Requirement of Pygopus 2 in breast cancer

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Abstract. The development of novel therapeutic strategies for breast cancer requires the identification of molecular targets involved in malignancy. Human Pygopus (Pygo)-1 and -2 are recently discovered components of the Wnt signaling pathway required for ß-Catenin/Tcf dependent transcription in embryos and colorectal cancer cells, but the role of these proteins in malignant cell growth and survival has not yet been determined. We report the expression and requirement for proliferation of hPygo2 in breast cancer cells. hPygo2 protein was overexpressed in malignant breast tumors and in the nuclei of five breast cancer cell lines, but was not expressed in the nuclei of non-malignant breast cells. Phosphorothioated antisense oligonucleotides were used to specifically knock-down expression hPygo2 in MCF-7 and MDA-MB-468 cell lines. hPygo2 was required for the growth, in tissue culture and anchorage-independent assays, of both cell lines and for the expression of the Wnt target gene Cyclin D1. We conclude that hPygo2 is highly expressed in, and required for the growth of breast carcinoma cells.

Introduction

The canonical Wnt/ß-Catenin signaling pathway is associated with the regulation of growth and differentiation events, primarily in the clonal expansion of cells (1). Consequently, mutations in its pathway constituents have been linked to a number of human cancers derived from colorectal, breast, ovarian, and neuroectodermal tissues (2-5).

Our understanding of canonical Wnt pathway stimulation and the consequent activation of cell growth-related target gene transcription is well-established. Secreted Wnt ligands initiate intracellular responses by binding to frizzled transmembrane receptors and to low-density lipoprotein (LRP5/6) co-receptors (6,7) which, in turn, activate the cytoplasmic intermediate, Dishevelled (8,9). Dishevelled subsequently binds Axin to inhibit phosphorylation of ß-Catenin by the Axin/adenomatous polyposis coli/glycogen synthase kinase-3ß tumor suppressor complex (10). Unphosphorylated ß-Catenin accumulates in the cytoplasm and nucleus where it interacts with T-cell factor/lymphoid enhancing factor (TCF/LEF), B-cell lymphoma-9 (Bcl-9) and Pygopus (Pygo) (11-13). Sequence-dependent enhancer binding via TCF/LEF initiates transcription of target genes involved in cell growth and proliferation, such as Cyclin D1 (14,15) and c-myc (16).

Since the Int1/Wnt1 oncogene was found to promote mammary tumors in mice (17), much attention has been focused on the role of Wnt/ß-Catenin signaling in breast cancer (18). For instance, many components of the Wnt signaling pathway become misexpressed in mammary gland tumors and contribute to their development and progression to adenocarcinoma (19-24). Consistently, cytoplasmic and nuclear ß-Catenin staining was observed in a significant number of breast tumors and was correlated with poor patient prognosis (19,21,25). In addition, a number of breast cancer cell lines exhibited high levels of Wnt dependent transcription resulting in the overexpression of the target gene Cyclin D1 (19).

In this study, we examined the expression and growth requirement of one of the Pygopus proteins, hPygo2, in breast cancer. Previous knockdown experiments in colorectal cancer cells suggested that both Pygo human family members, hPygo1 and hPygo2, may be involved in TCF/ß-Catenin driven transcription (12), and we previously demonstrated that hPygo2 was expressed in and required for the growth of epithelial ovarian cancer (26). Given the significant involvement of canonical Wnt signaling in breast cancer neoplasms, hyperplasia and adenocarcinomas (23), we hypothesized that, as in colorectal and ovarian cancer, hPygo2 would also have a prominent role in breast malignancy. Our results indicate that hPygo2 is highly expressed in and required for the growth of breast cancer cells.

Materials and methods

RT-PCR. Total RNA was extracted from cell lines using the Nucleospin RNA II Kit (Clontech). Semiquantitative RT-PCR

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was performed as previously described (27) using hPygo2 oligonucleotide primers described above, as well as primers for hPygo1 (F: 5'-GCCACGACAACCAAGAGGTG; R: 5'-CCA GTACAGATCCGATGAAACC) and GAPDH (28).

