Tissue-Engineered Mucosa:

Clinical Tool or *In Vitro* Model?

Wendy M.W. Tra

STELLINGEN

Tissue-Engineered Mucosa:
Clinical Tool or *In Vitro* Model?
Gekweekt wangslijmvlies: Klinische toepassing of *in vitro* model?

1. Fibroblasten zijn essentieel voor de ontwikkeling van nieuw epitheel (dit proefschrift).
2. Tissue-engineered mucosa (TEM) constructen zijn geschikt voor het bestuderen van de effecten van radiotherapie (dit proefschrift).
3. Gedeëpithelialiseerde dermis is de meest geschikte matrix voor TEM (dit proefschrift).
4. Hyperbare zuurstof therapie verhoogt de secretie van angiogene factoren, maar vernietigt de morfologie van TEM (dit proefschrift)
5. Het keratine expressie patroon van het epitheel van TEM is meer afhankelijk van de oorsprong van de matrix dan van de oorsprong van de cellen (dit proefschrift).
6. Keratinocyten en fibroblasten zijn als ying en yang, de een kan niet zonder de ander.
7. Immunohistochemie is een kwestie van trial-and-error (P.J. Canfield, Dev Comp Immunol 2000;24:455-71).
8. Het benodigd aantal proefdieren zal afnemen naarmate de weefselkweek zich verder ontwikkelt (A. El Ghalbzouri, Toxixol in Vitro 2008;22:1311-20).
9. Wetenschap is slechts een beeld van de waarheid (Francis Bacon, Engels filosoof en staatsman 1561-1626)
10. Als we wisten wat we deden, heette het geen onderzoek (Albert Einstein, Duits-Amerikaans natuurkundige 1879-1955).
11. Born to lose, live to win (Ian “Lemmy” Kilmister)

Wendy M.W. Tra

Tissue-Engineered Mucosa: Clinical Tool or *In Vitro* Model?

Wendy M.W. Tra

Financial support by the Erasmus MC for the publication of this thesis is gratefully acknowledged.

Printing of this thesis was kindley supported by Ipskamp Drukkers.

Cover: Wendy M.W. Tra
Design and Layout: Wendy M.W. Tra
Printing: Ipskamp Drukkers, Enschede

ISBN:

Tissue-Engineered Mucosa: Clinical Tool or *In Vitro* Model?

Gekweekt wangslijmvlies:
Klinische toepassing of *in vitro* model?

Proefschrift

ter verkrijging van de graad van doctor aan het

Erasmus Medisch Centrum Rotterdam

op gezag van de

rector magnificus

Prof.dr. H.A.P. Pols

En volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op

18 november 2014 om 11.30 uur

Door

Wendy Maria Wilhelmina Tra

Geboren te Tilburg

PROMOTIECOMMISSIE

Promotor:

Prof.dr. S.E.R. Hovius

Co-promotoren:

Dr. S. Perez-Amodio

Dr. J.W. van Neck

Overige leden:

Prof.dr. R.J. Baatenburg de Jong

Prof.dr. T.E.C. Nijsten

Prof.dr. D.F.E. Huylebroeck

Paranimfen:

B. Tuk

N.A.J. Cuppens-Tra

TABLE OF CONTENTS

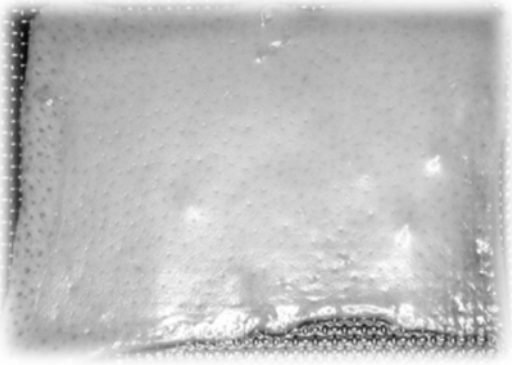
LIST OF ABBREVIATIONS

A/L	air/liquid interface
ANOVA	analysis of variance
BM	basement membrane
BSA	bovine serum albumin
CM	conditioned medium
DAB	3,3'diaminobenzidine
DED	de-epidermized dermis
DMEM	dulbecco's modified eagle medium
ECM	extracellular medium
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGM	endothelial growth medium
ELISA	enzyme-linked immunosorbent assay
FCS	fetal calf serum
FGF	fibroblast growth factor
H&E	hematoxylin eosin staining
HBO	hyperbaric oxygen
HG-DMEM	high glucose dulbecco's modified eagle medium
HGF	hepatocyte growth factor
HIF-1 alpha	hypoxia inducible factor- 1 alpha
HUVEC	human umbilical vein endothelial cells
IgG	immunoglobulin G
IL	interleukin
IR	ionizing radiation
KGF	keratinocyte growth factor
MMP	matrix metalloproteinase
MSC	mesenchymal stem cell
NGS	normal goat serum
NHP	normal human plasma
NNOM	native non-keratinizing oral mucosa
NS	not significant
OCT	optimal cutting temperature-medium

PAS	periodic acid-Schiff
PBS	phosphate-buffered saline
PI	proliferation index
PLGA-PCL	poly-lactic-co—glycolic poly-caprolactone
PlGF	placental growth factor
RT	room temperature
SD	standard deviation
SEM	standard error of the mean
TE	tissue engineering
TEM	tissue-engineered mucosal substitutes
TIMP	tissue inhibitor matrix metalloproteinase
TNF	tumor necrosis factor
TUNEL	terminal deoxynucleatidyl transferase dUTP nick end labeling
VEGF	vascular growth factor

CHAPTER 1

General introduction



CHAPTER 1

GENERAL INTRODUCTION

Reconstruction of large oral defects is challenged by the limited availability of mucosal grafts. This has initiated the search for alternative tools for grafting. In recent years the field of tissue engineering has significantly evolved and now tissue-engineered mucosal substitutes can be used for studying biological and pathobiological processes including radiotherapy, wound healing, oral mucositis, and fibrosis. The goal of this thesis was to develop a tissue-engineered mucosal construct (TEM) that strongly resembles native oral mucosa based on keratin expression pattern of the epithelium, presence of a basement membrane and composition of the underlying connective tissue. We assessed the suitability of this TEM construct for studying the potential of hypoxia and hyperoxia preconditioning, the acute effects of ionizing radiation and examined the potential of TEM as alternative tool for grafting.

COMPOSITION OF ORAL MUCOSA

Oral mucosa is the membrane that lines the inside of the oral cavity. The structure of the mucosa is determined by the functional demands and therefore depends on the regions of the oral cavity. Nevertheless, in all types the same three distinctive layers can be observed; epithelium, basement membrane and dermis or connective tissue (see Fig. 1) [1].

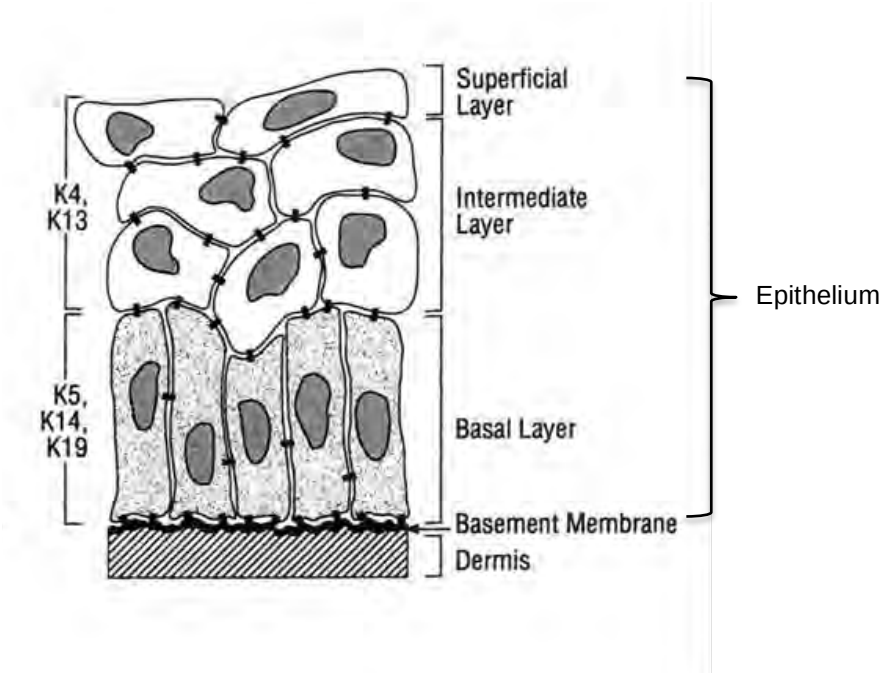


Figure 1. Schematic overview of non-keratinizing oral mucosa (source Presland and Dale [3]).

Epithelium

In the oral cavity two types of epithelium can be found; stratified squamous keratinizing and stratified squamous non-keratinizing epithelium [2, 3]. Stratified squamous non-keratinizing epithelium, i.e. of lining epithelium covering cheeks and floor of the mouth, consists of 3 layers; a basal, intermediate and superficial layer. The basal layer of non-keratinizing stratified epithelium consists of 2 to 3 layers of cells, or keratinocytes, that are neatly arranged, small cuboidal in shape and rapidly proliferate.

The predominant structural proteins found in the epithelial layer are keratins [3]. Depending on the location of the mucosa in the oral cavity, specific types of

keratins are expressed. In non-keratinizing epithelium the cells in the proliferating basal layer typically express keratin pair 5/14. Upon differentiation into the intermediate layer, the cells of the basal layer of non-keratinizing epithelium undergo several changes that include increased cell size, elongation and flattening of the cell, and expression of keratin pair 4/13. Cells of the superficial layer show membrane thickening, with a gradual decrease of volume which is followed by desquamation [1]. In buccal mucosa keratin 19 is additionally expressed in the basal layer, and keratins 6 and 16 are only expressed during hyper proliferation or hyperplasia [3].

The cell-cell attachment of epithelial cells is provided by two types of structures, desmosomes and adherents junctions. Desmosomes are highly organized, disc-shaped, intercellular junctions that provide strong adhesion between cells [4, 5] and give strength to tissues by linking intracellularly to the intermediate filament cytoskeleton to form adhesive bonds [5]. Desmosomes are important for maintaining tissue integrity of tissues that are exposed to mechanical stress such as the oral epithelium [6].

Basement membrane

The basement membrane (BM) is a thin layer that connects the epithelium to the underlying connective tissue. It provides support and functions as an anchoring membrane for the epithelium [7]. Electron microscopic images showed that the BM consists of three distinct layers, the lamina lucida, lamina densa and the fibroreticular lamina [8]. The BM is composed of several extracellular proteins including collagen type IV, laminin-332, fibronectin, proteoglycans and glycoproteins. Collagen type IV is the most abundant protein in the BM and can self-assemble into meshwork. Laminin-332, the predominant

non-collagenous protein, also self-assembles into a meshwork like collagen type IV [9, 10]. Collagen type IV is believed to be the initial scaffold upon which the laminin-332 mesh is deposited, however few studies suggest that laminin functions as the initial scaffold [9].

The attachment of the basal layer to the underlying BM occurs through specialized junctional complexes, called hemidesmosomes. Hemidesmosomes are small electron dense domains of the plasma membrane on the surface of cells like basal keratinocytes [11]. Hemidesmosomes provide stable adhesion to the BM and they contribute to the resistance of mechanical stress [12]. In complex epithelia and skin the hemidesmosome consists of integrin subunits alpha6 and beta4 (α6β4), BP180 and BP230. The integrin α6β4 functions as a trans membrane receptor for the extracellular matrix protein laminin-332. BP180 is believed to contribute to the structure of the anchoring filament and BP230 is involved in linking the hemidesmosome to the keratin cytoskeleton [13, 14].

Connective tissue and extracellular matrix

The basal layer is attached to the underlying connective tissue or lamina propria by collagen type VII anchoring fibrils that connect to the collagen fibers of the lamina propria [1]. The lamina propria has papillae [15] and consists of a dense fiber network. In the lamina propria of lining mucosa also elastic fibers can be found [1]. The papillae of the lamina propria interdigitate with the rete ridges of the epithelial layer. The dominant cell type in the lamina propria is the fibroblast, which is involved in producing and maintaining collagen fibers and the extracellular matrix. The extracellular matrix is a complex supramolecular

structure composed of collagens, glycoproteins, elastin and proteoglycans [16] which are secreted locally and assembled into a network to which cells can adhere [17].

TISSUE-ENGINEERED MUCOSA

Tissue engineering (TE) is a multidisciplinary field that combines engineering and life sciences to restore, maintain or improve function of tissue [18, 19]. Initially, the focus of many studies was on establishing TE substitutes for a variety of tissues such as bone, cartilage, adipose tissue, skin and oral mucosa. Tissue-engineered oral mucosal equivalents has been proven to be a useful for studying the dynamics of wound healing [20], fibrosis [21] and to test cytotoxicity and working mechanisms of new treatments [22]. Aside from these *in vitro* applications tissue-engineered mucosal constructs can be used in a clinical setting [23, 24]. To serve the above-mentioned purposes the structural features such as the presence of a multilayered epidermis, basal layer, basement membrane and underlying connective tissue as well as the expression pattern of cytokeratins of tissue-engineered mucosa should closely resemble that of native oral mucosa. We developed a tissue-engineered mucosal substitute that consists of an a-cellular de-epidermized dermal scaffold that is repopulated with primary keratinocytes and fibroblasts, as illustrated in fig. 2.

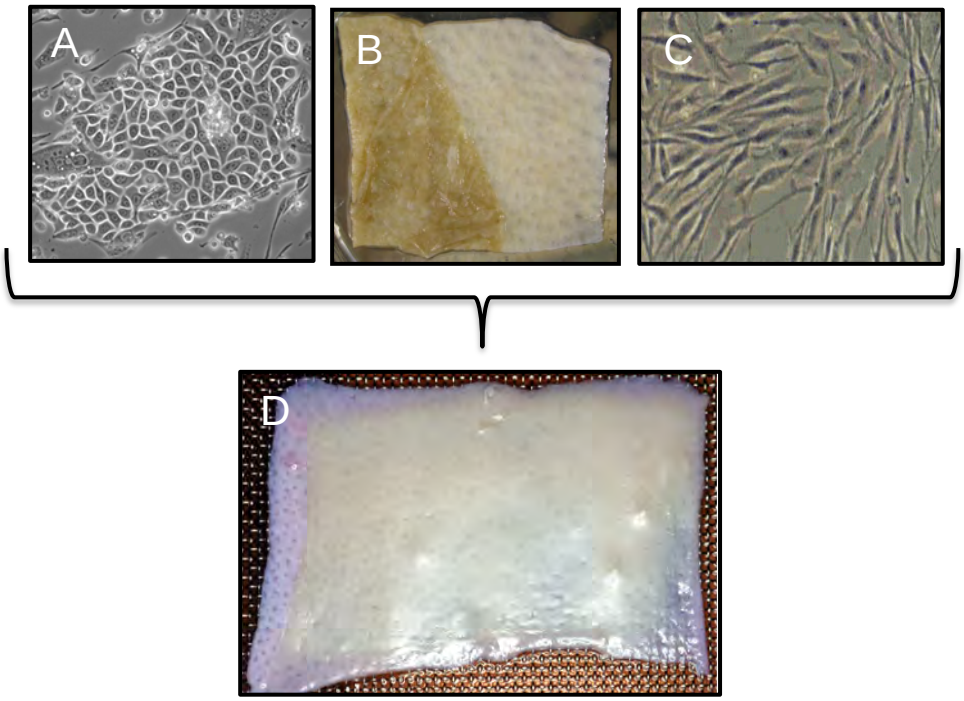


Figure 2. Composition of TEM. (A) Keratinocytes are seeded onto the papillary side of (B) de-epidermized dermis (DED) and (C) fibroblasts are seeded onto the lamina propria. (D) After 14 days of culture TEM is formed.

Cells

Oral mucosa consists primarily of keratinocytes and fibroblasts. Keratinocytes form the epithelial layer whereas fibroblasts are the predominant cell type found in the connective tissue. Tissue-engineered mucosal equivalents usually consist of keratinocytes alone or co-cultures of keratinocytes and fibroblasts seeded onto scaffolds [25]. Cells from different areas of the human body types such as gingivae, palatum, or skin can be used for tissue-engineered mucosal substitutes.

Scaffolds

A crucial component in tissue engineering is the material of the scaffold into which the cells are seeded. The choice for a certain type of scaffold is dictated by the type of tissue that is engineered and by mechanical properties, degradability, and contraction of the scaffold [26]. The number of scaffolds mentioned in literature is enormous as scaffolds can be made from natural materials such as collagen [27], fibrin [28] a-cellular dermis [25] , and synthetic materials such as polyglycolic acid [29], or PLGA-PCL (poly-lactic-co-glycolic poly-caprolactone) [30]. Many of these scaffolds are now commercially available such as a-cellular Matriderm [31] and Alloderm [32] or fibroblast-populated scaffolds such as Apligraf [33] or Dermagraft [34].

ORAL DEFECTS AND EXISTING TREATMENTS

Oral cancer is the sixth most common cancer worldwide and malignant tumors in the oral cavity make up approximately 30% of all cancers in the head and neck region. In the Netherlands approximately 2200 people every year are diagnosed with cancer in the head and neck region, and almost 30% of these people have cancer in the oral cavity [35]. The average 5-year survival rate is approximately 50%, which is correlated to the size of the primary tumor and the occurrence of metastases. Most often the treatment of these patients consists of oncological resection combined with radiotherapy or chemotherapy, often followed by reconstruction (fig. 3). Current techniques used for reconstruction use pedicle flaps or free micro vascular flaps [36]. Drawbacks of using these flaps are large scar formation of the donor site, perspiration, keratinization and hair growth in the flap [37]. Additionally, the limited amount of tissue available

to reconstruct the oral cavity has initiated the search for alternative tool to treat patients. One of these tools is tissue engineering.

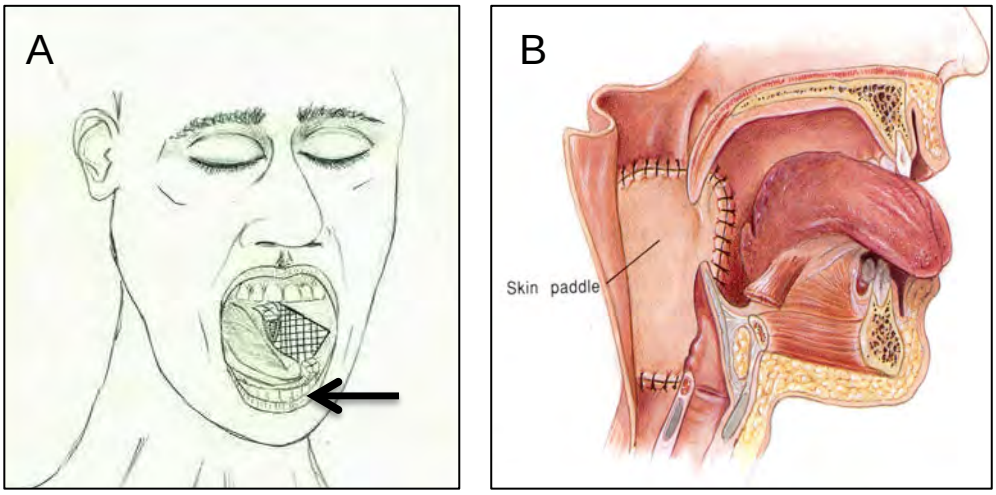


Figure 3. (A) Defect of the lateral mandible and soft tissue of the oral cavity (indicated with arrow; adapted from Source ePlasty © 2013 Open Science Co. LLC.) (B) Schematic representation of an oral defect reconstructed with a skin paddle (adapted from R.L.Ferris; source www.gbmc.org).

CLINICAL APPLICATION TISSUE-ENGINEERED MUCOSA

The earliest attempts to use tissue-engineered mucosa as an alternative source for grafting consisted of cultured epithelial sheets [38, 39]. The lack of supporting scaffolding made the epithelial sheets fragile and therefore difficult to handle. Consequently, more advanced tissue-engineered mucosal equivalents were developed. These equivalents consisted of keratinocytes seeded onto carrier biomaterials whether or not repopulated with fibroblasts. These more advanced full thickness mucosal equivalents have been used in for intra-oral grafting [23, 24]. However, a major problem in successfully using tissue-engineered equivalents in a clinical setting still is the lack of continuous oxygen supply due to the lack of vasculature.

PRECONDITIONING WITH HYPOXIA

As TEM was originally designed as an alternative source of tissue grafting, survival of the graft after implantation is crucial. The first days after implantation, the graft has to be able to survive without direct oxygen supply from blood vessels, or hypoxia. Prolonged exposure to an environment without oxygen will lead to cell death and eventually loss of the graft. Interestingly, it has been observed that low oxygen levels stimulate angiogenesis and stromal cells show increased angiogenic capacity. These observations resulted in the hypothesis that hypoxic priming of TEM before engraftment should accelerate the induction of blood vessel formation post-implantation thereby positively affecting the chances of TEM survival post-implantation.

HYPERBARIC OXYGEN TREATMENT

Hyperbaric oxygen (HBO) is the counterpart of hypoxia, and is currently used to improve wound healing of different types of wounds [40, 41]. Treatment with HBO is twofold: the oxygen levels are increased to 100% and the atmospheric pressure in increased to 2.4ATA [41]. By increasing these two components the oxygen levels in the capillaries rises resulting in the enhanced diffusion of oxygen in the surrounding tissues [42, 43]. Similar to hypoxia, HBO treatment has been shown to increase angiogenic factors. Additionally, HBO should stimulate proliferation of fibroblasts [44] and keratinocytes [45]. Therefore, the priming of TEM constructs before engraftment with HBO could accelerate the formation of blood vessel post-implantation.

RADIOTHERAPY

Most patients diagnosed with cancer in the head/neck region are treated with oncological resection combined with radiotherapy and/or chemotherapy. Aside from killing cancer cells radiotherapy is known to damage the healthy surrounding tissue resulting in many undesirable side effects. These side effects can be extremely severe resulting in interruption or termination of the radiotherapy treatment. The side effects can be divided into two groups; acute and late side effects. The acute effects include erythema, mucositis and xerostomia [41, 46]. The late effects include ulceration, telangiectasia, atrophy, soft tissue necrosis and fibrosis [47, 48]. Many studies on effects of radiotherapy have been done on monocultures of primary keratinocytes and fibroblasts or cell lines [49], but these monolayers are no realistic source of information, as they do not resemble the composition of native mucosa. As it is known that keratinocytes and fibroblasts affect each other in a paracrine fashion, it is essential that both cell types are included into a construct in order to study effects of radiation in a more realistically setting. More extensive knowledge of the acute response of both keratinocytes and fibroblasts on radiation might assist in accurate prediction of tissue developing severe of chronic side effects.

AIM OF THIS THESIS AND OUTLINE

The aim of this thesis was twofold:

1. To develop TEM that mimics native oral mucosa that can serve as an alternative source of tissue grafts for oral reconstruction.
2. To use this TEM for a variety of applications such as studying the effects of harmful or beneficial environments and the intrinsic repair capacity following ionizing radiation.

In chapter 2 we characterized our tissue-engineered mucosal substitute with extensive immunohistochemical staining. Although several studies have reported on mucosal substitutes [24, 27, 50], a detailed histological evaluation of a mucosal construct remained absent. Therefore we validated our mucosal construct by determining the expression of various components of the newly formed epithelial layer, basement membrane and underlying connective tissue and compared these results with those observed in native non-keratinizing oral mucosa.

To promote survival of TEM post-implantation we exposed TEM constructs in chapter 3 to hypoxia and to hyperbaric oxygen in chapter 4. Oxygen supply is an essential parameter for the survival of all tissue-engineered models. A lack of oxygen results in hypoxia, and when not restored can lead to graft failure or loss of the graft. A positive aspect of hypoxia however is that low oxygen levels stimulate angiogenesis and increase the angiogenic capacity of stromal cells. In chapter 3 we assessed the effect of hypoxia preconditioning on the angiogenic factors secreted in TEM.

Hyperbaric oxygen also promotes the secretion of angiogenic factors. In chapter 6 we examined whether HBO preconditioning of TEM increases the secretion of

angiogenic factors. Additionally, we evaluated whether HBO is beneficial for cell proliferation after TEM has been exposed to a single high dose of ionizing radiation, as HBO is believed to enhance wound healing.

As TEM was originally designed to serve as an alternative source for tissue grafting during oral reconstruction we evaluated the survival of TEM after implantation. In chapter 5 TEM was implanted into subcutaneous pockets on the back of nude mice. Three, 7, 14 and 28 days post-implantation the TEM constructs were harvested and evaluated.

Next we studied the effects of ionizing radiation on TEM in chapter 6. Radiotherapy is an essential element in the treatment of patients with cancer in the head and neck region. Aside from the beneficial effects such as killing of cancer cells, ionizing radiation is known to cause significant damage to the surrounding healthy tissue which can express themselves in acute of late effects. The late or chronic side effects are fibrosis, ulceration and loss or alteration of taste by damaged taste buds [45]. The early or acute side effects include oral mucositis and ulceration, both severe side effects that negatively affect the quality of life of treated patients. We hypothesized that more insight in the pathobiology of acute side effects might contribute to the prediction of the patients' reaction to ionizing radiation. In this chapter we examined the acute effects of ionizing radiation in TEM and compared these to the effects observed in native non-keratinizing oral mucosa. Hereby also determining the potential of TEM to function as an *in vitro* model to study effects of ionizing radiation.

In chapter 7 and 8 the findings of this thesis are summarized and discussed. In addition some suggestions for future research are given.

REFERENCES

1. Winning, T.A., G.C. Townsend (2000) Oral mucosal embryology and histology. Clin Dermatol *18(5)*: 499-511.
2. Janeway CA Jr, T.P., M. Walport, M.J. Shlomchik (2001) Immunobiology: The immune system in health and disease. *5th ed*.
3. Presland, R.B., B.A. Dale (2000) Epithelial structural proteins of the skin and oral cavity: function in health and disease. Crit Rev Oral Biol Med *11(4)*: 383-408.
4. Lewis, J.E., J.K. Wahl 3rd, K.M. Sass, P.J. Jensen, K.R. Johnson, (1997) Cross-talk between adherens junctions and desmosomes depends on plakoglobin. J Cell Biol *136(4)*: 919-34.
5. Garrod, D., M. Chidgey (2008) Desmosome structure, composition and function. Biochim Biophys Acta *1778(3)*: 572-87.
6. Green, K.J., C.L. Simpson (2007) Desmosomes: new perspectives on a classic. J Invest Dermatol *127(11)*: 2499-515.
7. Kannan, S., P. Balaram, G.J. Chandran, M.R. Pillai, B. Mathew (1994) Alterations in expression of basement membrane proteins during tumour progression in oral mucosa. Histopathology *24(6)*: 531-7.
8. Verma, G.P., (2001) Fundamentals of Histology. New Delhi: New Age International (P) Limited, Publishers. 449.
9. Rowe, R.G., S.J. Weiss (2008) Breaching the basement membrane: who, when and how? Trends Cell Biol *18(11)*: 560-74.
10. LeBleu, V.S., B. Macdonald, R. Kalluri (2007) Structure and function of basement membranes. Exp Biol Med (Maywood) *232(9)*: 1121-9.

11. Borradori, L., A. Sonnenberg (1999) Structure and function of hemidesmosomes: more than simple adhesion complexes. J Invest Dermatol *112(4)*: 411-8.
12. de Pereda, J.M., E. Ortega, N. Alonso-Garcia, M. Gomez-Hernandez, A. Sonnenberg (2009) Advances and perspectives of the architecture of hemidesmosomes: lessons from structural biology. Cell Adh Migr *3(4)*: 361-4.
13. Jones, J.C., S.B. Hopkinson, L.E. Goldfinger (1998) Structure and assembly of hemidesmosomes. Bioessays *20(6)*: 488-94.
14. Guo, L., L. Degenstein, J. Dowling, Q.C. Yu, R. Wollmann, *et al.* (1995) Gene targeting of BPAG1: abnormalities in mechanical strength and cell migration in stratified epithelia and neurologic degeneration. Cell *81(2)*: 233-43.
15. Junqueira, L.C., J. Carneiro (2004) Funtionele Histologie. Vol.10.: Elsevier gezondheidszorg.
16. Schonherr, E., H.J. Hausser (2000) Extracellular matrix and cytokines: a functional unit. Dev Immunol *7(2-4)*: 89-101.
17. Adams, J.C., F.M. Watt (1993) Regulation of development and differentiation by the extracellular matrix. Development *117(4)*: 1183-98.
18. Langer, R., J.P. Vacanti (1993) Tissue engineering. Science *260(5110)*: 920-6.
19. Pollok, J.M., J.P. Vacanti (1996) Tissue engineering. Semin Pediatr Surg *5(3)*: 191-6.

20. Boyce, S.T., G.D. Warden (2002) Principles and practices for treatment of cutaneous wounds with cultured skin substitutes. Am J Surg *183(4)*: 445-56.

21. Wang, X., Y. Liu, Z. Deng, R. Dong, Y. Liu, *et al*. (2009) Inhibition of dermal fibrosis in self-assembled skin equivalents by undifferentiated keratinocytes. J Dermatol Sci *53(2)*: 103-11.

22. Klausner, M., S. Ayehunie, B.A. Breyfogle, P.W. Wertz, L. Bacca, *et al.* (2007) Organotypic human oral tissue models for toxicological studies. Toxicol In Vitro *21(5)*: 938-49.

23. Lauer, G., R. Schimming (2001) Tissue-engineered mucosa graft for reconstruction of the intraoral lining after freeing of the tongue: a clinical and immunohistologic study. J Oral Maxillofac Surg *59(2)*: 169-75; discussion 175-7.

24. Izumi, K., S.E. Feinberg, A. Iida, M. Yoshizawa (2003) Intraoral grafting of an ex vivo produced oral mucosa equivalent: a preliminary report. Int J Oral Maxillofac Surg *32(2)*: 188-97.

25. Moharamzadeh, K., I.M. Brook, R. Van Noort, A.M. Scutt, M.H. Thornhill (2007) Tissue-engineered oral mucosa: a review of the scientific literature. J Dent Res *86(2)*: 115-24.

26. Patterson, J.M., A.J. Bullock, S. MacNeil, C.R. Chapple (2011) Methods to reduce the contraction of tissue-engineered buccal mucosa for use in substitution urethroplasty. Eur Urol *60(4)*: 856-61.

27. Kinikoglu, B., C. Auxenfans, P. Pierrillas, V. Justin, P. Breton, *et al.* (2009) Reconstruction of a full-thickness collagen-based human oral mucosal equivalent. Biomaterials *30(32)*: 6418-25.

28. Ahmed, T.A., E.V. Dare, M. Hincke (2008) Fibrin: a versatile scaffold for tissue engineering applications. Tissue Eng Part B Rev *14(2)*: 199-215.

29. Sachlos, E., J.T. Czernuszka (2003) Making tissue engineering scaffolds work. Review: the application of solid freeform fabrication technology to the production of tissue engineering scaffolds. Eur Cell Mater *5*: 29-39; discussion 39-40.

30. Ng, K.W., H.L. Khor, D.W. Hutmacher (2004) In vitro characterization of natural and synthetic dermal matrices cultured with human dermal fibroblasts. Biomaterials *25(14)*: 2807-18.

31. Golinski, P.A.,S. Groger, J.M. Herrmann, A. Bernd, J. Meyle (2011) Oral mucosa model based on a collagen-elastin matrix. J Periodontal Res *46(6)*: 704-11.

32. Peramo, A., C.L. Marcelo, S.E. Feinberg (2012), Tissue Engineering of Lips and Muco-Cutaneous Junctions: In Vitro Development of Tissue Engineered Constructs of Oral Mucosa and Skin for Lip Reconstruction. Tissue Eng Part C Methods *18(4)*: 273-82.

33. Karr, J. (2008) Utilization of living bilayered cell therapy (Apligraf) for heel ulcers. Adv Skin Wound Care *21(6)*: 270-4.

34. Marston, W.A. (2004) Dermagraft, a bioengineered human dermal equivalent for the treatment of chronic nonhealing diabetic foot ulcer. Expert Rev Med Devices *1(1)*: 21-31.

35. http://www.nwhht.nl.

36. Wehage, I.C., H. Fansa (2011) Complex reconstructions in head and neck cancer surgery: decision making. Head Neck Oncol *3*: 14.

37. Lauer, G., R. Schimming, N.C. Gellrich, R. Schmelzeisen (2001) Prelaminating the fascial radial forearm flap by using tissue-engineered mucosa: improvement of donor and recipient sites. Plast Reconstr Surg *108(6)*: 1564-72; discussion 1573-5.

38. Lauer, G., J.E. Otten, B.U. von Specht, W. Schilli (1991) Cultured gingival epithelium. A possible suitable material for pre-prosthetic surgery. J Craniomaxillofac Surg *19(1):* 21-6.

39. Ueda M., K. Ebata, T. Kaneda (1991) In vitro fabrication of bioartificial mucosa for reconstruction of oral mucosa: basic research and clinical application. Ann Plast Surg *27(6):* 540-9.

40. Kuffler, D.P. (2010) Hyperbaric oxygen therapy: an overview. J Wound Care *19(2)*: 77-9.

41. Spiegelberg, L., U.M. Djasim, H.W. van Neck, E.B. Wolvius, K.G. van der Wal (2010) Hyperbaric oxygen therapy in the management of radiation-induced injury in the head and neck region: a review of the literature. J Oral Maxillofac Surg *68(8)*: 1732-9.

42. Kairuz, E., Z. Upton, R.A. Dawson, J. Malda (2007) Hyperbaric oxygen stimulates epidermal reconstruction in human skin equivalents. Wound Repair Regen *15(2)*: 266-74.

43. Gill, A.L., C.N. Bell (2004) Hyperbaric oxygen: its uses, mechanisms of action and outcomes. QJM *97(7)*: 385-95.

44. Hollander, D.A., M.Y. Hakimi, A. Hartmann, K. Wilhelm, J. Windolf (2000) The Influence of Hyperbaric Oxygenation (HBO) on Proliferation and Differentiation of Human Keratinocyte Cultures In Vitro. Cell Tissue Bank *1(4)*: 261-9.

45. Sander, A.L., D. Henrich, C.M. Muth, I. Marzi, J.H. Barker, *et al.* (2009) In vivo effect of hyperbaric oxygen on wound angiogenesis and epithelialization. Wound Repair Regen. *17(2)*: 179-84.

46. Specht, L. (2002) Oral complications in the head and neck radiation patient. Introduction and scope of the problem. Support Care Cancer *10(1)*: 36-9.

47. Cooper, J.S., K. Fu, J. Marks, S. Silverman (1995) Late effects of radiation therapy in the head and neck region. Int J Radiat Oncol Biol Phys *31(5)*: 1141-64.

48. Tolentino Ede, S., B.S. Centurion, L.H. Ferreira, A.P. Souza, J.H. Damante, *et al.* (2011) Oral adverse effects of head and neck radiotherapy: literature review and suggestion of a clinical oral care guideline for irradiated patients. J Appl Oral Sci *19(5)*: 448-54.

49. Petit-Frere, C., E. Capulas, D.A. Lyon, C.J. Norbury, J.E. Lowe, *et al*. (2000) Apoptosis and cytokine release induced by ionizing or ultraviolet B radiation in primary and immortalized human keratinocytes. Carcinogenesis *21(6)*: 1087-95.

50. Rakhorst, H.A., S.J. Posthumus-Van Sluijs, W.M. Tra, J.W. van Neck, G.J. van Osch, *et al*. (2006) Fibroblasts accelerate culturing of mucosal substitutes. Tissue Eng *12(8)*: 2321-31.

CHAPTER 2

Characterization of a three-dimensional mucosal equivalent: similarities and differences with native oral mucosa

Wendy M.W. Tra[1], Johan W. van Neck[1], Steven E.R. Hovius[1], Gerjo J.V.M. van Osch[2,3], Soledad Perez-Amodio[1]

Department of Plastic & Reconstructive Surgery[1], Department of Otorhinolaryngology[2], Department of Orthopaedics[3], Erasmus MC, Rotterdam, the Netherlands

Published in *Cells Tissues Organs 2012; 195(3):185-96*

ABSTRACT

The aim of this study was to create and characterize a tissue engineered mucosal equivalent (TEM) that closely resembles native mucosa. TEM consists of human primary keratinocytes and fibroblasts isolated from biopsies taken from healthy donors and seeded onto a de-epidermized dermis and cultured for 14 days at the air/liquid interface. The structure of TEM was examined and compared with native nonkeratinizing oral mucosa (NNOM). The various components of the newly formed epidermal layer, basement membrane and underlying connective tissue were analyzed using immunohistochemistry. The mucosal substitute presented in this study showed a mature stratified squamous epithelium that was similar to that of native oral mucosa, as demonstrated by K19, desmoglein-3 and involucrin staining. In addition, the expression of basement membrane components collagen type IV, laminin-5 and integrin α6 and β4 in TEM proved to be consistent with native oral mucosa. The expression of PAS, Ki67, K10 and K13 however appeared to be different in TEM compared to NNOM. Nevertheless, the similarities with native oral mucosa makes TEM a promising tool for studying the biology of mucosal pathology such as oral mucositis or fibrosis as well as the development of new therapies.

INTRODUCTION

Tissue engineering of oral mucosa is a promising technique for studying the biology and pathology of oral mucosa, and can be used as a model to study dynamics of wound healing [1], fibrosis [2] and to test cytotoxicity and working mechanisms of new treatments [3]. Next to these *in vitro* applications, tissue engineered mucosal constructs might be used in a clinical setting to provide an alternative source for mucosal grafts for the reconstruction of large oral defects which form a major challenge in reconstructive surgery [4, 5].

To serve the above described purposes, tissue engineered mucosal substitutes should mimic native oral mucosa. Structural features of the tissue such as the presence of a multilayered epidermis, basal layer, basement membrane (BM) and underlying connective tissue as well as the expression pattern of cytokeratins should resemble that of native oral mucosa. Several studies have reported the development of mucosal substitutes, however only few of them included histological characterization of the construct [6-8]. Izumi *et al* [6] described the proliferative capacity and keratin 10/13 expression of their mucosal substitutes and Garzon *et al* [9] reported on the cytokeratin pattern in tissue engineered periodontal mucosa. Other studies described the pattern of cytokine release of mucosal substitutes [10] or the use of a variety of scaffolds to create tissue engineered mucosal substitutes [11]. All these studies focussed on some components of the construct, but to our knowledge none of these studies describe a detailed characterization of the mucosal construct.

The aim of the work described here was to engineer and thoroughly characterize a mucosal substitute that resembles non-keratinizing native oral mucosa (NNOM). This tissue engineered mucosal substitute (TEM) consists of primary human keratinocytes and fibroblasts isolated from oral mucosa and

seeded onto de-epidermized dermis (DED). DED was chosen over collagen-based scaffolds for its resemblance to native connective tissue, easy handling and non-contractile nature. The incorporation of fibroblasts into the scaffold has been proven to be essential for epidermal development [12], as they stimulate keratinocyte proliferation and migration by paracrine mechanisms [13]. The TEM construct was characterized by studying the expression of several components of the BM, the expression pattern of keratins, keratinocyte proliferation and differentiation. As cell-cell adhesion and epidermal attachment to the underlying connective tissue has been shown to be essential for functionality [14], the expression of desmosomes and hemidesmosomes was also evaluated.

MATERIALS & METHODS

Chemicals and culture media

Dulbecco's Modified Eagle Medium 4.5 g/l glucose (DMEM), Ham's F12 culture medium, penicillin, streptomycin, gentamicin, amphoceterin B, dispase, collagenase type I and trypsin/ ethylenediaminetetraacetic acid (EDTA) were purchased from Invitrogen (Breda, the Netherlands). Fetal calf serum (FCS) was purchased from PAA Laboratories (Cölbe, Germany). Bovine serum albumin (BSA), epidermal growth factor (EGF), keratinocyte growth factor (KGF) and other chemicals were purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands). Insulin was purchased from Eli Lilly (Houten, the Netherlands).

Cell culture

For this study punch biopsies were taken from healthy individuals, 3 males and 1 female, all Caucasian, aged 62.75 ± 6.39, upon approval of the Medical Ethics Committee (# MEC + 2007-282). Half of the biopsies were snap frozen with liquid nitrogen. The remaining biopsies were rinsed thoroughly with phosphate buffered saline (PBS) and DMEM. The epidermis was separated from the dermis by an overnight incubation in 2.5 mg/mL dispase solution. After rinsing the epidermal sheet with PBS, keratinocytes were isolated from the epidermis using 0.25 % trypsin-EDTA and the single cell suspension was seeded onto lethally irradiated 3T3 fibroblast feeder layers, according to the Rheinwald & Green protocol [15, 16]. Culture Medium A consisted of a 3:1 mixture of DMEM and Ham's F12 medium with 5 % FCS, 1 µM hydrocortisone, 1 µM isoproterenol, 0.1 µM insulin, 100 IU/mL penicillin, 100 ug/mL streptomycin, and 1 ng/mL EGF. Fibroblasts were isolated by mincing the dermis followed by incubation in collagenase/dispase (1.5 mg/mL / 2.5 mg/mL, respectively) solution for 2 hours at 37 ^{o}C. Next, the collagenase/dispase solution containing the dermis was filtered twice with a 100 µm cell strainer (Sigma-Aldrich, Zwijndrecht, the Netherlands), in order to obtain a single cell suspension. The isolated fibroblasts were cultured in fibroblast medium consisting of DMEM supplemented with 10 % FCS, 100 IU/mL penicillin and 100 µg/mL streptomycin. The cells used in this study were within passage 3 to 6.

De-epidermized dermis (DED)

Human cadaver skin, cryopreserved in 10% glycerol, and tested negative for cytomegalovirus, human immunodeficiency virus and hepatitis B, was obtained from the European Skin Bank (Beverwijk, the Netherlands). The epidermis was

removed from the dermis by gently shaking the skin in PBS supplemented with 200 IU/mL penicillin, 200 µg/mL streptomycin and 5 µg/mL amphoceterin B. The skin was kept in the PBS solution for 3 weeks and PBS was changed three times a week.

Tissue engineered mucosa (TEM)

TEM was created by spinning 1 x 10^5 fibroblasts gently into the lamina propria of the DED. For this procedure we used 15mL tubes filled with 13 mL 1 % agarose gel (Sigma Aldrich) on which the DED scaffold is placed, lamina propria side upwards. Next the fibroblasts are placed onto the DED. The tubes containing the DED and fibroblasts are placed in a centrifuge and the fibroblasts were gently spun into the DED, at 500 rpm for 60 minutes. Next, the DED containing the fibroblasts was placed onto a stainless steel grid and kept at an air/liquid interface with fibroblast medium for approximately 5 hours to ensure cell attachment to the DED. Finally the DED was flipped over and the keratinocytes were seeded onto the BM, and kept under submerged condition for 24 hours with Medium A, supplemented with 4 ng/mL KGF in stead of EGF. After 24 hours, the construct was raised to the air/liquid interface and Medium A was replaced by Medium B consisting of 3:1 mixture of DMEM and Ham's F12 supplemented with 1 % FCS, 24 µM BSA, 1 µM hydrocortisone, 1 µM isoproterenol, 0.1 µM insulin, 10 µM L-carnitine, 10 mM L-serine, 1 µM D-L-α-tocopherolacetate, fatty acid cocktail (15 µM linoleic acid, 7 µM arachidonic acid and 25 µM palmitic acid), 100 IU/mL penicillin, 100 ug/mL streptomycin, and 4 ng/mL KGF. Medium B was removed after 48 hours and replaced by Medium C consisting of Medium B in which FCS was omitted, linoleic acid concentration was increased to 30 µM and 50 µg/mL ascorbic acid was added.

