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p107-Dependent recruitment of SWI/SNF to the alkaline phosphatase promoter during osteoblast differentiation☆

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ABSTRACT

The retinoblastoma protein family is intimately involved in the regulation of tissue specific gene expression during mesenchymal stem cell differentiation. The role of the following proteins, pRB, p107 and p130, is particularly significant in differentiation to the osteoblast lineage, as human germ-line mutations of *RB1* greatly increase susceptibility to osteosarcoma. During differentiation, pRB directly targets certain osteogenic genes for activation, including the alkaline phosphatase-encoding gene *Alpl*. Chromatin immunoprecipitation (ChIP) assays indicate that *Alpl* is targeted by p107 in differentiating osteoblasts selectively during activation with the same dynamics as pRB, which suggests that p107 helps promote *Alpl* activation. Mouse models indicate overlapping roles for pRB and p107 in bone and cartilage formation, but very little is known about direct tissue-specific gene targets of p107, or the consequences of targeting by p107. Here, the roles of p107 and pRB were compared using shRNA-mediated knockdown genetics in an osteoblast progenitor model, MC3T3-E1 cells. The results show that p107 has a distinct role along with pRB in induction of *Alpl*. Deficiency of p107 does not impede recruitment of transcription factors recognized as pRB co-activation partners at the promoter; however, p107 is required for the efficient recruitment of an activating SWI/SNF chromatin-remodeling complex, an essential event in *Alpl* induction.

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Introduction

The retinoblastoma protein, pRB and its related family members p107 and p130 are important mediators of various cell processes including cell cycle progression, apoptosis and differentiation [1–3]. In particular, the retinoblastoma family is intimately involved in the regulation of tissue specific gene expression during mesenchymal stem cell differentiation. pRB is implicated in the differentiation of chondrocytes [4], myoblasts [5–8] adipocyctes [9–11] and osteoblasts [12–17]. The osteoblast differentiation function has particular relevance for the tumor suppressor role of pRB, as human germ-line mutations of the pRB-encoding gene *RB1* greatly increase susceptibility to osteosarcoma [18].

The molecular mechanisms by which pRB influences osteoblast differentiation are still emerging, but it is clear that certain osteogenic promoters are targeted directly by pRB for activation. The best studied is the *Bglap* gene, encoding osteocalcin. During late differentiation, pRB occupies the activated osteocalcin promoter. pRB forms a binding partnership with the osteogenic transcriptional regulator RUNX2 [13,19,20]. Another pRB activity involves releasing repression at promoters of certain osteogenic genes through displacement of the repressor KDM5A demethylase (syn.: RBP2). pRB physically associates with KDM5A, and after displacement of KDM5A, pRB returns to the target promoter in the company of activating factors including E2F1 [17,21,22]. The gene encoding the major early marker of osteoblast differentiation, alkaline phosphatase (*Alpl*) is another direct activation target of pRB [22]. Chromatin immunoprecipitation (ChIP) probes also revealed a converse p130-dependent repression of both *Alpl* and *Bglap* [23]. Regulation of *Alpl* and *Bglap* requires pRB and p130 respectively acting in concert with activator and repressor members of the E2F family to regulate tissue specific gene expression [17,23].

Mouse models indicate overlapping roles for pRB and p107 in bone and cartilage formation [24], and potential roles for p107 distinct from pRB [4,25,26]. However, beyond these biological phenotypes, very little is known about the potential direct tissue-specific gene targets of p107, or the consequences of targeting by p107. ChIP assays indicate that *Alpl* is targeted by p107 with the same dynamics as pRB (*i.e.* selectively during activation) [22], raising the possibility that p107 helps promote osteoblast differentiation by contributing to *Alpl* activation.

