Conditional inactivation of Myc impairs development of the exocrine pancreas

Hassan Nakhai, Jens T. Siveke, Lidia Mendoza-Torres and Roland M. Schmid*

Recent studies have shown that Wnt/β-catenin signaling is essential for development of the exocrine pancreas, but the role of β-catenin-dependent target genes such as Myc during pancreatic development is not well known. Here, we show that tissue-specific deletion of Myc causes a slightly accelerated differentiation of pancreatic epithelial cells into endocrine cells and perturbs the proliferation of pancreatic progenitors and acinar precursor cells during early development, resulting in a severe reduction of the epithelial cell mass of pancreatic buds and an extensive acinar hypoplasia. Loss of Myc does not affect the expression of the tissue-specific transcription factor PTF1a, which is required for the differentiation of acinar cells. In contrast to its role for exocrine cell growth, the development of endocrine cell lineages is not significantly disturbed. These data suggest that Myc is required for the expansion of the exocrine pancreas. Our observations are consistent with the findings in β-catenin-deficient pancreas, suggesting that Wnt/β-catenin signaling affects the proliferation of pancreatic epithelial cells and acinar precursors through its target gene Myc.

KEY WORDS: Myc, Pancreas, Development, Mouse

INTRODUCTION

Myc (previously known as c-Myc) is a transcription factor of the basic helix-loop-helix (bHLH) leucine zipper that has been extensively studied as a proto-oncogene but is also essential for normal cell cycle progression. Although Myc promotes cell growth and proliferation in several tissues, it induces or sensitizes cells to apoptosis in others (Dang, 1999).

Myc is a key target gene of the Wnt/β-catenin pathway (He et al., 1998) and is activated during pancreatic development (Dessimoz et al., 2005). In adult pancreata, accumulation of β-catenin through conditional inactivation of Apc leads to hyperplasia of acinar cells with increased expression of Myc (Strom et al., 2007). β-Catenin itself is a component of the E-cadherin-mediated cell-cell adhesion system (Lin et al., 2000) and a key effector of the Wnt signaling pathway, which plays a crucial role in growth, cell division and cell fate decisions during organogenesis (Orford et al., 1999; Peifer and Polakis, 2000; Polakis, 2000). In response to Wnt signals, β-catenin complexes with T-cell factor/lymphoid-enhancing factors (Tcf/Lef) and p300 to induce transcription of target genes known to be important in cell proliferation such as Myc (He et al., 1998) and cyclin D1 (Shutman et al., 1999).

Here, we show that Myc is essential for the proliferation of pancreatic epithelial and acinar precursor cells. Inactivation of Myc during pancreatic development results in the decreased size of pancreatic buds and severe pancreatic hypoplasia during organogenesis.

MATERIALS AND METHODS

Generation of genetically modified mice

Mice containing a floxed allele of Myc were a generous gift from Moreno de Alboran. The Myc allele in these animals contains LoxP sites in the first and third intron as described previously (de Alboran et al., 2001). To obtain conditional deletion of Myc within the pancreas, we used a Ptf1a-cre(ex1) knock-in mouse (Nakhai et al., 2007), previously reported to mediate efficient recombination in the developing mouse pancreas by embryonic day 10.5 (E10.5) (Nakhai et al., 2008). All mouse protocols were approved by the Faculty of Medicine, Technical University of Munich.

X-gal staining

β-gal activity was determined on whole-mount preparations according to Kawaguchi et al. (Kawaguchi et al., 2002).

BrdU labeling

In vivo pulse labeling with 5-bromo-2-deoxyuridine (BrdU) was used to mark newly synthesized DNA. BrdU (20 mmol/l, 5 ml/kg body weight) was injected i.p. into pregnant mice 2 hours before sacrifice.

