Mesenchymal Activin-A Overcomes Defective Human Trisomy 21 Trophoblast Fusion

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Placental development is markedly abnormal in trisomy 21 (T21) pregnancies. We hypothesized that abnormal paracrine cross talk between the fetal mesenchymal core and the trophoblast might be involved in the defect of syncytiotrophoblast formation and function. In a large series of primary cultured human cytotrophoblasts isolated from second-trimester control (n = 44) and T21 placentae (n = 71), abnormal trophoblast fusion and differentiation was observed in more than 90% of T21 cases. We then isolated and cultured villous mesenchymal cells from control (n = 10) and T21 placentae (n = 8) and confirmed their fetal origin. Conditioned medium of control mesenchymal cells overcame the abnormal trophoblast fusion of T21 cytotrophoblasts by activating the TGFβ signaling pathway, as shown by the phosphospecific protein microarray analysis and the use of TGFβ signaling pathway antagonists. Using protein arrays, we further analyzed the cytokines present in the conditioned medium from control and T21 mesenchymal cells. Activin-A was identified as strongly secreted by cells from both sources, but at a significantly (P < 0.01) lower level in the case of T21 mesenchymal cells. Recombinant activin-A stimulated T21 trophoblast fusion. Blocking activin-A antibody inhibited the fusion induced by conditioned medium and exogenous activin-A. Furthermore, follistatin, an activin-A binding protein largely secreted by T21 mesenchymal cells, inhibited the conditioned medium fusogenic activity. These results show that the defective trophoblast fusion and differentiation associated with T21 can be overcome in vitro and reveal the key role of the fetal mesenchymal core in human trophoblast differentiation. (Endocrinology 152: 5017–5028, 2011)

The chorionic villus is the structural and functional unit of the human placenta. Its mesenchymal core is covered by cytotrophoblastic cells forming a monolayer of epithelial cells attached to the villous basement membrane. These cells proliferate and differentiate by fusion, forming a syncytiotrophoblast that covers the entire surface of the villus, which bathes in the maternal blood (1). The syncytiotrophoblast plays a major role in fetomaternal exchanges throughout pregnancy, because it is the site of numerous placental functions including ion, nutrient, and gas exchanges, removal of waste products, and synthesis of steroid and peptide hormones required for fetal growth and development (2, 3). This cell fusion process can be reproduced in vitro. Purified cytotrophoblastic cells isolated from human placenta aggregate and then fuse, forming the multinucleated syncytiotrophoblast producing pregnancy-specific hormones [human chorionic gonadotropin (hCG) and human placental lactogen (hPL)].

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(4). Using this physiological model, we have previously demonstrated the direct involvement of the endogenous retroviral envelope protein Herv-W (syncytin-1) (5), gap junctional intercellular communication (6) via connexin 43 (7, 8), and the connexin 43-associated zona occludens-1 protein in cytotrophoblastic cell fusion (9). Studies with other models have also shown the involvement of phosphatidyserine flip (10), cadherin 11 (11), caspase 8 (12), 4F2 cell-surface antigen heavy chain (13), and disintegrin and metalloproteinase domain-containing protein 12 precursor (14) in this process.

Chromosome 21 trisomy (T21), which causes Down’s syndrome, is the leading genetic cause of mental retardation and affects about one in 800 live births. Although antenatal T21 screening is based on maternal serum markers of placental origin, little is known about placental development in this aneuploid disorder. Syncytiotrophoblast formation is defective in T21 placentae. Cultured cytotrophoblasts isolated from T21 placentae aggregate but fuse poorly or belatedly (15, 16). This abnormal trophoblast fusion is related to super oxide dismutase 1 (SOD-1) gene overexpression (17) and to the secretion of hyperglycosylated hCG with low bioactivity (18). A predominance of two-layered mononuclear cytotrophoblasts is observed in T21 placentae compared with controls (19, 20).

