Alternative splicing variants of proinsulin mRNA and the effects of excess proinsulin on cardiac morphogenesis

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ABSTRACT

Alternative forms of proinsulin mRNA with differential translational capacities and unknown significance are expressed in several developing tissues and in the adult pancreas. In the chick embryo developing heart, we observed low expression of the translationally active transcript of embryonic proinsulin (Pro1B), and predominant expression of the intron 1-unspliced variant, translationally inactive. In the embryonic mouse heart, intron 1-unspliced isoform appeared after E12.5. This tight regulation is required for normal development, since overexpression of Pro1B resulted in abnormal cardiac morphogenesis in 40% of chick embryos, and was accompanied by changes in gene expression of *Amhc1* and *Vmhc1*.

1. Introduction

In mammalian organisms, insulin, secreted mainly by the pancreas, acts as an anabolic hormone after birth, maintaining glucose homeostasis. In the last two decades, other developmental functions of insulin, its precursor proinsulin, and insulin-like peptides have emerged in vertebrates and invertebrates [1-3]. Moreover, extrapancreatic proinsulin mRNA expression and alternative mechanisms of postranscriptional regulation have been reported [4-8]. We previously characterized insulin gene expression in the chick embryo during early stages of development, prior to differentiation of the pancreas [3]. Prepancreatic proinsulin remains unprocessed [9] and its tissue levels are finely regulated through the expression of transcript variants (Fig. 1A) that differ in their translational activity [4, 5]. In the prepancreatic embryo, insulin gene transcription begins 32 nucleotides (nt) 5' upstream of the pancreatic transcription start site, generating a proinsulin transcript (Pro1B) with an extension of 32 nt in its 5' untranslated region (5'UTR). This transcript variant has lower translational activity than the pancreatic isoform (Pro1A) due to the presence of two upstream open reading frames [4]. An additional isoform, generated by the retention of intron 1 in the embryonic Pro1B transcript, is found in the prepancreatic embryo. The intron 1-containing transcript (Pro1B1) has a much longer 5'UTR (extra 717-nt) that largely blocks its translational activity [5]. In the human adult pancreatic islet, a partially-spliced intron 1 isoform with higher translational activity has been described [6]. Low levels of the intron 1-retained isoform have also been reported in the adult mouse pancreas, although with higher translational activity than the spliced isoform [8]. These observations indicate that intron 1 retention has differential effects on proinsulin biosynthesis in different species, and may respond to distinct functional requirements at the level of the tissue or organism.

In the chick embryo, tight regulation of proinsulin expression appears to be essential for normal neural tube formation. Treatment of the embryo during closure of the neural tube with proinsulin antisense oligonucleotides increased the proportion of apoptotic cells in the rostral part of the embryo, indicating that a physiological function of proinsulin is to regulate cell survival/death during neurulation [9]. Information is lacking on the consequences of alternative transcripts expression or abnormal proinsulin expression in other developing tissues. The expression of proinsulin transcripts changes during early cardiogenesis in chicks; in the precardiac region only the fully-processed

mRNA (Pro1B) is detected, while the intron 1-containing transcript (Pro1B1) is expressed upon fusion of the heart tubes [5]. The aim of the present study was to analyze the expression of proinsulin embryonic transcripts during later stages of cardiac morphogenesis in mouse and chick. In addition, we evaluated the pathophysiological consequences of overexpression of translationally active variants, which increased proinsulin exposure in chick during cardiac development. We found that mRNA expression of proinsulin[1] isoforms in the heart was developmentally regulated in both species. Moreover, the induction of inappropriately high proinsulin levels during cardiac morphogenesis in chick, resulted in major cardiac malformations. These alterations were paralleled by abnormal expression of atrial and ventricular myosine genes.

2. Materials and Methods

See Supplementary Data for full methods

2.1. Embryos and plasmid electroporation

All protocols for animal experiments were in accordance with EU guidelines for animal research and were approved by the CIB bioethics committee. Fertilized chicken eggs (Granja Santa Isabel, Córdoba, Spain) were incubated at 38ºC in forced-draft, humidified incubators. Mouse embryos (C57BL/6 and CD1) were removed from the uterus of pregnant females. The day of detection of the vaginal plug was designated as embryonic day (E) 0.5.

For gain-of-function experiments, stage 3 cultured chick embryos were injected with pCAGs-I-GFP (control), pCAGs-Pro1A-I-GFP or pCAGs-Pro1B-I-GFP in the region committed to form the heart and electroporated as described previously [10].

2.2. Whole-mount ISH, RNA isolation, PCR and Western Blot

All were performed following standard procedures. Specific modifications used and primer sequences are provided in Supplementary Data.

2.3. Plasmids

pCAGs-Pro1B-I-GFP, pCAGs-Pro1BV5-I-GFP, pCAGs-Pro1A-I-GFP and pCAGs-Pro1AV5-I-GFP were generated by excising the corresponding proinsulin

cDNA from previously described constructs [5] and cloning them into the pCAGs-I-GFP [10].

3. Results

3.1. Regulated expression of proinsulin transcript variants during cardiogenesis. Two embryonic proinsulin mRNA variants were previously shown to be dynamically regulated in the initial stages of cardiogenesis in the chick embryo, prior to pancreatic development [5]. The fully processed, translationally active, transcript was the predominant variant during specification of the cardiac fields. The percentage of intron 1-unspliced, translationally inactive transcript, subsequently increased, peaking during the formation of the linear heart tube (stage 10), the final stage analyzed in that study [5]. Here, we further characterized proinsulin mRNA expression during heart morphogenesis, from the heart tube stage through to ventricular and atrial chamber formation (stages 10 to 20 for chicken and E8.5-13.5 for mouse, see Supp. Fig. 1). At each stage studied in the chick embryo, the predominant form of proinsulin mRNA was the intron 1-unspliced variant (Fig. 1A and B). One of the first morphogenetic events after heart tube formation is the establishment of the anterior-posterior polarity of the tube, exemplified by the progressive restriction of the expression of the ventricular and atrial myosine heavy chain genes (*Vmhc1* and *Amhc1*) to the anterior and posterior portions of the heart tube, respectively [11]. We thus investigated whether the proinsulin transcript variants were preferentially distributed in the heart tube. The intron 1-unspliced variant Pro1B1 represented ~90% of the total proinsulin transcripts in both the atrial (posterior) and ventricular (anterior) regions, indicating no difference between regions in the expression of Pro1B1 at this stage (Fig. 1C).

