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ISOLATION OF HUMAN AMNIOTIC STEM CELLS FROM TERM PLACENTA AND THEIR IN VITRO DIFFERENTIATION IN AIRWAY EPITHELIUM TO CORRECT CF PHENOTYPE

Settore/i scientifico disciplinari di afferenza

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SUMMARY

ABSTRACT

Cystic fibrosis (CF) is a lethal autosomal recessive disorder due to mutations in the CF transmembrane conductance regulator (CFTR) gene, a cAMP-dependent chloride channel expressed on the apical side of epithelial cells. Although CF involves many organs with secretory/absorptive properties, including the liver, the main cause of morbidity and mortality is a chronic inflammatory lung disease. Because of its monogenic nature, and since the lung is easily accessible, CF has been a target disease for gene-based therapeutic intervention; however, this approach has given unsatisfied results in terms of efficiency of gene delivery to the lung and of efficacy outcomes. This partial success was due to the inefficiency of passing the mucus barrier overlying the epithelial cells and to the immune response against the gene therapy vectors. Cell therapy could be a more effective treatment because allogenic normal cells and autologous engineered cells express CFTR gene. Bone marrow-derived stem cells have been the first source evaluated for homing to the lung and curative potential, but the in vivo efficiency of bone marrow stem cells to differentiate in airways epithelium is very low (0.01–0.025%), as also demonstrated by different studies in CF mice.

Recently, new cell sources for CF treatment have been characterized; MSCs from cord blood and amniotic fluid stem cells can differentiate in vitro and in vivo in airway epithelium. Stemming from these results on MSCs, and based on the demonstrated high plasticity of amniotic derived stem cells, after an extensive characterization of the expression of phenotypic and pluripotency markers by hAMSCs and their differentiative potential, we preliminarily evaluated their usefulness in CF by in vitro experiments using cocultures of hAMSCs and CF-respiratory epithelial cells.

INTRODUCTION

1.1 Cystic Fibrosis

Cystic fibrosis (CF) is an autosomal recessive disease of epithelia in the airways, liver, pancreas, small intestine, reproductive organs, sweat glands and other fluid-transporting tissues (1, 2). In Caucasians the disease affects about 1 in 2500 live births and is the most common eventually lethal genetic disease (3). The cause of CF is different mutations in the *CFTR gene* (cystic fibrosis transmembrane conductance regulator), the product of which is a protein expressed in the apical membrane of most epithelia. This membrane protein, the cystic fibrosis conductance regulator (CFTR), is a cyclic AMP (cAMP) regulated chloride (Cl)-channel involved in different regulatory processes of the cell, e.g., both transcellular and paracellular ion and water transport (1, 4).

Chronic progressive obstructive lung disease and pancreatic insufficiency are the main clinical symptoms of CF, where pulmonary disease is the major cause (95%) of morbidity and mortality (5). The succession of events leading from the defective CFTR to the clinical symptoms is not completely understood. However, it is obvious that the abnormal ion transport with hyperabsorption of Na+ and impaired Cl- and HCO3- secretion in the airway epithelial cells leads to a disturbance and acidification of the thin fluid layer, the airway surface liquid (ASL) covering the airways (6-9).

Net vectorial transport of ion and water in human airways depends critically on the amiloride-sensitive epithelial sodium channel (ENaC) and CFTR operating in concert with the paracellular and transcellular pathways (4). This interaction is organ specific. In the airways, intestine and kidneys, CFTR inhibits ENaC (8, 10, 11), whereas in the sweat duct ENaC activity is dependent on and increases with CFTR activity (12). Thus in the airways, the absence of CFTR with its failure of cAMP-regulated Cl- secretion

produces upregulation of Na+ absorption and depletion of ASL height, leading to thickened mucus adhering to the glycocalyx coating ciliary shafts and impaired mucociliary clearance (13). It has also to be taken into account that signaling molecules within the ASL itself are controlling ASL volume (14).

These molecules are ATP and adenosine (15, 16) that via purinergic and adenosine receptors mediate inhibition of ENaC and activation of both CFTR and calcium-activated chloride channels (CaCC) (11, 14, 17, 18) (see 1.2.3).

The above communications go awry in CF partly because the relative activities of CFTR and ENaC become unbalanced. Certainly, CF airway epithelia release ‘normal’ amounts of ATP in response to shear stress, with subsequent regulation of ENaC and CaCC (14, 15, 19, 20). However, in contrast to normal airway epithelia, luminally formed adenosine does not activate Cl_ secretion and inhibits ENaC because of the absence of the target of adenosine receptor signalling (i.e., CFTR) (8). Furthermore, an abnormally low surface pH may harmfully affect mucus viscosity by altering exposure of hydrophobic regions of mucin molecules as well as changing the electrostatic charge of their carbohydrate side chains (21, 22). Failure to compensate for an intraluminal acid load may also increase the inflammatory response in the airway by interfering with bactericidal activity promoting secretion by immune cells of substances harmful to the lung (22-24). This may be one explanation for the neutrophil-dominated inflammation evident in the CF lung (25), where characteristic pathogens are Pseudomonas aeruginosa, Staphyococcus aureus and Heamophilus influenzae. Finally, experiments in our laboratory have previously shown that the apical fluid in CF-airway epithelial cell cultures, as well as the ASL in transgenic CF-mice and CF-patients has a higher salt content than that of their healthy counterparts (26). These findings are clinically relevant, since the activity of anti-bacterial substances in the ASL, such as defensins, is diminished when they are exposed to an environment rich in salt (27).

1.1.2 The gene

The *CFTR* gene was identified 1989 and this has sharply accelerated the research on CF. The gene, which is situated on the long arm of human chromosome 7 (7q31.2), spans approximately 250 kilobases (kb) of nucelotide sequences together with its promoters and regulatory regions. The 27 exons form a 6.5 kb long coding sequence, which is capable of encoding a protein of 1480 amino acids (28, 29).

The introns allow alternative splicing of CFTR messenger RNA (mRNA), which has clinical significance because it may decrease the amount of mature CFTR protein expressed, and thus be responsible for the variable severity of the disease. The quantity of correctly spliced transcripts may also differ among various organs of the same patient, contributing to differential organ disease severity (30, 31). Five percent correctly spliced CFTR Mrna appears to be sufficient to avoid the severe complications of the disease (32).

Since the CFTR gene has features of a houskeeping gene, CFTR, its gene product, is not limited to the cells of epithelial origin. In fact, CFTR mRNA transcripts and/or CFTR protein have been demonstrated in lung fibroblasts, blood cells, alveolar macrophages, and smooth muscle cells (33, 34). In addition to its typical plasma membrane location, CFTR was also found in membranous organelles such as lysosomes of alveolar macrophages (35) and in both apical and basolateral membrane of the sweat duct (36). Recently CFTR-like channels have also been found in basolateral membrane of human and bovine tracheal primary cell cultures (37).

Although over 1,500 different mutations in the CFTR gene are known (38, 39), approximately 66 % of the patients worldwide carry the ΔF508 mutation (a deletion of three nucleotides that results in a loss of phenylalanine at position 508 of the CFTR

structure) with somewhat higher prevalence in Western Europe and USA (40). This type of mutation causes an incorrectly assembled CFTR protein resulting in endoplasmatic reticulum (ER) retention and degradation of the protein (41) as well as defective regulation (42).

Patients homozygous for ΔF508 usually have more pronounced clinical manifestations compared to heterozygotes and genotypes without ΔF508 (43-45) although these differences are highly variable (46).

1.1.3 The CFTR gene product

Based on the amino-acid sequence and its structure, CFTR is identified as a member of the superfamily of ATP-binding cassette (ABC) transporters.

However, among the thousands of ABC family members, only CFTR is an ion channel (47, 48).

ABC transporters are ubiquitous in the entire animal kingdom due to their role in coupling transport to ATP hydrolysis. They also are involved in many genetic diseases (49).

Chloride channels provide the major route for transmembrane chloride transport. This transport is involved in key cellular events including cellvolume regulation, transepithelial fluid transport, muscle contraction and neuroexcitation. Within cells, chloride transport across organelle membranes is involved in endosomal, lysosomal and Golgi complex acidification. In contrast to cation channels, which often show high selectivity for a specific ion, chloride channels are also permeable to other anions including other halides, the pseudohalide SCN- and bicarbonate (50).

Whether CFTR is involved in all the above mechanisms or not has not yet been revealed. Although functioning as a channel rather than either an importer or an

exporter, CFTR is a member of the ubiquitous exporter class of ABC proteins, which may indicate the multifunctional character of the protein (51-53).

The biophysical properties of CFTR include a low halide conductance between 6 and 10 pS. The anion permeability sequence of CFTR is Br- >Cl->I- >F-. The channel is voltage independent and has a linear current-voltage relationship (54).

Besides its cAMP-induced chloride channel function, CFTR is reported to have important regulatory functions on other ion channels and transporters.

Below some of these interactions are presented: HCO3- is conducted from the cell into the lumen (55) through reciprocal regulatory interactions between CFTR and the SLC26 chloride/bicarbonate exchanger (56, 57) and loss of this mechanism contributes to both airway and pancreatic-duct disease in CF (55, 58). CFTR enhances ATP release by a separate channel (59), not yet identified (60). This CFTR mediated release, although debated, is thought to be stimulated by hypotonic challenge to strengthen autocrine control of cell volume regulation through a purinergic receptor-dependent signalling mechanism (59, 60). Furthermore, transport of glutathione is directly mediated by CFTR, which is essential for control of oxidative stress (61).

CFTR downregulates CaCC (62), and stimulates outwardly rectifying chloride channels (63). Other channels regulated are the volume-regulated anion channel (64) and ATP-sensitive KATP channels such as inwardly rectifying outer medullary potassium channels (65, 66). The interaction between CFTR and ENaC (described in 1.1) is of vital importance for lung disease development (67). Other mechanisms mediated by CFTR are: intracellular compartment acidification (68), vesicle trafficking (69), protein processing, mucus secretion, regulation of gap junctions, aquaporins (1, 70) and tight junctions (TJ) (4)

Regulatory sites on NBD-1 interact with several of the above processes.

For example, NBD-1 contains a CFTR-specific regulatory site that downregulates ENaC. This regulatory site is also needed for CFTR-mediated interactions with other

transporting membrane proteins (1, 70, 71). In addition direct cytoskeletal interactions have been demonstrated at the extreme N-terminus of CFTR (72). Several studies also have identified a short stretch of amino acids (-DTRL-) at the COOH terminal end, forming a PDZ binding domain (1, 73-76). This PDZ binding domain interacts with different PDZ-domain-containing proteins, anchors CFTR to the cytoskeleton and stimulates the channel activities through downstream signaling elements (Fig. 1) (1, 73, 75, 77). In the last years a new role for CFTR as a chloride-induced kinase controller has been proposed, involving a cascade of protein kinases bound to the channel, with ionic concentrations acting as a signal for efficient transmission of phosphate energy (78).

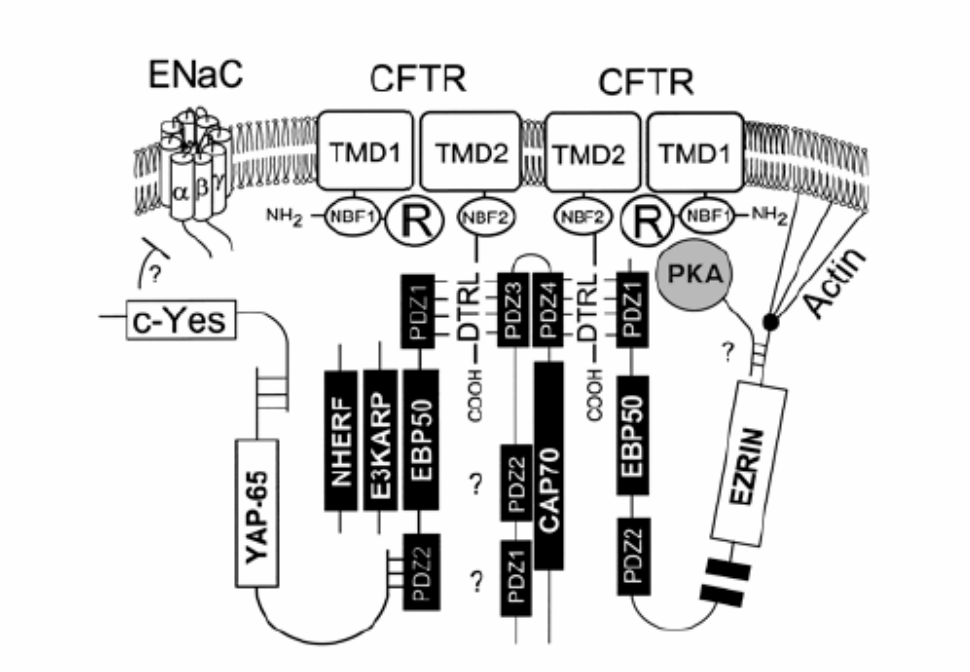

Figure 1. Schematic drawing of PDZ domain proteins regulating CFTR activity and participating in CFTR-dependent regulation of ENaC. The C-terminal end forms a PDZ-binding domain. This domain interacts with different PDZ-containing proteins, anchors CFTR to the cytoskeleton and stimulates the channel activities through downstream

signaling elements: CAP70, Na+/H+ exchange regulatory factor (NHERF), ezrin binding protein 50 (EBP50), or Na+/H+ exchange type 3 kinase. A regulatory protein (E3KARP) may be involved in regulation of CFTR activity by formation of a CFTR-CFTR dimer enhancing Cl- conductance. The figure is used by permission (1).

Like other ABC transporters CFTR contains two membrane-spanning domains (MSDs), each containing six hydrophobic α-helices (M1-M6 and M7-M12). The glycosylation site in wild-type CFTR is placed in the loop between M7 and M8 on the extracellular surface (79). Each MSD is followed by a hydrophilic nucleotide-binding domain (NBD) located at the cytoplasmic site of the protein. Since NBD1 and NBD2 are only 27% identical, the two halves (TMD-NBD) of CFTR are not symmetrical. This structural asymmetry of the two NBDs in CFTR likely accounts for the observation that only NBD2, but not NBD1, hydrolyzes ATP at an appreciable rate (80). The regulatory domain (R domain) located between the first NBD (NBD1) and the second MSD (MSD2) is unique in CFTR among ABC transporters. The domain contains several consensus phosphorylation sites for protein kinases A (PKA) and C (PKC) (28)

The MSDs assemble to form a transmembrane pore ~5.3-6Å (54) through which Cl- ions stream across the cell membrane (Fig. 2). It is presently not known how many TM domains contribute to the conduction pathway of CFTR. Indeed, it is also unclear whether a single CFTR channel is formed by a single CFTR peptide (81), or if the functional CFTR channel is constructed from a dimer of CFTR peptides. However, TM 6 with its four basic residues Lys 355, Arg 334, Arg 347, and Arg 352 is one important candidate for the formation of the pore (82-84). Other discriminating residues in TM6 are Thr 338 and Ser 341(85).

The opening and closing of the CFTR Cl- channel is tightly controlled by the balance of kinase and phosphatase activity within the cell and by cellular ATP levels (86). Activation of PKA causes the phosphorylation of multiple serine residues within the R domain. leading to conformational changes in the R domain (87) relieving its inhibitory

functions on CFTR channel gating (88). Phosphorylation of the human R domain by PKC only has a small influence on CFTR channel activity (87), but potentiates channel activation by PKA (89, 90).

Once the R domain is phosphorylated, channel opening requires binding of cytosolic ATP. NBD1-NBD2 dimerization due to hydrogen bond formation induces channel opening, whereas ATP hydrolysis at the NBDs induces dimer disruption and channel closure (47, 91) with the rate limiting step at NBD2 (92). Recently, interaction between the surface of NBD1 and a cytoplasmic loop (CL4) in the C-terminal membrane-spanning domain (MSD2) has been revealed, which is dynamically involved in regulation of channel gating (53). Finally, channel activity is terminated by protein phosphatases that dephosphorylate the R domain and return CFTR to its quiescent state (86).

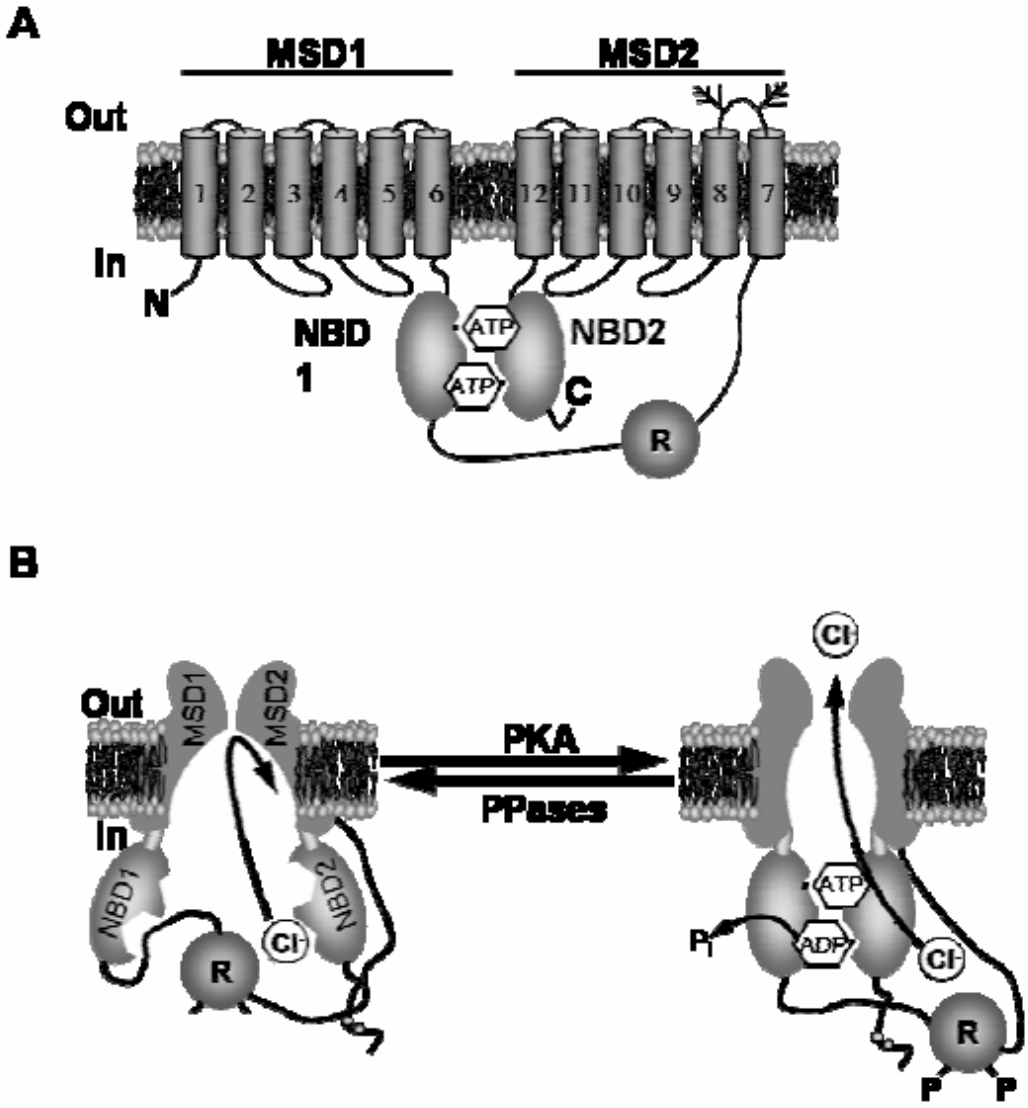

Figure 2. Schematic drawing of the regulation of CFTR by cAMP-dependent phosphorylation at the R domain and cycles of ATP binding and hydrolysis at the NBDs. Abbreviations: MSD, membrane-spanning domain; NBD, nucleotide-binding domain; P phosphorylation of the R domain; Pi, inorganic phosphate; PKA, protein kinase A; PPase, protein phosphatase; R, regulatory domain. Figure is used by permission (93).

