Chapter 1

Placenta-Derived Stem Cells as a Source for Treatment of Lung and Liver Disease in Cystic Fibrosis

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1. Introduction

In the first part of this chapter we will summarize the main clinical aspects of cystic fibrosis as well as the pathophysiology of lung and liver diseases, with particular reference to the role of airway and biliary duct epithelia, where the cystic fibrosis gene is expressed. In the second part we will describe the main features of placenta-derived stem cells and their potential use for the treatment of lung and liver diseases in cystic fibrosis.

1.1. Cystic fibrosis

Cystic fibrosis (CF) is an autosomal recessive disease of epithelia in the lung, 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* (cystic fibrosis transmembrane conductance regulator) gene, the product of which is a protein expressed in the apical membrane of most epithelia. This membrane protein 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]. However, liver disease is also increasing as the life span of these individuals becomes longer.

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 hyper-absorption of Na^+ and impaired Cl^- and $HCO3^-$ secretion in airway epithelial cells and cholangiocytes leads to a disturbance of the fluid lining the airways and the bile ducts [6-10].

1.1.1. The CFTR gene

The *CFTR* gene was identified in 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 nucleotide sequences together with its promoter 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 [11].

The *CFTR* 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, hematopoietic stem/progenitor stem cells (HSPC), alveolar macrophages, and smooth muscle cells [12-14]. In addition to its typical plasma membrane location, CFTR was also found in membranous organelles such as lysosomes of alveolar macrophages [15] and in both apical and basolateral membrane of the sweat duct [16].

Although over 1,900 different mutations in the *CFTR* gene are known (Cystic Fibrosis Mutation Database, http://www.genet.sickkids.on.ca/cftr/Home.html), approximately 66% of the patients worldwide carry the F508del mutation (a deletion of three nucleotides that results in a loss of phenylalanine at position 508 of the CFTR protein) with somewhat higher prevalence in Western Europe and USA [17]. This type of mutation causes an incorrectly assembled CFTR protein resulting in endoplasmatic reticulum (ER) retention and degradation of the protein [18] as well as defective regulation [19]. Patients homozygous for F508del usually have more pronounced clinical manifestations compared to heterozygotes and genotypes without F508del [20-22] although these differences are highly variable [23].

1.1.2. The CFTR protein

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 [24, 25]. 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 [26]. Like other ABC transporters CFTR contains two membrane-spanning domains (MSDs), two hydrophilic nucleotide-binding domains (NBDs) located at the cytoplasmic site of the protein, and, as a unique feature among ABC transporters, a regulatory domain (R domain) located between NBD1 and MSD2. The R domain contains several consensus phosphorylation sites for protein kinases A (PKA) and C (PKC) [27]. 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 [28]. Activation of PKA causes the phosphorylation of multiple serine residues within the R domain leading to conformational changes in this domain [29] relieving its inhibitory functions on CFTR

channel gating [30]. Once the R domain is phosphorylated, channel opening requires binding of cytosolic ATP. NBD1-NBD2 dimerization induces channel opening, whereas ATP hydrolysis at the NBD2 induces dimer disruption and channel closure [24, 31, 32]. Finally, channel activity is terminated by protein phosphatases that dephosphorylate the R domain and return CFTR to its quiescent state [28].

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 [33] through reciprocal regulatory interactions between CFTR and the SLC26 chloride/bicarbonate exchanger [34] and loss of this mechanism contributes to both airway and pancreatic-duct disease in CF [33, 35]. CFTR enhances ATP release by a separate channel [36], not yet identified [37]. 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 [36, 37]. Furthermore, transport of glutathione is directly mediated by CFTR, which is essential for control of oxidative stress [38]. The interaction between CFTR and epithelial sodium channel (ENaC) is of crucial importance for lung disease development (see below). CFTR downregulates calcium-activated chloride channels (CaCC) [39], and stimulates outwardly rectifying chloride channels [40]. Other channels regulated are the volume-regulated anion channel [41] and ATP-sensitive K_{ATP} channels such as inwardly rectifying outer medullary potassium channels [42].

Regulatory sites on NBD1 interact with several of the above processes. For example, NBD1 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, 43]. Several studies also have identified a short stretch of amino acids (-DTRL-) at the COOH terminal end, forming a PDZ binding domain [1, 44]. 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 [44, 45].

2. The 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 [46]. It can produce mucus, and can release mediators of the immune system such as lysozyme, lactoferrin, mucous glycoprotein, immunoglobulins, chemokines, cytokines, lectins and β-defensin (cationic antimicrobial peptides) [47, 48].

Furthermore, the airway epithelium produces antioxidants such as glutathione and ascorbic acid [49]. Aside from these protective functions it also regulates the airway physiology via

production of smooth muscle relaxant factors such as prostaglandin E_2, nitric oxide and enzymes, which catabolize smooth muscle contractile agonists [50, 51].

