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Concepts of Cardiac Development in Retrospect

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Abstract Recent research, enabled by powerful molecular techniques, has revolutionized our concepts of cardiac development. It was firmly established that the early heart tube gives rise to the left ventricle only, and that the remainder of the myocardium is recruited from surrounding mesoderm during subsequent development. Also, the cardiac chambers were shown not to be derived from the entire looping heart tube, but only from the myocardium at its outer curvatures. Intriguingly, many years ago, classic experimental embryological studies reached very similar conclusions. However, with the current scientific emphasis on molecular mechanisms, old morphological insights became underexposed. Since cardiac development occurs in an architecturally complex and dynamic fashion, molecular insights can only fully be exploited when placed in a proper morphological context. In this communication we present excerpts of important embryological studies of the pioneers of experimental cardiac embryology of the previous century, to relate insights from the past to current observations.

Keywords Cardiovascular development · Heart fields · Lineage · Proliferation · Morphology

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Introduction

The introduction of molecular techniques to developmental biology has greatly empowered research on cardiac development and has led to some important shifts of paradigm. For instance, it is now firmly established that the initially formed heart tube does not contain all prospective cardiac chambers, but that it is mainly fated to become the left ventricle, while the remainder of the myocardium is added during subsequent development (Fig. 1) [3]. Furthermore, the cardiac chambers were shown not to originate from circumferential segments around the early straight tubular heart, but to balloon out from the outer curvatures of the looping heart tube [22]. Remarkably, traditional morphological embryology, relying on limited experimental techniques, reached very similar conclusions, as we show in a few examples.

With the advent of molecular biology as a powerful and productive discipline in embryology, scientific emphasis became largely focused on cellular and molecular mechanisms that control the formation of the heart, while the morphological context of these processes became of lesser importance. Because the developing heart and its precursors rapidly transform in a spatially complex fashion, molecular data can only be interpreted in a proper three-dimensional (3D) context. It is, therefore, disappointing that morphological insights of heart formation are hardly incorporated into current-day molecular research on cardiac development. The complexity of interpreting morphological data using traditional 2D approaches may be an underlying cause. Fortunately, novel 3D reconstructions and other visualization techniques can fill this gap [31].

It is the goal of this article to place the new concepts of cardiac development in the context of classic morphological

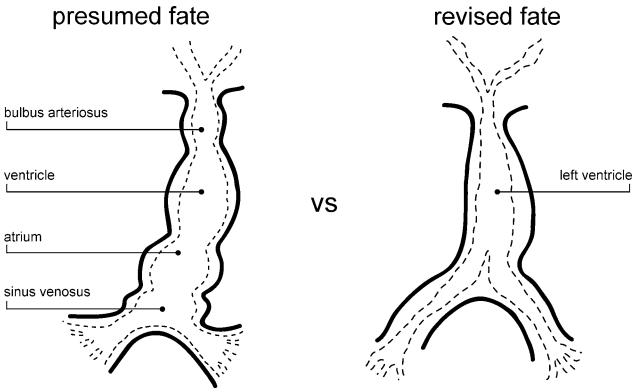

Fig. 1 Changing view of cardiac development. The illustration on the *left* shows a heart tube as it was previously often depicted: the straight heart tube containing all cardiac compartments as circumferential segments. The illustrations on the *right* show the straight heart tube with its revised fate: only precursors of the future left ventricle are present. The hatched lines indicate endocardial cell layers. The continuous *lines* indicate myocardial cell layers. *Source*: images are based on Patten and Kramer [26]

and physiological insights. We discuss excerpts of the meticulous work of pioneering researchers of cardiac development (namely, Bradley Patten, Robert DeHaan, and Victoria de la Cruz) to investigate whether their views harmonize with recent insights and to explore whether pre-existing morphological insights can stimulate the formulation of new hypotheses.

Fusion of the Vitelline Veins Forms the Early Heart

The current view on cardiac development is that the heart is formed by cells that originate from several embryonic fields [2]. Prior to this notion, however, only a single heart-forming region (HFR) was described [29, 30]. The classic consensus of the formation of the early heart tube from this HFR is depicted in Fig. 2. With gastrulation, intra-embryonic mesoderm is formed, which is then separated into a splanchnic and a somatic layer by formation of the coelomic cavity. The somatic mesoderm lines the ectoderm, and the splanchnic mesoderm lines the endoderm. Transplantation studies showed that the embryonic disc contains a left and a right heart-forming region HFR in its splanchnic mesoderm [29, 30]. Expression of important cardiac transcription factors such as *Nxk2.5* [21], *Gata4* [17], and *e/dHand* [40] underlines the cardiogenic capacity of this mesoderm.

