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Expression of cyclin D1 in the developing lens of c-maf <sup>-/-</sup> mice

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## Summary

The *maf* gene encodes a transcription factor protein containing a typical basic/leucine zipper domain structure, a motif for protein dimerization and DNA binding. It has been demonstrated that *maf* family genes have important roles in embryonic development and cellular differentiation. In this study, expression of cyclin D1, one of cell cycle-related molecules, was examined immunohistochemically in developing lens cells of *c-maf* knockout (-/-) mice. At embryonic day 14 in wild-type mice, lens cells consisted of round epithelial cells of a single layer and regularly arranged elongated lens cells, indicating primary lens fiber cells. Cyclin D1-positive nuclei were observed in the lens epithelial cells, whereas cyclin D1 was not expressed in the primary lens fiber cells. In *c-maf* -/- mice, a variety of round epithelial cells were located in the anterior and posterior lens. Many cyclin D1-positive nuclei were observed in lens epithelial cells as well as posterior lens cells. These results suggest that *c-maf* plays a role in the regulation of expression of cyclin D1 in developing lens cells.

**Key Words:** *c-maf*, cyclin D1, lens

## Introduction

Morphologic lens development begins with the head ectoderm responding to inductive signals from the underlying optic vesicle to form the lens placode. The lens placode subsequently invaginates to form the lens pit, which detaches from the surface ectoderm to form the lens vesicle. The lens vesicle develops into the lens when the anterior cells remain proliferative and form the lens epithelium, and then, the posterior cells exit from the cell cycle, elongate into lens fiber cells, and fill up the lumen of the lens vesicle. After the basic structure is established, the lens continues to grow throughout life through differentiation of the lens epithelial cells into fiber cells. Fiber cell differentiation is characterized by withdrawal from the cell cycle, cell elongation, and degradation of intracellular organelles, including the nucleus (Wride, 1996).

The musculoaponeurotic fibrosarcoma (*maf*) gene encodes a transcription factor protein containing a typical basic/leucine zipper (bZIP) domain structure, a motif for protein dimerization and DNA binding, and forms a heterodimer with *c-Jun* (Kataoka et al., 1994; Kerppola et al., 1994; Matsushima HY, 1998). Recently, *in vivo* study clearly disclosed that *c-maf* and *maf-B* genes had important roles in embryonic development and cellular differentiation (Kase et al., 2004; Ogata et al., 2004). We previously isolated *c-maf* cDNA clones from rat and have detected their expression in developing lens fiber cells (Sakai et al., 1997; Yoshida et al.,

1997; Sakai et al., 2001; Yoshida et al., 2001). In addition, proliferative cells were detected at the posterior region of the c-maf<sup>-/-</sup> lens, suggesting that c-maf plays a role in inhibition of the proliferation of lens fiber cells (Sakai et al., 1997; Yoshida et al., 1997). The mechanism in the regulation of lens cell growth involved by c-maf, however, has not been determined.

The inappropriate proliferation in lens fiber cells was also reported in retinoblastoma (Rb)-deficient embryos (de Bruin et al., 2003). Progression into the DNA synthesis phase of the mammalian cell cycle requires inactivation of the Rb protein via its phosphorylation by cyclin D-dependent kinases. Down-regulation of cyclin D in the postmitotic lens fiber cell is required for the maintenance of the postmitotic state (Gomez Lahoz et al., 1999). Screening of the cyclin D1 promoter revealed that it contains an AP-1-binding site sequence (Shiozawa et al., 2004). c-maf induces changes in nuclear DNA-binding activities at multiple sites containing AP-1 elements (Cao et al., 2002).

In this study, the expression of cyclin D1 in developing lens was examined using c-maf knockout (-/-) mice.

## Materials and methods

### Animals and tissues

All animal experiments conformed to the ARVO Resolution on the Use of Animals in Research. Six C57Bl6 mice were obtained from Hokudo Corp., Sapporo, Japan. The morning that the vaginal plug was detected was defined as embryonic day (E) 0. On E 14, the mice were anesthetized with an intraperitoneal injection of 6% sodium pentobarbital (0.1 ml/100g), and the embryos were surgically removed and then fixed transcardially with 4% paraformaldehyde in 0.1 M borate buffer (pH 9.5) for 2 hr and processed for paraffin sections.

Six *c-maf*<sup>-/-</sup> mice were produced as described previously (Yoshida et al., 2001). Briefly, *c-maf* genomic clones were isolated from a 129-genomic phage library and mapped by using several restriction enzymes. The targeting vector was linearized at the *NotI* site and electroporated into embryonic stem cells. After selection, Southern blotting identified correct replacement events, and homologous recombination events were shown to have occurred in embryonic stem cell lines. Finally, one line was obtained by germ-line transmission.