The role of p107 at the *Alpl* promoter was probed here in comparison with that of pRB using shRNA-mediated knockdown genetics in an osteoblast progenitor model. The results show that normal induction

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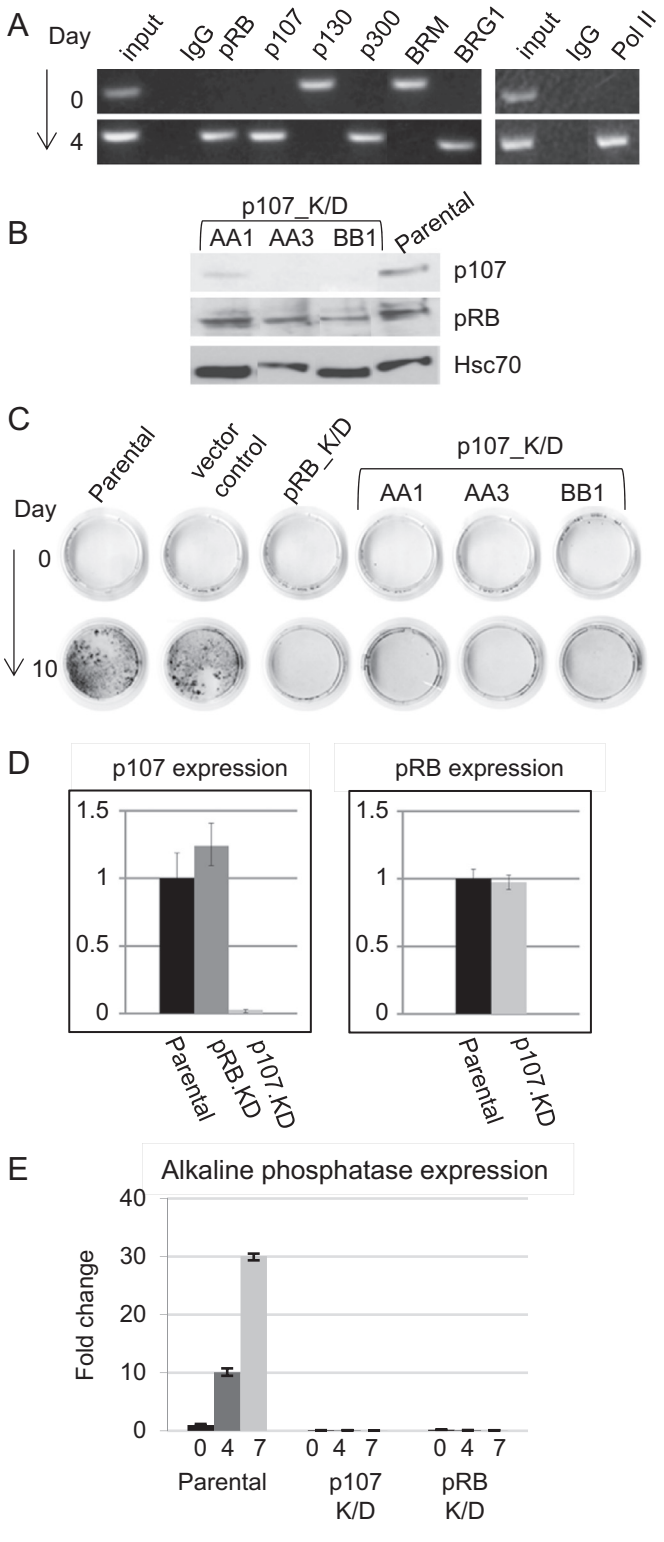
of *Alpl* is severely impaired in p107-depleted cells. Induction of other recognized targets of pRB-mediated activation, the osteocalcin and osteopontin encoding genes, is independent of p107. Thus, the requirement for cooperation between pRB and p107 for *Alpl* activation is a comparatively specific strategy. At the *Alpl* promoter, p107 is not required for recruitment of transcription factors recognized as co-activation partners with pRB, but p107 is required for efficient recruitment of an activating form of the SWI/SNF chromatin-remodeling complex, an essential event in *Alpl* activation.



