Nodal and ALK7 Inhibit Proliferation and Induce Apoptosis in Human Trophoblast Cells*

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Received for publication, January 20, 2004, and in revised form, May 12, 2004
Published, JBC Papers in Press, May 18, 2004, DOI 10.1074/jbc.M400641200

Nodal, a member of the transforming growth factor-β superfamily, is known to play critical roles in early vertebrate development, but its functions in extraembryonic tissues are unclear. ALK7 is a type I receptor for Nodal. Recently, we demonstrated that nodal mRNA and several ALK7 transcripts are expressed in human placenta throughout pregnancy (Roberts, H. J., Hu, S., Qiu, Q., Leung, P. C. K., Cannigia, I., Gruslin, A., Tsang, B., and Peng, C. (2003) Biol. Reprod. 68, 1719–1726). In this study, we determined the role of Nodal and ALK7 in trophoblast cell proliferation and apoptosis. Overexpression of Nodal in normal trophoblast cells (HTR8/SVneo) and several choriocarcinoma cell lines resulted in a significant decrease in the number of metabolically active cells. The effect of Nodal could be mimicked by constitutively active ALK7 (ALK7-ca), but was blocked by kinase-deficient ALK7. The growth inhibitory effect of Nodal was blocked by dominant-negative Smad2/3. Overexpression of Nodal and ALK7-ca induced apoptosis in trophoblast cells as determined by Hoechst staining, flow cytometry, and caspase-3 Western blotting. In addition, Nodal and ALK7-ca decreased the number of proliferating cells as measured by bromodeoxyuridine assays. Furthermore, overexpression of Nodal or ALK7-ca increased p27 expression, but reduced the levels of Cdk2 and cyclin D1. Taken together, this study demonstrates for the first time that Nodal, acting through ALK7 and Smad2/3, inhibits proliferation and induces apoptosis in human trophoblast cells. Our findings also suggest that the Nodal-ALK7 pathway inhibits cell proliferation by inducing G1 cell cycle arrest and that this effect is mediated in part by the p27-cyclin E/Cdk2 pathway.

Members of the transforming growth factor-β (TGF-β) superfamily regulate a variety of cellular functions and play critical roles in many developmental and physiological processes (1–3), including placental development (4–6). This family consists of a large group of peptide growth factors/hormones, including TGF-β, activins, bone morphogenetic proteins, growth and differentiation factors, as well as Nodal and its related proteins (1–3). The role of Nodal has been extensively studied in mouse, Xenopus, and zebrafish, in which Nodal and its related proteins have been found to be critical for mesoderm formation and left-right axis patterning during early development (7–9). The role of Nodal in adult physiological processes is unknown. Studies in rodents have shown that Nodal may inhibit the differentiation of stem trophoblast cells into giant cells. Homozygous mutations of the nodal gene in mice result in excessive numbers of trophoblast giant cells (10). On the other hand, overexpression of Nodal in the rat choriocarcinoma cell line Echo-1 decreases giant cell numbers (11). We have recently found that Nodal mRNA is expressed in human placenta from early to late gestation and in JEG-3 choriocarcinoma cells (12), suggesting that Nodal may regulate human placental development and functions.

ALK7 (activin receptor-Iike kinase-7) is a type I receptor belonging to the serine/threonine kinase receptor family. The serine/threonine kinase receptor family consists of two related groups of receptors termed type I and type II receptors. The type I and type II receptors form functional complexes to mediate signaling by members of the TGF-β superfamily (1–3). In mammals, seven type I (known as ALK1–7) and five type II receptors have been characterized. Both type I and type II receptors have similar structural features, including an extracellular ligand-binding domain, a transmembrane domain, and an intracellular serine/threonine kinase domain (1). In addition, type I receptors also have a GS box, which serves as an activation domain for their type II receptor partners (13). Upon activation, type I receptors phosphorylate two distinct sets of receptor-regulated Smad proteins, Smad1/5/8 (activated by ALK1/2/3/6) and Smad2/3 (activated by ALK4/5/7) (14–16). In turn, these activated receptor-regulated Smad proteins form complexes with Smad4 and, through their interaction with other transcription factors, regulate target gene expression (1–3, 14–16).

