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Strategies for Oral Mucosal Repair by Engineering 3D Tissues with Pluripotent Stem Cells

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Significance: Human-induced pluripotent stem cells (iPSC) can be differentiated into patient-specific cells with a wide spectrum of cellular phenotypes and offer an alternative source of autologous cells for therapeutic use. Recent studies have shown that iPSC-derived fibroblasts display enhanced cellular functions suggesting that iPSC may eventually become an important source of stem cells for regenerative therapies.
Recent Advances: The discovery of approaches to reprogram somatic cells into pluripotent cells opens exciting avenues for their use in personalized, regenerative therapies. The controlled differentiation of functional cell types from iPSC provides a replenishing source of fibroblasts. There is intriguing evidence that iPSC reprogramming and subsequent differentiation to fibroblast lineages may improve cellular functional properties. Augmenting the biological potency of iPSC-derived fibroblasts may enable the development of novel, personalized stem cell therapies to treat oral disease.
Critical Issues: Numerous questions need to be addressed before iPSC-derived cells can be used as a practical oral therapy. This will include understanding why iPSC-derived cells are predisposed towards differentiation pathways along lineages related to their cell of origin, screening iPSC-derived cells to ensure their safety and phenotypic stability and developing engineered, three-dimensional tissue models to optimize their function and efficacy for future therapeutic transplantation.
Future Directions: Future research will need to address how to develop efficient methods to deliver and integrate iPSC-derived fibroblasts into the oral mucosa. This will require an improved understanding of how to harness their biological potency for regenerative therapies that are specifically targeted to the oral mucosa.

Abbreviations and Acronyms

3D = three-dimensional
BMP = bone morphogenetic protein
c-Myc = Myelocytomatosis viral oncogene homolog
ECM = extracellular matrix
hESC = human embryonic stem cells
HSE = human skin-equivalent
iPSC = induced pluripotent stem cells
KLF4 = Kruppel-like factor 4
MSC = mesenchymal stem cells
OCT4 = Octamer-binding transcription factor 4
SOX2 = SRY (Sex determining region Y)-box 2

SCOPE AND SIGNIFICANCE

Human-induced pluripotent stem cells (iPSC) offer an alternative source of autologous cells for a range of human therapies that include repair and regeneration of the oral mucosa. After their reprogramming from somatic cell types, iPSC can be maintained indefinitely under defined culture conditions and subsequently differentiated into lineage-and patient-specific cells with a wide spectrum of cellular phenotypes. The realization that somatic cells are reprogrammable to a pluripotent state has resulted in novel approaches related to drug development and cell-based treatments for a range of diseases and tissue engineering strategies. Recent studies have shown that iPSC-derived cells may also display

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functional improvements when compared to the parental cells from which these iPSC were originally reprogrammed, indicating that they can be a preferred source for autologous cell-based therapies. For example, fibroblasts differentiated from iPSC displayed improved functional features, such as extended replicative potential, increased mitochondrial function,[1,2] and wound reparative potential.[3] Acquisition of these features suggests that iPSC are rapidly becoming a renewable source for regenerative therapies of the oral mucosa. This review will present an overview of the relevance of iPSC reprogramming for wound healing and regenerative therapies in a broad sense, describe the properties of iPSC-derived fibroblasts capable of healing and will discuss approaches through which iPSC-based technologies may be applied for future oral mucosal repair and regeneration.

TRANSLATIONAL RELEVANCE

The controlled differentiation of functional cell types from iPSC establishes a replenishing cell source for tissue repair. For regenerative therapies, iPSC-derived cells must demonstrate essential reparative phenotypes and functions and show long-term stability to ensure their safety after transplantation.[4] There is intriguing evidence that iPSC reprogramming may improve cellular function upon iPSC differentiation to a fibroblast lineage.[1,3,5] In this light, the use of iPSC-derived fibroblasts may circumvent the limitations of existing sources of fibroblasts that are currently used for tissue repair. This suggests that it may soon be possible to leverage the biological potential of iPSC-derived cells to improve current strategies for oral mucosal wound healing.