In normal human airways the surface epithelium is on average 50 μm thick and rests on a basement membrane. 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 [52].

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) [53]. In addition brush cells (columnar with microvilli only) have been identified in the respiratory tract from nose to alveoli [54]. Scattered along the respiratory tree, various progenitor niches are present in the airway epithelium [55].

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 the volume regulation of airway surface liquid (ASL) and CF [56-59]. However, these findings have later been debated. It has been demonstrated that normal (but not the F508del) 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 [60]. 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.

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 [61].

Fluid absorption is mainly controlled by the transport of Na^+ through apical ENaC, which is also the dominant basal ion transport process. *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 tight junctions [61].

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) [62]. 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 [63]. 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 [64]. 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^+ [65].

2.2. The airway surface liquid

ASL, the fluid covering the airway epithelium, consists of a periciliary layer (PCL), which is a watery layer surrounding the cilia, and of mucus on top of the cilia. 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) [66], whereas the ASL layer (mucus plus PCL) varies in thickness of 20-150 μm for different species (20-58 μm in humans) [67]. 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 [68], see also paragraph 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 epithelial 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 [69].

2.3. Pathogenesis of CF lung disease

The lung of CF patients is normal at birth, but soon after birth an endobronchiolitis ensues with surprisingly few pathogenic bacterial species (*Pseudomonas aeruginosa* in most cases), and which is associated with an intense neutrophilic response localized to the peribronchial and endobronchial spaces [70-72]. The neutrophil-dominated inflammatory response is harmful for the host by causing exaggerated production of inflammatory cytokines and proteases which may sustain infection [73]. CF primarily affects the airways and submucosal glands with sparing of the interstitium and alveolar spaces until late in the disease [74, 75]. The CF lung disease is characterized by a picture of airway epithelial injury [76] and remodeling, such as squamous metaplasia [77], cell hyperproliferation [78], basal and goblet cell hyperplasia, and hypersecretion of mucus due to the inflammatory profile [79-81]. 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 [82].

There are several hypotheses about the early pathogenetic steps in the CF lung disease and how defective CFTR leads to the airway disease:

- *The low ASL volume hypothesis* claims that the ASL is isotonic both normally and in CF. CFTR functions both as a Cl^- channel and as an inhibitor of the ENaC. 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 [83].

- *The high salt hypothesis* suggests that the ASL normally is hypotonic [84] 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 when CFTR is defective or absent [84].
- *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 [85]. All the above factors may inhibit mucociliary clearance (MCC) and thus elimination of bacteria from the airways [86].
- *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 *P.aeruginosa* [87].
- *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 [88].
- *The soluble mediator hypothesis* proposes that signalling molecules within the ASL itself are controlling ASL volume [89]. These molecules are ATP, which is breathing- or shear-stress induced [90], 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 [91, 92]. 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 [93]. In this tissue, CFTR is co-localized with AQP9 in the apical membrane, and this association promotes the activation of AQP9 by cAMP [94]. In a heavily debated study, concerning the clinical benefit of nebulized hypertonic saline in cystic fibrosis, an important role of amiloride-inhibitable AQP water channels in the generation of ASL was proposed [95]. 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 [96, 97] and is still open. Recently, it has been found that interleukin (IL)-13 enhances the expression of CFTR but abolishes the expression of AQP in airway epithelial cells [98]. In conclusion, the relation between CFTR and AQP needs further study.

The differences in the proposed hypotheses 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 there 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 [99].

Furthermore, 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 [100]. It is also possible that variant forms of ENaC or different regulatory components operate in different systems [101].

3. The biliary duct epithelium

The biliary tree is a complex network of conduits within the liver that begins with the canals of Hering and progressively merges into a system of ducts, which finally deliver bile to the gallbladder and to the intestine. Cholangiocytes are the epithelial cells forming the biliary epithelium which shows a morphological heterogeneity that is strictly associated with a variety of functions performed at the different levels of the biliary tree [102]. Thus, the canals of Hering, located at the ductular-hepatocellular junction, constitute the physiologic link of the biliary tree with the hepatocyte canalicular system and they are the site where a facultative progenitor cell compartment resides; these liver progenitor cells are variably elicited only after liver injury. Given the strong capacity of mature hepatocytes to proliferate, cholangiocyte ability to behave as liver progenitor cells becomes evident only when hepatocellular proliferation is hampered as a result of severe liver damage, as that induced by several toxins or drugs, or occurring under certain conditions, *i.e.* viral hepatitis or non alcoholic steatohepatitis [103]. Cells lining the intrahepatic biliary tree have different functional and morphological specializations: the terminal cholangioles (size <15 μm) have some biological properties such as plasticity (i.e., the ability to undergo limited phenotypic changes) and reactivity (*i.e.*, the ability to participate in the inflammatory reaction to liver damage); interlobular (15-100 μm) and large ducts (100 μm to 800 μm) modulates fluidity and alkalinity of the primary hepatocellular bile.

