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Original article

Stem/progenitor cells in the developing human lung

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Abstract

Human lungs are composed of more than 40 cell types. The lung is classified as a "conditionally renewing" tissue and is able of a quick response to cellular damage thanks to the presence of multiple stem/progenitor cells. Embryonic and fetal progenitors actively proliferate determining lung size, shape and cellular composition and could be of paramount importance in understanding lung development and mechanisms of congenital diseases. Furthermore, developmental molecular pathways may be chronically or aberrantly activated in tumorigenesis or in lung diseases later in life. Lungs have a mixed endodermal and mesodermal origin. Endoderm progenitors are early marked by TTF1. Other reported markers of endodermal respiratory progenitors are Sox2, Sox9 and Id2. Proximal versus distal differentiation is guided by the expression of Fgf10. Little is known about mesodermal stem/progenitor cells in the developing lung. A signaling interplay among endoderm, mesoderm and mesothelium plays a role in the signaling network. Our preliminary data stress the importance of the interaction of the endodermal component, giving rise to tubular structures, and the mesodermal component, that showed the

tendency to acquire a concentric onion-like or solid or nests arrangement. Subpleural niches appeared in strict contact with the thin pleural mesothelium and were formed by a complex array of cell types. At immunohistochemistry, nuclear reactivity for TTF1 was found in the interior layer of the epithelial tips, while the endodermal component of the developing lung showed positivity for SOX-2, and CD34 revealed the newly formed vascular structures inside the lung mesenchyme. In conclusion, the complexity of the histological picture of the developing human lung is emphasized by the multiple stem/progenitor cells. The endodermal and mesodermal ones, through complex processes of differentiation, act together in order to give rise to a huge number of cell types.

Keywords

Stem cell, progenitor, human, lung, endoderm, mesoderm, morphogenesis.

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Background

Human lungs are complex organs, composed of more than 40 cell types [1]. From a regenerative point of view, the lung is classified as a "conditionally renewing" tissue [2]. In fact, its cell turnover is very slow in basal conditions, but it speeds up in case of injury [3]. The lung surface area is constantly exposed to a large number of environmental hazards, including oxidants, pollutants, drugs and pathogens [4]. Adult lungs are therefore capable of a quick response to cellular damage thanks to the presence of multiple populations of resident lung stem/progenitor cells [4, 5]. Different regions of the lung are characterized by different niches of progenitors, and it has furthermore been postulated that lung stem/progenitor populations behaviour may differ in damage response and homeostasis mainteinance. These progenitor cells have been detected in different experimental models [2] and multiple niches of lung epithelial stem cells have been identified [6, 7]. Briefly, in the proximal airways of the mouse lung, basal cells and Clara cells are able to restore ciliated cells, while submucosal gland duct progenitors are able to give rise to submucosal gland cells. In the bronchioli, neuroendocrine cells are able to give rise to Clara and ciliated cells, while variant Clara cells can replenish Clara cells. At the broncho-alveolar duct junction (BADJ), besides variant Clara cells, bronchoalveolar stem cells (BASCs) can give rise to bronchiolar cells, but also to alveolar colonies. In the distal lung, alveolar type 2 cells are able to restore alveolar type 1 cells. Basal cells and alveolar type 2 cell have as well been confirmed as stem/progenitor cells in the adult human lung [2, 8]. It is interesting to notice that all niches reside in areas of abundant vascularization [8]. Less is known about mesenchymal stem/progenitor cells of lung structures such as vascular smooth muscle cells, airway smooth muscle cells, endothelial cells, and fibroblasts. In recent years, resident progenitors sharing some common features with bone marrow-derived mesenchymal stromal cells have been identified in the lung [9]. In summary, it has long been held that, in contrast to rapidly renewing organs, the adult lung is apparently able to maintain itself without the aid of an undifferentiated stem cell population [3]. On the contrary, a recent landmark and controversial work by Kajstura and coworkers has claimed the existence of c-kit+ uncommitted stem cells in the adult human lung [10]. However, there is no consensus on these results in the literature [11-15].

Turning to the developmental period, it is clear that stem cells should be viewed from a different perspective. While adult stem cells rarely activate in basal conditions and their aim is to maintain cell turnover homeostasis, embryonic and fetal progenitors actively proliferate and the number of their divisions determines lung size, shape and cellular composition. It has actually been postulated that adult lung epithelial stem cells and embryonic progenitors might represent distinct cell populations [16]. Embryonic stem/progenitor cells could be of paramount importance in understanding lung development and mechanisms of congenital diseases such as bronchopulmonary dysplasia and congenital lung hypoplasia, especially in preterm newborns, whose stem/progenitor cells prematurely come into contact with the postnatal environment and thus may not function properly [16, 17]. Furthermore, developmental molecular

pathways may be chronically or aberrantly activated in tumorigenesis or in lung diseases later in life, hence much is to be discovered in this research field [6]. The aim of this paper is to give an update on stem/progenitor cells in the developing human lung and, in particular, to focus on the morphological and immunohistochemical features of stem/progenitor cells that can be of great interest in the preterm human newborn.

Stem/progenitor cells in the developing human lung: who? where? how many? how to find them?

Lung morphogenesis has been extensively described in the mouse [18] and some data have been confirmed in humans. Lungs have a mixed origin: while endodermal-derived progenitors give rise to epithelial precursors and later bronchi, bronchioles, saccules and alveoli, mesodermal-derived mesenchymal precursors generate the non-epithelial structures of the mature lung, e.g. blood vessels, lymphatics, interstitial and muscular cells. Furthermore, endodermal-mesodermal interactions are necessary for adequate lung development. A few components of the mature lungs have a different origin, including nerves from ectoderm and alveolar macrophages from bone marrow-derived monocytes. In the following sections, endoderm and mesoderm progenitors will be taken into consideration (**Fig. 1**).

