UNIVERSITÉ DE STRASBOURG

ÉCOLE DOCTORALE PHYSIQUE, CHIMIE-PHYSIQUE

Inserm BioMatériaux et BioIngénierie UMR-S 1121

THÈSE présentée par :

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Soutenue le : **20 Septembre 2019**

Pour obtenir le grade de : **Docteur de l'Université de Strasbourg**

Discipline/ Spécialité : Biomatériaux et Bioingénierie

Utilisation d'hydrogels fonctionnalisés pour une ré-épithélialisation rapide des implants hybrides en ingénierie tissulaire

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Remerciements

A mon maître et directeur de thèse le Professeur Christian DEBRY,

Voilà bientôt 10 ans que j'ai intégré cette équipe d'ORL de Strasbourg, vous avez toujours été présent aussi bien pour ma formation chirurgicale que mes débuts dans la recherche. Je vous remercie et vous fais part de ma profonde reconnaissance. Vous m'avez permis de mener ce travail à terme avec tout l'encadrement nécessaire dans une formidable équipe solidaire et motivée. Vos capacités d'être sur de multiples fronts et votre enthousiasme pour cette belle épopée du larynx artificielle sont pour moi une source d'inspiration pour continuer l'aventure.

Au Professeur Pierre SCHAAF,

Je vous remercie de m'avoir accepté au sein de votre unité de recherche et je vous fais part de mon profond respect et de toute ma gratitude pour votre bienveillance.

Au Professeur Sybille FACCA,

Vous avez répondu présent à toutes les étapes clés de ce travail pour m'apporter vos bon conseils, vos critiques constructives et la motivation nécessaire. Je vous fais part de toute ma gratitude et vous remercie encore pour votre disponibilité et votre bienveillance.

Au Professeur Sébastien VERGEZ,

Je voulais vous faire part de mon profond respect et vous remercier d'avoir accepté de juger ce travail. Votre expertise en cancérologie ORL a été un apport indéniable et incontournable pour la problématique de ce travail.

Au Professeur Christophe EGLES,

Vous avez accepté de juger ce travail pour apporter votre expertise en sciences fondamentales, je tenais à vous faire part de toute ma reconnaissance. Je vous remercie et vous fais part de mon profond respect.

A mon maître le Professeur Philippe SCHULTZ,

Je voulais te faire part ici de tout mon respect et ma gratitude pour tout ce que tu m'as apporté en pratique clinique. Je te remercie également d'avoir accepté de juger ce travail. Je suis ravi de poursuivre dans le service sous ton œil bienveillant pour la chirurgie cervicale et reconstructrice.

Au Docteur Julien Barthès,

Je te remercie pour ces longues années passées à me guider et me soutenir. La collaboration avec toi au sein de l'unité 1121 a été une chance et un plaisir. Je te remercie également d'avoir accepté de juger ce travail. J'espère que l'on pourra discuter encore longtemps lors de pause-café, clope, défaire et refaire le monde.

A mon co-directeur de thèse le Docteur Nihal Engin VRANA,

Je te remercie pour tout ce que tu as fait pour moi. Sans toi je ne suis pas certain que j'aurais pu autant avancer dans ce travail. Je te fais part de mon profond respect et de toute ma reconnaissance. Je reste toujours aussi admiratif de tes capacités de travail surhumaines de ton génie et ta motivation. Encore un grand merci.

Au Docteur Philippe LAVALLE,

Je te remercie pour ton accueil et ta gentillesse. Tu as su me motiver dans les moments quelques peu difficiles, de doute… et il y en a eu. Ça y est c'est fait ! Je serai bientôt de retour pour de nouvelles aventures avec un esprit plus serein.

Au Docteur Dominique VAUTIER,

Merci pour ta gentillesse et ton soutien toute au long de ces années. C'est toujours un plaisir que de discuter avec toi lors des passages dans l'unité. L'aventure continue avec une équipe toujours agréable.

A toute l'équipe de l'unité INSERM 1121,

A Morgane … le Confocal est toujours entier et le laser fonctionne ! Merci beaucoup pour toutes tes formations gracieuses pour les différents appareils. Mais faudra remettre une couche à mon retour.

A Céline…, ton sens de l'organisation me laisse toujours admiratif. Faudra que je m'en inspire. Merci pour tout ce que tu as fait au pied levé pour moi.

A Héléna … désolé pour tout le matériel que je t'ai piqué dans le frigo. Merci pour ton aide et tes conseils.

A Camille…, merci pour tout ton aide.

A Bernard…, merci pour ton aide ta gentillesse sans pareil et les magnifiques boîtes à moustaches.

A toute l'équipe d'ORL de Strasbourg,

Au Docteur Patrick HEMAR,

Toujours disponible, toujours de bon conseil, et surtout solution à tout. Merci beaucoup pour tout ce que tu m'as apporté. Te savoir pas loin est source d'apaisement.

A Mon maître le Professeur Anne CHARPIOT,

Merci pour ton accompagnement, tes conseils et tout ce que tu m'as apporté en chirurgie de l'oreille.

Au Docteur Dominique ROHMER,

Merci pour toutes ces années et tout ce savoir que vous nous avez transmis toujours dans la joie et la bonne humeur. Vos enfants spirituels poursuivent le vertige dans tous les recoins de l'hôpital. Reposez en paix.

A tous mes co-internes, co-chefs

A mes Amis Guillaume, Sarah, Anne Sophie, Sofiane, Morgan, Wilfried, Mathieu, Stéphane, Joël, Jean Marc, Frédéric

Merci de me supporter comme je suis …. Je n'ai pas prévu de faire des efforts pour changer.

A mes Parents

A mes Frères,

A ma Sœur

A – Préambule

1- Résumé

Utilisation d'hydrogels fonctionnalisés pour une ré-épithélialisation rapide des implants hybrides en ingénierie tissulaire

INTRODUCTION

En pratique clinique, les carcinomes avancés du larynx sont traités par une laryngectomie totale qui conduit à une séparation des voies aérodigestives. Il en résulte une trachéostomie définitive avec une perte de la fonction de phonation. Cela impacte négativement la qualité de vie des patients. Pour pallier à cette perte de fonction de phonation, des travaux sur la réalisation d'un « larynx artificiel » sont en cours au Centre hospitalier et universitaire de Strasbourg. Le titane très largement utilisé dans les biomatériaux implantables a été choisi pour le prolongement de la section trachéale afin d'y connecter une valve multidirectionnelle. Dans le cadre du remplacement du larynx, le titane présente comme principal avantage de résister aux contraintes mécaniques qui lui sont appliquées ainsi qu'à la colonisation bactérienne et fongique. Cependant la colonisation tissulaire de cet implant en titane poreux conduit à un bourgeonnement de tissu conjonctif en endoluminal à l'origine d'une sténose luminale. Une des raisons avancées pour cet hyper bourgeonnement est l'absence de ré-épithélialisation rapide de la face endoluminale de cet implant.

L'objectif de ce travail de thèse était de développer un support pour permettre la ré-épithélialisation rapide des implants en bioingénierie tissulaire. Le choix s'est porté sur la repousse de l'épithélium respiratoire sur la face endoluminale de l'implant en titane poreux utilisé dans le larynx artificiel développé au CHU de Strasbourg.

RESULTATS

La première partie de ce travail a permis de démontrer la viabilité des cellules épithéliales dans un système de double couche de gélatine (dérivé de collagène dénaturé de matrice extracellulaire porcine) permettant de mimer une matrice extracellulaire artificielle. L'adhésion de ces films de gélatine au titane a été testé avec succès dans les conditions de culture cellulaire. L'expérimentation en culture cellulaire a permis de prouver que la viabilité cellulaire était assurée dans un tel système. Nous avons également testé la capacité de ces films de gélatine à être chargés avec des facteurs de croissance cellulaires et à les libérer progressivement dans le milieu de culture. Ces films ont été chargés avec de l'albumine de

sérum bovin avec un marqueur fluorescent (Fluoroisothiocynate), utilisé comme modèle protéique pour les essais de relargage. Les mesures de cette fluorescence dans le liquide du milieu de culture ont été effectués en spectrofluorométrie. Nous avons pu observer que ces films préalablement chargés pouvaient libérer les facteurs de croissance avec un relargage massif dans les 24 premières heures mais se poursuivait sur 2 semaines.
Dans la deuxième partie de ce travail, nous avons porté notre attention à l'optimisation de la fabrication de ces films. Les films utilisés dans la première partie étaient fabriqués manuellement. Dans cette seconde partie nous avons utilisé un spincoater (tournette) pour la production des films. Il s'agit d'un procédé qui permet d'étaler de façon homogène la gélatine à la surface de lamelles de verre par centrifugation. Nous avons étudié la variation de l'épaisseur des films en fonction des concentrations de gélatine utilisées et en fonction de la vitesse de rotation du spincoater. Nous avons conclu que l'épaisseur des films était conditionnée par la concentration en gélatine, plus que par la vitesse de rotation.

Pour assurer la stabilité des films dans les milieux de culture cellulaire et éviter leur dissolution rapide, nous avons testé la fabrication de films avec des gels interpénétrés de gélatine (14%) et d'acide hyaluronique-tyramine (HA-Tyr, 1%). Permettant ainsi une double réticulation enzymatique (transglutaminase A pour la gélatine B et Horse Radish Peroxydase pour l'HA-Tyr). Nous avons ainsi assuré une meilleure stabilité des films en milieu de culture.
Pour faciliter le décollement de ces films des lamelles de verre utilisées comme support pour le spincoater, une fine couche d'acétate de cellulose est déposée initialement sur les lamelles puis laissée à sécher. Le mélange gélatine et HA-Tyr est ensuite déposé sur ces lamelles déjà préparées. Ensuite la double réticulation est réalisée. Une fois séchées ces films de gélatine – HA-Tyr sont aisément décollées des lamelles de verre après immersion dans de l'eau, les interactions entre le film et verre étant devenues trop faible. Les films ainsi fabriqués peuvent être utilisées pour la culture cellulaire après rinçage en condition stérile. Ils ont été ensuite testés in vitro avec des cellules épithéliales carcinomateuse bronchiques humaines (A549).
L'analyse a été réalisée en videomicroscopie sur 24h pour évaluer la vitesse et la distance de migration des cellules sur un tel support en fonction de la constitution des films. L'analyse des données montre que la double réticulation des films n'impacte pas la migration des cellules sur la surface de ces films.

Lors de l'implantation d'un biomatériau au niveau du corps, l'intégration tissulaire passe par une réponse inflammatoire adaptée pour la cicatrisation. Cette réponse inflammatoire est en partie conditionnée par l'activité des macrophages. La différenciation des monocytes en macrophages se fait vers une voie de signalisation cellulaire M1 dite pro-inflammatoire ou bien M2 dite anti-inflammatoire ou pro-cicatrisante. Nous avons testé la gélatine pour encapsuler des macrophages. Sous l'action de l'IL-4 intégrée dans le milieu de culture nous avons pu orienter la réponse des macrophages vers une voie de signalisation pro-cicatrisante. La fonctionnalisation des films d'hydrogels avec des cytokines de la réponse inflammatoire adaptée permettrait ainsi d'optimiser la bio-intégration des matériaux utilisés en ingénierie tissulaire.

L'épithélium respiratoire étant une structure tissulaire avec plusieurs types cellulaires très bien différenciés, la régénération est limitée par le faible nombre de cellules de régénération spontanément disponibles dans les muqueuses respiratoires. Aussi une autre alternative évoquée pour une régénération de cet épithélium respiratoire est l'utilisation de cellules souches mésenchymateuses. Nous avons testé ces films d'hydrogels chargés avec des facteurs de croissance épithéliaux pour permettre une différenciation des cellules souches mésenchymateuses en cellules épithéliales. Ceci nous a permis de démontrer que sur une période de culture suffisamment longue (2-3 semaines), les cellules mésenchymateuses mises en culture sur les films de Gélatine-HA présentait des modifications morphologiques s'orientant vers les caractéristiques de cellules épithéliales. Progressivement ces cellules souches expriment de moins en moins les marqueurs (vimentine) propre aux cellules mésenchymateuses pour exprimer de plus en plus les marqueurs des cellules épithéliales (cytokératines). Ceci montre que ces films chargés avec les facteurs de croissance spécifiques dans un milieu de culture adapté permettent d'orienter la différenciation cellulaire vers une lignée de cellules souhaitée.

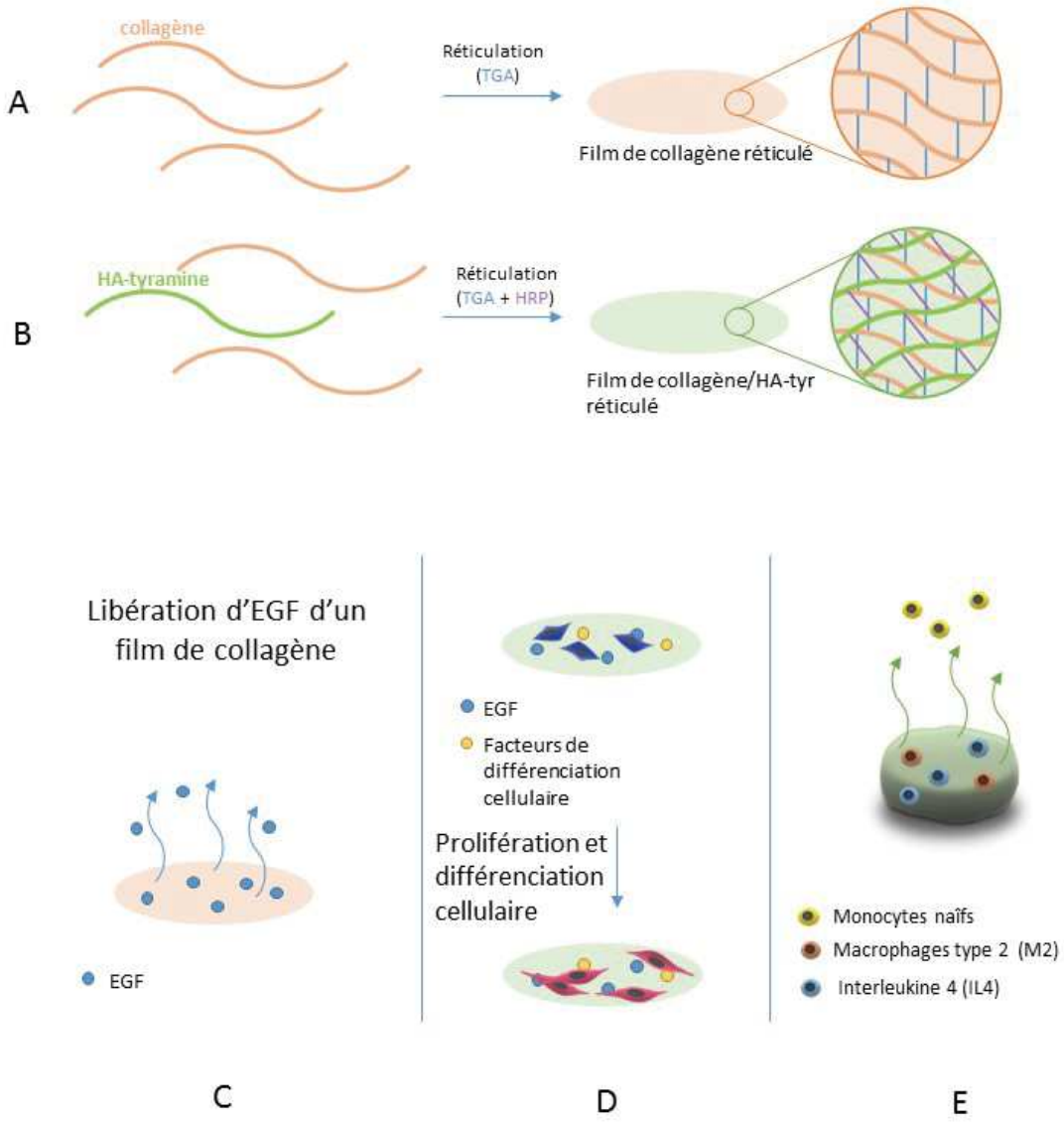

Figure 1 : Schématisation des objectifs attendus des films d'hydrogels. (A) Stabilisation par réticulation des films de gélatine. (B) Stabilisation des films d'hydrogels interpénétrés de gélatine et HA-tyr. (C) Hydrogels servant de plateforme de libération de facteurs préalablement chargés. (D) Hydrogels fonctionnalisés assurant une prolifération et une différenciation des cellules ensemencées. (E) Encapsulation de macrophages dans les hydrogels assurant une immunomodulation lors de l'implantation.

CONCLUSION

Au travers des différentes expérimentations réalisés nous avons pu prouver que l'utilisation d'hydrogels réticulés et fonctionnalisés était une voie de recherche pour le développement d'implants hybrides adaptés aux besoins de la clinique. En effet ces hydrogels stabilisés permettent une stabilité en milieu de culture cellulaire suffisamment longtemps pour avoir un impact sur la bio-intégration des matériaux utilisés en ingénierie tissulaire. De plus les hydrogels jouent le rôle d'une plateforme multimodale pouvant être fonctionnalisée avec les facteurs de croissance cellulaire pour orienter la différentiation cellulaire et des cytokines inflammatoires po-cicatrisantes pour améliorer l'intégration tissulaire. Ils agissent comme une matrice extracellulaire dont les caractéristiques pourraient être modulées pour les adapter en fonction du type histologique du site anatomique de l'implantation. Les améliorations possibles qui pourraient être apportés à ces films sont l'ajout d'agents antibiotiques et antifongiques pour limiter également la colonisation des implants.

2- Introduction Générale

Les maladies liées au système respiratoire et les cancers sont des causes de décès courantes. Les patients doivent faire face à une résection dramatique des voies respiratoires en raison de problèmes associés au cancer, entraînant une perte des fonctions associées (respiration, phonation et déglutition). Bien qu'il existe plusieurs méthodes bien établies pour surmonter la perte de tissus dans le cadre de cancers du larynx et de la trachée (telle que l'anastomose de la trachée après résection partielle), il existe toujours un besoin non satisfait de remplacements fonctionnels garantissant une qualité de vie pour les patients. La perte de la parole, la séparation permanente des systèmes respiratoire et digestif et les complications liées à la présence d'un trachéostome (ouverture pratiquée chirurgicalement reliant la trachée à l'air ambiant par la voie cervicale antérieure, pour remplacer la voie respiratoire normale) soulignent toutes la nécessité de développer un substitut fonctionnel tout en limitant les complications qui en découlent.

Afin de remédier à la pénurie de donneurs d'organes et au recours aux médicaments immunosuppresseurs notamment dans le cadre de cancers, une solution possible est le développement d'outils et de méthodes pour la fabrication de tissus de substitution personnalisés, qui est devenu un domaine de recherche bien développé. Dans le cas précis du remplacement du larynx, cet axe de développement de substitut tissulaire est encore plus important puisque la transplantation laryngée se résume à une transplantation d'organe non fonctionnel engendrant plus de complications comme des fausses routes massives ou bien une dyspnée inspiratoire. Ceci est directement lié au fait que la ré-innervation par les nerfs laryngés donne des résultats très médiocres. La médecine personnalisée ne se limite pas aux applications pharmacologiques et au progrès de l'utilisation de différents dosages chez des patients spécifiques, elle inclut également des traitements médicaux et des systèmes de diagnostic prenant en compte les caractéristiques génétiques, anatomiques et physiologiques de chaque patient. Dans le cas des dispositifs implantables, ceci consiste non seulement en une adéquation anatomique et biomécanique du dispositif avec chaque patient mais également l'adaptation de celui-ci au profil immunologique et aux capacités de régénération propre à chaque patient.

Dernièrement, dans le cadre de l'ingénierie tissulaire et de la médecine régénérative, la médecine personnalisée s'est étendue à la construction d'une solution spécifique pour chaque patient en utilisant des technologies de pointe telles que le prototypage rapide. Dans un proche avenir, il sera possible de développer un substitut d'organe qui corresponde à la taille

et à la composition tissulaire exacte de l'organe cible qui soit complètement adaptée à la capacité de régénération du patient. Le remplacement de la trachée est l'un des domaines où l'ingénierie tissulaire est devenue une réalité clinique, plusieurs implantations déjà réalisées ont été rapportées. Cependant, le larynx n'est pas ciblé en raison de sa structure et de son fonctionnement complexe, hormis quelques tentatives de transplantation. Dans les sections suivantes, nous décrivons ces deux organes en détail, expliquons les problèmes médicaux nécessitant leur résection totale ou partielle et développons les méthodes de remplacement en mettant l'accent sur les essais cliniques de larynx et de remplacement de la trachée.

2.1 Généralités en anatomie du larynx et de la trachée

Dans cette section, les bases de l'anatomie et de l'histologie laryngées et trachéales seront brièvement décrites. L'objectif est de fournir les principales données anatomiques afin de mieux comprendre les problèmes cliniques associés lors d'un remplacement du larynx et de la trachée.

2.1.1 Le larynx

Le larynx est un organe multifonctionnel situé dans la partie antérieure et médiale du cou, au niveau des vertèbres cervicales C5 – C6. Il établit la limite entre les voies respiratoires supérieures et inférieures. Il s'articule ensuite avec la partie cervicale de la trachée située à la base du cou. Il joue un rôle dans la respiration, la déglutition et la phonation. Le larynx a une partie cartilagineuse avec neuf composants connectés les uns aux autres, ce qui fournit le support nécessaire à sa fonction. Cette partie cartilagineuse du larynx comprend le cartilage thyroïdien, le cartilage cricoïde, les cartilages corniculés, l'épiglotte, les cunéiformes et les cartilages aryténoïdes.

Le cartilage thyroïdien est considéré comme le plus grand des cartilages. Il est composé de deux lames qui fusionnent à leurs parties antérieures pour former la proéminence du larynx. Cette proéminence est plus grande chez les hommes adultes que chez les femmes. Le cartilage cricoïde est un cartilage complet en forme d'anneau situé sous le cartilage thyroïdien et relié à ce dernier avec la membrane cricothyroïdienne dans la partie antérieure et les articulations cricothyroïdiennes dans la partie postérieure. La trachée cervicale est reliée à la partie inférieure du cartilage cricoïde. L'épiglotte est attachée à la face interne de la partie antérieure du cartilage thyroïdien. Il se présente sous la forme d'une « feuille » et est situé derrière la base de la langue. Les cartilages aryténoïdes sont pairs et triangulaires, ils reposent sur le

cartilage cricoïde au niveau des articulations cricoaryténoïdes situées sur son bord postérieur et supérieur. Ils régulent le niveau de tension et la position des cordes vocales. Les cartilages corniculés et cunéiformes sont situés au sommet des aryténoïdes.

Toutes ces structures sont maintenues ensemble avec des ligaments et soutenues par plusieurs insertions musculaires. Les muscles extrinsèques interviennent dans le maintien du larynx dans la partie centrale du cou et sa mobilisation au cours de ses différentes fonctions. Ils suspendent le larynx à l'os hyoïde. Les muscles intrinsèques jouent un rôle dans la mobilisation des cordes vocales, ils sont responsables de l'ouverture et de la fermeture des cordes vocales et de la régulation de la filière laryngée lors de la respiration, de la phonation et de la déglutition. La région des cordes vocales est aussi appelée glotte. À ce niveau, le tractus pharyngé se sépare en deux filières : la filière respiratoire (la trachée en avant) et la filière digestive (l'œsophage en arrière).

L'innervation sensorielle et motrice du larynx est assurée par des branches du nerf vague, avec les nerfs laryngés supérieurs et récurrents. La face interne du larynx est recouverte d'un épithélium malpighien. Le fonctionnement de la glotte et de l'épiglotte s'apparente à celui d'une valve multifonctionnelle.

Pendant la respiration, les cordes vocales sont maintenues ouvertes et l'épiglotte est en position relevée. Cela permet à l'air de circuler du tractus aérodigestif supérieur vers les voies respiratoires inférieures, à savoir la trachée. De même, pendant la phonation, l'épiglotte reste dans la même position surélevée et l'ouverture et la tension des cordes vocales sont contrôlées pour permettre leur vibration avec l'air expiré, produisant ainsi un son qui sera ensuite articulé au niveau de la cavité orale pour donner la parole. D'autre part, lors de la déglutition, le larynx joue un rôle crucial dans la protection des voies respiratoires inférieures, obtenue par une séquence d'actions automatiques et finement régulées. Le processus de déglutition commence par une phase contrôlée et volontaire (mastication), suivie d'une phase involontaire, automatique (déglutition). Il comprend une série d'événements : l'ascension laryngée, puis le recul de la base de la langue. Ensuite, les cordes vocales se placent en position fermée et l'épiglotte s'abaisse dans une position horizontale permettant le recouvrement des cordes vocales. Le bol alimentaire est ainsi dirigé vers l'œsophage par la contraction des muscles pharyngiens et l'ouverture du sphincter supérieur de l'œsophage. Toutes ces étapes sont contrôlées par l'innervation sensorielle et motrice et sont cruciales pour

un bon processus de déglutition. Cela signifie que si l'une de ces étapes manque ou est altérée, les fonctions normales qui leur sont associées seront compromises.

2.1.2 La trachée

La trachée, également appelée trachée-artère, débute à la partie inférieure du cartilage cricoïde, plus précisément au niveau de C6 et se poursuit dans le médiastin. Elle est classiquement décrite en deux parties: la trachée cervicale qui va de l'anneau du cartilage cricoïde jusqu'au niveau de T2, où elle pénètre dans la cage thoracique pour donner la trachée thoracique. Son diamètre interne varie en fonction du sexe. Il est plus grand chez les hommes adultes (~1,8 cm). La trachée se bifurque ensuite au niveau de T5 en deux bronches souches droite et gauche qui chacune à leur tour vont présenter une segmentation progressive. La trachée constitue la partie initiale des voies respiratoires inférieures, elle conduit l'air jusqu'aux alvéoles pulmonaires permettant, pendant les phases d'inspiration et d'expiration, les échanges gazeux pour assurer l'hématose (oxygénation du sang et élimination du dioxyde de carbone).

La trachée, qui mesure environ 15 cm de long, est constituée de 16 à 20 anneaux cartilagineux incomplets en forme de « C », empilés les uns sur les autres. Ces anneaux sont maintenus ensemble par un tissu fibreux dans les parties antérieure et latérales. La face postérieure de la trachée est constituée d'un tissu fibro-musculaire directement en contact avec la face antérieure de l'œsophage. La trachée passe dans le médiastin juste en arrière du sternum. Son armature cartilagineuse confère à la trachée la rigidité nécessaire pour éviter les phénomènes de collapsus pendant l'inspiration. Dans sa partie cervicale la trachée est en contact direct, à sa face antérieure, avec la glande thyroïde.

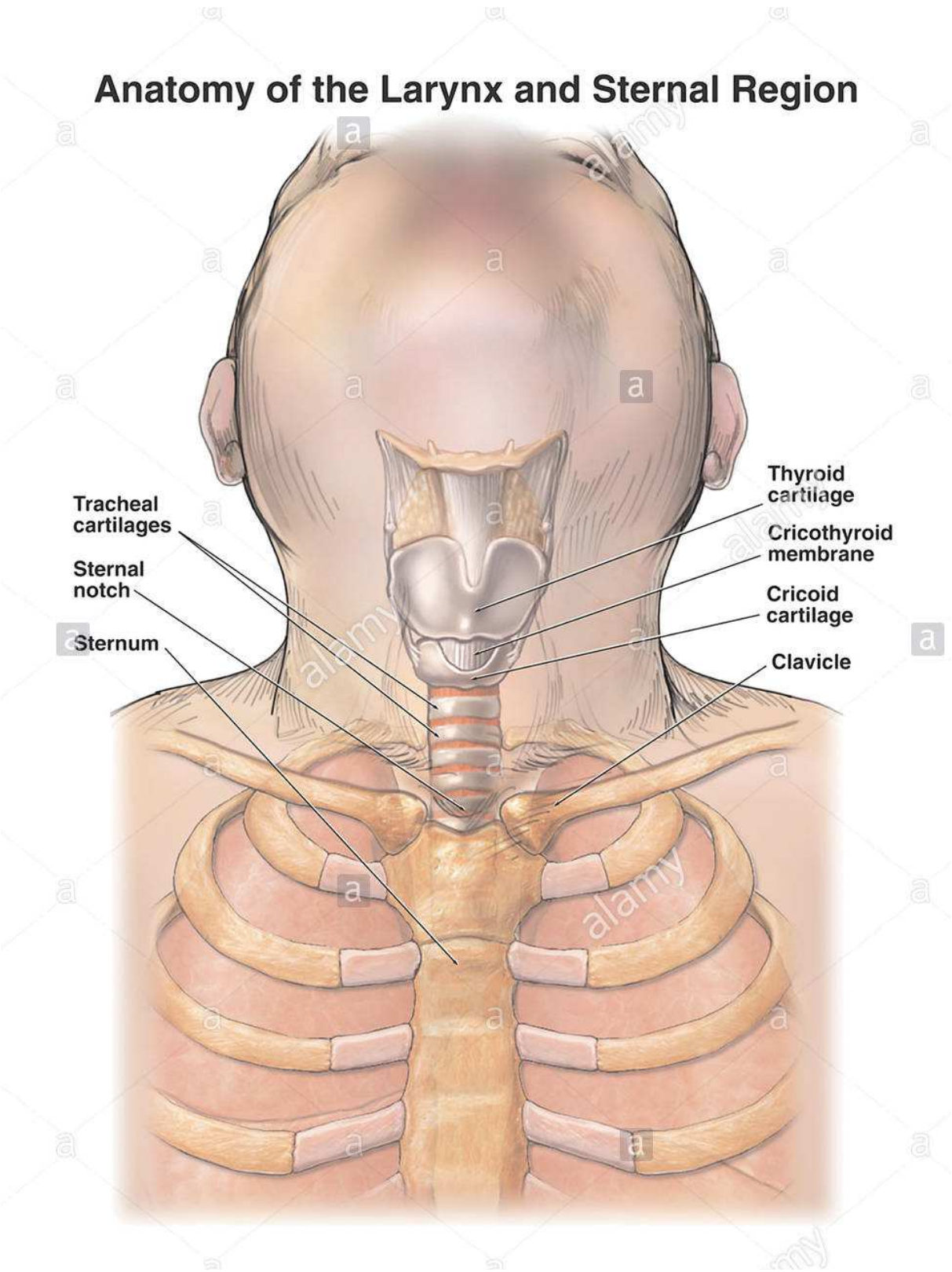

Figure 2 : Vue antérieure de la position du larynx au niveau cervical.

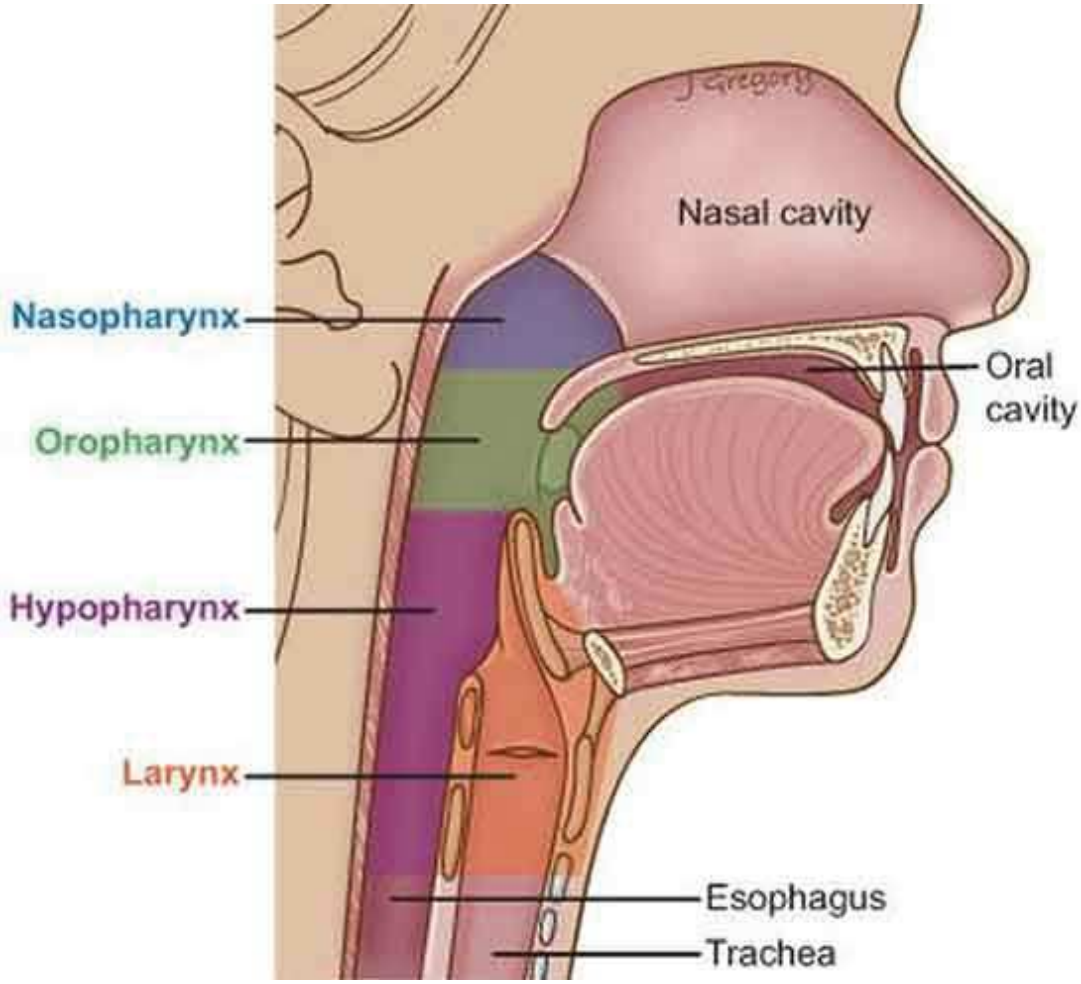

Figure 3 : Coupe sagittale médiane de l'extrémité céphalique. Structure anatomique des voies aéro-digestives supérieures.

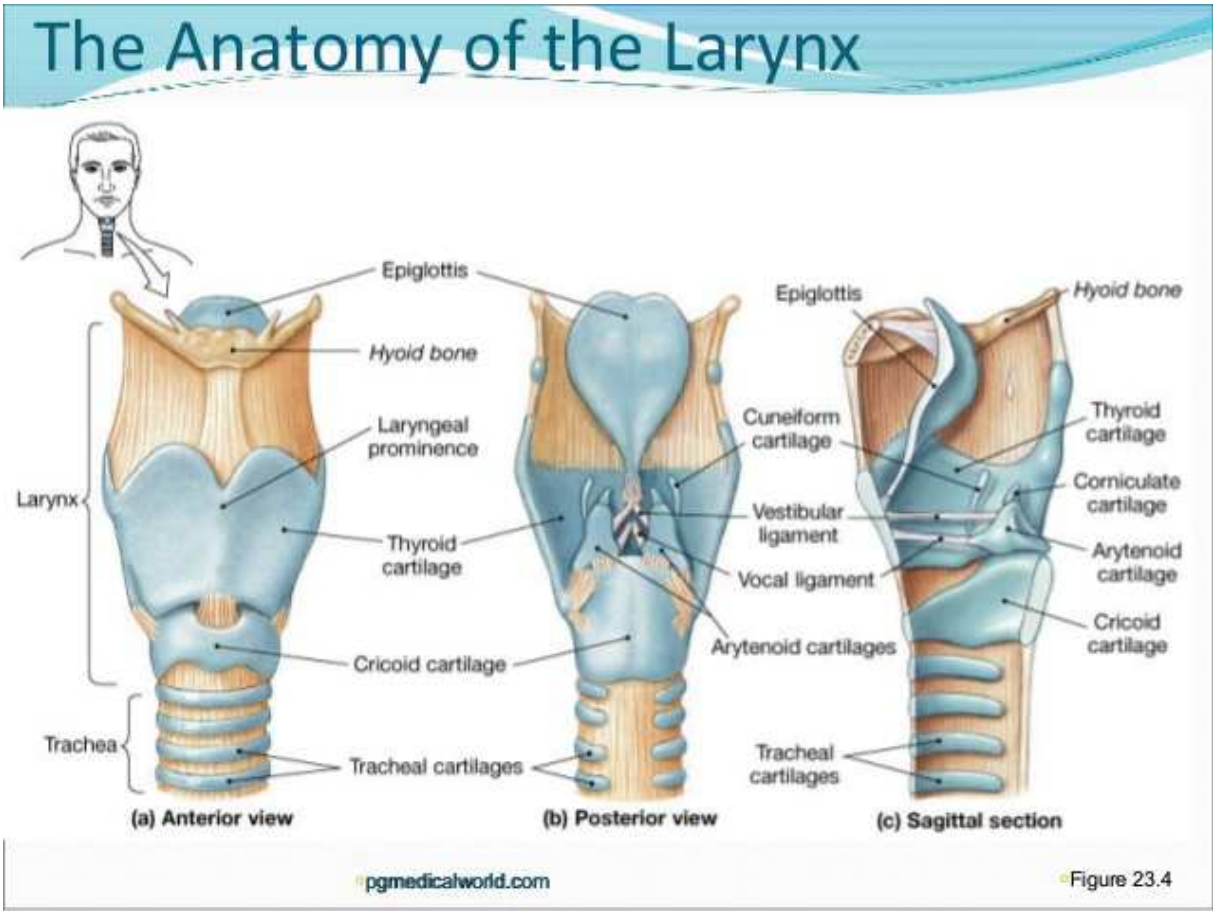

Figure 4 : Anatomie du larynx : (a) vue antérieure, (b) vue postérieure, (c) coupe sagittale.

