Antisense Suppression of Pygopus2 Results in Growth Arrest of Epithelial Ovarian Cancer

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Abstract

Purpose: The Pygopus proteins are critical elements of the canonical Wnt/β-catenin transcriptional complex. In epithelial ovarian cancer, constitutively active Wnt signaling is restricted to one (endometrioid) tumor subtype. The purpose of this study was to determine the level of expression and growth requirements of human Pygopus2 (hPygo2) protein in epithelial ovarian cancer.

Experimental Design: Expression and subcellular localization of hPygo2 was determined in epithelial ovarian cancer cell lines and tumors using Northern blot, immunoblot, and immunofluorescence. Immunohistochemistry was done on 125 archived patient epithelial ovarian cancer tumors representing all epithelial ovarian cancer subtypes. T-cell factor–dependent transcription levels were determined in epithelial ovarian cancer cells using TOPflash/FOPflash *in vivo* assays. Phosphorothioated antisense oligonucleotides were transfected into cell lines and growth assayed by cell counting, anchorage-independent colony formation on soft agar, and xenografting into severe combined immunodeficient mice.

Results: All six epithelial ovarian cancer cell lines and 82% of the patient samples overexpressed nuclear hPygo2 compared with control cells and benign disease. Depletion of hPygo2 by antisense oligonucleotides in both Wnt-active (TOV-112D) and Wnt-inactive serous (OVCAR-3, SKOV-3) and clear cell (TOV-21G) carcinoma cell lines halted growth, assessed using tissue culture, anchorage-independent, and xenograft assays.

Conclusions: hPygo2 is unexpectedly widely expressed in, and required in the absence of, Wnt signaling for malignant growth of epithelial ovarian cancer, the deadliest gynecologic malignancy. These findings strongly suggest that inhibition of hPygo2 may be of therapeutic benefit for treating this disease.

Deregulation of developmentally important genes in adults can contribute to malignant disease. For instance, the canonical Wnt/β-catenin pathway is normally required for embryonic development and for the controlled proliferation of adult stem cells (1–3) but is frequently abnormally activated in cancer. Determining critical components of this pathway, therefore, is an important means to identify novel therapeutic targets.

In canonical Wnt/β-catenin signaling, binding of the Wnt ligand to its receptor initiates an intracellular signaling cascade resulting in the stabilization of cytoplasmic β-catenin, allowing it to accumulate in the nucleus where it converts the lymphoid enhancer/T-cell factor (LEF)/T-cell factor (TCF)-1 complex from a gene transcriptional repressor into an activator. The *pygopus* genes encode proteins that have been shown to function in this pathway during early embryogenesis as a component of the LEF/TCF-1 complex (4–7). Pygopus is proposed to play a role in the activator function of the complex by acting either as a nuclear "escort" (8) or as a critical link in the complex that recruits the basal transcription machinery (9–11).

Pygopus is likely important for Wnt signaling in cancer cells. For example, transfection of siRNA sequences targeting Pygopus mRNA in established colorectal cancer cell lines resulted in inhibition of TCF/LEF–mediated transcriptional activation of reporter genes (7, 8). However, the levels of reduction of endogenous Pygopus protein were not assessed in these studies. Whether the depletion of Pygopus resulted in phenotypic changes to the malignant cells was also not determined. Indeed, the expression of endogenous Pygopus proteins has yet to be assessed in any malignancy. Understanding of the requirement of Pygopus proteins in cancer, therefore, is far from complete.

In humans, there are two *pygopus* genes, *hPygo1* and *hPygo2*, both of which are presumed to have overlapping, if not

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redundant, roles in TCF-mediated transcription. Epithelial ovarian cancer is the deadliest gynecologic malignancy, resulting from transformation of the ovarian surface epithelium. The pathology of epithelial ovarian cancer is diverse, with tumors forming at least five different subtypes, including serous, endometrioid, mucinous, clear cell, and undifferentiated. Although there are malignancies, such as colorectal (12) and breast (13) cancer, in which Wnt signaling has a significant role, most epithelial ovarian cancer tumors are derived from the subtypes in which the canonical Wnt pathway may not play a significant role (14). Only those of the endometrioid subtype seem to be constitutively active in Wnt signaling (15–17). The question remains, therefore, whether the expression and requirement for the Pygopus proteins are restricted to the endometrioid subtype, as expected, or whether they are expressed in and/or required for those subtypes in which Wnt signaling is not active.

Our results show that one of the Pygopus family members, hPygo2, is overexpressed in six malignant epithelial ovarian cancer cell lines in relation to nonmalignant immortalized and normal ovarian surface epithelium. hPygo2 was also unexpectedly overexpressed in the nuclei of a majority of patient epithelial ovarian cancer tumors, including those derived from subtypes of Wnt active (endometrioid) and those of Wnt-inactive (nonendometrioid) origin. Using hPygo2-specific antisense knockdown technology, we show a requirement of hPygo2 for growth of epithelial ovarian cancer cell lines derived from tumors of Wnt-inactive, serous, and clear cell origin, indicating that hPygo2 is required in the absence of Wnt signaling for epithelial ovarian cancer growth. Our findings suggest that the widespread nuclear expression among different epithelial ovarian cancer subtypes and requirement of hPygo2 for epithelial ovarian cancer growth indicate that it is a strong candidate for therapeutic targeting for this malignancy.

Materials and Methods

Cell culture. Cell lines were obtained from American Type Culture Collection (Manassas, VA) and were cultured in DMEM (Invitrogen, Burlington, Canada) containing 10% FCS, 100 units/mL penicillin, and 100 μg/mL streptomycin. Normal ovarian surface epithelial cells were obtained as described (18) in accordance with Memorial University of Newfoundland human investigation committee protocol 01.53 with patient consent, and cultured in MCDB105 (Sigma-Aldrich, Oakville, Canada) and 199 (Sigma; 1:1) containing 15% FCS, 100 units/mL penicillin, and 100 μg/mL streptomycin as described (18). Immortalized ovarian surface cells (iOSE397) were received as a gift from Dr. Nelly Auersperg (Department of Obstetrics and Gynecology, University of British Columbia, Vancouver, Canada). These cell lines were derived from normal ovarian surface epithelial cells that were transfected with SV40 large T-antigen and have characteristics of early neoplasms. Cells were counted using a hemacytometer.

