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Expression of retinoid receptors during rabbit lung development

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Rabbit lung is often used to study the pathophysiology of some congenital anomalies affecting the lung because its development is very comparable with that of human. Retinoids and the molecular transducers of the retinoic signal play a crucial role in mammalian lung development. In the rabbit, the molecular retinoic pathway has so far been poorly studied. As a first step in elucidating this process, we aimed to identify the retinoic acid receptors (RARs) and retinoid X receptors (RXRs). We cloned a part of the nuclear receptors (RARs and RXRs), and we used reverse transcription - polymerase chain reaction (RT-PCR) and immunohistochemistry assays to demonstrate the presence of RAR (α,β) and RXR (α, β) at all stages of rabbit lung development. Our results initiate further analysis into the molecular and genetic functions of retinoids during normal and pathological rabbit lung development including the surgical model of congenital diaphragmatic hernia.

Keywords: lung development, rabbits, retinoids, retinoic acid receptor, retinoid X receptor

Introduction

Many factors including hormonal, biochemical and physical factors have been identified that modulate growth and development of the lung (Keijzer *et al.*, 2000; Cardoso, 2001; Copland and Post, 2004; Rajatapiti *et al.*, 2005). Among the variety of biochemical agents identified so far, retinoids (active derivatives of vitamin A) play a clearly defined role in lung development at its different stages (Gallot *et al.*, 2005; Maden, 2004). Vitamin A (retinol) exerts important effects upon a vast number of development processes via signalling through heterodimerization complexes comprising retinoic acid receptors (RARs) and retinoid X receptors (RXRs) (Chambon, 1996). These receptors belong to the steroid/thyroid hormone receptor super family of ligand-inducible transcription regulators known to control the expression of target genes. All members of this family share a strongly conserved modular structure with discrete functional domains for ligand binding, DNA binding and transactivation. There are three RAR isotypes (RARα, β, γ) activated by both all-*trans* and 9-*cis* retinoic acid (RA) and three retinoid X receptors (RXRα, β, γ) only activated by 9-*cis* RA (Bastien and Rochette-Egly, 2004). The RAR and RXR proteins interact with each other to form RAR/RXR heterodimers which are able to bind to specific DNA sequences called RA responsive elements usually located within the 5′-regulatory region of retinoid-regulated genes.

The importance of vitamin A in vertebrate lung development was first demonstrated by the observation that foetuses from pregnant rats which were fed a vitamin A-deficient diet had either agenesis of the left lung or rudimentary lung buds (Warkany and Wilson, 1948; Chazaud *et al.*, 2003). Double mutant mice RARα, $\beta^{-/-}$ revealed defects comparable with the consequences of vitamin A deficiency including hypoplastic lungs (Mendelsohn *et al.*, 1994; Kastner *et al.*, 1997; Mascrez *et al.*, 1998). Abnormalities in the vitamin A status and/or metabolism may also play a role in the genesis of congenital diaphragmatic hernia (CDH). CDH can be induced in rodent by either maternal retinol deficiency during pregnancy (Anderson, 1941 and 1949; Warkany and Wilson, 1948; Wilson *et al.*, 1953; Mendelsohn *et al.*, 1994; Lohnes *et al.*, 1995), or administration of nitrofen, a retinal dehydrogenase type 2 (RalDH2) inhibitor reducing the RA production (Nakao and Ueki, 1987; Kluth *et al.*, 1990; Mey *et al.*, 2003). Therefore maternal administration of retinol or RA was recently proposed as a potential therapeutic measure to decrease foetal lung abnormalities in the rodent 'nitrofen-toxic' CDH

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model (Thebaud *et al*., 1999; Babiuk *et al*., 2004; Oshiro *et al*., 2005).

