DAVID T. WONG

Salivary Diagnostics

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Edited by
David T. Wong
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Contents

Contributors viii

Foreword xv

Preface xvii

Acknowledgment xviii

I Background and Foundation

1 Salivary Gland Development and Regeneration 3
Sarah M. Knox and Matthew P. Hoffman

2 Salivary Gland Physiology Relevant to Diagnostics 14
Victor G. Romanenko and James E. Melvin

3 Saliva: Properties and Functions 27
Arie van Nieuw Amerongen, Enno C.I. Veerman, and Arjan Vissink

4 Saliva Collectors 37
Arjan Vissink, Andy Wolff, and Enno C.I. Veerman

5 Salivary Secretion in Health and Disease 60
Michael D. Turner and Jonathan A. Ship

6 Processing and Storage of Saliva Samples 69
Enno C.I. Veerman, Arjan Vissink, David T. Wong, and Arie van Nieuw Amerongen

II Saliva Diagnostics

7 Historical Perspectives and Present 79
Frank G. Oppenheim

8 Diagnostics Other Than Blood 94
Sudhir Srivastava and Karl Krueger

9 Perceptions of Saliva: Relevance to Clinical Diagnostics 104
Sreenivas Koka and David T. Wong

10 Saliva-Based Diagnostic Technologies: Highlights of the NIDCR's Program 111
Eleni Kousvelari, John T. McDevitt, Daniel Malamud, David T. Wong, and David R. Walt

11 Human Salivary Proteomics 122
Shen Hu, Markus Hardt, Susan J. Fisher, David T. Wong, John R. Yates, James E. Melvin, and Joseph A. Loo

12 Genomic Targets in Saliva 129
Bernhard G. Zimmermann, Zhanzhi Hu, and David T. Wong

13 Point-of-Care Diagnostics for Infectious diseases 136
Paul L.A.M. Corstjens, Daniel Malamud

14 Dental Caries Risk Assessment 150
Paul C. Denny and Patricia A. Denny

15 Periodontal Disease 156
Christoph A. Ramseier, Thiago Morelli, Janet S. Kinney, Meghan Dubois, Lindsay Rayburn, and William Giannobile

16 Role of Saliva in Detection of Substance Abuse 169
Uttam Garg and Lance Presley

17 Head and Neck Cancer 180
Joseph A. Califano and Suhail K. Mithani

18 Sjögren's Syndrome 189
Philip C. Fox

19 Salivary Gland Dysfunction Associated with Systemic Disease 198
Nelson L. Rhodus

20 Progression and Treatment Evaluation in Diseases Affecting Salivary Glands 214
Jiska M. Meijer, Cees G.M. Kallenberg, and Arjan Vissink

21 The Uses of Saliva in Forensic Genetics 226
Jack Ballantyne and Jane Juusola

22 SPKB—Salivary Proteome Knowledge Base: A Platform for Collaborative Proteomics Research 233
Weihong Yan, Weixia Yu, Shawn Than, Renli Qiao, D. Stott Parker, Joseph A. Loo, and David T. Wong

III Saliva Diagnostics—A New Industry

23 Commercialization of Oral Fluid Products and Technologies 243
R. Sam Niedbala

24 Coverage and Reimbursement for Salivary Diagnostic Tests 255
James J. Crall

25 Saliva Diagnostics in the Dentist's and Physician's Office 263
Chakwan Siew and Milton V. Marshall

26 Salivary Hormones in Research and Diagnostics 270
Douglas A. Granger, Christine K. Fortunato, Leah C. Hibel

27 Crossroads Between Saliva Diagnostics and Salivary Gland Gene Therapy 278
Bruce J. Baum, Ana P. Cotrim, Corinne M. Goldsmith, Fumi Mineshiba, Senrong Qi, Gabor Z. Racz, Yuval Samuni, Takayuki Sugito, Antonis Voutetakis, and Changyu Zheng