CFTR is synthesized as an 130-150 kDa core-glycosylated protein in the ER (94) and the process takes about 10 minutes in most cell types tested (95).

Both ATP as well as association with cytosolic ER chaperones are required for CFTR to fold properly (94, 96). Such chaperones are calnexin, cytoplasmic HSC70, HSP90 and Hdj2 (97-100). Especially the activities of the isoforms HSC70 and HSP70 enhance CFTR maturation where HSP70 facilitates protein folding and HSC70 targets misfolding proteins for degradation. Wild-type CFTR is released from the chaperones and is transported from the ER to the Golgi apparatus, while HSC70 and calnexin remain bound to the mutant until degradation ensues (99, 100). In other cases, the folding and degradation targeting activities may reside in one chaperone complex. For example, HSP90 has been proposed to facilitate both CFTR maturation and degradation (97). One interpretation of these combined data is that the chaperone-CFTR complexes represent folding intermediates. This implies that multiple chaperones bind coordinately to nascent polypeptides and if one group fails to fold the polypeptide, another is readily available to target the substrate for destruction by the proteosome (96).

The process described above is highly inefficient with a majority of wt-CFTR (~75%) and about 99% of ΔF508 CFTR not meeting the quality control requirement of the ER quality control machinery, where different ubiquitin ligases act sequentially in the ER membrane and cytosol to monitor the folding status of CFTR and _F508-CFTR (95). The fraction that successfully has passed all steps in the ER is exported to the Golgi complex where it undergoes N-glycosylation and becomes a fully mature 170-180 kDa protein (94). Once in the cell membrane, CFTR has a relatively fast turnover with a half-life exceeding 48 hours for wt CFTR but only 4 hours for ΔF508-CFTR (101).

The protein is recycled by endocytosis or degraded by lysosomal proteinases.

Based on their effect on protein synthesis and function, mutations in CFTR are divided into six classes:

I Unstable mRNA production due to nonsense, frameshift and splice site mutations. No protein is synthesized.

II Abnormal CFTR processing and trafficking. Most CFTR mutations including ΔF508 belong to this class.

III Defective CFTR regulation, i.e., failure to respond to cAMP, but with normal trafficking. The dysregulation leads to decreased net Cl-transport through the pore.

IV Defective conductive properties but with a normal response to cAMP regulation.

V Reduced synthesis of normal CFTR due to incorrect splicing. Normal splicing can still occur in a small proportion of these transcripts and, as a consequence, a small amount of functional CFTR chloride channels will be generated.

Some mutations have features of more than one class of mutations. For example, ΔF508, the most frequent CFTR mutation, causes an arrest in maturation (class II) and defective regulation of the channel (class III) (42). The mutation classes III, IV and V result in a milder phenotype (42, 102).

Cross-species analysis shows significant conservation in structure between several vertebrate species, especially for some of the transmembrane domains, the region around human amino acid 508 at the NBD1, ATP binding loops at NBD1 and NBD2 (103), the phosphorylation sites of the R-domain, the extreme N-terminal residues (amino acids 1–25) and glycosylation sites and sequences within non-coding regions (possibly regulatory elements). Such conserved structures imply functional importance of the channel in evolutionary diverse organisms (72, 104, 105). It also argues in favor of using animal models in the study of cystic fibrosis, however, keeping in mind the tissue- and species-specific expression of the channel (30, 31, 104, 105).

1.1.4 CFTR inhibitors

Different CFTR inhibitors have been used extensively to block CFTR chloride-channel function in various cell culture, tissue and in vivo systems.

A traditional blocker is glibenclamide (106). Recently a more specific inhibitor, the thiazolidinone CFTRinh-172, has been identified (107) (Fig 3.).

This compound acts on the cytoplasmic side of the plasma membrane to block CFTR chloride conductance with a Ki value of ~300 nM. Patch-clamp analysis indicated a voltage-independent channel blocking mechanism with prolongation of mean channel closed time (108). This involves an interaction with arginine 347, which is located at the intracellular end of the CFTR pore (109). CFTRinh-172 has low toxicity, and undergoes renal excretion with minimal metabolism (110). One drawback is a limited water solubility; however, recent structure-activity studies have identified thiazolidinone CFTR-inhibitors that are more water soluble than CFTRinh-172 (111).

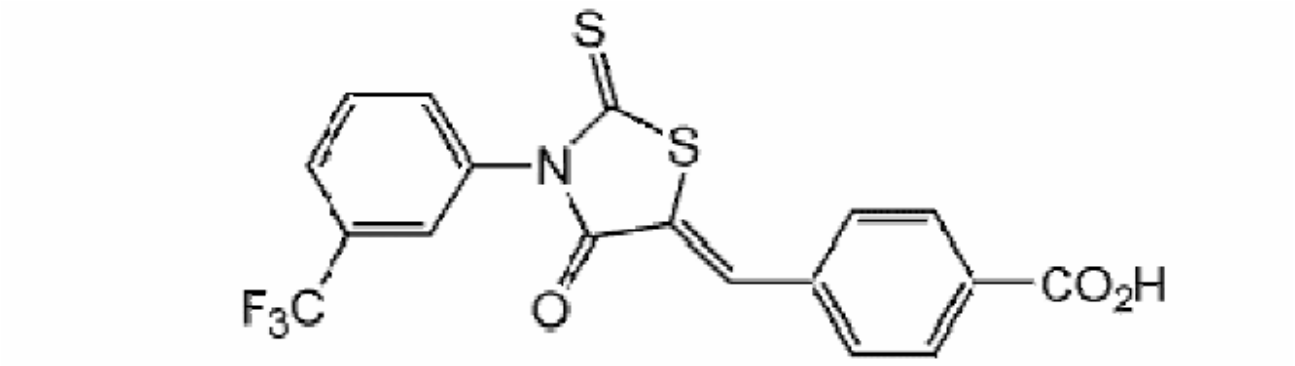

Figure 3. 3-[(3- trifluoromethyl)phenyl]-5-[(4-carboxyphenyl) methylene]-2-thioxo-4-thiazolidinone (CFTRinh-172), a specific inhibitor of CFTR (111)

1.2 Airway epithelium

The airway epithelium is a target for potentially noxious substances and pathogens. It plays a critical role in maintaining a sterile undamaged airway and also separates the connective tissue as well as the smooth muscle from the airway luminal contents. In addition to its barrier function, the airway epithelium has a regulated fluid and ion transport together with a secretory function, although its function is mainly absorptive (112). It can produce mucus, release mediators of the immune system such as lysozyme, lactoferrin, mucous glycoprotein, immunoglobulins, chemokines, cytokines, lectins and β-defensin (cationic antimicrobial peptides) (113-115).

Furthermore, it has a production of antioxidants such as glutathione and ascorbic acid (116). Aside from these protective functions there is also a regulation of the airway physiology via production of smooth muscle relaxant factors such as prostaglandin E2 and NO and enzymes, which catabolise smooth muscle contractile agonists (117, 118).

In normal human airways the surface epithelium is on average 50 μm thick and rests on a basement membrane (119, 120). The epithelium in the major bronchi and proximal bronchioles is ciliated pseudostratified with the main cell types: ciliated and secretory columnar cells, and underlying basal cells. In addition, immune cells, inflammatory cells and phagocytic cells migrate to and remain within the epithelium (114). More distally, in the terminal bronchioles, the epithelium changes towards a simple ciliated columnar and, finally, to simple cuboidal epithelium with ciliated and non-ciliated cells (Clara cells) (121). In addition brush cells (columnar with microvilli only) have been identified in the respiratory tract from nose to alveoli (122).

The ciliated (fluid-secreting) *columnar cells* are the predominant cell type within human airways accounting for over 50% of the epithelial cells. Each cell has about 300 cilia on its apical surface (123) with an average length of ~6 μm (124). The main function of the motile cilia is to provide a directional transport of mucus from the lung to the throat

(114). The ciliated cells are found down to the terminal bronchioles and they usually, like other columnar cells, reach the basal lamina by extending slender processes (125).

The secretory cells (goblet, serous, Clara, neuroendocrine and presecretory cells types) comprise 15-25% of the bronchial and nasal epithelium and are primarily involved in the production of airway secretion.

Goblet cells, interspersed among the ciliated cells, secrete high molecular weight mucopolysaccharides (mucus) (118, 126). The two major airway mucins MUC5AC and MUC5B are secreted predominantly by goblet cells and submucosal glands, respectively (127). Clara cells have a role in surfactant production and detoxification.

The pyramidal-shaped *basal cells* may serve to anchor the columnar cells to the basal lamina, as well as being precursor of columnar and secretory cells. They form desmosomes with the columnar cells and are themselves connected to the basal lamina via hemidesomsomes (118, 128).

At present, the function of pulmonary *brush cells* only can be guessed (122). Unmyelinated nerves found in association with the airway brush cells and possibly also with the alveolar brush cells (129), suggest a chemoreceptor function for these cells. On the basis of their high concentration at first alveolar duct bifurcations, it also has been suggested that they may play a role in detoxification or act as a sensor for alveolar fluid or alveolar air tension (130). Another possible function for brush cells that arises from their location in the lung is immune surveillance (122).

In CF there is a picture of airway epithelial injury (131) and remodeling, such as squamous metaplasia (132), cell hyperproliferation (133), basal and goblet cell hyperplasia, airway epithelial height increase (134), and hypersecretion of mucus due to the inflammatory profile (135-138). The epithelial regeneration characterized by successive steps of cell adhesion and migration, proliferation, pseudostratification, and terminal differentiation is disturbed and characterized by delayed differentiation, increased proliferation, and altered pro-inflammatory responses (139).

It has been widely accepted that acinar gland serous cells are the predominant site for CFTR expression in the human large airways, arguing for a dominant role of submucosal glands in ASL volume regulation and CF (140-144). However, these findings have later been debated. It has been demonstrated that normal (but not the ΔF508 mutation) surface airway epithelia express CFTR in every ciliated cell, also in glandular ducts, with decreased expression towards the distal airways. This suggests a key role for the superficial epithelium in the initiation of ASL volume depletion and as the site for early disease. (145). It also supports a role for CFTR in regulating glandular secretion homeostasis, but predominantly in the submucosal ducts rather than in the serous acini as was earlier proposed.

1.2.1 Ion and water transport in airway epithelium

Net vectorial fluid transport depends critically on ENaC and CFTR operating in concert with the paracellular and transcellular pathways. CFTR is also directly connected to the cytoskeleton through the N-terminus (72) and indirectly through a PDZ-binding domain at the C-terminus (1, 73-76).

Fluid absorption is mainly controlled by the transport of Na+ through apical ENaC, which is also the dominant basal ion transport process (146). *Fluid secretion* is regulated by cell-to-lumen movement of Cl-, via CFTR, CaCC and volume regulated chloride channel, and /or HCO3- via the interactions between CFTR and the SLC26 channel. In both cases the transport occurs along the electrochemical gradient and the movement of counterions likely takes place predominantly through leaky TJ (4). Over the basolateral membrane a Na+ gradient is maintained by the Na+-K+-ATPase, which pumps 3 Na+ ions out of the cell for every 2 K+ ions coming in. As a result the intracellular concentration of Na+ is low (20 mM), whereas the K+ concentration is high (150 mM) (147). In addition, the Na+-K+-2Cl- co-transporter moves Cl- against its electrochemical

gradient and accumulates Cl- inside the cell to be released via apical channels. Secretion of Cl- is electrically coupled to efflux of K+ through basolateral K+ conductance channels (148). Through the paracellular pathway, Cl- is absorbed or Na+ secreted and the water-flow is regulated by diffusion following osmotic gradients.

The maintenance of the electro-osmotic gradients is dependent on limiting back diffusion. The tightness of the paracellular barrier and the molecular selectivity together contribute to the overall epithelial transport characteristics (Mitic et al., 2000). In many epithelia the transport of different ions is performed by different cell types, however, in airway epithelia the ciliated cell is responsible for both secreting Cl- and absorbing Na+ (149).

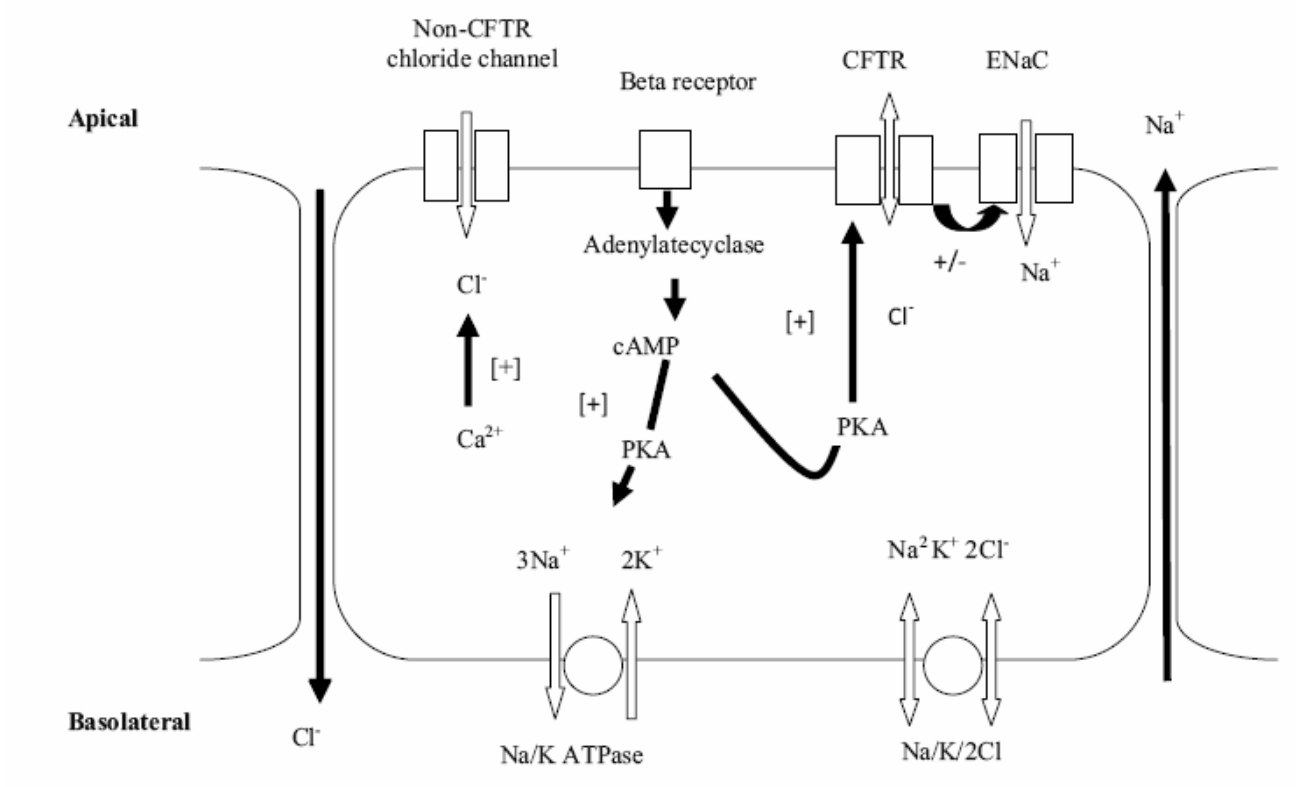

Figure 4. Ion transport in the airways

1.2.2 Tight junctions

The key function of TJ is to limit and control the paracellular permeability and to form a selective permeable barrier between the apical and basolateral compartments of the extracellular space (159). This specific barrier function can be characterized and quantified as transepithelial electrical resistance (TEER) as well as charge selectivity (160). Furthermore, they have a fence function restricting the movement of membrane proteins, thus creating a distinction between ion channels or receptors in the apical and basolateral membrane and maintaining cell polarity.

TJ are located in the most apical part of the cell and form a continuous belt-like region (100-800 nm deep) of intimate contact between the plasma membrane of adjacent cells (i.e., "membrane kisses"). The belt-like regions act as a seal where the number of strands and complexity of the network are correlated to TEER and thus the permeability of the epithelium. There is a substantial physiological variation in the "tightness" of TJ between different cell lines (161). It has been suggested that a minimum cut off value of TEER should be set for each cell monolayer, above which threshold the TJ are considered fully occluded (162).

The scaffold of the junctions is formed by three members of the membrane-associated guanylate kinase (MAGUK) homologue protein family (163), namely ZO-1, ZO-2, and ZO-3. Each of these scaffold proteins contains a sequence of protein-binding domains, including three Postsynaptic density proiten-95, PSD-95; Discs large, (Dlg); tumor suppressor A; Zonula occludens-1 protein (PDZ) domains, an Src homology 3 (SH3) and a guanylate kinase (GUK) domain (164). ZO-1, ZO-2, and ZO-3 connect to actin filaments, where they assemble a wide variety of structural and regulatory proteins via interaction with the PDZ domains. Such proteins are kinases and G-proteins together

with membrane proteins, which are essential for a proper function of the junction (165). Activation of pathways modulating the phosphorylation pattern of cytoskeletal proteins associated with TJ are important for assembly and remodeling of junctional structures and could change their permeability (166, 167)

The membrane proteins are the junctional adhesion molecule (JAM), occludin and claudin (168-170). Claudins and occludins form the strands of the TJ but claudins are the only junctional proteins known to have tissue specificity (164, 171). The physiological variation in the "tightness" of TJ between different cell lines may possibly be explained by this tissue specificity (172, 173). Occludin, the regulator protein of the permeability of TJ is controlled by phosphorylation (174, 175). It is also involved in the fence function (171). Incorporating different mixtures of claudins and occludins results in a flexible and regulatable scaffold where pores of varying size and charge properties can be assembled. The reported pore sizes vary widely (165). In epithelia most pores are in the range of 8–9 Å in diameter, which is much larger than are transmembrane pores and channels (176). JAM belongs to the immunoglobulin superfamily (177). This protein is involved in the formation of cell polarity through the polarity protein Par-3, which in turn forms a complex with Par-6 and atypical protein kinase C (aPKC) (178).

