RESEARCH ARTICLE

Gene expression profiling of postnatal lung development in the marsupial gray short-tailed opossum (Monodelphis domestica) highlights conserved developmental pathways and specific characteristics during lung organogenesis

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Abstract

Background: After a short gestation, marsupials give birth to immature neonates with lungs that are not fully developed and in early life the neonate partially relies on gas exchange through the skin. Therefore, significant lung development occurs after birth in marsupials in contrast to eutherian mammals such as humans and mice where lung development occurs predominantly in the embryo. To explore the mechanisms of marsupial lung development in comparison to eutherians, morphological and gene expression analysis were conducted in the gray short-tailed opossum (Monodelphis domestica).

Results: Postnatal lung development of Monodelphis involves three key stages of development: (i) transition from late canalicular to early saccular stages, (ii) saccular and (iii) alveolar stages, similar to developmental stages overlapping the embryonic and perinatal period in eutherians. Differentially expressed genes were identified and correlated with developmental stages. Functional categories included growth factors, extracellular matrix protein (ECMs), transcriptional factors and signalling pathways related to branching morphogenesis, alveologenesis and vascularisation. Comparison with published data on mice highlighted the conserved importance of extracellular matrix remodelling and signalling pathways such as Wnt, Notch, IGF, TGFβ, retinoic acid and angiopoietin. The comparison also revealed changes in the mammalian gene expression program associated with the initiation of alveologenesis and birth, pointing to subtle differences between the non-functional embryonic lung of the eutherian mouse and the partially functional developing lung of the marsupial Monodelphis neonates. The data also highlighted a subset of contractile proteins specifically expressed in Monodelphis during and after alveologenesis.

Conclusion: The results provide insights into marsupial lung development and support the potential of the marsupial model of postnatal development towards better understanding of the evolution of the mammalian bronchioalveolar lung.

Keywords: Lung, RNA-seq, Monodelphis domestica, Marsupial

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Background

During evolution mammals have developed a bronchoalveolar lung characterised in part by the presence of a large number of small alveoli. Eutherians and marsupials have evolved from common therian mammalian ancestors, but adopted different reproduction strategies [1]. Eutherians, including humans and mice, acquired a well-developed placenta which sustains the embryo throughout a long period of gestation; they give birth to a mature neonate [2]. In contrast, marsupials have retained a primitive form of placenta and after a short gestation give birth to immature neonates [3]. Therefore, organs in the marsupial newborns are generally at comparatively earlier stages of development at birth and the respiratory, digestive, neuronal, immune and respiratory systems are immature and still under the process of development [4].

In eutherians the lungs develop as a respiratory organ to exchange gases immediately after birth. In general, the majority of lung development occurs throughout intrauterine life and is driven, in part, by maternal factors delivered through the placenta [5–7]. Lung development is categorized into five morphological stages (embryonic, pseudoglandular, canalicular, saccular and alveolar) based on characteristic morphology [8, 9]. In eutherians the lungs of the newborn are predominantly at the transition between the saccular and alveolar stage of development with small terminal sacs and a well-developed bronchial system and the key changes during early postnatal life involve an increase in alveolar number and maturation of lung microvasculature [10]. Studies on the development of the lung have been performed previously in several marsupial species, including bandicoot (Isoodon macrourus) [11], Julia Creek dunnart (Sminthopsis douglasi) [12], tammar wallaby (Macropus eugeni), quokka (Setonix brachyurus) [13] and Monodelphis domestica [14]. At birth, the lungs are comprised of a small number of large air sacs providing limited surface area for respiration and are therefore considered functionally immature [15]. Studies of the respiratory mechanism of Julia Creek dunnart and tammar wallaby have demonstrated that neonates perform respiration through the skin during early postnatal development in order to fulfil the requirement for oxygen. However, this limited development changes gradually as lungs mature to perform efficient respiration [12, 16]. Marsupial neonates are similar in development to a late eutherian foetus corresponding to the 40–100 day old human foetal stage, foetal rat at E13-E14 or mice E12-E13 [17, 18], and the immature lung is required to develop further during early lactation to become fully functional. This provides improved opportunities to investigate the progressive changes in the gene expression of the postnatal lung and identify mechanisms and factors involved in lung maturation.

