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$Sox2^+$ Stem and Progenitor Cells in Tooth Renewal and Odontogenic Tumors

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Sox2^{+} stem and progenitor cells in tooth renewal and odontogenic tumors

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ACADEMIC DISSERTATION

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Abbreviations

ABCG2	ATP-binding cassette sub-family G member 2
APC	Adenomatous polyposis coli
Bcl11b	B-Cell CLL/Lymphoma 11B
Bmi1	BMI1 polycomb ring finger oncogene
Bmp	Bone morphogenetic protein
BrdU	5-bromo-2-deoxyuridine
cKO	Conditional knockout
CL	Cervical loop
Dkk1	Dickkopf WNT signalling pathway inhibitor 1
Dlx	Vertebrate homologue of Drosophila distal-less (Dll) gene
E	Embryonic day
Eda	Ectodysplasin
Edar	Receptor for ectodysplasin
Edaradd	Edar-associated adapter protein
EDTA	Ethylenediaminetetraacetic acid
ERM	Epithelial cell rests of Malassez
ES cell	Embryonic stem cell
FAP	Familial adenomatous polyposis
Fgf	Fibroblast growth factor
Fgfr	Fibroblast growth factor receptor
GFP	Green fluorescent protein
Gli1	GLI Family Zinc Finger 1
Gsk3ß	Glycogen synthase kinase-3 beta
H2B	Histone 2B
Hh	Hedgehog
HERS	Hertwig's epithelial root sheath
HMG	High-mobility-group
ICM	Inner cell mass
Igfbp5	Insulin-like growth factor binding protein 5
IEE	Inner enamel epithelium
iPS cell	Induced pluripotent stem cell
Klf4	Kruppel-like factor 4
Ldb1	LIM domain binding 1
Lef1	Lymphoid enhancer-binding factor 1
Lgr5	Leucine rich repeat containing G-protein coupled receptor 5
Lhx	LIM homeobox
LNA	Locked Nucleic Acid
LRC	Label-retaining cell
miRNA	MicroRNA
Msx	Vertebrate homologue of Drosophila muscle (Msh) segment gene
K14	Keratin 14
Oct4	Octamer-binding transcription factor 4
OEE	Outer enamel epithelium

P	Post natal day
Pax9	Paired box gene 9
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
Pitx	Paired-like homeodomain
RT-PCR	Reverse transcription polymerase chain reaction
Runx*2*	Runt domain transcription factor 2
Sfrp5	Secreted frizzled-related protein 5
SI	Stratum intermedium
Shh	Sonic hedgehog
Sox	Sry related HMG-box
Spry	Sprouty
SR	Stellate reticulum
Sry	Sex-determining region Y
TA	Transit amplifying
Tbx1	T-Box 1
Tgfß	Transforming growth factor beta
Tnf	Tumor necrosis factor
Wnt	Vertebrate homologue of the *Drosophila* Wingless gene
YAP	Yes-associated protein

Gene names are written in *italics* and protein names in roman. Mouse genes or proteins are in lower case letters and human in upper case letters.

List of original publications

This thesis is based on original publications, which in the text are referred to by their Roman numerals.

I Juuri E, Saito K, Ahtiainen L, Seidel K, Tummers M, Hochedlinger K, Klein OD, Thesleff I, Michon F (2012). Sox2+ stem cells contribute to all epithelial lineages of the tooth via Sfrp5+ progenitors. *DevCell* 23, 317-328.

II Juuri E*, Jussila M*, Seidel K, Holmes S, Wu P, Richman J, Heikinheimo K, Chuong C-M, Arnold K, Hochedlinger K, Klein O, Michon F, Thesleff I (2013). Sox2 marks epithelial competence to generate teeth in mammals and reptiles. *Development* 140, 1424-1432.

III Juuri E, Isaksson S, Jussila M, Heikinheimo K, Thesleff I (2013) Expression of the stem cell marker, SOX2, in ameloblastoma and dental epithelium. *Eur J Oral Sci,*121, 509-516.

*Authors contributed equally to the work.

Abstract

Teeth are an important model for the study of the development and renewal of ectodermal organs. Tooth renewal capacity varies greatly among species. Several modes to renew teeth have been evolved and common to all of them is the requirement of stem cells. Putative stem cells have been identified both in the rodent incisor, which grows and renews continuously, and in reptiles, which continuously replace their teeth. However, molecular markers to identify these stem cells are lacking. Stem cells are also associated with tumor formation. Odontogenic tumors are thought to derive from tissues related to the developing tooth germ but the role of stem cells in tumor pathogenesis is unknown.

In this thesis, a specific marker for epithelial stem cells in the continuously growing mouse incisor was sought and its association in the dental epithelium with continuous tooth formation, tooth replacement, and odontogenic tumor formation was investigated. By transcriptome microarray analysis, *in situ* hybridization, and genetic lineage tracing the transcription factor Sox2 was identified as a specific marker for the incisor epithelial stem cells. Further, *Sox2*$^{+}$ stem cells were shown to give rise to all epithelial cell lineages of the mouse incisor. As evidenced by *in vitro* inhibition assays and transgenic mouse models, *Sox2* expression is regulated by Fgfs and miRNAs. Analysis of the expression pattern of Sox2 during two different modes of successional tooth formation, 1) tooth replacement in human, ferret, and five reptilian species, and 2) distal molar addition in mouse and ferret, revealed that Sox2 marks the epithelial competence to generate teeth in both reptiles and mammals. Finally, immunostaining revealed that SOX2 is expressed in ameloblastoma, an odontogenic tumor, and that Sox2 expression persists in the dental lamina fragments of the third molar.

The findings indicate that Sox2-expressing stem and progenitor cells in the dental epithelium have the capacity to generate epithelial dental tissues in different modes of tooth renewal. Understanding the cellular and molecular mechanisms underlying the self-renewal and differentiation of dental epithelial stem and progenitor cells is essential for developing novel tooth regenerative therapies and unraveling the pathogenesis of odontogenic tumors.

1 Introduction

Organ renewal depends on multipotent stem cells that produce a constant supply of differentiating progeny. A major focus of developmental biology and regenerative medicine is the identification and characterization of these stem cells. The capacity for tooth renewal varies greatly across species. In general, it has decreased during evolution as the complexity of tooth shapes has increased: the simpler teeth of lower vertebrates such as fish and reptiles are continuously replaced whereas the morphologically more complex teeth of mammals display limited renewal capacity (Jernvall and Thesleff, 2012). The species in which teeth are continuously renewed by replacement or continuous growth offer valuable model systems to study the mechanisms of tooth renewal and the dental stem cells.

In humans, like in most mammals, the renewal capacity is limited to replacement of deciduous with permanent teeth. Rodent dentition is reduced in number due to specialized diet and is not replaced. Instead, rodent dentition is renewed by continuous growth to counteract the abrasive nature of their diet. In mice, incisors are able to grow continuously due to stem cells, which have been identified in the apical end of the incisor by their label-retaining property (Harada et al., 1999). The incisor epithelial stem cells reside in a niche that bears anatomical similarities to the well-studied epithelial stem cell niches of hair and intestine. However, examination of the incisor stem cells has been hindered by the lack of specific molecular markers allowing their localization and isolation. Label-retaining putative epithelial stem cells have also been localized in the dental epithelium in species with continuous tooth replacement such as in gecko, a reptilian (Handrigan et al., 2010). The capacity for tooth replacement is suggested to reside in the stem cells of dental epithelium associated with existing teeth (Smith et al., 2009).

The regulation of stem cells is crucial for tissue homeostasis and function. Disturbances in regulation may lead to excessive cell proliferation, and many tumors are originated and/or maintained by malfunctioning stem cells (Clevers, 2011). Stem cell markers are expressed in many tumor cells and they are involved in several steps of tumor formation (Klonisch et al., 2008). In odontogenic tumors, the formation of dental tissues is continuous, and they are believed to derive from cells associated with developing teeth (Philipsen and Reichart, 2004). The contribution of dental stem cells to the generation of odontogenic tumors is not known.

In this thesis, I present experimental findings that led to the identification of Sox2 as a specific marker for epithelial stem cells in continuously growing mouse incisors. Subsequently, these data led to the demonstration that Sox2 associates with tooth initiation and replacement as well as with ameloblastoma, an odontogenic tumor.

2 Review of the literature

2.1 Stem cells

Stem cells are defined as undifferentiated cells that are able to self-renew, divide without limit, and produce numerous differentiated progeny life long (Potten and Loeffler, 1990). Stem cells can be categorized based on their capacity to produce differentiated cell lineages. In the first stages of development, the embryo is composed of *totipotent* cells that have the potential to form all intra- and extraembyonic tissues. The embryonic cells that are capable of producing all germ layers of the embryo proper and cell types of mature tissues are called *pluripotent*. During development, the pluripotent cells go through multiple steps of cell fate determinations that narrow down their lineage possibilities. In adult, tissue-specific stem cells retain the capacity to self-renew: *unipotent* stem cells give rise to one and *multipotent* stem cells to several differentiated tissue-specific cell lineages (Jaenisch and Young, 2008).

Cells gradually lose their stem cell characteristics as they differentiate, and differentiation is normally a one-way process as specialized cells lose their stemness permanently. It is, however, possible to artificially induce pluripotency in adult somatic cells by defined factors (Takahashi and Yamanaka, 2006). These induced pluripotent stem (iPS) cells are in many ways similar to pluripotent embryonic stem (ES) cells that can be isolated from an early embryo.

2.1.1 Embryonic stem cells

After fertilization and the following cleavage divisions of the zygote, cells are committed to follow either intra or extraembryonic cell lineages. The outer layer of the early embryo gives rise to the trophoectoderm that forms the extraembryonic tissues, such as the placenta, and the inner cell population gives rise to the inner cell mass (ICM) that forms the embryo proper. Pluripotent embryonic stem (ES) cells can be derived from the ICM of a blastocyst stage embryo (Martin, 1981; Evans and Kaufman, 1981). They are plastic and have potentially unlimited capacity for self-renewal (Suda et al., 1987). ES cells express a conserved set of specific transcription factors, including *Oct4*, *Nanog*, and *Sox2*, that are associated with pluripotency and that positively regulate their own expression and repress the expression of genes that promote differentiation (Boyer et al., 2005; Wang et al., 2012). By introducing specific factors to ES cells, it is possible to induce them to differentiate towards distinct cell lineages such as cardiomyocytes, pancreatic beta cells, intestinal cells, liver cells, and nerve cells (Phillips et al., 2007; Hay et al., 2008; Chambers et al., 2009; Soong et al., 2012; Ogaki et al., 2013). The possibility to transplant differentiated cells, cultured from human ES cells, to replace lost or diseased cells in various organs is of great interest for medical applications.

2.1.2 Induced pluripotent stem (iPS) cells

The risk of rejection by the immune system and the ethical issues related to the use of human ES cells have motivated the search for different methods to obtain autologous pluripotent cells from adult tissues. A major breakthrough in the stem cell field was the discovery by Takahashi and Yamanaka (2006; 2007) to reprogram mouse and human fibroblasts into iPS cells by introducing transcription factors *Oct4*, *Klf4*, *c-Myc*, and *Sox2* into the fibroblasts. It is also possible to reprogram other cell types and more efficient reprogramming techniques utilizing for example miRNA technology have been developed (Robinton and Daley, 2012). This is a major advance

for bioengineering protocols since cells for clinical transplantations could be derived from iPS cells generated from the patient's own cells. At the moment, the first clinical study on iPS cell therapy is set up to treat macular degeneration in Japan (Cyranoski, 2013). The reprogrammed cells may, however, be more prone to genomic instability than ES cells and further research on the safety issues and characterization of iPS cells is required before stem cell therapy applications can be widely used in clinics (Robinton and Daley, 2012).

2.1.3 Organ renewal and tissue-specific adult stem cells

A small reserve of multipotent stem cells in possibly all adult tissues maintains tissue homeostasis by replenishing the cells lost or damaged by daily use, injury or disease. The rapidly renewing epidermis, intestine, and hematopoietic system are well-characterized examples of adult tissues that exist naturally in a state of dynamic flux and continually give rise to new cells with relatively short life to maintain proper function (Watt and Jensen, 2009; Wang and Wagers, 2011; Barker, 2014).

Adult stem cells are able to self-renew, meaning that the final outcome of the cell divisions keeps the pool of stem cells relatively unchanged. Different models for self-renewal have been proposed (Figure 1). In the invariant asymmetry model, stem cells divide into a stem cell identical to the original cell as well as a progenitor cell that differentiates (Klein and Simons, 2011). Nevertheless, recent evidence suggests that stem cells in some tissues follow the neutral drift model in which all stem cells have equal proliferative potential and can divide either by asymmetric or symmetric cell divisions. Some stem cells are lost in a stochastic manner and others multiply to replace them (Klein and Simons, 2011). If the balance of the homeostasis is perturbed, it may lead to pathological consequences such as leukemia, in which the abnormal increase in blood cell numbers is due to dysregulation of hematopoietic stem cell self-renewal (Rizo et al., 2006).

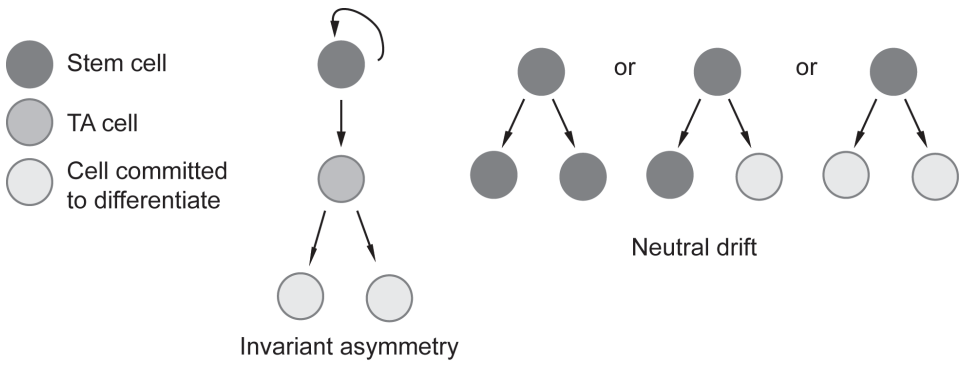

Figure 1. The invariant asymmetry and the neutral drift models for stem cell self-renewal. In the invariant asymmetry model, every division of a stem cell is asymmetric leading to a daughter cell, which maintains the stem cell identity, and to a transient amplifying daughter cell, which gives rise to two differentiating cells. In the neutral drift model, each stem cell has three possible division outcomes with equal probability: a stem cell can give rise to 1) two stem cells, 2) a stem cell and a differentiating cell, or 3) two differentiating cells. TA, transient amplifying cell

Adult stem cells are located in specific anatomical locations, called niches, that create a unique microenvironment for them (Hsu and Fuchs, 2012). For example, skin stem cells reside in the basal layer in the interfollicular epidermis (Blanpain and Fuchs, 2009), stem cells responsible for the hair growth in the bulge of the hair follicle (Cotsarelis et al., 1990; Tumbar et al., 2004), intestinal stem cells in the crypt bottom of the intestine (Barker et al., 2007), and hematopoietic stem cells in the bone marrow (Spangrude et al., 1988; Wang and Wagers, 2011). The niche regulates stem cell activity and influences the cell fate decision through the release of extrinsic signals including paracrine signals from local sources, structural constraints of the space in the niche, physical interactions with the basement membrane, metabolic products, and neural inputs (Scadden, 2006). The neighboring cells of the niche and the stem cell progeny also give inputs to the stem cells (Hsu and Fuchs, 2012). For example, the *Lgr5*$^{+}$ intestinal stem cells are maintained adjacent to the Paneth cells and require Wnt signaling (Clevers, 2013). Details of the structural organization of niches and the mechanisms of how they function to regulate stem cell proliferation and differentiation vary greatly among different organs (Snippert and Clevers, 2011). In each case, understanding how the niche elements work together is essential to elucidate the process of stem cell fate determination.

