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# Early Mesodermal Cues Assign Avian Cardiac Pacemaker Fate-Potential in a Tertiary Heart Field**

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## Abstract

Cardiac pacemaker cells autonomously generate electrical impulses that initiate and maintain the rhythmic contraction of the heart. Although the majority of the heart is thought to originate from the primary and secondary heart fields, we report that chick pacemaker cells arise from a discrete region of mesoderm outside of these fields. Shortly after gastrulation, canonical Wnts promote the recruitment of mesodermal cells within this region into the pacemaker lineage. These findings identify the ontogeny of cardiac pacemaker cells, suggesting that pacemaker cells are physically segregated and molecularly programmed in a tertiary heart field, prior to the onset of cardiac morphogenesis.

The rhythm of the heart is maintained by a specialized sub-class of myocytes known as cardiac pacemaker cells (PCs). These cells generate action potentials (APs) in a cyclic manner to stimulate cardiac contractions. The anatomic position of mature PCs, the sinoatrial node (SAN), was described more than 100 years ago (1), however, little is known regarding the ontogeny or molecular mechanisms that specify PCs during development. This study was designed to address the timing, location, and mechanisms of PC cell fate acquisition.

Electrophysiological studies (2, 3, 4) have mapped cells that initiate cardiac APs to the inflow region at heart tube, looping, and septation stages. However, recent evidence indicates that as the heart matures, it continually expands with cells being added to both the inflow and outflow segments (reviewed 5). To determine which, if any, of the previously identified developmental pacing centers give rise to the mature SAN, whole embryonic chick hearts were imaged using optical mapping (4). Coincident with the heart's first contractions at Stage 10 (Hambuger and Hamilton staging (6)) the AP initiation site was preferentially associated with the left posterior inflow segment of the heart (Figure 1A (red region), F). Left sided pacing remained dominant through the process of dextral looping, (Figure 1B,F). By late heart looping (St18), the AP initiation site shifted to the ventral surface of the right inflow, juxtaposed to and outside of the forming atria (Figure 1C). From this stage on, all hearts displayed a right-sided AP initiation site (Figure 1D, E, F, see also Supplementary Movies 1–5).

As the AP initiation site shifted from left to right, AP morphology changed substantially, displaying pronounced slow diastolic depolarization and shorter AP duration than earlier pacing centers (Supplemental figure 1). Right sided pacemakers additionally exhibited a

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unique expression profile, becoming enriched for genes associated with mature PC AP generation including Hcn4, Serca2, and Ryr2 (7,8,9,10), and co-expressing the atrial and ventricular muscle markers Amhc1 and Vmhc1 (Supplementary Fig 2). To determine whether these differences were due to the maturation of migrating earlier pacing cells, or were caused by the differentiation of a new cell population, vital lipophilic fluorescent dyes were used to trace the fates of early pacing cells. In no case did dye labeled cells from left sided AP initiation sites contribute to older pacing centers (Figure 1G, H, Supplementary Fig 3). In contrast, cells from the post looping, right inflow remained associated with the heart's pacing region at all subsequent stages examined, eventually integrating into the SAN region at the back of the right atria (Figure 1I, J, Supplementary Fig 4). These data demonstrate that PC precursors emerge from a population of cells that is electrically inactive during the initial stages of heart development and begin pacing the heart within about an 8 hour developmental window coinciding with late dextral looping.

To identify when the assignment of PC fate occurs, we determined the location of PC precursors at stages prior to their electrical activation. Since none of the above markers displayed similar enrichment at earlier time points (Supplementary Fig 2), we utilized a non-marker, direct cell labeling approach to create a series of geometric fate maps. Fate maps were scored based on labeled cell incorporation into the late looping stage PC region, and verified by whole mount in situ hybridization for Hcn4 (see Supplemental Figures 5,6). At each of the stages examined, including gastrulation stages (St5), neurula stages (St8), and heart tube stages (St10), PC precursors were mapped to a discreet region of the right lateral plate mesoderm (Figure 2A Blue = all tagged sites, Red =>10% PC). This region was posterior to the classical primary and secondary heart fields, as previously defined by cell tracing experiments and Nkx2.5 and Isl-1 expression (Figure 2B) (11–17). Due to the limitations of vital dye labeling, we cannot rule out that at each of these stages other regions contribute to the PCs. However, since labeled cells occupied the majority of the available area (Supplementary Figure 5) when present at the ventral right inflow outside contributions would have to be minor.

