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Ror2 Enhances Polarity and Directional Migration of Primordial Germ Cells

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Abstract

The trafficking of primordial germ cells (PGCs) across multiple embryonic structures to the nascent gonads ensures the transmission of genetic information to the next generation through the gametes, yet our understanding of the mechanisms underlying PGC migration remains incomplete. Here we identify a role for the receptor tyrosine kinase-like protein Ror2 in PGC development. In a Ror2 mouse mutant we isolated in a genetic screen, PGC migration and survival are dysregulated, resulting in a diminished number of PGCs in the embryonic gonad. A similar phenotype in Wnt5a mutants suggests that Wnt5a acts as a ligand to Ror2 in PGCs, although we do not find evidence that WNT5A functions as a PGC chemoattractant.

We show that cultured PGCs undergo polarization, elongation, and reorientation in response to the chemotactic factor SCF (secreted KitL), whereas Ror2 PGCs are deficient in these SCF-induced responses. In the embryo, migratory PGCs exhibit a similar elongated geometry, whereas their counterparts in Ror2 mutants are round. The protein distribution of ROR2 within PGCs is asymmetric, both in vitro and in vivo; however, this asymmetry is lost in Ror2 mutants. Together these results indicate that Ror2 acts autonomously to permit the polarized response of PGCs to KitL. We propose a model by which Wnt5a potentiates PGC chemotaxis toward secreted KitL by redistribution of Ror2 within the cell.

Introduction

Primordial germ cells (PGCs) are embryonic precursors of the gametes that arise before other major cell lineages in most multicellular animals [1]. This early specification necessitates a lengthy migration through the developing embryo in order to reach the nascent ovaries or testes. In mice, epiblast-derived cells seal their germline commitment at the embryo periphery ~e7.25, then enter the forming endoderm and travel through the elongating hindgut epithelium. PGCs make a coordinated exodus into the surrounding mesentery at e9.5 and then converge on the gonadal ridges between e10.5 and e11.5. Though exquisitely coordinated, this process is also imperfect; by e12 when migration is over, stragglers consistently remain outside the gonad in midline tissue, and are eliminated by apoptosis [2]. The importance of balanced regulation of PGC survival and migration is evident by the consequences of dysregulation: failure to survive or reach the gonad can lead to sterility, whereas inappropriate survival can lead to germ cell tumors [3,4]. The molecular mechanisms underlying the migration of these evolutionarily essential but relatively inaccessible cells remain largely unknown in the mammalian germline. Here we conducted a forward genetic screen for germ cell defects in mouse embryos and identified an allele of Ror2.

Ror2 is a highly conserved receptor tyrosine kinase with homologs in many metazoans from Aplysia to Drosophila to humans [5]. Widely expressed during development, Ror2 has been implicated in chondrocyte differentiation, cochlear, craniofacial, heart, limb and gut morphogenesis in mice and humans [6–9]. Work in a number of different organisms suggests that Ror2 signaling affects cell polarity. In the developing mouse gut epithelium, the protein exhibits apicolabasal polarity in its distribution [10]. Polarity is requisite for cells undergoing directed migration, cell division in a particular orientation, as in asymmetric divisions, and for the organization or shape of cells with respect to their neighbors, for example in convergent extension. Defects in cell shape and convergent extension have been reported in the mouse gut, organ of Corti, and Xenopus gastrula as a result of Ror2 signaling loss [9,11–13]. Ror2-mediated polarized cell division has been reported in C. elegans [14]. A role for Ror2 signaling in directional migration has been reported in the mammalian palate [15] and in several cell lines, via e-Jun N-terminal Kinase and the actin-binding protein FilaminA [16–10].
Author Summary

Egg and sperm derive from precursors in the early embryo called primordial germ cells (PGCs). The mechanisms underlying the migration of PGCs through the embryo to the forming gonads remain unclear. In a genetic screen, we identified a role for the receptor Ror2 and its ligand Wnt5a in promoting PGC colonization of the embryonic gonads. By ex vivo culture, we show that Ror2 acts autonomously in PGCs to enhance their polarized response to the chemotactic factor SFC. Asymmetric distribution of ROR2 within PGCs in vitro and in vivo suggests that signaling via Ror2 locally amplifies cell polarity in response to other directional cues. These studies identify a novel relationship between Ror2 and cKit signaling in polarized migration.

Phenotypic resemblance between mouse embryos with targeted deletions of Ror2 and those deficient for Wnt5a first suggested that these genes share a common pathway [6,8,17,19]. Biochemical approaches later confirmed ligand-receptor interactions between Wnt5a and Ror2 via the cysteine-rich (frizzled-like) extracellular domain of Ror2 [17]. Indeed, the expression patterns of Wnt5a and Ror2 virtually overlap in the primitive streak, tail mesoderm and limb buds of midgestation mouse embryos [19-21]. Wnt5a was similarly invoked in aspects of cell polarity, including orientation of cell division in the limb [22], convergent extension movements and cell shape in the Xenopus gastrula [23,24], and polarized migration in a melanoma cell line [25,26]. Many of these different Wnt5a-Ror2 pathway mutants exhibit similarly altered distribution of polarity mediators, such as Disheveled [23,27,28], the Dlg-Lgl complex [24,29], Van Gogh [29], or adhesion receptor complexes [26].

The identification of the Ror2Y324C mutant in an unbiased screen for PGC defects brings to light a previously unrecognized function of Ror2 in germ cell development. We show here that Ror2 and its putative ligand Wnt5a promote efficient migration of PGCs to the embryonic gonads. These studies demonstrate a cell intrinsic function for Ror2 in potentiating the polarized secretion of KitL, drawing a new link between Ror2 and Kit signaling in PGC migration.