Statistics. Means and SDs were used to derive *P* values calculated using the Student's *t* test and significance indicated using a confidence interval of 95%.

RNA extraction and Northern blotting. Total cellular RNA was extracted using RNeasy (Qiagen, Missisauga, Canada). Northern blot analysis was done at high stringency at 65°C for 15 minutes (19). Semiquantitative reverse transcription-PCR was done as described (19) using hPygo2 (forward: 5′-GCATCCAACCCTTTGAAGATGAC-3′; reverse: 5′-TCAGCCAGGGGGTGCCAAGCTGTTG-3′), hPygo1 (forward: 5′-GCCACGACAACCAAGAGGTG-3′; reverse 5′-CCAGTACAGATCCGATGAAACC-3′), and glyceraldehyde-3-phosphate dehydrogenase (20) oligonucleotides (Invitrogen).

Protein extraction and Western blotting. Eighty percent to 90% confluent transfected or untransfected cell monolayers were washed with cold PBS, lysed in 2× SDS loading buffer, passed through a 21 G needle, and loaded onto 10% to 12% SDS-denaturing polyacrylamide gel. Fractionated proteins were then transferred to Hybond enhanced chemiluminescent nitrocellulose membrane (Amersham Pharmacia Biotech, Montreal, Canada) under semidry conditions and detected using the enhanced chemiluminescence system (Amersham).

Rabbit anti-hPygo2 polyclonal antisera were produced as described (21) using an *in vitro* synthesized polypeptide corresponding to amino acid residues 89 to 328, lacking both NHD and PHD conserved regions of hPygo2 (5), generated by subcloning PCR-derived sequences from a human Pygo2 EST (I.M.A.G.E.) cDNA into pGEX4T1 (Amersham) and tested by immunoprecipitation and Western blot analysis. Anti–extracellular signal-regulated kinase-1 (ERK-1) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Immunohistochemistry. Five-micrometer sections were mounted on coated glass slides (Surgipath, Richmond, IL) and processed as described (22) with hPygo2 primary antisera diluted 1,000× in 1% bovine serum albumin (Sigma) in PBS, β-catenin mouse monoclonal primary antibodies diluted 1/2,000× (Santa Cruz Biotechnology), anti-mouse and anti-rabbit horseradish peroxidase–linked secondary antibodies diluted 1/250× (Amersham) detected using brown staining diaminobenzidine in the presence of H_2O_2 (Sigma). Sections were counterstained with hematoxylin and mounted in Permount (Fisher Scientific, Ottawa, Canada).

Immunofluorescence. Cells grown on glass chamber slides were fixed in 4% formaldehyde and processed for immunofluorescence using hPygo2 primary antisera, β-catenin primary monoclonal antibodies (Santa Cruz Biotechnology), FITC-donkey anti-rabbit (Amersham), or Cy3 donkey anti-mouse (Jackson Laboratories) secondary antibodies and mounted in 10% glycerol/PBS.

Antisense knockdown. Cells (5×10^4 to 6×10^4) were seeded into 12-well plates 24 hours before transfection using the OligofectAMINE Transfection kit (Invitrogen) or the Effectene Transfection kit (Qiagen) with 50 to 100 nmol/L of the following 19 mer HPLC-purified, phosphorothioated oligonucleotides, from oligos, etc. (* indicates a phosphorothioate bond): hPy-ON5 antisense hPygo2 oligo, 5′-G*G*C*TGAGCAAATCGTT*G*G*G; hPy-ON8 antisense hPygo2 oligo, 5′-C*C*T*CTGGCCAGAAAC*T*T*T; 4 base mismatch oligo, 5′-G*C*C*TGAGCTAATCATT*G*G*T (mismatches are in bold). The culture medium was changed 24 and 48 hours after transfection. Transfection efficiencies ranged from 48% (OVCAR-3) to 58% (SKOV-3), determined by the expression of cotransfected green fluorescence protein–expressing plasmids.

Rescue assays. SK-OV-3 cells (4.5×10^4) were seeded in 12-well plates 24 hours before transfection. pCS2+ vector (0.15 μg) harboring the hPygo2 coding region or pCS2+ empty vector were transfected into cells using Effectene. After 24 hours, 100 nmol/L hPy-ON8 antisense oligonucleotide or the mismatched control oligonucleotide were transfected into the cells.

In vitro *transcription assays.* Measurements of the activation of the TCF/LEF–dependent luciferase reporter gene, *TOPflash*, were done as described (16). The ratio of TCF-dependent TOPflash reporter expression to control FOPflash expression was measured 36 hours after transfection with Effectene and then normalized using a β-galactosidase expressing vector.

Soft agar (anchorage-independent) growth assays. An agarose underlying (0.7%) gel was prepared by mixing equal volumes of 2× medium containing 2× fetal bovine serum with melted 1.4% low melting temperature agarose in sterile water. Four hours after transfection, 1×10^3 cells were resuspended in 2× medium containing 2× fetal bovine serum, mixed with equal volumes of melted 0.7% low melting temperature agarose in sterile water, and seeded onto 1 mL of

the underlying layer of solidified agarose. Fresh medium was carefully added at 1 week intervals and colonies counted after 30 days.

