Pediatr Surg Int (2011) 27:181–185
DOI 10.1007/s00383-010-2797-9

ORIGINAL ARTICLE

Spatial and temporal patterns of c-kit positive cells in embryonic lungs

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Published online: 11 November 2010

Abstract
Background It has been reported that the smooth muscles in fetal airways exhibit spontaneous phasic contractions throughout gestation. However, the mechanism of these spontaneous contractions is unknown. In the bowel wall, interstitial cells of Cajal (ICCs), which are derived from c-kit positive precursor cells, play an important role as pacemaker cells responsible for the spontaneous, rhythmic activity in the smooth muscle cells. In this study, we investigated the spatial and temporal expression patterns of c-kit positive cells in the embryonic lung and its relationship to the smooth muscle cells surrounding the trachea and the bronchus.
Methods Rat fetuses were removed from timed pregnant dams on embryonic days (E) 11.5, 13.5, 15.5, and 17.5. Immunohistochemical studies with anti c-kit antibody and anti α-SMA antibody were carried out using frozen sections.
Results A small number of c-kit positive cells were observed in the mesenchyme of the lung bud on day E 11.5. They were markedly increased in number on day E 13.5. On day E 15.5 and on day E 17.5, strong c-kit expressions were observed on the vascular wall and moderate expressions in the mesenchyme. C-kit positive cells co-localized with α-SMA positive smooth muscle cells surrounding the airway epithelium.
Conclusion Co-localization of c-kit positive cells and airway smooth muscles in the fetal lung suggests that c-kit positive cells may play an important role in the spontaneous contractions of fetal airways. C-kit expressions in the fetal pulmonary vascular wall suggest that these cells may play an important role in vasculogenesis and angiogenesis of the embryonic lung.

Keywords Spontaneous contraction · Airway peristalsis · Airway smooth muscle · Interstitial cells of Cajal · Embryonic lung

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Introduction

Several physical and mechanical factors in embryonic lung development have been reported [1]. There have been recent reports suggesting that fetal airway peristalsis plays an important role in the maintenance of adequate alveolar distension and positive intraluminal pressure, which is considered to be one of the physical factors in embryonic lungs [2–6]. Airway peristalsis is the spontaneous rhythmic contraction of airway smooth muscles [5, 7]. These contractions in isolated fetal airways of chick embryos were first described by Lewis [8]. A similar phenomenon was reported in guinea pigs, humans, mice, pigs, and rabbits [2, 3, 9]. Airway smooth muscle development begins early in gestation. Smooth muscle cells are present in the human fetal trachea, primary and lobar bronchi by the sixth to the eighth week of gestation [10]. Studies using lung explant models have shown that spontaneous rhythmic contractions cause visible movement of intraluminal fluid along the length of the airways and distending to the distal ends [2–4]. The contractile activity of the airway smooth muscles in the fetal lung is phasic, just like in the gastrointestinal smooth

muscles. This is not surprising because intestines and lungs have common embryological origins, with the lung developing as an outgrowth of the foregut in the late embryonic stage [11]. The mechanisms of underlying spontaneous contractility of the airway smooth muscles in the fetal lung are unknown. Judging from the embryological findings mentioned above, it is highly likely that the mechanism of spontaneous airway contractility may be closely related to spontaneous intestinal contractility. Moreover, despite the presence of nerve fibers in embryonic lungs, spontaneous smooth muscle activity continues in the presence of atropine or tetrodotoxin [1, 3]. Furthermore, these spontaneous contractions are suppressed by calcium antagonists [1, 3]. These observations suggest that spontaneous rhythmic contractions in embryonic lungs are of myogenic origin.