28 Saliva Diagnostics—A New Industry 288
Stuart R. Smith, David T. Wong, and R. Michael Buch

Index 295

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Foreword

"... there is no disease that I spit on more than treachery."
Aeschylus (525-456 BC)

Saliva is one of those things you love or hate. Although historically scorned in literature, viewed by many cultures as the ultimate insult and clinically "damned," investigators, clinicians, and our patients are increasingly turning to saliva as a safe and "non-invasive" indicator of health and disease. Many biomarkers may be measured using oral fluids. This opens up the extraordinary opportunity of enhancing research conducted in the field or expanding the versatility of point-of-care diagnostics by using saliva as the diagnostic fluid.

Behavioral research that seeks to correlate physiological with psychological status has been revolutionized by the availability of assays that accurately measure such hormones as cortisol in saliva. Reaction (and sometimes aversion) to a needle stick for collection of blood has a far greater chance of disturbing the subject than does the collection of oral fluid. Accurate and rapid diagnostic tests that can be performed in the privacy of one's home or in a community or field setting are proving crucial to controlling a number of diseases and conditions. For example, such tests for sexually transmitted diseases can yield diagnosis in the early stages of the infection resulting in a decreased transmission from asymptomatic patients. Infectious diseases kill approximately 15 million persons worldwide each year. Saliva-based point-of-care diagnostic tests, which dispense with the need for a phlebotomist, have the potential to overcome some of the limitations and challenges of the modest medical infrastructures found in the developing world.

Continued advances in biomedical engineering coupled to enhance understanding of the salivary proteome will make it possible to one day implant biosensors directly in the mouth. These in-dwelling "labs on a chip" will help catalyze a shift from our current practice of disease detection to a health surveillance through detection and measurement of multiple, relevant biomarkers in saliva. With the advances of this technology comes the additional significant obligation to ensure the privacy of patients. While saliva collection is facile and anatomically non-invasive, oral fluids have sufficient quantities of DNA to decode genotypes. Thus, one must be equally discreet with saliva and blood samples.

The numerous contributors to this volume are experts in their respective areas and provide the historical background as well as the status of current research. They see salivary diagnosis developing into a new “industry;” they deal with its utilization in dentists’ and physicians’ offices and explore reimbursement mechanisms for the services. The projection into the future also considers testing at home and concerns regarding the commercial market.

Salivary Diagnostics provides insight into an area that will increasingly involve many disciplines.

Lawrence A. Tabak, DDS, PhD
Irwin D. Mandel, DDS

Preface

This book is dedicated to the visionaries who planted and nurtured the seeds for saliva diagnostics to flourish and bear harvest.

The publication of "Salivary Diagnostics" marks a defining moment. Revolutionary genome-wide research tools have spawned remarkable advances in human genomics, proteomics, and metabolomics. These interrelated developments, largely driven by the Human Genome Project, ushered a new dawn on the ancient study of saliva. We have learned that human saliva contains a repertoire of proteins, glycoproteins, lipids, metabolites, RNA and genomic information, in addition to the 700 microbial species and is endowed with many of the same diagnostic analytes inherent in other bodily fluids such as blood, cerebral spinal fluid, and urine.

The National Institute of Dental and Craniofacial Research charged into the inclement waters of risky science and planted its flag on the *terra incognita* of saliva omics. The completion of the human salivary proteome project is a landmark accomplishment marking the discovery of the first diagnostic alphabet in saliva. Now, the race is on for a self-contained diagnostic chip that can provide clinical information to a physician or dentist at point-of-care before a patient leaves the clinician's office during a regular visit.

From the adventurer's past, saliva diagnostics emerges into the clockmaker's present. Now is the time for us to exchange adventurer's leather jacket for a clockmaker's loupe. We are in the critical phase where the scientific foundations of saliva for clinical disease detection must be established beyond any doubt. Only on the broadest foundation of absolute scientific integrity can we build the highest edifice of saliva diagnostics in the shortest possible time.