In mammals, adult stem cells are able to produce only the cell types of the tissue in which they reside, whereas in some non-mammalian species the stem cells are more plastic. An extreme case of cell plasticity is present in the axolotl, a type of salamander, which is capable of regenerating many parts of its body, such as limbs, and their original complex structure even after major injury. This is due to the formation of a structure called blastema upon injury. The blastema contains a specific set of mesenchymal stem cells in which the organ structure is reprogrammed allowing the exact regeneration of the missing structures (McCusker and Gardiner, 2013). In mammals, the regeneration capacity of the limb is limited to the tips of digits and is based on nail stem cells that orchestrate the digit regeneration (Takeo et al., 2013).

2.1.4 Identification of adult stem cells

The identification of tissue-specific stem cells has been complicated because they are sparse and have different identities in each tissue or organ. Specific markers would allow the identification of stem cells within a given tissue, enabling their further characterization. However, as the known stem cell markers identify only one or few types of stem cells (Barker et al., 2007; Jaks et al., 2008; Barker et al., 2010), there is no general stem cell marker for the adult stem cells. Functional assays such as label-retaining techniques, genetic lineage tracing, and clonogenic and transplantation assays can be utilized for the identification of stem cells.

The slow division rate of stem cells has been used to identify them in different tissues. In classical label retention techniques, DNA analogues such as BrdU are incorporated into the cells. The label is diluted in rapidly dividing cells but retained in the slowly dividing cells. After prolonged chase periods this enables the visualization of the quiescent cells in fixed and permeabilized tissues (Cotsarelis et al., 1990). The label retention, however, reveals all cells that are dividing slowly whether they are stem cells or not. New transgenic approaches such as H2B-GFP method has emerged to fluorescently label and track slowly cycling cells *in vivo* (Tumbar et al., 2004). In H2B-GFP method, GFP linked to histone H2B is constitutively expressed under the control of a regulatory element, and after purposed termination of H2B-GFP expression, retained GFP can be visualized in slowly-dividing cells (Tumbar et al., 2004). The advantage of this method over traditional label retention is that it allows the isolation of live cells and their further characterization for stemness.

The genetic lineage tracing technique allows the visualization of stem-cell dynamics *in vivo*. This technique requires the identification of a stem cell-specific marker gene candidate and is based on Cre-ER-loxP system in mice that include a drug-inducible Cre recombinase together with a reporter system (Figure 2). By placing Cre recombinase under the control of a gene-specific promoter, the transient activation of Cre recombinase leads to the excision of a stop cassette in targeted cells and the permanent expression of a reporter construct in the targeted cells and their descendants. This technique has revealed the self-renewal capacity, multipotential nature, and growth kinetics of many stem cell populations for instance in the skin and intestine (Morris et al., 2004; Barker et al., 2007). Furthermore, multicolored reporters have been developed and can be used to study the individual behavior of stem cells within a niche (Snippert et al., 2010).

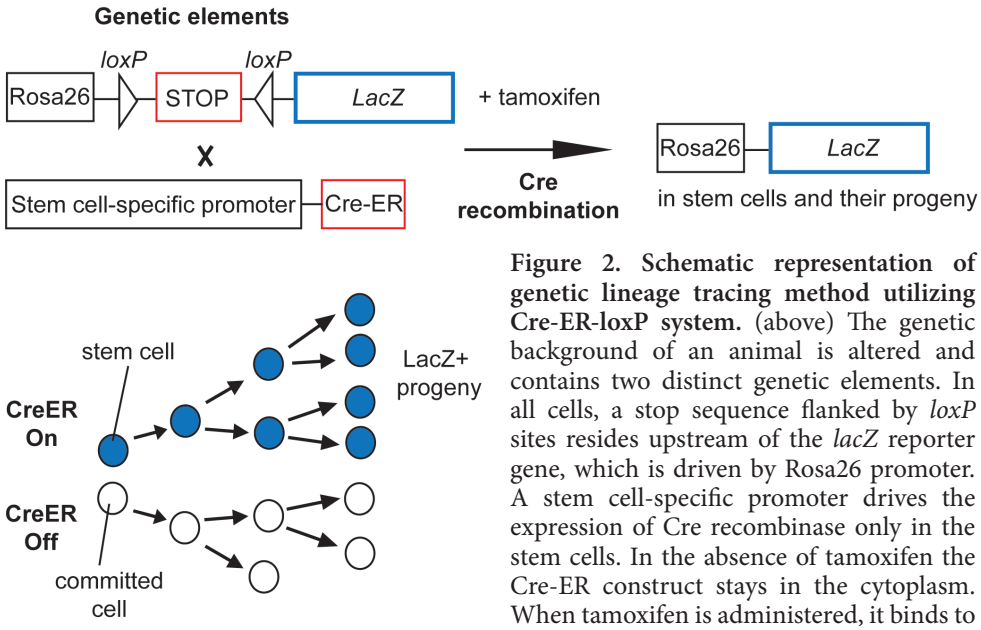

Figure 2. Schematic representation of genetic lineage tracing method utilizing Cre-ER-loxP system. (above) The genetic background of an animal is altered and contains two distinct genetic elements. In all cells, a stop sequence flanked by *loxP* sites resides upstream of the *lacZ* reporter gene, which is driven by Rosa26 promoter. A stem cell-specific promoter drives the expression of Cre recombinase only in the stem cells. In the absence of tamoxifen the Cre-ER construct stays in the cytoplasm. When tamoxifen is administered, it binds to the ER domain of the Cre-ER fusion protein and the CRE recombinase translocates into the nucleus and removes the stop sequence upstream of the *lacZ* reporter gene. After this, there is a permanent expression of LacZ in the stem cells and their progeny driven by Rosa26 promoter. (below) All progeny of the labeled stem cell will be LacZ-positive.

In vitro clonogenic assays, where single cells are cultured and assessed for their capacity to self-renew and differentiate, can also be used to identify stem cells. In addition, by culturing single, isolated stem cells in 3D culture placed in Matrigel, a gelatinous protein matrix, the potential of stem cells can be tested. For example, a single *Lgr5*$^{+}$ intestinal stem cell gives rise to a mini-gut in which all the characteristics of the normal intestinal epithelium are present (Sato et al., 2009). Moreover, transplantation assays where putative stem cells are transplanted to recipient mouse are used to measure the self-renewal capacity and the multipotent nature of the cells (Beck and Blanpain, 2013).

A combination of different functional assays is required for the identification of tissue-specific stem cells and for the analysis of their self-renewal capacity and multipotency. In addition to functional assays the identification of specific molecular markers for stem cells is

essential for their detailed characterization. The expression of pluripotency markers *Oct4*, *Sox2*, and *Nanog* defines the embryonic stem cells and the iPS cells (Jaenisch and Young, 2008), but it is not known if adult stem cells possess a molecular signature that is common to all adult stem cell populations. However, some markers, such as *Lgr5*, can identify different stem cell populations in the digestive tract (Barker et al., 2007; Barker et al., 2010) and in the epidermis (Jaks et al., 2008). The marker profiles discovered for certain adult stem cell populations are extremely variable and may indicate that the self-renewal and multipotency in different adult stem cells are maintained by a variety of mechanisms (Fortunel et al., 2003).

2.1.5 Dental stem cells and tooth bioengineering

The realization of the potential of stem cells for organ bioengineering has also raised interest for clinical applications in dentistry. Although the damaged, diseased, congenitally missing, or lost teeth can be replaced by osseo-integrated dental implants made of artificial materials, fully functioning tooth transplants would offer many benefits over current techniques. Bioengineering of a complete human tooth would require a source of stem cells and a detailed recipe to instruct them towards proper lineages.

Dental stem cells have been isolated from a variety of adult sources (Gronthos et al., 2000; Miura et al., 2003; Sonoyama et al., 2006; Sonoyama et al., 2008). For instance, human mesenchymal dental stem cells have been isolated from the dental pulp of third molars or exfoliated deciduous teeth (Gronthos et al., 2000; Miura et al., 2003), and epithelial stem cells have been successfully isolated from the roots of porcine teeth (Xiong et al., 2012). Yet, these stem cells may not have the potential to form a complete tooth, and in addition, harvesting them from the patient's teeth would present a serious problem. However, cells from embryonic mouse molars were used to build a tooth with its periodontal ligament and proper bone integration (Ikeda et al., 2009; Oshima et al., 2011). Here, the epithelial and mesenchymal cells were disintegrated, recombined, cultured *in vitro* and subsequently transplanted into an adult jaw (Ikeda et al., 2009). Recently, other tissues have been built from ES cells by directed differentiation in 3D culture, such as a multilayered cortex, optic cup with retinal tissue, and functional pituitary (Sasai, 2013). Moreover, human iPS cells were programmed to form hepatic endoderm cells by directed differentiation and these cells were grown *in vitro* to form liver buds. The buds were transplanted into mice and formed vascularized and functional liver tissue (Takebe et al., 2013).

In the case of tooth, there is a method for successful bioengineering from isolated cells, but so far, proper cell populations that have the odontogenic potential to form teeth are lacking. Induced pluripotent stem cells could be used for tooth bioengineering, if one knew how the odontogenic potential is established in the tooth forming cells in a physiological environment to trigger the tooth to develop. The continuously growing mouse incisor (see 2.4.1) is, at the moment, the best model to study *in vivo* the molecular cues necessary for dental stem cell maintenance and differentiation.

2.1.6 Cancer stem cells

In addition to the crucial role of stem cells in embryonic development and adult tissue homeostasis, their powerful mitotic properties may potentially participate in tumor formation. Tumors are either benign or malignant, and the malignant ones that grow without control and generate metastases are called cancer. Decades ago it was realized that the cells that form the tumor are not identical and that some tumor cells are more differentiated than others (Heppner,

1984). Moreover, a small proportion of undifferentiated tumor cells seems to posses stem cell-like characteristics, such as long-term renewal potential, the ability to produce different types of tumor cells, and to initiate new tumors in transplantation experiments (Pierce and Speers, 1988).

These findings led to the establishment of the cancer stem cell concept (Sell, 2010). The cancer stem cells are thought to possess characteristics of normal stem cells. The tumors also show hierarchical organization of cells resembling the cell hierarchy in normal tissue homeostasis with the exception that the transit-amplifying cells in the tumor do not undergo normal cell differentiation but continue to proliferate (Sell, 2010).

Different models for tumor growth have been proposed, and the cancer stem cell concept appears to fit these theories (Clevers, 2011). Functional assays have recently been assessed in studies concerning cancer stem cells. Several studies utilizing genetic lineage tracing in different mouse models have demonstrated the existence of cancer stem cells in solid tumors (Beck and Blanpain, 2013). For instance, genetic lineage tracing in the intestine showed that adenomas of the intestinal epithelium originate from the *Lgr5*$^{+}$ intestinal stem cells (Barker et al., 2009).

2.2 Transcription factor Sox2

Transcription factors of the Sox (Sry related HMG-box) family play widespread roles during development and regulate stem and progenitor cell fate decisions and differentiation (Sarkar and Hochedlinger, 2013). Sox proteins bind to their target DNA as complexes with other transcription factors, are regulated at multiple levels, and act in a dosage-dependent manner (Kamachi and Kondoh, 2013). So far, twenty *Sox* genes sharing a highly conserved HMG (high-mobility-group) DNA domain have been identified (Schepers et al., 2002). The HMG domain mediates DNA binding and was originally identified in the mammalian testis-determining factor *Sry* (Sex-determining region Y) gene (Sinclair et al., 1990). Based on the similarities in the HMG domain, *Sox* genes are divided into subgroups A to H. The members of the same subgroup share similar biochemical properties and have overlapping and redundant functions (Sarkar and Hochedlinger, 2013).

Sox2 is a member of SoxB1 family (Schepers et al., 2002). It plays key roles in the context of stem cell biology in many stages of mammalian development, in adult tissue homeostasis, and in pathological situations. During embryogenesis, Sox2 is expressed in the morula cells of the early embryo and it is required to maintain the pluripotent stage of the cells (Avilion et al., 2003). In fetal development, Sox2 regulates the lineage specification, morphogenesis, proliferation, and differentiation of cells in various tissues. *Sox2*$^{+}$ fetal progenitors are identified in all germ layers, endoderm, ectoderm, and mesoderm, as well as in the primordial germ cells (Sarkar and Hochedlinger, 2013). Sox2 influences cell fates by acting in a dosage-dependent manner by inhibiting transcription factors that specify alternative cell lineages during organogenesis (Kamachi and Kondoh, 2013). For instance, in the ectoderm, Sox2 promotes the neuroectodermal fate by suppressing the regulators that promote the mesoendodermal fate (Wang et al., 2012), thereby driving the cell fates towards neural specification (Pevny and Nicolis, 2010). In later development of central and peripheral nervous system, Sox2 promotes proliferation and differentiation of a variety of progenitor populations together with other SoxB1 factors (Kamachi and Kondoh, 2013).

In the adult, Sox2 is expressed in adult stem cells or early progenitor cells in various self-renewing epithelial tissues, for example in skin, trachea, stomach, brain, and testis (Suh et al., 2007; Arnold et al., 2011). Sox2 is required for tissue homeostasis as shown by functional studies

in the brain and trachea (Ferri et al., 2004; Que et al., 2009). Conditional deletion of *Sox2* in the neural progenitor cells leads to neurodegeneration and impaired neurogenesis (Ferri et al., 2004), and the deletion of *Sox2* in tracheal epithelium has revealed that Sox2 sustains homeostasis by regulating the number and type of the progenitor cells (Que et al., 2009).

In addition to the numerous roles of Sox2 in development and maintenance of adult tissue homeostasis, recent studies show that dysregulation of Sox2 may promote tumor growth and function at various levels of carcinogenesis. The *SOX2* locus is amplified in human squamous cell carcinomas of the lung and esophagus as well as in human small cell lung cancers, suggesting that *Sox2* acts as an oncogene (Liu et al., 2013). Sox2 is involved in various physiological activities related to cancer cells such as proliferation and growth, cellular invasion, and formation of metastasis (Liu et al., 2013). Sox2 also has an important role in maintaining the self-renewal of tumor-initiating cells in osteosarcomas (Basu-Roy et al., 2011).

Sox2 expression is regulated and modulated extra- and intracellularly (Sarkar and Hochedlinger, 2013). Extracellular signals belonging to major signaling pathways can positively or negatively control Sox2 expression levels (Sarkar and Hochedlinger, 2013). For instance, Sox2 expression is induced by Wnt signaling in the developing taste bud cells (Okubo et al., 2006), whereas overexpression of Sox2 in osteoblasts leads to inhibition of canonical Wnt signaling (Mansukhani et al., 2005). Sox2 also suppresses osteoblast differentiation by targeting *APC* and *GSK3ß* genes, which are negative regulators of the Wnt pathway (Seo et al., 2011). Moreover, Fgf signaling is associated with Sox2 regulation: Fgf signaling negatively regulates Sox2 during foregut development (Que et al., 2007), whereas in osteoblast progenitors, Fgf signaling positively regulates Sox2 (Mansukhani et al., 2005).

