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Cloning and developmental expression of mouse *pygopus* 2, a putative *Wnt* signaling component⋆

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Abstract

Recent studies in *Drosophila* identified *pygopus*, which encodes a PHD finger protein, as an additional nuclear component of the canonical *Wingless(Wg)/Wnt* signaling pathway. In this study, we describe the molecular cloning and expression analysis of a mouse *pygopus* gene, *mpygo*2. *mpygo*2 transcripts were detected in almost all adult mouse tissues examined, whereas transcripts of another mouse *pygopus* gene, *mpygo*1, were detected only in heart tissue. Abundant *mpygo*2 transcripts were observed during embryogenesis in multiple developmental sites. Consistent with the demonstrated role of the *Wnt*-β-catenin–LEF/TCF signaling pathway in mammalian skin development, *mpygo*2 expression was detected in the developing epidermis and hair follicles, which suggests that *mpygo*2 might mediate the effect of this signaling pathway in mouse skin.

Keywords

Wg/Wnt signaling; β-Catenin; LEF/TCF; *pygopus*; *mpygo*2; *mpygo*1; Skin; Hair follicles

Introduction

Wnt signaling is crucial for normal development and tumorigenesis [1–3]. The developmental processes regulated by this signaling event are diverse, ranging from patterning the body axis and the central nervous system to organogenesis that involves reciprocal epithelial-mesenchymal interactions [1,2,4]. Furthermore, *Wnt* signaling is implicated in the maintenance and differentiation of postnatal stem cells in epithelial tissues such as skin and intestine [1,2, 5]. An activated *Wnt* signal triggers several distinct intracellular signal transduction pathways [6]. The so-called canonical pathway, which is the most extensively characterized, involves a complex series of downstream events culminating in the stabilization of β-catenin, a protein with dual functions in cell–cell adhesion and signaling [3,6]. β-Catenin accumulates in the cytoplasm and enters the nucleus, where it binds to the LEF/TCF family of transcription factors and regulates gene expression.

The *Wnt* signaling pathway intersects with a complex network of molecular events both outside and inside the nucleus. Studies have shown that several nuclear factors, including Sox, Smad, CBP, CtBP, and Groucho, can associate with either β-catenin or LEF/TCF proteins to modulate the transcription regulatory activity of the β-catenin–LEF/TCF complex [7–14]. Distinct from these nuclear factors that display context-dependent modulatory activities, a recently identified

⋆Sequence data from this article have been deposited with the EMBL/GenBank Data Library under accession number AY486147 (mouse *mpygo*2 full-length cDNA).

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gene in *Drosophila*, named *pygopus* (*pygo*), appears to encode an obligatory and highly specific nuclear component of the β-catenin–LEF/TCF transcription regulatory complex [15–18]. Mutations in *pygo* specifically disrupt *Wg* (the fly *Wnt* counterpart) signaling throughout *Drosophila* development [15–18]. The Pygo protein contains a PHD domain, often found in chromatin remodeling factors that are thought to alter chromatin structure and thereby allow activation or repression of specific genes [19]. Pygo is recruited to β-catenin–LEF/TCF via an adaptor protein, Lgs/BCL9, and existing evidence suggests that Pygo is required for maximum activation of β-catenin–LEF/TCF-dependent promoters/genes in cell culture [15–18,20]. Pygo homologs have also been identified in *Xenopus*, and functional studies indicate that xPygo proteins are required for *Wg/Wnt* signaling during *Xenopus* embryogenesis, particularly in embryonic brain patterning [18,21].

Although previous studies have implicated the existence of two mouse *pygopus* genes, *mpygo*1 and *mpygo*2 [15,18], a full-length sequence of *mpygo*2 was not reported. Here we describe the cloning of a full-length *mpygo*2 cDNA and the structure of the *mpygo*2 gene. Our evidence shows that *mpygo*2 is expressed during embryonic development and in multiple adult tissues and suggests widespread involvement of *mpygo*2 in canonical *Wnt* signaling.

