnaud de Villeneuve (Montpellier) for providing placentas and endometrial specimens. We thank Thierry Gostan for performing statistical analysis and Jean Marie Blanchard, Delphine Haouzi, Robert Hipskind, Marion Peter, and Pierre Savagner for helpful discussion and critical reading of the manuscript.

Received February 22, 2008. Accepted May 13, 2008.

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This work was supported by Grant 3446 from the l'Association pour la Recherche sur le Cancer (to M.-L.V.).

Disclosure Statement: The authors have nothing to disclose.

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