3.1. Ion and water transport in cholangiocytes

In addition to funnelling bile into the intestine, cholangiocytes are actively involved in bile production. In humans, around 40% of the total bile production is of ductal origin. Cholangiocytes exert a series of reabsorptive and secretory process which dilute and alkalinize the bile during its passage along the biliary tract. Modifications of ductal bile appear to be tightly regulated by the actions of nerves, biliary constituents, and some peptide hormones like secretin [104]. Accordingly to *in vivo* and *in vitro* models, it is possible to distinguish between three different bile flow fractions: 1) the canalicular bile salt-dependent flow that is driven by concentrative secretion of bile acids by the hepatocytes followed by a facilitated efflux of water; 2) the canalicular bile salt-independent flow, which is also created by hepatocytes but through active secretion of both inorganic (bicarbonate) and organic (glutathione) compounds; and 3) the ductal bile flow, that is the bile salt-independent flow contributed by cholangiocytes, mainly through production of a bicarbonate-rich fluid in response to secretin and other regulatory factors. Cl^- secretion into the ductal lumen is the driving force of a chloride/

bicarbonate exchanger that exports HCO_3^- into the bile flowing into the biliary tree. Indeed, this AE (anion exchanger) activity is facilitated by the outside to inside transmembrane gradient of Cl^- at relatively high intracellular concentrations of $HCO3^-$, specially upon secretin stimulation. The AE activity in the liver is operated by AE2/SLC4A2 which is localized not only in the canaliculi but also in the luminal membrane of bile duct cells [105]. Experiments of RNA interference with recombinant adenovirus expressing short/small hairpin RNA have confirmed that AE2/SL4A2 is indeed the main effector of both basal and stimulated Na^+-independent Cl^-/HCO_3^- exchange in rat cholangiocytes [106]. Besides acid/base transporters cholangiocytes possess other ion carriers like those for Cl^-, Na^+, and K^+, which greatly contribute to intracellular pH regulation and bicarbonate secretion. Thus, CFTR had been localized at the apical side, where it plays a role in biliary excretion of bicarbonate [107, 108]. Although bicarbonate permeability through activated CFTR has been shown in several epithelia [109], its main contribution to biliary bicarbonate secretion appears to occur through a coordinated action with AE2/SL4A2 [106, 110, 111]. In addition to CFTR, cholangiocytes possess a dense population of Ca^{2+}-activated Cl^- channels. These channels are responsive to interaction of the purinergic-2 (P2) receptors with nucleotides (mainly ATP or UTP) [112, 113]. The apical fluxes of anions results in increased osmotic forces in the bile duct lumen which in the presence of AQPs contributes to water flux. AE2/SLC4A2 and CFTR colocalize with AQP1 in cholangiocyte intracellular vesicles wich coredistribute to the apical cholangiocyte membrane upon both cAMP and secretin stimulations [114].

3.2. The pathogenesis of CF liver disease

CF is associated with liver disease in almost 30% of all patients. In general, CF-associated liver disease develops during the first decades of life and does not progress rapidly. The diagnostic criteria were initially established by Colombo et al. [115]. Hepatobiliary disease in CF encompass a wide variety of complications, including steatosis, focal biliary cirrhosis (FBC), multilobular biliary cirrhosis (MBC), microgallbladder, distended gallbladder, cholelithiasis, intraheapatic sludge or stones, and cholangiocarcinoma [116]. The pathogenesis of steatosis (fatty liver) is not directly ascribed to the CFTR gene defect but has been attributed to malnutrition, essentially fatty acid deficiency, carnitine or choline deficiency, or insulin resistance [117].

With regarding to the pathogenesis of FBC and MBC, various hypotheses have been proposed [118, 119]:

- *The low chloride secretion hypothesis* proposes that loss of CFTR function leads to blocked biliary ductules with thick periodic acid-Schiff positive material leading to acute and chronic periductal inflammation, bile duct proliferation and increased fibrosis in scattered portal tracts. Hepatic stellate cells (important drivers of hepatic fibrosis) become activated to produce collagen and stimulate the bile duct epithelium to produce the profibrogenic cytokine TGF-β. The progression of FBC to MBC and portal hypertension, which occurs in up to 8% of patients, may take years to decades, and should be viewed as a continuum [120]. Considering CFTR as a driving force for Cl^-/HCO_3^- exchange, the postulated sequence of CF-associated hepatobiliary complications is that loss of functional CFTR protein in the

apical membrane of cholangiocytes presumably initiates a cascade of abnormal Cl^- and HCO_3^- secretion, decreased bile flow, bile duct plugging by thickened secretions, and cholangiocyte/hepatocyte injury [10].