The formation and maintenance of TJ are dependent on the cell-cell adhesion activity of E-cadherin and nectin (178). The connection between TJ and E-cadherin involves Ca2+, which is essential to maintain intercellular contacts and is important for the stability of mature TJ. Removal of extracellular Ca2+ causes the opening of previously formed TJ and prevents *de novo* formation (179). E-cadherin is linked to actin through peripheral membrane proteins such as α- and β-catenin, p120- catenin, α-actinin, and vinculin (180-183), that modulate the formation and maintenance of the epithelial junctional complex (182). Nectin is involved in the localization of JAM at TJ and co-localizes with E-cadherin where it associates with the actin cytoskeleton through the adaptor protein afadin (178). Formins are recruited by α-catenin to adherens junctions and have the

ability to increase the rate of actin filament elongation and mediate the active interplay between actin filaments and microtubules (184)

Inhaled antigens and resident inflammatory cells release mediators that can alter the TJ (185, 186). Also hyperosmolar solutions can alter the TJ (187)

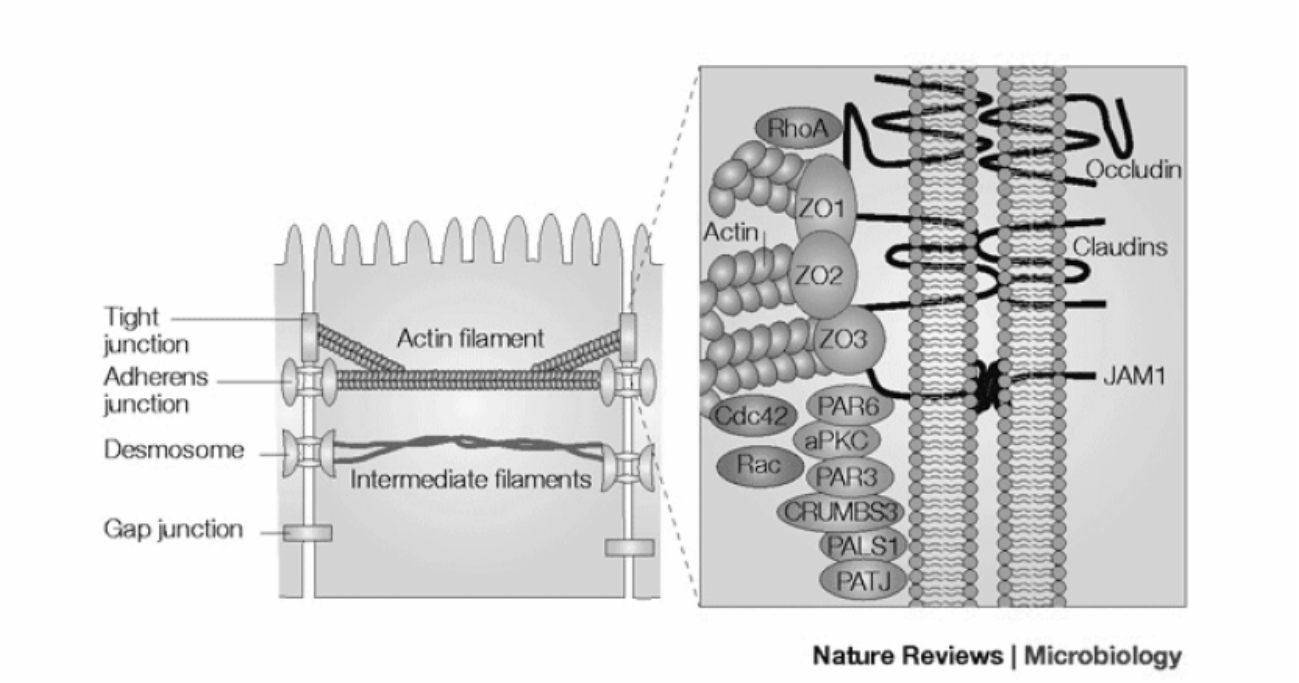

Figure 5 Tight junctions constitute the main barrier function of the epithelium and are connected with the actin cytoskeleton. Three types of transmembrane proteins are part of the tight junctions: occludin, claudins and JAMs, and they in turn are connected to adaptor proteins such as ZO-1, ZO-2, and ZO-3.

1.2.3 Airway surface liquid

The fluid covering the airway epithelium, the airway surface liquid (ASL), consists of a periciliary layer (PCL), which is a watery layer surrounding the cilia, and of mucus on top of the cilia. The PCL is produced by the serous cells. Mucus is produced mainly by the submucosal glands, while a small amount is produced by the goblet cells. In normal airways PCL height is defined as the length of an outstretched cilium (~6 μm) (124), whereas the ASL layer (mucus plus PCL) varies in thickness of 20-150 μm for different species (20-58 μm in humans) (188). ASL is the first line of defense against inhaled pathogens and is important for mucociliary clearance. It contains e.g., mucins, phospholipids, albumin, lactoferrin, lysozyme, proteases, defensins and other peptides, ions and water (189), see also 2.1. The composition, volume and physical properties of the ASL depend manly on secretions of the airway submucosal glands and the absorptive properties of the surface cells.

Regulation of the balance between absorption and secretion determines the net transport of ions across the epithelium through transcellular and paracellular pathways and, thus the mass of salt on an epithelial surface. (190)

There are several hypotheses about how defective CFTR leads to the airway disease:

- *The low ASL volume hypothesis* claims that the ASL is isotonic both normally and in CF (191). CFTR functions both as a Cl_ channel and as an inhibitor of the ENaC (inversely related communication). In CF airway epithelia, with an absence of either molecular or functional CFTR, there will be unregulated Na+ absorption and a decreased capacity to secrete Cl_. This leads to dehydration of the airway surface, with a collapsed PCL, concentration of mucins within the mucus layer, and adhesion of mucus to the airway surface (192).
- *The high salt hypothesis* suggests that that the ASL normally is hypotonic (193) and provides an optimal environment for defensins, According to this view the

ASL in CF patients would have a higher salt concentration than normal because the absorbing function of ENaC depends on the state of CFTR and cannot be activated where CFTR is defective or absent (parallel related communication) (12, 193).

- *The low pH hypothesis* focuses on the interactions between CFTR and the SLC26 and proposes an acidic ASL. This may compromise the function of airway immune cells and increase toxic oxidant species. Lowering the pH may also eliminate electrostatic repulsive charges between organisms and facilitate "tighter" biofilm formation as well as reduce electrorepulsive forces between bacteria and negatively charged mucins. Furthermore, ciliary beat frequency in bronchial epithelium is reduced when external pH falls (194). All the above factors may inhibit mucociliary clearance (MCC) and the clearance of bacteria (195, 196).
- *The low oxygenation hypothesis* postulates that the oxygen content of the ASL is low, due to build-up of mucus plugs, resulting in enhanced growth of the facultative anaerobic *Pseudomonas aeruginosa* (197).
- *The defect gland function hypothesis* suggests that the primary defect in CF is reduced fluid secretion by airway submucosal glands and possibly altered secretion of mucous glycoproteins (198).
- *The soluble mediator hypothesis* proposes that signalling molecules within the ASL itself are controlling ASL volume (14, 199). These molecules are ATP, which is breathing- or shear-stress induced (15, 16), and adenosine. ATP interacts with receptors such as the purinergic P2Y2 receptors and adenosine reacts with the adenosine A2b receptors, that mediate inhibition of ENaC and activation of both CFTR and CaCC (11, 14, 17, 18). This mechanism is also supposed to include PDZ interactions and cytoskeletal elements (1).

An interesting question is, what the role of aquaporins (AQP) is in the production of ASL, compared to paracellular water flow and CFTR. In the epididymis, CFTR appears to regulate AQP-mediated water permeability (200). In this tissue, CFTR is co-localized with AQP9 in the apical membrane, and this association promotes the activation of AQP9 by cAMP (201). In a heavily debated study, concerning the clinical benefit of nebulized hypertonic saline in cystic fibrosis, an important role of amilorideinhibitable AQP water channels in the generation of ASL was proposed (202). However, although the positive effect of hypertonic saline as such is not disputed, the question whether this effect is mediated by AQP has received conflicting answers (203, 204) and is still open. Recently, is has been found that interleukin-13 (IL-13) enhances the expression of CFTR but abolishes the expression of AQP in airway epithelial cells (205)(Skowronzwarg et al., 2007). In conclusion, the relation between CFTR and AQP needs further study.

The differences of opinion are due to difficulties in determining the accurate composition of the ASL because of the very small depth of the layer. Among the problems encountered are difficulties to collect an adequate amount of ASL without disturbing the epithelium and inducing secretion from submucosal glands or leakage of interstitial fluid into the lumen, which may modify the composition of the ASL (206). Furthermore, the importance of fluid secretion by submucosal glands differs markedly between mammalian species. For example, in transgenic mice that serve as animal models for CF, the fluid transport in the airways is much less affected than in CF patients. It is also possible that variant forms of ENaC or different regulatory components operate in different systems (12).

In line with the above-described difficulties, published data on the composition of the ASL are divergent and vary between species and also within a single species (depending on the collection technique). For mouse the variations are very large from strongly hypotonic, 6mM Na+ and 1 mM Cl- (207) to values in the isotonic range (~115-120 mM

Cl-) (208-210). For monkey and rabbit, near isotonic values have been reported (211). This was also the case for human ASL (211, 212). Hyperosmotic values were found in dogs (158 mM Na+ and 134 mM Cl-) (206). Tracheal or bronchial ASL has been collected both from humans and from other species, mainly from mouse. Different techniques for sampling and analysis have been used and it appears that the main source of variation is the different ways of sampling.

Research has been performed on cell cultures of airway epithelial cells as well. In a study by Matsui et al. (192) no evidence was found that liquids lining the airway surface were hypotonic or that salt concentrations differed between CF and normal cultures. However, an earlier study by Smith et al.(27) had shown that epithelial surface fluid in primary cell cultures from CF patients had higher salt concentrations than healthy cultures.

1.3 Model systems

1.3.1 Mouse models of CF

Gene targeting based on homologous recombination of the cloned and mutated murin *CFTR* gene have made it possible to generate CF mice (233-237).

Most CF mutations result in loss of function due to abnormal processing of CFTR and failure to insert CFTR in the plasma membrane. Therefore generating animals that mimic these forms of defects is of importance (235).

The importance of creating ΔF508 mice is that this mutation has the highest prevalence, about 70% (40). The murine CFTR gene is 78% homologous to its human counterpart and ΔF508 occurs in the same position in both species (238). Created ΔF508 mice models (234, 239, 240) exhibit the same CFTR processing defect as in humans.

Several studies have revealed high levels of CFTR mRNA in murine duodenum, jejunum, ileum, cecum and colon and it is well known that mice develop typical gastrointestinal symptoms similar to meconium ileus in human CF infants (233, 234). However, the absence of dramatic pathological alterations in the pancreas of CF-mice suggests that CFTR may play a smaller role in normal pancreatic function in mice than in humans. This is in agreement with low levels of CFTR mRNA expressed in the murine pancreas (233, 234)

Although it has been reported that long-living animals develop manifestations of CF-like disease in all organs that are pathologically affected in the human form of CF (241), it is generally accepted that CF mice do not develop the severe lung pathology in the lower airways to the same extent described in humans. It seems that CFTR does not play an important role in distal murine airways since the expression of CFTR is low (235). Deficient CFTR-mediated Cl- secretion is instead compensated by upregulation of CaCC (242). However, functional analysis of the ion transport in different regions of the airways in mice revealed that Na+ hyperabsorption and cAMPdependent Cl- secretion as well as the associated phenotype with depletion of ASL is seen in the upper airways. Thus murine nasal mucosa appears to be an excellent model for human CF airway tissue (235).

1.3.2 Transformed cell lines

Cultured well-differentiated primary epithelial cells have the benefit of flexibility, control of experimental conditions, and greater opportunities for interventions compared with *in vivo* studies. Different culturing methods are well described (243, 244). However, these cultures are difficult to handle, e.g., there might be limited availability of cells, significant variability between donors and a limited lifespan. Therefore, several

continuously growing cell lines have been developed. These cell lines have been derived from carcinomas or have been transformed using viral genes (245-248).
Although these cell lines are valuable, several, but not all, of them show limitations including differences in morphology and the lack of important functions such as functional tight junctions (249).

1.3.2.1 16HBE14o- cells

The cell line 16HBE14o- (expressing wt CFTR) is a postcrisis SV40 large Tantigen transformed epithelial cell line derived from normal human bronchiolar epithelium. The transformation was accomplished by using the replication defective pSVori- plasmid (246). When grown under submerged conditions (i.e., growth medium added both apically and basolaterally) 16HBE14o- cells form layers with a height of 1-5 cells comparable with bronchial epithelium *in vivo* and form well-defined TJ, adherence and gap junctions (250). The cell layers have directional ion transport, generate a well-developed transepithelial electrical resistance (TEER) and maintain cAMP- and Ca2+-dependent chloride currents (246). They are also known to bind lectins specific to bronchial epithelial cells (251). Because of the wellconserved phenotype the cell line is used as a model for bronchial epithelial cells *in situ* in studying different pathological and physiological conditions (250).

1.3.2.2 CFBE41o- cells

The CFBE41o- cell line was generated by the transformation of bronchial epithelial cells obtained from a CF patient. As in the 16HBE14o- cultures the transformation was accomplished with SV40 large T antigen. CFBE41ocultures have often been used in CF research (252-255). The cell line is homozygous for ΔF508 over multiple passages in

culture, where the localisation of the CFTR protein is mostly intracellular indicating the retention of mutant CFTR in the ER. Furthermore, CFBE41o- form electrically tight cell layers with functional cell-cell contacts and microvilli, when grown under immersed (but not air-interfaced) culture conditions (249).

It will, however, been shown in this thesis that the less than optimal performance with regard to the formation of functional cell-cell contacts under air liquid interphase conditions, reported above, is not in agreement with findings in our laboratory. We found that the CFTE41o- cells in air liquid interphase developed cilia, which implies fully developed polarity of the cell and functional cell-cell contacts (paper III).

Both 16HBE14o- and CFBE41o- express a number of proteins relevant for pulmonary drug absorption. Such proteins are for example Pglycoprotein P-gp (MDR1), lung resistance-related protein (LPR), and caveolin-1 (cav-1) (249, 256).

1.3.2.3 CFBE41o pCep4 cells

16HBE14o- cells are often used as healthy controls for the CFBE41o- cells.

However, these two cell lines do not originate from the same subject and therefore are not fully comparable (249). Instead the generation of a stable CF airway epithelial cell line complemented with a wtCFTR DNA construct may serve this purpose. CFBE41o pCep4 cells are CFBE41o- cells transfected with an episomal expression vector, which contains a full-length (6.2kb) wtCFTR cDNA and a hygromycin B resistance (HYGRB) gene. In these constructs, cAMP-stimulated CFTR Cl- currents are present at a significant level. However, the expression of apical wtCFTR channels per cell is lower than in 16HBE14o- cells (257).

1.4 Mesenchymal stem cell from human placenta as source for CF therapy

The placenta is a fetomaternal organ consisting of 2 components: the maternal component, termed the decidua, originating from the endometrium, and the fetal component, including the fetal membranes–amnion and chorion–as well as the chorionic plate, from which chorionic villi extend and make intimate contact with the uterine decidua during pregnancy.

Since the first studies on placenta-derived stem cells were reported, it has been recognized that, due to the complexity of the placental structure, there is a need for assigning proper terminologies to the different regions of this organ and to the various cell types that can be isolated from these regions. To this end, as reported by Parolini et al. [16], the First International Workshop on Placenta-Derived Stem Cells saw the following nomenclature proposed:

- human amniotic epithelial cells (hAEC),
- human amniotic mesenchymal stromal cells (hAMSC),
- human chorionic mesenchymal stromal cells (hCMSC),
- human chorionic trophoblastic cells (hCTC).

Furthermore, isolation protocols, phenotypic markers, and in vitro differentiation potential have been described for hAEC, hAMSC, and hCMSC [16].

Characterization of hAEC has shown that these cells express molecular markers of pluripotency, and can differentiate in vitro into cell types of all 3 germ layers [16].

More recently, comparative analysis of hAEC from amnion of early-stage pregnancies and from term amnion showed that expression of the stem cell-specifi c cell surface markers TRA1-60 and TRA1-81, and of the molecular markers of pluripotency Nanog and Sox2, are all significantly higher in fetal amnion, while expression of Oct-4 mRNA is similar between cells obtained either from fetal or term amnion [20].

hAMSC and hCMSC are defined as plastic-adherent cells that are capable of forming fibroblast colony-forming units and displaying a specific pattern of cell surface antigens comparable to that of bone marrow MSC ($CD90^+$, $CD73^+$, $CD105^+$, $CD45^-$, $CD34^-$, $CD14^-$, $HLA\text{-}DR^-$). These cells are also capable of differentiating toward one or more lineages including osteogenic, adipogenic, chondrogenic, and vascular/endothelial [16]. Furthermore, recent reports suggest that, like the amniotic epithelial fraction, the human amniotic mesenchymal region also harbors a multipotent side population showing multilineage differentiation potential [21].

Recent advances regarding the differentiation potential of placenta-derived cells have shown expression of major cartilage components by hAMSC after chondrogenic induction, with deposition of collagen II after implantation of these cells into the subfascial space of the abdominal muscle of mice [22]. Mesenchymal cells from the amniotic and chorionic membranes have also been recently shown to differentiate in vitro into a range of neuronal and oligodendrocyte precursors [23]. In addition, use of amniochorionic membrane as a scaffold has been proposed for improving osteogenic differentiation of chorionic membrane-derived cells (Mohr S. et al., submitted 2009). Meanwhile, it has been recently shown that hAEC display bifunctional hepatic differentiation potential in vitro, with the ability to differentiate into both parenchymal hepatocytes as well as cells expressing a molecular marker profi le consistent with biliary cells and which form tubular 3D structures reminiscent of bile ducts when cultured on extracellular matrix [24]. However, discrepant results have been reported for osteogenic and adipogenic differentiation of hAEC and hAMSC [25, 26], indicating the heterogeneous nature of these cell populations, and highlighting the need to develop better methods for selecting progenitor cells from placental tissues.

In exploring placental tissues as a source of progenitor cells, researchers have focused their attention mainly on cells derived from fetal tissue, in particular from amnion, chorion, and umbilical cord [16, 27, 28]. However, stem/progenitor properties of

placental cells of maternal origin have also been described [29]. Recently, a comparative phenotypical study between bone marrow- and placenta-derived mesenchymal cells has underlined the fact that the cell types have a very similar cell surface marker profile; however, they differ in their expression of the chemokine receptors CCR1 and CCR3 [30], which are only present on placenta-derived cells; meanwhile, other molecules such as CD56, CD10, and CD49d have been shown to be more highly expressed on placenta-derived mesenchymal cells [31], and differences in proliferation potential have been also observed between these 2 cell types [29].

However, based on the lack of significant differences between these above-mentioned cell types, as well as the fact that placental cells are plentiful and easily procured, a good manufacturing practice (GMP)-compliant facility has been established for isolating and expanding human placenta-derived MSC in a first clinical trial setup [32].

As a potential source of MSC, placental tissues have also attracted recent interest in the hematopoiesis field. During mouse embryogenesis, the placenta has been newly unveiled as an important niche for hematopoietic stem cell (HSC) development, although the origin of placental hematopoiesis remains undefined. Recent advances in this field come from a study using Ncx1 −/− embryos lacking a functional heart and circulation. Rhodes and colleagues [33] found hematopoietic progenitors with both myeloerythroid and lymphoid potential in the placenta of these embryos, as well as in dorsal aorta, yolk sac, and vitelline vessels, indicating that these cells arose in situ. Meanwhile, the potential role of human placenta in embryonic hematopoiesis has also been recently documented, together with development of procedures for processing and storing hematopoietic cells from placental tissue [34 , 35]. Specifically, $CD34^{+}CD45^{dim}$ cells isolated from human placenta were shown to contain myeloid and erythroid progenitors [34 , 35] and were capable of generating $CD56^{+}$ natural killer cells and $CD19^{+}CD20^{+}sIgM^{+}$ B cells in vitro [34]. More importantly, human placenta has been hown to contain bona fide HSCs throughout fetal development [36]. HSCs can be

detected as early as gestational week 6 (either $CD34^+$ or $CD34^-$), and most strikingly at term. At weeks 16–20, HSCs are more enriched in the CD34 + fraction.

After storage in liquid nitrogen, the placental cells retain their HSC potential, suggesting this tissue as an important source for banking and clinical application [36].

