Cdk4 Is Indispensable for Postnatal Proliferation of the Anterior Pituitary*

For proper development and tissue homeostasis, cell cycle progression is controlled by multilayered mechanisms. Recent studies using knock-out mice have shown that animals can develop relatively normally with deficiency for each of the G1/S-regulatory proteins, D-type and E-type cyclins, cyclin-dependent kinase 4 (Cdk4), and Cdk2. Although Cdk4-null mice show no embryonic lethality, they exhibit specific endocrine phenotypes, i.e. dwarfism, infertility, and diabetes. Here we have demonstrated that Cdk4 plays an essential non-redundant role in postnatal proliferation of the anterior pituitary. Pituitaries from wild-type and Cdk4-null embryos at embryonic day 17.5 are morphologically indistinguishable with similar numbers of cells expressing a proliferating marker, Ki67, and cells expressing a differentiation marker, growth hormone. In contrast, anterior pituitaries of Cdk4-null mice at postnatal 8 weeks are extremely hypoplastic with markedly decreased numbers of Ki67* cells, suggesting impaired cell proliferation. Pituitary hyperplasia induced by transgenic expression of human growth hormone-releasing hormone (GHRH) is significantly diminished in the Cdk4+-/+ genetic background and completely abrogated in the Cdk4+-/- background. Small interfering RNA (siRNA)-mediated knockdown of Cdk4 inhibits GHRH-induced proliferation of GH3 somato/lactotroph cells with restored expression of GHRH receptors. Cdk4 siRNA also inhibits estrogen-dependent cell proliferation in GH3 cells and closely related GH4 cells. In contrast, Cdk6 siRNA does not diminish proliferation of these cells. Furthermore, Cdk4 siRNA does not affect GHRH-induced proliferation of mouse embryonic fibroblasts or estrogen-dependent proliferation of mammary carcinoma MCF-7 cells. Taken together, Cdk4 is dispensable for prenatal development of the pituitary or proliferation of other non-endocrine tissues but indispensable specifically for postnatal proliferation of somato/lactotrophs.

Proliferation-stimulatory and -inhibitory signals control the G1 phase of the cell cycle, which is critical for development and homeostasis of essentially all animal tissues (1). Cyclin-dependent kinases (Cdks)1 activated by association with the regulatory cyclin subunits play central roles in promoting cell cycle progression, and Cdk4 is a critical regulator of the G1 phase (2). In complex with D-type cyclins, Cdk4 phosphorylates G1-specific substrates, including the retinoblastoma protein (Rb). Unphosphorylated or hypophosphorylated forms of Rb participate in transcriptional repressor complexes with E2F1-3. Rb phosphorylation in collaboration with cyclin D/Cdk4 (or closely related Cdk6) and cyclin E/Cdk2 results in release of Rb from the E2F complex, leading to transactivation of the E2F target genes important for the S-phase, e.g. cyclins E and A, dihydrofolate reductase and DNA polymerase-α (3). Furthermore, several Cdk inhibitor proteins, including p16INK4a, p27Kip1, and p21Cip1/Waf1, play roles in coordinating temporal activation of Cdk4 and Cdk2 by D-type and E-type cyclins, respectively (4). The multilayered G1-Cdk regulation ensures proper control of proliferation, as well as timely transition between proliferation and quiescence, and should be crucial for development and function of every tissue.

Two groups, including ours, recently demonstrated that Cdk4-deficient mice survive embryogenesis, suggesting that Cdk4 is dispensable for embryonic development (5, 6). Consistently, mouse embryonic fibroblasts (MEFs) prepared from Cdk4-null E12.5 embryos proliferate normally, with the exception of a modest delay in cell cycle entry from serum deprivation-induced quiescence. These data suggest that some other genes, possibly Cdk6 and Cdk2, may compensate for Cdk4 deficiency. However, studies on postnatal Cdk4-null mice (5, 6) unraveled unique phenotypes: 1) spontaneous degeneration of the pancreatic β cells, first detectable at several weeks of age, resulting in insulin-dependent diabetes mellitus; 2) postnatal growth retardation; and 3) infertility in both females and males. Cdk4+-/- male mice display severe testicular atrophy with a meiotic defect, whereas females exhibit normal ovulation and fertilization but fail implantation with complete sterility (7). Our data indicated that prolactin deficiency in Cdk4+-/- female mice, associated with pituitary hypoplasia, plays a major role in the sterility phenotype (7, 8). Thus, Cdk4 is required for homeostasis of particular endocrine tissues such as the pancreatic islet and pituitary even though Cdk4 is expressed ubiquitously.