And the future? With the potential to revolutionize delivery of diagnostic services both on the personal and public health levels, the future of saliva diagnostics is enormous.

David T. Wong, DMD, DMSc

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Part I

Background and Foundation

1 Salivary gland development and regeneration

Sarah M. Knox and Matthew P. Hoffman

INTRODUCTION

The formation of salivary glands throughout embryogenesis is exquisitely orchestrated so that at birth the organ system is primed and ready to produce salivary secretions under the control of autonomic innervation. An appreciation for the developmental mechanisms that give rise to this complex and highly regulated system is an important foundation to understanding the physiology and biochemistry of postnatal saliva production, which is the fluid required for salivary diagnostics. In this chapter, we compare what is known about human salivary gland development with mouse submandibular gland development and highlight human genetic conditions that provide profound insight into gland development. We have also included a section on potential strategies for salivary gland regeneration.

SALIVARY GLAND DEVELOPMENT

The salivary organ system is composed of three pairs of major glands: the submandibular (SMG), parotid, and sublingual (SLG), which together produce more than 90% of saliva, as well as numerous minor glands lining the oral cavity. The major salivary glands share homologous mechanisms of branching morphogenesis during organogenesis to produce an intricate branched structure of ducts with terminal buds that differentiate into acinar structures. They are also well supplied by the vascular system, which develops alongside, and is intimately associated with the branching epithelial structures. Little is known about vascular development in the salivary gland; salivary secretion is accompanied by increased blood flow to the glands, and an extensive capillary network is found around the striated duct where ion exchange occurs. The salivary glands are innervated by the parasympathetic and sympathetic branches of the autonomic nervous system, which are essential for postnatal saliva secretion and will be the focus of a section below. Human salivary glands are composed of different types of acinar cells that produce distinct types of saliva: parotid acinar cells are serous and secrete a watery serous saliva; SMG acinar cells are approximately 80% serous and also contain mucous secretory

units capped by seromucous demilunes, producing a seromucous saliva; SLG acinar cells are mixed secretory units but are mainly composed of mucous cells and produce a mucous saliva. The reader is directed to a textbook for a comprehensive histological description of cell types within the glands.[1]

The development of human and mouse salivary glands is similar in many aspects, and to highlight this point, we will compare human and mouse development in this chapter. There have been a few histological reports documenting human salivary gland development[2–4] that basically corroborate the early histological descriptions by Thoma in 1919, shown in Figure 1.1.[5] The parotid gland is derived from the oral ectoderm, and the mesenchyme is neural crest-derived ectomesenchyme. However, the origin of the SMG and SLG epithelium has not been clearly defined. Humans with mutations in the ectodysplasin A (*EDA*) gene develop hypohidrotic ectodermal dysplasia (HED), characterized by defects in teeth, hair, sweat glands, and salivary glands, and mice lacking the EDA receptor (*Edar*) have SMG aplasia or hypoplasia (recently reviewed in reference 6). Mice lacking p63, an ectodermal marker, have no salivary glands,[7] suggesting that the origins of the SMG and SLG are ectodermal.

Human salivary gland development begins with the thickening of the oral epithelium to form a placode at a specific site in the oral cavity. Figure 1.1 shows the parotid placode forming in the lateral border of the buccal sulcus of a human embryo. Factors that define the site of placode initiation of human and mouse salivary glands are not known. However, a recent review of salivary gland development in *Drosophila* describes how global patterning genes, such as the homeotic gene *scr*, define the site of salivary gland initiation, whereas other genes repress gland initiation in the anterior–posterior and dorsal–ventral axes.[8] Experimental evidence from *Drosophila* provides a paradigm for how gland initiation is likely to occur in humans and mice, and the reader is referred to Denny et al.[9] for discussion.

