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CRITICAL REVIEWS IN ORAL BIOLOGY & MEDICINE

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Applications of Microscale Technologies for Regenerative Dentistry

ABSTRACT

While widespread advances in tissue engineering have occurred over the past decade, many challenges remain in the context of tissue engineering and regeneration of the tooth. For example, although tooth development is the result of repeated temporal and spatial interactions between cells of ectoderm and mesoderm origin, most current tooth engineering systems cannot recreate such developmental processes. In this regard, microscale approaches that spatially pattern and support the development of different cell types in close proximity can be used to regulate the cellular microenvironment and, as such, are promising approaches for tooth development. Microscale technologies also present alternatives to conventional tissue engineering approaches in terms of scaffolds and the ability to direct stem cells. Furthermore, microscale techniques can be used to miniaturize many *in vitro* techniques and to facilitate high-throughput experimentation. In this review, we discuss the emerging microscale technologies for the *in vitro* evaluation of dental cells, dental tissue engineering, and tooth regeneration.

Abbreviations: AS, adult stem cell; BMP, bone morphogenic protein; ECM, extracellular matrix; ES, embryonic stem cell; HA, hydroxyapatite; FGF-2, fibroblast growth factor; iPS, inducible pleuripotent stem cell; IGF-1, insulin-like growth factor; PDGF, platelet-derived growth factor; PDMS, poly(dimethylsiloxane); PGA, polyglycolate; PGS, polyglycerol sebacate; PLGA, poly-L-lactate-co-glycolate; PLL, poly-L-lactate; RGD, Arg-Gly-Asp attachment site; TCP, tricalcium phosphate; TGF-β, transforming growth factor beta; and VEGF, vascular endothelial growth factor.

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INTRODUCTION

In oral surgery, teeth are likely candidates for replacement by artificial components (Esposito *et al.*, 2007) such as orthodontic implants. Overall, this approach is highly successful; however, restorative operations involving implants generally have a finite lifespan and may require replacement at a future time (Dodson, 2006; Jung *et al.*, 2008). Replacement of implants is undesirable for several reasons. First, while generally slight, all surgery involves some degree of risk, time for recovery, and pain. When surgery is undertaken, implanted components may fail to achieve fixation and may become infected, and, in the case of replacements, treatment options may be limited by the available or remaining bone stock (Lang *et al.*, 2000; Schwarz, 2000; Porter and von Fraunhofer, 2005; Clayman, 2006; Paquette *et al.*, 2006; Schwartz and Larson, 2007). As a result, regeneration-based approaches to tooth replacement are the subject of considerable interest.

Tooth regeneration offers new and innovative approaches to common problems encountered in oral and dental surgery and may eventually provide other alternatives to orthodontic surgery. For example, teeth generally last much longer than implants. Survival rates of healthy teeth are 99.5% over 50 years (92%-93% if periodontally compromised), compared with a 10-year survival rate of 82%-94% for orthodontic implants (Holm-Pedersen *et al.*, 2007). However, as a result of cost, placement of orthodontic implants is unlikely in developing countries. Furthermore, in the developed world, the treatment of dental caries and other dental maladies generally does not result in tooth loss. In cases where a tooth is lost, it may be replaced with an implant, bridge, or denture capable of mastication. However, in many developing countries, it is often simpler (and far more cost-effective) to remove the tooth (Peck and Peck, 1979; Walker *et al.*, 1982). Not surprisingly, the loss of numerous teeth is associated with an overall decrease in quality of life resulting from undesired movement of the surrounding teeth, difficulties in eating and speaking, and a significant loss of surrounding bone, limiting future options for surgical intervention (Steele *et al.*, 2004; Hashimoto *et al.*, 2006; Brennan *et al.*, 2008).

Strategies based upon regenerative medicine that facilitate the repair or replacement of damaged teeth may hold particular promise as a means to reduce the cost of dental care. According to the 2006 National Health Expenditure Accounts, the annual US expenditures on dental services totaled 91.5 billion dollars (NHEA, 2006). It is estimated that 90% of adults have caries lesions and that 40% of the Western population is missing one or more teeth (Beltran-Aguilar *et al.*, 2005; Garcia-Godoy and Murray, 2006). Tissue engineering strategies for tooth replacement could potentially account for 90 million instances of caries, 45 million fractured or avulsed teeth, and 21 million procedures for endodontic surgery each year in the US (Garcia-Godoy and Murray, 2006).

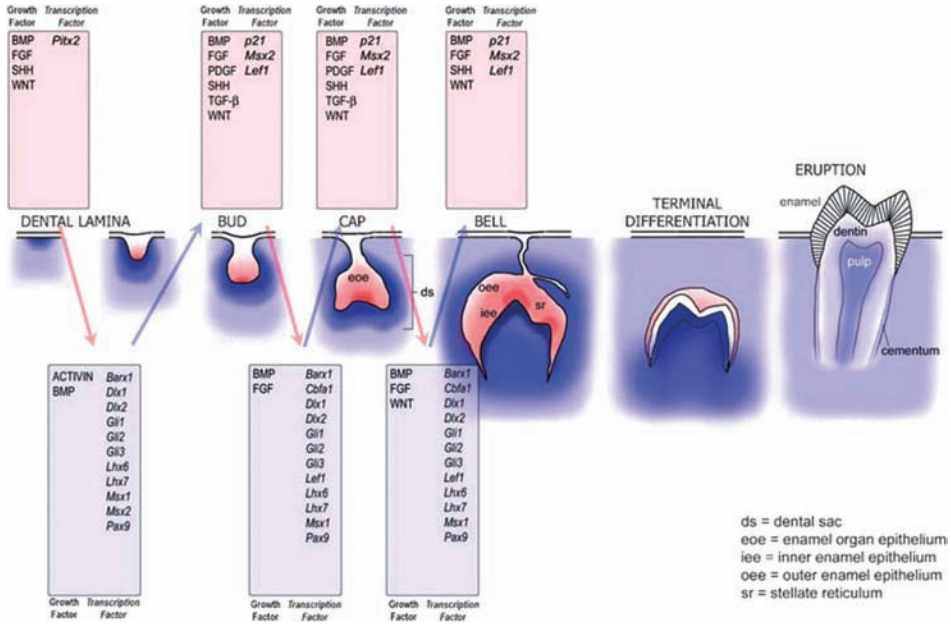

Figure 1. Tooth morphogenesis from the dental lamina to tooth eruption supported and directed by a complex network of signaling, signal transduction, and subsequent gene regulation (Slavkin and Bartold, 2006).

Dental maladies aside, the tooth is also a compelling candidate as a template for organogenesis which could have far-reaching implications for the field of regenerative medicine (Casasco *et al.*, 2007). In this regard, the tooth is well-suited for the study of organogenesis, because it is easily accessible and easily monitored, and tooth failure is not life-threatening (Sartaj and Sharpe, 2006). To advance therapeutic options in tissue engineering, a strong movement exists to progress from cell-seeded scaffolds to the development of complex, functional, and organized tissues. The field of regenerative dentistry draws upon knowledge from cellular, molecular, and developmental biology, tissue engineering, and stem cell biology. It is believed that the knowledge and skills gained from the development of an artificial tooth will be applicable to the generation of other organs (Sartaj and Sharpe, 2006; Nakahara and Ide, 2007; Nakao *et al.*, 2007).

