Examining the Roles of the Receptor Tyrosine Kinase PVR and the Bone Morphogenic Protein Receptor Thickvein in Regulating Self-Renewal and Division Frequency in the Cyst Stem Cells of the Drosophila Testis Niche

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This Dissertation by: Nastaran SoleimaniBarzi Mues


has been approved as meeting the requirement for the Degree of Doctor of Philosophy in College of Natural and Health Sciences in School of Biological Sciences

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Adult stem cells live in different tissues, and they support and regenerate both the tissue they reside in and themselves. The stemness behavior is tightly regulated by the niche. The *Drosophila* testis is a valuable model to study stem cells in their niche. In this niche, there are two populations of stem cells; germline stem cells (GSCs) and somatic cyst stem cells (CySCs). GSCs and CySCs provide the cellular structure required to maintain the production of sperm in *Drosophila* males. These stem cells co-mingle around a group of non-dividing somatic cells known as the hub, and is the niche that provides molecular signals to instruct the behavior of stem cells. The fate of GSCs and differentiating germ cells is dependent on CySCs and their descendants, cyst cells, because if these cells are blocked, GSCs would be unable to be maintained normally as stem cells or differentiate properly.

This dissertation describes the role of PVR and BMP signaling pathways in the CySCs of the *Drosophila* testis stem cell niche. PVR signaling regulates the cell division frequency of the CySCs in the stem cell niche. This was shown by loss of function experiments, as inhibiting PVR in the CySCs by RNA interference and dominant negative transgene expression resulted in significant reduction of CySCs at the niche and the cycling CySCs and disruption of transit amplifying germ cells non-autonomously. It
was also demonstrated by constitutive PVR expression, or ectopic Pvf₁ ligand expression, which resulted in tumorous accumulation of cyst lineage cells throughout the testis, and misregulated cycling of these cells outside of the niche. PVR might also play a role in stem cell competition at the niche. PVR mutant CySCs clones do not persist as stem cells in the testis and PVR mutant clones are out competed with the wildtype CySCs. Our result also demonstrated that when constitutively activate PVR is combined with tumor suppressor Merlin over-expression in the cyst cells lineage, Merlin suppresses PVR signaling and is able to prevent any tumor formation due to hyperproliferation of cyst cells in the testis.

BMP signaling pathway is the main self-renewal regulator in GSCs. We have examined the requirement for BMP signaling in the CySCs and demonstrated that BMP signaling pathway regulates stem cell maintenance in the *Drosophila* testis stem cell niche. When we ablated *tkv*, the type I receptor of BMP pathway in the cyst lineage cells by RNA interference, we observed a partial loss of CySCs and loss of differentiated cyst cells. In the absence of cyst cells, the germline cells were also incapable of completing differentiation and the number of GSCs also decreased in the manipulated testes showing the non-autonomous effect of ablating BMP pathway in CySCs on the population of GSCs.
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CHAPTER I
INTRODUCTION AND LITERATURE REVIEW

Introduction

Stem Cells and Their Niches

**Adult stem cells.** In adult bodies, each tissue holds small numbers of undifferentiated stem cells that are known as adult stem cells and they are vital components of the tissues. Adult stem cells are a precious source of new cells in tissues and organs, and play a crucial role in regeneration and homeostasis of the tissue they compose by replenishing dying cells. These stem cells use symmetric cell division to maintain their population. The daughter cells have the ability to self-renew thus upholding their stem cell identity in order to maintain their population. They are also inept to undergo asymmetric cell division in order to repopulate a specific cell type through their descendants and replenish and regenerate the tissue in which they reside (Ohlstein et al., 2004). The fate of these stem cells is determined by the stimuli and the extrinsic molecular signals that they receive from their in vivo microenvironment which is called the stem cell niche (Ferraro et al., 2010).

Adult stem cells can be unipotent which means that they can give rise to only one specific population of cells like germline stem cells or they can be multipotent. Multipotent stem cells are capable of giving rise to more than one type of cells like the hematopoietic stem cells.
The extrinsic signals provided by stem cell microenvironment, the niche, are responsible for regulation of maintenance and differentiation of these cells. The concept of the niche was first introduced by Schofield in 1978. He proposed that for a cell to be able to possess stem cell characteristics, it has to be in contact with other cells. He called these other cells the niche cells that are able to direct the performance of the stem cells (Schofield, 1978). To this day, adult stem cells population have been identified in many tissues and organs in different organisms from human to fruit flies including the mammalian hair follicle bulge, the mammalian bone marrow and the *Drosophila* testis (Leatherman, 2013).

Although research on understanding the function of stem cells has tremendously increased in the past decades, there are many aspects of adult stem cells that are poorly understood, including how multiple stem cells lineages coordinate proliferation specially in mammalian stem cells due to the complicated nature of their niches (Li and Xie, 2005). Breaking the stem cell niche coordination balance may disrupt the architecture of the tissue that these stem cells reside in and results in loss of adult stem cells in that tissue or organ. Having the potential use of these cells in regenerative medicine is also another reason of enhanced interest in doing research on genetics of these cells (Preston et al., 2003).

**Other types of stem cells.** There is another group of stem cells that are found in the inner mass cell of the 4-5-day old blastocyst and can be cultured in vivo. These cells are called Embryonic Stem cells (ES). ES cells are pluripotent stem cells and have the capacity to differentiate into any of the three germ layer cells (endoderm, mesoderm, and ectoderm) (Itskovitz-Eldor et al., 2000).
Another type of pluripotent stem cells that can be derived in cell culture called induced Pluripotent Stem cells (iPS). These cells have been generated through reprogramming of fully differentiated cells and their ability to differentiate into all three germ layer cells granting them more regenerative competence compare to adult stem cells (Rashidi et al., 2018).

There is a small subpopulation of cells found in the tumors that are able to self-renew, differentiate, and tumorigenice when transplanted into another animal host. These cells are referred to as Cancer Stem Cells (CSC). These cells are resistant to chemotherapy and radiation therapy and they are thought to be the source of cancer metastasis and cancer revert in patients after their treatment. There is much more attention regarding the importance of targeting these cells for anti-cancer drug innovations (Yu et al., 2012).

**The Drosophila Testis Stem Cell Niche**

**Structure and architecture of the testis.** The *Drosophila melanogaster* testis with two populations of stem cell and a simple niche, is one of the best characterized model systems to study stem cell niches (Hardy et al., 1979). The two *Drosophila* testes are coiled, blind-ended tubes that are located in the abdomen of the males. The niche resides at the apical tip of the testis and consists of 10-15 densely arranged, fully differentiated cells known as the hub cells. There are two types of stem cells associated with the hub cells. These cells physically adhere to the hub. The first population of stem cells is the sperm producing germline stem cells (GSCs) and the second population is the support somatic stem cells, known as the cyst stem cells (CySCs). When GSCs divide, they undergo an oriented cell division, and the daughter cell which detaches from the hub
will differentiate into a gonialblast (de Cuevas and Matunis, 2011; Yamashita et al., 2003) and the daughter cell that remains next to the hub undergoes self-renewal. CySCs also undergo oriented cell division, and the daughter cell moving away from the hub will differentiate into a cyst cell and the daughter cell that lingers next to the niche endures receiving stemness molecular signals from the hub (Cheng et al., 2011). Gonialblasts will undergo four transit-amplifying synchronous mitoses with partial cytokinesis, to create sixteen interconnected spermatogonia.

