University of Cincinnati

Date: 10/17/2011

I, Ying Gu , hereby submit this original work as part of the requirements for the degree of Doctor of Philosophy in Developmental Biology.

It is entitled:

A Traveling Niche: The Role of Steel Factor in Mouse Primordial Germ Cell Development

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2108

A Traveling Niche: The Role of Steel Factor in Mouse Primordial Germ Cell Development

A dissertation submitted to the

Graduate School

Division of Research and Advanced Studies

Of the University of Cincinnati

In partial fulfillment of the requirements for the degree of

DOCTORATE OF PHILOSOPHY (Ph.D.)

In the Graduate Program of Molecular and Developmental Biology

of the College of Medicine

2011

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Abstract

Primordial germ cells (PGCs) are the embryonic precursors of adult gametes. In the mouse, they arise around E7.5 in the allantois, and migrate through the developing hindgut and midline dorsal body wall mesenchyme to colonize the gonad primordia by E11.5. PGC behavior, including proliferation, survival, and motility, is controlled by cellular signaling during migration. Steel factor is known as an essential survival factor for PGCs. It is the protein product of the *Steel* locus, and the ligand for the receptor tyrosine kinase c-kit, which is expressed by PGCs throughout migration. Steel factor exists in two forms, membrane-bound and the soluble, generated by alternative splicing. This thesis addresses two general questions:

1) Is PGC behavior controlled by Steel factor from the beginning of their migration?
2) Do the two different forms of Steel factor control different aspects of PGC behavior?

Using the mouse reporter line *Stella-GFP,* in which PGCs express GFP under the control of the promoter of *Stella* gene, I demonstrate that PGC number is significantly reduced in *Steel*$^{-/-}$ embryos at E7.5. Similarly, in the absence of Steel factor, either by null mutation or antibody blockade, PGCs aggregate together and show dramatically decreased motility, but their directionality is maintained. These data indicate an essential role for Steel factor in PGC survival and motility. I then show that Steel factor-expressing cells surround PGCs from the time of their initial specification in the

allantois, to the time of their colonization of the gonad primordia, providing a "spatio-temporal niche" that travels with PGCs to regulate their survival, proliferation and motility throughout migration. Further, I show that these functions of Steel factor in PGC behavior are distributed between the membrane-bound and soluble forms by analyzing PGC behavior in *Steel-dickie* mutant embryos, which make only the soluble form. Soluble Steel factor alone is sufficient for PGC survival at E7.5. However, PGCs in E7.5 *Steel*$^{d/d}$ embryos aggregate and migrate at much slower rate, indicating that membrane-bound Steel factor is required for PGC motility. Addition of excess soluble Steel factor to *Steel*$^{d/d}$ embryos rescued PGC motility, suggesting that the membrane-bound form provides a higher local concentration of Steel factor that controls the balance between germ cell motility and aggregation. Taken together, this thesis demonstrates that the two forms of Steel factor control different aspects of germ cell behavior, and the membrane-bound Steel factor, by providing higher local ligand concentration, defines a "motility zone" for PGCs. This ensures that PGCs in appropriate locations maintain survival and motility, but ectopic PGCs cease motility and then die by apoptosis.

Acknowledgment

First of all, I would like to give my special thanks to my mentor, Dr. Christopher Wylie, for his continuous support of my PhD study and research. I feel very privileged and lucky to be trained under his supervision. With his inspiration, enthusiasm, and great patience, he helped me grow everyday as a researcher. I could not have come this far without his encouragement and guidance. I would also like to express my thanks to Dr. Janet Heasman for her valuable advice and support on my project.

I am also very grateful to all former and present lab members: Chris Runyan, Amanda Shoemaker, Matt Kofron, Chitra Dahia and Sang-wook Cha, for their great ideas and advice to make my experiments a success; Sumeda Nandadasa and Sha Wang for their company as my fellow graduate students; and all other members for making my graduate life so colorful and unforgettable.

I would like to express my sincere gratitude to my thesis committee members, Dr. Kenneth Campbell, Dr. Iain Cartwright, Dr. James Wells and Dr. Aaron Zorn for their encouragement, insightful comments and suggestions. I would also like to thank people in the MDB graduate program for their support on my graduate training.

Finally, I am deeply indebted to my parents for the encouragement, love and care they have shown to me. And I am most grateful to my husband Liang Chen, for his love, patience and support during these years.

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Chapter I

Introduction

Formation of Mouse Primordial Germ Cells

Primordial germ cells (PGCs) are the embryonic cells that give rise to adult gametes. In many species, PGCs are specified during gastrulation, and migrate through different tissues, while the embryo is undergoing organogenesis, to colonize the developing gonads, where they differentiate into either sperm or egg. In organisms such as *C.elegans*, *Drosophila* and *Xenopus*, PGCs are induced in an "inheritance" mode by the determinants in the "germ plasm". In mouse, no such germ plasm is present in the embryo. Instead, the signals from the extraembryonic tissues are responsible for the induction of the germ line in the early embryo during gastrulation (Lawson et al., 1999; Tremblay et al., 2001; Ying et al., 2000; Ying and Zhao, 2001). The bone morphogenetic protein 4 (BMP4) is expressed in the extraembyonic ectoderm immediately adjacent to the epiblast around E5.5-E6.0, and this ligand is shown to be essential for the generation of PGCs, given the fact that the *BMP4*$^{-/-}$ embryos lack PGC precursors, and therefore, contain no PGCs at later stages (Lawson et al., 1999; Ohinata et al., 2005). BMP2 and BMP8b, which are detected in the extraembryonic ectoderm and posterior mesoderm, respectively, are also indispensable for the formation of PGC precursors (Ying et al., 2000; Ying and Zhao, 2001). Consistent with these findings, embryos that are homozygous null for BMP downstream transducers, *smad1* and *smad5*, display severe reduction in PGC numbers (Tremblay et al., 2001). Upon the induction of the BMP signaling, the proximal epiblast cells begin to express *fragilis/Ifitm3,* an interferon-inducible transmembrane (IFITM) protein (Saitou et al., 2002). These *fragilis*-positive cells then move to the extraembryonic mesoderm and form a cluster of cells at the posterior end. At E6.25, only six cells within the *fragilis*-

positive cells start to express *Blimp1*, the marker for the lineage-restricted PGC precursors (Ohinata et al., 2005). Blimp1 is a potent transcriptional regulator which functions dominantly by repressing the expression of many somatic cell genes, such as *Hoxa1*, *Hoxb1*, *Lim1*, *Evx1*, *Fgf8* and *Snail* (Ancelin et al., 2006). Coupled with the down-regulation of somatic genes, there is also reacquisition of potential pluripotency and activation of PGC-specific genes in the Blimp-1 positive cells, which results in the establishment of the germ line (Kurimoto et al., 2008; Ohinata et al., 2005; Yamaji et al., 2008). Between E6.25 and E7.25, the number of PGC precursors increases from 6 cells to a cluster of around 40 cells at the base of the allantois, by signals which are yet unclear (McLaren and Lawson, 2005). This group of PGC founding cells becomes the initial cluster of the definitive PGCs at E7.25, characterized by their expression of PGC markers, such as tissue non-specific alkaline phosphatase (TNAP) and developmental pluripotency associated 3 (Dpp3 or Stella) (Ginsburg et al., 1990; Hayashi et al., 2007; Ohinata et al., 2005; Saitou et al., 2002).

Migration of Primordial Germ Cells

Immediately following their specification, the nascent PGCs start their migration through different tissues to colonize the gonads (**Figure 1**). Firstly, PGCs at the base of the allantois move proximally into the definitive endoderm, which forms the future hindgut. At E8.5, PGCs are found as a cluster of cells in the hindgut diverticulum, and the majority of them begin to migrate along the hindgut during its anterior extension. Between E9.0 and E9.5, PGCs leave the hindgut from its dorsal side and migrate into the midline structure of the dorsal mesenchyme. Once out of the hindgut, PGCs divide

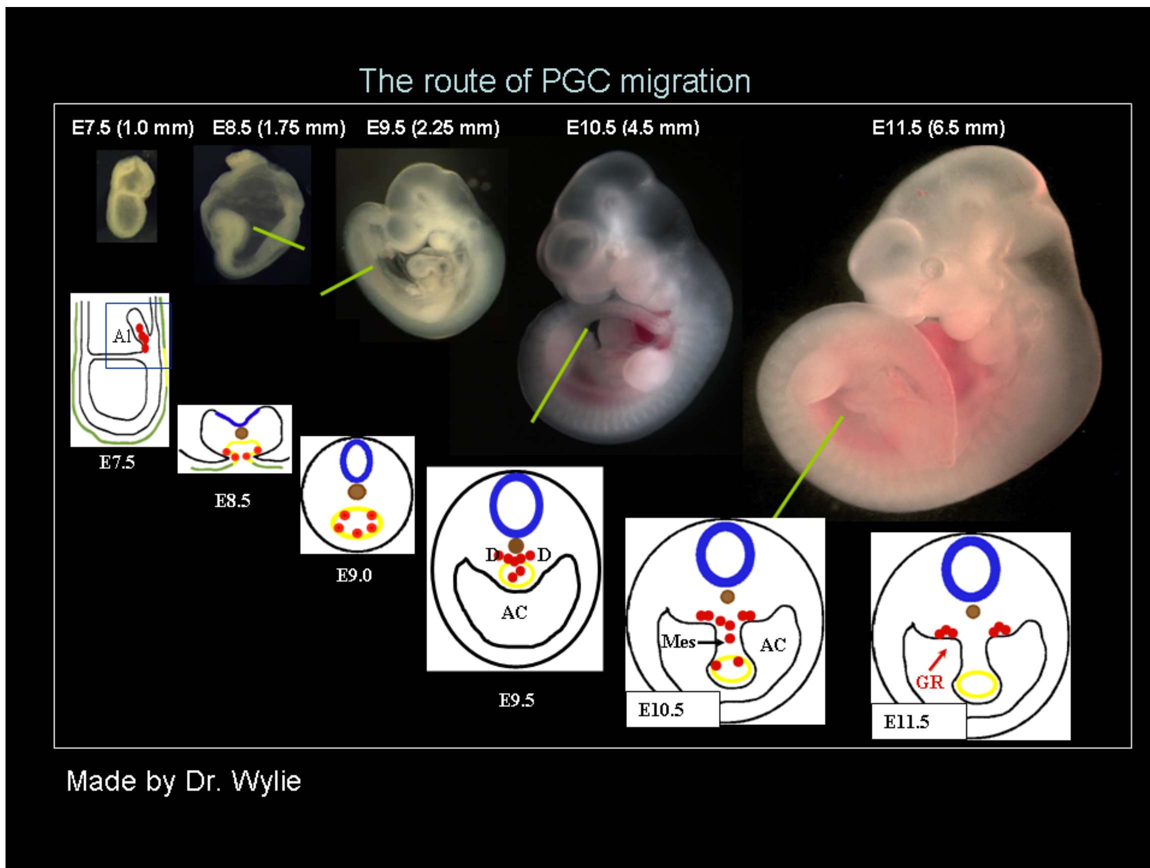

Figure 1: Illustration of the positions of PGCs at different stages of their migration in the mouse embryo.

Photographs of the embryos during this process indicate degree of embryo growth (the pictures are approximately to scale), and organogenesis that occurs during PGC migration. Red=PGCs, Yellow=gut endoderm, Green=Visceral endoderm. AL=allantois, D=Dorsal, AC=abdominal cavity, Mes=mesenchyme, GR=genital ridges. Green lines indicate regions of the transverse sections shown in the diagrams for E8.5 and later.

into left and right streams of individual cells that migrate laterally towards the genital ridges. At E10.5, PGCs close to the genital ridges continue to migrate into the genital ridges to form the primary sex cords, while PGCs remaining in the midline structures die by apoptosis. By E11.5, most germ cells have aggregated into clusters in the genital ridges to form the primary sex cords, which will differentiate later into the seminiferous tubules in the male, or ovarian follicles in the female (Anderson et al., 2000b; Clark and

Eddy, 1975; Molyneaux and Wylie, 2004; Molyneaux et al., 2001; Wylie, 1999). Throughout their journey from the allantois to the genital ridges, PGCs respond to a variety of signals from their somatic neighbors. These signals from surrounding tissues are thought to be essential to regulate PGC behavior along their migratory route.

Regulation of PGC motility and directionality during migration

The initial process of PGC migration was thought to be regulated by IFITM1 through repulsion of PGCs from the mesoderm to the endoderm; however, a more recent study on embryos containing a deletion of the *IFITM* gene family showed no defects in PGC migration, leaving the mechanism of motility initiation an open question (Lange et al., 2008; Tanaka et al., 2005).

In *zebrafish* and mouse, the main molecules that guide the direction of PGC migration are the stromal-derived factor 1 (SDF-1) and its receptor, the G-protein coupled receptor (GPCR) chemokine (CXC motif) receptor 4b (CXCR4b) (Doitsidou et al., 2002). In mouse, SDF-1 is detected along the dorsal body wall mesenchyme and the genital ridges at E10.5; while CXCR4 is expressed in the PGCs at the same time. Removal of either the ligand or the receptor causes a defect in PGC colonization in the genital ridges (Ara et al., 2003; Molyneaux et al., 2003).

Besides its important role in PGC specification, BMP signaling also performs a paracrine function in directing PGC migration towards the genital ridges (Dudley et al., 2010; Dudley et al., 2007). PGC movements are slowed and randomized by the addition of the BMP-antagonist Noggin to embryo slice cultures, resulting in a failure in PGC

genital ridge colonization (rDudley et al., 2007). Conditional knockout of BMP receptor 1a (BMPR1a) in the genital ridges leads to increased somatic cell death, as well as reduced gene expression of Steel factor and SDF-1, suggesting that BMP signaling regulates PGC migration indirectly, possibly through promoting somatic cell survival in the genital ridges (Dudley et al., 2010).

There is recent evidence for a role of cholesterol in PGC motility, showing that inhibition of cholesterol synthesis in a tissue culture system impairs PGC migration, while addition of both cholesterol and isoprenoids rescues the defect. Moreover, cholesterol is enriched in the genital ridges, further supporting its role in PGC migration (Ding et al., 2008).

Molecules mediating cell adhesive interactions also seem to be important for PGC migration. E-cadherin is expressed by PGCs as they emigrate from the hindgut, and disrupting E-cadherin function by a blocking antibody causes defects in PGC aggregation in the genital ridges, and in some cases, results in ectopic PGCs outside of the genital ridges (Bendel-Stenzel et al., 2000). Other adhesion molecules, such as platelet endothelial cell adhesion molecule (PECAM-1) and β-integrin are expressed in migratory PGCs as well, and are required for PGC migration into the genital ridges (Anderson et al., 1999; Wakayama et al., 2003).

Regulation of PGC Survival and Proliferation during migration

The definitive PGCs in mouse start with a cluster of only 40 cells at the base of the allantois at E7.25. With a doubling time of 16 hours, PGC number boosts rapidly to

~100 cells at E8.5, ~1,250 cells at E10.5 and ~ 25,000 cells in the E13.5 gonads, indicating that signals which improve PGC survival and proliferation are imperative for continuous reproductive function and success (Donovan et al., 1986; Mahakali Zama et al., 2005; Tam and Snow, 1981).

Steel factor, by activating its receptor c-kit expressed in PGCs, promotes PGC survival and proliferation both *in vivo* and *in vitro*, in addition to its role to regulate PGC migration in the mouse embryo (see section on **Steel factor in PGC development**) (Dolci et al., 1991; Donovan, 1994; Godin et al., 1991; Mahakali Zama et al., 2005; Matsui et al., 1991b; Mintz and Russell, 1957; Pesce et al., 1993; Runyan et al., 2006).

Many factors that are required for PGC migration also play roles in PGC survival, such as SDF-1 and BMP signaling. PGC counts at different stages in *CXCR4*$^{-/-}$ embryos indicate that the SDF-1/CXCR4 interaction mediate PGC survival while promoting PGC motility (Molyneaux et al., 2003). Addition of BMP or BMP antagonist Noggin to *in vitro* cultured embryo slices results in elevated PGC number or decreased PGC number, respectively, suggesting that BMP signaling also performs multi-functions in PGC development (Dudley et al., 2007).

Fibroblast growth factors (FGFs) are found to be expressed by somatic cells in the mesoderm along PGC migration routes, and to promote PGC survival and proliferation *in vitro* (Kawase et al., 2004; Matsui et al., 1992; Resnick et al., 1992). A recent study from our lab revealed that two FGF receptors, FGFR1-IIIc and FGFR2-IIIb, are expressed by PGCs at E10.5. Addition of FGF-7, the ligand for FGFR2-IIIb, increases PGC number in embryo slices culture, whereas FGF2, the ligand for FGFR1-IIIc, accelerates PGC motility. Moreover, PGC number is dramatically reduced in the

FGFR2-IIIb$^{-/-}$ embryo, further confirming that FGF signaling through FGFR2-III2b controls PGC number (Takeuchi et al., 2005).

It is crucial to maintain PGC survival during their journey. On the other hand, cell death also plays an important role in PGC development. As PGCs move through different tissues, the embryo is also undergoing rapid growth, tissue movements, and organogenesis, with the result that some PGCs diverge from their normal migratory route, or lag behind, and become out of range of the chemotactic factors from their targets and end up in ectopic locations. For example, ectopic PGCs are found in the allantois around E8.5. These have disappeared at later migratory stages, suggesting cell death occurred in these PGCs (Anderson et al., 2000a). In addition, PGCs are found in the hindgut and gut mesentery at E10.5 with apoptotic morphology (Molyneaux et al., 2001). A more recent study showed that only the PGCs close to the genital ridges successfully migrate to the targets at E10.5, whereas the ones in the midline no longer migrate laterally but instead die and disappear (Runyan et al., 2006). All these observations indicated that apoptosis is the mechanism that is responsible for the removal of 'ectopic' PGCs which fail to reach their migration target. The pro-apoptotic protein, Bax, is required for apoptosis of the ectopic PGCs, and *Bax*$^{-/-}$ embryos have many surviving ectopic PGCs along the migration path (Runyan et al., 2008; Runyan et al., 2006; Stallock et al., 2003).

Although many signaling molecules have been found to regulate PGC survival, proliferation and migration, experimental data on their function exists only for stages after PGCs migrate out of the hindgut, and little is known about their roles at earlier stages. How PGC behavior is controlled before hindgut colonization still awaits

experimental analysis.

Steel Factor in Mouse Primordial Germ Cell Development

Identification of Steel Factor and c-Kit Receptor

Steel factor, also known as Kit Ligand, Stem Cell factor (SCF) and Mast Cell Growth Factor, is a glycoprotein encoded at the *Steel* (*Sl*) locus on chromosome 12 in human, and chromosome 10 in mouse (Flanagan et al., 1991; Zsebo et al., 1990). The receptor for Steel factor is c-kit, a member of the tyrosine kinase receptor family (subclass III). It is a proto-oncogene encoded by the *W* (*Dominant White Spotting*) locus on chromosome 4 in human, and chromosome 5 in mouse (Qiu et al., 1988; Yarden et al., 1987). The extracellular domain of c-kit contains five immunoglobin (Ig)-like domains, and the intracellular domain consists of a tyrosine kinase domain split into two by an insertion region (**Figure 2**) (Qiu et al., 1988). Binding of the Steel factor homodimer mediates the dimerization of the c-kit receptor, which leads to autophosphorylation on the tyrosine residues in the intracellular domain (Broudy et al., 1998). The activated c-kit receptor provides anchoring points for adaptor proteins and leads to the activation of different signaling pathways (**Figure 2**). For example, the interaction between Phosphatidyl-inositol-3 (PI3) kinase and phosphorylated c-kit activates Akt, which subsequently phosphorylates the pro-apoptotic protein Bad to inhibit Bad activity, thereby promoting cell survival (Blume-Jensen et al., 1998). PI3-kinase, as well as Src kinase, induces the activation of GTP-binding protein Rac1 and JNK pathway to mediate cell proliferation (Timokhina et al., 1998). In addition, Src kinase also activates the small G-protein Ras and the mitogen-activated protein (MAP) kinase pathway,

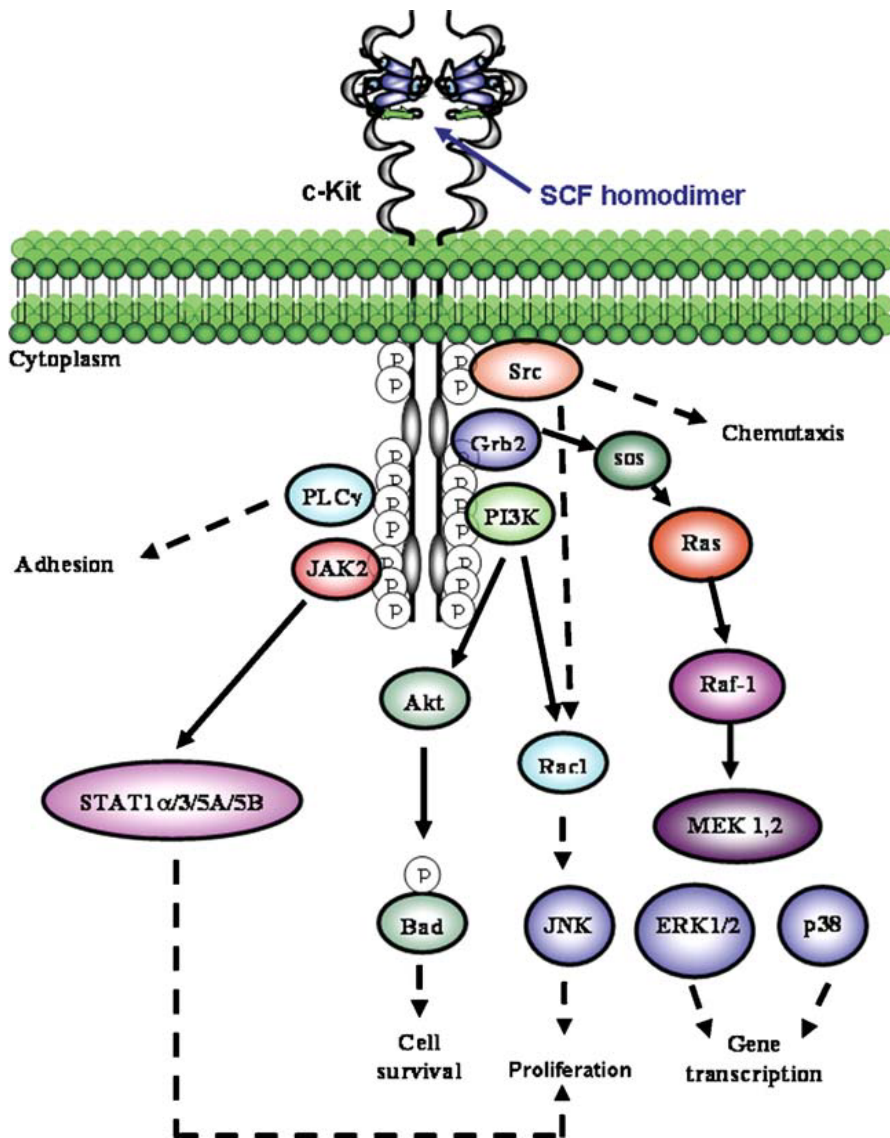

Image adapted from Reber, L., C.A. Da Silva, et al. (2006) Eur J Pharmacol 533(1-3): 327-340.

Figure 2: Signal transduction pathways of c-Kit receptor upon Steel factor binding.
Steel factor homodimer binds to c-kit receptor, inducing c-kit homodimerzation and autophosphorylation, and the activation of downstream signaling pathways.

which mediates Steel factor-induced gene transcription (Lennartsson et al., 1999).