A number of factors promoting or inhibiting cytotrophoblastic cell fusion and differentiation have been described (21). These include epidermal growth factor (22, 23), which acts through a membrane tyrosine kinase receptor, and activin (24) acting through a combination of type I and II transmembrane serine/threonine kinase receptors that stimulate trophoblast differentiation, whereas TGFB1 (25) and TNFα (26) are known inhibitors of trophoblast differentiation. The monomeric activin binding protein follistatin has been shown to regulate/inhibit the effects of activin by forming an inactive complex. Follistatin has been described in human placenta (27) and shown to neutralize the effects of activin on the endothocrine and molecular cytogenetic analyses were performed on cultured trophoblastic cells (27, 28). Several studies have also confirmed the importance of hCG and its membrane receptor in syncytiotrophoblast formation (29–31). Binding of hCG to its receptor activates adenylate cyclase, phospholipase C, and ion channels, which in turn control cellular cAMP, inositol phosphates, Ca^{2+}, and other second messengers (32, 33). cAMP, via cAMP-dependent protein kinases [protein kinase A (PKA)], promotes cytotrophoblast fusion in vitro (34), and also elevates mRNA levels of the fusogenic protein syncytin-1 in cultured trophoblasts (5).

Possible cross talk between trophoblastic and mesenchymal cells may play a major role in placental development but has rarely been explored (35–38). In this study, we identified activin-A as a soluble paracrine factor involved in the cross talk between trophoblastic and mesenchymal cells in normal and T21 placentae regulating the trophoblastic cell fusion.

**Materials and Methods**

**Materials**

The following were purchased from the indicated commercial sources: monoclonal antibodies anti-CD14 (PN6602622), anti-CD34 (IM0786), and antivimentin (IM1919) from Beckman Coulter Company, Marseille, France; anti-CD45 (31251A) from PharMingen International, San Diego, CA; anti-CD90 (ASO2) from Dianova, Hamburg, Germany; monoclonal antibody anti-CD31 (M0823) and anti-cytokeratin 7 (M7018) and rabbit polyclonal antibody anti-hPL (A0137) from Dako, Glostrup, Denmark; rabbit polyclonal antibody anti-actin (A2066) and monoclonal antibody antisemoplin (D1286) from Sigma-Aldrich, St. Louis, MO; human activin-A (338-AC) and monoclonal antibody anti-activin-A (MAB3381) from R&D Systems Europe, Lille, France; follistatin (F1175) from Sigma-Aldrich St. Louis, MO; 8-Br cAMP (B7880) and H89 dihydrochloride hydrate (B1427) from Sigma-Aldrich, St. Louis, MO; chelerythrine (C2932), herbimycin A (H6649), SB 525334 (S8822), A-83-01 (A5480), Tyr23 (T7165), Clostridium difficile B (C4102) from Sigma-Aldrich St. Louis, MO; signaling pathway antagonists LY294002 (440202), Go6976 (6976), and Y27632 (68800) from Calbiochem La Jolla, CA; TGFB signaling phospho-antibody array (PTG176) from Full Moon Biosystems, Inc., Sunnyvale, CA; and RayBio arrays (AAH-CYT-8) from RayBioTech, Inc., Norcross, GA.

**Placenta collection**

Second-trimester placentae were collected during termination of pregnancies at 12–25 wk of amenorrhea from T21 and non-T21 gestational-age-matched samples referred to as control throughout this study. Gestational age was confirmed by ultrasound measurement of crown-rump length at 8–12 wk. Fetal Down’s syndrome was diagnosed by karyotyping of amniotic fluid cells, chorionic villi cells, or fetal blood cells. Termination of the control pregnancies was indicated for severe bilateral or low obstructive uropathy or major cardiac abnormalities. Conventional and molecular cytogenetic analyses were performed on cultured trophoblastic cells (free trisomy 21 or normal karyotype) as previously described (39). A total of 71 T21-affected placentae (37 male and 34 female fetuses) and 44 gestational-age-matched control placentae (17 males and 27 females) were used for cell culture experiments. These biological samples were obtained after informed patient written consent and approval from our local ethics committee (CCPPRB, Paris Cochin, no. 18-05, Paris, France).

**Trophoblast cell culture**

Villus cytotrophoblasts from second-trimester placentae were isolated and purified as previously described (40). Cells were plated to a final density of 140,000 cells/cm².
Isolation and culture of mesenchymal cells

Mesenchymal cells were isolated by further trypsin-deoxyribonuclease digestion steps of the same second-trimester villi from control and T21 placenta used for trophoblastic cell isolation. Cells isolated by digestions 6–7 were pooled, diluted to a final density of 150,000 cells/cm² in 0.15 ml in supplemented DMEM (10% fetal calf serum, 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin). They were used after five passages. Conventional and molecular cytogenetic analyses were applied to cultured mesenchymal cells to detect chromosomes 21 and Y (39). To avoid contamination by cells of maternal origin, we used only control gestational-age-matched XY mesenchymal cells and XY mesenchymal cells harboring a free T21. Mesenchymal cells were used after five passages to avoid any trophoblastic cell contamination, and similar activin-A production was observed in cells derived between passages 2 and 7. Control mesenchymal cell-conditioned medium (CMC-CM) and T21 mesenchymal cell-conditioned medium (T21MC-CM) were collected and centrifuged at 200 x g for 10 min at 4°C; supernatants were kept frozen at −20°C until used.

