Fibroblast growth factor (FGF)-4 can induce proliferation of cardiac cushion mesenchymal cells during early valve leaflet formation

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Abstract

While much has been learned about how endothelial cells transform to mesenchyme during cardiac cushion formation, there remain fundamental questions about the developmental fate of cushions. In the present work, we focus on the growth and development of cushion mesenchyme. We hypothesize that proliferative expansion and distal elongation of cushion mesenchyme mediated by growth factors are the basis of early valve leaflet formation. As a first step to test this hypothesis, we have localized fibroblast growth factor (FGF)-4 protein in cushion mesenchymal cells at the onset of prevalve leaflet formation in chick embryos (Hamburger and Hamilton stage 20–25). Ligand distribution was correlated with FGF receptor (FGFR) expression. In situ hybridization data indicated that FGFR3 mRNA was confined to the endocardial rim of the atrioventricular (AV) cushion pads, whereas FGFR2 was expressed exclusively in cushion mesenchymal cells. FGFR1 expression was detected in both endocardium and cushion mesenchyme as well as in myocardium. To determine whether the FGF pathways play regulatory roles in cushion mesenchymal cell proliferation and elongation into prevalvular structure, FGF-4 protein was added to the cushion mesenchymal cells explanted from stage 24–25 chick embryos. A significant increase in proliferative ability was strongly suggested in FGF-4-treated mesenchymal cells as judged by the incorporation of 5'-bromodeoxyuridine (BrdU). To determine whether cushion cells responded similarly in vivo, a replication-defective retrovirus encoding FGF-4 with the reporter, bacterial β-galactosidase was microinjected into stage 18 chick cardiac cushion mesenchyme along the inner curvature where AV and outflow cushions converge. As compared with vector controls, overexpression of FGF-4 clearly induced expansion of cushion mesenchyme toward the lumen. To further test the proliferative effect of FGF-4 in cardiac cushion expansion in vivo (ovo), FGF-4 protein was microinjected into stage 18 chick inner curvature. An assay for BrdU incorporation indicated a significant increase in proliferative ability in FGF-4 microinjected cardiac cushion mesenchyme as compared with BSA-microinjected controls. Together, these results suggest a role of FGF-4 for cardiac valve leaflet formation through proliferative expansion of cushion mesenchyme.

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Introduction

It is generally acknowledged that the endocardial epithelium (endothelium) of specific segments of the primary tubular heart transforms into prevalvular tissue called “cushion mesenchyme” (Markwald et al., 1975, 1977; reviewed by Eisenberg and Markwald, 1995; Mjaatvedt et al., 1999; Kisanuki et al., 2001). Although always presumed to be the foundation of valve tissue, there is very little known about how mesenchymalized cushions become mature valve leaflets.

What is known is that two major cushion “pads” or swellings project into the AV canal, the inferior and superior AV cushions, which fuse to form the AV septum. Based on in vivo labeling, the septal leaflets for the mitral and
tricuspid valves arise as luminal outgrowth from the AV septum (de la Cruz et al., 1977; Lamers et al., 1995), whereas their mural leaflets arise from two lateral AV cushions which appear later in development (de la Cruz and Markwald, 1998). In the proximal portion of the outflow tract (OT), two large cushions (conal "ridges") also expand and fuse, but these cushions later become muscularized (myocardialized) to form the muscular outlet septum and a part of the posterior wall of the infundibulum (Qayyum et al., 2002). Only cushions that develop in the distal outflow tract (or truncus) form leafllets of the aortic and pulmonary outlet valves (Bouchey et al., 1996; Markwald et al., 1998; Qayyum et al., 2002). Thus, in the AV inlets and outlet segments, cushion mesenchyme distally elongates, producing bulges that project into each lumen. The distribution of proliferating mesenchymal cells is not uniform. For example, subpopulations of cushion cells immediately adjacent to the endocardium divide more rapidly than those at sites close to the AV myocardium (de la Cruz and Markwald, 1998). This suggested to us that proliferation within the cushion mesenchyme might produce the "force" for elongation into the lumen. However, the mechanisms regulating cushion expansion into the lumen have not been investigated.

