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Neural ectoderm-secreted FGF initiates the expression of *Nkx2.5* in cardiac progenitors via a p38 MAPK/CREB pathway

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ABSTRACT

Vertebrate heart development is derived from paired primordia of anterior dorsolateral mesoderm expressing Nkx2.5 and GATA4 transcription factors. Yet growth factors and intracellular pathways specifying heart precursor gene expression are poorly understood. In the present work, we investigated the signaling events initiating *Nkx2.5* expression in *Xenopus laevis*. We describe here that fibroblast growth factor (FGF) initiates the expression of *Nkx2.5* without affecting *GATA4*. At gastrula, FGF3 is expressed in anterior neural ectoderm, and results presented here indicate that this tissue is involved in the induction of *Nkx2.5* expression in neighboring lateral tissues. Further studies indicate that the intracellular p38 MAPK and the CREB transcription factor function downstream of FGF to initiate *Nkx2.5* expression. Activation of the p38 MAPK pathway and of the CREB protein is both necessary and sufficient for the initial expression of *Nkx2.5*. Therefore, we would like to suggest that FGF expressed in anterior neural ectoderm is a major inducer of *Nkx2.5* expression in neighboring cells. In these cells, FGF activates an intracellular p38 MAPK signaling pathway and its downstream target, the CREB transcription factor, all participating in the expression of *Nkx2.5* in cardiac progenitors.

Introduction

During gastrulation, bilateral symmetrical regions of anterior mesoderm become specified to the cardiac lineage. As neurulation proceeds, the presumptive cardiac mesoderm extends laterally to eventually fuse at the ventral midline. The signals that specify the cardiac progenitors originate at the Spemann organizer and its underlying endoderm. Surgical removal of the endoderm at the onset of gastrulation prevents heart formation (Cooke, 1989; Sater and Jacobson, 1989). Since early specification occurs dorsally, patterning signals antagonizing bone morphogenetic proteins (BMPs) and canonical Wnt pathways secreted by the Spemann organizer are necessary for the initial competence of heart primordia (Schneider and Mercola, 2001). Therefore, it is difficult to distinguish the direct effects of these morphogens on heart formation from indirect effects mediated by their role in body patterning. In *Xenopus*, the deep endoderm underlying the dorsal marginal zone (DMZ) secrete Cerberus, a BMP, Wnt and Nodal antagonist which is necessary for the induction of heart (Schneider and Mercola, 1999). Endoderm-secreted Cerberus and organizer secreted DKK1, a Wnt antagonist function in parallel to initiate cardiogenesis (Foley et al., 2007). Secretion of BMP ligands from endoderm at later stages is essential for later cardiac development, such as migration and fusion of heart primordia, cardiomyocyte differentiation (Walters et al., 2001) and heart looping (Breckenridge et al., 2001; Peterkin et al., 2003). In the *chick*, the ability of endoderm to initiate cardiac fate depends on the activity of BMPs (Schultheiss et al., 1995; Schultheiss et al., 1997) and of fibroblast growth factor 8 (FGF8) (Alsan and Schultheiss, 2002). Identifying molecules initiating cardiac specification in the frog took advantage of explant assays. Molecules such as Alk4 (TGFβ) (Logan and Mohun, 1993) and Wnt11 (non-canonical pathway) (Pandur et al., 2002) induced cardiogenesis of multipotent animal cap (AC) explants. The role of FGF signaling in the initiation of cardiac development in *Xenopus* was overlooked since early studies of Amaya and colleagues (1991, 1993) that convincingly demonstrated that the expression of a dominant-negative mutant form of the FGF receptor disrupted mesoderm formation altogether.

One of the earliest markers of cardiac fate is the homeobox transcription factor Nkx2.5, the homologue of *Drosophila* cardiac regulator Tinman, which expression is initiated at cardiac specification in the gastrula through the subsequent onset of cardiac differentiation in the tailbud embryo. Transcripts of *Nkx2.5* are first detected during late gastrulation (stage 12) as two symmetrical patches of lateral anterior mesoderm (Tonissen et al., 1994; Sparrow et al., 2000). *Nkx2.5* expression persists during the ventral expansion of cardiac progenitors and later at the forming heart tube and morphogenesis of the heart. Expression of *Nkx2.5* is not limited to the cardiac lineage only, since it is expressed in other regions such as

Abbreviations: VMZ, ventral marginal zone; DMZ, dorsal marginal zone; AC, animal cap; AMO, antisense morpholino oligonucleotide; MAPK, mitogen-activated protein kinase.

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anterior endoderm tissue (Cleaver et al., 1996). Nkx2.5 among other family members functions as an initial specification factor of cardiac lineage at gastrulae together with other transcription factors of the GATA family. The signals regulating the expression of *Nkx2.5* are only partially explored. BMP ligands are implicated in the maintenance of *Nkx2.5* expression at the ventral heart field region (Shi et al., 2000; Walters et al., 2001; Peterkin et al., 2003). Yet, the initial signals inducing *Nkx2.5* expression at the anterior-lateral mesoderm in *Xenopus* are less understood. While we have some knowledge of the extracellular ligands participating in cardiac *Nkx2.5* expression, we lack knowledge of the intracellular events.

In the present study, we asked what are the extra- and intracellular signals initiating the expression of *Nkx2.5*. We identified the intracellular signaling pathway of p38 MAPK phosphorylating the CREB transcription factor that together function to initiate the expression of *Nkx2.5* and later cardiac fate. This intracellular pathway is activated by FGF signaling that is expressed in anterior neural ectoderm of gastrulating embryos. We present evidence that anterior neural ectoderm induces the initial expression of *Nkx2.5* in neighboring lateral tissues.

Materials and methods

Embryonic manipulation

Ovulation, *in vitro* fertilization, embryo culture and dissections were carried out as described (Re'em-Kalma et al., 1995). Embryos were staged according to (Nieuwkoop and Faber, 1967). Embryos were taken at different stages and analyzed for immunohistochemistry and whole-mount *in situ* hybridization assays. Ventral marginal zone (VMZ) and dorsal marginal zone (DMZ, 60° in size) explants were removed at stages 10–10.25 and cultured to different stages as described (Bonstein et al., 1998). Animal cap explants were removed at stages 8–9 and cultured to different stages as described. In some experiments, explants were treated at different stages with LiCl (120 mM), bFGF (purified from bacteria, 150 ng\ml; Henig et al., 1998), the p38 inhibitor SB 203580 (30 μM), the FGF inhibitor SU 5402 (30 μM) and the ERK MAPK inhibitor U0126 (30 μM).

Microinjections

Capped synthetic RNA of activated MKK6 (MKK6E) (0.1 ng), inactive MKK6 (MKK6A) (0.1 ng), VP16-CREB (0.15 ng), Alk4 (25 pg), DN FGFR (XFD) (0.05 ng), FGF8 (0.1 ng), DN BMPR (0.05 ng) and two antisense morpholino oligonucleotides (AMO) were from Gene Tools, CREB (5′CACTGCCTCCTCTCCGTCACATTGG3′),

Xp38α (5′GACGTAAGATTGATTGGATGACATA3′) were injected into the marginal zone of one- or two-cell stage embryos in 0.3× MR containing 3% Ficoll. In some experiments, capped RNA (0.1 ng) encoding nuc-βgal was injected along with the MKK6E or VP16-CREB into the marginal zone of two-cell stage embryos later stained with x-Gal in order to identify the injected side of the embryo.

Western blot analysis

Protein extracts were prepared as described (Zetser et al., 2001). Equal amounts of extracted proteins were loaded (approximately 40 μg), separated by SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose filters by standard methods. Primary antibodies to CREB, phospho-CREB, ERK, phospho-ERK (Cell Signaling) and α-tubulin (Sigma) were used. Secondary HRP-conjugated antibody was detected using the Pierce SuperSignal chemiluminescence detection system.