PC precursors mapped to a region of the embryo that had not previously been identified as cardiogenic. Given the relatively large distance between PC precursors and the Nkx2.5/Isl1 expression domains currently associated with cardiac mesoderm, we wanted to determine the distribution of cell fates within this region where Nkx2.5/Isl1 were undetecable. A contour plot of the St 8 labeling data revealed that the region with the highest probability of PC fate was 100um in diameter centered 300um lateral to somite 3 (Figure 2C). The surrounding Nkx2.5/Isl1 negative mesoderm generated atria, atrioventricular junction, and the proepicardium (Figure 2D, Supplementary Figure 5), indicating large portions of the cardiogenic mesoderm does not express detectable levels Nkx2.5 or Isl1 at St 8. PC precursors did not substantially overlap with adjacent cardiac cell types, suggesting that heart precursors spatially segregate very early during lateral plate mesoderm formation.

Using the above fate mapping information, we examined when PC fate became specified. PC precursors and primary/secondary heart field cells (Nkx2.5/Isl1 positive domain) were isolated at the embryonic stages outlined above and allowed to differentiate ex vivo. The physiological and molecular identity of explants was then monitored. St 18 explants spontaneously initiated APs with a periodicity of 256 +/– 29ms and displayed phase 4 (diastolic) depolarization (Figure 3A,B, Supplementary Figure 7, Movie 7) distinct from stage matched working myocardial explants. PC AP characteristics were not present in St 5 explants (Figure 3A, B, Supplementary Figure 7, Movie 7). Explants from St 5 had large variations in interbeat intervals, slow beating rates, lacked phase 4 depolarization, and displayed an expression profile inconsistent with St18 PCs (Supplementary Figure 8). PC precursors from both St 8 and St 10, however, did differentiate into PC-like cells following

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culture. Both stages displayed clear phase 4 depolarization, which was absent in age matched primary/secondary heart field derived cells, (Figure 3A, B, Supplementary Figure 7, Movie 7), and spontaneously depolarized at intervals of 303 +/− 53 ms and 294 +/− 45 ms, respectively. Expression profiles of explants from St 8 were consistent with in vivo PC as described above, showing enrichment for Hcn4, Serca2, Ryr2, as well as co-expressing Amhc1 and Vmhc1 (Supplementary Figure 8).

These data suggest that by St 8, PC fate is already established in the Nkx2.5/Isl1 negative lateral plate mesoderm. To determine the spatial restrictions of PC specification, mesoderm directly adjacent to the PC precursors was isolated from the presumptive atrial, atrioventricular junction, and proepicardium (see Figure 3C). Only the PC region displayed elevated phase 4 depolarization and high rate AP production (Figure 3D, Supplementary Figure 7). These findings suggest that by St8, PC fate is specified in a highly restricted sub-domain of the right lateral plate mesoderm, and that the initial events dictating the functional divergence of PC-fate from the adjacent working myocardium must occur prior to this stage.

Additionally, by this stage, our data indicate a large region of mesoderm outside of the primary and secondary heart fields is already specified into working myocardial and PC fates. In order to delineate this mesodermal subdomain from the more classically defined heart fields, we refer to it as a tertiary heart field in chick. Although the precise boundaries of the primary and secondary heart fields remain controversial (18), our high resolution fate mapping reveals the distribution and boundaries of several subtypes of cardiac precursors within the tertiary heart field. Conservation of this field in other models systems will require further validation.