TOOTH STRUCTURE AND DEVELOPMENT

[AQ: 2]

The tooth is comprised of 4 major tissues: the enamel, dentin, cementum, and the dental pulp. The tooth is anchored to the bones of the jaw and protected by the tissues of the periodontium. For permanent teeth, the template for these tissues is established during fetal development around the 20th week. Tooth development is the cumulative result of spatial and temporal interactions between different tissues, namely, of mesoderm and ectoderm origins (Sharpe, 2001; Ohazama and Sharpe, 2004; Tucker and Sharpe, 2004), and progresses through 4 widely recognized stages of tooth development: the bud, cap, bell, and crown stages. Complex and repeated signaling interactions determine the formation, position, and overall shape of tooth development (Tucker and Sharpe, 1999; Sharpe, 2001; Thesleff, 2003; Coudert *et al.*, 2005; Honda *et al.*, 2005; Tompkins, 2006; Kapadia *et al.*, 2007; Salazar-Ciudad, 2008) (Fig. 1). Such interactions generally occur within length scales of tens to hundreds of microns, and microscale technologies are well-suited to recreating such spatial organization in three-dimensional (3D) environments.

GENERAL APPROACHES TO THE REGENERATION AND REPAIR OF DENTAL TISSUE

Tissue engineering is a term that describes the application or use of cells, scaffolds, and growth factors to restore, maintain, or enhance tissue function (Langer and Vacanti, 1993). As described below, a variety of strategies has been used to repair or supplement tissues of the periodontum and dental pulp to reduce the likelihood of tooth loss. When tooth loss does occur, regeneration of the entire tooth may be advantageous in comparison with replacement by implants.

Current efforts to reproduce a viable tooth can be broadly categorized as those based on tissue engineering techniques (scaffold-based) (Thesleff and Tummers, 2003; Duailibi *et al.*, 2004, 2008; Modino and Sharpe, 2005; Young *et al.*, 2005; Yelick and Vacanti, 2006; Xu *et al.*, 2008; Yen and Sharpe, 2008) or developmental biology (organogenesis- or germ-tissue-based) (Sharpe and Young, 2005; Sartaj and Sharpe, 2006; Nakao *et al.*, 2007). The tissue engineering approach commonly utilizes a cell-seeded scaffold to guide and support tooth formation, while the developmental or "organotype" approach facilitates development of a tooth from a collection of cells resembling the tooth germ. Recent advances in the understanding of tooth development, cellular interaction, and signaling, as well as some extraordinary experimental results, all suggest that the generation of biological tooth replacements may be possible (Duailibi *et al.*, 2004, 2006, 2008; Ohazama *et al.*, 2004; Tucker and Sharpe, 2004; Honda *et al.*, 2005; Modino and Sharpe, 2005; Sharpe and Young, 2005; Young *et al.*, 2005; Mikos *et al.*, 2006; Sartaj and Sharpe, 2006; Yelick and Vacanti, 2006; Nakahara and Ide, 2007; Yen and Sharpe, 2008). In the following sections, we discuss the current strategies in the regeneration and repair of various dental tissues, such as the periodontium (tissues anchoring and surrounding the tooth), the dental pulp (tissue within the tooth), or the entire tooth itself.

Regeneration of the Periodontium

The periodontium is comprised of tissues (cementum, periodontal ligament, alveolar bone, and gingiva) that surround, support, protect, and anchor the tooth. Loss of the tissue adjacent to the

tooth is broadly referred to as periodontal disease. Successful preclinical strategies for periodontal tissue regeneration have utilized collagen sponges seeded with cells derived from the periodontal ligament (Nakahara *et al.*, 2004) or hydroxyapatite/tricalcium phosphate (HA/TCP) scaffolds seeded with periodontal-ligament-derived stem cells (Liu *et al.*, 2008). Current clinical strategies for the treatment of periodontal disease prevent further regression of the periodontium while guiding its regeneration. Several clinically available 'cell-occlusive' devices and biomaterials (barriers) prevent ingress of epithelial and gingival cells while providing a protected niche for repair by periodontal cells (Taba *et al.*, 2005). In addition, several clinically available scaffold materials exist for periodontal repair, and growth factors such as bone morphogenic proteins (BMPs) 2 & 7, platelet-derived growth factor (PDGF), insulin-like growth factor-1 (IGF-1), and fibroblast growth factor-2 (FGF-2) have shown promise for periodontal repair (Taba *et al.*, 2005).

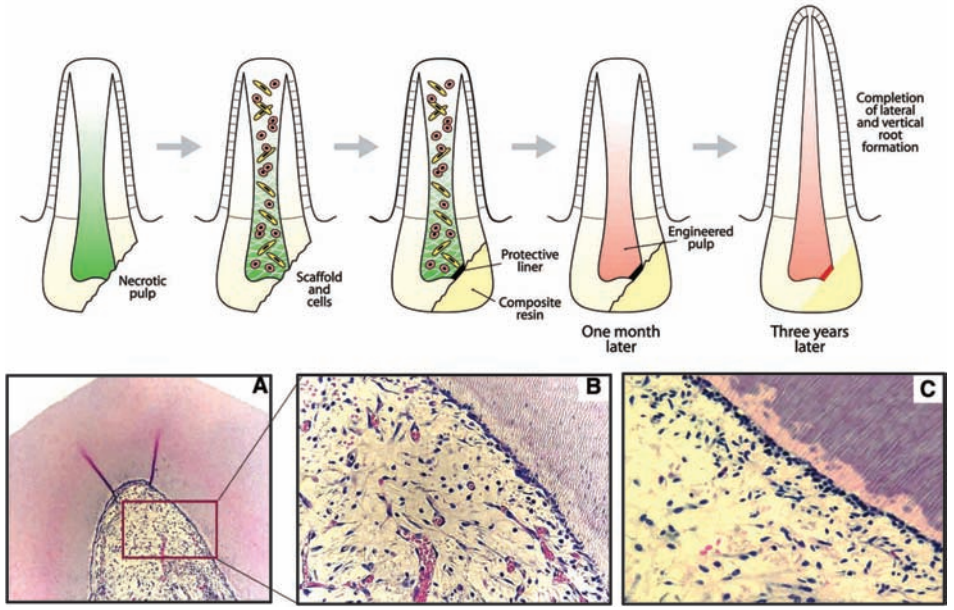

Figure 2. (top) Tissue engineering concept for dental pulp regeneration and maturation of damaged young tooth. **(bottom)** Engineering of representative dental pulp tissue at **(A)** low magnification (100x) and **(B)** high magnification (400x) grown in the mouse. **(C)** Histology of a dental pulp of a human third molar (control tooth) (Nör, 2006).

Microscale technologies that facilitate the controlled positioning and organization of multiple tissue types in close proximity may be of particular benefit to periodontal tissue engineering. For example, microscale technologies that direct and guide tissue formation and control local interactions among tooth, ligament, and bone are likely of interest for teeth generated *in situ* in the extracted socket.

