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Specificity protein 7 is not essential for tooth morphogenesis

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Abstract

Tooth formation is a multifaceted process involving numerous interactions between oral epithelium and neural crest derived ecto-mesenchyme from morphogenesis to cytodifferentiation. The precise molecular regulator that drives the cyto-differentiation and dynamic cross-talk between the two cell types has yet to be fully understood. Runx2 along with its downstream target Sp7 are essential transcription factors for development of the mineralizing cell types. Global knockout of the *Runx2* gene results in an arrest of tooth morphogenesis at the late bud stage. Like *Runx2*, *Sp7*-null mutants exhibit peri-natal lethality and are completely devoid of alveolar bone. However, the role of Sp7 in tooth development remains elusive. Here, we report the effects of *Sp7* deletion on tooth formation. Surprisingly, tooth morphogenesis progresses normally until the mid bell stage in *Sp7*-homozygous mutants. Incisors and multi-cusped first and second molars were noted in both littermates. Thus, formation of alveolar bone is not a prerequisite for tooth morphogenesis. Tooth organs of *Sp7*-null however, were significantly smaller in size when compared to WT. Differentiation of both ameloblasts and odontoblasts was disrupted in *Sp7*-null mice. Only premature and disorganized ameloblasts and odontoblasts were noted in mutant mice. These data indicate that Sp7 is not required for tooth morphogenesis but is obligatory for the functional maturation of both ameloblasts and odontoblasts.

Keywords

Ameloblasts differentiation; gene regulation; odontoblasts; osterix; tooth development

Introduction

Tooth formation is a complex process that is driven by dynamic interactions between multiple cell lineages derived from the ecto-mesenchyme and oral ectoderm. In mice, tooth development begins at embryonic day 10 (E10) and is completed in molars during week 3 of post-natal life (1). The entire process is divided into three broad stages of initiation, morphogenesis, and cyto-differentiation. The midpoint of morphogenesis, known as the cap stage, involves the invagination and wrapping of epithelium around the condensing mesenchyme at E14. During the cap stage, mesenchymal cells differentiate and morph into the dental papilla and the dental follicle, while the epithelium forms the primary enamel knot (1–3). The final stage of morphogenesis, the bell stage, starts at E16 when the epithelium divides into an inner and outer enamel epithelium, and the stellate reticulum (1–3).

Signals from the secondary enamel knots cause folding of the inner enamel epithelium and complete surrounding of the mesenchyme by the invaginating epithelium. During the bell stage, the inner dental epithelium terminally differentiates into enamel-producing ameloblasts while the dental papilla differentiates into dentin-producing odontoblasts. The cytodifferentiation and maturation process continues through early post-natal life. The epithelium–mesenchyme cellular crosstalk is regulated by secreted morphogens of the FGF, BMP, Shh, and Wnt families. These signaling molecules bind to their specific receptors on epithelial and mesenchymal cells and activate key transcription factors to either induce or repress transcription of target genes required for tooth development (3).

Transcription factors involved in the progression of tooth morphogenesis and maturation of ameloblasts and odontoblasts include Runx2, Msx, Dlx, and Pitx (1–4). Runx2 is considered a master transcription factor required for bone and tooth development as global deletion, in mice, results in a complete failure of bone tissue formation (4, 5). The second major transcription factor Sp7, also known as Osterix, is a downstream target of Runx2. Sp7 belongs to the zinc-finger containing Sp/KLF family of transcription factors. Sp proteins recognize GC-rich sequences in target gene promoters to induce or suppress their transcription during embryonic and post-natal development (6). Sp7 is required for maturation and maintenance of functional osteoblasts. *Sp7*-null mice lack mineralized bone tissue and die shortly after birth (7). Frame shift mutations in the human *Sp7* gene are associated with osteogenesis imperfecta (8). Mutations and haploinsufficiency of *Runx2* gene in humans are linked with cleidocranial dysplasia, characterized by supernumerary teeth and delayed tooth eruption (9). Interestingly, global deletion of *Runx2* gene in mice results in arrested development of the dentition at the late bud stage (4, 5). However, specific contributions of Sp7 during different stages of tooth development and formation of ameloblasts and odontoblasts lineage remain unknown. Here, we identify the developmental effects of *Sp7* gene ablation on tooth formation. Our data suggest that Sp7 is not required for tooth morphogenesis but is essential for maturation of dentin and enamel-producing cells.

Materials and Methods

Sp7-null mouse model

The *Sp7* heterozygous (*Sp7*^{+/-}) mice were generated and maintained on a C57BL/6 background (7). In these mice, the second exon of the *Sp7* gene that encodes all functional domains of the protein was replaced with a β -galactosidase gene. The *Sp7*^{+/-} mice were interbred to obtain wild-type (WT) and *Sp7*^{-/-} littermates. Deletion of *Sp7* was confirmed by PCR genotyping and Western blot analysis (7). All mouse work was approved by the Institutional Animal Care and Use Committee at University of Alabama at Birmingham and was in compliance with federal regulations.

