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PHENOTYPIC CHARACTERIZATION AND DIFFERENTIATION POTENTIAL OF CELLS FROM HUMAN PLACENTA: NEW APPROACHES OF STEM CELL THERAPY FOR CYSTIC FIBROSIS

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SUMMARY

Cystic fibrosis (CF) is the most frequent severe autosomal recessive disorder in the European population. The gene is located on the long arm of chromosome 7 and encodes for CFTR protein (Cystic Fibrosis Transmembrane Conductance Regulator). Despite the disease involves different organs, the most clinically relevant symptoms are found in the lung. Recently, given the monogenic nature of CF, different gene therapy strategies have been developed, but the results show that the correction of the defect is only temporary. Therefore, a novel approach based on stem cell-based therapy for reconstitution of the airway epithelium and CFTR expression should be explored.

CF is a potential model disease for stem cell therapy because of the persistent lung inflammation that leads to damage and remodeling, and can promote engraftment of stem cells. If stem cells, transferred through autologous or heterologous transplant, would harbour in stem niches of respiratory epithelium, then the restoration of genetic and cellular damage will be definitive.

In the last years, several cell populations derived from adult bone marrow, amniotic fluid or umbilical cord blood, have been shown to differentiate in different epithelia, including respiratory. Even if the *in vivo* efficiency of bone marrow stem cells to differentiate in airway epithelium is very low, recent studies demonstrate that a small percentage of corrected cells is needed to revert the defect. Bone marrow mesenchymal stem cells are not without drawbacks, e.g. they are limited in numbers and lack well-defined markers required for their purification. One approach to overcome these limitations is to consider alternative sources of stem cells capable of repopulating damaged respiratory epithelium.

In the present study, we evaluated the possibility of obtaining stem cells from an ethically acceptable source as human placenta.

As a potential source of cells for application in CF therapy, the placenta has the advantage of being a natural by-product of birth which is often simply discarded, while harvest of term amniotic membrane does not pose any risk to the mother or newborn.

We demonstrated that amniotic membrane is a fetal tissue rich in cells with stem cell characteristics and with the capacity to remain “plastic” in their differentiation options. Furthermore, amniotic cells display features as low immunogenicity and non tumorigenicity.

For the first time, our results provided evidence that human amniotic epithelial cells (hAECs) and amniotic mesenchimal stromal cells (hAMSCs) can be induced *in vitro* to express markers of airway epithelial phenotype, like CFTR and ZO-1, particularly when co-cultivated with CF human epithelial bronchial cells, and so have the potential to differentiate into airway epithelial cells.

Overall, our data showed that these cells may contribute to partial correction of the CF phenotype and are very likely an ideal candidate for cell-based therapy for CF.

Whether the beneficial effects of placenta-derived cells are due to differentiation of the transplanted cells themselves or to paracrine actions on the surrounding host tissue in order to reduce inflammation and promote regeneration remains to be fully elucidated. In any case, the promising data obtained to date constitute compelling evidence regarding the potential utility of these cells for clinical application.

Future studies testing these cells in *in vivo* model, will help in finding a strategy of cell therapy for the cure of respiratory disease of CF, directly transferable to CF patients.

RIASSUNTO

La fibrosi cistica (FC) è la più frequente malattia autosomica recessiva letale nella razza caucasica. Il gene responsabile è localizzato sul braccio lungo del cromosoma 7 e codifica per una proteina denominata CFTR (Cystic Fibrosis Transmembrane Conductance Regulator). Nonostante la patologia interessi diversi organi, i sintomi clinicamente più rilevanti si riscontrano a livello polmonare. Data la natura monogenica della FC, negli ultimi anni si stanno sviluppando diverse strategie di terapia genica; tuttavia poiché dai risultati finora disponibili la correzione del difetto di base sembra essere solo temporanea, si cerca di indagare nuovi approcci basati su cellule staminali che possano ricostituire l'epitelio danneggiato ed esprimere una proteina CFTR "wild-type". Se le cellule staminali, trasferite attraverso trapianto autologo o eterologo, localizzassero nelle "stem niches" dell'epitelio respiratorio, la riparazione del danno genetico e cellulare sarebbe definitivo.

Negli ultimi anni, diverse popolazioni cellulari (cellule staminali adulte da midollo osseo, liquido amniotico o sangue del cordone ombelicale) hanno mostrato la capacità di differenziare in diversi epiteli, tra cui il respiratorio.

Anche se l'efficienza delle cellule staminali da midollo osseo di differenziare *in vivo* in epitelio respiratorio è molto bassa, studi recenti hanno dimostrato che è necessaria una piccola percentuale di cellule per correggere il difetto. Vi sono però limiti circa l'utilizzo delle staminali da midollo osseo, come lo scarso numero e la mancanza di marcatori specifici per la loro purificazione. Un approccio valido per superare tali limiti è quello di considerare fonti alternative di cellule staminali capaci di ripopolare l'epitelio respiratorio danneggiato.

Questo studio intende valutare la possibilità di utilizzare cellule staminali pluripotenti ottenute dalla membrana amniotica della placenta, per la messa a punto di una terapia cellulare per la cura della FC.

La placenta ha il vantaggio di essere un tessuto generalmente eliminato dopo il parto il cui prelievo non comporta alcun rischio per la salute della madre o del neonato.

Abbiamo dimostrato che la membrane amniotica è un tessuto fetale ricco in cellule che possiedono caratteristiche tipiche delle cellule staminali e che hanno capacità di differenziare verso cellule dei diversi foglietti embrionali. Inoltre, le cellule della membrana amniotica mostrano proprietà immunomodulatorie e sembrano non indurre tumori negli animali trapiantati.

I nostri risultati hanno documentato per la prima volta che le cellule epiteliali amniotiche (hAECs) e mesenchimali stromali amniotiche (hAMSCs) possono essere indotte *in vitro* ad esprimere marcatori di epitelio respiratorio, come CFTR e ZO-1, in modo particolare quando sono co-coltivate

con cellule di epitelio bronchiale FC, dimostrando così la potenzialità di queste cellule di differenziare in cellule dell'epitelio respiratorio.

In conclusione, i nostri dati provano che queste cellule possono contribuire parzialmente alla correzione del fenotipo FC e quindi possono rappresentare un candidato ideale alla terapia cellulare per questa patologia.

Rimane da chiarire se gli effetti benefici delle cellule della membrana amniotica siano dovuti a differenziamento delle cellule stesse o ad effetti paracrini sul tessuto circostante, volti a ridurre l'infiammazione e a promuovere la rigenerazione. In ogni caso, i dati promettenti ottenuti fino ad oggi costituiscono una prova convincente della potenziale utilità di queste cellule per l'applicazione clinica.

Studi futuri volti a testare queste cellule in un modello *in vivo* sono necessari al fine di trovare una strategia di terapia cellulare per la cura della malattia respiratoria di FC, direttamente trasferibile ai pazienti.

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INTRODUCTION

1. INTRODUCTION

Basic and clinical research accomplished during the last few years on stem cells has constituted a revolution in regenerative medicine by generating multiple possible therapeutically useful cell types. Intense research on stem cells has provided important information on developmental, morphological and physiological processes that govern tissue and organ formation, maintenance, regeneration and repair after injury. More recently, significant advances in the understanding of stem cell biology have provoked great interest and hold high therapeutic promises based on the possibility of stimulating the ex vivo and *in vivo* expansion and differentiation into functional progeny that could regenerate the injured tissue/organ firstly in animals and then in humans (*Mimeault M et al, 2007*)

1.1 Cystic fibrosis

Cystic fibrosis (CF) is the most common fatal genetic disorder in Caucasians, with an incidence of 1 in 2,500-2,700 live births (*Ratjen and Doring, 2003*).

Cystic fibrosis (CF) is caused by mutations of the CF transmembrane conductance regulator (CFTR) gene, which encodes a chloride channel that is expressed on the apical membrane of secretory epithelia. The absence of functional CFTR in airway epithelia of patients with CF leads to abnormal airway surface liquid, which favors chronic infection with *Pseudomonas aeruginosa* (PA), *Staphylococcus aureus, Burkholderia cepacia* and other bacteria. This chronic infection in CF lungs is associated with an exaggerated inflammatory response leading to pulmonary disease, the first cause of death in CF.

Exocrine pancreatic insufficiency is present in about 90% of patients with cystic fibrosis. Pancreatic disease is thought to result from a reduced volume of pancreatic secretion with low concentrations of HCO_3^- (*Kopelman et al, 1985*) Without sufficient fluid and HCO_3^-, digestive proenzymes are retained in pancreatic ducts and prematurely activated, ultimately leading to tissue destruction and fibrosis. The resulting malabsorption contributes to the failure to meet raised energy demands caused by the hypermetabolic state associated with endobronchial infection.

Ninety-eight percent of men with cystic fibrosis are infertile, with aspermia secondary to atretic or absent vasa deferentia and dilated or absent seminal vesicles (*Dodge, 1995*). Female reproductive function is normal, although cervical mucus can be dehydrated, which might impair fertility (*Kopito et al, 1973*).

Life expectancy has increased dramatically in people with CF over the past several decades due to availability of intravenous antibiotics, pancreatic enzymes and nutritional support, improved standards of care, newborn screening and development of new therapies.
Indeed, many exciting discoveries have been made in the two decades since the gene responsible for CF was described. New therapies and approaches to treatment have also been developed, some of which may have applications in other types of genetic diseases.

1.1.1 Pathophysiology

CF is an autosomal recessive disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene located on the long arm of chromosome 7 (*Lommatzsch and Aris, 2009; Rowe et al. 2005*). The CFTR gene encodes for the CFTR protein which is a chloride channel present in the epithelial cells of many organs and an important contributor to salt and water balance (Figure 1).

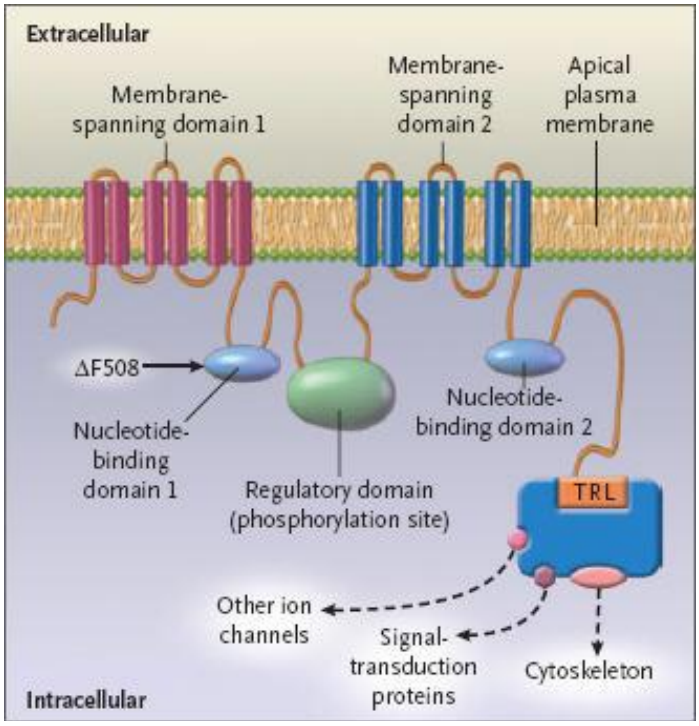


Figure 1- Scheme of the structure of CFTR

Approximately 4% of all Caucasians carry a single CFTR mutation and do not usually show any phenotypic abnormalities. However, when two mutations are present, CFTR protein does not function properly as a chloride channel with manifestations developing in multiple organs, such as lungs, pancreas, gastrointestinal tract, liver, sweat glands and reproductive tract. After description of the CF gene in 1989 (*Riordan et al, 1989*), it soon became clear that CF genetics was more complex than originally thought and there are now more than 1600 described mutations (www.genet.sickkids.on.ca). The most common mutation worldwide is deletion of phenylalanine in the 508 position (F508del) found in almost 70% of patients. This causes a misfolding of CFTR protein leading to degradation within the cell. CFTR mutations are grouped into six classes, depending on the alterations of CFTR production and function within the cell (Figure 2a). CFTR may not be synthesized (class I), have inadequate processing (class II), lack regulation (class III), have reduced conductance (class IV), have partially

defective production (class V) or accelerated turnover (class VI). F508del is a class II mutation and, in general, class I, II and III mutations are more likely to be associated with pancreatic insufficiency and more severe disease manifestations. When CFTR is absent or has decreased function in the apical membrane of the airway epithelium, the abnormalities in chloride and water flux are thought to cause a decrease in the airway surface liquid (*Ratjen, 2009*). This, in turn, leads to thick hyperviscous mucus, disruption in mucociliary clearance and a vicious cycle of neutrophil-dominant inflammation and bacterial colonization and chronic infection. Ongoing chronic infection and inflammation leads to tissue destruction and bronchiectasis with airway obstruction, a process that eventually ends with respiratory failure (Figure 2b).

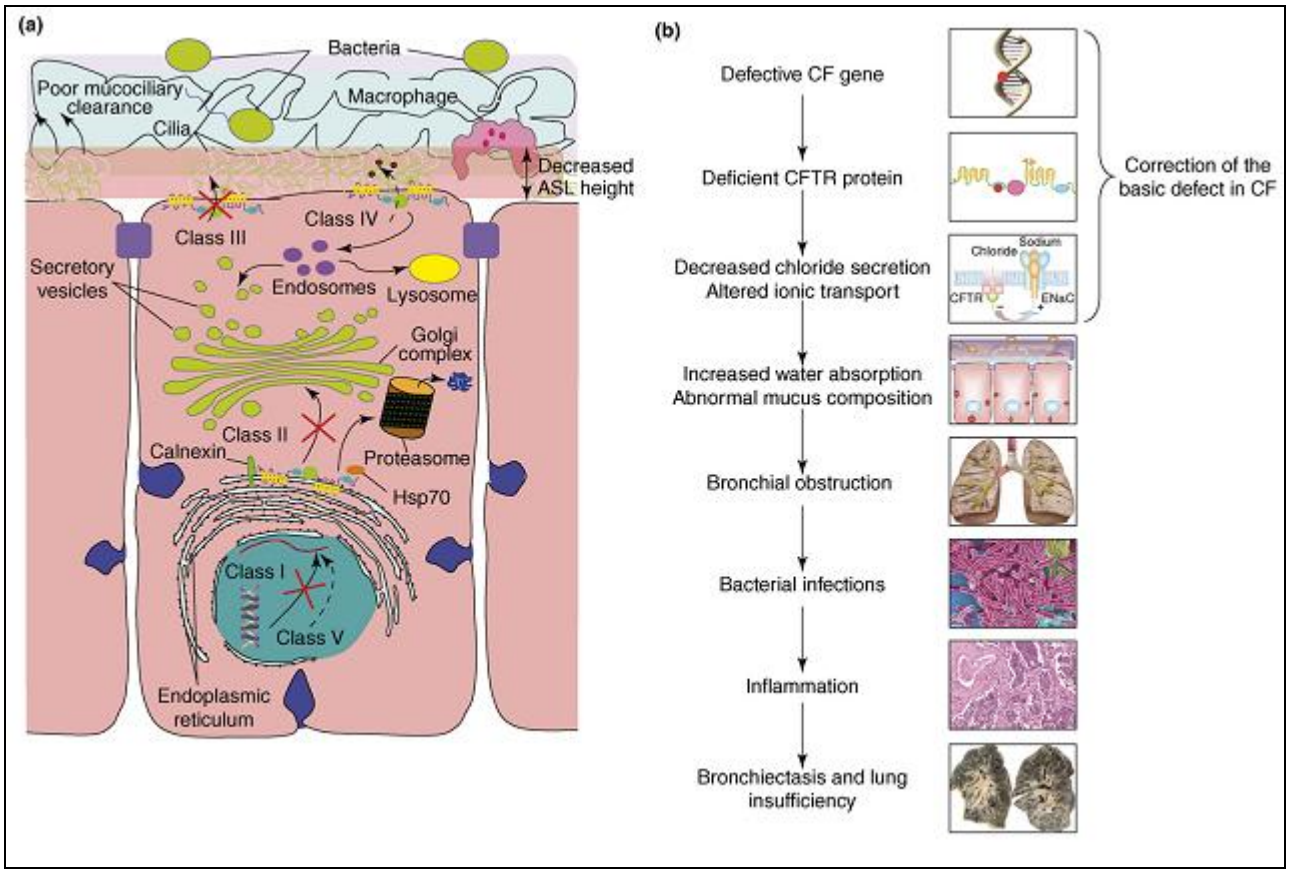


Figure 2 – Mechanism of CF dysfunction. (a) Classes of CF mutation that facilitate correction of the basic defect through a 'mutation-specific' approach (Box 1). (b) The CF pathogenesis cascade in the lung, from the primary CFTR gene defect to lung deficiency (*Amaral and Kunzelmann, 2007*).

The clinical course of CF lung disease is usually one of episodic exacerbations of pulmonary infection and inflammation interspersed with periods of relative stability. Airway microbiology may change over the lifetime of a person with CF, with *Staphylococcus aureus* and *Haemophilus influenzae* being the most common organisms isolated in infancy and childhood and *Pseudomonas aeruginosa* more likely to be cultured from adults. Treatment strategies may vary from center to

center, but common approaches include the use of aggressive airway clearance, inhaled mucolytics and airway rehydrators, antiinflammatory agents, and inhaled and intravenous antibiotics. While new therapies have become available to address evacuation of airway mucus and treat airway inflammation and infection, there are currently many more novel therapies in various stages of clinical development, targeted at all points of the disease cascade from CFTR abnormalities to chronic infection (*Ashlock et al, 2009*).

1.1.2 The respiratory system and the airway epithelium

The respiratory tract consists of upper and lower airways. The nose, followed by the sinuses, mouth, and the throat, make up the upper airways, while the trachea, bronchi, and lungs and their internal structures are contained in the lower airways. The bronchi branch into smaller bronchi and then the bronchioles, ending with air sacs called the alveoli where gas exchange takes place. These are all contained inside lobes of the lungs. The entire respiratory system is lined with epithelial cells containing a number of different cell types with specific structure and function. An anatomical overview of the airways is shown in Fig. 3.

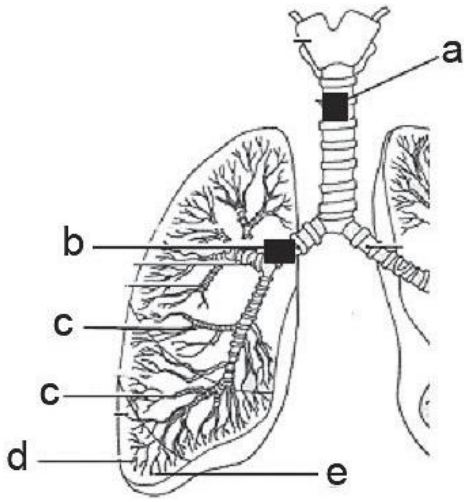


Figure 3. Anatomical overview of the lung and the putative stem/ progenitor cell niches in the (mouse) lung: (a) a ductal cell in the submucosal gland, (b) basal cells in the intercartilaginous zones of the lower trachea, (c) Clara cells in the bronchioles, (d) Clara cells at the bronchiolar-alveolar duct junctions, and (e) alveolar type II cells. Note that the mouse only has submucosal glands in the proximal trachea, in contrast to the human (*Roomans, 2010*).

The respiratory tree is lined with several specialized epithelial cell types that fulfill a number of functions to ensure lung homeostasis. There are at least 8 morphologically unique epithelial cell types, which can be classified into three categories: basal, ciliated columnar and non-ciliated secretory columnar cells (*Knight and Holgate, 2003*) depending on the region in which they are found in the respiratory system. Immune and inflammatory cells are also seen to migrate to and remain in the airway epithelium through the basement membrane to aid in epithelial cell function.

Basal cells are strongly attached to the basement membrane *via* specialized cell-extracellular matrix (ECM) junctions, termed hemidesmosomes, which confer additional mechanical integrity in the tissue (*Green and Jones, 1996*). Similarly, basal-columnar interactions are ensured *via* the intercellular adhesion molecules that belong to cadherin family (desmosomes), which also create trans-cellular networks throughout the tissue giving it the ability to resist mechanical stress (*Green*

and Jones, 1996). As for columnar-columnar interactions, these depend upon belt like tight junctions (zona occludens), responsible for cellular transport regulation, and adherence is also maintained *via* E-cadherin adherens junctions (*Roche et al, 1993*).

The wall of the trachea and bronchi in the normal human airway consists of a surface epithelium (with ciliated, brush, basal, and secretory cells), approximately 50 μm thick, resting on a basement membrane. Then follow layers of connective tissue and smooth muscle (not clearly separated), and finally, cartilage (Fig. 4).

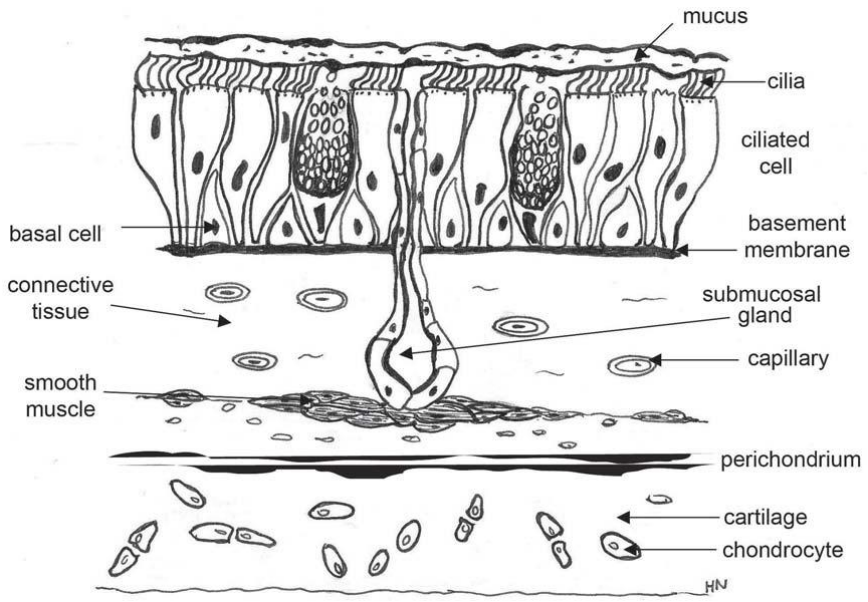


Figure 4 - Overview of the airway wall (*Roomans, 2010*).

The bronchioli lack cartilage. The epithelium in the major bronchi and proximal bronchioles (Fig. 5a) is a ciliated pseudostratified epithelium, with ciliated columnar cells as the predominant cell type; in the distal bronchioles the epithelium consists of a single cell layer (Fig. 5b). The ciliated columnar cells reach the basal lamina with slender basal processes (*Shebani et al, 2005*). The function of the brush cells of the airway epithelium is not completely known. These cells lack cilia, and may be involved in detoxification, act as a sensor for airway surface liquid, or may have a chemoreceptor function. The pyramid-shaped basal cells rest on the basal lamina but do not reach the airway lumen. The average half-life of the ciliated cells has recently been reported to be 6 months in the trachea and 17 months in the lung, which is much longer than previously estimated (*Rawlins and Hogan, 2008*). This makes the airway epithelium a tissue with a slow turn-over, which is relevant for its repair (*Snyder et al, 2009*). Different kinds of secretory cells (goblet cells in the trachea, bronchi and proximal bronchioles, Clara cells in the bronchioles, and neuroendocrine cells in the intrapulmonary airways) comprise around 20% of the bronchial and nasal epithelium and produce airway secretion (fluid and mucus). In addition, the Clara cell secretes Clara cell secretory protein (CCSP), an immunoregulatory protein (*Snyder et al, 2009*). The wall of the alveoli consists of squamous alveolar type I and surfactant-producing alveolar type II cells (Fig. 5c).

1.1.2.1 Basal cells

Basal cells are abundant in the conducting airways although their number decreases according to the size of the region (*Wang et al, 1992*). They have pyramidal shape with low cytoplasm to nucleus ratio (*Evans et al, 2001*). Historically, basal cells were thought to be the origin of stem cells in the airway epithelium, giving rise to ciliated and secretory columnar cells in larger airways (*Boers et al, 1998*). In addition to their possible progenitor role and attachment of superficial cells to the basement membrane, basal cells also secret a number of active molecules including cytokines, chemokines, and growth factors.

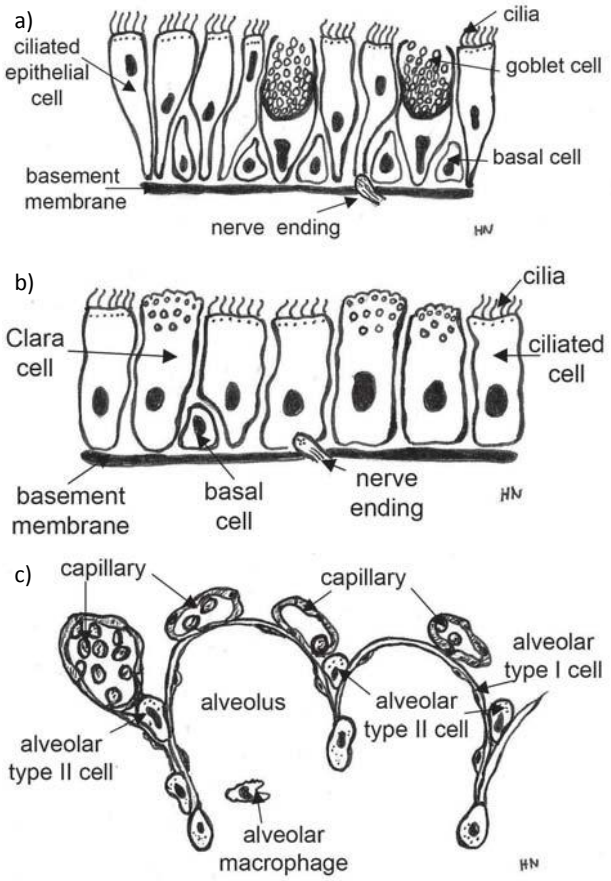


Figure 5 - **(a)** The bronchial epithelium, with ciliated and goblet cells, **(b)** the bronchiolar epithelium and **(c)** the alveoli (*Roomans, 2010*).

1.1.2.2 Columnar secretory cells

These cells are present at the apical surface of the epithelial layer. In large airways, mucus-secreting goblet cells are the predominant secretory cells and the main source of airway mucus. They are characterized by membrane-bound electron-lucent acidic mucin granules (*Jeffery and Li, 1997*). Mucus is secreted to trap any toxin and foreign molecules that the airway epithelium comes into contact with and thus these are the epithelial cell type involved in the mucociliary clearance function of the airway epithelium. These mucus goblet cells are seen in larger airways (trachea and bronchi), while Clara cells are found in smaller airways (terminal bronchioles). Mucous and Clara cells also contain electron dense granules (*Jeffery and Li, 1997*). Clara cells are believed to metabolize xenobiotic compounds by the action of p450 mono-oxygenases, and they produce bronchiolar surfactant (*Joan Gil, 1971*) as well as having ion absorbing and secreting properties (*Van Scott et al, 1987*). Moreover, Clara cell populations are thought to contain the stem or progenitor cells of the small airway epithelium where mucus goblet cells and basal cells are sparse (*Hong et al, 2001*). Another type of secretory cell is the serous cell, resembling mucus goblet cells with a difference in granule content where it is seen to be electron-dense. These cells have only

been found in rodent airways, but two populations of these rare cells have been observed in small airways of human lung (*Rogers et al, 1993*).

1.1.2.3 Columnar ciliated cells

Ciliated cells account for over 50% of all epithelial cells within the airways (*Knight and Holgate, 2003*). It is thought that these cells arise from secretory or basal cells and were initially thought to be terminally differentiated. Their primary role is the directional transport of mucus, or any foreign object, from the lung to the throat.

1.1.2.4 Alveolar type I cells

Covering 90-95% of the alveolar surface, alveolar type I cells are one of the two types of alveolar epithelial cells (*Crapo et al, 1982*). They are squamous thin cells that are very susceptible to injury. Alveolar type I cells are unable to divide, and form a thin blood-gas barrier where gas exchange occurs. They are important in maintaining the normal cellular composition and function of the alveolar epithelium (*Williams, 2003*). It has been clear that alveolar type I cells are involved in maintaining homeostasis in the alveolar epithelium (*Berthiaume et al, 2006*).

1.1.2.5 Alveolar type II cells

Type II cells are cuboidal in shape covering 5% of the distal lung. They serve as stem or progenitor cells for type I cells after alveolar injury and play a major role in gas exchange (*Sugahara et al, 2006*). One other major function of type II cells is their surfactant protein (SP) production (especially SPC), and phospholipids production which line the alveoli differentially reducing surface tension at different volumes contributing to alveolar stability (*Witherden et al, 2004; Mason, 2006*).

