Review

Recent update on craniofacial tissue engineering

Journal of Tissue Engineering
Volume 12: 1–25

DOI: 10.1177/20417314211003735
journals.sagepub.com/home/tej
SAGE

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Abstract

The craniofacial region consists of several different tissue types. These tissues are quite commonly affected by traumatic/pathologic tissue loss which has so far been traditionally treated by grafting procedures. With the complications and drawbacks of grafting procedures, the emerging field of regenerative medicine has proved potential. Tissue engineering advancements and the application in the craniofacial region is quickly gaining momentum although most research is still at early in vitro/in vivo stages. We aim to provide an overview on where research stands now in tissue engineering of craniofacial tissue; namely bone, cartilage muscle, skin, periodontal ligament, and mucosa. Abstracts and full-text English articles discussing techniques used for tissue engineering/regeneration of these tissue types were summarized in this article. The future perspectives and how current technological advancements and different material applications are enhancing tissue engineering procedures are also highlighted. Clinically, patients with craniofacial defects need hybrid reconstruction techniques to overcome the complexity of these defects. Cost-effectiveness and cost-efficiency are also required in such defects. The results of the studies covered in this review confirm the potential of craniofacial tissue engineering strategies as an alternative to avoid the problems of currently employed techniques. Furthermore, 3D printing advances may allow for fabrication of patient-specific tissue engineered constructs which should improve post-operative esthetic results of reconstruction. There are on the other hand still many challenges that clearly require further research in order to catch up with engineering of other parts of the human body.

Keywords

Craniofacial, tissue engineering, craniofacial bone

Date received: 27 October 2020; accepted: 26 February 2021

The craniofacial region is a complex network of several tissue types, including bone, cartilage, muscle, salivary glands, nerve tissue, teeth and the surrounding periodontium, and skin/mucosa. The loss of craniofacial tissue may be due to congenital causes, such as clefting[1] and craniofacial microsomia, or acquired conditions, such as facial trauma or tumor resection. The result of this loss is significant aesthetic, functional, and psychological affliction.[2]

Craniofacial reconstruction is challenging due to the complexity of the various structures involved. The various sources of infection in the region (oral/nasal infections) are also to be taken care of. Reconstruction of such defects has conventionally been approached using autologous, allogenic, or xenogeneic grafts to restore the missing tissue with the hope for long-term functional rehabilitation. Complications of this approach range from graft rejection, infection, increased morbidity, and prolonged hospital stays along with the economic burden of such complications.[3–5] With continually evolving tissue-engineering and stem cell technologies, the application of regenerative strategies has gained momentum among many research groups worldwide. Regenerative medicine is a broad term encompassing all efforts to reach the ultimate goal of tissue replacement clinically. Tissue engineering is used to denote production of the target tissue using one of many approaches, which all

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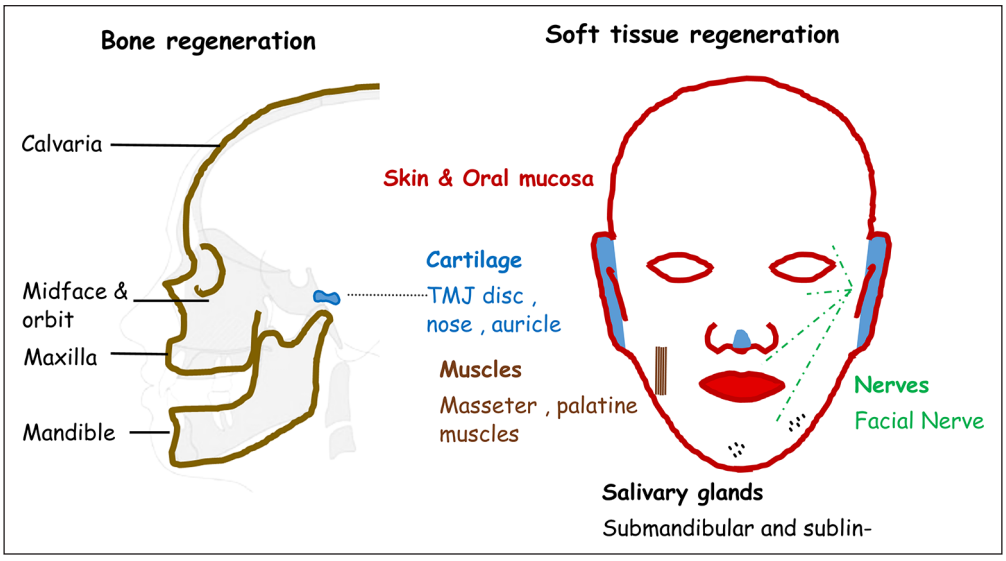

Figure 1. Diagrammatic representation of different craniofacial tissues.

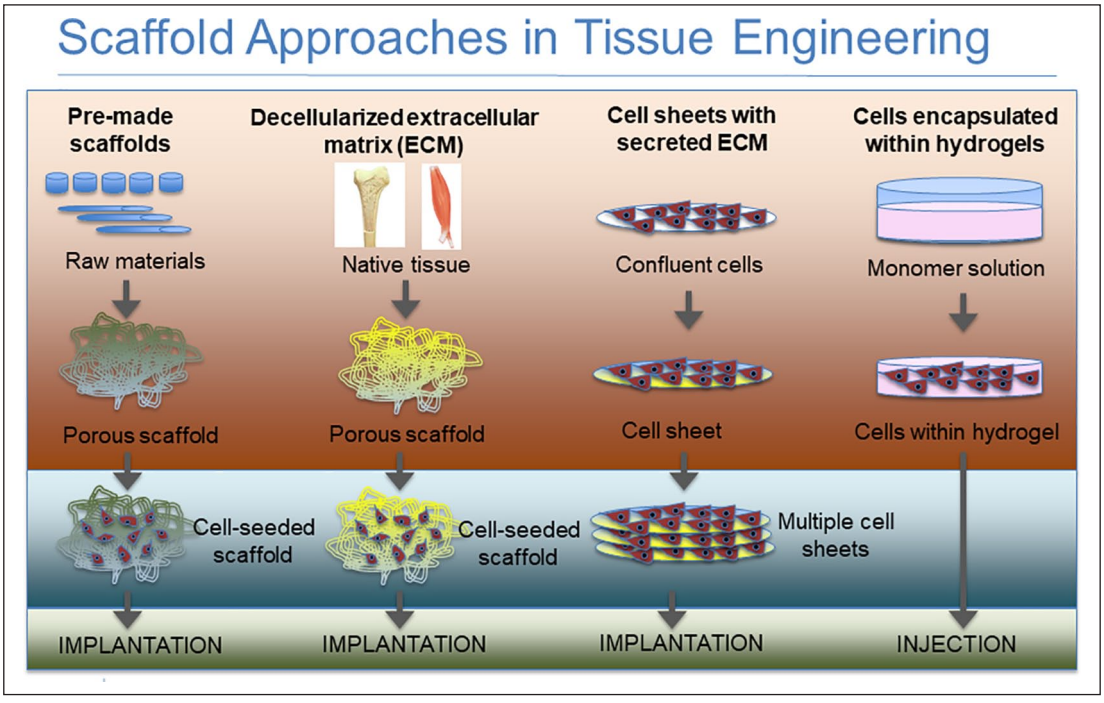

Figure 2. Diagrammatic representation of the different scaffold approaches used in tissue engineering.

follow similar principals. The most common pillars of the tissue engineering process are cells, scaffolds, growth factors, and gene modification to guide cellular differentiation and proliferation[6] (Figure 1). The use of drug molecules for tissue engineering and regenerative procedures has also been reported to be a logical and successful regenerative mechanism.[7] Cell transplantation is often used when the defect is challenging to repair using only the natural regenerative process. Cells are isolated from a donor biopsy and expanded in vitro before transplanting into the defect to effect repair and regeneration. Alternatively, expanded cells may be loaded into a bioactive scaffold. Following cellular adhesion and proliferation, the engineered tissue is then implanted into the defect. The scaffold gradually degrades to allow space for the regenerated tissue to fully integrate with the host to restore structure and function.[8,9] Different scaffolding techniques are used for tissue engineering applications, including the use of decellularized extracellular matrix (ECM) and sheets of cells on ECM (Figure 2). An added

advantage of the regenerative procedures is the ability of incorporation of antimicrobial materials to fight infection which may attack the implanted constructs from nasal or oral infections.[10]

The specific application of such strategies in the craniofacial region is relatively recent compared to the limb region. Craniofacial reconstructive procedures have transitioned from grafting of tissue to 3D-printed implants to tissue engineering techniques, or a combination of these.[11] This review aims to provide clinicians and researchers with a wide overview of what has been reported so far in the field of craniofacial tissue engineering. The latest evidence for each of the craniofacial tissue types will be presented with a representative image from discussed literature when possible (table 1). Tooth regeneration was left out of this review as it has been covered in depth in other previously published reviews.[12,13]

Craniofacial bone regeneration

The current "gold standard" for craniofacial bone tissue replacement is the use of autologous bone grafts from the rib, cranium, or iliac crest. These procedures are associated with several complications, including bone resorption, infection, donor site complication, and can only be used in relatively small defects.[14] Bone regeneration is a popular research topic and numerous reports have been published on novel approaches to promote the best outcomes.[15–17] The investigation of such techniques in the craniofacial region has been reported clinically, but is still in its infancy due to the difficulty of developing a robust in vivo model of a craniofacial bone defect[18] (Figure 3).

The innate regenerative process of healthy bone has been reported as early as 1992.[19] The role of the cellular constituents of the periosteum and their distinct functions at the time of bone injury dictate the regenerative process.[20] The resident bone-forming stem cells of the periosteum differ according to the type of bone, such that the craniofacial periosteum triggers intramembranous bone formation while long bone periosteum promotes endochondral ossification.[21] The employability of the endochondral route to craniofacial bone formation is also a logical approach according to the type of bone to regenerate.[22] The proven regenerative power of the periosteum has led to its use in the management of maxillofacial defects of relatively small size.[23–26] Bone engineering techniques may use a combination of cells (especially mesenchymal stem cells—MSCs), scaffolds and growth factors to manage larger defects. Although several sources of stem cells are known (embryonic; ESCs, Umbilical cord cells; UCSCs, Adult somatic; iPSCs and adult tissue cells) BM-MSCs and ADSCs remain the major sources of stem cells. MSCs are usually harvested from the bone marrow (BM-MSCs) or can be adipose-derived (ADSCs).[27] Bioactive scaffolds provide the proliferating cells with a framework for adhesion, proliferation, and consolidation. 3D printing technologies may be used to produce these scaffolds from materials, such as natural and synthetic polymers. Other recent technologies for scaffold fabrication reported include gas foaming, cryogelation, material extrusion, photopolymerization, electrospinning each with a set of materials that work with it.[28] The use of specific agents/materials/growth factors to enhance the bone-forming activity of differentiated osteoblasts is regularly