Results

Postnatal lung development in Monodelphis

Histology of lung samples collected from Monodelphis at post-natal days 1, 8, 14, 29, 35, 61 and adult stages (Fig. 1 and Additional file 1: Figure S1 for high resolution images) revealed that the lungs collected 1 day after birth consisted only of a few large air sacs surrounded by a thick layer of epithelial cells (Fig. 1a) typical of the late canalicular stage of development. Lung collected at day 8 consisted of large air sacs and the thickness of the walls had reduced (Fig. 1b). The sacs were lined with a single layer of cells and were connected with a single respiratory duct, indicating the lungs were transiting from the canalicular to the saccular stage. The number of air sacs had increased in the lungs collected at day 14 and the sacs were comparatively smaller in size, but the thickness of the air sac walls had increased and only partial regions consisted of a thin layer of epithelial cells (Fig. 1c). At day 29 the number of air sacs had increased and the airways extended into the terminal air sacs. The increase in the respiratory area by growth of air sacs was correlated with a gradual decrease of interstitial tissue (Fig. 1d). At day 35 the lungs were transitioning from the saccular to the alveolar stage with primitive alveolar sacs connected to air ducts (Fig. 1e). At this stage, the airway system and branching pattern was established throughout the lung. Finally, after a large increase in the number of alveoli at day 63 the lung was fully mature (Fig. 1f) and histological analysis showed no major difference in morphology in lungs from the adult (Fig. 1g).

The results are consistent with previous studies, confirming that lung development follows similar trajectory in all mammals examined and providing imaging at additional time points to improve our understanding of the slower developmental timing in Monodelphis and other marsupials compared to eutherians [14].

Transcriptomics of postnatal lung development in Monodelphis

Gene expression profiling of Monodelphis lung was conducted by RNA-seq of lung tissue collected from young at day 3, 8, 14, 29, 35, 61 and adults. After annotation and normalisation as described in methods, hierarchical clustering of the normalised quantitative expression data (Fig. 2a) suggested 3 major groups of samples, corresponding to three phases of lung development: 1) early postnatal development (day 3, 8 and 14), 2) mid-development (day 29 and 35), and 3) late development (day 63 and adult). This result was consistent with morphological analysis and confirmed that there are two major transitions in gene expression corresponding to the early canalicular-to-saccular and saccular-to-alveolar morphology. Principal component analysis (PCA) indicated that the greatest influence on gene expression was the age of the animal (51% of
variation) while the second greatest component (21% of variation) was apparently influenced by time to or from the transition into alveogenesis (Fig. 2b).

**Surfactant proteins and highly expressed genes relevant to lung function**

The surfactant protein genes *SFTPB* and *SFTPC* were amongst the most highly expressed genes in the RNA-seq dataset overall (rank max log expression 62 and 29 in any sample respectively; rank mean log expression 84 and 22) with a relatively constant expression throughout development. RNA-seq also confirmed high expression of surfactant *SFTPA* and *SFTPD* candidate genes on chromosome 1 throughout the postnatal period (Fig. 2c). Similarly, osteonectin (*SPARC*) encoding an acidic extracellular matrix glycoprotein that plays a vital role in cell-matrix interactions and collagen binding, was among the 26 most highly expressed genes. The top 100 expressed genes overall were, in majority, mitochondrial and ribosomal proteins genes but included keratins (15, 19, 35 and 4) with increasing expression during development (for example *KRT4* in Figs. 2c and 5b), and NADPH oxidase organizer 1 (*NOXO1*), a regulator of angiogenic capacity of lung endothelial cells [19] with constant expression. Other genes of relevance to lung physiology that were highly expressed (top 200 genes) included...
secretoglobin family 3A member 1 and 2 (SCGB3A1, SCGB3A2, Fig. 2c), resistin like beta (RETNLB), a mitogenic factor in lung cell induced in hypoxia, decorin (DCN), an extracellular matrix proteoglycan which affects airway mechanics, airway-parenchymal interdependence, airway smooth muscle proliferation, apoptosis and transforming growth factor-β bioavailability, ADA (adenosine deaminase), which has been associated with pulmonary inflammation [20], and EPAS1 (endothelial PAS domain protein 1) which is potentially involved in lung and vascular development [21].

**Lung-specific gene expression**

A recent comparative study of gene expression in different eutherian tissues has identified a set of 83 candidate genes with lung-specific expression, including 32 confirmed genes [22]. In *Monodelphis*, 20 out of the 32 confirmed gene set and 35 out of the 83 candidate gene set were identified and presented a variety of expression dynamics (heatmap of Fig. 2c). It is not clear if the remaining lung-specific candidate genes are not expressed in the lung, not yet annotated or absent from the *Monodelphis* genome.