2.2 Généralités histologie du larynx et de la trachée

Au niveau de la cage thoracique, la trachée est entourée d'importantes structures anatomiques telles que l'aorte, la veine cave supérieure et l'artère pulmonaire. La face endoluminale de la trachée est recouverte d'un épithélium respiratoire. Cet épithélium est pseudo-stratifié et cilié. L'épithélium respiratoire de la lumière de la trachée est composé des cellules suivantes qui sont alignées sur la lamina propria, qui est essentiellement un tissu conjonctif fibroélastique: (i) les cellules cylindriques ciliées, qui sont des cellules allongées ciliées au niveau apical. Elles sont responsables du déplacement du mucus à la surface de l'épithélium ; (ii) des cellules caliciformes, cellules exocrines productrices de mucus, disséminées individuellement dans l'épithélium ;

(iii) les cellules basales, qui sont des cellules rondes situées à la surface basale de l'épithélium, qui constituent essentiellement une réserve de cellules souches pour remplacer les cellules ciliées et caliciformes; (iv) les cellules en brosse, qui sont des cellules en colonnes avec des

microvillosités apicales dotées d'un rôle de récepteur sensoriel grâce aux synapses avec les terminaisons dendritiques des fibres nerveuses sensorielles; et (v) les cellules granulaires à noyau dense, qui sont des cellules endocrines qui semblent être liées au système d'absorption et de décarboxylation des précurseurs d'amine (APUD) et peuvent libérer des substances vasoactives.

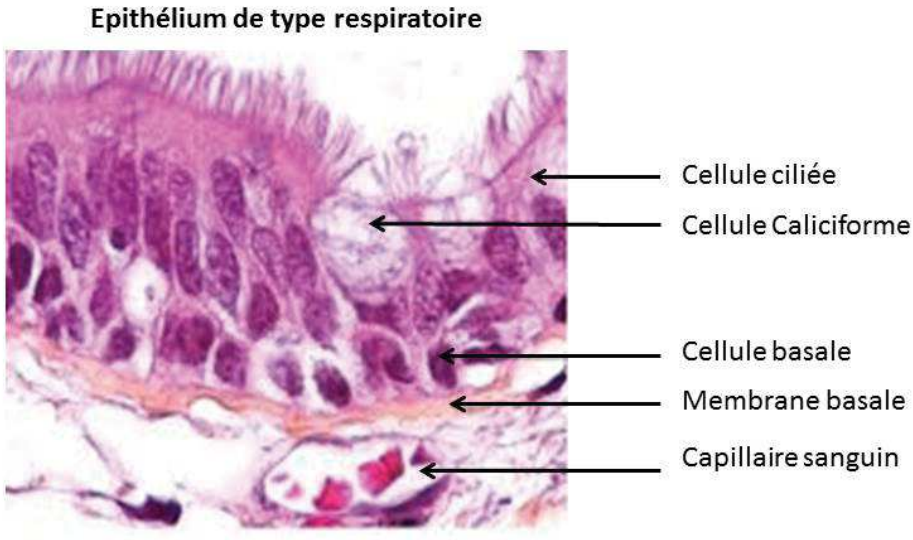

Figure 5 : Coupe histologique de l'épithélium respiratoire.

D'un point de vu physiologique, l'épithélium respiratoire joue un rôle de barrière avec le milieu extérieure mais pas seulement. Les cellules caliciformes produisent un mucus libéré au niveau apical qui tapisse la surface de cet épithélium. Les cellules ciliées quant à elles assurent le déplacement de ce tapis muqueux vers la partie proximale des voies aériennes jusqu'au niveau du plan glottique. Le mucus alors chargé en particules est expectoré, éliminant ainsi les particules en suspension dans l'air inhalé. Il s'agit là d'un fonctionnement complexe et synchronisé des cils vibratiles assurant ainsi une protection des voies aériennes basses. Toute atteinte physique ou bien anomalie fonctionnelle de ce tapis muco-ciliaire engendre une accumulation de mucus et de particules inhalées au niveau des voies aériennes provocant des infections pulmonaires itératives fragilisant l'état de santé des patients. Ainsi

dans le cadre de la mucoviscidose par exemple, la production d'un mucus dont la viscosité est augmentée est à l'origine des pneumopathies à répétition de ces patients. Car celui-ci moins fluide, stagne au niveau des bronches. La dyskinésie ciliaire primitive est également une maladie génétique ou ce sont les mouvements des cils vibratiles qui sont défectueux. Dans ce cas l'accumulation du mucus qui ne peut être éliminé par le tapis muco-ciliaire est également à l'origine de surinfections respiratoires. Au travers de ces deux exemples il est alors aisé de comprendre que la moindre dysfonction de cet épithélium respiratoire se répercute de façon directe sur le pronostic des patients. Aussi dans le cadre du développement d'un substitut au larynx et à la trachée en bioingénierie, le rôle de l'épithélium respiratoire ne peut être laissé au second plan.

2.3 La laryngectomie

2.3.1 Technique chirurgicale.

La laryngectomie totale est une intervention chirurgicale pratiquée sous anesthésie générale. Elle est encore pratiquée dans le cadre du traitement de certains cancers du larynx ou du pharyngo-larynx localement avancés. Elle consiste en l'ablation de l'ensemble de la structure du larynx par une voie d'abord cervicale. Cette exérèse implique une ouverture de l'hypopharynx. Une fois l'ablation du larynx réalisée, les parois de l'hypopharynx sont refermées sur elle-même par des sutures. Ainsi refermé, l'hypopharynx s'apparente à un « entonnoir » communicant en haut avec l'oropharynx, les cavités orales et nasales et se prolonge vers le bas avec l'œsophage. Les filières respiratoires et digestives sont donc définitivement séparées. La filière oro-pharyngée se résume alors à un tractus digestif, la fonction de respiration et phonation ne sont alors plus assurées par les voies aérodigestives supérieures. La trachée est directement abouchée à la peau au niveau de la région cervicale antérieure au-dessus de la fourchette sternale. Cette modification anatomique permet une respiration directement par l'orifice de trachéostomie en shuntant les voies aérodigestives supérieures ainsi que les fonctions de filtration, d'humidification et de réchauffement de l'air inspiré par les muqueuses des fosses nasales.

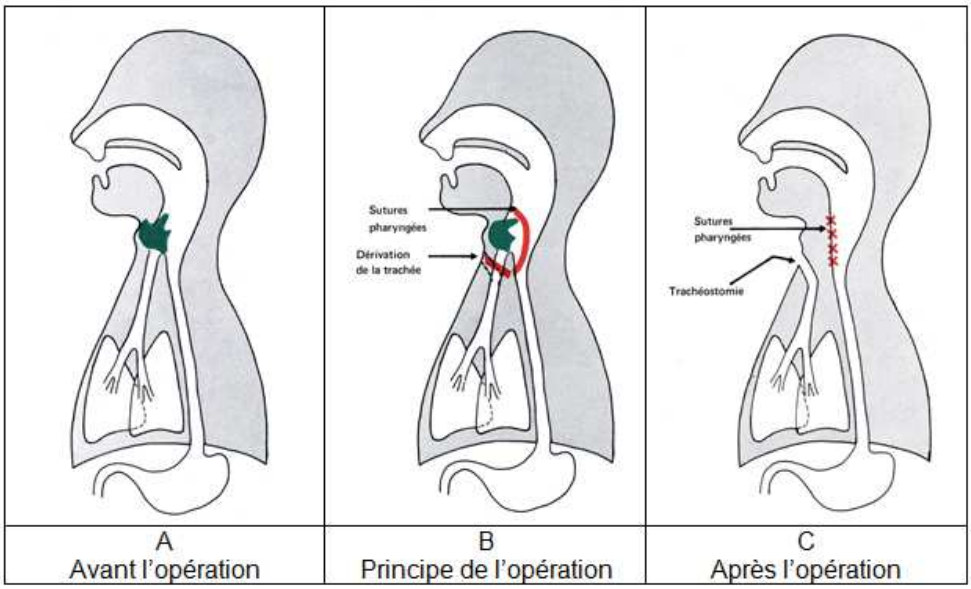

Figure 6 : Schématisation du principe de la laryngectomie totale.

2.3.2 Conséquences de la laryngectomie.

La séparation des filières respiratoires et digestives résultant de la laryngectomie totale n'est pas sans conséquences. En effet, bien que nécessaire pour le traitement de la pathologie carcinologique chez ces patients, elle se fait au prix d'une dégradation significative de la qualité de vie, impactant à des degrés divers les trois fonctions essentielles du larynx que sont la déglutition, la phonation et la respiration.

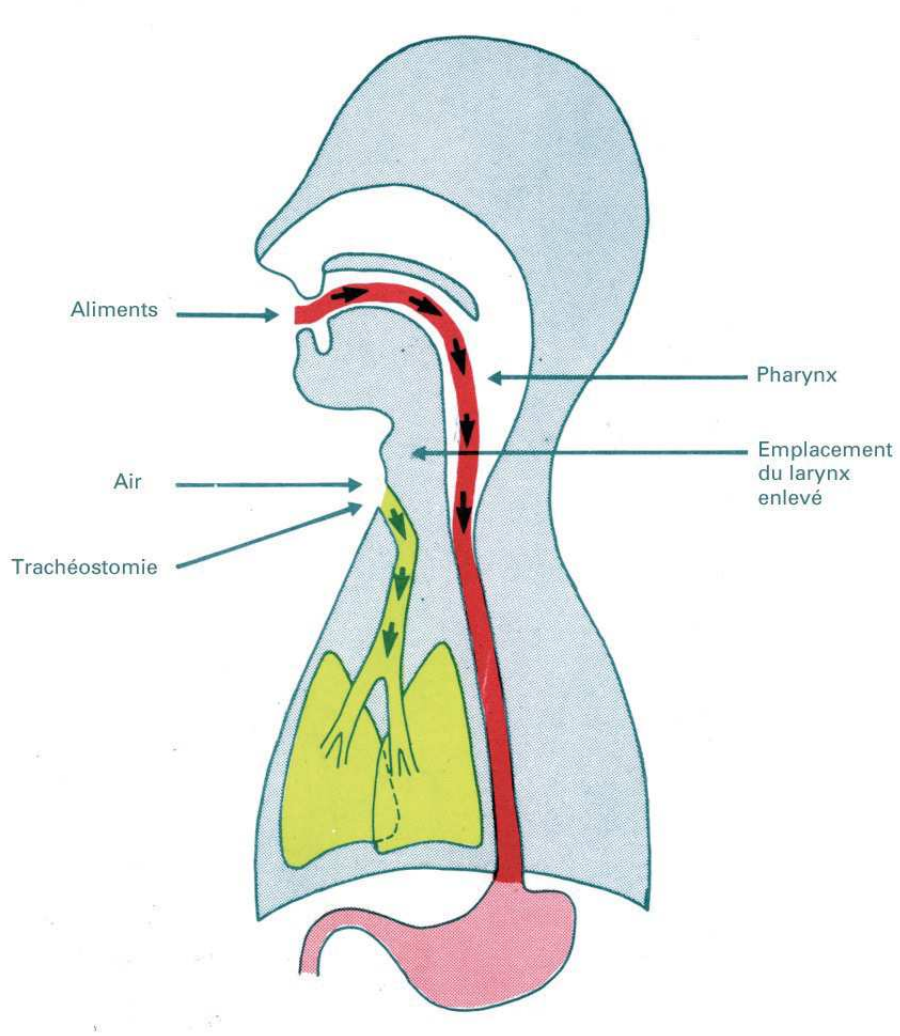

Figure 7 : Schéma montrant la séparation définitive des filières aériennes et digestives après laryngectomie totale.

2.3.2.1 La déglutition après laryngectomie totale.

La déglutition est encore la fonction la moins impactée par la laryngectomie totale. En effet l'alimentation se fait normalement par voie orale. Une fois respecté le délai d'environ dix jours de cicatrisation nécessaire pour les sutures du pharynx, le patient est autorisé à reprendre une alimentation quasi normale. Les principales complications rencontrées sont les pharyngostomes (issue de salive au travers des sutures pharyngées vers le cou) essentiellement en post opératoire précoce. Ceux-ci sont pour la majorité traités par des soins locaux moyennant une période de cicatrisation plus ou moins longue pendant laquelle l'alimentation orale n'est pas autorisée.

Une autre complication, d'apparition plus tardive, est la sténose pharyngée. Elle est liée soit à une résection trop importante de la muqueuse du pharynx lors de la laryngectomie totale soit à une cicatrisation défectueuse plus moins en lien avec une radiothérapie post opératoire. Son

traitement est possible avec des techniques plus ou moins lourdes qui vont de la simple dilatation à l'apport de nouveau tissu par des lambeaux pédiculés ou libres (avec micro-anastomoses).

Enfin en l'absence de flux d'air par les fosses nasales, le goût peut être altéré en relation directe avec l'altération de l'odorat. En effet le flux d'air au niveau des fosses nasales étant aboli, les molécules olfactives ne parviennent pas au niveau des récepteurs olfactifs des fosses nasales et celles-ci jouent un rôle important dans la perception du goût des aliments.

2.3.2.2 La respiration après laryngectomie totale.

Le larynx en situation normale s'apparente à une valve au niveau de l'hypopharynx. Lors de la respiration normale, les cordes vocales sont en position ouvertes et l'épiglotte relevée permettant ainsi le passage de l'air provenant des voies aérodigestives supérieures vers la trachée. Lors de la déglutition, cette valve est fermée pour empêcher le bol alimentaire de passer au niveau de la trachée et évite ainsi les fausses routes alimentaires et salivaires. Cette fermeture est assurée par le rapprochement des cordes vocales en position médiane et la bascule de l'épiglotte en position horizontale, venant couvrir le plan glottique.

L'air qui arrive au niveau pulmonaire est conditionné au niveau des fosses nasales. En effet l'air extérieur est humidifié et réchauffé au contact de la muqueuse des fosses nasales lors de son passage. De plus le mucus présent à la surface de cette muqueuse qui est une muqueuse respiratoire permet une filtration de l'air extérieur. Les particules fines en suspension dans l'air ambiant restent collées au mucus et ne peuvent ainsi pas arriver au niveau pulmonaire.

Ainsi lorsqu'une laryngectomie totale est réalisée, l'air extérieur arrive directement au niveau pulmonaire par l'orifice de trachéostomie. Cet air n'est donc pas conditionné puisque le passage par les fosses nasales est shunté. Les voies respiratoires inférieures sont donc directement en contact avec un air « impur ». Ceci est à l'origine de réactions inflammatoires des muqueuses respiratoires et provoque la formation de bouchons muqueux liés à la surproduction de mucus respiratoire et à l'assèchement de celui-ci.

Le shunt des voies respiratoires supérieures à également pour conséquence l'altération de l'odorat et du gout. Le passage de l'air ne se faisant plus par les fosses nasales, les composés volatils organiques ne rentrent plus en contact avec les récepteurs olfactifs des fosses nasales. Il en résulte une altération de l'odorat mais également du gout puisque l'odorat participe en partie à la sensibilité gustative.

Les patients chez lesquels une laryngectomie totale est pratiquée présentent une altération non négligeable de leur qualité de vie.

Des solutions pour améliorer la qualité de l'air inspiré existent sans être parfaites. La mise en place d'un échangeur d'humidité au niveau du trachéostome permet de conditionner l'air qui arrive au niveau pulmonaire. Il s'agit d'un dispositif avec une mousse à travers laquelle l'air est inspiré et expiré. Cette mousse, après plusieurs expirations, se charge en humidité et en chaleur provenant des poumons. Ainsi par la suite l'air inspiré qui passe au travers de cette mousse est également humidifié et réchauffé avant d'arriver au niveau pulmonaire. Les particules en suspension sont également piégées dans la mousse sans atteindre les voies respiratoires inférieures. Il s'agit d'une solution simple mais qui nécessite un entretient contraignant pour le patient. La mousse saturée en humidité et en mucus étant de moins en moins perméable au passage de l'air doit être régulièrement nettoyée ou bien changée.

2.3.2.3 La phonation après laryngectomie totale.

On peut aisément comprendre qu'après l'exérèse du larynx la phonation sera considérablement dégradée. Elle n'est néanmoins pas totalement impossible. La parole est le fruit de l'articulation du son émis par la vibration des cordes vocales sous la pression de l'air expiré. Cette articulation met en jeu différentes structures anatomiques des voies aériennes supérieur. Les cavités orale et nasales servent de caisse de résonnance. La langue, le voile ainsi que les muscles des joues et des lèvres assurent l'articulation du son émis. Lors de la laryngectomie c'est la production du son qui est impacté en premier lieu. Cette production sonore est non seulement dégradée par l'absence des cordes vocales mais également par le shunt du flux d'air de la respiration. En effet après la laryngectomie le passage de l'air se fait directement par le trachéostome et non plus par les voies aériennes supérieures. Un passage d'air au niveau des voies aériennes peut également produire un son non plus grâce aux cordes vocales mais par la vibration des structures muqueuses du néopharynx. Cet apport d'air peut se faire de deux façons. Le patient peut avaler de l'air vers l'œsophage puis l'éructe. Ceci permet une sortie d'air vers les voies aériennes supérieures avec une vibration de la muqueuse et produit ainsi un son. Celui-ci est articulé comme décrit précédemment et permet au patient de « parler ». Il s'agit d'une voie dite œsophagienne qui est nettement de moins bonne qualité mais qui permet au patient très bien entrainé de se faire comprendre. Une rééducation importante est nécessaire. La deuxième possibilité est la mise en place d'un implant phonatoire. Il s'agit d'une valve unidirectionnelle positionnée au niveau d'une fistule (communication) crée chirurgicalement entre la trachée et l'œsophage. Cette valve permet le

passage de l'air de la trachée vers l'œsophage mais ne permet pas le passage de la salive et du bol alimentaire des voies digestives vers la trachée. Elle nécessite également une surpression aérienne au niveau pulmonaire pour être activée. Cette surpression assurée grâce à une deuxième valve qui ferme l'orifice de trachéostomie. Il s'agit d'un dispositif le plus souvent collé sur l'abouchement de la trachée à la peau au niveau du cou. Ce système est équipé d'une valve qui permet un passage de l'air dans les deux sens lors de pressions de respiration normales. Lors que le patient veut parler il exerce une pression d'air plus élevée grâce aux muscles du diaphragme et intercostaux. Cette hyperpression aérienne pulmonaire et par conséquent trachéale enclenche la valve du bouchon de l'orifice de trachéostomie. Ceci engendrant sa fermeture. Ainsi l'air en hyperpression au niveau de la trachée ne peut plus sortir par l'orifice de trachéostomie et fuit vers l'œsophage par l'intermédiaire de la valve phonatoire. Ensuite la vibration de la muqueuse permet la production d'un son. La parole à l'aide d'un implant phonatoire est de meilleure qualité que la voix œsophagienne. Son utilisation est plus aisée mais nécessite également une rééducation chez certains patients.

Dans le cadre de l'amélioration de la qualité de vie des patients laryngectomisés, nous avons décrits les solutions existantes pour chacune des fonctions altérées telle que la déglutition, la phonation et la respiration. Cependant elles ne sont que partielles est contraignantes pour le patient. La proposition d'une solution globale pour ces patients passe par la mise en place d'un dispositif de substitution pour remplacer le larynx. La complexité de fonctionnement de cet organe rend plus que difficile ce remplacement et a pour conséquence l'absence de solution proposée et utilisée en pratique clinique courante. De nombreux travaux de recherche sur le remplacement laryngé ont été menés sans déboucher sur un dispositif commercialisé. D'autres sont toujours en cours. Le service d'Oto Rhino Laryngologie du centre Hospitalier et Universitaire de Strasbourg, sous l'égide du Professeur Debry, mène depuis une vingtaine d'années des travaux sur le développement d'un larynx artificiel en étroite collaboration avec l'unité de recherche INSERM 1121 Biomatériaux et Bioingénierie. Ce travail a été mené dans ce cadre afin d'apporter des améliorations nécessaires au dispositif développé.

Le dispositif mis au point dénommé ENTegral™, est un dispositif en titane. Il comporte 3 parties distinctes. La partie initiale est un anneau microporeux à base de microbilles de titane, il sert à assurer la connexion du dispositif avec la section trachéale réalisée lors de la laryngectomie. La partie intermédiaire est un tube en titane qui permet de prolonger la voie aérienne (trachée) jusqu'au niveau de la base de langue. La dernière partie est une valve multidirectionnelle qui est mise en place dans un deuxième temps opératoire par voie endoscopique transorale. Elle a pour but de mimer le fonctionnement du plan glottique et sus-glottique (cordes vocales et épiglotte) lors des mouvements de phonation, déglutition et respiration.

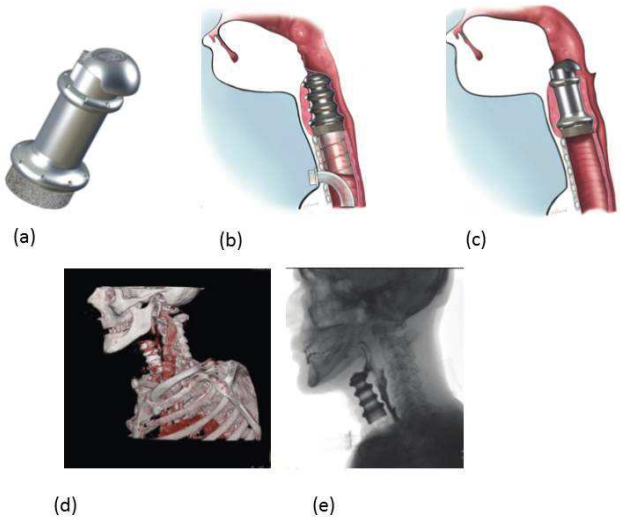

Figure 8 : Dispositif ENTegral™

(a) Dispositif avec les trois parties. (b) Schéma du dispositif implanté avant la mise en place de la valve. (c) Schéma du dispositif implanté complet (d) Reconstruction 3D d'une tomodensitométrie montrant le dispositif implanté au niveau cervical. (e) Image de vidéofluoroscopie de déglutition chez un patient implanté avant la mise en place de la valve multidirectionnelle.

Lors des différents essais cliniques menés dans le service d'Oto-Rhino-Laryngologie et chirurgie cervico-faciale des Hôpitaux Universitaires de Strasbourg, plusieurs améliorations ont été apportés notamment sur la technique chirurgicale d'implantation comme la mise en place d'un lambeau musculaire de grand pectoral autour du dispositif afin d'optimiser son intégration. Les principaux axes d'optimisation encore nécessaires ont découlé des observations cliniques réalisés lors du suivi post-opératoire de ces patients implantés. L'un des axes d'amélioration concerne la valve multifonctionnelle. En effet le dispositif implanté a permis une fonction de phonation et de respiration correcte mais il a été observé des fausses routes alimentaires résiduelles. Un deuxième axe d'amélioration concerne la bague en titane

poreux. Cette bague a été conçue à l'aide de microbilles de titane dont le diamètre permet de contrôler les dimensions des porosités. Ces porosités ont pour objectif d'être colonisées par du tissu conjonctif assurant ainsi une intégration du dispositif au niveau cervical. Cependant, malgré la création d'un gradient de porosité afin de contrôler la colonisation du tissu conjonctif, un hyper bourgeonnement de celui-ci en endoluminal créant ainsi une sténose endoluminal a été observé. Ceci a nécessité la mise en place d'un tube en silicone en intraluminal au niveau de la prothèse pour assurer la perméabilité de la voie aérienne. Cet hyper bourgeonnement de tissu conjonctif est directement lié à l'absence de ré-épithélialisation rapide et spontané de la face endoluminale du dispositif. De plus l'absence d'épithélium respiratoire fonctionnel est aussi à l'origine de la formation de bouchon muqueux pouvant causer l'obstruction de la prothèse.

L'objectif de ce travail de thèse est de mettre au point une solution pouvant assurer une ré-épithélialisation da la face endoluminale de ce larynx artificiel pour non seulement pallier les complications citées ci-dessus mais également limiter le potentiel risque de colonisation bactérienne et fongique de la face endoluminale de la prothèse afin de parfaire son intégration tissulaire.

B – Etat de l'art

Cette partie à fait l'objet d'une publication sous forme d'un chapitre dans un ouvrage intitulé « Tissue Engineering for Artificial Organs: Regenerative Medicine, Smart Diagnostics and Personalized Medicine » publié en 2017.

3 - Engineering Trachea and Larynx

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Tissue Engineering for Artificial Organs: Regenerative Medicine, Smart Diagnostics and Personalized Medicine,
First Edition. Edited by Anwarul Hasan.

3.1 Abstract

Upper respiratory airway is the point of entry and exit for the gas exchange processes that are essential for the whole organism. Moreover, larynx has several other functions such as protection of the respiratory system from aspiration of food. Moreover, it houses the vocal cords, which are responsible for the controlled production of sound and enable speech and other higher vocal capabilities such as singing. Congenital diseases, accidents, and cancer-related problems can significantly reduce the capacities of upper respiratory airways, which might result in significant decrease in the quality of life. In some cases, larynx and trachea might need to be surgically removed, totally or partially.

Replacement of trachea and larynx following resection are ongoing clinical problems with no established gold standards. In the last 10 years, there have been considerable advances in this field from implantation of several engineered tracheas to implantation of an artificial larynx system in human patients. However, there are still several problems associated with the development of trachea and larynx substitutes with methods in tissue engineering and regenerative medicine.

In this chapter we review the advances in the development of tracheal and larynx substitutes. First, we present a brief overview of the anatomy of the airway system, the physiological roles of these tissues, and the indications that would necessitate their resection and replacement. This will be followed by the definition of the current state of the art in tracheal and laryngeal replacement with special emphasis on the clinical replacement examples.

3.2 Introduction

Diseases related to the respiratory system and cancers are two of the most common causes of death worldwide. Patients may face dramatic airway resection due to the cancer-associated problems, which involves the loss of associated functions. Even though there are several well-established methods to overcome disease and cancer-related tissue loss for larynx and trachea (such as anastomosis of the tracheal sections following partial resection), there is still an unmet need for functional replacements for ensuring a high quality of life for the patients. Speech loss, permanent separation of the respiratory and digestive systems, and complications related to the presence of a tracheostoma (a surgically implemented opening connecting trachea directly with the ambient air to replace normal respiratory pathway) all underline the need to develop a functional replacement without any ensuing problems.

In order to overcome organ donor shortage, and the need of immunosuppressant drugs, one possible solution is the development of tools and methods for personalized, engineered substitute tissues, which has become a well-developed research area. Personalized medicine is not only limited to the pharmacological applications and to the advancement of using different dosages to specific patients but also include medical treatments and diagnostic systems that con- sider the genetic, anatomical, and physiological characteristics of each patient. Lately, within the context of tissue engineering and regenerative medicine, personalized medicine is extended to the construction of a specific solution to each patient by using advanced technologies such as rapid prototyping. In the near future, it will be possible to develop an organ substitute that will match the size and the exact tissue composition of the target organ that is completely adapted to the patient's regenerative capacity. Tracheal replacement is one of the areas where tissue engineering has become a clinical reality, with several successful implantations already reported [1, 2]. However, larynx is not aimed much because of its complex structure, but for a few transplantation attempts. In the following sections, we describe these two organs in detail, explain the medical conditions that necessitate their partial resection or removal, and elaborate on the methods for their replacement with a special focus on the clinical larynx and tracheal replacement trials.

3.3 Basic Anatomy and Histology of the Larynx and Trachea

In this section, the basics of the laryngeal and tracheal anatomy and histology will be described briefly. The goal is to provide the anatomical background in order to understand better the associated clinical problems that would require larynx and trachea replacement.

3.3.1 The Larynx

The larynx is a multifunctional organ located in the anterior and medial part of the neck at the level of C5–C6 cervical vertebrae. It establishes the limit between the upper and lower airways. It is followed by the cervical part of the trachea in the basement of the neck, which plays a role in breathing, swallowing, and phonation. The larynx hasa cartilaginous part with nine components connected each to other, which provides the necessary support for its function. This cartilaginous part of the larynx includes the thyroid cartilage, the cricoid cartilage, corniculate cartilages, the epiglottis, cuneiform, and the arytenoid cartilages.

The thyroid cartilage is considered the largest of the cartilages. It is composed of two laminae that fuse at their anterior parts to form the laryngeal pro-eminence. This pro-eminence is larger in adult males than females. The cricoid cartilage is a complete ring-shaped cartilage lying under the thyroid cartilage and connected to it with the cricothyroid membrane in the anterior part and the cricothyroid joints in the posterior side. The cervical trachea is connected to the inferior part of the cricoid cartilage. The epiglottis is attached to the internal side of the anterior part of thyroid cartilage. It is leaf-shaped and situated behind the base of the tongue. The arytenoid cartilages are paired and triangular and rest on the cricoid ring on the cricoarytenoid joints located at the posterior and upper side. They regulate the level of tension and position of the vocal cords. The corniculate and cuneiform cartilages are both paired and located at the apex of arytenoids.

All these structures are maintained together with ligaments and supported by several muscle insertions. The extrinsic muscles are important for positioning the larynx in the central part of the neck and its mobilization during its different functions. They suspend the larynx to the hyoid bone. In addition, the intrinsic muscles play a role in vocal cords mobilization, as they are the ones responsible for allowing the opening and closing of the vocal cords and the regulation of the laryngeal field during breathing, phonation, and swallowing. The vocal cord region is also called glottis. At this level, the pharyngeal tract is divided into the respiratory tract (trachea) and the digestive tract (esophagus).

The larynx's sensory and motor innervation is provided by the vagus nerve, with the superior and the recurrent laryngeal nerves. The internal side of the larynx is covered with the

malpighian epithelium. The functions of glottis and epiglottis can be compared to a multifunctional valve.

During breathing, the vocal cords are maintained open and the epiglottis is in the raised position. This allows air to flow from the upper aerodigestive tract to the lower airway, namely the trachea. Similarly, during phonation, the epiglottis stays in the same raised position, and the vocals cords' opening and tension are controlled to permit their vibration with expired air, resulting in sound pro- duction. On the other hand, during swallowing, the larynx plays a crucial role in the lower airway protection, which is achieved by a highly regulated sequence of actions. The swallowing process starts with a controlled, voluntary phase (mastication), which is followed by an involuntary phase (deglutition). The deglutition process is comprised of a series of events. First, the laryngeal ascension takes place, and then the recession of the root of the tongue occurs. Then, the vocal cords move to the closed position, and the epiglottis moves down to a horizontal position covering the vocal cords. Afterward, the alimentary bolus is directed to the esophagus by the contraction of pharyngeal muscles. All these steps are controlled by the sensory and motor innervation and are crucial for a good swallowing process. This means that if any of these parts are missing or impaired, the normal functions associated with them will be compromised.

3.3.2 The Trachea

The trachea, also called the windpipe, begins in the inferior side of the cricoid cartilage at cervical basement, more precisely at the C6 level, and runs in the mediastinum. It is classically described as two parts: cervical trachea from the cricoid ring to level T2, where it enters the rib cage. Its inner diameter changes in males and females. It is larger in the male adults (∼1.8 cm). It bifurcates at level T5 into two primary bronchi. The trachea, the initial part of the lower airway, conducts air to the lungs during inspiration and expiration for gas exchange.

The trachea, which is about 15 cm long, is constituted by 16–20 C-shaped, incomplete cartilage rings, stacked against each other. These rings are maintained together by the presence of fibrous tissue in the anterior and lateral parts of

the annular ligament of the trachea. The posterior part of the trachea is a fibro-muscular tissue that is attached to the anterior side of the esophagus. It runs

in the mediastinum just behind the breast bone. This cartilaginous scaffold prevents the trachea from collapsing during inspiration and expiration. The cervical part the trachea supports the thyroid gland on its anterior face, which is crucial for regulating metabolism,

growth, development, and the temperature. At the rib cage level, the trachea is surrounded by important anatomical structures such as the aorta, the superior vena cava, and the pulmonary artery. The inner lumen of the trachea is covered with the respiratory epithelium. This epithelium is pseudo-stratified, columnar, and ciliated. The respiratory epithelium of the inner lumen of the trachea is composed of the following cells that are lined on the lamina propria, which is basically a fibroelastic connective tissue: (i) ciliated columnar cells, which are elongated cells that are ciliated on the apical side. They are responsible for moving the mucus on the epithelium; (ii) goblet cells, which are exocrine, mucus-producing cells, individually disseminated in the epithelium; (iii) basal cells, which are round cells located at the basal surface of the epithelium, which basically are a reserve of stem cells for replacing ciliated and goblet cells; (iv) brush cells, which are columnar cells with apical microvilli with a sensory receptor role as they synapse with the dendritic endings of sensory nerve fibers; and (v) the dense core granule cells, which are endocrine cells that appear to be related to the amine precursor uptake and decarboxylation (APUD) system and may release vasoactive substances. From the description and explanations presented here, it can be appreciated that the trachea is more than a simple tube but is a complex organ with different tissues and cells. All these structures have specific functions. The pseudo-stratified, columnar, ciliated epithelium plays a role in heating the inspired air and also moistening it. The ciliated cells clear the airways by moving the produced mucus to the upper airways. In this way, the entrapped dust particles and exogenous microbial elements that are inhaled are eliminated.

Thus, in any laryngotracheal replacement, all these multiple functions need to be replaced and ensured. The biomaterial to be used needs to be compatible with the trachea's environment. The multiple functions of larynx (protection of the air- way from food aspiration while providing the route for breathing) and the actions of the pseudo-stratified, ciliated epithelium in the trachea (first line of defense against insults, removal of particle and bacteria, etc.) should be, at least minimally, achieved in an engineered tissue or implant for an optimal replacement.

3.4 Indications for Tracheal Resection

In this section, the conditions under which tracheal or laryngeal resections are necessary are described in detail.

For tumors, or tracheal lesions due to trauma involving more than 50% of the trachea in adult patients or one-third in children, direct anastomosis cannot be performed even with maximum tracheal mobilization. Extensive tracheal resection remains a persistent clinical problem in

terms of surgical reconstruction. The indications of tracheal resection include laryngotracheal stenosis, benign tracheal tumors, and malignant tracheal tumors either primary or secondary.

3.4.1 Laryngotracheal Stenosis

Laryngotracheal stenosis is the decrease of the caliber of the respiratory tree at the level of the larynx or trachea. Diagnosis and staging are based primarily on laryngotracheal endoscopy under general anesthesia. The severity of the stenosis is evaluated based on the degree of reduction of the cross-section of the stenotic segment (classified from grade I, which corresponds to reduction up to 50%, to grade IV, which corresponds to a total stenosis).

