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Negative Fgf8-Bmp2 feed-back is regulated by miR-130 during early cardiac specification

Carmen Lopez-Sanchez [a], Diego Franco [b], Fernando Bonet [b], Virginio Garcia-Lopez [c], Amelia Aranega [b], Virginio Garcia-Martinez [a,*]

[a] Human Anatomy and Embryology, Faculty of Medicine, University of Extremadura, 06006 Badajoz, Spain
[b] Cardiovascular Development Group, Department of Experimental Biology, University of Jaén, CU Las Lagunillas B3-362, 23071 Jaén, Spain
[c] Pharmacy Services, Minimally Invasive Surgery Centre, Cáceres, Spain

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A B S T R A C T

It is known that secreted proteins from the anterior lateral endoderm, FGF8 and BMP2, are involved in mesodermal cardiac differentiation, which determines the first cardiac field, defined by the expression of the earliest specific cardiac markers *Nkx-2.5* and *Gata4*. However, the molecular mechanisms responsible for early cardiac development still remain unclear. At present, microRNAs represent a novel layer of complexity in the regulatory networks controlling gene expression during cardiovascular development. This paper aims to study the role of miR130 during early cardiac specification.

Our model is focused on developing chick at gastrula stages. In order to identify those regulatory factors which are involved in cardiac specification, we conducted gain- and loss-of-function experiments in precardiac cells by administration of Fgf8, Bmp2 and miR130, through *in vitro* electroporation technique and soaked beads application. Embryos were subjected to *in situ* hybridization, immunohistochemistry and qPCR procedures. Our results reveal that Fgf8 suppresses, while Bmp2 induces, the expression of *Nkx-2.5* and *Gata4*. They also show that Fgf8 suppresses Bmp2, and vice versa. Additionally, we observed that Bmp2 regulates miR-130 -a putative microRNA that targets Erk1/2 (Mapk1) 3′UTR, recognizing its expression in precardiac cells which overlap with Erk1/2 pattern. Finally, we evidence that miR-130 is capable to inhibit Erk1/2 and *Fgf8*, resulting in an increase of *Bmp2*, *Nkx-2.5* and *Gata4*.

Our data present miR-130 as a necessary linkage in the control of Fgf8 signaling, mediated by Bmp2, establishing a negative feed-back loop responsible to achieve early cardiac specification.

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1. Introduction

During early chick gastrulation, groups of cells from the epiblast, specified as precardiac cells, invaginate through the primitive streak just caudal to its rostral end and Hensen's node (Garcia-Martinez and Schoenwolf, 1993; Garcia-Martinez et al., 1997; Lopez-Sanchez et al., 2001, 2009). These cells migrate in an anterior–lateral direction between the ectoderm and endoderm to form a crescent of mesoderm in the most anterior part of the embryo, known as precardiac mesoderm area (Schultheiss et al., 1995; Redkar et al., 2001). This region is also identified as “first (primary) cardiac field” (FCF; Harvey, 2002), in order to differentiate it from the secondary cardiac field, a new group of cells that also contribute to heart development after cardiac tube formation, involving some others morphogenetic mechanisms (Hurle et al., 1977; Hurle and Ojeda, 1979; Mjaatvedt et al., 2001; Waldo et al., 2001; Kelly et al., 2014).

Precardiac anterior lateral mesoderm is defined by the expression of transcription factors *Nkx-2.5* (Schultheiss et al., 1995) and *Gata4* (Laverriere et al., 1994), which are detected in a crescent shape pattern at stage 5-6 HH (Hamburger and Hamilton, 1951). Cardiac fate specification requires a close association between the precardiac mesoderm and the adjacent endoderm. Previous experimental evidence suggests that bone morphogenetic protein – BMP2, emanating from the adjacent endoderm, induces *Nkx-2.5* expression in the precardiac mesoderm (Schultheiss et al., 1995, 1997). In particular, misexpression experiments in chick embryos, by applying exogenous BMP2 -medial to the FCF, result in induction of *Nkx-2.5* expression (Schultheiss et al., 1997, Andrée et al., 1998). In addition, ectopic exogenous administration of BMP2 is also capable of inducing *Gata4* (Schultheiss et al., 1997, Andrée

* Corresponding author.
E-mail address: virginio@unex.es (V. Garcia-Martinez).

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et al., 1998) and *Fgf8* (Alsan and Schultheiss, 2002). It has also been shown that several members of the fibroblast growth factor family – FGF – induce cardiogenic markers in non-precardiac mesoderm, since FGF8 administration – lateral to the FCF – results in induction of *Nkx-2.5* – but not of *Bmp2*- expression (Alsan and Schultheiss, 2002). Moreover, we have previously shown that FGF4 is able to induce the expression of *Nkx-2.5* in non-precardiogenic tissue, at the level of the germinal cell crescent (Lopez-Sanchez et al., 2002). Interestingly, in some other non-precardiogenic tissues, such as explanted caudal lateral mesoderm, a combination of BMP2 and FGF4, but neither factor alone, is able to induce *Nkx-2.5* expression (Lough et al., 1996; Barron et al., 2000). All these data suggest that initial cardiac specification is regulated by complex relationships between Bmp and Fgf signaling pathways, cooperation which has been proposed at later stages of cardiac development during myocardial and epicardial formation (Kruithof et al., 2006).

However, the intrinsic mechanisms involved in the crosstalk between Fgf and Bmp signaling, responsible for the induction and transcription factor regulation, still remain unclear. It has been proposed that mitogen-activated protein kinases (MAPKs) are highly conserved family proteins that play an integral role in signal transduction and regulation during cardiac development (see Rose et al., 2010). Additionally, ERK1/2 (extracellular signal-regulated kinase), one of MAPK subfamilies, has been involved in Fgf signaling pathway, playing a crucial role in the interaction Fgf–Bmp signals during cardiac differentiation at later developing stages (van Wijk et al., 2009; Tirosh-Finkel et al., 2010; Hutson et al., 2010). Interestingly, by means of bioinformatic analysis through TargetScan prediction of microRNA targets (Lewis et al., 2003), we identified miR-130 as a putative microRNA that targets ERK1/2 3 ′UTR. Guided by this prediction, we focused on the above mentioned molecular factors for early cardiac specification. MicroRNAs represent a novel layer of complexity in the regulatory networks controlling gene expression, cell specification and differentiation (Choi et al., 2013; Hosoda, 2013). MicroRNAs constitute a subclass of non-coding RNAs, with approximately 22-24 nucleotides in length, which by base-pair complementarity can bind to target mRNA and either trigger transcript degradation or protein translational blockage (Bartel, 2004; Cao and Chen, 2012). Over the last few years, the role of microRNAs in cardiac development and disease has progressively emerged (Lozano-Velasco et al., 2011; Chen and Wang, 2012; Espinoza-Lewis and Wang, 2012; Porello, 2013; De Rosa et al., 2014). Moreover, microRNA microarray expression analyses have identified, during cardiac development, a large set of microRNAs with increasing expression during ventricular maturation, including miR-130 (Chinchilla et al., 2011). Although little is known about miR-130 function to date, it has been referred in several adult and embryonic cardiovascular processes (Svensson et al., 1999; Chen and Gorski, 2008; Jakob et al., 2012; Osbourne et al., 2014). Gene ontology analyses revealed that targeting Bmp signaling was selectively represented among those up-regulated microRNAs (Chinchilla et al., 2011).

In order to gain an insight into the role of these molecular factors during early cardiac development, in this work we have carried out a series of experiments based on gain- and loss-of-function of Fgf8 and Bmp2 to dissect their effects on miR-130 expression and function during cardiac specification. Our results provide evidence about the presence of a negative feed-back loop between Fgf8 and Bmp2, mediated by miR-130, constituting a crucial element involved on the establishment of the precise patterning of the early developing heart.

