Directionality of Heart Looping: Effects of Pitx2c Misexpression on Flectin Symmetry and Midline Structures

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A critical regulatory laterality gene expressed in the left side of the straight heart tube during development is Pitx2, which when mutated in humans underlies Rieger's Syndrome. Previously reported results have indicated that, when using gain-of-function and loss-of-function approaches of the chick cPitx2c isoform, this results in randomization of heart looping. To determine whether Pitx2c misexpression affects downstream morphogenesis by altering the expression of specific proteins in the myocardium during looping, after experimental manipulations, we analyzed immunohistochemically for the extracellular matrix molecule flectin that normally is expressed predominantly in the left lateral plate mesoderm (LPM) and left side of the straight chick heart tube before and during looping. We show here that the left-side predominance of flectin is due to a delay in the timing of expression in one heart field vs the other. Experimental results indicate that misexpression of Pitx2c in the heart field using antisense or retroviral delivery perturbs the normal temporal pattern of flectin expression in the left LPM relative to the right: abnormally leftward looping hearts show predominant right-sided flectin expression in the dorsal mesocardial regions around the foregut ventral midline. Additionally, Pitx2c misexpression affects the positioning of the developing foregut to more lateral areas, either on the right or left side of the embryonic midline. The position of the heart with respect to the embryo midline is defined by the position of the foregut. Incubating embryos in the presence of flectin antibody caused randomization of heart looping or no looping.© 2002 Elsevier Science (USA)

Key Words: flectin; Pitx2; left–right asymmetry; embryonic midline; foregut floor; chick embryo; dorsal mesocardium; heart looping.

INTRODUCTION

For studies on embryonic left–right axis determination, the rightward or dextral directionality of heart looping has served as a key downstream morphologic readout of the effects of manipulation of upstream genes associated with laterality pathways in the early chick embryo. Perturbation of upstream laterality genes, such as sonic hedgehog (Shh), activin β and the activin receptor IIA, nodal, Lefty1, and Lefty2, can result in randomization of the direction of heart looping. The bicoid-like homeobox laterality gene Pitx2, which is downstream of nodal in all vertebrates examined, is of particular interest in this study because it is first expressed in the left lateral plate mesoderm (LPM) and, unlike the more upstream signaling cascade of factors, it subsequently is expressed in the left side of the straight heart tube (Logan et al., 1998; Meno et al., 1998; Piedra et al., 1998; Ryan et al., 1998; St. Amand et al., 1998). This organotypic expression and functional analyses indicate that Pitx2 may serve to interface the left–right developmental program with morphogenesis to result in dextral looping (Campione et al., 1999; Logan et al., 1998; Ryan et al., 1998). Recently, it was shown that it is the Pitx2c isoform that is expressed in the chick left heart field and left heart tube and regulates rightward heart looping (Yu et al., 2001). The downstream targets of Pitx2, however, are currently unknown. Pitx2 encodes a transcription factor that preferentially binds to the bicoid homeodomain binding site and transactivates reporter genes containing this site (Amendt

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et al., 1998). The transactivation domain of Pitx2 has been mapped to the C terminus (Yu et al., 2001).

The early morphoregulatory events of heart development have been analyzed in the greatest detail in the chick. After cardiac cell compartmentalization takes place between stages 6 and 8 (Linask, 1992; Linask et al., 1997), the two bilateral cardiac myocardial compartments bend ventrally to form partial tubelike structures, which fuse in a cephalo-caudal manner to form a single straight heart tube which then begins to loop and beat. Directionality of looping is easily discernible at the 11-somite stage. Looping appears to be intrinsic to the heart (Nakamura and Manasek, 1978), but does not require the presence of the cardiac jelly (Baldwin and Solursh, 1989; Nakamura and Manasek, 1978), which is an extended highly organized extracellular matrix (ECM) compartment sandwiched between the endocardium and myocardium during the looping stages. Looping also does not appear to result from differential myocardial cell proliferation (Stalsberg, 1969, 1970).