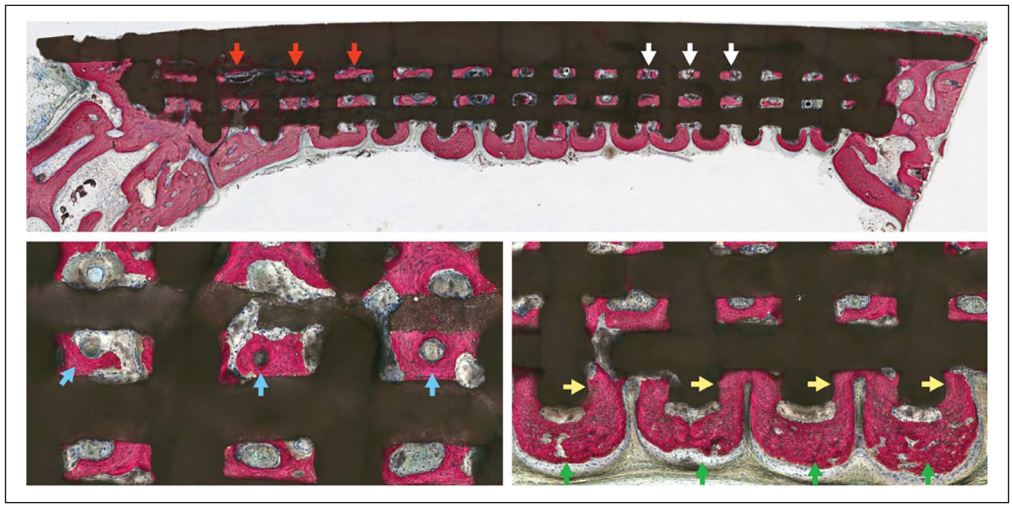

Figure 3. Dipyridamole coated β-TCP scaffold assessment: quadrant scaffold demonstrates bone regeneration through scaffold porosity, at both larger (*red arrows*) and smaller (*white arrows*) pore dimensions. (*Below*, *left*) Highly cellular and vascularized bone formation is seen within scaffold interstices. Intramembranous-like healing is observed with regions of mature, lamellar-like bone formation (*blue arrows*). (*Below*, *right*) Bone formation is guided by highly osteoconductive scaffold dimensions as new bone formation is directed from scaffold pore-to-pore (*green arrows*) while interacting with scaffold struts (*yellow arrows*). Adapted from Maliha et al.[18]

Table 1. Summary of the tissue engineering approaches in the craniofacial region.

Authors	Study type	Year	Regenerative construct	Study model	Reported results	References
Bone regeneration						
Maliha et al.	In vivo	2020	Dipyridamole coated 3D printed B-tricalcium phosphate with varying pore dimensions (220, 330, and 500 μm)	Calvarial defects in rabbits	Large pore scaffolds with Dipyridamole coating showed most bone growth	[18]
Francis et al.	Clinical retrospective trial	2012	Endoscopic craniofacial reconstruction with injectable calcium phosphate cement	Secondary craniofacial reconstruction	The study group showed efficacious, cost-effective reconstruction	[31]
Kirschner et al.	In vivo	2020	Carbonated calcium phosphate cement in craniectomy defects	Frontal cranial defects in immature piglets	The study group with the CRS showed promising bone healing without growth hinderance when compared to the negative control	[32]
Mediero et al.	In vivo	2016	Collagen sponge with CAM/ticagrelor 1 μm/10 μm	Calvarial defect in mice	Ticagrelor and CAM both showed more bone formation three dimensionally as compared to negative control (scaffold with saline). Comparable to the amount of bone when BMP was used	[35]
	In vivo	2016	3D printed collagen coated hydroxyapatite—βtricalcium phosphate scaffolds with ticagrelor 1 mM or CAM 1 mM	Calvarial defect in mice	Both ticagrelor and CAM showed significantly more bone formation than scaffold alone and comparable amount to the BMP treated defect	
Nokhbatolfoghahaei et al.	In vitro	2020	Gelatin/β-tricalcium phosphate scaffolds loaded with mesenchymal cells from the buccal fat pad and rotating-perfusion versus perfusion bioreactors	–	Rotating-perfusion bioreactor group showed higher RUNX2, OCN expressions and ALP and collagen one production increase when compared to the static and perfusion bioreactor	[36]
Lopez et al.	In vivo	2019	3D-printed bioceramic scaffolds with 1000 μm of dipyridamole/10,000 μm of dipyridamole/0.2 mg/ml of rhBMP-2	Alveolar clefts in white immature rabbits	Dipyridamole allowed bone healing comparable to the BMP group with the early suture closure seen with the latter. The formed bone in both groups were of mechanical properties comparable to that of the native bone	[37]
Wang et al.	In vivo	2019	Dipyridamole loaded 3D printed β-tricalcium phosphate scaffolds	Calvarial and alveolar defects in immature rabbits	The scaffolds showed significant bone formation in comparison to the gold standard bone graft	[38]
Zhao et al.	In vitro	2009	β-tricalcium phosphate mixed with fibrinogen and thrombin to make injectable scaffolds	–	Human mesenchymal stem cells showed cytoviability and cellular number increase in the scaffold. Increased β-TCP content enabled a higher elastic modulus of the final scaffold	[41]
Wang et al.	In vitro	2016	Injectable calcium phosphate cement scaffolds with different cell types hDPSCs, hiPSC-MSCs from bone marrow (BM-hiPSC-MSCs) and from foreskin (FS-hiPSC-MSCs) and hBMSCs	–	The scaffolds supported cell viability, osteogenic differentiation. All cell types showed expression of bone forming genes. FS-hiPSC-MSCs were reported to be relatively inferior to the rest of the cell types in osteogenesis	[42]
Hasani-Sadrabadi et al.	In vivo	2020	Injectable alginate-based hydrogel scaffold (AdhHG) with mesenchymal stem cells	Subcutaneous implantation in mice	The hydrogel was proven to be biocompatibility, biodegradable and osteoconductive	[43]
		2020	Injectable Alginate-based hydrogel scaffold (AdhHG) with gingival mesenchymal stem cells	Rat peri-implantitis models	Complete bone regeneration was achieved around failing dental implants	
Chen et al.	In vivo	2019	DBBM/collagen gel/DBBM + collagen gel	Rabbit calvarial model	Addition of DBBM significantly improved immature bone formation while the Gel group improved soft tissue healing. The combination treatment is the best way to manage multi-tissue regeneration	[47]

(Continued)

Table 1. (Continued)

Authors	Study type	Year	Regenerative construct	Study model	Reported results	References
Salamanca et al.	In vivo	2016	Freeze-dried porcine collagen membrane with bovine xenograft	Lateral alveolar ridge defects in beagle dogs	The new collagen membrane improves osteoconduction and reduces alveolar height resorption rate	48
Salamanca et al.	In vitro	2020	Collagenated porcine graft compared to porcine graft, HA/β-tricalcium phosphate with MG-63 osteoblast-like cell line	–	CPG group showed greater cell proliferation and osteoblastic differentiation. Gene sequencing showed stable bone formation markers and reduction of resorption makers	49
	In vivo	2020	Collagenated porcine graft compared to porcine graft, HA/β-tricalcium phosphate	Calvarial defects in adult male white rabbits	CPG group showed the highest new bone regeneration by osteoconduction	
Cassetta et al.	Clinical trial	2015	Augmentation using 100% autologous bone, 100% porcine graft, 50:50 mixture of both	Sinus augmentation	Porcine bone alone and with autologous bone showed osteoconductivty and biocompatibility	50
Ning et al.	In vivo	2019	LAGG-PM composite hydrogels with rat adipose-derived stem cells (rADSCs)	MRONJ induced rat model	LAGG-PM composite hydrogels were found to promote mucosal recovery, bone tissue reconstruction, and osteoclastogenesis	52
Rodrigues-Lozano et al.	In vivo	2020	Bone marrow derived-MSCs cultured on β-Tri calcium phosphate	MRONJ induced mouse model (maxillary alveolar sockets)	No MRONJ-related bone exposure was detected in the study group versus 33% exposure in the control (β-TCP and saline)	53
Sallstrom et al.	In vitro	2020	Zwitterionic sulfobetaine hydrogel with direct culture of neuroblastoma cell line VS indirect culture		The material seemed to support cellular growth and proliferation and that was supported by the appearance of extended neurites on the hydrogel surface	54
Diez-Escudero et al.	In vitro	2020	Porous polylactic acid scaffolds with Diamon/Gyroid/Schwarz internal configuration with pre-osteoblastic cell lines		No cytotoxicity was reported. The larger and multimodal porosity supported differentiation better	55
Muscle regeneration						
Manchineella et al.	In vitro	2016	Silk fibroin/melanin films and electrospun fiber sheets as scaffolds with C2C12 myoblast cell line	–	The scaffolds promoted the myoblast's assembly and differentiation and proved thermal stability provided by melanin	61
Vandenburgh et al.	In vitro	2008	Primary mouse myoblasts on polydimethylsiloxane (PDMS) attached to flexible microposts of varying diameters (300–800 μm), 4–5 mm tall, and 4 mm apart	–	The miniature bioartificial muscles generated active forces upon electric stimulation	62
Abou Neel et al.	In vitro	2005	Phosphate-based glass fibers (PGF) with different iron oxide (Fe_2O_3) molarity	–	PGF with larger diameters and 3–5 mol% Fe_2O_3 are more durable scaffolds that should allow for better initial myoblast attachment than others with 1 or 2 mol% Fe_2O_3	63
Farano et al.	In vitro	2018	Melt-quenched phosphate glasses were combined as powders with collagen fibers from bovine achilles tendon to make degradable scaffolds	Scaffold characterization	Characterization of the fabricated scaffolds showed interconnected porous structures and biodegradability. Bioactivity was proven by finding a Ca-P rich layer on all scaffolds' surfaces—whish was comparable to that formed by HA in one sample	64
Guo et al.	In vitro	2019	Injectable electroactive degradable hydrogels (dextran-graft-tetraaniline and N-carboxyethyl chitosan) with C2C12 myoblasts and human umbilical vein endothelial cells		Biocompatibility was confirmed Myoblasts showed linear like release	65
	In vivo	2019	Injectable electroactive degradable hydrogels (dextran-graft-tetraaniline and N-carboxyethyl chitosan) with C2C12 myoblasts and human umbilical vein endothelial cells	200 μL were injected subcutaneously in rat tibialis anterior defects	Due to it's injectability, the hydrogel allows non-surgical implantation high myofiber density, more capillaries, and centronucleated myofibers in the defect were detected in all study groups with significantly higher numbers of centronucleated myofibers in the 3% AT scaffolds	

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Table 1. (Continued)

