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Rb1 mRNA expression in developing mouse teeth

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Abstract

Rb1 is a tumor suppressor gene that regulates cell cycle progression through interactions with E2F transcription factors. In recent years, new roles for Rb1 in regulating cellular differentiation have also emerged. For example, it has been shown that Rb1 regulates osteoblast differentiation in a cell cycle independent manner, by binding to the transcription factor Runx2, and facilitating the up-regulation of late bone differentiation markers. Based on the facts that Runx2 also functions in tooth development, and that little is known about potential roles for Rb1 in mammalian tooth development, here we evaluated the expression of *Rb1* mRNA in developmentally staged mouse teeth. Our data show that *Rb1* mRNA is expressed in both dental epithelial and dental mesenchymal progenitor cells. In addition, *Rb1* mRNA appears upregulated in differentiating ameloblasts and odontoblasts, suggesting roles for Rb1 in tooth differentiation.

Keywords

Rb1; tooth development; dental epithelium; dental mesenchyme; cell-cell signaling

1. Introduction

Mammalian teeth develop through a series of well defined stages - epithelial thickening, bud, cap and bell stages (Caton and Tucker, 2009). Sequential and reciprocal interactions between the dental epithelium and the neural crest derived dental mesenchyme are required for proper tooth development (Thesleff and Aberg, 1999). Dental epithelial cells differentiate into ameloblasts which secrete enamel, while dental mesenchymal cells differentiate into odontoblasts which form dentin (Linde and Goldberg, 1993). Tooth formation is a complex and tightly regulated process, controlled by many signaling pathways and transcription factors (Mitsiadis and Graf, 2009; Thesleff and Aberg, 1999).

The Rb1 gene is the first identified tumor suppressor gene (Friend et al., 1986; Fung et al., 1987; Lee et al., 1987). Mutations in Rb1 in humans lead to the rare pediatric eye tumor, retinoblastoma (Friend et al., 1986; Fung et al., 1987; Lee et al., 1987). Moreover, mutations

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in Rb1 are present in 90% of osteosarcomas (Deshpande and Hinds, 2006). The most studied function of Rb1 is in the regulation of cell cycle progression, by binding to and repressing the function of E2F transcription factors (Sidle et al., 1996). The function of Rb1 is regulated in a cell cycle dependent manner through its cumulative phosphorylation by cyclin dependent kinases (cdk), in complex with cyclins (Chinnam and Goodrich, 2011). Rb1 is active and bound to E2F in hypophosphorylated form. Upon phosphorylation by several cyclin/cdk complexes including cyclin D1/cdk4, cyclin D1/cdk6, cyclin E/cdk2, and cyclin A/cdk2, Rb1 becomes hyperphosphorylated and can no longer bind E2F, freeing E2F to activate the transcription of downstream targets required for cell cycle progression (Chinnam and Goodrich, 2011).

In recent years it has become clear that in addition to functioning in G1 checkpoint, Rb1 plays a role in regulating cell differentiation and survival (Drysdale and Crosby, 2005). It is thought that Rb1 functions as a transcriptional co-factor that binds to a specific transcription factor, recruits chromatin remodeling proteins such as histone deacetylase (HDAC), the histone demethylase Kdm5b, and the SWI/SNF complex (Macaluso et al., 2006; Nijwenning et al., 2011), resulting in either an inhibitory or activating effect. It has recently been shown that Rb1 regulates cell differentiation by binding to tissue-specific transcription factors including MyoD, Pax8, Tbx2, and Gata1 (Gu et al., 1993; Kadri et al., 2009; Miccadei et al., 2005; Vance et al., 2010). More specifically, Rb1 promotes osteoblast differentiation in a cell cycle independent manner (Deshpande and Hinds, 2006). Rb1 physically interacts with Runx2, and upregulates the expression of late stage osteoblast differentiation markers (Thomas et al., 2001). Recently, it has been shown that Rb1 expression levels can direct mesenchymal progenitors to differentiate into either osteoblasts or adipocytes. In presence of Rb1, mesenchymal precursors adopt an osteoblastic fate, while in the absence of Rb1, they differentiate into adipocytes (Calo et al., 2010). Although bone and tooth development share many signaling pathways, it is unclear at this time whether Rb1 plays a role in tooth development. Although no published reports have described tooth defects caused by Rb1 gene mutations, altered methylation of the Rb1 gene has been associated with calcifying cystic odontogenic tumors (Moreira et al., 2009). Furthermore, the expression of Rb1 during tooth development is not known. In this study, we investigated the spatial-temporal expression patterns of *Rb1* in developmentally staged mouse tooth development. For reference, we have compared *Rb1* mRNA expression to the well characterized dental epithelial marker *Shh*, and the dental mesenchymal cell marker, *Runx2*.

