Folate rescues lithium-, homocysteine- and Wnt3A-induced vertebrate cardiac anomalies

Mingda Han1,*, Maria C. Serrano1,*, Rosana Lastra-Vicente1,*, Pilar Brinez1, Ganesh Acharya2, James C. Huhta1, Ren Chen3 and Kersti K. Linask1,‡

SUMMARY
Elevated plasma homocysteine (HCy), which results from folate (folic acid, FA) deficiency, and the mood-stabilizing drug lithium (Li) are both linked to the induction of human congenital heart and neural tube defects. We demonstrated previously that acute administration of Li to pregnant mice on embryonic day (E)6.75 induced cardiac valve defects by potentiating Wnt-β-catenin signaling. We hypothesized that HCy may similarly induce cardiac defects during gastrulation by targeting the Wnt-β-catenin pathway. Because dietary FA supplementation protects from neural tube defects, we sought to determine whether FA also protects the embryonic heart from Li- or HCy-induced birth defects and whether the protection occurs by impacting Wnt signaling. Maternal elevation of HCY or Li on E6.75 induced defective heart and placental function on E15.5, as identified non-invasively using echocardiography. This functional analysis of HCY-exposed mouse hearts revealed defects in tricuspid and semilunar valves, together with altered myocardial thickness. A smaller embryo and placental size was observed in the treated groups. FA supplementation ameliorates the observed developmental errors in the Li- or HCy-exposed mouse embryos and normalized heart function. Molecular analysis of gene expression within the avian cardiogenic crescent determined that Li, HCY or Wnt3A suppress Wnt-modulated Hex (also known as Hhex) and Islet-1 (also known as Is1l) expression, and that FA protects from the gene misexpression that is induced by all three factors. Furthermore, myo-inositol with FA synergistically enhances the protective effect. Although the specific molecular epigenetic control mechanisms remain to be defined, it appears that Li or HCY induction and FA protection of cardiac defects involve intimate control of the canonical Wnt pathway at a crucial time preceding, and during, early heart organogenesis.

INTRODUCTION
The heart is the first organ to develop in the embryo and different regions of the heart, including the right and left ventricles and the outflow tract, are already specified at the gastrulation stages. The development is complex, involving two heart fields and the sequential activation of multiple signaling pathways together with regulation of genetic cascades in the cardiogenic crescent. Genetic and environmental effectors can severely impact these pathways and lead to abnormal development. Two factors, namely an elevated level of the metabolic intermediary homocysteine (HCY) and the therapeutic drug lithium (Li), have been a focus of birth defect research for over 30 years. Low dietary folate (folic acid, FA) and mutations in methylenetetrahydrofolate reductase (MTHFR) lead to elevated maternal plasma HCY levels. Elevated HCY increases the risk for neural tube, neural crest, craniofacial and congenital heart defects in the offspring (Boot et al., 2004; Huhta et al., 2006; Rosenquist et al., 1996; Tang et al., 2004). The association with elevated HCY is particularly strong for specific outflow tract defects, including pulmonary valve stenosis, coarctation of the aorta, and aortic valve stenosis (Huhta et al., 2006). Maternal Li therapy for bipolar disorder is similarly associated with neural, skeletal, craniofacial and cardiac valve defects, specifically of the tricuspid valve in association with Ebstein’s anomaly (Iqbal and Mahmud, 2001; Yonkers et al., 2004). Despite Li and HCY being linked with neural tube and heart defects, the etiology of the induced congenital defects associated with these chemicals has remained unknown.

With a long-term focus on defining heart developmental mechanisms and, specifically in relation to β-catenin, the important downstream intermediary of Wnt signaling (Linask, 1992; Linask et al., 1997), Li and HCY became specific targets of our studies to define the underlying mechanisms of certain cardiac birth defects and to understand the molecular origins. We observed that the phenotypic defects induced after a single exposure to Li during gastrulation (Chen et al., 2008) are similar to those reported by others for elevated HCY in a transgenic mouse model (Tang et al., 2004). Because of the similarities in the cardiac and neural anomalies induced by either HCY or Li exposure, and because Li is known to mimic Wnt-β-catenin signaling, we hypothesized that both HCY and Li may target canonical Wnt signaling during the same early developmental window, but that this modulation of the pathway occurs at different regulatory levels.

Clinically, Li continues to be considered as only a modest risk factor for the development of birth defects with first-trimester embryonic exposure (Cohen et al., 1994; Jacobson et al., 1992). Our experimental evidence, relating peak cardiac defects to an early timing of exposure during vertebrate gastrulation stages (Chen et al., 2008; Mansastry et al., 2006), means that the most susceptible exposure period for heart defects occurs before most women are aware they are pregnant. Moreover, evidence published since the mid-1990s suggests that the risk of birth defects arising from Li exposure during early pregnancy is high and can result in early embryonic lethality. This evidence includes: (1) Li mimics canonical Wnt signaling by inhibiting glycogen synthase kinase-3 (GSK-3)
defects indicates that FA metabolism intersects with canonical Wnt pathway in early cardiogenesis and neurogenesis. This pulse of both potentiate the canonical Wnt pathway, which is a crucial models, demonstrate that the two dissimilar factors Li and HCy exposure. Our results, which are based on two different vertebrate species, that were exposed to elevated HCy (50 μM) displayed cardiac abnormalities, which were defined by immunolocalization of sarcomeric myosin heavy chain using MF20 antibody (Fig. 1). Exposure at HH stages 6 and 7 led to normal development. The main embryonic defects related to the timing of embryonic exposure; the regions that were primarily affected related to an anterior-to-posterior (AP) wave of development along this embryonic axis. The exposure effects ranged from early effects (e.g. no cardiac tissue, heart development occurring anterior to neural tissue, cardiabifida) to later effects, such as wide hearts and left looping (Table 1). The cardiac phenotypes observed with early HCy exposure were similar, although not identical, to those obtained from our studies of Li exposure on avian heart development (Manisastry et al., 2006).