1.1.2.6 Pulmonary neuroendocrine cells (PNEC)

In addition to the above main epithelial cell types, pulmonary neuroendocrine cells (PNEC) are present throughout the bronchial tree either clustered in neuroepithelial bodies (NEB) or as individual cells (*Gosney et al, 1988*). These cells are thought to be very rare, increasing in number in chronic bronchitis. PNEC are thought to be involved in the secretion of a number of amines and peptides vital for foetal lung growth and airway function (*Reynolds et al, 2000*) and may play role in localized epithelial cell regeneration.

1.1.2.7 Other cell types surrounding the airway epithelium

The airway epithelium is underlined by (myo) fibroblasts, smooth muscle cells, blood vessels, afferent nerve endings, and immune cells. Fibroblasts are present beneath the basement membrane where the epithelium rests. They play a role in tissue repair and airway remodelling by secreting growth and attachment factors (*Pollak et al, 1992*) and chemotactic cytokines stimulating the bronchial epithelium to migrate following injury and thus contributing to the repair mechanism (*Shoji et al, 1989*). Surrounding the airway wall are airway smooth muscle cells (ASMC) from the trachea down to the alveolar duct controlling contraction and relaxation of the airway walls. These responses are regulated by intracellular signalling pathways (*Hall, 2000*) achieved *via* a series of homeostatic mechanisms though phospholipase C, adenyl cyclase, and ion channel pathways.

1.1.2.8 Basement membrane (BM)

Basement membrane (BM), also called the basal lamina, is a thin sheet-like membrane (40-120 nm thick), upon which basal epithelial cells adhere, consisting of ECM proteins, anchoring filaments of laminin 5, collagen IV, and proteoglycans including perlecan (*Altraja et al, 1996*). BM consists of a lamina lucida and a lower lamina densa. Below the BM, the reticular basement membrane – a layer of collagenous matrix – is found where fibroblasts are embedded. Collagen, proteoglycans, and glycoproteins, are present in all layers of the BM in differing combinations. BM plays a role in holding all the epithelial cells together and also in epithelial-mesenchymal interactions. In addition to its structural support role, BM facilitates adhesion and migration of epithelial cells through ECM protein interactions and acts as a reservoir for growth factors. It is also required for the establishment of correct epithelial polarity (*Howat et al, 2001*). Recently, pores which traverse the whole BM thickness have been observed, wide enough to allow the cells from the lamina propria to cross into the epithelium without matrix degradation (*Howat et al, 2001*).

1.1.2.9 Embryonic development of the lung

In the human, the embryonic lung starts to develop during the fourth week of embryonic development, when a ventral protuberance in the foregut is formed, the respiratory diverticulum (Fig. 6a) (*Larsen, 1993*). This entodermal protuberance is covered by mesodermal splanchnopleure, which will develop first into the connective tissue lamina propria, and then into the smooth muscle and cartilage of the airway wall, whereas the blood vessels develop from the aortic arch. On day 26-28, the protuberance splits into two primary bronchial protuberances (the origin of the two lungs), and during the fifth week secondary bronchial protuberances (three on the right side and two on the

left side: the lung lobes, Fig. 6b) are formed. During the sixth week the tertiary bronchial protuberances are formed that will give rise to the lung segments (Fig. 6c). The protuberances continue to divide during weeks 26-28; the 16th division forms the terminal bronchioles that subsequently give off the respiratory bronchioles and alveolar saccules (week 26) (Fig. 6d). From about week 32, primitive alveoli are formed. Between week 36 and birth, the alveoli (Fig. 6e) mature and from parturition on until the age of 8-10 years, new respiratory bronchioles and alveoli are formed. Only at this age the lungs are fully developed (although the lungs retain regenerative capacity at an advanced age, e.g., after surgical resection).

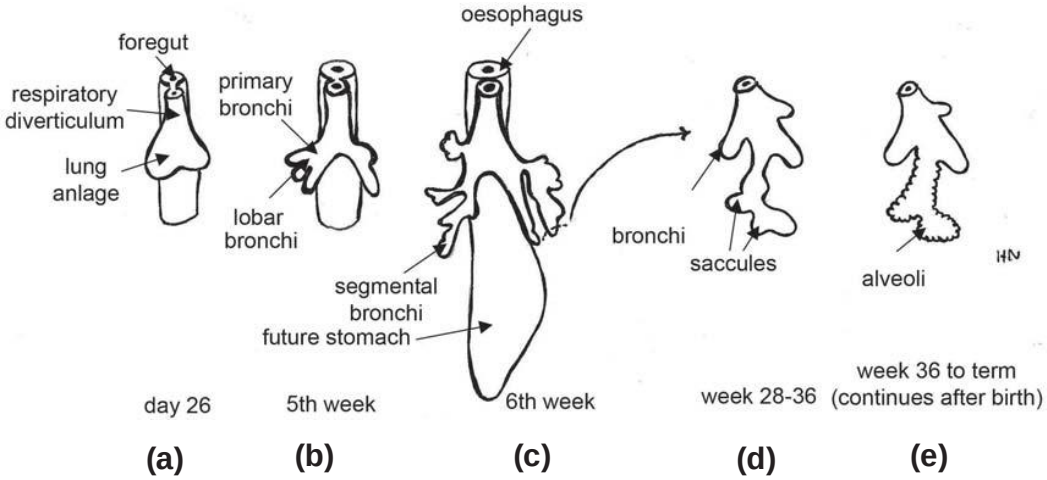


Figure 6 - Embryonic development of the lungs. **(a)** Formation of an entodermal diverticulum (respiratory diverticulum) (from the foregut) and the two anlage for the right and left lung, **(b)** the lung lobes with primary and lobar bronchi, **(c)** the segmental bronchi; behind the lung are the oesophagus and the stomach, **(d)** the bronchial tree and the formation of alveolar saccules, **(e)** the alveoli (development of the alveoli continues after birth until about 8 years of age) (*Roomans, 2010*).

Organ morphogenesis is, in general, controlled by cell-cell signaling between the epithelium, the mesenchyme, and the vasculature. In practice, therefore, repair of a defect in the airway wall will require, at a minimum, progenitors of epithelial cells, basement membrane, and fibroblasts, as well as cells able to form blood vessels. Damage to the airway epithelium may be the result of a number of airway diseases. In addition, extraneous chemicals, e.g., air pollution from traffic or industrial activities, may cause damage to the epithelium. However, even under normal conditions, there has to be a turn-over of the cells of the airway wall. Tissues with a relatively slow turn-over, use the self-renewal of differentiated cells as a strategy for tissue regeneration (*Rawlins and Hogan, 2006*). In addition, damage to the airway epithelium occurs frequently, e.g., due to components of smoke, air pollution, or to bacterial and viral infection, either from direct effects of these microorganisms or due to the inflammatory reaction that follows infection. Normally, in the case of small lesions, the

airway epithelium possesses repair mechanisms that can restore the normal structure. If the damage is more extensive, the repair process is more extensive, and is called airway remodeling (*Folli et al, 2008*). Airway repair appears to be controlled both by bronchial epithelial cells and by endothelial cells of the perfusing bronchial vasculature (*Zani et al, 2008*).

1.1.3 Target cells for gene transfer

Because the pulmonary disease in CF, which is characterized by abnormal mucus secretion, chronic bacterial infection, and airway inflammation, is the major cause of morbidity and mortality, the lung was the first target organ for gene replacement. To have successful CFTR gene transfer, the biology of CFTR and its function and the necessary target cell(s) for gene therapy need to be understood. At the cellular level, CFTR is an apical membrane protein found in several types of lung epithelial cells that serves as a regulated chloride channel (*Rich et al, 1990*). Through interactions between CFTR and the amiloride-sensitive epithelial sodium channel (ENaC), absence of functional CFTR in CF epithelial cells results in sodium hyperabsorption and lack of cyclic adenosine monophosphate–mediated chloride secretion (*Boucher, 1994*). CFTR is important for other cellular functions, including post-translational processing of high-molecular-weight glycoconjugates and cell surface receptors, pH regulation of intracellular organelles and airway surface liquid, regulation of membrane trafficking, secretion of mucus, and regulation of glutathione transport (*Metha, 2005*). There are increasing data demonstrating that constitutive and stimulated release of soluble inflammatory mediators is increased from CF airway epithelial cells. This may reflect, in part, increased basal and stimulated cell signaling resulting from increased nuclear factor–kB activity, AMP-dependent kinase activity, or altered antioxidant homeostasis in cells with defective CFTR (*Machen, 2006*). Although the mechanisms for many of these effects remain incompletely understood, they provide evidence of the complexity and multiple cellular effects that need to be considered with CFTR gene replacement. At the organ level, CFTR in lung is localized primarily at the ciliated cells of the proximal airways and the ciliated cells of the submucosal glands, which are located primarily in the proximal airways. Whether CFTR is substantially expressed in other epithelial cell types remains unclear. Earlier studies suggesting expression in non-ciliated airway epithelial cells of the airways and glands, including basal epithelial cells, have been contradicted by more recent studies demonstrating that, although CFTR mRNA may be found in these cells, levels of CFTR protein expression are low and of uncertain significance (*Anson et al, 2006; Kreda et al, 2005*). Moreover, although CFTR can be detected in type 2 alveolar epithelial cells, CFTR expression is generally highest in proximal as compared with distal airways and alveoli (*Anson et*

al, 2006). This suggests that targeting the ciliated cells, primarily in the submucosal glands, will have most effect on regulation of mucus and airway surface liquid and presumably ameliorate the most relevant pathophysiologic respiratory effects of defective CFTR. However, there is turnover of the differentiated ciliated epithelial cells, and targeting the underlying basal progenitor epithelial cells may be a more viable approach to provide for longer- lasting or even indefinite expression. Progress has been made toward identifying endogenous progenitor cells resident in proximal and distal airways, but no viable strategy for specifically targeting these cells with gene transfer vectors has been identified. Targeting defective CFTR alone may not fully correct airway disease. This is exemplified by the development of the ENaC over-expressing mouse (*Mall et al, 2004*). Unlike the CFTR knockout or F508del transgenic mice, which do not develop substantial airway disease, increased airway sodium absorption in the airways of the ENaC mice resulted in airway surface liquid volume depletion, increased mucus concentration, delayed mucus transport, and mucus adhesion to airway surfaces (*Mall et al, 2004*). The mice also developed severe spontaneous lung disease, comparable to that in patients who have CF, including mucus obstruction, goblet cell metaplasia, neutrophilic inflammation, and poor bacterial clearance. This provides evidence of the important role of ENaC in the development of many of the clinical manifestations of CF lung disease. Whether replacing defective CFTR results in appropriate regulation of ENaC remains unclear. Further, because none of the vectors used or currently being used in human trials efficiently achieves gene transfer to submucosal glands, if normal CFTR and thus normal ENaC function in submucosal glands are necessary for normal airway clearance, current approaches are not likely to affect disease pathogenesis in the proximal airways. However, efficient gene transfer to surface epithelium in more distal airways could ameliorate distal airway disease and thereby affect the clinical course of the disease without necessarily achieving a "cure".

1.1.4 Stem cells and cell-based therapy for cystic fibrosis lung disease

In cystic fibrosis two important requisites for a disease to be considered as an ideal candidate for gene therapy are fulfilled: it is a monogenic disorder and the main affected organ is the lung, which is relatively accessible via topical administration. Although initially successful gene transfer in animal models generated enthusiasm, later studies in primate models and in patients were discouraging (*Sueblinvong et al, 2007*). This has led to a search for alternative approaches including the use of stem cell populations. CF is a potential model disease for stem cell therapy because of the persistent lung inflammation that leads to damage and remodeling, and can promote engraftment of stem cells (*Piro et al, 2008*).

A developing potential therapeutic approach for CF and other lung diseases has been stimulated by recent reports demonstrating that several cell populations derived from adult bone marrow or from umbilical cord blood, including stromal derived mesenchymal stem cells (MSCs), endothelial progenitor cells, and circulating fibrocytes, can localize to a variety of organs and acquire phenotypic and functional markers of mature organ-specific cells (*Korbling and Estrov, 2003; Prockop, 2003; Herzog et al, 2003; Weiss et al, 2006*). Whether the cells used in these studies were truly ‘‘stem’’ cells has not been rigorously demonstrated, and some of these studies are controversial (*Weiss et al, 2006; Wagers et al, 2002; Neuringer and Randell, 2006*). Additionally, embryonic stem cells can be induced in culture to develop markers of lung epithelium, and a human embryonic stem cell line containing the F508del CFTR mutation has been established (*Pickering et al, 2005; Denham et al, 2006; Mateizel et al, 2006; Coraux et al, 2005*). Investigations using human embryonic stem cells are limited by scientific, ethical, and political considerations (*Brown et al, 2006*). In lung, in vitro studies demonstrate that adult marrow–derived and cord blood–derived cells can be induced to express markers of airway or alveolar epithelial phenotype (*Weiss et al, 2006; Neuringer and Randell, 2006*). In some cases, the acquisition of functional phenotype in vitro has been demonstrated. For example, human marrow–derived MSCs co-cultured with primary human airway epithelial cells express several airway epithelial markers, including cytokeratin, occludin, and CFTR (*Wang et al, 2005*). MSCs obtained from the bone marrow of patients who have CF and transduced ex vivo to express wild-type CFTR partly correct defective CFTR-mediated chloride conductance when co-cultured with primary airway epithelial cells obtained from patients who have CF (*Wang et al, 2005*). Parallel in vivo studies in mouse models have suggested that bone marrow– derived cells can localize to lung and acquire phenotypic markers of airway and alveolar epithelium, vascular endothelium, and interstitial cells (*Weiss et al, 2006; Neuringer and Randell, 2006*). In humans, lung specimens from clinical bone marrow transplant recipients demonstrate chimerism of epithelial and endothelial cells (*Suratt et al, 2003; Mattsson et al, 2004*). Similarly, lung specimens from lung transplant patients demonstrate chimerism of lung epithelium (*Kleeberger et al, 2006; Spencer et al, 2005*). Many of these reports are based on sex-mismatched transplantation and *in situ* demonstration of Y chromosome–containing donor marrow–derived cells in recipient lungs by fluorescence *in situ* hybridization followed by immunohistochemical phenotyping of the donor-derived cells (*Trotman et al, 2007*). As analytical techniques have improved, despite earlier reports demonstrating substantial engraftment with donor-derived cells, more recent reports demonstrate that only small numbers of transplanted adult marrow–derived cells engraft in recipient lungs, particularly in airway or alveolar epithelium (*Weiss et al, 2006;*

Chang et al, 2005). This includes only rare engraftment and CFTR expression in airway and intestinal epithelium after transplantation of adult marrow–derived cells containing wild-type CFTR to CFTR knockout mice (*Loi et al, 2006; Bruscia et al, 2006*). Further, the mechanisms by which marrow-derived or cord blood–derived cells are recruited to lung and acquire airway or alveolar epithelial phenotype are poorly understood. Thus, although airway or alveolar epithelial engraftment by marrow-derived cells occurs, it is rare and has not been linked to potential therapeutic benefit. Despite rare engraftment of airway or alveolar epithelium, there are an increasing number of studies demonstrating a functional role of adult marrow–derived cells in the mitigation of lung injury. This has been described in models of lung inflammation, emphysema, and fibrosis (*Ishizawa et al, 2004; Yamada et al, 2004; Ortiz et al, 2003*). These effects have been observed with several cell populations, including MSCs. Systemic administration of MSCs immediately after intratracheal bleomycin administration decreased subsequent lung fibrosis and collagen accumulation (*Ortiz et al, 2003*). Another population of marrow-derived cells contributed to airway epithelial re-epitheliazation in a tracheal allograft model (*Gomperts et al, 2006*). Conversely, marrow-derived cells, including circulating fibrocytes, may contribute to the development of lung fibrosis (*Epperly et al, 2003; Hashimoto et al, 2004; Schmidt et al, 2003*). The mechanisms for these effects are unknown but strongly suggest that marrow-derived cells can participate in lung injury and repair. More recently, several reports have demonstrated that intratracheal, rather than systemic, administration of adult marrow–derived MSCs can mitigate lung injury. Intratracheal administration of MSCs 4 hours after intratracheal endotoxin administration decreased mortality, pulmonary edema, and BALF levels of the proinflammatory cytokines tumor necrosis factor–a and MIP-1b compared with endotoxin-only–treated mice (*Gupta et al, 2006*). There was little structural engraftment of airway or alveolar epithelium by the MSCs. Comparably systemic administration of MSCs to mice with bleomycin-injured lungs decreased levels of proinflammatory cytokines and levels of matrix metalloproteinase 2 and 9 in the lungs (*Ortiz et al, 2003*). The mechanisms for these effects are unknown, but accumulating data suggest that MSCs may have significant immunomodulatory effects in the lung. Whether these cells can be used to modulate airway inflammation in CF or whether engraftment of marrow or cord blood–derived cells in airway epithelium can be used to correct defective CF airway epithelium are areas of active study but have not yielded viable therapeutic strategies. Efforts to better identify and characterize resident progenitor cells and progenitor cell niches in the upper and lower airways and

in alveoli may provide targets for sustained gene expression. Evidence in mice and humans suggests that there are regional niches of airway and submucosal gland progenitor cells with different phenotypic and functional characteristics (*Reynolds et al, 2000; Hong et al, 2001; Giangreco et al, 2002; Schoch et al, 2004; Engelhardt et al, 1995; Zepeda et al, 1995; Hollande et al, 2004*). Alveolar epithelium in patients who have CF contains primitive cuboidal cells that express primitive cell markers, including thyroid transcription factor and cytokeratin 7. This suggests that endogenous progenitor cell pathways in CF lungs may be altered, but this has not been extensively investigated. Progress toward identifying a specific pluripotential stem cell in the airway may allow for targeting with gene transfer vectors that provide sustained expression. Targeting of endogenous progenitor cells also requires methods to overcome the barriers to gene delivery.

1.2 Regenerative medicine

Recently, in addition to the current therapeutic modalities, such as medical therapy, surgery, organ transplantation, and mechanical assist devices, regenerative medicine is being focused on as a potential alternative to complicated tissue/organ transplantation. Regenerative medicine is a newly emerging and multidisciplinary field which draws on biology, medicine and genetic manipulation for the development of strategies aimed at maintaining, enhancing or restoring the function of tissues or organs which has been compromised through disease or injury. Due to their ability to differentiate toward multiple cell types, stem cells will undoubtedly play a key role in the development of such strategies.

People with end-stage lung diseases could potentially benefit from regenerative medicine since the current best treatment available is lung transplantation. Regenerative medicine offers great potential for future medical care.

1.2.1 Tissue engineering

Tissue engineering is an innovative technology employing biomedically engineered cells and scaffolds to create artificial organs and tissues such as skin, bone and heart valves. The most challenging aspect of tissue engineering is the creation of an artificial organ/tissue that has similar biological composition and physiological functions to the native organ/tissue. This may be why tissue engineering remains a colossal challenge in the lung. Recapitulating the cellular components involved in immunity, the mechanical functions of breathing and the intricate physiology of gas exchanges are factors that overwhelm tissue engineering efforts in the lung. Although no one has ever been able to generate an entire lung through tissue engineering approaches, a few studies have

shown some success in creating parts of the large airways (*Choe et al, 2006; Yang et al, 2003*) or lung parenchyma (*Chen et al, 2005; Shigemura et al, 2006*). The combination of stem/progenitor cells with bioengineering has shown promise in generating "lung equivalents" comprised of lung-like cells (*Andrade et al, 2007; Coraux et al, 2005; Cortiella et al, 2006*) since stem cells have great potential to differentiate into most if not all cell types. Bioengineering of lung cells *in vitro* with embryonic stem cells (ESC) (*Rippon et al, 2006*) is another promising effort for cell-based therapies. Although tissue engineering is a highly desirable long-term goal for lung regeneration, it remains rudimentary.

1.2.2 Gene therapy

Gene therapy involves the insertion of a therapeutic gene into cells to treat diseases, that engage replacing a mutant allele with a normal one. The discovery of CFTR has stimulated intensive research into therapeutic gene therapy approaches to replace the defective gene in CF. The first clinical trial for CF began in 1992 where adenovirus-mediate gene transfer effectively restored Cl^- conductance in the nasal epithelium of cystic fibrotic patients (*Zabner et al, 1993*). Since then, much progress has been made in terms of using gene therapy as a technique to replace the defective gene in affected cells (*Alton et al, 1993; Chow et al, 1997; Drumm et al, 1990; Flotte et al, 1993; Flotte et al, 2003; Koehler et al, 2003; Moss et al, 2004*). Many animal models of cystic fibrosis have also been created to evaluate and delineate the pathogenesis of the disease. The first and most common is the CFTR knockout mice model which most closely resembles certain features of the disease in humans (*Snouwaert et al, 1992*). Various gene delivery methods such as adenovirus (*Johnson et al, 1996; Zabner et al, 1993*), retrovirus (*Olsen et al, 1992*) and adeno-associated viruses (*Flotte et al, 1993; Flotte et al, 2003; Moss et al, 2004*) have been studied extensively for treatment of CF. These methods of gene delivery have proven to be somewhat effective, yet concerns remain over the long-term efficacy and safety of using viral-based vectors in humans. Liposomes have shown limited success in gene delivery (*Alton et al, 1993; Noone et al, 2000; Yatvin, 2002*). These vectors are unstable and have poor transfection efficiencies in vivo (*McLachlan et al, 1995*). Extracellular and intracellular barriers hinder successful gene transfer. The lack of viral receptors on the apical surface of the airway epithelium for example limits viral transfection with the transgene to the target cell when delivered topically through the airways. Systemic delivery increase risks of multiorgan toxicity and most often results in transfection of the respiratory epithelium than the airway epithelium. Furthermore, the inability of vectors to evade the host immune response limits the efficiency of successful transfection. Retroviral and adenoviral

methods of gene delivery are often accompanied by significant inflammatory responses due to these vectors. The random delivery of the transgene by viruses poses another risk of insertional mutagenesis which can affect the expression of other important genes. In addition, a study has shown the presence of viral genetic material incorporated into host genome (*Eissa et al, 1994*). In this study, about 13% of the E1 genes (responsible for viral replication) were found in the affected epithelium of patients that received adenovirus-mediated gene transfer of CFTR. Intracellular barriers include cytoplasmic clearance mechanisms (eg. Ubiquination and cytoplasmic protease digestion) that can digest the transgene preventing it from entering the nuclei (*Kitson et al, 1999*). Uptake of DNA into the nuclear compartment may also be an important barrier in terminally differentiated, non-dividing cells. Although attempts to increase nuclear uptake of oligonucleotides and plasmids by adding nuclear localization signals to the DNA (*Zanta et al, 1999*) have shown some improvements, sustained gene expression may remain a problem in slow-cycling cells.

1.2.3 Cell-based gene therapy

Cell-based gene therapy is another field of regenerative medicine. It promises the use of a host's own cells, after genetic manipulations, to deliver the therapeutic protein/DNA to the site of injury for repair and/or regeneration. Since it uses the host's own cells as vehicles for gene delivery, it avoids the immunological barriers that are often associated with viral-based vectors. In addition, this cell-based approach eliminates the risk of DNA degradation as observed in naked-DNA delivery.

1.2.4 Stem cells

Stem cells (see section 1.3) have received incredible attention within the past decade. Numerous studies have suggested the potential of stem cells to regenerate tissue/organs. The mechanism of stem cell-mediated regeneration remains controversial. While some studies suggest stem cells contribute to tissue cell lineages upon homing and lodging in the injured site by transdifferentiation and/or fusion (*Kotton et al, 2001; Krause et al, 2001; Nygren et al, 2004; Reinecke et al, 2004; Rizvi et al, 2006; Shi et al, 2004; Wang et al, 2005*), others suggest that stem cells do not contribute to tissue cell lineages but provide a pro-regenerative milieu (*Fazel et al, 2006*) for repair of injured tissue with local progenitor cells or by attenuation of immune/inflammatory responses to injury (*Ayach et al, 2006a; Ortiz et al, 2007; Xu et al, 2007b*). Functional tissue have been created through the use of stem cell manipulation/transplantation such as repair of the infarcted myocardium (*Fazel*

et al, 2005), axonal regeneration following spinal cord injury (*Kim et al, 2007*), alveolar regeneration (*Ishizawa et al, 2004a*) and the intestinal epithelium (*Bruscia et al, 2006*).

1.2.5 Rationale for stem cell-mediated regenerative medicine

The best current treatment for lung diseases such as end-stage lung diseases or CF is lung transplantation. Not only is a lack of organ donation an obstacle to treatment, but also graft rejection/failure is a major limitation for long-term success in lung transplantation. Stem cell-mediated regenerative medicine offers great promise for treatment of diseases that involves cell-based gene correction and replacement of a particular cell such as in cystic fibrosis. Successful long-term restoration of continuously self-renewing tissues such as the lung depends on the use of self-renewing stem cells. In addition, the pluripotent/multipotent differentiation capabilities of stem cells allows for potential functional replacement and restoration of tissue components. Therefore stem cells provide appropriate targets for prospective cell-based gene therapies and cell replacement strategies.

1.3 Stem cells

1.3.1 Stem cells

A stem cell is defined as an undifferentiated cell capable of long-term self renewal and multi-lineage differentiation potential. Under homeostatic conditions, stem cells are slow-cycling and/or quiescent. Upon stimulation such as during tissue injury, stem cells can give rise to daughter cells called transient amplifying (TA) cells that generates sufficient specialized progenies for tissue maintenance. Stem cells can undergo symmetric cell division whereby a stem cell divides and gives rise to 2 daughter stem cells identical to itself. Stem cells can also undergo asymmetric cell division in which one of the daughter cell loses some of the stem cell characteristics of the parent cell and begins the process of cell lineage commitment.

1.3.2 Progenitor cells and transient amplifying cells

A progenitor cell has more limited differentiation potential but may still be multipotent. Progenitor cells may have self-renewal capacity but, unlike stem cells, progenitor cells do not have unlimited self-renewal capabilities. Transient amplifying cells are intermediary cells between a stem cell and its final differentiated progeny. These cells are highly proliferative and can give rise to one or more differentiated cell type. It remains unclear whether TA cells are the same as progenitor cells.

1.3.3 Stem cell characteristics

1.3.3.1 Self-renewal

Self-renewal is defined as the ability of a cell to give rise to daughter cells that retain the properties of the parent cell. The process has been rigorously studied and involves several intrinsic signals such as the proto-oncogene Bmi-1 (*Park et al, 2003*) which encodes for polycomb group complex proteins that repress genes involved in differentiation, the transcription factor HoxB4 (*Sauvageau et al, 1995*), and signaling molecules Notch (*Varnum-Finney et al, 2000*), Sonic hedgehog (*Bhardwaj et al, 2001*) and Wnt (*Austin et al, 1997; Nemeth and Bodine, 2007; Reya et al, 2003*). Activation of Wnt signaling is also important in mesenchymal stem cells proliferation and mesenchymal lineage specification (*Etheridge et al, 2004*). Telomerase activity is associated with increased cell proliferation. Telomeres are regions of highly repetitive DNA found at the end of linear chromosomes that cannot be replicated by the DNA polymerase complex. Therefore, with every replication of the chromosome, it will lose a small portion of the genetic information. Telomerase is a subgroup of reverse transcriptase enzymes known as TERT that can extend telomeres. The decrease in telomerase activity has been linked with aging (*Hornsby, 2007*) while an increase in telomerase activity has been associated with cancer (*Deng and Chang, 2007*).

1.3.3.2 Development of diverse phenotypes

Cells can develop and mature through a lineage program through many pathways. The most common pathway is differentiation of an immature cell such as a progenitor cell into a cell with more specialized function(s) and greater level of complexity. The process may require several rounds of cell division going through several intermediary cell types before becoming the fully mature cell type. Transdifferentiation occurs when a cell becomes another cell type outside of its already established differentiation pathway. Dedifferentiation is the process by which a terminally differentiated cell reverts to a cell of an earlier developmental stage. Cell fusion may be another pathway by which a cell acquires the phenotype of another cell. Bone marrow cells have been shown to give rise to lung epithelial, cardiomyocyte, and muscle cells by cell fusion with resident tissue cells (*Nygren et al, 2004; Shi et al, 2004; Spees et al, 2003; Terada et al, 2002; Wong et al, 2007*).

Epigenetic regulation plays a role in stem cell differentiation. The three major epigenetic mechanisms that have been shown to control stem cell differentiation are DNA methylation, Histone modification, and non-coding RNA-mediated regulatory events.