2. Materials and methods

Experimental protocols with animals were performed in agreement with the Spanish law in application of the EU Guidelines for animal research, and conformed to the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH Publication no. 85-23). Approval by the University of Extremadura bioethics board was obtained prior to the initiation of the study.

For further details on the methodology see Appendix A.

2.1. Early chick whole embryo culture

Fertilized eggs (Granja Santa Isabel, Córdoba, Spain) were incubated at 38 °C in forced-draft humidified incubators. Embryos were staged (PS stages: Lopez-Sanchez et al., 2005; HH stages: Hamburger and Hamilton, 1951) and subjected to early chick (EC) embryo culture (Chapman et al., 2001).

2.2. Embryo electroporation of precardiac primitive streak cells

Cultured embryos were injected and electroporated in precardiac primitive streak cells. For gain-of-function experiments, three different groups of embryos were electroporated, with *Fgf8* expressing construct (pCS2-Fgf8), *Bmp2* expressing construct (pIRES-Bmp2-EGFP) and pre-miR-130, respectively. Likewise, for loss-of-function experiments, two different groups of embryos were electroporated, with *Fgf8* siRNA expressing construct (pSilencer-Fgf8) and anti-miR-130, respectively. For control embryos, EGFP expressing construct (pCAGGs-EGFP), or CFDA, was electroporated. Embryos were additionally incubated for 14–16 h.

2.3. Bead implantation

For gain-of-function experiments, beads were soaked either in FGF8 or in BMP2 solutions. In the same way, for loss-of-function experiments, beads were soaked either in noggin (an antagonist of BMP signals) or in SU5402 (FGF receptor inhibitor) solutions. Soaked beads were implanted at the desired site, and the embryos were additionally incubated for 6–8 h.

After the experimental procedure, all embryos were fixed overnight in 4% PFA, dehydrated in methanol and stored at −20 °C. Alternatively, embryos were selected and processed for whole mount *in situ* hybridization, while others were processed for immunohistochemistry.

2.4. Whole-mount in situ hybridization (ISH) and sectioning

Embryos were hydrated by incubation in graded methanol/PBT (PBS, 0.1% Tween 20) steps to a pure sterile PBT solution. Embryos were processed for ISH following standard procedure (Chapman et al., 2002) using antisense-*Fgf8, -Bmp2, -Nkx-2.5,* and *-Gata4* labeled probes, respectively. Double ISH (Chapman et al., 2002) was also performed using antisense-*Fgf8* and *-Bmp2* probes. A group of embryos was processed (Darnell et al., 2006) for ISH with LNA-labeled microRNA probes (Exiqon) against miR-130.

For histology, embryos were dehydrated with an ethanol series, cleared in isopropanol and processed for paraplast embedding, obtaining 15 µm transverse serial sections.

2.5. Immunohistochemistry (IMH)

Whole mount IMH was performed (Lopez-Sanchez et al., 2004) by using policlonal rabbit phospho-P44/42 MAPK/ERK1/2 antibody (Cell Signaling), followed by anti-rabbit IgG-HRP antibody (Chemicon).

2.6. RNA isolation and qRT-PCR

Pre-miR-130, anti-miR-130 and control (CDFA) electroporated embryos were collected and the primitive endocardial tubes were carefully dissected. Total RNA isolation was performed using Trizol reagent (Roche) according to the manufacturer's guidelines. For qRT-PCR analyses, DNAse treatment was performed during 1 h at 37 °C prior to cDNA synthesis using SuperScript RT III (Invitrogen) detection system. Real time PCR experiments were performed with 1 μL of cDNA, SsoFast EvaGreen mix and corresponding primer sets. All qPCRs were performed using a CFX384TM thermocycler (Bio-Rad) following the manufacturer's recommendations. The relative level of expression of each gene was calculated (Livak and Schmittgen, 2001) by using *Gapdh* and *Gusb* as internal control. Each PCR reaction was carried out in triplicate and repeated in at least three distinct pooled biological samples to obtain representative means.

A number of 12–15 embryos – successfully developed – were selected for every particular procedure.

3. Results

Previous studies have shown a relevant role for FGF8 and BMP2 proteins, secreted from the endoderm, as inductive signals of cardiogenesis on the adjacent precardiac mesodermal cells (Schultheiss et al., 1997; Alsan and Schultheiss, 2002). However, the precise spatio-temporal relationship between these two inductive signals during heart development remains rather elusive. It was previously reported (Lopez-Sanchez and Garcia-Martinez, 2011) that *Fgf8* and *Bmp2* are firstly expressed at the level of the primitive streak, and subsequently on the endoderm adjacent to the precardiac mesoderm (see Supplementary Fig. 1). Interestingly, using double ISH at later stages, we observed that *Fgf8* displays a more medial expression as compared to *Bmp2* expression pattern (Fig. 1). Such a partial overlap is consistently observed as well at later developmental stages, just after the primitive endocardial tubes are formed.

3.1. *Fgf8* modulates cardiac specific markers in a topologically dependent manner

Fgf8 gain-of-function experiments, by electroporation of the prospective cardiogenic primitive streak cells at PS4 stage as previously described (Lopez-Sanchez et al., 2005), demonstrate that expression of cardiac specific markers such as *Nkx-2.5* and *Gata4* is significantly suppressed at the level of primitive endocardial tubes (Fig. 2A). Importantly, *Bmp2* expression is also diminished by *Fgf8* overexpression. In order to further support these findings, FGF8-soaked beads were applied specifically inside FCF at PS11 stage. As expected, FGF8 suppresses *Bmp2*, *Nkx-2.5* and *Gata4* expression (Fig. 2B) at the level of the ipsilateral endocardial tube. Remarkably, application of FGF8 -soaked beads- laterally to FCF leads to *Nkx-2.5* induction (Fig. 2C), in line with previous reports (Alsan and Schultheiss, 2002).

In order to further explore the role of Fgf8 in early cardiogenesis, Fgf8 loss-of-function complementary approach was carried out, through electroporation with *Fgf8* siRNA expressing construct, within the prospective cardiogenic cells at primitive streak stages (PS4). Our results demonstrate (Fig. 3A) that *Fgf8* expression is inhibited, and concomitantly, *Bmp2*, *Nkx-2.5* and *Gata4* expression is increased.

FGF signaling is mediated via tyrosine kinase receptors (FGFRs) acting through a number of diverse transduction pathways (Böttcher and Niehrs, 2005), such as the highly conserved Ras-Erk1/2/MAPK (mitogen-activated protein kinase) cascade. It has