Mesenchymal cell characterization

Mesenchymal cells were fixed, permeabilized as previously described (40), and characterized by indirect immunofluorescence staining using antibodies anti-CD16 (6 μg/ml), anti-CD31 (4 μg/ml), anti-CD34 (4 μg/ml), anti-CD45 (6 μg/ml), anti-CD90 (2.5 μg/ml), antivimentin (2.5 μg/ml), anti-cytokeratin 7 (2 μg/ml), and A488-labeled goat antimouse IgG (3 μg/ml). Controls (omission of the primary antibody or use of a nonspecific IgG of the same isotype) were all negative.

Trophoblast fusion assay

Syncytium formation was followed by monitoring the cellular distribution of desmoplakin and nuclei. The fusion index (9) were determined as previously described (30, 34).

Cells were cultured for 18 h after plating and treated with 1 mM 8-Br cAMP, 3 μM H89 dihydrochloride hydrate, recombinant human activin-A, or recombinant human follistatin. TGFβ signaling pathway antagonists were used on trophoblastic cells at their respective IC₅₀, LY 294002 (5 μM), G66976 (100 nM), tyrphostin 23 (Tyr23, 50 μM), chelerythrine (30 μM), herbimycin A (12 μM), A-83-01 (90 nM), SB 253534 (28 nM), and C. difficile B (50 μM). Cell viability after treatment was quantified with Trypan blue (41). To neutralize the bioactivity of activin-A, 5 ng/ml recombinant activin-A or CMC-CM was incubated with 2 μg/ml monoclonal antihuman activin-A antibody or with 1 μg/ml human recombinant follistatin for 1 h at 37°C and then added to cultured T21 cytotrophoblasts.

Phosphospecific protein microarray analysis for TGFβ signaling

Phosphospecific protein microarrays were performed according to the manufacturer’s instructions. Protein microarray analysis was applied to trophoblast cells derived from T21 (n = 3) and matched control placentae (n = 3) treated with CMC-CM for 0, 20, and 60 min. Briefly, T21 placentae (n = 3) or control placentae (n = 3) were pooled, respectively. Cell lysate (100 μg protein) was collected in 50 μl reaction mixture and labeled with 1.5 μl biotin/DMF (N,N-dimethylformamide, 10 μg/μl). The bound biotinylated proteins were detected with Cy3-streptavidin (0.5 mg/ml), and the signal intensity was determined on a Typhoon Imager (model 9410; GE Healthcare, Piscataway, NJ) using ImageJ software (Rasband, W.S., National Institutes of Health, Bethesda, MD). The immunoreactivity of phosphoprotein was analyzed by subtracting the background and normalizing to the positive markers provided on the same slide. Then the relative protein intensity of each spot and for each membrane was illustrated using Adobe Photoshop software version CS3.

Intracellular cAMP assay

Cells (2.8 × 10⁶) were plated in 60-mm dishes and cultured as described above. After 24 h, the cells were preincubated with 10 mM isobutylmethylxanthine for 1 h to prevent cAMP degradation and were then stimulated for 20 or 60 min with CMC-CM. Intracellular cAMP was assayed as previously described (30).

Protein array

Media conditioned for 24 h by subconfluent mesenchymal cells isolated from T21 placentae (T21MC-CM, n = 5) or controls (CMC-CM, n = 5) were pooled and concentrated 4-fold on Amicon Ultra-15 columns. RayBio arrays were used to detect cytokines, and the membranes were processed according to the manufacturer’s recommendation. Briefly, the membranes were blocked and incubated with biotin-conjugated antibodies and finally incubated with horseradish peroxidase-conjugated streptavidin.

Chemiluminescence was detected with a LAS-1000 device (Fujifilm), and the data were digitized and analyzed with ImageQuant software in volume integration mode (Molecular Dynamics, Sunnyvale, CA). By subtracting the background staining and normalizing to the positive controls on the same membrane, relative protein densities for each membrane were obtained, and the fold difference in protein concentration was calculated.