The transformation of the HFR into a heart tube is morphologically complex. As illustrated by the transverse sections in Fig. 2, the lateral limits of the HFR luminize [9] and make endothelial cells [6]. This forming lumen is caudally contiguous with the vitelline veins, which cover the yolk sac. Cranially, the walls of these primitive vitelline veins will start to express sarcomeric proteins [9, 44] and will fuse in the embryonic midline to form the embryonic heart tube [7, 15, 26, 46, 47]. Shortly after fusion, the ventral side of the heart tube starts to twitch [32], which is followed shortly by rhythmic peristaltic contractions originating from the venous pole of the heart tube [20, 26].

The fact that cardiac and vessel precursors reside in neighboring developmental territories was also found to be of importance for the molecular control of their development. Knockdown of *scl* or *etsrp,* transcription factors that promote vessel formation, enlarged the heart field and increased the number of cardiomyocytes of zebrafish embryos. Conversely, overexpression of these "vessel-genes" reduced the heart field and the number of cardiomyocytes [35].

Transformation of the Heart-Forming Region with Folding

A new and important concept in heart development are the proposed multiple heart-forming fields. Cardiac precursors were shown to be added to both poles of the initially formed heart tube [3]. These precursors are thought to take origin from a second heart-forming field, primarily located in the coelomic wall that overlies the foregut [2]. In the previous century a large body of research was invested into the delineation of cardiac precursors within the embryonic disc, and into the transformation of these cells into the heart tube [29, 30, 42]. These studies, however, did not lead to the proposition of multiple heart-forming fields.

As previously pointed out, an explanation for not describing a second source of cardiac precursors might lie in the inability to culture embryos up to stages when addition of cells to the heart was completed [25]. This experimental disadvantage has most likely hampered the observation of the full extent of the original HFR. Nevertheless, addition at both poles of the heart tube does occur during the time frame of culturing. De la Cruz et al. excised the early heart tube of an embryo in culture. With culturing the pericardial cavity filled at both the venous and the arterial pole with newly forming myocardium [11]. This indicates that addition from what is currently called the second heart field could have been observed. Why it was not denoted as such may lie in the intricacy of the morphological transformations of the HFR during embryonic folding.

Folding can be regarded as the process by which both the cranial and the lateral aspects of the embryonic disc bend inward (Fig. 2) [42]. By this process the foregut is formed as a pocket in the endoderm and the left and right HFRs swing toward midline, thus forming the heart tube (Fig. 2). Inspection of the morphogenetic transformation of

