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Canonical Wnt Signaling Sets the Pace

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In this issue of *Developmental Cell*, Ren et al. (2019) identify the embryonic origin of cardiac pacemaker cells in zebrafish and implicate Wnt5b in promoting their differentiation. Furthermore, canonical Wnt activation in human stem cell-derived cardiac progenitors produces functional pacemaker cells *in vitro*, advancing the therapeutic potential of biological pacemakers.

Heart rate is controlled by a subclass of cardiomyocytes termed pacemaker cells that coalesce during embryogenesis to form the sinoatrial node (SAN) in the wall of the right atrium (Liang et al., 2017). These cells spontaneously generate electrical impulses that travel throughout the heart by way of the conduction system to stimulate cardiac contractions in a predictable and cyclic manner. Disturbances in SAN formation or function result in abnormally slow heart rates (i.e., bradycardia) that compromise blood circulation. Regardless of whether these disturbances are inherited or acquired, treatment is determined by the presence of associated symptoms and the degree of heart rate slowing. Since the late 1950s, implantation of electronic pacemakers has been the mainstay of therapy, with more than 200,000 patients receiving devices annually in the U.S. alone (Greenspon et al., 2012). Although artificial pacemakers continue to evolve, they still present significant limitations including device failures, device-related infections, the need for battery exchange, the inability to provide a true autonomic response, and the incapacity for growth in pediatric patients (Cingolani et al., 2018). As such, an unmet clinical need exists to generate implantable pacemakers with improved therapeutic properties.

Promising alternatives to mechanical devices are biological pacemakers where nodal cardiomyocytes, derived from human pluripotent stem cells (hPSCs), are implanted into the right atrium of the affected heart to serve as a new SAN (Cingolani et al., 2018). However, realizing this goal requires that the natural framework of pacemaker cell development be elucidated. Specifically, identifying the progenitor populations that give rise to the SAN during embryogenesis and the signaling pathways that orchestrate pacemaker cell differentiation are critical for bioengineering clinically effective SANs with appropriate automaticity, adrenergic responsiveness, and sustained rhythm maintenance.

Previously, the progenitor populations giving rise to pacemaker cells have been investigated in both rodent and avian species. Fate-mapping studies in mice have suggested that pacemaker cardiomyocytes derive from a cardiac progenitor population that transiently expresses the transcription factor Nkx2.5 prior to differentiation (Mommersteeg et al., 2010). In fact, a defining molecular feature of mature pacemaker cardiomyocytes is the absence of Nkx2.5 expression, which distinguishes them from their force-generating counterparts in the working myocardium (Christoffels et al., 2006). By contrast, pacemaker cardiomyocytes were reported to derive from Nkx2.5-negative progenitor cells in an avian fate-mapping study (Bressan et al., 2013). While seemingly in conflict, these observations are in fact compatible if species-specific differences exist or the SAN originates from multiple progenitor sources.

Regarding the signaling pathways regulating SAN morphogenesis, retinoic acid (RA) (Protze et al., 2017), BMP (Protze et al., 2017), and canonical Wnt signaling (Bressan et al., 2013) have all been implicated in this process, but many details remain obscure. As examples, the specific Wnt ligand(s) that promotes pacemaker differentiation and the conservation of canonical Wnt signaling for directing human pacemaker differentiation were not previously explored. In this issue of *Developmental Cell*, Ren et al. (2019) took advantage of the embryonic accessibility of the zebrafish model system coupled with the translatable attributes of hPSC-derived cardiomyocytes to provide a more refined paradigm for how Wnt signaling establishes pacemaker cell fate.

First, Ren et al. (2019) employed a lineage tracing system to track the fates of *nkx2.5*-expressing progenitors in the zebrafish heart field. Photo-labeled cells in the lateral most regions gave rise to pacemaker cardiomyocytes, as revealed by their subsequent positioning in the inflow tract, upregulation of Isl1, and downregulation of Nkx2.5, consistent with previous findings in mice (Mommersteeg et al., 2010). Next, the authors investigated a potential role for Nkx2.5 in establishing the SAN lineage by quantifying pacemaker cell numbers in *nkx2.5* mutants and transgenic animals inducibly overexpressing Nkx2.5. Similar to null mice (Mommersteeg et al., 2007), *nkx2.5* mutant zebrafish contained an overabundance of pacemaker cells, demonstrating that, although pacemakers derive from Nkx2.5+ progenitors, Nkx2.5 function is dispensable for establishment of this lineage. By contrast, induced overexpression of Nkx2.5, to counteract its natural disappearance prior to differentiation, caused a reduction in pacemaker cell number. Overall, these studies demonstrate that the transient expression of Nkx2.5 in SAN progenitors during normal embryonic development serves two reciprocal purposes. The initial appearance of Nkx2.5 restricts the number of pacemaker cells, whereas its subsequent downregulation promotes pacemaker

identity. Despite these observations, the molecular and cellular mechanisms underlying the seemingly complex role played by Nkx2.5 in the regulation of pacemaker cell development remain to be fully elucidated.

Using an array of genetic mutants and small molecules to perturb Wnt signaling in zebrafish embryos, Ren et al. (2019) demonstrated that Wnt5b, a ligand typically associated with non-canonical Wnt signaling (Kilian et al., 2003), induced canonical Wnt signaling within a subset of Nkx2.5+ atrial myocardial progenitors to promote pacemaker differentiation. Supporting this conclusion, *wnt5b* mutants contained fewer pacemaker cells and showed a concomitant increase in atrial cardiomyocytes. Conversely, embryos overexpressing *wnt5b* contained more pacemaker cells and fewer atrial cardiomyocytes. Interestingly, *wnt5b* appeared to be co-expressed with *nkx2.5* in the majority of cardiac precursors at the 16-somite stage. However, only a small number of these cells activated canonical Wnt signaling and became pacemaker cardiomyocytes, suggesting that additional regulatory layers must spatially restrict Wnt pathway activation. As such, it will be important to learn whether Wnt receptors are highly localized, if Wnt inhibitors are present, and/or whether progenitors that give rise to other regions of the heart show active Wnt repression.

Lastly, Ren et al. (2019) tested whether their findings in zebrafish translate to the hPSC model. hPSCs were differentiated to an *NKX2.5*-expressing cardiac progenitor stage, and canonical Wnt signaling was activated by treatment with a small molecule or recombinant Wnt5b. As in zebrafish, Wnt activation caused an impressive induction of pacemaker cell fate, as evidenced by characteristic marker expression, electrophysiologic properties, and the ability to pace three-dimensional bioprinted hPSC-derived cardiomyocytes *in vitro*.

Despite the advances made by this work, future studies will be necessary to resolve some important unanswered questions. What are the genetic relationships between Wnt activation and other signaling pathways (BMP and RA) (Protze et al., 2017) or transcription factors (*NKX2.5*, *TBX18*, *TBX3*, and *SHOX2*) (Liang et al., 2017) that regulate pacemaker cell induction? Could these relationships be exploited to achieve synergies for maximizing the efficiency of *in vitro* pacemaker cell differentiation? Are the pacemaker cardiomyocytes derived through Wnt activation capable of curing bradycardia in large animal models? If so, then these discoveries would bring us one step closer to the prospect of implanting biological pacemakers for therapeutic benefit in the human population.

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