

1 *Article*

2 **Spontaneous Left Cardiac Isomerism in Chick**
3 **Embryos: Case Report, Review of the Literature, and**
4 **Possible Significance for the Understanding of**
5 **Ventricular Non-Compaction Cardiomyopathy in the**
6 **Setting of Human Heterotaxy Syndromes**

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10

11 **Abstract:** Except for a few species, the outer shape of vertebrates normally is
12 characterized by bilateral symmetry. The inner organs, on the other hand,
13 normally are arranged in bilaterally asymmetric patterns, which are of special
14 importance for the normal function of the cardiovascular system of lung-breathing
15 vertebrates. Deviations from the normal organ asymmetry can occur in the form of
16 mirror imagery of the normal arrangement (*situs inversus*), or in the form of
17 arrangements that have the tendency for development of bilateral symmetry,
18 either in a pattern of bilateral left-sidedness (left isomerism) or bilateral
19 right-sidedness (right isomerism). The latter two forms of visceral *situs* anomalies
20 are called “heterotaxy syndromes”. During the past 30 years, remarkable progress
21 has been made in uncovering of the genetic etiology of heterotaxy syndromes.
22 However, the pathogenetic mechanisms causing the spectrum of cardiovascular
23 defects found in these syndromes remain poorly understood. In the present report,
24 a spontaneous case of left cardiac isomerism found in a HH-stage 23 chick embryo
25 is described. The observations made in this case suggest that hearts with left
26 cardiac isomerism may have the tendency for development of a non-compaction
27 cardiomyopathy caused by defective development of the proepicardium.

28 **Keywords:** heterotaxy syndromes, cardiac isomerism; chick embryo; *Pitx2*;
29 proepicardium; non-compaction cardiomyopathy

30

31 **1. Introduction**

32 Except for a few species, the outer shape of vertebrates normally is
33 characterized by bilateral symmetry. The inner organs, on the other hand, normally
34 are arranged in bilaterally asymmetric patterns, which are of special importance for
35 the normal function of the cardiovascular system of lung-breathing vertebrates.

36 Congenital deviations from the normal organ asymmetry can occur in the form
37 of mirror imagery of the normal arrangement - so-called “*situs inversus*” - or in the
38 form of arrangements that have the tendency for development of bilateral

39 symmetry, either in a pattern of bilateral left-sidedness (left visceral isomerism) or
40 bilateral right-sidedness (right visceral isomerism). The latter two forms of visceral
41 situs anomalies are usually classified as “visceral heterotaxy syndromes” or
42 “heterotaxy syndromes” [1]. They are typically associated with complex
43 cardiovascular malformations [2].

44 During the past 30 years, remarkable progress has been made in uncovering of
45 the genetic etiology of visceral heterotaxy syndromes [2, 3]. However, the
46 pathogenetic mechanisms causing the spectrum of cardiovascular defects found in
47 these syndromes still remain poorly understood.

48 Studies on chick embryos have significantly contributed to the discovery of the
49 genetic and molecular background of the normal as well as abnormal development
50 of the visceral situs of vertebrates [3]. In the present report, a spontaneous case of
51 left cardiac isomerism found in a HH-stage 23 chick embryo is described. The
52 observations made in this case suggest that hearts with left cardiac isomerism may
53 have the tendency for the development of a non-compaction cardiomyopathy
54 caused by defective development of the proepicardium.