Simple monitoring is sufficient if the degree of reduction of the cross-section is less than 50%. Otherwise, the stenotic segment should be addressed surgically. Depending on the characteristics of the stenosis, treatment can be performed either endoscopically by a laser (carbon dioxide (CO2) or potassium titanyl phosphate (KTP)) or by using other external approaches. While addressing such lesions, two principles are adopted depending on anatomical considerations: widening of the stenosed segment, in which case laryngotracheoplasty using mostly costal cartilage; or excision of the pathological segment completely by cricotracheal resection anastomosis or pure tracheal resection anastomosis. Historically, acquired stenosis was mainly secondary to infectious diseases, trauma, hamartomas, or amyloidosis. Currently, the leading cause of acquired laryngotracheal stenosis is iatrogenic, that is, prolonged intubation or too high a tracheostomy (performed close to the cricoid cartilage). The estimated risk of laryngotracheal stenosis after prolonged intubation varies from 6% to 21% of cases and after a tracheostomy between 0.6% and 21% of cases [3]. The use of low-pressure, high-volume cuffs in endotracheal intubation resulted in decreased risk [4].

3.4.2 Benign Tumors of the Trachea

The treatment of these tumors is generally endoscopic, with the necessity, in some advanced cases, for partial or circumferential tracheal resection. In pediatric population, benign tumors represent ~90% of tracheal tumors [5]. Up to 20% of them are inflammatory pseudotumors. Hamartomas, including chondroid hamartomas, are the second most common ones [6]. Diffuse papillomatosis is rare (about 2%) and is seen in children ranging from 18 months to 3 years of age. In adults, benign tumors of the trachea and bronchi represent only 4.7% of tracheobronchial tumors. They can be of various origins: epithelial, mesenchymal, nervous, or mixed. Mixed tumors (inflammatory tumors or granulomas) are among the most common

benign tumors. They are usually associated with chronic irritation (foreign body, bronchopulmonary infection), but can also be specific (due to tuberculosis or syphilis). Chondroid hamartomas are due to an original embryonic injury. The average age of diagnosis is between 60 and 70 years, with a male predominance. Malignant transformation is rare. Epithelial tumors include papilloma in adults (usually solitary), which is more likely to originate in the larynx or bronchi than the trachea. Adenoma is also exceptional. Among the mesenchymal tumors is the granular cell tumors (Abrikossoff tumor) of neural origin developed from Schwann cells. There is also neurofibroma, schwannoma, fibroma, leiomyoma, fibroleiomyoma, and chondroma. They can be also benign and localized in trachea through infiltration in diseases such as amyloidosis, sarcoidosis, Wegener's granulomatosis, and tracheopathia osteochondroplastica.

3.4.3 Primary Malignant Tumors of the Trachea

In these kinds of tumors, the main surgical technique is tracheal resection– anastomosis. In the absence of a tracheal substitute and if an extended tracheal resection is needed, treatment is palliative. For trachea, primary malignant tumors are rare and represent ∼2% of malignant tumors of the respiratory tree. In 90% of cases, it is either squamous cell carcinoma or adenoid cystic carcinoma [7, 8]. Squamous cell carcinomas are the most common primary tumors in adults with a male predominance and a peak incidence between 60 and 80 years. Smoking is the main risk factor. Adenoid cystic carcinomas (ACCs) or cylindromas represent 0.1% of cancers of the respiratory tract. They are the second most common primary malignant tumor of the trachea, of which the most common origins are the major and accessory salivary glands. These tumors are observed more frequently in young patients than other malignancies, with no gender predominance. They are not associated with smoking [9]. Other primary malignancies of the trachea include, in the order of decreasing frequency, carcinoid tumors (typical and atypical), adenocarcinomas, anaplastic carcinomas, small-cell carcinomas, mucoepidermoid carcinomas, and melanomas.

3.4.4 Secondary Malignant Tumors of the Trachea

The secondary tumors of the trachea result either from a locoregional invasion or by distant metastasis. Locoregional invasions can be linked to laryngeal, bronchogenic, thyroid, or esophageal cancers, which typically occur through a tracheoesophageal fistula. Distant metastases are frequently hematogenous. Their sites of origin include breast cancer, kidney, colon, hepatocellular carcinoma, and melanoma.

3.5 Indications of Total Laryngectomy

Total laryngectomy is the complete excision of the larynx, from the cricoid cartilage to the hyoid bone. Once the pharynx is closed by several mucosal and muscular planes, the digestive tract becomes completely separated from the respiratory tract. The proximal end of the trachea is sutured to the skin to form a permanent tracheostomy. The International Association of Laryngectomees reports that 57 000 laryngectomees are performed in the United States (http://www.theial.com). At the moment, a definitive laryngeal replacement without considerable decrease in the quality of life of the patient is not available [10]. Total laryngectomy is indicated for larynx or hypopharynx cancers, in advanced cases of larynx benign tumors, and in nonfunctioning larynx either post-traumatic or due to neurological diseases.

3.5.1 Cancer of Larynx or Hypopharynx

Total laryngectomy is performed in more than 98% of cases in the treatment of advanced cases of laryngeal or hypopharyngeal cancer that is classified T3 or T4. In over 95% of cases, it is squamous cell carcinoma. When the local extension of the primary tumor contraindicates the performing of functional partial laryngectomy, and when organ preservation protocol (chemoradiotherapy) is impossible or has failed, total laryngectomy becomes the treatment of choice. The 5-year survival of laryngectomees, all stages, is over 43% [11]. Although this surgery gives a satisfactory life expectancy, it causes major difficulties in the daily lives of patients.

3.5.2 Benign Tumors of the Larynx

The treatment of benign tumors tends to be more conservative: that is, endoscopic excision or partial laryngectomy. Total laryngectomy can be considered only in progressive tumors that are very large and which cause complete stenosis of the larynx, or when it is impossible to deal with them with a more conservative approach. Its indication is thus exceptional. It has been reported in the literature in cases of chondroma [12], rhabdomyoma, and Abrikossoff tumor [13].

3.5.3 Nonfunctioning Larynx

Surgeries are resorted to when there are major swallowing disorders with aspiration (mainly in neurological diseases) in order to protect the lungs by separating the respiratory and digestive tracts. The options are closing the laryngotracheal tract, tracheal diversion, or total

laryngectomy. Total laryngectomy, although effective in controlling aspiration, implies a mutilating surgery with an irreversible effect on the voice quality. Currently, it is replaced by other equally effective procedures such as laryngeal prosthesis or closed tracheal diversion [14, 15]. Very rare cases of severe external trauma to the larynx (gunshot or highway accidents) may require total laryngectomy. It is the same for severe necrosis of larynx after radiotherapy for a laryngeal or hypopharyngeal cancer [16].

3.6 Available Remedies Following Total Laryngectomy

Currently, there are four ways for restoring the laryngeal functions after total laryngectomy: (i) laryngeal transplantation, (ii) grafts, (iii) biomaterial-based solutions (implants), and (iv) tissue engineering.

3.6.1 Laryngeal Transplantation

There are only a few examples of laryngeal transplantation in humans. Trans- plants have been mostly carried out on animals, particularly on rats [17], dogs, and pigs [18]. A percentage of animals survived in these trials, but nerve sutures were either not performed or nonfunctional. Thus the main objective of the trans- plantation cannot be attained: that is, obviating the need for a tracheostomy. Also, animals are not the best models for conducting such research because of the voluntary control of swallowing in humans on one hand and anatomical differences on the other. Strome et al. [19] managed to transplant a human larynx. The graft was viable and resulted in good voice quality. The tracheostomy tube was, however, left in place because of incomplete motor function of the trans- plant. Their experience confirms the two obstacles associated with this surgical procedure: the need to maintain immunosuppressant therapy, and satisfactory re-innervation of the larynx or stimulation of nerve branches.

3.6.2 Grafts

3.6.2.1 Implantation of Nonviable Tissues

Bio-prostheses consisting of lyophilized tissue [20], frozen or chemically fixed, have been tested. Tracheal allografts treated with glutaraldehyde in rats and pigs, lyophilized aortic grafts in dogs, and trachea fixed with formaldehyde in humans have been implemented without success. This kind of treatment can avoid rejection, but these nonviable tissues are not revascularized and eventually become necrotic. Regrowth of the epithelium from both

tracheal ends leading to formation of a granulation bed is also another associated problem.

Tracheal Transplantation and Autografts (Viable Tissues) Nonvascularized grafts, either in the form of autografts or allografts, of tracheas have led generally to failures either by stenosis or necrosis of the graft. On the contrary, the use of a tracheal autograft, for example, the resection of some tracheal rings followed by their grafting, has demonstrated the viability of such grafts provided that the graft has a vascular support. But tracheal transplantation with its vascular pedicle is particularly difficult because of the caliber of anastomotic vessels. All trials encountered such problems of vascular anastomoses except in cases where tracheal transplantation was associated with pulmonary and bronchial transplantation, leading inevitably to the use of immunosuppressive therapy.

The trachea does not have an extensive vascular supply. It is connected with the vasculature by a network of vessels, the origins of which are the bronchial and the right inferior thyroid arteries. Thus, the provision of vascular support, particularly in the case of allogenic replacements, is highly challenging [21, 22]. Additional constraints to successful tracheal tissue engineering applications should be resolved to make it a reality, such as the absence of a fully functional respiratory epithelium; foreign body response or other immunologic complications; local infection; biomaterial failure; and repeated operations, including tracheostomy [23]. Moreover, unlike the liver, heart or kidney, tracheal trans- plantation is not possible because of the number and size of its vascular supply. It can only be transplanted together with the adjacent organs such as the esophagus or thyroid, which have a common blood supply [22] due to their adjacent location.

3.6.2.2 Allografts

Despite all these problems, Rose et al. reported in 1979 the first case of allogenic transplant of the trachea. First, a tracheal graft from a cadaver was transplanted into the sternocleidomastoid muscle in order to achieve revascularization (3 weeks). Afterward, the vascularized tracheal graft was implanted. A second case was described in 1993 by Levashov et al. In order to achieve vascularization indirectly, they performed omentopexy in a single-stage transplantation. Though this case seemed promising at the time, the allograft viability and functionality were not reported subsequently. Ten years later, Kleteptko et al. reported heterotypic vascularization in the momentum of an allogenic graft. The functional and structural integrity of the graft was maintained. However, all allogenic transplantations require lifelong immunosuppression, which has considerable side effects. It also presents a

significant risk for patients with malignancies. However, immunosuppression is inevitable because the tracheal epithelial cells and the chondrocytes can trigger acute inflammation, which can lead to chronic inflammation/rejection [21].

Cryopreservation of the grafts can decrease their antigenicity, as demonstrated by Murakawa et al., who compared allografts (fresh, frozen, and cryopreserved) in a primate. Although cryopreservation resulted in amelioration, there was still infiltration by mononuclear cells after 1 year, which points out that, in the long term, immunosuppression is still necessary [24].

3.6.2.3 Autografts

Another possibility is to use non-tracheal autologous tissues. These are tissue grafts from the same individual. This kind of transplantation can take place either with or without a vascular pedicle. Autologous tissues without a vascular pedicle are used primarily to repair tracheal defects. The most widely used tissues are fascia, cartilage, and periosteum, which ultimately are expected to turn into cartilage [25]. The precarious nature of the vascularity of tissues in contact with the flap often causes necrosis of the implanted tissue, leading to anastomotic dehiscence, infection, and formation of fibrosis. As it is difficult to consider using an aortic autograft in humans, allografts of fresh aortas were taken to be implanted to replace trachea in sheep [26, 27]. The results were similar to those reported in autologous reconstruction, and a gradual transformation of the aortic graft was noted between the third and the sixth month. After extensive inflammation of the tissue, the vessel was re-colonized by a differentiated ciliated epithelium, and cartilage islands appeared in the connective tissue. No immunosuppression was required because of the disappearance of the vascular tissue [28, 29]. These results are quite encouraging. This strategy opens a new avenue for tracheal or laryngeal reconstruction but needs further studies and confirmation. Transplantation of autologous tissue with vascular pedicle allows larger reconstructions with a decreased risk of necrosis. They may also be associated with biomaterials. These techniques take into account the quality and the long-term functional outcomes; rejection is not possible as all tissues are autografts. Nevertheless, these techniques require a long surgery time, they are tedious, and they not easy to repeat.

3.6.3 Biomaterials

What kind of biomaterials can be used for the restoration of a rigid structure [30]? A few teams are currently working on the design of an artificial laryngeal pros- thesis. The benefits of such a prosthesis are as follows: (i) the prosthesis can be placed during same

operation, just after the total laryngectomy; (ii) proper bio- materials that are not considered as a contraindication for adjuvant radiotherapy or magnetic resonance imaging (MRI) can be used; and/or (iii) there would be no need for immunosuppressants. However, there are many steps to consider for an artificial larynx system: first, the need to validate the acceptance of a long-term implanted material in a potentially septic environment; second, the design and manufacture of a triple sphincteric valve that can replace the functions of phonation, swallowing, and respiration; and finally, the surgical restoration of the pharynx. Despite these challenges, an implantable laryngeal prosthesis has been gradually developed by our team [31, 32] by combining a biocompatible titanium immovable prosthesis of alternating smooth and porous parts for the anastomoses, which could replace laryngeal cartilage and/or the tracheal tube, with a removable functional structure (valves synchronizing the functions of respiration and swallowing), intended to be fixed on the immovable prosthesis. The valves have been developed to be replaced under laryngoscopy suspension. Many materials have been used primarily for tracheal reconstruction in animal studies (dogs, sheep, goats, pigs, rabbits, rats) and in humans. As there exists no animal model for a complete laryngeal replacement [33], we have conducted experiments based on tracheal replacements to judge the tissue acceptance.

Surface treatment processes have become of major importance for many industrial processes, particularly in the biomedical field. Thus, the great challenge now lies in the development of new surface structures whose reactivity can be controlled and modulated to induce specific and appropriate cellular responses (adhesion, proliferation, differentiation, etc.). Currently, nanoscience and nanotechnology present a unique opportunity to achieve such properties and to modify the surfaces of biomaterials in use. In our system, they have been especially considered for the porous titanium structures. In vivo acceptance of such prosthesis, supporting active valves as described above, may be potentiated by nanostructuration of the surface of any type of porous structure of a polymeric product, associated with biological factors. It seems possible, in the light of the work in both small and big animals (i) to improve the sealing of the porous titanium prosthesis during the first days/weeks after implantation to limit the deposition of mucus and bacterial colonization from the tracheal lumen, and (ii) to

accelerate cell colonization by stimulating the colonization of porous titanium beads. Thus, it is possible to improve the material response by combining surface treatments with the use of degradable and functional components.

Even if laryngeal transplantation problems, such as the need for immunosuppressants and nerve anastomoses, are solved in the future, the limited number of donors still remains a limiting factor to solve this medical problem. In fact, one of the limiting causes is actually the shortage of donor organs for this kind of applications. To recreate a larynx, the trachea should be elongated by an integrated hollow structure, which is vascularized by the surrounding tissues. Porous titanium seems to fit to the demands of such an implant, allowing our team to per- form the first implantations with an artificial larynx (ENTegralTM, Figure 11.1a–e). ENTegralTM is composed of two parts: (i) a permanent tracheal prosthesis containing a porous titanium connector which is in contact with trachea, and (ii) a removable valve-based system that provides the route for inspiration and expiration. The implantation of the valve part is done endoscopically. Recently, we have reported the implantation of this system in a 65-year-old patient who had the artificial larynx system for 8 months [10] (Figure 11.1). Our main objective is to implant the artificial larynx just after total laryngectomy in order to avoid delays in postoperative radiotherapy. However, further research is necessary for the regular implantation of the artificial larynx in the clinic. We hope that the artificial larynx design can be incorporated in the tissue-engineered trachea development, which is covered in the next sections.

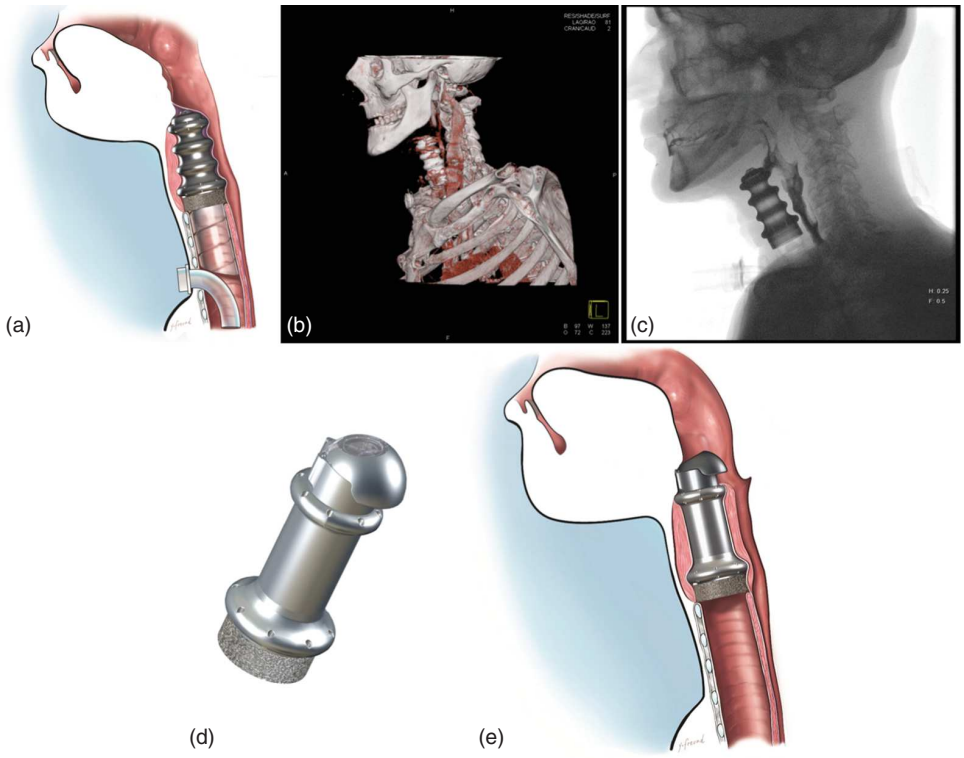

Figure 11.1 ENTegral™ Artificial Larynx system. (a) ENTegral after the first surgical step (implantation of the permanent part). (b) 3D CT scan shows the positioning of the implant. (c) Barium swallow test confirms the absence of fistulas. (Reprinted with permission from [10], Head and Neck.) (d) The overall structure of the implant with a removable valve system to restore the breathing and swallowing functions and a permanent part with a porous tracheal connector to ensure the proper integration of the implant with the airway. (e) The full implant in its orthogonal position. (Courtesy of Protip Medical.)

3.7 Regenerative Medicine Strategies and Tissue Engineering Tools for Tracheal and Larynx Replacement

Replacement of the respiratory tissue is needed after a tumor resection, trauma, stenosis, or in tracheal defects (such as tracheomalacia or tracheal strictures), which causes high levels of morbidity [21, 23, 34]. Around 150 000 patients have complications due to mechanical ventilation or endotracheal intubation every year in the United States. In the case of brain or spinal cord injuries, the complications are more common, as long-term ventilator support and intubation is often necessary. Chronic airway problems result in high mortality rates (11–24%), which poses an important clinical challenge due to the limitations of surgical resection, as will be explained below [35]. Currently, the only curative treatment for primary cancers of trachea is resection [21]. Tracheal replacement still is a major challenge in thoracic surgery and also in regenerative medicine. Over the past 60 years, Grillo and several surgeons world-wide have solved the different problems associated with tracheal surgery in order to provide standardized approaches for airway diseases (Grillo 2004). Thus, the majority of tracheal lesions requiring surgery can be now treated by resection followed by primary end-to-end anastomosis. Surgical techniques have been also developed for cases with laryngeal or cardinal extension. However, the resection of tracheal lesions extended to more than half the trachea is not recommended because reconstruction using direct anastomosis is not possible. Large airway defects still are a significant clinical problem, as patients cannot survive if tracheal resection is necessary for more than 6 cm in length in adult patients or 30% of the total tracheal length in pediatric patients. For these patients, we need of a tracheal substitute.

3.7.1 Replacement of the Trachea

In the last chapter of his reference textbook, Grillo detailed the results of the five schematic ways of research that have been explored by numerous teams to find a valuable tracheal substitute (Grillo 2004). An ideal tracheal substitute should (i) be a strong, flexible, tubular biocompatible structure, (ii) must facilitate epithelialization, (iii) must integrate with adjacent tissues, (iv) should not cause stenosis, and (v) must resist bacterial biofilm formation. The use of various foreign materials has caused serious complications such as granulation tissue formation, undesired movement of the prosthesis, and infection. A few studies have evaluated nonviable tissues with limited functionality. Tracheal allotransplantations generally lead to necrosis and stenosis. Also, allotransplatations are not suitable for patients with malignant lesions. Because of their complications, the aforementioned methods have been largely abandoned. Use of autologous tissues such as the pericardium, skin, costal cartilage, bowel,

esophagus, or bladder involve long and complex procedures and did not provide prospective clinical trials. Based on the pioneering work by Vacanti and colleagues, tissue engineering techniques have become a potential solution [36, 37] (Figure 11.2). The challenge is represented by the creation of cartilaginous tubes covered with respiratory cells [38].

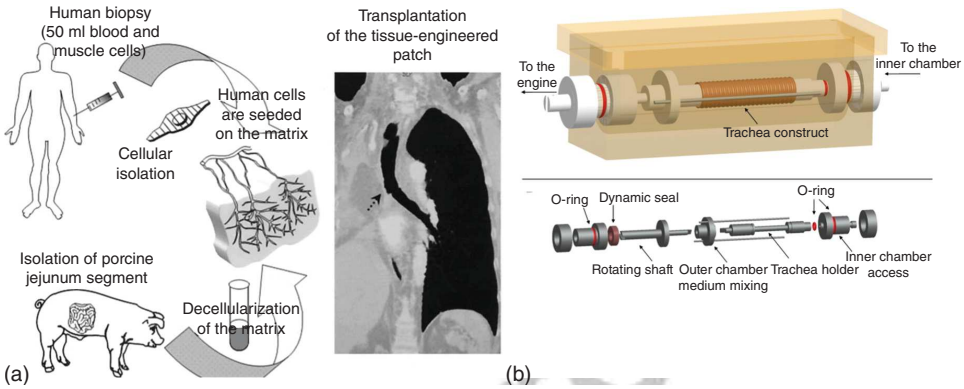

Figure 11.2 (a) The overall route of airway tissue engineering. Several different variations of this scheme have been achieved clinically during the last 10 years. (Macchariani 2004. Reproduced with permission of Elsevier.) (b) A double-chamber rotating bioreactor design to fulfill the requirements of two different cell types necessary for the tissue engineering of trachea. The outer part ensures the microenvironment for differentiation of patient's MSCs to chondrocytes within the scaffold, whereas the rotation of the system provides the air–liquid interface for the epithelial cells seeded to the scaffold lumen. (Asnaghi 2009. Reproduced with permission of Elsevier.)

3.7.2 Ex vivo Tissue Engineering

Birchall's group in London reported in 2008 the first tissue-engineered tracheal implantation in a patient who suffered from post-tuberculosis end-stage bronchomalacia [39]. In vitro, recipient' epithelial cells and chondrocytes derived from recipient's mesenchymal stem cells were cultured and then seeded onto and into (epithelial cells) a decellularized human trachea in a bioreactor system that allowed air/liquid interface culture for epithelial cells. Four months after implantation, the clinical evaluation of the engineered tissue was favorable. The shortage of human tracheal donors is, however, a major limitation of the technique. Furthermore, because of the use of the patient's epithelial cells, this method is not suitable for cancer

lesions. Also, the time required (few months) for obtaining the graft is prohibitive for its widespread use. In 2011, some members of Birchall's group replaced the decellularized trachea with a nanocomposite scaffold to circumvent the donor shortage problem [40]. The nanocomposite scaffold was seeded with the patient's bone-marrow mononuclear cells and matured in a bioreactor. The first patient implanted had recurrent and extensive cancer. The clinical observations demonstrated neovascularization, extracellular remodeling, and wound repair and also homing of stem cells in vivo after a follow-up of 5 months. The stem cell mobilization was boosted via growth factor administration. In 2012, the first case of a pediatric patient with tissue-engineered tracheal replacement (with autologous stem cells), with 2 years of follow-up, was published by Elliott et al. [41]. In 2010, a novel surgical procedure performed in a patient with extensive post-traumatic stenosis was described by Delaere et al. [42]. Briefly, under immunosuppression, a tracheal allograft was wrapped in the forearm fascia of the recipient. The tracheal allograft was revascularized in the fascia, and eventually it was fully lined with donor respiratory epithelium and recipient buccal mucosa. Following the cessation of immunosuppressive therapy at month 4, the tracheal allograft with the intact vasculature was moved to its orthogonal position. A 1-year follow-up gave satisfactory results. However, the need for immunosuppressive therapy and other problems in this method are similar to those of ex vivo engineered airways. Also, currently there are some ongoing investigations on the first engineered trachea implantations carried out in Karolinska Institute by Prof. Paolo Macchariani regarding the obtainment of patient consent and representation of the clinical results, which makes it harder to assess the real benefit of these implantations. However, recently a follow-up study of the implantation of an engineered trachea in a pediatric patient has again shown the great promise of this method for replacement, particularly in pediatric patients (Figure 11.3).

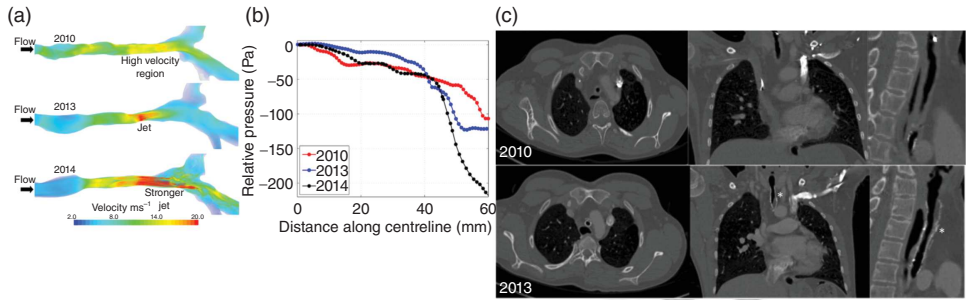

Figure 11.3 (a) Flow velocities in the engineered trachea as a function of time. Even though there is an area of constriction after 4 years, the engineered segment still allows the passage of air. (b) Relative pressure at three time points. (c) Axial, coronal, and sagittal section (CT images from 2010 and 2013, respectively. The transplanted segment (narrowed) can be seen in both the 2010 and 2013 images. (Hamilton [1],

3.7.3 In vivo Tissue Engineering

In 1997, we proposed to evaluate the use of aortic grafts as matrices for airway transplantation. In successive experimental studies, we demonstrated encouraging results with the use of aortic autografts and fresh or cryopreserved allografts [27, 38, 43–45]. We observed in vivo tissue engineering with epithelial and cartilage regeneration from bone-marrow stem cells [46]. This led to the first clinical applications in humans for extensive tracheal malignant tumors and conservative lung cancer surgery [2, 47, 48]. A prospective clinical trial (NCT01331863) is in progress. In 2014, the International Society of Cell Therapy organized the first meeting with the leading clinicians and biologists involved in airway tissue engineering to debate the issues and propose recommendations for the future [49].

3.8 Inherent Needs of Trachea Tissue Engineering

Although it would appear that trachea is only a simple tube that will need to be replaced, and for this reason eminently suited for tissue engineering applications, in actuality trachea is a complex organ composed of different sophisticated structures with several cellular components. Tracheal regeneration requires a cartilaginous tube lined with viable respiratory mucosa (Figure 11.2b).

3.8.1 Developing Strategies for Trachea Regeneration

As previously mentioned, an efficient tracheal substitute has strict requirements. Moreover, in pediatric patients, the graft should be able to keep up with somatic growth. For example, an acellular bioartificial airway patch was implanted in a case where tuberculosis damaged the patient's tracheobronchial tree [50]. How- ever, this method cannot be applied to all airway replacement scenarios. To overcome the different hurdles in airway engineering, several new routes are under investigation. There are other techniques, aside the ones described in the previous sections, that have not reached clinical settings but have been shown to be feasible in animal models. For example, chondrocytes (autologous) in a fibrin/hyaluronan gel have been used in rabbits to regenerate circumferential tracheal cartilage segment defects 0.5 cm wide and cervical defects 1 cm long [23]. The graft lumen was covered with a ciliated epithelium without any inflammatory reactions. The beating frequency of cilia was close to that of a healthy respiratory epithelium. Moreover, after the engineered graft implantation, the recipient rabbits breathed without stridor and there were no wound infection. In addition, in all animals entering in this preliminary study, no stenosis or granulated tissue formation was observed, nor stenosis or granulation of the regenerated trachea. Recently, the same group has made advances in the reconstruction of the trachea. Lately, a system based on a porous, degradable scaffold (poly L-lactic-co-glycolic acid) (PLGA)) and fibrin/hyaluronic acid (HA) composite hydrogel has been tested for the partial reconstruction of trachea with allogenic chondrocytes [35]. The chondrocytes were obtained from rabbit articular cartilage, and they were encapsulated within fibrin/HA hydrogels. The cell-laden structures were injected into the PLGA scaffold. Following in vitro culture (4 weeks), the structures were implanted in eight rabbits with tracheal defects. The engineered tissue was evaluated 6 and 10 weeks after the operation. A functional ciliated epithelium was established as a regenerative event, which contributed to the absence of signs of respiratory distress on the experimental animals. Moreover, there was no stenosis, and the defects recovered well. The advantages of this new approach to trachea regeneration can be seen in comparison to a previous preliminary study, where the first fibrin/HA gel-only system was not able to maintain long-term survival of chondrocytes and did not induce neocartilage formation in vivo [35].

For an efficient tracheal reconstruction, there are some requirements, such as the mechanical stability to withstand negative pressure during inhalation; a respiratory epithelium as a first line of defense against infection and also to prevent the formation of intraluminal granulation tissue; and vascularization to ensure graft viability [22]. The presence of a functional

epithelium is crucial, and luminal coverage with ciliated, mucus-secreting respiratory epithelium is indispensable for successful airway tissue regeneration [51]. In addition, an intact epithelial line is crucial to prevent the in-growth of granulomatous tissues and fatal airway obstruction. The cilia are necessary for expelling microorganisms and other nano/microscale materials via the movement of the mucosal fluid. This is why tracheal tissue engineering strategies are based on the re-epithelialization of the tracheal tubes, which are crucial for the whole organ to function.

Different tissue engineering approaches for the replacement of trachea have been used in case of disease or tissue defects [21]. A recent article on the protocols on tracheal replacement models in animals demonstrates the feasibility of different approaches for such surgeries (Figure 11.4). However, the paucity of long-term data regarding the use of these strategies makes it very difficult to con- duct a conclusive evaluation of which is the best in terms of clinical practice. In 2005, Omori et al. transplanted a long Marlex mesh tube that was covered by a collagen foam [52]. Within 2 months, epithelialization was observed, and further re-epithelialization was detected after 20 months. Walles et al. [53] used a decellularized a porcine jejunum patch as a carrier for cells, as the decellularization process leaves the vascular organization relatively intact. This technique has been applied so far to small defects, but its main disadvantages are the inability produce whole tracheal segments (rings) and the time required for the proper decellularization and re-cellularization of the patch [21]. In 2011, Jungebluth reported the use of a CT scan of the patient to reconstruct a resected section of the trachea with high precision. To enhance the functionality of the synthetic graft, neovascularization has been promoted using different growth factors delivered in the graft and/or administered systemically. But the use of growth factors in cancer patients carries certain risks, and therefore this approach cannot be used for some patients. Besides, omentopexy was also performed as described for other strategies mentioned here.

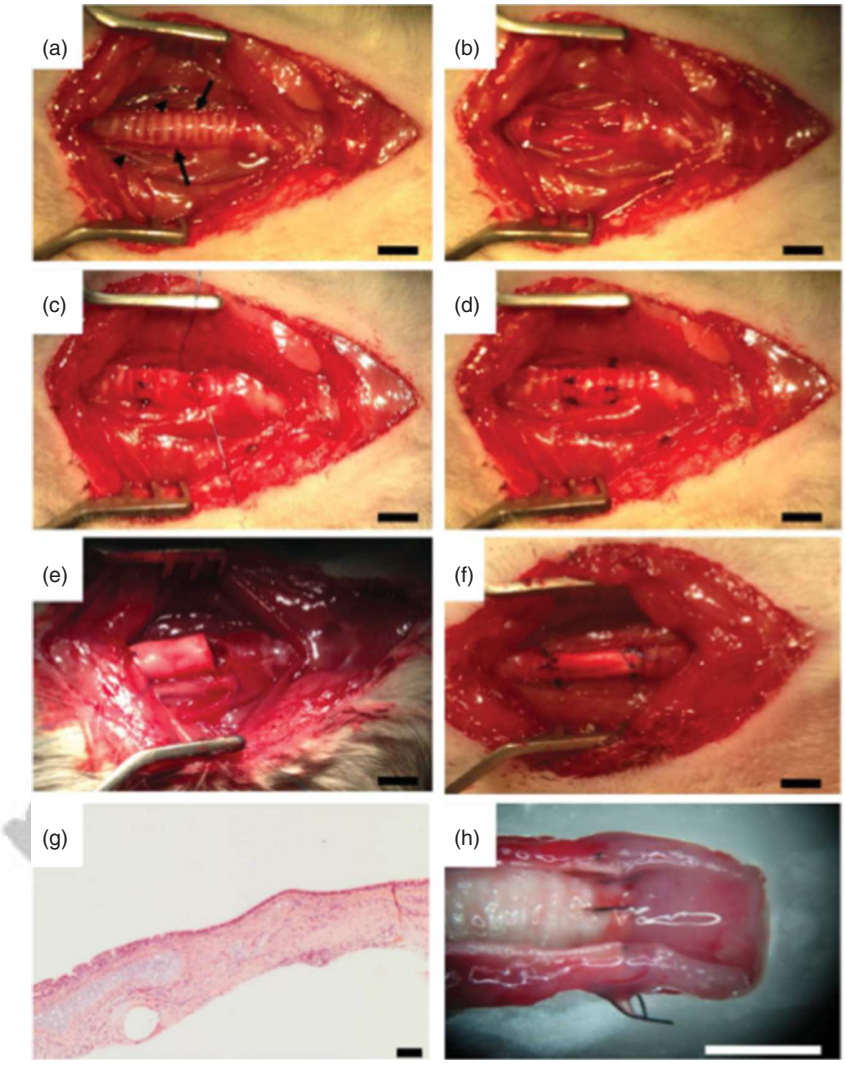

Figure 11.4 Representative examples of the application of tracheal replacements in rats. (a) Exposure of the tracheal segment. (b) Removal of the tracheal segment. (c) Replacement of the resected trachea with an allogenic decellularized rat trachea. (d) Replacement with a bioengineered trachea. (e) Replacement with a polymer-scaffold-based engineered trachea. (f) Position of the implant prior to wound closure. (g) Histological evaluation of the engineered trachea. (h) Postmortem evaluation of the engineered trachea. (Jungebluth 2014 [64]. Reproduced with permission of Nature Publishing Group.)

Autologous tissues are the gold standard in many cases in reconstructive surgery. This is also the case for tracheal reconstruction, as several reports that have used experimental tracheal reconstruction with allotransplants have not demonstrated enough clinical amelioration for human use. As cartilage is an avascular tissue, engineered cartilage does not require large-scale vascular supply, which simplifies its production procedure. In 2012, Jungebluth et al. reported the concept of in vivo airway tissue engineering [40]. In their study, the authors tested the concept of a "natural bioreactor," that is, using own body of the animal for engineered tissue maturation. They used decellularized pig tracheas without recellularization before transplantation. These tracheas were treated with mononuclear cells and growth factors during implantation. During the postoperative period, the in-situ regeneration (taking also advantage from their own bodies' capabilities) was boosted as a result of the administration of bioactive molecules, which promoted the peripheral mobilization of stem cells that differentiated once within the engineered tissue and facilitated the regeneration of the implanted structure. After 2 weeks, the implanted structure had an intact respiratory epithelium with a well-defined cartilaginous component. In addition, because of the mobilization of the progenitor cells into the peripheral circulation in consequence to the administration of the growth factors and bioactive molecules, there was also an upregulation of the antiapoptotic genes. With the advances in cell biology, and in stem cell biology in particular, new strategies have been developed in airway tissue engineering [54]. Stem cells are cells with high levels of self-renewal and differentiation capacity, and have become an indispensable tool in regenerative medicine [50]. Development of robust methods for obtaining induced pluripotent stem cells (iPSCs) has expanded the possible cell sources for tissue engineering. Recent findings related to the no immunogenicity of these stem cells further support their widespread use.

