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Review Article

Mapping the milestones in tooth regeneration: Current trends and future research

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ABSTRACT

Research into finding the perfect replacement for lost dentition is an ever-evolving and rapidly advancing subject involving many scientific disciplines. The present consensus appears to be that regeneration of tooth in morphological and functional form is the ideal answer to lost tooth replacement. This article traces the milestones in this elusive search for the ultimate tooth replacement. The various research developments are highlighted that are aimed at the final goal of being able to “re-grow a natural tooth”. Whole tooth regeneration is technically challenging and further research into this field of complex molecular biology, embryology, biomaterials and stem cells is required to answer the unsolved questions. However, the milestones that have been crossed in the attempts at whole tooth regeneration have been remarkable and the future is quite promising. This article highlights the noteworthy research work that is being done in the field of whole tooth regeneration with a view to not only inform the clinicians of the significant developments but also inspire them to actively participate in this rapidly evolving field.

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Introduction

Regeneration of lost tissue or organs for rehabilitation of patients has been the ultimate dream of every clinician and healthcare researcher. Due to the unique, multifarious role of the teeth and associated structures there has been a sustained effort to replace the missing dentition over many centuries. From the prehistoric attempts of wiring together extracted teeth, various denture designs and materials to the development of dental implants the search has had many unique

breakthroughs and path-breaking developments. Despite all its advantages, dental implants (currently considered to be the best alternative) have certain inherent drawbacks. They lack the 3-dimensional structure of natural teeth and consequently their functionality, such as a periodontal ligament which gives a sense of proprioception and cushioning effect; pulpal tissue that act as a reparative source of cells and the lack of thermal stimuli through nerve endings' that all of which have got a protective role teleologically.

The consensus, therefore, has been that regeneration of tooth in morphological and functional form is the only perfect

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answer to lost tooth replacement. This article traces the milestones in this elusive search for the ultimate tooth replacement. We attempt to highlight the various research developments that are aimed at the final goal of being able to “re-grow a natural tooth”. This article highlights the noteworthy research work that is being done in the field of whole tooth regeneration with a view to not only inform the clinicians of the significant developments but also inspire them to actively participate in this rapidly evolving field.

Background

To understand the models of whole tooth regeneration, we need to appreciate certain concepts of evolutionary biology and embryology. These biological processes may be replicated in the laboratory via tissue engineering process using stem cells.

Evolutionary biology

As we move up the evolutionary chain from fish to reptiles to mammals, we see that the following characteristics are observed: the total number of teeth decrease (from polyodonty to oligodonty); morphological complexity increases (from homodonty to heterodonty) and tooth regeneration capability also decreases (from polyphyodonty to di- and/or monophyodonty).¹ Many non-mammalian vertebrates are polyphyodonts and have cyclical rounds of tooth regeneration throughout life e.g. – in an adult cichlid fish each tooth is replaced every 30–100 days. However, most mammals like humans are diphyodont and gets only two sets of teeth in a life span. In contrast, mice have one set of dentition which comprises one incisor and three molars in a single row in each quadrant of the jaw. These incisors have a self-renewal capacity with the continuous deposition of enamel labially. This self-renewal or self-regenerative ability of tooth is due to presence of stem cells in post-embryonic tissues.²

Many explanations have been postulated of the manner in which the evolutionary process has made its imprint into the biomolecular level, for evolving vertebrates from polyphyodonty to di- and/or monophyodonty. This may be via, a change in genetic expression, followed by modification in post-transcription and translational pathway leading to modification of signaling.³

Embryology and signaling

Not only are the teeth functionally and morphologically unique in the human body, but even the process of odontogenesis is remarkable in the sense of its functional outcome i.e. the teeth are region-specific (incisor and canines in anterior to premolars and molars in posterior regions of the jaws). The stages of tooth development have been classically described, from early to late as: Lamina, Bud, Cap and Bell stages (early and late stage), by the morphologic changes which are discernible under the microscope.⁴

