Developmental Phenotypes and Reduced Wnt Signaling in Mice Deficient for Pygopus 2

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Received 14 November 2006; Revised 7 March 2007; Accepted 16 March 2007

Summary: Canonical Wnt signaling involves complex intracellular events culminating in the stabilization of β-catenin, which enters the nucleus and binds to LEF/TCF transcription factors to stimulate gene expression. Pygopus was identified as a genetic modifier of Wg (Wnt homolog) signaling in Drosophila, and encodes a PHD domain protein that associates with the β-catenin/LEF/TCF complex. Two murine pygopus paralogs, mpygo1 and mpygo2, have been identified, but their roles in development and Wnt signaling remain elusive. In this study, we report that ablation of mpygo2 expression in mice causes defects in morphogenesis of both ectodermally and endodermally derived tissues, including brain, eyes, hair follicles, and lung. However, no gross abnormality was observed in embryonic intestine. Using a BAT-gal reporter, we found Wnt signaling at most body sites to be reduced in the absence of mpygo2. Taken together, our studies show for the first time that mpygo2 deletion affects embryonic development of some but not all Wnt-requiring tissues. genesis 45:318–325, 2007. © 2007 Wiley-Liss, Inc.

Key words: pygo2; pygopus; Wnt/β-catenin signaling

INTRODUCTION

The secreted Wnt proteins trigger several intracellular signaling pathways, of which the most extensively studied is canonical Wnt signaling (Logan and Nusse, 2004; Veenman et al., 2003). The canonical Wnt pathway functions in normal development and adult homeostasis of myriad tissues and organs, and is associated with diseases of the bone, brain, eye, skin, and heart, as well as cancer (Clevers, 2006; Logan and Nusse, 2004). Wnt signaling has been shown to direct patterning and cell fate decisions during skin appendage formation and lung morphogenesis (Andl et al., 2002; Chu et al., 2004; Gat et al., 1998; Huelsken et al., 2001; Shu et al., 2005), while during intestinal and lymphocyte development, it regulates proliferation and survival of stem/progenitor cells (Korinek et al., 1998; Reya et al., 2000; Timm and Grosschedl, 2005).

The critical event of activated Wnt signaling is stabilization of β-catenin, which then enters the nucleus and binds a member of the LEF/TCF family of transcription factors to stimulate expression of target genes involved in diverse cellular processes. Pygopus, a PHD domain protein identified by genetic screens in Drosophila, is suggested to function as a devoted coactivator for the β-catenin/LEF/TCF complex, and/or to facilitate nuclear retention of the complex (Kramps et al., 2002; Krieghoff et al., 2006; Stadeli and Basler, 2005; Thompson, 2004; Townsley et al., 2004). The in vivo role of mammalian pygopus genes in development and in Wnt signaling has not been explored. Of the two mammalian pygopus paralogs, murine pygopus 2 (mpygo2) is more broadly expressed than pygopus 1 (mpygo1) during embryonic development and in adult tissues (Li et al., 2004).

To investigate the in vivo function of mpygo2, we used Cre/loxP technology to generate mice with deficient or conditional alleles of the gene. LoxP sites were introduced in positions flanking exon 3, which encodes amino acids 52–405 of the protein, including a conserved PHD finger domain (Fig. 1a) (Li et al., 2004). Homologous recombination followed by Cre-mediated excision in ES cells gave rise to clones containing either an mpygo2 deletion allele (where both exon 3 and neo are removed; referred to herein as “mutant” or “−/−”), or a “floxed” allele (where neo is removed and exon 3 is

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Published online in Wiley InterScience (www.interscience.wiley.com).

DOI: 10.1002/dvg.20299
flanked by LoxP sites), as confirmed by Southern blot analyses, and small arrows above the targeted locus indicate positions of primers used in PCR genotyping (see Methods). (b) Southern and (c) PCR analysis of E14 ES cell clones containing wild-type or mutant alleles. (d) Southern and (e) PCR genotyping of embryos from intercrosses of heterozygous mutants containing the "-" allele. (f) Northern blot analysis of RNA from +/- and +/- E18.5 skin using a cDNA probe specific for exon 1. Note absence of the 3.2-kb mpygo2 transcript in the mutant (arrow). The same blot was stripped and reprobed with GAPDH to control for loading. (g) Western blot analysis of +/- and +/- E18.5 skin extracts using the α-mPygo2 antibody. An anti-actin antibody was used as loading control (bottom).