Xenograft assays. SK-OV-3 epithelial ovarian cancer cells were transferred s.c. into severe combined immunodeficient mice (Charles River Laboratories, Saint-Constant, Quebec, Canada) essentially as described (23). Cells were transfected with the antisense oligonucleotides as described above. Four hours after transfection, cells were trypsinized, resuspended in an equal volume of Matrigel at 18°C for a final volume of 0.5 mL, and injected s.c. in mice. Animals were monitored daily for tumor growth and sacrificed after 17 weeks. The maximum tumor length was measured using precision digital calipers (Lee Valley Tools, Ottawa, Canada). Tumors were dissected and weighed using an analytic balance.

Results

hPygo2 is overexpressed in epithelial ovarian cancer cell lines. The expression of hPygo2 in epithelial ovarian cancer cell lines was investigated by Northern blot, immunoblot, and immunocytochemical analyses. We found that *hPygo2* mRNA levels were significantly higher in the six cancer cell lines we tested, relative to that of normal surface epithelial cells (Fig. 1A). In addition, whereas hPygo2 protein was found in epithelial ovarian cancer cells, it was barely detectable in the normal primary cultured ovarian surface epithelial cells (Fig. 1B). These observations suggested that overexpression of hPygo2 is a characteristic of epithelial ovarian cancer cell lines.

Previous studies indicated that Pygopus proteins were active in the nuclei of embryonic cells (8). We therefore examined the subcellular localization of endogenous hPygo2 in three different epithelial ovarian cancer cell lines and in a nonmalignant, immortalized ovarian epithelial cell line (iOSE397). Nuclear localization of hPygo2 was confirmed by staining OVCAR-3 cells with a DNA-specific fluorescent dye (Fig. 1C, *Nucl*). Nuclear expression of hPygo2 was consistent in two other malignant lines, TOV21G and TOV112D (Fig. 1D). hPygo2 was also expressed in a perinuclear pattern in the immortalized, nonmalignant control cell line (Fig. 1D, *iOSE397*). In OVCAR-3 cells (Fig. 1C), β-catenin was localized mainly to the plasma membrane, consistent with previous findings (24). β-Catenin was localized mainly to the plasma membrane in immortalized iOSE397 cells as well (Fig. 1D) and was not detectable in the TOV-21G cells, consistent with previous findings (15–17). β-catenin was, however, expressed at relatively high levels in the cytoplasm and nuclei of TOV-112D (endometrioid) cells, as expected, because this cell line has high levels of constitutive canonical Wnt signaling (15). These observations suggest that although β-catenin has variable subcellular expression, hPygo2 is consistently expressed in nuclei of epithelial ovarian cancer cells. Thus, hPygo2 and β-catenin do not necessarily colocalize to nuclei in epithelial ovarian cancer cell lines.

Nuclear localization of hPygo2 in epithelial ovarian cancer. To determine the prevalence of hPygo2 expression in epithelial ovarian cancer disease, we analyzed the *in situ* localization of hPygo2 protein in 125 tumors from archived surgical samples. We semiquantitatively assessed expression based on intensity and percentage of tumor cells stained in both the cytoplasm and nuclei (Fig. 1E). All epithelial ovarian cancer histologic tumor subtypes, including serous, mucinous, clear cell, endometrioid, and undifferentiated were represented in our sample set, but the majority, as expected, were of the serous subtype (25, 26). The tumor subtypes and distribution of level and localization (either nuclear or cytoplasmic) of hPygo2 staining for each subtype is displayed in Table 1.

hPygo2 was undetectable or very weakly expressed in the nuclei of nonmalignant or benign ovarian tumors, such as cystadenomas (Fig. 1E, −). On the other hand, as shown in Table 1 ("total" column for nuclear staining), 82% of the tumors with a pathologic diagnosis consistent with malignant epithelial ovarian cancer had moderate ("++", 52%) to strong ("+++", 30%) nuclear accumulation of hPygo2 protein. This abnormal accumulation of hPygo2 protein was observed in tumor cells but not in the surrounding stromal cells (Fig. 1E and F, *red arrows*). In addition, hPygo2 was found in the nuclei of tumors representing all of the epithelial ovarian cancer histologic subtypes (Table 1). These observations are consistent with a role for hPygo2 as a nuclear protein in malignant epithelial ovarian cancer disease.

β-Catenin subcellular localization was compared with nuclear hPygo2 staining in adjacent sections from 87 of the 125 tumor specimens from the patients diagnosed with malignant epithelial ovarian cancer collected above (Fig. 1F; Supplementary Table S2). Eight (9.2%) tumors stained positive for nuclear β-catenin, a result similar to previous findings (24). Eighty-four (97%) tumors stained positive for nuclear hPygo2, eight (9.2 %) of which simultaneously stained for nuclear β-catenin. None of the tumors stained exclusively for nuclear β-catenin and only one tumor did not stain for either protein. These observations indicated that relative to β-catenin, hPygo2 is highly expressed in the nuclei of epithelial ovarian cancer cells. Thus, as with the cell lines, hPygo2 and β-catenin do not necessarily colocalize in epithelial ovarian cancer tumors.

hPygo2 knockdown causes growth arrest of Wnt-inactive epithelial ovarian cancer cell lines. The observation that hPygo2 was more frequently expressed in nuclei than β-catenin in epithelial ovarian cancer cell lines and in tumors with diverse subtypes raised the hypothesis that it has activity in cancer cells that are not active in canonical Wnt signaling. We confirmed and extended the findings of others (16, 24) that TCF-dependent transcriptional activity was absent in two serous carcinoma–derived cell lines (SKOV-3 and OVCAR-3) and one clear cell carcinoma line (TOV-21G) but, in comparison, significantly active ($P < 0.00001$, $n = 9$) in cell lines of endometrioid (TOV112D) origin (Fig. 1G).

