Biochemical and Biophysical Research Communications 463 (2015) 280–284

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

# Disruption of Smad4 in odontoblasts and dental epithelial cells influences the phenotype of multiple keratocystic odontogenic tumors☆

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ARTICLE INFO

*Article history:*
Received 29 April 2015
Available online 20 May 2015

*Keywords:*
Keratocystic odontogenic tumors
Knockout mouse
Smad4
Hh signaling pathway

ABSTRACT

Keratocystic odontogenic tumors (KCOTs) are cystic epithelial neoplasms with a high recurrence rate. The molecular mechanisms underlying the initiation and progression of KCOTs are still largely unknown. Previous research showed that specific ablation of Smad4 in odontoblasts and dental epithelia resulted in spontaneous KCOTs in mice, and that constitutively activated Hedgehog (Hh) signaling was detected in the cyst epithelia of both $Smad4^{Co/Co}$ OC-Cre and $Smad4^{Co/Co}$ K5-Cre mice. Here, we ablated Smad4 in mouse odontoblasts and dental epithelia and compared the sizes and numbers of KCOTs. Both the number and size of KCOTs in $Smad4^{Co/Co}$ OC-Cre mice were larger than those in $Smad4^{Co/Co}$ K5-Cre mice, suggesting that paracrine signals from root odontoblasts play a more important role than those from Hertwig's epithelial root sheath (HERS) cells.



## 1. Introduction

Keratocystic odontogenic tumors (KCOTs) are benign uni- or multicystic, intraosseous tumors of odontogenic origin that account for 11% of all oral cysts. In contrast to other maxillary cysts, such as periodontal lateral cysts, epidermoid cysts, and radicular cysts, KCOTs stand out for their characteristic parakeratinized stratified squamous epithelial lining, high recurrence rate, and potential for aggressive, infiltrative behavior [1–4]. Human KCOTs occur either sporadically or as part of nevoid basal cell carcinoma syndrome.

In addition to KCOTs initiated by environmental factors, a subset of human KCOTs may harbor PTCH1 or PTCH2 mutations, which may lead to the derepression of Smoothened (Smo) and to constitutive Hedgehog (Hh) signaling activity [5–8]. In accordance with clinical findings, overexpression of the Hh transcriptional effector Gli2 within dental epithelium is sufficient for the induction of highly penetrant KCOTs in K5-*Gli2* transgenic mice [9]. To date, other signal transduction events that occur during KCOT initiation and progression remain largely unknown.

Smads are primary cytoplasmic signal transducers of the transforming growth factor-beta/bone morphogenic protein (TGF-β/BMP) signaling pathway, which is required for organ development and is commonly involved in disease [10]. Previous research demonstrated that the conditional deletion of Smad4 in odontoblasts and dental epithelia resulted in spontaneous KCOTs in mice, accompanied by constitutively activated Hh signaling. To more fully understand the role of Smad4-mediated TGF-β/BMP signaling in KCOT progression, we specifically deleted the Smad4 gene in odontoblasts and dental epithelia using the Cre-LoxP

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http://dx.doi.org/10.1016/j.bbrc.2015.05.051
0006-291X/

system in the well-established OC-Cre and K5-Cre transgenic strains [11]. We then compared the size and number of KCOTs that developed in these two types of mice. Our data showed that the KCOT stroma, as well as providing structural support for the cyst wall, plays an important role in the neoplastic behavior of this tumor type.

## 2. Materials and methods

### 2.1. Mouse strains and genotyping

The Smad4$^{Co/Co}$ OC-Cre and Smad4$^{Co/Co}$ K5-Cre mice were generated in the State Key Laboratory of Proteomics, Genetic Laboratory of Development and Diseases, Institute of Biotechnology, Beijing P.R. China and were described previously [11–13]. Offspring were genotyped by PCR analyses using primers described previously [12,14]. Control littermates were used in all experiments. All experimental protocols were designed according to the recommendations of the Beijing Experimental Animal Regulation Board (SYXK/JING/2005/0031).

### 2.2. Microradiological and histological studies

Mandible specimens were prepared from Smad4$^{Co/Co}$ OC-Cre and Smad4$^{Co/Co}$ K5-Cre mice (3.5 months; female). The tissue specimens were fixed in 10% neutral buffered formalin for several days, and soft X-ray radiographs were obtained in lateral projection. After decalcification in 0.4 M ethylenediamine tetraacetic acid (EDTA) solution, they were routinely embedded in paraffin wax, and 30–50 serial 3- um thick sections were prepared from the tissue blocks for histological, immunohistochemical analyses. Every fifth section was stained with hematoxylin and eosin (HE). Control specimens of mandibular bone from 15 Smad4$^{Co/Co}$ mice (3.5 months; female) were prepared and examined similarly.