Briefly, sonic hedgehog (Shh) is a secreted signaling factor expressed in dental epithelial cells of the early forming tooth bud (Bitgood and McMahon, 1995; Dassule and McMahon, 1998). In cap stage teeth, Shh expression is confined to the enamel knot, the signaling center regulating tooth shape (Bitgood and McMahon, 1995; Dassule and McMahon, 1998). At later developmental stages, Shh is expressed in ameloblasts (Bitgood and McMahon, 1995; Dassule and McMahon, 1998). Targeted knockout of Shh from dental epithelial cells revealed that Shh is essential for tooth growth and morphogenesis (Dassule et al., 2000).

The transcription factor Runx2, a master regulator of osteoblast differentiation (Ducy et al., 1997), also regulates odontoblast differentiation and subsequent dentin formation (Camilleri and McDonald, 2006). Runx2-null mice showed a complete lack of bone formation due to

maturational arrest of osteoblasts (Komori et al., 1997; Otto et al., 1997). In humans, haploinsufficiency of Runx2 leads to Cleidocranial Dysplasia (CCD), characterized by bone defects (absence of clavicles) and a variety of dental disorders including supernumerary teeth, abnormal tooth eruption, and tooth hypoplasia. (Komori et al., 1997; Lee et al., 1997; Mundlos et al., 1997). Tooth development in Runx2-null mice is arrested at cap/early bell stages (D'Souza et al., 1999). It has been shown that Runx2 is expressed in dental mesenchyme (Yamashiro et al., 2002), suggesting roles in odontoblast differentiation, and in the reiterative signaling between the dental epithelium and dental mesenchyme throughout tooth development. As mentioned above, the recently characterized interactions of Rb1 and Runx2 in osteoblast differentiation (Calo et al., 2010) prompted us to examine the co-expression of *Rb1* and *Runx2* mRNAs in tooth development.

2. Results

2.1. Rb1 mRNA expression during mouse molar tooth development

To define the tissue specificity of *Rb1* gene expression during tooth development, we performed sectioned *in situ* hybridization on developmentally staged mouse teeth. Alternate sections were probed with *Runx2* as a marker for dental mesenchymal cells, and *Shh* as a marker for dental epithelial cells. We first confirmed the integrity of our probe by examining *Rb1* mRNA expression in tissues known to express it. As previously described (Jiang et al., 1997) we detected *Rb1* mRNA in the eye lens and vibrissae of P0 mice (Figure 1A and 1B). We also detected *Rb1* mRNA expression in the prefrontal bone (Figure 1C), where Runx2 was also expressed (Figure 1D), consistent with Rb1 function in osteoblast differentiation, and with cranial bone defects observed in conditional Rb null mice (Calo et al., 2010; Gutierrez et al., 2008).

In bud (E12.5) and cap (E14.5) staged teeth, *Rb1* mRNA was barely detectable in dental mesenchyme and dental epithelium (Figure 2A and 2D). At these stages, *Runx2* was strongly detected in the dental mesenchyme (Figure 2B and 2E), and *Shh* was expressed in dental epithelial cells at E12.5, and in the enamel knot at E14.5 (Figure 2C and 2F).

In bell stage teeth (E16.5), *Rb1* was detected in the inner enamel epithelium and the dental sac (Figure 3A, A1, and A2). *Runx2* mRNAs were robustly expressed in pre-odontoblasts, and exhibited diffuse expression in dental pulp cells (Figure 3B, B1, and B2). *Shh* exhibited strong expression in the inner enamel epithelium (Figure 3C, C1, and C2).