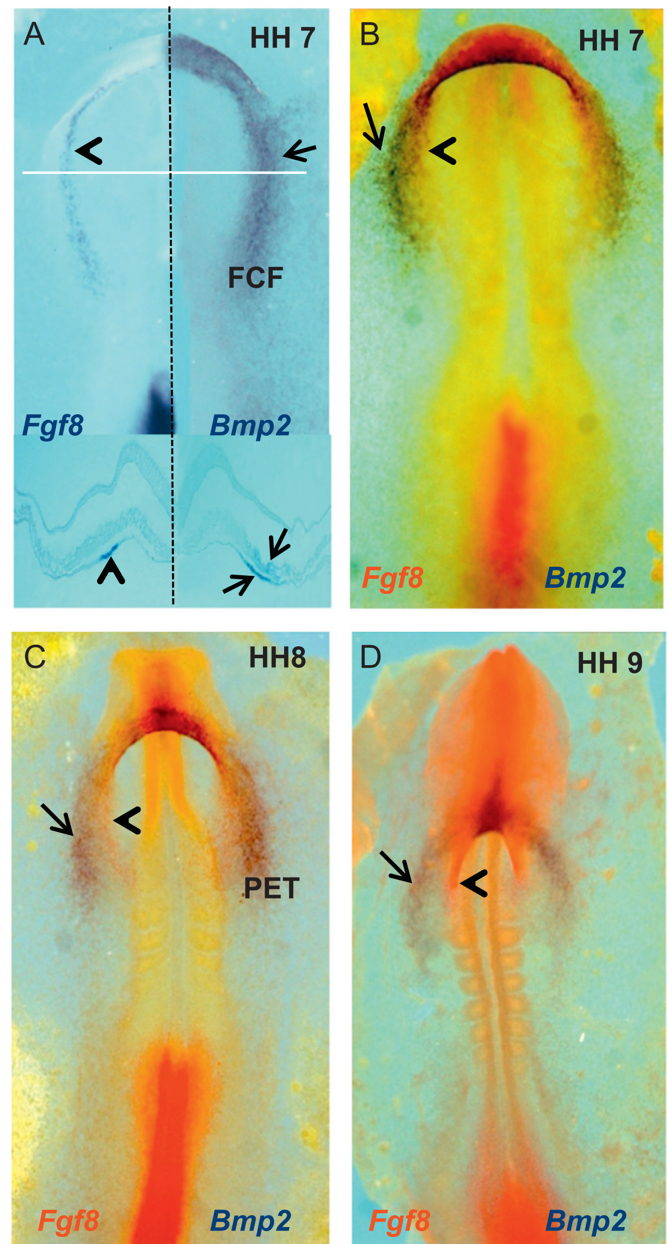

Fig. 1. Whole-mount ISH for *Fgf8* and *Bmp2* during early chick development in control embryos. Note *Fgf8* expression (arrowhead) medially to *Bmp2* (arrow) at primitive endocardial tube level. (A) After sagital section, the same embryo was processed for ISH. The right half (left image) was processed for *Fgf8*, and the left half (right image) was processed for *Bmp2*. Note the different topological expression pattern. The white line indicates the transverse section level, to show *Bmp2* expression in the precardiac mesoderm and the underlying endoderm (arrows), and *Fgf8* expression just in the underlying endoderm (arrowhead). (B)–(D): Double ISH for *Fgf8* (in red) and *Bmp2* (in blue), simultaneously. FCF: first cardiac field. PET: primitive endocardial tube.

been previously reported (Echevarria et al., 2005) that Fgf8 signals are mediated by Erk1/2 (MAPK). In this regards, we therefore explored the Erk1/2 (MAPK) response after our Fgf8 loss-of-function experiments, showing a markedly decreased expression of Erk1/2 protein (Fig. 3A).

Remarkably, application of SU5402 –soaked beads- specifically inside FCF (a chemical blocker of FGF signal by specifically binding to FGFR1; Eblaghie et al., 2003), leads to similar observations as those obtained with *Fgf8* siRNA electroporation, including both *Bmp2* and *Gata4* increased expression as well as supressed *Fgf8* and Erk1/2 expression (Fig. 3B), at the level of the ipsilateral

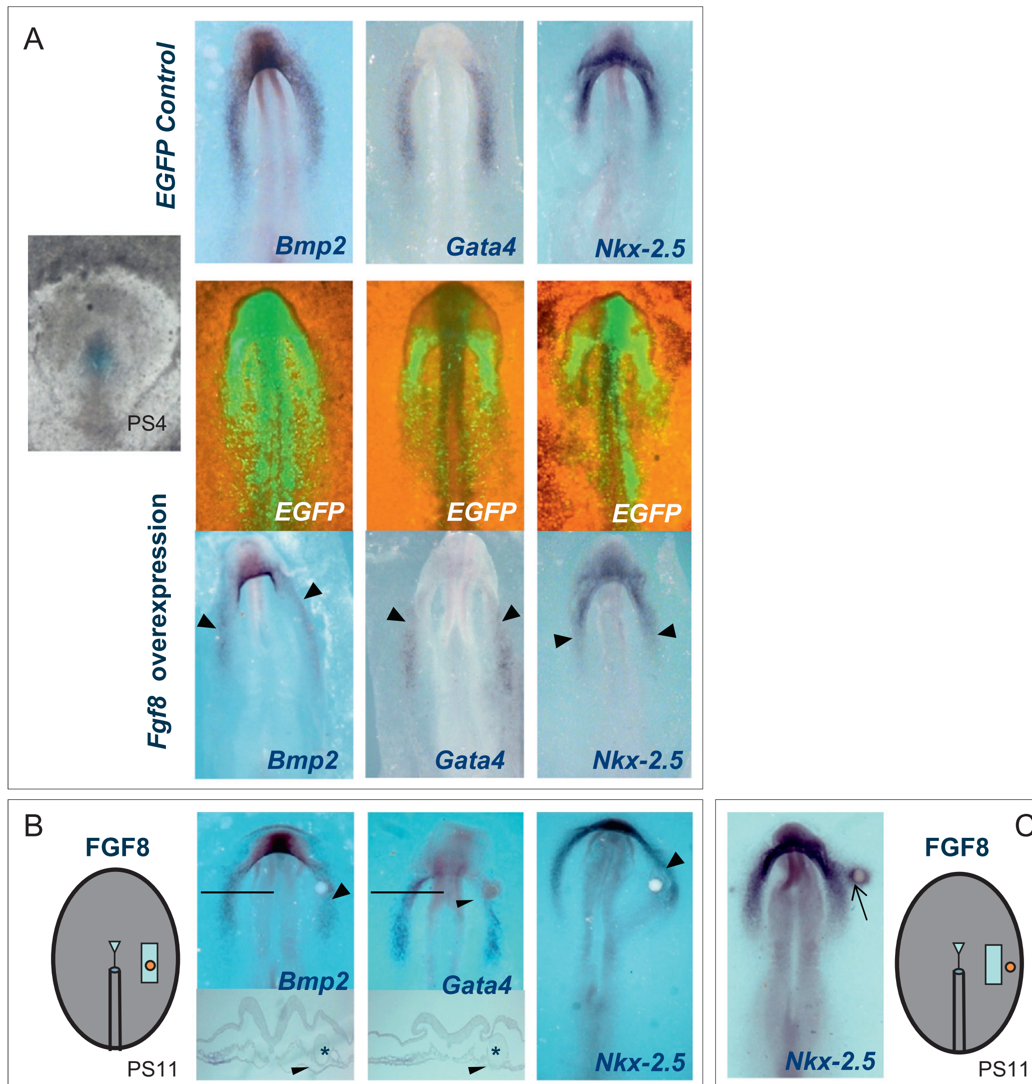

Fig. 2. Effect of Fgf8 gain-of-function on primitive endocardial tube specification. Whole-mount ISH for *Bmp2*, *Gata4* and *Nkx-2.5*. (A)**:** Embryos electroporated at the level of the primitive streak precardiac cells (left image), either with the control construct or *Fgf8* expressing construct. Note their markedly suppressed endocardial tube expression (arrowheads). EGFP expression of experimental embryos is shown in the immediate upper panel. (B): After FGF8 soaked bead, applied just inside FCF (left drawing), *Bmp2*, *Gata4* and *Nkx-2.5* are diminished at the ipsilateral endocardial tube level (arrowheads). The black line indicates the transverse section level (the asterisk indicates the bead). (C): FGF8 soaked bead, applied laterally to FCF (right drawing), induces *Nkx-2.5* expression (arrow).

endocardial tube. However, no significant change is observed for *Nkx-2.5* expression.

Therefore, these data indicate that the topological application is critical in Fgf8 signaling, determining the induction or/and inhibition of cardiac specific markers.