Endoderm progenitors

TTF1 (thyroid transcription factor-1, also called Nkx2.1) is generally considered the earliest marker of endodermal respiratory commitment and pulmonary specification in the ventral foregut. In the mouse embryo it appears as early as E 8.5 days, while in the human lungs its expression starts after 28 days of gestation [18]. A member of the homeodomain-containing transcription factor family, TTF1 plays a pivotal role in lung development, particularly in the process of branching morphogenesis [19]. In humans, TTF1 protein has been detected by immunohistochemistry in the tips of lung buds at 11-12 weeks of gestation. In particular, immunostaining for TTF1 has been reported in subsets of epithelial cells, i.e. nonciliated bronchiolar cells, distal air spaces lining

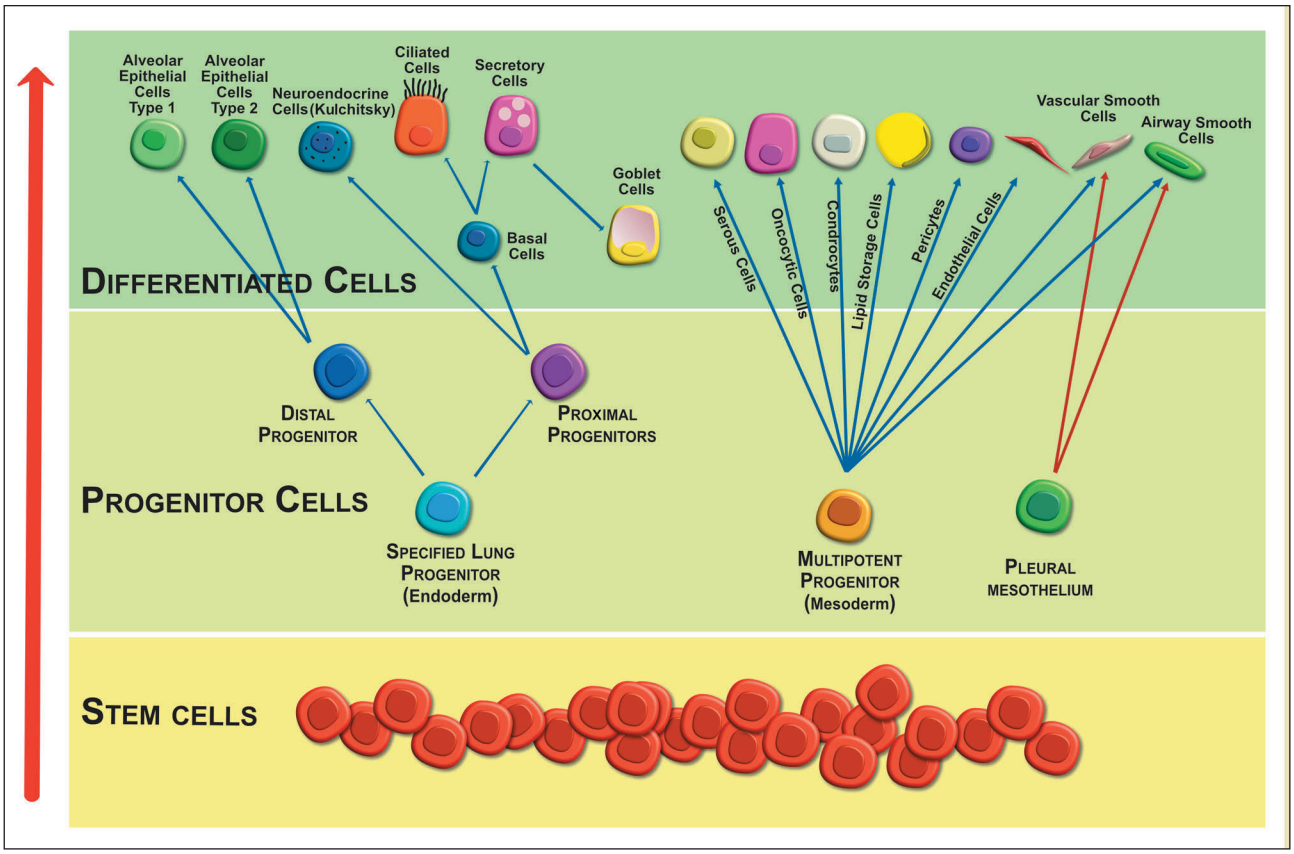

Figure 1. Schematic rappresentation of the differentiation process of stem/progenitor cells in the developing human lung.

cells, and rarely in nonciliated bronchial cells. By midgestation, TTF1 expression has been reported in epithelial cells of distal bronchiolar and terminal airways, while at term it has been described in subsets of nonciliated distal bronchiolar cells and alveolar type 2 cells [20-22].

Other markers of endodermal respiratory progenitors well characterized in the mouse embryo are Sox2, Sox9 and Id2 [16, 18]. During branching morphogenesis, starting at 6 weeks in the human embryo, multipotent cells at the distal tip of the lung buds start to give rise to distinct proximal and distal stem/progenitor cells niches, leading to distinct cell lineages. In the mouse, Sox 2+ proximal progenitors give rise to neuroendocrine (Kulchitsky) cells and non-neuroendocrine cells, including ciliated, secretory (club) and goblet cells. Sox 9+/Id2+ distal progenitors generate alveolar epithelial type 1 and type 2 cells [16, 18]. Proximal versus distal differentiation is guided by the expression of Fgf10 in the surrounding mesenchyme and entails a relative movement of distal Sox9+ epithelial progenitor cells, that assume more proximal positions along the developing airway while the epithelial bud grows, becoming Sox2+ and Sox9- [6, 23]. As stem/progenitor cells differentiate, a pool of them is preserved in the distal lung, at the tip of the branching airways, in order to ensure continued growth [9, 24].

Mesoderm progenitors

As for adults, less is known about mesodermal stem/progenitor cells in the developing lung. A signaling interplay between endoderm and mesoderm is once more of paramount importance for the development of mesodermal structures [1]. Furthermore, mesothelium also plays a role in the signaling network. Along this line, a model of separate early mesenchymal compartments has been proposed, as mesodermal submesothelial and subepithelial areas are exposed to and respond to different signaling factors [25]. Mesoderm stem/progenitor cells have been described in the mouse at the distal tip of branching airways. These Twist2+, Foxf1+ and Tbx4+ cells are supposed to give rise to Pdgfra+ airway smooth muscle cells, Pdgfrb+ pericytes of blood vessels, endothelium and vascular smooth muscle cells [1]. The development of pulmonary vasculature is still debated and is probably a result of angiogenesis arising from embryonic vessels present at the distal tip of branching airways, and of vasculogenesis from mesodermal hemangioblasts [26]. CD31+ endothelial cells have been described in human embryos as soon as at 28 days of gestation in the mesenchyme surrounding the ventral diverticulum. Furthermore, ephrin B4 and ephrin B2 have been described as markers of lung arteries and veins in human embryos, starting at 44-47 and 56-84 days of gestational age, respectively [27]. Undifferentiated mesodermal precursors in close proximity to the endothelium plexus would also give rise to vascular smooth cells [1]. Still, some Authors propose the existence of a common multipotent mesodermal precursor for both heart and pulmonary vascular systems [28]. Recent conflicting evidence in mouse regarding a possible derivation of mesenchymal cell lineages from pleural mesothelium has sparked interest. According to this hypothesis, pleural mesenchymal cells would undergo mesothelial-mesenchymal transition and generate smooth muscle cells of vessels and airways [17, 29, 30]. Finally, the group of Kajstura et al. reported the existence of uncommitted stem cells in human embryos as well. These c-kit+ cells, expressing NANOG, OCT3/4m SOX2 and KLF4, have been found in combination with progenitors of epithelium, endothelium and smooth muscle progenitors and would give rise to cell lineages of both endodermal and mesodermal origins [10]. However, a subsequent work identified c-kit expression in early fetal lung as a marker restricted to a progenitor population of cells of endothelial committment, coexpressing CD34, VEGFR2 and Tie 2 [31]. Clearly, further research is needed to clarify these promising yet contrasting data.