Mammalian lung development can be divided into five stages (embryonic, pseudo-glandular, canalicular, saccular and alveolar). Lung development in rabbits is relatively comparable with that of humans. The pseudoglandular phase extends over more than 75% of gestation (23 to 24 of 31 to 33 days). Being quite long, it allows for feasible surgical procedures on foetuses sufficiently large. The next three stages of development are gone through in 3 to 4 days time (Kikkawa *et al*., 1968). Alveolisation starts prior to birth and is completed postnatally (Kikkawa *et al*., 1968; Pringle, 1986). The rabbit is an inexpensive, widely available animal with limited housing demands and short gestational duration. A surgical CDH rabbit model has been described inducing the morphological lung changes observed in the human conditions both in the airways and in the vasculature (Fauza *et al*., 1994; Wu *et al*., 2000; Roubliova *et al*., 2004). However the molecular retinoic pathway has been poorly studied in the lung parenchymal tissue of this specie. The aim of this study was to determine the presence and expression of RARs and RXRs during the different stages of rabbit lung development.

Material and methods

Animal and tissues collections

The Ethical Committee for Animal Experimentation of the Katholieke Universiteit Leuven approved the experiments and all animals were treated according to current guidelines on animal welfare. New Zealand rabbits were housed in separate cages at normal room temperature and daylight, with free access to food and water. They were euthanased with a 3 ml slow intravenous injection of a mixture of embutramine 200 mg/ml, mebezonium iodide 50 mg/ml and tetracaine hydrochloride 5 mg/ml (T61; Marion Roussel, Brussels, Belgium). Tissue samples used were entire embryos (8th and 11th day of gestation), foetal lung (21st, 26th, 28th, 31st day of gestation) and post-natal lung (1st and 6th week of life) from New Zealand rabbits. These were either frozen at −80°C for cloning and reverse transcription-polymerase chain reaction experiments or placed in moulds with embedding medium and frozen on the surface of dry ice for immunohistochemistry assays.

Partial cloning of retinoids receptors

A Dounce homogeniser was used for tissue disruption and Trizol (Invitrogen®, Cergy-Pontoise, France) to extract total RNA. The cDNA was generated using superscript first-strand synthesis system for the reverse transcription - polymerase chain reaction (RT-PCR) (Gibco-BRL®, Cergy-Pontoise, France). Using the highly conserved nature of the nuclear retinoid receptors (RARs and RXRs) gene family and of the house keeping gene 36B4, consensus PCR primers were designed (Web program 'Primer3') against regions of high homology between the mouse, rat and human sequences. These primers were used to amplify the portions of the coding regions using the cDNA of entire rabbit embryos. PCR amplification was carried out in an Eppendorf tube with initial denaturation at 95°C for 5 min, followed by denaturation at 95°C for 45 s, annealing at 53°C for 45 s, and extension at 72°C for 60 s (45 cycles), terminated by a final extension of 72°C for 7 min. The PCR products were separated on a 2% agarose gel, ligated into the plasmid pCRII-TOPO (Invitrogen®, Cergy-Pontoise, France) and sequenced on both strands by using the DNA dye terminator cycle sequencing kit (Applied Biosytems®, Courtaboeuf, France) and the Applied Biosystems model 377 DNA sequencer.

RT-PCR amplifications of the retinoids receptors

The temporal distribution of the RARα, β, γ and RXRα, β, γ mRNA in the lung samples was studied by RT-PCR assays. A Dounce homogeniser was used for tissue disruption and Trizol (Invitrogen®, Cergy-Pontoise, France) to extract total RNA. The cDNA was generated using superscript first-strand synthesis system for RT-PCR (Gibco-BRL®, Cergy-Pontoise, France). The RNA quantity was determined by spectrophotometeric measurement at 260 and 280 nm (ratio with proteins). The RNA quality was studied by the RNA/protein ratio (260 nm / 280 nm) and by gel electrophoresis (2% agarose) to observe the presence of intact 28S and 18S RNA bands. The oligonucleotide primers to amplify rabbit RARα, β, γ and RXRα, β, γ and 36B4 are described in Table 1. Two microlitres of cDNA from specimen harvested at the 21st (late pseudoglandular stage), 26th (canalicular stage), 28th (saccular stage), 31st (alveolar stage) day of gestation as well as the 1st and 6th week of life were amplified by initial denaturation at 95°C for 10 min followed by denaturation at 95°C for 45 s, annealing at 55°C for 45 s and extension at 72°C for 1 min (32 cycles), followed by a final extension of 72°C for 7 min in a Eppendorf mastercycler. The PCR products were analysed by electrophoresis on a 12% polyacrylamide gel and sequenced as described earlier (Goncalves-Mendes *et al*., 2004). Amplification of the housekeeping gene acidic ribosomal phosphoprotein P0 (36B4) was used as positive control. A negative control for amplimer contamination was set up using a complete PCR reaction mix without cDNA.