2.3 Tooth development

Teeth are ectodermal organs among hair, scales, feathers, beaks, and exocrine glands such as mammary and sweat glands. The development of all ectodermal organs is very similar in the early phases and sequential epithelial-mesenchymal interactions regulate their formation. These interactions are mediated by conserved signaling pathways, which regulate the expression of numerous genes. Among the identified pathways are Hedgehog (Hh), Wnt, Fibroblast growth factor (Fgf), Transforming growth factor beta (Tgfß), Bone morphogenetic protein (Bmp), and Ectodysplasin (Eda). Although most knowledge regarding tooth development and its cellular and molecular mechanisms comes from studies on mice, much of this knowledge is applicable to other species, such as human, as well as to other organs.

2.3.1 Developmental anatomy

The developmental anatomy of teeth described here concerns mammalian teeth that have roots. Although the teeth show extreme diversity across species, the basic steps of tooth morphogenesis are similar in all vertebrates (Jernvall and Thesleff, 2012). Teeth start to form very early during fetal development. In human, the formation of primary teeth begins in the 6th-8th gestational week and that of the permanent teeth in the 20th week (Ten Cate et al., 2003). In mice, the first sign of tooth formation is observed at embryonic day 11 (E11).

The dental epithelium is derived from the ectoderm of the first branchial arch and the frontonasal prominence. The mesenchyme is derived from the cranial neural crest cells, which arise from the margins of the neuro-epithelium and migrate to the facial prominences (Noden and Schneider, 2006).

The dental lamina (also known as odontogenic band) is the first morphological sign of tooth development and it appears as a horseshoe-shaped thickening of the oral epithelium. The development of individual teeth begins as the placodes appear in the dental lamina. This tooth-forming epithelium undergoes morphological changes that can be divided into stages (initiation, bud, cap, and bell) named according to their morphological appearance (Figure 3).

In the initiation stage, the placode invaginates into the underlying mesenchyme and forms an epithelial bud. In the following bud stage, the mesenchyme condenses around the epithelial bud and forms the dental mesenchyme (for review, see Thesleff and Tummers, 2009). The primary enamel knot, which is a cluster of non-dividing epithelial cells, forms in the tip of the bud and serves as signaling center, expressing multiple signals that belong to the conserved signaling molecule families (Jernvall et al., 1994; Thesleff, 2003). The epithelium flanking the enamel knot continues to proliferate and grows downwards leading to a cap-like appearance of the tooth germ.

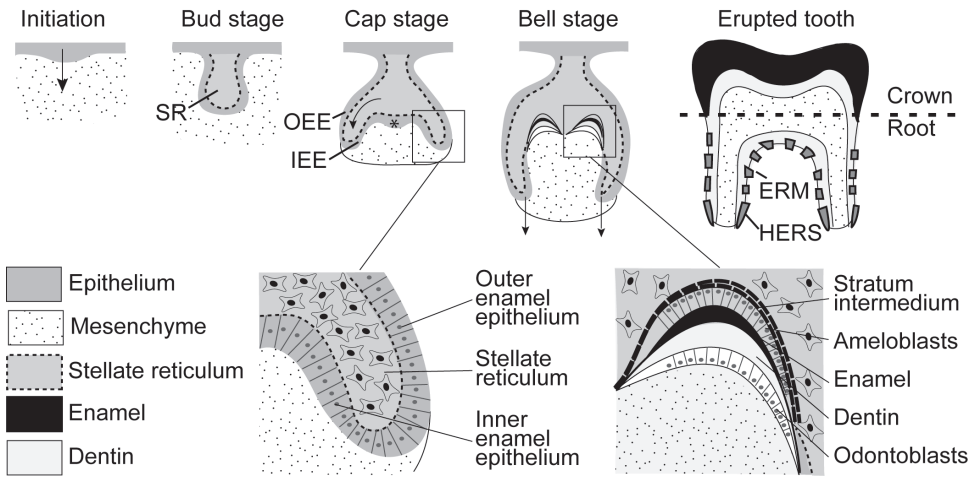

Figure 3. Schematic presentation of mammalian tooth development. The epithelium induces the mesenhcyme (arrow) to form the dental mesenchyme. During the bud stage, the epithelium invaginates into the mesenchyme, and is composed of the basal epithelium and stellate reticulum cells (SR). In the cap stage, the enamel knot (*) forms, and inner and outer enamel epithelia (IEE and OEE) form cervical loops, which grow down in the mesenchyme (arrow). During the bell stage, ameloblasts differentiate from the inner enamel epithelium and odontoblasts from the dental mesenchyme. The stratum intermedium layer differentiates from the SR next to the ameloblast layer. Deposition of the extracellular matrices of dentin and enamel begin from cusp tips. Cervical loops form Hertwig's epithelial root sheath (HERS) that guides the root development (arrows). When the tooth erupts, the epithelial cell layers that cover the tooth crown are degraded and lost. HERS fragments and forms the epithelial cell rests of Malassez (ERM) that remain on the root surface.

In the cap stage, the epithelium matures to form three separate epithelial layers that form the epithelial structure known as enamel organ (Figure 3). The stellate reticulum (SR) forms in the middle of the enamel organ and consists of star-shaped epithelial cells with mesenchymal-like appearance. The SR is surrounded by the enamel epithelium, which is composed of the inner enamel epithelium (IEE), which faces the dental mesenchyme, and the outer enamel epithelium

(OEE), which faces the dental follicle. The enamel organ proliferates and the IEE and OEE form loop-like structures called cervical loops (Figure 3). The proliferation of the epithelium continues in these structures and guide morphogenesis and size of the tooth crown.

In the following bell stage, the secondary enamel knots form in the IEE and specify the sites of the future cusps in teeth with multiple cusps, such as molars (Jernvall et al., 1994). The secondary enamel knots do not form in unicuspid teeth, such as incisors. The differentiation of tooth-specific cells, the ameloblasts and odontoblasts, begins at the cusp tips and is followed by the deposition and mineralization of dental hard tissues (Figure 3). Odontoblasts develop from mesenchymal cells, which are located adjacent to the IEE, and secrete predentin, which later mineralizes into dentin. Ameloblasts develop from the IEE, which differentiates to preameloblasts and later to ameloblasts, which start to secrete the extracellular matrix of enamel. Stratum intermedium (SI) cells that develop from SR and are positioned next to the ameloblast layer participate in enamel production, but their exact role has remained unknown. The ameloblasts and other epithelial structures of the crown form the reduced enamel epithelium that participate in enamel maturation and are lost when the tooth crown erupts into the oral cavity.

The differentiation of the cells and the hard tissue deposition proceed in the cervical direction. When crown development is complete and mineralization has reached the cervical loops, root development begins in the teeth that have roots. At the same time the process of tooth eruption starts. The crown-to-root transition begins when the SR cells from the cervical loops disappear and the IEE and OEE come into contact and form a bilayer called Hertwig's epithelial root sheath (HERS). HERS is an epithelial structure that guides the root development and is later fragmented into the epithelial cell rests of Malassez (ERM). ERM cells form an epithelial cell network on the root surface and most likely regulate and maintain the periodontal space. The dental follicle cells, which are derived from the mesenchymal cells that surround the tooth germ, differentiate into cementoblasts. These secrete cementum, which covers the dentin of the roots. The periodontal ligament, which anchors the tooth to the alveolar bony socket, is also formed from the dental follicle.

2.3.2 Epithelial-mesenchymal interactions

Epithelial-mesenchymal interactions play a key role in all steps of tooth development (Thesleff and Tummers, 2003). The potential to form teeth is established very early in odontogenic tissues. It was demonstrated by studies long ago that early tooth development is not dependent on the surrounding connective tissue or vascularization or innervation since a tooth germ undergoes normal morphogenesis when cultured *in vitro* in suitable organ culture conditions (Glasstone, 1936; Lumsden, 1979). Also, when an early molar bud is isolated and placed in the anterior chamber of the eye all three molars develop (Lumsden, 1988), indicating an inherent developmental potential of the tooth forming tissues.

Classical tissue recombination experiments demonstrated that it is the epithelium that gives the first inductive signal for tooth formation. Tooth formation was induced when the oral epithelium from mouse embryos (E9 and E11.5) was separated from the mesenchyme and placed against non-dental mesenchyme (Mina and Kollar, 1987; Lumsden, 1988). After the formation of the placode (E11.5), the early signaling center in the dental lamina, the inductive potential shifts to the underlying mesenchyme. After this shift, the dental mesenchyme is able to induce tooth formation when placed against non-dental epithelium (Kollar and Baird, 1970). Thereafter the mesenchyme controls the advancing morphogenesis of the tooth and also defines the tooth identities (Kollar and Baird, 1969).

Reciprocal interactions between the epithelium and mesenchyme also guide the later stages of tooth development such as the differentiation of ameloblasts and odontoblasts at the interface between the epithelium and mesenchyme (Bei, 2009a).

2.3.3 Molecular regulation

Complex gene regulatory networks regulate tooth morphogenesis; many genes act at specific stages of tooth development to regulate tooth patterning and cell differentiation processes. The expression data of many of the molecular factors can be found on publicly available databases bite-it (http://bite-it.helsinki.fi) (Nieminen et al., 1998) and ToothCode (O'Connell et al., 2012). The genes encode diverse proteins, including soluble ligands, cell membrane receptors, intracellular signaling factors, transcription factors, as well as co-factors and antagonists that belong to the four conserved signaling pathway families Bmp, Fgf, Shh and Wnt. The same conserved signaling pathway families regulate the development of other organs as well. Importantly, there are no tooth-specific genes identified and this same genetic tool-kit regulates all developmental processes during embryogenesis.

The conserved signaling pathways such as Bmp, Fgf, Shh, Wnt, and Eda are repeatedly used during tooth development to mediate the epithelial-mesenchymal interactions in all phases from the early initiation stage to the cell differentiation stage (Figure 4). Studies using transgenic mice have shown that if any of the signaling pathways is blocked, tooth formation is severely affected or arrested, leading to malformations or complete lack of teeth (Tummers and Thesleff, 2009; Bei, 2009b). The arrest of tooth development most commonly occurs in the early stage of tooth development: either before placode formation and budding, or before morphogenesis of the bud into cap stage (Tummers and Thesleff, 2009).

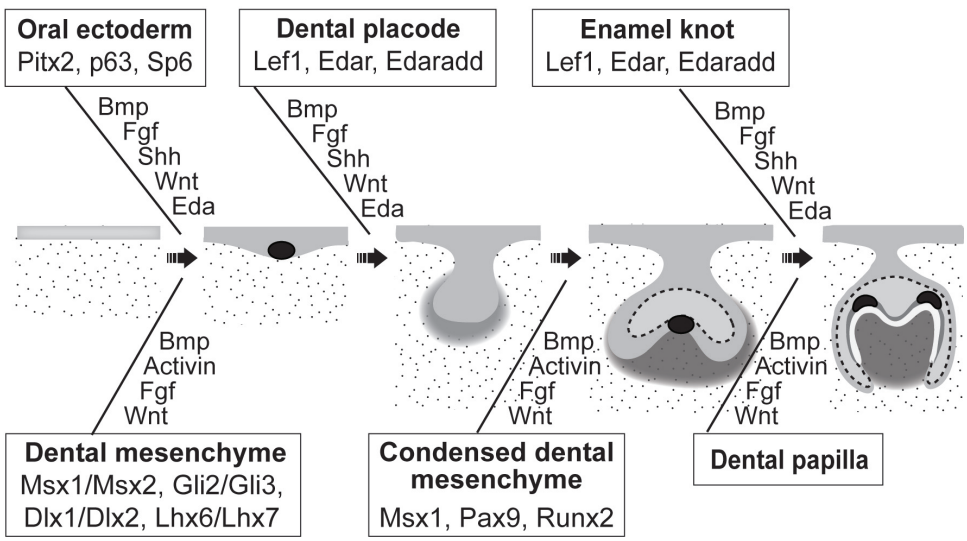

Figure 4. Tooth development is regulated by sequential and reciprocal interactions between the dental epithelium and mesenchyme. The same signaling pathways regulate tooth development in all stages. The signaling centers (black spots) in the placode and subsequently in the enamel knots are key regulators of tooth development. The genes indicated in the boxes are necessary for normal tooth morphogenesis as shown by knockout mice studies.

The three transient signaling centers, dental placodes and primary and secondary enamel knots, have key roles in the regulation of tooth patterning and morphogenesis (Figure 4). Several signaling pathways converge in these signaling centers, which express largely the same set of genes. For example Fgfs, Shh, Bmps, and several Wnts as well as their targets such as *Lef1* (Wnt pathway), are expressed in the enamel knots (Thesleff and Mikkola, 2002). Enamel knots also express Edar and Edaradd that belong to the Eda signaling pathway, which has a particular role in placode formation and is a specific regulator of ectodermal organ development (Mikkola, 2009).

Transcription factors mediate the coordinated interplay of signals at all steps of tooth development. Some of the important factors are shown in Figure 4. In the oral ectoderm the earliest transcription factor expressed is *Pitx2*, which is a target of the Fgf pathway (Mitsiadis et al., 1998; St Amand et al., 2000). *Sp6* (*Epiprofin*) is expressed in the dental epithelium during early stage of development and its absence leads to supernumerary tooth formation (Nakamura et al., 2008). Finally, *p63* is required for placode formation in all ectodermal organs (Laurikkala et al., 2006).

A number of transcription factors are expressed in the dental mesenchyme. These include targets of the Bmp pathway (*Msx1*, *Msx2*, *Dlx1*, and *Dlx2*), targets of Shh pathway (*Gli2* and *Gli3*) and targets of Fgf pathway (*Lhx6* and *Lhx7*, *Pax9*, and *Runx2*) (Bei, 2009b). Many knockout mouse models have shown that the loss of function of these transcription factors leads to an arrest of tooth development. Some of the transcription factors have redundant functions, and in some cases loss of two factors in the same family is needed to obtain arrested tooth development (Tummers and Thesleff, 2009; Bei, 2009b).

A fine balance between signaling ligands, their receptors, inhibitors and activators, as well as transcription factors that regulate tooth development in all aspects is essential (Tummers and Thesleff, 2009). For example, Follistatin, which antagonizes Activin and Bmp signaling, is essential for the formation of proper crown patterning (Wang et al., 2004). Its deletion or overexpression leads to aberrant cusp patterns in mice (Wang et al., 2004). Similarly, deletion of *Sostdc1*, which is an inhibitor of the Bmp and Wnt pathways, affects cusp patterning (Kassai et al., 2005). Interestingly, the modulation and fine-tuning of enamel knot signaling that establish the patterning of the crown create the diversity of the tooth crowns observed in various animals (Jernvall and Thesleff, 2012). A morphogenetic mathematical model can predict the specific crown patterns observed during evolution by adjusting the balance between different modulators (Salazar-Ciudad and Jernvall, 2002). This is supported by recent *in vitro* studies where the complexity of mouse tooth can be increased substantially by adjusting multiple signaling pathways simultaneously (Harjunmaa et al., 2012).