Our preliminary data

Our preliminary data are relative to the histological and immunohistochemical analysis of the lungs in four human fetuses, ranging from 12 to 14 weeks of gestation. At these gestational ages, the human lung appeared characterized by the interaction of two main components: the endodermal component, giving rise to tubular structures, whose lumen appeared covered by large epithelial precursors, and the mesodermal component that, under induction of endodermal cells, showed the tendency to acquire a concentric onion-like arrangement (**Fig. 2**). In the interstitial spaces, at some distance from the dichotomously proliferating epithelial ducts, it was possible to identify some nests of undifferentiated

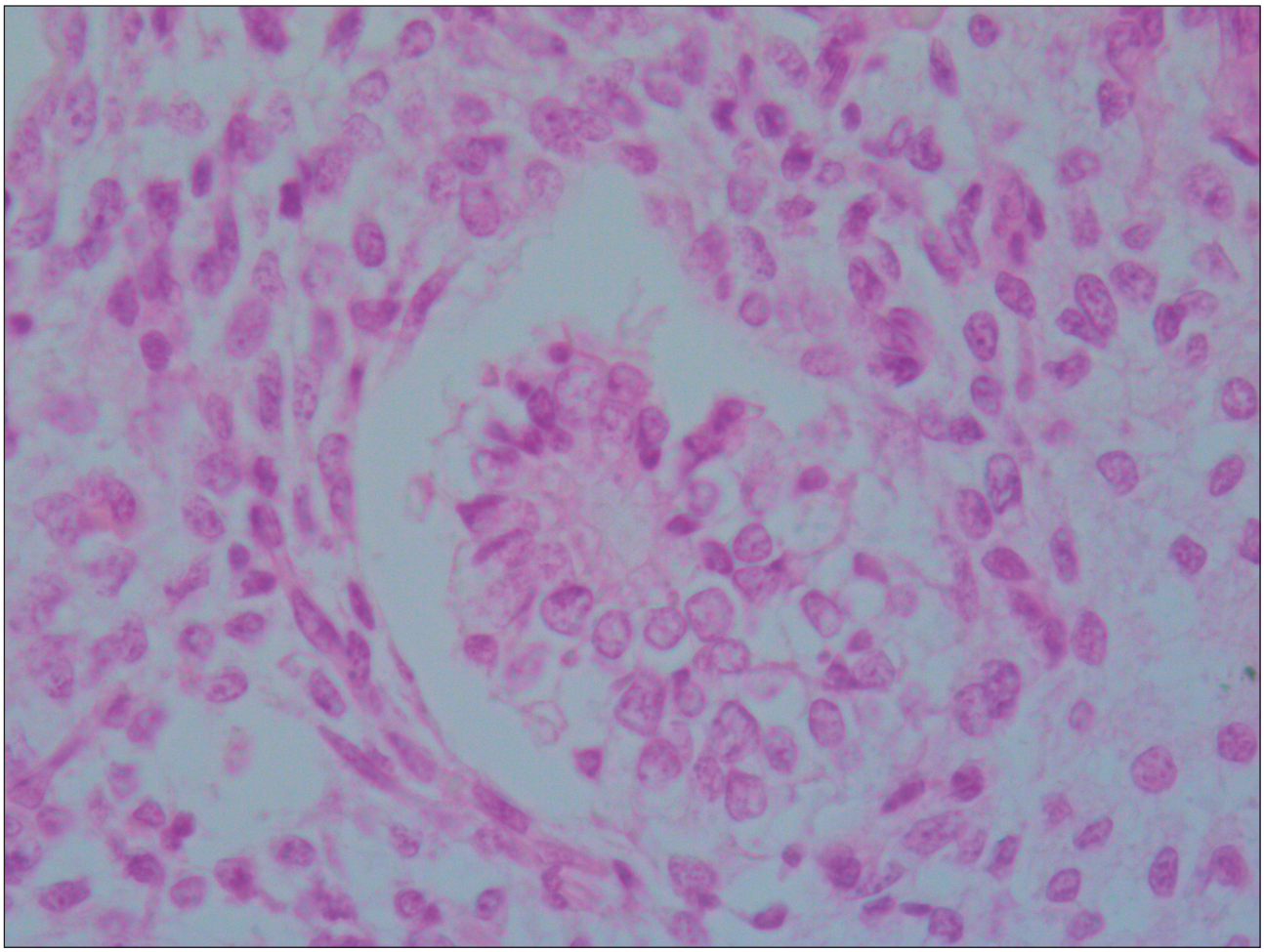

Figure 2. The concentric onion-like arrangement of mesodermal component under induction of endodermal cells.

mesenchymal stem/progenitor cells (**Fig. 3**). Pulmonary stem/progenitor cells were characterized by a large nucleus, with open chromatin, and showed the tendency to overlap. Stem cell borders were indistinct. Among these stem/progenitor cells, it was possible to identify small cells with dark nuclei, putatively representing stromal cells involved in the composition of the cell niche of these endoderm-derived progenitors (**Fig. 3**). When we focused the histological analysis on the periphery of the developing lungs, it was possible to easily identify the majority of pulmonary stem cell niches (**Fig. 4**). Subpleural niches appeared in strict contact with the thin pleural mesothelium and were formed by a complexity of cell types. In the center of the niches, we frequently observed the bud of epithelial precursors, around which the mesenchymal precursors gave rise to a concentric arrangement (**Fig. 4**). In some niches, the endodermal-derived cell component of the lung niche was characterized by a solid pattern, in the absence of a clearly defined lumen (**Fig. 5**). The mesenchymal precursor component of the stem cell niche showed a complex composition: large cells with oval clear nuclei, putatively stem/precursor cells, were admixed with smaller cells with roundish nuclei and with elongated fibroblast-like cells, probably representing different stages of differentiation toward the multiple non-epithelial components of the mature human lung (**Fig. 5**). Other sub-pleural niches showed a completely different pattern: they were characterized by a roundish or oval shape, had a superficial location and pushing margins on the pleural surface and were formed exclusively by mesenchymal cells, in the absence of any epithelial component (**Fig. 6**). The tips of the endoderm-derived tubular structures, one of the main component of the lung stem cell niche, appeared in strict contact with the mesenchymal stem/progenitor cells (**Fig. 7**). In the absence of any well-formed basal membrane, it was impossible, at histology, to detect a border between the epithelial and the mesenchymal progenitors. Moreover, focusing on the endoderm-