Immuolocalization of the retinoids receptors

The translation into RARα, β, γ and RXRα, β, γ proteins was demonstrated by immunohistochemistry. Sectioning and immunohistochemistry were performed as previously described (Goncalves-Mendes *et al*., 2004) using six primary polyclonal specific epitope antibodies against RARα, β, γ and RXRα, β, γ, respectively Santa-Cruz (sc) 551, 552, 550, 553, 831, 555 (SantaCruz®, Tebu-France, Le Perray en Yvelines, France). The cryosections of lung were fixed in 4% paraformaldehyde in PBS (pH 7.4) at 25°C for 10 min, rinsed three times with PBS, and incubated in PBS with 3% bovine serum albumin (Sigma Aldrich, Saint-Quentin-Fallavier, France) at 25°C for 30 min. Using the Historodent® kit

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Table 1 *Sequence and characteristics for PCR amplification of RARs, RXRs and 36B4*[†]

Primer	Sequence (3′-5′)	Tm (°C)	Length of amplicon (bp)
Retinoic acid receptor alpha (Sense)	CTTGCTTTGTTTGTCAAGA	52.35	106
Retinoic acid receptor alpha (Anti-sense)	TACACCATGTTCTTCTGGAT	52.84	
Retinoic acid receptor beta (Sense)	TGCAGAAGTGCTTTGAAGT	54.58	80
Retinoic acid receptor beta (Anti-sense)	CTTTGAAGGCTCCTTCTTT	53.87	
Retinoic acid receptor gamma (Sense)	AGTACACCACGAACTCCAG	53.40	100
Retinoic acid receptor gamma (Anti-sense)	ACAATCTTGATGATGCATTT	52.99	
Retinoid X receptor alpha (Sense)	ATCTTTGACAGGGTGCTAA	53.20	100
Retinoid X receptor alpha (Anti-sense)	GGTTGAACAGGACAATGG	54.49	
Retinoid X receptor beta (Sense)	GTTTAATCCAGATGCCAAG	53.18	73
Retinoid X receptor beta (Anti-sense)	TGAGGCGTACACCTTCTC	54.57	
Retinoid X receptor gamma (Sense)	ACCAAAGACCGAATCCTAC	54.07	107
Retinoid X receptor gamma (Anti-sense)	ACTCAACAAGGGTGAAGAG	52.51	
36B4 (Sense)	GGAAGTCCAACTACTTCCTT	52.55	256
36B4 (Anti-sense)	GAGGTCCTCCTTGGTGAA	52.90	

[†] Specific primers for retinoic acid receptors (RARα, β and γ), retinoid X receptors (α, β and γ) and 36B4 (ribosomal house-keeping gene) were designed using the strong sequence homologies found in human, rat and mouse species. The calculated values of optimal hybridisation temperature (Tm) and the predictive length (base pairs, bp) were noted respectively for each primer and each potential amplified sequence.

(Cliniscience, Montrouge, France), the antibodies diluted at 1/200 were incubated 15 min at room temperature followed by incubation with anti-rabbit IgG FITC-conjugated secondary antibody during 5 minutes at room temperature. Histological examination was performed after DAPI (nuclear) staining (1 min, dilution in PBS: 1/500) using a Zeiss Axiophot microscope (Carl Zeiss, Oberkochen, Germany).

