Effects of the peroxisome proliferator-activated receptor \( \gamma \) agonist rosiglitazone on foetal lung development in an experimental rat model of congenital diaphragmatic hernia

A thesis submitted by

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Candidate thesis declaration

I declare that this thesis, which I submit to RCSI for examination in consideration of the award of a higher degree of Master of Science by research, is my own personal effort. Where any of the content presented is the result of input or data from a related collaborative research programme this is duly acknowledged in the text such that it is possible to ascertain how much of the work is my own. I have not already obtained a degree in RCSI or elsewhere on the basis of this work. Furthermore, I took reasonable care to ensure that the work is original, and, to the best of my knowledge, does not breach copyright law, and has not been taken from other sources except where such work has been cited and acknowledged within the text.

Signed

Student Number 1240787

Date 28/04/2014
Dedication

This work is dedicated to my mother, who has been a constant source of love, affection and encouragement, and to my late father, whom I dearly miss.

The summit is just a halfway point.

Edmund Viesturs
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Summary

Congenital diaphragmatic hernia (CDH) is a prenatal defect in the integrity of the developing diaphragm, which results in severe pulmonary hypoplasia (PH) with alveolar immaturity. Peroxisome proliferator-activated receptor γ (PPARγ) plays a key role in foetal alveolarization by coordinating alveolar cell proliferation and differentiation, which results in enhanced expression of alveolar lipid-containing interstitial fibroblasts (LIFs). Furthermore, PPARγ increases the lipid content of alveolar LIFs by upregulation of adipocyte differentiation-related protein (ADRP), a lipogenic marker for alveolar mesenchymal differentiation and physiological determinant for the synthesis of surfactant phospholipids. Since PPARγ transcripts and alveolar LIFs are significantly decreased in hypoplastic rat lungs, the overall aim of this work was to investigate the hypothesis that the synthetic PPARγ agonist rosiglitazone (RGZ) has the potential to improve foetal alveolar development in the nitrofen rat model of CDH-associated PH.

The first objective of this work was to examine the in vitro effects of RGZ treatment in an explant culture of foetal rat lungs. Morphometric analysis revealed that daily administration of RGZ enhances alveolarization in nitrofen-exposed hypoplastic lungs compared to Placebo-treated lung explants, which was further supported by a significantly increased radial alveolar count and decreased mean linear intercept.
The second objective was to evaluate the *in vivo* effects of prenatally administered RGZ on alveolar development and maturation in foetal rats with CDH. Immunohistochemical/-fluorescence analysis demonstrated that maternal administration of RGZ shortly before birth stimulates epithelial and mesenchmal differentiation in nitrofen-induced PH compared to Placebo-treated foetuses, as evidenced by increased lamellar body count and ADRP expression. There was also a substantial accumulation of cytoplasmatic lipid droplets in alveolar interstitial cells, which was accompanied by a markedly increase of LIFs in the mesenchymal compartments of distal alveolar walls.

Taken together, these results suggest that RGZ has a therapeutic potential in attenuating foetal PH in rats with nitrofen-induced CDH through accelerating epithelial-mesenchymal interactions, which may result in enhanced expression of LIFs and thus alveolar maturation.
**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>αSMA</td>
<td>Alpha smooth muscle actin</td>
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<tr>
<td>ADRP</td>
<td>Adipocyte differentiation-related protein</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>AST</td>
<td>Alveolar septal thickness</td>
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<td>CDH</td>
<td>Congenital diaphragmatic hernia</td>
</tr>
<tr>
<td>DAP</td>
<td>Diaminobenzidine</td>
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<td>DAPI</td>
<td>4′,6-diamidino-2-phenylindole</td>
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<td>LIF</td>
<td>Lipid-containing interstitial fibroblast</td>
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<td>MLI</td>
<td>Mean linear intercept</td>
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<tr>
<td>ORO</td>
<td>Oil-red-O</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
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<tr>
<td>PH</td>
<td>Pulmonary hypoplasia</td>
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<td>PHMP</td>
<td>Posthepatic mesenchymal plate</td>
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<td>PPF</td>
<td>Pleuroperitoneal fold</td>
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<td>PPARγ</td>
<td>Peroxisome proliferator-activated receptor γ</td>
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<tr>
<td>PPHN</td>
<td>Persistent pulmonary hypertension of the newborn</td>
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<td>RGZ</td>
<td>Rosiglitazone</td>
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Chapter 1

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Introduction
1.1 Congenital diaphragmatic hernia

1.1.1 Definition

Congenital diaphragmatic hernia (CDH) is a prenatal defect in the integrity of the developing diaphragm, which is characterized by absence of one or more of the diaphragmatic leaflets, causing intrathoracic herniation of abdominal organs and thus disturbing normal lung development.

