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Abnormal Mammary Gland Development in MMTV-CBLC Transgenic Mouse

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Abstract. *The CBL family of E3 ubiquitin ligases regulates cell signaling in a number of tissues by promoting degradation of tyrosine kinase receptors such as epidermal growth factor receptor. CBLC, the third member of the CBL family, is expressed in epithelial tissues, including the mammary gland. A transgenic mouse strain expressing a tetracyclin-inducible CBLC in the mammary gland was derived. It was found that CBLC transgene expression reduces the number and length of ducts during the development of the gland. In vivo results support the concept of CBLs as negative regulators of cell proliferation. Alternatively, the phenotype may be due to increased apoptosis. This mouse model may be used to further study regulatory components of the CBL pathway and may be crossed with mice susceptible to develop mammary tumors.*

During mouse puberty the mammary gland develops from a rudimentary tree that undergoes extensive expansion leading to a branched epithelial network of ducts that can support alveolar development and subsequent milk production during pregnancy and lactation. The ductal pattern is created by the invasion of a highly proliferative structure called the terminal end bud (TEB) into the surrounding fat pad. Stimulated by ovarian hormones at puberty, bulbous TEBs consisting of relatively undifferentiated epithelial cells form at the tips of the ducts and penetrate further into the fat pad as the ducts elongate. TEBs are composed of an outer layer of highly proliferative cells called the Cap cells and a multilayered inner mass containing body cells, some of which undergo apoptosis to give rise to the hollow mammary ducts. Several signaling pathways play a role during mammary gland development, including the epidermal growth factor receptor (EGFR), fibroblast growth factor receptor (FGFR), insulin-like growth factor one receptor (IGF1R) and WNT pathways.

Members of the CBL protein family, CBL (also known as c-CBL or CBLA), CBLB and CBLC (also known as CBL3), control multiple cellular processes by acting as ubiquitin ligases and multifunctional adaptor molecules (1). The three CBLs regulate receptor tyrosine kinase (RTK) ubiquitination and downregulation. Because signaling pathways involving RTKs (*e.g.* EGFR, ERBB2, IGF1R) are important for mammary gland development (2) downstream regulation of their networks by CBL proteins must also be important (3, 4).

Here, a transgenic animal model that overexpresses the wild-type mouse CBLC in the mammary gland in a doxycycline (Dox) -inducible manner is described. Findings indicate that CBLC overexpression impairs mammary duct development.

Materials and Methods

Generation and genotyping of transgenic mice. To generate the tet-inducible Cblc transgenic mice a mouse Cblc cDNA with three in-frame hemagglutinin (HA) epitope tags at its N-terminus was subcloned into the pBI-G vector (Clontech-Takara Bio Europe 78100 Saint-Germain-en-Laye, France). This construct, called pTet-Gal-CBL3 (Figure 1A) can be used to express HA-CBLC and β-galactosidase from a bidirectional tet-responsive promoter.

Pronuclear injection of the purified linearized pTet-Gal-CBL3 construct into fertilized oocytes from B6/SJL mice was carried out at the Clinique de la souris (Strasbourg, France). Animals were screened for transgene integration by PCR using lacZ primers, done on genomic DNA isolated from tail snips. Southern blot analysis using a Cblc cDNA probe was done to confirm the PCR identification of the seven transgenic animals.

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To determine which of the seven male founder mice were capable of doxycycline-regulated expression of the transgene, embryonic fibroblasts established in culture from their litters were transfected with the pTet-On vector (Clontech) coding for the rtTA activator, cultured in the presence of 1 mg/mL doxycycline for 48 hours and stained for β-galactosidase activity. Four founders carried a doxycycline inducible transgene, among them two (C43 and C52) were selected for further analysis. These founder mice were backcrossed to FVB mice for at least eight generations and maintained on this background. C43 and C52 were crossed to MTB mice carrying construct MMTV-rtTA-pA (5) to get bi-transgenic mice identified by PCR analysis done on genomic DNA isolated from tail snips using LacZ and rtTA primers. Transgene expression in bi-transgenic mice, called D43 or D52, was induced by adding 2 mg/mL doxycycline to the drinking water changed twice a week.