Recent efforts have also been dedicated to optimizing isolation, culture, and preservation methods for placenta-derived cells. These include a study aimed at defining cell yields obtainable from the amniotic epithelial and mesenchymal regions [26], while others have proposed long-term expansion methods to allow thorough analysis of cellular material before use in cell-based therapies. Immortalized hAMSC have been established through ectopic expression of the human telomerase catalytic subunit, and compared to the parent population, the resultant cells displayed unaltered surface marker profile, morphology, karyotype, and differentiation potential for up to 87 population doublings, as well as similar or reduced immunogenic/immunosuppressive properties [37]. Alternatively, it has also been shown that in vitro life span can be dramatically extended by optimizing expansion conditions. For example, when cultured with animal-free culture supplements such as human platelet lysate (PL), a suitable alternative to fetal calf serum (FCS) for MSC cultures [38 , 39], hAMSC have been shown to exhibit an increased proliferation potential and in vitro life span compared to cells cultured with FCS (Wolbank, unpublished data). However, although different culturing methods may influence cell behavior, it should be noted that in vitro culture itself can also cause some alterations, as highlighted by a study in which hAEC displayed reduced osteogenic potential associated with a phenotypic shift after culturing [25].

Finally, recent attempts to improve cryopreservation of placenta-derived cells have demonstrated that vitrification, which uses a high cryoprotectant concentration and does not require a programmable temperature-decreasing container, represents a fast preservation method. This has proved reliable and effective for long-term preservation

of hAMSC, showing retention of surface marker expression and differentiation potential after thawing [40].

In summary, since the first studies on placenta-derived stem cells were published, much knowledge has been gained regarding the characteristics, handling methods, and potential of these cells. However, to maintain uniformity and clarity in the field, precise descriptions of the placental regions from which different cell populations are isolated are paramount, along with extensive phenotypic analyses of these cells.

1.4.1 Immunomodulatory Properties of Placenta-Derived Cells

One of the most intriguing features of MSCs is that they escape immune recognition and can inhibit immune responses [13].

1.4.1.1MSCs modulate T cell proliferation and function

Numerous studies have demonstrated that MSCs can suppress the T lymphocyte proliferation induced by alloantigens, mitogens and anti-CD3 and anti-CD28 antibodies *in vitro* in humans, baboons and mice [14–20]. MSCs have a similar effect on memory and naive T cells [20], as well as CD4+ and CD8+ T cells [21], of a murine model. In addition, this suppressive effect did not require major histocompatibility complex (MHC) restriction and could also be mediated by allogeneic MSCs [15,20]. This effect may be attributed to the inhibition of cell division, which is evidenced by the accumulation of cells in the G0/G1 phase of the cell cycle [21].At the molecular level, cyclin D2 expression is down-regulated, whereas p27 expression is up-regulated; this may explain why T cell proliferation, rather than activation, and interferon (IFN)-g production are affected by MSCs[21]. Inhibition of T cell proliferation by MSCs appears

to be mediated by both cell–cell interaction [17,22,23] and release of soluble factors such as IFN-g and interleukin (IL)-1b [24,25]. Some studies have indicated that soluble factors are essential for enhancing the suppressive effect of human MSCs, while the effect of rodent MSCs is mediated by cell–cell contact [14,17,20,26].

Transforming growth factor (TGF)-b1, hepatocyte growth factor (HGF) [14], indoleamine 2,3-dioxygenase (IDO) [27] and prostaglandin E2(PGE2) [28] represent MSC-derived molecules that are believed to have immunomodulatory activity on T cell responses. Neutralizing antibodies against TGF-b andHGFcan restore the MSC-induced suppression of T cell proliferation [14]. Treatment with IFN-g causes MSCs to express the protein IDO and exhibit functional activity of IDO, which in turn degrades essential tryptophan and results in kynurenine synthesis and thereby suppresses lymphocyte proliferation [27].Co-culturing T cellswith MSCs resulted in elevated levels of PGE2,and treatmentwith inhibitors of PGE2 production mitigated the MSC-mediated immune modulation [28]; however, the mechanism underlying the immunosuppressive effect of PGE2 is poorly understood. The production of nitric oxide (NO) by MSCs has also been implicated as a potential mechanism by whichMSCsinhibitT cell proliferation [29].NO inhibits the proliferation of T cells by suppressing the phosphorylation of signal transducer and activator of transcription-5 (STAT5), a transcription factor crucial for T cell activation and proliferation [30] (Fig. 1). Ding *et al*. reported that matrix metalloproteinases (MMPs), in particularMMP-2 andMMP-9 secreted by MSCs, mediate the suppressive activity of MSCs via reduction of CD25 expression on responding T cells in amodel of allogeneic islet transplant [31]. The secretion of human leucocyte antigen-G5 (HLA-G5) by MSCs is reported to be essential for the following effects of MSCs: suppression of T cell and NK cell function, shift of the allogeneic T cell response to a T helper type 2 (Th2) cytokine profile [32] and induction of CD4+CD25highforkhead box P3 (FoxP3+) regulatory T cells (Tregs) [33].

MSCs do not express MHC class II and co-stimulatory molecules such as CD80, CD86 or CD40 [9,17,34], and it is believed that T cell activity may result in anergy, which is reflected as immune tolerance. Le Blanc and co-workers reported that when MSCs are treated with IFN-g, which is up-regulated in inflammation, they express MHC class II [15]. In an experimental arthritis model, MSCs decreased antigen-specific Th1/Th17 cell expansion and decreased the production of cytokines released by Th1/Th17 cells, such as IFN-g and IL-17, and caused the Th2 cells to increase production of IL-4 [20,28,35] and IL-10 in lymph node joints [36]. T cell inhibition by MSCs is not due to the induction of apoptosis, but by the inhibition of cell division and probably by the production of soluble factors [14]. However, a recent study reported that MSCs could induce apoptosis in activated T cells [CD3(+) and bromodeoxyuridine (BrdU)(+)], but not in the resting T cells [CD3(+) and BrdU(-)]; this leads to marked attenuation of delayed-type hypersensitivity (DTH) response *in vivo* by inducing NO production [37].
Moreover, MSCs can inhibit the cytotoxic effects of antigenprimed cytotoxic T cells (CTLs) [16] by suppressing the proliferation of CTLs, rather than by direct inhibition of cytolytic activity [26,38].
A recent study showed that the negative co-stimulatory molecule B7-H4 was involved in the immunosuppressive effect of MSCs on T cell activation and proliferation via induction of cell cycle arrest and inhibition of the nuclear translocation of nuclear factor (NF)-kappa B [39]. Some studies revealed that the absence of T cell response in the presence of MSCs was transient and could be restored after the removal of MSCs [14,20]; however, others reported that T cell tolerance was induced by MSCs in murine models [35].
Although some of the mechanisms underlying the immunosuppressive effects of MSCs on T cells have been elucidated previously, the molecular mechanisms underlying this effect remain controversial. It is believed that the mechanisms underlying the suppressive effect of MSCs may differ by species. Ren and colleagues demonstrated that

mouse MSCs and human MSCs utilize different effector molecules in suppressing immune reactions [40]. Immunosuppression by human- or monkey-derived MSCs is mediated by IDO, whereas mouseMSCsexert their effect viaNOunder the same culture conditions. Immunosuppression by human MSCs was not intrinsic, but was induced by inflammatory cytokines and was chemokine-dependent, as it is in mouse [40]. The degree of the suppressive effect depends on the concentration of the MSCs. The high MSC/lymphocyte ratio is associated with the inhibitory effect of MSCs, while a low MSC/lymphocyte ratio is often accompanied by enhanced proliferation [41]. In this setting,MSCsmay act synergisticallywith HLA-DR antigens upon mitogenic stimulation.However, the exact mechanisms need to be investigated further.

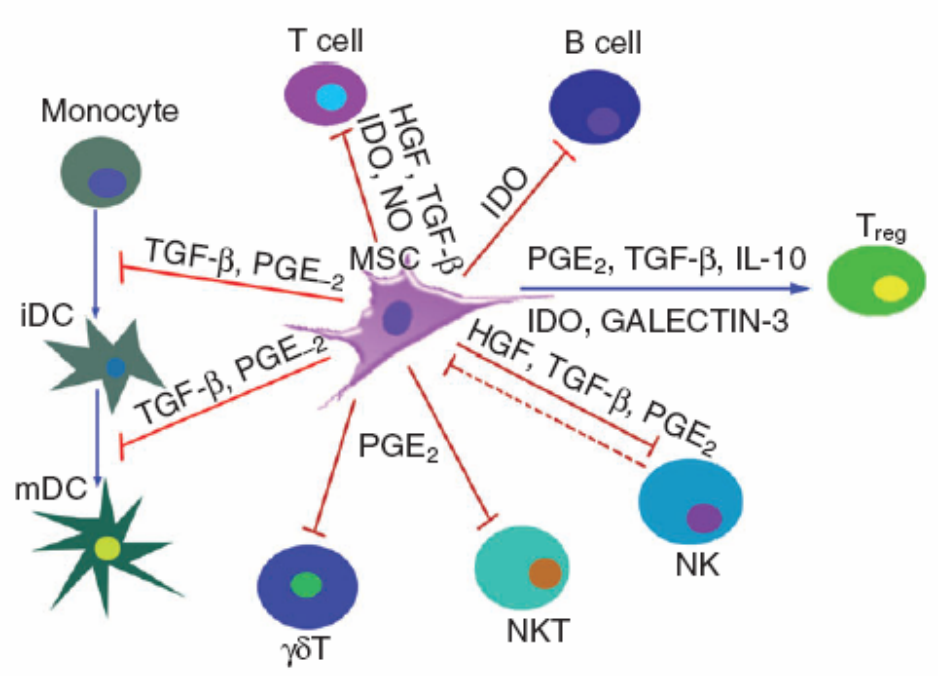

Fig. 1. Effects of mesenchymal stem cells (MSCs) on immunocytes. MSCs modulate the immune response by their interaction with a wide range of immune cells, including T cells, B cells, dendritic cells (DCs), regulatory T cells (T), natural killer (NK), NK T and gdT cells. Inhibitory role by MSCs is dependent on cell–cell contact and soluble factors released by MSCs. HGF: hepatocyte growth factor; iDC: immature dendritic cell; IDO: indoleamine 2, 3-dioxygenase; IL-10: interleukin-10; mDC: mature dendritic cell; NO: nitric oxide; PGE2: prostaglandin E2; TGF-b: transforming growth factor b.

1.4.1.2 MSCs modulate B cell proliferation and function

In murine models, MSCs have been shown to inhibit the proliferation of B cells when stimulated with anti-CD40L and IL-4 [21] or pokeweed mitogen [42]. Similarly, in humans, MSCs have also been shown to inhibit the proliferation of B cells activated with anti-immunoglobulin (Ig) antibodies, anti-CD40L antibody and cytokines (IL-2 and IL-4) [43]. In addition, the B cell functions of antibody production and secretion of the chemokine receptors CXCR4, CXCR5 and CCR7, which are responsible for chemotaxis to CXCL12 and CXCL13, were impaired by MSCs; however, the expression of B cell co-stimulatory molecules and cytokine production were not affected by MSCs [43]. MSCs inhibited the proliferation of B cells only in the presence of IFN-g, which probably implies that IFN-g causes MSCs to produce IDO, which in turn suppressed the proliferation of effector cells through the tryptophan pathway [24] (Fig. 1).

The nature of the mechanism involved in this inhibitory effect of MSCs has not yet been elucidated completely. Thus far, the major mechanism of B cell suppression by MSCs is attributed partly to the physical contact between MSCs and B cells and in part to the

soluble factors released by MSCs; this leads to the blocking of B cell proliferation in the G0/G1 phase of the cell cycle with no apoptosis [21,42,43], unlike the case with T cells. Deng *et al*. reported that allogeneic MSCs inhibited the activation, proliferation and IgG secretion of B cells in BXSB mouse models of human systemic lupus erythematosus (SLE) [44]. In addition, MSCs enhanced the CD40 expression and CD40 ligand ectopic hyperexpression on the B cells of BXSB mice [44].

1.4.1.3 MSCs modulate the functions of natural killer cells

Numerous studies have shown that MSCs suppress NK cell proliferation and IFN-g production driven by IL-2 or IL-15, but only partially inhibit the proliferation of activated NK cells [22,26,28,38,45]. Rasmusson *et al*. reported that MSCs did not inhibit the lysis of freshly isolated NK cells [26] and that MSCs were not lysed by allogeneic NK cells [26]. Conversely, Krampera *et al*. reported that NK cells cultured for 4–5 days with IL-2 in the presence of MSCs showed reduced cytolytic potential against K562 target cells and that this suppressive effect might be attributed to the IFN-g produced by NK cells [24]. Exposure to IFN-g did not ablate MSCinduced inhibition of T cell proliferation, but triggered the expression of HGF and TGF-b1 secreted by MSCs at concentrations that suppressed alloresponsiveness [45] (Fig. 1).

Furthermore, a study indicated that MSCs suppressed NK cell cytotoxicity against HLA class I-positive cells more effectively than HLA class I-negative cells [22]. Low HLA class I expression makes allogeneic as well as syngeneic MSCs more susceptible to lysis by activated NK cells [22,46,47]. Incubation of MSCs with IFN-g decreased their susceptibility to NK cell-mediated lysis because of the up-regulation of HLA class I expression on MSCs [46]. The mechanisms underlying the immunosuppressive effects of MSCs are still unclear and several different, sometimes contradictory, theories have

been proposed. Soluble factors such as TGF-b1 and PGE2 are believed to play a role in the MSC-mediated suppression of NK cell proliferation [22].

The physiological interactions between MSC and NK cells would be the reciprocal effects exerted by the two cell types, in particular the ability of activated NK cells to kill MSC.

Some studies showed that IL-2-activated NK cells can effectively lyse MSCs [22,46,47] because MSCs express ligands (ULBP, PVR and nectin-2) that are recognized by activated NK receptors (NKp30, NKG2D and DNAM-1), which in turn trigger NK cell alloreactivity [46,48].

Recently, Prigione *et al*. found that the inhibitory effect of MSCs on the proliferation of invariant NK T (iNK T, Va24+Vb11+) and gdT (Vd2+) cells in the peripheral blood is mediated by releasing PGE2, rather than IDO and TGF-b1 (Fig. 1); however, cytokine production and cytotoxic activity of the cells were only partially affected by MSCs [49]. Vd2+ cells also serve as professional antigen-presenting cells for naive CD4+ T cell response, and MSCs did not inhibit antigen processing/presentation by activated Vd2+ T cells to CD4+ T cells [49].

1.4.1.4 Interaction between MSCs and dendritic cells (DCs)

MSCs impaired the differentiation of monocytes or CD34+ haematopoietic stem cells into dendritic cells (DCs) by inhibiting the response of the former to maturation signals, reducing the expression of co-stimulatory molecules and hampering the ability of the former to stimulate naive T cell proliferation and IL-12 secretion [23,50,51]. In addition, this inhibitory effect might be mediated via soluble factors and may be dose-dependent [50]. Spaggiari *et al*. showed that MSCs strongly inhibited the maturation and functioning of monocyte-derived DCs by interfering selectively with the generation of immature via inhibitory mediator of MSCderived PGE2, but not IL-6 [52] (Fig. 1).

However, the mechanism underlying the up-regulation of PGE2 in monocyte–MSC co-cultures remains unclear. Ramasamy *et al*. reported that the cell cycle in DCs was arrested in the G0/G1 phase upon interaction with MSCs [53]. A recent study reported that MSCs isolated from human adipose tissue were more potent immunomodulators for the differentiation of human DCs than MSCs derived from the bone marrow [54].

1.4.1.5 Tregs induced by MSCs

MSCs may also modulate immune responses via the induction of Tregs. MSC can induce the generation of CD4+CD25+ cells displaying a regulatory phenotype (FoxP3+) in mitogenstimulated cultures of peripheral blood mononuclear cells [28,38], although the functional properties of these cells have not yet been elucidated. For example, depletion of CD4+CD25+ Tregs had no effect on the inhibition of T cell proliferation by MSCs [20]. However, a recent study reported that MSCs could induce kidney allograft tolerance by inducing the generation of CD4+CD25+FoxP3+ Tregs *in vivo* [55]. Additionally, MSCs have been reported to induce the formation of CD8+ Tregs that are responsible for the inhibition of allogeneic lymphocyte proliferation [19].

In a recent study, Ghannam *et al*. found that under inflammatory conditions, MSCs prevented the differentiation of naive CD4+ T cells into Th17 cells and inhibited the function of Th17 cells *in vitro* by secreting PGE2. Moreover, MSCs could induce the Treg phenotype in Th17 cells, which can inhibit the proliferative responses of activated CD4+ T cells *in vitro* [56]. Tipnis *et al*. reported that umbilical cordderived MSCs (UC-MSCs) constitutively express B7-H1, which is a negative regulator of T cell activation. In addition, B7-H1 expression was increased and IDO expression was induced in UC-MSCs after IFN-g treatment. Furthermore, UC-MSCs inhibited the differentiation and maturation of monocyte-derived DCs and augmented the generation of Tregs [57].

These immunosuppressive effects of UC-MSCs are mediated largely by cell–cell contact [57].

The induction of Tregs by MSCs involves not only involves direct contact between MSCs and CD4+ cells, but also the secretion of soluble factors such as PGE2 and TGF-b1 [58].

Human gingiva-derived MSCs (GMSCs) can induce IL-10, IDO, inducible NO synthase (iNOS) and cyclooxygenase 2 (COX-2) and thereby serve as immunomodulatory components in the treatment of experimental inflammatory diseases [59] (Fig. 1). A study has shown that the immunosuppressive effect of MSCs is mediated by the secretion of galectin-3, a protein known to modulate T cell proliferation, gene expression, cell adhesion and migration [60]. Madec *et al*. reported that MSCs prevent autoimmune B cell destruction and subsequent diabetes in NOD mice by inducing Tregs [61]. The effect of MSCs in the treatment of autoimmune diseases may be through the induction of *de novo* generation of antigen-specific CD4+CD25+FoxP3+ Treg cells [36,62].

However, a recent study reported that MSCs could sustain or suppress T cell proliferation depending on their concentration, and a low MSC/T-cell ratio might support T cell proliferation [63]. A recent study indicated that MSCs could stimulate the activation and proliferation of resting T cells and generate Tregs [64]. These data suggested that the culture conditions play an important role in the clinical application of MSCs [63].

1.4.10.6 Clinical application of MSCs for immune-mediated diseases

Over the past 3 decades, numerous efforts have beenmade to explore the therapeutic applicability of MSCs. In pathological conditions, MSCs migrate preferentially into lymphoid organs, allografts, injured and/or inflammatory tissue sites after systemic

transfusion, where MSCs interact with the activated immune cells and modulate their function [65,66].

The *in vivo* immunomodulatory properties of MSCs were first described in a baboon model of skin transplantation [18]. The therapeutic potential of MSCs in immunomodulation is being explored currently in several Phase I, II and III clinical trials [67], many of which have recently been completed or are under way, as reported in the clinical trials website of the United States sponsored by the National Institutes of Health (http://clinicaltrials.gov). Because of their immunosuppressive properties, MSCs are believed to play a role in the maintenance of peripheral tolerance and the induction of transplantation tolerance, and they are considered potential candidates in cellular therapy for graft-*versus* host disease (GVHD) and autoimmune diseases and in protecting solid-organ grafts from being rejected [39].

Table 1 presents data on the several applications of MSCs as immunosuppressants as studied in clinical trials (data available at the time of the preparation of this manuscript).

Recently, Le Blanc *et al*. reported that MSCs obtained from HLA-identical sibling donors, haploidentical donors and third-party HLA-mismatched donors infused in 55 patients with steroid-refractory acute GVHD elicited a response in more than half the patients; the study showed that MSCs exerted their therapeutic effect in the case of both HLAmatched and HLA-unmatched donors.However, for GVHD, the use of MSCs is a double-edged sword, because the prevention of GVHD was associated with a high incidence of leukaemia relapse, which is the result of the non-specific immunosuppressive effect of MSCs on graft-*versus*leukaemia [68,69]. Liang *et al*. reported that allogeneic MSC transplantation in patients with refractory SLE resulted in the amelioration of disease activity, improvement in the levels of serological markers and stabilization of renal function without the occurrence of serious adverse events [70].