ALK7 was initially cloned from the rat and found to be predominantly expressed in the central nervous system (17, 18). Nodal has been recently identified to be the physiological ligand of ALK7 (19). The kinase domain of ALK7 is closely related to that of ALK4 and ALK5 (12, 17, 18, 20). Similar to ALK4 and ALK5, ALK7 also activates the Smad2/3 signaling pathway (20–22). The human ALK7 cDNA has been recently cloned by us (12) and another group (20). We found that alternative splicing of the ALK7 gene generates four transcripts, designated ALK7-1, -2, -3, and -4 (12). ALK7-1 encodes full-
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Progression of cells through the cell cycle requires sequential activation of cyclin-dependent kinases and their regulatory subunits, cyclins (26–28). During progression through G1 phase, expression of D-type cyclins (D1, D2, and D3) increases, and they then associate with and activate Cdk4/6 (27). On the other hand, an increase in cyclin E/Cdk2 activity is required for commitment from G1 to S phase (27, 28). The Cdk4/6 and Cdk2 activities are controlled by two groups of cell cycle inhibitors, the Ink4 and Cip/Kip family, respectively (26, 27). The Ink4 family is composed of several proteins, including p15, p16, p18, and p19 whereas the Cip/Kip family has three members, p21, p27, and p57 (28). Many of these cell cycle inhibitors, such as p21, p27, and p15, have been shown to be regulated by TGF-β, and are involved in TGF-β-induced cell growth arrest (29).

In this study, we investigated the role and signaling of Nodal and ALK7 in normal and tumor trophoblast cell lines. We demonstrated that overexpression of Nodal and constitutively active ALK7 inhibited proliferation and induced apoptosis. The effect of Nodal is mediated by ALK7 and Smad2/3, as the dominant-negative forms of these molecules blocked the effect of Nodal on trophoblast cell growth. Furthermore, we identified several genes that are involved in cell cycle progression as targets of Nodal and ALK7.

EXPERIMENTAL PROCEDURES

Cell Lines and Cell Culture—The human choriocarcinoma cell lines JEG-3, JAR, and BeWo were obtained from American Type Culture Collection (Manassas, VA). The immortalized first trimester trophoblast cell line HTR8/SVneo was established from normal human trophoblast cells as described previously (30). A stable JAR cell line overexpressing Smad3 (JAR-Smad3/c) was developed as described previously (31). JEG-3, JAR, and Smad3/c, and HTR8/SVneo cells were cultured in RPMI 1640 medium (Invitrogen), whereas BeWo cells were cultured in Ham’s F-12 medium and Dulbecco’s modified Eagle’s medium (1:1; Hyclone Laboratories, Logan, UT) supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin (Invitrogen) in the presence of 10% fetal bovine serum (Sigma).

RNA Extraction and RT-PCR—Total RNA was extracted using TRIzol reagent (Invitrogen) following the manufacturer’s protocols and stored at −80 °C until RT-PCR analysis. Five micrograms of total RNA were reverse-transcribed into cDNA in a total volume of 50 μl using 0.5 μg of oligo(dT) primer (Amersham Biosciences) and 500 units of Moloney murine leukemia virus reverse transcriptase (New England Biolabs Inc., Mississauga, Ontario, Canada). The reaction was carried out at 37 °C for 2 h in 1× reaction buffer (50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, and 10 mM dithiothreitol) containing 0.5 mM dNTPs, 10 mM dithiothreitol, and 50 units of RNase inhibitor (RNAGuard, Amersham Biosciences) and terminated by heating the mixture at 95 °C for 5 min. An aliquot of the cDNA sample (2 μl) was subjected to PCR, which was performed in the presence of 10 mM Tris-HCl (pH 8.3), 2.0

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**TABLE I**

| Target mRNA          | Forward primer | Sequence (5' → 3')                  |
|----------------------|----------------|-------------------------------------|
| ALK7-1, -3, -4 (AY127050) | Forward primer | GCACTTCAAAGGGTGTCG                  |
| Reverse primer       |                | GACATTCCAGCAGATGTC                  |
| ALK7-2 (AF525679)    | Forward primer | CGAATTCGTCCGCAAGGTGCA               |
| Reverse primer       |                | GTGCAAGTTGGTGGTGGTGGTC              |
| ALK7-1 (AY127050)    | Forward primer | ATGACCGGGCGCTCTGCTCA                |
| Reverse primer       |                | ATACGTGAGCAGTGACGTA                 |
| ALK7 expression construct (AY127050) | Forward primer | AGACATCATCAGCCAGGCTACA             |
| Reverse primer       |                | GTCACATTGAAACCGCTTACAG              |
| ALK7-1, -3, -4 (AY127050) | Forward primer | GCACATCCGAGGAAGGGAGTG               |
| Reverse primer       |                | GACATTCCAGCAGATGTC                  |
| p15 (BC014469)       | Forward primer | TGAGGGAATGAACTTCGCCCT              |
| Reverse primer       |                | TTGGGCTCTCCTTCGCCCT                 |
| p21 (U03106)         | Forward primer | TGCCGAAGTTACTACAGA                  |
| Reverse primer       |                | TTGGGGACAAGTCGTGAAAC                |
| p27 (U10906)         | Forward primer | AAGTCTACCTTGGACGTGAC                |
| Reverse primer       |                | ACGGCTTCGTACACCCTCTCT               |
| GAPDH (M33197)       | Forward primer | CCGAAATCCGAGGGAACGGCTCTGCT         |
| Reverse primer       |                | TCCCGGGGCTCTTTGAGCTCTTGGCACCA       |
| Nodal expression construct (AB067630) | Forward primer | CGGAATTCGACCAGGCGTCCTGCT           |
| Reverse primer       |                | CCGGAATTCGAGGAGGACACCCACATTCTCCAC  |