The most recently appreciated modulators of signal pathways associated with tooth development are miRNAs. They constitute a class of short regulatory RNA molecules that exert post-transcriptional control on gene expression at the level of translation in many tissues (Singh et al., 2008) and they have essential roles in various developmental events in vertebrates (Miska, 2005). Recently, miRNAs were shown to be involved in tooth morphogenesis and patterning as well as in the terminal differentiation of cells; the conditional deletion of Dicer-1, which processes pre-miRNAs into mature miRNAs, in transgenic mouse dental epithelium led to aberrations in the molar shape and cusp pattern as well as defects in the structure of enamel (Michon et al., 2010; Cao et al., 2010).

2.4 Tooth renewal

Teeth are found in almost all vertebrates and appear in a great variety of shapes, sizes, and numbers. The teeth of lower vertebrates such as fish and reptiles are continuously replaced. The capacity for tooth replacement has decreased during evolution when the complexity of teeth has increased (Jernvall and Thesleff, 2012). Therefore, the more complex teeth of mammals have limited capacity for tooth replacement. Most mammals replace part of their dentition only once: having deciduous or primary (milk) teeth, which are replaced by a secondary (permanent) dentition.

Another way to renew teeth is to increase the growth of individual teeth. To counterbalance the reduction of tooth height caused by chewing hard food, specialized dentitions have evolved in some mammals such as rodents. For example, mouse teeth are not replaced but mice have continuously growing incisors. Although molars do not grow continuously in mice, they do so in other rodents such as voles, as well as in rabbits and quinea pigs. The continuous growth as well as the continuous replacement of teeth require stem cells. Most studies on dental stem cells have used the continuously growing mouse incisor as a model organ.

2.4.1 Continuously growing mouse incisor

Mouse dentition consists of one continuously growing incisor and three molars separated by a toothless diastema in each jaw quadrant (Figure 5). The early development of the incisor follows the same morphological events as the molar development discussed above. At the cap stage, the incisor tooth germ, however, rotates anteroposteriorly and the epithelial cervical loops start to develop asymmetrically, leading to a smaller lingual (tongue side) and bigger labial (lip side) cervical loops (Mucchielli and Mitsiadis, 2000). The formation of the cervical loops is crucial for the development of the renewal capacity of the incisor.

The continuous generation of dental hard tissues forms a gradient of epithelial and mesenchymal cell differentiation from the apex towards the incisor tip. The proliferation of the progenitor cells takes place in the apical region, and the differentiating cells move toward the incisal direction like a conveyer belt and differentiate into tall, polarized ameloblasts that secrete enamel and odontoblasts that secrete dentin (Thesleff and Tummers, 2009). The incisor is asymmetric in the deposition of dental hard tissues. Due to this, the labial side of the tooth resembles the crown and is therefore called crown-analog, whereas the lingual side consisting of root structures is called the root-analog (Amar et al., 1986) (Figure 5). The ameloblasts are produced only from the labial cervical loop, and the SR cells are maintained and surrounded by basal epithelium formed by IEE and OEE. The SR cells in the thinner lingual cervical loop are mostly depleted in the adult incisor. Therefore, the lingual side of the incisor is enamel-free and the lingual cervical loop is thought to give rise to the ERM cells. The odontoblasts produce dentin, which underlies the enamel on the labial side. The cementum in the lingual side is derived from the mesenchyme. The follicle cells are also derived from the early dental mesenchyme and give rise to the periodontal ligament that connects the lingual surface to the jawbone.

The asymmetric deposition of enamel is functionally important since the consequent differences in tissue hardness lead to a self-sharpening system. When the rodent gnaws hard food, the tips of the incisors are constantly abraded leading to the need of constant production of new cells. The continuous growth of the incisor requires the production of all cell types of the tooth, both mesenchymal and epithelial of origin. This production of cells is fueled by epithelial and mesenchymal stem cells that reside in the apical end of the incisor.

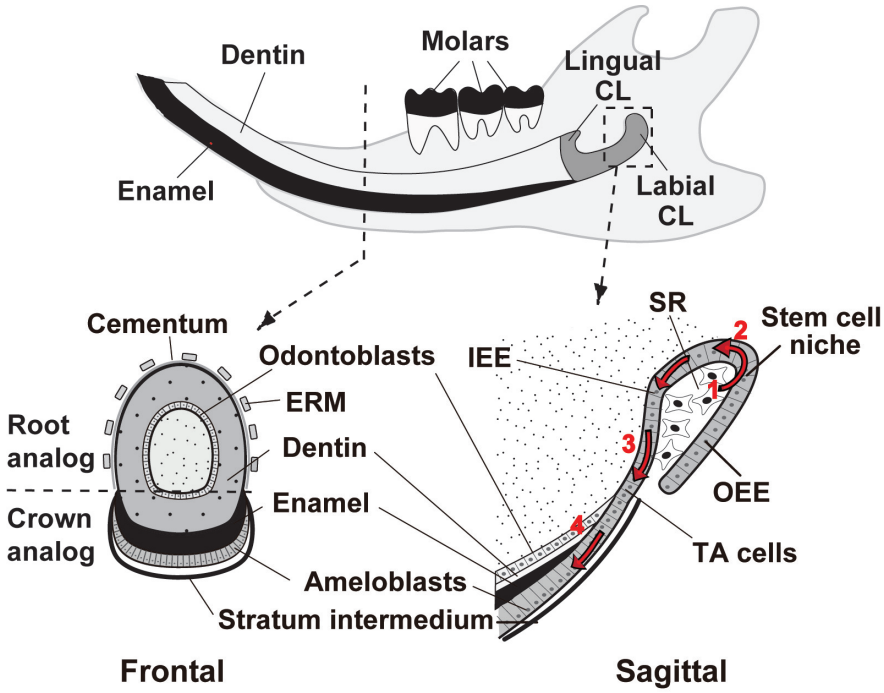

Figure 5. Structure of the mouse lower jaw and incisor. Each jaw quadrant has three molars and one continuously growing incisor separated by a toothless diastema. Frontal section from the middle part of the incisor illustrates the root- and crown-analog and the cell types that are characteristic of the root and crown. Sagittal section from the proximal end of the incisor illustrates the labial cervical loop, which houses the epithelial stem cells. A putative model for the dynamics of the stem cell is indicated by arrows: a stem cell in the stellate reticulum divides (1) and gives rise to a progenitor cell, which is inserted in the basal enamel epithelium (2). The progenitor cell divides and forms the TA cell population (3), which starts to differentiate into ameloblasts (4) that eventually deposit the enamel matrix. CL, cervical loop; ERM, epithelial cell rests of Malassez; IEE, inner enamel epithelium; OEE, outer enamel epithelium; SR, stellate reticulum; TA, transient amplifying cells. Modified from Study I.

2.4.1.1 Incisor stem cells

The epithelial stem cells reside in the cervical loops in the proximal end of the incisor and give rise to the rapidly proliferating transit amplifying (TA) cells, which differentiate to ameloblasts and to the cells of stratum intermedium (SI) (Figure 5) (Harada et al., 1999). The mesenchymal stem cells that give rise to the dentin-producing odontoblasts reside in the mesenchymal compartment between the epithelial cervical loops, but their exact location is unknown and they have remained uncharacterized (Seidel et al., 2010; Feng et al., 2011; Lapthanasupkul et al., 2012).

The first clues that the labial cervical loop contains a stem cell population that is responsible for the supply for rapidly proliferating progenitor cells in the rodent incisor came from label-retaining experiments that examined the proliferation dynamics (Smith and Warshawsky 1975, Smith, 1980). Later, BrdU incorporating LRCs were detected in the SR compartment of mouse incisor labial cervical loop (Harada et al., 1999). Furthermore, *in vitro* tissue ablation experiments showed that the dissected cervical loop from the postnatal (P2) mouse continues to produce

dental tissues, indicating that cells with significant growth and differentiation potential reside there (Harada et al., 1999). More recently, a technique utilizing H2B-GFP technology revealed that LRCs in the adult mouse labial cervical loop were located in specific areas, including parts of the SR and OEE (Seidel et al., 2010). The molecular identity of the epithelial stem cells in the labial cervical loop has remained unknown but some general stem cell marker genes such as *Lgr5* (Suomalainen and Thesleff, 2010), *ABCG2*, *Bmi-1*, *Oct-3/4*, and *Yap* (Li et al., 2011) have been shown to be expressed in these cells.

Although the labial cervical loop contains stem cells, it is not known if the lingual cervical loop contains stem cells that produce the ERM cells in the lingual side of the incisor. However, some LRCs have been identified in the lingual cervical loop (Seidel et al., 2010).

2.4.1.2 Molecular regulation of incisor epithelial stem cells

In the last 15 years significant progress has been made on understanding how the epithelial stem cells are maintained and regulated. It has become clear that the epithelial stem cells are regulated by complex interactions between the cervical loop epithelium and the underlying mesenchyme, involving several conserved signaling pathways, transcription factors, cell adhesion molecules, and, miRNAs, as observed most recently (Hu et al., 2013).

Several signaling pathways such as Fgf, Bmp, Shh, Wnt, and Notch are associated with the formation and maintenance of the incisor epithelial stem cells during development and in adult homeostasis (Hu et al., 2013). The most studied is the Fgf pathway (Harada et al., 2002; Klein et al., 2008; Tummers and Thesleff, 2009). Expression of several Fgf ligands and their receptors localizes in the proximal part of the incisor during development and in adult (Harada et al., 1999; Kettunen et al., 2000; Harada et al., 2002; Wang et al., 2007). For instance, *Fgf10* and *Fgf3* are expressed in the dental mesenchyme and have an overlapping expression pattern (Harada et al., 1999). *Fgf3* expression is restricted to the mesenchyme next the labial cervical loop while *Fgf10* is expressed in a wider area, consisting of the mesenchyme next to both cervical loops (Harada et al., 1999). *Fgf9* is expressed in the IEE and TA cells (Yokohama-Tamaki et al., 2008), and *Fgf8* in the SR (Wang et al., 2009). The Fgf receptors, *Fgfr1b* and *Fgfr2b*, are enriched in the cervical loop epithelium and in the TA region (Harada et al., 1999).

Functional studies have shown that Fgf10 is required for the development of the cervical loops and for the formation of the epithelial stem cell niche (Harada et al., 2002). *Fgf10*-deficient mice die at birth, but the analysis of postnatal *Fgf10*$^{-/-}$ incisors cultured under kidney capsule revealed a loss of the epithelial stem cell compartment and a transition of the incisor crown-analog to root-analog (Yokohama-Tamaki et al., 2006). Notably, *Fgf3*-deficient mice do not show any gross phenotype, but *Fgf3*$^{-/-}$;*Fgf10*$^{-/+}$ compound mutants have hypoplastic cervical loops, indicating that the precise levels of Fgf signaling are important for the regulation of the epithelial stem cell pool (Wang et al., 2007). In line with this is that the conditional removal of *Fgfr2b* in the incisor epithelium leads to impaired growth, failure of enamel formation, and degradation of the incisors (Parsa et al., 2010). The survival of stem cells is, however, not compromised when *Fgfr2b* is transiently knocked out (Parsa et al., 2010).

A fine balance of Fgf signaling is important for the maintenance of the asymmetric deposition of enamel and for the limitation of the production of ameloblasts on the labial aspect of the incisor while their formation on the lingual side is prevented. *Sprouty* genes (*Spry*), which encode intracellular antagonists of Fgfs, prevent the generation of lingual ameloblasts by controlling an Fgf-mediated epithelial-mesenchymal signaling loop (Klein et al., 2008).

Downregulation of *Sprouty* genes in *Spry4*$^{-/-}$;*Spry2*$^{+/-}$ mutants leads to ectopic production of ameloblasts on the lingual side and development of incisors to a tusk-like form (Klein et al., 2008). Follistatin inhibits ameloblast differentiation in the lingual side by antagonizing Bmp signaling from odontoblasts, and therefore, ectopic ameloblasts on the lingual side are also observed in mice lacking *Follistatin* (Wang et al., 2004). Transcription factor Bcl11b is also required for the establishment and maintenance of labial-lingual asymmetry by controlling the expression of several key signaling molecules (Kyrylkova et al., 2012). Transcription factor Tbx1, which is regulated by Fgf signaling, is required for ameloblast formation in the labial cervical loop, and its deletion leads to enamel-free incisors (Catón et al., 2009).

Several pathways seem to converge on Fgf signaling, especially via regulation of Fgf3, to control the maintenance of the epithelial stem cells in the labial cervical loop. A complex signaling network involving Activin, Bmp, Fgf, and Follistatin ensure the maintenance of Fgf3 expression in the labial mesenchyme that promotes stem cell proliferation (Wang et al., 2007). The loss of Tgfß receptor I (Alk5) in the dental mesenchyme affects the maintenance of epithelial stem cells by downregulation of Fgf3, Fgf9, and Fgf10 (Zhao et al., 2011). This defect was, however, rescued *in vitro* by adding Fgf10 into incisor culture (Zhao et al., 2011). E-cadherin, which is regulated by Fgfs, is yet another important regulator of incisor stem cells and its conditional inactivation leads to decreased numbers of label-retaining stem cells and reduced cell migration (Li et al., 2012).

Incisor stem cells also require Notch pathway activity, and several Notch receptors and ligands are expressed in the cervical loop region (Harada et al., 1999). Inhibition of Notch signaling in the incisor leads to a reduced size of the labial cervical loop, indicating a possible association between Notch signaling and epithelial stem cell maintenance (Harada et al., 1999; Felszeghy et al., 2010).

Moreover, stem cells in the labial cervical loop are responsive to Shh, which is expressed by their differentiating progeny (Seidel et al., 2010). When Hedgehog (Hh)-signaling was blocked, ameloblast (but not SI cell) production stopped, indicating a positive feedback loop between the Hh-responsive stem cells and their progeny (Seidel et al., 2010). Interestingly, Hh-responsive stem cells were not only detected in the epithelial cervical loops but were also detected in the mesenchyme (Seidel et al., 2010).

Wnt signaling is associated with the regulation and maintenance of several adult epithelial stem cells, including those of the hair follicle and the intestine (Schuijers and Clevers, 2012). In the incisor, however, active Wnt/ß-catenin signaling is not detected in the epithelial stem cells but instead in the lingual cervical loop epithelium, suggesting that Wnt signaling inhibits ameloblast formation and epithelial stem cell proliferation (Suomalainen and Thesleff, 2010). Indeed, overexpression of Wnt3 leads to a progressive loss of ameloblasts in the mouse incisor (Millar et al., 2003).

Finally, miRNAs have also been associated with the regulation of stem cells due to their ability to control the maturation and differentiation of cells (Gangaraju and Lin, 2009). MicroRNAs play a critical role in tooth development by regulating ameloblast differentiation and the proliferation of epithelial progenitors in the incisor cervical loop (Michon et al., 2010; Cao et al., 2010). The deletion of *Dicer-1* in the epithelium by using Pitx2-Cre led to multiple, branched, enamel-free incisors and molars (Cao et al., 2010). A milder tooth phenotype was observed when *Dicer-1* was deleted by using K14-Cre, the expression of which starts later in development than Pitx2-Cre (Michon et al., 2010). Several miRNAs are also expressed in the labial cervical loop stem cell niche and most likely take part in the regulation of epithelial stem cells (Jheon et al., 2011).

2.4.2 Tooth replacement

Vertebrates have different capacities to replace their teeth. Most fish, sharks, amphibians, and reptiles have large numbers of simple teeth and have the capacity to renew their dentition several times throughout life and are therefore called polyphyodont. Mammals have generally more complex teeth and replace their teeth only once (diphyodont) or not at all like monophyodont rodents (Jernvall and Thesleff, 2012).