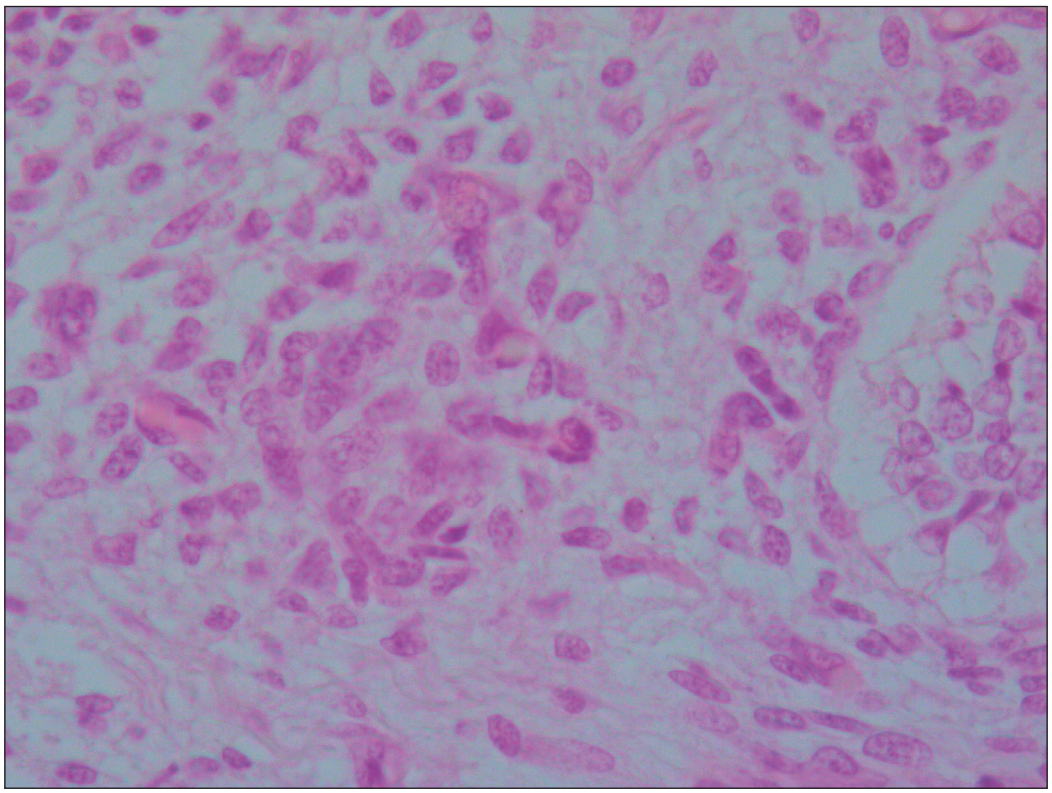

Figure 3. Nests of undifferentiated mesenchymal stem/progenitor cells (characterized by a large nucleus, with open chromatin, tendency to overlap, indistinct cell borders) and small stromal cells (with dark nuclei, putatively involved in the composition of the cell).

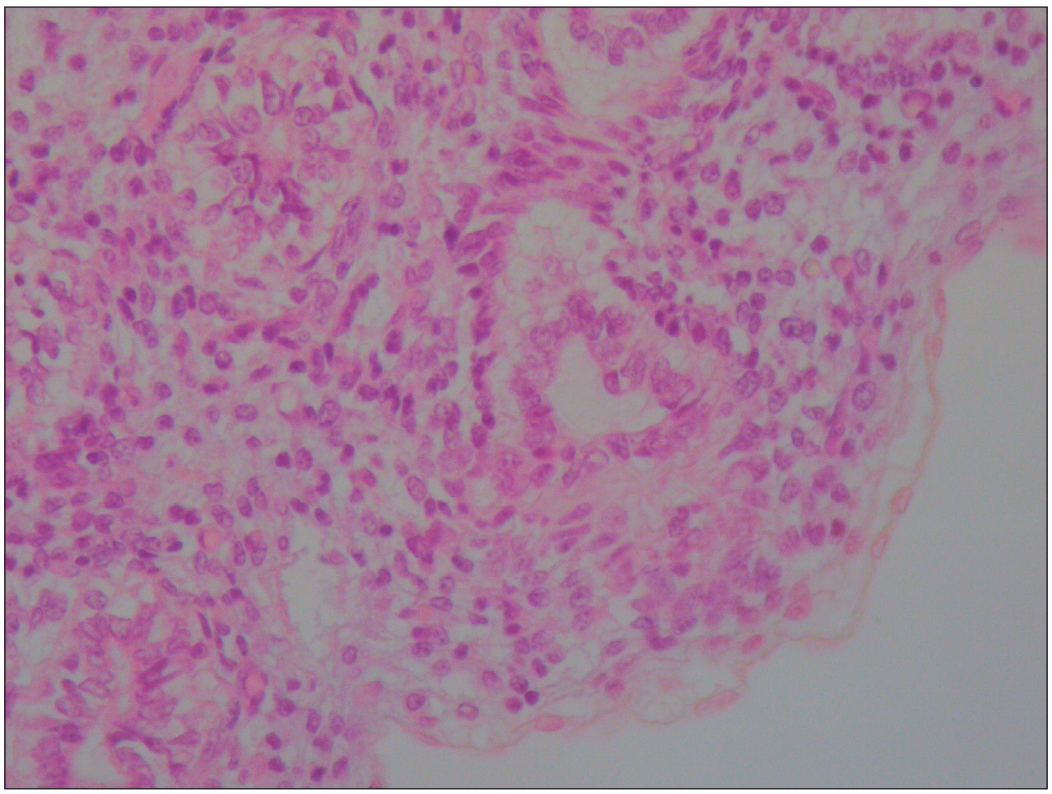

Figure 4. The periphery of the developing lungs, where the majority of pulmonary stem cell niches are easily identified, formed by a complexity of cell types, with the bud of epithelial precursors in the center of the niche and the mesenchymal precursors giving rise to a concentric arrangement.