### 2.3. Immunohistochemical analysis

Tissue sections were deparaffinized and immersed in methanol with 0.3% hydrogen peroxide to eliminate endogenous peroxidase activity. After treatment with normal serum for 30 min to block non-specific binding, the sections were incubated with primary antibodies at 48 °C overnight. Rabbit polyclonal antibodies were used against Patched (1:50; ab39266; Abcam, Cambridge, UK), Smo (1:50; ab113438; Abcam), and Gli-1 (1:50; ab151796; Abcam). Streptavidin–biotin–peroxidase complexes were deposited using the Histofine SAB-PO kit (Nichirei, Tokyo, Japan). Reaction products were visualized by immersing the sections for 3–5 min in 0.03% diaminobenzidine (DAB) solution containing 2 mM hydrogen peroxide. Nuclei were lightly counterstained with 1% methyl green. For control studies of the antibodies, serial sections were treated with phosphate-buffered saline, normal rabbit IgG, and normal goat IgG in place of the primary antibodies, and the sections were confirmed to be unstained.

### 2.4. Statistical analyses

Results are presented as means ± standard deviations (SD). All statistical analyses were performed using Excel software. Statistical differences were determined by Student's t test. $P$ values $\leq 0.05$ were considered statistically significant in all experiments.

## 3. Results

### 3.1. Typical KCOT phenotypes in Smad4$^{Co/Co}$ OC-Cre and Smad4$^{Co/Co}$ K5-Cre mice

Distinctive features, including origin in epithelial rests of Malassez (ERM), a stratified squamous epithelial lining, corrugated corneal layers, and an aggressive nature, strongly suggested that the cysts in mutant mice were odontogenic keratocysts. Human KCOTs have been associated with Patched gene mutations, which lead to the pathological activation of Hh signaling [5,6]. By immunohistochemical analyses, we observed remarkable upregulation of the expression of a series of Hh signaling components, including Patched, Smo, and Gli1, in the cysts of Smad4$^{Co/Co}$ K5-Cre mice (Fig. 1). Based on this evidence for dramatic activation of Hh signaling within the hyperplastic epithelium, the cysts in Smad4$^{Co/Co}$ OC-Cre and Smad4$^{Co/Co}$ K5-Cre mice were classified as typical KCOTs.

### 3.2. Histological and microradiological findings

No apparent histopathological changes in the mandible were observed in any of the 30 control Smad4$^{Co/Co}$ and Smad4$^{Co/Co}$ K5-Cre mice (Fig. 2A and C). Protrusions on the mandibles were observed in Smad4$^{Co/Co}$ OC-Cre mice (Fig. 2E).

Soft X-ray microradiographs of mandibles from all 30 Smad4$^{Co/Co}$ and Smad4$^{Co/Co}$ K5-Cre mice showed no apparent pathological changes in the alveolar or periodontal regions of incisors or molars (Fig. 2B and D). Radiographic analysis demonstrated cystic lesions within the mandible of Smad4$^{Co/Co}$ OC-Cre mice, as indicated by expansile changes and a thinning of the mandibular cortex (Fig. 2F).

### 3.3. Hematoxylin-eosin staining

Cystic lesions close to the incisors were not observed in any of the mice examined (Fig. 3A and C). Histologically, the mandibular cysts were lined by thin parakeratotic stratified squamous epithelium with capsular fibrous connective tissue and contained keratinized material (Fig. 3B and D). The cyst lining did not appear to be odontogenic epithelium but was similar to that seen in the enamel organ or dental lamina.

### 3.4. Comparison of KCOT numbers and sizes in Smad4$^{Co/Co}$ OC-Cre and Smad4$^{Co/Co}$ K5-Cre mice

We counted KCOTs in HE-stained sections from Smad4$^{Co/Co}$ OC-Cre and Smad4$^{Co/Co}$ K5-Cre mice. Solitary mandibular cysts were found in seven Smad4$^{Co/Co}$ K5-Cre mice (46.6%), while multiple mandibular cysts occurred in all Smad4$^{Co/Co}$ OC-Cre mice (100%). The mean number of KCOTs was 5.18 in 15 Smad4$^{Co/Co}$ OC-Cre mice and 1.45 in 15 Smad4$^{Co/Co}$ K5-Cre mice (Table 1). ($P$ values<0.05).