Analysis of proinsulin expression in equivalent cardiac developmental stages in the mouse embryo revealed the presence of a single form of proinsulin mRNA from the earliest stage analyzed (E8.5, heart tube stage), corresponding to the fully spliced mRNA. An additional proinsulin transcript variant was detected from E12.5 onwards (Fig. 2B). The shortest molecular weight PCR band corresponded to the fully spliced isoform and the largest to the intron 1-unspliced isoform (all data confirmed by DNA sequencing). By contrast, in the developing mouse pancreas, at the level of sensitivity of semi-quantitative PCR, we only detected expression of the fully processed proinsulin

transcript (Fig. 2C). These results indicate that post-transcriptional regulation of heart proinsulin mRNA occurs during mouse cardiac morphogenesis, although at later developmental stages than in the chick.

We next investigated the potentially deleterious effects of supraphysiological embryonic proinsulin in cardiogenesis in the chick embryo model. Before evaluating the embryonic phenotype, two constructs carrying a V5 tag were electroporated into gastrulating chick embryos (stage 3), and were tested for their ability to translate *in vivo*. Analysis of whole embryo protein extracts showed that these constructs gave rise to high (Pro1A) or moderate (Pro1B) levels of proinsulin (Fig. 3). Note that the molecular weight of the protein yield produced by the electroporated constructs corresponded to that of unprocessed proinsulin, as does the endogenously expressed protein at these embryo stages [9].

3.2. Insulin receptor mRNA is expressed in the developing heart. We next analyzed the expression of insulin receptor (IR) as a potential mediator of proinsulin signaling. IR expression was detected in the developing heart in chick and mouse embryos at all ages studied (Fig. 4). One single band was PCR-amplified in chick, in agreement with our previous finding of only one IR isoform (corresponding to the IR-A isoform) in this species [12]. This semi-quantitative approach revealed IR expression at all ages tested, between stages 10 and 20 (Fig. 4A).

In the embryonic mouse heart, both IR-A and IR-B isoforms were identified from E8.5 onwards, with the IR-A form increasing its expression slightly in the second half of gestation (Fig. 4B).These findings suggest that both the chick IR and mouse IR-A can mediate proinsulin signaling, as the IR-A isoform has been recently shown to bind proinsulin with high affinity and trigger intracellular signaling pathways upon autophosphorylation [13].

3.3. Overexpression of proinsulin mRNA induces abnormal cardiac gene expression and morphogenesis. We previously demonstrated that while some proinsulin signaling is essential during early development, proinsulin overexpression is deleterious for neural tube development [9]. We thus analyzed the effect of supra-physiological levels of embryonic proinsulin on chick cardiogenesis. The two constructs producing high (Pro1A) and moderate (Pro1B) proinsulin levels were electroporated into gastrulating chick embryos and morphological evaluation was performed. Overexpression of Pro1A

and Pro1B induced cardiac malformations in different proportions of up to 61%, while only 8% of electroporated control embryos exhibited abnormalities (see Supp. Table 1). The malformations observed included cardia bifida, aberrant torsion and a "globular" aspect of the heart tube (Fig. 5A). Moreover, the severity of cardiac malformation was correlated with the translational activity of the transcripts. Whereas 38% of highly proinsulin expressing embryos (Pro1A-electroporated) showed absence of a beating heart formed, all of the moderately proinsulin expressing embryos (Pro1B-electroporated) displayed a beating, although abnormal, heart tube (Supp. Table 1).

Cardiac gene markers were visualized by *in situ* hybridization in control (GFP) and moderately high proinsulin expressing embryos. Significant changes in the expression of the atrial and ventricular chamber genes were observed. In embryos overexpressing Pro1B, *Amhc1* expression was markedly decreased, while a concomitant increase in *Vmhc1* expression was observed, which in some cases extended posteriorly (Fig. 5B). Embryos overexpressing Pro1A were excluded from the analysis as the severity of the malformations induced confounded analysis. A few embryos were sectioned and examined by optical microscopy (Fig. 5C). In the control embryos (GFP) there was a gradient in the expression of *Amhc1* but all cells were positive. In contrast, Pro1B electroporated embryos had dramatically decreased expression and in some cases discontinuity in *Amhc1* signal, with the negative cells located at the equator part of the tube in these horizontal sections (Fig. 5C). This "butterfly" image suggests either abnormal or absence of differentiation of a subset of precursors into cardiomyocytes. Our results thus show that, in the maternally-independent chick embryo model, supra-physiological proinsulin levels during cardiogenesis (glucose-independent) alter the dynamic pattern of gene expression and impair cardiac chamber formation (Fig. 5).

4. Discussion

In vertebrate organisms, insulin expression, regulation, active form and main function differ between developing and postnatal stages [for reviews see [1, 3]]. The postnatal pancreas abundantly expresses proinsulin mRNA and secretes mature insulin. The production and secretion of insulin is primarily regulated by glucose, and its main targets are metabolic tissues such as liver, muscle and fat. By contrast, prior to differentiation of the pancreas, proinsulin is the active protein form, and is produced in low abundance by several extrapancreatic tissues [9]. The expression of embryonic extrapancreatic proinsulin mRNA is independent of glucose levels, but depends on the presence of different transcript isoforms, which mediate distinct protein translation capabilities and tissue- and time-specific proinsulin availability [3]. The main aim of the present study was to characterize the alternative splicing pattern of proinsulin mRNA in the developing heart and determine the impact of inappropriately high proinsulin embryo exposure. In the chick embryo, the predominant proinsulin mRNA isoform detected during cardiac morphogenesis was the intron 1-unspliced transcript. This isoform is translationally inactive, suggesting that inactivation of proinsulin production is required during these stages of cardiac formation. Indeed, moderate increases in proinsulin levels induced by overexpression of the Pro1B proinsulin transcript dramatically interfered with the cardiac morphogenesis (see later). Here, we demonstrate that proinsulin production can be abrogated in the heart by regulating intron 1 processing, rather than completely switching off insulin gene expression. Previous studies have demonstrated that the splicing pattern of intron 1 is altered in response to growth factor deprivation, increasing proinsulin availability in the stressed embryo [5]. We thus speculate that maintenance of proinsulin gene in a transcriptionally active but translationally inactive state may represent a reversible back-up mechanism that allows embryonic tissues to adapt to environmental or developmental changes.

