Reptilian heart development and the molecular basis of cardiac chamber evolution

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Author Contributions. K.K-T. performed reptile histology and gene expression studies; A.D.M., B.K., T.S., and B.G.B. performed mouse experiments; J.C.-T. and S.F.G. obtained turtle specimens and isolated T. scripta Tbx5 cDNA; S.L. and L.B. isolated Anolis specimens under direction of J.W.; B.K. acquired and reconstructed OPT images; R.O.G. performed Tbx5 immunohistochemistry under direction of M.N.; R.M.H. directed initial mouse embryo OPT; J.K.T. obtained chick and mouse specimens; R.O.G., M.N., B.L.B., and E.N.O. provided genetically modified mice prior to publication; B.G.B. conceived and directed the project, and wrote the paper. All authors contributed to the written manuscript.

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Abstract

The emergence of terrestrial life witnessed the need for more sophisticated circulatory systems. This has evolved in birds, mammals, and crocodilians into complete septation of the heart into left and right sides, allowing separate pulmonary and systemic circulatory systems, a key requirement for the evolution of endothermy 1–3. However, the evolution of the amniote heart is poorly understood. Reptilian hearts have been the subject of debate in the context of the evolution of cardiac septation: do they possess a single ventricular chamber or two incompletely septated ventricles 4–7? We examined heart development in the red-eared slider turtle, *Trachemys scripta elegans* (a chelonian), and the green anole, *Anolis carolinensis* (a squamate), focusing on gene expression in the developing ventricles. Both reptiles initially form a ventricular chamber that homogenously expresses the T-box transcription factor gene *Tbx5*. In contrast, in birds and mammals, *Tbx5* is restricted to left ventricle precursors 8,9. In later stages, *Tbx5* expression in the turtle (but not anole) heart is gradually restricted to a distinct left ventricle, forming a left-right gradient. This suggests that *Tbx5* expression was refined during evolution to pattern the ventricles. In support of this hypothesis, we show that loss of *Tbx5* in the mouse ventricle results in a single chamber lacking distinct identity, indicating a requirement for *Tbx5* in septation. Importantly, misexpression of *Tbx5* throughout the developing myocardium to mimic the reptilian expression pattern also results in a single mispatterned ventricular chamber lacking septation. Thus, ventricular septation is established by a steep and correctly positioned *Tbx5* gradient. Our findings provide a molecular mechanism for the evolution of the amniote ventricle, and support the concept that altered expression of developmental regulators is a key mechanism of vertebrate evolution.
transcription factors of the T-box family are important regulators of heart formation. One T-box gene, Tbx5, has an expression pattern that suggests a role in the evolution of cardiac septation (see Supplementary note 1). In amphibians, Tbx5 is expressed throughout the developing heart. In birds and mammals, there is a steep gradient of Tbx5 expression from high levels in the prospective left ventricle (LV) to low levels in the prospective right ventricle (RV). Reduced dosage of Tbx5 in humans and mice leads to defects in interventricular septum (IVS) formation and patterning, suggesting that a steep gradient of Tbx5 is critical for IVS formation. The evolutionary role of Tbx5 in septation is unknown.

We examined cardiac embryology of the red-eared slider turtle, T. scripta elegans (a chelonian), and the green anole, A. carolinensis (a squamate), focusing on the ventricles. Although the phylogenetic relationship of turtles to other reptiles is controversial based on anatomical considerations, molecular phylogenies consistently group turtles with the archosaurs (birds and crocodiles) and anoles are considered to be more basal than archosaurs. The post-hatching anole heart has a thick muscular ridge (Fig. 1a–d and Supplementary Fig. 1) that separates a proximal outflow tract, or cavum pulmonale, from the main ventricular chamber. Turtles have a smaller muscular ridge and are thought to have a primitive IVS-like structure, as we determined by three-dimensional reconstructions revealing a dense coalescence of trabeculae spanning the full depth of the heart (Fig. 1e–h and Supplementary Fig. 1). Initially, developing turtle and anole hearts showed no clear evidence of ventricular septation (Fig. 1i, and Supplementary Figs. 2–4). In contrast, the chick has a well-developed IVS at comparable early stages (Figs. 1i and Supplementary Fig. 3). In the turtle, a structure resembling an IVS appears only at stage 21 (Fig. 1i). Alligator embryos have a muscular ridge and a distinct IVS. The muscular ridge has been interpreted as analogous to the IVS, leading to the impression that reptiles have multiple septa. We speculate that the development of the muscular ridge in reptiles reflects persistent growth of the proximal outflow tract, as seen transiently in chick heart (Fig. 1i–k and Supplementary Fig. 3).