For solid organ transplantation, the beneficial effect of MSCbased immunosuppressive therapy is debatable. The application of calcineurine inhibitors (CNIs) would abrogate

the immunosuppressive effect of MSC therapy. In addition, CNIs cause renal failure, hypertension and hyperglycaemia and increase the risk of malignancy; therefore, efforts have been made to minimize the use of CNIs treatment in organ transplantation protocols. Consequently, it may be worthwhile to compare the usefulness of combining CNI treatment and MSC therapy in organ transplantation [71].

Conversely, non-selective immunosuppression would have affected patients' antiviral immunity equally [70].

In the future, well-designed preclinical trials should be conducted to explore the clinical applicability of MSCs.

Thereafter, randomized trials comparing treatment with infusions of MSCs and conventional drug-based therapies should be undertaken to confirmthe therapeutic potential of these cells, as it is important not to overestimate the potential therapeutic effects of MSCs. Information gathered over such studies would help to develop innovative cell-based therapies for the treatment of diseases characterized by exaggerated immune responses.

Table 1. Clinical trials using mesenchymal stem cells (MSCs) as immunosuppressants.

Clinical trial	Disease	Cell type/source	Status	Sponsor	ClinicalTrials.gov identifier
Autologous mesenchymal stem cells from adipose tissue in patients with secondary progressive multiple sclerosis	Multiple sclerosis	Adipose tissue-derived autologous MSCs	Recruiting	Fundacion Progreso y Salud, Spain	NCT01056471
Mesenchymal stem cell infusion as prevention for graft rejection and GVHD	GVHD	BM-derived autologous MSCs	Recruiting	University Hospital of Liege, Belgium	NCT00504803
Mesenchymal stem cell transplantation in the treatment of chronic allograft nephropathy	Chronic allograft nephropathy	BM-derived autologous MSCs	Not yet recruiting	Fuzhou General Hospital, China	NCT00659620
Mesenchymal stem cells and subclinical rejection	Renal transplantation	BM-derived allogenic MSCs	Not yet recruiting	Leiden University Medical Center, the Netherlands	NCT00734396
Safety and efficacy study of umbilical cord blood-drived mesenchymal stem cells to promote engraftment of unrelated haematopoietic stem cell transplantation	GVHD	Human umbilical cord blood-derived MSCs	Not yet recruiting	Medipost Co. Ltd, Korea	NCT00823316
Safety and efficacy study of allogenic mesenchymal stem cells to treat extensive chronic GVHD	Chronic GVHD	BM-derived allogenic MSCs	Not yet recruiting	Guangdong General Hospital, China	NCT00972660
Evaluation of the role of mesenchymal stem cells in the treatment of GVHD	Steroid-resistant GVHD	BM-derived autologous MSCs	Recruiting	Christian Medical College, Vellore, India	NCT00314483
Mesenchymal stem cell infusion as treatment for steroid-resistant acute GVHD or poor graft function	GVHD, poor graft function	BM-derived allogenic MSCs	Recruiting	University Hospital of Liege, Belgium	NCT00603330
Safety and efficacy study of adult human mesenchymal stem cells to treat acute GVHD	GVHD	BM-derived allogenic MSC (prochymal)	Completed	Osiris Therapeutics, USA	NCT00136903
Treatment of refractory (acute or chronic) GVHD by the infusion of expanded *in-vitro* allogenic mesenchymal stem cell	GVHD	BM-derived allogenic MSCs	Recruiting	University of Salamanca, Spain	NCT00447460
Mesenchymal stem cells for treatment of amyotrophic lateral sclerosis	Amyotrophic lateral sclerosis	Adipose tissue-derived autologous MSCs	Not yet recruiting	Mayo Clinic, USA	NCT01142856
Mesenchymal stem cells under basiliximab/low-dose RATG to induce renal transplant tolerance	Kidney transplant	BM-derived autologous MSCs	Recruiting	Mario Negri Institute for Pharmacological Research, Italy	NCT00752479
Mesenchymal stem cells transplantation for refractory systemic lupus erythematosus	Refractory systemic lupus erythematosus	BM-derived allogenic MSCs	Recruiting	Nanjing Medical University, China	NCT00698191
Efficacy and safety of adult human mesenchymal stem cells to treat patients who have failed to respond to steroid treatment for acute GVHD	GVHD	BM-derived allogenic MSC (prochymal)	Completed	Osiris Therapeutics, USA	NCT00366145
Donor mesenchymal stem cell infusion in treating patients with acute or chronic GVHD after undergoing a donor stem cell transplant	GVHD	BM-derived allogenic MSCs	Not yet recruiting	Case Comprehensive Cancer Center	NCT00361049
Safety and efficacy of prochymal for the salvage of treatment-refractory acute GVHD patients	GVHD	BM-derived allogenic MSCs	Completed	Osiris Therapeutics, USA	NCT00284986
Safety and efficacy of human mesenchymal stem cells for treatment of liver failure	Liver failure	Human umbilical cord-derived MSCs	Recruiting	Beijing 302 Hospital, China	NCT01218464
Umbilical cord mesenchymal stem cells for immune reconstitution in HIV-infected patients	HIV	Human umbilical cord-derived MSCs	Recruiting	Beijing 302 Hospital, China	NCT01213186

BM: bone marrow; GVHD: graft-*versus*-host disease; HIV: human immunodeficiency virus; RATG: rabbit anti-human thymocyte globulin.

1.4.2 The Utility of Placenta-Derived Cells for Treating Inflammatory Diseases

In 1910, Davis was the first to report the use of fetal membranes in skin transplantation [65], prompting subsequent applications that demonstrated the utility of these membranes for treating other conditions including leg ulcers [66 , 67] and burns [68 , 69], as well as for applications in ophthalmology [70–72]. One century later, although fetal membranes in toto continue to be applied therapeutically in some settings, the focus of scientific investigations has turned to the various cell populations present in these membranes, with accumulating evidence now lending support to the hypothesis that placental cells may be useful for treating a range of pathologic conditions.

Ocular surface disorders

The first clinical applications of amniotic membrane were reported for treatment of ocular surface disorders in the 1940s, and after a hiatus, use of this membrane was reintroduced to the field in 1995 by Kim and Tseng [73].

Today, use of the stromal matrix of the amniotic membrane, rather than of cells derived from it, continues to represent the main strategy by which placental tissues are applied in ophthalmology.

Use of the amniotic membrane in this field is based on several mechanisms mediated by various cytokines and enzymes, ultimately conferring beneficial effects including enhanced epithelialization and wound healing, suppression of inflammation and fibrosis, and inhibition of angiogenesis.

In this context, it is noteworthy that the angiogenic profile secretome largely depends on the preparation method of amniotic membrane, which should therefore be considered when selecting an amniotic product for clinical application [74].

The anti-scarring properties of amniotic membrane are mediated by the presence of fetal hyaluronic acid in the stromal matrix, which suppresses TGF-β signaling, cell proliferation, and myofibroblastic differentiation of normal corneal and limbal fibroblasts, as well as of normal conjunctival and pterygium fibroblasts [75]. The amniotic stromal matrix also suppresses expression of inflammatory cytokines originating from the ocular surface epithelia, including IL-1α, IL-1β [76], and IL-8 [77], and up-regulates the expression of IL-1 receptor antagonist [76]. Such suppression of inflammation is paramount for preventing corneal and conjunctival scarring, neovascularization, and fibrosis. Several clinical applications in ocular surface reconstructive surgery have now been developed based on these properties [73].

The most important ophthalmology-based application of amniotic membrane is as a temporary bandage after acute chemical burns of the ocular surface [78]. In this scenario, the membrane serves as an anti-inflammatory agent during acute phases of chemical and thermal burns, limiting corneal and limbal inflammation and angiogenesis [79] and reducing symblepharon formation. A possible anti-inflammatory mechanism of the amniotic membrane may be trapping of inflammatory cells infiltrating the ocular surface after a chemical burn, whereby as these cells are caught in the membrane, they undergo apoptosis [80]. This hypothesis is in accordance with a study showing that both epithelial and mesenchymal cells of the amniotic membrane express Fas ligand (CD95), a cell surface receptor that mediates apoptosis [81].

Additionally, various other studies report significant reductions in inflammation when transplantation of amniotic membrane is applied during treatment of ocular surface disorders, including pterygium surgery [82], fornix reconstruction, and symblepharon repair [83], reconstruction of deep corneal ulcers and persistent epithelial defects in autoimmune and inflammatory disorders of the ocular surface [84 , 85], and in treatment of chronic pseudophakic corneal edema [86].

Neurological disorders

Neurological disorders represent a significant burden to western societies, highlighting the need to develop effective therapies. Cell replacement therapy has been proposed as a basis for new treatment strategies for a broad range of neurological diseases; however, the paucity of suitable cell types has so far hampered the development of this promising therapeutic approach [87]. In this context, placenta-derived cells have been investigated for their potential to confer beneficial effects in a range of neurological disorders.

In preclinical studies using animal models of Parkinson's disease [52 , 88–90] and ischemia [53], hAEC have been found to offer neuroprotection and functional recovery. The observed therapeutic effects are likely mediated by secretion of diffusible factors, including neurotransmitters [91–93] and many neurotrophic and growth factors [94 , 95].

The potential utility of placenta-derived cells has also been investigated for treatment of spinal cord injury (SCI), a condition in which inflammation-mediated "secondary injury," rather than the primary physical force, has been implicated for many of the devastating effects observed [96]. Therefore, counteracting these cell death cascades with a suitable therapy at the earliest possible time postinjury would likely translate into a successful treatment. Similarly, the tissue damage caused by the host's immune response following injury may be suppressed by the anti-inflammatory properties of placental cells, which further make them attractive candidates for SCI treatment. Indeed, hAEC transplantation has been shown to produce beneficial effects after transection SCI in bonnet monkeys and rats [55 , 97] with functional improvement observed in rats. Similarly, when co-transplanted with neural stem cells, AEC have been shown to enhance recovery after contusive SCI in rats [51]. Despite

these promising data, however, there still appear to be critical gaps in the knowledge that would be required to allow commencement of clinical trials using placental stem cells for SCI repair. For example, of the many placental cell types

showing stem cell features [16 , 98 , 99], only hAEC have been tested for SCI repair to date and much work is still required to fully prove their effi cacy and the mechanisms underlying their functional benefi ts in this setting. Meanwhile, a study in which hAMSC were transplanted into an experimental model of SCI showed chondrogenic differentiation of these cells (Sankar et al., unpublished data). This could be due to release of TGF-β by host cells as part of the inflammatory reaction following SCI [100 , 101], which is also known to be a powerful inducer of stem cell chondrogenic differentiation [102]. Therefore, as for all applications in which placenta-derived cells have been proposed for therapy, use of these cells for SCI treatment clearly requires further validation.

Stroke is another serious neurological disorder representing a current unmet medical condition of significance worldwide, and for which placenta-derived cells may offer new hope for therapy. In the United States, stroke is the third leading cause of death and the primary cause for disability.

Because inflammation is a major contributor to the secondary cell death cascade following the initial stroke episode, transplanted cell-mediated abrogation of such inflammatory deleterious side effects should directly alter stroke progression. A major caveat for this anti-inflammatory mechanism to effectively mitigate cell therapy and stroke outcome is demonstrating robust and stable secretion of anti-inflammatory factors by transplanted cells at the appropriate timing post-injury. Although inflammation is shown to exacerbate stroke, early pathological inflammatory cues, such as stromal derived factor-1, serve as a migratory guide for transplanted cells to reach the ischemic tissue [103]. These time-dependent positive and negative effects of inflammation may be circumvented by direct intracerebral transplantation of cells into the ischemic penumbra; however, in the acute setting of stroke, a minimally invasive peripheral cell administration may be more practical. Thus, the challenge for cell therapy to reconcile the double-edged sword feature of inflammation is to find the optimal therapeutic

window when elevated inflammatory migratory signals can direct cell migration toward the ischemic brain and thereafter for the cells to subsequently suppress inflammation.

Cell therapy has been proposed as a novel treatment for acute [104 , 105], subacute [106 , 107], and chronic stroke [108–110]. Transplantation of human placenta-derived cells has been shown to exert beneficial effects in a rodent stroke model. Specifically, transplantation of hAEC or hAMSC at Day 2 poststroke attenuated both motor and neurological deficits associated with occlusion of the middle cerebral artery at days 7 and 14 compared to the vehicle-infused stroke group. Following the last behavioral test at Day 14 poststroke, histology via Nissl staining revealed transplantation of hAEC or hAMSC at Day 2 poststroke increased the number of healthy looking cells (>75% of the intact brain) in the ischemic penumbra compared to the vehicle-infused stroke group. These positive behavioral and histological effects were achieved when 400,000 human placenta cells were transplanted directly into the presumed ischemic penumbra in the absence of immunosuppression [111].

That placenta-derived cells display transplantable cell properties including their ability to secrete anti-inflammatory factors [112] will benefit from optimizing the timing and route of cell delivery after transplantation.

Pulmonary fibrosis

In a mouse model of bleomycin-induced lung injury, treatment with a mixture of fetal membrane-derived mesenchymal and epithelial cells from either human or mouse have both been shown to cause a reduction in severity and extent of lung fibrosis [9]. These antifibrotic effects were observed at Day 14 after bleomycin instillation, by which time maximal lung fi brosis is observed [113 , 114] and notably, these effects were seen regardless of the cell source (allogeneic or xenogeneic) or administration route (systemic: intravenous or intraperitoneal; local: intratracheal).

Treatment with placenta-derived cells also resulted in evident reductions in the numbers of infiltrating neutrophils in the lungs of bleomycin-injured mice at Day 14, which could be partly responsible for the observed reduction in fibrosis, as the presence of neutrophils is known to be associated with poor prognosis in idiopathic pulmonary fibrosis in humans [115]. Whether the transplanted fetal membrane-derived cells released soluble factors that acted to down-regulate neutrophil recruitment remains to be elucidated, although this possibility is supported by reports that soluble factors released by these cells can inhibit T-cell proliferation [44] and dendritic cell differentiation and function [47] in vitro,
while these cells also display anti-inflammatory effects in clinical settings [116].
Although both allogeneic or xenogeneic cells were detectable in the lungs of bleomycin-treated mice through DNA microchimerism and immunohistochemistry until Day 14, previous studies have shown that only a very small percentage of placenta-derived cells engraft and survive long term after transplantation [45]. Further studies are therefore warranted to elucidate the mechanisms of action of placenta-derived cells in this model. In addition, the recent indication that MSC from both mouse bone marrow and human umbilical cord blood produce factors that stimulate proliferation and matrix production by lung fibroblast cells [117], which could potentially exacerbate existing fi brotic damage, suggests that caution should also be exercised when proposing placental mesenchymal cells for treatment of pulmonary fibrosis. Thus, although promising, the data discussed above represent preliminary evidence that would require further validation in order to verify whether transplantation of placenta-derived cells does indeed represent a viable treatment option for pulmonary fibrotic disease.

Critical limb ischemia

Recently, Pluristem Therapeutics Inc. has investigated 3D expanded human term placenta-derived cells, termed PLXPAD, for their potential to treat critical limb ischemia [118], whereby cells were administered via local intramuscular injection 5 h after induction of hind limb ischemia in mice through a previously established method [119]. During a follow-up period of 21–28 days, blood flow in ischemic limbs of cell-treated mice was significantly elevated compared to non-cell-treated mice, and this was accompanied by a significant decrease in the rate of cell necrosis in these animals.
Furthermore, immunohistochemical analysis of tissues from limbs of cell-treated animals demonstrated a statistically significant increase in the number of new capillaries supplying the limb, while reduced levels of nitrotyrosine, an indicator of oxidative stress, and also of VCAM-1, an indicator of endothelial inflammation, were also observed in cell-treated animals compared to controls. Importantly, no treatment-related adverse effects were reported in PLXPAD- injected mice. Based on these results, PLX-PAD is now being assessed in clinical trials for critical limb ischemia in the United States and European Union.

Inflammatory bowel disease

Chronic relapsing and remitting inflammation of the intestinal tract is the hallmark trait of ulcerative colitis (UC) and Crohn's disease (CD), collectively termed inflammatory bowel diseases (IBD). In genetically susceptible individuals an aberrant immune response, probably triggered by commensal bacteria or luminal antigens, is accompanied by impairments in tissue repair processes, ultimately leading to loss of tissue architectural organization, ineffective ulceration healing, and fibrosis [120 , 121].
Current therapies, ranging from anti-inflammatory drugs to immunosuppressive regimens, are often inadequate to control IBD and possess severe long-term side effects. As for other inflammatory-based diseases, cell therapies based on the

immunomodulatory properties of MSC have been proposed for IBD and are currently under evaluation in phase III clinical trials for IBD manifestations using bone marrow and adipose tissue-derived MSCs [120].

Recently hAMSC were shown to possess trophic effects upon intestinal epithelial cells, stimulating architectural organization and polarized differentiation (Lanzoni et al., unpublished data). These findings suggest that hAMSC may be useful for treating IBD; while their angiogenic potential may aid in ameliorating perfusion and healing, the paracrine activity of these cells may be beneficial in inducing ulcer re-epithelialization. Finally, their immunomodulatory effects may facilitate in restoring a correct balance between inflammatory cell activation and suppression in the intestinal mucosa, thereby preventing further damage.

Liver-based metabolic diseases

Several preclinical studies reported to date provide promising evidence regarding the potential of human amniotic membrane-derived cells to perform hepatic functions in vivo.

The first such evidence came from a study whereby hAEC that had been transduced with the β-galactosidase gene were transplanted into the livers of SCID mice, resulting in detection of integrated transplanted α-fetoprotein- and albumin positive cells in the hepatic parenchyma at both 1 and 2 weeks after cell injection, suggesting that hAEC could serve as transgene carriers after transplantation into the liver [90]. Later, it was shown that after transplantation of human amniotic membrane into the peritoneum of SCID mice, human albumin could be detected in the sera and peritoneal fluid of these animals from Day 1 until Day 7 (duration of the study) [122]. In another study, transplantation of hAEC into immunodeficient mice resulted in detection of human α-1

antitrypsin circulating in the serum of recipient animals, confirming that hAEC can perform this important hepatic function in vivo [123].

More recently, it has been shown that 2 weeks after transplantation of hAEC into the livers of SCID/Beige mice that had been pretreated with CCL4 in oil, human cytokeratin-positive cells could be detected in the bile ducts of these animals, with some bile ducts appearing to be completely humanized (Strom et al., unpublished data).

These studies provide compelling evidence in support of the bifunctional hepatic potential of hAEC in vivo, with demonstration of differentiation toward both parenchymal hepatocytes and cells with characteristics of bile ductular epithelial cells, thereby supporting the potential of hAEC as a useful tool for liver regeneration in the future.

Cardiac ischemia

Even though their ability to differentiate toward cardiomyocytes is still debated [8 , 54], several lines of evidence suggest that transplantation of isolated amniotic and chorionic cells can improve cardiac function. For example, Ventura et al. have reported improved myocardial function for up to 4 weeks after intramyocardial injection of fetal membrane-derived cells into infarcted rat hearts [8].