Secreter growth factors are reasonable candidates for regulating these early events in valvulogenesis based on their well known role in embryonic development. For example, fibroblast growth factors (FGFs) belong to a structurally related heparin-binding protein family that regulate tissue differentiation and patterning, such as mesoderm formation, neural induction, and patterning of the primary body axis and limb mesoderm (see review by Szébenyi and Fallon, 1999). It is well accepted that FGFs are potent mitogens for endothelial and mesenchymal cells (Folkman and Klagsbrun, 1987; Rifkin and Moscatelli, 1989; Goldfarb, 1990; Gospodarowicz, 1991; Sugi et al., 1993, 1995;
Cohn et al., 1995; Zhu et al., 1996; Choy et al., 1996; Dealy et al., 1997). FGFs bind to and signal through low- and high-affinity FGF receptors (FGFRs) (Szebenyi and Fallon, 1999). To date, four high-affinity FGF receptors have been identified. They are structurally similar transmembrane receptor tyrosine kinases, and their extracellular portions contain two or three immunoglobulin-like motifs (Ig loops) that are important for ligand binding.

In the present study, we have localized mRNA of FGFR1, 2, and 3, and FGF ligand, FGF-4, in developing cardiac cushion tissue during the initial proliferative period of early valvulogenesis. FGF-4 binds to all three of the receptors except for a FGFR-IIIb splice variant. One significance of our findings is that expression of FGFRs correlates well with the proliferative zones of AV cardiac cushion (de la Cruz and Markwald, 1998). Based on in vivo expression

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Fig. 2. Localization of FGF receptor messenger RNA expressions in stage 22 chick heart by in situ hybridization (dark field view). (A) FGFR1 expression was detected throughout the heart, including myocardium and cushion mesenchyme. (C) FGFR2, however, was expressed intensely in the cushion mesenchymal cells (arrows), but little signal was detectable in the myocardium. (E) Most strikingly, FGFR3 expression was confined to the endocardium of the cardiac cushion (arrows). (A, C, E) Correspond to sections hybridized to antisense probes and (B, D, and F) sections hybridized to control sense probes.
patterns, we hypothesize that FGF-4 modulates cardiac cushion mesenchymal cell proliferation through FGFRs expressed in the cushion mesenchymal cells and overlying endocardium. FGF-4−/− embryos die on embryonic day (ED) 5.0 (Feldman et al., 1995), while FGFR2-deficient mice embryos die at ED 10-11 (Xu et al., 1998). Thus, these mutant mice embryos die before or at the onset of valve formation, which begins at around ED 10.5, making the study of the roles of FGF-4 and/or FGFR2 in cardiac valvulogenesis difficult in these animal models. Therefore, in our current work, we exogenously added or overexpressed FGF-4 in avian cardiac cushion tissue to test our hypothesis. Our data indicated that exogenous FGF-4 can promote proliferation of AV cushion mesenchymal cells in culture and in vivo (ovo), while virally supported, overexpression of FGF-4 in cardiac cushion mesenchymal cells induced a precocious expansion of cushion mesenchymal pads toward and into the lumen. These results are the first to suggest a potential role for a specific growth factor FGF-4 in signaling cardiac valve leaflet formation.

Materials and methods

**Chick embryos**

Viral-free fertilized eggs of White Leghorn (*Gallus gallus domestica*) chicken were purchased from Spafas (Preston, CN) and incubated in a humid atmosphere at 37.5°C. Stages of embryonic development were determined by using the criteria of Hamburger and Hamilton (1951).

**Immunohistochemical localization of FGF-4**

Anti-FGF-4 antibody was purchased from Santa Cruz Biotechnology, Inc. (Cat. No. sc-1361, Santa Cruz, CA). It is an affinity-purified goat polyclonal antibody raised against a peptide mapping at the carboxy terminus of the FGF-4 of human origin (Taira et al., 1987), which is identical to corresponding mouse and rat sequences and has 83% homology with corresponding chick sequences (Niswander et al., 1994). Specificity of anti-FGF-4 antibody was tested by Western blotting by the manufacturer. Anti-FGF-4 antibody reacts with FGF-4 of mouse, rat, and human origin. In the present study, specificity of FGF-4 in immunostaining of the chick embryonic tissue was verified by immunohistochemical localization. Anti-FGF-4 antibody was diluted to 1:100 (final concentration of IgG was 2 μg/ml) and preabsorbed with 100 μg/ml immunogenic peptide (Santa Cruz, Cat. No. sc-1962p) for 30 min at room temperature, followed by clarification at 13,000g. This treatment abolished antibody staining in chick tissue sections (Fig. 1).