Western blotting. Total protein from tissue culture cells was extracted in protein sample buffer. As a positive control, *in vitro* translated hPygo2 protein was prepared using the transcription/ translation coupled cell-free system (Promega). Approximately 50 μg of total cell lysate was separated by SDS-PAGE, transferred to nitrocellulose membranes (Hybond-ECL™; Amersham) blotted with polyclonal hPygo2, monoclonal or polyclonal ß-Catenin (Santa Cruz Biotechnology) or monoclonal Cyclin D1 (BD Biosciences) antibodies and visualized by enhanced chemiluminescence (Amersham). Blots were reprobed with monoclonal anti-ß-actin (Sigma) to confirm equal loading of protein as described (26).

Antisense oligos. Highly specific phosphorothioated antisense oligonucleotides (Invitrogen) against hPygo2 were designed and tested as we described previously (26). The sequences used were: hPygo2 antisense oligo, 5'-G*G*C*TGAGCAAAT CGTT*G*G*G; non-specific control oligo, 5'-T*T*T*GCGCC GTTTCTT*C*T*C; 4 base mismatch oligo, 5'-G*C*C*TGAGC TAATCATT*G*G*T (mismatches are underlined, * indicates a phosphorothioate bond).

Cell culture and transfection. All cell lines, except normal endocervical (HEN) (29), were purchased from the American Type Culture Collection. T98G and Sk-N-Sh cells were maintained in minimal essential media (Gibco) supplemented with 10% fetal bovine serum. HEN cells were maintained in Keratinocyte Serum Free Media (Gibco). All remaining cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco).

All transfections utilized Oligofectamine (Invitrogen) as per the manufacturer's instructions, replacing the growth media every 24 h. hPygo2 antisense/control oligonucleotides were transfected to a final concentration of 250 nM and siRNAs to a final concentration of 100 nM in MCF-7 cells. hPygo2 antisense/control oligonucleotides were transfected to a final concentration of 67.5 nM. For RT-PCR analysis, cells were seeded at a density of $1.5x10^5$ cells/well in 6-well plates and were harvested 24 h after transfection for RNA extraction. For Western blot analysis cells were seeded at a density of 10^5 cells/well in 12-well plates and were harvested 48 or 72 h after transfection. For cell growth analysis, cells were seeded in triplicate at a density of $7.5x10^4$ cells/well in 12-well plates, and were counted with a hemacytometer 48 and/or 72 h after transfection using trypan blue exclusion (Sigma). Anchorage-independent colony-forming assays were performed as described (26).

Immunofluorescence and immunohistochemistry was performed as described (26). Breast tumor sections assessed by a pathologist (Dr J.D. Robb, Division of Laboratory Medicine) were from archived samples with no patient or physician identifiers obtained under Memorial University of Newfoundland human investigation committee protocol #01.53. Normal breast tissue microarrays (CHTN2002N1) were purchased from the Cooperative Human Tissue Network (Bethesda, MD).

Results

Expression of hPygo2 in breast cancer. The expression of both *Pygopus* mRNAs, *hPygo2* and *hPygo1*, in normal and malignant breast cell lines was initially determined using RT-PCR. A variety of breast cancer cell lines (Bt-20, Bt-474, MCF-7, MDA-MB-231, MDA-MB-468, Hs-578T), as well as normal breast cell lines (Hs-574, Hs-578-Bst) were used in the analysis. The expression of *hPygo2* mRNA was highest in six of the seven malignant breast cell lines and showed a lower expression level in Hs-578T and the normal breast cell lines (Hs-574, Hs-578-Bst). On the other hand, the expression of *hPygo1* mRNA was was not specifically overexpressed in the malignant cell lines. Furthermore, the expression of *hPygo1* mRNA showed little correlation with the expression of hPygo2 (Fig. 1a).