DNA methylation occurs mainly at CpG dinucleotides. Hypermethylation is associated with gene silencing whereas hypomethylation is associated with gene expression. DNA methylation is primarily associated with parental-specific imprinting during gametogenesis (*Kaneda et al, 2004*) and silencing of genes on the inactivated X-chromosome (*Maatouk et al, 2006*) but it also involved in cell differentiation (*Takizawa et al, 2001*). Post-translational modification of histones by acetylation of lysine residues on the amino end of the chromatin is another mechanism that controls cells differentiation (*Cheung et al, 2000*). Lysine acetylation by histone acetyltransferases (HATs) is associated with gene activation, whereas deacetylation with histone deacetylases (HDACs) is associated with gene inactivation. Histone methylation however is associated with transcriptional repression whereas demethylation is associated with transcriptional activation. Finally, post-transcriptional gene regulation by small non-coding RNAs or microRNAs is a newly emerging epigenetic mechanism. These RNAs are distinct from siRNA (small interfering RNA) in that they are transcribed products produced from RNA-coding genes. These microRNAs can then bind to mRNA sequences that share some complementary sequences to the mRNA preventing the mRNA from being translated. The outcome is reduced expression of a particular group of genes. MicroRNAs have been shown to be involved in embryonic stem cells development (*Chen et al, 2004*).

1.3.3.3 Plasticity

Plasticity is defined as the ability of a cell to produce progenies that cross lineage boundaries. There is a hierarchy of stem cell plasticity. Totipotent cells can give rise to all cells in the body as well as the placenta and embryonic cells (ESC and germ cells). These cells are considered to be the most primitive stem cells and exists in the first few cell divisions of the fertilized egg. Pluripotent stem cells are descendents of totipotent stem cells and can give rise to cells of all germ layers. These cells are found in the inner cell mass of the blastocyst and while ESC are the best known, there are others such as primordial germ cells, and possibly certain adult stem cells such as bone marrow-derived very small embryonic-like stem cells. Multipotent stem cells have more restricted differentiation potential than pluripotent stem cells. As the progeny become more lineage-committed or determined, the ability of the cell to give rise to various cell lineages becomes restricted down to the unipotent progenitor cell which can only give rise to one particular cell phenotype. It has long been felt that multipotent stem cells can only give rise to cells within a lineage. However, there is growing evidence that cells that were once thought to be multipotent can give rise to cells outside of their traditional lineage commitment.

1.3.4 Types of stem cells

1.3.4.1 Embryonic stem cells

Embryonic stem cells (ESC) are pluripotent cells derived from the inner cell mass of the blastocyst within the first 5-7 days after an egg is fertilized by sperm (*Thomson et al, 1998*). In other words, they can produce cells from all three germ layers: endoderm, ectoderm and mesoderm. These cells transiently exist in the developing embryo. These cells can be isolated and cultured ex vivo as cell aggregates called embryoid bodies and maintained in an undifferentiated state indefinitely through artifactual conditions using leukemia inhibitory factor containing media and on feeder layers. A recent study showed that ESC can also be cultured as single cell suspensions in an undifferentiated state using a Rho-associated kinase inhibitor (*Watanabe et al, 2007*). ESC express several markers associated with self-renewal and pluripotency namely OCT-4, Rex-1, Nanog, SSEA-1 (mouse) and SSEA3/4 (humans) and the presence of alkaline phosphatase and telomerase activity (*Armstrong et al, 2005; Babaie et al, 2007; Pan and Thomson, 2007; Wobus et al, 1984*). ESC has been shown to differentiate into cells of neuronal (*Bibel et al, 2004*), cardiac (*Guo et al, 2006; Reppel et al, 2004*), retinal (*Tabata et al, 2004*), hepatic (*Shirahashi et al, 2004*), and lung epithelial (*Samadikuchaksaraei et al, 2006*) cells.

1.3.4.2 Adult stem cells

Adult stem cells are multipotent in that they have less self-renewal capacity and their potential for differentiation was believed to be limited to their tissue of origin. However, recent evidence have suggested that certain adult stem cells may have pluripotent characteristics (*Devine et al, 2003; Jiang et al, 2002a; Krause et al, 2001; Kucia et al, 2006a*).

1.3.5 Stem cell challenges

Stem cells isolated from adults or developing embryos are currently thought to be a source of cells for regenerative medicine. However, despite their therapeutic potential, both adult and ES cells present a number of challenges associated with their clinical application and are thus not in general use yet (*Smith et al, 2006*). For example, although adult stem cells can be directly isolated from the patient and are therefore immunologically compatible with the patient, they are generally hard to isolate and grow in culture; and moreover, transplantation of a sufficient number of cells to adult tissue needs a large-scale cell supply. In contrast, human ES cells can proliferate very fast in culture and differentiate into cells of all adult tissues, but additional research is required to control the

growth and to overcome the risks of tumor formation by undifferentiated ES cells and graft rejection. It is also necessary to resolve the ethical issues surrounding the use of materials from embryos.

Embryonic stem cells require destruction of the human embryo for their procurement and are associated with a high rate of tumor induction (teratoma) following transplantation, while mesenchymal stromal cells from bone marrow carry with them a risk of viral infection (*Eichna et al. 2008*), and the differentiation capacity of these cells has been seen to decrease with donor age (*D'Ippolito et al, 1999; Mareschi et al, 2006*). The need therefore remains to identify a source of stem cells that is safe, easily accessible, provides a high cell yield and for which cell procurement does not provoke ethical debate.

Recently, the multipotent differentiation ability of amnion-derived cells has been reported and these cells have attracted a lot of attention as a cell source for cell transplantation therapy.

In addressing the complex scenario described above, several groups have recently turned their attention to the human term placenta as a possible source of progenitor/stem cells. The fact that placental tissues originate during the first stages of embryological development supports the possibility that these tissues may contain cells which have retained the plasticity of the early embryonic cells from which they derive. Meanwhile, the fact that the placenta is fundamental for maintaining fetomaternal tolerance during pregnancy suggests that cells present in placental tissue may have immunomodulatory characteristics. These two key aspects make cells from placenta good candidates for possible use in cell therapy approaches, with the possibility of providing cells that are capable of differentiating into multiple different cell types, and which also display immunological properties that would allow their use in an allo-transplantation setting. Furthermore, given that the placenta is generally discarded after birth, this tissue is available in large supply, the recovery of cells from this tissue does not involve any invasive procedures for the donor, and their use does not pose any ethical problems.

The amniotic membrane has already been applied in medicine, for example, in burn lesion treatment, surgical wound covering to avoid collusion (*Trelford et al, 1979*), and ocular surface reconstitution, although uncertainty remains regarding the mechanism of its effects.

1.4 The placenta

1.4.1 Placental Compartments

The fetal adnexa is composed of the placenta, fetal membranes, and umbilical cord. The term placenta is discoid in shape with a diameter of 15–20 cm and a thickness of 2–3 cm. From the

margins of the chorionic disc extend the fetal membranes, amnion and chorion, which enclose the fetus in the amniotic cavity, and the endometrial decidua. The chorionic plate (Fig. 7) is a multilayered structure that faces the amniotic cavity.

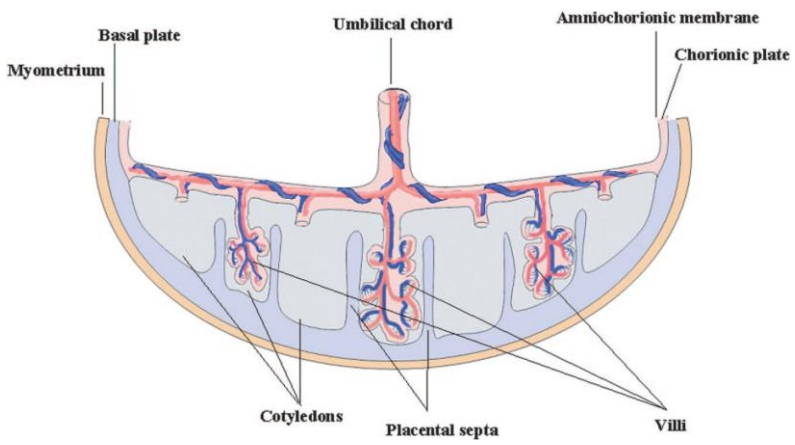


Figure 7- Schematic section of the human term placenta. During placenta formation, from the maternal side, protrusions of the basal plate within the chorionic villi produce the placental septa, which divide the parenchima into irregular cotyledons. Each cotyledon contains several villi, which originate from the chorionic plate. Fetal blood vessels are located within the branches of the villi.

It consists of two different structures: the amniotic membrane (composed of epithelium, compact layer, amniotic mesoderm, and spongy layer) and the chorion (composed of mesenchyme and a region of extravillous proliferating trophoblast cells interposed in varying amounts of Langhans fibrinoid, either covered or not by syncytiotrophoblast). Villi originate from the chorionic plate and anchor the placenta through the trophoblast of the basal plate and maternal endometrium. From the maternal side, protrusions of the basal plate within the chorionic villi produce the placental septa, which divide the parenchyma into irregular cotyledons (Fig. 7). Some villi anchor the placenta to the basal plate, whereas others terminate freely in the intervillous space. Chorionic villi present with different functions and structure. In the term placenta, the stem villi show an inner core of fetal vessels with a distinct muscular wall and connective tissue consisting of fibroblasts, myofibroblasts, and dispersed tissue macrophages (Hofbauer cells). Mature intermediate villi and term villi are composed of capillary vessels and thin mesenchyme. A basement membrane separates the stromal core from an uninterrupted multinucleated layer, called syncytiotrophoblast. Between the syncytiotrophoblast and its basement membrane are single or aggregated Langhans cytotrophoblastic cells, commonly called cytotrophoblast cells. Fetal membranes continue from the edge of the placenta and enclose the amniotic fluid and the fetus. The amnion is a thin, avascular membrane composed of an epithelial layer and an outer layer of connective tissue, and is contiguous, over the umbilical cord, with the fetal skin. The amniotic epithelium (AE) is an uninterrupted, single layer of flat, cuboidal and columnar epithelial cells in contact with amniotic fluid. It lies on top of a mesodermal layer consisting of an upper acellular compact layer and a deeper layer containing dispersed fibroblasts.

The chorionic membrane consists of a mesodermal layer (AM) and a layer of extravillous trophoblast cells. (Fig. 8).

In the amniotic mesoderm closest to the epithelium, an acellular compact layer is distinguishable, composed of collagens I and III and fibronectin. Deeper in the AM, a network of dispersed fibroblast-like mesenchymal cells and rare macrophages are observed. Very recently, it has been reported that the mesenchymal layer of amnion indeed contains two subfractions, one having a mesenchymal phenotype, referred as amniotic mesenchymal stromal cells, and the second containing monocyte-like cells (*Magatti et al, 2007*). A spongy layer of loosely arranged collagen fibers separates the amniotic and chorionic mesoderm (Fig. 8). The chorionic membrane (chorion leave) consists of mesodermal and trophoblastic regions (Fig. 8). Chorionic and amniotic mesoderm are similar in composition. A large and incomplete basal lamina separates the chorionic mesoderm from the extravillous trophoblast cells. The latter, similar to trophoblast cells present in the basal plate, are dispersed within the fibrinoid layer and express immunohistochemical markers of proliferation. The Langhans fibrinoid layer usually increases during pregnancy and is composed of two different types: a matrix type on the inner side (more compact) and a fibrin type on the outer side (more reticulate). At the edge of the placenta and in the basal plate, the trophoblast interdigitates extensively with the deciduas (*Cunningham et al, 1997; Benirschke and Kaufmann, 2000*).

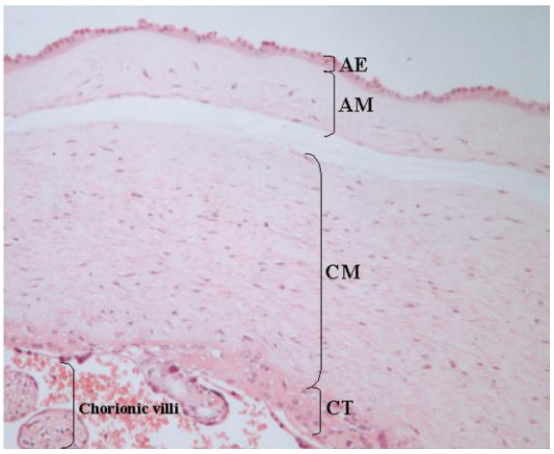


Figure 8- Cross-sectional representation of human fetal membranes (amnion and chorion). Abbreviations: AE; amniotic epithelium; AM, amniotic mesoderm; CM, chorionic mesoderm; CT, chorionic trophoblast.

1.4.2 Embryological Development

In humans, by days 6–7 after fertilization (during the implantation window), the blastocyst implants and placenta development begins. At this stage, the blastocyst is flattened and composed of an outer wall (trophoblast) that surrounds the blastocystic cavity. A small group of larger cells, the inner cell mass, is apposed to the inner surface of the trophoblastic vesicle. The embryo, umbilical cord, and amniotic epithelium are derived from the inner cell mass. As the blastocyst adheres to the endometrium, invading trophoblasts erode the decidua, facilitating implantation of the blastocyst. By 8–9 days after fertilization, trophoblastic cells at the implanting pole of the blastocyst proliferate robustly, forming a bilayered trophoblast. The outer of the two layers becomes the

syncytiotrophoblast by fusion of neighboring trophoblast cells, whereas the inner cells (cytotrophoblast) remain temporally unfused. The proliferating cytotrophoblasts and the syncytiotrophoblasts give rise to a system of trabeculae intermingled with hematic lacunae. From these trabeculae are generated the primordial villi that are distributed over the entire periphery of the chorionic membrane. Villi in contact with the decidua basalis proliferate to form the leafy chorion or chorion frondosum, whereas villi in contact with the decidua capsularis degenerate into the chorion leave. At day 8–9 after fertilization, the inner cell mass differentiates into two layers: the epiblast and the hypoblast. Subsequently, from the epiblast, small cells that later constitute the amniotic epithelium appear between the trophoblast and the embryonic disc and enclose a space that will become the amniotic cavity. On the opposite side, between the hypoblast and cytotrophoblast, the exocoelomic membrane and its cavity modify to form the yolk sac. The extraembryonic mesoderm arranges into a connective tissue that surrounds the yolk sac and amniotic cavity, giving rise to amniotic and chorionic mesoderm (Fig. 9).

Gastrulation, the process through which the bilaminar disc differentiates into the three germ layers (ectoderm, mesoderm, and endoderm) and develops a defined form, with a midline and cranio-caudal, right-left, and dorsal-ventral body axes, occurs during the 3rd week after fertilization (*Benirschke and Kaufmann, 2000; Moore et al, 1998*).

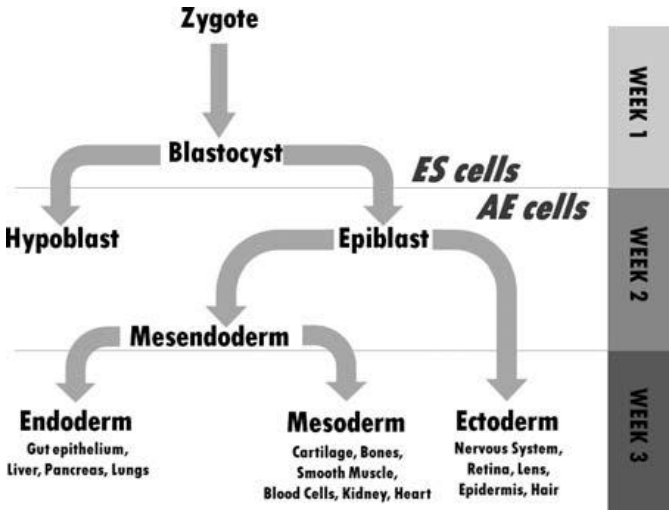


Figure 9 – Diagram of embryogenesis from fertilization to gastrulation

1.4.3. Basic structure and function of amniotic membrane

The amniotic membrane is a tissue of fetal origin and is composed of three major layers: a single epithelial layer, a thick basement membrane, and an avascular mesenchyme (Fig. 10) (*Benirschke*

and Kaufmann, 2000). There are no nerves, muscles, or lymphatics in the amnion. It is adjacent to the trophoblast cells and lines the amniotic cavity. It can be easily separated from the underlying chorion, with which it never truly fuses at the cellular level. The amnion obtains its nutrition and oxygen from the surrounding chorionic fluid, the amniotic fluid, and the fetal surface vessels.

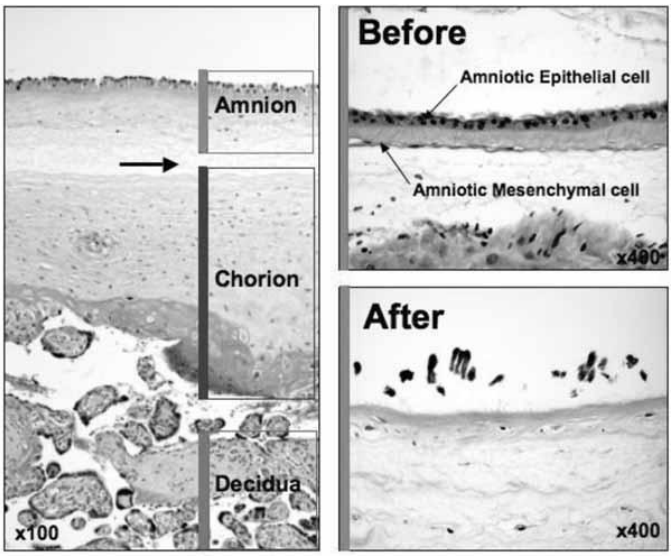


Figure 10 - Histology of amnion. Normal human placenta sample as sectioned and stained with hematoxylin and eosin. In the left panel, the three major components, amnion, chorion, and decidua, were shown. The arrow indicates the coarse intermediate layer of connective tissue. The right panel shows photos of membrane "before" and "after" trypsinization. Only amniotic epithelial cells were dissociated by trypsinization. Although not shown, subsequent collagenase digestion would release the amniotic mesenchymal cells from the connective tissue.

One of the basic functions of the amniotic membrane is to provide the developing embryo with protection against desiccation and an environment for suspension in which the embryo can grow without distortion by pressure from surrounding structures. The amnion also plays an important role during parturition. In the initiation and maintenance of uterine contraction, prostaglandins play a pivotal role. The amniotic epithelium is not only one of the main sources of prostaglandins, especially prostaglandin E2 (*Okazaki et al, 1981*), but also expresses prostaglandin-biosynthesis enzymes such as phospholipase, prostaglandin synthase, and cyclooxygenase (*Bryant-Greenwood et al, 1987*). Moreover, these enzymes are regulated by human chorionic gonadotropin (hCG), the receptors of which are found on the amniotic epithelium (*Toth et al, 1996*). Amniotic epithelium is metabolically highly active throughout gestation, and it is also responsible for regulating the pH of the amniotic fluid, keeping it constant at about 7.10.

From a structural perspective, the amniotic cells are connected to each other by numerous desmosomes; however, tight junctions occluding the lateral intercellular spaces and thus limiting paracellular transport can not be observed between the cells (*King, 1982*). Consequently, the intercellular clefts may represent an effective route for paracellular transfer of macromolecules. The basal lamina contains large quantities of proteoglycans, rich in heparan sulfate, that may serve as a permeability barrier to amniotic macromolecules (*King, 1985*). Wolf et al. reported that in the amniotic epithelium, the specialized arrangement of intracellular cytoskeletal filaments such as actin, α- actinin, spectrin, ezrin, cytokeratins, vimentin, and desmoplakin indicates their role in the structural integrity and modulation of cell shape as well as in junctional permeability (*Wolf et al,*

1991). Laminin is one of the main components of the basement membrane and it critically contributes to cell differentiation, cell shape and movement, maintenance of tissue phenotypes, and promotion of tissue survival via cell surface receptors such as integrins and dystroglycans (*Akashi et al, 1999*).

1.4.4 Pluripotency of amnion-derived cells

Regarding differentiation potential, it is expected that the amniotic membrane might maintain pluripotent properties. Developmentally, the inner cell mass of the blastocyst, from which ES cells are derived, gives rise to both the epiblast (from which the embryo is derived) and the hypoblast (from which the yolk sac is derived). From this epiblast (embryonic ectoderm) the amniotic epithelial layer (amniotic ectoderm) is derived on about the 8th day after fertilization, while the mesenchymal cells (amniotic mesenchyme) are from extraembryonic mesoderm of the primitive streak (*Enders and King, 1988*). Considering that the epiblast also gives rise to all of the germ layers of the embryo, amniotic epithelial cells are also expected to give rise to three germ layers. Moreover, the amniotic epithelial layer is derived before gastrulation. Considering that pluripotent embryonal carcinoma cells can only be generated from cells derived before gastrulation (*Diwan and Stevens, 1976*), indicating the importance of gastrulation in the differentiation and specification of cell fate, it is expected that amniotic epithelial cells might maintain the plasticity existing in the cells in the pregastrulation embryo (*Miki et al, 2005*). Several reports demonstrated that both human amniotic epithelial cells (hAECs) and amniotic mesenchymal stromal cells (hAMSCs) express several stem cell markers such as octamer-binding transcription factor (OCT)-4, which is specifically expressed in ES cells and germ cells; GATA-4, which is a marker of definitive embryonic and visceral (extra-embryonic) endoderm; hepatocyte nuclear factor-3β (HNF-3β), which is a marker of definitive endoderm; nestin, which is an intermediate protein and a neural stem cell-specific marker; and nanog (*Miki et al, 2005; Wei et al, 2003; Takashima et al, 2004*). These facts suggest that hAECs and hAMSCs possess pluripotency.

1.4.5 Anti-inflammatory and low immunogenic characteristics of amniotic membrane /amnion-derived cells

Amniotic membranes/amnion-derived cells have been considered to be suitable tissue or cells for allotransplantation, based on their anti-inflammatory effects and low immunogenicity. Much evidence supporting these ideas has been accumulated. As to anti-inflammatory effects, Hao et al. reported that both hAECs and hAMSCs express various antiangiogenic and anti inflammatory

proteins such as interleukin (IL)-1 receptor antagonist; tissue inhibitors of metalloproteinase (TIMPs)-1, -2, -3, -4; and IL-10 (*Hao et al, 2000*). Amniotic membrane stromal matrix markedly suppressed lipopolysacharide-induced upregulation of both IL-1α and -1β in human corneal limbal epithelial cells cultivated on it (*Solomon et al, 2001*). In addition, amniotic membrane stromal matrix also suppressed DNA synthesis and subsequent differentiation of myofibroblasts obtained from human cornea and limb, through suppressing the TGF-β signaling system (*Tseng et al, 2009*). hAECs expressed mRNA of tumor necrosis factor (TNF)α, Fas ligand, TNF-related apoptosis-inducing ligand (TRAIL), TGF-β, and macrophage migration-inhibitory factor (MIF) (19). The supernatants from hAECs cultures inhibited the chemotactic activity of neutrophils and macrophages toward macrophage inflammatory protein 2, reduced the proliferation of T- and B-cells after mitogenic stimulation, and induced apoptosis of T and B cells, but not of corneal epithelial cells (*Li et al, 2005*), and also suppressed corneal neovascularization and migration of major histocompatibility complex (MHC) class II+ antigen-presenting cells (APCs) in cauterized mouse corneas (*Kamiya et al, 2005*). As to antibacterial properties, human amniotic membrane reduces bacterial counts and promotes healing of infected wounds (*Talmi et al, 1991*). As to the low immunogenicity, clinical signs of acute rejection were not observed when amniotic membrane was transplanted into volunteers (*Akle et al, 1981*). The expression level of MHC class I antigens is still controversial. Although it was reported that HLA-A, -B, -C, and –DR were not detected in cultured amniotic epithelium (*Adinolfi et al, 1982*), the detection of class I antigen in almost all cells in the amniotic membrane has been reported since then (*Hammer et al, 1997; Kubo et al, 2001*). In contrast, class II antigen was expressed only in some fibroblasts in human amniotic membrane (*Kubo et al, 2001*). Several studies examined the fate of amniotic membrane grafts. Wang et al. (*Wang et al, 2006*) studied allogeneic GFP+ mouse amniotic membrane grafts heterotopically transplanted in the eye. Kubo et al. studied xenotransplanted human amniotic membrane in the eye of rats (*Kubo et al, 2001*). These experiments showed that the fate or allogenicity of amniotic epithelial cells depended on the graft site. For example, grafts implanted in the anterior chamber or intracornea survived longer than those transplanted on the ocular surface. The authors mentioned that because of the short period of viability of donor-derived amniotic epithelial cells grafted on the ocular surface, these cells had already lost viability and thus were unable to display enough antigens to represent a target for effector CD4+ and CD8+ T cells. As a result, long-term memory of sensitization was not acquired (*Wang et al, 2006*). In addition, from the observation that amniotic cells disappeared without a rejection reaction, the authors speculated that the short existence of donor derived AE cells on the ocular surface might be due to the process of apoptosis (*Kubo et al,*

2001; Wang et al, 2006). Actually, both hAECs (*Li et al, 2005; Runic et al, 1998*) and hAMSCs (*Kubo et al, 2001*) were reported to express Fas ligand, which may be easily released from apoptotic amniotic cells as the soluble form, and may exert an immunosuppressive effect. Fetal nonclassic HLA-G (class Ib antigen) is also expressed in the human amniotic membrane (*Hammer et al, 1997; Kubo et al, 2001*). Because the HLA-G molecule has low polymorphism compared to with class Ia antigen, aggression against the fetus is not easily initiated by HLA-G expression in the fetal-maternal interface. Kubo et al. noted that the expression of HLA-G in the amniotic membrane implies two possibilities for the host immune system. First, HLA-G may play the role of a tolerogenic peptide, and the host lymphocytes or dendritic cells may be inactivated by HLA-G's binding to inhibitory receptors. Secondly, HLA-G may be recognized by certain T cells because CD8 can bind to HLA-G, and these cells may have a suppressor function (*Kubo et al, 2001*). The expression of these immunosuppressive and immunoregulatory factors may also explain it in part. Thus, the problem of immune rejection could be overcome by the use of amniotic membrane. However, it was also reported that in presensitized recipient mice and recipients that underwent repeated amniotic epithelial implantation, graft survival was markedly shorter than in normal recipients, suggesting that fresh allogeneic mouse amniotic epithelium expressed immunogenicity (*Wang et al, 2006*). Therefore, at present, amniotic membrane and the cells derived from it seem to be suitable tissue for transplantation because of their anti-inflammatory and low immunogenic characteristics; however, in clinical applications, the risk of immunogenicity depending on the transplantation-site or of rejection, occurring after repeated amniotic membrane transplantation from the same donor, should not be ignored.

1.4.6 Non-tumorigenicity

There was no evidence of tumorigenicity in humans when isolated amniotic cells were transplanted into human volunteers to examine their immunogenicity or into patients in an attempt to correct lysosomal storage diseases (*Akle et al, 1981; Scaggiante et al, 1987; Sakuragawa et al, 1992*). However, trisomy mosaicism in the amnion has been reported, especially in amniotic epithelial cells. Robinson reported that in 16 (48%) of 33 cases of prenatally diagnosed trisomy mosaicism, trisomy was confirmed to be present by molecular analysis of the amnion, although the trisomy was absent from most fetal tissues (*Robinson et al, 2002*). In the cases in which trisomy mosaicism in the amnion was not noticed because the fetal tissue was normal, whether or not some biological effects would arise in clinical application of these amnions is unclear.

1.4.7 Little ethical problems with usage

Because the amniotic membrane is discarded after parturition, it is easy to obtain without harming mothers or babies and would thereby overcome the ethical issues associated with the use of ES cells. However, it is still in the possession of the mother, so the use of human amniotic membrane had been approved by the Ethics Committee of each Institution and written informed consents had been obtained from the mothers. Based on these considerations, human amniotic membrane / amnion-derived cells are considered to be a useful biological material and also a novel cell source for cell transplantation.

AIM OF THE STUDY

2. AIM OF THE STUDY

The present study aims at evaluating the possibility of using pluripotent stem cells derived from human placenta (amnion) in gene and cell therapy for Cystic Fibrosis (CF).

So far, most experience in preservation of placental tissue derived cells has been gained with cord blood, which contains both hematopoietic and mesenchymal stem cells. When cord blood transplantation proved effective, many cord blood banks were established, offering collection and banking for public (allogenic) or private (autologous or allogenic) use. In contrast to cord blood, fetal membrane stem cells are presently preserved mainly for research. However, as these cells gain interest for their regenerative and immunomodulatory properties, future medical needs may require concomitant application of cord blood and placental cells from the same donor. Stem cells studies are in rapid evolution and new sources are investigated in order to find a major "cell plasticity".

It is well established that human placenta may represent a reserve of progenitor/stem cells. Because the amniotic membrane is discarded after delivery, it is easy to obtain without harming mothers or babies and would thereby overcome the ethical issues associated with the use of embryonic stem cells. Based on these considerations, human amniotic membrane/amnion-derived cells are considered to be a useful biological material and also a novel cell source for cell transplantation.