Authors	Study type	Year	Regenerative construct	Study model	Reported results	References
Jung et al.	In vivo	2017	Pulp cells extracted from adult human premolars treated with 5-Aza	Gastrocnemius and masseter muscles of male mice	The epigenetic modification with 5-Aza stimulated muscle regeneration in vivo	[70]
Brady et al.	In vitro	2008	Human myogenic and non-myogenic muscle-derived cells (MDC) seeded in 3D collagen constructs		Non-myogenic cells can be used for 3D myogenic differentiation, force generation and matrix remodelling The mix of cell origins had a synergistic effect on peak force and MMP-2 mRNA expression	[71]
Shah et al.	In vitro	2004	Human masseter derived cells cultured on phosphate-based glass fibers of different orientations		3D mesh arrangement of the glass fibers supported the best cell attachment and proliferation Increasing seeding density and adding ILGF-1 and Matrigel enhanced prototypic muscle fiber formation	[72]
Zhang et al.	In vivo	2019	Human amniotic mesenchymal cells with the DNA demethylating agent 5-azacytidine	Volumetric muscle loss in rat tibialis anterior muscle	The rat model showed improved local tissue repair and increased angiogenesis	[74]
Cartilage regeneration						
Vinatier et al.	In vivo	2009	Autologous rabbit nasal chondrocytes (RNC) associated with an injectable self-setting cellulose-based hydrogel (Si-HPMC)	Rabbit articular cartilage defect	The defect treated with RNC showed formation of repair tissue organized similar to normal cartilage The regenerated tissue was histologically hyaline-like cartilage	[77]
Ahtiainen et al.	In vivo	2013	Bi-layer polylactide (PLA) discs and autologous adipose stem cells (ASCs) with TGF-β1 for TMJ disc regeneration	Rabbit temporomandibular joints	ASC—PLA discs pre-treated with TGF-β1 improved condylar integrity Histologically, no inflammation, infection or foreign body reactions were detected	[78]
Vapniarsky et al.	In vivo	2018	Scaffold-free tissue constructs from passaged costal chondrocytes	Intralaminar implantation in TMJ discs of minipigs	The tissue engineered construct group showed better healing of the defect than the empty control. Histologically the cartilaginous formation and collagen content change was noted, while the mechanical properties of the constructs were also acceptable. Necropsy revealed no signs of cell damage/inflammation/neoplastic changes	[79]
Cakmak et al.	In vivo	2013	Injectable tissue engineered cartilage within a fibrin glue with/without aprotinin, different concentrations of thrombin and fibrinogen. (chondrocytes harvested from auricle/costa/nasal septum)	Subcutaneous injection interocular and forehead of white rabbits	Inflammatory reactions, abscess formation, and foreign body reactions around the new cartilage tissue of tissue-engineered cartilage The different groups (concentrations of constituents/cell sources) showed no statistically significant differences	[80]
Kim et al.	In vivo	2019	Human umbilical cord matrix-mesenchymal stem cells (hUCM-MSCs) for the treatment of TMJ-osteoarthritis in comparison to other MSCs origins	Intra-articular injection in rabbit models with induced TMJ osteoarthritis	Regenerative and anti-inflammatory capacity of the hUCM-MSCs was clear hUCM-MScs anti-inflammatory effect was comparable to that of dexamethasone Moreover, only hUCM-MSCs showed potential for chondrogenesis.	[82]

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Table 1. (Continued)

Authors	Study type	Year	Regenerative construct	Study model	Reported results	References
Cui et al.	In vivo	2020	Human dental pulp stem cells (DPSCs) were injected into the articular cavity to treat rat TMJ arthritis	Local injection in arthritic temporomandibular joints of female rats	Local injection of DPSCs in rats with arthritic joints of rats relieved hyperalgesia, synovial inflammation, reduced cartilage degradation, and enhanced bone regeneration	[83]
Ogasawara et al.	In vivo	2020	IV injection of conditioned media from human exfoliated deciduous teeth stem cells (SHED-CM)	Injection in induced osteoarthritic mouse model	Suppressed temporal muscle inflammation, and improved bone integrity and surface smoothness of the destroyed condylar cartilage	[84]
Zhang et al.	In vivo	2019	Mesenchymal stem cells' exosomes injection	Intra-articular injection in 8-week old rats' osteoarthritic TMJ	MSC exosomes promoted TMJ repair and regeneration in OA The cell-free ready-to-use exosome-based therapeutic potential for treating TMJ pain and degeneration is significant	[85]
Kuznetsov et al.	In vivo	2019	Undifferentiated bone marrow stromal cells (BMSCs) on fibrin microbeads (FMBs)	Subcutaneous injection in immunocompromised mice	Significant amounts of hyaline-like cartilage were reported when BMSCs were attached to hyaluronic acid coated FMBs	[86]
Chen et al.	In vivo	2020	3D fabricated decellularized bone scaffolds with autologous adipose-derived chondrogenic and osteogenic cells.	Ramus-condyle defect models in minipigs	The fabricated RCUs maintained their structure and cartilage was regenerated over the underlying bone more than the bone only and acellular scaffold comparators	[87]
Park et al.	In vivo	2017	3D-printed PolyCaproLactone implants	Septal grafting for nasal reshaping in white rabbits	The implants retained their location Histologically, the implant retained its morphology with significant fibrovascular ingrowth and minimal inflammation	[89]
Reuther et al.	In vitro	2014	Human septal chondrocytes expanded and resuspended in alginate on transwell clear polyester membrane insert		The expanded constructs were histologically similar to those of the standard size	[90]
Mendelson et al.	In vivo	2014	Alginate containing gelatin microspheres encapsulating cytokines on PLGA base (with r-TGFβ3 at different concentrations)	Rhinoplasty model in rats	Cartilage-like tissue formation was enhanced by increasing doses of TGFβ3 This technique may be a successful alternative for augmentative and reconstructive rhinoplasty	[91]
Yi et al.	In vivo	2019	3D model of customized nasal implant with injected hydrogel containing human adipose-derived stem cells	Subcutaneous implantation in mice	Maintenance of the exquisite shape and structure, and striking formation of the cartilaginous tissues for 12 weeks	[92]
Cao et al.	In vivo	1996	PGA-PLA scaffolds with chondrocytes isolated from bovine articular cartilage	Subcutaneous pockets on dorsa of athymic mice	Morphologic and histologic assessment showed the formation of new cartilage The overall geometry resembled that of an infant auricle	[94]
Morrison et al.	In vivo	2016	Human auricular chondrocytes (hAuC) and human mesenchymal stem cells (hMSC) encapsulated into type I collagen hydrogels shaped like full scale-ear constructs	Subcutaneously implanted in mice dorsa	The construct showed cartilage microstructure The human ear constructs maintained shape, projection, and flexibility	[95]
Kagimoto et al.	In vivo	2016	Xenotransplantation of progenitor cells to reconstruct ear cartilage.	Subcutaneous region of a craniofacial defect in a monkey	Elastic cartilage was regenerated Mature elastic cartilage with newly formed perichondrium was successfully detected	[96]
Liao et al.	In vivo	2015	A chondrocyte membrane on an ear-shaped Ti model	Implanted in dorsal pockets of nude mice	Histologically the newly formed tissue was confirmed to be elastic cartilage	[97]

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Table 1. (Continued)

Authors	Study type	Year	Regenerative construct	Study model	Reported results	References
Matuska et al.	In vitro	2018	Effect of delipidation on decellularized porcine TMJ disc with seeded human MSCc		A combination of solvents and surfactant treatment no cytotoxicity or residual lipid content was noted	98
Nerve regeneration						
Binnetoglu et al.	In vivo	2019	Bacterial cellulose conduits for nerve regeneration with or without primary suturing	Main trunk of facial nerve in female rats	The number of myelinated fibres was significantly higher with the placement of bacterial cellulose conduits	107
Piao et al.	In vivo	2020	Collagen conduits with collagen-binding domain (CBD)-human basic fibroblast growth factor (bFGF)	Buccal branch of facial nerve injury model in white rabbits	CBD-bFGF enhanced functional facial nerve regeneration	108
Watanabe et al.	In vivo	2017	Silicone conduits with differentiated and undifferentiated Adipose derived stem cells (ADSCs) embedded in a collagen gel	Nerve defect in the buccal branch of the facial nerve of rats	Functional nerve regeneration was evident in all groups comparable to results of autologous nerve grafts	110
Sasaki et al.	In vivo	2011	Degradable PLGA tubes filled with dental pulp cells (DPCs) embedded in collagen gel	Nerve defects in the buccal branch of mandibular nerve of adult rats	The PLGA tubes resorbed in vivo Tuj-1 positive axons were noted 2 months after transplantation	111
Costa et al.	In vivo	2013	Bone marrow stem cells in Polyglycolic acid tube conduits with BMSCs/Schwann-like cells differentiated from BMSCs	Mandibular branch of facial nerve defects in rats	Facial nerve regeneration was improved by PGAt and the Schwann-like cells enhanced the regeneration potential	112
Xiao et al.	In vitro	2017	Dental pulp cell spheroids on matrigel in vitro		DPCs differentiated into neuronal lineage under neuronal inductive conditions They can stimulate neurogenesis in mouse hippocampal slices in vitro	113
Salivary gland regeneration						
Joraku et al.	In vivo	2005	Primary human salivary gland cells grown expanded and seeded on Polyglycolic acid scaffolds	Subcutaneous implantation in mice	Histologically acinar gland-like structures were noted in the regenerated tissue Expression of human salivary type of α-amylase mRNA was confirmed	121
Joraku et al.	In vitro	2007	Human salivary cells cultured, expanded and seeded on a 3D collagen-based gel scaffold	–	Functional, differentiated salivary units containing acini and ducts were reported	122
Nam et al.	In vivo	2019	Submandibular gland cell sheets (single vs multiple layers)	Direct placement into the wounded submandibular glands of mice	Single layer cells retained the cell-to-cell junctions. The double layer sheets formed glandular like structures in vitro.	125
Ogawa et al.	In vivo	2013	Bioengineered gland germ from cells from submandibular, sublingual and parotid glands of mice with PGA extension into the parotid duct	Implanted atop the masstere muscle after extraction of salivary glands in female mice	Salivary flow and content was comparable of that in normal mice	124
Nam et al.	In vivo	2017	Submandibular gland cells on Fibrin Hydrogels with LI peptide conjugation	Submandibular gland wound models in mice	Organized salivary tissue was formed with good collagen organization was noted in the group with the FH scaffolds	126

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Table 1. (Continued)