Medium was changed three times a week and TEM construct was harvested after 14 days. In this study keratinocytes and fibroblasts isolated from biopsies from different donors were never pooled.

Morphology and Immunohistochemistry

Harvested TEM and NNOM biopsies were snap frozen using liquid nitrogen. Sections (6 µm) were cut, and overall morphology was assessed by Haematoxylin Eosin (H&E) and Periodic Acid & Schiff (PAS) stain. For immunohistochemical analysis of epidermal components K10, K13 and K19, cryo sections were air dried and fixed for 10 minutes in acetone. After incubation with the primary antibodies, frozen sections were incubated with Link/Label complex (BioGenex, San Ramon, USA) according manufacturers instructions. Finally, sections were stained with New Fuchsine substrate and Haematoxylin. For immunohistochemical analysis of the BM and underlying connective tissue, frozen sections were stained for collagen type IV, laminin, involucrin, vimentin, desmoglein-3, integrin α6 and β4. For antibodies and concentrations used in this study see Table 1. After incubation with the primary antibodies, sections were incubated with secondary antibody biotin labelled Goat anti-mouse of Goat anti-Rabbit (both 1/200, DAKO), followed by incubation with Streptavidin-HRP (DAKO) and Diaminobenzidin (DAB) substrate. The presence of proliferative cells was assessed using Ki67 antibody, using the protocol as described above. All sections were counterstained with Haematoxylin. Controls were included with each staining performed. As a negative control, the primary antibody was replaced by PBS. For the positive control cryosections of normal oral mucosa were used. An isotope control was also included, for which the primary antibody was replaced by Mouse anti-

human IgG or Rabbit anti-human IgG (DAKO), depending on the origin of the primary antibody it replaced. The concentration was similar to that of the primary antibody.

Table 1. Primary antibodies used for immunohistochemistry

Antibody	Concentration	Source
Collagen type IV	1/500	Euro-Diagnostica, Nijmegen, The Netherlands
Desmoglein-3	1/500	Novus Biologicals, LLC., Littleton, Colo., USA
Integrin α6	1/1,000	Novus Biologicals, LLC., Littleton, Colo., USA
Integrin β4	1/2,000	Novus Biologicals, LLC., Littleton, Colo., USA
Involucrin	1/500	Novus Biologicals, LLC., Littleton, Colo., USA
K10	1/200	Euro-Diagnostica, Nijmegen, The Netherlands
K13	1/200	Euro-Diagnostica, Nijmegen, The Netherlands
K19	1/500	Novus Biologicals, LLC., Littleton, Colo., USA
Ki67	1/200	DAKO, Glostrup, Denmark
Laminin-5	1/1,000	Abcam, Cambridge, Mass., USA
Vimentin	1/200	Euro-Diagnostica, Nijmegen, The Netherlands

Epidermal thickness

Average epidermal thickness was determined by measuring the epidermis at 12 randomly selected regions for both NNOM and TEM, using the NDPI Viewer (Hamamatsu Photonics, Germany). This was repeated for 4 independent experiments, which were done in triplicate. Statistical significance was determined using the Mann-Whitney U test.

Quantification of cell proliferation

To determine the proliferation index (PI), the basal layer of the epithelium was analyzed. Images were taken from 12 randomly chosen microscopic views using 100x magnification. The PI was established as the ratio of the positive cells to all

cells of the basal layer (x 100%), and results were displayed as mean ± SD. Experiments were done in triplicate. Statistical significance was determined using the Mann-Whitney U test.

RESULTS

Morphology

The overall morphology of TEM was assessed by Haematoxylin Eosin staining. The newly developed TEM construct had a well developed, multilayered and stratified squamous epithelium, consisting of a basal, intermediate and superficial layer (Fig. 1A) and resembles NNOM (Fig. 1B). The epidermis of TEM was para-keratinized, as pyknotic cells were present in the epithelial layer. The basal layer was neatly arranged in two to three layers, with cubodial shaped cells. The epidermal layer of NNOM has an average thickness of 194.3 ± 59.3µm which is significantly thicker compared to the epidermis of TEM, which has an average thickness of 72.6±26.9µm (Fig. 1C) $P < 0.001$. Vacuoles were observed in the epithelial cells in the intermediate layer of NNOM and TEM. Glycogen content in NNOM was indicated by PAS staining, showing glycogen in the intermediate layer (Fig. 1E). PAS staining was also present in the intermediate layer in TEM albeit less intense than in NNOM (Fig. 1D).

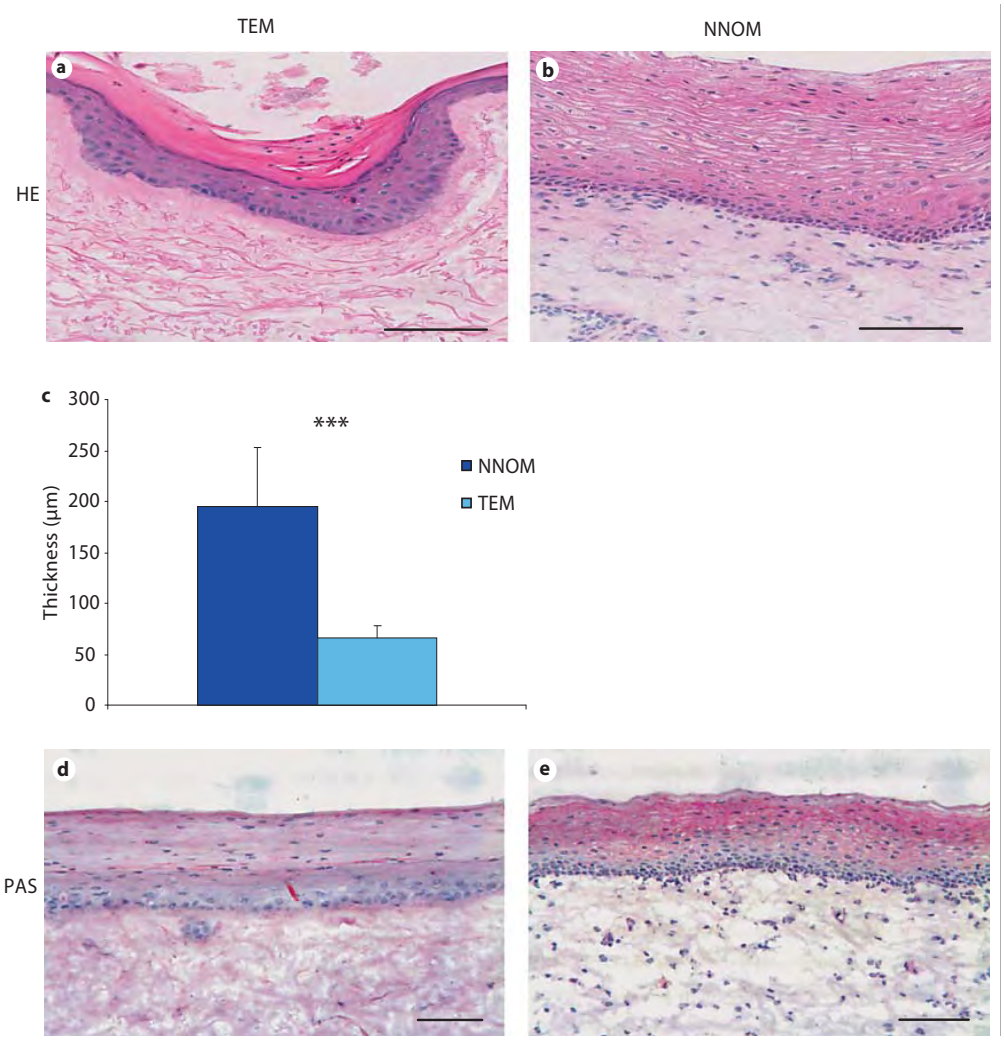


Figure 1. Hematoxylin-eosin staining of a cross section of (A) TEM and (B) NNOM. TEM resembles overall morphology of NNOM, and shows a well-formed, multilayered, parakeratinized epidermis. (C) The epidermis was significantly thinner in TEM (72.6 ± 26.6 µm) when compared to NNOM (194.3 ± 59.3 µm). Data are presented as mean ± SD (***$p < 0.001$). The keratinocytes in the intermediate layer seemed to have vacuoles. PAS staining showed lower glycogen content in (D) TEM compared with (E) NNOM. All pictures are representative for the staining performed. Scale bars = 100 µm.

Epidermal differentiation

Keratin expression in the epidermis was assessed by staining for K10, K13 and K19. TEM showed expression of K10 in the superficial layer only, whereas no expression of K10 was seen in NNOM (Fig. 2A-B). K13 stained weakly in the intermediate layer of TEM (Fig. 2D), and in both the intermediate and superficial layers of the epithelium of NNOM (Fig. 2E). Expression of K19 was observed in the basal layer only of the epithelium of TEM and NNOM (Fig. 2G-H). Close observation showed that not every basal cell is positive for K19. Analysis of the basal layer of TEM and NNOM showed that TEM has an average of 74.85 ± 12.84 positive cells, which is significantly lower compared to NNOM (88.03 ± 6.41) $P < 0.001$. Involucrin was observed in all layers except the basal layer in both TEM and NNOM (Fig. 2J-K).

Proliferation

Cells in the basal layer are typically the only cells that proliferate in the epithelium. Proliferation was assessed by Ki67, a protein present in all proliferating cells**.** In both TEM only (Fig. 3A) and NNOM proliferating cells were observed in the basal layer only (Fig. 3B). The PI was significantly lower in TEM (36.6 ± 11.1) compared to NNOM (55.8 ± 8.3) (Fig. 3D) $p < 0.001$.

Connective tissue

Fibroblasts are the predominant cell type in the dermal layer. The presence and distribution of fibroblasts was assessed by vimentin staining. In TEM, fibroblasts were found in the lamina propria, where they were mainly located on the bottom surface of the lamina propria (Fig. 3E). In NNOM, the fibroblasts were found distributed homogeneously in the connective tissue (Fig. 3F).

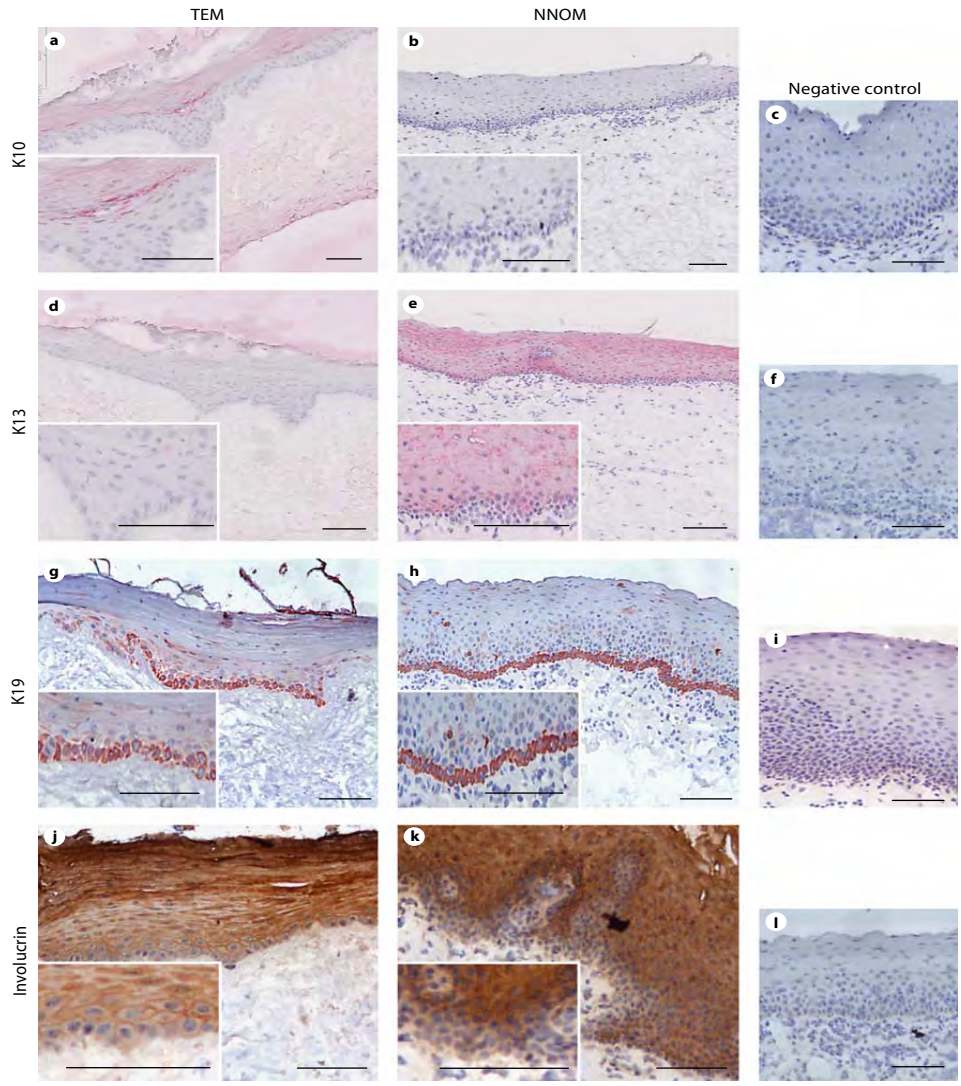


Figure 2. Keratin expression was assessed in TEM and NNOM. K10, present in keratinized mucosa, was present in the superficial layer of the epidermis of (A) TEM but was not observed in (B) NNOM. K13, present in nonkeratinizing epithelium, was weakly expressed in (D) TEM but was strongly expressed in (E) NNOM. K19, present in the basal layer of nonkeratinizing of the oral cavity, was observed in the basal layer of (G) TEM and (H) NNOM. The expression pattern of involucrin in (J) TEM proved to be similar to (K) NNOM. (C, F, I and L) Negative controls of all staining are included. All pictures are representative for the stainings. Scale bars = 100 µm.

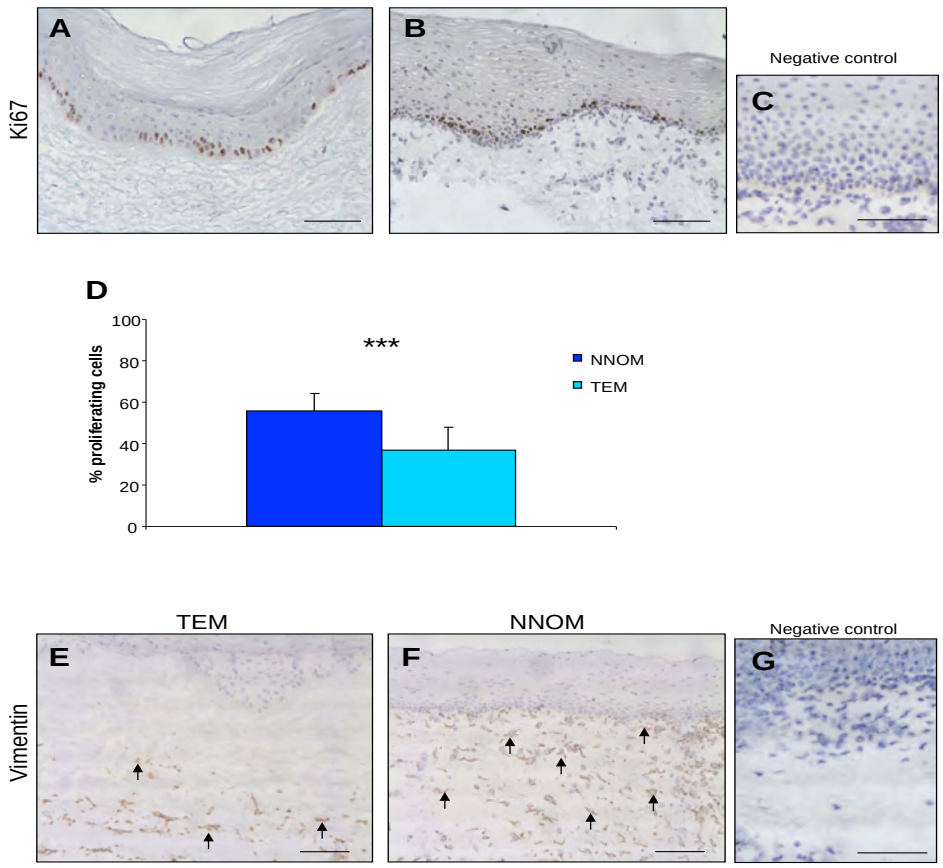


Figure 3. Cell proliferation in (A) TEM was compared to (B) NNOM. Proliferating cells were only observed in the basal layer of both NNOM and TEM. (D) The number of proliferating cells in TEM (36.6 ± 11.1) was significantly lower compared to NNOM (55.8 ± 8.3). Data are presented as mean ± SD (***$p < 0.001$). Fibroblast distribution was assessed by vimentin staining. (E) In TEM, fibroblasts (indicated by arrows) were seen only on the lower surface of the lamina propria, whereas in (F) NNOM, fibroblasts were seen throughout the connective tissue. (C and G) Negative controls of both stainings are included. All pictures shown are representative for the stainings. Scale bars = 100 µm.

Basement membrane

The presence of basement membrane components collagen type IV and laminin 5 were first determined in the DED scaffold. Collagen type IV is a component of basal lamina and laminin 5 is a component of the anchoring filaments. Both components were expressed in the scaffold along the whole dermal-epidermal junction before seeding of fibroblasts and keratinocytes (Fig. 4A-E). The expression of collagen type IV and laminin 5 was restricted to the basement membrane. The addition of fibroblasts and keratinocytes did not alter the expression of collagen type IV (Fig. 4B) or laminin 5 (Fig. 4F) in TEM. Collagen type IV expression was higher in TEM (Fig. 4B) than in NNOM (Fig. 4C). Laminin 5 was more abundantly expressed in NNOM (Fig. 4G) than in TEM (Fig. 4F).

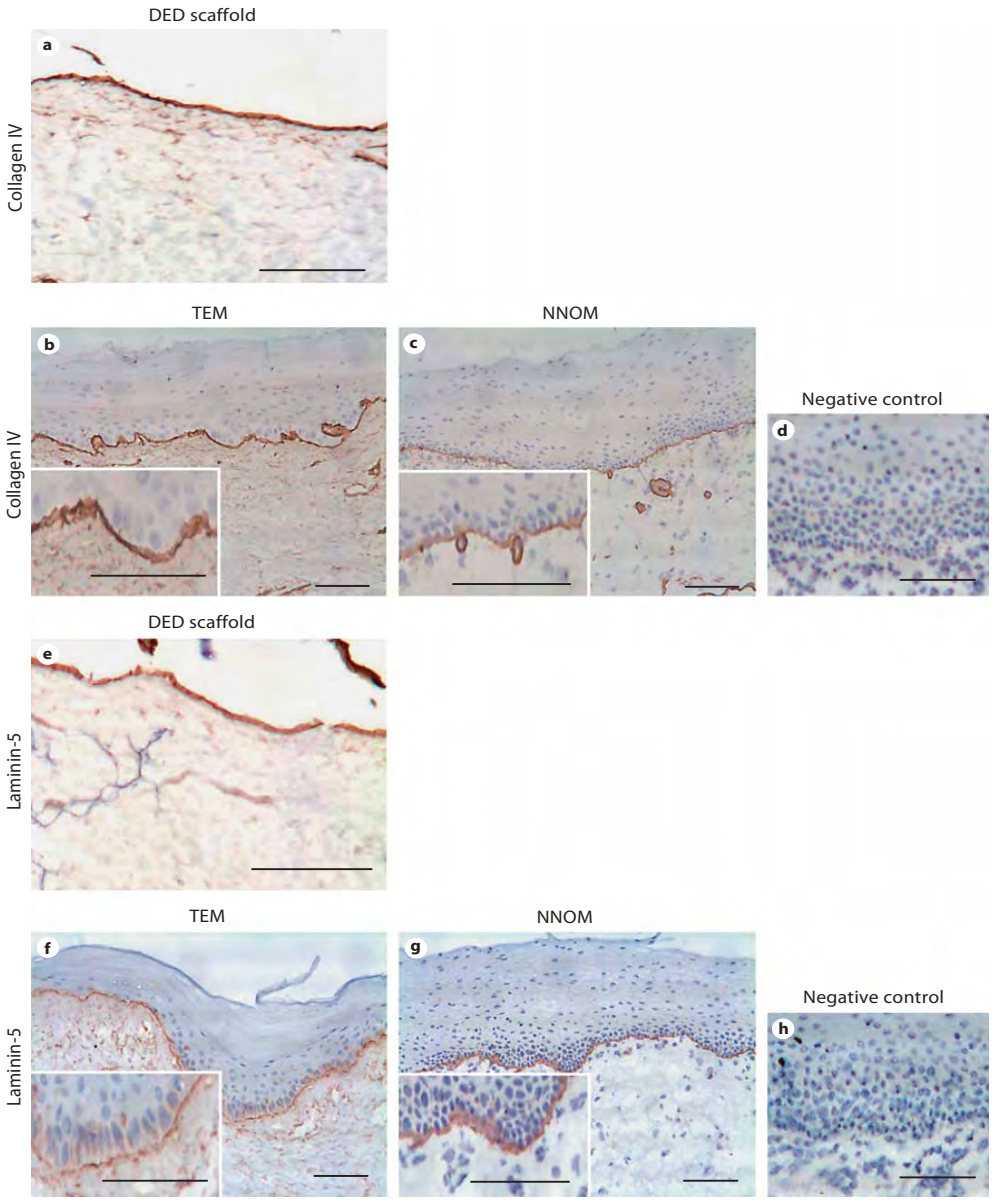


Figure 4. BM components (A) collagen type IV and (B) laminin-5 (E) were present in the DED scaffold before seeding of keratinocytes and fibroblasts. Collagen type IV and laminin-5 were equally expressed in (B and F) TEM and (C and G) NNOM). (D and H) Negative controls are included. All pictures shown are representative for the stainings. Scale bars = 100 µm.

Dermal-Epidermal junction

Desmosomes and hemidesmosomes are responsible for cell-cell attachment and the attachment of the epithelial layer to the underlying connective tissue. Cell-cell attachment by desmosomes was assessed by desmoglein-3, a glycoprotein component of desmosomes. Cells in the basal and intermediate layer of TEM and NNOM were equally positive for desmoglein-3 (Fig. 5A-B). Before the seeding of fibroblasts and keratinocytes, the hemidesmosomal subunits integrin alpha6 and beta4 were not present in the DED scaffold (Fig. 5D-E). Integrin alpha6 was expressed along the whole dermal-epidermal junction, predominantly in the BM, in both TEM (Fig. 5F) and NNOM (Fig. 5E). Integrin beta4 was also present along the whole dermal-epidermal junction, predominantly in the BM of both TEM (Fig. 5G) and NNOM (Fig. 5H), albeit the intensity of integrin beta4 was lower in TEM compared to NNOM. In all sections used, the newly formed epithelium was attached to the underlying connective tissue and detachment was never seen.

Table 2. Summary of epidermal characteristics of TEM and NNOM

	Epidermis	
	TEM	NNOM
Tissue architecture	BL, IL, SL	BL, IL, SL
Number of living cell layers	5–10	15–20
K10	SL	absent
K13	SL	IL, SL
K19	BL	BL
Ki67	BL, ++	BL, ++++
Desmoglein-3	BL, IL	BL, IL
Integrin α6	BL	BL
Integrin β4	BL	BL
Collagen type IV	BL	BL
Laminin-5	BL	BL
Involucrin	BL, IL, SL	BL, IL, SL

BL = Basal layer; IL = intermediate layer; SL = superficial layer.

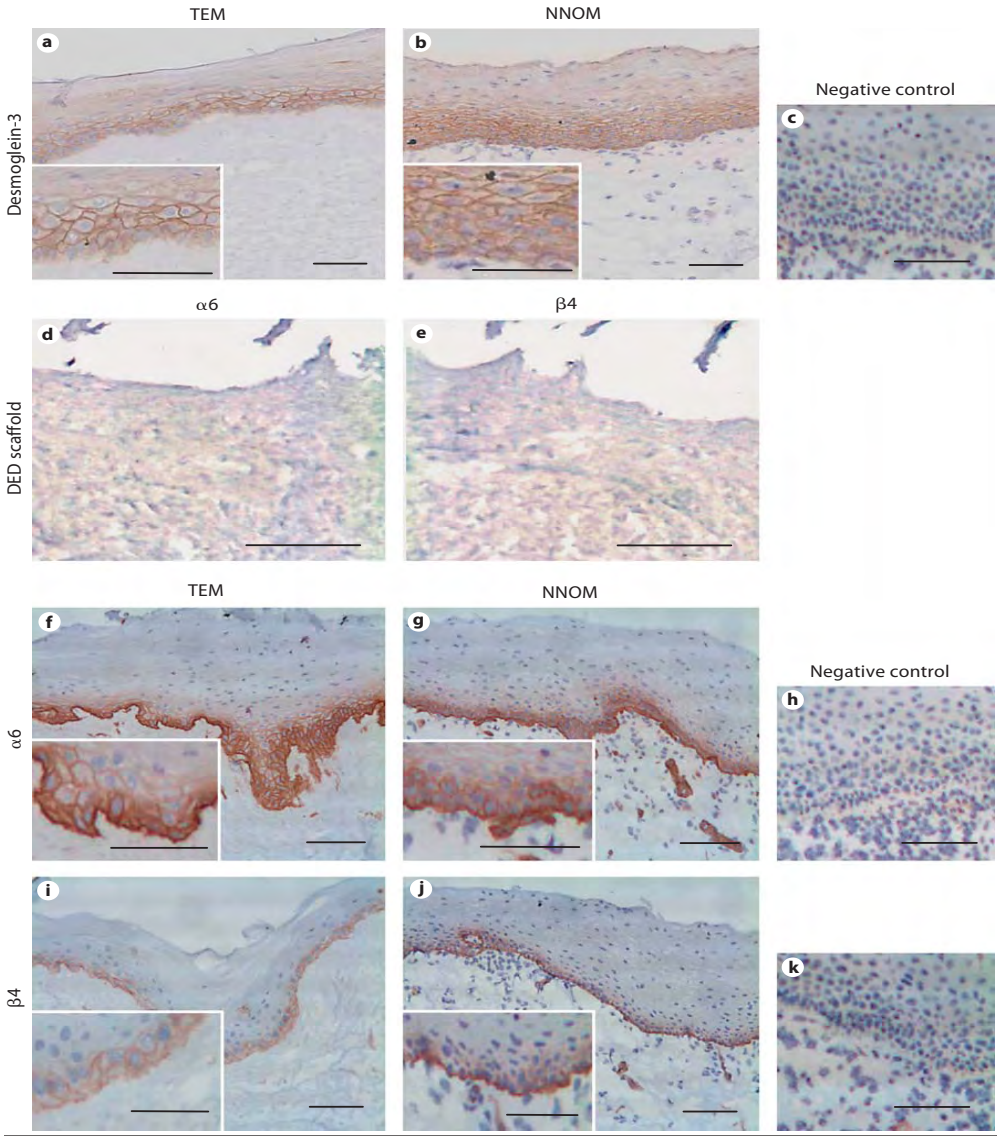


Figure 5. Cell-cell adhesion was assessed by desmoglein-3, a glycoprotein of desmosomes. In both (A) TEM and (B) NNOM desmoglein-3 was observed in the basal layers of the epidermis. Hemi-desmosomal subunits (D) α6 and (E) β4 were not expressed in the DED scaffold. A similar expression pattern for integrin α6 was observed in (F) TEM and (G) NNOM. Integrin β4 expression was seen in the basal layer of (I) TEM and (J) NNOM, although the intensity in TEM was lower than in NNOM. (C, K and H) Negative controls are included. All pictures shown are representative for the stainings. Scale bars = 100 µm.

DISCUSSION

The aim of this study was to create a mucosal construct that resembles native oral mucosa. Therefore, we engineered TEM by seeding oral keratinocytes and fibroblasts onto DED. Next, TEM and NNOM were compared on epidermal structure, basement membrane and underlying connective tissue organization. Here we show an oral mucosal construct composed of a multi-layered epithelium with many morphological and immunological characteristics observed in NNOM. No morphological differences were observed between the TEM constructs from different donors. Keratin 19, Ki-67, involucrin, integrin α6 and β4, vimentin, collagen type IV and laminin-5 were present in our TEM construct (See table 2). Overall morphology of TEM was observed to be similar to NNOM, although the epidermal layer of TEM was found to be significantly thinner compared to NNOM. Despite the epidermis being thinner in TEM, essential components such as a basement membrane, a proliferating basal layer and a differentiated epidermis were present in the epidermal layer, and resembled NNOM. The presence of involucrin, a marker for terminal differentiation [17], demonstrated that a mature epithelium was formed in TEM. The DED scaffold used in this study was chosen for its easy handling, non-contractile nature and firmness. The scaffold has to be able to withstand mechanical stress, as the oral mucosa is continuously exposed to some degree of mechanical stress [18]. We also found the presence of basement membrane components an advantage as the BM connects the epithelium to the underlying connective tissue. The seeded cells do not have to deposit these BM components themselves before developing the new epithelial layer.

Oral epithelium has a generally high number of proliferating cells. In buccal mucosa the turnover rate is approximately two to three times that of epidermis

of skin [19], and a proliferation index (PI) of 58.8 has been reported in native buccal mucosa [20]. After three weeks of culture our TEM model showed proliferating cells in the basal layer of the construct, indicating a self-renewal capacity of TEM. The PI of TEM was found to be significantly lower than that of NNOM. The lower PI of TEM was in accordance with the lower staining intensity of PAS indicating less glycogen storage in TEM than in NNOM. About the number of proliferating cells in mucosal equivalents many differences have been reported. Some studies report hyperproliferation [6, 21], slightly reduced proliferation [8] or strongly reduced to no proliferation [22]. It has been shown that the number of keratinocytes expressing Ki-67 progressively decrease over time, showing maximum proliferative capacity in the first week of culture to a few cells at the end of the third week of culture [22, 23], the latter is in accordance with our observations. The discrepancies between the studies might be a result of the differences in scaffold, which varies from DED to fibrin, and the composition of the culture media used to construct the mucosal substitutes, which are very variable between different studies.

Keratins are the predominant cytoskeletal proteins in epithelia and depending on the stage of differentiation and cell type different cytokeratins are expressed [18]. The differentiation pattern of our TEM construct was assessed by keratins 10, 13 and 19. K19 was observed in the majority of the cells of the basal layer of the epithelium of TEM, indicating a non-keratinizing nature of the epithelium of our construct. K13, a keratin present in the intermediate and superficial layer of the non-keratinizing epithelium, was weakly expressed in TEM. Interestingly, K10, an early differentiation marker of keratinizing epithelium, was found to be expressed in the superficial layer of the epidermis in TEM, which is unusual as K10 is normally expressed in the spinous layer of keratinizing epithelia. We

hypothesize that this K10 expression in the superficial layer of TEM is the result of the fact that we handle TEM gently and apply minimal mechanical trauma, meaning that this layer was never rubbed off, as would happen in native oral epithelium.

Previous studies have shown that connective tissues are responsible for the final pattern of oral epithelium differentiation [21, 24-25]. The DED scaffold used to create the TEM construct originates from donor skin, which might explain the keratin expression pattern in TEM. The use of fibroblasts taken from buccal mucosa incorporated in TEM did not lead to direct expression of K13. This suggests that the connective tissue has a higher influence on the differentiation pattern of the epidermis than the incorporated fibroblasts or the seeded keratinocytes. This finding is in line with a study by Rakhorst *et al* [7] which showed that incorporation of fibroblasts in TEM did not affect the differentiation pattern of the keratinocytes. In addition, other studies suggested that intrinsic and extrinsic factors influence the differentiation process of the epithelium and that the expression of K10 and K13 might be determined by the connective tissue whereas K19 expression might be determined by the epithelium [26]. Another explanation for the differences in keratin expression could be the location of the fibroblasts; keratin expression may vary with the distribution of fibroblasts, which are, in the TEM model, not present close to the epithelium.

In this study, the presence and distribution of fibroblasts in TEM and NNOM was assessed by vimentin staining. In TEM, fibroblasts were found to be on the lower half of the connective layer, whereas in NNOM they are dispersed homogenously in the connective layer. Several studies have shown the necessity of incorporation of fibroblasts into the scaffold for epidermal development, as

they stimulate keratinocyte proliferation and migration [7, 12-13]. In addition, the presence of fibroblasts is known to lead to a well developed epidermal layer as these cells secrete growth factors and the paracrine cross talk between keratinocytes and fibroblasts is essential for the formation of a new epidermis [27]. Even though the fibroblasts were not homogenously distributed in TEM, the construct displays a well-developed, multi-layered epidermis.

Cell-cell adhesion and attachment of the epidermal layer to the connective tissue is essential for correct functionality of NNOM and therefore also for mucosal substitutes [14, 18]. Desmosomes are complexes that link the keratinocytes to each other. An important component of desmosomes is the protein desmoglein-3. Immunohistochemistry proved that desmoglein-3 was present in TEM and a similar expression pattern was found in NNOM. This suggests that the cells were actively synthesizing components to ultimately form desmosomes for cell-cell adhesion to maintain structural stability and functionality. Hemidesmosomes are responsible for the adhesion of the epithelial layer to the basement membrane of the connective layer. Integrin α6 and β4, subunits of hemidesmosomes, were found to be similarly expressed in NNOM and TEM construct. This corresponding expression of these integrins and desmosomal protein desmoglein-3 implies that TEM constructs form a number of proteins necessary for the formation of desmosomes and hemidesmosomes in order to form a tight barrier that mimics native oral mucosa and also might mimic its functionality. However, this specific aspect remains to be proven.

The applications for TEM range from transplantation into the oral cavity to studying oral mucositis or fibrosis. These applications require further investigation. Before TEM can be used for transplantation, we have to test it *in vivo* to determine the quality of TEM post-transplantation by, for example

assessing viability and angiogenesis. To study oral mucositis the TEM construct has to be extended as no immune cells are present in the current model. Addition of immune cells such as dendritic cells or Langerhans cells would be required [28]. Or TEM could be treated with pro-inflammatory cytokines modifying TEM to create an equivalent mimicking oral mucositis [29].

In summary, the TEM construct developed in this study resembles NNOM. TEM is an oral mucosal construct composed of a multi-layered epithelium with many morphological and immunological characteristics observed in NNOM. Based on the results of this study, we are convinced that TEM holds promise for future research, for example the early mechanisms leading to oral mucositis and fibrosis.

ACKNOWLEDGEMENTS

This study was supported by a grant from Nuts-OHRA Foundation (#0802-105)

REFERENCES

1. Boyce, S.T., G.D. Warden (2002) Principles and practices for treatment of cutaneous wounds with cultured skin substitutes. Am J Surg *183(4)*: 445-456.
2. Wang, X., Y. Liu, Z. Deng, R. Dong, S. Hu, *et al.* (2009) Inhibition of dermal fibrosis in self-assembled skin equivalents by undifferentiated keratinocytes. J Dermatol Sci *53(2)*: 103-111.
3. Klausner, M., S. Ayehunie, B.A. Breyfogle, P.W. Wertz, L. Bacca, *et al.* (2007) Organotypic human oral tissue models for toxicological studies. Toxicol In Vitro *21(5)*: 938-949.
4. Lauer, G., R. Schimming (2001) Tissue-engineered mucosa graft for reconstruction of the intraoral lining after freeing of the tongue: a clinical and immunohistologic study. J Oral Maxillofac Surg *59(2)*: 169-175; discussion 175-167.
5. Izumi, K., S.E. Feinberg, A. Iida, M. Yoshizawa (2003) Intraoral grafting of an ex vivo produced oral mucosa equivalent: a preliminary report. Int J Oral Maxillofac Surg *32(2)*: 188-197.
6. Izumi, K., J. Song, S.E. Feinberg (2004) Development of a tissue-engineered human oral mucosa: from the bench to the bed side. Cells Tissues Organs *176(1-3)*: 134-152.
7. Rakhorst, H.A., S.J. Posthumus-Van Sluijs, W.M. Tra, J.W. Van Neck, G.J. Van Osch, *et al.* (2006) Fibroblasts accelerate culturing of mucosal substitutes. Tissue Eng *12(8)*: 2321-2331.

8. Kinikoglu, B., C. Auxenfans, P. Pierrillas, V. Justin, P. Breton, *et al.* (2009) Reconstruction of a full-thickness collagen-based human oral mucosal equivalent. Biomaterials *30(32)*: 6418-6425.

9. Garzon, I., M.C. Sanchez-Quevedo, G. Moreu, M. Gonzalez-Jaranay, M. Gonzalez-Andrades, *et al.* (2009) In vitro and in vivo cytokeratin patterns of expression in bioengineered human periodontal mucosa. J Periodontal Res *44(5)*: 588-597.

10. Xu, Q., K. Izumi, T. Tobita, Y. Nakanishi, S.E. Feinberg (2009) Constitutive release of cytokines by human oral keratinocytes in an organotypic culture. J Oral Maxillofac Surg *67(6)*: 1256-1264.

11. Moharamzadeh, K., I.M. Brook, R. Van Noort, A.M. Scutt, K.G. Smith, *et al.* (2008) Development, optimization and characterization of a full-thickness tissue engineered human oral mucosal model for biological assessment of dental biomaterials. J Mater Sci Mater Med *19(4)*: 1793-1801.

12. El-Ghalbzouri, A., S. Gibbs, E. Lamme, C.A. Van Blitterswijk, M. Ponec (2002) Effect of fibroblasts on epidermal regeneration. Br J Dermatol *147(2)*: 230-243.

13. El Ghalbzouri, A., E. Lamme, M. Ponec (2002) Crucial role of fibroblasts in regulating epidermal morphogenesis. Cell Tissue Res *310(2)*: 189-199.

14. Green, K.J., J.C. Jones (1996) Desmosomes and hemidesmosomes: structure and function of molecular components. FASEB J *10(8)*: 871-881.

15. Rheinwald, J.G., H. Green (1975a) Formation of a keratinizing epithelium in culture by a cloned cell line derived from a teratoma. Cell *6(3)*: 317-330.

16. Rheinwald, J.G., H. Green (1975b) Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. Cell *6(3)*: 331-343.

17. Alaminos, M., I. Garzon, M.C. Sanchez-Quevedo, G. Moreu, M. Gonzalez-Andrades, *et al.* (2007) Time-course study of histological and genetic patterns of differentiation in human engineered oral mucosa. J Tissue Eng Regen Med *1(5)*: 350-359.

18. Presland, R.B., R.J. Jurevic (2002) Making sense of the epithelial barrier: what molecular biology and genetics tell us about the functions of oral mucosal and epidermal tissues. J Dent Educ *66(4)*: 564-574.

19. Winning, T.A., G.C. Townsend (2000) Oral mucosal embryology and histology. Clin Dermatol *18(5)*: 499-511.

20. van Oijen, M.G., M.M. Gilsing, G. Rijksen, G.J. Hordijk, P.J. Slootweg (1998) Increased number of proliferating cells in oral epithelium from smokers and ex-smokers. Oral Oncol *34(4)*: 297-303.

21. Chung, J.H., K.H. Cho, D.Y. Lee, O.S. Kwon, M.W. Sung, *et al.* (1997) Human oral buccal mucosa reconstructed on dermal substrates: a model for oral epithelial differentiation. Arch Dermatol Res *289(12)*: 677-685.

22. Pena, I., L.M. Junquera, A. Meana, E. Garcia, V. Garcia, *et al.* (2010) In vitro engineering of complete autologous oral mucosa equivalents: characterization of a novel scaffold. J Periodontal Res *45(3)*: 375-380.

23. Tomakidi, P., D. Breitkreutz, N.E. Fusenig, J. Zoller, A. Kohl, *et al.* (1998) Establishment of oral mucosa phenotype in vitro in correlation to epithelial anchorage. Cell Tissue Res *292(2)*: 355-366.

24. Karring, T., N.P. Lang, H. Loe (1975) The role of gingival connective tissue in determining epithelial differentiation. J Periodontal Res *10(1)*: 1-11.

25. Mackenzie, I.C., M.W. Hill (1984) Connective tissue influences on patterns of epithelial architecture and keratinization in skin and oral mucosa of the adult mouse. Cell Tissue Res *235(3)*: 551-559.

26. Kautsky, M.B., P. Fleckman, B.A. Dale (1995) Retinoic acid regulates oral epithelial differentiation by two mechanisms. J Invest Dermatol *104(2)*: 224-230.

27. El Ghalbzouri, A., M. Ponec (2004) Diffusible factors released by fibroblasts support epidermal morphogenesis and deposition of basement membrane components. Wound Repair Regen *12(3)*: 359-367.

28. Bechetoille, N., C. Dezutter-Dambuyant, O. Damour, V. Andre, I. Orly, *et al.* (2007) Effects of solar ultraviolet radiation on engineered human skin equivalent containing both Langerhans cells and dermal dendritic cells. Tissue Eng *13(11)*: 2667-2679.

29. Tjabringa, G., M. Bergers, D. van Rens, R. de Boer, E. Lamme, *et al.* (2008) Development and validation of human psoriatic skin equivalents. Am J Pathol *173(3)*: 815-823.

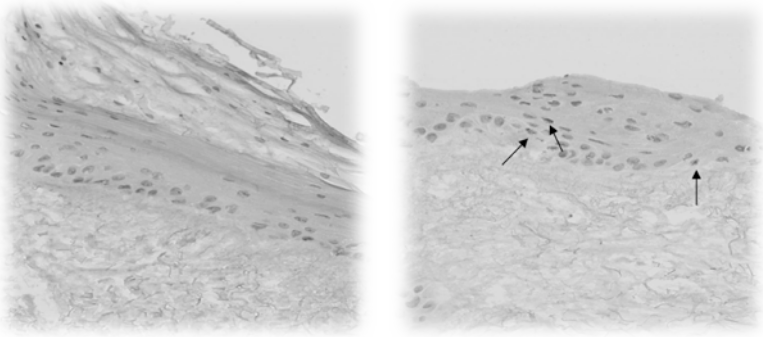
CHAPTER 3

Hypoxia preconditioning of tissue-engineered mucosa enhances its angiogenic capacity *in vitro*

Soledad Perez-Amodio, Wendy M.W. Tra, Hinne. A. Rakhorst, Steven E.R Hovius, Johan W. van Neck.