The next stage in development is the expansion and invagination of the placode as an epithelial bud into a condensing mesenchyme at around 6–8 weeks in humans (Fig. 1.2) and embryonic day 12 (E12) in mice.[4,5] The condensing mesenchyme provides growth factors and extracellular matrix that play an instructive role in branching morphogenesis of the mouse salivary gland (reviewed in references 6 and 10). The human SMG epithelium then undergoes successive rounds of branching morphogenesis, which involves cleft formation, end bud expansion, and duct elongation, finally appearing as solid end buds and cords of epithelial cells (Fig. 1.3) at 10 weeks,[2,5] similar to an E14 mouse SMG (Fig. 1.2). By 14 weeks, functional differentiation of the gland has started with branches and terminal buds forming lumens to establish presumptive ducts and acini.

Branching morphogenesis continues until approximately 12 weeks where lumenization of ducts and polarization of the end bud epithelium begins, similar to E15 in the mouse. However, from approximately 12 to 28 weeks, cellular differentiation begins with polarization of the end buds and lumenization of the ducts with secretory material appearing in the lumens of the ducts and end buds, similar to E17 in the mouse SMG (Fig. 1.2). Also, during mouse SMG development, the gene expression of secretory products, such as parotid secretory protein (PSP) and submandibular gland protein C (SMGc), is barely detectable