- *The cholangiocyte damage hypothesis* has been put forward by the studies of Freudenberg et al. in the F508del mouse model for CF [121]. These mice present with increased fecal loss of bile acids and a higher bile salt-to-phospholipid ratio in cell membranes, which was found to be associated with damage to intrahepatic bile ducts determining increased permeability of unconjugated bilirubin into cholangiocytes. They suggest that cholangiocytes injury is caused by a more hydrophobic bile acid pattern and an increased detergency from augmented bile salt-to-phospholipid ratio caused by hyperbilirubinbilia. In addition, lower gallbladder pH values and elevated calcium bilirubinate ion products in bile of CF mice raise the likelihood of supersaturating bile and forming black pigment gallstones [122].
- *The purinergic hypothesis* suggests that CFTR regulates the release of ATP into the bile duct lumen which regulates cholangiocyte secretion via the activation of the purinergic P2Y receptors [123]. Accordingly, Fiorotto et al. [124] have demonstrated that the choleretic effect of ursodeoxycholic acid (UDCA) is mediated via CFTR-dependent ATP secretion.
- *The mechanosensitive pathway hypothesis* indicates that the mechanical effects of fluid flow or shear stress at the apical membrane of biliary epithelial cells results in stimulation of ATP release and Cl^- secretion [123, 125]. The decreased bile flow due to CFTR dysfunction may be associated with alterations in mechanosensitive pathways which exacerbate abnormalities in Cl^- secretion and bile formation [123, 125].
- Finally, *the biliary HCO_3^- umbrella hypothesis* postulates that adequate apical biliary HCO_3^- secretion would appear crucial for protection of cholangiocytes against uncontrolled invasion of protonated bile acid monomers from bile via apical membranes into the cholangiocyte interior, inducing damage and apoptosis [126]. The Cl^-/HCO_3^- exchanger AE2/SLC4A2 and an intact glycocalyx appear to be crucial for the biliary HCO_3^- umbrella [127].

4. Placenta-derived stem cells

The placenta is a highly specialised organ, about 15 to 25 centimetres in diameter, that plays an important role in maintaining normal pregnancy and supporting the normal growth and development of the fetus. It is made up of a fetal and a maternal component: the fetal component include amnion and chorion as well as the chorionic plate, from which chorionic villi extend and make intimate contact with the uterine decidua during pregnancy; the maternal part of the placenta is the decidua basalis and it derived from endometrium.

As reported by Parolini et al. [128], different cell types can be isolated from the regions of the placenta:

- human amniotic epithelial cells (hAEC),
- human amniotic mesenchymal stromal cells (hAMSC),

- human chorionic mesenchymal stromal cells (hCMSC),
- human chorionic trophoblastic cells (hCTC).

In several studies hAEC, hAMSC, and hCMSC have been isolated and characterized for phenotypic and pluripotency molecular markers; moreover, has been demonstrated that these cells display differentiation potential and immunomodulatory effects [129].

hAEC express a pattern of mesenchymal markers while are negative for those of hematopoietic origin ($CD90^+$, $CD73^+$, $CD105^+$, $CD44^+$, $CD29^+$, $CD45^-$, $CD34^-$, $CD14^-$, $HLA\text{-}DR^-$), and these cells are capable to differentiate in vitro into cell types of all 3 germ layers [128]. Like the amniotic epithelial fraction, the human amniotic and chorionic mesenchymal regions display the same pattern of phenotypic markers of bone marrow (BM) MSC, also displaying the expression of pluripotency markers (such as *Oct*-4) and the capability to differentiate toward different lineages including osteogenic, adipogenic, chondrogenic, and vascular/endothelial [128].

Placenta-derived stem cells seems to have a multipotent potential towards other cell types different from mesenchyme cells. hAMSC and hCMSC were shown to differentiate in vitro into a range of neuronal, oligodendrocyte and astrocyte precursors [130-132]. In addition, the use of amniochorionic membrane as a scaffold has been proposed for improving osteogenic differentiation of chorionic membrane-derived cells [133]. Alviano and colleagues reported that hAMSC display the ability to differentiate into endothelial cells in vitro [134]. Recently it has been shown that hAEC can differentiate in vitro in cells with hepatic characteristics, in particular in cells with the ability to differentiate into parenchymal hepatocytes as well as biliary cells that form duct-like three-dimensional structures when cultured on extracellular matrix [135]. hAMSC were demonstrated to differentiate into hepatocyte-like cells as judged by functional and phenotypic markers [136].

As regard the osteogenic and adipogenic differentiation of hAEC and hAMSC, discrepant results have been reported [137, 138], most likely due to the heterogeneous nature of these cell populations and due to the need to isolate the right population of progenitor cells from placental tissues. In this respect, recent efforts have been dedicated to optimizing isolation, culture, and preservation methods for placenta-derived cells; these include a study to determine the quantity and quality of amnion cells after isolation and culture [138], while other studies aimed to define long-term expansion methods to obtain a large cell population for analysis before use in cell-based therapies.

