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Steel factor controls primordial germ cell survival and motility from the time of their specification in the allantois, and provides a continuous niche throughout their migration

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Steel factor is an essential survival and proliferation factor for primordial germ cells (PGCs) during their migration in the early mouse embryo. PGCs arise during gastrulation, and migrate into the posterior endoderm that becomes the hindgut. Previous reports have suggested that PGCs become dependent on Steel factor when they colonize the hindgut. However, in the absence of a good marker for living PGCs, their behavior before hindgut colonization has not been previously studied. We report here the normal behavior of PGCs in live embryos before hindgut colonization, and the roles of Steel factor, using a reporter line in which GFP is driven by the promoter of the *Stella* gene, whose activation accompanies the initial specification of PGCs. We show first that PGCs are surrounded by Steel factor-expressing cells from their first appearance in the allantois to the time they enter the genital ridges. Second, fewer PGCs are found in the allantois in *Steel*-null embryos, but this is not due to a failure of PGC specification. Third, the analysis of cultured *Steel*-null early embryos shows that Steel factor is required for normal PGC motility, both in the allantois and in the hindgut. Germ cells migrate actively in the allantois, and move directionally from the allantois into the proximal epiblast. In the absence of Steel factor, caused by either null mutation or antibody blockade, PGC motility is dramatically decreased, but directionality is maintained, demonstrating a primary role for Steel factor in PGC motility. This was found both before and after colonization of the hindgut. These data, together with previously published data, show that PGCs are Steel factor dependent from their initial specification until they colonize the genital ridges, and suggest the existence of a 'spatio-temporal niche' that travels with this important pluripotential cell population in the embryo.

KEY WORDS: Primordial germ cell, Steel factor, Stem cell niche, Mouse

INTRODUCTION

Primordial germ cells (PGCs) are the embryonic precursors of the female and male gametes. In mouse, PGC competence is induced in the proximal epiblast at pre-gastrula stages (E6.0-E6.5) in response to signals from the extra-embryonic ectoderm and visceral endoderm (Lawson and Hage, 1994; Saitou et al., 2002; Tanaka and Matsui, 2002). Within this group of epiblast cells, about six cells activate the expression of Blimp1 (Prdm1 – Mouse Genome Informatics), a marker of lineage-restricted PGC precursors, whilst neighboring cells acquire a somatic fate. The Blimp1-positive PGC precursors increase in number as they move to the posterior primitive streak and allantois, where they become specified at E7.25 to form exclusively PGCs, and express markers of specified PGCs, such as alkaline phosphatase (AP) and Stella (Dppa3 – Mouse Genome Informatics) (Hayashi et al., 2007; Ohinata et al., 2005). The details of PGC behavior between E7.25 and E8.5 are not clear. However, by E8.5, PGCs are localized around the hindgut diverticulum, and by E9.0 they are incorporated into the hindgut epithelium (Anderson et al., 2000). PGCs emigrate from the dorsal aspect of the hindgut between E9.0 and E9.5, separate into left and right streams of individual cells, and migrate laterally across the dorsal body wall. At E10.5, PGCs close to the genital ridges continue to migrate, singly or in clusters, into the genital ridges to form the primary sex cords, while PGCs remaining in the midline structures die by apoptosis (Molyneaux et al., 2001).

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Steel factor (also known as stem cell factor, kit ligand or mast cell growth factor) is the product of the *Steel* locus and a member of the short-chain helical cytokine family. It has been shown by many studies to be an essential survival factor for PGCs (De Felici and Pesce, 1994; Dolci et al., 1991; Godin et al., 1991; Matsui et al., 1991). There are two forms of Steel protein, generated by alternative splicing: soluble Steel factor and membrane-bound Steel factor. The membrane-bound form lacks an extracellular domain containing a proteolytic cleavage site, which normally causes release of the extracellular region of the protein (Flanagan et al., 1991; Huang et al., 1992). The receptor for Steel factor is the product of the *W* locus, c-Kit, a tyrosine-kinase receptor of the PDGFRB superfamily that is expressed in PGCs throughout migration from E7.25 (Loveland and Schlatt, 1997; Yabuta et al., 2006). In the absence of either ligand or receptor, mice are sterile, and reduced numbers of PGCs are seen during migration. In addition, surviving PGCs in $Steel^{-/-}$ embryos are often described as being retarded in their migration, clumped, and in ectopic locations (Bennett, 1956; Mahakali Zama et al., 2005; McCoshen and McCallion, 1975; Mintz and Russell, 1957). Recent work from our laboratory has revealed novel aspects of Steel factor function in regulating migratory PGC behavior (Runyan et al., 2006). At E9.5, Steel factor is expressed in midline structures as well as in the genital ridges. At E10.5, Steel factor expression is lost from midline structures, but is enriched in the genital ridges. The change in location of Steel factor expression causes PGCs in the midline to die through the intrinsic apoptotic pathway, but the survival of PGCs closest to the genital ridges is maintained. Steel factor functions at close range, as the genital ridges are only about 100 μm away from the midline at E10.5. Analysis of embryos in which PGC apoptosis was blocked showed that, in addition to its role in PGC survival, Steel factor is also required between E9.0

and E10.5 for PGC proliferation and migration (Runyan et al., 2006). These data suggest the existence of a 'traveling niche' of Steel factor-expressing cells that control many aspects of PGC behavior.

Little is known about when the association between PGCs and Steel factor-expressing cells begins, or whether it exists throughout PGC migration. Classical studies of W and Steel mutants have reported that PGCs only become Steel factor-dependent in the hindgut (Bennett, 1956; Mahakali Zama et al., 2005; McCoshen and McCallion, 1975; Mintz and Russell, 1957). However, the inability to visualize PGCs in living embryos before gut colonization has made this period of their development inaccessible to study. In this paper, a novel reporter mouse line expressing GFP under the *Stella* promoter (Payer et al., 2006) has been used to visualize PGCs from the time of their first appearance in the embryo. We show that PGCs are surrounded by Steel factor-expressing cells, from the time they first turn on expression of *Stella* in the allantois to the time they colonize the genital ridges. Fewer PGCs are found in the allantois in *Steel*-null mutant embryos when compared with their wild-type littermates, indicating that Steel factor is required to maintain PGC numbers as early as E7.5. Moreover, PGCs in *Steel*$^{-/-}$ embryos show reduced motility and increased clump formation, but the direction of PGC migration is not randomized. Although germ cells are known to express E-cadherin (Okamura et al., 2003), clumping is not due to an upregulation of this adhesion protein. The results were further confirmed by the acute loss of Steel signaling following the use of an Ack2 antibody, an antibody that blocks the c-Kit receptor. Analyses of PGC migration in E9.0 embryos revealed that Steel factor is also required for normal PGC motility at this stage, in addition to its role in regulating PGC survival and proliferation. These data show that a wave of Steel factor expression moves with the PGCs, from the time of their specification to their arrival in the genital ridges, thereby forming a traveling niche for this important population of migrating pluripotential cells.