Many studies have identified factors necessary for inducing myocyte specification in the primary and secondary heart fields. To determine factors that may play a role in inducing PC fate within the tertiary heart field, we examined the expression of several factors thought to positively or negatively influence myocyte specification during the developmental window outlined above. Expression of a canonical Wnt, Wnt8c, was detected in the region of pre-specified PCs but not the more anterior heart fields (Supplementary Fig 9). Additionally, PC precursors, but not heart field cells, displayed nuclear accumulation of beta-catenin suggesting active Wnt signaling (Supplementary Figure 9I–L). We found this surprising since Wnts have previously been identified as inhibitory for heart field specification (19, 20).

To determine whether Wnt signaling is required to promote PC fate, cells expressing the soluble Wnt antagonist Crescent (19, 20) were microinjected adjacent to PC precursors prior to their specification. Following 8hrs, PC precursors were explanted and allowed to differentiate ex vivo. Exposure to Crescent decrease the slope of PC phase 4 depolarization by 65% when compared to control injections (Figure 4B, D). These experiments cannot rule out that Crescent is interacting with factors not associated canonical Wnt signaling, however. Therefore, to further demonstrate that Wnt signaling was capable of inducing PC fate, we injected Wnt expressing cells into the presumptive heart fields. This resulted in a a 69% increase in phase 4 slope (Figure 4C, D). Further we activated Wnt signaling in the heart field using a pharmacological GSK3 inhibitor, Bio, which has previously been shown to stabilize beta-catenin (21,22). Consistent with above, 10uM Bio increased diastolic slope in heart field explants when compared to control (Supplementary Figure 10).

When we allowed injected embryos to develop to late looping stages, aberrant Wnt signaling led to severe morphological defects, consistent with previous reports (Figure 4F, H) (23). Crescent injection adjacent to PC precursors led to the ectopic expression of Nkx2.5 in PC at St 18, which is in agreement with a conversion of PC into a more working myocardial fate

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(24) (Figure 4E, F). Approximately 35% of Wnt injected embryos survived to heart looping stages. Wnt introduction into the primary and secondary heart field mesoderm, resulted in irregularly contracting hearts, with decreased Nkx2.5 expression on the injected side of the embryo (Figure 4G, H). To confirm that these Nkx2.5 negative regions were still electrically active, we preformed optical mapping. Consistent with a Wnt-based conversion of working myocardium into PC-like cells, retrograde propagation (outflow towards inflow) as well as ectopic pacemaker sites were detected (Supplementary Movie 8). These ectopic sites, were restricted to the Wnt injected side of the embryo and demonstrated AP shapes similar to control PCs (Figure 4I, J, Supplementary Movie 8).

These findings suggest that early mesodermal, Wnt-mediated, cues are sufficient to induce pacemaker-like fates that do not manifest until late looping stages. However, since Wnts are broadly and bilaterally expressed in the posterior mesoderm, additional cues are most likely required to restrict PC fate, including laterality genes (25, 26). The early diversification of PC fate from the working myocardium, however, suggests that fate specification is assigned directly in the lateral plate mesoderm, and is not the result of the specialization of an already functional embryonic myocyte. These data establish a framework through which PC development should be viewed providing a foundation for tissue engineering and stem cell-based approaches for PC generation.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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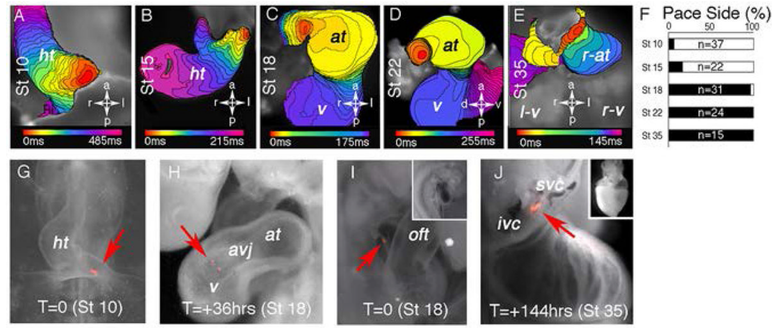