Sources such as amnion tissue offer outstanding possibilities for allogeneic transplantation due to their high differentiation potential and their ability to modulate immune reaction. Limitations, however, concern the reduced replicative potential as a result of progressive telomere erosion, which hampers scalable production and long-term analysis of these cells. The establishment and characterization of human amnion-derived stem cells lines immortalized by ectopic expression of the catalytic subunit of human telomerase (hTERT) resulted in continuously growing stem cells lines that were unaltered concerning surface marker profile, morphology, karyotype, and immunosuppressive capacity with similar or enhanced differentiation potential for up to 87 population doublings [139].

Interestingly, two groups found a more reliable and unlimited non-animal source for large-scale expansion of hMSC for future allogeneic clinical use: they cultured MSC with animal-free culture supplements such as human platelet lysate (PL), a suitable alternative to fetal calf serum (FCS) showing that these cells exhibit an increased proliferation potential and in vitro life span compared to cells cultured with FCS [140, 141]. On the other hand, it has been demonstrated that phenotypic shift of hAEC in culture is associated with reduced osteogenic differentiation in vitro, therefore different culturing methods may influence cell behavior [137].

In a recent comparative phenotypical study, BM- and placenta-derived mesenchymal cells has been shown that have a very similar morphology, size and cell surface phenotype for characteristics MSC markers [142]; in contrast, differences in proliferation potential have been observed between these two cell types [142]. Another study found different expressions of the chemokine receptors CCR1 and CCR3, which are only present on placenta-derived cells, while the adhesion molecules such as CD56, CD10, and CD49d have been shown to be more highly expressed on placenta-derived mesenchymal cells [143]. On the basis of numerous studies in the literature which clearly show the lack of significant differences between BM- and placenta-derived mesenchymal cells types, and on the basis of the fact that placenta is readily and widely available, a good manufacturing practice-compliant (GMP) reagents and protocols has been established for isolating and expanding human placenta-derived MSC that can be directly translated to the clinical trial setup [144].

4.1. Immunomodulatory features of placenta-derived stem cells

Since the placenta is fundamental for maintaining fetomaternal tolerance during pregnancy, the cells present in placental tissue may have immunomodulatory characteristics; this aspect contributes to make cells from placenta good candidates for possible use in cell therapy approaches, with the possibility of providing cells that display immunological properties that would allow their use in an all-transplantation setting.

It has been demonstrated that cells derived from placenta are negative for the expression of major histocompatibility complex (MHC) class II and for co-stimulatory molecules; all this is reflected as immune tolerance [128, 145]. Furthermore, these cells possess remarkable immunosuppressive properties and can inhibit the proliferation and function of the major immune cell populations, including dendritic cells (DCs), T cells, B cells and natural killer (NK) cells. Most of these studies have been recently summarized in up-to-date reviews [146-148]. Here, we give a brief account of the major findings concerning hAMSC.

Numerous studies showed that amniotic and chorionic membrane-derived cells can suppress the T lymphocyte proliferation induced by alloantigens, mitogens, anti-CD3 and anti-CD28 antibodies in *in vitro* and *in vivo* models [149-152]. The suppression of lymphocyte population was shown to be not dependent on cell death but on decreased proliferation and increased numbers of regulatory T cells [145]. Inhibition of T cell proliferation by placenta-derived stem cells appears to be mediated by both cell–cell interaction [153] and release of soluble factors such as indoleamine 2,3-dioxygenase (IDO), transforming growth factor β (TGF-β), and IL-10 [145, 154, 155]. The immunosuppressive activity of hAMSC on T cells seems to be not only direct but involves also DCs. Indeed, cells derived from the

mesenchymal region of human amnion impaired the differentiation of monocytes into DCs by inhibiting the response of the former to maturation signals, reducing the expression of co-stimulatory molecules and hampering the ability of monocytes to stimulate naive T cell proliferation [156]. The mechanism involved is not known, however, this inhibitory effect might be mediated via soluble factors, like IL-6, and may be dose-dependent, as it has been shown for BM-derived MSCs [157] (Figure 1).

This immune-privileged status of placenta-derived stem cells has been indicated as the cause of lack of rejection in allo- and xeno-transplantation settings. In this regard, several studies examined the fate of amniotic membrane derived stem cells grafts. Wang et al. [158] studied allogeneic GFP^+ mouse intact amniotic epithelium grafts heterotopically transplanted in the eye. Kubo et al. [159] studied xenotransplanted human amniotic membrane in the eye of rats. Several preclinical studies have already reported prolonged survival of human placenta-derived cells after xenogeneic transplantation into immunocompetent animals including swine [152] and bonnet monkeys [128], with no evidence of immunological rejection.