Janus Kinase (JAK) is shown to be associated with c-kit to induce the phosphorylation

of the STAT pathway, which is responsible for cell proliferation and differentiation (Linnekin et al., 1996). However, the biological functions of these signaling pathways are still only partially understood, particularly in PGC development.

Biological functions of Steel factor/c-kit signaling

Mice containing mutations at either the *W* or *Sl* locus display a white-coat phenotype, and are sterile and anemic, indicating that Steel factor/c-kit signaling is involved in the development of melanocytes, germ cells, and hematopoiesis (Russell, 1979).

In mouse embryos, c-kit receptor is expressed in a subpopulation of neural crest cells (melanocyte precursors) right before their migration onto the dorsolateral migration pathway. The expression pattern of Steel factor is correlated with melanocyte migration and localization. Loss of function studies using mice lacking either the ligand or the receptor showed that the Steel factor/c-kit signaling is essential for the migration and survival of the melanocyte precursors (reviewed in (Wehrle-Haller, 2003)).

In bone marrow, c-kit receptor is expressed in early multi-potential hematopoietic progenitor cells. Upon the maturation of the progenitor cells into their respective lineages, the c-kit expression is down-regulated except in the mast cell lineage. Signaling pathways downstream of the Steel factor/c-kit are shown to be essential for hematopoietic progenitor cell survival, proliferation, and differentiation (reviewed in (Edling and Hallberg, 2007; Sharma et al., 2006)). In the mast cell linage, Steel factor acts as an important growth factor to promote survival via suppression of apoptosis. It also induces mast cell migration and mediates their adhesion to the extracellular matrix.

Moreover, Steel factor stimulates mast cell degranulation, leading to expression and release of histamine, pro-inflammatory cytokines and chemokines (reviewed in (Reber et al., 2006)).

Steel factor/c-kit signaling in PGC development

Steel factor has been known for many years to be essential for fertility. The sterility of *W* and *Sl* mutant mice revealed an important role that Steel factor/c-kit signaling plays in PGC development (Bennett, 1956; Mintz and Russell, 1957). When culturing migratory PGCs isolated from wild type embryos *in vitro*, addition of Steel factor greatly increases the survival of PGCs, but it does not stimulate PGC proliferation, shown by bromodeoxyuridine (BrdU) labeling (Dolci et al., 1991; Godin et al., 1991; Matsui et al., 1991a). Blockade of Steel/c-Kit signaling by the addition of a c-Kit blocking antibody, Ack2, decreases PGC number in culture, confirming that Steel factor is a pro-survival factor for PGCs (Matsui et al., 1991b).

When looking at PGC development in the embryos with mutations in the *W* or *Sl* locus, there are fewer PGCs found in the developing genital ridges, but PGC numbers during the period from E8.0 to E9.0 are similar in the mutants to those in the wild type embryos (Bennett, 1956; Mahakali Zama et al., 2005; McCoshen and McCallion, 1975; Mintz and Russell, 1957). These data suggest that PGCs become dependent on Steel factor only after their colonization of the hindgut. However, a more recent study from our lab showed that PGC numbers are already significantly reduced in E9.0 *Steel-null* embryos, raising the possibility that Steel factor is required for PGC survival from the time that they become motile in the allantois (Runyan et al., 2006). In support of this, the mRNA

of Steel factor is expressed by cells that found along the PGC migration route (Keshet et al., 1991). The receptor, c-kit, is found to be expressed in migratory PGCs from the time they initiate migration (E7.5), to the time they colonize the gonad (Manova and Bachvarova, 1991; Matsui et al., 1990; Yabuta et al., 2006). It would be exciting to elucidate when PGCs start to be Steel factor-dependent.

Steel factor regulates PGC survival as a short-range signal, revealed by studying the midline PGC death in E10.5 embryos (Runyan et al., 2006). From E10.5 to E11.5, PGCs that are still in the midline structures no longer migrate to the genital ridges, but instead die and disappear (Molyneaux et al., 2001). At E9.5, antibody staining showed that PGCs in the midline are surrounded by Steel factor protein as they emigrate from the hindgut. By E10.5, Steel factor expression is lost from the midline structures, but is upregulated laterally around the genital ridges. The withdrawal of Steel factor from midline structures causes the PGCs in the midline to die through the intrinsic apoptotic pathway (Runyan et al., 2006). Consistent with this, addition of exogenous Steel factor rescued midline PGC death, whilst addition of the c-kit blocking antibody Ack2 caused PGC death everywhere in the embryo (Runyan et al., 2006). These data showed that the change in location of Steel factor expression leads to the apoptosis of the midline PGCs. Those PGCs cannot receive the pro-survival signal from the genital ridges which are only 100 μm away from them, indicating that Steel factor functions in a short range to regulate PGC survival (Runyan et al., 2006). New aspects of Steel factor regulation in PGC development were also revealed in this study, when PGC apoptosis was rescued by removing the pro-apoptotic protein Bax. At E10.5, PGCs in wild type embryos already start to migrate along the dorsal body wall towards the genital ridges. However,

PGCs in Bax/Steel double null embryos failed to migrate out of the hindgut, and remained in ectopic locations (Runyan et al., 2006). When stained for the mitosis marker, phospho-histone H3, PGC mitoses were found dramatically reduced in Bax/Steel double null embryos at E10.5, suggesting that Steel factor is required for PGC proliferation as well (Runyan et al., 2006). Altogether, these findings suggest that Steel factor is a multi-functional factor which functions at short range to regulate several aspects of PGC behavior during their migration from the midline to the genital ridges, and the ectopic PGCs are cleared by apoptosis when Steel factor expression turns off in the microenvironment surrounding those PGCs. Whether this Steel factor-expressing microenvironment always exists around PGCs to offer short range signal to control PGC survival, proliferation and migration will be a fascinating question to address.

The downstream pathways activated by Steel factor/c-kit signaling have been intensively studied in other cell populations such as hematopoietic stem cells, but little is known about their roles in PGC development in mammals. An early study using retroviral-mediated gene transfer demonstrated that AKT phosphorylation, through the action of mTOR, is required for Steel factor-dependent PGC survival (De Miguel et al., 2002). However, a recent study showed that PI3K and Src kinase are responsible for the Steel factor-dependent PGC migration and AKT activation, while p38 and mTOR seem to have no effect on PGC behavior in transwell chamber migration assays (Farini et al., 2007). It would be good to know how different downstream effectors are involved in regulating different aspects of PGC behavior upon the activation of the same signaling receptor.

Soluble and membrane-bound Steel factor

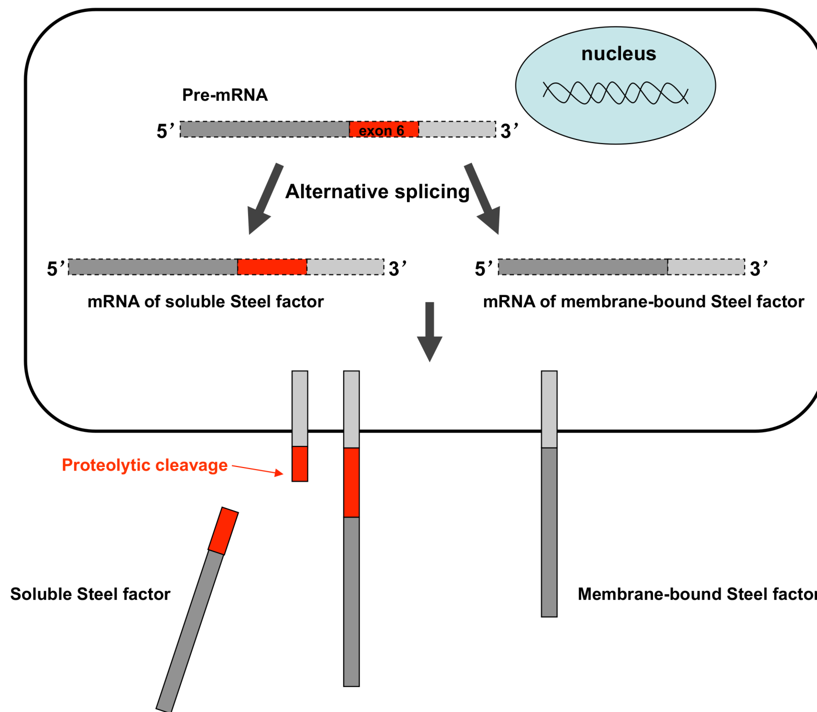

Figure 3: The production of the two forms of Steel factor.

The soluble and membrane-bound forms of Steel factor arise from alternative splicing of the proteolytic cleavage site encoded by exon 6 on the pre-mRNA.

The protein of Steel factor is expressed in two forms as a result of alternative splicing and proteolytic cleavage (**Figure 3**) (Anderson et al., 1991; Flanagan et al., 1991). The soluble form is generated from an integral protein precursor expressed transiently on the cell surface, by proteolytic cleavage at a site located in the proximal extracellular domain (Huang et al., 1992). The cleavage site is encoded by a short 84 bp-long alternative exon (exon 6), and is removed from the mRNA of the membrane-bound form by alternative splicing, causing the membrane-bound protein product to be sustained on

the cell surface (Flanagan et al., 1991). This cleavage can be mediated by several factors, such as the phorbol 12-myristate 13-acetate (PMA) and calcium ionophore A23187 (Huang et al., 1992), the cell-associated serine proteinase (Pandiella et al., 1992), the mast cell chymase (de Paulis et al., 1999; Longley et al., 1997), and the matrix metalloproteinase (MMP) (Chesneau et al., 2003; Gallea-Robache et al., 1997).

The *Steel-dickie* (*Steel*$^{d/d}$) mutation offers a great tool to study the biological functions of the two Steel factor isoforms. Different from the *Steel-null* mutation, which carries a big deletion of the entire *Steel* gene (Runyan et al., 2006), the *Steel-dickie* mutation has a deletion of a 4.0-kb sequence that encode the intracellular and transmembrane domains of Steel factor (Brannan et al., 1991). As a result of this deletion, the *Steel-dickie* mutant only produces a soluble truncated protein, which is as biologically active as the wild type soluble Steel factor (Brannan et al., 1991). The homozygotes of *Steel-dickie* mutants are sterile, have abnormal pigmentation, decreased erythrocyte cell number and display macrocytic anemia at birth, suggesting a requirement of membrane-bound Steel factor in PGC, melanocyte and hematopoietic cell development (Brannan et al., 1991; Mahakali Zama et al., 2005; Rajaraman et al., 2002).

Many studies have focused on understanding the indispensable role which membrane-bound Steel factor plays in the development of these cell linages. It is known that the two isoforms of Steel factor have similar binding affinities to the c-kit receptor, and either form is capable of activating c-kit tyrosine kinase (Flanagan and Leder, 1990; Miyazawa et al., 1995). However, the membrane-bound Steel factor induces more persistent activation of c-kit than the soluble form, probably due to the membrane-bound form maintaining a prolonged life span, whilst the soluble form triggers rapid internalization

and degradation of c-kit receptor upon binding (Miyazawa et al., 1995). This provides a possible answer for why the two forms have different effects. In support of this, the membrane-bound Steel factor is shown to be required for long-term growth of hematopoietic cells in culture, while the soluble form supports only short-term proliferation of hematopoietic cells (Friel et al., 1997). In addition, membrane-bound Steel factor induces greater proliferation and lower incidence of apoptosis in erythroid cells than the soluble form (Kapur et al., 2002; Kapur et al., 1998). Similarly, fibroblasts, which were transfected with constructs expressing membrane-bound Steel factor, but not the soluble form, promote oocyte growth and survival *in vitro* (Thomas et al., 2008). Whether membrane-bound Steel factor is specifically required for PGC survival and proliferation is not yet clear.

Membrane-bound Steel factor may also play an important role in mediating cell adhesion. It has been shown that c-kit-expressing mast cells undergo adhesion to COS cells which express membrane-bound Steel factor, but not soluble Steel factor (Flanagan et al., 1991). It also stimulates the binding of mast cells to cell matrix (Kinashi and Springer, 1994). In mouse gametogenesis, the adhesion between Sertoli cells and germ cells is mediated through the membrane-bound form of Steel factor, but not the soluble form, given the fact that Sertoli cells from *Steel*$^{d/-}$ mutants are unable to bind germ cells (Marziali et al., 1993). Furthermore, a study using E11.5 PGCs in culture reported that PGCs prefer to adhere to somatic cells expressing membrane-bound Steel factor than those expressing the soluble form (Pesce et al., 1997). It is likely that the role played by membrane-bound Steel factor in promoting survival, proliferation and migration of these cells may depend on the ability of the membrane-bound form to

directly mediate adhesion to the surrounding cells; however, further studies need to be performed to test this possibility.

Although *Steel-dickie* mutants are sterile, as they are in the *Steel-null* mutants, there are more PGCs found in the genital ridges in *Steel*$^{d/d}$ embryos than in the *Steel*$^{-/-}$ embryos, suggesting that some activity of the Steel factor signaling pathway remains in these mutants through its soluble form (Mahakali Zama et al., 2005). It has been shown previously that both forms of Steel factor are essential for melanocyte development, with the soluble Steel factor required for the initiation of melanocyte precursor dispersal onto the lateral pathway, while the membrane-bound form is required for their subsequent survival in the dermis (Wehrle-Haller and Weston, 1995). However, it is not known why the membrane-bound and soluble forms have different properties in these model systems. Steel factor has multiple functions in PGC behavior, including survival, proliferation and migration. It is possible that the membrane-bound form of Steel factor is specifically required for some, but not all aspects of PGC behavior. It is also possible that the properties of the two forms are the same, but in *Steel*$^{d/d}$ embryos the soluble form diffuses away from the secreting cells, making the local concentration sub-optimal, thus causing the defects seen in the *Steel-dickie* mutations. These questions are waiting to be answered.

There is another puzzling aspect of the *Steel*$^{d/d}$ mutation. Steel factor is known to be an essential survival factor for both PGCs and hematopoietic cells. However, *Steel-null* mutants are sterile, and usually die around E15 due to severe anemia, while *Steel-dickie* homozygotes are sterile but viable, and most of them survive to P18, similar to wild type (Bennett, 1956; Rajaraman et al., 2002). This suggests that Steel factor plays

some role in PGC behavior that is not shared with hematopoietic cells, but the underlying mechanism remains unknown.

The regulation of Steel factor expression

The important biological functions of Steel factor have been studied for years in various cell populations. Surprisingly little is known about how Steel factor expression is controlled during development. Previous studies have suggested that interleukins (ILs), such as IL-1β, 4, 6, 13 and 18, are capable of inducing Steel factor expression, possibly through activation of p38 MAPK or NF-κB pathways (Bouchelouche et al., 2006; Hue et al., 2005; Peters et al., 2001; Reber et al., 2009). It has also been shown that Activin A signals via SMAD2/3 to repress Steel factor expression in cultures of human fetal ovaries (Childs and Anderson, 2009; Coutts et al., 2008). However, most of these studies have been done either in cell lines, or in overexpression assays; therefore, the results may not perfectly reflect the regulation of endogenous Steel factor *in vivo*.

In PGC development, Steel factor is enriched in midline structures at E9.5 when PGCs are migrating through the midline mesenchyme. At E10.5, PGCs migrate laterally to colonize the genital ridges, and the high-level Steel factor expression moves from the midline to the genital ridges. This change in location of Steel factor expression results in the PGCs left in the midline dying by apoptosis (Runyan et al., 2006). This observation suggests that the Steel factor is expressed in a spatial-temporal manner that is crucial for proper Steel factor functions. Although it has been reported that BMP4 treatment elevates Steel factor expression in the somatic cells surrounding PGCs in organ culture

of E9.5 embryo slices, and reduced BMP signaling leads to decreased Steel factor expression in the genital ridges at E10.5 (Dudley et al., 2010; Dudley et al., 2007), whether the change in location of Steel factor expression is caused by BMP signaling remains ambiguous. Future studies investigating how this dynamic expression is regulated will greatly benefit our understanding of the regulation in PGC development, as well as in many other cell populations such as hematopoietic stem cells and melanocytes.

Specific Aims:

The focus of my graduate research is described in the following aims:

Aim 1: To test the hypothesis that PGC behavior is controlled by Steel factor from the beginning of their migration.

Aim 2: To test the hypothesis that the two forms of Steel factor control different aspects of PGC behavior.

Aim 3: To design the Steel factor-reporter construct using Bacteria Artificial Chromatin (BAC) strategy, in order to make transgenic reporter mouse strain to study how Steel factor expression is controlled.

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Chapter II

Steel factor is required for normal PGC behavior throughout their migration

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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Published in *Development* 136(8): 1295-303

Abstract

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 failure of PGC specification. Third, analysis of cultured Steel factor-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, either by 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.

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 (E6.0-6.5) in response to signals from 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 expression of Blimp1, 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 (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 to 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).

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., 1991a). 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 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 lab 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 the genital ridges. At E10.5, Steel factor expression is lost from midline structures, but 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 maintains survival of PGCs closest to the genital ridges. Steel factor functions at close-range, since the genital ridges are only some 100 μm away from the midline at E10.5. Analysis of embryos in which PGC apoptosis was blockaded 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 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 WT littermates, indicating that Steel factor is required to maintain PGC numbers as early as E7.5. Moreover, PGCs in the *Steel*$^{-/-}$ embryos show reduced motility and increased formation of clumps, 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 up-regulation of this adhesion protein. The results were further confirmed by acute loss of Steel signaling using Ack2 antibody, the blocking antibody to 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, forming a “travelling niche” for this important population of migrating pluripotential cells.

Materials and methods

Mouse breeding, embryo preparation and genotyping

Mouse embryos were obtained by crossing male homozygotes for the *Stella-GFP* transgene (Payer et al., 2006) on a B6/CBA background, with 6-8 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, *Oct4PE:GFP* transgenic mice (Anderson et al. 1999) 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’; *Oct4PE: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 $Kitl^{Sl}$, wildtype and deleted alleles were determined by 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. *Oct4PE: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 which contains 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. 10 μg/ml mouse IgG (JacksonImmunoResearch) was used as 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 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 unpaired, two-tailed Student's t-test with equal variances.

RT-PCR

For RT-PCR analysis, allantoids from E7.5 embryos, and genital ridges from E10.5 embryos were dissected, and RNA extracted from dissected tissues using RNeasy Kit (Qiagen). 10ng of RNA was reverse-transcribed using Superscript III First-Strand Synthesis Systems (Invitrogen) per 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 (2x10 minutes), blocked in PBSST (PBS/0.3% TX-100 with 5% goat or donkey sera, 2x 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% TX-100, 2x 15 minutes, then 3x 1 hour) at 4°C, incubated overnight at 4°C in PBSST with Cy3 or Cy5-conjugated secondary antibodies (Jackson Immuno Research), washed in PBST (2X15 minutes, then 3x 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 RT), and incubated with primary antibody overnight at 4°C. The next day, slides were washed with PBST (3x 15 minutes), incubated in PBSST with secondary antibody (2 hours), washed with PBST (2x 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 Immuno Research) were used at the following dilutions: 1:300 for Cy5 donkey anti-goat; 1:300 Cy5 goat anti-rabbit; 1:300 Cy5 goat anti-rat; 1:300 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 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 (arrowed in **Fig. 4A-C**). These data are supported by the single cell RT-PCR analysis in a recent study (Yabuta et al., 2006), which showed that Steel factor was expressed in PGCs at E7.25, but was down-regulated 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. 4A-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. 4I**). Whole-mount staining of E8.5 embryos showed that Steel protein was strongly expressed in the cells of visceral endoderm, as well as the adjacent hindgut diverticulum containing the PGCs (**Fig. 4D**). Transverse sections through the hindgut diverticulum at E8.5 showed that PGCs only occupied the ventral aspect of the hindgut epithelium, which was strongly stained for Steel factor, but not the dorsal part, which lacked Steel factor expression (**Fig. 4E and 4F**). At E9.0, Steel factor was expressed by all the cells of the hindgut, and PGCs now occupied the dorsal, as well as the ventral hindgut (**Fig. 4G**). At E10.0, Steel factor staining was reduced or absent in the hindgut epithelium, and enriched in tissues surrounding the PGCs in the midline dorsal body wall and the coelomic angles (**Fig. 4H**). 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 **Figure 4B and 4C**, 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 better cross reaction with the anti-Steel factor antibody. TCA fixation causes loss of signal from the GFP, so PGCs could not be identified. However, a group of cells in the core of the allantois showed strong membrane staining of Steel factor (arrow, **Fig. 4J**). 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 RT-PCR confirmed the presence of both soluble and membrane-bound Steel factor in the allantois at E7.5 (**Fig. 4K**).

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 making time-lapse movies during these time periods. **Figure 5A** shows a sequence of frames from

one movie started at E7.25 (see also Supplementary Data, Movie 1). 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. 5A**). 7 out of 8 movies started at E7.25 showed PGC migration in this direction. Figure 5B shows a movie started at E7.5 (see also Supplementary Data, Movie 2). More PGCs were seen at the beginning of movies 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 8 out of 10 movies started at E7.5, we observed the PGC behavior shown in **Figure 5B**.

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. 6A**). In 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., 1991a) and survival (Godin et al. 1991, Dolci et al. 1991, Matsui et al. 1991a, 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 for mitosis and the cleaved-PARP antibody for 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 at this stage made it difficult to perform statistical analysis.

Recent work from our lab 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. 6A**). 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. 6B**. These data show that Steel factor is an essential survival factor for PGCs even before they enter the hindgut, and that PGCs die through 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, since the number of PGCs increased in Steel-null embryos when apoptosis was inhibited.

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. 7A and B**). The movies themselves are shown as Supplementary Data Movies 3-5. 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. 7C and D**), and calculated the velocities and displacements of PGC movement. These are shown in **Fig 7E**. PGCs in $Steel^{+/+}$ embryos were highly motile, with a maximum velocity of 51.4±3.1 μm/h, an average velocity of 24.7±1.2 μm/h and an average total displacement during the time of filming, of 83.1±8.9 μm. The maximum velocity (50.1±3.3 μm/h), average velocity (23.6±1.3 μm/h) 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/h), average velocities (17.5±0.8 μm/h), and displacements (44.9±7.7 μm) compared to PGCs in $Steel^{+/+}$ (p=1.0x10^{-6}, 3.8x10^{-10}, and 1.2x10^{-7}, respectively) and $Steel^{+/-}$ littermates (p=1.8x10^{-7}, 7.4x10^{-12}, and 2.2x10^{-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 start-point of each PGC path on the same point (**Fig. 7D**) to generate a windrose diagram for the average trajectories of PGCs in embryos of each genotype (**Fig. 7F**). The directionality was altered, but not randomized, by 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 Figure 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 in **Fig. 7C**) 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 factor (**Fig. 7G**), which was significantly reduced when compared with *Steel*$^{+/+}$ (66.8±5.4%, p=3.7x10^{-5}) and heterozygous littermates (51.4±4.0%, p=0.0001). 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 7A, 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. 7H**). Nearly 58.3% of PGCs in *Steel*$^{-/-}$ embryos were in clusters compared with 18.7% in *Steel*$^{+/+}$ embryos (p=6.4x10^{-5}). Loss of one allele of *Steel* also increased the number of clustered PGCs compared with Steel WT 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). **Fig. 8A** shows that E-cadherin was not up-regulated by PGCs in Steel$^{-/-}$ embryos at this stage. It is possible that expression of other adhesion molecules is responsible for clumping, in addition to failed motility. However, these have not yet been characterized in early migrating germ cells.

To exclude the possibility that 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. Loss of one allele of Bax rescues germ cell numbers (**Fig. 6**), but does not rescue germ cell motility (**Fig. 9**).