Studies on other knock-out mice deficient in the G1 control pathways also suggest that the pituitary gland is extremely

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1 The abbreviations and trivial terms used are: Cdk, cyclin-dependent kinase; Rb, retinoblastoma gene product; MT, metallothionein; GH, growth hormone; GHRH, GH-releasing hormone; Ad GHRH-R, adeno-virus encoding GHRH receptor; siRNA, small interfering ribonucleic acid; MEF, mouse embryonic fibroblast; BrdUrd, bromodeoxyuridine; TUNEL, terminal deoxynucleotidyltransferase-mediated nick-end labeling; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; E2, estrogen.

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¶ To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Genetics, University of Illinois College of Medicine, 900 S. Ashland Ave., M/C 669, Chicago, IL 60607-7170. Tel.: 312-355-1601; Fax: 312-413-2028; E-mail: kiyokawa@uic.edu.
sensitive to perturbation of G1 regulation. Mice heterozygous for Rb deletion develop pituitary tumors originating from the melanotrophs in the intermediate lobe (9). Moreover, mice deficient for p27^Kip1 or p18^INK4a also develop melanotroph adenomas (reviewed in Ref. 10). Interestingly, double deficiency for p27^Kip1 and p18^INK4a or Rb results in more aggressive pituitary tumors, suggesting genetic cooperation. Thus, proper control of the Cdk/Rb-dependent G1 regulatory pathway seems to be critical for homeostasis and tumor suppression in the pituitary gland, although the mechanism of this unique tissue specificity remains to be defined.

Here we have reported that Cdk4 is required specifically for hormonally regulated proliferation of somatotrophs and lactotrophs in postnatal pituitary glands, whereas Cdk4 is dispensable in embryonic development of the pituitary. Together with the requirement of Cdk4 for maintenance of postnatal pancreatic β cells (11, 12), the data suggest a unique nature of cell cycle control in particular endocrine tissues.

**EXPERIMENTAL PROCEDURES**

**Animals—**Cdk4-deficient mice and transgenic mice expressing hGHRH (MT-hGHRH) were described previously (5, 13). Mice were cross-bred under a 13-h light, 11-h dark cycle and fed ad libitum with a pelleted diet. To isolate E17.5 pituitaries, embryos were obtained from Cdk4+/− females at day 17.5 p.c. MEFs were prepared from day 12.5 mouse embryos as described previously (5).

**Cell Culture—**GH3 and GH4 cell lines were a gift from Dr. Eun Jig Lee and J. Larry Jameson, Northwestern Medical School. MCF-7 cells were a gift from Dr. William T. Beck, University of Illinois at Chicago. GH3 and GH4 cell lines were cultured in 1:1 DMEM/Ham’s F12 (Cambrex, Walkersville, MD) supplemented with 10% FBS (Sigma) and penicillin/streptomycin. MCF-7 cells and MEFs were cultured in DMEM, supplemented with 10% FBS, and penicillin/streptomycin.

**Immunohistochemistry—**Pituitary tissues were processed and sectioned as described previously (5). Sections were dewaxed in Safeclear II (Fisher Scientific, Pittsburgh, PA) at 1:1,000. Slides were then treated for 30 min with 1.5% normal goat serum and incubated for 30 min with biotinylated secondary antibody (Roche Applied Science) and fluorescein isothiocyanate-conjugated anti-mouse antibody (Vector Laboratories). Cells were incubated for 48 h for the expression of hGHRH-R before being replated in 6-cm culture dishes in the medium supplemented with or without GHRH (Sigma). Cdk4 siRNA knockdown and cell proliferation assay were performed 24 h later. MEF cells were cultured in DMEM + 10% FBS and infected with Ad GHRH-R. Cell numbers were determined 4 days after the treatments of 50 nM GHRH.

**Apoptosis Assay—**Apoptotic cells were detected in paraffin-embedded sections by the terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) assay using the Apoptosis Detection System, Fluorescein (Promega, Madison, WI) according to the manufacturer’s protocol.

