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Tissue Engineering of a Tracheal Equivalent

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Dedication

There is a Chinese saying
May You Live in Interesting Times

Like science, it is both a blessing and a curse

This thesis is dedicated to the former inmates of the Subiaco house of madness. It was indeed a case of living in interesting times

Aknowledgements

Should i write down all the people whom in some way ,minutesor or great, that have made this possiblity a reality, this work would resemble a whos-who in the world of Brian Ziegelaar, rather than thesis of science So i will mention only a few.

Those unmentioned, remember you may not be recorded, but you are far from forgotten.

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Abstract

Lectin histochemistry and scanning electron microscopy (SEM) was used to assess the growth and characterise the differentiation of human respiratory epithelial cells (REC) cultured on two biomaterial scaffolds. The first scaffold, based on a hyaluronic acid derivative, was observed to be non-adhesive for REC. This lack of adhesion was found to be unrelated to the presence of the hyaluronic acid binding domain on the surface of isolated REC. The other scaffold, consisting of equine collagen, was observed to encourage REC spreading and adhesion. Positive Ulex Europaeus agglutinin (UEA) lectin staining of this preparation indicated the presence of ciliated REC on the scaffold surface. However, the marked decrease in peanut agglutinin (PNA) positive staining, relative to that of control cultures and native tissue, indicates a dedifferentiation of the secretory cells in monolayer. SEM analysis of REC cultured on the collagen scaffold confirmed the presence of ciliated cells thereby validating the UEA positive staining. The presence of both established and developing cilia was also verified. This indicates that collagen biomaterials are appropriate for the tissue engineering of REC. Furthermore, that UEA and PNA staining is a useful tool in the characterisation of cells cultured on biomaterials, therefore helpful in identifying biomaterials that are suitable for specific tissue engineering purposes.

The culture of REC at an air liquid interface (ALI) was investigated. Both conventional ALI inserts and the Biofleece scaffold were used. The cells grown the on conventional inserts became multilayered and showed some degree of ciliation after the period of ten days. The cells grown on the Biofleece scaffold became necrotic

and died due to nutrient deprivation. The use of ALI culture techniques on scaffold materials needs to be adjusted to allow for sufficient nutrient supply to the cells.

The Biofleece scaffold was found to be suitable for the tissue engineering of cartilage in vitro. Constructs with a cartilage-like morphology were generated with the scaffold after two weeks in culture. The tissue-engineered cartilage was found to contain a higher number of cells and less extracellular matrix (ECM) than the native tissue controls. Suction seeding techniques were used to improve the distribution of cells within the scaffold and thereby increase the overall efficiency of cartilage tissue engineering within the scaffold. Alcian blue (AB) and Papanicolau (PN) stains of the tissue engineered cartilage described two distinct regions within the constructs, namely the developed cartilage-like region and the developing region. The latter is thought to be areas in which the cartilage cells are yet to fully remodel the scaffold material and deposit their own “native” ECM. However, the Biofleece scaffold material was observed to loose 40-50% of its initial volume during the tissue engineering process over a period of two weeks. Thus the degradation of the Biofleece scaffold exceeds the rate of maturation of the cartilage tissue within the scaffold. This rapid biodegradation is most likely a result of matrixmetalloproteinase (MMP), in particular collagenase, production by the maturing chondrocytes. This reduction in size means that the Biofleece scaffold is not an appropriate material for the tissue engineering of a trachea. The optimal biomaterial for the tissue engineering of a trachea would degrade at a rate equal too, or slower than, the time taken for the cells within the scaffold to mature into functional tissue.

The co-culture of REC and chondrocytes was achieved through the use of matrigel as a basement membrane replacement (note that direct growth of REC on cartilage tissue has been observed to be difficult). The co-cultured constructs were not stable because the Biofleece scaffold degrades at a high rate in the presence of both cell

types. The constructs were observed to shrink to approximately 35-30% of the original dimensions in a period of 3-7 days. The reason for this accelerated degradation is not known but is most likely the result of severe MMP production by the two cell types when in combination.

It was concluded that the characterisation procedures used in this study (histochemical staining, fluorescent staining and scanning electron microscopy) for both REC and chondrocyte tissue engineered constructs are appropriate for this and further studies. The chondrocyte seeding methodologies in particular are a useful tool for tissue engineering. This study succeeds in many ways to investigate the tissue engineering of a tracheal substitute by detailing how REC and chondrocytes can be cultured on biomaterials and assessed for tissue development. However, the study does not deliver such a viable substitute as an end product. The primary reason for this outcome is the rapid degradation of the Biofleece scaffold material.

Zusammenfassung

Lectin Histochemie und Elektronenmikroskopie wurden benutzt, um das Wachstum von humanen respiratorischen Epithelzellen (RECs), welche auf zwei Biomaterialien kultiviert wurden, festzusetzen und ihren Differenzierungsgrad zu bestimmen. Das erste Trägermaterial, welches auf einem Hyaluronsäurederivat basiert, ließ keine Anheftung der RECs zu. Diese fehlende Anheftung ließ sich jedoch nicht zurückführen auf das Vorhandensein der Hyaluronsäure bindenden Domaine auf der Oberfläche isolierter RECs. Das andere Trägermaterial, aus Pferdekollagen hergestellt, zeigte dagegen eine verstärkte Teilungsaktivität und Anheftung der REC. Die positive Ulex Europaeus Agglutinin (UEA) Lectin Färbung dieser Proben ließ die Anwesenheit von mit Zilien versehenen RECs auf der Trägerstoffoberfläche vermuten. Darüber hinaus weist das im Vergleich zu Kontrollkulturen und nativem Gewebe deutliche Nachlassen der positiven Peanut Agglutinin–Färbereaktion auf eine Dedifferenzierung der sekretorischen Zellen in der Monolayer-Kultur hin. Die rasterelektronenmikroskopische Untersuchung der auf dem Kollagenbiomaterial kultivierten RECs bestätigte das Auftreten von Zellen mit Zilien und damit auch die Aussagekräftigkeit der positiven UEA–Färbung. Dies zeigt somit, dass Biomaterialien aus Kollagen für das Tissue Engineering von RECs geeignet sind und dass sowohl die UEA–als auch die PNA–Färbung geeignete Methoden zur Charakterisierung von Zellen darstellen, die auf Biomaterialien kultiviert wurden. Somit helfen sie bei der Identifizierung von Biomaterialien für bestimmte Einsatzgebiete im Tissue Engineering.

Des weiteren wurde die Kultivierung von RECs auf einem Air liquid interface (ALI) untersucht, wobei sowohl der konventionelle ALI–Einsatz als auch das

Biovliesmaterial zum Einsatz kamen. Dabei wuchsen die Zellen auf dem konventionellen Einsatz in Multilayern und zeigten nach einem Zeitraum von 10 Tagen einen bestimmten Anteil an Ziliierung. Die Zellen auf dem Biovlies dagegen wurden nekrotisch und gingen schließlich an Nahrungsmangel ein. Deshalb muss der Einsatz von ALI–Kulturtechniken bei Trägermaterialien dementsprechend modifiziert werden, dass eine ausreichende Versorgung der Zellen mit Nährstoffen gewährleistet ist.

Für das in vitro–Tissue Engineering von Knorpel erwies sich das Biovlies jedoch als geeignet. Mit ihm konnten nach zwei Wochen Kulturzeit Konstrukte mit einer knorpelähnlichen Morphologie erzeugt werden. Dabei zeigte sich, dass der Tissue Engineering–Knorpel eine höhere Zellzahl bei reduzierter extrazellulärer Matrix (ECM) aufwies als vergleichbares natives Kontrollgewebe. Dabei wurden Saugtechniken benutzt, um die Verteilung der Zellen im Trägerstoff zu verbessern. Die Alzian – Blau – Färbung (AB) und Papanicolau – Färbung (PN) zeigten bei dem Tissue Engineering–Knorpel zwei unterschiedliche Regionen innerhalb des Konstrukts, nämlich eine knorpelähnliche bereits entwickelte Region und eine sich entwickelnde Region. Bei letzterer dürfte es sich wohl um Gebiete handeln, in denen Zellen noch im Begriff sind, den Trägerstoff vollends umzubauen und ihre eigene „native“ ECM abzulagern. Nichtsdestoweniger büßte das Biovlies während des Tissue Engineering Prozesses über einen Zeitraum von zwei Wochen annähernd 40-50 % seines anfänglichen Volumens ein. Somit übersteigt das Ausmaß der Degradation des Biovlieses das des Heranreifens von Knorpelgewebe in dem Trägermaterial. Diese schnelle Biodegradation ist am ehesten das Ergebnis der Aktivität von Matrixmetalloproteinasen (MMP), insbesondere der Kollagenase, welche von reifenden Chondrozyten produziert wird. Diese Schrumpfung bedeutet also, dass das Biovlies kein geeignetes Material für das Tissue Engineering der

Trachea darstellt. Denn ein optimales Biomaterial für das Tissue Engineering der Trachea sollte sich innerhalb derselben Zeit bzw. über einen längeren Zeitraum hinweg abbauen, als innerhalb desjenigen, den die sich in dem Trägermaterial befindlichen Zellen benötigen, um zu funktionalem Gewebe heranzureifen.

Durch den Einsatz von Matrigel als Ersatz für die Basalmembran konnte eine Kokultur aus RECs und Chondrozyten etabliert werden (wobei anzumerken ist, dass sich direktes Wachstum von RECs auf Knorpelgewebe als problematisch erweist). Die Konstrukte aus Kokulturen waren nicht stabil, da das Biovlies in Anwesenheit beider Zelltypen hochgradig abgebaut wird. Innerhalb von 3–7 Tagen schrumpften die Konstrukte auf ca. 35–50 % ihrer Ausgangsgröße zusammen. Der Grund für diesen beschleunigten Abbau ist unbekannt, jedoch ist am ehesten eine ausgeprägte Produktion von MMP durch die beiden Zellarten anzunehmen, sobald diese in Kombination vorliegen.

Insgesamt lässt sich sagen, dass die Methoden zur Zell- und Gewebecharakterisierung, welche in dieser Studie benutzt wurden (histochemische Färbungen, Fluoreszenzfärbung und Elektronenmikroskopie) sowohl für mit RECs als auch mit Chondrozyten hergestellte Konstrukte für die vorliegende Arbeit als auch zukünftige Studien als geeignet anzusehen sind. Diese Studie hat in vielerlei Hinsicht erfolgreich das Tissue Engineering einer Luftröhre untersuchen können, indem sie im Detail aufzeigt, wie RECs und Chondrozyten auf Biomaterialien kultiviert und für das Tissue Engineering eingesetzt werden können. Trotzdem kann diese Arbeit kein einsetzbares Ersatzmaterial als Endprodukt liefern. Der Hauptgrund für dieses Ergebnis ist in erster Linie in dem schnellen Abbau des Biovlieses als Trägermaterial zu sehen.

Declaration

I B. W Ziegelaar Declare that this work was conducted by myself for the purpose of submission as a doctoral thesis and is not a reproduction of any previous work by myself or other authors. Furthermore this work has not been submitted in any form to another university for promotion.

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CHAPTER 1

Introduction

1.0 Introduction

To date, many attempts have been made to create non-autologous tracheal prosthetics for use in reconstructive surgery. The use of a variety of "non-degradable" materials such as polytetrafluorethylene (PTFE), composite Teflon-Marlex mesh and silicone have yielded varied results [1-3]. All have failed to reach successful clinical application due to complications such as, formation of granulation tissue, stenosis and tissue melting proximal to the implant site [1-3]. Investigations into the use of autologous grafts have been conducted. Carbignani et al. experimented with the use of cryopreserved aortic allografts as tracheal replacements after wide resections [4]. They reported suitable neovascularisation, low rejection but high proliferation of fibroblasts on the luminal side of the implant which was somewhat limited by insertion of a silicone tube inside the allograft. Similarly, cartilaginous allografts have also been used to repair long segment tracheal defects in piglets with some success and the lumen of the grafts were found to be lined with respiratory epithelium in which ciliation was present [5]. The use of allografts has some drawbacks such as graft availability and the use of non- human tissues for surgical procedures. However, they do indicate that the idea of tissue-based therapies is a promising one. Therefore, an investigation into the feasibility of engineering an autologous tracheal replacement from patient tissue is pertinent. Indeed, this has already been recognised by others in the field of tissue engineering [6]. The aim of this study is to investigate the use of biodegradable scaffolds for tissue engineering a tracheal prosthetic.

1.1 What is tissue engineering?

Vacanti defines tissue engineering as, "an interdisciplinary field in which the principles of engineering and the life sciences are applied toward the generation of biological substitutes aimed at the creation, preservation or restoration of lost organ

function“ [7]. However, a more pragmatic definition of tissue engineering is, the generation of functioning human tissue replacements through the use of modern cell culture techniques and biodegradable “scaffold” materials.

To date tissue engineering has been applied to many of the bodies organ systems, the generation of connective tissues, such as cartilage and bone, being the most widely publicised [8, 9]. However, tissue engineering of neurons for maxillofacial surgery, encapsulation of islet cells for diabetic disorders, liver cells, heart valves, muscle tissue, tendon and ligament generation, and a host of other applications are currently being investigated [7 and 10, 11, 12, 13].

1.2 Cells and scaffolds

The object of tissue engineering is to create tissue replacements that are adaptable to the physical and biological environment in vivo. To further explain, we first need to look at the components of the tissue engineering approach. There are two primary components; these are the cells and the biodegradable scaffolds into which the cells are to be placed. The normal starting point of tissue engineering is the harvesting of the type of cells needed for seeding into the scaffold. It is at this point that two problems arise, that of cell number and cell dedifferentiation.

1.2.1 Cell Sources

The standard method to generate cells for tissue engineering is to take a small biopsy from the donor (in most cases the patient requiring implantation of a tissue engineered product) and isolate the cells using an enzymatic digest to release the cells from the surrounding tissue of the biopsy. This biopsy is often of the same

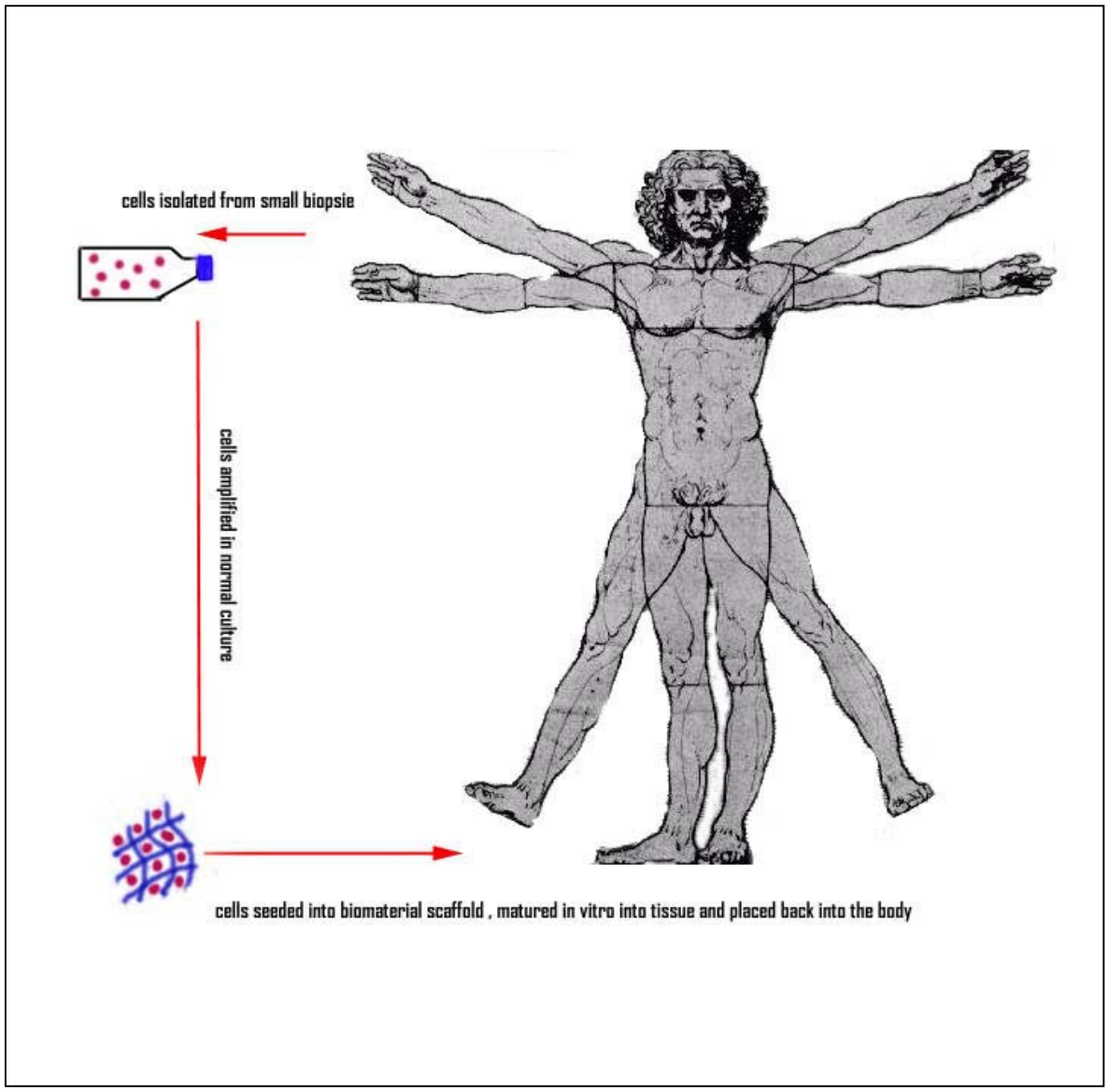
tissue type, which is to be tissue engineered. The digestion of such biopsies results in a small cell harvest, which does not often allow for the optimal seeding of a scaffold. Naturally, a bigger biopsy would generate more cells following a digest but a biopsy is a wound all of its own and to create a big wound to fix a defect in the body is just not sensible. For example, if one were to generate cartilage for a knee reconstruction of the left knee it would be pointless to take a large cartilage biopsy from the patients right knee in order to have enough cells to reconstruct the left. Therefore, harvested cells are amplified in number by conventional tissue culture techniques in vitro and then seeded into the biodegradable scaffold. This has been the central dogma of tissue engineering for many years and will henceforth be referred to as simple tissue engineering throughout this work.

1.2.2 Simple tissue engineering

Simple tissue engineering can be considered a three-step process. First, the desired cells are isolated from a small biopsy from the patient; these are then amplified in number using traditional cell culture techniques. The second step involves the impregnation of these cells into a biodegradable scaffold. This scaffold gives the cells a temporary structure in which to further proliferate and form into functioning organ-like units. The third and final step is the maturation of the cell-biomaterial construct into a functional tissue mass. This maturation process is dependent on many factors, which will be addressed briefly. For now it is sufficient to say, that the biomaterial must have favourable properties to encourage the formation of the desired functioning tissue mass by the impregnated cells. In addition, the culture conditions to which the cell-biomaterial construct is subjected must also be favourable to the maturation process. One example of tissue created in this way is the keratinocyte

based artificial skin used extensively in the treatment of burn wounds [14, 15] .The work contained in this thesis is primarily of the simple tissue engineering approach.

Figure 1. Tissue engineering in its simple form.

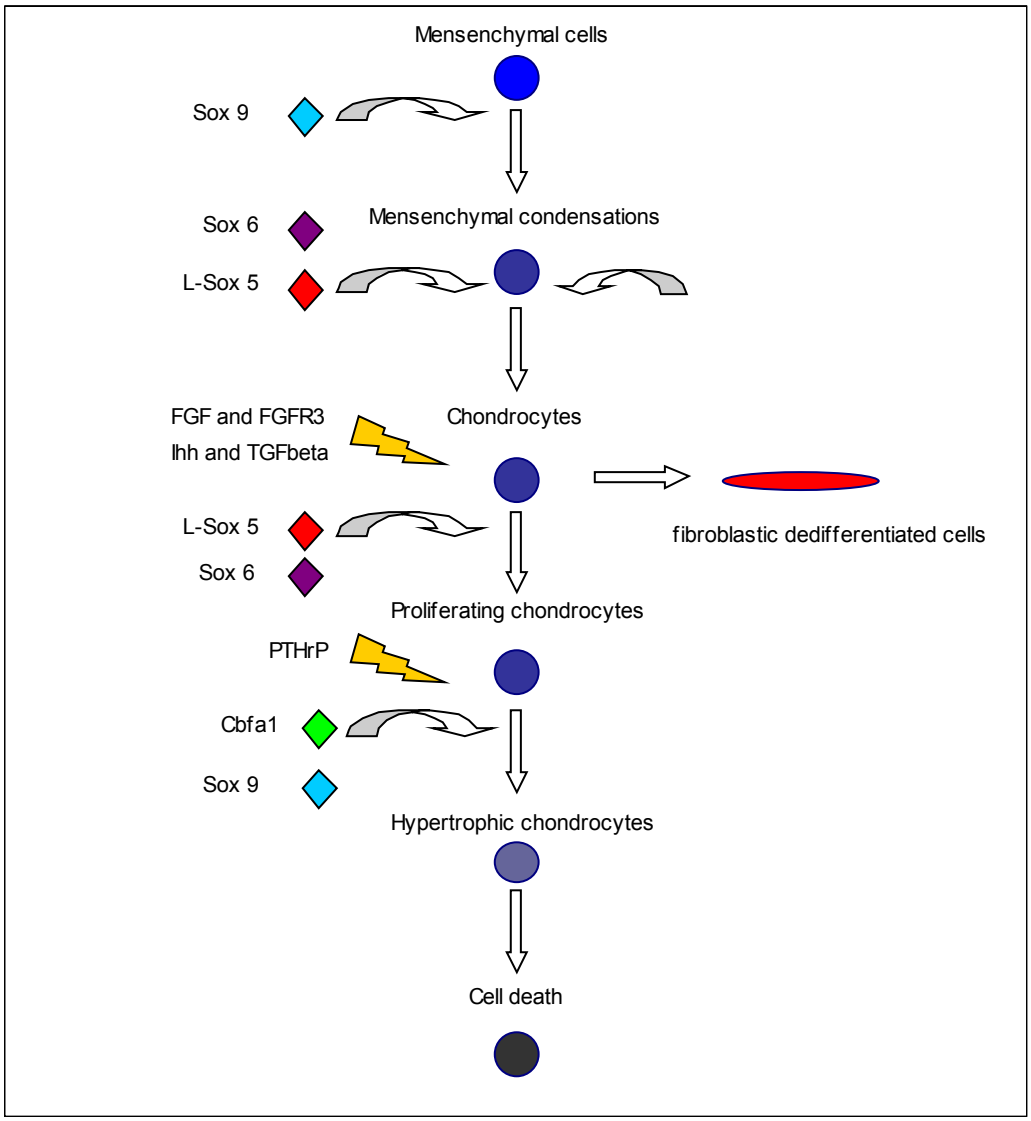


The problem with the simple tissue engineering approach is that cells outside of their normal in vivo environment tend to dedifferentiate [16]. The process of dedifferentiation is a constant problem when amplifying cells from primary tissue biopsies by conventional cell culture techniques. Differentiation can be simply defined

as the process of cell maturation from a progenitor cell to a fully functioning adult cell type that expresses certain specific cell markers and carries out certain specific biochemical, structural, and biological functions. This process involves the influence of many growth factors, signalling pathways and sometimes the interaction with other cell types. An example of how complex the process of differentiation can be readily seen in figure two, which summarises cartilage differentiation. The sox family of transcription factors is vital to cartilage development [17]. These combined with the fibroblastic growth factors, the transforming growth factors and the effect of the various ECM components direct the differentiation and proliferation of cartilage cells [17,18]. The parathyroid hormone related proteins and the cbaf1 are involved the formation of hypertrophic chondrocytes [17]. Dedifferentiation is the process whereby cells loose the mature adult status. In most cases this is not a backwards step down the process of maturation but more of a sideward step to an alternative cell form. Dedifferentiated cells loose many of the biochemical, physical and morphological characteristics which allowed them to function as an integral part of an organ system. For example, dedifferentiated chondrocytes change shape to similar to that of a fibroblast and loose the potential to produce collagen type II an integral extracellular matrix (ECM) component of mature cartilage [19-21]. Likewise, respiratory epithelial cells (REC) loose the ability to produce mucus and become fibroblastic and non ciliated [22, 23]. However, the process of differentiation in most cases is not terminal if some criteria are met during the amplification procedure. First, the amplification of the primary cells must be limited in number and time [16]. Second, the use of correct growth media with additional supplements that maintain cellular differentiation is also important. In recent years the development of special media for particular primary cells has progressed significantly. Finally, the culture of cells under correct conditions, those approximating the in vivo environment, also inhibits

dedifferentiation. For example, the growing of respiratory cells on collagen coated plates and at air-liquid interfaces (ALI) has been shown to inhibit dedifferentiation during amplification [24]. Furthermore, dedifferentiated REC can be encouraged to redifferentiate to ciliated REC if grown on a collagen basement membrane like structure at an air liquid interface [24,25]. However, highly amplified REC tend toward terminal dedifferentiation and remain fibroblastic even when cultured under these favourable conditions.

Figure 2. Differentiation of Chondrocytes (adapted from de Crombrugghe et al. Curr. Opin. Cell. Biol. 2001).



1.2.2 Alternative cell sources

One alternative to simple tissue engineering is the use of universal cell types such as mesenchymal stem cells from the bone marrow. Recent research indicates that the bone marrow, the peripheral blood and most mature tissues contain resident progenitor cells that can be cultured and used for generating new tissue [26-28]. Stem cells are unique because unlike differentiated cells they have a capacity to transdifferentiate to cells of a wide variety of tissues. After embryonic development most tissues are fully differentiated and any further growth or repair is undertaken by resident stem cells within the tissues [27,28]. The best-characterised population of stem cells are the hematopoetic stem cells, which replenish all of the differentiated cells of the blood. The identification, isolation and culture of stem cells allows for the generation of large cell pools that are then able to be used to generate a wide variety of tissues due to the inherent plasticity of these cells.