We next performed immunofluorescence on select Wnt-requiring embryonic tissues (DasGupta and Fuchs, 1999; Merrill et al., 2001; Mucenski et al., 2003) to examine whether mpygo2 is expressed there. Nuclear mPygo2 protein was detected in epithelial cells of the developing pelage and whisker hair follicles (Fig. 2a,c), respectively. Within the pelage hair follicle, the protein was primarily localized in the presumptive bulge, in matrix/precortex cells, and in dermal cells adjacent to these epithelial compartments. In E18.5 lung, most prominent nuclear staining was seen in a subset of epithelial cells lining the larger terminal airways (Fig. 2e); however, scattered mPygo2-positive cells were also observed in epithelium of the smaller, peripheral terminal airways, as well as in the mesenchyme (Fig. 2e). In E18.5 intestine, weak, but detectable nuclear staining was seen in cells of the villous epithelium, where future crypts form, and in a subset of mesenchymal cells (Fig. 2g). No specific staining was observed in mpygo2-deficient tissues (Fig. 2b,d,f,h).

Consistent with the role of Wnt signaling in multiple developmental processes, we observed a pleiotropic phenotype in mpygo2-/- animals. Most mutants were born slightly runted (with an average reduction of 15–20% in body weight and ~10% in length) and all exhibited perinatal lethality. Approximately 14% of 63 mutant animals examined between E12.5 and term displayed an enlarged brain and exencephaly or a domed head (Fig. 3b,d). Furthermore, macroscopic analysis of mutant pups revealed ~25% of mutants had smaller eyes; often these were distinct from the pups with brain defects (Fig. 3f,h). Histological analysis of eyes at different embryonic stages revealed several abnormalities. First, at all stages examined, mutant embryos contained smaller lens than the wild type or no lens at all (Fig. 3i,k,n,o). At E15.5, the wild-type lens was well-differentiated with cuboidal epithelial cells restricted to the anterior part of the lens (or capsule) (Fig. 3l, left). In contrast, the mu-
mPygo2-expressing intestinal epithelial and mesenchymal cells, respectively. Arrow and ''*'' in (e) point to strongly stained lung epithelial cells and mesenchymal cells, respectively. Arrow and arrowhead in (g) indicate bulge and precortex of a pelage follicle, respectively. Arrow in (c) indicate mPygo2 expression in outer root sheath cells of a whisker follicle.

Red, so that nuclei of mPygo2-expressing cells were yellow. Arrow and arrowhead in (a) indicate mPygo2 expression in the presumptive high magnification images showing nuclear presence of the protein. White lines denote basement membrane. Bar: 65 μm in a, b; 20 μm in c, d; 25 μm in e, f; 50 μm in g, h.

**FIG. 2.** mPygo2 protein expression in embryonic tissues. Shown are results of immunofluorescence analysis of E18.5 pelage hair (a, b) and whisker (c, d) follicles, lung (e, f), and intestine (g, h) using a rabbit α-mPygo2 antibody (green). DAPI staining was artificially colored red, so that nuclei of mPygo2-expressing cells were yellow. Arrow and arrowhead in (a) indicate mPygo2 expression in the presumptive bulge and precortex of a pelage follicle, respectively. Arrow in (c) indicates mPygo2 expression in outer root sheath cells of a whisker follicle. Arrow and ''*'' in (e) point to strongly stained lung epithelial cells and mesenchymal cells, respectively. Arrow and arrowhead in (g) indicate mPygo2-expressing intestinal epithelial and mesenchymal cells, respectively. ''*'' in (g) indicates nonspecific signals. Insets in (a) and (c) are high magnification images showing nuclear presence of the protein. White lines denote basement membrane. Bar: 65 μm in a, b; 20 μm in c, d; 25 μm in e, f; 50 μm in g, h.