To observe molecular patterning of reptile ventricles, we examined expression of Tbx5. In mammals and birds, Tbx5 mRNA and protein are highly enriched in the prospective LV (Fig. 2b,c and Supplementary Fig. 5). At looping heart tube stages, Tbx5 was broadly expressed throughout the embryonic turtle and anole hearts (Fig. 2a,d), similar to Xenopus Tbx5 (Ref 13), but unlike its early restricted expression in chick and mouse (Fig. 2b,c). In the anole, Tbx5 expression extended to the boundary of the ventricle and outflow tract, where the muscular ridge forms. At later stages, Tbx5 expression in turtle (stage 15) and anole (stage 13) remained homogeneous throughout the ventricle (Fig. 2e,h and data not shown). In comparable stages in chick, it was sharply restricted to LV primordium. At stages 17–18 in the turtle, Tbx5 mRNA levels decreased in RV primordium, remaining enriched in LV primordium, creating a steep left-right gradient, although not as sharply defined as in chick (Fig. 2f,g,h,i and Supplementary Figs. 6,7). This gradient was maintained at stage 21 (Fig. 2h,i). Tbx5 expression in Anolis was not restricted in the ventricle (Fig. 2f,i and Supplementary Fig. 7). We examined expression of Tbx5 target genes expressed in trabeculae but excluded from mammalian IVS myocardium. Bmp10 was...
expressed throughout the early turtle and anole trabeculae, but was excluded in turtles at Stage 17–18 from an expansion of the compact myocardium corresponding to presumptive IVS precursors, correlating with the boundary of Tbx5 expression (Fig. 2j and Supplementary Fig 6). This suggests a conserved molecular transition in the trabeculae that form the IVS. Turtle Nppa (not found in anoles23), formed a gradient similar to Tbx5 (Fig. 2k). Thus, turtle ventricles, but not those of Anolis, acquire distinctions between left and right components late in development.

A steep Tbx5 gradient in chick and mouse may have evolved to pattern the ventricles. Reducing Tbx5 levels supports this1,4,16,17. To address a potential role for Tbx5 in septation, we deleted Tbx5 from segments of developing mouse ventricles, using a conditionally deletable Tbx5 allele (Tbx5<sup>LDN</sup>)16, and ventricular myocyte-specific Nkx2.5::Cre mice24 (Fig. 3a). These mice (Nkx2.5::Cre<sup>0/0</sup>;Tbx5<sup>LDN/LDN</sup> mice, or Tbx5<sup>V-del</sup> mice) lacked morphological distinctions between the LV and RV that were obvious in wild-type embryos by embryonic day (E) 9.5 (Fig. 3b). Embryos with this univentricular phenotype persisted until E11.5 (Fig. 3c). Expression of Nppa and Bmp10, normally excluded from the interventricular groove, was expanded throughout the single ventricle of Tbx5<sup>V-del</sup> embryos (Fig. 3e,f). Hand1 was expressed at lower levels, but in its normal domains, the LV and RV primordia (Fig. 3g). Thus, loss of Tbx5 from developing ventricles results in a single mispatterned ventricle.

To determine if a steep Tbx5 gradient at the interventricular midpoint is critical for IVS formation, we deleted Tbx5 with Mef2cAHF::Cre mice25 (Fig. 3h). Since Mef2cAHF::Cre is active in RV and IVS precursors, but not in the LV free wall, the Tbx5 expression boundary is shifted leftward. Tbx5<sup>LDN/LDN</sup>;Mef2cAHF::Cre (Tbx5<sup>AHF-del</sup>) mice lacked an IVS (Fig. 3i–k). Gene expression analysis showed that a distinction between LV and RV was maintained (Fig. 3l,m), but a clear absence of IVS-enriched markers (Irx2, Dkk3) at the ventricular midpoint, while maintained in the adjacent trabeculae, emphasize the absence of ventricular septation (Fig. 3n, Supplementary Fig. 8). Thus, a boundary of cells expressing high Tbx5 levels is necessary within a segment of myocardium where IVS outgrowth will occur. This implies a prepattern within which Tbx5 must function; the nature of this prepattern is unknown (See supplementary note 2). Tbx5 expression and additional patterning cues may have co-evolved, or the prepattern may exist in all amniotes. Regardless, IVS formation requires a sharp Tbx5 boundary indicating that Tbx5 patterning was a major factor in evolution of septation.