**Figure 1.** *Drosophila* testis stem cell niche architecture. The hub cells (blue) reside at the apical niche of the testis surrounding the germline stem cells (GSCs, light purple) and cyst stem cells (CySCs, light green). The GSCs and CySCs divide and the daughter cells either stay in the niche and remain as stem cells or they move away from the niche and differentiate. The GSCs daughter cell that moves away from the hub give rise to the spermatogonia (dark purple) which eventually differentiate into sperms. The CySCs that move away from the hub differentiate into cyst cells (dark green), which envelope and encapsulate the germ cells and guide them through their differentiation.

All along, two cyst cells will fully envelope and encapsulate each gonialblast and support the appropriate cell division and differentiation program (Figure 1). Moving further away from the testis apex and the hub, cysts of sixteen spermatogonia will mature.
and differentiate into spermatocytes. Spermatocytes then massivly grow and expand approximately 25 times in volume which will undergo meiosis to eventually create 64 spermatids. These spermatids then complete their transformation and create 64 haploid sperm (Davies and Fuller, 2008). Successful spermatogenesis process directly depends on the cooperation between the germ cells and the cyst cells. If at any point this cooperation is disrupted by deleting cyst cells from the testis or their ability to encapsulate the germ cells, these germ cells would fail to finish their differentiation past their early stages of spermatogonia (Kiger et al., 2000; Lim and Fuller, 2012; Tran et al., 2000).

**The stem cell niche formation and the origin of cells during development.** The niche of the stem cells in *Drosophila* testis is defined in early embryonic stages even before the testes come together (Okegbe and DiNardo, 2011). The hub cells derive from a group of somatic gonadal precursor cells (SGPs). SGP cells originate from mesodermal cells located in embryo parasegments 10 and 11. Different genetics and epigenetics stages during the embryo development divide both the ectoderm and the mesoderm of the embryo into 14 parasegments. Each of these parasegments will create a precise distinct parts of the larva and adult (Martinez-Arias and Lawrence, 1985)

Notch signaling gets activated in SGPs via ligand Delta while these cells migrate along midgut during embryogenesis. Notch signaling helps with specification of the hub cells and if the ligand Delta, gets eliminated from the testis, Notch signaling will be disrupted and a significant decrease of the hub cells will be observed (Okegbe and DiNardo, 2011). Once the testis has formed, Epidermal growth factor (EGFR) pathway become activated in SGPs through primordial germ cells (PGCs) secreting the EGFR ligands Spitz and Sevenless, at the posterior end of the testis and this pathway inhibits the
remaining SGPs to become hub cells. If the EGFR signaling pathway is disrupted in SGPs, it causes a significant increase in the number of hub cells at the expense of the cyst cells. This results show that EGFR signaling is enough to inhibit the hub cells formation stimulated by Notch signaling (Kitadate and Kobayashi, 2010; Kitadate et al., 2007). The location of PGCs at the posterior end of the testis ensures inhibition of hub cells expansion to the anterior of the testis (Kitadate and Kobayashi, 2010). Ten to 15 of the SGP cells become the hub cells and the rest of these cells become CySCs and cyst cells and envelope PGCs. Although all these cells (hub cells, GSCs, and CySCs) support each other the presence of the germ cells is not required for the maintenance of the CySCs but the CySCs are sufficient to support stemness maintenance of the GSCs (Gonczy and DiNardo, 1996; Leatherman and DiNardo, 2008).

The stem cell niche structure becomes visible by embryonic stage 15 with a group of SGP cells expressing high levels of cell adhesion proteins such as DE Cadherin, DN Cadherin, and Fasciclin III at the anterior side of the testis. These SGP cells undergo a mesenchymal to epithelial transition (MTE) and adhere tightly to each other and create the rosette shape of the niche (Le Bras and Van Doren, 2006). The hub cells attach to the apical end of the testis extracellular matrix via integrin protein (Tanentzapf et al., 2007). The hub cells also adhere to the adjacent SGPs and PGCs with a Cadherin-based adhesion and these cells are likely to become the CySCs and GSCs (Leatherman and DiNardo, 2010; Stine et al., 2014; Voog et al., 2008). The exterior area around the hub cells will determine the number of GSCs and CySCs surrounding the hub cells as these cells need to physically attach to the hub cells to maintain their stemness behavior (Hardy et al., 1979; Resende et al., 2013). The complete structure of the testis niche becomes
complete by the end of embryonic stage 17 and it continues with the same structure throughout adulthood. The number of the hub cells will remain stable through adulthood but the number of CySCs and GSCs reduces by aging but remain in an approximate ratio of 2 to 1 (Amoyel et al., 2014; Sheng et al., 2009). GSCs and CySCs remain mitotically active to produce cells for differentiation but the hub cells do not undergo mitosis under normal circumstances and this stability of the hub cells provide a steady anchor for the GSCs and CySCs in the Drosophila testis stem cell niche (DiNardo et al., 2011; Gonczy and DiNardo, 1996; Hardy et al., 1979; Hétié et al., 2014; Voog et al., 2008).

**Signaling Pathways in the Drosophila Testis Stem Cell Niche**

**Stem cell maintenance.** The stem cell maintenance is depending on both the signals and the structure of the niche (Morrison and Spradling, 2008). The two stem cell populations physically attach to the hub cells and this attachment keep them close enough to the niche environment and the cells continue receiving signals from the hub cells and there is a competition between CySCs and GSCs for space around the hub (Hardy et al., 1979; Issigonis et al., 2009). Hub cells secrete molecular ligands that signal the stem cells to maintain their stemness and it represses their differentiation (Leatherman and DiNardo, 2010; Ma and Xie, 2011; Zhang et al., 2013). CySCs physical attachment to the hub cells is by adherens junctions and possibly integrin adhesions (Stine et al., 2014; Voog et al., 2008). The DE-Cadherin expression in the CySCs is partly regulated by activation of receptor Roundabout 2 (Robo2) when it binds to its ligand Slit. Hub cells express Slit on their plasma membrane (Stine et al., 2014). These adherens junctions are known to regulate the asymmetric division of the GSCs but their role in asymmetric division of the CySCs is unclear (Inaba et al., 2010).
The stem cell asymmetric cell division results in one daughter cell displaced from their niche and lose contact with the hub cells and thus it does not receive the stemness molecular signals from the hub and it is poised to differentiate (Cheng et al., 2011; Yamashita et al., 2003). Therefore, the unique architecture of the stem cell niche in the Drosophila testis can lead to both maintenance and differentiation of the stem cells.