Histological & statistical analysis

Heads from new born WT and *Sp7*^{-/-} mutants were de-skinned and fixed in 4% PFA for 12 h at 4 °C and then processed for paraffin embedding. Heads were sectioned sagittally at 7 μ M thickness. Serial sections of WT and homozygous mutant littermates were then cleared in xylene, rehydrated, and processed for hematoxylin and eosin. Parallel slides were stained with DAPI solution (300nM DAPI in PBS) for 5 min. Images were captured with Nikon Eclipse 90i at indicated magnifications and analyzed using NIS Elements software. Tooth size measurements were taken from nine serial sections surrounding the central point of each tooth from at least two independent wild-type and homozygous littermates. Average height and width values were pooled from independent mice. The average values of maxillary and mandibular molars from wild-type were set as 100 and the percent difference in homozygous was calculated.

Results

Sp7 deficient ecto-mesenchyme undergoes normal lineage commitment

To assess *Sp7* mediated regulatory control of tooth development, we have established a colony of *Sp7*-null mice. Global deletion of *Sp7* was confirmed by PCR and Western blot analysis (data not shown). The homozygous *Sp7*^{-/-} mutant mice were uniformly dwarfed and died within minutes after birth. Failed osteogenesis in *Sp7*-null mice was confirmed by skeletal preparations from newborn littermates stained with alizarin red and alcian blue. The well developed and calcified skeletons and mineralized alveolar bone noted in WT mice was completely absent in *Sp7*-null littermates (data not shown). To evaluate tooth development, heads from newborn mice were sectioned sagittally. Surprisingly, homozygous mutants showed normal tooth number and morphogenesis (Figure 1A). These data demonstrate that alveolar bone is not a prerequisite for tooth morphogenesis. To better understand progression of cyto-differentiation, selected areas of incisors were analyzed at high magnification (Figure 1B). Age appropriate uni-cusp incisors and multi-cusped first and second molars were present in both WT and homozygous littermates. Maxillary and mandibular first molars progressed to the bell stage in both mice. However, incisors and molar tooth organs were consistently smaller in homozygous mutants. Maxillary and mandibular molars of mutants were 22% and 18% smaller when compared to WT littermates. The WT incisors clearly exhibited odontoblasts and ameloblasts that were uniformly elongated and well-polarized. Normal progression of tooth differentiation was

evident with presence of dentin, pre-dentin, and enamel layers. Interestingly, *Sp7*^{-/-} tooth organs also showed commitment of stem cells to pre-odontoblast and pre-ameloblast, and a pulp chamber. However, mesenchymal portions became cuboidal, aggregated, and completely unorganized from the middle of the tooth organ through the cuspal tip (Figure 1B). When compared with WT, the nuclei of *Sp7*^{-/-} mutant cells exhibit no alignment, polarity, or organization especially in mesenchyme layer (Figure 1C). Moreover, only a poorly formed pre-dentin-like layer was noted in incisors and molars. Thus, global deficiency of *Sp7* does not impair the ability of ecto-mesenchymal cells to commit toward the odontoblast and ameloblast cell lineages but disrupts their maturation.

Tooth development is arrested at different stages in *Runx2* and *Sp7* null mice

Failed skeletogenesis and complete absence of alveolar bone formation is observed in *Runx2* and *Sp7*-null mice. However, tooth formation is arrested at different stages in both models. To better understand differences in the disruption of tooth development by *Runx2* and *Sp7* gene ablation, we show a summary of the respective null phenotype (Figure 2). Normal progression of tooth formation that occurs in sequential stages signified by key morphological features is noted in wild-type mice. The bud stage involves the condensing of dental mesenchyme followed by the further invagination of the oral epithelium and the appearance of the primary enamel knot at the cap stage. At E18.5 the ameloblast and odontoblast layers are formed along with secondary enamel knots at the early bell stage. At birth, WT teeth possess a clear pre-dentin, dentin, and enamel layers that exhibit a concomitant thickness and mineralization relative to the size and maturity of the cells secreting these matrices (Figure 2, top panel).

Global deletion of the *Runx2* gene results in an arrest of tooth morphogenesis at the late bud to early cap stage (4, 5). This developmental arrest is characterized by a complete lack of epithelial and mesenchymal cell polarity as well as no deposition of dentin and enamel matrix (Figure 2, middle panel). In this study, we show that tooth development in *Sp7*-null is arrested at the early bell stage (Figure 1). Pre-ameloblasts and pre-odontoblasts like cell layers are established but they remain disorganized and cuboidal shaped and cells never mature (Figure 2, bottom panel). Higher magnification representations are shown at the cuspal tip of null incisors. Taken together our results reveal that although both *Runx2* and *Sp7*-null mice show failed osteogenesis, these genes have a different role in tooth formation as developmental arrest is noted at very different stages.