Amniotic epithelial cells (hAECs) and amniotic mesenchymal stromal cells (hAMSCs) are known to have unique characteristics, such as low level expression of major hystocompatibility complex antigens, and a less restricted differentiation potential (*Miki and Strom, 2006*). In culture, hAECs and hAMSCs differentiate into cell types from all the three germ layers. The availability of hAECs and hAMSCs and the lack of ethical concerns for this source of stem cells are considered advantageous for their widespread use and acceptance. Studies reported the differentiation of embryonic stem cells in epithelial tissue composed of basal cells, ciliated cells, intermediate cells, nonciliated Clara cells, and similar to the epithelium covering the human bronchioles (*Miki et al, 2009*).

Although gene therapy is easier to perform and has the advantage to transfer Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) wild-type gene into CF airways and to restore the correct chloride ion flux through the epithelium, its effect is only temporary. Cell therapy could be a more effective treatment because allogenic normal cells and autologous engineered cells may reconstitute the damaged epithelium and express the CFTR gene.

Recently, new cell sources for CF treatment have been characterized: mesenchymal stem cells from cord blood (*Sueblinvong et al, 2008*) and amniotic fluid stem cells (*Carraro et al, 2008*) can differentiate in vitro and in vivo in airway epithelium. The in vivo efficiency of bone marrow stem cells to differentiate in airway epithelium is very low (0.01-0.025%) (*Piro et al, 2008*), as

demonstrated by different studies in mice (*Loi et al, 2006; Bruscia et al, 2006*); in order to facilitate cell homing and epithelium differentiation of these cells, pulmonary damage can be induced by chemical or physical agents, as bleomycin or X-ray radiation. Recent studies demonstrate that with 0.1% of donor cells that became respiratory host cells a partial correction of the electrophysiological defect has been obtained (*Bruscia et al, 2006*). On the contrary, other studies demonstrate that a higher percentage (6-20%) of corrected cells is needed to revert the defect (*Farmen et al, 2005*).

In the present study, we evaluated the possibility of obtaining epithelial and mesenchymal stem cells from an ethically acceptable source as human placenta. After the isolation of these cells, we characterized them and expanded in culture. We finally evaluated the ability of amniotic epithelial and mesenchymal cells in differentiating in vitro in airway epithelium for the cure of cystic fibrosis.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1. Isolation of Human Amniotic Epithelial and Mesenchymal Stromal Cells

Human amniotic epithelial and mesenchymal stromal cells (hAECs, hAMCs) were isolated from term placentas which would normally be discarded after delivery. For sterility purposes, placentas were normally obtained from caesarean section; however, theoretically, any placenta should be useful for amniotic cell isolation. Tissues were obtained under appropriate Ethical Committee approval and signed informed consent (see appendix). All infectious pathogen–positive deliveries including those involving HBV, HCV, and HIV, as well as cases of prediagnosed genetic abnormalities, were excluded. Even with these precautions, standard precautions for safe use of human tissue must be followed. Placenta samples were procured immediately after delivery and kept in a sterile beaker. Once in the laboratory, placentas were transferred on a sterile field under flow laminar cabinet.

3.1.1 Isolation of hAECs

Reagents and solutions preparation (*Miki et al, 2010*):

Pre-digestion buffer

Prepare 1000 ml calcium- and magnesium-free HBSS (CMF-HBSS; Lonza, cat. no. 04-315Q) supplemented with 0.5 mM EGTA (Sigma, cat. no. E4378). Store up to 4 weeks at 4°C.

Standard culture medium

430 ml DMEM (Invitrogen, high-glucose formulation; cat. no. 11960044)

5 ml 100 mM (100×) sodium pyruvate (Invitrogen, cat. no. 11360-070)

50 ml heat-inactivated fetal bovine serum (Invitrogen, cat. no. 16141; 10% v/v final)

5 ml 100 mM (100×) nonessential amino acids (Invitrogen, cat. no. 11140-050)

5 ml 200 mM L-glutamine (Cellgro, cat. no. 25-005-CI)

500 µl 55 mM 2-mercaptoethanol (Invitrogen, cat. no. 21985-023)

5 ml antibiotic-antimycotic solution (Cellgro, cat. no. 30-004-CI)

10 ng/ml human recombinant epidermal growth factor (EGF; Sigma, cat. no. E9644)

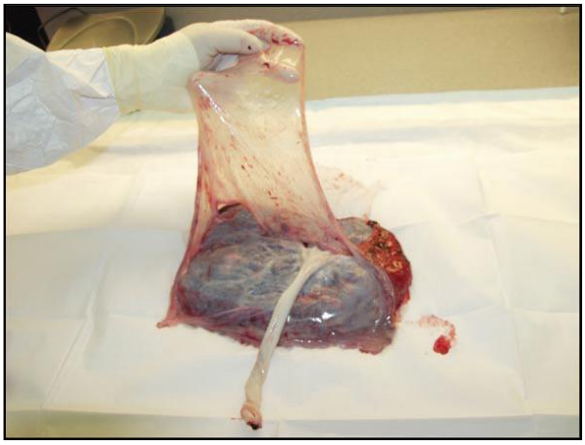
Store up to 15 days at 4°C

In this standard culture medium the cells grow for 2 to 6 passages, a passage being approximately a 1:3 split when the cells approach confluence. Removal of the EGF results in a rapid decrease in proliferation and what appears to be terminal differentiation. Even in the continued presence of

serum and EGF, the cells do not normally proliferate past passage 6 under the culture conditions described here.

Carry out dissection:

- Wearing sterile gloves, place the whole placenta on the sterile field (Fig.11).



The maternal surface (rough surface) should be facing down on the paper, with the smooth surface bearing the umbilical cord facing up. In this position, the amnion membrane will lay across the upper surface of the placenta.

Figure 11- Placenta with amnion membrane facing up

- Trim the umbilical cord close to the placental surface and cut an X-shaped incision into, but not through, the placental tissue (Fig.12). The X should intersect at the position of the umbilical cord. It may be easiest to start the incision in the region of the umbilical cord. After this step there could be an excess of blood on top of the amnion. If that is the case, pour some CMF-HBSS on top of the membrane and gently massage to remove blood clots.

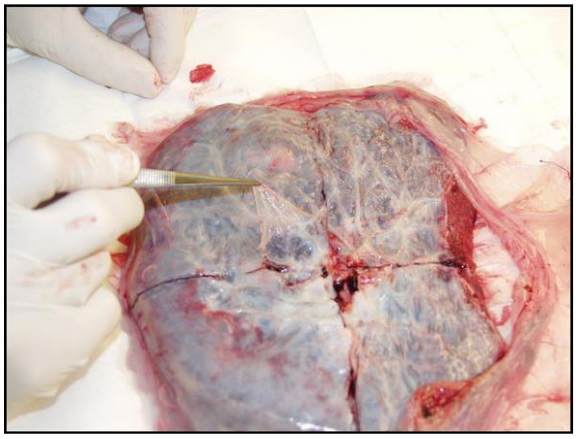


Figure 12- placenta with an X-shaped incision

- Peel the amnion membrane from the underlying chorion layer of the placenta body. Start from the cut edge (middle of the placenta body) and peel the membrane from the placenta. In order to avoid taking extra tissue, pinch the rough part with a second pair of forceps and only collect the amnion. This phenomenon is more common in tissue from lengthy labor or when the amnion is partially detached from the discoid placenta.

- Place the amnion in the sterilized 500-ml glass beaker containing 200 ml CMF-HBSS (Fig.13), discarding the remaining part of the placenta.

Figure 13- Washing the amniotic membrane in HBSS

- Wash the amnion two to three times, each time by moving the amnion to a clean 500-ml beaker containing 200 ml fresh CMF-HBSS. This washing step is crucial for the trypsin to work properly. Blood clots will reduce the efficiency of the trypsin. Ideally, all clots on the membrane will be cleared.

Digest the membrane

- Thaw and prewarm the trypsin/EDTA solution to 37°C in a water bath.
- Place the membrane in a 50-ml centrifuge tube and add 20 ml pre-digestion buffer. Gently rock the membrane in the solution for 30 sec, then transfer the membrane with forceps to two new 50-ml tubes. Discard the buffer.
- Add 20 ml of pre-digestion buffer to the tissue in the tubes. Incubate 10 min at 37°C.
- Transfer the membrane into new tubes (discarding the pre-digestion buffer) and add 30 ml trypsin/EDTA solution. Incubate for 40 min at 37°C. Save the trypsin digest.
- Transfer the membrane into new 50-ml tubes, add 30 ml fresh trypsin/EDTA, and incubate for an additional 40 min. Save the trypsin digest.
- Add two volumes of standard culture medium to both the two trypsin digests and centrifuge 10 min at 200 × *g*, 4°C.
- Decant the supernatant and resuspend the pellet in appropriate volume of standard culture medium. The cell pellet might appear like a compact tissue-like aggregate. Pipet up and down several times in order to release the cells. Discard any remaining small cell aggregate or filter the cell preparation through a 100-µm filter. If necessary, centrifuge one or two more times at 200 × g, 4°C.

Prepare cultures

- Count the cell number.
- If cells are to be cultured, plate up to 1×10^5 cells per cm^2 in standard culture medium.

3.1.2 Isolation of hAMSCs

Reagents and solutions preparation (*Marongiu et al, 2010*):

Digestion solution

500 ml EMEM (with 25mM HEPES buffer without L-glutamine, with Earle's BSS; Lonza, cat. no. 12-136F) supplemented with:

1 mg/ml Collagenase type IV (Sigma, cat. no. C5138)

25 μg/ml DNase I (Sigma, cat. no. DN25)

To be prepared fresh in a volume proportional to the amount of tissue.

Standard culture medium

430 ml DMEM (Lonza, cat. no. 12-604F, with 4.5 g of glucose/liter)

5 ml sodium pyruvate (Invitrogen, cat. no. 11360-070)

50 ml 10% (v/v) heat-inactivated fetal bovine serum (Invitrogen, cat. no. 16141)

5 ml 100 mM non-essential amino acid (Invitrogen, cat. no. 11140-050)

5 ml 200 mM L-glutamine (Cellgro, cat. no. 25-005-CI)

500 μl 55 mM 2-mercaptoethanol (Invitrogen, cat. no. 21985-023)

5 ml antibiotics solution (Cellgro, cat. no. 30-004-CI)

10 ng/ml epidermal growth factor (EGF; Sigma, cat. no. E9644, human recombinant)

Store up to 2 weeks at 4°C

The isolation of hAMSCs is performed after the isolation of hAECs as follows:

- Transfer the membrane into a 500-ml beaker with cold HBSS.
- Wash the amnion two to three times with 200 ml HBSS, each time by moving the amnion to a clean beaker.

Digest the membrane

- Transfer the membrane into two (or more) 50-ml centrifuge tubes, allowing excess HBSS to drip from the membrane. The amount of wet tissue will be increased after trypsin digestion. In order not to dilute the collagenase solution excessively, divide the tissue as needed so as not to have more than 20 ml of wet tissue in 50 ml final volume.
- Add the digestion solution to completely fill the tubes. Incubate on a rotator 1 hr at 37°C.
- The incubation time varies according to the membrane thickness, ranging between 45 min to 1.5 hr. It is wise to occasionally check the status of digestion after the first 30 min, and stop the incubation as soon as the tissue is completely dissolved.
- Equally divide the contents of each tube into two new 50-ml tubes.
- Add an equal volume of cold HBSS and centrifuge 5 min at 200 × *g*, 4°C.

- Discard the supernatant. Resuspend the pellet with fresh HBSS to fill the tube and centrifuge again 5 min at 200 × *g*, 4°C.
- Resuspend the pellets in a small volume of standard culture medium and combine them in a single solution.
- Count the cell number.
- If cells are to be cultured, plate up to 1×10^5 cells per cm^2 in standard culture medium.

3.1.3 Purification of amniotic mesenchymal stromal cells from amniotic epithelial cells

Although the isolation of hAECs by trypsin/EDTA digestion yields a virtually pure preparation, this is not the case for hAMSCs isolation; a considerable number of hAECs may still be attached to the membrane before collagenase digestion, and this may result in the presence of hAECs in the hAMSCs preparation.

Considering the slow adhesion of hAECs to culture substrates, as opposed to the fast attachment of hAMSCs, it is good practice to change the culture medium 1 to 2 hr after plating. This will remove many of the hAECs that might be present in the preparation. However, significant contamination of hAMSCs with hAECs will frequently occur. The morphological differences between the two cell types will allow to easily determine whether the contamination with hAECs is substantial or not after 24 hr in culture.

3.1.4 Determination of cell counts

Cell counts were routinely determined prior to seeding cells into fresh media. After harvesting and re-suspending, 10 µl aliquots of cells were placed in the chambers of a hematocytometer. Cells in each corner quadrant of the grid were counted and multiplied by 10^4 to give the number of cells per ml since the area of the 4 corner squares is 1 mm^3 (0.1 mm^3 x 10^4 = 1 ml). Volumes of cells were then adjusted to give the desired seeding densities.

3.1.5 Cryopreservation of cells

The cells harvested were cryopreserved re-suspending the pellet in cold 10% dimethyl sulphoxide (DMSO, diluted in 1ml of the desired media). Cell suspensions were placed in a sterile cryovial and immediately stored at -80°C for freezing for four hours then transferred to liquid nitrogen (-179°C).

3.2 Characterization of human Amniotic Epithelial and Mesenchymal Stromal Cells

3.2.1 Flow cytometry

Flow cytometric analyses of hAECs and hAMSCs were performed at different passages.

Cultured cells were characterized by means of flow cytometry for membrane antigens. Cells were detached from culture flask using trypsin and, after washing, were incubated with 4% normal mouse serum/PBS/NaN_3 for 20 minutes at 4°C in order to block non-specific Fc receptor sites on cell membrane. Cells were then stained in the dark at 4°C for 20 minutes with 7-amino actinomycin-D to discriminate viable cells from fragments and dead cells and with monoclonal antibodies conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE) or PE-Cyanin 7 (PE-Cy7) or allophycocyanin (APC) or APC-Cyanin 7 (APC-Cy7). The cells were characterised at different passages using the following monoclonal antibodies (moabs): anti-epithelial cell adhesion molecule (EpCAM), anti-Thy-1, anti-ATP binding cassette-G2 (ABCG2), anti-CD45, anti-CD34, anti-CD44, anti-CD49f, anti-CD56 (Neural Cell Adhesion Molecule, NCAM), anti-CD13, anti-CD166 (Leukocyte Cell Adhesion Molecule, LCAM), anti-CD146, anti-CD105, anti-CD29, anti-CD73, anti-CD31, anti-SSEA4 (Becton Dickinson Biosciences, BD, Franklin Lakes, NJ, USA), anti-CD133-1 (Miltenyi Biotech, Bergisch Gladbach, Germany).

For internal labeling, cells were fixed at room temperature with 4% paraformaldehyde (PFA) for 10 min and permeated with 100% ethanol for 2 min after washing with PBS. Cells were incubated with 10% FBS to block non-specific binding, followed by primary antibody Oct-4 and Nanog (Santa Cruz Biotechnology, Inc. USA) for 1 hour; secondary FITC-conjugated antibody (Molecular Probes) was applied for 30 min. Mouse or rat non-immune immunoglobulins were always used as controls. The fluorescence threshold between negative and positive cells was set on the basis of the reactivity of appropriate non-specific fluorochrome-conjugated isotypical controls. No lysing or fixing procedures were applied to cell suspensions to avoid possible changes on antigen expression and cellular alterations. At least 10^6 cells were finally analysed using a FACSCanto II equipped with FACSDiva software (BD).

3.2.2 Immunofluorescence Microscopy of Cultured Cells

Fixed plated cells (4% paraformaldehyde or 70% ethanol) were permeabilized and then immunostained with anti-EpCAM, anti-cytokeratin (CK)-18, anti-alpha-foetoprotein (Sigma), anti-CK19 (Novocastra, Newcastel, UK), anti-albmin (Dako-Cytomation, Milan, Italy), anti-CK7, anti-CD49f, anti-CD29, anti-S100A4, anti-CD90, anti-CD31, anti-CD146, anti-ZO-1, anti-fibronectin, anti-alpha1-antitrypsin, anti-E-cadherin, and anti-Beta-catenin (BD).

The following procedure was applied:

- Wash the plated cells three times in PBS;
- Fix the plated cells in 4% paraformaldehyde or 70% ethanol for 30 min.;
- Wash the cells twice in PBS;
- Permeabilize using HEPES-Triton X-100 buffer 0.25% in PBS;
- Wash the cells twice in PBS;
- Incubated with a permeabilisation Horse (or Horse-Goat) Serum Dilution Buffer containing 0.5 M NaCl, 20 mM NaHPO4, 0.1% Triton X-100 and 30% horse (or horse and goat) serum for 30 min.;
- Wash the cells several times in PBS;
- Add the primary antibodies diluted in PBS and incubate for 2 h at room temperature;
- Wash the cells several times in PBS;
- Add fluorescence-conjugated specific secondary antibodies and incubate for 1 h at RT in a dark room;
- Wash the cells several times in PBS;
- Stain the cell nuclei with 4'- 6'-diamidino-2-phenylindone DAPI (Sigma) for 5 min. in a dark room;
- Wash the cells several times in PBS.

Images were taken using a Leica Microsystems DM IRE 2 microscope and analysed with the FW4000I software (Leica Microsystems, Milan, Italy).

3.2.3 Cytogenetic analysis

Chromosome analysis of amniotic epithelial and mesenchymal cells at different passages were performed.

- After removing culture medium, add 3 ml of hypotonic solution (0.6 g trisodium citrate and 0.15 g KCl in 100 ml distilled water) and incubate 10 minutes at room temperature.
- Remove hypotonic solution and add 3 ml Ibraimov solution (acqueous acetic acid 5%); incubate 5 minutes at room temperature.
- Remove Ibraimov solution and add 3ml of fresh fixative (4:1 methanol - acetic acid).
- Remove the fixative and add 3 ml of new fixative solution.
- Remove the glass slide from the capsule, remove the excess of liquid and dry it in Optichrome at 28°C 42% humidity.

The cells on the slides were observed and analyzed with a fluorescence microscope Olympus BX 41: mercury vapor lamp HBO 100 W high pressure, excitation filter 425-503 nm, barrier filter 415 nm. The source is a mercury vapor lamp characterized by a linear characteristic spectrum, with high emission intensity in some spectral bands (ultraviolet, blue and green).
The image analyzer is connected to the microscope for cytogenetics through a camera and the camera is connected to a digital processing system. The analyzer used in our laboratory is Cytovision ™ System, an analytical tool that assists the cytogeneticist in the observation of the metaphases of chromosomes and in detecting cell abnormalities.

3.4 Cell differentiation

Differentiation towards different lineages was investigated for both hAECs and hAMSCs.
Bone marrow stem cells were used as positive control for differentiation; experiments were repeated with different samples.

Adipogenic differentiation

To induce adipogenic differentiation, 1 to 3 passages cells were plated at $4x10^3$ cells/cm^2 density on tissue culture dishes (Falcon, BD) and treated with adipogenic medium for three weeks. Medium changes were carried out 3 times a week and adipogenisis was assessed at 21 days. Adipogenic medium consisted of DMEM low glucose (Sigma) supplemented with 10% FBS (Gibco), 0.25mM IBMX (Sigma), 0.1μM dexamethasone (StemCell Technologies Inc.), 66nM insulin (MP Biomedicals, Germany), 1nM Triiodothyronine T3 (Sigma), 10μM rosiglitazone (Avandia®, GlaxoSmithKline, United Kingdom), 10mM hepes (Sigma), 170μM pantotenate, 33μM biotin (Ink Biomedicals, USA), 10μg/ml transferrin (MP Biomedicals), 1% penicillin/streptomycin and 1% L-glutamine (Gibco).

Osteogenic differentiation

To induce osteogenic differentiation, 1 to 3 passages cells were plated at $4x10^3$ cells/cm^2 density on tissue culture dishes (Falcon, BD) and treated with osteogenic medium for 3 weeks with medium changes 3 times a week. Osteogenesis was assessed at 21 days. Osteogenic medium consisted of DMEM low glucose (Gibco) supplemented with 100nM dexamethasone, 10mM β-glycerol phosphate, 50μM ascorbic acid 2 phosphate (StemCell Technologies Inc.), 1% penicillin/streptomycin (Gibco).

Hepatocyte differentiation

A simple protocol for hepatic differentiation is shown in the schematic form in Fig.14. For hepatic differentiation, hAECs and hAMSCs were plated on type 1 collagen-coated culture dishes in standard culture media supplemented with neaa, glu, 2ME and EGF and steroid hormones such as hydrocortisone (HydC) or dexamethasone (Dex) (*Miki et al, 2009*).

Figure 14- Protocol for hepatic differentiation

3.4.1 Airway epithelium differentiation

To induce airway epithelium differentiation, the following protocol was applied (*Karp et al, 2002*).

Reagents and solutions preparation:

Airway medium 1: this medium is for cell seeding and the first culture day only. It is composed of a ratio of a 1:1 of DMEM and Hams F-12 supplemented with 5% FBS (not heat-inactivated) and 1% MEM non essential amino acids solution (Life technologies, cat. No. 11140-050)

Airway medium 2: this medium is for use after culture d1 and then continuously. It consists of a 1:1 ratio of DMEM and Hams F-12 supplemented with 2% Ultroser G.

Permeable membrane support for culture epithelia: Costar Transwell-Clear, 0.4 um pore size, 6.5mm diameter (Costar, Cat. No. 3470) (Fig. 15).

Figure 15 – Plate with permeable supports

Preparation of permeable membranes:

Coat the membrane insert with a solution of human placental collagen (60 µg/ml) for a minimum of 18 hours:

- use a ratio of 30 mg collagen with 50 ml deionized water and 100 µl glacial acetic acid.
- Cover the holding beaker with parafilm and stir moderately at about 37°C until collagen strands are dissolved.
- Dilute the filtered collagen stock 1:10 with deionized sterile water. This diluted collagen stock is the working solution (60 µg/ml) for coating plastic and membrane surfaces.
- Filter sterilize with a 0.2 µm membrane. If the filter membrane quickly plugs, the collagen strands have not fully dissolved.
- Collagen-coat the surface of membrane for a minimum of 18 hours at room temperature. The collagen improves cell attachment efficiency and proliferation.
- On the day of cell seeding remove the liquid collagen from surface and air-dry the membrane surface.
- Rinse at least twice with sterile PBS on both side of membrane support. It is important to remove all trace of the liquid collagen. Residual liquid collagen can be toxic to cells.

Seed the cells

- the seeding range should be $2.5x10^5$ to $5x10^5$ cells/cm^2.
- The cell suspension seeded onto the top of the permeable membrane insert should be of sufficient volume, 100-400 µl, to insure a uniform distribution of cells settling upon the membrane surface. The volume of the medium under the membrane insert should be sufficient (250-500 µl) to immerse the membrane bottom without floating the insert.
- After seeding, make sure that the membrane supports are level to insure uniform cell attachment during the first 12 hours.
- Leave the cluster dishes with the seeded membrane supports undisturbed for a minimum 18-24 hours in a CO_2 incubator at 37°C and 8 to 9% CO_2. The higher CO_2 increases successful achievement of confluence.
- The day after seeding, change the airway medium 1 on both sides of the membrane to airway medium 2 (2% Ultroser G).
- Remove the top medium with vacuum suction and rinse the top membrane surface once with airway medium 2 to remove unattached cells (Fig. 16).
- Then remove the medium from the top of the membrane surface (air interfacing) so that medium is present only on the bottom of the membrane insert. When cells grow at the air interface, they form a confluent sheet with tight junctions and no visible fluid on top. The air interface allows the confluent sheet to better differentiate as a barrier separating air on top

from the liquid media immersing the bottom. This air interface condition is comparable to the air-covered surface of the airway epithelium in vivo.

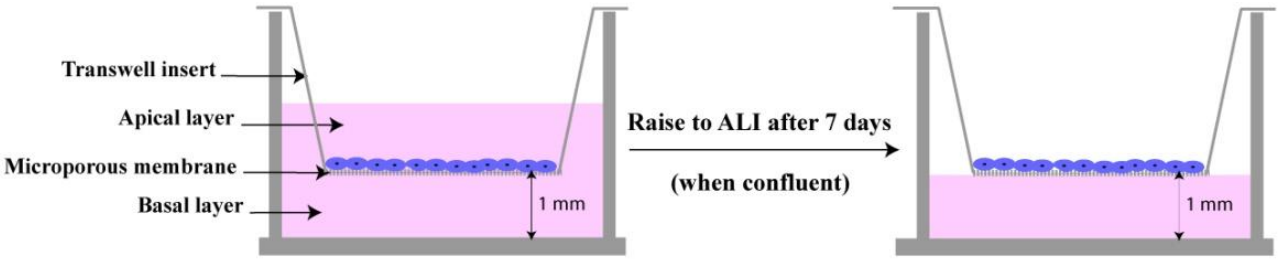


Figure 16 – hAECs and hAMSCs were cultured in vitro on microporous polyester membranes in submerged culture using airway medium 1 for the first day of culture after which they were raised to air liquid interface (ALI) for a further 15-20 days to differentiate (right).

- Remove any liquid form the top surface once daily until the membrane culture remains visibly dry on top, which usually occurs 3-6 days after seeding in 8 to 9% CO_2.
- Maintain the airway medium 2 on the bottom side of the membrane insert for the entire culture duration to prevent the culture form drying and the cells dying.
- After the cells achieve the confluence, the polarized sheet of cells will regulate the minimal fluid level and content on top. The amount of liquid is so small that is not visible to the eye.
- After the first 4-6 days of culture and once the air interface can be maintained by the cells, keep the culture in a 5%CO_2 humidified atmosphere at 37°C.
- Remove any liquid on the top surface daily, until the epithelium becomes confluent and can maintain the air interface.

3.4.2 Stem cell labelling

hAECs and hAMSCs were labelled with Chloromethylbenzamido (CellTrackerTM CM-DiI) (Fig. 17). CM-DiI incorporates a mildly thiol-reactive chloromethyl substituent that confers aldehyde fixability via conjugation to thiol-containing peptides and proteins.

Stock solutions of CM-DiI were prepared in dimethylsulfoxide (DMSO) at 1 ng/µl. Immediately before labeling, the stock solution was diluted up to a final concentration of 0.005 ng/µl into Dulbecco Modified Eagle's Medium (DMEM) without phenol red. Cells grown at confluency in a T25 flask were washed with phosphate-buffered saline (PBS) and then incubated with the dye working solution for 30 minutes at 37°C. After labelling, cells were washed twice with PBS, then incubated at 37°C 5% CO_2 for at least 24h in the presence of fresh medium (Fig. 18).

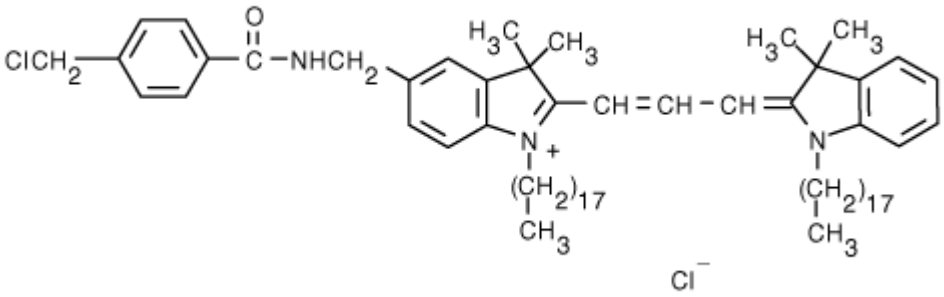


Figure 17: Chemical structure of CellTrackerTM CM-DiI.

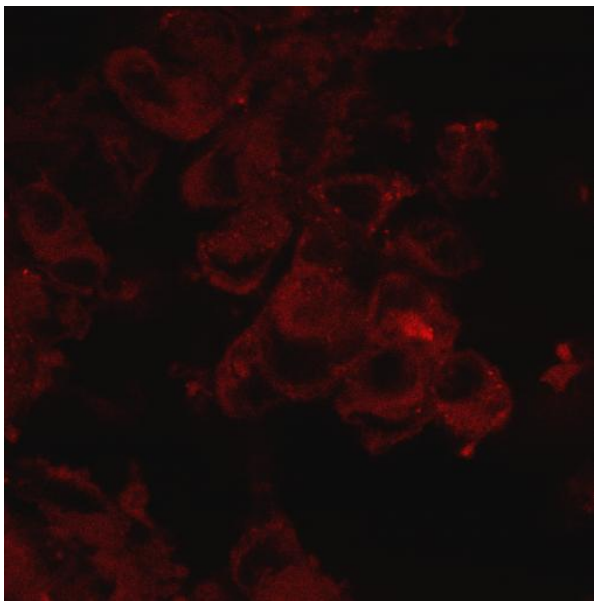


Figure 18: hAMSCs labelled with CM-DiI after isolation.