Whole-mount mammary gland staining. For whole-mount staining, the fourth mammary glands were spread on microscope slides, fixed and defatted in acetone for 48 hours, rehydrated and stained with Mayer's hemalun. After dehydration samples were cleared overnight in Histolemon (Carlo Erba Reactifs Val De Reuil, France), mounted under coverslip with Pertex mounting medium (CellPath, UK) and photographed with a digital camera. For each gland, the ductal length was determined by measuring the distance between the middle of the lymph node and the end of the longest duct and the number of ductal branches counted in an arbitrary rectangle drawn besides the lymph node.

LacZ expression on whole-mount mammary glands was monitored by incubating the glands in Xgal staining solution (1xPBS, 2 mM $MgCl_2$, 0.01% sodium deoxycholate, 20 mM Tris-HCl pH 7.3, 30 mM K3FeCN6, 30 mM K_4FeCN_6, 1 mg/mL Xgal) overnight at 37˚C. Glands were then rinsed in PBS, immersed in acetone for 24 hours and then cleared and mounted as above.

Results

Characterization of rtTA Cblc/lacZ mice. To experimentally determine whether CBLC affect mammary development, transgenic mice that overexpress the full-length mouse *Cblc* gene in a conditional manner were generated. A plasmid harboring a Dox-dependent, bidirectional promoter was constructed (Figure 1A). In one direction a minimal CMV promoter drives the expression of the *LacZ* reporter gene and in the opposite direction the expression of an HA-CBLC fused protein. This Tet-On bidirectional *LacZ/HA-Cblc* construct was microinjected into pronuclei of fertilized mouse oocytes. Founder mice were screened by PCR analysis using tail DNA samples and confirmed by Southern blot analysis (data not shown). Embryonic fibroblasts were established from seven independent founder lines and transfected with a tTA plasmid, cultured in the presence of Dox and assessed for the expression of the *LacZ* gene revealed by the presence of blue cells after X-Gal staining. Stable transmission of the transgene was established in four separate founders. The highest expression of the LacZ protein was achieved in lines C43 and C52, which were subsequently analyzed in more details.

Mice overexpressing *Cblc* in the mammary epithelium were generated by crossing C43 and C52 founder lines to the MMTV-rtTA transgenic mouse (5). Double transgenic mice *LacZ-HA-Cblc+/-/MMTV-rtTA+/-* were produced to determine the expression pattern of exogeneous *Cblc* in the mammary gland revealed by LacZ expression after a short Dox induction (72 hr). Positive LacZ staining was apparent in most ducts from whole-mounts of mammary glands of 6-week-old virgin mice (Figure 1B). LacZ staining of sections showed that this expression was patchy, labeling only some epithelial cells (Figure 1C).

CBLC transgene expression affects mammary gland development. Given the ability of MMTV-rtTA to induce *Cblc* expression in mammary epithelium whether overexpression of *Cblc* affected mammary gland development was assessed in bi-transgenic mice. Heterozygous *LacZ/HA-Cblc* transgenic mice were treated with Dox and mated with the MMTV-rtTA male. Treatment was continued during pregnancy and suckling. Mammary glands from their monotransgenic and bitransgenic litters were analyzed at 6, 7 and 8 weeks after birth. At 6-weeks age whole-mounted-stained mammary glands dissected from bigenic mice from founders C43 and C52 (Figure 2Ab-d) showed impaired duct development as compared to control mammary glands (Figure 2Aa). Because both founders had the same phenotype, the results from C43 and C52 were pooled. Quantification indicated that overexpression of CBLC significantly reduced the length of the longest duct (4.47 mm±0.37 in bigenic mammary glands versus 6.21 mm±0.23 in controls) (Figure 2B) and the number of branch points per unit area (19.18±1.35 in bigenic mammary glands versus 28.36±1.19 in controls) (Figure 2C). Ductal inhibition in bigenic mice decreased with age (Figures 2A and B). At 7 weeks after birth, ductal length progressed in bigenic and control mice: 7.58 mm±0.6 and 9.66 mm±0.5, respectively. At 8-weeks age, ductal development was nearly similar whatever the genotype, indicating that the phenotype induced by CBLC overexpression was transient. However, a variation between samples from mammary glands of the same genotype and of the same age was observed as illustrated in Figure 2A, by comparing panel j with a strong reduction of duct development to panel l with a nearly normal development.