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Nodal expression construct (AB067630)

Length ALK7, whereas the other transcripts encode three novel ALK7 isoforms (12). These isoforms include a truncated receptor missing the ligand-binding domain and two soluble proteins that have the ligand-binding domain, but no transmembrane domain (12). Unlike rat ALK7, human ALK7 is widely distributed in various tissues, including brain, pancreas, kidney, ovary, and placenta (12). Using reverse transcription (RT)-PCR and Western blot analysis, we have found that ALK7 and its isoforms are expressed in placenta from early to late gestation (12), suggesting a role for ALK7 during pregnancy. The role of ALK7 is largely unknown. In the rat neuronal cell line PC12, ALK7 has been found to arrest cell proliferation and to induce morphological differentiation (21).

Nodal has been shown to interact with two types of receptor complexes. First, Nodal can bind to the activin receptor complex containing activin receptors IIB and IB (ALK4) (19, 23, 24). Signaling of Nodal through this receptor complex requires Cripto since, in the absence of Cripto, Nodal loses its signaling activity through the activin receptor complex (19, 23–25). The other receptor complex that mediates Nodal action is activin receptor IIB-ALK7, which was performed in the presence of 10 mM Tris-HCl (pH 8.3), 2.0
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DNA was diluted into 150 mM NaCl. After incubation at room temperature for 10 min, these two solutions were mixed and further incubated at room temperature for 20 min. The polyethyleneimine/DNA mixture was then diluted into Opti-MEM and added to the cells. After overnight incubation at 37 °C, the culture medium was changed to RPMI 1640 medium supplemented with 10% fetal bovine serum. Transfection efficiency, estimated by transfecting the cells with pEGFP, was >60% for 100-mm dishes, 40–50% for cells in 24-well plates, and 30% for cells cultured in 96-well plates.

**Determination of Cell Growth**—Cell growth was determined either by direct cell counting or by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. For cell counting experiments, JEG-3, JAR, and JAR-Smad3/c cells were cultured in 24-well culture plates at a cell density of 2 × 10^5 cells/well. Cells were transiently transfected with 1 µg of pcDNA4, wild-type ALK7 (ALK7-wt), and ALK7-ca plasmid DNA (n = 6). Cells were trypsinized 48 h after transfection, and cell number was counted using a hemocytometer. Each experiment was repeated three times. For MTT assays, JEG-3, JAR, JAR-Smad3/c, and HTR8/SVneo cells were seeded on 96-well plates at a density of 10^4 cells/well. After 16–20 h of incubation, cells were transfected with different constructs (0.3 µg of pcDNA4, 0.18 mM polyethyleneimine (0.28 µl/µg of DNA), and 0.18 mM polyethyleneimine (0.28 µl/µg of DNA) before being used in the experiments. All constructs were fully sequenced.

**Transient Transfection**—Transient transfection was carried out using 25-kDa polyethylenimine (Sigma) as described previously (32). Briefly, cells were seeded at 50% cell density on tissue culture dishes (Sarstedt, Inc., Montreal, Quebec, Canada) and allowed to adhere and grow overnight. The cells were washed, and the culture medium was replaced with reduced serum Opti-MEM (Invitrogen) 2 h prior to transfection. Plasmid DNA and 0.18 µl polyethylenimine (0.28 µl/µg of DNA) were mixed with 0.18 µl polyethyleneimine (0.28 µl/µg of DNA) and were incubated at room temperature for 20 min. The polyethyleneimine/DNA mixture was then diluted into Opti-MEM and added to the cells. After overnight incubation at 37 °C, the culture medium was changed to RPMI 1640 medium supplemented with 10% fetal bovine serum. Transfection efficiency, estimated by transfecting the cells with pEGFP, was >60% for 100-mm dishes, 40–50% for cells in 24-well plates, and 30% for cells cultured in 96-well plates.

**Flow Cytometry**—JEG-3 cells were plated on 100-mm dishes and transfected with 15 µg of pcDNA4, ALK7-wt, ALK7-ca, or Nodal con-
Experiments were repeated twice with similar results. *, p < 0.05 versus the respective pcDNA control.