The requirement of hPygo2 in epithelial ovarian cancer cells was determined by transfecting phosphorothioated antisense oligonucleotides to knock down hPygo2 mRNA and its protein, initially in the serous tumor-derived epithelial ovarian cancer cell lines OVCAR-3 and SKOV-3. Both hPygo2 mRNA and protein levels were significantly reduced by an antisense oligonucleotide, hPy-ON5, without affecting the expression of hPygo1 RNA or β-catenin protein (Fig. 2A). The sequence of hPyOn5 is complementary to nucleotides at positions 634 to 652 (from the start of translation), within the coding region of hPygo2 mRNA. Of the two cell lines tested, the antisense oligonucleotide reduced endogenous hPygo2 protein by ~40% in SK-OV-3 cells and 30% in OVCAR-3 cells when compared with controls (Fig. 2A), which included untransfected cells (*Cont.*), cells treated only with transfection reagents (*Reag.*), and cells transfected with the mismatched control oligo. To confirm the specificity of the antisense knockdown, we

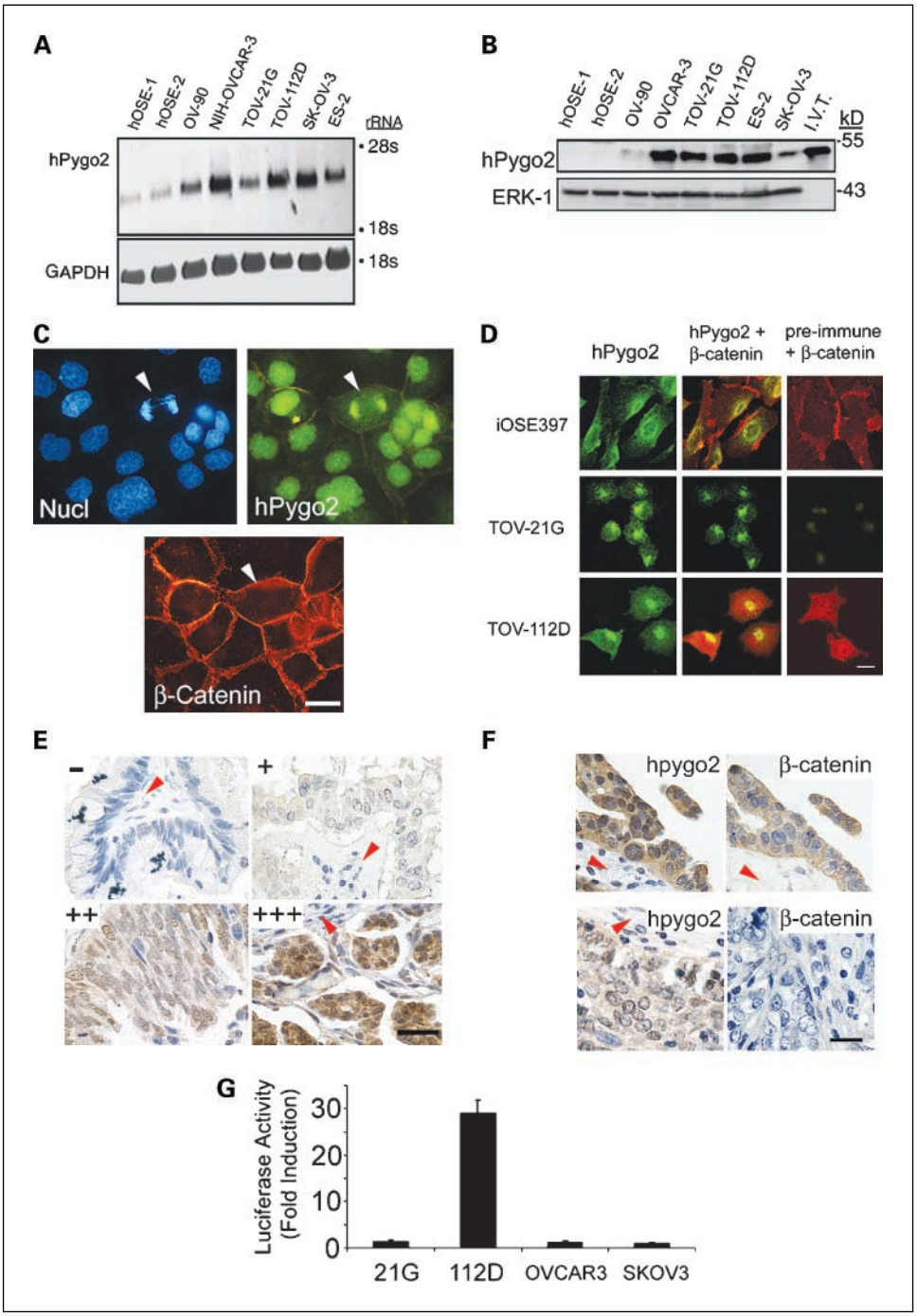

Fig. 1. Expression analyses of hPygo2 and TCF-dependent transcription in epithelial ovarian cancer tumors and cell lines. *A,* expression of hPygo2 RNA in epithelial ovarian cancer cell lines using Northern analysis. Positions of the 28S and 18S rRNA are shown. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a loading control. *B,* expression of hPygo2 protein in epithelial ovarian cancer and human ovarian surface epithelial cell lines using immunoblots. *In vitro* synthesized hPygo2 (*I.V.T.*) was used as a positive control. ERK-1 is used as a loading control. Molecular weight is expressed in kilodaltons (*kD*). *C,* OVCAR-3 cells were stained simultaneously with nuclear-specific Hoecsht 33342 dye (*Nucl*), hPygo2 antisera (*hPygo2*) detected with fluorescein-conjugated secondary antibodies, and β-catenin monoclonal antibodies detected with Texas red – conjugated secondary antibodies (*β-catenin*) and viewed under a fluorescence microscope. Arrowhead, position of an anaphase stage cell. Bar, 5 μm. *D,* immortalized epithelial ovarian cells (*iOSE397*) and two epithelial ovarian cancer cell lines (*TOV-21G* and *TOV-112D*) were processed for confocal immunofluorescent detection of hPygo2 (*green fluorescence*) and β-catenin (*red fluorescence*). Left column, expression of hPygo2 alone; middle column, additional expression of β-catenin; right columns, expression of β-catenin alone. Bar, 20 μm. *E,* hPygo2 protein expression in tumors was graded according to intensity and frequency of tumor cell nuclei staining (*brown*) with hPygo2 antisera in relation to background staining of surrounding stromal tissue (*red arrows*). All nuclei were stained in blue with hematoxylin. Some visible features of malignant cells used in the analysis include larger than normal nuclei, disorganized cellular arrangement, and invasion of the stroma. Nonmalignant ovarian epithelial adenomas are negative (−) for hPygo2. Staining of malignant tumors ranged from weak (+, light staining or <60% nuclei stained) to moderate (++, intermediate intensity staining, 80% nuclei stained) to strong (+++, intense staining with >90% nuclei stained). Bar, 30 μm. *F,* examples of hPygo2 and β-catenin expression in adjacent tumor sections. Top pair, strong nuclear staining of hPygo2 in a tumor coincident with weak nuclear and moderate cytoplasmic β-catenin staining. Bottom pair, coincident moderate hyPgo2 nuclear staining and negative β-catenin staining. Bar, 22 μm. *G,* activation of TCF-dependent transcription in TOV112D endometrioid adenocarcinoma cells, TOV21G clear cell carcinoma, and SKOV-3 and OVCAR-3 serous carcinoma cells. Columns, means of three independent experiments repeated in triplicate; bars, SD.

Table 1. Frequency distribution of histologic tumor subtypes (percentages in parentheses) according to level (− to +++, as indicated in Fig. 1E) of nuclear and cytoplasmic hPygo2 staining