In P0 M1 molar teeth, *Rb1* mRNA appeared highly expressed in differentiated ameloblasts, and relatively weakly expressed in less differentiated pre-ameloblasts (Figure 4A, and A1, arrows). In contrast, *Rb1* expression appeared stronger in less differentiated odontoblasts, as compared to differentiated odontoblasts (Figure 4A, A1). *Rb1* mRNA was also expressed in osteoblasts in the surrounding alveolar bone, and was weakly expressed in the dental pulp (Figure 4A, A1). *Runx2* expression in odontoblasts was relatively weak at this stage, as previously reported, but robust *Runx2* expression was detected in the surrounding alveolar bone (Figure 4B and B1). *Shh* was highly expressed in dental epithelial cell derived ameloblasts (Figure 4C and C1).

2.2. Rb1 expression in P0 incisor teeth

We also assessed *Rb1* mRNA expression in the continuously erupting P0 mouse incisor. Similar to M1 molar teeth, *Rb1* was detected in dental epithelial and dental mesenchymal derived tissues, clearly seen on the labial side of the incisor (Figure 5A, A1, and A2). As observed in M1 molar teeth, *Rb1* mRNA expression appeared stronger in differentiated ameloblasts as compared to less differentiated pre-ameloblasts, and was more highly expressed in undifferentiated dental mesenchyme adjacent to the labial cervical loop as compared to differentiated odontoblasts (Figure 5A2). *Rb1* was also detected along the terminally differentiated lingual side of the incisor, in the undifferentiated dental epithelium of the cervical loop, and in the adjacent dental mesenchyme and alveolar bone (5 A, A1, and A2). *Runx2* was strongly detected in the alveolar bone surrounding the incisor, and in dental mesenchyme and odontoblasts (Figure 5B, B1, and B2). Robust *Shh* expression was detected in labial side pre-ameloblasts and differentiated ameloblasts (Figure 5C, C1, and C2).

3. Discussion

In this study, we characterized the expression of *Rb1* mRNA in developing mouse teeth, using the well characterized expression patterns of *Runx2* and *Shh* for comparison. Our results indicate that *Rb1* is barely detected in early stage E12.5 and E14.5 teeth (Figure 2A and D). In E16.5 bell stage teeth, *Rb1* expression was detected in differentiating dental epithelium and mesenchyme (Figure 3A, A1 and A2). Interestingly, in P0 differentiation stage M1 molar and incisor teeth, *Rb1* mRNA exhibited relatively stronger expression in differentiated ameloblasts as compared to less differentiated pre-ameloblasts, and appeared stronger in less differentiated dental mesenchyme as compared to differentiated odontoblasts (Figure 4A and A1). Together, these results support roles for *Rb1* in both dental epithelial and dental mesenchymal cell differentiation. It is also interesting that dental mesenchymal *Runx2* expression is similar to that of *Rb1*, in that it is expressed in less differentiated dental mesenchymal tissues (Figure 2B and E), as compared to differentiated odontoblasts (Figure 4A and A1). Roles for *Runx2* in tooth development have been well characterized, and *Runx2* null mouse teeth are arrested at cap/early bell stage, prior to odontoblast differentiation (D'Souza et al., 1999). *Runx2* overexpression inhibits odontoblast terminal differentiation (Li et al., 2011; Miyazaki et al., 2008), resulting in the elaboration of abnormally thin dentin that is deposited around odontoblasts, rather than in a distinct dentin layer (Li et al., 2011; Miyazaki et al., 2008).

Rb1 has been shown to physically interact with *Runx2* in osteoblasts, and *Rb1* function is required for osteoblast terminal differentiation (Thomas et al., 2001). *Rb1*-null mice exhibit reduced expression of late osteoblast differentiation markers, and have larger osteoblast progenitor cell populations (Gutierrez et al., 2008). *In vivo* studies demonstrate that *Rb1* promotes osteogenic cell fates, while reduced *Rb1* expression levels promote adipogenic cell fates (Calo et al., 2010). It is interesting that both *Rb1* and *Runx2* exhibit relatively higher expression levels in undifferentiated dental mesenchymal tissues as compared to differentiated dental mesenchyme derived odontoblast and alveolar bone tissues.