1.1.2 Historical background

The earliest description of diaphragmatic defects was by Hippocrates (460-370 BC), who reported fatal perforations caused by sword and knife injuries [1]. The term diaphragmatic hernia was introduced in 1575 by the French surgeon Ambroise Paré, who described two autopsy cases of traumatic diaphragmatic defects [2]. Following a post-mortem examination of a 24-year-old man, the French physician Lazare Rivièrè discovered the first case of CDH, which he published in 1679 [3]. In 1701, Sir Charles Holt reported the first paediatric case of CDH [4]. The Italian anatomist Giovanni Battista Morgagni differentiated in his monograph from 1761 various types of CDHs, including the anterior defect that bears his name [5]. In 1819, the French physician René Laennec demonstrated that the diagnosis of CDH could easily be made by chest auscultation and also suggested that laparotomy might be the correct approach for hernia repair [6]. Sir Astley Paston Cooper published in 1827 the first comprehensive report on classification, symptoms and pathology of CDH [7]. The first cohort series of patients with CDH was collected by Henry Bowditch in 1847 at the
Massachusetts General Hospital in Boston, emphasizing the clinical criteria for diagnosis [8]. In 1848, the Czech anatomist Vincent Alexander Bochdalek accurately described a posterolateral defect in the diaphragm, which carries his name today [9]. However, his understanding of the embryology of CDH was incorrect as he speculated that the hernia resulted from an intrauterine rupture of the membrane in the lumbocostal triangle. In 1888, the Swedish surgeon Naumann proposed a 2-cavity approach after unsuccessfully operating on a 19-year-old patient with an infarcted bowel that had herniated through a diaphragmatic defect [10]. The first, but unsuccessful repair in a 3-year-old infant with CDH was performed by the American surgeon O'Dwyer via an abdominal approach [11]. The first report of a successful repair in an adult was by the German surgeon Aue in 1901 [12], followed by Heidenhain in 1905, who successfully repaired a CDH in a 9-year-old boy [13]. In a comprehensive review from 1925, the American surgeon Hedblom showed that 75% of untreated cases with CDH died in the newborn period, suggesting that an earlier intervention might improve survival [14]. After that, Bettman and Hess presented in 1929 the youngest patient with CDH, who had successfully been operated on aged 3.5 months [15]. Greenwald and Steiner reviewed symptoms of infants and children with CDH, concluding that this condition might not be as infrequent as it was generally believed [16]. Surgical repair of CDH remained often unsuccessful until 1940, when Ladd and Gross reported 9 of 16 patients surviving surgery, the youngest being 40 hours old [17]. Robert Gross subsequently performed in 1946 the first successful repair in a neonate with CDH less than 24 hours after birth [18]. In 1950, Koop and
Johnson proposed a transthoracic approach as a means of closing the CDH under more direct vision [19]. As the surgical expertise improved further, several innovative techniques were introduced to address large diaphragmatic defects, including the use of muscle flaps [20] and prosthetic patches [21]. All these findings and advances have contributed to the significant reduction in mortality of newborns with CDH.

1.1.3 Classification

In humans, CDHs are classified according to their presumed anatomical location and three different types of hernia can be distinguished: (1) a posterolateral Bochdalek-type (~90-95%), (2) an anterior Morgagni-type (~2%) and (3) a central, septum transversum-type (very rare) [22]. Approximately 80% of the hernias are left-sided, 19% right-sided and 1% bilateral [23]. However, close scrutiny of various diaphragmatic defects has demonstrated wide phenotypic variations in shape, size and location [24], suggesting that a clear distinction among the different types can be problematic.

1.1.4 Epidemiology

With an estimated incidence of 1 in 2000 to 4000 newborns, CDH is a relatively common birth defect that accounts for approximately 8% of all major congenital malformations [25,26]. Recent population-based studies from the USA and Western Australia have found prevalence rates ranging between 2.4 and 3.8 cases per 10,000 total births [27-30]. Similar prevalence rates were reported by European registry-based studies, currently
affecting between 2.55 and 2.7 cases per 10,000 births [31,32]. However, the incidence of CDH in stillborns and abortions seems to be less well documented. It can be estimated that approximately one third of infants with CDH are stillborn, which is mainly a result of the associated fatal congenital anomalies [33] and adds a “hidden mortality” to the operative and postoperative deaths [34]. Thus, the true incidence of CDH is considerably higher than seen in the neonatal surgical practice [35].

1.2 Normal embryological development of the diaphragm and lung

Due to highly complex spatiotemporal processes involving multiple regulatory factors, normal embryological development of diaphragm and lung remains poorly understood. In humans, the diaphragm starts to develop at approximately 4 weeks of gestation, which is equivalent to embryonic day 12.5 in rats. The mesenchymal-derived pleuroperitoneal fold (PPF) has recently been identified as a key structure during normal diaphragmatic development [36,37]. However, it remains controversial if the muscle precursor cells and phrenic axons, which originate from the PPF, form the diaphragm alone or how much the posthepatic mesenchymal plate (PHMP) contributes to the closure of the pleuroperitoneal canal [38,39].

Normal lung development begins around the same time with outgrowth of two bronchial buds and is divided into five histological stages: embryonic, pseudoglandular, canalicular, saccular and alveolar [40,41].
During this period, the lung develops by dichotomous branching from two primitive endodermal buds to a functional organ with a large surface area and highly differentiated alveoli.

1.3 Pathogenetical and -physiological aspects of congenital diaphragmatic hernia

Although it is relatively easy to repair the diaphragmatic defect either by primary closure or with a patch, the main problem is the associated disturbed lung development, resulting in severe pulmonary hypoplasia (PH) and persistent pulmonary hypertension of the newborn (PPHN) [42]. Both conditions occur to a variable extent in patients with CDH and due to the absence of sufficient lung-protective strategies, most of the newer treatment modalities such as exogenous surfactant, inhaled nitric oxide, high-frequency oscillation and extracorporeal membrane oxygenation have replaced mortality with a higher rate of long-term morbidity from bronchopulmonary dysplasia and chronic lung disease [43,44]. Therefore, CDH remains one of the major therapeutic challenges in neonatal intensive care units [45].