CLINICAL RELEVANCE

After iPSC are differentiated to specified cell lineages, such as fibroblasts and keratinocytes, they can be incorporated into an *in vitro* tissue engineered microenvironment to support their cellular functions and to enable host integration after transplantation. Tissues containing iPSC-derived fibroblasts have been shown to support the development of a well-differentiated, stratified squamous epithelium[6] and stimulate re-epithelialization after wounding.[3] Constructing *in vivo*-like tissues using iPSC-derived fibroblasts will provide a platform that can facilitate the clinical testing and optimization of repair-competent cells that can accelerate oral wound healing through regenerative therapy approaches.

DISCUSSION

Generation of oral cell types from iPSC sources

Reprogramming somatic cells to iPSC. The breakthrough discovery of reprogramming somatic cells into pluripotent cells with properties of human embryonic stem cells (hESC) has opened dramatic new opportunities to transform human health.[7,8] iPSC were first generated by delivering four transcription factors using a viral vector (*Oct4/Sox2/Klf4/c-Myc, OSKM)* to skin-derived fibroblasts that were able to reprogram them to a pluripotent state.[7] More recently, small molecules have been used as an alternative method for reprogramming in the hope of avoiding complications linked to delivering reprogramming factors using viral vectors. Zhang *et al.*[9] and Kahler *et al.*[10] have showed that adding a combination of small molecules such as sodium butyrate and SB431542 during reprogramming of somatic cells can efficiently generate human iPS cells. Recently, Hou *et al.*[11] reported substituting chemical compounds for the four transcription factors to generate mouse iPS cells that were designated as "chemically induced pluripotent stem cells" (CiPS cells). Attempts towards deriving human CiPS are currently ongoing.

iPSC demonstrate many features similar to hESC, including cellular morphology and growth kinetics, unlimited expansion potential, global patterns of gene expression that are similar to hESC, and the capacity to differentiate into cells from all germ layers.[12] Alternative techniques for reprogramming are being developed to improve efficiency and to directly convert one cell type to another.[8] Cellular reprogramming techniques used to generate iPSC have established that terminal differentiation of adult somatic cells is reversible; thus, opening many new avenues of research and transforming perceptions of what confers cellular identity. The generation of embryonic-like, pluripotent cells is now being used to generate patient-specific tissues and cells,[8] that will be an important additional avenue for cell-based regenerative medicine applications.

Oral tissues have been shown to be a very useful source of cells that can be targeted for reprogramming to iPSC. Dental pulp stem cells, stem cells from exfoliated deciduous teeth, stem cells from apical papilla, as well as oral mucosal fibroblasts, have all shown a high efficiency of reprogrammed to iPSC.[13,14] Oral hard and soft tissues may prove to be a very useful source of cells for reprogramming, as oral biopsies and extracted teeth are easily accessible for cell isolation and expansion.

iPSC are known to harbor a residual DNA methylation signature related to their cell-of-origin, termed "epigenetic memory," that predisposes iPSC-derived cells towards differentiation pathways along lineages related to the cell type from which the iPSC were initially derived.[15,16] This further supports the use of reprogrammed cells from the oral cavity to generate iPSC-derived cells that will enable their differentiation to oral cell types and phenotypic stability upon long-term therapeutic applications in the oral and craniofacial complex (Fig. 1).

Differentiation of iPSC to fibroblast lineage fate. To realize the potential of pluripotent stem cells, it is necessary to predictably and reproducibly control their differentiation to specific cell types and cell lineages. The directed differentiation of human pluripotent cells to a spectrum of cell types recapitulates many of the *in vitro* fate decisions that occur during human embryonic development *in vivo*.[2,17] One important variable in establishing differentiation approaches is the type of surface on which these cells grow. It is known that protein substrates or cell feeder layers contribute to essential cell-cell or cell-matrix interactions needed to direct differentiation responses of pluripotent stem cells.[18,19] Another factor directing pluripotent stem cell differentiation is the presence of soluble growth factors.[20] These and other studies have shown that iPSC differentiation is mediated by a relatively small number of soluble factors, including Wnt, Nodal, and bone morphogenetic protein (BMP), which are dynamically coordinated during development to endodermal, ectodermal, or mesenchymal cell fates.

