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MicroRNA processing machinery in the developing chick embryo

Gil Carraco [a,b,1], Ana N. Gonçalves [a,b,1], Carlos Serra [a,b], Raquel P. Andrade [a,b,*,2]

[a] Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, 4710-057 Braga, Portugal
[b] ICVS/3B's – PT Government Associate Laboratory, Braga/Guimarães, Portugal

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ABSTRACT

Gene expression regulation during embryo development is under strict regulation to ensure proper gene expression in both time and space. The involvement of microRNAs (miRNA) in early vertebrate development is documented and inactivation of different proteins involved in miRNA synthesis results in severe malformations or even arrests vertebrate embryo development. However, there is very limited information on when and in what tissues the genes encoding these proteins are expressed. Herein, we report a detailed characterization of the expression patterns of DROSHA, DGCR8, XPO5 and DICER1 in the developing chick embryo, from HH1 (when the egg is laid) to HH25 (5-days incubation), using *whole mount in situ* hybridization and cross-section analysis. We found that these genes are co-expressed in multiple tissues, mostly after stage HH4. Before early gastrulation DICER1 expression was never detected, suggesting the operation of a Dicer-independent pathway for miRNA synthesis. Our results support an important role for miRNAs in vertebrate embryo development and provide the necessary framework to unveil additional roles for these RNA processing proteins in development.

Vertebrate organisms employ efficient molecular mechanisms to regulate gene expression in cells, tissues and organs. Some of these mechanisms have been classically studied, such as transcription factor-mediated regulation or morphogen signalling cascades, among others, while novel mechanisms are emerging. Gene expression regulation by non-coding RNAs (ncRNA) has been gaining importance in the last few years (reviewed in Patil et al., 2014) and among the most studied regulatory RNA molecules are the microRNAs (miRNAs), small ncRNAs with 21–23 nucleotides (Bartel, 2004). In 1993, Lee et al. discovered the first miRNA (*lin-4*) in the development of *C. elegans* (Lee et al., 1993) and 7 years later, Pasquinelli et al. described the second miRNA, *let-7*, also in *C. elegans* (Pasquinelli et al., 2000). These two miRNAs are crucial for timing *C. elegans* development. In fact, loss-of-function of *lin-4* leads to a delay in larval stage transitions and *let-7* loss-of-function delays the shift from larval stage L4 to the adult organism (Grishok et al., 2001). From then on, thousands of miRNAs were discovered (Kozomara and Griffiths-Jones, 2011) and a recent study has suggested that miRNAs are responsible for the regulation of more than 60% of the mammalian protein coding genes (Friedman et al., 2009). miRNAs regulate target mRNAs by blocking translation or even leading to mRNA degradation (reviewed in Moor et al., 2005). For that propose, the miRNA present in the RNA-induced silencing complex (RISC) binds the 3′UTR of the target mRNA, where a five or six nucleotide sequence (seed sequence) is particularly important for recognition of their target (reviewed in Moor et al., 2005).

There are several miRNA biosynthesis pathways (reviewed in Winter et al., 2009). The most common and best characterized, also known as the canonical pathway, begins with the transcription of an RNA molecule by RNA polymerase II (Lee et al., 2004). This RNA can be originated from a coding or non-coding gene, or even from an intron (Tang and Maxwell, 2008). The immature transcript, called primary miRNA (pri-miRNA), forms a stem-loop structure that is processed in the nucleus by an enzymatic microprocessor, composed of the RNAse Drosha and its co-enzyme DGCR8 (*DiGeorge* syndrome critical region 8), releasing the stem-loop structure with approximately 70 nucleotides, called precursor miRNA (pre-miRNA) (Denli et al., 2004; Lee et al., 2003; Yeom et al., 2006). Exportin-5, encoded by XPO5 gene, is responsible for the transport of pre-miRNAs from the nucleus to the cytoplasm (Yi et al., 2003). In the cytoplasm, another class II RNAse III, Dicer and TAR RNA binding protein, bind the pre-miRNA and excise the loop off of the hairpin, forming a double-stranded RNA molecule of nearly 22 nucleotides (Daniels et al., 2009; Koscianska et al., 2011). Finally, Argonaute proteins bind the double stranded RNA to form the RISC

Abbreviations: ncRNA, non-coding RNA; miRNA, microRNA; RISC, RNA-induced silencing complex; pri-miRNA, primary miRNA; pre-miRNA, precursor miRNA; cKO, conditional KO; HH, Hamburger and Hamilton; PSM, presomitic mesoderm; P-PSM, prospective presomitic mesoderm; LPM, lateral plate mesoderm.