In situ hybridization (ISH) and immunohistochemistry

ISH was carried out with digoxigenin-labeled probes (Hemmati-Brivanlou et al., 1990; Harland, 1991; Zetser et al., 2001) Embryos were cultured to different stages and subsequently fixed for *in situ* hybridization. *XNkx2.5*, *XGATA4*, *Xmhcα* and *FGF3* specific probes were used. Whole-mount immunostaining was performed as described (Brivanlou and Harland, 1989). ISH on slides was preformed as was described before (Butler et al., 2001). For immunohistochemistry on sections, slides were deparaffinized and hydrated with a decreased alcohol gradient. Then sections were blocked with goat serum for 1 h following 12-h incubation with the primary antibody (Troponin I). Slides were incubated with a secondary rhodamine-conjugated antibody, counterstained with TO-PRO-3 and were visualized by confocal microscopy. Alternatively, deparaffinized paraffin sections were reacted for 14 h at 4 °C with antibody to pCREB (1:40). This was followed by incubation with an appropriate biotinylated secondary antibody, streptavidin–peroxidase conjugate and *S*-(2-aminoethyl)-L-cysteine (AEC) as a substrate (Histostain-SP kit; Zymed Laboratory, San Francisco, CA, USA); counterstaining was done with hematoxylin. Immunostaining of whole embryos (St.16) with anti phospho-CREB antibody was performed; prior to antibody incubation, embryos were incubated with H_2O_2:methanol (1:10) to inactivate endogenous peroxidase. Bound antibody was detected with horseradish–peroxidase (HRP)-conjugated secondary antibody (goat anti-rabbit IgG;Pierce) diluted 1:100 and was visualized with HRP staining.

Histological procedure

Slides were deparaffinized and hydrated with a decreasing alcohol concentrations. Then slides were submitted to histological analysis by optical microscopy. The hematoxylin–eosin method was used to stain the tissue.

RT-PCR

RT-PCR was performed as described (Wilson and Melton, 1994), except that random hexamers (100 ng per reaction) were used for reverse transcription. Primers for *XHis4* (Gawantka et al., 1995), *MHCα*, *MLC2*, *GATA4*, *Nkx2.5*, *Myocardin* (Small et al., 2005), *FGF3*, *FGF4*, *FGF8*, *FGFR1*, *FGFR3* and *FGFR4a* (Monsoro-Burq et al., 2003) were described.

Results

Knockdown of p38α prevents the formation of the heart

In previous studies, we analyzed the role of p38 MAPK in the Spemann organizer (Keren et al., 2008) and in skeletal myogenesis (Keren et al., 2005). One effect of morpholino-mediated p38α knockdown noticed in these studies was the absence of beating hearts in tadpoles. To further investigate this phenomenon, morpholino-injected embryos as well as control uninjected embryos were transverse sectioned at the heart plane (Fig. 1A). The heart morphology with its distinct tissues observed in control embryo is completely lost in the knockdown embryo. Instead, an unorganized group of cells is apparent. Interestingly, the foregut wall appears relatively unaffected in the knockdown embryo compared to the control embryo. Co-injection of transcripts encoding mouse p38 together with p38α AMO partially rescued the heart tube morphology (Fig. 1A). Troponin I immunostaining of embryos is indicative of a complete absence of myocardium in the knockdown embryo (Fig. 1B). Similarly, a significant reduction in the expression of cardiac isoform of myosin heavy chain (MHCα) by *in situ* hybridization and semi-quantitative RT-PCR analyses is observed (Fig. 1C). Together, these

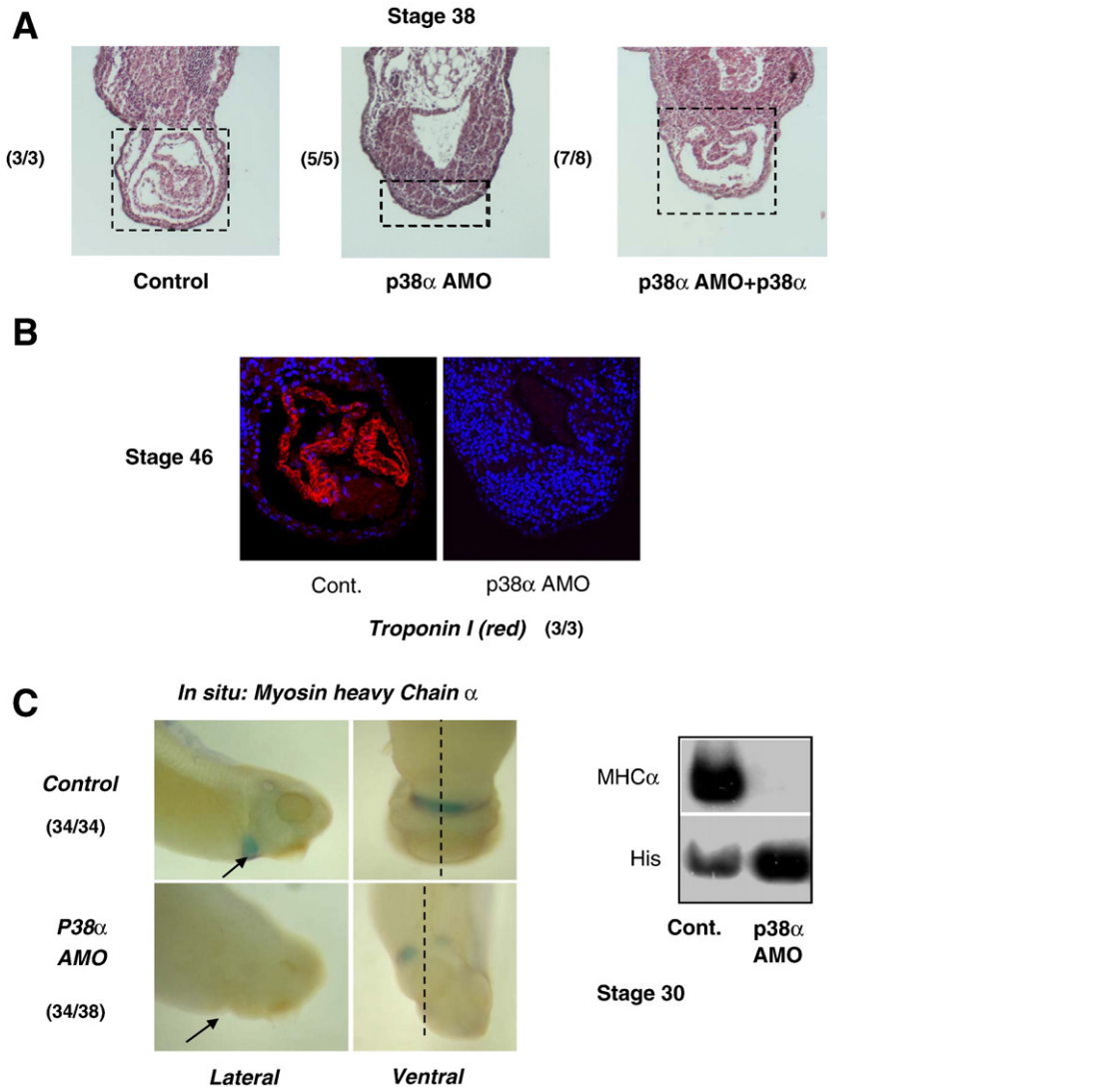

Fig. 1. Knockdown of p38α prevents heart development. (A) Anterior transverse sections of stage 38 control embryos with ventral heart structures, embryos injected with p38α AMO missing heart structures and embryos injected with p38α AMO and a transcript encoding mouse p38α with some heart structures. Sections were hematoxylin–eosin stained. (B) Anterior transverse sections of stage 46 control embryos or of embryos injected with p38α AMO. Sections were immunostained with antibodies to Troponin I (red) and counterstained with TO-PRO-3 (nuclear, blue). (C) Left panel: *In situ* hybridization staining of stage 30 embryos with a probe detecting myosin heavy chain α. At the two-cell stage, one blastomer was injected with p38α AMO ($n=38$). Control group ($n=34$) was not injected. Right panel: Embryos were injected with p38α AMO, DMZ explants ($n=18$) were isolated and grown to stage 30. Total RNA was isolated and RT-PCR analysis was performed.

data support the involvement of p38 MAPK in heart development. It is important to note that the effect of p38-knockdown was not specific only to the heart formation, since we could detect mild defects in the anterior structures of the eyes and brain (not shown). This is not surprising since p38 MAPK affects also embryo patterning at an earlier developmental stage (Keren et al., 2008). We relate to the specificity of p38 hereafter (see Supplementary Fig. 4).