Regeneration of the Endodontium

Regenerative endodontics (repair of the dental pulp) is a likely near-term dental treatment that will bring widespread application of tissue engineering principles to regenerative dentistry (Murray *et al.*, 2007; Sloan and Smith, 2007; Huang, 2008). The objectives of pulp replacement procedures are to regenerate the pulp-dentin complex, regenerate damaged coronal dentin, and regenerate resorbed root and cervical or apical dentin (Cotti *et al.*, 2008; Gotlieb *et al.*, 2008; Huang, 2008). Tissue engineering approaches may include the use of growth factors for revascularization, as well as stem cells and scaffolds for pulp tissue regeneration (Murray *et al.*, 2007; Sloan and Smith, 2007; Tecles *et al.*, 2008). Pulp regeneration may be a particularly beneficial treatment for damaged developing adult teeth (Fig. 2), as has been demonstrated experimentally with tooth slices and cells implanted subcutaneously into a murine model (Nör *et al.*, 2001; Nör, 2006). Pulp regeneration may be restricted by the anatomy of the tooth, specifically, the single point of vascular access at the tooth apex. As a result, microscale technologies that provide open channels or the ability to guide vascular ingress from the apex through the pulp may be of particular benefit.

Scaffold-based Tooth Regeneration

Tooth-like tissues have been generated by the seeding of different cell types on biodegradable scaffolds (Table). A common methodology is to harvest cells, expand and differentiate cells *in vitro*, seed cells onto scaffolds, and implant them *in vivo;* in some cases, the scaffolds are re-implanted into an extracted tooth socket or the jaw. In one of the earliest examples of this approach, Young and colleagues generated mineralized tooth-like structures by seeding porcine tooth bud cells on poly(L-lactide-co-glycolide) (PLGA) scaffolds. Although the resulting structures did not conform to the shape of the implanted scaffolds, this example demonstrated that the fabrication of engineered biological tissues may be possible (Young *et al.*, 2002). In their subsequent work, Young *et al*. seeded porcine tooth bud cells on PLGA scaffolds and implanted them into the omenta of athymic adult rats (Young *et al.*, 2005). After 4 wks, each scaffold with the tooth bud cells was sutured to a scaffold containing bone marrow progenitor cells and re-implanted into the omenta of athymic adult rats for an additional 8 wks. This resulted in the generation of bioengineered dental tissues that roughly conformed to the size and shape of the scaffold and produced tissue that was organized into layers identified as dentin, cementum, pulp, and the periodontal ligament. The co-development of a tooth/bone complex demonstrated the potential for the engineering of an implantable tooth with periodontal fixation and an osseous bed for transplantation. Furthermore, Xu and co-workers seeded tooth bud cells from the rat on scaffolds fabricated from silk fibroin with 2 different pore sizes that were

Table. Selected Approaches to Regeneration of Dental Tissues

Approach	Cell Source	Technique	Biomaterial	Relevance	Reference
Periodontal regeneration	Canine (beagle)	Harvest cells, seed onto collagen sponge, implant against periodontium	Collagen sponge (70% Type 1, 30% Type 2)	Potential of *in situ* tissue engineering using autologous cells for the regeneration of periodontal tissues	Nakahara *et al.*, 2004
	Stem cells from periodontal ligament of miniature pig	Harvest cells, expand *ex vivo*, seed onto hydroxyapatite /tricalcium phosphate scaffold	Hydroxyapatite/tricalcium phosphate scaffold	Feasibility of using stem cell-mediated tissue engineering to treat periodontal diseases	Liu *et al.*, 2008
Endodontal regeneration	Human stem cells from exfoliated teeth (SHED)	Seed cells on to scaffold and place in prepared canals of human teeth	D,D-L,L-polylactic acid scaffold	Possible to implant tissue engineered pulp into teeth after cleaning and shaping	Gotlieb *et al.*, 2008
Hard tissue	Apical pulp derived cells, human molar	Harvest of human apical pulp, expansion *in vitro*, seed onto hydroxyapatite scaffold, implant subcutaneously in nude mice	Porous hydroxyapatite scaffold	The human tooth with an immature apex is an effective source of cells for hard tissue regeneration	Abe *et al.*, 2008
Scaffold-based tooth regeneration	Tooth bud cells, rat pups	Harvest, *in vitro* expansion, seed on scaffold for *in vivo* maturation	Porous hexafluoroisopropanol (HFIP) silk scaffolds (± RGD binding sequence) in 250- and 550-μm pore sizes	Generation of mineralized tissues for tooth tissue engineering; use of silk scaffold	Xu *et al.*, 2008
	Tooth bud cells, rat pups	Harvest, *in vitro* expansion, seed on scaffold for *in vivo* maturation	PGA and PLGA scaffold materials	Tooth-tissue engineering methods can be used to generate both pig and rat tooth tissues	Duailibi *et al.*, 2004
	Tooth bud cells, porcine crown	Harvest, seed onto PGA mesh, implant in omentum of rat	PGA fiber mesh	Development of tissue engineered teeth closely resembles the pattern of odontogenesis	Honda *et al.*, 2005
	Tooth bud cells, porcine molar	Harvest, seed tooth cells onto scaffold, implant in omentum of rat, join with bone grown in bioreactor regrow in rat	PGA and PLGA scaffold materials	Generation of hybrid tooth–bone for the eventual clinical treatment of tooth loss accompanied by alveolar bone resorption	Young *et al.*, 2005
Organotype-based tooth regeneration	Dissociated single cells from epithelial and mesenchymal tissues, recombined dissociated cells	Harvest of murine tooth bud cells, implant in tooth socket	Collagen	Proximity of ectodermal and mesenchymal cells necessary for tooth development; generation of a structurally correct tooth with penetration of blood vessels and nerve tissue	Nakao *et al.*, 2007
	Tooth bud cells, rat pups	Isolation of tooth bud cells and co-culture with dental pulp stem cells, pelletize and culture in renal capsule	N/A	Mimic the dentinogenic microenvironment from tooth germ cells *in vitro*. Demonstrate that soluble factors can produce a conditioned medium beneficial for *in vitro* growth	Yu *et al.*, 2006
	Rat marrow stromal cells, mouse embryonic stem cells, mouse embryonic neural stem cells	Cultured embryonic oral epithelium with other mesenchymal cells, transfer tooth primordium to jaw to grow tooth. Cell pellet wrapped in epithelium	N/A	Embryonic oral tissue can guide differentiation of other stem cells to odontoblasts; embryonic primordium can develop in the adult environment; generation of a functional tooth	Ohazama *et al.*, 2004

either used as fabricated or treated with the RGD binding peptide (Xu *et al.*, 2008). These tissue-engineered constructs were placed in the omenta of athymic adult rats for 20 wks prior to analysis. The larger pore sizes, as well as scaffolds treated with RGD, resulted in more mineralized osteodentin-like tissue. Using a similar technique, Duailibi *et al*. developed mature tooth-like structures from single-cell suspensions (Duailibi *et al.*, 2004). They also determined that the point of tooth bud harvest (maturation) has a significant impact on the quality and extent of tissue formation in the resulting tissue-engineered construct. Subsequent work by demonstrated their ability to form tooth-like structures using cell-seeded scaffolds implanted directly into extraction sockets in the jaw, bypassing a previous maturation step in the omentum (Duailibi *et al.*, 2008). This is a significant step toward the clinical application of tissue-engineered teeth.