Results

Cloning and molecular structure of mpygo2

BLAST searches of the GenBank database using the sequences of *Drosophila pygo*, human *pygo*1 (*hpygo*1), and human *pygo* 2 (*hpygo*2) revealed two mouse cDNA sequences representing *mpygo*1 and *mpygo*2, respectively. Although *mpygo*1 encodes a full-length protein of 417 amino acids, the *mpygo*2 cDNA thus identified encodes only what may be a truncated open reading frame (ORF) of 338 amino acids. We therefore cloned a full-length *mpygo*2 cDNA by compiling PCR-amplified cDNA fragments, and the sequence of this cDNA is shown in Fig. 1A. This full-length *mpygo*2 cDNA encodes an ORF of 405 amino acids. The putative mPygo2 protein shares 81% amino acid sequence identity with hPygo2, whereas mPygo1 shares 56% overall amino acid sequence identity with hPygo1. Like other Pygo proteins, mPygo2 contains a predicted nuclear localization signal (NLS) sequence in its N-terminal region and a Cys_4-His-Cys_3 PHD finger domain in its C-terminal region (Fig. 1A). The amino acid sequence alignment of all Pygo proteins identified thus far reveals two well-conserved domains across species: a domain rich in proline and charged amino acids at the N-terminus, previously referred to as the N-terminal homology domain (NHD [17]), and the PHD finger domain at the C-terminus (Fig. 1B).

The structure of the *mpygo*2 gene was revealed by a comparison between the *mpygo*2 genomic and cDNA sequences. The gene consists of three exons that span approximately 4.9 kb (Fig. 1C). The predicted physical location of *mpygo*2 is on the F2 band of mouse chromosome 3, between D3Mit49 and D3Mit341 and approximately 90.5 cM from the centromere. Searches against various available mouse genomic sequence databases consistently identified only one hit on chromosome 3. Southern blot analysis using an *mpygo*2 genomic fragment also revealed a single band (data not shown). Taken together, these results indicate that *mpygo*2 is a single gene in the mouse genome.

mpygo2 displays a broader expression spectrum than mpygo1 during development and in adult tissues

The tissue distribution of *mpygo*2 and *mpygo*1 expression was determined. Northern blot analysis identified *mpygo*2 transcripts in almost all adult mouse tissues examined, including brain, heart, kidney, liver, lung, skin, small intestine, spleen, stomach, testis tissue, and thymus (Fig. 2A). All expressing tissues contained a major *mpygo*2 transcript of ~3.2 kb, confirming

that the cDNA we cloned is indeed full-length, whereas testis contained an additional transcript of 4.5 kb. In contrast, a *mpygo*1 transcript of 7.5 kb was detected only in heart tissue even after prolonged exposure.

The expression of *mpygo*2 and *mpygo*1 was also examined during development using a blot containing poly(A)$^+$ RNA prepared from developing mouse embryos. *mpygo*2 transcripts were detected in embryos of all ages examined, including E7, E11, E15, and E17 (Fig. 2B), with the expression level peaking at E17. In contrast, weak *mpygo*1 expression was detected in E15 and E17 embryos. Therefore, *mpygo*2 appears to be expressed more broadly in postnatal tissues and at a higher level in developing embryos than *mpygo*1.

mpygo2 is expressed in multiple embryonic tissues

To determine the tissue distribution of *mpygo*2 during development, we performed in situ hybridization on developing embryos. At E12.5, *mpygo*2 expression was detected nearly throughout the embryo but was strongest in the mesencephalon of the brain and in the spinal cord (Fig. 3A). A similarly broad pattern of expression was also observed in embryos at E13.5, E15.5, and E16.5 (data not shown). These results are in agreement with the widespread expression of *mpygo*2 in adult tissues.

Higher magnification images revealed additional details of *mpygo*2 expression in selected tissues such as brain (Figs. 3C and 3D), spinal cord (Fig. 3E), lung (Fig. 3F), intestine (Fig. 3G), and liver (Fig. 3H). In the developing brain, *mpygo*2 hybridization signals were observed in multiple regions including the telencephalon, diencephalon, mesencephalon, metencephalon, myelencephalon, cerebral peduncle, and lamina terminalis, but expression was considerably stronger in the mesencephalon (Figs. 3C and 3D). In the developing lung, *mpygo*2 transcripts were detected in the bronchus epithelium (arrow in Fig. 3F). In the developing intestine, *mpygo*2 transcription was observed in the epithelium of the future colon (Fig. 3G).