Immunohistochemistry and morphometric analysis

Dissected tissues were fixed in ice-cold 4% paraformaldehyde, paraffin-embedded and cut into 2-3 μm sections. Immunohistochemistry was performed using primary antibodies (details can be provided on request). For immunoperoxidase detection, Vectastain ABC kit (Vector Labs) was used according to the manufacturer’s instruction. For double-immunofluorescence staining, the primary antibodies were followed by incubation with secondary antibodies conjugated with fluorescent Alexa 488 or Alex 568 (Molecular Probes). Sections were mounted with Vectorshed mounting medium (Vector Laboratories) and examined using an Axiovert 200M (Zeiss) fluorescent inverse microscope equipped with the Axiovision version 4.4 software (Zeiss). For morphometric analyses, the pancreatic buds were immunostained with anti-PDX1 and analyzed using the AxioVision Image analysis software (Zeiss). To calculate the number of PHH3- and neurogenin3-positive cells, the whole pancreatic buds of three control and three PMycko embryos were cut into 3 μm serial sections. Every fifth section was stained and the number of PHH3+ cells and glucagon+ cell area were counted and calculated relative to the whole area of PDX1+ pancreatic epithelium in every section. The endocrine cell mass at E18.5 was calculated as the ratio of each hormone-positive cell area to the total area of the pancreas section using AxioVision Image analysis software (Zeiss, Germany). For each group three mice were used. The measurement and calculation were carried out with three sections from each mouse. The sections were far apart from one another. The whole area of each section was investigated, and the insulin- or glucagon-positive cell area relative to the whole pancreatic area was determined. All values are expressed as mean±s.e.m. Statistical significance was tested using Student’s t-test.

*Author for correspondence (e-mail: roland.schmid@lrz.tum.de)

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RESULTS AND DISCUSSION

Tissue-specific deletion of Myc results in severe pancreatic hypoplasia and neonatal lethality

We achieved pancreas-specific deletion of Myc by breeding a Ptf1a-cre(ex1) strain (Nakahai et al., 2007) with a strain containing a floxed Myc allele. Recombination between the two loxP sites mediated by the Cre recombinase leads to the complete inactivation of the Myc gene via deletion of exons 2 and 3 (de Alboran et al., 2001). Ptf1a-cre(ex1) mice heterozygous for the floxed Myc allele displayed no apparent abnormalities in either the embryonic or adult pancreas. However, mice with conditional inactivation of both Myc alleles in the pancreas (Myc<sup>F<sup>-</sup></sup>/Ptf1a<sup>-cre(ex1)</sup>) died at birth, most probably due to defects in the neural system. For simplicity, Myc<sup>F<sup>-</sup></sup>/Ptf1a<sup>-cre(ex1)</sup> mice will be termed PMycKO (pancreas specific Myc knockout) and heterozygote littermates and littermates not expressing Cre recombinase will be termed PMyc<sup>+/-</sup>and wild-type mice, respectively.

To identify the possibility of mosaic Cre-induced recombination, we crossed the PMycKO mice to Gi (ROSA) 26Sortm1Sor (R26R) reporter mice (Soriano, 1999) to generate a PMycKO;R26R strain. In these mice, Ptf1a-driven Cre expression causes inactivation of Myc as well as expression of β-galactosidase, which can be identified by X-gal staining. Using this method, we did not observe X-gal-negative exocrine cells in embryonic pancreata (Fig. 1B,D). At E18.5, the latest stage characterized, pancreatic X-gal staining of PMycKO;R26R and Myc<sup>F<sup>-</sup></sup>/R26R embryos revealed a severe pancreatic hypoplasia in Myc-deficient but not heterozygous littermates (Fig. 1A,B). Histological analysis showed poorly branched pancreatic ducts and disruption of exocrine pancreas formation in PMycKO;R26R embryos compared with PMyc<sup>+/-</sup>;R26R littermates (Fig. 1C,D). Immunohistological analysis using antibodies against acinar cell markers such as amylase and PTF1a confirmed a severe reduction of acini in Myc-deficient pancreata compared with heterozygous littermates (Fig. 1E,F,H,I, arrows). Although the majority of the acinar cells were absent, it was striking that there were clusters of normal appearing acini in mutant pancreata. Double-immunofluorescence staining for β-galactosidase and amylase confirmed Cre activity in amylase-positive acinar cells (Fig. 1G, arrow).

Depletion of pancreatic acini is caused by decreased cell proliferation and accelerated differentiation of epithelial cells into endocrine cells

PTF1a is an essential transcription factor for differentiation of pancreatic precursors into acinar cells (Kawaguchi et al., 2002; Krapp et al., 1998). The expression of this factor in Myc-deficient pancreata at E18.5 suggests that the loss of Myc activity may not impair acinar specification and differentiation. To determine the stage at which the exocrine pancreatic development is perturbed, we analyzed E10.5-E13.5 PMycKO embryos by immunohistochemistry. At E12.5, immunolabeling of pancreatic buds for Myc displayed expression in pancreatic epithelial cells of wild-type embryo (Fig. 2A) and confirmed the loss of Myc protein in PMycKO embryos (Fig. 2B). At this age, staining of pancreatic epithelial cells for PDX1 revealed a significantly reduced epithelial mass with weakly branched structures in both buds of PMycKO embryos and those of wild-type littermates (Fig. 2C,D), suggesting that Myc is essential for the expansion of the early pancreatic epithelium. A possible cause for this reduction of pancreatic epithelium (Fig. 2N) could be the premature differentiation of pancreatic progenitor into endocrine cells. As reported previously in Rbpsi-deficient buds (Fujikura et al., 2006), accelerated differentiation of endocrine cells leads to an increase in glucagon-expressing cells and a decreased number of neurogenin 3<sup>+</sup> (Ngn3<sup>+</sup>) endocrine precursor cells early in pancreatic development.