Forty-eight hours after transfection, proliferation and apoptosis assays were performed. Proliferation was determined by measuring incorporation of bromodeoxyuridine (BrdUrd) into DNA. Cells were incubated with 10 µM BrdUrd (Sigma) for 1 h, trypsinized, washed twice with phosphate-buffered saline (PBS), and fixed in ethanol. The cell suspension was then treated with 2 N HCl at 37 °C for 30 min, washed with 0.1 N NaOAc, and resuspended in PBS containing 0.5% Tween 20 and 1% bovine serum albumin to a density of 10^6 cells/100 µl. Cells were further incubated with 10 µl of fluorescein isothiocyanate-conjugated mouse anti-BrdUrd monoclonal antibody (Dako Corp., Mississauga) for 30 min. After washing, the cells were resuspended in PBS containing 5 µg/ml propidium iodide and immediately analyzed using a FACScan (BD Biosciences). Apoptosis was assayed using an annexin V apoptosis detection kit (Santa Cruz Biotechnology Inc., Santa Cruz, CA) following the instructions of the manufacturer. Briefly, cells were trypsinized and then pelleted by centrifugation. The cells were washed twice with cold PBS and resuspended in the assay buffer to obtain a density of 10^6 cells/ml. Annexin V-fluorescein isothiocyanate was added to cell aliquots (2 × 10^6 cells), followed by incubation at room temperature in the dark for 15 min. The cells were then washed with cold PBS and subjected immediately to FACScan analysis.

**Hoechst Staining—JEG-3 cells** were seeded on 100-mm culture dishes and transfected with pcDNA4, ALK7-wt, ALK7-ca, or Nodal plasmid DNA (15 µg/dish). Forty-eight hours after transfection, cells were subjected to trypsin treatment at 37 °C for 3 min, fixed with 4% formalin in PBS, and washed with PBS. Cells were then incubated with 0.1 µg/ml Hoechst 33342 (bisbenzimide, Sigma) and spotted on slides for microscopy. Nuclear morphology was observed and photographed using a Zeiss Axiosvert 35 fluorescent microscope.

**Protein Extraction and Western Blot Analyses—HTR8/SVneo and JEG-3 cells** cultured in 100-mm dishes were transfected with 15 µg/dish plasmid DNA (pcDNA4, ALK7-ca, ALK7-kd, Nodal, or ALK7-kd). Forty-eight hours after transfection, cells were washed twice with ice-cold PBS and lysed with radiolmmune precipitation assay buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, and 1% SDS) containing 1 mM dithiothreitol, 1 mM Na3VO4, 5 mM NaF, 100 mM EDTA, 10 mg/ml aprotinin, and 100 mM phenylmethylsulfonyl fluoride. Protein samples were subjected to SDS-PAGE and blotted onto a nitrocellulose membrane. The membrane was blocked with TBST (10 mM Tris-Cl (pH 8.0), 150 mM NaCl, and 0.05% Tween 20) containing 5% nonfat dry milk powder (TBSTBM) at room temperature for 30 min. The membrane was then incubated for 2 h at room temperature with a primary antibody (rabbit anti-cleaved caspase-3 (Asp175) polyclonal antibody (1:2000) (Cell signaling Technology); goat anti-Cdk2 polyclonal antibody (1:500), mouse anti-cyclin D1 monoclonal antibody (1:2000), mouse anti-cyclin E monoclonal antibody (1:2000) (all from Santa Cruz Biotechnology Inc.); or goat anti-p27 polyclonal antibody (1:2000) (BD Signal Transduction)) prepared in TBSTBM. The membranes were washed three times with TBST for 15 min each and then incubated for 2 h with a horseradish peroxidase-conjugated secondary antibody (donkey anti-rabbit IgG, goat anti-mouse IgG, or rabbit anti-goat IgG antibody (1:5000)) in TBSTBM. After washing as described above, signals were detected using an ECL kit (Amersham Biosciences) according to the instructions of the manufacturer.

**Statistical Analysis**—Differences among several groups were determined by one-way analysis of variance, followed by Student-Newman-Keul’s test using GraphPad InStat software. For comparison between two groups, Student’s t test was used. p < 0.05 was considered significant.

**RESULTS**

**Expression of ALK7, Nodal, and Smad mRNAs in Trophoblast Cell Lines**—The mRNA expression of ALK7, Nodal, Smad2, and Smad3 was determined in several trophoblast cell lines, including JEG-3, JAR, and BeWo choriocarcinoma cells; a stable JAR cell line overexpressing Smad3; and an immortalized normal trophoblast cell line, HTR8/SVneo. Using a pair of primers spanning exons III and IV of the ALK7 gene, which are known to be alternatively spliced to generate different transcripts (12), we detected three DNA fragments corresponding to the expected sizes of ALK7-1, -3, and -4 in all trophoblast cell lines tested (Fig. 1A). Similarly, when a pair of primers specific for ALK7-2 was used in PCR, a DNA fragment of the expected size was obtained (Fig. 1A).