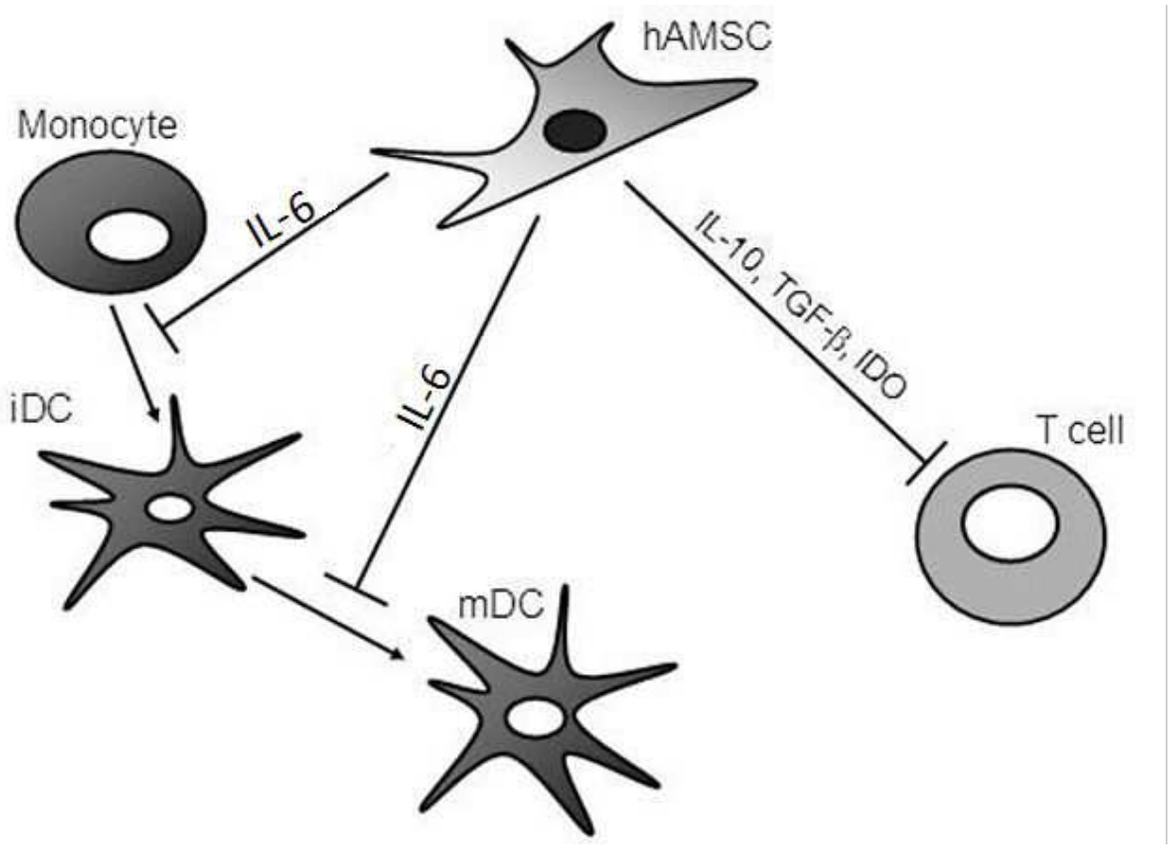

Figure 1. Effects of placenta-derived stem cells on immunocytes. Placenta-derived cells exert immunomodulatory effects both on dendritic cells and T cells. Their inhibitory role is dependent on cell–cell contact and secreted soluble factors. Since most of the studies have focused on hAMSC, this cell type is represented in the scheme. iDC: immature dendritic cell; IDO: indoleamine 2, 3-dioxygenase; IL-6: interleukin-6; IL-10: interleukin-10; mDC: mature dendritic cell; TGF-β: transforming growth factor β.

4.2. Clinical application of placenta-derived stem cells

More than once century ago, Davis was the first to report the use of the amniotic membrane (AM) to heal skin wounds [160], prompting subsequent applications in the treatment of leg ulcers [161, 162] and burns [163], as well as for applications in ophthalmology [164]. These studies have suggested that placenta-derived stem cells may be useful for treating a range of pathologic conditions, including neurological disorders [165-167], spinal cord injury [128, 168], critical limb ischemia [169], inflammatory bowel diseases [170], and myocardial infarction [171]. Here, we will focus on the potential application of placenta-derived stem cells to lung and liver, the major organs interested by CF.