One general shortcoming of the scaffold-based approaches to tooth regeneration has been the size of the resulting tooth-like structures. While promising, the overall size of most tissue-engineered constructs is small (1-2 mm) and does not mimic the 3D complexity of the adult human tooth (Duailibi *et al.*, 2004; Xu *et al.*, 2008). This size limitation may be a consequence of the animal model or directly related to mass transfer. In the body, most cells are located near blood vessels, but with non-vascularized scaffold structures, diffusion of nutrients and metabolites is generally limited to the periphery. As a consequence, animal studies using scaffold-based approaches often rely upon *in vivo* maturation (Ohazama *et al.*, 2004; Young *et al.*, 2005) of a small scaffold in an environment such as the renal capsule or omentum, followed by implantation into the jaw to support and develop a tooth-like structure. *In vitro* approaches to overcome the problem of limited diffusion generally rely upon perfusion or flow-based bioreactors that facilitate a deeper exchange of molecules within the scaffold (Timmins *et al.*, 2007; Jaasma *et al.*, 2008). Microscale technologies that support vascularization and enhance diffusion may be of benefit for both the *in vivo* and *in vitro* development of sizeable tooth-like structures. Tissue engineering strategies to generate a functional tooth also require appropriate cell-cell interactions with highly regulated spatial organization, which also may be fabricated by microscale technologies.

Scaffold-free Regeneration of the Tooth

Organs originate from germ tissue present in the developing embryo. An understanding of embryotic development and the reciprocal, local interaction between the cells in these developing tissues is beneficial for the recreation of biomimetic tooth organs (Sharpe and Young, 2005). Much experimental work to this effect has shown that genetic regulators such as the Barx1 homeobox gene (Thomas and Sharpe, 1998; Ferguson *et al.*, 2000; Miletich *et al.*, 2005) are important for directing the formation and location of teeth from the tooth germ (Tucker and Sharpe, 2004; Mitsiadis and Smith, 2006). Several other genes, important to tooth morphogenesis and development, have also been identified (Thesleff and Åberg, 1999; Tucker and Sharpe, 1999; Fukumoto and Yamada, 2005; Ryoo and Wang, 2006; Tompkins, 2006; Foster *et al.*, 2007; Hu and Simmer, 2007; Kapadia *et al.*, 2007; Thesleff *et al.*, 2007).

In addition to appropriate developmental signals, the ability of the local environment to support repeated interaction between epithelial and mesenchymal tissue has also been identified as an important aspect for organotype tooth development (Thesleff and Åberg, 1999; Thesleff, 2003; Yen and Sharpe, 2008). The spatial orientation of cells—specifically, the relative number of each population (epithelial-mesenchymal cell ratios)—can direct cell differentiation and crown morphogenesis, perhaps as a result of the relative concentrations of local factors and signals (Yu *et al.*, 2008).

An early study in this area utilized a murine model to study stem-cell-based tooth regeneration (Ohazama *et al.*, 2004), to generate an organ (tooth) from primordial tissue *in vitro* and successfully complete development in the jaw. In this study, embryonic epithelial oral tissue was harvested and recombined with non-dental cells (neural and mixed population obtained from the bone marrow) to generate germ tissue for transplantation to the renal capsule of the mouse for maturation before implantation into the jaw. Rudimentary teeth were generated from both cell types, indicating that embryonic epithelial oral tissue can direct the maturation of dental-like tissue from non-dental cells. Furthermore, Nakao *et al*. found that dissociated and re-aggregated single-cell populations from the tooth bud (epithelial or mesenchymal cells) were unable to generate a correct tooth structure when cultured alone. However, co-cultures of both epithelial and mesenchymal cells with each group of cells, physically separated in a collagen gel but grown in close proximity to facilitate temporal signaling, resulted in the formation of a tooth germ. Temporary maturation of these constructs in the renal capsule, followed by transplantation into tooth cavities, resulted in the generation of a correct tooth-like structure (Nakao *et al.*, 2007) (Fig. 3).

Nearly all scaffold-free approaches to tooth regeneration need to be placed in the body for maturation. Ideally, the development of suitable *in vitro* environments and scaffolds with appropriate microstructures to facilitate vascularization as well as length scales and spatial organization of different cell types that facilitate and support tooth development would be advantageous.

There seems to be no clear indication of which approach will provide a better clinical outcome for tooth regeneration. Given the small size, limited vascular access, and potential difficulty anchoring a tooth regenerated *in vitro*, it seems that, at this time, the tooth will require maturation in the host in the desired location. Because it is presently unclear if scaffold-based teeth formed in the jaw will erupt into the oral cavity and develop into mature teeth, it seems that the scaffold may need to mature *in situ* in its final shape and desired location. As a result, scaffold-based approaches that mature in the oral cavity need to overcome challenges associated with infection, attachment to the jaw, repetitive movement, and ability to withstand load during maturation; however, the potential for rapid formation of a functional tooth of the correct shape and in the desired location is promising. Scaffold-free approaches that are seeded in an extraction socket or in a defect in the jaw and covered with a layer of protective tissue may avoid some of the aforementioned potential complications; however, precise control over tooth development (shape and orientation) and acquisition and direction of suitable stem cells are areas of ongoing research.