Since ALK7 has been shown to mediate the effect of Nodal in mesoderm induction (19) and to activate Smad2 and Smad3 (23, 25), we also examined mRNA expression of Nodal, Smad2, and Smad3. Similar to our previous studies that showed that Nodal mRNA is expressed in JEG-3 cells and in normal placenta (12), RT-PCR detected Nodal mRNA in JEG-3, JAR, JAR-Smad3/c, and HTR8/SVneo cells (Fig. 1A). Both Smad2 and Smad3 mRNAs were detected in the trophoblast cell lines (Fig. 1B). Whereas Smad2 mRNA levels were similar in all cell lines tested, Smad3 mRNA levels were much lower in JEG-3 and JAR cells than in the other cell lines, confirming our previous findings (33).

**Nodal and ALK7 Inhibit Trophoblast Cell Growth via Smad2/3**—To determine the function of ALK7 in trophoblast
cells, we first evaluated its effect on cell growth. JEG-3, JAR, and JAR-Smad3/c cells were transiently transfected with ALK7-wt, ALK7-ca, or the empty vector as a negative control, and cell number was determined 2 days after transfection. Overexpression of ALK7-wt slightly reduced cell numbers, whereas overexpression of ALK7-ca significantly inhibited cell growth. A 27.5% decrease in cell number was observed in JEG-3 cells overexpressing ALK7-ca compared with the empty vector control. ALK7-ca caused 30.7 and 31.5% inhibition in JAR and JAR-Smad3/c cells, respectively (Fig. 2). The growth inhibitory effect of ALK7 was further examined in JEG-3 and JAR cells using the MTT method. In these experiments, cells in 96-well plates were transiently transfected with different amounts of ALK7-ca or empty vector cDNA, and the number of metabolically active cells was determined 48 h after transfection. A dose-dependent inhibition of cell growth was observed in ALK7-ca-transfected cells compared with the vector control in both JEG-3 and JAR cells (Fig. 3A). Time course studies were also performed, and the effect of ALK7-ca on cell growth was observed 24–72 h after transfection, with the most significant effect of ALK7-ca seen 48 h after transfection (data not shown).

To determine whether Nodal can act through ALK7 to regulate trophoblast cell growth, JEG-3 and JAR cells were transiently transfected with different amounts of empty vector or Nodal cDNA, and the number of metabolically active cells was determined 48 h after transfection. A dose-dependent inhibition of cell growth was observed in all three cell lines tested. Moreover, Nodal overexpression blocked the growth inhibitory effect of ALK7-ca in all three cell lines tested. In HTR8/SVneo cells, both dominant-negative Smad2 and Smad3 blocked the growth inhibitory effect of Nodal and ALK7-ca; however, in JEG-3 cells, only dominant-negative Smad2 was able to neutralize the effect of Nodal and ALK7-ca.

Fig. 4. A kinase-deficient mutant of ALK7 blocks the inhibitory effect of Nodal on cell growth. JEG-3 (upper panel), JAR (middle panel), and HTR8/SVneo (lower panel) cells were transfected with 0.15 μg of Nodal alone or together with 0.15 μg of ALK7-kd. ALK7-ca and pcDNA4 were included as positive and negative controls, respectively. Cell growth was determined using MTT assays 48 h after transfection. Data represent means ± S.E. (n = eight wells). The experiment was repeated three times with similar results. Different letters denote statistical significance (p < 0.05). ALK7-kd blocked the inhibitory effect of Nodal on cell growth in all three cell lines tested.

Fig. 5. Effects of dominant-negative Smad2 and Smad3 on Nodal- and ALK7-ca-inhibited cell growth. HTR8/SVneo (A) and JEG-3 (B) cells were transiently transfected with 0.15 μg of Nodal or ALK7-ca either alone or in combination with 0.15 μg of dominant-negative (DN) Smad2 or Smad3. Cell growth was determined using MTT assays 48 h after transfection. Data represent means ± S.E. (n = eight wells). The experiment was repeated twice with similar results. Different letters denote statistical significance (p < 0.05). In HTR8/SVneo cells, both dominant-negative Smad2 and Smad3 blocked the growth inhibitory effect of Nodal and ALK7-ca; however, in JEG-3 cells, only dominant-negative Smad2 was able to neutralize the effect of Nodal and ALK7-ca.
either alone or in combination with ALK7-wt or ALK7-kd. In all three cell lines, overexpression of Nodal resulted in a significant decrease in the number of metabolically active cells compared with the empty vector control. Similarly, overexpression of ALK7-ca had an inhibitory effect on cell growth. However, ALK7-kd, the dominant-negative form of ALK7 that was generated by mutating the ATP-binding site of the kinase domain, completely abolished the inhibitory effect of Nodal on cell growth (Fig. 4).

