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Materials and Methods

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Retinoic Acid Signaling Restricts the Cardiac Progenitor Pool

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Organogenesis begins with specification of a progenitor cell population, the size of which provides a foundation for the organ's final dimensions. Here, we present a new mechanism for regulating the number of progenitor cells by limiting their density within a competent region. We demonstrate that retinoic acid signaling restricts cardiac specification in the zebrafish embryo. Reduction of retinoic acid signaling causes formation of an excess of cardiomyocytes, via fate transformations that increase cardiac progenitor density within a multipotential zone. Thus, retinoic acid signaling creates a balance between cardiac and noncardiac identities, thereby refining the dimensions of the cardiac progenitor pool.

Generation of the proper number of organ progenitor cells is likely to involve interplay between inductive and repressive signaling pathways. Key inductive mechanisms have been identified for many organs, including the heart, but mechanisms for repressing progenitor fate assignment are poorly understood. Several factors, including Bmp2, Fgf8, Nodal, and Wnt11, are implicated in promoting the initial selection of myocardial progenitor cells from a multipotential popu-

lation (1). Although convergence of inductive signals might be sufficient to delimit the number of progenitor cells, opposing signals could also be necessary to restrict myocardial specification. Prior studies have suggested mechanisms for inhibiting cardiomyocyte differentiation within the anterior lateral plate mesoderm (ALPM), by means of Notch signaling (2) or interactions with the notochord (3), but little is known about whether repressive pathways limit the initial assignment of myocardial identity.

We find that reduction of retinoic acid (RA) signaling causes formation of an excess of cardiomyocytes. The zebrafish mutation *neckless* (*nls*) disrupts function of the *retinaldehyde dehydrogenase 2* gene (*raldh2*), which controls a rate-limiting step in RA synthesis (4, 5). *nls* mutants exhibit an increased number of cells expressing *nkx2.5*, a marker of the bilateral populations of precardiac mesoderm within the ALPM (Fig. 1A). Although *nkx2.5*

expression appears expanded in anterior, posterior, and lateral directions (Fig. 1, A and B), we do not observe an increase in the overall size of the ALPM in *nls* mutants (fig. S1A). As myocardial differentiation proceeds, *nls* mutants exhibit a surplus of cardiomyocytes, identifiable by their expression of *cardiac myosin light chain 2* (*cmlc2*) (Fig. 1B; fig. S1B). Formation of this myocardial surplus depends on the conventional myocardial differentiation pathway (1), which requires the activity of the growth factor Fgf8 and the transcription factors Hand2 and Gata5 (fig. S2). Consistent with a repressive influence of RA on cardiomyocyte formation, exposure to the pan-retinoic acid receptor (RAR) antagonist BMS189453 (6, 7) causes expansion of *nkx2.5* and *cmlc2* expression (Fig. 1, A and B). Conversely, exposure to exogenous RA results in a reduced number of cardiomyocytes (fig. S1C) (8). Together, these data demonstrate that cardiomyocyte population size within the ALPM is inversely related to the level of RA signaling.

The *raldh2* gene is expressed throughout early zebrafish embryogenesis (4, 5): In the blastula, *raldh2* is found at the embryonic margin; during gastrulation, *raldh2* is in involuting mesendoderm; and, after gastrulation, *raldh2* is in both lateral and paraxial mesoderm. To investigate when RA influences cardiomyocyte number, we tested the effectiveness of BMS189453 during different time intervals, initiating exposure at various stages and later assessing *nkx2.5* or *cmlc2* expression (Fig. 1, C and D; fig. S3). Addition of BMS189453 before gastrulation [40% epiboly, 5 hours post fertilization (hpf)] causes a myocardial expansion, whereas addition of BMS189453 during gastrulation (75% epiboly, 8 hpf) results in a more modest increase.