Hormone assays

hCG concentration was determined in triplicate in culture medium after 72 h of culture using an immunoassay developed on the Advia Centaur XP system (Siemens Healthcare Diagnostics, Deerfield, IL) (detection limit of 2 mIU/ml).

Activin-A concentration was determined in triplicate in cell-conditioned medium of T21 (n = 8) and control (n = 10) placental mesenchymal cell cultures, using the activin-A assay kit (MCA1426KZZ) from R&D Systems Europe (detection limit <78 pg/ml). Follistatin concentration was determined in triplicate in culture medium of T21 (n = 5) and control (n = 5) placental mesenchymal cell cultures, using the human follistatin assay kit (DFN00) from R&D Systems Europe (detection limit <29 pg/ml).

Immunoblotting

Cell extracts were prepared as previously described (42). The membranes were immunoblotted with a rabbit polyclonal antibody against hPL (2 μg/ml) or with a mouse antibody against human activin-A (0.5 μg/ml); the specific band was detected by chemiluminescence (West Pico Chemiluminescent; Pierce, Rockford, IL) after incubation with an antirabbit or antimouse peroxidase-coupled antibody according to the primary antibody. Actin was used as housekeeping control, and the membranes were immunoblotted using a rabbit polyclonal anti-actin antibody (A2066; 0.5 μg/ml) and detected with an antirabbit peroxidase-coupled antibody.
Results

Cultured T21 cytotrophoblastic cells do not fuse and differentiate poorly in vitro

During a 4-yr period, we collected 71 second-trimester placentae with T21 and 44 gestational-age-matched control placentae with a normal karyotype. Villous cytotrophoblastic cells were isolated and cultured in vitro for 3 d (4). We found that mononucleated cytotrophoblastic cells isolated from control placentae aggregated and fused to form a syncytiotrophoblast after 72 h culture (Fig. 1A). This was associated with a large increase in hCG secretion into the culture medium, from 7.4 ± 2.3 mIU/10⁶ cells at 24 h to 1015 ± 31 mIU/10⁶ cells at 72 h. In contrast, in 67 of 71 cultures of cytotrophoblastic cells isolated from T21 placentae, cytotrophoblastic cells aggregated but fused inefficiently, forming only a few small syncytiotrophoblasts after 3 d of culture; the majority of the cells remained aggregated (Fig. 1A). Four cultures of cytotrophoblastic cells isolated from T21 placentae were able to fuse to form a syncytiotrophoblast. The fusion index was significantly lower in trophoblastic cells isolated from T21 placentae (P < 0.001, Fig. 1A). This defective syncytiotrophoblast formation was associated with significantly lower (P < 0.001) hCG secretion into the culture medium compared with control cells (2.8 ± 1 and 166 ± 12 mIU/10⁶ cells at 24 h and 72 h; Fig. 1A).

Characterization of control and T21 placental mesenchymal cells

Mesenchymal cells and cytotrophoblasts were isolated and purified from the same placental villi. The fetal origin of the mesenchymal cells was determined by conventional and molecular cytogenetic analyses. We confirmed the presence or absence of three chromosomes 21 and a Y chromosome. No difference in the shape (Fig. 1B) or growth rate (data not shown) of control and T21 mesenchymal cells was observed. After five passages, subconfluent control and T21 mesenchymal cells were allowed to condition the culture medium for 24 h. As illustrated in Fig. 1B, the mesenchymal cells used in this study stained positively for CD90 and vimentin (fibroblast markers) and negatively for CD14 (macrophage marker), CD31 (endothelial cell marker), CD34 (endothelial and hematopoietic stem cell marker), and cytokeratin 7 (trophoblastic cell marker).
Mesenchymal cell-conditioned medium induces T21 trophoblastic cell fusion

We confirmed that 8-Br cAMP stimulated T21-cytotrophoblast fusion (30, 31), as shown by the significant ($P < 0.001$) increase in the fusion index (Fig. 2, A and B). Mesenchymal cell-conditioned medium also induced T21-cytotrophoblast fusion. Indeed, T21-cytotrophoblast cultured for 72 h contained five times more mononuclear cells in control conditions (58.2 ± 0.6% vs. 12% in untreated cells) (Fig. 2B). Interestingly, T21MC-CM induced syncytia formation as well, but only 50% of nuclei participated ($P < 0.001$ vs. T21 untreated cells). Thus, mesenchymal cells secrete soluble factors that are able to induce syncytialization.