Department of Plastic and Reconstructive Surgery, Erasmus Medical Centre, Rotterdam, The Netherlands

Published in *Tissue Engineering Part A 2011; 17(11-12):1583-1593*



ABSTRACT

Improving vascularization of tissue engineered oral mucosa (TEM) is a major challenge in the field of plastic surgery. Hypoxia is a stimulator of angiogenesis through a number of mechanisms. Therefore, hypoxia is a critical parameter that can be controlled in an effort to improve angiogenesis. In the present study we studied the secretion of a number of angiogenic factors during hypoxia exposure and evaluated the effect of TEM conditioned medium on endothelial cells. TEM was constructed by seeding human oral mucosa keratinocytes and fibroblasts on a-cellular human donor skin. TEM was exposed to hypoxia during 6, 12 and 24 hours. Cellular hypoxia was assessed by immunolocalization of the hypoxia inducible factor-1 α (HIF-1 α). Secretion of vascular endothelial growth factor (VEGF), placental growth factor (PGF), tissue inhibitors of matrix metalloproteinases-1 and -2 (TIMP-1 and TIMP-2), and the activity of matrix metalloproteinase 9 (MMP-9) significantly increased during hypoxia exposure. Moreover, conditioned medium from hypoxic TEM strongly enhanced endothelial cell proliferation and migration. *In vitro* exposure of TEM to hypoxia improves its capacity to support endothelial cell proliferation and migration, which suggests that hypoxia preconditioning of TEM potentially improves angiogenic responses for *in vivo* implantation.

INTRODUCTION

The limited availability of mucosal grafts turns the reconstruction of oral defects into a major challenge in plastic surgery. Oral defects resulting, for example, after oncological resection or cleft palate reconstruction are usually covered by skin grafts or vascularized skin flaps [1]. However, skin grafts result in donor site morbidity and the grafted skin maintains perspiration and hair growth, all undesirable and uncomfortable for patients [2-4].

Tissue engineered mucosa (TEM), using keratinocytes from different sites of the oral cavity [5-6] and a variety of scaffolds [7-8], is a promising technique for reconstruction of oral defects. Recently, we and others have shown that substitutes, composed of oral mucosa keratinocytes and -fibroblasts cultured on an a-cellular dermis, possess histological and immunohistochemical characteristics close to normal oral mucosa [7, 9-10].

However, so far, intra-oral implantation of mucosal substitutes in animal models, showed a relatively poor long-term viability resulting in loss of grafts [11]. The primary reason for graft failure appears to be the lack of adequate and timely graft vascularisation [12-13]. Usually, the survival of cells within the engrafted substitute is limited by diffusion of nutrients and oxygen from the underlying wound site, an in view of the typical graft size, this diffusion mechanism is inadequate for sustained survival. One approach to increase graft acceptance is to precondition the mucosal substitute to encourage rapid vascularisation from the patient's wound bed.

The observation that low oxygen levels stimulate angiogenesis [14-15] and increase the angiogenic capacity of stromal cells [16-17] has resulted in a novel and relatively simple approach for inducing post-implantation blood vessel formation in tissue-engineered grafts by hypoxic priming before engraftment.

The aim of this study was to investigate whether hypoxia preconditioning of tissue engineered mucosa increases the secretion of important angiogenic factors, and to evaluate whether these changes affect the proliferation and migration of endothelial cells *in vitro*.

MATERIALS AND METHODS

Chemicals and culture media

Dulbecco's Modified Eagle Medium 4.5 g/l glucose (DMEM), Ham's F12 culture medium, human endothelial serum-free medium, penicillin, streptomycin, amphoceterin B, dispase, collagenase type I and trypsin/ethylenediamine-tetraacetic acid (EDTA) were purchased from Invitrogen (Breda, The Netherlands). Fetal calf serum (FCS) was purchased from PAA Laboratories (Cölbe, Germany). Bovine serum albumin (BSA), Epidermal growth factor (EGF), Keratinocyte Growth Factor (KGF) and other chemicals were purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands). Vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF-2) were purchased from Peprotech EC (London, UK). Insulin was purchased from Eli Lilly (Houten, the Netherlands).

Cell culture

Keratinocytes and fibroblasts were isolated from buccal biopsies that were obtained from three patients upon approval of the Medical Ethics Committee (# MEC + 2007-282). Biopsies were rinsed in DMEM with 100 IU/mL penicillin, 100 µg/mL streptomycin, 50 µg/mL gentamicin and 2.5 µg/mL amphoceterin B. The epidermis was separated from the dermis by overnight incubation in 2.5 mg/ml dispase solution. After rinsing the epidermal sheet with phosphate buffered saline (PBS), keratinocytes were isolated from the epidermis using

0.25 % trypsin-EDTA and the single cell suspension was seeded onto lethally irradiated 3T3 fibroblast feeder layers, according to Rheinwald and Green [18]. The keratinocyte culture medium consisted of a 3:1 mixture of DMEM and Ham's F12 medium with 5 % FCS, 1 µM hydrocortisone, 1 µM isoproterenol, 0.1 µM insulin, 1 % penicillin/streptomycin, and 1 ng/mL epidermal growth factor (EGF). Fibroblasts were isolated by mincing the dermis followed by incubation in collagenase/dispase (1.5 mg/mL / 2.5 mg/mL, respectively) solution for 2 hours at 37 ^{o}C. Afterwards, a single cell suspension was obtained by filtering the collagenase/dispase solution containing the dermis, with a 100 µm cell strainer (Sigma-Aldrich). The isolated fibroblasts were cultured in DMEM containing 10 % FCS and 1 % penicillin/streptomycin. Passages two to five were used for the experiments.

Preparation of a-cellular dermis.

Human cadaver skin, cryopreserved in 85 % glycerol, and cytomegalovirus, human immunodeficiency virus and hepatitis B negative, was obtained from the European Skin Bank (Beverwijk, the Netherlands). The epidermis was removed from the dermis after incubating in PBS with 1 % penicillin/streptomycin, 100 µg/mL gentamicin and 5 µg/mL amphoceterin B for three weeks at 37 °C [19]. The dermis was divided into 1.0 cm^2 sections and stored in DMEM supplemented with 1% penicillin/streptomycin, 0.5 % gentamycin and 5 µg/mL amphoceterin B until use.

Tissue-Engineered Mucosa

TEM was prepared as described previously [9, 20]. Briefly, 5 x 10^4 fibroblasts were seeded onto the a-cellular dermis using a centrifugal seeding technique

[21]. Subsequently, 1 x 10^6 keratinocytes were seeded into a stainless steel ring placed on top of the dermis and incubated overnight in keratinocyte culture medium. Subsequently, the cultures were fed with the same keratinocyte medium containing 2.4 x 10^{-5} M bovine serum albumin, 1 µM hydrocortisone, 1 µM isoprotenerol, 0.1 µM insulin, 1 x 10^{-5} L-carnitine, 1 x 10^{-2} L-serine, 1 µM D-L-tocopherol-acetate, and a lipid supplement containing 25 µM palmitic acid, 15 µM linoleic acid, 7 µM arachidonic acid, 1 % penicillin/streptomycin, 1 ng/mL EGF, 4 ng/mL KGF and cultured under submerged conditions for an additional two days. Thereafter, the cultures were lifted to the air/liquid (A/L) interface and cultured for 14 days in the same medium except that serum was omitted, the concentration of linoleic acid was increased to 30 µM and 100 mg/ml ascorbic acid phosphate, 1 ng/mL EGF and 4 ng/mL KGF were added. The medium was changed twice a week.

Oxygen systems

The standard oxygen level was defined as the pO2 which exists in a standard, conventional, humidified tissue culture incubator at 37 °C (20 %). The low (1.5 %) oxygen system was established in a humidified environmental chamber set at 37 °C. This incubator uses an oxygen analyzer to monitor and maintain the selected chamber oxygen concentration. This oxygen concentration was maintained with a calibrated gas mixture consisting of 95 % nitrogen and 5 % carbon dioxide.

After 2 weeks of culture at the air/liquid media, in normoxic conditions, the media were changed to air/liquid media without EGF and KGF and incubated for 24h in normoxic conditions, prior to continuing incubation under hypoxic

conditions for 6, 12 and 24 h. Parallel tissues were maintained for identical time periods under normoxia as controls. In another experiments, hypoxia exposure of TEM was extended up to 48 h.

Collection of TEM-conditioned media and histological preparation

Conditioned media from TEM exposed to hypoxia/normoxia for 6, 12, and 24 h were collected, centrifuged at 400 *g* for 5 min at 4 °C and stored at -80 °C until further analysis. Hereafter, TEM was harvested and prepared for histological analysis. For histological determinations, samples were snap frozen with liquid nitrogen for cryo-sectioning. Sections (6 µm) were cut and stained with Haematoxylin and Eosin (H&E) (Klinipath, Duiven, the Netherlands). Stained sections were viewed using a light microscope with an Olympus eyepiece micrometer (A × 0071, 20.4 mm square; Olympus, Tokyo, Japan).

Immunohistochemistry

For Ki-67 and HIF-1 α staining, cryo sections were fixed for 10 min with acetone, washed with 3 changes of PBS and blocked with 10% normal goat serum (NGS) and 10% normal human plasma (Sanquin, Rotterdam, the Netherlands). Tissue sections were incubated with anti-Ki67 (1:200) (DAKO, Heverlee, Belgium) or anti HIF-1α (1:100) (Novus Biologicals, Littleton, CO) in PBS with 10% normal human plasma (Sanquin, Rotterdam, the Netherlands) for 1 hour at room temperature (RT). Slides were washed and incubated with goat anti-mouse or goat anti-rabbit biotin-labelled antibodies (DAKO) in 2% NGS, 2% normal human plasma and 5% BSA (Sigma) in PBS for 30 minutes at RT, followed by incubation with Streptavidin-ABC-HRP (DAKO) in PBS for 30 minutes at RT. For visualization of the Ki-67 and HIF-1α positive cells, the slides were incubated for 5 minutes at

RT with a substrate which consisted of 5 % 3,3'-Diaminobenzidine tetra hydrochloride hydrate (DAB, Sigma-Aldrich), PBS and 30 % H_2O_2. After washing the slides thoroughly with tap water, sections were counterstained with Haematoxylin for background visualization and coverslipped with Vectamount mounting media and examined with a light microscope. Control slides were incubated with an irrelevant mouse IgG.

Apoptosis assay

To determine the number of apoptotic cells, The DeadEnd Colorimetric TUNEL assay kit (Promega, Madison, WI) was used. Cryo-sections were fixed and stained according to the manufacturer's instructions. Sections were counterstained with Haematoxylin for background visualization and coverslipped with Vectamount mounting media and examined with a light microscope. Control sections were incubated with PBS.

Quantification of hypoxic cells, cellular proliferation and apoptosis

Immunostaining for HIF-1α, Ki67, and apoptotic assay were performed on tissues from three individual experiments from each hypoxic and normoxic time point. Quantification of hypoxic cells was done by counting the number of HIF-1α-positive cells within twelve random microscopic fields (magnification 200 x). The amount of HIF-1α-positive cells was expressed as a percentage of the total number of cells. For quantification of proliferative cells, the number of Ki67-positive basal cells within twelve random microscope fields (final magnification 200 x) was manually counted. The number of Ki67 positive nuclei from the total number of basal cells (x 100 %) was used to determine the proliferation index. For quantification of apoptotic cells, the number of apoptotic nuclei found

within the length of the entire epidermal (basal layer) tissue section was manually counted. Two observers, who were blinded to the conditions, carried out the quantification independently.

ELISA assay of conditioned medium

Concentration of TEM-secreted angiogenic factors in the conditioned medium was measured using commercially available sandwich ELISA kits according to the manufacturer's instructions (VEGF, PlGF, bFGF, HGF, TIMP-1, and TIMP-2; R&D Systems, Abingdom, UK). Results are expressed as ng or pg/cm^2 tissue with each sample consisting of 4 ml supernatant derived from 1 cm^2 tissue.

Zymography for MMP-9 and MMP-2

Gelatinolytic proteinases in TEM conditioned medium were assayed by gelatin-substrate zymography. Aliquots of 15µl of conditioned medium were diluted 1:1 with sample buffer (0.1 M Tris-HCl, 4 % SDS, 20 % glycerol, 0.005 % bromophenol blue, and 10 mM EDTA) and electrophoresed through a 10 % polyacrylamide gel containing 2 % gelatin as substrate. Following SDS-PAGE, SDS was removed from the gels by 2.5 % (v/v) Triton-X-100 washes (2 × 20 min), and the gels incubated in assay buffer (50 mM Tris-HCl, 1 % Triton X-100, and 5 mM $CaCl_2$). After an overnight incubation at 37 °C, the gel was stained with 0.1 % Coomassie Brilliant Blue and cleared with 7 % acetic acid and 5 % methanol. MMP-2 and MMP-9 were visualized as unstained bands. Gelatinolytic activity was detected as clear bands against the aqua-blue stained gelatin background. As a marker for electrophoretic mobility of gelatinases in zymograms, the pro- and active forms of MMP-2 and MMP-9 (Calbiochem, La Jolla, CA) were used.

Gels were scanned by Kodak Image Station 440CF (Kodak, Rochester, NY) and the relative intensity of each band was quantified using the NIH ImageJ software (http://rsb.info.nih.gov/ij/).

Human umbilical vein endothelial cells (HUVEC) proliferation assay

Isolated HUVEC at passage 4 were seeded at a density of 3 x 10^4 cells/well in 48-well plates in endothelial growth medium (EGM) consisting of human endothelial-SFM supplemented with 20 % FCS, 10 % human serum, 20 ng/ml FGF-2, and 100 ng/ml EGF for 24h. The next day the cells were starved with HG-DMEM supplemented with 0.5 % FCS for 24 h. Cells were washed with PBS and treated with conditioned medium from TEM exposed to hypoxia/normoxia (1:1 diluted with HG-DMEM supplemented with 0.5 % FCS). Control HUVEC cultures received EGM and HG-DMEM supplemented with 0.5 % FCS.

Cells were harvested after 48h with 0.05 % trypsin/EDTA, resuspended in medium and stained with 0.4 % trypan blue. Cell counts were done in triplicate in a Neubauer chamber. Each assay was performed in duplicate. Data from three independent experiments was pooled for statistical analysis.

HUVEC migration assay

Migration assays were performed in transwell plates (Costar, Cambridge, MA) of 6.5 mm filters with a pore size of 8µm. The filters were coated with growth factor-reduced Matrigel (Becton Dickinson Labware, Bedford, MA) for 30 min at 37 °C. HUVEC at passage 4 were seeded at a density of 5 x 10^4 cells in the upper compartment in 100 µl of EGM. The same medium (600 µl) was added to the lower compartment. Cells were allowed to adhere for 2 h, and then the medium in the upper and lower compartment was replaced with HG-DMEM

supplemented with 0.5 % FCS. The inserts were transferred to new 24-well plates containing 600 µl of conditioned medium from TEM who has been exposed to hypoxia/normoxia; EGM and HG-DMEM supplemented with 0.5 % FCS in the lower compartment. The cells were allowed to migrate for 6 h at 37 °C. The non-migrated cells were removed from the upper surface by scraping with a cotton swab. Migrated cells were fixed in absolute methanol for 2 min at room temperature and stained with Giemsa (1:20) (Sigma-Aldrich) for 15 minutes. Quantification of migrating cells on the lower surface of each filter was done by counting five random microscopic fields under a light microscope (final magnification 200 x). Each assay was performed in duplicate. Data from three independent experiments was pooled for statistical analysis. HUVEC proliferation and migration assays are schematically depicted in Fig. 1.

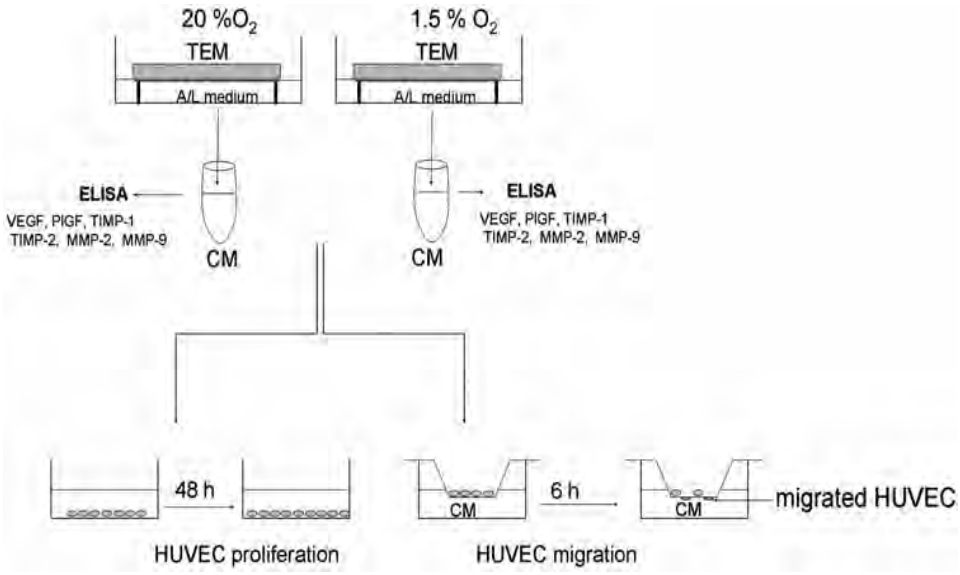


Figure 1. Schematic representation of HUVEC proliferation and migration assay in the presence of conditioned medium (CM) obtained from TEM exposed to normoxia or hypoxia. HUVEC, human umbilical vein endothelial cell; TEM, tissue-engineered oral mucosa.

Effect of VEGF on HUVEC proliferation and migration

The role of VEGF on the enhanced proliferation and migration of HUVEC observed with conditioned medium from hypoxic TEM was analyzed. Therefore, proliferation and migration assays were performed using conditioned medium from TEM exposed to normoxia supplemented with 0.6 ng/ml, an amount equal to the maximum amount of VEGF measured in conditioned medium from TEM exposed to hypoxia for 24 h, conditioned medium from TEM exposed to hypoxia for 24 h or EGM supplemented with 20 pM FGF and 10 pM VEGF.

Statistical analysis

The data are presented as the mean ± standard error of the mean. Statistical analyses were conducted using one-way analysis of variance (ANOVA) using GraphPad Prism software (San Diego, CA). Statistical difference was defined as $p \leq 0.05$. Comparisons between group means were made with the Tukey-Kramer test for multiple comparisons.

RESULTS

Confirmation of cellular hypoxia

To confirm that TEM metabolically responded to lowered oxygen conditions, we assessed whether cells in TEM exposed to 1.5 % O_2 activated HIF-1α, a central regulator of the cellular response to hypoxia and ubiquitously expressed in mammalian cells and degraded when exposed to normoxia. As illustrated in Fig. 2A-D, HIF-1α-positive cells were detected in the hypoxic samples. Exposure to hypoxia for 12 and 24 resulted to a 2- and 2.6-fold increase in the number of

HIF-1 alpha-positive cells, respectively, as compared to the normoxic samples (Fig. 2E).

No obvious changes in the morphology of the epidermis were observed at the light microscope level (Fig. 3A, B). At 24 h of oxygen deprivation, a displacement of nuclei was observed in a number of cells within all living cells of the epidermis. Nuclear material was shifted to the perimeter of the nucleus, resulting in vacuoles.

In all samples, Ki67-positive cells (proliferative cells) were found in the basal layer (Fig. 3C, D). Compared with normoxic controls, no changes in keratinocyte proliferation were observed after tissues were exposed to hypoxia for up till 24 hours (data not shown). As shown in figures 3 E, F few apoptotic cells were detected in TEM exposed to hypoxia. No differences in the number of apoptotic cells were found in TEM exposed to hypoxia compared to the normoxic controls (data not shown).

In order to analyse whether prolonged hypoxia exposure periods may affect the viability of TEM, in another experiments hypoxia exposure was extended for up to 48 h. Longer periods of hypoxia resulted in a 3.5-fold increase in the number of HIF-1α-positive cells when comparing to the normoxic controls. In addition, TEM showed cells with pyknotic nuclei as well as alterations in epidermal attachment to the underlying connective tissue that was not found at 24 h of hypoxia. Exposure of TEM to hypoxia for 48h resulted in a significant decrease in the number of proliferating cells compared to 24 h of hypoxia exposure (TEM exposed to hypoxia for 24h, 36.8 ± 2.3; TEM exposed to hypoxia for 48h, 12.5 ± 1.4; mean ± SEM, $p < 0.001$).

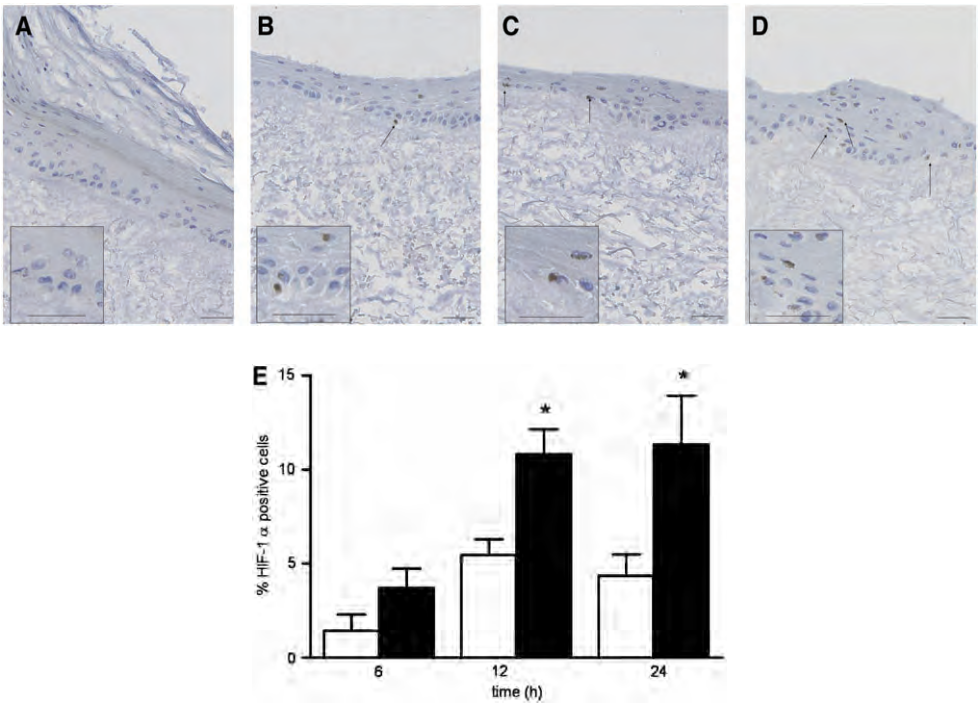


Figure 2. Confirmation of cellular hypoxia. TEM was exposed to (A) normoxia and hypoxia (B) for 6 h, (C) 12 h, and (D) 24 h, and cross sections were immunostained with anti-human HIF-1α antibody (brown). Scale bar = 50 µm. HIF-1α-expression was found in the epidermis of TEM exposed to hypoxia (arrows). (E) The number of HIF-1α-positive cells was significantly higher in TEM exposed to hypoxia (closed bars) for 12 and 24 h compared to TEM exposed to normoxia (open bars). Data are expressed as mean ± SEM in TEM from different donors. *$p < 0.05$. HIF, hypoxia-inducible factor.

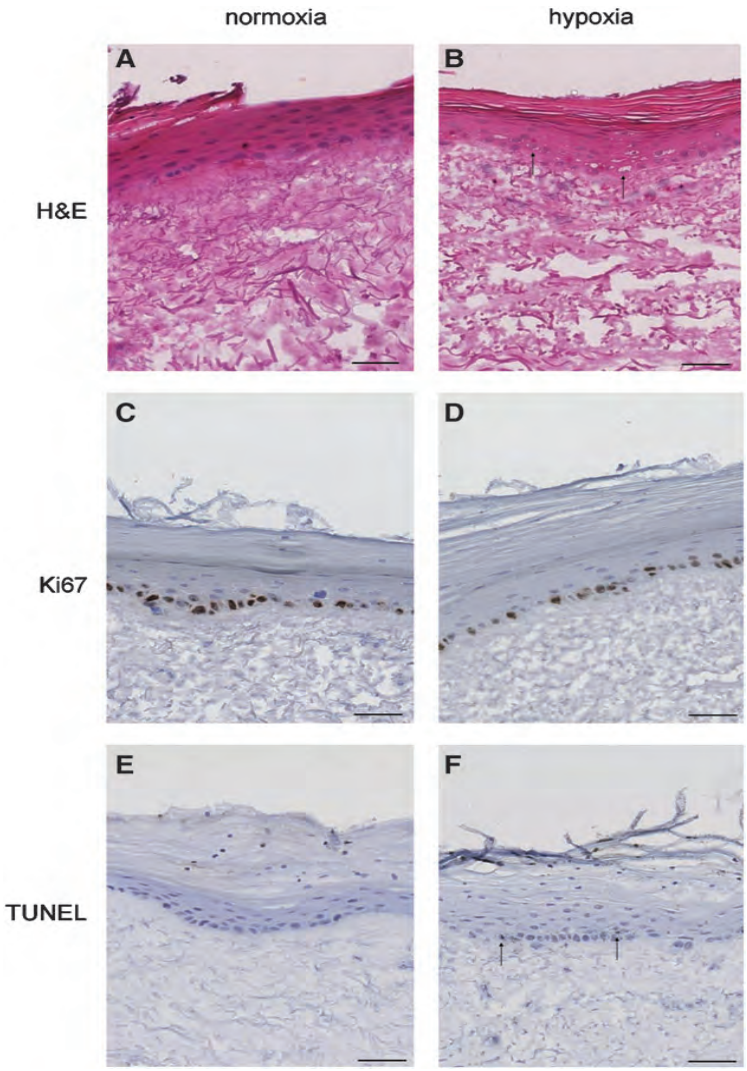


Figure 3. Histological appearance of TEM exposed to (A) normoxia and (B) hypoxia for 24 h (hematoxylin and eosin staining). Note a displacement of nuclei in some cells in TEM exposed to hypoxia (arrows). (C, D) Cell proliferation was observed using a antibody against Ki67 (brown). TUNEL staining (E, F) showed that positive cells were most prevalent in the basal cells of the epidermis of TEM exposed to hypoxia (arrows) (F). Scale bar = 50 µm.

Assessment of angiogenic factors in TEM exposed to hypoxia

To study the effect of hypoxia on the angiogenic capacity of TEM, the secretion of a number of angiogenic factors was measured in the conditioned media of TEM. Hypoxia exposure of TEM for 12 and 24 h resulted in a 35% increase in the amount of secreted VEGF compared to the normoxic controls. Hypoxia also increased the secretion of PlGF (2.1-fold), TIMP-1 (1.6-fold) and TIMP-2 (2.0-fold) in TEM after 24 h of incubation (Fig. 4). If present, the amount of HGF and bFGF in the conditioned medium were below the detection limits of the ELISA used. None of the angiogenic factors were detected in unconditioned culture media (data not shown).

Since longer hypoxia periods may influence VEGF production by TEM, the secretion of this growth factor was analyzed in conditioned medium obtained after 48 h of hypoxia. Exposing TEM to hypoxia for up to 48 h resulted in a slightly increase in the levels of secreted VEGF compared to the amounts of VEGF secreted at 24 h hypoxia exposure however, these differences proved not to be statistically significant (secreted VEGF after 48 h of hypoxia, 262.6 ± 38.6 pg/cm^2 tissue; secreted VEGF after 24 h of hypoxia, 168.1 ± 2.0 pg/cm^2 tissue). In addition, HGF and bFGF were not detected after exposing TEM to 48 h of hypoxia (data not shown).

Given the elevated levels of TIMPs in the medium of TEM exposed to hypoxia, we also analyzed the activity of MMPs in our cultures by gelatin zymography. Fig. 5 A shows a representative zymography of conditioned medium from TEM exposed to normoxia/hypoxia that are quantified in Fig. 5 B-E.

Zymography gels show that TEM produces gelatinase activity in bands at 92, 82, 72 and 68 kDa (Fig. 5). The bands at 92 and 82 kDa correspond to the pro- and the active forms of MMP-9, respectively, and the bands at 72 and 66 kDa to the

pro-and the active forms of MMP-2 (Fig. 5A). The stimulating effect of hypoxia on the level of MMP-9 activity was particularly evident in media from TEM exposed to hypoxia for 12 and 24h (Fig. 5A, B). Higher levels of MMP-2 were also found in TEM exposed to hypoxia for 24 h compared to the normoxic controls (Fig. 5 D, E).

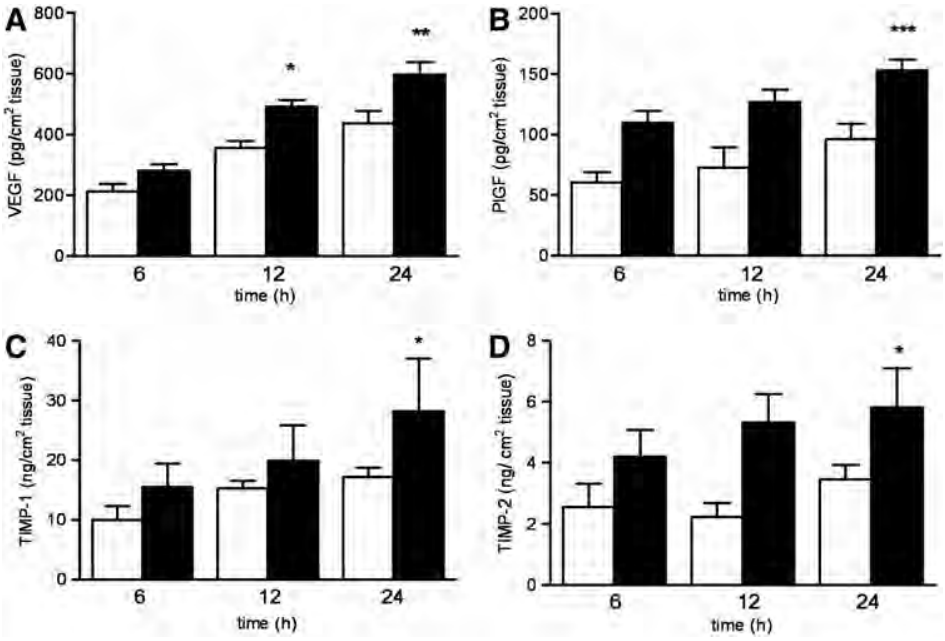


Figure 4. Release of angiogenic factors by TEM. The secretion of (A) vascular endothelial growth factor (VEGF), (B) placental growth factor (PlGF), tissue inhibitors of matrix metalloproteinase-1 (C) (TIMP-1), and -2 (D) (TIMP-2) by TEM exposed to normoxia (open bars) and hypoxia (closed bars) for 6, 12, and 24 h. Conditioned medium of TEM derived from three different donors were assayed in duplicate. Data are expressed as mean expression ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

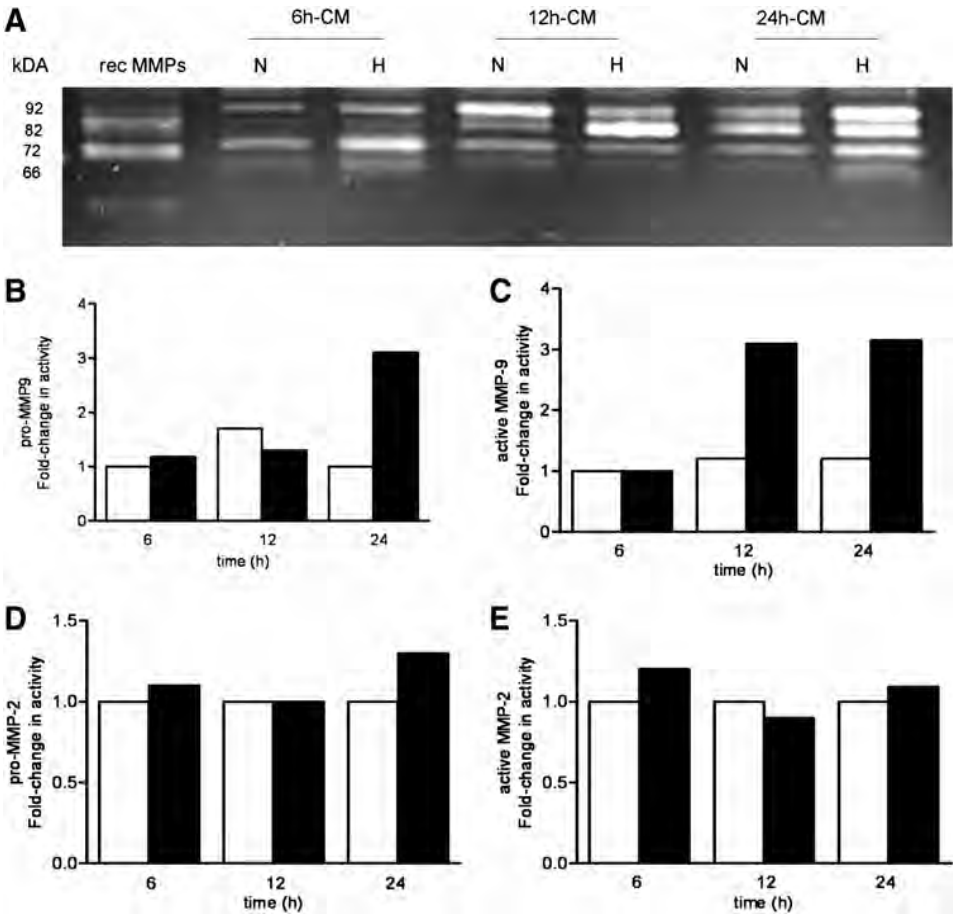


Figure 5. Increased MMP-2 and MMP-9 activity under hypoxic conditions TEM was exposed to normoxia (N) or hypoxia (H) for 6, 12, and 24 h. The medium was collected and MMP-9 and MMP-2 activities were measured by gelatin zymography. (A) Gelatin zymograms showing increased MMP-2 (72 and 68 kDa; inactive proenzyme form and active form, respectively) and an increased level of MMP-9 (92 and 82 kDa inactive proenzyme form and active form, respectively). (B-C) Quantification of MMP-9 activity. (D-E) Quantification of MMP-2 activity. The fold-change in MMP-9 and MMP-2 activities under hypoxia (closed bars), as assessed by densitometry compared to normoxia (open bars). Conditioned medium of TEM derived from three different donors were assayed in triplicate. MMP, matrix metalloproteinase.

Effects of hypoxic TEM-conditioned medium on endothelial cell numbers and migration

To verify the biological activity of the factors secreted in the conditioned medium, the effects of conditioned medium derived from hypoxic TEM on human endothelial cells growth were analyzed. Conditioned medium from hypoxic TEM, collected at 6 and 12 h, equally stimulated endothelial cell growth. This in contrast to conditioned medium derived from 24h hypoxic TEM that resulted in higher numbers of endothelial cells compared to medium conditioned from TEM exposed to normoxia (Fig. 6 A). Besides proliferation, also migration of endothelial cells is an important aspect in angiogenesis. Therefore, we analyzed whether conditioned medium from hypoxic TEM was capable of attracting endothelial cells *in vitro*. Conditioned medium from TEM exposed to hypoxia for 6 and 12 h had no significant effect on the attraction of endothelial cells. However, conditioned medium from 24h hypoxic TEM increased the migration of endothelial cells 2-fold compared to conditioned medium exposed to normoxia (Fig. 6 B). Conditioned medium collected from TEM exposed to normoxia at the different time points did not affect endothelial cell migration.

Since VEGF is thought to be the primary cytokine responsible for initiating angiogenesis, we examined whether the secreted VEGF levels of TEM exposed to hypoxia influenced HUVEC proliferation and migration by performing these assays with normoxic TEM-conditioned medium supplemented with VEGF and hypoxic TEM-/normoxic TEM-conditioned medium. When cultured with normoxic TEM-conditioned medium supplemented with VEGF, no additional effects on HUVEC proliferation or migration over the controls were observed

(Fig. 6 C,D). Fig. 6 C, D also shows that culturing with EGM supplemented with VEGF and FGF significantly increased the number of proliferating and migration HUVEC.

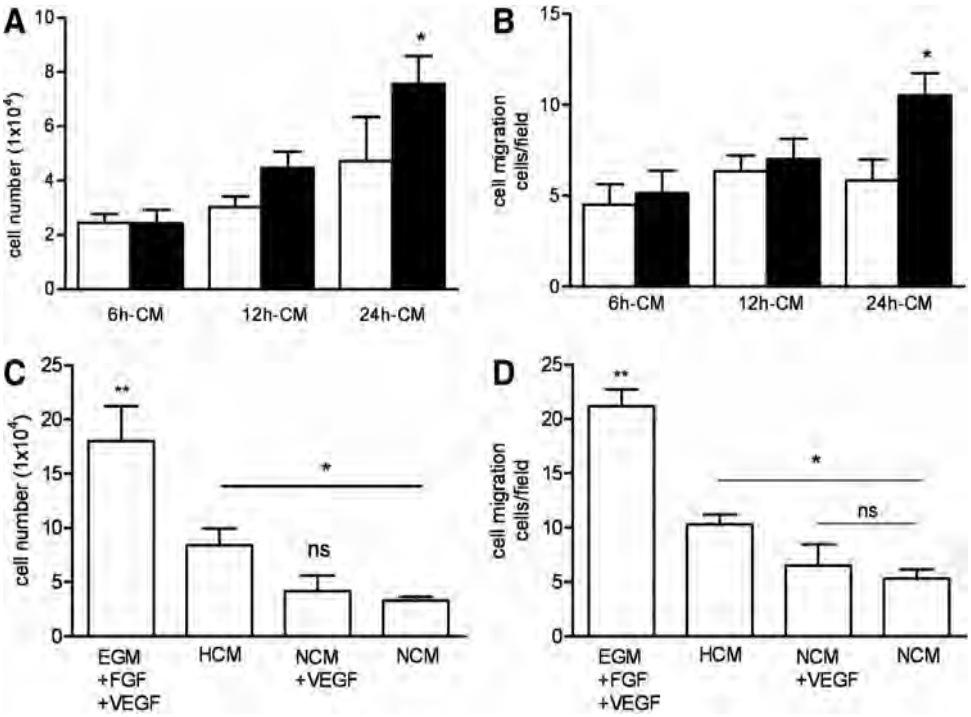


Figure 6. (A) Endothelial cell proliferation after 48 h of incubation with conditioned medium from TEM exposed to normoxia (open bars) and hypoxia (closed bars) collected after 6 (6h-CM), 12 (12h-CM), and 24 h (24h-CM) of incubation. The number of endothelial cells was significantly higher after incubation with conditioned medium obtained from TEM exposed to normoxia for 24 h compared to the corresponding normoxic conditioned medium. (B) Migration of endothelial cells toward conditioned medium obtained from normoxia (open bars) and hypoxia (closed bars) collected after 6 (6h-CM), 12 (12h-CM), and 24 h (24h-CM). The number of migrating cells was significantly higher toward conditioned medium from TEM exposed to hypoxia for 24 h. (C) Endothelial cell proliferation after 48 h of incubation with EGM supplemented with FGF and VEGF, conditioned medium from TEM exposed to hypoxia for 24

h, conditioned medium from TEM exposed to normoxia supplemented with 0.6 ng/mL VEGF, and conditioned medium from TEM exposed to normoxia. Addition of VEGF to conditioned medium from TEM exposed to normoxia resulted in lower numbers of endothelial cells compared to conditioned medium from hypoxic TEM. The number of endothelial cells was higher in the presence of EGM supplemented with FGF and VEGF compared to conditioned medium from hypoxic TEM. (D) Migration of endothelial cells toward EGM supplemented with FGF and VEGF, conditioned medium from TEM exposed to hypoxia for 24h, conditioned medium from TEM exposed to normoxia supplemented with 0.6 n/mL VEGF, and conditioned medium from TEM exposed to normoxia. Migration of endothelial cells toward conditioned medium from normoxic TEM supplemented with 0.6 ng/mL was lower compared to conditioned medium from hypoxic TEM. The number of migrating cells was higher in the presence of EGM supplemented with FGF and VEGF compared to conditioned medium from hypoxic TEM, Conditioned medium of TEM derived from three different donors were assayed in duplicate. Data are expressed as mean expression ± SEM. *p < 0.05, **p < 0/001, ns: not significant. FGF, fibroblast growth factor; EGM, endothelial growth medium.

DISCUSSION

In the present study, we demonstrated that hypoxia preconditioning of TEM enhances the secretion of angiogenic factors increasing the proliferation and migration of endothelial cells *in vitro.* The normal morphology preserved by the construct and the absence of basal cell degeneration indicate that no profound structural changes occurred by exposing TEM to short periods of low oxygen concentrations.

This capacity to adapt to external hypoxic stress would be specifically important at sites of implantation, where the ingrowth of vessels into the engineered construct requires days to weeks, and hence, an inadequate supply of oxygen to cells after transplantation must be assumed [22].

The secretion of the angiogenic factor VEGF by oral mucosal substitutes has been reported before [23-24]. To our knowledge however, this is the first study

in which the secretion of angiogenic factors by tissue engineered oral mucosa, other than VEGF, is analyzed. Several studies have shown that hypoxia strongly stimulates the secretion of angiogenic factors in a variety of cells such as adipose derived stromal cells, smooth muscle cells, human umbilical vein endothelial cells and endothelial progenitor cells [14, 17, 25]. We therefore analyzed the secretion of several factors known to influence angiogenesis. We found significantly higher levels of VEGF, PlGF, TIMP-1 and TIMP-2 in the conditioned medium of TEM exposed to hypoxia compared to conditioned medium of TEM cultured under normoxia.

Both VEGF and PlGF are known to stimulate endothelial cell proliferation and induce angiogenesis [26-27] whereas TIMP-1 and TIMP-2 are known to regulate the activities of matrix metalloproteinases (MMPs) [28-29], and to modulate the growth, differentiation and migration of cells [30]. The fact that high levels of TIMP-1 and TIMP-2 were found in the conditioned medium of TEM exposed to hypoxia, prompted us to investigate whether active MMPs were present in this media. Zymography analysis indicated increased MMP-2 and MMP-9 levels in conditioned media of TEM exposed to hypoxia compared to the normoxic controls. TIMPs and MMPs play an important role in angiogenesis since they activate and modify angiogenic growth factors and cytokines, remove matrix proteins needed for endothelial cell migration and create space in the matrix to allow generation of endothelial cell tubules [31-33].

The above-described results suggested that hypoxia- preconditioned TEM might have a higher angiogenic capacity than normoxic TEM. To investigate this further, we obtained conditioned medium from TEM exposed to hypoxia for 6, 12, and 24 hours and found that 24 hour-hypoxic medium significantly increased the number of endothelial cells *in vitro*, compared to normoxic

medium. Since not only proliferation of endothelial cells but also its migration is important for angiogenesis, we assessed HUVEC migration toward the medium conditioned by hypoxic and normoxic TEM. Our results indicate that conditioned medium obtained from TEM exposed to hypoxia for 24 h stimulated endothelial cells migration *in vitro*.