The expression of Nkx2.5 is affected by the p38 MAPK pathway

Two families of transcription factors, GATA and Nkx, are among the first to be expressed in heart progenitors and function at different stages of heart development (reviewed in Latif et al., 2006). Next, we analyzed how the p38 pathway affected the expression of two prototypic members of these families, *GATA4* and *Nkx2.5* (Fig. 2). Similar results to those presented below were obtained with other members, *GATA5* and *Nkx2.3* (not shown). Injection of p38α morpholino into one of two blastomere embryos abolished the expression of *Nkx2.5* without affecting *GATA4* expression at the injected side of stage 13 embryos (Fig. 2A, upper panel). At this stage, *Nkx2.5* and *GATA4* are co-expressed as bilateral anterior patches. At later tailbud stage, knockdown of p38α prevented *Nkx2.5* but did not affect *GATA4* expression at the ventral heart field (Fig. 2A, lower panel). The effect of p38α knockdown on the expression of *GATA4* and *Nkx2.5* was further studied in several explant assays (Fig. 2B). In one, DMZ explants of native cardiac progenitors were isolated at stage 10.25 (Sater and Jacobson, 1990). In another, pluripotent animal cap explants from Alk4 (Activin type I receptor)-injected embryos were isolated at stage 8 (Logan and Mohun, 1993). Activin signaling induces different mesodermal fates including cardiac gene expression in isolated animal caps (Ariizumi et al., 1991; Gurdon et al., 1994). In another, VMZ explants were isolated at 10.25 from LiCl-treated embryos (Slack et al., 1988). In all cases, injection of p38α morpholino reduced *Nkx2.5* expression without affecting the expression of *GATA4* (Fig. 2B). The specificity of p38α morpholino was investigated by the co-injection transcripts encoding a non-targetable mouse p38α (Supplementary Fig. 1). Expression of mouse p38α rescued the expression of *Nkx2.5* in p38α morpholino-treated embryos. A second approach was used to reduce p38 activity. An inactive mutant of MKK6 (MKK6A) was injected to embryos. Its expression significantly

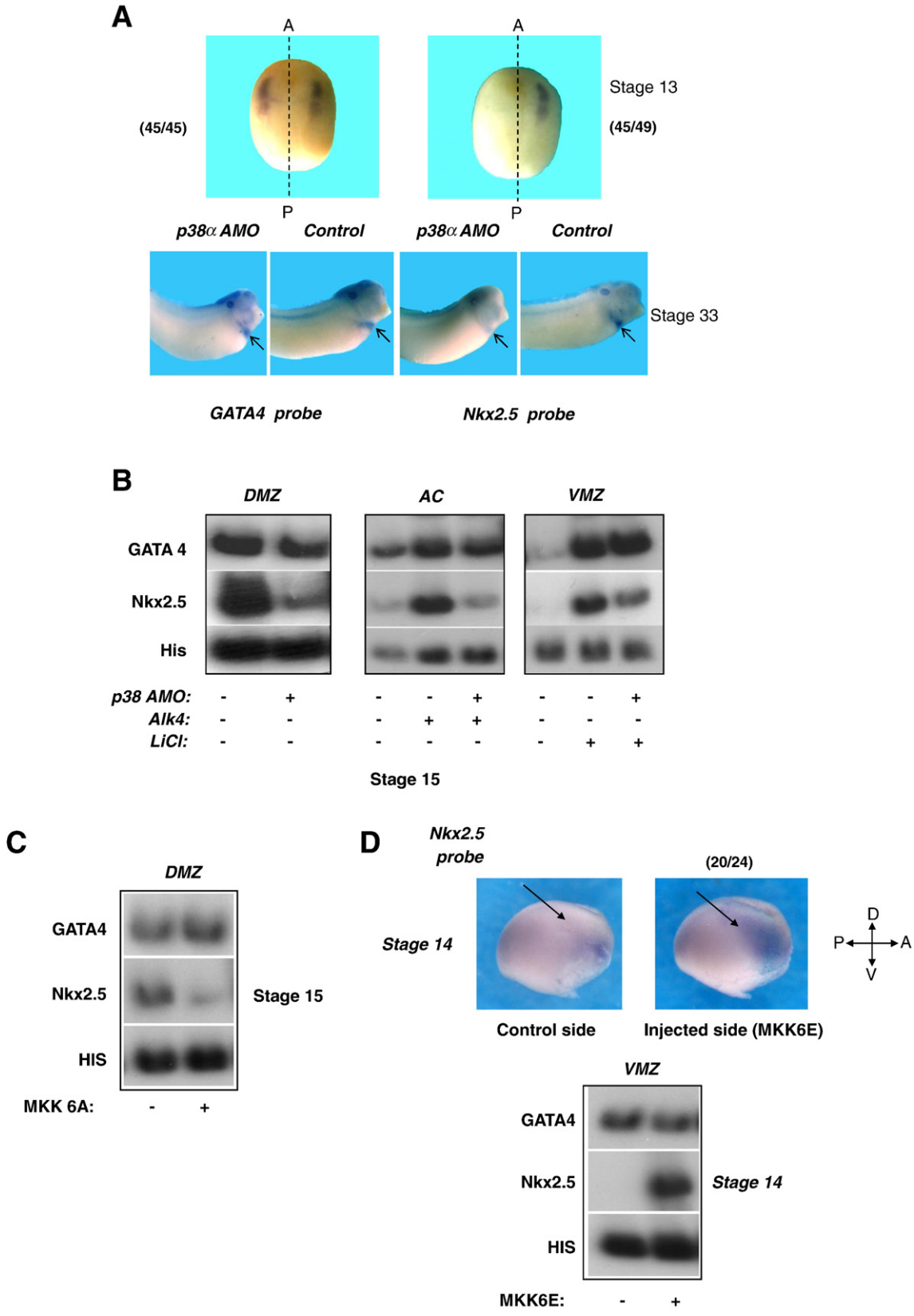

Fig. 2. The p38 MAPK pathway is involved in the early expression of *Nkx2.5*. (A) One of two blastomeres (upper panel) or two of two blastomeres (lower panel) were injected with p38α AMO, and the expression of *Nkx2.5* and *GATA4* was detected by *in situ* hybridization of stage 13 (top) and stage 33 (bottom) embryos. (B) p38α AMO was injected to embryos, and gene expression was assessed in three cardiac explant assays; DMZ explants (left), AC explants from Alk4-expressing embryos (middle) and VMZ explants from LiCl-treated embryos (right). At stage 15, total RNA was isolated and RT-PCR analysis was performed. (C) Transcripts encoding inactive MKK6 (MKK6A) were injected to one cell embryos, and DMZ explants ($n = 18$) were isolated and were grown to stage 15. Total RNA was isolated and RT-PCR analysis was performed. (D) Upper panel: Transcripts encoding MKK6E were injected to one of two blastomere embryos ($n = 24$). Embryos were allowed to develop to stage 14 and *in situ* hybridization was performed with a probe to *Nkx2.5*. The injected side was recognized by the staining of a β-Gal lineage tracer. Lower panel: Transcripts encoding MKK6E were injected to one cell embryos, VMZ explants ($n = 18$) were isolated and were grown to stage 14. Total RNA was isolated and RT-PCR analysis was performed.

reduced *Nkx2.5* but not *GATA4* in DMZ explants (Fig. 2C). Therefore, loss of p38 expression or activity reduces Nkx2.5 expression.

To induce p38 activity, transcripts encoding constitutive active mutant of MKK6 (MKK6E) were injected to embryos. In one experiment, transcripts were injected to one of two blastomere embryos and *Nkx2.5* expression was analyzed by *in situ* hybridization (Fig. 2D, upper panel). Expanded expression domain of *Nkx2.5* was observed in the injected side of embryos relative to the uninjected side. In another experiment, the expression of *Nkx2.5* was induced in VMZ explants isolated from MKK6E-injected embryos (Fig. 2D, lower panel). Basal level of *GATA4* expression in VMZ explants was not further induced by ectopic MKK6E. Together, these results indicate that the p38 MAPK pathway affect the early expression of *Nkx2.5* without affecting the expression of *GATA4*.