Our loss-of-function experiments demonstrate a requirement for Tbx5 in IVS formation distinct from a more global role in differentiation. These results do not address the evolutionary role of Tbx5 patterning; in particular, whether the broad expression of Tbx5 observed in anole and turtle would preclude IVS formation. Previous misexpression attempts yielded variable results ranging from no effect to severely malformed hearts (Ref. 9 and J.K.T., unpublished data). We misexpressed Tbx5 in the ventricles by crossing a mouse line bearing a stable Cre-activatable transgene expressing moderate Tbx5 levels upon induction (CAT-Tbx5)26 with Mef2cAHF::Cre or Nkx2.5::Cre mice (Fig. 4). CATTbx5; Mef2cAHF::Cre embryos survived until E11 and had a single ventricle at E10.25. Molecular analysis revealed expanded expression of Tbx5, Nppa, and Bmp10 across the interventricular
groove of CAT-Tbx5;Mef2cAHF::Cre embryos (Fig. 4b). CAT-Tbx5;Nkx2.5::Cre embryos survived longer (until E12), presumably because this manipulation avoided secondary effects of Tbx5 overexpression in cardiac progenitors. CAT-Tbx5;Nkx2.5::Cre embryos at E11.5 also had defective ventricular septation and mispatterned gene expression (Fig. 4c and Supplementary Fig. 9). Interestingly, due to the mosaic expression of Tbx5 by Nkx2.5::Cre, some embryos had no septum at all, while others with a more graded expression of Tbx5 had a rudiment of a septum in which not all genes were mispatterned (Fig. 4c and Supplementary Fig. 9). Thus, misexpression of Tbx5 in a pattern reminiscent of the reptilian heart leads to loss of IVS patterning and morphogenesis, further supporting a role for Tbx5 patterning in the evolution of septation.

Our results provide evidence that the reptilian heart, although evolved to function physiologically under conditions particular to reptilian life, is an evolutionary intermediate between amphibian and avian/crocodilian hearts in its ventricular development. The dynamic expression of Tbx5 and its leftward restriction suggest a temporal refinement model in which early restriction of Tbx5 expression to LV precursors, as seen in chick and mouse, provides a robust patterning cue for ventricular septation. In this model (Fig. 4d), a quantitative gradient of Tbx5 is essential for proper formation and patterning of the IVS. Our mouse genetic analyses, including decreased dosage, are consistent with an important role for a steep gradient of Tbx5 in chamber patterning and IVS formation. In the reptilian heart, the delayed and less pronounced establishment of this patterning may contribute to varying degrees of septation. Therefore patterning of Tbx5, in the archosauromorph and synapsid lineages, is likely to be an important mechanism in the convergent evolution of septation. Our findings generally support the concept that altered expression of developmental regulators is an important aspect of morphological evolution.

METHODS SUMMARY

Embryos were isolated from T. scripta elegans eggs (Kliebert Turtle and Alligator Farm, Hammond, LA). Green anole (A. carolinensis) embryos were collected in captivity. Mouse strains were described. Whole-mount and section in situ hybridizations were performed using standard protocols. Immunohistochemistry and OPT were performed as previously described. For all mouse experiments, at least 3 embryos were examined for each genotype at each stage, all with comparable results.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. Farmer CG. Evolution of the vertebrate cardio-pulmonary system. Annu Rev Physiol. 1999; 61:573–592. [PubMed: 10099702]

2. Hillenius WJ, Ruben JA. The evolution of endothermy in terrestrial vertebrates: Who? When? Why? Physiol Biochem Zool. 2004; 77:1019–1042. [PubMed: 15674773]

3. Olson EN. Gene regulatory networks in the evolution and development of the heart. Science. 2006; 313:1922–1927. [PubMed: 17008524]

4. Holmes EB. A reconsideration of the phylogeny of the tetrapod heart. J Morph. 1975; 147:209–228.

5. Webb GJW. Comparative Cardiac Anatomy of the Reptilia. III. The heart of crocodilians and an hypothesis on the completion of the interventricular septum of crocodilians and birds. J Morph. 1979; 161:221–240.