Experiments using the same concentrations of VEGF as the concentrations present in the hypoxic conditioned medium to induce HUVEC proliferation and migration, showed lower numbers of proliferating and migrating HUVEC than the ones found by using hypoxic conditioned medium. This finding indicates that the increased secretion of VEGF upon hypoxia exposure did not only account for the increased HUVEC proliferation and migration and suggests that other factors among which, PlGF, TIMP-1 and TIMP-2 may play an important role. These results are in line with previous work that showed that soluble factors derived from hypoxic fibroblasts, other than VEGF and bFGF are necessary to induce angiogenesis *in vitro* [34]. Also, in agreement with Griffith and George [34], bFGF seemed not to play a role in the enhanced HUVEC proliferation and migration observed in the presence of conditioned medium from hypoxic TEM since its production was not upregulated upon hypoxia.

We confirmed that TEM was exposed to hypoxia by the detection of HIF-1 α protein, an ubiquitous expressed mediator of the cellular response to hypoxia in mammalian cells, which plays a pivotal role in the anaerobic metabolism, angiogenesis, erythropoyesis, and vasodilatation [35]. The HIF-1 α protein is degraded when exposed to oxygen through its oxygen-dependent degradation domain. Under hypoxic conditions, the HIF-1 α protein is translocated into the nucleus where it dimerises with HIF-1 β to its active form [36-37-38]. In our study, significantly higher numbers of abundant HIF-1α-positive cells were

detected when TEM was exposed to hypoxia for 12 and 24h. Interestingly, HIF-1α-positive cells were also observed in the normoxic samples. Oxygen concentrations have been seen to decline rapidly from the exterior to the interior of grafts [39-40] thus it is likely that at least some cells in the normoxic engineered mucosa construct are hypoxic.

Oxygen tension is known to influence proliferation of cells [41]. Incubation for up to 24 hours under hypoxic conditions did not influence cell proliferation in TEM, however, longer incubation periods resulted in a decrease in proliferative capacity as well as in morphological alterations of the epidermis of the engineered mucosa construct. These observations are supported by previous studies culturing skin substitutes at low oxygen concentrations [42-43] and indicate that 24 hours is the maximal period to expose TEM to hypoxia. The oxygen tension we have used in our work was based on studies reporting increases in VEGF production in keratinocytes and fibroblasts that were exposed to low oxygen concentrations [44-46]. It could be that the oxygen tension that was chosen for this study is not the most optimal, however, based on our results we can conclude that exposure of engineered mucosal substitutes to 1.5% of oxygen for 24 hours upregulate the secretion of angiogenic mediators and stimulate endothelial proliferation and migration *in vitro*. Whether other oxygen concentrations may improve the angiogenic capacity of engineered mucosa constructs need to be clarified in future studies.

Taken together, our results show that engineered mucosa constructs produce soluble mediators that are upregulated under hypoxia conditions and that these mediators are responsible for angiogenesis related events *in vitro*. Since VEGF, PGF, TIMP-1, TIMP-2, MMP-2 and MMP-9 were upregulated under hypoxia, it is likely that these mediators play a role in the enhanced proliferation and

migration of endothelial cells observed in our study. This is in agreement with studies directed at improving tissue regeneration *in vivo*. In these studies, hypoxia preconditioning of human mesenchymal stem cells (hMSC) promoted post-implantation blood vessel formation [47-48]. Although in our study hypoxia exposure of TEM shows to be beneficial for endothelial cell proliferation and migration *in vitro*, higher effects were found when stimulating with endothelial medium supplemented with VEGF and bFGF. This observation has to be considered when pre-treating engineered mucosa constructs with hypoxia prior implantation

Clinical applications of *in vitro* engineered oral mucosa tissue are still limited. For engineered mucosa tissue constructs to become a viable option for future clinical use, they must be able to survive *in vivo*. For this, the presence of an adequate blood supply is necessary. Several approaches to improve vascularization of tissue-engineered constructs have been used, including incorporation of dermal microvascular endothelial cells [49-52] or adipose derived stromal cells [53], and overexpression of VEGF in modified engineered substitutes [54]. Combining such techniques with hypoxia preconditioning to hypoxia may improve the survival of oral mucosa engineered constructs in vivo.

ACKNOWLEDGEMENTS

We are grateful to Joost Rens (Department of Experimental Surgical Oncology, Erasmus MC) for providing the hypoxic culture facilities and to Dr. G. van Osch (Departments of Orthopaedics & Otorhinolaryngology, Erasmus MC) for her comments regarding this manuscriptThis work was supported by the Vanderes Foundation (contract # 155).

AUTHOR DISCLOSURE STATEMENT

No competing financial interest exist

REFERENCES

1. Neligan, P.C., P.J Gullane, R.W. Gilbert (2003) Functional reconstruction of the oral cavity. World J Surg *27(7)*: 856-62.
2. Avery, C.M., J. Pereira, A.E. Brown (2001) Suprafascial dissection of the radial forearm flap and donor site morbidity. Int J Oral Maxillofac Surg *30(1)*: 37-41.
3. Graham, B., P. Adkins, L.R. Scheker (1992) Complications and morbidity of the donor and recipient sites in 123 lateral arm flaps. J Hand Surg Br *17(2)*: 189-92.
4. Kimata, Y., K. Uchiyama, S. Ebihara, M. Saikawa, R. Hayashi, *et al.* (2000) Postoperative complications and functional results after total glossectomy with microvascular reconstruction. Plast Reconstr Surg *106(5)*: 1028-35.
5. Cho, K.H., H.T. Ahn, K.C. Park, J.H. Chung, S.W. Kim, *et al.* (2000) Reconstruction of human hard-palate mucosal epithelium on de-epidermized dermis. J Dermatol Sci *22(2)*: 117-24.
6. Yoshizawa, M., S.E. Feinberg, C.L. Marcelo, V.M. Elner (2004) Ex vivo produced human conjunctiva and oral mucosa equivalents grown in a serum-free culture system. J Oral Maxillofac Surg *62(8)*: 980-8.
7. Ophof R., R.E. van Rheden, H.J. Von den, J. Schalkwijk, A. M. Kuijpers-Jagtman (2002) Oral keratinocytes cultured on dermal matrices form a mucosa-like tissue. Biomaterials *23(17)*: 3741-8.
8. Moharamzadeh, K., I.M. Brook, R. Van Noort, A.M. Scutt, M.H. Thornhill (2007) Tissue-engineered oral mucosa: a review of the scientific literature. J Dent Res *86(2)*: 115-24.

9. Rakhorst, H.A., S.J. Posthumus-Van Sluijs, W.M. Tra, J.W. Van Neck, G.J. Van Osch, *et al.* (2006) Fibroblasts accelerate culturing of mucosal substitutes. Tissue Eng *12(8)*: 2321-31.

10. Izumi K., J. Song, S.E. Feinberg (2004) Development of a tissue-engineered human oral mucosa: from the bench to the bed side. Cells Tissues Organs *176(1-3)*: 134-52.

11. Ophof R., J.C. Maltha, A.M. Kuijpers-Jagtman, J.W. Von den Hoff (2008) Implantation of tissue-engineered mucosal substitutes in the dog palate. Eur J Orthod *30(1)*: 1-9.

12. Boyce S.T., A.P. Supp, M.D. Harriger, D.G. Greenhalgh, G.D. Warden (1995) Topical nutrients promote engraftment and inhibit wound contraction of cultured skin substitutes in athymic mice. J Invest Dermatol *104(3)*: 345-9.

13. Sumi Y., K.I. Hata, Y. Sawaki, H. Mizuno, M. Ueda (1999) Clinical application of cultured oral epithelium for palatal wounds after palatoplasty: a preliminary report. Oral Dis *5(4)*: 307-12.

14. Kelly B.D., S.F. Hackett, K. Hirota, Y. Oshima, Z. Cai, *et al.* (2003) Cell type-specific regulation of angiogenic growth factor gene expression and induction of angiogenesis in nonischemic tissue by a constitutively active form of hypoxia-inducible factor 1. Circulation research *93(11)*: 1074-81.

15. Kroon M.E., P. Koolwijk, B. van der Vecht, V.W. van Hinsbergh (2001) Hypoxia in combination with FGF-2 induces tube formation by human microvascular endothelial cells in a fibrin matrix: involvement of at least two signal transduction pathways. Journal of cell science *114(Pt 4):* 825-33.

16. Thangarajah, H., D. Yao, E.I. Chang, Y. Shi, L. Jazayeri, *et al.* (2009) The molecular basis for impaired hypoxia-induced VEGF expression in diabetic tissues. Proceedings of the National Academy of Sciences of the United States of America *106(32):* 13505-10.
17. Thangarajah, H., I.N. Vial, E. Chang, S. El-Ftesi, M. Januszyk, *et al.* (2009) IFATS collection: Adipose stromal cells adopt a proangiogenic phenotype under the influence of hypoxia. Stem cells *27(1)*: 266-74.
18. Rheinwald, J.G., H. Green, (1975) Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. Cell *6(3)*: 33143.
19. Ponec M., A. Weerheim, J. Kempenaar, A.M Mommaas, D.H. Nugteren (1988) Lipid composition of cultured human keratinocytes in relation to their differentiation. J Lipid Res *29(7)*: 949-61.
20. Rakhorst, H.A., W.M. Tra, S.T. Posthumus-Van Sluijs, S.E. Hovius, P.C. Levendag, *et al.* (2006) Quantitative analysis of radiation-induced DNA break repair in a cultured oral mucosal model. Tissue Eng *12(12)*: 3395-403.
21. El Ghalbzouri, A., E. Lamme, M. Ponec (2002) Crucial role of fibroblasts in regulating epidermal morphogenesis. Cell and tissue research *310(2)*: 189-99.
22. Malda, J., T.J. Klein, Z. Upton (2007) The roles of hypoxia in the in vitro engineering of tissues. Tissue Eng *13(9)*: 2153-62.
23. Nakanishi, Y., K. Izumi, M. Yoshizawa, C. Saito, Y. Kawano, *et al*. The expression and production of vascular endothelial growth factor in oral mucosa equivalents. Int J Oral Maxillofac Surg *36(10)*: 928-33.

24. Xu, Q., K. Izumi, T. Tobita, Y. Nakanishi, S.E. Feinberg (2009) Constitutive release of cytokines by human oral keratinocytes in an organotypic culture. J Oral Maxillofac Surg *67(6)*:, 1256-64.

25. Abaci, H.E., R. Truitt, E. Luong, G. Drazer, S. Gerecht (2010) Adaptation to oxygen deprivation in cultures of human pluripotent stem cells, endothelial progenitor cells, and umbilical vein endothelial cells. Am J Physiol Cell Physiol *298(6)*: C1527-37.

26. Carmeliet, P., L. Moons, A. Luttun, V. Vincenti, V. Compernolle, *et al.* (2001) Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. Nature medicine *7(5)*: 575-83.

27. Asahara, T., T. Takahashi, H. Masuda, C. Kalka, D. Chen, *et al*. (1999) VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. EMBO J *18(14):* 3964-72.

28. van Hinsbergh, V.W., M.A. Engelse, P.H. Quax (2006) Pericellular proteases in angiogenesis and vasculogenesis. Arterioscler Thromb Vasc Biol *26(4)*: 716-28.

29. Brew, K., D. Dinakarpandian, H. Nagase (2000) Tissue inhibitors of metalloproteinases: evolution, structure and function. Biochim Biophys Acta *1477(1-2)*: 267-83.

30. Brew, K., H. Nagase (2012) The tissue inhibitors of metalloproteinases (TIMPs): an ancient family with structural and functional diversity. Biochim Biophys Acta *1803(1)*: 55-71.

31. Egeblad, M., Z. Werb (2002) New functions for the matrix metalloproteinases in cancer progression. Nat Rev Cancer *2(3)*: 161-74.

32. Mott, J.D., Z. Werb (2004) Regulation of matrix biology by matrix metalloproteinases. Curr Opin Cell Biol *16(5):* 558-64.

33. van Hinsbergh, V.W., P. Koolwijk (2008) Endothelial sprouting and angiogenesis: matrix metalloproteinases in the lead. Cardiovasc Res *78(2)*: 203-12.

34. Griffith, C., S. George (2009) The effect of hypoxia on in vitro prevascularization of a thick soft tissue. Tissue Eng Part A *15(9):* 2423-34.

35. Semenza, G.L. Hypoxia-inducible factor 1: master regulator of O2 homeostasis. Curr Opin Genet Dev *8(5)*: 588-94.

36. Trentin, D., H. Hall, S. Wechsler, J.A. Hubbell (2006) Peptide-matrix-mediated gene transfer of an oxygen-insensitive hypoxia-inducible factor-1alpha variant for local induction of angiogenesis. Proc Natl Acad Sci U S A *103(8):* 2506-11.

37. Ke, Q., M. Costa (2006) Hypoxia-inducible factor-1 (HIF-1). Mol Pharmacol *70(5):* 1469-80.

38. Semenza, G.L. (1999) Regulation of mammalian O2 homeostasis by hypoxia-inducible factor 1. Annu Rev Cell Dev Biol *15:* 551-78.

39. Kellner, K., G. Liebsch, I. Klimant, O.S. Wolfbeis, T. Blunk, *et al.* (2002) Determination of oxygen gradients in engineered tissue using a fluorescent sensor. Biotechnol Bioeng *80(1)*: 73-83.

40. Radisic, M., J. Malda, E, Epping, W. Geng, R. Langer, *et al.* (2006) Oxygen gradients correlate with cell density and cell viability in engineered cardiac tissue. Biotechnol Bioeng *93(2)*: 332-43.

41. Das, R., H. Jahr, G.J. van Osch, E. Farrell (2010) The role of hypoxia in MSCs: considerations for regenerative medicine approaches. Tissue Eng Part B *16(2)*: 159-68.

42. Straseski, J.A., A.L. Gibson, C.L. Thomas-Virnig, B.L. Allen-Hoffmann (2009) Oxygen deprivation inhibits basal keratinocyte proliferation in a model of human skin and induces regio-specific changes in the distribution of epidermal adherens junction proteins, aquaporin-3, and glycogen. Wound Repair Regen *17(4)*: 606-16.

43. Gill, E.M., J.A. Straseski, C.A. Rasmussen, S.J. Liliensiek, K.W. Eliceiri, *et al.* (2010) Visualization of morphological and molecular features associated with chronic ischemia in bioengineered human skin. Microsc Microanal 16(2): 117-31.

44. Oberley, C.C., F. Gourronc, S. Hakimi, M. Riordan, S. Bronner, *et al.* (2008) Murine epidermal side population possesses unique angiogenic properties. Exp Cell Res *314(4)*: 720-88.

45. Detmar, M., L.F. Brown, B. Berse, R.W. Jackman, B.M. Elicker, *et al.* (1997) Hypoxia regulates the expression of vascular permeability factor/vascular endothelial growth factor (VPF/VEGF) and its receptors in human skin. J Invest Dermatol, *108(3):* 263-8.

46. Steinbrech, D.S., M.T. Longaker, B.J. Mehrara, P.B. Saadeh, G.S. Chin, *et al.* (1999) Fibroblast response to hypoxia: the relationship between angiogenesis and matrix regulation. J Surg Res *84(2):* 127-33.

47. Li, T.S., K. Hamano, Suzuki, H. Ito, N. Zempo, N., *et al.* (2002) Improved angiogenic potency by implantation of ex vivo hypoxia prestimulated bone marrow cells in rats. Am J Physiol Heart Circ Physiol *283(2)*: H468-72.

48. Rosova, I., M. Dao, B. Capoccia, D. Link, J.A. Nolta (2008) Hypoxic preconditioning results in increased motility and improved therapeutic potential of human mesenchymal stem cells. Stem cells *26(8):* 2173-82.

49. Black, A.F., F. Berthod, N. L'Heureux, L. Germain, F.A. Auger (1998) In vitro reconstruction of a human capillary-like network in a tissue-engineered skin equivalent. FASEB J *12(13)*: 1331-40.

50. Supp, D.M., K. Wilson-Landy, S.T. Boyce (2002) Human dermal microvascular endothelial cells form vascular analogs in cultured skin substitutes after grafting to athymic mice. FASEB J *16(8):* 797-804.

51. Sahota, P.S., J.L Burn, N.J. Brown, S. MacNeil (2004) Approaches to improve angiogenesis in tissue-engineered skin. Wound Repair Regen *12(6)*: 635-42.

52. Ponec, M., A. El Ghalbzouri, R. Dijkman, J, Kempenaar, G. van der Pluijm, *et al.* (2004) Endothelial network formed with human dermal microvascular endothelial cells in autologous multicellular skin substitutes. Angiogenesis *7(4)*: 295-305.

53. Trottier, V., G. Marceau-Fortier, L. Germain, C. Vincent, J. Fradette (2008) IFATS collection: Using human adipose-derived stem/stromal cells for the production of new skin substitutes. Stem Cells *26(10):* 2713-23.

54. Supp, D.M., S.T. Boyce (2002) Overexpression of vascular endothelial growth factor accelerates early vascularization and improves healing of genetically modified cultured skin substitutes. J Burn Care Rehabil 23(1): 10-20.

CHAPTER 4

Hyperbaric oxygen treatment of tissue-engineered mucosa enhances secretion of angiogenic factors *in vitro*

Wendy M.W. Tra[1], Linda Spiegelberg[2], Bastiaan Tuk[1], Steven E.R. Hovius[1], Soledad Perez-Amodio[1]

Department of Plastic & Reconstructive Surgery[1], Department of Oral and Maxillofacial Surgery[2], Erasmus MC, Rotterdam, the Netherlands

Published in *Tissue Engineering Part A 2014; 20(9-10):1523-1530*

ABSTRACT

The survival of tissue-engineered mucosa (TEM) after implantation is mostly dependent on the presence of blood vessels for continuous oxygen supply. Therefore the stimulation of vascularization of TEM is essential to improve survival *in vivo*. Hyperbaric oxygen treatment (HBO), used to improve wound healing, stimulates the secretion of angiogenic factors. In this study we evaluated the effect of daily HBO treatments on TEM for 1, 3 or 5 consecutive days. Overall histology with hematoxylin-eosin staining showed no apparent changes after 1 treatment. After 3 and 5 HBO treatments, the basal layer became irregular and pyknotic cells were observed. Measurements of the viable epithelium showed significant thinning after 1 and 5 treatments, however proliferation was not affected. The angiogenic factors keratinocyte growth factor (KGF), hepatocyte growth factor (HGF), basic fibroblast growth factor (FGFbasic), and placental growth factor (PlGF) were significantly increased after 1 HBO treatment, whereas after 3 treatments a significant decrease of FGFbasic and PlGF was seen. After 5 treatments KGF, PlGF and vascular endothelial growth factor (VEGF) were significantly increased. One HBO treatment of TEM enhances the secretion of important angiogenic factors, hereby potentially improving the survival rate after *in vivo* implantation.

INTRODUCTION

The reconstruction of large oral defects as a result of trauma or oncological resection is limited by the availability of mucosal grafts, initiating the search for alternative grafts. We and others have developed TEM using keratinocytes and fibroblasts seeded onto a scaffold. Essential for all studies using tissue-engineered constructs is that the construct has a similar morphology to native oral mucosa [1-3].

Oxygen is a critical component in the wound healing process as it is involved in re-epithelization, collagen synthesis, angiogenesis and oxidative killing of bacteria [4, 5]. Cells need a constant supply of oxygen, as they cannot store oxygen for long periods of time. The lack of oxygen results in hypoxia, which causes cell death and impairs wound healing [4, 6]. HBO can be used to improve healing of different types of wounds [5, 7]. HBO treatment is twofold; oxygen levels increase to 100% and atmospheric pressure is increased, to i.e. 2.4ATA. By increasing these two components the oxygen levels in the capillaries rises resulting in the enhanced diffusion of oxygen into surrounding tissues [4, 8]. Although the exact working mechanism of HBO is not clearly understood, HBO has shown to increase the release of transforming growth factors β1 and β2 (TGF β1 and β2) and (VEGF), an important angiogenic factor [9, 10]. Furthermore, TGFβ1 and 2 have been reported to stimulate fibroblast proliferation in the tissue surrounding wounds and also to have angiogenic activity. These observations prompted us to use this novel and relatively simple approach to treat TEM with HBO in order to increase important angiogenic factors in TEM before engraftment. The aim of this study was to evaluate whether HBO treatment of TEM induces the secretion of angiogenic factors.

MATERIALS & METHODS

Cell culture

Biopsies of approximately 1 cm^2 were taken from the cheeks of four healthy individuals upon informed consent. Single cell suspensions of keratinocytes and fibroblasts were obtained as described before [1, 11]. Briefly, keratinocytes were isolated from the epithelial sheet by overnight incubation in 0.25% trypsin-EDTA (Invitrogen) and the single cell suspension was seeded onto lethally irradiated 3T3 fibroblast feeder layers, according to the Rheinwald & Green protocol [12]. Fibroblasts were isolated by mincing the dermis with scalpels, followed by incubation in collagenase/dispase (1.5 mg/mL / 2.5 mg/mL, respectively) (Invitrogen) solution. The cells used in this study were within passage 3 to 6.

Tissue-engineered mucosa

TEM was created as described previously [1, 13]. Briefly, per construct $1x10^5$ fibroblasts were spun into the lamina propria of the DED [11] and $1x10^6$ keratinocytes were seeded into a steel ring (diameter 10 mm) placed onto the papillary side of the DED. After culturing under submerged conditions for 24 hours, the constructs were raised to the air/liquid interface and cultured with A/L culture medium consisting of 3:1 Dulbecco's Modified Eagle Medium 4.5 g/l glucose: Ham's F12 supplemented with 24 µM bovine serum albumin, 1 µM hydrocortisone, 1 µM isoproterenol, 0.1 µM insulin, 10 µM L-carnitine,
10 mM L-serine, 1 µM D L-α-tocopherolacetate, fatty acid cocktail (30 µM linoleic acid, 7 µM arachidonic acid and 25 µM palmitic acid), 50 µg/ml ascorbic acid 100 IU/ml penicillin, 100 µg/ml streptomycin, for an additional 14 days. The medium was changed 3 times a week.

Hyperbaric oxygen treatment

TEM constructs were treated with HBO using the hyperbaric oxygen chamber as illustrated in fig. 1A-B [14]. TEM was treated on a daily basis up to 5 consecutive days. The chamber was flushed with pure oxygen for 10 minutes while pressure was increased to 2.4 ATA. This condition was maintained for 90 minutes. Next, pressure was decreased again to 1.0 ATA in 5 minutes. Immediately after HBO treatment TEM constructs were harvested. The following groups were included; one group received a single HBO treatment; one group received 3 HBO treatments; one group received 5 treatments. The control group did not receive HBO treatment and was harvested at the same 3 time points as the HBO treated groups. Each group consisted of 4 TEM constructs and three independent experiments were performed.

Collection preconditioned media and histology

Culture media was collected prior to harvesting of TEM, centrifuged at 400*g* for 5 minutes at 4 ^{o}C and stored at -80 ^{o}C until further analysis. Next, TEM was harvested by snap freezing with liquid nitrogen. Cryosections (6 µm) were stained with Hematoxylin-Eosin (HE) (Klinipath and Sigma, respectively) and overall morphology was assessed using a light microscope (Olympus). The thickness of the viable epithelium was determined from 2 consecutive images and the average thickness (µm) was measured using Hamamatsu software (Hamamatsu Photonics) by averaging 12 measurements per image, the results were displayed as mean ± SEM.

Immunohistochemistry

Staining for keratin 10, 13 and 19 was done as described before [1, 13]. For staining of Ki67 (1:200; DAKO), vimentin (1:200; Euro Diagnostica), collagen type III (1:200; Abcam), or collagen type IV (1:200; Euro Diagnostica), cryosections were fixed for 10 minutes with acetone, washed 3 times with PBS, and blocked for 30 minutes with 10% goat serum in PBS/1 % BSA. After incubation with primary antibodies slides were washed 3 times with PBS and incubated with goat anti-mouse or goat anti-rabbit biotin-labeled (both antibodies 1:200; DAKO) in PBS/1 % BSA for 30 minutes at RT, followed by incubation with Streptavidin-HRP (1:200; Southern Biotech) for 30 minutes at RT. 5 % 3,3'-Diaminobenzidine tetra hydrochloride hydrate (Sigma-Aldrich) in PBS supplemented with 30 % H_2O_2 was used for visualization of positive cells. After rinsing thoroughly with tap water, slides were stained with hematoxylin for background observation. Next, sections were air-dried and coverslipped using Vectamount (Vector) and sections were assessed using a light microscope. Negative control slides were incubated with an irrelevant mouse IgG.

Quantification of proliferation

To determine the proliferation index (PI), the basal layer of the epithelium was analyzed. Images were taken from 12 randomly chosen microscopic views using a 100x magnification. The PI was established as the ratio of the Ki-67 positive cells to all cells of the basal layer (x 100 %), and results were displayed as mean ± SEM.

ELISA assay on conditioned medium

Concentration of TEM-secreted angiogenic factors in the conditioned medium was measured using sandwich ELISA kits according to manufacturer's instructions. Factors that were studied include VEGF, PIGF, HGF, KGF and FGFbasic (R&D Systems). Results are expressed as ng or pg/cm^2 tissue with each sample consisting of 4mL supernatant derived from 1cm^2 tissue.

Proliferation of human umbilical vein endothelial cells

Proliferation rate of human umbilical vein endothelial cells (HUVEC) when exposed to culture media was assessed as described previously [13]. Briefly, HUVECs were seeded in 48-well plates in endothelial growth medium (EGM; consisting of human endothelial-serum free medium supplemented with 10 % FBS, 20 ng/mL FGF2 and 100 ng/mL EGF). Next, cells were cultured in starving medium (DMEM with 0.5 % FCS). After 24 h, cells were cultured with air/liquid culture medium supplemented with KGF (940 pg/cm^2), FGFbasic (75 pg/cm^2), and HGF (25 ng/cm^2) as measured with ELISA in conditioned media obtained from TEM exposed to 1 HBO treatment. After 48 h, cell numbers were analyzed using the CYQUANT proliferation assay (Molecular Probes) following manufacturer's instructions. The positive control consisted of endothelial growth medium or EGM without growth factor supplements. The negative control consisted of A/L medium without growth factor supplements. Each assay was done in triplicate.

Statistical Analysis

Data are presented as mean ± SEM. Tests of normality were performed using the Shapiro-Wilk test. Statistical analyses were performed using Student *t* test or Mann-Whitney U test. Statistical differences were defined as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, ns = not significant.

RESULTS

Overall morphology after hyperbaric oxygen treatment

The effect of HBO treatment on TEM overall morphology was assessed using HE staining. No apparent changes in overall morphology were observed after 1 HBO treatment (Fig. 1C-D). After 3 and 5 consecutive HBO treatments, the basal layer became irregular and pyknotic cells were observed in the basal and intermediate layer (Fig. 1E-H). Additionally, the attachment of the epithelial layer to the underlying connective tissue appeared to be affected as gaps between these layers became apparent. Measurements of the viable epithelium showed significant thinning after 1 and 5 treatments with HBO (Fig. 1 I).

Epithelial differentiation was determined using antibodies against keratins; the predominant component of the epithelial layer. Expression of K10 (Fig. 2A), K13 (Fig. 2B) and K19 (Fig. 2C) was not significantly changed by HBO treatment.

Collagen type IV, expressed along the whole basement membrane in all control groups and after 1 HBO treatment, proved to be no longer expressed after 3 treatments. After 5 treatments collagen type IV was again observed in irregular intervals in the basement membrane (Fig. 3A). Staining of collagen type III was more intense near the basement membrane in untreated TEM and after 5 HBO treatments more intense staining was observed at the lower half of the lamina propria (Fig. 3B).

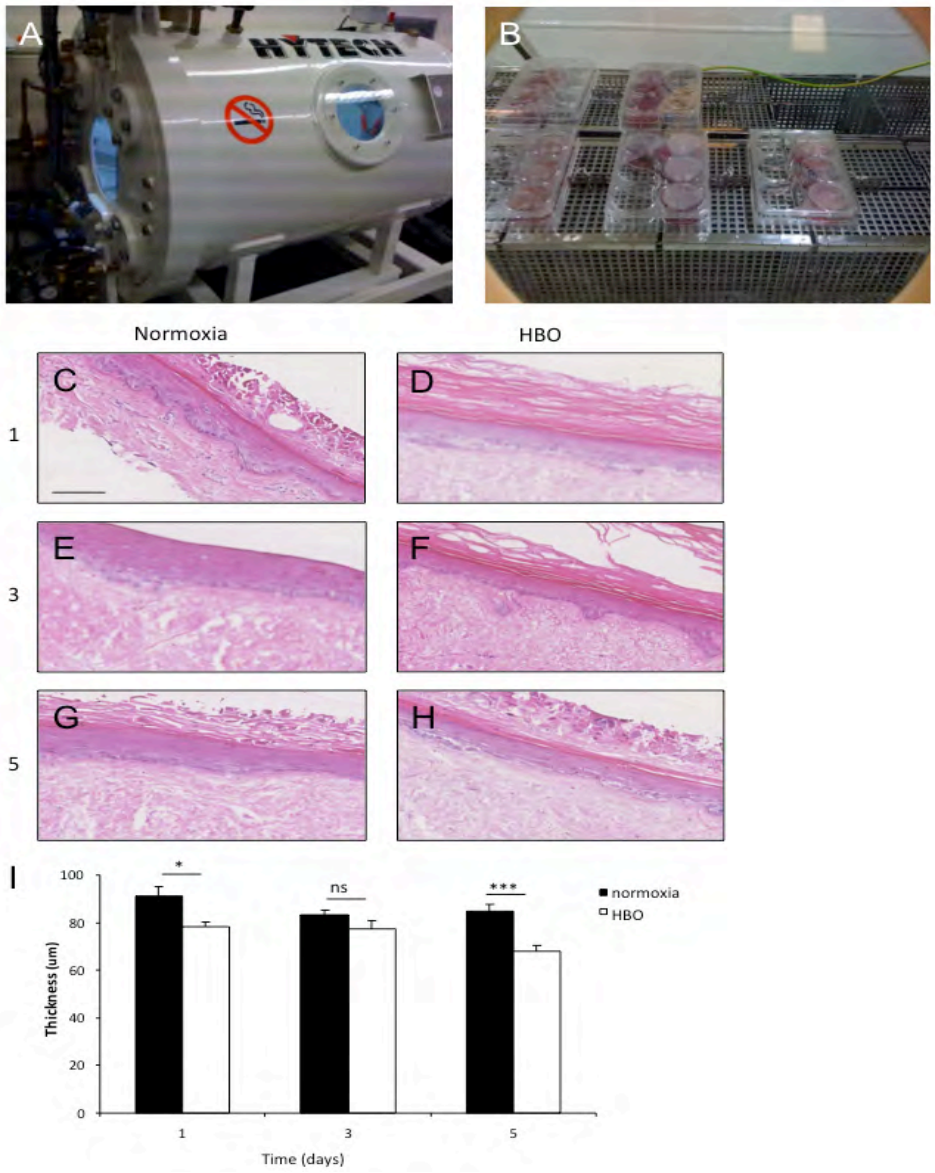


Figure 1. (A-B) Images of the HBO chamber used in this study. Histological appearance of TEM exposed to normoxia and HBO after (C-D) 1, (E-F) 3 or (G-H) 5 consecutive treatments. (I) Thickness of the viable epithelium decreased at all time points measured. Scale bar = 100µm.
ns = not significant; *$p < 0.05$; ***$p < 0.001$.

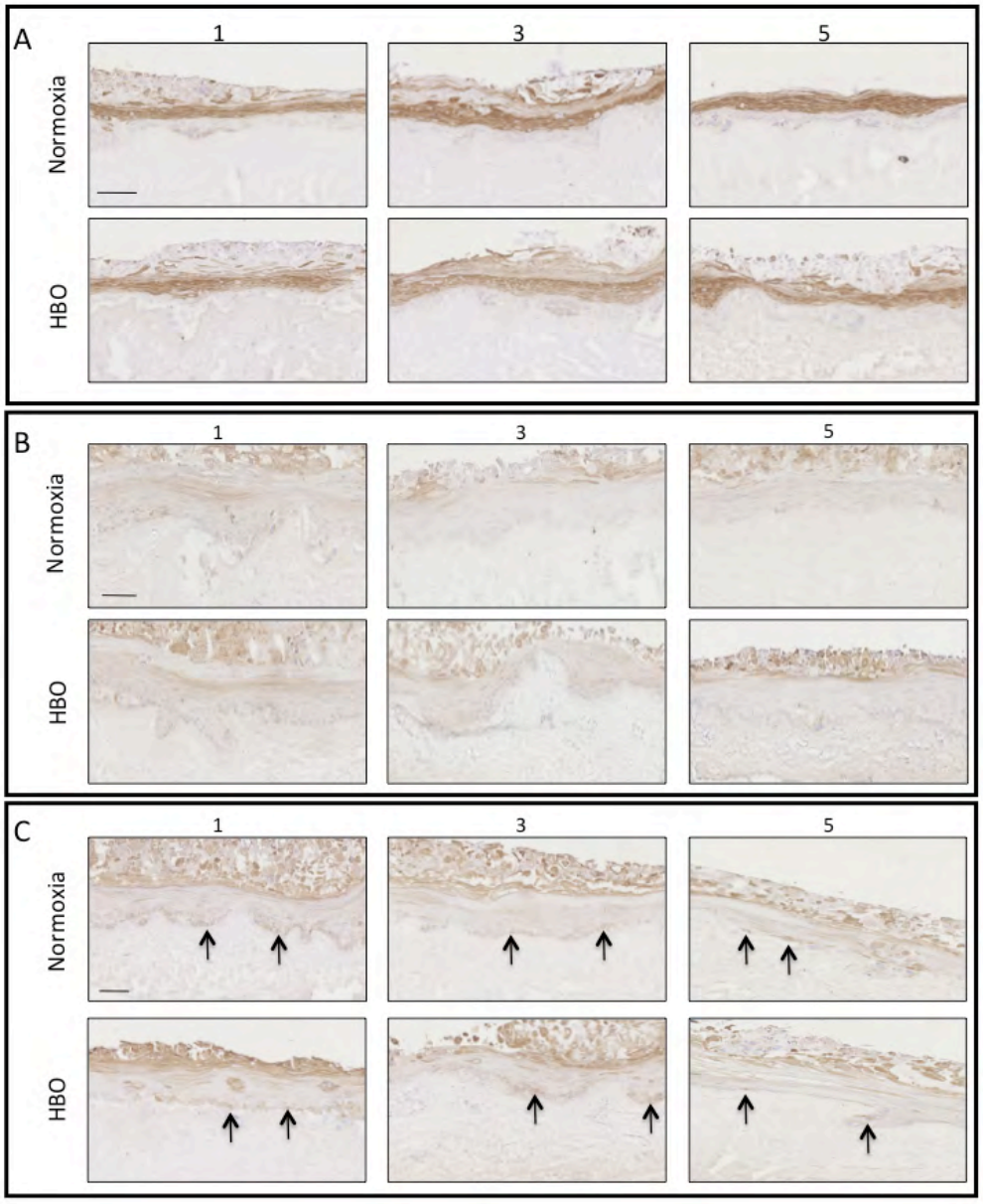


Figure 2. Histological appearance of TEM exposed to normoxia or HBO. Epithelial differentiation was assessed using antibodies against (A) K10, (B) K13 and (C) K19 (positive cells indicated with arrows). Scale bar = 100µm

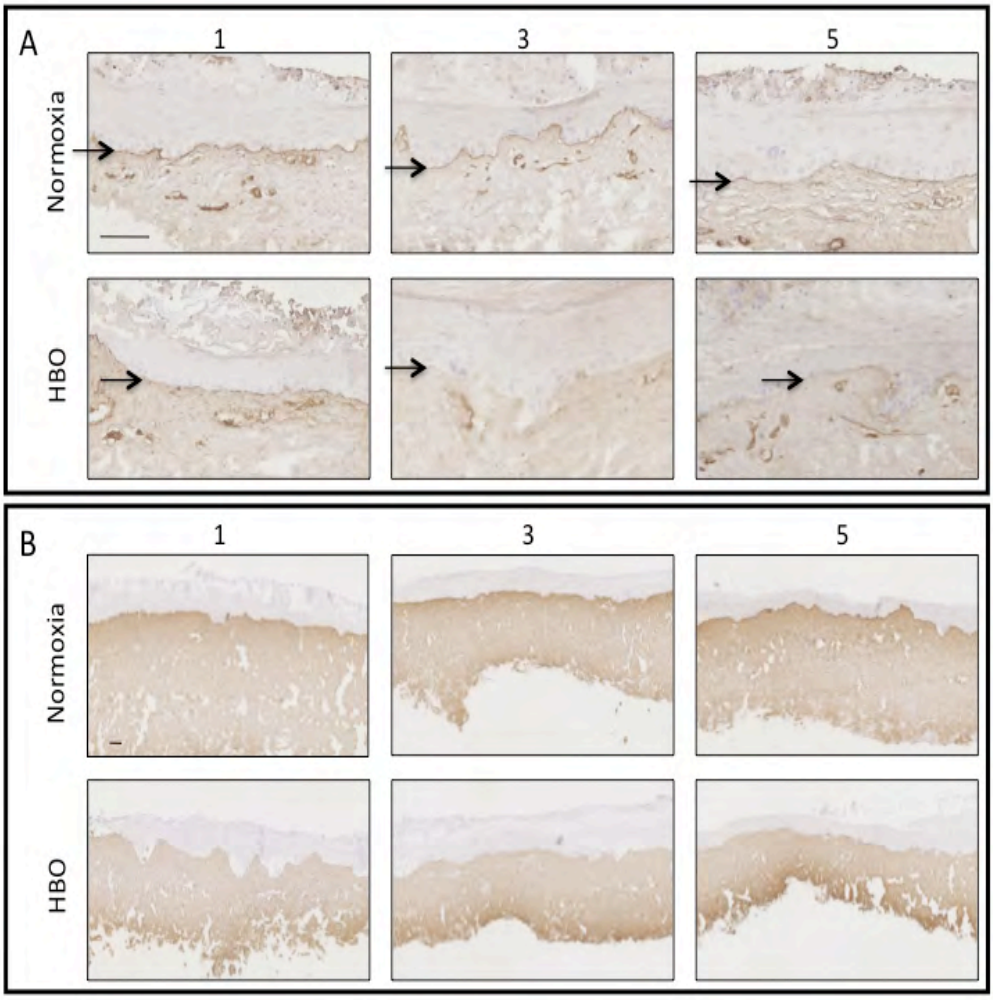


Figure 3. (A) Collagen type IV was irregularly expressed in the basement membrane (indicated with arrows) after HBO treatment when compared with normoxia. (B) Collagen type III deposition was altered after HBO treatment when compared with normoxia. Scale bar = 100µm

The distribution of fibroblasts and fibrocytes studied using vimentin staining was not altered after HBO treatment. Distinction between fibroblasts and fibrocytes was established morphologically. The fibroblast/fibrocyte ratio was affected by

HBO treatment as more fibrocytes relative to fibroblasts were observed after each treatment (data not shown).

Proliferating cells were observed in the basal layer of the epithelium only (Fig. 4A). After 1, 3 and 5 consecutive HBO treatments the number of proliferating cells increased when compared to untreated TEM (Fig. 4B) albeit this increase was not statistically significant.

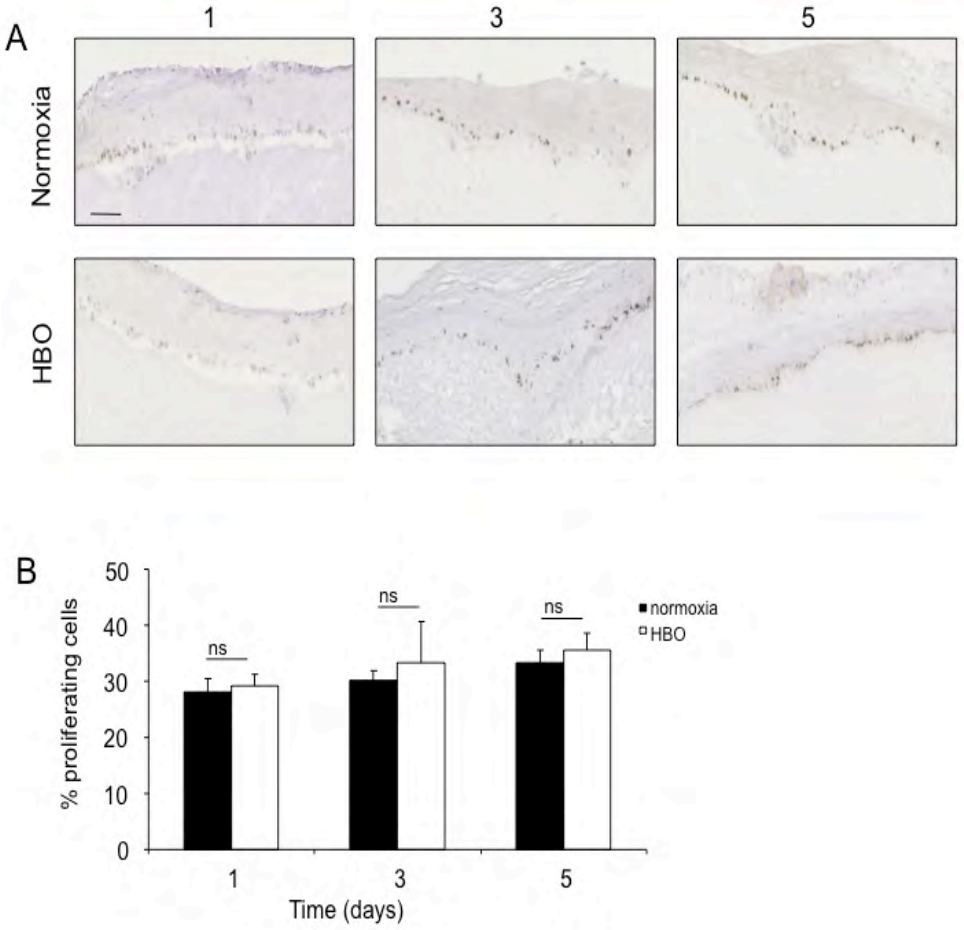


Figure 4. (A) Cell proliferation was observed in the basal layer of the epithelium. (B) HBO treatment slightly stimulated cell proliferation. Scale bar = 100µm. ns = not significant.

Assessment of angiogenic factors after HBO treatment

To determine the effect of HBO on the secretion of angiogenic factors by TEM, a number of angiogenic factors were measured in conditioned media (Fig. 5A-E). A single treatment with HBO resulted in a significant increase of KGF (2.6-fold), HGF (1.4-fold), FGFbasic (2.0-fold) and PlGF (3.2-fold) when compared to untreated TEM (Fig. 5). The levels of VEGF in the conditioned media did not change after a single HBO treatment. After 3 HBO treatments no changes in the levels of KGF, HGF and VEGF were observed and levels of FGF basic (1.4-fold) and PlGF (1.8-fold) were decreased when compared with untreated TEM. After 5 consecutive HBO treatments a significant increase in the secretion of KGF (4.2-fold), PlGF (1.7-fold) and VEGF (1.2-fold) was observed whereas HGF secretion was similar to that in untreated TEM. Secretion of FGFbasic significantly decreased 1.7-fold when compared to untreated TEM.