The expression of hPygo2 protein correlated with its mRNA and was overexpressed in the majority of malignant breast cancer cells. hPygo2 showed the highest protein expression levels in Bt-20, Bt-474, MCF-7, MDA-MB-157, MDA-MB-231 and MDA-MB-468 as compared to Hs-578T and normal breast cells Hs-574 and Hs-578-Bst (Fig. 1c). hPygo2 protein levels were higher in ovarian (Sk-Ov-3, Es-2), cervical (CaSki) and neuroblastoma (T98G) cell lines relative to normal breast cells (Hs-574) but lower than in malignant breast cells (Bt-474, MCF-7). These protein levels appeared to correlate with ß-Catenin protein levels as expected, except in the normal breast cell lines which showed higher levels of ß-Catenin than hPygo2, and in the MDA-MB-231 breast cancer cell line, which showed reduced ß-Catenin and higher levels of hPygo2. These results provide evidence that the upregulation of *hPygo2* expression may play an important role in breast cancer.

We next confirmed expression of hPygo2 in breast cancer tumors. Its *in situ* localization was analyzed in normal breast tissue and in malignant tumors by immunohistochemical staining. While hPygo2 was absent from normal connective (stroma) tissue and adipose tissue of the breast, it did accumulate weakly but asymmetrically in the cytoplasm of ductal epithelium (Fig. 2a). Most of the 22 archived breast tumor sections that we stained for hPygo2 protein were invasive ductal carcinomas (Fig. 2b-d). In 14 (64%) tumors, hPygo2 was localized to malignant cells, but not in the surrounding non-tumor cells. Of the 14 positively stained specimens, 8 (57%) tumors had only cytoplasmic hPygo2 staining (Fig. 2b and c) whereas the remaining 6 (43%) showed strong specific nuclear and cytoplasmic staining for hPygo2 (Fig. 2d).

Pygopus proteins are proposed to act in concert with legless/BCL-9 by anchoring TCF/Lef-1/ß-catenin activating complexes to the nucleus (30) and as an adaptor protein to allow or promote transcription from the complex (30-33). Indeed, pygopus proteins are required in nuclei of colorectal cancer cells for TCF/ß-catenin mediated transcriptional regulation (12,34). Thus the nuclear localization of hPygo2 would be consistent with its activity in canonical Wnt signaling. We next determined, therefore, the subcellular localization of hPygo2 relative to ß-Catenin in normal and malignant breast cell lines using co-immunofluorescence confocal microscopy. Endogenous hPygo2 protein was predominantly found in nuclei of the breast carcinoma cell lines Bt-474, MCF-7, MDA-MB-231 and MDA-MB-468 while it was weakly

