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Regulation of Rb family proteins by Cdk6/Ccnd1 in growth plates

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The growth plate lacks vascular tissues and is solely composed of chondrocytes. The growth plate is composed of three layers, i.e., the resting, proliferating and hypertrophic chondrocyte layers. The chondrocytes in the resting and proliferating layers proliferate and then they uniformly maturate toward the diaphysis to become hypertrophic chondrocytes, which lose the ability to proliferate. As the growth plate lacks vasculature, apoptotic chondrocytes are not phagocytosed, and the fragmented DNA detected by TUNEL remains until they reach the bone marrow. Therefore, the growth plate is an appropriate tissue to use for evaluation of cell proliferation, differentiation, and apoptosis.

We examined the effects of Cdk6 and Ccnd1 (cyclin D1) in chondrocyte proliferation, differentiation, and apoptosis.[1] Transgenic mice overexpressing either *Cdk6* or *Ccnd1* in chondrocytes show no increase in the number of BrdU-positive cells, and the chondrocytes normally maturate, although *CCND1* is a well-established human oncogene. In contrast, transgenic mice overexpressing both *Cdk6* and *Ccnd1* show dwarfism. In *Cdk6*/*Ccnd1* double transgenic mice, chondrocyte maturation to hypertrophic chondrocytes is inhibited, and the number of BrdU-positive cells is increased, but the proliferation of chondrocytes is not enhanced, and the frequency of apoptotic chondrocytes is greatly increased. The apoptosis is rescued by *p53* deletion, but chondrocyte proliferation is still not enhanced in $p53^{-/-}$*Cdk6*/*Ccnd1* double transgenic mice.

Cdk6 and Ccnd1 regulate the activity of E2fs by phosphorylating Rb family proteins, Rb, p107, and p130, and trigger progression through the G_0/G_1 and G_1/S transitions of the cell cycle. The effects of targeted deletions of Rb family genes in chondrocytes were reported by two groups.[2-4] *p107*-knockout mice show most apparent inhibition of chondrocyte differentiation among the knockout mice of Rb family genes. Double knockout mice of *p107*/*p130* or *p107*/*Rb* show more severe inhibition of chondrocyte differentiation and chondrocyte proliferation is enhanced without an increase in chondrocyte apoptosis. Therefore, all of the Rb family proteins are involved in chondrocyte proliferation and differentiation, and p107 plays a major role among them.

When we generated *Cdk6*/*Ccnd1* double transgenic mice, we expected that overexpression of both *Cdk6* and *Ccnd1* would result in the inactivation of all Rb family proteins by their phosphorylation. However, the phenotype of *Cdk6*/*Ccnd1* double transgenic mice was different from those of *p107*/*p130* or *p107*/*Rb* double knockout mice. BrdU-positive cells are increased, and chondrocyte differentiation is inhibited in *p107*/*p130* and *p107*/*Rb* double knockout mice and *Cdk6*/*Ccnd1* double transgenic mice. However, chondrocyte proliferation is enhanced without an increase in apoptosis in *p107*/*p130* and *p107*/*Rb* double knockout mice, whereas chondrocyte proliferation is not enhanced, and apoptotic chondrocytes are greatly increased in *Cdk6*/*Ccnd1* double transgenic mice. In *Cdk6*/*Ccnd1* double transgenic mice, Rb but not p107 is highly phosphorylated, and mRNA and protein levels of p107 are increased.[1] *p107* transcription is regulated by E2f, and p107 is upregulated in $Rb^{-/-}$ and $p130^{-/-}$ mouse embryonic fibroblasts.[5-7] Therefore, the phenotypic differences between *p107*/*p130* and *p107*/*Rb* double knockout mice and *Cdk6*/*Ccnd1* double transgenic mice could be explained by the absence or presence of p107.