Results

Rabbit RARs and RXRs mRNA fragments were obtained by RT-PCR, using entire embryos mRNA: DQ102471 / RARα, DQ102472 / RARβ, DQ102474 / RARγ, DQ102473 / RXRα, DQ102475 / RXRβ and DQ102476 / RXRγ? (Figure 1a). Rabbit RAR and RXR cDNA sequences displayed a strong homology to those reported for mouse, rat and human (87 to 100% for RARs and 84 to 95% for RXRs; see Figure 2). Similar homology was found in terms of predictive amino acid sequence (96 to 100% for RARs and 93 to 100% for RXRs, data not shown). At all developmental stages RARα, β and RXRα, β mRNA were detected by RT-PCR assays. In contrast, the transcripts of RARγ and RXRγ were not detected at any stage of lung development (Figure 1b). The developmental expression pattern was similar in the left and right lung (Table 2). No postnatal modification of RARs and RXRs transcript expression was detected at the 1st or 6th week of life. Translation into RARα, β and RXRα, β proteins was demonstrated by immunohistochemistry at all developmental stages analysed by the RT-PCR approach. All of them presented similar bronchial expression (Figure 3 and data not shown).

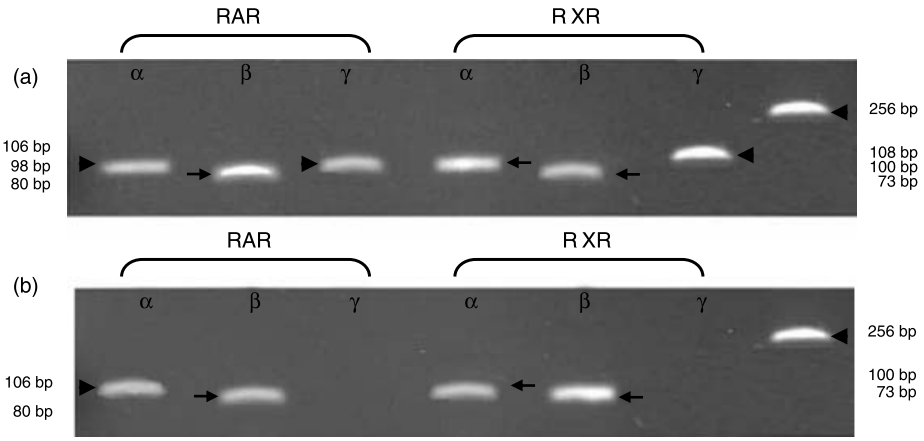

Figure 1 (a) RT-PCR analysis of RARs and RXRs mRNA expression at 11th day of rabbit total embryo.(b) RT-PCR analysis of RARs and RXRs mRNA expression at day 31 of rabbit lung development. Specific fragments (verified by systematic sequencing) of rabbit RARα (106 bp), RARβ (80 bp), RARγ (98 bp), RXRα (100 bp), RXRβ (73 bp) and RXRγ (108 bp) were detected using RT-PCR analysis on foetal (day 31) lung rabbit. Rabbit 36B4 (256 bp) was used as a positive control for the RT-PCR assay and showed positive signals in all samples.