To ascertain whether the number of glucagon<sup>+</sup> cells was increased and the number of endocrine precursors was decreased in the absence of Myc, pancreatic buds were respectively analyzed for glucagon- and Ngn3-positive cells. At E10.5, double-immunofluorescence staining of PMycKO and wild-type littermates for glucagon and PDX1 showed a 1.4 times increase in glucagon<sup>+</sup> cell area in the dorsal bud of knockout embryos (Fig. 2E-G). At E12.5, pancreatic buds of PMycKO and wild-type littermates were immunolabeled for Ngn3. This analysis revealed that the number of Ngn3<sup>+</sup> cells decreased by about 1.2 times (20%) in PMycKO compared with control embryos, suggesting partial premature differentiation of endocrine precursor cells (Fig. 2H-J). In order to determine whether the loss of Pdx1<sup>+</sup> epithelial cells in PMycKO embryos was also related to a decrease in proliferation of Myc-deficient cells, we performed immunostaining of PMycKO and wild-type embryos at E12.5, using an anti-phosphohistone H3 (PHH3) antibody and assessed the number of PHH3<sup>+</sup> cells per Pdx1<sup>+</sup> pancreatic area (Fig. 2K,L, arrows). These studies revealed a significantly decreased proliferation rate (to ~40%)
in *Myc*-deficient versus wild-type buds (Fig. 2M), suggesting that *Myc* plays an important role in controlling proliferation of pancreatic epithelial cells. Several *Myc* target genes involved in cell proliferation are expressed in the wild-type pancreatic epithelium at this stage, including cyclin D1 (Fernandez et al., 2003) and CDK4 (Hermeking et al., 2000). Although expression of cyclin D1 appeared to be unaffected in *PMycKO* littermates (Fig. 2N,O), CDK4 was downregulated in the absence of *Myc* (Fig. 2P,Q). Regarding the role of *Myc* as regulator of apoptosis, we found no evidence to suggest that apoptosis contributed to early pancreatic hypoplasia (data not shown).

### Myc is required for proliferation of acinar precursor cells

Exocrine differentiation, termed the secondary transition, begins at E14.5 and continues until birth (Pictet et al., 1972). By E14.5, cells in the periphery of the branching epithelial tree begin to adopt a pyramidal shape and organize into discrete clusters, representing nascent acinar structures. At this time, nuclear Myc immunoreactivity remained evident in nascent acini of wild-type embryos, which also expressed acinar cell markers such as amylase (Fig. 3A,C, arrows). In serial sections of *PMycKO* embryos, we detected some amylase+ acinar cells that did not express Myc (Fig. 3D, arrows).
pancreatic area in multiple pancreatic sections. The relative cell areas of β and α cells were not significantly altered in PMycKO pancreata (Fig. 4Q,R).

**Conclusions**

The generation of PMycKO mice via a Cre-mediated conditional gene-targeted deletion strategy allowed us to define the role of Myc during pancreatic development. Pancreata lacking Myc were smaller than those of wild-type embryos, with dramatically fewer differentiated acinar cells. This effect can be attributed mainly to a decrease in the proliferation rate of early progenitors and partially to premature differentiation of these cells to endocrine cells.

Although an accelerated premature progenitor cells conversion into endocrine cells as a predominant effect has been observed in mice with modulation of the Notch signaling pathway, this mechanism is not predominantly involved into the reduced acinar cell mass in Myc-deficient pancreata, as we observed only about 20% fewer Ngn3+ endocrine precursor cells but a 40% reduction in proliferating epithelial cells in PMycKO pancreata. Thus, the main reason for the impaired development of the exocrine compartment in PMycKO pancreata is the reduced ability of epithelial and acinar precursor cells to proliferate. The ability of Myc to promote cell-cycle reentry is, in part, due to its ability to directly induce the transcription of Cdk4 (Hermeking et al., 2000). The loss of CDK4 expression in Myc-deficient pancreata reflects a direct effect of Myc on progenitor and nascent acinar cell proliferation, although no pancreatic exocrine phenotype was reported in mice lacking Cdk4 (Mettus and Rane, 2003; Rane et al., 1999). Thus, besides the loss of CDK4 expression, other Myc-dependent factors probably contribute to the impaired proliferation. In contrast to CDK4, the expression of cyclin D1 in PMycKO embryos was not affected, suggesting that Myc-deficient epithelial cells do not have a general defect in their mitogenic signaling cascades. Furthermore, the expression of the tissue-specific transcription factor PTF1a in Myc-deficient acinar precursor cells suggests that Myc is required for expansion of exocrine cells, rather than for development of progenitor into acinar cells.