### Immunohistochemistry

The slides were deparaffinized and rehydrated, and then rinsed with phosphate-buffered saline (PBS) twice for 10 min and incubated with the normal goat serum for 20 min and then with a mouse monoclonal antibody against cyclin D1 (1:50; Santa Cruz Biotech, Santa Cruz, CA) at room

temperature for 2 hr. Binding of the primary antibody was localized using FITC-conjugated goat anti-mouse IgG (1:100; Jackson Immuno Research Laboratories Inc. West Grove, PA) for 30 min. Nuclei were then stained with PBS containing 4'6-diamidino-2-phenylindole (DAPI)(4-10 g/ml) for 5 min. The specificity of the immunostaining was confirmed by using Tris-buffered saline instead of the primary antibody for cyclin D1 (negative control).

## Results

At E 14 in wild-type mice, lens cells consisted of round epithelial cells in a single layer (Fig. 1 a, arrowhead) and regularly arranged elongated lens cells, which were considered to be primary lens fiber cells (Fig.1 a, arrow). Cyclin D1-positive nuclei were observed in the lens epithelial cells, whereas cyclin D1 was not expressed in the primary lens fiber cells (Fig.1 b). In *c-maf*<sup>-/-</sup> mice, a variety of round epithelial cells were located in the anterior and posterior lens. Lens epithelial cells proliferated and formed a few layers. The elongation seen in lens cells of wild-type mice failed to occur, resulting in a small hollow lens cavity (Fig. 1 c). Many cyclin D1-positive nuclei were observed in lens epithelial cells as well as posterior lens cells (Fig.1 d). In both wild-type and *c-maf*<sup>-/-</sup> mice, many neuroblastic cells of the developing retina also expressed cyclin D1 in the nuclei.

## Discussion

Proliferative cells were located in the anterior epithelium but were absent from the posterior region of the developing lens (Yoshida et al., 2001). Maf encodes a transcription factor protein containing a bZIP domain structure, a motif for protein dimerization and DNA binding (Kataoka et al., 1994; Kerppola et al., 1994). In our previous studies, we showed that c-maf was not expressed in the lens epithelial cells, but was expressed in the equatorial region and posterior portion of the lens (Sakai et al., 1997; Yoshida et al., 1997). This study clearly demonstrated that cyclin D1-positive nuclei were observed in the lens epithelial cells, whereas cyclin D1 was not expressed in the primary lens fiber cells (Fig.1 b). The lack of cyclin D1 where the c-maf expression occurred prompted us to hypothesize that c-maf plays a role in the regulation of expression of cyclin D1. Hence, we examined the role of c-maf in the expression of cyclin D1 using c-maf<sup>-/-</sup> mice in this study.

c-maf induces changes in nuclear DNA-binding activities at multiple sites containing AP-1 elements (Cao et al., 2002). Screening of the cyclin D1 promoter revealed that it contains an AP-1-binding site sequence (Shiozawa et al., 2004). In this study, many cyclin D1-positive nuclei were observed in posterior lens cells in c-maf<sup>-/-</sup> mice (Fig.1 d), suggesting that c-maf inhibits the transcriptional activation of cyclin D1.

In fact, c-maf was able to suppress the *IL-12 p40* promoter, and resulted in a strong inhibition of mRNA expression in some cell types (Cao et al., 2002). Inhibition of the gene expression by c-Maf requires the N-terminal transactivation domain (Cao et al., 2002), suggesting an indirect mechanism of transcriptional inhibition of cyclin D1 involving the induction of an unidentified repressor.

We previously demonstrated that proliferative cells were detected at the posterior region of the developing lens in c-maf <sup>-/-</sup> mice (Yoshida et al., 2001). In an *in vitro* study, it was shown that down-regulation of cyclin D in postmitotic lens fiber cells was required for the maintenance of the postmitotic state (Gomez Lahoz et al., 1999). The continued expression of cyclin D1 in conjunction with proliferation in the posterior region of the c-maf<sup>-/-</sup> lens (Yoshida et al., 2001) suggested that down-regulation of cyclins D was also required for the maintenance of the postmitotic state in the lens fiber cell *in vivo*. Proliferation of the lens epithelium and migration of lens epithelial cells to beneath the posterior lens capsule result in a pulverulent cataract (Smith et al., 1997). Elucidation of the mechanism by which c-maf regulates the protein level of cyclin D1 should prove valuable in delineating the molecular pathways underlying the pulverulent cataract found in the maf-mutant lens (Jamieson et al., 2003).

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## Figure Legends

Fig.1. DAPI nuclear staining (a, c) and immunoreactivity for cyclin D1 (b, d) in developing lens cells on embryonic day (E) 14 in wild-type mice (a, b) and *maf*<sup>-/-</sup> mice (c, d). At E 14, lens cells consist of epithelial cells (a, arrowheads) and primary lens fiber cells (a, arrows). Nuclear immunoreactivity for cyclin D1 is noted in the lens epithelial cells (b, arrowheads). In contrast, cyclin D1 is not expressed in the primary lens fiber cells (b, arrows). In *c-maf*<sup>-/-</sup> mice, a variety of round epithelial cells are located in the anterior and posterior lens. Lens epithelial cells proliferate and form a few layers. Lens epithelial cells do not show differentiation into fiber cells (c). Many cyclin D1-positive nuclei are observed in lens epithelial cells as well as posterior lens cells (d). Bar equals 50  $\mu$ m.