Mesenchymal cell-conditioned medium does not stimulate trophoblast hormone secretion

Stimulation of syncytiotrophoblast formation by soluble factors of mesenchymal origin was not accompanied by secretion of the hormones specifically synthesized by the syncytiotrophoblast, contrary to the effect induced by 8-Br cAMP (Fig. 2B).

hCG secretion increased from 75 ± 3 mIU/10^6 cells in untreated condition to 2000 ± 23 mIU/10^6 cells in the presence of 8-Br cAMP at 72 h culture ($P < 0.001$). No increase in hCG secretion was observed when T21-cytotrophoblast cells were cultured with mesenchymal cell-conditioned medium for 72 h (Fig. 2B).

hPL was weakly detected in T21 trophoblastic cells at 72 h culture using immunoblot despite the formation of a syncytiotrophoblast in contrast to T21 cells treated with 8-Br cAMP (Fig. 2C; $P < 0.001$). No increase in hPL was observed when T21 cytotrophoblasts were cultured with mesenchymal cell-conditioned medium (Fig. 2C).

Thus, the production of mesenchymal cell-derived soluble factors that induced syncytialization was not accompanied by the increase in hormones of pregnancy (hCG and hPL), as usually observed during syncytium formation.

TGFβ signaling but not cAMP signaling is involved in the stimulation of T21 trophoblast cell fusion by mesenchymal cell-derived soluble factors

Two major signaling pathways have been implicated in trophoblastic cell fusion: the cAMP signaling pathway and signaling associated with TGFβ and EGF. T21 trophoblastic cells were cultured with H89 (a specific inhibitor of the cAMP-dependent protein kinase), either alone or together with control or T21MC-CM. H89 had no effect on the fusion index of cells treated with mesenchymal cell-conditioned medium (Fig. 3A). Likewise, no significant difference in intracellular cAMP levels between trophoblastic cells from control and T21 placentae was observed after incubation for 20 or 60 min with mesenchymal cell-conditioned medium (Fig. 3B). The results ruled out involvement of the cAMP signaling pathway.
pathway. We then used an antibody microarray strategy to determine which proteins of the TGFβ/H9252 signaling pathway induced cell fusion. T21 cytotrophoblasts were stimulated with CMC-CM for 20 or 60 min. As illustrated in Fig. 4A, some proteins showed an increase in phosphorylation or expression after 20 min, suggesting a role in syncytialization. After 60 min stimulation, these candidate proteins returned mostly to their basal level of expression. These proteins were regrouped (group I–VII) based on their known interactions described in the literature, with group I corresponding to the TGFβ family members and their receptors. CMC-CM activated via the TGFβ receptor (group I), the Rac-cdc43/p21-activated kinase 1/c-Abl pathway (group II), the phosphatidylinositol 3-kinase (Pi3K)/akt/mammalian target of rapamycin pathway (group III), and the adaptor proteins Shc (group IV). PKC (group V), the MAPK signaling pathway (MKK6/p38; group VI), and some Smad family proteins (group VII) were also activated. The Ras and RhoA pathways did not seem to be involved. Thus, CMC-CM appeared to activate specific downstream effectors of the TGFβ signaling pathway.

Effect of specific TGFβ signaling pathway inhibition on trophoblastic cell fusion induced by CMC-CM

To identify which downstream effectors of the TGFβ signaling pathway were involved in the trophoblastic cell fusion induced by CMC-CM, we used specific inhibitors. T21 cytotrophoblasts were cultured with CMC-CM to induce cell fusion, and the impact of specific inhibitors was measured in a fusion assay (Fig. 4B). TGFβ receptor (ALK4, -5, and -7) inhibitors (A-83-01 and SB 525334), specifically from group I, presented a 50% reduction of fusion index (P < 0.001) compared with cells treated with CMC-CM or with 8-Br cAMP. This observation was also made by the use of specific TGFβ1 and -β2 blocking antibodies (data not shown). Interestingly, the C. difficile B (a group II inhibitor, Rho/Rac/Cdc42 antagonist; C4102), a Pi3K inhibitor (group III inhibitor, LY294002), and another PKC antagonist (group V antagonist, Go6976) presented as well a significant inhibition in cell fusion (up to 50%) induced by CMC-CM (P < 0.001). Finally, the use of group VI inhibitors (tyrosine kinase inhibitors Tyr23 and herbimycin A) and another PKC antagonist (group V, chelerythrine) presented an inhibition in cell fusion as well (data not shown).
Protein array analysis of conditioned medium identifies activin-A as largely produced by mesenchymal cells