3.2. Bmp2 signaling positively regulates cardiac specific markers and negatively regulates Fgf8 expression in first cardiac field

Given the observations obtained by Fgf8 analysis, we similarly dissected the role of Bmp2 in cardiogenesis. Gain-of-function experiments, by means of *Bmp2* electroporation within the prospective cardiogenic primitive streak cells at PS4 stage, resulted in an increased expression of *Nkx-2.5* and *Gata4* at the level of primitive endocardial tubes (Fig. 4A). Curiously, both *Fgf8* and Erk1/2 expressions were significantly diminished by *Bmp2*. Further support on the up-regulation of *Nkx-2.5* and *Gata4* and down-regulation of *Fgf8* and Erk1/2 expression by Bmp2 was obtained by applying BMP2 soaked beads inside FCF (Fig. 4B). Importantly, after the application of BMP2 –soaked beads- medially to FCF (Fig. 4C), *Nkx-2.5, Gata4* and *Fgf8* expression was induced, in line with previous authors (Schultheiss et al., 1997; Andrée et al., 1998; Schlange et al., 2000; Alsan and Schultheiss, 2002).

Remarkably, loss-of-function Bmp2 experiments (Fig. 4D), through the application of noggin – soaked beads – inside FCF, led to down regulation of *Bmp2*, *Nkx-2.5* and *Gata4* expression, while no significant changes were observed for *Fgf8* and Erk1/2 expression. Therefore, these data further support the notion that topological application of both Fgf8 and Bmp2 is critical to induce or inhibit cardiac specific markers.

3.3. miR-130 Regulates Fgf8–Bmp2 signaling during early cardiac development

As we have mentioned above, Erk1/2 signaling decreases in both types of experiments: those based on Fgf8 loss-of-function (Fig. 3) and those involving Bmp2 gain-of-function (Fig. 4). Through bioinformatic analyses by using Target-Scan on-line software we identified miR-130 as a putative microRNA that targets Erk1/2 (MAPK) 3´UTR (Supplementary Fig. 2), thus suggesting

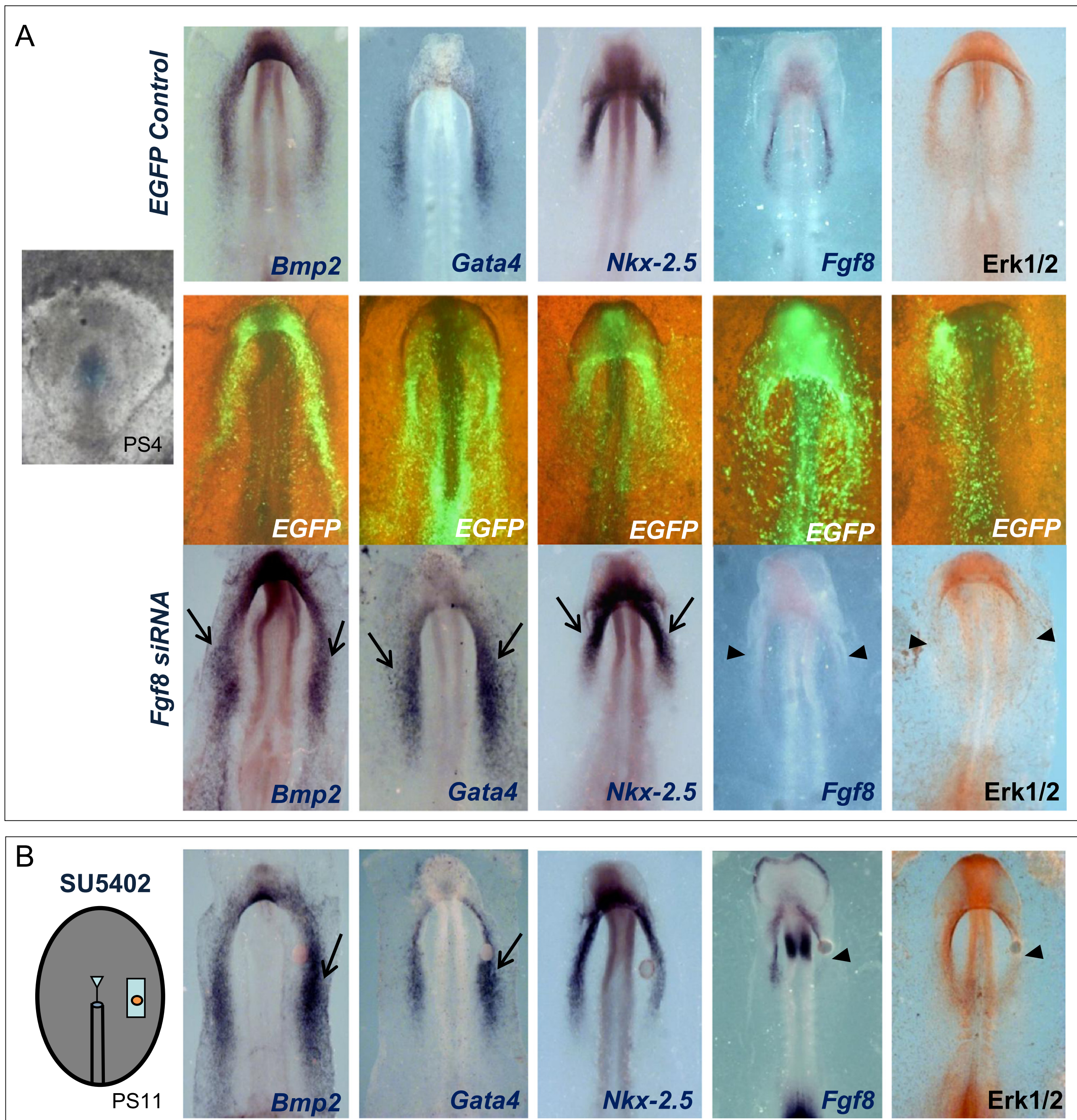

Fig. 3. Effect of Fgf8 loss-of-function on primitive endocardial tube specification. Whole-mount ISH for *Bmp2*, *Gata4*, *Nkx-2.5* and *Fgf8*, and IMH for Erk1/2. (A): Embryos electroporated at the level of the primitive streak precardiac cells (left image), either with the control construct or *Fgf8* siRNA expressing construct. Note that at the primitive endocardial tube level *Bmp2*, *Gata4* and *Nkx-2.5* are markedly increased (arrows), whereas *Fgf8* and Erk1/2 expressions are dramatically reduced (arrowheads). EGFP expression of experimental embryos is shown in the immediate upper panel. (B): After SU5402 soaked bead, applied just inside FCF (left drawing), *Bmp2* and *Gata4* are increased at the ipsilateral endocardial tube level (arrows), whereas *Fgf8* and Erk1/2 are significantly reduced (arrowheads).

that it might exert post-transcriptional regulation on Erk1/2 expression. Taking these data into account, by using ISH we have performed an analysis of miR-130 expression from primitive streak stages to primitive cardiac tube formation, and we have also studied its involvement in the Fgf8/Bmp2 interaction.

Our results revealed that miR-130 expression starts at PS4 stage in the primitive streak, followed by its expansion towards both sides of the mesodermal and endodermal layers of the embryo at PS8 stage. Later on, from PS13 onwards, miR-130 expression becomes restricted to precardiac mesoderm and underlying endoderm (Fig. 5A). Interestingly Erk1/2 IMH analyses demonstrated a complementary pattern (Fig. 5B), suggesting a plausible regulatory link during early cardiogenesis. Additionally, we have observed miR-130 expression in non precardiac regions (Supplementary Fig. 3), including neural plate and notochord, as well as emerging paraxial mesoderm and vessels, locations which are overlapped with Erk1/2 signaling previously described (Lunn et al., 2007).