It has been shown that ALK7 activates Smad2 and Smad3 (20–22). We therefore determined whether the effect of Nodal and ALK7 on cell growth is mediated by Smad2 and/or Smad3. In HTR8/SVneo cells, which express both Smad2 and Smad3, overexpression of either dominant-negative Smad2 or Smad3 blocked the effect of Nodal and ALK7-ca on cell growth (Fig. 5A). In JEG-3 cells, which have a very low level of Smad3 mRNA (Fig. 1) (33) and no detectable Smad3 protein (32), overexpression of dominant-negative Smad3 had little effect on either Nodal- or ALK7-ca-induced cell growth arrest. However, overexpression of dominant-negative Smad2 completely eliminated the effect of Nodal and ALK7-ca (Fig. 5B).

Nodal and ALK7 Induce Apoptosis and Inhibit Proliferation—Since the decrease in cell number after Nodal and ALK7-ca treatments could be the result of a decrease in cell proliferation and/or an increase in apoptosis, we subsequently determined whether Nodal and ALK7-ca affect cell proliferation and apoptosis. JEG-3 cells were transiently transfected with pcDNA4 (control), ALK7-wt, ALK7-ca, or Nodal. Overexpression of ALK7 and Nodal was confirmed by RT-PCR analysis using primers specific for ALK7-1 and primers that span the entire coding region of Nodal. In cells transfected with Nodal cDNA, there was a much higher level of Nodal mRNA; and in cells transfected with either ALK7-ca or ALK7-wt, the mRNA level of ALK7-1 was greatly increased (Fig. 6). The cell density and cell morphology of the ALK7-wt-transfected cells were similar to those of the control cells (empty vector-transfected). However, in the Nodal- or ALK7-ca-transfected cells, there was a dramatic decrease in cell density. Also, cells appeared to form aggregates (Fig. 6).

The number of cells undergoing apoptosis was measured by annexin V staining, followed by flow cytometry. There was no difference in the number of apoptotic cells between pcDNA4- and ALK7-wt-transfected cells. However, the number of cells undergoing apoptosis was 4-fold higher in Nodal- or ALK7-ca-transfected cells than in the control cells (Fig. 7A). To confirm that Nodal and ALK7 induce apoptosis in trophoblast cells, Hoechst staining was also performed, and typical morphological features of apoptotic nuclei, such as DNA condensation and fragmentation and nuclear shrinkage, were observed in ALK7-ca- and Nodal-transfected cells (Fig. 7B). Using an antibody specific for the cleaved caspase-3 fragments, we detected an increase in active caspase-3 levels in Nodal- and ALK7-ca-transfected cells (Fig. 7C). Analysis of cell proliferation by staining actively proliferating cells using BrdUrd revealed that the number of proliferating cells (i.e. in S phase of the cell cycle) in pcDNA4- or ALK7-wt-transfected cells ranged from 30 to 40%, whereas in Nodal- or ALK7-ca-transfected cells, only 5–8% of the cells were actively proliferating (Fig. 8A).

Nodal and ALK7 Up-regulate p27 and Down-regulate Cyclin D1 and Cdk2—To further understand how Nodal and ALK7 regulate trophoblast cell proliferation, we used RT-PCR to measure mRNA levels of three cell cycle regulators, p15, p21, and p27. Overexpression of Nodal and ALK7-ca slightly increased p21 mRNA levels, but significantly increased p15 and p27 mRNA levels (Fig. 8B). Since p27 was most strongly induced by Nodal and ALK7-ca, Western blot analysis was conducted to confirm its up-regulation by Nodal and ALK7-ca. As shown in Fig. 9, significant increases in p27 protein levels after Nodal and ALK7-ca transfection were observed in both JEG-3 and HTR8/SVneo cells. Overexpression of ALK7-kd alone did not affect p27 expression; however, it neutralized the effect of Nodal. On the other hand, Nodal and ALK7-ca decreased Cdk2 levels in both cell lines tested, and the effect of Nodal was blocked by cotransfection with ALK7-kd (Fig. 9). There were some differences in cyclin E expression between JEG-3 and HTR8/SVneo cells. The major form detected in JEG-3 cells was the unphosphorylated form, whereas in HTR8/SVneo cells, the predominant form was phosphorylated cyclin E. Also, overexpression of Nodal or ALK7-ca in JEG-3 cells resulted in a reduction in the phosphorylated cyclin E levels, but no significant effect was observed in HTR8/SVneo cells (Fig. 9). Treatment by Nodal and ALK7-ca also significantly inhibited cyclin D1 expression (Fig. 9). In these experiments, equal loading of protein samples was confirmed by Western blotting and probing with an anti-β-actin antibody.