**Jak/Stat signaling pathway.** The hub cells regulate the maintenance of the stem cells through secreting molecular ligands which activate signaling pathways in the neighboring stem cells to alter gene expression. The cytokine Unpaired (Upd) is produced from the hub, and it can activate the Janus Kinase (Jak)/Stat signaling pathway (Kiger et al., 2001; Tulina and Matunis, 2001). Jak is a cytoplasmic tyrosine kinase which performs by binding on the cytoplasmic site of the cytokine receptors on the cell surface. Upon binding of Upd to the receptor Domeless (Dome), the two monomers of the receptor dimerize and this dimerization brings the Jak proteins in correct proximity of each other and they can trans-phosphorylate each other. Jak proteins also phosphorylate the tyrosine on the cytoplasmic domain of the cytokine receptor to create docking sites for transcriptional regulators called Stats. Upon Stats binding to the receptor, Jak proteins phosphorylate the Stats. Phosphorylated Stats then dissociate from the receptor and migrate to the nucleus and change gene transcription (Figure 2) (Bustin, 2015). One of these target genes is socs36E which codes a negative feedback to the Jak receptor. GSCs and CySCs competition over the space around the hub was first shown by inhibiting the Soc36E protein and persistence activation of Jak/Stat pathway (Issigonis et al., 2009; Singh et al., 2010).

Jak/Stat signaling pathway has two different roles in CySCs and GSCs. In GSCs,
Jak/Stat signaling is responsible for adhering the GSCs to the hub cells (Leatherman and DiNardo, 2010). The GSC hub cells interaction is maintained by protuberant cell-cell adhesions. Jak/Stat signaling pathway is responsible for these cell-cell adhesions (Leatherman and DiNardo, 2010). Upd ligand is released from the hub cells and binds to its receptor Dome on the GSCs. Activation of Jak/Stat signaling results in an increase in the expression of the adherens junction protein DE-Cadherin in GSCs, and it ensures that they stay attached to the hub cells (Leatherman and DiNardo, 2010). The DE-Cadherin based junctions are also involved in controlling and ensuring the asymmetric cell division of GSCs by locating their centrosomes during their mitosis as described above (Inaba et al., 2010). Thus, Jak/Stat signaling ensures both the GSCs maintenance and the disposition of gonialblasts from the hub where they are positioned to differentiate. In CySCs, transcriptional Stat target genes Zfh-1 and Chinmo, promote self-renewal in CySCs (Flaherty et al., 2010; Kiger et al., 2001; Leatherman and DiNardo, 2008; Tulina and Matunis, 2001).
CySCs are maintained by receiving signals from the hub cells. One of these signals that is required and sufficient for the maintenance of the CySCs is the Jak/Stat signaling pathway described above. This pathway drives the expression of the two transcription factors Zfh1 and Chinmo that facilitate the self-renewal of CySCs as mentioned above (Flaherty et al., 2010; Leatherman and DiNardo, 2008). When Jak/Stat signaling is eliminated from the CySCs, these cells differentiate and the population of CySCs in the testis is lost while by constitutively activating Jak/Stat signaling, all the cyst

**Figure 2.** Jak/Stat signaling pathway activated by cytokine Upd. Upon binding Upd to the receptor, the Domeless receptors dimerize and bring Jak proteins in close proximity to trans-phosphorylate each other and creating binding sites for Stats. Jak phosphorylate Stat in which they dissociate from the receptor and dimerize and enter the nucleus and control gene expression of their target genes. Adapted from the Molecular Biology of the Cell (Bustin, 2015).
cells in the testis act as efficient CySCs (Kiger et al., 2001; Leatherman and DiNardo, 2008; Tulina and Matunis, 2001).

The idea of CySCs being part of the GSCs niche was first introduced by the evidence that both zfh-1 and chinmo expressing CySCs could lead the GSCs to self-renew away from the hub cells (Flaherty et al., 2010; Leatherman and DiNardo, 2008). Later, when scientists ablated CySCs from the niche, half of the testes lost their GSCs population and in the other half, the early germ cells were accumulated away from the hub, suggesting that CySCs, in addition to the hub, are important in maintaining the GSC population (Lim and Fuller, 2012). Moreover, it is well-established that CySCs play a crucial role in providing guidance and information to the GSCs (Kiger et al., 2000; Tran et al., 2000). Thus, CySCs and their differentiating cells, cyst cells, are required for appropriate maintenance of GSCs and their differentiating daughter cells, the germline cells (Leatherman and DiNardo, 2010; Lim and Fuller, 2012). Hence, the cyst lineage cells can be also considered as a part of the germline’s niche. How the cyst lineage cells behave and how their behavior influences the germline cells is a key to understanding the regulation of this niche.

**Hedgehog signaling pathway.** One of the other signals secreted from the hub is the ligand Hedgehog (Hh). Hh binds to its receptor Patched on the CySCs and upon binding the ligand to Patched, this receptor is internalized and degraded in the lysosomes. Degradation of Patched allows the G protein coupled receptor Smoothened to be recruited to the plasma membrane and be phosphorylated. Smoothened recruits protein complexes that inhibit degradation of transcription factor Ci (Figure 3) (Amoyel et al., 2013). In the absence of Hh ligand, Patched inhibits the pathway receptor Smoothened,
permitting Ci to be phosphorylated by different kinases. These phosphorylation of Ci leads to a partial cleavage and allows for formation of the repressor form of Ci. Binding of the ligand Hh to Patched receptor and co-receptor iHog put a stop to Patched inhibition on Smoothened. As a result, Smoothened accumulates on the cell membrane and allows the activation of Ci and its function as a transcription inducer of the Hh pathway target genes (Figure 3) (Bustin, 2015).

Hh signaling is similar to Jak/Stat signaling in that it averts the differentiation of the CySCs. Unlike Jak/Stat signaling, Hh is not sufficient to induce CySCs identity. Evidence shows that when Hh is expressed ectopically, CySC differentiation in the testis is delayed and the over activation of this pathway results in an increase in CySCs proliferation, whereas CySCs mutant clones for Hh signaling pathway differentiate and are lost from the niche (Amoyel et al., 2013; Michel et al., 2012; Zhang et al., 2013).

The transcription activator Zfh1 is considered to be a joint target of both the Hh and Jak/Stat signaling pathways in the CySCs (Albert et al., 2018). A recent study has shown that Zfh1 acts by binding to the two tumor suppressors genes 12alvador and kibra and downregulating their expression. These two tumor suppressors function by inhibiting the pro-proliferation factor Yorkie. Thus, Zfh1 is competent to inhibit the tumor suppressors and allow proliferation of Zfh1+ cyst stem cells in the niche (Albert et al., 2018).

The fact that the hub cells are the only source of the ligands for Jak/Stat and Hh signaling pathways, possibly show that only the cyst cells that are in close proximity to the hub cells are able to receive these signals and are competent to activate these pathways and maintain their stemness characteristics. This arrangement also suggests that
the distant cyst cells from the hub cells are not able to receive the stemness signals and this plays a role as a controlling element in their differentiation.