6. Farrell, AP.; Gamperl, AK.; Francis, ETB. Biology of the Reptilia. Gans, C.; Gaunt, AS., editors. Vol. Vol. 19. Ithaca, NY: Soc. fort he Study of Amphibians and Reptiles; 1998. p. 375-424. (Morphology G)

7. Hicks JW. The physiological and evolutionary significance of cardiovascular shunting patterns in reptiles. News Physiol Sci. 2002; 17:241–245. [PubMed: 12433978]

8. Bruneau BG, Logan M, Davis N, et al. Chamber-specific cardiac expression of Tbx5 and heart defects in Holt- Oram syndrome. Dev Biol. 1999; 211:100–108. [PubMed: 10373308]

9. Takeuchi JK, Ohgi M, Koshiba-Takeuchi K, et al. Tbx5 specifies the left/right ventricles and ventricular septum position during cardiogenesis. Development. 2003; 130:5953–5964. [PubMed: 14573514]

10. Seymour RS, Bennett-Stamper CL, Johnston SD, et al. Evidence for endothermic ancestors of crocodiles at the stem of archosaur evolution. Physiol Biochem Zool. 2004; 77:1051–1067. [PubMed: 15674775]

11. Greil A. Beitrage zur vergleichenden Anatomie und Entwicklungsgeschichte des Herzens und des Truncus arteriosus der Wirbeltiere. Morphol. Jahrb. 1903; 31:123–310.

12. Stennard FA, Harvey RP. T-box transcription factors and their roles in regulatory hierarchies in the developing heart. Development. 2005; 132:4897–4910. [PubMed: 16258075]

13. Horb ME, Thomsen GH. Tbx5 is essential for heart development. Development. 1999; 126:1739–1751. [PubMed: 10079235]

14. Bruneau BG, Nemer G, Schmitt JP, et al. A murine model of Holt-Oram syndrome defines roles of the T-box transcription factor Tbx5 in cardiogenesis and disease. Cell. 2001; 106:709–721. [PubMed: 11572777]

15. Bruneau BG. The developmental genetics of congenital heart disease. Nature. 2008; 451:943–948. [PubMed: 18288184]

16. Mori AD, Zhu Y, Vahora I, et al. Tbx5-dependent rheostatic control of cardiac gene expression and morphogenesis. Dev Biol. 2006; 297:566–586. [PubMed: 16870172]

17. Koshiba-Takeuchi K, Takeuchi JK, Arruda EP, et al. Cooperative and antagonistic interactions between Sall4 and Tbx5 pattern the mouse limb and heart. Nat Genet. 2006; 38:175–183. [PubMed: 16380715]

18. Rieppel O. Turtle origins. Science. 1999; 283:945–946. [PubMed: 10075558]

19. Lyson T, Gilbert SF. Turtles all the way down: Loggerheads at the root of the chelonian tree. Evolution and Development. 2009; 11:133–135. [PubMed: 19245543]

20. Hedges SB, Poling LL. A molecular phylogeny of reptiles. Science. 1999; 283:998–1001. [PubMed: 9974396]

21. Shedlock AM, Botka CW, Zhao S, et al. Phylogenomics of nonavian reptiles and the structure of the ancestral amniote genome. Proc Natl Acad Sci U S A. 2007; 104:2767–2772. [PubMed: 17307883]
22. Chen H, Shi S, Acosta L, et al. BMP10 is essential for maintaining cardiac growth during murine cardiogenesis. Development. 2004; 131:2219–2231. [PubMed: 15073151]

23. Trajanovska S, Donald JA. Molecular cloning of natriuretic peptides from the heart of reptiles: loss of ANP in diapsid reptiles and birds. General and comparative endocrinology. 2008; 156:339–346. [PubMed: 18295764]

24. McFadden DG, Barbosa AC, Richardson JA, et al. The Hand1 and Hand2 transcription factors regulate expansion of the embryonic cardiac ventricles in a gene dosage-dependent manner. Development. 2004; 132:189–201. [PubMed: 15576406]

25. Verzi MP, McCulley DJ, De Val S, et al. The right ventricle, outflow tract, and ventricular septum comprise a restricted expression domain within the secondary/anterior heart field. Dev Biol. 2005; 287:437–449.