3.4.3 Co-culture of hAMSC with CFBE41o-

Labelled hAMSCs were mixed with CFBE41o- (cystic fibrosis human epithelial bronchial cell line) homozygous for the F508del allele (F508del/F508del), (generous gift of Professor D. Gruenert, University of California at San Francisco, USA) at different ratios (1:5 , 1:10, 1:15, 1:20) and, in order to obtain polarized co-cultures, cells were seeded on 6.5-mm diameter Snapwell, 0.4-μm pore size (Corning, Acton, MA, USA) at 1 X 10^5 per filter coated with a solution of 10 μg/ml Fibronectin (BD Biosciences, CA, USA), 100 μg/ml albumin from bovine serum (Sigma-Aldrich, Milan, Italy), and 30 μg/ml bovine collagen type I (BD Biosciences) dissolved in Mimimum Essential Medium (MEM). Co-cultures were maintained at 37°C 5% CO_2 at least for six days in order to obtain polarization.

3.5 Cell differentiation analyses

Oil-Red-O staining. The presence of adipose elements in induced and control cultures was determined by Oil-Red-O staining (Sigma). The cells were washed in PBS, then fixed in 10% formalin for 1 hour, washed in isopropanol 60% and air-dried. The cells were incubated with Oil-Red-O staining solution for 10 minutes, then washed several times in PBS.

Alizarin Red staining. The presence of calcium deposits in induced and control cultures was determined by Alizarin Red at 2% in water at pH 4.2. The cells were fixed in 10% formalin for 1 hour, then washed in deionized water and incubated for 30 min at room temperature with Alizarin Red. The cells were finally washed several times to remove the excess of staining.

Evaluation of cytochrome P450 dependent mixed function oxidases. One of the functions in cultured hepatocytes is that of the cytochrome P450 dependent mixed function oxidases (MFO). Diethoxy (5,6) chloromethylfluorescein is a probe suitable for use as an *in situ* stain for MFO activity. It is postulated that the ethoxy groups are removed by MFO activity, and the chloromethyl moiety reacts with reduced glutathione to form the fluorescent conjugate which is retained within the cell (*Anderson et al, 1998*). The presence of cytochrome P450 in induced and control cultures was determined by specific probe hybridization and visualized by fluorescence microscopy.

5mg of probe was eluted in 1143µl DMSO (Stock solution 10 mmol). Test medium was prepared as follow: 987µl di RPMI, 12µl HEPES 1M (12 mmol final), and 1µl probe 10 mmol (10 µmol final). Control medium was prepared as follow: 987µl RPMI, 12µl HEPES 1M (12 mmol final), and 1µl DMSO. The cells were washed in PBS and incubated with the test (or control) medium for 2 hours at 37°C in a dark room. Viable cells incorporate the probe and they metabolize it in a green fluorescent compound. The fluorescent compound can be visualized by fluorescence microscopy.

3.5.1 Differentiation into airway epithelium

3.5.1.1 Transepithelial resistance

The evaluation of the polarized cultures is critically important to determine that they have the phenotype associated with airway epithelia. This can be done by routinely check the transepithelial resistance and morphology.

Transepithelial resistance (TER) can be monitored using a portable voltohmmeter attached to dual “chopstick” electrodes (Fig. 19). Each of the two electrode stem contains at their tip a Ag/Ag Cl-electrode for measuring voltage and a concentric spiral of silver wire for passing current across epithelium.

TER was measured by the following steps:

- Sterilize the electrode tips in 70% alcohol before use.
- In order to measure electrical properties, place 300-400 µl of medium on the apical surface. It is removed after measurement.

Figure 19 – Voltohmmeter with "chopstick" electrodes.

- Place the shorter electrode tip into medium on top of the apical surface and place the longer electrode tip into the external bathing medium.
- TER values higher than the background fluid resistance indicate a confluent airway epithelium with tight junctions.

3.5.1.2 Transepithelial Fluid Transport Measurement

To quantify fluid absorption, the cells were cultured as explained above. Six days after seeding, the apical surface of epithelia was washed with a saline solution containing (in mM): 137 NaCl, 2.7 KCl, 8.1 Na_2HPO_4, 1.5 KH_2PO_4, 1 $CaCl_2$, 0.5 $MgCl_2$, with or without 100 µM amiloride. The apical medium was removed, then 500 µl of room temperature saline solution was added to the apical surface. Filters were rotated gently to remove the medium remaining at the walls of the cup, and then the fluid was recovered and eliminated. This process was repeated three times. After washing, the apical side of the epithelium was covered with 50 µl of the same solution and 150 µl of mineral oil to prevent evaporation. Cells were maintained at 37°C in 5% CO_2. After 24 hours, the apical fluid was carefully removed, centrifuged to separate the mineral oil, and the residual volume of aqueous phase measured.

3.5.1.3 CFTR cytofluorimetric assay

Co-coltures of hAMSCs with CFBE41o- (at different ratios) were first detached with trypsin-EDTA treatment and fixed in PBS containing 2% paraformaldehyde for 5 min. After centrifugation at 1500 rpm the resulting pellets were washed and resuspended in PBS. The cells were then incubated with CFTR antibody MAB25031 mouse IgG2a (R&D Systems, Minneapolis, MN) used at 1:20 dilution for 1h at 4°C. After wash in PBS, the cells were incubated with the FITC-conjugated secondary antibody (anti mouse used at 1:100; Sigma) for 1h at 4°C, followed by two washes in PBS, and analyzed by flow cytofluorimetry. Data were collected using a Coulter Epix XL flow cytometer (Beckman Coulter, Fullerton, CA) and analyzed with WinMDI 2.9 (www.cyto.purdue.edu/flowcyt/software/Winmdi.htm). Ten thousand cells were examined in each experiment. Analysis was performed by plotting the FLH-1 channel (525 nm) against the FLH-2 channel (575 nm).

3.5.1.4 Confocal analysis

3.5.1.4.1 ZO-1 Immunodetection

Polarized cells were washed three times with PBS, fixed in 3% paraformaldehyde, 2% sucrose for 5 minutes at room temperature, and permeabilized with ice cold Triton Hepes buffer (20 mM Hepes, 300 mM sucrose, 50 mM NaCl, 3 mM $MgCl_2$, 0.5% Triton X-100, pH 7.4) for 5 min at room temperature. Cells were washed three times with BSA 0,2% in PBS and then incubated with blocking solution (2% BSA, 2% FBS in PBS), for 15 min at 37°C. Cells were then incubated with FITC-conjugated mouse anti-ZO-1 antibody in PBS 0.2 % BSA (Zymed Laboratories Inc., San Francisco, CA, USA) (dilution 1:50) for 30 min at 37°C, and finally were rinsed three times with 0.2% BSA in PBS. Filters were excised and placed side up on a glass slide, and overlayed with a drop of Mowiol (Calbiochem, San Diego, CA, USA) followed by a coverslip. Cells were analyzed using Nikon TE2000 microscope coupled to a Radiance 2100 confocal dual-laser scanning microscopy system (Bio-Rad, Segrate, Italy). Specimens were viewed through a 60X oil immersion objective. Digital images were processed using the program Laser Sharp 2000 (Bio-Rad).

3.5.1.4.2 CFTR Immunodetection

Polarized cells were washed three times with PBS, and incubated in PBS 2% BSA for 30 minutes on ice. Cells were incubated with CFTR antibody MAB25031 diluted 1:20 in PBS containing 0.2% BSA for 1h on ice. Cells were rinsed three times with PBS, and incubated with the FITC-conjugated secondary antibody (Sigma) diluted 1:100 in PBS added with 0.2% BSA for 30 minutes

on ice. After two washes in PBS, cells were fixed in 3% paraformaldehyde, 2% sucrose for 10 minutes. After three washes in PBS, filters were excised and placed side up on a glass slide, and overlayed with a drop of Mowiol followed by a coverslip. Cells were analyzed as described before for ZO-1.

3.6 RNA extraction and RT-PCR

Total RNA was isolated from undifferentiated and differentiated cells with TRIZOL® Reagent (Invitrogen), according to the manufacturer's protocol.

The reagent is a mono-phasic solution of phenol and guanidine isothiocyanate. During sample homogenization or lysis, TRIZOL® Reagent maintains the integrity of the RNA, while disrupting cells and dissolving cell components. Addition of chloroform followed by centrifugation, separates the solution into an aqueous phase and an organic phase. RNA remains exclusively in the aqueous phase. After transfer of the aqueous phase, the RNA is recovered by precipitation with isopropyl alcohol. The RNA pellet is washed in Ethanol 70% in order to remove the impurities and then rehydrated with diethylpyrocarbonate (DEPC) water, which contains RNase inhibitors. All the following procedures must be performed in ice:

- Lyse cells directly in a culture dish by adding 1 ml of TRIZOL® Reagent to a 3.5 cm diameter dish, and passing the cell lysate several times through a pipette. The amount of TRIZOL® Reagent added is based on the area of the culture dish (1 ml per 10 cm^2) and not on the number of cells present. An insufficient amount of TRIZOL® Reagent may result in contamination of the isolated RNA with DNA.
- Incubate the homogenized samples for 5 minutes at 15 to 30°C to permit the complete dissociation of nucleoprotein complexes. Add 0.2 ml of chloroform per 1 ml of TRIZOL® Reagent. Cap sample tubes securely.
- Shake tubes vigorously by hand for 15 seconds and incubate them at 15 to 30°C for 2 to 3 minutes.
- Centrifuge the samples at no more than 12,000 × g for 15 minutes at 2 to 8°C. Following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. The volume of the aqueous phase is about 60% of the volume of TRIZOL® Reagent used for homogenization.
- Transfer the aqueous phase to a fresh tube.

- Precipitate the RNA from the aqueous phase by mixing with isopropyl alcohol. Use 0.5 ml of isopropyl alcohol per 1 ml of TRIZOL® Reagent used for the initial homogenization.
- Incubate samples for 10 minutes and centrifuge at no more than 12,000 × g for 10 minutes at 2 to 8°C. The RNA precipitate, often invisible before centrifugation, forms a gel-like pellet on the side and bottom of the tube.
- Remove the supernatant.
- Wash the RNA pellet once with 75% ethanol, adding at least 1 ml of 75% ethanol per 1 ml of TRIZOL® Reagent used for the initial homogenization.
- Mix the sample by vortexing and centrifuge at no more than 7,500 × g for 5 minutes at 2 to 8°C.
- At the end of the procedure, briefly dry the RNA pellet (air-dry or vacuum-dry for 5-10 minutes). Do not dry the RNA by centrifugation under vacuum. It is important not to let the RNA pellet dry completely as this will greatly decrease its solubility. Partially dissolved RNA samples have an A260/280 ratio < 1.6.
- Dissolve RNA in RNase-free water or DEPC water (0,01%) by passing the solution a few times through a pipette tip, and incubating for 10 minutes at 55 to 60°C.

In order to verify purity and quantity, the absorbance of RNA at 260nm and 280nm was analysed by spectrophotometer and the RNA was evaluated on agarose gel.

3.6.1 RT-PCR

1µg of RNA was reverse-transcribed into first strand cDNA with the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems) using random primers following manufacturer's instructions.

- Prepare the reaction mix in ice:

RT buffer 10X	2 µl
dNTPs 100mM	0.8 µl
Random primers	2 µl
Multiscribe enzime	1 µl
RNAse inhibitor	1 µl
Sterile water	3.2 µl

- Aliquote the reaction mix into eppendorf tubes and add 5 µl RNA 200 ng/µl;
- PCR reaction:

	Step 1	**Step 2**	**Step 3**	**Step 4**
Temperature (°C)	25	37	85	4
Time	10’	120’	5’’	∞

3.6.2 cDNA processing

3.6.2.1 α , β , γ ENaC expression

In order to analyze the expression of α, β, and γ ENaC subunits by RT-PCR, 100 ng of cDNA was added in a final volume of 25 µl of reaction buffer containing 200 nM dNTPs, 10 pM of each primer (Table 1), 0.3 U Taq-DNA-polymerase, and $MgCl_2$ (Invitrogen). Cycling conditions consisted of 1 cycle at 95°C for 6 minutes, 35 cycles consisting in denaturation at 95°C for 30 seconds, annealing at 58°C for 1 minute and elongation at 72°C for 2 minutes, and 1 final cycle at 72°C for 7 minutes.

cDNA from H441 (human lung adenocarcinoma epithelial cell line) treated with dexamethasone were used as positive control for ENaC analysis. PCR products were evaluated on 1.5% agarose gel electrophoresis.

3.6.2.2 CFTR expression

In order to analyze the expression of CFTR gene, 100ng of cDNA was used in a final volume of 25ul with 200nM dNTP, 10pM of each outer primer (Table 1), 0.3U Taq-DNA-polymerase, reaction buffer, and MgCl2 (Invitrogen). A second nested PCR was performed using inner primers (Table 1). Cycling conditions consisted of 95°C for 30 seconds, annealing at 60°C for 1 minute and elongation at 72°C for 2 minutes. Cycle numbers consisted of 35 cycles.

cDNA from nasal brushing from healthy subject was used as positive control for CFTR analysis. Nested PCR products were evaluate on 1.5% agarose gel electrophoresis.

Table 1 - Primer sequences for ENaC and CFTR analysis

Gene	Forward Primer	Reverse Primer	Product Lenght (bp)
Alpha-ENaC	CCTGGAATCAACAACGGTCT	ATGTTGACTTTGGCCACTCC	614
Beta-ENaC	CCCCGTCCAAAACTTCTACA	GATCTCCCCAAACTCGATGA	526
Gamma- ENaC	TCTACCTCCTGCAGCCAACT	AAGCAGGCTTTTTGGTCAGA	845
CFTR outer primers	CGAGAGACCATGCAGAGGTC	GCTCCAAGAGAGTCATACCA	1108
CFTR inner primers	CGAGAGACCATGCAGAGGTC	TGTACTGCTTTGGTGACTTCCCC	301

3.6.3 Real-Time PCR

In conventional PCR, the amplification product is evaluate on agarose gel at the end of the reaction. On the contrary, real time PCR is a technique used to monitor the progress of a PCR reaction in real time. Real Time PCR is based on the detection of the fluorescence produced by a reporter molecule which increases, as the reaction proceeds. The fluorescence emitted by the reporter molecule manifolds as the PCR product accumulates with each cycle of amplification. These fluorescent reporter molecules include dyes that bind to the double-stranded DNA (i.e. SYBR® Green) or sequence specific probes (i.e. Molecular Beacons or TaqMan® Probes).

Real time PCR facilitates the monitoring of the reaction as it progresses. One can start with minimal amounts of nucleic acid and quantify the end product accurately. Moreover, there is no need for the post PCR processing which saves the resources and the time. These advantages of the fluorescence based real time PCR technique have completely revolutionized the approach to PCR-based quantification of DNA and RNA. Real time PCR assays are now easy to perform, have high sensitivity, more specificity, and provide scope for automation.

Real-Time PCR focuses on the exponential phase of PCR reaction because it provides the most precise and accurate data for quantitation. Within the exponential phase, the real-time PCR instrument calculates two values. The Threshold line is the level of detection at which a reaction reaches a fluorescent intensity above background. The PCR cycle at which the sample reaches this level is called the Cycle Threshold, Ct. The Ct value is used in downstream quantitation or presence/absence detection. By comparing the Ct values of samples of unknown concentration with a series of standards, the amount of template DNA in an unknown reaction can be accurately determined.

Every real-time PCR reaction contains a fluorescent reporter molecule, a TaqMan® Probe, to monitor the accumulation of PCR product. As the quantity of target amplicon increases, so does the amount of fluorescence emitted from the fluorophore.

TaqMan® Gene Expression Assays consist of a pair of unlabeled PCR primers and a TaqMan® probe with a FAM™ or VIC® dye label and minor groove binder (MGB) moiety on the 5' end, and non-fluorescent quencher (NFQ) dye on the 3' end.

An oligonucleotide probe is constructed containing a reporter fluorescent dye on the 5' end and a quencher dye on the 3' end. While the probe is intact, the proximity of the quencher dye greatly reduces the fluorescence emitted by the reporter dye by fluorescence resonance energy transfer (FRET) through space. If the target sequence is present, the probe anneals downstream from one of the primer sites and is cleaved by the 5' nuclease activity of Taq DNA polymerase as this primer is extended. This cleavage of the probe:

- Separates the reporter dye from the quencher dye, increasing the reporter dye signal.
- Removes the probe from the target strand, allowing primer extension to continue to the end of the template strand. Thus, inclusion of the probe does not inhibit the overall PCR process.

Additional reporter dye molecules are cleaved from their respective probes with each cycle, resulting in an increase in fluorescence intensity proportional to the amount of amplicon produced.

When calculating the results of real-time PCR experiment, it can be used either absolute or relative quantitation. In absolute quantitation, unknowns are quantitated based on a known quantity, through the creation of a standard curve. In relative quantitation, changes in gene expression in a given sample relative to another reference sample (such as an untreated control sample) are analyzed.

The comparative Ct method compares the Ct value of one target gene to another (using the formula: $2^{\Delta\Delta CT}$) - for example, an internal control or reference gene (e.g., housekeeping gene) - in a single sample. For the comparative Ct method to be valid, the efficiency of the target amplification (your gene of interest) and the efficiency of the reference amplification (your endogenous control) must be approximately equal.

In the present study, the following TaqMan probes have been used:

- Hs02387400_g1 (NANOG), a transcription factor critically involved with self-renewal of undifferentiated embryonic stem cells. NANOG is a gene expressed in embryonic stem cells (ESCs) and is thought to be a key factor in maintaining pluripotency. NANOG is thought to function in concert with other factors such as POU5F1 and SOX2 to establish ESC identity. These cells offer an important area of study because of their ability to maintain pluripotency.

- Hs00360675_m1 (SALL4), a gene part of a group of genes called the SALL family. These genes provide instructions for making proteins that are involved in the formation of tissues and organs during embryonic development. SALL proteins are transcription factors, whose exact function remains unclear. Based on the functions of similar proteins in other organisms (such as zebrafish and mice), the SALL4 protein appears to play a critical role in the developing limbs. This protein may also be important for the development of nerves that control eye movement and for the formation of the walls (septa) that divide the heart into separate chambers.
- Hs00195591_m1 (SNAI1). The Drosophila embryonic protein snail is a zinc finger transcriptional repressor which downregulates the expression of ectodermal genes within the mesoderm. The nuclear protein encoded by this gene is structurally similar to the Drosophila snail protein, and is also thought to be critical for mesoderm formation in the developing embryo. At least two variants of a similar processed pseudogene have been found on chromosome 2.
- Hs99999909_m1 (HPRT) used as reference. The *HPRT1* gene provides instructions for producing an enzyme called hypoxanthine phosphoribosyltransferase 1. This enzyme allows cells to recycle purines, a type of building block of DNA and its chemical cousin RNA. Manufacturing purines uses more energy and takes more time than recycling purines, which makes recycling these molecules more efficient. Recycling purines ensures that cells have a plentiful supply of building blocks for the production of DNA and RNA. The process of recycling purines is also known as the purine salvage pathway.

PCR reaction was performed as follow:

	TEMPERATURE (°C)	**TIME**	**NUMBER OF CYCLES**
Enzyme activation	50 95	2’ 10’	1
Denaturation, *annealing* and extension	90 60	15’’ 1’	40

RESULTS

4. RESULTS

4.1 Isolation and characterization of cells from human amnion

The collection of human term placentas after birth was carried out in the Department of Gynaecology and Obstetrics of Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico of Milan, after obtaining informed consent from the mother. Through mechanical separation and successive enzymatic digestions, two cellular fractions from the amnion were isolated: human amniotic epithelial cells (hAECs), and amniotic mesenchymal stromal cells (hAMSCs).

At least 65 x 10^6 hAECs (range: 6-130 x 10^6) were recovered in each isolation with a viability of 75-85%.

At least 33 x 10^6 hAMSCs (range 26-160 x 10^6) were recovered in each isolation with a viability of 85-90%.

4.1.1 Morphology

In the presence of EGF, hAECs proliferated robustly and formed a confluent monolayer of cobblestone-shaped epithelial cells (Fig. 20A and 20B).

The mesodermal fraction of the amnion (hAMSCs) was characterized after plastic adhesion by a fibroblastic morphology very similar to that described for mesenchymal cells isolated from bone marrow (Fig. 20C and 20D).

4.1.2 Growth kinetics

Isolated hAECs readily attached to plastic or basement membrane-coated culture dishes and could be kept until six passages before proliferation ceased. Approximately one cell doubling was observed over 15 days, giving these cells an average doubling time of 20.35 in the presence of EGF. Without EGF, the proliferation stopped early and giant multinuclear cells were formed. If cells were cultured in low densities, the senescence occurred before, apparently due to less cellular interaction and complex molecular steps related to EGF receptor and integrins.

hAMSCs adhered and proliferated on tissue culture plastic and could be kept until passages 5-10. Proliferation slowed beyond passage two. Approximately two cell doublings were observed over 15 days, giving these cells an average doubling time of 18.03.

Growth curves for hAECs and hAMSCs are presented in Figure 21.

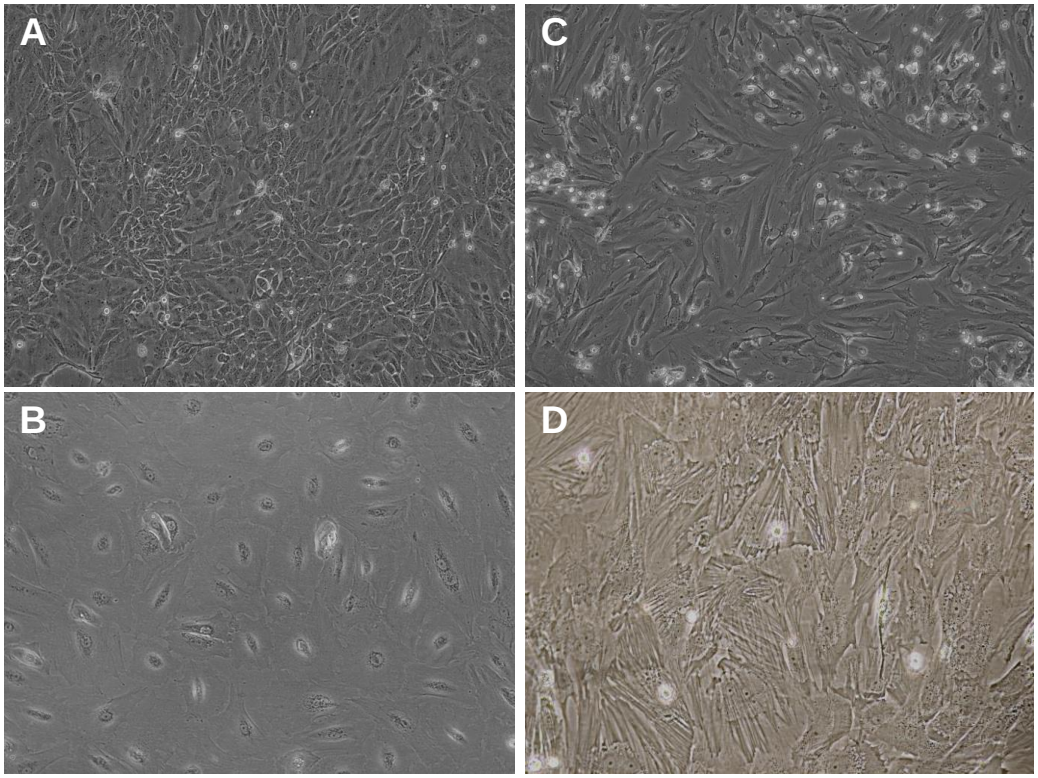


Figure 20 –Culture of placenta derived stem cell types (phase contrast micrograph pictures); magnification ×10. A) hAECs passage 1; B) hAECs passage 3; C) hAMSCs passage 1; D) hAMSCs passage 3

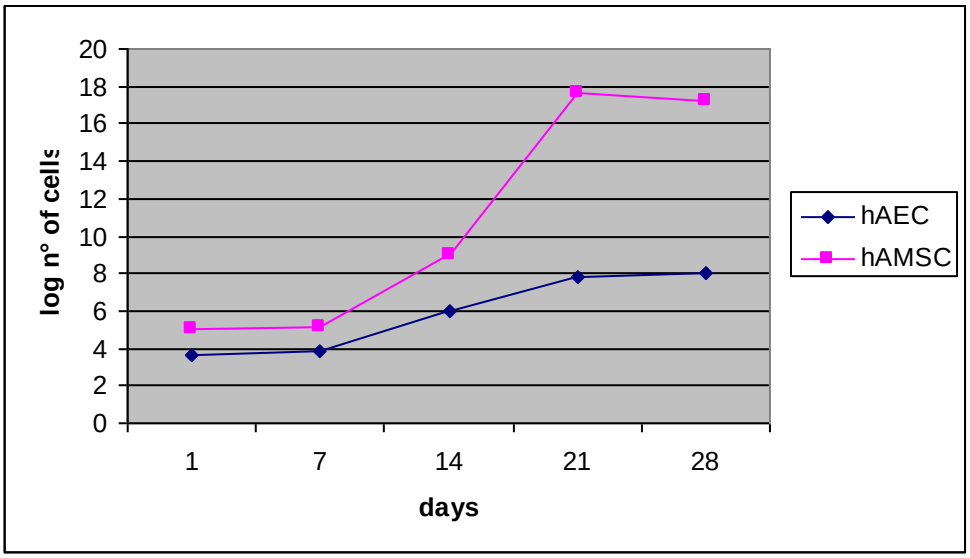


Figure 21 – Growth kinetics of hAEC (blue) and hAMSC (pink)

4.1.3 Flow cytometry analysis

Although the hAEC had a homogeneous morphology appearance, the isolated cells showed a heterogeneous phenotype, suggesting that might be different subpopulations of cells. The hAECs showed antigens such as the EpCAM, CD49F, CD29, CD73, CD166 (Table 2). They did not express CD34 (marker of hematopoietic and endothelial stem cells) and CD45 (pan leukocyte marker), nor CD133 (expressed in hematopoietic stem cells, endothelial cells, glioblastoma cells). They did not show mesenchymal markers as CD105, at least in the first passages, while the expression of CD90 (Thy-1) was stable during culture.

The hAMSCs showed surface marker associated with mesenchymal cells form bone marrow as CD105, CD73 and CD90. They do not express hematopoietic markers (CD34 and CD45). Fresh isolated hAMSC showed relatively low expression of epithelial markers (EpCAM and 49F), which decreased after the first passage (Table 3). Since these markers were expressed by a separate population, we hypothesized a possible epithelium contamination.

Representative flow analysis diagrams of hAECs and hAMSCs are shown in Figure 22.

hAECs and hAMSCs showed surface markers associated with embryonic stem cells such as SSEA4 (stage specific surface embryonic antigen 4), while no expression by flow cytometry of molecular markers associated to pluripotent stem cells (Nanog, Oct-4) was observed (Fig. 23).

4.1.4 Immunofluorescence analysis

Immunohystochemistry stains showed that virtually all cultured hAECs were positive to low molecular weight cytokeratin, EpCAM, Beta-catenin, E-chaderin, ZO-1 and alpha1-anti trypsin. The amniotic epithelial cells expressed not only epidermal markers and general epithelial markers, but also expressed vimentin. (Fig. 24A, 24B and 24C).

Human amniotic mesenchymal cells (hAMSCs) were positive for CD90, CD29, fibronectin and vimentin (Fig. 24A, 24B and 24C).

These facts suggested that the amnion derived cells had not completely differentiated into epithelial or mesenchymal cells. Another explanation from the embryological point of view is that the epithelial-mesenchymal transition (EMT) may occur in the amniotic membrane.