**Differential expression**

Differential expression analysis revealed 1242 genes were uniquely or differentially expressed at specific time points during postnatal development as represented in the heat map of Fig. 3 (*p* < 0.05). Sequencing results were validated by RT-PCR as described in methods. A set of 10 genes was selected to represent differential expression clusters (genes chosen from profiling of Fig. 3). Although this is a limited validation of 10 genes and there are a few discrepancies at particular time points such as, for example, day 8 for SERPINC1 (serpin family C member 1), FGA (fibrinogen alpha chain) and HPX (hemopexin), in general there is a high concordance between the temporal expression trends obtained by qPCR and RNA-seq results (Fig. 4). It is unclear if discrepancies are due to variation between animals with a single animal used for sequencing or transitional variation around particular time points of rapid changes such as day 8 for SERPINC1 and FGA. Functional classification of differentially expressed genes highlighted genes encoding for components of cellular processes (446 genes), binding activity (363 genes), developmental processes (298 genes) and immunity (273 genes) (Additional file 2: Figure S2). Developmental processes included system development (190 genes) related to various systems (nervous system, heart, muscle and haematopoiesis). Associated functional categories were also related to embryonic development (ectoderm and mesoderm) and cell differentiation. Cellular processes were mainly related to cell communication (251 genes) while others were related to cell cycle and proliferation. Many (44%) of the genes listed in response to stimulus were related to immune (114 genes) and cellular defence responses (57 genes). Genes involved in signalling included components of the WNT, PDGF, TGFβ, NOTCH, VEGF and Retinoic acid (RA) pathways (see discussion in Additional file 3). Differential expression of genes related to the extracellular matrix (ECM) and signalling (also discussed in Additional file 3) are represented in Fig. 5a and b respectively. It can be seen that these genes exhibit a variety of expression profiles.
and that a broader subset of ECM components display decreased expression over time, likely to reflect the active establishment of complex tissue connectivity in early stages.

**Gene expression clustering**

Gene expression clustering of differentially expressed genes produced 4 major temporal expression patterns (Fig. 3, clusters A, B, C and D). A large set of genes presented a gradual increase in expression as the lung developed (cluster A, 523 genes) with high expression during the later alveolar stage at day 63 and in the adult. This gene set was enriched in genes associated with muscle contraction (enrichment score (ES) 8, \( p = 2E-16 \)), calcium binding (ES 4, \( p = 1.2E-5 \)) and epithelial development (ES 4, \( p = 1.4E-4 \)). In contrast, a second set of genes was specifically expressed during the early phases of development with lower expression as lung matured (cluster C, 293 genes). This cluster was enriched in secreted and extracellular matrix components (ES 18, \( p = 1.5E-28 \)), plasma proteins, innate immunity and response.
to wound healing (ES 6, \(p = 1E-31 \sim E-13\)). Other genes were specifically expressed at particular time points, mainly including day 14, 29 and 35, with increasing (cluster B) or decreasing (cluster D) expression during the course of development. Cluster B (166 genes with peak expression at saccular stage days 29 and 35, Fig. 3b) was enriched in immune genes involved in T cell activation (ES 7, \(p = 1E-17\)), cell surface signalling (ES 4, \(p = 4.5E-7\)) and the regulation of apoptosis (ES 3, \(p = 8.4E-5\)). Finally, cluster D contained 260 genes highly expressed during early stages from day 3 to day 29 with decreased expression from day 35 (Fig. 3d) and was enriched in extracellular matrix (ES 17, \(p = 6E-25\)) and cell cycle (ES 8, \(p = 1.7E-12\)). Overall the results confirm that temporal differential gene expression is associated with morphological changes during development, revealing gene markers and providing insight into the development processes with active cell division, extracellular matrix deposition and establishment of innate immunity in early development followed by establishment of adaptive immunity and, finally, epithelial proliferation and, more surprisingly, muscle development.

Comparative analysis of lung development transcriptomes in *Monodelphis* and mice

Published gene expression studies of lung development in mouse [23] and human [24] lung tissue have previously identified a molecular signature of time-to-birth supported by principal component analysis. In *Monodelphis*, principal component analysis similarly indicated that the first component represented the age of the animal (Fig. 2b). However, in this case the second component was not influenced by time from birth but was instead apparently influenced by the time to and from the saccular-alveolar transition, a period overlapping the time of birth in mice.
but occurring only after birth around day 35 postnatal in *Monodelphis*.

To compare further the mammalian lung development programs of *Monodelphis* and mice, two murine gene expression microarray datasets were retrieved from the Gene Expression Omnibus database (GEO dataset GSE20954 and GSE74243) [25, 26]. Combining *Monodelphis* RNA-seq and mice microarray dataset GSE20954, the first principal component (37% variation) remained mainly influenced by age, providing an approximate alignment of development time between the species (Fig. 6a). Not surprisingly, the second component (22% variation) represented variation between species. Clustering of the combined dataset resulted in co-clustering of the two species at: 1) early time points (mouse day E16 and *Monodelphis* days 3 and 8), 2) intermediate time points (*Monodelphis* days 14 and 29 and mouse day P2 and P10 postnatal), 3) late time points (mouse day P30 postnatal and *Monodelphis* day 63 and adult), and, 4) *Monodelphis* day 35 and mouse day E18 (Fig. 6c). Integration with GSE74243 recapitulated these results (Fig. 6b and d) showing overall a broadly conserved temporal signature of gene expression during mammalian lung development.