The role of intestinal cells of Cajal (ICCs) as coordinators for intestinal motility is well recognized. In the bowel wall, ICCs play an important role as pacemaker cells responsible for spontaneous, rhythmic activity in smooth muscle cells [12–15]. The ICCs are derived from c-kit positive precursor cells, which also have the potential to develop into smooth muscle cells [12–15]. However, the presence of ICCs in embryonic lungs is unclear. We hypothesized that rhythmic contractions of embryonic lungs are derived from ICCs. In this study, we investigated the spatial and temporal expression patterns of c-kit positive cells in embryonic lungs and their relationship to smooth muscle cells, using the earliest smooth muscle cell markers, α-smooth muscle actin (α-SMA) [16].

Materials and methods

Animals

Female (220–250 g) and male (250–300 g) Sprague–Dawley rats were mated overnight and the morning of finding a vaginal plug was counted by day 0.5 of gestation. Timed-pregnant dams were euthanized with cervical dislocation under isoflurane inhalation on 11.5, 13.5, 15.5, and 17.5 days (day E 11.5–day E 17.5) of gestation. The fetuses were removed by cesarean section. All experiments carried out in compliance with current European Union regulations for animal investigations (B100/3439) and prior ethical approval under license from the Department of Health, Ireland.

Organ culture

Fetal rat lung culture was performed to examine the spontaneous contraction of airways. We used fetal rat lungs obtained from day E 13.5 fetuses. The lungs were placed on translucent polycarbonate culture dish inserts (3.0 μm pore size, 12 mm diameter; Transwell®, Costar, UK) and cultured with serum-free DMEM/F12 at 37°C in 21% O_2, 5% CO_2 for 48 h. The airway contraction was recorded by video-assisted recorder (NV-DJ100, Panasonic, Japan) after 48 h culture.

Specimens

The fetuses were fixed in freshly prepared 4% (v/v) paraformaldehyde in phosphate-buffered saline (PBS) for 1–4 h at 4°C. They were then dehydrated in 20% sucrose/PBS overnight at 4°C. The fetuses were individually embedded in O.C.T. compound (VWR International, UK), frozen in isopentane that had been chilled in liquid nitrogen, and stored in −80°C. Serial axial cryosections of 6-μm thickness were obtained on Superfrost® slides (VWR International, UK). In preparation for immunohistochemistry, sections were air-dried and fixed for 2 min in acetone at −20°C. The slides were stored −80°C until immunostaining was performed.

Antibodies

Rabbit polyclonal antibody against c-kit (c-19) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA,

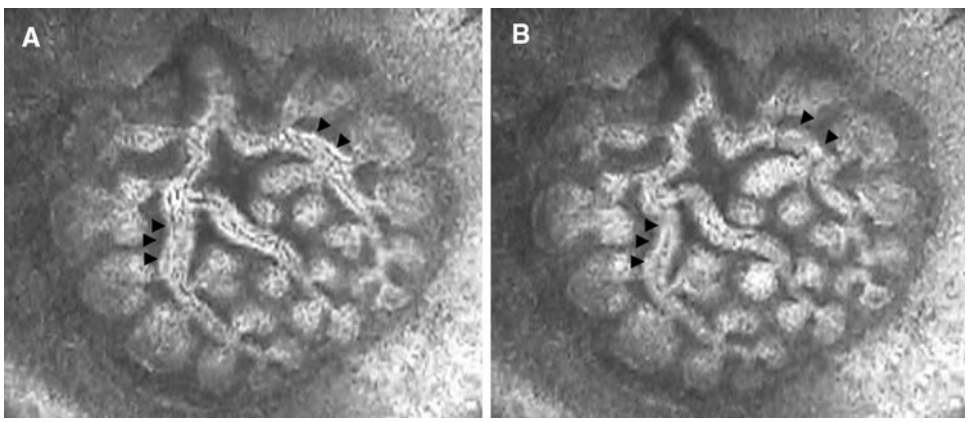

Fig. 1 Representative photomicrographs of phasic airway contraction on day E 13.5 fetal rat lung cultured for 48 h. **a** Relaxation phase, **b** contraction phase. The airway narrowed in contraction phase compared to the relaxation phase (*arrows*)