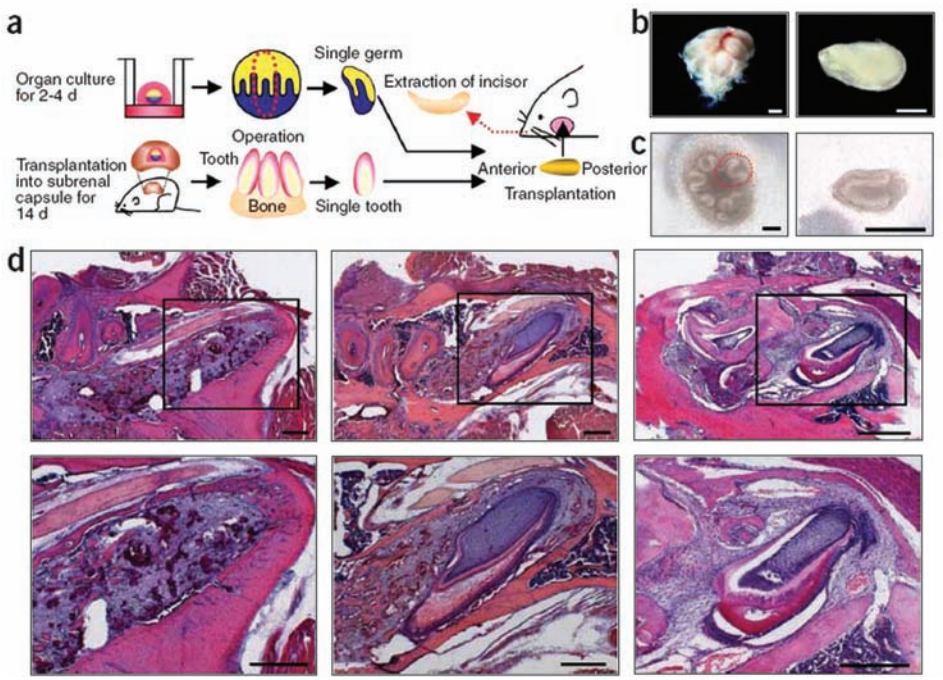

Figure 3. Development of a bioengineered mouse incisor. **(a)** Schematic of the procedure. Reconstituted tooth germ cells cultured for 2 days were separated into single primordia prior to implantation into the subrenal capsule, then transplanted into a tooth cavity. **(b)** A bioengineered incisor developed in a subrenal capsule environment for 14 days (left) and a tooth separated from reconstituted tissue in the subrenal capsule and used for transplantation (right). **(c)** Separation of individual primordia (dotted circle) from a bioengineered tooth germ that had been cultured for 2 days. **(d)** Histological images of the explants at 14 days after transplantation into a tooth cavity. Images from the control experiment (left) and transplants isolated from a single incisor primordium (center) and a single tooth developed in the subrenal capsule (right) are shown and at higher magnification (boxes) (Nakao *et al.*, 2007).

CELL SOURCES FOR DENTAL TISSUE REGENERATION

While advances in engineering scaffold architecture have yielded results, a suitable source of cells for dental tissue regeneration has so far eluded researchers. This is because cells harvested from the dental tissue may not be expanded easily *in vitro*. An alternative source of cells is stem cells, which have an extensive ability to self-renew or differentiate. There are two main types of stem cells: embryonic stem (ES) cells, which are derived from blastocysts; and adult stem (AS) cells, which are derived from adult tissues. Both ES cells and AS cells have been shown to be capable of differentiating toward dental cells. In the clinical setting, the use of ES cells is the subject of ethical concerns, and AS cells can be difficult to isolate, expand, and differentiate *in vitro*. One promising alternative may be inducible pluripotent stem (iPS) cells. iPS cells are reprogrammed cells derived from adult tissue, usually by the addition of several promoters (Chang and Cotsarelis, 2007; Pera and Hasegawa, 2008).

Ongoing work with different cell types indicates that a growing number of cell sources may be suitable as precursors for the generation of dental tissues (Zhang *et al.*, 2005; Maria *et al.*, 2007; Yen and Sharpe, 2008). Cells with regenerative capacity and a suitable phenotype for dental tissue engineering have been derived from the apical pulp (Abe *et al.*, 2008), dental pulp (Prescott *et al.*, 2008), cranial neural crest (Jiang *et al.*, 2008), periodontal ligament (Ballini *et al.*, 2007), bone marrow (Hu *et al.*, 2006), dental follicle (Yao *et al.*, 2008), and cells surrounding the vasculature (Murray and Garcia-Godoy, 2004). There appears to be no consensus regarding a preferred cell source for tooth regeneration; however, differences in odontogenic capacity between stem cells derived from the dental pulp and those derived from the bone marrow have been noted (Yu *et al.*, 2007). Odontoblasts and ectomesenchymal cells are difficult to obtain in the clinical setting (Yen and Sharpe, 2008). However, stem cells derived from the dental pulp can be directed to develop into odontoblast-like cells by being cultured in conditioned media from the tooth germ, again indicating the importance of extracellular signaling (Yu *et al.*, 2006). Primary teeth have also been identified as a potential source of stem cells (Miura *et al.*, 2003), and conservation of exfoliated deciduous teeth may provide a future source of dental stem cells.

There are concerns regarding the development and differentiation of stems cells in non-fetal environments such as the adult mouth; however, a review of the literature suggests that adult tissues are capable of odontogenesis (Yen and Sharpe, 2008). In terms of providing a suitable developmental environment, it has also been demonstrated that the oral mesenchyme can be replaced with epithelial cells obtained from another source (Ohazama *et al.*, 2004), a promising finding for both the tissue engineering and organotype approaches to tooth regeneration.

In this review, we will discuss the application of microscale technologies to address the current challenges in dental tissue engineering. One such challenge is scaffold vascularization, and microscale technologies offer promising approaches to guide vascular formation and create vascular networks. Control of scaffold features at the micro and nano levels presents new opportunities to control the cellular microenvironment and to direct cell fate. Similarly, the high-resolution modification of scaffold properties by incorporation of growth factors, molecules, and cell ligands can also provide other avenues for the control of tissue development. Microscale technologies also offer the ability to culture cells in close proximity, facilitating communication and spatial interaction, the importance of which has been demonstrated for tooth development. We also discuss the use of microscale technologies

to create large-scale, homogeneous arrays of stem cell bodies that facilitate the high-throughput evaluation of culture conditions to control stem cell differentiation. The ability to co-culture different cell types in controlled microenvironments also facilitates the study of cell-cell interactions as they relate to tooth development.

MICROSCALE TECHNOLOGIES FOR DENTAL TISSUE ENGINEERING AND REGENERATION

Techniques commonly used in the micro-electronics and semiconductor industries to fabricate miniaturized structures are being increasingly utilized to study cellular events and interactions, as well as to generate scaffolds and cell environments with micron-scale resolution (Kane *et al.*, 1999; Whitesides *et al.*, 2001; Khademhosseini *et al.*, 2006c). Soft lithography is one technique that has emerged whereby patterned silicon wafers are used as master casting templates to mold elastomeric materials such as poly(dimethylsiloxane) (PDMS). Soft lithography has been used to "print" or mold surfaces with chemical and topographical patterns (at resolutions as low as tens of nanometers) (Kane *et al.*, 1999), as well as to pattern cells selectively (Rozkiewicz *et al.*, 2006), rapidly, and inexpensively. Photolithography is another technique used to create microscale features in scaffolds. In this approach, a light-sensitive solution is selectively exposed to light by means of a photomask. The exposed solution undergoes a polymerization or crosslinking reaction, and the unpolymerized ('masked') solution can subsequently be washed away. Such approaches can be used to pattern substrates in 2D or can be layered to achieve structures with a 3D architecture, useful for the generation of tissue-engineered scaffolds or micro-channels to support vascular ingress (Zhang *et al.*, 2003; Kim *et al.*, 2006; Rozkiewicz *et al.*, 2006; Borenstein *et al.*, 2007; Wong *et al.*, 2008). The development of microengineered scaffolds with patterns of progenitor cells of dental-specific tissue types, growth factors, and cues to direct cell behavior, supported by a controlled micro-vasculature, may also offer more rapid and robust methods for the generation of teeth *in vitro*.