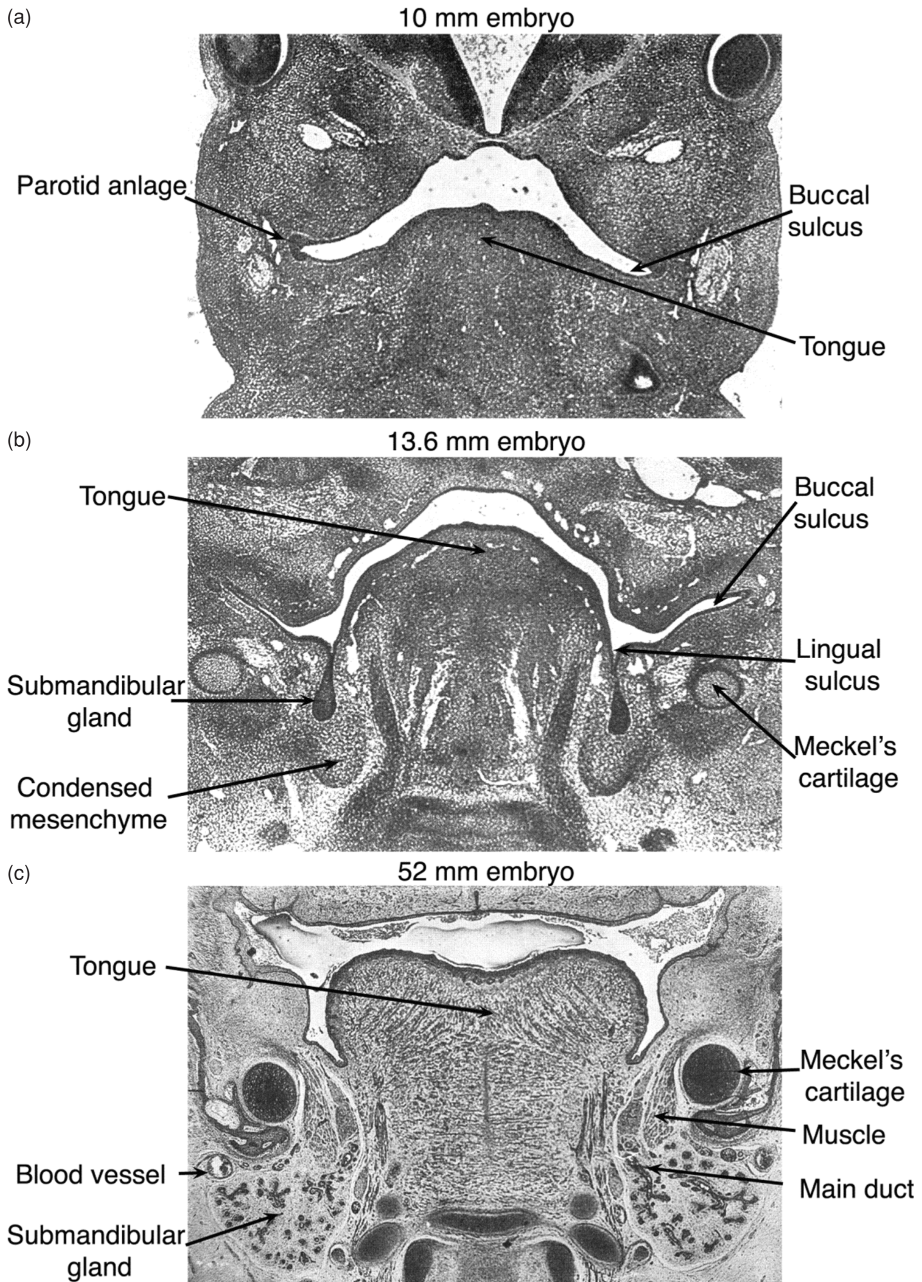

Figure 1.1 (a) Early development of the human parotid gland. A section from an approximately 6–8-week-old human embryo shows the thickening of the parotid placode, which forms in the major groove of the buccal sulcus. (b) Development of the human SMG. At approximately 6–8 weeks, but later than that in part (a), the SMG rudiment extends into a condensed mesenchyme capsule that provides growth factors and matrix, which stimulate branching morphogenesis. (c) At 10 weeks, the SMG has undergone extensive branching within the mesenchymal capsule. The main duct is visible, and lumens can be seen in some minor ducts. (All figures from Reference 5.)

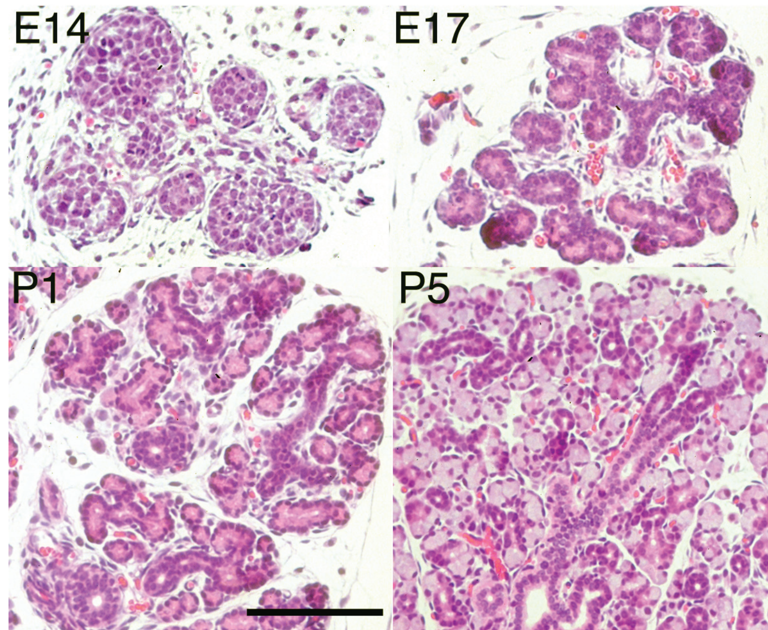

Figure 1.2 Development of the mouse SMG. At E14, the branched epithelium consists of solid end buds and ducts. By E17, functional differentiation has begun with lumen formation and end bud polarization. At P1, the acinar cells are differentiated, but connective tissue remains around the parenchyma. By P5, the functioning acini have continued to enlarge, and ductal differentiation is apparent. Bar = 200 μm.

by real-time polymerase chain reaction (PCR) at E15, but increase at E17 and after birth at postnatal day 1 (P1) (Fig. 1.3). By 13–16 weeks in human SMGs, cells adjacent to lumens show the presence of desmosomes and numerous microvilli projecting into the intercellular space. The cytoplasm of some cells contained serous, but not mucous, granules, and the luminal contents were strongly positive for glycosaminoglycans.[11] The epithelium is surrounded by a well-developed basal lamina and contains a few elongated cells that appear similar to myoepithelial cells. By 16 weeks, both striated ducts and intercalated ducts are distinguishable. From 20 to 24 weeks, acinar cells begin to predominate; in rodents (E18), granular convoluted tubules (GCTs) appear. By 28 weeks there is a marked increase in the amount of acini (and GCT cells in rodents),