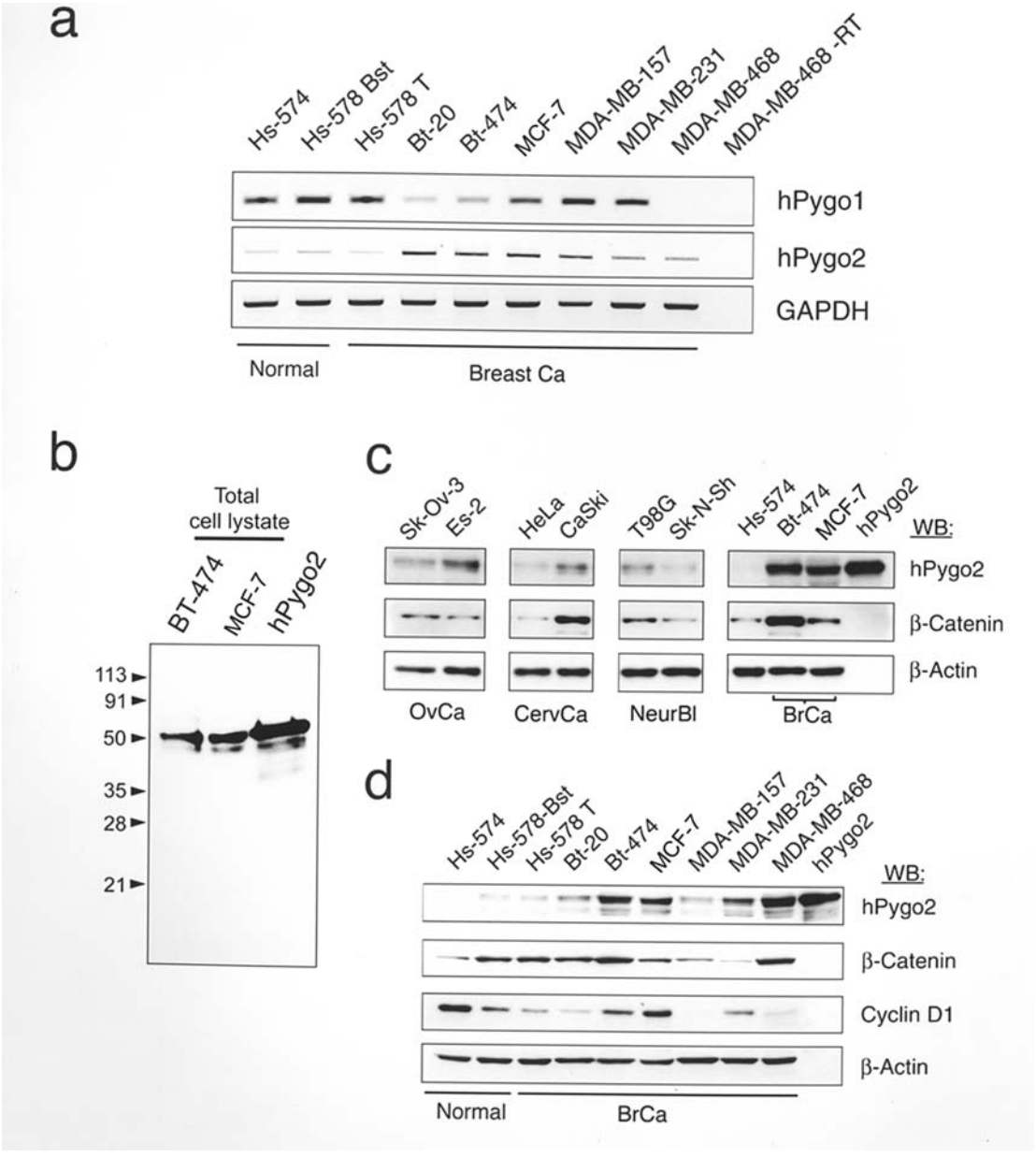

Figure 1. Expression of hPygo2 and ß-Catenin in cell lines. Levels of RNA and protein were standardized using GAPDH and ß-actin, respectively. (a) Expression of human Pygo RNAs was analyzed by RT-PCR in breast cancer cell lines. -RT, negative control, without reverse transcriptase. (b) Immunoblot showing specificity of antibody for the hPygo2 protein in BT-474 and MCF-7 breast cancer cell lines. The approximate size of hPygo2 protein is 50 kDa, as indicated by molecular weight markers shown on the left. *In vitro* transcribed and translated full length hPygo2 protein (hPygo2) was used as a positive control. (c) Expression of hPygo2 and ß-Catenin by Western blot analysis of total lysate from cell lines derived from non-breast malignant tumors. Samples were run on the same gel to ensure equal loading, but separated according to tumor type in the figure for clarity of illustration. (d) Expression of hPygo2 ß-catenin and cyclin D1 by Western blot analysis of total lysate from cell lines derived from breast cancer cell lines.

expressed in the cytoplasm of normal Hs-574 cells (Fig. 2e), an observation consistent with that seen in normal breast epithelium (Fig. 2a). ß-Catenin was consistently found in the cytoplasm and associated with the inner cell membrane of the normal and cancer cell lines, except for MDA-MB-231 cells, which also expressed high levels of nuclear ß-catenin (Figs. 1b and 2e). The high levels of cytoplasmic ß-catenin and nuclear hPygo2 are consistent, therefore with their proposed roles in canonical Wnt-signaling in breast cancer.