Materials for Dental Tissue Engineering

Suitable scaffolds for the regeneration of dental tissue can be fabricated from several materials; however, polymers are often selected because their biological, chemical, and mechanical properties can be controlled. Polymers can be classified as natural or synthetic materials. Natural polymers (such as collagen, chitosan, silk, and fibrin) and synthetic polymers (such as polyglycolide [PGA], PLGA, and polyglycerol sebacate [PGS]) are commonly used as scaffolds for tissue engineering (Vozzi *et al.*, 2003; Young *et al.*, 2005; Chevrier *et al.*, 2007). Hydrophilic polymers may be processed into the form of a hydrogel, a network of water-insoluble polymer chains suspended in water. Hydrogels have several desirable properties, such as high water content (up to 99%) and mechanical characteristics similar to those of native tissue. The addition of recognized cytoskeletal binding sites, such as the RGD sequence, to various polymers can be used to enhance cell adhesiveness (He *et al.*, 2008; Jabbari *et al.*, 2008). For enhancement of the mechanical properties of the hydrogels, the degree of crosslinking of the polymer chains within the hydrogel can be increased. Also, the development of novel, collagen-based gels, containing nano-hydroxyapatite particles crosslinked with non-collagenous bone peptides similar to osteonectin, represents a promising approach to the goal of generating biomimetic load-bearing hydrogels for bone tissue engineering (Sarvestani *et al.*, 2007, 2008). Additionally, bone-like scaffolds comprised of microvascular networks in a collagen-hydroxyapatite matrix have been developed to address problems of limited nutrient transfer issues in moderate-sized (>2 mm) tissue-engineered constructs and have an obvious application to tissue engineering of the tooth, where vascularized, mineralized, and load-bearing structures are required (Sachlos *et al.*, 2006).

Spatially Regulated Hydrogels and Scaffolds

Perhaps the most obvious application of microscale technologies is the generation of tissue-engineered constructs with properties and architecture similar to those of native tissue (Faraj *et al.*, 2007; Murugan and Ramakrishna, 2007). In terms of tooth development, strict control of scaffold architecture and tissue organization will likely be of fundamental importance for the generation of complex, mineralized load-bearing structures. With microfabrication techniques, a variety of functional structures ranging from a few to hundreds of micrometers in size can be created in hydrogels (Choi *et al.*, 2007; Khademhosseini and Langer, 2007; Ling *et al.*, 2007). The ability to alter substrate architecture by the incorporation of micro- and nano-scale features provides another avenue to direct and control cell development and activity (Curtis and Wilkinson, 1999; Webster *et al.*, 2000). In this regard, surface topography has a profound effect on cell behavior (Hacking *et al.*, 2008), migration and alignment (Curtis and Wilkinson, 1997), and tissue formation (Hacking *et al.*, 2002), as do scaffold pore size and geometry (Bobyn *et al.*, 1980, 1999). Such spatial cues and features will likely be of benefit for scaffold optimization for dental tissue regeneration, where control of a variety of cell types in close proximity is required (Curtis and Riehle, 2001). Spatial control has also been extended to the development of hydrogels with gradients of adhesive or signaling molecules to direct cell growth and guide tissue formation (Burdick *et al.*, 2004). Further control over cell activity, such as cell adhesion and cell-scaffold interaction, can be achieved by the incorporation into the scaffold of various biological ligands, such as the adhesive peptide RGD, which is derived from fibronectin (Evangelista *et al.*, 2007; Morgan *et al.*, 2008).

Many biological processes are regulated by soluble signals, which often occur locally. Therefore, growth factor delivery can be utilized to modulate cellular behavior, maturation, and tissue formation. The ability to sequester and deliver growth factors locally from within the scaffold at appropriate times would enable the generation of scaffolds that may be beneficial to tooth regeneration. Several growth factors have demonstrated application in tissue engineering of the tooth. For example, BMPs have been successfully applied for the regeneration of periodontal tissue (Ripamonti, 2007), and other factors, such as PDGF, IGF-1, FGF-2, TGF-β, and BMPs (Taba *et al.*, 2005), have demonstrated

utility in tooth tissue engineering. Often as a result of their physiologic solubility, growth factors like BMPs are applied at levels in excess of their endogenous expression (McKay and Sandhu, 2002). These higher loading levels can result in unwanted side-effects and limited spatial control. Microencapsulation (Carrasquillo *et al.*, 2003) or binding of these factors to the scaffold (Lin *et al.*, 2008) can relieve problems related to loss of activity of diffusion of the molecules from the scaffold (Downs *et al.*, 1992).

Microparticles containing growth factors or drugs are another example of the use of microscale technologies to control the activity of cells (Cheng *et al.*, 2006). For example, PLGA microspheres that release vascular endothelial growth factor (VEGF) have been delivered into a porous scaffold to provide sustained growth factor release for up to 21 days (Ennett *et al.*, 2006). Compared with scaffold-immobilized VEGF, the release from microspheres lasted longer and provided sustained levels of VEGF, resulting in significantly enhanced angiogenesis.

In terms of tooth tissue engineering or regeneration of the dental pulp, fabrication of vascularized scaffolds is likely a key requirement. Compared with other organs, the tooth may be smaller, but it is encased in an impermeable material that prevents large-scale diffusion of nutrients or metabolites. Blood supply to the interior of the tooth and dental pulp is achieved by vessels at the apex of the tooth root. The ability of perfused agarose hydrogels containing microfluidic channels to support cell metabolism has been demonstrated (Ling *et al.*, 2007). Interestingly, it was demonstrated that encapsulated cells within 200 micrometers of the microfluidic channels generally had the best survival, suggesting that microchannels can be used to deliver oxygen and nutrients to cells to maintain cell function.

Microfabrication has been increasingly used to fabricate tissue-engineered scaffolds with micro-engineered capillary beds (Tan and Desai, 2005; Borenstein *et al.*, 2007). The incorporation of microvascular networks into tissue-engineered constructs is a promising advance toward a tissue-engineered tooth. Polymers such as PLGA can be microengineered and seeded with cells to produce endothelialized capillary networks (Fidkowski *et al.*, 2005; Ryu *et al.*, 2007). Early work in the field demonstrated the possibility of generating 2D microvascular networks of endothelial cells that could be lifted off and stacked to generate vascularized tissues (Kaihara *et al.*, 2000; Ogawa *et al.*, 2004). Also, larger tissue can be engineered by superpositioning and stacking multiple layers of fabricated scaffolds (Vozzi *et al.*, 2003). Encapsulated cells in such structures remain viable by diffusion of oxygen and nutrients from micro- and nanochannels (Kim *et al.*, 2006; Ling *et al.*, 2007), thus providing evidence that microfluidic channels can support cells in tissue-engineered constructs (Fig. 4). Also, collagen scaffolds reinforced with biomimetic hydroxyapatite crystals with microchannels have been fabricated. Although these approaches have focused on other tissues, these techniques are directly applicable to the tissue engineering of the tooth (Sachlos *et al.*, 2006).

The ability to pattern scaffolds and create microchannels in the construct permits the development of 3D structures with the potential for rapid vascularization or fluid exchange. This is especially important for larger, more complex structures such as a tooth, since not only is the diffusion of nutrients and metabolites often a critical factor limiting tissue-engineered construct size, tissue organization, and viability, but also there is only one point of vascular access at the apex of the tooth root (Nör, 2006).