In this context, miR-130 overexpression by means of electroporation led to inhibited Erk1/2 signal and *Fgf8* expression, and

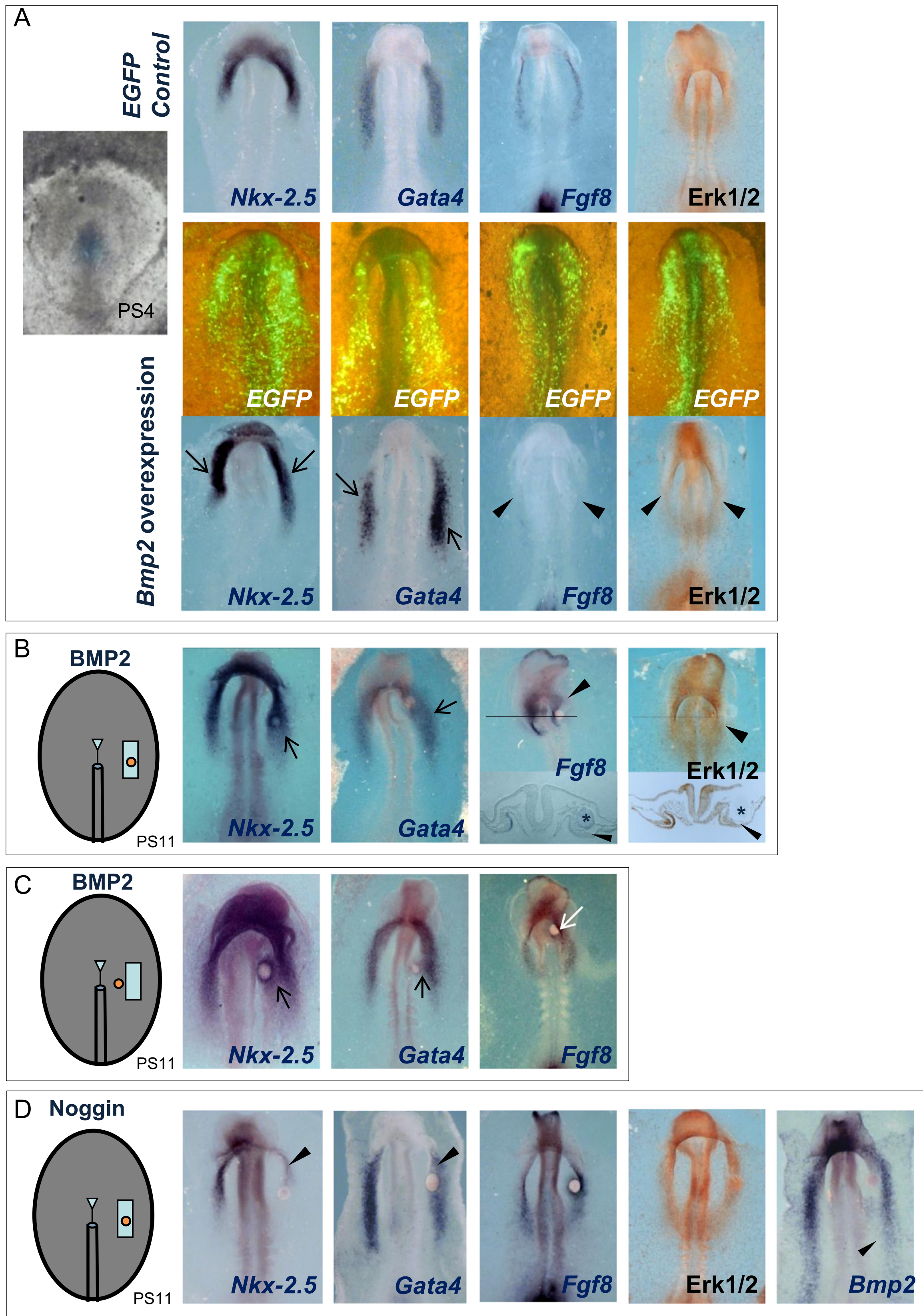

Fig. 4. Effect of Bmp2 gain- and loss-of-function on primitive endocardial tube specification. Whole-mount ISH for *Nkx-2.5, Gata4, Fgf8* and *Bmp2*, and IMH for Erk1/2. (A): Embryos electroporated at the level of the primitive streak precardiac cells (left image), either with the control construct or *Bmp2* expressing construct. Note at the primitive endocardial tube level a marked increase of *Nkx-2.5* and *Gata4* (arrows), while *Fgf8* and Erk1/2 are dramatically reduced (arrowheads). EGFP expression of experimental embryos is shown in the immediate upper panel. (B): After BMP2 soaked bead, applied right inside FCF (left drawing), *Nkx-2.5* and *Gata4* are increased at the ipsilateral endocardial tube level (arrows), whereas *Fgf8* and Erk1/2 are reduced (arrowheads). The black line indicates the transverse section level (the asterisk indicates the bead). (C): BMP2 soaked bead, applied medially to FCF (left drawing) induces *Nkx-2.5*, *Gata4* and *Fgf8* (arrows). (D): After noggin soaked bead, applied just inside FCF (left drawing), *Nkx-2.5*, *Gata4* and *Bmp2* are diminished at the ipsilateral endocardial tube level (arrowheads).

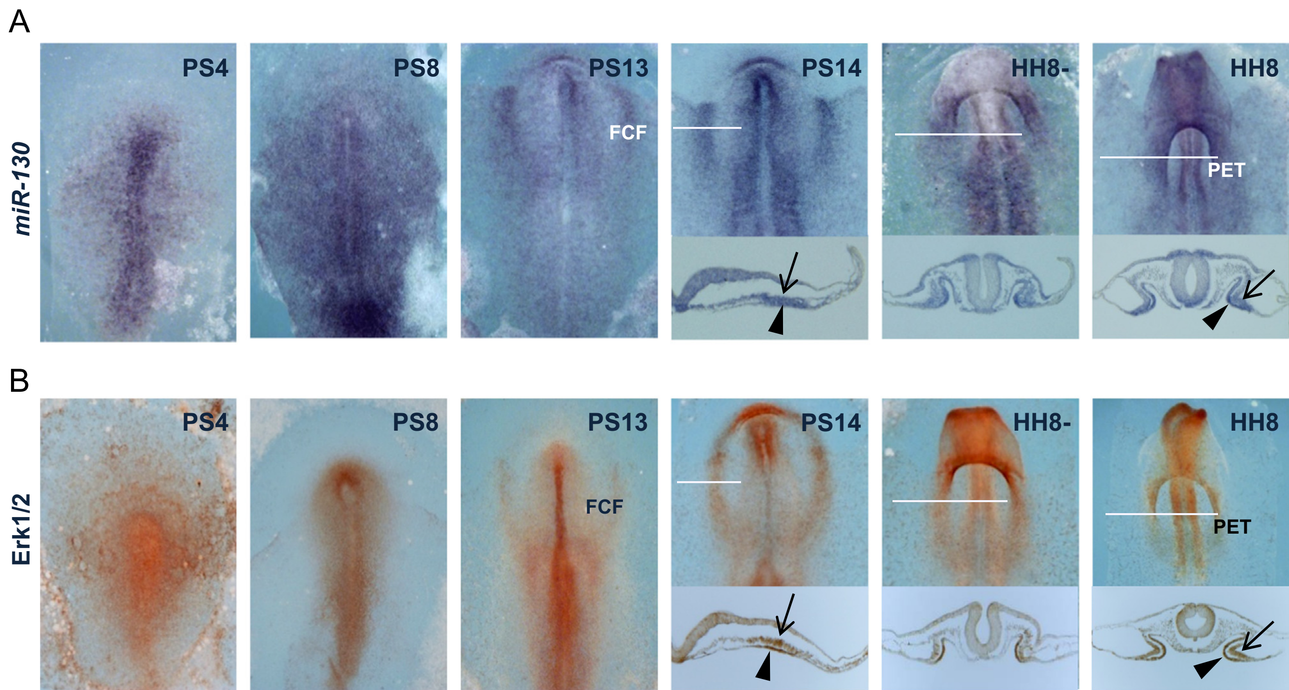

Fig. 5. Whole-mount ISH analysis for miR-130 and IMH for Erk1/2 during early chick development in control embryos. (A): Expression profile of miR-130 from stage PS4 to HH8. miR-130 is highly expressed from the epiblast and primitive streak -through FCF- to the primitive endocardial tube (PET) level. (B): Erk1/2 shows a similar, overlapped, distribution pattern. The white lines indicate the transverse section level, showing miR-130 expression and Erk1/2 activity in the precardiac mesoderm (arrows) and the underlying endoderm (arrowheads).

increased *Bmp2*, *Nkx-2.5* and *Gata4* expression (Fig. 6A). As expected, the electroporation of anti-miR-130 resulted in Erk1/2 and *Fgf8* increase, whereas *Bmp2*, *Nkx-2.5* and *Gata4* were suppressed (Fig. 6B). Moreover, qRT-PCR analysis showed a marked increase of *Nkx-2.5* and *Gata4* levels in endocardial tubes after miR-130 overexpression, as well as a substantial decrease after anti-miR-130 electroporation (Fig. 6C). Thus, these findings corroborate the results obtained by ISH.