Expression of mpygo2 in developing and adult hair follicles

Hair follicle development in mice represents a well-studied system in which *Wnt* signaling components are functionally required [22]. We therefore examined *mpygo*2 expression in skin within the developmental window (E12.5–E16.5) during which hair follicle development initiates and proceeds [23]. At E12.5, hair follicle placodes are normally only present in the whisker pad region, and *mpygo*2 expression was detected in these developing vibrissae placodes (Fig. 4A). Furthermore, *mpygo*2 expression was seen in cells scattered throughout the surface ectoderm (arrowhead in Fig. 4B). By E13.5, *mpygo*2 transcripts appeared throughout the ectoderm, which is still single-layered at this stage, and in emerging pelage hair placodes (Fig. 4C). As development proceeds, *mpygo*2 expression expanded to include the future suprabasal layers of the epidermis and the entire epithelial compartment of the hair germ (Figs. 4D and 4E). Expression was also observed in the dermal condensates around the developing pelage placodes that will form the future dermal papilla of adult hair follicles (arrows in Figs. 4C–4E).

The widespread expression of *mpygo*2 throughout the epithelial cells of the epidermis and hair follicles persisted in postnatal skin (Figs. 4F, 5A, and 5B and data not shown). Although comparable levels of expression were detected between the hair bulb and the upper portion of the mature hair follicles at P9 (Figs. 5A and 5C), hybridization signals appeared particularly prominent in the matrix of the early-(P23; Fig. 5E) and mid-anagen (P28; Fig. 5F) hair follicles of the new hair cycle. Weaker expression was also observed in the dermal papilla of these follicles. During telogen, *mpygo*2 transcripts were detected in the ball-shaped dermal papilla

at the base of the resting hair follicles and in the epithelial cells including the stem cell-containing bulge that lines the club hair (Fig. 5D).

Discussion

It is not uncommon that multiple homologs of a single gene in *Drosophila* exist in higher organisms such as mouse and human. In the case of *Drosophila* pygo, a newly identified nuclear component of the canonical *Wnt* signaling pathway, two mouse and human *pygopus* genes, namely *mpygo*1/*hpygo*1 and *mpygo*2/*hpygo*2, have been identified [15,17,18]. Are both paralogs required for mediating *Wnt* signaling in mammalian development? Results of our expression studies revealed a broad transcription pattern for *mpygo*2 but a restricted pattern of distribution for *mpygo*1 transcripts. *mpygo*2 expression occurs in multiple adult and developing tissues, many of which have been shown to require *Wnt* signaling activity for proper development, morphogenesis, and maintenance [6]. Overall, the embryonic and adult expression pattern of *mpygo*2 is more consistent than that of *mpygo*1 with the demonstrated widespread involvement of *Wnt* signaling in development and morphogenesis. Similarly, in the NCBI-UniGene database, *hpygo*2 (Hs.172084) cDNAs have been detected in a much broader range of embryonic and adult tissues, including various types of tumor tissues and cells, than *hpygo*1 (Hs.256587). The strongest embryonic *mpygo*2 expression was observed in the developing nervous system of the embryo. A functional involvement of *Wnt* signaling components in the developing and mature central nervous system is well-documented Conventional *Wnt*1 and conditional β-catenin knockout mice both show brain malformation [24–27]. *Wnt*1/*Wnt*3a double mutant mice show additional spinal cord defects [28]. The abundant expression of *mpygo*2 in the developing brain and spinal cord, together with the finding that a depletion of *xpygo*2 gene products in *Xenopus* led to brain defects [21], suggests that *mpygo*2 is an essential component of the *Wnt*-β-catenin–LEF/TCF signaling pathway in the development of the central nervous system in mice.