Before and during heart bending, the bilateral plate mesoderm and subsequently the tubular heart show asymmetric localization of several ECM proteins, such as flectin (Tsuda et al., 1996, 1998), Lamp1, and JB3 (Smith et al., 1997). In this study, we show that the myocardial, including

### TABLE 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of embryos</th>
<th>R</th>
<th>L</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td>RCAS-Pitx2</td>
<td>16</td>
<td>5 (3)</td>
<td>6 (4)</td>
<td>5 (3)</td>
</tr>
<tr>
<td>RCAS-GFP</td>
<td>11</td>
<td>9 (3)</td>
<td>2 (2)</td>
<td>0</td>
</tr>
<tr>
<td>Antisense oligo</td>
<td>14</td>
<td>8 (4)</td>
<td>5 (3)</td>
<td>1 (1)</td>
</tr>
<tr>
<td>Random oligo</td>
<td>5</td>
<td>4 (2)</td>
<td>1 (1)</td>
<td></td>
</tr>
</tbody>
</table>

Note. All embryos were immunostained and analyzed. Numbers in parentheses indicate embryos sectioned. R, rightward looping; L, leftward looping; M, midline not looping.

![FIG. 1. Expression of flectin at approximately the four-somite stage in the heart fields. (A) Immunolocalization. (B) Computer-assisted intensity profile of image in (A) with white designating highest intensity, blue lowest. In this ventral view of the embryo, flectin first is synthesized chiefly in a localized region in the anterior left (L) heart-forming region. A small amount of flectin is detectable across the midline and in the right (R) lateral plate mesoderm at this time. Several hours later at stage 9 (C), flectin on the left is now detectably localizing further caudal than on the right. The horizontal lines demarcate the left-right posterior-most limits of its detection at this stage. By stage 10+/H11001 at the straight heart tube stage (D), flectin continues to localize in an asymmetric manner as can be seen in this confocal optical section with flectin localization indicated by red signal and at a higher intensity, by yellow. Thus, the asymmetry arises from a delay in timing of flectin expression in the right as compared with the left LPM.](image-url)
FIG. 2. Expression patterns of cPitx2c mRNA by whole-mount in situ hybridization after retroviral or antisense manipulations of early chick embryos. Whole-mount in situ hybridization was performed as described previously (St. Amand et al., 1998). In each panel, a ventral view of stage 11 hearts is shown with the right (R) and left (L) axes indicated. Arrow points to the heart. (A) Expression in the left LPM and left side of the heart tube in a control heart. (B) cPitx2c is now ectopically expressed in the right side of the heart tube as well, after RCAS-Pitx2c infection in the right heart field at stage 3. This heart exhibited a reversed looping directionality. (C) Embryo shown had been treated with antisense oligonucleotides specific to Pitx2c and showed a leftward looping heart with no Pitx2c expression within the heart forming regions. However, expression of Pitx2c in the head mesoderm remains unaffected.

FIG. 3. Flectin localization in normal, right-looping hearts of control embryos. (A) Right heart fields were injected with RCAS-GFP. Hearts loop normally. Left myocardial wall has rotated to form the outer curvature and shows more flectin expression than on the opposite side, specifically in the myocardial region. Arrows point to the dorsal mesocardial areas. Asterisk pinpoints the midline of the floor of the foregut to which the endocardial cells attach via matrix molecules. White diagonal line through the floor of neural tube and notochord area depicts embryo midline. Foregut midline is displaced slightly right of embryonic midline. Magnification bar, 50 μm. (B) Embryos were incubated in the presence of random sequence oligonucleotides. In the majority of embryos, normal right looping occurred as seen in this embryo. More flectin is apparent in the left myocardial wall that forms the outer curvature. Asymmetry of flectin expression is readily apparent in the dorsal mesocardial folds.
dorsal mesocardial, ECM asymmetry around the cardiac midline, may be associated with the development of specific biophysical parameters within the heart tube to result in dextral bending.

The downstream targets of laterality genes and why randomization of the direction of heart looping occurs when these genes are misexpressed have remained unknown. The question of whether perturbation of upstream genes within the laterality pathways affect matrix-related morphoregulatory events of heart looping is addressed here by ectopically expressing cPitx2c in the right LPM by retroviral infection or by eliminating cPitx2c expression from the left LPM using antisense oligonucleotides. The effects of these manipulations on looping direction, flectin expression, and midline structures were then assessed. In addition, we show that if flectin interactions are blocked by the F22 flectin antibody, looping direction becomes randomised or hearts do not loop.