**Figure 3.** *Drosophila* Hh signaling pathway. A) In the absence of the ligand Hedgehog, Patched keeps Smoothened inactive. Ci is phosphorylated and cleaved in the proteasomes and form a transcriptional repressor and helps keeping Hh signaling pathway target genes inactivated. B) In the presence of the ligand Hedgehog, it binds to iHog and patched and it takes away the inhibition of smothened by patched. Phosphorylation and activation of Smoothened allows the Ci to remain unprocessed and act as a transcriptional activator of the Hh signaling pathway target genes. Adapted from the Molecular Biology of the Cell (Bustin, 2015).
**Epidermal growth factor receptor signaling pathway.** Epidermal Growth Factor Receptor (EGFR), is a member of the Receptor Tyrosine Kinase (RTK) family. EGFR signaling pathway, like many other RTK pathways, functions by activating different pathways including the MAPK/ERK pathway (Mitogen Activated Protein Kinase/ Extracellular signal Regulated Kinase) (Kiger et al., 2000; Sarkar et al., 2007; Tran et al., 2000). Upon binding the ligand Epidermal Growth Factor (EGF) to its receptor, the EGFR kinase domain is activated and auto phosphorylates on the cytosolic domain creating docking sites for other proteins such as Ras protein and other adaptor proteins to bind to the receptor and be phosphorylated. Activated Ras then activates the MAPK pathway and changes the gene transcription in the testis stem cells (Figure 4) (Bustin, 2015; Kitadate and Kobayashi, 2010). MAPK acts through a progressive phosphorylation of different kinases in this pathway. Ras phosphorylates Raf (MAPKKK), Raf phosphorylates Mek (MAPKK), and Mek phosphorylates the Erk (MAPK). Erk then phosphorylate transcription factors or other kinases (38).

EGF is secreted from the germ cells, and activates EGFR on the cyst cells, promoting differentiation of the cyst cells (Sarkar et al., 2007; Schulz et al., 2002). Inhibiting egfr or raf in the cyst cell lineage results in maintenance of the population of cyst cells in the testis but a non-autonomous accumulation and growth of undifferentiated germ cells was observed (Amoyel et al., 2016; Chen et al., 2013; Parrott et al., 2012). This evidence led to the speculation that cyst cells promote germline cell differentiation through the EGFR/MAPK signaling pathway (Kiger et al., 2000b; Sarkar et al., 2007; Schulz et al., 2002; Tran et al., 2000).
Also, EGFR signaling is required for encapsulation of the germ cell via activating Rac1 protein in the cyst cell lineage. Rac1 protein is a GTPase and is responsible for the cyst cells growth cone persistence and correct enclosure of germline cells by cyst cells and it gets inhibited by the Rho protein. Rho protein is also a GTPase and activating Rho results in cyst cells fail to envelope the germ cells (Bustin, 2015; Gupta et al., 2018).

![Figure 4. RTK/Ras/MAPK signaling pathway. Binding the growth factor ligand to the RTK brings the receptor monomers together and creates a dimer which leads to transphosphorylation and activation of the receptor and creates docking sites for other proteins and activation of the Ras protein which phosphorylate and activate the MAPK pathway cascade. Upon phosphorylation and activation of Erk, it is released and moves to the nucleus and changes the MAPK signaling pathway target genes. Adapted from the Molecular Biology of the Cell (Bustin, 2015).](image)

Studies have also shown that EGFR/MAPK signaling pathway is also operating and required for CySCs maintenance as stem cells as diphosphorylated MAPK (dpErk) accumulates in the CySCs and egfr mutant clones failed to be maintained in the testis niche by not be able to compete for the niche and they were replaced by the wildtype.
CySCs (Amoyel et al., 2016; Kiger et al., 2000). Activation of ERK downstream targets in CySCs is necessary for synchronizing the mitotic divisions of transit amplifying germ line cells and subsequently their division (Gupta et al., 2018).

**PVR signaling pathway.** PVR (PDGF/VEGF homolog) is a receptor tyrosine kinase. Vascular Endothelial Growth Factor (VEGF) and Platelet-Derived Growth Factor (PDGF) receptors are members of RTKs family, which are involved in many processes in development, cancer, and metastasis. PDGF receptors (PDGFRs) are crucial for cell survival, proliferation, and migration (Heldin and Westermark, 1999; Hoch and Soriano, 2003) and VEGF receptors (VEGFRs) are essential for embryonic vasculogenesis and angiogenesis in humans (Ferrara et al., 2003; Olsson et al., 2006). In *Drosophila*, PVR is required in embryonic dorsal closure and in pupal thorax closure (Garlena et al., 2015; Ishimaru et al., 2004). PVR signaling pathway is also involved in ovarian border cells in female ovaries, hemocyte cells, and glial cells migration (Duchek et al., 2001; Learte et al., 2008; McDonald et al., 2003; Wood et al., 2006). In *Drosophila* intestines, PVR is involved in stem cells mitosis regulation and homeostasis. When PVR was inhibited in the *Drosophila* intestines stem cells, it resulted in a significant decrease in their population and by constitutively activating PVR in these stem cells, an over-proliferation of cells was observed in the intestine (Bond and Foley, 2009). The previously described CySCs stem cell factor, Zfh-1 has been reported to influence PVR-dependent map kinase signaling (MAPK/ERK) (Park et al., 2009; Sims et al., 2009). Thus, these published articles suggest that PVR might play a role in maintenance and or cycling of CySCs.
**Transforming growth factor β signaling pathway.** The TGFβ (Transforming Growth Factor beta) signaling pathway in *Drosophila* contains three ligands: *decapentaplegic (dpp)*, *glass bottom boat (gbb)*, and *screw (scw)*. The TGFβ pathway has two type-II cytokine receptors called *punt (put)* and *wishful thinking (wit)*. It consists of three type-I receptors called *thick veins (tkv)*, *saxophone (sax)*, and *baboon (babo)* and two receptor-activated intracellular transducers called *Mothers against decapentaplegic (Mad)*, and *Smad on X (Smox)*, one intracellular co-transducer called *Medea (Med)*, and one inhibitory transducer called *Daughters against decapentaplegic (Dad)*. The TGFβ ligands will dimerize together prior to binding type-I and type-II receptors on the outside surface of the plasma membrane. Both types of receptors dimerize simultaneously and type-I and type-II dimers further oligomerize to form a quaternary, ligand-bound structure. When the receptor structure is assembled, the receptors phosphorylate and activate Smads. The receptor-activated Smad proteins are the signal transducers and transcriptional modulators. Smads are phosphorylated at the receptor complex and then travel to the nucleus to elicit transcriptional effects. These intracellular Smads can bind each other and recruit a co-Smad to form trimeric transcription factor complexes (Figure 5). Depending on Smads’ phosphorylation patterns and other activating or deactivating modifications, these structures can further bind to a variety of factors. In the nucleus, activated complexes are capable of binding to other transcription factors in order to change the transduced signal in different ways. One typical target is i-Smad genes; i-Smads are expressed with BMP (Bone Morphogenic Protein) signaling activation and proceed to repress the BMP activation in a negative feedback cycle (Leatherman and DiNardo, 2008; Schulz et al., 2004; Shivdasani and Ingham, 2003).
The TGFβ signaling pathway in the testis niche has been shown to act at two places; first, the BMP signaling pathway has an identified role in regulating GSCs and is required for self-renewal of these cells (Kawase et al., 2004; Shivdasani and Ingham, 2003). The ligands for this pathway, dpp and gbb, are expressed in the hub, and gbb is also expressed in the CySCs. Previous studies have shown that by over-activation of the BMP signaling pathway genes in germline lineage cells, the number of GSCs were mildly increased, and by ablating this pathway genes, the testes lost their GSCs (Bunt and Hime, 2004; Kawase et al., 2004; Michel et al., 2011; Schulz et al., 2004; Shivdasani and Ingham, 2003). Inhibition of the gene bag-of-marbles (bam), a gene that is repressed by the BMP signaling pathway, was found to be essential for GSC self-renewal. Bam misexpression in GSCs promotes their differentiation (Kawase et al., 2004).
Figure 5. The TGFβ/Activin signaling pathway. Binding the TGFβ dimer allows the assembly of the tetrameric receptors. Type II receptors phosphorylate type I receptors and results in activation of type I kinase domain which leads to phosphorylation of Smads. Smads form a complex that then moves to the nucleus and change the transcription of the TGFβ pathways target genes. Adapted from the Molecular Biology of the Cell (Bustin, 2015)