Outcomes of exposure of pluripotent stem cells to such signaling mediators is temporally controlled as well, as BMP signaling during the early stage of differentiation induces ectodermal specification, inhibits neuronal differentiation during later stages of differentiation, and promotes selection of definitive ectoderm or epidermal lineages.[21] By providing specific growth substrates and a well-defined soluble growth milieu in this temporal setting, it is possible to provide a controlled environment that can be monitored and fine-tuned, based on cell morphology, protein expression profiles and the need to meet the criteria necessary for differentiation to the cell type of interest (Fig. 2). Thus, well-defined protocols have been developed to differentiate iPSC to a broad variety of cell types shown to function in a number of experimental contexts. Specifically, these differentiation approaches can now generate cells with properties of repair-competent fibroblasts using well-defined growth conditions.[3,6,22]

The stromal fibroblast would be a valuable cell type to derive from iPSC for regenerative strategies targeted to the oral mucosa. Fibroblasts represent a diverse population of mesenchymal stromal cells that play a central role in regulating

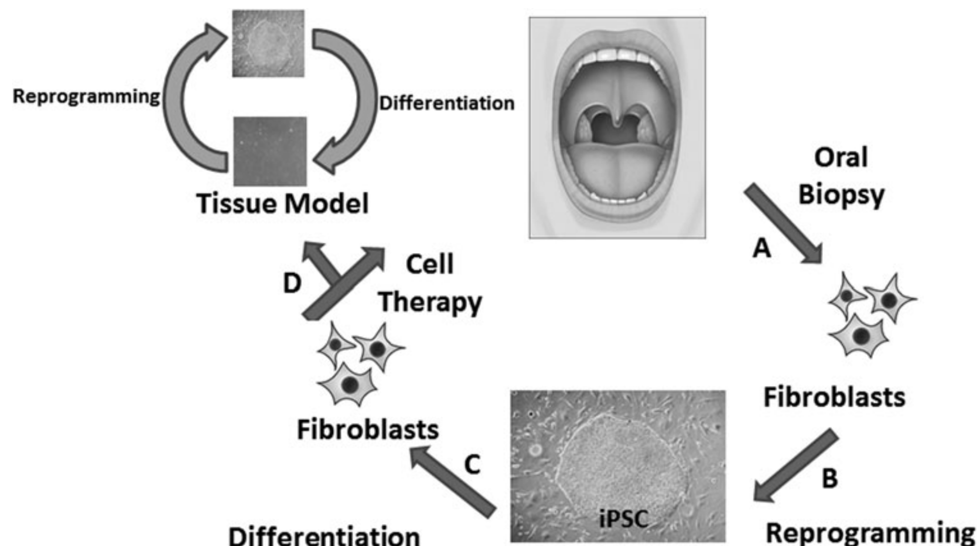

Figure 1. Reprogramming of oral fibroblasts to induced Pluripotent Stem Cells (iPSC) and their applications. Patient fibroblasts derived from an oral cavity biopsy **(A)** can be expanded and reprogrammed using four transcription factors to a pluripotent state (iPSC) **(B)**. Using highly-defined protocols, these iPSC can be differentiated to mature, functional cells types **(C)** that can be used for the generation of tissue models that mimic the features of the oral mucosa (tissue model) that can be used for modeling oral disease or for screening drug compounds **(D)**. In the future, tissues harboring these cells can be used for therapeutic transplantation (cell therapy).