Microscale technologies are becoming increasingly used as tools for the development and investigation of tissue regeneration, where spatial control of cells is of primary interest (Khademhosseini *et al.*, 2006a,b,c; Khademhosseini and Langer, 2007). Cell-laden, microfabricated scaffolds provide the means to bring cells, potentially of different origins, together so that they can communicate and interact during tissue formation and maturation, much as they would during embryonic development. Such cell-cell interactions and repeated temporal signaling are known to be important for the development and maturation of a tooth, making such approaches of interest to tissue engineers (Ohazama *et al.*, 2004; Nakao *et al.*, 2007).

One approach to control cell-cell interactions is the use of cell-laden, microfabricated hydrogels that are made from the self-assembly of small blocks of encapsulated cells that can be assembled into larger tissue constructs (Du *et al.*, 2008). Microfabricated hydrogels possessing complementary shapes can be fabricated to facilitate specificity during assembly. Such "bottom-up" approaches are promising for the formation of large, multicellular scaffolds such as a tooth. Interlocking and self-assembling microfabricated components may facilitate the fabrication of dental pulp containing microchannels covered with endothelial cells for vascularization and interlocking with components containing dental pulp cells. This scaffold block may then be surrounded by layers of microfabricated hydrogels, delivering and spatially organizing cells suitable for the formation of the dentin and enamel. Finally, more layers of microfabricated hydrogels, containing cells necessary for the formation of the periodontal ligament and associated tissues, could conceivably be added, providing a template for a tissue-engineered tooth consisting of multiple cell types, all present in a well-defined geometry and spatial arrangement.

High-throughput Applications for Dental Tissue Investigation

Microscale technologies can also be used to study the effects of new biomaterials on cell behavior by miniaturizing assays. High-throughput techniques facilitate the rapid assessment of one or many factors in a well-controlled environment with minimal use of reagents. Such tests enable large-scale, rapid assessment of biomaterials, drugs, or other compounds to be conducted in a parallel and reproducible fashion. Microengineering approaches can be used to generate arrays of homogeneous cell clusters and also provide large, well-controlled environments for the investigation of cellular activity. Both are highly relevant to the assessment of biomaterials and the development of culture conditions for dental tissue regeneration.

Arrays to assess the effects of material composition on cell behavior consist of multiple, uniformly sized and spaced spots, each of which contains different materials such as hydrogels or extracellular matrix (ECM) components printed on a surface (Anderson *et al.*, 2005). Cells can then be seeded on these arrays, and their response (*e.g.*, growth or differentiation) can be assessed.

Biomaterial arrays have been used to evaluate cellular interactions with various components of the ECM. Using a modified DNA spotter, Flaim *et al.* evaluated the effects of several combinations of collagen I, collagen III, collagen IV, laminin, and fibronectin on hepatocyte cell function and ES cell differentiation (Flaim *et al.*, 2005). Combinations of ECM proteins that supported both hepatocyte activity and differentiation were identified. This approach has been extended to include the simultaneous evaluation of growth factors as well as ECM proteins on stem cell activity (Flaim *et al.*, 2008). Similar techniques can be applied to the evaluation of dental stem cells and function to refine or optimize scaffold design and culture conditions.

A major challenge facing regenerative techniques is the ability to obtain a sufficient number of autogenous cells for scaffold seeding (Pittenger *et al.*, 1999). One reason may be because cells isolated from adult tissues are often difficult to expand *in vitro* and generally do not maintain their phenotype (Avital *et al.*, 2002). While the use of stem cells is promising, in the context of dental applications, many questions remain regarding their controlled differentiation to specific lineages. Conventional methods for investigating the responses of stem cells to various agents and environments are generally laborious, limited by the number of variables evaluated and the inability to generate consistent cell aggregates for repeated analysis. Microscale technologies that facilitate high-throughput approaches are of particular interest for stem cell evaluation for dental tissue regeneration (Anderson *et al.*, 2004; Kim *et al.*, 2007).

Microscale technologies can be used to produce relatively reproducible ES cell aggregates for evaluation (Moeller *et al.*, 2008). This is particularly desirable, because the development of artificial stem cell environments or 'niches' may be an effective means to differentiate stem cells efficiently and reproducibly into a variety of lineages (Dang *et al.*, 2004; Bauwens *et al.*, 2008; Chung *et al.*, 2008) for tissue engineering or for high-throughput analysis. Fabrication of microbioreactor arrays (Figallo *et al.*, 2007) that provide myriad functionalities to