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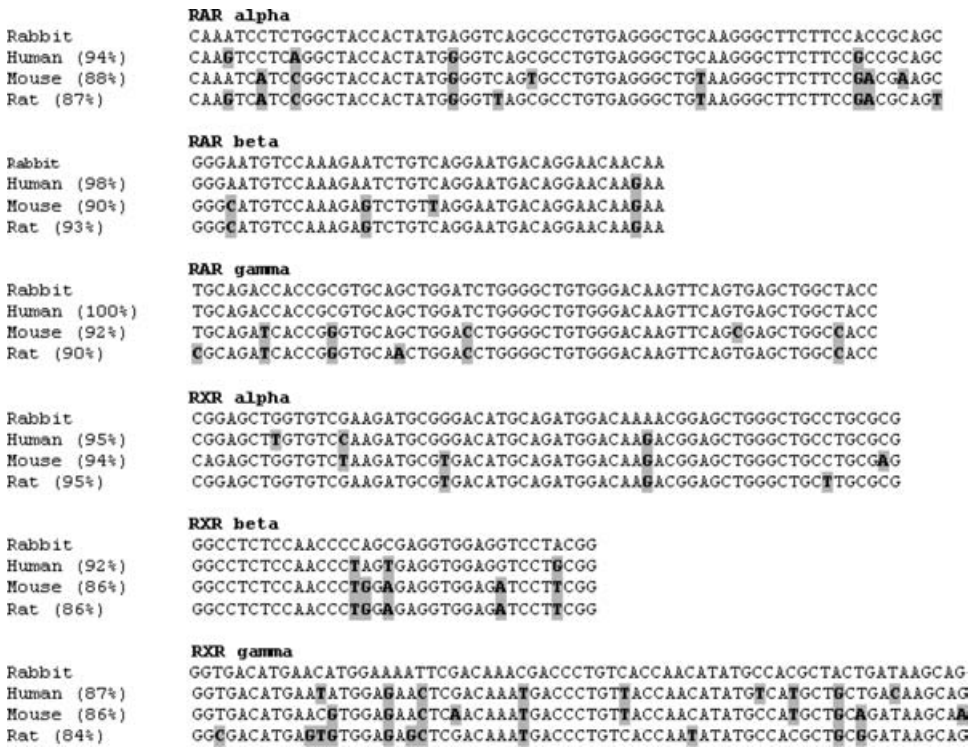

Figure 2 Sequence alignment RAR (α, β and γ) and RXR (α, β and γ) from rabbit, human, mouse and rat origins. The percentage of homology between the rabbit and the other species was calculated using the Blast program. The nucleotide changes between the different species are bold and underlined in grey.

Discussion

Our cloning results established the presence of all six nuclear retinoid receptors in the New Zealand rabbit embryo, completing the previous description of one single sequence (AF126242 / rabbit kidney) related to nuclear retinoid pathways. The identification of the retinoid receptors in the rabbit species supports the molecular actions of RA previously described in rabbit trachea (Nervi *et al.*, 1991; Inayama *et al.*, 1996) and rabbit stomach development (Karam *et al.*, 2004). The necessity to design the primers in highly conserved regions in order to clone the rabbit retinoid receptors probably contributed to the high degree of sequence homology with the other species. Nevertheless this strong homology for each RAR and RXR was anticipated by the phylogenic studies of Escriva *et al.* (2004). Similar homology was found in terms of predictive amino acid sequence (96 to 100% for RARs and 93 to 100% for RXRs, data not shown).

We demonstrated the expression of RARα, β and RXRα, β mRNA during the pseudo-glandular, canalicular, saccular and alveolar stages of the rabbit lung development. Immunohistochemistry confirmed the strong correlation between the mRNA and the proteins pattern of expression for retinoid receptors. The presence and co-localization of RAR and RXR in the developing rabbit lung supports the formation of RAR/RXR heterodimers, the molecular actors of nuclear RA signalling pathway (Bastien and Rochette-Egly, 2004). Data from a variety of studies conducted in the mouse model established that retinoids play a crucial role during lung development. At the embryonic stage, retinoids participate in the initial budding of the lungs from the foregut (Dolle *et al.*, 1990 and 1994; Mendelsohn *et al.*, 1994; Mascrez

Table 2 *RT-PCR analysis of RARs and RXRs mRNA expression during rabbit lung development*

	RARα		RARβ		RARγ		RXRα		RXRβ		RXRγ	
Side of the lung[†]	L	R	L	R	L	R	L	R	L	R	L	R
Day 21 (Late pseudo − glandular stage)	+	+	+	+	−	−	+	+	+	+	−	−
Day 26 (Canalicular stage)	+	+	+	+	−	−	+	+	+	+	−	−
Day 28 (Saccular stage)	+	+	+	+	−	−	+	+	+	+	−	−
Day 31 (Alveolar stage)	+	+	+	+	−	−	+	+	+	+	−	−

[†] L: left lung; R: right lung.