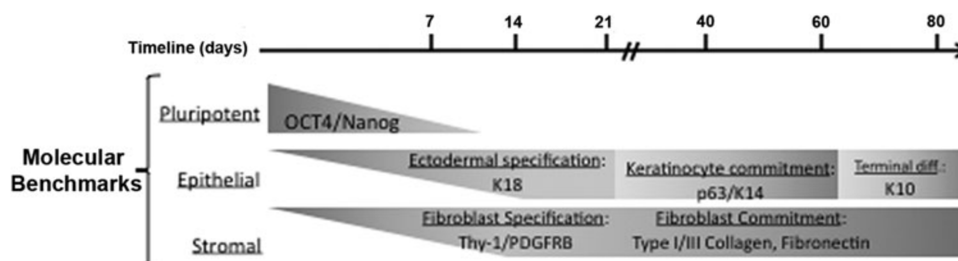

Figure 2. Molecular benchmarks can be used to identify production of iPSC-derived fibroblasts. iPSC lose pluripotency markers Oct4/Nanog at very early time points of differentiation. Generation of functional oral tissue cell types from pluripotent stem cells can be detected via molecular markers to define specific cell types. For example, epithelial cell demonstrate upregulation of specific cytokeratin markers (K18, K14, K10), while stromal cells begin to express specific growth factor and extracellular matrix proteins (collagen type I, collagen type III and fibronectin).

tissue homeostasis and wound repair.[23] These cells produce and organize elements of extracellular matrix (ECM) and also degrade structural elements of the ECM during reparative processes. Fibroblasts secrete a complex mixture of growth factors, cytokines and chemokines; as they control the growth and migration of cell types, including other mesenchymal cells, as well as epithelial, neural, and immune cells.[3,24] These interactions are also reciprocal, as fibroblasts can respond to signals from epithelial and other cell types to stimulate wound healing.[25] Thus, fibroblasts represent a critical cell type in different types of tissues that have broad impact on tissue repair and regeneration.

However, the utility of fibroblasts isolated from adult tissues for wound repair therapy is currently limited by difficulties to acquire sufficient numbers of donor cells upon their *ex vivo* expansion and by a level of cellular heterogeneity that may lead to unpredictable clinical outcomes.[24,26,27] Fibroblasts in a chronic wound environment suffer from a lack of robust provisional matrix production and often show defects in cell migration.[28,29] Chronic wound conditions, such as those seen in periodontal disease exist within the oral cavity and may benefit from cell- and/or tissue-based strategies to enhance tissue repair. In this light, the development of approaches aimed at generating clinically relevant quantities of fibroblasts with significant repair potential from iPSC may provide a reliable and alternative source of fibroblasts for oral tissue repair and regeneration.

To be utilized for regenerative therapies, fibroblasts derived from iPSC must demonstrate functional properties of fibroblasts, as well as a long-term stability that will ensure their safety after transplantation[4] (Fig. 3). The future use of iPSC-derived fibroblasts for oral mucosal repair has been supported by the dramatic improvement in cell function seen when fibroblasts are differentiated from iPSC and compared to the original cell type before reprogramming.[3] This suggests that iPSC reprogramming may reset the cellular

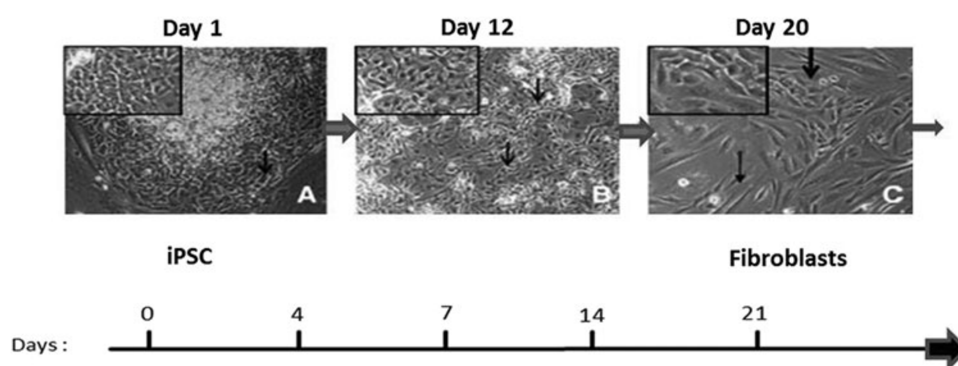

Figure 3. Differentiating iPSC to fibroblasts. iPSC were induced to differentiate through sequential developmental stages in a variety of media and substrate conditions. **(A)** iPSC were grown in the presence of growth factors and developed into cells with features of neural cells (arrows, **B**). By day 21, features of fibroblasts were apparent (arrows, **C**).