Results

An ENU allele of Ror2 and its expression in PGCs

As an unbiased approach to identifying new genes involved in mouse germ cell development, we conducted a genome-wide recessive ethylnitrosourea (ENU) mutagenesis screen for PGC defects in e9.5 embryos [30]. One of the mutations identified based on the presence of ectopic PGCs mapped to the region of Ror2. An A to G transition in exon 7 at nucleotide 1203 causes a tyrosine to cysteine substitution at position 324 (Y324C) of the ROR2 predicted protein (Figure 1A). This missense mutation falls in the kringle domain, a conserved structural motif in the ROR2 extracellular domain. Ror2Y324C homozygous embryos exhibit defects in tail elongation (Figure 1B, 1C) and somite segmentation, similar to the Ror2 targeted deletion allele (Figure 1D) [8,17]; like the knockout, Ror2Y324C mutants die perinatally. Ror2 immunoblotting on e10.5 embryo lysates revealed a double band at approximately 200 kD; both bands were present in similar amounts between WT and Ror2Y324C mutants (Figure 1E). In humans, missense mutations in the kr2 cysteine rich, kringle and tyrosine kinase domains that are associated with Robinow syndrome cause the protein to be retained in the endoplasmic reticulum [31]. We examined the expression of ROR2 at e11.5 by intracellular staining with an antibody directed against the cytoplasmic tail of the receptor; by flow cytometry signal was present at similar levels in WT and Ror2Y324C (Figure 1F, right). These experiments suggest that the mutation does not affect protein stability but do not discriminate between its normal or abnormal subcellular localization.

To determine whether Ror2 is expressed in PGCs, we employed a transgenic mouse strain, Oct4APE-EFP, which expresses Enhanced Green Fluorescent Protein (GFP) under a modified Oct4 reporter that is specific to PGCs during mid-gestation [10,32] (Figure 1F). By flow cytometry, ROR2 intracellular staining was present within the GFP+ population at e11.5 (Figure 1F). Furthermore, when Oct4APE-EFP+ PGCs were purified by flow cytometry, Ror2 transcript could be detected by semi-quantitative RT-PCR; more transcript appeared to be present in GFPnegative cells from embryo tails (denoted ‘soma’; Figure 1G), where high levels of Ror2 have been previously detected by in situ hybridization [8]. The purity of sorted PGCs was confirmed by RT-PCR for Oct4, which was absent in somatic cells, and KitL, which was confined to soma (Figure 1G). ROR2 protein was similarly detected in histologic sections with two different antibodies; signal appeared to be concentrated at the apical surface of the hindgut and somites [33] and in the ventral neural tube in wild type embryos (Figure 1H–1H′), as previously reported [34]. ROR2 was also present throughout the e10.5 dorsal mesentery and enriched at the membrane of wild type PGCs (Figure 1I, I′). These studies confirm the expression of Ror2 mRNA and protein in migratory and postmigratory PGCs, as suggested by previous microarray data [35], and demonstrate the stable expression of Ror2Y324C mutant protein.

A major ligand for Ror2 is believed to be Wnt5a. Wnt5a mRNA expression in the tail and hindgut of the embryo overlaps that of Ror2, although precisely which cells secrete Wnt5a remains unclear [19-21]. By RT-PCR we determined that Wnt5a transcript is present in sorted Oct4APE-EFP+ PGCs, although it is more abundant in GFPnegative somatic cells of the tail and hindgut (Figure 1G). In histological sections stained with a WNT5A antibody, we observed bright foci as well as intercellular signal in the intestine and gonadal ridges (Figure 1J–1J′), which both lie on the PGC migratory route. Upon closer examination, WNT5A could be detected at variable levels at or near the surface of PGCs (Figure 1J, inset). These results collectively identify a role for Ror2 in PGC development and raise the possibility that PGCs perceive paracrine or autocrine WNT5A signals via the Ror2 receptor.

PGCs are gradually depleted in Ror2 and Wnt5a mutants

We next characterized the phenotypes of PGCs in Ror2Y324C mutants. In e10.5 embryos stained with SSEA1 antibody [36,37], PGCs can be visualized migrating through the dorsal mesentery (Figure 2A). In Ror2Y324C mutants, PGCs do not migrate rostrally, but remain in the mesentery surrounding the caudal hindgut (Figure 2B), as well as on the surface of the tail and in the allantois (Figure S3B, S3C). At e11.5, immunostaining with GCNA (a marker of postmigratory PGCs [38]) revealed a reduction in the number of PGCs within Ror2Y324C gonad primordia compared to wild type; furthermore, the distribution of Ror2Y324C PGCs was skewed toward the caudal end of the gonad and extragonadal PGCs were increased in midline tissues (Figure 2E, 2F). At e12.5, male and female Ror2Y324C gonads appeared less densely populated with PGCs (Figure 2I, J), female not shown.

We developed techniques for the quantification of PGCs in the entire embryo or embryonic gonad with confocal imaging and 3D analysis (see Methods). The mean number ± standard deviation of PGCs in mutants at e10.25 (443±73) was similar to wild type
Increased programmed cell death of PGCs in \textit{Ror2} and \textit{Wnt5a} mutants

To investigate the cellular mechanism underlying the PGC deficit in \textit{Ror2} and \textit{Wnt5a} mutants, we extended our quantitative imaging in the embryonic gonad to include markers of proliferation and death. We performed triple immunofluorescence for GCNA, as well as phospho-histone H3 (PHH3), and cleaved PARP to quantify subsets of mitotic and apoptotic PGCs, respectively (Figure S2). No differences were observed in cleaved PARP expression among postmitotic PGCs in wild type, \textit{Ror2}, or \textit{Wnt5a} gonads (Figure 3). However, analysis of e10.5 embryo sections revealed an increase in cleaved PARP expression among still migratory PGCs in wild type, \textit{Ror2}, or \textit{Wnt5a} gonads (Figure 3). This discrete wave of apoptosis preceded any observed loss in cell number in \textit{Ror2} mutants. We next compared the frequencies of programmed cell death between PGCs within and outside the e11.5 gonad. Caspase3 staining in histologic sections revealed similar frequencies in the PGC compartment, which corroborates their function there as ligand and receptor.