**Figure 1.**

PCs begin pacing heart rhythm during late looping stages: A–E) Isochronal maps denoting AP initiation site (red) and propagation pattern, St10–St35. F) Pacemaker side spanning 5 progressive embryonic stages (white = left-sided, black = right-sided pacemaker). **G, H**) Labeling of St 10 AP initiation site (arrow) developed for 36hrs to St 18. I, J) Labeling of St 18 AP initiation site (arrow) developed 144hrs to St 35. Insets show low magnification images of labeled embryo. a-anterior, p-posterior, r-right, l-left, ht- heart tube, v-ventricle, at- atria, avj- atrioventricular junction, r-at- right atria, lv- left ventricle, rv- right ventricle, oft - outflow tract, svc- superior vena cava, ivc-inferior vena cava.

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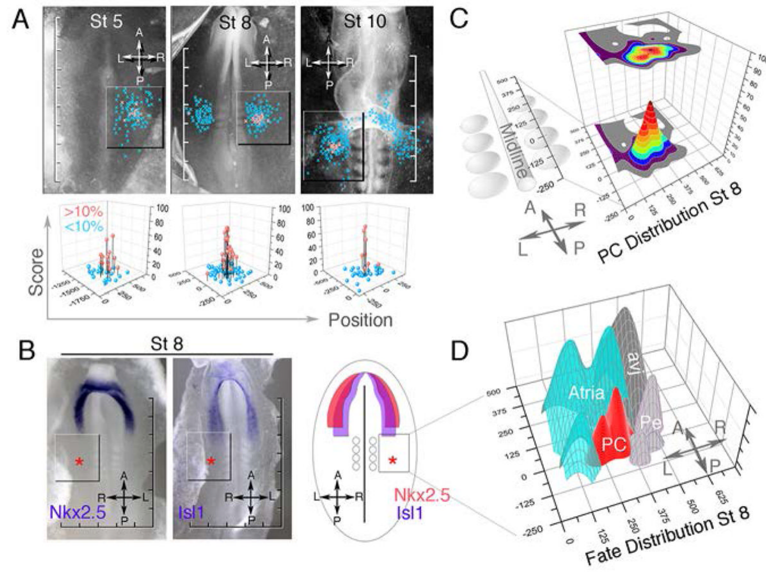


**Figure 2.**

PCs originate from mesoderm posterior to the heart fields. A) Fate maps depicting the location of PC progenitors at St 5, St 8, and St 10 (St 5 and 8 dorsal view, St 10 ventral view). Scale = 250um/division. Quantification of labels in boxed region is shown below (see materials and methods/supplemental Figure 5). B) In situ hybridization (dorsal view) and schematic (ventral vie) for heart field markers Nkx2.5 and Isl1 at St 8. asterisks = PC region. C) Best fit contour plot of data from St 8 fate mapping. D) Overlays of surface plots indicating location of atrial, atrioventricular junction, proepicardial, and pacemaker precursors. A-Anterior, P-Posterior, R-Right, L-Left.