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 Ack2 antibody, which has been shown to effectively block Steel signaling, including the blockade of Steel factor function in PGCs (Nishikawa et al., 1991; Runyan et al., 2006; Stallock et al., 2003). As shown in **Fig. 10**, both the velocity and displacement of PGCs were significantly decreased by treatment with 10 μg/ml Ack2 for 6 hours compared with control embryos (p<0.01, **Fig. 10A**). In addition, the proportion of PGCs entering the posterior embryo was significantly decreased by Ack2 treatment (25.5±9.2% compared to 53.4±6.9%, p=0.003, **Fig. 10B**). PGCs also clumped more in the presence of Ack2, with 42.3±7.5% of PGCs in clusters,

than those in control embryos, with a percentage of 15.3±5.6% (p=0.002, **Fig. 10C**). Movies of E7.5 Ack2-treated, and control embryos incubated in the same concentrations of a non-immune IgG, which did not affect PGC behavior, are shown as Supplementary Data Movies 6 and 7. Treatment with the Ack2 antibody did not alter PGC numbers, probably due to the short time-period (6 hrs) 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; a deficiency of PGC specification).

Steel factor is required for PGC motility in the hindgut

We reported previously that rescue of PGC apoptosis in *Steel*$^{-/-}$ embryos by 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 either to 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. 7**). To test whether the same is true once PGCs have colonized the hindgut, time-lapse movies were made using E9.0 *Oct4PE: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. 11A**) and time=420 minutes (**Fig. 11B**), and the migration directions of individual PGCs are plotted using the method

described above (**Fig. 11C**). **Fig. 11D** shows that PGCs in *Steel*$^{-/-}$ embryos had significantly decreased displacement (22.6±5.22 μm) compared to *Steel*$^{+/+}$ (56.8±5.8 μm, p=6.93x10^{-8}) and *Steel*$^{+/-}$ (65.1±8.1 μm, *p*=1.89x10^{-9}) littermates. They also showed a decreased maximum velocity (11.8 ±1.4 μm/h), compared with *Steel*$^{+/+}$ (22.2 μm/h, *p*=2.49x10^{-10}) and *Steel*$^{+/-}$ (20.9±1.7, *p*=1.00x10^{-9}), and a decreased average velocity (5.0±0.5 μm/h) compared to *Steel*$^{+/+}$ (9.6±0.6 μm/h, *p*=3.48x10^{-12}) and *Steel*$^{+/-}$ (10.1±0.8 μm/h, *p*=1.24x10^{-12}). Windrose assays (**Fig. 11E**) showed that all the PGCs migrated in a net dorsal and slightly rostral direction (right panel in **Fig. 11E**), regardless of *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., 1991a). Early studies reported that PGC numbers in Steel mutants are similar to those in WT 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 the 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 they first turn on *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 of the allantois. 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 as well as RT-PCR analyses at E7.5 shows that PGCs become surrounded by cells expressing membrane-bound Steel factor when they are in the allantois, suggesting that the PGC behaviors may be dependent on this close-range signaling as they first form in the embryo. It is possible that membrane-bound Steel protein controls different aspects of PGC behaviors from 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, since only the 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 for cell death, or phospho-histone H3 staining for mitosis, extremely difficult. However, in the five

embryos examined so far that are *Steel*$^{-/-}$ and *Bax*$^{+/-}$, PGC numbers were dramatically increased by 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 movie frames starting at E7.5 revealed that both the velocities and 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 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 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 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 due to the fact that germ cells are migrating 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*, *Oct4*, *Sox2*, *nanos3*, and *nanog* (reviewed in Saitou et al. 2005). This process takes place in the allantois, and both the sources and 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, 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 the other signaling ligands that control PGC behavior, will be interesting questions to answer in future studies.

Acknowledgments

The authors would like to thank the Cincinnati Children's Hospital Research Foundation for financial support for this project, and Dr. Fred Finkelman for providing the Ack2 antibody.

Supplementary Materials

Supplementary movies are available at:

http://dev.biologists.org/content/136/8/1295/suppl/DC1

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Figures and Figure Legends

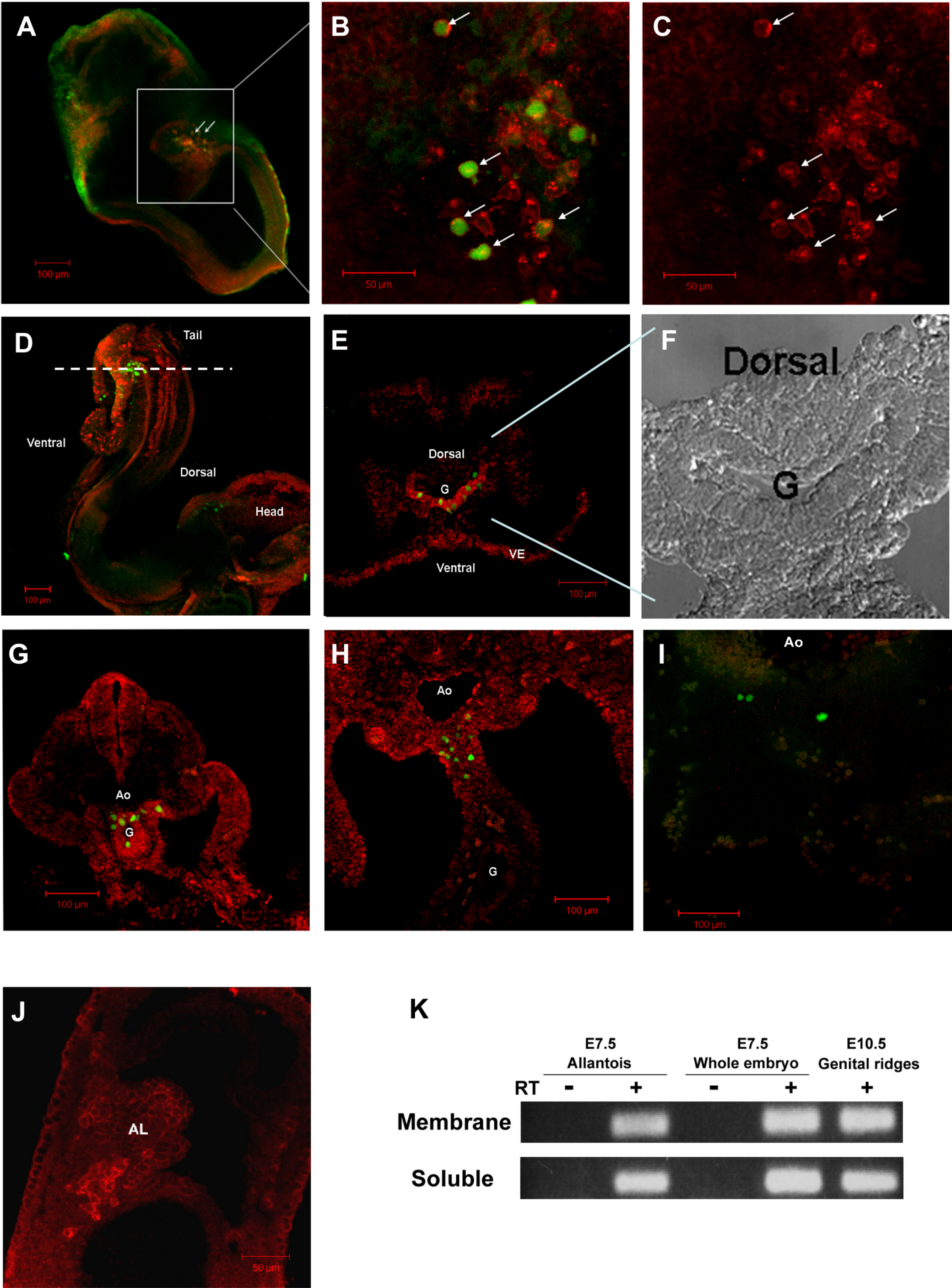


Figure 4: Expression of Steel factor

(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 (B), without the green channel, to allow visualization of Steel factor staining in the PGCs (arrowed). (D) Whole-mount staining of 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 E8.5 hindgut diverticulum (shown by the dashed line in C). 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 E9.0 hindgut. Both the ventral and dorsal hindgut is stained for Steel factor, and PGCs have moved to the dorsal region of the gut. (H) Transverse section through E10.0 embryo. Steel staining is lost from the hindgut, but enriched in the dorsal mesentery and body wall. (I) Immunostaining of a section from E10.5 *Steel*$^{-/-}$ embryo demonstrates the antibody specificity (lack of red signal). (J) Whole-mount immunostaining of Steel factor in E7.5 embryo fixed in 2% TCA, which increases antibody reaction with Steel factor. (K) RT-PCR analysis of membrane-bound and soluble Steel factor. cDNA was prepared from dissected E7.5 allantoids, whole embryo and E10.5 genital ridges. AL: allantois, Ao: aorta, G: hindgut, VE: visceral endoderm

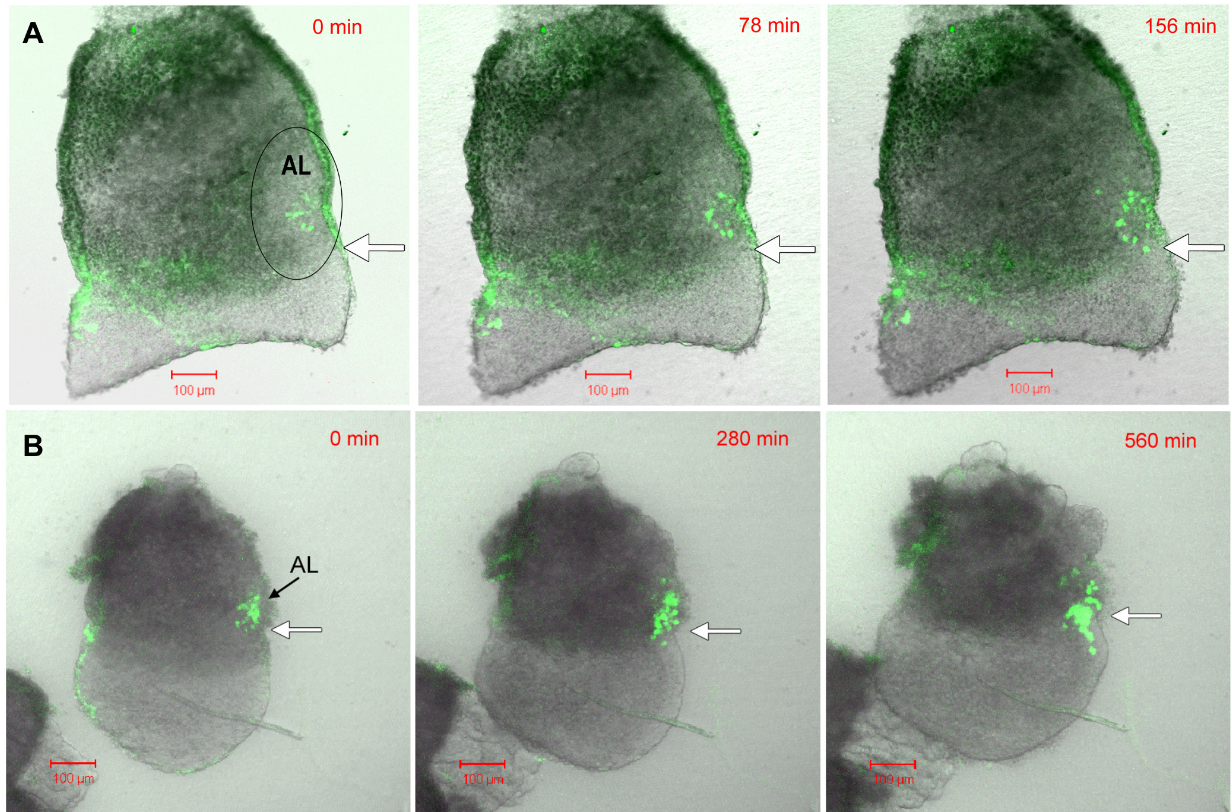


Figure 5: Time-Lapse frames of E7.25 and E7.5 Stella-GFP embryo.

(A) Three frames at t=0, t=78 minutes, and t=156 minutes of a movie started at E7.25. PGCs are in the allantois (AL). Residual expression of *Stella* is also seen elsewhere in the embryo. PGCs appear in the allantois by switching on *Stella*, and start to spread towards the epiblast (down). Arrow indicates approximate boundary between allantois and proximal epiblast. (B) Three frames at t=0, t=280 minutes, and t=560 minutes, starting 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 allantois and proximal epiblast. PGC population expands into proximal epiblast. AL: allantois.

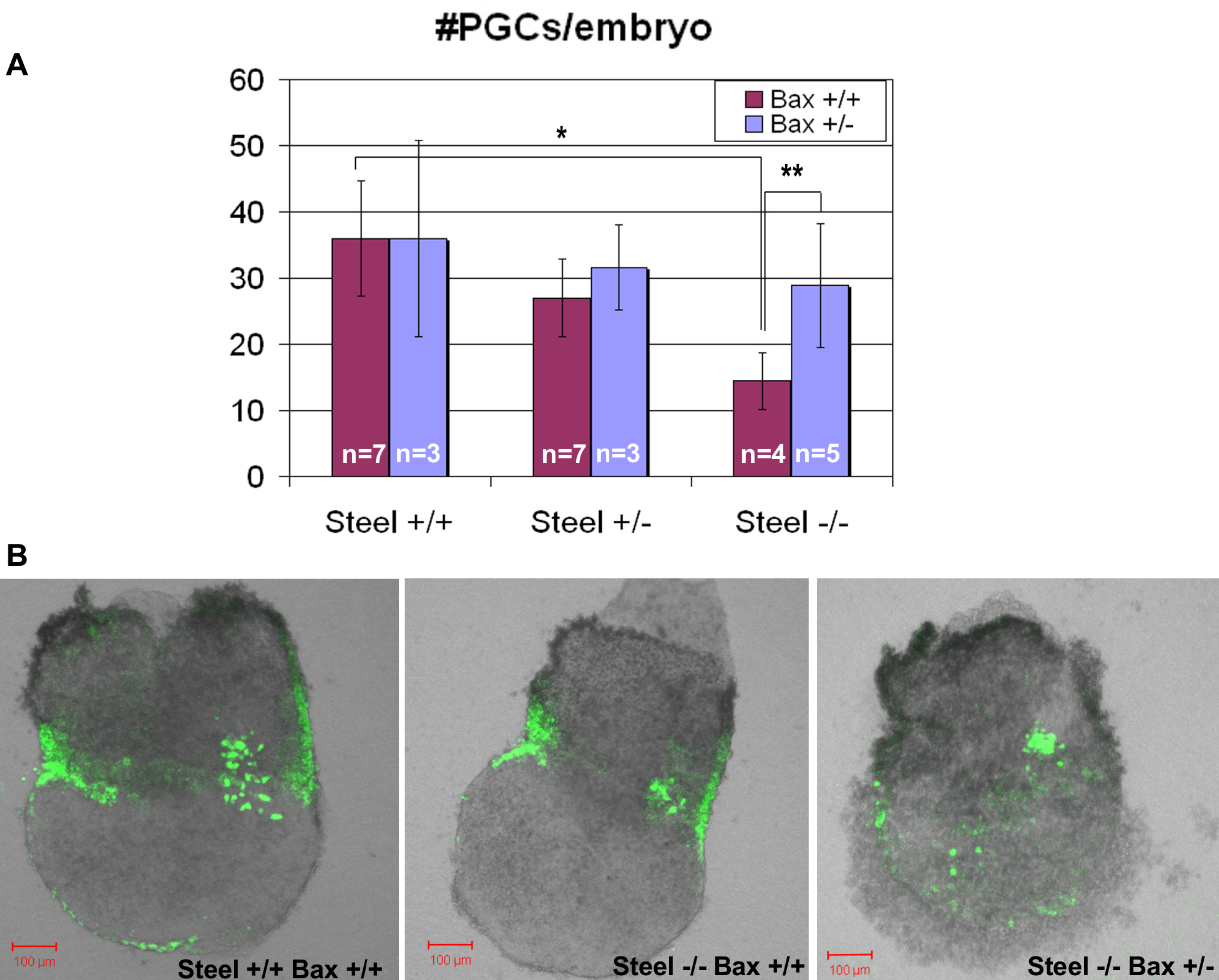
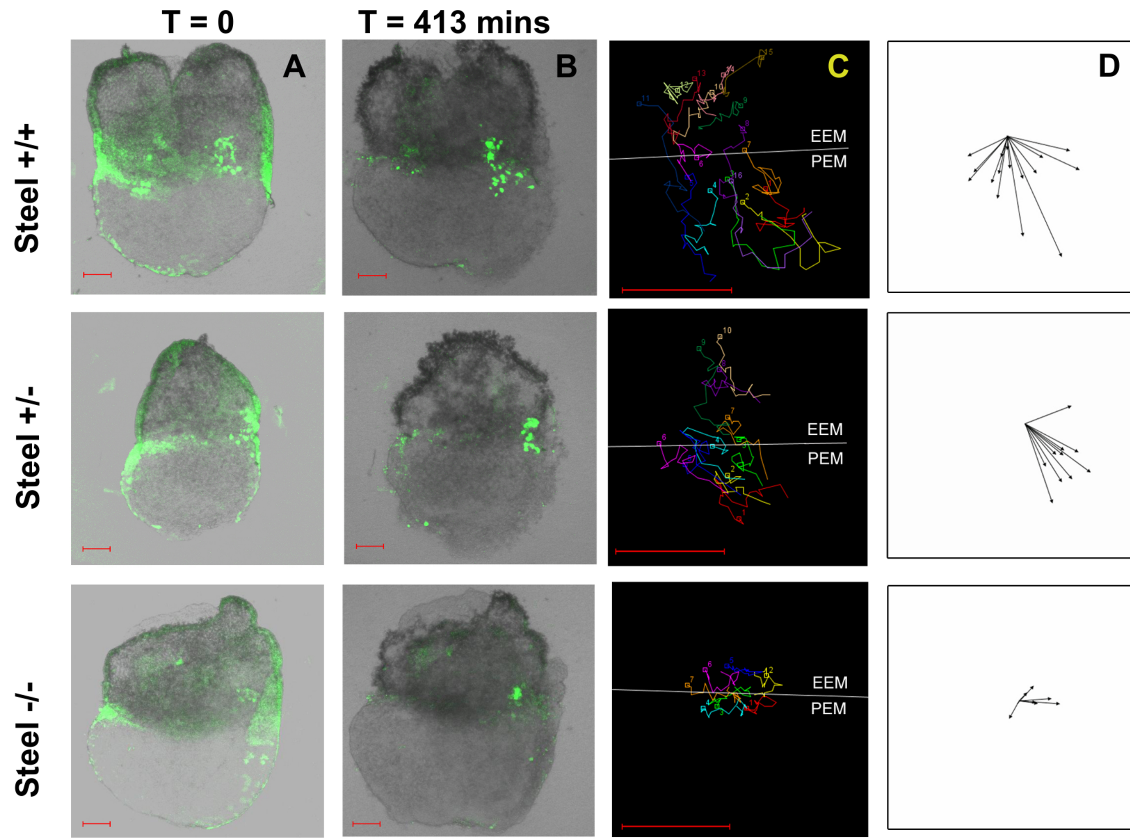
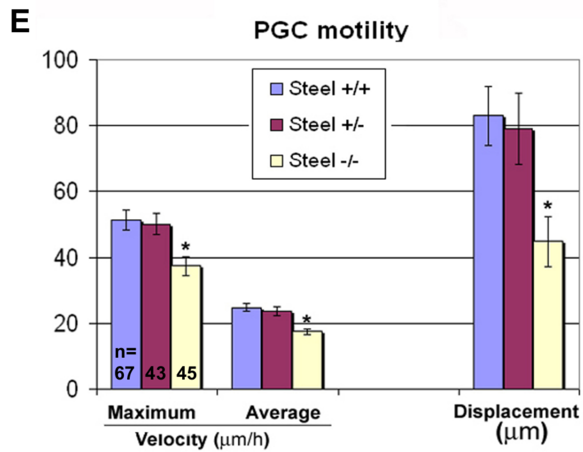
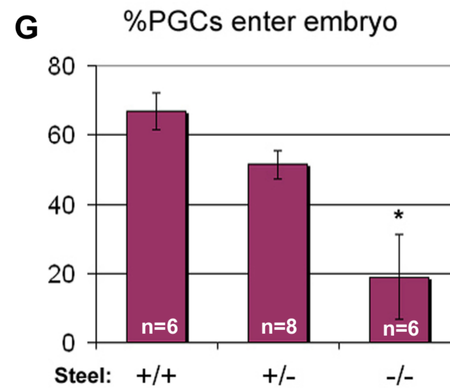


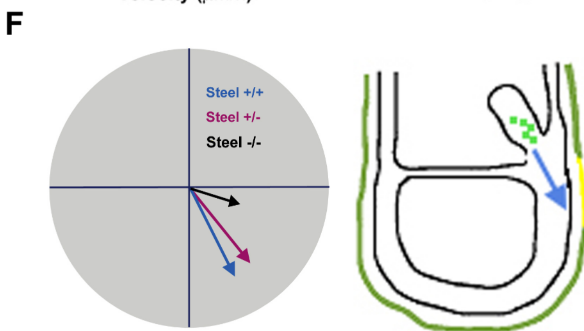
Figure 6: Effects of Steel factor on PGC numbers at E7.5.

(A) PGC numbers at E7.5 were significantly reduced in *Steel*$^{-/-}$ embryos compared to *Steel*$^{+/+}$ and *Steel*$^{+/-}$ littermates (red bars). PGC numbers were significantly rescued in *Steel*$^{-/-}$ embryos by 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) shows examples of embryos of each genotype used to generate the PGC counts.









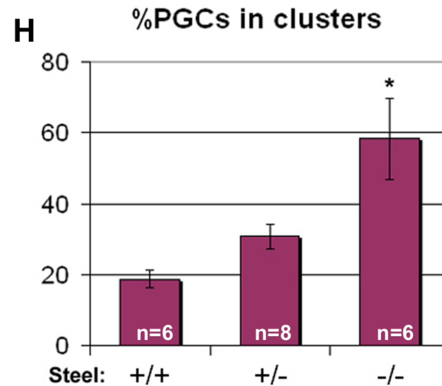


Figure 7: Effects of Steel factor on PGC migration at E7.5.

Frames at t=0 (column A), and t=413 minutes (column B) from movies of E7.5 *Stella-GFP* embryos with different *Steel* genotypes. PGCs are green. Column C shows 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. Column D shows the overall directions of movement of individual PGCs, made by placing the starting point of each PGC track on 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” indicates the number of PGCs used for quantitation. Units on the “Y” axis vary based upon parameter, and are indicated below the bar charts. (F) Windrose diagram shows that directionality of PGC migration is altered in *Steel*$^{-/-}$ embryos, but not randomized (colored arrows correspond to colored text for genotype). 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 which 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 which form clusters is markedly higher in the absence of Steel factor. “n” indicates the number of embryos used for quantitation for (G) and (H). *=p<0.01, Bars represent mean±s.e.m.