Avian model
Hcy or Li exposure in avian embryos during Hamburger-Hamilton (HH) stages 3+ to 5 induced similar heart defects
The peak time of sensitivity corresponded to HH stages 3+/4 to stage 5, in which 78% (n=128) and 68% (n=74) of embryos, respectively, that were exposed to elevated HCy (50 μM) displayed cardiac abnormalities, which were defined by immunolocalization of sarcomeric myosin heavy chain using MF20 antibody (Fig. 1). Exposure at HH stages 6 and 7 led to normal development. The main embryonic defects related to the timing of embryonic exposure; the regions that were primarily affected related to an anterior-to-posterior (AP) wave of development along this embryonic axis. The exposure effects ranged from early effects (e.g. no cardiac tissue, heart development occurring anterior to neural tissue, cardiabifida) to later effects, such as wide hearts and left looping (Table 1). The cardiac phenotypes observed with early HCy exposure were similar, although not identical, to those obtained from our studies of Li exposure on avian heart development (Manisastry et al., 2006).

Hcy and Li suppress Hex and Islet-1 (Isl1) gene expression in the avian heart fields
To extend our previous finding that Li suppresses Hex gene expression in the chick primary heart fields (Chen et al., 2008), we analyzed and compared the effects of HCy on the patterning of

Fig. 1. Effects of exogenous Hcy on early chick heart development after a 24-hour incubation. Immunolocalization of MF20 defines the presence of cardiac tissue (arrows). (A,B) Two extremes of cardiabifida: in A, two small cardiogenic regions are differentiating bilaterally; in B, the two heart fields have moved close to the midline, are almost touching, and are ready to fuse. (C) Light microscopic view of an embryo displaying a severely truncated neural tube with cardiac tissue differentiating cephalad to the neural area (D). (E) Bright-field ventral view of a normal, control, right-looping heart and (F) fluorescence view showing MF20 localization in the heart shown in E. In all panels, the embryonic anterior is at the top. Bars, 300 μm.
cardiac gene expression. The avian model allows for more precise targeting of exposure to specific early stages of development. Mouse embryos within a litter can vary widely in their developmental stages, leading to misinterpretation of results when, at the time of the acute exposure, the window of susceptibility for some embryos has already passed. In the Li study, we detected that the viable defects in the mouse embryo were related to derivatives of the SHF. Therefore, we also analyzed the effects of HCY and Li exposure on \textit{Isl1} expression, a SHF marker and one that is modulated by β-catenin in the canonical Wnt signaling pathway (Cai et al., 2003; Lin et al., 2007).

Chick embryos (HH stages 3+/4) were incubated on agarose-albumin supplemented with HCY (50 μM). Instead of giving a single injection in ovo, embryos were exposed to HCY for 8 hours in order to define the effect on early cardiac gene expression. Another group of embryos were exposed to HCY for 24 hours at which time the effects on gene expression were determined. To analyze the effects during the looping stages, incubation was stopped after between 22 and 24 hours. After the 8-hour incubation, Li and HCY suppressed \textit{Hex} and \textit{Isl1} expression in the cardiogenic crescent (Fig. 2). In the untreated control embryo, \textit{Hex} expression was apparent in the Hensen’s node region, anteriorly in the prechordal plate, and extending laterally (Fig. 2A). By 24 hours, \textit{Hex} was still apparent in the anterior intestinal portal (AIP) endoderm in a looping HH stage 14 heart (Fig. 2B). Exposure to either Li (Fig. 2C) or HCY (Fig. 2E) for 8 hours decreased \textit{Hex} expression in the prechordal plate. After exposure to Li for 24 hours, \textit{Hex} was expressed in the AIP and cardiogenic regions of a developmentally delayed embryo (Fig. 2D). After exposure to HCY for 24 hours, the embryo had a looping heart with a truncated neural tube, and the AIP region displayed only diffuse \textit{Hex} expression (Fig. 2F). By contrast, the control embryo had developed to a late-looping stage (Fig. 2B). If HCY or Li exposure occurred at HH stages 6 or 7, \textit{Isl1} expression was already activated and was similar to control embryos (not shown). In summary, 8 hours of Li or HCY exposure during the early stages of chick development repressed \textit{Hex} and \textit{Isl1} expression. By 24 hours, \textit{Isl1} gene expression had recovered but heart development and neural tube development were abnormal and remained delayed.

FA protects against HCY- and Li-induced adverse effects in early chick cardiac development

Since FA can partially rescue HCY-induced neural- and neural crest-related defects in the mouse (Zhu et al., 2007), we determined whether cardiac defects could be rescued in the avian culture system. FA-supplemented medium rescued HCY- and Li-induced heart defects in embryos that were exposed between HH stages 3 and 5 (Table 2). At HH stages 3 and 4, FA supplementation at 10 μg/ml provided protection in embryos exposed to HCY (50 μM) or Li, with 54% and 48% of HCY- and Li-exposed embryos, respectively, displaying normal heart development. Without FA supplementation, only 22% and 28% of HCY- and Li-exposed embryos, respectively, had normal heart development. Of the HH stage 3/4 control embryos that had been treated with physiological saline, 48% showed normal development.