		Tumor subtype						Total
		Benign	Mucinous	Endometrioid	Serous	Clear cell	Undifferentiated	
Nuclear	−	7 (100)	3 (21)	0	2 (2)	0	0	12 (10)
	+	0	1 (7)	1 (12)	8 (9)	0	0	10 (8)
	++	0	7 (50)	5 (63)	46 (53)	4 (80)	3 (75)	65 (52)
	+++	0	3 (21)	2 (25)	31 (36)	1 (20)	1 (25)	38 (30)
	Total	7 (100)	14 (100)	8 (100)	87 (100)	5 (100)	4 (100)	125 (100)
Cytoplasmic	−	5 (72)	4 (29)	0	12 (14)	0	0	21 (17)
	+	0	6 (43)	5 (63)	53 (61)	5 (100)	1 (25)	70 (56)
	++	1 (14)	3 (21)	3	21 (24)	0	2 (50)	30 (24)
	+++	1 (14)	1 (7)	0 (37)	1 (1)	0	1 (25)	4 (3)
	Total	7 (100)	14 (100)	8 (100)	87 (100)	5 (100)	4 (100)	125 (100)

repeated the above experiments using an antisense oligonucleotide (hPy-ON8) targeting the 3′ untranslated region of *hPygo2* mRNA, spanning nucleotides 1,644 to 1,662 from the start of translation. The levels of hPygo2 protein reduction using this antisense oligonucleotide were similar to the levels when we used hPy-ON5 (Fig. 2B).

The two cell lines we initially tested for knockdown of hPygo2 by antisense oligonucleotides underwent significant reduction in cell number for SK-OV-3 cells at 48 hours (P = 0.0003, n = 12) and 72 hours (P = 0.002, n = 12) posttransfection and for OVCAR-3 cells 48 hours (P = 0.001, n = 12) and 72 hours (P = 0.0005, n = 12) posttransfection using hPy-ON5 (Fig. 3A) compared with cells transfected with the mismatched control oligonucleotide. Similar results were obtained using hPy-ON8 (Fig. 3B, all $P < 0.005$, n = 12).

Antisense knockdown of hPygo2 is reversible. We next attempted to rescue the effect of the antisense treatment by cotransfection of hPygo2 to further confirm that the knockdown we observed was due to specific reduction of wild-type hPygo2 protein. To restore hPygo2 protein expression reduced by the antisense treatment, hPy-ON8 antisense oligonucleotides were cotransfected with an expression vector (pCS2+) containing only the coding region of hPygo2. Cotransfection of this plasmid with hPy-ON8 antisense oligonucleotides that target noncoding sequences restored the levels of hPygo2 protein close to control levels compared with cells cotransfected with empty vector and hPy-ON8, which showed significant hPygo2 protein reduction (Fig. 3C).

The significant reduction in cell numbers by treatment with hPy-ON8 (Fig. 3D) at 48 hours (P = 0.0004, n = 9) and 72 hours (P = 0.01, n = 9) was restored to levels of the controls (empty vector plus mismatched control oligo) at 48 hours (P = 0.94, n = 9) and 72 hours (P = 0.96, n = 9) by exogenously introducing the coding region sequences of *hPygo2* (Fig. 3D). It is likely, therefore, that the effects on cell numbers by transfection of hPy-ON8 were specific to reduction of endogenous hPygo2 RNA and hPygo2 protein as opposed to off-target effects. These results indicate that hPygo2-specific antisense sequences inhibit epithelial ovarian cancer cell growth by specifically reducing endogenous hPygo2 protein levels.

hPygo2 is required for growth of a β-catenin-deficient cell line. The growth requirements for hPygo2 in SKOV-3 and OVCAR-3 cells, both of which are derived from serous tumors, suggested that hPygo2 functions in cells not active in Wnt signaling. Unlike endometrioid cells, these cell lines do not seem to constitutively activate TCF/LEF-1 – dependent transcription but it is possible that Wnt activation occurs transiently so the detection of nuclear β-catenin, for instance, would be easily missed in these cells. To confirm that hPygo2 has activity in non-Wnt active epithelial ovarian cancer cells, we determined the effect of depleting hpygo2 in the clear cell tumor-derived TOV-21G cell line, which does not express β-catenin and in which TCF-dependent transcription is imperceptible, if not absent (Fig. 1G). As a comparison, we determined the effect of knocking down hPygo2 in TOV-112D cell lines, which show