As a putative central component of the *Wnt* signaling pathway, *mpygo*2 is expected to be expressed during skin development, particularly during development of the hair follicle. The actual sites in skin where active *Wnt* signaling occurs have been elegantly mapped using transgenic mice expressing TOP-GAL, a LEF/TCF-responsive β-gal reporter [29,30]. As hair follicle morphogenesis initiates from the pluripotent ectoderm, *Wnt* signaling is activated in the forming placodes and in the underlying dermal condensates [30]. In postnatal hair follicles, LEF1 transcripts were detected primarily in the hair matrix and precortex cells, whereas TOP-GAL is activated in the precortex [30]. We observed active *mpygo*2 transcription in all of these locations, placing *mpygo*2 in the right place and at the right time to mediate *Wnt* signaling in these developmental sites. Interestingly, *mpygo*2 transcripts were also detected in the developing interfollicular epidermis, where canonical *Wnt* signaling does not appear to be required. Overall, *mpygo*2 appears to be expressed more ubiquitously than the actual sites of active *Wnt* signaling. This is also true for *Drosophila pygo* [16], yet *pygo* action appears to be highly specific to *Wg* signaling [15–18]. Studies are ongoing to examine the function of *mpygo*2 in the development and differentiation of epidermis and its appendages and to address whether it is involved in both *Wnt*-dependent and *Wnt*-independent pathways.

Materials and methods

Cloning and sequence analysis

*mpygo*2 genomic sequence was obtained from the Ensembl database and can be found in the GenBank database (accession number NT_078386). A full-length *mpygo*2 cDNA of 3198 bp was obtained by RT-PCR on RNAs isolated from adult mouse skin using the following primers: 5′ TGTCGAGTGGTGCGTTCCACTCT 3′ (5′PY2–4) and 5′ GGATTTTCAAGTCATTTTATTAATC-CAACCG 3′ (PY2–3′). The design was based on

*mpygo*2 genomic sequence and an existing *mpygo*2 EST sequence (GenBank accession number. BC025531). Sequencing was performed using this cDNA as well as cDNA fragments obtained by RT-PCR using several internal primer pairs, and a full-length sequence was assembled from that of overlapping fragments. mPygo2 protein sequence analysis was conducted using Pfam, Prosite, and Predict NLS server.

Northern blot analysis

Northern blot containing poly(A)$^+$ RNA from various adult mouse tissues was purchased from Origene (Rockville, MD). Northern blot containing poly(A)$^+$ RNA from four embryonic stages was purchased from Clontech (Palo Alto, CA). These blots were hybridized with a random primed, ^{32}P-labeled 531-bp probe containing *mpygo*1 cDNA sequence (GenBank accession number AK011208; probe corresponds to positions 1054–1584 of the sequence) or a 894-bp probe containing the 3′ UTR sequence of *mpygo*2 (corresponds to positions 1927–2820 of the *mpygo*2 cDNA sequence in Fig. 1A) using standard procedures.

In situ hybridization

Digoxigenin-labeled cRNA sense and antisense probes were synthesized from either a 894-bp *mpygo*2 fragment corresponding to the 3′ UTR region or a 1215-bp ORF-encoding *mpygo*2 fragment corresponding to positions 357–1571 of the *mpygo*2 cDNA sequence in Fig. 1A. Identical results were obtained using these two different probes. In situ hybridizations with these cRNA probes were performed using a procedure adapted from Deng and Lin [31]. Specifically, freshly dissected embryos were fixed in 4% paraformaldehyde/PBS overnight at 4 °C and subsequently passed through a series of sucrose/PBS solutions of increasing sucrose concentration. After an overnight incubation in 30% sucrose/PBS:OCT (1:1), the samples were frozen in 30% sucrose/PBS:OCT (1:3); 10-μm sections were then cut and dried at 50 °C for 2 hours, followed by drying at room temperature overnight. Sections were treated with Proteinase K (30 μg/ml in PBS) for 5 min at room temperature, rinsed briefly in PBS, and refixed in 4% paraformaldehyde/PBS for 7 min. For postnatal skin, 10- to 15-μm frozen sections were fixed in 4% paraformaldehyde/PBS for 10 min at room temperature. Postfixation washes, probe hybridizations, posthybridization washes, and immunological detection were as described in reference 31, followed by a colorimetric reaction using the NBT/BCIP substrates.

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Fig. 1.

Sequence and structural analysis of *mpygo*2 and its gene products. (A) *mpygo*2 cDNA sequence and the deduced amino acid sequence. The putative NLS sequence is boxed. The PHD finger domain is underlined. (B) Amino acid sequence alignment of the N-terminal (top) and C-terminal (bottom) domains conserved in Pygo proteins from mouse and human *Drosophila* and *Xenopus*. Identical residues are highlighted. NLS residues are marked by black dots under the sequence. Structural residues of the PHD domain are marked by black stars under the sequence. (C) Exon-intron structure of the *mpygo*2 gene. Rectangular boxes indicate exons (solid boxes, coding sequences; open boxes, noncoding sequences). Horizontal lines indicate introns. Sizes of exons and introns are indicated. D3Mit49 and D3Mit341 are markers on chromosome 3 that flank the *mpygo*2 gene, and chromosomal locations are indicated in cM. GenBank accession numbers of sequences used in alignment follow: Pygo, NM_143615; xPygo, AF521655; mPygo1, AK011208; hPygo1, AF457207; hPygo2, BC032099.