## 4. Discussion

Mutations of human PTCH genes which lead to pathological activation of Hh signaling [6–8] have frequently been found to be involved in nevoid basal cell carcinoma syndrome and in sporadic KCOTs [7,8]. However, there is no persuasive evidence indicating that the epithelium plays a significant role in KCOT formation. Increasing evidence supports the argument that the tumor microenvironment plays a major role during all phases of tumorigenesis, including initiation, progression, maintenance, and metastasis, and it may also influence therapeutic outcomes [15–17].

The KCOT stroma has been reported to promote tumor progression [18–22]. In 1975, Browne first proposed that the

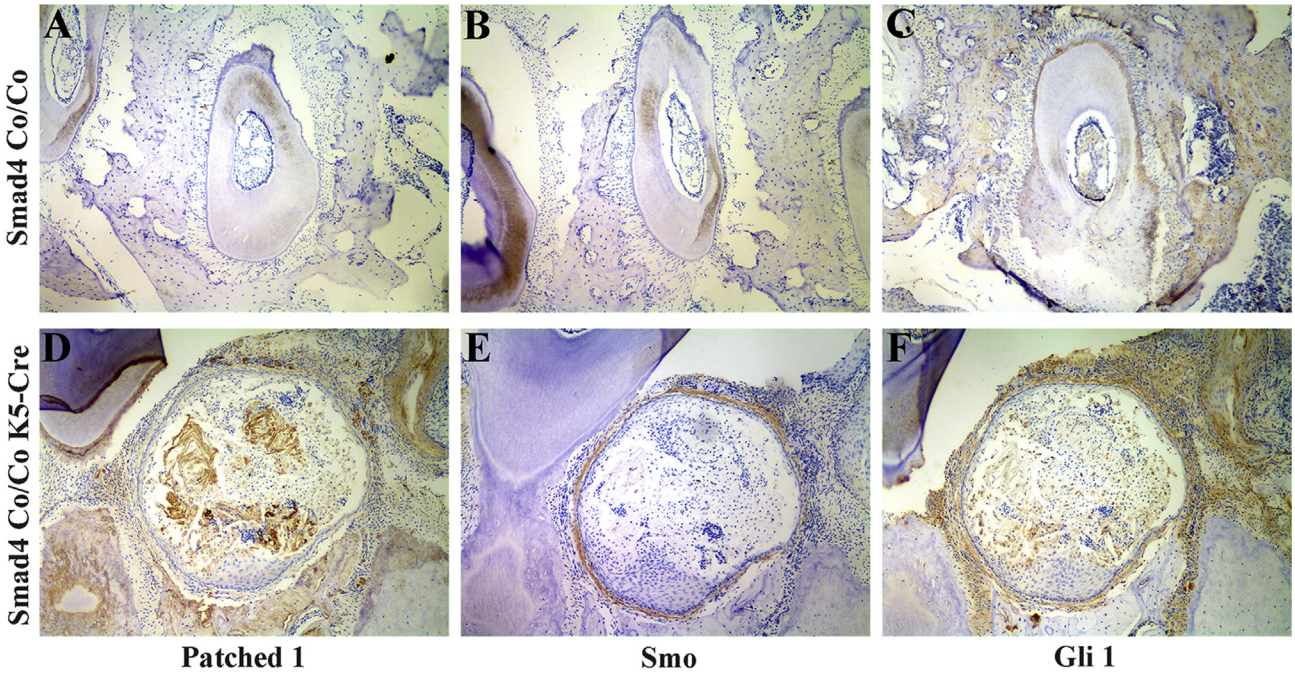


**Fig. 1.** Immunohistochemical expression of Patched 1, Smo, and Gli1 proteins in mandibular cysts of Smad4$^{Co/Co}$ K5-Cre mice and control littermates. There is no apparent staining in the control littermates (A, B and C). The remarkable upregulation of the expression a series of Hh signaling components, Patched 1, Smo, and Gli1, in the cysts of Smad4$^{Co/Co}$ K5-Cre mice (D, E and F) (magnification × 100).

connective tissue wall plays a significant role in the pathogenesis of KCOT [23]. Recently, differences in angiogenesis, collagen fibers, and in osteoclastogenic ability have been identified between the stroma of KCOTs and control tissues [18,20,24,25]. However, the mechanisms by which the stroma influences the progression of KCOT remain elusive. To investigate how mesenchymal cells affect KCOT progression, we ablated Smad4 in epithelial and ectomesenchymal tissues.