Fish and reptiles have proved to be useful models to study tooth replacement because of the presence of several stages of replacement tooth development at any time point (Smith et al., 2009; Richman and Handrigan, 2011). In reptiles, teeth form a single marginal row in the lower jaw, whereas in the upper jaw the dental pattern can vary from a single row of teeth, as in crocodilians, to the multiple rows of teeth observed in snakes (Richman and Handrigan, 2011).

Mammals have a regionalized dentition possessing different tooth types, which are divided into groups (incisors, canines, premolars, and molars) based on their morphology. Most mammals replace their antemolar teeth only once and molar teeth are not replaced. Although the mouse has been a valuable model to study the molecular regulation of primary tooth development, its teeth are not replaced and are considered deciduous teeth (Moss-Salentijn, 1975). Diphyodont models such as ferret, minipig, and shrew, have emerged more recently to study mammalian tooth replacement (Järvinen et al., 2008; Järvinen et al., 2009; Stembírek et al., 2010).

The dental lamina is thought to possess the capacity for tooth replacement in all vertebrates (Järvinen et al., 2009; Smith et al., 2009; Richman and Handrigan, 2011). The primary teeth develop from the primary dental lamina, which grows down into the mesenchyme and remains as an interdental lamina connecting the tooth germs in most mammals and reptiles (Figure 6) (Ooë, 1981; Whitlock and Richman, 2013; Jussila et al., 2014). The primary dental lamina forms an extension, called the successional dental lamina, on the lingual side of the primary teeth. The replacement teeth in reptiles and mammals form from this successional dental lamina.

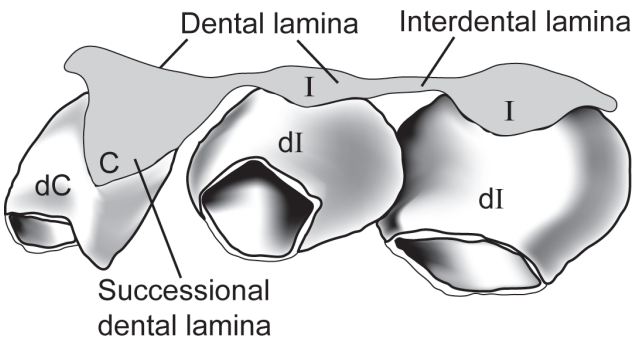

Figure 6. Illustration of the human deciduous tooth germs and the dental lamina. The interdental lamina connects the individual tooth germs. Replacement teeth are initiated from the successional dental lamina, which forms on the lingual aspect of the deciduous teeth. Reconstruction is adapted from (Ooë, 1981) and shows the lingual view of anterior tooth germs in the lower jaw. dI and dC; deciduous incisor and canine. I and C; permanent incisor and canine. Modified from Study II.

In reptiles, the dental lamina and successional dental lamina remain as a continuous stripe from the oral epithelium to the dental mesenchyme, connecting all future replacement teeth within a family (Figure 7) (Whitlock and Richman, 2013). In mammals, the dental lamina fragments and loses its connection to the oral epithelium, and later the successional dental lamina is fragmented between the primary and secondary teeth (Figure 7) (Ooë, 1981; Jussila et al., 2014). There is, however, evidence that a new successional lamina for the third tooth generation forms in the permanent incisor and premolar tooth germs in humans (Ooë, 1981). In

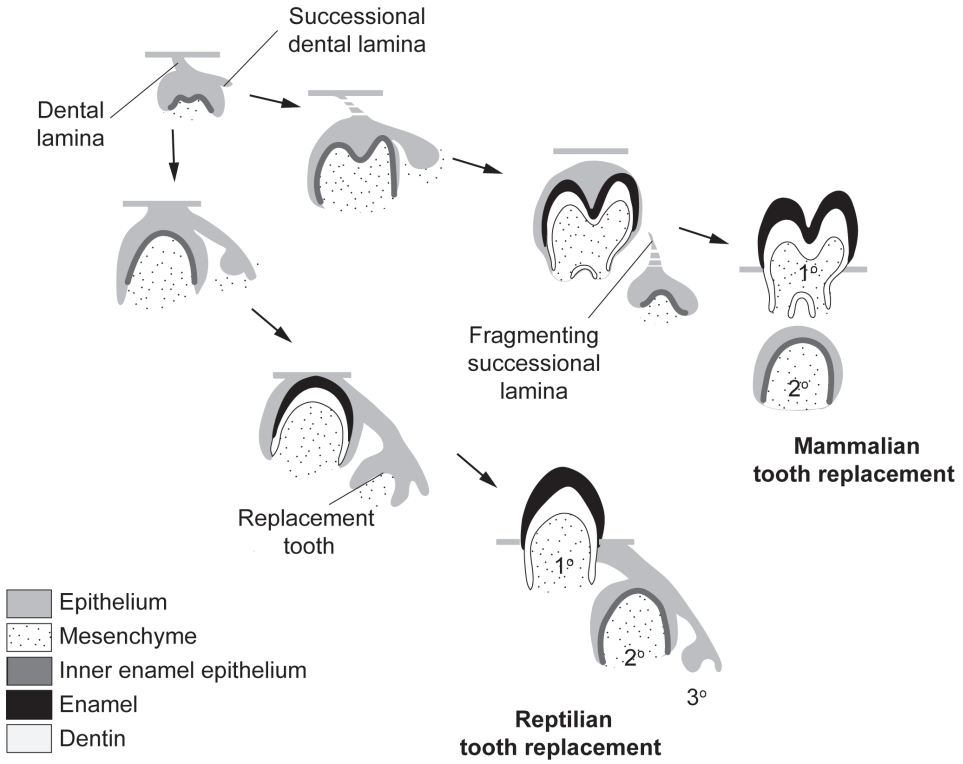

Figure 7. Schematic illustration of mammalian and reptilian tooth replacement. In mammals, replacement tooth formation is initiated when the primary tooth has reached the cap stage. The dental lamina on the lingual side of the primary tooth buds and forms the successional dental lamina. The successional lamina grows into the underlying mesenchyme towards the apex of the deciduous tooth. The development of the permanent tooth begins and the successional lamina is later degraded.
In reptiles, the replacement tooth formation is a very similar process. However, in reptiles the dental lamina remains in contact with the oral epithelium and forms a continuous stripe that connects several generations of teeth (1°, 2°, 3°). The dental lamina also contains putative stem cells identified in reptiles. In mammals, the dental lamina connecting the primary teeth to oral epithelium and the successional lamina connecting the primary and the secondary teeth is degraded. Modified from Jernvall and Thesleff, 2012.

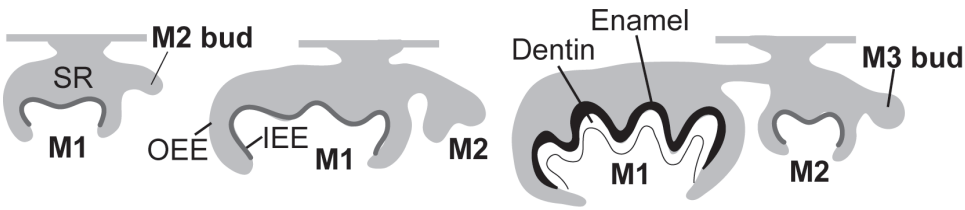

Figure 8. Schematic presentation of the serial addition of molars in mouse. The dental epithelium at the distal aspect of the first molar (M1) gives rise to a bud from which the second molar (M2) forms. The third molar (M3) is formed by the same sequence of steps.
M, molar; IEE, inner enamel epithelium; OEE, outer enamel epithelium; SR, stellate reticulum. Modified from Study II.

the monophyodont mice, molars develop by serial addition, and the only site where the dental lamina persists is the distal aspect of the molars (Figure 8).

It has been suggested that the capacity for continuous replacement requires the integrity of the dental and successional lamina, but recently it was reported this is not the case at least in the polyphyodont alligator, in which the dental lamina loses its connection with the oral epithelium (Wu et al., 2013).

2.4.2.1 Molecular regulation of replacement

The molecular regulation of tooth replacement and the formation of the successional lamina are still poorly understood. Studies on the diphyodonts shrew and ferret have identified only a few genes, such as *Axin2* and *Sostdc1*, that are regionally restricted to the site where replacement tooth formation occurs (Järvinen et al., 2008; Järvinen et al., 2009). Molecular studies on reptiles have only given some cues that the conserved signaling pathways that regulate primary tooth formation such as Shh, Bmp, and Wnt, also participate in the replacement tooth formation (Handrigan and Richman, 2010). Shh is, however, probably not directly linked to replacement tooth formation (Buchtová et al., 2008; Järvinen et al., 2009), and Bmps might only affect the proliferation of the successional lamina (Handrigan and Richman, 2010). The best candidate associated with replacement tooth formation seems to be Wnt signaling. In the gecko, Wnt signaling is associated with the tip of the successional lamina where the replacement tooth originates (Handrigan and Richman, 2010). In line with this is the reduction in Wnt signaling in the bearded dragon (a reptile), which replaces its teeth only once (Richman and Handrigan, 2011).

The variations in the signaling pathway activities can induce the formation of new teeth also in species that do not normally replace their teeth. For instance, the role of Wnt signaling in activating replacement tooth formation is supported by various studies from transgenic mice and manifestations of human syndromes. Supernumerary teeth form in the mouse when Wnt signaling is stimulated in the oral epithelium (Järvinen et al., 2006; Liu et al., 2008; Nakamura et al., 2008; Wang et al., 2009). Similarly, supernumerary teeth develop in human syndromes where Wnt signaling is affected. Familial adenomatous polyposis (FAP) is caused by a mutation in the *APC* gene (Groden et al., 1991), which is a tumor suppressor gene associated with the stabilization of the ß-catenin that is an intracellular transducer of Wnt signaling. A variant of FAP called Gardner syndrome displays dental abnormalities including impacted supernumerary teeth and odontomas (Gardner, 1962). Moreover, cleidocranial dysplasia is caused by mutations on the *RUNX2* gene, which is also linked to Wnt signaling, leading to the formation of multiple successional generations of teeth (Jensen and Kreiborg, 1990; Mundlos et al., 1997; Otto et al., 1997).

It is more common to have missing teeth than supernumerary teeth. Tooth agenesis, a failure of one or several teeth to develop, is the most common developmental anomaly in humans. The most frequently missing teeth are permanent teeth and those that develop last within the tooth family: the lateral incisors, the second premolars, and the third molars. The specific vulnerability of the last forming member may be explained by the development of the teeth in each family from a common placode (Nieminen, 2009). Half of the patients with oligodontia (more than 6 teeth missing excluding wisdom teeth) have mutations in the *WNT10A* gene (van den Boogaard et al., 2012). Other genes associated with tooth agenesis involve *EDA*, *EDAR*, *EDARADD*, *MSX1*, and *PAX9* (Nieminen, 2009). In addition, mutation in *AXIN2*, which is also a component of

the WNT signaling pathway, leads to agenesis of several permanent teeth, suggesting that particularly tooth replacement is impaired (Lammi et al., 2004).

The continuous replacement requires stem cells. In the polyphyodont gecko, putative stem cells are identified in the lingual side of the dental lamina and these cells express some known stem cell markers such as *Lgr5*, *Dkk1*, and *Igfp5* (Handrigan et al., 2010). Recently, putative stem cells were localized in the alligator, in the tip of the successional lamina (Wu et al., 2013). It has been suggested that the capacity for tooth replacement is still present in mammals in the form of stem cells embedded in the dental lamina (Smith et al., 2009).

2.5 Odontogenic tumors

Odontogenic tumors are a heterogeneous group of rare lesions of the jaw that are thought to originate from the primordial tooth-forming tissues. Odontogenic tumors are usually benign and are often associated with impacted or missing teeth. Some tumors, such as odontomas that consist of multiple small teeth, are nowadays considered as developmental malformations or hamartomatous lesions rather than true neoplasms. Classification of odontogenic tumors is based on their behavior and the benign tumors are further subdivided into groups based on their histology and derivation form epithelial, ectomesenchymal/or mesenchymal elements of the developing tooth germ (Philipsen et al., 2005).

The most frequently encountered and clinically significant odontogenic tumor is the ameloblastoma. Ameloblastoma is classified as benign, but it is locally invasive, often infiltrates the bone, has high recurrence, and may become malignant (Mendenhall et al., 2007). Treatment is surgical with wide margins. There appears to be no preference for gender and the average age of patients at the time of diagnosis is 36 years (Reichart et al., 1995). Ameloblastomas are most frequently found in the molar region of the mandible, and are often associated with the presence of unerupted teeth (Reichart et al., 1995).

Ameloblastoma is thought to arise from the epithelial cells of the developing tooth. It consists of epithelial cells only, and histologically the ameloblastoma epithelium resembles the enamel organ of a bell staged tooth germ (Philipsen and Reichart, 2004). The central cells of ameloblastoma islands resemble the SR cells of the tooth germ, and the peripheral epithelial cells resemble preameloblasts. However, ameloblastoma cells do not differentiate further and dental hard tissues do not form. Ameloblastomas are divided into four subtypes based on their clinical, radiographic, histopathologic, and behavioral aspects (Philipsen et al., 2005). Ameloblastomas are also categorized by their growth patterns, but often mixtures of different patterns are observed within a lesion. There are two main basic growth patterns of ameloblastoma: 1) the follicular, in which neoplastic epithelium appears as tumor islands, and 2) the plexiform pattern, in which the epithelium appears as anastomosing strands and cords of tumor cells (Philipsen et al., 2005).

Although the pathogenesis of the ameloblastoma has been increasingly studied in recent years (Gomes et al., 2010), the origin of the neoplastic epithelium has remained unknown. It has been suggested that it may arise directly from the enamel organ of a developing tooth, from the remnants of the odontogenic epithelium, from the lining of an odontogenic cyst, or even from the basal layer of oral mucosa. It is most likely that the ameloblastoma epithelium originates from the epithelial cells of the developing tooth because of its histological resemblance to the tooth germ. However, the ERM cells, which are the only dental epithelial cells present in adult teeth, have also been considered as the tumor-initiating cells (Takeda and Yamamoto, 1990). The genetic changes underlying the pathogenesis of ameloblastoma are poorly understood. Several

microarray studies have been conducted to identify such molecular factors, but the results have been inconclusive (Heikinheimo et al., 2002; Lim et al., 2006; DeVilliers et al., 2011). Only until recently, a high-frequency mutation in *BRAF* oncogene was identified in ameloblastoma (Kurppa et al., 2014).

3 Aims of the study

Dental tissues that continuously renew, such as the ever-growing rodent incisor and the continuously replaced reptilian dentition, require stem cells to supply these organs with new differentiating cells. However, the study of epithelial dental stem cells has been hindered by a lack of a specific marker.

The aims of my thesis were to:

1. Find a specific marker for the epithelial stem cells in the continuously growing mouse incisor

After the specific stem cell marker, Sox2, was identified the further aims of my thesis were to:

2. Identify the progeny of *Sox2*-expressing cells in the incisor and explore the regulation of Sox2 expression.
3. Study whether Sox2 could be used as marker to identify the progenitor cells of replacement teeth in reptiles and mammals.
4. Investigate the involvement of *Sox2* in odontogenic tumor formation.

4 Materials and methods

The methods used in this thesis are described in detail in the Methods section in each original publication. The mouse strains that were used are listed in Table 1, the methods are listed in Table 2, and the probes used in *in situ* hybridization are listed in Table 3.