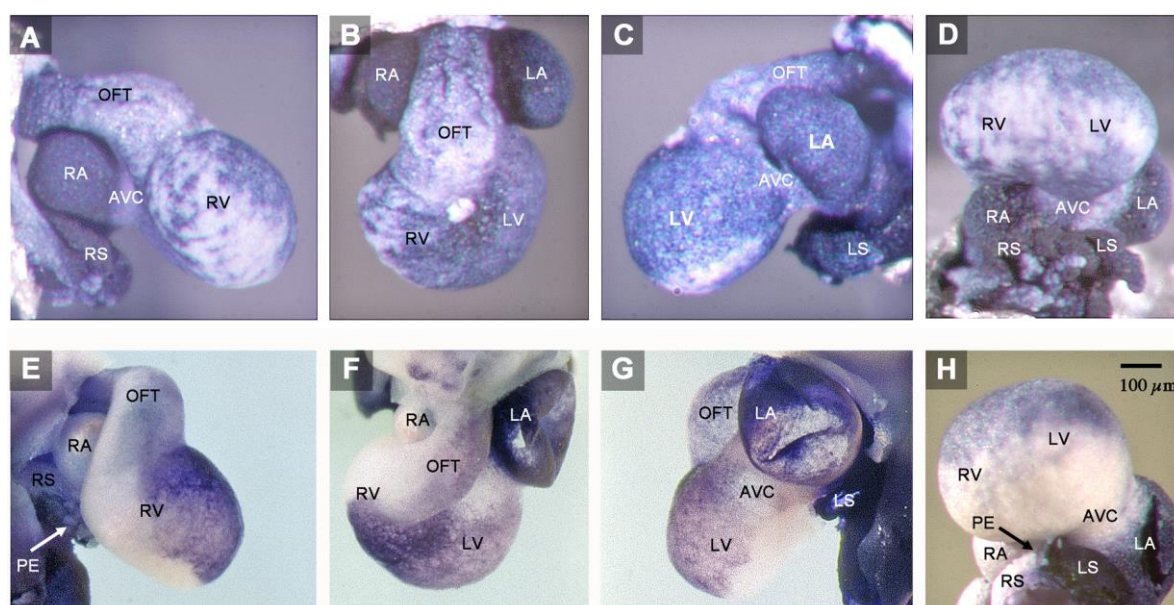
55 2. Materials and Methods

56 The abnormal chick heart presented in this report was a lucky find. It belonged
57 to a series of embryonic chick hearts that were prepared for a left-right lineage
58 tracing study by a PhD student. For this study, fertilized chicken eggs (White
59 Leghorn) were obtained from the Georg-August University research farm and
60 incubated at 38°C and 75% humidity. Chick embryos of normal external
61 morphology were fixed for whole mount in situ hybridization (ISH) with the
62 left-lineage marker *Pitx2* [4] at various developmental stages (incubation days 3 to
63 5; stages 15 to 25 according to Hamburger and Hamilton (HH), [5]). Eggs and
64 embryos were handled in accordance with the Declaration of Helsinki and the local
65 animal protection laws, which did not claim the approval of the study by an
66 institutional review board or ethics committee. Prior to fixation of the embryos, the
67 hearts were arrested in an end-diastolic state [6]. After fixation, a tissue block
68 consisting of the heart and the mediastinum was dissected free from the embryo.
69 Whole mount ISH of the tissue blocks was carried out according to established
70 protocols [6]. Subsequent to whole mount ISH, the stained specimens were critical
71 point dried in order to facilitate stepwise dissection of dried heart specimens with
72 fine tungsten needles in alternate with “gross morphological” analyzes under a
73 dissection microscope. For histological analyzes of the abnormal heart specimen,
74 the dried specimen was re-transferred into a fluid medium (methylbenzoate) and
75 prepared for histological analyzes according to established protocols [6].

76 3. Results

77 The abnormal heart under discussion was obtained from a four-day-old chick
78 embryo (HH-stage 23). The heart was first noticed as representing an unusual
79 specimen after whole mount ISH with the *Pitx2* probe. The specimen differed from

80 all other hearts of the series by an intense blue staining of its entire wall (Fig. 1A-D).
 81 The normal hearts of the series showed the typical Pitx2 expression pattern, which
 82 was characterized by blue staining of only the left heart field-derived portions of
 83 the heart (Fig. 1E-H). Having noticed the unusual staining pattern of the heart
 84 specimen, its external morphology and Pitx2 expression pattern were carefully
 85 analyzed under a dissection microscope and compared with the morphology and
 86 Pitx2 expression pattern of normal HH-stage 23 heart specimens. The analysis of
 87 the external shape was followed by the analysis of the internal morphology on
 88 serial histological sections. Microscopical analyzes disclosed several abnormalities,
 89 which are presented here in a sequential segmental order along the physiological
 90 flow path, starting at the venous heart pole.