The interspecific correlation of temporal gene expression was estimated by matching the gene expression average of each species during early, intermediate and late development. Of 510 differentially expressed genes identified in each species during early, intermediate and late development in both species, 207 genes had highly correlated expression profiles (Pearson product-moment correlation coefficient [cor] > 0.8, including 142 genes with cor > 0.9). This included lung specific genes (*AGER* and *PRX*) and genes with established roles in mammalian lung development. The gene set was associated with secreted glycoprotein and the extracellular matrix (Enrichment Score: 6, corrected p-value 1E-5 ~ 8.4E-7), cellular division, insulin-like binding proteins and EGF signalling, proteolysis, collagen metabolism and immune response (Enrichment Score: 3), immune system process, system development, T cell regulation (Enrichment Score: 2), cell-cell adhesion, blood circulation, cell cycle, WNT signalling, development, muscle fibres and transcription (Enrichment Score < 3). It can be seen in Fig. 7 that early *Monodelphis* day 3, 8 and 14 co-clustered with embryonic E16 to E18.5 in mice. *Monodelphis* D29 co-clustered with P3 to P13 in mice while *Monodelphis* day 35 co-clustered in a late cluster from day P13–14 postnatal in mice and included the late samples from both *Monodelphis* (day 63 and adult) and mice (up to day P56). This alignment of the major phases of lung development in mice and *Monodelphis* further supports the conserved progression of a significant proportion of the lung transcriptome during development.

**Differential temporal expression between *Monodelphis* and mice**

In Fig. 7, the major specific difference was during the perinatal phase in mice with a clear separation between
E19-P2 and P3-P5. The perinatal period E19.5-P2 was unusual in the mouse with an apparent temporary downregulation of genes involved in cellular division and upregulation of genes involved in muscle formation. In contrast, no such downregulation was seen in Monodelphis until day 35 with the activation of alveologenesis, although we cannot exclude that a similar effect occurs at an intermediate time between day 14 to 35 in Monodelphis.

More generally, genes with low temporal correlation between the species were associated with secreted and signaling proteins (Enrichment Score: 10, corrected $p$-value $1E^{-9}$ to $1E^{-16}$), immunity (adaptive and innate, Enrichment Score: 6, corrected $p$-value $1E^{-5}$ to $2.4E^{-7}$), muscle proteins (Enrichment Score: 3.5, corrected $p$-value $1E^{-5}$ to $6E^{-9}$), proteolysis (Enrichment Score: 3.5, corrected $p$-value $1.5E^{-2}$ to $7.8E^{-5}$), extracellular matrix (Enrichment Score: 3, corrected $p$-value $6.3E^{-9}$) and weakly associated with platelet activation and positive regulation of vascular endothelial growth factor production. To retrieve genes differentially expressed between the species a second approach was employed. Genes with high loadings on the second PC component (representing species variation) were retrieved (77 genes with absolute loadings above 0.04, including 59 genes identified as differentially expressed in Monodelphis with 32 genes differentially expressed in both species). Functional enrichment analysis identified two clusters. One cluster was enriched in genes associated with blood coagulation (VTN, FGA, FGB, FGG, AMBP, APOH, fold enrichment 28.81, $p$-value $3.72E^{-02}$) representing genes highly expressed in the early postnatal period and the functionalization of the lung in both species and therefore directly correlated with time of birth rather than development progress. This cluster also contained ANG (angiogenin), an important regulator of angiogenesis, and WISP1 (WNT1-inducible signaling protein-1), a gene known to be important in lung maintenance and repair through the WNT signalling pathway [27]. WISP1 was highly expressed postnatally from day 3 to 35 in Monodelphis and from P3 to P14 in the mouse. The second cluster was enriched in genes associated with muscle contraction (10 genes, fold enrichment 23.48, $p$-value $4.16E^{-09}$; myosin light chain 2 [MYL2], small muscle protein, X-linked SMPX, myomesin 2 MYOM2, myosin light chain, phosphorylatable, fast skeletal muscle MYLPC, myosin light chain 1 MYLI, myosin binding protein C, slow type MYBPC1, troponin T1, slow skeletal type TNNT1, tropomodulin 4 TMOD4, leiomodin 3 LMOD3 and myosin light chain 10 MYL10), which are highly expressed in late stages (day 35 to adult) in Monodelphis and generally poorly expressed in mice. These results therefore also point to a differential regulation of blood coagulation factors and muscle fibres between the two species.
Discussion