Importantly, *Bmp2* overexpression in precardiac primitive streak cells increased miR-130 at the primitive endocardial tube level (Fig. 7A), and likewise, the application of noggin-soaked beads, specifically into the FCF, reduced miR-130 expression in the ipsilateral endocardial tube (Fig. 7B).

Therefore, our findings indicate that miR-130 is an intermediate effector on the Fgf8-Bmp2 signaling during early cardiogenesis.

4. Discussion

Transcription factors *Nkx-2.5* (Schultheiss et al., 1995) and *Gata4* (Laverriere et al., 1994) are known to be the first markers identifying cardiac specification at mesodermal level (first cardiac field: FCF). It has been proposed that these earliest markers are induced by *Bmp2* and *Fgf8*, both expressed in the underlying endoderm (Schultheiss et al., 1997; Andrée et al., 1998; Schlange et al., 2000; Alsan and Schultheiss, 2002; Brand, 2003). In this work, a detailed analysis – by means of double ISH – has revealed that *Fgf8* is expressed medially in the endoderm underlying FCF, while *Bmp2* is expressed laterally, establishing two specific – yet overlapping – areas of expression. Additionally, although *Bmp2* expression was initially observed only in the underlying endoderm (Schultheiss et al., 1997), it was further noted (Schlange et al., 2000; Lopez-Sanchez and Garcia-Martinez, 2011) that *Bmp2* is also expressed in precardiac mesodermal cells during early cardiac specification. In this sense, it is important to mention that *Fgf8* is expressed only in the endoderm in chick development, whereas mouse *Fgf8* expression is observed at both endodermal and precardiac mesodermal levels (Crossley and Martin, 1995), as previously described in zebrafish as well (Reifers et al., 2000). In this study, we propose that this topographical distribution of gene expression pattern in the embryonic layers plays an important role as an inductive/repressive factor of the endoderm over the precardiac mesoderm.

4.1. *Fgf8* suppresses while *Bmp2* induces early cardiac markers

In our *Fgf8* overexpression experiment – through electroporation of primitive streak precardiac cells – we observed inhibition of mesodermal cardiac marker *Nkx-2.5* and *Gata4*. Additionally, when we administrated FGF8 right inside FCF, it also resulted in *Nkx-2.5* and *Gata4* inhibition. Since these results apparently disagree with previous experiments, in which FGF8 was able to induce *Nkx-2.5* (Alsan and Schultheiss, 2002), we performed the same experiments under the same conditions: we applied FGF8 just laterally to FCF, equally obtaining *Nkx-2.5* ectopic induction. All these results consequently demonstrate that topological location of the inductive signals is crucial in this context. As an additional result, our loss-of-function experiments gave rise to *Gata4* increase, both through *Fgf8* siRNA electroporation – an inhibitor of *Fgf8* signaling – and SU5402 administration – a chemical FGFR inhibitor, while *Nkx-2.5* increases or maintains its expression, respectively, this last result coinciding with previous experiments (Alsan and Schultheiss, 2002). Thus, these data demonstrate the disability to inhibit cardiac specific markers expression when using a number of inhibitors of *Fgf8* signaling during early cardiac specification. In addition, it has been reported that FGF8 administration at later stages of cardiogenesis represses *Nkx-2.5* expression in the anterior heart field, whereas SU5402 induces its expression (Tirosh-Finkel et al., 2010). Moreover, studies in *acerebellar/fgf8* -a zebrafish mutant with no expression of either *Nkx-2.5* or *Gata4* (Reifers et al., 2000) reveal that the injection of *Fgf8* is