There was an association between the severity of defects and the developmental stage at exposure. HCY exposure (50 μM) beginning at HH stage 5 resulted in normal cardiac development in 32% of embryos. With FA supplementation, 73% of HH stage 5 embryos had normal cardiac development. In summary, FA rescued cardiac defects, but was less effective at the early stages of development when the HCY- or Li-induced defects would be embryonic lethal.

Inclusion of FA and inositol is additive, and increases the percentage of Li-exposed embryos that display normal development

In addition to mimicking canonical Wnt signaling, Li modulates phosphatidylinositol signaling (Belmaker et al., 1998). We thus...
FA rescues Li-, HCy- and Wnt3A-induced misexpression of *Hex* and *Isl1* at the gastrulation stages

We analyzed whether FA rescues the downregulation of *Hex* and *Isl1*. Chick embryos were exposed to Li, HCy or Wnt3A, as above, with and without 10 μg/ml of FA; the experiments were terminated after either an 8-hour or 24-hour exposure period, at which time in situ hybridizations for gene expression were performed. After 8 hours of exposure, Li, HCy and Wnt3A all suppressed *Hex* (Fig. 5D,G,J) and *Isl1* (Fig. 6D,G,J) compared with the control embryonic expression patterns (Fig. 5A; Fig. 6A). FA supplementation for 8 hours resulted in normalized gene expression patterns and the expression was at higher levels than in control embryos [Fig. 5B,E,H,K (for *Hex*); Fig. 6B,E,H,K (for *Isl1*)]. By 24 hours, normal beating hearts had formed and normal *Hex* (Fig. 5C,F,I,L) and *Isl1* (Fig. 6C,F,I,L) gene expression was apparent in the developing atrial region in the SHF. Thus, FA supplementation suppressed the potentiation of the inhibitory Wnt signaling, allowing for normal induction of *Hex* and *Isl1* gene expression.

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**Table 2. FA or inositol supplementation increases the percentage of normal heart development**

<table>
<thead>
<tr>
<th>HH stage</th>
<th>Treatment*</th>
<th>Number of embryos</th>
<th>Abnormal embryos</th>
<th>Normal embryos</th>
<th>Normal hearts (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3+/4</td>
<td>Control (NaCl)</td>
<td>87</td>
<td>45</td>
<td>42</td>
<td>48%</td>
</tr>
<tr>
<td>3+/4</td>
<td>HCy</td>
<td>128</td>
<td>100</td>
<td>28</td>
<td>22%</td>
</tr>
<tr>
<td>3+/4</td>
<td>HCy + FA</td>
<td>57</td>
<td>26</td>
<td>31</td>
<td>54%</td>
</tr>
<tr>
<td>5</td>
<td>Control (NaCl)</td>
<td>42</td>
<td>11</td>
<td>31</td>
<td>74%</td>
</tr>
<tr>
<td>5</td>
<td>HCy</td>
<td>74</td>
<td>50</td>
<td>24</td>
<td>32%</td>
</tr>
<tr>
<td>5</td>
<td>HCy + FA</td>
<td>22</td>
<td>6</td>
<td>16</td>
<td>73%</td>
</tr>
<tr>
<td>3+/4</td>
<td>FA only</td>
<td>11</td>
<td>5</td>
<td>6</td>
<td>55%</td>
</tr>
<tr>
<td>3+/4</td>
<td>Inositol only</td>
<td>5</td>
<td>2</td>
<td>3</td>
<td>60%</td>
</tr>
<tr>
<td>3+/4</td>
<td>Li only</td>
<td>29</td>
<td>21</td>
<td>8</td>
<td>28%</td>
</tr>
<tr>
<td>3+/4</td>
<td>Li + FA</td>
<td>40</td>
<td>21</td>
<td>19</td>
<td>48%</td>
</tr>
<tr>
<td>3+/4</td>
<td>Li + inositol</td>
<td>30</td>
<td>18</td>
<td>12</td>
<td>40%</td>
</tr>
<tr>
<td>3+/4</td>
<td>Li + FA + inositol</td>
<td>34</td>
<td>9</td>
<td>25</td>
<td>74%</td>
</tr>
</tbody>
</table>

*Concentrations used: HCy, 50 μM; Li, 50 μM; FA, 10 μM; inositol, 50 mM.

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Folate rescues Li-, HCy- and Wnt3A-induced cardiac defects

Since both HCy and Li seemed to modulate the canonical Wnt pathway during the cardiac specification stages, we speculated and confirmed that FA should rescue the deleterious effects of canonical Wnt3A (Manisastry et al., 2006) on heart development (Figs 3 and 4).

The minimal effective doses of Wnt3A that induced cardiac and neural anomalies were 2 ng/ml and 10 ng/ml, respectively. Using these concentrations, we then analyzed whether FA supplementation of media at 10^3-fold higher concentrations, that is 2 μg/ml or 10 μg/ml, would provide protection against the augmentation of canonical Wnt3A signaling. With only Wnt3A exposure at HH stages 3+/4, 18% of embryos had normal hearts; Wnt3A media supplemented with FA rescued normal heart development (54%) to near control levels (58%) (Figs 3 and 4). A normal looping control heart is shown with MF20 immunolocalization of sarcomeric myosin heavy chain (Fig. 4A). The addition of FA alone, at a concentration of 2 μg/ml or 10 μg/ml, resulted in normal heart development (Fig. 4B,C, respectively). Embryos exposed to 2 ng/ml of Wnt 3A (Fig. 4D) displayed incomplete cardiac tube formation, whereas those exposed to 10 ng/ml displayed cardiabifida (Fig. 4G). Wnt3A (2 ng/ml) in the presence of 2 μg/ml (Fig. 4E) or 10 μg/ml (Fig. 4F) of FA supplementation resulted in normal heart development. At the higher Wnt 3A concentration (10 ng/ml) (Fig. 4G-I), FA supplementation at the 2 μg/ml level provided only partial rescue of tube formation (Fig. 4H), and left-looping hearts were observed occasionally. The higher FA concentration (10 μg/ml) also rescued heart development in the high Wnt3A exposure group (Fig. 4I). To summarize, FA suppressed the effects of direct Wnt3A potentiation of canonical Wnt signaling and normalized development.
Mouse model