In other studies, injection of rat amnion-derived cells into syngeneic animals with an acute infarcted left ventricular myocardium following permanent ligation of the proximal left coronary artery prevented ventricle dilatation, while contractile function was maintained between 2 and 6 weeks after transplantation. Histological assessment revealed that the amniotic cell-treated myocardium had reduced scar areas and fibrosis, with increased left ventricle myocardial wall thickness [124].

Finally, application of a human amniotic membrane fragment onto the left ventricle of rats that had undergone ischemia through left anterior descending coronary artery

ligation has been shown to significantly reduce postischemic cardiac dysfunction [125]. Echocardiographic assessment of morphological and functional cardiac parameters performed over a 3-month period demonstrated that membrane-treated rats showed higher preservation of cardiac dimensions and improved cardiac contractile function in terms of higher left ventricle ejection fraction, fractional shortening, and wall thickening [125]. In this study, no engraftment of amniotic cells was detected in host cardiac tissues, again supporting the hypothesis also suggested by other reports that the beneficial effects observed are likely due to paracrine secretion by amniotic cells of soluble factors that promote protection and regeneration of host tissues, rather than differentiation of the transplanted cells themselves.

2. Materials and Methods

2.1. Isolation and Culture of Human Amniotic Mesenchymal Stromal Cells.

Human amniotic mesenchymal stromal cells (hAMSCs) were isolated from term placentas (n = 3) which would normally be discarded after delivery. Tissues were obtained under appropriate Ethical Committee approval and signed informed consent. All infectious pathogen-positive deliveries including those involving HBV, HCV, and HIV, as well as cases of prediagnosed genetic abnormalities, were excluded. Placenta samples were procured immediately after delivery and processed under sterile conditions.

After peeling from the placenta and washing with calcium- and magnesium-free HBSS (CMF-HBSS, Lonza, Treviglio, Italy) supplemented with 0.5 mM EGTA (Sigma, Milan, Italy), amnion membranes were processed to remove epithelial cells as previously reported [12]. Once epithelial cells were removed, the amniotic membranes were digested in order to collect hAMSCs [13]. Briefly, amniotic membranes were washed three times with cold HBSS, cut into pieces, and transferred into 50-mL centrifuge tubes, containing about 30–40 mL of digestion solution composed by EMEM (Lonza) supplemented with 25 mM HEPES buffer without L-glutamine (Lonza), 1 mg/mL collagenase type IV, and 25 μg/mL DNase I (both from Sigma, Milan, Italy). Membranes were incubated on a rotator between 45 min to 1.5 h, depending on tissue thickness, at 37^0C. After blocking the enzymatic reaction with cold HBSS, cell suspensions were centrifuged 2 times for 5 min at 200 ×g, 4^0C and counted using a Burker chamber. ¨

After isolation, DNA was obtained from hAMSCs and hAECs by phenol/chlorophorm extraction. Purified DNA was investigated for most frequent mutations in CFTR gene

using a commercial kit (Inno-Lipa CFTR19, Inno-Lipa CFTR17+TnUpdate, Inno-Lipa CFTR-Italian Regional-Innogenetics, Ghent, Belgium).

hAMSCs were plated at a density of 1×10^5 cells per cm^2 in standard culture medium composed by DMEM (Lonza) supplemented with 1% sodium pyruvate, 10% (v/v) heatinactivated fetal bovine serum (FBS), 1% nonessential amino acid, 55 μM β-mercaptoethanol (all by Invitrogen, Milan, Italy), 1% L-glutamine, 1% antibiotics solution (both by Cellgro, Manassas, VA, USA), and 10 ng/mL epidermal growth factor (EGF, Sigma), according to the previously reported protocol [13]. Medium was replaced 2 h after plating in order to remove unattached contaminating epithelial cells and then every 2 days.

Every time cells reached 80% of confluence, cells were detached with trypsin-EDTA (Invitrogen), washed, counted with a Burker chamber, and replated in a new plastic flask at a density of 1×10^5 cells per cm^2 in order to calculate their growth curve. Doubling time was calculated inserting times and cell counts on the website http://www.doubling-time.com/compute.php.

2.2. Characterization of hAMSCs

2.2.1. Flow Cytometry.

Flow cytometry analyses of hAMSCs were performed immediately after dissociation and at second culture passage as previously described [14]. Briefly, cells were detached from culture flask using trypsin and, after washing, were incubated with 4% normal mouse serum/PBS/N_aN_3 for 20 minutes at 4^0C in order to block nonspecific sites on cell membrane. Cells were then stained in the dark at 4^0C for 20 minutes with 7-amino actinomycin-D (7AAD) to discriminate viable cells from fragments and dead cells and with the following monoclonal antibodies (moabs): against CD13, CD29, CD31, CD34,

CD44, CD45, CD49f, CD73, CD90, CD105, CD146, CD166, EpCAM, SSEA4 (all from Becton Dickinson Biosciences, BD, Franklin Lakes, NJ, USA), and CD133-1 (Miltenyi Biotech, Bergisch Gladbach, Germany). Moabs were conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE) or PE-Cyanin 7 (PE-Cy7) or allophycocyanin (APC) or APC-Cyanin 7 (APC-Cy7).

For internal labelling, 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 nonspecific binding, followed by primary antibodies against Oct-4 and Nanog (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h; secondary PE-conjugated antibody (Invitrogen) was applied for 30 min. The fluorescence threshold between negative and positive cells was set on the basis of the reactivity of appropriate nonspecific fluorochrome-conjugated isotypic controls. At least, 10^6 cells were finally analysed using a FACSCanto II equipped with FACSDiva software (BD).

2.2.2. Immunofluorescence Microscopy of Cultured Cells.

Plated cells were stained as reported elsewhere [14]. Fixed (4% PFA or 70% ethanol for 30 min) and permeabilized (HEPESTriton X-100 buffer 0.25% in PBS for 20 min) cells were incubated with a blocking buffer containing 0.5 M NaCl, 20 mM NaHPO4, 0.1% Triton X-100, and 30% horse and goat serum for 30 min (all reagents were from Sigma) and then immunostained with the following primary moabs: anti-EpCAM, cytokeratin (CK)18, alpha-fetoprotein (Sigma), CK19 (Novocastra, Newcastle, UK), albumin (DakoCytomation, Milan, Italy), CK7, CD49f, CD29, S100A4, CD90, CD31, CD146, zonula occludens-1 (ZO-1), fibronectin, alpha1-antitrypsin, E-cadherin, and beta-catenin (BD) for 2 h.

After washing, cells were incubated with the appropriate secondary FITC or Texas Red-conjugated antibodies (BD) for 1 h in the dark. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI, Sigma) for 5 min in the dark.
Images were taken using a Leica Microsystems DM IRE 2 microscope and analysed with the FW4000I software (Leica Microsystems, Milan, Italy).

2.2.3. Reverse-Transcriptase (RT)-Polymerase Chain Reaction (PCR).

CFTR mRNA expression was investigated by semiquantitative RT-PCR. Total RNA was isolated from freshly isolated and cultured cells with TRIzol Reagent (Invitrogen), according to the manufacturer's protocol. One μg 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. In order to analyze the expression of CFTR gene, 100 ng of cDNA was used in a final volume of 25 μL with 200 nM dNTP, 10 pM of each outer primer (Table 1), 0.3 U Taq-DNA-polymerase, reaction buffer, and MgCl2 (Invitrogen). A second nested PCR was performed using inner primers (Table 1). Cycling conditions consisted of 95^0C for 30 seconds, annealing at 60^0C for 1 min and elongation at 72^0C for 2 min. Cycle numbers consisted of 35 cycles. cDNA from nasal brushing from healthy control was used as positive control for CFTR analysis; no reverse-transcribed sample was used as negative control.
In parallel, β-actin was used as house-keeping gene (Table 1). PCR products were evaluated on 1.5% agarose gel electrophoresis.

Table 1. Primer sequences for CFTR RT-PCR analysis.

Gene	Forward Primer	Reverse Primer	Product length (bp)
CFTR			
Outer primers	CGAGAGACCATGCAGAGGTC	GCTCCAAGAGAGTCATACCA	1108
Inner primers	CGAGAGACCATGCAGAGGTC	TGTACTGCTTTGGTGACTTCCCC	301
□actin	CAACTGGGACGACATGGA	ACGTCACACTTCATGATGGA	610

2.3. Differentiation of hAMSCs Towards Different Lineages

2.3.1. Adipogenic and Osteogenic Differentiation.

To induce adipogenic and osteogenic differentiation, cells at passages 1–3 were harvested and plated on tissue culture dishes (BD) at a density of 4×10^3 cells per cm^2. Cells were then treated with either adipogenic or osteogenic differentiation media (Lonza) for three weeks. The adipogenic protocol consisted of 4 rounds of adipogenic induction medium for 2 days followed by adipogenic maintenance medium for 3 days. The presence of adipose elements in induced cultures was determined by Oil-Red-O (Sigma) staining as follow: cells were washed in PBS, then fixed in 10% formalin for 1 h, washed in isopropanol 60%, and air dried. Cells were then incubated with OilRed-O staining solution for 10 min, then washed several times in PBS, and observed with an inverted microscope Eclipse TS100 (Nikon, Tokyo, Japan) equipped with a DS-FI1 CCD camera (Nikon).

In order to induce osteogenesis, cells were treated with osteogenic medium for 3 weeks with medium changes 3 times a week. The presence of calcium deposits in induced cultures was determined by Alizarin Red (Sigma) staining

as follow: cells were fixed in 10% formalin for 1 h, then washed in deionized water, and incubated for 30 min at room temperature with Alizarin Red 2% in water at pH 4.2. The cells were finally washed several times to remove the excess of staining and analyzed as described above.

2.3.2. Hepatocyte Differentiation.

A simple protocol [15] was used for hepatic differentiation of hAMSCs; cells were plated on type 1 collagen-coated culture dishes in DMEM supplemented with 10% FBS, 1% nonessential aminoacids, 1% Lglutamine, beta-mercaptoethanol, and 10 ng/mL of EGF for 8 days and then with IMDM with the same compounds plus 10^{-7}M dexamethasone (Sigma) for 6 days. One of the functions in cultured hepatocytes is that of the cytochrome P450-dependent mixed function oxidases (MFOs). Diethoxy (5,6) chloromethylfluorescein (Invitrogen) is a probe suitable for use as an in situ stain for MFO activity since this colorless molecule is metabolized in a fluorescent green compound retained in the cells [16]. Five mg of probe was eluted in 1143 μL DMSO (stock solution 10 mmol). Test medium was prepared as follow: 987 μL of RPMI, 12 μL HEPES 1 M (12 mmol final), and 1 μL probe 10 mmol (10 μmol final).

Control medium was prepared as follow: 987 μL RPMI, 12 μL HEPES 1 M (12 mmol final), and 1 μL DMSO. The cells were washed in PBS and incubated with the test (or control) medium for 2 h at 37^0C in a CO_2 incubator. Cells were analyzed using a Leica Microsystems DM IRE 2 microscope.

After differentiation for 21 days (8 days in DMEM supplemented as above +13 days in IMDM supplemented with dexamethasone), cells were stained by means of immunofluorescence as reported above in order to verify the expression of epithelial markers.

2.3.3. Cholangiocyte differentiation.

Cells were kept in DMEM/F-12 (Sigma-Aldrich, St. Louis, MO) containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 1X insulin/transferrin/ selenium (Invitrogen), 10 mM nicotinamide (Wako Pure Chemicals, Osaka, Japan), 0.1 uM dexamethasone (Sigma-Aldrich), 5 mM l-glutamine, and 5 ng/ml hepatocyte growth factor (HGF) and epidermal growth factor (EGF). Cells were suspended in type I collagen gel or mixture of type I collagen and Matrigel at a density of 4 X 10^4 cells/ml. One hundred fifty microliters of the cell suspension was added to each 1-cm-diameter culture insert (Millipore, Billerica, MA). After incubation at 37°C for 2 h to solid the gel, 500 ul of DMEM/F-12 with or without growth factors was added on top and under the insert.

2.4. hAMSC Labelling

Passage two hAMSCs were labelled with chloromethylbenzamido (CellTracker CM-DiI) [17].

Stock solutions of CM-DiI were prepared in dimethylsulfoxide (DMSO) at 1 ng/μL. Immediately before labelling, the stock solution was diluted up to a final concentration of 0.005 ng/μL in DMEM without phenol red. Cells grown at confluence in a T25 flask were washed with phosphate-buffered saline (PBS) and then incubated with the dye working solution for 30 min at 37^0C. After labelling, cells are washed twice with PBS, then incubated at 37^0C 5% CO_2 for at least 24 h in the presence of fresh medium.

2.5. Cultures of Airway Epithelial Cells.

16HBE14o- and CFBE41o- are human epithelial bronchial cell lines, wild type

and homozygous for the F508del allele (F508del/F508del), respectively, a generous gift of Professor D. Gruenert (University of California at San Francisco, USA). Epithelial cells were grown in MEM, 10% FBS, 100 U/mL penicillin, and 100 U/mL streptomycin, alone or in Coculture with hAMSCs.

2.6. Coculture of hAMSCs with CFBE41o- Cells.

Labelled hAMSCs were mixed with CFBE41o- cells at different ratios (1 : 5, 1 : 10, 1 : 15, and 1 : 20) and, in order to obtain polarized cocultures, cells were seeded on 6.5-mm diameter Snapwell, 0.4-μm pore size (Corning, Acton, MA, USA) at 1×10^5 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) dissolved in MEM. As controls, hAMSCs and CFBE41o- were seeded at 2.5×10^4 and 1×10^5 per filter, respectively. Cocultures were maintained at 37^0C 5% CO_2 for at least 6–8 days.

Separate cocultures were obtained by seeding hAMSCs onto the filter and CFBE41o- cells onto the bottom of the lower chamber. To obtain 1 : 5 and 1 : 10 ratios, hAMSCs were seeded at 2×10^4 and 1×10^4 and CFBE41o- cells at 8×10^4 and 9×10^4, respectively. As controls, hAMSCs were seeded at 1×10^5 per filter. Medium was changed daily in each chamber for 5 days, and cultures were analyzed at day 6.

2.7. CFTR Cytofluorimetric Assay.

Cells were detached with trypsin-EDTA treatment and fixed in PBS containing 2% PFA for 5 min. After centrifugation at 250 ×g, 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, USA) used at 1 : 20 dilution for 1 h at

4^0C. After washing in PBS, the cells were incubated with the FITC-conjugated secondary antibody (anti-mouse used at 1 : 100; Sigma) for 1 h at 4^0C, followed by two washes in PBS, and analyzed. As a background control, cocultures were incubated with secondary antibody only, and the resulting fluorescence was subtracted from the analyzed samples incubated both with primary and secondary antibodies. Data were collected using a Coulter Epix XL flow cytometer (Beckman Coulter, Fullerton, CA, USA) and analyzed with WinMDI 2.9 (http://www.cyto.purdue.edu/flowcyt/software/Winmdi.htm). Ten thousand cells were examined in each experiment. Since physical parameters (forward scatter and side scatter) did not allow us to distinguish hAMSCs from CFBE41o- cells, specific expression of CFTR on hAMSCs was detected in the CM-DiI-labelled cells. Analyses were performed by plotting the FLH-1 channel (525 nm) against the FLH-2 channel (575 nm), identifying the CFTRspecific green signal and the red-labelled hAMSCs, respectively. The vitality was evaluated by trypan blue exclusion assay and resulted to be >98%.

2.8. Confocal Analysis of CFTR Protein

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

After two washes in PBS, cells were fixed in 3% PFA and 2% sucrose for 10 min. After three washes in PBS, filters were excised and placed side up on a glass slide and overlaid with a drop of Mowiol (Calbiochem, San Diego, CA, USA) followed by a coverslip. Cells were analyzed using a 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).

2.9 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 Na2HPO4, 1.5 KH2PO4, 1 CaCl2, 0.5 MgCl2, 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% CO2. After 24 hours, the apical fluid was carefully removed, centrifuged to separate the mineral oil, and the residual volume of aqueous phase measured.

2.10 Fluorescence Measurements of Apical Chloride Efflux

Chloride efflux was measured using the Cl-sensitive dye MQAE. Confluent cell monolayers were loaded overnight in culture medium containing 5 mM MQAE at 37°C in a CO2 incubator and then inserted into a perfusion chamber that allowed independent perfusion of apical and basolateral cell surfaces.

The apical Cl- efflux measurements were performed when the confluent cell monolayers reached a transepithelial resistance >300 Ω X cm2 measured with the Millicell-ERS; Electrical Resistance System, Millipore). Fluorescence was recorded with a Cary Eclipse spectrofluorometer (Varian, Palo Alto, CA). To measure chloride efflux rate across the apical membrane, the apical perfusion medium was changed with a medium in which chloride was substituted with iso-osmotic nitrate. All experiments were performed at 37°C in HEPESbuffered bicarbonate-free media [Cl-medium: 135 mM NaCl, 3 mM KCl, 1.8 mM CaCl2, 0.8 mM MgSO4, 20 mM HEPES, 1 mM KH2 PO4 , 11 mM glucose, and Cl- free-medium: 135 mM NaNO3, 3 mM KNO3, 0.8 mM MgSO4, 1 mM KH2 PO4 , 20 mM HEPES, 5 mM Ca(NO3)2, and 11 mM glucose). We measured the apical CFTR-dependent chloride secretion as described previously (Guerra et al., 2005): CFTR-dependent chloride secretion was calculated as the difference in the rate of change of FSK- plus IBMX-stimulated fluorescence in the absence or presence of apical treatment with the specific CFTR inhibitor CFTRinh-172 (Ma et al., 2002; Taddei et al., 2004) (Supplemental Figure 3S).

2.11. Statistical Analysis.

Statistical significance of differences was evaluated by a two-tailed unpaired Student's t-test. Data were analyzed using Prism 4 (GraphPad Software, Inc., La Jolla, CA, USA). P values of less than 0.05 were considered significant.

2. Results

3.1. Isolation and Characterization of hAMSCs from Human Amnion. At least 33 x 10^6 hAMSCs (range 26-160 x 10^6) were recovered in each isolation (n=3) with a viability of 85-90%. Inno-lipa screening revealed the absence of most frequent mutation of CFTR (86% of detection rate) in hAMSCs used in this study. After plastic adhesion, hAMSCs were characterized by a fibroblastic morphology very similar to that described for mesenchymal cells isolated from bone marrow (Fig. 1A and B) and could be kept in culture until passages 5-10. Proliferation slowed beyond passage two. In the exponential growth phase, approximately two cell doublings were observed over 15 days, giving these cells an average doubling time of 18.03 days calculated over 28 days of culture. An example of a growth curve for hAMSCs is presented in Figure 1C. As it has been shown previously [18], the number of cells reached a plateau after 21 days in culture.

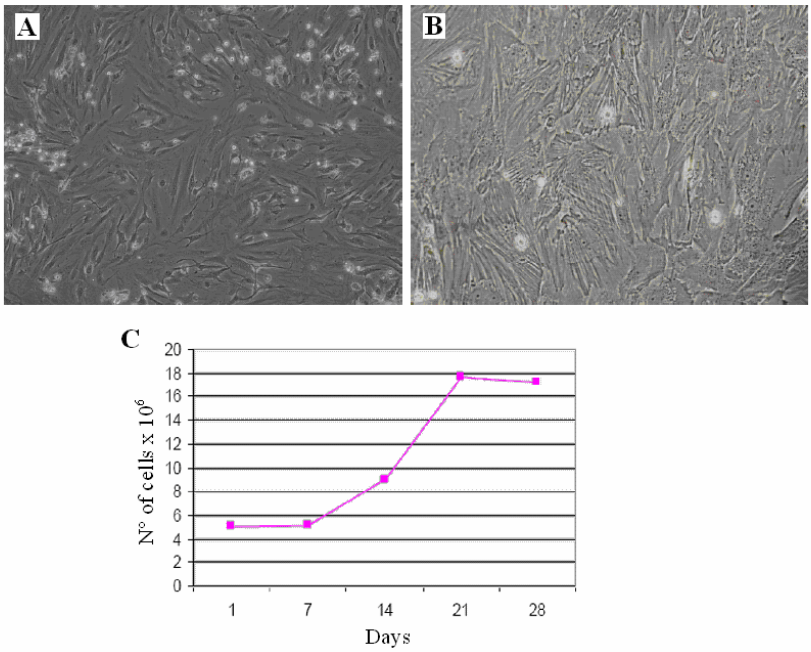

Figure 1. hAMSCs morphology and growth. Cell morphology at passage one (A) and passage three (B), original magnification 10X. Growth kinetics of hAMSCs in culture (C).