The insulin gene is part of a phylogenetically conserved synteny formed by the tyrosine hydroxylase (*Th*), insulin and *Igf2* genes. We previously demonstrated that unusual chimeric RNA transcripts are generated by *Th* and insulin genes during embryonic development, representing an additional post-transcriptional mechanism to regulate expression of the two adjacent genes [14]. Although the function of the chimeric RNA is unknown, alteration of TH levels in the chick embryo induces cardiac defects [10].

In mouse embryos, a proinsulin mRNA intron 1-containing isoform was observed at later stages of cardiac morphogenesis than in chicks. In contrast to the effects of intron 1-retention in chicks, the two alternative proinsulin transcripts generated in mammals by differential processing of intron 1 have been reported to display enhanced translational activities [6, 8]. Sequence and structural motifs in the UTR have a major impact on translational regulation [15, 16]. Among those 5'UTR structural features known to control translation, long and energetically stable secondary structures impair translation. Comparison of the secondary structure prediction for the 5'UTR of the chick fully-spliced (Pro1B) and intron 1-unspliced (Pro1B1) transcripts (Supp. Fig. 2) revealed a notable change in free energy (ΔG = -29 to -256 Kcal/mol) due to intron 1 retention, which includes a very long and stable stem-loop structure; thus this structural feature may account for the impaired proinsulin translation of the Pro1B1 mRNA. By contrast, mouse intron 1-retention resulted in a less stable stem-loop structure (change in free energy from ΔG= -5.5 to -37 Kcal/mol; Supp. Fig. 2), which appeared not to impair proinsulin translation.

Interestingly, intron 1-containing proinsulin mRNA isoform is much more abundant in the heart than in the pancreas in embryonic stages. Indeed, we detected no expression of the intron 1-containing transcripts in the developing mouse pancreas at any of the stages analyzed, in agreement with our findings in the chick [5]. This result is compatible with the very low abundance of intron 1-unspliced transcript described in adult mouse and human pancreatic islets [6, 8]. Our findings thus support the functional significance of RNA processing in the fine tuned proinsulin regulation required during embryonic development. The differential effects of intron 1 retention on translational activity in chicks and mice may reflect the distinct proinsulin requirements of each species during cardiac development.

The additional post-translational regulation specific to the pancreatic islets (*i.e.,* cleavage of proinsulin into mature insulin) further differentiates the pancreatic hormone from the growth factor-like activity of extrapancreatic proinsulin. While an anti-apoptotic role in neurulating chick embryos has been previously demonstrated [9], proinsulin may mediate other physiological effects in the precardiogenic area. Insulin receptor A isoform is expressed throughout cardiac development in mice and chicks, and can bind proinsulin with high affinity to predominantly activate mitogenic pathways [13]. We observed a deleterious effect on chick cardiogenesis when the physiological

decrease in proinsulin signaling was bypassed by forced expression of the most translationally active transcript, Pro1A, or the intermediate active transcript, Pro1B. These effects were accompanied by significant alterations in the expression of atrial and ventricular chamber genes. It is unlikely that increased proliferation or inhibition of apoptosis is implicated (preliminary results not shown). In view of the observed changes in *Amhc1* and *Vmhc1*, particularly the lack of expression of *Amhc1* in groups of cells (Fig. 5C) when proinsulin remains high, it is possible that proinsulin downregulation is part of the heart differentiation or morphogenetic program; further studies are required to investigate this hypothesis.

A complex network of molecular signals modulates cardiac morphogenesis and cardiomyocyte differentiation during development [17]. Cardiac malformations in mice and humans have been reported in response to dysregulation of transcription factors and morphogenetic molecules [18]. As mentioned above, we recently reported that the catecholaminergic pathway initiated by early developmental expression of TH plays an important role in regulating the gene network involved in antero-posterior patterning of the primitive heart tube [10]. Thus molecules such as proinsulin and catecholamines, well known for their roles in other contexts in developed organisms, may play a regulatory role in early cardiogenesis.

Understanding the impact of high proinsulin levels during development is clinically relevant, as there is a potential implication of proinsulin in fetal malformations in infants of obese and diabetic mothers. Maternal overweight and obesity are associated with an increased risk of fetal anomalies, including cardiac malformations [19, 20]. They could be additional factors to maternal hyperglycaemia contributing to that teratogenesis found in type 2 diabetes [21]. Insulin resistance and beta cell dysfunction, typically associated with increased proinsulin/insulin concentrations [22, 23], are common to obesity and type 2 diabetes, and thus both proteins are potential teratogens. We wish to place the present results in the context of the malformations found more frequently in infants of diabetic and obese mothers and speculate that hyperproinsulinemia may contribute to the cardiac malformations in their infants. Further human studies, performed early in pregnancy, are required to test this hypothesis.

Acknowledgements

Supplementary data associated with this article can be found in the online version.

Footnote[1]: we use the term 'insulin gene' as used in databases, but 'proinsulin mRNA' and 'proinsulin protein' to refer to the embryonic expression products.