Embryos were fixed in methanol at −20°C overnight and embedded in low melting temperature (50–54°C) paraffin, Paraplast X-TRA, to preserve antigenicity. Embedded embryos were stored at −20°C until sectioned at 5 μm. Sections were deparaffinized with xylene and rehydrated in decreasing ethanol series. After rinsing with distilled water and phosphate-buffered saline (PBS, pH 7.2), the sections were incubated at room temperature with 3% bovine serum albumin (BSA)–PBS for 15 min to block nonspecific binding. Sections were then incubated with primary antibody, anti-FGF-4 (1:100 in 1% BSA–PBS/0.02% NaN3), primary antibody preabsorbed with immunogenic peptide as described above or normal goat IgG (2 μg/ml) in 1% BSA–PBS/0.02% NaN3, as a negative control in a humid chamber at 4°C for 4–16 h. After rinsing with 1% BSA/PBS, FITC-labeled rabbit anti-goat IgG (ICN Pharmaceuticals, Aurora, OH) was applied for 1 h. For double immunostaining, MF20 (anti-sarcomeric myosin) immunostaining was performed after FGF-4 immunostaining. The samples were rinsed and incubated with MF20 followed by RITC-labeled secondary antibody (ICN, Aurora, OH). The hybridoma for MF20 was obtained from the Developmental Studies Hybridoma Bank. After rinsing, samples were mounted in DABCO (Sigma, St. Louis, MO) in 90% glycerol/PBS. Immunostained sections were examined under a Leica BMLB fluorescent microscope.

**In situ hybridization of FGF receptors**

Isolation for FGF receptor (FGFR)1, FGFR2, and FGFR3 cDNA fragments from chick embryos and details of the hybridization procedure were reported in a previous communication (Szebenyi et al., 1995). Briefly, probes for in situ hybridization were labeled with 35S[UTP] in vitro transcription reactions (Promega, WI). The C-terminal half of the third Ig loop of the FGFR1, 2, and 3 is encoded by alternatively spliced exons resulting in either IIIb or the IIIc splice variants. The probe we used in this study hybridizes to both IIIb and IIIc variants of each FGFR (see previous communication, Szebenyi et al., 1995). Chick embryos
Fig. 4. Exogenously added FGF-4 stimulates BrdU incorporation by cardiac cushion mesenchymal cells in culture. Cushion mesenchymal cells were from stage 20–25 chick AV canal region and were cultured with serum-free-defined medium (SFM; medium 199 supplemented only with insulin, transferrin, and selenium) or SFM containing added FGF4 (50 ng/ml). FGF4 (50 ng/ml in SFM) was added daily. After 48 h, cultures were treated with 50 μM 5′-bromodeoxyuridine (BrdU) for the last 2 h of the culture period, followed by fixation and immunostaining using an anti-BrdU monoclonal antibody. Note that the increased incidence of BrdU-positive (green) nuclei in the FGF4-treated culture (D) compared with the control culture with SFM (B). (A, C) Phase contrast photomicrograph.

AV cushion mesenchymal cell culture and exogenous FGF treatment

AV cushion tissues were dissected out from stage 24–25 chick embryos and dispersed with 0.25% trypsin-PBS at 37°C. Trypsinization was stopped by adding chick serum (Sigma) to a final concentration of 10%. Dispersed cells were resuspended in serum-free medium (SFM), medium 199 (Gibco/BRL, Grand Island, NY) supplemented with ITS (insulin, transferrin, and selenium; Collaborative Research), and 100 units/ml penicillin and 100 μg/ml streptomycin (Gibco/BRL). The dissociated cells were plated at the density of 25,000 cells/well in eight-well Lab-Tek chamber slides (Nunc) that had been precoated with fibronectin (bovine plasma fibronectin; Sigma). After attachment of the cells to the well, medium was replaced with SFM or recombinant human FGF-4 (R&D Systems, Minneapolis, MN) in SFM at final concentrations of 10, 25, 50, 100, or 250 ng/ml. FGF-4 protein is conserved among species, and the recombinant human FGF-4 acts as a differentiation inducer in
chick mesoderm culture (Zhu et al., 1996). Mesenchymal cells were observed daily by using Hoffman optics, and the effect of added growth factors were evaluated at 48 h of culture.