MATERIALS AND METHODS

Mouse breeding, embryo preparation and genotyping

Mouse embryos were obtained by crossing males homozygous for the *Stella-GFP* transgene (Payer et al., 2006) on a B6/CBA background with 6- to 8-week old CD1 females (Charles River). Embryonic day 0.5 (E0.5) was assumed to be noon of the morning a vaginal plug was observed. *Stella-GFP* mice were crossed with *Kitl*^{Sl} heterozygotes (Jackson Laboratories, Stock number 000693) to obtain mice that were *Stella-GFP*$^{+}$, *Steel*$^{+/-}$. These were interbred to yield *Stella-GFP*$^{+}$, *Steel*$^{-/-}$ embryos. To generate *Steel/Bax* double mutants, *Stella-GFP*+, *Steel*$^{+/-}$ mice were bred with *Bax* heterozygotes (Knudson et al., 1995). For E9.0 studies, *Oct4ΔPE:GFP* transgenic mice (Anderson et al., 2000) were used instead of *Stella-GFP* mice. Genomic DNA was isolated from tails snips (adults), embryo halves (E7.5 embryos) and heads (E9.0 embryos), and genotypes were determined by PCR. Genotyping primers used were as follows: *Stella-GFP*, F-5′-TGCATCGGTAACCCACAGTA-3′, R-5′-GAACTTCAGGGTCAGCTTGC-3′; *Kitl*^{Sl}, Common-F-5′-CGGGGTTTATGAGGGTAGGA-3′, WT-R-5′-TTGGGCCTGTGTGACAAACT-3′, DEL-R-5′-ACTTCCTAGGGCTGGAGAGATG-3′; *Bax* (Deckwerth et al., 1996), EX5-F-5′-GAGCTGATCAGAACCATCATG-3′, IN5-R-5′-GTTGACCAGAGTGGCGTAGG-3′, NeoPGK-R-5′-CCGCTTCCATTGCTCAGCGG-3′; *Oct4ΔPE:GFP* (Yeom et al., 1996), F-5′-GGAGAGGTGAAACCGTCCCTAGG-3′, R-5′-GCATCGCCCTCGCCCTCGC-3′. *Stella-GFP* expression was determined by the presence of a 289-bp fragment. For *Kitl*^{Sl}, wild-type and deleted alleles were determined by the presence of 294- and 646-bp fragments, respectively. For genotyping of *Bax*, wild-type and mutant alleles were determined by the presence of 304- and 507-bp fragments, respectively. *Oct4ΔPE:GFP* transgene expression was determined by the presence of a 250-bp fragment.

Embryo slice culture

E7.25 and E7.5 embryos were cut into halves along the sagittal axis using a scalpel. One half of each embryo was placed onto a millicell CM organ culture insert (Millipore) pre-coated with collagen IV (BD) and the other half was used for genotyping. For E9.0 embryos, the caudal halves were cultured on the inserts as described previously (Molyneaux et al., 2001). The millicell organ inserts were then placed into a metal stage that contained glass-bottom chambers, and incubated in 600 μl Hepes-buffered DMEM/F-12 (Gibco) medium with 0.04% lipid-free BSA and 100 U/ml penicillin/streptomycin (Gibco). To generate an acute loss of Steel signaling, 10 μg/ml c-Kit blocking antibody, Ack2 (a kind gift from Dr Fred Finkelman, CCHMC), was added to the slice culture medium. Mouse IgG (10 μg/ml, Jackson ImmunoResearch) was used as a control. Embryo slices were maintained at 37°C by placing the metal stage in a temperature-controlled stage (Zeiss) and maintaining humidity with wet paper towels placed in a 100 mm culture dish fastened over the organ culture chambers.

Time-lapse analysis of migrating PGCs

Slices were filmed with a Zeiss LSM510 confocal system attached to a Zeiss axiovert microscope. Pictures were acquired every 5 minutes for 6 to 10 hours, and movies were analyzed using NIH image as described (Molyneaux et al., 2001). Briefly, all cells that remained in focus for the duration of the filming were traced, and the average velocity, maximum velocity and displacement were measured for each of these cells using two cell-tracking macros written for the NIH ImageJ software by Kathy Molyneaux or by Erik Meijering. Their directionality was also recorded and the net trajectory for all cells in each embryo was plotted on a windrose diagram. Experiments were repeated at least three times, and three to eight embryos were analyzed per group. For statistical comparisons, the data were analyzed using an unpaired, two-tailed Student's *t*-test with equal variances.

RT-PCR

For RT-PCR analysis, allantoides from E7.5 embryos, and genital ridges from E10.5 embryos were dissected, and RNA extracted from dissected tissues using the RNeasy Kit (Qiagen). RNA (10 ng) was reverse transcribed by using Superscript III First-Strand Synthesis Systems (Invitrogen), following the manufacturer's recommendations. PCR reactions were performed using Redmix Plus (GeneChoice). Primers used were as follows: membrane-bound Steel factor, F-5′-TCCCGAGAAAGGGAAAGC-3′, R-5′-CTGCCCTTGTAAGACTTGACTG-3′ (predicted fragment length, 149 bp); soluble Steel factor, F-5′-TTATGTTACCCCCTGTTGCAG-3′, R-5′-CTGCCCTTGTAAGACTTGACTG-3′ (predicted fragment length, 195 bp).

Immunofluorescence analysis on whole-mount embryos or frozen sections

Embryos were fixed in 4% paraformaldehyde (PFA). For whole-mount staining, embryos were then washed in 0.5% NP-40 (2×10 minutes), blocked in PBSST (PBS/0.3% Triton X-100 with 5% goat or donkey sera, 2×1 hour washes) and incubated overnight at 4°C in PBSST with primary antibody. The following day, the embryos were washed in PBST (PBS/0.3% Triton X-100, 2×15 minutes, then 3×1 hour) at 4°C, incubated overnight at 4°C in PBSST with Cy3- or Cy5-conjugated secondary antibodies (Jackson ImmunoResearch), washed in PBST (2×15 minutes, then 3×1 hour) and cleared in 50%, then 90%, glycerol for imaging. Embryos to be sectioned were dehydrated in sucrose and mounted in OCT compound (Tissue-Tek) for cryosectioning. Sectioned-embryos were rehydrated with PBST (10 minutes), blocked with PBSST (1 hour at room temperature), and incubated with primary antibody overnight at 4°C. The next day, slides were washed with PBST (3×15 minutes), incubated in PBSST with secondary antibody (2 hours), washed with PBST (2×15 minutes) and mounted with DABCO (Sigma) for imaging.

Primary antibodies were used at the following dilutions: 1:50 for anti-Steel factor (R&D Systems), 1:200 for anti-cleaved-PARP (Cell Signaling), 1:2000 for anti-phospho-histone H3 (Upstate), 1:250 for anti-E-cadherin (a kind gift from Dr Masatoshi Takeichi, RIKEN Center for Developmental Biology, Japan). Secondary antibodies (Jackson ImmunoResearch) were

used at the following dilutions: 1:300 for Cy5 donkey anti-goat; 1:300 for Cy5 goat anti-rabbit; 1:300 for Cy5 goat anti-rat; 1:300 for Cy3 goat anti-mouse. Images were captured using a Zeiss LSM 510 confocal system.