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Fig. 1. Cardiomyocyte number increases when RA signaling is reduced. Expression of (A and C) *nkx2.5* and (B and D) *cmlc2*, at indicated somite (som) stages. Dorsal views, anterior to the top, comparing wild-type and *nls* mutant siblings, as well as untreated and BMS189453-treated wild-type siblings. (A and B) RAR antagonism has a more potent effect than *raldh2* loss of function, consistent with the presence of low levels of RA in *raldh2* mutants (5, 10, 18, 19). (C and D) RA signaling is required before tailbud stage to prevent expansion of *nkx2.5* and *cmlc2* expression.

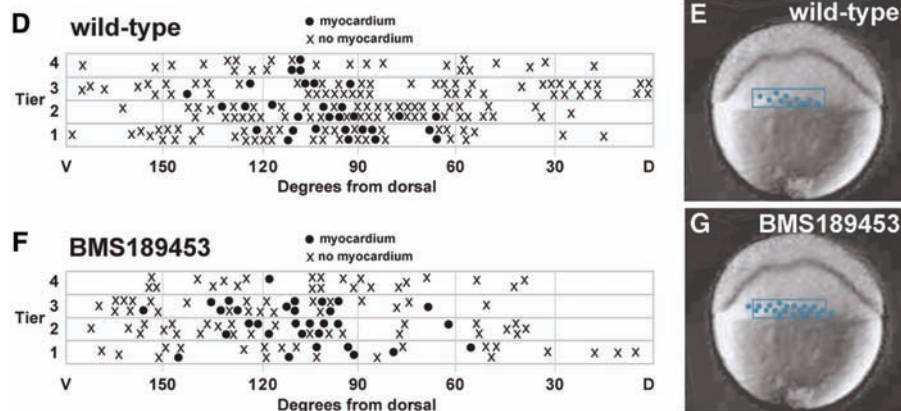
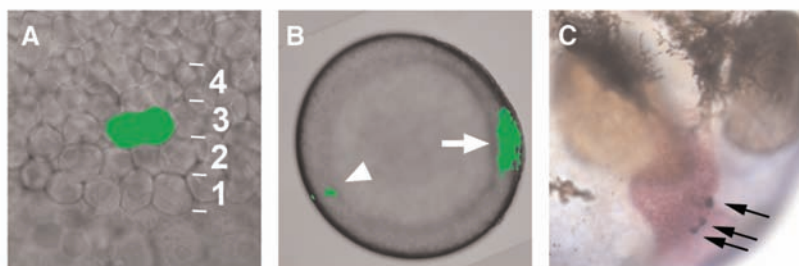
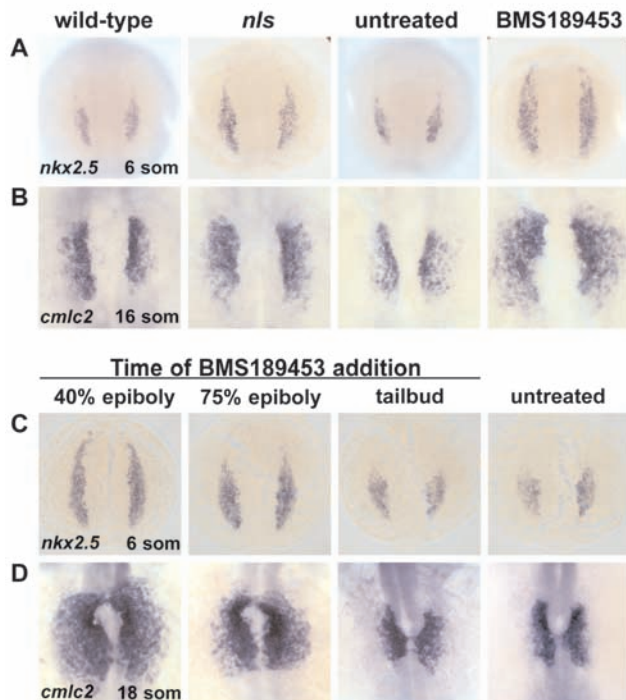


