Developmental Biology 318 (2008) 224–235

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

Analysis of *mPygo2* mutant mice suggests a requirement for mesenchymal Wnt signaling in pancreatic growth and differentiation

Nicolas Jonckheere [a,1], Erin Mayes [a,1], Hung-Ping Shih [a], Boan Li [b,2], Oleg Lioubinski [a,3], Xing Dai [b], Maike Sander [a,*]

[a] *Department of Developmental and Cell Biology, University of California Irvine, Irvine, CA 92697-2300, USA*
[b] *Department of Biological Chemistry, University of California Irvine, Irvine, CA 92697-1700, USA*

ARTICLE INFO

Article history:
Received for publication 23 October 2007
Revised 10 February 2008
Accepted 3 March 2008
Available online 20 March 2008

Keywords:
Pygopus
Wnt
Pancreas development
Mesenchyme
Islet
Endocrine
Proliferation
Differentiation
Mouse

ABSTRACT

Pygopus has recently been identified in *Drosophila* as an essential component of the nuclear complex required for canonical Wnt signaling. Here, we have investigated the role of the mammalian *pygopus* ortholog, *mPygo2*, in pancreas development. We show that a null mutation of *mPygo2* in mice causes pancreas hypoplasia due to decreased progenitor cell proliferation after embryonic day (e) 12.5. During the same time window, *mPygo2*-deficient embryos begin to display a reduction in endocrine progenitors and consequently a decrease in islet endocrine cell mass. Consistent with its function after e12.5, late-developing endocrine cell types, such as beta, delta and PP cells, are specifically reduced, while the earlier-forming alpha cells develop normally. We find canonical Wnt signaling to be predominantly active in the mesenchyme at the time when mPygo2 is required and demonstrate the dependence of Wnt signal transduction on mPygo2. Furthermore, conditional deletion of $mPygo2^{flox}$ allele in the pancreatic epithelium does not phenocopy the defects in *mPygo2*-null mutants. Since mPygo2 is expressed in the pancreatic mesenchyme and the role of the mesenchyme in epithelial progenitor cell expansion is well documented, our findings suggest an indirect role for mPygo2 in epithelial growth and differentiation through regulation of mesenchymal signals. Together, our data suggest a previously unappreciated role for mesenchymal Wnt signaling in regulating pancreatic organ growth and cell differentiation.

Introduction

In mammals, the pancreas develops as separate ventral and dorsal evaginations from the gut endoderm (Slack, 1995). As the dorsal and ventral pancreatic buds appear at embryonic day (e) 9.5 and e10.5, respectively, they each become surrounded by a cap of dense mesenchyme. Subsequent proliferation of the epithelial progenitors rapidly leads to formation of a branched epithelium that gives rise to an endocrine and an exocrine tissue compartment. The pancreatic endocrine compartment is comprised of glucagon-producing alpha, the insulin-producing beta, the somatostatin-producing delta and pancreatic polypeptide (PP)-producing cells that form sequentially during development and cluster in so-called islets of Langerhans shortly before birth. The exocrine acinar cells and ducts become visible as distinct structures by about e14.5.

All pancreatic cells arise from a common pool of pancreatic epithelial progenitor cells that are marked by the transcription factor Pdx1 (Gu et al., 2002). Expression of the transcription factors Ngn3 and after e13.5, Ptf1a, subsequently restrict the developmental potential of these pluripotent progenitors to an endocrine and exocrine fate, respectively (Gu et al., 2002; Zhou et al., 2007). Since the pancreas has limited capacity for adaptive growth, the final size of the endocrine and exocrine cell compartments at birth is largely determined by the size of the highly proliferative pluripotent progenitor cell pool at the pancreatic bud stage (Stanger et al., 2007). Mutations that affect progenitor cell expansion early in development therefore commonly result in peri- and postnatal pancreatic hypoplasia. Proliferation and branching of the epithelial buds is controlled by both autonomous cues in the epithelium as well as non-autonomous signals from the surrounding mesenchyme (Murtaugh, 2007). When cultured in the absence of mesenchyme, epithelial buds show minimal growth and branching, which is restored upon recombination with mesenchyme (Golosow and Grobstein, 1962; Wessells and Cohen, 1967). The mesenchyme not only controls progenitor cell expansion, but also promotes both endocrine and exocrine cell differentiation (Attali et al., 2007; Li et al., 2004b; Scharfmann, 2000).

Previous studies have provided evidence that the Wnt signaling pathway controls several aspects of pancreatic development. Early in

* Corresponding author. Fax: +1 949 824 4709.
E-mail address: msander@uci.edu (M. Sander).
[1] These authors contributed equally.
[2] Present address: School of Life Sciences, Xiamen University, Xiamen, Fujian 361005, P. R. China.
[3] Present address: Max-Delbrück Center for Molecular Medicine, Robert-Rössle-Str. 10, 13092 Berlin, Germany.