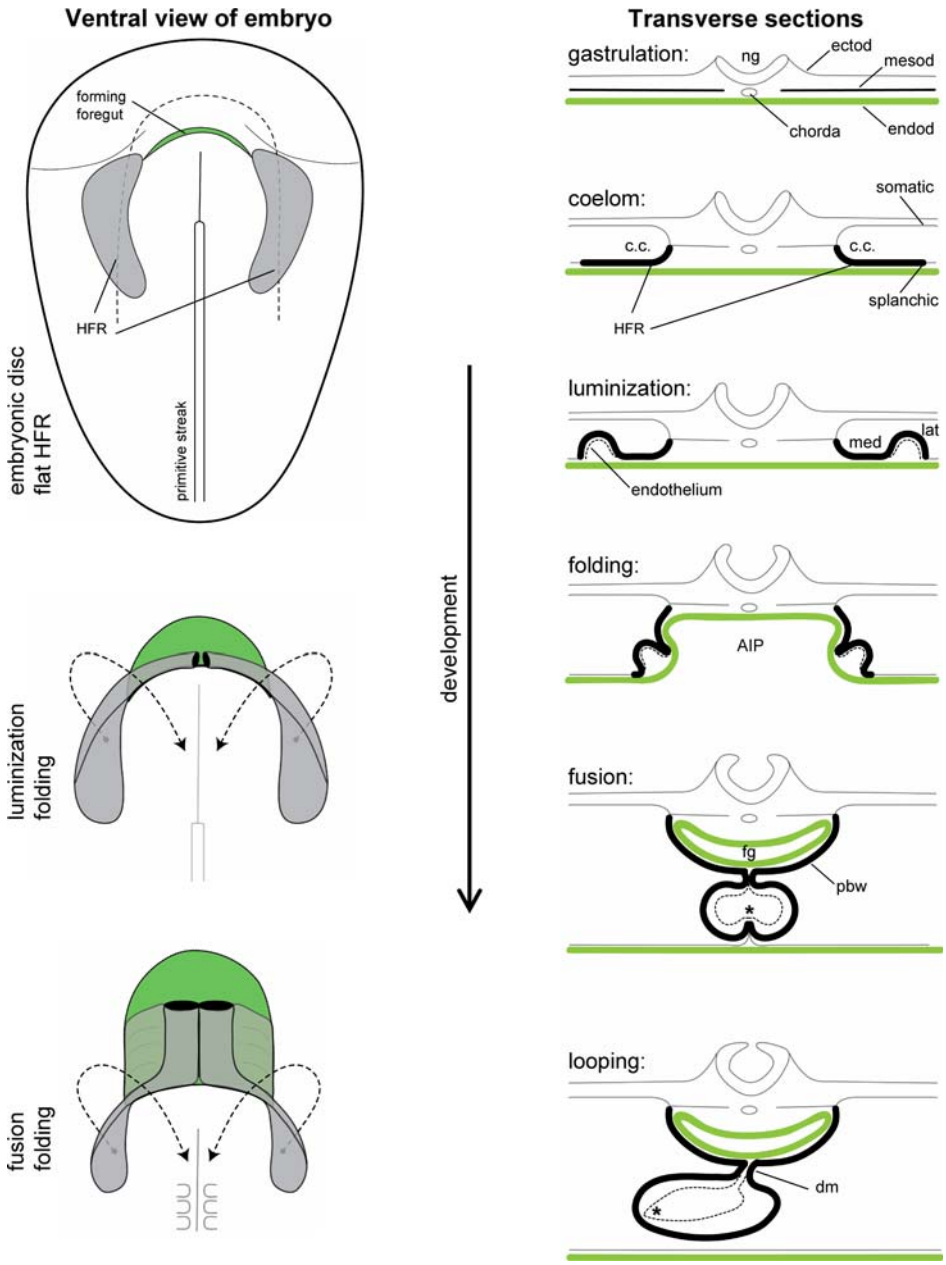

Fig. 2 Morphological changes during early heart development. The illustrations on the left show how, by folding of the embryo, a foregut (show in *green*) is formed, and how the bilateral heart-forming region (shown in *gray*) swings toward ventral and medial to progressively fuse at the midline. The illustrations on the right show schematic transverse sections of the changes that occur in the embryo during folding. *AIP* anterior intestinal portal, *c.c.* coelomic cavity, *dm* dorsal mesocardium, *ectod* ectoderm, *endod* endoderm, *fg* foregut, *HFR* heart-forming region, *lat* lateral, *med* medial, *mesod* mesoderm, *ng* neural groove, *pbw* pericardial back wall, * contact between endocardium and myocardium. *Source*: images are based on Stalsberg and De Haan [42] and De Jong et al. [9]

the HFR (sections in Fig. 2) shows that the lateral parts of the HFR fuse and luminize to form the ventral aspect of the heart tube. The medial parts of the heart fields, however, remain to contact the endoderm and will become the pericardial back wall. It is this back wall that is now said to contain the second heart field. The contact between the medial and the lateral mesoderm can later be recognized as the dorsal mesocardium. After rupture of this mesocardium, the medial mesoderm contacts the heart tube only at its venous and arterial poles.

These morphological details indicate that the original HFR is not the equivalent to the first heart field, and contains (at least a part of) the recently described second heart field. In line with this notion, previous radiolabeling of one side of the "classic" HFR showed unilateral marking of the endocardium, the myocardium, and the pericardial back wall [41]. Moreover, migration studies by Rosenquist and DeHaan already clearly showed that the original HFR contributes to both poles of the heart [30].

Developmental Plasticity of the Heart-Forming Region

A reason for classical embryologists not to segregate the HFR into more fields might be that they adhered to a stricter definition of an embryonic field. Such a field was defined to be an "area of tissue within which a certain process, such as [induction of an organ] occurs" [37]. In

other words, cells within a limb field are committed to form a limb; cells within an eye field, to form an eye; and cells within a heart field, to form a heart. Therefore, if one would propose multiple fields within the HFR, this, by definition, would imply each proposed field to be committed to a specific fate. This, however, did not appear to be the case.