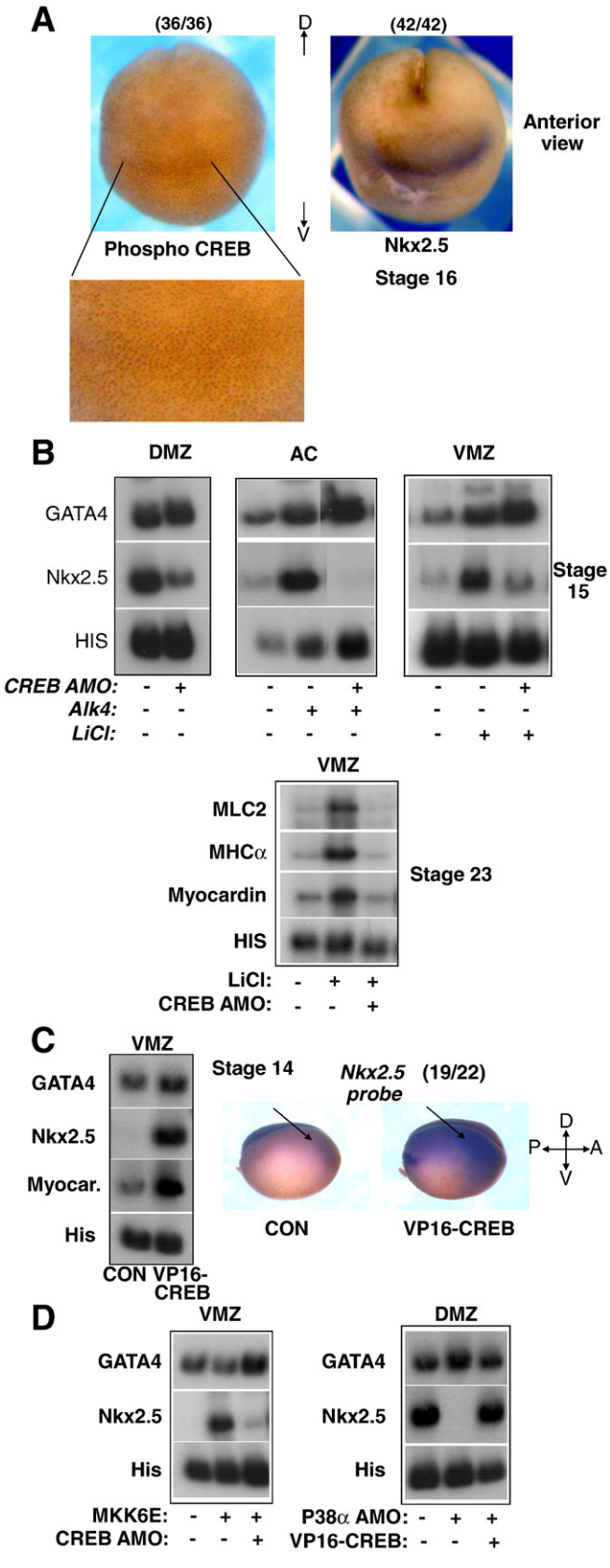

Fig. 3. The CREB protein is involved in the expression of *Nkx2.5* downstream of p38 MAPK. (A) Stage 16 embryos were immunostained using an antibody to phospho-CREB (S133) (left) or analyzed by *in situ* hybridization with a probe to *Nkx2.5* (right). (B) CREB AMO was injected to embryos, and gene expression was assessed in three cardiac explant assays; DMZ explants (left), AC explants from Alk4-expressing embryos (middle) and VMZ explants from LiCl-treated embryos (right). At stage 15 or stage 23, total RNA was isolated and RT-PCR analysis was performed. (C) Transcripts encoding VP16-CREB were injected to one of two blastomeres (right panel) or to two of two blastomeres (left panel). The expression of *Nkx2.5* was detected by *in situ* hybridization (right panel) or by RT-PCR analysis on VMZ explants ($n=18$) (left panel). (D) Different combinations of transcripts encoding activated MKK6 and CREB AMO were injected to embryos and VMZ explants were isolated and grown to stage 14 (left panel). Different combinations of transcripts encoding VP16-CREB and p38α AMO were injected to embryos and DMZ explants were isolated and grown to stage 14 (right panel). Total RNA was isolated and RT-PCR analysis was performed.

The CREB transcription factor functions downstream of p38 MAPK and affects cardiac expression

In our previous study, CREB was identified as a target of p38 MAPK at the Spemann organizer (Keren et al., 2008). Phosphorylation of CREB at serine 133 may therefore serve as an indicator for the activity of p38 MAPK. Next, we analyzed the role of CREB in cardiac gene expression. First, CREB phosphorylation at serine 133 was analyzed by immunostaining of stage 16 embryos (Fig. 3A). Phospho-CREB is detected by a typical spotted-nuclear staining. It was scattered in many regions of embryos, mostly in the epidermis (A.K. unpublished results), but its staining was intensified in a furrow-like structure at the anterior ventral part of the embryo. *In situ* staining of stage 16 embryos detected similar pattern of *Nkx2.5* expression (Fig. 3A). Thus, expression of phospho-CREB appears to overlap with the expression of *Nkx2.5* at this particular stage and also at an earlier stage (see Fig. 4A). To determine possible involvement of CREB in *Nkx2.5* expression, CREB AMO was injected to embryos and several explants assays were used to assess the expression of cardiac *Nkx2.5* (Fig. 3B). In all three explant assays, knockdown of CREB significantly reduced *Nkx2.5* expression while did not affect and even induced the expression of *GATA4*. Reduced expression of *Nkx2.5* was correlated with later loss of cardiac gene expression since CREB knockdown inhibited late expression of cardiac differentiation markers (Fig. 3B, lower panel). Next, we analyzed whether CREB activity was sufficient to induce *Nkx2.5* expression in VMZ explants (Fig. 3C). Transcripts encoding activated form of CREB (VP16-CREB) were injected to embryos and the expression of several cardiac genes was analyzed in VMZ explants. Expression levels of *Nkx2.5* and *Myocardin* were induced while that of *GATA4* was not affected (Fig. 3C, left panel). In a similar experiment, VP16-CREB was expressed in half embryos and *Nkx2.5* was identified by *in situ* hybridization (Fig. 3C, right panel). The expression domain of *Nkx2.5* was expanded in the injected side relative to the uninjected side of the embryo. These results indicate that CREB is sufficient to induce *Nkx2.5* expression. To determine whether p38 and CREB share the same pathway, the following experiments were performed (Fig. 3D): In the first, ectopic expression of MKK6E induced *Nkx2.5* in VMZ explants. Simultaneous knockdown of CREB prevented *Nkx2.5* expression while augmented *GATA4* expression (left panel). In the second, knockdown of p38α inhibited *Nkx2.5* expression in DMZ explants and simultaneous expression of VP16-CREB rescued the expression of