→

Figure 1.3 Differential gene expression of secretory products highlights the development of secretory function during mouse SMG development. FGF2 expression is increased during branching morphogenesis, whereas PSP and SMGc expression begins after E15 when functional differentiation begins. During postnatal development, EGF and NGF expression dramatically increases after puberty. Gene expression, normalized to the housekeeping gene 29S, was measured by real-time PCR, over a developmental time course from E12 to adult (Adt).

Fold change in gene expression

FGF2
E12 = 26.9

PSP
E15 = 32.1

SMGc
E15 = 30.9

EGF
E15 = 34.7
17,000

NGFa
P5 = 37.4

29S
E12 = 23.7

E12 E13 E14 E15 E17 P1 P5 Adt

although less secretory products are apparent.[2] At birth, only serous cells are present, with connective tissue spaces between the acini, similar to P1 of mouse SMG development (Fig. 1.2). In humans, prenatal growth and differentiation continue after 28 weeks such that the glands are capable of secreting saliva in response to parasympathetic stimulation at P1. In comparison, postnatal mouse SMG development, particularly the differentiation of the GCTs by puberty, is evidenced by expression of GCT secretory gene products such as EGF, which is weakly expressed from E17 and at birth but dramatically increases after puberty, and NGF, which is barely detectable at P5 but also dramatically increases with postnatal ductal differentiation (Fig. 1.3).

DEVELOPMENT OF THE AUTONOMIC INNERVATION OF SALIVARY GLANDS

Innervation of salivary glands by the parasympathetic and sympathetic branches of the autonomic nervous system is essential for secretion and tissue homeostasis because denervation results in loss of saliva production and atrophy of the tissue. For a comprehensive review of salivary gland innervation, the reader is directed to Proctor and Carpenter (2007).[12] Embryonic innervation of the human salivary glands has been described from histological sections,[4] but most experimental investigations have used rodent SMGs.

There are important differences between sympathetic and parasympathetic nerve development in salivary glands. First, sympathetic innervation is postnatal, around P3–P5. In contrast, parasympathetic innervation develops at the same time as the gland and requires an interaction with the salivary epithelium.[13] Second, the sympathetic efferent nerves, which release norepinephrine, arise from the superior cervical ganglion located away from the gland in the thoracic spinal cord. However, the parasympathetic nerves secrete acetylcholine and extend either from the otic ganglion, which innervates the parotid, or from the submandibular ganglion, which innervates both the SMG and SLG. The submandibular ganglion lies within the SMG, whereas the otic ganglion is separate from the parotid. Interestingly, branching morphogenesis of the parotid gland does not begin until otic neurons reach the initial epithelial bud. Most of what is known about embryonic development of the glandular parasympathetic ganglions is derived from early studies on mouse SMG development.[14,15]

HUMAN GENETIC CONDITIONS AFFECTING SALIVARY GLAND DEVELOPMENT

There are a few human genetic conditions that affect salivary gland development leading to aplasia or hypoplasia. However, two of the most informative are due to mutations in genes that affect fibroblast growth factor (FGF) receptor signaling. Mutations in FGF10, resulting in haploinsufficiency, have been linked to aplasia of lacrimal and salivary glands (ALSGs) syndrome (OMIM