Effect of growth factors secreted by TEM on endothelial cell proliferation

In order to analyze whether the increased concentration of angiogenic factors found in conditioned media of HBO treated TEM was capable to stimulate endothelial cell proliferation, HUVECs were cultured with air/liquid media supplemented with either KGF, HGF or FGFbasic. Endothelial cell proliferation appeared to increase after incubation with A/L medium supplemented with FGF and HGF, although no statistical difference was found. A/L medium supplemented with KGF did not stimulate cell proliferation when compared with standard A/L medium (Fig. 5F).

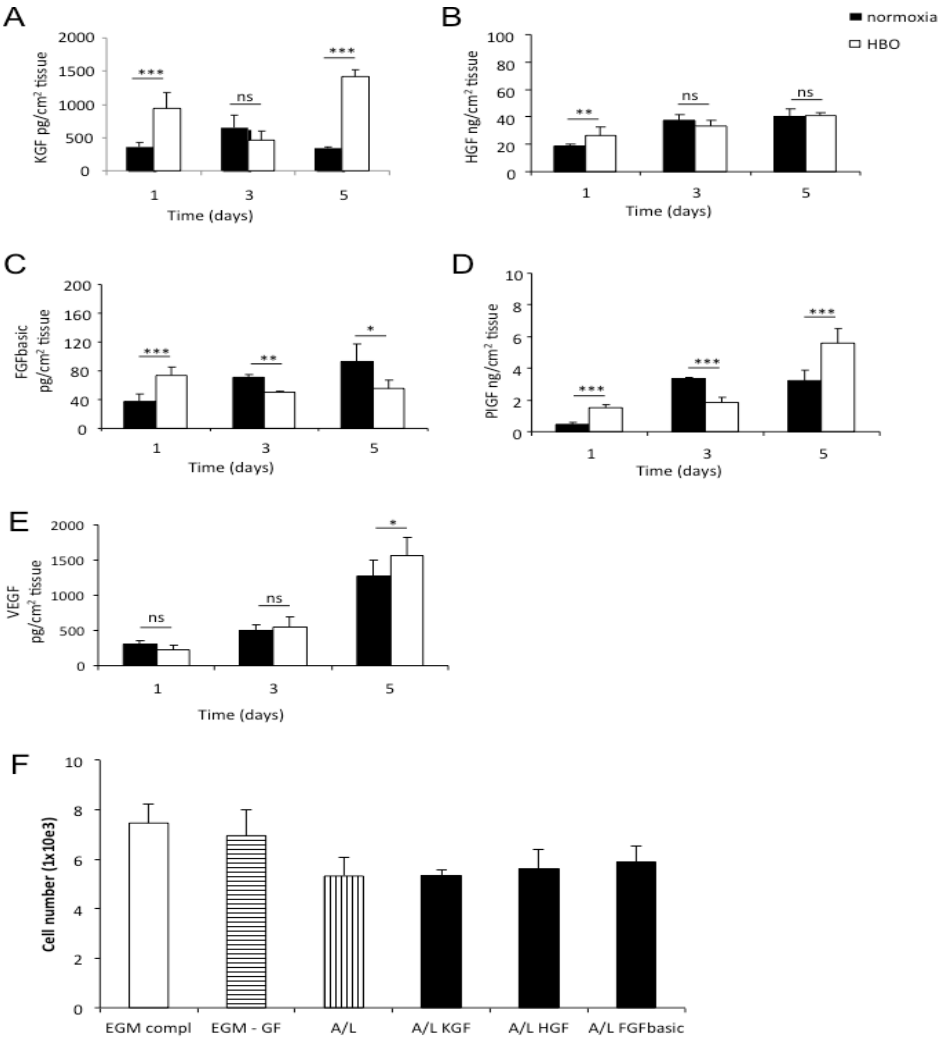


Figure 5. Release of angiogenic factors by TEM constructs. The secretion of (A) KGF, (B) HGF, (C) FGFbasic, (D) PlGF and (E) VEGF by TEM exposed to normoxia (closed bars) or HBO (open bars). Conditioned culture media from three different donors were assayed in triplicate. (F) Endothelial cell proliferation after 48h of incubation with A/L culture medium supplemented with KGF, HGF and FGFbasic. The number of endothelial cells appeared to increase when A/L medium was supplemented with HGF or FGFbasic when compared with normal A/L medium, but this difference was not statistically significant. Data are expressed as mean ± SEM in pg or ng/cm^2.
ns = not significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

DISCUSSION

In this study we demonstrated that treatment of TEM with HBO stimulates the secretion of multiple angiogenic factors. Overall morphology was maintained and the basal cell layer remained well organized after 1 HBO treatment. Minor structural changes where observed in TEM after 3 and 5 HBO treatments as the first morphological changes became apparent suggesting a potential harmful effect of HBO, for example oxygen toxicity.

Oxygen tension is known to influence cell proliferation [15, 16]. Previous studies have reported that the balance between oxygen tension and cell proliferation is delicate [9]. *In vitro* studies with keratinocyte monocultures reported inhibition of keratinocyte proliferation by HBO, whereas in the same study they reported that cells seeded into 3D skin equivalents did increase proliferation due to HBO demonstrated by increased thickness of the epidermis [17]. Even though our results showed a slight stimulating effect of HBO on cell proliferation, the thickness of the viable epithelium decreased. This could indicate accelerated maturation of the epithelium and earlier onset of epithelial stratification [4]. Previous studies by Hollander *et al* [6] and Dimitijevich *et al* [17] reported on the observation that HBO treatment resulted in increased keratinocyte differentiation. Our finding that the number of cells expressing K19 in HBO treated TEM was diminutive to that in the normoxia TEM might be due to the accelerated maturation, which results in a depletion of cells numbers in the basal layer [17].

HBO is known to influence collagen synthesis [18], although studies report contradicting findings. Studies reported on an increase of collagen deposition [4] whereas others report a decrease in collagen deposition [17]. Our results showed the expression of collagen type IV after 1 HBO treatment and in all

normoxia treated TEM. After 3 consecutive HBO treatments collagen type IV was no longer expressed whereas after 5 consecutive treatments collagen type IV was again observed. Previous work in our laboratory showed, prior to cell seeding, donor collagen type IV deposition along the whole basement membrane in the DED scaffold [1]. The observation that collagen type IV deposition was not seen after 3 treatment to appear again after 5 treatment suggests active remodeling of the basement membrane. However, as it is not possible to distinct between the donor collagen and the collagen deposited by the seeded cells, we cannot rule out the possibility of retained collagen from the donor.

The overall effect of HBO treatment on tissue-engineered skin equivalents has been studied before [4, 17] and the secretion of angiogenic factors by oral mucosal equivalents has been shown [19]. However, to our knowledge this is the first study in which the secretion of multiple important angiogenic factors by TEM after HBO treatment is studied. Treatment with HBO is known to increase VEGF secretion in wound fluid [20] and HUVEC [21]. Aside from VEGF, HBO has been shown to induce other angiogenic factors in hind limbs of mice [22] and HUVECs [23]. We therefore studied the secretion of angiogenic factors in TEM and found significantly higher levels of PlGF, KGF, HGF and FGFbasic in conditioned culture media of TEM after 1 HBO treatment when compared to conditioned culture media of TEM exposed to normoxia. Endothelial cell proliferation is considered to be an important component of angiogenesis [24]. Our finding that multiple angiogenic factors were increased after 1 HBO treatment, prompted us to study whether these increased concentrations were capable of inducing endothelial cell proliferation. KGF is known to be an important angiogenic factor for neovascularization. Our finding that KGF did not

induce proliferation of HUVECs was also reported by Rubin *et al* who showed that KGF lacked mitogenic activity on endothelial cells from large vessels [25] and by Gillis *et al* who showed that KGF induced proliferation of cultured capillary endothelial cells but not HUVECs [26]. Additionally, the lack of induced proliferation could be due to the absence of the FGFR2b receptor on HUVECs, being exclusively expressed on epithelial cells, as KGF specifically acts on this receptor. Both basic FGF and HGF are known to affect proliferation and migration of endothelial cells. Our results showed that HGF alone could not significantly increase endothelial cell proliferation. This is in line with the finding of Ding *et al* who reported that proliferation of HUVECs is increased by HGF in a dose-dependent manner, albeit that cell density is critical in the responsiveness of HUVEC to HGF [27]. Although HGF and FGFbasic individually did not improve endothelial cell proliferation, it could be that a combination of these factors may increase angiogenesis post-implantation [28]. Additionally, the need for oxygen is critical for survival of the graft and HBO preconditioning might shorten the hypoxic period after implantation by inducing endothelial cell proliferation and neovascular growth.

As continuous oxygen supply is essential for survival after implantation, it is necessary that blood vessels are quickly formed after implantation. HBO treatment has shown to stimulate the secretion of important angiogenic factors in TEM without effecting epithelial morphology. All together, our results suggest that pre-conditioning of TEM constructs with 1 HBO treatment prior to implantation might increase the survival rate of TEM grafts therefore making TEM an alternative tool in the reconstruction of large oral defects.

ACKNOWLEDGEMENTS

The authors would like to thank Dr. A. Seynhaeve, for her kind gift of HUVECs and EGM, and the Erasmus MC Tissue Bank, department of Pathology for facilitating the virtual microscope.

AUTHOR DISCLOSURE STATEMENT

No competing financial interest exists.

REFERENCES

1. Tra, W.M., J.W. van Neck, S.E. Hovius, G.J. van Osch, S. Perez-Amodio (2012) Characterization of a three-dimensional mucosal equivalent: similarities and differences with native oral mucosa. Cells Tissues Organs *195(3)*: 185-96.
2. Ophof, R., R.E. van Rheden, H.J. Von den, J. Schalkwijk, A.M. Kuijpers-Jagtman (2002) Oral keratinocytes cultured on dermal matrices form a mucosa-like tissue. Biomaterials *23(17)*: 3741-8.
3. Izumi, K., J. Song, S.E. Feinberg (2004) Development of a tissue-engineered human oral mucosa: from the bench to the bed side. Cells Tissues Organs *176(1-3)*: 134-52.
4. Kairuz, E., Z. Upton, R.A. Dawson, J. Malda (2007) Hyperbaric oxygen stimulates epidermal reconstruction in human skin equivalents. Wound Repair Regen *15(2)*: 266-74.
5. Kuffler, D.P. (2010) Hyperbaric oxygen therapy: an overview. J Wound Care *19(2)*: 77-9.
6. Hollander, D.A., M.Y. Hakimi, A. Hartmann, K. Wilhelm, J. Windolf (2000) The Influence of Hyperbaric Oxygenation (HBO) on Proliferation and Differentiation of Human Keratinocyte Cultures In Vitro. Cell Tissue Bank *1(4):* 261-9.
7. Spiegelberg, L., U.M. Djasim, H.W. van Neck, E.B. Wolvius, K.G. van der Wal (2010) Hyperbaric oxygen therapy in the management of radiation-induced injury in the head and neck region: a review of the literature. J Oral Maxillofac Surg *68(8)*: 1732-9.

8. Gill, A.L., C.N. Bell (2004) Hyperbaric oxygen: its uses, mechanisms of action and outcomes. QJM *97(7)*: 385-95.
9. Kang, T.S., G.K. Gorti, S.Y. Quan, M. Ho, R.J. Koch (2004) Effect of hyperbaric oxygen on the growth factor profile of fibroblasts. Arch Facial Plast Surg *6(1)*: 31-5.
10. Wu, D., J. Malda, R. Crawford, Y. Xiao (2007) Effects of hyperbaric oxygen on proliferation and differentiation of osteoblasts from human alveolar bone. Connect Tissue Res *48(4)*: 206-13.
11. El Ghalbzouri, A., E. Lamme, M. Ponec (2002) Crucial role of fibroblasts in regulating epidermal morphogenesis. Cell Tissue Res *310(2)*: 189-99.
12. Rheinwald, J.G., H. Green (1975) Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. Cell *6(3):* 331-43.
13. Perez-Amodio, S., W.M. Tra, H.A. Rakhorst, S.E. Hovius SE, J.W. van Neck (2011) Hypoxia preconditioning of tissue-engineered mucosa enhances its angiogenic capacity in vitro. Tissue Eng Part A *17(11-12):* 1583-93.
14. Djasim, U.M., L. Spiegelberg, E.B. Wolvius, K.G. van der Wal (2012) A hyperbaric oxygen chamber for animal experimental purposes. Int J Oral Maxillofac Surg *41(2):* 271-4.
15. Balin, A.K., L. Pratt (2002) Oxygen modulates the growth of skin fibroblasts. In Vitro Cell Dev Biol Anim *38(5)*:305-10.
16. Tuncay, O.C., D. Ho, M.K. Barker (1994) Oxygen tension regulates osteoblast function. Am J Orthod Dentofacial Orthop *105(5)*:457-63.
17. Dimitrijevich, S.D., S. Paranjape, J.R. Wilson, R.W. Gracy, J.G. Mills (1999) Effect of hyperbaric oxygen on human skin cells in culture and in human dermal and skin equivalents. Wound Repair Regen *7(1)*: 53-64.

18. Al-Waili, N.S., G.J. Butler (2006) Effects of hyperbaric oxygen on inflammatory response to wound and trauma: possible mechanism of action. ScientificWorldJournal *6:* 425-41.
19. Nakanishi, Y., K. Izumi, M. Yoshizawa, C. Saito, Y. Kawano, *et al* (2007) The expression and production of vascular endothelial growth factor in oral mucosa equivalents. Int J Oral Maxillofac Surg *36(10)*: 928-33.
20. Sheikh, A.Y., M.D. Rollins, H.W. Hopf, T.K. Hunt (2005) Hyperoxia improves microvascular perfusion in a murine wound model. Wound Repair Regen *13(3)*: 303-8.
21. Lee, C.C., S.C. Chen, S.C. Tsai, B.M. Wang, Y.C. Liu, *et al* (2006) Hyperbaric oxygen induces VEGF expression through ERK, JNK and c-Jun/AP-1 activation in human umbilical vein endothelial cells. J Biomed Sci *13(1)*: 143-56.
22. Asano, T., E. Kaneko, S. Shinozaki, Y. Imai, M. Shibayama, *et al* (2007) Hyperbaric oxygen induces basic fibroblast growth factor and hepatocyte growth factor expression, and enhances blood perfusion and muscle regeneration in mouse ischemic hind limbs. Circ J *71(3)*: 405-11.
23. Lin, S., K.G. Shyu, C.C. Lee, B.M. Wang, C.C. Chang, *et al* (2002) Hyperbaric oxygen selectively induces angiopoietin-2 in human umbilical vein endothelial cells. Biochem Biophys Res Commun *296(3)*: 710-5.
24. Machado, M.J., M.G. Watson, A.H. Devlin, M.A. Chaplain, S.R. McDougall SR, *et al* (2011) Dynamics of angiogenesis during wound healing: a coupled in vivo and in silico study. Microcirculation *18(3):* 183-97.
25. Rubin, J.S., H. Osada, P.W. Finch, W.G. Taylor, S. Rudikoff, *et al* (1989) Purification and characterization of a newly identified growth factor specific for epithelial cells. Proc Natl Acad Sci U S A *86(3):* 802-6.

26. Gillis, P., U. Savla, O.V. Volpert, B. Jimenez, C.M. Waters, *et al* (1999) Keratinocyte growth factor induces angiogenesis and protects endothelial barrier function. J Cell Sci 112(Pt 12): 2049-57.

27. Ding, S., T. Merkulova-Rainon, Z.C. Han, G. Tobelem (2003) HGF receptor up-regulation contributes to the angiogenic phenotype of human endothelial cells and promotes angiogenesis in vitro. Blood *101(12):* 4816-22.

28. Castellon, R., H.K. Hamdi, I. Sacerio, A.M. Aoki, M.C. Kennedy, *et al* (2004) Effects of angiogenic growth factor combinations on retinal endothelial cells. Exp Eye Res *74(4)*: 523-35.

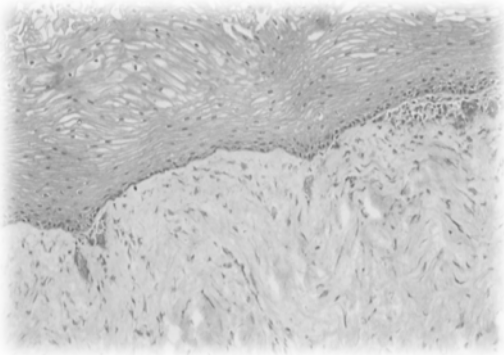
CHAPTER 5

Survival of tissue-engineered mucosal equivalents in immunodeficient mice

Wendy M.W. Tra, Bastiaan Tuk, Johan W. van Neck, Steven E.R. Hovius, Soledad Perez-Amodio

Department of Plastic & Reconstructive Surgery, Erasmus MC, Rotterdam, the Netherlands

Submitted in *Tissue Engineering Part A*



ABSTRACT

Limited availability of oral grafting material is a major challenge in reconstructive surgery, and therefore alternative tissue sources are needed. Tissue-engineering has proven to be an excellent method of constructing grafts using autologous cells *in vitro*. In the present study we assessed the survival of tissue-engineered mucosal equivalents (TEM) after implantation in nude mice. TEM was constructed by seeding oral keratinocytes and fibroblasts on acellular de-epidermized dermis (DED). TEM was implanted into subcutaneous dorsal pockets and harvested after 3, 7, 14 or 28. Evaluation of the constructs after harvest showed that half of the implanted TEMs had survived. Epithelial maturation was assessed using markers for keratin 5 and 13, and showed that some TEM had lost the epithelium, whereas other TEM constructs had a mature multi-layered epithelium with a regular basement membrane (BM) as illustrated by collagen type IV expression. Proliferation marker Ki67 showed a slight increase of proliferating cells in the first 14 days. CD31 was used to assess the presence of murine endothelial cells in the TEM. Although some positive cells in the connective tissue were found, no tube-like structures were observed. The results indicated that TEM can survive implantation and suggest that TEM has the potential to become an alternative source for grafts.

INTRODUCTION

The reconstruction of large oral defects, as a result of oncological resection or trauma, is often challenged by the limited availability of mucosal grafts. Current techniques for covering large oral defects use free vascularized skin flaps [1]. Drawbacks of these skin grafts include hair growth, perspiration and donor-site morbidity. Each of these drawbacks is highly uncomfortable and reduces the quality of life of treated patients [2, 3].

Tissue engineering of oral mucosa with autologous cells, such as keratinocytes and fibroblasts, on varying scaffolds [4, 5] resemble native oral mucosa [6-8]. These equivalents have successfully been used to study wound healing [9], the effects of radiotherapy [10] or to test cytotoxicity [11].

Contradicting results have been published on the outcome after intraoral grafting. Some studies have reported on a successful intraoral engraftment [12, 13], whereas others showed a relatively poor survival of the grafts post-implantation [14]. The loss of the graft could be the result of inadequate vascularization [15]. For all tissue-engineered mucosal constructs to survive upon implantation, oxygen and nutrient supply by blood vessels is essential as a lack of oxygen, or hypoxia, can lead to loss of the graft [16]. However, a study reported on by our group showed that TEM couldn't only survive a certain period of hypoxia, but that the secretion of important angiogenic growth factors such as VEGF is increased [17].

As TEM was originally designed for clinical applications, it is essential to study the survival of TEM post-implantation. Therefore, we evaluated TEM after implantation into subcutaneous skin pockets on the back of nude mice by studying epithelial cell viability, stratification of the epithelial layer, remodelling of the basement membrane and underlying connective tissue. Accordingly, we

studied the ingrowth of murine vascular structures into the TEM grafts and the survival of the TEM grafts post-implantation.

MATERIALS & METHODS

Primary cell culture

Biopsies of 1cm^2 were taken from the cheeks of four healthy individuals upon informed consent. Single cell suspensions of keratinocytes and fibroblasts were obtained as described before [6]. Briefly, keratinocytes were isolated from the epithelial sheet by overnight incubation in 0.2% trypsin-EDTA (Invitrogen). Fibroblasts were isolated by mincing the dermis followed by incubation in collagenase/dispase (1.5mg/mL / 2.5mg/mL, respectively) (Invitrogen) solution. The cells used in this study were within passage 3 to 6.

Tissue-engineered mucosa

TEM was created as described before [6, 18]. Briefly, 1 cm^2 DED scaffolds (EuroSkin bank) were obtained by removing the epidermis by gentle shaking with PBS. Per construct 1 x 10^5 fibroblasts were spun into the lamina propria of DED [18] and 1 x 10^6 keratinocytes were seeded into a steel ring (diameter 10mm) placed onto the papillary side of the DED. After culturing under submerged conditions for 24 hours, the constructs were raised to the air/liquid interface and cultured with A/L culture medium consisting of 3:1 Dulbecco's Modified Eagle Medium 4.5 g/l glucose: Ham's F12 supplemented with 24 µM bovine serum albumin, 1 µM hydrocortisone, 1 µM isoproterenol, 0.1 µM insulin, 10 µM L-carnitine, 10 mM L-serine, 1 µM D L-α-tocopherolacetate, fatty acid cocktail (30 µM linoleic acid, 7 µM arachidonic acid and 25 µM palmitic

acid), 50 µg/ml ascorbic acid 100 IU/ml penicillin, 100 µg/ml streptomycin, for an additional 14 days. The medium was changed 3 times a week.

Evaluation TEM pre-implantation

To determine the quality of all TEM constructs, a small slice was taken from each construct and snap frozen with liquid nitrogen. Overall morphology of the TEM constructs was assessed using Haematoxylin-Eosin staining (HE). Only TEM constructs consisting of a well-developed multi-layered epithelium and a neatly arranged basal layer were used for *in vivo* grafting. The thickness of the viable epithelium was determined from 2 consecutive images and the average thickness was measured using Hamamatsu software (Hamamatsu Photonics) by averaging 12 measurements per image. The results were displayed as mean ± SEM.

In vivo implantation

Twenty 6-week-old athymic male nude mice (NMRI-nu/nu, Taconic, Hudson, New York) were used in this study upon approval of the animal ethics committee. The mice were placed under general anaesthesia with 2.5 % isoflurane and an incision was made through the dorsal skin. Next, a subcutaneous pocket was prepared by blunt dissection of the subcutaneous tissue and one TEM construct per pocket was implanted. Pockets were closed using discontinuous sutures. Three, 7, 14 or 28 days after implantation, the mice were sacrificed and the TEM constructs were harvested.

Histology

Harvested TEM constructs were fixed in 10% formalin in PBS and embedded in paraffin or directly embedded in OCT-medium (Sakura) and snap frozen using liquid nitrogen. Paraffin-embedded sections (5µm) were deparaffinized and rehydrated. Cryosections (6µm) were fixed with acetone for 15 minutes. To determine the quality of TEM post-implantation, overall morphology and the thickness of the viable epithelium and total scaffold (determined as described above) was assessed using H&E staining.

Evaluation TEM post-implantation

Maturation of the epithelial layer was determined using monoclonal mouse anti-human antibodies against keratin-5 (1:200; Euro-Diagnostica), keratin-13 (1:200; Euro-Diagnostica), and keratin-19 (1:500; Novus Biologicals). Next, sections were incubated using Link/Label complex (BioGenex) according manufacturer's instructions. Finally, sections were stained with New Fuchsine substrate and counterstained with Haematoxylin and sealed using Vectamount. For analysis of the BM and underlying connective tissue, frozen sections were fixed for 10 minutes with acetone, blocked with 1%BSA/PBS for 30 minutes and incubated for 60 minutes at RT with antibodies against either collagen type III (1:200; Abcam), collagen type IV (1:200; Euro-Diagnostica) or Ki67 (1:200 dilution in PBS/1% BSA, Clone MIB-1, DAKO). After incubation with secondary biotin-conjugated goat anti-mouse or rabbit anti-mouse (both 1:200; DAKO), sections were incubated with Streptavidin-horseradish peroxidise (1:300 dilution in PBS/1%BSA; DAKO) for 30 minutes. Diaminobenzidine (DAB; Sigma Aldrich) substrate was used for visualization. Haematoxylin was used to visualize the background and sealed with Vectamount (Brunschwig Chemical). Positive

controls consisted of native oral mucosa sections and negative controls were incubated with an irrelevant IgG.

Assessment of vascular structures post-implantation

To determine the ingrowth of vascular structures into TEM, monoclonal CD31 antibody was used. After fixation with acetone, cryosections were blocked with PBS /1%BSA. After incubation with CD31 (1:500; Abcam), sections were incubated with secondary biotin-conjugated goat anti-mouse (1:200; DAKO) and Streptavidin-HRP (1:200; DAKO). To visualise CD31 positive cells, DAB substrate was used. Slides were stained with hematoxylin for background observation.

Statistical analysis

Data are presented as mean ± SEM. Tests of normality were performed using the Shapiro-Wilk test. Statistical analyses were performed using the one-way analysis of variance using GraphPad InStat software. Statistical differences were defined as $p \leq 0.05$. Comparisons between group means determined using the Tukey-Kramer t test for multiple comparisons.

RESULTS

Characterization of TEM pre-implantation

The quality of all TEM constructs was assessed before implantation. Therefore, cross-sections stained with H&E were used to study the overall morphology. H&E staining showed that all constructs consisted of a neatly arranged basal layer and a multi-layered epithelium before implantation (Fig. 2A). The average thickness of the viable epithelium was 81.6 ± 2.0 µm (Fig. 2F).

Evaluation of TEM following implantation

TEM constructs were harvested 3, 7, 14 and 28 days after implantation. Gross examination revealed that the constructs had maintained their normal shape at day 3 and 7 post-implantation (Fig. 1A-B). Additionally, on day 3 and 7 post-implantation small blood vessels of the surrounding murine tissue were adjacent to the TEM constructs. On 14 and 28 days post-implantation gross examination showed that all constructs had a thickened and rounded form (Fig. 1C-D). Dissection of these latter constructs showed that the centre cavity was filled with blood (Fig. 1E). Similar to day 3 and 7, at day 14 and 28 blood vessels from the surrounding murine tissue were adjacent to the TEM constructs.

Post-implantation, H&E staining showed that 11 of the 20 implanted TEM constructs had lost the epithelial layer. The remaining 9 TEM constructs had maintained the epithelial layer, including regular basal layer (Fig. 2B-E). These constructs were used for further analysis. Measurements of the viable epithelium showed that the epithelium became significantly thinner after 3 (49.1 ± 1.1µm), 7 (58.2 ± 2.7µm), 14 (79.6 ± 10.4µm) and 28 (70.6 ± 8.3µm) days post-implantation when compared with TEM prior implantation (81.6 ±2.0µm) (Fig. 2F).

We also measured the thickness of the implanted TEM whether epithelium was or was not present. The constructs with epithelium were 659.6 ± 49.9µm at day 3, 402.5 ± 21.8µm at day 7, 861.7 ± 79.2µm at day 14 and 413.2 ± 38.0µm at day 28. The constructs without epithelium measured 495.0 ± 21.9µm at day 3, 604.1 ± 27.3µm at day 7, 727.7 ± 71.8µm at day 14, and 616.4 ± 33.7µm at day 28 (Fig. 2G).

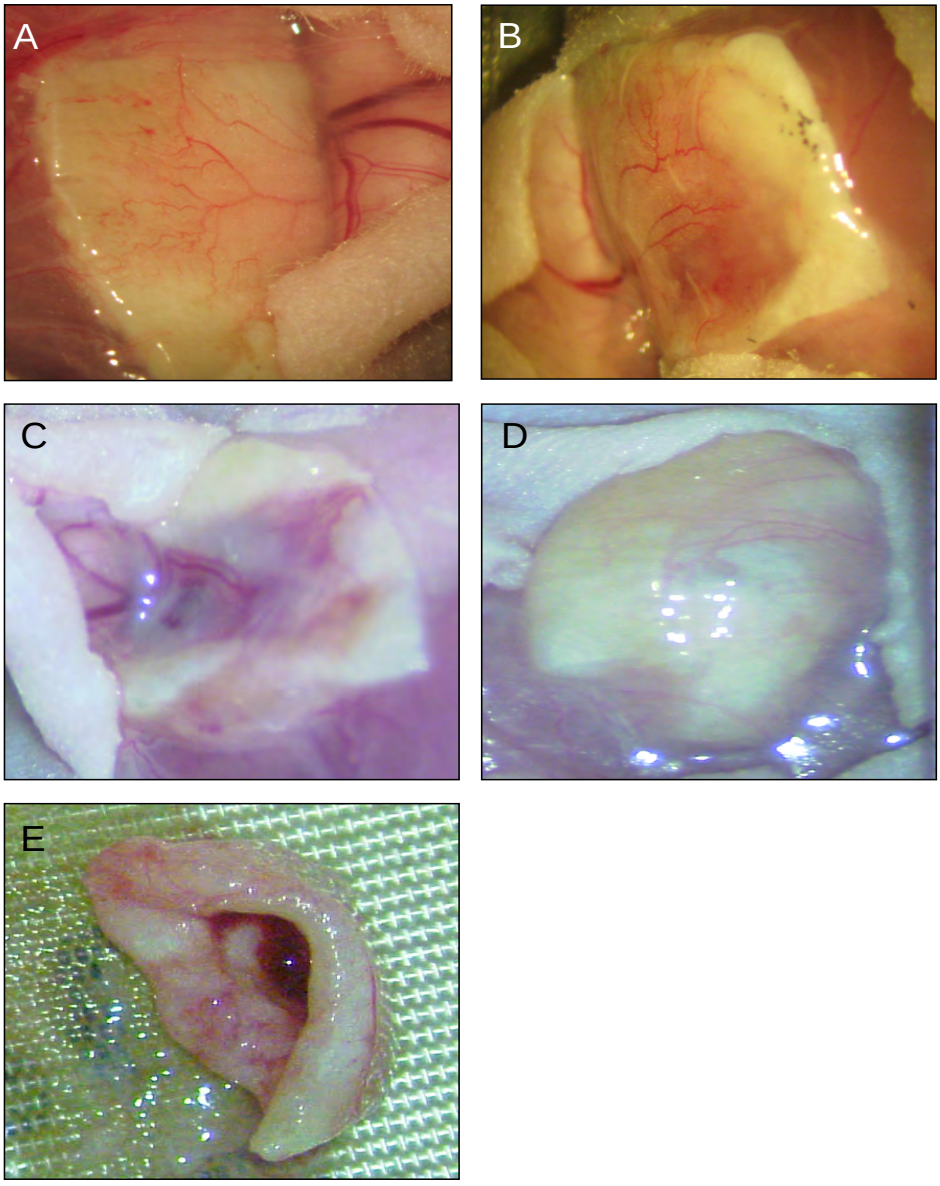


Figure 1. Gross morphology of TEM constructs (A) 3, (B) 7, (C) 14 and (D) 28 days post-implantation. (E) After 14 and 28 days the TEMs were thickened in the centre of the construct. Dissection of these thickened constructs showed a cavity filled with blood.

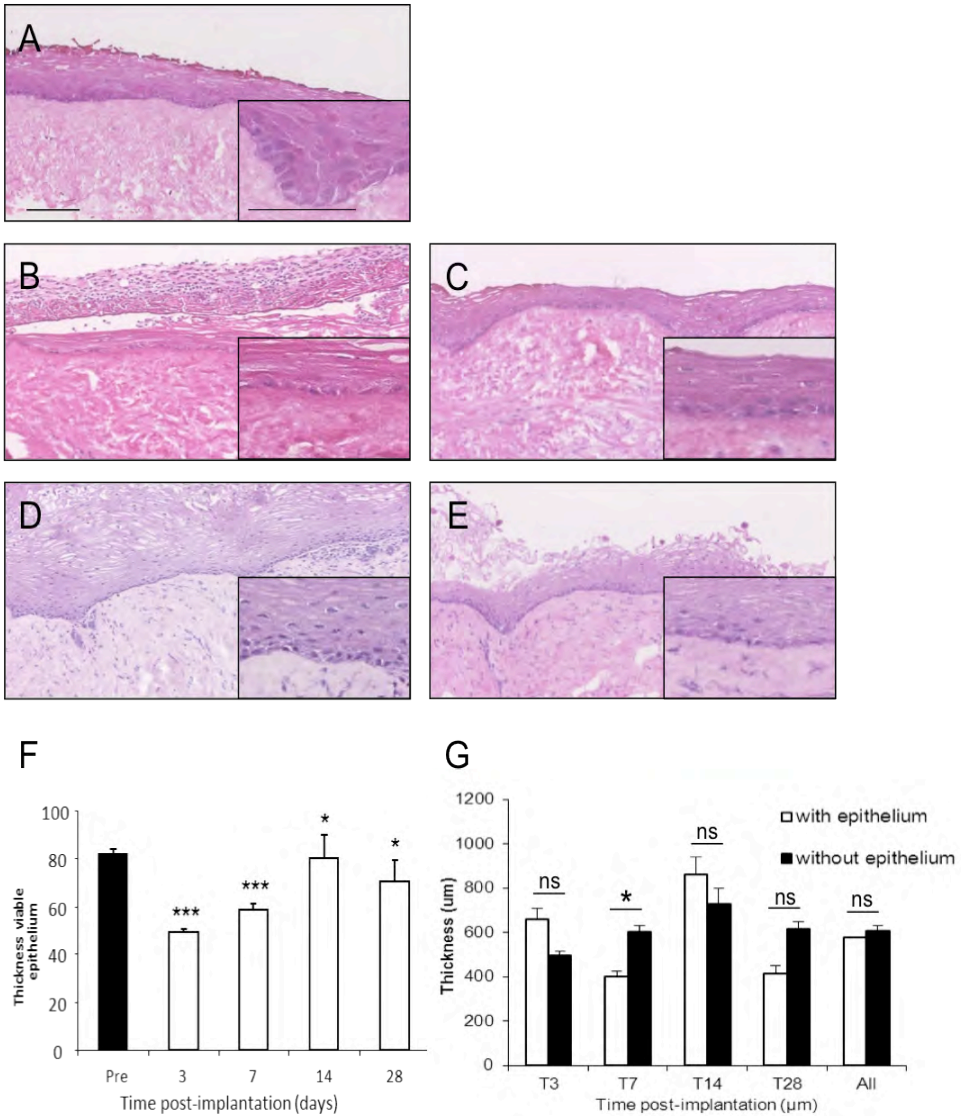


Figure 2. Hematoxylin and Eosin staining was used to assess overall morphology (A) prior to implantation and (B) 3, (C) 7, (D) 14 or (E) 28 days post-implantation. (F) Measurements of the viable epithelium showed that the thickness of the epithelium reduced when compared with the control TEMs. (G) Measurements of the construct with epithelium (open bars) and without epithelium (closed bars) showed inconclusive data as the thickest average measurements alternated between the construct with and without epithelium. Magnification 100x. Scale bar = 100µm. ns = not significant; *p<0.05; ***p<0.001.

Assessment epithelium post-implantation

To determine the viability of TEM post-implantation the number of proliferating cells was assessed using Ki67. Proliferating cells were observed in the basal layer of the epithelium and in the connective tissue (Fig. 3A-D). Quantification of the proliferation index showed that the number of proliferating cells did not change 3 (15.0 ± 1.6) or 7 (15.8 ± 1.0) days post-implantation. After 14 days the number of proliferating cells increased (19.5 ± 3.0), however this increase was not significant. After 28 days (11.3 ± 0.8) cell proliferation significantly decreased when compared with 14 days post-implantation (Fig. 3E).

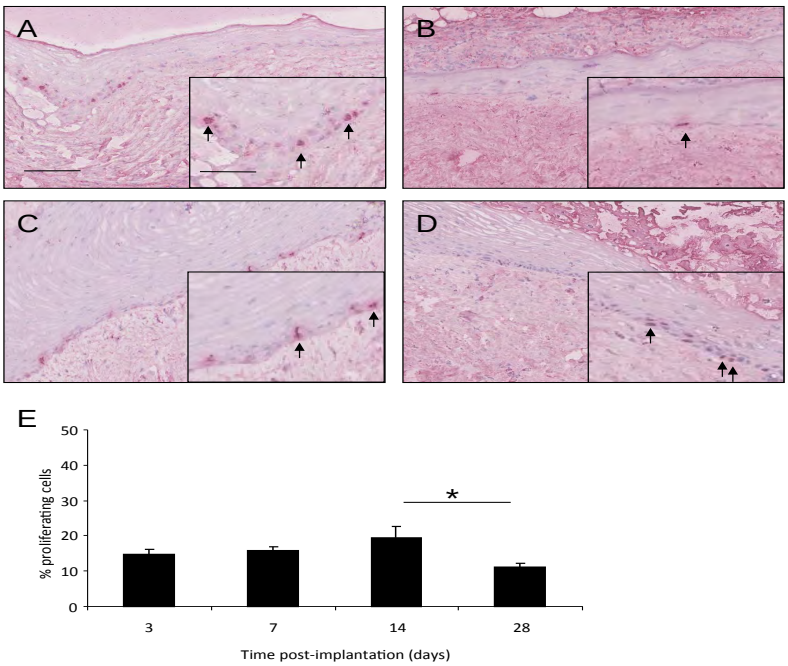


Figure 3. Cell proliferation was visualized using an antibody against Ki67. Proliferating cells (red; indicated with arrows) were observed in the basal layer of TEM constructs after (A) 3, (B) 7, (C) 14 or (D) 28 days post-implantation. (E) Quantification of the number of proliferating cells showed an increase in the first 14 days post-implantation. After 28 days the number of proliferating cells significantly decreased. Magnification 100x. Scale bar = 100µm. *$p < 0.05$.

Keratin 13, normally expressed in the intermediate layer of non-keratinizing oral mucosa, was observed in the intermediate and superficial layer of the epithelium of the TEM constructs at 3, 7, 14 and 28 days post-implantation (Fig. 4A-D). K5 and K19, normally expressed in the basal layer of the epithelium, were used to determine the maturation of the epithelial layer. After 3 and 7 days post-implantation the expression of K5 was observed in the basal layer of the epithelium, whereas after 14 and 28 days K5 was not detectable (data not shown). K19 was observed in the basal layer of the epithelium of TEM harvested at all time points (Fig. 4E-H).

Basement membrane and connective tissue

Before implantation all TEM constructs showed a continuous expression of collagen type IV, a major component of the basement membrane. After 3 days, collagen type IV was not observed (Fig. 5A). After 7 days collagen type IV was only limited expressed in the basement membrane (Fig. 5B). After 14 (Fig. 5C) and 28 (Fig. 5D) days collagen type IV was found expressed throughout the whole basement membrane. No differences in expression of collagen type III, a major component of the underlying connective tissue, were observed after implantation at all-time points studied (data not shown).

Assessment of vascular structures post-implantation

The presence of blood inside the cavities in some constructs prompted us to determine the presence of murine vascular structures adjacent to TEM constructs. Therefore we performed an immunohistochemical staining using an anti-CD31 antibody. The results showed no tube like structures in the tissue surrounding the TEM construct or within the constructs. Instead we observed

some CD31 positive cells in the underlying connective tissue only as illustrated in figure 5E-H.

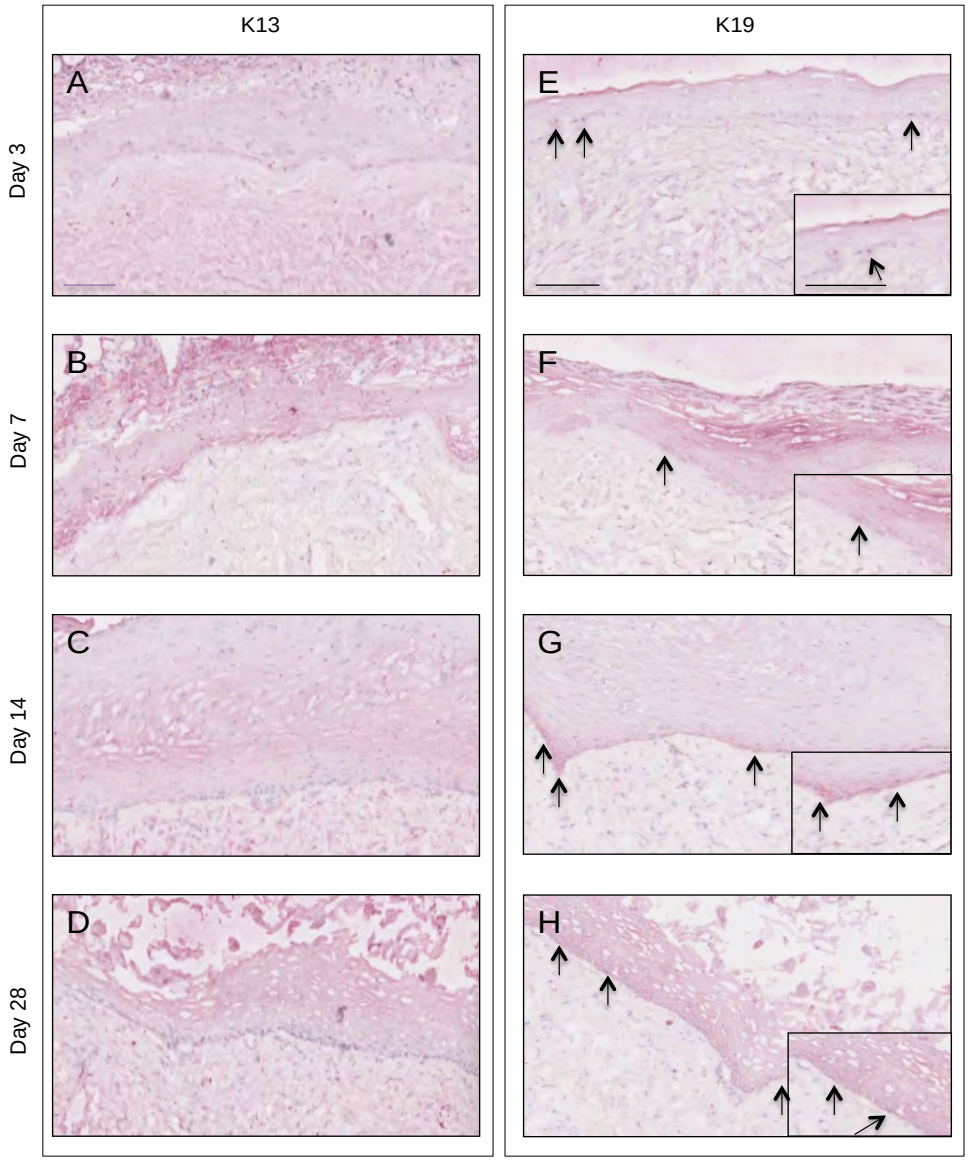


Figure 4. Maturation of the newly formed epithelial layer was determined using (A-D) K13 and (E-H) K19 after 3, 7, 14 or 28 days of implantation. Magnification 100x. Scale bar = 100 µm.