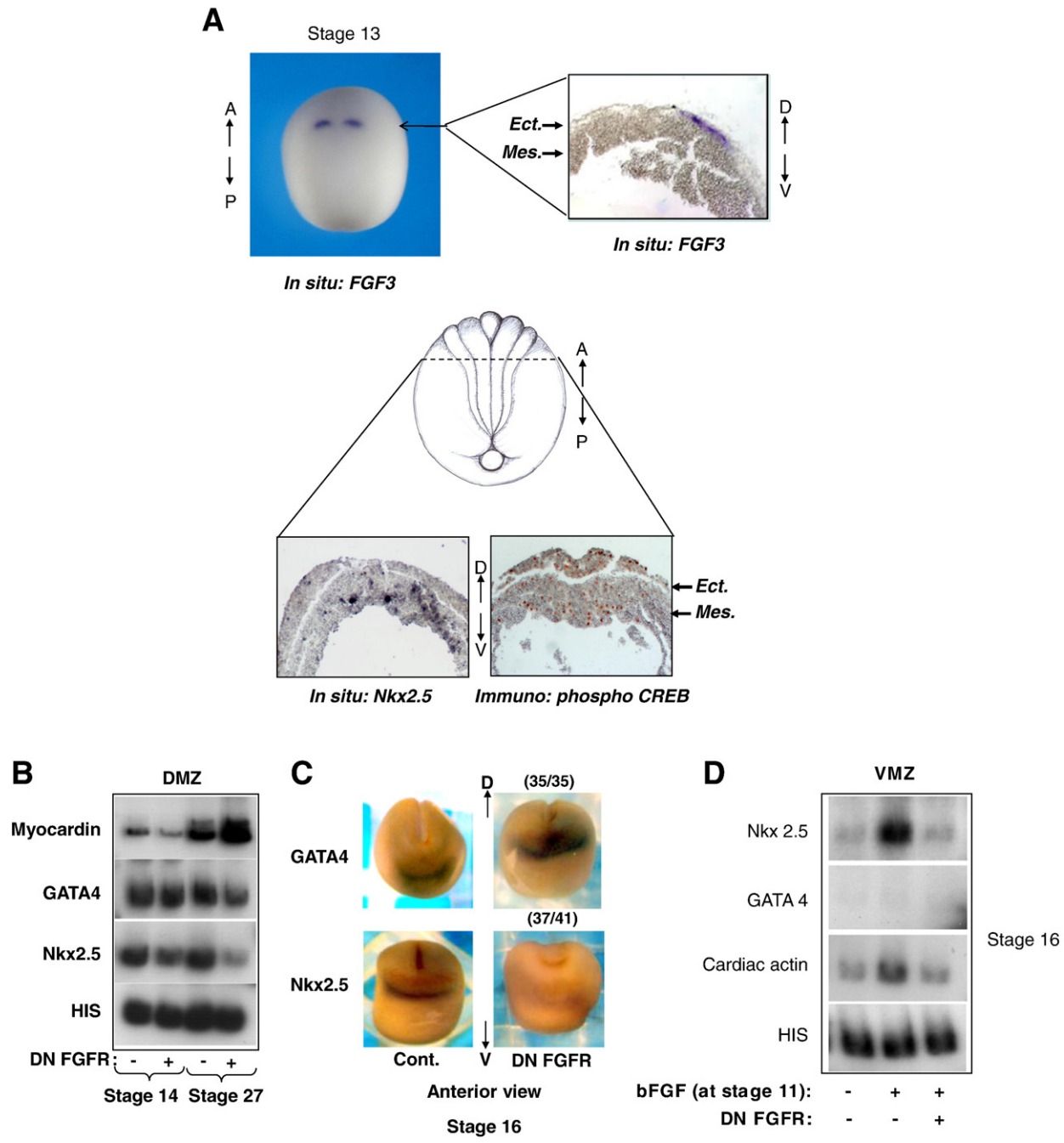

Fig. 4. FGF induces *Nkx2.5* gene expression without affecting *GATA4* gene expression. (A) Expression of FGF3 was detected on stage 13 embryos by whole-mount *in situ* hybridization ($n=20$) (left). Stained embryos were transversely sectioned at the stained plane. One section is presented (right). Anterior transverse sections in stage 13 embryos followed by *in situ* staining with a probe to *Nkx2.5*. *Nkx2.5* is expressed in lateral mesoderm of anterior section (bottom left). Adjacent sections immunostained with antibodies to phospho-CREB (S133). Red dots represent nuclear phospho-CREB staining. The embryo scheme indicates the plane section. (B) Transcripts encoding truncated FGF receptor (DN FGFR) were injected to embryos and DMZ explants ($n=36$) were isolated and grown to stages 14 and 27. Total RNA was extracted and RT-PCR analysis was performed. (C) Transcripts encoding DN FGFR were injected to one cell embryos and the expression of *GATA4* and *Nkx2.5* was analyzed by *in situ* hybridization of stage 16 embryos. (D) VMZ explants were dissected from embryos injected with transcripts encoding DN FGFR or from control embryos. At stage 11, basic FGF (150 ng/ml) was added and explants were grown to stage 16. Total RNA was extracted and RT-PCR analysis was performed.

Nkx2.5 (right panel). Together these data support p38α and CREB functioning in the same pathway in which p38α is upstream of CREB.

FGF signaling is involved in initiating Nkx2.5 expression

Next, we asked which of the secreted ligands involved in cardiogenesis may affect the intracellular pathway initiating *Nkx2.5* expression. Since FGF signaling was implicated in the initiation of cardiac gene expression both is *zebrafish* (Reifers et al., 2000) and in *chick* (Alsan and Schultheiss, 2002), we investigated its involvement in initiating *Xenopus Nkx2.5* expression. To establish possible involvement of FGF, the expression of *FGF3* was analyzed by whole-mount *in situ* of stage 13 embryos (Fig. 4A, left panel). FGF3 expression appears as two symmetrical stripes at the anterior part of the embryo. Transverse sections of the same embryos indicate that the expression is specific to neural ectoderm of the future mid-hind brain (Fig. 4A, right panel) (Tannahill et al., 1992). Ectoderm expression of *FGF3* was approximate to the expression zone of

Nkx2.5 that was detected mostly in the mesoderm and endoderm of adjacent sections and to phosphorylated CREB in dorsal and lateral mesoderm and endoderm (Fig. 4A, lower panel). Expression of *FGF3* and possibly other FGF ligands (Lea et al., 2009) at the immediate vicinity of *Nkx2.5* expression may reflect functional relationships between these molecules. To elucidate a possible role of FGF signaling in the initiation of *Nkx2.5* expression, FGF signaling activity was inhibited by the expression of truncated receptor of FGF (XFD) (Amaya et al., 1993). Since XFD affects early events of mesoderm formation and gastrulation movements, it was injected at low levels to allow development of embryos to neurulation. Expression of early cardiac markers was analyzed by RT-PCR (Fig. 4B) and *in situ* hybridization (Fig. 4C). The levels of *Nkx2.5* were reduced while those of *GATA4* were barely affected (Fig. 4B). The levels of *Myocardin* were reduced at early stage while induced at late-stage embryos. Inhibition of *Nkx2.5* expression was better detected at stage 16 embryos analyzed by *in situ* hybridization (Fig. 4C). The expression of *GATA4* was not affected. The role of FGF in *Nkx2.5* expression was further investigated; stage 11 VMZ explants were incubated with basic FGF (FGF2) (150 ng/ml), and the expression of pre- cardiac markers was analyze by RT-PCR (Fig. 4D). Basic FGF induced the expression of *Nkx2.5* and *cardiac actin* without affecting *GATA4* gene expression. *Nkx2.5* was not induced by bFGF in explants expressing a truncated FGF receptor. The activity of bFGF was confirmed by its ability to induce the phosphorylation of ERK MAPK (Supplementary Fig. 2). Therefore, introduction of FGF at the proper developmental time (stage 11) induces *Nkx2.5* expression in ventral mesoderm.

Anterior neural ectoderm induces Nkx2.5 expression in cardiac progenitors

Next, the possible involvement of anterior neural ectoderm in the induction of *Nkx2.5* expression was investigated. Antagonism of BMP transforms ectodermal AC explants into anterior neural ectoderm (Lamb et al., 1993). Hence, transcripts encoding truncated BMP receptor (tBR) were injected to embryos and AC explants were isolated. Expression of anterior neural markers (*Otx2, Krox20*) was induced in these explants (data not shown). Interestingly, BMP antagonism induced the expression of *FGF3* and *FGF8*, as expected for an acquired non-autonomous activity of this tissue (Fig. 5A). Moreover, expression of *Nkx2.5* but not of *GATA4* was induced in these explants. This raised the possibility that an autocrine FGF signal was established and induced the expression of *Nkx2.5*. For that reason, the expression of several FGF receptors was analyzed. *FGFR1* was expressed in control AC explants while the expression of *FGFR3* and *FGFR4a* was significantly induced in neuralized explants (Fig. 5A). Co-injection of truncated FGF receptor (XFD) reduced expression of *Nkx2.5*. Mesoderm markers were not identified in these explants (not shown). Therefore, induction of anterior neural ectoderm in AC explants generates FGF signaling and *Nkx2.5* expression. In addition,