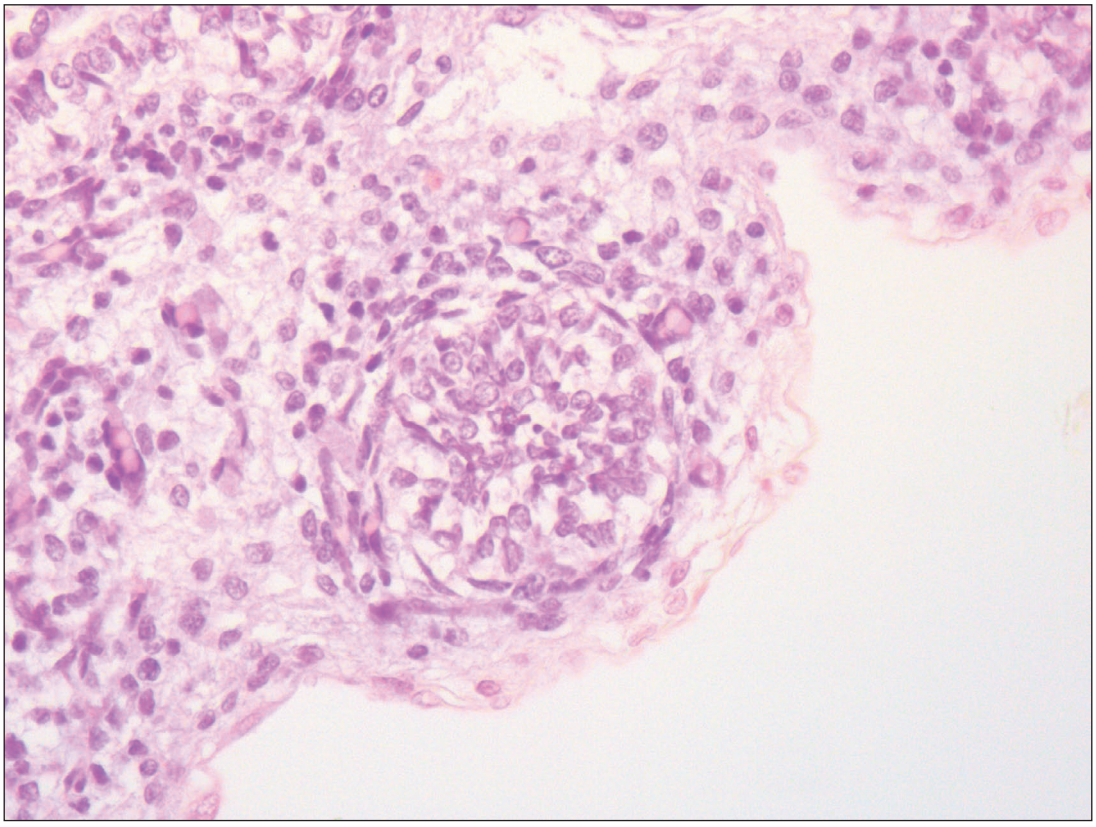

Figure 5. The solid pattern with the absence of a clearly defined lumen of the endodermal-derived cell component of some of the lung niches.

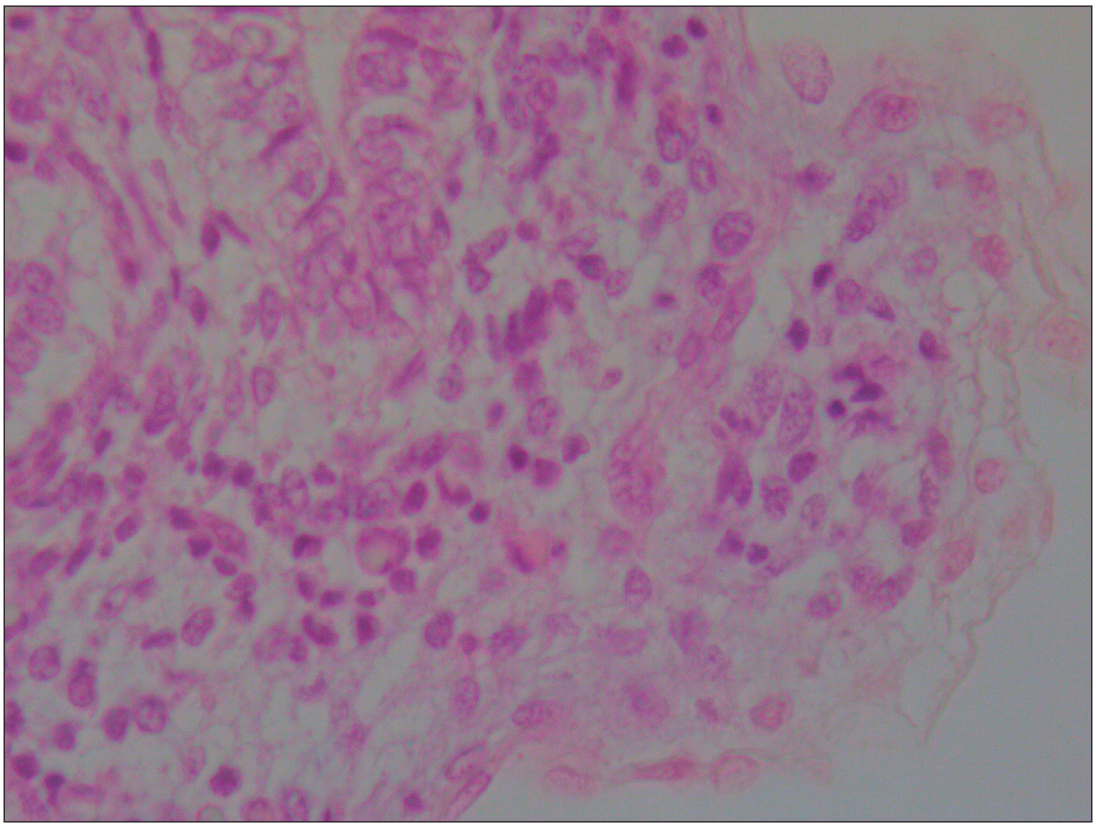

Figure 6. Sub-pleural niches characterized by a roundish or oval shape, superficial location and pushing margins on the pleural surface, formed exclusively by mesenchymal cells, in the absence of any epithelial component.