Doppler ultrasound assessment of Li and HCY exposure on embryonic mouse heart function

To determine whether our results in the avian system would extend to the mammalian system, we conducted studies in the mouse embryo. We studied the effects of increased maternal HCY serum levels by acute targeting of the same E6.75 developmental window that induced cardiac defects with Li exposure. Doppler ultrasound was used to monitor non-invasively the effects on heart and placental function.

The maternal and embryonic hemodynamic variables measured by Doppler ultrasonography on E15.5 embryos are shown in Fig. 7, and a comparison between the HCY and Li echo data is provided in Tables 3 and 4. The significant differences are highlighted below.

Li group compared with control group. Based on the median values shown in Table 3, the maternal heart rate was higher and the uterine artery pulsatility index was lower in the Li group versus the control group. With Li exposure, 63% (n=43/68) of embryos displayed valve regurgitation (Fig. 7A, normal pattern; Fig. 7B-D, abnormal pattern); semilunar (SL) valve regurgitation (Fig. 7C) occurred in 34 (50%) of the embryos, and both SL and atrioventricular (AV) valve regurgitation occurred in 9 (13%) of the embryos. The increased atrial contractility may be a compensatory mechanism, since compliance of the ventricles was reduced.

HCY group compared with control group. Except for the structural and valve defects described below, there were few statistically significant differences in myocardial performance between the HCY and control groups. Fig. 7E-M depicts the typical waveforms that were obtained. Thirty-nine (66%) embryos exposed acutely to HCY displayed valve regurgitation. SL valve regurgitation occurred in 53% of embryos, AV valve regurgitation in 10%, and both AV and SL valve regurgitation occurred in 3% of embryos. All control animals had normal Doppler patterns (Fig. 7A). In summary, cardiac valve defects developed at a similar rate in embryos following a single exposure to Li or HCY on E6.75.
Based on statistically significant differences, acute Li exposure results in poorer myocardial performance than that observed with HCY.

Following echo monitoring on E15.5, placental weight and morphometric measurements of crown-rump length and body weight were obtained. With a single exposure to Li or HCY, all three morphometric measurements were significantly decreased compared with control embryos (Table 4). There were also significantly more resorptions with HCY (46%, n=27/59 embryos) than with Li exposure (24%, n=16/68 embryos). Some litters were allowed to develop to E18.5. No recovery of development was apparent and the same valve defects were seen on E18.5 (data not shown) compared with those seen earlier in gestation, indicating that it was not only a delay in valve development that was monitored on E15.5.

Pathological assessment of HCY-exposed embryonic hearts
On E15.5 (Fig. 8A-C, control heart), HCY exposure was associated with right and left side cardiac hypertrophy, as evidenced by an increase in myocardial wall thickness (Fig. 8D-L). The lumen often had a spongy appearance and was not distinctly evident. In embryos that displayed AV valve regurgitation upon echo monitoring, most tricuspid valve leaflets had not formed normally: the septal leaflet had not delaminated and it remained attached to a wide interventricular septum (compare the normal AV valves in Fig. 8A with the AV valve anomalies that are apparent in Fig. 8D,G). The embryo shown in Fig. 8A-C displayed an echo pattern indicating both AV and SL valve regurgitation. Malformed and small aortic valves (AoV in H) and pulmonary valves (PV in I) were apparent. The embryonic heart shown in the sections in Fig. 8J-L displayed...
Table 3. Comparison between maternal and embryonic hemodynamic parameters obtained by Doppler ultrasonography of mice at E15.5, after exposure at E6.75 to Li, HCY or NaCl (control)

<table>
<thead>
<tr>
<th>Maternal variables</th>
<th>HCY (n=59)</th>
<th>NaCl (n=42)</th>
<th>Li (n=68)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate (beats/minute)</td>
<td>458</td>
<td>421</td>
<td>491 †</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Uterine artery PI</td>
<td>2.62</td>
<td>2.77</td>
<td>2.12 ‡</td>
<td>0.0001*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Embryonic variables</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate (beats/minute)</td>
<td>181</td>
<td>169</td>
<td>195 †</td>
<td>0.0004*</td>
</tr>
<tr>
<td>Umbilical artery PI</td>
<td>1.55</td>
<td>1.68</td>
<td>1.76</td>
<td>NS</td>
</tr>
<tr>
<td>Ductus venosus PI</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>NS</td>
</tr>
<tr>
<td>Descending aorta PI</td>
<td>1.99</td>
<td>1.94</td>
<td>2.11</td>
<td>NS</td>
</tr>
<tr>
<td>ICT (%)</td>
<td>6.56</td>
<td>7.27</td>
<td>7.54</td>
<td>NS</td>
</tr>
<tr>
<td>IRT (%)</td>
<td>11.93</td>
<td>12.30</td>
<td>16.82 †</td>
<td>0.0025*</td>
</tr>
<tr>
<td>ET (%)</td>
<td>42.51</td>
<td>41.71</td>
<td>41.26</td>
<td>NS</td>
</tr>
<tr>
<td>MPI</td>
<td>0.44</td>
<td>0.48</td>
<td>0.60 †</td>
<td>0.0049*</td>
</tr>
<tr>
<td>E velocity (cm/second)</td>
<td>12.25</td>
<td>11.32</td>
<td>11.46</td>
<td>NS</td>
</tr>
<tr>
<td>A velocity (cm/second)</td>
<td>40.14</td>
<td>37.27</td>
<td>41.36 †</td>
<td>0.0214*</td>
</tr>
<tr>
<td>E/A ratio</td>
<td>0.30</td>
<td>0.31</td>
<td>0.27 ‡</td>
<td>0.0065*</td>
</tr>
<tr>
<td>OF peak velocity (cm/second)</td>
<td>39.92</td>
<td>36.56</td>
<td>41.05 †</td>
<td>0.0476*</td>
</tr>
</tbody>
</table>