**MATERIALS AND METHODS**

**Chick embryos.** Fertilized White Leghorn chick eggs were used for the experimental manipulations.

**Retrovirus construction and infections.** These were carried out as previously described (Yu et al., 2001). Briefly, Pitx2c sequences were cloned into a ClaI2 adapter plasmid. The ClaI fragments containing Pitx2c sequence were cloned into the RCAS retroviral vector. Orientation was determined by PCR using an upper primer targeting the retroviral sequence and a lower one targeting the ClaI sequence. Generation and concentration of viral supernatants were done according to Logan and Francis-West (1999). RCAS retrovirus infection was performed by multiple points of injection in the right side of the blastoderm of stage 3-4 chick embryos (Hamburger and Hamilton, 1951) set up in New culture (New, 1955). Control embryos similarly were injected with RCAS vectors expressing green fluorescent protein (GFP). Antisense oligonucleotide treatment was performed on stage 6-7 embryos set up in New culture as described earlier (Isaac et al., 1997; Srivastava et al., 1995). The Pitx2c antisense oligonucleotides has the sequence 5'-GGTCTCAGATGATGCTG-3' targeted to the N-terminal region. The random oligonucleotide, 5'-AGGCTCGAACTCAGATT-3', was used as the control. The oligonucleotides were synthesized as phosphorothioate derivative and HPLC purified (IDT, Corvalle, IA). Oligonucleotides were diluted to a concentration of 40 μM in lipofectamine (Gibco/BRL). Approximately 10 μl of oligonucleotide/lipofectamine mixture was applied directly onto the cultured embryo within the ring.

Table 1 shows the numbers of embryos treated and the directionality of heart looping at the end of the experiments. The percentages of R/L looped hearts were similar to what had been reported earlier (Yu et al., 2001). All embryos were immunostained and analyzed in whole-mount. Embryos for sectioning indicated in parentheses were picked at random within each treatment and within the group showing either a right- or left-looping heart.

**Flectin antibody perturbation experiments.** Antibody perturbation was performed by using stage 5 embryos set up on filter paper rings as described earlier (Linask and Lash, 1988). This method is a modification of the New culture technique. The flectin F-22 monoclonal antibody was diluted 1:1 with normal 2:2:1 medium made up as described in the above citation, using an F12X-based medium. Controls were set up in normal medium or in mouse whole serum diluted similarly. Embryos were incubated overnight for 24 h and heart loop directionality was scored. The embryos were fixed in 4% paraformaldehyde/PBS, permeabilized, and immunostained with antibody MF-20 (Developmental Studies Hybridoma Bank, IA University) [sarcosomic myosin heavy chain as a marker for the myocardium to analyze more precisely heart morphology and the directionality of looping in whole mounts.](#)

**In situ hybridization and immunostaining.** All embryos were allowed to develop to stage 11, and were then scored for directionality of heart looping. Subsequently, randomly picked embryos showing left- or right-looping hearts in each class of treatments were fixed and immunostained, embedded in plastic, and serially sectioned through the heart from anterior to posterior at 2-μm thickness, as previously described (Linask and Tsuda, 2000; Tsuda et al., 1996). This provides high resolution, detailed analyses of heart morphogenesis and protein localization. To determine efficiency of retroviral infection and antisense elimination, treated embryos and control embryos at stage 11 also were randomly picked and whole-mount in situ hybridization was carried out by using Pitx2c full-length probe, as described previously (St. Amand et al., 1998).

**Microscopy and photography.** All sections were observed with a Nikon Optiphot II fluorescence microscope. Digitized images were captured directly with a Princeton Micromax cooled CCD (mesocardial regions between arrow and arrowhead contrasted). The foregut midline, indicated by the asterisk, is displaced slightly left of embryonic midline (denoted by white solid line). Arrow at outermost loop in (B) shows region where endocardium associates with the myocardial wall. Magnification bar, 50 μm. R, right side; L, left side; FG, foregut; NT, neural tube; EN, endocardial cells; MY, myocardium. **FIG. 4.** Flectin expression in embryos injected into the right lateral plate mesoderm with RCAS-Pitx2c. (A) In a right-looping heart, more flectin is present in the left myocardium (arrow). In addition, the ventral midline floor of the foregut (shown by arrowhead) is displaced to the right of the embryo midline (indicated by the white solid line). This displacement, shown by broken white line, is more than that observed in control embryos. (B) In a leftward looping heart, the displacement of the foregut is to the left (L; compare position of broken line relative to axis of embryo midline (solid line). Arrowhead depicts the midline of the foregut floor. The embryo midline is shown by solid white line drawn through notochord. Definitive dorsal mesocardial folds are lacking and intensities of flectin localization signal appear to be relatively equal on both sides of the myocardium with more extending up into the right myocardial wall (arrows) than on the left. In embryos in which hearts looped to the left, the neural tissue often has remained as a flat sheet and has not folded to form a tube (see also Fig. 5B). (C, D) Respective whole mounts of hearts that were sectioned and shown in (A) and (B). Broken line in (D) outlines edge of notochord.