3.8.2 Laryngeal Tissue Engineering

The larynx is a multifunctional organ that has a function in voice production, coughing, swallowing, and breathing [55]. It is the voice box [54] of humans, and so it should be regarded with extra care in airway tissue engineering, as it allows one to speak, to sing, and to communicate. It is therefore essential for human lives. Each year, about 136 000 individuals worldwide are diagnosed with laryngeal carcinoma, some of which will be treated with total laryngectomy [56–59]). In the European Union alone, 11 826 new cases of laryngeal cancers were registered in 2006 [55].

Functional laryngeal reconstruction after total laryngectomy remains one of the most challenging problems. The routine treatment after a laryngectomy in clinical medicine is to perform pharynx-to-esophagus anastomosis and tracheostomy to maintain breathing, causing great pain to the patients. So far, the optimal treatment for laryngeal functional reconstruction has been laryngeal transplantation [60]. Once the larynx is transplanted, patients must face lifelong immunosuppression and live with the risk of tumor recurrence, metastasis, and multiple infections. For this reason, researchers and medical doctors have paid much attention in exploring new avenues of treatment for these clinical cases. Theoretically speaking, the creation of a bioartificial larynx that does not elicit the immunological problems mentioned previously would solve the problem [58]. One of the main requirements of laryngeal function restoration is the presence of functional vocal cords. The muscular actions pertaining to the larynx are extremely complex, which is indirectly evidenced by the density of motor axons in the recurrent laryngeal nerve. The larynx has one of the most sophisticated actions of any muscular-based organ in the human body, as may be appreciated by listening to high-performance singing. This complexity is reflected by the comparatively high density of motor axons in the recurrent laryngeal nerve with respect to the relative size of the innervated muscles. Thus, re-innervation of any implanted larynx substitute is highly important. This can be achieved by the incorporation of neurotrophic factors or engineered nerve grafts [55, 61]. In fact, only a few cases of allotransplants have been documented, and the clinical acceptance of the method is poor because of the difficult procedure [57]. According to Baiguera et al. (as covered in detail in a previous section), there are two documented laryngeal transplants in humans to date [60], which seems a very small number compared to the required actual needs. This is already an indication of how difficult it is to achieve a successful transplant and how much there is still to be done. Therapies based on tissue engineering, using either biological or synthetic scaffolds, are therefore desired. Recently, the human larynx has been decellularized and characterized for its anatomical, physiologic, and biomechanical properties. The technology to develop a larynx exists, but further efforts must be made before a translation to the clinic would be possible. The development of a bioartificial cricoarytenoid unit that needs to be implanted in patients who need a total laryngectomy would probably be the most relevant undertaking. To overcome the immunogenicity problem, a new tissue engineering solution has been developed. The extracellular matrix (ECM) has been successfully used as a biological scaffold derived from decellularized organs and tissues in both preclinical and animal studies. The ECM has low immunogenicity; thus its use decreases the need for immunosuppressants. Moreover, it provides a perfect three-dimensional

architecture for tissue regeneration and induces the adherence and proliferation of cells. In order to reconstruct a full, functional bioartificial larynx, one has to take into account the fact that larynx is a complicated organ comprising multiple tissues. It has been proven that cartilage is an organ with low immunity, and therefore larynx immunogens are positioned mainly in the mucosa and muscles. Hou et al. in a preliminary study recellularized the decellularized laryngeal muscle with mesenchymal stem cells and constructed a low-immunity, heterogenic laryngeal graft [58]. Currently, there is a clinical trial going on regarding the efficiency and safe use of stem-cell-based engineered airways in the United Kingdom (RegenVOX study). The tested airway replacements are decellularized laryngotracheal grafts seeded with autologous stem cells. Ten patients (inclusion criteria: severe, acquired laryngotracheal stenosis (Myer–Cotton Grade 3 or 4), idiopathic, traumatic, iatrogenic, or inflammatory caused with no possible conventional treatment method) will have been implanted with such structures during the trial. A 2-year follow-up of the patients is foreseen [54]. Further studies have been conducted with bone-marrow mesenchymal stem cells (BM-MSCs) transplanted with an artificial ECM for regenerating a scarred vocal fold in vivo. It was shown that the groups of rats with the transplanted BM-MSCs proliferated and showed low levels of apoptosis or myofibroblast differentiation markers. The group with BM-MSCs showed increased levels of expression of procollagen type III, fibronectin, and TGB-β1. The injections of BM-MSCs in the vocal cords of rat promoted new ECM deposition, as evidenced by the presence of fibronectin, as previously mentioned. Grafting of BM-MSCs laden HA-based artificial ECM-based scaffolds in injured vocal folds is a promising method for vocal cord regeneration [18].

A recent clinical transplant in minipigs has been reported to be a success. Bir- chall et al. reported an early immunological implication (48 h and 1 week) in larynxes to seven pairs of NIH minipigs, which were homozygous at the major histocompatibility (MHC) locus. This is a robust model of laryngeal transplantation in the NIH minipigs, and they did not show a strong immunological response [18]. New insights seem to be available on the microstructure physiology of the vocal folds. This new knowledge, together with new surgical techniques developed such as the modern phonomicrosurgery, will provide the necessary tools for the regeneration of the vocal folds [62]. Treatments included in the laryngeal tissue engineering field comprise also the use of growth factors, implantable scaffold, or cell therapy used alone or in com- bination. Gugatschka et al. used side population cells, as they are considered to present high content of stem cells, to repair a rat vocal fold injury for a period of 5 weeks. Interestingly, the number of this specific cell population increased over time, and an early

vocal fold wound healing was reported, which seems promising in larynx tissue engineering [63].

3.9 Conclusions and Future Directions

One of the problems related to the transplantation and engineering of the trachea is its shared vascularization with the esophagus. In some cases, the replacement of trachea and esophagus might be required at the same time, because of metastasis. In cases of stenosis of the esophagus, or cancer that is untreatable by chemoradiotherapy, the only possible treatment would be resection. In some circumstances, reconstruction of the esophagus is feasible by gastric advancement, colon or jejunal interposition, or a free jejunal flap. Each of these interventions has potential risks and complications especially in fragile patients, and sometimes may increase morbidity and even mortality. In patients in whom no intervention is possible, oral feeding is definitely prohibited for lifetime, being replaced by the need to use a gastrostomy tube for alternative feeding. For this reason, the creation of an engineered esophagus would be a necessary step in several cases of tracheal replacement cases. Tissue engineering would offer the possibility of preparing such systems, which would need to be pliable enough to limit the risk of rupture of the great vessels, given the proximity of the esophagus to the aorta, and rigid enough to allow the passage of food without the risk of obstruction due to collapse. As for the artificial and engineered larynx, total laryngectomy is generally associated with pharyngectomy, and hence it is called total pharyngolaryngectomy (except for strictly glottic cancers). Therefore, the remaining pharyngoesophageal (neopharynx) segment is limited in size, which complicates the use of artificial larynx or an engineered larynx, as the food bolus descends down slowly or may remain stuck around the prosthesis. Technical manipulations like using a vascularized flap can, however, increase the space of the pharynx under certain con- ditions. The possibility of producing a complete prosthesis or engineered tissue (a pharyngolaryngeal implant) reconstructing the larynx while maintaining a wide pharynx is very interesting, yet it remains under study. A more biomimetic artificial larynx, for its tracheal part, where titanium and stem cells will be present, is an objective of our team, and seems a very promising line of research. Valves allowing breathing and swallowing in the upper airway are also being studied to optimize the function of this prosthesis. It is not innate for patients rehabilitated by an artificial larynx to block their breathing during swallowing. Thus, a major rehabilitation is necessary, which is sometimes impossible for certain patients. An automated control system for opening/closing valves (pacemaker) is being considered to avoid this, sometimes tedious, rehabilitation. It is therefore in hands of researchers and clinicians to dedicate time and effort to these questions

in order to enable the clinical transfer of the technologies described above and their timely application to afflicted patient populations. However, given the recent clinical data, one can be hopeful about the use of the currently available techniques and strategies for decreasing the suffering of so many patients that each day face the difficulties of loss of a part of the respiratory system.

C – Résultats

4 - Double thin film-based sandwich-cell carrier design for multicellular tissue engineering

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Dans cette première partie nous avons voulu démontrer qu'un film d'hydrogel à base de collagène pouvait être utilisé comme support pour une repousse de l'épithélium respiratoire sur la face endoluminale de la prothèse de larynx en titane. Dans ce but défini il fallait développer un film suffisamment stable et résistant à l'hydrolyse pour durer assez longtemps lors de l'implantation afin de mimer une matrice extracellulaire artificielle et assurer l'adhésion des cellules épithéliales. Cette stabilité a été assurée par une réticulation enzymatique avec de la transglutaminase. De plus la capacité de ces films à être chargés avec des facteurs de croissance spécifiques et à les libérer progressivement a été testée. L'effet de l'adjonction des facteurs de croissance sur la prolifération cellulaire a également mis en évidence une augmentation de l'activité cellulaire. Ces films ont ensuite été testés pour assurer une adhésion sur une surface en titane.

A l'issu de ces premiers essais nous avons démontré qu'un film d'hydrogel à base de collagène d'origine porcine réticulé avec de la trasnglutaminase pouvait être utilisé comme support pour une greffe ou une repousse rapide d'épithélium respiratoire. Ces films ont également été utilisés dans un système sandwich pour se rapprocher au mieux d'une matrice extracellulaire. Des essais de culture cellulaire en co-culture ont été réalisés dans ce but. Des fibroblastes ont été ensemencés entre deux films d'hydrogels et à la surface du système des

cellules épithéliales respiratoires d'origine carcinomateuses. Cela a permis de prouver la viabilité des deux populations cellulaires dans un tel montage. Les résultats de cette première partie ont fait l'objet d'une publication dont les résultats sont détaillés ci-dessous.

4.1 Abstract

Many organs are multicellular and each cell type requires a different microenvironment. Thus, there is a need for modular structures where the microenvironment of each cell type can be tuned separately. Herein, we describe enzymatically crosslinked gelatin based double layered film structures where each layer can be loaded with growth factors separately. As a model, we have developed a bi-layer system to produce a respiratory epithelium. This system constitutes an in vitro "epithelial patch" that can be adhered to the lumen of any implant. Crosslinking of the patches with transglutaminase resulted in 7 days of stability at 37 °C. The film layer was first used to release growth factors and it was shown that the release significantly improved the proliferation over 5 days. A549 human lung epithelial cells were used and under the release of an epithelial growth supplement mix, there was a significant improvement on the epithelial proliferation ($p<0.01$). The designed substrate was successfully attached to titanium implants and we demonstrated the stability of the epithelial patch under in vitro conditions for 7 days without deterioration. Under co-culture conditions for three days both cell types were alive. Such patches can be used to obtain fast epithelialization of large implant surfaces.

4.2 Introduction

During, the embryonic development in vertebrates, the invagination of blastophore in the gastrula leads to three layers: the endoderm is the inner layer, the ectoderm is the outer layer and the mesoderm is the middle layer. In organogenesis all organs developed from these 3 layers of cells. The nervous system, the skin and hair originate from the ectoderm. Bone, muscles and vascular system are developed from the mesoderm. The endoderm leads to the formation of the lining of tube shaped organs like part of digestive tract or respiratory tract. The association of mesodermal cells and endodermal cells and their differentiation pro- vides a functionality to different organs such as respiratory tract or urinary bladder.

A clinically relevant example is the tracheal respiratory epithelium which is a pseudostratified ciliated columnar epithelium that contains five different cell types: 1) ciliated columnar cells, 2) goblet cells, 3) basal cells, 4) brush cells, and 5) dense core granulation cells [65]. The ciliated columnar cells are elongated cells with apical cilia responsible for moving mucus

along the surface of the epithelium. Goblet cells are disseminated individually within the epithelium, they are mucus secreting exocrine cells that produce the large part of the mucus covering the epithelial surface [66]. The basal cells are rounded cells located in the basal surface of the epithelium. Moreover, they are the reserve stem cell population which replaces lost ciliated cells and goblet cells. The brush cells are columnar cells with apical microvilli instead of cilia, they synapse with the dendritic endings of sensory nerve fibers playing a sensory receptor role. The dense core granule cells are endocrine cells that release vasoactive substances [67]. All these cells types are lined on the lamina propria which consists in a thin layer of fibroelastic connective tissue rich in elastic fibers. The collagen and glycoproteins in the basement membrane are produced in a part by the epithelial cells and in other part by connective tissue especially by the fibroblasts [68].

The respiratory epithelium has several dynamic functions such as 1) warming the inspired air by heat conduction from blood flow in the vascularized connective tissue; 2) moistening inhaled air by evaporation of water from mucous or serous glandular secretions; and 3) removal of the exogenous particles such as dusts or bacterial materials by trapping them in the sticky mucous layer and then transport them to hypopharynx by the ciliary movements [69]. This overview of the respiratory epithelium's functions demonstrates that the deficiency of one of these roles may lead to pathological situations. For example, the primary ciliary dyskinesia that affects the cilia motility or the cystic fibrosis leads to a thick and viscous mucous secretion responsible for respiratory infections because of mucus stagnation [70].
The complexity of these physiological functions makes the development of tracheal substitutes more difficult. Indeed, although the need to develop a tracheal substitute, to cure expansive tracheal defects, is not new [71], the optimal technique or biomaterial that can be used in routine clinical applications have not been found yet. This is due to the structure and composition of the trachea itself which is a multi-cellular organ composed of cartilaginous, connective and epithelial tissues [72]. Mimicking the interactions of the connective tis- sue ECM secretions and with the epithelial lining is an important point to consider in tissue regeneration [73]. All the different techniques using prosthetic replacements, allografts, composite auto- grafts, proposed over the years to replace trachea have shown limitations [74]. The absence of functional respiratory epithelium in these current solutions has been considered as one of the limits in prosthetic replacement and tissue grafts. The main problems following the lack of epithelial layer are bacterial and/or fungal colonization of the prosthesis

lumen, mucous plugs, and connective tissue hypertrophy resulting in endoluminal stenosis [75]. A typical example clinical issue concerned by these problems in head and neck surgery is the replacement of larynx.

The late stage laryngeal carcinomas are most commonly treated by laryngectomy. Separation of digestive and respiratory tracts results from this surgical intervention with a definitive tracheostomy and the loss of the capacity of phonation. These changes deteriorate the patient's life quality significantly. One of the working axes to remedy this situation is the development of an artificial larynx, where titanium can be utilized due to its mechanical stability [76]. Our group has recently reported the first successful implantation of such a system clinically [77]. This artificial larynx is designed in two parts: a multifunctional valve to replace the larynx's function it- self (particularly the function of epiglottis) and a tracheal substitute to connect remaining trachea and the multifunctional valve. For this tracheal substitute we developed a porous microbead-based titanium implant that provides better integration with host tissues [78]. The integration happens through the migration of the cells from the surrounding connective tissue into the porous titanium structure. But in animal studies, we were confronted with the same problems such as endoluminal stenosis and mucous plugs, both phenomenon due essentially to the absence of re-epithelialization of the endoluminal side of the titanium implants [79]. In in vivo animal trials we could observe often spontaneous epithelialization only in the case of small animals like rats [80]. But there was no epithelialization either in the case of bigger animals such rabbits and sheep, nor in human transplantations [81], even though no side effects due to this absence was observed. Thus, the further clinical improvement of the artificial larynx seems to depend, in part, to the ability to regenerate a functional respiratory epithelium.

In order to solve this problem we propose a multilayered epithelial cell delivery system, where separate substrates for the epithelial cells and underlying connective tissue can be delivered simultaneously and where each component of the carrier can be modularly produced [82]. For this end, we developed a double layered crosslinked gelatin film based constructs where the physical and chemical properties of the parts facing the epithelial cells or the connective tissue cells can be separately controlled (Fig. 1). Gelatin, a natural polymer which is obtained by denaturation of collagen, is a widely used biocompatible material. Its ability to form thermoreversible gels and the presence of RGD sequences within its chains due to its collagenous origin, makes it a versatile material for development of tissue engineering scaffolds, delivery systems and cell growth substrates [82,83]. However, gelatin is highly soluble in aqueous solutions and gelatin based films need to be stabilized for long-term

applications. There are several ways to crosslink gelatin such as use of chemical crosslinking agents like EDC/NHS or genipin. A more versatile and specific way is the use of transglutaminase enzymes, which in nature crosslink collagen molecules [84]. To reach this purpose, we emit the hypothesis that a functionalized biodegradable gelatin hydrogel patch (where the degradation is con- trolled by crosslinking degree via transglutaminase) could support the epithelial cell grafts until degradation of the synthesized material by connective tissue. These hydrogels will serve as a feeder layer and will release growth factors by mimicking an artificial basement membrane. This basement membrane can be further supported by an additional patch layer containing connective tissue cells to mimic the actual epithelial layer. In order to test the efficacy of such a design we tested the stability, controlled release capacity and feasibility of the system as a multicellular delivery system with model cell types.

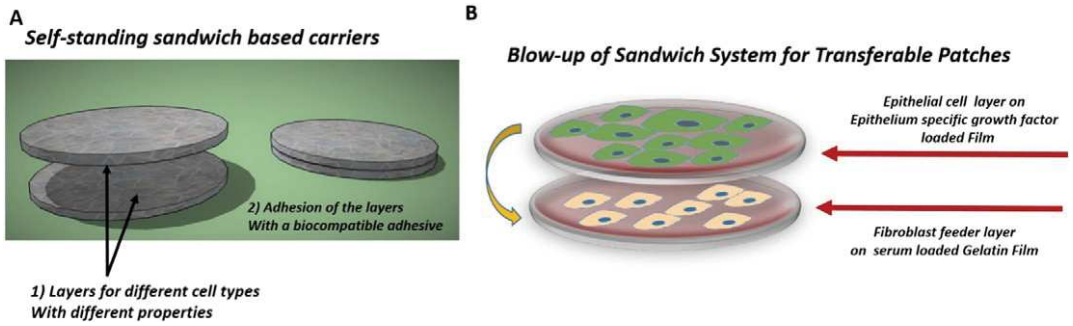


Fig. 1. Sandwich like double-layered patches for transfer of epithelial cells in the presence of a connective tissue. The two layers are put together with a wet, non-cytotoxic adhesive which provides stability over a week. Each layer has its own physical properties adjusted for the cells they carry: i) the upper layer will contain the epithelial cells and specific growth factors loaded and ii) the bottom part will integrate fibroblasts with serum components loaded.

4.3 Materials

Gelatin from porcine skin and BSAFITC (Mw = 6.6 × 104 Da, pI = 4.7–4.9) were purchased from Sigma-Aldrich (Germany). For cell culture Phosphate Buffered Saline (PBS), RPMI 1640 cell culture basal medium, penicillin/streptomycin mixture, fungizone, and trypsin–EDTA were obtained from General Electrics Healthcare (USA). Fetal Bovine Serum (FBS) was provided by Gibco (France). SupplementMix airway epithelial cell growth medium was purchased from PromoCell (Germany). Composition of SupplementMix airway epithelial cell growth medium are given in Table S1. Microbial transglutaminase was kindly provided by Ajinomoto (Japan). Cell proliferation was an- alyzed with Alamar Blue test (Promokine, Germany). Fluorescence readings were performed with Xenius® XC (SAFAS, Monaco). For cell viability determination, Vybrant® cell adhesion assay (Invitrogen, USA) was used. Live cells were labeled with calcein-AM and dead cells with Sytox® Green available in the same kit (Molecular probes, Life technologies, USA).Cells staining were performed with Hoechst 33358 and phalloidin provided by Sigma-Aldrich. Images were obtained with fluorescence microscopy Nikon Eclipse-Ti (Japan). Images were then processed with ImageJ (NIH, USA).

4.4 Methods

4.4.1 Gelatin film fabrication and crosslinking

Porcine gelatin was dissolved in sterile, deionized water at 37 °C at the content of 5%, 7.5% and 10% w/v. For convenience, these three conditions can also be referred as 5%, 7, 5% and 10% in the paper. Metallic rings with 8 mm inner diameter where used as mold on hydrophobic substrates (parafilm). 100 µL of gelatin solution was poured in the molds and left to air-dry for 24 h. Then a solution of 10% (w/v) of microbial Transglutaminase (TGA) in PBS was prepared. 100 µL of this solution was incubated on gelatin films of each concentration for 1 h, 2 h and 4 h. Then two rinsing steps of 5 min were performed by adding 100 µL of PBS each time and the films were air-dried for 24 h. The films were removed from molds after being completely dried.

4.4.2 Gelatin film physical characterization

The release of (BSAFITC) (Fluorescein isothiocyanate labeled bovine serum albumin) was monitored with SAFAS Genius XC spectrofluorimeter (Monaco). The gelatin patches crosslinked with microbial transglutaminase were made according to the protocol above.

Then the patches were put in a solution of BSAFITC (1 mg·mL−1 in PBS) for 30 min. After that, the release experiments were carried out at 37 °C in 1 mL of PBS. The supernatants were analyzed with the spectrofluorimeter. The wavelength of excitation and emission for BSAFITC are λex/ λem = 495 nm/520 nm. After each analysis, a new PBS solution was added and so a cumulative release was performed.

The thickness of each rate of dry gelatin film and their mass were measured before and after 2 and 4 h of crosslinking time (for statistics n ≥ 6 different samples were measured for each gelatin film composition and each different crosslinking time) The thickness of dry gelatin crosslinked for 1 h have not been performed because the film was fully dissolved after 48 h. For thickness a digital vernier caliper was used and 3 different measurements for each sample were performed before and after crosslinking. Then to study the film degradation by hydrolysis, each type of film, not crosslinked and for 1, 2 or 4 h of crosslink, were incubated at 37 °C and 5% CO2 in 500 µL sterile PBS (n ≥ 3). The mass of each film was measured in dry form before hydrolysis. After 7 days of degradation the samples were removed from PBS, dried completely overnight and then weighed again.

4.4.3 Patch adhesion to titanium implants

A 10% porcine gelatin solution was prepared as previously described. 50 µL of this solution was poured on porous, acid etched micro beads based, titanium implants. Then a dried, 2 h crosslinked, 7.5% gelatin film was put on this gelatin droplet that acts as a glue between crosslinked gelatin film and titanium. The patches attached to titanium were placed in 12 well plates. 1 mL of sterile PBS was added in each well and left in incubation at 37 °C in a cell culture incubator for 7 days. After 7 days of incubation the PBS was removed. For each sample we con- trolled if the gelatin film was still adherent to the titanium.

4.4.4 Production of the sandwich system

To mimic the pseudostratified structure of the respiratory epithelium, we designed a sandwich system with two gelatin patches. These two patches are linked through an adhesive made of gelatin type A from porcine skin crosslinked with a solution of microbial transglutaminase (10% w/v in PBS).

4.4.5 Cell culture

3T3 fibroblasts and A549 human lung carcinoma epithelial cells where cultured in RPMI 1640 basal medium completed with 10% v/v Fetal bovine serum, 1% v/v Pen-Strep and 1%

v/v Fungizone. 75 cm2 tissue culture plates were used for incubation at 37 °C with 5% CO2. These cells were passaged at near confluence.

For cells seeding trials the same protocol was used. Culture medium was removed and cells were washed with 10 mL of PBS. Then cells were detached with 0.05% trypsin during 5 min. Cell concentration was calculated by a hemocytometer and the final cell number was adjusted to obtain the concentration of 50 000 cells in 30 µL by centrifugation and volume adjustment. Then the samples of each concentration of (5%, 7.5%, 10% w/v) gelatin films were prepared in a 24 wells plate. All gelatin films used for cell seeding were cross-linked for 2 h. They were sterilized with UV exposure for 30 min. Then 30 µL suspended cell solution was seeded on each film. The films were incubated without culture medium for 30 min to permit cells to attach gelatin films and then 1 mL of complete RPMI medium was added in each well. The samples were then left to incubation at 37 °C with 5% CO2 for 7 days. Culture medium was changed every 48 h.

3T3 fibroblast seeding

Two groups were performed with 3T3 fibroblasts. The first group was performed with 5%, 7.5% and 10% gelatin films only (n = 7). For the second group the gelatin films of each concentration were previously incubated in pure FBS for 30 min. Then the films were placed in the well plate and cells were seeded as described above (n = 7).

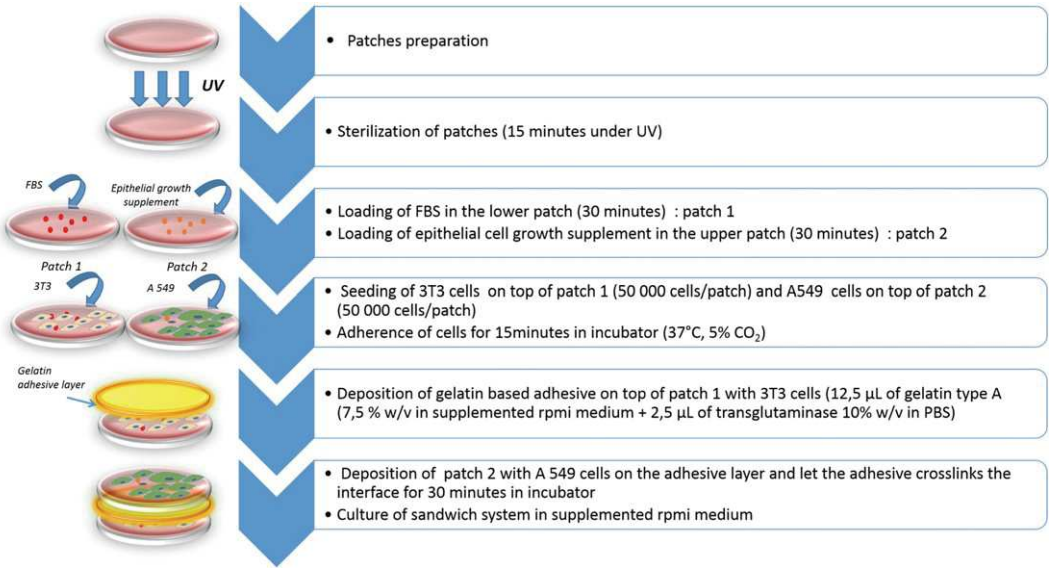
A549 respiratory epithelial carcinoma cell seeding

A549 epithelial cells were suspended as previously described and seeded on 7.5% gelatin films. Two configurations of A549 seeding were performed with cells seeded on samples of 7.5% gelatin films: i) either with samples without growth factor on the films ii) or with samples previously incubated in epithelial growth medium supplement, and with growth factors. The experiment was then performed for 7 days in both cases.

3D cell culture in the sandwich system

These two patches can be loaded with different growth supplements with respect to the need of the different cell types seeded on top of them (FBS for the lower patch with fibroblasts and epithelial growth supplement for the upper patch with epithelial cells). For all 3D experiments gelatin patches made of gelatin type A from porcine skin were used (7.5% w/v in PBS). To perform 3D cell culture experiment, the following protocol was used. The lower gelatin patch was sterilized with UV for 15 min and then incubated in FBS solution for 30 min. The patch was air dried for 15 min and then the cells were seeded on top of the patch at a density of

50,000 cells. The cells were left for 15 min in incubator to ensure adherence (37 °C, 5% CO2) and afterwards, the adhesive was deposited on top of the cells. First 12.5 µL of gelatin type A (7.5% w/v in supplemented RPMI culture medium previously sterilized in UV) was introduced on top of the cells and 2.5 µL of microbial transglutaminase (10% w/v in PBS previously sterile-filtered at 0, 22 µm) was added to crosslink the gelatin. Finally the upper patch was put in contact with the lower patch with the adhesive and the interface between the two patches was crosslinked for 30 min in incubator prior to the addition of the culture medium in the culture plate. In this configuration, the fibroblasts were encapsulated in gelatin matrix at the interface between the two gelatin patches. For the complete sandwich as described above, 3T3 cells were seeded on a patch then a second patch that was seeded with A 549 cells was deposited on the top. These constructs were kept in culture for 3 days in supplemented RPMI medium (Scheme 1).



Scheme 1. Different steps for sandwich structure development.

Cell characterization on the patches

Cell proliferation was determined by Alamar Blue method (resazurin based assay, Promokine, Germany). As previously explained, the cell experiment was performed for 7 days and the metabolic activity was recorded at day 1 and day 7. 500 µL of Alamar Blue solution (10% v/v in supplemented RPMI medium) was incubated for 2 h in each well containing the samples. These samples were previously placed into a new well plate to avoid counting cells that were attached to well plate and not on gelatin films. After 2 h of incubation, 100 µL of the solution for each well was transferred into a 96 well plate for fluorescence readings. The fluorescence intensity of the solutions was monitored with a spectrofluorimeter (Excitation λ: 560 nm Emission λ: 590 nm). As a positive control TCPS was used ($n \geq 6$). For determining the viability of the adhered cells on the patches, Vybrant cell adhesion assay was used. Live Cells were labeled with Calcein-AM (Molecular probes, Life technologies, USA), according to the provider's instructions. Dead Cells were labeled with Sytox Green. Calcein AM stock solution was diluted 1:200 times in cell culture medium and the cells were incubated with Calcein-AM solution for 30 min at 37 °C. Cell counts per image were conducted using a fluorescence microscope ($n \geq 6$) and with ImageJ software.

All cell culture analyses were performed after cell fixation and staining. After two rinsing steps of 5 min with PBS, 400 µL of PFA solution (4% w/v in PBS) was added in each well for 15 min. Then the PFA was removed. After two rinsing steps with PBS, the samples were left for 5 min in Triton X solution (0,1% v/v in PBS) and then a new rinsing step was performed. 200 µL of the Hoechst 33358 solution (20 µg in 1mL PBS was added in each well for 10 min. Then the samples were rinsed two times with PBS. Then 200 µL of the phalloidin solution (5 µg in 1mL PBS) was added in the wells with samples for 45 min and the samples were rinsed again two times with PBS before storage in PBS at 4 °C. The analyses were performed with fluorescence microscopy. The images were processed with ImageJ software. For 3D cultures the incubation periods were modified as follows: Triton-X was incubated for 10 min and phalloidin for 1 h.

4.4.6 Statistical analysis

Statistical analyses were carried out by the Microsoft Analysis Toolpak Add-in for Excel/Microsoft. Student's t-test was used for comparison of two conditions whereas one-way Analysis of Variances (ANOVA) was used for comparison of 3 conditions. p was set as < 0.05 for statistical significance.

4.5 Results

We first entrapped fibroblasts within the sandwich system in order to see their 3D distribution under this culture condition (Fig. 2A). Cells were observed in a section of 80–120 μm showing that they were distributed within the volume in between the patches. DAPI/phalloidin staining demonstrated that fibroblasts exhibit spread morphology probably due to the simultaneous dorsal and ventral contact with ECM containing surfaces in the sandwich condition (Fig. 2B). Fibroblasts proliferated in the first 3 days of culture ($p < 0.05$) but then the metabolic activity stayed stable.

In order to see whether the films have the capacity to release bioactive agents previously loaded such as components of Fetal Bovine Serum (FBS) we have selected a protein of FBS, Bovine Serum Albumin (BSA), as the model protein and quantified its release over 5 days (Fig. 3A). After an initial burst release, a steady release of BSA was observed from the patches over several hours. Then we tested whether the patches can support fibroblast and epithelial cell attachment. 3T3 fibroblasts seeded on gelatin patches, without additional FBS loaded in the patch, were already alive and attached on films after 7 days of incubation. The cell activity measured with Alamar blue assay didn't show any increase in proliferation between the day 1 and day 7 (Fig. 3B). The cell activity was similar at day 1 and day 7 for all the different gelatin films (5, 7.5 and 10%) crosslinked for 2 h ($p > 0.05$). As an additional facilitator, we checked the effect of loading a cocktail of growth factors from FBS within patches on the proliferation of the cells. When the gelatin patches were previously loaded with FBS, Alamar Blue® assay shows a significantly increased cell proliferation between day 1 and day 7 (Fig. 3C). For all groups (5%, 7.5% or 10% gelatin) cell proliferation significantly increases about 3 times from day 1 to day 7 ($p < 0.05$). The A549 respiratory epithelial cell cultures on 7.5% gelatin films that were crosslinked 2 h show similar results, i.e. a strong increase in proliferation between day 1 and day 7 when the patches are previously loaded with epithelial growth supplement mix ($p < 0.05$) (Fig. 3D). The growth factor loading had a positive effect on the epithelial cells' adhesion and proliferation (Fig. 3E).

In order to obtain a double layer composed of fibroblasts and epithelial cells, a sandwich system based on 2 patches (7.5%) was constructed. The lower part is for supporting epithelial tissue by providing the means for epithelial/connective tissue interactions via the presence of fibroblasts (replenishment of ECM components, secretion of growth factors etc.). The upper part is for supporting the respiratory epithelial cells. In this configuration the fibroblasts were in between two patches whereas the epithelial cells covered the top patch. To check if there is

any effect of this configuration on cell viability, the cell viability tests were done with A 549 epithelial cells on top and 3T3 fibroblasts between the 2 gelatin patches. The live cells were stained with Calcein-AM and the dead cells were stained with Sytox. Therefore, it was possible to observe that the majority of the cells that attached the film's surface were alive (Fig. 4A, B). Indeed, in the same batches of samples, there were more fibroblasts or epithelial cells stained with Calcein than Sytox for both cases.

As a proof of concept we also demonstrated the adhesion of the patches on porous titanium disks (Fig. 4C). After 7 days of incubation in PBS under cell culture conditions, the 7.5% gelatin films, previously crosslinked for 2 h, were still adherent to the porous titanium samples. There was no spontaneous detachment with 12 samples tested. A manually applied force was necessary to remove the film from titanium implants.

We then checked the effect of the crosslinking duration on the stability of the patches. When left to degradation by hydrolysis (PBS, at 37 °C), non-cross-linked patches totally dissolved in the first few hours. 5% and 7.5% (w/v) gelatin patches crosslinked 1 h totally dissolved after 48 h. The 10% gelatin films crosslinked 1 h dissolved partially at 48 h in the form of remnant small pieces. The 10% gelatin films that were crosslinked 2 and 4 h were still intact after 7 days of incubation in PBS even though they have lost weight (Fig. 5A and B). There was no difference between 2 and 4 h crosslinking time, the mass loss was similar in each concentration of gelatin films ($p > 0.05$). Gelatin films thickness and mass increased after crosslinking with transglutaminase ($p < 0.05$) (Fig. 5C). This trend was observed in all concentration of gelatin films after 2 h and 4 h crosslinking time. This was probably due to the entrapped dextran (which is a biocompatible additive in the transglutaminase formulation) and adsorption transglutaminase within the film structure.

In order to see morphology of the cells on patches, fluorescence microscopy was used. With the DAPI/phalloidin staining, it was also possible to have a visual confirmation of the results provided by Alamar blue® assays. The addition of growth factors (FBS for 3T3) in gelatin patches prior to cell culture experiment improved both the number of 3T3 initially attached to the films and their spreading on the surface compared to the patches without addition of growth factors (Fig. 5D).