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doi:10.1016/j.ydbio.2008.03.014

development, the level of endodermal Wnt activity appears to control the specification of intestinal, liver and pancreatic fates in foregut endoderm (Heller et al., 2002; McLin et al., 2007; Ober et al., 2006). Gain- and loss-of-function studies in mice further indicate that during pancreas morphogenesis, Wnt signaling is required for growth and differentiation of the epithelium (Dessimoz et al., 2005; Heiser et al., 2006; Murtaugh et al., 2005; Papadopoulou and Edlund, 2005; Wells et al., 2007). Additional roles for Wnt signaling have been further suggested during the perinatal and postnatal periods in islet formation, islet cell proliferation and beta cell function (Fujino et al., 2003; Kim et al., 2005; Rulifson et al., 2007).

While these studies have established that Wnt signaling is important in the pancreas, the spatial and temporal pattern of Wnt signal transduction and the specific requirements for Wnt activity in the epithelium and mesenchyme have remained elusive. Notably, Wnt signals can be intracellularly transduced through a so-called canonical (Wnt/β-catenin) pathway or non-canonical (Wnt/Ca^+ and Wnt/planar cell polarity) pathways (Miller, 2002). The existence of parallel pathways leaves ambiguity as to which intracellular signals control the phenotype when Wnt signaling is perturbed at the Wnt ligand or frizzled receptor level. As conditional deletion of the Wnt-effector *β-catenin* in pancreatic epithelial progenitor cells causes defects in progenitor cell expansion and exocrine cell differentiation, it has been recently suggested that normal pancreas morphogenesis requires canonical Wnt signaling autonomously in the epithelium (Murtaugh et al., 2005; Wells et al., 2007). However, interpretation of the mechanisms that underlie these phenotypic changes is complicated by the fact that β-catenin also has Wnt-independent functions in cell adhesion (Nelson and Nusse, 2004). To better understand the role of canonical Wnt signaling in pancreas development, it is therefore necessary to compare the effects of perturbing signal transduction at several levels of the pathway.

In response to Wnt signaling, cytoplasmic β-catenin becomes stabilized and enters the nucleus, where it functions in a complex with Tcf/Lef transcription factors to activate Wnt target genes (Nelson and Nusse, 2004). Recently, *pygopus* has been identified as an additional core component of this complex in *Drosophila* and appears to act as a nuclear anchor as well as coactivator for the β-catenin/Tcf/Lef complex (Kramps et al., 2002; Krieghoff et al., 2006; Stadeli and Basler, 2005; Thompson, 2004; Townsley et al., 2004a, 2004b). Analysis of loss-of-function phenotypes in *Drosophila* and *Xenopus* have shown that *pygopus* is an essential regulator of canonical Wnt pathway responses (Belenkaya et al., 2002; Lake and Kao, 2003; Parker et al., 2002; Thompson et al., 2002). The murine genome contains two *pygopus* orthologs, *pygopus 1* (*mPygo1*) and *pygopus 2* (*mPygo2*). Consistent with the broader expression of *mPygo2* than *mPygo1* during development and in adult tissues (Li et al., 2004a), loss of mPygo2 function causes severe developmental defects in various Wnt-dependent tissues, while mPygo1-deficient mice exhibit no apparent abnormalities (Li et al., 2007; Schwab et al., 2007; Song et al., 2007). Therefore, *mPygo2* appears to be the functionally important ortholog in mice.

We show here that germline mutation of *mPygo2* in mice causes pancreatic hypoplasia and a specific defect in endocrine cell differentiation. We find that these defects result from decreased proliferation of undifferentiated pancreatic progenitors and reduced formation of endocrine progenitors after e13. Since the pancreas develops normally in mice with a pancreatic epithelial-specific deletion of *mPygo2*, our data suggest that mPygo2 controls mesenchymal signals required for pancreatic growth and differentiation. Accordingly, we find that canonical Wnt signaling is active in the pancreatic mesenchyme at the time *mPygo2* mutant mice first show a phenotype and demonstrate dependence of this signal on mPygo2 function. Therefore, our results suggest that expansion and differentiation of pancreatic epithelial progenitors requires canonical Wnt signaling in the mesenchyme.

Materials and methods

Mice

Mice carrying the null allele for *mPygo2* were generated by Cre-mediated recombination in ES cells (Li et al., 2007). For pancreas-specific deletion of *mPygo2*, mice with a floxed *mPygo2* allele (*mPygo2^{flox}*) (Li et al., 2007) were crossed with *Pdx1-Cre* mice (Gu et al., 2002). In these experiments, *mPygo2$^{flox/+}$*; *Pdx1-Cre* or *mPygo2$^{+/-}$*; *Pdx1-Cre* littermates served as controls. PCR detection of the *mPygo2^{flox}* allele was performed using primers forward 5′-AGC GTG TCT AAG GTC AGC CAG AGG TTT G-3′ and reverse 5′-GTA AAG CGT TGG GGG AGA GGA GGA GGA C-3′. *Axin2$^{+/lacZ}$* and *BatGAL* mice have been previously described (Lustig et al., 2002; Maretto et al., 2003).

All strains were maintained on a C57BL/6J background. Experiments were performed with the approval of the UC Irvine Institutional Animal Care and Use Committee protocol 2002-2420. Noon of the vaginal plug day was considered as e0.5. To label S-phase nuclei, some mice were injected with 50 g/g body weight of BrdU i.p. 45 min prior to sacrifice.