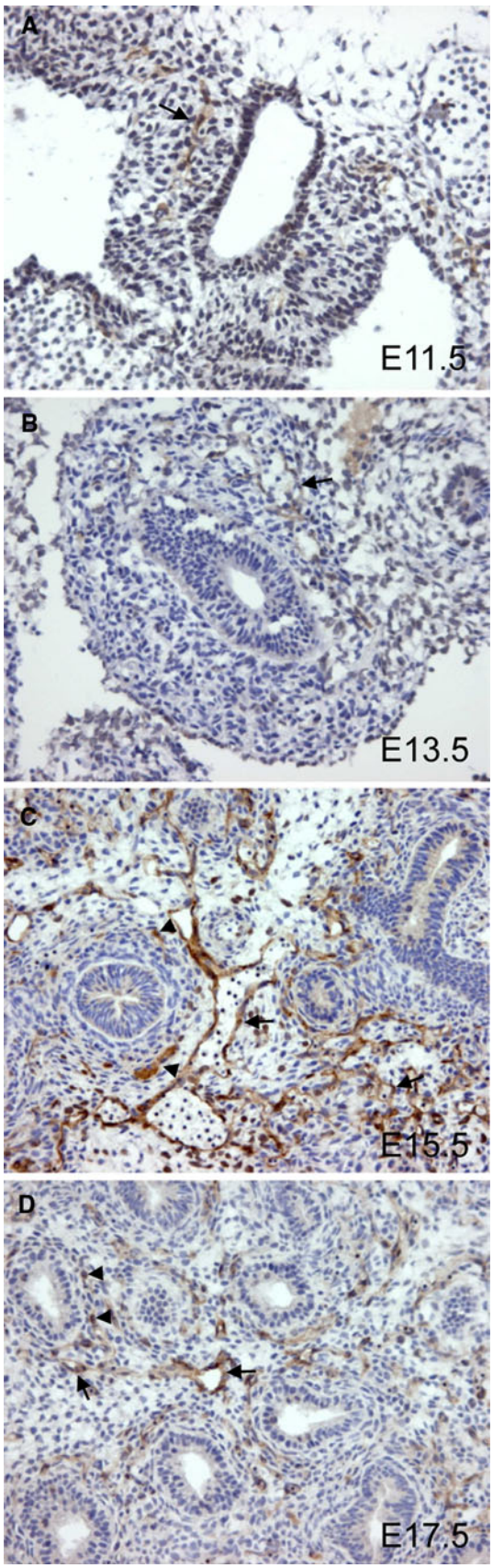

Fig. 2 Distribution of c-kit positive cells in the pseudoglandular stage lung (day E 11.5–day E 17.5): C-kit positive cells were present in the mesenchyme throughout the stage (**a**–**d**). C-kit positive cells are prominently expressed in the vascular wall even in the small vessels (*arrows* in **c**, **d**). *Solitary* and *clustered* c-kit positive cells were seen around airways (*arrow heads* in **c**, **d**)

USA). Mouse monoclonal antibody against α-smooth muscle actin (α-SMA) was purchased from DAKO Cytomation (Glostrup, Denmark).

Immunohistochemistry for c-kit

Antigen retrieval was performed by boiling the sections in 0.01 mol/l citrate buffer pH 6.0 for 2 min in a microwave at medium–high output. Endogenous peroxidase activity was quenched with 0.3% (v/v) hydrogen peroxide in methanol for 30 min at room temperature (RT). Nonspecific binding site were blocked using 10% (v/v) normal goat serum, 1% (w/v) bovine serum albumin and 10% (v/v) fetal calf serum for 30 min at RT. The slides were then incubated at 4°C overnight with c-kit antibody (1:1,000, dilution). The sections were incubated with Histofine Simple Stain MAX-PO(R), (Nichirei Corporation, Tokyo, Japan) for 30 min at RT. Immunoreacted cells were then visualized with DAB solution (Vector Laboratories, CA, USA). The sections were counterstained by hematoxylin.