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camera interfaced with MetaMorph (Universal Imaging, West Chester, PA) image processing analysis software.

RESULTS

A Timing Delay of Flectin Protein Expression in Right Heart Field Results in Asymmetry

In order to understand the basis for the observed asymmetry of flectin protein expression, we first determined whether the timing of the appearance of the protein was the same on both sides, with more protein being expressed over time on the left relative to the right. Alternatively, the protein could first be expressed on the left and, after a delay on the right, also resulting in the observed asymmetry. To resolve the correct scenario, we analyzed embryos for the earliest expression of flectin protein by using immunohistochemistry. The localization pattern indicates that the flectin asymmetry is due to a timing delay in expression, appearing first in a localized area in the left heart forming region at approximately the 5- to 6-somite stage (Fig. 1A; relative intensity levels shown in Fig. 1B, with white indicating highest level of intensity). In this embryo, a low level of expression is seen across the midline and sparse localization is detectable in the right heart field. Once this initial asymmetric pattern is established, it is maintained throughout looping (Figs. 1C and 1D). Several hours of development later (e.g., in a stage 9 embryo shown in Fig. 1C), flectin is expressed more caudal in the left side with respect to the right (relative caudal limits of flectin expression demarcated by horizontal white lines). Figure 1D is a confocal micrograph showing the typical left-dominant expression pattern at the “straight heart tube” stage 10. After looping, the total expression diminishes rapidly to a low level within the myocardium (Tsuda et al., 1996). In situ messenger RNA analysis by in situ hybridization of stage 9 embryos provided similar results as in Fig. 1C in that the left heart-forming region expressed message more caudal than on the right (not shown).

Misexpression Experiments

To determine whether alterations of Pitx2c expression which result in randomization of heart looping changes the pattern of flectin expression, localization patterns of flectin were analyzed in experimental embryos and compared with controls. The predominant localization of flectin in the left myocardium/dorsal mesocardium and little in the right before and during looping suggested that flectin’s asymmetry may be regulated indirectly by Pitx2, a left-sided-only regulatory gene. Regulation can only be indirect, since flectin is expressed in the right heart field, as well as left.

In experimental embryos, Pitx2c was misexpressed by using either RCAS retroviral vectors in a gain-of-function approach to ectopically express Pitx2c in the right LPM or by using antisense oligonucleotides in a loss-of-function manner in the left LPM. The experimental approaches to misexpress Pitx2c were confirmed by whole-embryo in situ hybridization for Pitx2c expression by random selection of embryos from the experimental groups (Figs. 2A–2C). Control groups included use of RCAS-GFP constructs and random oligonucleotides sequences. In control embryos, the characteristic left-sided expression was seen (Fig. 2A). In RCAS-Pitx2c gain-of-function, Pitx2c message was present nearly equally on both sides of the heart (Fig. 2B). Loss-of-function via antisense oligonucleotides resulted in no detectable Pitx2c expression in the embryonic heart region. Expression in the brain region remained unaffected. Randomization of looping directionality in the experimental embryos occurred as previously described (Yu et al., 2001). Embryonic hearts were analyzed further for flectin expression along the anterior/posterior and left/right axes in stage 11 embryos, a time point at which looping directionality can be clearly defined. We were especially interested in flectin expression patterns in relation to the cardiac and embryo midlines, as previous results have suggested that the dorsal mesocardial region at the midline plays an important role in looping (Taber et al., 1995; K.K.L., unpublished observations).