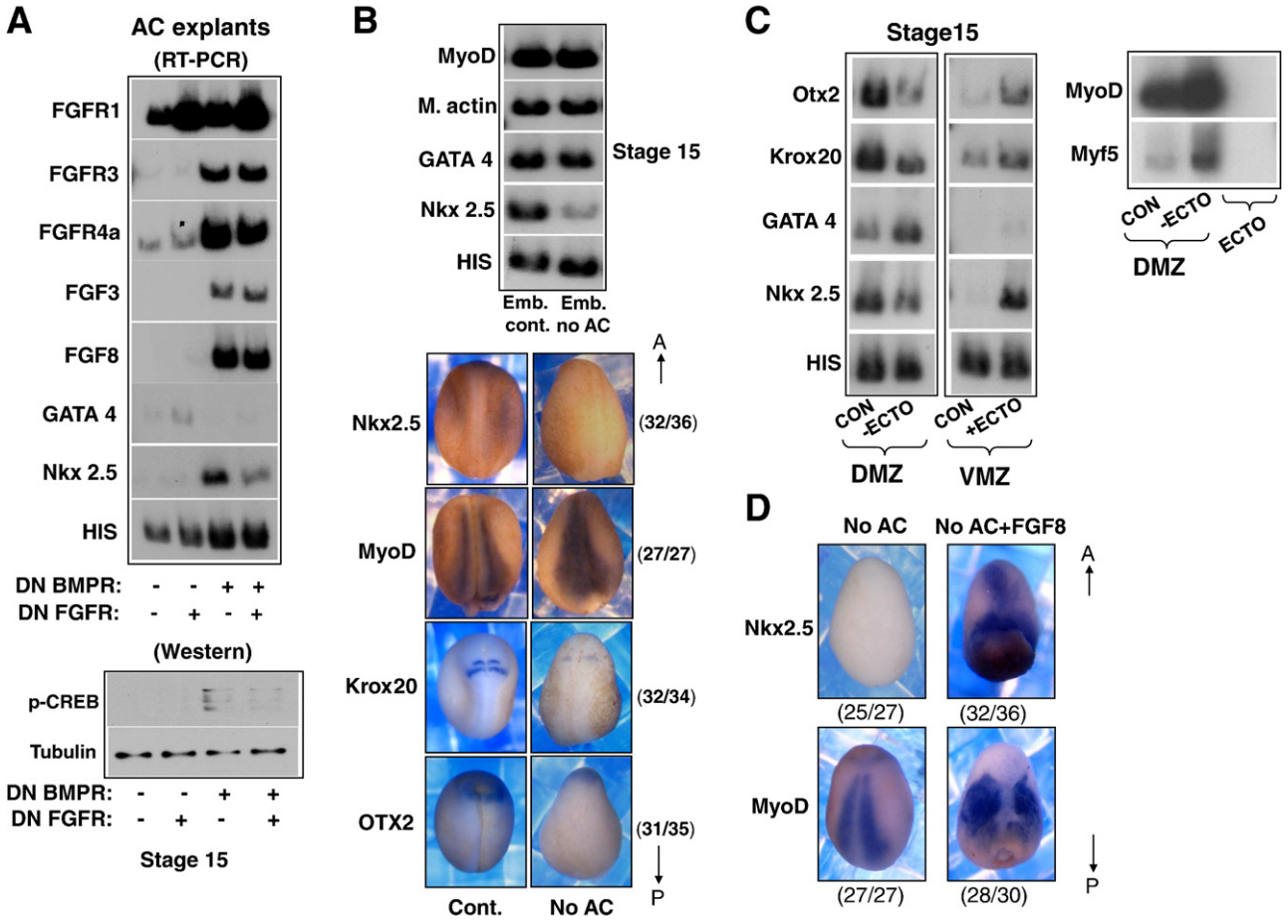

Fig. 5. Anterior neural ectoderm is necessary to induce mesodermal *Nkx2.5* expression. (A) Transcripts encoding truncated BMP receptor were injected and AC explants ($n=36$) were isolated and grown to stage 15. Half of the explants were used to extract RNA for RT-PCR analysis (upper) and the other half to extract proteins for Western analysis (lower). (B) Animal caps were removed at stages 8–9 from whole embryos. "Mildly affected" embryos were allowed to grow to stage 15, and whole embryos were extracted and subjected to RT-PCR analysis of their RNA (upper) or to *in situ* hybridization with probes to *Nkx2.5*, *MyoD*, *OTX2* and *Krox20* (lower). (C) DMZ and VMZ explants were dissected at stage 10.25. Ectoderm was removed from some of the DMZ explants and was recombined with VMZ explants (pairs of VMZ: dorsal ectoderm) or grown alone (ecto.). At stage 15, total RNA was extracted and RT-PCR analysis was performed. (D) Several embryos were injected with mRNA encoding FGF8, and animal caps were removed at stages 8–9. Embryos were allowed to grow to stage 15 and *in situ* hybridization with probes to *Nkx2.5* and *MyoD* was performed.

BMP antagonism induced the phosphorylation of CREB and co-injection of truncated FGF receptor reduced its level (Fig. 5A, lower). The several bands detected with anti phospho-CREB antibody may represent alternative splice products and/or close family members like ATF1 (Mayr and Montminy, 2001). Next, we investigated the effect of ectoderm ablation on *Nkx2.5* expression in whole embryos. Animal caps were surgically removed (stages 8–9), and embryos were developed to stage 15 (Fig. 5B). Embryos lacking ectoderm failed to express *Nkx2.5*, but not *GATA4* (see RT-PCR and *in situ* hybridization results). The expression of paraxial mesoderm marker *MyoD* was not affected indicating that early mesoderm development was not affected while the expression of anterior neural ectoderm markers Krox20 and Otx2 was significantly reduced confirming that ectoderm was considerably removed (*in situ* results). Therefore, ectoderm is necessary for the early expression of cardiac *Nkx2.5* but is not required for early paraxial mesoderm gene expression (*MyoD*). Next, we established a microsurgical technique to selectively remove ectoderm from DMZ explants (stage 10). The removal of ectoderm was validated by the reduced expression of anterior neural markers (*Otx2, Krox20*) normally expressed in DMZ explants. The removed ectoderm was conjugated to VMZ explants of the same embryos, and explants were cultured to stage 15 (Fig. 5C). Removal of ectoderm from DMZ explants reduced the expression of *Nkx2.5* while induced the expression of *GATA4*. The expression of other lateral mesoderm genes, such as *Myf5* and *MyoD*, was even induced in the absence of ectoderm, excluding a possible accidental removal of mesoderm in this procedure (Fig. 5C, right panel). On the other hand, the expression of *Nkx2.5* was induced while that of *GATA4* was not induced in recombinant VMZ/dorsal ectoderm explants. In a control experiment, the expression of mesoderm markers, MyoD and Myf5, was not detected in isolated ectoderm explants. These data suggest that neural ectoderm is the inducer *Nkx2.5* expression in mesodermal explants. Next, we analyzed whether ectopic expression of FGF could rescue *Nkx2.5* expression in AC-deficient embryos (Fig. 5D). As shown in Fig. 5B, surgical removal of AC from embryos prevented *Nkx2.5* expression. However, injection of mRNA encoding FGF8 to embryos that later underwent the same microsurgical procedure induced significant expression of *Nkx2.5* throughout the dorsal axis and posterior region. Ectopic expression of FGF also expanded the lateral staining of MyoD. Therefore, ectopic FGF expression induced dorsal expression of *Nkx2.5* in whole embryos missing ectodermal structures.

FGF affects Nkx2.5 expression via p38 MAPK and not ERK MAPK

To investigate the relationship between FGF and p38 MAPK/CREB signaling in the expression of *Nkx2.5*, DMZ explants were incubated with pharmacological inhibitors of several signaling molecules (Fig. 6).