BrdU incorporation assay in cultured cushion mesenchymal cells

5′-bromodeoxyuridine (BrdU; Sigma) was added to a final concentration of 50 μM for 2 h before the termination of the culture. Mesenchymal cells were rinsed with PBS, fixed with ice-cold 100% ethanol, and rehydrated in decreasing ethanol series. Immunohistochemistry to assess BrdU incorporation was described previously (Sugi et al., 1993). Briefly, after rinsing with PBS, cultured cells were incubated in 2 N HCl containing 0.5% Tween 20 for 8 min at 37°C, washed with 0.5% Tween 20/PBS, and incubated in a 1:2.5 dilution of monoclonal anti-BrdU (Becton-Dickinson, Cat. No. 7580) for 1 h at room temperature. After rinsing with PBS, the cells were stained with FITC-conjugated goat anti-mouse IgG (ICN, Aurora, OH) for 30 min at room temperature. Lastly, to stain all nuclei in the mesenchymal cells, 0.1 μg/ml propidium iodide (Molecular Probes) in distilled water (DW) was added for 1 min. After rinsing with DW, the cells were mounted in DABCO (Sigma) in 90% glycerol/PBS and examined under a Zeiss Axioscope.

Propagation of virus

The retroviruses used for the present study are replication-defective variants of the spleen necrosis virus. Construction of the viral vectors and propagation of the virus have been described previously (Mima et al., 1995a,b; Mikawa, 1995; Itoh et al., 1996). Briefly, after removal of polyadenylation signals, the cDNA of FGF4 was inserted between the 5′LTR and a bacterial β-galactosidase (β-gal) gene of a vector plasmid, pSNZ (Mikawa et al., 1992). As a negative control, a retrovirus CXL (Mikawa et al., 1991) expressing only β-gal was used. Each viral construct was transfected into packaging cells, D17.2G. After propagating recombinant viruses, 10^8 virions/ml for CXL and FGF-4 were obtained on average from ultracentrifugation/concentration of the supernatant of the virus-producing packaging cells. The biological activities of the FGF-4 virus have been examined by infecting somatic mesoderm, mesenchyme in limb bud, cardiogenic mesoderm and coronary vascular cells, and assaying the effects on morphology, differentiation, and cell proliferation as described (Mima et al., 1995a,b; Mikawa, 1995; Ito et al., 1996).

Microinjection of virus and morphological analysis

A small window of less than 5 mm diameter was opened in the shell at the blunt end of the egg, and the underlying shell membrane was removed. Embryos were staged after introducing biologically inert dye, India ink (5% in Tyrode’s solution), beneath the embryos. Viruses were microinjected into the cushions developing along the inner curvature at stages 17–18. At these stages, the superior AV cushion (SAVC) and the sinistroventral conal cushion (SVCC) merge along the inner curvature but do not normally form luminal expansions until stages 25–28. Virus solutions of 1–5 nl containing 100 μg/ml of polybrene were pressure injected into the cushion mesenchyme in ovo under the control of Picospritzer II (General Valve Co., NJ). After the eggs were resealed with Parafilm, each injected embryo was returned to a humidified incubator at 37.5°C and incubated for 2 additional days (approximate stages 23–25). Embryos were harvested and fixed with 2% paraformaldehyde in PBS at 4°C overnight. Embryos were then rinsed with PBS at 4°C and processed in whole mount for 5-bromo-4-chloro-3-indolyl β-d-galactopyranoside (X-gal) histochemistry overnight, as previously described (Mikawa et al., 1992). After taking whole-mount images under a stereomicroscope, MZ12 (Leica) equipped with digital microscope camera (Polaroid), embryos were subsequently embedded in paraffin and serially sectioned at 5–6 μm thickness. Sections were deparaffinized, counterstained with nuclear fast red, and observed under a light microscope, Axioscope (Zeiss).