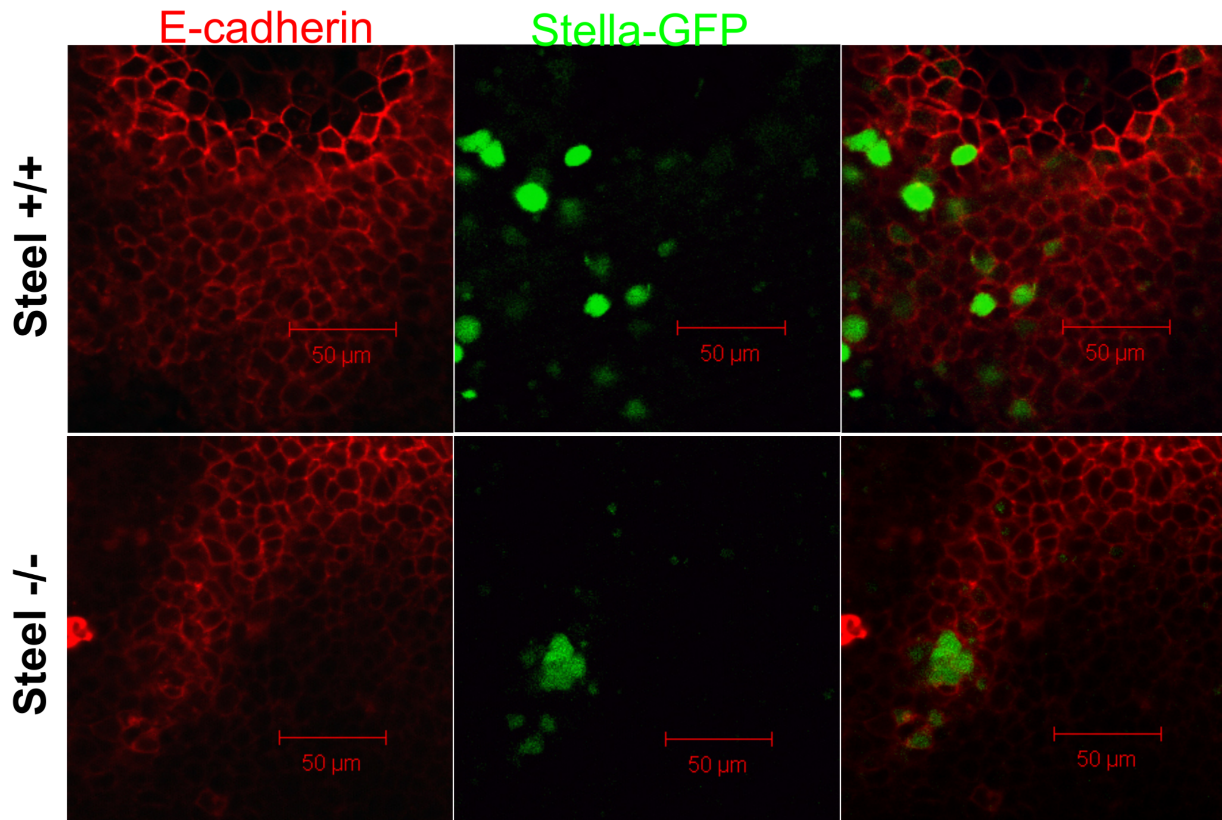


Figure 8: PGC clumping in Steel mutant embryos is not due to up-regulation of E-cadherin.

Upper panels show E-cadherin (red) and PGCs (green) in *Steel*$^{+/+}$ E7.5 embryos, individually and combined. The lower panels show the same data for *Steel*$^{-/-}$ embryos. No difference in expression of E-cadherin was observed in different *Steel* genotypes.

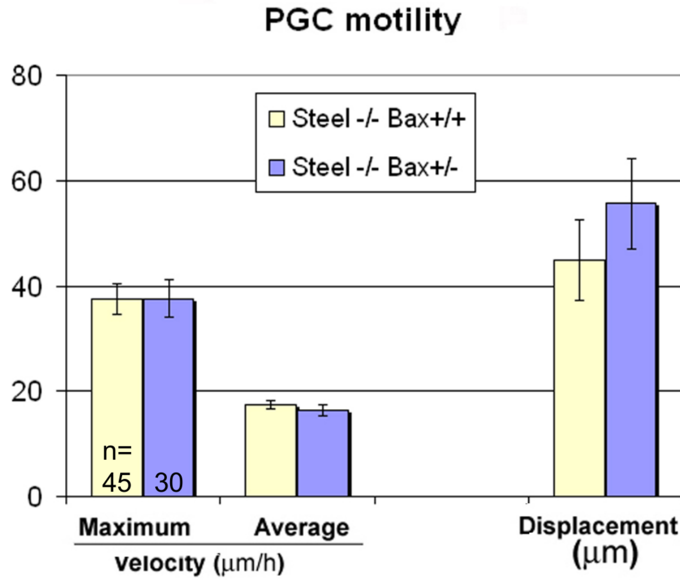


Figure 9: Loss of an allele of Bax does not rescue PGC motility.

Velocities and displacements of PGCs in movies from *Steel*$^{+/+,\ +/-,}$ and $^{-/-}$ 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” indicates the number of PGCs used for quantitation. Units on the “Y” axis vary based upon parameter, and are indicated below the bar charts.

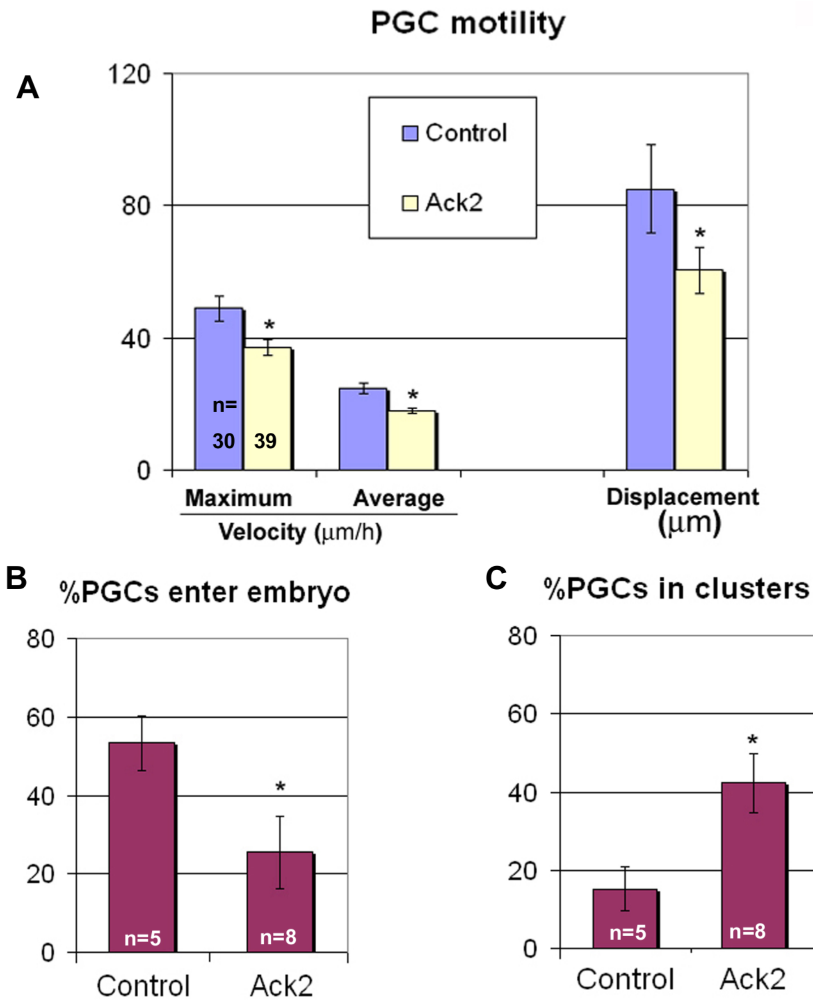
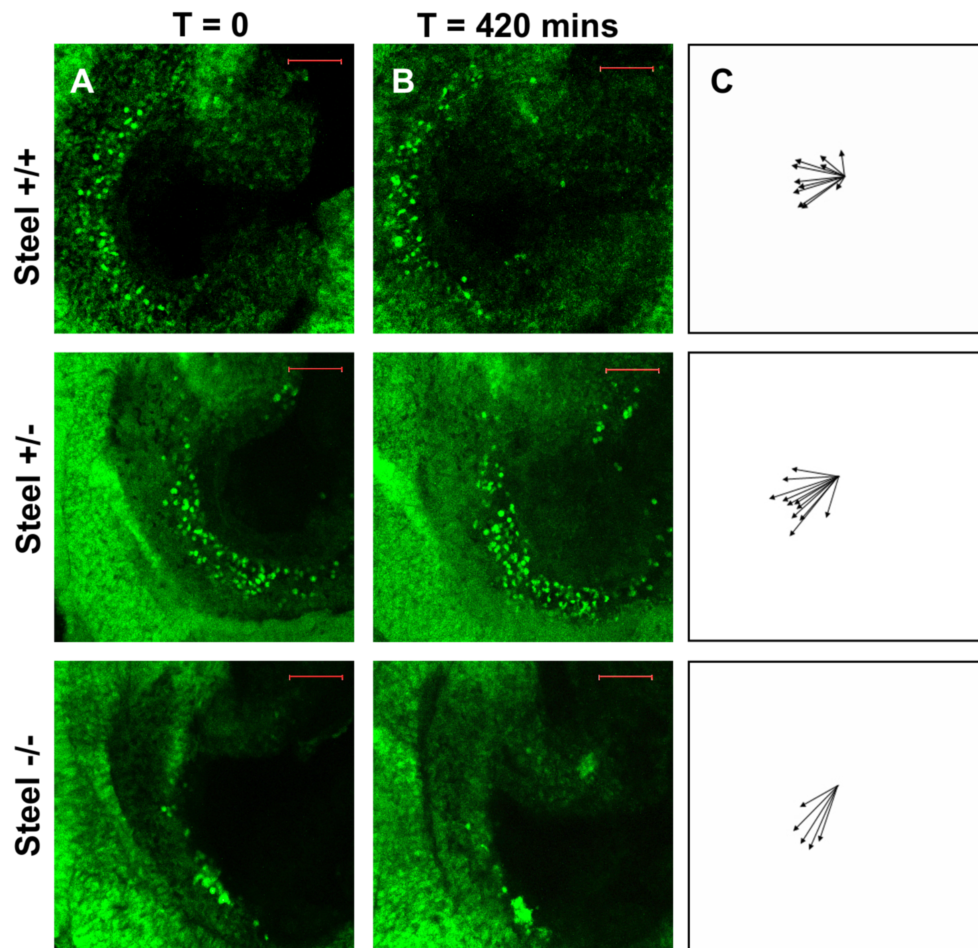
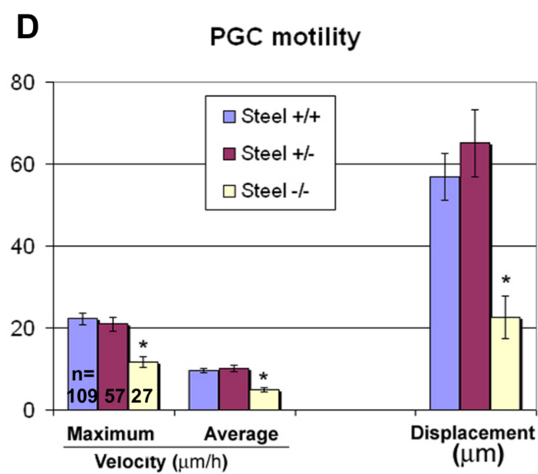


Figure 10: Effects of Ack2 blocking antibody on PGC motility at E7.5.

(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” indicates 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 is significantly lower when cultured 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) and (C). *=p<0.01, Bars represent the mean±s.e.m.





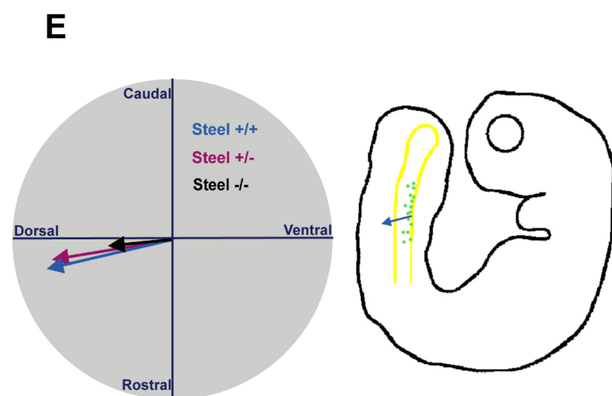


Figure 11: Effects of Steel factor on PGC migration at E9.0.

Movie frames of E9.0 *Oct4PE:GFP* embryos with different *Steel* genotypes are shown in

(column A) t=0, and (column B) t=420 minutes. PGCs are green. Scale bars in (A) and (B): 100 μm. (C) Shows 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” indicates 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 that directionality of PGC migration (colored arrows correspond to colored text for genotype). 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.

Chapter III

The role of membrane-bound and soluble Steel factor in PGC development

Membrane-bound Steel factor maintains a high local concentration for mouse primordial germ cell motility, and defines the region of their migration

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Published in *PLoS ONE 6(10): e25984.*

Abstract

Steel factor, the protein product of the *Steel* locus in the mouse, is a multifunctional signal for the primordial germ cell population. We have shown previously that its expression accompanies the germ cells during migration to the gonads, forming a “travelling niche” that controls their survival, motility, and proliferation. Here we show that these functions are distributed between the alternatively spliced membrane-bound and soluble forms of Steel factor. The germ cells normally migrate as individuals from E7.5 to E11.5, when they aggregate together in the embryonic gonads. Movie analysis of *Steel-dickie* mutant embryos, which make only the soluble form, at E7.5, showed that the germ cells fail to migrate normally, and undergo “premature aggregation” in the base of the allantois. Survival and directionality of movement is not affected. Addition of excess soluble Steel factor to *Steel-dickie* embryos rescued germ cell motility, and addition of Steel factor to germ cells in vitro showed that a fourfold higher dose was required to increase motility, compared to survival. These data show that soluble Steel factor is sufficient for germ cell survival, and suggest that the membrane-bound form provides a higher local concentration of Steel factor that controls the balance between germ cell motility and aggregation. This hypothesis was tested by addition of excess soluble Steel factor to slice cultures of E11.5 embryos, when migration usually ceases, and the germ cells aggregate. This reversed the aggregation process, and caused increased motility of the germ cells. We conclude that the two forms of Steel factor control different aspects of germ cell behavior, and that membrane-bound Steel factor controls germ cell motility within a “motility niche” that moves through the embryo with

the germ cells. Escape from this niche causes cessation of motility and death by apoptosis of the ectopic germ cells.

Introduction

Primordial germ cells (PGCs) are the embryonic precursors of the gametes, and therefore play a central role in biology. In mice, PGCs are first specified in the extraembryonic allantois around E7.25, as a small group of cells that express characteristic markers such as alkaline phosphatase (AP) and Stella (Hayashi et al., 2007; Ohinata et al., 2005). They then migrate proximally into the posterior region of the embryo and become incorporated into the developing hindgut (Anderson et al., 2000; Gu et al., 2009). Between E9.0 and E9.5, PGCs emigrate from the dorsal aspect of the hind gut and then migrate laterally through the dorsal body wall mesenchyme where they cease migration and aggregate together into clumps in the embryonic gonads (Molyneaux et al., 2001). During the four-day period of PGC migration, the embryo is undergoing rapid growth and organogenesis. The embryo grows more than six-fold in length during this period, and new tissues arise around PGCs as they migrate. PGC behavior, including proliferation, survival, motility and homing, are likely to be controlled by short-range signals in such a rapidly changing environment. We have shown previously that Steel factor provides such a short-range signal throughout the migratory period (Gu et al., 2009; Runyan et al., 2006; Stallock et al., 2003).

Mutations at the *Dominant white spotting* (*W*) and *Steel* (*Sl*) loci in the mouse cause failure of PGCs to colonize the embryonic gonads (Bennett, 1956; Besmer et al., 1993;

Buehr et al., 1993; Mahakali Zama et al., 2005; McCoshen and McCallion, 1975; Mintz and Russell, 1957). The *W* gene encodes c-kit, a cell-surface receptor tyrosine kinase expressed by PGCs throughout migration. The *Steel* gene encodes its protein ligand; Steel factor (also known as Stem Cell Factor, Kit-ligand, or Mast Cell Growth Factor). Previous studies from our laboratory and others revealed that Steel factor is an essential survival factor for PGCs in vitro (Dolci et al., 1991; Godin et al., 1991; Matsui et al., 1991). More recently, we have shown that it also controls survival in vivo, and that its down-regulation in the midline at E10.5 causes death by apoptosis of ectopic PGCs still located there (Runyan et al., 2006). In addition to survival, Steel factor also controls motility and proliferation of the PGCs during migration (Gu et al., 2009; Runyan et al., 2006). Steel factor is expressed by somatic cells immediately surrounding PGCs throughout their migration, from the time of PGC specification in the allantois, to the time they colonize the genital ridges, and is lost from these tissues after PGCs have moved on to the next location. Since PGC survival, proliferation and motility are all controlled by Steel factor signaling throughout migration (Gu et al., 2009), this suggests the existence of a “traveling niche” in which the Steel factor-expressing cells provide a continuous short-range signal to control different aspects of PGC behavior throughout their migration. This observation is supported by the fact that down-regulation of Steel factor in the midline at E10.5 causes the death by apoptosis of any ectopic germ cells still present in the midline (Runyan et al., 2006). However, the mechanism by which Steel factor plays such different roles in PGC behavior is still not understood.

The precursor RNA of Steel factor can be alternatively spliced to produce both soluble and transmembrane forms of the protein. The soluble form contains an extracellular

domain with a proteolytic cleavage site which allows release of the extracellular region of the protein. The membrane-bound form lacks this domain, and therefore remains associated with the cell surface (Flanagan et al., 1991; Huang et al., 1992). Previous studies have shown that both forms of Steel factor are capable of activating c-kit, though the membrane-bound form tends to induce more persistent tyrosine kinase activation than the soluble form (Miyazawa et al., 1995). In some cell types, the two forms are thought to play different roles. For example, mast cells (which express c-kit) undergo initial adhesion to COS cells transfected with membrane-bound, but not soluble, Steel factor cDNA (Flanagan et al., 1991). Similarly, membrane-bound Steel factor stimulates adhesion between hematopoietic stem cells and extracellular matrix (Kinashi and Springer, 1994). It has also been reported that membrane-bound Steel factor, but not the soluble form, induces long-term proliferation of $CD34^+$ cells (Friel et al., 2002). In neural crest migration, the soluble form is essential for the initiation of melanocyte precursor dispersal onto the lateral pathway, while the membrane-bound form is required for their subsequent survival in the dermis (Wehrle-Haller and Weston, 1995). It is not known why the membrane-bound and soluble forms have different properties in these model systems, or in most cases whether they do, in fact, play different roles in vivo. Steel factor has multiple functions in PGC behavior during migration, including survival, proliferation and motility, and it is not yet clear whether these different functions require specific forms of Steel factor.

The *Steel-dickie* (*Steel*$^{d/d}$) mutation offers an opportunity to address this issue. This mutation carries a deletion of sequences that encode the intracellular and transmembrane domains of Steel factor, and therefore only produces a soluble

truncated protein that lacks both the cytoplasmic and transmembrane domains (Brannan et al., 1991). *Steel*$^{d/d}$ mice are sterile, as they are in *Steel-null* (*Steel*$^{-/-}$) mutants that lack the entire Steel gene. However, more PGCs are found in the gonad primordium in *Steel*$^{d/d}$, suggesting some activity of the Steel factor signaling pathway remains in these mutants through its soluble form (Brannan et al., 1991; Mahakali Zama et al., 2005). In addition, *Steel*$^{d/d}$ mice are viable, which has suggested that Steel factor plays some role in PGC behavior that is not shared with hematopoietic cells. This has been a puzzling aspect of the *Steel*$^{d/d}$ mutation, since Steel factor is known to be a survival factor for both cell types.

In the present study, we first show by RT-PCR that both the membrane-bound and the soluble forms of Steel factor transcripts are expressed along the PGC migratory route. We then show that there is no significant change in PGC numbers between *Steel*$^{d/d}$ embryos and their wild type littermates at E7.5, suggesting that PGC survival requires only the soluble form of the protein at this stage. However, PGC numbers start to decrease in E8.0 *Steel*$^{d/d}$ embryos. Therefore some explanation must be found for the fact that they survive at E7.5, but die at later times. Time-lapse analysis of embryos at E7.5 shows that PGCs in *Steel*$^{d/d}$ embryos migrate at dramatically reduced rates, and instead aggregate into clumps in the allantois. As a result, they fail to migrate normally into the hindgut. This change in motility is the same as previously seen in Steel-null embryos, showing that PGC migration specifically requires the membrane-bound form of the protein. There are two possible reasons for this; either membrane-bound Steel factor confers a specific function on the cells adjacent to germ cells, perhaps altering adhesive properties for example, or it simply provides a higher local concentration of the

protein immediately adjacent to the PGCs. To distinguish between these possibilities, we added increasing concentrations of recombinant soluble Steel factor to *Steel*$^{d/d}$ embryos cultured at E7.5, and showed that it rescued the defects of PGC motility in cultured *Steel*$^{d/d}$ embryos. Moreover, addition of soluble recombinant Steel factor into wild type embryo slices at E11.0 resulted in the reacquisition of motility in PGCs that had ceased migrating and aggregated in the genital ridges, and migration away from these sites of aggregation. Together, these data suggest that the primary role of membrane-bound Steel factor for PGC migration in vivo is to maintain an optimal local ligand concentration that promotes motility and inhibits aggregation in a restricted area of the embryo. It also shows that Steel factor controls different aspects of germ cell behavior at different concentrations. Low concentrations are sufficient to maintain survival, while higher local concentrations provided by the membrane-bound form are required for motility.

Results

Both soluble and membrane-bound forms of Steel factor are present around PGCs throughout migration

Our previous study showed that both forms of Steel factor mRNA were present in the allantois at E7.5 by RT-PCR (Gu et al., 2009). To investigate the expression pattern at later stages, we extracted RNA from tissues occupied by PGCs at all stages of migration and performed RT-PCR using primers that distinguish the two forms of Steel factor transcripts. Both transcripts were found in E7.5 allantoides, E8.5 hindgut

endoderm, E9.5 hindguts and dorsal body walls, and E10.5 genital ridges (**Fig. 12A**), showing that both forms of Steel factor are present around PGCs throughout migration.

The *Steel-dickie* mutant makes only the soluble form. It has been shown previously by northern analysis that the *Steel-dickie* allele is expressed at the mRNA level at similar levels as the wild type (Brannan et al., 1991; Flanagan et al., 1991); however, the protein levels were not previously assayed. We performed a western blot analysis of supernatant fractions of E12.5 embryos of different genotypes after triton extraction and high speed spin to remove the membranes. As shown in **Fig. 12B and 12C**, there is no loss of Steel factor protein in *Steel*$^{d/d}$ embryos compared to their littermates, suggesting that there is no defect in mRNA translation in the *Steel*$^{d/d}$ embryos.

Soluble Steel factor is sufficient to maintain PGC number at E7.5

Steel factor controls several aspects of PGC behavior, and both forms of the protein surround them during migration. This suggests the hypothesis that different forms of the protein play different roles. To test this, we used *Steel*$^{d/d}$ embryos, in which the membrane-bound form is not present. We bred the *Steel-dickie* mutation into the Stella-GFP mouse line in which PGCs express GFP under the control of the *Stella* promoter. We then counted PGC numbers in bisected E7.5 *Steel*$^{d/d}$*, Stella-GFP* embryos under the confocal microscope. Interestingly, there was no significant decrease of PGC numbers in the E7.5 *Steel*$^{d/d}$ embryos (23.3±2.1 per embryo) compared to wild type (26±3.6 per embryo, p=0.229) and heterozygous littermates (25.4±4.5 per embryo, p=0.446). This result is shown in **Fig. 13A** and indicates that the soluble Steel factor produced in the *Steel-dickie* mutants is sufficient to maintain PGC numbers immediately

after their specification in the allantois. These data should be compared with those from Steel-null embryos at the same stage (Gu et al., 2009), in which the PGC numbers were already reduced by E7.5, and could be rescued by removing an allele of *Bax*, indicating that they were already starting to undergo apoptosis in the absence of Steel factor.