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Figure Legends

Figure 1. Expression of proinsulin mRNA transcripts in the developing chick heart. A) Schematic organization of the chick insulin gene and proinsulin mRNA transcript variants. The three exons (E) and two introns (I) of the insulin gene and primers for standard PCR are indicated. The right primer spans the exon 2-3 splicing junction. The Pro1B transcript exhibits a 5' extension (clear grey box) as compared with the pancreatic transcript Pro1A. The Pro1B1 transcript resembles Pro1B but retains intron 1. B) RT-PCR for proinsulin mRNA transcripts in chick heart. RT was performed with RNA from pools of approximately 30-40 (stages 10 to 14) or 10-15 (stages 16 and 20) hearts. C) Expression of proinsulin transcripts in the ventricular and atrial regions of the embryonic chick heart. Quantitative RT-PCR of RNA was performed from pools of 9 ventricles and 9 atria from stage 12-13 hearts. The levels of each proinsulin transcript were normalized to GAPDH mRNA levels and the results expressed as a percentage with respect to the total (Pro1B+Pro1B1). A control (C) sample lacking cDNA is included in B. The PCR results were replicated several times with the same pools of hearts and once with another set of embryos.

Figure 2. **Expression of proinsulin mRNA transcripts in the developing mouse heart and pancreas** A) Schematic organization of the insulin 2 gene, indicating the three exons (E) and two introns (I) and the primers used for standard PCR. B) RT-PCR for proinsulin mRNA transcripts in mouse heart. RT was performed with RNA from pools of hearts harvested at embryonic days (E) 8.5 (n = 24), E9.5 (n = 28), E10.5 (n = 14), E12.5 (n = 14) and E13.5 (n = 7). The arrow indicates the fully spliced transcript and the arrowhead indicates the amplified band corresponding to unspliced intron 1 isoform. The PCR results were replicated once. C) RT-PCR for proinsulin mRNA transcripts in mouse pancreas. RT was performed with RNA from pools of 4-8 pancreas, except from E15.5-E16.5 stages when 2 pancreas were pooled. A control (C) sample lacking cDNA is included in B and C.

Note that the intron 1 is 114 nt, slightly longer than the 102 nt reported by Minn et al. 2005 [6], and we consider the initiation of transcription as mapped by Wentworth et al., 1986 [24].

Figure 3. Proinsulin translation levels after electroporation of whole chick embryos. Whole chick embryo lysates were resolved by SDS-PAGE and immunoblotted sequentially with three antibodies. The embryos had been collected directly from the incubated egg (*in ovo*) or after electroporation with plasmids (all were pCAGS-I-x-GFP) either control (GFP), pancreatic proinsulin-V5 transcript (Pro1AV5) or embryonic proinsulin-V5 transcripts (Pro1BV5). For this particular experiment, both proinsulin transcripts were fused to the V5 epitope to facilitate detection of the proinsulin protein. The anti-V5 recognizes the V5 tag linked to proinsulin which is not processed to insulin in the embryo. Anti-GFP immunoblotting indicates electroporation efficiency and anti-GAPDH was used as a loading control. Note that Pro1BV5-overexpressing embryos (lysates 1 and 2 from two different embryos) displayed much lower proinsulin levels than the Pro1AV5-overexpressing embryo lysate, despite their higher (sample 1) or similar (sample 2) GFP levels. The blot shown is representative of three experiments.

Figure 4. Expression of insulin receptor transcripts in the developing heart. RT-PCR for insulin receptor (IR) transcripts in embryonic chick (A) and mouse (B) hearts. A control (C) sample lacking cDNA is included. RT-PCR was performed with RNA from pools of hearts as described in the legend to Fig. 1 for chick; for mouse embryos, pools of 20-28 hearts (E8.5-E11.5) or 10-15 hearts (E12.5-E15.5) were used. Note that the two spliced mRNA variants of the mouse insulin receptor (IRA and IRB, arrowheads) were expressed at all embryonic stages analyzed in mice, while only one form of IR (corresponding to the IRA isoform) exists in chicks.

Figure 5. Proinsulin overexpression in chick embryos alters anterior-posterior heart tube patterning, resulting in cardiac malformations. A) Representative light microscopy images of proinsulin (pCAGs-Proinsulin-I-GFP)-electroporated chick embryos (three examples) exhibiting cardiac malformations, compared with a control GFP (pCAGs-I-GFP)-electroporated embryo. The shape of the heart tube is outlined with yellow dots. B) Whole-mount ISH for *Amhc1* and *Vmhc1* in electroporated chick embryos. GFP fluorescence images of the same embryos are shown in the corresponding panels on the left. The normal limit of *Vmhc1* expression is outlined with yellow dots. On the isolated panel on the right, double ISH showing normal expression

of *Vmhc1* (red) and *Amhc1* (purple) in a stage 12 embryo. C) Whole-mount and sections of ISH for *Amhc1* of proinsulin (pCAGs-Pro1B-I-GFP)-electroporated chick embryos (two different embryos), compared with a control GFP (pCAGs-I-GFP)-electroporated embryo. The black dotted line indicates the level of the histological section shown below each panel. In these sections, viewed by optical microscopy (20x objective), the discontinuity of the expression of *Amhc1* in the more central located cells of the heart tube is evident. The selected embryos are representative of three to six per group subjected to ISH after electroporation. D) Schematic showing the proposed effect of proinsulin overexpression on atrio-ventricular markers in the developing chick heart.
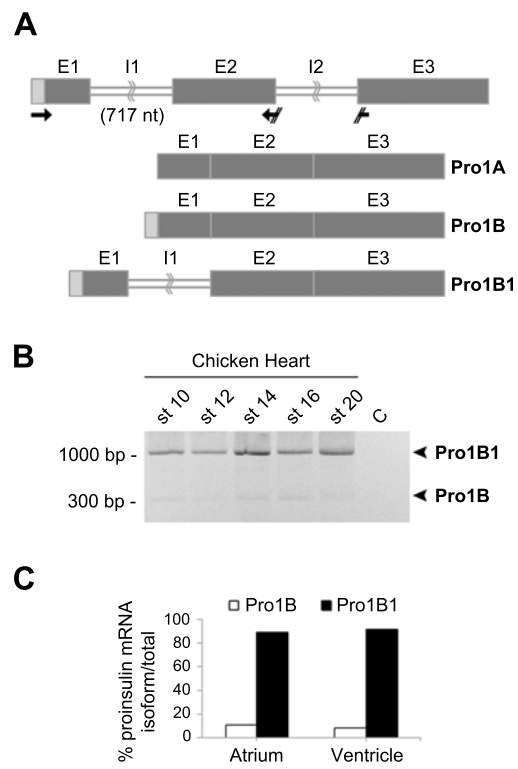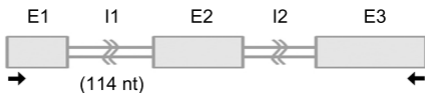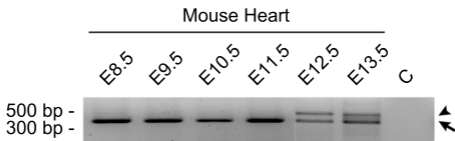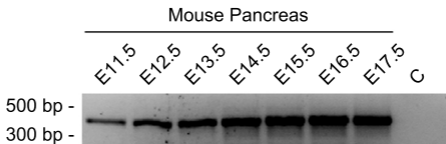
A