* Corresponding author. Life and Health Sciences Research Institute (ICVS), School of Health Sciences, Campus de Gualtar, University of Minho, 4710-057 Braga, Portugal. Tel.: +351 253604865; fax: +351 289800076.
E-mail address: raquel.pego.andrade@gmail.com; rgandrade@ualg.pt (R.P. Andrade).

[1] These authors contributed equally to this work.

[2] Present address: Regenerative Medicine Program, Departamento de Ciências Biomédicas e Medicina, Universidade do Algarve, 8005-139 Faro, Portugal.

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loading complex (MacRae et al., 2008). After this step, other protein factors join the complex and one of the miRNA strands is chosen to form the active miRISC complex (Miyoshi et al., 2009).

Gene expression regulation mediated by miRNAs play a crucial role during embryo development (reviewed in Campos et al., 2011). For instance, Mir-9 is a crucial player in nervous system development by mediating repression of *hes1* expression by mRNA degradation (Bonev et al., 2012). Mir-430 is decisive for zebrafish maternal-to-zygote transition and gastrulation (Bazzini et al., 2012; Giraldez et al., 2006) and Mir-125a-5p participates in timing somite formation in chicken by repressing *Lunatic fringe* by translation repression and mRNA degradation (Riley et al., 2013). Moreover, inactivation of the proteins involved in miRNA processing and maturation also impact embryo development. Conditional KO (cKO) studies demonstrated that DGCR8 is required for proper central nervous system development in *Drosophila* (Fenelon et al., 2011) and cKO of *DGCR8* in the mouse vascular system evidenced that embryos die in early developmental stages (Chen et al., 2012). DICER1 KO embryos die early in development, depleted of stem cells (Bernstein et al., 2003), evidencing an essential role for DICER1 in embryo development. DICER1 cKO in vascular smooth cells also lead to a development delay and premature embryonic death (Pan et al., 2011). Dicer has also been shown to participate in the anterior–posterior positioning of the limb buds (Zhang et al., 2011), as well as in the correct morphogenesis of these structures, since cKOs present smaller limbs (Harfe et al., 2005). It was also demonstrated that without DICER1 oligodendrogenesis and astrogliogenesis are impaired in brain development (Zheng et al., 2010).

Although the involvement of miRNAs and their processing machinery is now well established in embryo development, the expression patterns over time and space of the genes encoding these RNA processing enzymes remain largely unknown, particularly in early stages of development. In this study, we have characterized the expression of four crucial genes of the canonical miRNA biosynthesis pathway: DROSHA, DGCR8, XPO5 and DICER1. The aim of this work is to understand when and where the genes of the miRNA processing machinery are expressed during chick embryo development. These findings will provide novel insight on miRNA-mediated gene expression regulation in different tissues over development.

1. Results

1.1. DROSHA

A detailed characterization of the patterns of expression of four genes involved in the processing and maturation of miRNAs – DROSHA, DGCR8, XPO5 and DICER1 – was performed by whole mount *in situ* hybridization analysis and cross section histology in the developing chick embryo. DROSHA is expressed in the early embryo, presenting widespread expression in the epiblast in late blastula (stage HH1, $n = 8/9$) and also along the primitive streak at HH4 (gastrulation stage, $n = 6/6$) (Fig. 1A). At stage HH6 DROSHA is expressed along the primitive streak and anterior part of the embryo and is less expressed in the remaining epiblast (22-25h of incubation, $n = 4/4$). By stage HH8 (4-somite stage, Fig. 1A and B), DROSHA transcripts are localized in the head process mesoderm (Fig. 1Bi), anterior neural tube (Fig. 1Bii), central neural tube (Fig. 1Biii), neural plate (Fig. 1Biv) and prospective presomitic mesoderm tissue (P-PSM) at the level of the primitive streak. At this stage, DROSHA is not expressed in the notochord. At stage HH9$^-$ ($n = 5/5$), HH11 ($n = 4/4$) and HH13$^-$ ($n = 3/4$), DROSHA expression is maintained in the head, neural plate/tube and somites. It is also expressed in the lateral plate mesoderm (LPM) (Fig. 1A). Stages HH18 (3-day incubation, $n = 6/6$) and HH23 (4–4.5 day incubation, $n = 4/4$) embryos present expression in the brain, pharyngeal arches, somites, tail bud and limb buds (Fig. 1A, C, D). Transversal sections of HH18 embryo evidence DROSHA expression in all the mesencephalon in a salt-and-pepper manner (Fig. 1Ci), in the retina, rhombencephalon, hypophysis and in the trigeminal ganglion (Fig. 1Cii), in oesophagus and pharyngeal arches, the neural tube (Fig. 1Ciii), nephric ducts, somites, notochord (Fig. 1Civ) and wing buds (Fig. 1Cv). At HH23 (Fig. 1D) it is also possible to see expression in somites, wing and leg buds, telencephalon (Fig. 1Di), pharyngeal arcs (Fig. 1Dii), retina and tail bud (Fig. 1Diii).