In the past, it has been assumed that the different parts of the diaphragm fail to fuse properly [46]. Consequently, the pleuroperitoneal canal does not close and PH was believed to be the result of mechanical compression by herniation of abdominal viscera into the thoracic cavity [47]. It has also been suggested that a primary disturbance of the pulmonary
development may cause CDH [48]. However, it has been demonstrated by combining teratogenic and transgenic animal models that this hypothesis is not true [49]. Moreover, Fgf10 knockout mice do not have any lungs but show normal diaphragmatic development [50]. The so-called "dual-hit hypothesis" implicates a primary disruption in bilateral lung development before closure of the diaphragm combined with a second ipsilateral insult caused by intrathoracic herniation and thus interference with foetal breathing movements [51]. Recently, a proliferative abnormality of the PPF has been postulated for the insufficient formation of the diaphragm [52]. Surprisingly, the origin of the diaphragmatic defect lies in the amuscular mesenchymal component of the PPF, supporting the hypothesis that CDH occurs independently of myogenesis and lung formation [38]. Regardless of all these theories, the exact pathogenesis of CDH and associated pulmonary malformation remains unclear.

Hypoplastic lungs in CDH are characterized by immaturity and smaller size with a significantly decreased number of terminal airway generations, thickened alveolar walls, increased interstitial tissue, diminished alveolar airspaces and reduced gas-exchange surface area [53-55]. Apart from the gas-exchange layer, well-documented changes are present in the vascular components consisting of arterial media hyperplasia, peripheral muscularisation of smaller pre-acinar vessels and adventitial thickening [56-58].
1.4 Animal models of congenital diaphragmatic hernia

Most of our current knowledge about the structural and morphological changes in hypoplastic lungs associated with CDH has originated from experimental animal models, in which the diaphragmatic defect is either surgically, transgenically or toxicologically created [59,60].

1.4.1 Surgical models

Surgically created diaphragmatic defects in foetal sheep and rabbits are useful for investigating interventional treatment strategies such as in utero repair and tracheal occlusion [61,62], but are less helpful in studying the earlier pathogenesis of PH in CDH.

1.4.2 Genetic models

Over the last decade, several knockout models for genes involved in embryonic mouse development, such as for Wt1 [63], Shh [64], Gli2/Gli3 [65], Slit3 [66], Fog2 [67], Gata4/Gata6 [68,69], COUP-TFII [70], Pdgfra [71] and RARs [72] have been established, which show various phenotypes of CDH and disruption in lung branching morphogenesis. However, until now only the Fog2 gene mutation has been found in a patient with non-syndromic CDH [71].
1.4.3 Nitrofen model

Administration of the herbicide nitrofen (2,4-dichlorophenyl-p-nitrophenyl ether) to pregnant rodents has been found to cause different developmental anomalies in heart, lung and diaphragm [73]. Maternal exposure during midgestation results in CDH in approximately 70% and PH in 100% of the offspring [74]. Therefore, the toxicologically introduced nitrofen model has widely been used to investigate the CDH-associated anomalies, since timing of the diaphragmatic insult and bilateral PH are remarkably similar to the human situation [74,75]. Nevertheless, the significance of these potential teratogenic effects has never been proven in humans.

Although none of the available animal models are perfect in mimicking the human diaphragmatic defect, they all have provided new insights into the underlying pathogenesis and pathophysiological mechanisms of CDH. Moreover, genome-wide arrays may be useful to identify potential CDH-related genes in humans.

1.5 Peroxisome proliferator-activated receptor γ

and its role in foetal lung development

Peroxisome proliferator-activated receptor γ (PPARγ) is a ligand-activated transcription factor that belongs to the superfamily of nuclear hormone receptors [76-78], which are involved in various developmental
processes such as cell proliferation and differentiation [79-81]. PPARγ expression has been found in a broad range of developing organs, including the lung [82]. In foetal rat lungs, PPARγ expression increases 3-5 times between gestational day 18 and term (day 22), with peaking just before birth [83,84]. Several studies have evaluated the role of PPARγ in lung maturation, demonstrating its important function by stimulating the alveolar epithelial-mesenchymal paraocrine signalling [85,86]. PPARγ is known to be highly expressed in lipid-containing interstitial fibroblasts (LIFs), which play a critical role in foetal alveolarization by promoting alveolar epithelial cell proliferation and differentiation [86-88]. However, the expression of alveolar LIFs requires upregulation of PPARγ, which in turn stimulates epithelial-mesenchymal interactions in the developing lung [89,90]. The lipid content of LIFs is controlled by the lipogenic marker adipocyte differentiation-related protein (ADRP), which is a known downstream target of PPARγ [91-93]. Moreover, it has been shown that the expression of ADRP and LIFs is significantly decreased in CDH-associated PH, suggesting a disturbance of normal LIF functioning in hypoplastic lungs [94]. Conditional knock-out of PPARγ in conducting airway epithelium has also been demonstrated to result in a hypoplastic phenotype with abnormal alveolar maturation and decreased airspaces [80]. Recent work from our laboratory has provided strong evidence that PPARγ transcripts are significantly decreased in nitrofen-induced PH, while in control lungs relative mRNA expression peaks normally on gestational day 21 [95], thus indicating that upregulation of PPARγ at this specific time-point may be critical for foetal lung development and maturation in rats with CDH.
Furthermore, it has been pointed out that prenatal administration of the PPARγ agonist rosiglitazone (RGZ) significantly enhances lung maturation without affecting neonatal [96] and long-term metabolic profile [97], suggesting a therapeutic potential in attenuating CDH-associated PH by stimulating alveolar maturation through an increase in LIFs. RGZ, a member of the thiazolidinedione class of drugs, is a high-affinity synthetic ligand that has the ability to activate PPARγ [77,98,99].
1.6 Aims and objectives

The overall aim of this work was to investigate the effects of the synthetic PPARγ agonist RGZ on foetal lung development in the nitrofen-induced rat model of CDH. Based on previous studies [96,97], it can be assumed that RGZ may have the ability to enhance alveolar maturation in foetal rats with CDH-associated PH. In order to prove this hypothesis, *in vitro* and *in vivo* treatment studies with RGZ were performed.