Tissue processing, immunohistochemistry and microscopy

Pancreata were fixed in 4% paraformaldehyde in PBS for 4 h and immunofluorescence was performed on 10 m cryopreserved or 7 m paraffin sections. Staining with hematoxylin/eosin (Fisher) was performed according to the manufacturer's instructions. For immunostaining we followed a previously described protocol (Sander et al., 1997). The following antibodies were used: rabbit anti-mPygo2 (1:500; Li et al., 2007), guinea pig anti-insulin (1:5000; Linco), mouse anti-glucagon (1:50; Sigma), mouse anti-somatostatin (1:3000; gift from Palle Serup, Hagedorn Institute), rabbit anti-pancreatic polypeptide (1:2000; Dako), rabbit anti-amylase (1:500; Sigma), armenian hamster anti-MUC1 (1:200; Lab Vision Corporation), mouse anti-β-catenin (1:100; Sigma), guinea pig anti-Pdx1 (1:5000; gift from Chris Wright, Vanderbilt University), guinea pig anti-Ngn3 (1:1000; Henseleit et al., 2005), rabbit anti-Ptf1a (1:2000; gift from Helena Edlund, Umea University), mouse anti-BrdU (1:100; Chemicon), rat anti-E-cadherin (1:2000; Sigma), rabbit anti-mPygo1 (1:3000; Schwab et al., 2007), guinea pig anti-Pbx1 (1:400; Kim et al., 2002), guinea pig anti-Isl1 (1:5000; gift from Johan Ericson, Karolinska, Stockholm), rabbit anti-Sox9 (1:2000; Chemicon). For immunohistochemistry, biotinylated anti-mouse or anti-guinea pig IgG antibodies were used (1:200; Vector Laboratories). For immunofluorescence, antigen–antibody complexes were detected with Cy3- (1:2000; Jackson Laboratory) or Alexa488-conjugated (1:2000; Molecular Probes) goat-raised secondary antibodies. For detection of Pdx1, Ptf1a, Ngn3 and BrdU, antigen retrieval was performed by incubation in pH 6.0 citrate buffer at 37 °C for 1 h. Glucagon and BrdU were detected using the M.O.M. Immunodetection Kit (Vector Laboratories). TUNEL assays were performed using the ApopTag® Plus Peroxidase In Situ Apoptosis Detection Kit (Chemicon). Using 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) as a substrate, whole-mount X-gal staining was performed on either whole embryos or isolated abdominal organs as described (Mombaerts et al., 1996). Images were collected on a Zeiss Axioplan2 microscope with a Zeiss AxioCam driven by Zeiss AxioVision v. 4.0 software.

Morphometric quantification of cell numbers and area

For morphometric analysis, all pancreata were embedded in the same orientation and serial sections were collected from the entire organ. Following immunostaining, images from an entire pancreas section were acquired and if necessary assembled in Adobe Photoshop. Cell counting and morphometric analysis were performed on every fifth section. E-cadherin was used to visualize the entire pancreatic epithelial area and DAPI staining for detection of individual nuclei. Mesenchymal cell area was measured by subtracting the epithelial cell area from the total pancreatic area, including the mesenchyme. Morphometry was conducted using Image-Pro Plus v. 5.0.1 (Media Cybernetics). The data are displayed as averages± standard error of the mean. Statistical significance was determined using Student's *t*-test (Minitab v. 14.20).

RNA preparation and RT-PCR and real-time quantitative PCR

Total RNA from dissected pancreatic anlagen was extracted with the RNeasy kit (Qiagen) and treated with DNAse. cDNA was prepared by *in vitro* transcription using the iScript cDNA synthesis kit (Biorad). The following primers were used for PCR: for detection of *mPygo2* forward 5′-GGA GCG AAG AAA GTC CAA TAC-3′ and reverse 5′-GTT AGA AGC GAC CAG ATG ATC-3′ (75 bp product); for detection of *mPygo1* forward 5′-GTG GTG ACA GTG GAC TGG ATG-3′ and reverse 5′-CTG AGT GAG TAA GGA CCA CAG-3′ (323 bp product); for detection of β-actin as an internal control forward 5′-TGT TAC CAA CTG GGA CGA CA-3′ and reverse 5′-GGG GTG TTG AAG GTC TCA AA-3′. Amplification was performed for 35 PCR cycles.

For quantitative RT-PCR analysis, all transcripts were amplified with 1× SYBR Green PCR master mix (Applied Biosystems), as described previously (Kioussi et al., 2006). Listed 5′ to 3′, primer sequences were as follows: mPygo1 forward: CCATCGCGCTGAAGAGAGTTA; mPygo1 reverse: GTGTGTTGGCCTTGCGTTTT; mPygo2 forward: AAGGCCGGTCTGCAAATGAA; mPygo2 reverse: GTTAGAAGCGACCAGATGATCC; Axin2 forward: TGACTCTCCTTCCAGATCCCA; Axin2 reverse: TGCCCACACTAGGCTGACA.