Tant lens was poorly differentiated, contained few lens fibers, and was surrounded by cuboidal epithelial cells (Fig. 3l, right). These defects persisted until later, as cuboidal cells with nuclei were still seen at both the anterior and posterior parts of the mutant lens at E18.5 (Fig. 3q). Such eyelid development was delayed in the mutant, as at E15.5 mutant eyelids failed to form and close properly (Fig. 3k, arrowhead). Third, the cornea did not form properly at E15.5 (Fig. 3k1; a single-layered surface ectoderm was seen where cornea was expected), and appeared deformed at E18.5 (Fig. 3n, q) in mutant embryos. Finally, the hyaloid cavity was not present and the retina was abnormally organized and often convoluted (Fig. 3i–o). Hair follicle defects were also seen but were relatively mild. A single mutant newborn showed an arrest of hair follicle development at the bud stage; however, most mutants examined contained grossly normal or slightly less elongated follicles than the wild type (Fig. 3k, a, b, and data not shown). An ~30% decrease in hair follicle density was observed in the mutant (Fig. 4c–e). These results demonstrate that mpygo2 is required for the proper morphogenesis of several ectodermally-derived tissues.

Endodermally derived tissues were also examined. Lungs from E18.5 and newborn mpygo2–/– animals were often pale and smaller than the wild type (data not shown). Examination of hematoxylin/eosin (H/E)-stained sections revealed that at this stage wild-type lung contained well-developed terminal airways, characterized by the presence of small, irregularly shaped sacs lined with a wavy epithelium (Fig. 5a, c). In contrast, mutant lung contained airways that terminate in large, smooth-walled cylindrical structures (Fig. 5b, d). These defects are reminiscent of the reduced number of airway sacsules observed in mice overexpressing Dkk1, a Wnt inhibitor (Shu et al., 2005), and suggest that loss of mpygo2 also inhibited distal airway branching and formation in the embryonic lung. Furthermore, the expected thinning of the interstitial tissue, which normally occurs upon septation, appeared defective in the mutant (Fig. 5d). In the intestine, previous studies of TCF-4 knockout mice implicate Wnt signaling in the maintenance/proliferation of progenitor cells located in the intervillous intestinal epithelium (Korinek et al., 1998). Surprisingly, the intervillous region of E18.5 and newborn mpygo2–/– animals appeared indistinguishable from the wild type (Fig. 5f, compare with 5e). By BrdU labeling, comparable levels of progenitor cell proliferation were observed between wild-type and mutant animals (Fig. 5g, h). Furthermore, histological staining for goblet and enteroendocrine cells did not reveal significant alterations in the mutant intestine (data not shown). Nearly all E18.5 mutant embryos (>90%) showed an average of ~10–15% reduction in the length of their small intestine compared to littermates. However, the correlation of this reduction to that in body weight/length makes it unlikely that this is a specific defect.

To address whether mpygo2 ablation affects Wnt signaling, we generated double transgenic mice carrying a transgenic BATgal Wnt reporter gene and wild-type or mutant mpygo2 alleles. In BATgal transgenics, LacZ is under the control of LEF/TCF-responsive elements, allowing β-galactosidase activity to be detected at sites where canonical Wnt signaling is active (Maretto et al., 2003). Whole-mount staining for β-galactosidase activity revealed a general reduction of BATgal expression in mutant embryos (Fig. 6a, b, and data not shown). Section-
FIG. 3. Brain (a–d) and eye (e–q) defects in mpygo2−/− mice. Shown are morphology (a–h) and histology (i–q) of wild-type (+/+) and mutant (−/−) animals at E12.5 (a–b), E13.5 (i), E15.5 (e–f, j–l), E18.5 (c–d, g–h, m–q). (l) contains high magnification images of boxes shown in j (left) and k (right). (p) and (q) are high magnification images of boxes shown in (m) and (n), respectively. Note that lens (indicated by “*”) is smaller in one mutant (n) but absent in another (o). Also note the presence of cuboidal epithelial cells at the posterior (P) part of the mutant lens (l, q). A, anterior part of the lens; HC, hyaloid cavity. Arrows indicate cornea, which is defective in the mutant. Bar: 130 μm in i; 225 μm in j, k; 55 μm in l; 285 μm in m–o; 75 μm in p, q.