Double staining

Double staining was performed to examine co-localization of both c-kit and α-SMA. After antigen retrieval and blocking of endogenous peroxidase activity, nonspecific binding sites were blocked with 10% (v/v) goat serum for 30 min at RT. The slides were incubated at 4°C overnight with α-SMA antibody (1:500 dilution). The sections were incubated with Histofine Simple Stain MAX-PO(M), (Nichirei Corp, Tokyo, Japan) for 30 min at RT. Immunoreacted cells were then visualized with DAB solution. Nonspecific binding sites were blocked using 10% (v/v) normal goat serum, 1% (w/v) bovine serum albumin and 10% (v/v) fetal calf serum for 30 min at RT. The slides were incubated at 4°C overnight with c-kit (1:1,000 dilution). The sections were incubated with Histofine Simple Stain MAX-PO(R), (Nichirei) for 30 min at RT. Immunoreacted cells were then visualized with VIP (Vector Laboratories, CA, USA). The sections were counterstained by methyl green.

Results

Spontaneous and phasic contractions were observed in the lung explant cultured for 48 h. The airway narrowed in

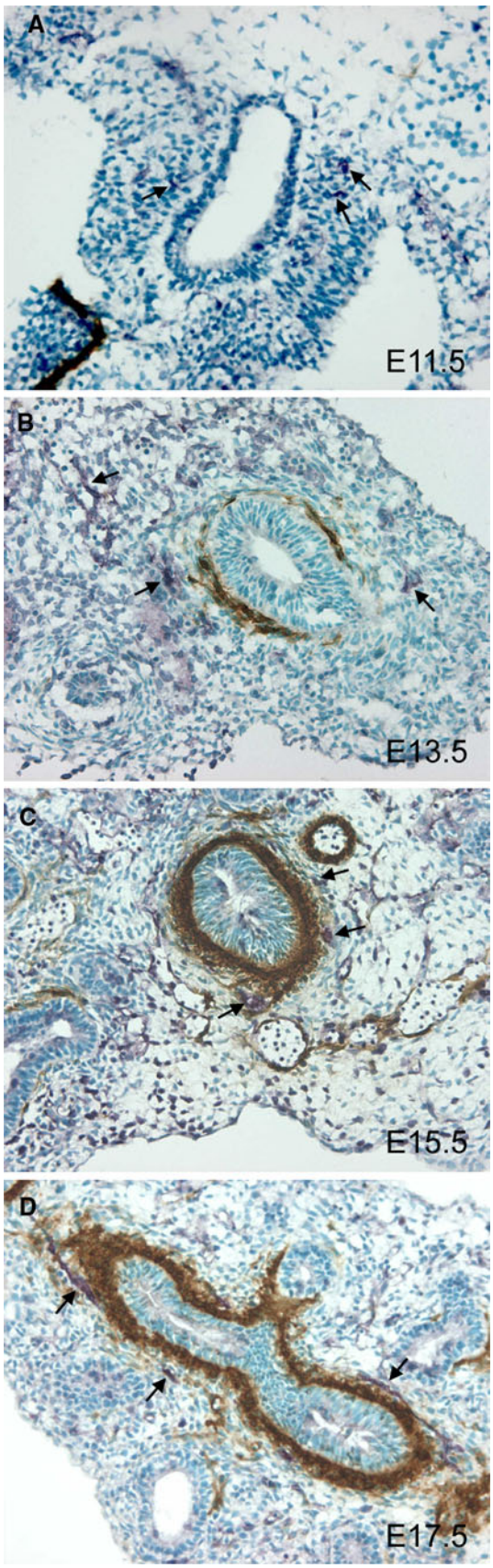

Fig. 3 Co-localization of c-kit positive sells with α-SMA (**a**–**d**): C-kit ► positive cells were stained in *purple*. *Solitary* and *clustered* c-kit positive cells (*arrows*) were co-localized with airway smooth muscles which were expressed α-SMA immunoreactivity (*brown*). These findings were found from day E 13.5 to day E 17.5 (*arrows* in **b**–**d**). The co-localization was prominent on day E 15.5 and on day E 17.5. Some c-kit positive cells were observed as fiber-like projection on day E 17.5. On day E11.5, α-SMA immunoreactivity was not observed around foregut (**a**)