DISCUSSION

This study has demonstrated for the first time that Nodal, acting through ALK7, is involved in the regulation of trophoblast cell proliferation and apoptosis and that its effect on cell proliferation may be mediated in part by the p27-cyclin E/Cdk2 pathway.

Previously, we have shown that there are four ALK7 transcripts derived from alternative splicing of the ALK7 gene (12). ALK7-1, -2, -3, and -4 encode the full-length receptor, the truncated receptor lacking part of the ligand-binding domain, and two soluble proteins that have no transmembrane and GS domains, respectively (12). In this study, all four ALK7 transcripts and Nodal mRNA were detected in all trophoblast cell
ALK5 (20) pathway involving Smad2 and Smad3, similar to ALK4 and ALK7. It has been found that ALK7 activates a signaling
Nodal (19), indicating that Nodal is a physiological ligand of
receptor IIB and ALK7 can form a functional receptor complex
previous studies (33). A recent study has shown that activin
cells. These results are in agreement with those from our
levels of Smad3 mRNA (33), but not Smad3 protein (32), can be
in the growth inhibitory effect of Nodal between cells that have
detected (32), both dominant-negative Smad2 and Smad3
ants. The constitutively active
form of ALK7 mimics the activity of Nodal in mesoderm induc-
whereas dominant-negative ALK7 abolishes the effect of
Nodal (19), indicating that Nodal is a physiological ligand of
ALK7. It has been found that ALK7 activates a signaling
pathway involving Smad2 and Smad3, similar to ALK4 and
Thus, the expression of these molecules in trophoblast cells suggests that the Nodal-ALK7 signaling pathway exists in human trophoblast cells.

The data obtained in this study indicate that Nodal, acting through the ALK7 pathway, has an inhibitory effect on trophoblast cell growth. To determine the role of Nodal-ALK7 pathway in trophoblast cells, we first generated constitutively active ALK7 that can function in the absence of the ligand. Overexpression of ALK7-ca significantly reduced cell numbers compared with the empty vector control. Similarly, overexpression of Nodal also inhibited cell growth. To investigate whether the effect of Nodal is mediated by ALK7, we transfected cells with Nodal alone or in combination with kinase-deficient ALK7 that dominantly negatively blocks ALK7 signaling (20, 21). In both normal trophoblast and choriocarcinoma cell lines, over-expression of ALK7-kd completely abolished the inhibitory effect of Nodal on cell growth, demonstrating that Nodal acts through ALK7 to inhibit trophoblast cell growth. This notion is further supported by the findings that ALK7-kd also blocked the effect of Nodal on p27, Cdk2, and cyclin D1 expression. Nodal is also known to act through ALK4 in a Cripto-dependent manner (23–25). Whether or not ALK4 and Cripto are involved in the growth inhibitory action of Nodal in trophoblast cells requires further investigation.

Smad2 and Smad3 act downstream of ALK7, as activation of ALK7 results in phosphorylation of Smad2 and Smad3 (20–22) and induction of Smad2/3-dependent reporter constructs (22, 24). Similarly, Nodal has been shown to induce the transcriptional activity of Smad2/3-dependent promoters (33). To confirm the involvement of Smad2/3 in Nodal-ALK7-induced cell growth arrest, we examined the interaction between dominant-negative Smad2/3 and Nodal or ALK7-ca. In HTR8/SVneo cells, in which both Smad2 and Smad3 mRNAs and proteins have been detected (32), both dominant-negative Smad2 and Smad3 mutants significantly reduced the effect of Nodal and ALK7-ca on cell growth inhibition. However, in JEG-3 cells, only dominant-negative Smad2 (but not Smad3) could completely block the effect of Nodal and ALK7-ca. This result is consistent with the finding that Smad2 expression in JEG-3 cells is comparable with that in normal trophoblasts (32, 33), whereas only low levels of Smad3 mRNA (33), but not Smad3 protein (32), can be detected. Our findings confirm that Smad2 and Smad3 are downstream signaling molecules of Nodal-ALK7. These results, together with the observation that there is no difference in the growth inhibitory effect of Nodal between cells that have normal levels of Smad3 mRNA (33), but not Smad3 protein (32), can be detected. Our findings confirm that Smad2 and Smad3 are downstream signaling molecules of Nodal-ALK7. These results, together with the observation that there is no difference in the growth inhibitory effect of Nodal between cells that have normal levels of Smad3 (HTR8/SVneo) and no detectable Smad3 (JEG-3 and JAR), suggest that Smad2 can compensate for the loss of Smad3 in Nodal signaling.