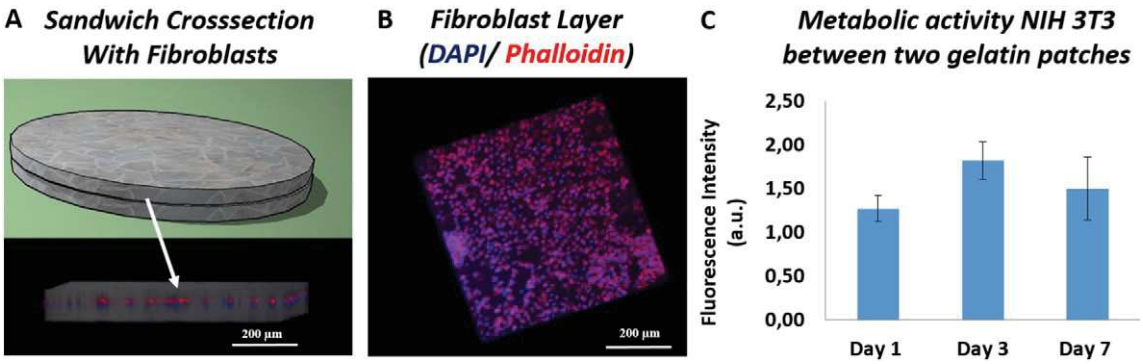


Fig. 2. A) Schematic illustration of location of cells and 3D confocal images of the sandwich structure made with two gelatin patches (7.5%) (transmission mode) with the fibroblast cells in between stained with DAPI (blue channel) and phalloidin (red channel). B) 3D reconstruction of the fibroblast cells (stained with DAPI/phalloidin) encapsulated in the sandwich structure after 7 days of culture; a significant portion of the volume in between the cells have been filled by day 7. C) Metabolic activity of the cells in sandwich configuration.

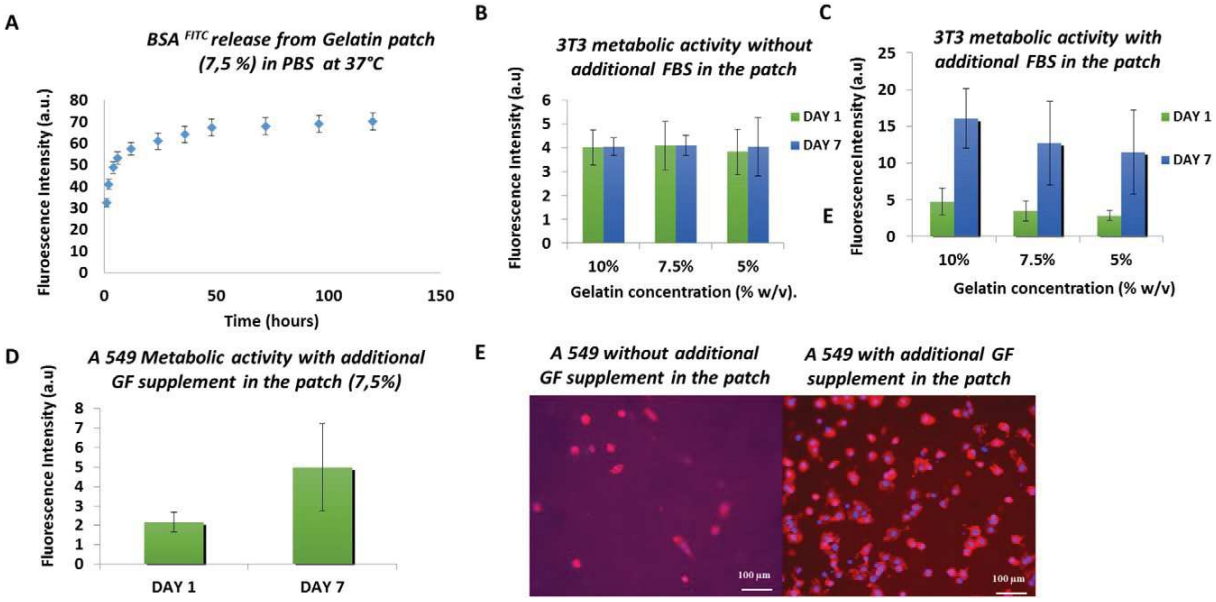


Fig. 3. A) Release profile of FITC labeled BSA from a single patch. B, C) Metabolic activity of 3T3 cells seeded on crosslinked gelatin patches of different concentrations, B) without or C) with loading of FBS into the patch structure. D) Proliferation of A549 cells over 7 days in the presence of Epithelial Cell Growth Supplement Mix. E) DAPI/phalloidin staining of A549 epithelial cells on 7.5% gelatin patches without and with epithelial growth factor supplement (magnification 10×) after 7 days of culture.

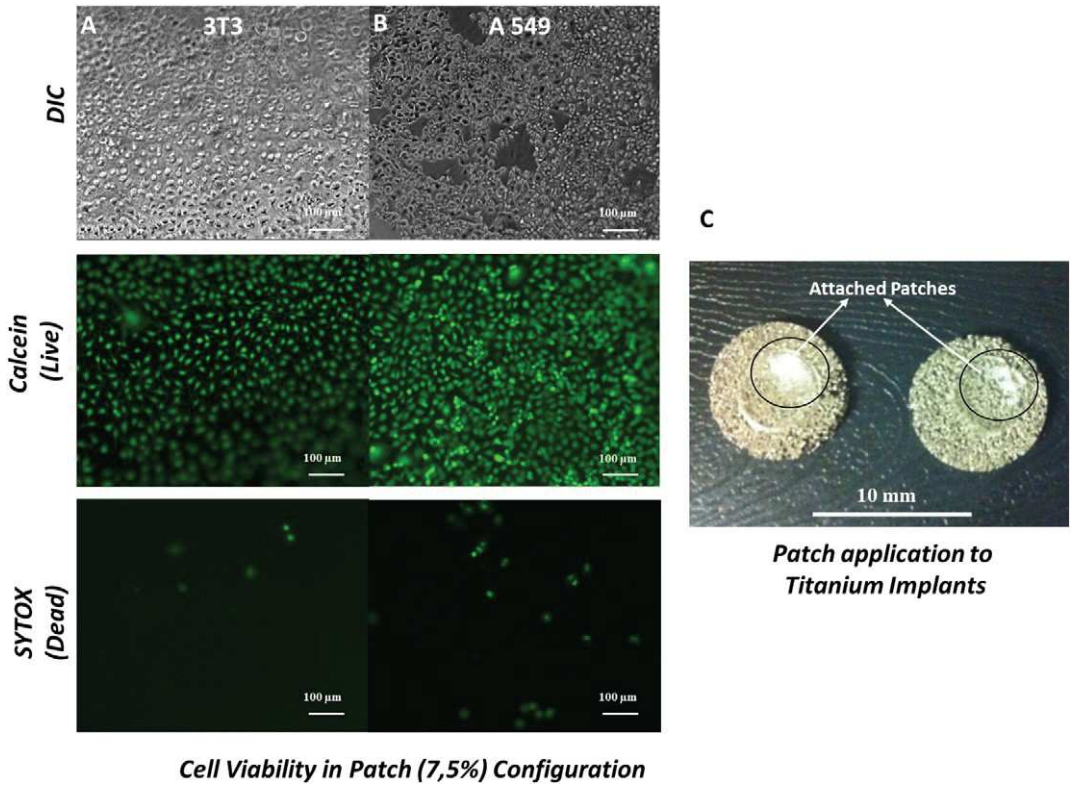


Fig. 4. A, B) Viability of fibroblasts (3T3) and epithelial cells (A549) in patch configuration. From top to bottom: DIC images of the cells showed confluent layers in patch configuration. Cells were alive as evidenced by calcein staining and for both cases there were only few dead cells observed by Sytox Green. C) The attachment of patches on titanium implants; the patches were stable on the implants after 7 days of incubation in PBS at 37 °C.

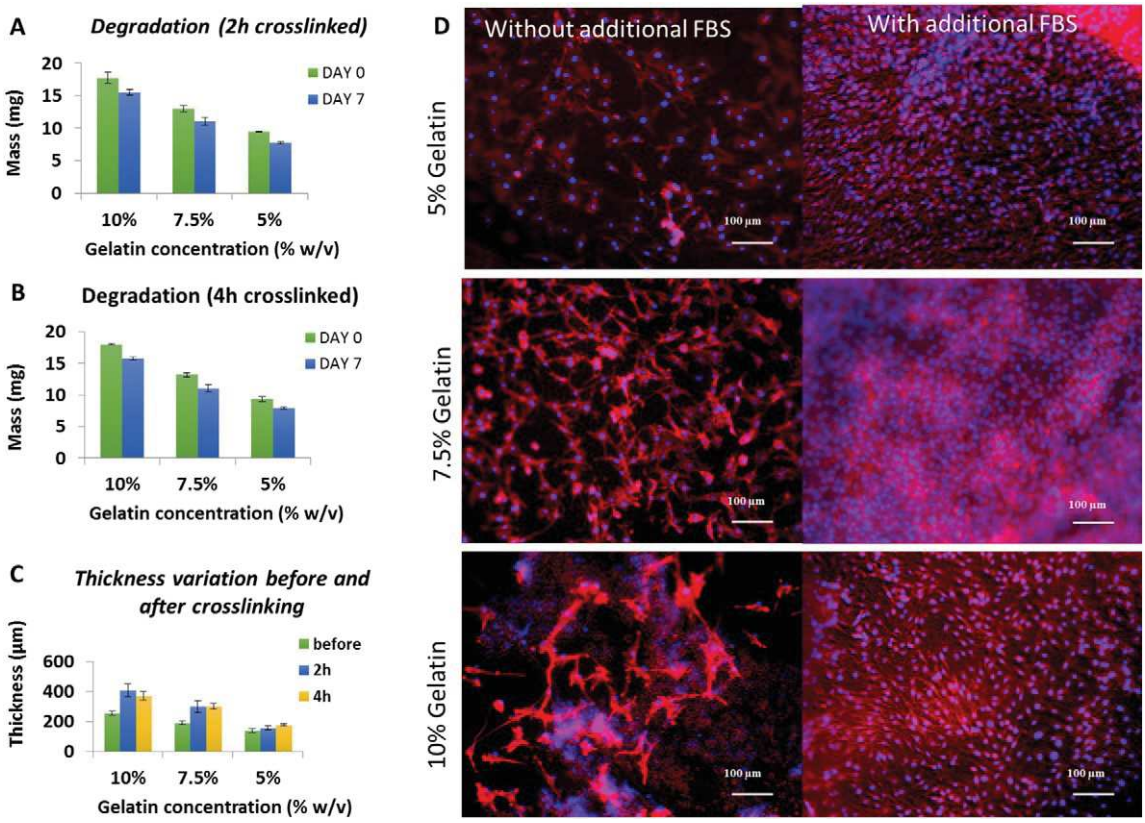


Fig. 5. A) Mass loss over 7 days in PBS at 37 °C of gelatin patches of different composition crosslinked with transglutaminase for A) 2 h and B) 4 h. Both durations led to patches which resist to hydrolytic degradation. C) Thickness increase in the crosslinked patches before and after crosslinking. D) DAPI/phalloidin staining of 3T3 cells on transglutaminase crosslinked gelatin patches without or with loading of FBS within the patch. Loading of FBS has a significant effect on cell number and spreading (magnification 10×).

4.6 Discussion

Direct use of epithelial layers for regeneration of epithelial tissue is a feasible technique [85]. Such techniques known as cell sheet engineering, used for example to regenerate cornea [86] or retina [87] have been successfully applied in clinic. However when the need for functional epithelium covering large areas, such as in the case of tracheal replacement a carrier system is necessary. The gelatin based hydrogels have shown their ability to be used as a feeder layer for several cell type's culture. They provide a biocompatible and biodegradable temporary substratum with a weak inflammatory reaction [88]. Here we confirm that such structures can be a good support for epithelial cell grafts in the cases of bioengineered prosthetic implants. But the film structure cannot be held in place without crosslinking step. For this crosslinking step we to use microbial transglutaminase, which has already been shown to be versatile, robust crosslinker [89] and which is also non-cytotoxic. The crosslinking period shouldn't be shorter than 2 h to held gelatin films structure in place in cell culture medium. In the meantime, we showed that crosslinking more than 2 h didn't increase significantly the films stability, thus 2 h is a plateau of crosslinking which is optimal that is why we chose this condition for cell culture experiment. As the crosslinking causes the shrinkage of the gels, to have a control over the exact size of the patches a mold is necessary. We prevent the circumferential shrinkage by using metallic rings as a template. Indeed the gelatin adheres to metallic ring and this eliminates the shrinkage by crosslinking. Afterwards, the crosslinked and dried films could be removed easily and had better dispositions for cell seeding. Using the templates also permit to control the films diameter.

It has been shown that the connective tissue begins invading the micro porous titanium pores in the first days [78]. We have observed cellular presence in the cross-section of porous titanium implants after one week of implantation in Wistar rats [90]. Thus, a one week of gelatin film stability time will be enough for establishment of a fibrovascular tissue beneath the epithelial patches. This will permit epithelial cells to survive and after the establishment of proper vascularization the provision of nutrition will be taken over by the invading connective tissue's vascularization [91]. However, this needs to be proven with animal studies; which is planned to be carried out in New Zealand White rabbits with tracheal re-epithelialization after de-epithelialization in line with our previous work [79].

Gelatin is a naturally adhesive molecule and it has been used in different forms as a tissue adhesive or coating [92]. By applying a small amount of solution on titanium surface we were able to obtain a stable attachment of the films to the implants. The patch can be easily applied

on other surfaces. For better attachment several other options can be used such as the use of catechol moieties, biomimicking surface patterning of the gelatin film etc. [93,94]. These are our future study directions to improve the adhesive properties of the patch structure.

Gelatin films ability to stock and then release gradually drugs have already been demonstrated [95]. As one of the main aims of the project is to obtain fast epithelialization, we tested the capacity of the patches to deliver growth factors. For fibroblasts we used FBS and for epithelial

cells we used a commercially available epithelial growth factor supplement mix. In both cases we obtained better proliferation in the presence of the growth factors, demonstrating the ability of the films to be loaded and then to release necessary growth factors. In this way we will accelerate the endoluminal epithelialization of the titanium based tracheal implant and thus protect the surrounding tissues from bacterial and fungal colonization. By releasing mixes of growth factors instead of a single growth factor such as EGF, we also aim to promote the growth of the different cell types in adequate proportions necessary to regenerate a respiratory epithelium which can fully undertake the mucus secretion, ciliary movement and replenishment functions.

Previously, it has been shown that respiratory epithelial cells, co-cultured on a fibroblast including matrix, tend to form a columnar pseudostratified epithelium [96]. By having a sandwich based system, the growth factor loading and the physical characteristics of the films can be separately controlled to achieve the physical requirement of the related cell type. We successfully cultured 3T3 fibroblasts seeded between 2 gelatin films. This sandwich based system can be used for primary fibroblast and epithelial cells co-culture. Fibroblasts cultured between 2 gelatin films serve as an artificial extra cellular matrix for epithelial cell grafting. This will also improve the integrity and thus the ease of the handling of the structure. These patches can be modified both biochemically with the loading of different growth factors and biophysically with the exogenous addition of nanoparticles in the bulk material to modulate substrate stiffness [97]. This sandwich system based on ECM mimicking component is a versatile system since it can be very easily modified respectfully to the cells needs in term of substrate stiffness or biochemical needs with growth factors. So this procedure can be used not only for respiratory epithelium but also to mimic other stratified tissues in conjunction with porous microfluidic scaffolds [98]. A weak point of the current study is the use of adenocarcinoma cells instead of primary respiratory epithelial cells. This was done to ensure the determination of the optimal conditions of the sandwich system prior to the utilization of

fragile primary cells. Our current work also focuses on the use of stem cell sources for the patches to obviate the challenge of limited supply of autologous epithelial cells.

4.7 Conclusions

Here, we demonstrated the development of a double layer sandwich patch system with an epithelial layer and underlying fibroblast feeder layer. Such a system can be used in situ for epithelialization of implant surfaces such as tracheal implants without a need to wait for a long term in vitro epithelialization. Moreover, it can be used for epithelialization of engineered tissues which has an epithelial component or for studying mesenchymal/epithelial cell interactions in miniaturized tissues. Recent reviews have emphasized the importance of re- epithelialization particularly in the regenerative medicine in respiratory system [99,100]; thus modular epithelialization system can prove to be highly useful in regenerative medicine context. Current work focuses on the in vivo/in vitro determination of the necessary number of patches for a given area of implants and a given geometry and on testing of the system with primary human respiratory epithelial cells.

Supplementary data :

Airway epithelial cell growth supplement composition	Concentration after addition to the medium
Bovine Pituitary Extract	0,004 mL/mL
Epidermal Growth Factor (recombinant human)	10 ng/mL
Insulin (recombinant human)	5µg/mL
Hydrocortisone	0,5 µg/mL
Epinephrine	0,5 µg/mL
Triiodo-L-thyronine	6,7 ng/mL
Transferrin, holo (human)	10 µg/mL
Retinoic Acid	0,1 ng/mL

Figure S1. Airway epithelial cell growth supplement composition provided by PromoCell.

5 - A composite Gelatin/hyaluronic acid hydrogel as a basement membrane mimic for developing mesenchymal stem cell derived epithelial tissue patches

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Les travaux de cette deuxième partie avaient pour objectif l'optimisation de la fabrication des films d'hydrogels et leur utilisation comme plateforme multifonctionnelle pour l'obtention d'un épithélium respiratoire à partir de cellules souches mésenchymateuses.

Afin d'augmenter la stabilité des films d'hydrogels nous avons réalisé les expérimentations avec des films d'hydrogels interpénétrés de gélatine et d'acide hyaluronique soumis à une double réticulation enzymatique. Ces films ont été produits à l'aide d'un spincoater (système de dépôt à la tournette). Différents paramètres de ce spincoater ont été étudiés afin de vérifier leurs effets sur l'épaisseur des films. La capacité de relargage de facteurs de croissance préalablement chargés a également été étudiée pour ces films à base de gélatine et d'acide hyaluronique. Les expérimentations de culture cellulaire ont démontré que la nouvelle formulation de ces gels n'avait pas d'impact négatif sur l'adhésion et la migration cellulaire par rapport aux films de gélatine seule. Les expérimentations avec les cellules souches mésenchymateuses ont démontré qu'avec un milieu de culture et des facteurs de croissance adéquats, ces films pouvaient servir de support pour le développement d'un épithélium respiratoire à partir de cellules souches.

L'ensemble des résultats de cette deuxième partie sont détaillés ci-dessous sous la forme d'une publication qui est en cours de révision.

5.1 ABSTRACT

In this study we report fabrication of Gelatin based biocomposite films and their potential application in developing epithelial patches. The films could be loaded with an epithelial cell growth factor cocktail and were used as a basement membrane mimic for in vitro regeneration of organised respiratory epithelium using Calu-3 cell line as well as mesenchymal stem cells (MSCs). Our data show full differentiation of Calu-3 cells on composite films including ciliary differentiation and barrier formation. We also show the suitability of the composite films as a biomimetic scaffold and growth factor delivery platform for differentiation of human MSCs to epithelial cells. Stem cell differentiation to the epithelium lineage was confirmed by staining for epithelium and stem cell specific markers using fluorescent microscopy. Our data show that the MSCs acquire the epithelial characteristics after two weeks of culture with significant reduction in vimentin and an increase in pan cytokeratin expression as well as morphological changes. However, despite the expression of epithelial cell lineage markers these cells did not seem to form fully functional tight junctions as evidenced by low expression of junctional protein ZO1. Further optimisation of culture conditions and growth factor cocktails is required to enhance tight junction formation in MSCs derived epithelial cells on the composite hydrogels. Nevertheless, our data clearly highlight the possibility of using MSCs in epithelial tissue engineering and the applicability of the composite hydrogels as transferrable basement membrane mimics and delivery platforms with potential applications in regenerative medicine and in vitro modelling of barrier tissues.

Keywords
mesenchymal stem cell, Gelatin/HA, hydrogel, growth factors, controlled release, epithelium differentiation

5.2 Introduction

Respiratory tissue injuries, infections and degeneration lead to several serious pathologies such as Chronic Obstructive Pulmonary Disease, Asthma, and Chronic Bronchitis. The bronchial epithelium plays a critical role in maintaining the integrity and functionality of the respiratory system. The regeneration of airway epithelium is a complex phenomenon, and several parameters need to be considered to optimise the epithelium like organised tissue regeneration including epithelial migration, induction of differentiation of different cell types of functional epithelium, in-vivo epithelium migration, epithelium interaction with other tissues (immune cells), and effects of underlying tissues on epithelium functionality [101]. Thus far the in-vitro development of functional epithelium has not been achieved due to its complex nature, limited differentiation ability of epithelial basal cells and formation of a non-organized epithelium even when cells are cultured at air-liquid interface (ALI) [102]. Various studies recommend the potential use of stem cells (embryonic stem cells, induced pluripotency stem cells; and adult stem cells) for epithelium development using biochemical approaches, however these methods need several cumbersome steps and long culture time to differentiate and thus are unattainable for lab-bench to clinical translation or easy to use in vitro model systems [103].

Stem cells are key elements of tissue engineering and offer hope as a therapeutic avenue. However, major consideration when using stem cells for respiratory epithelium development is the identification of the correct stem cells and their capacity for organised epithelial differentiation [104]. Mesenchymal stromal cells (MSCs) are one of the most widely used cells and their presence also have been reported in lung tissue and have role is tissue regeneration in-vivo. However, MSCs have shown limited application in-vitro respiratory tissue regeneration. For example, the MSCs support the development of respiratory mucosa-like tissue in co-culture with normal human bronchial epithelial (NHBE) but did not acquire the epithelial characteristics [105].

The interaction of basement membrane ECM with epithelium stem cells/progenitor cells promotes the airway epithelium repair in-vivo by modulating epithelial cell migration and proliferation via differentiation of these progenitors cells to the epithelium subtypes [106]. The comparative study of lung and bone marrow (BM) derived MSCs suggest that the epithelial differentiation of BM-MSCs can be achieved in the presence of retinoic acid, however this effect was minimal compared to lung derived MSCs or epithelium basal cells [107]. Thus, mesenchymal to epithelial transition is still controversial; however, if successful,

this phenomenon can significantly accelerate the respiratory epithelium development using MSCs.

Biomaterial based scaffolds have been used for the development of various tissues [108,109,110,111]. However, there is still a need to develop application specific biomaterials with appropriate mechanical properties and capacity to support cell growth, migration and proliferation. Novel Extracellular matrix (ECM)-based delivery platforms carry and deliver the therapeutic agents (e.g. growth factor) in a controlled manner, and simultaneously protect them from fast degradation [112]. The mechanical properties of in-vitro microenvironment could also direct the stem cell differentiation to a specific cell lineage [113,114,115,116]. Moreover, the extracellular matrix (ECM) based scaffolds are potential tools for in vitro tissue development due to their ability to mimic the native microenvironment, e.g. collagen type I, collagen IV, laminin, and glycoproteins [117]. These ECM components induce epithelial migration via integrin signalling and are through to play a key role in directing epithelial repair. Thus the airway epithelial regeneration in the presence of thin biomaterial substrates is considered as one of the possible methods to induce airway epithelium formation for developing robust models which have the basement membrane component [118]. In this context, Gelatin based biomaterials have been used in supporting the growth of liver [119], bone [120], cardiac [121] and skin [122] tissues. The mechanical properties of Gelatin can be further manipulated using cross linking and/or encapsulating nanoparticles [123]. Controlled cross linking of Gelatin-based scaffolds also induces the porous structures, conductive to enhanced cell attachment and differentiation [119]. The advantage of patch based delivery is to ensure the positioning of the epithelial cells. We envision an endoscopic delivery with a releasable clamp that holds the patch in place; with fibroscopy the positioning of the patch can be ensured and once in the correct location the clasp is release to apply the patch to the target surface. As both gelatin and HA are adhesive molecules by their nature, the establishment of the interface would not be problematic. If need be, a layer of wet adhesive as we have described previously can be added [124]. Another advantage of the transfer with patches is that, the MSCs can continue their differentiation with the right polarity (as the substrate defines their positioning) in a microenvironment that is particularly suitable for respiratory epithelium differentiation.

In this study, we describe fabrication of Gelatin based biocomposite films loaded with an epithelial cell growth factor cocktail for developing epithelial patches using BM-MSCs. It was hypothesised that growth factor loaded composite films can act as a basement membrane mimic that is able to facilitate the BM-MSCs differentiation to multiple respiratory

epithelium. First, we demonstrated the feasibility of supporting an epithelial layer with a respiratory epithelial cell line (Calu 3 cells). Calu-3 is a highly studied respiratory epithelial cell line which is able to make the tight junctions in vitro and this factor allows it to be potentially used for modeling the airway epithelial barrier in respiratory system research [125]. Moreover, the system was used to assess whether it is capable to support the differentiation of the BM-MSCs towards respiratory epithelium lineage.

5.3 Materials and methods

All tissue culture plastics were purchased from Sarstedt and Nunc. Tissue culture inserts were purchased from Costar Corning. The FGF- 7 & FGF-10 were purchased from Peprotech UK. The bronchial epithelium media and stem cell media were purchased from Promocell. The bronchial differentiation media was prepared using epithelium growth medium from Lonza UK without adding triiodothyronine. All primary and secondary antibodies were purchased from Abcam UK and Thermo Fisher Scientific UK respectively. Gelatin type B (Mw =2-2.5×104 Da, pI = 4.7-5.2) from bovine skin, Fluorescein isothiocyanate labeled bovine Albumin (BSAFITC, Mw = 6.6×104 Da) were purchased from Sigma Aldrich (St. Quentin Fallavier, France). Microbial Transglutaminase (M-TG) (Mw =3.8×104 Da, pI = 9) was a kind gift of Ajinomoto (Japan). Hyaluronic acid (HA, Mw = 3 x105Da) and HA-Tyramine (HA-Tyr, Mw = 3.1 x105 Da), were produced and characterized by CONTIPRO (Czechia). All other chemicals, cell culture media and reagents were purchased from Sigma Aldrich unless otherwise stated.

5.3.1 Development of Gelatin based film

Spin coating of Gelatin and Gelatin-HA-tyr films

Gelatin and Gelatin-Hyaluronic Acid-Tyramine films (Gelatin; Gelatin-HA-Tyr), were made using the spin coating method (spin coater WS-650Mz-23NPP; Laurell). Powders of Gelatin (15% w/v) or Gelatin mixed with or HA-Tyr (1% w/v) were dissolved in 0.15 M NaCl/10 mM Tris solutions (pH = 7.4). The solution was heated to 50°C with constant stirring. Then 200µL of Gelatin solution was dropped on a glass slide (previously installed in the spin coater) and the spin coating program was started. To obtain the self-standing films 60µL of cellulose acetate (1 % w/v in acetone) was deposited on top of the glass slide prior to spin coating process and the sample were dried for one day at 4°C. The parameters for spin coating were 2500 rpm with an acceleration of 1250rpm for 2 minutes time. Afterwards, the films were kept dry at 4°C for at least 3 hours before cross linking. All solutions were

prepared using ultrapure water (Milli Q-plus system, Millipore) with a resistivity of 18.2 MΩ.cm and filtered using 0.22µm filter before use.

Crosslinking of Gelatin and Gelatin-HA-Tyr films

A solution of 10% (w/v) of transglutaminase (TGA) in PBS was prepared. The Gelatin and Gelatin-HA-Tyr (Gelatin-HA) films were incubated in 100µL of this solution for 30 minutes. The non-crosslinked ingredients were washed out using two rinsing steps with 100µL of PBS each time for 5 minutes. The Gelatin-HA-Tyramine film an additional crosslinking step was performed for the dimerization of tyramine to make dityramine using horseradish peroxidase (HRP) mediated reaction. For this, the solution of H2O2: HRP (10:1) was prepared with 0.24mg/mL of HRP in PBS and 0.1M H2O2. 100µL of this solution were than incubated on Gelatin/HA films for further 30 minutes. Again, the non-crosslinked ingredients were washed out using two rinsing steps by incubating with 100µL of PBS each time for 5 minutes.

Thickness determination

The thickness of Gelatin and Gelatin/HA films were estimated with confocal microscope (ZEIS LSM 710). To visualize the film and estimate the thickness, BSAFITC solution (green fluorescent probe) was used to label the films. By reconstructing the whole film thickness using multiple Z-stacks can be visualized, hence allowing the determination of the thickness of the film with a 20 x objective.

5.3.2 Film transfer on transwell and growth factors loading

For the biocomposite delivery system, the films were prepared according the protocol described above. A 6% w/v solution of Gelatin type A was prepared in MilliQ water. Gelatin solution was put in water bath at 50°C until complete dissolution. At the same time a 20% w/v TGA solution was prepared in PBS. Then 5 µL of TGA solution and 25 µL of Gelatin solution was added on top of transwell (Costar Transwell®, 0.4µm) just before the transfer of crosslinked film in order to attach the film on transwell insert. The transwell with attached film was then put in incubator at 37°C for at least 15minutes. The non-crosslinked Gelatin washed out using 100mL PBS washing (2X) under aseptic conditions. The growth factors; Recombinant Human FGF-10; FGF10 (10µL, [25µg/mL]) and Recombinant Human KGF; FGF7 (10µL, [10µg/mL]) solutions both prepared in MilliQ water and were incubated on top of each film overnight at 4°C (previously sterilized 15 minutes under UV).

5.3.3 Release profile of the film

Release experiment from Gelatin-HA-tyramine (Gelatin-HA) was performed using a fluorescently labeled protein (BSAFITC: Bovine serum albumin labelled with fluorescein isothiocyanate). BSAFITC solution was prepared in PBS solution at 1mg/mL. Then 100µL BSAFITC solution was incubated on top of Gelatin-HA film crosslinked with TGA and HRP overnight at 4°C. The release experiments were performed at 37 °C in a PBS solution (1mL). The supernatant was analyzed with a spectrofluorimeter (SAFAS Genius XC, Monaco). A new PBS solution (1 mL) was added after each analysis. For BSAFITC the wavelength parameters was λex/λem = 495 nm/520 nm.

5.3.4 A549 cells experiment & cellular migration (Time lapse microscopy)

A549 human lung carcinoma epithelial cells were used as model of respiratory epithelial cells. They were cultured in RPMI 1640 basal medium supplemented with 10% v/v Fetal bovine serum, 1% v/v Pen-Strep and 1% v/v Fungizone. After trypsinization, 50,000 cells prepared in 30µL of medium were deposited on top of the Gelatin-HA film previously crosslinked with TGA and HRP and then UV treated for 15 minutes. After seeding, samples were then left to incubate at 37 °C with 5% CO2 for 7 days. Culture medium was changed every 48h. Metabolic activity was determined with a resazurin-based test (Sigma Aldrich) and checked at day 1, 3 and 7 to evaluate the proliferation. DAPI/Phalloidin (F-actin) staining were performed after 7 days of culture. After fixation with paraformaldehyde (3.7% v/v in PBS for 15 minutes), cells were incubated with Triton X-solution (0.1% in PBS for 5 minutes) and BSA solution (1% v/v in PBS for 20 minutes). Then samples were incubated for 1 hour with phalloidin (Alexa Fluor 568 phalloidin [6.6µm], Molecular Probes Life Technologies) at a dilution of 1/40 in BSA solution (1% v/v in PBS). After that, two rinsing steps in PBS were performed and the samples were incubated 5 minutes in Hoechst 33342 solution (20µg/mL). Finally, samples were visualized with confocal microscope.

For cellular migration analysis, A549 cells were stained with Hoechst 33358 solution (20µg/mL) for 30 minutes in a T75 cm2 for 30 minutes. Then they were trypsinized and seeded on the different Gelatin based films (15000 cells/films) for 15 minutes and mounted in a Ludin Chamber (Life Imaging Services, Basel, Switzerland) at 37°C, 5% CO2. Then time-lapse experiment was performed on a Nikon Ti-E microscope equipped with a 10x objective and with an Andor Zyla sCMOS camera and driven by the Nikon NIS-Elements Ar software. Images were acquired every 10 min for 24 h simultaneously by phase contrast and by

fluorescence microscopy with nucleus staining by Hoechst 33342. Cell tracking by the software "NIS-Elements Ar 3D tracking" (Nikon) was carried out in different fields of the substrates. First, the software detected objects "nuclei" by thresholding and they were afterwards tracked over the 24 hours. Phase contrast images were used to check the viability of the followed cells. Cells that died during the experiment were eliminated.

5.3.5 MSCs cell culture & epithelium differentiation on Gelatin based film

To get enough number of cells the MSCs (Promocell, Germany) were expanded in MSCs medium (Promocell Germany). The cells were routinely cultured at 37 °C and 5% CO2 in stem cell media (consist of basal media and medium supplement) as per the manufacturer's protocol. The medium was changed routinely in every 2-3 days up to the confluence. For all experiments the lower passage MSCs (p4-p5) were used.

The MSCs were seeded on Gelatin based films (100,000 cells per inserts) on a transwell insert in epithelium media (Promocell). The cells were left for 2-3 weeks in submerged culture followed by two weeks culture at air-liquid interface (ALI) in epithelium differentiation media (Lonza). For this, the medium from upper chamber was removed and 500µl differentiation medium was used in lower chamber. The experiment was also repeated using DMEM-F12 media to assess the impact of the Gelatin films in the absence of growth factor in the differentiation media. Moreover, to assess the impact of Rho-associated protein kinase (ROCK) inhibition on MSCs's differentiation towards respiratory epithelium; 1µL/ml of Rho kinase inhibitor Y-27632 was added throughout the culture in a separate experiment. In each case, the medium was changed every 2-3 days.

5.3.6 Calu-3 cell seeding and differentiation on gelatin based film

Calu-3 cells were used as a model epithelial cell to assess the ability of ciliary differentiation. Briefly, Calu-3 cells were seeded on Gel-HA film (within transwell) at 200,000 cells/CM2 in DMEM-F12 medium with 10% FBS, 1% P/S, 100 mM non-essential amino acid and 100mM of L glutamine supplement. The cells were transferred to ALI after growing in submerged conditions for a week.

5.3.7 Evaluation of MSCs and Calu 3 differentiation on film

Immunofluorescence for epithelium differentiation

After submerged culture of MSCs for 2-3 weeks the expression of epithelium and MSCs markers was assessed using various epithelium markers; wide spectrum cytokeratin, pan cytokeratin and cytokeratin 18 (1:100 dilution) and mesenchymal marker; vimentin (1:200 dilution) antibodies (all antibodies from abcam). At the end of each culture, the cells were fixed with 3% formaldehyde in PBS (pH 7.4) for 30 minutes and were permeabilised using the 0.25% Triton X 100 solution for 20 minutes. After each step, the samples were washed three times in PBS (5 minutes). The samples were further incubated with 3% BSA for 1 hour to stop the non-specific binding of proteins. Cell layers were incubated with primary antibodies diluted in PBS for 90 minutes at room temperature. After primary incubation, the samples were incubated with secondary antibody for 45minutes at room temperature. The secondary antibodies used were Alexa Fluor® 488, chicken anti rabbit 1:250 dilution in PBS or rhodamine red anti mouse 1:250 dilution in PBS). Each antibody incubation was followed by three washes in PBS for 5 minutes. For nuclear staining, DAPI (4' 6-diamidino-2-phenylindole) was used at 1: 4000 dilution (Invitrogen, UK) for 15 minutes and followed by 3 PBS washing for 5 minutes. Finally, the samples were mounted on glass slides with VectaShield (Vector Laboratories, UK) for direct observation. The images were taken using Leica DMRB fluorescence microscope using 10X or 40X objective. The intensity of the fluorescence images were evaluated using the image J software.

Trans-Epithelial Electrical Resistance (TEER) Measurements

The trans-epithelial electrical resistance (TEER) measurements across the cell monolayer cultured at the ALI were performed according to the method described elsewhere [126]. Briefly; the measurements were performed using an EVOM volt-ohm-meter and STX2 chopstick electrodes (World Precision Instruments, U.K.). The cell culture media was added to the upper chamber (500 µL) and lower chamber (1.5 mL total volume) and allowed to equilibrate for 30 minutes prior to measurements (37 °C, 5% CO2). The chopstick electrodes were sterilized using 70% v/v ethanol in distilled water. Control measurements were performed using Calu 3 cell lines cultured in a similar way.