To further confirm that soluble Steel factor is sufficient to maintain PGC number, we cultured isolated PGCs on primary MEFs derived from Steel-null embryos (ΔMEF), and added increasing concentrations of soluble recombinant mouse Steel factor into the culture medium. After culture for 24 hours, PGC numbers in Steel factor-treated cultures were significantly higher than those without Steel factor (**Fig. 13B**). There were no obvious differences between different Steel factor concentrations, and the lowest concentration (50ng/ml) was enough to show the effect (**Fig. 13B**). To test whether membrane-bound Steel factor alone is able to maintain PGC number, we cultured isolated PGCs on M220 cells which express only membrane-bound Steel factor (Matsui et al., 1991; Toksoz et al., 1992). PGC numbers on M220 cells were similar to those on ΔMEF cells with added Steel factor after 24 hours culture (**Fig. 13B**), and significantly higher than those in the Steel factor-free groups (**Fig. 13B**). These data show that either form of Steel factor is capable of maintaining PGC survival in culture.

Steel-dickie mutants are sterile, and the number of PGCs colonizing the genital ridges is dramatically reduced (Brannan et al., 1991; Mahakali Zama et al., 2005). To investigate when PGC number starts to decrease, we counted PGC numbers in E8.0 embryos. There were 50.5±7.5 PGCs per embryo found in wild type embryos, and this number was significantly reduced to 32±9.0 in *Steel*$^{d/d}$ mutants (p=0.02) (**Fig. 13C**). Given that soluble Steel factor is sufficient to maintain PGC numbers at E7.5, the reduction of PGC

number may be caused by a decrease in proliferation. Another possibility is that the PGCs fail to migrate in *Steel*$^{d/d}$ embryos, which causes some of them to remain in the allantois when Steel factor is turned off there (Gu et al., 2009), and thus die by apoptosis, in the same manner which occurs later to PGCs left in the midline (Runyan et al., 2006).

Steel-dickie mutants show defective PGC motility

To test whether membrane-bound Steel factor is required for normal PGC migration, time-lapse analyses were carried out using sagittally bisected E7.5 *Stella-GFP*$^{+}$*, Steel*$^{d/d}$ embryos and their littermates. The time-lapse movies are accessible as Video S1-S3, and the examples of movie frames are shown at time=0 and time=6.0 hours (**Fig. 14A and B**). The movements of individual PGCs were manually traced in serial confocal images from each embryo. Tracings were taken only from PGCs that remained in focus and could be distinguished from others for the duration of the filming (**Fig. 14C**). The PGCs aggregate together in *Steel*$^{d/d}$ embryos; therefore, not all of the PGCs in *Steel*$^{d/d}$ embryos were suitable for analysis, and therefore fewer tracks are shown from *Steel*$^{d/d}$ embryos in **Fig. 14C**. The velocities and displacements of PGCs were calculated based on these tracings (**Fig. 14D**). Although PGC numbers in *Steel*$^{d/d}$ embryos remained similar to those of wild type littermates, the maximum velocity (51.7±3.2 μm/h), the average velocity (22.8±1.5 μm/h) and the displacement (34.3±3.7 μm) of PGCs in E7.5 *Steel*$^{d/d}$ embryos were all significantly reduced when compared to wild type (Maxi V=65.9±4.5 μm/h, $p=3.3 \times 10^{-6}$; Ave V=29.2±1.2 μm/h, $p=2.3 \times 10^{-7}$; Dis=72.7±9.2 μm, $p=8.5 \times 10^{-11}$). Since soluble Steel factor was present in these embryos, and PGC

numbers did not change, these data show that the membrane-bound form of Steel factor is specifically required for normal PGC motility at the beginning of their migration.

We then scored the numbers of PGCs that migrated across the boundary from the extraembryonic allantoic region (EEM) into the posterior end of the embryo (PEM) in embryos of different genotypes. As a result of reduced PGC motility, the percentage of PGCs that reached the posterior epiblast was significantly decreased in $Steel^{d/d}$ embryos (30.8±2.9%) when compared with wild type (50.5±6.0%, p=0.002) and heterozygous littermates (51.7±10.4%, p=0.009) (**Fig. 14E**). These data demonstrate that decreased motility of the PGCs in the absence of membrane-bound Steel factor leads to a dramatic decrease in the numbers of PGCs reaching the hindgut endoderm.

Unlike PGCs in wild type embryos which were highly motile and moved individually, PGCs in E7.5 *Steel*$^{d/d}$ embryos aggregated together into clumps in the proximal region of the allantois (**Fig. 14A, B**) in the same way as previously seen in *Steel*$^{-/-}$ embryos (Gu et al., 2009). The percentages of PGCs in clusters of more than 3 PGCs were scored in E7.5 embryos of each genotype (**Fig. 14F**). In wild type embryos, around 6.5±4.5% of PGCs form clusters. This number was significantly increased to 36.2±4.7% in the Steel-dickie mutants ($p=2.2x10^{-6}$).

We then looked at the PGC locations in E8.0 embryos, when PGCs normally enter into the hind gut and migrate along it anteriorly (as shown in the cartoon in **Fig. 14G**, arrow indicates the direction of migration). In wild type embryos, most PGCs were found localized along the hindgut diverticulum extending anteriorly from its posterior margin towards the mid gut (**Fig. 14G**). In contrast, the majority of PGCs in *Steel*$^{d/d}$ embryos were observed in a big cluster at the junction of the posterior margin of the hindgut

diverticulum and the allantois, indicating a failure of their anterior migration at this stage (**Fig. 14G**). These data further confirm that membrane-bound Steel factor is required for normal PGC migration in vivo.

Addition of soluble recombinant mouse Steel factor rescues PGC motility defects at E7.5

There are two obvious possible mechanisms whereby membrane-bound Steel factor could be required for PGC motility. First, it may play a specific role on the cell surface of the secreting cell, perhaps mediating the adhesiveness of PGCs to their surrounding cells. Second, it may sustain a higher local ligand concentration, which is essential for normal PGC motility, while the concentration of soluble Steel factor is lower, due to diffusion away from the secreting cells. To distinguish between the two possibilities, we added increasing amounts of soluble recombinant Steel factor to *Steel-dickie* mutant embryos at E7.5. If the Steel factor specifically functions as a membrane component, this should not rescue the motility defects seen in *Steel-dickie* mutant embryos. If the primary function of membrane-bound Steel factor is to maintain a high level of ligand immediately around the PGC, it should rescue the motility defects. The time-lapse movies are accessible as Video S4 and S5, and the examples of movie frames are shown at time=0 and time=6.0 hours (**Fig. 15A and B**), and the velocities and displacements of PGC migration were calculated based on the tracks of individual PGC movement (**Fig. 15C**).

Addition of 200 ng/ml Steel factor significantly increased the motility of PGCs in E7.5 *Steel*$^{d/d}$ embryos (**Fig. 15D**). The maximum velocity was increased from 51.7±3.2 μm/h

to 70.2±4.3 μm/h ($p=2.4 \times 10^{-9}$), the average velocity was increased from 22.8±1.5 μm/h to 32.7±1.5μm/h ($p=6.0 \times 10^{-15}$), and the displacement of PGC movement was increased from 34.3±3.7 μm to 60.9±7.2 μm ($p=2.2 \times 10^{-8}$). These findings suggest that the primary role of membrane-bound Steel factor is to maintain an optimal local ligand concentration for PGC migration, and that Steel factor activates two different functions in PGCs at different concentrations.

To further confirm that higher concentrations of Steel factor activate PGC motility, we cultured PGCs in vitro on Steel-null MEFs with the addition of increasing concentrations of soluble Steel factor. In contrast to the data in **Fig. 13B**, which show that 50 ng/ml soluble Steel factor was sufficient for maintaining PGC number (there were no differences in the survival effects between different concentrations of Steel factor), higher doses of added Steel factor (≥ 200 ng/ml) were required to show a significant increase of both PGC velocity and displacement compared to those without Steel factor (**Fig. 15E**, $p<0.05$) (There were no significant differences in the motility effects between the concentrations higher than 200ng/ml). Together, these data suggest that Steel factor controls different aspects of germ cell behavior at different concentrations. Low concentrations are sufficient to maintain PGC survival, while higher concentrations provided by the membrane-bound form activate both survival and motility.

Global addition of soluble recombinant mouse Steel factor caused distal PGCs to migrate randomly at E7.5

The addition of soluble Steel factor to *Steel*$^{d/d}$ embryos in culture dramatically increased PGC motility (**Fig. 15**). An interesting feature of this was revealed by indicating the

overall direction of their movements by drawing an arrow to link the start point and the end point of individual PGC movement tracks. The results are shown in **Fig. 16A** (Column I, II, and III are representative images from 3 different embryos). Without Steel factor addition, most of PGCs migrated more slowly than in wild type embryos, but in the correct direction; proximally towards the posterior region of the embryo. However, in *Steel*$^{d/d}$ embryos with added soluble Steel factor, some PGCs located in distal portion of the allantois moved in the wrong directions, while PGCs closer to posterior embryo migrated correctly (**Fig. 16A**). The most likely explanation for this is that distally located PGCs are out of range of the directionality cues that guide PGCs into the posterior endoderm at this stage. These PGCs would normally die by apoptosis as Steel factor is turned off, but are maintained by the addition of exogenous Steel factor. The alternative explanation, that membrane-bound Steel factor itself provides guidance information seems less likely, since the germ cells closest to the embryo do continue to migrate in the right direction.

To investigate whether a globally higher concentration of Steel factor has the same effect in a wild type background, we added 200ng/ml soluble recombinant Steel factor into E7.5 wild type embryos and analyzed the directions of PGC migration. PGCs in wild type embryos with added Steel factor also showed randomized migration only when they were located in the most distal region of the allantois, while PGCs without Steel factor addition predominantly migrate towards the correct target (**Fig. 16B**). We also traced individual PGC movements in wild type embryos after addition of soluble Steel factor. The maximum velocity and average velocity of PGC motility were accelerated with Steel factor addition ($p=1.7\times10^{-4}$, 1.1×10^{-7}, respectively) (**Fig. 16C**), suggesting that

PGC motility responds to Steel factor in a dose-dependent manner. There was no significant increase in the displacement of PGC migration with Steel factor addition (p=0.44). PGCs in embryos with Steel factor addition migrated forwards and backwards, but were still in a restricted region of the embryo.

Global addition of soluble recombinant mouse Steel factor caused PGCs in the genital ridges to reacquire motility.

PGCs aggregate together and stop migrating when they reach the genital ridges around E11.0 (Molyneaux et al., 2001). The requirement for Steel factor for motility raised the hypothesis that the cessation of PGC migration could be due to down-regulation of Steel/c-kit signaling. To test this hypothesis, we added 200 ng/ml soluble recombinant Steel factor into E11.0 wild type embryo slices and made time-lapse movies. The movies are available as Video S6 and S7, and the examples of movie frames are shown at time=0, 6.0, and 12.0 hours (**Fig. 17 A-C**). Without addition of Steel factor, PGCs moved slowly and gradually coalesced with each other to form big clusters in the genital ridges within this time period. In slices with global addition of soluble Steel factor, there was an obvious acceleration of the motility in some of the PGCs, which migrated out of the genital ridges into surrounding tissues. These data suggest that the normal cessation of PGC migration, and their aggregation, may be caused by regulation of Steel factor signaling. One obvious possibility is that membrane-bound Steel factor expression is down regulated in the genital ridges. To test this, we dissected the genital ridges from E10.5 embryos, and both genital ridges and dorsal midline regions from E11.5 embryos. qPCR was used to assay the expression levels of both forms (**Fig.**

17D). Consistent with previously published data, the expressions of both forms of Steel mRNA were significantly reduced in the E11.5 midline compared to E11.5 genital ridges ($p<0.05$) (Runyan et al., 2006). However, no decrease in either form of Steel mRNA was observed in E11.5 genital ridges compared to E10.5 genital ridges. Instead, the membrane-bound mRNA was significantly increased ($p<0.01$). This suggests that the cessation of PGC migration is not due to reduced expression of membrane-bound Steel factor in the genital ridges at E11.5.

E-cadherin is expressed by migrating PGCs, and blockade of its function by the ECCD-1 blocking antibody is known to cause a similar spreading of the PGCs into adjacent tissues at E11.5 (Bendel-Stenzel et al., 2000). To examine whether the addition of Steel factor promotes PGC motility through down-regulating E-cadherin expression, we applied antibody staining for E-cadherin on embryo slices after treatment with soluble Steel factor. As shown in **Fig. 17E and F**, there is no significant change of E-cadherin expression in PGCs between the groups, suggesting that the maintenance of motility, rather than aggregation, by Steel factor is not by control of E-cadherin expression.

Discussion

Many studies in the past have reported that Steel factor plays an essential role in PGC development (Dolci et al., 1991; Godin et al., 1991; Mahakali Zama et al., 2005; Matsui et al., 1991; McCoshen and McCallion, 1975; Runyan et al., 2006). Most recently, work from our lab revealed that the Steel factor-expressing cells establish a “traveling niche” surrounding migratory PGCs which provides a short-range signal to regulate PGC

survival, proliferation and motility throughout their migration (Gu et al., 2009). However, how these different behaviors are regulated by the same ligand, and how Steel factor functions as a short-range signal remain uncertain. In other cell populations, the two forms of Steel factor, membrane-bound and soluble form, have been shown to have different functions (Flanagan et al., 1991; Friel et al., 2002; Kinashi and Springer, 1994; Miyazawa et al., 1995; Wehrle-Haller and Weston, 1995), although the mechanisms whereby they do so have remained unclear. This suggested that different aspects of PGC behavior might also be regulated through different forms of Steel factor. The *Steel-dickie* mutation, which only produces soluble Steel factor due to a deletion in cytoplasmic and transmembrane domain of Steel allele, offers a powerful tool to address this question. Previous work has shown that transcription from the *Steel-dickie* allele is at the same level as wild type. Here we showed, by western blotting using supernatant fractions of E12.5 whole embryos, that Steel factor protein is produced in the mutant embryos, and that there is an increased amount of the soluble form, compared to the wild type embryo. This is the expected result if the mutation generates only the soluble form, the translation levels are similar, and if protein stability is not altered. In this case, all the translated protein will be in the soluble form in the *Steel-dickie* mutant embryo. To test the functions of the two forms, we analyzed PGC number and motility in E7.5 $Steel^{d/d}$ embryos. The results showed that the soluble form of Steel factor present in $Steel^{d/d}$ embryos maintained the survival but not the motility of PGCs. This shows that Steel factor does not need to be membrane-bound for PGC survival, but that the membrane-bound form is essential for their motility.

The results presented here also offer a mechanistic explanation of the somewhat

puzzling effects of the *Steel-dickie* mutation. It has long been known that this mutant is viable but sterile, implying that membrane-bound Steel factor plays different roles in the PGCs and hematopoietic cell lineages. However, Steel factor is known to be a survival factor in both cell types (Dolci et al., 1991; Galli et al., 1992; Godin et al., 1991; Iemura et al., 1994; Yee et al., 1994), and the soluble Steel factor protein present in *Steel*$^{d/d}$ embryos is clearly sufficient to maintain their survival (shown in this work). We propose that the difference is due to the fact that membrane-bound Steel factor provides a niche for maintaining active motility, and in its absence, the PGCs lose motility, and do not move to the next location in the pathway. We have shown previously that Steel factor maintains PGC survival only at short range (Runyan et al., 2006), and that expression of Steel factor moves along the PGC migratory pathway (Gu et al., 2009). With reduced motility, PGCs in *Steel*$^{d/d}$ embryos will not keep up with the wave of Steel factor expression, and will die because this essential survival factor is turned off before they have moved to the next site of expression. All the observations to date support this hypothesis. First, in E7.5 *Steel*$^{d/d}$ embryos, no obvious reduction of PGC number were observed, implying that the soluble Steel factor produced in the *Steel-dickie* mutants is adequate to maintain PGC survival at this stage. Although there is no significant decrease in cell number, PGCs in *Steel*$^{d/d}$ embryos show remarkably reduced motility and instead form a large clump of non-motile cells. Second, *Steel*$^{d/d}$ PGCs remain clustered at the boundary between allantois and hindgut at E8.0, while most PGCs in wild type embryos have already migrated anteriorly along the hindgut endoderm. Third, many PGCs are found in ectopic locations with apoptotic morphology in *Steel*$^{d/d}$ embryos (Mahakali Zama et al., 2005).

The finding that addition of soluble Steel factor to E7.5 *Steel*$^{d/d}$ embryos is capable of restoring PGC motility is interesting, because it suggests the membrane-bound Steel factor is essential to maintain an optimal local concentration in a defined region, rather than play a distinct role to regulate PGC motility. The addition of large amounts of soluble recombinant Steel factor can restore PGC motility in *Steel*$^{d/d}$ embryos, showing that the local ligand concentration is crucial for Steel factor regulation. In support of this view, a previous study showed that in vivo administration of soluble recombinant Steel factor into *Steel*$^{d/-}$ mice caused a notable reduction of the severity of their macrocytic anemia and a great increase of mast cell number at the injection sites (Zsebo et al., 1990). The high levels of Steel factor required for PGC motility is normally maintained not by increased synthesis, but by maintenance of a high local concentration by presenting it on the membranes of adjacent cells in a restricted area, thus defining the region in which PGCs can maintain their motility. Movement of this "motility niche" would thus create the pathway along which PGCs are able to move.

When treating the E7.5 *Steel*$^{d/d}$ and wild type embryos with added soluble Steel factor, some PGCs in the most distal region of allantois tended to migrate randomly, instead of moving proximally towards the posterior epiblast. It may be that these PGCs are already too far away from the normal chemotactic signals, which attract PGCs to the posterior epiblast. Without the added soluble Steel factor, these cells would normally die by apoptosis because they are in an ectopic location where Steel factor expression is being turned off. However, the globally added soluble Steel factor allowed them to survive and migrate randomly. These findings accentuate the importance of maintaining a high local concentration only in a defined region to make sure that PGCs which

receive the chemotactic signals maintain motile, whereas the ones out of the range of direction cues cease motility and die.

One observation that remains unexplained is the formation of large clumps of PGCs in the allantois in both *Steel*$^{-/-}$ and *Steel*$^{d/d}$ embryos. We have previously shown that PGCs activate expression of E-cadherin when they leave the hindgut. There is a failure of the normal aggregation of germ cells as they enter the genital ridges when E-cadherin function is blocked (Bendel-Stenzel et al., 2000). In a previous study of *Steel-null* mutant embryos, we stained for E-cadherin protein in PGCs of different genotypes to check whether PGC clumping at E7.5 was due to an upregulation of E-cadherin in the absence of Steel factor. However, no significant change in E-cadherin expression level was observed (Gu et al., 2009). To further confirm that E-cadherin is not responsible for the increased cluster formation and decreased motility, we treated wild type E7.5 embryos with Ack2, the inhibitor of c-kit receptor, and ECCD1, an antibody which blocks the function of E-cadherin, to test whether loss of E-cadherin function could rescue the defects caused by loss of Steel factor signaling. Compared to embryos which were only treated with Ack2, the dual treatment group did not show any significant change in motility, displacement and cluster formation (data not shown), indicating the defects shown in *Steel*$^{-/-}$ and *Steel*$^{d/d}$ embryos are not due to a premature up-regulation of E-cadherin function. The mechanism of PGC cluster formation in *Steel-null* and *Steel-dickie* mutants needs to be investigated in future studies.

It is important to maintain PGC motility during their migration. It is also crucial to ensure PGCs stop migration when they coalesce in the genital ridges. Interestingly, addition of soluble Steel factor into embryo slices at E11.0 resulted in reacquisition of motility in

PGCs, indicating that PGCs can still respond to high levels of Steel factor, and that modulation of Steel/c-kit signaling may control the ending of migration. However, there is no decrease in the expression of Steel factor mRNA in E11.5 genital ridges as determined by real-time RT-PCR, suggesting that the ending of PGC migration is not due to down-regulation of Steel factor expression at transcriptional level. There are a number of possible explanations for this paradoxical finding. First, PGCs could still be motile in the E11.5 genital ridge, but are constrained by other factors. Second, there may be less membrane-bound Steel factor at the protein level. Third, an inhibitor of Steel factor signaling could be present in the genital ridges, which acts independently of Steel factor expression level. Fourth, when PGCs arrive in the genital ridges, the amount of membrane-bound Steel factor available per PGC may decrease. As PGCs adhere to each other, they will be less likely to be adjacent to a membrane-bound Steel factor-expressing somatic cell. Whatever the mechanism of clumping, it is overcome by addition of increased soluble Steel factor, so is likely to include the inhibition of Steel factor signaling. E-cadherin function has been shown to be essential for PGC coalescence in the genital ridges (Bendel-Stenzel et al., 2000). Although there is no obvious change in E-cadherin expression in PGCs with Steel factor addition, we cannot exclude the possibility that Steel factor promotes PGCs to migrate out of the cluster by interrupting E-cadherin function without affecting its membrane expression. Further studies need to be performed to address these questions.

These studies reveal two important aspects of the “traveling niche”. First, the Steel factor activates two different functions in PGCs at different concentrations, and second that the moving expression of membrane-bound Steel factor maintains PGC motility in a

defined region of the embryo. PGCs escaping from this region, or that get left behind on the migratory route, cease motility, and are removed by apoptosis as the expression wave moves on towards the genital ridges. Thus the colonization of the genital ridges is as much due to the control of survival and motility as it is to chemotactic signals.

Materials and Methods

Ethics Statement

All experiments were carried out in strict accordance with institutional guidelines under Institutional Animal Care and Use Committee (IACUC) approval at Cincinnati Children's Hospital Research Foundation (CCHRF). IACUC at CCHRF approved the study described in this manuscript with Animal Use Protocol number 9A02019.

Mouse breeding, embryo preparation and genotyping

Stella-GFP transgenic mice (Payer et al., 2006) on a B6/CBA background were crossed with *Steel-dickie* ($Kitl^{Sl\text{-}d}$ or $Steel^{d/d}$) heterozygotes (Jackson Laboratories, Stock number 000160) on a C57BL/6J background to obtain mice that were $Stella\text{-}GFP^{+}$, $Steel^{d/+}$. These mice were interbred to yield $Stella\text{-}GFP^{+}$, $Steel^{d/d}$ embryos. Embryonic day 0.5 (E0.5) was assumed to be noon of the morning a vaginal plug was observed. Genomic DNA was isolated from tail snips (adults), embryo halves (E7.5 embryos) and heads (E8.0 and later stages), and genotypes were determined by PCR. Genotyping primers used were as follows: Stella-GFP: F-5’TGCATCGGTAACCCACAGTA-3’, R-5’ GAACTTCAGGGTCAGCTTGC 3’; Steel-Dickie: WT-F-

5’TGCAAAGGCTGCACAGTAAG-3’, DEL-F-5’GGTGTGAGTGAGCCAAGAGC-3’, Common-R-5’CCAAGCCTTTCTCCAGTCAT-3’. Stella-GFP expression was determined by the presence of a 289 bp fragment. For Steel-dickie, wild type and deleted alleles were identified by 180 and 770 bp fragments, respectively.