Based mainly on studies in mouse, Xenopus, and zebrafish, Nodal has been identified to be a critical regulator of early vertebrate development. Nodal may also be involved in regulating placental function since, in the rat, it has been suggested that Nodal may inhibit the differentiation of stem trophoblast cells into giant cells (11). Recently, Nodal mRNA has been detected in a number of cell lines originating from mouse mammary gland (34) and human ovary (35), suggesting that Nodal also plays a role in adult life. However, no functional studies have been reported on the role of Nodal in mammalian cells.
during adult life. Similarly, the function of ALK7 is poorly understood. ALK7 has been shown to mediate the effect of Nodal in mesoderm induction during embryonic development (19). It also inhibits proliferation in PC12 cells (21). During the revision of this manuscript, Kim et al. (36) reported that ALK7 induces apoptosis in the rat hepatoma cell line FaO. In the present study, we found that Nodal, acting through ALK7 and Smad2/3, induced cell growth arrest. Using flow cytometry, we further demonstrated that Nodal and ALK7 inhibited cell proliferation and induced apoptosis in trophoblast cells. We have observed similar effects of Nodal-ALK7 on proliferation and apoptosis in human ovarian cancer cells.2 Taken together, these findings suggest that, similar to TGF-β, Nodal may be a multifunctional cytokine involved in the regulation of various cellular activities in both prenatal and postnatal stages.

2 G. Xu, Y. Zhong, S. Munir, B. Yang, B. K. Tsang, and C. Peng, unpublished data.
blocked the effect of Nodal. Since p27 is known to inhibit the activity of cyclin E/Cdk2 (37–39), these results suggest that Nodal, acting through ALK7, up-regulates p27, which, in turn, inhibits cyclin E/Cdk2, leading to the inhibition of cell entry into S phase.

Although we have demonstrated that p27 is a target gene of the Nodal-ALK7 pathway, it is possible that other cyclin-dependent kinase inhibitors are also involved in the anti-proliferative action of Nodal-ALK7. In PC12 cells, overexpression of ALK7-ca leads to induction of p15 and p21 promoter activities (21). In this study, we also observed a significant increase in p15 mRNA levels and a slight increase in p21 mRNA levels, suggesting that these proteins may play a role in mediating the effect of Nodal and ALK7. Also, p27 is regulated by multiple mechanisms, including transcriptional, translational, post-transcriptional, and subunit assembly (26–28, 41). Therefore, more studies are required to fully understand the mechanisms underlying the inhibition of cell cycle progression by the Nodal-ALK7 pathway.

The loss of responsiveness to growth inhibitory effects of growth factors, such as TGF-β, is a common phenomenon during tumorigenesis (42–44). In normal trophoblast cells, TGF-β inhibits proliferation; however, in choriocarcinoma cell lines, such as JEG-3 and JAR, TGF-β loses its growth inhibitory effect (45–47). Although the mechanism underlying the resistance of choriocarcinoma cells to TGF-β is unclear, it is unlikely that this is entirely due to defects at the level of the receptor or Smad signaling pathway, as TGF-β is functional in regulating
Steroid hormone production (47). Furthermore, TGF-β responses are incompletely restored in Smad3-restituted JAR cells (48). It is therefore possible that the resistance occurs at the level of target genes. Interestingly, we found that Nodal and ALK7-ca inhibited cell growth in both normal and tumor trophoblast cell lines. There were no major differences in the response to Nodal or ALK7-ca among different cell lines examined. These results suggest that the mechanisms whereby the Nodal-ALK7 pathway induces cell growth arrest are different from those of TGF-β.

In conclusion, we have demonstrated that activation of the Nodal-ALK7 pathway results in inhibition of proliferation and induction of apoptosis in human trophoblast cells. We have further shown that p27 is up-regulated, whereas Cdk2 and cyclin D1 are down-regulated by signaling of Nodal through the Nodal-ALK7 pathway results in inhibition of proliferation and induction of apoptosis in human trophoblast cells. We have

Acknowledgment—We thank Dr. B. Loughton for critically reviewing the manuscript.

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Steroid hormone production (47). Furthermore, TGF-β responses are incompletely restored in Smad3-restituted JAR cells (48). It is therefore possible that the resistance occurs at the level of target genes. Interestingly, we found that Nodal and ALK7-ca inhibited cell growth in both normal and tumor trophoblast cell lines. There were no major differences in the response to Nodal or ALK7-ca among different cell lines examined. These results suggest that the mechanisms whereby the Nodal-ALK7 pathway induces cell growth arrest are different from those of TGF-β.

In conclusion, we have demonstrated that activation of the Nodal-ALK7 pathway results in inhibition of proliferation and induction of apoptosis in human trophoblast cells. We have further shown that p27 is up-regulated, whereas Cdk2 and cyclin D1 are down-regulated by signaling of Nodal through ALK7, suggesting that the Nodal-ALK7 pathway induces G2 cell cycle arrest in part via p27. Our studies suggest that Nodal is a multifunctional cytokine involved in regulating a variety of cellular activities.

Acknowledgment—We thank Dr. B. Loughton for critically reviewing the manuscript.