FIG. 4. Reduced hair follicle density in mpygo2 mutant. Longitudinal (a, b) and cross-sections (c, d) of E18.5 skin were stained with hematoxylin/eosin. Cross-sections immediately underneath the skin surface were taken to quantify the total number of hair follicles (including those that may show delayed development), and values per 2.6 × 10^5 μm^2 were graphically represented in (e) (P = 0.0017, n = 20). Bar: 80 μm in a, b; 120 μm in c, d.
ing through stained E11.5 embryos revealed BAT-gal expression primarily in the mesenchymal cells between surface ectoderm and the neural tube and a decreased number of LacZ⁺ cells in the mutant (arrow in Fig. 6d; compare with Fig. 6c). A subset of surface ectodermal cells above the neural tube were also positive, and again the number of positive cells appeared to be reduced in the mutant (Fig. 6e,f). The lateral surface ectoderm in

**FIG. 5.** *mpygo2*⁻/⁻ embryos show abnormal lung morphology but no apparent anomaly of presumptive intestinal crypts. Shown are results of histological analysis of lung (a–d) and intestine (e, f), and of BrdU-labeling experiments of intestine (g, h). Arrows in (g, h) point to BrdU-labeled cells in the intervillous region. Bar: 85 μm in a, b; 30 μm in c, d; 33 μm in e, f; 50 μm in g, h.

**FIG. 6.** Reduced Wnt signaling in some *mpygo2*-deficient embryonic tissues. (a, b) Whole-mount LacZ staining of E11.5 wild-type (+/+ ) and mutant (−/−) embryos. Note that reduction is particularly striking in posterior structures of the mutant, but is also visible in the head region (arrows). (c–h) H/E counter-stained sections of E11.5 embryos at positions indicated by lines in (a–b). (e) and (f) show high magnification images of boxed areas in (c) and (d), respectively. Arrows in (c–d) and (g, h) point to LacZ⁻ mesenchymal and LacZ⁻ surface ectodermal cells, respectively. (i, j) Prolonged LacZ staining to show expression in eyelids of E11.5 embryos. Note reduced staining in some (arrowhead) but not all areas of the mutant. (k, l) Whole-mount LacZ expression analysis showing stained hair follicles on back skin of E15.5 embryos. (m) Assays of β-galactosidase activity in extracts of skin (light purple) and lung (burgundy) from E18.5 embryos of the indicated genotypes. Values for the wild-type animals were set as 1, and * indicates statistically significant (P < 0.06) differences from the wild type. Bar: 125 μm in c, d; 55 μm in e, f; 55 μm in g, h.
widespread effects on the development of various tissues, including brain, eyes, hair follicles, and lung. Curiously, BAT-gal expression was predominantly observed in pigmented cells of retina, a layer that was apparently unaffected by mpigo2 deletion (Fig. 3k), and no reduction of lacZ staining was seen in the mutant (data not shown). Prolonged incubation with β-galactosidase substrates helped to visualize BAT-gal expression in surrounding eyelids, and it appeared that fewer cells were LacZ-positive in the mutant (arrowhead in Fig. 6j). Decreased BAT-gal expression in mutant hair follicles was evident in E15.5 embryos (Fig. 6k,l). Quantitative assay of β-galactosidase activity was performed on extracts of dorsal skin and lung from E18.5 embryos, and mutant tissues displayed considerably lower (>two-fold) activity than the wild type (Fig. 6m). Interestingly, despite the absence of any apparent phenotype in heterozygous mutants, a slight reduction in β-galactosidase activity was also seen in their skin and lung extracts (Fig. 6m), suggesting that morphogenic consequences occur only when signaling activity falls below a certain critical threshold. A very high level of β-galactosidase activity was detected in wild-type intestinal extracts, and no decrease was observed in the mpigo2 mutants (data not shown). Taken together, our results indicate that mpigo2 is required in vivo for maximum Wnt signaling in some but not all tissues.