Fig. 2. Reduction of RA signaling increases myocardial progenitor density. (A) Lateral view, 40% epiboly, animal pole to the top. Latitude is measured in tiers (white bars and numerals), defined as cell diameters from the embryonic margin (9). Here, we labeled two neighboring blastomeres (green) in tier 3. (B) Animal view, dorsal to the right. Longitude is measured as angular distance along the embryonic circumference, with the dorsal midline [marked by the center of *Tg(gsc:gfp)* expression; arrow] defined as 0° (9). Here, labeled cells (arrowhead) are 153° to 160° from dorsal. (C) Frontal view at 44 hpf, head to the top. Here, we detected three labeled cardiomyocytes (blue cells, arrows; pink, *cmlc2* expression). (D and F) Myocardial fate maps in (D) wild-type and (F) BMS189453-treated embryos. Each symbol represents the location of labeled blastomeres in an individual experiment and indicates whether these cells contributed to the myocardium. (E and G) Schematics depicting myocardial progenitor distribution in the zebrafish blastula. Reduction of RA signaling increases the number of blastomeres in the LMZ (blue box) that become myocardial progenitors (blue dots). Images in (D) and (E) adapted from (9).

In contrast, adding BMS189453 at the end of gastrulation (tailbud, 10 hpf) does not alter total cardiomyocyte number. Therefore, RA signaling is required during gastrulation for its effects on cardiomyocyte population size.

Because of the early influence of RA on cardiomyocyte formation, we hypothesized that inhibition of RA signaling creates a myocardial surplus through fate transformation, rather than through myocardial proliferation. To test this model, we generated a myocardial fate map in BMS189453-treated embryos. By adapting a technique that we have used in wild-type embryos (9), we examined the frequency with which cardiomyocytes originate from particular locations within the blastula. Using laser-mediated activation of caged fluorescein (6), we labeled selected blastomeres at 40% epiboly. We recorded the positions of labeled blastomeres using latitude and longitude coordinates (Fig. 2, A and B) (9) and then treated embryos with BMS189453. Later, we evaluated the fates of labeled cells, with an emphasis on their cardiac contributions (Fig. 2C). In 60 of our 120 labeling experiments (Fig. 2F), labeled blastomeres were located in the lateral marginal zone (LMZ). This zone, defined as the first three tiers of blastomeres between 60° and 140° from the dorsal midline, is the most common region of origin for myocardial progenitors in wild-type embryos (Fig. 2D) (9).

Fate map comparisons (Fig. 2, D and F) reveal that RA affects the number of LMZ blastomeres that become myocardial progenitors. In both wild-type and BMS189453-treated embryos, myocardial progenitors are found throughout the LMZ (Fig. 2, D and F). However, in BMS189453-treated embryos, LMZ blastomeres generate myocardial progenitors twice as often as they do in wild-type embryos [labeled cardiomyocytes detected in 45% (27 out of 60) of BMS189453-treated embryos (Fig. 2F) versus 20% (20 out of 100) of comparable wild-type embryos (Fig. 2D) (9); binomial test, $P < 0.05$]. This substantial increase in progenitor density is accompanied by a slight expansion of the region containing myocardial progenitors; in BMS189453-treated embryos, we occasionally detect myocardial progenitors just outside of the dorsoventral boundaries of their typical wild-type locations (Fig. 2, D and F). Although BMS189453-treated embryos appear to contain an increased number of myocardial progenitors, the individual progenitors each seem to produce the same number of cardiomyocytes as their wild-type counterparts. On the assumption that only one progenitor is labeled in each experimental embryo (9), the average myocardial contribution per progenitor is 4.3 cardiomyocytes in wild-type embryos (9) and 4.8 cardiomyocytes

in BMS189453-treated embryos (table S1) (t test, $P > 0.2$). Therefore, reduction of RA signaling does not appear to affect cardiomyocyte proliferation during the stages examined. Altogether, the fate map data demonstrate that reduction of RA signaling causes a fate transformation that generates an excess of myocardial progenitors.