The abnormally, leftward, looping hearts provided the most information on looping in regards to the effects of misexpressing Pitx2c. Pitx2c misexpression altered the sidedness of flectin expression, as well as sidedness of the position of the foregut. The position of the developing heart was seen to be related directly to the midline of the ventral floor of the foregut, rather than the embryo midline. These results are shown in Figs. 3A and 3B (hearts of control embryos), Figs. 4A and 4B (gain-of-function study), Figs. 5A–5C, and Figs. 6A and 6B (loss-of-function study).

Control embryos. Control embryonic hearts in Fig. 3, i.e., RCAS-GFP-injected embryo (Fig. 3A) and random oligonucleotide-treated embryo (Fig. 3B), show the normal left (L)-sided (i.e., outer curvature) predominance of flectin expression around the cardiac midline. The initial left side of the heart, as rotation occurs, becomes the convex outer curvature on the ventral side. Note the contrast of flectin expression levels in regions demarcated by small arrows and arrowheads in the left and right mesocardial folds at the foregut region. As described earlier, the endocardium extends from the ventral midline of the foregut (asterisk) to a region at the outermost part of the loop (white arrow, Fig. 3B). Solid white diagonal lines drawn through the plane connecting the ventral floor of the neural tube and notochord regions denote the embryo midline. In the control embryos, the ventral midline of the foregut (asterisk) is positioned relatively close to the embryo midline, but not exactly at the midline. In control embryos, the ventral floor of the foregut is usually seen positioned slightly to the left of the midline, but occasionally to the right (Fig. 3A). The heart tube and endocardial attachments develop in relation to the midline foregut floor. Directionality of looping is associated with the predominance of flectin in the outer curvature or initial left side of the heart and left dorsal mesocardial fold.
FIG. 5. Comparison of two antisense oligonucleotide-treated embryos showing opposite directionality of heart looping: a rightward (normal direction, A) and leftward (abnormal direction, B). The asymmetry of flectin within the dorsal mesocardial folds (see arrows pointing to side of higher level of expression) close to the cardiac midline is consistent with the direction of looping: More flectin in the left (L) fold is consistent with the heart looping to the right; if more in the right (R) fold, the heart loop has looped to the left. White solid line drawn through floor of neural tube and notochord depicts embryonic midline. Arrowhead shows the midline of the floor of the foregut; broken line shows amount and direction of displacement of foregut from embryonic midline. In embryos with leftward looping hearts, displacement of foregut is often much farther to the side than in right looping hearts. In (B), embryonic midline is at the upper left edge of photograph (small arrow). Magnification bar in (B) for both panels, 25 μm. N, neural tube; FG, foregut; EN, endocardium; MYO, myocardium.

FIG. 6. Flectin expression in leftward looping heart of embryo treated with antisense oligonucleotides. The same heart is shown at different levels as heart is sectioned serially from anterior to posterior. (A) Arrows point to the dorsal mesocardial area flanking the ventral floor of the foregut (FG) in anterior region of heart. There is more flectin apparent in the right (R) mesocardial fold than in the left (L; see arrows for regions to contrast). Otherwise, intensity of localization in the right and left side of the tubular heart wall is not very different. (B) A more midlevel region of the same heart that is now beginning to show predominant localization of flectin in the left myocardial wall which is seen at even higher intensity in (C) in a more caudal section. The asymmetry around the midline in (B) and (C) is not apparent, as in (A). Thus, directionality appears to be determined by the anterior cardiac region, and is maintained during looping, regardless of asymmetry posteriorly. Directionality apparently is not changed, once it is established and may be constrained by the extraembryonic splanchnopleural membrane (sm) apposed close to the myocardium throughout looping. NT, neural tube; EN, endocardial cells; MY myocardium. Magnification bar in (B), 25 μm.
Ectopic expression of Pitx2c in the right LPM. As reported earlier, randomization of heart looping occurs upon misexpression of Pitx2c (Yu et al., 2001). In embryos in which the hearts continued to loop to the right (shown in Fig. 4A, sectioned; Fig. 4C, whole mount), there continues to be predominant expression of flectin in the left myocardial wall. The dorsal mesocardial folds often are developed and positioned abnormally, as are the endocardial extensions and associations with the foregut and myocardial wall. The heart has a more a more prominent appearance than that seen in control embryos. Note that the ventral midline floor of the foregut (arrowhead) is positioned in this embryo to the right of the embryo midline (designated by the solid white line cutting through plane of notochord and floor of neural tube). In some embryos, the foregut may be positioned even more laterally than seen here or in control embryos. Displacement of foregut is indicated by the broken arrow. Flectin localization around the notochord to the extent apparent in this embryo is sometimes observed.