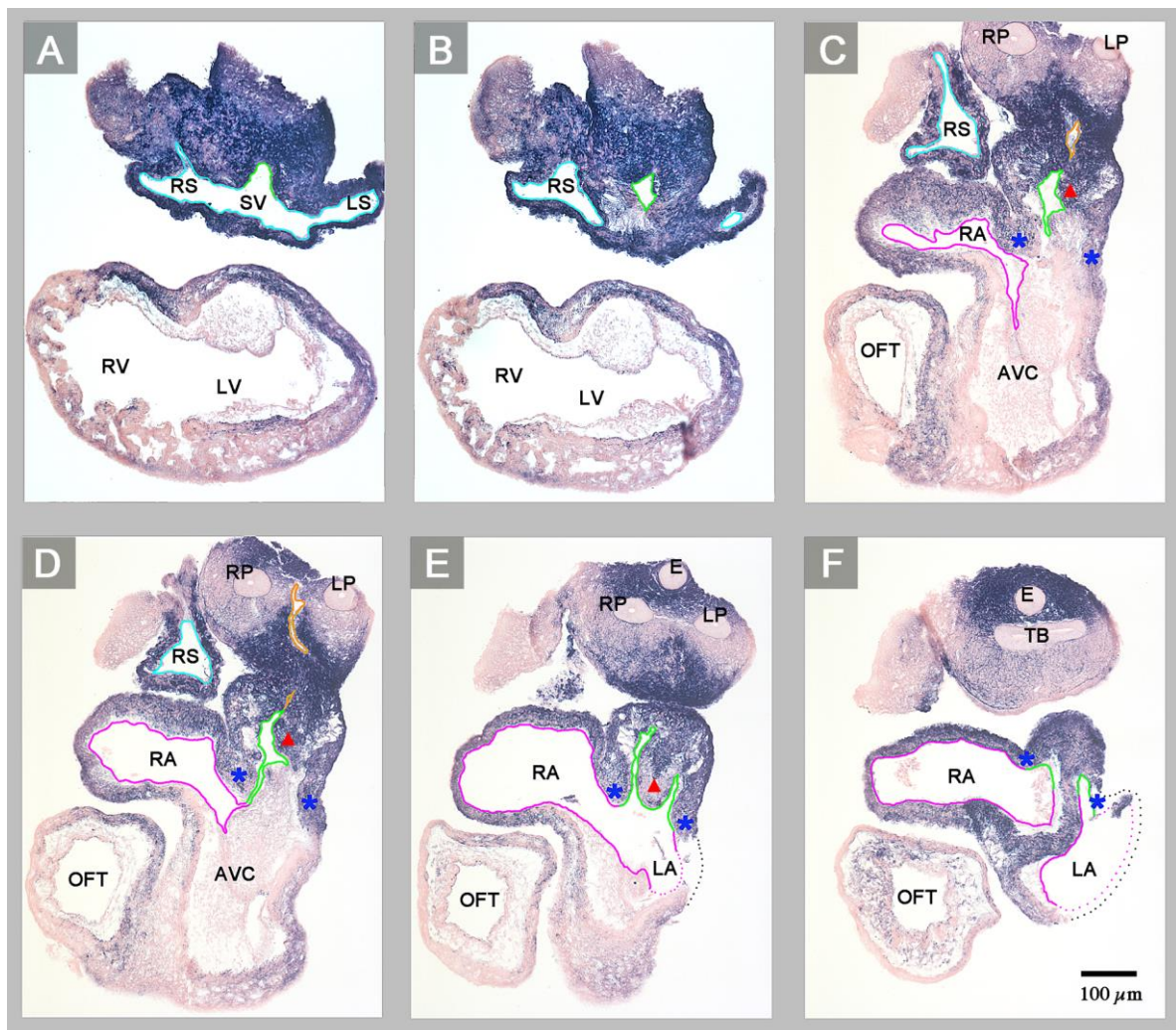


91 **Figure 1.** These microphotographs depict the outer shape and Pitx2 expression pattern of the
 92 abnormal HH-stage 23 heart with left cardiac isomerism (A-D) and of a normal HH-stage 23
 93 embryonic chick heart (E-H). Specimens are shown in right lateral views (A,E), cranio-ventral views
 94 (B,F), left lateral views (C,G), and caudal views (D,H). The abnormal heart shows a global Pitx2
 95 expression (blue staining), whereas the normal heart expresses Pitx2 only in those areas that are
 96 derived from the left heart fields. The abnormal heart, furthermore, shows a tendency for bilaterally
 97 symmetric development of its components, which is especially prominent at the sinus venosus and
 98 the atria. The left and right atrium of the abnormal heart have almost the same size and shape (B),
 99 whereas there is a marked difference in size between the two atria of the normal heart (F). Note also
 100 that the abnormal heart lacks a proepicardium-derived tissue bridge (A, D), which normally bridges
 101 the pericardial cavity between the right sinus horn and the dorsal wall of the embryonic ventricles (E,
 102 H). The proepicardium is a morphological marker for right-sidedness. Abbreviations: AVC =
 103 atrioventricular canal; LA = left atrium; LS = left sinus horn; LV = embryonic left ventricle; OFT =
 104 outflow tract; PE = proepicardium-derived tissue bridge; RA = right atrium; RS = right sinus horn; RV
 105 = embryonic right ventricle.