Our findings are in agreement with those of other groups, who noted a severe loss of exocrine pancreatic tissue following conditional ablation of β-catenin (Dessimoz et al., 2005; Murtaugh et al., 2005; Wells et al., 2007). Our results suggest that the loss of Myc expression in β-catenin-deficient pancreata may be the causal defect, leading to severe acinar hypoplasia. Additional support for Myc as a main β-catenin target gene in the embryonic pancreas comes from a recent study in which the effect conditional deletion of adenosomatous polyposis coli (Apc) was investigated (Strom et al., 2007). Loss of Apc led to an accumulation of β-catenin and to hyperplasia of acinar cells in adult Apc-deficient pancreata accompanied by increased Myc expression. As acinar hyperplasia could be reversed by additional conditional inactivation of Myc, these results suggest that Myc is responsible for acinar cell hyperplasia mediated by β-catenin. However, accumulation of β-catenin was observed only postnatally, despite embryonic inactivation of Apc and, consequently, reversal of the phenotype by Myc ablation was reported in adult pancreata. In this study, no phenotype upon deletion of Myc alone was reported. Our results are the first to report the essential role of Myc during early pancreatic development and substantiate a functional Wnt/β-catenin/Myc axis not only in the adult but also in the embryonic exocrine pancreas.

**Endocrine cell development in Myc-deficient pancreas**

Most mature endocrine cells appeared after E13.5 in PMycKO embryos, similar to wild-type controls. At this stage, we identified the first insulin-expressing β cells in wild type, as well as in PMycKO embryos (Fig. 4A,B). At E18.5, we could detect all endocrine cell lineages, glucagon-producing α cells, insulin-containing β cells, somatostatin-δ cells and pancreatic polypeptide+ (PP) cells, in Myc-deficient embryos (Fig. 4D,F,H,J).

Although no mosaic pattern of recombination was apparent by X-gal staining of acinar cells in pancreatic section from Ptf1aCre(ex1);R26R and PMycKO;R26R mice at E18.5, the endocrine cells were only partially positive for X-gal in both pancreata (Fig. 4K-P). To quantify the endocrine cell mass in PMycKO and wild-type pancreata, we estimated the hormone-positive area per total

**Fig. 3. Acinar cells in PMycKO embryos.** Serial sections of E14.5 wild-type and PMycKO embryos stained for Myc (A,B, black, arrows) and amylase (C,D, brown, arrows). (E,F) Double-immunofluorescence staining of wild-type and PMycKO sections for amylase (green) and BrdU (red) at E15.5. Arrows indicate the BrdU-positive and arrowheads the BrdU-negative acinar cells. (G) BrdU+ nuclei were counted in amylase immunoreactive cells. Inserts in A and C represent a 4× enlargement and a 2× enlargement in E. Abbreviations: ac, acini; l, liver; p, pancreas. Scale bar: 50 μm.
Interestingly, inactivation of Myc did not affect development of the endocrine pancreas, similar to results observed in pancreata that lack β-catenin (Murtaugh et al., 2005; Wells et al., 2007). Although it has been reported that Wnt/β-catenin signaling regulates pancreatic β-cell proliferation (Rulifson et al., 2007), we did not find a decrease in β cell numbers in Myc-deficient pancreata. A reason for this could be the only partial activity of Ptf1a observed in endocrine progenitors from our Ptf1a-cre(ex1) knock-in mouse. It is conceivable that the decreased proliferation of Myc-deficient endocrine progenitors would have been masked by the proliferation of Myc-expressing endocrine progenitor cells.

In summary, our studies support a model wherein the Wnt/β-catenin signaling pathway regulates proliferation of the developing exocrine pancreas through activation of Myc as its key target gene. Further studies are required to define the precise role of Wnt and Myc-dependent target genes crucial to proliferation and differentiation of pancreatic progenitors into acini.

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References


essential for the formation of the exocrine and the correct spatial organization of the endocrine pancreas. Genes Dev. 12, 3752-3763.