The first objective of this work was to determine the impact of RGZ application on alveolarization in a foetal explant culture of control- and nitrofen-exposed rat lungs by using morphometric analysis techniques.

The second objective was to evaluate the efficacy of prenatally administered RGZ on alveolar development in control and nitrofen-exposed foetuses with and without CDH. Therefore, following maternal RGZ administration shortly before birth, lung maturation was assessed by examining the expression of key markers for alveolar epithelial and mesenchymal differentiation that are the hallmarks of alveolar development in foetal lungs, including lamellar body protein and ADRP expression.
Chapter 2

Material and methods
2.1 Animal model and experimental design

2.1.1 Animal protocol

After obtaining ethical approval (Ref. REC668b) from the local research ethics committee, pathogen-free Sprague-Dawley\textsuperscript{®} rats (Harlan Laboratories, Shardlow, UK) were kept in a well-controlled environment (50-55\% humidity, 19-21°C, 12-h light period, food and water \textit{ad libitum}) and females were checked daily for presence of vaginal plug after overnight mating. The day of plugging was defined as embryonic day 0.5 (E0.5) and timed-pregnant animals were randomly divided into two groups ("Nitrofen" and "Control"). On E9.5, dams were briefly anaesthetized with 2\% volatile isoflurane (Piramal Healthcare Ltd, Morpeth, UK) and 100 mg nitrofen (2,4-dichlorophenyl-p-nitrophenyl ether) (Wako Chemicals GmbH, Neuss, Germany) was administered in 1 ml olive oil via oral-gastric lavage, while control animals received vehicle alone.

For all \textit{in vitro} and \textit{in vivo} treatment studies with RGZ (Cayman Chemical, Ann Arbor, USA), foetuses were delivered via caesarean section and sacrificed by decapitation (E18.5 and E21.5, respectively). After laparotomy, diaphragms were inspected under a Leica S8AP0 stereomicroscope (Leica Microsystems AG, Heerbrugg, Switzerland) for CDH and whole lungs were dissected under sterile conditions.

All animal procedures were performed following current guidelines for management and welfare of laboratory animals and were approved by the Department of Health and Children (Ref. B100/4378) under the Cruelty
to Animals Act, 1876 (as amended by European Communities Regulations 2002 and 2005), in accordance with the ethical standards laid down in the 1964 declaration of Helsinki.

2.1.2 *In vitro* treatment studies of foetal lung explants with rosiglitazone

Isolated E18.5 lungs from control and nitrofen-treated foetuses were kept in ice-cold Hank’s balanced salt solution (Gibco® by Life Technologies™, Carlsbad, USA) until explant culture was initiated. Briefly, the surrounding tissue was gently removed and whole lungs were separated into single segments. The trimmed specimens were then transferred into Costar® culture clusters (Corning Life Sciences, Corning, USA) containing 4 ml Dulbecco’s modified eagle medium/nutrient mixture F-12 (Gibco® by Life Technologies™, Carlsbad, USA) supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin (Life Technologies™, Carlsbad, USA). Based on previous studies [100], explants were treated either with 10⁻⁵ mol/l RGZ, 10⁻⁵ mol/l RGZ plus 10⁻⁵ mol/l of the specific PPARγ antagonist GW9662 (Cayman Chemical, Ann Arbor, USA) or with diluents alone. This set of experiments created the following treatment groups: Control+Placebo (*n* = 8), Control+RGZ (*n* = 8), Control+RGZ+GW9662 (*n* = 8), Nitrofen+Placebo (*n* = 8), Nitrofen+RGZ (*n* = 8), Nitrofen+RGZ+GW9662 (*n* = 8). All *in vitro* studies were conducted in an atmosphere of 5% CO₂-air at 37°C for 72 h with daily changes of the culture medium. The cultured lung explants were then fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) solution (Santa
Cruz Biotechnology Inc, Santa Cruz, USA) for histological analysis (Fig. 1).

**Fig. 1** Experimental design for *in vitro* treatment studies with rosiglitazone (RGZ), GW9662 and placebo.

### 2.1.3 *In vivo* treatment studies with rosiglitazone

On E18.5, control and nitrofen-treated rats were randomly assigned to one of the four treatment groups: Control+Placebo (*n* = 2), Control+RGZ (*n* = 2), Nitrofen+Placebo (*n* = 2) and Nitrofen+RGZ (*n* = 2). Based on previous studies [97], 3 mg/kg RGZ (Cayman Chemical, Ann Arbor, USA) was administered intraperitoneally in 100 µl volumes under short anaesthesia once daily on E18.5 and E19.5, while control animals received diluent alone. The dams were then anaesthetized on the selected end point E21.5 and foetuses were weighed before the whole lung was dissected. After weighing, lung specimens were fixed in 4% PFA in PBS solution (Santa Cruz Biotechnology Inc, Santa Cruz, USA) for morphological and
immunohistochemical analysis. A total of 16 foetal lungs were used for each treatment group (7-9/litter) (Fig. 2).