In embryos in which the hearts looped abnormally to the left (shown in Fig. 4B, sectioned; Fig. 4D, whole mount), noticeable differences are apparent: The hearts may show nearly equal levels of flectin on the right and left sides often with slightly more flectin on the right side, which becomes the outer curvature in the abnormally left-looping hearts. More flectin can be seen extending into the right dorsal mesocardial fold than seen in left fold. In this embryo, the mesocardial folds appear very abnormal. Here, the folds are abnormally widely separated, and on the left side, it appears to be associating with the lateral body fold rather than with the foregut. The foregut midline (see arrowhead) in left-looping hearts is displaced off to the far left side of the embryo. The heart and endocardial attachments develop in relation to the midline of the floor of the foregut. In the leftward looping situation, neural tube closure was also often abnormal. Whether misexpression of Pitx2c has a general effect on bending or looping within the neural region, body folds, as well as of the heart, cannot be ruled out.

Antisense loss-of-Pitx2c expression in left LPM. Randomization of heart looping is also observed in loss-of-function studies. Similar to the results described above, when Pitx2c expression is repressed or decreased within the heart fields in the left lateral plate mesoderm, right-looping hearts can develop (Fig. 5A). In these embryos, as in controls, flectin is expressed at higher levels in the left (L) dorsal mesocardial area (white arrow) and in the left myocardial wall that becomes the outer curvature. Displacement of foregut is to the right (R). In contrast, in leftward looping hearts (Fig. 5B), flectin is predominant in the right (R) mesocardial fold and right side of the heart extending from the fold region. The right side of the heart due to rotation becomes the outer curvature during leftward looping. The foregut has been displaced far to the left. In Fig. 5B, the arrow in the upper left side of photograph indicates the position of the notochord, which depicts the embryo midline. The arrowhead indicates the midline of the floor of the foregut. The broken line indicates the displacement of the foregut to the embryonic left. The endocardial attachment and cardiac midline are situated according to the midline of the foregut.

Quantification of the displacement of the foregut and statistical analysis of this displacement to the left or to the right in the various treatments cannot be applied for primarily two reasons: One can never be assured that the developmental window is exactly the same at time of exposure to the molecular treatments. Two embryos may look the same, but heart development can be off by 3–4 h between embryos. This can make a difference in the degree of perturbation. In addition, there is no way to control that efficiency of retroviral infection or antisense penetration is always the same between embryos. Variability will be part of these types of experiments. One can, however, determine a pattern to the observed effects, as shown here. We are also seeing similar affects by perturbing another gene associated with the laterality pathway and that affects Pitx2 expression and flectin (unpublished observations).

That the asymmetry within the dorsal mesocardial folds...
in the anterior part of the heart appears to define the directionality of heart looping is apparent from the cephalo-caudal sequence of sections from the same heart that is shown in Figs. 6A–6C. In this embryo treated with antisense oligonucleotides in the left heart field, abnormal leftward looping was observed. In anterior regions of the heart, both sides of the myocardial wall showed similar flectin expression levels and localization. However, within the dorsal mesocardial area, more right (R)-sided asymmetry is present in the two mesocardial folds (compare regions indicated by arrows). More posteriorly, shown in Fig. 6B and even further caudal in Fig. 6C, there is a detectable shift of expression levels to the left side of the heart tube with little, if any, flectin apparent in the dorsal mesocardium. This shift in expression may be due to a slow degradation of the perturbing antisense oligonucleotides, as differentiation proceeds in an anterior-posterior wave across the heart-forming regions. The left dominant expression seen caudal would be more characteristic of rightward looping hearts associated with normal and control embryos. The heart tube, however, did not change the directionality of looping posteriorly, but remained in a leftward loop position. Note also that the extraembryonic splanchnic membrane (sm) is always closely apposed to the myocardial wall, possibly helping to constrain the heart to the direction that is initially taken anteriorly.