5. Potential application of placenta-derived stem cells to CF

5.1. Placenta-derived stem cells for lung diseases

The first report demonstrating a therapeutic effect of placenta-derived stem cells in lung diseases is that by Cargnoni and colleagues [172]. In a mouse model of bleomycin-induced lung injury, transplantation of fetal membrane-derived cells resulted in a reduction in the severity of pulmonary fibrosis. This result was obtained when cells were administered either systemically (intravenous or intraperitoneal) or locally (intratracheal) 15 min after intratracheal bleomycin instillation and in two different settings, *i.e.* either using allogeneic or xenogeneic (a mixture of 50% human amnion/chorion mensenchymal stem cells and 50% hAEC) cells. Although the inflammatory score was not decreased, a reduction in the number of infiltrating neutrophils was observed. It is worth noting that that the presence of neutrophils is known to be associated with poor prognosis in idiopathic pulmonary fibrosis in humans [173]. The question arises whether these anti-inflammatory and anti-fibrotic effects may be due to the engraftment of placenta-derived stem cells or to the secretion of soluble factors. In this study allogeneic or xenogeneic cells were detected in the injured lung of transplanted mice, although not in a quantitatively fashion, by means of PCR analysis, and these results are in accordance with those obtained by Bailo and colleagues, who demonstrated microchimerism upon transplantation of human amnion and chorionic cells in neonatal swine and rats [152]. The release of soluble factors has been addressed in a further study. The administration of conditioned medium generated from hAMSC to bleomycin-treated mice determined a reduction in lung fibrosis scores in terms of fibrosis distribution, fibroblast proliferation, collagen deposition and alveolar obliteration [174]. This study support the increasing evidence that MSC isolated from various sources produce bioactive molecules, so that injection of conditioned medium obtained from MSC could be an effective experimental treatment for different tissue injuries [175, 176]. Further studies are therefore warranted to elucidate the mechanisms of action of placenta-derived cells in this model, in particular paracrine factors that act to down-regulate neutrophil recruitment.

It has to be said that the role of exogenous stem cells in pulmonary fibrosis is controversial, meaning that some studies have demonstrated that these cells can act as a potential source of fibroblast, which may accentuate the fibrotic process [177]. Since these findings were obtained

with BM-derived stem cells, it should be further assessed if a similar behaviour is presented by amniotic-derived stem cells. Of note, placenta-derived cells did not exert any profibrotic effect after their transplantation [172].

In vitro studies have so far demonstrated that co-cultures of hAMSC and CF epithelial cells originated from bronchi can elicit CFTR protein expression in 33-50% hAMSC, in front of 6% prior to the co-cultures, and the lower the hAMSC:CFBE41o- ratios the lower the CFTR expression in hAMSC [136]. Indirect co-cultures data indicate that this effect is primarily due to the contact between hAMSC and epithelial cells, and not due to factors acting by a paracrine manner. BM-MSC 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 [178]. Preliminary analysis of the chloride transport defect in co-cultures between CF cells and hAMSC showed a partial correction of the chloride efflux (Carbone et al., unpublished results). Furthermore, since only 6-20% of corrected cells is needed to revert the basic defect in chloride secretion [179], our data showing that 33-50% of hAMSC acquired CFTR expression shed a positive light on the use of amnion MSCs in the CF treatment. Overall, these data point out to a cross talk between amniotic and epithelial cells, for which a critical number of hAMSC is needed. Indeed, in other co-culture systems, developed with MSC 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 [180, 181]. 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 MSC and resident epithelial cells [182].

Overall, the potential usefulness of placenta-derived stem cells in CF lung disease might be either in the correction of the early basic defect (chloride transport) or in late remodelling events (pulmonary fibrosis).

5.2. Placenta-derived stem cells for liver diseases

Several preclinical studies have reported to date that placenta-derived stem cells can engraft into the liver and perform hepatic functions *in vivo*. Takashima and colleagues [183] showed 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. Sakuragawa and colleagues [184] showed that the transplantation of hAEC transduced with the β-galactosidase gene into the livers of SCID mice resulted in detection of β-galactosidase-positive cells at 1 week after transplantation, indicating that the transplanted cells had been integrated into the hepatic parenchyma within a few days [184]. More recently, it has been shown that six months after transplantation of hAEC into the livers of SCID/beige mice that had been pretreated with retrorsine, most mature liver genes were expressed at levels comparable to those of authentic human adult livers, including the major CYP genes, other metabolic enzymes, plasma proteins, and hepatocyte-enriched transcription factors and genes encoding hepatic-transporter proteins [185].

These studies provide compelling evidence in support of the functional hepatic potential of hAEC *in vivo*, thereby supporting the potential of hAEC as a useful tool for liver regeneration in the future.

MSC represent an alternative tool for the establishment of a successful stem-cell-based therapy of liver diseases [186] with preliminary clinical improvements in acute and chronic hepatic diseases [187, 188]. To date, several studies on animal models reported the beneficial effects of MSC in promoting hepatic tissue regeneration [189]. Overall, a number of different mechanisms contribute to the therapeutic effects exerted by MSC, among which their differentiation into functional hepatic cells. However, these studies have not provided definitive evidence that MSC have a capability to differentiate into functional hepatocytes *in vivo* [190]. Rather, the observed improvements could be attributed to the known property of MSC to produce a series of growth factors and cytokines that could suppress inflammatory response, reduce hepatocytes apoptosis, regress liver fibrosis, and enhance hepatocytes functionality [191, 192].