1.2. DGCR8

DGCR8 is typically not expressed at stage HH1 ($n = 10/12$). However, it could be observed in the epiblast of late HH1 embryos ($n = 2$) (Fig. 2A). At stage HH5 *dcgr8* expression is detected in all the epiblast and strongly along the primitive streak ($n = 8/8$). HH8 embryos present expression in the head neural folds, neural tube, somites, neural plate and in the caudal epiblast and primitive streak ($n = 7/7$) (Fig. 2A). Cross section analysis of the HH8 embryo evidenced DGCR8 expression in the dorsal neural tube (Fig. 2Bi) and neural plate (Fig. 2Bii). As previously observed for DROSHA, DGCR8 is also excluded from the notochord (Fig. 2Bii). DGCR8 is further expressed in the posterior neural plate and lateral plate mesoderm (Fig. 2Biii) and primitive streak (Fig. 2Biv). At stages HH10$^+$ ($n = 5/7$) and HH14 ($n = 5/6$) it is possible to see expression in all developing brain structures, namely, forebrain, midbrain and hindbrain (Fig. 2A). DGCR8 is also expressed in the prospective heart region. More posteriorly, expression is observed in the somites, PSM, neural tube, intermediate mesoderm and tail bud. At stage HH17 (Fig. 2C) DGCR8 expression is present in the head structures, including eyes and otic vesicles. It is expressed also in the somites, limb buds and neural tube ($n = 7/7$). Transverse section analysis evidenced DGCR8 transcripts in the rhombencephalon, Rathke's pouch, notochord, lens vesicle, retina, infundibulum and prosencephalon (Fig. 2Ci), in the neural tube, pharyngeal arches and telencephalon (Fig. 2Cii). In the posterior portion of the embryo, is possible to see expression in the leg bud, somites, notochord and neural tube (Fig. 2Ciii). This expression pattern is mostly maintained at stage HH19 (Fig. 2A); however the expression spreads along the entire wing and leg buds ($n = 7/7$). At stage HH23, DGCR8 is expressed in the head, pharyngeal arches, limb buds, dorsal root ganglia and tail bud ($n = 6/6$) (Fig. 2A and D). Fig. 2D shows DGCR8 mRNA in the telencephalon, wing bud and pharyngeal arches (Fig. 2Di), dorsal root ganglia (Fig. 2Dii), diencephalon, myotome (Fig. 2Diii), retina and tail bud (Fig. 2Div).

1.3. XPO5

XPO5 has widespread expression in the early chick embryo (Fig. 3), namely in the epiblast of HH1 ($n = 4/4$) and HH4 ($n = 3/3$) embryos. By HH6, XPO5 exhibits stronger expression in the head process, neural tube and primitive streak ($n = 7/7$). At stage HH9$^-$, XPO5 expression is present in the head, neural tube, somites, neural plate, lateral plate mesoderm and primitive streak. As for the previous genes, there is no expression in notochord ($n = 5/5$). Transverse sections confirmed this analysis (Fig. 3D): XPO5 is observed in the neural folds (Fig. 3i), neural tube and somites (Fig. 3Dii) and neural plate (Fig. 3Diii and iv). At stage HH11 the expression of XPO5 is seen in the developing brain, epithelial somites, PSM, intermediate mesoderm, lateral plate mesoderm, neural tube and also in the remaining primitive streak ($n = 5/5$). A similar expression pattern was observed at stage HH13. At stage HH17 (Fig. 3C), XPO5 expression is visible in the retina and lens vesicle (Fig. 3Ci), in the rhombencephalon, Rathke's pouch, prosencephalon, optic stalks and trigeminal ganglia (Fig. 3Cii), limb buds, somites and neural tube (Fig. 3Ciii). Similar patterns were obtained for HH23 and HH25

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