**Assessment of Antibody Perturbation of Flectin Interactions**

As has been reported, flectin F-22 antibody recognizes a unique site on the protein that is highly conserved in all 11 species that have been analyzed (Mieziewska et al., 1994, abstract). We used this antibody to assess whether flectin may have a functional role in heart looping, as suggested by the misexpression experiments and by flectin expression patterns before and during looping. Chick embryos (n = 30 in triplicate experiments) set up in ring cultures (Linask and Lash, 1988) were exposed to the antibody at stages 5–7 and were incubated overnight to stages 11–12 next day at which time looping directionalility can be established. Based upon its localization pattern, the heart region would be most accessible to the antibody. After incubation, the embryos were fixed and immunostained for cardiac myosin heavy chain using MF20 antibody (Figs. 7A–7D). This allowed for more precise determination of cardiac morphology than only bright field observation of whole-mount embryos. When single tubular structures had formed, heart looping in antibody-treated embryos became randomized, either looping to the right (Fig. 7A; 35% of the embryos) or to the left (Fig. 7B; 30%). In the leftward looping heart shown here, the posterior region appeared somewhat bifurcated. Some tubular hearts did not loop and remained straight, but had fused at the midline (Fig. 7C; 10%). Some hearts (25%), which appeared under brightfield to be single, wide hearts at the midline, were actually bifid hearts and fusion had not taken place (Fig. 7D). These bifid structures are not closed tubes, but in sections are open, partial tube-like, structures. In control embryos, 90% (n = 10) looped normally to the right. Therefore, flectin appears functionally to have a role in direction of heart looping and in fusion of the bilateral heart compartments.

**DISCUSSION**

Flectin is an extracellular matrix molecule first characterized in the interphotoreceptor matrix of the eye (Mieziewska et al., 1994) and which may be involved in the morphoregulation of a number of tissues and cell types, including the neural crest. It is often, but not exclusively, seen in embryonic tissues that undergo bending. Characterization of the flectin cDNA sequence and protein has been done by a different laboratory and will be reported separately (Yoon et al.). The molecule appears to be a member of a recently emerging class of extracellular proteins, known as matricellular proteins, all of which are functionally similar, but molecularly distinct. The matricellular proteins mediate cell–matrix interactions, can interact with other ECM molecules, including growth factors, and are expressed chiefly in tissues undergoing changes in cell–matrix and cell–cell associations, such as occurs during tissue remodeling, tissue renewal, and development (reviewed in Brekken, 2001). When flectin interactions were perturbed by exposing stage 5 embryos to flectin F-22 monoclonal antibody, fusion of heart tubes were often abnormal or looping direction became randomized. Presently, the specific biochemical interactions or cell signaling mediated by flectin within the myocardium and dorsal mesocardial folds are unknown. However, flectin participates in morphoregulatory pathways involved in coordinating looping and is modulated downstream by Pitx2c.

Vertebrates display bilateral asymmetry by the positioning of organs to the left or right of the body midline. Individual organs display an intrinsic asymmetry during their development, such as occurs in the brain, heart, and lungs. How this intrinsic asymmetry is established is a question that has attracted much interest, and great advances have been made towards characterizing upstream molecular pathways that underlie early left–right patterning in the embryo (reviewed by Harvey, 1998). A prominent downstream regulatory homeobox gene, Pitx2, has emerged as an important player in chick and mouse heart laterality. It is expressed in the left lateral plate mesoderm before the tubular heart forms and later continues to be expressed in the left side of the straight heart tube. An interesting question from our perspective was: Are laterality genes such as Pitx2c interfaced with organ morphogenesis by modulating downstream morphoregulatory ECM molecules in the heart? The results of the present study suggest that this is the case. Flectin expression and localization...
patterns are altered by Pitx2c misexpression. Flectin asymmetry within the mesocardial folds at the cardiac midline and within the myocardium in anterior regions of the heart appears to have an important role in establishing looping directionality. During randomization, mesocardial/myocardial asymmetry showing left-sided predominance will define the rightward movement of the heart, as is seen in the normal embryo. Once defined, looping direction appears not to change, even if flectin expression and localization is altered from its normal pattern more posteriorly in the same heart. If the asymmetry is altered to show right-sided predominance in more anterior regions, the hearts then show leftward looping. Based on our analyses, the left–right protein difference in expression does not have to be large. Apparently, a small instability on one side relative to the other is all that is needed for looping directionality to be defined. That the dorsal mesocardium has an important role in looping was suggested earlier based on biomechanical and mathematical concepts (Taber et al., 1995). Once directionality is established, the extracellular membrane of splanchnic mesoderm closely apposed to the myocardial wall may aid in facilitating the bending of the heart tube in the direction already prescribed.