Discussion

Sp7, a downstream target of *Runx2*, begins to express in calvaria and perichondrium at E13.5. *Runx2* is required for the priming of chondro-progenitor cells commitment to osteoprogenitors, while *Sp7* is obligatory for carrying out the terminal differentiation towards osteoblasts. Thus, the global deletion of *Sp7* results in a complete lack of bone tissue formation caused by an arrest in the maturation of cells of chondrogenic and osteogenic lineages (7). Here, we report that during tooth formation the differentiation of the ectomesenchyme is disrupted. However, unlike *Runx2*, defects in *Sp7* mutants are noted at a much later developmental time point of bell stage.

Homozygous Runx2 mutants exhibit arrest of tooth development at the bud-cap stage. However, it remains unclear if dental phenotype in *Sp7*-null mice reflects a mere delay or total arrest of tooth development. Future studies will include the use of an *ex vivo* organ culture to help elucidate this question. Our data suggest that both Runx2 and Sp7 are obligatory for the development of dentition. Furthermore, Runx2 is required for the commitment of the cells of the ecto-mesenchyme towards ameloblasts and odontoblasts while Sp7 is essential for the maturation of both cell types in either a direct or indirect manner. Expression of *Sp7* is seen throughout the development of osteoblasts and alveolar bone yet its expression has not been thoroughly investigated in teeth (7). Recent studies of selective deletion of *Sp7* with a collagen-I Cre line identified Sp7 role in formation of cellular cementum in post-natal tooth, however Sp7 expression in the primary cells involved in dental formation at birth has yet to be examined (10). Further research is required to identify if the failed maturation of both ameloblasts and odontoblasts in *Sp7*-null mice is due to direct regulatory requirement of Sp7 or simply represent a failed epithelial–mesenchymal interaction.

Conclusions

Sp7 is not required for initial tooth morphogenesis but is essential for differentiation and maturation of both ameloblasts and odontoblast *in vivo*.

Acknowledgments

Declaration of interest

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Abbreviations

Runx2	Runt-related transcription factor 2
Sp7	Specificity protein 7
FGF	Fibroblast growth factor
BMP	Bone morphogenetic protein
Shh	Sonic hedgehog
Wnt	Wingless
Msx	Msh homeobox
Dlx	Distal-less homeobox
Pitx	Paired-like homeodomain
OD	Odontoblast
AM	Ameloblast

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Figure 1. Deletion of *Sp7* gene does not impair tooth number or morphogenesis

Deletion of *Sp7* gene does not impair tooth number or morphogenesis. Histological analysis of dental tissue from WT and *Sp7*-null mice. Heads from new born littermates were dissected, separated sagittally, fixed and embedded in paraffin. Serial sections (sagittal plane) were taken at 7 mm and stained with either H & E or DAPI to reveal cell nuclei. (A) The molars and mandibular incisor regions are shown. The first/second molars are denoted as M1 and M2. Arrows point out presence and absence of alveolar bone in wild-type and *Sp7*-null, respectively. All images were captured at 4x magnification. Scale bar; 500 μ m. (B) The middle portions of incisors in boxed area are shown at 60x magnification. The ameloblasts (AM), odontoblast (OD), pre-dentin (PD), dentin (D) and pulp (P) are indicated. Lack of dentin and disorganization reflective of failed odontoblast maturation in the *Sp7*-null tooth is identified by an asterisk. (C) Monochrome image of DAPI stained nuclei close to cervical loop (pre-mature) and adjacent to cuspal tip (mature) are shown. The disorganized and un-polarized odontoblasts lining the pulp in *Sp7*-null mice are indicated by an asterisk. All images were captured at 40x magnifications. Scale bar; 100 μ m.

Figure 2. Comparison of tooth phenotype in *Runx2* and *Sp7*-null mice

Different stages of tooth development are illustrated diagrammatically. Top panel shows normal progression of tooth formation characterized by key developmental stages at specific time points. At 13.5, mesenchyme (blue/black) condenses around the invaginating epithelium (light orange/grey). The primary enamel knot (green/black oval) appears during the cap stage followed by secondary knots at the early bell stage. Right panel depicts the status of ameloblast and odontoblast maturation located at the cuspal tip of incisors at high magnification. Mesenchymally derived odontoblasts (blue/dark grey) are elongated with processes projecting through pre-dentin (PD) and dentin (D) layers. Ameloblasts from epithelial lineage (light orange/grey) are polarized with shorter Tomes processes projecting into the enamel (E) layer. The middle row shows *Runx2* deletion causes arrested tooth development at the cap stage. Cells at the cuspal tip show a separation of cellular layers however cells are severely disorganized and do not polarize nor secrete any dentin or enamel matrix. The bottom row shows the phenotype revealed in this study. Tooth organs from *Sp7*-null progress to the early bell stage and have normal cusp number. However, higher magnification reveals cells are organized into distinct layers but the ameloblasts and

odontoblasts do not exhibit elongation or polarity. Only a poorly secreted pre-dentin-like matrix is observed in *Sp7*^{-/-} teeth.