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REGULAR ARTICLE

Characterization of miRNA processing machinery in the embryonic chick lung

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Abstract Lung development is a very complex process that relies on the interaction of several signaling pathways that are controlled by precise regulatory mechanisms. Recently, microRNAs (miRNAs), small non-coding regulatory RNAs, have emerged as new players involved in gene expression regulation controlling several biological processes, such as cellular differentiation, apoptosis and organogenesis, in both developmental and disease processes. Failure to correctly express some specific miRNAs or a component of their biosynthetic machinery during embryonic development is disastrous, resulting in severe abnormalities. Several miRNAs have already been identified as modulators of lung development. Regarding the spatial distribution of the processing machinery of miRNAs, only two of its members (*dicer1* and *argonaute*) have been characterized. The present work characterizes the expression pattern of *drosha*, *dgcr8*, *exportin-5* and *dicer1* in early stages of the embryonic chick lung by whole mount in situ hybridization and cross-section analysis. Overall, these genes are co-expressed in dorsal and distal mesenchyme and also in growing epithelial regions. The expression pattern of miRNA processing machinery supports the previously recognized regulatory role of this mechanism in epithelial and mesenchymal morphogenesis.

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Introduction

Pulmonary development is tightly controlled by genetically conserved intercellular signaling pathways (such as FGF, Wnt, Shh and Notch) that regulate cell proliferation, fate and differentiation, leading to branching morphogenesis and eventually to proper lung formation (reviewed by Herriges and Morrisey 2014). More recently, microRNAs (miRNAs) have arisen as new players in respiratory development (Lü et al. 2005; Harris et al. 2006). In fact, several studies have reported the temporal–spatial expression of numerous miRNAs throughout lung development (reviewed by Khoshgoo et al. 2013). Moreover, misexpression of miRNAs has also been described to be involved in some pulmonary diseases, namely bronchopulmonary dysplasia (Bhaskaran et al. 2012) and pulmonary fibrosis (Dakhlallah et al. 2013).

MicroRNAs (miRNAs) are small single-stranded non-coding RNA fragments of about 22 nucleotides (Bartel et al. 2004) that silence gene expression by targeting specific mRNAs and therefore control innumerable cellular processes in plants and animals (Ouellet et al. 2006; Stefani and Slack 2008). Canonical miRNA biogenesis is a multistep process that involves different enzymes and proteins (Nana-Sinkam et al. 2009). In most eukaryotic organisms, miRNAs are encoded in the genome as introns in protein coding genes, as independent transcription units or in polycistronic clusters (Stefani and Slack 2008). After being transcribed by an RNA polymerase, primary miRNAs (pri-miRNAs) are processed, still in the nucleus, by Drosha (RNase III)/DGCR8 complex-originating miRNA precursors (pre-miRNAs) with about 60–70 nucleotides. Pre-miRNAs are subsequently exported out of the

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nucleus by Exportin-5 (Xpo5). In the cytoplasm, these pre-miRNAs are then cleaved by Dicer (a cytoplasmatic RNase III) to form double-stranded miRNAs. The next step is the separation and selection of a mature chain of about 22 nucleotides that will associate with a multi-protein complex called the RNA-induced silencing complex (RISC), which contains a member of the Argonaute protein family (Ouellet et al. 2006). Gene silencing occurs when this complex loaded with miRNA binds to target mRNA transcripts specifically repressing its translation, or leads to the cleavage and degradation of transcripts, compromising protein synthesis in both scenarios (Johanson et al. 2013). In addition to the canonical miRNA biogenesis pathway, there are several other miRNA biosynthesis pathways that are Drosha/DGCR8-independent or Dicer-independent (Abdelfattah et al. 2014).