In recent years, with a better understanding of molecular control, the process of odontogenesis can be broadly described

as three closely-linked processes – initiation, patterning and morphogenesis which are all integrally associated with sequential differentiation. All these stages are controlled by highly complicated, sequential mutual interactions between two major cell types: stomodeal ectoderm and ectomesenchyme (derived from neural crest cells). These mutual interactions are regulated by growth factors, transcription factors (approx. 100) and odontogenic genes (approx. 300). The site of tooth formation, morphological differences (incisor or molar) and other phenotypical characteristics are controlled by different spatiotemporal expression of odontogenic genes.^{1,5}

Initiation of teeth and determination of position

All teeth, including supernumeraries, develop only in the dental lamina. Initially FGF8 (Fibroblast Growth Factor) activity in the presumptive dental epithelium determines the oral-aboral axis of 1st branchial arch. Later, FGF & BMP (Bone Morphogenetic Protein) signaling in epithelium determine the tooth-forming site by regulating genetic expression of signaling molecules such as Pax9 in mesenchyme & Pitx2 in dental lamina.⁵ Studies, indicate *Wingless* (Wnt) and *Sonic hedgehog* signaling (*Shh*) pathway also play a role in positional specification. Initially *Shh* is only expressed at regions where the tooth formation is desirable, while *Wnt7b* is expressed in the other areas of the oral epithelium. Ectopic expression of *Wnt7b* in dental epithelium represses *Shh* signaling, leading to inhibition of tooth formation. This highly coordinated genetic interaction helps to limit the tooth formation to specific regions in the jaws.⁶

Tooth identity or patterning

Tissue recombination experiments have confirmed that early information for patterning of tooth identity (incisor vs molar) remains in the presumptive dental epithelium even before neural crest-derived cells migrate to the branchial arch. Again FGF8 and BMP4 are master molecules in induction of the earliest patterning information in the ectomesenchyme by a spatial pattern of genetic expression called 'odontogenic homeobox code'.^{5,7} This odontogenic homeobox code basically consists of a specific combination of homeobox genes in the ectomesenchyme that determines type and shape of teeth. Expression of *Dlx* and *Barx1* in ectomesenchyme are specific for molar teeth pattern, whereas *Msx1*, *Msx2* are determinants for incisor formation.⁸

Tooth morphogenesis

The determination of tooth-crown shape occurs at the time of bud stage. The 'odontogenic homeobox code' specifies tooth crown shape and regulates epithelial folding at the transition of Bud to Cap stage. The transition from Bud to Cap stage is one of the most critical steps in morphogenesis – when the enamel knot forms in dental epithelium and acts as a signaling center for control of crown shape. Nearly 10 signaling molecules belonging to BMP, FGF, Wnt and *Shh* families, are expressed in

enamel knot. After disappearance of the primary knot, secondary enamel knot develops at future cusp tips in multi-cusped teeth and controls the pattern of cusp shapes.⁵ Another important family of protein is found in enamel knot signaling center, that is Tumor Necrosis Factor family, which has been studied in *Tabby*, *Downless* and *Crinkled* mutant mice where size and number of molar cusps are significantly reduced with missing incisors and molars.^{9,10}

Tissue recombination technique: understanding odontogenesis

Early experiments (Table 1) with tissue recombination, not only unfolded mystery of complex epithelial-mesenchymal interaction during formation of tooth, but helped in the generation of tooth outside oral cavity. Huggins¹¹ in 1934 successfully generated dentin and enamel ectopically after transplantation of tooth germs in abdominal wall of pups. An interesting finding in their experiments was that segregated

epithelium in isolation failed to develop an enamel structure, proving the combined importance of dental ectomesenchyme in guiding the formation of tooth. Later many recombination experiments were done by isolating tooth germ elements from different developmental stages in various spatial and temporal relations. Mina et al.¹² in 1987 formed dental papilla from non-odontogenic ectomesenchyme of second arch when combined with epithelia of mandibular arch before embryonic day 12 in mouse and proved that odontogenic potential is present in the epithelium even before Bud stage of tooth development. The ground-breaking experiments of Kollar et al.^{13,14} with Cap and Bell stage tooth germs of mouse revealed the subsequent switching of the odontogenic potential to the dental papilla which later guides the formation of tooth and its eventual shape. The role of dental ectomesenchyme to induce tooth formation was unequivocally established when Kollar et al.¹⁵ in 1970 and later in 1980 Kollar et al.¹⁶ regenerated tooth structure in anterior chamber of eye by recombining mouse dental papilla with epithelium from foot and snout of mouse and later with chick oral epithelium.¹⁶

Table 1 – Summary of studies on tooth development.