106

107 (1.) The confluence of the systemic veins (sinus venosus) of HH-stage 23 hearts
 108 normally is characterized by bilateral asymmetry. The sinus venosus normally
 109 drains exclusively to the right-sided atrium and the Pitx2 expression normally is
 110 confined to the wall of the left sinus horn. The Pitx2 negative right sinus horn

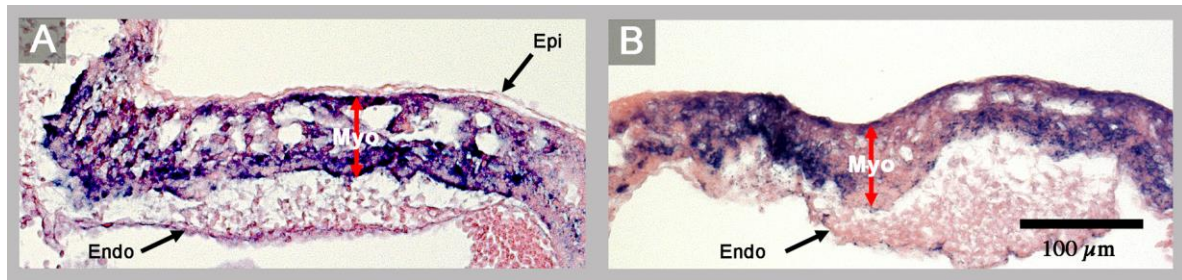
111 harbors a Pitx2 negative proepicardium-derived tissue bridge, which connects its
 112 ventral wall with the dorsal wall of the ventricular bend (Fig. 1E and H). The left
 113 sinus horn of chick embryos normally does not form a proepicardium-derived
 114 tissue bridge [6, 7]. In contrast to the normal situation, the sinus venosus of the
 115 abnormal HH-stage 23 chick heart showed a bilaterally symmetric arrangement
 116 with respect to molecular expression patterns as well as morphology. Pitx2 was
 117 expressed in the walls of the left as well as the right sinus horn, and both sinus
 118 horns did not show any trace of a proepicardium-derived tissue bridge (Fig. 1A, C,
 119 D and Fig. 2A, B). Absence of the proepicardium-derived tissue bridge was
 120 associated with defective formation of the epicardium (Fig. 3). The left and right
 121 sinus horns were of the same size and drained to a narrow midline canal (Fig. 2A,
 122 B). This canal was continuous with a dorsal component of the developing atria (Fig.
 123 2A-E), which we called the “atrial inflow component” [8].
 124