Authors	Study type	Year	Regenerative construct	Study model	Reported results	References
Maruyama et al.	In vitro	2015	Combination of laminin and a feeder layer of human hair follicle derived mesenchymal stem cells (hHF-MSCs)	–	hHF-MSC conditioned medium improved cellular orientation and allowed acinar and ductal structure formation	127
Su et al.	In vivo	2020	Labial stem cells from human labial glands were extracted and expanded, the extract (LSCE) after centrifugation was used to regenerate irradiated salivary glands	Irradiated mice were injected with the LSCE through the tail vein	50%–60% increase in salivary flow was noted in LSCE treated mice in comparison to the control group Histologically a comparable number of acinar and neurovascular components was noted	129
Skin, mucosa, and periodontal regeneration						
Gielkins et al.	In vivo	2008	Poly (DL-lactide-e-caprolactone) (PDLLCL) membrane versus collagen and expanded polytetrafluoroethylene (ePTFE) membranes in implant defects	Mandibular angle defects in male rats	PDLLCL membranes showed less bone formation than the collagen and ePTFE membranes	132
Duskova et al.	In vivo	2006	Resorbable collagen membranes (single-layer and double-layer); porcine collagen type I and III membrane versus atelocollagen membrane	Clinical alveolar defects with cancellous bone grafts	No statistically significant difference was found between the groups although the double membrane was more expensive	134
Cortellini et al.	Clinical in vivo	2011	Non-resorbable/bio-resorbable barrier membranes; enamel matrix derivative (EMD)/a combination of bio-resorbable membranes and a bovine xenograft of bovine origin/a combination of EMD and alloplastic biomaterials/a combination of bio-resorbable membranes and EMD versus extraction and restoration of hopeless teeth	Hopeless teeth with perio-endo lesions	92% of the teeth treated with regeneration protocols lasted throughout the 5-year follow-up Most of the regenerated teeth showed reduction in mobility	135
Liu et al.	In vitro	2020	Assessment of potential use of Human periodontal ligament stem cells (hPDLSCs) to differentiate into different cell lineage	–	hPDLSCs were able to differentiate into bone-, fiber- and cementum-forming cells, and so can be used for regeneration of periodontium—bone-PDL-cementum complex specifically	136
Guo et al.	In vivo	2017	Dental follicle cell (DFC) sheets and periodontal ligament cell (PDLC) sheets in periodontal defects	Healthy beagle dogs with simulated periodontal defects	Periodontal attachment was noted in both groups. Periodontal ligament–cementum complex structure and better alveolar bone height was only noted in the DFC sheet group DFC sheets are more effective for periodontal regeneration	137
Xue et al.	Clinical trial	2018	Human acellular amniotic membrane (HAAM) with Vaseline gauze	Full-thickness defects in the lower third of the nose in humans	HAAM improved hemostasis and accelerated pain reduction. Lower infection rates and scar incidence were also noted	140
Chen et al.	Clinical trial	2018	Bioengineered dermal substitute (dermal regeneration template)	Human traumatic periocular tissue loss	Defects either healed completely (50%), one case showed significant improvement not requiring secondary reconstructive procedures, and one other case showed significant reduction in defect size	142
Rhee et al.	Clinical trial	1998	Acellular dermal matrix in comparison to split thickness skin grafting	Intraoral mucosal defects in humans	Graft take was successful in 90% of the cases	143

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Table 1. (Continued)

Authors	Study type	Year	Regenerative construct	Study model	Reported results	References
Seol et al.	In vivo	2018	BioMask—a customized bioengineered skin substitute which fits perfectly onto facial wounds	Face defects in mice	Skin regeneration was noted at the dermis and epidermis levels According to patient's CT; wound dressing material and cell-laden hydrogels are accurately printed in a layer-by-layer way	[144]
John et al.	In vitro	2019	De-epithelialization of human amniotic membrane as a cellular scaffold as a skin substitute	–	Trypsin and cell scraper provided best de-epithelialization results but showed tissue strain Culturing of keratinocytes and fibroblasts on the membrane was successful and resulted in a mostly keratinized surface	[145]
Roh et al.	In vivo	2017	Mucosa and skin equivalents were produced from cultured fibroblasts and autologous fibrin and seeding keratinocytes	Full-thickness excisional wounds of rat skin	The cell sheets enhanced healing with earlier wound closure and less scarring Lower TGF-β1, α-smooth muscle actin, and fibronectin mRNA expression was also noted	[147]
Suzuki et al.	In vitro	2020	Fish scale type I collagen scaffolds as oral mucosa equivalent	–	Histologically, a fully differentiated epithelial layer was noted indicating that the microstructured fish scale collagen scaffolds can be used to fabricate tissue-engineered oral mucosa equivalents for clinical use	[150]
Engineering of multiple tissues						
Costa et al.	In vivo	2014	Biphasic scaffold with a bone compartment (coated with a calcium phosphate (CaP) layer) and a periodontal PCL compartment	Subcutaneous implantation dorsally in nude male rats	The CaP compartment showed significant ALP activity while the PCL compartment showed with the larger pores allowed better vascularization and periodontal attachment	[153]
Lee et al.	In vivo	2014	PCL-HA scaffolds with three phases (100 mm microchannels for cementum/dentin interface, 600 mm microchannels for PDL, and 300 mm microchannels for alveolar bone) with DPSCs, PDLSCs, and ABSCs	Subcutaneous pouches in immunodeficient mice	Properly oriented PDL-like collagen fibers, bone sialoprotein-positive bone-like tissue and putative cementum matrix/dentin tissues were found indicating success of the multiphasic scaffold	[154]

reported in literature. These agents may enhance cellular recruitment and adhesion to the scaffold, promote proliferation then differentiation of specific cells important for bone regeneration and inhibit antagonistic activity (such as that of osteoclasts).[15–18,29,30]

Calcium phosphates are widely used for bone reconstruction.[31,32] 3D-printed β-tricalcium phosphate (β-TCP) scaffolds soaked in collagen and coated with dipyridamole have been investigated for bone regenerative purposes. Dipyridamole is a known osteogenic agent that increases osteoblastic differentiation and inhibits osteoclastic activity and inflammatory responses.[33–35] Scaffolds of varying pore dimensions (220, 330, and 500 μm) impregnated with different concentrations of dipyridamole (100, 1000, and 10,000 μm) were placed within critical-sized calvarial defects in 5-week-old rabbits. Optimal bone growth and scaffold biodegradation were reported with larger pore sizes and the highest dipyridamole concentration. Histological and radiographical assessment showed vascularized woven and lamellar bone along with initial formation of vascular canals (denoting angiogenesis) within the scaffold lattice and patent calvarial sutures, an important requirement for calvarial reconstruction/regeneration.[35] Other researchers have also reported success with 3D-printed β-TCP scaffolds coated with dipyridamole.[36,37] Wang et al.[38] compared autologous bone grafts with 3D-printed β-TCP dipyridamole-coated scaffolds in alveolar clefts and calvarial defects created in immature rabbits. At 24 weeks, the bioactive scaffolds showed better osteogenic generation than the autologous graft in both the alveolar and calvarial defect sites. In addition, the regenerated bone in both sites showed resemblance to the native bone in terms of organization of trabeculae and mechanical characteristics. The patency of the sutures was validated radiographically at 6 months.

A firm, solid cell-scaffold construct can prove difficult to handle and may not fit a defect easily. Injectable scaffolds have several advantages over stiffer scaffolds.[39] The ability to inject a semi-solid or gel-like material into a defect and have it set in position in vivo supports a less invasive clinical approach.[40] Injectable tricalcium phosphate scaffolds were tested in vitro within a fibrin gel and were proposed as an osteogenic property-enhancer.[41] Injectable hydrogels containing calcium phosphate cement have proven to possess superior mechanical properties and allow better cellular adhesion in vitro.[42] Research on methods to enhance the mechanical properties of the setting scaffolds is currently ongoing. Such bioactive hydrogels have been investigated for craniofacial bone regeneration applications.[43] Injectable alginate hydrogels loaded with hydroxyapatite, bone morphogenic proteins, and gingival mesenchymal cells were used in peri-implantitis defects created in the maxilla of mice. Once injected, final setting of the alginate hydrogel was achieved by photopolymerization. In vivo assessment showed that more than 50% of the alginate had dissociated by 6 weeks. Clinical and micro-CT assessment revealed fewer inflammatory mediators, better bone recovery and implant survival in defects managed with the hydrogel compared to controls. The option to alter the degradation rate of the hydrogel by changing its molecular weight opens up the potential for regeneration of tissues other than bone.[43] Hydroxyapatite (HA)—which is a naturally occurring calcium phosphate—has been commonly used to enhance osteoconductivty of regenerative constructs is a commonly followed strategy.[44] HA and other biphasic calcium phosphates have been reported to be a successful addition to β-TCP in experimental maxillofacial models, clinical socket preservation, sinus lift procedures, and crestal height and width augmentation.[45] The use of synthetic biomimetic calcium phosphate (SBCP) granules in rat calvarial defects showed bone regrowth that was comparable to that resulting from deproteinized bovine bone material (DBBM). SBCP led to faster bone regeneration. This was thought to be due to the microstructure and higher total porosity of SBCP. The SBCP showed superior results in terms of vertical bone growth which is of great clinical importance in alveolar augmentation.[46]

Collagen is an integral constituent of bone. Thus, many groups have utilized this natural biomaterial combined with xenogeneic bone material to mimic bone structure and facilitate regeneration. Collagen gels combined with DBBM were used to reconstruct critical-sized calvarial defects in adult rabbits. The presence of DBBM allowed maturation of the formed bone such that it resembled the composition and mechanical properties of native bone. The collagen gel supported better soft tissue healing (in the calvarial skin flap) when compared to the autologous bone grafting; which adds to the scaffold advantages regarding multi-tissue regeneration.[47] Collagen combined with porcine bone particles was studied and reported to enhance bone regeneration and reduce bone loss in alveolar defects in beagle dogs.[48] Moreover, the collagen-porcine bone particle scaffolds demonstrated greater bone regeneration of critical-sized calvarial defects in adult white rabbits as compared to β-TCP/hydroxyapatite scaffolds.[49,50]

In cases of bone osteonecrosis-specifically MRONJ (medication related osteonecrosis of the jaw) the use of stem cell therapies to reduce the effect of the drug on the bone, reducing inflammatory reactions and promoting healing. One study identified that MSCs in a mouse MRONJ model improved the healing capacity of the affected MSCs and so provided a better therapeutic benefit. The effect of MSCs in MRONJ treatment may be attributed to their capability of secreting immunomodulatory factors and being immunopriviliged.[51] On the other hand, the local delivery of ASCs and BMP-2 in a hydrogel vehicle reinforced with hemicellulose polysaccharide fibers, showed better mucosal recovery, bony reconstruction,

and formation of new osteoclasts in a rat model.[52] A more recent report also confirmed the effectiveness of BM-MSCs cultured on β-TCP in improving mucosal coverage with no bone exposure and better bony healing in comparison to the negative control in rat models.[53] This gives hope for future better management of MRONJ cases which are a clinical dilemma for craniofacial surgeons. The application of bone engineering technologies in the craniofacial region has had reasonable success. The multiple cell types, scaffolds and additions (e.g. growth factors) allow for testing of different combinations to promote bone deposition and maturation. The use of 3D printing technologies and computer-aided design of scaffolds with specific micro- and macro-structure of the scaffolds is now possible and has a promising future. A recent study reported acceptable primary results using a syringe-extruded hydrogel scaffold which was then cured to final setting. Although elastic modulus and tensile strengths of the final material still needs to be improved; the cellular viability and proliferation of the used neuronal cells in this study prove potential for further use.[54] Moreover, the further utilization of additive manufacturing and computer-aided designing (CAD) technologies will allow the specific designing of internal micro and macro porosity which could be tweaked according to the necessary tissue types. This was tested on a PLA printed scaffold with different internal pore morphologies and the seeded pre-osteoblastic line showed promising proliferation and activity.[55]

Further research on the application of these technologies is expected soon. The possibilities of specific designing of the internal architecture of scaffolds to mimic the extracellular matrix of target defects in patients are encouraging. Combining these methods with more conventional grafting procedures or with vascularized grafts may also be a possibility in large defects to combine the advantages of each of the techniques. Clinically oriented research should soon be able to provide evidence on techniques suitable for the different types of craniofacial bony defects.