Breathing with immature lungs

In eutherians such as humans, a primitive lung structure is present in highly preterm young and the mortality rate in these infants is higher, especially in very preterm infants [28, 29]. There are important determinants such as country of birth, race and socioeconomic status that affect the rate [30–32], but the underlying pathology is most often due to immature lung development in the preterm infants [33–35]. In the infants that survive, the consequence of immaturity of the lungs at birth may be significant developmental problems [30, 32, 36]. At birth, the lung of the newborn opossum is comprised of thin-walled large air sacs with few sacs occupying the whole organ. Until day 8 of post-natal development, the lungs contain few air sacs with walls of epithelial cells connected by primitive respiratory ducts. Overall, marsupial opossum neonatal lungs are immature at birth and the respiratory tree is still undergoing considerable post-natal development during the lactation period. In contrast to eutherian newborns, respiration in marsupial neonates occurs through the skin and low metabolic activity allows neonatal survival in the absence of fully functional lungs [12, 14]. Here, morphology and gene expression profiles of embryonic mice E16 and Monodelphis days 3 to 8 were the most similar in the pre-saccular stage.

Signaling pathways and their significance in lung development

Lung organogenesis is primarily dependent on epithelial-mesenchymal interactions [37–39]. These interactions are mainly mediated by secretory factors released from epithelial and mesenchymal cells [40]. Transcriptional factors, growth factors, ECM proteins and MMPs mediate the interactions and participate in regulatory feedback loops [37–39]. Many of the genes contributing to pathways known to influence lung development in eutherians are also regulated in Monodelphis, indicating a large overlap between mechanisms of lung development in marsupials and eutherians. These pathways include Wnt [41, 42], retinoic acid [43], TGFβ [44] and NOTCH signalling pathways [45]. Although it is difficult to draw conclusions from bulk organ gene expression profiling, some candidate genes possibly contributing to these developmental pathways, angiogenesis and ECM remodelling are discussed in more details in Additional file 3.

Conserved processes of lung development

There are limitations in the comparative analysis of gene expression during lung development in Monodelphis and mice. For technical reasons the study had to focus on the variation of gene expression and relied on current annotation. However a significant proportion (~ 20–30%) of RNAs identified remains anonymous. Despite these limitations and the potential difference in specific lung development dynamics, the study has shown largely conserved transcriptome dynamics during mammalian lung development allowing the filtering of some of the most important putative signalling factors. The data also indicated the major influence on bulk RNA-seq of the transition from saccular to alveolar stages rather than time from birth, as has been previously suggested from eutherian studies alone [23].

Specific differences and their significance in lung development

Discordant expression patterns between the species were likely to be related to physiological differences between the embryonic eutherian and the partially functional lung of marsupial neonates. Differences related to blood circulation, immunity, enrichment in platelet activation and vascular proliferation probably reflect the activation of lung circulation at birth following constriction of the ductus arteriosus shortly after birth, which takes place at different stages of development in marsupial and eutherians. This is also supported by recent characterisation of the role of the lung as a major site of platelet biogenesis and a reservoir for haematopoietic progenitors [46]. Differences in the extracellular matrix are likely to denote the evolutionary flexibility of this multi-component system made in part of collagens, proteases and protease inhibitors. This is also supported by reports of differential expression of ECM components by different strains of mice and between mice and human lung in embryonic stages [26]. Similarly, enrichment in muscle contraction is compatible with more extensive differentiation of smooth muscle cells in lung tissue of marsupials compared to eutherians [13]. Specific up-regulation from the onset of alveologenesis to the mature stage only in Monodelphis is supported by qPCR validation of myotilin (MYOT) expression (n = 3 at each time points) and included a number of contractile protein candidate genes such as actin and myosins (e.g., ACTA1, MYL1, MYL2, MOYM2). Because the lung is isolated by pleural membranes, contamination of samples from skeletal muscle tissue is unlikely, especially in older animals with larger lungs. This observation raises the issue of the role of contractile fibres in the origin and evolution of the mammalian bronchoalveolar lung. Indeed, a variety of muscular lung morphological associations have been described in the multicameral lung of non-mammal tetrapods which is regarded as the precursor of the mammalian bronchoalveolar lung [47]. It has been argued that, like the appendix, lung muscle cells are a vestigial remnant without function in the lung [48]. However, recent studies have supported the role of smooth muscle cells and myofibroblasts in lung development and remodelling, including the essential role of
localized smooth muscle cell differentiation for epithelial bifurcation during branching morphogenesis [49] and the role of YAP (YY1 associated protein 1) in regulating mechanical force through the phosphorylation of myosin light chains [50]. Alveolar myofibroblasts make an essential contribution to alveolar septal formation during alveologensis [51] and 3D microscopy has recently shown how myofibroblasts deposit overlapping fishnet-like networks of actin and elastin fibres to define the walls of the developing alveoli, emphasising the crosstalk between the contractile properties of myofibroblasts and the mechanical properties of the extracellular matrix [52]. These observations clearly establish the role of muscular contraction in lung morphogenesis and support the concept that mechanical forces may have contributed to the evolution of the bronchoalveolar lung of mammals. Interestingly, the expression of skeletal myosin heavy chains was observed in rat lung myofibroblasts in vitro, and the expression control differed from that in muscle [53], suggesting that eutherian lung myofibroblasts have the capacity to be reprogrammed to express a skeletal muscular protein. However, eutherian alveolar myofibroblasts typically apoptosis after alveolarisation, although studies have implicated these cells in lung tissue repair and a number of serious medical conditions characterised by elastin fibre deposition. The lung contains about 40 cell types and myofibroblasts represent 10% of mature lung cells. As the normal rate of regeneration is estimated at 5% lung/week, a better understanding of the functional evolution of the mammalian lung myofibroblast could further improve our understanding of lung physiology. However, the exact production, localisation and function of these putative contractile proteins in Monodelphis and other species remain to be fully established.