All data represent median values.
*Parameters showing significant values for Li when compared with the control group, as based on the non-parametric Kruskal-Wallis test.

FA supplementation results in normal cardiac function in Li- and HCY-exposed mouse embryos

We next determined whether rescue of normal cardiac function and valve defects is possible using FA supplementation with Li or elevated HCY exposure. Pregnant females were administered concomitantly with FA (125 μl at 75 μM) and HCY (75 μM), both intraperitoneally (i.p.), on E6.75. FA supplementation resulted in normal valve development and cardiac function, as determined by echocardiography in the HCY-exposed group (100%, n=27/27 embryos). However, FA supplementation provided on E6.75 normalized the effects of HCY, but not Li, exposure. Protection from Li exposure was obtained when FA was administered in the diet at 10 mg/kg throughout gestation, beginning with the presence of the vaginal plug on the morning of E0.5. Cardiac development then remained protected following Li exposure on E6.75. Normal valve formation and cardiac function were observed in 40 out of 41 embryos monitored (97.6%) (Table 4). The one embryo that did not have a normal echo pattern displayed mild SL valve regurgitation. In summary, FA rescued cardiac function in the mouse embryo after acute Li or HCY exposure; however, Li-induced defects required earlier and higher levels of FA supplementation for rescue.

DISCUSSION

Our results indicate that Li and elevated HCY pose a serious threat to embryonic heart development, and that heart defects can be induced by only a single exposure during gastrulation and cardiac specification. In the early embryo, Wnt–β-catenin signaling in the cardiogenic crescent is required to maintain the undifferentiated state of cells. A key downstream intermediary of the active pathway is β-catenin, which accumulates in the cytoplasm and translocates to the nucleus to activate target genes. Preceding specification, the mesendoderm of the bilateral heart fields begins to express the Wnt antagonists Crescent and Dickkopf-1 (Dkk-1). These antagonists suppress canonical Wnt signaling, resulting in decreased β-catenin levels. As a result, genes associated with the induction of cardiogenesis, such as Hex and Isl1, are upregulated. Hex is

Table 4. Summary of FA rescue of the Li or HCY effects on mouse heart development

<table>
<thead>
<tr>
<th></th>
<th>Valve regurgitation</th>
<th>CRL (mm)</th>
<th>Body weight (g)</th>
<th>Placenta weight (g)</th>
<th>Resorptions</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCY</td>
<td>39/59 (66.1%)</td>
<td>13</td>
<td>0.35</td>
<td>0.11</td>
<td>27/59 (45.7%)</td>
</tr>
<tr>
<td>Li</td>
<td>43/68 (63.2%)</td>
<td>13.8</td>
<td>0.37</td>
<td>0.11</td>
<td>16/68 (23.5%)</td>
</tr>
<tr>
<td>NaCl</td>
<td>0/42</td>
<td>15.2</td>
<td>0.41</td>
<td>0.13</td>
<td>0/42</td>
</tr>
<tr>
<td>HCY + FA rescue</td>
<td>0/27</td>
<td>14.8</td>
<td>0.42</td>
<td>0.12</td>
<td>0/27</td>
</tr>
<tr>
<td>Li + FA rescue</td>
<td>1/30 (3.3%)</td>
<td>14.6</td>
<td>0.40</td>
<td>0.11</td>
<td>1/30 (3.3%)</td>
</tr>
</tbody>
</table>

CRL, crown-rump length.
expressed in the prechordal plate and primary heart field and Islet-1, a marker for the SHF, is synthesized (Cohen et al., 2007; Foley and Mercola, 2005). Both Li and HCy act to augment canonical Wnt signaling intracellularly, and are able to bypass the extracellular Dkk-1 or Crescent antagonism. Therefore, both Li and HCy exposure cause the Wnt–β-catenin pathway to remain active at a time when it would normally be downregulated in the early embryo. Thus, Li and HCy repress and delay the induction of Hex and Islet-1.

An association between HCy and FA deficiency and the Wnt–β-catenin pathway has been suggested previously from microarray analysis (Ernest et al., 2006; Liu et al., 2007). Additionally, analysis of tumorigenesis indicates that histone and DNA methylation are fundamental processes in the Wnt–β-catenin pathway and in gene target regulation (Sierra et al., 2006; Wohrle et al., 2007), including by β-catenin, adenomatous polyposis coli (APC) and Dkk-1 regulation (Csepregi et al., 2007; Willert and Jones, 2006). HCy and FA are central molecules in the synthesis path for S-adenosylmethionine (SAM), the universal methyl donor for biological methylation. FA deficiency causes HCy accumulation and produces a cellular deficit of SAM, thus limiting transmethylation reactions in the nucleus (Williams and Schalinske, 2007). Since β-catenin/lymphoid-enhancer factor (LEF) also regulates Islet-1 expression (Lin et al., 2007), we suggest that elevated HCy may, through an epigenetic mechanism involving methylation, modulate the Wnt–β-catenin pathway and its target gene expression. As a corollary, FA rescue of the effects of Li and Wnt3A could be either at the level of methylation of β-catenin nuclear-binding proteins and/or at the level of DNA methylation, which affects the expression and synthesis of the antagonist Dkk-1. Either possibility can lead to potentiation of β-catenin target genes.