Craniofacial skeletal muscle regeneration

Muscles of the craniofacial region are of great importance for function and esthetic appearance.[56,57] These muscles may be subject to trauma, cancer, generalized muscle disorders, surgical resections, or autoimmune diseases necessitating grafting/regenerative procedures. Orofacial congenital defects, such as cleft lip and/or palate, are associated with impaired muscle regeneration and fibrosis after surgery also requiring repair.[58]

For engineered muscle to be an acceptable option for muscle regeneration, the tissue has to have similar to native architecture and possess acceptable mechanical properties. The muscle healing process starts by activation of quiescent myogenic progenitor cells followed by proliferation and differentiation.[59] To resemble the regenerative process on a larger scale, muscle progenitor cells can be extracted and expanded in vitro. The confluent cells may then be loaded onto a scaffold, which guides and supports early muscle formation and maturation.[30] Growth factors may also be used to support the muscle regenerative process and include fibroblast growth factor (FGF), hepatocyte growth factor (HGF), insulin-like growth factor (IGF), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and stromal cell–derived factor (SDF).[60]

The fabrication of a scaffold mimicking the ECM of the native muscle tissue is necessary to enable proper growth and angiogenic activity of the central core to provide nutrients to the newly formed muscle and the proliferating cells. There are several different biomaterials that have been investigated for muscle tissue engineering. Biodegradable polyester scaffolds used for muscle regeneration include natural compounds, such as α-hydroxy acids (polyglycolic acid, poly-L-lactic acid, and polycaprolactone) and silk-fibroin.[61] Synthetic biomaterial scaffolds, such as polyurethanes, polypropylene, silicone,[62] and phosphate-based glasses[63,64] are alternative options.[65] Injectable hydrogels have further allowed for simpler delivery to sites of defect.[65]

Different cell sources have also been investigated for muscle regenerative approaches including satellite cells, ADSCs, BM-MSCs, PVSCs, iPSCs, ESCs, and UC-MSCs.[66] Umbilical cord mesenchymal stem cells (UC-MSC) are a promising source. These can be harvested from the umbilical cord without the need for a separate biopsy procedure from the child. The umbilical cord blood and tissue contain a heterogeneous mixture of stem and progenitor cells at different stages of differentiation.[67,68] The use of UCMSCs in cleft lip and palate surgery together with anti-inflammatory and antifibrotic agents is a promising method.[69]

Pulp stem cells were also reported to have myogenic potential when pretreated with 5-Aza (5-aza-2'-deoxycytidine; a modified demethylation agent) in vitro. Treatment of the pulp cells with 5-Aza stimulated myotube formation, myogenic differentiation associated with desmin and myogenin expression, and a degree of scaffold contraction. The epigenetic modification of these cells (collected from premolars) stimulated craniofacial muscle regeneration in the masseter and gastrocnemius of adult mice in vivo.[70]

Human myogenic and non-myogenic craniofacial muscle-derived cells (MDC) extracted from biopsies and seeded onto 3D collagen constructs expressed myogenin, indicative of myogenic differentiation. Furthermore, there was a synergistic effect as the heterogeneous co-culture of myogenic and non-myogenic cells generated the highest peak force (muscle function) and expressed the most MMP-2 mRNA compared to isolated individual cell populations.[71] This may guide further research on muscle regeneration by starting off with a mixed co-culture if that enhances the final engineered muscle activity. Human

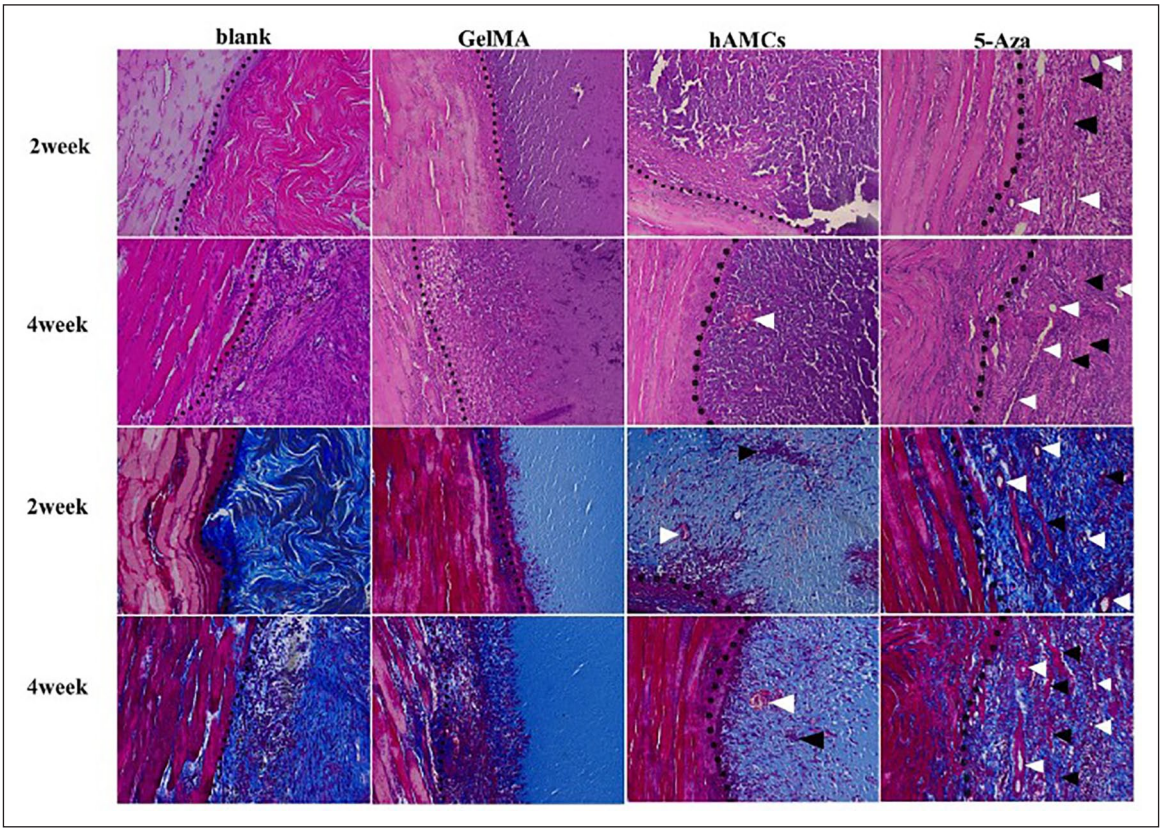

Figure 4. hAMCs improved tissue repair on VML. H&E and Masson trichrome staining of 2 and 4 weeks after establishment of VML model (blank group), implantation of GelMA gel (GelMA group), GelMA + hAMCs (hAMCs group), and GelMA + 5-Aza-inducted hAMCs (5-Aza group). The dotted line is the boundary between normal muscle tissue and defect. White arrow shows the neovascularization, black arrow shows fused hAMCs and fiber-like tissue. 200×. Adapted from Zhang et al.[74]

masseter muscle-derived cells were also used with phosphate-based glass fiber scaffolds in vitro. The scaffolds were fabricated with different internal configurations; bundle alignment, spread-out, and mesh-like arrangement. Microscopic imaging showed that the mesh arrangement led to optimal cell attachment and proliferation which may have been due to the macrotopography of the scaffolds which provided more delicate spaces allowing better cellular grouping and adhesion.[72]

Creation of a robust craniofacial muscle defect in animal models has proved challenging—for example, a soft palate surgical site was tested in adult rats, but proved too small and difficult to handle.[58] Therefore, the most common region to test muscle engineering applications are the limb muscles. Although the structural similarities of craniofacial and limb muscle are undeniable, cellular constituents vary.

Clinical application of muscle regeneration spans a wide range of defects with volumetric muscle loss being the type of defect immensely requiring intervention whether as in grafting with the complications of that[73] or regeneration.[66,74] A recent study reported encouraging results in Volumetric Muscle Loss (VML) of rat tibialis anterior muscle using amniotic mesenchymal cells. Muscle specific markers (MyoD and desmin) were detected at the end of the study along with improved angiogenesis and local tissue repair[74] (Figure 4).

Craniofacial muscle engineering efforts are starting to gain more attention with the important clinical solutions they offer. Challenges still exist regarding the conductivity of scaffolds, vascular ingrowth within the engineered constructs and transfer of the in vitro outcomes to the in vivo environment to further test the proposed methods.

Cartilage

The craniofacial region has several cartilaginous structures, such as the articular disc of the temporomandibular joint (TMJ), the nose and ears. The avascular nature of cartilage and its resulting poor regenerative capacity has made the reconstruction of cartilage defects/deformities an important area of research.

The TMJ disc is a cartilaginous structure, which is integral to the normal mandibular movements pertaining to function. Traditional approaches to manage common destructive conditions of the TMJ disc, include non-invasive and invasive joint procedures associated with high complication and failure rates including diminished

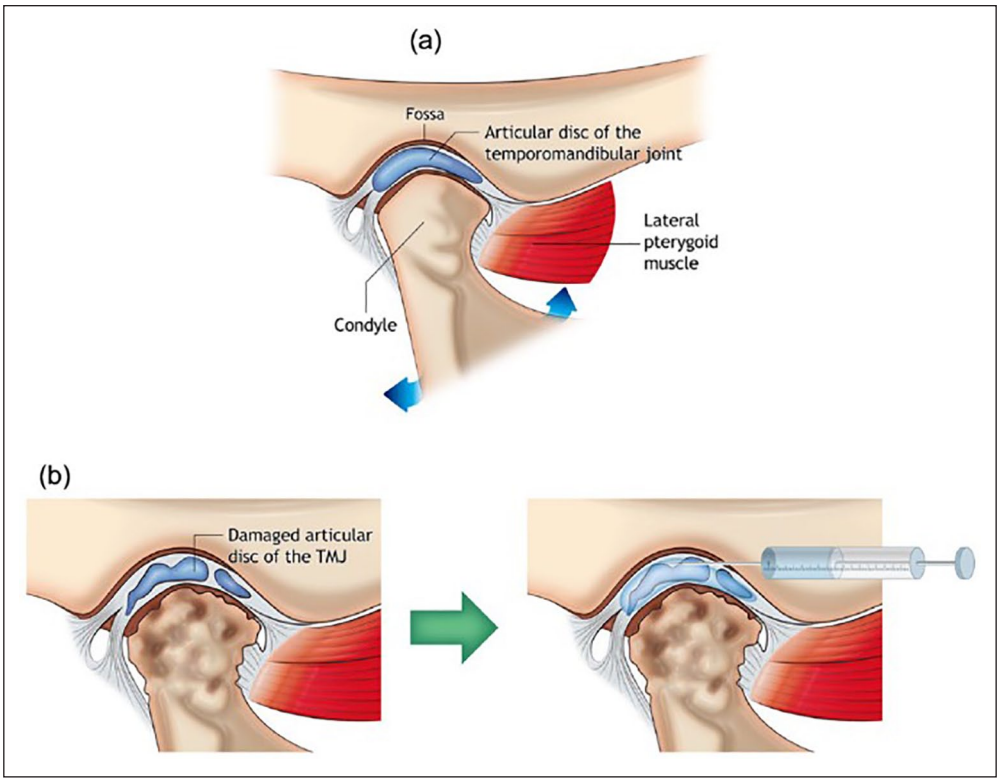

Figure 5. (a) Schematic image of the anatomical structure of temporomandibular joint (TMJ) and the most common target sites for treating temporomandibular disorder (TMD). The image shows components of normal joint anatomy, including the articular disk of TMJ, mandibular fossa, the head of the mandibular condyle, lateral pterygoid muscle, and TMJ capsule enclosing the disk. (b) TMD morphology; the head of the mandibular condyle and the articular disk lose their structures and functions. Intra-articular injection: injection with syringe and needle can deliver proper biomolecules into TMJ capsule for treating TMD as adapted from Dashnyam et al.[81]