**RESULTS**

**Cdk4 Is Dispensable for Prenatal Pituitary Development and Somatotroph Differentiation—**Somatotrophs and lactotrophs of the pituitary in adult Cdk4−/− mice are severely hypoplastic, whereas gonadotrophs remain undisturbed (8). However, small numbers of growth hormone (GH)- and prolactin-immunoreactive cells are present in Cdk4−/− pituitaries, suggesting that cell fate determination to the somatotroph/lactotroph lineages is still functional in the absence of Cdk4. To examine whether Cdk4 deficiency affects embryonic development of the pituitary, we examined the morphology of pituitaries from Cdk4−/− and Cdk4+/+ embryos at day 17.5 post coitus (E17.5). We chose the developmental stage in which the lineage commitment of all anterior pituitary cells is completed (16). However, at E17.5 the somatotroph population has yet to be fully expanded because the expression of the somatotroph-specific mitogen, growth hormone-releasing hormone (GHRH), is not increased until a later stage of development (17, 18). Interestingly, transverse sections of heads demonstrated that pituitaries of Cdk4−/− embryos were morphologically indistinguishable from those of wild-type embryos (Fig. 1, A–F). Immunohistochemistry showed that GH expression was observed in similar numbers of cells in E17.5 Cdk4−/− and Cdk4+/+ anterior pituitaries, indicating that differentiation of the somatotroph lineage takes place normally at this developmental stage (Fig. 1, A–F). In addition, proliferation in E17.5 Cdk4−/− anterior pituitaries appeared intact. Immunohistochemistry for a proliferation marker, Ki67 (19), showed similar degrees of proliferation in both E17.5 Cdk4−/− and Cdk4+/+ anterior pituitary (Fig. 1, G and H). Especially at the area of residual Rathke’s pouch, similar numbers of cells displayed Ki67 immunoreactivity in wild-type and mutant pituitaries. These data indicate that Cdk4 is dispensable for pituitary morphogenesis up to E17.5 and for the commitment to the somatotroph lineage.

**Pituitary Cells in Cdk4−/− Mice Are Insensitive to Mitogenic Signals by a GHRH Transgene—**The complex endocrine network involving the hypothalamus and target organs regulates the homeostasis of the postnatal pituitary (20). Hypoplastic changes of somatotrophs and lactotrophs observed in Cdk4−/− pituitaries could result from perturbation of mitogenic stimuli from the endocrine network, insensitivity of pituitary cells to mitogenic signals, or both. To assess the mechanism, we examined effects of ectopic expression of GHRH upon pituitaries of Cdk4−/− mice. Cdk4-null mice were cross-bred with human GHRH transgenic mice under the control of the metallothi-
changes were within the anterior lobe, displaying increased numbers of GH-immunoreactive cells (Fig. 2, C and D). Counting monodispersed pituitary cells (Fig. 2E) showed that cell numbers of the somatotroph (GH⁺), lactotroph (PRL⁺), and thyrotroph (TSH⁺) in Cdk4+/+ MT-hGHRH mice were increased by more than 2-fold compared with wild-type mice without the transgene (Fig. 2E, Cdk4+/+). The MT-hGHRH transgene did not significantly increase numbers of lactotrophs, somatotrophs, or thyrotrophs in Cdk4−/− mice. To further address the mechanism of negative impact of Cdk4 deficiency on GHRH-induced hyperplasia, we examined proliferation and apoptosis in transgenic pituitaries by Ki67 immunohistochemistry and TUNEL assay, respectively. Pituitaries of MT-hGHRH;Cdk4+/+ mice at 8 weeks of age showed more proliferating (Ki67⁺) cells than those of Cdk4+/+ mice without the transgene (Fig. 2, A and B). In contrast, pituitaries of Cdk4−/− mice exhibited minimum numbers of Ki67⁺ cells regardless of the absence or presence of the transgene. Pituitaries of Cdk4+/+ and Cdk4−/− mice, with or without the MT-GHRH transgene, did not show any difference in apoptosis according to the TUNEL assay (data not shown). These data suggest that Cdk4 is essential and rate limiting for GHRH-induced proliferation in the postnatal pituitary.

GHRH-induced Proliferation of Cultured Somato/Lactotroph Cells Depends on Cdk4.—The MT-GHRH transgenic study suggests that impaired somatotroph proliferation in Cdk4−/− mice is related to insensitivity of somatotrophs to the specific mitogen, GHRH. To further examine the role of Cdk4 in proliferation of the somatotroph lineage in an environment without a complex endocrine network, we used rat somato/lactotroph GHR3 cells. This immortalized cell line exhibits characteristics of both somatotroph and lactotroph and expresses detectable levels of GH and PRL, whereas it shows minimum expression of GHRH receptors (22, 23). Thus, to study GHRH-mediated proliferation in vitro, we restored the expression of GHRH receptors in GHR3 cells by Ad GHRH-R (15). Infection with Ad GHRH-R sensitized GH3 cells to the GHRH-mediated proliferation of MEFs. Upon ectopic expression of GHRH receptors, fibroblasts are capable of proliferating in response to GHRH (24). Using Ad GHRH-R, we expressed GHRH receptors in MEFs prepared from Cdk4−/− and Cdk4+/+ E12.5 embryos and then cultured cells without or with GHRH (50 nM) for 4 days. GHRH treatment significantly increased cell numbers in Ad GHRH-R-infected MEF cultures regardless of the Cdk4 status (Fig. 4D). These observations indicate that, unlike GH3 cells, MEFs do not depend on Cdk4 for GHRH-mediated proliferation.