The continuously erupting mouse incisor provides a unique opportunity to study odontoblast and ameloblast differentiation, due to the fact that, in comparison to the terminally differentiated and enamel-free lingual side, labial cervical loops do not terminally differentiate, but rather maintain an active stem cell niche, and form thick enamel layers (Thesleff et al., 2007)(Harada et al., 1999; Tummers and Thesleff, 2008). As such, labial and lingual cervical loops differ in size and proliferative capacity. Dental epithelial stem cells in the labial cervical loop actively proliferate, migrate out as transit amplifying cells, and eventually differentiate into ameloblasts (Harada et al., 1999). In contrast, mouse incisor lingual cervical loops contain few proliferating stem cells, consistent with the absence of ameloblasts and enamel on the lingual side (Harada et al., 1999). We found that *Rb1* mRNAs exhibited reduced expression in terminally differentiated, lingual side odontoblasts, and appeared upregulated in undifferentiated labial side odontoblasts. These results indicate that, similar to Rb1 function in osteoblast cell differentiation, lower Rb1 expression levels may be required to maintain dental mesenchymal stem cell populations, while higher Rb1 expression results in odontoblast differentiation.

In conclusion, our studies characterizing *Rb1* mRNA expression in mouse tooth development have led to some surprising findings. First, we determined that *Rb1* mRNA is dynamically expressed in both dental epithelial and dental mesenchymal cell differentiation, but in a reciprocal manner. We found that *Rb1* mRNA was more highly expressed in differentiated versus undifferentiated dental epithelial cells, while in contrast, *Rb1* mRNAs were expressed at lower levels in differentiated odontoblasts, as compared to undifferentiated pre-odontoblasts. *Rb1* mRNA expression in odontoblast differentiation resembles that of the Rb1 interacting transcription factor, *Runx2*, which is reduced in differentiated odontoblasts as compared to less differentiated progenitor cells. Based on recently published roles for Rb1 and Runx2 interactions in osteoblast differentiation, our future studies will investigate potential interactions of Rb1 and Runx2 in odontoblast differentiation. We will also investigate Rb1 function in ameloblast differentiation, based on our observation that *Rb1* is more robustly expressed in differentiated, as compared to undifferentiated, dental epithelial tissues.

4. Experimental procedures

4.1. Animal husbandry and dental tissue harvesting and preparation

All experimental procedures involving the use of animals were approved by the Tufts University IACUC. Wild type C57BL/6J mice were mated overnight, and the presence of a vaginal plug was designated as embryonic day (E) 0.5. Pregnant mice were sacrificed at embryonic stages E12.5, E14.5, and E16.5. The heads of E16.5 embryos and new born (NB) pups were cut sagittally into two halves to allow better solution penetration. Heads were fixed in 10% buffered formalin (NBF) (Fisher Diagnostics, Pittsburgh, PA, USA) overnight at 4°C, washed in PBS, incubated with 30% sucrose, embedded in OCT media and frozen. E12.5 and E14.5 samples were embedded for coronal sectioning. E16.5 and NB heads were embedded for sagittal sectioning.

4.2. In situ Hybridization

The OCT embedded samples were cryosectioned at 10µm. The *in situ* hybridization was performed as previously described (Connerney et al., 2006). The following primers were used to amplify the mouse *Rb1* gene by Polymerase Chain Reaction (PCR): sense CTT TTT TGT AAA ACA GGG CAA GGG; antisense TAT CTT TCT CCT CTA AGA TCT CCA AAG. The 145 bp PCR product was cloned into TOPO-PCR Blunt II vector (In Vitrogen, Carlsbad, CA, USA). DIG-labeled antisense riboprobes corresponding to *Rb1*, *Runx2* (gift from M. H. Drissi), and *Shh* (gift from J. K. Yoon) were generated DIG RNA Labeling Kit (Roche Applied Science, Indianapolis, IN, USA).

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Abbreviations

E	embryonic
P0	postnatal day 0
Rb1	retinoblastoma gene
Shh	sonic hedgehog

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Highlights

- Rb1 mRNA is expressed in dental epithelium and dental mesenchyme.
- Rb1 mRNA is enriched in differentiated dental epithelial cells as compared to undifferentiated progenitor dental epithelial cells.
- Rb1 mRNA is enriched in dental mesenchymal cell progenitor cells as compared to differentiated dental mesenchymal cells.
- Rb1 may function in dental epithelial and dental mesenchymal cell differentiation.