hPygo2 is required for growth of MCF-7 and MDA-MB-468 breast cancer cells. The requirement of hPygo2 for growth of breast cancer cells was determined using antisense knockdown experiments. We used antisense oligonucleotides (α-hpy2) that we previously demonstrated to specifically target hPygo2 mRNA in epithelial ovarian cancer cells (26). In MCF-7 cells, α-hpy2 knocked down endogenous hPygo2 mRNA levels to <50% of the controls, without affecting the expression of hPygo1 (Fig. 3). Transfection of α-hpy2 into both MCF-7 (Fig. 4a) and MDA-MB-468 (Fig. 4b) breast cancer cells also significantly reduced hPygo2 protein levels compared to control ONs, while ß-Catenin levels remained unaltered. There was a considerable reduction of cell growth after transfection with the hPygo2-specific ON, in tissue culture growth assays

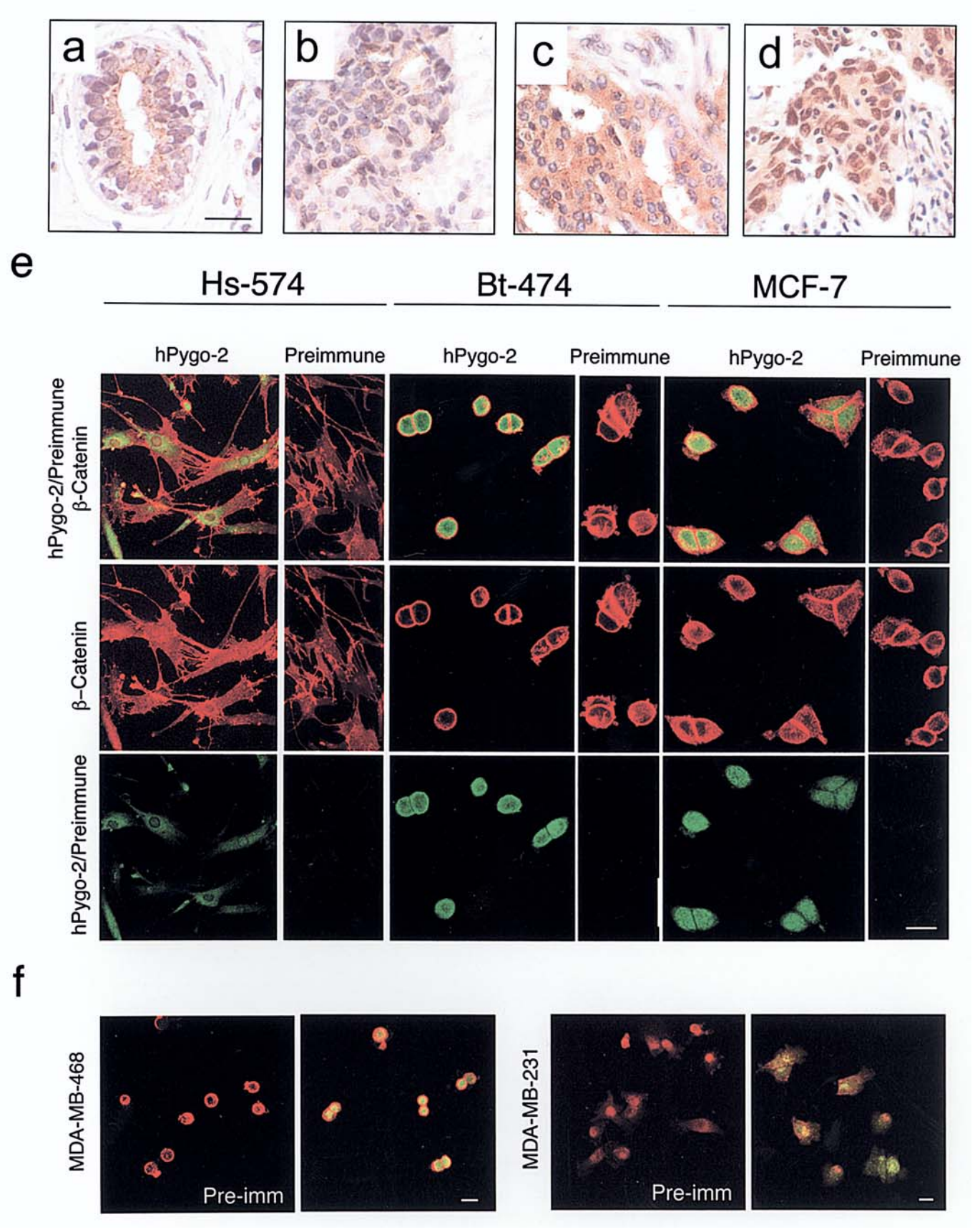

Figure 2. Subcellular localization of hPygo2 in breast tumors and cell lines. (a) Normal breast tissue showing assymetrical cytoplasmic hPygo2 in ductal breast epithelium. (b-d) Infiltrating ductal carcinomas stained with hPygo2 demonstrating weak cytoplasmic hPygo2 staining (b), strong cytoplasmic hPygo2 staining (c), and strong nuclear and moderate cytoplasmic hPygo2 staining (d). (e and f) Subcellular localization of hPygo2 (green fluorescence) and ß-Catenin (red fluorescence) in an immortalized normal breast ductal epithelial cell line (Hs-574) and in malignant breast cancer cell lines Bt-474, and MCF-7 (e), and MDA-MB-468 and MDA-MB-231 (f) using confocal microscopy. Preimmune serum was used at the same dilution as hPygo2 immune serum for a negative control. Scale [a or (b, c, and d)] = 100 μm.

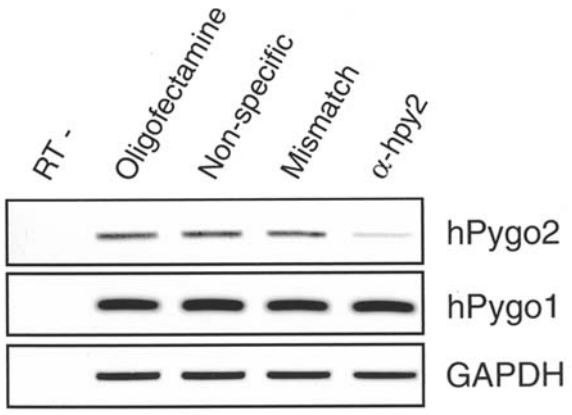