In summary, our data demonstrate that mpigo2 is required for a subset of mammalian developmental processes that require Wnt signaling, particularly the proper morphogenesis of brain, eyes, hair follicles, and lung. This work is the first to address the in vivo function of a mammalian pygopus gene. Because mpigo2<sup>-/-</sup> animals die perinatally, these experiments focused on embryonic defects caused by the deficient allele. Availability of the “floxed” mpigo2 allele as reported here will facilitate future analysis of mpigo2 function in specific adult tissues. Our observation of reduced BAT-gal expression in most mpigo2-deficient tissues suggests that mpigo2 functions in vivo to modulate Wnt signaling. However, phenotype appearance does not correlate 100% with reduced BAT-gal expression, particularly for eyes and brain. It is possible that Wnt signaling is affected in these tissues at an earlier developmental stage not examined here, or alternatively, that mpigo2 acts in these tissues in a Wnt-independent manner. Whether mpigo2 functions cell-autonomously and via protein–protein interactions to potentiate transcriptional activation by β-catenin/LEF/TCF complexes, as previously proposed (Kramps et al., 2002; Kriehoff et al., 2006; Stadeli and Basler, 2005; Thompson, 2004; Townsley et al., 2004), or that it regulates intermediate pathways or factors that in turn activate Wnt signaling remains to be addressed using more direct methods. With multiple attempts, we have not been able to detect nuclear β-catenin in wild-type tissues under study; thus we could not address a previously raised notion that Pygopus proteins facilitate the nuclear accumulation of β-catenin (Thompson et al., 2002; Townsley et al., 2004). A considerable level of Wnt signaling remained in the absence of mpigo2; consistently, mpigo2-deficient animals do not present a phenotype expected for a mutant with a complete loss of Wnt signaling. The early Wnt signaling-associated phenotypes, such as body axis and mesodermal defects displayed by the β-catenin knockout animals (Haegele et al., 1995; Huelsken et al., 2000), were not observed in mpigo2-deficient embryos. While β-catenin ablation or Dkk1 overexpression abolishes hair follicle initiation (Andl et al., 2002; Huelsken et al., 2001), mpigo2 mutant newborns are able to produce hair follicles, albeit at a reduced density. Furthermore, unlike TCF-4 (Korinek et al., 1998), mpigo2 does not appear to be required for the formation and proliferation of intestinal stem cells in developing embryos. However, inactivation of β-catenin or overexpression of Dkk1 in lung epithelium results in expanded proximal airway formation at the expense of distal airways (Shu et al., 2005), a phenotype shared by the mpigo2 mutant embryos. While flies have a single pygopus gene, mammals have two pygopus paralogs. It is possible that mpigo1 plays a partially redundant role for mpigo2 during early developmental processes and in tissues that are least affected by the loss of mpigo2, although we did not observe any significant increase in the level of mpigo1 transcripts in mpigo2-deficient embryonic skin (data not shown). Definitive evidence awaits the generation and analysis of mpigo1 and mpigo2/mpigo1 double mutant mice. Alternatively, pygopus genes in mammals may have evolved to play a nonessential but augmenting role in Wnt signaling, and requirements for such an augmentation role vary by tissue type. For example, mpigo2 may only be needed when a particular LEF or TCF factor is in use at a particular tissue site. In light of our findings and a recent study that describes Wg-independent association of Drosophila Pygo with target genes of dTCF (de la Roche and Bienz, 2007), it becomes clear that further studies are needed to fully understand the cellular and molecular function of mammalian pygopus genes and their relationship with Wg/Wnt signaling.