Microinjection of FGF-4 protein and morphological analysis

Chick embryos were prepared in the same way as described for the virus injection study. Recombinant human FGF-4 was microinjected into the cushions developing along the inner curvature at stages 17–18. One to five nanoliters of FGF-4 solutions (50 μg/ml) with 0.1% BSA (carrier protein) in PBS or control BSA (0.1%) in PBS were pressure injected into the cushion mesenchyme in ovo under the control of Picospritzer II (General Valve Co., NJ). Embryos were resealed and incubated another 18 h. Ten micrograms of BrdU/200 μl Tyrodes were topically applied to the embryos for 3 h before the termination of the incubation following the methods published elsewhere (Sedmera et al., 2002). Embryos were harvested and fixed with cold 100% Methanol overnight, embedded in paraffin, and serially sectioned at 6 μm thickness in sagittal planes. Four serial sections were mounted on each slide, and slides were serially numbered. From each sample, sections spanning the entire proximal-distal lengths of the injected cushions were carefully selected under light microscope, and odd-numbered slides (4–5 slides from each sample) were subjected for immunofluorescent microscopy and quantitative evaluation of BrdU incorporation. Sections were deparaffinized and immunostained with MF20 followed by Cy5-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). Sections were then incubated in 2 N HCl containing 0.5% Tween 20 for 30 min at room temperature, washed with 0.5% Tween 20/PBS, and
incubated in a 1:2.5 dilution of monoclonal anti-BrdU (Becton-Dickinson, Cat. No. 7580) for 1 h at room temperature. After rinsing with PBS, the sections were stained with FITC-conjugated goat anti-mouse IgG (ICN, Aurora, OH) for 1 h. Lastly, to stain all nuclei, 0.1 g/ml propidium iodide (Molecular Probes) in distilled water was applied for 1 min. After rinsing and mounting in DABCO (Sigma) in 90% glycerol/PBS, sections were examined under a Leica TCS SP2 AOBS confocal microscope.

**Results**

**Immunohistochemical localization of FGF-4 at the onset of valve formation**

FGF-4 protein expression in myocardial precursors and developing myocardial cells has previously been demonstrated in chick hearts from stages 5 to 27 (Zhu et al., 1996). Our present work focuses on cardiac cushion mesenchymal cells and their antecedent, endocardial endothelial cells along the inner curvature of the heart at the onset of valve formation (reviewed in Mjaatvedt et al., 1999). In agreement with Zhu et al. (1996), we found that FGF-4 was localized in atrial and ventricular myocardium adjacent to the forming AV cardiac valves (Fig. 1A and B). Cytoplasmic expression of FGF-4 was detected in AV and OT cushion mesenchymal cells as well as AV endocardial endothelial cells (Fig. 1B). Immunostaining of FGF-4 was abolished by preabsorption of the FGF-4 antibody with immunogenic peptide as described in Materials and methods (Fig. 1C and D), which verified specificity of FGF-4 immunostaining in chick embryos.

**Spatial expression patterns of FGFR1, 2, and 3 at the onset of cardiac valve formation**

In situ hybridization was performed to examine the expression of FGFR1, 2, and 3 transcripts prior to and after the onset of cardiac valve formation (i.e., stages 18 vs 25–26). We specifically focused on the AV canal region of the inner curvature. Regarding the FGFRs, all stages examined exhibited similar patterns of expression. Fig. 2 indicates FGFR1, 2, and 3 expression patterns in a stage 23 chick heart. FGFR1 mRNA was distributed throughout the heart, including epicardium, myocardium, endocardium, and cardiac cushion mesenchyme (Fig. 2A). Note that, even though FGFR1 was expressed throughout the heart, expression in the cushion mesenchyme appeared higher than in any other tissue of the heart. Unlike FGFR1, both FGFR2 and FGFR3 exhibited distinctive regional expression patterns in the heart. FGFR2 mRNA was exclusively expressed in the AV cushion mesenchyme with little expression found in the myocardium (Fig. 2C, arrows). Most strikingly, FGFR3 mRNA expression was restricted primarily to AV endocardial endothelial cells (Fig. 2E) with little, if any, expression in the AV cushion mesenchymal cells (Fig. 3).
Effect of exogenously added FGF-4 on BrdU incorporation by cultured cardiac cushion mesenchymal cells