1.2.3 Scaffolds

It must be stressed tissue engineering is different from the field of biomaterials. Biomaterial is a field in which artificial substances are used to replace tissue functions. Some examples of successful uses of biomaterial substitutes are the artificial heart [29] and the artificial cornea (Chirila Keratoprostheis) [30, 31]. In both these cases polymeric materials have been use to create a device that effectively replaces the non-functioning tissue. The key word in tissue engineering is “biodegradable”. The artificial scaffold that nurtures the dissociated cells to maturation must, in the end, be completely degraded leaving only a mass of functioning generated tissue. As can be expected many of the problems associated with the field of biomaterials are present in the field of tissue engineering, namely those of biocompatibility and structural integrity. Hutmacher, detailed that a scaffold

must ideally have the following characteristics; (i) three dimensional structure, with highly porous network for cell growth and flow of nutrients; (ii) biocompatiblibility and bioresorbility with a controllable degradation and resorption rate to match cell/tissue growth in vitro and in vivo; (iii) suitable surface chemistry for cell attachment, proliferation and differentiation and (iv) mechanical properties to match those of the tissue at the site of implantation [32].

1.2.4 Scaffolds must be porous

The balance of scaffold integrity, nutrient supply and cellular invasion is paramount to the success of a tissue-engineered product. This balance is regulated primarily by the pore size and the frequency of pores (porosity) of the scaffold. For example, Poly-hydroxymethylmethacrylate (PHEMA) scaffolds were observed to allow for cell invasion with pore sizes as low as 40 microns. However, it was found that optimal cell invasion occurred in scaffolds with a pore size of 80-120 microns [30]. The latter scaffold being rendered less clinically applicable by the resulting decrease in mechanical strength by the increased number and size of interconnected pores within the scaffold structure. Similarly, if a scaffold is not porous enough the nutrient supply to cells in the scaffold will be limited and the cells will die. This is particularly the case if fluids are unable to diffuse through the scaffold material itself. For example, a porous scaffold made of fibrin glue would allow nutrients supply through the pores and by diffusion through the fibrin material itself. Alternatively, a scaffold made of titanium would only allow for nutrient supply only via the pores. In this case nutrient supply and pore size would be an important consideration. The correct nutrient supply to cells within a scaffold is extremely important pre and post implantation.

1.2.5 Tissue maturation and mechanical strength

Biological tissues and organs consist of specialised living cells arrayed within a complex structural and functional framework, generally known as the extracellular matrix (ECM). The ECM is a major contributor to the mechanical and functional properties of tissues and its composition in various tissues differs. The diversity in protein content of the ECM gives rise to particular properties of many tissues. For example, the ECM contributes to rigidity of bone, the resilience of cartilage and the flexibility of tendons. The ECM of tissues is dynamic, in that it can be degraded and deposited by the cells localised within the tissues. In this way, cells of tissues are specialised to create the functional and mechanical properties of the tissue in which they reside. It is this dynamic ability of the ECM that makes it important in both tissue growth and wound healing. In the field of tissue engineering the term tissue maturation is synonymous with ECM remodelling. For example, cartilage consists of few cells surrounded by a rich ECM consisting of collagens, hyaluronic acid, laminins, glycosaminutesoglycans (GAGs) and many other proteins. Chondrocytes seeded into a biomaterial scaffold are initially devoid of this ECM and the progress of a tissue engineered cartilage construct is usually assessed by the deposition of this ECM within the scaffold over time [18, 33, 34]. The eventual aim is the complete disappearance of the scaffold and its replacement by ECM deposited by the cartilage cells. So in retrospect, a biomaterial scaffold must be conducive to the deposition of ECM and also in some cases be a substitute for the mechanical and functional properties while the ECM develops. For this reason one of the big questions in the creation of a tissue engineered product is “how long should the scaffold remain while the tissue develops?” The answer to this question is dependent on the type of product and where it will be implanted. For example, two cases of cartilage tissue

engineering are the generation of cartilage for knee reconstruction and that for auricle reconstruction. The requirement of these two products differs due to the environment of the implantation site. In the case of the auricle reconstruction it is optimal that the scaffold provides some elastic and shape maintaining properties after implantation while the cartilage matures and the deposited ECM is able to provide these properties intrinsically for the tissue. Practitioners in our laboratory propose that such a process could last up to four or six months and that in this time the external structure of the scaffold must not shrink or deform terminally in any fashion. An alternative situation is the model of tissue-engineered cartilage for use in knee reconstructive surgery [35]. This involves the implantation of an established avascular tissue engineered cartilage product under a periosteal flap in the knee joint. In this procedure an atelocollagen scaffold is loaded with amplified cartilage cells in the fashion of "simple tissue engineering" and matured in vitro for 3 weeks prior to implantation. The matured block of cartilage is then surgically shaped to cover the lesion in the knee joint and secured by covering with a periosteal flap. In this scenario the scaffold is not required to be present within the construct post implantation. So the scaffold is only required on a short-term basis, long enough for the cartilage to mature in vitro. An extreme case of short-term scaffold requirement is that of the experimental use of fibrin glue loaded with epithelial cells for the immediate coverage of surgical sites within the Fallopian tubes or as an alternative to normal suture methods [36 and 37]. The fibrin glue-cell mix is used to coat the sites of surgical procedures to encourage normal re-epithelialisation and discourage the formation of scar tissue, which can be detrimental to the chances of later pregnancy. In this case, researchers envisage that the fibrin glue (the scaffold component) needs only to provide a short-term basis for closing the sutureless or reduced suture anastomosis and to encourage the epithelial cells to re-cover the wound site. This

has been observed to be achievable in a matter of 10 days with complete repithelialisation and ciliogenesis present at the wound site [36]. Therefore, the scaffold needs not be preserved beyond this point.

1.2.6 What is biodegradation?

It is pertinent at this point to describe what is meant by biodegradation, or more simply put, what causes a biomaterial scaffold to degrade? In this context the term biodegradable is misleading. A biodegradable material is defined as a solid polymeric material that breaks down due to macromolecular degradation with dispersion but not necessarily elimination within the body [32]. The subsequent loss in molecular mass labels the material as degradable.

The main cause of biodegradation of a polymer within the body can be attributed to the presence of hydrolysable bonds (usually an ester bond) within the polymer structure. A hydrolysable bond is one that is susceptible to nucleophillic attack by a water molecule. Owing to the aqueous nature of the physiological environment a polymer containing a high number of such bonds will be degraded very quickly. The presence of hydrolysable bonds within a biomaterial guarantees that the polymer will be biodegradable. However, the macromolecular degradation of a polymer can also be attributed to other agents, such as, enzymes, super oxides, phagocytic attack or even mechanical stress.

1.2.6.1 Enzymatic degradation

Enzymes within the body are able to aid in the chemical degradation of polymers. An extreme example is the surface cracking of poly(ether urethanes) (PEU) pacemaker

leads. PEU a non-degradable polymer was found to degrade in the presence of esterases such as, cathepsin C and papain. In addition lysosomal enzymes from the liver were also able to degrade PEU [39]. It is thought that these enzymes are able to attack the eurea bond within the polymer. Similar studies on other supposedly non-degradable polymers show that the degradative activities of enzymes are specific to each polymer. The process of how these enzymes degrade such non-degradable polymers is not well defined. Another example of how enzymes degrade biomaterials is that of the so-called bioresorbable materials. This group of materials can be completely digested by the naturally occurring enzymes of the body. Two of the most commonly used resorbable materials in cartilage tissue engineering are collagen and hyaluronic acid. These are degraded by the matrix metalloprotease(MMP) family of enzymes and hyaluronidase respectively [ref for both]. Both collagen and hyaluronic acid are primary components of the ECM in many tissues. Thus the deposition and degradation of these substances is tightly regulated by the localised production of MMPs and hyaluronidase from the cells of these tissues. In the form of a biomaterial these substances can be completely resorbed by these enzymes so that no detectable trace of the original material can be located within the body. Such resorbable materials are thus the focus of much experimentation in the field of tissue engineering.

1.2.6.2 Phagocytic attack

PHEMA is known as a non-degradable substance. That is to say it degrades extremely slowly in its solid hydrogel form. However, PHEMA in the particulate sponge form is susceptible to mechanical and cellular attack, with the bulk polymer mass being reduced in size and particles of the polymer able to be located in nearby

giant cells formed from macrophages that have engulfed part of the material [39]. Similarly, poly(1-vinyl-2-pyrrolidinone) hydrogel (PVP), used as a vitreous substitute in the eye has also observed to be susceptible to phagocytic attack [40] .

1.2.6.3 Oxidative attack

Oxidative degradation of biomaterials is a subject of intense study. Free radicals produced in the body by leukocytes and macrophages are able to aid in the degradation of biomaterials. For example, the surface of biodegradable suture materials were observed to be degraded in the presence of superoxides and that the degradation increased with increasing superoxide concentration [41]. In addition, the degradation of poly(etherurethane)urea (PEUU) was catalysed in the presence of hydroxyl radicals and it was concluded that these radicals are the prime reason for the degradation of PEUU post implantation [42].

1.2.7 Biocompatibility

Biocompatibility has been defined as the ability of a material to carry out a desired function within the body without eliciting and unfavourable host response [43]. Briefly, this means that a material must carry out its function without causing a major inflammatory response from the body's immune system and a major enzymatic response from the cells proximal to the site of implantation. Thus, it is reasonable to suggest that any biomaterial for use in tissue engineering need be only biocompatible for its desired functional duration. For example, in the case of the fibrin glue coating of fallopian tubes the biomaterial need only be biocompatible over a short period. It is

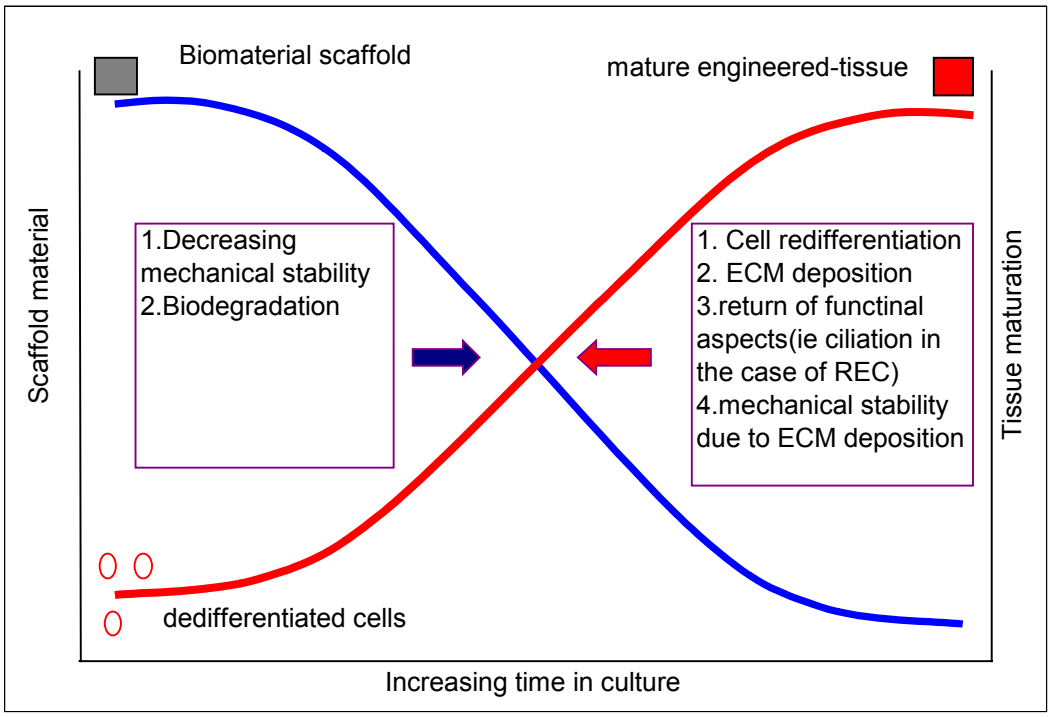
adequate that the fibrin glue in this case does not elicit an immediate and rapid classical inflammatory response. In contrast a biomaterial scaffold that will be retained post implantation for longer periods would also have to be resistant to the slower build-up of unfavourable responses such as tissue melting proximal to the implant site, encapsulation by immune cells recruited to the site of implantation by the complement binding pathways and a host of other potentially detrimental responses.

Another aspect of biocompatibility is that a scaffold should encourage the formation of the desired tissue in vitro and in vivo. This is an often-overlooked aspect of biocompatibility. For example, hyaluronic acid based scaffolds have been observed to limit and even discourage the dedifferentiation of isolated cartilage cells [19]. In the context of producing quality engineered cartilage the inhibition of dedifferentiation is of paramount importance. Dedifferentiated cartilage cells loose the ability to synthesise collagen type II that is a primary component of the ECM in cartilage tissue [20, 44]. The inability to synthesise collagen type II leads to the formation of fibro-cartilage with an ECM high in collagen type I. This alters the mechanical and functional aspects of the cartilage away from what is normal and desired. It should be noted that an in-depth discussion of biocompatibility is beyond the scope of this study.

1.3 Tissue engineering is a balancing act

In summary, tissue engineering can be considered a balancing act between cell growth, cellular dedifferentiation, tissue maturation, scaffold mechanical stability, biocompatibility and biodegradation. Figure three is a summary of the processes involved in this balancing act.

Figure 3. Summary of the aspects of tissue engineering.

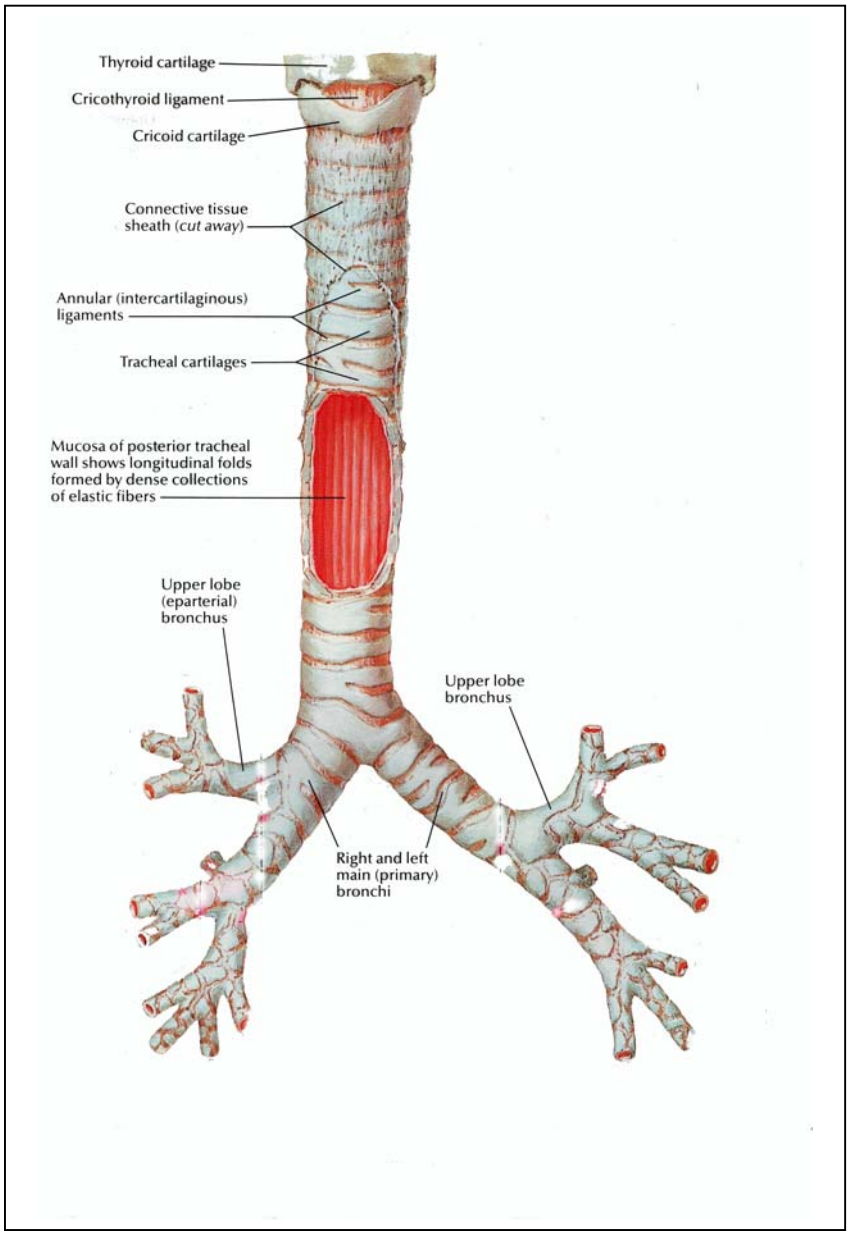


1.4 The human trachea

The trachea is a cartilaginous and membranous tube extending from underneath of the larynx, on level with the six cervical vertebrae to the upper boarder of the fifth thoracic vertebra. The trachea is composed of non-symetrical rings of hyaline cartilage, muscular tissue, fibrous tissue, glandular tissue and a mucous membrane (Figure 4). The cartilage rings of the trachea vary in number from sixteen to twenty. These rings measure about 4mm in height and 1mm in thickness, the inner areas of the rings being thicker than the outside area of the rings. The outer surfaces of the rings are flattened and the inner surfaces are convex. The cartilage rings are enclosed in a fibrous membrane that consists of two layers. At the upper and lower ends of the trachea these layers blend to form a single layer. The muscular tissue

consists of two layers of non-striated muscle which run both transverse and longitudinally.

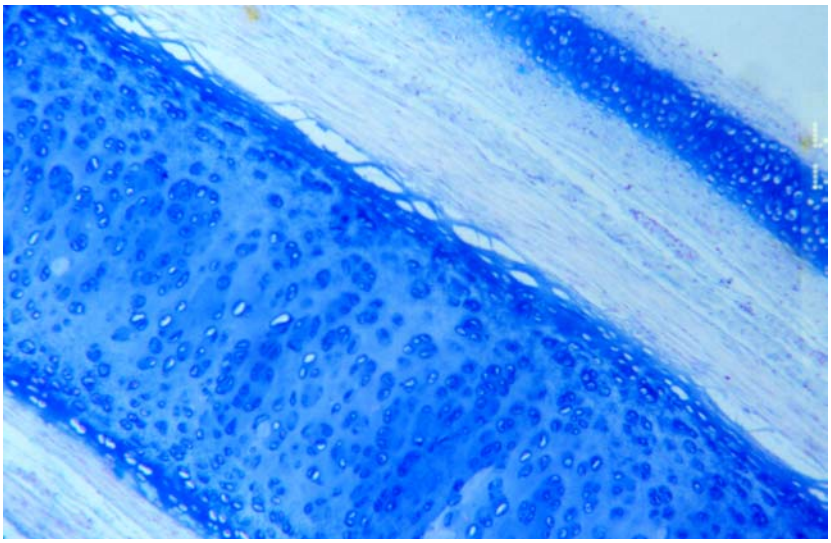
Figure 4. The human trachea (adapted from the Atlas of Human Anatomy: Netter) Showing the cartilaginous rings and gross structure of the trachea.



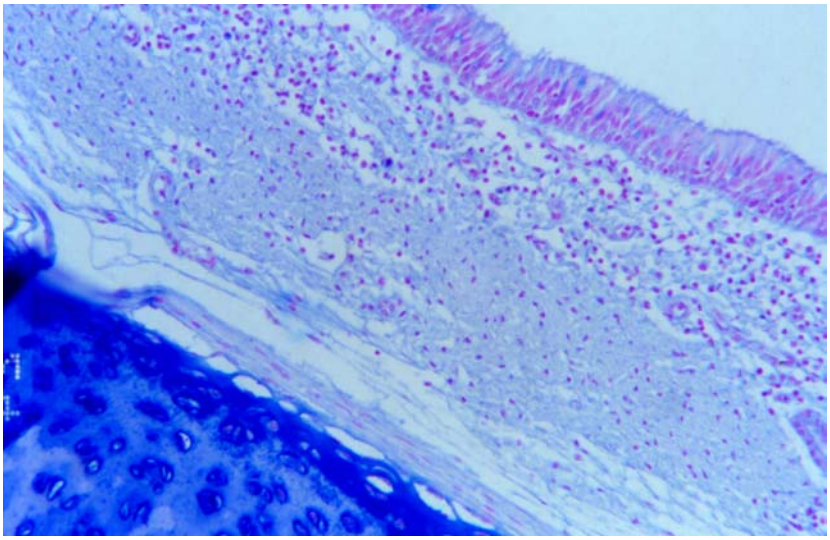
The mucous membrane component (mucosa) consists of respiratory and lymphoid tissue and presents a well-defined basement membrane, which supports a stratified epithelium. The surface of this epithelium consists of columnar cells, which are

ciliated, while the deeper layers consist of more rounded or oval shaped cells these being the basal, and para-basal layers of the respiratory epithelium. Beneath the basement membrane is a loose network of connective and vascular tissue which adjuncts to the hyaline cartilage rings via the two fibrous layers previously mentioned. The cartilage rings consist of relatively few cells encased in an extensive ECM (Figure 5).

Figure 5. The human trachea in transverse section.



5(a) Detailing the morphology of the cartilage surrounded by ECM (stained blue).

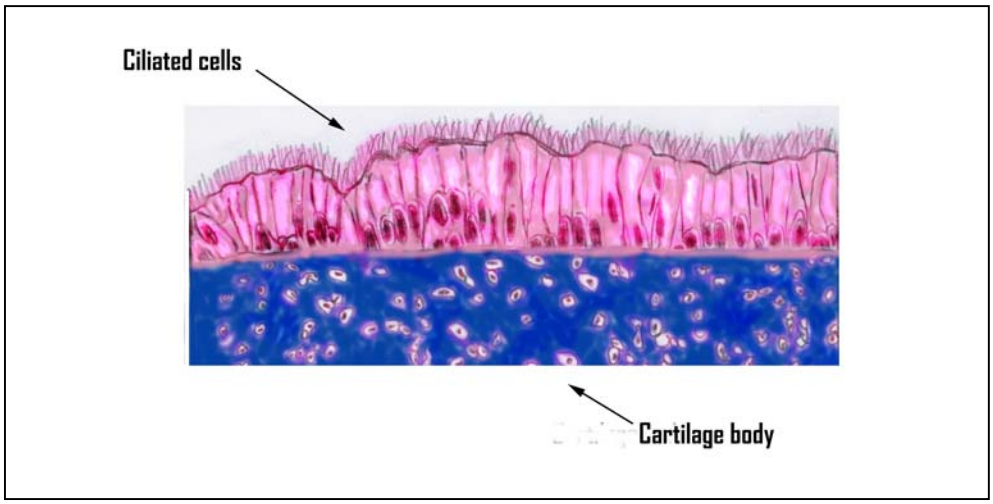


5(b) shows the fibrous vascular layer that is covered by respiratory epithelium at the luminutesal surface of the trachea.

1.5 A simple model for tissue engineering of a tracheal tissue

The aim of this study is to investigate the use of biodegradable scaffolds for tissue engineering a tracheal replacement material. The simplest form of such a trachea prosthetic would be a cartilage body covered by a multilayer of respiratory epithelial cells that could be implanted to replace small excised areas of the trachea thereby avoiding the need for a full thickness resection. Figure 6 summarises this simple proposed model of a tissue engineered tracheal prosthetic using a porous biomaterial scaffold.

Figure 6. A simple model for engineered tracheal tissue.



Key Points

1. **Tissue engineering is engineering is, the generation of functioning human tissue replacements through the use of modern cell culture techniques and biodegradable "scaffold" materials**.

2. **The aim of this study is to investigate the use of various biodegradable scaffolds to engineer a simplified tracheal construct consisting of a cartilage body surfaced with respiratory epithelial cells.**

3. **The process of simple tissue engineering is used throughout this study**.

4. **Tissue engineering is a balancing act of biodegradation and tissue maturation.**

CHAPTER 2

Materials and Methods

2.1. Tissue source for the harvesting of respiratory epithelial cells

Inferior nasal turbinates were obtained from male and female patients ranging in age from 22 years to 55 years, mean age 41. All subjects enrolled in this research have responded to an Informed Consent, which has been approved by the University of Munich Ethical Committee that found this protocol acceptable. The turbinates were placed in phosphate buffered saline (PBS) following surgical extraction. These were then transferred into Dulbecco´s modification of Eagles medium with HAMS-F12 at 50% v/v (DMEM/HAMS-F12) (Biochrom, Germany) supplemented with 10% v/v fetal calf serum (FCS) (Biochrom, Germany), penicillin, streptomycin, L-glutaminutese (PAA Laboratories, Austria), nonessential aminuteso acids, insulin, transferrin, selenium (Sigma, USA) all at 1% v/v. The turbinates were washed in this media three times to reduce the erythrocyte and mucus content on the tissue surface. The turbinates were then placed in fresh DMEM/HAMS-F12 for cell isolation.

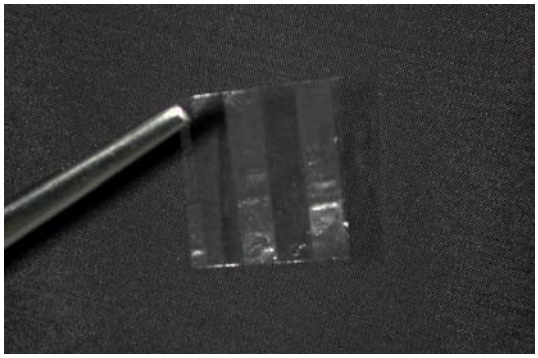
2.2. Biomaterials used in this study

Two biomaterial scaffolds were used in this study: a hyaluronic acid derivative (Hyaff^{TM}, Fidia, Italy) and one constructed from collagen (Biofleece^{TM}, Baxter, USA). For ease of reference we will henceforth refer to these scaffolds as hyaff and Biofleece respectively. The hyaff material is produced in the form of a perforated membrane approximately 1 mm thick and is constituted of 90% esterified hyaluronic acid. The Biofleece material is a composite comprising of a non-woven scaffold, approximately 5 mm thick, with a solid membrane surface on one side. This material consists of native equine collagen types I and III. The membrane surface was used for culture of the REC in this study. All the biomaterial samples used in this study

were cut into squares of approximately 1cm by 1cm. Note that the thickness of the materials was predetermined by company manufacture as detailed above.

Figure 7: Macroscopic images of the two biomaterials used in this study.