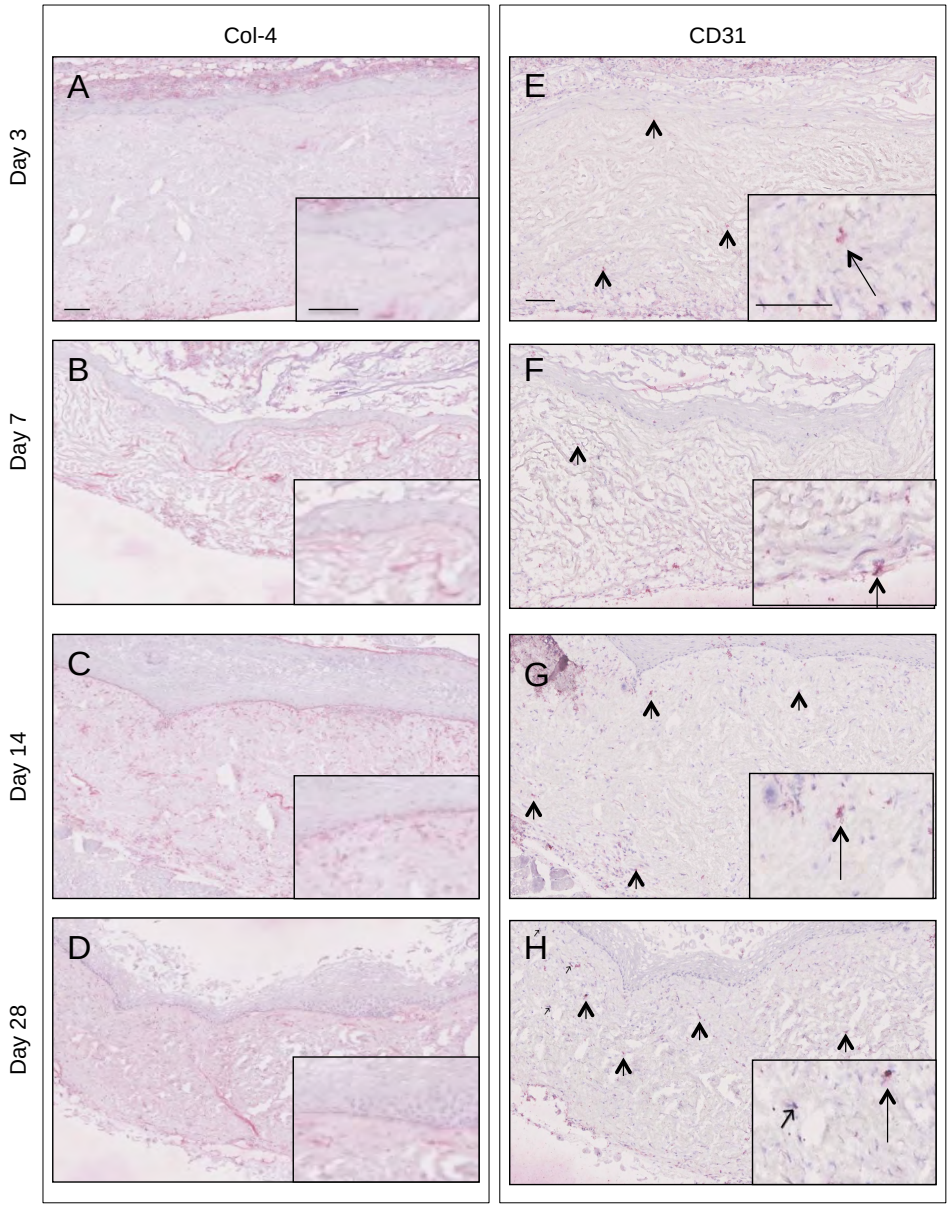


Figure 5. The expression pattern of basement membrane component collagen type IV was determined (A) 3, (B) 7, (C) 14 and (D) 28 days post-implantation. The results showed that collagen type IV was detected after the first week, but was not continuous until 28 days post-implantation. The presence of endothelial cells was determined (E) 3, (F) 7, (G) 14 and (H) 18 days post-implantation.

DISCUSSION

In this study we demonstrated that TEM constructs could survive prolonged periods of time after implantation into subcutaneous pockets on the back of nude mice. Histological analysis showed that approximately half of the TEM constructs survived the implantation. Overall morphology was maintained and the basal cell layer remained well organized up to 28 days. Structural changes were observed in TEM as soon as 3 days after implantation as the first morphological changes became apparent.

Not all the implanted TEMs survived implantation, as some TEMs no longer had an epithelial layer, whereas other TEMs had a very thin epithelium of 2 to 3 cell layers left. The usage of subcutaneous pockets was first described by Barrandon *et al* [19] and is a much used technique. Other groups using this technique have reported on partial detachment of the epithelium, but none mentioned the complete loss of the epithelial layer [20, 21]. The loss of the epithelium could be caused by continuous friction of the TEM with the surrounding murine tissue or by the harvesting procedure. We only harvested the TEM construct, whereas others collected not only the construct but also part of the surrounding murine tissue. By not taking out the surrounding murine tissue we may have damaged the fragile epithelial layer.

As shown by our results, approximately half of our TEM constructs survived prolonged periods of time post-implantation. Jain *et* al reported that a tissue-engineered construct thicker than 100-200 µm requires vascularization for the continuous supply of oxygen and nutrients [22]. The thickness of the scaffolds, with or without epithelium, well exceeds this 100-200 µm suggesting that the presence of blood vessels and vascularization of the TEM is paramount. Even though some CD31-positive cells in the connective tissue were found, no tube-

like structures were observed within the TEM construct. This could be an indication to why half of the TEM constructs did not survive after implantation. The other TEMs might have survived by the presence of relatively large murine veins in the tissue directly surrounding the construct.

Cell viability was assessed using proliferation marker Ki-67. Contradicting findings on the expression of Ki67 post-implantation have been reported. Some studies reported that during time after implantation the numbers of proliferating cells increased [20, 23], whereas decreasing cell proliferation was also reported [24]. Our results showed a decrease of proliferating cells starting after 14 days, which is in accordance with the findings reported on by Tomakidi *et al* [24]. The expression pattern of the proliferating cells suggests that TEM upon implantation needs time to adapt and then proliferation rate increases during the first 7 days. After this brief period, the cells of the basal layer actively proliferate to maintain a multilayered epithelium and that after 14 days the proliferation rate decreases to baseline and equilibrium is established.

It is well known that the basement membrane is essential for maintaining structural integrity [25, 26]. As collagen type IV is a major component of the basement membrane we assessed it's expression pattern in the harvested TEM constructs. A previous study performed in our laboratory showed that collagen type IV is expressed along the whole basement membrane of the scaffold and of TEM after a 14 day culture period. We could detect collagen type IV 7 days post-implantation, but it was not untill 28 days post-implantation that collagen type IV was expressed continously along the whole basement membrane. These results suggests active remodelling of the basement membrane upon implantation.

As the results in immunodeficient mice were positive, the next step is to make TEMs suitable for clinical application. In several publications tissue engineered mucosal equivalents have been implanted into the oral cavity of patients diagnosed with oral squamous cell carcinoma or oral premalignant lesions such as dysplasia [21, 27-28]. Izumi for instance reported on a more mature, differentiated epithelium and inflammatory response within the dermal layer after implantation in biopsies taken four weeks post-implantation. Their histological findings are very similar to the overall histology of our TEMs at four weeks post-implantation. These similarities suggest that TEM might act accordingly after implantation into the oral cavity of patients thereby supporting the clinical potential of our model.

ACKNOWLEDGEMENTS

The authors would like to thank Iris Janssen for her expertise while carrying out the animal experiments, and the Erasmus MC Tissue Bank, department of Pathology for facilitating the virtual microscope.

REFERENCES

1. Neligan, P.C., P.J. Gullane, R.W. Gilbert (2003) Functional reconstruction of the oral cavity. World J Surg *27(7):* 856-62.
2. Avery, C.M., J. Pereira, A.E. Brown (2001) Suprafascial dissection of the radial forearm flap and donor site morbidity. Int J Oral Maxillofac Surg *30(1):* 37-41.
3. Kimata, Y., K. Uchiyama, S. Ebihara, M. Saikawa, R. Hayashi, *et al* (2000) Postoperative complications and functional results after total glossectomy with microvascular reconstruction. Plast Reconstr Surg *106(5)*: 1028-35.
4. Kriegebaum, U., M. Mildenberger, U.D. Mueller-Richter, U. Klammert, A.C. Kuebler, *et al* (2012) Tissue engineering of human oral mucosa on different scaffolds: in vitro experiments as a basis for clinical applications. Oral Surg Oral Med Oral Pathol Oral Radiol 114(5 Suppl):S190-8.
5. Moharamzadeh, K., I.M. Brook, R. Van Noort, A.M. Scutt, M.H. Thornhill (2007) Tissue-engineered oral mucosa: a review of the scientific literature. J Dent Res *86(2):* 115-24.
6. Tra, W.M., J.W. van Neck, S.E. Hovius, G.J. van Osch, S. Perez-Amodio (2012) Characterization of a three-dimensional mucosal equivalent: similarities and differences with native oral mucosa. Cells Tissues Organs *195(3):* 185-96.
7. Ophof, R., R.E. van Rheden, H.J. Von den, J. Schalkwijk, A.M. Kuijpers-Jagtman (2002) Oral keratinocytes cultured on dermal matrices form a mucosa-like tissue. Biomaterials *23(17):* 3741-8.

8. Izumi, K., J. Song, S.E. Feinberg (2004) Development of a tissue-engineered human oral mucosa: from the bench to the bed side. Cells Tissues Organs *176(1-3):* 134-52.
9. Boyce, S.T., G.D. Warden (2002) Principles and practices for treatment of cutaneous wounds with cultured skin substitutes. Am J Surg *183(4):* 445-56.
10. Tra, W.M., B. Tuk, J.W. van Neck, S.E. Hovius, S. Perez-Amodio (2013) Tissue-engineered mucosa is a suitable model to quantify the acute biological effects of ionizing radiation. Int J Oral Maxillofac Surg *42(8):* 939-48.
11. Klausner, M., S. Ayehunie, B.A. Breyfogle, P.W. Wertz, L. Bacca, *et al*. (2007) Organotypic human oral tissue models for toxicological studies. Toxicol In Vitro *21(5):* 938-49.
12. Pena, I., L.M. Junquera, S. Llorente, L. de Villalain, J.C. de Vicente, *et al* (2012) Clinical outcomes after the use of complete autologous oral mucosa equivalents: preliminary cases. Oral Surg Oral Med Oral Pathol Oral Radiol *113(5):* e4-e11.
13. Sauerbier, S., R. Gutwald, M. Wiedmann-Al-Ahmad, G. Lauer, R. Schmelzeisen (2006) Clinical application of tissue-engineered transplants. Part I: mucosa. Clin Oral Implants Res *17(6):* 625-32.
14. Ophof, R., J.C. Maltha, A.M. Kuijpers-Jagtman, J.W. Von den Hoff (2008) Implantation of tissue-engineered mucosal substitutes in the dog palate. Eur J Orthod *30(1)*: 1-9.
15. Sumi, Y., K.I. Hata, Y. Sawaki, H. Mizuno, M. Ueda (1999) Clinical application of cultured oral epithelium for palatal wounds after palatoplasty: a preliminary report. Oral Dis *5(4):* 307-12.

16. Boyce, S.T., A.P. Supp, M.D. Harriger, D.G. Greenhalgh, G.D. Warden (1995) Topical nutrients promote engraftment and inhibit wound contraction of cultured skin substitutes in athymic mice. J Invest Dermatol *104(3):* 345-9.
17. Perez-Amodio, S., W.M. Tra, H.A. Rakhorst, S.E. Hovius, J.W. van Neck (2011) Hypoxia pre-conditioning of tissue-engineered mucosa enhances its angiogenic capacity in vitro. Tissue Eng Part A *17(11-12):* 1583-93.
18. El Ghalbzouri, A., E. Lamme, M. Ponec (2002) Crucial role of fibroblasts in regulating epidermal morphogenesis. Cell Tissue Res *310(2):* 189-99.
19. Barrandon, Y., V. Li, H. Green (1988) New techniques for the grafting of cultured human epidermal cells onto athymic animals. J Invest Dermatol *91(4):* 315-8.
20. Pena, I., L.M. Junquera, A. Meana, E. Garcia, C. Aguilar, *et al* (2011) In vivo behavior of complete human oral mucosa equivalents: characterization in athymic mice. J Periodontal Res *46(2):* 214-20.
21. Izumi, K., S.E. Feinberg, H. Terashi, C.L. Marcelo (2003) Evaluation of transplanted tissue-engineered oral mucosa equivalents in severe combined immunodeficient mice. Tiss Eng *9(1):* 163-74.
22. Jain, R.K., P. Au, J. Tam, D.G. Duda, D. Fukumura (2005) Engineering vascularized tissue. Nat biotechnol *23(7):* 821-3.
23. Yoshizawa, M., S.E. Feinberg, C.L. Marcelo, V.M. Elner (2004) Ex vivo produced human conjunctiva and oral mucosa equivalents grown in a serum-free culture system. J Oral Maxillofac Surg *62(8)*: 980-8.
24. Tomakidi, P., D. Breitkreutz, N.E. Fusenig, J. Zoller, A. Kohl, *et al* (1998) Establishment of oral mucosa phenotype in vitro in correlation to epithelial anchorage. Cell Tissue Res *292(2):* 355-66.

25. Paulsson, M. (1992) Basement membrane proteins: structure, assembly, and cellular interactions. Crit Rev Biochem Mol Biol *27(1-2):* 93-127.

26. Kairuz, E., Z. Upton, R.A. Dawson, J. Malda (2007) Hyperbaric oxygen stimulates epidermal reconstruction in human skin equivalents. Wound Repair Regen *15(2):* 266-74.

27. Hotta, T., S. Yokoo, H. Terashi, T. Komori (2007) Clinical and histopathological analysis of healing process of intraoral reconstruction with ex vivo produced oral mucosa equivalent. Kobe J Med Sci *53(1-2):* 1-14.

28. Lauer, G., R. Schimming (2001) Tissue-engineered mucosa graft for reconstruction of the intraoral lining after freeing of the tongue: a clinical and immunohistologic study. J Oral Maxillofac Surg *59(2):* 169-75; discussion 75-7.

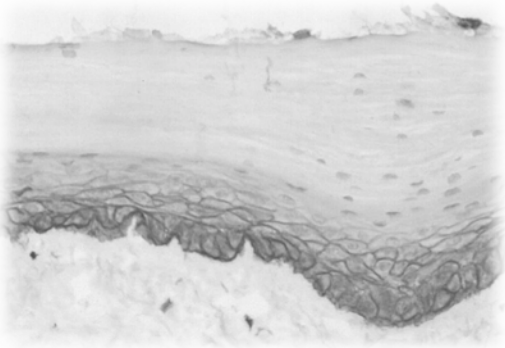
CHAPTER 6

Tissue-engineered mucosa is a suitable model to quantify the acute biological effects of ionizing radiation

Wendy M.W. Tra, Bas Tuk, Johan W. van Neck, Steven E.R. Hovius, Soledad Perez-Amodio

Department of Plastic & Reconstructive Surgery, Erasmus MC, Rotterdam, the Netherlands

Published in *International Journal Oral & Maxillofacial Surgery 2013; 42(8): 939-948*



ABSTRACT

The aim of this study was to evaluate the suitability of tissue-engineered mucosa (TEM) as a model for studying the acute effects of ionizing radiation (IR) on oral mucosa. Therefore, TEM and native non-keratinizing oral mucosa (NNOM) were exposed to a single dose of 16.5Gy and harvested at 1, 6, 24, 48 and 72 hours post-irradiation. DNA damage induced by IR was determined by using 53BP1 and DNA repair was determined using Rad51. Various components of the epithelial layer, basement membrane (BM) and underlying connective tissue were analysed using immunohistochemistry. The expression of cytokines IL-1β and TGFβ1 was analysed using ELISA. The expression of DNA damage protein 53BP1 and repair protein Rad51 were increased post-irradiation. The expression of K19, vimentin, collage type IV, desmoglein-3, integrin α6 and β4 were altered post-irradiation. Proliferation significantly decreased at 24, 48 and 72h post-irradiation in both NNOM and TEM. IR increased the secretion of IL-1β whereas TGFβ1 secretion was not altered. All observed IR induced alterations in TEM were also observed in NNOM. Based on the similar response of TEM and NNOM to IR we consider our TEM construct a suitable model to quantify the acute biological effects of IR.

INTRODUCTION

The field of tissue engineering of mucosal equivalents has significantly developed during the last 10 years. We and others have reported on developing tissue-engineered mucosal constructs using autologous keratinocytes seeded onto biomaterials whether or not repopulated with fibroblasts [1-3]. Tissue-engineered mucosal constructs (TEM) have been successfully used to study dynamics of wound healing [4] and to test cytotoxicity and working mechanisms of new treatments [3]. A major advantage of TEM over the use of NNOM is in limited availability of NNOM tissue, as from a single biopsy many cells can be cultured and multiple TEM constructs of consistent quality can be engineered, therefore many different parameters can be studied in a single experiment.

Radiotherapy, essential during treatment of oral cancer, is well known to not only kill tumor cells but also to damage the surrounding healthy tissue. This damage to healthy tissue manifests in a certain order. The first stage, characterized by erythema, is followed by the manifestation stage, sub-acute stage, chronic stage and finally the late stage each with their specific characteristics. Many studies have reported on the side effects of IR in oral mucosa. These side effects include erythema, dry or moist desquamation or ulceration [5, 6] and they can be very severe resulting in interruption or termination of the radiation treatment.

Immediately after exposure to IR, several biological processes occur. Certain proteins such as 53BP1 and γH2Ax are elevated within 5 minutes after IR, both indicating the presence of DNA double strand breaks, a well documented result of IR [7, 8]. Additionally a number of cytokines, such as interleukin (IL)-1, IL-6, IL-8, tumor necrosis factor (TNF)-α, transforming growth factor (TGF)-β, are increased, thereby initiating the biological response to IR [9].

Keratins are the predominant proteins found in the epithelial layer and as the expression of certain keratins is a clear indication of the maturation and stratification of the epithelium, we studied keratin expression in the epithelium in non-irradiated and irradiated TEM [10]. As mentioned earlier, radiotherapy is known to severely damage tissues, we used markers for cell proliferation and apoptosis to evaluate the viability of non-irradiated and irradiated TEM. To study if radiotherapy affects the structural integrity of TEM we evaluated the expression of (hemi-) desmosomal proteins, and the expression of major basement membrane component collagen type IV.

The aim of this study is to evaluate the suitability of TEM as a model for studying the acute effects of IR on oral mucosa. Therefore, we evaluate epithelial proliferation and differentiation, expression of basement membrane components, cell-cell adhesion, attachment of the epithelium to the underlying connective tissue and DNA damage and repair on TEM. Additionally, we evaluate the presence of pro-inflammatory cytokines in the conditioned culture media of irradiated and non-irradiated TEM. Finally, the results observed in TEM are compared with those observed in NNOM.

MATERIALS AND METHODS

Cell culture

Biopsies of approximately 2 cm^2 buccal tissue were taken from 4 healthy individuals (3 males and 1 female, all Caucasian, aged 62.75 ± 6.39) upon informed consent. Single cell suspensions of keratinocytes or fibroblasts were obtained as described before [2]. Briefly, keratinocytes were isolated from the epithelial sheet by incubation in trypsin-EDTA and the single cell suspension was seeded onto lethally irradiated 3T3 fibroblast feeder layers, according to the

Rheinwald & Green protocol [11, 12]. Fibroblasts were isolated by mincing the dermis using scalpels. This was followed by incubation in collagenase/dispase (1.5 mg/mL / 2.5 mg/mL, respectively) solution to obtain a single cell suspension. The cells used in this study were within passage 3 to 6.

Preparation of de-epidermized dermis

Human cadaver skin, cryopreserved in 10% glycerol, and tested negative for cytomegalovirus, human immunodeficiency virus and hepatitis B, was obtained from the Euro Skin Bank (Beverwijk, the Netherlands). The epidermis was removed by gently shaking the skin in PBS supplemented with 200 IU/ml penicillin, 200 µg/ml streptomycin and 5 µg/ml amphotericin B. The skin was kept in the PBS solution for 3 weeks and PBS was changed 3 times a week. The de-epidermized dermis (DED) was trimmed in pieces of approximately 1 cm^2.

Tissue engineered mucosa (TEM)

TEM was created as described before [2]. Briefly, per construct 1 x 10^5 fibroblasts were spun into the lamina propria of the DED [1] and 1 x 10^6 keratinocytes were seeded into a steel ring (diameter 10 mm) placed onto the papillary side of the DED. This was kept under submerged conditions for 24 hours. Next, the construct was raised to the air/liquid interface and cultured for 14 days before harvesting. Each test was done in triplicate and three independent replicates of the experiment were performed.

Radiation protocol

TEM and NNOM were gamma-irradiated with 16.5Gy, a dose based on preliminary studies in our laboratory (unpublished data), using a 137-Cs source.

The control group was not irradiated. TEM constructs and NNOM were cultured at 37^{o}C, 10% CO_2 and harvested 1, 6, 24, 48 or 72 hours post-irradiation by snap freezing with liquid nitrogen for cryo-sectioning.

Histology and collection TEM-conditioned culture media

Culture media was collected prior to harvesting of TEM, centrifuged at 400*g* for 5 minutes at 4^{o}C and stored at -80^{o}C until further analysis. Cryosections (6µm) were stained with Hematoxylin-Eosin (HE) (Klinipath, Duiven, the Netherlands) and overall morphology was assessed using a light microscope (Olympus). The thickness of the viable epithelium was determined from 2 consecutive images and the average thickness (µm) was measured using Hamamatsu software (Hamamatsu Photonics, Japan) by averaging 12 measurements per image.

Immunohistochemistry

For immunohistochemical analysis, sections were fixed for 10 minutes with acetone. After incubation with primary antibodies for K10 (1:200; Euro-Diagnostica), K13 (1:200; Euro-Diagnostica, K19 (1:500; Novus Biologicals, collagen type IV (1:500; Euro-Diagnostica), Ki-67 (1:200; DAKO), cleaved caspase-3 (1:1500; Abcam), vimentin (1:200; Euro-Diagnostica), desmoglein-3 (1:500; Novus Biologicals), integrin α6 (1:1000; Novus Biologicals) or β4 (1:2000; Novus Biologicals), sections were incubated with diaminobenzidin (DAB) substrate or New Fuchsin substrate. All sections were counterstained using Mayer's Haematoxylin. To visualize DNA damage and repair sections were fixed with 4% formaldehyde in PBS for 15 minutes. After incubation with primary antibody for either 53BP1 or Rad51, sections were stained with secondary antibody Alexa Fluor 594 (1/1000, Goat anti-Rabbit IgG, Molecular Probes,

Leiden, the Netherlands). Finally the sections were mounted using DAPI/DAPCOA/VectaShield to visualize cell nuclei. Positive controls were biopsies of NNOM and for the negative controls PBS replaced the primary antibody.

Quantification of radiation damage and repair

DNA damage was quantified by counting the number of cells containing DNA DSBs in both the epithelial layer and the connective tissue in 12 randomly chosen microscopic views (100 x magnification). The index was established as the ratio of the positive cells to all the cells in the basal layer or connective tissue (x 100 %). Quantification of repair, using Rad51, was done similarly. Only sections from TEM harvested at 1, 6 and 24h were quantified as the characteristic kinetics of both 53BP1 and Rad51 take place within 24h post-irradiation.

Proliferation and apoptosis index

To determine the proliferation index (PI), the basal layer of the epithelium was analyzed. Images were taken from 12 randomly chosen microscopic views using a 100 x magnification. The PI was established as the ratio of the Ki-67 positive cells to all cells of the basal layer (x 100 %). Apoptotic cells were detected using an antibody against cleaved caspase-3. The apoptotic index (AI) was established as the ratio of the caspase-3 positive cells to all the cells in the basal layer or connective tissue (x 100 %).

ELISA assay on conditioned media

Concentration of interleukin-1 beta (IL1-β), TGFβ, tissue inhibitor of matrix metalloproteinase (TIMP-1 and TIMP-2) in the conditioned culture media was measured using the DuoSet sandwich ELISA kits according to the manufacturers instructions (R&D Systems). Results are expressed as pg or ng/cm^2 tissue with each sample consisting of 4 mL supernatant derived from 1 cm^2 tissue.

Zymography for MMP-2 and MMP-9

Gelatinolytic proteinases in conditioned culture media were assessed using zymography. Samples of 10 µL conditioned media were 1:1 diluted with sample buffer (0.1 M Tris-HCl, 4 % SDS, 20 % glycerol, 0.00 5% bromopherol blue,
10 mM EDTA) were loaded onto a 10 % polyacrylamide gel containing 2 % gelatin. After electrophoreses gels were washed with 2.5 % (v/v) Triton X-100 to remove the SDS. After overnight incubation at 37 ^{o}C in incubation buffer consisting of 50 mM Tris-HCl, 1 % Triton X-100 and 5 mM $CaCl_2$ gels were stained with Coomassie brilliant blue. MMP-2 and MMP-9 appeared as unstained bands. Gels were scanned using a Kodak Image Station 440CF (Kodak) and relative intensity of each band was quantified.

Native oral non-keratinizing mucosa (NNOM)

To determine whether the observations in TEM regarding proliferation and expression patterns of components of the epithelium, BM and connective tissue post-irradiation, are similar to those in native oral mucosa we exposed biopsies of native non-keratinizing oral mucosa (NNOM) to 16,5 Gy and assed the above mentioned components 1, 6, 24, 48 and 72h post-irradiation using immunohistochemistry.

Statistical Analysis

All data are expressed as mean ± SEM. Tests of normality were done using the Shapiro-Wilk W test using SPSS software (IBM Nederland, the Netherlands). Statistical analyses were performed using Student *t* test using SPSS software. Comparisons between group means were made using Tukey-Kramer test using GraphPad Instat software (GraphPad Software, USA). *P*-values ≤ 0.05 were considered significant.

RESULTS

Confirmation radiation damage

To confirm that a single dose of 16.5 Gy did result in DNA double strand breaks (DSBs), the number of cells containing 53BP1 foci was analyzed. As shown in fig. 1A the number of 53BP1 positive cells in irradiated TEM significantly increased 1.7-fold 1 h post-irradiation. After 6 and 24 h, a 1.3-fold and 1.6-fold increase was observed, respectively, when compared with non-irradiated TEM. Additionally, DNA repair was quantified and as shown in fig. 1B, 1 and 6 h post-irradiation a 1.3-fold and 1.7-fold increase, respectively, in irradiated TEM was observed as compared to non-irradiated TEM. After 24 h the number of Rad51 positive cells in irradiated TEM returned to baseline.

Overall morphology of irradiated TEM was not affected 1 and 6 h post-irradiation (Fig. 2A-B) when compared to non-irradiated TEM (Fig. 2F-G). First changes in morphology were observed 24 h post-irradiation (Fig. 2C and H) as the basal layer became irregular and pyknotic cells appeared the basal and intermediate layer. These changes became more pronounced 48 and 72 h post-irradiation (Fig. 2D-E) when compared to non-irradiated TEM (Fig. 2I-J).

Measurements of the viable epithelium showed a significant decrease in the thickness of irradiated TEM 24, 48 and 72 h post-irradiation as compared with non-irradiated TEM (Fig. 2K).

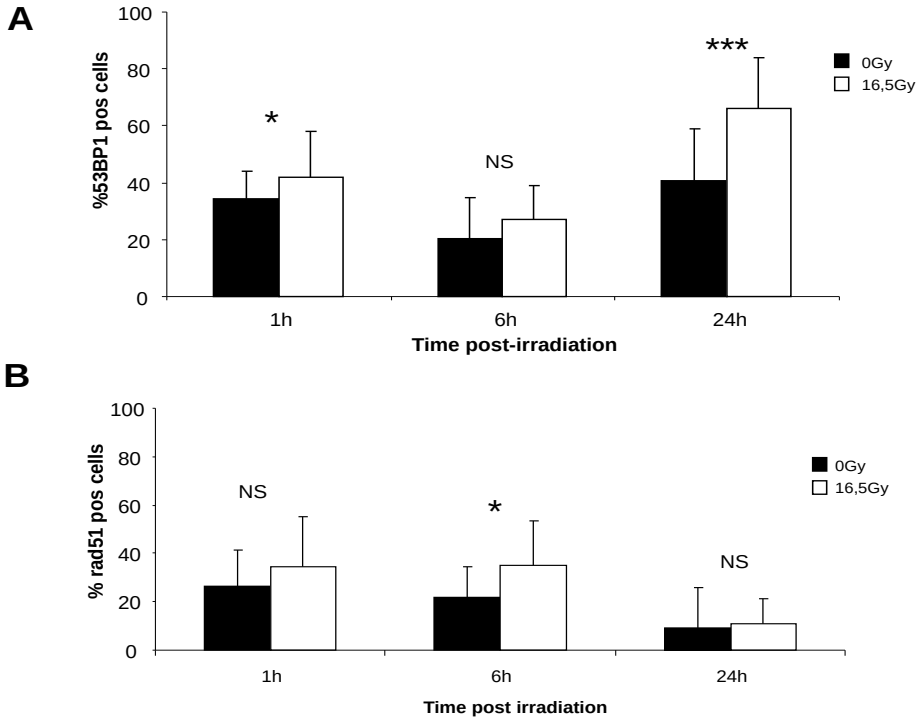


Figure 1. DNA damage was assessed with (A) 53BP1. The number of 53BP1-positive cells in both native non-keratinizing oral mucosa (NNOM) and tissue-engineered mucosa (TEM) significantly increased post-irradiation. DNA repair was assessed using (B) Rad51.

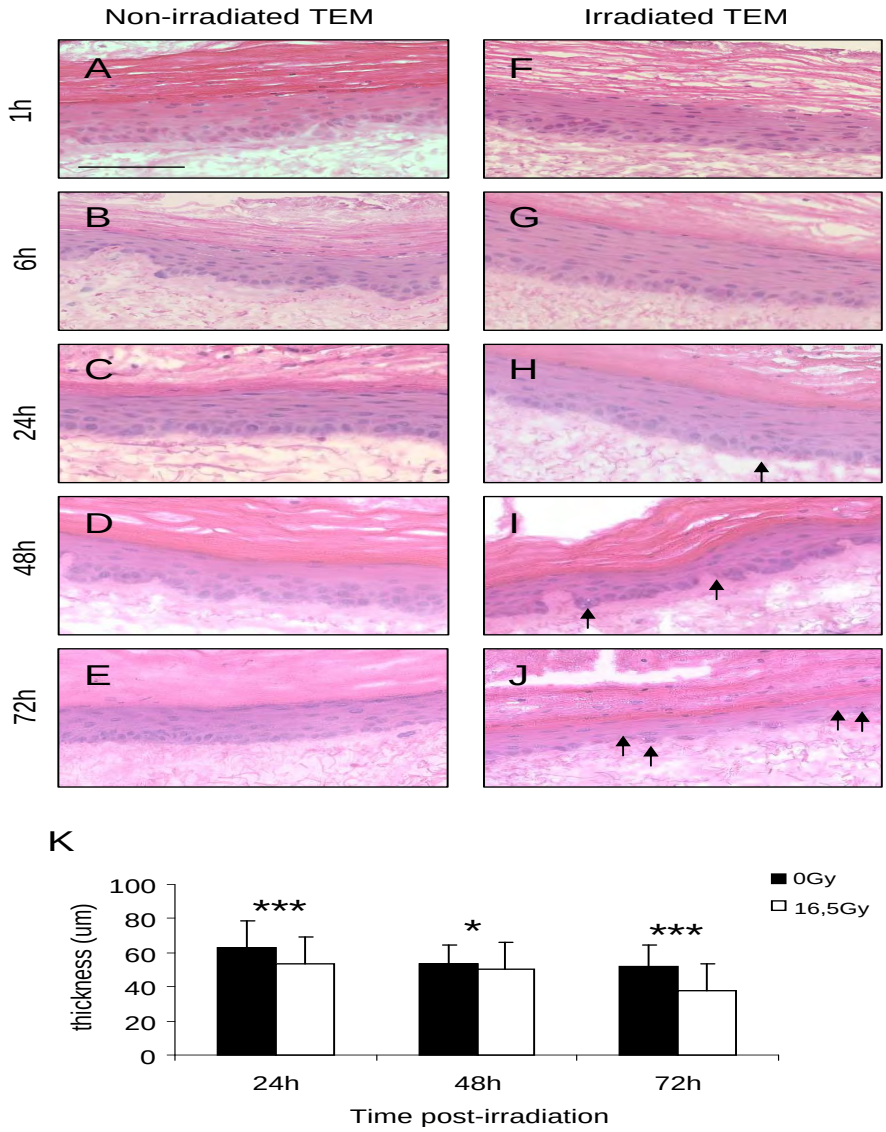


Figure 2. Overall morphology (assessed by hematoxylin-eosin staining) showed a multi-layered, parakeratinized epithelium in (A-E) non-irradiated tissue-engineered mucosa (TEM) and (F-J) irradiated TEM. Note the appearance of irregular-shaped cells and pyknotic cells in the basal and intermediate layer of the epithelium of irradiated TEM at (H) 24 h, (I) 48 h, and (J) 72 h post-irradiation (arrows). (K) The thickness of the viable epithelium significantly decreased post-irradiation. Magnification 100x. Scale bar = 100 µm

Proliferating cells were observed in the basal layer only of TEM and IR did not change this pattern (Fig. 3A; indicated with arrows). After exposure to IR, the PI significantly decreased at all time points compared with non-irradiated TEM (Fig. 3B). Analysis of the cleaved caspase-3 staining revealed only a few apoptotic cells in the basal layer and the connective tissue of TEM after irradiation at all time points. After IR the number of apoptotic cells increased in irradiated TEM when compared with non-irradiated TEM but these differences were not significant (data not shown).

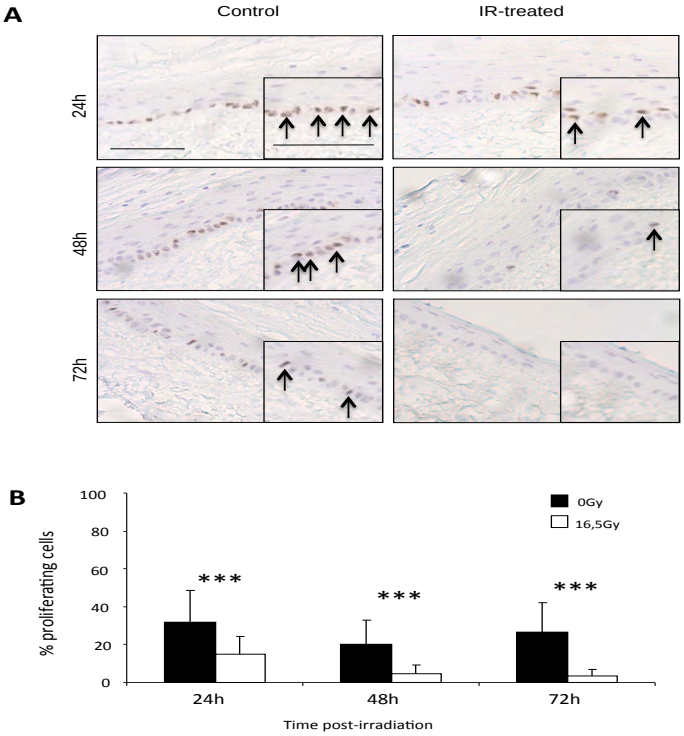


Figure 3. Proliferating cells were observed in the basal layer of both (A) non-irradiated tissue-engineered mucosa (TEM) and irradiated TEM. (B) Quantification of the number of proliferating cells showed a significant decrease at 24, 48, and 72 h post-irradiation. Scale bar = 100 µm.

Immunohistochemical staining for cytokeratins 10 and 13 revealed no differences in epithelial differentiation between irradiated TEM and non-irradiated TEM (data not shown). Keratin 19 positive cells were observed in the basal layer of the epithelium (Fig. 4A-B; indicated with arrows). Expression decreased after exposure to IR and quantification of K19 positive cells showed a 1.2-fold, 1-fold and 1.2-fold decrease at 24, 48 and 72 h post-irradiation, respectively (data not shown).

Assessment basement membrane and connective tissue

Collagen type VI was present along the whole dermal-epithelial junction in TEM and this expression pattern remained unchanged up to 24 h. Forty-eight and 72 h post-irradiation the staining intensity decreased and in small regions of the BM collagen type IV was not detectable (Fig. 4C-D).

Hemidesmosomal proteins integrin α6 and β4 were also expressed along the whole dermal-epithelial junction of TEM. Irradiation did decrease staining intensity and reduced the number of cell layers expressing integrin α6 (Fig. 4E-F) and β4. Desmosomal protein desmoglein-3 was expressed in the basal and intermediate layer of the epithelium of TEM and this expression pattern did not change up to 24 h post-irradiation in irradiated TEM when compared with non-irradiated TEM. After 48 and 72 h, in irradiated TEM desmoglein-3 is no longer expressed in the intermediate layer as compared to non-irradiated TEM (Fig. 4G-H).

Vimentin staining showed that fibroblast distribution in the connective tissue in irradiated TEM was similar to that observed in non-irradiated TEM. From 24h up

to 72 h post-irradiation, vimentin positive cells were observed in the epithelial layer of irradiated TEM, whereas no vimentin positive cells were observed in the epithelial layer of non-irradiated TEM (Fig. 4I-J).

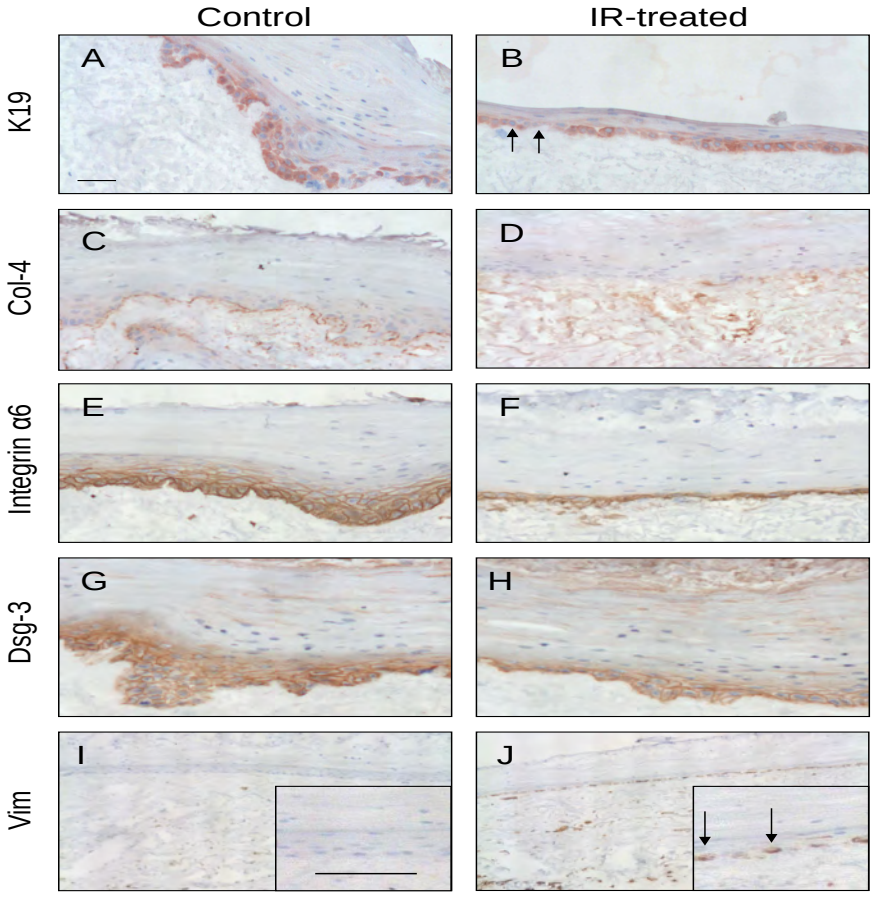


Figure 4. (A-B) Histological appearance of non-irradiated tissue-engineered mucosa (TEM) and irradiated TEM at 72 h post-irradiation showed that the number of K19-positive cells decreased post-irradiation (indicated with arrows). (C-D) Collagen type IV deposition in the basement membrane was not present at 72 h post-irradiation. (E-F) Integrin α6 and (G-H) desmoglein-3 expression was reduced after ionizing radiation (IR). (I) Vimentin was not observed in the epithelium of TEM. (J) Exposure to IR induced vimentin expression in the basal layer of irradiated TEM (indicated with arrows). Scale bar = 100 µm.

Assessment cytokines and TIMP in irradiated TEM

To study the effect of IR on secretion of inflammatory cytokines, the secretion of IL-1β and TGFβ1 was measured in conditioned culture media of TEM. IR increased the secretion of IL-1β after 1 h (1.6-fold), 6 h (1.4-fold), 24 h (1.1-fold) and 72 h (1.5-fold) when compared with non-irradiated TEM (Fig. 5A). The activation of TGFβ1 was increased after IR at 1 h (1.1-fold), 6 h (1.1-fold), 24 h (1.0-fold), 48 h (1.1-fold) and 72 h (1.2-fold) (Fig. 5B). Total TGFβ1 activation showed no differences between irradiated and non-irradiated samples and total TGFβ1 levels did not change during culture up to 72 h after treatment (data not shown). IR decreased TIMP-1 secretion after 6 h (1.3-fold), 24 h (2.8-fold), 48 h (1.3-fold) and 72 h (1.1-fold) as compared with non-irradiated TEM (Fig. 5C). TIMP-2 secretion was not increased after IR (Fig. 5D).

Zymography MMP-2 and MMP-9

As IR can increase the secretion of matrix metalloproteinases (MMP) through TGF-β, we analyzed the activity of MMPs in TEM by gelatin zymography. The gels as shown in figure 4E showed bands at 92 and 82 kDa, pro-active MMP-9 and active MMP-9 respectively for non-irradiated TEM (indicated with -) and irradiated TEM (+). Bands were also observed at 72 and 66 kDa indicating pro-active MMP-2 and active MMP-2 respectively. Quantification of zymography gels showed that IR decreased the activity of pro-MMP-9 or active MMP-9 (Fig. 5F-G). Pro-MMP-2 activity was increased 72 h post-irradiation only when compared to non-irradiated TEM, whereas active MMP-2 levels were only elevated 48h post-irradiation (Fig. 5H-I).