![Diagram of experimental design](image)

**Fig. 2** Experimental design for *in vivo* treatment studies with rosiglitazone (RGZ) and placebo.

### 2.2 Preparation of lung samples for morphological and immunohistochemical analysis

After the *in vitro* and *in vivo* experiments, PFA-fixed foetal lungs were washed in cold PBS to remove exterior debris, followed by embedding in O.C.T. compound mounting medium (VWR International Ltd, Dublin, Ireland) and snap freezing in liquid nitrogen. Frozen blocks were then stored at -80°C until cryosectioning was performed. All lung specimens were cut transversely at a thickness of 10 μm using a CM1900 cryostat (Leica Microsystems GmbH, Nussloch, Germany) at -20°C and serial sections were mounted on SuperFrost® Plus microscopy glass slides (VWR International Ltd, Dublin, Ireland).
2.3 Assessment of alveolar maturation and lung morphology

Two independent-blind investigators unaware of the treatment group performed lung morphometry, which was objectively assessed by determining the radial alveolar count (RAC) and mean linear intercept (MLI) on haematoxylin- and eosin-stained (Sigma Aldrich, Saint Louis, USA) sections. Fifty randomly selected, non-overlapping fields from serial sections obtained from frozen blocks from each experimental group were investigated under a Leica DM LB research microscope (Leica Microsystems GmbH, Wetzlar, Germany). Each field was viewed at 40-fold magnification, and the image was digitized and projected on a computer screen by using a Leica DC300F digital camera (Leica Microsystems AG, Heerbrugg, Switzerland). For each field, the number of alveoli was counted visually and RAC was performed by identifying respiratory bronchioles, as described by Randell et al. [101]. Briefly, the number of distal air sacs that were transacted by a line drawn from a terminal respiratory bronchiole to the nearest pleural surface was counted. No counts were made if the respiratory bronchiole was nearer to the edge of the slide than to the nearest connective tissue septum. The MLI represents the average alveolar diameter, alveolar septal thickness (AST) and tissue density, which is the proportion of the field occupied by tissue (area occupied by tissue/area occupied by lung tissue + alveoli). All images were analyzed with ImageJ 1.47a, a public domain, Java™-based image processing and analysis software program (National Institute of Health, Bethesda, USA).
2.4 Immunohistochemical stainings

2.4.1 Lamellar body staining

Lamellar body protein expression was determined by labelling for p180 lamellar body, a specific marker for alveolar epithelial cells type II. Briefly, thawed tissue sections were incubated with PBS containing 1.0% Triton X-100 (Sigma Aldrich Ltd, Arklow, Ireland) for 20 min to improve cell permeabilization. In order to prevent nonspecific absorption, sections were blocked with 10% normal goat serum (Sigma Aldrich, Saint Louis, USA) for 30 min, followed by incubation with affinity-purified mouse anti-p180 lamellar body protein antibodies (ab24751, 1:100) (Abcam plc, Cambridge, UK) at 4°C overnight. On the next day, sections were washed in FBS + 0.05% Tween (Sigma Aldrich, Saint Louis, USA) and incubated with Alexa Fluor® 488 goat anti-mouse secondary antibodies (A11029, 1:100) (Bio-Sciences Ltd, Dun Laoghaire, Ireland) at room temperature for 30 min. The sections were then counterstained with DAPI (10236276001, 1:1000) (Roche Diagnostics GmbH, Mannheim, Germany) for 10 min to visualize double-stranded DNA. Following coverslipping with fluorescent mounting medium (DAKO Ltd, Cambridgeshire, UK), two investigators independently evaluated sections with a LSM 700 confocal microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany). All images were analyzed with ZEN, an image processing and analysis software program (Carl Zeiss MicroImaging GmbH, Jena, Germany).
2.4.2 Adipocyte differentiation-related protein staining

Alveolar mesenchymal differentiation was ascertained by labelling for ADRP, a key marker for alveolar mesenchymal differentiation and downstream target of PPARγ. Briefly, thawed sections were incubated with phosphate buffered saline (PBS) containing 1.0% Triton X-100 (Sigma Aldrich, Saint Louis, USA) for 20 min to improve cell permeabilization. In order to avoid masking of antigenic sites, sections were immersed in heated Target Retrieval Solution® (DAKO Ltd, Cambridgeshire, UK) in a microwave oven at 750 W for 15 min. Next, endogenous peroxidase activity was blocked with Peroxidase Block® (DAKO Ltd, Cambridgeshire, UK) for 5 min. To prevent nonspecific absorption, sections were blocked with 10% normal goat serum (Sigma Aldrich, Saint Louis, USA) for 30 min, followed by incubation with affinity-purified rabbit ADRP antibodies (sc-32888, 1:50) (Santa Cruz Biotechnology Inc, Santa Cruz, USA) at 4°C overnight. On the next day, sections were washed in PBS + 0.05% Tween and incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibodies (K4011, 1:100) (DAKO Ltd, Cambridgeshire, UK) at room temperature for 30 min. The antibody-antigen complexes were then visualized by staining with diaminobenzidine (DAB) + Substrate Buffer® and DAB + Chromogen® (DAKO Ltd, Cambridgeshire, UK) for 30 s. After counterstaining with haematoxylin (Sigma Aldrich, Saint Louis, USA) for 10 s, sections were coverslipped using DPX Mountant for histology (Sigma Aldrich, Saint Louis, USA). All sections were independently evaluated by two investigators with a Leica DM LB research microscope (Leica Microsystems GmbH, Wetzlar, Germany) using the image processing and
analysis software program Leica IM50 version 1.20 (Leica Microsystems AG, Heerbrugg, Switzerland).