Biofleece collagen scaffold



Hyaluronic acid scaffold

2.3. Isolation and culture of respiratory epithelial cells on biomaterials scaffolds and tissue culture plastic

REC were isolated from turbinates using a modified dispase dissociation technique. Turbinates were incubated in DMEM/HAMS F12 supplemented medium containing dispase (1 U/ml) for approximately 35-40 minutes at 37^{o}C in an atmosphere of 5% CO_2. After which the turbinates appeared white in color and fluffy in texture. The turbinates were then placed in 10 ml fresh DMEM/HAMS F12 medium, supplemented with 10% v/v fetal calf serum (FCS), L-glutaminutese, penicillin, streptomycin, non essential amino acids, insulin transferrin and selenium at 1% v/v. Sheets of REC were gently brushed off the surface of the turbinates using the back edge of a scalpel. The remaining turbinate tissue was then discarded leaving the cell suspension for use in experiments. Due to the short time duration of the dispase treatment, large sheets of cells, rather than individuals or groups were isolated. Other reported isolation methods use 5 U/ml dispase overnight at 2-4^{o}C, which in our experience yields a much more dissociated cell population [22]. Sterile scaffolds of

approximately 1.3 cm in diameter were pre-incubated for 2 hours in the supplemented DMEM/HAMS F12 as described previously at a temperature of 37 ^{o}C in an atmosphere of 5% CO_2. The biomaterials were then placed in a 24-well plate (Falcon, USA) and 2 ml suspension of REC sheets at an approximate concentration of 10^5 cells/ml was applied to the surface of the scaffolds. In addition, a cell suspension was also placed into an empty well for the purpose of a control, using only tissue culture plastic (TCP) as the culture surface. Cell number and viability was assessed by toluidine blue staining using a standard 1 μl counting chamber (Madaus Diagnostic, Germany). The cell number is an approximation as it is difficult to assess the number of cells contained in an epithelial sheet. Cells were cultured on the scaffolds for 5 days during which the media was changed only on day 3.

2.4. Preparation of native nasal turbinates for a native tissue control

Two sets of Nasal turbinates that were not subjected to the dispase treatment were prepared as a comparative control for the cultured REC. Whole turbinates were placed in cryomoulds and immersed in Tissue-TEK embedding compound (O.C.T, USA). These were then frozen in liquid nitrogen and stored at -20^{o}C before cryosectioning. Sections of 10 μm of the prepared turbinates were made on a Frigocut 2800 cryostat (Reichert-Jung, Germany). These were further stored at -20^{o}C until evaluation by lectin staining and fluorescence microscopy.

2.5. Cell staining with lectins

Two lectins, Ulex Europaeus agglutinin (UEA) directly conjugated with rhodamine and peanut agglutinin (PNA) direct conjugated with fluorescein (Vector Laboratories,

USA), were used in the assessment of both native tissue and cultured REC. Five separate stainings were conducted for both native tissue and cultured REC. Cells cultured on TCP and the biomaterial scaffolds were fixed by immersion for 5 minutes in an 70% v/v ice cold ethanol solution made up in Hank´s phosphate buffered saline containing calcium. They were then washed three times for 5 minutes in (PBS, pH 7.4) and incubated at room temperature for 1 hour with both lectin probes at a concentration of 20 μg/ml. After incubation, the fixed cells were washed three times for 5 minutes in PBS. The samples were then analysed on an Axiovert 135 microscope (Zeiss, Germany) with positive UEA stained cells appearing red and PNA staining appearing green. REC growth on the scaffolds was further analysed by the use of a Hoechst 33258 counterstain (Sigma, USA), which stains the cell nuclei blue. In this procedure, a 1:100 v/v-diluted solution of Hoechst counterstain at a concentration of 1 mg/ml in PBS was applied to the specimens for 1 minute. The specimens were then washed with fresh PBS to avoid overstaining and observed by fluorescence microscopy.

2.6. Assessment of hyaluronic acid binding domain of respiratory epithelial cells in culture and in native tissue

An investigation into the expression of the hyaluronic acid binding domain on REC was conducted using three REC cultures isolated from three separate turbinates. REC cultured for 5 days on 4 separate Hyaff scaffolds were analysed for the hyaluronic acid binding domain using a mouse antihuman IgG monoclonal antibody specific for the domain (H4C4, hybridoma studies bank, USA) [47, 48]. This antibody was further conjugated with a secondary goat anti mouse antibody with Oregon red fluorescence (Alex^{TM} 594, Molecular Probes, USA). In this procedure cultured REC were washed three times in calcium-containing PBS (pH 7.4) for a period of 5

minutes. The cells were then fixed with ice cold 70% ethanol for 10 minutes at room temperature. Following this, the cells were washed three times in PBS in rapid succession to remove the excess of ethanol. The fixed cells were then incubated for 1 hour at room temperature in a 1:100 dilution of the primary HC4H antibody made up in PBS (pH 7.4). The specimens were then washed in PBS three times for a period of 5 minutes, after which they were incubated in a 1:150 dilution of the secondary antibody made up in PBS (pH 7.4) for a further hour. This incubation was carried out at room temperature and in a covered box to avoid degradation of the Oregon red fluorescence conjugate. The specimens were washed a further three times for 5 minutes and analysed for the presence of the hyaluronic binding domain using a Axiovert 135 fluorescent microscope (Zeiss, Germany). A Hoechst counterstain (blue nuclei) was utilised for better visualisation of the cellular morphology. This procedure was repeated with 3 cryosections of native tissue from different turbinates and three 5-day cultures of human epithelial cell line FaDu (laryngeal carcinoma, ATCC, USA).

2.7. Scanning electron microscopy

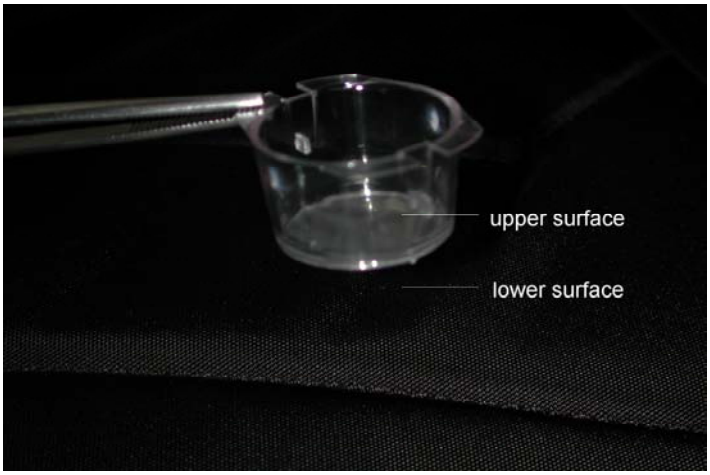
Six samples of REC grown on Biofleece and three native tissue controls were prepared for SEM analysis. The samples were fixed in 5% glutaraldehyde in PBS (pH 7.4) 2 hours at room temperature. The samples were then dehydrated using a graded ethanol series from 10% to 100%, with three times 10-minutes incubations at each step. Dehydration was then completed by critical point drying using CO_2. The mounted specimens were sputter-coated with gold and viewed on an ISM-35FC scanning electron microscope (Jeol, Japan).

2.8 Respiratory epithelial cell growth at an air liquid interface (ALI)

REC were isolated from turbinates using the previously described modified dispase dissociation technique. The isolated cell-sheets were then placed in a culture flask and cultured using a specialised bronchial epithelial growth medium (BECGM) (Promocell, Germany) at a temperature of 37^{o}C in an atmosphere of 5% CO_2. Sheets were observed to adhere to the flask surface over the period of 2-3 days after which the initial medium was replaced with fresh BECGM to aid in the removal of any non-adherent cells, blood cells and debris. Passage zero REC Cells cultured in BECGM as described above were trypsinised in 2.5% trypsin EDTA (PAA Laboratories, Austria) and then placed on pre-incubated ALI cellagen inserts for six well plates (ICN, USA). Pre-incubation of the inserts involved both the lower and upper face of the membrane insert being immersed in a Dulbecco´s modification of Eagles medium with HAMS-F12 at 50% v/v (DMEM/HAMS-F12) (Biochrome, Germany) supplemented with 2% v/v ULTROSER growth supplement (ICN, USA) and penicillin streptomycin at 1% v/v. The immersed membrane inserts were then placed in an incubator at 37 ^{o}C for 1 hour prior to seeding. The REC were seeded on the upper side (Fig 8.) of the inserts at a density of approximately 5x10^{5} cells/ml in a solution of ULTRASER supplemented DMEM/HAMS-F12 and then placed in an incubator for culturing at 37^{o}C in an atmosphere of 5% CO_2 overnight. The medium on the upper surface of the membranes was removed following this period and the cell surface washed once in phosphate buffered saline (PBS) ph 7.2 (PAA laboratories, Austria). The washing step is to remove any non-adhered cells from the cell-membrane surface. Following this washing period, the now air exposed REC layer was cultured at 37^{o}C in an atmosphere of 5% CO_2 utilising the aforementioned modified DMEM/HAMS-F12 medium as the growth medium placed at the lower side of the cell

insert. Regular medium changes were made each day to ensure correct ph and growth conditions. This is a necessary procedure since the small volume of medium able to be placed under the cell insert undergoes rapid pH changes as indicated by the phenol red indicator of the growth medium. In total 33 such cultures were conducted using cells isolated from 22 turbinates form 11 different patients. These cultures were analysed by normal phase microscopy, SEM analysis (see section 2.7 of this chapter) and by a live-dead vitality stain. The large number of test samples used in this study results from the difficulty encountered in preparing the ALI-membrane samples for SEM analysis.

Figure 8. Cellagen cell insert showing upper and lower surfaces of the membrane.



2.8.2 Live-Dead vitality staining of REC grown at the ALI

Samples were placed in live-dead incubation fluid consisting of 50μM carboxymethylfluorescein diacetate and 50mM ethidium bromide homodimer-1 (both supplied by Molecular Probes, USA) made up in PBS. The samples were incubated at room temperature in this solution for approximately 30 seconds and then analysed by fluorescent microscopy using an Axiovert 135 microscope (Zeiss, Germany). An

approximation of the percentage of dead cells in each culture was assessed by comparing live to dead cells within three fields of view in each culture.

2.8.3 Growth of respiratory epithelial cells on Biofleece at the ALI

RECS cultured as above were placed on the Biofleece membrane by the drop-wise method at a concentration of 10^5 cells/ml so as to correlate with the previously detailed method (see section 2.2). The cell-seeded Biofleece was then placed in submerged culture of BECGM overnight in a 6 well plate (Falcon, USA) and was subsequentially exposed to an ALI. The ALI was achieved simply by removing enough media from the 6 well plate to expose only the surface of the Biofleece-cell construct to the air leaving the sides and bottom of the scaffold covered by the medium. Nine such studies were conducted each using three Biofleece samples. These were analysed by SEM microscopy using the method previously described in section 2.7 of this chapter.

2.9 Harvesting and culture of primary Chondrocytes

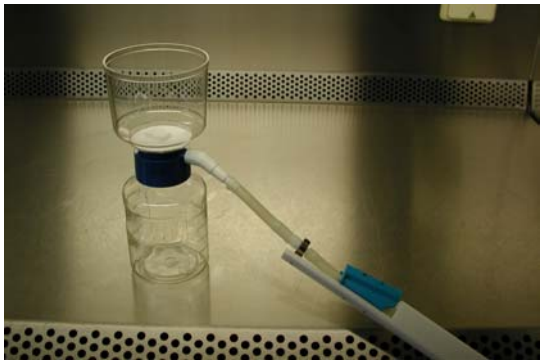
Chondrocytes (cartilage cells) were harvested from nasal septal cartilage of patients varying in age from 20-50 years. The cartilage was first washed in DMEM medium containing 2% v/v penicillin-streptomycin to lower the chance of infections. The washed cartilage was then scraped clean of perichondrium and connective tissue using a scapel. This was to ensure no contamination of the culture by other cell types during the amplification of the chondrocytes. The cartilage was then digested in a 2U/ml collagenase type II solution (GibcoBRL, USA) overnight for approximately seventeen hours. After digestion the cell-enzyme solution was centrifuged at 1000rpm for ten minutes. The supernatant was then removed and the cells re-suspended in DMEM/HAMS F12 medium, supplemented with 10% v/v FCS, L-

glutamine, penicillin, streptomycin, non essential amino acids, insulin transferrin and selenium at 1% v/v. The cells were then amplified in number by traditional tissue culture techniques at a temperature of 37^{o}C in an atmosphere of 5% CO_2.

2.10 Seeding of the Biofleece scaffold with chondrocytes to create cell biomaterial constructs.

Chondrocytes were seeded into the biomaterial by two methods; the conventional drop-wise method and a second method involving the suction of cartilage cells into the fleece by the use of a disposable filtered-vacuum pump for normal filter sterilisation of solutions such as media and buffers (Falcon, USA) (Fig 9.).

Figure 9. Suction apparatus for the seeding of cartilage cells.



Chondrocytes were seeded into the fleece at a concentration of 4x10^5 cells in a volume of 200µl of media, which was added drop-wise to the surface of the fleece in two 100µl units while under suction. Numerous studies were conducted using the drop-wise method of seeding and 18 studies using 6-3 Biofleece samples were conducted using the described suction method.

2.11 Histological analysis of paraffin embedded sections

The cell-biomaterial constructs were placed in 10% formalin (Merck, Germany) and embedded in paraffin and sectioned at a thickness of 4 μm. The sections were then analysed using the alcian blue (AB), haematoxylin eosin (H&E) and papanicolaou (Pap) stains. All sections were prepared for staining with a 5minutes xylol (Sigma, Germany). Following this, the sections were sequentially rehydrated in alcohol at concentrations of 96%, 86% and 70 % v/v for a period of 5 minutes. Following staining all sections were covered with Eukitt (Kindler, Germany) and cover-slipped. All sections were then analysed by bright field microscopy using a Zeiss standard 25 histological microscope (Zeiss, Germany).

2.11.1 Haematoxylin and eosin staining of the treated paraffin sections

The sections were washed in aqua-dest pH 7.0 for 5 minutes after which they were incubated in the haemaloun stain (Merck, Germany) for 5 minutes. The stained sections were then washed 3 times in normal tap water for a period of 5 minutes after which they were incubated in the eosin stain for 5 minutes. The sections were then rinsed once in aqua-dest and subjected to alcohol dehydration by incubation at 70% v/v alcohol for 10 sec; 80% v/v for 30 sec; 96% v/v for 1 minutes; 100% v/v for 5minutes and finally in xylol for a further 5 minutes [44]

2.11.2 Alcian blue (AB) staining of the treated paraffin sections

The sections were washed in aqua-dest pH 7.0 twice for a period of 5 minutes after which they were placed in 3% v/v acetic acid for 3 minutes. The sections were then stained for 30 minutes in alcian blue (Chroma, Germany). The stained sections were

washed in tap water for 2 minutes and counterstained with nuclear red (Chroma, Germany). Following this the sections were washed once more in aqua-dest pH 7.0 and subjected to alcohol dehydration by incubation at 70% v/v alcohol for 10 sec; 80% v/v for 30 sec; 96% v/v for 1 minutes; 100% v/v for 5minutes and finally xylol for a further 5 minutes [44]

2.11.3 Papanicolau (PN) staining of the treated paraffin sections

The sections were washed in tap water for a period of 2 minutes and placed in a haematoxylin stain (Merk, Germany) for a period of 4 minutes. The stained sections were rinsed quickly in tap water and placed into an acid alcohol solution (60ml glacial acetic acid, 100 ml water and 21 ml absolute ethanol) for 5 seconds. The sections were then placed in placed in blue stain with tap water tap water for 10 minutes and sub sequentially incubated in an alcohol series for dehydration for a period of 30 seconds at each step (50% v/v, 70%v/v, 80% v/v and 96% v/v). The sections were then stained with orange G (Merk, Germany) for 10 seconds and rinsed in absolute alcohol twice for a period of 30 sec. Following this the sections were placed in EA 50 stain (Merk, Germany) for 2minutes, rinsed in absolute alcohol for 30 sec and incubated in xylol twice for a period of 2 minutes [44]

2.11.4 May-Grünwald-Giemsa (MG) stainining of the treated paraffin sections

The sections were placed in May-grünwald (Merk, Germany) for approximately 25minutes at a temperature of 60 ^{o}C. These were then placed immediately in aqua dest ph 7.0 for 1 minute. These were then washed quickly in aqua dest pH 7.0 and placed in Giemsa stain (Merck , Germany) for 35 minutes at a temperature of 60 ^{o}C. The sections were then placed briefly in a solution of aqua dest and glacial aceatic acid (Merck, Germany) and then dehydrated by incubation in 70% v/v alcohol for 10

sec; 80% v/v for 30 sec; 96% v/v for 1 minutes; 100% v/v for 5minutes and finally xylol for a further 5 minutes [44].

2.11.5 PeriodicAcid Schiffs (PAS) staining of cellagen ALI inserts

The inserts were rinsed in aqua dest at ph 7.2 and then treated with 1% periodic acid for 10 minutes. These were then washed briefly with aqua dest at ph 7.2 3 times. These were then treated with Schiffs reagent (Merck, Germany) for ten minutes and washed in tap water 3 times for a period of 3 minutes. The nuclei of the cells were stained with Carazzi haematoxylin (Merck, Germany) for a further 2 minutes and then rinsed in alcohol for differentiation allowed to air dry. Following which the inserts were covered with Eukitt (Kindler, Germany) and cover-slipped. All sections were then analysed by bright field microscopy using a Zeiss standard 25 histological microscope (Zeiss, Germany) [44].

2.12 Co-culture of respiratory epithelial cells and chondrocytes

Chondrocytes at a concentration of $4x10^5$ cells were seeded by the suction method onto 6 precut Biofleece scaffolds as previously described (see section 2.10). These constructs were then coated with a cooled (4 ^oC) solution (1 part matrigel 2 parts DMEM-HAMS media without FCS) of the basement membrane-like material matrigel (Sigma-Aldritch, USA). The constructs were then placed in a small amount of the previously described modified DMEM-HAMS F12 media (see section 2.10) taking care not to submerge the surface that was coated in matrigel. The constructs were then placed in an atmosphere of 5% CO_2 at 37 ^oC for 30minutes to allow the matrigel to set and harden. Following this REC at a concentration of 10^5 cells in modified DMEM-HAMS F12 were seeded onto the matrigel surface in two 50µl units. The REC were allowed to air dry for a further 5 minutes and then placed in a 6 well plate for normal culture using the aforementioned modified DMEM-HAMS F12 media. These

samples were cultured for the period of two weeks and analysed by the Pap and MG stains described in sections 2.11.3 and 2.11.4 respectively.

CHAPTER 3

The Growth of Respiratory Epithelium on Resorbable Scaffolds

3.1 Introduction

The functional aspects of the tracheal epithelium are a very important consideration with respect to creating a viable tissue engineered tracheal replacement. In particular, the mucocillary transport of particles and liquids is of primary importance, as are the ciliated cells that are involved in this process. The in vitro culture of human respiratory epithelial cells (REC) in monolayers often results in the loss of cilia function and/or the dedifferentiation of ciliated cells themselves [22,23]. The use of extracellular matrix proteins as culture beds for cells in order to inhibit, to some extent, dedifferentiation is well described [22,23,24]. REC have been cultured on collagen disks and collagen coated plates in order to maintain cellular morphology and help promote ciliogenesis at air-liquid interfaces [24,25]. In our laboratory, chondrocytes expanded in vitro redifferentiate back from a fibroblastic phenotype to the normal cell phenotype after culture on hyaluronic acid scaffolds [19]. The use of collagen coated culture plates has also been reported to maintain phenotypic characteristics of isolated corneal epithelial cells [19, 46, 47]. The observed inhibition of dedifferentiation and the fact that such ECM proteins are, by their nature, degradable, has made materials such as collagen and hyaluronic acid viable candidates for many tissue engineering applications [47,48].

During this investigation lectin histochemistry and scanning electron microscopy (SEM) was used to assess the growth and to characterise the differentiation of the cultured REC. Lectins are glycoproteins that have a high affinity for specific carbohydrates, such as those located in glycosaminoglycans (GAG) expressed on cellular surfaces [49, 50, 51]. Changes in the carbohydrate expression on a cell surface are often associated with cellular differentiation. Therefore, lectin staining provides a useful tool for studying the subtle changes in carbohydrate structure under

the varying conditions of cell culture, which may cause cellular dedifferentiation [50,51]. As stated previously, the dedifferentiation of REC often results in a loss of ciliation, and subsequent changes in the lectin staining pattern of cultured REC should reflect this. In addition, we have chosen to correlate the lectin staining data with our observations on cell growth by SEM.

In this chapter we investigate the culture of REC on two commercially produced scaffolds; one made from a hyaluronic acid derivative (Hyaff^{TM}, Fidia, Italy) and one made from equine collagen (Biofleece^{TM}, Baxter, USA). As previously mentioned the use of collagen as a substrate for the culture of respiratory epithelium has been reported to promote ciliogenesis and maintenance of cellular morphology [24,25]. As such the use of the Biofleece membrane was a logical choice. The Biofleece material is a composite comprising of a non-woven scaffold with a solid membrane surface on one side and thus has the future ability to accommodate experiments into co-culture with cartilage cells being seeded into the fleece and epithelial cells cultured on the membrane. Similarly, in the future context of generating a tracheal replacement consisting of both a cartilage and respiratory epithelium, the use of the Hyaff material is also justified as work done in our laboratory, has reported that the fleece form of this material is suitable for the tissue engineering of human cartilage [19]. The aim of this chapter is to identify which of these materials will support a viable respiratory epithelium thereby ascertaining the suitability of these materials for tracheal tissue engineering.

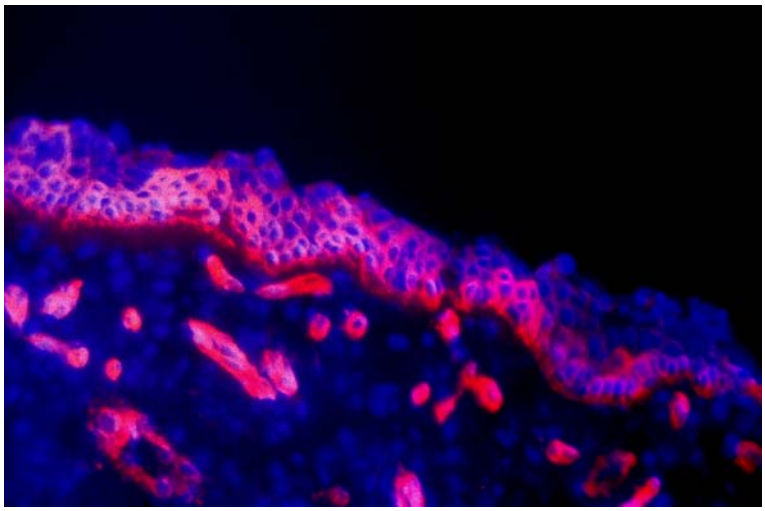
3.2 Results

3.2.1 Native tissue control

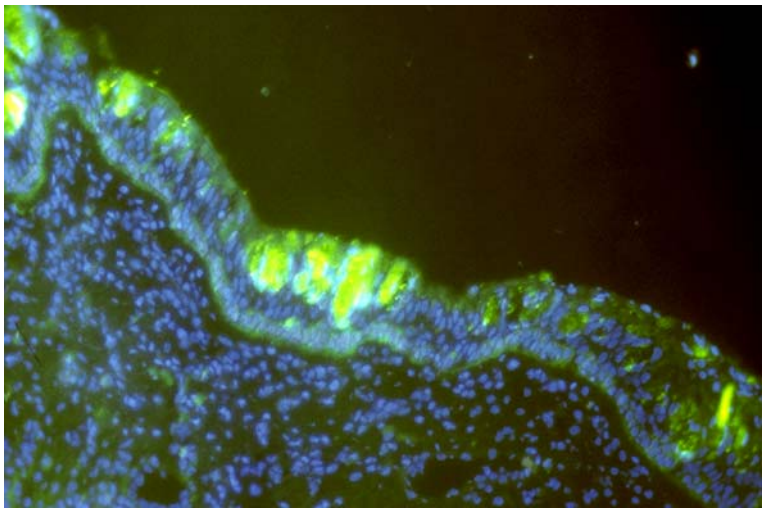
The pattern of PNA and UEA lectin staining for cryosections of untreated nasal turbinates is shown in Fig. 10. Note that the two figures shown are representatives of

five separate stainings from 2 separate turbinates (see materials and methods section 2.4). The rhodamine (red) conjugated UEA lectin clearly binds to cells in the epithelial layer of the native tissue sections. Furthermore, this stain is present in the membrane of the cells as can be seen by the red outline surrounding the blue stained cell nuclei, indicating that this is the positive staining of ciliated cells in the respiratory epithelium.

Figure 10: Micrographs of PNA and UEA staining of native tissue.



10(a) The UEA (red) staining of the ciliated cells in the epithelial layer (x30 Mag).Note the unstained secretory cells in the right corner of the epithelium.



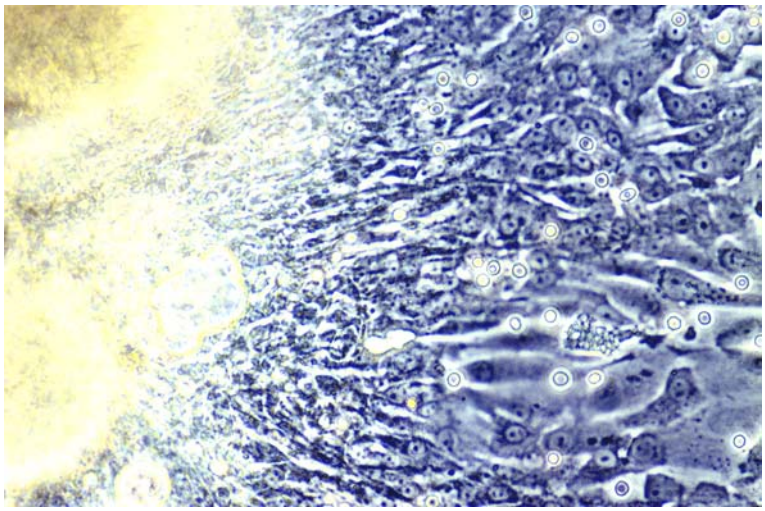
10(b) The PNA(green) staining of the basement membrane the columnar secretory cells and the surface layer of mucus of the epithelium (x30 Mag).