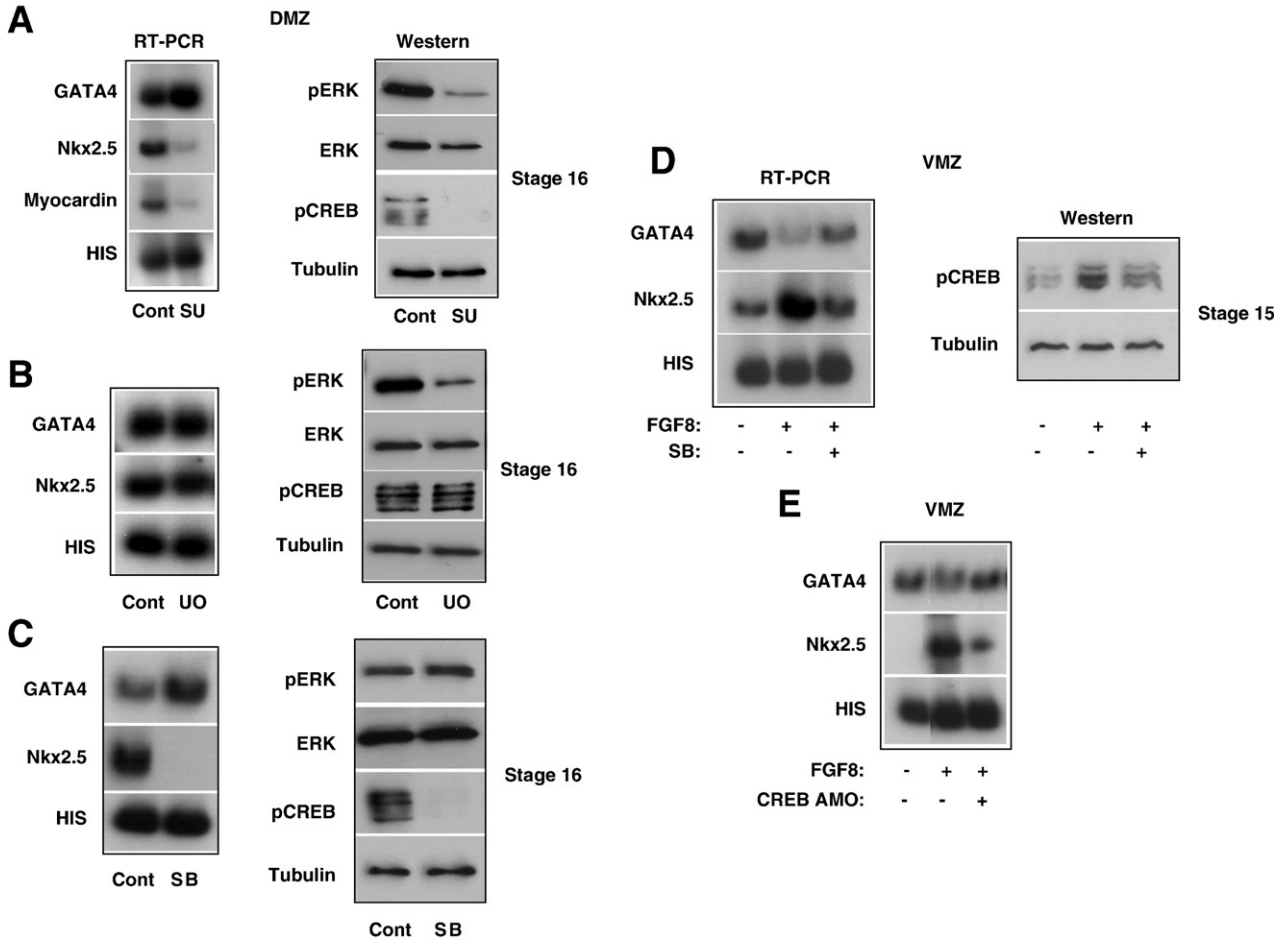

Fig. 6. Induction of *Nkx2.5* by FGF is mediated by p38 MAPK and not by ERK MAPK. (A) DMZ explants ($n=36$) were dissected and at stage 11 were cultured in SU 5402 (30 μM) and allowed to develop to stage 16. Half of the explants were used to extract RNA for RT-PCR analysis (left) and the rest were used to extract proteins for Western analysis (right). (B) DMZ explants were cultured and processed as above, except their incubation in U0126 (30 μM). (C) DMZ explants were cultured and processed as above, except their incubation in SB203580 (30 μM). (D) Transcripts encoding FGF8 were injected and VMZ explants ($n=36$) were dissected and gown from stages 11 to 15 in the absence or presence of SB203580 (30 μM). Half of the explants were used to extract RNA for RT-PCR analysis (left) and the rest were used to extract proteins for Western analysis (right). (E) Different combinations of transcripts encoding FGF8 and CREB AMO were injected to embryos, and VMZ explants were dissected at stage 10+. RNA was extracted at stage 15 and RT-PCR analysis was performed.

To inhibit FGF signaling, SU 5402, an inhibitor of FGF receptor tyrosine kinase activity was added at stage 11 to DMZ explants (Mohammadi et al., 1997). Incubation of DMZ explants with the inhibitor reduced the expression of *Nkx2.5* and *Myocardin* without affecting *GATA4* (Fig. 6A). This result links FGF signaling to the initiation of *Nkx2.5* expression by bypassing its earlier effects on mesoderm specification. Expectedly, SU 5402 inhibited phosphorylation of ERK MAPK, a known intracellular target of FGF (Fig. 6A). It also prevented the phosphorylation of CREB (phospho-S133), indicating that CREB was phosphorylated in response to FGF signaling. Similar results were observed with Alk4-expressing AC explants (Supplementary Fig. 3). Addition of SU 5402 prevented CREB phosphorylation and the induced expression of *Nkx2.5* but not of *GATA4*. The ERK MAPK pathway was considered before as the major effector of FGF signaling in *Xenopus* development (reviewed in Tsang and Dawid, 2004). To inhibit the ERK MAPK pathway, U0126 (MEK inhibitor) was added to DMZ explants (Fig. 6B). Phosphorylation of ERK was largely prevented in the presence of U0126, confirming its activity. Interestingly, the inhibitor failed to affect the phosphorylation of CREB, relative to its phosphorylation in control DMZ explants. Therefore, ERK MAPK is likely not the kinase phosphorylating CREB. Furthermore, the expression of *Nkx2.5* was not affected by U0126 (Fig. 6B). Thus, ERK MAPK is not the intracellular pathway downstream of FGF affecting *Nkx2.5* expression. Since our previous studies (Keren et al., 2005, 2008) and results presented here (Fig. 3D) indicate that CREB is affected by the p38 MAPK pathway, we incubated DMZ explants in the presence of SB 203580, an inhibitor of p38α and β (Fig. 6C). Expression of *Nkx2.5* was abolished (left panel) and CREB phosphorylation was prevented (right panel) in the presence of SB 203580. Together, these results illuminate the roles of FGF and p38 MAPK signaling in the initiation of *Nkx2.5* expression while excluding a possible role of ERK MAPK in the process. Since p38 knockdown may affect some anterior structures other than the heart (see Fig. 1), we took advantage of the explant assay to investigate if inhibition of FGF or p38 affected anterior neural expression. Treatment of DMZ explants in SU 5402 or SB 203580 did not affect the expression of anterior markers such as Otx2 and Krox20 and XAG1 suggesting that inhibition of *Nkx2.5* by FGF or p38 inhibitors in this assay was independent of possible anterior neural defects (Supplementary Fig. 4).

To further examine the relations of FGF and the intracellular p38/CREB pathway, transcripts encoding FGF8 were injected to embryos and the expression of cardiac markers was analyzed in VMZ explants (Figs. 6D, E). The activity of injected FGF8 was confirmed by the induction of ERK MAPK phosphorylation (Supplementary Fig. 2). FGF8 induced the expression of *Nkx2.5* in VMZ explants and reduced *GATA4* expression in those explants. Additional treatment of explants with p38 MAPK inhibitor (SB) at stage 10.25 reduced *Nkx2.5* and induced *GATA4* expression, restoring their levels in control VMZ explants (Fig. 6D). Similarly, co-injection of CREB morpholino with transcripts encoding FGF8 prevented the expression of *Nkx2.5* that was induced by FGF8 alone (Fig. 6E). We conclude that p38 and CREB activities are necessary for the induction of *Nkx2.5* expression by FGF signaling.

Discussion

The anterior bilateral expression of *Nkx2.5* in gastrulating embryos is correlated with cardiac competence of these cells. Therefore, knowledge of the signals initiating *Nkx2.5* expression can shed light at the earliest events specifying cardiac fate.

Intracellular signaling pathway initiating Nkx2.5 expression in cardiac progenitors

Previous studies focused at the extracellular ligands involved in cardiac expression. The participation of FGF and BMP signals in *Nkx2.5* expression at different cardiac developmental stages was demonstrated in deferent vertebrate model systems (Schultheiss et al., 1997; Barron et al., 2000; Reifers et al., 2000; Walters et al., 2001; Abu-Issa et al., 2002; Alsan and Schultheiss, 2002). Still, the intracellular mediators remain relatively unexplored. We demonstrate the involvement of p38 MAPK and the CREB transcription factor, sharing the same pathway regulating the initial expression of *Nkx2.5*. The involvement of this pathway in the expression of *Nkx2.5* is demonstrated by loss and gain of function experiments. Loss of function was achieved by knocking down p38α (Keren et al., 2005) and CREB (Keren et al., 2008) using antisense morpholino oligonucleotides, the expression of inactive variant of MKK6 and a pharmacological inhibitor of p38 MAPK. Gain of function was achieved by expression of constitutive active variants of MKK6 (the activating kinase of p38 MAPK) and VP16-CREB. Using different whole embryo and explant assays, we find that modulation of these proteins affect the expression of *Nkx2.5* but not of *GATA4*. The finding that *GATA4* expression is not affected by these proteins emphasizes the specificity of this pathway. *GATA4* expression is initiated at blastula in prospective mesoendoderm mostly at the dorsal side (Fletcher et al., 2006), while *Nkx2.5* expression is initiated at late gastrula (Tonissen et al., 1994; Sparrow et al., 2000) and its expression is confined to anterior-lateral mesoderm. At this stage, *GATA4* expression is at list partially overlapping with that of *Nkx2.5*. Therefore, it might not be surprising that different signaling pathways participate in the initiation of *Nkx2.5* and *GATA4* gene expression. Since p38/CREB function in mesoderm patterning (Keren et al., 2008), it was important to elucidate its independent function in the initiation of *Nkx2.5* expression. The results presented here indicate a patterning-independent function of this pathway in the induction of *Nkx2.5* expression. First, the observation of overlapping expression of phospho-CREB (active) and *Nkx2.5* in embryos at initial stages of *Nkx2.5* expression. Second, knockdown of p38 or CREB was performed with limited morpholino concentrations that did not affect early patterning in any significant manner and may therefore allow detect their effect on later developmental stages. Third, specific inhibition of *Nkx2.5* expression was observed in explants that were incubated with a pharmacological inhibitor of p38 (SB 203580) at the proper developmental stage (late gastrula). Regulation of *Nkx2.5* expression by CREB was suggested before (Sundaram et al., 2003). However, in that study, it was suggested that maternal but not zygotic CREB controlled initiation of *Nkx2.5* expression. We would like to suggest that the results in that study reflect earlier patterning defects than the results presented in our study. Future studies will prove whether CREB functions directly at the *Nkx2.5* promoter as it may happen in *drosophila* (Venkatesh et al., 2000).