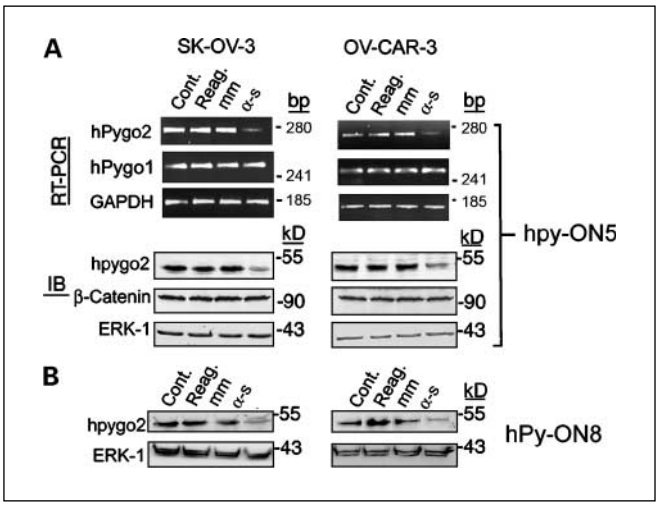

Fig. 2. Knockdown of hPygo2 protein in SK-OV-3 and OV-CAR-3 epithelial ovarian cancer cells by antisense oligonucleotides. *A,* cells were transfected with the hPygo2 antisense oligonucleotide, hPy-ON5 (*α-s*), or mismatched control (*mm*) oligonucleotides and compared with untransfected (*Cont.*) or mock-transfected (*Reag.*) cells and assayed by reverse transcription-PCR (*RT-PCR*) and immunoblotting (*IB*) for hPygo2, hPygo1, and β-catenin expression. Glyceraldehyde-3-phosphate dehydrogenase expression was used as a loading control for reverse transcription-PCR and ERK-1 for immunoblots. Amplification product sizes are expressed in bp. *B,* cells were transfected with the hPygo2 antisense oligonucleotide, hPy-ON8, or mismatched control oligonucleotides and compared with untransfected or mock-transfected cells and assayed by immunoblot with ERK-1 as a loading control.

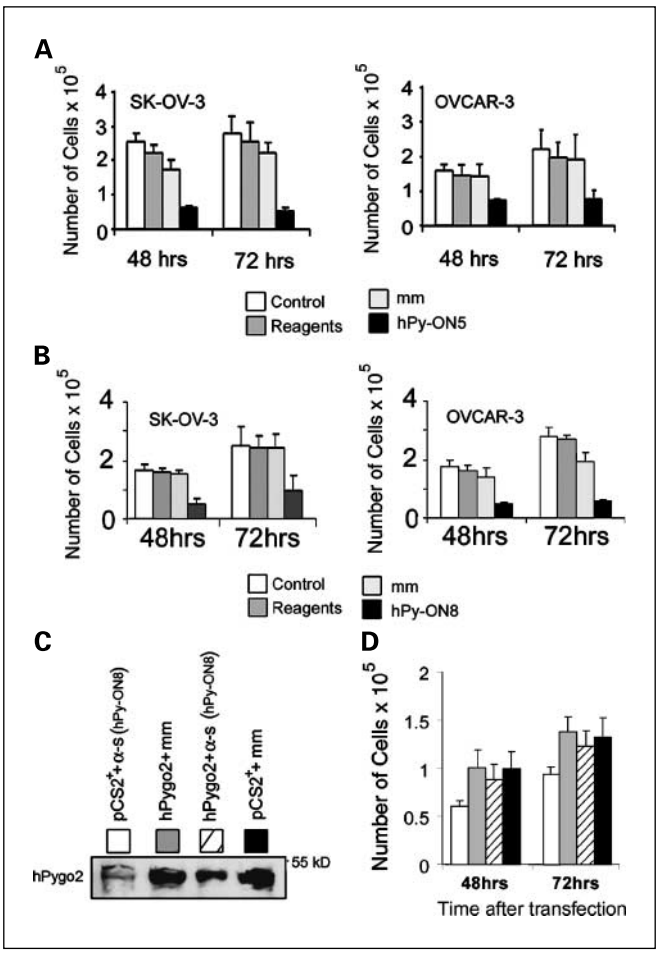

Fig. 3. Knockdown of hPygo2 reduces epithelial ovarian cancer cell numbers. *A,* results of growth assays of SK-OV-3 and OV-CAR-3 cells transfected with the hPy-ON5 antisense oligonucleotide and mismatched control oligonucleotides 48 and 72 hours after transfection. Columns, mean; bars, SD. *B,* results of growth assays of SK-OV-3 and OV-CAR-3 cells transfected with the hPy-ON8 antisense oligonucleotide and mismatched control oligonucleotides 48 and 72 hours after transfection. Columns, mean; bars, SD. *C,* hPygo2 cotransfection restores protein levels in cells depleted of hPygo2 by the hPy-ON8 antisense oligonucleotide (*α-s*). SKOV-3 cells were transfected with the hPy-ON8 antisense oligonucleotide (*hPy-ON8*), which targets 3′ untranslated sequences of hPygo2 or the mismatched control oligonucleotide together with either an expression plasmid vector containing hPygo2 coding sequence (*hPygo2*) or empty vector ($pCS2^+$). Transfected cells were assayed for expression of hPygo2 using Western (immunoblot) analysis 48 hours after transfection. Equal amounts of total protein, as determined by measuring absorbance, were loaded for each sample. *D,* exogenous hPygo2 rescues cell number reduction caused by depletion of endogenous hPygo2. Columns, mean; bars, SD. SKOV-3 epithelial ovarian cancer cells were transfected with antisense oligonucleotides and the numbers of cells were counted (*Y axis*) 48 and 72 hours posttransfection. Histograms, data corresponding to the treatments shown in (*C*).

constitutive activation of Wnt signaling (Fig. 1G). We also confirmed the lack of association of hPygo2 with β-catenin in the TOV-21G cell line compared with the TOV112D line using coimmunoprecipitation (data not shown).