S. no	Year	Author	Source of cells	Age/stage	Method	Outcome
1.	1934	Huggins ¹¹	Epithelial and mesenchymal layers of developing tooth germs of young dogs	3–6 weeks	Recombined in various relationship, and transplanted into abdominal wall	Epithelial layer did not produce enamel in absence of mesenchymal layer
2.	1969	Kollar et al. ¹³	Dental epithelium and papilla from mouse embryo	Embryonic day (ED) 13–16	64 types of tissue re-combinations were done	Odontogenic potency exists in mesenchyme
3.	1970	Kollar et al. ¹⁴	1. Incisor enamel organ/lip-furrow epithelium 2. Incisor/molar papilla of mouse	Embryonic day ED 14–17	Tissue recombination and transplant in anterior chamber of eye	Successful generation of tooth structure when epithelium combined with dental papilla; tooth shape (incisor/molar) is governed by the dental papilla
4.	1970	Kollar et al. ¹⁵	1. Epithelium from foot plate and snout 2. Incisor/molar mesenchyme of mouse	Embryonic day 14–16	Tissue recombination and transplant in anterior chamber of eye	Proved inductive role of dental mesenchyme unequivocally
5.	1980	Kollar et al. ¹⁶	1. Epithelium from 1st, 2nd pharyngeal arches of chick 2. Mesenchyme from 1st molar of mouse	1. 5 days old embryo 2. ED 16–18	Xenoplastic tissue recombinations and grafting into eye of athymic mouse	Complete teeth developed in 4 grafts out of 55 recombinations
6.	1987	Mina et al. ¹²	Epithelium and mesenchymal component of 1st and 2nd branchial arch of mouse	ED 9–13 days	Heterotypic recombinations of mandibular arch and second branchial arch tissues	Before ED 12, mandibular arch epithelia, possess odontogenic potential and can induce the formation of a dental papilla in non-odontogenic, mesenchymal cells of the second arch

Regeneration of tooth

Inspired by the understanding of odontogenesis process, in recent years, researchers have tried to regenerate tooth and dental tissues. Tooth regeneration techniques can be broadly divided into two methods (1) scaffold-based tooth regeneration or (2) scaffold-free regeneration (Table 2).

Scaffold based technique

Stem cells collected from post-natal tooth buds of animals are seeded into a biodegradable scaffold. For maturation *in vivo*, seeded scaffolds are transplanted either into renal capsule or omentum.

The pioneering work using bio-scaffold was done by Young et al.¹⁷ in 2002. They seeded poly (L-lactide-co-glycolide) (PLGA) scaffolds by single cell suspension dissociated from third molar tooth buds of pig. After growing for 20–30 weeks in immunodeficient rat hosts, mineralized tooth structures were found to be formed. Although peridontium and a complete tooth were not formed in their experiments, the results were promising. Duailibi et al.¹⁸ in 2004 did similar experiments on polyglycolic acid (PGA) or PLGA scaffold, using cultured tooth bud cells taken from 3 to 7 days old post-natal rats.

In 2005, Honda et al.¹⁹ tried to characterize tooth regeneration process using immunohistochemistry in a PGA scaffold. They showed that although the pattern of regeneration closely mimicked that of natural odontogenesis, however the control over the size of the teeth and more significantly control of shape, timing of development e.g. cementum developing over radicular dentin and enamel over coronal dentin, was not possible. They postulated that following dissociation of tooth-bud cells, the natural regulation of cellular differentiation was lost. The cervical epithelium in mouse molar switches over from crown to root formation by loss of stellate reticulum, but in tissue engineering both the processes appeared to occur at the same time.