Embryo and slice culture

Embryo culture was used as described previously (Gu et al., 2009). E7.5 embryos were cut sagittally into halves using a scalpel. One half of each embryo was used for culture and the other half was used for genotyping. The embryo halves were put onto millicell culture inserts pre-coated with collagen IV (BD), and the inserts were then placed into a metal stage which contains glass-bottom chambers with 600 µl culture medium (Hepes-buffered DMEM/F-12 (Gibco) medium with 0.04% lipid-free BSA and 100 U/ml penicillin/streptomycin (Gibco)). For the Steel factor addition assay, 200 ng/ml soluble mouse recombinant Steel factor (R&D Systems) was added into the embryo culture medium immediately before time-lapse analysis was started. Embryo halves were maintained in a humidity-controlled environment, and at 37°C throughout time-lapse analysis. For E11.0 embryos, embryo slices were prepared as described previously (Runyan et al., 2006), and cultured under the same condition as embryo culture.

Time-lapse analysis of migrating PGCs

Time-lapse analyses were carried out using a Zeiss LSM510 confocal system attached to a Zeiss axiovert microscope. Embryo slice images were acquired every 5 minutes for 6 hours, and movies were analyzed using NIH Image J as described (Gu et al., 2009;

Molyneaux et al., 2001). Experiments were repeated at least three times, and three to eight embryos were analyzed per group.

Isolation of PGCs

Embryos from Stella-GFP mice were harvested between E9.5 to E10.5, and tissues containing PGCs were dissected in PBS, washed 3 times in PBS, and trypsinized by adding a small volume of 0.25% trypsin-EDTA (Gibco) and incubated at 37°C for 5 minutes. Tissues were then pipetted in 200 μl PBS containing 1% Fetal Bovine Serum (FBS) (Gibco) for about 50 times using a 200 μl tip to make single cell suspension. The single cell suspension was then passed through a cell trainer cap into a 12x75 mm Falcon test tube (Falcon) and analyzed by Flow cytometry. PGCs were isolated based on their GFP expression into PGC culture medium (phenol red-free L-15 medium (Gibco) with 20% knockout serum replacement (Gibco), 0.1mM non-essential amino acids (Gibco), 0.1 mM 2-mercaptoethanol (Gibco), and 100 U/ml penicillin/streptomycin (Gibco)).

In vitro culture of PGCs

Primary mouse embryonic fibroblast cells and M220 cells (kindly provided by Dr. David A. Williams) were routinely grown in DMEM with 10% FBS (Gibco), 100 U/ml penicillin/streptomycin (Gibco), and 2mM L-glutamine (Gibco). Confluent cell monolayers were inhibited from proliferation by treatment with 5μg/ml mitomycin C (Sigma) for 2 hours at 37°C. Cells were then washed with PBS, harvested by

trypsinization, and adjusted to a concentration of 2×10^5 cells/ml, and 300 μl per chamber were plated into LabTek II 8 chamber coverglass (Nunc). Flow-sorted PGCs were added onto the treated feeder cell layers 24 hours after plating and cultured in PGC culture medium. PGC number was counted under fluorescent microscopy, and PGC motility was analyzed by time-lapse movies.

Western Blots

To detect levels of Steel factor protein, embryos were harvested at E12.5 and each embryo was homogenized with 50 µl lysis buffer (1% Triton X-100, 1mM PMSF and 1:100 dilution of protease inhibitor cocktail (PIC, Sigma) in ice-cold PBS). Lysates were cleared by centrifugation at 12,000 rpm for 5 minutes at 4^0C. The supernatants were then transferred into clean 1.5 ml microfuge tubes. 10 µl of 4x sample buffer was added to 20 µl supernatants, mixed and boiled for 5 minutes. The samples were then loaded on 12% SDS PAGE gels and separated for 2 hours at 90 volts. Gels were blotted onto nitrocellulose membranes and all the membranes were blocked with 2% BSA in PBS with 0.1% Tween 20 for 2 hours at room temperature. For Steel factor detection, membranes were incubated with goat anti-mouse Steel factor antibody (R&D Systems) at a dilution of 1:1000 in blocking buffer overnight at 4^oC. Donkey anti-goat HRP secondary antibody was used at a dilution of 1:5000. Signal detection was carried out using ECL developing solution (Amersham). As a loading control, membranes were stripped after signal detection and incubated with anti-α-tubulin antibody at 1:10,000.

RT-PCR and real-time RT-PCR

For RT-PCR analysis, allantoides from E7.5 embryos, hindguts from E8.5 embryos, hindguts and dorsal body walls from E9.5 embryos, and genital ridges from E10.5 embryos were dissected. For real-time RT-PCR, genital ridges from E10.5 and E11.5 embryos, and midline region of dorsal body walls from E11.5 embryos were dissected. RNA was extracted from the dissected tissues using RNeasy Kit (Qiagen) and reverse-transcribed using Superscript III First-Strand Synthesis Systems (Invitrogen). Regular PCR reactions were performed using Redmix Plus (GeneChoice). Real-time RT-PCR was performed using a LightCycler (Roche). Water-blank and RT-minus controls were included in all runs. All real-time RT-PCR results are presented as percentage compared with the level in E10.5 genital ridges after normalization to the expression of glyceraldehyde 3-phosphate dehydrogenase (GADPH). 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)

GAPDH: F-5'ACCACAGTCCATGCCATCAC-3', R-5'TCCACCACCCTGTTGCTGTA-3' (predicted fragment length: 452 bp)

Statistical analysis

All results are expressed as mean values ± SEM. Statistical significance was determined with two-tailed paired Student's *t*-test and a probability of $p<0.05$ was considered to be statistically significant.

Acknowledgments

The authors would like to thank Dr. David A. Williams from Children's Hospital Boston for providing M220 cells.

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Supplementary Materials

Supplementary movies are available at:

http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0025984

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Figures and Figure Legends

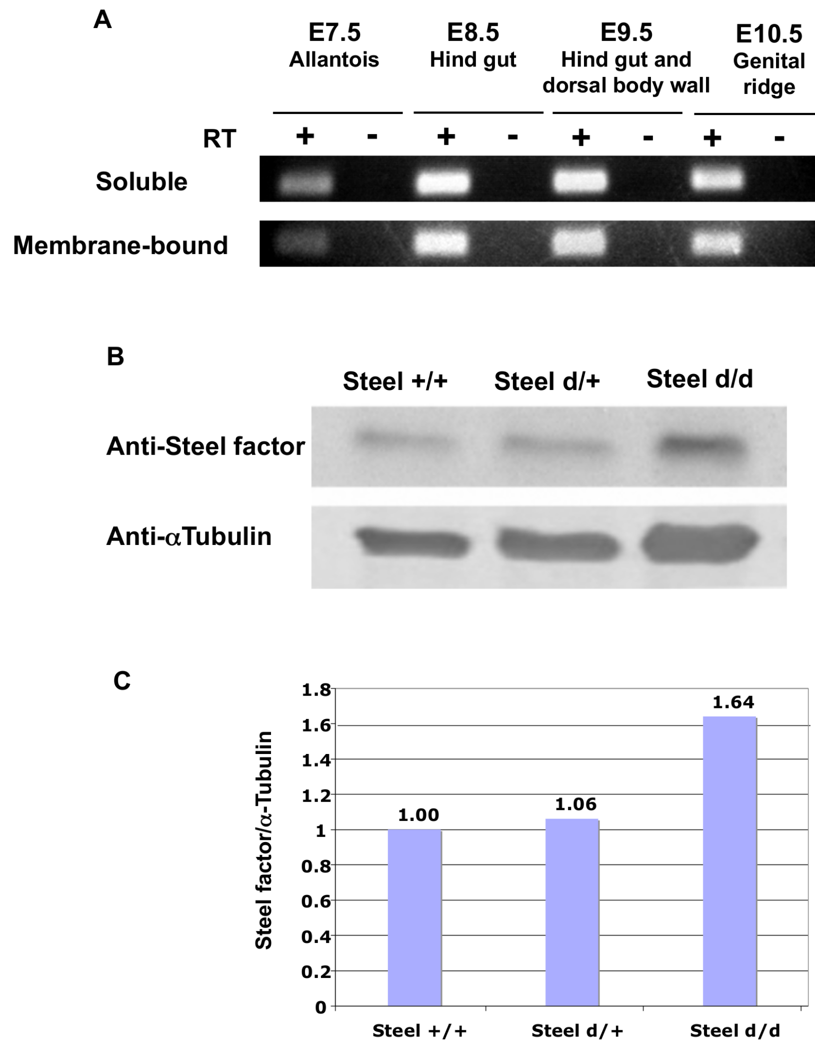


Figure 12: Expression of Steel factor in mouse embryos.

(A) RT-PCR analysis of membrane-bound and soluble Steel factor. cDNA was prepared from dissected E7.5 allantois, E8.5 hind gut, E9.5 hind gut and dorsal body wall, and E10.5 genital ridge. (B) Expression levels of soluble Steel factor protein in E12.5 embryos of different *Steel-dickie* genotypes measured by western blot. (C) Densitometric analysis of western blots in (B). α-tubulin antibody was used as a loading control.

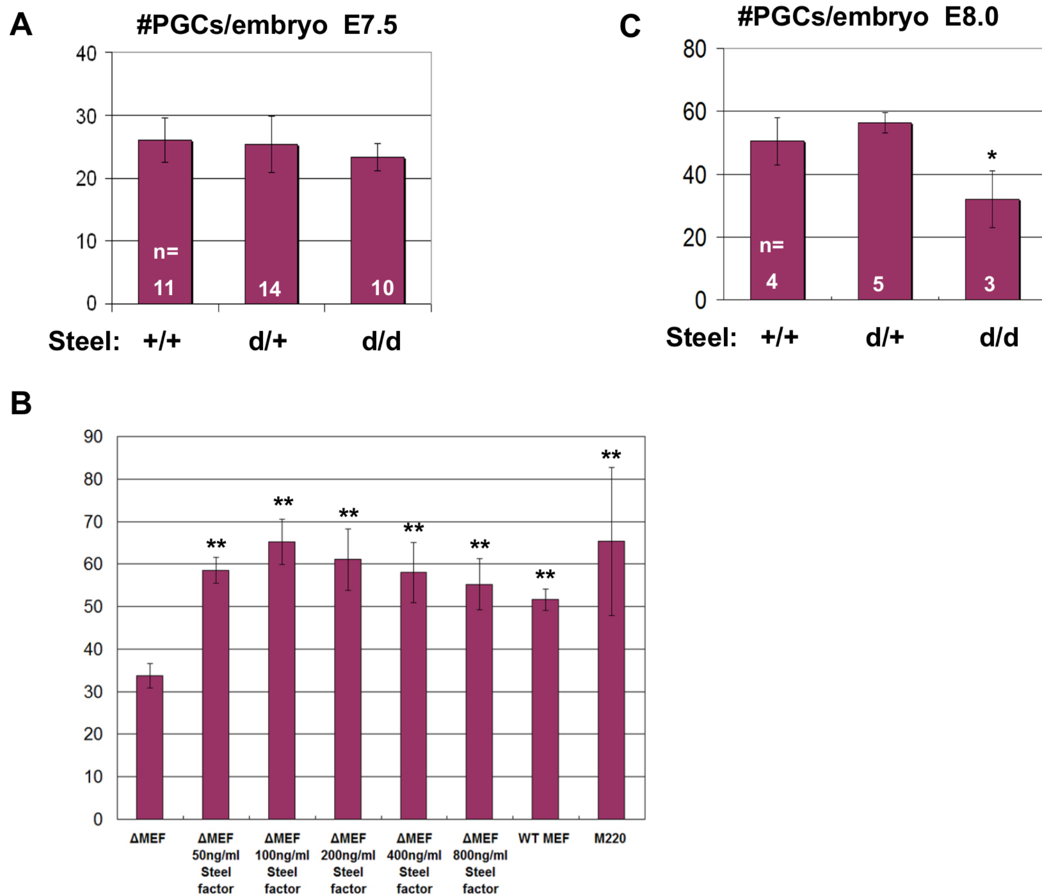
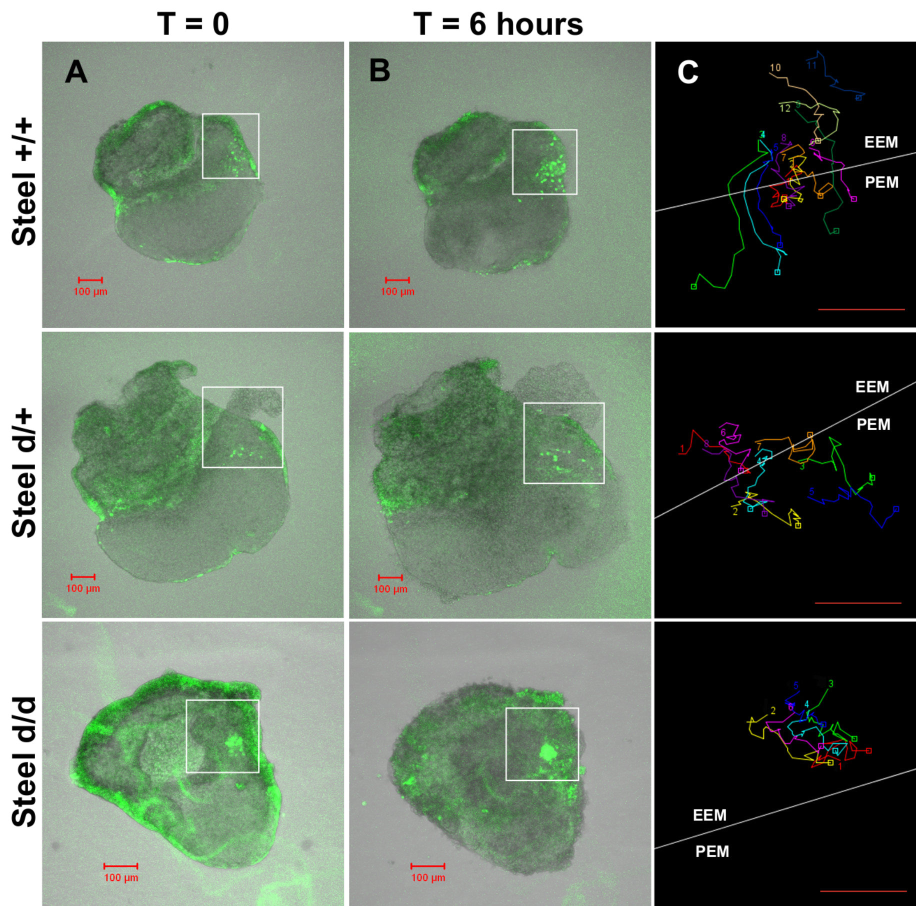
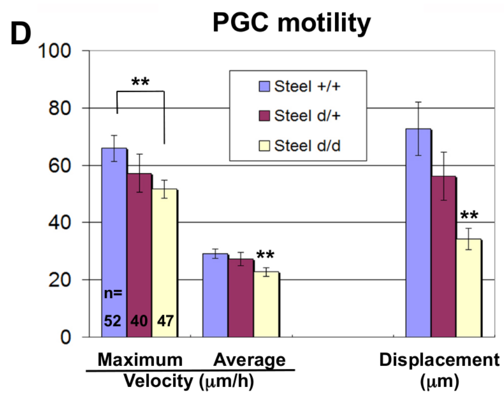
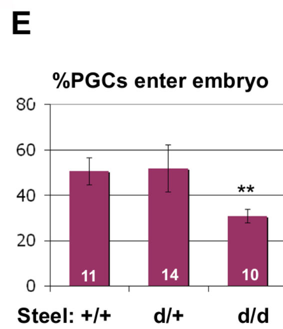


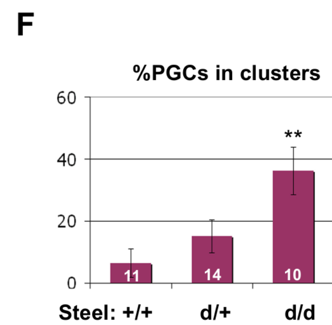
Figure 13: PGC number in Steeld/d embryos and in vitro culture.

(A) There was no significant change in PGC numbers at E7.5 in *Steel*$^{d/d}$ embryos compared to their littermates. "n" indicates the number of embryos used for quantitation.. (B) PGC number after 24 hours in vitro culture in medium with or without soluble recombinant Steel factor on different feeder layer cells. Y axis represents the ratio of PGC number 24 hours after plating versus 3 hours after plating. ΔMEF: primary MEF from *Steel-null* embryos. M220: stromal cell line express only membrane-bound Steel factor. **=p<0.01. (C) PGC number reduced significantly in E8.0 *Steel*$^{d/d}$ embryos compared to their littermates. "n" indicates the number of embryos used for quantitation. *=p<0.05.









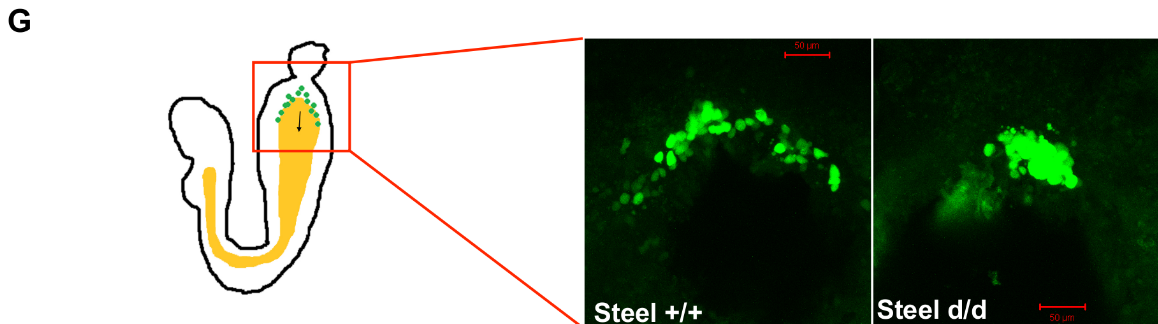
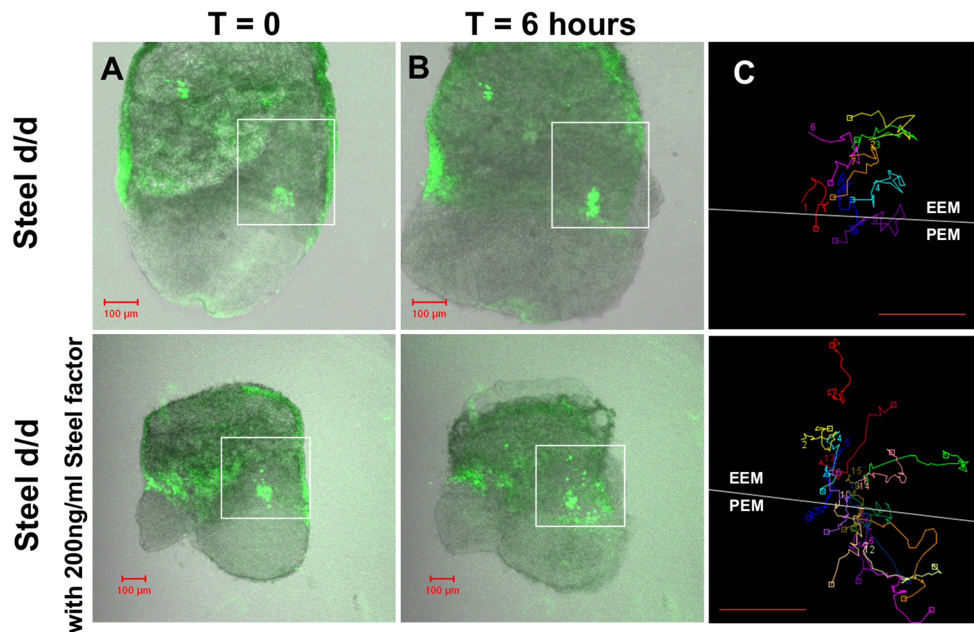
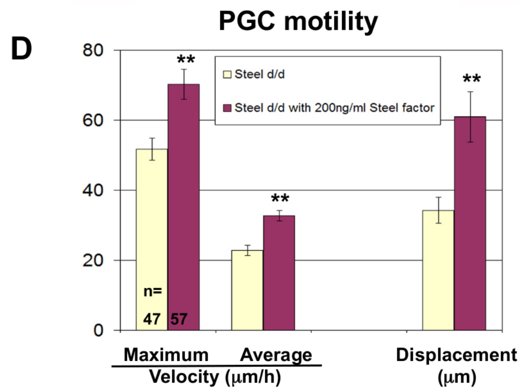


Figure 14: PGC migration in Steeld/d embryos at E7.5.

(Column A, B) Frames at t=0 and t=6 hours respectively from movies of E7.5 embryos with different *Steel-dickie* genotypes. (Column C) Tracks were made from PGCs in the allantois (white boxes) that remained in the plane of the confocal image throughout the movies. The white line in C indicates the boundary between the extraembryonic tissues (EEM), and the posterior end of the embryo (PEM). Scale bars in (A-C): 100 μm. (D) The maximum velocity, average velocity, and displacement of E7.5 PGCs with different *Steel-dickie* genotypes. PGCs in $Steel^{d/d}$ embryos showed significantly decreased velocities and displacement compared to wild type littermates. “n” indicates the number of PGCs used for quantitation. Units on the “Y” axis vary based upon parameter, and are indicated below the bar charts. **=p<0.01. (E) The percentage of PGCs which enter the posterior of the embryo is significantly reduced in $Steel^{d/d}$ embryos. (F) The percentage of PGCs which form clusters is dramatically increased in $Steel^{d/d}$ embryos. “n” indicates the number of embryos used for quantitation for (E) and (F). **=p<0.01. (G) PGCs in E8.0 wild type and $Steel^{d/d}$ embryos. The left diagram shows an E8.0 embryo with PGCs migrating along the hindgut (yellow). Arrow shows the direction of PGC migration. Red box indicates the area shown in the image on the right.





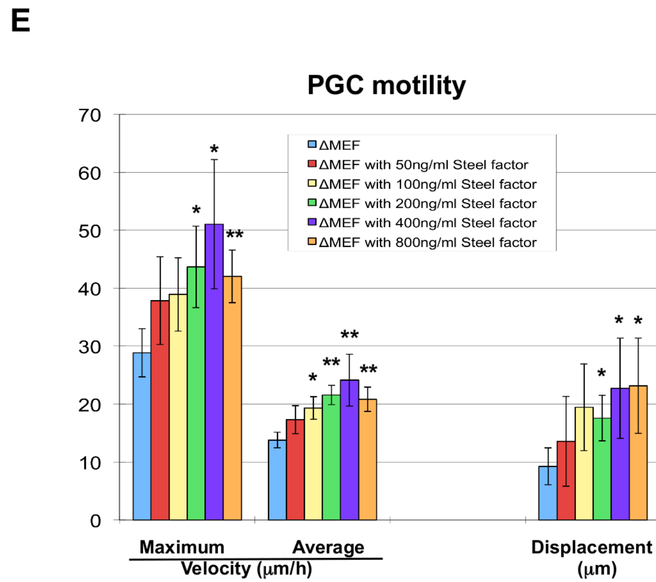


Figure 15: Effects of soluble recombinant Steel factor on PGC motility in Steel$^{d/d}$ embryos at E7.5 and in vitro culture.

(Column A, B) Frames at t=0 and t=6 hours respectively from movies of E7.5 *Steel*$^{d/d}$ embryos with or without addition of 200 ng/ml soluble recombinant Steel factor. (Column C) Tracks were made from PGCs in the allantois (white boxes) that remained in the plane of the confocal image throughout the movies. The white line indicates the boundary between the extraembryonic tissues (EEM), and the posterior end of the embryo (PEM). Scale bars in (A-C): 100 μm. (D) The maximum velocity, average velocity, and displacement of PGCs in E7.5 *Steel*$^{d/d}$ embryos were significantly increased by adding of 200 ng/ml soluble recombinant Steel factor into culture medium for 6 hours. “n” indicates the number of PGCs used for quantitation. Units on the “Y” axis vary based upon parameter, and are indicated below the bar charts. **=$p<0.01$. (E) The maximum velocity, average velocity, and displacement of PGCs after 24 hours in vitro culture with increasing concentration of soluble recombinant Steel factor on Steel-null MEFs (ΔMEF). Units on the “Y” axis vary based upon parameter, and are indicated below the bar charts. *=$p<0.05$, **=$p<0.01$.