"biological clock" of the somatic cells from which iPSC were initially reprogrammed. For example, it has been shown that iPSC reprogrammed from senescent fibroblasts showed an improvement on a spectrum of functions upon their subsequent differentiation to a fibroblast lineage, that included evasion of cell senescence, elongation of telomeres, and improved mitochondrial function.[1] Additional evidence that an improved repair potential can be acquired after reprogramming and subsequent differentiation were seen in iPSC-derived mesenchymal stem cells (MSC), which showed improved vascular rescue of limb ischemia when compared to adult-derived MSC.[5]

Fibroblasts derived from iPSC also have been shown to produce greater amounts of ECM proteins than the parental cells from which they were derived.[3] These observations provide the foundation for future of oral therapies that may use iPSC technologies to reverse a senescent phenotype and shift fibroblasts to a more functional state to restore tissue's health. By understanding mechanisms linked to the improved biological potential of iPSC-derived fibroblasts, we will continue to take important steps towards understanding how these cells may be optimized for improved efficacy and function for future regenerative oral therapies.

Significant early efforts in deriving skin-like keratinocyte cells from mouse ESC was able to show definitive differentiation of mesenchymal fibroblast-like cells within the same cultures,[30] indicating that both of the major cell types needed for skin development could be generated from a single derivation protocol. Further refinements of this technique have allowed for enrichment of mesenchymal, epithelial, or peripheral nerve cell differentiation using a similar protocol.[31] Together, these studies provide the framework to controlling skin differentiation from iPSC-derived early ectodermal lineages, and understanding the key regulatory signals for selecting specific skin tissue-specific cell types. Additional studies will be needed to optimize the derivation of oral mucosal epithelium with specific functional properties that can be incorporated into three-dimensional (3D) tissues.

Tissue engineering of complex tissues using iPSC-derived cells

It has become apparent that 3D model systems will be needed to test the behavior of cells derived from pluripotent sources, such as hESC or iPSC, to characterize the function of cells and tissue derived from pluripotent cells. After their differentiation from iPSC, fibroblasts and other cells will need to demonstrate the functionalities necessary for regenerative medicine applications and will need to demonstrate features comparable to these seen in adult cell types and organs (Fig. 4). To show this, iPSC-derived cells can be incorporated into *in vitro,* 3D-engineered tissues to monitor the capacity of these cells to contribute to normal tissue morphogenesis. Since pluripotent stem cells can be differentiated and expanded to a variety of specialized cell lineages, their incorporation into such an optimal engineered, 3D microenvironment may allow these cells to self-organize into multicellular tissue structures that mimic the morphology and functional features of their *in vivo* counterparts.[32]

Formation of skin-like tissues is an example of the robust ability of cells to self-organize. Fabricated skin-like tissues have demonstrated functional organ development. Skin tissues are comprised of two major compartments, the epidermis which harbor a stratified squamous epithelia of keratinocytes and the dermis, containing fibroblasts, that need to interact to develop normally.[33] By constructing 3D-stratified epithelial skin tissues incorporating fibroblasts and keratinocytes, it is possible to study paracrine signaling during tissue repair and remodeling, as well as determine the ability of cells to contribute to tissue development (Fig. 4). Similar tissues have been

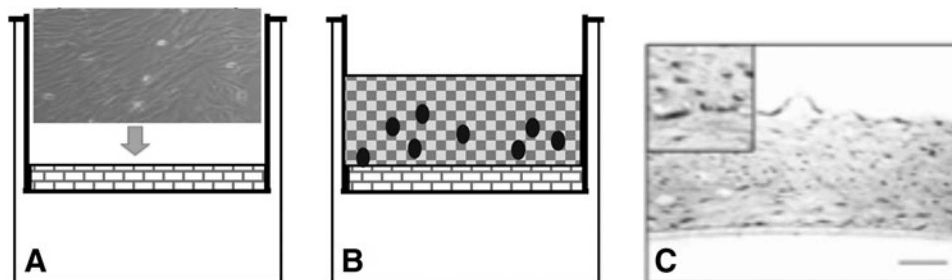

Figure 4. Stromal tissue formation from fibroblast. **(A)** Fibroblasts were seeded directly onto a polycarbonate membrane and grown in media containing ascorbic acid for 5 weeks. Stromal tissues with features of granulation tissue developed **(B)**, which were populated with fibroblasts (inset, **C**).