The fluorescein (green) conjugated PNA lectin binds to the basement membrane of the native tissue and also to columnar cells present in the upper layer of the respiratory epithelium. It is also present as a thin surface coating on the top of the cells, which corresponds to the mucus layer covering the epithelium. It should be noted that not all cells at the surface of the epithelium stain positive for UEA (red) staining (Fig. 10a). These areas are likely to be secreatory cells also located at the surface as are shown in Fig 10(b).

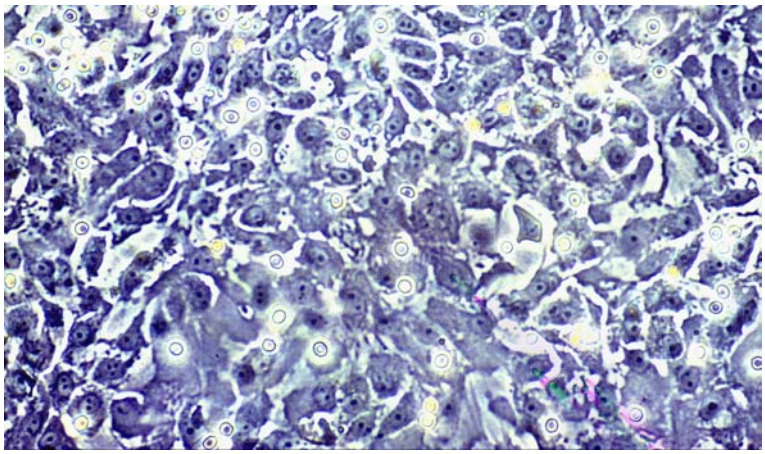
3.2.2 Tissue culture plastic control culture of REC

The growth of dispase isolated REC on the TCP control surface is documented by phase microscopy in Fig. 11. Fig. 11a shows the initial lay-down of REC sheets obtained by the modified dispase technique. For ease of description the label “centre” will designate these areas of the cell culture. This group of cells is highly ciliated, and is representative of the epithelium on native tissue. Fig. 11b, or the “middle” area, shows cells spreading out from the initial lay-down. This area still contains some ciliated cells. In the “periphery” of this outgrowth the cells become more fibroblastic and no ciliated cells are present (Fig. 11c). It should be noted that outgrowth occurred from the sites of initial lay-down, within the first 24 hours of culture, and the time taken to confluence differed from patient to patient.

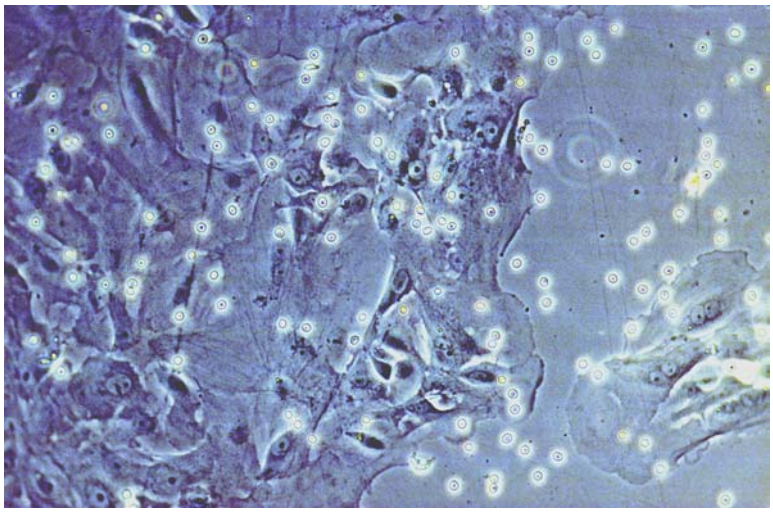
Figure 11: Phase microscopy of the outgrowth of REC cultured on TCP.



11(a) The "center" area of initial lay-down, which is ciliated (x 20 Mag).



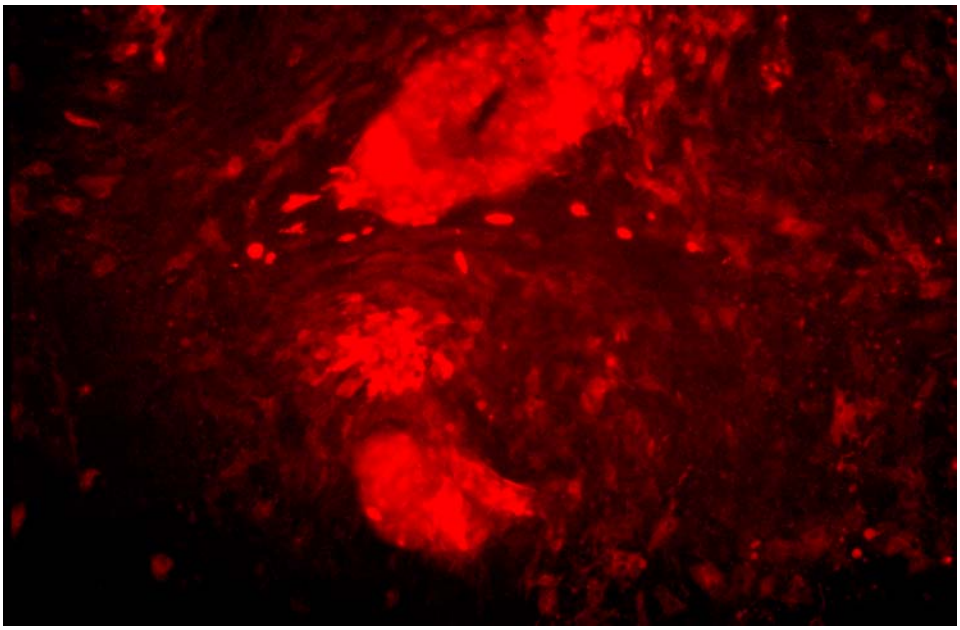
11(b) The outgrowth from the initial lay-down in the "middle" area, which contains some ciliated cells (x20 Mag).



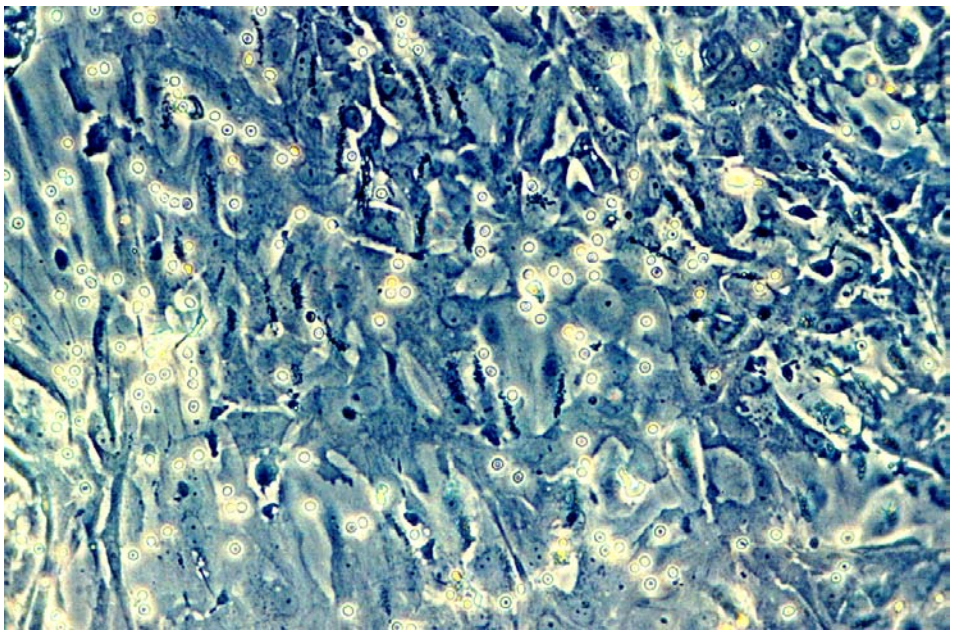
11(c) Fibroblastic cells at the "periphery" of the outgrowth (x30 Mag).

Lectin staining of representative "centre" and "middle" areas shows that ciliated REC stain positive for the UEA lectin, as is shown by positive staining of the elongated cells and the area of initial lay down (Figs. 11a and 11b). Fig. 12a shows two areas of initial lay-down that stain positive for UEA (red). In addition several elongated spots of positive staining are present around these two areas. A high magnification phase contrast photograph of this area shows the presence of such elongated ciliated cells proximal to an area of initial lay-down (Fig. 12b). This localised UEA with "known" areas of ciliation were observed consistently in numerous cultures.

Figure 12: Micrographs of UEA staining of isolated REC cultured on TCP.



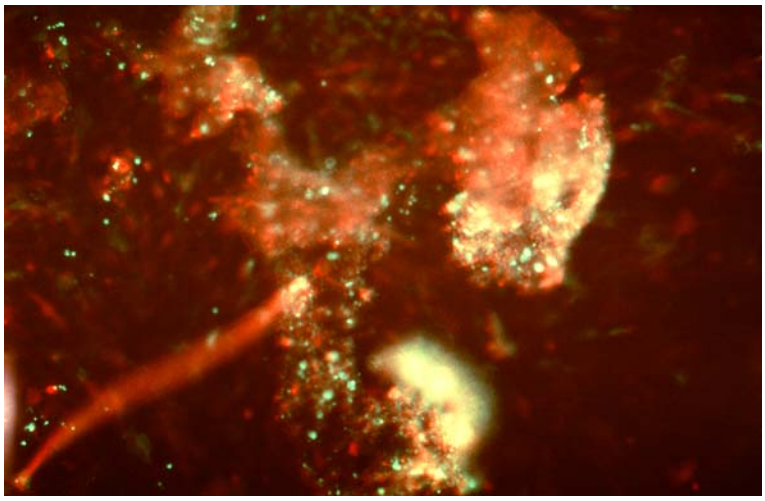
12(a) Both the initial lay-down of the "center" area and some isolated elongated ciliated cells in the immediate outgrowth that stain positive for UEA (x10 Mag).



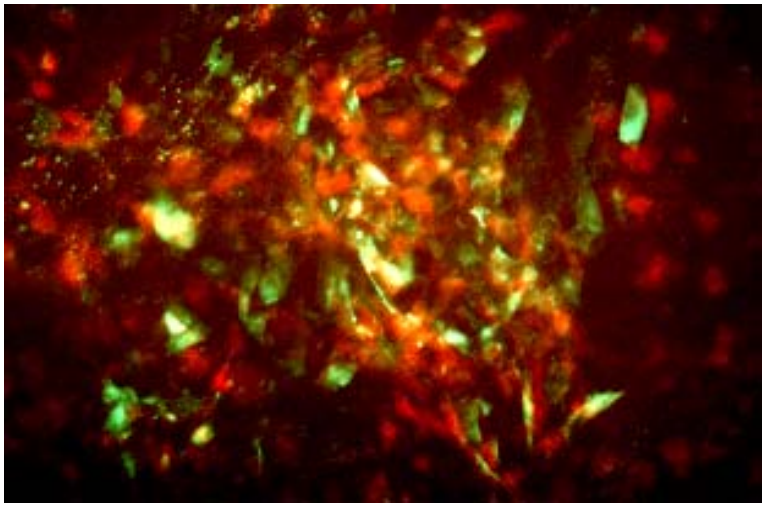
12(b) Phase contrast micrograph showing the cilia of the isolated elongated ciliated cells (x20 Mag).

The simultaneous UEA (red) and PNA (green) staining of REC cultured on TCP for 5 days indicates that there is a progression away from dual staining (green and red) to singular PNA (green) staining from the "centre" to the "periphery"(Figs. 13a, 13b and 13c) The "centre" and "middle" areas bind both lectins, as is indicated by positive green and red staining. However, only PNA (green) staining is present in the "periphery" where the cells are more fibroblastic in morphology (Fig. 11c).

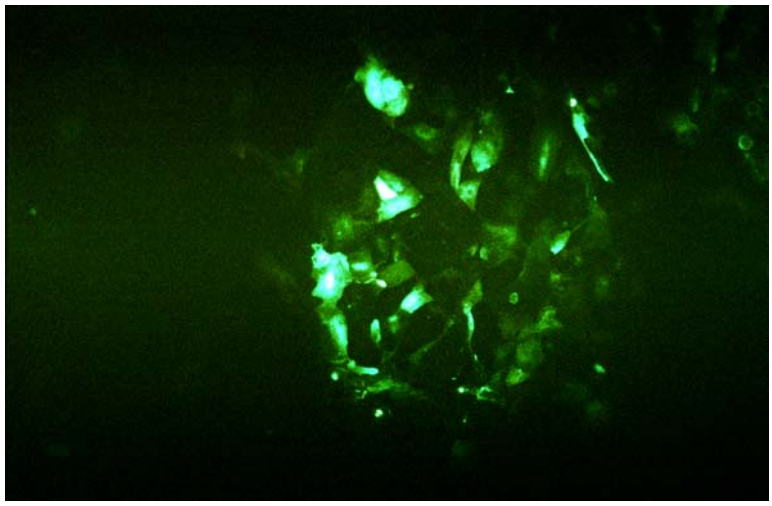
Figure 13: Double staining of REC cultured on TCP with the UEA and PNA lectins.



13(a) The dual staining of the sheet of cells at the point of initial lay-down indicating presence of both ciliated and secretory cells (x20 Mag).



13(b) The "middle" area of immediate outgrowth also stains for both lectins (x20Mag).

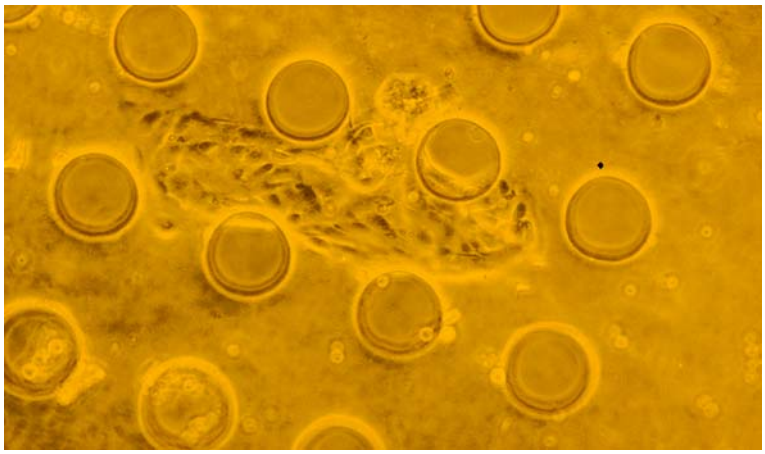


13(c) Cells at the "periphery" of the outgrowth are fibroblastic and stain only for the PNA lectin (green) (x20 Mag).

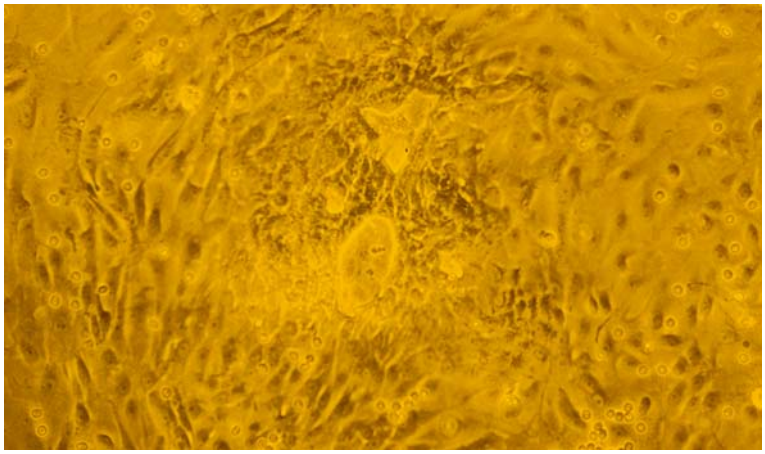
3.2.3 Cell growth on hyaluronic acid scaffold

The Hyaff scaffold was found to be consistently non-adhesive for REC in the majority of test cultures. In the few cases where an isolated REC sheet managed to adhere, the Hyaff membrane was observed to limit cell spreading when compared to that of the TCP control seeded at the same time (Figs. 14a, b).

Figure 14: REC growth on TCP and the Hyaff membrane.



14(a) REC cultured on Hyaff were observed not to spread even after 20 days in culture (x20 Mag).



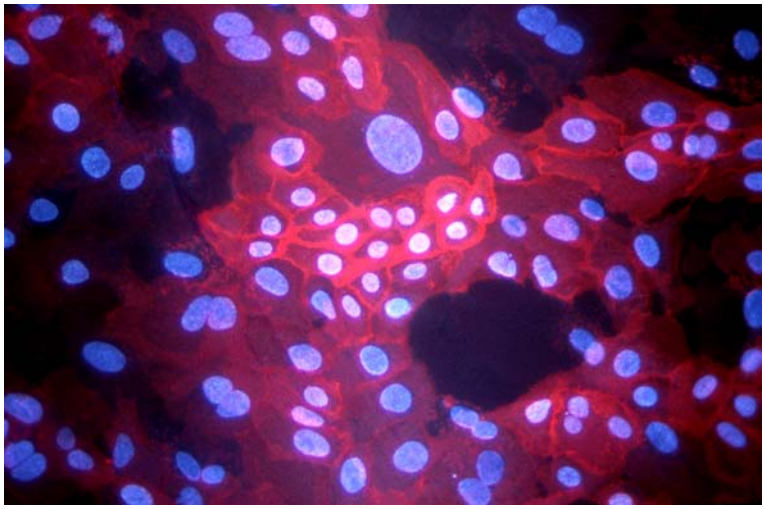
14(b) REC cultured on TCP were confluent at day 6 (x20 Mag).

Furthermore, the spreading of REC on the Hyaff membrane progressed little over the period of 20 days whilst the TCP control plate was confluent at day 6 (Figs 14b).

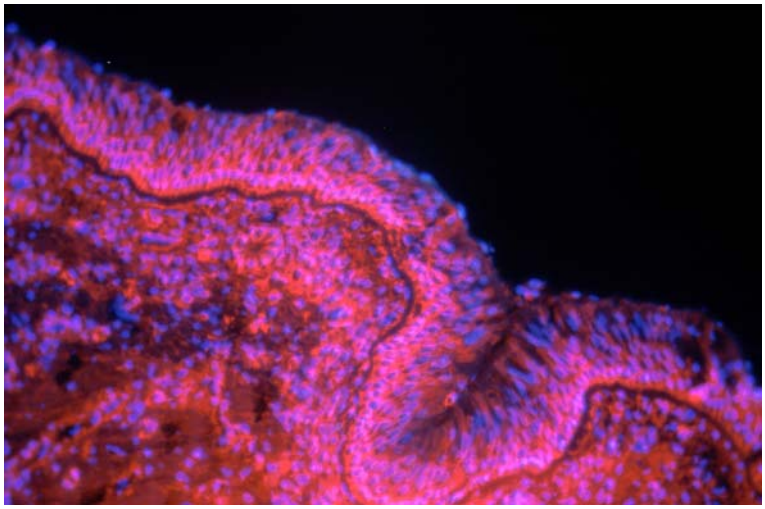
3.2.4 Staining for the presence of the Hyaluronic acid binding domain with the H4C4 CD44 antibody

The lack of adhesion of REC on Hyaff prompted an investigation into whether or not the culture of isolated REC caused a loss in the expression of the CD 44 hyaluronic acid binding domain on the cell surface, which would explain the lack of adhesion and cell spreading. Due to the lack of adhesion of RECs to hyaff the lectin staining pattern investigation was not performed for cells on this material. It was observed that the cultured REC, native tissue and the FaDu cell line all stained positive for the hyaluronic binding domain (Figs. 15a, b, c). In particular, H4C4 staining was highly localized in the cell membranes of the cultured RECs and FaDu cells. The FaDu epithelial cell line was used as a control to check whether or not the limitation on cell spreading was isolated only to cultured REC. We observed that FaDu cells were able to adhere and spread rapidly over the Hyaff membrane and that the membrane was entirely covered after five days in culture (Fig. 16).

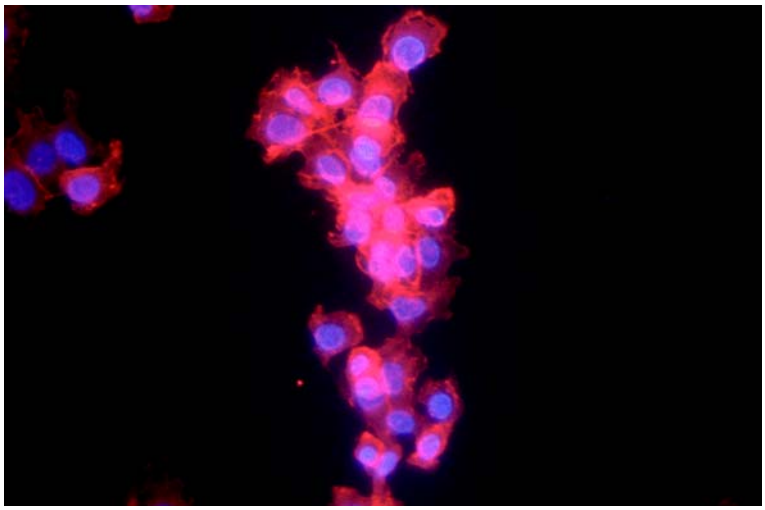
Figure 15: Presence of the Hyaluronic acid binding domain.



15(a) Isolated REC cultured on TCP showing positive staining for the hyaluronic acid (CD44) binding domain in the cell membranes (x40 Mag).

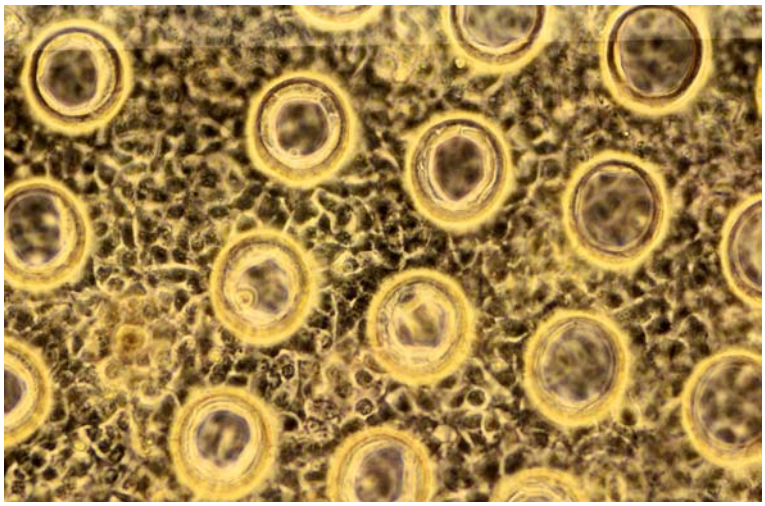


15(b) Native tissue showing positive staining for the hyaluronic acid (CD44) binding domain (x20 Mag).



15(c) FaDu cell line with positive staining for the hyaluronic acid (CD44) binding domain in the cell membrane(x30 Mag).

Fig. 16. Phase micrograph of FaDu cells.



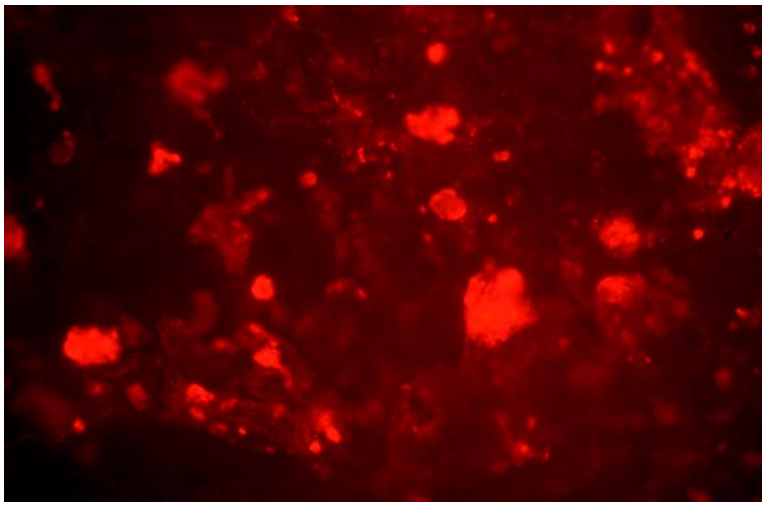
FaDu epithelial cells cultured on the Hyaff membrane spread well and are confluent by day 5 (x20 Mag).

3.2.4 Lectin staining and cell growth on the collagen scaffold

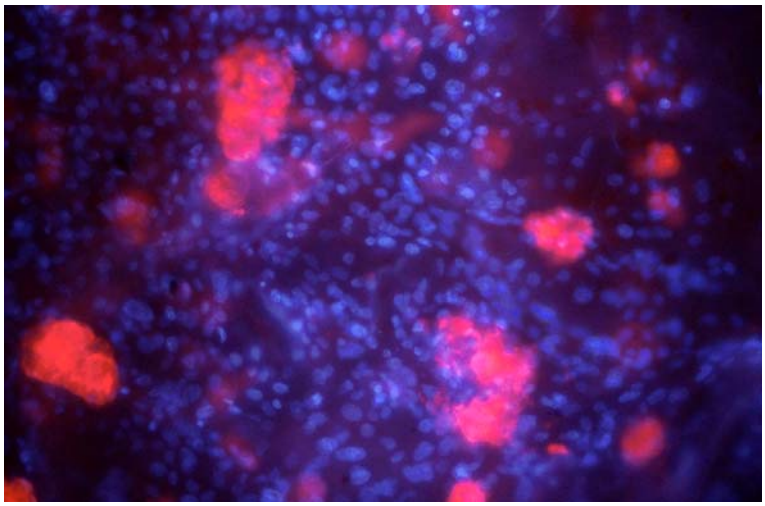
Homogenous REC adherence was observed for cells cultured on the Biofleece collagen membrane in all five of the test samples (Fig. 17a, b, c). Lectin staining of these cells revealed a strong positive UEA signal. However, UEA staining appeared in this case, to be more evenly spread and not mainly localised to cell clumps formed

by the initial lay-down of REC sheets from the primary preparation as was observed in the lectin staining of REC cultured on TCP control. Limited PNA staining relative to that of the TCP control was observed on some areas of the collagen scaffold (Fig. 17c).

