Int. J. Dev. Biol. 45 (S1): S99-S100 (2001)

Short Report

Selective inhibition of gene expression by RNAi in chick embryos *in ovo*

VICTOR HERNÁNDEZ-HERNÁNDEZ*, JUANA FERNÁNDEZ, ALBERT CARDONA, RAFAEL ROMERO and DAVID BUENO

Departament de Genètica, Facultat de Biologia, Universitat de Barcelona, Catalonia, Spain

ABSTRACT Selective inhibition of gene expression using double-stranded RNA is a technique currently used to study gene function in many biological systems, from plants (post-transcriptional gene silencing) to animals. This cellular mechanism, called RNA interference (RNAi), consists of the targeted degradation of the mRNA into 21-23 nucleotide fragments by the action of a specific endonuclease complex, and is directed by the presence of the homologous double-stranded RNA (dsRNA). It has been postulated that in the case of vertebrates, RNAi mechanisms may not be specific, as the presence of any dsRNA triggers a vehement antiviral response mediated by a double-stranded RNA dependent protein-kinase, which inhibits transcription and induces apoptosis. Studies on zebrafish and mouse embryos in which a specific dsRNA was injected into the cytoplasm of oocytes, zygotes or blastomers, did not solve the question, as the information about mutant phenotypes obtained was difficult to understand, causing some contradictory reports. To clarify this question we microinjected chicken *fibroblast growth factor-8* (*fgf8*) dsRNA into the neural tube of 2-day-old chick embryos which were killed and analysed two days later (four days of *in ovo* development). The whole mount *in situ* RNA hybridisation with a *fgf8* antisense riboprobe revealed severe inhibition of *fgf8* expression and many morphological malformations in organs and structures where *fgf8* is known to be active. We conclude that the RNA interference technique could be used in chick embryos *in ovo* to study the function of genes involved in development.

Introduction One of the main goals of developmental biology is to analyse gene function during development. Recently, specific mRNA degradation mediated by double-stranded RNA (dsRNA), which is termed RNA interference (RNAi), has been used to inhibit gene expression in invertebrates, such as *C.elegans* (Fire, 1999) and planarians (Sánchez-Alvarado and Newmark, 1999). Experiments both *in vivo* and *in vitro* demonstrated that a specific nuclease cleaves both sense and antisense strands of the dsRNA into pieces of 21-23 nucleotides (Elbashir *et al.,* 2001), which remain bound to a hypothetical protein complex. These dsRNA pieces then template sequence-specific cleavage of the cellular homologous mRNA, and the sense strand is replaced by the mRNA strand. This regeneration of the small dsRNA pieces after mRNA degradation explains how relatively small amounts of dsRNA degrade large amounts of the corresponding mRNA, and the ability of dsRNA to cross cell boundaries and spread throughout the organism.

The specific ability of dsRNA to silence genes in vertebrates has been the focus of an intense debate, with some contradictory reports (Li *et al.,* 2000, and Oates *et al.,* 2000). It has been postulated that RNAi mechanisms may not be specific in vertebrates, as the presence of any unspecific dsRNA triggers a vehement antiviral response, mediated by a double-stranded RNA dependent protein-kinase, which inhibits transcription and induces apoptosis. Currently, however, it is generally accepted that RNAi in vertebrates degrades the corresponding mRNAs, causing specific effects of loss-of-function. Both the presence of multiple sites for single-gene expression at different developmental stages and the presence of gene families with redundant functions, which hinder the analysis of loss-of-function phenotypes, may account for these contradictory reports. DsRNA has been used to silence genes in zebrafish, *Xenopus* and mice by micro-injection into the cytoplasm of oocytes, zygotes or blastomers, specifically to degrade mRNAs involved in early developmental or dormant maternal mRNAs stages (Li *et al.,* 2000, and Oates *et al.,* 2000).

In this work we have tested the possibility of degrading targeted chicken mRNAs with RNAi by microinjecting chicken *fgf8* dsRNA *in ovo*. *Fgf8* is one of the members of the *fgf* gene family, which is expressed at different sites and at different developmental stages during chick embryogenesis (i.e. midbrain-hindbrain junction, apical ectodermal ridge [AER], brachial arches, anterior telencephalon, and several organs; Crossley *et al.,* 1996; Shamin *et al.,* 1999).