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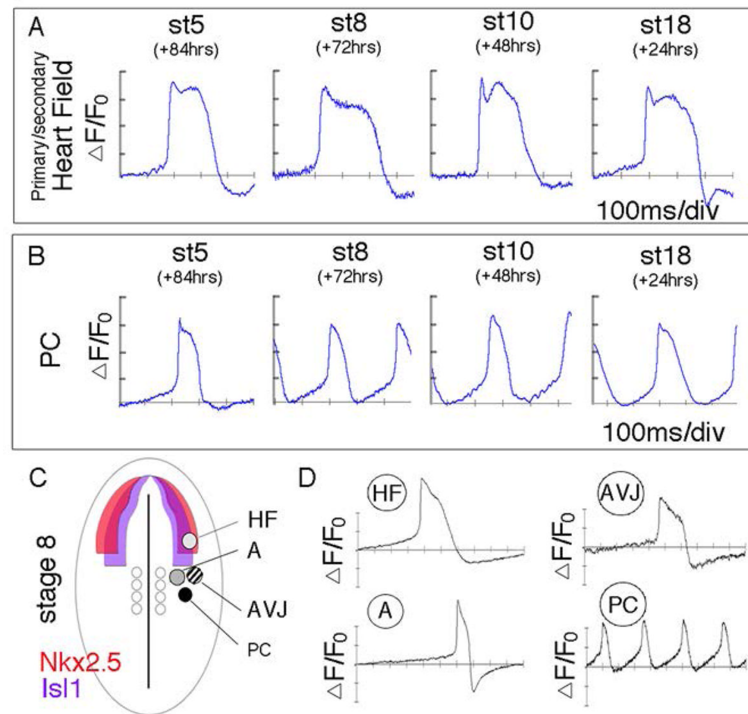


**Figure 3.**

PC fate is specified by St 8. A) Membrane depolarization recorded from heart field and (B) PC precursors isolated from indicated stages. C) Schematic indicating sites of mesodermal isolation from St 8 embryos relative to the Nkx2.5 and the Isl1 expression. D) Representative optical tracings of membrane potential from regions indicated in (C) following 72 hrs of culture.

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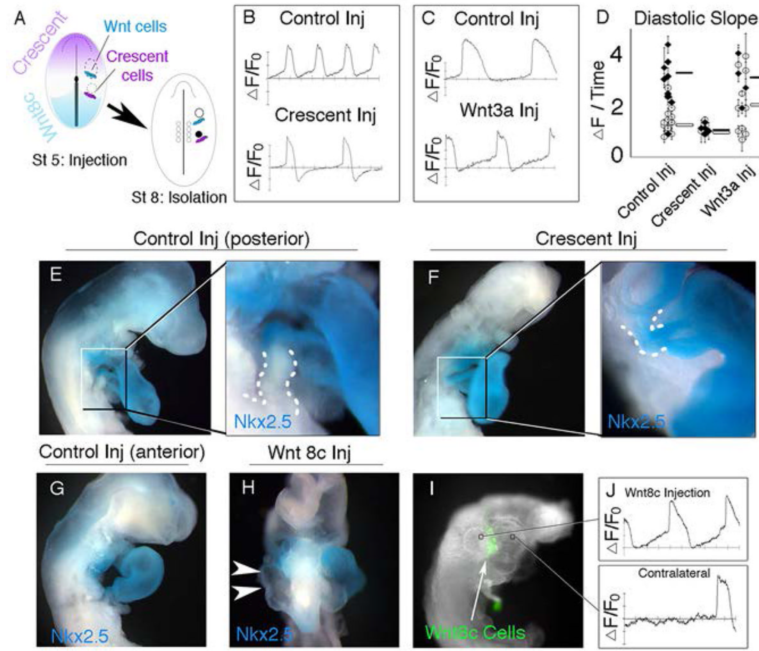


**Figure 4.**

Canonical Wnt signaling promotes PC fate. A) Schematic of stage 5 embryo indicating Crescent and Wnt8c expression domains. B, C) Following Crescent or Wnt expressing cell injections, embryos were developed to St 8, when PC or 1/2 Heart field precursors were isolated and placed in culture. Optical Membrane potential recordings from PC or heart field cultures following culture. D) Quantification of diastolic slope from PC (black diamonds) or heart field cells (white circles). E–H) Nkx2.5 expression is expanded in to the PC region following Crescent expressing cell injection (dashed area of high magnification insets from E–F ), and is lost in the heart following Wnt cell injection (arrow in H) compared to control cells injection. I) Location of Wnt8C expression relative to ectopic PC-like AP generation (J).