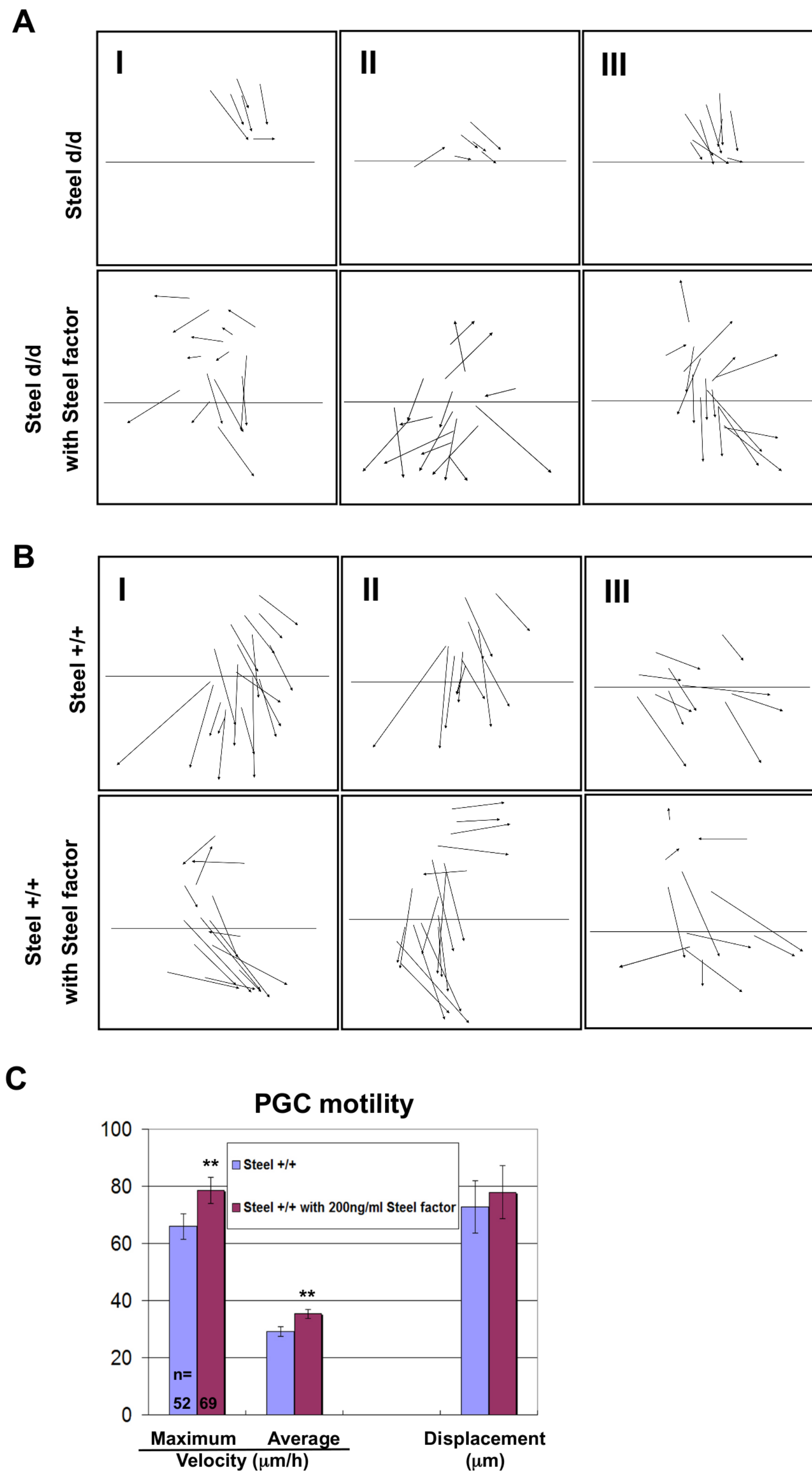


Figure 16: Effects of soluble recombinant Steel factor on PGC directions.

Directions of individual PGC migration in *Steel*$^{d/d}$ embryos (A) or wild type embryos (B) with or without addition of 200ng/ml soluble recombinant Steel factor. The boundary between the extraembryonic tissues (EEM), and the posterior end of the embryo (PEM), is marked by a line. Column I, II, and III are representative images from 3 different embryos of the same genotype labeled on the left. (C) The maximum velocity, average velocity, and displacement of PGCs in E7.5 wild type embryos with or without addition of 200 ng/ml soluble recombinant Steel factor into culture medium for 6 hours. “n” indicates the number of PGCs used for quantitation. Units on the “Y” axis vary based upon parameter, and are indicated below the bar charts. **=$p<0.01$.

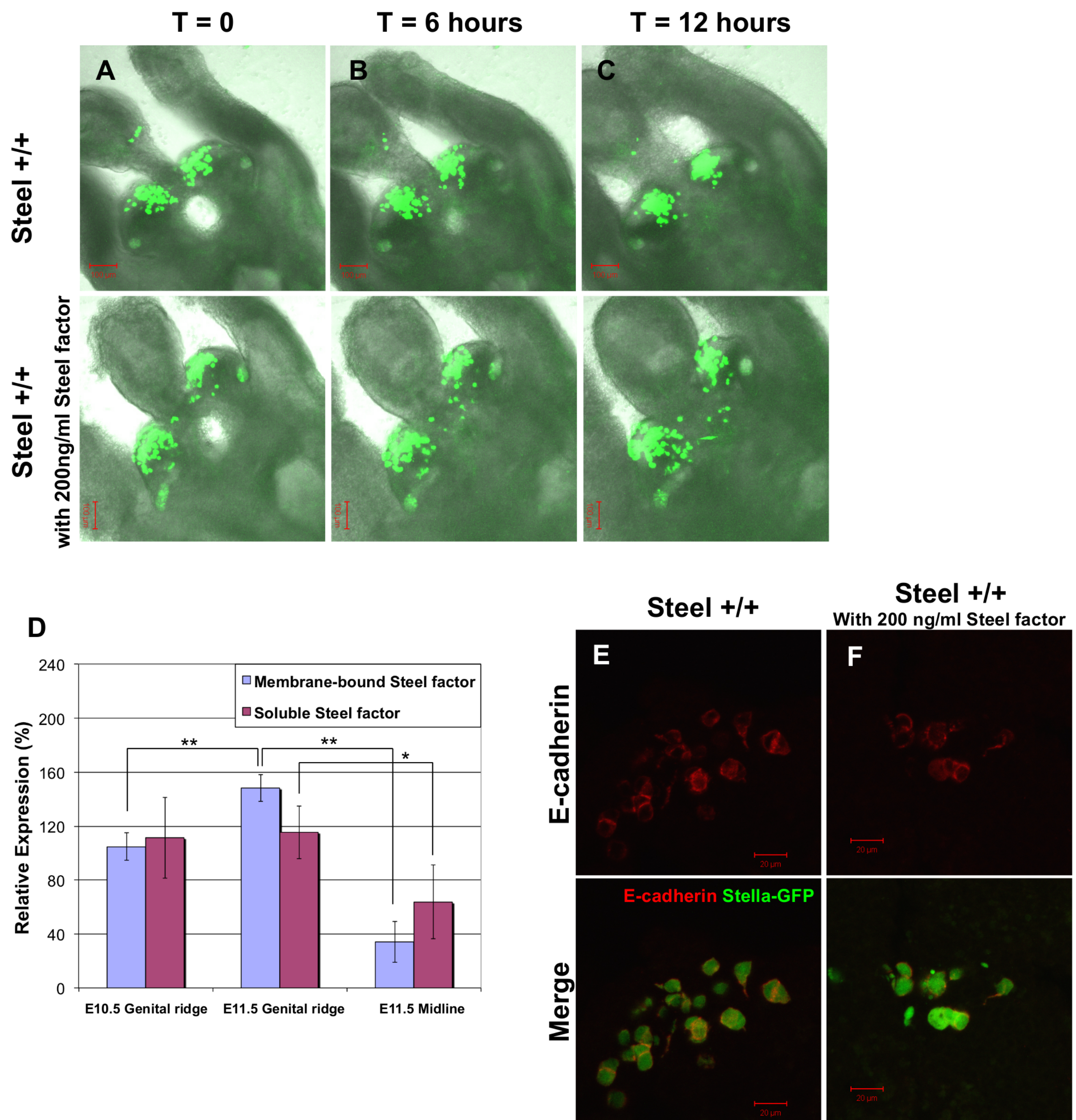


Figure 17: Effects of soluble recombinant Steel factor on PGC motility in wild type embryos at E11.0.

(Column A-C) Frames at t=0, t=6 and t=12 hours respectively from movies of E11.0 wild type embryo slices with or without addition of 200 ng/ml soluble recombinant Steel factor. (D) The relative expression level of membrane-bound and soluble Steel factor

mRNA in E10.5 genital ridges, E11.5 genital ridges and E11.5 midline mysenchyme as determined by real-time RT-PCR. *$p<0.05$, **$p<0.01$. (E and F) E-cadherin expression in PGCs without (E) or with (F) addition of Steel factor. Upper panels show E-cadherin (red) staining. The lower panels show the merged images with PGC marker Stella-GFP (green). No difference in expression of E-cadherin was observed between groups.

Chapter IV

Generation of *Steel-d2EGFP* reporter construct via Bacterial Artificial Chromosome strategy

Abstract

Steel factor is an essential factor for primordial germ cell (PGC) development. We have shown previously that the Steel factor-expressing somatic cells surround PGCs throughout their migration, providing a “traveling niche” that controls PGC motility, survival and proliferation. However, how the spatio-temporal pattern of Steel factor expression is established remains unclear. To study the mechanism that regulates Steel factor expression in the mouse embryo, we generated a transgene construct using BAC recombineering strategy. We used a BAC clone (RP23-353C6) which contains the coding sequences, >100kb upstream and ~22kb downstream sequences of *Steel* gene. We first generated the 5’ and 3’ homologous arms of the BAC DNA by PCR. We then inserted the destabilized form of EGFP at the *Steel* transcription start site of the 5’ homologous arm, and cloned the modified homologous arms into PL451 vector. The recombinant BAC was then generated by an inducible lambda phage recombinase system in the SW105 bacteria. The *Steel-d2EGFP* BAC construct will be used to generate a transgenic reporter mouse line to visualize the endogenous expression of Steel factor, thus offering a powerful tool to study the regulation of Steel factor expression.

Introduction

Steel factor is the ligand for the c-kit receptor, a receptor tyrosine kinase that is expressed by migratory primordial germ cells (PGCs) (Yabuta et al., 2006). The Steel

factor/c-Kit signaling plays essential roles to regulate PGC survival, proliferation and motility from the time of their specification in the allantois, to the time of their colonization in the genital ridges (Gu et al., 2009; Runyan et al., 2008). During PGC migration, the Steel factor-expressing cells are found surrounding PGCs on their migratory route, and the expression of steel factor turns off when PGCs move to the next site of their migration, establishing a “traveling niche” to regulate PGC behavior (Gu et al., 2009). PGCs that are left behind or migrate out of the niche will end in ectopic places, and are removed by apoptosis when they lack Steel factor (Gu et al., 2009; Runyan et al., 2008; Stallock et al., 2003). Therefore, the spatio-temporal pattern of Steel factor expression is fundamental for its function in PGC development. However, the dynamic regulation of Steel factor expression in the mouse embryo is poorly understood.

Analysis of gene expression *in vivo* has been greatly advanced by the use of transgenic reporter technology, which requires the insertion of a reporter gene, such as GFP, into the regulatory elements of the gene of interest. The spatial and temporal control of gene expression can be mediated by promoter elements close to 5’ start site. But recent studies revealed that the *cis* regulatory modules at a great distance from the start site of transcription also contribute to the regulation of gene expression pattern (reviewed in (Long and Miano, 2007)). The bacteria artificial chromosomes (BACs) usually contain large fragments of genomic DNA (>100kb), and include both the distal and the proximal regulatory information required for the endogenous gene expression. Therefore, transgenic mice generated from the BACs may resemble the endogenous expression pattern more accurately, compared to mice generated from transgenes that only contain

the proximal regulatory elements (Gong et al., 2003).

To study the spatial and temporal regulation of Steel factor in the mouse embryo, we generated a transgene construct using BAC DNA containing the *Steel* locus. Considering the large size of BACs, the method for the insertion of DNA fragments into BAC relies on homologous recombination, rather than restriction enzyme digestion and ligation. Here we used a BAC clone (RP23-353C6) containing the coding sequences and >100kb upstream and ~22kb downstream sequences of Steel factor, and amplified the 5' and 3' homologous arms of the BAC DNA by PCR. We then introduced the destabilized form of EGFP (d2EGFP, half life ~2 hours) at the *Steel* transcription start site of the 5' homologous arm by splicing overlap extension PCR, and cloned the 5' arm-d2EGFP and 3' arm into PL451 vector. The recombinant BAC was then generated by an inducible lambda phage recombinase system in the SW105 bacteria. If possible, this construct will be used for generation of reporter mice to monitor the dynamic expression of Steel factor in live embryos, and to isolate Steel factor-expressing cells via FACS; thus to investigate the signals which contribute to the regulation of Steel factor expression.

Materials and Methods

Materials:

The murine BAC clone RP23-353C6 containing the *Steel* locus was obtained from BACPAC Resources Center (BPRC) (http://bacpac.chori.org/home.htm). The SW105 bacterial strain was obtained from Biopharmaceutical Development Program (BDP) at the National Cancer Institute Frederick campus (NCI-Frederick). The TOP-GFPd2

plasmid containing destabilized GFP (d2EGFP) was kindly provided by Dr. Xinhua Lin (Cincinnati Children's Hospital Medical Center). The PL451 vector was kindly provided by Dr. Steven Potter (Cincinnati Children's Hospital Medical Center).

Methods:

Transform BAC into SW105

Protocols were modified from the ***Recombineering and Gap Repair Protocol by Nicolas Vodovar.***

1. BAC miniprep and BAC isolation

BAC clone was cultured in LB with 12.5 μg/ml chloramphenicol overnight at 37 ^{o}C, and then harvested by centrifugation in a 15 ml Falcon tube. The pellet was resuspended in 250 μl P1 buffer (from Qiagen miniprep Kit) with RNase A. 250 μl P2 (from Qiagen miniprep Kit) buffer was added into the tube and incubated at room temperature for 5 minutes. 350 μl N3 buffer was then added and incubated on ice for 5 minutes. The mixture was then centrifuged for 4 minutes at 14000 rpm. The supernatant was transferred into a clean new tube. 750 μl isopropanol was added into the tube, and incubated at room temperature for 10 minutes. The tube was then centrifuged for 10 minutes at 14000 rpm. The pellet was washed with 70% ethanol, air-dried, and resuspended in 25 μl nuclease-free water.

For BAC isolation, the BAC clone was cultured, harvested and processed using the NucleoBond BAC 100 Kit (Clontech) under manufacturer's instructions.

2. Transformation of BAC from DH10B into SW105

One colony of SW105 cells was grown in LB without antibiotics at 30 ^{o}C. At the end of the overnight culture, 1 ml of cultured SW105 bacteria was added into 50 ml new LB and cultured for another 3-4 hours at 30 ^{o}C until OD_{600} reached 0.6. The cultured bacteria was then incubated on ice/waterbath slurry for 5 minutes, and centrifuged at 3200 rpm for 10 minutes at 4 ^{o}C. The pellet was washed with 5 ml of autoclaved ice-cold nano-pure water, and centrifuged at 3200 rpm for 10 minutes at 4 ^{o}C. The washing step was repeated 3 times, and at the final time, 1 ml autoclaved ice-cold nano-pure water was used to resuspend the pellet. The resuspension was then transferred to a clean 1.5 ml tube, and centrifuged at 4000 rpm for 10 minutes at 4 ^{o}C. 150 μl autoclaved ice-cold nano-pure water was added into the tube to resuspend the pellet. 1μg of BAC DNA was mixed with 50 μl SW105 bacteria, and used for electroportation in a 0.1 cm cuvette at 25 μF, 1.8 kV and 200 Ω. After electroporation, the bacteria were recovered in 1 ml LB for 1 hour at 30 ^{o}C. After recovery, the bacteria was plated on LB plates with 12.5 μg/ml chloramphenicol, and incubated at 30 ^{o}C for 24-48 hours.

PCR amplification of homologous arms

1. PCR amplification of 5' and 3' homologous arms

The homologous arms were generated by PCR (Figure 18). A KpnI site was engineered at the 5' end on the 5' homologous arm to facilitate subcloning. The ATG (Figure 18, in red) of d2EGFP (Figure 18, in green) cassette was fused in frame with the endogenous ATG start codon of *Steel* (Figure 18, in light brown) at the 3' end of the 5' homologous arm. This step was designed to avoid deletion of any sequences from the *Steel* locus.

The primers used to generate 5' homologous arm were as following:

Forward: 5'- ATGGTACCGGCGGGAGGGAGCTGTATAAAAA -3'

Reverse: 5'- TGCTCACCATAAGGAAAGGCAGCGCTGCGATCC -3'

BamHI and SacII sites were engineered at the 5' end and 3' end, respectively, on the 3' homologous arm to facilitate subcloning. The primers used to generate 3' homologous arm were as following:

Forward: 5'- TAGGATCCAAGACACAAGTGAGTAGGGCAC -3'

Reverse: 5'- ATATCCGCGGAAACTTGCCACAGGCTCACTCT -3'

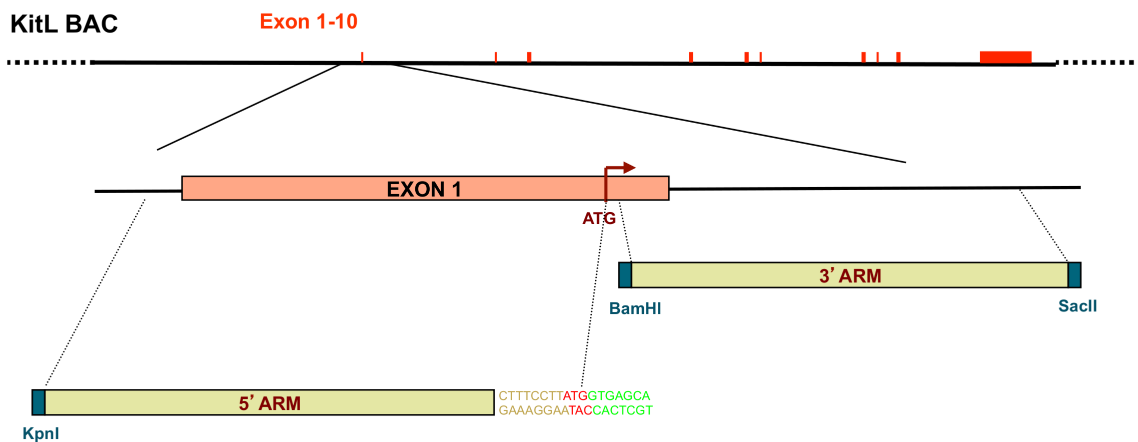


Figure 18: PCR amplication of 5' and 3' homologous arms.

2. <u>Fusion of *d2EGFP* gene with 5' homologous arm via Splicing Overlap Extension (SOE) PCR</u>

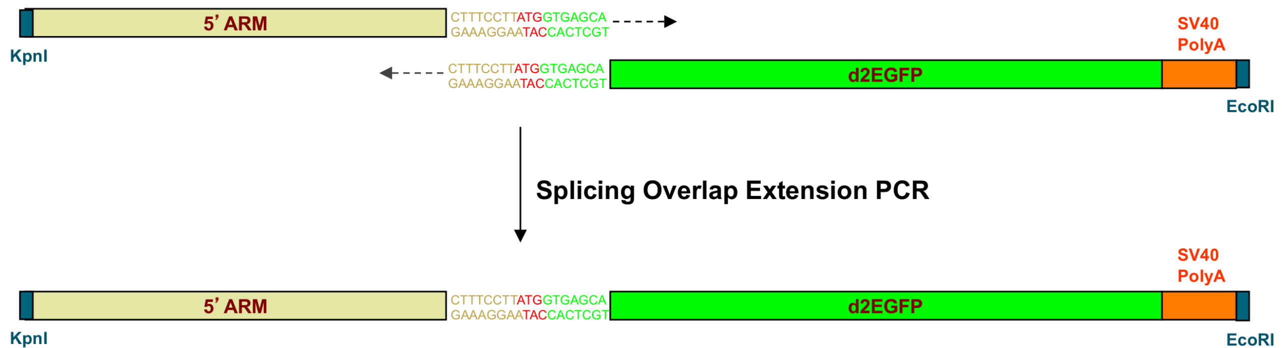


Figure 19: Fusion of d2EGFP gene with 5' homologous arm via SOE PCR.

The d2EGFP cassette was fused with 5' homologous arm via splicing overlap extension (SOE) PCR as previously described (Horton et al., 1993) (**Figure 19**). Briefly, the 5' homologous arm and the d2EGFP cassette were first amplified in separate tubes. The primers used to generate 5' arm were listed above. The primers used to amplify the d2EGFP cassette were as following:

Forward: 5'- CTTTCCTTATGGTGAGCAAGGGCGAGGAGC -3'

Reverse: 5'- AAGAATTCTACCGGGCCCAATGCATTGGC -3'

The two PCR products were then gel-purified, and used as the two templates for SOE PCR. The SOE PCR was performed under the same conditions as the regular PCR with the forward primer for 5' homologous arm and the reverse primer for d2EGFP cassette.

Preparation of vector for homologous recombination

The 3' homologous arm was subcloned into BamHI/SacII cut PL451 vector to generate the *3' Steel-d2EGFP*-PL451 vector. The 5' arm-d2EGFP fusion cassette was then subcloned into KpnI/EcoRI cut *3' Steel-d2EGFP*-PL451 to generate the *5' Steel-*

d2EGFP-3' Steel-PL451 vector (Figure 20). This vector was then linearized by

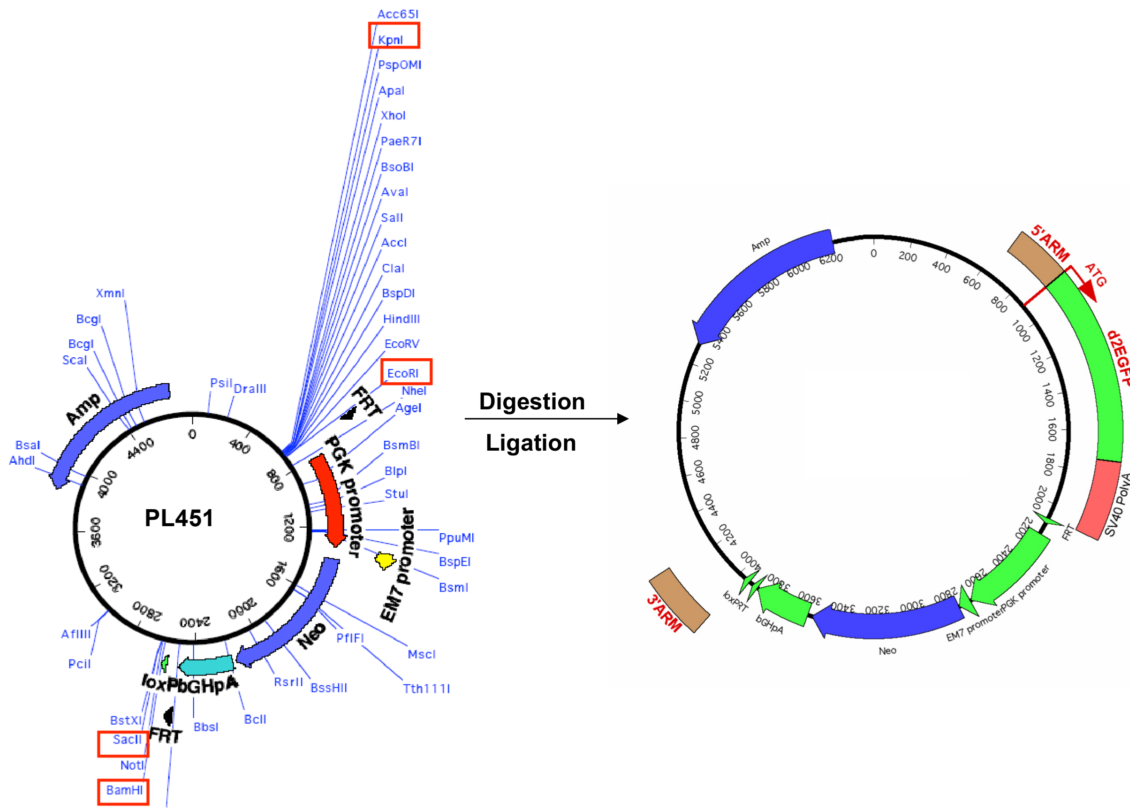


Figure 20: Preparation of PL451 vector for homologous recombination.