\textit{Ror2} is required for efficient PGC colonization of the gonads

Restriction of the observed burst of programmed cell death to migratory PGCs, together with its absence in gonadal PGCs, suggested that the location of mutant germ cells could be a factor in their elimination. On one hand, migrating mutant PGCs could be more sensitive to the reduced levels of survival factors such as KITL and SDF1 in the dorsal mesentry as compared to

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Figure 1. A \textit{Ror2} ENU allele and expression in PGCs. (A) Schematic of the \textit{Ror2} gene product with indicated mutation at nucleotide 1203 and predicted amino acid change. (B–D) \textit{Ror2} \textit{Y324C} homozygotes and (C) \textit{Ror2} null embryos (D) at e10.75 exhibit a short tail (arrow). (E) Whole embryo lysates from e9.5 immunoblotted for \textit{Ror2} and \text{β}-Tubulin indicate that protein is present in \textit{Ror2} \textit{Y324C} homozygous mutants. (F) PGCs at e11.5 flow cytometrically identified by the Oct4\textit{PE-GFP} transgene (left) show comparable levels of \textit{ROR2} intracellular staining (right). (G) RT-PCR from WT sorted Oct4\textit{PE-GFP}+ (denoted PGC) and GFP\textit{−} (soma) cells, and no cDNA controls (--) at e10.0 show the presence of \textit{Ror2} and \textit{Wnt5a} in both populations. The purity of Oct4\textit{PE-GFP}+ cells was confirmed by the presence of Oct4 and absence of \textit{KitL}. Both images are from the same gel, with 100 bp ladder shown at right. (H–J) \textit{ROR2} immunostaining (green) in the ventral neural tube, hindgut and somites (arrowheads) of WT e10.5 sections. Scale bar = 24 \text{um}. (J–J’) \textit{ROR2} immunostaining (green) was present throughout WT e10.5 dorsal mesentry and appeared enriched on the surface of PGCs, coincident with SSEA1 (red). Scale bar = 24 \text{um}. (J–J’) In e11.5 transverse sections, WNT5A immunostaining (green) was enriched in the intestine (arrow) and gonadal ridges (dashed lines) where the majority of PGCs (red) reside. Nuclei are shown in grey. Variable levels of WNT5A signal were observed in PGCs, such as these two adjacent examples (inset, 5 × magnification). Scale bar = 118 \text{um}. doi:10.1371/journal.pgen.1002428.g001
the gonad [2,41,42,43], where they are more protected from death. On the other hand, inefficient migration may lead to an accumulation of ectopic Ror2 PGCs, which die in an environment lacking survival factors [2]. To distinguish between these possibilities, we rescued PGC apoptosis in Ror2 mutants by generating double mutants with a targeted knockout of the pro-death gene Bax. Previous work established an increase in ectopic PGCs in e11.5 Bax single mutants due to the lack of apoptosis of mis-migrated PGCs, although the total number of PGCs remained unchanged [2]. Genetic ablation of Bax in Ror2Y324C mutants increased the number of midline and ectopic PGCs, but did not restore the number of PGCs in the gonads. At e11.5, 1815 ± 362 PGCs were counted in Ror2; Bax double mutant gonads, which did not differ from 1275 ± 636 in stage matched Ror2 littermates (p = 0.07; Figure 3D). Although Bax does not rescue PGCs in Ror2 gonads, a significant increase in the total number of PGCs in the entire aorta-gonad-mesonephros region of double mutants compared to Ror2 single mutants (p = 0.036; data not shown) reflects rescue of ectopic PGC death throughout the midline in Ror2; Bax (Figure 3G, compared to Figure 3E, 3F). This result suggests that defects in migration are primary to the defects in PGC survival in Ror2 mutants.

We next directly compared the efficiency of PGC migration in mutants. When quantified in histological sections at e11.5 (Figure 3H, 3I), ectopic (extragonadal) PGCs comprised over 70% of the total PGCs in Wnt5a mutants, and 30% in Ror2Y324C, compared to less than 5% in wild type (Figure 3J). Poor cell trafficking could therefore account for the loss of gonadal PGCs of both mutants at e11.5. However, it remained unclear whether morphologic differences in the caudal hindgut of both mutants cause the observed migration defects. Indeed, morphological and molecular analysis revealed a shortening and widening of the Ror2Y324C caudal hindgut at e9.5 (Figure 1E, Figure S4), which corresponds to the PGC exodus from the hindgut. Upon examining embryos before hindgut formation, we confirmed that the location and number of early PGCs were indistinguishable from wild type in Ror2 as well as Wnt5a at e7.5–8.0 (Figure S5). By e9.0, we observed ectopic PGCs accumulated in the allantois, throughout the tail mesoderm, and caudal hindgut of Ror2Y324C mutants (Figure S6A–S6C). However, this phenotype does not distinguish between the possibilities of an intrinsic PGC migration defect versus a structural abnormality that hindered the passage of PGCs from the allantois into the hindgut pocket. [44].

Given the previously demonstrated expression of Wnt5a throughout the allantois and primitive streak [19], we wondered whether it...
could act chemotactically to draw PGCs from the allantois into the hindgut. To address this possibility, we implanted beads coated with WNT5A into the caudal region of e8.0 embryos. Control BSA-coated beads delivered to the hindgut pocket did not disrupt embryo or PGC development over 24 h culture (Figure S6D, S6G). Beads soaked in recombinant WNT5A or concentrated conditioned medium did not alter the course of PGCs, whether placed directly on embryo posteriors and maintained on Matrigel in serum-free media. Without the support of feeder cells, which provide growth factors, survival was poor and PGCs appeared round and devoid of filopodia (Figure 4A). As reported [46], the addition of SCF elicited cytoskeletal changes and membrane protrusions in wild-type PGCs over a short period [46]. We replicated this result using flow cytometrically purified Oct4-EGFP+ cells from e9.5 embryo posteriors and maintained on Matrigel in serum-free media. The source of SCF (schematized in Figure 4H). When SCF was provided as a chemotactic gradient. Using the long cellular axis (Figure 4D–4D’). Following 7 hours of culture without SCF, a mean Elongation Index (EI) of 0.044 was observed in wild type PGCs, which increased to 0.088 in the presence of SCF (p = 0.0005; Figure 4E). PGC elongation continued to increase in culture up to 20 hours in SCF, to a mean EI of 0.169, (Figure 4F). By contrast, Ror2 PGCs mutants cultured in parallel exhibited a mean EI of 0.114 in SCF, which is significantly lower than mixed wild type and heterozygous PGCs (p = 0.005). When SCF was excluded from the media, but a strip of Matrigel was introduced along one side of the culture well to produce a gradient, the elongation response of WT PGCs was similar to that in static SCF, with a mean EI of 0.20; the graded source of SCF did not increase the EI of Ror2 PGCs: mean EI of 0.11, p = 0.0004. Short axis dimensions did not differ between wild-type and mutant PGCs (data not shown), but as we did not assess the z-axis length, these results do not exclude the possibility that Ror2 PGCs occupy less volume instead of remaining more spherical than wild type following SCF treatment. We also examined the capacity of PGCs to align with a chemotactic gradient. Using the long cellular axis explained above, we measured the angle between this axis and a line orthogonal to the source of SCF (schematized in Figure 4H). When SCF was uniformly present in the media (here termed static), the orientation of WT PGCs was randomly distributed between 0 and 90° from recombinant WNT5A, suggesting that WNT5A is biologically active (Figure S6F). Collectively these results could indicate that WNT5A may not act as a direct chemotactic cue for PGCs; rather, they suggest that Wnt5a and Ror2 could have a permissive role to allow the response of PGCs to other navigation signals.