Figure 1. *Rb1* expression in P0 mouse eye, vibrissae and prefrontal bone

Rb1 mRNA was detected in: (A) the eye lens; (B) hair follicle of vibrissae; and (C) in the prefrontal bone where it co-localized with (D) *Runx2* expression. P0 mouse heads were sectioned sagittally. Abbreviations: c, capsule; hf, hair follicle; i, iris; l, lens; pb, prefrontal bone.

Figure 2. *Rb1*, *Runx2* and *Shh* expression in E12.5 and E14.5 mouse M1 molar teeth

Rb1 mRNA was virtually undetectable in E12.5 (A) and E14.5 (D) tooth buds. *Runx2* was strongly expressed in the dental mesenchyme of E12.5 (B) and E14.5 (E) stage teeth, while *Shh* was detected in the dental epithelium of E12.5 (C) and the enamel knot of E14.5 (F) stage teeth. Embryos were sectioned coronally. Abbreviations: de, dental epithelium; dm, dental mesenchyme; ek, enamel knot; ide, inner dental epithelium, ode, outer dental epithelium; tb, tooth bud.

Figure 3. *Rb1*, *Runx2* and *Shh* expression in E16.5 M1 molar teeth

At E16.5, *Rb1* was easily detected in the inner enamel epithelium, outer dental epithelium, and dental mesenchyme (A, A1, A2). *Runx2* was expressed in pre-odontoblasts and dental sac (B, B1, B2). *Shh* was clearly expressed in the inner enamel epithelium (C, C1, C2). A1, B1, and C1 present higher magnification images of maxillary M1 molar teeth in boxed area 1 in panels A, B and C, respectively. A2, B2, and C2 display higher magnification images of mandibular M1 molar teeth in boxed area 2 in Panels A, B and C, respectively. Embryos were sectioned sagittally. Abbreviations: dp, dental pulp; ds, dental sac; iee, inner enamel epithelium; od, odontoblasts; pod, pre-odontoblasts.

Figure 4. *Rb1*, *Runx2* and *Shh* expression in newborn (P0) mouse M1 molar teeth

Rb1 was detected in differentiated ameloblasts, somewhat reduced in differentiated odontoblasts, and appeared weakly expressed in the dental pulp (A, A1). *Runx2* mRNAs were weakly expressed in differentiated odontoblasts, in dental pulp tissue, and in surrounding alveolar bone (B, B1). *Shh* exhibited robust expression in dental epithelium and ameloblasts (C, C1). A1, B1, and C1 present higher magnification images of the boxed areas in the panels A, B and C, respectively. P0 mouse heads were sectioned sagittally. Abbreviations: ab, alveolar bone; am, ameloblasts; dp, dental pulp; od, odontoblasts; pam, pre-ameloblasts; pod, pre-odontoblasts.

Figure 5. *Rb1*, *Runx2* and *Shh* expression in P0 mouse incisor teeth

Rb1 was detected in the dental epithelium and dental mesenchyme (A, A1, A2). *Rb1* expression appeared higher in differentiated ameloblasts as compared to undifferentiated dental epithelial cells (A2). In contrast, *Rb1* expression appeared higher in labial side undifferentiated odontoblasts, as compared to terminally differentiated lingual side odontoblasts (A1 versus A2). *Runx2* was detected in the dental papilla and pre-odontoblasts (B, B1, B2), while *Shh* mRNA was detected in labial side terminally differentiated ameloblasts (C, C1, C2). A1, B1, and C1 present higher magnification images of the lingual side cervical loop boxed area 1 in Panels A, B and C, respectively. Similarly, A2, B2, and C2 present higher magnification images of the labial side cervical loop boxed area 2 in Panels A, B and C, respectively. Pups were sectioned sagittally. Abbreviations: am, ameloblasts; la-cl, labial cervical loop; li-cl, lingual cervical loop; dp, dental pulp; od, odontoblasts.