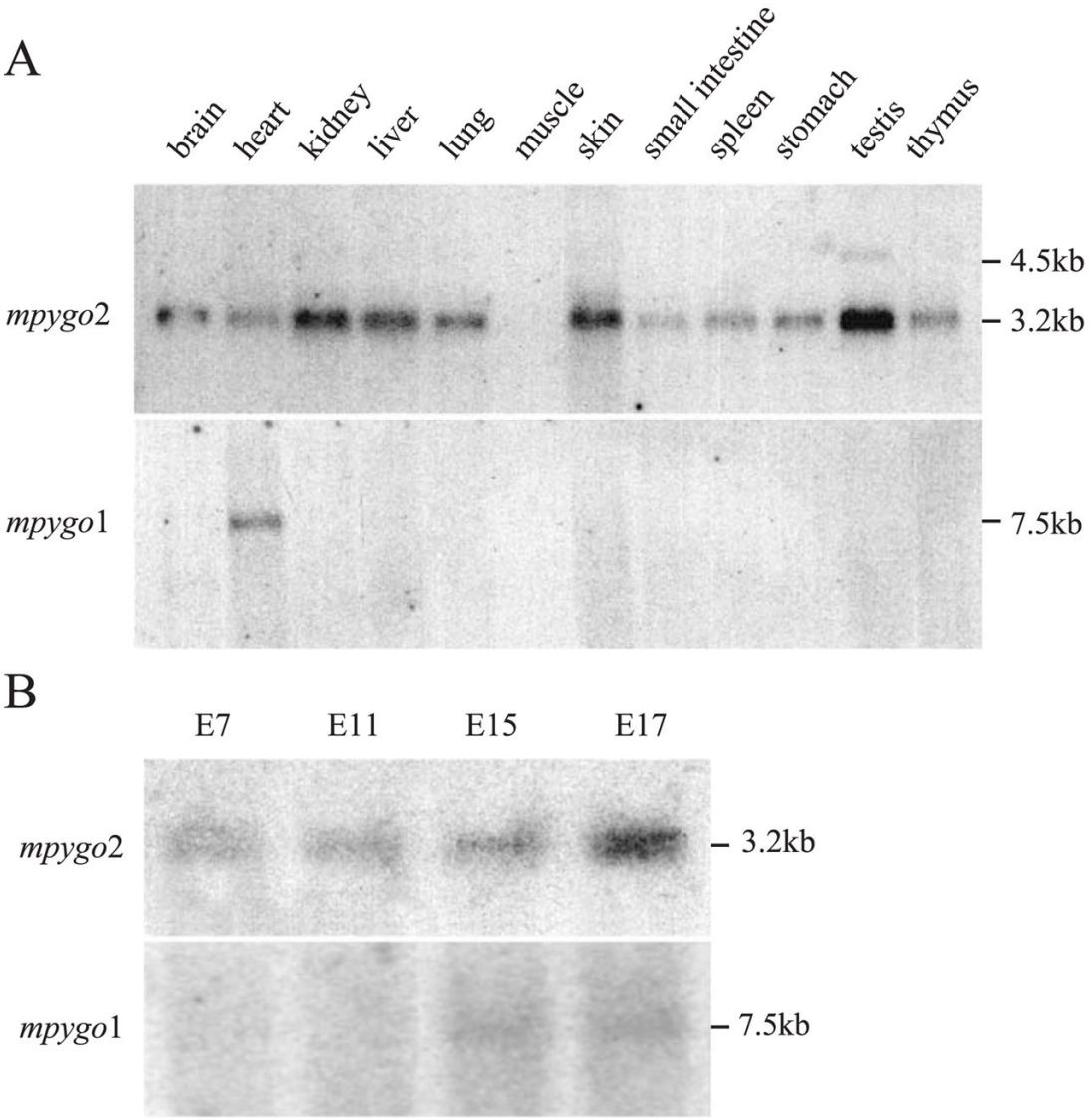

Fig. 2.