RESULTS

Steel factor is expressed by cells surrounding PGCs throughout migration

We first analyzed the expression pattern of Steel factor from E7.5 to E10.0 by using anti-Steel factor antibody on *Stella-GFP* embryos. At E7.5, cells expressing both Stella and Steel factor could be seen in the allantois (arrow, Fig. 1A-C). These data are supported by the single cell RT-PCR analysis carried out in a recent study (Yabuta et al., 2006), which showed that Steel factor was expressed in PGCs at E7.25, but was downregulated within 24 hours. Somatic cells immediately adjacent to PGCs also showed high levels of Steel factor staining on their cell surfaces at E7.5 (Fig. 1A-C). Diffuse staining was seen in other regions. We interpret this to be soluble Steel factor, on the basis that the *Steel*$^{-/-}$ embryos completely lacked this diffuse staining (Fig. 1I). Whole-mount staining of E8.5 embryos showed that Steel protein was strongly expressed in cells of the visceral endoderm, as well as the adjacent hindgut diverticulum containing the PGCs (Fig. 1D). Transverse sections through the hindgut diverticulum at E8.5 showed that PGCs occupied only the ventral aspect of the hindgut epithelium, which was stained strongly for Steel factor, but not the dorsal part, which lacked Steel factor expression (Fig. 1E,F). At E9.0, Steel factor was expressed by all cells of the hindgut, and PGCs now occupied the dorsal, as well as the ventral, hindgut (Fig. 1G). At E10.0, Steel factor staining was reduced or absent in the hindgut epithelium, and was enriched in tissues surrounding the PGCs in the midline dorsal body wall and the coelomic angles (Fig. 1H). As published previously (Runyan et al., 2006), Steel factor expression is subsequently lost in midline structures at E10.75 and becomes restricted bilaterally to the genital ridges. The data presented here, together with the previously published results, show that PGCs are immediately surrounded by cells expressing Steel factor from the time they first appear in the allantois to the time they colonize the genital ridges.

As shown in Fig. 1B,C, cell membrane-localized Steel factor was seen in the allantois at E7.5. To confirm the presence of membrane-bound Steel factor in the allantois, we fixed E7.5 embryos with 2% trichloroacetic acid (TCA), which gave a better cross-reaction with the anti-Steel factor antibody. TCA fixation causes loss of the GFP signal, so PGCs could not be identified. However, a group of cells in the core of the allantois showed strong membrane staining of Steel factor (Fig. 1J, arrow). We also dissected allantoides from E7.5 embryos, and performed RT-PCR using primers that distinguish membrane-bound and soluble Steel factors. The results of the RT-PCR confirmed the presence of both soluble and membrane-bound Steel factor in the allantois at E7.5 (Fig. 1K).

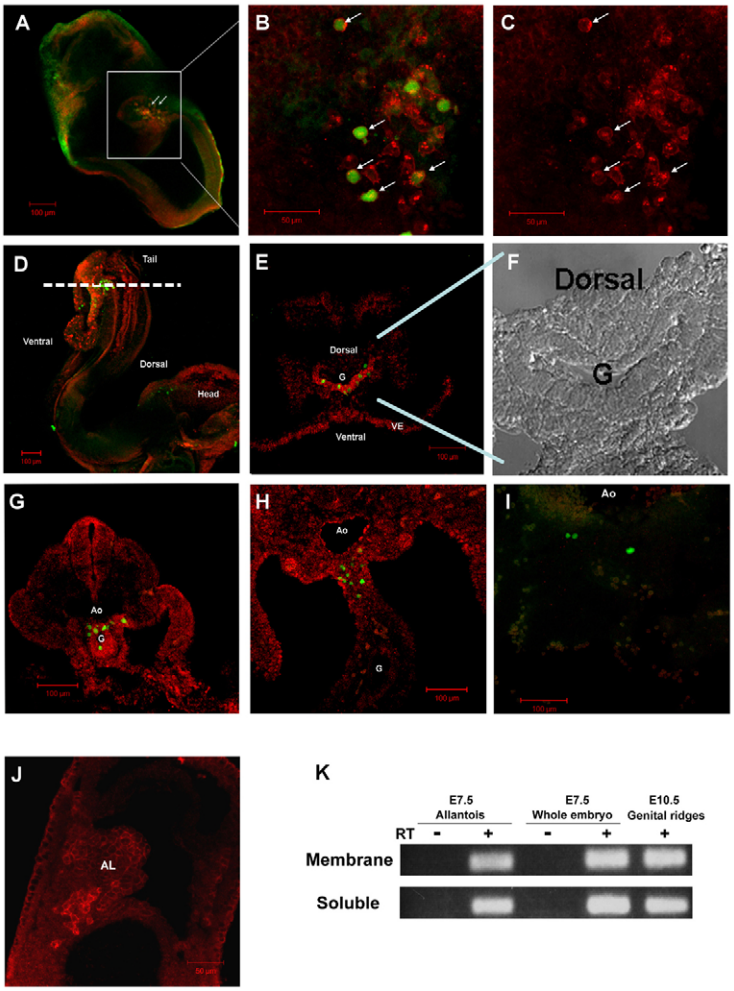

Fig. 1. Expression of Steel factor. (**A**) Whole-mount Steel factor (red) staining of E7.5 *Stella-GFP* embryos; PGCs in the allantois (boxed) are green. Residual Stella expression in other regions of the embryo at this early stage is also green. (**B**) Higher magnification views of whole-mount Steel factor staining (red) of E7.5 embryos. PGCs express the *Stella-GFP* transgene, as well as Steel factor at this stage (arrows in B). Steel factor-expressing cells surround the PGCs. (**C**) Same image as in B, without the green channel, to allow visualization of Steel factor staining in the PGCs (arrows). (**D**) Whole-mount staining of an E8.5 *Stella-GFP* embryo. PGCs are clustered in the hindgut diverticulum, immediately adjacent to Steel factor-expressing cells of the visceral endoderm. (**E**) Transverse section through an E8.5 hindgut diverticulum (shown by the dashed line in D). The ventral aspect of the hindgut is strongly stained for Steel factor, and PGCs are confined to this ventral region of the gut. (**F**) Differential interference contrast image of the region labelled 'Dorsal' and 'G' in E, to show outline of the whole hindgut. (**G**) Transverse section through an E9.0 hindgut. Both the ventral and dorsal hindgut is stained for Steel factor; PGCs have moved to the dorsal region of the gut. (**H**) Transverse section through an E10.0 embryo. Steel staining is lost from the hindgut, but is enriched in the dorsal mesentery and body wall. (**I**) Immunostaining of a section from an E10.5 *Steel*$^{-/-}$ embryo demonstrating the antibody specificity (lack of red signal). (**J**) Whole-mount immunostaining of Steel factor in an E7.5 embryo fixed in 2% TCA, which increases the antibody reaction with Steel factor. (**K**) RT-PCR analysis of membrane-bound and soluble Steel factor. cDNA was prepared from dissected E7.5 allantoides, whole embryo and E10.5 genital ridges. AL, allantois; Ao, aorta; G, hindgut; VE, visceral endoderm. Scale bars: 100 μm in A,D,E,G-I; 50 μm in B,C,J.