E1   I1   E2   I2   E3

(717 nt)

E1   E2   E3   **Pro1A**

E1   E2   E3   **Pro1B**

E1   I1   E2   E3   **Pro1B1**

B

Chicken Heart

st 10   st 12   st 14   st 16   st 20   C

1000 bp -   ◄ **Pro1B1**

300 bp -   ◄ **Pro1B**

C

□ Pro1B   ■ Pro1B1

% proinsulin mRNA isoform/total

100
80
60
40
20
0

Atrium   Ventricle

Figure 1

A

E1   I1   E2   I2   E3

(114 nt)

B

Mouse Heart

E8.5 E9.5 E10.5 E11.5 E12.5 E13.5 C

500 bp -
300 bp -

C

Mouse Pancreas

E11.5 E12.5 E13.5 E14.5 E15.5 E16.5 E17.5 C

500 bp -
300 bp -

Figure 2

Figure 3

A

Chick Heart

| st 10 | st 12 | st 14 | st 16 | st 20 | | C |

200 bp -
100 bp -

IR

B

Mouse Heart

| E8.5 | E9.5 | E10.5 | E11.5 | E12.5 | E13.5 | E14.5 | E15.5 | | C |

200 bp -

◄ IRB
◄ IRA

Figure 4

A

pCAGs-I-GFP   pCAGs-Proinsulin-I-GFP

B

pCAGs-I-GFP   pCAGs-Pro1B-I-GFP

GFP   *Amhc1*   GFP   *Amhc1*

VMHC1

GFP   *Vmhc1*   GFP   *Vmhc1*

AMHC1

C

pCAGs-I-GFP   pCAGs-Pro1B-I-GFP

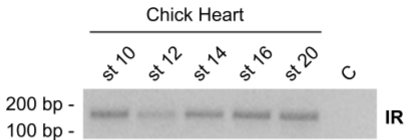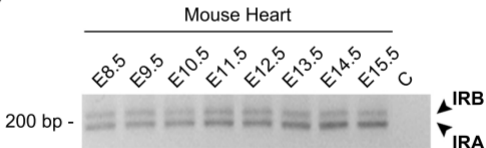*Amhc1*   *Amhc1*   *Amhc1*
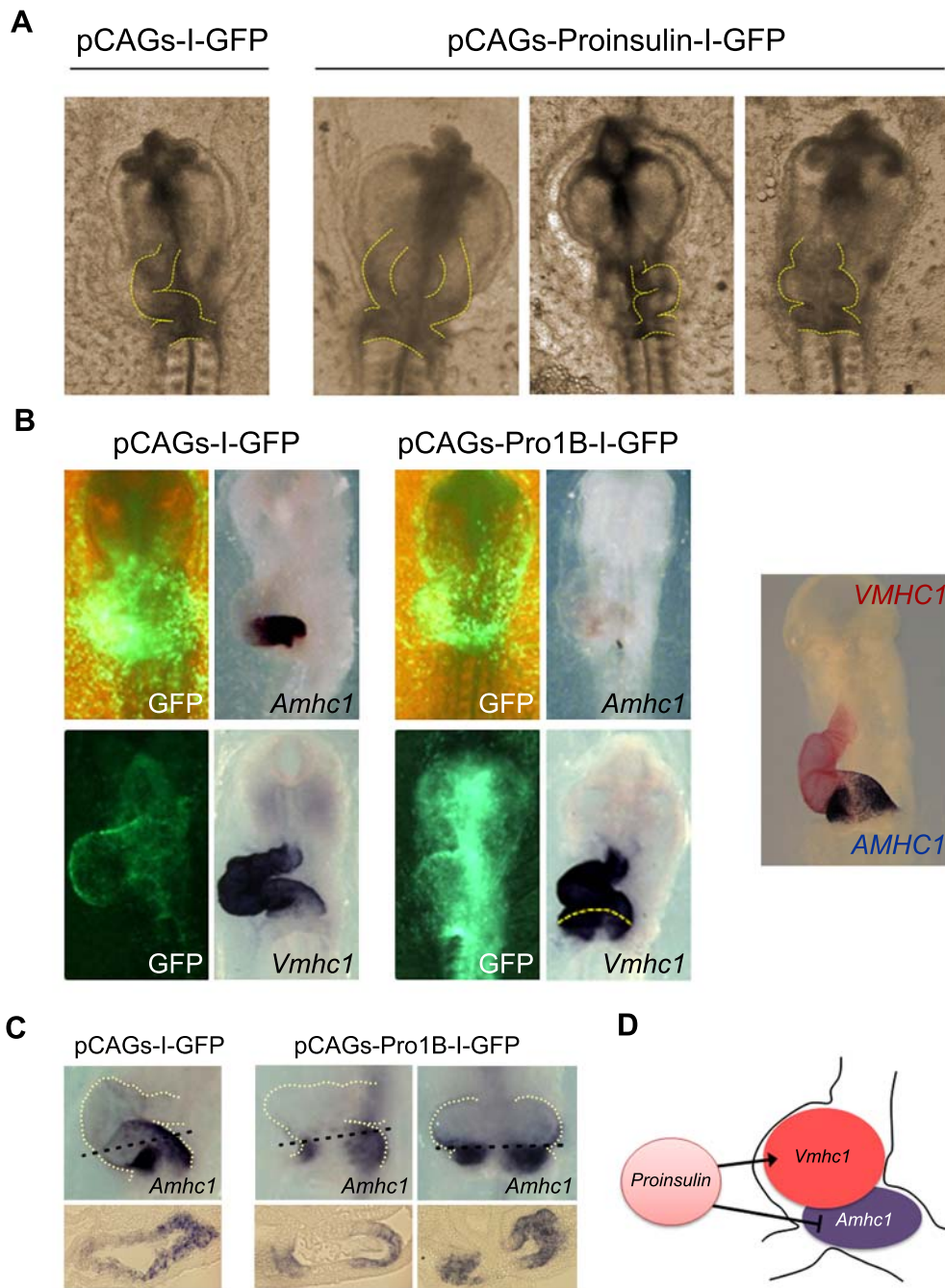
D