Tissue distribution and embryonic expression of *mpygo*2 and *mpygo*1. (A) Northern blot analysis of adult mouse tissue poly(A)$^+$ RNA. (B) Northern blot analysis of poly(A)$^+$ RNA from developing embryos at the indicated ages.

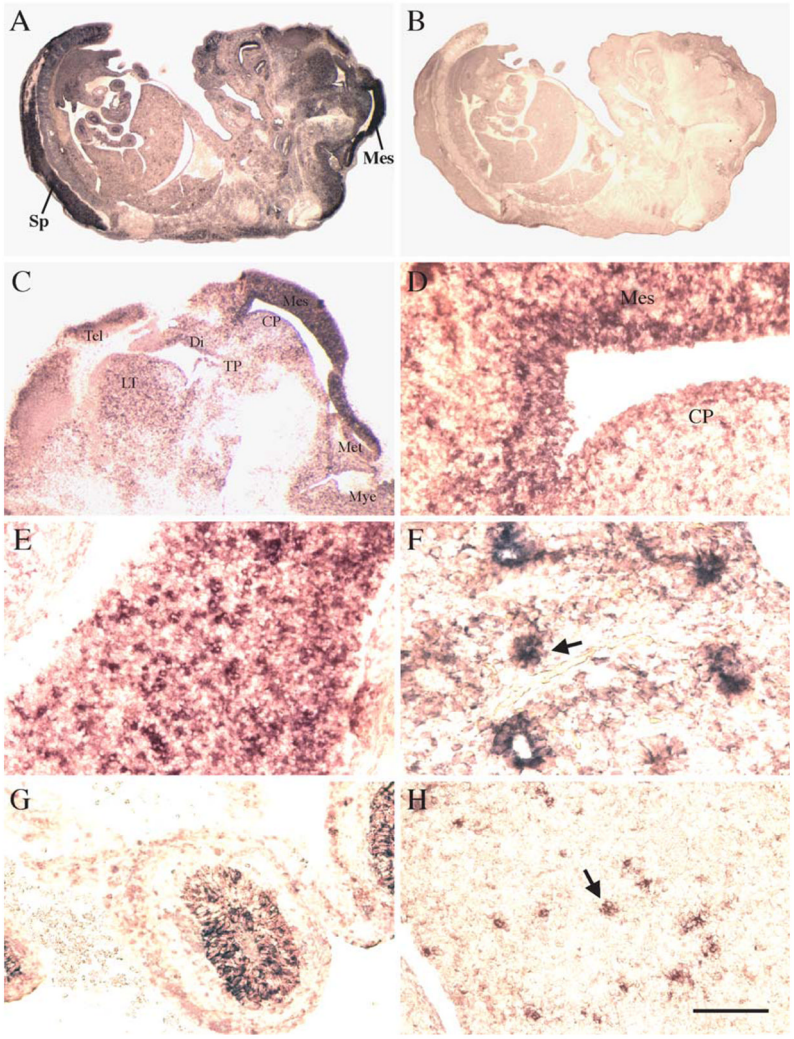

Fig. 3.

Developmental expression of *mpygo*2. Results are from in situ hybridization of E12.5 embryos using antisense (A, C–H) and sense (B) *mpygo*2 probes. (C–H) High-magnification images of selected developing tissues including brain (C and D), spinal cord (E), lung (F), intestine (G), and liver (H). Sp, spinal cord; Mes, mesencephalon; Tel, telencephalon; Di, diencephalon; Met, metencephalon; Mye, myelencephalon; CP, cerebral peduncle; TP, tuberculum posterius; LT, lamina terminalis. Arrow in F indicates bronchus epithelium of the developing lung. Arrow in H indicates *mpygo*2 expression in cells scattered throughout fetal liver. Bar, 2 mm in A and B; 800 μm in C; 120 μm in D, E, G, and H; 60 μm in F.