26. Georges R, Nemer G, Morin M, et al. Distinct expression and function of alternatively spliced Tbx5 isoforms in cell growth and differentiation. Mol Cell Biol. 2008; 28:4052–4067. [PubMed: 18391012]

27. Carroll SB. Evo-devo and an expanding evolutionary synthesis: a genetic theory of morphological evolution. Cell. 2008; 134:25–36. [PubMed: 18614008]

28. Lickert H, Takeuchi JK, von Both I, et al. Baf60c is essential for function of BAF chromatin remodelling complexes in heart development. Nature. 2004; 432:107–112. [PubMed: 15525990]
Figure 1. Reptilian heart development

a–h. Optical projection tomography of post-hatching anole (a–d) and turtle (e–h) hearts. a,e external view, b,c,f,g chamber fill; d,h histology. i. Histological analysis of heart development in turtle, anole, and chick embryos. Four representative stages shown are equivalent between species. arrow: interventricular groove. j. Histology of stage 21 embryonic alligator heart. k. OPT of st. 17 (left) and st. 21 (right) turtle hearts. In all reptile embryos (i–k), note close apposition of mr and ot. a: atrium, IVS?: IVS-like structure, la:
left atrium, lv: left ventricle, mr: muscular ridge, ot: outflow tract, ra: right atrium, rv: right ventricle.
Figure 2. Gene expression in amniote embryos

a–c, *Tbx5* expression in turtle, chick, and mouse. Left panels: whole-embryo views. He: heart, fl: forelimbs, dr: dorsal retina, hl: hindlimbs. Right panels: closeup ventral views of embryonic hearts. In c, bottom panel shows *Tbx5* immunohistochemistry; red arrowheads: rv/lv junction; purple arrowhead: epicardium. la: left atrium, lv: left ventricle, ot: outflow tract, ra: right atrium, rv: right ventricle. d–g, Expression of *Tbx5*. As: atrial septum; avc: atrioventricular cushion. Arrowheads mark the boundary between lv and rv, or V and OT for Anole in d. h. Quantitation of *Tbx5* mRNA levels in turtle LV and RV; data are mean ± SD.
normalized to St 15 RV. *P<0.005 by t-test. i. Ratio of Tbx5 mRNA levels between the LV and RV. j. Bmp10 expression in turtle and Anole hearts. Arrowheads: interventricular groove and septum. Brackets: thickness of Bmp10-negative area. k. Nppa expression in the turtle is in a left-right gradient similar to Tbx5.
Figure 3. Ventricle-restricted deletion of mouse Tbx5

a, Strategy for ventricular deletion of $Tbx5$. b–d, OPT of wild-type (WT) and $Tbx5^{vdel/vdel}$ embryos and hearts at E9.5 (b) and E11.5 (c, d). Arrowheads indicate position of the IVS.

e,f,g: Gene expression for indicated transcripts.

h, Strategy for $Tbx5$ deletion in anterior heart field derivatives.

i–k, OPT of wild-type (WT) and $Tbx5^{AHFdel/AHFdel}$ hearts at E10.5.

i: external view, j: chamber fill, k: virtual sections.

l,m,n: Gene expression for indicated transcripts.
Figure 4. Misexpression of Tbx5 results in loss of IVS patterning

a. Strategy for ventricular misexpression of Tbx5. b. Morphology and gene expression in CAT-Tbx5;Mef2cAHF::Cre embryos for indicated transcripts. Brackets: IVS region, magnified in lower panels. Arrowheads: trabecular Bmp10 expression. c. Morphology and gene expression in CAT-Tbx5;Nkx2.5::Cre embryos at E11.5. Orange arrows: interventricular septum region (IVS). Brackets show a rudimentary septum in a mutant embryo. d. Diagrammatic representation of embryonic heart structures and patterns of Tbx5.
expression (blue) in vertebrate evolution. la: left atrium, lv: left ventricle, ot: outflow tract, ra: right atrium, rv: ventricle, v: ventricle.