used successfully for skin transplantation and as biologically-active tissues for the healing of chronic wounds.[34] Such tissue-based *in vitro* systems can be used to study many aspects of normal skin and mucosal development, including basement membrane formation and paracrine crosstalk.[35] This platform for studying tissue biology *in vitro* provides a powerful tool to understand the behavior of pluripotent-derived cells within a complex 3D microenvironment

Most cells and tissue types needed for construction of engineered oral replacement mucosal tissues have previously been fabricated *in vitro* and *in vivo*. Tissue generated in this manner have been used for autologous tissue grafts for many years.[36] Oral tissues can be generated by harvesting cells from the oral cavity, expanding them, and growing them in a 3D tissue before engraftment.[37,38] Recently, 3D, skin-like tissues have been approved for clinical use in the treatment of periodontal disease.[39]

Levenberg *et al.* first established proof-of-concept that ectodermal and mesenchymal cells differentiated from hESCs could assemble into tissues displaying *in vivo*-like features and could integrate into the host vasculature.[40] In addition, tissues that show fully-mature, functional skin upon engraftment to mice have been engineered from hESC-derived keratinocytes.[41] Both iPSC- and hESC-derived cells displaying characteristics of stromal fibroblasts have also been shown to support complex, epithelial tissue development and to enhance re-epithelialization of wounds in skin equivalent tissues.[3,6] The ability of iPSC-derived fibroblasts to direct the development of engineered skin in an *in vitro* tissue model that mimics many of the *in vivo* features of the tissue supports the feasibility of constructing complex, stratified epithelial tissues with iPSC-derived cells for future oral regenerative therapies.[22] Generation of iPSC-derived fibroblasts and keratinocytes may each provide functional features that can be well-suited for specific clinical indications and wound healing functions. iPSC-derived fibroblasts would likely be well-adapted for filling large, mucosal defects due to their synthetic functions and ECM production. On the other hand, iPSC- derived keratinocytes may be tailored for primary wound closure by optimizing the migratory functions of these cells. Such tissue surrogates serve as a platform to assess the developmental capacity of iPSC-derived cells to acquire cellular function, as that will optimize their use for future oral regenerative therapies.

FUTURE CONSIDERATIONS

The generation of iPSC from patients with a spectrum of oral diseases may enable the differentiation of iPSC-derived cells to recapitulate key stages of disease pathogenesis in ways that can be monitored *in vitro*. Such application of iPSC-based technology facilitates the development of *in vitro*