mobility, prolonged pain episodes, scarring, numbness, and bleeding.[75] Therefore, investigation into disc regeneration utilizing tissue engineering approaches has become essential. The direct injection of human mesenchymal cells into arthritic joints showed better articular cartilage repair and slowing down of the arthritic progression.[76] However, there is still a need to produce cartilage discs in the presence of advanced destruction, and many approaches have also utilized scaffolds loaded with cells. Nasal chondrocytes extracted from nasal cartilage of adult female white rabbits were reported to successfully treat a knee articular disc defect in an adult rabbit model when used with an injectable hydrogel scaffold. Although this was tested in an osteochondral knee defect; the principle was reported to be applicable in other similar joints.[77] Moreover, Chondrogenic differentiation of rabbit adipose stem cells seeded on PLA scaffolds enabled fabrication of articular discs.[78]

Scaffold-free tissue engineered implants were constructed from costal chondrocytes of minipig ribs. After ex vivo testing of the technique of the tissue-engineered construct implantation; the constructs were placed in a designed intralaminar defects of TMJ discs of minipigs. The efficacy of these constructs to repair thinning discs was assessed by gross inspection, histologic assessment, and osteoarthritis scoring. All these criteria showed better disc repair using the tissue engineered constructs as opposed to the empty controls.[79]

An injectable mixture of fibrin, thrombin, and differentiated chondrocytes was reported to enable fabrication of cartilage-like structures in adult rabbit heads. The origin of these chondrocytes was septal/auricular and the recipient sites were the forehead and interocular regions.[80] The inflammatory events that occur within the temporomandibular joint are now proven to be a primary cause of the pain, functional limitation, and degenerative procedures that occur. The use of cell-laden biomaterials as vehicles for drug delivery intra-articularly has shown superior results to conventional injections due to the longevity of its action and the regenerative capacity it enforces[81] (Figure 5). Moreover, hUC-MSCs injected into osteoarthritic rabbit joints, showed significant regenerative capacity and anti-inflammatory action comparable to that of the Dexamethasone injection control. Growth factors, ECM markers and anti-inflammatory cytokines exhibited upregulated expression while pro-inflammatory cytokines

expression was reduced.[82] This was also proven with Dental Pulp stem cells in arthritic rat joints[83,84] human shed deciduous SCs in mice arthritic joints[84] and human-derived ESCs in rats.[85] Although clinical trials have not yet reported the use of these technologies to fabricate human cartilage clinically; reports of the differentiation and implantation of human bone marrow cells to engineer cartilage have emerged.[86]

The regeneration of ramus-condyle units (RCU) has been reported to be the solution in cases of TMJ degenerative disease. Scaffolds were milled into anatomically identical forms using decellularized bone matrix and impregnated with adipose-derived chondrogenic and osteogenic cells. The constructs were implanted in minipig's jaws after culturing for 5 weeks. The constructs maintained their forms and showed better full-thickness regeneration and mechanically comparable cartilage formed over a bony stump than acellular scaffolds and bone-only engineered grafts. This provided an opportunity for multiple tissue regeneration. Inclusion of the adjacent tissues such as soft connective tissues and the TMJ disc could further extend the functional integration of engineered RCUs with the host.[87]

Nasal reconstruction historically involved autogenous grafting to attain an aesthetic alar/nostril configuration especially in cleft patients.[88] This has evolved recently with the use of 3D printing technologies to improve post-surgical outcome.[89] Septal chondrocytes harvested from healthy human candidates showed regenerative capacity and resulted in neocartilage constructs of significant volume in vitro.[90] A cell-homing procedure was reported where nasal dorsum progenitor cells were recruited and chondrogenically differentiated onto a bi-layered alginate and PGLA scaffold leading to the formation of cartilage-like constructs 10 weeks later in rat models.[91] A recent report on a 3D printed nasal cartilage augmentation technique has shown promising results when the printed scaffold impregnated with hASCs implanted subcutaneously in female athymic mouse backs. The use of such technology carries a promise for better postoperative esthetics and shorter healing periods which are crucial in esthetic surgery.[92]

With the improved esthetics and patient satisfaction, similar techniques were used for auricular cartilage reconstruction.[93,94] Human auricular chondrocytes (hAuC),[95] monkey-derived perichondrium progenitor cells,[96] and porcine chondrocytes were all tested for their regenerative capacity.[97] The cells in these studies were harvested, grown in vitro then implanted in vivo. The harvested tissue showed organized arrangement of cartilage mimicking auricular composition. Ex vivo testing of scaffolds versus scaffold-less injectable techniques showed primary results of the possibility of using such techniques for articular regeneration.[98]

Although research is targeted towards cartilage regeneration in several parts of the human body (in vitro and in vivo preclinical testing), the applications in the craniofacial region are in the early stages. Clinical reports on TMJ disc regeneration are yet to provide evidence of success. Improving mechanical properties of the engineered cartilage and further testing of its long-term survival and function is also yet to be improved.

Craniofacial nerve tissue engineering

Peripheral nerve injuries generally require a complicated grafting procedure to return to pre-injury sensation/function in sensory and motor neurons, respectively. The grafting procedures carry increased risk of infection, graft rejection, and donor site morbidity.[99,100] Craniofacial nerves are of specific importance. They are either sensory, motor, or carry both types of fibers. These nerves play an integral part in the sensory detection and reaction to internal and external stimuli. They also have a key role in motor control of the masticatory muscles, extraocular muscles, and muscles of facial expression.[101] Craniofacial nerve injury is not uncommon due to the prevalence of facial trauma/tumors affecting the related neural structures.[102] The facial nerve, in particular, is responsible for facial expression—injury of the facial nerve causes facial asymmetry and esthetic deficits, which are managed by primary surgical nerve repair or nerve grafting.[103,104] These techniques are quite complex and require expensive equipment and training to enable the surgeon to perform the delicate microsurgical anastomosis.[105,106]

An intact endoneurium is necessary to bridge gaps between the distal and proximal edges of a nerve injury. The use of conduits for neural repair is a common strategy where different materials and/or grafts are implanted at the injury site to guide the neuronal regeneration.[107] Different additives have been tested on the fabricated scaffolds for neuronal regeneration, such as fibroblast growth factor,[108] IL-6, neurotrophins, glial-derived neurotrophins, and persephin.[109] Most of the reported studies have used pluripotent stem cells because they are easy to harvest and readily differentiate to nerve tissue. A few studies have reported the use of ADSCs, BMSCs, or dental pulp cells in facial nerve regeneration with varying success.[110–112] Dental pulp stem cells have a common ectodermal origin making these cells a logical source for nerve tissue engineering. These cells were tested in vitro and showed encouraging results in terms of stimulating neurogenesis in adult mice ex vivo.[113,114] Isolated muscle stem cells have also shown ability to regenerate nerve cells in murine xenotransplantation models.[115,116] Moreover, recent murine studies have demonstrated that cells of the immune system, specifically regulatory T cells, play a critical role in nerve regeneration following acute chemical injury.[115–118] The utilization of recent technologies has not been disregarded in neural regeneration attempts; recently a 3D engineered functional human peripheral nerve was tested on a novel nerve-on-a-chip platform with very

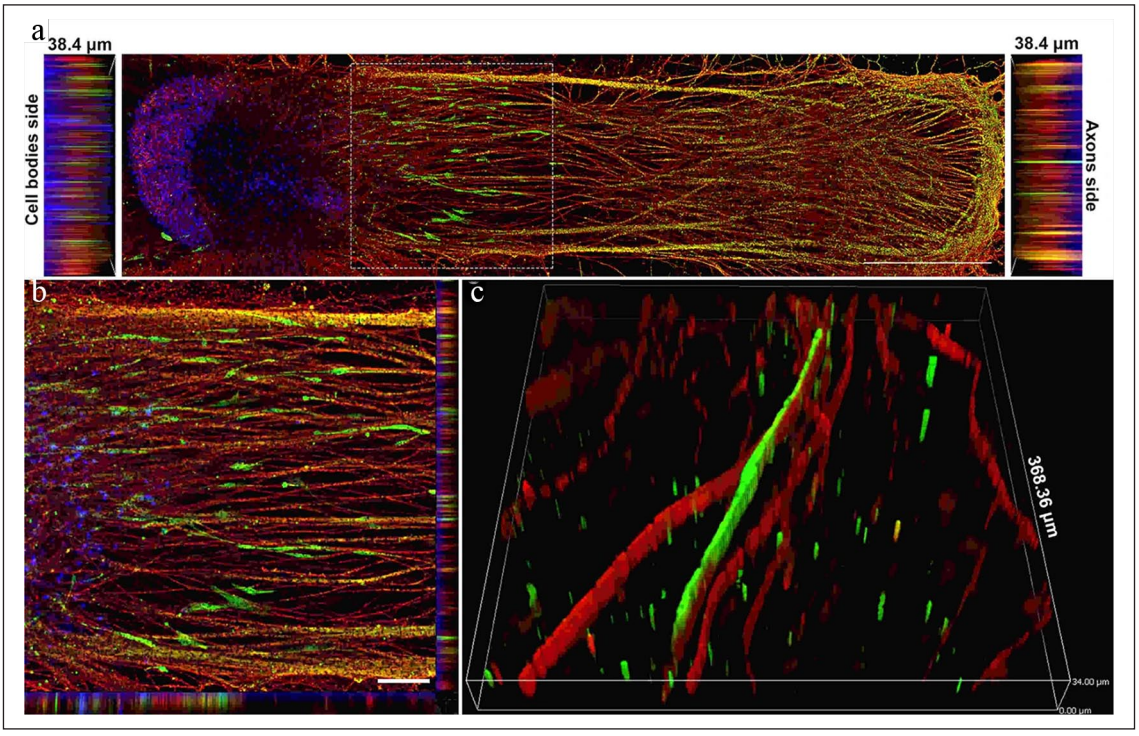

Figure 6. Schwann cells migrated out of the spheroid and elongated along the axons. (a) Image showing how human Schwann cells (hSCs) stained for the hSC marker S100 (green) migrated out of the spheroid along with growing axons stained for βIII-tubulin (red) over a period of 4 weeks. Nuclei were labeled with DAPI (blue). Scale bar: 1000 µm. (b) High-magnification image of inset from image A. Scale bar: 25 µm. (c) 3D image showing close-up of the relationship between hSCs (green) and myelinated axons (red). Slice size was 368.36 × 368.36 × 34.00 µm. Adapted from Sharma et al.[119]

encouraging results[119] (Figure 6). With most of these trials, especially targeting craniofacial nerve engineering, being in vitro; translational and in vivo trials are much needed. Further assessment of the proposed methods, their longevity and long-term results and whether they may also aid in myogenic regeneration of target muscles is crucial. Moreover, clinical application remains largely unattained to with it being the most important target of all neural regeneration research.