Formation of extra myocardial progenitors within the LMZ is likely to occur at the expense of another progenitor population. Some LMZ lineages seem unaffected by inhibition of RA signaling; for example, we labeled endocardial progenitors in 26% (26 out of 100) of wild-type embryos (9) and 25% (15 out of 60) of BMS189453-treated embryos (table S2). In contrast, other LMZ lineages (9) appear considerably diminished when RA signaling is reduced, including pancreas (10), pharyngeal pouches (4), and pectoral fin mesenchyme (4, 5). All of these cell types are sensitive to RA signaling during the same time interval when RA influences myocardial progenitors (5, 10) (fig. S3). The differential effects of reducing RA signaling during gastrulation suggest that RA influences the balance between cardiac and noncardiac progenitor populations that arise from within the LMZ (Fig. 2, E and G).

Taken together, our data indicate that RA signaling has a potent repressive role during cardiac specification. It is evident that this early role of RA is one of many functions for this signaling pathway during cardiogenesis; previously established roles for RA include influences on cardiac chamber identity (8, 11–13), terminal myocardial differentiation (14), cardiac looping (12, 15), and ventricular maturation and growth (12, 16, 17). Beyond uncovering an additional role for RA, our data also suggest a new means by which RA regulates fate assignment. Several previous studies, including analyses of the zebrafish hindbrain (4, 5) and gut (10), have suggested that RA has a posteriorizing influence during the establishment of anterior-posterior coordinates. However, reduction of RA signaling does not appear to cause a unidirectional shift in the location of myocardial progenitors (Fig. 2). Instead of defining spatial boundaries for myocardial specification relative to the embryonic axes, RA signaling seems to impose limits on the density of myocardial progenitor cells intermingled within an eligible population. We propose that RA, synthesized within or near the LMZ (4, 5), biases fate assignments in this area, restricting the opportunities for becoming myocardial. Thus, the repressive function of RA during cardiac specification provides an example of a previously unappreciated mode of selecting organ progenitors from within a multipotential pool.

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Global Circumnavigations: Tracking Year-Round Ranges of Nonbreeding Albatrosses

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Although albatrosses are paradigms of oceanic specialization, their foraging areas and migration routes when not breeding remain essentially unknown. Our continuous remote tracking of 22 adult gray-headed albatrosses for over 30 bird-years reveals three distinct strategies: (i) Stay in breeding home range; (ii) make return migrations to a specific area of the southwest Indian Ocean; and (iii) make one or more global circumnavigations (the fastest in just 46 days). The consistencies in patterns, routes, and timings offer the first hope of identifying areas of critical habitat for nonbreeding albatrosses, wherein appropriate management of longline fisheries might alleviate the plight of the world's most threatened family of birds.

Albatrosses are the world's most threatened family of birds (with 19 of 21 taxa on the International Union for the Conservation of Nature Red List). Recent catastrophic population decreases are mainly a consequence of incidental mortality in longline and trawl fisheries (1). Knowledge of the at-sea distribution of albatrosses is thus critical to their conservation, yet few data from birds of known status and provenance are available outside the breeding season. Regular successive recoveries have been made off eastern Australia of wandering albatrosses (*Diomedea exulans*) banded at South Georgia (54°00'S, 38°03'W), showing that some birds migrate great distances, but more recent tracking data

from the same species breeding in the Indian Ocean indicate that this population is much more sedentary during the nonbreeding period (2–4). Satellite tracking of postbreeding birds of other species has confirmed several long-distance migrations (5, 6), but the brevity of these studies and the small samples mean that range and behavior during the nonbreeding season is, by comparison, very little known.

We deployed leg-mounted light level loggers (7) on 47 gray-headed albatrosses (*Thalassarche chrystostoma*) that were rearing chicks at the end of the austral summer (April 1999) at Bird Island, South Georgia. This species breeds biennially when successful, and 35 of the loggers were retrieved between September and November 2000, of which 22 provided complete data throughout the approximately 18-month interval between breeding events (corresponding to 11,034 bird-days) and 13 failed to download.

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