ZO-1 and Mucin staining

At the end of ALI culture, the samples were fixed with 3% formaldehyde in PBS (pH 7.4) for 30 minutes and were permeabilized using the 0.25% Triton X 100 solution for 20 minutes. The samples were further incubated with 3% BSA for 30 minutes to stop the non-specific binding of proteins. Cell layers were incubated with primary antibodies diluted in PBS for 90 minutes at room temperature. After each step, the samples were washed three times in PBS (5 minutes). The primary antibody used was rabbit anti ZO1 and Mucin5A/C from Abcam (1:100 dilution in PBS). After primary incubation, the samples were incubated with secondary antibody for 45 minutes at room temperature. The secondary antibodies used were Alexa Fluor® 488, chicken anti rabbit 1:250 dilution or anti mouse Rhodamine red. For nuclear staining, DAPI (4' 6-diamidino-2-phenylindole) was used at 1: 4000 dilution (Invitrogen, UK). Finally, the samples were transferred on glass slide and the cover slips were mounted on samples with VectaShield (Vector Laboratories, UK) for direct observation. The images were taken using a Leica DMRB fluorescence microscope using 40X objective.

5.3.8 Statistical analysis

The statistical analysis was performed using GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla California USA (www.graphpad.com). All results are shown as mean ± standard deviation (SD) from three independent experiments. Statistical differences were determined using the student t-test or one-way ANOVA method with Tukey post-hoc testing. A p - value <0.05 was considered statistically significant.

5.4 Results and discussion

Composite film fabrication and their protein release profile

The epithelium stem cells are scarce and have limited regenerative capacity for epithelium tissue regeneration thus there is an imperative need for alternative cell sources which can potentially be used for airway epithelium development [102]. The current literature suggests the significant contribution of various stem cells in epithelium regeneration e.g. MSCs, ESCs and iPSCs. The MSCs have been widely used for the development of bone, cartilage and adipose tissue, originated from the mesodermal layer of embryo. Recent literature also provides some evidence for MSCs differentiation towards non-mesodermal cell and tissue development e.g. neuronal, liver, epithelium and pancreatic tissue. Some recent studies also suggested the differentiation capability of BM-MSCs in respiratory epithelium in vitro [127] and in vivo [128]. They acquire phenotypic and functional epithelial characteristics when co-

cultured with airway epithelial cells [129,130]. Providing appropriate growth factors and mechanical and biochemical cues that simulate extracellular matrix in the basement membrane could potentially support MSCs differentiation towards epithelial cells, removing the need for complex co-cultures. Thus, we proposed to use a Gelatin based bio-composite film capable of controlled release of epithelium inducing growth factors (FGF-7 + FGF10) as a basement membrane mimic for developing a respiratory epithelium patch using BM-MSCs.

Gelatin, as a natural biomaterial, has been used in various tissue regeneration applications in two dimensional and three dimensional cross-linked form [131]. The physiochemical properties of the Gelatin can be controlled easily for development of substrates for cells, e.g. the controlled cross linking of Gelatin based scaffold induces porous structure, conductive to enhanced cell attachment and differentiation [119]. The hyaluronic acid (HA) is an important component of extra cellular matrix in various tissues in human body, such as connective, epithelial, and neural tissues. Due to its high water retention capacity, the presence of HA provides the advantage of increased volume for the substrate which substantially increase its ability to be loaded with growth factors. Moreover, as a component of ECM, HA has intrinsic interactions with most of the growth factors together with its polyanionic nature under physiological conditions which provides additional electrostatic retention for particularly positively charged growth factors. Thus, Gelatin (15%) and Gelatin-HA (14%+1%) films were developed using the spin coating methods for the in vitro development of respiratory epithelium.

In the first part of this study, the fabrication method of Gelatin film has been optimized to determine the main parameters that will influence the thickness of the film. To do that, different Gelatin concentrations and rotation speeds have been tested to spin coat and the thickness of the resulting film after crosslinking with transglutaminase has been estimated with confocal microscope after the loading of BSA-FITC to visualize the cross section (Figure 1A). Our data clearly show that the main parameter that influences the thickness of the film is Gelatin concentration. The difference in gel thickness between three different rotation speeds for the same concentration was not significant. The only trend observed was the increase in the thickness with Gelatin concentration. To have better stability and thicker film, we worked with 15% w/v Gelatin concentration and used a rotation speed of 2500 rpm for the rest of the study. To better simulate the composition of the basement membrane in vivo, we incorporate hyaluronic acid in the film formulation. In a previous work from our lab (Knopf-Marques et al., 2017), we have demonstrated that the stability of Gelatin/HA membrane films can be improved using HA derivative such as HA-tyramine by creating an

interpenetrated network through a double crosslinking step. Gelatin is crosslinked with transglutaminase to create amide bond between amine groups on lysine residues and carboxamide groups on glutamine residues and HA-tyramine is crosslinked through the formation of dityramine groups in the presence of HRP (Horseradish peroxidase). HA-tyramine was incorporated in the film formulation with the following ratio (Gelatin 14%/ HA-tyramine 1% w/v) and referred to as Gelatin-HA. The addition of hyaluronic acid in the structure resulted in an increase in the film thickness, from about 10µm for Gelatin to 15.5µm for Gelatin/HA (Figure 1B). This difference in thickness can be attributed to the intrinsic capacity of hyaluronic acid to absorb large amount of water.

As these Gelatin based membranes are supposed to release growth factors for the differentiation of MSC to epithelial cells, the release property of these materials was tested using a fluorescently labelled model protein BSAFITC. The release property of Gelatin membrane was tested in a previous work from our group [132] and we reproduced this experiment with Gelatin/HA film and the cumulative release of BSAFITC was followed at 37°C in PBS solution by quantifying the fluorescence in the supernatant using a spectrofluorimeter (Figure 1C). Both materials have shown the ability for the loading and the release of bioactive molecules for at least two weeks after an initial burst release.

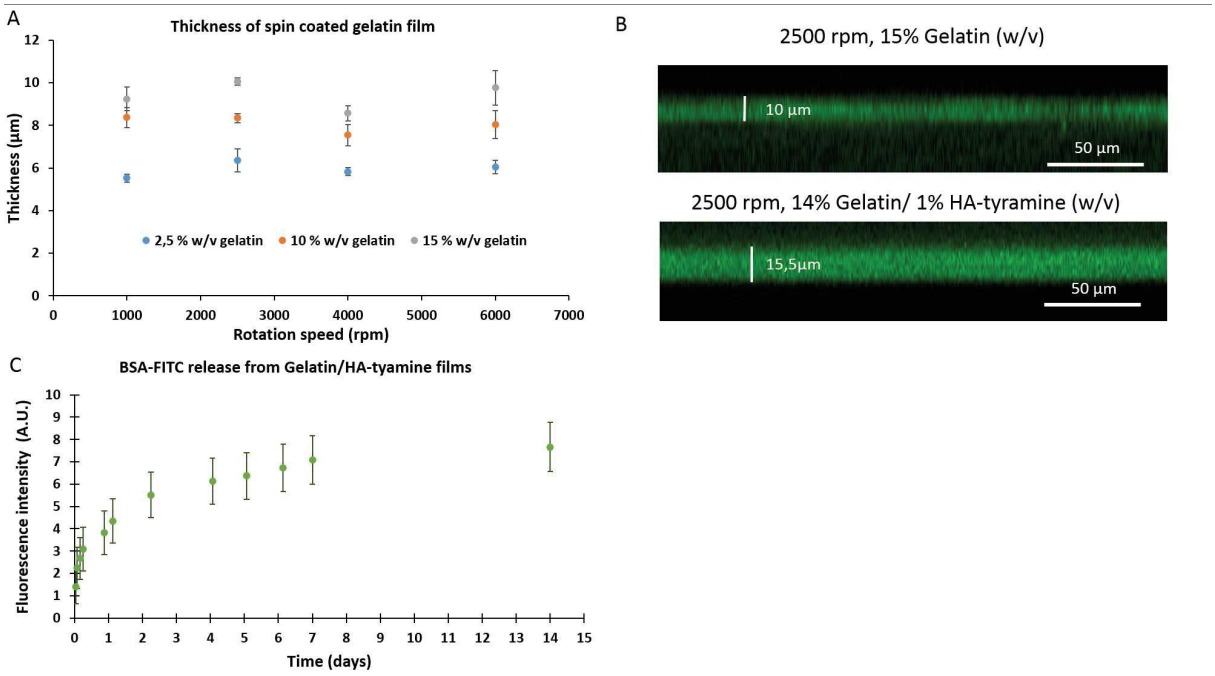


Figure 1. A) Thickness of Gelatin film made by spin coating process using different Gelatin concentrations and different rotation speeds. The thickness was characterized with confocal microscope after incubation of BSAFITC in the film to observe the xy section. B) Confocal pictures (xy section) of Gelatin film loaded with BSAFITC to estimate film thickness (20x magnification). C) Release experiment of BSAFITC performed at 37°C from Gelatin-HA film with spectrofluorimeter.

The composite bio-film supports migration, growth and differentiation of alveolar epithelial cells

The next step was to compare the behaviour of both film formulations (Gelatin vs Gelatin-HA) on A549 epithelial cells in terms of migration distance and migration speed to see if the addition of HA in the formulation had an effect. A549 cells are selected due to their aggressively migratory nature with the hypothesis that with a more migratory cell type the effect of the substrate will be accentuated and easier to quantify. This experiment was carried out using Time Lapse microscopy for 24 hours. It was shown that the addition of HA in the formulation did not have an effect on both cell migration distance and cell migration speed. Both materials exhibited the same response toward A549 epithelial cells (Figure 2A and B). Moreover EGF (Epidermal Growth Factor) was also loaded in Gelatin-HA film and the same experiment of migration was repeated. The presence of this growth factor also did not affect cell migration and cell migration distance when we compare to both Gelatin and Gelatin-HA film. The proliferation of A549 epithelial cells on Gelatin material has been studied in a

previous work from our group [133] and we have repeated the same experiment with Gelatin-HA film (Figure 2C). The metabolic activity was followed for 7 days and a significant increase was seen between day 1, day 3 and day 7 meaning that cells were proliferating on the film. DAPI/F-actin staining was performed at day 7 to check the morphology and the confluence of the cells on top of the film (Figure 2D). After 7 days of culture a confluent layer of epithelial cells was observed in top the film. These experiments have shown that Gelatin-HA film did not have any negative effects on epithelial cell attachment, migration and proliferation compared to pure Gelatin film while having advantages in having a HA component, higher thickness for increased volume for growth factor loading and improved stability. Moreover, as the films are detachable, it enables to perform ALI cultures in the presence of a basement membrane mimicking structure.

Based on the application of Gelatin and hyaluronic acid in respiratory epithelium development, the growth factor loaded films were developed for the controlled BM-MSCs differentiation towards respiratory epithelium lineage.

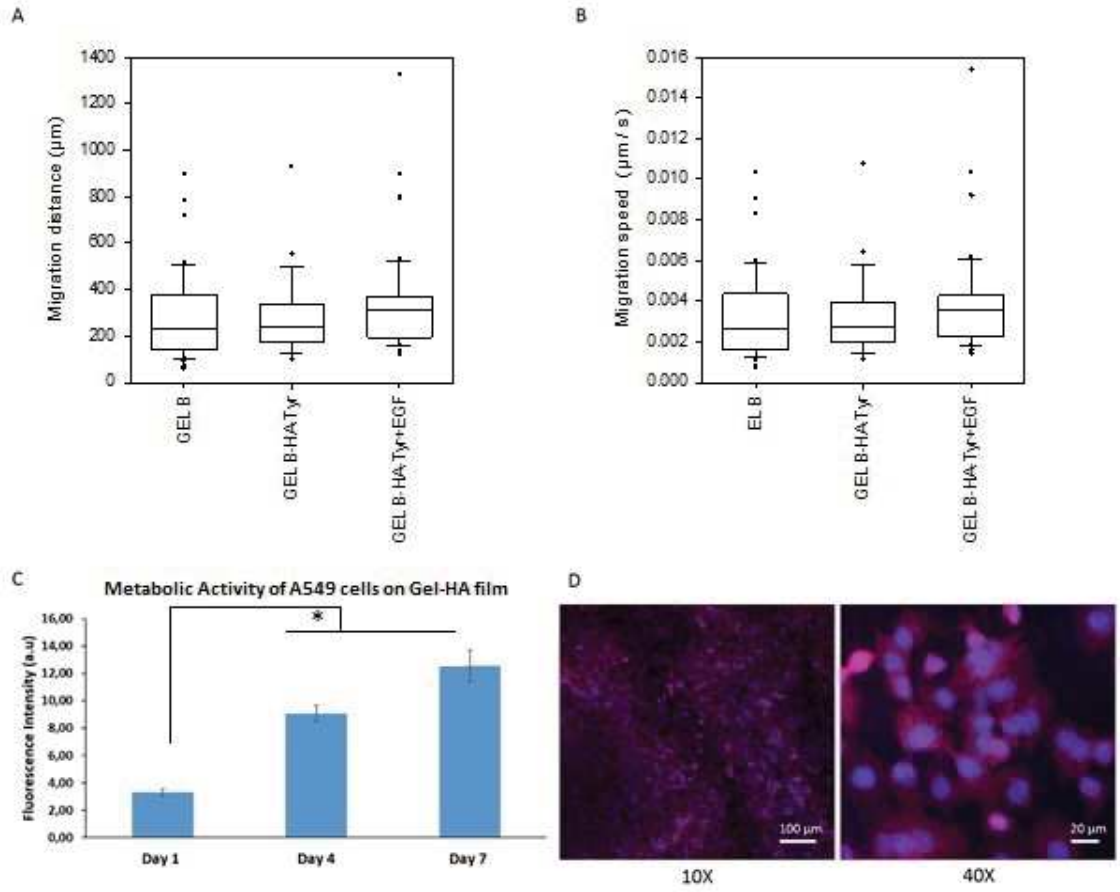


Figure 2. A and B) Comparison of Gelatin and Gelatin-HA on the migration of A549 cells. The effect of the loading of EGF in the film was also tested. These experiments were carried out with Time lapse microscopy and the migration was followed for 24 hours. C) Metabolic activity of A549 cells cultivated on Gelatin-HA film for 7 days. D) DAPI/F-Actin staining of A549 cells cultivated on Gelatin-HA after 7 days of culture. The images are representative of 3 independent experiments (n=3)

MSCs growth and differentiation to epithelial like cells on gelatin based films

The MSCs were seeded on Gelatin and Gelatin-HA films and cultured in submerged cultures for up to three weeks to adapt to epithelium environment using epithelium media. It is evident that the MSCs seeded on various films adhered well on films and were viable even up to 3-4 weeks of culture. The epithelium cells are cobblestone shaped cell smaller in size than the mesenchymal cells. The morphology of BM-MSCs seeded on transwell or Gelatin based films changed into round shape morphology, similar to epithelium. However, the stem cells in standard medium still demonstrate the spindle morphology (Figure 3a). The cells on Gelatin based film without the growth factors also demonstrate the smaller size due to the emulation

of softer substrate similar to native epithelium ECM and thus the physicochemical and mechanical properties of culture microenvironment could control the MSCs cell behaviour [134]. The mechanical and degradation properties of the described films have been previously reported [135].

The MSCs grown under submerged culture in epithelium medium on Gelatin films also expressed lower vimentin and there was a further reduction in vimentin expression on stem cells grown in growth factors loaded films (Figure 3b). The expression pattern of pan cytokeratin and wide spectrum cytokeratin is opposite to the vimentin (Figure 3c, 4d).

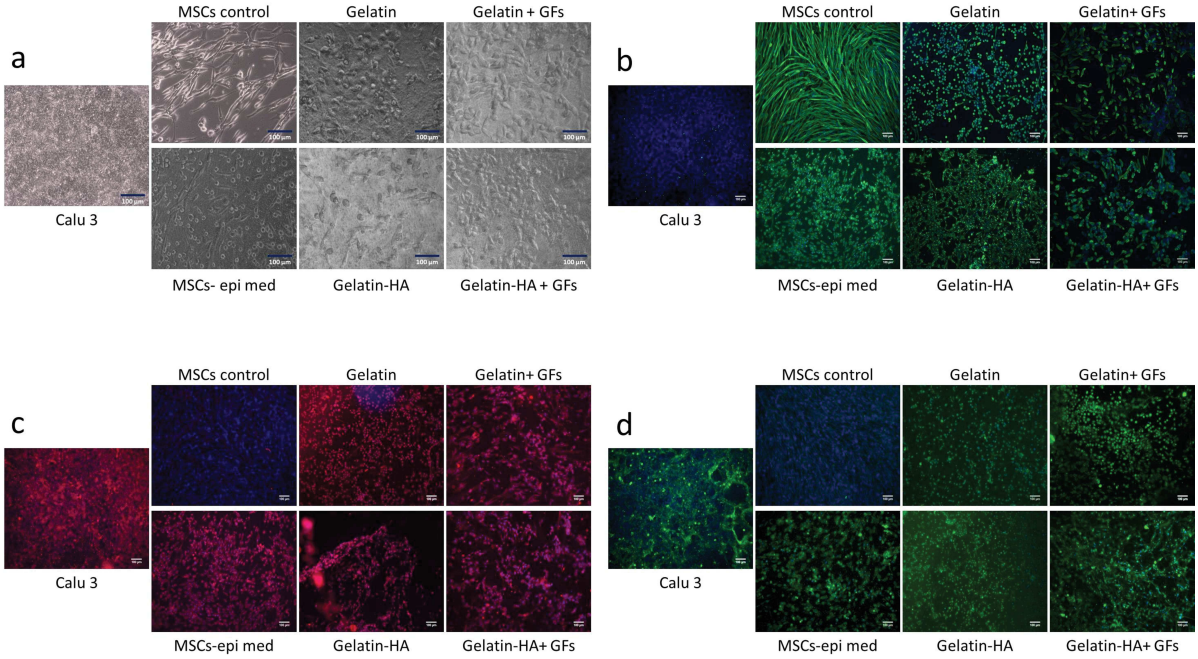


Figure 3. The MSCs cultured on Gelatin and Gelatin-HA film. The effect of growth factors released from Gelatin based film on morphology (a) clearly suggest the change in spindle shaped MSCs morphology to epithelial morphology in the presence of growth factors encapsulated film. The effect of growth factors released from Gelatin based film on MSCs marker; vimentin (b), and epithelial markers; pan cytokeratin (c) and wide spectrum cytokeratin (d) expression in the presence of epithelium medium. The corresponding fluorescent intensity (Figure S1) suggest the reduction in vimentin and increase in pan cytokeratin as well as wide spectrum epithelium (n=3).

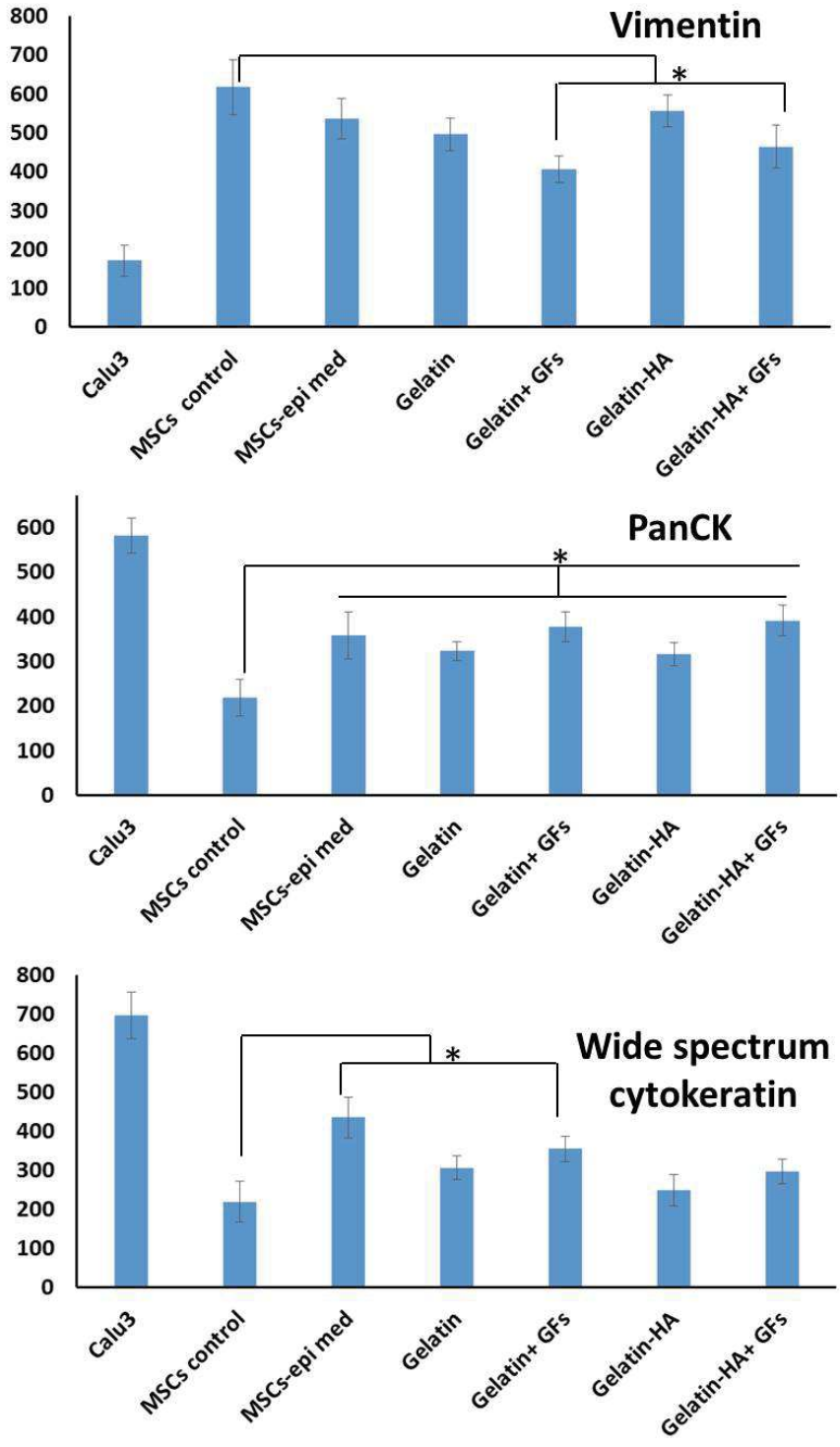
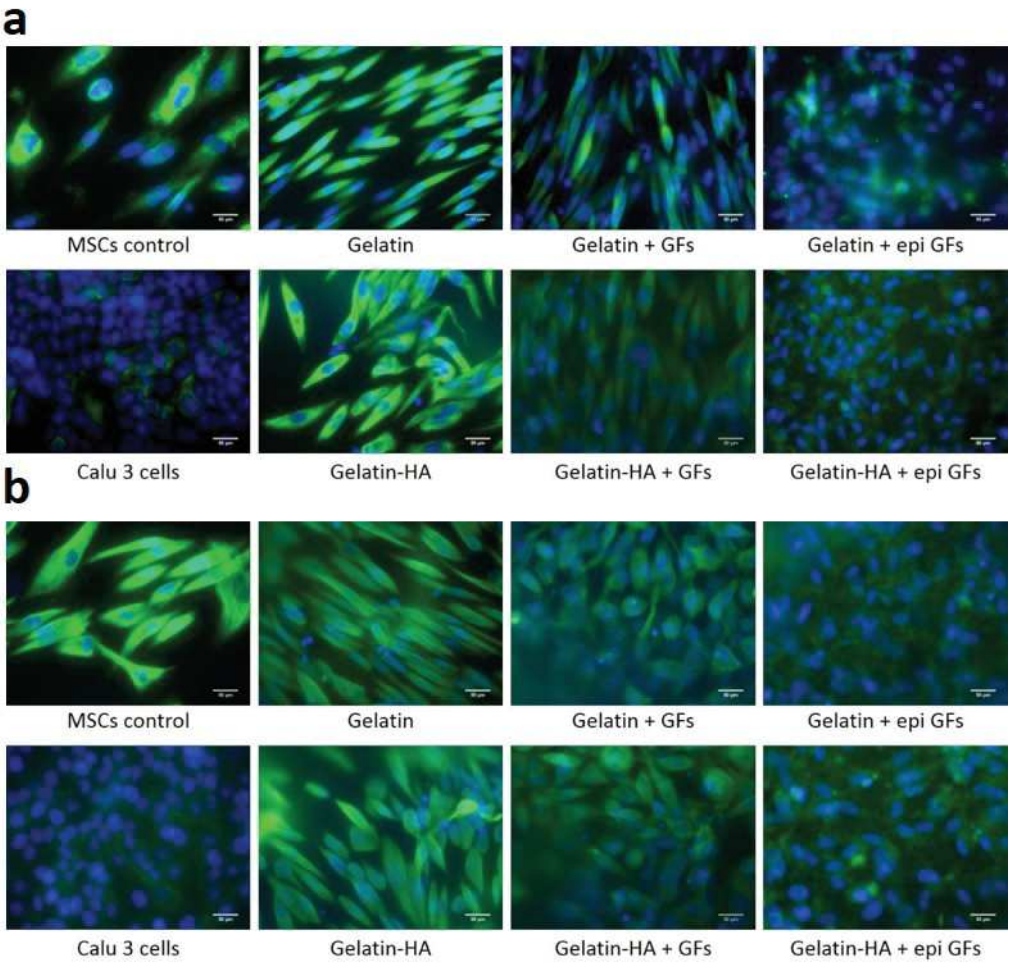


Figure S1: The fluorescence intensity of stem cells and epithelium markers suggest the reduction in mesenchymal characteristics (Vimentin) and up regulation in pan cytokeratin and wide spectrum cytokeratin epithelium markers (n=3). MSCs control = mesenchymal cells cultured using MSCs medium, MSCs-epi med= the mesenchymal cells grown on epithelium medium without using any film, Gelatin= MSCs grown on epithelium medium on gelatin film, Gelatin-HA= MSCs grown on epithelium medium on gelatin-hyaluronic acid film and Gelatin-HA+ GFs= MSCs grown on epithelium medium on gelatin-hyaluronic acid film loaded with growth factors.

There is an upregulation of the epithelial markers expression in stem cells grown on growth factors loaded Gelatin based film, which suggested the differentiation of MSCs towards epithelium like cells in response to growth factor release over time. These experiments were also performed using the DMEM-F12 media to assess the role of GFs loaded Gelatin films in the absence of defined epithelium media. Data from these experiments indicated that even in the absence of the epithelium-defined media the composite films support epithelium differentiation of MSCs as evidenced by upregulation of pan cytokeratin and downregulation of vimentin in cultured MSCs over the time. However, the morphological changes (spindle-round shape) were not as prominent as seen in the presence of epithelium medium (Figure S2) and the presence of tight junction protein ZO-1 was not observed (Figure S3).



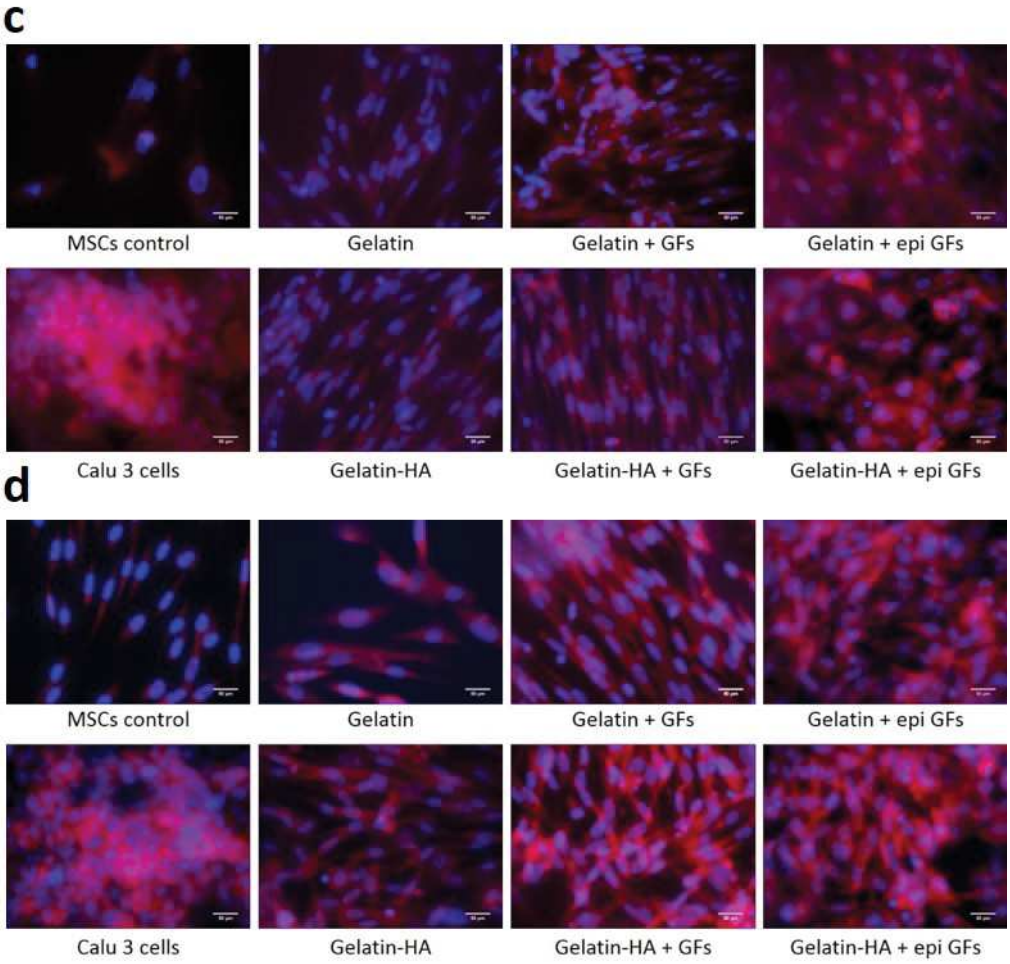
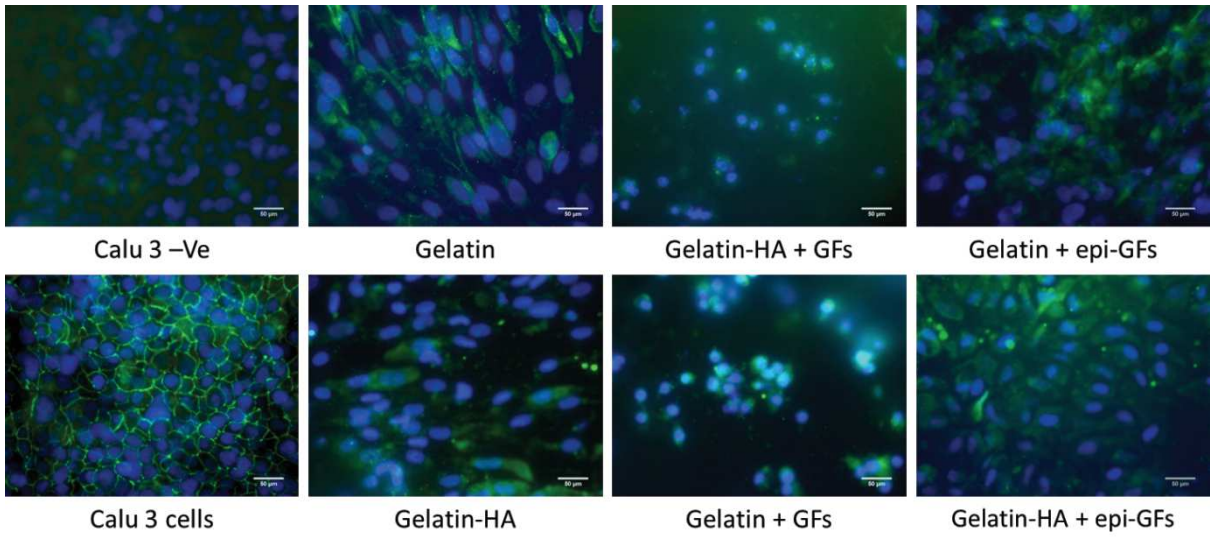


Figure S2: The biocomposite film encapsulated with GFs also favour the MSCs- epithelium differentiation in the presence of DMEM-F12 medium. The expression of MSCs marker vimentin is downregulated with the time (a & b; 2 & 3 weeks respectively) and increase in pan cytokeratin markers was reported (c & d; 2 & 3 weeks respectively) (n=3). MSCs control= mesenchymal cells cultured using MSCs medium (control), Gelatin= MSCs grown DMEM-F12 medium on gelatin film, Gelatin+ GFs= MSCs grown on DMEM-F12 medium on gelatin film loaded with growth factors, Gelatin+ epi GFs= MSCs grown on epithelium medium on gelatin film loaded with growth factors, Gelatin-HA= MSCs grown DMEM-F12 medium on gelatin-hyaluronic acid film, Gelatin-HA+ GFs= MSCs grown on DMEM-F12 medium on gelatin hyaluronic acid film loaded with growth factors, Gelatin-HA+ epi GFs= MSCs grown on epithelium medium on gelatin hyaluronic acid film loaded with growth factors.



Supplementary Figure S3: There was no formation of tight junctions after 2 weeks ALI culture as no change in TEER measurement and no expression of ZO-1 observed in MSCs derived epithelial like cells in the presence of DMEM-F12 medium(n=3). MSCs control= mesenchymal cells cultured using MSCs medium (control), Gelatin= MSCs grown DMEM-F12 medium on gelatin film, Gelatin+ GFs= MSCs grown on DMEM-F12 medium on gelatin film loaded with growth factors, Gelatin+ epi GFs= MSCs grown on epithelium medium on gelatin film loaded with growth factors, Gelatin-HA= MSCs grown DMEM-F12 medium on gelatin-hyaluronic acid film, Gelatin-HA+ GFs= MSCs grown on DMEM-F12 medium on gelatin hyaluronic acid film loaded with growth factors, Gelatin-HA+ epi GFs= MSCs grown on epithelium medium on gelatin hyaluronic acid film loaded with growth factors.

More importantly, the expression of pan cytokeratin increases with time in culture. However, in all experiments the MSCs still express the vimentin markers and thus complete epithelial differentiation of MSCs was not achieved using the controlled growth factors delivery approach from the Gelatin based film. Further, two week of ALI culture of these MSCs derived epithelium do not help in the formation of tight junctions and no expression of ZO1 was observed in all samples other than control Calu 3 control cells (Figure 4a). The TEER measurement of MSCs was significantly lower compared to the Calu 3 cells. However, higher TEER was detected in the MSCs derived epithelium cultured on films compared to the MSCs on tissue culture insert alone or films without cells (Figure 4b). These data suggest that despite slight increase in TEER reading, that could indicate the beginning of barrier formation, after 2 weeks of ALI culture the MSCs differentiated to the epithelium still show

very low expression of tight junction protein ZO-1. These results also verify the previous study, where it is reported that human bone marrow-derived MSCs are able to differentiate into epithelial-like cells in vitro in the presence of growth factor enriched medium [127]. However, the authors did not report the formation of intercellular tight junctions.