PGCs first express *Stella* in the extraembryonic allantois, and migrate proximally into the posterior epiblast

It has been reported that PGCs are specified in the allantois around E7.25 (Hayashi et al., 2007; Ohinata et al., 2005), but little is known about the behavior of PGCs after their specification. To study PGC behavior before they colonize the hindgut, we developed a method of culturing bisected *Stella-GFP* embryos at E7.25 and E7.5, and made time-lapse movies during these time periods. Fig. 2A shows a sequence of frames from one movie started at E7.25 (see also Movie 1 in the supplementary material). During the time period of this movie (2.5 hours), PGCs turned on *Stella* expression in the extraembryonic mesenchyme of the allantois, and migrated proximally towards the embryo (Fig. 2A). Seven out of the eight movies started at E7.25 showed PGC migration in this direction. Fig. 2B shows a movie started at E7.5 (Movie 2 in the supplementary material). More PGCs were seen at the beginning of movies started at this stage. During the culture period, PGCs moved from the allantois (AL) into the posterior epiblast (EP) of the embryo. They came to occupy a region that included the proximal epiblast and proximal allantois. Once in this region, they migrated in random directions within it. PGCs that migrated to the edges of the region turned back again, indicating a mechanism for retention of the PGCs in this posterior region of the embryo. In eight out of 10 movies started at E7.5, we observed the PGC behavior shown in Fig. 2B.

Steel factor signaling controls PGC numbers before entry into the hindgut

To test for Steel factor function in the allantois, we bred the *Steel* null mutation ($Kitl^{Sl}$) into the *Stella-GFP* mouse line and counted PGC numbers in bisected E7.5 embryos under confocal microscope. $Steel^{-/-}$ embryos had a statistically significant reduction in PGC number (15±4.3 per embryo) compared with $Steel^{+/-}$ (27±5.9 per embryo, P=0.028) and $Steel^{+/+}$ (36±8.7 per embryo, P=0.016) embryos (Fig. 3A). By contrast, the small difference in PGC numbers between $Steel^{+/-}$ and $Steel^{+/+}$ embryos was not statistically significant (P=0.09). These results indicate that as early as E7.5, before PGCs colonize the hindgut, their numbers are dependent on Steel factor. Many previous observations have demonstrated that Steel factor is required for PGC proliferation (Bennett, 1956; Buehr et al., 1993; Matsui et al., 1991) and survival (Godin et al., 1991; Dolci et al., 1991; Matsui et al., 1991; De Felici et al., 1999; Stallock et al., 2003). To distinguish between effects on proliferation and apoptosis of PGCs in the absence of Steel factor, whole-mount embryos at E7.5 were stained using the phospho-histone H3 antibody to detect mitosis and the cleaved-PARP antibody to detect apoptosis. We observed that a few PGCs were positively stained for phospho-histone H3 or cleaved-PARP in the embryos (data not shown). However, the low PGC numbers present at this stage made it difficult to perform statistical analysis.

Recent work from our laboratory showed that loss of Steel factor leads to PGC apoptosis beginning on or before E9.0, which can be rescued by removal of the pro-apoptotic protein Bax (Runyan et al., 2006). We therefore examined embryos from *Steel*/*Bax* crosses at E7.5. In five $Bax^{+/-}$, $Steel^{-/-}$ embryos examined, loss of one allele of *Bax* rescued the decrease of PGC number in $Steel^{-/-}$ embryos at E7.5 (Fig. 3A). In $Steel^{-/-}$ embryos, PGC numbers increased from 15±4.3 to 29±9.3 (P=0.041) in the absence of one allele of *Bax*. Sample embryos from which these counts were made are shown in Fig. 3B. These data show that Steel factor is an essential survival factor for PGCs even before they enter the hindgut, and that PGCs die through the Bax-dependent apoptotic pathway at E7.5 when they lack Steel factor. The data also exclude the possibility that Steel factor is required for initial PGC specification, as the number of PGCs increased in Steel-null embryos when apoptosis was inhibited.

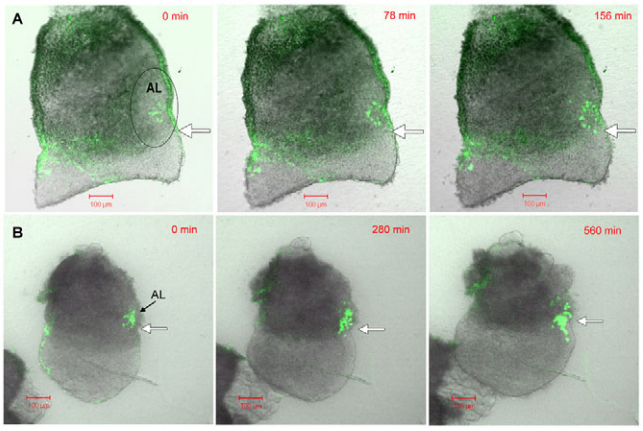

Fig. 2. Time-lapse frames of E7.25 and E7.5 Stella-GFP embryos. (**A**) Three frames at t=0, t=78 minutes and t=156 minutes of a movie started at E7.25. At t=0, PGCs, detected by the expression of *Stella* (green), are visible in the allantois (AL). Residual expression of *Stella* is also seen elsewhere in the embryo. PGCs start to spread downwards towards the epiblast. Arrow indicates approximate boundary between the allantois and the proximal epiblast. (**B**) Three frames at t=0, t=280 minutes and t=560 minutes of a movie started at E7.5. PGCs move from the allantois into the posterior epiblast of the embryo during the movie time period. Arrow indicates approximate boundary between the allantois and the proximal epiblast. The PGC population expands into proximal epiblast. Scale bars: 100 μm.

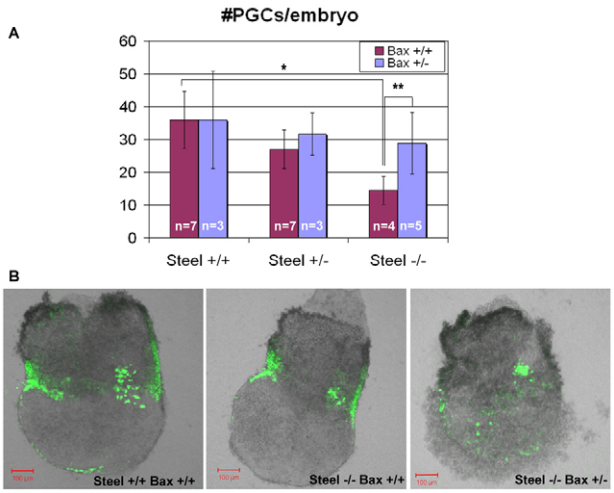

Fig. 3. Effects of Steel factor on PGC numbers at E7.5. (**A**) PGC numbers at E7.5 were significantly reduced in $Steel^{-/-}$ embryos compared with in $Steel^{+/+}$ and $Steel^{+/-}$ littermates (red bars). PGC numbers were significantly rescued in $Steel^{-/-}$ embryos by the loss of a single allele of *Bax* (blue bars). n indicates the number of embryos used for quantitation. * and **, P<0.05. Bars represent the mean±s.e.m. (**B**) Examples of embryos of each genotype used to generate the PGC counts. Scale bars: 100 μm.