miRNAs play essential roles as regulators of numerous biological processes, such as, for instance, cellular differentiation and apoptosis, developmental timing, immune responses, nerve system patterning and organogenesis (reviewed by Sayed and Abdellatif 2011). Their importance is further reinforced if one considers the severe developmental abnormalities observed in *dicer1* knockout mouse that leads to lethality at early embryonic developmental stages due to stem cell depletion (Bernstein et al. 2003). Regarding pulmonary development, conditional inactivation of *dicer* in lung epithelium leads to branching impairment and abnormal growth of the epithelial tube, suggesting a crucial role for miRNA regulation in epithelial lung morphogenesis (Harris et al. 2006). Moreover, miRNA expression profile studies have demonstrated that different miRNA families are temporally expressed in embryonic and adult lung and that they modulate fetal lung development (Williams et al. 2007; Lu et al. 2007; Bhaskaran et al. 2009; Dong et al. 2010; Yang et al. 2012; Mujahid et al. 2013). Nevertheless, regarding miRNA processing machinery, only *dicer1* and *argonaute* expression patterns have been described in fetal mammalian lung (Lü et al. 2005; Harris et al. 2006).

Avian and mammalian adult lungs present a dramatically different architecture. Despite these dissimilarities, the molecular events and signaling pathways underlying lung development at early stages seem to be conserved in both phyla (Moura et al. 2011, 2014). Therefore, in this study, we used the embryonic chick lung to characterize, by in situ hybridization, the expression pattern of *drosha*, *dgcr8*, *xpo5* and *dicer1* that are essential for canonical miRNA biogenesis, in early stages of chick lung development. Furthermore, with this report, we aim to fill in a blank regarding the expression of miRNA processing machinery during early lung development, and ultimately contribute to better comprehension of the mechanisms of miRNA-dependent gene regulation.

Materials and methods

The work presented in this manuscript was performed in the chick model, at early stages of development, which does not need ethical approval from review board institutions or ethics committees.

Tissue collection

Fertilized chick (*Gallus gallus*) eggs, obtained from commercial sources, were incubated for 4–6 days in a 49 % humidified atmosphere at 37 °C. Embryonic lungs were carefully dissected under a dissection microscope (Olympus SZX16, Japan), classified in stages b1, b2, and b3 taking into account the number of secondary buds formed (Moura et al. 2011), which corresponds to the developmental window between stage 24 to 26, according to the Hamburger and Hamilton classification (Hamburger and Hamilton 1992), and then processed for whole mount in situ hybridization.

In situ hybridization

Whole mount in situ hybridization (*n*=15 per stage and probe) was performed as previously described (Henrique et al. 1995). Each set of lungs/probe was processed simultaneously and developed for the same amount of time, and then photographed with an Olympus U-LH100HG camera coupled to an Olympus SZX16 stereomicroscope. Antisense digoxigenin-labeled RNA probes for *drosha*, *dgcr8*, *exportin-5* and *dicer1* were produced as previously described (Carraco et al. 2014).

Cross-section preparation

Hybridized chick lungs were fixed in 4 % paraformaldehyde, embedded in 2-hydroxyethyl methacrylate (Heraeus Kulzer, Germany) and processed for sectioning (coronal and axial) at 25 μm using a rotary microtome (Leica RM 2155, Germany). Lung sections were photographed with an Olympus DP70 camera coupled to an Olympus BX61 microscope. Afterwards, coverslips were removed, sections were rehydrated, Hematoxylin-Eosin (HE) staining was performed, and the sections were photographed again.

Results

drosha expression pattern

drosha mRNA is located in the mesenchyme surrounding the secondary bronchi, from the proximal-most onward (Fig. 1b, dark arrowhead) and also in the distal mesenchyme (Fig. 1c, open arrowhead). It seems to be absent in the upper epithelial