In a previous study, it was demonstrated that disruption Smad4 in odontoblasts impaired mesenchymal BMP signaling pathways, altering the fate of ERM (epithelial rests of Malassez) and leading to the onset of multiple KCOTs [26], which exhibited pathological characteristics resembling those of human KCOTs. Similar KCOTs have been confirmed in a few other mouse models. For example, 25.4% of heterozygous *Ptc* knockout mice and 95–100% of K5-*Gli2* transgenic mice developed KCOTs, which arose from mutant ERM due to activation of Hh signaling [9,27].

By immunohistochemistry, we observed remarkable upregulated expression of the Hh signaling components Patched 1, Smo, and Gli1 in the cysts of Smad4$^{Co/Co}$ K5-Cre mice (Fig. 1D, E and F).

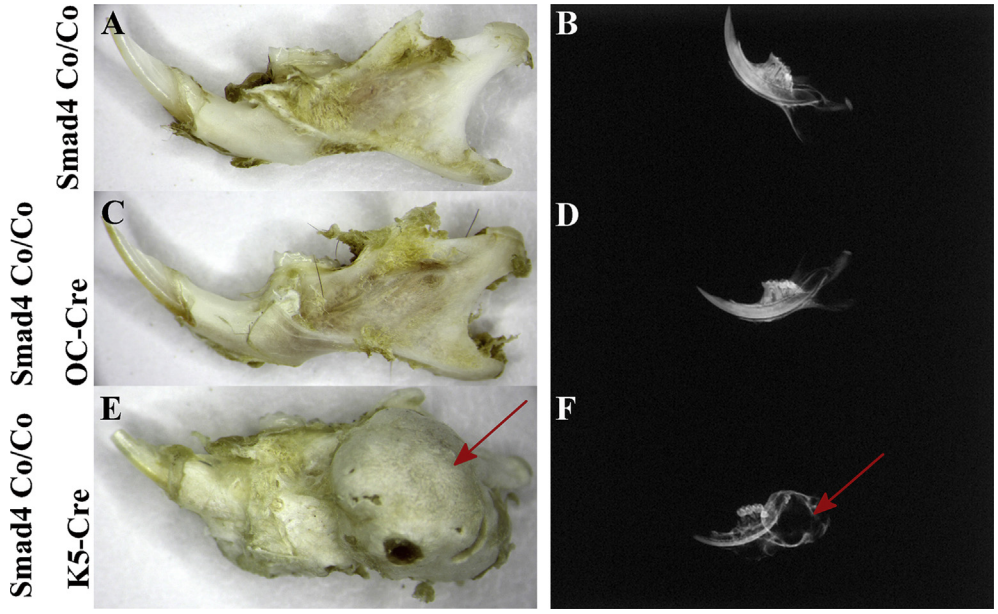


**Fig. 2.** Gross appearance (left) and microradiographic view (right) of mandibles from Smad4$^{Co/Co}$ OC-Cre, Smad4$^{Co/Co}$ K5-Cre, and Smad4$^{Co/Co}$ mice older than 3 months. Apparent pathological change cannot be seen in Smad4$^{Co/Co}$ K5-Cre and Smad4$^{Co/Co}$ mice (A, B, C and D). The large cyst was seen in Smad4$^{Co/Co}$ OC-Cre mice. The arrows point to the protrusion on the mandible of Smad4$^{Co/Co}$ OC-Cre mice (E and F).