Young et al.²⁰ in 2005 developed a hybrid bioengineered tooth and bone constructs which had the potential to regenerate both tooth and lost alveolar bone. Initially PGA and PLGA scaffolds were seeded with cells from porcine 3rd molar tooth buds and were grown in omentum of adult rat hosts. A separate bone implant scaffold containing bone marrow progenitor cells was grown in a rotational oxygen-permeable bioreactor for 10 days. After 4 weeks both these scaffolds were harvested, sutured together, transplanted and grown in omentum of rats for additional 8 weeks. Histochemical studies revealed, regenerated tissues comprising enamel, primary and reparative dentin in the tooth part of the hybrid and osteocalcin, sialoprotein-positive bone in the bone part of the hybrid construct.

All these experiments using scaffolds mostly produced multiple small teeth-like structures and number, shapes and sizes were not controllable. In 2007 Honda et al.²¹ developed a unique method by sequential seeding of epithelial cells and mesenchymal cells from dissociated third molar tooth buds of pig. After further growth in athymic rats, constructs were analyzed and found to form single tooth structure conforming

to the natural tooth. Duailibi et al.²² tried to implant cultured rat tooth bud cells seeded in PGA/PLGA/PLLA (poly-L-lactide) tooth-like mold directly in the jaw. After 12 weeks radiographic and immunohistochemical studies demonstrated nearly similar organized tissue formation similar to their previous experiments which were grown in omentum.¹⁸

Although both omentum and extraction socket of mandible supported formation of dental tissues in seeded scaffold, the omental site appeared to form more organized tissue in the series of studies of Honda and co-workers. However, the disadvantage of scaffold-based technique is that the size of the developed teeth is often very small; size and shape rarely simulates a natural tooth.

Scaffold free technique

Ohazama et al.²³ in 2004 developed tooth primordia by recombination technique using mouse oral embryonic epithelium (embryonic day: ED10); and cell aggregates comprising non-dental cell population prepared from embryonic stem cell, neural stem cell, bone marrow derived cells. They also for the first time transplanted ED14.5 mouse tooth primordia within diastema region of adult mice, which successfully developed normal structures histologically.

Nakao et al.²⁴ in 2007 developed a novel 3D organ culture method where they co-cultured epithelial and mesenchymal cells in close approximation within a collagen gel. Regenerated tooth germ was allowed to mature in the renal capsule and later transplanted to the jaw. Structural similarity was found with natural tooth with demonstration of growth of blood vessels and nerve fibers.

In 2009 Ikeda et al.²⁵ followed same 3D organ culture method of Nakao et al.,²⁴ and first prepared bioengineered molar tooth germ reconstituted from epithelial and mesenchymal cells of ED14.5 embryonic molar tooth germ of mice. The tooth germ was transplanted into upper first molar region of alveolar bone with proper orientation after 5–7 days in an organ culture, and the reconstituted germ developed into early bell stage with mean length of $534.4 \pm 45.6 \mu\text{m}$. In 56.6% of transplantation, the bioengineered tooth showed exposure of cusp tips around 36.7 ± 5.5 days and after 49.5 ± 5.5 days occluded with opposing molar counterpart. The bioengineered tooth demonstrated correct structure, comprising enamel, dentin, cementum, pulp chamber and periodontal ligament space. However the tooth was smaller in size than the natural tooth demonstrating that overall crown width, cuspal position and tooth patterning are poorly controlled after *in vitro* cell manipulation technique. The Knoop hardness number of enamel and dentin of bioengineered tooth was found to be within normal range of natural tooth. Histological response was found to be identical with natural tooth, when subjected to mechanical stress and the ability to perceive noxious stimuli, such as stimulation of pulp or orthodontic treatment, was also demonstrated by the expression of galanin, a neuro peptide involved in pain transmission.

Oshima et al.²⁶ in 2011 followed 3D organ culture technique and tried to develop a mature tooth in sub-renal capsule of a mouse by utilizing size control method. In their experiment, a single, width-controlled correctly structured single mature

Table 2 – Summary of studies on whole tooth regeneration.