**Ror2 autonomously enhances chemotactic response of PGCs to SCF in vitro**

Reduced PGC colonization of the gonads in Ror2 and Wnt5a mutants could result from disruptions in hindgut architecture or from intrinsic defects in PGC migration. The expression of Ror2 in both PGCs and their surrounding tissues does not provide any insight. In fibroblasts, previous work showed that WNT5A induces motility, cell shape change, and chemotaxis via Ror2 [16,45]. We did not observe PGC chemotaxis toward a WNT5A source in cultured embryos. Other work shows that WNT5A polarizes melanoma cells when a chemotactic gradient is present [26]. We sought a direct test of migratory capacity of isolated Ror2 PGCs. However, when sorted from e9.5–10.5 embryos using the Oct4-EGFP reporter, we did not observe any migration of wild type PGCs toward SDF1 or SCF in a transwell assay, as previously reported [46]. However, Farini et al. also showed that SCF elicited cytoskeletal changes and membrane protrusions in isolated PGCs over a short period [46]. We replicated this result using flow cytometrically purified Oct4-EGFP+ cells from e9.5 embryo posteriors and maintained on Matrigel in serum-free media. Without the support of feeder cells, which provide growth factors, survival was poor and PGCs appeared round and devoid of filopodia (Figure 4A). As reported [46], the addition of SCF induced morphological changes in PGCs, including the acquisition of membrane protrusions and ellipsoid shape (Figure 4B). We noted that the shape assumed by Ror2 PGCs cultured in these conditions differed from wild type, and therefore endeavored to quantify this morphology. Using phalloidin to define the F-actin cytoskeleton, we measured the longest cellular axis and the orthogonal short axis of the cell body; we then computed an Elongation Index $I_{long} - I_{short}/(I_{long} + I_{short})$, which approaches zero for round cells, such as the example in Figure 4C. Elongated cells often extended filopodia or lamellododia, which were not included in the measurement, but which usually aligned with the long axis (Figure 4D–4D’). Following 7 hours of culture without SCF, a mean Elongation Index (EI) of 0.044 was observed in wild type PGCs, which increased to 0.088 in the presence of SCF (p = 0.0005; Figure 4E). PGC elongation continued to increase in culture up to 20 hours in SCF, to a mean EI of 0.169, (Figure 4F). By contrast, Ror2 PGCs mutants cultured in parallel exhibited a mean EI of 0.114 in SCF, which is significantly lower than mixed wild type and heterozygous PGCs (p = 0.005). When SCF was excluded from the media, but a strip of Matrigel was introduced along one side of the culture well to produce a gradient, the elongation response of WT PGCs was similar to that in static SCF, with a mean EI of 0.20; the graded source of SCF did not increase the EI of Ror2 PGCs: mean EI of 0.11, p = 0.0004. Short axis dimensions did not differ between wild-type and mutant PGCs (data not shown), but as we did not assess the z-axis length, these results do not exclude the possibility that Ror2 PGCs occupy less volume instead of remaining more spherical than wild type following SCF treatment.

We also examined the capacity of PGCs to align with a chemotactic gradient. Using the long cellular axis explained above, we measured the angle between this axis and a line orthogonal to the source of SCF (schematized in Figure 4H). When SCF was uniformly present in the media (here termed static), the orientation of WT PGCs was randomly distributed between 0 and 90° from...
Figure 4. Impaired elongation and alignment with an SCF gradient in cultured Ror2\textsuperscript{Y324C} PGCs. (A–B) Sorted e9.5 Oct4\textsuperscript{-}PE-GFP\textsuperscript{+} PGCs cultured 7 h on trigel without SCF (A) and with 50 ng/ml SCF (B), inset magnified 10x. (C–G) Cell axis measurements of the largest plane of the cell performed after staining with Phalloidin and DAPI are shown for representative round (C) and elongated (D) PGCs cultured ex vivo. A maximal
Ror2 and PGC Migration

Ror2 promotes coordinated PGC elongation and polarized positioning of the Golgi apparatus