A second place that TGFβ signaling is required is during the differentiation process. Activin-type signaling is required in the differentiating cyst cells to encyst germ cells and this correct encasement is required for germline cells to perform precise mitotic division transition and differentiation from spermatogonia to spermatocyte (Li et al., 2007; Matunis et al., 1997). Activin type signaling is distinct from BMP-type signaling, in the way that it uses a different receptor-activated Smad called Smox. If expression of
smox, the type II receptor punt, or the mad gene cofactor schnurri, are inhibited in the
cyst cells, the germ cells will over-proliferate in response (Li et al., 2007).

**Contact Dependent Inhibition of Proliferation**

Contact dependent inhibition of proliferation has recently been proposed as a
mechanism that regulates CySCs division and proliferation, via the function of the tumor
suppressor factor Merlin (Mer) (Inaba et al., 2017). Contact dependent inhibition of
proliferation has been most carefully studied in cell culture, and is characterized by
proliferation arrest when the cells reach confluency, elicited by cell-cell contact via
adherens junctions (Chiasson-MacKenzie et al., 2015). Deficiency in contact-dependent
inhibition of proliferation is one of the indications of tumor cells (Hanahan and
Weinberg, 2011; Polyak et al., 1994). Merlin is a homolog of the neurofibromatosis 2
(Nf2) tumor suppressor gene and it encodes a protein that is closely related to 4.1, Ezrin,
Radixin, and Moesin (FERM) cytoskeletal linker superfamily proteins which are required
in cellular structures and cell adhesion (Sherman and Gutmann, 2001). Merlin localizes
primarily at the plasma membrane and cytoskeleton, and it binds to several
transmembrane receptors and intracellular proteins (Stamenkovic and Yu, 2010). Growth
factor receptors including receptor tyrosine kinases such as EGFR are inhibited by the
contact dependent inhibition of proliferation through Mer even with continuous supply of
their ligands (McClatchey and Yap, 2012). The anti-proliferation function of Mer is
triggered by cell adhesion and control of the availability and function of membrane
receptors such as EGFR in mammalian cells and *Drosophila*. In mammalian cell culture
Mer negatively regulates the EGFR Ras Erk pathway by blocking the internalization of
activated EGFR (Ammoun et al., 2008; Chiasson-MacKenzie et al., 2015; Cooper and
Giancotti, 2014; Curto et al., 2007; Curto and McClatchey, 2008; Jin et al., 2006; Maitra et al., 2006).

Merlin plays a role as an anti-mitogenic factor in tight-junctions. Hence, inactivating Mer can lead to mitogenic signaling and neoplastic over-proliferation of cells. Recent study has shown the role of Mer in preventing CySCs from excess proliferation in association with GSCs in the Drosophila testis. Mer has been shown to localize to the cell membrane of the cyst cell lineage. In testes that were mutated for Mer, CySCs could not uniformly coordinate their proliferation with GSCs and the number of cyst cells were reported higher (Inaba et al., 2017). This lack of coordination was highlighted when the Mer mutation was combined with dpp stimulation and it led to proliferation of cyst lineage cells. They further modified major components of adherens junctions, E-cadherin and Armadillo protein, in the absence of Mer in the cyst lineage cells in the testis. In Merts1 mutants, a temperature sensitive mutant allele of mer, E-cadherin was less marked in between cyst cells compared to the wildtype and GFP-Armadillo was lost in between cyst cells. Based on these evidence, the researchers concluded that the phenotype of mer mutant testes comes from destabilizing the adherens junctions (Inaba et al., 2017).

**Scopes of Dissertation**

The goal of this dissertation was to deliver insights into the mechanisms and different genetic pathways in which were involved in the maintenance and cell division rate of the CySCs in the niche. First, I identified and described the PVR pathway in the testis and determined their role in regulating the CySCs maintenance and division frequency and its relationship with the tumor suppressor Merlin. Second, I examined the
role of the BMP signaling pathway in maintenance of the CySCs and the non-autonomous effect of this pathway on the GSCs. Overall, I demonstrated the importance of PVR and BMP signaling pathways that mediated the CySCs maintenance and division rate in the *Drosophila* testis stem cell niche.
CHAPTER II

THE ROLE OF RECEPTOR TYROSINE KINASE PVR
IN REGULATING CELL DIVISION FREQUENCY
AND MAINTENANCE OF STEM CELLS
IN THE DROSOPHILA TESTIS
STEM CELL NICHES

Introduction

Stem Cells and Their Niches

Adult tissues maintain long term homeostatic function by sustaining a population of stem cells. These stem cells are distinguished by their ability to keep a proper balance between self-renewal and differentiation. Adult stem cells mostly reside in a specialized area known as the niche. The stem cell niche provides the molecular signals that (affects the fate) regulates self-renewal and expansion of the stem cells and is required for promoting the characteristics of stemness while at the same time inhibiting excess proliferation (de Cuevas and Matunis, 2011; Ohlstein et al., 2004). After the stem cell symmetric cell division, the daughter cell will remain next to the niche and is able to receive the molecular signals from the niche and maintain its stemness. Asymmetric stem cell division results in removal of the transit amplifying daughter cell from the niche, resulting in a reduced volume of received signals from the niche (Morrison and Spradling, 2008; Resende et al., 2013). This results in differentiation of the daughter cells. A single stem cell niche is capable of supporting one or more types of stem cells.
(Morrison and Spradling, 2008; Yamashita et al., 2003). The regulation of these stem cells by the niche and coordination of this balance and whether one stem cell is maintaining the other stem cell is poorly understood. Although the volume of research aimed at understanding the function of stem cells and their niches has increased in the past decades, there are many characteristics of adult stem cells that require more research to understand different aspect of stem cell regulation, including how multiple stem cell lineages residing in a single niche coordinate proliferation (Li and Xie, 2005). Breaking the niche coordination balance may disrupt the architecture of the tissue that these stem cells reside in and this results in increasing decay of the tissue regenerative abilities (Behrens et al., 2014).

Stem cells have great capacity to be used in regenerative medicine and acquiring anti-cancer therapies that eradicate cancer stem cells (Preston et al., 2003). All these findings about adult stem cells will help us to put aside the ethical issues of using embryonic stem cells in regenerative medicine. Besides all the possible assets of using stem cells in regenerative medicine, the discovery that cancer cells frequently have significant similarities to normal stem cells has raised the clinical importance toward investigating the nature and essence of these cells (Preston et al., 2003; Reya et al., 2001).