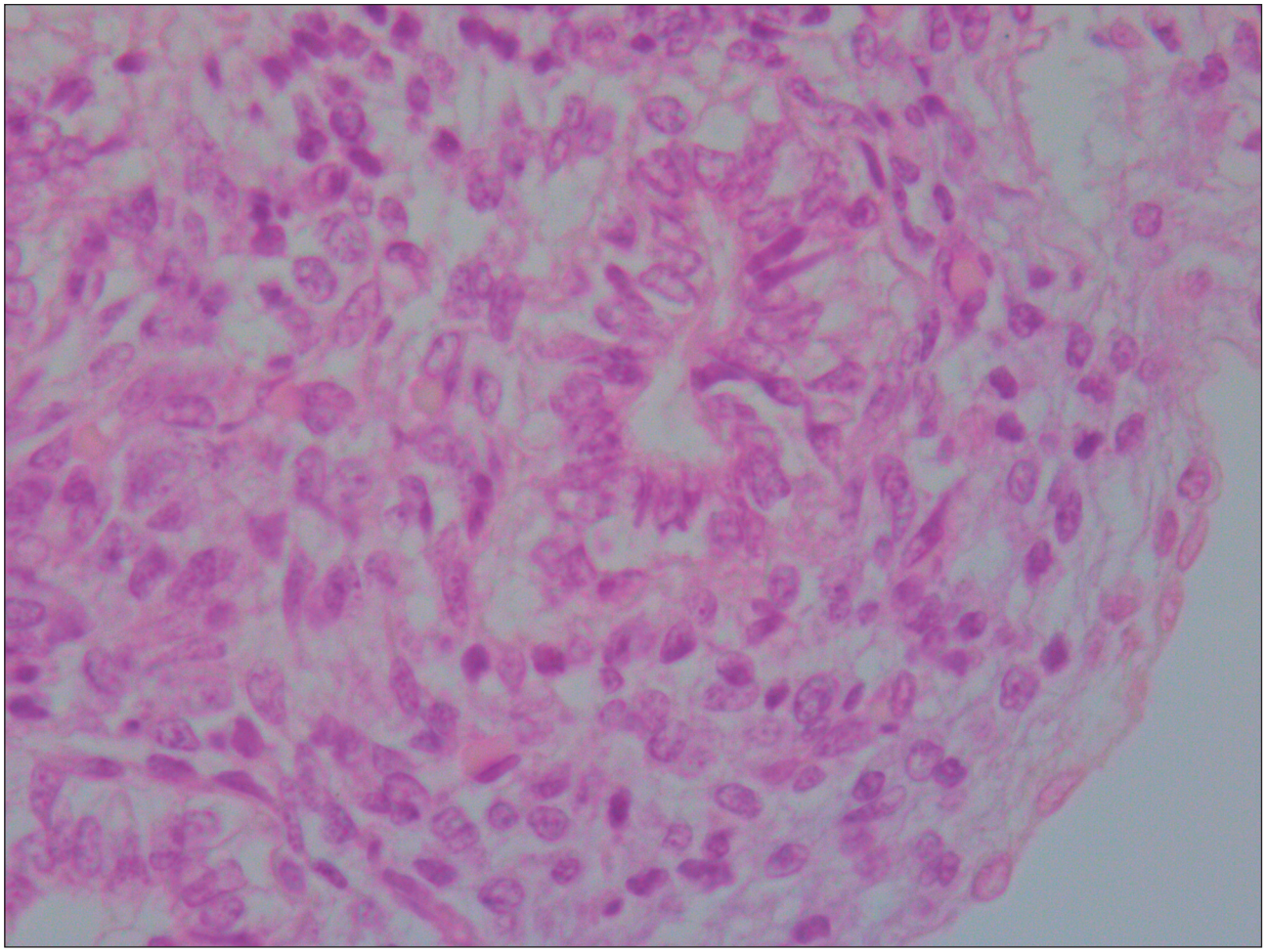

Figure 7. The tips of the endoderm-derived tubular structures in strict contact with the mesenchymal stem/progenitor cells and multiple cell types detected, suggestive of an ongoing differentiation process inside the epithelial lung precursors.

derived cells, it was possible to detect multiple cell types, a finding suggestive for an ongoing differentiation process inside the epithelial lung precursors (**Fig. 7**).

At immunohistochemistry, reactivity for TTF1 was restricted to the nuclei of the interior layer of the epithelial tips (**Fig. 8** and **Fig. 9**), in the absence of any reactivity in the branching intrapulmonary tubular structures and in the mesenchymal component. As a consequence, TTF1 expression was a marker of the pulmonary stem cell niches. A different pattern of reactivity was observed for SOX-2, another marker of the endodermal component of the developing lung. SOX-2 expression was not found in the tips, being restricted to the epithelial precursors of the proximal tubules, as well documented in **Fig. 10**. As a consequence, SOX-2 expression, in this study, was not detected inside the stem cell niches. The mesenchymal component, and in particular mesenchymal cells surrounding the proliferating tubules, were well marked by immunostaining for Wilms Tumor-1 (Wt1). This marker appeared very useful for the identification of the mesenchymal component undergoing induction by the endodermal cells, since mesenchymal cells at some distance from the tubules did not react significantly with the anti-Wt1 antibody. Interesting data were obtained with the anti-CD34 antibody, which revealed the newly formed vascular structures inside the lung mesenchyme. CD34-reactive small vessels were highly represented inside the subpleural stem cell niches and were mainly distributed at the periphery of the niche, putatively indicating the external border of the structure (**Fig. 11**).

Conclusions

Our preliminary data show that the human developing lung represents a relevant source of stem/progenitor cells. These lung progenitors have been identified, in this study, in two main locations:

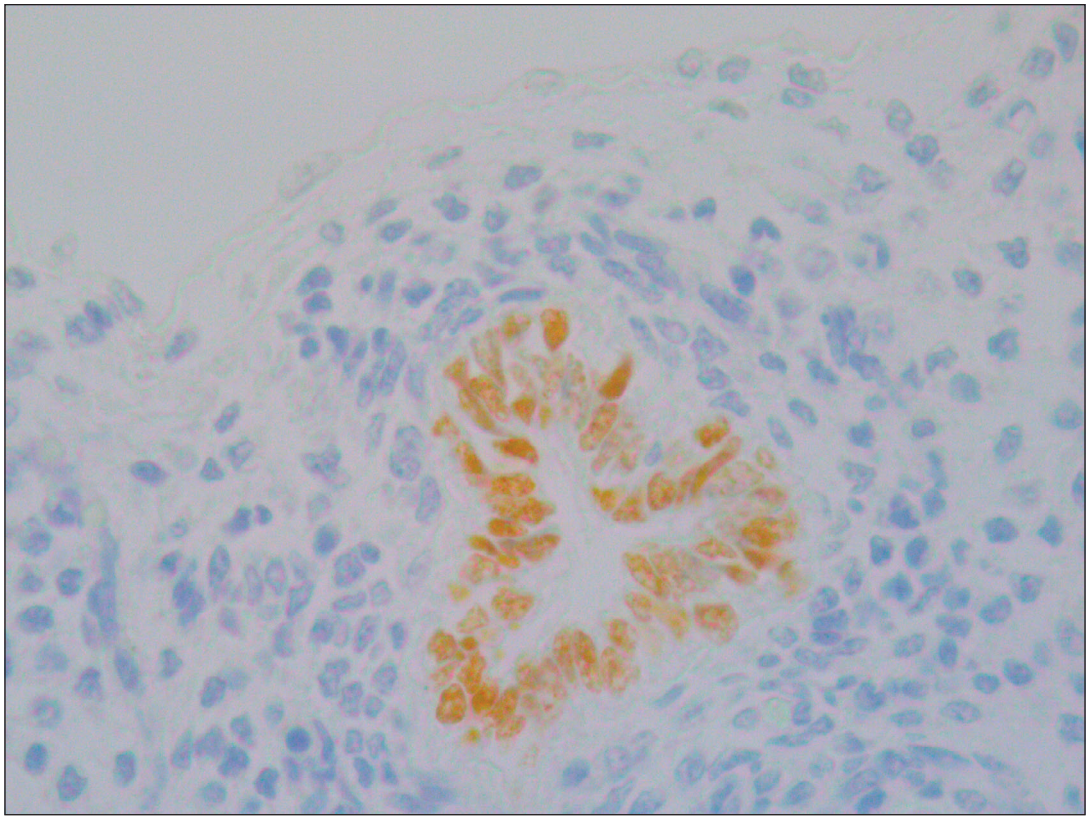

Figure 8. Nuclear immunohistochemistry reactivity for TTF1, restricted to the interior layer of the epithelial tips, and absence of any reactivity in the branching intrapulmonary tubular structures and in the mesenchymal component.

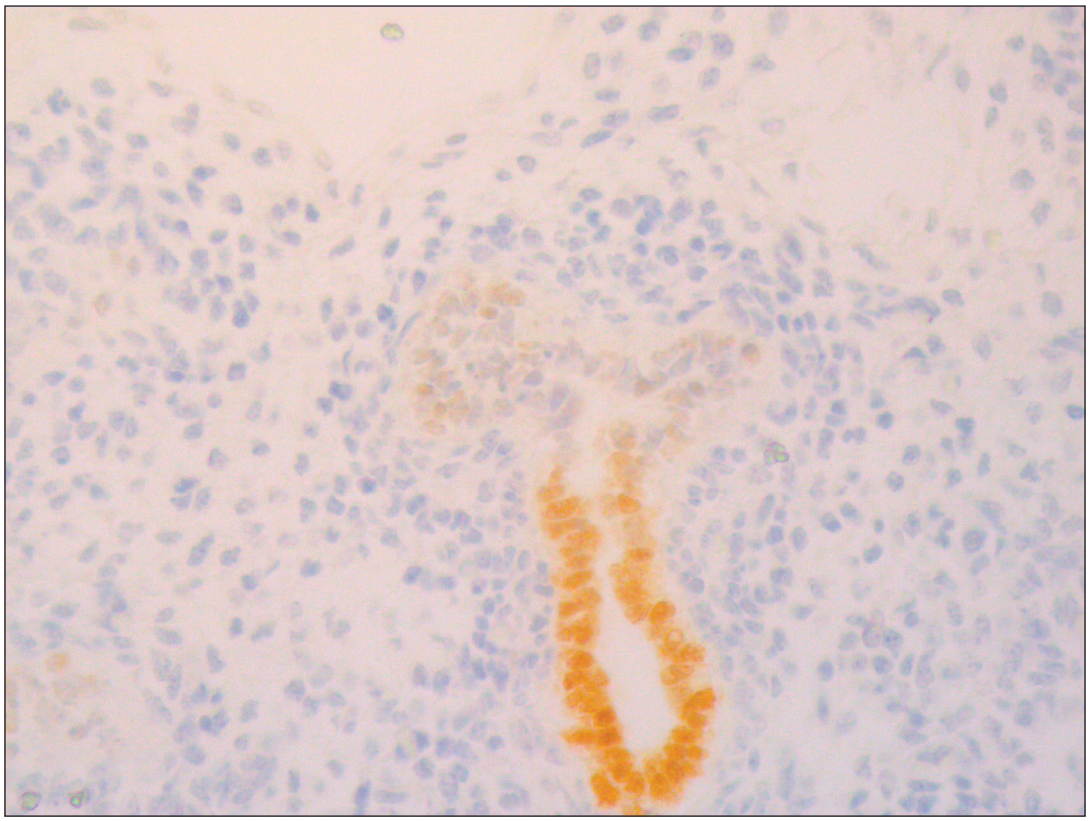

Figure 9. Nuclear immunohistochemistry reactivity for TTF1, restricted to the interior layer of the epithelial tips, and absence of any reactivity in the branching intrapulmonary tubular structures and in the mesenchymal component.

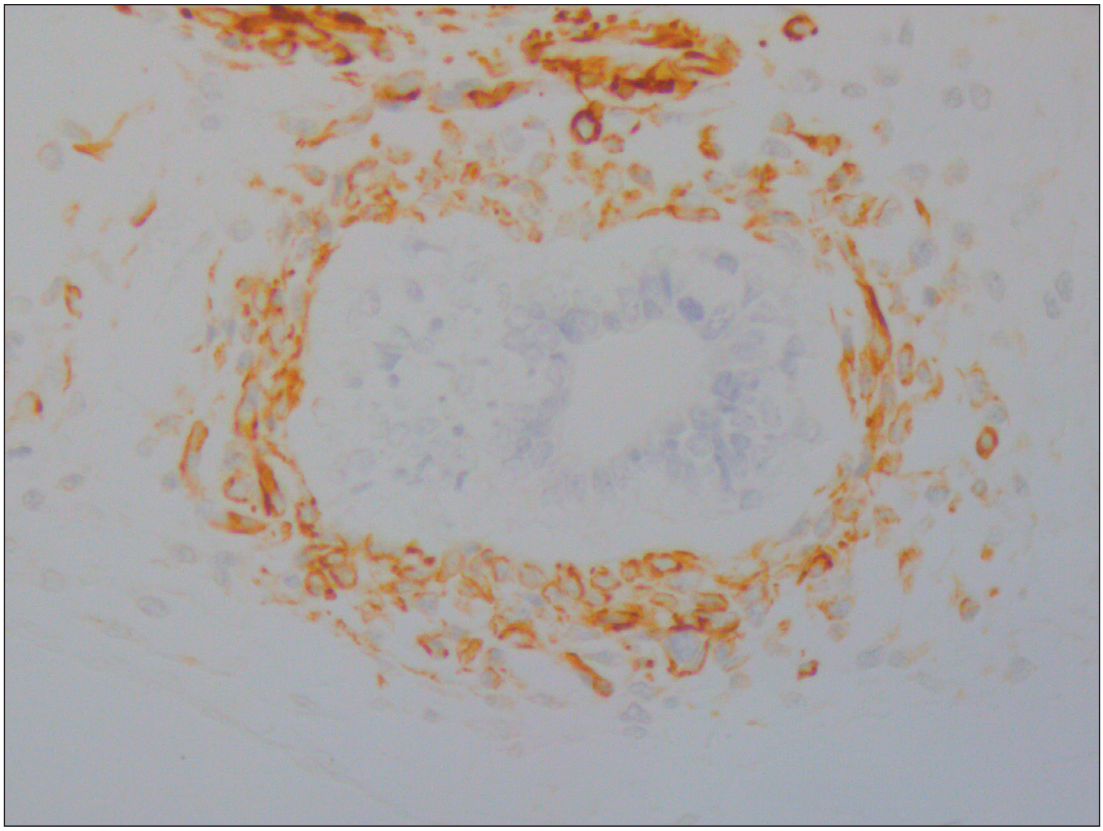

Figure 10. Reactivity for SOX-2 restricted to the epithelial precursors of the proximal tubules not found in the tips.