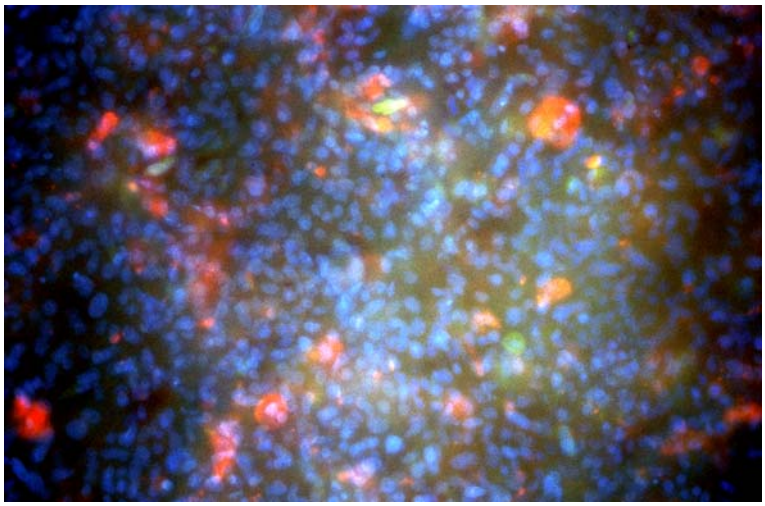
Figure 17: Micrographs of UEA and PNA lectin staining of REC cultured on the Biofleece membrane.



17(a) Strong positive UEA signal evenly spread over field of view (x 20 Mag).



17(b) UEA positive signal associated with cells as indicated by Hoechst counterstain (x20 Mag).

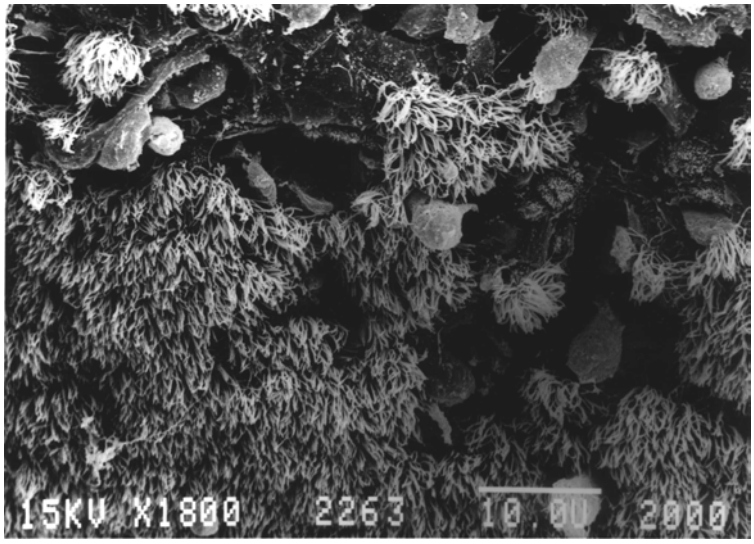


17(c) PNA and UEA double stain indicating very low levels of PNA positive staining (x20 Mag).

3.2.5. Scanning electron microscopy analysis of cells cultured on the collagen membranes

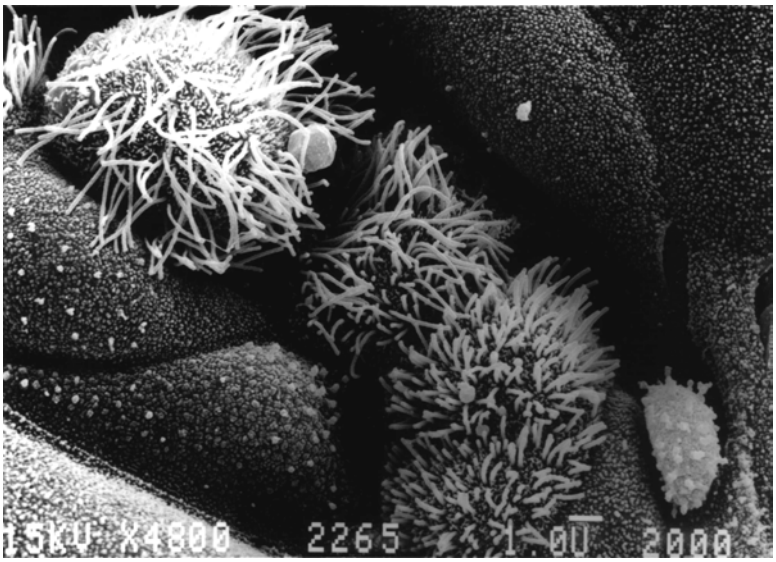
The SEM analysis of the native tissue clearly shows large areas densely covered with cilia (Fig. 18a) The epithelial cells cultured on the Biofleece membrane presented both long established cilia, equivalent to that observed in the native tissue and shorter microvilli (Fig. 18b). A low magnification scanning electron micrograph of the Biofleece surface shows complete coverage by REC. These are either ciliated or presenting short microvilli-like structures (Fig 18c). However, the density of the established ciliated areas on the collagen membranes are lower than those observed in the native tissue control.

Figure 18: SEM micrographs of REC cultured on the Biofleece membrane.



18(a) Micrograph of native tissue showing dense areas of ciliation on the surface of the nasal turbinate.

18(b) A low magnification micrograph of REC cultured on the Biofleece membrane showing complete coverage of the membrane by REC. Note the presence of clumps of established cilia and the abundance of short microvilli on the surface of these cells.



18(c) High magnification micrograph showing in detail the established cilia and short microvilli.

3.4 Discussion

The isolation of REC by the modified dispase technique generates cell cultures similar to those generated by traditional explant outgrowth methods. The major difference is the time taken for outgrowth to initiate. In the dispase cultures, outgrowth starts within the first 24 hours and proceeds rapidly afterwards. Our experience with explant cultures is that the outgrowth takes several days to establish. Furthermore, the possibility of an explant not sticking to the TCP is a common problem with traditional explant cultures. This particular problem is not an issue when using the dispase culture system. The lectin staining pattern of native tissue observed in this study agrees with that reported by Hassid et al. [51]. They also found that UEA lectins bind to ciliated cells and the PNA lectin binds to the basal membrane, secretory cells and the mucus itself of the epithelial layer. This indicates that the use of these two lectins in identifying the functional characteristics of the respiratory epithelium is valid. The localisation of the UEA stain with ciliated cells is further corroborated by the staining of the elongated ciliated cells cultured on TCP

control (Fig. 12). This further highlights the ability of UEA staining to identify ciliated cells with an REC culture.

It is evident from the studies of REC cultured on TCP that the further a cell is from the point of initial lay-down (center region of culture) the more fibroblastic it is likely to be (Fig. 11). This is not a unique finding and has been reported by several other investigators and represents the limitation of outgrowth-like cultures [22,23 and 51]. In addition it has also been reported that longer culture periods (more than the five days culture of this study) are accompanied by complete loss of ciliation and a change to fibroblastic morphologies [22,23,52]. The subsequent loss of ciliation in the "periphery" region as observed in this study is therefore predictable, since the fibroblastic morphology indicates a dedifferentiation from the normal state. This loss of ciliation is corroborated by the lack of UEA lectin staining of the cells within the "periphery" region of outgrowth (Fig. 13c). However, the positive PNA staining of these fibroblastic cells is interesting. As stated previously, the secretory and basal cells of a respiratory epithelium stain positive for the PNA lectin only (Fig. 10). Therefore, it is possible that these fibroblastic cells have a similar GAG expression to the basal and secretory cell types. Other investigators have proposed that ciliated cells are generated from the basal cells and or secretory cells [53, 54]. Thus, it is not surprising that a dedifferentiation of these cells, in this short-term culture, would result in a similar lectin-staining pattern to that of the basal cells and secretory cells. It is not known whether in a longer-term culture that this PNA staining of fibroblastic cells would be conserved. However, I hypothesise that as cells progress to a more dedifferentiated (fibroblastic) state over longer culture periods, that this PNA (secretory cell) staining would be lost entirely.

The lack of adhesion of isolated REC to the Hyaff membrane was interesting (Fig. 14). Hyaluronic acid derivative scaffolds have been used extensively in the culture of

many different cell types including chondrocytes and as previously mentioned in the introduction, therefore made a logical choice for the tissue engineering of REC constructs [19,48, 55]. This lack of adhesion prompted us to investigate whether or not the culture of isolated REC caused a loss in the expression of the CD 44 hyaluronic acid binding domain on the cell surface. In vivo, cells bind to Hyaluronic acid (HA) by the ubiquitous 34 kDa cell surface receptor known as CD 44 [56]. CD 44 contains a HA binding domain that is similar to the ones localised in many ECM proteoglycans [56]. Thus, if the isolated and cultured cells lost the ability to produce the CD 44 surface receptor the inability of REC to adhere to the Hyaff could be explained. Isolated REC, native tissue and the FaDu epithelial cell line all stained positive for the hyaluronic acid binding domain (Fig. 15). So it is clear that no loss in hyaluronic acid binding domain expression is occurring in isolated REC over the culture period of five days and that the REC of the native tissue do express the binding domain. The FaDu cell line was found to be adherent and spread well over the Hyaff membrane (Fig. 16). It is unclear, however, why REC did not do the same, as we would expect similar adhesion mechanisms to be at work in both cell types since they are both of epithelial origin. Similarly, our previous experience showing that chondrocytes cultured on Hyaff adhere well also indicates that adhesion mechanisms required to be satisfied for REC culture on Hyaff are somewhat different to other cells [19]. At this point it is imperative to point out the high specificity of the H4C4 antibody used in the detection of the binding domain is evidenced by studies done on knockout mice among others [57,58]. This previously established high specificity, rules out to some degree, that the positive staining for the binding domain on the cells and tissues is totally unspecific and therefore not representative of the presence of the domain. It is evident that the presence of the hyaluronic acid binding domain alone is not sufficient to promote cellular adhesion. I hypothesise that the

inability of REC to adhere to the Hyaff material may be due in some part to an inability of the some integral proteins to adhere to the esterified biomaterial. For example, if the Hyaff does not bind the proteins that are specific for the integrin receptors of the REC this would deter adhesion. Integrins are large transmembrane glycoproteins that attach cells to ECM proteins or ligands on other cells [59]. If Hyaff inhibits the binding of specific proteins that the integrins of the REC bind too, the lack of adhesion of REC to Hyaff could be explained. This topic requires further investigation but was not within the scope of this study.

In contrast, REC adhered well to the collagen-based scaffold (Biofleece), which is not surprising since collagen is well known to promote adhesion and cell spreading [47, 60]. Complete coverage of the membrane-like surface of the scaffold by cells is indicated by the Hoechst staining of cell nuclei and the SEM analysis (Figs.17 and 18). UEA positive staining was predominant in these preparations, which indicates the presence of ciliated cells. The Biofleece also promotes a much more even distribution of UEA positive cells than that observed in the TCP control (Figs. 13 and 17). This indicates that the collagen biomaterial is promoting the maintenance of ciliated cells that are not associated with the clumps of primary tissue formed in the initial lay-down of REC sheets from the primary isolation as is the case in the cultures on TCP. As stated previously, the maintenance of the ciliated cells is important when attempting to create a functional tracheal replacement because of the need for the transport of particles and liquids within the trachea. However, there are also large areas of the membrane that are covered by cells that exhibited no lectin positive staining. (Fig.17). This would indicate a distinct dedifferentiation of these REC and is not an optimal finding. Furthermore, the low levels of PNA lectin staining would indicate that the numbers of secretory and basal cells present in these cultures are low. This also indicates the dedifferentiation of the cell population when grown on the

collagen scaffold. It may be that the secretory and basal cells are more susceptible to dedifferentiation during the culture period of five days than the ciliated cells. We find it unusual that PNA staining is abundant in REC cultures grown on TCP and not so in those grown on the Biofleece membrane. Many researchers have reported that cells grown on collagen are less likely to dedifferentiate [22, 44, 46, 47]. However, it appears that in a complex multi-cell type system, such as a primary culture of REC, the inhibition of dedifferentiation by collagen is somewhat selective. It is also possible that the surface topography of the membranes encourages the dedifferentiation of these cells. TCP is a smooth surface while that of Biofleece is relatively rough. The effect of biomaterial surface topography on cell differentiation is well documented [60,61]. It is possible that the basal and secretory cells are more prone to dedifferentiation induced by a rough surface topography than are the ciliated cells. It should also be pointed out that collagen membranes are autoflourescent under the fluorescence lamp and perhaps the number of visible PNA positive cells is reduced by this effect.

The SEM analysis of REC cultured on the Biofleece membrane confirms the presence of ciliated cells, further validating the use of the UEA lectin as a detection system. The density of ciliated cells on the membrane was not as high when compared to that of the native tissue control (Figs. 18). However, the presence of short microvilli on the surface of the “nonciliated” cells is encouraging (Fig. 18b and c). Jorrisen et al. reported that these short microvilli were present in the first days of suspension cultures of REC and they developed into established cilia after the period of 10 days [62]. This would suggest that given a longer culture period than 5 days the density of ciliated cells on the surface of the membrane would be higher. It remains unclear whether the established ciliated cells are derived from “developed” cilia or ciliated cells that adhered during the primary culture procedure. It would be logical to

suggest that the established ciliated cells are from the primary source. However, other investigators have shown that ciliogenesis is both a rapid and varied process, with the time to completion varying from cell to cell and even within one cell [52]. The abundance of microvilli on the cell surfaces of the REC cultured on the Biofleece scaffold may also provide an explanation for the lack of PNA staining detected in these cell cultures. If the secretory cells are encouraged to develop cilia by culture on this membrane, then this would accompany a loss in PNA positive staining and a subsequent shift to UEA positive staining. This may explain why the REC cultured on TCP maintained the PNA staining for as mentioned previously, the culture of REC on TCP is not conducive to the process of ciliation.

Key Points

1. **Lectin staining is a useful tool for characterisation of respiratory epithelial cells**

2. **The collagen based Biofleece scaffold was found to maintain a ciliated epithelial layer as observed by the positive UEA staining and the observed ciliated cells by SEM analysis**

3. **The hyaluronic acid based scaffold was found to be non-adherent for respiratory epithelial cells.**

4. **The non-adherence of the hyaluronic acid scaffold was not related to the loss of the hyaluronic acid binding domain of the REC during culture.**

5. **Surface topography and chemical groups play a role in adhesion and the dedifferentiation of REC.**

CHAPTER 4

The growth of respiratory epithelium at an air liquid interface

4.1 Introduction

As mentioned previously (see chapter 3) the growth of REC at an air liquid interface (ALI) has been found to encourage ciliogenesis [22,24 and 25]. In these studies it is common to culture the REC on a semi permeable collagen membrane of which one side is exposed to the air and the other exposed to a liquid nutrient medium. Researchers have found that this type of culture results in a multilayered epithelium that is homogeneously populated by ciliated cells in the upper layers [22, 24 and 25]. Since the Biofleece is a collagen based scaffold it was prudent to investigate if the extent and homogeneity of ciliation of REC cultured on the fleece could also be encouraged by an air liquid interface culture method.

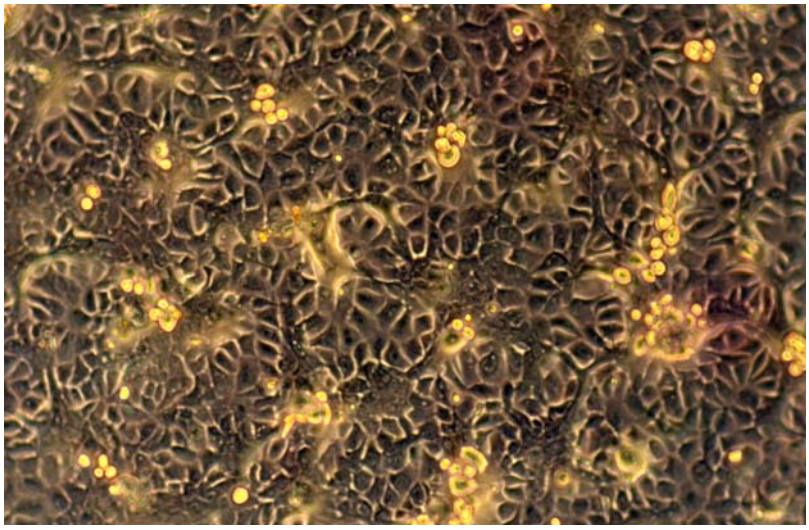
4.2 Results

4.2.1 Control study: growth on cellagen inserts

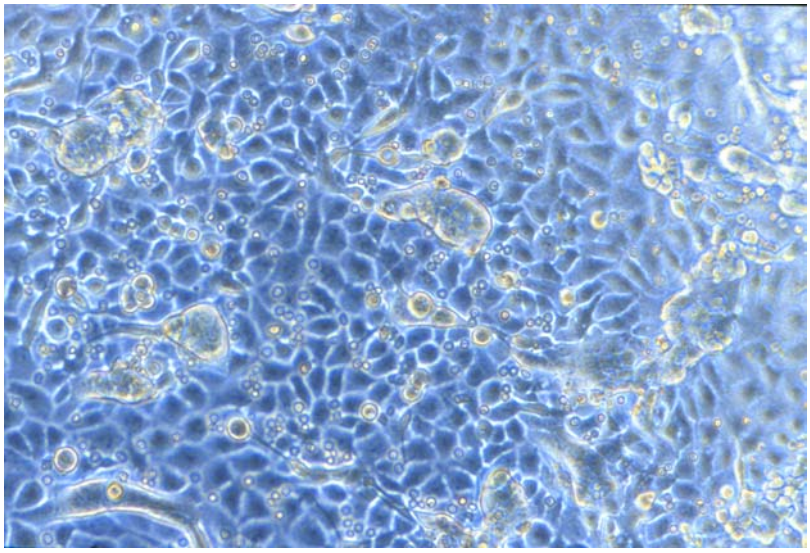
In all test samples REC were found to spread rapidly to confluence on the cellagen inserts over a period of 1-2 days (Fig 19). The morphology of the cells when grown on the collagen inserts and in the culture flasks (TCP) were similar, both being cobblestone in appearance and totally confluent. At this stage no ciliation was present in the cultures. Following exposure to the air interface and subsequent culture for a period of ten days the cells grown on the cellagen insert appeared multilayered in areas as observed by phase microscopy. Due to the raised nature of the inserts it was unable to be determined whether the REC had become ciliated at this point by phase microscopy. The inserts had also become more opaque due to multilayering and the cells appeared to have a slight white coloured mucus layer on their surface. The presence of this mucus layer was confirmed by a PAS staining for mucus detection that stained an insert as positive for mucus (purple) (Fig 19d). SEM

analysis of the cellagen insert cultures after the ten day period show that the cells are ciliated in some areas and are multilayered (Fig. 20).

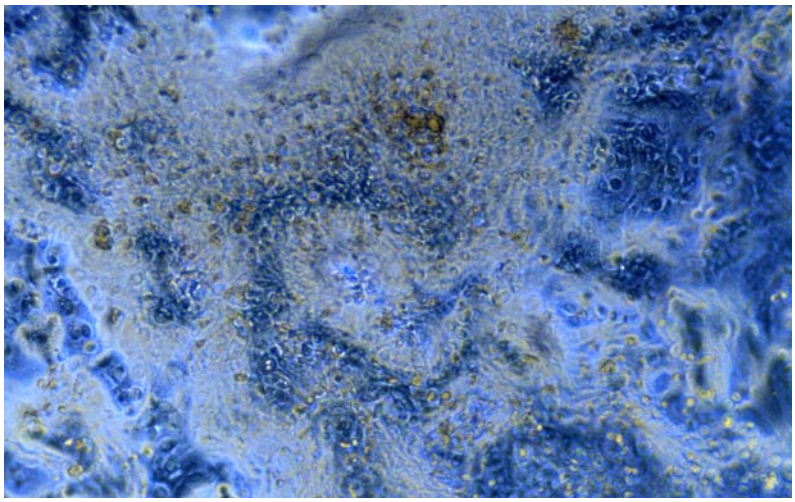
Figure 19. Phase micrographs of REC grown on the cellagen membrane and in normal culture flasks.



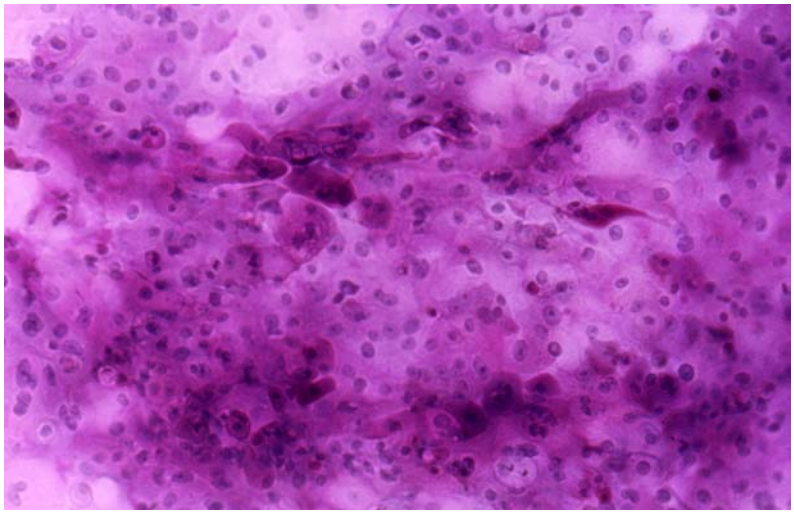
19(a) REC cultured on TCP showing the multilayered cobblestone morphology of the cells and the lack of fibroblastic cells in the culture



19(b) REC cultured on the cellagen membrane prior to exposure to the air interface. Note the multilayered cobblestone morphology of the cells and the lack of fibroblastic cells

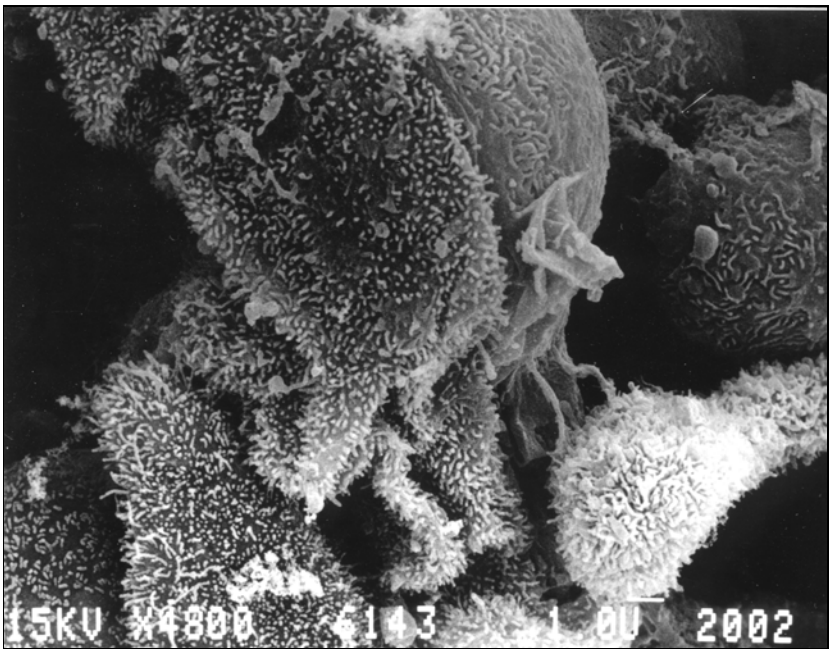


19(c) REC at day 1, after exposure to the liquid interface showing the extent of multi-layering (center of figure) after 10 days at the ALI.

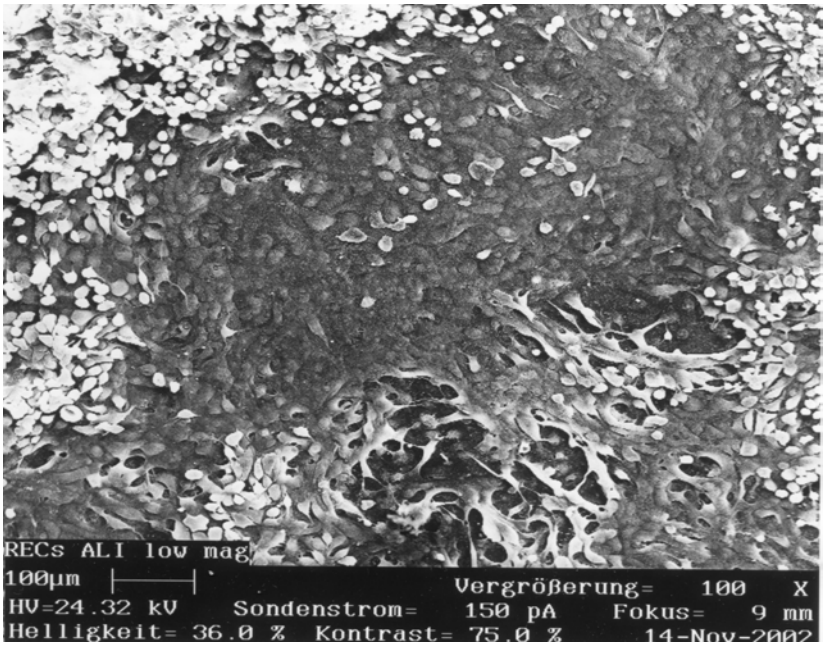


19(d) PAS staining of a cellagen insert with RECs cultured at the ALI. Positive purple staining is distributed on the surface of the cells indicating the presence of mucus. This staining appears darker in areas of multilayering.

Figure 20. SEM Micrographs of cells grown on cellagen membranes following 10 days culture at the ALI.