When *Cdk6* and *Ccnd1* are overexpressed, Rb, p107, and p130 should be phosphorylated, leading to the release of repressing E2fs (E2f4, 5) and activation of activating E2fs (E2f1–3), both of which enhance the transcription of E2f target genes including *p107* (**Fig. 1A and B**). Therefore, phosphorylation of Rb, p107, and p130 enhances the transcription of *p107* by E2f and unphosphorylated p107 is continuously supplied. This is the reason why unphosphorylated p107 is dominant in *Cdk6*/*Ccnd1* double transgenic mice.[1] Although p107 binds to repressing E2fs at the physiological condition, upregulation of p107 allows complex formation of activating E2fs with p107.[8] Therefore, the increased amount of unphosphorylated p107 associates not only with repressing E2fs, but also with activating E2fs (**Fig. 1C**). As p107 plays a major role in cell cycle regulation among Rb family proteins in chondrocytes, the association of p107 with repressing and activating E2fs would affect the expression of E2f target genes. It occurs in *Cdk6*/*Ccnd1* double transgenic mice as shown by the downregulation of *cyclin E*, *dhfr*, *cdc25a*, and *B-Myb*. Further, chondrogenic ATDC5 cells overexpressing *Cdk6* and *Ccnd1* also had downregulated expression of these genes, and siRNA of *p107* reversed the downregulated expression of these genes. Therefore, phosphorylation of Rb, p107, and p130 upregulates *p107* expression, which dysregulates E2f target gene expression leading to p53-dependent apoptosis (**Fig. 1B and C**). *p53* deletion in addition to Rb inactivation is still not sufficient for enhancement of chondrocyte

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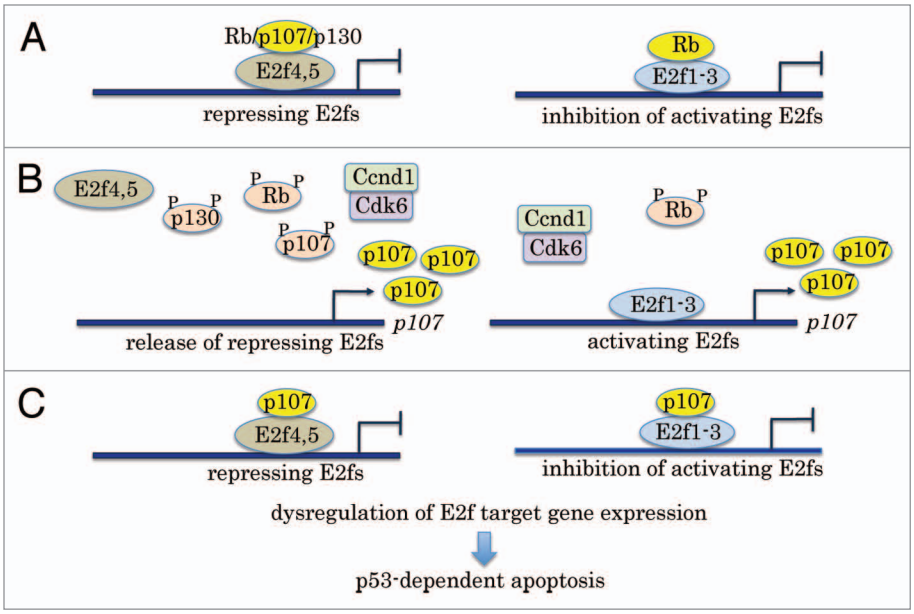

Figure 1. Regulation of Rb family proteins by Cdk6/Ccnd1. (**A**) Rb, p107, and p130 associate with repressing E2fs (E2f4 and E2f5) which are located in the cytoplasm, the complexes translocate to the nucleus, and they bind to E2f target genes and inhibit their expression (left). Rb associates with activating E2fs (E2f1, E2f2, and E2f3) and inhibits the activity of E2fs (right). (**B**) Overexpression of *Cdk6* and *Ccnd1* phosphorylates Rb, p107, and p130, the phosphorylated Rb, p107, and p130 dissociate from the repressing E2fs, and the E2fs translocate to the cytoplasm (left). The phosphorylated Rb dissociates from the activating E2fs, and the E2fs are activated (right). In both cases, the repression of genes necessary for progression through the cell cycle is lifted. However, the expression of *p107*, which is regulated by E2f, is also upregulated. (**C**) Unphosphorylated p107 associates with repressing E2fs and suppresses the expression of E2f target genes (left). Unphosphorylated p107 also associates with activating E2fs and inhibits them (right). In both cases, the expression of E2f target genes is inhibited and the dysregulation of E2f target genes results in p53-dependent apoptosis.

proliferation, suggesting that p107 contributes to the low incidence of chondrosarcoma as an anti-oncogenic protein, because the frequency of chondrosarcoma is much less than those of sarcomas arising in soft tissues.

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