Fig 1 *drosha* expression pattern in early stages of chick lung development. Representative examples of whole mount in situ hybridization (**a–c**) of stages b1, b2 and b3, sections of hybridized lungs (**d–f**) and correspondent HE-stained sections (**g–i**). *Dark arrowhead* dorsal mesenchyme. *Open arrowhead*. *Asterisk* distal epithelium. *Black arrow* secondary bronchi epithelium. The *black* and *dashed rectangles* indicate the coronal and axial region, respectively, shown in the corresponding slide sections. *Scale bars* (**a–c**) 2000 μm; (**d–i**) 100 μm
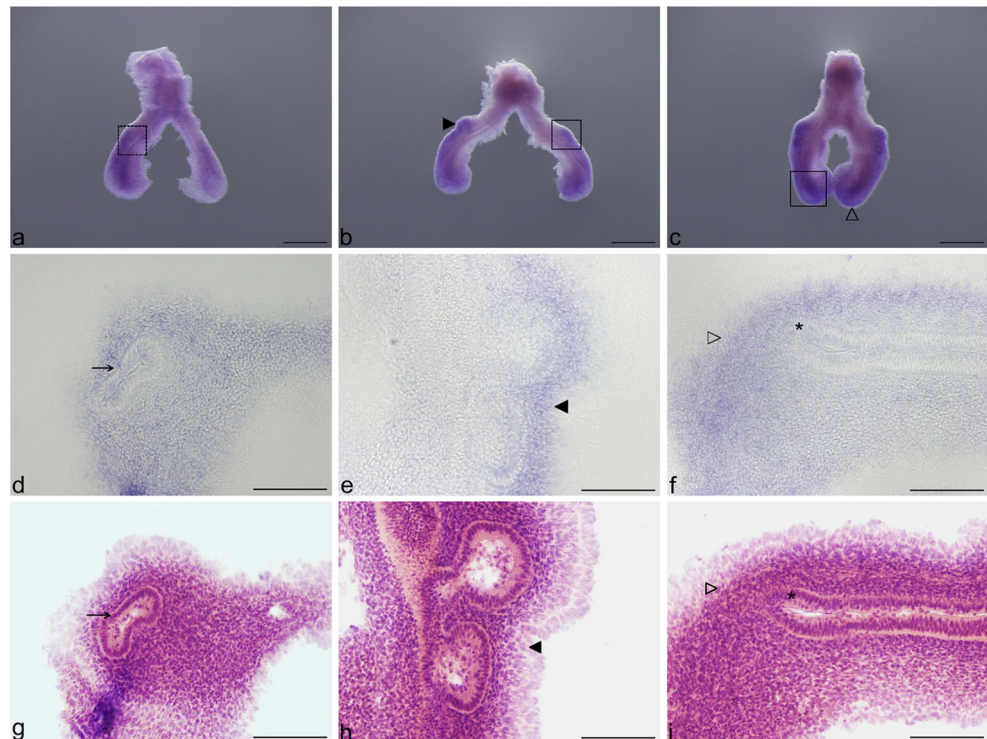
and mesenchymal compartment. No differences were observed in *drosha* expression pattern in all stages studied. Sections of hybridized lungs confirmed the dorsal (Fig. 1e, dark arrowhead) and distal mesenchymal expression (Fig. 1f, open arrowhead). Moreover, a weak epithelial expression was observed in the main bronchus (Fig. 1f, asterisk) and in the secondary bronchi (Fig. 1d, black arrow). The expression in both cases seems to be more restricted to the inner lining of the epithelial tube. Epithelial and mesenchymal structures are clearly visible in HE-stained sections (Fig. 1g–i).