S. no	Year	Investigator	Source of cells	Scaffold	Technique	Outcome
1.	2002	Young et al. ¹⁷	Porcine third molar tooth buds	PLGA	Maturation in immunodeficient rat for 20–30 weeks	Recognizable tooth structures formed that contained dentin, odontoblasts, well-defined pulp chamber
2.	2004	Duailibi et al. ¹⁸	Rat tooth bud cells	PGA PLGA	Omenta of adult rat hosts for 12 weeks	Rat 4-day post-natal (dpn) tooth bud cells seeded for 1 h onto PGA or PLGA scaffolds generated bioengineered tooth tissues most reliably
3.	2004	Ohazama et al. ²³	Mouse oral embryonic epithelium (ED10); non-dental cell population from ES cell, neural stem cell, BMD cells	Scaffold free	1. Tissue recombination followed by transplant in kidney for 10–14 days 2. Implanted ED14.5 molar rudiments in diastema region of adult mice	1. Teeth (crown) developed associated with bone and soft tissue 2. Ectopic tooth was of normal size and demonstrated normal histology
4.	2005	Honda et al. ¹⁹	Porcine third molar tooth buds	PGA mesh	Immunohistochemical analysis of regenerative process following transplanting seeded scaffold into omentum of athymic rats for 2–25 weeks	Regeneration process parallels natural odontogenesis. Tooth size, shape and root formation timing differs
5.	2005	Young et al. ²⁰	Porcine third molar tooth buds, induced osteoblasts from bone marrow progenitor cells	PGA PLGA	1. Seeded tooth scaffold grown for 4 weeks in adult rats 2. Induced osteoblast seeded onto PLGA scaffolds, grown in bioreactor for 10 days joined tooth-bone constructs are grown in rat omentum	Generation of hybrid tooth bone constructs histochemically resembling bone and periodontal tissues
6.	2007	Honda et al. ²¹	Porcine third molar – epithelium, mesenchyme	Collagen sponge	Novel cell-seeding technique: sequential seeding of mesenchymal and epithelial cells on scaffold	In vivo tooth morphology was similar to that of natural tooth, and one tooth structure formed in each scaffold
7.	2007	Nakao et al. ²⁴	Murine tooth bud embryonic stem cells	Scaffold free	3D cell manipulation technique: reorganizes epithelial and mesenchymal embryonic stem cells by increasing cell density and compartmentalizing	Multiple structurally correct tooth was generated both in vitro and in vivo after transplantation in jaw
8.	2008	Duailibi et al. ²²	Rat 4-dpn tooth bud cells	PGA/PLLA & PLGA	Harvested, seeded onto scaffold, then implanted into fresh extraction socket for 12 weeks	Many small tooth crowns, containing enamel, dentin, pulp and periodontal tissues were regenerated. Less organized than grown in the omentum
9.	2009	Ikeda et al. ²⁵	Mouse ED14.5 molar tooth germ cells	Scaffold free	Transplantation of bioengineered tooth germ (3D organ germ method) into the jaw of adult mouse	Bioengineered tooth fully erupted, occluded with opposing tooth. Structure and hardness had similarity with natural tooth. Response to noxious stimuli was present

Table 2 (Continued)

S. no	Year	Investigator	Source of cells	Scaffold	Technique	Outcome
10.	2011	Oshima et al. ²⁶	Mouse ED14.5 molar tooth germ cells	Scaffold free	By following 3D organ culture methods, tried to develop mature tooth in sub-renal capsule followed by transplantation into lost tooth model of mouse	Single, width controlled correctly structured mature tooth developed with periodontal tissue After transplantation in the jaw, alveolar bone regeneration was observed. Tooth also responded to mechanical and noxious stimuli
11.	2013	Cai et al. ³⁰	Integration-free human urine derived iPSCs (ifhU-iPSCs), mouse ED14.5 molar mesenchyme	Scaffold free	1. Differentiation of ifhU-iPSCs to epithelial sheets and recombination with mouse molar mesenchyme 2. Sub-renal culture of recombinant in nude mice for 3 weeks	Intact tooth like structure formed in the recombinant. Molecular marker confirmed differentiation of ifhU-iPSCs into ameloblast Raman spectroscopy and nano-indentation demonstrated nearly identical physical property of enamel

tooth developed with periodontal tissue. After transplantation into the jaw, alveolar bone regeneration was observed. Tooth also responded to mechanical and noxious stimuli. Their study was significant in the sense that they transplanted a fully functional matured tooth in the jaw, which showed full structural and functional integration.