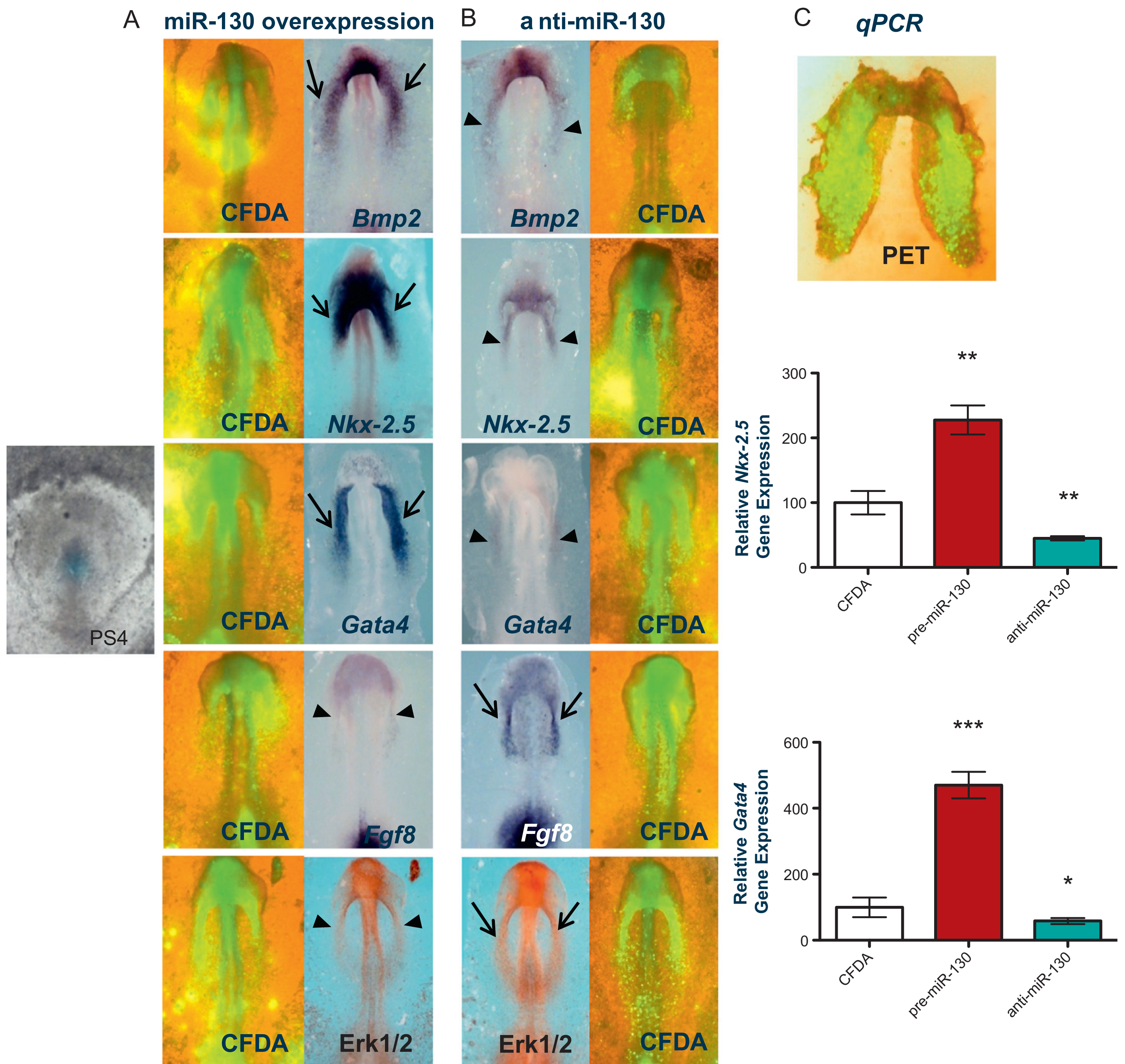

Fig. 6. Effect of miR-130 gain- and loss-of-function on primitive endocardial tube specification. Whole-mount ISH for *Bmp2*, *Nkx-2.5*, *Gata4* and *Fgf8*, and IMH for Erk1/2. Embryos electroporated with pre-miR-130 (A) and anti-miR-130 (B) at the primitive streak precardiac cell level (left image). Visualization of CFDA in the immediate left or right panels, respectively. (A): Note that at the primitive endocardial tube level *Bmp2*, *Nkx-2.5* and *Gata4* are markedly increased (arrows), whereas *Fgf8* and Erk1/2 are dramatically reduced (arrowheads). (B): Note that at the primitive endocardial tube level *Bmp2*, *Nkx-2.5* and *Gata4* are markedly suppressed (arrowheads), while *Fgf8* and Erk1/2 are increased (arrows). (C): RT-qPCR of RNA from dissected primitive endocardial tube (PET) areas (upper image) in embryos electroparated either with CFDA, pre-miR-130 or anti-miR-130. Overexpression of miR-130 leads to increased *Nkx-2.5* and *Gata4* transcripts, whereas miR-130 inhibition leads to decreased transcripts. Standard deviations are from three independent experiments. $^{*}P<0.01$, $^{**}P<0.001$ and $^{***}P<0.0001$.

able to restore *Gata4* but not *Nkx-2.5*. On the other hand, experiments based on removal of the anterior endoderm (Alsan and Schultheiss, 2002) result in loss of *Nkx-2.5* expression (but not *Gata4*), and this deficiency is rescued by applying FGF8 alone. However, it is yet to be considered the possibility of an endodermal regeneration influenced by FGF8, a known cellular proliferative factor (Engelmann et al., 1993; Choy et al., 1996). Interestingly, noggin administration – a Bmp2 inhibitor – did not modified *Fgf8* expression, while *Nkx-2.5* and *Gata4* expression were diminished. All the above data support that Fgf8 alone is incapable to act as a direct cardiac inductive factor in FCF. Therefore, this study brings new data with respect to the regulation process in cardiogenesis during chick development.

In reference to our *Bmp2* overexpresion experiments, through primitive streak precardiac cells electroporation, we observed an increase in expression of *Nkx-2.5* and *Gata4*. Furthermore, at the ipsilateral primitive endocardial tube level, *Nkx-2.5* and *Gata4* expression areas were increased when administering BMP2, and diminished when applying noggin beads, both inside FCF. It has been shown that BMP2 administration, just medial to FCF, has the ability to induce specific cardiac markers *Nkx-2.5* and *Gata4* ectopically (Schultheiss et al., 1997; Andrée et al., 1998; Schlange et al., 2000; Alsan and Schultheiss, 2002), results that we also obtained after reproducing the same experiment. The fact that

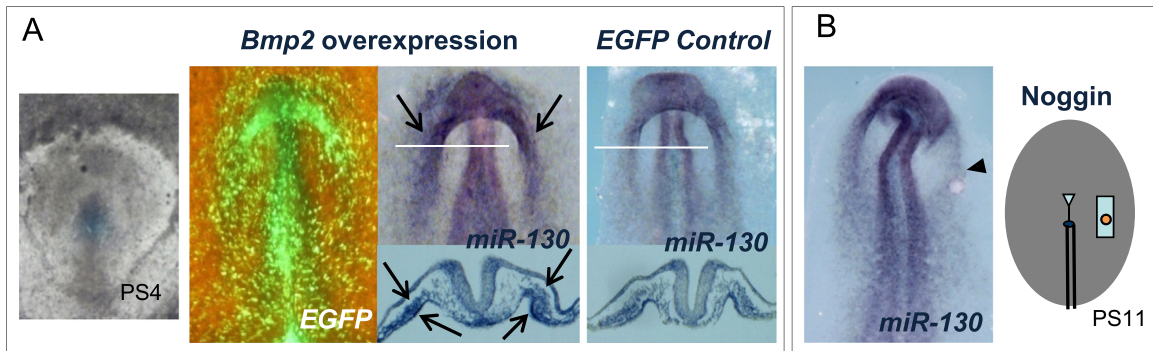

Fig. 7. Effect of Bmp2 gain- and loss-of-function on primitive endocardial tube specification. Whole-mount ISH for miR-130. (A): Embryo electroporated at the level of the primitive streak precardiac cells (left image), either with the control construct or *Bmp2* expressing construct. Note a marked increase of miR-130 (arrows) at the primitive endocardial tube level. The white lines indicate the transverse section level. EGFP expression of experimental embryo is shown in the immediate left panel. (B): After noggin soaked bead, applied just inside FCF (right drawing), miR-130 is diminished at the ipsilateral endocardial tube level (arrowhead).

Bmp2 is capable to induce *Nkx-2.5* and *Gata4* expression, both at FCF and ectopic locations, supports that Bmp2 plays a key role in cardiogenic inductive differentiation processes.

4.2. Fgf8–Bmp2 displays a negative feed-back loop in early cardiogenesis

Another relevant result directly derived from our *Bmp2* gain-of-function experiments showed *Fgf8* inhibition at primitive endocardial tube level. This was obtained through *Bmp2* electroporation -at precardiac primitive streak level- as well as by applying BMP2 beads inside FCF. Nevertheless, when we applied BMP2 beads medial to FCF, we observed ectopic *Fgf8* expression, result which is in agreement with previous studies (Alsan and Schultheiss, 2002). Additionally, in this work we show the capability of Fgf8 to inhibit *Bmp2* expression by using gain-of-function experiments, supported by Fgf8 loss-of-function experiments, which generate an increase in Bmp2 at primitive endocardial tube level. This is the first time that there is clear evidence of a negative regulation of Bmp2 mediated by Fgf8 during early cardiogenesis, aspect which has been pointed out at later stages of cardiac development in a different experimental model *in vitro* (Kruithof et al., 2006). The specific results we obtained – suppression of *Fgf8* by *Bmp2*, suppression of *Bmp2* by *Fgf8*, and separated topographical location of *Bmp2* and *Fgf8* endodermal expression – reveal the intervention of a negative Fgf8-Bmp2 feed-back mechanism responsible to define the first cardiac field. Thus, from the underlying endoderm, Bmp2 signaling determines the first step to induce mesoderm cardiac specification, whereas Fgf8 delimits medially the first cardiac field, by means of an inhibitory effect. At later stages of cardiac development, it has also been proposed that Fgf8 regulates the position of the boundary between myocardium and pericardial mesoderm in *in vitro* explant assay (Kruithof et al., 2006). All the above data are supported by previous papers focused on different experimental models (Prall et al., 2007; Tirosh-Finkel et al., 2010), describing that Fgf8 is mainly involved in cell proliferation and Bmp2 is related to cell differentiation.

Our hypothesis is supported by the fact that *Bmp2* is capable to induce both *Nkx-2.5* and *Gata4* by overexpressing the signal just in FCF, and also when BMP2 is administrated medially to FCF. This probably indicates that *Fgf8* is expressed in response to specific levels of Bmp2 signals. Although *Fgf8* is only able to induce *Nkx-2.5,* and just laterally to FCF, we do not discard its possible role of counteracting other endogenous factors which inhibit cardiac differentiation laterally (Brand, 2003; Lopez-Sanchez and Garcia-Martinez, 2011). Therefore, our model sets the bases for the initial steps in cardiac specification.