FGF functions to initiate Nkx2.5 expression

Several signaling ligands were proposed to promote cardiac development in vertebrates. The signals affecting *Xenopus* cardiac development include antagonists of the canonical Wnt pathway (Marvin et al., 2001; Schneider and Mercola, 2001; David et al., 2008) and at the same time agonists of non-canonical Wnt pathway such as Wnt11 (Pandur et al., 2002; Garriock et al., 2005; Afouda et al., 2008). BMP signaling is required in *Xenopus* at several stages of cardiac development like cell migration, differentiation and heart looping, but is dispensable for the initial expression of cardiac regulators (Breckenridge et al., 2001; Walters et al., 2001; Peterkin et al., 2003). FGF signaling is required during the initiation of cardiac gene expression in *zebrafish* (Reifers et al., 2000) and *chick* (Lough et al., 1996; Alsan and Schultheiss, 2002). A recent *Xenopus* study that followed later-stage heart development (from stage 22 on) indicated a role of FGF signaling in cardiomyocyte survival and proliferation (Langdon et al., 2007). Interestingly, the role of FGF signaling in early cardiac development was not considered in *Xenopus*. We show for the first time that FGF signaling is necessary for the initiation of *Nkx2.5* expression. Expression of dominant-negative receptor of FGF prevents

Nkx2.5 and heart formation in whole embryos. The incubation of stage 11 DMZ explants with a pharmacological inhibitor of FGF receptor (SU 5402) prevented *Nkx2.5* expression, further illuminating that FGF signaling is necessary at the proper developmental stage of *Nkx2.5* initiation. Alternatively, incubation of stage 11 VMZ explants with bFGF induced *Nkx2.5* gene expression. These results dissociate the direct effect of FGF on cardiac progenitors from a possible general patterning of Spemann organizer's secreted ligands. BMP and Wnt antagonists of the Spemann organizer are necessary for the induction of cardiac progenitor cells, though their role in cardiac development cannot be separated from their function in patterning. The distinct expression of FGF3 in proximity and during the initiation of *Nkx2.5* expression indicates of a more direct role of this pathway. Interestingly, in *Xenopus* as in the *chick* (Alsan and Schultheiss, 2002), FGF selectively affected the expression of *Nkx2.5* and not the expression of *GATA4*.

It should be emphasized that FGF ligands other than FGF3 may be involved in *Nkx2.5* expression. A recent study that systematically investigated the expression of 17 FGF ligands in the developing *Xenopus tropicalis* reveals a complex expression pattern (Lea et al., 2009). For example, FGF8, like FGF3, is expressed at late gastrula at the mid-hindbrain boundary and may function redundantly with FGF3. Indeed, in our preliminary studies, morpholino-mediated knockdown of FGF3 did not affect *Nkx2.5* expression, indicating the involvement of additional FGF ligands (A.K.P., unpublished results). Moreover, our results indicate that different FGF ligands, bFGF (FGF2) and FGF8, induce the expression of *Nkx2.5*. Therefore, we should consider the involvement of more than one FGF ligand in the initiation of *Nkx2.5* expression.

Anterior neural ectoderm is the source of FGF signal initiating Nkx2.5 expression

The next question of interest is which tissue at the environment of lateral mesoderm expressing *Nkx2.5* secretes FGF ligands that could initiate the intracellular signaling events. Analysis of the expression of *FGF3* by whole-mount *in situ* hybridization and in transverse section of stage 13 embryos detected specific expression in anterior neural ectoderm (neural plate). Mesoderm (paraxial and lateral) in this region expresses FGF receptors (Monsoro-Burq et al., 2003), indicating that all elements necessary for signaling are expressed around the time of *Nkx2.5* initiation. Prior to the present work, two germ layers, dorsal mesoderm (Spemann organizer) and the underlying endoderm were considered as the only signaling tissues inducing cardiac progenitors (Jacobson and Sater, 1988; Sater and Jacobson, 1990; Nascone and Mercola, 1995; Schultheiss et al., 1995; Foley et al., 2007). We would like to suggest anterior neural ectoderm as an additional inducer of cardiac progenitors. We present several lines of evidence supporting this assumption: (a) surgical removal of animal caps resulted with no expression of *Nkx2.5* and heartless embryos. (b) Neuralized animal cap explants (expressing DN BMPR) express FGF ligands, FGF receptors and *Nkx2.5*. Induction of *Nkx2.5* expression is mediated by autocrine FGF signaling since the co-expression of a truncated FGF receptor inhibited *Nkx2.5* gene expression. (c) Removal of ectoderm cells from DMZ explants decreased the expression of *Nkx2.5*. (d) Recombining an ectoderm tissue removed from DMZ explants with VMZ explants induced the expression of *Nkx2.5*. Therefore, at the earliest specification stages, anterior ectoderm provides the signal for *Nkx2.5* expression. Interestingly, two reports indicating the involvement of anterior ectoderm on *Xenopus* heart development suggested a dorsal inhibitory signal that restricted heart development only to the far ventral region (Raffin et al., 2000; Garriock and Drysdale, 2003). Raffin and colleagues (2000) suggest that an inhibitory signal emanating from the neural tube limits the expression domain of *Nkx2.5* and differentiation of the lateral heart field expressing *Nkx2.5* while enabling differentiation of the far ventral heart field. Garriock and colleagues (2003) recombined whole embryos (twins) at the end of gastrulation and showed that the dorso-anterior surface of one embryo inhibited the expression of Nkx2.5 and GATA4 at the ventral cardiac field of a second embryo. These inhibitory effects were detected in late neurulation at the *ventral* heart field and therefore may reflect a later function of the dorsal neural ectoderm to limit heart development to the ventral end. The positive effect of neural ectoderm that we describe is likely earlier than those described in the other studies. At stage 12, when *Nkx2.5* expression is initially detected as two anterior dorsal lateral patches, it is located proximal to the anterior neural plate. It is more likely that at this stage of *Nkx2.5* initiation, the adjacent neural tissue plays an inductive rather than a suppressive role. Whether neural ectodermal induction of cardiac progenitors is unique to *Xenopus* or represents a broader evolutionary event in vertebrates remained to be investigated. A similar expression pattern of FGF8 in the mid-hind brain boundary at tailbud stage close to the expression domain of *Nkx2.7* which includes cardiac precursors in *zebrafish* may indicate of a more general function of anterior neural ectoderm in the initiation of *Nkx* expression (Lee et al., 1996; Reifers et al., 2000).

FGF-p38-CREB relations in cardiac development

The involvement of FGF signaling in developmental events such as mesoderm induction and neural specification are mediated by Ras-ERK MAPK signaling (Gotoh et al., 1995; Uzgare et al., 1998). More recently, studies of Amaya and colleagues indicated that regulators of receptor tyrosine kinases from the Sprouty and Spred families modulate different signaling pathways downstream of the FGF receptor (FGFR) and consequently different developmental processes (Sivak et al., 2005). In the present study, we suggest the involvement of a new pathway, p38 MAPK/CREB, downstream of FGF in cardiac specification. Experiments presented here indicate that FGF activates ERK and p38 MAPK pathways and possibly other intracellular events. However, our results suggest that p38 MAPK is the mediator of FGF in *Nkx2.5* expression, while ERK is not involved in the process. Activation of p38 MAPK by FGF signaling was reported in many cellular systems, though the mechanism was not elucidated. A complex array of signaling networks is emerging from many developmental studies. It is obvious that crosstalk between signaling pathways could occur both non-autonomously, by induction of secreted ligands affecting other cells or intracellularly. For example, crosstalk between FGF, BMP and Wnt was described in several developmental processes (Shimogori et al., 2004; Hong et al., 2008). Thus, future studies will elucidate the mechanism by which FGF induces p38 MAPK pathway.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2009.09.012.

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