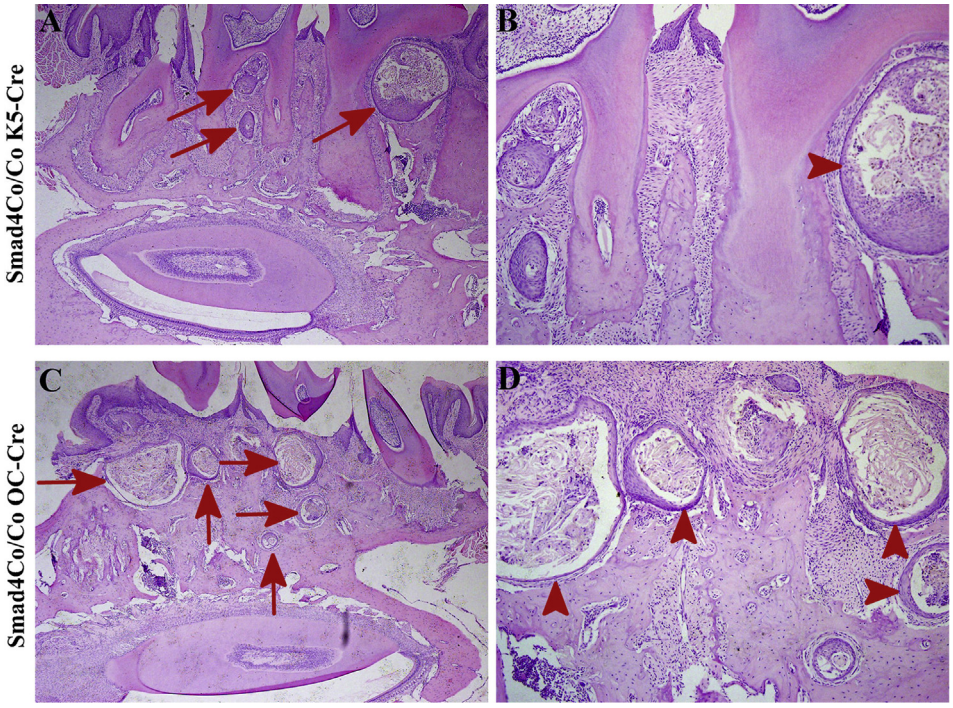


**Fig. 3.** Representative hematoxylin-eosin-stained sections of the mandible of Smad4$^{Co/Co}$ OC-Cre and Smad4$^{Co/Co}$ K5-Cre mice. The number of KCOTs in Smad4$^{Co/Co}$ OC-Cre mice was higher than that in Smad4$^{Co/Co}$ K5-Cre mice ($P$ values<0.05). The arrows indicate the KCOTs in Smad4$^{Co/Co}$ OC-Cre and Smad4$^{Co/Co}$ K5-Cre mice (A and C) with variable thickness of Epithelial cell linings (arrowheads, B and D).

**Table 1**
The number of KCOTs in Smad4$^{Co/Co}$ OC-Cre and Smad4$^{Co/Co}$ K5-Cre mice.

| | n | Mean | Standerd deviation | Minimum | Maximum | Total |
|---|---|---|---|---|---|---|
| OC-Cre/Smad4 | 15 | 4.33 | 1.72 | 3 | 6 | 65 |
| K5-Cre/Smad4 | 15 | 1.80 | 0.82 | 1 | 4 | 27 |

Immunohistochemical analyses of Smad4$^{Co/Co}$ OC-Cre mice have been reported previously by our group [26]. The evidence of mandibular cysts lined by thin parakeratotic stratified squamous epithelium with capsular fibrous connective tissue containing keratinized material (Fig. 3), as well as the reactivation of Hh signaling within the hyperplastic epithelium, indicated that the cysts in Smad4$^{Co/Co}$ K5-Cre mice were also KCOTs.

While it is widely accepted that the development of KCOT is largely due to the accumulation of somatic mutations in epithelial cells, we have demonstrated that normal ERM epithelial cells that fail to receive the appropriate paracrine signals from root odontoblasts continue to proliferate and form KCOTs. We compared the numbers and sizes of KCOTs between Smad4$^{Co/Co}$ OC-Cre and Smad4$^{Co/Co}$ K5-Cre mice. Both were higher in Smad4$^{Co/Co}$ OC-Cre mice than in Smad4$^{Co/Co}$ K5-Cre mice (Table 1 and Figs. 2, 3). These results indicated that the TGF-β pathway signaling in mesenchymal cells plays a more important role than that in epithelial cells. Additionally, the observation of identical KCOTs in Smad4$^{Co/Co}$ K5-Cre mice with Smad4-deficient ERM epithelium conclusively demonstrated that unimpeded TGF-β/BMP signaling was required to regulate the activity of Hh signaling in ERM, to direct their fates, and finally to inhibit KCOT formation. Our data provide convincing evidence of a pivotal role for ectomesenchymal tissues in the progression of KCOT.

In summary, our findings support the hypothesis that the stroma of KCOT plays an important role in the neoplastic behavior of this tumor type, in addition to providing structural support for the cyst wall. Due to the limited number of mice used, further studies using additional methods and larger numbers of mice will be required to clarify the role of the stroma in KCOT.

## Acknowledgments

This work was supported by the National Natural Science Foundation of China (grant numbers 81030018, 30872900, 81241062, 81200762), the National Basic Research Program of China (2012CB966904), the Doctoral Fund of Ministry of Education of China (grant number 20120001110043), and the Science Foundation of the Third Dental Center, Peking University School and Hospital of Stomatology (grant numbers 011401). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

## Conflict of interest

The authors declare that there is no conflict of interest in this work.

## Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.05.051.

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