125 **Figure 2.** Starting at the level of the confluence of the systemic veins (sinus venosus), this sequence of
 126 transverse histological sections depicts the veno-atrial connections of the abnormal HH-stage 23
 127 heart with left cardiac isomerism. The sinus venosus (endothelial lining marked in light blue) drains
 128 to a narrow midline canal (A, B) that belongs to the atrial inflow component (endothelial linings
 129 marked in green). The atrial inflow component is connected to both atria (endothelial linings marked

130 in magenta) and incompletely divided into left and right portions by a rudimentary interatrial
 131 septum (red arrowhead marks the crest of the septum) (E, F). The atrial inflow component is
 132 demarcated from the rest of the atria by two myocardial folds (marked by blue asterisks). Note that
 133 the common pulmonary vein (endothelial linings marked in orange) is connected to the right portion
 134 of the atrial inflow component (C, D). Abbreviations: E = esophagus; LP = left lung bud; PV =
 135 common pulmonary vein; TB = tracheal bifurcation; RP = right lung bud; SV = sinus venosus; other
 136 abbreviations as used before.

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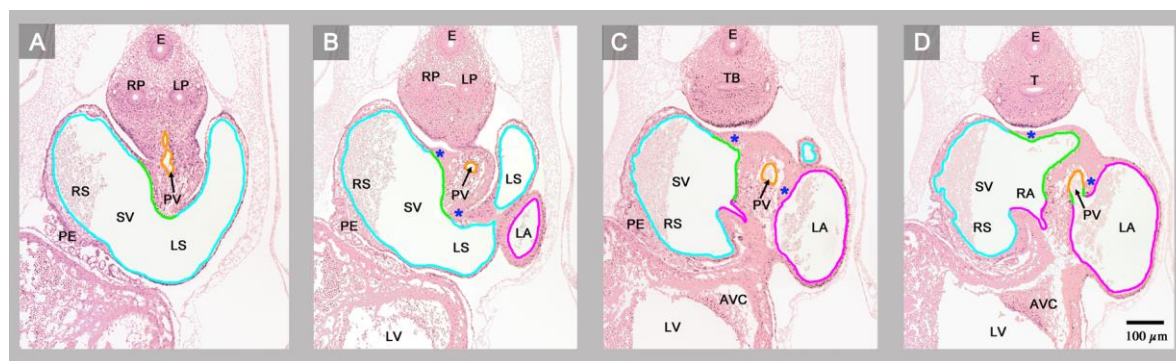


138 **Figure 3.** These histological sections show the ventricular wall architecture of a normal HH-stage 23
 139 embryonic heart (A) and of the abnormal HH-stage 23 heart with left cardiac isomerism (B). A. The
 140 normal ventricular wall consists of three-layers: the epicardium (Epi), the myocardium (Myo), and
 141 the endocardium (Endo). B. The ventricular wall of the abnormal heart lacks the epicardium.

142

143 (2.) The atrial segment of HH-stage 23 hearts normally is characterized by
 144 bilateral asymmetry. There is a marked difference in size between the two atria - the
 145 right atrium is smaller than the left - and *Pitx2* expression is confined to the left
 146 atrium (Fig. 1E-H). The sinus venosus is connected to the right atrium via the right
 147 portion of the atrial inflow component, while the stem of the pulmonary veins is
 148 connected to the left atrium via the left portion of the atrial inflow component (Fig.
 149 4). In contrast to the normal situation, the atrial segment of the abnormal HH-stage
 150 23 chick heart showed bilateral symmetry. Both atria were of almost the same size
 151 and *Pitx2* was expressed in the left as well as the right atrium (Fig. 1A-D). The sinus
 152 venosus drained via a narrow midline canal to the atrial inflow component, which
 153 was connected to both atria (Fig. 2A-E). The stem of the pulmonary veins was
 154 abnormally connected to the right portion of the atrial inflow component (Fig. 2C,
 155 D).