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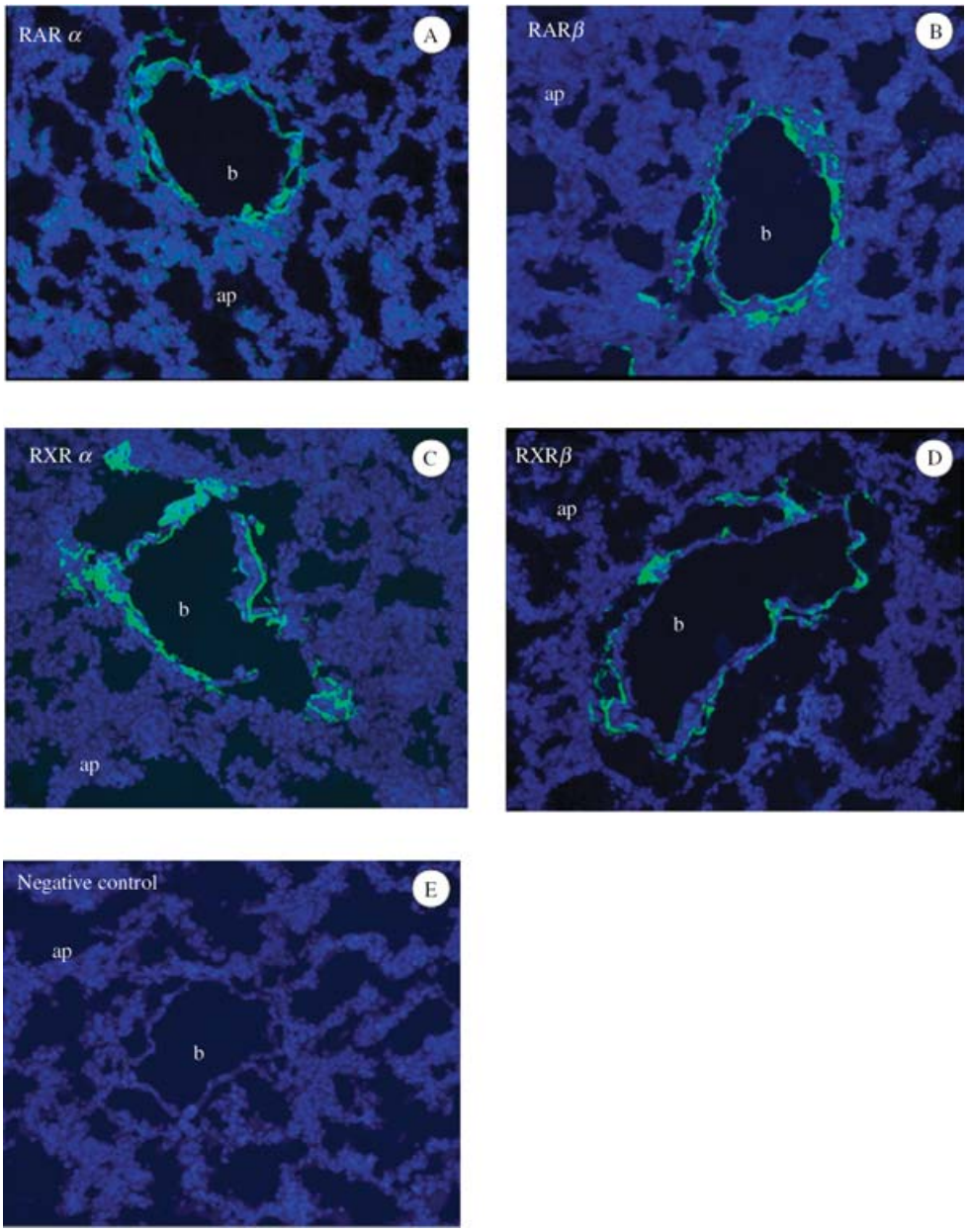