Discussion

The CBL family of E3-ubiquitin ligases plays a major role in the inactivation of RTKs, especially EGFR, through ubiquitin-mediated endocytosis and lysosomal degradation. This applies more particularly to CBLC (6), which is only expressed in epithelial tissues (7, 8). Thus, in the mammary gland *Cblc* is expressed in epithelial cells but not in the fat

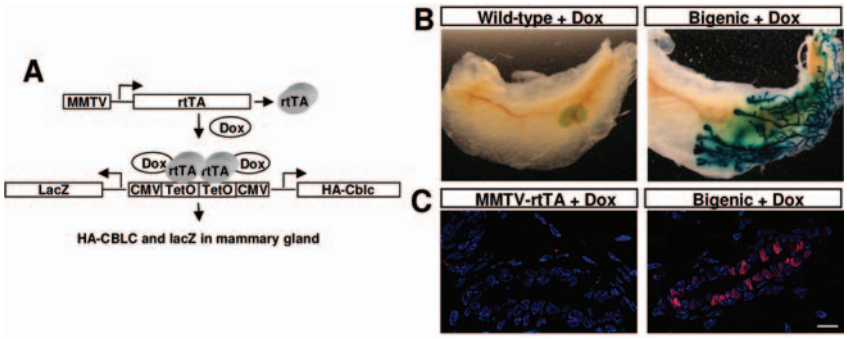

Figure 1. *Generation of a CBLC transgenic mouse. (A) Schematic drawing of the two constructs used to generate bitransgenic tet-regulated Cblc-overexpressing mice. Bigenic MMTV-rtTA/LacZ HA-Cblc mice were generated by crossing heterozygous or homozygous LacZ HA-Cblc with MMTV-rtTA mice. (B) Specific induction of LacZ in mammary epithelial cells of bigenic mice was confirmed by X-Gal staining of whole inguinal mammary glands from 6-week-old wild type or bigenic virgin mice fed with Dox-water for 2.5 weeks. Only bigenic mammary glands are positive. (C) Sections through 5-week-old mammary glands from MMTV-rtTA or bigenic mice fed with Dox-water for 72 h were stained with anti-LacZ antibody. A patchy LacZ induction is observed in bigenic mammary epithelium. Bar: 10 μm.*