contraction phase compared to the relaxation phase (Fig. 1a, b). C-kit immunoreactivity was present in the mesenchyme of lung buds throughout on day E 11.5 to on day E 17.5. On day E 11.5, it was difficult to identify the origin and potential of c-kit positive cells, because of their immaturity (Fig. 2a). C-kit expression increased in number on day E 13.5 near the carina, sparsely distributed around bronchiolar walls and on vessel walls (Fig. 2b). On day E 15.5 and on day E 17.5, strong c-kit expressions were observed on the vascular wall and moderate expression in the mesenchyme (Fig. 2c, d). C-kit positive solitary and clustered cells were observed in peribronchial region and also around distal airways on day E 15.5 and on day E 17.5.

To further characterize the localization of c-kit and α-SMA, we performed double staining. Solitary and clustered c-kit positive cells were co-localized with airway smooth muscles expressing α-SMA immunoreactivity. These findings were found from day E 13.5 to day E 17.5 (Fig. 3b–d). On day E 11.5, α-SMA immunoreactivity was not observed around the foregut (Fig. 3a). Thus, it was difficult to define their co-localization on day E 11.5. However, the co-localization was prominent on day E 15.5 and on day E 17.5. Some c-kit positive cells were observed as fiber-like projection on day E 17.5 (Fig. 3d).

Discussion

Rat lung development begins on day E 11, when the lung buds emerge from the foregut and have divided into four chronological stages similar to the stages of human lung development [17].

In this present study, we investigated the spatial and temporal expression patterns of the c-kit positive cells from the early to late pseudoglandular stage of developing fetal rat lung and examined its co-localization with airway smooth muscles. We found that c-kit positive cells were present in the mesenchyme of the lung bud from day E 11.5 of the early pseudoglandular stage. C-kit positive cells were prominently distributed in vessel walls on day E 15.5 and on day E 17.5, both were on the large branches of pulmonary vessels as well as on the small vessels in the lung parenchyma. During embryogenesis, lung vascular

development is achieved through vasculogenesis and angiogenesis. Vasculogenesis is defined as the trans-differentiation and organization of endothelial cells into vascular structure. Angiogenesis is the process of formation of blood vessels that sprout from pre-existing vessels [18]. Bernex et al. [19] showed c-kit expression in endothelial cells of the intersomatic arteries of day E 9.5 in mice. Our data suggested that c-kit is expressed during both vasculogenesis and angiogenesis and may play an essential role in vessel formation in the embryonic lung.

In embryonic lung, c-kit expressing cells were detected in the respiratory system using c-kit mutant mice $W^{lacZ}/+$ embryos [19]. However, their report did not describe in detail the distribution of c-kit positive cells nor the co-localization with airway smooth muscle in the embryonic lung. In the gastrointestinal tract, c-kit is a specific marker of ICCs responsible for the spontaneous rhythmic activity in smooth muscle cells. Jesudason et al. [5] suggested that airway smooth muscles of right lung behave as pace maker of the phasic contractions, and the contraction was independent of c-kit expression, because of the absence of c-kit immunoreactivity in embryonic lungs. The presence of c-kit positive cells observed in the fetal lung and their co-localization with smooth muscle cells in our study suggests that c-kit positive cells may play a role of the pacemakers of fetal airway contraction. The present study provides the first evidence of solitary and clustered c-kit positive cells co-localizing with smooth muscle cells surrounding the airways on day E 15.5 and on day E 17.5.

In order to find the interaction with c-kit positive cells and airway smooth muscles in the embryonic lung, it is necessary to examine the functional activity in future by using electrical recordings of the slow waves in the c-kit positive cells.

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