Results

p107 is required for efficient activation of the Alpl promoter

The mouse calvaria-derived MC3T3-E1 cell line has a pre-osteoblast phenotype suitable for studying induction of osteoblast-specific gene expression. When stimulated by bone anabolic agents, these non-transformed precursor cells undergo terminal differentiation. The full differentiation program unfolds over more than two weeks in a highly coordinated successive pattern of gene expression [27,28]. Induction of alkaline phosphatase (*Alpl*) is an early stage event, coordinated closely with differentiation-associated cell cycle arrest. The presence of p107 on the active *Alpl* promoter with the same dynamics as pRB was noted earlier [22] (Fig. 1A) in a pattern suggesting p107 may contribute to *Alpl* activation. Selective pRB knockdown has already shown p107 to be insufficient for *Alpl* activation in pRB-deficient cells [22] but those experiments do not indicate whether p107 is necessary. This issue was addressed here using a parallel shRNA approach to create stable knockdown of p107 in the osteoblast precursors.

Three independent lines were isolated, designated p107_K/D.AA1, p107_K/D.AA3, and p107_K/D.BB1. Western blots show that expression of pRB is relatively unaffected in the p107-depleted lines (Fig. 1B). An *in situ* staining assay shows severely impaired induction of alkaline phosphatase activity in each depleted line (Fig. 1C), to an extent similar to that reported previously with pRB depletion (Fig. 1C and [22]). A representative line (p107_K/D.BB1) was selected for further study here. Quantitative analysis by qRT-PCR shows sharply decreased expression of p107 with no significant effect on pRB expression (Fig. 1D). Conversely, the pRB-depleted line shows normal expression of p107. Side-by-side comparison of *Alpl* expression in induced pRB-depleted and p107-depleted cells shows comparably severe impairment (Fig. 1E). Thus p107 plays a largely non-redundant role with pRB in activation of *Alpl*.

Osteopontin and osteocalcin are induced independently of p107

The osteocalcin and osteopontin encoding genes (*Bglap* and *Spp1*, respectively) are other key osteoblast genes that have been identified as direct pRB targets in differentiating osteoblasts [13]. They were screened here to determine whether they are p107 targets as well. Osteopontin expression is already active in committed pre-osteoblasts, and a chromatin immunoprecipitation (ChIP) assay (Fig. 2A) shows pRB on the promoter in non-induced (day 0) cells, while p107 association was not detected even after induction of differentiation (day 7). Osteopontin expression increases several-fold more as the committed cells progress through differentiation; this increase is pRB-dependent,

Fig. 1. Depletion of p107 in osteoblast progenitors impairs induction of the alkaline phosphatase gene *Alpl*. A. ChIP analysis conducted on the *Alpl* promoter in normal parental pre-osteoblasts before and after induction of differentiation (4 and 7 days post-induction) shows recruitment of the pRB and p107 proteins specifically on the activated promoter, indicated by the presence of the p300 co-activator and RNA polymerase II (pol II). During activation the promoter changes from a repressed form characterized by the presence of p130 and BRM-SWI/SNF to an activated form characterized by recruitment of BRG1-SWI/SNF. The dynamics, assayed in parallel provide strong negative controls for the antibodies. B. Western blot analysis shows reduced levels of p107 in each of three cell lines isolated independently after stable transfection with a plasmid encoding a p107-targeting shRNA sequence. Constitutively expressed heat shock protein hsc70 was used as a loading control. C. Alkaline phosphatase activity, monitored colorimetrically *in situ*, shows sharply increased activity by day 10 post-induction in parental cells, or cells stably transfected with a vector control. The three independently isolated p107-depleted (p107_K/D) cell lines all show impaired induction similar to that seen with pRB deficiency (pRB_K/D). D. p107 and pRB gene expression determined by qRT-PCR in a representative p107 knockdown line is shown relative to normal parental cells and the pRB knockdown line. E. Alkaline phosphatase gene expression determined by qRT-PCR before (day 0) and at 4 and 7 days post-induction in differentiating osteoblast progenitors. The induction pattern in a p107 knockdown line is shown relative to the patterns in normal parental cells and the pRB knockdown line.

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