De Haan and coworkers transplanted tissue within the classic HFRs. In normal development, caudal tissue from the HFR forms myocardium that expresses an atrium-specific myosin and has a relatively high beat rate, while cranial tissue will form ventricular myosin-expressing myocardium with a lower beat rate. Interestingly, relocated cells adapted to the phenotype of their new surroundings: cranial tissue increased in beat rate when grafted caudally, while caudal tissue gave rise to ventricular myosin-expressing myocardium after being placed cranially [33, 34]. From these experiments DeHaan and coworkers concluded that "although pre-cardiac mesoderm is spatially organized to form particular cardiac tissues … the cells are not irreversibly committed … to a pre-determined pattern of physiological differentiation" [8]. This argues against a subdivision of the original HFRs into distinct fields.

The current separation of the HFR into multiple fields has, however, proven to be useful. It has led to knowledge of the molecular control of the formation of cardiomyocytes from precursors, which might be translated to regeneration-based therapeutics for heart disease. The underlying morphological mechanism of these observations might possibly be better explained otherwise. Classic studies support a model of a gradual formation of the heart from a single HFR, which, with embryonic folding, is molded in such a way that it can only add cells to the heart via its inflow and its outflow. The concept of just a single heart field is also supported by recent observations showing that marker genes of the second heart field are already expressed in the first heart field [1, 27, 48]. Our own lineage and proliferation studies also indicate a gradual formation of the heart tube from a single focus of rapidly proliferating cells in the splanchnic mesoderm [45].

The Fate of a Gradually Lengthening Heart Tube

As a consequence of the ongoing recruitment to the heart, the initially formed heart tube does not contain all prospective cardiac components. Recent data have shown that this early tube only gives rise to the future left ventricle [3]. An important parameter for understanding the growth of the heart tube is the regionalization of proliferation. In other words, is the inherent proliferation rate of early myocardium sufficient to account for the growth of the heart?

Previous publications with respect to this subject are scarce and contradicting, stating slow [36] as well as rapid [43] proliferation of the early heart tube. However, novel developed techniques [39] have enabled us to show that newly forming myocardium of embryos of both chicken [38, 45] and mouse (unpublished results) embryos does not proliferate. These studies further emphasize that early cardiac growth can only be achieved by recruitment of cardiomyocytes. This, and the notion of the left ventricular fate of the early heart, can also be deduced from classic observations, as we show below.

Figure 3 summarizes several experiments by De la Cruz et al. [12–14]. The early heart was labeled at both its arterial (●) and its venous edges (▲), i.e., at the pericardial reflections. The initially placed cranial label could be observed to move caudal. This movement was recently also observed in the developing mouse heart [49]. Further tracing such an early cranial label (●) showed its presence in the ventricular septum. This observation was confirmed by recent cell tracings at our lab [28]. A label placed in the outflow tract at a later stage (○) ended up in the right ventricular free wall, showing that the right ventricle is formed by cardiomyocytes that are added to the heart at the

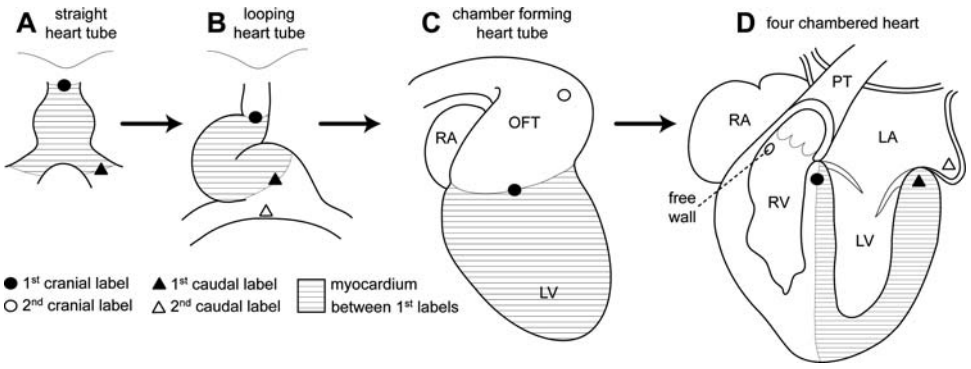

Fig. 3 The fate of the early heart tube. A summary of experiments by De la Cruz et al. [12–14]. The early heart tube was labeled and reincubated. Ventral views of a straight (**a**) and a looping (**b**) heart tube. **c** Right view of a chamber-forming heart. **d** Ventral view of a four-chambered heart, showing the inflow of the left ventricle (*LV*) and the outflow of the right ventricle. *LA* left atrium, *PT* pulmonary trunk, *RA* right atrium, *RV* right ventricle