Proinsulin

Vmhc1

Amhc1

Figure 5

Supplementary Data

Materials and Methods

Embryos and plasmid electroporation

All protocols for animal experiments were in accordance with EU guidelines for animal research and were approved by the CIB bioethics committee. Fertilized chicken eggs (Granja Santa Isabel, Córdoba, Spain) were incubated at 38ºC in forced-draft, humidified incubators. Embryos were staged according to [1], and cultured as described previously [2]. Mouse embryos (C57BL/6 and CD1) were removed from the uterus of pregnant females. The day of detection of the vaginal plug was designated as embryonic day (E) 0.5.

For gain-of-function experiments, stage 3 cultured chick embryos were injected with pCAGs-I-GFP (control), pCAGs-Pro1A-I-GFP or pCAGs-Pro1B-I-GFP in the region committed to form the heart and electroporated as described previously [2]. After an additional 28-36 hours of incubation (stage 11-12), embryos were either fixed in 4% PFA and processed for whole-mount ISH or collected for protein assay by Western blot following standard procedures. For proinsulin Western blot, the pCAGs-I-GFP (control), pCAGs-Pro1AV5-I-GFP or pCAGs-Pro1BV5-I-GFP vectors were electroporated into chick embryos.

Whole-mount ISH and double ISH

Whole-mount ISH was performed using digoxigenin-labelled chick *Amhc1 and Vmhc1* probes, as previously described [2]. Double whole-mount ISH was performed using digoxigenin-labelled chick *Amhc1* and fluorescein-labelled *Vmhc1* (Roche) probes, as described [3], with some modifications. The addition of 1 μ g/ml of both probes was followed by overnight incubation at the appropriate hybridization temperature. Embryos were then processed until the desired staining intensity for *Amhc1* was achieved as described [2], and subsequently fixed. The fixative was removed and the embryos washed at room temperature in PBT (2 x 30 min) followed by MABT (2 x 30 min), in MABT at 63ºC (1 x 30 min), and again in MABT at room temperature (2 x 30 min). For fluorescein detection, nonspecific binding was blocked by incubation in blocking solution (2 x 1 hr) and the embryos were incubated overnight at 4°C, with agitation, with AP-conjugated anti-fluorescein antibody (1:500 in blocking solution; Roche). The embryos were then washed at room temperature in MABT (6 x 1 hr, followed by overnight incubation), and finally rinsed (2 x 20 min) in TBST (100

mM Tris-HCl [pH 8.2-8.5], 0.25% Tween 20). AP-conjugated anti-fluorescein antibody was detected using the Vector Red Alkaline Phosphatase Substrate Kit I (Vector Laboratories) in TBST. The reaction was stopped by washing in PBT once the desired staining intensity for *Vmhc1* was achieved, and the embryos were fixed in 4% PFA overnight and photographed. Selected embryos were embedded in paraffin, sectioned (14 µm), mounted and photographed under optical microscopy.

RNA isolation and standard and quantitative PCR

Total RNA from the whole heart or dissected heart regions and from the pancreas was isolated with the Trizol reagent (Invitrogen) and 2.5 µg of RNA was typically reverse transcribed (RT) using the Superscript III Kit and random primers (all from Invitrogen). Quantitative PCR (qPCR) was performed with the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Weiterstadt, Germany) using TaqMan Universal PCR Master Mix, No-AmpErase UNG (Applied Biosystems) and probes from the Universal Probe Library (URL, Roche Applied Science) for detection. Chick (c) and mouse (m) primer sequences and the respective URL probes are listed below.

Primers and URL probes for qRT-PCR:

Gene	Primers	URL
cPro1B	GCAAACTTCTCTGCATCTCTTTCT (left)	136
	CCAGAAGAGGCAGTGATCG (right)	
cPro1B1	TGGTAAAGAGCTGGCTTGGT (left)	72
	GCAATGTGCTCAAAGACCTG (right)	
cGapdh	GTCCTCTCTGGCAAAGTCCA (left)	49
	ACCATGTAGTTCAGATCGATGAAG (right)	

Primers for standard RT-PCR:

Gene	Primers
cIns	GAATGGGGAAATTTCTACCAGT (left)
	GACTGCTCACTAGGGGCTGC (right)
cIR	TGAGAGTGCAGAGGAGCAGA (left)
	GGCACTGACATAAGCTGCAA (right)
mIns2	TAACTGATCCGCTACAATCA (left)
	CAGTAGTTCTCCAGCTGGTA (right)
mIR	GGCCAGTGAGTGCTGCTCATGC (left)
	TGTGGTGGCTGTCACATTCC (right)

2.4. Plasmids

pCAGs-Pro1B-I-GFP, pCAGs-Pro1BV5-I-GFP, pCAGs-Pro1A-I-GFP and pCAGs-Pro1AV5-I-GFP were generated by excising the corresponding proinsulin cDNA from previously described constructs [4] and cloning them into the pCAGs-I-GFP [5].