Transfection of anti-β-catenin antisense oligonucleotides caused close to 60% reduction in endogenous β-catenin protein in TOV-112D cells, whereas transfection of hPygo2 antisense oligonucleotides (hPY-ON8) caused ~50% reduction in hPygo2 levels in both TOV-112D (Fig. 4A) cells and TOV-21G cells (Fig. 4A). The anti-β-catenin antisense oligonucleotides did not have an effect on TOV-21G cell growth (Fig. 4B, *right*), as expected, as these cells do not express β-catenin (Figs. 1D and 4A). There was, on the other hand, a ~3-fold reduction in cell number at 48 hours (P = 0.00002, n = 12) and 72 hours (P = 0.00004, n = 12) after transfection of anti-β-catenin antisense oligonucleotides into TOV-112D cells (Fig. 4B). hPygo2 antisense oligonucleotides caused significant growth inhibition of TOV-112D cells (Fig. 4B) after 48 hours (P = 0.00001, n = 12) and 72 hours (P = 0.00001, n = 12) and TOV-21G cells (Fig. 4B) after 48 hours (P = 0.0001, n = 12) and 72 hours (P = 0.003, n = 12) when compared with the mock-transfected and mismatched control-transfected cells. These results indicated that hPygo2 is required for growth of both constitutively Wnt-active and Wnt-inactive (β-catenin deficient) cell lines.

hPygo2 is required for anchorage-independent growth and growth in severe combined immunodeficient mice. We next tested the ability for transfection of antisense oligonucleotides to reduce anchorage-independent growth. hPy-ON5 and hPy-ON8 antisense oligonucleotides (50 nmol/L each) were pooled for transfection. In three separate experiments reproduced in triplicate, there was a significant decrease in the size (Fig. 5A) and number (Fig. 5B, $P < 0.00001$, n = 9) of colonies grown in soft agar formed by SK-OV-3 cells transfected with the pooled hPygo2 antisense oligonucleotides, compared with those transfected with the mismatched control oligo.

Our *in vitro* growth inhibition findings were complemented with *in vivo* growth assays to determine whether the *in vivo* culture environment might reverse the initial effects of hPygo2 antisense oligonucleotides. hPygo2 antisense oligonucleotides or mismatched control oligos were transiently transfected into SK-OV-3 cells and then implanted s.c. into severe combined immunodeficient mice. Measurable tumors appeared 11 weeks after transfection in the mice implanted with the control cells (Fig. 5C), whereas mice implanted with

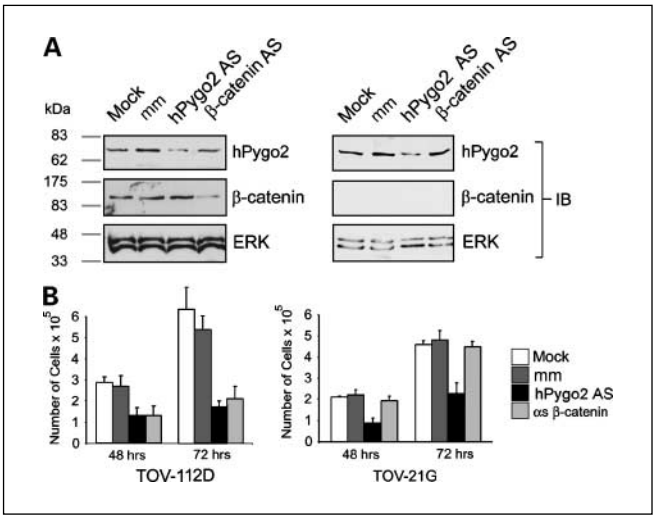

Fig. 4. hPygo2 is required for growth of both Wnt-constitutive and Wnt-inactive (β-catenin defective) cell lines. *A,* TOV112D and TOV21G cells were untransfected (*Mock*) or transfected with antisense hPygo2 (*hPygo2 AS*), β-catenin (*β-catenin AS*), or mismatched control oligonucleotides and assayed for knockdown of endogenous protein by immunoblotting using antibodies against hPygo2, β-catenin, and ERK as a loading control. *B,* number of cells was determined at 48 and 72 hours after transfection. Columns, mean; bars, SE.