180920),[16] an autosomal dominant anomaly with aplasia or hypoplasia of the lacrimal and salivary systems. Importantly, mice also missing one copy of FGF10 or its receptor, FGFR2b, have severe salivary gland hypoplasia. Additionally, lacrimoauriculodentodigital (LADD) syndrome is an autosomal dominant disorder characterized by aplasia or hypoplasia of the lacrimal and salivary systems as well as abnormalities of the face, ears, eyes, mouth, teeth, digits, and genitourinary system. LADD syndrome occurs as a result of mutations in fibroblast growth factor receptors 2 and 3 (FGFR2, FGFR3), as well as in FGF10.[17,18] ALSG and LADD syndrome are thought to be allelic disorders with variable expressivity, possibly due to different types of FGF10 mutations.[18]

Other genetic diseases showing salivary gland phenotypes include mutations in EDA and its receptor, EDAR, which result in HED, characterized by abnormal ectodermal organs such as hair, teeth, sweat glands, and salivary glands. Similar phenotypes occur in *Tabby* (Eda^{Ta}) and *Downless* ($Edar^{dl}$) mutant mice.[19] Eda^{Ta} SMGs are hypoplastic, while $Edar^{dl}$ SMGs are dysplastic. Eda/Edar signaling is essential for branching morphogenesis, lumen formation, and differentiation, but not for initial gland formation.[20]

MODEL SYSTEMS TO STUDY SALIVARY GLAND DEVELOPMENT

As described earlier, there are many similarities in the development of the mouse SMG compared to human salivary glands, both in the histological development and also in the development of the secretory function of the gland. A major advantage of using mouse SMG to model human gland development is the ability to experimentally manipulate the mouse genome. Ex vivo mouse SMG organ culture allows the investigation of branching morphogenesis in a complex system that contains mesenchyme, epithelium, parasympathetic neurons, and endothelial cells forming the developing vasculature (Fig. 1.4). E12 SMGs, beginning with a single epithelial bud in a condensed mesenchyme, display reproducible branching morphogenesis in serum-free culture and appear similar to SMGs in vivo. SMG organ culture has been used extensively to explore the molecular mechanisms of epithelium–mesenchyme interactions, and a broad range of molecules have been identified that regulate epithelial branching, including constituents of the extracellular matrix, cell adhesion receptors, proteases, and growth factors. The reader is referred to recent detailed reviews on mouse SMG branching morphogenesis.[6,10]

THE GOAL OF FUNCTIONAL SALIVARY GLAND REGENERATION

An irreversible loss of salivary gland function often occurs in humans after removal of salivary tumors, after therapeutic radiation of head and neck tumors, as a result of Sjögren’s syndrome, and in certain rare genetic syndromes affecting FGF signaling. The loss of salivary gland function impairs the oral health of