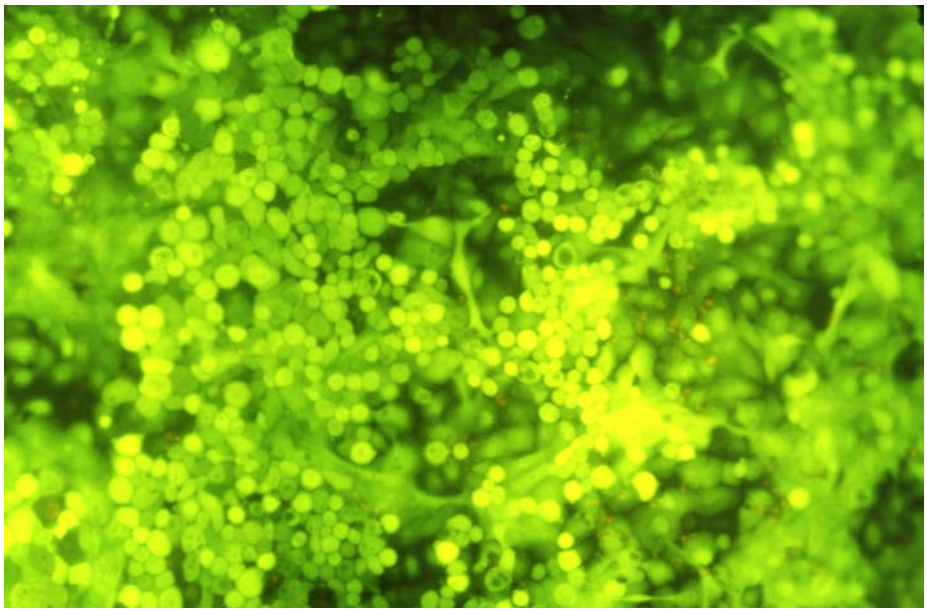
20(a) High magnification showing in detail the multilayered cell morphology with "short cilia" and microvilli present. Note the short nature of the cilia.



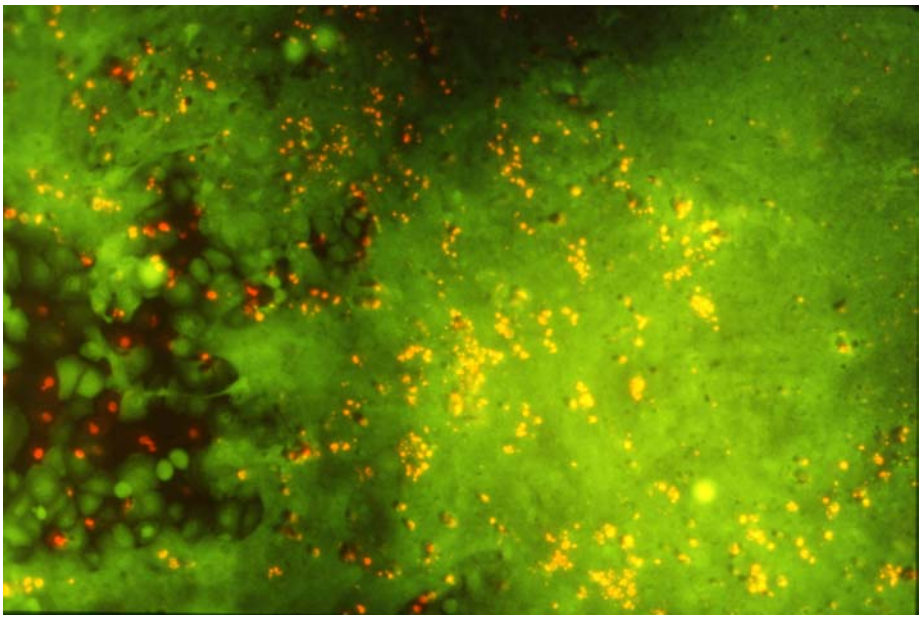
20(b) Low magnification showing flattened cobblestone shaped cells (center of picture), multilayered spreading cells (bottom right of picture) and mucus deposition (top left corner of picture).

The live-dead stains of REC on cellagen membranes after ten days at the ALI confirm the findings of the SEM data. The cellagen membranes were densely populated by live cells following the ten-day period (Fig. 21a). Furthermore, the cells on the very top of the multilayers appear to be rounded in morphology and less flat when compared to those of the bottom layer, a distinction that is readily visible in the center area of figure 21(a). Very few dead cells were observed and large parts of the cellagen membrane were covered in multilayers of densely packed cells that appear as green stained carpet-like areas on the membrane (Fig 21b). Areas of single non-multilayered cells were visible in-between these carpeted areas. Dead cells were homogeneously distributed throughout the culture, these being relatively low (approximately 15%) in number when compared to the live cells in all cultures. Note that the approximation of the percentage of dead cells in each test sample was difficult due to the multilayering of the cells

Figure 21: Live-Dead micrographs of cells grown on cellagen membranes following 10 days culture at the ALI.



21(a) High magnification micrograph showing in detail the multilayering of the cells (center of picture) and abundant live cell staining (as indicated by the green fluorescence).



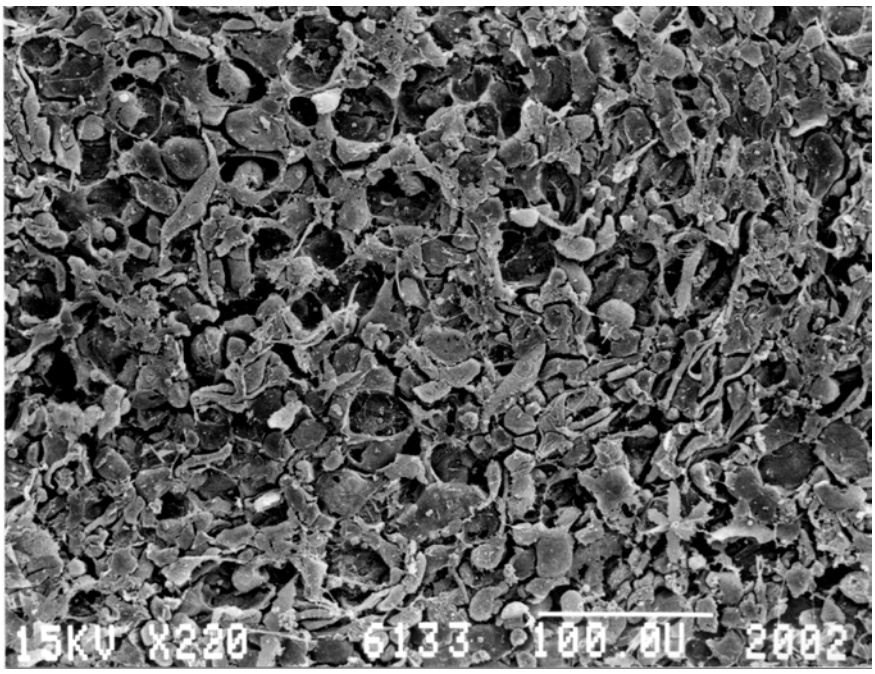
21(b) Low magnification showing large carpet-like areas of multilayered with relatively few dead cells visible. Note red-orange staining indicates dead cells and green staining indicates live cells.

4.2.2 REC cultured on Biofleece scaffold at the ALI

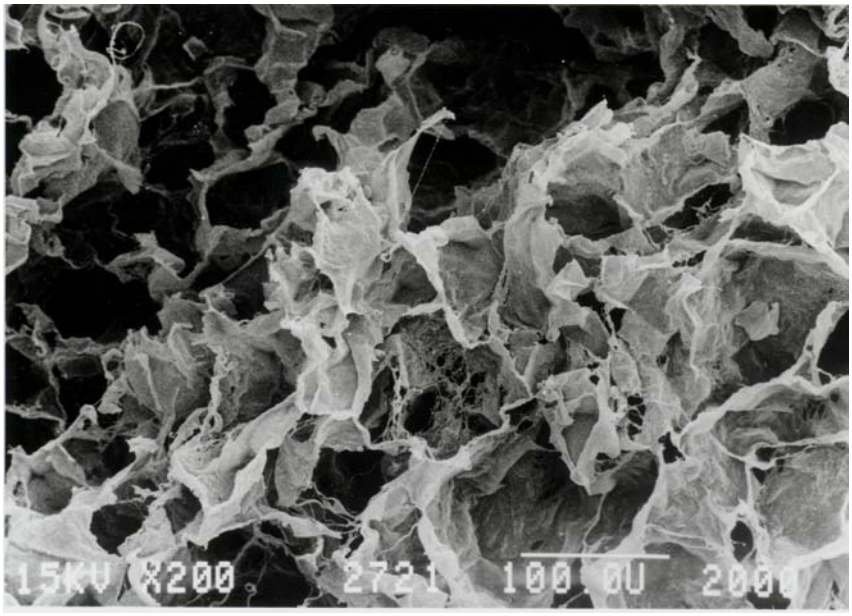
Phase microscopic analysis of the REC grown on the surface of Biofleece at an ALI was not possible due to the opaque-like nature of the material. However, small bulges of cell growth were observed around the periphery of the material and in one case the “bulge” was populated with small and actively beating cilia (results not shown due to the problems of photographing small moving cilia with still-photography).

SEM analysis of the surface of the material revealed that the vast majority of the fleece was covered in dead cellular material and that few if any cells remained viable on the cell surface after exposure to the air interface (Fig. 22).

Figure 22: SEM micrographs of cells grown on the Biofleece Scaffold following 10 days culture at the ALI.



22(a) Low magnification image showing necrotic cells on the surface of the Biofleece scaffold. Note the still apparent cobblestone morphology from the cells established on the surface before exposure to the air interface.



22(b) Comparative micrograph showing the surface of the Biofleece without cells, which emphasises the presence of necrotic looking, cells in figure 22a.

4.3 Discussion

The isolation and subsequent culture of cells in the specialised BECGM (chapter 2 section 2.8) allows for the amplification of REC in culture while minimising the effects of dedifferentiation. REC cultured in the BECGM maintained cobblestone morphology throughout the growth period to confluence after which a multilayer of the cells was apparent (Fig. 19). The ability to amplify the REC in number without apparent dedifferentiation (as would be indicated by a fibroblastic morphology), is an advantage when considering tissue engineering of larger implants than are investigated in this study. Also, it is possible that only a small biopsy of respiratory epithelium is obtainable from the patient, so once again the amplification of cell number without dedifferentiation is very valuable. Conversely, RECs cultured in conventional DMEM-HAMS media tend towards fibroblast morphology during long-term traditional submerged culture. This was observed and discussed previously in chapter three where RECs at the periphery of outgrowth from a sheet of initial lay-down were observed to be fibroblastic. As emphasised throughout this study, the inhibition of dedifferentiation and the associated loss of cell-specific functionality is of primary importance to any tissue-engineering endeavour.

The SEM analysis of REC culture at an ALI on the cellagen membranes confirms the presence of ciliated cells in a multilayered configuration (Fig. 20). This indicates that the REC which were previously non-ciliated in conventional BECGM culture have an ability to regain ciliation when placed an ALI. Furthermore, these cells appear to be producing mucus during the period of culture as was confirmed by the PAS stain and the SEM analysis (Fig 19d and 20b). These results concur with other research in this field, although some researchers report better ciliation than is presented here [63,64]. The presence of microvilli indicates that the degree of ciliation may improve with

increased culture times. Jorrisen etal. reported that these microvilli are pre-cilia and develop into cilia over time [62]. Some researchers have maintained ciliated REC cultures at an ALI for a period of 20 days and in suspension cultures for up to six months [64, 65]. Where cells are not in multilayers the cobblestone morphology is conserved (Fig. 20b). Furthermore, the live-dead studies also show that in areas of multilayering cells appear to be more rounded and columnar in shape (Figure 21). This indicates that the cultured epithelial layer may be assembling much like that of native tissue with columnar cells at the surface supported by a network of squamous and flattened cells. These observations show that the culture at ALI on cellagen membranes aids in the construction of a multilayered “native tissue-like” epithelium. This ability to reacquire functional aspects such as mucus production and ciliation after submerged culture indicates that a REC layer on a tissue engineered tracheal replacement may do the same once placed in the air-liquid environment of the respiratory tract.

In contrast the SEM analysis of the Biofleece-cell construct shows that cells were able to be cultured on the membrane in submerged culture and that these cells became necrotic after they were placed at an air interface (Fig. 20). The dead cells could be fixed by the SEM preparation procedure and the cobblestone morphology of these cells is still visible (Fig. 22a). This point is emphasised when comparing the morphology of the Biofleece without cells to that with cells grown exposed to the ALI (Fig 22a and 22b). One plausible explanation for this observation is that cells on the surface of the membrane do not receive enough nutrition because of lack of exposure to the media. This would also explain the presence of the cell bulges on the sides of the fleece, as this would be the only area in direct contact with the media and its nutrients. Perhaps, a Biofleece scaffold sitting on the bottom of a culture vessel only allows limited diffusion through its side areas and this may not be

sufficient for cell growth. Similarly, it is hard to keep the media at a level equal too (but not above) the surface of the Biofleece construct due to evaporation of the liquid media. Thus, the nutrient supply to the surface of the scaffold is decreased in another way. Other researches have indicated that cells need to be within 200 μm of a blood-nutrient supply to be sufficiently supplied with nutrition [66]. The diffusion of liquid media to the centre of the Biofleece membranes surface would appear to be limited and thus detrimental to cell growth. This is not the case with the cellagen membrane that receives a uniform diffusion from under the membrane and not merely from the sides, as is the case with the Biofleece. It is evident from the live-dead studies on the cellagen membranes that sufficient nutrient supply is available. A new method for placing the Biofleece onto an air liquid interface while allowing for adequate nutrition from the media is required. An apparatus that allowed for periodical media coverage of the cells with the nutrient media might solve these issues by decreasing the time that cells are exposed to low nutrient supply while at the same time maintaining an intermittent air-liquid environment for the positive differentiation of the REC layer. However, the eventual problem of nutrient supply in vivo still remains. The normal REC layer of the trachea obtains most of its nutrients from the vascularised fibrous layer of tissue that is located between the basement membrane and the cartilage rings. In order for a tissue engineered REC layer to survive in vivo the problem of nutrition must be solved. The optimal solution would be to encourage the rapid vascularistion of the construct from nearby tissue post-implantation. This next level of tissue engineering needs to be investigated further and would require the construction of novel “tailor-made” biomaterial scaffolds and perhaps the use of growth factors that encourage vascularisation.

Key Points

1. **The use of BECGM in culture of REC helps maintain the phenotypic morphology of the cells.**

2. **Cells grown at the air liquid interface re-ciliate after a period of ten days.**

3. **Cells grown at the air liquid interface produce mucus.**

4. **The air liquid interface culture on the Biofleece scaffold receives too little nutrition to be viable.**

5. **The problem of vascularisation for nutrient supply of the REC layer in a tissue engineered tracheal construct is an important issue and requires further investigation.**

CHAPTER 5

The growth of chondrocytes in the Biofleece scaffold

5.1 Introduction

In chapter 3 I have shown that the collagen biomaterial (Biofleece) is appropriate for the growth and maintenance of respiratory epithelial cells. Therefore, this chapter investigates whether the scaffold is appropriate for cartilage tissue engineering, which is the next step towards the goal of a co-cultured epithelial and chondrocytes (cartilage cell) construct (see chapter 1 section 1.5).

Damaged cartilage has a limited ability to repair itself in adults [35, 67, 68]. For this reason the tissue engineering of cartilage is a widely investigated field. Collagen based biomaterials have shown promising properties with regards to cartilage tissue engineering and thus the choice of Biofleece as a material has some basis [69,70,71]. The cell density in mature cartilage tissue is low and unlike the respiratory epithelium, (particularly the ciliated and goblet cells), the functional aspects of mature cartilage are derived more from the ECM than the cells themselves. It is the composition of collagens, fibronectins, elastins and glycosaminoglycans (GAGs) that confer elasticity, mechanical strength, compressibility and tensile strength to cartilage [72,73,74]. GAG content is known to directly affect water retention in cartilage tissues, which is an important factor that controls the compressibility of cartilage [72,74]. It is important to note that the compositional mix of the ECM is different for each cartilage type. For example, cartilage of the ear pinna (elastic cartilage) has higher elastin content than that of nasal septal(hyaline) cartilage [74,75]. Thus the ear is more flexible than the nasal septum. However, the common components of all cartilage types are collagen type II and the presence of GAGs, which are both, recognised as important markers for differentiated chondrocytes [76, 77, 78]. The subgroups of cartilage; elastic cartilage, fibrous cartilage and hyaline cartilage have all been found to differ in ECM components in some ways but complete characterisation of these differences is an ongoing process.

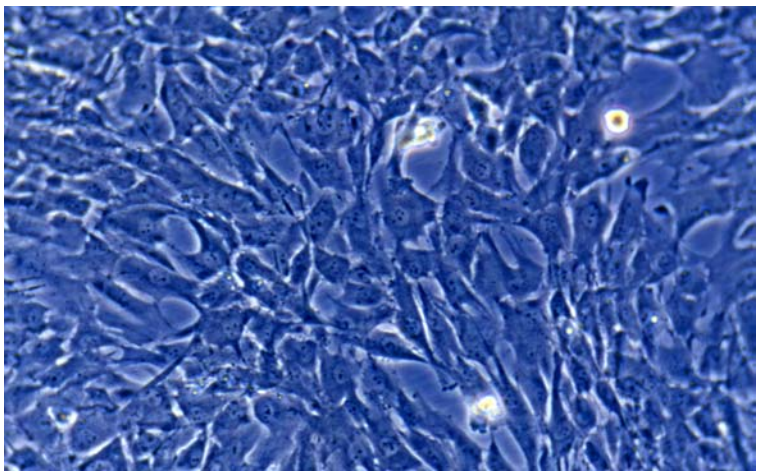
This chapter aims to assess the potential of Biofleece as scaffold for cartilage tissue engineering with respect to utilising a small number of cartilage cells to generate mature-like cartilage tissue.

5.2 Results

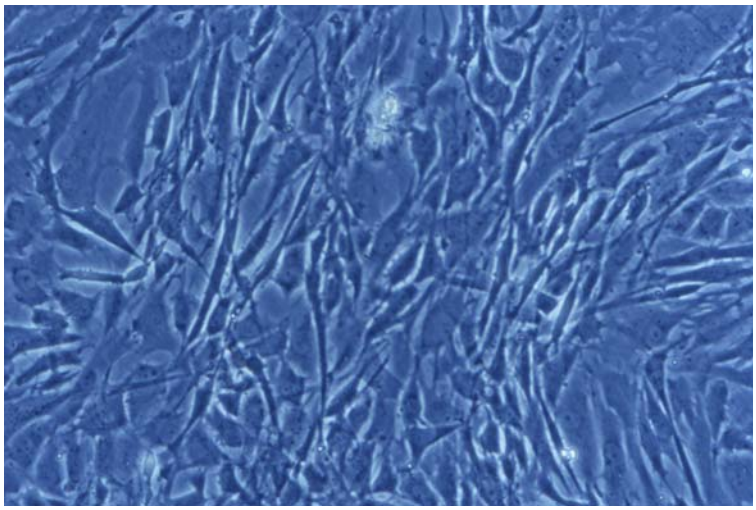
5.2.1 Isolation of septal chondrocytes and their growth on TCP

Isolated chondrocytes grown with the supplemented DMEM-HAMS F12 media (10% FCS, 1% v/v penicillin streptomycin, L-Glutaminutese, ITS and NEAA) displayed a rounded morphology, with some evidence of multilayering after passage two (Fig 23a). In contrast, cartilage cells grown with the conventional DMEM (10% FCS, 1% v/v penicillin streptomycin, L-Glutaminutese) were observed to be fibroblastic in normal culture after two passages (Fig 23b).

Figure 23: Cartilage cell morphology cultured on TCP.



23(a) Phase contrast micrograph of passage two chondrocytes cultured in DMEM-HAMS F12 supplemented medium showing the "rounded" morphology of cartilage cells (x20 Mag).

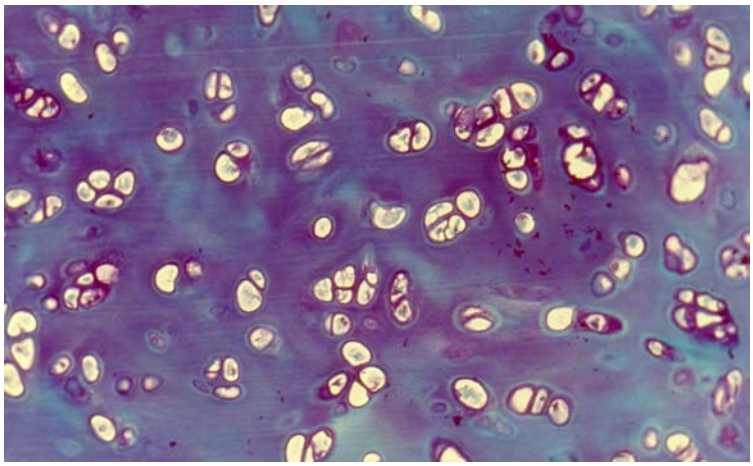


23(b) Phase contrast micrograph of passage two chondrocytes cultured with normal DMEM medium. Note the fibroblastic morphology of the cells.

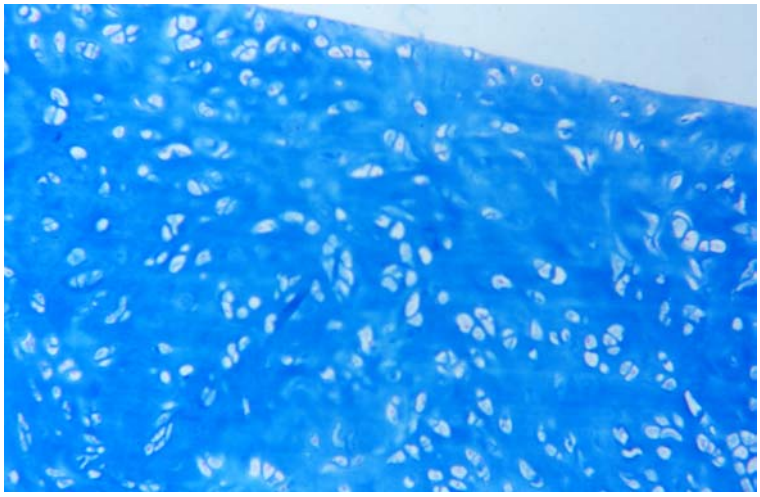
5.2.2 Native septal cartilage stained with the AB and PN for comparison to the tissue engineered constructs.

Two stains were used to assay the native septal cartilage for comparison to the cartilage-Biofleece constructs (Fig24). The typical cartilage morphology, of cells in islets surrounding by stained ECM is observed in both sections. PN stains the ECM purple close to the cells with an overall aqua green background. The AB stains the ECM around the cartilage cells light blue.

Figure 24: Histological staining of native septal cartilage.



24(a) PN staining of native septal cartilage detailing the differential staining (purple close to the cells on a background of aqua green)(Mag x20).

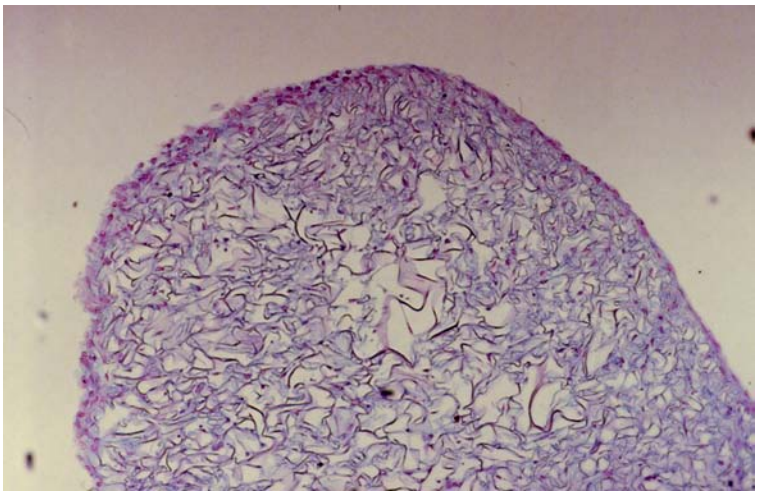


24(b) AB staining of native septal cartilage showing the positive staining for GAGs (Mag x10).

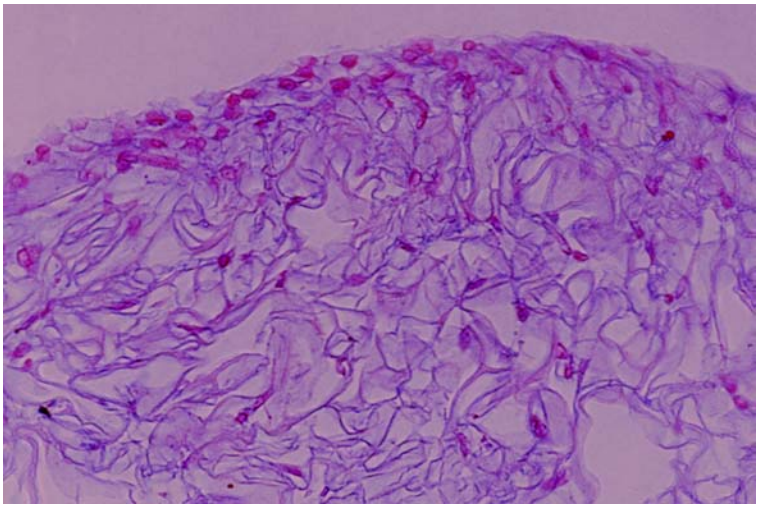
5.2.2 Chondrocytes seeded by the drop-wise method

The penetration of chondrocytes to the Biofleece scaffold by the drop-wise method was minimal. The histological analysis of drop-wise seeded scaffolds show a thin line of chondrocytes on the surface of the scaffold and very few cells within the scaffold (Fig. 25).

Figure 25:AB staining of cartilage seeded by the drop-wise method.



25(a) Low magnification micrograph showing the thin line of chondrocytes on the surface of the Biofleece scaffold (x10 Mag).