Polarized cell migration depends upon the perception of an extracellular chemotactic gradient, the acquisition of polarized molecular or membrane components, and ensuing changes in cellular organization, including cytoskeletal elements and organelles [47]. We observed an overall reduction in Ror2+ PGC shape change and alignment in the presence of an SCF gradient compared to wild type. This phenotype could result from the impaired perception of a chemotactic cue or diminished capacity to respond. As little is known about PGC polarity, we first examined the localization of two subcellular structures involved in polarized responses of migratory cells, the Golgi apparatus and the centrosome; identified here by GM130 (Golgi) and Pericentrin (centrosome) immunofluorescence, these organelles are positioned by microtubules in response to polarity cues [48]. Following SCF exposure, cultured wild type and Ror2+ PGCs both elaborated F-actin-rich extensions (Figure 5A, 5B, 5C). GM130 and Pericentrin staining was observed colocalized in three discrete cellular geometries. Asymmetric localization of GM130 and Pericentrin to one extreme of the nucleus in elongated cells was denoted Class I (Figure 5A). Central positioning of GM130 and Pericentrin adjacent to or above the nucleus was denoted Class II (Figure 5B and 5C). Class III included geometrically rounded cells with eccentric GM130 and Pericentrin (Figure 5D). Finally, GM130 was occasionally observed as dispersed foci (not shown), Class IV, which is most likely the configuration in mitotic cells [48]. The tabulated results of several experiments are shown (Figure 5E). A similar frequency of wild-type Class I PGCs was observed in static and graded SCF (69% and 73%, respectively). This distribution of the Golgi and centrosome appears to be nonrandom given the relatively large cellular area occupied by the PGC nucleus. Strikingly, a significant overall reduction of Class I Golgi position was observed in Ror2+: 45% in static SCF and 48% in a gradient, both of which differ from wild type (p = 0.005). This result suggests that the coordination of centrosome/Golgi position and cell shape is affected in Ror2 PGCs. However, if we consider the Golgi position apart from cell shape—since the rounded Class III cells could retain molecular and organelle polarization—it becomes apparent that the cells in Class III also exhibit Golgi/centrosome asymmetry. In this line of reasoning, we find that the frequency of combined Class I and III PGCs does not differ between wild type and Ror2 in graded SCF (p = 0.23) and is barely significant in static SCF (p = 0.04). This analysis could suggest that Ror2 PGCs are defective in cell elongation, but not polarized positioning of the centrosome and Golgi. Conversely, if we compare only geometrically elongated cells, or those in Classes I and II, we find a decreased incidence of polarized Golgi position (Class I) of Ror2 PGCs cultured in static SCF (p = 0.029), but not in gradient SCF (p = 0.18) compared to wild type. Given that the majority of Ror2 PGCs elongate to some degree in SCF, this discrepancy in Golgi position could reveal a more subtle defect in their polarized response. Finally, although a rare class, the incidence of Class IV or dispersed GM130 appears elevated in Ror2 PGCs (Figure 5E). This uptick could reflect a slight increase in proliferation of the mutant PGCs that we have observed in vitro (Figure S7). Taken together these experiments demonstrate a decoupling between cell elongation and polarized position of the Golgi and centrosome in Ror2 mutant PGCs; however with the alternate interpretations of Class III cells as either randomly positioned or polarized Golgi/centrosome within a rounded cell, it remains possible that Ror2 acts as a cell polarity effector or else in the associated cell shape changes.

ROR2 exhibits a polarized distribution in migratory PGCs in vivo and in vitro

Several previous studies have implicated Ror2 in cell polarity, including polarized cell division in C. elegans [14], directional migration in the limb and several mammalian cell lines [18,26,49], and apicobasal polarity in the mouse gut [9]. A polarized distribution of ROR2 within the developing gut epithelium [9] prompted us to examine ROR2 localization in PGCs following culture in SCF. Immunofluorescence revealed asymmetry of ROR2 within the cytoplasm as well as on the surface membrane of PGCs. The distribution of ROR2 at one extreme of the cell coincided with GM130 (Figure 5F, 5F). ROR2 was also observed prominently on the membrane protrusions of cultured PGCs. Returning to the embryo, we examined the subcellular distribution of ROR2 and GM130 in PGCs in vivo. In e10.25 histologic sections, immunostaining revealed an apical enrichment of ROR2 in the hindgut and dorsal neural tube, colocalized with GM130 (Figure 6A–6A). At this stage, PGCs identified by the expression of Stella are migrating through the dorsal mesentery toward the gonadal ridges (Figure 6A). Within these PGCs, ROR2 appeared to be enriched on one side in most instances (Figure 6B, 6C, 6D). This enrichment was coincident with GM130 (Figure 6F, 6F) and, unexpectedly, Stella (Figure 6B, 6C). As a polarized Stella distribution has not been previously reported, we wondered whether this pattern could reflect the plane of section. When PGCs were instead immunostained with the SSEA-1 antibody, the asymmetric distribution of ROR2 persisted (Figure 6D), but SSEA-1 appeared to be localized consistently around the PGC border (Figure 6D), as did β-catenin (Figure 6D). Together, these data demonstrate a polarized distribution of ROR2 in PGCs that are responding to chemotactic cues in vitro and migrating in vivo. Its localization on filopodia and segregation on the same side of the cell as the Golgi suggests Ror2 could be important in the polarization response of the cell in response to SCF. Upon examining histologic sections from Ror2 embryos, we did not observe a comparable degree of asymmetric ROR2 distribution or Stella distribution in PGCs, and GM130 staining was...
Figure 5. Reduced polarization by SCF in cultured Ror2Y324C PGCs. (A–D) Actin, centrosome and Golgi positions were examined in 20 h ex vivo cultured Oct4-ΔPE-GFP+ PGCs in the presence of SCF by GFP, Phalloidin, Pericentrin and GM130 immunofluorescence. (A–A”) An example of
asymmetrically distributed GM130 and Pericentrin of an elongated cell, categorized as Class I. Class II contains elongated cells with centrally located GM130 and Pericentrin (B–B′). We located the largest cellular cross section for measuring the longest cellular axis and the short axis orthogonal to this one (Figure 7A, 7D). In migratory wild-type PGCs, we measured a mean EI of 0.12 that was determined in two dimensions from SCF-treated embryos (Figure 7E). This in line with the mean EI of 0.20±0.12 that was determined in two dimensions from SCF-gradient cultured PGCs. In Ror2−/− embryos, migratory PGCs exhibited a mean EI of 0.09±0.07, which is significantly less than in wild type (p<0.0001). For comparison, we examined PGCs located within the gonads of e10.75–11.5 embryos, since previous studies reported that following their arrival in the gonad, PGCs acquire a rounded morphology [50]. Wild-type postmigratory PGCs measured 0.08±0.05 mean EI. Together these in vivo observations suggest that Ror2 enhances the polarized response that leads to cell elongation of migratory PGCs.