**Drosophila Testis Stem Cell Niche**

The *Drosophila* testis stem cell niche provides an exceptional in vivo model to study stem cells in their niches at a molecular and cellular level (Cheng et al., 2011; Sheng et al., 2009). *Drosophila* testis is a blind ended coiled tube and the niche of the stem cells is located at the apical end of the testis. The niche is made up of 10 to 15 tightly packed completely differentiated somatic cells that are
termed the hub cells. This niche supports two population of stem cells which are physically attached to the niche cells by adherens junctions (Hardy et al., 1979). On the condition that these stem cells are physically attached to the hub, they can remain as stem cells and by being removed from the hub, the stem cells are incapable of receiving signals from the hub and they start to differentiate. Recent studies demonstrated that there is a selection for the fittest stem cell that can maintain its population and proliferate for the tissue homeostasis and development and thus a competition between stem cells to remain in the niche and the

_Drosophila_ testis stem cells are no exception to this rule (Issigonis et al., 2009; Singh et al., 2010).

There are two population of stem cells residing in the niche. The germline stem cells (GSCs) that are attached to the hub cells by direct connections through cadherin based cell adhesions molecules and cyst stem cells (CySCs) that are in contact with the hub by their cellular extensions (Davies and Fuller, 2008; de Cuevas and Matunis, 2011; Ohlstein et al., 2004). The GSCs undergo asymmetric cell division with mitotic spindles perpendicular to the hub (Cheng et al., 2011; Yamashita et al., 2003). The daughter cell that stay in contact with the hub is competent to receive the stemness molecular signals from the hub and remains as a stem cell and the one that is removed from the hub will differentiate into gonialblast owing to the deficiency of stemness ligands concentration. Similarly CySCs daughter cell that moved away from the hub differentiates into a cyst cell (Gonczy and DiNardo, 1996; Hardy et al., 1979). Cyst cells do not divide further, but they continue to expand their cellular extensions around the germline cells.
(Cheng et al., 2011; de Cuevas and Matunis, 2011). Gonialblast then complete four incomplete rounds of mitosis and create 16 interconnected spermatogonia while the two cyst cells encapsulate these germ cells and guide them through their differentiation (Gonczy and DiNardo, 1996). Then, these germ cells grow in size up to 25 times, become transcriptionally highly activated, and become spermatocytes which enter meiosis and complete their differentiation into sperms (Voog et al., 2008; Yamashita et al., 2003).

Appropriate differentiation of germline cells is reliant on correct encapsulation by cyst cells. Early germline cells secrete an EGF like ligand Spitz, which activates the EGFR signaling pathway in the cyst cells (Kiger et al., 2000; Tran et al., 2000). EGFR signaling pathway functions by activating PI3K signaling pathway in the cyst cells to facilitate enveloping the germ cells through encapsulation by activating Rac1(DiNardo et al., 2011; Sarkar et al., 2007). By disrupting the EGFR signaling pathway through loss of egfr or raf, encapsulation of germline cells by cyst cells fail and the spermatogonia are incapable of completing their differentiation and turn into spermatocytes (Kiger et al., 2000; Sarkar et al., 2007; Schulz et al., 2002; Tran et al., 2000). These spermatogonia without correct encapsulation will undergo extra rounds of cell division and accumulation of early staged germline cells in the testes occurs (Gupta et al., 2018; Kiger et al., 2000).

**Epidermal Growth Factor Receptor Signaling Pathway**

EGFR/MAPK signaling pathway in CySCs is not only required for promoting differentiation in the germline cells, but also autonomously is required for the maintenance of CySCs as dpERK (diphosphorylated and active form of ERK) accumulates in the CySCs and egfr mutant clones do not persist in the stem cell niche. A
recent study showed the role of EGFR/MAPK signaling pathway by ablating egfr or MAPK signaling pathway components resulting in significant decrease of the population of Zfh1+ CySCs in the testes (Amoyel et al., 2016). It is also well established that modulation of MAPK signaling levels in the CySCs is able to affect how competitive the CySCs are for a spot in the niche, next to the hub. This is likely the reason why individual mutant egfr cells (“mutant clones”) in an otherwise heterozygous background are not maintained in the niche, in contrast to the maintenance of egfr mutant CySCs in the niche when all the CySCs are mutant (Amoyel et al., 2016; Qian et al., 2014). Several other studies have demonstrated that when MAPK signaling is increased, those CySCs are able to better compete for a spot in the niche (Amoyel et al., 2016; Hou and Singh, 2017; Issigonis et al., 2009; Singh et al., 2016). Specifically, loss of socs36E, a negative inhibitor of the self-renewal Jak/Stat signaling pathway in the CySCs, resulted in CySCs outcompeting GSCs for a position next to the hub. In the CySCs with socs36E inhibition, dpERK was accumulated in the cells indicating that the mediation of MAPK signaling pathway resulted in an increase in CySCs competency to stay in the niche and out competed the GSCs (Amoyel et al., 2016; Amoyel et al., 2014). Another factor important in niche occupancy competition by stem cells is the tumor suppressor Mlf1-adaptor molecule (Madm). When Madm is knocked down, and the EGFR signaling pathway ligand is overexpressed, it can promote CySCs over-proliferation and increase their ability to outcompete GSCs from the niche (Singh et al., 2016).

This complicated setting where EGFR and the MAPK/ERK signaling can promote differentiation in the germline cells, but also contributes to maintaining CySCs stemness raised the question of how the signaling output may be modified to promote
these different outcomes. We speculated that another RTK pathway may alter the signaling output in one cell population, but not the other. Microarray experiments have previously identified Pvf₁ as a gene with enriched expression in the testis niche (Terry et al., 2006). Therefore, we decided to investigate a potential role for this RTK in the testis niche. In addition, the previously described CySC stem cell factor, Zfh-1, has been reported to specifically potentiate PVR-dependent map kinase signaling (MAPK/ERK) (Park et al., 2009; Sims et al., 2009). Thus, we speculated that the RTK PVR may play a role in the CySCs, in either their maintenance as stem cells or their cycling behavior.

A recent study has shown that the tumor suppressor Merlin, the Neurofibromatosis type 2 (NF2) homolog, is responsible for regulating proliferation and cell division of the CySCs in the Drosophila testis niche by the contact dependent inhibition of proliferation mechanism (Inaba et al., 2017). Contact dependent inhibition of proliferation has been extensively studied in cell culture (Chiasson-MacKenzie et al., 2015). This mechanism acts by cell-cell contact via adherens junctions and halt proliferation when cells attain confluence (Hanahan and Weinberg, 2011; Polyak et al., 1994). Merlin inhibits cell over-proliferation even with continuous supply of EGF (McClatchey and Yap, 2012). In mammalian cell culture Merlin, which localizes at the plasma membrane and cytoskeleton, negatively regulates the EGFR/MAPK signaling pathway by blocking the internalization of activated EGFR (Ammoun et al., 2008; Chiasson-MacKenzie et al., 2015; Cooper and Giancotti, 2014; Curto et al., 2007; Curto and McClatchey, 2008; Jin et al., 2006; Maitra et al., 2006; Stamenkovic and Yu, 2010). A recent study highlighted the role of Merlin in regulating CySCs proliferation. Merlin resides on the cell membrane of the CySCs and in Merlin mutants that the mitogen dpp
(ligand for BMP signaling pathway) was overexpressed, CySCs tumors were observed (Inaba et al., 2017). These data suggests that Merlin plays a role in downregulating the mitogenic pathways in the CySCs hence the role of this tumor suppressor in regulating other mitogenic pathways remains unknown.