4.3. miR130 Regulates negative Fgf8–Bmp2 feed-back

In order to gain a deeper insight into the mechanism involved in negative Fgf8-Bmp2 feed-back loop, we have explored some other different pathways responsible for cardiac specification. It is well known (Eblaghie et al., 2003; Echevarria et al., 2005; Lunn et al., 2007) that Fgf signaling is mediated via tyrosine kinase receptors (FGFRs) by acting through a number of transduction pathways, such as the highly conserved Ras-ERK mitogen-activated protein kinase (MAPK). In this work, there is evidence to show the existence of a direct link between *Fgf8* and Erk1/2 by obtaining Erk1/2 inhibition at primitive endocardial tube level, after both Fgf8 loss-of-function and Bmp2 gain-of-function experiments. These interesting results show Erk1/2 as a regulatory factor in the cardiac induction process. In this sense, previous authors have proposed a similar active role of Bmp in the regulation of FGF/ERK pathway at later stages of cardiovascular development, i.e., during cardiomyocyte differentiation of the anterior heart field (Tirosh-Finkel et al., 2010; Hutson et al., 2010), and during modulation of myosin in vascular smooth muscle cells (Zhang et al., 2014). Taking into account all the above data, we have analyzed the possible influence of some additional factors in order to establish a mechanism which justifies a control pathway of FGF/MAPK from Bmp2 during early cardiac specification. Interestingly, we identified miR-130 – through Target-Scan program analysis – as a putative microRNA that targets ERK1/2 3′UTR. MicroRNAs have shown to play a crucial role in several cardiac (Chinchilla et al., 2011; Malizia and Wang, 2011; Chen and Wang, 2012) and angiogenic (Chen and Gorski 2008; Jakob et al., 2012) developmental models. In particular, miR-130 has been predicted to target a conserved region of FOG-2 3′UTR (Kim et al., 2009), being FOG-2 a transcriptional co-factor critical for mouse cardiac development, modulator of *Gata4* (Svensson et al., 1999).

Our detailed analysis of miR-130 expression pattern during early gastrula stages revealed its distribution at the primitive streak level, including the precardiac cells, followed by its expression in the precardiac mesoderm as well as in the underlying endoderm. The fact that this expression pattern is coincident with Erk1/2 signal at these developing stages evidence a close relationship between miR-130 and Erk1/2 during cardiogenesis.

After our miR-130 gain-of-function experiments, we observed that Erk1/2 signal diminishes, followed by a decrease of *Fgf8*. Interestingly, *Bmp2* expression increased, and consequently, cardiac markers *Nkx-2.5* and *Gata4* increased as well. These data are supported by the results we obtained from miR-130 loss-of-function experiments, where we observed that Erk1/2 and *Fgf8* increase, whereas *Bmp2* diminishes, and consequently, *Nkx-2.5* and *Gata4* decrease as well. Additionally, our *Bmp2* overexpression

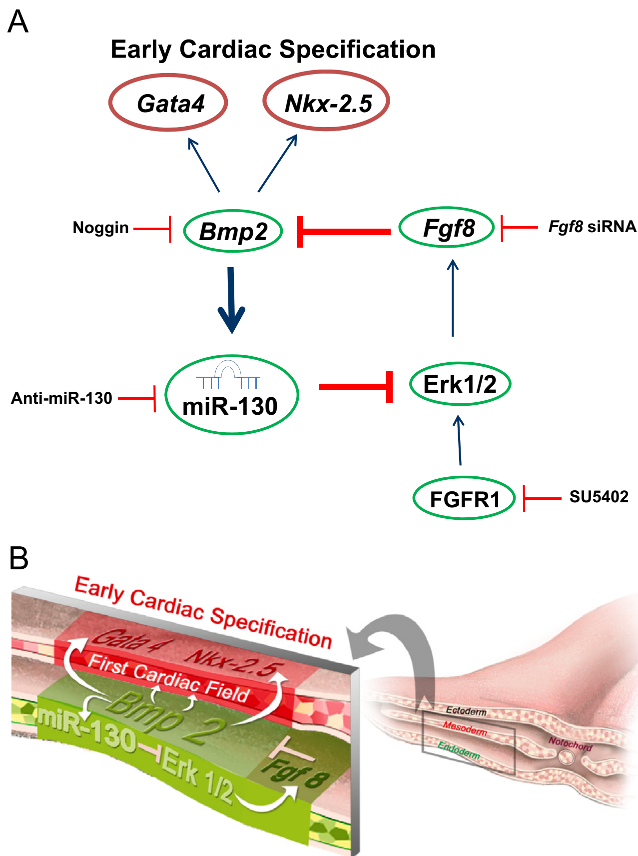

Fig. 8. Proposed model for early cardiac specification. Our model indicates that the earliest cardiac markers -*Nkx-2.5* and *Gata4*- are induced by *Bmp2*. *Fgf8* represses *Bmp2*. Moreover, *Bmp2* induces miR-130, which represses Erk1/2. Since *Fgf8* is controlled via Erk1/2, our model presents *Bmp2* as a modulator of *Fgf8* expression through miR-130. Thus, miR-130 acts as necessary linkage in the control of Fgf8 signaling, mediated by Bmp2, establishing a negative feed-back loop responsible to achieve the initial cardiac specification.

experiments resulted in an increase of miR-130 expression. In agreement with that, after noggin administration, we obtained an inhibition of miR-130 expression. Our data support that miR-130, upstream-regulated by Bmp2, controls Erk1/2 signaling, either repressing its translation and/or transcript stability. In our illustrative Diagram (Fig. 8A) we draw the various links proposed in early cardiac specification. We could hypothesize a pathway in which Bmp2 signal controls Fgf8 via Erk1/2, mediated by miR-130, which is acting as a regulator in cardiac specification. Finally, since Fgf8 constitutes an essential factor involved in MAPK/ERK intracellular signaling (Echevarria et al., 2005), it is reasonable to consider additional factors involved in this pathway, such as FGF receptors, which play a significant role as early steps in the Fgf8/ERK signaling pathway (Hadari et al., 1998; Dell'Era et al., 2003). Although a relevant effect of those receptors has not yet been demonstrated during cardiac induction, some authors have identified FGFR1 in the endoderm underlying the precardiac mesoderm (Sugi et al., 1995; Lunn et al., 2007). However, its functional role has been reported in growth and proliferation rather than in differentiation (Zhu et al., 1999). In this sense, it has been previously reported that cardiomyocyte proliferation decreases after miR-133 overexpression (Liu et al., 2008). In line with this, we have observed that miR-133 is expressed in all the precardiac areas during early cardiogenesis (Lopez-Sanchez et al., submitted), expression pattern which is comparable to distribution FGFR1 (Sugi et al., 1995; Lunn et al., 2007). In order to gain a deeper insight into the linkage between miR-133 and FGFR1, we developed additional experimental designs, showing a reciprocal repression between Fgf8 and miR-133. We propose that this cooperation plays a crucial role in Fgf8/ERK pathway cascade, probably mediated by FGFR1, through Bmp2 signaling.

In conclusion, our study reveals for the first time a key role of miR-130 in molecular regulation during early cardiogenesis. This model proposes a regulation of Erk1/2 (MAPK) through miR-130. Therefore, this microRNA functions as a necessary linkage in the control of Fgf8 signaling mediated by Bmp2. This mechanism establishes a negative feed-back loop responsible for *Nkx-2.5* and *Gata4* induction to achieve the early cardiac specification (Fig. 8B).

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2015.07.007.

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