2.4.3 Immunofluorescence-double-staining

The presence of alveolar LIFs was evaluated by immunofluorescence-double-staining with oil-red-O and alpha smooth muscle actin (αSMA) in order to determine localization and lipid content of this specific cell type. Briefly, sections were incubated with PBS containing 1.0% Triton X-100 (Sigma Aldrich Ltd, Arklow, Ireland) for 20 min to improve cell permeabilization. An oil-red-O solution was prepared by slowly dissolving 0.7 g oil-red-O powder (Sigma Aldrich, Saint Louis, USA) in 100 ml propylene glycol, while heating to 100°C for a few minutes. This solution was filtered twice and cooled down before further use. In order to prevent nonspecific absorption, sections were blocked with 10% normal goat serum (Sigma Aldrich, Saint Louis, USA) for 30 min, followed by incubation with oil-red-O solution and affinity-purified mouse anti-αSMA antibodies (M0851, 1:500) (DAKO Ltd, Cambridgeshire, UK) at 4°C overnight. On the next day, sections were washed in PBS + 0.05% Tween and incubated with Alexa Fluor® 488 goat anti-mouse secondary antibodies (A11029, 1:100) (Bio-Sciences Ltd, Dun Laoghaire, Ireland) for 30 min. The sections were then counterstained with DAPI (10236276001, 1:1000) (Roche Diagnostics GmbH, Mannheim, Germany) for 10 min to visualize double-stranded DNA. Following coverslipping with fluorescent mounting medium (DAKO Ltd, Cambridgeshire, UK), two investigators independently evaluated sections with a LSM 700 confocal microscope (Carl Zeiss MicroImaging
GmbH, Jena, Germany). All images were analyzed with ZEN, an image processing and analysis software program (Carl Zeiss MicrolImaging GmbH, Jena, Germany).

2.5 Statistical analysis

Data was analyzed using GraphPad Prism 5 (GraphPad Software Inc, La Jolla, USA) and tested for Gaussian distribution with a Kolmogorov-Smirnov test. All results are presented as means ± standard error of the mean (SEM). In order to determine any statistical differences between the experimental groups, one-way ANOVA with Tukey's test for post-test analysis was performed. A $P$ value < 0.05 was considered as statistically significant.
Chapter 3

Results
3.1 Effect of \textit{in vitro} treatment with rosiglitazone on foetal lung development

3.1.1 Effect of \textit{in vitro} treatment with rosiglitazone on lung morphometry

Morphometric analysis of foetal lung explants revealed a significant advance in alveolar development after daily treatment with RGZ. Nitrofen-exposed lungs that were treated with RGZ showed after 72 hours in culture an enhancement of alveolarization, which was expressed in a significant increase in RAC (6.00 ± 1.2 per mm$^2$ vs. 4.32 ± 1.1 per mm$^2$; $P < 0.0001$) and decrease in MLI (39.26 ± 1.8 μm vs. 43.13 ± 3.1 μm; $P < 0.0001$) compared to placebo-treated lung explants. Furthermore, co-culture with the specific PPARγ antagonist GW9662 blocked the RGZ-mediated improvements in alveolar maturation in nitrofen-exposed lungs, without any significant changes in RAC (4.37 ± 1.3 per mm$^2$ vs. 4.32 ± 1.1 per mm$^2$; $P = 0.7732$) and MLI (43.02 ± 29.2 μm vs. 43.13 ± 3.1 μm; $P = 0.6472$) compared to placebo-treated lung explants (Fig. 3).
Fig. 3  Effect of *in vitro* treatment with RGZ on lung morphometry. Daily treatment resulted in a significantly increased alveolarization in nitrofen-exposed lungs compared to placebo-treated lung explants, while co-culturing with the specific PPARγ antagonist GW9662 blocked the RGZ-mediated improvements. Control+Placebo (A), Control+RGZ (B), Control+RGZ+GW9662 (C), Nitrofen+Placebo (D), Nitrofen+RGZ (E), Nitrofen+RGZ+GW9662 (F). Representative H&E stained lung section and corresponding histograms of radial alveolar count (G) and mean linear intercept (H) are shown. Magnification x40. (**p < 0.0001, ns = not significant; vs. Nitrofen+Placebo).
3.2 Effect of *in vivo* treatment with rosiglitazone on foetal lung development

3.2.1 Effect of *in vivo* treatment with rosiglitazone on body and lung weight

Since body and lung weight is a reflection of overall development, the *in vivo* effect of RGZ on body and lung weight was determined. There was a significant increase in body (4.06 ± 0.12 g vs. 3.53 ± 0.05 g; *P* < 0.01) and lung (72.95 ± 3.25 mg vs. 55.89 ± 4.78 mg; *P* < 0.01) weight after prenatal administration of RGZ in Nitrofen+RGZ compared to Nitrofen+Placebo treatment group, which was also shown in a significantly increased lung/body weight ratio (2.03 ± 0.03 % vs. 1.77 ± 0.08 %; *P* < 0.01) (Fig. 4).

![Graph showing lung/body weight ratio](image)

**Fig. 4** Effect of *in vivo* treatment with RGZ on lung/body weight ratio. (**P* < 0.01; vs. Nitrofen+Placebo).
3.2.2 Effect of *in vivo* treatment with rosiglitazone on lung morphometry

Morphometric analysis of foetal lungs on E21.5 revealed a significant advance in alveolar development after prenatal administration of RGZ. Nitrofen-exposed foetuses that received RGZ application shortly before birth showed an enhancement of alveolarization, which was expressed in a significant increase in RAC (6.66 ± 1.3 per mm² vs. 5.70 ± 1.2 per mm²; *P* < 0.0001) and decrease in MLI (42.44 ± 1.5 μm vs. 45.06 ± 1.3 μm; *P* < 0.0001) compared to placebo-treated foetuses (Fig. 5).
Fig. 5 Effect of *in vivo* treatment with RGZ on lung morphometry. Prenatal administration resulted in a significantly increased alveolarization in nitrofen-exposed lungs compared to placebo-treated lungs. Control+Placebo (A), Control+RGZ (B), Nitrofen+Placebo (C), Nitrofen+RGZ (D). Representative H&E stained lung section and corresponding histograms of radial alveolar count (E) and mean linear intercept (F) are shown. Magnification x40. (***P < 0.0001, vs. Nitrofen+Placebo).
3.2.3 Effect of \textit{in vivo} treatment with rosiglitazone on alveolar epithelial differentiation