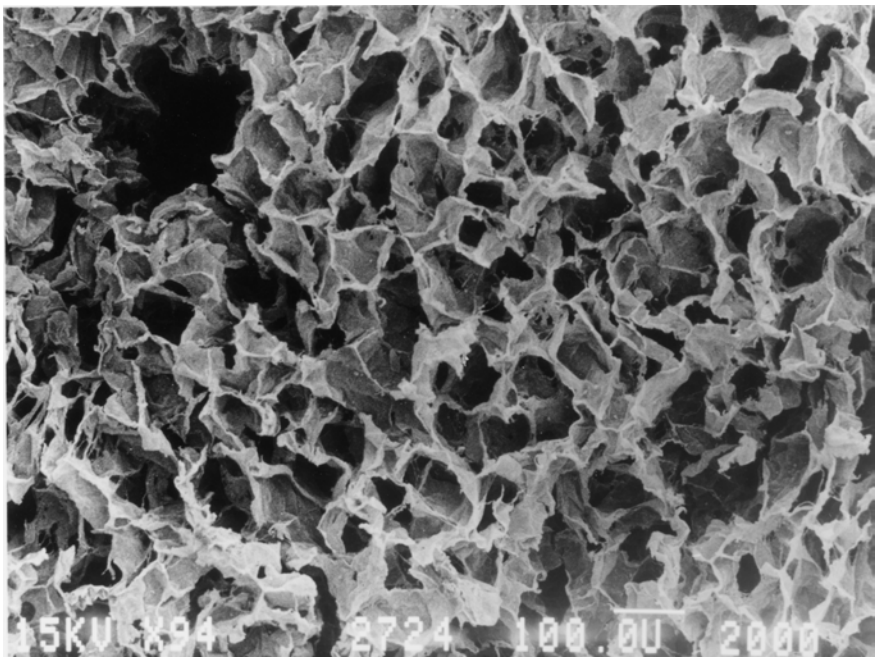


25(b) Higher magnification micrograph showing the lack of penetration of chondrocytes into the Biofleece scaffold (x20 Mag).

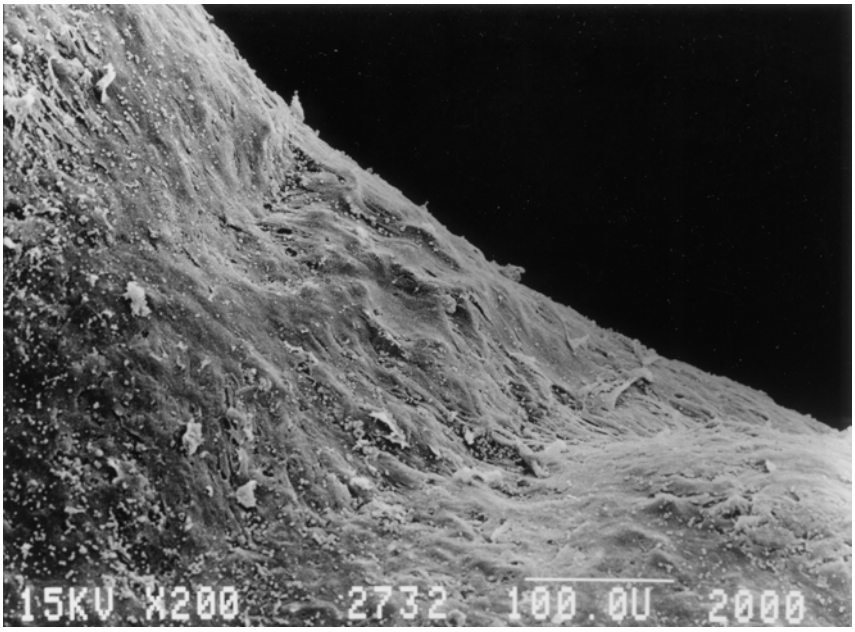
SEM analysis of drop-wise seeded scaffolds were used to further confirm that the chondrocytes formed a thin layer on the surface of the construct. Figure 26 compares micrographs of the scaffold without cells to that with cells at the time points of one and two weeks. A hole was made purposely in the scaffold to see whether cells would invade into this hole and cover the material. The micrograph of the Biofleece without cells shows that the size of holes in which the cells are seeded range from 50-150 microns (Fig. 26a). The SEM study revealed that at two weeks the scaffold is

entirely covered by chondrocytes (Fig. 26b). Furthermore, chondrocytes filled the hole made purposely in the scaffold within 1 week of seeding (Fig. 26c) indicating that the cells can fill large holes in the non-woven Biofleece scaffold.

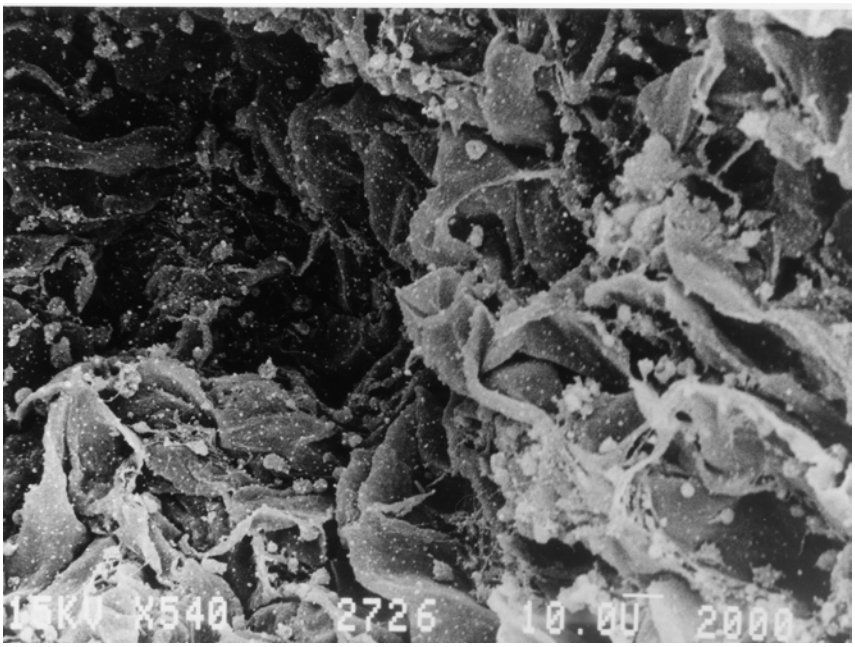
Figure 26: SEM analysis of chondrocytes seeded by the drop-wise method.



26(a) High magnification micrograph of Biofleece scaffold showing the holes within the non-woven material.



26(b) Low magnification image showing the scaffold surface completely covered by cartilage cells after 2 weeks.

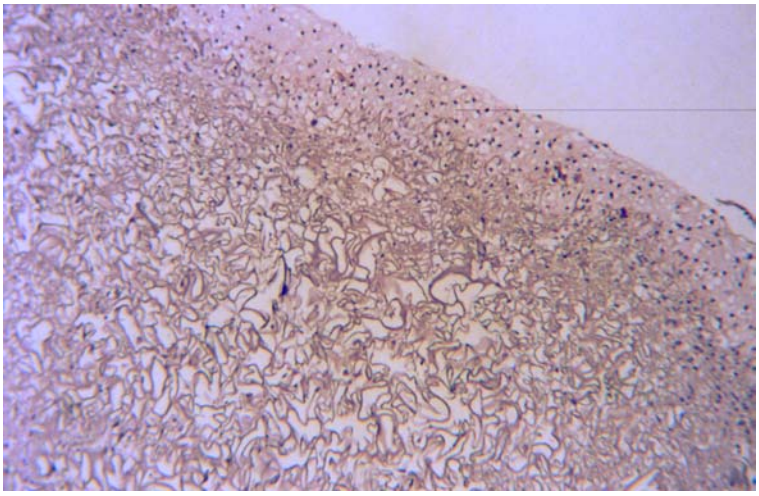


26(c) High magnification image showing that cartilage cells are able to invade and fill the defect hole.

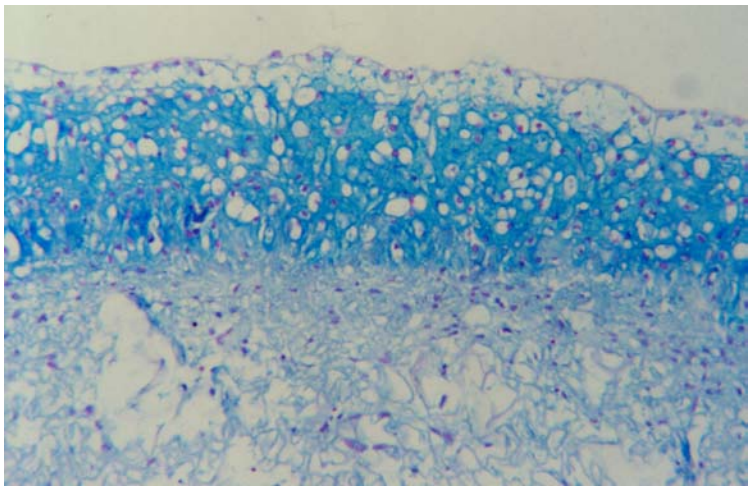
5.2.3 Chondrocytes seeded by the suction method

Chondrocytes seeded into the scaffold by the suction method were observed to penetrate further into the fleece than those by the drop-wise method. However, the penetration was not homogenous and most of the cells were located in a broad band on the surface of the scaffold. Figure 27 shows the location of these cells within the scaffold using three different histological stains. The HE stained section clearly indicates the broad band of cells by the dark staining of the cell nuclei. Unfortunately, it is hard to distinguish singular cells deeper within the scaffold from the background colour and distortions created by the staining of the Biofleece scaffold itself.

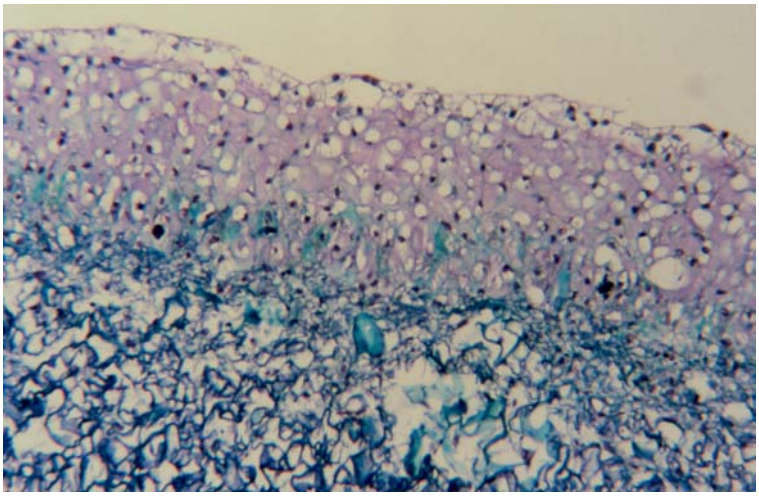
Figure 27: Histological analysis of chondrocytes seeded by the suction method.



27(a) HE staining clearly indicating the broad band of cells on the surface of the scaffold (x10 Mag).



27(b) AB staining showing that the cells in the band of tissue are cartilage-like in morphology and surrounded by ECM (x20 Mag).



27(c) PN staining showing the different areas; developed cartilage (purple colour), developing cartilage (aqua green coloured) and scaffold material (dark blue-purple coloured) (x20 Mag).

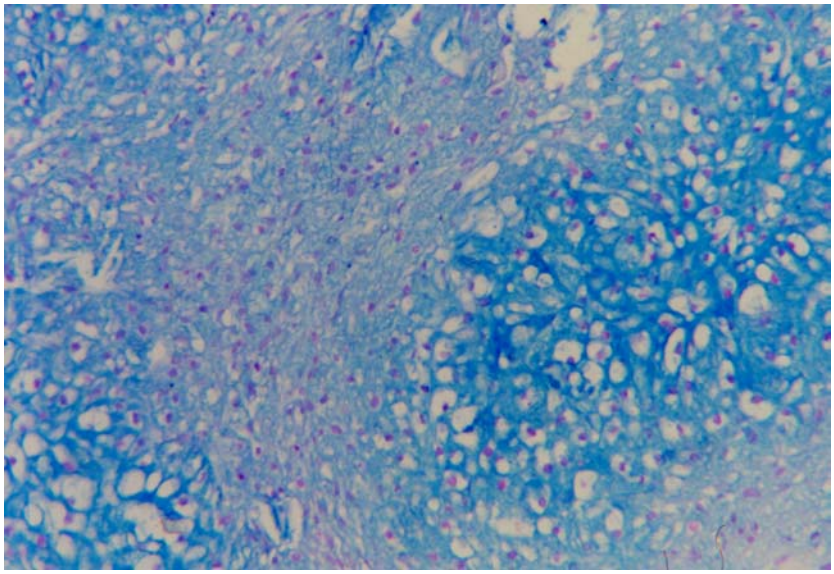
AB staining of a similar section reveals that the broad band of cells on the surface of the scaffold has developed a distinctive cartilage-like morphology akin to that seen in the native septal cartilage control. The cells are located within islets surrounded by ECM. This ECM is distinctive from the scaffold as it stains light blue with alcian while the fleece material stains blue grey. The cells are clearly visible by obtaining a red-pink stain. Close inspection of this section also shows a distinct area of “developing”

cartilage just below the light-blue stained “developed” cartilage-like area. This “developing” region differs from the internal regions of the scaffold as it has more cells and less fleece material evident, which gives the impression that this area is darker stained than that of the less cell-populated areas. The PN stained section reveals that there are indeed three distinct areas within this construct. The broad band of cells that is definitively cartilage-like in morphology as detected in the AB stain is purple-violet in colour. The area directly beneath this established cartilage stains a lighter aqua green and corresponds to the developing cartilage area of the AB stained section. The internal structure of the fleece attains a dark blue-purple colour and is easily distinguishable from the two other described regions. Cell nuclei are stained dark-purple or black with this stain.

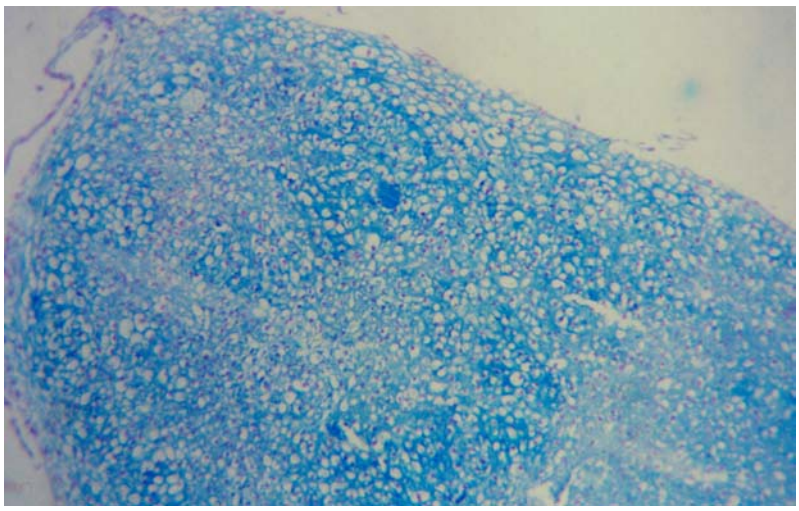
5.2.4 Chondrocytes seeded by the suction method using cut scaffolds

Macroscopic inspection of the Biofleece scaffold revealed that the surface of the scaffold had a thin crust-like layer (probably caused by the manufacturing technique of the material). This layer was cut away using a scalpel and the inner more porous part of the scaffold was exposed. This crust-like surface is thought to be the cause of the sub-optimal cartilage cell penetration reported thus far. The scaffolds that have this area removed will henceforth be referred to as cut scaffolds and those without the surface removed as uncut scaffolds. Figure 28 shows the results of the seeding of cartilage cells by the suction method on scaffolds with this surface cut away. The AB stain reveals that chondrocytes were able to invade deeply into the cut scaffold.. Large areas of the scaffold are completely cartilage-like in morphology and stain blue indicating the presence of GAGs around the cells (Fig 28 a and b). On average approximately 80% of the full scaffold was populated with cells when the suction method of seeding was used.

Figure 28: Histological analysis of chondrocytes seeded by the suction method into cut Biofleece scaffolds.



28(a) AB staining staining detailing the difference in the staining of the cartilage-like areas (light blue) and the developing areas(blue grey) (x20 Mag).



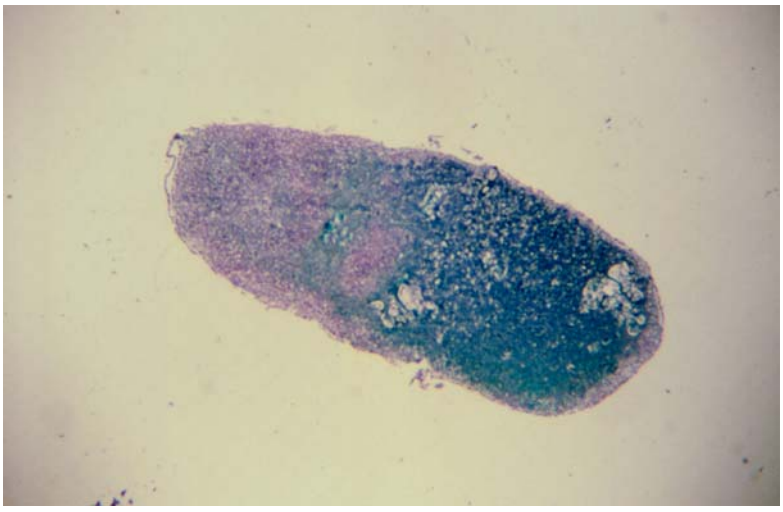
28(b) AB staining showing the extent to which the cells invade the cut scaffolds and the presence of ECM (indicated by the light blue staining) in the cartilage-like areas (x10Mag).

Once again, two distinct regions are identifiable from this analysis, the blue stained cartilage-like area and the blue grey stained area (figure 28a). The later region is

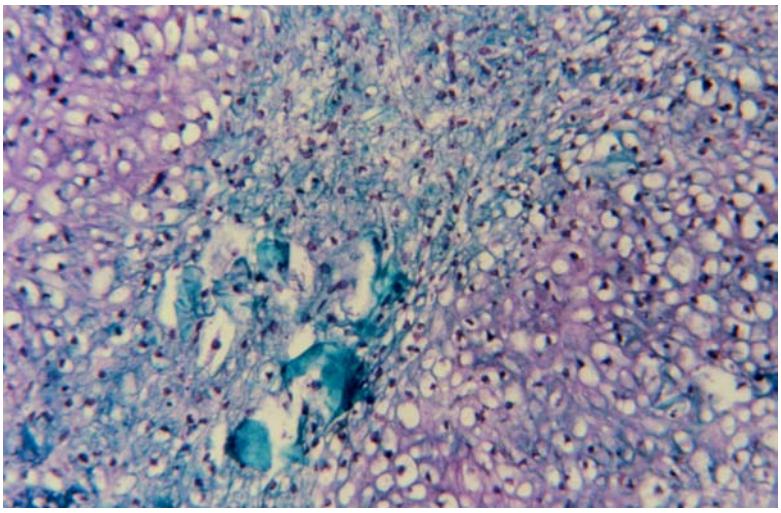
densely populated by cells and would appear to contain remnant fleece material after the culture period of two weeks.

The PN staining also indicates the scaffold is invaded with cells; which is to be expected. The cartilage-like areas in using this stain are clearly distinguishable from the developing areas and the remaining scaffold material. Figure 29a, a low magnification micrograph, shows the cartilage-like areas staining violet-purple; the "developing" areas of cartilage are aqua-green in colour and the remaining scaffold material are dark blue-purple. A higher magnification micrograph of the PN stained construct clearly demonstrates that the cell density in the "developing" areas and the "cartilage-like" areas are similar (Fig. 29b). However, the area, which is predominately scaffold material, has fewer cells than the "cartilage-like" and "developing cartilage" areas (Fig 29c).

Figure 29: Histological analysis of chondrocytes seeded by the suction method into cut scaffolds.



29(a) Low magnification micrograph showing the distinctive differential staining pattern of the PN stain. Cartilage-like area is blue-purple in co lour, developing cartilage area is aqua green in colour and the remaining fleece materials stains dark blue-black (x2.5 Mag).



29(b) High magnification micrograph detailing the difference of the developing cartilage areas (aqua green colour) to that of the cartilage-like areas (violet-purple colour) (x20 Mag).

5.2.5 Biofleece degradation over the period of two weeks

The control Biofleece scaffold without cells was found to be stable when placed in culture medium. However, when impregnated with cells the scaffold lost approximately 40-50% of its volume over the two-week period. Many cells were observed to be growing on the bottom of the culture flask after impregnation even following transfer of the constructs into new culture plates. These cells were not the typical “outgrowth” of cells from the implant, which are seen often, but more so clumps of cells that appeared to have fallen out of the degrading scaffold material.

5.3 Discussion

The use of insulin-supplemented DMEM-HAMS media appears to ensure the chondrocyte phenotypic morphology in culture. Other researchers have reported that tissue engineered cartilage grown with insulin showed an improved morphological appearance and an improved ECM deposition [79]. These observed effects of insulin were found to be similar to those observed in cartilage cells treated with insulin like

growth factor (IGF). It is plausible that the insulin in the supplemented DMEM-HAMS media used in this study is helping to maintain the phenotypic morphology of cultured chondrocytes. However, a study investigating which component of the supplemented DMEM-HAMS F12 media is responsible for maintaining the chondrocytic phenotype is required to confirm this hypothesis. It is clear that this property is beneficial in the context of this study, for it is logical that the less dedifferentiated a cell type is before tissue engineering, the more likely it is that these cells will produce the desired tissue product. Therefore, like the use of BECGM to maintain the REC phenotype (see chapter 4) the use of DMEM-HAMS supplemented medium to maintain the chondrocytes phenotype during cell amplification is also important.

The results of the drop-wise seeding experiments indicate that this seeding method is not applicable for the Biofleece scaffold. This is especially evident when comparing the results of the drop-wise seeded constructs to that of the suction seeded constructs using uncut Biofleece scaffolds (Figures 25 and 27). The increases in cell impregnation by the suction seeding method emphasises the need for more advanced seeding methodologies when creating a cell-biomaterial construct. It is worth noting that the suction seeding techniques were able to generate cartilage-like material while utilising a relatively small number of cells ($1x10^5$ cells per 1cm x 1cm x 0.5cm height). Aigner et al. used $20x10^6$ cells to seed a $1cm^3$ HYAFF non-woven fleece to generate a cartilage-like construct [19]. The ability to use a relatively small number of cells is an advantage when the size of patient biopsies, time taken to generate cells, and the use of low-passage cells for seeding are considered. The observation that the suction seeded scaffolds produced areas of distinctly cartilage-like morphology, while the drop-wise method did not, is easily explained. Isolated chondrocytes are known to require a three-dimensional (3-D) structure in order to redifferentiate and produce cartilage specific ECM components [80, 81]. The drop-

wise method allows little cell impregnation into the 3-D area of the scaffold. Subsequently, the cartilage cells do not redifferentiate and form only a monolayer on the scaffold surface effectively similar to that of cells in normal culture. In retrospect, it is evident that the suction method of seeding is better than the conventional method of seeding with respect to the Biofleece scaffold. It should be stressed that a less “tight” scaffold with a more open and porous structure may not seed so well under suction, since the cells may be sucked right through the scaffold. A comparison of the suction seeded cut and uncut scaffolds reveals that exposing the pore-like structure of the scaffold allows for better cell distribution. Uneven distribution of cells still occurs (Fig. 29a) and in some constructs only 80% of the scaffold was seeded. This is probably linked to the unevenness of the applied suction. The suction apparatus consists of a series of slits through which the suction is applied. If part of the scaffold is not placed on one of these slits, the resulting uneven suction causes an uneven cell distribution to occur.

The AB stain is a common stain for the detection of GAGs and mucus [82, 83]. The positive (blue) staining of the suction seeded scaffolds indicates that the areas of cartilage like morphology are producing their own ECM, which includes GAGs. The light blue staining of these “developed” cartilage-like areas concurs with that seen in the AB staining of native tissue (Figs 24(b), 27 and 28). Recall, that native cartilage is rich in GAGs and that these GAGs are responsible for some of the mechanical properties of cartilage, namely compressibility [72, 81, 84]. The indication that the engineered cartilage-like tissue is developing both towards a native cartilage morphology and ECM composition is encouraging. The grey blue defined “developing” areas are most likely regions where the cells are yet to fully modify their environment by degrading the scaffold material and replacing it with endogenously produced ECM. The presence of both the developed and developing regions

represents an opportunity to study the differences in ECM components within these areas and warrants further investigation.

The PN staining of these scaffolds also concurs with the corresponding staining of native tissue. The easily distinguishable and defined regions of “developed” cartilage like morphology (staining violet-purple), the “developing” regions of cells and fibrous tissue (staining aqua green) and the remaining scaffold material (staining blue-purple) are an advantage of the PN stain over that of the AB stain. The PN (or Pap stain as it is commonly known) is what is termed an “oversight stain”, meaning, that it stains the overall morphology of tissues [85]. The primary, use of the stain is to differentiate between cancerous, pre-cancerous and non-cancerous cells in the cervix by the Pap smear procedure [85]. This differential staining ability is also established in this study by the ease in which different regions of the tissue-engineered constructs could be defined. The differential staining appears to be related to the ECM content of the area. This is emphasised when comparing the purple colour of the cartilage-like area to the aqua-green colour of the developing regions. The purple stained cartilage-like tissue has been assessed as positive for GAGs by the AB stain. The corresponding aqua green stained region stained negative for GAGs with the AB staining of an equivalent section (Fig. 28). Therefore, it can be projected that this difference in ECM content between the two regions (with or without GAGs; purple stained or green stained respectively) is in part responsible for the differential staining by the PN stain. It is almost sure that the differential staining is not due to GAG content alone but a combination of GAG content and other ECM components. Furthermore, the PN of native septal cartilage shows that areas closer to the cells stain purple with the overall staining of the ECM being aqua green. The ECM content proximal to the cartilage cells of native tissue is known to be different in composition to that of the ECM more distal to the cells [86, 87 ,88].

Further immunological and histochemical assessment is needed to investigate these differential-staining patterns of the PN stain with regards to engineered tissue. However, the sensitivity of the PN staining allows for quick assessment of tissue-engineered constructs and is therefore an advantageous tool for future studies.

The remaining scaffold material and the presence of the developing areas observed in the suction seeded cut-scaffolds indicates that the culture period of two weeks may not be long enough to allow full maturation of the construct in vitro. It is possible that with an increased culture period there would be more cartilage-like tissue in these constructs and less areas of the developing tissue and remaining scaffold material.

In comparison, the cartilage-like areas of the tissue-engineered constructs have a higher cell number and less ECM than that of the native tissue. It is unknown if this cell number would decrease as the tissue engineered constructs matured. One hypothesis is that this initial high cell density is representative of cartilage in its foetal state and that the ratio of cell number over ECM content would decrease in time. This hypothesis yet to be proven and both in vitro and in vivo long-term studies are needed to investigate this fully.