Cdk4 Is Required for Estrogen-mediated Proliferation of Pituitary Cell Lines—Estrogen (E2) is a potent mitogen that stimulates lactotroph proliferation, mediating physiological lactotroph hyperplasia during pregnancy. We previously demonstrated that E2 administrations into mature Cdk4−/− female mice failed to increase numbers of lactotrophs, whereas

![Figure 1](image-url)
the same treatment effectively induced lactotroph hyperplasia in Cdk4−/− females (8). Thus, similar to GHRH-induced somatotroph hyperplasia, E2-induced lactotroph hyperplasia may require Cdk4. To elucidate whether estrogen signaling in lactotrophs requires intrinsic Cdk4 for proliferative response, we performed siRNA-mediated knockdown of Cdk4 in E2-treated GH3 cells. We also examined closely related GH4 somato/lactotroph cells (25) and human mammary carcinoma MCF-7 cells as a non-endocrine cell type responsive to estrogen. All of these cell lines depend largely on E2 for proliferation, and culture under E2-deprived conditions using charcoal-treated serum markedly inhibits proliferation (Fig. 5A). We also examined expression of G1-Cdk’s and D-type cyclins in E2-stimulated GH3 and MCF-7 cells (Fig. 5B). GH3 cells exhibited high levels of Cdk4, Cdk6, and Cdk2, whereas Cdk6

Cdk4+/+, Cdk4+/−, and Cdk4−/− mice without the MT-hGHRH (open bars) and with the MT-hGHRH transgene (closed bars). Data are expressed as means + S.E. (n = 4). *, p < 0.005. C, hematoxylin-eosin staining of pituitaries from Cdk4+/+ (left), Cdk4+/− (center), Cdk4−/− (right) mice without (upper) and with (lower) the MT-hGHRH transgene. a, anterior pituitary; i, pars intermediate; n, pars nervosa. D, high magnification view of GH-immunostained pituitary sections from Cdk4+/+ (center) and Cdk4−/− (right) mice without (upper) and with (lower) the MT-hGHRH transgene. neg., negative control without primary antibody. The arrows indicate representative GH− cells with immunoreactivity in the cytoplasm. E, numbers of anterior pituitary cells isolated from 8-week-old Cdk4+/+, Cdk4−/−, and Cdk4+/− female mice without (open bars) and with (closed bars) the MT-hGHRH transgene. Cells were analyzed by immunohistochemistry for the hormones indicated. The data are expressed as means + S.E. (n = 4).

FIG. 2. Cdk4 is required for GHRH-induced anterior pituitary hyperplasia. A, pituitaries isolated from Cdk4+/+ (left), Cdk4+/− (center), Cdk4−/− (right) mice without (upper) and with (lower) the MT-hGHRH transgene at 8 weeks of age. B, pituitary weights of

FIG. 3. Impaired proliferation in Cdk4−/− pituitaries. A, Ki67 immunohistochemistry of paraffin-embedded anterior pituitary sections from 8-week-old Cdk4+/+, Cdk4−/−, Cdk4+/−;MT-hGHRH, Cdk4−/−;MT-hGHRH female mice. B, numbers of Ki67+ cells were quantified by examining five random fields (40×) of pituitary sections from three different mice/group. Data are expressed as means + S.E. *, p < 0.005.
Cdk4 was undetectable in MCF-7 cells. Cyclin D1 was up-regulated by E2 in GH3 cells. Cyclin D3 levels were not altered by E2 in GH3 and MCF-7 cells, whereas cyclin D2 was barely detectable. GH4 cells showed similar expression patterns of these G1-regulatory proteins as GH3 cells (data not shown). Transfection with Cdk4 siRNA effectively decreased Cdk4 levels and brought down numbers of GH3 and GH4 cells in the S-phase (BrdUrd/H11001) by 40 and 75%, respectively (Fig. 6A). We also found that the Cdk4 siRNA did not affect continuous proliferation of MEFs (Fig. 6B) despite 80% reduction in Cdk4 protein levels (data not shown). Although human Cdk4-specific siRNA decreased Cdk4 protein levels by 90% in MCF-7 cells, it did not alter BrdUrd incorporation under E2-induced or -deprived conditions (Fig. 6B). These data suggest that Cdk4 is dispensable for E2-responsive proliferation of MCF-7 cells. Consistently, stable silencing of Cdk4 using retrovirus-based Cdk4 siRNA did not impair proliferation of MCF-7 cells. More-