**Discussion**

The trafficking of PGCs through the embryo is a widely conserved process across many vertebrates and invertebrates [1]. Success is critical for fertility of the organism and propagation of its genome, and thus subject to selection. Failure has been linked to the development of germ cell tumors, because surviving mislocalized PGCs are subject to transformation [2,51]. Our knowledge of the mechanisms underpinning PGC migration in mammals is limited [52]. Through forward genetics, we have implicated a new pathway in the migration of PGCs. Here we have identified an ENU allele of the putative ligand Ror2 that leads to a diminution of PGCs in the fetal gonads. By contrast, in Steel mutants, which lack both membrane and secreted KitL, one or two Bax null alleles is sufficient to rescue KitL−/− gonadal PGCs [2]. Together these results argue that migration is the primary defect in Ror2 PGCs, and apoptosis in the periphery arises as a consequence of reduced survival factor exposure. The possibility remains that ectopic Ror2 PGCs are increasingly sensitive to the withdrawal of survival factors. KITL and SDF1, known to be the most important PGC survival factors [42,43,44], are both concentrated in the e11.5 gonads and their absence in peripheral tissues likely leads to Ror2 PGC death.

**Does Wnt5a function as a chemoattractant in PGC migration?**

In the developing mammalian palate, a series of bead and cell implantations suggest that WNT5A is sufficient for directional movement of cells via Ror2 [15]. However, similarly implanted beads coated with WNT5A did not divert the migration of PGCs in our studies (Figure S6), and we could not detect any role for Wnt5a as a chemoattractant. Instead, we suggest that Wnt5a may act permissively in PGC migration. This is not unprecedented, as in a melanoma cell line, Witz et al. showed that WNT5A acts permissively to regulate the polarized distribution of adhesion receptors in response to a chemokine gradient [26]. In PGCs, the known chemoattractive factors include SDF1 [42,43] and KitL [2,41,55]. Recognized as the most critical growth and survival factor for PGC, KitL, was first postulated as a guidance cue for PGCs from the analysis of the Steel−/− mutant [56]; SCF (secreted KitL) was later shown to induce PGC migration ex vivo, as well as inducing cell shape changes [46,57]. Aberrant cell shape was previously noted in PGCs from Steel mutants [58]. When the survival of Steel PGCs was restored in compound mutants with Bax, functions for KitL in motility, adhesion and colonization of the gonad were identified [2,41]. The resemblance of these cellular phenotypes to what we observed in Ror2−/− embryos prompted us to ask whether Ror2 could enhance the response to KitL chemoattractant cues.

**What is the role of Ror2 in PGC migration?**

Our ex vivo experimental approach address three separate aspects of cell migration: polarity, cell shape change and orientation toward a chemoattractant cue. We find that a gradient of SCF induces geometric elongation as well as a nonrandom alignment of wild type PGCs within the field. This chemotrophic function of SCF was previously recognized in mast cells but not PGCs [59,60]. Similar to a recent report [57], our results also reveal a chemokinetic, or non-directional function of SCF in...
Figure 6. Asymmetric localization of ROR2 in polarized migratory PGCs in vivo. (A–E) Histologic sections from e10.25 WT embryos were immunostained with Stella, Ror2 and GM130 antibodies and PGCs migrating through the dorsal mesentery (A–A’) were examined (arrowheads).
migration, as wild-type PGCs assumed an elongated, polarized morphology when SCF was uniformly present. This morphology was accompanied by a polarization of the Golgi apparatus, which is typical of migratory cells [61]. In all of these cellular behaviors, Ror2 PGCs exhibit a mitigated response to SCF; their orientation is randomized instead of aligned with respect to the gradient, they elongate less, and the frequency of polarized Golgi distribution reduced. We find, strikingly, a similar difference in the shape of PGCs in Ror2 mutant embryos. This result confirms in vivo a function for Ror2 in the polarized migration of PGCs. Taken together, the in vivo and in vitro experiments suggest that Ror2 signaling enhances the chemotactic response of PGCs to KitL emanating from the gonadal ridges. Although the precise function of Ror2 in this polarized migration remains to be determined, its nonrandom protein distribution throughout migratory PGCs may provide a clue. The observed pattern of Ror2 within the cytoplasm and near the cell membrane is reminiscent of the asymmetry within the hindgut epithelium [9].

The potential colocalization with the Golgi apparatus is intriguing and warrants further investigation. On the other hand, the distribution of Ror2 on membrane protrusions is reminiscent of the reported expression on the dendrites of hippocampal neurons [62]. The altered distribution of ROR2 in Ror2Y324C PGCs argues for its specificity and functional significance, and leads us to propose that ROR2 distribution becomes polarized in response to directional KitL cues and thus reinforces the polarization of the cell. In other words, Ror2 might potentiate asymmetry in Kit signaling, or even transform it from a general signal to a polarized signal. It is also possible that the localization of ROR2 on protrusions promotes the growth and selection of filopodia into a clear lamellipodia or a leading edge, similar to the axonal path finding function of the homolog in C. elegans [7]. Elucidating the dynamic distribution of ROR2 in PGCs undergoing polarized responses will be an important future pursuit.

How does Ror2 enhance the response to KitL?

The connection established in these studies between Ror2 and SCF-induced cell polarization is new, and the molecular nature of the relationship is unclear. The robust evidence for the specificity of SCF for cKit rules out the possibility of biochemical interaction between SCF and Ror2 [63]. However, the absence of feeder cells or serum and purity of sorted PGCs in our culture system demonstrates that both proteins are acting in the same cell. Based on the detection of Wnt5a transcript and protein in PGCs, a plausible scenario would involve autocrine WNT5A secreted from PGCs and near the cell membrane is typical of migratory cells [61]. In all of these cellular behaviors, Ror2 PGCs exhibit a mitigated response to SCF; their orientation is randomized instead of aligned with respect to the gradient, they elongate less, and the frequency of polarized Golgi distribution reduced. We find, strikingly, a similar difference in the shape of PGCs in Ror2 mutant embryos. This result confirms in vivo a function for Ror2 in the polarized migration of PGCs. Taken together, the in vivo and in vitro experiments suggest that Ror2 signaling enhances the chemotactic response of PGCs to KitL emanating from the gonadal ridges. Although the precise function of Ror2 in this polarized migration remains to be determined, its nonrandom protein distribution throughout migratory PGCs may provide a clue. The observed pattern of Ror2 within the cytoplasm and near the cell membrane is reminiscent of the asymmetry within the hindgut epithelium [9].