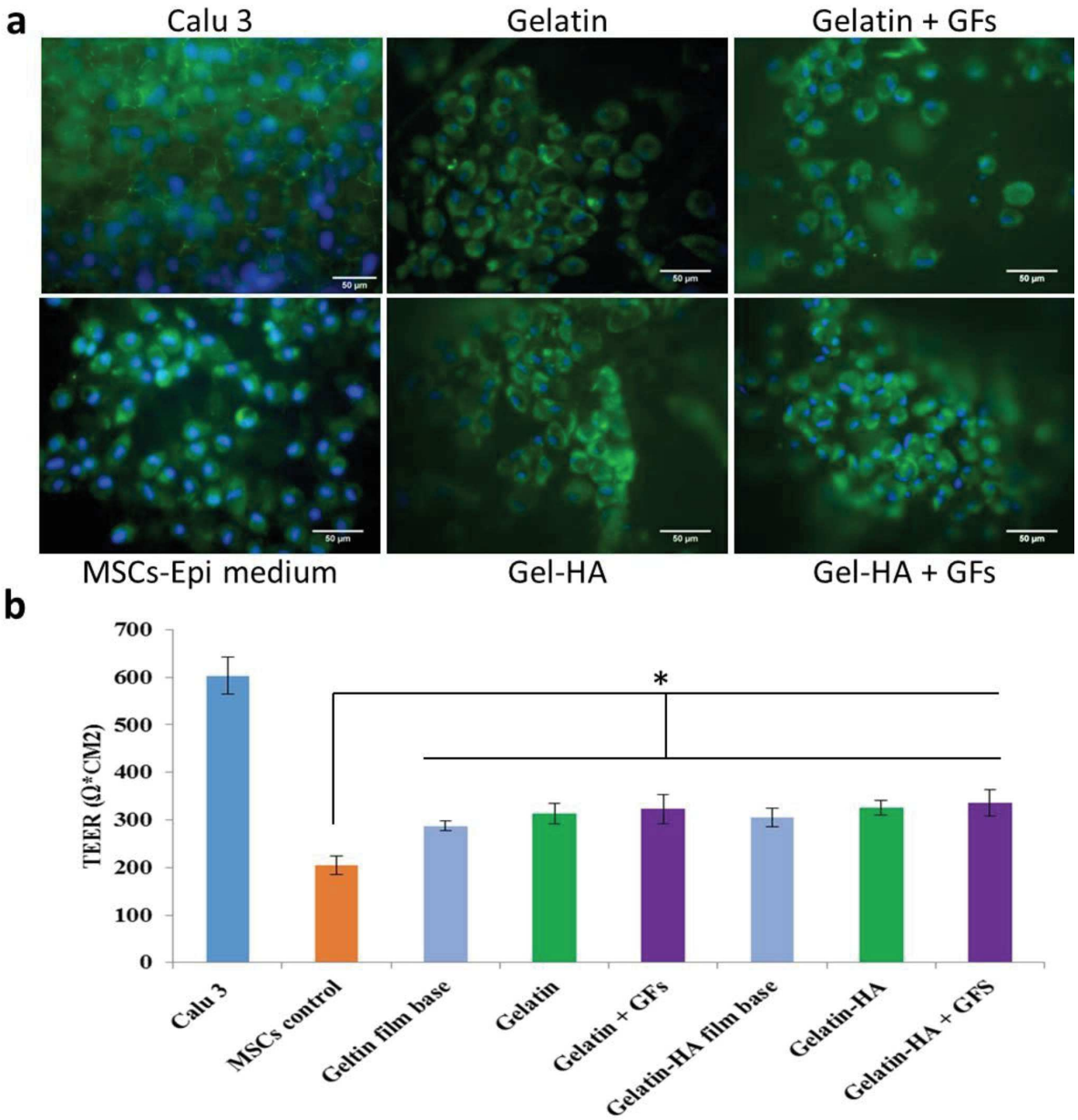


Figure 4. No expression of ZO1 was observed after two weeks of ALI culture of MSCs, however the Calu 3 cell line strongly demonstrated the tight junction formation as expressed by ZO1 expression (5a) and significantly higher TEER measurement (5b) (n=3). The bar represent average of 3 experiments ±SD

Impact of Rho-associated protein kinase (ROCK) inhibition on MSCs full differentiation to epithelial cells.

In order to enhance the differentiation of MSCs to respiratory epithelium we investigated the impact of ROCK inhibition. ROCK inhibition using Rho kinase inhibitors like Y-27632 has been shown to promote the induction efficiency, self-renewal and differentiation of MSCs towards the keratinocytes like cell with good expression of cytokeratin 14 and cytokeratin 5 in the presence keratinocyte-conditioned medium [136]. Thus, it was envisaged that this approach can also further push the epithelial differentiation of MSCs in the presence of biocomposite films. Accordingly, we performed the above experiments in the presence of Y-27632, a well-established Rho kinase inhibitor. Data from these experiments indicated better epithelium differentiation with adequate epithelial morphology and better expression of cytokeratin markers; cytokeratin 18 and pan cytokeratin. However, this effect was not statistically significant (Figure 5a and S4). Despite changes in linage marker expression in the presence of the ROCK inhibitor, suggesting more efficient differentiation to epithelial cells, the MSCs derived epithelial like cells still did not show barrier function as evidenced by low or no Mucin5A/C or ZO1 expression (Figure 5b).

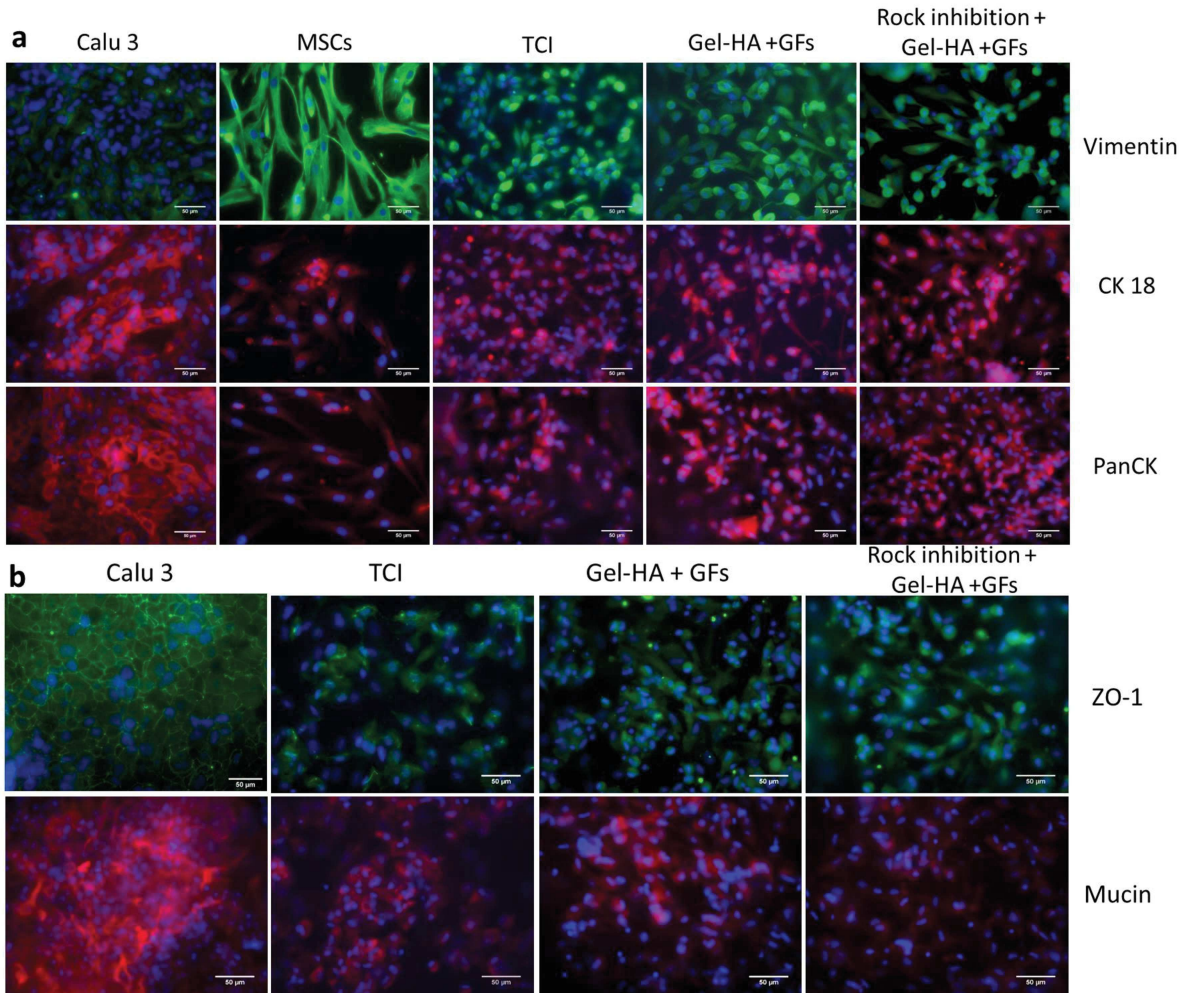


Figure 5. ROCK inhibition of MSCs further induces the cytokeratin and pan cytokeratin expression but could not induce the tight junctional barrier formation, as evidenced by absence of ZO1 and Mucin5A/C after 2 weeks of ALI (n=3).

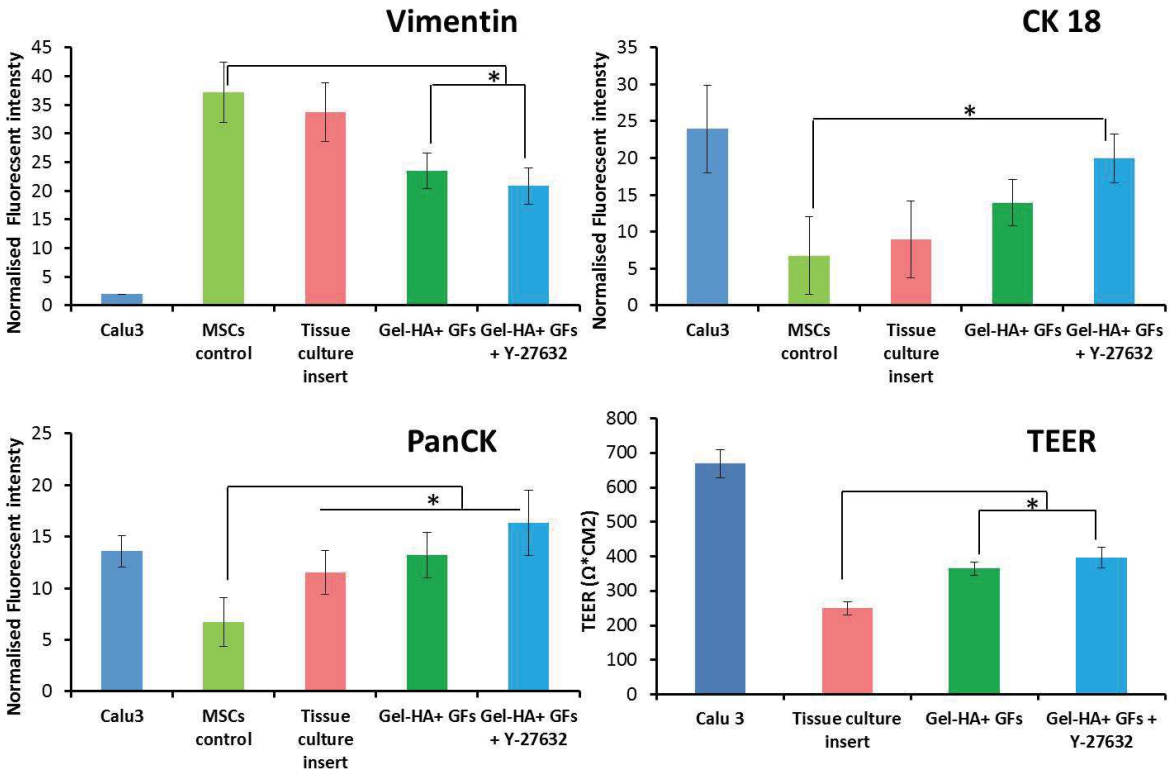


Figure S4: The fluorescence intensity of BM-MSC for mesenchymal marker (vimentin) and epithelium markers Pan cytokeratin and cytokeratin 18) in the presence of Rho kinase inhibitor Y-27632 on GFs loaded gelatin-HA film. The TEER measurement further confirmed the formation of intercellular tight junctions. However the effect were not significant more than GFs loaded film (n=3). MSCs control = the mesenchymal cells cultured using MSCs medium, Gelatin-HA+ GFs= MSCs grown on DMEM-F12 medium on gelatin hyaluronic acid film loaded with growth factors, Gelatin-HA+ GFs= MSCs grown on epithelium medium on gelatin hyaluronic acid film loaded with growth factors in the presence of Rho kinase inhibitor Y 27632.

MSCs as a potential cell sourced for developing personalised epithelia patches

Given their more accessible nature and availability in larger numbers (e.g. compared to epithelial cells from nasal turbinate), MSCs could be potentially used for development of personalised epithelial models. The provision of an ECM like basement membrane layer with appropriate mechanical properties further provides a means to mimic the effects of basement membrane on the behaviour of healthy and diseased epithelial cells. However, for a more robust model further development that would induce better barrier function and cell-cell contact is required. In order to see if the developed composite basement membrane can be used for more general respiratory epithelium models, we also cultured a well-established epithelial cell line, Calu 3 (Figure 6) on these films. Staining for Mucin and ZO-1 demonstrated mucin secretion and tight junction formation by the cells on the surface of basement membrane mimics. This further highlights the potential application of the biocomposite films for developing respiratory epithelium models.

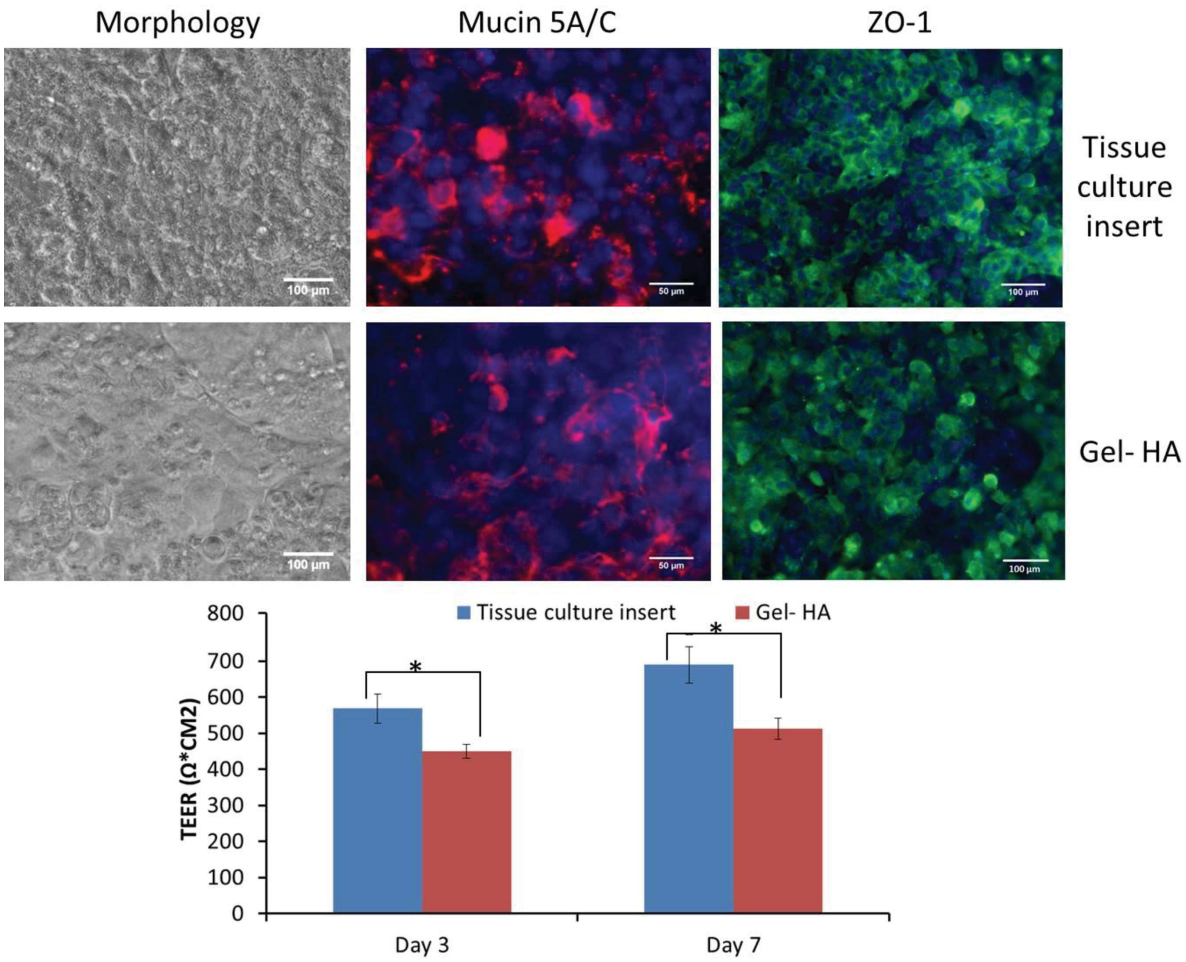


Figure 6: Application of Gel-HA films for the Calu-3 cell differentiation confirmed the supportive role of Gelatin based films for the successful differentiation (n=3) with high expression of mucin, ZO1 and increase in TEER value with time. The error bars represent average value of 3 independent experiment± SD

5.5 Conclusion

Using MSC derived epithelial cells would provide a larger cell pool (compared to primary respiratory epithelium) and patient specificity (compared to respiratory epithelium cell lines) that could enable development of more personalised tissue models with clear benefits for disease modelling or testing new drug leads. Such endeavour also requires basement membrane mimicking substrates that are adaptable to air liquid interface culture conditions to provide more physiological relevance. Herein, we describe composite film that mimics the basement membrane with high stay stability for long term culture periods and the capacity to release growth factors under ALI conditions. MSCs cultured on these substrates in the presence of growth factors showed substantial decrease in mesenchymal marker expression and increased epithelial marker expression. ROCK inhibition provided a more advanced differentiation. This study demonstrates the feasibility of using growth factor loaded biocomposite films and MSCs for development of in vitro respiratory epithelial models. Our future work will focus on optimisation of culture conditions including modifying the composition of the films and GF delivery conditions to induce better mucin secretion and epithelial barrier function.

Acknowledgements
This work was supported by CASCADE-FELLOWS Scheme of EU Marie Curie COFUND in association to the University of Nottingham and EU FP7 IMMODGEL (602694) project. This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 760921 (PANBioRA).

Conflict of interest statement
The authors declare no conflict of interest in this study.

6 - Incorporation of Resident Macrophages in Engineered Tissues: Multiple Cell Type Response to Microenvironment Controlled Macrophage-laden Gelatin Hydrogels

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Dans cette troisième partie l'objectif était de développer une modélisation in vitro de l'interaction entre les cellules colonisant un biomatériau implanté et les cellules responsables de la réponse inflammatoire qui se produit suite à une implantation. Lors de la mise en place d'un biomatériau dans le corps, une réaction inflammatoire se déclenche autour du dispositif. Cette réaction inflammatoire peut être à l'origine de la cicatrisation et intégration du biomatériau mais également de rejet de celui-ci. Il s'agit d'une réaction principalement médiée par les macrophages dont le phénotype oriente vers une cicatrisation et une intégration du dispositif implanté ou bien vers son rejet.

Ici nous avons voulu montrer que l'incorporation de macrophages encapsulés dans un hydrogel et l'orientation de leur phénotype pouvait permettre une immunomodulation vers des propriétés pro-cicatrisantes. Ce qui serait un bénéfice considérable lors de la mise en place d'un dispositif implantable.

Les résultats de ces travaux présentés ci-dessous ont fait l'objet d'une publication.

6.1 Abstract

The success of tissue engineering strategy is strongly related to the inflammatory response, mainly through the activity of macrophages that are key cells in initial immune response to implants. For engineered tissues, the presence of resident macrophages can be beneficial for maintenance of homeostasis and healing. Thus, incorporation of macrophages in engineered tissues can facilitate the integration upon implantation. In this study, we developed an in-vitro model of interaction between encapsulated naive monocytes, macrophages induced with M1/M2 stimulation and incoming cells for immune assisted tissue engineering applications. To mimic the wound healing cascade, Naive THP-1 monocytes, endothelial cells, and fibroblasts were seeded on the gels as incoming cells. The interaction was first monitored in the absence of the gels. In order to mimic resident macrophages, THP-1 cells were encapsulated in the presence or absence of IL-4 to control their phenotype and then these hydrogels were seeded with incoming cells. Without encapsulation, activated macrophages induce apoptosis in endothelial cells. Once encapsulated no adverse effects were seen. Macrophage-laden hydrogels attracted more endothelial cells and fibroblasts compared to monocytes-laden hydrogels. The induction (M2 stimulation) of encapsulated macrophages did not change the overall number of attracted cells; but significantly affected their morphology. M1 stimulation by a defined media resulted in secretion of both pro and anti- inflammatory cytokines compared to M2 stimulation. We demonstrated that there is a distinct effect of encapsulated macrophages on the behavior of the incoming cells; this effect can be harnessed to establish a microenvironment more prone to regeneration upon implantation.

Keywords: Macrophage, Hydrogel, Cell/Cell interactions, Foreign Body Response, Gelatin, Microenvironment

6.2 Introduction

Once a material is implanted into the body, adverse immune reactions can be triggered. Cells receive a diverse range of signals from their surrounding microenvironment and adjust their responses accordingly. [137] The immune system recognizes the material as foreign, initiating a macrophage-mediated acute inflammatory phase. [138] Inflammatory cells initially are recruited to the site of inflammation due to the endothelial response to injury Monocyte responds to monocyte chemoattractant protein-1 (MCP-1), which is secreted by cytokine-stimulated endothelial and smooth muscle cells in the intima. In response to cytokines secreted by endothelial cells, fibroblasts, and other cells, the incoming monocyte at the site of injury differentiates into macrophages. [139] This chain of events makes macrophages an important factor in the acceptance or rejection of implanted biomaterials. Another important aspect of the fate of engineered tissues is their integration with the host vasculature; as angiogenesis is involved in wound healing by promoting the outgrowth of new blood vessels from preexisting vasculature. Inflammation also plays a major role in physiological angiogenesis. Inflammatory cells such as macrophages secrete a multitude of inflammatory mediators of which may influence angiogenesis. [140] Thus it is important to establish a strong control over the cross-talk between endothelial cells and macrophages in the vicinity of engineered tissues. The success of the tissue engineering strategy is strongly related to the inflammatory response, mainly through the activity of macrophages. They can be polarized either into M1 or M2 phenotype, depending on the activation signals. [137] M1 macrophages are considered to be pro-inflammatory, while M2 macrophages promote tissue regeneration. However, the exploitation of these cells to control engineered tissue integration has not been widely studied. [141] Resident macrophages are cells observed in most of tissues with a heterogenous phenotype. [142] They are involved in wound healing and resolution of inflammation; thus encapsulation of monocytes or pre-differentiated macrophages in hydrogels can mimic this function in tissue engineering applications. Some examples of resident macrophages are microglia, Kupffer cells, alveolar macrophages and osteoclasts. [143]

One of the main means for macrophages to control the microenvironment around the implanted biomaterials is the cytokine production, M1 macrophages produce larger amounts of the pro-inflammatory cytokines: TNF-α (tumor necrosis factor alpha), IL-12, and IL-1β. TNF-α is a master pro-inflammatory cytokine involved in chronic inflammation. [144] IL-12

promotes TH1 cell-mediated responses, which have been implicated in the pathogenesis of a number of inflammatory and autoimmune diseases. [145] IL-1β is an important mediator of the inflammatory response. [146] M2 macrophages produce anti-inflammatory cytokines such as IL-1RA, CCL18. IL-1RA is a natural inhibitor of the pro-inflammatory effect of IL-1β. IL-1RA is used to treat autoinflammatory diseases such as rheumatoid arthritis and juvenile idiopathic arthritis. [146] CCL18 is a chemokine, i.e. a cytokine with chemotactic activity. [147] CCL18 is produced by myeloid cells and induces chemotaxis of lymphocytes and immature DCs, as well as collagen deposition by fibroblasts. [148] Although, it is not one of the main components of macrophage secretome; Interleukin 4 (IL-4) is one of the main factors in macrophage polarization. It drives the differentiation of naive T helper (TH) cells to TH2 effector cells and monocytes and macrophages to an M2 (or alternatively) activated phenotype. [149] As can be seen from the aforementioned activities of these cytokines, the cytokine microenvironment around an engineered tissue would significantly affect its remodeling and integration and needs to be controlled. [150]

Wound healing cascade can be divided in three steps. The initial inflammatory step is characterized first by the formation of blood clot and the mobilization of neutrophils and then macrophages to the site of injury. [151] Then there is a "proliferative phase" with the formation of a granulation tissue. At this stage, endothelial cells are crucial because neovascularization will enable the procurement of the nutrients to the wound and this will lead to the formation of a new stroma. The last stage is the "maturation phase" which is characterized by the transition from granulation tissue to scar formation. [152,153] In this step, fibroblasts are of interest because they will secrete collagen and they will be the key players to remodel extracellular matrix. In all these three stages and especially in the last two, macrophages will continuously secrete growth factors that will stimulate angiogenesis and collagen deposition and that is why these three cell types are the main actors for tissue remodeling after injury.

One way to understand and control these complex interactions is to develop simplified in vitro models. However, currently there are no models that directly deal with the interaction of immune cells with connective tissue cells in tissue engineering context. A recent example of monitoring of cell-cell interactions is an intestine organ-on-chip study by Kim and colleagues (2016) [154]. The cells are exposed to physiological peristalsis-like motions and fluid flow. The systems involve co-culture human intestinal endothelial and immune cells, in the

presence of bacteria in order to elucidate how they contribute to host tolerance of infection and disease inflammation. [154]. However, in order to assess regeneration in a multi-cellular environment a model should integrate extracellular matrix component. One way to achieve this is to encapsulate cells in ECM like hydrogels as the three-dimensional (3D) cell culture in vitro environment is a crucial key for acquiring phenotypes and for responding to stimuli analogous to in vivo biological systems. [155]

The main objective of this study is to develop an in-vitro model of interaction between incoming cells and encapsulated macrophages as a model of inclusion of resident macrophages in engineered tissues. Fibroblasts and endothelial cells have been selected in co-culture condition with macrophages due to their key role in wound healing process. Thus, in a tissue engineering setting it is important to control the incoming cells to have a precise control over the remodeling of the implanted engineered tissue. For this end, we observed the attachment, morphology and cytokine profile in different settings pertaining to wound healing cascade in the presence of macrophages in M1/M2 phenotype inducing microenvironments. Through this model, we aim to define the optimal inclusion conditions for macrophages in engineered tissues.

6.3 Materials

Gelatin Type A from porcine skin and PMA were purchased from Sigma-Aldrich (St Quentin Fallavier, France). M1 and M2 macrophage generating media, Interleukin-4, HUVEC cells, Endothelial cell growth medium, Apoptotic/Necrotic/Healthy Cell kit, Fluorimetric cell viability kit I and DAPI were purchased from Promocell (Heidelberg, Germany). THP-1 (human) and NIH-3T3 (murine) cells were purchased from ATCC in frozen form (Manassas, US). RPMI-1640 and DMEM media, Dulbecco's Phosphate buffered saline, Fetal bovine serum, 0.05% trypsin/0.02% EDTA, TripLETM Express (1x), β-mercaptoethanol, Cell trackerTM and Vybrant® Cell Adhesion Assay Kit were obtained from Life Technologies (Carlsbad, USA). Transwell plates were purchased from Millipore. Bacterial transglutaminase was kindly provided by Ajinomoto Inc (Tokyo, Japan).

6.4 Methods

The cell culture was done with different cell types: naïve THP-1, 3T3 fibroblasts, HUVEC, THP-1 cells in M1 and M2 macrophage polarization media. The experiments were done under two different conditions: (A) non-contact cell co-culture, it means with a transwell (Figure 1a) and (B) contact cell co-culture, with cells encapsulated in a gel, as shown in (Figure 2a).

The THP-1 (a human monocytic cell line) were cultured in RPMI 1640 GlutaMAX supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 0.2% fungizone and 0.05 mM 2-mercaptoethanol. To differentiate the monocytes to macrophages, cells were treated with 50 ng/ml of phorbol myristate acetate (PMA) which is dissolved in media (RPMI 1640 without 2-mercaptoethanol) for 24 h at 37 °C, 5% CO2. Unattached cells were removed after washing with DPBS. PMA activated THP-1 cells were detached using TripLE TM Express (1x), centrifuged and re-suspended in media (without 2-mercaptoethanol).

NIH-3T3 cells were cultured in RPMI 1640 (Gibco Life Technologies, USA) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 0.2% fungizone at 37 °C in a 5% CO2 atmosphere. Prior to seeding, cells were harvested using 0.05% trypsin/0.02% EDTA, centrifuged and re-suspended in media.

Human Umbilical Vein Endothelial Cells (HUVEC) were used at passages between 4 and 8. The culture media used were endothelial cell growth medium supplemented with Supplement Mix C-39215 (mainly composed of heparin, hydrocortisone, fetal calf serum, basic fibroblast growth factor, epithelial growth factor and endothelial cell growth supplement) and 1% penicillin/streptomycin. Prior to seeding, cells were harvested using 0.05% trypsin/0.02% EDTA, centrifuged and re-suspended in media.

M1 and M2 phenotypes are generally characterized by specific cytokine inductions of a variety of phenotype marker expression, such as cytokines, surface markers, bioactive molecules such as ROS, NO and chemokines. The commercially available M1 and M2 generation media from PromoCell (Heidelberg, Germany) contains the necessary cytokines to induce M1 or M2 differentiation. In this configuration, they were used to induce a specific

stimulation microenvironment. The cells were pre-treated with the differentiation medium prior to the seeding of incoming cells. [156]

6.4.1 Non-contact cell co-culture

Transwell® permeable supports (3-µm porous polyethylene terephthalate (PET) inserts) (Millicell®, Millipore, France) were utilized for non-contact cell co-culture. THP-1 cells (1.106 cells.mL-1) were added to mono-culture wells (except for the condition "no THP-1"), and 10 000 HUVEC cells were added in the Transwell® inserts (Figure 1A). THP-1 cells were either naive THP-1 or activated directly (presence of PMA in the culture medium: SET A) or pre-activated one day before the experiment (Activation in PMA containing medium before co-culture experiments, to avoid the effects of PMA on the other cell type: SET B). SET C: THP-1 cells were either naive THP-1 or activated directly (presence of IL-4 in the culture medium) or pre-activated for six days before the experiment.

6.4.2 Contact cell co-culture

For all cell experiments, the same protocol of seeding was used.

Gelatin type A solution was prepared in cell culture medium (RPMI 1640 GlutaMAX for THP-1) under sterile condition. All the solutions, 6% gelatin (w/v) and 20% (w/v) of TGA solution in PBS were filtered prior to use (0,22 µm). THP-1 cells were centrifuged in order to get cells pellet. Then gelatin type A solution was added in order to get a cell density of 6x106 cells.mL-1 solution and kept in water bath at 37°C. 50 µL of gelatin solution with encapsulated cells (300 000 cells/hydrogel) were then deposited on top of 10 µL of TGA solution in order to get homogeneous crosslinking and then put in incubator at 37°C for at least 15 minutes prior to add cell culture medium in each well. The encapsulated cells were cultured for 3 days in different media (RPMI-1640 with or without 10 ng/mL of IL-4, M1 or M2 media). We have previously demonstrated that transglutaminase crosslinked gelatin structures can be loaded and can release growth factors and cytokines [157] On the 3rd day, the cell culture medium was removed and 50 000 or 100 000 incoming cells (HUVEC, THP-1 or 3T3) were added to all the samples. Cells were cultured for a further 3 days or 6 days. The metabolic activity of those cells was measured by Alamar Blue assay to assess cell

proliferation, and cytokine (IL-1ß, IL-1RA, IL-4, IL-10, IL-12, CCL-18 and TNF-α) analysis of supernatants was done by ELISA.

6.4.3 Metabolic activity

To assess metabolic activity, samples were incubated with 10% v/v Resazurin (Fluorometric cell viability kit I, PromoKine, Germany) in cell culture medium for 2 h. The substrate will become fluorescent (red) when incubated with viable cells due to reduction. The amount of fluorescence was monitored with a SAFAS Xenius XML fluorescence reader (SAFAS, Monaco) at excitation 560 nm and emission wavelength 590 nm.

6.4.4 Apoptotic/Necrotic/Healthy Cell Assay

Apoptotic/Necrotic/Healthy cell assay allows to detect apoptotic (green), necrotic (red) and healthy (blue only) cells using fluorescent microscopy. Staining was made according to the manufacturer's procedure (PromoCell, Germany). Briefly, samples were washed twice with binding buffer. Incubated for 15 min at RT and protected from light in a solution containing FITC-Annexin V, Ethidium homodimer III and Hoechst 33342. For 100 µL of binding buffer 5 µL of each dye was added. Samples were washed again twice and kept in binding buffer for imaging. Fluorescence images were performed using Nikon Eclipse Ti-S with a 10x PL Fluor (0.30 NA) objective equipped with Nikon Digital Camera (with NIS-Elements software), and processed with ImageJ.

6.4.5 Cytokine detection by ELISA

Cell culture media was collected at days 3 and 6 and the cytokine amounts in the media was quantified by ELISA developer kits. As the concentration of specific cytokines are not known for M1 and M2 media, standard curves were done using the relevant sample media type. Absorbance measurements were done @450 nm. The cytokine amounts were calculated using the standard curves. OD cut-off is set as the OD value for standard concentration of 0 ng/mL. Following cytokines were quantified: IL-1ß, IL-1RA, IL-4, IL-10, IL-12, CCL-18 and TNF-α.

6.6.6 Cell Pre-Labelling

In order to differentiate the encapsulated cells from the incoming cells, a pre labelling was done with cell tracker or calcein-AM. Briefly, to stain 1 million cells, either 5µL calcein or

1µL cell tracker was added to 1 mL serum free medium. Cells were centrifuged in order to get cells pellet and resuspended in staining solution. Incubated for 30 minutes at 37°C in the dark and washed with PBS using centrifugation. Cells were resuspended in gelatin for encapsulation or in culture medium for the seeding on top of the gel. Fluorescence images were performed using Nikon Eclipse Ti-S with a 10x PL Fluor (0.30 NA) objective equipped with Nikon Digital Camera (with NIS-Elements software), and processed with ImageJ.

6.6.7 Scanning electron microscopy (SEM).

The samples were fixed with 4% glutaraldehyde. The specimen were washed with DPBS prior to following a dehydration protocol using an alcohol series of increasing concentration (70%, 95% and 2 × 100%), with incubation periods of 5 min for each. Subsequently, samples were incubated in 100% ethanol/hexamethyldisilazane (HMDS) (1:1) for 5 min, then only in HMDS for 2x 5 min and dried overnight. Samples were adhered onto titanium discs using a carbon tape and coated with gold/palladium in a sputter coater. The samples were sputtered at 7.5 mA for 3 min under argon atmosphere and images were acquired using a scanning electron microscope (Hitachi TM1000).

6.6.8 Statistical Analysis

The statistical significance of the obtained data was assessed using the t-test or Mann-Whitney test ($n \geq 3$). The error bars are representative of Standard Deviation (SD). Differences at $p \geq 0.05$ were considered statistically not significant.

6.5 Results and Discussion

Any implanted cell-containing material in vivo will be in contact with several incoming cell types including monocytes/macrophages, endothelial cells and connective tissue cells such as fibroblasts. Our working hypothesis is that addition of a macrophage component in such systems to mimic “resident macrophages” and their role in wound healing will help the regeneration process. As macrophage function is tightly linked to their physicochemical niche, encapsulation in hydrogels might provide the necessary signal for encapsulated macrophages to assume a more resident macrophage-like phenotype. In order to assess the effects of the macrophage presence, we have developed a system where pre-labelled cells of different types can be directly added onto macrophage-laden hydrogels in order to assess their

interaction with the encapsulated macrophages. In order to determine the effect of encapsulation, the interaction was first monitored in the absence of the gels with a transwell system (paracrine interactions).

6.5.1 Transwell system

In order to see the extent of the reaction of HUVECs to the presence of THP-1 cells without cell to cell contacts; naive and activated THP-1 cells were put into co-culture conditions with HUVECs with the help of a transwell inserts (Figure 1A). In the absence of THP-1 cells, HUVECs formed a monolayer by 5 days with an increase in metabolic activity (Figure 1B); in the presence of THP-1 cells however, particularly with activated THP-1 cells; HUVECs had significantly lower metabolic activity (Figure 1B) with substantial number of cells going through apoptosis as evidenced by positive Annexin-V staining (Figure 1C, D). In order to see whether this effect has any direct relation with the macrophage phenotype; the macrophages were put in contact with IL-4 (M2 inducer) (Figure S1); aside from M2 inducing capacity of IL-4; it has also been shown that IL-4 dependent proliferation can be seen in resident macrophages. [142] Addition of IL-4 did not have a significant effect on metabolic activity of HUVECs; however the number of alive attached cells were significantly higher in the presence of IL-4 (Figure S1 B and C). The IL-4 has been shown to induce V-CAM expression by vascular endothelial cells [158], but there are no reports on the effect of IL-4 on HUVEC proliferation; thus the differences in the metabolic activity can be attributed to the secretions of monocytes that changes with the presence of IL-4.