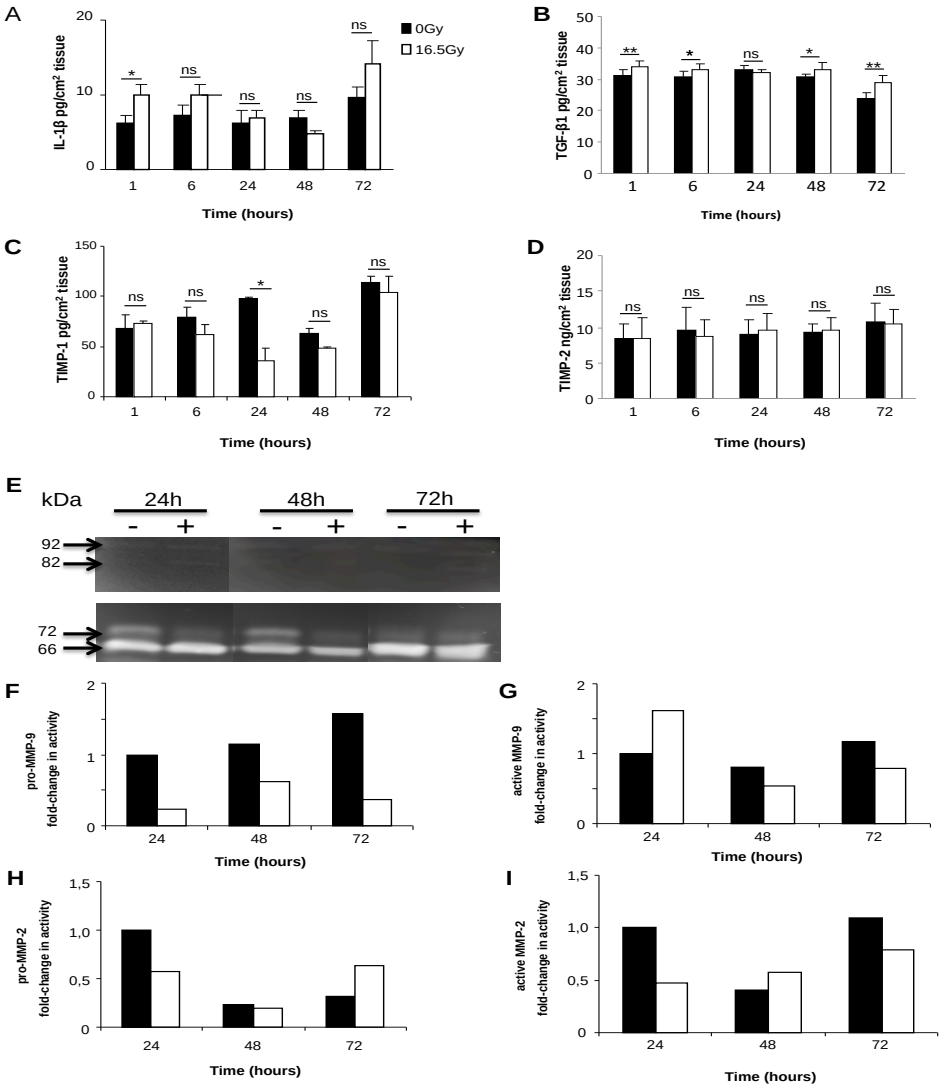


Figure 5. Secretion of (A) IL-1β, (B) TGF-β1, (C) TIMP-1 and (D) TIMP-2 by non-irradiated TEM (closed bars) and irradiated TEM (open bars) measured at 1, 6, 24, 48, and 72 h post-irradiation. (E) Gelatin zymograms showed decreased MMP-9 (92 and 82 kDa inactive proenzyme and active form, respectively) and MMP-2 (72 and 66 kDa inactive proenzyme and active form, respectively). The fold-change in (F-G) MMP-9 and (H-I) MMP-2 activity in non-irradiated TEM (closed bars) and irradiated TEM (open bars) was determined using densitometry. Il., interleukin; TGF, transforming growth factor; TIMP, tissue inhibitor of matrixmetalloproteinase; MMP, matrix metalloproteinase.

Assessment effects IR on NNOM

To verify the effects of IR on TEM, all above-mentioned parameters were assessed in irradiated NNOM and compared these with non-irradiated NNOM. First DNA damage in NNOM was assessed and irradiated NNOM revealed a 1.7-fold and 1.5-fold increase of 53BP1 positive cells 1 and 6 h post-irradiation, respectively, as compared with non-irradiated NNOM (data not shown).

Next, analysis of the HE stain revealed that first changes in overall morphology of NNOM post-irradiation were observed 24 h post-irradiation as from 24 up to 72 h post-irradiation, pyknotic cells were observed in the basal layer (Fig. 6A-B). Compared to non-irradiated NNOM, thickness of the viable epithelium decreased 1.3-fold 24 h post-irradiation and 1.7-fold and 2.6-fold 48 and 72 h post-irradiation, respectively (data not shown). Similarly to TEM, K10 and K13 expression (Fig. 6C-D) were unchanged post-irradiation. Quantification of the number of K19 positive cells showed a 1-fold, 1.2-fold and 1.1-fold decrease when compared with non-irradiated NNOM (data not shown). The number of proliferating cells in irradiated NNOM decreased 1.9-fold 24 h post-irradiation and 1.4-fold and 1.1-fold at 48 and 72 h post-irradiation, respectively, when compared to non-irradiated NNOM. Comparable to our observations in TEM, only few apoptotic cells were observed in both non-irradiated and irradiated NNOM (data not shown). Additionally, assessment of BM components and connective tissue revealed that collagen type IV staining intensity in non-irradiated NNOM (Fig. 6E) was more intense as compared to irradiated NNOM (Fig. 6F). Integrin α6 and β4 were observed along the whole basement membrane. Post-irradiation the number of cell layers expressing integrin α6 and β4 decreased as illustrated in figure 6G-H. Desmoglein-3, expressed in the basal and intermediate layer of non-irradiated NNOM (Fig. 6I), was no longer

expressed in the intermediate layer in irradiated NNOM 72 h post-irradiation (Fig. 6J). Additionally, vimentin staining, only observed in the connective tissue in non-irradiated NNOM, was observed in the basal layer from 24 up to 72 h post-irradiation as shown in figure 6K-L.

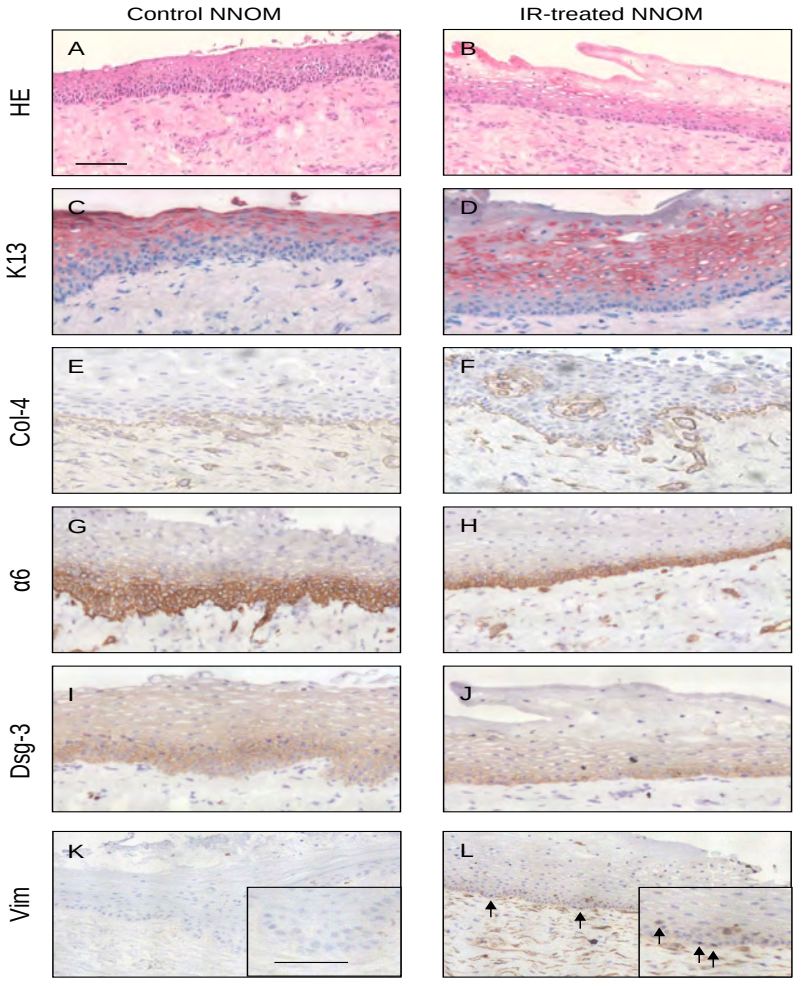


Figure 6. (A-B) Histological appearance of native non-keratinizing oral mucosa (NNOM) at 72 h post-irradiation after hematoxylin-eosin staining. (C-D) K13 expression and (E-F) collagen type IV deposition was unaltered at 72 h post-irradiation. (G-H) Integrin α6 and (I-J) desmoglein-3 expression was reduced after IR. (K-L) In irradiated NNOM, vimentin-positive cells were observed in the basal layer. Scale bar = 100 µm.

DISCUSSION

In this study we demonstrated that structural changes in oral mucosa induced by IR can be studied using TEM as IR affects TEM in a similar manner as native oral mucosa. The normal morphology of TEM is preserved up to 24h post-irradiation as shown by HE stain and expression of keratin 10 and 13. Thereafter basal cell degeneration, decreased proliferation, thinning of the epithelium and reduced K19 and collagen type IV expression indicated profound structural changes after exposure to one single high dose of ionizing radiation.

The presence of 53BP1 foci has been used to determine the degree of damage in cells and 53BP1 plays an important role in the repair of DNA DSBs as it interacts with DNA repair proteins. 53BP1 foci have been described to form within 5 minutes post-irradiation reaching a maximum at 15 to 30 minutes, and then decreases to baseline levels within 16h post-irradiation [7]. Interestingly, this characteristic kinetic pattern was not observed, as after 24 h the number of 53BP1 foci increased in TEM and NNOM. The presence of persistent 53BP1 foci in a tissue-engineered construct has been shown before [13] and their appearance could indicate irreparable DNA DSBs that most often result in apoptosis. Therefore the presence of cleaved caspase-3, an executioner caspase that becomes active upon the first signaling events of apoptosis, was studied. We found that the number of apoptotic cells increased post-irradiation, confirming that cells containing persistent 53BP1 foci will undergo programmed cell death. To be able to survive IR, TEM has to be capable to respond to damaging external influences. The finding that the presence of DNA repair protein Rad51 was more abundant post-irradiation suggests that TEM is able to respond to the DNA damage, but that the damaged induced by a single high dose of IR could not be completely repaired.

It is well known that cell-cell adhesion and attachment of the epithelium to the underlying connective tissue is essential for structural integrity [14]. Desmosomes are complexes of cell adhesion proteins involved in cell-cell attachment [14] and provide a transcellular network to resist mechanical stress [15]. The observation that desmoglein-3 and integrin α6 and β4 expression decreased post-irradiation could indicate a loss of functionality or integrity and might ultimately lead to ulceration op the epithelium [14]. As the basement membrane is also important for maintaining structural integrity [16] we assessed the expression pattern of collagen type IV, a major component of the BM. Previous study showed that collagen type IV was expressed along the whole basement membrane [2]. The observation that collagen type IV was not detectable 48 and 72h post-irradiation in both irradiated TEM and NNOM could indicate that IR induced remodeling of the basement membrane.

Morphological observations have indicated that alterations in the epithelial layer are preceded by changes in the connective tissue [17]. This prompted us to study the distribution of fibroblasts in the connective tissue post-irradiation. Our results indicated that IR did no affect fibroblast distribution. Interestingly, we did find vimentin positive cells in the epithelium post-irradiation. Normally, oral epithelium does not express vimentin, as observed in non-irradiated TEM and NNOM. However, when exposed to stress epithelial cells have been shown to express vimentin [18]. The presence of vimentin expression in the epithelial layer of TEM and the NNOM confirms the extensive stress in both models after exposure to IR.

The role of TGF-β1 in the cascade of events immediately following IR has been proven in multiple tissues including skin, lung and intestine [19, 20]. In skin, IR is known to activate TGF-β1. Our results showed that a single dose of 16.5 Gy did

increase the levels of activated TGF-β1. This observation is confirmed by studies performed using irradiated pigskin that showed that doses of 16 Gy and higher induced TGF-β1 expression on both mRNA and protein level [20].

The observation that IL-1β secretion was increased after exposure to IR indicates the initiation of the biological response in TEM [9]. If the increased levels of IL-1β are maintained for prolonged periods of time this could be an important indicator of disturbed MMP secretion and regulation that may lead to fibrosis [9]. The observation that MMP-2 and MMP-9 activity was not increased after irradiation suggests that the extracellular matrix is not actively remodeling. Additionally, TIMP-1 and TIMP-2 levels did not change after IR, suggesting that the elevated IL-1β secretion after a single high dose of IR could not disrupt the homeostasis of the extracellular matrix of TEM.

In summary, our data suggest that TEM responds in a similar way to IR as NNOM. Therefore, TEM might help to study the underlying mechanisms regarding oral mucositis and radiation induced fibrosis. However, as TEM is engineered does not contain immune cells, like dendritic cells and Langerhans cells, or endothelial cells the total effects of irradiation cannot be studied yet. The inclusion of these cells is important, as radiation is known to alter immune functions, cytokine secretion for instance, of certain immune cells [21]. Future research would therefore include the addition of immune cells to TEM to obtain a more complete view of the acute biological effects of radiation and to study the effects of novel radiation protective agents or novel therapeutic treatments for oral mucositis and fibrosis.

Competing interests: None declared

Funding: Nuts Ohra

Ethical approval: This research was performed upon approval of the Medical Ethical Committee of the Erasmus MC. (#MEC + 2007-282).

REFERENCES

1. El Ghalbzouri A., E. Lamme, M. Ponec (2002) Crucial role of fibroblasts in regulating epidermal morphogenesis. Cell Tissue Res *310(2)*: 189-99.
2. Tra W.M., J.W. van Neck, S.E. Hovius, G.J. van Osch, S. Perez-Amodio (2012) Characterization of a three-dimensional mucosal equivalent: similarities and differences with native oral mucosa. Cells Tissues Organs *195(3)*:185-96.
3. Klausner M., S. Ayehunie, B.A. Breyfogle, P.W. Wertz, L. Bacca, *et al.,* (2007) Organotypic human oral tissue models for toxicological studies. Toxicol In Vitro *21(5):* 938-49.
4. Boyce S.T., G.D. Warden (2002) Principles and practices for treatment of cutaneous wounds with cultured skin substitutes. Am J Surg *183(4):* 445-56.
5. Sonis S.T. (2004) Pathobiology of mucositis. Semin Oncol Nurs *20(1):* 11-5.
6. Dorr W., C.S. Hamilton, T. Boyd, B. Reed, J.W. Denham (2002) Radiation-induced changes in cellularity and proliferation in human oral mucosa. Int J Radiat Oncol Biol Phys *52(Supll 1):* 911-7.
7. Mochan T.A., M. Venere, R.A. DiTullio Jr, T.D. Halazonetis (2004) 53BP1, an activator of ATM in response to DNA damage. DNA Repair (Amst) *3(8-9):* 945-52.
8. Rappold I., K. Iwabuchi, T. Date, J. Chen (2001) Tumor suppressor p53 binding protein 1 (53BP1) is involved in DNA damage-signaling pathways. J Cell Biol *153(3):* 613-20.

9. Muller K., V. Meineke (2007) Radiation-induced alterations in cytokine production by skin cells. Exp Hematol *35(4 Suppl 1):* 96-104.
10. Moll R., M. Divo, L. Langbein (2008) The human keratins: biology and pathology. Histochem Cell Biol *129(6):* 705-33.
11. Rheinwald J.G., H. Green (1975) Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. Cell *6(3):* 331-43.
12. Rheinwald J.G., H. Green (1975) Formation of a keratinizing epithelium in culture by a cloned cell line derived from a teratoma. Cell *6(3):* 317-30.
13. Su Y., J.A. Meador, C.R. Geard, A.S. Balajee (2010) Analysis of ionizing radiation-induced DNA damage and repair in three-dimensional human skin model system. Exp Dermatol *19(8):* 16-22.
14. Donetti E., M. Bedoni, G. Guzzi, P. Pigatto, C. Sforza (2009) An in vitro model of human oral explants to study early effects of radiation mucositis. Eur J Oral Sci *117(2):* 169-74.
15. Green K.J., J.C. Jones (1996) Desmosomes and hemidesmosomes: structure and function of molecular components. FASEB J *10(8):* 871-81.
16. Paulsson M. (1992) Basement membrane proteins: structure, assembly, and cellular interactions. Crit Rev Biochem Mol Biol *27(1-2):* 93-127.
17. Sonis S.T. (2004) The pathobiology of mucositis. Nat Rev Cancer *4(4):* 277-84.
18. Hansson A., B.K. Bloor, Z. Sarang, Y. Haig, P.R. Morgan, *et al.* (2003) Analysis of proliferation, apoptosis and keratin expression in cultured normal and immortalized human buccal keratinocytes. Eur J Oral Sci *111(1):* 34-41.

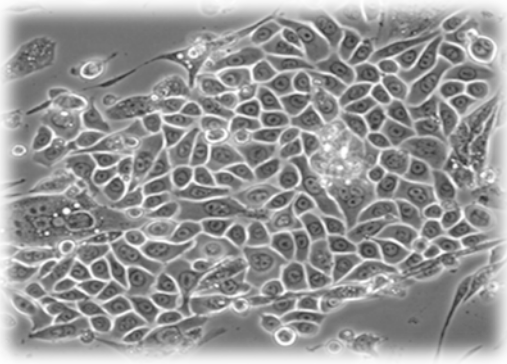
19. Martin M., M.C. Vozenin, N. Gault, F. Crechet, C.M. Pfarr, *et al.* (1997) Coactivation of AP-1 activity and TGF-beta1 gene expression in the stress response of normal skin cells to ionizing radiation. Oncogene *15(8):* 981-9.

20. Martin M., J. Lefaix, S. Delanian. TGF-beta1 and radiation fibrosis: a master switch and a specific therapeutic target? Int J Radiat Oncol Biol Phys *47(2):* 277-90.

21. Bechetoille N., C. Dezutter-Dambuyant, O. Damour, V. André, I. Orly, *et al.* (2007) Effects of solar ultraviolet radiation on engineered human skin equivalent containing both Langerhans cells and dermal dendritic cells. Tissue Eng *13(11)*: 2667-79.

CHAPTER 7

Summary, general discussion and future perspectives



CHAPTER 7

SUMMARY, GENERAL DISCUSSION AND FUTURE PERSPECTIVES

The aim of this thesis was to develop a tissue-engineered mucosal construct that 1) could be used for clinical applications and 2) is a representative model for in vitro research regarding harmful or beneficial environments as for instance radiation or hyperbaric oxygen therapy. Tissue engineering has already been proven a useful tool to successfully develop tissue equivalents as skin [1], bone [2], cartilage [3], blood vessels [4] and soft tissues [5]. We have developed a tissue-engineered mucosal equivalent (TEM) that strongly resembles native oral mucosa based on structure and functionality (Chapter 2). In this thesis we studied the potential of preconditioning TEM with either hypoxia or hyperbaric oxygen (HBO) treatment to enhance the secretion of angiogenic factors (Chapter 3 and 4). A critical obstacle in the clinical application of TEM is the survival of TEM after implantation (Chapter 5). Additionally, the suitability of TEM to function as in vitro model for studying treatments like radiotherapy was explored (Chapter 6).

DEVELOPING TISSUE ENGINEERED ORAL MUCOSA

To engineer a mucosal equivalent several aspects have to be taken into account, as the graft has to withstand speech, passing of food and fluids and movement of the tongue [6]. The mechanical stress in the oral cavity is high and therefore, the choice of scaffold depends on mechanical properties, degradability and contraction. The demand for a sturdy

scaffold is illustrated by the earliest grafts, consisting of cultured epithelial sheets, which were fragile and difficult to handle due to a lack of supporting connective tissue [7]. Our first step towards developing a tissue-engineered mucosal equivalent was deciding what biomaterial we wanted to use for our scaffold (CHAPTER 2). Many different materials are currently used for scaffolding [8], varying from de-epidermized dermis (DED) to synthetic scaffolds and have reported on TEM equivalents constructed with collagen-based scaffolds [9]. However, major disadvantages of a collagen-based scaffold are the prompt degradation and the inadequate ability to withstand mechanical stress. In addition, they are likely to contract, especially with the number of fibroblasts increasing and aligning [7].

The DED scaffold is obtained from split thickness skin grafts taken from donors, of which the epidermal layer is gently removed. A major advantage of DED is the high similarity with the underlying connective tissue of oral mucosa as the extracellular matrix and basement membrane components [10]. This prompted us to choose DED as the scaffold for our mucosal equivalent. Indeed, the presence of basement membrane components has shown to increase keratinocyte attachment and improve epithelial morphology [11]. The use of a-cellularized scaffolds has recently gained interest with the finding that a scaffold consisting of only the extracellular matrix (ECM) can be repopulated to rebuild the original tissue [12]. A recent publication by Maher showed that the ECM of a rodent heart was repopulated with human cardiac

cells resulting in a newly formed beating heart revealing the potential of the ECM as a scaffold [13].

The second step in the development of TEM was to decide on the cell types we wanted to seed onto the DED scaffold and are needed for optimal resemblance to native mucosa. We decided to use the predominant cell types in the epithelial and submucosa, namely keratinocytes and fibroblasts. The decision to incorporate fibroblasts next to keratinocytes was also based on the results of earlier research published by us and others reporting on the incomplete development of a new epithelium when only keratinocytes were used [14, 15]. Even though keratinocytes are able to synthesize most components of the basement membrane, such as collagen type IV and laminin-332 [16], a continuous and mature basement membrane is only formed in the presence of both keratinocytes and fibroblasts [11].

The next step was to compare our TEM with native non-keratinizing oral mucosa by determining the presence of structural features such as a multilayered epidermis, basal layer, basement membrane and underlying connective tissue as well as the expression pattern of cytokeratins in the epithelial layer. We demonstrated that our TEM has a strong morphologic resemblance with native oral mucosa, with a similar expression pattern of various keratins and basement membrane components (CHAPTER 2).

In the clinical situation where patients with oral cancer are locally deprived of normal mucosa and are treated with radiotherapy, the next

step was to focus are experimental work on the preconditioning of our mucosal construct (TEM). In the following chapters therefore the suitability to use TEM for studying the potential of hypoxia and hyperoxia preconditioning, the acute effects of ionizing radiation and examining the potential of TEM as alternative grafting material.

PRECONDITIONING OF TISSUE-ENGINEERED MUCOSA WITH HYPOXIA

It is well established that all tissue-engineered constructs require oxygen and nutrients supplied by blood vessels to survive upon implantation [17-19] and that the lack of oxygen results in hypoxia, which can lead to cell death and ultimately loss of the graft [20, 21].

Conversely, the term hypoxia is somewhat ambiguous as it refers to a condition where partial oxygen pressure (pO_2) is lower than the normal oxygen pressure, or physioxia, but is often used when the O_2 concentration is lower than the standard culture conditions of 20% O_2. Physioxia usually ranges between 1 and 10% oxygen depending on the tissue [22, 23]. The pO_2 in skin, which has a structure similar to mucosa, varies from 1% O_2 in the epidermis to approximately 5% O_2 in the dermis [24].

In chapter 3 we exposed our TEM constructs to 1.5% O_2 for 6, 12, 24 or 48 h and determined cellular hypoxia, morphology and secretion of angiogenic factors. We demonstrated that overall morphology of the construct was maintained and that no profound structural changes occurred by exposing TEM to hypoxia for short periods of time.

Cellular hypoxia was assessed using Hypoxia Inducible Factor 1 alpha (HIF-1α), a transcriptional activator that is up-regulated in cells under hypoxic conditions [23]. The HIF-pathway mediates the cellular responses to hypoxia, and is responsible for the adaption to hypoxia. During hypoxia, HIF-1α accumulates in the cell nucleus [25]. Nuclear HIF-1α expression in our TEM constructs significantly increased with increasing exposure time, thereby confirming cellular hypoxia.

HIF-1α is considered to be a key factor in the regulation of the secretion of important angiogenic factors, including VEGF, PlGF and bFGF [25], and it has been reported that hypoxia increases the secretion of angiogenic factors in a variety of cells including stromal cells [26-28], HUVECs [29, 30], and endothelial progenitor cells [31]. Therefore, hypoxia induced secretion of multiple angiogenic factors by TEM was assessed. We found that exposure to hypoxia significantly increased the secretion of VEGF and PlGF, both known to induce angiogenesis and stimulation of endothelial cell proliferation [32-34].

Even though the results of this study showed that TEM produce factors under hypoxic conditions that contribute to angiogenesis *in vitro*, this angiogenic capacity *in vivo* needs to be clarified.

PRECONDITIONING OF TISSUE-ENGINEERED MUCOSA WITH HYPERBARIC OXYGEN THERAPY

Opposite of hypoxia is hyperbaric oxygen (HBO) treatment. During HBO the oxygen levels are increased from 20% to 100% and atmospheric pressure is increased from sea level pressure, 1 ATA, to 2.4 ATA [35]. Adjunctive HBO therapy has been used for a wide variety of afflictions, including healing of (chronic) wounds [36-40], treatment of diabetic foot ulcers [41], traumatic brain injury and stroke [42], and to promote the viability of vascularized flaps [43]. HBO is known to increase the secretion of growth factors such as transforming growth factors β1 and β2 (TGF β1 and β2) [44], which are associated with wound healing [45, 46], and important angiogenic factors including VEGF in fibroblasts [44].

These characteristics prompted us to evaluate the effect of HBO on our TEM construct (CHAPTER 4). For this, TEM constructs were treated with 100% oxygen and 2.4 ATA for up to 5 consecutive days. Histology revealed that overall morphology was maintained after 1 HBO treatment. However, after 3 and 5 consecutive treatments the TEM constructs were affected as the epithelium became significantly thinner. Additionally, detachment of the epithelium from the underlying connective tissue was seen, which is probably caused to remodeling of the basement membrane, as illustrated by the changes in collagen type IV deposition [47]. These results suggest that the cells have yet to adapt to the HBO conditions or a potential harmful effect of HBO as for instance oxygen toxicity [48, 49].

As mentioned earlier, HBO is known to increase the secretion of angiogenic factors in wound fluid [50], hind limbs of mice [51], HUVECs [52-54] and fibroblasts [44]. Therefore, we assessed the secretion of VEGF, PlGF, HGF and bFGF by TEM after treatment with HBO and found significantly higher levels of these angiogenic factors in conditioned medium after a single HBO treatment. As the migration and proliferation of endothelial cells is important for angiogenesis, the effect of these angiogenic factors on HUVECs was determined. Our results did show a slight increase in endothelial cells proliferation when exposed to culture medium supplemented with HGF and bFGF, albeit this was not significant. Additionally, migration of HUVECs was not induced after exposure to conditioned culture medium supplemented with these factors (unpublished data). These results indicate that the formation of functional blood vessels requires more than the simple addition of only one or two growth factors. This is supported by the work of Roukema *et al* who reported that the addition of unbalanced levels of just one growth factor results in incomplete and non-functional vessels [55]. Taken together, our results showed that HBO treatment of our TEM constructs upregulated the secretion of certain growth factors that mediate angiogenesis *in vitro*. This is in agreement with studies intended for the survival of tissue-engineered constructs.

IMPLANTATION OF TISSUE-ENGINEERED MUCOSA

Aside from their potential to function as a model for scientific research, ultimately our TEM constructs were developed for a clinical application. We want TEM to serve as an alternative source of grafts that are to be used during reconstruction of large oral defects. The need for an alternative grafting material become clear as the number of patients with head-neck cancer has increased over the last 10 years and has become the sixth most occurring cancer type in Europe. In the Netherlands it is estimated that every year 3000 new patients are diagnosed with head-neck cancer (http://www.vumc.nl/afdelingen/CCA-V-ICI/nieuws/hoofd-halskanker/). The majority of the patients will be treated with a combination of radiotherapy and chemotherapy, followed or preceded by oncological resection. Currently, large defects are reconstructed with free vascularized soft tissue flaps taken from arm or leg, either or not with bone when a defect in the mandible is present [56]. Disadvantages of the skin covering these soft tissue flaps include hair growth, perspiration, and donor site morbidity [57]. With TEM we aim to provide a suitable alternative grafting material for the top skin layer covering soft tissue without these side effects.

We implanted TEM constructs into subcutaneous pockets on the back of nude mice and harvested the constructs 3, 7, 14 or 28 days post-implantation (CHAPTER 5). Analysis of the harvested constructs showed that a number of the TEMs survived implantation up to 28 days. Unfortunately, not all TEM constructs had the desired structure upon

harvesting, as approximately 50% of the implanted constructs had, partially, lost the epithelial layer. The usage of subcutaneous pockets was first described by Barrandon *et al* [58]. The finding that the epithelium was partially detached in several constructs is similar to the findings of other groups who used this technique and who likewise reported on partial detachment of the epithelium [59, 60]. The loss of the epithelial layer in some constructs could be caused by the lack of hemidesmosomes, by the harvesting procedure or could be due to degeneration of the epithelium [60].

Upon harvesting, the surviving TEM constructs did show structural similarities with the tissue-engineered mucosal equivalents that have been implanted into the oral cavity of patients or immunodeficient mice [60-62]. Izumi *et al* for instance reported on a mature, differentiated epithelium in their constructs taken four weeks post-implantation, which is in line with our findings post-implantation [60]. Peña *et al* reported that collagen type IV expression in their construct was not continuous until 3 weeks post-implantation [59]. This pattern was similar to that observed in our TEM post-implantation.

Additionally, we studied the presence of murine endothelial cells in TEM using endothelial cell marker CD31 as we hypothesized that the survival of a number of TEMs could be explained by the presence of murine blood vessels. The results of this staining showed that there was no detectable ingrowth of murine vascular structures into any the TEM constructs. So

apparently, some TEMs were able to survive without the presence of neovascularization or ingrowth of murine blood vessels.

Even though the results in immunodeficient mice were promising, it has become clear that our TEM constructs need adjustment in order to improve the survival rate post-implantation and before TEM can be used in any clinical setting. For instance, our *in vitro* studies showed that preconditioning with hypoxia significantly induced the secretion of angiogenic factors. The presence of these factors might induce neovascularization and attract blood vessels from the surrounding tissue. Both phenomena should improve survival of TEM after implantation. Additionally, our research showed that HBO could improve the survival of TEM. As HBO is known to improve wound healing, exposure to HBO directly after implantation could provide the oxygen levels necessary for TEM to survive without vascularization.

RADIATION OF TISSUE-ENGINEERED MUCOSA

After we established the close resemblance of our TEM model with native oral mucosa in chapter 2, we hypothesized that TEM could also serve as an *in vitro* model, this being the second aim of this thesis. We chose to use TEM to study the acute biological changes following radiotherapy (CHAPTER 6). Radiotherapy is essential for the treatment of cancer in the oral cavity. A major disadvantage of radiotherapy is the damage of the healthy tissue surrounding the tumor.

Many papers have been published on the long-term side effects in

patients but little is known about the acute effects [63-67]. We are convinced that more detailed knowledge of the acute biological changes of this healthy tissue might help to study the underlying mechanisms regarding oral mucositis and radiation-induced fibrosis. The last decade new insights in the pathogenesis of oral mucositis have arisen as it became clear that aside from direct cell damage, a complex cascade of biological events in cells and surrounding tissue lead up to mucositis [68, 69].

Recently, research has focused on studying biological changes in tissues and targeted therapies [70]. Additionally, the alternative approaches regarding mucositis risk prediction are studied. The need to increase the knowledge on the pathobiology of oral mucositis is emphasized by the number of patients suffering from this side effect. It is estimated that approximately 80% of patients with malignancies in the head – neck region that are treated with radiotherapy are affected by mucositis [71].

Before any of these side effects can be addressed we had to validate our model. In CHAPTER 6 we validated TEM by irradiation with a single high dose and demonstrated that the normal morphology of TEM is preserved up to 24h post-irradiation. Thereafter profound structural changes such as basal cell degeneration, decreased proliferation and thinning of the epithelium were observed. Even though the response of TEM to radiation was similar to that in biopsies of native oral mucosa we were not able to study oral mucositis yet. In order to make TEM a representative model to study oral mucositis, adaptions to our model are necessary. By treating

TEM with pro-inflammatory cytokines, we might be able to create an equivalent mimicking oral mucositis [72]. The inclusion of immune cells such as dendritic cells, or their epithelial subpopulation called Langerhans cells, could be crucial as these cells are regarded as the key mediators of the mucosal immune responses [73, 74].

Radiation is known to activate TGF-β in skin, and in our mucosal model we also observed an increase of activated TGF-β after IR. TGF-β is essential for the migration of immature Langerhans cells into skin [74] and is an important in the induction and maintenance of immunological tolerance [75]. This, together with the incorporation of Langerhans cells in TEM, would make it very interesting to study the effects of radiation and might actually mimic oral mucositis. However, one should realize that the immune system in the oral cavity is especially complex and still cannot be completely simulated in a multicellular tissue-engineered model.

CONCLUSION

In conclusion, we were able to develop a TEM construct that mimics native oral mucosa based on structure and functionality. We also showed that TEM constructs could be cultured and implanted into immunodeficient mice where it survives and even further differentiates and matures. This last result is very promising and suggests that we might be able to reach our ultimate goal: to serve as an alternative graft for clinical applications.

The second aim of this thesis was to develop a construct that is a representative model for in vitro research regarding harmful or beneficial environments as for instance radiation or hyperbaric oxygen therapy. We were able to study the effects of preconditioning of TEM with hypoxia and hyperbaric oxygen treatment, and established that this preconditioning induces the secretion of angiogenic factors. Furthermore, we demonstrated that TEM mimics the response of native oral mucosa to ionizing radiation, confirming the potential of TEM to function as an *in vitro* model.

FUTURE PERSPECTIVES

Future perspectives to further enhance the stability and viability of the construct could be:

I. *Inclusion of other cell types to the construct (immune cells, dermal endothelial cells, stem cells)*

When designing our TEM construct we strived to keep the model straightforward, sturdy and easy to handle. We decided to incorporate the two main cell types only, namely fibroblasts and keratinocytes. With the knowledge accumulated through the experiments described in the preceding chapters, we are certain that expanding the number of cell types could make TEM more effective when implanted or more complete as an *in vitro* model. For instance the inclusion of dermal endothelial cells could improve the survival of TEM post-implantation. These cells are

capable of forming tube like structures that together with the appropriate stimuli can potentially shorten the period of hypoxia post-implantation. Additionally, the addition of a combination of mesenchymal stem cells and endothelial cells should also result in tube-like structures [76]. With the proper seeding concentration, the addition of these cell types might result in prevascular structures in our TEM construct prior to implantation, which in turn could stimulate vascularization upon implantation [77].

The inclusion of immune cells such as dendritic cells, or their epithelial subpopulation called Langerhans cells, could be crucial as these cells are regarded as the key mediators of the mucosal immune responses [74]. Radiation is known to activate TGF-β in skin, and in our mucosal model we also observed an increase of activated TGF-β after IR. TGF-β is essential for the migration of immature Langerhans cells into skin [74] and is an important in the induction and maintenance of immunological tolerance [75]. This, together with the incorporation of Langerhans cells in TEM, would make it very interesting to study the effects of radiation and might actually mimic oral mucositis.

II. *Stimulate vessel formation in TEM construct*

In chapter 5 we showed that approximately 50% of the implanted TEMs survived post-implantation. Therefore, methods to improve this success rate need to be explored. In chapter 3 we showed that preconditioning with hypoxia significantly increased the secretion of angiogenic factors.

This should be beneficial for vascularization, which is critical for the survival of most tissue-engineered constructs post-implantation. Additionally, the addition of a combination of mesenchymal stem cells and endothelial cells should result in tube-like structures [76]. With the proper seeding concentration, the addition of these cell types might result in prevascular structures in our TEM construct prior to implantation. This in combination with hypoxia-induced angiogenic factors might result in increased graft survival.

III. *Adjusting TEM for clinical application*

Before TEM can be used in any clinical setting several aspects of the current culture protocol, such as use of serum and feeder layer and laboratory facility, have to be addressed. In order for cells to grow and proliferate various essential nutrients and growth factors have to be present. Fetal calf serum (FCS) is the most often used supplement in culture media [78]. However, the use of FCS is undesirable due to the ethical objections regarding the cruel harvesting procedure of serum from unborn calves [79, 80]. Clinical objections to the use of FCS include possible presence of animal pathogens [81], transmission of viruses and prions, high batch-to-batch variation, and the potential immunological response of the host [82, 83].

Therefore, studies have focused on finding and evaluating suitable substitutes for FCS. These substitutes include autologous serum [84], platelet lysate [85, 86] or platelet rich plasma [87]. Additionally,

chemically defined culture media [88, 89] and chemical substitutes have been explored, including Ultroser G [90, 91]. As most of this research regarding FCS substitutes has focused on the expansion of mesenchymal stem cells, multiple substitutes should be tested in order to select the most appropriate substitute for our multicellular construct.

IV. *Radioprotective agents*

In this thesis, we showed that TEM could be used as a model for *in vitro* research regarding harmful or beneficial environments. Based on the results of this thesis, and on publications by other groups on tissue-engineered constructs that were used as an *in vitro* model to study wound healing, cytotoxicity or working mechanisms of new treatments [92, 93], we believe that our TEM construct can be used in a similar manner. As mentioned earlier in chapter 6 the side effects of radiotherapy can be severe and diminishes the patients' quality of life. This prompted the search for new treatments including so-called radioprotective agents [94, 95] as methionine [96], pentoxifylline [97, 98] and amifostine [99]. Currently, palifermin has gained interest in the treatment of oral mucositis and is the first FDA- approved agent [100]. It would be interesting to use TEM to evaluate the effects of new radioprotective agents and determine the best treatment for individual patients.

V. *Larger constructs or larger quantities*

Currently, our TEM construct is approximately 1cm^2. However, the average oral defect is much larger and cannot be covered with a single TEM. Other groups have reported on the intra-oral grafting with small tissue-engineered constructs but one can imagine that implanting one or two larger constructs is easier that implanting multiple smaller constructs. Nonetheless, the surface area of the construct cannot be expanded easily. Potential limitations that should be addressed include handling, the number of autologous cells needed, and culture time. TEM constructs are thin and flexible, and when lifted from the supporting mesh they are prone to shrivel. The larger the construct the harder it will be to transfer to the acceptor site in reconstruction.

Another factor that needs to be addressed is the number of autologous cells that are required to engineer larger constructs or larger quantities of TEM [101]. To construct 1cm^2 TEM we used approximately 1x10^6 cells, and with increasing surface area the number of cells increases linearly. The limiting factor here would the available donor material as little healthy buccal mucosa remains after oncological resection. Therefore, alternative cell sources or culture methods need to be taken into consideration.

REFERENCES

1. Kamel, R.A., J.F. Ong, E. Eriksson, J.P. Junker, E.J Caterson (2013) Tissue engineering of skin. J Am Coll Surg *217(3)*: 533-55.
2. de Peppo, G.M., P. Thomses, C. Karlsson, R. Strehl, A. Lindahl, *et al.* (2013) Human progenitor cells for bone engineering applications. Curr Mol Med *13(5):* 723-34.
3. Johnstone, B., M. Alini, M. Cucchiarini, G.R. Dodge, D. Eglin, *et al.* (2013) Tissue engineering for articular cartilage repair--the state of the art. Eur Cell Mater 25: 248-67.
4. Nemeno-Guanzon, J.G., S. Lee, J.R. Berg, Y.H. Jo, J.E. Yeo, *et al.* (2012) Trends in tissue engineering for blood vessels. J Biomed Biotechnol 2012: 956345.
5. Tay, C.Y., S.A. Irvine, F.Y. Boey, L.P. Tan, S. Venkatraman (2011) Micro-/nano-engineered cellular responses for soft tissue engineering and biomedical applications. Small *7(10):* 1361-78.
6. Presland, R.B., B.A. Dale (2000) Epithelial structural proteins of the skin and oral cavity: function in health and disease. Crit Rev Oral Biol Med *11(4):* 383-408.
7. Moharamzadeh, K., I.M. Brook, R. Van Noort, A.M. Scutt, M.H. Thornhill (2007) Tissue-engineered oral mucosa: a review of the scientific literature. J Dent Res *86(2):* 115-24.
8. Kriegebaum, U., M. Mildenberger, U.D. Mueller-Richter, U. Klammert, A.C. Kuebler, *et al.* (2012) Tissue engineering of human

oral mucosa on different scaffolds: in vitro experiments as a basis for clinical applications. Oral Surg Oral Med Oral Pathol Oral Radiol *114(5 Suppl):* S190-8.

9. Rouabhia, M., N. Deslauriers (2002) Production and characterization of an in vitro engineered human oral mucosa. Biochem Cell Biol *80(2):* 189-95.
10. Pianigiani, E., F. Ierardi, B. Mazzanti, R. Saccardi, C. Cuciti, *et al. (*2010) Human de-epidermized dermis as a stem cell carrier. Transplant Proc *42(6):* 2244-6.
11. Ralston, D.R., C. Layton, A.J. Dalley, S.G. Boyce, E. Freelander, *et al.* (1999) The requirement for basement membrane antigens in the production of human epidermal/dermal composites in vitro. Br J Dermatol *140(4):* 605-15.
12. Ott, H.C., T.S. Matthiesen, S.K. Goh, L.D. Black, S.M. Kren, *et al.* (2008) Perfusion-decellularized matrix: using nature's platform to engineer a bioartificial heart. Nat Med *14(2):* 213-21.
13. Maher, B. (2013) Tissue engineering: How to build a heart. Nature *499(7456):* 20-2.
14. Rakhorst, H.A., S.J. Posthumus-van Sluijs, W.M. Tra, J.W. van Neck, G.J. van Osch, *et al.* (2006) Fibroblasts accelerate culturing of mucosal substitutes. Tissue Eng *12(8):* 2321-31.
15. El Ghalbzouri, A., E. Lamme, M. Ponec (2002) Crucial role of fibroblasts in regulating epidermal morphogenesis. Cell Tissue Res *310(2)*: 189-99.

16. Larjava, H., T. Salo, K. Haapasalmi, R.H. Kramer, J. Heino (1993) Expression of integrins and basement membrane components by wound keratinocytes. J Clin Invest *92(3):* 1425-35.

17. Lovett, M., K. Lee, A. Edwards, D.L. Kaplan (2009) Vascularization strategies for tissue engineering. Tissue Eng Part B Rev *15(3):* 353-70.

18. Novosel, E.C., C. Kleinhans, P.J. Kluger (2011) Vascularization is the key challenge in tissue engineering. Adv Drug Deliv Rev *63(4-5):* 300-11.

19. Sahota, P.S., J.L. Burn, N.J. Brown, S. MacNeil (2004) Approaches to improve angiogenesis in tissue-engineered skin. Wound Repair Regen *12(6):* 635-42.

20. Malda, J., T.J. Klein, Z. Upton (2007) The roles of hypoxia in the in vitro engineering of tissues. Tissue Eng *13(9)*: 2153-62.

21. Ophof, R., J.C. Maltha, A.M. Kuijpers-Jagtman, J.W. Von den Hoff (2008) Implantation of tissue-engineered mucosal substitutes in the dog palate. Eur J Orthod *30(1)*: 1-9.

22. Carreau, A., B. El Hahny-Rahbi, A. Matejuk, C. Grillon, C. Kieda (2011) Why is the partial oxygen pressure of human tissues a crucial parameter? Small molecules and hypoxia. J Cell Mol Med *15(6)*: 1239-53.

23. Hadjipanayi, E., R.A. Brown, V. Mudera, D. Deng, W. Liu, *et al.* (2010) Controlling physiological angiogenesis by hypoxia-induced signaling. J Control Release *146(3)*: 309-17.

24. Wang, W., C.P. Winlove, C.C. Michel (2003) Oxygen partial pressure in outer layers of skin of human finger nail folds. J Physiol *549(Pt 3)*: 855-63.