Figure 3. hPygo2 mRNA depletion by antisense in breast carcinoma cell lines. RT-PCR analysis of both human Pygo family members that were treated with an hPygo2 antisense oligonucleotide, showing specific knockdown of hPygo2. RT-, negative control, without reverse transcriptase.

using both cell lines (Fig. 4). This reduction in cell number was associated with a decrease in the level Cyclin D1, a cell cycle regulatory protein and target of Wnt signal transduction, in MCF-7 cells (Fig. 4a) and MDA-MB-468 cells (not shown). These results are consistent with a role for hPygo2 in Wnt signaling and the growth of breast cancer cell lines.

To confirm the effect of hPygo2 depletion on MCF-7 cell growth, soft agar colony forming assays were performed over several weeks, as described (26). The long-term culture conditions in these assays served to demonstrate any latent effects of depletion of these proteins and on anchorage-independent growth, a malignant property of these cells. As shown in Fig. 5, α-hpy2, but not the control oligonucleotides significantly and consistently reduced MCF-7 colony formation over the long-term. These experiments indicated that the effect of hPygo2 depletion was persistent on growth of breast cancer cells.

Discussion

Our results are the first to demonstrate a requirement for hPygo2 in breast cancer. hPygo2 protein, which is normally present at low levels in the cytoplasm of normal adult cells was found at high levels in the nuclei of breast cancer cells, but not in normal breast ductal cells. In addition, we demonstrated that hPygo2 was required for expression of the Wnt target gene Cyclin D1 and consequent growth of mammary carcinoma cells. Because the antisense knockdown of hPygo2 did not significantly reduce levels of ß-catenin, these findings support a role for hPygo2 in mediating downstream responses to canonical Wnt/ß-catenin signal transduction.