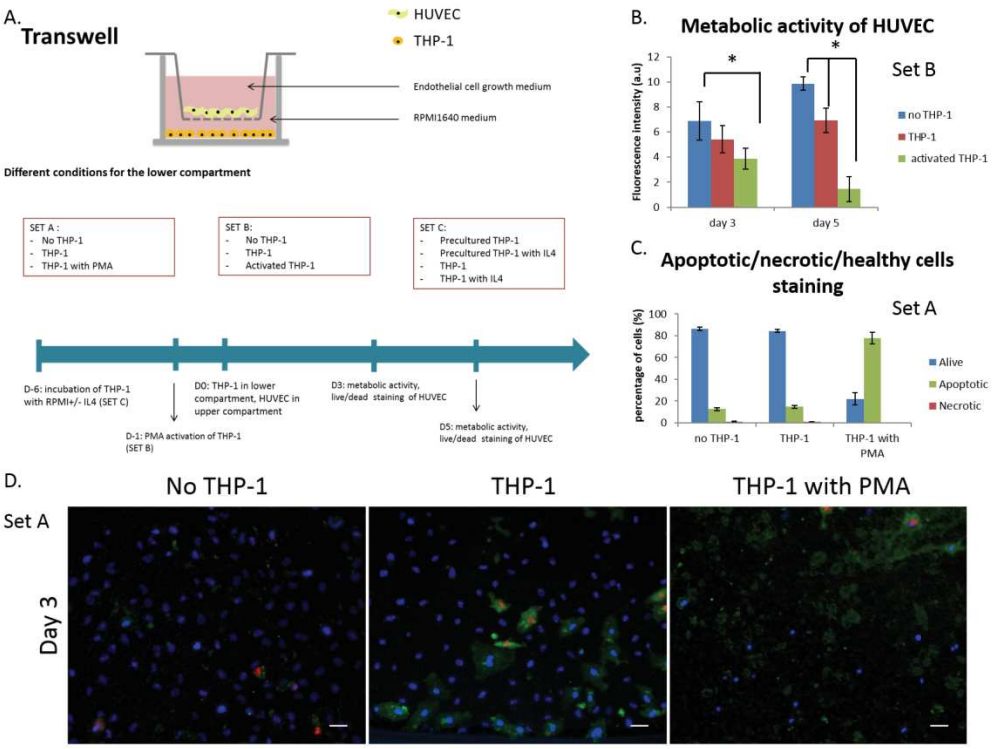

Figure 1. A) Experimental design of Transwell based co-culture of HUVECs with phenotype controlled THP-1 cells.
B) Metabolic activity of HUVECs in the Transwell co-culture system (set B).
C) Quantification of HUVECs viability after three days of culture using Apoptotic / Necrotic / Healthy cells kit (set A).
D) Apoptotic (green)/Necrotic (red)/Live (blue) staining of HUVECs on Transwell after three days of culture in the presence of THP-1 cells (set A). Scale bar = 50 µm.

6.5.2 Contact cell co-culture

In order to see the effect of encapsulated THP-1 cells on different cell types implicated in wound healing, (endothelial cells, fibroblasts and incoming monocytes/macrophages), we encapsulated THP-1 cells in the presence or absence of IL-4 to control their phenotype. Then we seeded these hydrogels with naive monocytes, endothelial cells and fibroblasts (Figure 2A). The presence of IL-4 does not have an effect on the metabolic activity of encapsulated THP-1 cells (Figure 2B). After 6 days, the number of attached incoming fibroblasts were significantly higher compared to monocytes and endothelial cells (Figure 2C and D). Even though there was no significant difference between the number of attached cells with respect to the IL-4 presence; in the case of fibroblasts, the cells on the gel containing IL-4 were more

spread compared to no IL-4 conditions (Figure 2D). As IL-4 is known to induce synthesis of fibronectin [159] by fibroblasts; the increase in spreading can be attributed to the presence of more self-secreted ECM proteins on the gelatin substrate induced by either directly via IL-4 presence or the secretions of macrophages activated with IL-4. When the cross-sections of the hydrogels are observed; THP1 cells could be seen as individual cells within the hydrogel structure and the incoming cells on the surface of the structure. When the gel surfaces were observed with SEM; macrophages which escaped from the gel were visible together with individual endothelial cells (which had a more defined shape in the case of no IL-4, (Figure 3A) and well spread, spindle shaped fibroblasts with significant granular material around them; which could be new ECM secretion. The organization of fibroblasts were more apparent in the case of IL-4 treatment (Figure 3B). In both cases no significant in-growth of the incoming cells were observed in the given timeframe. On day 6 the IL-4 treated group had significantly more release of two important anti-inflammatory cytokines IL-1RA and CCL-18 (Figure 3C). The release profile of these cytokines were even more profoundly affected when the incoming cells are monocytes, due to the additional secretion by the incoming cells after paracrine interaction with the encapsulated macrophages (Figure S2B).

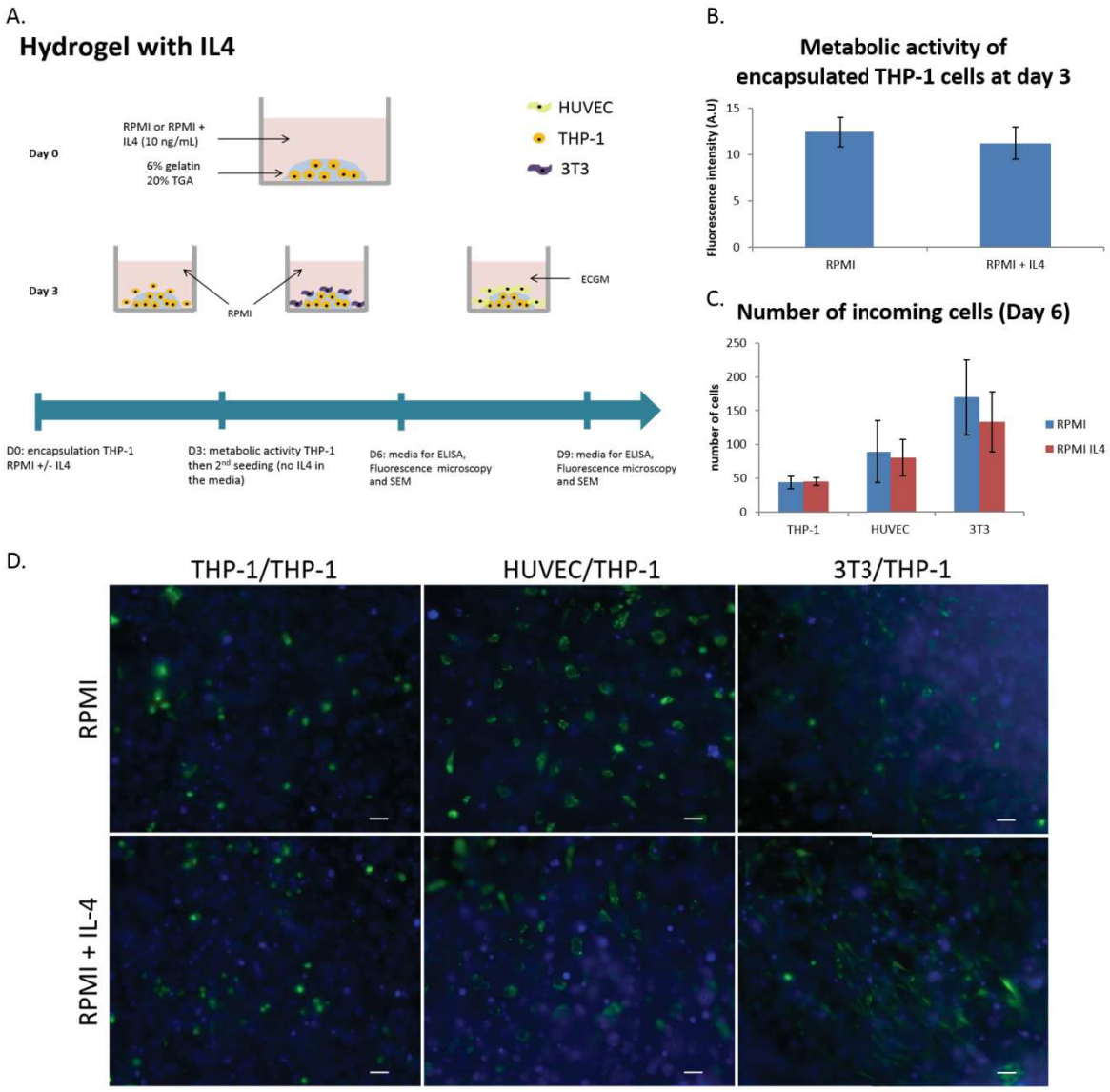

Figure 2. A) Experimental design of the hydrogel based co-culture system with pre culture of encapsulated THP-1 cells in RPMI medium with or without supplementation of IL-4.
B) Metabolic activity after three days of culture of encapsulated THP-1 cells in the presence or absence of IL-4 in the medium.
C) Average number of incoming cells. Incoming cells were pre-labelled with calcein-green and pictures were taken with epifluorescence microscope (10x) and cells per image were counted with four pictures.
D) Epifluorescence pictures (10x) at day 6 showing the interaction between pre-labelled (calcein-green) THP-1, HUVECs or 3T3 cells with encapsulated THP-1 cells. Cells were cultured with or without IL-4 in the medium. Scale bar = 50 µm

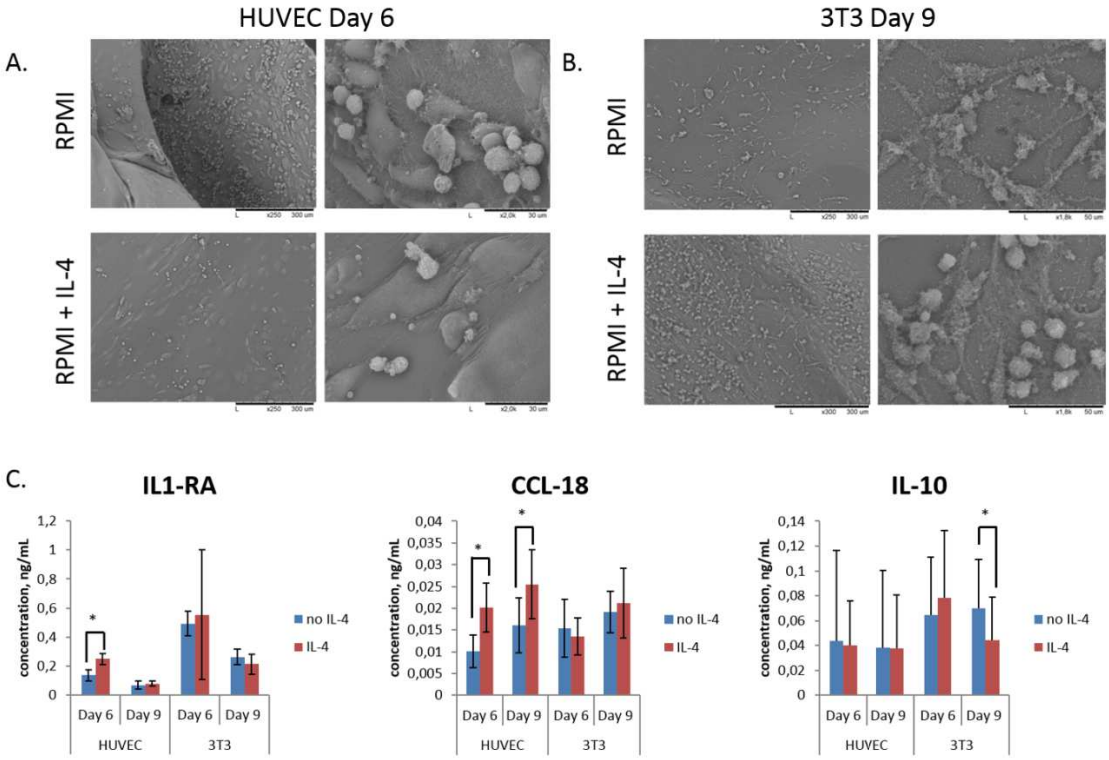

Figure 3. SEM pictures of incoming:
A) HUVECs (Day 6)
B) 3T3 cells (Day 9) on encapsulated THP-1 cells. THP-1 were pre cultured with or without IL-4 in the medium.
C) ELISA quantification of anti-inflammatory cytokines in the supernatant at day 6 and day 9 of culture. Statistics were performed using t-test or Mann-Whitney ($p<0,05$).

In tissue, following the initial immune reaction, resident macrophages contribute to the resolution of inflammation by IL-10 and TGF-beta dependent pathways; thus the presence of macrophages within an implanted tissue can facilitate the resolution process by having an indirect effect on the incoming cells. In order to see the distinct effect of M1/M2 inducing microenvironments, we have used specific media that has M1 and M2 macrophage polarization inducing properties (Figure 4A). The medium composition in these conditions has a significant effect on encapsulated macrophage metabolic activity within M1 induction conditions the metabolic activity was higher (Figure 4B). M1/M2 induction has opposing effects on fibroblast and endothelial cell attachment; where in M1 induction case more

fibroblasts were seen to spread on the surface of the hydrogels; particularly at the borders (Figure 4C, D), in M2 induction the effect was more apparent on endothelial cells where in M2 conditions a nearly confluent layer of endothelium was formed on the gel surface (Figure 4 C). M1/M2 media induced the organization of the attached cells more apparently compared to no induction or IL-4 induction conditions. However, direct M1/M2 induction does not have a significant effect on cytokine secretions, as all tested cytokines except CCL18 were below detection limits (data not shown).

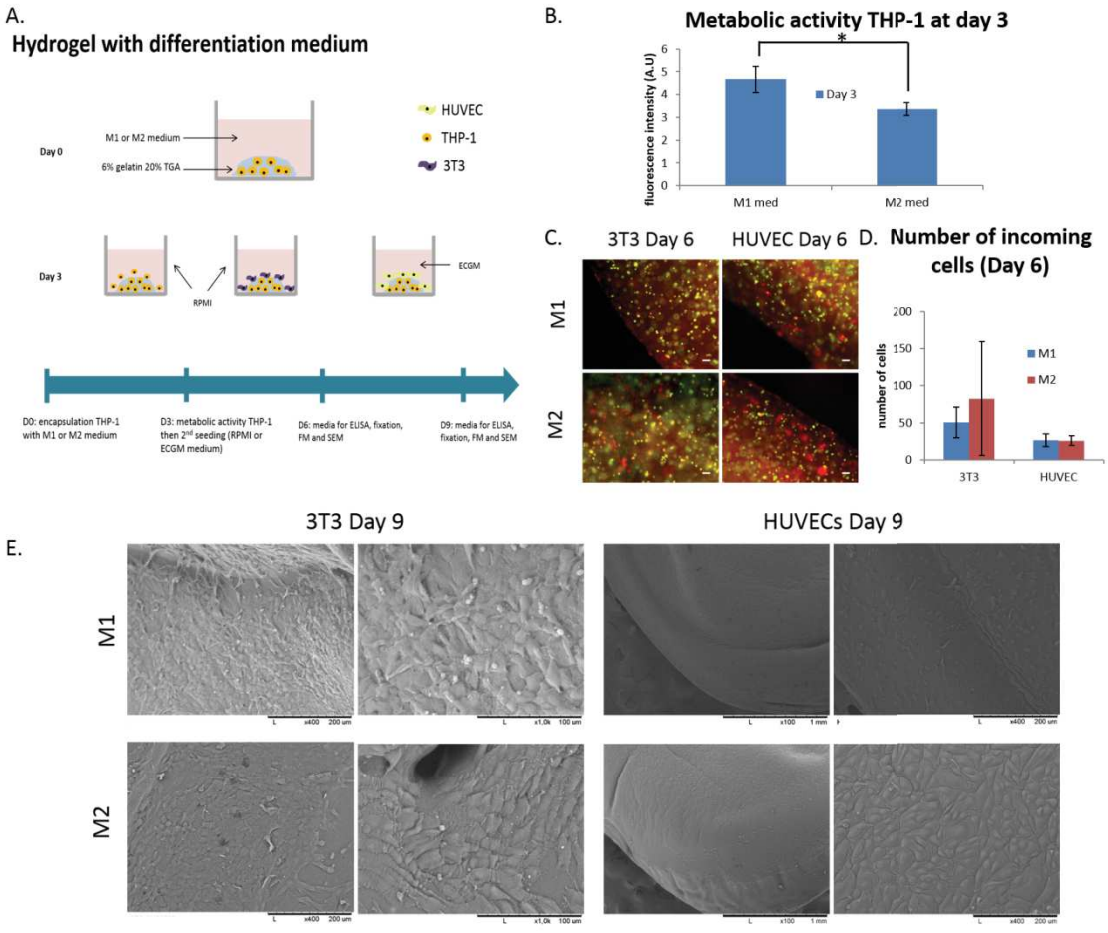

Figure 4. A) Experimental design of the hydrogel based co-culture system with pre culture of encapsulated THP-1 cells in M1 or M2 differentiation medium.
B) Metabolic activity after three days of culture of encapsulated THP-1 cells in differentiation medium.
C) Epifluorescence pictures (10x) at day 6 showing the interaction between incoming pre-labelled (red cell tracker) 3T3 cells or HUVECs in contact with pre-labelled (calcein green) encapsulated THP-1 cells. Scale bar = 50 µm.
D) Average number of incoming cells. Incoming cells were pre-labelled with (red cell tracker) and pictures were taken with epifluorescence microscope (10x) and cells per image were counted with 5 pictures.
E) SEM pictures of incoming 3T3 cells or HUVECs at day 9 in contact with encapsulated THP-1 cells. THP-1 were pre cultured with M1 or M2 differentiation medium.

The observations here are in line with previous simulation work where [160] developed a mathematical model describing angiogenesis in soft tissue wound healing. This model is based on the evolution of capillary-tip endothelial cells and the formation of new blood vessels in the direction of the wound site. Through a system of partial differential equations, they hypothesize that the migration of endothelial cells and the blood vessel density is deeply correlated to the local concentration of chemoattractant secreted by macrophages present in the wound site. Endothelial cells migrate in the direction of increasing macrophage derived chemoattractant which lead to the increase of blood vessel density. They also took into account a self-regulatory mechanism that will decrease the secretion of chemoattractant once blood vessel density reach a critical value. This example show the cross talking between endothelial cells and macrophage which is crucial for angiogenesis. With our system based on the encapsulation of macrophages, we aim to exert more control over the secretions of the chemoattractant by inducing a specific microenvironment for incoming macrophages. This should accelerate angiogenesis which is necessary to supply nutrient in the wound site. In the current model developed we demonstrated that the nature of induction (single stimulant or specific polarization inducing media) had a direct effect on incoming cell attachment and can be used to regulate the chemokine profile secreted by macrophages. With the aim of observing if M1/M2 stimulated macrophages have a pronounced effect on the cytokine profiles of incoming monocytes, the model was also done by incoming naive THP-1 cells; which induced moderate attachment of incoming cells. The M1/M2 induction resulted in similar amount of THP-1 cell attachment on gel surface (Figure S2); however there was a significant difference in the total amount of secreted cytokines (Figure 5). For both pro- and anti-inflammatory cytokines M1 induction medium was more potent in induction of secretion; for the case of pro-inflammatory cytokines there is an initial high secretion which then decreased (TNF-alpha and IL1beta); for anti-inflammatory IL1RA a similar trend was observed whereas for IL-10 and CCL-18 there is a time dependent manner increase . For both IL1RA and CCL-18 the secreted amount was higher than IL-4 induction cases; which demonstrates the specific advantage of using more defined induction cocktails. It should be pointed out that, due to the presence of a surrounding 3D protein structure, the polarization effect of M1/M2 induction media did not create specific macrophage phenotypes. Thus, the effects observed were more linked to the cytokine microenvironment created than the specific phenotype of encapsulated macrophages (which can be expected to have a crosslinked gelatin microenvironment specific signature).

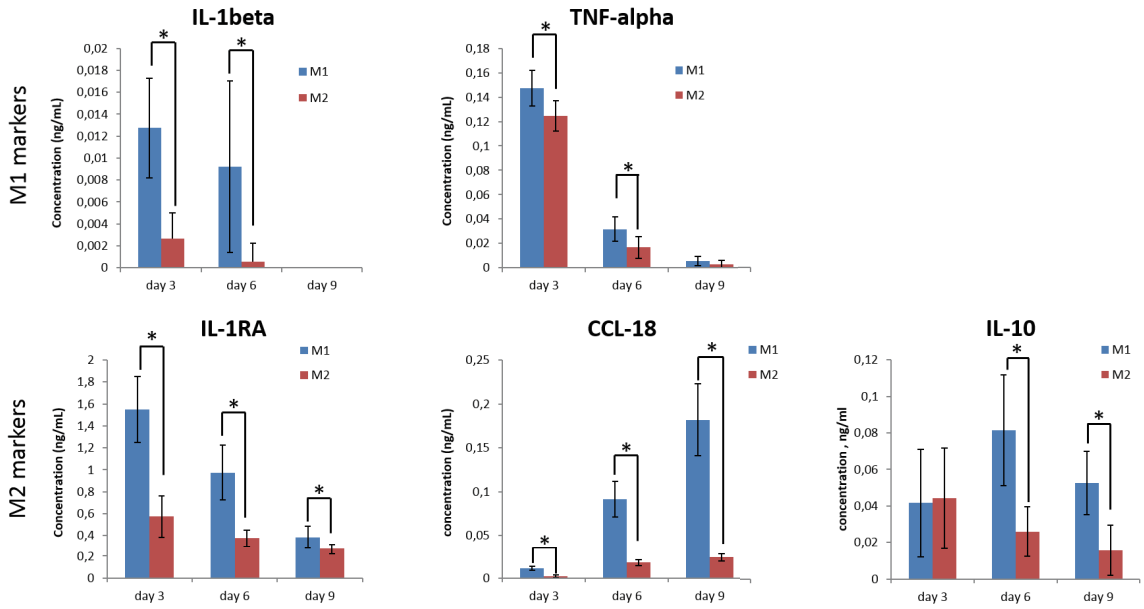

Figure 5. ELISA quantification of pro-inflammatory (M1) and anti-inflammatory (M2) cytokines in the supernatant at day 3, 6 and 9 of culture for hydrogel based co-culture system with incoming THP-1 cells in contact with encapsulated THP-1 cells pre cultured in M1 or M2 differentiation medium. Statistics were performed using t-test or Mann-Whitney ($p<0,05$).

Finally, for modelling the movement of the cells from surrounding tissues; macrophage containing hydrogels are put in contact with endothelial cell laden hydrogels (Figure 6A). Over the course of two weeks, endothelial cells moved more towards the macrophage laden hydrogels in M2 media microenvironment (Figure 6B). Just as in direct contact conditions, M1 microenvironment was more potent in inducing both pro- and anti-inflammatory cytokine secretion (Figure 6C). For both conditions, after an initial high levels of pro-inflammatory TNF-alpha and IL-1beta release, the microenvironment has become more pro-regeneration with the presence of IL-1RA, CCL-18 and IL-4. It has been previously shown that the presence of endothelial cells improves the viability of cardiomyocytes in 3D hydrogel configuration [161]; thus establishment of a microenvironment that can induce the in-growth of endothelial cells would be advantageous. Also, the migration in the presence of two gels

demonstrate that the effect of the encapsulated macrophages is not limited to the cells in suspension.

In regenerative medicine, the final aim is the complete integration of the implanted artificial tissue over time. This implies that the artificial tissue should integrate with the host circulatory system, nervous system and also the immune system. The immune system integration can be triggered by addition of macrophages in the artificial tissue formulation, in the manner described in this study, which can facilitate the establishment of a resident macrophage population. This can also facilitate the healing process and the resolution of inflammation induced by implantation.

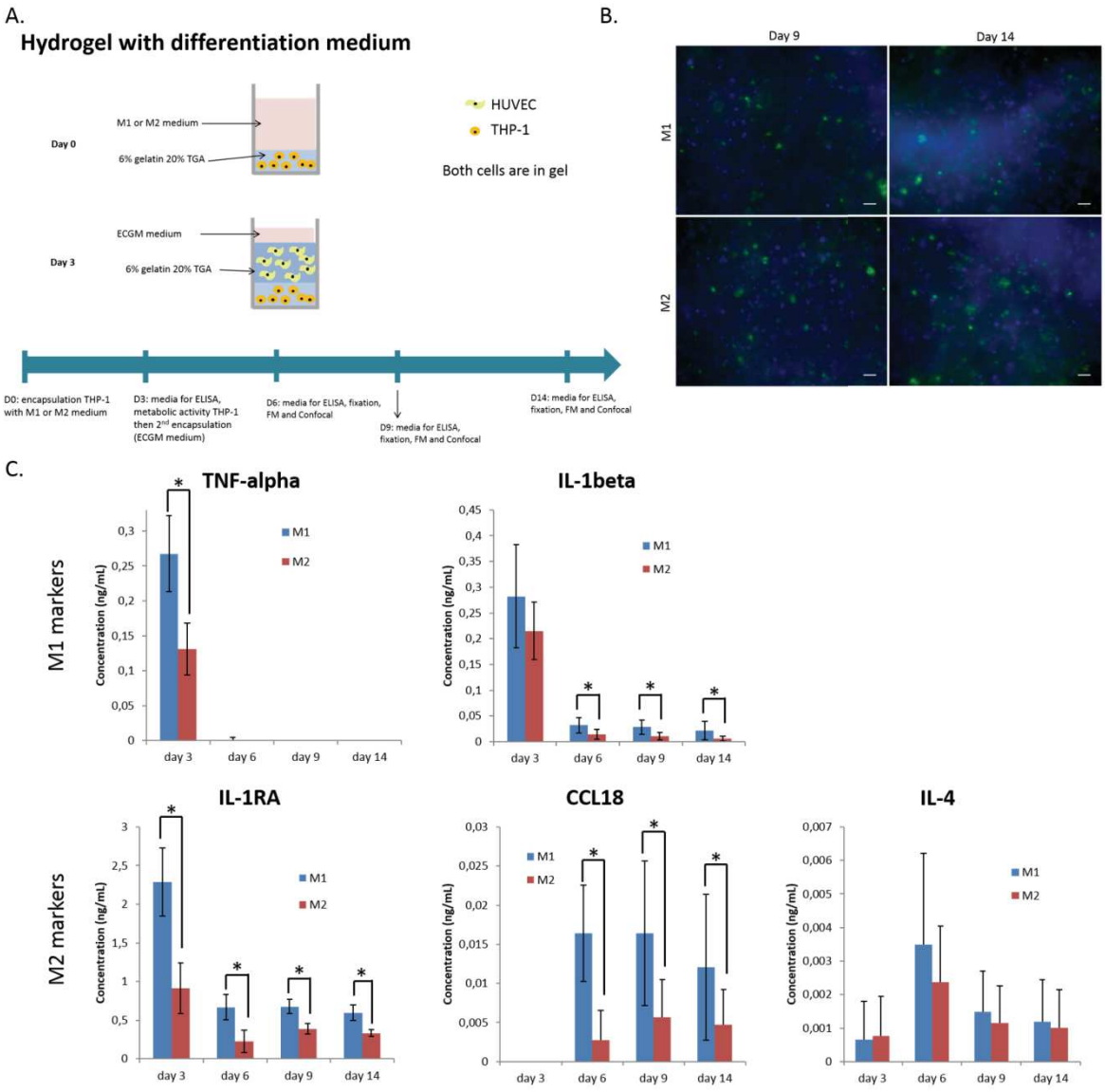

Figure 6. A) Experimental design of the hydrogel based co-culture system with pre culture of encapsulated THP-1 cells in M1 or M2 differentiation medium. Incoming HUVECs and encapsulated THP-1 cells are in a different gel.
B) Epifluorescence pictures (10x) at day 9 and 14 showing the interaction between pre-labelled (calcein green) encapsulated HUVECs with pre-labelled (blue cell tracker) encapsulated THP-1 cells pre cultured in M1 or M2 differentiation medium. Scale bar = 50 µm.
C) ELISA quantification of pro-inflammatory (M1) and anti-inflammatory (M2) cytokines in the supernatant at day 3, 6, 9 and 14. Statistics were performed using t-test or Mann-Whitney ($p<0,05$).

6.6 Conclusion

The design and maturation of an engineered tissue is important for its integration within the host. Here we demonstrated in an in vitro model that the encapsulated macrophages, mimicking resident macrophages, have an impact on the behavior of incoming cells in a cell-type specific manner. The presence of pro- or anti-inflammatory cytokines can be impacted by the conditioning of the encapsulated macrophages. In the future, the incorporation of phenotype controlled macrophages in engineered tissues has an important potential the initial immune reaction upon implantation. Controlled delivery of polarization inducing cytokines could achieve partially a similar effect; but incorporation of macrophages would provider an active source of secretion for a variety of cytokines and bioactive agents for a longer term. Such a model can be used to better understand possible immunoengineering strategies using macrophages and also studies of immune response to xenogenic and allogenic cells in a controlled manner. Our future studies will focus on the elucidation of the encapsulation conditions on macrophage polarization for potential regenerative medicine applications.

Acknowledgements

This project has received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration under grant agreement no. 602694 (IMMODGEL).

Author Contributions:

Dollinger C, Ciftci S, Knopf-Marques, Guner Rabia and Barthès J performed experiments. Dollinger C, Knopf-Marques H, Barthès J and Vrana NE designed experiments. Ghaemmaghami AM, Vrana NE, Debry C supervised experiments. Ghaemmaghami AM,

Vrana NE, Debry C, Dollinger C, Barthès J and Knopf-Marques H analyzed and interpreted data. Dollinger C, Barthès J, Knopf-Marques H and Vrana NE wrote the manuscript. All authors revised and corrected the final manuscript.

Competing Financial Interests:

Authors declare that there are no competing financial interests.

D - Conclusion Générale

Dans le cadre du développement d'un larynx artificiel en bioingénierie, un des éléments clés à prendre en compte est l'épithélium respiratoire. Celui-ci joue un rôle primordial non seulement pour la biointégration optimale du dispositif lors de son implantation mais également sur le caractère fonctionnel de celui-ci par la suite. Le développement d'hydrogels à dégradation controlée et fonctionnalisés est une voie de recherche tout à fait adaptée dans ce but. En effet ces hydrogels présentent comme avantage de pouvoir être stabilisés suffisamment longtemps pour accomplir leur rôle de matrice extracellulaire artificielle et servir de support optimisés pour la repousse rapide d'un épithélium respiratoire. De par leur structure ils ont également la capacité d'être chargés avec divers facteurs protéiques lesquels diffuseront sur le site d'implantation. L'utilisation de facteurs de croissance cellulaire épithéliaux sur ces hydrogels permet une augmentation de l'activité des cellules colonisant la surface des films démontrant ainsi la possibilité de moduler l'activité des cellules de l'hôte au niveau du site d'implantation. La fonctionnalisation de ces hydrogels ouvre le champ à de multiples possibilités. Avec l'utilisation de facteurs adaptés il est possible non seulement d'accélérer la colonisation par des cellules épithéliales mais également d'envisager une différenciation de cellules souches mésenchymateuses vers des cellules épithéliales. L'encapsulation de macrophages et l'utilisation de cytokines de l'inflammation adaptées dans ces hydrogels permettent d'orienter l'activité des cellules inflammatoires qui colonisent le dispositif implantable. Cette immunomodulation lors de l'utilisation d'un dispositif est un atout majeur pour pallier les risques de rejet des implants en orientant la réaction inflammatoire vers une voie pro-cicatrisante.

Ces hydrogels se présentent dès lors comme des plateformes servant de support pour une repousse de l'épithélium mais qui régulent et orientent également l'activité des cellules colonisant le dispositif.

Dans la perspective de l'utilisation des hydrogels en pratique clinique, différentes étapes seront néanmoins encore nécessaires. Les interactions entre les différents facteurs de fonctionnalisation ajoutés devront être étudiées avant de débuter les essais in-vivo avec des expérimentations animales.

A terme ces films développés pour accélérer la ré-épithélialisation de la face endoluminale du larynx artificiel offrent d'autres champs d'application. En oto-rhino-laryngologie et chirurgie cervico-faciale ils pourraient être utilisés dans le cadre de myringoplasties en remplacement de greffons d'aponévrose musculaire ou bien de périchondre. Leur utilisation dans le

traitement des perforations du septum nasale est également une piste d'utilisation en pratique clinique courante. Dans ces champs d'application, la forme la plus pertinente de commercialisation semble être l'utilisation des hydrogels déshydratés et stérilisés qui seraient fonctionnalises au moment du geste chirurgical par adjonction des différents facteurs sous forme liquide.

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Listes des publications

Double thin film-based sandwich-cell carrier design for multicellular tissue engineering : **Ciftci, S.**, Barthes, J., Lavalle, P., Özçelik, H., Debry, C., Dupret-Bories, A., & Vrana, N. E. (2016) *Materials & Design*, *95*, 648-655.

Engineering Trachea and Larynx (book chapter for inclusion in "Tissue Engineering for Artificial Organs".) :
Marta B. Evangelista, PhD; **Sait Ciftci**, MD; Peter Milad, MD; Emmanuel Martinod , MD, PhD; Agnes Dupret-Bories, MD,
PhD; Christian Debry, MD, PhD; Nihal Engin Vrana, PhD (Wiley)

Incorporation of Resident Macrophages in Engineered Tissues: Multiple Cell Type Response to Microenvironment Controlled Macrophage-laden Gelatin Hydrogels.
Dollinger C, **Ciftci S**, Knopf-Marques H, Guner R, Ghaemmaghami AM, Debry C, Barthès J, Vrana NE. J Tissue Eng Regen Med. 2017 May 8.

A composite Gelatin/hyaluronic acid hydrogel as a basement membrane mimic for developing mesenchymal stem cell derived epithelial tissue patches

Pramod. Kumar, **Sait. Ciftci**, Julien. Barthes, Helena. Knopf-Marques, Céline. Blandine Muller, Christian. Debry, Nihal Engin Vrana & Amir M. Ghaemmaghami
J Tissue Eng Regen Med (accepted in September 2019)

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Utilisation d'hydrogels fonctionnalisés pour une ré-épithélialisation rapide des implants hybrides en ingénierie tissulaire.

Résumé

Dans le cadre du développement d'un larynx artificiel, les expérimentations sur l'animal et les essais cliniques ont mis en évidence un défaut de ré-épithélialisation de la face endoluminale de la prothèse. Cet épithélium respiratoire est absolument nécessaire pour obtenir un dispositif implantable totalement intégré dans le corps mais également pour la fonctionnalité d'un tel implant. Dans ce travail nous avons développé de nouveaux films d'hydrogels de collagène et d'acide hyaluronique interpénétrés et réticulés pour assurer une repousse épithéliale rapide. Ces films d'hydrogels optimisés ont une résistance suffisante à l'hydrolyse pour limiter leur dégradation précoce une fois implantés. Ils ont été fonctionnalisés par des facteurs de croissance et de différenciation cellulaire libérés de façon progressive avec un résultat objectivé sur la prolifération cellulaire. L'encapsulation de cellules immunitaire et l'utilisation de cytokines dans ces gels permettent également de moduler la réponse inflammatoire vers un processus de cicatrisation plutôt que de rejet.

Mots clés : Larynx, Epithélium Respiratoire, Hydrogels, Intégration, Ingénierie tissulaire, Implants.

Summary

As part of the development of an artificial larynx, in vivo experiments and clinical trials have revealed a defect in re-epithelialization of the endoluminal side of the prosthesis. This respiratory epithelium is absolutely necessary to obtain an implantable device fully integrated into the body but also for the functionality of such an implant. In this work we have developed patches of interpenetrated and reticulated hydrogels based on collagen and hyaluronic acid to ensure rapid epithelial regrowth. These optimized hydrogel patches have sufficient resistance to hydrolysis to limit their early degradation once implanted. They have been functionalized by growth and cell differentiation factors that are released gradually with an objectified result on cell proliferation. Encapsulation of immune cells and the use of cytokines in these gels also modulate the inflammatory response towards a healing process rather than rejection.

Key words: Larynx, Epithelium, Respiratory, Hydrogels, Integration, Implants, Tissue engineering