156



157 **Figure 4.** Starting at the level of the confluence of the systemic veins (sinus venosus), this sequence of
 158 transverse histological sections depicts the veno-atrial connections of a normal HH-stage 23
 159 embryonic chick heart (sections belong to the chick embryo collection of the Institute of Anatomy
 160 and Embryology). The sinus venosus drains to the right atrium via the right portion of the atrial
 161 inflow component (C). The common pulmonary vein drains to the left atrium via the left portion of
 162 the atrial inflow component (D). Note that the ventral wall of the right sinus horn harbors the
 163 proepicardium-derived tissue bridge. Color code for endothelial linings as used before.
 164 Abbreviations: PV = common pulmonary vein; T = trachea; other abbreviations as used before.

165 (3.) The atrio-ventricular canal (AVC), the primitive left and right ventricles,
 166 and the outflow tract (OFT) of early embryonic hearts normally undergo a process
 167 of ventricular looping morphogenesis, which changes the original form and
 168 position of these embryonic heart segments [9]. Ventricular looping generates a
 169 helically wound bend whose outer curvature normally points toward the right
 170 body side (ventricular D-loop). This sets the scene for development of the normal
 171 topographical relationship of the two ventricles. Looping toward the left body side
 172 (ventricular L-looping) is abnormal and sets the scene for development of a
 173 ventricular topology that is the mirror image of the normal one. In the setting of
 174 human cardiac isomerism, ventricular looping is said to occur in a random fashion,
 175 what means that only 50% of the cases develop ventricular D-loop topology while
 176 the remaining 50% develop ventricular L-loop topology [10]. The abnormal
 177 HH-stage 23 chick heart presented here had a ventricular D-loop. The configuration
 178 and *Pitx2* expression pattern of this D-loop, however, differed from the normal one
 179 (Fig. 1A,B). Compared to normal HH-stage 23 hearts, the ventricular D-loop of the
 180 abnormal heart specimen was mainly characterized by an abnormal tendency for
 181 ventral positioning of the embryonic right ventricle and straightening of the OFT.
 182 Abnormal *Pitx2* expression was found along the entire wall of the AVC, primitive
 183 ventricles and OFT (Fig. 1A-D). Normal *Pitx2* expression in the wall of these
 184 embryonic heart segments is mainly confined to the ventral walls (Fig. 1E-H).
 185

186 4. Discussion

187 The abnormal HH-stage 23 embryonic chick heart presented in this report
 188 showed a tendency for bilaterally symmetric - "isomeric" - formation of its
 189 components, which was especially prominent at its venous pole (sinus venosus,

190 atria). The bilateral expression of *Pitx2*, which is a molecular marker for
191 left-sidedness [11], and the bilateral absence of a proepicardium-derived tissue
192 bridge, which is a morphological marker for right-sidedness in avian embryos [6, 7,
193 12], suggest the presence of bilateral left-sidedness. The tendency for development
194 of bilateral left-sidedness is usually found in a subset of the visceral heterotaxy
195 syndrome that has been termed “polysplenia syndrome” [13], “left isomerism” [13,
196 14], “left cardiac isomerism” [15], “left atrial isomerism” [16], or “left isomerism of
197 the atrial appendages” [1, 17, 18]. This diagnosis is furthermore supported by the
198 fact that the abnormal embryonic chick heart had a striking hypoplasia of its
199 systemic venous component (sinus venosus) in combination with anomalous
200 pulmonary venous drainage to the right-sided atrium (Fig. 2A-D). Such features are
201 typically found in human hearts with left cardiac isomerism as well as in mouse
202 models for this subset of the visceral heterotaxy syndrome [18-20].