Figure 3 Immunohistochemistry showing expression of RAR and RXR proteins at day 31 of rabbit lung development. Rabbit RARα (A), RARβ (B), RXRα (C) and RXRβ (D) proteins were detected in foetal rabbit lung on the 31st day of gestation. Primary antibodies free incubations (E) were present as negative controls. Magnification × 60. Abbreviations: ap, alveolar parenchyma and b, bronchia.

et al., 1998). No lung abnormalities have been described in other species like rabbits or humans after maternal retinol deficiency or impairment of the retinoid signalling pathway during the embryonic stage (Wilcox *et al.*, 1996). Early maternal administration of nitrofen, a toxic reducing RA synthesis, only induces lung hypoplasia and diaphragmatic hernia in the murine model. This lack of evidence for an early critical role of retinoids in rabbit lung development led us to give up microdissection of the rabbit lungs at day 8 and 11 (retinoid receptors expression demonstrated in the total embryo). During the subsequent stages, RARα, β and RXRα, β were expressed. During the pseudo-glandular stage, the mouse model established that RA works as an inhibitor in distal branching (Malpel *et al.*, 2000). This activity is supported by the transcriptional regulation of RA target genes implicated in lung development such as transforming growth factor (TGF) β3, Sonic hedgehog, fibroblast growth factor (FGF) 10, Homeobox genes (a2, a4, a5, b5 and b6), receptor of epidermal growth factor (EGF) or midkine (Maden, 2004,). RARα, RXRα and β are associated with epithelial cell differentiation and structural changes during the transition from the glandular to the canalicular stage of lung development (Belloni *et al.*, 2000). In human, ultrasonographic prenatal screening usually occurs at 22 weeks of gestation, *i.e.* after the transition from the glandular to the canalicular stage. Therefore, most of lung

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abnormalities are detected at this stage of development. It enlightens the interest to demonstrate retinoid receptors expression after the pseudo-glandular stage rather than earlier because retinoids as a potential therapy could not be used earlier when the diagnosis is still not established. During the saccular period, RA stimulates proliferation of the stem cells of the alveolar epithelium through an *EGF*-mediated pathway (Schuger *et al*., 1993; Massaro and Massaro, 1996). During the alveolar period, retinoids are effective in promoting alveolar subdivision or septation (Hind *et al*., 2002; Biesalski and Nohr, 2003).The absence of RXRγ and RARγ expression was already established during lung development in mice (Dolle *et al*., 1990 and 1994) and rats (Grummer *et al*., 1994) even if some studies supported the presence of some isoforms of RARγ? (Grummer *et al*., 1994; McGowan *et al*., 1995; Hind *et al*., 2002; Biesalski and Nohr, 2003). Three RARs and three RXRs have been demonstrated in the developing human lung (Rajatapiti *et al*., 2005). Our results complete the list of nuclear receptors previously described to be expressed in the rabbit developing lung including pregnane X receptor (Savas *et al*., 2000), glucocorticoid receptor (GR) (Hummelink and Ballard, 1986), androgen receptor (Giannopoulos and Smith, 1982), thyroid receptor (TR) (Ballard *et al*., 1984), progesterone receptor (Camacho-Arroyo *et al*., 1994) and peroxisome proliferator activated receptor (Michael *et al*., 1997). In both humans (Rajatapiti *et al*., 2005) and rabbits, developing lungs express receptors TR, RAR, RXR and GR.

In conclusion, our study demonstrates the partial cloning, the presence of retinoid receptors and their expression during lung development in rabbits. These results are a crucial step to determine the role of retinoids at a molecular level in the pathophysiology of lung development including the surgical model of congenital diaphragmatic hernia.

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