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Materials and Methods

Animals

All animal work was carried out in compliance with care and use standards at each institution. Ror2Y324C, was identified in a recessive ENU screen at e9.5 for mouse mutants with PGC defects [23]. Other mouse strains used included: Bax (MGI:1857429), Wnt5a (MGI:1857617), and Oct4-ΔPE-GFP [67] with genotyping performed as described elsewhere [19,50]. Mice were maintained on C3H or mixed C3H/FvB genetic backgrounds. Embryos were generated in timed matings by monitoring for copulatory plugs. Pregnant females were sacrificed and embryos staged by the following anatomic landmarks: 23–33 somite pairs was designated e10.0, 34–39 somite pairs as late e10.5, 45–48 somite pairs and the appearance of the otic vesicle as e11.5, and the presence of embryonic kidneys designated e12.5; gonad sex was determined by the appearance of tubules and the coelomic vessel in e12.5 males, and Sry genotyping [68].

Mapping

MIT SSLP markers were used to map Ror2Y324C to chromosome 13 to a 10 Mb interval between D13 MIT 176 and D13 MIT 13. Sequencing of the Ror2 ORF revealed an A to G transition at position 1203, which creates a restriction site. T324C genotyping was carried out by PCR amplification of a 238 bp fragment in 25 μl reactions heated to 95°C for 3 min, followed by 45 cycles of 94°C for 30 sec, 57°C for 1 min, 72°C for 30 sec, and a 7 min hold at 72°C using the following primers: 5′-ACC AGT GCT ACA ACG GCT CT-3′ and 5′-AGT TCC ACG CTG TTT TT-3′. Subsequent digestion 5 h with 3 U HpyCh4 V (NEB) produced fragment sizes 152 and 86 bp for the wild type allele, 152, ~50 and ~30 bp for mutant allele.

Flow cytometry and RT–PCR

Embryos were dissected at e9.5–11.5 in cold PBS/0.2% BSA and the posterior fragment or gonads dissociated in 0.25% trypsin/EDTA for 3–5 minutes at 37°C followed by 1 mg/mL DNaseI for 5 min. For Ror2 intracellular flow cytometry, cells were prepared using the Cytofix/cytoperm Kit (Beckton Dickson) and stained at 1:50 (Santa Cruz Biotech A17). Live cell staining was carried out in phenol red-free DMEM/2% fetal bovine serum/10 mM EDTA. Dead cells were excluded on the basis of Sytox Blue (Invitrogen) signal. PGCs, delineated as Oct4-(PE)-GFP were sorted directly into lysis buffer and extracted with RNeasy Kit (Qiagen), DNase I treated, and reverse-transcribed with qScript (Quanta Biosciences) or Superscript III (Invitrogen). PCR primers were designed with Primer Express software.
Figure 7. Reduced elongation of migratory $Ror^2Y324C$ PGCs in vivo. (A) WT migratory PGC stained with SSEA1 (green) and DAPI (grey) shown in five sections at different levels through a confocal stack. The axes are measured (blue lines) in the largest plane of the cell, shown in the middle.
panel, resulting in an EI = 0.23. (B) A confocal stack from a WT e10.25 embryo section stained with SSEA1 and E-cadherin to delineate the hindgut (left). Autofluorescent erythrocytes are present in the lower right quadrant. Filopodia and lamellopodia can be seen on many PGCs; the cell in (A) is marked.

(Applied Biosystems). Amplification was carried out using 50 or 100 cell equivalents of cDNA on a Mastercycler EP (Eppendorf) using the following primer sets: 5'-GACCTGACAGCACTGCTGCCAC-3' and 5'-TTCCAGGACCTGTTGCTGTGA-3' for Gapdh; 5'-AATGGCAGACATTGCGATCTCC-3' and 5'-AGGATGCCTAGACTACTGGAAAA-3' for Wnt5a; 5'-AGTGGAGACCGATTTGGAGAAG T-3' and 5'-TACTCTTTCTTGTTGGGATACCTCAATA-3' for Oct4; 5'-GAGATCATGCCTGTTCCAC-3' and 5'-AGCATGCGCCTGTTGGCGG-3' for Ror2, 5'-GAGAGCCGACCGCTGTGATT-3' and 5'-CCAATCTGCGTCCAGTT-3' for Wnt5a.

Immunostaining

For Western blotting, day 9.5 embryos were lysed in RIPA buffer containing 1% Nonidet P-40, 0.25% Deoxycholate acid, 150 mM NaCl, 0.1% SDS, 50 mM HEPES (pH 7.4) and proteinase inhibitor cocktail (Roche). 40 pg protein was separated by SDS-PAGE gel and probed with anti-ROR-2 antibody (Santa Cruz Biotech, H-76).

For immunofluorescence histology, embryos fixed in 4% paraformaldehyde were embedded in OCT and cryosectioned at 10 um. Slides were blocked 1 h in 10% calf serum overnight in PBS-BSA, 25 ug/mL SCF (RnD), 25–50 ug/mL SDF1 (RnD), 50 ug/mL WNT5a (RnD) or 20–50 fold concentrated WNT5A conditioned media, washed, and implanted into the proximal allantois, hindgut pocket or axial mesoderm with microfroreces. Embryos were cultured in organ culture dishes (Falcon) containing 50% DMEM HG with Pen-Strep/50% Heat inactivated Rat Serum (Taconic) at 37°C in 5% CO2, for 24 hours.

Alkaline phosphatase activity

Fast red staining was carried out as detailed elsewhere [70].

Image collection and analysis

Brightfield imaging was performed on an Olympus MVX10 stereomicroscope. Confocal imaging was carried out with a 10x, 20x or 63x objective on a Leica SP5 TCS microscope equipped with 405, 488, 543, 594, and 633 nm lasers. Stacks were analyzed using Velocity (Improvision).