Material and Methods *Fgf8* dsRNAs were synthesised according to Sánchez-Alvarado and Newmark (1999), and were microinjected into the neural tube of 2-days chick embryos *in ovo*. *Tcen49* dsRNA from a planarian gene not homologous to any vertebrate gene was used as a negative control. After two days of *in ovo* development (4-day developing embryos), embryos were sacrificed and fixed in paraformaldehyde 4%, and then hybridised with a *fgf8* antisense riboprobe according to Bueno *et al.* (1995). Sections were obtained by embedding the embryos in paraffin, and were subsequently stained with Haematoxylin-Eosin.

Results and Discussion

Inhibition of fgf8 expression. Two days after *fgf8* dsRNA microinjection, the expression of *fgf8* was greatly reduced in most of the microinjected embryos, as revealed by *fgf8 in situ* hybridisation (Fig. 1), although some variability was observed, from embryos with a complete inhibition of *fgf8* expression to a few embryos with no significant reduction of its expression. Control embryos microinjected with the solvent (DMEM) or with *tcen49* dsRNA did not show any reduction of *fgf8* expression. These results indicate that the inhibition

***Address correspondence to:** Victor Hernández-Hernández. Departament de Genètica, Facultat de Biologia, Universitat de Barcelona, Av. Diagonal 645, 08028 Barcelona, Catalonia, Spain. e-mail: victor@porthos.bio.ub.es

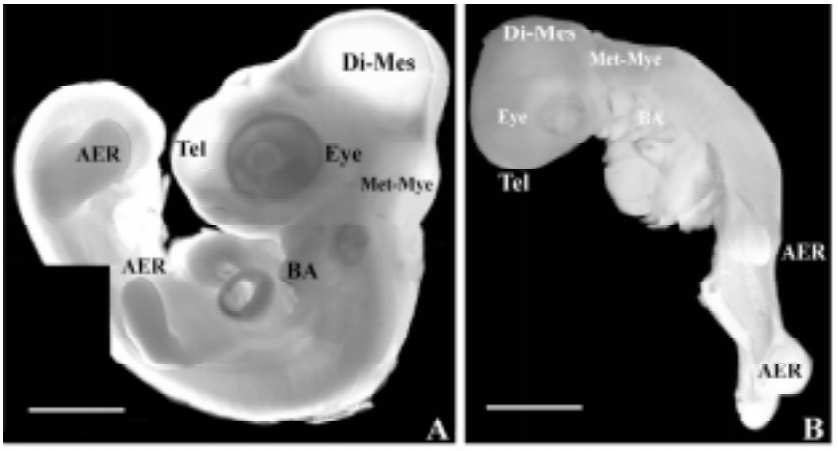

Fig. 1. Whole mount *in situ* hybridisation with *fgf8* riboprobe on 4 day developing embryos. (A) *Chick embryo microinyected with dsRNA from tcen49. No inhibition of fgf8 expression nor tissue malformations were observed.* **(B)** *Chick embryo microinjected with fgf8 dsRNA. Note that limbs and wings have grow normally, but the other structures are abnormal. Scale bar, 2mm.*

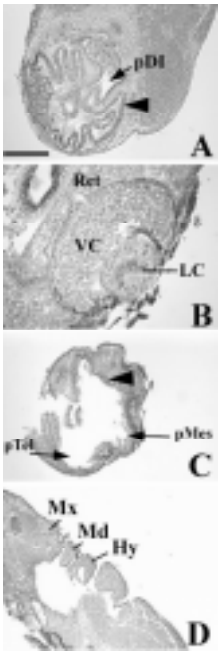

Fig. 2. 10 mm sections from Fig.1 B. (A) *The growth of the diencephalic neuroepitelium is abnormal.* **(B)** *Abnormal chick eye development.* **(C)** *Abnormal mesencephalic and telencephalic development.* **(D)** *Reduced pharyngeal arches. Scale bar, 1 mm. Abbreviations: AER, Apical Ectodermal Ridge; Arrowhead, Neuroepitelium; BA, Pharyngeal Arches; Di-Mes, Diencephalon-Mesencephalon; Hy, Hyoids; LC, Lens and Cornea; Md, Mandibulary arch; Met-Mye, Metencephalon-Myelencephalon; Mx, Maxillary arch; pDi, presumptive Diencephalic cavity; pMes, presumptive mesencephalic cavity; pTel, presumptive Telencephalic cavity; Ret, Retina; Tel, Telencephalon; VC, Vitreous chamber.*

of *fgf8* expression and the observed malformations (see below) are not an unspecific response due to the manipulation of the embryos (DMEM control) nor to the presence of an unspecific PKR activation by an exogenous dsRNA (*tcen49* dsRNA control).