Engineering the salivary glands

The craniofacial region has multiple major and minor salivary glands, which may be affected by conditions, such as auto-immune disorders (Sjogren's disorder) and tumors. The result of these conditions is salivary gland dysfunction leading to hyposalivation or xerostomia. Patients have a poorer quality of life, because of poor masticatory and taste ability, and the increased risk of fungal infections and dental caries due to loss of the protective and lubricant quality of saliva. Conventional management is with sialogogues and salivary substitutes, which require strict patient compliance. Sialogogues are associated with complications, such as muscle aches and pains.[120]

Attempts to regenerate the damaged salivary parenchyma started by isolation of primary human-salivary cells; the culture of isolated cells was reported as early as 2007.[121,122] Parotid specimens of healthy consenting adults were collected and the epithelial cells were isolated and cultured on collagen scaffolds. A rise in amylase production was noted in the cultured salivary epithelial cells indicating the organization and activity of acinar cells. These were compared with human bladder cells as a control group over a 6-day culture period. Ogawa et al. (2015) isolated germ cells from the submandibular, parotid, and sublingual glands of mice and demonstrated epithelial bud formation after 2 days in organ culture.[123] When transplanted back into salivary gland defects in mice, better salivary flow was noted when compared to the no-intervention control.[123] A mixture of epithelial and mesenchymal cells extracted from submandibular, parotid, and sublingual salivary glands of adult mice were used to regenerate an organ germ which was then implanted onto a masseter defect in female mice after extraction of their submandibular, sublingual, and parotid glands. The implanted gland had an extending Polyglycolic acid (PGA) guide which was inserted into the host parotid duct. Saliva was collected from the oral cavity with and without stimulation proving the functional capacity of the bioengineered salivary glands. No significant difference in salivary flow and content between the bioengineered glands and those in natural mice was noted (Figure 7).[124] Correct acinar/glandular cellular orientation

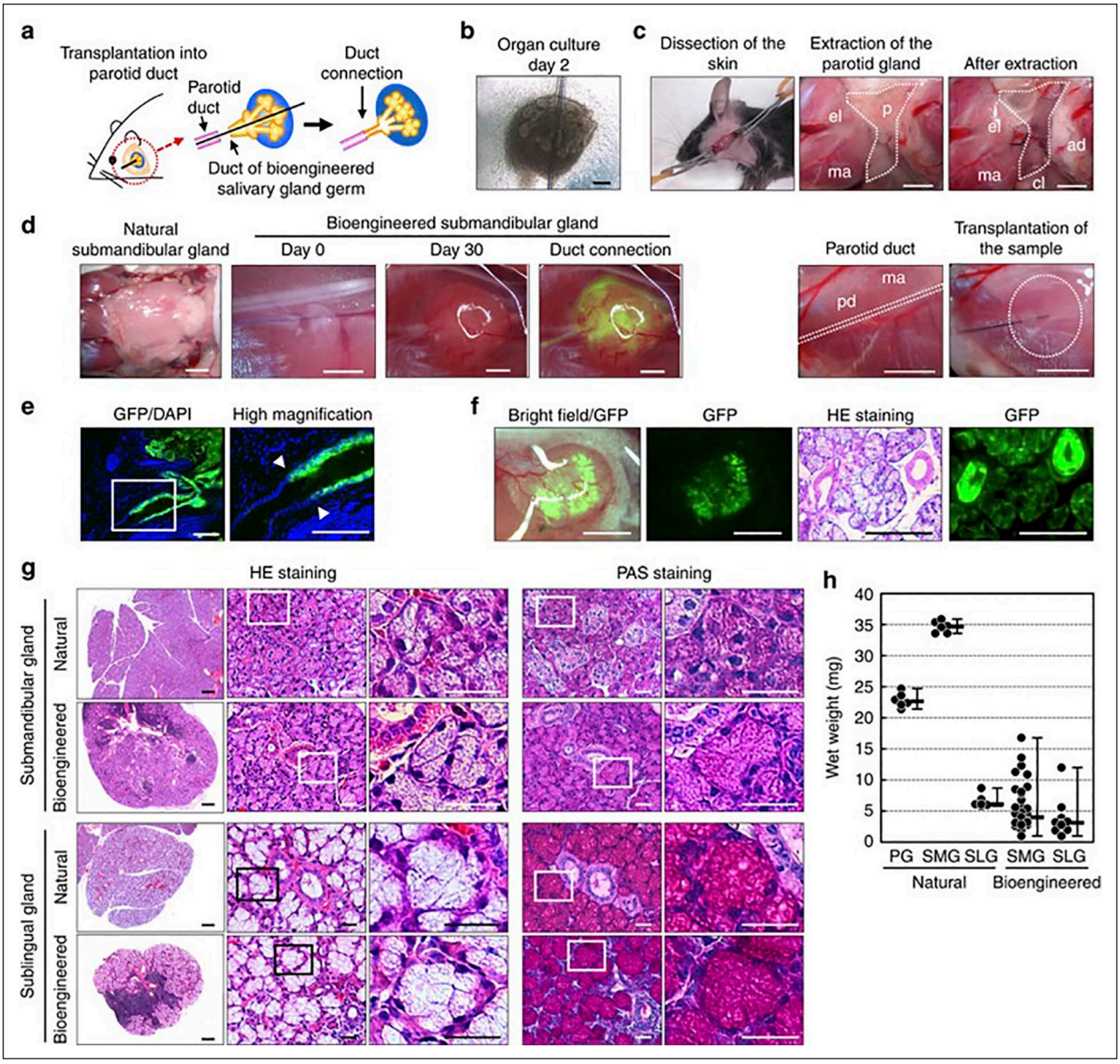

Figure 7. (a) Schematic representation of the transplantation procedure using the interepithelial tissue-connecting plastic method with the bioengineered salivary gland germ. (b) Phase-contrast images of the bioengineered salivary gland germ containing a PGA monofilament guide. Scale bar, 200 μm. (c) Photographs of bioengineered salivary gland germ transplantations in salivary gland defect mice. The three major salivary glands were extracted and a bioengineered salivary gland germ was transplanted. Scale bar, 1 mm. (d) Photographs of the natural submandibular gland (left) and the bioengineered salivary gland at days 0 and 30 after transplantation (second and third figure from the left). FITC-gelatine conjugate was injected into the bioengineered submandibular gland from the host parotid duct (right). Scale bar, 1 mm. (e) Histological images of the duct connection between the host duct and epithelial duct of the GFP-labelled bioengineered salivary gland (left). Higher magnification images in the box area are shown (right). Bioengineered salivary glands developed in vivo with the correct connection to the recipient parotid gland duct (arrowhead). Scale bar, 150 μm. (f) Photographs of the bioengineered salivary gland, which was reconstituted from GFP-transgenic mice-derived epithelial cells and normal mice-derived mesenchymal cells (left: merged with the stereomicroscope image and the GFP image, second figure from the left: GFP image). Scale bar, 1 mm. The section images of hematoxylin and eosin (HE) staining (third figure from the left) and GFP fluorescence (right) are shown. Scale bar, 200 μm. (g) Histological analysis of the submandibular gland (upper columns) and the sublingual gland (lower columns), including the natural (upper) and bioengineered (lower) salivary glands. Images of HE staining (left three) and periodic acid and Schiff (PAS) staining (right two) are shown. Higher magnification images in each box area are shown (second and third panels from the left, right figure). Scale bar, 100 μm in the left column and 25 μm in the second and subsequent columns. (h) Wet weights of natural and bioengineered salivary glands. The data are presented as the median ± max, min; $n = 6$ for the natural parotid, submandibular and sublingual glands, $n = 20$ for the bioengineered submandibular glands and $n = 9$ for the bioengineered sublingual glands. PG: parotid gland; SLG: sublingual gland; SMG: submandibular gland. Adapted from Ogawa et al.[124]

is crucial to provide proper salivary function. Researchers have been able to achieve this through the layering of cells. When implanted into a mouse model, a double layer of cultured submandibular gland cells showed superior regenerative properties with organization of the cells into acini-like structures compared to a single layer of cells.[125]

Hydrogel scaffolds were used in mouse submandibular gland injury models to prove their efficiency in promoting regeneration of salivary gland injury. Laminin-fibrin hydrogel scaffolds were used in a wound-healing model of mouse submandibular glands.[126] Laminin is a protein of the extracellular matrix that showed capacity to improve growth organization and differentiation of salivary cells in vitro.[127] Histologic assessment showed that the laminin-fibrin hydrogels guided organized salivary regeneration as opposed to the disorganized collagen formation in the untreated model. The defect treated with fibrin hydrogel alone showed some salivary organization, but was said to be worse than that seen with the laminin-fibrin group.[126] Any scaffolds to be used for salivary regeneration must allow for proper structural integrity for the heterogenic population the naturally occurring salivary glands. This was reported to be achieved by nature-inspired catechol-conjugated hyaluronic acid environment (NiCHE) formation. This was said to mimic the hyaluronic acid rich mesenchymal environment in embryonic submandibular glands. When tested on previously discussed scaffolds (PCL, hydrogels, polycarbonate membrane) led to better cellular adhesion, proliferation, angiogenesis, and structural branching in vitro.[128] Surgical access to cells of the minor salivary glands of the lips and cheeks is easier and safer than those of the parotid, submandibular, and sublingual salivary glands. Recently, cultured stem cells extracted from human labial tissue discarded during dental surgery were injected into the blood stream of irradiated mice. Better salivary flow was noted in the salivary glands of the injected subjects at 9 weeks than those of the sham control group.[129]

The use of different stem cell reservoirs to enable salivary differentiation and the combinations with different scaffold materials allows for a great deal of flexibility in research design. With the promising results seen in large animal studies, the transition into clinical trials is in the near-future and may finally provide a satisfactory solution for patients with salivary hypofunction.