2.5. Western immunoblot

Individual embryos were lysed in a buffer containing 50 mM Tris-HCl (pH 6.8), 10% (v/v) glycerol, 2% (w/v) SDS, 10 mM DTT and 0.005% (w/v) bromophenol blue. Half total protein lysate from each embryo was resolved on a 15% SDS-PAGE gel and transferred to nitrocellulose membranes (Protran, Whatman). Membranes were treated with 5% non-fat dry milk, 0.05% Tween 20 in PBS for 1-2 h at room temperature (RT), and sequentially incubated overnight at 4ºC with primary antibodies against V5 (1/1000) (Life Technologies); GFP (1/1000) (Clonthech); and GAPDH (1:20000, Abcam). Antibodies were detected with appropriate horseradish peroxidase-labeled secondary antibodies (Pierce) and visualized with the Super Signal West Pico chemiluminescent substrate (Pierce). GAPDH was used as protein loading control.

References

1.   Hamburger V & Hamilton HL (1951) A series of normal stages in the development of the chick embryo. *J Morphol* **88**, 49-92.
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5.   Fior R & Henrique D (2005) A novel hes5/hes6 circuitry of negative regulation controls Notch activity during neurogenesis. *Dev Biol* **281**, 318-333.
6.   Minn AH, Lan H, Rabaglia ME, Harlan DM, Peculis BA, Attie AD & Shalev A (2005) Increased insulin translation from an insulin splice-variant overexpressed in diabetes, obesity, and insulin resistance. *Mol Endocrinol* **19**, 794-803.
7.   Wentworth BM, Schaefer IM, Villa-Komaroff L & Chirgwin JM (1986) Characterization of the two nonallelic genes encoding mouse preproinsulin. *J Mol Evol* **23**, 305-312.
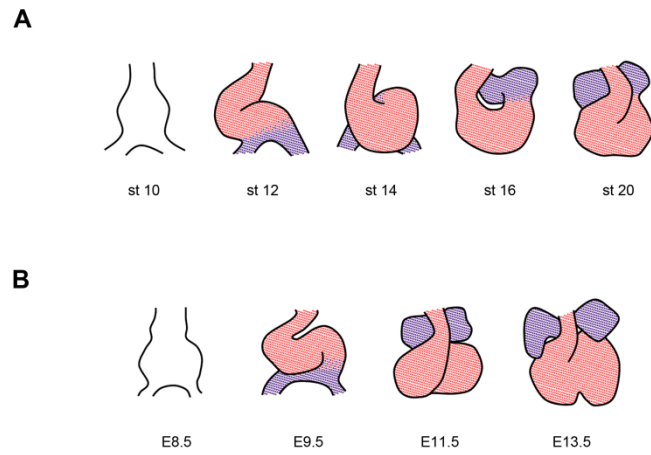
Figure 1S: Scheme depicting chick (A) and mouse (B) embryo heart at the corresponding indicated stages of development. Chronologically, the chick stage (st) 20 corresponds to 3.0-3.5 days of incubation, and the mouse E13.5 stage to 13.5 days of embryonic development. Ventricular regions are colored in red and atrial regions in blue. The youngest age is not colored since chamber regions are not yet well defined.
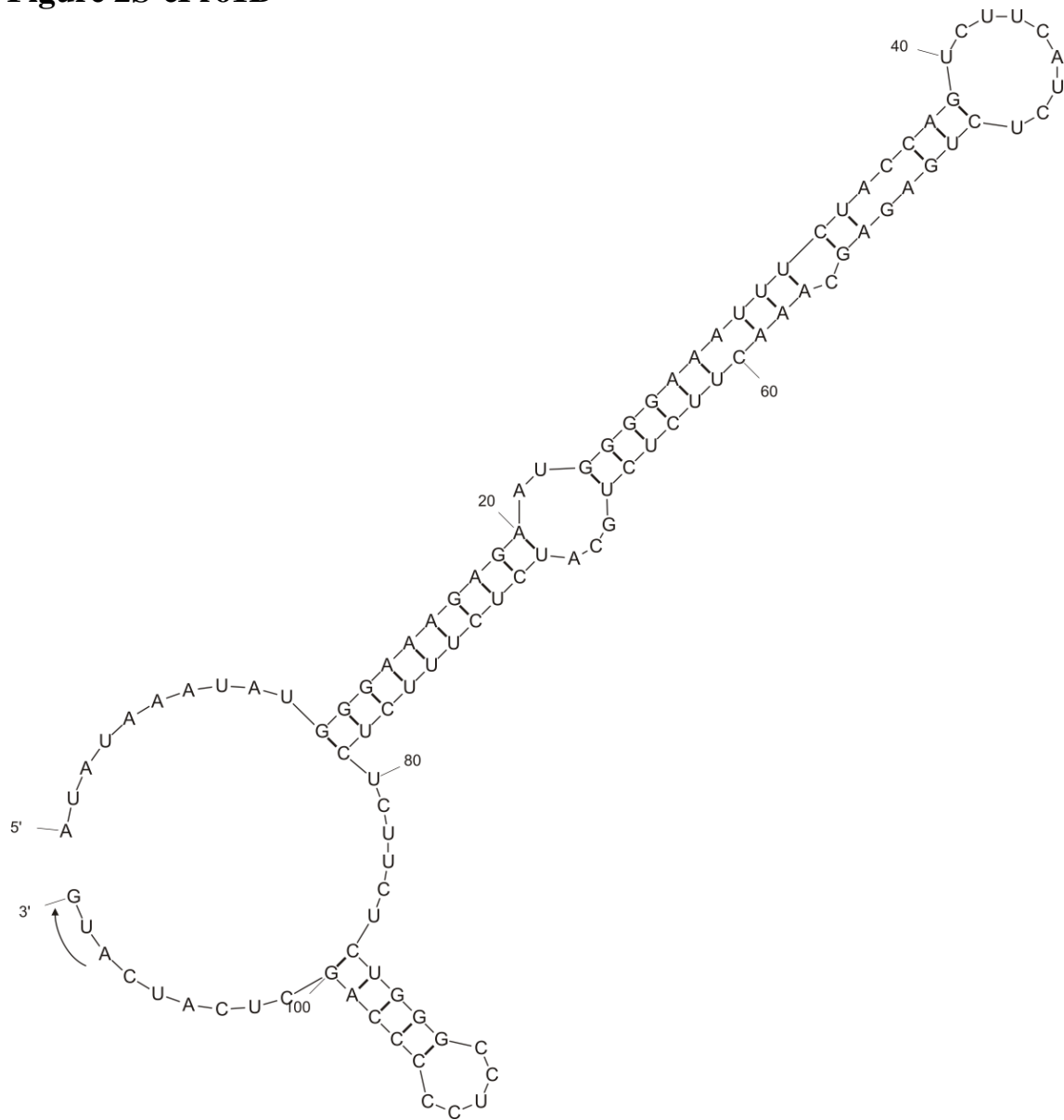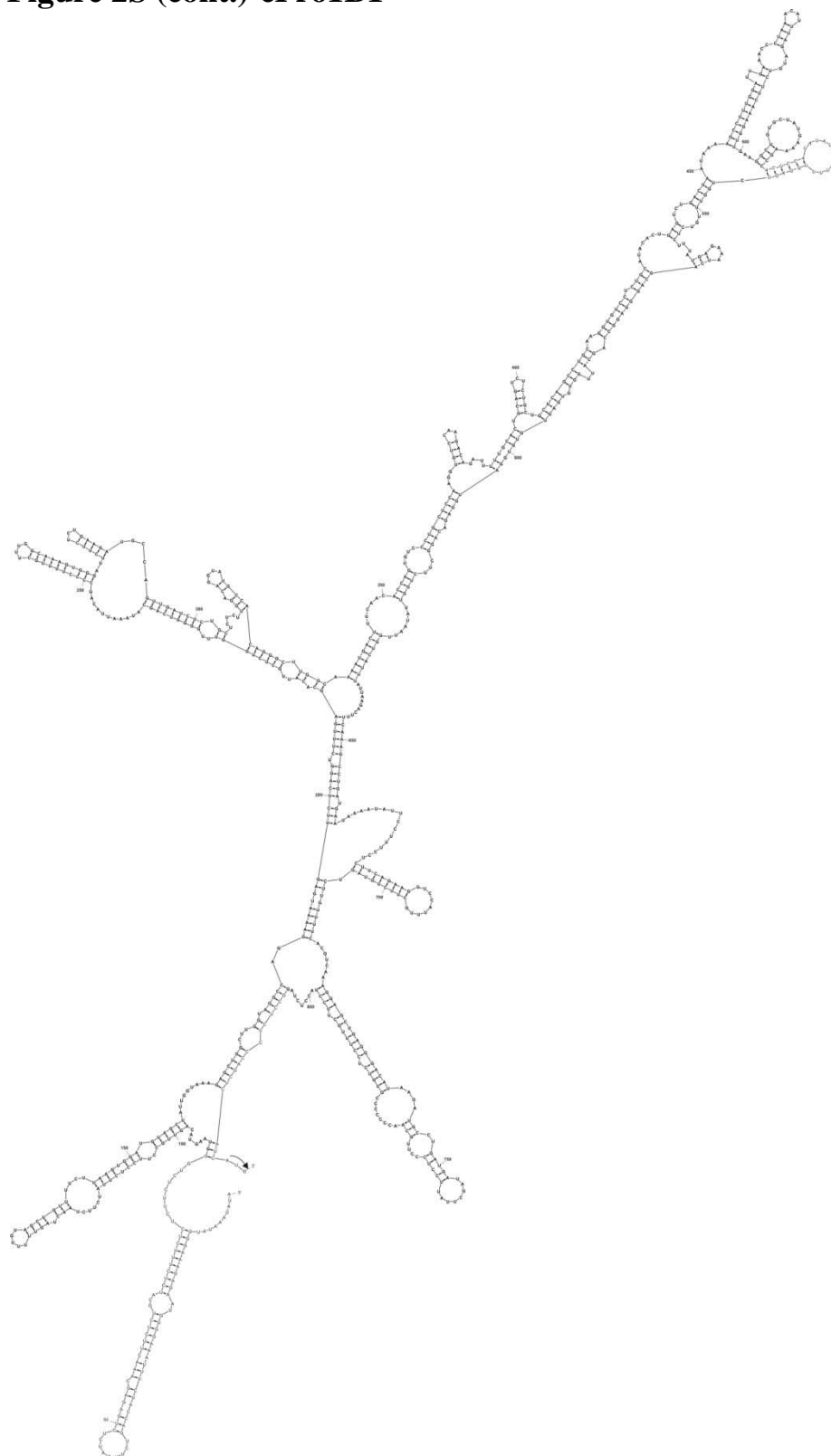
Figure 2S-cPro1B