arterial pole. At the venous pole, an early label could be traced to the left ventricular free wall of the four-chambered heart, while the later label (Δ) was found in the left atrium, upstream of the mitral valve. So these tracing experiments not only show addition of myocardium at both poles of the heart tube, but also demonstrate that the initial tube is fated to become the left ventricle.

Because the heart tube is gradually formed by a caudal progression of the fusion of the left and right HFRs (Fig. 2), the fate of a caudally placed label will depend on the time of placement. The initial point of fusion, however, will form the ventricular septum, as indicated by the earliest cranial label (●). Recent genetic lineage analysis in mouse, performed in our lab, reached a similar conclusion (Aanhaanen, Christoffels, and coworkers; in preparation].

Chamber Formation: The Ballooning Model

In the previous paragraphs we discussed that not all cardiac chambers are present in the myocardium of the early heart tube. Further complicating the concept of heart development is that not all myocardium of the growing heart tube will form chambers. Although often schematically represented, the chambers do not differentiate as circumferential segments along the length of the heart tube but, rather, as modules perpendicular to the axis of the looping heart tube. This mechanism of heart development was dubbed the *ballooning model* [22]. Although conceptually more complex than most schematics, the ballooning model offers an excellent example of how a morphological model can lead to the unraveling of molecular mechanisms underlying heart formation.

Apart from displaying slow proliferation, newly formed myocardium is also poorly differentiated. It has underdeveloped sarcomeres and is weakly electrically coupled, leading to a sluggishly contracting tube. The phenotype of this myocardium resembles the nodes of the adult conduction system [22]. During development, a subset of the heart tube further differentiates into the working myocardium of the chambers. This process initiates specifically at the outer curvature of the looping heart tube [4, 23]. Why this process initiates at this location might be explained by the local intimate association of the endocardium and myocardium.

As illustrated in the sections in Fig. 2, the fusion of the primitive vitelline veins occurs in the ventral midline of the