To determine whether FGF-4 exerted an effect on AV cushion mesenchymal cell proliferation, we added FGF-4 to the medium of cultured AV cushion mesenchymal cells. Just prior to the onset of valve leaflet formation, AV cushion mesenchymal cells at stages 24–25 were dissociated by trypsinization and cultured in SFM (medium 199 supplemented only with insulin, transferrin, and selenium) with or without FGF-4. As indicated in Fig. 4, mesenchymal cells grow well in SFM alone without serum supplement. Final concentrations of 10, 25, 50, 100, or 250 ng/ml FGF-4 were added daily to test dose-dependence effects. After 48 h, the culture received 50 μM 5′-bromodeoxyuridine (BrdU; Sigma) for the last 2 h of the culture period. The percentage of nuclei that contained BrdU indicates the percentage of the cells entering S-phase of the cell cycle, which provides an index of proliferation activity. As little as 10 ng/ml of FGF-4 caused a statistically significant, 50% increase in the proliferative activity of mesenchymal cells (Fig. 5).

Microinjection of FGF-4 retrovirus into cushion tissue

To evaluate the effect of FGF-4 on cardiac valve formation in vivo, FGF-4 retrovirus was pressure injected at stages 17–18 into the cushions of the inner curvature of the heart and embryos were further incubated to reach stage 23–25. At these stages, the inner curvature is completely lined with cushion tissue that formed by merging of the SVCC and the SAVC (Markwald et al., 1998), but luminal expansions have not normally begun (de la Cruz and Markwald, 1998). Replication-defective retrovirus, variants of the spleen necrosis virus, pSNZ (Mikawa et al., 1992) was generated to express both FGF-4 and β-galactosidase (Mima et al., 1995a, 1995b; Mikawa, 1995). Therefore, virus-infected, FGF-4-secreting cells are detectable by X-gal histochemistry for β-galactosidase. Another virus that expressed only β-galactosidase (β-gal) was used as a control. Cardiac mesenchymal cells infected in vivo with FGF-4 virus indicated dense populations of β-gal viral marker-positive cells detected in the paraffin sections (blue color in Fig. 6B) and expansion of mesenchyme into the lumen (arrow in Fig. 6A), suggesting proliferative expansion of the cushion. Control β-gal virus when injected into cushion mesenchyme showed normal development (Fig. 6C). A total of 17 chick embryos were successfully microinjected with virus as judged by formation of β-galactosidase-positive colonies confined to cardiac cushions. Eight out of 17 embryos were microinjected with FGF-4 + β-gal virus, and 9 were microinjected with control β-gal virus. None of the 9 β-gal virus-microinjected embryos showed any luminal expansion of cushion mesenchyme. Seven out of 8 of the FGF-4 + β-gal virally injected embryos clearly demonstrated luminal expansion of β-gal-positive cushion mesenchyme. Similar morphological changes were found in both SVCC and SAVC mesenchyme.

Microinjection of FGF-4 protein into cushion tissue

To further evaluate the in vivo effect of FGF-4 on cardiac cushion mesenchymal cell proliferation, recombinant human FGF-4 protein was pressure injected into the cushions of the inner curvature at stages 17–18. Embryos were re-sealed and incubated another 18 h to reach stages 19–20. Eighteen hours of treatment were chosen for the in ovo assay since biological effects of administered FGFs have been previously reported with this time frame (8–30 h) (Alsan and Schultheiss, 2002; Vogel and Tickle, 1993). BrdU was topically applied to the embryos for 3 h before the termination of the incubation. BrdU incorporation into the nuclei appeared to occur evenly in all parts of the embryos (not shown). As shown in Fig. 7B, FGF-4-injected cushion mesenchyme had a significant increase in the percentage of BrdU-labeled mesenchymal cells (indicated by the yellow color in Fig. 7), including those seen in cushions that protruded toward the lumen as compared with BSA-injected control (Fig. 7A). Quantitative evaluation of the BrdU incorporation assay shows that FGF-4 microinjection into the cushion tissue caused a statistically significant increase in BrdU incorporation of mesenchymal cells as compared with BSA-injected controls (Fig. 8). Moreover, BSA-injected control samples tended to show more BrdU-positive cells near the luminal surface of the cushion, while FGF-4-injected samples show more BrdU-positive cells in the central part of the cushion, which suggests that FGF-4 injection altered (expanded) the normal distribution of BrdU-positive cells. These data clearly support the hypothesis that FGF-4 can induce expansion of the cushion mesenchyme through proliferation of cushion mesenchymal cells in vivo (ovo).