METHODS

Generation of mpigo2 Mutant Alleles

mpigo2 genomic fragments were obtained by PCR using E14 ES cell DNA as a template and were cloned into the pPGKneobAlox2PGKDTA vector (a generous gift of Phil Soriano) to generate a targeting construct. Electrotransformation into E14 ES cells, screening for recombinants, and the generation of chimeric mice and germline mutants were performed as described (Mackay et al., 2006). PCR genotyping was performed using the following primers: (a) for the detection of targeted, floxed, and “allele, respectively, in ES DNA: 5′-CTCTAGCGTGTC-TAACGTAGCCAGACGG-3′ (primer no. 2 in Fig. 1a) and 5′-AGGGCAAGCTGCCATGTCACTTC-3′ (primer no. 323 DOI 10.1002/dvg
After three washes in 0.1 M phosphate buffer, pH 7.3, MgCl₂, 5 mM EGTA for 10–45 min at room temperature.

Histology and Immunostaining

Embryos or tissues were fixed in Bouin’s fixative for 24 h, processed and embedded in paraffin wax, sectioned at 6 μm, and stained with hematoxylin/eosin. For quantification of the number of hair follicles, cross-sections of E18.5 skin were prepared and hair follicles were counted in an area of 2.6 × 10³ μm². Statistical significance was determined using Student’s t-test. Indirect immunofluorescence of embryonic tissues was performed as described (Dai et al., 1998) using rabbit α-mPygo2 antiserum that was preadsorbed with tissue powder prepared from E15.5 mpygo2⁻/⁻ embryos. This antiserum was generated to a GST fusion protein containing amino acids 6–115 of mPygo2 (Harlan Bioproducts for Science) and affinity-purified.

BrdU-Labeling and Immunodetection

Pregnant mice were injected intraperitoneally with 50 μg/g body weight of BrdU in PBS. Embryos were taken 1 h after injection, cryosectioned at 10 μm, and fixed in 4% paraformaldehyde for 10 min, followed by immunohistochemistry as described (Li et al., 2005).

Analysis of BAT-gal Expression in Wild-Type and mpygo2-Deficient Embryos

mpygo2²/²⁻ mice were bred with BAT-gal transgenics (Maretto et al., 2003) to obtain mpygo2²/²⁻/⁻ BAT-gal offspring, which were subsequently bred with mpygo2²/²⁻/⁻ mice to generate mpygo2²/²⁻/⁻ BAT-gal and mpygo2²/²⁻/⁻-BAT-gal littersmates for analysis. E11.5 to E15.5 embryos were subjected to whole-mount staining for β-galactosidase activity as follows: embryos were fixed in 0.1 M phosphate buffer, pH 7.3, 0.2% glutaraldehyde, 2 mM MgCl₂, 5 mM EGTA for 10–45 min at room temperature. After three washes in 0.1 M phosphate buffer, pH 7.3, 2 mM MgCl₂, 0.01% sodium deoxycholate, 0.02% NP-40, they were transferred into freshly prepared X-gal staining solution containing 0.5 mg/ml X-gal, 10 mM potassium hexacyanoferrate (III), 10 mM potassium hexacyanoferrate (II), 5 mM EGTA, and 2 mM MgCl₂ in potassium phosphate buffer, and stained for 30 min at room temperature in the dark. After rinsing with PBS, embryos were postfixed in 4% paraformaldehyde and examined under the microscope for photography. For quantification of β-galactosidase activity, extracts were prepared by homogenizing E18.5 embryonic tissues in a lysis solution containing 100 mM potassium phosphate (pH 7.8) and 0.2% Triton X-100 (10 ml/g tissue), followed by three freeze-thaw cycles. After centrifugation at 4°C, β-galactosidase activity was measured from the supernatant using a chemiluminescence assay kit (Galacto-Light Kit from Tropix). β-galactosidase activity was normalized against protein concentration for each sample, and expressed as fold change over wild-type control. Statistical significance was determined using Student’s t-test.

ACKNOWLEDGMENTS

We thank the UCI Transgenic Mouse Facility for technical assistance with ES cell electroporation (Michele Musacchio), and blastocyst injection (Tom Fielder and Kai-Xian Shi). We are grateful to Qian-Chun Yu for advice on histological analysis and to Maike Sander for help with the BAT-gal mice.

LITERATURE CITED