203 During the past three decades, remarkable progress has been made in the
204 elucidation of the genetic and molecular control of the development of the visceral
205 situs of vertebrates [2, 3]. This progress led to the generation of several genetically
206 modified mouse models for visceral heterotaxy syndromes with left- [15, 21, 22] or
207 right cardiac isomerism [23-29]. This list of mouse models for visceral heterotaxy is
208 completed by mouse strains carrying spontaneous mutations of situs-relevant
209 genes [17, 30] as well as by teratogen-induced mouse models [19, 31, 32]. In view of
210 this fact, the question may arise as to why I do report on this single case of an
211 abnormal embryonic chick heart? Regarding this question, I should note that
212 studies on chick embryos have significantly contributed to the discovery of the
213 genetic and molecular background of the normal as well as abnormal development
214 of the visceral situs of vertebrates [3]. Furthermore, the chick embryo is a
215 well-established model for studying the normal as well as abnormal embryonic
216 development of the heart of higher vertebrates [33, 34]. Each animal model for a
217 human disease has its own specific advantages and drawbacks. We, therefore,
218 should keep in mind that studying the etiopathogenesis of heterotaxy syndromes
219 only on mouse models might hamper the discovery of some pathogenetic processes
220 acting in human heterotaxy syndromes. Studies on chicken models for cardiac
221 isomerism may help to discover pathogenetic mechanisms that possibly cannot be
222 easily uncovered in mouse models for human heterotaxy syndromes.
223 Unfortunately, however, the currently available experimental chicken models for
224 visceral heterotaxy syndromes do not facilitate analyzes of abnormal cardiogenesis
225 beyond the early stages of cardiac looping morphogenesis. Thus, at the present
226 time, the possibility to analyze the hearts of chicken with visceral heterotaxy
227 syndromes at advanced stages of cardiogenesis depends on the availability of
228 spontaneous cases, only. Such cases are rare findings. A careful search for reports
229 on spontaneous cases of cardiac isomerism in chicken disclosed only two reports
230 written in French [35, 36], but no report written in English or other languages. In the
231 two papers written in French, Dor and co-workers have documented six
232 spontaneous cases of cardiac isomerism found in four-day-old chick embryos.
233 Unfortunately, these authors did not distinguish between left and right cardiac

234 isomerism. They simply classified all of their cases as “atrio-ventricular situs
235 ambiguus” on the basis of patho-morphological analyzes using scanning electron
236 microscopy. Since these specimens were analyzed before the discovery of the
237 molecular markers for left-sidedness, there was no molecular evidence supporting
238 the correct assignment of these cases either to the group of left cardiac isomerism or
239 right cardiac isomerism. Thus the present case seems to be the first example of an
240 abnormal embryonic chick heart displaying molecular and morphological left
241 isomerism at an advanced stage of embryonic cardiogenesis. Comparison of the
242 morphological features of this case with those published by Dor and co-workers
243 shows that at least one of their cases (see Fig. 58 in [36]) seems to fit to the diagnosis
244 left cardiac isomerism.

245 It is the question as to whether the present case may have any relevance for the
246 understanding of human congenital heart defects in the setting of visceral
247 heterotaxy syndromes? The answer to this question may be found at the sinus
248 venosus of this heart, which shows a bilateral absence of a proepicardium-derived
249 tissue bridge (Fig. 1A, C, D). The proepicardium (PE) is a primarily extracardiac
250 population of embryonic progenitor cells that normally provides the epicardial
251 mesothelium, the subepicardial and intramyocardial fibroblasts, and several cell
252 lineages of the coronary blood vessels [7]. It forms in the area of the sinus venosus,
253 where it is found as a cauliflower-shaped accumulation of villous protrusions of the
254 pericardial coelomic epithelium. The first PE-derived cells normally reach the
255 originally naked myocardial surface of the embryonic heart during the second
256 phase of cardiac looping (S-looping; HH-stage 17 in chick embryos). They form the
257 primitive epicardium, which then provides mesenchymal cells that colonize the
258 subepicardial and myocardial wall layers where they differentiate into fibroblasts,
259 coronary smooth muscle cells, and coronary endothelial cells. In amphibian,
260 reptilian and avian embryos, the transfer of PE cells to the developing heart
261 normally is accomplished via a secondary tissue bridge, which is formed by the
262 firm attachment of the PE to the dorsal surface of the developing ventricles. The PE
263 arises from bilaterally paired anlagen [6]. In avian and amphibian embryos, PE
264 development normally shows a visible pattern of bilateral asymmetry. Here, only
265 the right-sided PE anlage normally undergoes a remarkable growth in size and
266 becomes the functioning PE that finally forms a PE-derived tissue bridge. The
267 left-sided PE anlage normally remains in a rudimentary state and does not
268 significantly contribute to heart development [6, 37]. Thus, the PE and the
269 PE-derived tissue bridge of chick embryos are features of morphological
270 right-sidedness. The complete absence of a PE-derived tissue bridge found in the
271 present chick heart with left cardiac isomerism, thus, can be interpreted as a feature
272 of bilateral left-sidedness. The consequences of defective formation of the PE have
273 been demonstrated by experimental studies on chick embryos [38]. The
274 experimentally induced loss of the PE or the prevention of the formation of the
275 PE-derived tissue bridge lead to a severe delay in the formation of the epicardium
276 combined with deficient formation of the subepicardial and intramyocardial
277 connective tissue, and coronary vessel defects. The experimentally induced “loss of