Steel factor is required for normal PGC migration before entry into the hindgut

Time-lapse movies were made using sagittally bisected E7.5 *Steel* null embryos and their littermates. Examples of movie frames from embryos of different genotypes are shown at time=0 and time=413 minutes (Fig. 4A,B; from Movies 3-5, see supplementary material). We manually traced PGCs in time-lapse movies, obtained trajectories of all the PGCs whose migratory routes remained in the confocal plane throughout the movie (Fig. 4C,D), and calculated the velocities and displacements of PGC movement (Fig. 4E). PGCs in $Steel^{+/+}$ embryos were highly motile, with a maximum velocity of 51.4±3.1 μm/hour, an average velocity of 24.7±1.2 μm/hour and an average total displacement during the time of filming of 83.1±8.9 μm. The maximum velocity (50.1±3.3 μm/hour), average velocity (23.6±1.3 μm/hour) and displacement (78.9±10.8 μm) of PGCs in $Steel^{+/-}$ embryos were not significantly changed from those in $Steel^{+/+}$ embryos (P=0.58, 0.23 and 0.57, respectively). However, PGCs in $Steel^{-/-}$ embryos had significantly decreased maximum velocities (37.4±2.9 μm/hour), average velocities (17.5±0.8 μm/hour) and displacements (44.9±7.7 μm) compared with PGCs in $Steel^{+/+}$ (P=1.0×10^{-6}, 3.8×10^{-10} and 1.2×10^{-7}, respectively) and $Steel^{+/-}$ littermates (P=1.8×10^{-7}, 7.4×10^{-12} and 2.2×10^{-6}, respectively). These data indicate that Steel factor is necessary for active PGC motility before entry into the hindgut.

To test the directionality of PGC migration, we placed the starting point of each PGC path on the same point (Fig. 4D) to generate a windrose diagram for the average trajectories of PGCs in embryos of each genotype (Fig. 4F). The directionality was altered, but not randomized, by the loss of Steel factor. In all embryos, PGCs migrated along a similar trajectory from the distal end of the allantois into the posterior primitive streak region of the embryo, as shown by the arrow in Fig. 4F. These data show that Steel factor is required for PGC motility, but not directionality, in the allantois.

One result of decreased PGC motility was that fewer PGCs reached the posterior epiblast. To show this, the positions of individual PGCs at the end of the movies were scored. PGCs that had migrated across the boundary (white line, Fig. 4C) between the extraembryonic region (EEM) and the posterior end of the embryo (PEM) were scored as 'enter embryo'. Only 18.8±12.3% of PGCs entered the posterior region of the embryo in the absence of Steel

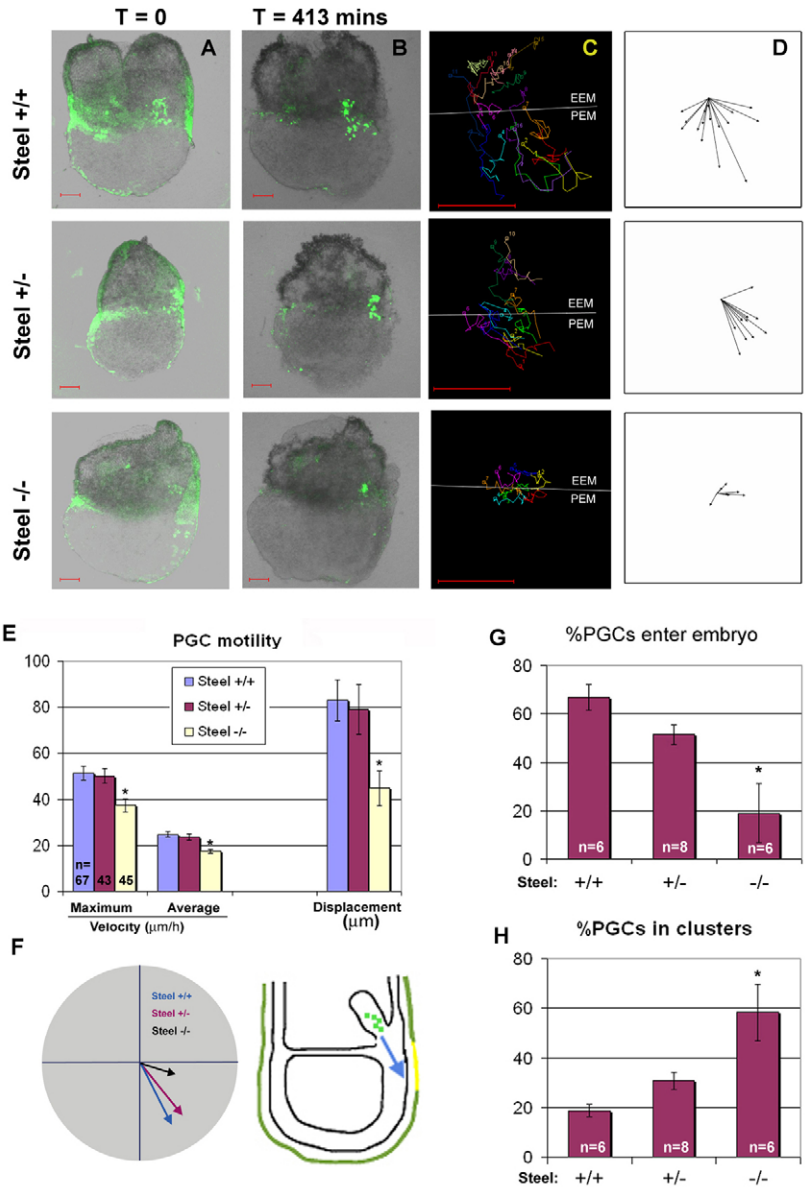

Fig. 4. Effects of Steel factor on PGC migration at E7.5 (**A**,**B**) Frames at t=0 (A) and t=413 minutes (B) from movies of E7.5 *Stella-GFP* embryos with different *Steel* genotypes. PGCs are green. (**C**) The tracks of all PGCs that remained in the plane of the confocal image throughout the movies. The boundary between the extraembryonic tissues (EEM; in the posterior region of the allantois) and the posterior end of the embryo (PEM) is marked by a white line. (**D**) The overall directions of movement of individual PGCs, made by placing the starting point of each PGC track onto the same point. Scale bars in A-C: 100 μm. (**E**) The maximum velocity, average velocity, and displacement of E7.5 PGCs with different *Steel* genotypes. Both the velocities and the displacements are significantly decreased in the absence of Steel factor. n, number of PGCs used for quantitation. Units on the y-axis vary based upon the parameter, and are indicated below the bar charts. (**F**) Windrose diagram shows that the directionality of PGC migration is altered in $Steel^{-/-}$ embryos, but not randomized. The accompanying diagram shows the trajectory (blue arrow) in the vectorial diagram that allows PGCs to leave the allantois and enter the posterior embryo and the hindgut endoderm (yellow). (**G**) The percentage of PGCs that enter the posterior of the embryo within the time frame of the movies (413 minutes), starting at E7.5, is significantly reduced in *Steel*-null embryos. (**H**) The percentage of PGCs that form clusters is markedly higher in the absence of Steel factor. n, the number of embryos used for quantitation for G and H. *P<0.01; bars represent the mean±s.e.m.