4.1 Tissue specimens and processing

Mice

Wild-type NMRI mice were used at various embryonic and postnatal stages. The day when a vaginal plug was detected was counted as embryonic day (E)0, and the day of birth was considered as postnatal day (P)0. The stages of the embryos were confirmed according to morphological criteria. The different mouse strains used and their references are shown in Table 1.

Table 1. Mouse strains used in Studies I-III

Mouse strains	*Reference*	*Used in*
ß-cat$^{\Delta ex3fl}$	Harada et al., 1999	III
BAT-gal	Maretto et al., 2003	I
Dicer-1$^{fl/fl}$	Harfe et al., 2005	I
Fucci-red	Sakaue-Sawano et al., 2008	I
K14Cre	Huelsken et al., 2001	III
NMRI		I, II, III
R26RlacZ	Soriano, 1999	I, II
Shh::GFPCre	Harfe et al., 2004	I, II
Sox2CreERT2	Arnold et al., 2011	I, II
Sox2$^{fl/fl}$	Smith et al., 2009	II
Sox2-GFP	D'Amour and Gage, 2003	I, II
TOPGAL	DasGupta and Fuchs, 1999	I

The genotyping was performed as previously described (each reference). For genetic lineage tracing, Sox2CreERT2 and R26RlacZ mice were crossed as described previously (Arnold et al., 2011). Sox2-GFP mice were used in organ cultures to detect Sox2 expression (I, II) and for inhibition assays (I). The conditional Sox2 knockout mouse line Shh::GFPCre;Sox2$^{fl/fl}$ was generated by crossing Shh::GFPCre with Sox2$^{fl/fl}$ mice (II). Conditional Wnt activation in the epithelium in the K14Cre; ß-cat$^{\Delta ex3fl}$ mouse line was generated by crossing K14Cre line with ß-cat$^{\Delta ex3fl}$ line as described previously (Järvinen et al., 2006)(III).

Fucci-red (mKO2-hCdt1) mice were crossed with Sox2-GFP mice for the *in vitro* confocal imaging (I). For deletion of miRNAs in the epithelium, Dicer-1$^{fl/fl}$ was crossed with Shh::GFPCre (I). TOPGAL and BAT-gal mice were used to detect activity of Wnt signaling (I).

The teeth from the lower jaw were dissected in Dulbecco's PBS, pH 7.4, for culture and histology. For histology, tissues were fixed in 4% PFA, decalcified in EDTA-PFA, dehydrated, embedded in paraffin, and sectioned at 5-7 µm in frontal or sagittal planes for *in situ* hybridization, immunohistochemistry, and hematoxylin and eosin staining. The dissection of E14 molars from the K14Cre/+; ß-cat$^{\Delta ex3fl}$ and kidney capsule transplants has been described previously (Järvinen et al., 2006).

All aspects of mouse care and experimental protocols that required the use of laboratory animals were performed according to the ethical guidelines of the European Convention and approved by the National Board of Animal Experimentation.

Ferret

Ferret embryos were collected from pregnant ferrets that were euthanized according to the guidelines for fur animals in fur farming research station of Agricultural Research Centre of Finland (Kannus, Finland). Embryos were collected at E28, E32, and E34, fixed, decalcified in Morse's solution, and embedded in paraffin.

Reptiles

Ball python, corn snake, and gecko embryos were provided by Triple-R-Corns (Aldergrove, BC, Canada), fertilized alligator eggs were collected in the Rockefeller Wildlife Refuge (Louisiana), and iguanas were from a local vendor and kept in the University of Southern California (USC) animal facility. The snakes and geckos were sacrificed according to procedures approved by the University of British Columbia animal ethics committee, protocol number A11-0352, and the alligators and iguanas were sacrificed according to the procedure approved by the Institutional Animal Care and Use Committee (IACUC) at USC. The snakes and geckos were fixed in 4% PFA; geckos were further decalsified in 7% EDTA in 2% PFA for 8 weeks, and tissues were embedded in paraffin and sectioned.

Human and tumor samples

Embryonal human jaw sections from 13-week-old fetuses were obtained from a human fetal collection owned by the University of Turku, Finland. Ameloblastoma samples from primary mandibular tumors of the solid/multicystic type were obtained during surgery at the Department of Oral Diseases, Turku University Central Hospital, Finland. Ethical approval was obtained from the Joint Authority for the Hospital District of Southwest Finland Ethics Committee, Turku, Finland (reference number 1/11 March 2007), and from the Finnish National Supervisory Authority for Welfare and Health (VALVIRA, reference number 648/32/300/05). The patients' written informed consent was obtained in accordance with the Helsinki Declaration of 1975, as revised in 1983. The tumor samples were snap-frozen in isopentane/liquid nitrogen, and 5 μm cryosections were cut and fixed in 4% PFA for immunostaining.

Table 2. Methods used in Studies I-III

Methods	*Used in*
Transcriptome microarray analysis	I
Radioactive *in situ* hybridization on sections	I, II, III
Whole mount *in situ* hybridization	II
miRNA *in situ* hybridization	I
Immunostaining	I, II, III
3D reconstruction of gene expression patterns	I
Organ culture	I, II
Preparation of living tissue slices	I
3D time lapse imaging and cell migration analysis	I
Hanging drop cultures and qRT-PCR	I
Apoptosis detection	I
Genetic lineage tracing	I, II

4.2 Transcriptome microarray analysis

The epithelium of the labial cervical loops and the whole proximal area containing both the labial and lingual cervical loops of the incisor were dissected from P2 NMRI mice. The labial cervical loop epithelium formed the first sample and the whole basal area formed the second sample, and biological triplicates for each sample were analyzed. The quality and concentration of RNA were monitored using a 2100 Bioanalyzer (Agilent Technologies). Biomedicum Genomics (Helsinki, Finland) processed and hybridized RNAs on the Agilent Mouse Genome 44K array, and GeneSpring GX11.0 software (Agilent) was used for data analysis. Limma software (Smyth and Speed, 2003; Smyth, 2004; Ritchie et al., 2007) was used to sort the functional enrichment of gene ontologies.

4.3 *In situ* hybridization

Radioactive *in situ* hybridization on 7 µm paraffin sections was carried out according to previously described standard protocols (Wilkinson and Green, 1990). RNA probes that were labeled with ^{35}S-UTP (Amersham) were used to detect expression of mouse *Sox2*, *Sfrp5*, *Fgf3*, *Fgf10*, *Fgf8*, *FgfrIc*, *Axin2*, *CD9,* and *Ldb1*.

MicroRNA *in situ* hybridization was performed on 7 µm paraffin sections as previously described (Michon et al., 2010) using double DIG-labeled LNA probes for U6 (positive control), scramble (negative control), mmu-miR-200b, and mmu-miR-720 (Exiqon).

Whole mount *in situ* hybridization was performed on PFA-fixed mouse lower jaws using InSituPro robot (Intavis AG). Digoxigenin-labeled probes were used to detect *Sox2* expression and BM Purple AP Substrate Precipitating Solution (Boehringer Mannheim) was used for visualization.

Table 3. Probes used in in situ hybridizations in Studies I-III

Probe	*Reference*	*Used in*
Axin2	Lustig et al., 2002	I
Fgf3	Kettunen et al., 2000	I
Fgf8	Kettunen and Thesleff, 1998	I
Fgf10	Kettunen et al., 2000	I
Fgfr1b	Kettunen et al., 1998	I
Ldb1	Zhao et al., 2007	I
Sfrp5	Witte et al., 2009	I
Sox2	Ferri et al., 2004	I, II, III

4.4 Reconstruction of gene expression patterns from *in situ* hybridization

The expression patterns of *Sox2* and *Sfrp5* were reconstructed in 3D from serial, frontal, and identical sections of P2 mouse incisor. The original size of the incisor was sustained by doubling the distance between the identical sections (14 µm). Photoshop CS4 software was used to import the images as a stack, and images were aligned using the midline of the jaw as the reference point. The shape of the epithelium was manually traced and filled white. ImageJ software (NCBI) was used to reslice the aligned images as sagittal sections. The jagged epithelial shape was

smoothened in Photoshop with a batch command: select white, expand two pixels, smooth 10 pixels. The filled epithelial shape was chosen with the color range tool and transferred into the path (tolerance 2 pixels), and the contour line was traced by the pencil tool onto a new layer. The isolated expression patterns of *Sox2* and *Sfrp5* were treated in the same sequence of steps as the epithelial shape and projected on the corresponding slice of the epithelial shape. The 3D images of the *Sox2* and *Sfrp5* expressions were superimposed on the epithelium.

4.5 Immunohistochemistry

Immunostaining was performed on paraffin and cryo sections. Rehydrated or melted sections were heated in a microwave in 10 mM sodium-citrate buffer (pH 6.0). Ultravision Large Volume Detection System Anti-Rabbit-kit, rabbit anti-Sox2 antibody (1:500-1:2000, Millipore) and anti-cytokeratin IgG (1:500 dilution; Abcam, Cambridge, UK), HRP Kit (Thermo Scientific) and the DAB Peroxidase Substrate Kit (Vector Laboratories, SK4100) were used to detect the expression of Sox2 and cytokeratin, respectively. Methyl Green was used to counterstain the sections.

4.6 Organ and live tissue slice culture

Embryonic and postnatal mouse molars and incisors were dissected and cultured in Trowell type organ culture (Sahlberg et al., 2002). The culture medium was changed every second day and contained DMEM and F12 (Ham's Nutrient Mix: Life Technologies) (1:1) supplemented with 10% fetal calf serum (PAA Laboratories), 150 mg/ml ascorbic acid, glutamine, and penicillin-streptomycin.

For confocal microscopy and DiI injections, the proximal area of the incisors from P2 Sox2-GFP and Fucci-red;Sox2-GFP mice were dissected in 2% glucose/PBS containing 100 U/ml penicillin and 100 mg/ml streptomycin and embedded in 5% low-melting point agarose (TopVision, Fermentas). Vibratome slices of 150 μm thickness were cut in sagittal or coronal orientation and tissue slices from Sox2-GFP mice were counterstained with BodipyTR (Invitrogen) and Draq5 (Biostatus). For time lapse imaging, the slices were allowed to recover from sectioning for a minimum of 2 hours and were maintained in DMEM/F12 without phenol red and supplemented with 5% serum, antibiotics, and 15 mM HEPES (GIBCO). The DiI labeling was performed by microinjections of fluorescent DiI (Invitrogen) to coronal slices of the incisor proximal area.

4.7 Confocal microscopy and migration analysis

Sagittally oriented tissue slices of the proximal end of P2 incisor were placed on a filter on a grid at the air-liquid interface for imaging with an upright Leica SP5 laser scanning confocal microscope equipped with an environmental chamber (5% CO_2 humidified and maintained at 37°C) and HC PL APO 103/0.4 air objective. Tissues were imaged every 20 minutes for up to 20 hours with low laser power (<10%, 600 Hz scanning speed), and confocal images were acquired as Z-stacks at 2 mm intervals. Suboptimal sampling by averaging of two scans was used to reduce extended-illumination-induced cell damage. The health of the tissue was confirmed by the frequency of mitoses and lack of pyknotic nuclei. AutoQuant software (Media Cybernetics) was used to deconvolute images, and Imaris 7.2.1 software (Bitplane) was used to analyze and perform the quantitative measurements. Prism 5 (GraphPad) and Sigmaplot 11.0 (Sigmaplot) softwares were used to statistically analyze and process the data. The angle of cell movement with respect to the labial-lingual axis was measured for individual cells, and the track length,

duration, cell net displacement distance, mean speed, and track straightness as the ratio of the displacement to total distance travelled were analyzed. A box-and-whiskers plot represented the minimum, 25th percentile, median, 75th percentile, and maximum values for the data set, and the nonparametric Mann-Whitney U-test was used for statistical analysis.

4.8 Inhibition assays and detection of apoptosis

Dissected cervical loops of P2 Sox2-GFP mice were cultured as previously described (Harada et al., 1999). To inhibit Fgf signaling globally 25 μM InSolution SU-5402 (Calbiochem) was added in standard medium (DMEM/F12 + 10% FCS). Monoclonal antibodies anti-Fgf8 (R&D) (200 μg/ml) with heparin (0.1 μg/μl) or anti-Fgf10 (C-17): sc-7375 (50 μg/ml; Santa Cruz Biotechnology, inc.) were included in the medium without serum to block specific Fgfs. Apoptosis was detected with ApopTag Red *In Situ* Apoptosis Detection Kit (Chemicon) from paraffin sections.

4.9 Hanging drop cultures and quantitative RT-PCR

Dissected E14.5 wild type incisors were used to analyze the induction of *Sox2* expression by Fgf8, Fgf9, and Fgf10. Tissues were allowed to recover for 30 minutes before placing in hanging drops. Tissues were collected into 700 μl TriReagent after 2, 4, and 6 hours and a minimum of triplicate samples was assayed each time. RNeasy Mini Kit (Qiagen, DE) was used to isolate total RNA and Nanodrop spectrophotometer was used for quantifying. cDNA synthesis and quantitative PCR were carried out as previously described (Fliniaux et al., 2008). The data were normalized against *Actin* expression and the standard deviation was calculated and the Student t-test was used to determine p-values for each triplicate. The p-value of $p < 0.01$ was deemed to be significant.

4.10 Genetic lineage tracing and X-Gal staining

Genetic lineage tracing of *Sox2*$^{+}$ cells was performed in embryos and posnatal Sox2CreERT2;R26RlacZ mice. For the embryonal lineage tracing 10 mg tamoxifen in corn oil (Sigma T-5648; Sigma-Aldrich) was given by oral gavage to pregnant females to induce the Cre driver in E13 Sox2CreERT2;R26RlacZ embryos. For the postnatal lineage tracing 0.3 mg tamoxifen in corn oil/pup was injected once (P2) or twice (P2 and P4), and for adult animals (5 weeks of age) 10 mg tamoxifen was given by oral gavage. Mice lacking the Cre-driver were used as controls. The whole mount X-Gal staining was carried out as previously described (Seidel et al., 2010), tissues were processed into paraffin, sectioned, and counterstained with Fast Red (Sigma-Aldrich). The research protocol involving the use of live animals was approved by the Animal Research Committee at Children's Hospital Los Angeles.

5 Results and discussion

5.1 *Sox2*⁺ stem cells of the mouse incisor give rise to all epithelial cell lineages of the tooth (I)

The continuous growth of the mouse incisor throughout life is fueled by stem cells that are located at the proximal end of the tooth. Although slowly cycling LRCs were identified in the incisor more than a decade ago (Harada et al., 1999), further identification and characterization of these stem cells has been hindered by lack of a specific marker. To find such a marker, a microarray gene-expression profile was conducted of the incisor labial cervical loop. Among the genes that were enriched in the labial cervical loop was *Sox2*, which marks the embryonic stem cells as well as various adult stem cell populations in many tissues (Avilion et al., 2003; Arnold et al., 2011; Sarkar and Hochedlinger, 2013) and is an iPS cell co-inducer (Takahashi et al., 2007).

Sox2 mRNA and protein expression analyses by *in situ* hybridization and immunohistochemistry, respectively, in the incisor at embryonic and postnatal stages revealed a dynamic expression pattern of Sox2 during incisor morphogenesis. At the initiation stage of incisor development, Sox2 was expressed in the oral epithelium and in the epithelium between the developing incisor placodes. At the cap stage, Sox2 expression was detected throughout the enamel organ, whereas at the bell stage, expression remained intense in the labial cervical loop while decreasing in the lingual cervical loop. During subsequent incisor morphogenesis, Sox2 expression in the enamel organ became gradually confined to a restricted area of the labial cervical loop containing a subset of cells from the SR and adjacent enamel epithelium (Figure 9). The lingual cervical loop did not express Sox2.