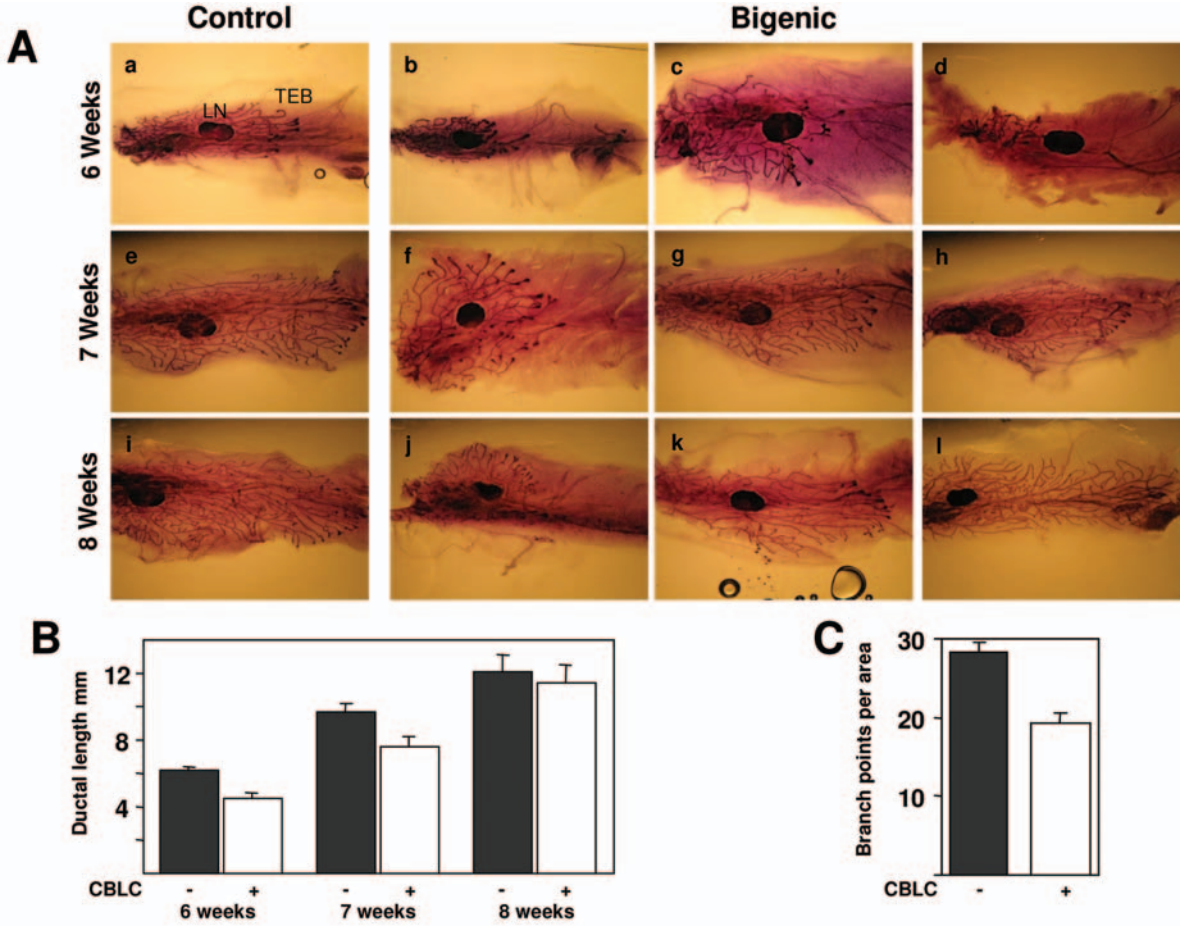

Figure 2. *Impaired development in CBLC-overexpressing mammary glands. (A) Mammary glands from 6-, 7- and 8-week-control and bitransgenic mice were whole-mounted to analyze the extent of ductal growth. Shown are representative mammary glands for each time point. LN, lymph nodes; TEB, terminal end buds. (B) Ductal invasion in bigenic and control mammary glands, represented as distance between the distal end of the lymph node to the distal end of the longest duct. Data are mean±SEM 6 weeks: p<0.001, control, n=54; bigenic, n=53; 7 weeks p=0.02, control n=7, bigenic n=9; 8 weeks, non significant, control, n=10; bigenic n=12. CBLC overexpression significantly induces reduction of ductal length measured at 6 and 7 weeks (paired student t test; **p<0.001, *p=0.02. (C) Number of branch points beyond the lymph node in an arbitrary square determined in mammary glands from 6-week-old control and bigenic mice. Data are mean±SEM using 47 control mammary glands and 53 bigenic mammary glands.*

pad (8). Despite this, *Cblc* knock-out mice exhibit normal epithelial development and cell proliferation (8). However, long-term effects of the absence of CBLC in *Cblc* -/- mouse have not been monitored. Increased ductal branching and density have been observed in the mammary glands of *Cbl* -/- mouse (9). This suggests that in the mammary gland CBLC could be compensated by CBL whereas CBL could not be compensated by CBLC. In *Drosophila*, lack of CBL in the eye leads to overgrowth due to increased activity of EGFR (10). It is shown here that, in contrast, an excessive and/or constitutive expression of CBLC delays mammary duct development. This phenotype is the opposite of the *Cbl* -/- mammary gland phenotype. The phenotype is also reminiscent of that observed after downregulation of EGFR in transgenic mice (11). However, epithelial EGFR signaling does not seem to be essential for mammary duct development (12, 13). It is thus possible that CBLC overexpression targets a receptor other than EGFR. The RET receptor is a candidate (14) and the IGF1R receptor, which has an important role in the mammary gland, might be one too (15).

The *in vivo* results support the concept of CBLs as negative regulators of cell growth (16). The mouse model may be used to further study regulatory components of the CBL pathway (3) and may also be crossed with mice susceptible to developing mammary tumors.

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