Understanding the function of PVR in the CySCs of the *Drosophila* testis stem cell niche helps us to gain a better understanding of the stem cell regulation in their niches. With the intention of further examining the role of PVR in the testis, we first examined expression of PVR and its ligands. We then performed PVR loss of function and PVR gain of function experiments. For the loss of function experiments, three different techniques were used—RNA interference, express of a dominant negative transgene, and mutant clones. For the gain of function experiment, a constitutively activated PVR transgene was used.

**Materials and Methods**

**Fly Stocks and Husbandry**

The following stocks were used as they are described in Flybase and they were grown in room temperature 25°C: C587 Gal4, C587 Gal4; Tub Gal80ts/CyO, yw; Tj Gal4/CyO, w; UAS DN PVR, w; UAS PVR RNAi/CyO, w; UAS λ PVR, c306; pvr[C1195] FRT 40A/CyO, w; PVR[5] FRT 40A/CyO, PVR[1] FRT 40A/CyO, UAS Pvf1/CyO; UAS Pvf1/TM6b, w; P[lacZ] Pvf1 [G0146]/FM7c (Hilton et al., 2016), ywhsflp; Ubi-GFP FRT40A/CyO, w; P[w+ 36 F] FRT 40A, w; UAS EGFR RNAi, yw: UAS EGFR DN; UAS EGFR DN, UAS Mer. The w; UAS λ PVR/ CyO; UAS Mer/Tm6b was generated by multiple crosses. Crosses were grown at 25°C except for the crosses with C587 Gal4; Tub Gal80ts/ CyO driver that were grown at 18°C and all F₁ generation
progenies were aged at 29°C after eclosion. For the control the w; P[w+ 36 F] FRT 40A transgene was used.

**Upstream Activation Sequence Galactose4 System**

UAS Gal4 system is a transgene activation mechanism that uses tissue-specific promoter sequences positioned directly upstream of a Gal4 transcriptional activator gene to promote Gal4 expression in the target tissues only. Gal4 binds the upstream activation sequence (UAS) enhancer, causing transcription of genes placed immediately downstream of it. Aging the flies that have UAS and Gal4 components at 29°C improves the Gal4/UAS system and allows for greater expression of the anticipated transgene (Brand and Perrimon, 1993; Duffy, 2002; Fischer et al., 1988). In some crosses, a UAS Gal4; Gal80ts was used to express the manipulation only in adult flies. Gal80ts is the Gal4 repressor and repress the expression of Gal4 target genes in 18°C. When the F1 generation eclosed, the temperature was shifted from 18°C to 29°C and this shift of temperature inactivated the temperature sensitive Gal80ts and thus activating Gal4 and gene transcription (Figure 6).
Three Different Techniques to Inhibit PVR in the Cyst Stem Cells

1. The PVR RNAi transgene (Duchek et al., 2001). RNA interference (RNAi) is a natural endogenous mechanism in the cell that degrades the messenger RNA (mRNA) transcripts using an RNA guide (Clemens et al., 2000). A Gal4 driver was used to express a PVR RNAi transgene specifically in the cyst lineage cells. Also, EGFR transcripts was inhibited by using EGFR RNAi transgenes in the cyst lineage cells. The testes were aged for 8 and 12 days, then dissected and immunostained. For the control, the Gal4 driver crossed with wildtype flies was used in which the F1 progenies had no genetic
manipulations.

2. The second technique used to knock down PVR was the dominant negative PVR transgene. Dominant negative PVR was expressed in cyst lineage cells using the Gal 4 UAS system (Sheppard, 1994). Dominant negative transgene inhibits the function of PVR protein. Dominant negatives function by expression of a mutant receptor protein which acts antagonistically to the wildtype protein. The assembly of the mutated receptor with the wildtype receptor impairs the correct function of the PVR receptor dimer in the cell (Elefant and Palter, 1999). The F1 generation was aged for 8 and 12 days, then testes were dissected. Also, EGFR dominant negative was expressed as a comparison to the PVR inhibition.

3. Mitotic recombination is a technique that has been developed for use in *Drosophila*, in which homozygous mutant cells for a particular gene are induced in an otherwise heterozygous background (Golic and Lindquist, 1989). The reason this technique was employed was because the individuals with the homozygous genotype for the mutant alleles would not survive as it is homozygous lethal in early stages of development in fruit flies (Theodosiou and Xu, 1998). For this purpose, the FLP/FRT system was used which is originated in *Saccharomyces cerevisiae* (Golic and Lindquist, 1989). This system is a site directed recombination technology that works through targeting the flippase (Flp), a recombination enzyme, to its target FRT (flippase recognition target) to mediate mitotic recombination in a controlled manner. In this system, the heterozygous individual’s dividing cells are forced to undergo mitotic recombination, and if this recombination occurs during G2 of the cell cycle, the subsequent mitosis has the potential to produce two homozygous daughter cells, one
which is homozygous mutant, and the other which is homozygous wildtype. lacks functional allele at the specific locus. The inclusion of a GFP transgene on the wildtype chromosome allowed cells that were homozygous mutant to be visualized by the lack of GFP expression after the recombination. Also, using the FLP under the control of a heat shock promoter gives us the advantage to control the time of clone induction. Like other molecular techniques, this technique has its own limitations. One limitation is that the mitotic recombination is not cell specific. So, except for the hub cells that are in quiescence, both dividing GSCs and CySCs will undergo mitotic recombination. The other limitation for this system is that it only affects the cells that are undergoing mitosis (Blair, 2003).

Three different mutant allele of PVR was used for this experiments. PVR<sub>5</sub> (a hypomorph mutated allele of PVR gene, which is not a total loss of PVR gene function), PVR<sub>1</sub>, and PVR<sup>c1197</sup> (which are PVR total loss of function mutated alleles of PVR). The F<sub>1</sub> generation was heat shocked and males were dissected on 0, 2, 4, 8, 12, 16, and 20 days after the clone induction. All mutant clones were randomly induced at 37°C for 1 hour, 1 hour rest in room temperature, and one more hour at 37°C using hs-FLP. Then immuno-stained the samples and imaged them and analyzed the results. The control of this experiment was the FRT driver crossed with the wildtype flies.

The mutant clones were marked negatively by not expressing green fluorescent protein (GFP). GFP was expressed under transcription control of the lacZ promoter. In <i>Drosophila</i> testis, lacZ was used as an enhancer trap for GFP in all cells and wherever the random mitotic recombination occurred, lacZ expression and thus GFP expression was attenuated in those cells. Consequently, the mutant clones were shown by lack of GFP
(Chalfie et al., 1994; O'Kane and Gehring, 1987; Yeh et al., 1995). All testes were analyzed for the presence of the CySCs, GSCs, or both cell type mutant clones and if no mutant clones were observed in the testes, it was assumed that the mitotic recombination did not occur in those testes. For the control, the only thing that the mutant clones were lacking was GFP expression but otherwise acting like wildtype cells.