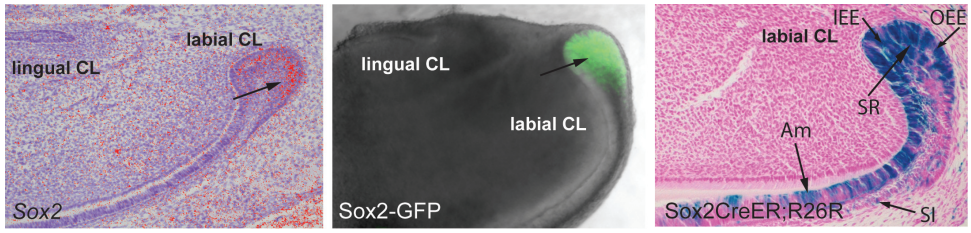

Figure 9. Sox2 expression and progeny of Sox2⁺ stem cells in the labial cervical loop of mouse incisor. (left) Sox2 mRNA (red) expression in a subset of SR cells and adjacent enamel epithelium in the labial cervical loop (arrow). (middle) GFP expression (green) in the labial cervical loop of the Sox2-GFP reporter mouse incisor in tissue culture. (right) LacZ-expressing (blue) progenitors from Sox2⁺ stem cells one month after tamoxifen induction. The distinct epithelial cell types derived from Sox2⁺ cells include SR, IEE, OEE, ameloblasts, and SI cells (arrows). Am, ameloblasts; CL, cervical loop; IEE, inner enamel epithelium; OEE, outer enamel epithelium; SI, stratum intermedium; SR, stellate reticulum

The Sox2-GFP reporter mouse made it possible to detect Sox2-GFP in incisor organ culture. The Sox2-GFP expression in the developing incisor showed similar localization to Sox2 mRNA and protein expression (Figure 9). To follow the movements of individual *Sox2*⁺ cells, a culture system was established allowing confocal time-lapse imaging of live tissue slices and 3D time-lapse tracking of cells. Living tissue slices were sectioned from the labial cervical loop of transgenic Fucci-red;Sox2-GFP mouse and individual *Sox2*⁺ cells were tracked over many hours.

Cell movement analysis revealed that *Sox2*$^+$ cells preferentially migrated from the SR toward the enamel epithelium, consistent with the previously presented model of stem cell movements in the labial cervical loop (Thesleff and Tummers, 2009). The results show that during development, the expression of Sox2 confines to a subset of SR cells in the postnatal labial cervical loop that corresponds to the location of the putative stem cells previously defined by LRCs and lineage tracing (Harada et al., 1999; Seidel et al., 2010), suggesting that Sox2 serves as a marker for the incisor stem cells.

Genetic lineage tracing of *Sox2*$^+$ cells in the incisor confirmed that *Sox2*$^+$ cells in the incisor are true stem cells. We genetically labeled *Sox2*-expressing cells and traced their descendants using an inducible Sox2CreERT2 strain crossed to a Cre-responsive R26RlacZ reporter line. The histological analysis after different lengths of cell chase revealed that *Sox2*$^+$ cells produced all the epithelial cell lineages of the incisor including IEE, OEE, SI, SR, ERM, and ameloblasts, showing the multipotent nature of these cells (Figure 9). After one month of chase, the progeny of the labeled *Sox2*$^+$ cells covered almost the entire labial cervical loop and the progeny showed clonal origin (Figure 9). These results demonstrate conclusively that stem cells in the incisor are *Sox2*$^+$. However, all *Sox2*$^+$ cells in the labial cervical loop are not necessarily stem cells. Additionally, it is also possible *Sox2*-negative stem cell populations within the labial cervical loop exist.

Interestingly, although the *Sox2*$^+$ stem cells produced plentiful amounts of ameloblasts and other cell types of the incisor crown-analog, we did not detect many labeled ERM cells in the lingual root-analog of the incisor. Thus, most ERM cells are likely produced by another stem cell pool. Indeed, a small number of LRCs have been detected in the lingual cervical loop (Seidel et al., 2010) but not much is known about these cells and whether they possess stem cell characteristics.

Among the genes enriched in the labial cervical loop was *Sfrp5,* an antagonist of non-canonical Wnt signaling. It showed a peculiar expression pattern restricted to a specific area in the border between OEE and IEE that surrounded the open proximal end of the incisor. The 3D reconstruction of *Sox2* and *Sfrp5* expression revealed complementary expression pattern of these genes and showed that *Sfrp5* transcripts were absent from the SR (Figure 10).

Surprisingly, these expression patterns were reminiscent of the distribution of two 3H-thymidine LRC populations previously described by Smith (1980) (Smith, 1980). Smith proposed in his study that the labeling dynamics of these two cell populations reflects the derivation of a more actively dividing progenitor pool from the slowly-dividing stem cells located in the labial cervical loop of the rodent incisor. Interestingly, the LRCs that Smith detected 2 days after the chase corresponded to the *Sox2*$^+$ cells in the labial cervical loop and the LRCs detected after 4 days chase corresponded to the *Sfrp5*$^+$ area of the IEE/OEE border suggesting that *Sfrp5* is a marker for the early progeny of the *Sox2*$^+$ stem cells. Injection of DiI label to *Sox2*$^+$ cells and the tracing of cells to the area of *Sfrp5* expression confirmed that *Sox2*$^+$ cells give rise to the *Sfrp5*$^+$ progenitors in the incisor. Interestingly, Sfrp5 regulates the active and quiescent stem cell pools in the intestine (Li and Clevers, 2010), suggesting that in the incisor it may have a similar function regulating the incisor stem cell progenitors prior to their differentiation towards TA cells.

Wnt signaling is associated with the maintenance of the quiescent and active stem cells in the intestine and in the hair bulge (Li and Clevers, 2010). Interestingly, both Sox2 and Sfrp5 are involved in Wnt pathway inhibition (Kelberman et al., 2008; Li et al., 2008). In the incisor, active canonical Wnt signaling is absent as demonstrated by studies on Wnt pathway effectors (Suomalainen and Thesleff, 2010) and reporter mice (Suomalainen and Thesleff, 2010), this

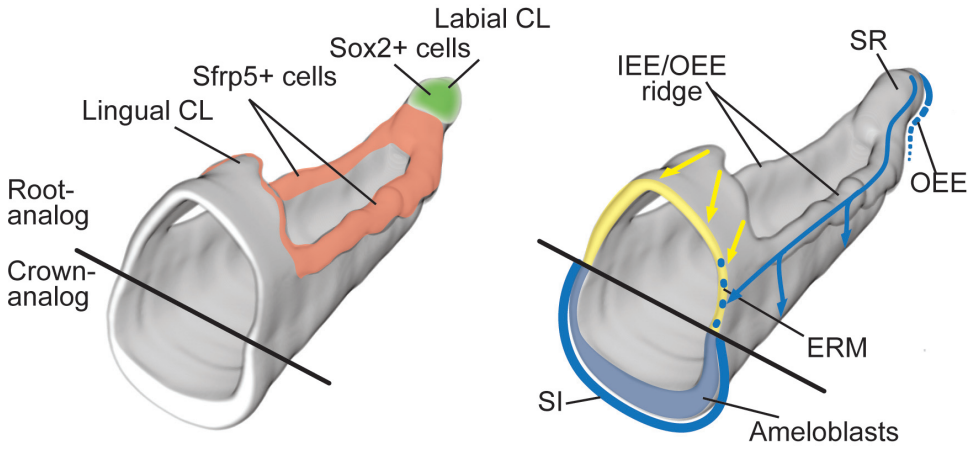

Figure 10. Schematic presentations of Sox2 and Sfrp5 expression patterns and the generation of distinct epithelial cell types from Sox2⁺ stem cells. (left) 3D-reconstruction of Sox2 (green) and Sfrp5 (red) expression patterns from *in situ* hybridization. Sfrp5 expression forms a circle marking the cells of the IEE/OEE ridge in the proximal opening of the incisor. Sox2 expression is restricted to the tip of the labial cervical loop and does not overlap with Sfrp5 expression. (right) Sox2⁺ stem cells give rise to progeny (blue) that go through the Sfrp5⁺ area and form the ameloblasts, SI, SR and OEE cells, and part of the ERM. The lingual cervical loop probably gives rise to most of the ERM cells of the root-analog (yellow).
CL, cervical loop; ERM, epithelial cell rests of Malassez; IEE, inner enamel epithelium; OEE, outer enamel epithelium. Modified from Study I.

study). In addition, we identified two Wnt inhibitors, *Ldb1* and *CD9* in the labial cervical loop. Our results suggest that downregulation of Wnt signaling is important in the incisor stem cell niche, possibly preventing cell differentiation and thus keeping the cells in an undifferentiated naive state. Further studies on Wnt signaling and Sfrp5 are required to understand their association with incisor epithelial stem and progenitor cells.

After the Study I was published, it was suggested that the mouse incisor indeed contains both slow-cycling LRCs and a more active *Lgr5*⁺ population of dental epithelial stem cells (Chang et al., 2013b). Both of these populations were isolated by sorting CD49f/integrin α6 –positive cells; they were able to self-renew and expressed characteristic genes of all dental epithelial cell lineages. In addition, *Bmi1* was identified as a marker for the epithelial stem cells of the labial cervical loop and it was shown to be required for the maintenance of these cells (Biehs et al., 2013). The actions of Bmi1 are mediated through repression of Ink4a/Arf expression to permit stem cell self-renewal and suppression of Hox genes to prevent inappropriate cell differentiation (Biehs et al., 2013). In our study, *Sox2* was expressed in the P2 incisor in a wider area including the more anterior part of the IEE, compared to *Bmi1*, *Gli1*, and *Lgr5* (Seidel et al., 2010; Chang et al., 2013b; Biehs et al., 2013). Notably, however, the expression patterns of these other stem cell markers were obtained from adult incisor (P28-P70) (Seidel et al., 2010; Chang et al., 2013b; Biehs et al., 2013). The P2 stage was selected in the beginning of our study because the incisor is relatively easy to dissect, and this stage had been previously used in many studies on stem cells. The adult stage would, however, most probably give a more realistic view of the function of stem cells in adult homeostasis and of the role of Sox2 in this process. It would

be interesting to compare the expression of all known dental epithelial stem cell markers at the same developmental stage of the incisor to better understand the relationship of these stem cell populations and their different properties.

5.2 *Sox2*$^+$ stem cells are regulated by Fgf8 and miRNAs (I)

During development, stem cell homeostasis, and tissue regeneration, Sox2 expression levels can be regulated positively and negatively by the molecules of the major signaling pathways (Takemoto et al., 2006; Domyan et al., 2011). Epithelial stem cells in the mouse incisor are regulated by Fgf signaling (Harada et al., 2002; Klein et al., 2008; Tummers and Thesleff, 2009). *Fgf3* and *Fgf10* are expressed in the mesenchyme in close proximity to the labial cervical loop (Harada et al., 1999), whereas *Fgf9* is expressed in the epithelial TA cells (Yokohama-Tamaki et al., 2008). Receptors for Fgf10, *Fgfr1b* and *Fgfr2b,* are coexpressed in the stem cell area (Harada et al., 1999). To study the effects of the Fgf signaling pathway on Sox2 expression in the incisor stem cells, we used a Sox2-GFP reporter mouse and followed the changes in GFP expression *in vitro* in tooth organ culture.

Global inhibition of Fgf signaling in the labial cervical loop led to the disappearance of Sox2 expression, demonstrating that Fgfs are required for Sox2 expression. Since global inhibition does not differentiate between separate Fgfs, we inhibited the actions of individual Fgfs in culture by adding antibodies against Ffg10 and Fgf8. Fgf10 is essential for the epithelial stem cell maintenance in the labial cervical loop (Harada et al., 2002), and *Fgf8* is expressed in the SR of the postnatal incisor (Wang et al., 2009) and it is essential during early tooth development (Trumpp et al., 1999). Fgf8 is also known to induce Sox2 expression in the olfactory epithelium (Tucker et al., 2010). Surprisingly, blocking Fgf10 in incisor culture did not have any effect on Sox2 expression although its receptors are expressed in the labial cervical loop. Instead, inhibition of Fgf8 decreased *Sox2* expression in a subset of *Sox2*$^+$ cells located in the OEE and part of the SR. To verify the role of Fgf8 on *Sox2* expression we cultured incisors in hanging drops to which Fgf8, Fgf9, and Fgf10 proteins were added. This experiment demonstrated that Fgf8 and Fgf9 could directly activate *Sox2* expression, whereas Fgf10 had no effect on *Sox2* expression. The reason for lack of regulation of *Sox2*$^+$ cells by Fgf10 might be that Fgf10 affects a distinct population of stem/progenitor cells in the labial cervical loop and that *Sox2*$^+$ stem cells do not belong to that cell pool. Indeed, a recent report showed that Fgf signaling is essential for self-renewal of the active *Lgr5*$^+$ stem cells but not of the slowly cycling population of stem cells of the cervical loop (Chang et al., 2013a). However, they did not study the separate actions of different Fgf proteins on these two stem cell populations (Chang et al., 2013a).

MicroRNAs are small non-coding RNAs that regulate signaling pathways during morphogenesis and organogenesis and take part in the control of embryonic and adult stem cells (Pauli et al., 2011; Yi and Fuchs, 2012) as well as facilitate reprogramming of somatic cells to iPS cells (Anokye-Danso et al., 2012). miRNAs also take part in tooth development and in differentiation of tooth-specific cell lineages (Michon et al., 2010; Cao et al., 2010). MicroRNA microarray profiling of the incisor revealed specific miRNAs that are enriched in the labial cervical loop (Michon et al., 2010). Among these were miR720 and miR200b. By luciferase assay we demonstrated that miR720 regulates Fgf8 and that miR200b regulates Sox2 expression. LNA *in situ* hybridization confirmed that both of these miRNAs are specifically expressed in the IEE and OEE of the labial cervical loop. To test if the global deletion of all miRNAs in the dental epithelium has an effect on Sox2 and Fgf8 expression we generated a mouse line in

which Dicer-1 was conditionally knocked out in the epithelium. In the mutant mice, the dental epithelium formed numerous extra foldings and ectopic *Sox2* expression was detected in the lingual cervical loop and in the TA and ameloblast regions of the labial side of the incisor. We did not detect any clear difference in *Fgf8* expression between the mutant and the control animals. Our demonstration that Fgf8 and Sox2 are regulated by miR720 and miR200b, respectively, and that deletion of miRNAs leads to loss of homeostasis in the dental epithelium is further supported by our finding that ectopic and increased *Sox2* expression was detected in the labial and lingual cervical loop of the Dicer-1 conditional knockout mouse. Consistent with our results, a recent study showed that miRNAs in the miR200 family directly repress Sox2 in neural stem/progenitor cells, suggesting that the miR200:Sox2 feedback loop might be a general mechanism to control the transition of cells from undifferentiated to a more differentiated state (Peng et al., 2012). Moreover, the Pitx2:miR200c:noggin pathway regulates Bmp signaling and ameloblast differentiation in the incisor (Cao et al., 2013), supporting our observations of the role of the miR200 family in the regulation of incisor epithelial stem cells.