Finally, growth and development of the marsupial neonate may, in part, be regulated by the timely delivery of maternal signalling factors supplied through milk [54–56]. Remarkably, day 60 tammar milk induced the differentiation of mouse lung mesenchyme cell cultures into invasive cells resembling smooth muscle cells or myofibroblasts [57]. Whether this differentiation results in the activation of muscular protein gene expression remains to be investigated.

Conclusion
Marsupials provide a unique animal model to improve our understanding of lung development. Gene expression profiling of postnatal lung development in Monodelphis has identified markers and candidate genes with putative physiological or regulatory roles in lung development. However, the full extent of the contribution of many of these genes is still unknown and additional studies will be required to fully assess their role and improve the temporal resolution with additional time points or single cell transcriptomics. Overall, the study has shown a large overlap in gene expression during lung development of the marsupial Monodelphis and the eutherian mouse despite differences mainly due to the timing of birth and contractile protein gene expression. The study shows how similar pathways are likely involved in the control of lung development of marsupials and eutherians and highlights the distinctive value of marsupial models towards understanding the evolution of mammalian lung development and the further identification of marsupial milk factors and their putative uterine equivalent in eutherians.

Methods
Lung tissue sample collection and ethics statement
The South American gray short-tailed opossum were provided by the colony established at Melbourne University. Lungs were collected from neonates on day 1, 3, 6, 8, 12, 14, 18, 24, 29, 35, 41, 51, 61, 100 of age and from an adult (Animal Ethics approval ID 1112115 from the University of Melbourne Animal Ethics committee Anatomy & Neuroscience, Pathology, Pharmacology and Physiology). The age of the neonates was determined by checking the breeding females on a daily basis after mating and removing young at appropriate ages after birth. Whole lungs were isolated by dissection and washed in PBS to remove any blood cells before further processing of the tissue.

Tissue processing for histology
For histology, whole lungs collected from one animal at each time point (postnatal day 1, 3, 6, 12, 18, 24, 35, 41, 51, 61 and adult) were fixed in 10% neural buffered formalin for 24–48 h and left in 70% ethanol prior to further processing. All tissue samples were routinely processed using ascending ethanol series and xylene before embedment in paraffin wax. Tissue samples were sectioned on a microtome in 4–6 μm thick slices and stained with Gill’s haematoxylin & eosin stains.

RNA sequencing
After PBS washing of whole lung dissected from one animal at each time point (day 3, 8, 14, 29, 35, 63 of development and adult), clean scalpel blades were used to cut the lung into smaller pieces before homogenisation. RNA isolation was performed using an Ambion RNA isolation kit and the quality and integrity were confirmed using the Agilent 2100 Bioanalyser RNA Nano Chip. RNA samples were sequenced using the Illumina Hiseq 2000 RNA-seq sequencing platform at BGI Co., Ltd. From 24 to 38 million Illumina paired-end reads pairs were obtained in each sample. Paired-end raw RNA-seq reads were cleaned by removing reads with low quality, adaptors only reads or reads with unknown nucleotides larger than 5% of read length. Filtered RNA reads were then aligned to the
Monodelphis genome (Monodelphis_domestica_broad05_67.gtf annotation from Ensembl) by, samtools-0.1.18, bowtie2-2.0.0-beta6, tophat-2.0.3 and Cufflinks2.1.1.1 software tools. From 20 to 31 million read pairs could be successfully mapped and, out of 33,996 referenced genes in the genome reference, 30,750 genes were identified, including 25,854 genes annotated with an official gene symbol (82%). The mapped RNA-seq data was then analysed using SeqMonk (version 0.24.1). The data was subjected to the RNA-Seq pipeline to define probes and quantify expression; the probes were defined through the probe generator, selecting mRNA features and removing exact duplicates and probes with no data, and the RNA-seq quantification pipeline was used for quantification (including mRNA features selection, log transform of normalised counts and including duplicate reads only once). Statistical filtering by intensity difference ($P < 0.05$) identified 1242 genes as significantly differentially expressed. Exploratory gene expression clustering was performed in SeqMonk, Hierarchical Clustering Explorer and R software.