The loss of 40-50% of the initial scaffold volume during the culture period of two weeks is not encouraging. The observation that cells are growing in the culture flasks after seeding indicates that cells have “fallen out” of the scaffold due to degradation of the material. This means that the degradation of the material is exceeding the maturation of the tissue within the scaffold and the subsequent ECM deposition. This rapid biodegradation is most likely a result of MMP and serine protease production by the maturing cartilage cells. MMP´s 1 and 13 are produced by chondrocytes and are involved in the degradation of collagens of collagens I, II and X [89, 90]. The optimal scaffold material would degrade at a rate close to, or equivalent to, the deposition of the ECM (see chapter 1 section 1.3) In this way, the mechanical properties of the

cartilage can develop while the construct maintains its dimensions. This loss in size of the scaffold is a barrier to the use of the Biofleece material in applications where the dimensions of the tissue-engineered product are of importance.

Key Points

1. **The use of insulin in the culture medium may help maintain the phenotypic morphology of chondrocytes.**

1. **Cell impregnation of the scaffold is improved using the suction seeding method.**

2. **Cartilage-like tissue engineered constructs could be generated using the Biofleece scaffold after two weeks in culture.**

3. **The suction apparatus needs to be optimised for even application of suction.**

4. **The Papanicolau stain allows the quick assessment of cartilage constructs because of its differential staining of developed and developing areas within the tissue engineered constructs.**

5. **The Biofleece scaffold looses 40-50% of its initial size during the tissue engineering of cartilage in a period of two weeks.**

CHAPTER 6

The co-culture of chondrocytes and respiratory epithelial cells

6.1 Introduction

The final aim of this study is to realise the very simplified tissue engineered trachea model proposed earlier in this work (chapter 1 section 1.8). In this model the simple co-culture of a chondrocyte impregnated Biofleece scaffold with a layer of REC on the apical surface was explained. Experience with co-culture studies in this laboratory has identified a flaw in this model. Respiratory epithelial cells do not appear to grow and spread directly over a cartilage layer. The inhibition of REC growth on cartilage has been reported by other researchers in the field [91]. In vivo, RECs exist on a basement membrane that effectively separates the cartilage body from the epithelial layer (see chapter 1 section 1.4). The work of this chapter investigates the use of a basement membrane replacement (matrigel) in order to create a co-cultured tissue engineered construct that in some ways mimics the native tracheal tissue.

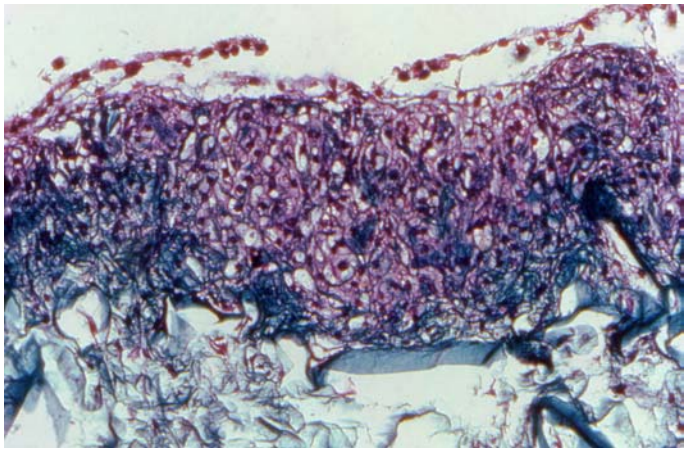
6.2 Results

6.2.1 The co-culture of REC and Chondrocytes using matrigel as a basement membrane substitute.

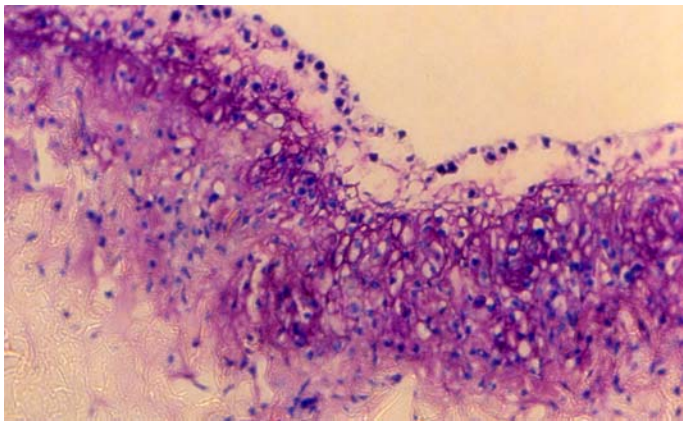
The results of the preliminary investigation (only 4 samples tested) into the co-culture of REC and chondrocytes in the Biofleece scaffold were not encouraging. The Biofleece scaffold was observed to degrade rapidly in the first 1-5 days of culture to approximately 30-35% of the original dimensions. The suction seeded chondrocytes were observed to “fall out“ from the rapidly degrading scaffolds and continue growing on the inner surface of the culture vessel. This was most notable in the first five days of culture after which the construct remained stable in its “shrunken state“. As was observed in the studies of chapter 5 the cells were observed to fall out of the scaffold

in clumps often associated with pieces of collagen. The loss of the chondrocytes was observed by placing the constructs in fresh culture dishes overnight and checking for the presence of cells growing in the bottom of the dish the following morning (see chapter 2 section 2.12) Figure 30 shows a section of one of the co-cultured test samples after the culture period of two weeks. These samples were stained with the PN stain used in chapter 5 and the MG stain (see chapter 2 section 2.12). The MG stain was used to better visualise the distribution of the cells within the scaffold since the cell nuclie are very easily distinguishable from the scaffold material with this stain. In contrast to the observations of chapter 5 the interior of the scaffolds of the co-cultured constructs retained few cells.

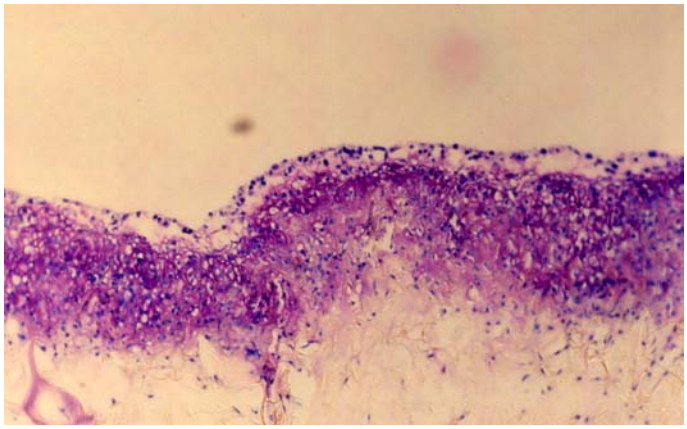
Figure 30. Stained sections of co-cultured REC and chondrocytes seeded into the Biofleece scaffold.



30(a) PN staining of co-cultured REC and chondrocytes detailing the broad band of cartilage-like tissue surfaced with matrigel and a single thin layer of REC. Note the cartilage-like tissue is stained purple as identified in the previous chapter (x20 Mag).



30(b) MG staining of co-cultured REC and chondrocytes detailing the band of cartilage-like tissue surfaced by matrigel and a single layer of REC with a lack of cells within the interior region of the scaffold (Mag x20).



30(c) A low magnification micrograph of the MG staining of co-cultured REC and chondrocytes showing the lack of chondrocytes within in the scaffold (Mag x10).

The PN staining shows that a band of cartilage-like tissue has developed proximal to the surface of the scaffold over the two-week period. This band of tissue is stained purple and correlates with the findings of chapter 5. A thin squamous layer of REC is also visible covering this band of tissue. The REC layer is only in contact with the band of tissue at a few points and is in the most part separated by a small space which is unstained or very lightly stained. It may be that these attachment points did

not survive the sectioning procedure. In addition small areas of aqua-green stained "developing regions" are also visible under the cartilage-like tissue, which correlates with the findings of the previous chapter. The remaining scaffold material is easily seen if somewhat over-stained.

The MG staining of an analogous section shows better the distribution of cells within the co-cultured constructs (Fig 30b and c). The cell nuclei are stained bright blue and this helps confirm the lack of cells deeper within the scaffold. Once again a band of cartilage-like tissue is identifiable as is the thin squamous layer of REC on the surface of the construct. The space between this thin layer and the cartilage body is stained light pink. This layer is most likely a mix of remaining matrigel and ECM deposited by the REC over the culture period of two weeks.

6.3 Disscussion

This preliminary investigation into the co-culture of REC and chondrocytes in the Biofleece scaffold indicates once again the shortcomings of the Biofleece as a scaffold material for tracheal tissue engineering. The Biofleece material was found to be prone to rapid degradation in the presence of REC and chondrocytes. This degradation is most likely caused by severe MMP production as both cell types remodel the scaffold and attempt to establish as tissues. MMP´s are produced by REC and chondrocytes and are involved in the degradation of collagens [89, 90, 92, 93]. It is a point of contention whether the lack of cells deeper within the scaffold is due to a defective suction seeding of these samples or due to the observed "falling out" of cells over the culture period. However, when taking into account the relatively good seeding of the scaffolds reported in the previous chapter and the severe degradation of the scaffolds observed in this study, it is reasonable to suggest the latter as the cause.

The rapid degradation of the scaffold by approximately 65-70% over the initial 3-7 days of culture makes the use of the scaffold impracticable where size of the implant is to be predetermined by the patients needs. It would perhaps be possible to create a large mass of tissue with the Biofleece scaffold that is then cut to size post-development. This is not an optimal solution when looking at replacing a full section of the trachea, which is circular, and of a defined diameter. Furthermore, the loss of cells from the scaffold during this rapid degradation makes the use of the scaffold inefficient when taking into account the number of cells used versus the number of cells which develop into the desired mature cartilage-like tissue. It is obvious from this study that a balance between Biofleece scaffold degradation and tissue maturation is not readily achieved (see section 1.3 chapter 1). A possible solution to improve the rate of tissue development is to use growth factors such as those belonging to the transforming growth factor beta family (TGFß) to improve cartilage maturation [83,94]. Alternatively, the use of inhibitors of collagenases such as the protease inhibitor Tetracycline could be used to slow down the active degradation of the scaffold while the tissue develops [90]. The use of Trasylol-Aprotinin (Bayer, Germany) to inhibit protease activity is being investigated in this laboratory for use in fibrin glue based tissue engineered constructs. Yet another solution would be to use a scaffold that degrades at a slower rate than the Biofleece. This is by far the simplest solution to the problem of rapid degradation. Finally, the procedure of co-culture may need to be altered to allow the cartilage body to develop prior to seeding with REC. Biofleece seeded with chondrocytes could be allowed to develop in vitro for the period of two weeks after which the surface of the construct would be coated with matrigel and then seeded with RECs. In this way the degradation associated with two cell types attempting to establish as tissue could be decreased since the REC are unlikely to degrade established cartilage tissue to the same degree as the

Biofleece scaffold seeded with un-established chondrocytes. Further studies are needed to verify this theory.

The formation of a band of cartilage-like tissue (Fig. 28) covered by a thin layer of REC indicates that the co-culture of the two cells is achievable when the REC are provided with a basement membrane substitute (matrigel) on which to grow. Other researchers have used similar methods to co-culture these two cell types. The most successful to date being a construct of cartilage surfaced by a layer of REC cells (a double layer in some places) utilising fibrin glue as the basement membrane replacement, which remained stable for five days [95]. The eventual aim would be the replacement of the basement membrane substitute with ECM deposited by the REC. As previously mentioned in the results section, it is unclear whether the matrigel remains after the culture period of two weeks. However, matrigel is nominally stable for only two weeks in culture [Sigma, Specification sheet, for matrigel, 2002] so it is most likely that the reported light pinked stained area beneath the layer of the REC is a mix of remaining matrigel and natively deposited ECM from the REC layer (Fig. 30b). A range of immunological and histochemical staining would be needed to confirm this finding and this was not within the scope of this preliminary study.

Optimally the co-cultured construct would have more than a single layer of REC on its surface as was achieved in this study (Fig. 30). REC are known to multilayer at the air liquid interface [22, 24, 25] or when given the certain growth medium such as BECGM (see chapter 4). This co-culture study utilises the medium used for the culture of chondrocytes (supplemented DMEM-HAMS F12). It is possible that the use of BECGM in the co-culture would improve the multilayering of the surface REC.. Therefore the effect of BECGM on chondrocytes needs to be investigated to establish whether this growth media has an effect on the establishment of cartilage-

like tissue by the seeded chondrocytes. A multilayer of REC pre-implantation is an advantage when considering that a single layer would be prone to damage during implantation. Furthermore, a multilayer of REC is more representative of native tissue. It is envisaged that the REC layer on the co-cultured construct may ciliate and redifferentiate once the construct is inside the patient and exposed to the airway as per the normal “native” respiratory epithelium. Thus the establishment of a REC layer that is at least representative of the native epithelium in some way is an important consideration.

Key Points

1. **The co-culture of REC and chondrocytes was achievable through the use of matrigel as a basement membrane replacement.**

2. **A thin layer of squamous epithelial surfacing a band of cartilage-like tissue was generated in this study.**

3. **The Biofleece scaffold degrades by approximately 65-70% in the first 3-7 days of the co-culture period.**

CHAPTER 7

Conclusions

7.1 Conclusions

It is evident that tissue engineered constructs consisting of more than one cell type present a unique and difficult challenge to the field of tissue engineering. The conditions that help one cell type to prosper may not be ideal for another cell type. This makes the prospect of tissue engineering very complex multicellular organs like the trachea difficult. However, a number of conclusions can be drawn from this study about how such a project can be approached.

Lectin staining was found to be a useful tool for the characterisation of cells cultured on biomaterials and is therefore helpful in identifying biomaterials that are suitable for tissue engineering of REC layers. The collagen-based membrane was found to be more suitable for the growth of respiratory epithelial cells than the hyaluronic acid-based membrane because of the adhesion qualities of the latter. The use of a collagen membrane as a culture substrate promotes the maintenance and development of ciliated cells generated from primary cultures. Furthermore, the secretory and basal cells of the respiratory epithelium appear to be more prone to dedifferentiation than are the ciliated cells when cultured on the Biofleece scaffold. Thus for REC engineering, the choice of culture substrate, i.e. collagen or hyaluronic acid is not the only variable that should be taken into account when choosing a material suitable for the chosen tissue engineering purpose and other factors such as surface topography and porosity should also be considered.

The use of ALI culture for the redifferentiation of REC shows great promise. The ability of REC to regain functionality, such as mucus production and ciliation is an important post-implant consideration when tissue engineering a construct. REC grown on a construct in submerged culture and then placed in the body as part of a tracheal substitute may undergo similar redifferentiation due to the inherent air-liquid

environment. However, the problem of nutrition needs to be solved for this to be realised. It is evident that without adequate nutrient supply any attempt at an REC layer on the surface of a tracheal construct would fail. It is the opinion of the author that this nutrient supply must come from the invasion of blood vessels into the construct from neighboring tissue and-or by the use of specially constructed biomaterials with inbuilt channels allowing the diffusion of nutrients up to the REC layer.

The suction seeding of the collagen scaffold allows for a better distribution of chondrocytes within the scaffold when compared to that of the conventional drop-wise seeding method. This seeding method allows for the development of cartilage-like tissue that produces its own ECM components. The PN staining provides a method to differentiate between cartilage-like tissue, developing cartilage-like tissue and scaffold material. The use of insulin in the growth media maintains low passage cartilage cells in a rounded morphology, as opposed to the fibroblastic morphology that is associated with cells cultured in medium without insulin. This indicates that insulin may act as an inhibitor of dedifferentiation for cartilage cells in culture and therefore its use in the culture of cells requires a more intensive study.

The degradation of the Biofleece scaffold exceeds the rate of maturation of the cartilage tissue within the scaffold. This leads to the reduction in the size of the scaffold by approximately 40-50% as reported in this study. This rapid biodegradation is most likely a result of matrixmetalloproteases (MMP), in particular collagenses, production by the maturing cartilage cells. The reduction in size means that the Biofleece scaffold is not an appropriate material for the tissue engineering of a trachea. The optimal biomaterial for the tissue engineering of a trachea would degrade at a rate equal too, or slower than, the time taken for the cells within the scaffold to mature into functional tissue. In this way the size of the engineered

trachea can be predetermined by the size of the scaffold material into which the chondrocytes are seeded. This allows for the engineered trachea to be easily fitted to a particular patient. A small reduction in size could most likely be accommodated for. However, the 40-50% size reduction as seen with the Biofleece presents too many complications. While, the Biofleece scaffold material is suitable for both the growth of REC and chondrocytes it is not appropriate for the engineering of a tracheal tissue for use in full thickness replacement surgeries.

The co-culture of REC and chondrocytes was achievable through the use of matrigel as a basement membrane replacement. However, the co-cultured construct is not stable and the Biofleece scaffold degrades at a high rate in the presence of both cell types. The end result being that the construct shrinks to approximately 35 – 30% of its original dimensions in a period of 3-7 days. The reason for this accelerated degradation is not known but is most likely the result of severe MMP production by the two cell types when in combination. This degradation makes the use of the Biofleece scaffold even less viable for tracheal tissue engineering than was concluded following the chondrocyte studies of chapter 5.

7.2 Summary

To summarise, it can be concluded that the characterisation procedures (histochemical staining, fluorescent staining and scanning electron microscopy) for both REC and chondrocyte tissue engineered constructs used in this study are beneficial and of use for further studies. The chondrocyte seeding methodologies (see chapter 5) in particular are a useful tool to be used in further studies. While this study succeeds in many ways to investigate the tissue engineering of a tracheal substitute it does not deliver such a viable substitute as an end product. The primary reason for this is the degradation of the scaffold material at a rate faster than that of

tissue maturation. It is the opinion of the author that the correct choice of scaffold it the most important step in any tissue-engineering endeavor.

7.2 Future Work

Much further work is to be conducted on the creation of tracheal tissue in this laboratory. First and foremost, work is progressing in this laboratory to make and identify suitable scaffold materials for future tracheal tissue engineering. The current methodology is to use a combination of a slow degrading scaffold made from poly-caprolactone and fibrin glue gel for the tissue engineering of a trachea. The slow degrading scaffold we are investigating has large pores into which a fibrin glue gel loaded with chondrocytes can be easily seeded. In this system the slower degrading caprolactone based scaffold acts like the exoskeleton of an insect, conferring strength and elasticity to the construct. Molding technologies can shape these scaffolds and these molds can be shapped by 3-D prototyping. Thus the problem of sizing a construct to a patient may be marginalised. The fibrin glue gel-chondrocyte component is the “active part” of the construct. The fibrin glue degrades more rapidly than the scaffold allowing the chondrocytes within the glue to replace the fibrin with “natively” produced ECM and develop into cartilage-like tissue. The molding technology may also allow for the creation of nutrient channels in order to supply the REC layer with nutrients during development and also allowing the possibility of placing this layer at an ALI while the entire tissue develops. Finally the use of chemical surface modification and basement membrane substitutes to enhance REC growth on the construct will be further investigated.

CHAPTER 8

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8.1 References

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CHAPTER 9

Papers published from this work by the author and the curriculum vitae of the author

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Personal Details

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- **Date of birth :** 17.11.71
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Education

1992-1995 University of Western Australia (UWA)Nedlands, Australia
Bachelors Degree in Science (BSc)

- Major in Biochemistry (Pass with distinction)
- Major in Mammalian Cell Biology (Pass with Credit)

1996–1998 University of Western Australia (UWA)Nedlands, Australia
Masters of Medical Science Degree in (Medicine) (MSc)

- Thesis title: Cellular Enzymatic Responses to PHEMA Hydrogels for use as Keratoprosthetic (artificial cornea) Materials

1999–2003 Ludwig Maximillians University (LMU) Munich , Germany
(submitted) Doctor of Human Biology (Medicine) (PhD)

- Thesis title: Tissue Engineering of a Tracheal Equivalent

Experience

1991-1992 BHP IRON ORE Ltd . Port Hedland, Western Australia

- **Title: Analytical Chemist**
- **Occupation:** Routine analysis of iron ores for sampling from trains and stockpiles

prior to shipping

- **Experiences, Equipment used Techniques Learnt:**

XRF analysis of ores

1991-1993 BHP IRON ORE Ltd. Port Hedland, Western Australia

- **Title: Analytical Chemist**
- **Occupation:** Research project on new ways of measuring of chemically bou

water in ores (loss on ignition) using Fourier Transform Infrared spectroscopic(FTIR) analysis.

- **Experiences, Equipment used Techniques Learnt:**

FTIR spectroscope

1993-1994 Department of Biochemistry (UWA) Western Australia

- **Title: Laboratory Assistant**
- **Occupation:** Basic lab maintenance
- **Experiences, Equipment used and Techniques Learnt:**

XRF analysis of ores

1995-1998 Lions Eye Institute Sir Charles Gardner Hospital , Western Australia

- **Title: Research Assistant**
- **Occupation:** Research project, to improve the biocompatibility of artificial corneas for use in first phase clinical trails by decreasing induced collagenase production proximal to the implant site.
- **Experiences, Equipment used and Techniques Learnt:**

Scanning Electron Microscopy (SEM)
Cell culture of primary rabbit epithelial and keratocyte cells
Cell culture of the 3T3 and L929 cell lines
Fluorescence microscopy
Setting up and running of an FDA approved clean room
General cytotoxicity testing for artificial corneas for use in first phase clinical trials
Zymography and reverse Zymography
Western Blot Analysis
ELISA based cell viability assays
Basic polymer production and handling skills
Basic animal handling and surgery skills (rabbits)
Cryosectioning

1999-2002 Klinikum Grosshadern, Klinik und Poliklinik für HNO-Kranke

- **Title: PhD student**
- **Occupation:** Research project, Tissue engineering of tracheal replacements for use in reconstructive surgery.
- **Experiences, Equipment used and Techniques Learnt:**

SEM
Cell culture and isolation of human respiratory epithelial cells
Cell culture and isolation of human cartilage cells
Cell culture and isolation of stem cells
Cell culture and isolation of respiratory basal cells
Immuno-histochemistry
Immuno-fluorescence cell sorting
Confocal Microscopy
Paraffin sectioning and Basic histology
MACS

Papers Published

B. W. Ziegelaar, J. Aigner, R. Staudenmaier, K. Lempart, B. Mack, T. Happ, M. Sittinger, M. Endres, A. Naumann, E. Kastenbauer and N. Rotter. The Characterisation of human respiratory epithelial cells cultured on resorbable scaffolds: First steps towards a tissue engineered tracheal replacement. Biomaterials, 23 1425-1438, **2002**.

B. W. Ziegelaar, J. H. Fitton, A. Clayton, S. Platten, M. Maley, T. Chirila. The modulation of corneal keratocyte and epithelial cell responses to poly(2-hydroxylethyl methacrylate) hydrogel surfaces: phosphorylation decreases collagenase activity in vitro. Biomaterials, 20 1979-1988, **1999**.

B. W. Ziegelaar, J. H. Fitton, A. Clayton, S. Platten, J. Steer, T. Chirila. The modulation of cell responses to poly(2-hydroxylethyl methacrylate) hydrogel surfaces: phosphorylation decreases macrophage collagenase production in vitro. British Journal of Biomaterials Science, Polymer Edition, 9 849-862, **1998**.

J. H. Fitton, **B. W. Ziegelaar**, C. Hicks, A. Clayton, I. Crawford, I. Constable, T. Chirila. Assessment of anticollagenase treatments after insertion of a keratoprosthetic material in the rabbit cornea. Cornea, 17 108-114, **1998**.

C. Hicks, G. Crawford, T. Chirila, S. Wiffen, S. Vijaysakerran, X. Lou, J. Fitton, M. Maley, A. Clayton, P. Dalton, S Platten, **B. W. Ziegelaar**, Y. hong, A. Russo and I Constable. Clinical results of the Chirila keratoprosthesis in rabbits. British Journal of Ophthalmology. 82 3-4, **1998**.

Y Hong, T Chirila, J. H Fitton, **B. W. Ziegelaar**, I Constable. Effect of crosslinked poly(1-vinyl-2-pyrrolidinone) gels on cell growth in static cell culture. Biomedical Materials Engineering, 7 35-47, **1997.**

Patents

Cross-linking of collagen in situ and uses thereof in wound healing. Inventors: J. H. Fitton, T. V. Chirila, C. R. Hicks, I. J. Constable, G.J. Crawford, **B. W. Ziegelaar**

Int. Pat. WO 98/22153, 28 May 1998 and Aust. Pat. 719661, 10 June 1998

Presentations

Tissue engineering of respiratory epithelial cells for use in reconstructive surgery. **B. W. Ziegelaar**, J. Aigner, R. Staudenmaier, K Lempart, B. Mack, T. Happ, M. Sittinger, M. Endres, A. Naumann, E. Kastenbauer and N. Rotter. European Society for Tissue Engineering. Innsbruck, Austria, **2001.**
Improving the biocompatibility of the artificial cornea. **B. W. Ziegelaar**, J. H. Fitton, A. Clayton, S. Platten, J. Steer, T. Chirila. Annual Australian Society for Biomaterials Conference, Canberra, Australia, **1998.**
Reducing the inflammatory response to PHEMA materials for use in the chirila Kpro **B. W. Ziegelaar**, J. H. Fitton, A. Clayton, S. Platten, J. Steer, T. Chirila. Annual. Australian Society for Biomaterials Conference, Victoria, Australia, **1997.**

Cellular responses of corneal keratocyte and epithelial cells to PHEMA materials fro use in the artificial cornea. **B. W. Ziegelaar**, J. H. Fitton, A.

Clayton, S. Platten, J. Steer, M, Mailey, T. Chirila. Annual Australian Society for Biomaterials Conference, Canberra, Australia, **1996.**
Improving the biocompatibility of the chirila keratoprosthesis. J. H. Fitton, **B. W. Ziegelaar**, C. Hicks, A. Clayton, I. Crawford, I. Constable, T. Chirila. World Biomaterials Conference, Toronto, Canada, **1996**.

Awards

1999 Best student presentation at the Annual Australian Society for Biomaterials meeting. Presentation titled “Increasing the biocomapibility of the Chirila keratoprosthesis”