278 PE function syndrome”, additionally, includes a severe growth defect of the
279 compact layer of the ventricular myocardium resembling the so-called “ventricular
280 non-compaction cardiomyopathy” found in human beings. Corresponding
281 observations were made in mice with embryonic epicardial defects and it has been
282 shown that the non-compaction cardiomyopathy found in epicardium-deficient
283 embryos resulted from the lack of trophic signals normally provided by the
284 embryonic epicardium [39, 40]. Based on these experimental data, it can be stated
285 that the abnormal embryonic chick heart presented here, which lacks a PE-derived
286 tissue bridge and the normal epicardial covering of its ventricular myocardium
287 (Figs. 1A, D; 2A, B; and 3), shows an early stage of development of the loss of PE
288 function syndrome. It is evident that this heart would have developed the full loss
289 of PE function syndrome if the embryo were allowed to survive up to
290 developmental stages when the myocardial non-compaction phenotype usually
291 becomes apparent (~ HH-stages 29/30). Thus, the present case of an embryonic
292 chick heart with left cardiac isomerism strongly suggests that chicken heterotaxy
293 syndromes with left cardiac isomerism have the tendency for the development of
294 the loss of PE function syndrome including non-compaction cardiomyopathy.
295 Chicken heterotaxy syndromes with right cardiac isomerism, on the other hand,
296 should not have this tendency, since it is to be expected that the affected embryos
297 have the tendency for the development of two functioning PEs. Based on these
298 reflections, it is tempting to speculate that human heterotaxy syndromes with left
299 cardiac isomerism may also have the tendency for the development of a
300 non-compaction cardiomyopathy caused by defective development of the
301 proepicardium. A web-based search for articles reporting on the occurrence of
302 non-compaction cardiomyopathy in the setting of human heterotaxy syndromes,
303 indeed, disclosed several cases with left cardiac isomerism but not a single case
304 with right cardiac isomerism [41-45].

305 I should finally note that the development of the PE in mammalian embryos
306 differs from the situation found in amphibian and avian embryos. Mammalian
307 embryos do not develop a morphological asymmetry of their functioning PE. The
308 PE of mammalian embryos normally is formed by the union of the left and right
309 PE-anlagen, and both halves of the PE seem to provide equal amounts of
310 PE-derived cells to the developing heart [6]. On the first view, this fact seems to
311 conflict with the speculation described above. We should not forget, however, that
312 the presence of morphological symmetry not necessarily means that the two halves
313 of the mammalian PE harbor functionally equivalent cell populations. It is
314 conceivable that the molecular signals that normally control body sidedness might
315 also cause side-specific differences in the composition of PE cell populations, so that
316 normally only the right halve of the mammalian PE may harbor the cell population
317 that will later provide trophic signals to the compact layer of the developing
318 myocardium.

319

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