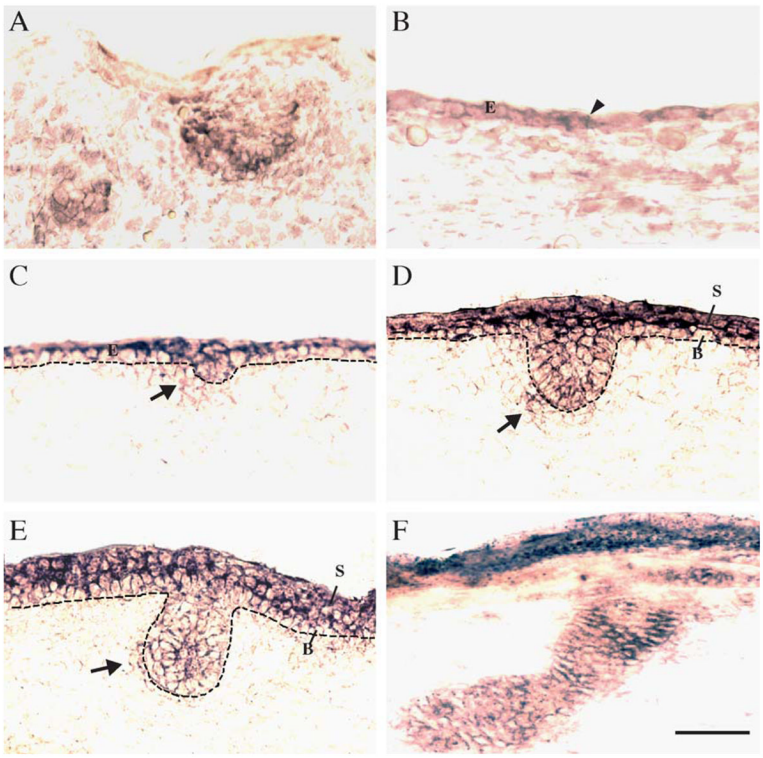

Fig. 4.

Expression of *mpygo*2 during skin development. Results are from in situ hybridization of developing skin at E12.5 (A and B), E13.5 (C), E15.5 (D), E16.5 (E), and newborn (F) using a *mpygo*2 cRNA antisense probe. Sense probes showed no specific hybridization signal (not shown). (A) *mpygo*2 expression in the developing vibrissae. (B–F) *mpygo*2 expression in developing pelage hair follicles. E, ectoderm; B, basal layer; S, suprabasal layers. Dotted line denotes the basement membrane. Arrows indicate hybridization signals in the dermal condensates. Bar, 50 μm.

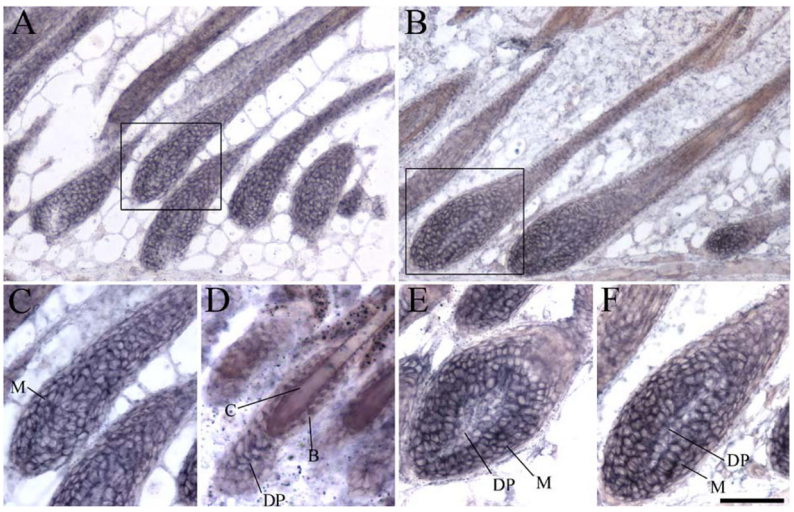

Fig. 5.

Expression of *mpygo*2 in mature and cycling hair follicles. Results are from hybridization of postnatal skin at the age of P9 (A and C), P21 (D), P23 (E), and P28 (B and F) using a *mpygo*2 cRNA antisense probe. (C and F) High-magnification images of the boxed areas in A and B, respectively. M, matrix; D, dermal papilla; B, bulge; C, club hair. Bar, 100 μm in A and B; 60 μm in C, E, and F; 40 μm in D.