dgcr8 expression pattern

dgcr8 expression is evident in the dorsal mesenchyme, specifically in the region adjacent to the secondary buds (Fig. 2c, dark arrowhead), along with the main bronchus and also in the distal-medial region (Fig. 2b, open arrowhead). Mesenchymal expression seems to inaugurate with the appearance of the most proximal bud. Conversely, *dgrc8* seems to be absent from the epithelium. This pattern is maintained in the three stages studied; however, a slight increase in the expression is observed from the b1 to b2 stages. Sectioning of the hybridized lungs confirmed the mesenchymal expression (Fig. 2d, dark arrowhead; f, open arrowhead). Furthermore, it revealed a faint distal epithelial expression (Fig. 2f, asterisk) and also in the secondary bud epithelium (Fig. 2d, e, black arrow). HE staining undoubtedly displays the epithelial and mesenchymal compartment (Fig. 2g–i).
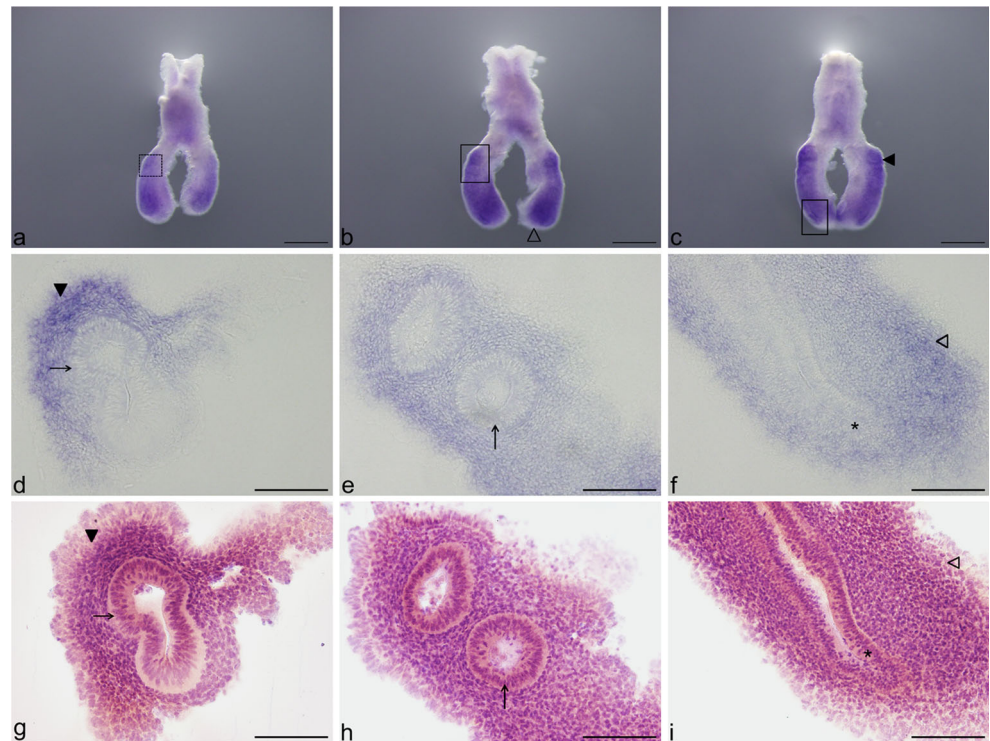
exportin-5 expression pattern

exportin-5 expression in the chick embryonic lung appears to be present mainly in the dorsal and distal mesenchyme (Fig. 3a, dark arrowhead; c, open arrowhead). Conversely, it seems absent from the proximal most mesenchymal region, and it seems that its dorsal expression initiates at the level of the first bud formed. This pattern is consistent in the three stages studied. Slide sectioning revealed that *xpo5* expression is not restricted to the dorsal and distal mesenchyme (Fig. 3d, e, dark and open arrowheads, respectively), but it is also present in the epithelial compartment particularly in the secondary bronchi (Fig. 3d, f, black arrow) and also distally in the main bronchus (Fig. 3e, asterisk). Interestingly, in the main bronchus, the epithelial expression is absent from the proximal region, and as it occurs with mesenchymal expression, its expression begins at the level of the most proximal bud. Mesenchyme and epithelium are evident in the HE-stained sections (Fig. 3g–i).

dicer1 expression pattern

dicer1 is detected primarily in the distal mesenchyme (Fig. 4a, open arrowhead) and it seems to be expressed in the epithelium at the growing tips of secondary and primary bronchi (Fig. 4). The expression pattern is, in general, similar in the three stages studied. Histological sectioning of hybridized lungs confirmed this expression pattern. It is clear that *dicer1* is expressed only in the epithelium of primary and secondary