Canonical Wnt signaling has been studied extensively in breast cancer. While ß-Catenin has been reported to have

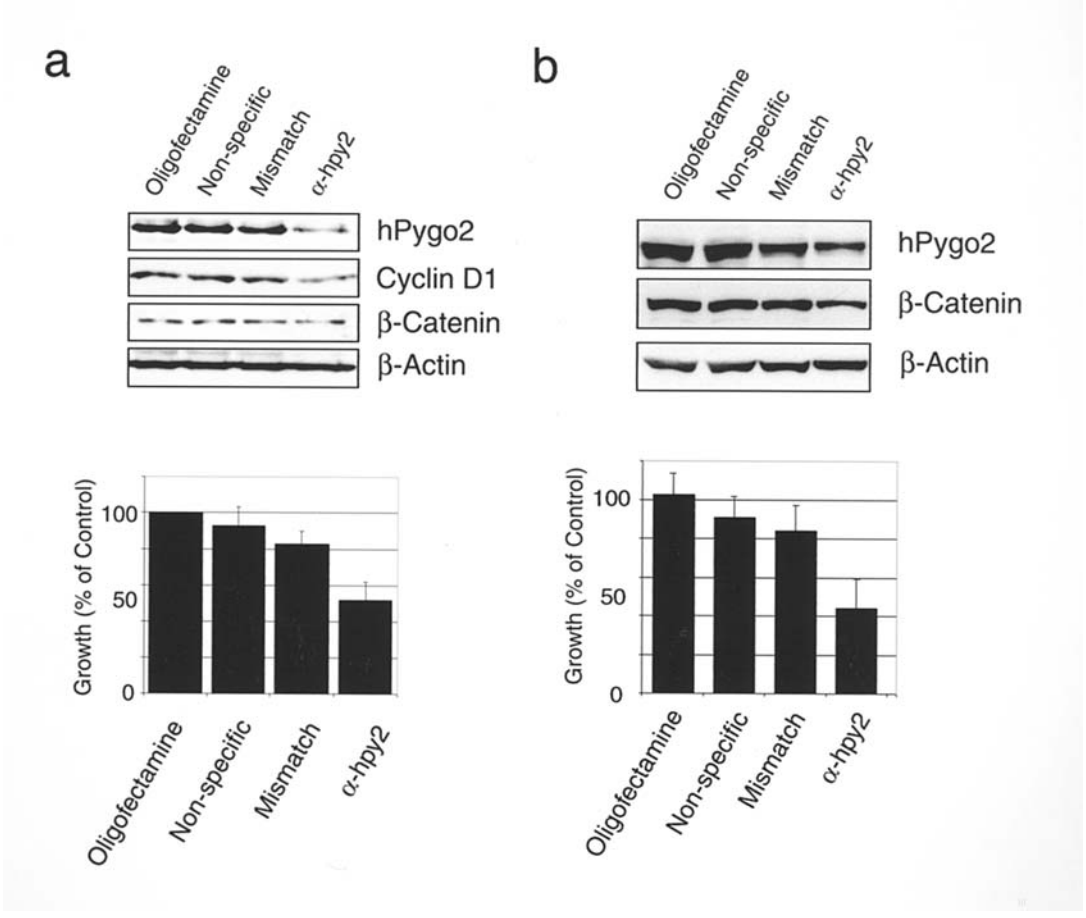

Figure 4. Reduction of hPygo2 protein by antisense causes growth inhibition of breast cancer cell lines. Depletion of hPygo2 inhibits growth of (a) MCF-7 and (b) MDA-MB-468 breast cancer lines. Upper figure shows Western blot analyses of hPygo2 protein in cell extracts at 24 h after transfection of antisense hPygo (α-hpy-2), the non-specific oligo control, the 4-base mismatched oligo control and reagents only (oligofectamine). Cyclin D1 and ß-catenin levels were also analyzed for MCF-7 cells in (a). Lower figures represent growth assays at 48 h after transfection. Error bars represent standard deviation.