3.2. Flow Cytometry and Immunofluorescence Analysis. hAMSCs showed an immunophenotypic profile very similar to that of mesenchymal stem cells derived from bone marrow, i.e. they are positive for CD29, CD44, CD73, CD90 and CD105, and negative for the hematopoietic markers CD34, CD133 and CD45. Freshly isolated hAMSCs showed a low expression of epithelial markers (EpCAM and CD49f), which decreased up to null expression after the first passage (Table 2).

Table 2. hAMSCs membrane marker expression.

	FRESHLY ISOLATED		PASSAGE TWO	
Surface antigens	Median %	Range	Median %	Range
CD45	2	0-4	4	3-4
CD34	0	0-1	0	0-1
CD133	0	0-1	0	0-1
CD13	80	70-89	95	89-99
CD44	81	71-90	90	89-92
CD73	90	88-91	94	88-99
CD90	79	69-89	94	89-99
CD29	76	66-86	98	95-99
CD105	49	30-66	58	40-76
CD166	83	71-95	85	71-98
CD49f	16	13-31	3	2-5
EpCAM	16	12-20	0	0-1
CD31	0	0	ND	ND
CD146	0	0	ND	ND

Data were expressed as median percentage and ranges of three different experiments.

hAMSCs showed the embryonic stem cell associated surface marker SSEA4 (Fig. 2F), while very low expression of molecular markers associated with pluripotent stem cells (Nanog and Oct-4) by flow cytometry was observed (Fig. 2D,E).

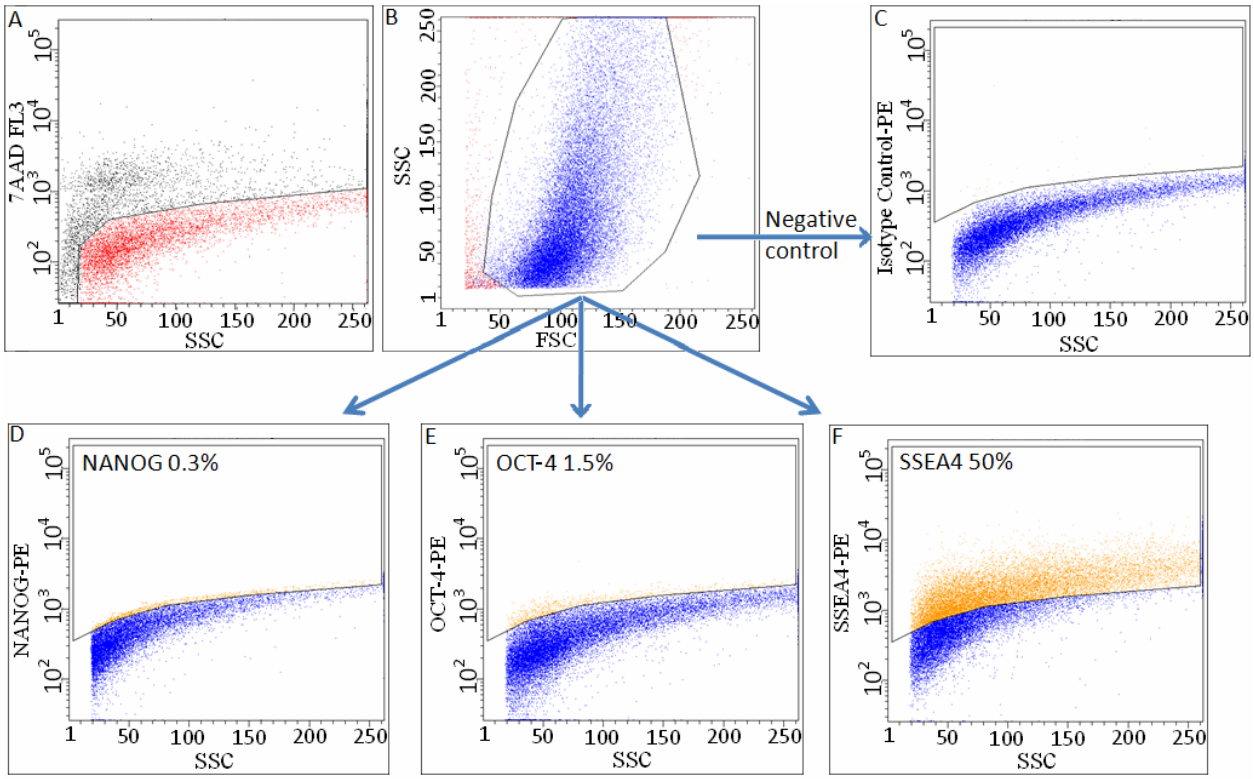

Figure 2. hAMSCs pluripotent stem cells and ESC marker expression. Flow cytometry representative expression of the pluripotent and embryonic stem cells markers in freshly isolated hAMSCs. A) For each staining, a gate on viable cells (red) was drawn; B) hAMSCs were gated on the basis of morphological features; C) cells incubated with isotypic control were used as negative controls; D) Nanog, E) Oct-4 and F) SSEA4 expression in hAMSCs.

Fluorescence microscopy confirmed the positivity for CD29 and CD90, and revealed the expression of other mesenchymal markers such as fibronectin and vimentin (Fig. 3). hAMSCs were almost negative for ZO-1, a marker of tight junctions and cytokeratin (CK) 7, while stained positive for CK18.

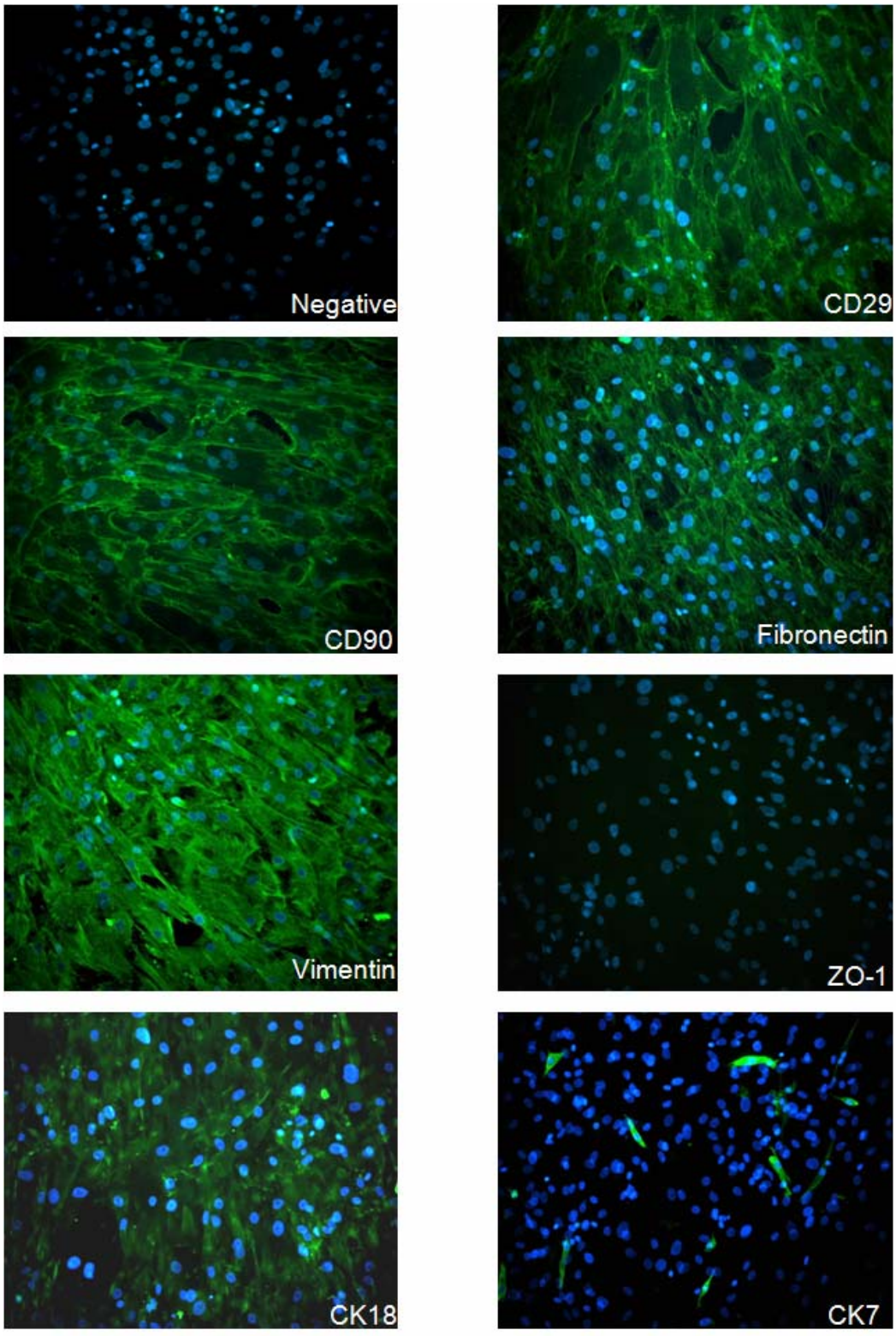
Negative
CD29
CD90
Fibronectin
Vimentin
ZO-1
CK18
CK7

Figure 3. Immunofluorescence characterization of hAMSCs. Representative images of fluorescence microscopy staining. The upper left panel (denoted as "negative") shows cells incubated with the secondary antibody only**.** hAMSCs were positive for CD29, CD90, fibronectin, vimentin, CK 18 and negative for ZO-1 and CK 7. Nuclei were counterstained with DAPI. Original magnification 20X.

3.3. Cell Differentiation Ability. To determine whether hAMSCs could differentiate into adipocytes, cells were allowed to grow to 70% confluence prior to induction. Morphological changes as well as formation of lipid droplets within the cells were noticeable starting from one week post induction and were visualized by Oil-Red-O staining (Fig. 4B). Cells maintained in control medium did not show any sign of adipogenic differentiation (Fig. 4A).

To investigate the osteogenic potential hAMSCs, cells were cultured under appropriate condition for differentiation. The presence of calcium deposits in induced and control cultures was determined by Alizarin Red (Fig. 4D). Cells maintained in control media did not show any change in their morphology and no calcium deposit (Fig. 4C).

Hepatocyte differentiation of hAMSCs was evaluated after 14 days of induction. Cells were incubated for 2 h with diethoxy (5,6) chloromethylfluorescein. The generation of fluorescent products was evaluated by fluorescence microscopy. Although hAMSCs were of mesenchymal origin, they showed signs of hepatocyte differentiation (Fig. 4F). Cells maintained in control medium did not show any sign of hepatocyte differentiation (Fig. 4E).

Moreover, we performed cell immunophenotyping after hepatocyte induction (Fig. 5). After hepatocyte differentiation, the number of cells expressing CK7 increased, while some cells expressed albumin and, weakly, alpha1-antitrypsin. Finally, we observed also the presence of CK19 positive cells. No alpha-foetoprotein expression was detected (not shown).

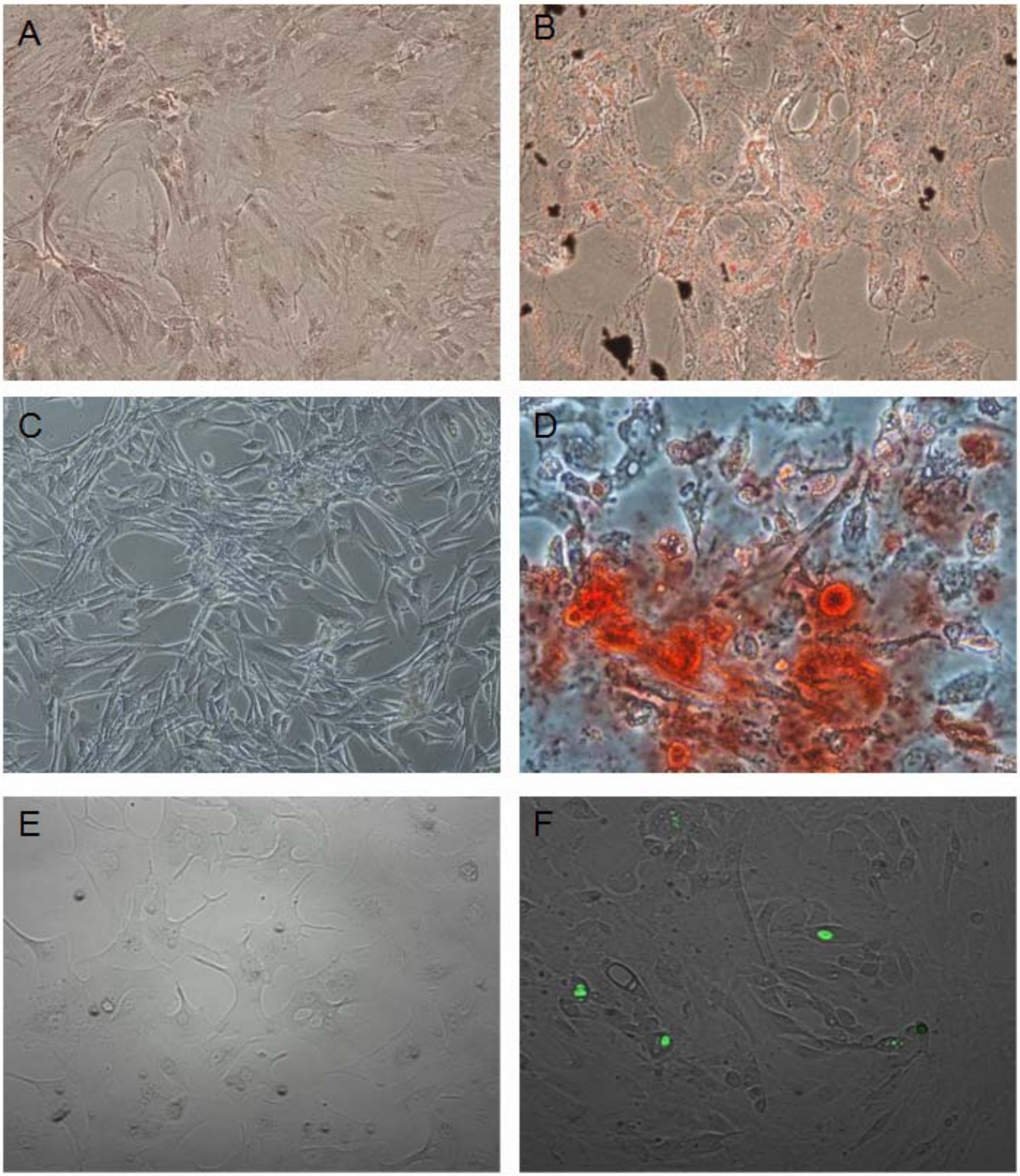

Figure 4. In vitro differentiation capability. Representative images of in vitro osteocyte (B), adipocyte (D) and hepatocyte (F) differentiation of hAMSCs. A), C), and E) represent respective negative controls (i.e. uninduced cells). Original magnification 20X.

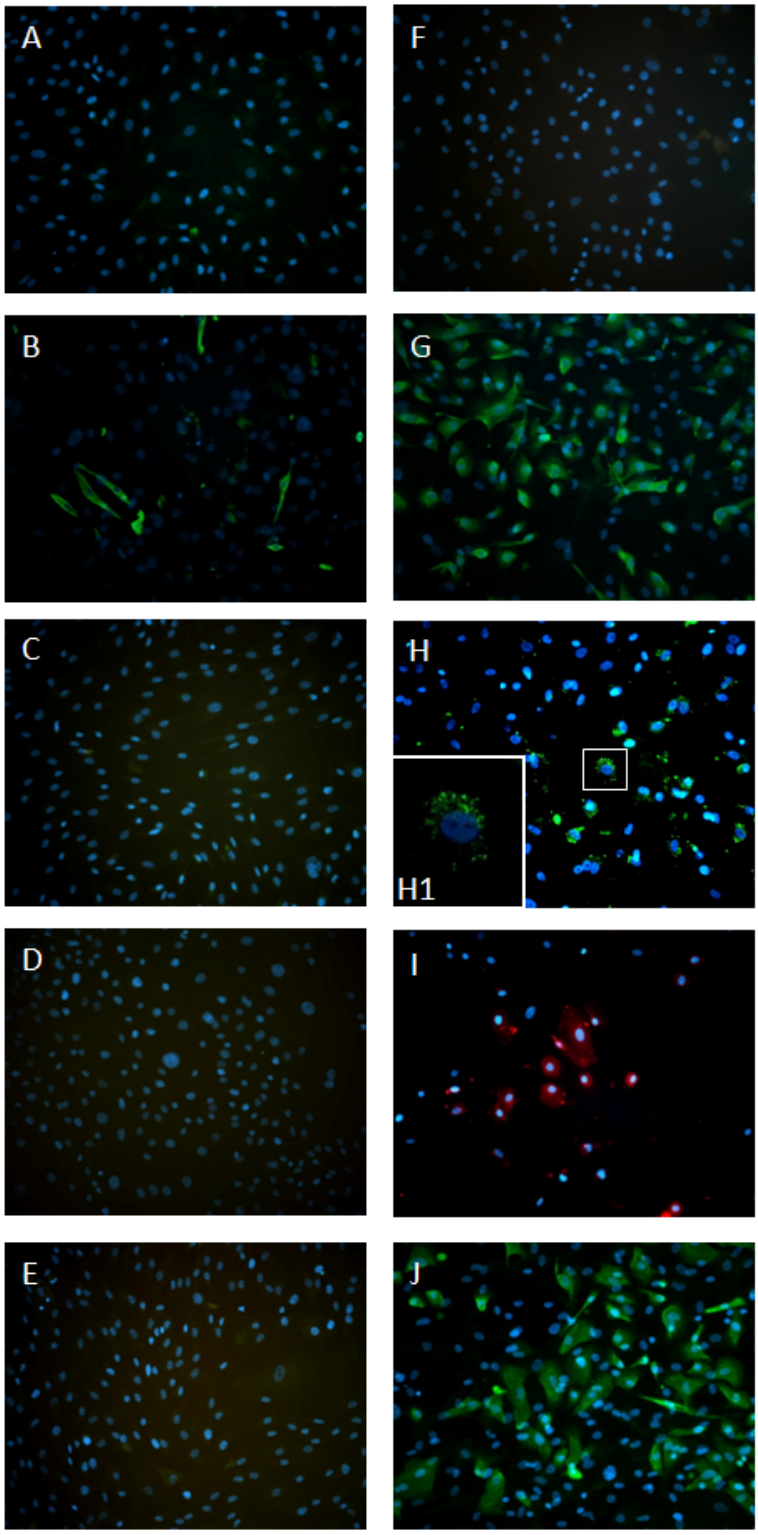

Figure 5. Immunophenotype of mesenchymal stem cells prior (left) and after (right) hepatocyte differentiation for 21 days. (A-F) negative controls, (B-G) CK7, (C-H) albumin, (D-I) alpha1-antitrypsin, (E-J) CK19. Original magnification 20X. The insert H1 represent an enlargement of the cell highlighted by the white square.