Fig 2 *dgcr8* expression pattern in early stages of chick lung development. Representative examples of whole mount in situ hybridization (**a**–**c**) of stages b1, b2 and b3, sections of hybridized lungs (**d**–**f**) and correspondent HE-stained sections (**g**–**i**). *Dark arrowhead* dorsal mesenchyme. *Open arrowhead*. *Asterisk* distal epithelium. *Black arrow* secondary bronchi epithelium. The *black* and *dashed rectangles* indicate the coronal and axial region, respectively, shown in the corresponding slide sections. *Scale bars* (**a**–**c**) 2000 μm; (**d**–**i**) 100 μm

bronchi (Fig. 4d, asterisk; f, black arrow); moreover it is possible to observe that it is also expressed in the mesenchyme surrounding the secondary buds in addition to the distal mesenchyme (Fig. 4d–f, dark arrowhead). Epithelial and mesenchymal structures are clearly visible in HE-stained sections (Fig. 4g–i).

Discussion

The relevance of miRNAs in fetal lung development is well recognized. miRNas have been described as key regulators of homeostasis and lung development (Lu et al. 2007), inflammation (Banerjee et al. 2010) and many pulmonary diseases,

Fig 3 *exportin-5* expression pattern in early stages of chick lung development. Representative examples of whole mount in situ hybridization (**a**–**c**) of stages b1, b2 and b3, sections of hybridized lungs (**d-f**) and correspondent HE-stained sections (**g-i**). *Dark arrowhead* dorsal mesenchyme. *Open arrowhead* distal mesenchyme. *Asterisk* distal epithelium. *Black arrow* secondary bronchi epithelium. The *black* and *dashed rectangles* indicate the coronal and axial region, respectively, shown in the corresponding slide sections. *Scale bars* (**a**–**c**) 2000 μm; (**d–i**) 100 μm