Pitx2 appears to be able to modulate flectin expression levels and localization patterns indirectly by affecting the timing of flectin expression. Analysis of in ovo, untreated embryos indicates that asymmetry is due to a timing delay of protein synthesis on one side relative to the other: Normally, flectin is first expressed on the left side in a localized region, and later a wave of lower level of expression progresses across the midline in some unknown manner to the right, as shown diagrammatically, to result in the observed asymmetric expression (Fig. 8A; also see Figs. 1A and 1B). Thus, Pitx2 may activate another intermediary molecule shown as “X,” or alternatively, represses a repressor “X” that is present in both fields or is only present at the midline, which then activates/derepresses flectin expression on the contralateral side. Because Pitx2 is in the left heart field, this trigger occurs in the left field first and then is relayed to the right. This type of left–right relay in signaling results in a sufficient delay to produce the observed asymmetry of flectin. If Pitx2 is misexpressed in the right field early, then timing of flectin expression would occur earlier on both sides to result in either nearly equal expression on both sides of the heart wall/mesocardium, or more expression on right to the extent it allows looping direction to be altered. When antisense treatment decreases Pitx2 expression on left, then this allows the endogenous right-sided expression to become dominant and can result in leftward looping. Since both antisense and retroviral approaches in chick embryos do not always result in complete gain-of-function or loss-of-function of expression, this suggests that Pitx2 function is dependent on specific threshold concentrations being reached to properly exert its effect. Phenotypic characteristics of Rieger Syndrome in humans, where Pitx2 is mutated, are considered to be due to haplo-insufficiency, which would also argue for the importance of maintaining adequate levels of Pitx2.

Not only does Pitx2c misexpression alter directionality of heart looping, but it also can alter positioning of the foregut from its normal position slightly off the midline to far more lateral left or right positions with respect to the embryo midline. This occurs independently from the positioning of the notochord underlying the neural tube, which can be used as a marker for the embryo midline. The heart, however, positions itself relative to the midline of the ventral floor of the foregut. Possibly the left expression of Pitx2c in the splanchopleura of the heart and gut fields not only determines leftness within the heart and gut position, but also serves to define the foregut midline boundary within a specific range of its concentration. Any misexpression of these molecules, as shown here with Pitx2c, that alters the normal balance of concentrations on one side relative to the other can shift the boundary to one side or the other of the embryo midline. The foregut may align itself within a specific concentration range of laterality specifying molecules. The myocardium and endocardium, responding to specific signals emanating from the midregion of the ventral floor of the foregut, become aligned relative to that region. Although a morphological effect on the midline and positioning of the foregut is apparent, whether a midline molecule is affected by altering normal Pitx2 concentrations remains unknown. Midline signaling by the ventral floor of the foregut, as it forms in an anterior/posterior direction, is superimposed upon left/right fields to regulate subsequent steps (see also Bisgrove et al., 2000). Candidate midline molecules shown to affect looping are GATA-4 (Ghatpande et al., 2000; Molkentin et al., 1997) and CFC (Schlange et al., 2001).

Heart looping is a complex, multifactorial process. Our analysis of this process has made use of an extracellular matrix protein flectin as a marker to follow downstream events related to heart looping in the chick embryo. Differential regulation of the timing of molecular expression in a left–right (L-R) manner in the heart fields, as shown here for flectin, achieves bilateral asymmetry within a few hours and provides a means of coordinating complex processes with possibly fewer numbers of molecules than otherwise would be necessary. In the heart fields, the timing of cell differentiation in an anterior–posterior wave is superimposed upon the timing of expression of ECM and cellular components in a L-R manner to achieve at times subtle, but definite, left–right differences of protein expression (see also, in mouse, Tsuda et al., 1998). Concomitantly, the timing of mesocardial attachment and detachment areas occurs in a dorsal–ventral (D–V) manner. Heart development, from its early induction by the anterior mesoderm, is tied closely with development of the foregut throughout looping. Thus, key morphoregulatory molecules are being...
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