Table 2 – Phenotypic characterization of hAECs at passage 1and 3 by flow cytometry

Surface antigens	PASSAGE 1 Median % (n=6)	PASSAGE 1 Range	PASSAGE 3 Median % (n=6)	PASSAGE 3 Range
CD 90	42	27-95	46	12-59
ABCG2	0	0-1	0	0-3
CD 34	1	0-7	2	0-10
CD133	0	0-1	0	0-2
EpCAM	94	78-100	93	92-95
CD 166	100	77-100	99	94-100
CD 44	39	13-78	60	29-99
CD 49f	98	91-99	100	95-100
CD 29	97	95-100	100	98-100
CD 105	4	0-52	38	3-52
CD 73	100	95-100	100	97-100
CD 31	2	0-7	2	0-4
CD146	1	0-1	3	1-5
CD 13	12	2-24	2	1-4
CD 45	0	0-5	2	1-2

Table 3 – Phenotypic characterization of hAMSCs freshly isolated and at passage 2 by flow cytometry

Surface antigens	FRESHLY ISOLATED Median % (n=3)	FRESHLY ISOLATED Range	PASSAGE 2 Median (n=3)	PASSAGE 2 Range
CD105	49	30-66	58.4	40-76
CD90	79	69-89	94.1	89-99
CD34	0	0-1	0.5	0-1
CD133	0	0-1	0.5	0-1
EpCAM	16	12-10	0.3	0-1
CD166	83	71-95	84.8	71-98
CD44	81	71-90	90.3	89-92
CD49f	16	13-31	2.5	2-5
CD73	90	88-91	93.7	88-99
CD31	0	0	ND	
CD146	0	0	ND	
CD13	80	70-89	94.6	89-99
CD45	2	0-4	4.3	3-4

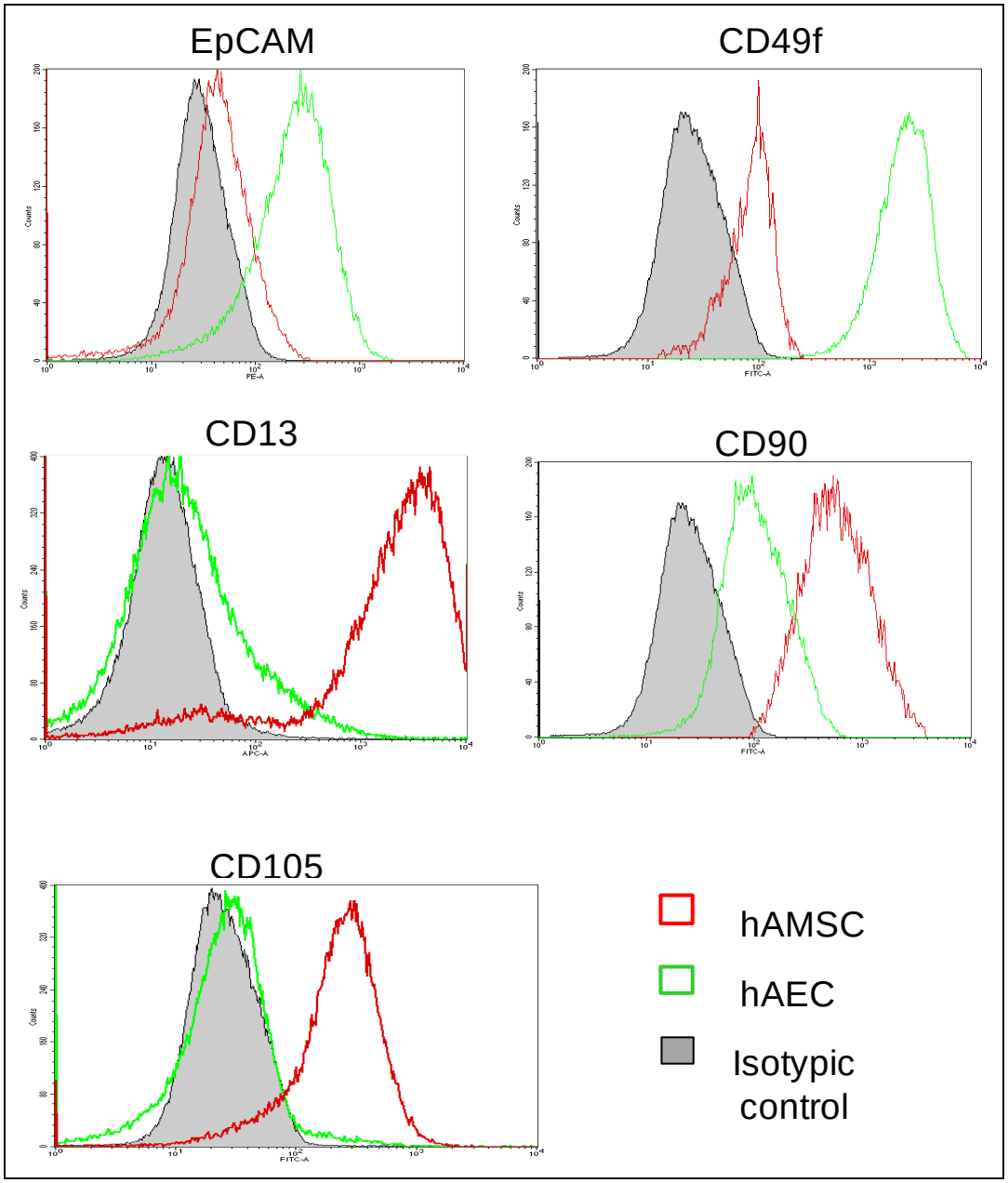


Figure 22 – Representative flow analysis diagrams of hAECs (green lines) and hAMSCs (red lines). The percentage of the positive fraction is relative to isotype controls.

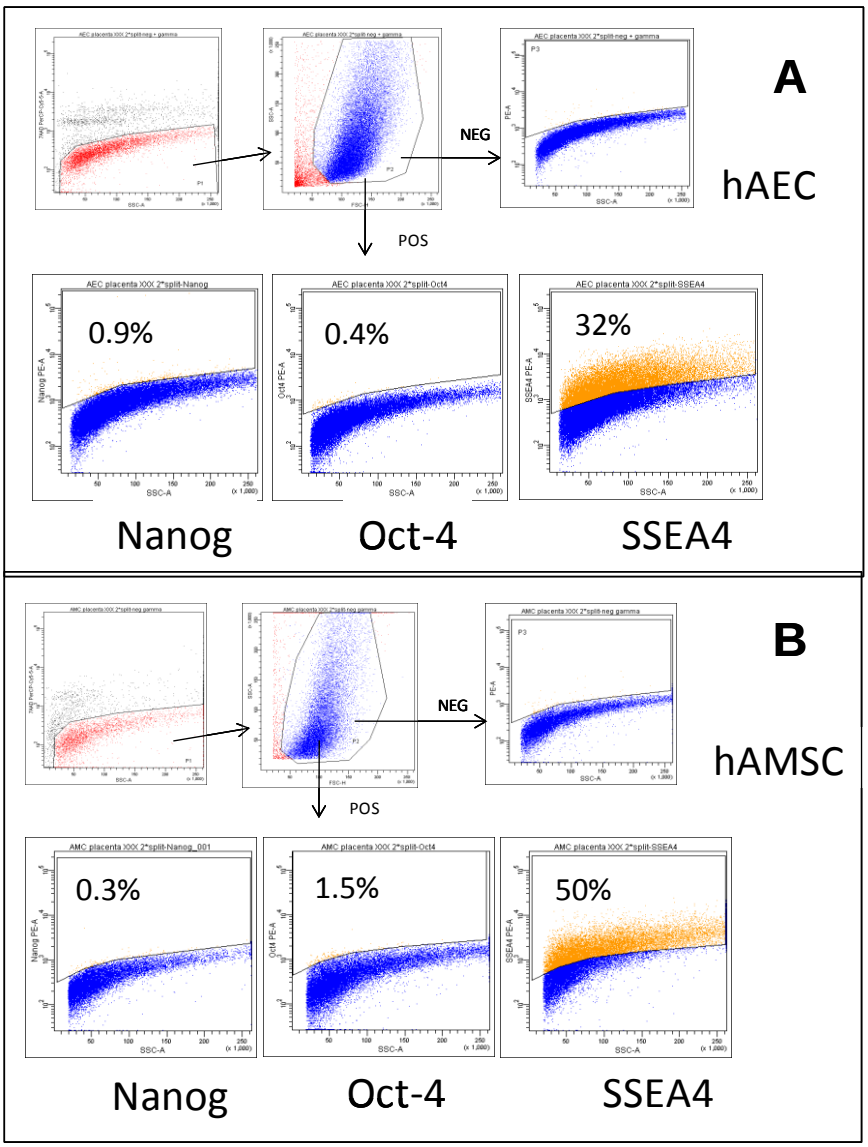


Figure 23 – Fluorescence-activated cell sorting analysis for molecular markers of pluripotent stem cells (Nanog and Oct-4) and surface marker of embryonic stem cells (SSEA4) for hAECs and hAMSCs.

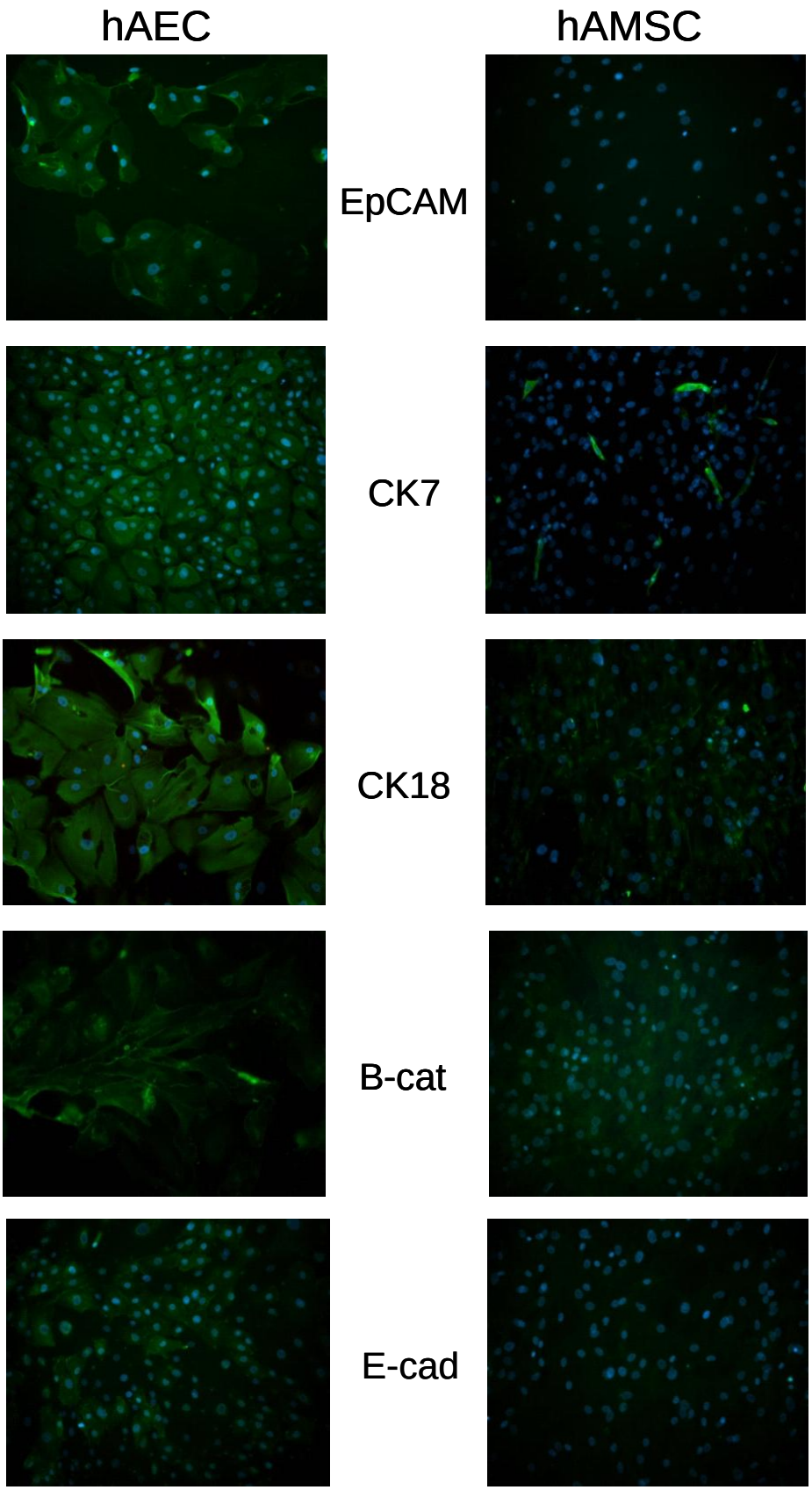


Figure 64A - Immunohystochemistry stains for hAECs and hAMSCs.

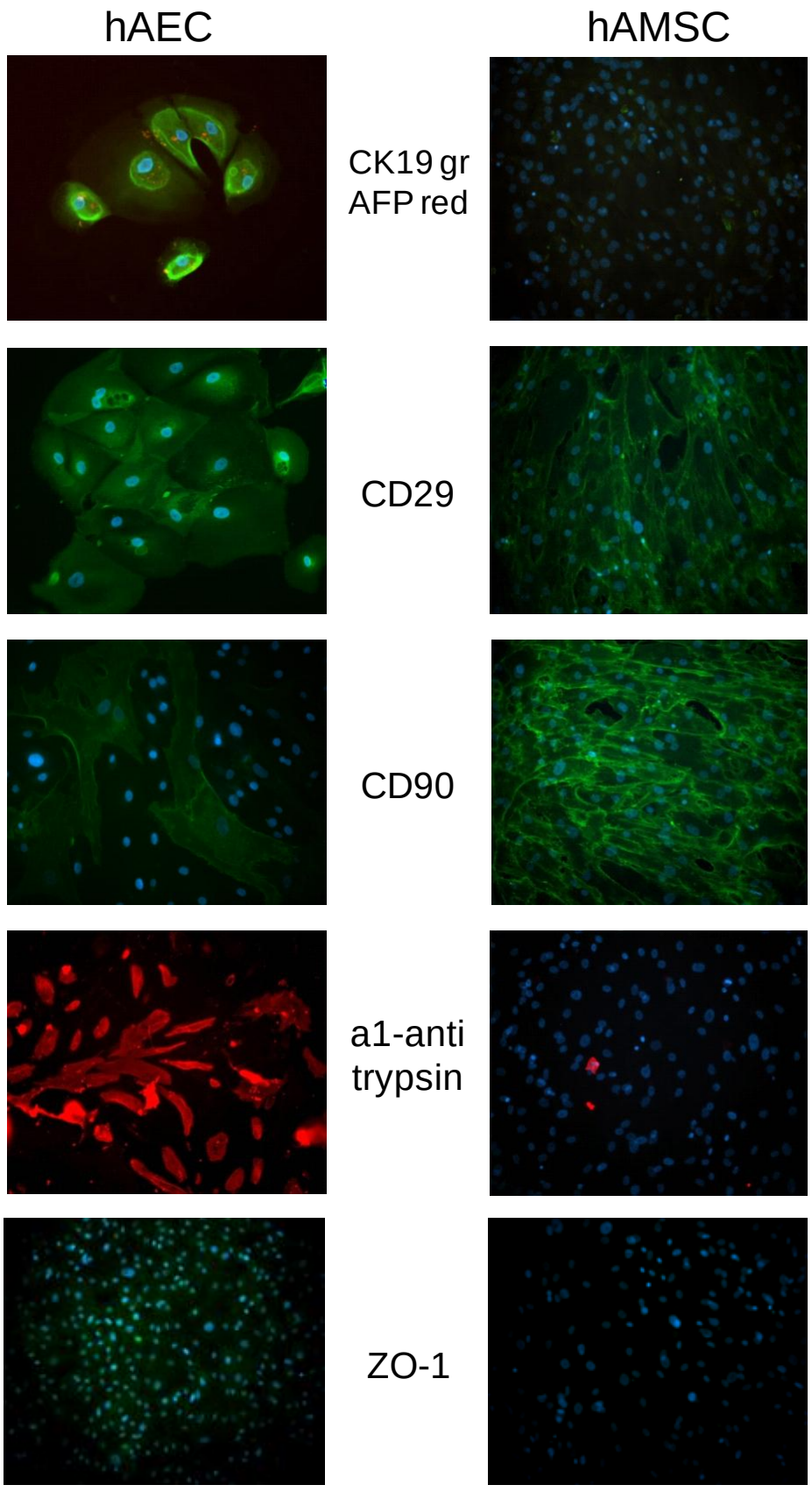


Figure 24B - Immunohystochemistry stains for hAECs and hAMSCs.

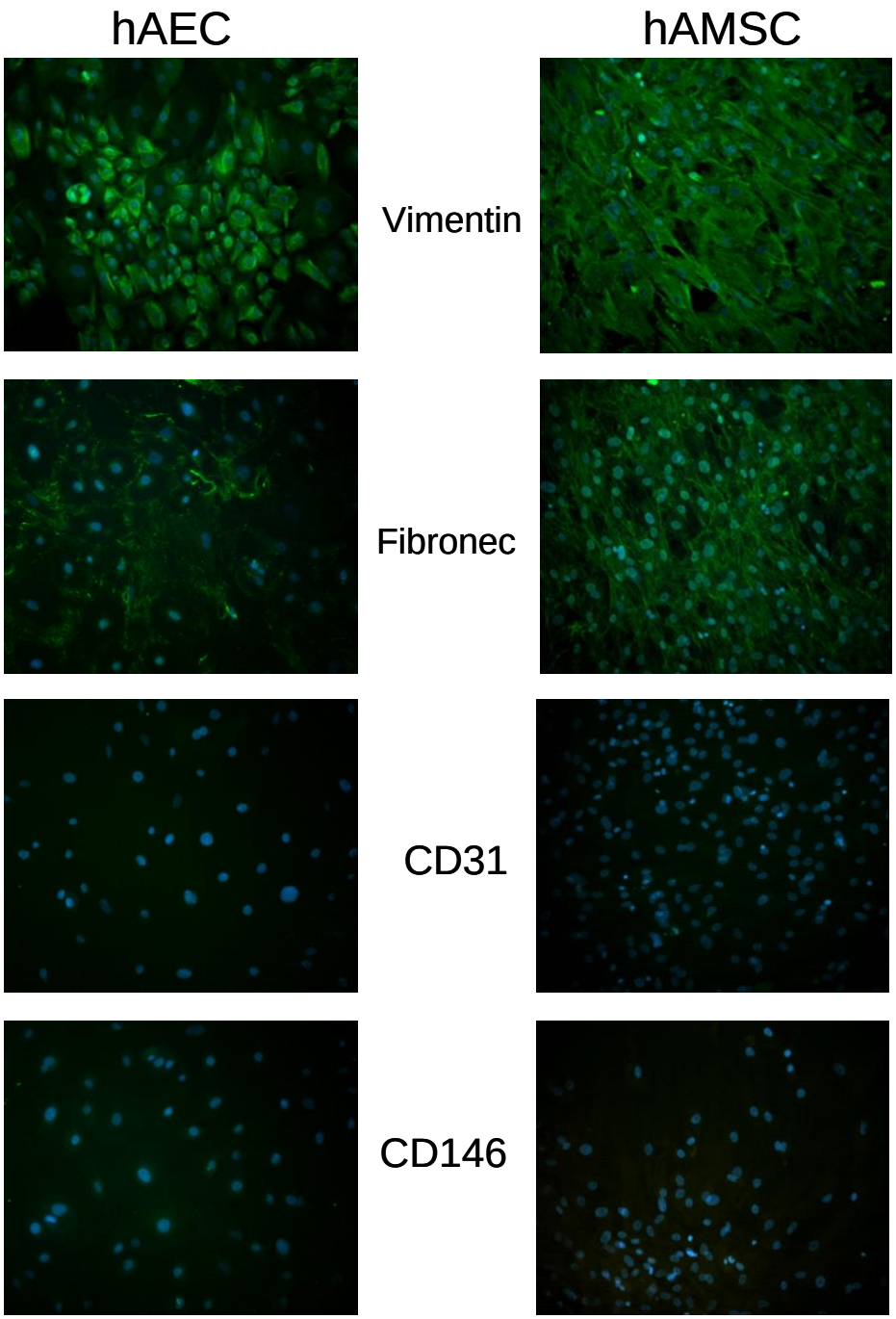


Figure 24C – Immunohystochemistry stains for hAECs and hAMSCs.

4.1.5 Cytogenetic analysis

Karyotypes were normal 46, XX or 46, XY (Fig.25) in all test samples and the analyses revealed no variation at different passages. Karyotype analyses confirmed the fetal origin of both hAECs and hAMSCs.

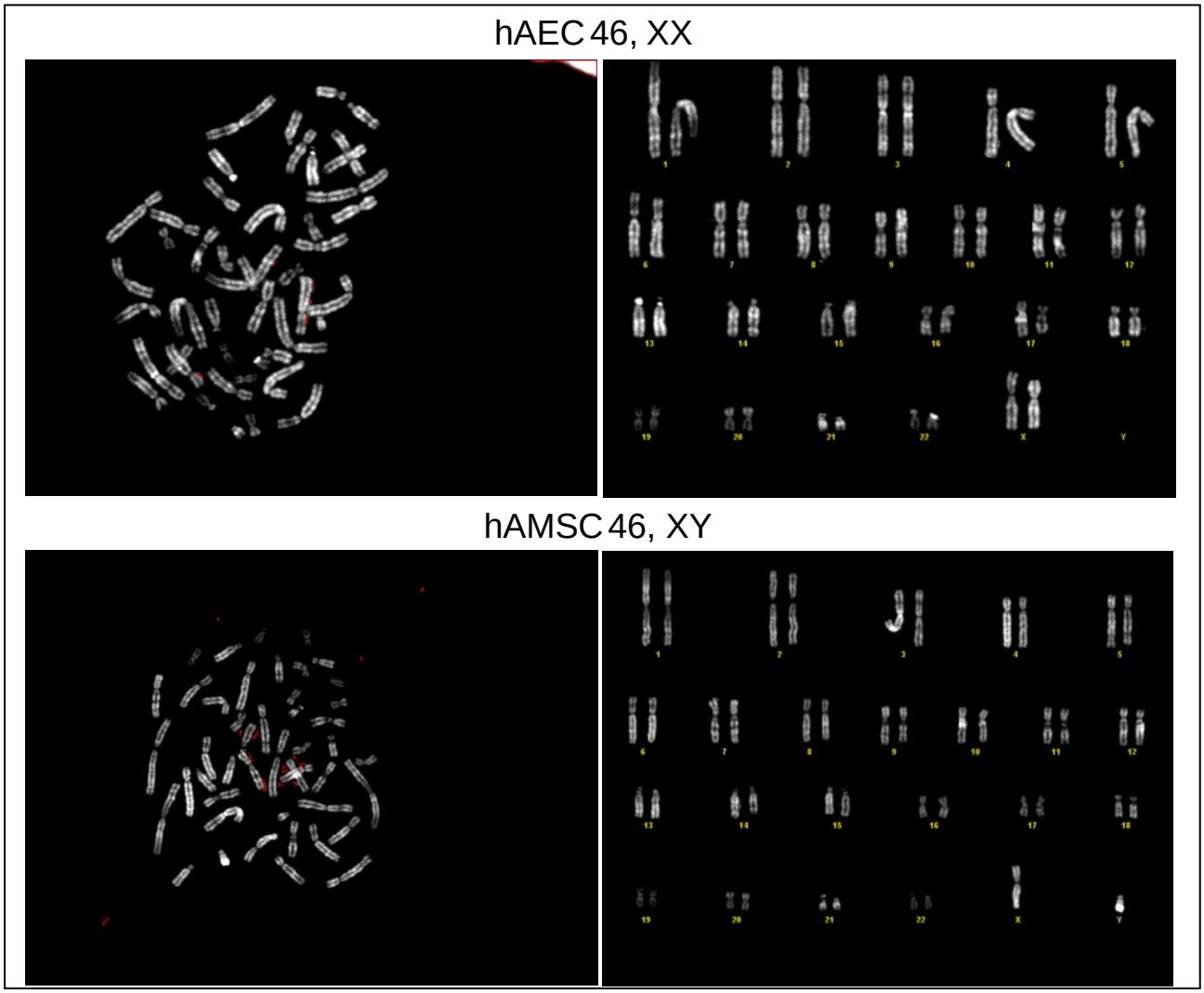


Figure 25 - All cell lines displayed normal karyotype: hAECs (passage 1), hAMSCs (passage 2).

4.1.6 Real time PCR

RNA extracted from cells was quantified by biophotometer and visualized on agarose gel electrophoresis (Fig. 26). A mean of 370 ng/µl RNA was extracted for each sample, with a purity (Absorbance 260/280) higher than 1.98.

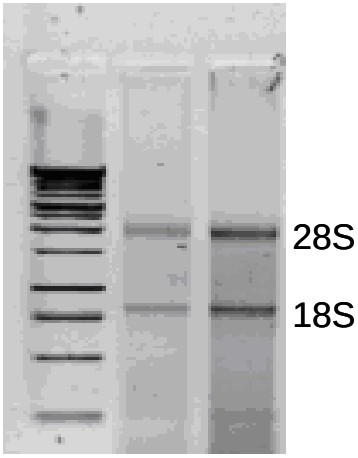


Figure 26 - Visualization on agarose gel electrophoresis of RNA samples extracted from cells. Most abundant RNA species are 28S and 18S.

Real time PCR was executed on RNA samples extracted from cultured amniotic cells at different passages. RNA from Embryonic Stem Cells (generous gift of Prof. E. Cattaneo, University of Milan) was used as positive control; RNA from human lymphocytes was used as negative control.

Real time PCR was performed in order to verify the mRNA expression of NANOG, SALL4 and SNAI1. HPRT was used as housekeeping gene.

At mRNA level, both hAECs and hAMSCs showed NANOG expression, a transcription factor critically involved with self-renewal of undifferentiated embryonic stem cells (Fig.27A), even if at lower levels than ES. Interestingly, the expression of this gene in hAECs decreased during the passages, while in hAMSCs the expression remained stable.

The expression of SALL4, a gene involved in the formation of tissues and organs during embryonic development, appeared to be significantly lower than that of ES. We observed a slight increase of the expression of this gene during the culture of hAMSC (Fig.27B)

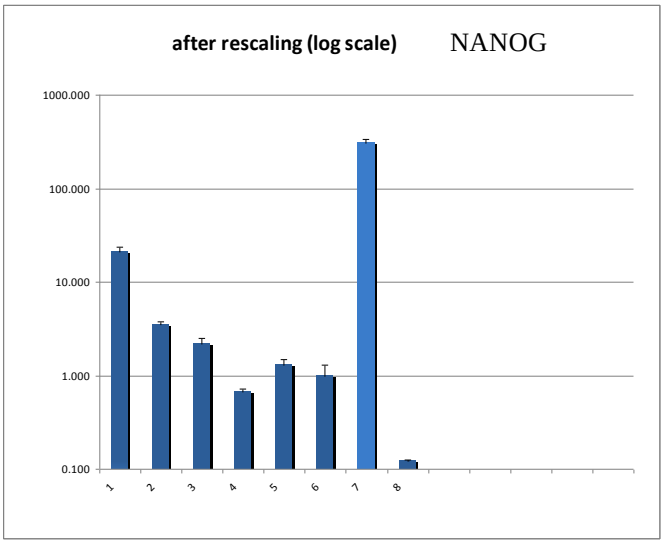
The expression of SNAI1, transcriptional repressor which downregulates the expression of ectodermal genes within the mesoderm appeared to be obviously stable in hAMSCs. In hAECs, we observed an increase in SNAI1 expression during the passages, probably do to epithelial-mesenchymal transition that may occur in the amniotic membrane (Fig.27C).

Data were confirmed by different experiments.