In order to evaluate the \textit{in vivo} effects of prenatally administered RGZ on alveolar epithelial differentiation, foetal lung sections were labelled for p180 lamellar body, a specific marker for alveolar epithelial cells type II. Sections of nitrofen-exposed foetuses that received RGZ treatment shortly before birth showed a markedly increased lamellar body staining and count (19.48 ± 2.3 per mm$^2$ vs. 10.54 ± 1.9 per mm$^2$; $P < 0.0001$) on E21.5, thus reflecting enhanced alveolar epithelial differentiation compared to placebo-treated foetuses (Fig. 6).
Fig. 6  Effect of in vivo treatment with RGZ on alveolar epithelial differentiation. Prenatal administration resulted in a significantly increased lamellar body staining in nitrofen-exposed lungs compared to placebo-treated lungs, as determined by specific labelling for the lamellar body marker p180. Control+Placebo (A), Control+RGZ (B), Nitrofen+Placebo (C), Nitrofen+RGZ (D). Representative cryostat sections of fixed lung tissue labelled with p180 lamellar body (arrow) and corresponding histogram of lamellar body count (E) are shown. Magnification x40. (**P < 0.0001, vs. Nitrofen+Placebo).
3.2.4 Effect of *in vivo* treatment with rosiglitazone on alveolar mesenchymal differentiation

To assess the *in vivo* effects of prenatal administration of RGZ on alveolar mesenchymal differentiation, foetal lung sections were investigated by labelling for ADRP, a key marker for alveolar mesenchymal differentiation. Sections of nitrofen-exposed foetuses that received RGZ treatment shortly before birth showed a markedly increased ADRP expression on E21.5, thus reflecting enhanced alveolar mesenchymal differentiation compared to placebo-treated foetuses (Fig. 7).

**Fig. 7** Effect of *in vivo* treatment with RGZ on alveolar mesenchymal differentiation. Prenatal administration resulted in markedly increased ADRP staining in nitrofen-exposed lungs compared to placebo-treated lungs, as determined by specific labelling for the mesenchymal differentiation marker ADRP. Control+Placebo (A), Control+RGZ (B), Nitrofen+Placebo (C), Nitrofen+RGZ (D). Representative cryostat sections of fixed lung tissue labelled with ADRP (*arrow*) are shown. Magnification x40.

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3.2.5 Effect of *in vivo* treatment with rosiglitazone on the expression of alveolar lipid-containing interstitial fibroblasts

The *in vivo* effects of prenatailly administered RGZ on the expression of alveolar LIFs were evaluated by immunofluorescence-double-staining with oil-red-O and αSMA in order to determine localization and lipid content of this specific cell type. Since αSMA is characteristically absent in alveolar LIFs, the combination of oil-red-O- and αSMA-immunofluorescence staining facilitated the analysis of LIF distribution within foetal alveolar tissue. Lung sections of nitrofen-exposed foetuses that received RGZ treatment shortly before birth showed a markedly increased LIF expression in mesenchymal and interstitial compartments of distal alveolar walls compared to Placebo-treated foetuses on E21.5, which was associated with a substantial accumulation of cytoplasmatic lipid droplets within these specific alveolar interstitial cells (*Fig. 8*).
Fig. 8 Effect of *in vivo* treatment with RGZ on alveolar LIF expression. Prenatal administration resulted in a markedly increased LIF expression in alveolar mesenchymal and interstitial compartments in nitrofen-exposed lungs compared to placebo-treated lungs, which was associated with a substantial accumulation of cytoplasmatic lipid droplets within these specific alveolar interstitial cells. Control+Placebo (A), Control+RGZ (B), Nitrofen+Placebo (C), Nitrofen+RGZ (D). Representative cryostat sections of fixed lung tissue double-stained with oil-red-O (*red* staining) and αSMA (*green* staining) are shown. Magnification ×40.
Chapter 4

Discussion
4.1 Discussion

Alveolarization and associated distal airway maturation is a fundamental phase during foetal lung development that requires spatiotemporal expression of many regulatory factors, which in turn stimulates epithelial-mesenchymal interactions [102]. It is well-known from the teratogenic nitrofen model that any disturbance of alveolar formation contributes to the development of PH in CDH [42]. Because PH is the main cause of neonatal mortality and long-term morbidity in infants with CDH, decades of research have focussed on attempts to improve lung maturation in these patients. This has led to the widespread use of exogenous surfactant, inhaled nitric oxide, high-frequency oscillation and extracorporeal membrane oxygenation [103]. On the other hand, due to the absence of sufficient lung-protective strategies, most of these newer treatment modalities have replaced mortality with a higher rate of chronic lung disease and impaired neurodevelopment [104,105]. Despite the necessity to find an optimal treatment for lung immaturity in CDH, extensive research in the field has not succeeded yet.