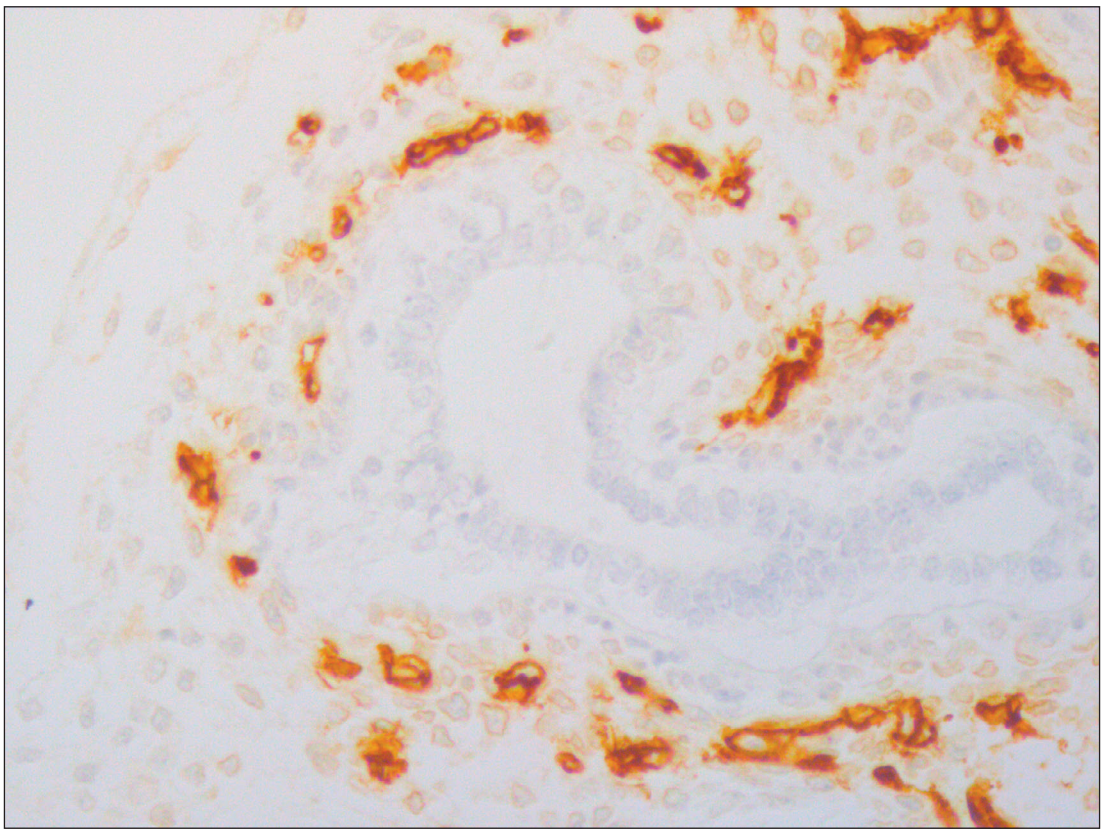

Figure 11. CD34 reactivity revealing the newly formed vascular structures inside the lung mesenchyme and highly represented inside the subpleural stem cell niches at the periphery of the niche.

a) inside the interstitial mesenchyme, arranged in small nests, without any clear-cut border with the surrounding mesenchyme; b) in close proximity to the pleural envelope, at the periphery of the developing lungs.

The architecture of the pulmonary stem cell niches was different, from one niche to the next. Some showed a solid structure, in the absence of any epithelial component; others were centered in the tip of a branching tubular structure, around which the inducted epithelium assumed an onion-like disposition. The different structures might represent different stages of stem cell niche differentiation. In all niches, a strict relationship was found between the epithelial and the mesenchymal component. This finding parallels recent reports on the renal stem cell niche [32], in which nano-channels extending from the epithelial cells of the ureteric bud tips and entering the cytoplasm of the surrounding mesenchyme have been reported. Even in the developing lung, in some niches we did not succeed in detecting a clear-cut border between the epithelial and the mesenchymal cells (see **Fig. 7**). This finding appears indicative of the existence of an anatomical bridge between these two structures even inside the lung stem cell niche.

The preliminary immunohistochemical data stress the necessity of further analyses, aimed at better clarifying the complexity of cell types inside the developing human lung. TTF1, as expected, was highly expressed in the most peripheral parts of the proliferating tubules, being restricted to the nuclei of the tip cells. According to these data, TTF1 might be utilized for marking the subpleural stem cell niches. On the contrary, SOX2, a typical marker of the endodermal component of the developing lung, was esclusively expressed at nuclear level, by the proximal component of the tubular component, being absent in the tips. According to these findings, SOX-2 should not be considered as a component of the stem cell niches, at least in the early stages of lung development. Intersting data emerge from immunostaining for Wt1, a marker previously reported in many human organs [33]. Wt1 was highly expressed in the lung mesenchyme, demonstrating a major role in lung development. Moreover, Wt1 was restricted to the mesenchymal cells induced by the proliferating tubules, and arranged in an onion-like appearance around them. This finding indicates Wt1 as a marker of mesenchymal induction, and a marker of advanced differentiation of the mesoderm-derived cells toward one of the multiple cell types that characterize the interstitium of the mature lung. Interesting data also emerge from the immunostaining for CD34. CD34-reactive small vessels were particularly numerous around the proliferating tubules. Moreover, they were found in high number inside the stem cell niches, showing a tendency to organize a plexus-like structure at the periphery of the stem cell niches.

In conclusion, our preliminary data stress the complexity of the histological picture of the developing human lung, due to the multiple stem/progenitor cells of endodermal and mesodermal origin, all acting together and giving rise to a huge number of cell types through complex processes of differentiation. The limits of morphology in the interpretation of this complex scenario are evident, and the lack in the literature of significant immunohistochemical data regarding the human lung is noticeable. As a consequence, further studies, mainly based on immunohistochemistry, appear mandatory for a better definition of all cell types present inside the stem cell niches and participating to lung development. A better knowledge of the physiological processes at the basis of embryology of the lung might help to develop new strategies for an efficient regenerative medicine for adult patients affected by chronic lung disease.

Declaration of interest

The Authors declare that there is no conflict of interest.

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