A

NANOG

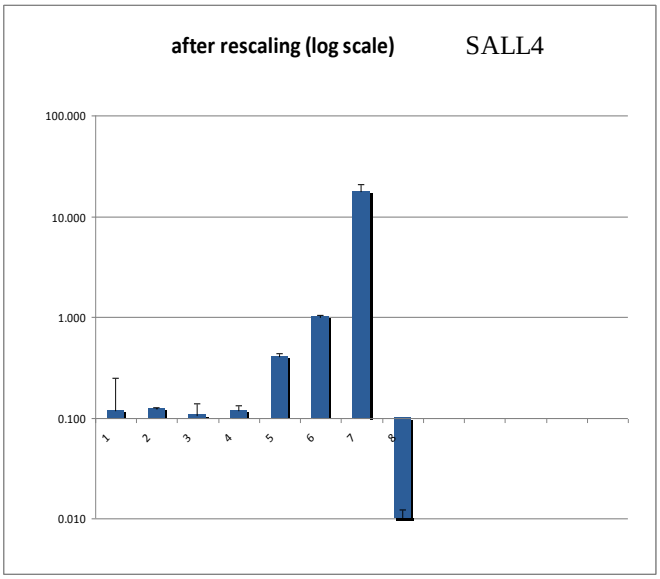
Sample ID	Sample	Expression level	SD
1	hAEC T0	21.187	2.488
2	hAEC P1	3.522	0.276
3	hAEC P3	2.195	0.304
4	hAMSC T0	0.668	0.055
5	hAMSC 1P	1.306	0.196
6	hAMSC 3P	1.000	0.285
7	ES	307.114	32.706
8	Neg CTR	0.122	0.004



B

SALL4

Sample ID	Sample	Expression level	SD
1	hAEC T0	0.118	0.132
2	hAEC P1	0.124	0.003
3	hAEC P3	0.105	0.034
4	hAMSC T0	0.118	0.013
5	hAMSC 1P	0.408	0.025
6	hAMSC 3P	1.000	0.046
7	ES	17.564	3.102
8	Neg CTR	0.010	0.002



C

SNAI1

Sample ID	Sample	Expression level	SD
1	hAEC T0	0.114	0.072
2	hAEC P1	0.164	0.045
3	hAEC P3	0.350	0.164
4	hAMSC T0	1.107	0.121
5	hAMSC 1P	1.421	0.166
6	hAMSC 3P	1.000	0.195
7	ES	0.283	0.054
8	Neg CTR	0.094	0.017

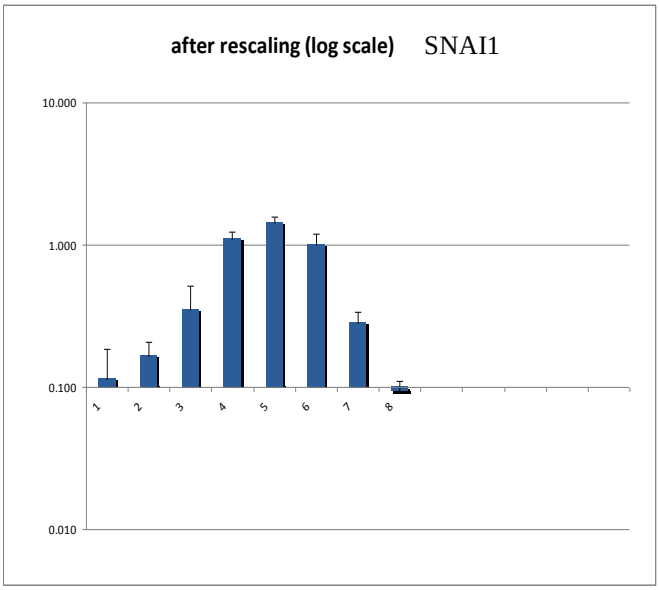
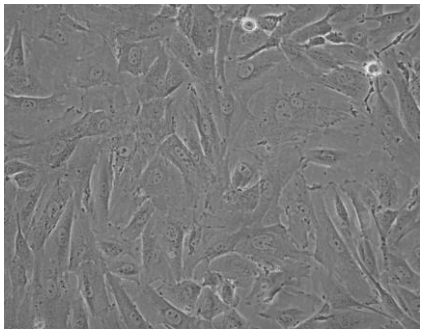


Figure 27 – Rescaled normalized expression of A) NANOG, B) SALL4 , and C) SNAI1 in hAECs and hAMSCs at different passages. Embryonic Stem cells (ES) were used as positive control. RNA from lymphocytes was used as negative control**.**

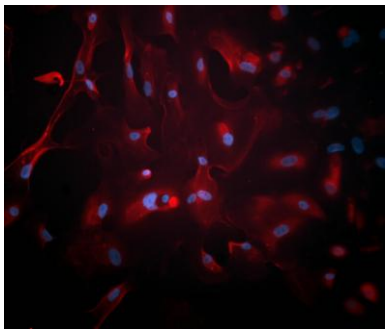
4.2 Epithelial to mesenchymal transition

In 3 cases, hAECs underwent epithelial to mesenchymal transition (EMT) acquiring a more spindle-shaped morphology (Fig. 28A) and expressing typical mesenchymal markers as CD105 and S100A4 (Fig. 28B). However, the expression of epithelial markers was maintained (Fig. 28C).

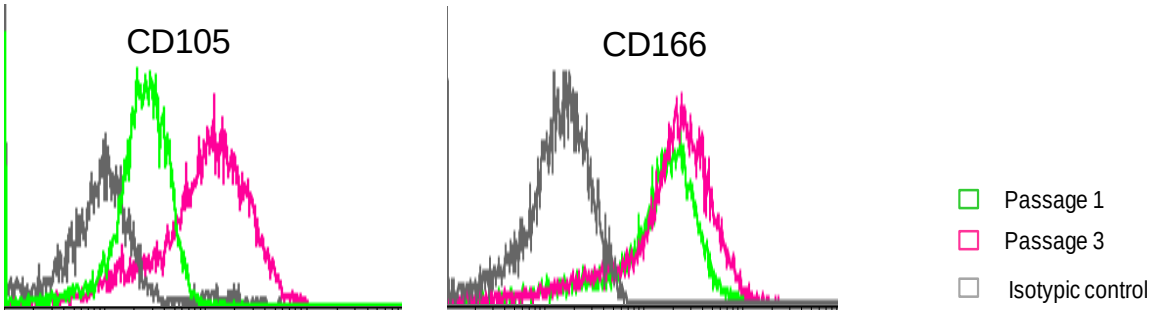
A



B



C



D

Surface antigens	Flow cytometry
EpCAM	0.3
CD166	84
CD49f	7
CD29	98
CD73	97
CD105	67

Intracellular antigens	IF staining
CK7	++
CK18	++
CK19	++
AFP	-
S100A4	++

Figure 28 - Characterization of hAECs underwent EMT at 3rd split by flow cytometry and immunofluorescence: (A) cellular morphology (20x); (B) expression by IF of S100A4 (DAPI blue, 20x); (C) comparison between expression by flow cytometry of CD105 at 1st (green) and 3rd split (pink) and (D) expression of CD166 at 1st (green) and 3rd split (pink) In gray: isotypic control; (D) membrane antigens expression (left table) and intracytoplasmic antigens (right tab).

4.3 Cell differentiation ability

4.3.1 *In vitro* differentiation into osteocytes and adipocytes

To determine whether hAECs and hAMSCs could differentiate into adipocytes, cells were allowed to grow to 70% confluence prior to induction. Morphological changes as well as formation of lipid vacuoles within the cells were noticeable one week post induction and were visualized by Oil-Red-O staining (Fig. 29B and 29C). Cells maintained in control medium did not show any sign of adipogenic differentiation (Fig. 29A).

To investigate the osteogenic potential of hAECs and hAMSCs, cells were cultured under appropriate condition for differentiation. The presence of calcium deposits in induced and control cultures was determined by Alizarin Red at 2% in water at pH 4.2 (Fig. 29E and 29F). Cells maintained in control media did not show any change in their morphology and no calcium deposit (Fig. 29D).

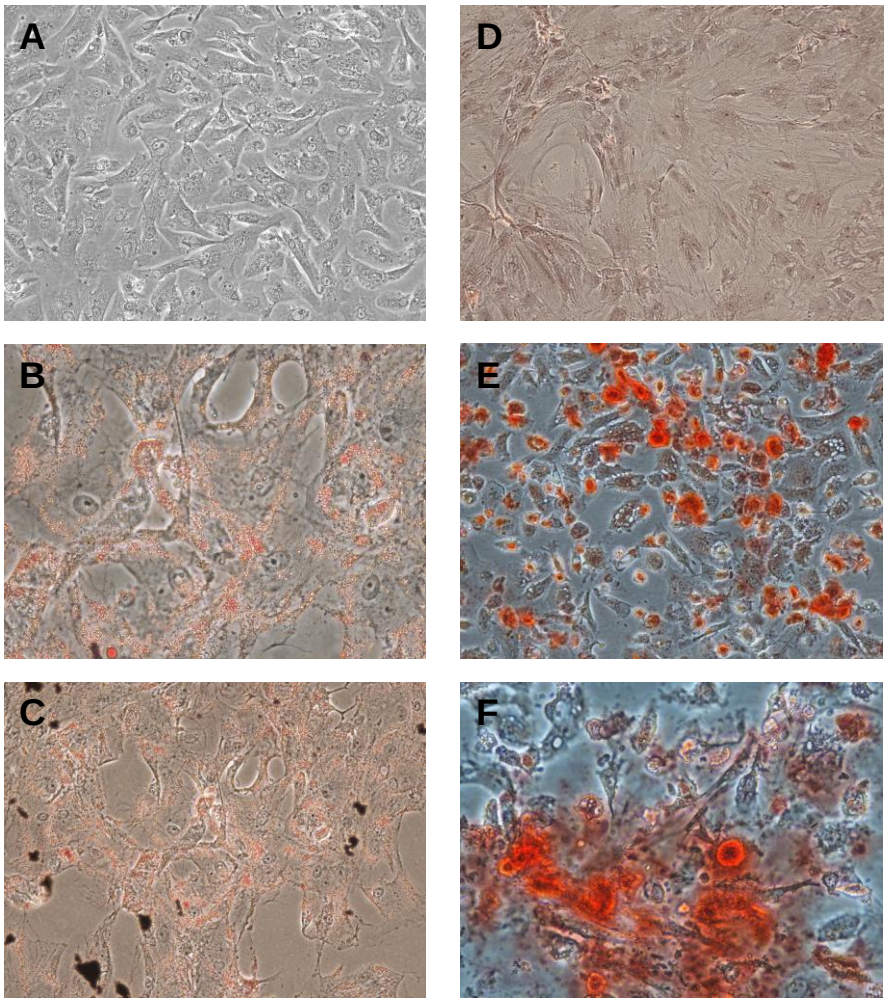


Figure 29 – Adipocyte (left column) and osteocyte (right column) differentiation of hAECs and hAMSCs. Phase contrast micrograph of hAECs cultured in A) basal medium stained with Oil-red-O and D) hAMSCs cultured in basal medium stained with Alizarin Red. Light microscope showed lipid vacuole after adipogenic stimulation of hAEC (B) and hAMSCs (C) and calcium deposit after osteogenic strimulation for hAECs (E) and hAMSCs (F).

4.3.2 *In vitro* differentiation into hepatocytes

To determine whether hAECs and hAMSCs could differentiate into hepatocytes, cells were allowed to grow to 8 days prior to induction. After 10 days of induction, the cells were incubated for 2 hours with Diethoxy (5,6) chloromethylfluorescein. The generation of fluorescent products was evaluated by fluorescence scanning microscopy. Both hAECs and hAMSCs showed few signs of hepatocyte differentiation (Fig. 30). Cells maintained in control medium did not show any sign of hepatocyte differentiation (Fig. 30A).

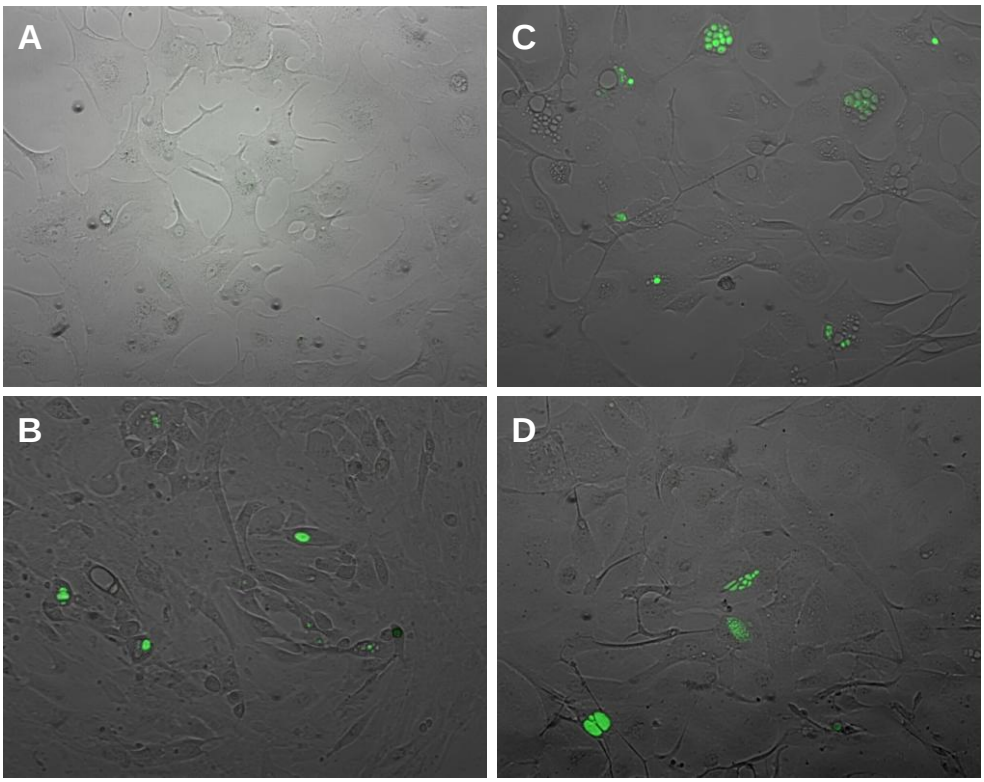


Figure 30 – Hepatocyte differentiation of hAECs and hAMSCs. A) Cells grown in basal medium as control; B) hAMSCs , C) and D) hAECs grown in induced medium in the presence of Diethoxy (5,6) chloromethylfluorescein.

4.4 Differentiation into airway epithelium

4.4.1 CFTR protein expression by flow cytometry

hAMSCs stained with CM-DiI (as described in Materials and Methods section) were mixed with CFBE41o- cells at different increasing ratios (1:20, 1:15, 1:10 and 1:5) and seeded onto semi-permeable filters (Transwells). After 7 days of co-culture, different parameters were studied, including CFTR expression and ZO-1 localization at the cell junctions.

In order to analyze the CFTR protein expression in hAMSC-CFBE41o- co-cultures at different ratios, a flow cytometric assay was performed on day 7 of co-cultivation. This mixed population was analyzed after labelling with CFTR antibody MAB25031 mouse IgG2a in the absence of permeabilization followed by incubation with FITC-conjugated secondary antibody. As a background control, co-cultures were incubated with secondary antibody only, and the resulting fluorescence was subtracted from the analyzed samples incubated both with primary and secondary antibodies. As a positive control, CFTR labelling was assessed in normal human airway 16HBE14o- cells, resulting in 60-70% of positive cells (data not shown). CFBE41o- cells did not show any CFTR-specific labelling, consistent with the lack of CFTR transport on the plasma membrane which is a characteristic of these cells (Fig. 31). Flow cytometry data showed an increase of both green (given by CFTR) and red (given by CM-DiI) fluorescence percentage at all hAMSC-CFBE ratios as compared with CFBE41o- cells, demonstrating that a specific signal due to CFTR expression is associated with hAMSCs labelled with the vital dye. In particular, at the ratio of 1:5, 10.6% of the whole population showed both green and red positive staining, and this value decreased by reducing hAMSC number in co-cultures. Interestingly, this value corresponds to approximately 50% of the total MSC population (Table 4). The percentage of CFTR-positive hAMSCs in the whole hAMSC population increased at lower cell ratios to reach 70-80% of the whole hAMSC population (Table 4), probably because a better performance of the antibody on less hAMSCs. Overall, these data show that at least 50-80% of hAMSCs has acquired a detectable CFTR expression above the CFBE background.

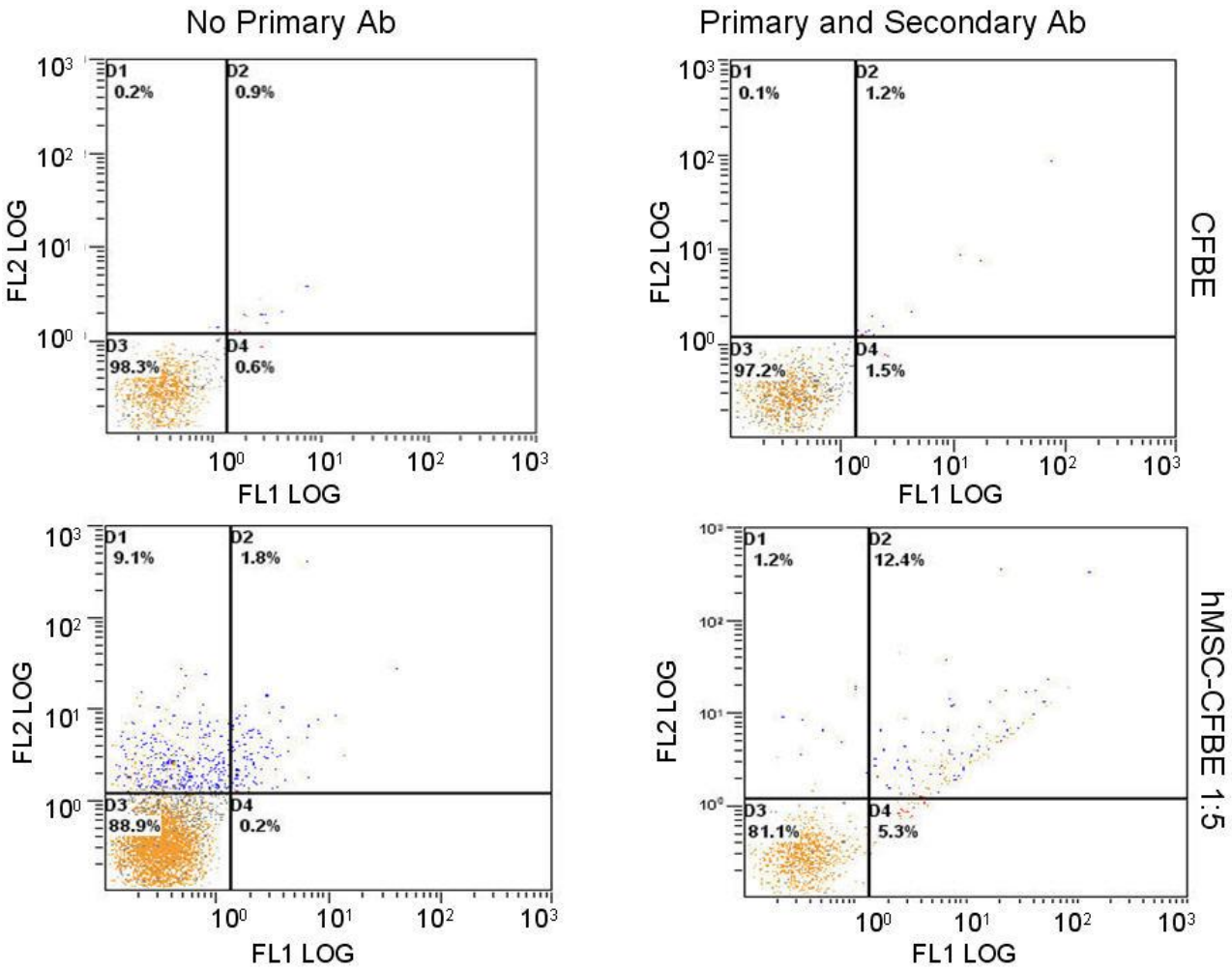


Figure 31 - CFTR expression in CM-DiI labelled hAMSCs co-cultured with CFBE41o- cells by cytofluorimetric analysis. FL1 indicates green fluorescence (CFTR), while FL2 the red fluorescence (CM-DiI)

Table 4. Percentages of CFTR+ hAMSCs labelled with CM-DiI in co-cultures with CFBE 41o- cells.

	CM-DiI+/CFTR+ cells	% of CFTR+ in whole CM-DiI+ population
CFBE	1.2 %	0 %
hAMSC-CFBE 1:5	10.6 %	53 %
hAMSC-CFBE 1:10	6.9 %	69 %
hAMSC-CFBE 1:15	5.3 %	79 %
hAMSC-CFBE 1:20	3.4 %	68 %

4.4.2 CFTR expression and localization by confocal microscopy

To confirm cytofluorimetric data and to analyze CFTR expression in cell compartments, hAMSCs-CFBE co-cultures were assayed by means of confocal microscopy. In previously published work (*Guerra et al, 2005*), it has been demonstrated that CFTR protein is expressed on the apical side of 16HBE14o- cells, while CFBE 41o- cells display only intracellular staining. After 7 days of co-cultivation, CFTR expression and localization was evaluated by epifluorescence with a protocol which allows detecting only surface and not intracellular CFTR (see Materials and Methods section), followed by confocal microscopy analysis. As can be seen in Figure 32, CFTR was highly expressed on the apical membrane of some hAMSCs since red labelled cells showed a green staining at membrane level, whereas CFBE41o- monolayers in absence of hAMSCs showed a background signal for CFTR expression, consistent with the lack of CFTR transport to the apical membrane in CF cells. These data confirm cytofluorimetric analysis as to the surface expression of CFTR in labelled hAMSCs.

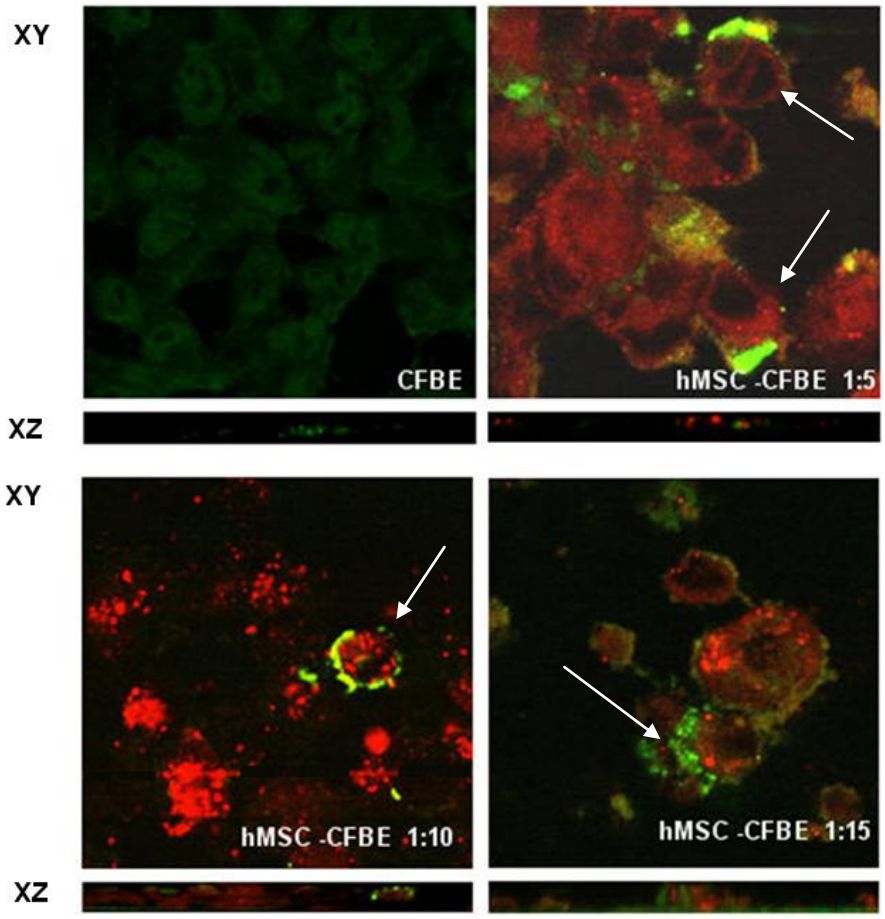


Figure 32 - CFTR immunodetection by confocal analysis. Confocal scans are shown in the horizontal cross-section (xy) plane and vertical cross-section (xz) plane. The white arrows point to CM-DiI labelled hAMSCs expressing CFTR on their membrane (green signal).

4.4.3 ZO-1 expression and organisation

hAMSC-CFBE41o- co-cultures at different ratios were examined by confocal analysis for the expression of structural markers involved in tight junction (TJ) organization, in particular we focused on ZO-1 protein. In CFBE41o- cells cultured without hAMSCs, the immunostaining showed that ZO-1 was not properly localized at TJ level as compared with wild-type 16HBE14o- cells (Fig. 33). In particular, in 16HBE14o- cells, ZO-1 staining produced a typical chicken wire-like pattern, indicating the presence of intact TJs. Moreover, we observed that co-culturing the CFBE41o- with hAMSCs induced a small re-organisation of ZO-1 at the level of TJs at the highest ratio. In particular, in presence of hAMSCs the junctional ZO-1 localization is slightly more distinctive at 1:5 (Fig. 33) and 1:10 (not shown) ratios, but still in part disorganized, while it resulted not properly organized at 1:15 and 1:20 (Fig. 33) ratios. However, it can be seen that ZO-1 expression increased in all co-culturing conditions, indicating that the presence of hAMSCs has induced a higher expression and a better reorganization of ZO-1 at cell borders.

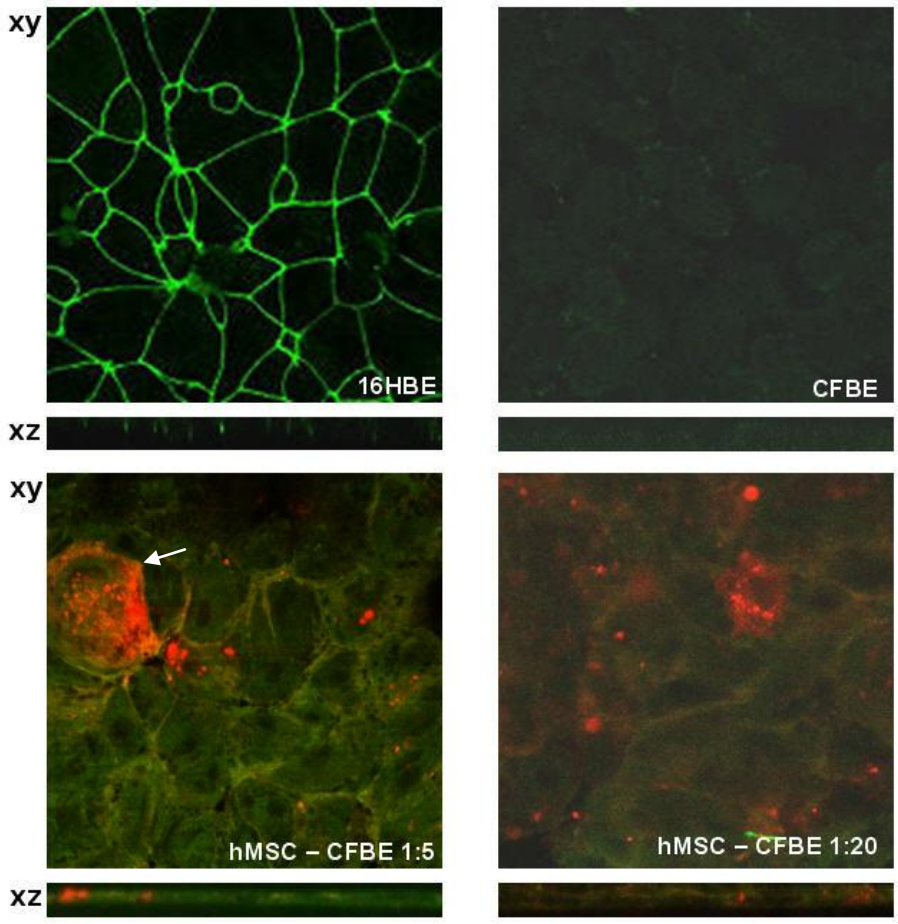


Figure 33- ZO-1 immunodetection by confocal analysis. Confocal scans are shown in the horizontal cross-section (xy) plane and vertical cross-section (xz) plane. In the lower left panel, the white arrow points to a CM-DiI labelled hAMSC. Please note that ZO-1 expression (green signal between the cells) is higher and more precisely delineating cell borders in the 1:5 ratio than in the 1:20 ratio.

4.4.4 Transepithelial resistance measurement

To evaluate the polarization of hAECs and hAMSCs, transepithelial resistance (TER) was measured at different days of culture, both for isolated cultivation (Fig. 34) and for hAMSC-CFBE41o- co-cultures at different ratios (Fig. 35). First, we measured TER in polarized non-CF 16HBE14o- control cells, and on CFBE41o- cells, hAMSCs, and hAECs alone. Non-CF 16HBE14o- showed a higher TER than CFBE41o- cells or hAMSCs and hAECs. Both hAECs and hAMSCs showed a slight increase in TER during culture, even if the value remained lower than that of 16HBE14o- (Fig. 34).

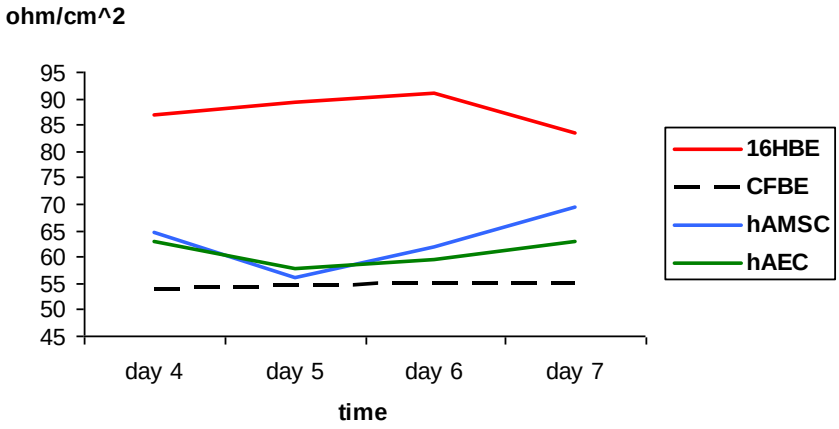


Figure 34 – TER in 16HBE14o-, CFBE41o-, in hAECs and in hAMSCs cells.

Then we measured TER on co-cultures of hAMSC-CFBE41o- cells at different ratios. CFBE41o- cells showed a lower TER as compared with 16HBE14o-, consistent with a non polarization of CF cells. Starting from day 6 of culture, TER slightly increased when CFBE41o- were co-cultured with hAMSCs respect to CFBE41o- cells alone, above all with higher hAMSC-CFBE ratios of 1:5. These results could indicate a partial correction of the CF phenotype, as evaluated by TER, in the presence of hAMSCs at least in higher ratios (Fig. 35).