Challenges in tooth regeneration

Tooth regeneration research is not without challenges. The major hurdles are first – finding an appropriate cell source which is clinically feasible, second – induction of 'odontogenic potency' to those cells and third – hastening the whole development process by gene-manipulation.²⁷

Tooth regeneration research has been carried out mainly by using either embryonic stem cells or adult stem cells. Along with ethical concerns regarding research with embryonic stem (ES) cells, it is difficult to find suitable source of ES cells for application in a patient. If xenogenically derived ES cells (non-human species) are used there is a chance of immune rejection. Autologous post-natal stem cells from tooth germ are difficult to isolate and expand in vitro and may not be feasible for use clinically. Moreover after in vitro expansion, these cells lose 'odontogenic potency' and may not serve as a suitable source for regeneration of an accurately structured tooth.²⁸ Mature tooth can also serve as a clinically suitable source of mesenchymal stem cells and 5 different types of stem cells have been identified so far- stem cells from exfoliated deciduous teeth, periodontal ligament stem cells, dental pulp stem cells, dental follicle progenitor cells (DFPCs) and stem cells from the apical papilla. As ameloblasts are lost via apoptosis after eruption, epithelial cells cannot be harvested from a tooth. However adult stem cells are not pluripotent as embryonic stem cells.²⁸

The limitations of ES cells regarding ethical concerns and limited pluripotency of the adult stem cells were resolved by

the ground-breaking discovery of induced pluripotent stem cells (iPSC) by Takahashi et al. in 2006, which changed the track of stem cell research and won them the Nobel Prize in 2012.²⁹ They induced pluripotency (similar to ES cells) into somatic cells by genetic expression of four factors, Oct3/4, Sox2, c-Myc, and Klf4 through transfection using a retro virus. Recently Cai et al.³⁰ in 2013 generated tooth-like structure using integration-free human urine-induced pluripotent stem cells (ifhU-iPSCs). However, use of iPSC has some concerns with regard to cancer-like growth in host tissue.²⁸

Although odontogenic mesenchymal stem cells, iPS cells or adult cells can function as a suitable source of cells for tooth regeneration, but conferring 'odontogenic potential' to these cells remains a formidable challenge till date.²⁷ Further research in molecular biology of tooth development may solve the unanswered questions of whole tooth regeneration – the ultimate goal of regenerative dentistry.

In near future it may be possible to unite iPSC derived epithelial cells and iPSC derived mesenchymal cells with 'odontogenic potential' to form a tooth bud in scaffold-free 3D organ culture technique. After growing in vitro up to late Bud stage, they can be transplanted into a patient's jaw. The differentiation and morphogenesis may need to be hastened genetically to develop a regenerated tooth faster than the normal process of tooth development.²⁷

Conclusion

As clinicians the goal of successful rehabilitation is to replace the lost teeth with morphologically and functionally similar substitutes. Despite the popularity and success of titanium implants, they still have many limitations. The consensus appears to be that the only long-term solution is to be able to regenerate the lost tooth in its entirety. This article endeavors to present an overview of the attempts and the current status of the research in this regard. We have confined our review of

the current research trends to primarily whole tooth regeneration. However, it is pertinent to point out that there is also concurrent research into various other odontogenic tissue regeneration attempts for clinical applications. Whole tooth regeneration is technically challenging and complex. None of the researchers till date have been able to successfully regenerate an entire tooth with acceptable morphology and size for clinical application. However, the milestones that have been crossed in the attempts at whole tooth regeneration have been remarkable and the future is quite encouraging. The most promising scenario appears to be with the efforts to unite iPSC derived epithelial and mesenchymal cells with 'odontogenic potential' to form a tooth bud in 3D organ culture technique. Further research into this field using our understanding of molecular biology, embryology, biomaterials and stem cells are required to answer the yet unsolved questions. The ultimate goal is to integrate all these techniques to have a fully functional and viable tooth replacement that is functionally and morphologically indistinguishable from its predecessor.

Note: Interested readers may refer to an Internet database of gene expression during tooth development, at <http://bite-it.helsinki.fi/> for comprehensive further reading.

Conflicts of interest

The authors have none to declare.

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