Alveolar LIFs, which account for about 50% of resident alveolar wall cells in immature lungs [106], have been reported to play an essential role in alveolar maturation by promoting alveolar epithelial cell proliferation and differentiation [107]. However, the expression of alveolar LIFs requires upregulation of PPARγ to stimulate the epithelial-mesenchymal interactions [86,90]. Normal expression of PPARγ is therefore crucial during this critical period of foetal lung development and it has further been shown that
hypoplastic rat lungs exhibit decreased PPARγ transcripts, while relative mRNA expression normally peaks in control lungs just before birth [95]. In addition, it has recently been demonstrated that the expression of alveolar LIFs is also significantly decreased in rats with CDH-associated PH [94], suggesting a disruption in PPARγ signalling and thus normal LIF functioning in hypoplastic lungs. Alveolar LIFs normally arise in foetal rat lungs during the late canalicular stage of pulmonary development with a significant increase over the last few days of gestation [108]. It has been indicated that alveolar LIFs contain large, cytoplasmatic lipid droplets, which enable their histological detection within the walls of developing foetal alveoli [91,93]. Furthermore, alveolar LIFs are characterized by a relatively high expression of ADRP immediately before birth [109], which is most pronouncedly in foetal and newborn lungs [110]. This key marker for alveolar mesenchymal differentiation mediates the uptake of lipid droplets by LIFs and their subsequent transport to alveolar epithelial cells type II [109]. Consequently, ADRP reflects the content of lipid droplets in alveolar LIFs [111] and is a physiological determinant for the synthesis of surfactant phospholipids in alveolar epithelial cells type II [93]. Treatment strategies aiming to increase the PPARγ-regulated expression of alveolar LIFs therefore represent a promising therapeutic approach to enhance alveolar development and thus lung maturation in CDH-associated PH.

Although a variety of structurally distinct molecules have been shown to bind and activate PPARγ, due to extensive clinical experience with the synthetic thiazolidinedione compound RGZ, most studies focussed
on RGZ and its role in perinatal lung maturation [96,112]. Prenatal administration of RGZ has recently been demonstrated to result in a significant enhancement of pulmonary maturation without affecting neonatal [96] and long-term [97] metabolic profile. However, it is important to note that its efficacy has never been investigated in an experimental model for CDH. Therefore, dose and number of days used in our in vitro and in vivo treatment studies with RGZ was based on previous studies [97,100].

In the present study, we observed increased alveolarization in nitrofen-exposed foetal lung explants after 72 hours in culture with RGZ, which was supported by significantly increased RAC and decreased MLI compared to placebo-treated lungs. Furthermore, the RGZ-mediated improvements in alveolar development in nitrofen-exposed lungs were blocked in co-culture with the specific PPARγ antagonist GW9662, without any significant changes in RAC and MLI compared to placebo-treated lung explants. As expected, similar results were seen in foetal lungs after prenatal treatment with RGZ. Based on these findings, we investigated the alveolar maturity on cellular level and found advanced epithelial and mesenchymal differentiation in lungs of nitrofen-exposed foetuses that received prenatally RGZ treatment, as evidenced by increased lamellar body count and ADRP expression. In addition, there was a substantial accumulation of cytoplasmatic lipid droplets in alveolar interstitial cells of nitrofen-exposed foetuses that were treated with RGZ shortly before birth, which was accompanied by a markedly increase of LIFs in the mesenchymal and
interstitial compartments of distal alveolar walls. This possibly provides an integrated basis for enhanced alveolar development in nitrofen-induced PH after prenatal administration of RGZ by coordinated expression of alveolar LIFs through stimulation of epithelial-mesenchymal interactions. Therefore, it can be speculated that the synthetic PPARγ agonist RGZ is utilized by alveolar LIFs of immature lungs for regulation of many PPARγ-responsive genes, thus contributing to the formation of new alveolar septa and surfactant protein synthesis in alveolar epithelial cells type II. As normal functioning LIFs are able to synthesize PPARγ, it is not surprising that we did not find any structural and morphological differences between Control+RGZ and Control+Placebo treatment group.

RGZ has initially been developed to reduce the insulin resistance in patients with type 2 diabetes mellitus [113]. The glucose-lowering effects of this synthetic PPARγ agonist eventually result from significant improvements in insulin sensitivity [114]. However, data from various animal studies in the past decade have demonstrated that the therapeutic potential of RGZ reaches far beyond its use as an insulin sensitization since it also has other benefits on the cardiovascular system such as improvement of contractile dysfunction [115], inhibition of the inflammatory response by reducing neutrophil and macrophage accumulation [116], and the protection of myocardial injury during ischemic/reperfusion [117]. Due to its known anti-inflammatory properties, RGZ has recently been successfully used in the treatment of murine systemic lupus erythematosus [118] as well as to reduce pulmonary inflammation in porcine models of acute lung injury
[119]. In addition, RGZ has recently been identified as neuroprotective agent by promoting synaptic plasticity and neurite outgrowth in several animal models [120], suggesting that it may also been used as a therapeutic target for improvement of cognitive performance. Despite these benefits, inconsistent findings have been reported from various animal models and growing evidence has demonstrated adverse effects of RGZ on the cardiovascular system, including increased risk of acute myocardial infarction [121], cardiac hypertrophy [122] and heart failure [123].

4.2 Future directions

A sound understanding of the aetiology and pathogenesis of CDH together with PH and PPHN is fundamental in order to prevent these children from the severe sequelae of this congenital malformation. Prenatal intervention by minimally invasive techniques such as foetal endoscopic tracheal occlusion and regenerative tissue engineering combined with stem cell therapy offers potential for future treatment strategies [124-126]. Collaborative translational research therefore represents an essential element in our quest for new treatment options for CDH-associated PH. The experiments performed in this work have allowed us to identify alveolar LIFs as a potential target for prenatal treatment with the synthetic PPARγ agonist RGZ to improve alveolar development in hypoplastic lungs. However, to get a more comprehensive knowledge of the underlying epithelial-mesenchymal interactions, further functional studies will need to be carried out.

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4.3 Conclusions

The results presented in this study provide strong evidence that prenatal administration of the synthetic PPARγ agonist RGZ can stimulate alveolarization and alveolar maturation in the nitrofen rat model of CDH-associated PH. These findings further suggest that systemically maternal treatment with RGZ shortly before birth may have a therapeutic potential in attenuating foetal PH in rats with CDH through accelerating epithelial-mesenchymal interactions, which results in an enhanced expression of alveolar LIFs.
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