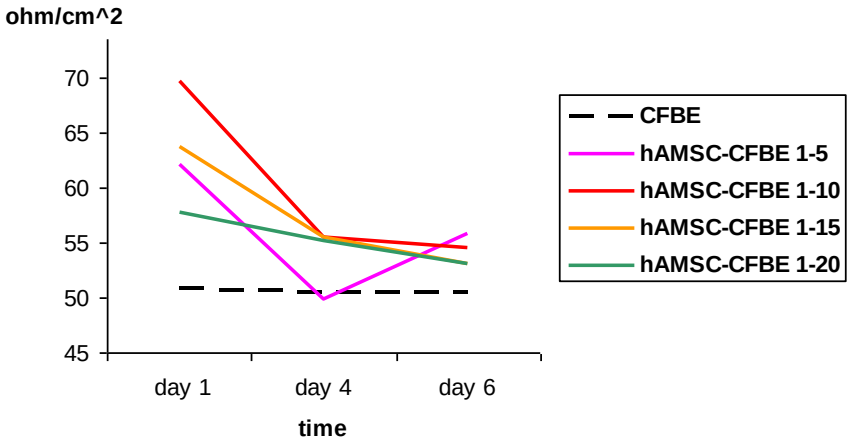


Figure 35 - TER in CFBE41o-, and in co-cultures of hAMSC-CFBE41o- cells.

4.4.5 Fluid Absorption

To evaluate the effect on the physiology of CFBE41o- cells by culturing them with hAMSCs at different ratios, we measured fluid absorption. First, we measured fluid transport in polarized non-CF 16HBE14o- control cells, incubated in the absence or in the presence of 10 mM or 100 mM amiloride for 24 h. Amiloride is a known inhibitor of ENaC (*Caci et al, 2009*). As shown in Figure 36, addition of amiloride reduced with a dose dependency ENaC-dependent fluid absorption from the apical side of the epithelium up to 80%. Then we measured fluid absorption on CFBE41o- cells alone, or on co-cultures of hAMSC-CFBE41o- cells. CFBE41o- cells showed a higher fluid absorption as compared with 16HBE14o-, consistent with a higher ENaC activity. Fluid absorption slightly decreased when CFBE41o- were co-cultured with hAMSCs respect to CFBE41o- cells alone, above all with higher hAMSC-CFBE ratios of 1:5 and 1:10. In these experiments we used amiloride at 100 mM, which was the most effective concentration that inhibited fluid absorption in 16HBE control cells. Amiloride treatment strongly decreased fluid absorption at higher ratios tested. These results strongly indicate a partial correction of the CF phenotype, as evaluated by fluid absorption, in the presence of hAMSCs.

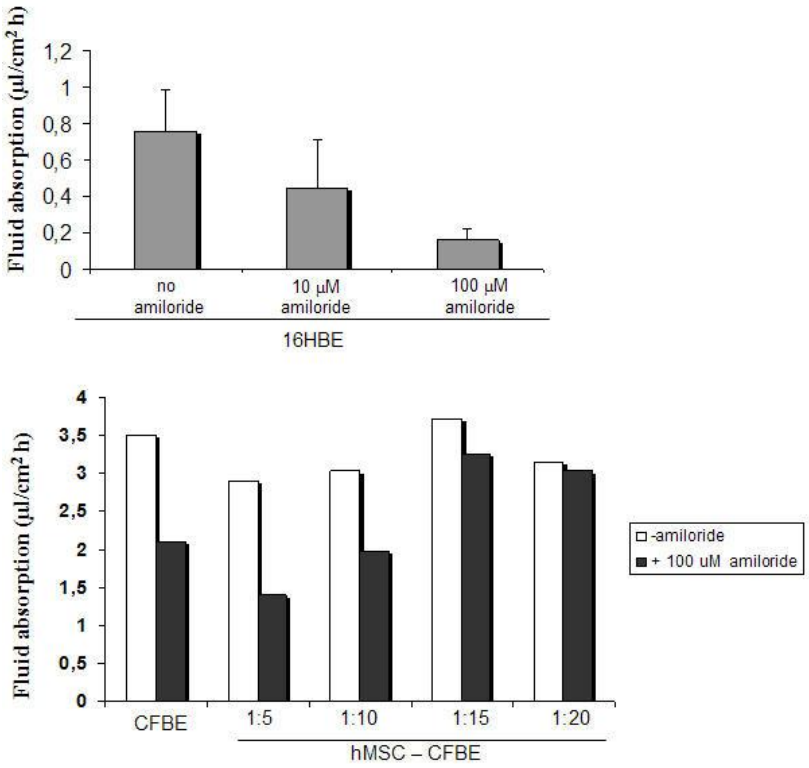


Figure 36- Fluid absorption in 16HBE14o-, CFBE41o-, and in co-cultures of hAMSC-CFBE41o- cells.

4.4.6 RT-PCR

To demonstrate that hAECs and hAMSCs can express ENaC subunits, RT-PCR analysis was performed. hAMSCs expressed α and β subunits of EnaC, but not γ subunit (Fig. 37).

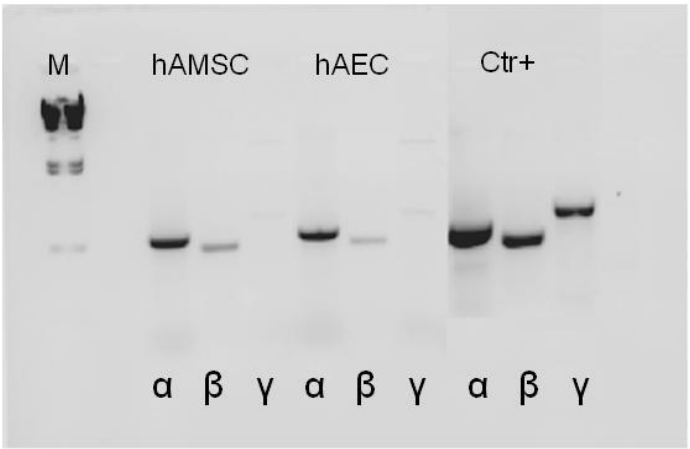


Figure 37 - RT-PCR for α, β, and γ ENaC subunits on hAECs and hAMSCs. M molecular weight markers; Ctr+ positive control (H441 treated with dexamethasone).

CFTR was detected in hAECs and in hAMSCs only after nested PCR. Moreover, while hAEC expressed CFTR at different passages, CFTR expression in hAMSC decreased dramatically during culture (Fig. 38).

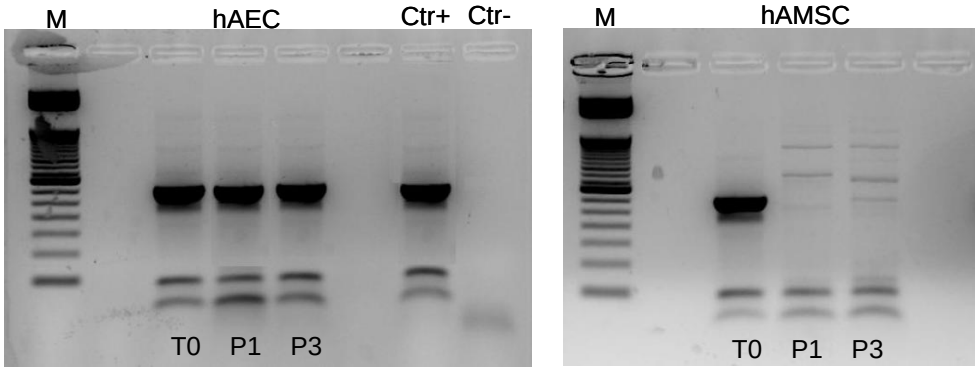


Figure 38 – RT-PCR for CFTR on hAECs and hAMSCs at different passages. M molecular weight markers; Ctr+ positive control; Ctr- negative control.

DISCUSSION

5. DISCUSSION

Cystic fibrosis (CF) is the most frequent severe autosomal recessive disorder in the European population. There are a number of reasons why CF should be an ideal disease for the application of gene therapy (*Conese and Rejman, 2006*). First of all, it is caused by a single-gene defect. Secondly, the main pathology is in the lung, an organ which is relatively easily accessible for treatment. In addition it offers a therapeutic window for treatment since CF patients demonstrate an almost normal phenotype at birth. Finally, it has been suggested that restoration of CFTR function to a level of only 5–10% of normal will be sufficient to adequately reverse the chloride transport dysfunction (*Johnson et al, 1992; Ramalho et al, 2002*) and give complete recovery of the intestinal disease in CF mice (*Dorin et al, 1996*). However, despite these encouraging features, clinical trials of gene therapy in CF patients have only resulted in inconsistent and low levels of vector-specific CFTR expression and at best minor functional changes towards normality (*Davies et al, 2001*).

This has led to a search for alternative approaches including the use of stem cell populations. CF is a potential model disease for stem cell therapy because of the persistent lung inflammation that leads to damage and remodeling, and can promote engraftment of stem cells (*Piro et al, 2008*).

A developing potential therapeutic approach for CF and other lung diseases has been stimulated by recent reports demonstrating that several cell populations derived from adult bone marrow, from amniotic fluid or from umbilical cord blood, including stromal derived mesenchymal stem cells (MSCs), endothelial progenitor cells, and circulating fibrocytes, can localize to a variety of organs and acquire phenotypic and functional markers of mature organ-specific cells (*Korbling and Estrov, 2003; Prockop, 2003; Herzog et al, 2003; Weiss et al, 2006; Sueblinvong et al, 2008; Carraro et al, 2008*).

The in vivo efficiency of bone marrow stem cells to differentiate in airway epithelium is very low (0.01-0.025%) (*Piro et al, 2008*), as demonstrated by different studies in mice (*Loi et al, 2006; Bruscia et al, 2006*). Importantly, it has been reported that circulating bone marrow-derived stem cells preferentially home to the damaged respiratory epithelium undergoing regeneration (*Theise et al, 2002; Kotton et al, 2001*). In order to facilitate cell homing and epithelium differentiation of these cells, pulmonary damage can be induced by chemical or physical agents, as bleomycin or X-ray radiation.

Recent studies demonstrate that with 0.1% of donor cells that became respiratory host cells a partial correction of the electrophysiological defect has been obtained (*Bruscia et al, 2006*). On the contrary, other studies demonstrate that a higher percentage (6-20%) of corrected cells is needed to revert the defect (*Farmen et al, 2005*).

The results published by Wang (*Wang et al, 2005*) and Loi (*Loi et al, 2006*) strongly suggest that the population of bone marrow cells relevant for repopulating the lung epithelium may be found in the plastic adherent stromal cell compartment. Yet, mesenchymal stem cells are not without drawbacks, e.g. they are limited in numbers and lack well-defined markers required for their purification (*Herzog et al, 2003*). One approach to overcome these limitations is to consider alternative sources of stem cells capable of repopulating damaged respiratory epithelium.

In the present study, we propose human placenta as an ethical source of stem cells for CF therapy.

It is well established that human placenta may represent a reserve of progenitor/stem cells. As a potential source of cells for application in regenerative medicine, the placenta has the advantage of being a natural by-product of birth which is often simply discarded, while harvest of term amniotic membrane does not pose any risk to the mother or newborn. Amniotic epithelial cells (hAECs) and amniotic mesenchimal stromal cells (hAMSCs) are known to have unique characteristics, such as low level expression of major hystocompatibility complex antigens, and a less restricted differentiation potential (*Miki and Strom, 2006*). The availability of hAECs and hAMSCs and the lack of ethical concerns for this source of stem cells are considered advantageous for their widespread use and acceptance.

Isolated cells readily attached to plastic dishes and they can be kept in culture in a simple medium supplemented with EGF. hAECs displayed typical cuboidal epithelial morphology and can be kept until 2-6 passages before proliferation ceased. hAECs presented different surface markers, suggesting some heterogeneity of phenotype (*Parolini et al, 2008*). Immediately after isolation, hAECs appear to express low molecular weight cytokeratin, EpCAM, integrin α6 (CD49f), integrin β1 (CD29), E-cadherin, zonula adherens-1 (ZO-1). The expression of CD90 (Thy-1) antigens appeared to be stable during culture. hAECs expressed not only epidermal markers and general epithelial markers, but also expressed vimentin. We also demonstrated that mRNA expression of SNAI1, a transcriptional repressor which downregulates the expression of ectodermal genes within the mesoderm, increased in hAEC during passages. These facts suggested that the epithelial-mesenchymal transition (EMT) may occur in the amniotic membrane (*Parolini et al, 2008*). Indeed, in three cases, hAECs underwent EMT, acquiring a more spindle-shaped morphology and expressing typical mesenchymal markers.

hAMSC displayed a fibroblastic morphology and can be kept until passages 5-10 in culture. hAMSCs presented surface markers associated with mesenchymal cells from bone marrow, as CD105, CD73, and CD90, and mesenchymal specific marker as vimentin. They also displayed positivity for CK18, and CD29. This hybrid phenotype of hAMSCs is interpreted as a sign of

multipotentiality, and suggests that the amnion derived cells had not completely differentiated into epithelial or mesenchymal cells (*Parolini et al, 2008*).

Cytogenetic analyses of hAECs and hAMSCs revealed that these cells are of fetal origin and maintain normal karyotypes at different passages.

As shown here, the amniotic cells derived from term placenta seem to remain somewhat "plastic" in their differentiation options and maintain the capability to differentiate and contribute to cells from different germ layers. In particular, we demonstrated that hAECs and hAMSCs differentiate into mesodermal (adipocyte and osteocyte) and endodermal (hepatocyte) lineages.

Because amniotic cells can differentiate to different types, we examined them with antibodies directed against well-known surface markers characteristic of ES cells. Like ESCs, amniotic cells express SSEA-4, although the relative proportion of SSEA-positive cells in initial isolates of amniotic cells is lower than that observed with ESCs. We speculate that more differentiated cells in the amnion may lose stem cell surface markers. In addition to characteristic stem cell surface markers, amniotic cells show mRNA expression of Nanog, a transcription factor with an expression pattern previously reported to be restricted to pluripotent stem cells (*In't Anker et al, 2003; Prusa et al, 2003*), and Sall4, a member of spalt-like protein family, which seems to interact with Nanog *in vitro* and exist as a complex with Nanog (*Wu et al, 2006*).

Both genes were readily detected in hAECs and hAMSCs cells at the time of isolation, and during culture, even if their expression seems to decrease with time, probably due to the fact that more differentiated cells may lose pluripotency markers during culture passages. Based on the expression of Oct-4, SSEA-4, Nanog and Sall4, amniotic membrane may contain around 10% stem cell marker-positive cells. These findings are consistent with those reported by a recent paper (*Miki et al, 2009*), which suggested that in terms of stem cell characteristics and differentiation potential, amniotic cells may be somewhere between pluripotent ESC clusters and multipotent neural stem/progenitor cell spheres.

Furthermore, for the first time our results provide evidence that hAECs and hAMSCs can be induced in vitro to express markers of airway epithelial phenotype, including ZO-1 and CFTR, and so have the potential to differentiate into airway epithelial cells.

To evaluate this, we performed a dose-dependent experiment: hAMSCs stained with CM-DiI were mixed with CFBE41o- cells at different increasing ratios (1:20, 1:15, 1:10 and 1:5) and seeded onto semi-permeable filters (Transwells). CFBE41o- cells did not show any CFTR-specific labelling, consistent with the lack of CFTR transport on the plasma membrane which is a characteristic of these cells. Flow cytometry data showed that CFTR and CM-DiI fluorescence increase in hAMSCs-

CFBE co-cultures, demonstrating that a specific signal due to CFTR expression is associated with hAMSCs labelled with the vital dye. In particular, at the ratio of 1:5, 10.6% of the whole population showed both CFTR and CM-DiI positive staining, and this value decreased by reducing hAMSC number in co-cultures. Overall, our data showed that at least 50-80% of hAMSCs has acquired a detectable CFTR expression above the CFBE background.

To confirm cytofluorimetric data and to analyze CFTR expression in cell compartments, hAMSCs-CFBE co-cultures were assayed by means of confocal microscopy. CFTR was highly expressed on the apical membrane of some hAMSCs, whereas CFBE41o- monolayers in absence of hAMSCs showed a background signal for CFTR expression, consistent with the lack of CFTR transport to the apical membrane in CF cells.

CFTR expression was evaluated by PCR in hAECs and in hAMSCs. While hAEC expressed CFTR at different passages, CFTR expression in hAMSC is low, and decreased dramatically during culture. Overall, the data suggest that low CFTR expression in hAMSCs can be increased by co-cultivation with CFBE, probably due to paracrine or autocrine signaling.

Afterwards, we examined hAMSC-CFBE41o- co-cultures at different ratios by confocal analysis for the expression of structural markers involved in tight junction (TJ) organization, in particular ZO-1 protein. In CFBE41o- cells cultured without hAMSCs, ZO-1 was not properly localized at TJ level as compared with wild-type 16HBE14o- cells, where it produced a typical chicken wire-like pattern, indicating the presence of intact TJs. Moreover, we observed that co-culturing the CFBE41o- with hAMSCs induced a small re-organisation of ZO-1 at the level of TJs at the highest ratio, and not at the lowest ratios. However, it can be seen that ZO-1 expression increased in all co-culturing conditions, indicating that the presence of hAMSCs has induced a higher expression and a better reorganization of ZO-1 at cell borders.

We then demonstrated that hAECs and hAMSCs can polarize when grown onto semi-permeable filters at the air-liquid interface. In order to evaluate the polarization, we measured transepithelial resistance (TER) at different days of culture and we observed that amniotic cells presented a slight increase in TER during culture, suggesting the establishment of tight junctions across the monolayer, even if the value remained lower than that of non-CF 16HBE14o- cells.

After that, we measured TER on co-cultures of hAMSC-CFBE41o- cells at different ratios. CFBE41o- cells showed a lower TER as compared with 16HBE14o-, consistent with a non polarization of CF cells. Starting from day 6 of culture, TER slightly increased when CFBE41o- were co-cultured with hAMSCs respect to CFBE41o- cells alone, above all with higher hAMSC-CFBE ratios.

Overall, these results could indicate a partial correction of the CF phenotype in the presence of hAMSCs at least in higher ratios, suggesting a directly proportional effect of the percentage of hAMSCs in co-cultivation. Still, it seems that only a subpopulation of hAMSCs (50-80%) are capable of contributing to the establishment of respiratory epithelial cell monolayer in this system.

Recent reports have suggested two possible mechanisms to explain the plasticity of mesenchymal stem cells: transdifferentiation and cell fusion (*Spees et al, 2003*). A previous paper suggested that cell fusion is a rare event in the air-liquid interface system and, even if mesenchymal stem cells undergo cell fusion with epithelia, they still can be therapeutically beneficial if the corrected CFTR gene is expressed (*Wang et al, 2005*).

To evaluate the effect on the physiology of CFBE41o- cells by culturing them with hAMSCs at different ratios, we measured fluid absorption.

The volume of the surface fluid covering the airways is maintained through a fine balance between ion and water secretion and absorption. This is obtained by exerting a tight control of the activity of ion channels and transporters localized on the apical and basolateral membranes of epithelial cells. In particular, Na^+ absorption through the epithelial Na^+ channel (ENaC), localized in the apical membrane, and the Na/K-ATPase, in the basolateral membrane, is in equilibrium with Cl^- secretion through the CFTR and other Cl^- channels in the apical membrane, and the Na-K-Cl (NKCC) cotransporter in the basolateral membrane (*Boucher, 1994*). An appropriate volume of periciliary fluid is essential not only to allow cilia beating, but also to maintain the hydration and therefore the proper visco-elastic characteristics of mucus. The correct performance of these two processes, absorption and secretion, ensures an effective mucociliary clearance. In CF, the equilibrium between absorption and secretion is disrupted by mutations in the CFTR Cl^- channel. As a consequence, Cl^- secretion is strongly reduced and Na^+ absorption becomes predominant. Accordingly, airways of patients with CF are dehydrated, obstructed by thick mucus, inflamed, and frequently infected (*Matsui et al, 1998*).

The negative effects of the disequilibrium between Na+ absorption and Cl- secretion has been also demonstrated by the production of a transgenic mouse that hyperexpresses the β subunit of the ENaC (*Mall et al, 2004*). In this mouse, the increased Na^+ and water absorption produces a CF-like lung disease, characterized by surface liquid depletion, increased mucus concentration and stasis, inflammation, and poor bacterial clearance. It has been suggested that down-regulation of ENaC may help to restore airway hydration and mucus clearance, and to reverse, at least partially, the airway phenotype in patients with CF. Amiloride is a known inhibitor of ENaC (*Caci et al, 2009*).

The reduction of ENaC activity does have functional consequences on the airways, as demonstrated by the fluid measurements.

First, we measured fluid transport in polarized non-CF 16HBE14o- control cells, incubated in the absence or in the presence of 10 mM or 100 mM amiloride for 24 h. Addition of amiloride reduced with a dose dependency ENaC-dependent fluid absorption from the apical side of the epithelium up to 80%. Then we measured fluid absorption on CFBE41o- cells alone, or on co-cultures of hAMSC-CFBE41o- cells. CFBE41o- cells showed a higher fluid absorption as compared with 16HBE14o-, consistent with a higher ENaC activity. Fluid absorption slightly decreased when CFBE41o- were co-cultured with hAMSCs respect to CFBE41o- cells alone, above all with higher hAMSC-CFBE ratios. In these experiments we used amiloride at 100 mM, which was the most effective concentration that inhibited fluid absorption in 16HBE control cells. Amiloride treatment strongly decreased fluid absorption at higher ratios tested.

To demonstrate that hAECs and hAMSCs can express ENaC subunits, RT-PCR analysis was performed. hAMSCs expressed α and β subunits of ENaC, but not γ subunit. The relative expression found here, characterized by a preponderance of α subunit followed by β and with low levels of γ, is similar to that described previously in human airways (*Burch et al, 1995*). Explanations for the unbalanced expression could be that excess in α subunit, the only subunit able to form an active channel, might assure a certain level of ENaC function. In contrast, the low expression levels of β and γ could suggest that they are more prone to modulation by external factors, such as hormones, cytokines, and so on (*Caci et al, 2009*).

Such experiments show a reduction in fluid absorption through the airway epithelium monolayer, suggesting that the periciliary fluid volume would increase and, as a consequence, there would be an improvement in mucociliary clearance.

CONCLUSION AND FUTURE PERSPECTIVES

6. CONCLUSION AND FUTURE PERSPECTIVES

The opportunity of having a fetal tissue rich in cells with stem cell characteristics and the capacity to differentiate towards the three germinal cells lines, with low immunogenicity, non tumorigenicity and non ethical barriers, make the amniotic membrane an alternative source of stem cells particularly interesting in the context of cell based therapy applications such as transplantation and regenerative medicine.

We have identified cells in the human amniotic membrane with characteristics of airway epithelial progenitors expressing CFTR and ZO-1, particularly when co-cultivated under air-liquid interface cultures with CF human epithelial bronchial cells. Amniotic cells has stem cell characteristics including self-renewal and multi-epithelial lineage differentiation potential.

Overall, our data show that these cells may contribute to partial correction of the CF phenotype and are very likely an ideal candidate for cell-based therapy for CF.

Whether the beneficial effects of placenta-derived cells are due to differentiation of the transplanted cells themselves or to paracrine actions on the surrounding host tissue in order to reduce inflammation and promote regeneration remains to be fully elucidated, although current evidence seems to lend greater support to the latter of these hypotheses. In any case, the promising data obtained to date constitute compelling evidence regarding the potential utility of these cells for clinical application.

Surely the true potential of amniotic cells for CF therapy requires testing in *in vivo* models. Future studies aiming at evaluating the *in vivo* ability of engraftment and differentiation of amniotic cells in NOD-SCID mice are needed. The NOD-SCID mouse represents a pre-clinic model used for the evaluation of the therapeutic effect of human stem cells. Despite rare engraftment of airway epithelium, there are an increasing number of studies demonstrating a functional role of stem cells in the mitigation of lung injury. After the optimization of the transplant conditions, the ability of reverting the electrophysiological defect of the cells should be evaluated in a model of CF mouse.

The use of a in-vivo model will help in finding a strategy of cell therapy for the cure of respiratory disease of CF, directly transferable to CF patients.

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APPENDIX

FONDAZIONE IRCCS CA' GRANDA
OSPEDALE MAGGIORE POLICLINICO

U.O.C. CENTRO FIBROSI CISTICA
DIRETTORE PROF. CARLA COLOMBO
VIA COMMENDA 9, 20122 MILANO
TEL 0255032456 FAX 0255032814

Consenso informato alla conservazione di campioni biologici in vista di studi futuri

INFORMAZIONE

Gentile Sig.ra,
La informiamo che, se presta il Suo consenso, dopo il parto saranno prelevati alcuni campioni della placenta, tessuto generalmente eliminato dopo il parto, che verranno processati/conservati presso l'U.O. 517 e/o presso la Biobanca Italiana, una struttura pubblica per la raccolta di campioni biologici (ad esempio, sangue, cellule, tessuti) con sede presso la Fondazione IRCCS Ospedale Maggiore Policlinico, Mangiagalli e Regina Elena di Milano.

Modalità di prelievo dei campioni

Tali campioni saranno ricavati dal materiale biologico che residua dopo il parto.
Tale prelievo non comporterà alcun rischio per la Sua salute o quella del neonato.

Finalità e natura degli studi

I campioni prelevati potranno essere utilizzati, anche in futuro, per compiere indagini di carattere anatomopatologico, molecolare o genetico. Tali studi saranno finalizzati ad un miglioramento delle metodiche di caratterizzazione e utilizzo delle cellule staminali.

Sviluppi correlati agli studi

I risultati acquisiti dal compimento di tali indagini potranno essere utilizzati, in forma anonima, in pubblicazioni scientifiche. Inoltre essi potranno contribuire allo sviluppo di nuove modalità terapeutiche. Nel rispetto delle normative vigenti i proventi ricavati dallo sviluppo di tali prodotti potranno essere impiegati ad esclusivo beneficio della collettività.

Tutela della riservatezza

I Suoi campioni e i dati personali e clinici ad essi associati verranno trattati dai ricercatori e dal personale incaricato in modo da garantire il rispetto della Sua riservatezza e potranno essere condivisi, sempre in forma anonima, con altri ricercatori.

Informazioni sui risultati degli studi e su eventuali indagini future

Se lo desidera, potrà ricevere informazioni sull'andamento degli studi che verranno compiuti usando i Suoi campioni e sui risultati ottenuti sino a quel momento.
In futuro i Suoi campioni potranno essere utilizzati per proseguire tali studi o per intraprendere nuove indagini ad essi correlate, senza necessità di chiedere un nuovo consenso.

Revoca del consenso
In qualunque momento e per qualunque motivazione potrà revocare il Suo consenso alla conservazione dei campioni e/o al loro utilizzo e potrà chiedere che i Suoi campioni e i dati clinici e personali ad essi associati siano eliminati. Tali scelte non avranno alcun effetto negativo sulla Sua assistenza medica. Esse, tuttavia, potranno impedire la continuazione degli studi intrapresi o l'avvio di nuovi studi correlati.

CONSENSO

In base alle informazioni che ho ricevuto e avendo avuto la possibilità di chiedere spiegazioni,

ACCONSENTO che:

1. I campioni biologici prelevati siano conservati nelle sedi segnalate.

   ☐ SI ☐ NO

2. I campioni biologici e i dati personali e clinici ad essi associati siano utilizzati per compiere in futuro indagini finalizzate al miglioramento di metodiche analitiche e terapeutiche

   ☐ SI ☐ NO

3. I campioni e i dati personali e clinici ad essi associati siano inoltre utilizzati per proseguire tali studi e/o compiere nuove indagini ad essi correlate, senza necessità di prestare un nuovo consenso

   ☐ SI ☐ NO

4. I campioni biologici e i dati personali e clinici ad essi associati vengano utilizzati in forma anonima dai ricercatori e dal personale incaricato e siano condivisi, sempre in forma anonima, con altri ricercatori

   ☐ SI ☐ NO

5. I risultati acquisiti dal compimento di tali indagini possano essere utilizzati in forma anonima in pubblicazioni scientifiche e/o possano contribuire allo sviluppo di nuove modalità terapeutiche derivanti da tali indagini

   ☐ SI ☐ NO

DICHIARO di:

6. aver ricevuto informazioni sulla possibilità di revocare il consenso alla conservazione e/o utilizzo dei miei campioni e dei dati ad essi associati in qualunque momento e per qualunque motivazione

   ☐ SI ☐ NO

7. voler ricevere informazioni sui risultati confermati dagli esami analitici compiuti durante lo svolgimento degli studi e su eventuali nuovi risultati e/o possibilità diagnostiche/terapeutiche derivanti da tali indagini

   ☐ SI ☐ NO

Data_____

Il paziente_____ **Firma**_____

Il presente consenso è stato raccolto da:

Dr _____ Firma_____

Qualifica _____

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Valentina