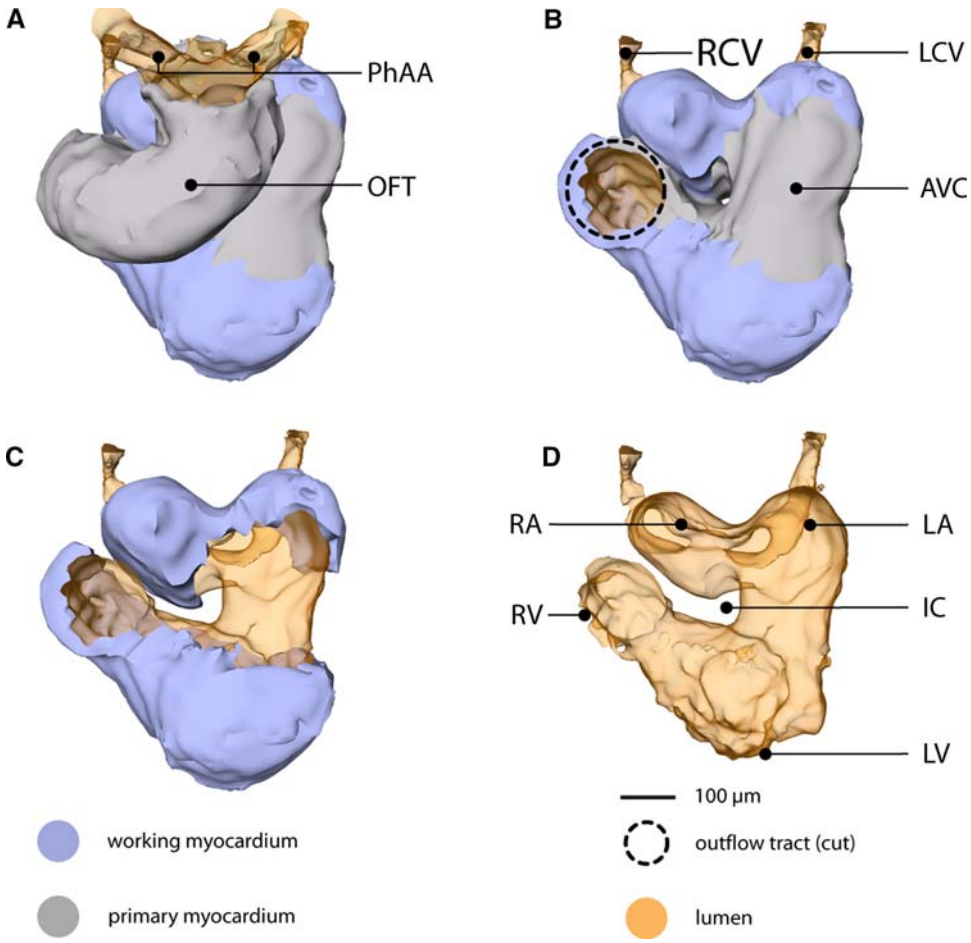

Fig. 4 The ballooning model. A reconstruction of Cx40 expression (shown in *blue*) in the heart of a mouse ED 9.5. **a** The entire reconstruction, in **b**–**d** the OFT is removed. *AVC* atrioventricular canal, *IC* inner curvature, *LA* left atrium, *LCV* left caval vein, *LV* left ventricle, *OFT* outflow tract, *PhAA* pharyngeal arch arteries, *RA* right atrium, *RCV* right caval vein, *RV* right ventricle

embryo. With this fusion, the endothelial layers also adjoin ventrally, where they contact the forming myocardium. In the heart, the endothelial cell layer is called the endocardium. With looping of the heart tube, the contact between endocardium and myocardium becomes located at the outer curvature. It has recently become clear that *Notch* signaling from the endocardium causes the myocardium to trabeculate [16], a hallmark of ventricular differentiation and occurring only at the outer curvature. Notch signaling depends on cell-cell contact, thus limiting this mechanism to the outer curvature.

Recent work from our lab showed that, prior to trabecularization, myocytes of the outer curvature of the looped heart enlarge in volume and then reinitiate proliferation [38]. This observation may suggest that cell-size-controlled pathways are important for myocyte proliferation and concomitant differentiation. At the proliferating outer curvatures, the myocardium also shows a local increase in conduction velocity [10], coinciding with the initiation of expression of gap-junctional proteins, such as Cx40 (Fig. 4). Also, the contractile apparatus of the chambers further develops at the outer curvatures [23]. Myocardium of the inner curvature, the atrioventricular canal, and the outflow tract remains poorly differentiated, resembling nodal myocardium.

The inner/outer curvature differentiation of the heart tube is tightly regulated by T-box transcription factors [24]. Tbx2 and Tbx3 were found to be specifically expressed in the above-described underdeveloped regions of the heart tube. Overexpression of Tbx2 in mice resulted in a failure of chamber differentiation [5]. Tbx3 is closely related to Tbx2 and is expressed in the developing conduction system of the heart [18]. Ectopic expression of this transcriptional repressor in working myocardium of the atria provoked an up-regulation of sinus node specific genes. Moreover, electrophysiological analysis of these atria showed the presence of ectopic nodal tissue [19].

The ballooning model is based on the recognition that cardiomyocytes of the initially formed heart tube resemble the nodes of the adult conduction system. The notion that, after looping of the heart tube, this phenotype is retained at the inner curvatures was supported by the observation that transcriptional repressors were expressed at the inner curvatures. These observations, and further functional analyses, provided insights into the formation of the sinus node which may offer clinical inroads regarding the development of bio-artificial pacemakers [19].

Conclusion

The ballooning model shows that the integration of morphological and physiological insights with recent molecular findings can lead to the unraveling of developmental mechanisms. Unfortunately, these old insights seldom are combined with current research on cardiac formation from precursors. The view that classic embryologists had of the developing heart is strikingly similar to the currently proposed models. This is nicely illustrated by a quote from an article by Bradley Patten in 1933: "The tubular heart is not formed all at once …. We must clearly recognize the fact that the part of the heart which we know in comparative anatomy as the sinus venosus is not established until after the ventricle and the atrium have been formed" [26].

Although lacking the power of molecular techniques, classic experiments have resulted in solid insights into the development of the heart, which are still proving to be valid. It is therefore worthwhile to incorporate molecular observations in a morphological framework, rather than using such observations for the postulation of new models of heart formation.

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