To identify soluble paracrine factors produced by control and T21 mesenchymal cells, cytokine arrays were applied to control and T21MC-CM. Six soluble factors showed markedly different signal intensities between control and T21 (Fig. 5A). These factors were activin-A, a growth factor (TGFβ2), latency-associated peptide (LAP), a cytokine and its receptor (IL-18 receptor IIB) and matrix metalloproteinases (MMP1 and MMP3). Semiquantitative analyses showed approximately 2-fold higher TGFβ2, IL-18 receptor IIB, and MMP1 protein levels and 4- to 10-fold lower MMP3, LAP, and activin-A levels in T21 mesenchymal cell culture supernatants than in controls.

T21 mesenchymal cells secrete less activin-A than control mesenchymal cells

As shown in Fig. 5B, activin-A levels measured with an ELISA were significantly higher in CMC-CM (5 ± 0.5 ng/10^6 cells) than in T21MC-CM (3 ± 0.4 ng/10^6 cells; P < 0.05). Interestingly, the extracellular amount of follistatin, the activin biological antagonist, quantified by ELISA (Fig. 5C) presented a 10-fold significant increased in T21MC-CM compared with control (11.2 ± 1.1 and 1.3 ± 0.1 ng/10^6 cells, respectively; P < 0.001).

Immunoblotting showed significantly (P < 0.01) lower activin levels in T21 cell extracts than in control cell extracts (Fig. 5, D and E). Moreover, the amount of extracellular follistatin produced by control and T21 cytотrophoblastic cells were observed to be nonsignificant and as low as the CMC-CM production (0.38 ± 0.1 and 1.1 ± 0.3 ng/10^6 cells, respectively; data not shown).

Activin-A present in mesenchymal cell-conditioned medium stimulates T21 trophoblast fusion

We first showed that recombinant activin-A stimulated syncytiotrophoblast formation (Fig. 6A). Three concentrations of activin-A (from 1–50 ng/ml) were tested (data not shown). In the presence of 5 ng/ml recombinant activin-A, the fusion index of the cells increased from 16 ± 2% to 70 ± 2% (Fig. 6A). This increase was not associated with a significant increase in hCG (data not shown). An
activin-blocking antibody partially inhibited the effect of exogenous activin-A on trophoblast fusion (fusion index was 70 ± 2% with activin-A alone and 38 ± 1% with activin-A and the blocking antibody; \( P < 0.001 \)). The blocking antibody also significantly inhibited (\( P < 0.001 \)) the effect of CMC-CM on T21 trophoblastic cell fusion (fusion index was 78 ± 2% with CMC-CM and 58 ± 2% with CMC-CM plus the blocking antibody; \( P < 0.001 \)). Finally, follistatin, first known as a potent biological inhibitor of activin and then described as an activin-binding protein, was used to confirm the role of activin-A in the cell fusion induced by mesenchymal cell-conditioned medium. The presence of follistatin (Fig. 6B) did not modify the fusion index compared with T21 untreated cells (19 ± 2 and 21.5 ± 2%, respectively). However, T21 trophoblastic cells treated with a human recombinant follistatin (1 \( \mu \)g/ml) and mesenchymal cell-conditioned medium (Fig. 6B) presented a significant reduction (46%; \( P < 0.001 \)) of fusion index (23 ± 1%) compared with T21 trophoblastic cells treated with mesenchymal cell-conditioned medium alone (69 ± 2%).

**Discussion**

Numerous histomorphological studies of chorionic villi obtained after miscarriage or termination of pregnancy during the first trimester of pregnancy have shown qualitative differences between control and T21 pregnancies (43–49). T21 is associated with villous hypovascularity, intrastromal cytotrophoblastic cells, persistence of nucleated red blood cells, and abnormalities of the trophoblastic layer, suggesting a delay in villous maturation. Few studies have focused on second-trimester placentae (50). Here we used primary cultures of villous trophoblastic cells isolated from second-trimester control and T21 placentae. We confirm in this large and unique collection of samples that syncytiotrophoblast formation is defective and hCG secretion is subnormal in T21 placentae. T21 cytotrophoblastic cells adhered well in culture (data not shown) and aggregated but fused poorly or not at all.