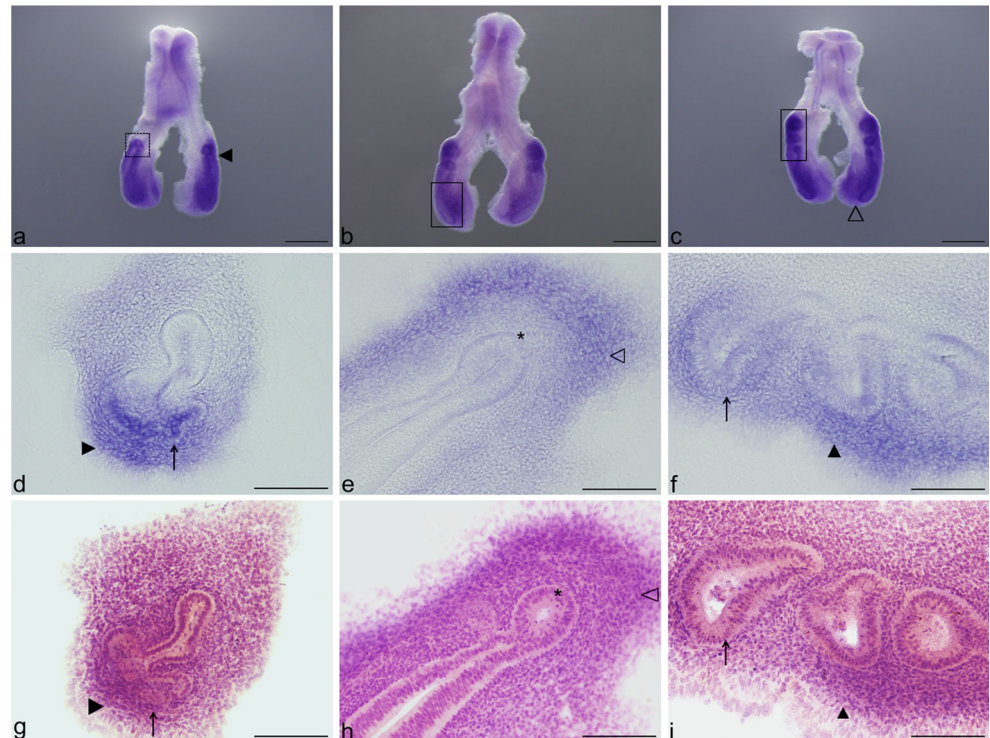
Fig 4 *dicer1* expression pattern in early stages of chick lung development. Representative examples of whole mount in situ hybridization (**a–c**) of stages b1, b2 and b3, sections of hybridized lungs (**d–f**) and correspondent HE-stained sections (**g–i**). *Dark arrowhead* dorsal mesenchyme. *Open arrowhead*. *Asterisk* distal epithelium. *Black arrow* secondary bronchi epithelium. The *black* and *dashed rectangles* indicate the coronal and axial region, respectively, shown in the corresponding slide sections. *Scale bars* (**a–c**) 2000 μm; (**d–i**) 100 μm
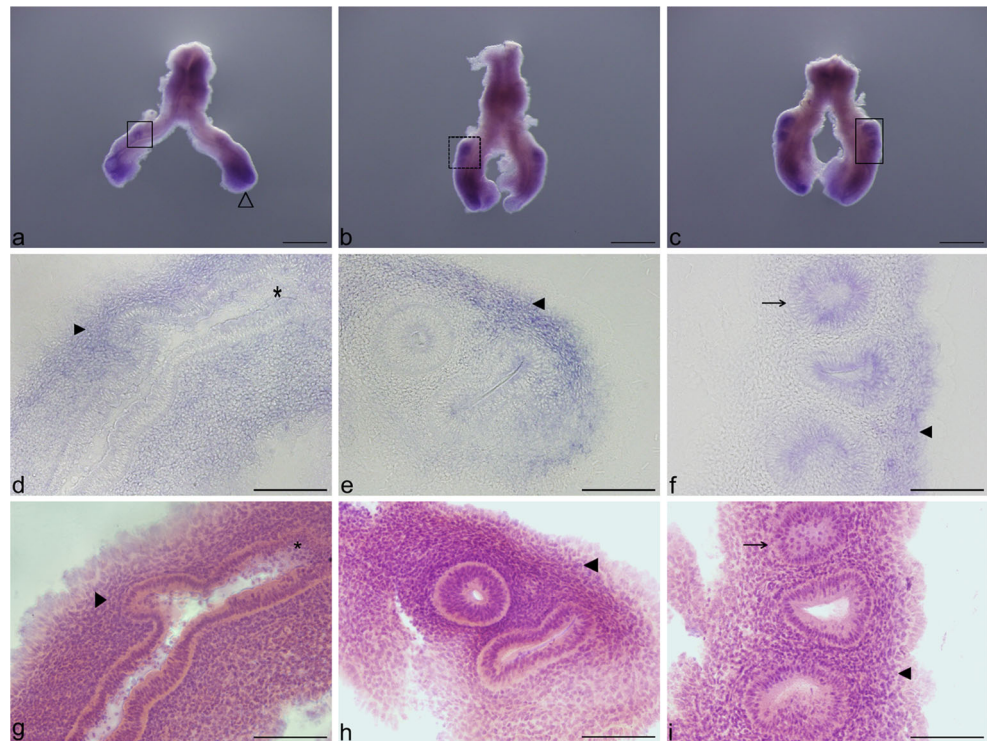
namely cancer (Yanaihara et al. 2006) and chronic obstructive pulmonary disease (Sato et al. 2010); nevertheless, the temporal–spatial distribution of their processing machinery remains mostly unknown. In this study, we have examined transcript location, by in situ hybridization, of genes coding for the canonical biosynthesis processing machinery in chick developing lung. At early stages of chick lung development, branching is monopodial, meaning that secondary buds emerge laterally of each primary bronchus (Hogan 1999), as it occurs in early mammalian lung development (Metzger et al. 2008; Warburton et al. 2010). For this reason, only the initial stages of chick lung branching (b1–b3) were characterized.