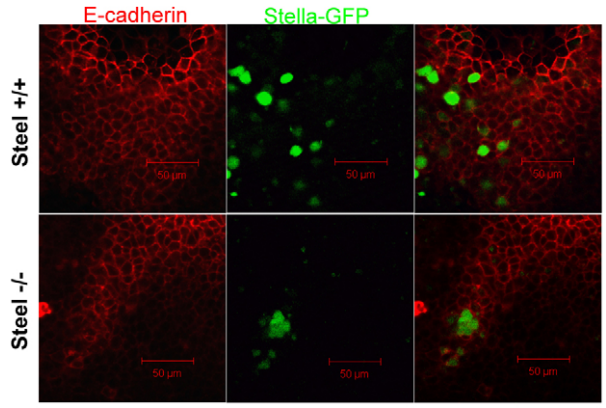

Fig. 5. PGC clumping in *Steel* mutant embryos is not due to upregulation of E-cadherin. (Top) E-cadherin (red) and PGCs (green) in *Steel*$^{+/+}$ E7.5 embryos, individually and combined. (Bottom) The same staining but for *Steel*$^{-/-}$ embryos. No difference in expression of E-cadherin was observed in different *Steel* genotypes. Scale bars: 50 μm.

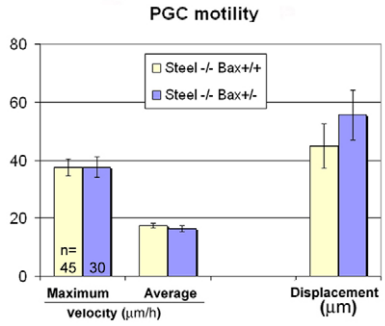

Fig. 6. Loss of an allele of *Bax* does not rescue PGC motility. Velocities and displacements of PGCs in movies from *Steel*$^{+/+}$, *Steel*$^{+/-}$ and *Steel*$^{-/-}$ embryos with and without loss of one *Bax* allele were assayed as in Fig. 4. No statistically significant differences in PGC motility were seen in *Steel*$^{-/-}$ embryos when one allele of *Bax* was lost. *n*, number of PGCs used for quantitation. Units on the *y*-axis vary based upon parameter, and are indicated below the bar charts.

factor (Fig. 4G), which was a significant reduction when compared with *Steel*$^{+/+}$ (66.8±5.4%, $P=3.7\times10^{-5}$) and heterozygous (51.4±4.0%, $P=0.0001$) littermates. This result confirmed the hypothesis that Steel factor is required for normal PGC migration before they enter into the hindgut.

Interestingly, PGCs in *Steel*$^{-/-}$ embryos also failed to move away from each other, and instead formed clusters in the proximal region of allantois (Fig. 4A,B). To quantify this, clusters were defined as clumps containing more than three PGCs, and these were scored in the end-point frames from E7.5 movies of each genotype (Fig. 4H). Nearly 58.3% of PGCs in *Steel*$^{-/-}$ embryos were in clusters compared with 18.7% in *Steel*$^{+/+}$ embryos ($P=6.4\times10^{-5}$). Loss of one allele of *Steel* also increased the number of clustered PGCs compared with *Steel*$^{+/+}$ littermates (30.8%, $P=0.0003$). The adhesion glycoprotein E-cadherin has been previously reported to be expressed by PGCs (Okamura et al., 2003; Bendel-Stenzel et al., 2000). To test whether PGC clumping is due to precocious expression of E-cadherin, we stained wild-type and *Steel*$^{-/-}$ embryos at E7.5 as whole mounts with the ECCD2 antibody against E-cadherin (Shirayoshi et al., 1986). E-cadherin was not upregulated by PGCs in Steel$^{-/-}$ embryos at this stage (Fig. 5A). It is possible that the expression of other adhesion molecules is responsible for clumping and for the failed motility. However, other adhesion molecules have not yet been characterized in early migrating germ cells.

To exclude the possibility that the failure of PGC motility was due to apoptosis, we analyzed maximum and average velocities, and total displacements of PGCs in E7.5 *Steel*$^{-/-}$, *Bax*$^{+/-}$ embryos. The loss of one allele of Bax rescues germ cell numbers (Fig. 3), but does not rescue germ cell motility (Fig. 6).

To exclude the possibility that the defects of PGC migration observed in *Steel*$^{-/-}$ embryos were consequences of a previous requirement for Steel factor before migration in the allantois, we carried out an 'acute' blockade to Steel factor signaling by culturing bisected E7.5 *Steel*$^{+/+}$ embryos in the presence of Ack2 antibody, which has been shown to effectively block Steel signaling and Steel factor function in PGCs (Nishikawa et al., 1991; Runyan et al., 2006; Stallock et al., 2003). As shown in Fig. 7, both the velocity and displacement of PGCs were significantly decreased by treatment with 10 μg/ml Ack2 for 6 hours when compared with those in control embryos ($P<0.01$; Fig. 7A). In addition, the proportion of PGCs entering the posterior embryo was significantly decreased by Ack2 treatment (25.5±9.2% compared with 53.4±6.9%, $P=0.003$; Fig. 7B). PGCs also clumped more in the presence of Ack2 (42.3±7.5% of PGCs in clusters) than did those in control embryos (15.3±5.6%, $P=0.002$; Fig. 7C). Movies of E7.5 Ack2-treated embryos and control embryos incubated in the same concentration of a non-immune IgG, which did not affect PGC behavior can be found in the supplementary material (see Movies 6 and 7). Treatment with the Ack2 antibody did not alter PGC numbers, probably because of the short time-period (6 hours) of the experiment. The results of this acute blockade of Steel factor signaling confirm that it is required for PGC migration at E7.5, and that the defects seen at this stage in *Steel*$^{-/-}$ embryos were not due to a previous requirement for Steel factor (for example, in PGC specification).

Steel factor is required for PGC motility in the hindgut

We reported previously that the rescue of PGC apoptosis in *Steel*$^{-/-}$ embryos by the removal of Bax revealed roles for Steel factor in both proliferation and migration between E9.0 and E10.5 (Runyan et al., 2006). The defects in later migration in *Steel/Bax* mutant embryos could have been due to either a failure of motility or directionality. In this study, we have shown that at an earlier stage, before colonization of the hindgut, Steel factor is required for motility but not directionality of the PGCs (Fig. 4). To test whether the same is true once PGCs have colonized the hindgut, time-lapse movies were made using E9.0 *Oct4ΔPE:GFP* embryos from *Steel/Bax* crosses. No differences in migration were observed between *Bax*$^{-/-}$ and *Bax*$^{+/-}$ PGCs, so the data from these two groups were combined and the embryos were grouped based upon their Steel genotype. Frames from the time-lapse movies are shown at time=0 (Fig. 8A) and time=420 minutes (Fig. 8B); the migration directions of individual PGCs were plotted using the method described above (Fig. 8C). Fig. 8D shows that PGCs in *Steel*$^{-/-}$ embryos had significantly decreased displacement (22.6±5.22 μm) compared with those in Steel$^{+/+}$ (56.8±5.8 μm, $P=6.93\times10^{-8}$) and Steel$^{+/-}$ (65.1±8.1 μm, $P=1.89\times10^{-9}$) littermates. They also showed a decreased maximum velocity (11.8±1.4 μm/hour), compared with Steel$^{+/+}$ (22.2±1.4 μm/hour, $P=2.49\times10^{-10}$) and Steel$^{+/-}$ (20.9±1.7 μm/hour, $P=1.00\times10^{-9}$),