This abnormal fusion and differentiation of trophoblastic cells isolated from T21 placentae was overcome in vitro. As previously shown (30, 31), 8-Br cAMP and bio- synthetic hCG induced T21 trophoblast fusion and differentiation. It is well established that agents that increase cellular levels of cAMP promote cytotrophoblast fusion in vitro (34). Here we report for the first time that medium conditioned by mesenchymal cells can overcome the abnormal fusion of T21 cytotrophoblasts in a cAMP-independent manner. cAMP acts through several effectors, including PKA, exchange protein activated by cAMP, and cyclic nucleotide-gated ion channels (51). We found that the specific cAMP-dependent protein kinase inhibitor H89 had no effect on trophoblastic cell fusion induced by mesenchymal cell-derived factors. This suggests that a signaling pathway other than the one activated by PKA is involved in trophoblastic cell fusion (30, 31, 34). Moreover, the noninvolvement of PKA in this process is confirmed by the absence of any increase in intracellular cAMP during trophoblastic cell fusion induced by conditioned medium. This also rules out the involvement of other cAMP-downstream effectors such as exchange protein activated by cAMP, which was recently shown to be involved in trophoblastic cell fusion (52).

The in vitro reversibility of abnormal T21 trophoblast differentiation points to abnormal regulation of the dynamic process leading to cell-cell fusion and differentiation rather than to a defect of major factors involved in trophoblast fusion and differentiation due to the genetic defect. To fuse in vitro, trophoblastic cells must exit the proliferative stage, express genes and proteins involved in the fusion process, and then recognize and interact with their fusion partners. This dynamic process is likely to be finely regulated and coordinated.

In vivo, cytotrophoblastic cells are in close contact with the underlying mesenchymal core. The villous chorionic core is composed of mesenchymal cells, Hofbauer macrophages (35), and fetal vessels. It is possible that the mesenchymal core plays a major role in villous development (35). Indeed, macrophage-conditioned medium has been shown to stimulate syncytiotrophoblast formation, an effect associated with a large increase in hCG secretion (53). Directional contact between the cytotrophoblast and syncytiotrophoblast is important for regulating the relative abundance of the two cell populations (54). Previous studies have shown that the extracellular matrix deposited by fibroblasts and mesenchymal cell-derived IGF-I stimulate trophoblast migration into the anchoring villi (37, 38).

We used mesenchymal cells derived only from male placentae and confirmed the presence of three chromosomes 21 and a Y chromosome. We also obtained XX cells not affected by T21 and therefore of maternal origin. These cells may be endothelial progenitor cells circulating in the intervillous space or endothelial cells coating the basal plate (55, 56). Only the cells of placental origin expressed fibroblastic markers.

Despite their similar growth rates and shapes, control and T21 mesenchymal cells produced different levels of cytokines and other soluble factors. The secretion of three factors was significantly lower in T21 mesenchymal cells. MMP3 is strongly expressed in the placenta and involved in matrix degradation and trophoblast invasion (for review see Ref. 57). It is also expressed by skin fibroblasts...
and type II activin receptors, in target cells (66). The type transmembrane serine/threonine kinase receptors, type I
onic stem cell pluripotency and in endoderm differentia-
(63, 64). Activin has also a critical role in human embry-
tation, differentiation, apoptosis, metabolism, homeostasis,
pituitary, has numerous biological roles in prolifera-
the biosynthesis and secretion of FSH by the anterior
chymal cells. Activin, initially identified as a regulator of
differentially secreted between control and T21 mesen-
cell fusion process. In this work, activin-A was found to be
activated (61). Together, these results are in keeping with transcriptomic analysis of T21 tissues (62) and suggest that the modulation, composition, and maintenance of the extracellular matrix secreted by mesenchymal cells might be altered in T21 pregnancies and contribute to the delayed placental maturation.