Li action starts in the cytoplasm but, through β-catenin, it also has a nuclear effect on gene expression. Li potentiates Wnt–β-catenin signaling by inhibiting GSK-3 and thereby stabilizing cytoplasmic β-catenin. In addition, Li suppresses the inositol-signaling pathway by inhibiting inositol monophosphatase and inositol polyphosphate 1-phosphatase (Belmaker et al., 1998). Wnt3A triggers G-protein-linked phosphatidylinositol signaling, transiently generating inositol polyphosphates, including inositol pentakisphosphate (IP_5, also known as Ins(1,3,4,5,6)P_5). IP_5, in turn, inhibits GSK-3β activity (Gao and Wang, 2007). Blocking IP_5 formation blocks β-catenin accumulation (Gao and Wang, 2007). Excess inositol may decrease IP_5 formation by increasing the generation of di- and tri-polyphosphates. The association between the inositol phosphatidyl pathway and its intermediates with canonical Wnt signaling would explain the level of severity and the
varied phenotypic cardiac defects that are induced by Li exposure in comparison with HCY. The additive effect of myo-inositol and FA in protection from Li exposure suggests the involvement of this second messenger pathway in Wnt signaling. We propose that the FA effect occurs primarily at the nuclear level by protecting transmethylation reactions and by replenishing nucleotide pools, whereas the inositol effect is cytoplasmic, involving inositol polyphosphate signaling. As a result, the protective effects of FA and myo-inositol are additive.

In addition to the cardiac hypertrophy, the viable cardiac valve anomalies seen on E15.5 following a single HCY exposure on E6.75 are related primarily to derivatives of the SHF (i.e. the outflow, the right ventricle, and the tricuspid and SL valves). In the embryonic heart, the valves are derived from the endocardial cushions, including in the outflow (Anderson et al., 2003). Expression of Isl1, a marker for the SHF, is suppressed for a time period by a pulse of Li or HCY, suggesting that the tricuspid and SL valve structures are affected downstream because these structures are derivatives of the right ventricle and outflow, respectively. Published evidence, using regulatory elements from the mouse Mef2c gene to direct the expression of Cre recombinase exclusively in the anterior heart field (AHF) and in its derivatives, indicates that the AHF enhancer is active in the future right ventricle, which includes the tricuspid valve region, and in the outflow tract, including the pulmonary valve (Verzi et al., 2005). Verzi et al. also reported that the endocardial endothelial cells of the right ventricle and outflow tract, which contribute to cushion formation, are marked by the Mef2c-AHF-Cre transgene. This cell lineage analysis showed that near the base of the heart, both Wnt1-Cre- and Mef2c-AHF-Cre-derived cells contributed to the smooth muscle cells of the pulmonary trunk and to the cells of the pulmonary valve leaflets. Wnt signaling plays an important role in controlling endothelial-to-mesenchymal transition (EMT) and in proliferation of the endocardial cushion and valve leaflets. Wnt signaling may be a target for other environmental factors that induce cardiac and neural birth defects, for example alcohol (ethanol). Since epigenetic processes are sensitive to change, they may have implications for FA protection as well. Additionally, Wnt-β-catenin signaling may be a target for other environmental factors that induce cardiac and neural birth defects.

METHODS
Vertebrate models and dose
Chick embryos
Pathogen-free White Leghorn chick (Gallus gallus; Charles River, MA) and quail (Coturnix coturnix japonica; Strickland Farms, GA) embryos were used to analyze the effects of HCY on early embryogenesis. The early processes of heart development are well conserved among vertebrates and the avian model allows for more precise timing of exposure in early embryos. The incubation methodology using agarose-albumin has been described previously (Darnell and Schoenwolf, 2000). Briefly, the experimental paradigm involved exposing specific early stages of chick embryos, at HH stages 4 to 7 (Hamburger and Hamilton, 1951), to different concentrations of HCY (L-homocysteine thiolactone hydrochloride, Sigma) made up in physiological saline. Final concentrations of HCY included 30 μM, 50 μM, 75 μM and 100 μM. The minimal teratogenic dose was determined empirically to be 50 μM. This fitted within the concentration range of 30 μM to 300 μM used by others that induced heart defects at later stages of development (Boot et al., 2004). Control embryos were incubated using the vehicle, physiological saline, in the agarose-albumin medium. Control and experimental cultures were incubated on the agarose-albumin plates for either 8 or 24 hours.
Carrier-free, recombinant mouse Wnt3A was purchased from R&D Systems (Minneapolis, MN). We empirically tested Wnt3A concentrations to find those that would induce cardiac defects but maintain embryonic viability. Wnt3A was tested at concentrations of 2 ng, 10 ng, 20 ng, 50 ng and 100 ng per ml. Concentrations of 2 ng/ml and 10 ng/ml were both effective in inducing cardiac defects. Both Wnt3A concentrations were used in the FA rescue experiments, as described. For the FA rescue experiments, folate (folic acid, Sigma-Aldrich) was added at a concentration of 2 μg, 5 μg or 10 μg per ml. In subsequent experiments, we used 10 μg/ml FA, which consistently rescued the heart defects induced by Li, HCy or Wnt3A in the avian model. For the experiments using inositol (myo-inositol, Sigma) supplementation, a concentration of 50 mg/ml was used.