Although numerous studies have reported that BM-derived MSC can reduce carbon tetrachloride (CCl_4)-induced liver fibrosis in mice, the mechanism by which MSC repair the fibrosis is unclear, and the results are controversial [190, 193-197]. One possibility is that MSC differentiate into hepatocytes, because of the *in vivo* niche, and secrete growth factors that promote liver regeneration. Another possibility is that MSC suppress hepatic stellate cells activity and secrete metalloproteinases (MMPs), thereby eliminating deposition of extracellular matrix [198]. It has been demonstrated that fibrosis, infiltration of neutrophils, synthesis of collagen I and α-smooth muscle actin (α-SMA), and expression of inflammatory were all reduced by infusion of isogenic MSC [199]. It is possible that these responses were partly due to the upregulation of cytoglobin expression by hepatic stellate cells, which protect against oxidative stress and controls tissue fibrosis and at the same time inhibits the activation of those cells to become myofibroblasts [200]. Finally, it has been demonstrated that intravenous administration of MSC caused an increase in IL-10 mRNA in the liver and protein in the blood in a CCl_4-induced liver fibrosis rat model [201]. IL-10 is an inhibitor of many cytokines that stimulate liver fibrosis, such as IL-6, TNF-α and TGF-β, all downregulated by the MSC infusion. In addition, IL-10 can suppress tissue inhibitor of metalloproteinase (TIMP)-1 expression and thereby relieve MMP-1 to degrade liver collagen deposits [202, 203].

In a recent study, hAMSC were infused in mice with CCl_4-induced hepatic cirrhosis and exerted various beneficial effects such as reduction of hepatic stellate cell activation, decrease of hepatocyte apoptosis, and reduction of hepatic fibrosis [204]. Infusion of hAMSC also depressed hepatocyte senescence and resulted in engraftment of hAMSC into the host liver as judged by the expression of the hepatocyte-specific markers, human albumin and α-fetoprotein. Finally, a study demonstrated that human AM, when applied as a patch onto the liver surface, reduced progression of experimental biliary fibrosis induced in rats by the biliary duct ligation procedure [205]. Again, a beneficial effect related to the release of soluble factors by the human AM patch has been invoked, since no massive (or at least very low/undetectable) engraftment of AM-derived cells occurred in the host liver.

6. Conclusion

Placenta-derived stem cells are endowed with interesting features that are important for choosing them as a source for approaches aimed to regenerative medicine: immune-privileged status, secretion of biomolecules with anti-scarring and anti-inflammatory properties, and, least but not last, no ethical concerns. Although the AM and AM-derived stem cells have been used in the clinics for over one hundred years, their employment in lung and liver diseases is coming on the stage only in the last few years. Placenta-derived stem cells have been recently more thoroughly characterized for their phenotype, multipotency and expression of pluripotency genes.

In CF, lung disease has been the target first of gene therapy approaches brought to the clinical stage [206, 207], hesitated in a slow progression due to limited efficiency of gene transfer vectors and pathophysiological barriers, and then of stem cell-based experimental treatments in mice [208]. Despite a very low level of engraftment of donor HSPC into the nose and the gut, significant CFTR mRNA expression and a measurable level of correction of the electrophysiological defect were observed after transplantation of wild-type marrow cells into CF mice [209]. It is uncertain whether this effect is due to the presence of CFTR-expressing epithelial cells derived from donor cells or to the paracrine effects of transplanted cells. Other sources, such as umbilical cord blood, embryonic stem cells, and induced pluripotent stem cells are being evaluated [210, 211]. Recent *in vitro* data on the acquisition of CFTR expression by hAMSC indicate placenta-derived stem cells as a possible source for treating the early phases of CF lung disease. Anyhow, caution should be taken when stem cell-based therapies are proposed for an inflammatory disease like that of CF lung, in view of the fact that these cells could be immunosuppressive and/or contribute to the inflammatory process. There is no available information concerning the immunomodulatory effects of placenta-derived stem cells in CF lungs.

Liver fibrosis is a common outcome of a variety of chronic liver diseases following different insults, including the biliary disorder occurring in CF. Orthotopic liver transplantation remains the only viable therapeutic option to treat CF patients with hepatic cirrhosis, and hepatocyte transplantation has never been attempted in this disease. The use of progenitor cell transplantation is emerging as a potential alternative, and several potential sources have been identified for the isolation of these cells [212]. For the treatment of liver cirrhosis, this approach has been performed mainly with BM-derived MSC [213, 214]. Given the drawbacks related to the use of BM-derived MSC (limited frequency, invasive procedure, age and disease state affecting the collection of healthy autologous BM), placenta-derived stem cells could represent a prime candidate for the treatment of liver fibrosis, since they are immunotolerated, can be isolated and produced at high yield, and do not provoke ethical debate. AM and AM-derived stem cells have been demonstrated to halt the progression of liver fibrosis and its evolution towards cirrhosis, but the long-term safety and therapeutic efficacy are not known yet, which warrant further studies. Moreover, optimal therapeutic regimens for clinical application of placenta-derived stem cells, such as optimal doses, transplantation route and interval period for transplantation should be evaluated in detail [215].

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