Mucosa, periodontal, and skin tissue engineering

The periodontal interface consists of bone, dentin, cementum, and the periodontal ligament. Periodontitis is a chronic disorder with progressive inflammation and damage of the tooth-supporting structures, eventually leading to tooth loss.[130] Treatment of the resulting defects must include regeneration of the alveolar bone and adjacent periodontal ligament (PDL). One of the earliest forms of craniofacial tissue engineering was the guided regeneration achieved by using membranes (such as GorTEX) in periodontal defects whereby unwanted cells were excluded from the regenerative field and the healing process was "guided" as needed. Several reports claim it a possible method of PDL regeneration.[131–134] A 5-year clinical trial reported the successful restoration of periodontal defects with the use of GorTEX membranes, bovine xenograft, enamel derivative, or a combination.[135] Regeneration of periodontal defects is a distinct process due to the complexity of the structures of the periodontal interface. Young native PDL stem cells (PDLSCs) derived during tooth extraction were investigated for their ability to regenerate the PDL structures. These cultured cells showed the ability to differentiate into osteogenic, cementogenic, and fibrogenic lineages enabling periodontal regeneration.[136] PDLSCs were also compared with dental follicle cells (DFCs) in their ability to restore periodontal defects in adult dogs. Although periodontal reattachment was seen in both groups, it was reported that the DFC group showed more organized periodontal regeneration.[137]

Regeneration of facial skin has a great impact on the patient's quality of life and psychological health. Skin engineering was one of the earliest tissues investigated. As a result, skin tissue grafts are commercially available reducing the need for autologous grafts from other sites and the associated morbidity.[138,139] However, limitations will always exist necessitating continual improvement. Acellular membranes, such as human acellular amniotic membranes separated from consenting mother placentas, have been used in clinical trials. When compared to Vaseline® gauze treatment, the human acellular membranes showed better hemostasis and pain scores.[140] Unfortunately, due to their acellular nature, these grafts show high resorption and infection rates.[141] Dermal substitutes, such as Integra®, have been used successfully in cases of traumatic defects of large areas of facial skin. Defect size shrinkage of up to 40% have been reported, but graft take, infection, time to treatment, and cost are some of the disadvantages.[142] Allograft dermal tissue is another method for mucosal defect coverage in various locations, such as the tongue, vestibule, floor of the mouth, palate, and lips. Although 90% of the patient population showed complete graft take and epithelialization, contracture, scarring, pain, and infection were also reported.[143] To further improve facial aesthetics and patient satisfaction, CAD/CAM applications and recent 3D bioprinting were used to produce patient-specific facial skin based on a CT images. Researchers have bioprinted "facial masks" composed of a tri-layer of polyurethane (PU), a keratinocyte-laden hydrogel, and a fibroblast-laden hydrogel and tested it within facial defects in adult mice with promising results (Figure 8).[144] Recently, culturing of keratinocytes and fibroblasts was reported on de-epithelialized human amniotic membranes with favorable results. A largely keratinized layer

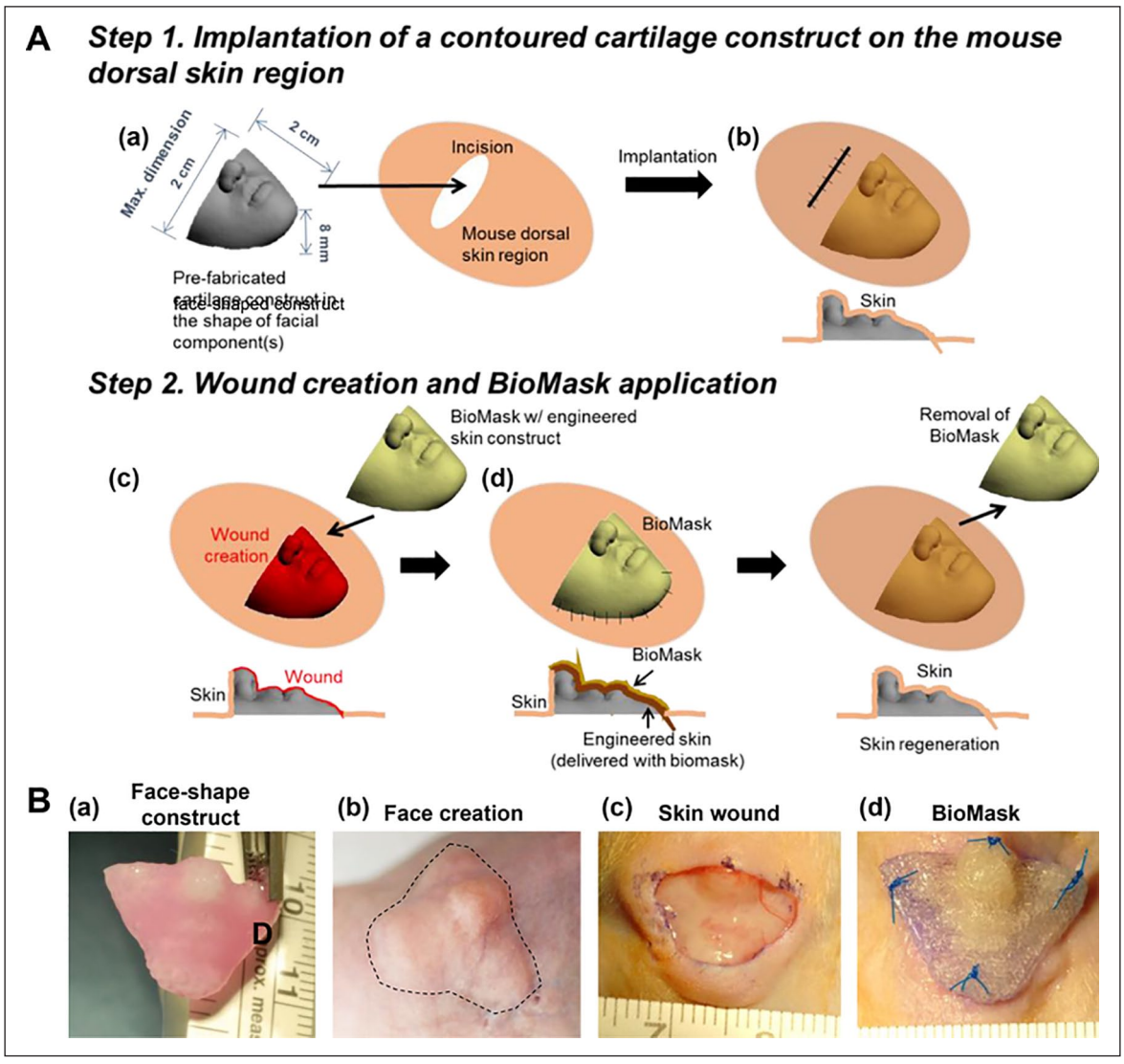

Figure 8. (A) Schematic illustration of facial skin wound animal model creation and implantation: (a) fabrication and (b) implantation of pre-fabricated face-shaped construct, (c) wound creation on the face-shaped construct after 4-week implantation, and (d) BioMask application. (B) Surgical procedure of BioMask application: (a) face-shape construct, (b) face creation after 4-week implantation, (c) 70% skin wound on the face-shaped construct, and (d) BioMask application. Adapted from Seol et al.[144]

was achieved on the epidermal surface of the membrane with a structured fibrous surface on the other side.[145]

Wound healing of the oral mucosa is generally faster and associated with less scarring than in the skin. This may be attributed to the simple epithelial differentiation and lower inflammatory cascades that occur.[146] Culture of mucosal cells (whether in vitro or in vivo) are therefore a logical goal. Oral mucosa cell sheets were grown from human and rat donor keratinocytes and fibroblasts. These were isolated from oral mucosal, pharyngeal, esophageal, and neck skin biopsies. Implantation in a skin model wound of adult rats demonstrated enhanced primary wound healing with less scar tissue formation in comparison to the no intervention control group.[147] Cell feeder systems were also proposed to fabricate mucosal tissue[148] with long-term cryogenic storage (up to 204 days) of fabricated oral mucosa epithelial cell sheets opening up more possibilities in clinical application.[149]

Recently, a biomimetic engineered human mucosal equivalent was produced from fish collagen. Histologic assessment of the produced construct showed a fully differentiated and stratified epithelial layer and a dermal-epidermal junction similar to that of human oral mucosal tissue.[150] The enhancement of the physical properties of this construct remains an issue that must be solved before clinical use.

Although there has been expansive research in skin/mucosal tissue engineering; the integration of these techniques clinically will only be acceptable when issues of infection, scarring, and cost are completely resolved. On

the other hand, periodontal tissue regeneration is becoming more and more predictable in preventing tooth loss secondary to periodontal disease.

Engineering of multiple tissues

The craniofacial region as discussed earlier consists of several different tissue types. Regeneration procedures in many of the clinical scenarios requires regeneration of more than a single tissue type. The most common explain for this is the engineering of the periodontium. In this case a bone, periodontal ligament, dentin, and cementum should be regenerated. Research has proven that is possible especially with the advances in scaffold fabrication. Multilayering of scaffolds to fit the different necessities for each tissue type has proven to be of acceptable results. Different materials and fabrication technologies have been used to fabricate multilayered scaffolds and all showed primary promising results in vitro.[151,152] An earlier attempt reported the use of a biphasic scaffold to engineer the bony and periodontal components of a periodontal defect. The scaffold consisted of an FDM fabricated part coated with Calcium phosphate, seeded with osteoblasts and cultured for 6 weeks. The resulting construct was then augmented with PDL cell sheets onto the electrospun scaffold surface and implanted into rats for a total of 8 weeks. The results showed good regeneration in the bony and periodontal compartments and high vascularization.[153] Fabricating scaffolds such that each part fits the target tissue needed. This was tested by fabricating PCL-HA scaffolds in three different phases each with internal porosities differing according to the tissue type and DPSCs, PDL stem cells, ABSCs were cultured. The results of this study also supported the evidence suggesting that multi-layered/configured scaffolds can indeed be used to produce constructs of different tissue types.[154]

The concept of simultaneous multi-tissue engineering is of great clinical importance. Most craniofacial defect caused by tumor ablation or trauma consist of several tissue types requiring regeneration whether bone, PDL, and mucosa or muscle and skin for example. Providing clinicians with well tested choices to regenerate these tissues rather than resort to much dreaded grafting procedures is target to lots of ongoing research.

Conclusions and future perspectives

The clinical condition of patients with craniofacial defects necessitates reconstruction utilizing the most cost-efficient and cost-effective approaches. Complexity of the reconstruction is paramount due to the different tissue types usually involved in such defects. The necessity for simple effective regenerative procedures is paramount and the results discussed in this review confirm the potential of craniofacial tissue engineering strategies as an alternative to avoid the problems of currently employed reconstructive/grafting techniques (Table 1). With the variety of scaffold materials, cell origins, growth factors, drug molecules, and gene modification possibilities; a wide range of application is feasible. Research targeting specific tissue type or a multi-tissue engineering are to be proven in vivo. Furthermore, recent advances in 3D printing and scanning technologies open the opportunity for fabrication of patient-specific tissue engineered constructs. This should allow launching of in vivo and clinical trials assessing what has already been proven in vitro. The translation of the research has shown that some of the successful in vitro approaches do not really work in vivo due to the complexity of natural cellular and extracellular microenvironments. The importance of vascular ingrowth (angiogenesis) in vivo is another area that needs more work. It remains one of the main reasons scaffold-cell construct implantations fail due to the necrosis of the inner portions of the constructs not receiving sufficient blood supply from the surrounding host tissue. There are still many challenges ahead and it is clear that further research is essential in order to catch up with engineering of other parts of the human body.

Acknowledgements

This review was completed during a Fulbright Visiting Scholar fellowship sponsored by the Fulbright Commission in Egypt.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: Part of Dr Emara's Fulbright Visiting Scholar Fellowship

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