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Cdk4 siRNA decreases BrdUrd incorporation in GHRH-treated GH3 cells. GHRH receptor-restored GH3 cells were cultured in phenol red-free DMEM/F12 minimal medium supplemented with 10% charcoal/dextran-treated FBS. Cells were then transfected with 200 μM Cdk4 siRNA or NS siRNA control in the medium with 1 nM GHRH or without GHRH. Data are expressed as means ± S.E. from three independent experiments. *, p < 0.05.
The central machinery of the cell cycle is highly conserved in all eukaryotic cells. Indeed, mammalian Cdk1 (Cdc2) and Cdk2 can complement the fission yeast cdc2 mutant and budding yeast Cdc28 mutant (26, 27). In contrast to the single Cdk protein functioning in yeast cells, multiple Cdk proteins appear to collaborate in the mediation of the cell cycle progression in mammalian cells. The observations that Cdk4-null mice are viable and develop almost normally except for particular endocrine organs (5, 6, 8) imply that presumably other Cdkks compensate for the absence of Cdk4. Cdk2-null mice and Cdk6-null mice are also viable (28–30), as are mice deficient in cyclin D1, D2, D3, E1, or E2 (31–35). These data further support the notion that the mammalian Cdks and cyclins play overlapping roles.


discussion

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During embryogenesis, precursor cells for the anterior pituitary lineages undergo dynamic regulation of proliferation and differentiation (36). Precursor cells committed to the anterior pituitary actively proliferate in the Rathke’s pouch, which is controlled by the transcription factor Pitx2 with influences of growth factors, including BMP2 and FGF8. Subsequent regulation by lineage-specific transcription factors, such as Prop-1, Pit-1, and GATA-2, govern the cellular commitment process to each pituitary lineage. Apparently normal development of the embryonic pituitary in Cdk4-null mice indicates that Cdk4 is dispensable for proliferation associated with this series of developmental events. After terminal differentiation of each pituitary lineage, a variety of extracellular signals regulate homeostasis of the tissue. The pituitary gland responds to both hypothalamic and peripheral signals by undergoing reversible and adaptive changes in proliferation. Multiple lines of evidence suggest that differentiated pituitary cells with expression of specific hormone markers can continue mitosis. For example, adult pituitary glands slowly undergo hyperplastic changes during pregnancy, associated with estrogen-mediated proliferation of lactotrophs. It has been described that about 30% of rat pituitary cells arise from “adaptive” proliferation of terminally differentiated cells, whereas others are originated from undifferentiated precursors or stem cells (20). The proliferation defects in somatotrophs and lactotrophs of postnatal Cdk4-null mice suggest that proliferation of adult pituitary cells, possibly by mitosis of differentiated cells, depends on Cdk4 in a non-redundant manner. Similarly, the proliferation defect of Cdk4-null pancreatic β cells is restricted to postnatal animals (11, 12). Proliferation of terminally differentiated β cells has recently been clearly demonstrated (37, 38). This unique requirement of the endocrine tissues for Cdk4 may imply that slow adaptive cell cycle progression is controlled by a mechanism distinct from the rapid cell cycle of regenerative tissues, e.g. the skin and digestive tracts. Possible fundamental differences in cell cycle control between differentiated endocrine cells and non-endocrine epithelial cells might be consistent with clinical data that tumors that arise from the pituitary or pancreatic islet rarely exhibit malignant phenotypes.

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2 D. Moons and H. Kiyokawa, unpublished data.

3 S. Jirawatnotai, P. Kaldis, and H. Kiyokawa, unpublished observation.
whereas carcinomas can frequently develop from the regenerative tissues. Although the Rb/E2F pathway had been long thought to be the only target of Cdk4, a recent report described that Cdk4 phosphorylates and regulates Smad3, a transcription factor mediating the transforming growth factor β/activin signaling (39). Thus, it will be important to determine whether Cdk4 regulates proliferation and function of postnatal endocrine tissues in a manner dependent on Rb or on other undefined substrates specific to the cell lineages. Studies on the genetically engineered mice with genomics and proteomics should provide us with further insight into tissue-specific control of the mammalian cell cycle.

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