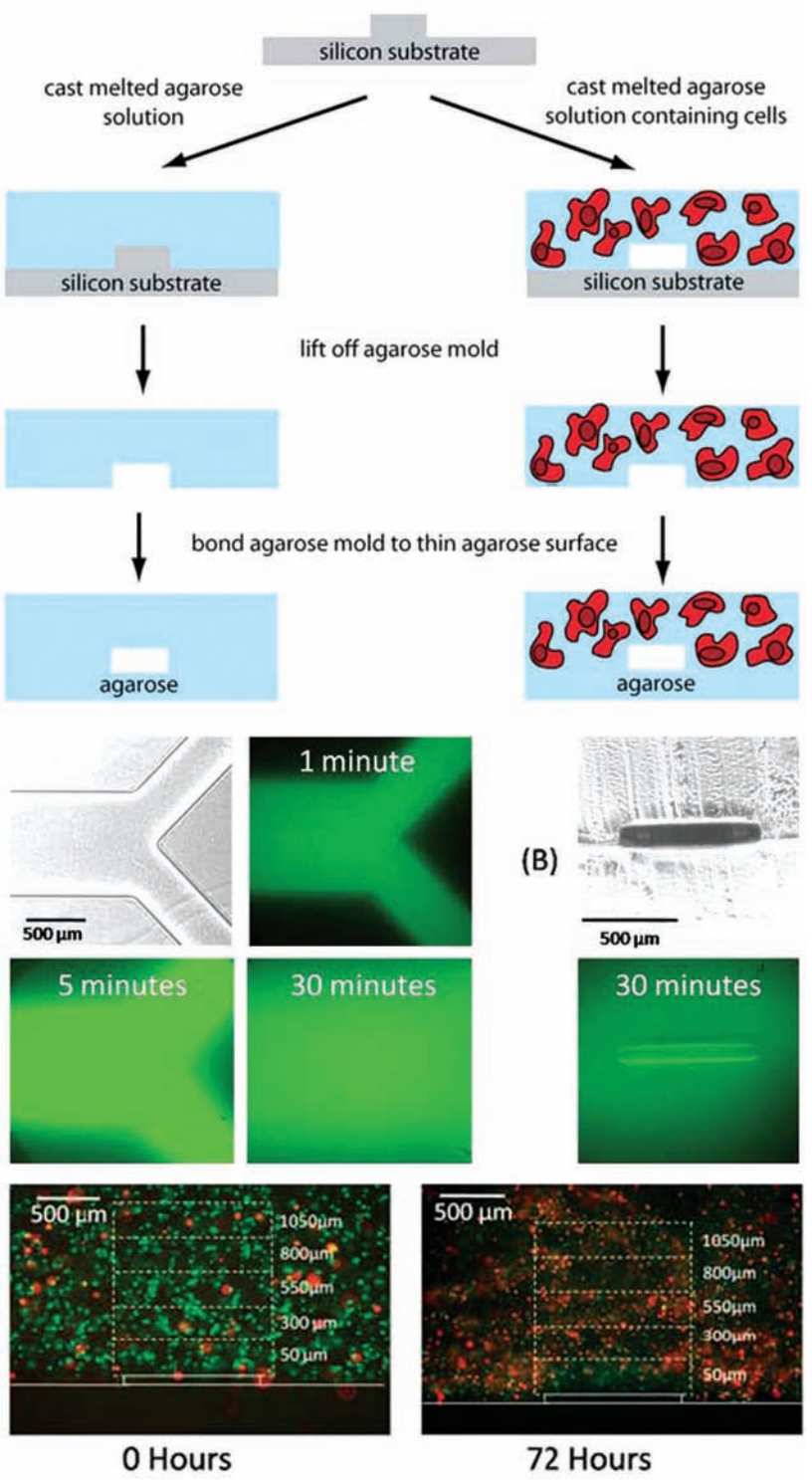

Figure 4. (Top) Fabrication of hydrogel microfluidic devices without (left) and with cells (right). **(Middle)** Diffusion of fluorescent dye from a microchannel within a hydrogel **(A),** also shown in cross-section **(B). (Bottom)** Cell viability of AML-12 murine hepatocytes encapsulated in agarose channels after 0 (left) and 3 days (right). Live (green)/dead (red) staining. Survival decreases with increasing distance from the microchannel. The microchannel is shown in cross-section and outlined for visibility as a small white rectangle at bottom of the image (Ling *et al.*, 2007).

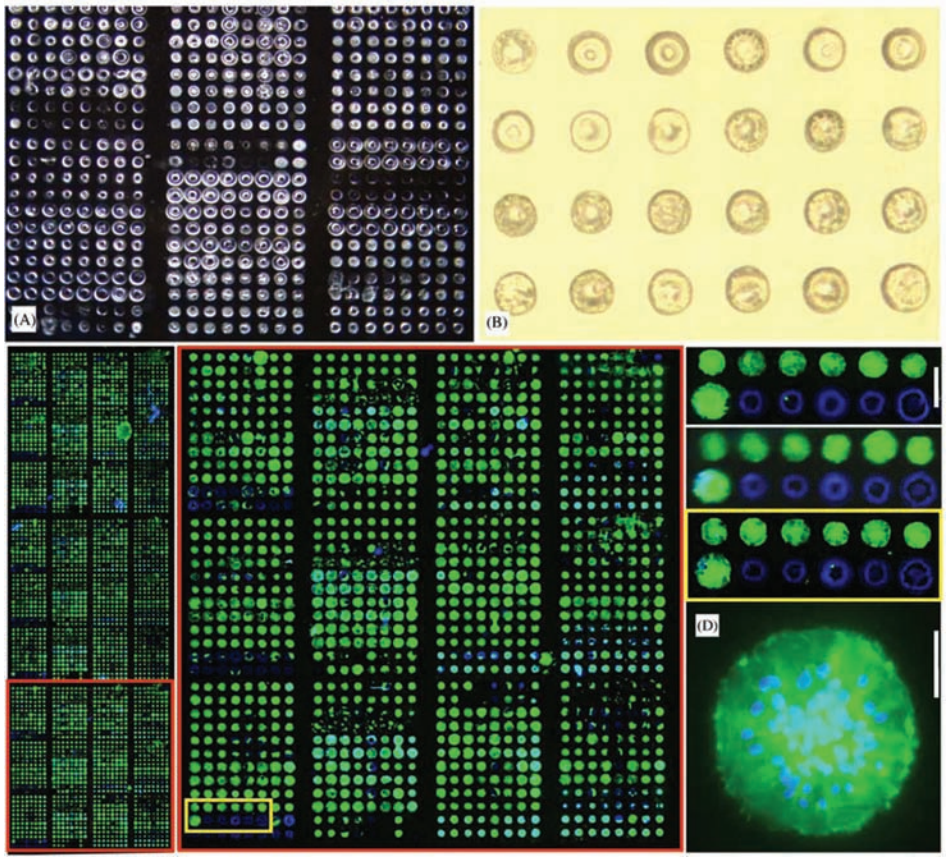

Figure 5. Rapid screening of a variety of polymer biomaterials is made possible by the use of biomaterial microarrays. **(Top A, B)** Light microscopy of 500-micrometer spaced polymer spots. Human mesenchymal stem cells grown on the polymer array and stained with phalloidin for F-Actin. Approximately 60 cells *per* polymer spot (Anderson *et al.*, 2005). **[AQ 3]**

monitor and control cell growth are technologies that will likely advance the field. Also, reproducible cellular patterning (Rosenthal *et al.*, 2007; Wright *et al.*, 2008), patterned co-cultures (Wright *et al.*, 2007), and control of the microenvironment over large areas permit arrays of cell constructs to be assessed in a high-throughput manner (Moeller *et al.*, 2008). Such techniques permit the assessment of a variety of growth factors, biomaterials (Anderson *et al.*, 2005), and substrate and cellular interactions, alone or in combination (Khademhosseini *et al.*, 2005; Chung *et al.*, 2008) (Fig. 5).

Microscale technologies also offer unique approaches to some of the obstacles and scientific challenges associated with tooth regeneration. It is well-known that tooth development is the result of the continued reciprocal interaction between epithelial and mesenchymal cells in distinct, but local, environments (Kapadia *et al.*, 2007; Salazar-Ciudad, 2008). Such conditions can be recreated in a controlled manner by the use of microscale techniques to isolate, seed, and study single cells or collections of cells (Khademhosseini *et al.*, 2005; Rosenthal *et al.*, 2007; Wright *et al.*, 2007, 2008). Thus, microscale techniques may provide new tools for the exploration of tooth development.

CONCLUSIONS

With respect to dental tissue engineering and regeneration, microscale technologies offer compelling benefits in terms of controlling scaffold architecture, biomechanics, growth factor delivery, vascularity, spatial orientation of cells, and temporal signaling. The application of microscale technologies will likely help to advance the technology and knowledge associated with dental tissue regeneration. Microscale technologies are likely to advance scaffold development and increase stem cell sources for dental tissue regeneration. Microscale scaffolds with controlled properties and architecture may facilitate the generation of complex, cell-laden, load-bearing vascularized scaffolds for hard tissue regeneration and the directed neo-vascularization essential for the *in vitro* development of a tooth. Also, microscale technologies will likely be of benefit to support the reciprocal temporal signaling and spatial organization of developing tissues and organs from a collection of germ cells, essential to tooth development. High-throughput tools have been developed to facilitate the rapid screening and optimization of biomaterials for dental tissue regeneration. Similarly, high-throughput techniques have been used to evaluate stem cells and their responses to numerous conditions in a manner directly applicable to the regeneration of dental tissues.

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