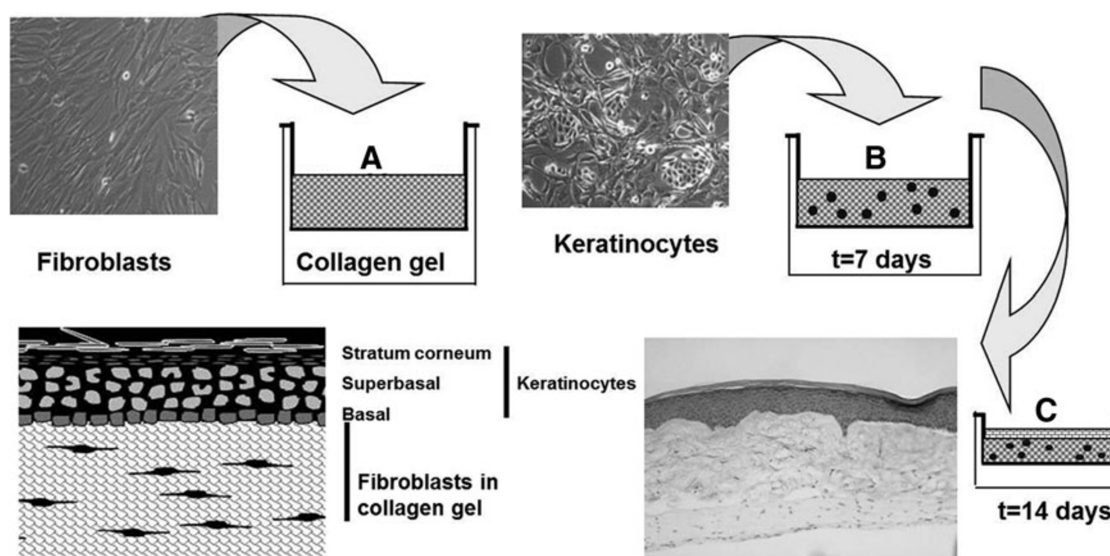

Figure 5. Fabrication of human skin-equivalent (HSE) tissues. Assembly of HSE involves preparation of cellular collagen layer, which serves as a dermal equivalent containing fibroblasts **(A)**. After 7 days, keratinocytes are seeded on top of this contracted cellular layer **(B)**. Developing tissues are maintained submerged in media for 7 days, and then lifted to an air-liquid interface to allow full stratification **(C)**.

disease models that will lay the groundwork for "disease in a dish" cultures used for screening of drug compounds that can be targeted to disrupt disease progression.[42] Through continued refinement of disease modeling using cells differentiated from oral derived and patient-specific iPSC, these techniques will eventually offer an unprecedented opportunity to develop new treatment strategies targeted to specific patient populations (Fig. 5). In addition to its pluripotent nature, iPSC provides a common source of cells for generating complex multicellular tissues that can be used for autologous transplantation. Refinement of existing differentiation and culture strategies might also allow for the *in vitro* fabrication of increasingly complex oral tissues, such as gingiva and teeth. There is an enormous need to advance the generation of such engineered tissues after reconstructive surgery and injury.

Despite their potential, the application of cells derived from iPSC for oral therapies faces many barriers that limit their implementation due to concerns related to their safety, purity, and immunogenicity.[8,43] It has been demonstrated that reprogramming of iPSC may result in accumulation of genetic mutations and aberrant karyotypes after prolonged culture that may alter their phenotype and differentiation potential.[44–46] Another concern is that some populations of cells derived from iPSC may contain small numbers of undifferentiated, pluripotent iPSC that can persist in long-term differentiated populations and lead to teratoma formation after transplantation *in vivo*.[47] Despite extensive screening, this risk still seems to be a major complication of pluripotent-based therapies in animal transplantation models.[48] This suggests that evaluation of the biological potential of iPSC-derived cells will require the development of reliable methods to evaluate their functional properties before they can be translated for oral repair and regeneration strategies.

The discovery and implementation of iPSC-based approaches for regenerative medicine is an exciting and rapidly evolving field, and one that can be applied to many clinical indications. A central challenge that must be overcome before therapeutic application of iPSC-derived cells is the development of reliable and sensitive methods to evaluate their safety and efficacy by developing tools and platforms to evaluate their stability in an *in vivo*-like tissue context. It is therefore, critical to fully-characterize the properties of differentiated cells derived from iPSC by developing preclinical, engineered tissue models that will better predict cell behavior before therapeutic transplantation to the oral cavity and other sites.

TAKE-HOME MESSAGES

Basic science advances

- Reverting somatic cells to an embryonic-like state presents a novel paradigm for recapitulating and advancing critical steps in cellular and developmental biology.
- Cellular reprogramming provides an unlimited source of pluripotent cells for regenerative medicine following the directed differentiation of iPSC to specific cell types.

Clinical science advances

- Strategies for the *in vitro* generation of oral mucosa could be improved using iPSC–derived fibroblasts and keratinocytes.
- Wound-repair modeling using patient-specific iPSC can lead to the development of novel screening platforms to test new drugs directed to therapeutic targets important for wound healing.

Relevance to clinical care

- Oral mucosal repair can be dramatically advanced by future therapeutic approaches using iPSC-derived cells that allow for the generation of an unlimited supply of patient-specific cells and tissues.
- Engineered tissues harboring iPSC-derived fibroblasts create microenvironment that simulate the *in vivo* behavior of the oral mucosa and can accelerate the development of these cells for repairing the oral tissues and also improving the aesthetic outcome in human cell therapies.

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