C-DNA synthesis and q-PCR
One microgram of total RNA per sample was used to synthesise cDNA. Superscript III™ Reverse Transcriptase (Invitrogen) was used to synthesise cDNA following manufacturer instructions. Real time Q-PCR was performed using SsoFast EvaGreen Supermix (Bio-Rad) and CFX96™ Real-Time PCR Detection System (Bio-Rad). The reaction mix contained diluted cDNA, 1X master mix and Forward and Reverse primers (Table 1). Sequencing results were validated by RT-PCR. A set of 10 genes was selected to represent differential expression clusters (genes chosen from profiling of Fig. 3). In addition, 6 housekeeping genes (GAPDH, 18S, RPL19, PPIL4, GUSB and ACTB2) were used as control for data normalisation. After standardization of housekeeping gene expression, 3 genes were retained for qPCR normalisation (18S, RPL19 and PPIL4). At each time point, PCR assays were performed on three individual whole lung samples from different animals.

### Table 1
| Gene Title | Forward Primer | Reverse Primer |
|------------|---------------|---------------|
| MYOT       | GCCTCAGATGCGGAACCTTAT | CCAAGAATATCTTGTGGAGT |
Abbreviations
ACOX1: Acyl-CoA oxidase like; ACTA1: Actin, alpha 1, skeletal muscle; ACTA2: Actin, alpha 2, smooth muscle, aorta; ACTB2: Actin Beta 2; ACTG2: Actin, gamma 2, smooth muscle, enteric; ADA: Adenosine deaminase; ADSS1: Adenosylcynohydrin synthase like 1; AGER: Advanced glycosylation end-product specific receptor; AKAP5: A-kinase anchoring protein 5; ALDH1A2: Aldelyde dehydrogenase 1 family member A2; AMBP: Alpha-1-microglobulin/bikunin precursor; ANG: Angiogenin; ANG1: Angiotensin 1; ANGI: Transmembrane 7 superfamily member 2; ANG2: Angiotensin 2; VPS51, GARP complex subunit; ANGPTL3: Angiopoietin-like 3; ANGPTL4: Angiopoietin-like 4; APOH: Apolipoprotein H; ARSF: Aryl sulfatase F; BGI: Beijing Genomic Institute; BMP: Bone morphogenic protein; BPIFA1: BPI fold containing family member 4; cDNA: Complementary deoxyribonucleic acid; CCL20: C-C motif chemokine ligand 20; CCN1: Cysteine rich angiogenic inducer 61; CDH11: Cadherin 11; CDHR3: Cadherin related family member 3; CDHR4: Cadherin related family member 4; cDNA: Complementary deoxyribonucleic acid; CYP2F1: Cytochrome P450 family 2 subfamily F member 1; CYR61: Cysteine rich angiogenic inducer 61; DCN: Decorin; DMR98: Dehydrogenase/reductase 9; DLL4: Delta like canonical Notch ligand 4; DSG5: Desmoglein-5; ECM: Extracellular matrix; EGF: Epidermal growth factor; ER: Endothelial PAS domain protein 1; ES: Enrichment score; FAM92B: Family with sequence similarity 92 member B; FGA: Fibrinogen alpha; FGF: Fibroblast growth factor; FGF1: Fibroblast growth factor 1; FGF2: Fibroblast growth factor 2; FGF3: Fibroblast growth factor 3; FGF4: Fibroblast growth factor 4; FGF5: Fibroblast growth factor 5; FGF6: Fibroblast growth factor 6; FGF7: Fibroblast growth factor 7; FGF8: Fibroblast growth factor 8; FGF9: Fibroblast growth factor 9; FGF10: Fibroblast growth factor 10; FGF11: Fibroblast growth factor 11; FGF12: Fibroblast growth factor 12; FGF13: Fibroblast growth factor 13; FGF14: Fibroblast growth factor 14; FGF15: Fibroblast growth factor 15; FGF16: Fibroblast growth factor 16; FGF17: Fibroblast growth factor 17; FGF18: Fibroblast growth factor 18; FGF19: Fibroblast growth factor 19; FGF20: Fibroblast growth factor 20; FGF21: Fibroblast growth factor 21; FGF22: Fibroblast growth factor 22; FGF23: Fibroblast growth factor 23; FGF24: Fibroblast growth factor 24; FGF25: Fibroblast growth factor 25; FGF26: Fibroblast growth factor 