Discussion

It has been always presumed that cardiac cushion mesenchyme is “remodeled” into valve tissue. However, little is known as to how cushions form mesenchymal expansions that progressively project into the lumen and subsequently differentiate into mature valvular tissue. Based on morphological studies (de la Cruz and Markwald, 1998), we propose that the following morphogenetic steps sequentially serve to transform a primitive cushion pad into an elongated and radially attenuated leaflet: (1) distal outgrowth, elongation, and spreading of cushion mesenchyme upon a substratum of ventricular myocardium (Oosthoek et al., 1998), (2) differentiation of multipotent cushion cells into fibroblastic/myofibroblastic tissue (Icardo and Colvee, 1995), and finally, (3) “delamination” of the leaflets from the associated ventricular myocardium, creating in the process both free edges and a persisting supportive, suspensory apparatus (de
Fig. 6. FGF-4 overexpression induces expansion of cardiac cushion mesenchyme in ovo. Replication-defective retrovirus encoding FGF-4 and bacterial β-galactosidase (FGF-4 + β-gal) or β-galacosidase (β-gal) was microinjected into the sinistroventral conal cushion (SVCC) of the heart of stage 17 chick embryos. Whole embryos were incubated to stage 24, then processed with X-gal histochemistry to detect viral reporter β-galactosidase expression. A total of 17 chick embryos were successfully microinjected with virus only into cardiac cushions. Eight out of 17 embryos were FGF-4 + β-gal virus microinjected, and 9 of them were β-gal-microinjected. None of 9 β-gal-microinjected embryos showed expansion of cushion mesenchyme. Seven out of 8 FGF-4 + β-gal virus-injected embryos clearly demonstrated luminal expansion of β-gal-positive cushion mesenchyme. (A) The arrow indicates intense viral β-galactosidase expression.
la Cruz and Markwald, 1998; Oosthoek et al., 1998). The present work focuses on what we believe must be a part of the initial step in these valvulogenic processes, the distal outgrowth of cushion mesenchyme into the lumen of the primary heart tube.

Because the initial expansion of cushions resembles that of limb buds, we considered that an analogy to limb morphogenesis might provide clues as to the molecular candidates that could serve to initiate the formation of prevallcular leaflets. In this paper, we focus on FGFs, which are known mitogens for limb mesoderm that play a key role in the distal expansion of the limb buds (reviewed by Szebenyi and Fallon, 1999). Recent work using conditional disruption of FGF-8 in mice indicated that inactivating FGF-8 in early limb ectoderm caused a reduction in limb-bud size, altered expression of sonic hedgehog (shh), bone morphogenetic protein (BMP)-2, and other FGF genes, such as FGF-4 (Lewandoski et al., 2000; Moon and Capecchi, 2000). FGF-4 knockout mice die at ED 5, indicating the importance of FGF-4 signaling during early morphogenesis (Feldman et al., 1995). More recently, conditional inactivation of FGF-4 using Msx2-cre mouse indicated that the mice survive longer and formed limbs in the absence of FGF-4 (Sun et al., 2000). However, these recent findings did not shed much light on the role of FGF-4 in cushion and valve morphogenesis because Msx2-cre mouse is based on a putative limb-specific Msx2 regulatory element in the Msx2 promoter, and moreover, Msx2 is expressed mostly in AV and OT myocardium not in the cushion mesenchyme, which express Msx-1 (Chan-Thomas et al., 1993).

FGF signaling pathways are also known to be intricate and intertwined with other regulatory molecules, e.g., insulin-like growth factor (IGF), transforming growth factor (TGF)-β, BMP and vertebrate homologue of Drosophila wingless activated pathways (reviewed by Szebenyi and Fallon, 1999). Interaction of FGFs and BMPs has been proposed in limb formation (Dahn and Fallon, 1999; Moon and Capecchi, 2000). FGF-4 and BMP-2 appear to have opposite effects on limb distal growth (Niswander and Martin, 1993). For example, inhibiting BMPs induced FGF-4 expression and established a shh/FGF4 feedback loop in regulating limb bud formation (Zuniga et al., 1999). BMP-2 transcripts (Lyons et al., 1990) are known to be expressed in AV myocardial cells prior to endocardial to mesenchymal transformation at ED 8.5–9.0 in mice. BMP-2 protein is also localized in AV and OT cushion mesenchymal cells at ED 10.5–16, (unpublished data). Our present work indicated that FGF-4 was expressed in cushion mesenchymal cells in the chick (Fig. 1), that exogenously added FGF4 induced proliferation of cushion mesenchymal cells in vitro (Figs. 4 and 5) and in vivo (ovo) (Figs. 7 and 8), and that FGF-4 overexpression induced precocious extension of cushion mesenchyme into the lumen (Fig. 6), indicating a role of FGF pathway in early leaflet formation. However, it remained to be determined whether FGFs also interact with