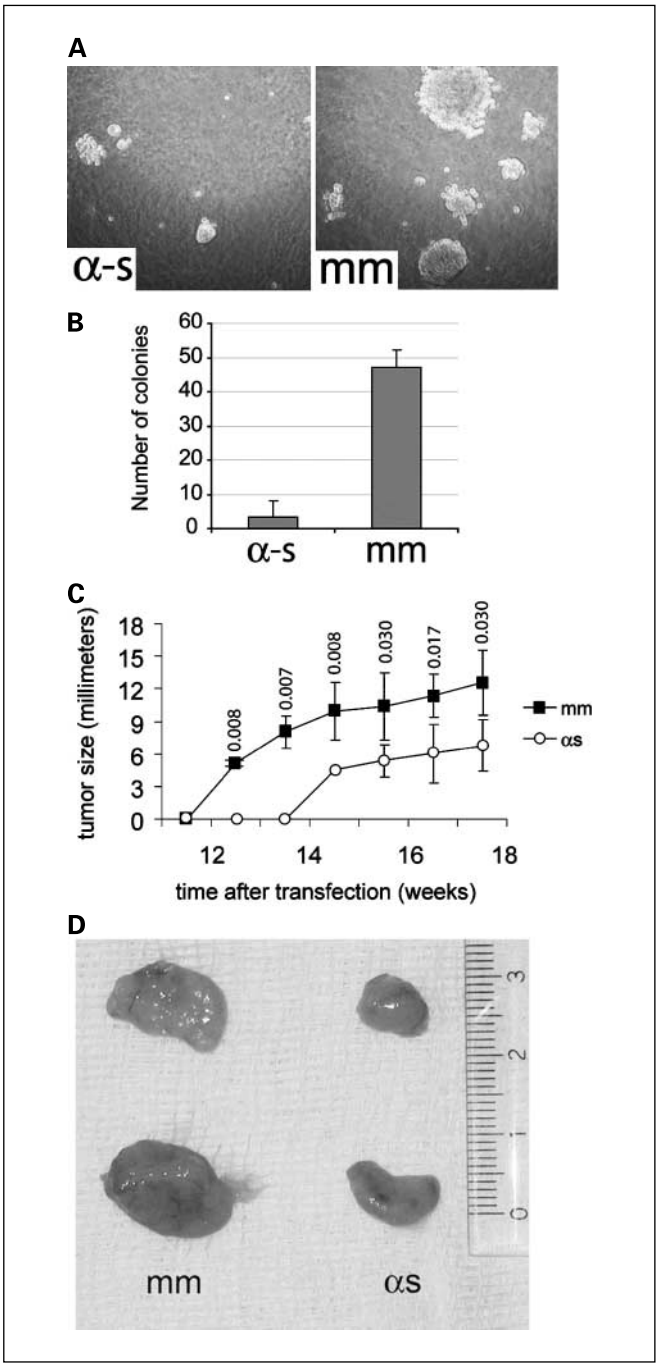

Fig. 5. Loss of anchorage-independent growth and growth *in vivo* by antisense hPygo2. *A,* SK-OV-3 cells were transfected with a pooled mixture of hPy-ON5 and hPy-ON8 antisense oligonucleotides and assayed for growth in soft agar. Note the reduction in size of colonies resulting from antisense transfection. *B,* colony numbers of cells transfected with hPygo2 antisense oligonucleotides or the mismatched control recorded 30 days after transfection. *C,* tumors from SK-OV-3 cells transfected with hPygo2 antisense oligonucleotide are growth retarded relative to those from cells transfected with mismatched control oligos (*P* values shown over sample points). *D,* examples of xenografted tumors dissected from animals sacrificed 18 weeks after transfection.

hPygo2 antisense oligonucleotide–treated cells developed tumors after 14 weeks. In all cases, antisense oligonucleotide–transfected cells formed smaller tumors than mismatched control transfected cells throughout the measurable growth period (Fig. 5C, $P < 0.03$, $n = 3$). After 18 weeks, there was a visible difference (Fig. 5D) in tumor mass ($P = 0.002$, $n = 3$) between mice implanted with cells transfected with hPygo2 antisense oligonucleotides (0.21 g) and those implanted with cells transfected with the mismatched control oligos (0.62 g). These results indicated that *in vivo* culture of hPygo2-transfected cells does not reverse the effect of growth inhibition by antisense hPygo2 antisense oligonucleotides.

Discussion

Our severe combined immunodeficient mice xenograft assays indicate that the *in vivo* microenvironment does not reduce the effect of depletion of hPygo2 by antisense oligonucleotides. We note, however, that there was not a complete elimination of colony growth or tumor formation and the growth differences between control and hPygo2 antisense oligonucleotide–transfected tumors in the xenograft *in vivo* assay seem to persist many weeks after the initial transfection event. This long-lasting effect would not be expected using the single transient transfection method. We suggest, however, that the immediate effect of knockdown of hPygo2 in cells may be permanent and that the formation of smaller tumors in hPygo2-transfected cells compared with control transfected cells is likely due to a small number of cells that escaped transient transfection. Thus, the effect we observed was equivalent to a smaller seeding of the initial tumor cells. Although we did not achieve full elimination of tumors by transient transfection, our findings necessarily provide the initial results upon which to base more advanced preclinical studies to assess other effects of continuous treatment.

There is much evidence that the canonical Wnt pathway does not likely play a role in epithelial ovarian cancer tumors that are not of endometrioid origin (14, 27, 28). Our results were consistent with this established observation. Because of its previously described dedicated role in canonical Wnt signaling, however, it would not be expected that hPygo2 would play a significant role in this malignancy. We are compelled to hypothesize, therefore, that Pygopus may have functions outside of Wnt signaling, or its constitutive localization to the nuclei of epithelial ovarian cancer cells is sufficient to activate downstream effectors of Wnt signaling in the absence of upstream signaling components.

Other components of the Wnt pathway, such as β-catenin, axin, and APC, are engaged in critical cellular functions apart from canonical signaling (29, 30). We previously showed that mutant Pygopus2 with a deletion of the domain that interacts with the TCF/LEF complex could activate genes associated with vertebrate embryonic nervous system formation (19), suggesting that Pygopus might have undescribed functions. Likewise, our present observations that hPygo2 was expressed in and required for growth of (Wnt inactive) serous and clear cell tumor lines suggests either that overexpressed hPygo2 can function as a downstream effector of the canonical Wnt pathway without interaction with the β-catenin complex, or that it has additional cellular roles. It is equally feasible that hPygo2 may interact with other transcriptional complexes, a possibility we are investigating.

The necessity to identify novel markers and therapeutic targets for epithelial ovarian cancer has provided strong

impetus to search for genes that are important for this disease (31). A variety of mRNAs are now being used as targets for antisense therapy in early phase clinical trials (32), including p53 (33), survivin (34), raf-1 (35), X-linked inhibitor of apoptosis protein (36), and telomerase (37, 38). The overexpression of hPygo2 in epithelial ovarian cancer cell lines and tumors relative to normal epithelia and its requirement for growth strongly suggest that it too is important in epithelial ovarian cancer and can be added to the growing armamentarium of therapeutic targets.

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