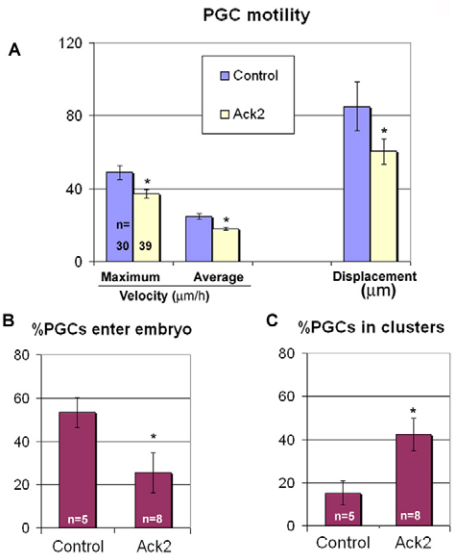

Fig. 7. Effects of Ack2 blocking antibody on PGC motility at E7.5. (**A**) The maximum velocity, average velocity, and displacement of E7.5 PGCs were significantly decreased by 10 μg/ml Ack2 treatment for 6 hours. *n*, the number of PGCs used for quantitation. Units on the *y*-axis vary based upon parameter, and are indicated below the bar charts. (**B**) The percentage of PGCs leaving the allantois and entering the posterior embryo within the timescale of movies (6 hours) started at E7.5 was significantly lower following treatment with 10 μg/ml Ack2. (**C**) PGCs clump more in the presence of 10 μg/ml Ack2 at E7.5. *n*, indicates the number of embryos used for quantitation for B,C. **P*<0.01; bars represent the mean±s.e.m.

and a decreased average velocity (5.0±0.5 μm/hour) compared with Steel$^{+/+}$ (9.6±0.6 μm/hour, $P=3.48\times10^{-12}$) and Steel$^{+/-}$ (10.1±0.8 μm/hour, $P=1.24\times10^{-12}$). Windrose assays (Fig. 8E) showed that all of the PGCs migrated in a net dorsal and slightly rostral direction (right panel, Fig. 8E), regardless of the *Steel* gene dosage. These results suggest that Steel factor plays an essential role in PGC motility, both in the allantois and in the hindgut, in addition to its role in PGC survival.

DISCUSSION

Steel factor has been known for many years to be a necessary survival signal for PGCs (Dolci et al., 1991; Godin et al., 1991; Matsui et al., 1991). Early studies reported that PGC numbers in Steel mutants are similar to those in wild-type embryos during the period E8.0 to E9.0, suggesting that PGCs are independent of Steel factor until colonization of the hindgut (Bennett, 1956; Mahakali Zama et al., 2005; McCoshen and McCallion, 1975; Mintz and Russell, 1957). However, recently published data suggest the need for a reappraisal of this. First, PGC numbers are already dramatically reduced at E9.0 in *Steel*-mutated embryos (Runyan et al., 2006). Second, Steel expression in the allantois, and c-Kit expression by PGCs, have recently been found to commence at E7.25, the time of PGC appearance (Yabuta et al., 2006). In this paper, we have shown that PGCs are surrounded by Steel factor-expressing cells from the time that they first express *Stella*, and that normal PGC behavior is controlled by Steel factor from the time of their first appearance in the embryo. Together with previous work, this shows that PGCs require Steel factor throughout their migration, from the time of formation to the time of colonization of the gonads.

In a previous paper, we showed that PGCs are surrounded by Steel factor at E9.5, when emigrating from the hindgut, and at E10.5, when in the genital ridges (Runyan et al., 2006). Here, we show that Steel factor expression surrounds PGCs at all earlier stages. First, in the allantois, where *Stella* expression is first activated in PGCs, Steel factor is expressed in a group of cells in the mesodermal core. PGCs also initially express Steel factor at this time, consistent with RT-PCR results from a previous study (Yabuta et al., 2006). By E8.5, PGCs occupy the ventral aspect of the hindgut epithelium, which also stains strongly for Steel factor. At E9.0, both the ventral and the dorsal gut epithelium express Steel factor, and as described before (Molyneaux et al., 2001), PGCs have now moved dorsally in the hindgut. At E10.0, when PGCs are concentrated in the dorsal midline, Steel factor is enriched in the midline, but has been lost from the hindgut. These data show that migratory PGCs are in a Steel factor-enriched microenvironment from the time of their first appearance to the time they colonize the gonads.

There are two forms of Steel protein, generated by alternative splicing of Steel precursor RNA. The transmembrane form lacks an extracellular domain containing a proteolytic cleavage site, which normally causes release of the extracellular region of the protein (Flanagan et al., 1991; Huang et al., 1992). The Steel-dickie (*Steel$^{d/d}$*) mutation, in which only soluble Steel factor is made, is sterile (Brannan et al., 1991), suggesting that the membrane-bound form is essential at some stage of PGC differentiation. Moreover, PGC numbers are already significantly reduced in *Steel$^{d/d}$* embryos at E9.5 (Mahakali Zama et al., 2005), indicating a requirement for membrane-bound Steel factor earlier than this. In our study, the staining pattern of Steel factor and the RT-PCR analyses at E7.5 show that PGCs become surrounded by cells expressing membrane-bound Steel factor when they are in the allantois, suggesting that PGC behaviors may be dependent on this close-range signaling as they first form in the embryo. It is possible that the membrane-bound Steel protein controls different aspects of PGC behaviors from the soluble Steel factor. It is also possible that the higher local concentration of membrane-bound Steel makes it indispensable for PGC development. Future work will focus on trying to distinguish between these possibilities.

In the *Steel$^{-/-}$* embryos examined, PGC numbers were already reduced (~40% of control numbers) at E7.5, suggesting that PGC numbers are controlled by Steel factor as soon as they appear in the embryo. This is almost certainly a direct interaction, as only PGCs express c-Kit in the allantois at this stage (Yabuta et al., 2006). The reduction of PGC numbers in the absence of Steel factor could be caused in three ways: increased apoptosis, decreased proliferation, or defects in PGC specification. The low PGC numbers at this stage make statistical analysis of cleaved-PARP staining (cell death) or phospho-histone H3 staining (mitosis) extremely difficult. However, in the five embryos examined so far that were *Steel$^{-/-}$* and *Bax$^{+/-}$*, PGC numbers were dramatically increased by the removal of one allele of *Bax*, implying that Steel factor is not required for PGC specification, but is required for their survival. This result does not exclude the possibility that PGCs also require Steel factor for their proliferation at this stage. Further investigations will be performed in a *Bax*-null background to study the role of Steel factor on PGC proliferation.