26; FGF27: Fibroblast growth factor 27; FGF28: Fibroblast growth factor 28; FGFR1: Fibroblast growth factor receptor 1; FGFR2: Fibroblast growth factor receptor 2; FGFR3: Fibroblast growth factor receptor 3; FGFR4: Fibroblast growth factor receptor 4; FGR: Friend retrovirus; FGF: Fibrinogen gamma chain; FGD2: Frizzled class receptor 2; GDPD1: Glyceroldehyde-3-phosphate dehydrogenase; GEO: Gene expression omnibus; GNR2: Gastrokine 2; GUSB: Glucuronidase; GP: Hemopexin; HTRA1: Serine protease HTRA1; IGF: Insulin growth factor; IGFBP1: Insulin like growth factor 1 mRNA binding protein 1; IGFBP2: Insulin like growth factor 2 mRNA binding protein 2; IGFBP3: Insulin like growth factor 2 mRNA binding protein 3; IGFBP1: Insulin like growth factor binding protein 1; IGFBP2: Insulin like growth factor binding protein 2; IGFBP3: Insulin like growth factor binding protein 3; IGF1: Insulin like growth factor binding protein 1; IGF2: Insulin like growth factor binding protein 2; IGF3: Insulin like growth factor binding protein 3; KRT4: Keratin 4; LAMA4: Laminin alpha 4; LAMP3: Lysosomal associated membrane protein 3; LDLRA1: Low density lipoprotein receptor class A domain containing 1; LEP: Lymphoid enhancer binding factor 1; LMOD3: Leiomodin 3; LRNR4: Leucine rich repeat neuronal 4; MMP14: Matrix metallopeptidase 14; MMP16: Matrix metallopeptidase 16; MMP2: Matrix metallopeptidase 2; MMP8: Matrix metallopeptidase 8; MMP9: Matrix metallopeptidase 9; MPP3: Matrix metallopeptidases; MOYM2: M-protein, also known as myomesin-2; MS4A15: Membrane spanning 4-domains A15; MUC5B: Mucin 5B, oligomeric mucus/gel-forming; MYBPC1: Myosin binding protein C, slow type; MYH7: Myosin heavy chain 7; MYH7B: Myosin heavy chain 7B; MYL1: Myosin light chain 1; MYL10: Myosin light chain 10; MYLF2: Myosin light chain 2; MYLPF: Myosin light chain, phosphorylatable, fast skeletal muscle; MYOM2: Myomesin 2; MYOT: Myotilin; NAPSA: Napsin A aspartic peptidase; NFAT: Nuclear factor of activated T-cells; NOTCH: Notch signaling pathway; NOCTH4: Notch 4; NOXO1: NADPH oxidase organizer 1; PAS: Per-Arnt-Sim domain; PCA: Feline leukemia virus subgroup C cellular receptor 1; PCDH18: Protocadherin 18; PCR: Polymerase chain reaction; PDFG: Platelet-derived growth factor; PIPL4: Peptidylprolyl isomerase like 4; PRK: Peroxiredoxin 6; qPCR: Quantitative PCR; RA: Retinoic acid; RETNLB: Resistin like beta; RNA: Ribonucleic acid; RNAseq: Ribonucleic acid sequencing; RPL19: Ribosomal protein L19; RTNK2: Rhotekin 2; RTPCR: Reverse transcriptase polymerase chain reaction; SGB3: Secretoglobin family 3A member 1; SGBP3A2: Secretoglobin family 3A member 2; SECC4L3: SEC4 like lipid binding 3; SPER1C: Serpin family C member 1; SRFP: Secreted frizzled related protein; SRFP1: Secreted frizzled related protein 1; SRFP2: Secreted frizzled related protein 2; SFTP: Surfactant protein B; SFTP: Surfactant protein C; SLC4A14: Solute carrier family 6 member 14; SWPX: Small muscle protein, X-linked; SNTN: Senta, cilia apical structure protein; SPARC: Secretened protein acidic and cysteine rich; SPINK: Serine protease inhibitor Kazal-type; TGF: Transcription factor 7; TGF: Transforming growth factor beta 2; TMOD4: Tromodulin 4; TNC: Tenascin C; TNFAP2: TNF alpha induced protein 2; TNNT1: Troponin T1, slow skeletal type; VEGF: Vascular endothelial growth factor A; VTN: Vimentin; WIF1: Wnt inhibitor factor 1; WISP1: Wnt inducible signaling pathway protein 1; Wnt: Wnt signaling pathways; YAP: Yap associated protein 1
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