In general, all the components of miRNA processing machinery present a similar expression pattern. Considering the fact that these proteins function in tandem to process miRNAs, their co-expression seems coherent. Their expression matches the previously described *ago1/ago2* expression patterns that also contribute to the overall miRNA biosynthetic process (Lü et al. 2005).

drosha and *dgcr8* are mainly expressed in the dorsal and distal mesenchyme at the level of the first secondary bud formed, and they are absent from the most proximal region of the lung. This might indicate a possible regulatory role in the branching process by guiding mesenchymal morphogenesis. In both cases, only a faint epithelial expression, restricted to the inner lining of the epithelial tube, is observed.

xpo5 and *dicer1* are clearly expressed in the mesenchymal compartment (dorsally, from the proximal-most bud onward,

and distally), but also in the epithelium (mainly in the apical side). In the murine lung, *dicer1* is expressed in the distal mesenchyme and epithelium (Lü et al. 2005), which is in agreement with the results obtained for the chick lung. It is known that *dicer* plays an important role in lung epithelial morphogenesis, since *dicer* conditional knockout mice lungs fail to branch properly (Harris et al. 2006). The same authors provide evidence of a regulatory mechanism based on FGF10 signaling. It seems that the mesenchymal expression of FGF10, a chemotactic factor for lung epithelial outgrowth, is somehow regulated by miRNAs produced by the epithelium (Harris et al. 2006). Additional studies have suggested that a miR-17-92 cluster, expressed in the mesoderm and endoderm (apical region), promotes the proliferative and undifferentiated state of lung epithelial progenitor cells, at early stages of lung development (Lu et al. 2007). Further studies have shown that, in the pseudoglandular stage, the mir-17 family of miRNA modulates FGF10-FGFR2b downstream signaling, hence regulating E-Cadherin expression, which in turn modulates epithelial bud morphogenesis in response to FGF10 signaling (Carraro et al. 2009). Taking into account that the expression pattern of both *dicer1* and *fgf10* (Moura et al. 2011) in the developing chick lung is similar to their mammalian counterparts, one can speculate that this regulatory mechanism is probably preserved in the avian model. Additionally, the mesenchymal expression of the microRNA processing machinery correlates with the expression of several miRNAs specifically expressed in this compartment, such as miR-326, which is known to be regulated by Shh activity (Jiang et al. 2014),

and miR-142-3p that regulates the proliferation of mesenchymal progenitors by controlling the level of WNT signaling (Carraro et al. 2014). The vast majority of miRNAs are synthesized by the canonical processing machinery which requires the microprocessor components (Drosha and DGCR8) to generate precursor miRNA, Exportin-5, to export it to the cytoplasm, and Dicer to form mature miRNA (Ouellet et al. 2006). In the embryonic chick lung, these four genes are expressed in the mesenchyme, mainly from the most proximal bud onwards, which might indicate that the canonical miRNA biogenesis pathway is probably involved in mesenchymal morphogenesis by regulating the expression of specific genes.

On the other hand, epithelial expression is somewhat different and the four genes do not colocalize: *xpo5* and *dicer1* are expressed in the epithelial tips, while *drosha* and *dgcr8* are basically absent. This dissimilar expression points toward the existence of a non-canonical miRNA pathway that most likely bypasses the microprocessor processing step of the canonical biosynthetic pathway. Actually, an alternative nuclear pathway for miRNA biogenesis, known as the mirtron pathway, has been described in invertebrates (Okamura et al. 2007; Ruby et al. 2007). Subsequently, deep sequencing technology and computational methods have permitted the identification of mirtrons in mammals (Berezikov et al. 2007) and in chicken (Glazov et al. 2008). Mirtrons are pre-miRNA short introns that use splicing machinery to generate miRNA hairpins directly suitable for Dicer cleavage. In this sense, the requirement for the Drosha/DGCR8 complex is circumvented and the mirtron pathway then merges with the canonical miRNA pathway at the Exportin-5-bound transport stage (Okamura et al. 2007). Taking into account the absence of *drosha* and *dgcr8* in lung epithelium, and the existence of mirtrons in the chick embryo, it is plausible to think that in this compartment a non-canonical miRNA biosynthetic pathway might be active.

In conclusion, the mesenchymal and epithelial expression of microRNA processing machinery in developing chick lung support the previously recognized regulatory role of this mechanism in epithelial and mesenchymal morphogenesis. These findings also suggest a similar role for some specific miRNAs in the chick lung.

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