Steel factor has been considered an essential factor for PGC survival and proliferation in previous studies. However, the precise role played by Steel factor in PGC migration has not been clear. Here, we show that germ cells are actively migratory before hindgut colonization, and that Steel factor is required for their motility, but not their directionality, at this stage. Tracing individual PGCs on

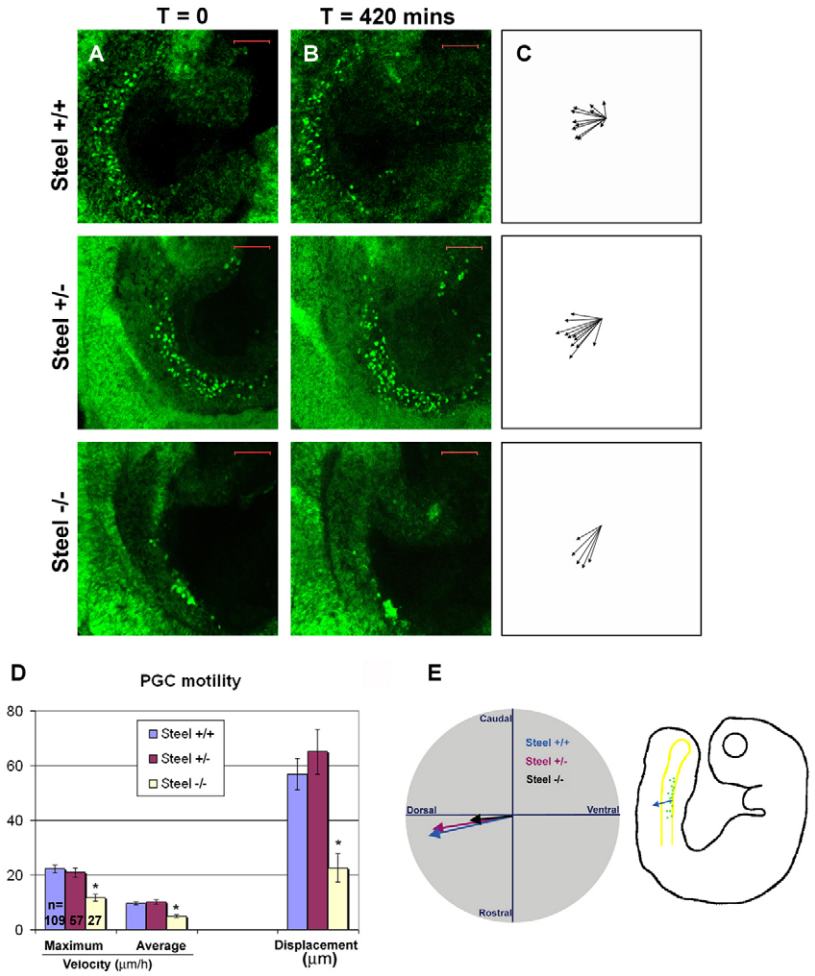

Fig. 8. Effects of Steel factor on PGC migration at E9.0. (**A**,**B**) Movie frames of E9.0 *Oct4ΔPE:GFP* embryos with different *Steel* genotypes are shown at (A) *t*=0 and (B) *t*=420 minutes. PGCs are green. Scale bars: 100 μm. (**C**) The direction of movement of individual PGCs during the movie period. (**D**) Both the velocities and the displacements of PGCs are significantly decreased in *Steel*$^{-/-}$ embryos. *n*, the number of PGCs used for quantitation. Units on *y*-axis vary based upon parameter, and are indicated below charts. **P*<0.01; bars represent the mean±s.e.m. (**F**) Windrose diagram shows the directionality of PGC migration. As the blue arrow shows in the accompanying diagram, all PGCs migrated in a net dorsal and slightly rostral direction in the hindgut (yellow), regardless of Steel gene dosage.

movie frames starting at E7.5 revealed that both the velocities and the displacements of PGCs were significantly decreased in *Steel*-null embryos. PGCs also failed to move away from each other in *Steel*$^{-/-}$ embryos, and instead formed clusters in the proximal region of the extraembryonic allantois. It is not clear why PGCs should adhere to each other without Steel factor. It may be that germ cells become specified as a group, and decreased motility causes them to fail to move away from the group. Alternatively, Steel factor may inhibit the expression of adhesion proteins. Acute blockade of Steel factor signaling by culturing E7.5 bisected embryos in the presence of Ack2 antibody confirmed the effects of Steel factor on PGC motility at this stage, demonstrating that the motility defects of PGCs are not consequences of a previous requirement for Steel factor.

The result of lower PGC motility in *Steel*$^{-/-}$ embryos is that fewer PGCs leave the allantois and enter the posterior region of the embryo. This explains the reduced PGC numbers seen in the hindgut at E9.0, when there are fewer than 25% of the wild-type numbers of PGCs in the hindgut in *Steel*-null embryos (Runyan et al., 2006). Because of these small numbers, the *Bax*-null mutation was bred into *Steel* mutants in order to reduce PGC apoptosis and generate enough PGCs for motility analyses. The defects in PGC migration in the hindgut in the absence of Steel factor were the same as those found in the allantois earlier. Although PGCs in all genotypes migrated in a net dorsal and slightly rostral direction in the hindgut at E9.0, both the velocities and the displacements of PGCs were significantly decreased in the absence of Steel factor. These data may explain why clumps of PGCs are found in structures ventral to the gut in both *Steel* and *W* mutant embryos after E9.0 (Buehr et al., 1993; Mahakali Zama et al., 2005; Runyan et al., 2006). Previously published data show that Steel factor is also required for later stages of PGC migration. In E10.5 *Steel/Bax* double-null embryos, germ cells were not found in their normal site, the dorsal body wall, but instead were found clumped around the hindgut (Runyan et al., 2006).

In vitro, using modified Boyden chambers, it has been reported that Steel factor has a chemotactic function (Farini et al., 2007). However, the in vivo experiments described here at two stages, before and after colonization of the hindgut, show that Steel factor is essential for PGC motility, but that the direction of movement is not randomized in the absence of Steel factor. This discrepancy could be due to other guidance cues being available in the absence of Steel factor, or to a requirement for Steel factor for guidance later in germ cell migration, which was not tested here. At E7.5, during migration from the allantois to the posterior epiblast, the directionality of PGCs in *Steel*-null embryos is altered a little. This could be because the germ cells migrate more slowly in these embryos, and are therefore affected more by other morphogenetic movements taking place at this time, although this possibility could not be tested.

An essential feature of PGC specification is the turning off of genes that initiate somatic cell fate, including *Evx1*, Hox genes and brachyury, and the maintenance (or switching on) of genes that maintain pluripotency in the germ cell lineage, including *Stella*, *fragilis* (*Ifitm3* – Mouse Genome Informatics), *Oct4*, *Sox2*, *Nanos3*

and *Nanog* (reviewed by Saitou et al., 2005). This process takes place in the allantois, and both the sources and the natures of the signals that control it are poorly understood. In well-characterized stem cell populations, such as male and female stem cells in the gonad, epidermal stem cells and hemopoietic stem cells, signals that control pluripotency and behavior are provided by the 'niche' in which the stem cells reside. However, germ cells are migratory and have no obvious niche, although the maintenance of all aspects of their behavior, including proliferation rate, migration and survival, are all continuously controlled (Kunwar et al., 2006; Runyan et al., 2006; Surani et al., 2007). Our results, together with previously published data, show that PGCs are surrounded by cells releasing Steel factor throughout their migration, from the time they first form in the allantois to the time they colonize the gonads, and that normal PGC behaviors, including survival, proliferation and motility, are all controlled by this close-range signaling. This suggests the existence of a 'traveling niche' in which the Steel factor-expressing cells provide a spatio-temporal environment along the migratory route to retain the normal properties of PGCs as they occupy different regions of the embryo. How this Steel factor niche is established and maintained, and whether the Steel factor-expressing cells also release other signaling ligands that control PGC behavior, are interesting questions to answer in future studies.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/8/1295/DC1

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