25. Krock, B.L., N. Skuli, M.C. Simon (2011) Hypoxia-induced angiogenesis: good and evil. Genes Cancer *2(12)*: 1117-33.

26. Hsiao, S.T., Z. Lokmic, H. Peshavariya, K.M. Abberton, G.J. Dusting, *et al.,* (2013) Hypoxic conditioning enhances the angiogenic paracrine activity of human adipose-derived stem cells. Stem Cells Dev *22(10)*: 1614-23.

27. Wei, L., J.L. Fraser, Z.Y. Lu, X. Hu, S.P. Yu (2012) Transplantation of hypoxia preconditioned bone marrow mesenchymal stem cells enhances angiogenesis and neurogenesis after cerebral ischemia in rats. Neurobiol Dis *46(3)*: 635-45.

28. Liu, L., J. Gao, Y. Yuan, Q. Chang, Y. Liao, *et al.* (2013) Hypoxia preconditioned human adipose derived mesenchymal stem cells enhance angiogenic potential via secretion of increased VEGF and bFGF. Cell Biol Int *37(6)*: 551-60.

29. Zeng, R., X.F. Jiang, Y.C. Chen, Y.N. Xu, S.H. Ma, *et al.* (2013) VEGF, not VEGFR2, is associated with the angiogenesis effect of mini-TyrRS/mini-TrpRS in human umbilical vein endothelial cells in hypoxia. Cytotechnology *66(4)*: 655-65.

30. Dal Monte, M., D. Martini, C. Ristori, D. Azara, C. Armani, *et al.* (2011) Hypoxia effects on proangiogenic factors in human umbilical vein endothelial cells: functional role of the peptide

somatostatin. Naunyn Schmiedebergs Arch Pharmacol *383(6)*: 593-612.

31. Abaci, H.E., R. Truitt, E. Luong, G. Drazer, S. Gerecht (2010) Adaptation to oxygen deprivation in cultures of human pluripotent stem cells, endothelial progenitor cells, and umbilical vein endothelial cells. Am J Physiol Cell Physiol *298(6)*: C1527-37.
32. Wu, Y., M.A. Al-Ameen, G. Ghosh (2014) Integrated Effects of Matrix Mechanics and Vascular Endothelial Growth Factor (VEGF) on Capillary Sprouting. Ann Biomed Eng *99:* 1024-36.
33. Cai, J., W.G. Jiang, A. Ahmed, M. Boulton (2006) Vascular endothelial growth factor-induced endothelial cell proliferation is regulated by interaction between VEGFR-2, SH-PTP1 and eNOS. Microvasc Res *71(1)*: 20-31.
34. Accornero, F., J.H van Berlo, M.J. Bernard, J.N. Lorenz, P. Carmeliet, *et al.* (2011) Placental growth factor regulates cardiac adaptation and hypertrophy through a paracrine mechanism. Circ Res *109(3)*: 272-80.
35. Shah, J. (2010) Hyperbaric Oxygen Therapy. J Am Col Certif Wound Spec *2(1)*: 9-13.
36. Goldstein, L.J. (2013) Hyperbaric oxygen for chronic wounds. Dermatol Ther *26(3)*: 207-14.
37. Howard, M.A., R. Asmis, K.K. Evans, T.A. Mustoe (2013) Oxygen and wound care: a review of current therapeutic modalities and future direction. Wound Repair Regen *21(4)*: 503-11.

38. Dauwe, P.B., B.J. Pulikkottil, L. Lavery, J.M. Stuzin, R.J. Rohrich (2014) Does hyperbaric oxygen therapy work in facilitating acute wound healing: a systematic review. Plast Reconstr Surg *133(2)*: 208e-15e.

39. Dequanter, D., D. Jacobs, M. Shahla, P.Paulus, C. Aubert, *et al.* (2013) The effect of hyperbaric oxygen therapy on treatment of wound complications after oral, pharyngeal and laryngeal salvage surgery. Undersea Hyperb Med *40(5)*: 381-5.

40. Kranke, P., M.H. Bennett, M. Martyn-St James, A. Schnabel, S.E. Debus (2012) Hyperbaric oxygen therapy for chronic wounds. Cochrane Database Syst Rev *4*: CD004123.

41. Londahl, M. (2013) Hyperbaric oxygen therapy as adjunctive treatment of diabetic foot ulcers. Med Clin North Am *97(5)*: 957-80.

42. Efrati, S., E. Ben-Jacob (2014) Reflections on the neurotherapeutic effects of hyperbaric oxygen. Expert Rev Neurother *14(3)*: 233-6.

43. Larson, J.V., E.A. Steensma, R.M. Flikkema, E.M. Norman (2013) The application of hyperbaric oxygen therapy in the management of compromised flaps. Undersea Hyperb Med *40(6)*: 499-504.

44. Kang, T.S., G.K. Gorti, S.Y. Quan, M. Ho, R.J. Koch (2004) Effect of hyperbaric oxygen on the growth factor profile of fibroblasts. Arch Facial Plast Surg *6(1)*: 31-5.

45. Wang, X.J., G. Han, P. Owens, Y. Siddiqui, A.G. Li (2006) Role of TGF beta-mediated inflammation in cutaneous wound healing. J Investig Dermatol Symp Proc *11(1)*: 112-7.

46. O'Kane, S., M.W. Ferguson (1997) Transforming growth factor beta s and wound healing. Int J Biochem Cell Biol *29(1)*: 63-78.

47. Kairuz, E., Z. Upton, R.A. Dawson, J. Malda (2007) Hyperbaric oxygen stimulates epidermal reconstruction in human skin equivalents. Wound Repair Regen *15(2)*: 266-74.

48. Seidel, R., C. Carroll, D. Thompson, R.G. Diem, K. Yeboah, *et al.* (2013) Risk factors for oxygen toxicity seizures in hyperbaric oxygen therapy: case reports from multiple institutions. Undersea Hyperb Med *40(6)*: 515-9.

49. Plafki, C., P. Peters, M. Almeling, W. Welslau, R. Busch (2000) Complications and side effects of hyperbaric oxygen therapy. Aviat Space Environ Med *71(2)*: 119-24.

50. Sheikh, A.Y., M.D. Rollins, H.W. Hopf, T.K. Hunt (2005) Hyperoxia improves microvascular perfusion in a murine wound model. Wound Repair Regen *13(3)*: 303-8.

51. Asano, T., E. Kaneko, S. Shinozaki, Y. Imai, M., Shibayama, *et al.* (2007) Hyperbaric oxygen induces basic fibroblast growth factor and hepatocyte growth factor expression, and enhances blood perfusion and muscle regeneration in mouse ischemic hind limbs. Circ J *71(3)*: 405-11.

52. Godman, C.A., K.P. Chheda, L.E. Hightower, G. Perdrizet, D.G. Shin, *et al.* (2010) Hyperbaric oxygen induces a cytoprotective and angiogenic response in human microvascular endothelial cells. Cell Stress Chaperones *15(4)*: 431-42.

53. Lee, C.C., S.C. Chen, S.C. Tsai, B.W. Wang, Y.C. Liu, *et al.* (2006) Hyperbaric oxygen induces VEGF expression through ERK, JNK and c-Jun/AP-1 activation in human umbilical vein endothelial cells. J Biomed Sci *13(1)*: 143-56.

54. Lin, S., K.G. Shyu, C.C. Lee, B.W. Wang, C.C. Chang, *et al.* (2002) Hyperbaric oxygen selectively induces angiopoietin-2 in human umbilical vein endothelial cells. Biochem Biophys Res Commun *296(3)*: 710-5.

55. Rouwkema, J., N.C. Rivron, C.A. van Blitterswijk (2008) Vascularization in tissue engineering. Trends Biotechnol *26(8)*: 434-41.

56. Xiao, Y., J. Zhu, X. Cai, J. Wang, F. Liu, *et al.* (2013) Comparison between anterolateral thigh perforator free flaps and pectoralis major pedicled flap for reconstruction in oral cancer patients--a quality of life analysis. Med Oral Patol Oral Cir Bucal *18(6)*: e856-61.

57. Tra, W.M., J.W. van Neck, S.E. Hovius, G.J. van Osch, S. Perez-Amodio (2012) Characterization of a three-dimensional mucosal equivalent: similarities and differences with native oral mucosa. Cells Tissues Organs *195(3)*: 185-96.

58. Barrandon, Y., V. Li, H. Green (1988) New techniques for the grafting of cultured human epidermal cells onto athymic animals. J Invest Dermatol *91(4)*: p. 315-8.

59. Pena, I., L.M. Junquera, A. Meana, E. Garcia, C. Aquillar, *et al.* (2011) In vivo behavior of complete human oral mucosa equivalents: characterization in athymic mice. J Periodontal Res *46(2)*: 214-20.

60. Izumi, K., S.E. Feinberg, H. Terashi, C.L. Marcelo (2003) Evaluation of transplanted tissue-engineered oral mucosa equivalents in severe combined immunodeficient mice. Tissue Eng *9(1)*: 163-74.

61. Hotta, T., S. Yokoo, H. Terashi, T. Komori (2007) Clinical and histopathological analysis of healing process of intraoral reconstruction with ex vivo produced oral mucosa equivalent. Kobe J Med Sci *53(1-2)*: 1-14.

62. Lauer, G., R. Schimming (2001) Tissue-engineered mucosa graft for reconstruction of the intraoral lining after freeing of the tongue: a clinical and immunohistologic study. J Oral Maxillofac Surg *59(2)*: 169-75; discussion 175-7.

63. Cooper, J.S., K. Fu, J. Marks, S. Silverman (1995) Late effects of radiation therapy in the head and neck region. Int J Radiat Oncol Biol Phys *31(5)*: 1141-64.

64. Ray-Chaudhuri, A., K. Shah, R.J. Porter (2013) The oral management of patients who have received radiotherapy to the head and neck region. Br Dent J *214(8)*: 387-93.

65. Rutten, H., L.A. Pop, G.O. Janssens, R.P. Takes, S. Knuijt, *et al.* (2011) Long-term outcome and morbidity after treatment with accelerated radiotherapy and weekly cisplatin for locally advanced head-and-neck cancer: results of a multidisciplinary late morbidity clinic. Int J Radiat Oncol Biol Phys *81(4)*: 923-9.

66. Combs, S.E., B. Salehi-Allameh, D. Habermehl, K.A. Kessel, T. Welzel, *et al.* (2014) Clinical response and tumor control based on long-term follow-up and patient-reported outcomes in patients with chemodectomas of the skull base and head and neck region treated with highly conformal radiation therapy. Head Neck *36(1)*: 22-7.

67. Mortensen, H.R., J. Overgaard, K. Jensen, L. Specht, M. Overgaard, *et al.* (2013) Factors associated with acute and late dysphagia in the DAHANCA 6 & 7 randomized trial with accelerated radiotherapy for head and neck cancer. Acta Oncol *52(7)*: 1535-42.

68. Sonis, S.T. (2004) The pathobiology of mucositis. Nat Rev Cancer *4(4)*: 277-84.

69. Sonis, S.T. (2011) Oral mucositis. Anticancer Drugs *22(7)*: 607-12.

70. Al-Dasooqi, N., S.T. Sonis, J.M. Bowen, E. Bateman, N. Blijlevens, *et al.* (2013) Emerging evidence on the pathobiology of mucositis. Support Care Cancer *21(11)*: 3233-41.

71. Chiappelli, F. (2005) The molecular immunology of mucositis: implications for evidence-based research in alternative and

complementary palliative treatments. Evid Based Complement Alternat Med *2(4)*: 489-94.

72. Tjabringa, G., M. Bergers, D. van Rens, R. de Boer, E. Lamme, *et al.* (2008) Development and validation of human psoriatic skin equivalents. Am J Pathol *173(3)*: 815-23.
73. Bechetoille, N., C. Dezutter-Dambuyant, O. Damour, V. Andre, I Orly, *et al.* (2007) Effects of solar ultraviolet radiation on engineered human skin equivalent containing both Langerhans cells and dermal dendritic cells. Tissue Eng *13(11)*: 2667-79.
74. Cutler, C.W., R. Jotwani (2006) Dendritic cells at the oral mucosal interface. J Dent Res *85(8)*: 678-89.
75. Radeke, H.H., H. von Wenckstern, K. Stoidtner, B. Sauer, S. Hammer, *et al.* (2005) Overlapping signaling pathways of sphingosine 1-phosphate and TGF-beta in the murine Langerhans cell line XS52. J Immunol *174(5)*: 2778-86.
76. Verseijden, F., S.J. Posthumus-van Sluijs, P. Pavljasevic, S.O. Hofer, G.J. van Osch, *et al.* (2010) Adult human bone marrow- and adipose tissue-derived stromal cells support the formation of prevascular-like structures from endothelial cells in vitro. Tissue Eng Part A *16(1)*: 101-14.
77. Verseijden, F., S.J. Posthumus-van Sluijs, E. Farrell, J.W. van Neck, S.E. Hovius, *et al.* (2010) Prevascular structures promote vascularization in engineered human adipose tissue constructs upon implantation. Cell Transplant *19(8)*: 1007-20.

78. Gstraunthaler, G. (2003) Alternatives to the use of fetal bovine serum: serum-free cell culture. ALTEX *20(4)*: 275-81.

79. van der Valk, J., D. Mellor, R. Brands, R. Fischer, F. Gruber, *et al.* (2004) The humane collection of fetal bovine serum and possibilities for serum-free cell and tissue culture. Toxicol In Vitro *18(1)*: 1-12.

80. Jochems, C.E., J.B. van der Valk, F.R. Stafleu, V. Baumans (2002) The use of fetal bovine serum: ethical or scientific problem? Altern Lab Anim *30(2)*: 219-27.

81. Naaijkens, B.A., H.W. Niessen, H.J. Prins, P.A. Krijnen, T.J. Klokhuis, *et al.* (2012) Human platelet lysate as a fetal bovine serum substitute improves human adipose-derived stromal cell culture for future cardiac repair applications. Cell Tissue Res *348(1)*: 119-30.

82. Mannello, F., G.A. Tonti (2007) Concise review: no breakthroughs for human mesenchymal and embryonic stem cell culture: conditioned medium, feeder layer, or feeder-free; medium with fetal calf serum, human serum, or enriched plasma; serum-free, serum replacement nonconditioned medium, or ad hoc formula? All that glitters is not gold! Stem Cells *25(7)*: 1603-9.

83. Tuschong, L., S.L. Soenen, R.M. Blaese, F. Candotti, L.M. Muul (2002) Immune response to fetal calf serum by two adenosine deaminase-deficient patients after T cell gene therapy. Hum Gene Ther *13(13)*: 1605-10.

84. Stute, N., K. Holtz, M. Bubenheim, C. Lange, F. Blake, *et al.* (2004) Autologous serum for isolation and expansion of human mesenchymal stem cells for clinical use. Exp Hematol *32(12)*: 1212-25.

85. Rauch, C., E. Feifel, E.M. Amann, H.P. Spotl, H. Schennach, *et al.* (2011) Alternatives to the use of fetal bovine serum: human platelet lysates as a serum substitute in cell culture media. ALTEX *28(4)*: 305-16.

86. Shahdadfar, A., K. Fronsdal, T, Haug, F.P. Reinholt, J.E. Brinchmann (2005) In vitro expansion of human mesenchymal stem cells: choice of serum is a determinant of cell proliferation, differentiation, gene expression, and transcriptome stability. Stem Cells *23(9)*: 1357-66.

87. Anitua, E., R. Prado, G. Orive (2013) Safety and efficient ex vivo expansion of stem cells using platelet-rich plasma technology. Ther Deliv *4(9)*: 1163-77.

88. van der Valk, J., D. Brunner, K. De Smet, A. Fex Svenningsen, P. Honegger, *et al.* (2010) Optimization of chemically defined cell culture media--replacing fetal bovine serum in mammalian in vitro methods. Toxicol In Vitro *24(4)*: 1053-63.

89. Brunner, D., J. Frank, H. Appl, H. Schoffl, W. Pfaller, *et al.* (2010) Serum-free cell culture: the serum-free media interactive online database. ALTEX *27(1)*: 53-62.

90. Coolen, N.A., M. Verkerk, L. Reijnen, M. Vlig, A.J. van den Bogaerdt, *et al.* (2007) Culture of keratinocytes for transplantation without the need of feeder layer cells. Cell Transplant *16(6)*: 649-61.

91. Ahrari, I., N. Purhabibi Zarandi, M. Khosravi Maharlooei, A. Monabati, A. Attari, *et al.* (2013) Adipose Tissue Derived Multipotent Mesenchymal Stromal Cells Can Be Isolated Using Serum-free Media. Iran Red Crescent Med J *15(4)*: 324-9.

92. Wang, X., Y. Liu, Z. Deng, R. Dong, Y. Liu, *et al.* (2009) Inhibition of dermal fibrosis in self-assembled skin equivalents by undifferentiated keratinocytes. J Dermatol Sci *53(2)*: 103-11.

93. Klausner, M., S. Ayehunie, B.A. Breyfogle, P.W. Wertz, L. Bacca, *et al.* (2007) Organotypic human oral tissue models for toxicological studies. Toxicol In Vitro *21(5)*: 938-49.

94. Grdina, D.J., J.S. Murley, Y. Kataoka (2002) Radioprotectants: current status and new directions. Oncology *63 Suppl 2*: 2-10.

95. Hosseinimehr, S.J. (2007) Trends in the development of radioprotective agents. Drug Discov Today *12(19-20)*: 794-805.

96. Vuyyuri, S.B., D.A. Hamstra, D. Khanna, C.A. Hamilton, S.M. Markwart, *et al.* (2008) Evaluation of D-methionine as a novel oral radiation protector for prevention of mucositis. Clin Cancer Res *14(7)*: 2161-70.

97. Laurent, C., J.P. Pouget, P. Voisin (2005) Modulation of DNA damage by pentoxifylline and alpha-tocopherol in skin fibroblasts exposed to Gamma rays. Radiat Res *164(1)*: 63-72.

98. Laurent, C., P. Voisin, J.P. Pouget (2006) DNA damage in cultured skin microvascular endothelial cells exposed to gamma rays and treated by the combination pentoxifylline and alpha-tocopherol. Int J Radiat Biol *82(5)*: 309-21.

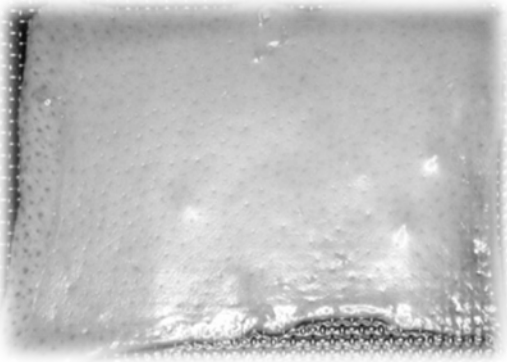
99. Bensadoun, R.J., M.M. Schubert, R.V. Lalla, D. Keefe (2006) Amifostine in the management of radiation-induced and chemo-induced mucositis. Support Care Cancer *14(6)*: 566-72.

100. Vadhan-Raj, S., J.D. Goldberg, M.A. Perales, D.P. Berger, M.R. van den Brink (2013) Clinical applications of palifermin: amelioration of oral mucositis and other potential indications. J Cell Mol Med *17(11)*: 1371-84.

101. Catalano, E., A. Cochis, E. Varoni, L. Rimondini, B. Azzimonti (2013) Tissue-engineered skin substitutes: an overview. J Artif Organs *16(4)*: 397-403.

CHAPTER 8

Nederlandse samenvatting



CHAPTER 8

NEDERLANDSE SAMENVATTING

Het herstel van grote defecten in de mondholte wordt belemmerd door een tekort aan geschikt lichaamseigen wangslijmvlies. Het tekort aan donorweefsel maakt de reconstructie van dergelijke defecten tot een grote uitdaging in de plastische chirurgie. Dit heeft de zoektocht naar een goed alternatief geïnitieerd. Weefselkweek (ook wel tissue engineering genoemd) is het kweken van weefsels buiten het lichaam, en een goede methode om nieuw weefsel te maken. In het laboratorium worden inmiddels huid, bot en zachte weefsels zoals vet succesvol gekweekt.

WEEFSELKWEEK WANGSLIJMVLIES

In hoofdstuk 2 hebben we gekweekt wangslijmvlies (tissue-engineered mucosa; TEM) gemaakt door de 2 belangrijkste cellen van wangslijmvlies, keratinocyten en fibroblasten, aan te brengen op a-cellulaire donorhuid. Na 3 weken kweken vormen deze 2 celtypes en de donorhuid samen een nieuw stukje wangslijmvlies. Dit nieuwe stukje TEM hebben we vergeleken met normaal slijmvlies zoals dit aanwezig is in de wang. Hiervoor hebben we gekeken naar cel proliferatie en cel differentiatie, de expressie van componenten van het basaal membraan en de verdeling van fibroblasten in het onderliggende bindweefsel. Daarnaast hebben we ook gekeken naar de onderlinge hechting van de keratinocyten en de hechting van het nieuwe epitheel aan het basaal membraan. Uit het onderzoek bleek dat het TEM construct grote gelijkenis vertoont met het normale wangslijmvlies. We zagen dat de expressie van belangrijke eiwitten in het epitheel, zoals keratine, gelijk was aan dat in normaal wangslijmvlies. Ook de celdeling van keratinocyten en de hechting van de

keratinocyten onderling was gelijk aan dat in normaal wangslijmvlies. De expressie van de belangrijkste componenten van het basaal membraan, collageen type 4 en laminine-332, en de hechting van het nieuwe epitheel aan het basaal membraan kwam overeen met dat in normaal wangslijmvlies. Bovengenoemde resultaten leidden tot de conclusie dat het TEM op de belangrijkste punten overeenkomt met het normale wangslijmvlies.

In de volgende hoofdstukken hebben we ons TEM gebruikt om de mogelijk gunstige eigenschappen van zuurstofgebrek (ook wel hypoxie genoemd) en hyperbare zuurstof therapie op het gebied van vascularisatie te onderzoeken. Daarnaast hebben we het TEM behandeld met radiotherapie. Tevens hebben we het overleven van TEM na implantatie bestudeerd.

PRECONDITIONERING GEKWEEKT WANGSLIJMVLIES

Alle gekweekte weefsels moeten kunnen overleven na implantatie. Om te kunnen overleven hebben cellen, en dus ook gekweekte weefsels, een continue toevoer van zuurstof nodig afkomstig van de bloedvaten in het omliggende weefsel. Een tekort aan zuurstof leidt tot zuurstofgebrek of hypoxie en een langdurig blootstelling aan hypoxie resulteert in celdood en uiteindelijk het afsterven van het geïmplanteerde weefsel. Echter, hypoxie bevordert ook de vorming van nieuwe bloedvaten (ook wel angiogenese genoemd) doordat het een gunstig effect heeft op de secretie van belangrijke angiogene factoren zoals bijvoorbeeld de vasculaire endotheliale groeifactor (VEGF). Gebaseerd op deze observatie, verwachten we dat het pre-conditioneren van gekweekt wangslijmvlies met hypoxie de angiogene capaciteit van het wangslijmvlies verhoogd en dat hierdoor de overleving na implantatie van het gekweekte wangslijmvlies verbeterd. In hoofdstuk 3 hebben we TEM gedurende 6 tot 24

uur blootgesteldaan hypoxie. Uit de resultaten bleek dat hypoxie niet leidde tot een verhoogde celdeling van keratinocyten of leidde tot celdood. De normale morfologie van TEM bleef intact na behandeling met hypoxie. Daarnaast zagen we dat dankzij blootstelling aan hypoxie de secretie van meerdere angiogene factoren omhoog ging, inclusief de groeifactoren VEGF en PlGF. Ook induceerde het geconditioneerde kweekmedium de proliferatie en migratie van endotheel cellen. Op basis van de bovengenoemde resultaten concluderen we dat het TEM relatief korte periodes van hypoxie kan overleven, wat veelbelovend is voor de overleving van TEM na implantatie. De verhoogde secretie van belangrijke angiogene factoren is zeer waarschijnlijk van belang voor de overleving van TEM na implantatie, omdat deze factoren bloedvaten uit het omliggende weefsel aan zullen trekken en hierdoor de toevoer van zuurstof aan het construct verbeteren.

Hyperbare zuurstof behandeling (HBO) is tegenovergesteld aan hypoxie. Tijdens HBO stijgt het percentage zuurstof van 21% naar 100% en wordt de normale atmosferische druk van 1 atmosfeer (ATA) verhoogd tot 2.4 ATA. Vergelijkbaar met hypoxie stimuleert HBO de secretie van diverse angiogene factoren. In hoofdstuk 4 hebben we TEM 1, 3 of 5 maal behandeld met hyperbare zuurstof. Uit histologisch onderzoek bleek dat de normale morfologie behouden bleef na 1 HBO behandeling. Echter, na 3 of 5 opeenvolgende behandelingen werd het epitheel zichtbaar dunner en liet het epitheel los van het basaal membraan. Deze resultaten suggereren het TEM construct zich nog aan moet passen aan de HBO condities. De toename in de secretie van angiogene factoren als gevolg van HBO, zou mogelijk de overleving van de TEM na implantatie bevorderen, op een gelijke wijze als pre-conditionering met hypoxie.

IMPLANTATIE VAN GEKWEEKT WANGSLIJMVLIES

Het TEM is oorspronkelijk ontwikkeld om te dienen als een alternatief transplantatie materiaal voor de reconstructie van grote orale defecten. In hoofdstuk 5 hebben we TEM constructen geïmplanteerd in onderhuidse envelopjes op de rug van naakte muizen. Deze constructen werden vervolgens na 3, 7, 14 of 28 dagen verwijderd. Na histologisch onderzoek bleek dat een aantal TEM constructen 28 dagen na implantatie nog vitaal waren. De dikte van het epitheel van deze constructen was significant minder vergeleken met de dikte van de constructen voorafgaand aan implantatie. Het dunner worden van het epitheel kan veroorzaakt worden door de afname van het aantal proliferende keratinocyten in de basale laag. De observatie dat het normale expressie patroon van de keratinen veranderde, is een indicatie dat implantatie veel stress veroorzaakt in de TEM constructen, resulterend in afname in de kwaliteit van de constructen. De waarneming dat de expressie van collageen type 4, een belangrijke component van de basaal membraan, 3 dagen na implantatie verschilde met de expressie op 14 of 28 dagen is een duidelijke indicatie voor remodelering van het basaal membraan. Om na te gaan waarom een aantal TEM constructen wel overleven na implantatie, hebben we de aanwezigheid van murine endotheel cellen in het TEM bepaald met de endotheel cel marker CD31. Na analyse van de kleuring met de CD31 marker bleek dat er geen detecteerbare ingroei van murine vasculaire structuren in het TEM was. De resultaten suggereren dat TEM constructen tot 28 dagen kunnen overleven na implantatie. Dit is een duidelijke indicatie dat TEM constructen een bepaalde periode van hypoxie kunnen overleven, een duidelijke bevestiging van de *in vitro* resultaten van de hypoxie studie. Echter, de exacte reden voor de overleving of verlies van TEM constructen is niet gevonden.

RADIOTHERAPIE

Radiotherapie is een essentieel onderdeel in de behandeling van patiënten met tumoren in het hoofd- halsgebied. Een ernstig neveneffect van radiotherapie is dat de straling niet alleen de tumorcellen doodt, maar ook het gezonde, omliggende weefsel beschadigd. Daarnaast kan radiotherapie ernstige bijwerkingen hebben zoals ontsteking van het wangslijmvlies, zweren, en fibrose. De exacte oorzaak van deze bijwerkingen is tot op heden niet bekend. In hoofdstuk 6 hebben we gekweekt wangslijmvlies en normaal wangslijmvlies behandeld met radiotherapie door het bloot te stellen aan een hoge dosis straling. Daarna hebben we veranderingen in het gekweekte en normale wangslijmvlies als gevolg van de bestraling bestudeerd. Hierbij hebben we gekeken naar veranderingen in de expressie patronen van belangrijke componenten van het epitheel, basaal membraan en het onderliggende bindweefsel. De resultaten lieten zien dat het epitheel na bestraling significant dunner werd, en dat dit veroorzaakt werd doordat de celdeling van de keratinocyten significant afnam. Ook werd de expressie van vimentine in de keratinocyten geobserveerd, een indicatie dat de keratinocyten van het epitheel ernstig beschadigd zijn. Onze resultaten laten zien dat de veranderingen in structuur en morfologie, als gevolg van radiotherapie, in gekweekt wangslijmvlies gelijk zijn aan de veranderingen in normaal wangslijmvlies. Uit bovengenoemde resultaten kunnen we concluderen dat het gekweekte wangslijmvlies een goed model kan zijn om de acute effecten van radiotherapie in detail te bestuderen.

CONCLUSIE

Op basis van deze bovengenoemde resultaten kunnen we concluderen dat we een construct hebben ontwikkeld dat sterk overeenkomt met origineel wangslijmvlies, qua structuur en functionaliteit. In deze dissertatie hebben we laten zien dat gekweekte TEM constructen geïmplanteerd kunnen worden in immunodeficiëntie muizen, waar de constructen vervolgens kunnen overleven en ontwikkelen. Met name dit laatst genoemde resultaat is veelbelovend en suggereert dat het mogelijk moet zijn om het uiteindelijke doel te halen: het gebruik van TEM constructen in een klinische setting voor de behandeling van patiënten.

DANKWOORD

Na vier jaar onderzoek en wat extra tijd is het eindelijk zover; mijn proefschrift is af. Een woordzoeker was een leuk idee maar niet toereikend, vandaar dat ik de volgende mensen via deze weg wil bedanken voor hun bijdrage aan de totstandkoming van dit proefschrift:

Allereerst wil ik mijn promotor, Prof.dr. S.E.R. Hovius bedanken. Prof. Hovius, ik wil u bedanken dat u mij de kans heeft gegeven om het stokje van Hinne over te nemen. U heeft mij in alle vrijheid mij het onderzoek laten doen zonder daarbij het einddoel uit het oog verliezen.

Mijn co-promotoren, dr. Soledad Perez-Amodio en dr. Han van Neck.

Sole, bedankt voor alles! Ik heb ontzettend veel van je geleerd, van het opzetten van experimenten tot het schrijven van artikelen. Ondanks je drukke baan en leven in Spanje ben je nog steeds betrokken bij mijn werk en maak je tijd voor mij vrij. Je adviezen en commentaren zijn altijd waardevol gebleken en ik ben er van overtuigd dat je mijn onderzoek naar een hoger niveau hebt getild.

Han, bedankt voor je adviezen en kritische opmerkingen bij het schrijven van mijn artikelen.

Graag wil ik mijn collega's bedanken met wie ik de afgelopen jaren samengewerkt heb. Te beginnen met mijn collega's van de Plastische & Reconstructieve Chirurgie:

Dr. Hinne Rakhorst, de initiatiefnemer van het mucosa onderzoek. Ik ben er trots op dat ik deel heb kunnen uit maken van het mucosa project en zonder jou was dit promotieonderzoek niet mogelijk geweest.

Linda Spiegelberg, een jaar na mij begonnen aan de uitputtingsslag die promoveren heet. Je hebt bent als verstekeling van de Kaakchirurgie bij mij op de kamer terechtgekomen en we hebben het ontzettend gezellig gehad. Het ziet er zelfs naar uit dat we heelhuids ons 'zinkende schuitje" verlaten!

Charlotte de Saint Aulaire, ik ken niemand die zoveel balletjes in de lucht houdt als jij; promotieonderzoek, in opleiding tot KNO-chirurg, moederschap, etc. Absoluut bewonderingswaardig. Ik heb erg fijn met je samen gewerkt!

Ook dr. Femke Verseijden, Sandra Posthumus – van Sluijs, dr. Miao Tong, dr. Urville Djasim, Antoinette van Driel, Ineke Henning-Weijma, Esther Fijneman en iedereen die tijdens mijn promotieonderzoek werkzaam was op het lab van de plastische in de "toren", dank voor de samenwerking.

Met veel plezier heb ik samengewerkt met "de 16de", ofwel de mensen van het KNO/Orthopaedie lab onder leiding van Prof.dr. Gerjo van Osch. Gerjo, ik wil je bedanken dat je ondanks je overvolle agenda toch tijd voor mij hebt gemaakt. Je adviezen zijn ontzettend waardevol gebleken

voor mijn onderzoek. Met name wil ik ook Wendy en Nicole bedanken voor hun betrokkenheid, luisterend oor en humor. Jullie grenzeloze kennis van alles wat met het lab te maken heeft was van onschatbare waarde. Tevens wil ik Ruud en Eric bedanken voor de gezelligheid op het lab, tijdens congressen en niet te vergeten bij Boudewijn!

Graag wil ik ook alle KNO chirurgen en assistenten bedanken voor hun inzet en bereidheid voor het afnemen van wangslijmvlies.

Uiteraard wil ik mijn nieuwe collega's van Xpand biotechnology bedanken voor hun interesse tijdens het schrijfproces.

I would like to thank prof. Dr. Douglas Taatjes and Marilyn Wadsworth for their patience and supervising during my internship at the MIC. It was my first internship ever, and thanks to you it was one of the best times in my life. I also want to thank Janet Schwarz, Michele von Turkovich, Anthony Quinn and Nicole Bishop for making my internship fun and a time I will never forget.

Bas, voor jou was een kort "bedankt" genoeg, maar wat meer tekst is toch echt noodzakelijk! De afgelopen jaren hebben we ontzettend veel gelachen samen, maar we hebben ook elkaars dieptepunten meegemaakt. Je hebt mijn hele promotieonderzoek van dichtbij meegemaakt en staat nog steeds altijd voor me klaar. Je bent zonder

twijfel de beste collega die er is en de enige ter wereld de me “serpent” mag noemen! Niet voor niets ben je een van mijn paranimfen.

Edwin & Tanja, Richard & Kim, Marco, Theo, Niels, Bart & Marina, Patricia & Martijn, Jos & Annette, met jullie is het altijd weer gezellig op feestjes, Graspop en daar buiten. If you ain’t a metalhead you might as well be dead!!

Angela, onze liefde voor de zware metalen muziek zorgde voor een innige vriendschap. Je begrip, luisterend oor, relativeringsvermogen en gevoel voor humor maken je tot mijn beste vriendin. Ik geniet van je gezelschap en onze uitstapjes. Wordt toch eens tijd dat we die Metal Quiz winnen!

Ik wil graag mijn gehele (schoon-) familie bedanken voor hun interesse in mijn onderzoek.

De laatste paragrafen zijn natuurlijk voor de mensen die het belangrijkst voor mij zijn, mijn familie.
Lieve pap en mam, jullie hebben me geleerd om alle kansen te grijpen en hard te werken. Jullie onvoorwaardelijke steun, motivatie en liefde hebben er voor gezorgd dat dit proefschrift daadwerkelijk geschreven is.
Lieve Natasja, als ik iets van jou kan leren is het wel dat je met doorzettings-vermogen en veerkracht alles kunt bereiken. Ik kan me geen betere zus wensen en ik ben blij dat je mijn paranimf wilt zijn. Willem, ik

ben blij dat mijn zus in jou haar lief heeft gevonden. “Bobbi”, zo ontzettend welkom in onze familie en ik kan niet wachten tot je er bent!

Evi, met Dennis was je een “package-deal”, erg wennen maar nu een onverwacht cadeautje.

Dennis, mijn lief, als iemand weet hoeveel tijd en energie er in dit proefschrift zit ben jij het wel. Je weet me als geen ander te kalmeren en te motiveren. Samen staan we aan de vooravond van wat weleens ons grootste avontuur kan worden en ik kijk uit naar onze toekomst.

Lieve ☆ **,** Dennis en ik zijn dolgelukkig met jou en ik kan niet wachten tot ik je eindelijk vast mag houden!

CURRICULUM VITAE

Wendy M.W. Tra was born on April 2nd 1981 in Tilburg, the Netherlands. After graduation at the Mill Hill College in 1999, she went to study Biology and Medical Laboratory research at the Faculty Technique and Nature of the Hogeschool Brabant where she received her Bachelors degree in 2003. In 2003 she started as a research technician at the department of Gastroenterology and Hepatology of the Erasmus Medical Centre, where she worked for the liver transplantation team on hepatitis B. From 2004 to 2008 she worked as a research technician at the department of Plastic and Reconstructive Surgery (Erasmus MC) under the supervision of Hinne A. Rakhorst on the development of tissue-engineered mucosa. After 4 years as a research technician, she started as a PhD-student at the department of Plastic and Reconstructive Surgery under the supervision of Prof.dr. S.E.R. Hovius, Dr. S. Perez-Amodio and Dr. J.W. van Neck. The subject of her PhD research project was developing tissue-engineered mucosa, and evaluating the tissue-engineered mucosa as an *in vitro* model. From November 2012, she started as a R&D Scientist at Xpand Biotechnology, Bilthoven.

PHD PORTFOLIO SUMMARY

Name PhD student:	Wendy M.W. Tra
Erasmus MC Department:	Plastic and Reconstructive Surgery
PhD period:	June 2008 - June 2012
Promotor:	Prof. Dr. S.E.R. Hovius
Co-promotors:	Dr. S. Perez-Amodio and Dr. J.W. van Neck

PhD-training	**Year**	**ECTS**
In-depth courses		
▪ Confocal Microscopy (EUR)	2008	0.5
▪ Scientific Writing in English for publication-writing to be read (EUR)	2009	1
▪ Hallmarks of Biology	2010	1.5
(Inter-)national conferences poster presentations		
▪ Effects of radiation on tissue engineered mucosa at the Tissue Engineering and Regenerative Medicine Society (TERMIS-EU), Porto, Portugal.	2008	3
▪ Tissue engineered mucosa: a 3D model to quantify the effects of ionizing radiation, DNA-repair congress, Crete, Greece.	2009	3
(Inter-)national conferences podium presentations		
▪ Effect of Radiation on Tissue Engineered Mucosa, World Biomaterials Congress, Amsterdam, the Netherlands	2008	1
▪ DNA repair in 3D tissue engineered mucosa: fundamental research to improve radioprotection at the Rotterdamse Werkgroep Hoofd-Hals Tumoren (RWHHT), Rotterdam, the Netherlands	2008	1

▪ Biological response of tissue engineered mucosa to ionizing radiation: a time study, European Conference of Scientists and Plastic Surgeons (ECSAPS), Helsinki, Finland.	2010	1,5
▪ Biological response of tissue engineered mucosa to ionizing radiation: a time study, TERMIS-EU, Galway, Ireland.	2010	2
▪ Biological response of tissue engineered mucosa to ionizing radiation: a time study, RWHHT, Amsterdam, the Netherlands.	2011	1
▪ Early effects of ionizing radiation in tissue engineered mucosa, TERMIS-EU, Granada, Spain.	2011	2
▪ Early effects of ionizing radiation in tissue engineered mucosa, European Tissue Repair Society (ETRS), Amsterdam, the Netherlands.	2011	1,5

Teaching activities:

▪ Supervising 3rd year medical students during practical laboratory assignment	2008	2
▪ Lecture “Tissue engineered mucosa”, 3rd year medical students.	2008	0,5
▪ Teaching keratinocyte and fibroblast isolation and culture experiments, visiting post doc.	2009	1
▪ Lecture “Tissue engineered mucosa: fundamental research on the effects of ionizing radiation”, 3rd year medical students.	2010	1
▪ Supervising wikipedia-assignment, 3rd year medical students.	2011	1

Nominations:

▪ Nominated for reaching the top 50 of best abstracts submitted at the TERMIS-EU, Galway, Ireland.	2010

LIST OF PUBLICATIONS

- **Tra WM,** Spiegelberg L, Tuk B, Hovius SE, Perez-Amodio S. *Hyperbaric oxygen treatment of tissue-engineered mucosa enhances secretion of angiogenic factors in vitro*. Tissue Eng Part A. 2014 May;20(9-10):1523-30.
- **Tra WM**, Tuk B, van Neck JW, Hovius SE, Perez-Amodio S. *Tissue-engineered mucosa is a suitable model to quantify the acute biological effects of ionizing radiation.* Int J Oral Maxillofacial Surg. 2013 Aug;42(8):939-48.
- **Tra WM**, van Neck JW, Hovius SE, van Osch GJ, Perez-Amodio S. *Characterization of a three-dimensional mucosal equivalent: similarities and differences with native oral mucosa.* Cells Tissues Organs. 2012;195(3):185-96.
- Perez-Amodio S, **Tra WM**, Rakhorst HA, Hovius SE, van Neck JW. *Hypoxia preconditioning of tissue-engineered mucosa enhances its angiogenis capacity in vitro.* Tissue Eng Part A. 2011 Jun;17(11-12):1583-1593.
- Zuijdendorp HM, **Tra WM**, van Neck JW, Mollis L, Court JH. *Delay of denervation athrophy by sensory protection in an end-to-side neurorrhaphy model: a pilot study.* J Plast Reconstr Aesthet Surg. 2010 Dec;63(12):1949-1952.

- De Wit T, de Putter D, **Tra WM**, Rakhorst HA, van Osch GJ, Hovius SE, van Neck JW. *Auto-crosslinked hyaluronic acid gel accelerates healing of rabbit flexor tendons in vivo.* J Orthop Res. 2009 Mar;27(3):408-415.
- Bosma BM, Metselaar HJ, **Tra WM**, Mancham S, Kuipers EJ, Tilanus HW, Kwekkeboom J. *Impairment of circulating myeloid dendritic cells in immunosurpressed liver transplants recipients.* Clin Exp Immunol. 2007 Sep;149(3):525-534.
- Rakhorst HA, **Tra WM**, Posthumus-van Sluijs ST, Hovius SE, Levendag PC, Kanaar R, Hofer SO. *Quatitative analysis of radiation-induced DNA break repair in a cultured oral mucosal model.* Tissue Eng. 2006 Dec;12(12):3395-3403.
- Rakhorst HA, Posthumus-van Sluijs SJ, **Tra WM**, van Neck JW, van Osch GJ, Hovius SE, El Ghalbzouri A, Hofer SO. *Fibroblasts accelerate culturing of mucosal substitutes.* Tissue Eng. 2006 Aug;12(8):2321-2331
- Rakhorst HA, **Tra WM**, Posthumus-van Sluijs SJ, de Groot E, van Osch GJ, van Neck JW, Hofer SO. *Mucosal keratinocyte isolation: a short comparative study on thermolysin and dispase.* Int J Oral Maxillofac Surg. 2006 Oct;35(10):935-940.
- Wadsworth MP, Sobel BE, Schneider DJ, **Tra W**, van Hirtum H, Taatjes DJ. *Quantitative analysis of atherosclerotic lesion composition in mice.* Methods Mol Biol. 2006;3(19):137-152.

- Moons LM, Kusters JG, Bultman E, Kuipers EJ, van Dekken H, **Tra WM**, Kleinjan A, Kwekkeboom J, van Vliet AH, Siersema PD. *Barrett's oesophagus is characterized by a predominantly humoral inflammatory response.* J Pathol. 2005 Nov;207(3):269-276.
- Kwekkeboom J, Tha-In T, **Tra WM**, Hop W, Boor PP, Mancham S, Zondervan PE, Vossen AC, Kusters JG, de Man RA, Metselaar HJ. *Hepatitis B immunoglobulins inhibit dendritic cells and T cells and protect against acute rejection after liver transplantation.* Am J Transplant. 2005 Oct;5(10):2393-2402.