In situ hybridization. In situ hybridizations on control and experimentally manipulated chick embryos were carried out using digoxigenin-labeled riboprobes with alkaline phosphatase detection (Linask et al., 2001). Images of whole-mounted chick embryos were taken with a Nikon DS-L2 camera unit. Probes for chick Hes were provided by Parker Antin, University of Arizona and probes for chick Iset-1 (Isl1) were provided by Thomas Jessell, Columbia University, NY.

Mice

All mice were maintained according to protocols approved by the Institutional Animal Care and Use Committee at the University of South Florida. The C57BL/6 mouse strain (Jackson Laboratories, Bar Harbor, ME) was used throughout this study. E0.5 was defined as the morning when the vaginal sperm plug was detected. Timed pregnant mice were randomly allocated to receive a single dose of 100 μl of 6.25 mg/ml Li chloride, as determined previously (Chen et al., 2008), 100 μl of 75 μM HCy, or 100 μl of 6.25 mg/ml sodium chloride (control group); all treatments were given i.p. On E15.5, the utero-placental circulation of the pregnant mice, and the central and peripheral circulations of the embryos were examined non-invasively in utero using Doppler ultrasonography (Giui et al., 1996; Linask and Huhta, 2000). Sixty-eight embryos were exposed to Li, 59 to HCy and 42 to physiological saline.

An HCy concentration of 75 μM, used for maternal exposure, was determined empirically using different doses of HCy (i.e. 150, 75, 50 and 15 μM). The minimal teratogenic dose was determined to be 75 μM. An acute, single dose of HCy at E5.5 resulted in embryonic lethality (79% of embryos; there were 11 resorptions and 3 small, but viable, embryos). The single i.p. injection of HCy at E6.75 produced a high number of valve defects (66%), as well as resorptions (46%). An i.p. injection on E7.75 did not cause any valve defects (n=16). Because not all embryos within litters are at exactly the same stage of development, the variability of effects seen with either Li or HCy probably reflects the differences in developmental stage. Hence, the variable effects that were observed on heart and valve development were expected.

FA diet for rescue of cardiac defects in mouse embryos

We supplemented animal chow with 10.5 mg/kg of FA, which was the concentration used in trial human population studies. As a control diet (i.e. normal mouse chow), mice continued to receive 3.3 mg/kg of FA as the baseline to maintain the health of the female mice. The baseline supplementation does not rescue cardiac defects. The animal chow supplemented with 10.5 mg/kg of FA was prepared for us by Harlan Laboratories. As recommended by Harlan, our calculations for the FA level in the special diet are based on the metabolic body weight of mice. Because of the obvious large difference in body weight between humans and mice, and because of the large difference in metabolic rate, Harlan use the metabolic weight as a method of scaling these differences between species; for mice this was calculated to be BW^{0.75} (i.e. metabolic weight equals body weight to the 0.75 power). Each mouse consumes approximately 4 g of chow per day; therefore, to obtain the desired dose of 10.5 mg/kg, we added 7.2 mg/kg of FA to the normal 3.3 mg/kg that is present in commercial chow. Pregnant mice were divided randomly into the experimental group that was supplemented with 10.5 mg/kg of FA and the control group of mice that did not receive any additional FA. On the morning of the plug date (E0.5), the pregnant mice were placed on the defined Harlan chows and maintained on this diet throughout the study. On E6.75 (at 15:30 PM) all pregnant females received Li (6.25 mg/125 μl), by an i.p. injection, which consistently induced valve and heart defects, as detected by echo on E15.5.

Doppler ultrasonography

Doppler ultrasonographic examinations were carried out using the Philips Sonos 5500 (Andover, MA) with a 12 MHz transducer and, more recently, with a Vevo 770 (VisualSonics) system. Both instruments provided similar echo patterns. On E15.5, the pregnant mice were anesthetized using a Surgivet Tech 4 anesthesia system (Waunakesa, WI) with inhalation anesthesia, consisting of 3% isoflurane, administered through a nose cone; the mice were then maintained on 1% isoflurane with 21% oxygen. The mother was placed supine on a heating pad with electrode footpads. The heart rate and temperature of the pregnant mice were monitored using a THM100 (Indus Instruments, Houston, TX) during scanning. The body temperature was maintained at 37°C. Maternal uterine artery blood flow velocity waveforms were obtained and the pulsatility index was calculated. The embryos were visualized and their position mapped in each uterine horn. Blood flow in the heart and blood vessels was detected in each embryo using color Doppler, and blood flow velocity waveforms were obtained using pulsed wave Doppler. Once the embryonic heart was identified by color Doppler, the sample volume of the pulsed Doppler was placed over the entire heart to obtain blood velocities. The gate length was adjusted to completely insonate the beating embryonic heart. The high-pass filter was at its lowest setting of 50 Hz. The embryonic heart was examined from several angles of insonation to obtain the maximal velocities and inflow and outflow waveforms. The pulsatility index was calculated from the descending aorta, umbilical artery and ductus venosus blood flow velocity waveforms. The cardiac cycle time intervals were measured from the inflow-outflow velocity waveforms, and the index of global myocardial performance (Tei index) was calculated as: (ICT+IRT)/ET, where ICT is the isovolemic contraction time, IRT is the isovolemic relaxation time and ET is the ejection time (Fig. 7). The presence of any valvular regurgitation was recorded. Holosystolic AV valve regurgitation was defined as regurgitation that occurred from the closure to the opening of the AV valve and with a peak velocity higher than 50 cm/second. SL valve regurgitation was identified as the
diastolic blood velocity waveform that is usually superimposed on the inflow waveforms. During echo, although the diastolic regurgitation jet of the SL valve is usually superimposed on the inflow waveform, by manipulating the ultrasound transducer and sample volume of the Doppler gate it is possible to obtain the inflow waveforms in the same Doppler envelope, thus allowing the measurement of IRT. After the ultrasonographic examination, the female mouse was euthanized and the fetuses within the uterine horns were identified according to their location during the ultrasonographic examination. The embryos were removed and processed for paraffin sectioning.