Developmental malformations induced by fgf8 expression inhibition. *Fgf8* microinjected embryos displayed a set of developmental malformations in the areas, structures and organs where *fgf8* has been reported to be active. Embryos with a complete or a major reduction of *fgf8* expression show greater malformations than embryos with a partial reduction of its expression (Figs. 1,2). We observed malformations in the formation of the diencephalon and telencephalon, where it has been reported that *fgf8* plays a role in maintaining and patterning their growth. The diencephalic-mesencephalic neuroepithelium grew normally, but the ectoderm and mesoderm that form the midbrain cavity did not grow, so the neuroectoderm was folded in on itself inside a reduced midbrain cavity. On the other hand, the telencephalic neuroepithelium and the ectoderm and mesoderm that form the telencephalic vesicles were equally reduced, so the telencephalic neuroepithelium was not folded inside the reduced telencephalic vesicles. This suggests a different role for *fgf8* in the patterning of telencephalic and diencephalic-mesencephalic vesicles (Shamim *et al.,* 1999). We also observed malformations in the patterning of the eyes, where the presence and action of several *fgfs* has been reported, and in the brachial arches, which were reduced. The mutant eyes exhibited a complete disorganisation of growth and patterning; the morphology of the lens cells was abnormal and the camera vitrea was full of round-shaped cells. The morphology of the retina cells was also abnormal and showed a significant decrease in pigment cells.

The pattern of wings and limbs is normal. It has been reported that *fgf8* is expressed in the AER of limbs and wings, promoting their growth and patterning. Although the expression of *fgf8* in the AER of the mutant embryos was greatly reduced or completely inhibited, these structures were not affected, and both grew normally (Fig. 1). This agrees with reports that show that the limbs of *fgf8* hypomorphic mouse mutants are normal. This may be due to the redundancy of function with other *fgfs* present in the AER, i.e. *fgf10*. *Fgf8* is sufficient but not necessary for limb and wing buds to grow normally (Meyers *et al.,* 1998).

RNAi in ovo. One of the disadvantages of introducing dsRNA in oocytes, zygotes or blastomers is the progressive accumulation of mutant phenotypes that hinder the analysis of the gene function at one specific point. The advantage of introducing dsRNA *in ovo* is that this can be done at later developmental stages, so the interference does not affect the previous stages. This enables us to define the function of critical genes used by the embryos at distinct sites at different developmental stages, so avoiding the progressive accumulation of mutant phenotypes. However, it is still impossible to silence gene expression at a single site of the embryo by directed dsRNA micro-injection, as RNAi effects spread all over the embryo, given its remarkable ability to cross cell boundaries.

References

BUENO, D., ABUD, H., SKINNER, J. and HEATH, J.K. (1995). Double *in situ* hybridisation on mouse embryos for detection of overlapping regions of gene expression. *TIGs* 12: 385-387.

CROSSLEY P.H., MINOWADA, G., MACARTHUR, D.A. and MARTIN, G.R. (1996). Roles for *fgf8* in the induction, initiation, and maintenance of chick limb development. *Cell* 84: 127-136.

ELBASHIR, S.M., LENDECKEL, W. and TUSCHL, T. (2001). RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes Dev.* 15: 188-200.

FIRE A. (1999). RNA-triggered gene silencing. *Trends Genet* 15: 358-363.

LI YX, FARRELL MJ, LIU R, MOHANTY N. and KIRBY ML. (2000). Double-stranded RNA injection produces null phenotypes in zebrafish. *Dev. Biol.* 217: 394-405.

MEYERS, E.N., LEWANDOSKY, M. and MARTIN G. (1998). An *fgf8* mutant allelic series generated by Cre- and Flp-mediated recombination. *Nature Genet.* 18: 136-141.

OATES AC, BRUCES AEE and HO RK. (2000). Too much interference: injection of double-stranded RNA has non-specific effects in the zebrafish embryo. *Dev. Biol.* 224: 20-28.

SÁNCHEZ-ALVARADO, A and NEWMARK, P.A. (1999). Double-stranded RNA specifically disrupts gene expression during planarian regeneration. *Proc. Natl. Acad. Sci.* USA 96: 5049-5054.

SHAMIM, H., MAHMOOD, R., LOGAN, C., DOHERTY, P., LUMSDEN, A. and MASON, I. (1999). Sequential roles for *fgf4*, *en1* and *fgf8* in especification and regionalisation of the midbrain. *Development* 126: 2549-2561.