Mesenchymal cell-conditioned medium stimulated the TGFβ signaling pathway as indicated by activation of spe-
cific downstream effectors such as PI3K, tyrosine kinases, and PKC and an increase in Smad2 and Smad3 phosphor-
ylation, suggesting that members of the TGFβ family are present in this medium. Interestingly, proteins shown to be
activated on protein arrays under stimulation with mes-
enchymal cell-conditioned medium were all found to be
involved in the cell fusion process by using specific TGFβ
signaling pathway antagonists, confirming the concor-
dance of the data obtained from these two approaches.
Then, it becomes complicated to define precisely which
downstream effectors of the TGFβ signaling pathway
could be responsible for the cell fusion. Indeed, this pro-
cess could be induced by cross talk between these effectors as presented in several systems or by the activation of mul-
tiple downstream effectors as suggested here by the effects of TGFβ signaling pathway antagonists on cell fusion. Interestingly, TGFβ1 has been shown to inhibit tropho-
blastic cell differentiation (25), whereas activin (24), a
TGFβ family member, has been described to stimulate the
cell fusion process. In this work, activin-A was found to be
differentially secreted between control and T21 mesen-
chymal cells. Activin, initially identified as a regulator of
the biosynthesis and secretion of FSH by the anterior pituitary, has numerous biological roles in prolifera-
tion, differentiation, apoptosis, metabolism, homeostasis,
immune function, wound repair, and endocrine functions
(63, 64). Activin has also a critical role in human embry-
onic stem cell pluripotency and in endoderm differentia-
tion (65). Activin-A signals are transmitted through two transmembrane serine/threonine kinase receptors, type I
and type II activin receptors, in target cells (66). The type I receptor is phosphorylated and activated by type II
receptor kinase. Activin-specific smads, Smad2 and -3,
are phosphorylated by the activated type I receptor. In
the nucleus, Smad2 and -3 complexes recruit additional
transcriptional activators and repressors to regulate tar-
get genes.

In the placenta, activin is produced by cytотrophoblast
cells (67, 68), which also possess activin receptors (69) and therefore appear to be a major local regulator of placental
development (70). Previous studies have shown that ac-
tavin addition stimulates hCG and progesterone secretion
by isolated cytotrophoblast cells in culture (71). In this
study, we found that exogenous activin-A strongly stim-
ulated the fusion of T21 cytотrophoblastic cells in culture.
This effect was not specific to aneuploid trophoblastic
cells, because it was also observed on control cytотrophob-
blasts isolated from second-trimester and term placentae
(data not shown). Interestingly, CMC-CM accelerated the
cell fusion process of control cytотrophoblasts, pointing to
the presence in the cell medium of a fusogenic and para-
crine compound produced by the mesenchymal cells. We
used in this study the well-established biological model of
defective fusion observed in T21 cytотrophoblastic cells to
easily characterize and quantify the fusogenic effect of the
mesenchymal cell-conditioned medium. The intensity of the
activin-A stimulatory effect on T21 trophoblast fusion
was similar to that of cAMP and biosynthetic hCG. How-
ever, the stimulation of trophoblast fusion by activin-A
was not associated with an increase of either hCG or hPL
secretion. These results conflicted with those of previous
studies, possibly because we used biosynthetic activin-A
and not purified activin from porcine origin (72) or be-
cause we used purified villous cytотrophoblasts and not a
mixed population of trophoblastic cells (71). Moreover,
follistatin inhibited the T21 cytотrophoblastic cell fusion
induced by mesenchymal cell-conditioned medium,
whereas follistatin had no effect in cell fusion by itself,
pointing again to the potential role of activin-A as a mes-
enchymal paracrine factor involved in cytотrophoblastic

cell fusion.

Activins are dimeric proteins comprising two β-sub-
units and containing a backbone of a cysteine-knot fold,
with the monomers linked by a single covalent disulfide
bond (63). Inhibin and activin share a common β-subunit,
inhibin constituting a dimer of a related α-subunit linked
to the β-subunit (73). Thus, regulation of α-subunit bio-
synthesis and dimerization with the β-subunit can alter
activin biosynthesis in cells that synthesize both subunits,
such as ovarian granulosa cells (74). We therefore checked
for the presence of inhibin-A. The assays developed on the
Accessory System (Beckman) did not detect inhibin-A in con-
tral or T21MC-CM. Similarly, immunoblot of mesenchy-
mal cell lysates detected no inhibin-A (data not shown).

In conclusion, this study highlights that trophoblast
fusion, differentiation, and therefore regeneration depend
on different signaling events arising from the mesenchy-
mal core of the villi. Interestingly, activin, which seems to play a major role in embryonic stem cell differentiation, appears from these in vitro studies to be a potential major regulator of trophoblast differentiation during the second trimester of pregnancy. The well-established model of T21 trophoblastic and placental mesenchymal cells should help to decipher the mechanisms of abnormal cell behavior in T21 as well as paracrine cross talk involved in placental development.

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