KpnI/BsaI enzyme digestion, and gel-purified.

Induction of homologous recombination in SW105

The SW105 containing the *Steel* BAC was cultured overnight at 30 °C with 12.5 μg/ml chloramphenicol. At the end of the overnight culture, 1 ml of cultured SW105 containing the BAC was added into 50 ml new LB and cultured for another 3-4 hours at 30 °C until the OD_{600} reached 0.6. The cultured bacteria were then divided into two groups. 25 ml of the bacteria was transferred into a new 100ml flask and incubated for 15 minutes

exactly at 42 °C in a shaking waterbath. The remaining 25ml of bacteria was incubated at 30 °C as uninduced control. The electrocompetent cells were prepared as described above (section "Transformation of BAC from DH10B into SW105"). 300ng of the linearized DNA of *5' Steel-d2EGFP-3' Steel*-PL451 was transformed into induced and uninduced bacteria by electroporation in a 0.1 cm cuvette at 25 μF, 1.8 kV and 200 Ω (**Figure 21**). After electroporation, the bacteria were recovered in 1 ml LB for 1 hour at 30 °C. After recovery, the bacteria was plated on LB plates with 12.5 μg/ml chloramphenicol and 50 μg/ml kanamycin, and incubated at 30 °C for 24-48 hours. Successful targeted homologous recombination resulting in the *Steel-d2EGFP* BAC was first identified by screening for neomycin resistance and subsequently confirmed by PCR.

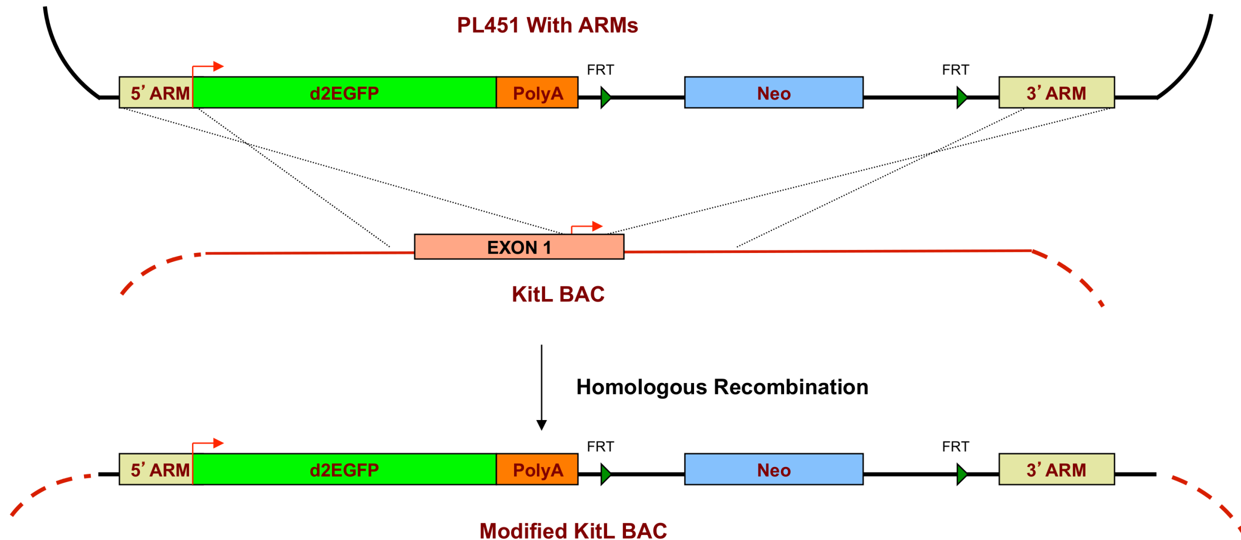


Figure 21: Homologous recombination of Steel BAC in SW105.

Discussion

In our previous study, we found that the expression of Steel factor moves with PGCs in all the tissues they migrate through, and this important feature of Steel factor expression pattern is essential for their regulation of PGC development (Gu et al., 2009; Runyan et al., 2008). However, little is known about how this spatial-temporal expression pattern of Steel factor is established. There is an EGFP transgenic mouse strain generated from a BAC clone (RP24-73F12) which contains ~55kb upstream and ~90kb downstream sequences of *Steel* gene, which shows expression pattern of Steel factor in adult mouse brain consistent with in situ data (information from GENSAT (Gene Expression Nervous System Atlas)). However, the expression of Steel factor in other tissues in this transgenic mouse has not been reported yet.

To study the spatial and temporal regulation of Steel factor expression in the mouse embryo, we generated a transgene construct via BAC recombineering strategy. The d2EGFP we used is a destabilized form of GFP protein which has a short half-life of about 2 hours; therefore, this transgenic mouse strain will be able to accurately visualize the endogenous pattern of Steel factor expression. This transgenic mouse strain, if generated successfully, can be used in many directions of future study. First, utilizing the embryo culture systems and time-lapse movie protocols developed in our lab, we can test whether Steel factor-expressing cells really move along PGC migration route. Also, we can screen for the pathways which may regulate Steel factor expression, by sorting out Steel factor-expressing cells via FACS based on their GFP expression and performing microarray analyses. We can also add either the activators or the inhibitors of candidate signaling pathways into culture medium, and investigate whether the

treatments change GFP expression. Furthermore, we can validate the results from signaling pathway screening genetically by breeding the *Steel-d2EGFP* BAC transgenic mouse strain into mice containing mutations of the components of the candidate pathways. To study whether PGCs themselves provide signals to induce Steel factor expression, we can breed the *Steel-d2EGFP* BAC transgenic mouse strain into Kit^{we} mutants, which have significantly decreased PGC number (Buehr et al., 1993), to investigate whether reduced PGC number may leads to a reduction of Steel factor expression. Altogether, the *Steel-d2EGFP* BAC transgenic mouse will offer us a powerful tool to study the regulation of Steel factor expression *in vivo*.

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Chapter V

Discussion

A "traveling niche" for PGC development

Many studies have focused on the signaling molecules which regulate PGC behavior during their migration. As a result, many factors have been found to be essential for PGC survival, proliferation and migration, providing useful insights into the field of PGC research. However, most of the findings are revealed by examining PGCs at certain stage of their migration, or in certain tissues along their migration route. As a population of cells which travel for a 4-day time period in a rapidly changing environment, how PGC behavior is dynamically controlled in a spatial-temporal manner is an interesting question to ask.

Here in my thesis work, I found, by studying the expression pattern and function of Steel factor in PGC development, that the Steel factor-expressing somatic cells established a "travelling niche" for migratory PGCs to regulate their behavior from the beginning of their migration in the extraembryonic allantois, to the end of their migration in the gonads. I also showed that the two forms of Steel factor contribute differently in regulating PGC behavior. Although the soluble Steel factor is sufficient to maintain PGC survival, membrane-bound Steel factor is required to maintain a high local concentration in a defined region in which PGCs are motile, receive the right chemoattractant signals, and do not aggregate (**Figure 22A**). PGCs escaping from the niche, or left behind in their migratory route, stop migration because they are out of the motility zone provided by the membrane-bound Steel factor, and therefore become ectopic when the rest of the PGCs and the expression of Steel factor move to the next site of PGC migration (**Figure 22B**). Later, these ectopic PGCs die by apoptosis due to lack of Steel factor in their surrounding tissues (**Figure 22C**, red dots represent PGCs that die by apoptosis).

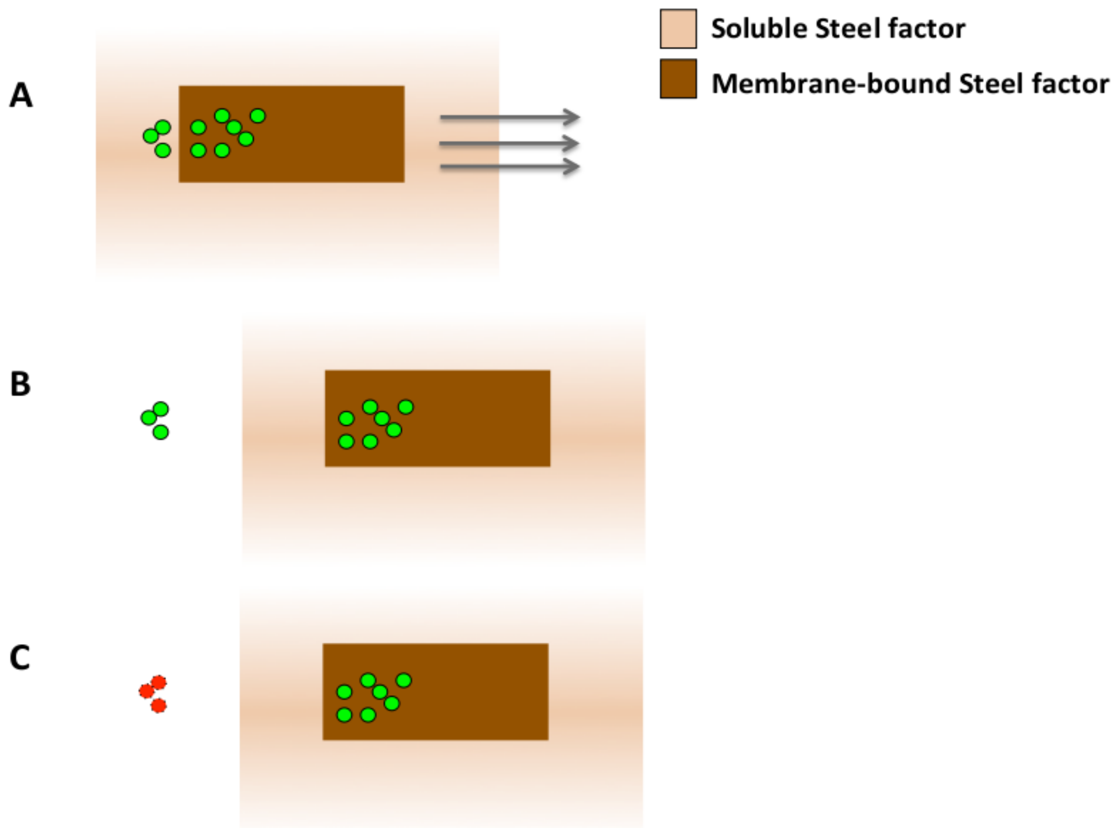

Figure 22: The model of Steel factor regulation in PGC migration in wild type embryos.
(A) Membrane-bound Steel factor defines a region in which PGCs are motile and do not aggregate. (B) PGCs escaping from this region, or left behind in their migration route, stop their migration and become ectopic. (C) These ectopic PGCs die by apoptosis due to lack of Steel factor. Green dots: Live PGCs; Red dots: PGCs undergoing apoptosis; Gray arrows indicate the direction of PGC migration.

The concept of a niche arose from studies on stem cells to describe the microenvironment in which their behavior and pluripotency are controlled by short-range signals. Now for the first time, this concept is extended to the population of migrating cells, the PGCs, to show how their behavior is controlled by Steel factor in a spatial-temporal manner. Steel factor is also known to be essential to several other migratory cell populations, such as hematopoietic stem cells and melanocyte precursors (Edling and Hallberg, 2007; Reber et al., 2006; Wehrle-Haller, 2003). Whether this “traveling

niche" model also applies to the regulation of Steel factor in other migrating cell populations is a fascinating question to ask in the future.

Understanding the control of PGC behavior during migration is also important for the prevention of one of the most common tumors of infancy, extragonadal germ cell tumors (EGCT). Germ cell tumors represent 7% of all tumors of children under the age of 20, and 66% of germ cell tumors in infancy are EGCTs (Bernstein L, 1999). These tumors are thought to arise from PGCs that became ectopic during migration and failed to die (Beard, 1904; Grosfeld and Billmire, 1985). Indeed, when apoptosis is blocked by removal the pro-apoptotic protein Bax, ectopic PGCs are found in many sites of extragonadal germ cell tumors (Runyan et al., 2008). The "traveling niche" model proposed here provides a mechanism in which these ectopic PGCs are normally cleared in the embryo by the regulation of Steel factor expression. During PGC migration, membrane-bound Steel factor defines the "motility zone" to ensure that the PGCs in the appropriate locations maintain survival and motility, while the ectopic PGCs which travel out of this motility zone lose their motility and stop migrating, and consequently die by apoptosis due to lack of survival factor (**Figure 22**).

Understanding the *Steel-dickie* mutation

There is an unanswered question about the *Steel-dickie* mutation, in which only the solube Steel factor is produced. PGCs are nearly absent in the gonads of *Steel-dickie* embryos, which is similar to *Steel-null* mutation, but no severe anemia is observed in the *Steel-dickie* embryos as the case in the *Steel-null* mutants (Bennett, 1956; Rajaraman et al., 2002). Steel factor is known to be required for the survival of both

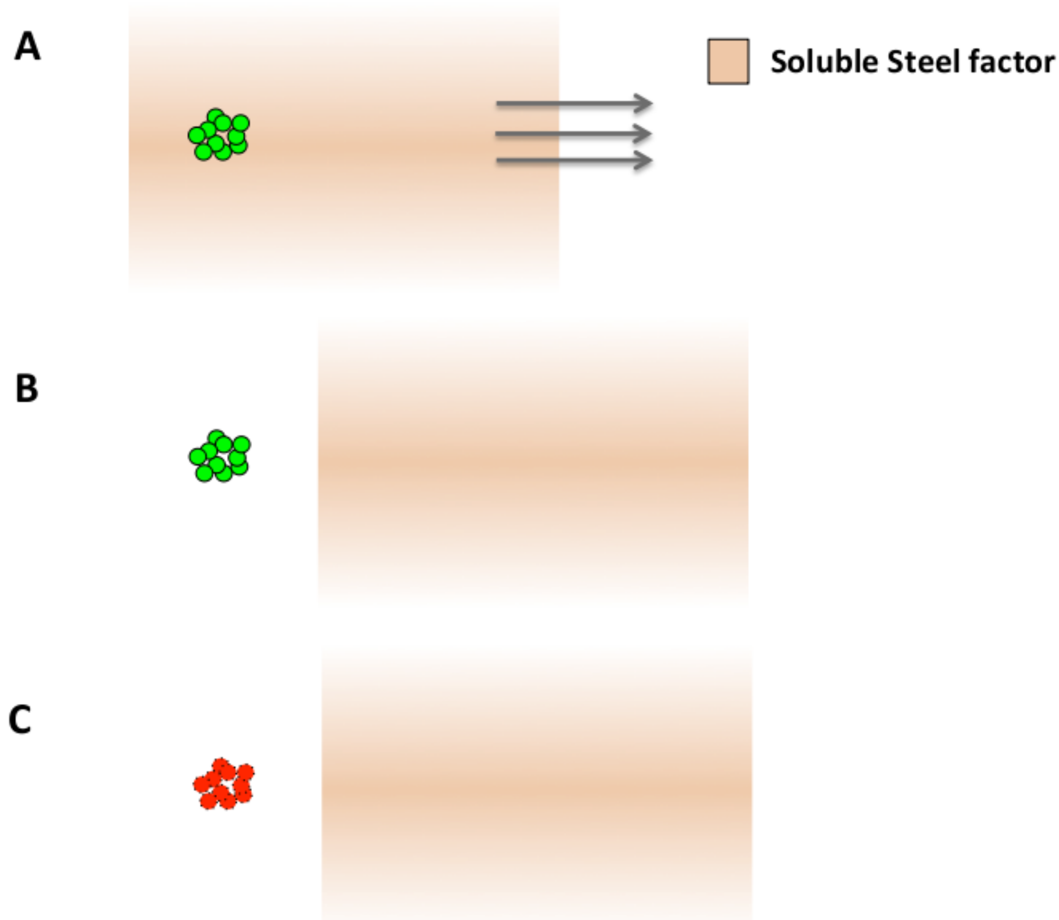

Figure 23: The model of Steel factor regulation in PGC migration in Steel-dickie embryos.

(A) Although the soluble Steel factor is sufficient for PGC survival, PGCs in the Steel-dickie embryos show defects in motility and aggregate together. (B) As a result of decreased motility, PGCs in the Steel-dickie embryos are left behind in their migration route, and become ectopic. (C) These ectopic PGCs die by apoptosis due to lack of Steel factor. Green dots: Live PGCs; Red dots: PGCs undergoing apoptosis; Gray arrows indicate the direction of PGC migration.

PGCs and hematopoietic stem cells, but why the survival of the two cell populations is affected differently in the *Steel-dickie* mutants is not understood. My study of the functions of the two forms of Steel factor provides an explanation for this puzzling question. In contrast to the *Steel-null* embryos, which show a significant decrease in PGC numbers as early as E7.5, no reduction in PGC numbers was observed in the

Steel$^{d/d}$ embryos at E7.5. This suggests that the soluble Steel factor made in the *Steel-dickie* mutants is capable of maintaining the survival of PGCs, as it does the hematopoietic stem cells. However, without membrane-bound Steel factor, the local concentrations of Steel factor in *Steel-dickie* embryos are not adequate to keep PGCs motile, or to prevent them from aggregation. As a result, PGCs in *Steel*$^{d/d}$ embryos are defective in motility and form a big clump in the allantois (**Figure 23A**). When the expression of Steel factor moves to the next site of PGC migration, the cluster of PGCs become ectopic and does not have Steel factor to maintain their survival, and they therefore die by apoptosis (**Figure 23B and C**). This suggests that the PGC death is not directly triggered by lack of membrane-bound Steel factor, but instead, is a consequence of the motility defect.

How is Steel factor expression established?

In our immunostaining data, we observed that the expression of Steel factor moves with PGCs in all the tissues they migrate through. This is one of the most important features of the “traveling niche” model. However, how this spatial-temporal expression pattern of Steel factor is established remains unclear. It is likely that the factors regulating PGC behavior during migration also control the expression of Steel factor, such as BMP and SDF-1. BMP signaling has been shown to regulate PGC survival and migration, and affects the expression of Steel factor mRNA between E9.5 and E10.5 (Dudley et al., 2010; Dudley et al., 2007). SDF-1 signaling, on the other hand, is also known to increase Steel factor expression at the transcriptional level in E9.5 embryo slice culture (previous unpublished data from our lab). In addition, the expression of SDF-1

correlates with PGC positions from the time when PGCs appear in the hindgut (Stebler et al., 2004). Together, these data raise the possibility that these signaling molecules may also be components of the “traveling niche”, and contribute to the establishment of Steel factor expression pattern along PGC migration route. There are other possibilities of how the “traveling niche” is established. For example, PGCs themselves may provide signals to induce Steel factor expression as they migrate. Or, the Steel factor-expressing somatic cells themselves may migrate with PGCs. The generation of a *Steel*-reporter mouse strain may help to distinguish between these possibilities. Here we made the *Steel-d2EGFP* BAC construct in order to generate transgenic reporter mice (described in **Chapter IV**). The d2EGFP we used is a destabilized form of GFP protein which has a short half-life of about 2 hours; therefore, this transgenic mouse strain, if made successfully, will be able to accurately visualize the endogenous pattern of Steel factor expression. Utilizing the embryo culture systems and time-lapse movie protocols developed in our lab, whether the Steel factor-expressing cells really migrate will easily be investigated. Also, adding either the activators or the inhibitors of candidate signaling pathways into culture medium, and then visualizing the change of GFP expression in the transgenic mouse tissue can, easily test whether certain signaling pathways are involved in regulating Steel factor expression. Considering the important role of Steel factor in the development of several cell populations, finding out how Steel factor expression is controlled will not only benefit our understanding of PGC development, but also advance our knowledge in other fields of research, such as research in the development of hematopietic stem cells and melanocytes.

What is downstream of c-kit receptor?

Based on our results, Steel factor plays different roles at different local concentrations. Lower local concentration is sufficient for PGC survival, while a higher local concentration provided by membrane-bound Steel factor is required for PGC motility (discussed in **Chapter III**). What are the downstream pathways triggered by different ligand concentrations that control different aspects of PGC behavior? This question invites follow up studies to determine the signal transduction pathways that lie between c-kit receptor activation and PGC survival, proliferation and motility. However, PGCs do not survive well in culture without feeder layer cells; on the other hand, it is difficult to interpret whether the effects of the inhibitors are PGC autonomous when PGCs are cultured with feeder layer cells. Therefore, it is very hard to investigate the signaling pathways downstream of c-kit receptor by inhibitor screening. Although previous studies using PGC *in vitro* culture have shown that AKT phosphorylation through the action of mTOR is required for Steel factor-dependent PGC survival (De Miguel et al., 2002), and PI3K and Src kinase are responsible for the Steel factor-dependent PGC migration (Farini et al., 2007), it is very important to confirm these targets genetically. Because most of the downstream pathways have indispensable roles in embryogenesis, and may cause early embryonic lethality if knocked out globally, generation of PGC specific knockout mouse strains will be necessary for this purpose. TNAP (Tissue Non-specific Alkaline Phosphatase)-Cre mouse line has been reported to drive Cre recombinase expression in migratory PGCs (Lomeli et al., 2000), but the Cre excision does not start until E9.0, and Cre activity is only seen in ~50% of E13.5 PGCs. Thus, the TNAP-Cre may not be suitable for PGC study at early stages of migration. Recently, two new PGC

specific lines have been generated. The *Stella-Cre* mouse line shows a100% Cre-LoxP recombination efficiency at E12.5 (Liu et al., 2011). The *Stella-MCM* (MER Cre MER) transgenic mouse line enables drug-inducible gene knockout in PGCs, and shows Cre-LoxP recombination activity as early as E7.5 (Hirota et al., 2011). These lines offer valuable opportunities to explore the possible downstream targets of c-kit in both loss-of-function and gain-of-function experiments in PGC development, and may help us to understand how different ligand concentrations trigger different signal transduction responses.

Summary

In summary, this work describes a “travelling niche” model in which Steel factor controls PGC behavior throughout their migration. Furthermore, it reveals that the membrane-bound Steel factor, by providing higher local ligand concentration, defines the “motility zone” for PGCs, to ensure PGCs in appropriate locations can maintain survival and motility, while ectopic PGCs cease motility and subsequently die by apoptosis. These findings offer a mechanism of how the migrating PGCs are precisely controlled in a spatial-temporal manner, and how the dangerous ectopic PGCs are eliminated to avoid tumor formation.

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