The number of PGCs in wholemount e10.5 immunostained embryos or e11.5–12.5 gonads was estimated using a measurement protocol created in Velocity 5.0 acquisition software. Objects were identified by in the SSEA1 or GCNA channel using the “Find Objects Using Standard Deviation (SD) Intensity” task, with a lower limit of 3.2–3.7 SDs above the mean. Holes were filled in objects, and those under 20 mm3 were excluded, and touching objects separated using a size guide of 200 mm3 in the GCNA channel or 750 mm3 in the SSEA1 channel. “Exclude Objects by Size” task was repeated to eliminate objects less than 20 mm3 created by the previous command. Objects were visually inspected to determine the approximate size cutoff for single objects. For gonads colabeled with antibodies against PHH3 or cleaved PARP, subsequent selection of colocalized objects was carried out using the intensity and colocalization functions. All measurement results were exported to Excel (Microsoft) for calculations. Clustered objects exceeding the defined threshold of single PGCs were summed and divided by the average PGC size. For instances in which over 20% of the total measured GCNA volume remained clustered, the quantity of PGCs was estimated by dividing this total volume over the average object size in well scattered specimens (300 um3 used here for GCNA).

Cell axes lengths were quantified in confocal stacks visualized in Velocity using the measurement function and exported to Excel for analysis. Image reconstructions with the long axis marked were exported to ImageJ (NIH) for angle measurements.

Supporting Information

Figure S1 PGCs are depleted in mutants homozygous for Ror2 targeted deletion allele. Gonads from age matched Ror2+/− and WT littermates on the G57Bl/6 background were stained in wholemount with GCNA antibody and PGCs quantified from e9.75–10.75 histologic sections. Postmigratory WT e10.75 (“Gonadal”) PGCs are shown at right in grey and the mean EI in red.

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confocal stacks. A significant deletion was observed in homozigous mutants (purple triangles) compared to WT (black diamonds) at e1.125 and e12.25 (A). At e11.5, compared to WT (B), *Ror2*−/− (C) gonads exhibit a skewed distribution of PGCs at the posterior (bottom), similar to *Ror2*Y324C. (TIFF)

**Figure S2** PGC proliferation and apoptosis in whole gonads. (A) Apoptosis and proliferation were simultaneously visualized in whole gonads, shown at e12.25 stained with GCNA (red), PHH3 (blue) and cleaved PARP (green) antibodies. PGCs were modeled as GCNA-delineated, size selected objects (B), and the subset of PHH33 nuclei (C) and cPARP5 (D) objects were quantified (frequency of total GCNA3 objects indicated as percentage). (TIFF)

**Figure S3** Mitotic and dying PGCs can be identified in histological sections. Sagittal section through a WT e10.75 embryo stained with (A) SSEA1 (green), PHH3 (blue) and cPARP (red) antibodies reveals proliferating (arrowheads) and apoptotic (arrows) PGCs in the dorsal mesentery. Nuclear staining with DAPI (grey) confirms nuclear localization of PHH3 (B) and cPARP (C) signals, with the overlay of all channels shown in (D). Scale bar = 39 um. (TIFF)

**Figure S4** Posterior hindgut morphogenesis is abnormal in *Ror2* mutants. Hematoxylin and eosin staining of transverse sections of lower trunk level (A) and tail bud level (A′) in wild-type embryos (arrows). However, the hindgut ring was dilated at all equivalent positions in *Ror2* mutant embryos (arrows in B, B′, B″). (TIFF)

**Figure S5** PGCs are specified normally in *Ror2*Y324C and *Wnt5a* mutants. Embryos collected at ~e7.5–8.0 were staged and alkaline phosphatase-stained PGCs were quantified. (A) *Ror2*Y324C did not affect the number of PGCs in heterozygotes (unfilled triangles) or homozygotes (blue triangles) compared to wild type (black triangles). Similarly, *Wnt5a* haploinsufficiency (unfilled circles) or ablation (pink circles) had no affect on PGCs at this stage [B]. (TIFF)

**Figure S6** PGCs migrate inefficiently in *Ror2* and *Wnt5a*, but *Wnt5a* is not a chemoattractant. (A–C) Embryos at e9.0 (12–14 somites) stained for alkaline phosphatase (AP) activity (red, arrowhead) reveal excessive ectopic PGCs throughout the allantoids (B) and tail mesoderm (C) of *Ror2*Y324C (insets 2× magnification). (D–F) Beads impregnated with either BSA, SCF, SDF1 or WNT5A conditioned medium were implanted into e9.0 embryos before 24 hours culture and AP staining, and shown in ~8 somite stage (D–F) and ~12 somite stage embryos (G–H). PGC migration was not disrupted by the bead’s presence (left) or altered by Wnt5a-heads (E,H). By contrast, SDF1 and SCF were capable of diverting the migration of PGCs at close range (F) and red arrows in n>3 embryos. (J) Culture of sorted e9.5 Oct4−APE-GFP+ WT PGCs for 20 h on Matrigel in presence of Wnt5a (250 ng/ml) revealed a significant increase in PGC number (p = 0.007 by paired t-test). (TIFF)

**Figure S7** Increase in ex vivo *Ror2*Y324C PGC proliferation measured by BrdU incorporation. Unsorted PGCs mechanically dissociated from e9.5 embryos were cultured for 24 hours, with 1 hour in Bromodeoxyuridine (BrdU). After fixation, PGCs were identifiable by SSEA immunofluorescence (A) with 5 PGCs in the field (arrows). (B) Anti-BrdU staining shows incorporation by many of the feeder cells and one PGC (arrowhead), with overlaid channels in (C). Scale bar = 100 um. (D) Quantification of the % of PGCs that incorporate BrdU reveals a slight increase (p = 0.05) in *Ror2*Y324C compared to mixed WT and heterozygous PGCs. (TIFF)

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**Author Contributions**

Conceived and designed the experiments: DJL, KVA, MDK, SA-K, XZ. Performed the experiments: DJL, MDK, SA-K, XZ. Analyzed the data: DJL, KVA, MDK, SA-K, XZ. Contributed reagents/materials/analysis tools: DJL, KVA, MDK, SA-K, XZ. Wrote the paper: DJL, KVA, SA-K.
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