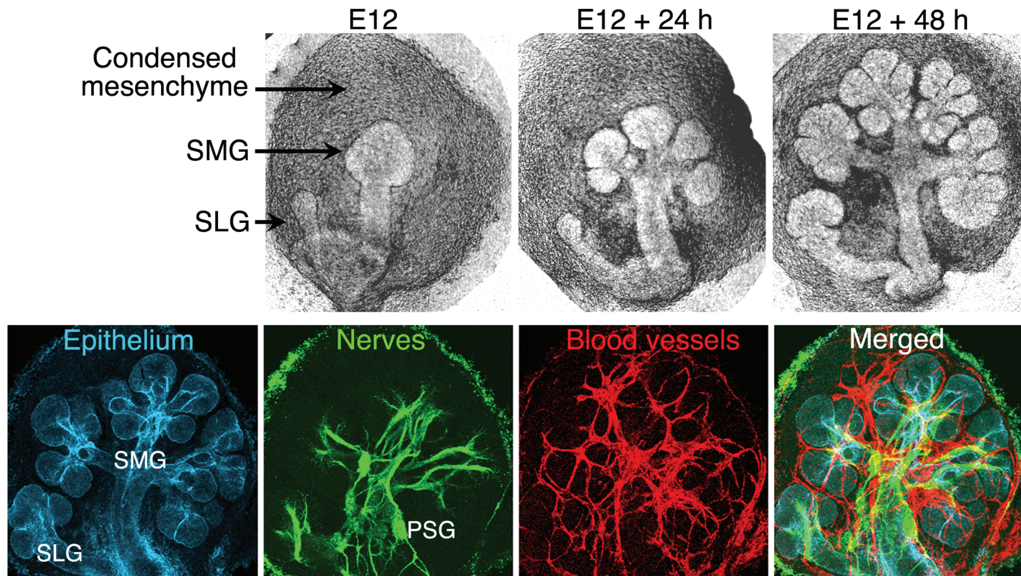

Figure 1.4 Ex vivo organ culture of E12 mouse SMGs for 48 h in serum-free media recapitulates the development of the gland in vivo. A single epithelial bud undergoes branching morphogenesis. The lower panel highlights the cellular complexity of the SMG. The epithelium (blue, laminin α1) is surrounded by nerves (green, β3-tubulin) that extend from the parasympathetic submandibular ganglion at the base of the primary duct. The mesenchyme contains a large network of blood vessels (red, PECAM). The image is a projection of a confocal stack.

these patients and significantly affects their quality of life. Currently, patients are treated with parasympathetic mimetics such as pilocarpine, which rely on some gland function. Patients with some remaining salivary tissue could benefit from genetic reengineering to restore tissue function or from a regenerative approach to repair the gland. Patients with little or no functional gland tissue may need regenerative therapy or replacement with artificial salivary glands.[21]

Animal models used to investigate and develop techniques for functional regeneration of salivary glands include rodents, minipigs, and primates. Rat models have been used to investigate the effects of irradiation,[22] denervation, and ductal ligation,[12] and intraductal adenovirus injection for gene therapy.[23] Minipigs are a large animal model whose SMGs resemble the human gland and are useful for studying changes after gland irradiation[24] and for preclinical applications of gene transfer to salivary glands.[25]

Genetic reengineering

Most research to date has focused on the reengineering and replacement of salivary glands. Reengineering of salivary glands is based on inductive gene transfer technology using recombinant viruses. A number of genes have been delivered successfully to nonhuman salivary glands in vivo, including human aquaporin 1 (*Aqp1*)[26] and aquaporin 5 (*Aqp5*).[27] Aquaporins are water channels located in acinar cells that facilitate the movement of water across the plasma bilayer. Aqp1 has been shown to be acutely downregulated after radiation

therapy. Gene transfer of Aqp1 using adeno-associated virus into salivary glands partially transformed ductal cells into functional secretory cells in rats and minipigs.[25,28] Aqp1 gene therapy on irradiated human patients using adeno-associated virus is currently in clinical trials. This topic is elaborated in Chapter 27.

Regenerate and repair

The use of stem cells to regenerate tissue is increasingly being utilized in many organ systems. Recently, Lombaert and coworkers[29] used GFP-labeled bone marrow stem cells (BMSCs) to regenerate irradiated mouse SMGs. They measured an increase in salivary secretion after mobilization of BMSCs in the irradiated tissue. However, the stem cells were not part of the epithelial compartment of the gland, but were exerting their effect from the mesenchyme.[29] Potentially, the effect may be mediated by repopulation of the vasculature or nerves to increase blood supply or innervation, as a reduction in either of these structures leads to gland atrophy.[12]

Replace

Patients who have lost parenchyma because of disease, radiation therapy, or surgery are not candidates for gene transfer or regenerative/repair strategies, and total organ replacement is required. Organ replacement is hampered by the cellular complexity of the salivary gland and the necessity to regenerate functional secretory epithelium, nerves, blood vessels, and stromal tissue in an encapsulated organ space. Engineering of an "artificial" salivary gland may involve the use of synthetic or ECM-derived 3D scaffolds onto which cells are seeded to form a polarized secretory epithelium.[26,30]

In conclusion, understanding how salivary glands develop may facilitate gland regeneration and/or tissue-engineering approaches to restore secretory function in adult glands. Fundamental scientific knowledge provides a foundation for therapeutic interventions and will benefit from the translational and clinical utilities of saliva and its analytes, the thematic topic of this book.

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