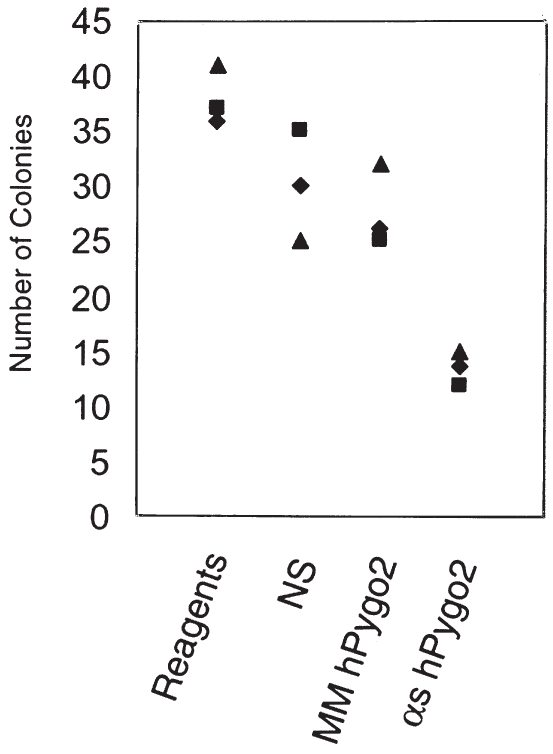

Figure 5. Knockdown of hPygo2 causes reduction of MCF-7 colony formation in soft agar assays. Three independent experiments, the data for each experiment represented by an individual shape (squares, triangles or diamonds), were plotted against the number of colonies counted after 30 days in soft agar culture. The treatments in each experiment, plotted on the horizontal axis are: mock transfected (reagents), non-specific control oligo (NS), mismatched antisense hPygo2 oligo (MM hPygo2) and antisense hPygo2 oligo.

nuclear/cytoplasmic staining in approximately 60% of breast tumors in two independent studies (19,21), mutations in Wnt pathway components that activate ß-catenin occur at relatively low frequencies (4,35,36). It has been suggested that over-expression of Wnt ligands or reduced expression of Wnt antagonists may lead to the nuclear stabilization of ß-Catenin in mammary carcinogenesis (18). Alternatively, this phenomenon may be due to Wnt independent regulation of ß-Catenin, such as the regulation of ß-Catenin by fibroblast growth factor (37), estrogen receptor (38) and epidermal growth factor (39) family members, as well as the Wnt independent regulation of GSK3 (40). Regardless of the mechanism of regulation of ß-catenin in breast malignancies, our finding that hPygo2 was over-expressed in nuclei of tumor samples and cell lines relative to normal and other cancers and its requirement for growth strongly suggest that it is essential for breast cancer.

Our results are also consistent with immunohistochemical studies of infiltrating ductal carcinomas that were shown to exhibit Cyclin D1 and ß-Catenin expression (41). In addition to its regulation by Wnt/ß-Catenin signaling, Cyclin D1 expression has been shown to be regulated in MCF-7 cells, through the Estrogen receptor (42) and peroxisome proliferator-activated receptor γ (PPARγ) (43,44). It is possible that other downstream effectors directly activate Cyclin D1, which might include hPygo2.

It will be of interest to determine the range of malignancies in which hPygo2 is overexpressed and required for growth. Wnt-dependent transcription was shown to require Pygopus in colorectal cancer cells (12), and, while not assayed, growth of these cells is predicted to be inhibited by Pygo knockdown consistent with that shown for ß-Catenin (45-47). Our previous findings that hPygo2 was expressed in and required for the growth of a diversity of epithelial ovarian cancer subtypes, some in which the canonical Wnt pathway is constitutively active and others in which ß-catenin is absent or transcriptionally inactive (26), suggests the potential for a more global involvement of hPygo2 in cancer. Our results presented here suggest a role for hPygo2 in the growth of breast cancer and indicates that it may be of therapeutic importance in combating this disease.

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