3.4. CFTR mRNA Expression. In order to see whether hAMSCs express CFTR mRNA, a semi-quantitative RT-PCR assay was carried out. CFTR was detected in hAMSCs by RT-PCR only after nested PCR (Fig. 6). The expression of CFTR in hAMSC appeared to decrease dramatically during culture. hAECs showed a similar expression of CFTR mRNA when studied upon isolation (Fig. 6).

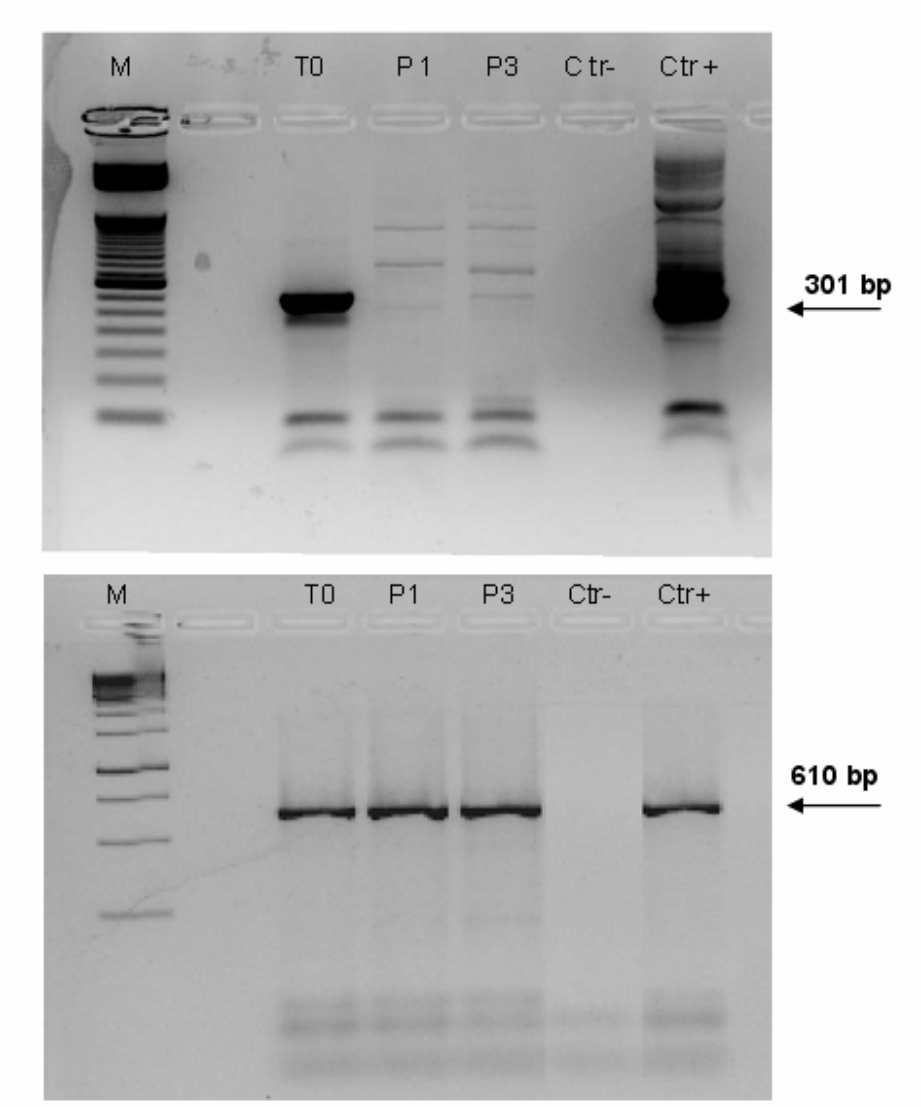

Fig. 6. CFTR mRNA expression in hAMSCs and hAECs. CFTR on hAMSCs and hAECs upon isolation (T0) and on hAMSCs at passages one (P1) and three (P3). Upper panel: CFTR; lower panel: β-actin. M: molecular weight markers; Ctr+: positive control (nasal brushing); Ctr-: negative control (no RT). On the right, arrows indicate the specific band along with PCR-product length.

3.5. 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. In order to analyze the CFTR protein expression in hAMSC-CFBE41o- co-cultures at different ratios, a flow cytometric assay was performed. This mixed population was analyzed after labelling with the CFTR antibody MAB25031 in the absence of permeabilization followed by an incubation with FITC-conjugated secondary antibody. As a positive control, CFTR labelling was assessed in normal human airway 16HBE14o- cells, resulting in 50±5.0% of positive cells, as previously shown [19]. CFBE41o- cells showed less CFTR-specific labelling on the membrane (11% of positive cells), consistent with the lack of CFTR transport on the plasma membrane which is a characteristic of these cells. Plasma membrane CFTR expression was detected in only 6.2% of hAMSCs (Table 3). It was possible to detect an increase of CFTR-specific signal in CM-DiI-labeled cells at all hAMSC-CFBE ratios as compared with hAMSCs cells alone. The lower the ratio of hAMSCs:CFBE41o- the lower the increase in CFTR-specific signal in CM-DiI-labeled cells, these data indicating that a critical number of hAMSCs is important in order to obtain a meaningful effect on CFTR expression. Overall, these data show that a population of hAMSCs with low CFTR expression have increased this expression upon co-cultures with CF epithelial cells.

Table 3. Percentages of CFTR+ hMSCs labelled with CM-DiI in co-cultures with CFBE41o- cells.

	% of CM-DiI$^+$ CFTR$^+$ cells	% of CFTR$^+$ in whole CM-DiI$^+$ population	P
hAMSCs	_	6.2 ±2.0	_
CFBE	_	11.2±1.3	0.0006
hAMSC-CFBE 1:5	12.1±2.5	50.0±6.1	<0.0001
hAMSC-CFBE 1:10	7.5±2.1	46.7±9.3	<0.0001

hAMSC-CFBE 1:15	3.0±0.4	33.2±6.5	<0.0001
hAMSC-CFBE 1:20	2.2±0.9	34.6±8.7	<0.0001

Co-cultures were compared with either unlabelled hAMSCs or unlabelled CFBE cells. Therefore, only the percentages of $CFTR^+$ cells in co-cultures were calculated on the whole $CM\text{-}DiI^+$ population overall. Data are shown as the mean±SD of five experiments. Significance is referred to $CFTR^+$ cells in the whole $CM\text{-}DiI^+$ population in all conditions as compared with hMSCs alone.

To investigate the mechanism underlying the expression of CFTR in hAMSCs after co-cultures with CFBE41o-cells, we performed separate co-cultures of hAMSCs and CFBE41o- cells. Thus, hAMSCs were seeded onto the filter whereas CFBE41o- cells onto the bottom well. After 6 days of culture, hAMSCs were analyzed for CFTR expression by cytofluorimetry. Results showed that at the hAMSCs:CFBE41o- ratios of 1:5 and 1:10 the percentages of CFTR+ hAMSCs were 10.5±3.8 and 11.6±5.0 respectively (n=3). These data, compared with those obtained in direct co-culture conditions (column "% of $CFTR^+$ in whole $CM\text{-}DiI^+$ population" of Table 3), indicate that a direct contact between hAMSCs and CFBE41o- is necessary to obtain a significant increase of CFTR-specific signal in hAMSCs.

3.6. 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 [20], we showed that CFTR protein is expressed on the apical side of 16HBE14o- cells, while CFBE41o- cells display only intracellular staining. CFTR expression and localization was evaluated by epifluorescence with a protocol which allows to detect only surface and not intracellular CFTR (see Materials and Methods section), followed by confocal microscopy analysis. As can be seen in Figure 7, CFTR was highly expressed on the apical membrane of some hAMSCs since red labelled cells showed a green staining at membrane level (Fig. 7B-D), whereas CFBE41o- monolayers in absence of hAMSCs showed essentially no specific signal for CFTR expression (Fig. 7A), consistent with the lack of CFTR transport to the apical membrane in CF cells.

hAMSCs showed a very faint signal related to CFTR (Fig. 7E). These data confirm cytofluorimetric analysis as to the plasma membrane expression of CFTR in labelled hMSCs which increases when co-cultured with CF cells.

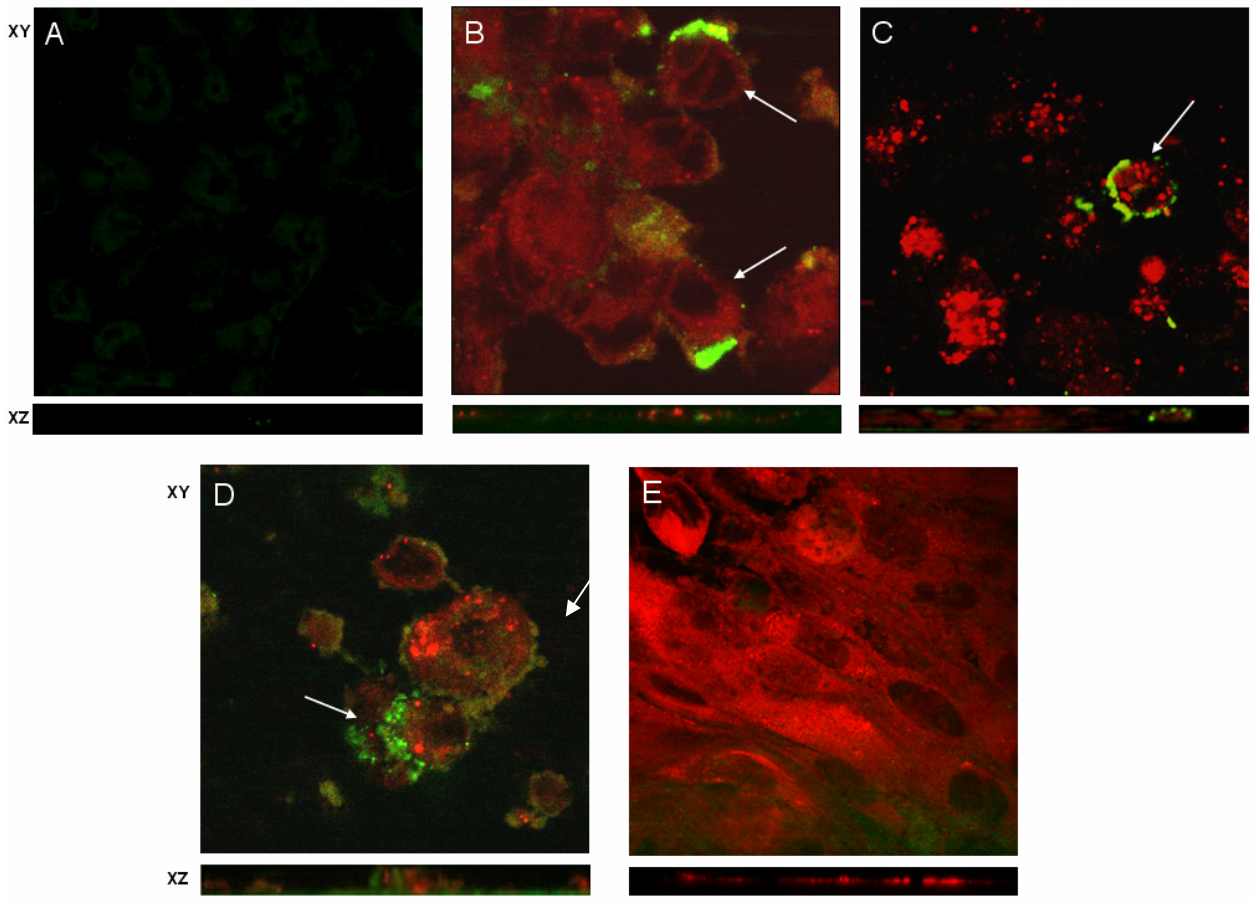

Fig. 7. CFTR immunodetection by confocal analysis. Confocal scans are shown in the horizontal cross-section (xy) plane and vertical cross-section (xz) plane. A) CFBE41o- cells; B) hAMSCs-CFBE 1:5 ratio; C) hAMSCs-CFBE 1:10 ratio; D) hAMSCs-CFBE 1:15 ratio; E) hAMSCs alone. The white arrows point to CM-DiI labelled hAMSCs expressing CFTR on their membrane (green signal). Note in D) that hAMSCs harbour some CFTR-specific signal in discrete regions under the apical plasma membrane.

4. Discussion

Human MSCs are pluripotent stem cells initially identified in postnatal bone marrow (BM) [21], which is the most common source used in clinical settings [22]. However, the use of BM has some limitations, including the low frequency of MSCs and the invasive procedure for obtaining them. Moreover, the age and disease state may affect the collection of sufficient healthy autologous BM for transplantation [23-25]. Finally, expansion of autologous BM cells could represent a cumbersome and low-yield approach. In the present study, we directed our attention on a source, the amniotic membrane, which is rich in MSCs [26], is easily accessible and ethically acceptable, since the term placenta is discarded after delivery. hAMSCs have been shown to be superior in proliferation and differentiation potential to BM cells [18] and to display differentiation potential towards mesoderm lineages (osteogenic, chondrogenic, and adipogenic) similar to BM cells [18, 26, 27]. Importantly, various studies have reported differentiation of hAMSCs to ectoderm (neural) [27, 28], mesoderm (skeletal muscle, cardiomyocitic, and endothelial) [18, 29-31], and endoderm (pancreatic) [32] lineages.

In the present study, we show that hAMSCs displayed a fibroblastic morphology and presented surface markers expressed also by BM-MSCs such as CD29, CD44, CD105, CD73, CD90, and vimentin. They also displayed positivity for the epithelial markers CD49f and CK18; since these markers are lost upon culture, they could represent a small contamination by epithelial cells. This hypothesis is corroborated by CFTR mRNA expression in hAECs upon their isolation from the placenta (as shown in Fig. 6). In alternative, this hybrid phenotype of hAMSCs is interpreted as a sign of pluripotency, and suggests that the amnion-derived cells had not completely differentiated into epithelial or mesenchymal cells [3]. Nevertheless, as shown here, the amniotic cells derived from term placenta seem to remain somewhat “plastic” and maintain the capability to differentiate and contribute to cells from different germ layers. In

particular, we demonstrated that hAMSCs can differentiate into mesodermal (adipocyte and osteocyte) and, although to a lower extent, into endodermal (hepatocyte) lineages. To the best of our knowledge, only one previous study has demonstrated that hAMSCs can differentiate into hepatocyte-like cells, although only at gene-expression level [33].

Because amniotic cells can differentiate to different cell types, we examined them with antibodies directed against well-known surface markers characteristic of embryonic stem cells. Amniotic cells express the stage specific embryonic antigen SSEA-4 [34], although the relative proportion of SSEA-4-positive cells in initial isolates is lower than that observed with embryonic stem cells [35]. In addition to characteristic stem cell surface markers, amniotic cells show very low expression of Oct-4 and Nanog, transcription factors involved in regulating ES cells' self-renewal and differentiation, as it has been previously shown for freshly isolated MSCs obtained from bone marrow, adipose tissue and heart [36]. Further studies are needed to understand whether these genes are regulated during the in vitro culture conditions, so to identify regulatory pathways that mimic in vivo activation.

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 [7]. 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 MSCs, endothelial progenitor cells, and circulating fibrocytes, can localize to the lung and acquire phenotypic and functional markers of mature lung-specific cells [10, 11, 37]. The results published by Wang et al. [38] and Loi et al. [8] strongly suggest that the population of BM cells relevant for repopulating the lung epithelium may be found in the plastic adherent stromal cell compartment. Besides the drawbacks presented by BM-MSCs discussed above, amniotic fluid contains a

heterogeneous population of cells from fetal origin [26], whereas MSCs could not be reliably isolated from all term umbilical cordon blood samples [10, 39-41].

In the present study, we propose human placenta as an ethical source of MSCs for CF therapy. The first goal was to investigate the CFTR expression in these cells. At the earliest stages of human development, CFTR protein and function have been detected in early blastocysts in the apical membrane of trophoectoderm cells, while its expression at mRNA level has been shown in first trimester placenta (8 weeks gestation) [42]. However, no data are available concerning its mRNA and protein levels in specific cell types of term placenta. In this study, we show that a nested RT-PCR was necessary for obtaining a detectable signal from freshly isolated hAMSCs, indicating very low levels of CFTR mRNA in these cells. We have recently shown that also hematopoietic stem/progenitor cells display such low levels upon purification from the bone marrow [19]. The reason why CFTR must be kept at low expression levels in stem/progenitor cell compartments is not known at the moment. Although freshly isolated hAMSCs did show CFTR expression by semi-quantitative RT-PCR, CFTR mRNA was barely present at passages one and three. Confocal microscopy confirmed these results at the protein level. Notably, CFTR was re-expressed by hAMSCs upon co-culture with epithelial cells, as demonstrated unequivocally by flow cytometry and confocal microscope analysis. At this stage, we do not know why the lower the hAMSCs:CFBE41o- ratios the lower the CFTR expression in hAMSCs. It can be speculated that this effect might be due to cross talk between amniotic and epithelial cells, for which a critical number of hAMSCs is needed. Indeed, in other co-culture systems, developed with MSCs and chondrocytes, it has been shown universally that the more chondrocytes the lower the expression of extracellular matrix genes and functional properties of engineered cartilage [43, 44].

Also, the mechanism underlying this effect is to be discovered yet. However, indirect co-cultures data give us an indication that this effect is primarily due to the contact

between amnion MSCs and epithelial cells, and not to factors acting by a paracrine manner. Lung morphogenesis is an orchestrated molecular and cellular process controlled by cellular interactions with growth factors and morphogenic factors [45]. Since the cellular interactions between epithelial and mesenchymal cells in monolayer co-culture are likely to be bi-directional, a possible mode of action could be cross talk between cells via gap junctions, which has been observed in vivo in the lung between transplanted MSCs and resident epithelial cells [46]. Recently, it has been found that MSCs could be induced to differentiate into corneal epithelium [47] or endothelium [48] in co-culture condition, but not in the indirect co-culture system where MSCs and endothelial cells were cultured in separate inserts [48]. More importantly, BM-MSCs acquired an airway epithelium phenotype when co-cultured with respiratory epithelial cells and determined a partial resumption of the chloride secretion defect in CF epithelia [38]. Although we have not analyzed the correction of the chloride transport defect in CFBE14o- monolayers by hAMSCs, based on the work by Wang et al. [38], it can be anticipated that we should see the same effect on the basic electrophysiological defect. Furthermore, since only 6-20% of corrected cells is needed to revert the basic defect in chloride secretion [49], our data showing that 33-50% of hAMSCs acquired CFTR expression shed a positive light on the use of amnion MSCs in the CF treatment.

5. Conclusions

Our data indicate hAMSCs as a novel, promising, readily accessible, and ethically compatible source of pluripotent cells, that could be used in regenerative medicine. In this respect, hAMSCs present promising features as indicated by their expression of embryonic stem cell markers such as SSEA4 and by their differentiation potential towards mesodermal and endodermal lineages.

Although CF is a clinically heterogeneous disease caused by a defect in the CFTR gene affecting multiple organ systems, major morbidity and mortality are given by the lung disease; however hepatobiliary complications of CF are increasingly common and clinically relevant as the age of patients increases [50, 51]. This study shows the differentiative potential of hAMSCs towards airway epithelium and hepatocytes, which might be useful in CF, and highlights the need for further investigations to elucidate the mechanism mediating CFTR expression in hAMSCs upon cell to-cell interactions.

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