staining in the heart (indicated in blue color). (B) A paraffin section of the same embryo shows the outgrowth of a bud-like expansion of cushion tissue stained with X-gal. (C) Control embryos infected with CXL virus encoding only bacterial β-galactosidase did not show this extra outgrowth from the cushion tissue. Fig. 7. FGF-4 protein injection stimulates BrdU incorporation by cardiac cushion mesenchymal cells in ovo. Recombinant human FGF-4 was microinjected into cushion mesenchyme of the inner curvature at stages 17–18. Embryos were incubated another 18 h to evaluate the proliferative effect of FGF-4. BrdU was applied topically to the embryo for 3 h before the termination of the incubation in ovo. After the fixation and paraffin embedding, sections were immunostained with an anti-BrdU monoclonal antibody (indicated as green color). Myocardium was immunostained with MF 20 (indicated as blue color), and all nuclei were stained with propidium iodide (red color). Note that the increased incidence of BrdU-positive (yellow color, after overlay of triple labeled immunofluorescent microphotographs) nuclei in the FGF-4-injected cushion mesenchyme (B) as compared with the BSA-injected control (A). BSA-injected control samples tends to show more BrdU-positive cells near the luminal surface of the cushion (A), while FGF-4-injected samples shows more BrdU-positive cells in the central part of the cushion (B).
other candidate regulatory molecules, such as Bmps, to regulate/coordinate mesenchymal cell proliferation and/or differentiation and gene expression necessary for further differentiation of cushions into valve leaflets.

Our in situ hybridization data for localizing FGF receptors also support a signaling role for FGF ligands in valvulogenesis. Specifically, our findings indicate a spatially restricted pattern for FGF expression in AV cushion tissue (Figs. 2 and 3). The expression of FGFRs correlated well with the proliferative zone previously seen in AV cardiac cushion. While FGFR1 expression was detected throughout the heart at all stages examined (stages 14–27), FGFR2 was confined to just the cushions in the heart. Most striking, however, was FGFR3, whose expression was uniquely restricted to the endocardium overlaying the cushions. It is presently unclear how these distinctively restricted patterns of expression for FGF receptors might interact to regulate various phases of early valvulogenesis, particularly FGFR3. It is possible that this receptor merely serves to keep the endocardium growing at a pace commensurate with cushion mesenchymal growth. Yet, the similarity of FGFR3 expression to that of nuclear factor of activated T cells (NFATc1) may suggest another role more akin to that of nuclear factor of activated T cells (NFATc1) in the mesenchymalized cushions failing to initiate elongation (Ranger et al., 1998; de la Pompa et al., 1998).

Rather, in mutant mice deficient for this transcription factor, the mesenchymalized cushions fail to initiate elongation into prevallcular leaflets and death occurs at ED 14 or 15. If NFATc1 deficiency disrupts cushion valvulogenesis is not known. If early valvulogenesis shares any similarities to early limb bud outgrowth, then one possibility is that the endocardium following FGF signaling secretes a paracrine signal that initiates or sustains cushion elongation. Thus, spatially and temporally restricted FGFR3 signaling of cushion endocardium may be linked in some unknown way to the mechanisms whereby endocardial upregulation of NFATc1 imparts an endocardial morphogenetic influence on the mesenchymalized primordia for future valvular leaflets. In addition to simply promoting proliferation, it will be of interest to know how FGF signaling modulates or interacts with other regulatory molecules to potentially regulate further morphological changes in valve formation, such as delamination from myocardium, differentiation into myofibroblastic lineage, or formation of the supporting tension apparatus.

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