5.3 *Sox2*$^+$ cells of the dental lamina have competence for tooth generation in different species (II)

The capacity for tooth replacement is restricted in mammals, which can normally replace their teeth only once. However, many vertebrates such as fish and reptiles have the capacity for continuous tooth replacement. In addition to the continuous growth of rodent incisors, the epithelial stem cells are required for continuous tooth replacement, and label-retaining putative stem cells have been identified in the dental epithelium of gecko and alligator (Handrigan et al., 2010; Wu et al., 2013). Because Sox2 was identified as a marker for epithelial stem cells in the continuously growing mouse incisor (Study I) we examined if *Sox2*$^+$ cells exist in epithelial progenitors during tooth initiation. Sox2 was expressed in the mouse primary dental lamina, which appears in the embryonic jaws in locations of future dental arches prior to tooth initiation. This primary dental lamina represents the origin of all teeth epithelia and is proposed to house stem cells for all dental epithelial tissues (Smith et al., 2009). Although mouse teeth are not replaced, during mouse molar development, Sox2 expression specifically localized to the lingual dental lamina, which is the known site for replacement tooth formation in other species (Ooë, 1981; Järvinen et al., 2009; Handrigan et al., 2010). Since Sox2 expression localized to this specific site of the mouse dental lamina, we explored its expression in the dental lamina in species that replace their teeth.

Ferret teeth are replaced once as in human. Interestingly, Sox2 expression was detected in the lingual side of the dental lamina similarly as in mice, but it was also detected in the successional dental lamina that gives rise to the replacement teeth and forms at the lingual side of the developing primary teeth. *Sox2*$^+$ cells were detected on the lingual edge along the successional lamina but Sox2 was absent from the free end of the lamina. A continuous stripe of *Sox2*$^+$ cells formed between the primary and secondary teeth starting from the dental lamina and OEE of the primary tooth continuing to the successional lamina and OEE of the secondary tooth.

To study the expression of Sox2 in the dental lamina of teeth that are continuously replaced, we obtained samples from alligator, iguana, leopard gecko, ball python, and corn snake. In alligator, iguana, and gecko, Sox2 expression was similar to that in the ferret: restricted to the dental lamina and the successional lamina. In the snakes, two differences in Sox2 expression were detected: there was no lingual shift of *Sox2*$^+$ cells in the successional lamina and the free

end of the successional lamina was Sox2 positive. Sox2 expression in the lingual dental lamina coincided with the localization of the label-retaining putative stem cells of gecko (Handrigan et al., 2010), although Sox2 was expressed in a wider area. In alligator, the LRCs are located in the distal tip of the successional dental lamina (Wu et al., 2013). However, *Sox2*$^{+}$ cells were not abundant in that area.

Even though mouse teeth are not replaced and all its teeth are primary teeth, successional tooth formation occurs in mice when the three molars develop in a row by serial addition. The distal aspect of the dental epithelium of the first molar gives rise to an epithelial bud, which forms the second molar, and the third molar originates in the same way from the second molar. Successional tooth formation also occurs within primary tooth families. For instance, premolars are sequentially added in other species, such as ferret, in a process similar to molar addition (Jussila et al., 2014).

During the successional formation of mouse molars, Sox2 expression localized to the distal part of the first molar dental lamina, which is the site for the budding of the epithelium that gives rise to the second molar. This expression pattern was repeated in the second molar as the third molar developed. In addition to localization of the Sox2 mRNA and protein in sections, we followed the dynamic expression of Sox2 in molar culture of a Sox2-GFP reporter mouse. Sox2-GFP expression was consistent with the mRNA and protein expression pattern and was detected at the sites where additional tooth formation took place during the serial addition of molars.

To demonstrate that *Sox2*$^{+}$ cells of the dental lamina of the first molar have the capacity for tooth formation, we took advantage of the same genetic inducible lineage tracing method that was used in Study I and showed that *Sox2*$^{+}$ cells from the mouse first molar give rise to the epithelial cells of the second and third molar. *Sox2*$^{+}$ cells gave rise to the ameloblasts, OEE and IEE, and the dental lamina cells of the second and third molars, indicating that *Sox2*$^{+}$ cells of the dental lamina are able to produce the different epithelial cell lineages required for the formation of a complete tooth.

To investigate the role of Sox2 in the tooth-forming epithelium, we generated the Shh::GFPCre;Sox2$^{fl/fl}$ mouse line. Our analyses were restricted to the embryonic stages of tooth development since these mice died at birth. Surprisingly, the molar phenotype was mild. The dental cord and the dental lamina were hyperplastic, but no gross differences in the tooth number or morphology were detected. This might be due to the low activity or mosaic activation of the recombinase in the targeted epithelial cells. Also, redundant functions of other Sox proteins could have led to this mild phenotype. Given the following pieces of evidence, we suggest that Sox2 may regulate the amount of dental epithelium. First, we observed hyperplastic epithelium in the *Sox2* cKOs. Second, *SOX2* mutation is associated with supernumerary tooth formation in humans (Numakura et al., 2010). Finally, Sox2 is a Wnt inhibitor and Wnt over-activation leads to supernumerary tooth formation as evidenced in many studies (Järvinen et al., 2006; Wang et al., 2009).

After Study II was published, Gaete and Tucker similarly showed that in the corn snake, Sox2 is expressed in the dental lamina whereas *Lef1*, a Wnt/β-catenin pathway target gene, was expressed in the successional lamina adjacent to the Sox2-expressing area of the dental lamina (Gaete and Tucker, 2013). Furthermore, activation of Wnt/β-catenin signaling in snake produced supernumerary teeth in an ectopic position of dental lamina (Gaete and Tucker, 2013).

Although the formation of molars stops after the third molar has formed, supernumerary 4th molars occasionally develop in mouse and human (Shahzad and Roth, 2012). An extreme example of successional molar formation occurs in the dentition of the silvery mole rat

(*Heliophobius argenteocinereus*) (Rodrigues et al., 2011). It continues to produce new molars distally while the most anterior teeth in the tooth row are shed away, resembling the process of continuous replacement described in reptiles (Whitlock and Richman, 2013). It is possible that the capacity for continuous tooth formation exists in mammals. This is supported by the fact that several generations of replacement teeth as well as supernumerary successional molars develop in patients with cleidocranial dysplasia and that several mutations lead to supernumerary tooth formation in mice and humans (Wang and Fan, 2011). The two processes, tooth replacement and successional molar formation, resemble each other and share similarities in morphology, developmental timing, and Sox2 expression. It can be suggested that these two modes of tooth addition represent variations of the same developmental process, differing only in the orientation and direction of new tooth formation.

In conclusion, Sox2 expression is conserved in mammals and reptiles and it is associated with the sites of dental epithelium where the generation of new teeth occurs. At least some of the *Sox2*$^{+}$ cells in the dental lamina are stem cells and these cells form the capacity for continuous tooth replacement and serial addition of teeth. Our results detailing Sox2 expression and genetic lineage tracing of *Sox2*$^{+}$ cells suggest a connection with *Sox2*$^{+}$ cells and the epithelial competence for tooth formation.

5.4 Ameloblastomas and dental lamina fragments express Sox2 (III)

Ameloblastoma is an odontogenic tumor, which is thought to derive from the rests of dental epithelium but its specific origin has remained unknown (Philipsen et al., 2005). Histologically ameloblastoma tumor islands resemble bell-stage tooth germs with stellate reticulum-like cells surrounded by basal epithelium. The ameloblastoma epithelium does not differentiate into secretory ameloblasts, there is no hard-tissue formation, and the fibrous stromal tissue of ameloblastoma has no characteristics of dental mesenchyme (Philipsen et al., 2005).

The findings in Studies I and II led us to explore whether Sox2 expression is also associated with the formation of ameloblastomas. The epithelial structures of the ameloblastoma closely resemble the labial cervical loop of the mouse incisor, where *Sox2*$^{+}$ stem cells are detected. Moreover, in ameloblastoma, the production of preameloblast-like cells is similarly continuous as in the mouse incisor. Indeed, Sox2 was expressed in the tumor epithelium in two different types of ameloblastomas, the follicular and the plexiform ameloblastoma. Sox2 expression was located to the palisade epithelial layers of the epithelial islands as well as to the SR-like cells. The surrounding mesenchyme was mostly negative for Sox2.

Since ameloblastoma most often occurs in the region of the lower third molar, we followed in mice the fragmentation of the dental lamina that connects the third molar to the second molar by histological sections. Interestingly, intense Sox2 expression was detected in the fragments of the third molar successional lamina. Sox2 expression was also detected in the posterior aspect of the third molar, which is the site for the development of an occasional 4th molar. The dental lamina of a human primary molar also expressed SOX2. The epithelial cells that cover the root part of the tooth, the HERS and later ERM, and have also been suggested as possible origins of ameloblastomas (Takeda and Yamamoto, 1990), but Sox2 was not expressed in these epithelial cells.

Supernumerary tooth formation is induced when Wnt signaling is constitutively activated in the epithelium of K14cre/+;β-cat$^{\Delta ex3fl}$ mouse line. *Sox2* expression was associated with the epithelial sites where supernumerary tooth formation occurred. When a single molar bud from

the K14cre/+;β-cat$^{\Delta ex3f}$ mouse is transplanted under a kidney capsule, dozens of teeth develop (Järvinen et al., 2006). This tumor-like mass of teeth resembles the odontoma tumor in humans. In these tumors, *Sox2*$^{+}$ cell clusters were detected between the supernumary teeth, suggesting that these *Sox2*$^{+}$ cell clusters could be the initiative cells for tumor growth.

The findings in Study I and II that Sox2 is an epithelial stem cell marker and that *Sox2*$^{+}$ cells have the capacity for tooth formation support the idea that the *Sox2*$^{+}$ cells in the dental lamina could be the founder cells of the ameloblastoma. In addition, the location of Sox2 expression in the dental lamina associated with the third molar, the typical site for ameloblastoma, hints toward this possibility of tumor origin. As the third molars develop late in the course of the development of human dentition, the dental lamina may therefore be more likely to accumulate mutations that initiate tumor growth. In addition, the abundance of *Sox2*$^{+}$ cell clusters in the induced odontomas is in line with the *Sox2*$^{+}$ origin of the tumor. However, to show that ameloblastomas originate from *Sox2*$^{+}$ dental lamina cells would require genetic lineage tracing experiments.

6 Concluding remarks

Recent advancements in the incisor stem cell field have lead to the identification of several stem cell-specific markers, including *Sox2* (Study I) (Chavez et al., 2013; Chang et al., 2013b; Biehs et al., 2013). The results from several studies point out that the incisor stem cell niche likely contains distinct stem cell subpopulations with differing dynamics (Study1) (Seidel et al., 2010; Chang et al., 2013b). We have shown that the *Sox2*$^+$ population is regulated by Fgf8, but not Fgf10, and thus we suggest that these subpopulations are most likely regulated by different factors. Indeed, in other epithelial organs stem cells with different roles are proposed to associate with distinct niche compartments (Greco and Guo, 2010). In the rapidly renewing organs such as intestine and hair, the stem cell niche is organized into two compartments: one that is engaged with immediate, rapid new growth, and one that contributes later to long-term growth (Greco and Guo, 2010). In addition, it was recently shown that the location of the stem cells in the niche can predict the cell fate in hair follicle (Rompolas et al., 2013). To determine whether similar features govern the incisor stem cell niche it would be essential to compare the location, the molecular and functional differences, and the colony forming ability of the subpopulations. How *Sox2*$^+$ cells are related to the stem cell subpopulations is a subject for further studies.

Sox2 expression is also associated with the two forms of tooth renewal, the successional tooth formation in tooth replacement and serial addition of primary teeth (Study II). The finding that Sox2 is located within a specific site in the dental lamina both in reptiles with continuous tooth replacement and in mammals with one round of replacement implies that the function of Sox2 has been conserved during evolution. Based on the morphological studies and the conserved expression pattern of Sox2 we suggest that tooth replacement and serial addition of primary teeth represent variations of the same developmental process. Hence, the serial addition of mouse molars may be used as a model to understand the signaling networks that are required for replacement tooth development. The molecular mechanisms underlying the activation of the successional dental lamina to form replacement teeth is not known at present, but it can be speculated that the dental lamina has an intrinsic ability to form new teeth. Our results indicate that the Sox2$^+$ dental epithelium has competence for successional tooth formation and that Sox2 regulates the progenitor state of dental epithelial cells. Together, this data supports the notion that there is dormant capacity in mammals for continuous tooth renewal embedded in the dental lamina.

An interesting question is whether the *Sox2*$^+$ stem cells are similar in the incisor cervical loop where they fuel continuous tooth growth and in the dental lamina where they contribute to successional tooth formation. Do they originate from the same fetal stem cells and how do they home to their niches? How are the stem cell niches established in the first place? Are they set aside during development or are they established later from the early progenitor cells by the cues coming from the niche? A recent study showed that early *Sox2*$^+$ fetal progenitors are the precursors for *Sox2*$^+$ adult stem cells in some organs such as stomach, tongue, lungs, and brain (Arnold et al., 2011). Our Sox2 *in situ* hybridization supported this study since during incisor morphogenesis Sox2 expression was gradually restricted to the labial cervical loop stem cell niche (Study I).

Sox2 is associated with the cells that have the capacity to renew epithelial dental tissues (Study I, Study II) and in line with this are our results from odontogenic tumors. Sox2 expression was detected in odontomas induced in transgenic mice and in human ameloblastomas, in which preameloblast-like epithelial tumor cells are continuously produced (Study III) (Figure 11).

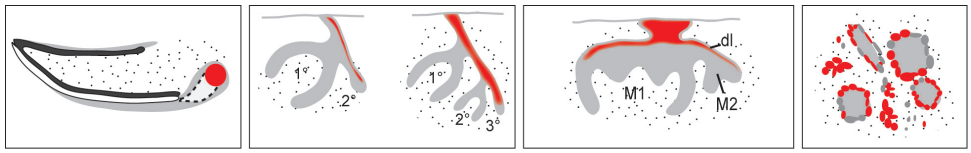

Figure 11. Sox2 expression is associated with the capacity to generate epithelial dental tissues. Sox2 expression (red) is localized to stem cells of the continuously growing mouse incisor, dental lamina that participates in tooth replacement and in serial addition of molars, and ameloblastoma epithelium. dl, dental lamina; M, molar; sl, successional lamina; 1°, 2°, 3°, tooth generation

Since Sox2 is a pluripotency marker and expressed in multipotent adult stem cells, SOX2 expression in ameloblastoma may be associated with the stem cell-like characteristics of the tumor cells, and $SOX2^+$ tumor cells might participate in the formation of recurrent ameloblastomas. Moreover, Sox2 is linked to the progression of various tumors and promotes tumor growth (Sarkar and Hochedlinger, 2013). Whether Sox2 is already expressed in the cell of origin for ameloblastoma or whether it is activated ectopically is a subject for further studies.

How stem cells are maintained and how their proliferation and differentiation are regulated are biologically important questions with wide implications. As such, the results of my thesis provide valuable new insights into the role of stem cells in health and disease. In addition, this study potentially has a high societal impact, as basic research of dental stem cells is a prerequisite for paving the way for clinical applications such as bioengineering of teeth. The iPS cells could be used for tooth bioengineering but it would require the knowledge how to program iPS cells to dental epithelial and mesenchymal fates to trigger the tooth to develop. In the future, the results of my thesis can aid in efforts to grow replacement teeth from human iPS cells.

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Emma Juuri

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