Figure 2S (cont.)-cPro1B1

Figure 2S (cont.)-mIns2SPV

Figure 2S: Secondary structure predictions of chick and mouse proinsulin mRNA isoforms. The secondary RNA minimun free energy structures of the chick, cPro1B and cPro1B1, and the mouse, mNAT and mSPV, proinsulin 5' UTR were predicted by the RNAfold program (Vienna RNA Package, http://www.tbi.univie.ac.at/~ivo/RNA/). Their resulting connect (.ct) format files were displayed by the Structure Display

application from the mfold web server (http://mfold.rna.albany.edu/?q=mfold/Structure-display-and-free-energy-determination) using its default parameters.

Note that in the mouse insulin 2 gene, intron 1 is depicted with 114 nt, slightly longer than the 102 nt reported by Minn et al. 2005 [6], and we consider the initiation of transcription as mapped by Wentworth et al., 1986 [7]. Curved arrows point to the AUG translation initiation codon.

Table 1S

Construction:	pCAGS-I-GFP	pCAGs-Pro1A-I-GFP	pCAGs-Pro1B-I-GFP
Total malformed embryos	5/62 (8%)	22/36 (61%)	8/20 (40%)
Major malformation	0/62	14/36	0/20
Lack of endocardial tubes fusion	0/62	10/36	0/20
Minor malformation	5/62	8/36	8/20
Altered anterior region	3/62	6/36	6/20

Major malformation: embryos in which heart tube formation failed to occur, some cases with lack of fusion of the two endocardial tubes into a single tube, or in which an aberrant non-beating heart tube was observed.

Minor malformation: embryos in which an abnormal but beating heart tube was observed. In these cases, the predominantly affected region was the anterior (future ventricle and outer flow tract) part.