Statistical analysis
Statistical analysis was performed using SAS version 9.1 (Cary, NC). We tested whether there was any significant difference among the three groups (global test) using the non-parametric Kruskal-Wallis test. If the global test indicated that a significant difference existed, pair-wise comparisons were carried out to test which pair(s) had a significant difference, again using the non-parametric Kruskal-Wallis test. Three possible pairs were analyzed: control versus HCy, control versus Li, and Li versus HCy. The significant differences are highlighted in Table 3. P<0.05 was considered statistically significant.

Histology and microscopy
Li- and HCy-exposed embryos, and control NaCl-exposed embryos, were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS, pH 7.4) and paraffin sectioned. Sections were stained with hematoxylin-eosin, and some were stained with Toluidine Blue, and examined. Cardiac regions were analyzed for the presence of any obvious malformations using a Nikon SMZ1500 fluorescent stereomicroscope, and digitized images were obtained using a Nikon DS-L2 camera unit.

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COMPETING INTERESTS
The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS
M.H. carried out chick embryo analysis of HCy exposure; the in situ hybridization within the avian heart-forming regions demonstrate that Li, Hcy and Wnt3A suppress Wnt-modulated Hex and Isl1 expression, and that FA prevents this effect. Inclusion of myo-inositol with FA supplementation potentiates the protection of Li-induced errors.

Impact and future directions
FA supplementation has been used since 1998 to reduce the occurrence of neural tube defects. The underlying basis of the protection is not known. Our results demonstrate that FA deficiency- and elevated HCY-associated birth defects involve Wnt-β-catenin signaling, and that prophylactic FA supplementation, at levels that are higher than those used currently for neural tube defect prevention, can prevent cardiac defects. Histone and DNA methylation are fundamental processes in the Wnt-β-catenin pathway and gene target regulation. Hcy and FA molecules are intermediaries in the S-methylation and α,β-gastrulation induces cardiac defects accompanied by potentiation of the Wnt-β-catenin signaling pathway. This suggests that elevated Hcy, associated with cardiac defects, may also target the Wnt-β-catenin pathway during neural tube and craniofacial development. It is well known that folic acid (FA) supplementation can reduce the incidence of neural tube defects, but the cause for its preventative action is not known. This work sought to address the ability of FA supplementation to prevent cardiac defects induced by Li, Hcy or Wnt3A in the vertebrate embryo.

Results
This study uses Doppler ultrasound to identify functional heart and placental defects in utero on embryonic day (E)15.5 after pregnant mice received a single exposure to Hcy or Li on E6.75. Histological analysis shows defects in tricuspid and semilunar valves and altered myocardial thickness in Hcy- and Li-treated embryos. These embryos, and their placentas, were also smaller than control animals. When FA supplementation was started immediately after fertilization, it prevented these developmental errors. Gene expression studies within the avian heart-forming regions demonstrate that Li, Hcy and Wnt3A suppress Wnt-modulated Hex and Isl1 expression, and that FA prevents this effect. Inclusion of myo-inositol with FA supplementation potentiates the protection of Li-induced errors.

Clinical issue
Congenital birth defects can arise with embryonic exposure to therapeutic drugs, high levels of normal plasma metabolites, or other environmental factors. Congenital cardiac defects arising from lithium (Li) exposure, a drug used for management of mood disorders, or from elevated plasma homocysteine (HCy) often involve tricuspid, pulmonary or aortic valve defects; a thickened heart wall; and/or defects in the outflow tract. Cardiac abnormalities are accompanied by neural tube defects and craniofacial anomalies that occur through unknown mechanisms. Lithium exposure during gastrulation induces cardiac defects accompanied by potentiation of the Wnt-β-catenin signaling pathway. This suggests that elevated H cycl, associated with cardiac defects, may also target the Wnt-β-catenin pathway during neural tube and craniofacial development. It is well known that folic acid (FA) supplementation can reduce the incidence of neural tube defects, but the cause for its preventative action is not known. This work sought to address the ability of FA supplementation to prevent cardiac defects induced by Li, Hcy or Wnt3A in the vertebrate embryo.

TRANSLATIONAL IMPACT
Clinical issue
Congenital birth defects can arise with embryonic exposure to therapeutic drugs, high levels of normal plasma metabolites, or other environmental factors. Congenital cardiac defects arising from lithium (Li) exposure, a drug used for management of mood disorders, or from elevated plasma homocysteine (HCy) often involve tricuspid, pulmonary or aortic valve defects; a thickened heart wall; and/or defects in the outflow tract. Cardiac abnormalities are accompanied by neural tube defects and craniofacial anomalies that occur through unknown mechanisms. Lithium exposure during gastrulation induces cardiac defects accompanied by potentiation of the Wnt-β-catenin signaling pathway. This suggests that elevated Hcyc, associated with cardiac defects, may also target the Wnt-β-catenin pathway during neural tube and craniofacial development. It is well known that folic acid (FA) supplementation can reduce the incidence of neural tube defects, but the cause for its preventative action is not known. This work sought to address the ability of FA supplementation to prevent cardiac defects induced by Li, Hcy or Wnt3A in the vertebrate embryo.

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Competing interests
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