**Tissue Fixation and Staining**

The same dissection and staining technique was used for all experiments. Male flies were dissected in Ringer’s solution (130 mM NaCl, 2 mM KCl, 1.8 mM CaCl$_2$, 4 mM MgCl$_2$, 35.5 mM Sucrose, 5 mM Hepes pH 6.9). Tissue samples were fixed in 4% formaldehyde for 20 minutes then were washed three times for 15 minutes in 1X PBS with 0.1% Triton X-100 (PBTx). Samples were blocked in 4% donkey serum in PBTx for at least an hour. Samples were incubated in primary antibody diluted in blocking solution overnight at 4°C, then washed 3 times in PBTx for at least 10 minutes each time. Next, secondary antibody was applied for at least an hour, followed by several PBTx washes. Tissues were stained with Hoechst 33342 dye at 1ug/mL in PBTx to stain the DNA in the cells. Finally, testes were equilibrated in 50% glycerol solution for at least an hour before fine dissection and mounting. Testes were mounted on slides in 90% glycerol with N-propyl gallate as an anti-bleaching agent. Slides were stored in the -20°C freezer to preserve the fluorescence stain.

The mouse anti-Fas3 antibody (Developmental Studies Hybridoma Bank, used at 1:50 dilution) was used to stain the hub cells, goat anti-Vasa (Santa Cruz, 1:300 dilution) was used to stain the germline cells, rabbit anti-Zfh1 (a kind gift from Ruth Lehmann, 1:5000 dilution) was used to stain CySCs, guinea pig anti-Tj (a kind gift from Dorothea
Godt, 1:10,000 dilution) was used to stain the cyst lineage cells, rat anti-PVR (a generous gift from Benny Shilo, 1:300 dilution) was used to stain PVR, and mouse anti-Pvf1 (a kind gift from Pernille Rørth, 1:200 dilution) was used to stain Pvf1 (Li et al., 2003). Testes were mounted on slides, and imaged on a Zeiss 700 confocal microscope. Testis images were quantified for the number of CySCs (Zfh1 positive cell) and the number of CySCs in S phase (EdU+ Zfh-1 positive cells) in the testis for all time points. CySCs were defined by staining positive for Zfh1 and not being more than a two germ cell distance away from the niche. The ICY software (Institute Pasteur) was used to visualize and analyze the images using the spot detector option.

To verify the cycling rate of cells in the testis, an EdU staining kit from Thermo Fisher was used (catalog number C10337). The kit marks cells that are undergoing S phase by integration of the thymidine analog 5-ethynyl-2'-deoxyuridine (EdU) in to the DNA while it is replicating. This process works by adding two steps to the regular dissection technique where testes tissues were incubated in EdU-containing Ringer’s (1:1,000 dilution) before being fixed, along with a ‘click-it’ reaction at the end of the staining procedure to visualize the EdU-labeled cells.

**Statistical Analysis**

All the quantifications for PVR loss of function experiments using dominant negative and RNA interference were statistically analyzed with unpaired Students’ t-test for the experimental group vs the control in Excel. To analyze the mutant clones experiments, Chi-square test was employed using GraphPads.
Results

PVR Localizes to the Cell Membrane of the Cyst Stem Cells and Cyst Cells

The anti-PVR antibody was used to determine the expression pattern of PVR in the testis. We found that PVR localizes to the membrane of cyst lineage cells. Cyst cells were detected by the expression of the Tj (Figure 7A and 7B). The cyst cells encapsulate each individual germ line stem cell, but also each cyst of differentiating germ cells. Since the PVR stain surrounds the groups of germ cells, not each individual differentiating germ cell, it is clear that PVR is being expressed only in the cyst lineage cells. To examine the expression of the PVR ligand, Pvf1, a lac Z enhancer trap and an anti-Pvf1 antibody was used. Pf1 antibody stain showed that Pf1 accumulated in the hub, shown by colocalization with the hub marker, fasciclin 3 (Figure 7C and 7D). The Pf1 enhancer trap (w; P[lacZ] Pf1 [G0146]/FM7c) showed lacZ expression in the hub, but also in the late cyst cells (Figure 7E and 7F). Since the late cyst cells did not stain with both methods, this staining location may be an artifact of the enhancer trap line. The fact that Pf1 is available in the hub and PVR is being expressed on the cell membrane of the cyst cell lineages suggests that PVR signaling may only be activated in the CySCs (and possibly auto-activated in the hub itself), but not in the cyst cells.
Figure 7. PVR and Pvf₁ expression pattern. PVR accumulates in hub and cyst lineage cells (A) PVR shown in white, (B) PVR is shown in green. Fas3 marks hub in magenta, the cyst cells are marked by Tj in white. Pvf₁, the soluble ligand for PVR, accumulates in the hub. (C) Pvf₁ shown in white, (D) Pvf₁ shown in green, Vasa marks germ cells in magenta. This experiment was performed by Dr. Leatherman. Pvf₁ lacZ enhancer trap line shows lacZ accumulation in the hub, and also faintly in differentiating cyst cells. (E) Pvf₁ shown in white, (F) Pvf₁ shown in green. Vasa marks germ cells in magenta. The hub in all images is outlined by a dashed line and a star.
RNA Interference Inhibition of PVR in the Cyst Stem Cells Significantly Reduces the Number of Cyst Stem Cells and Their Cell Division Rate in the Testes

To examine the role of PVR in the CySCs, PVR was inhibited by RNA interference using the tissue specific driver C587 Gal4. To compare the results of PVR inhibition, EGFR was also inhibited in the cyst lineage cells. We also simultaneously inhibited PVR and EGFR in the cyst lineage cells to compare the loss of both RTKs in the testes. All crosses were raised at room temperature and after the F1 progeny eclosed, they were shifted to 29°C, and aged for different numbers of days. To assess possible changes in the number and cell division rate of CySCs and GSCs, antibody staining for Vasa, Zfh1, and Fasciclin 3, and EdU labeling, were used (Figure 8A-H).
Figure 8. PVR inhibition in the cyst cells significantly reduced the CySCs and cycling CySCs population. A) Control testis niche for day 8. B) Control testis niche for day 12. C) PVR RNAi transgene stem cell niche for day 8. D) PVR RNAi transgene stem cell niche for day 12. E) EGFR RNAi transgene testis for day 8. F) EGFR RNAi transgene testis for day 12. G) PVR EGFR double RNAi testes for day 8. H) PVR EGFR double RNAi testes for day 12. DNA is stained in blue, Hub in white, germ cells in magenta, and cyst cells in green. The hub is shown by a white star. All samples were aged in restrictive temperature of 29°C for 8 and 12 days. Scales bar = 10 µm for all images.
In control testes, after aging the testes for 8 days, the normal architecture of the niche was observed, with an average of 28 CySCs per testis (Figure 9A, Table 1). In contrast, the number of CySCs in PVR RNAi testes averaged 21, which was significantly lower than that found in control testes after 8 days of aging in 29°C (Figure 9A, Table 1, Table 2). The number of CySCs per testis that were positive for EdU were also assessed, and we observed a significant decrease between EdU-positive cells in control (average of 11 CySCs per testis) and PVR RNAi (average of 8 CySCs per testis) after 8 days of aging (Figure 9C, Table 1, Table 2). To figure out whether this significant decrease in EdU$^+$ CySCs was due to the decrease in total number of CySCs, or lower cell division frequency of CySCs, the ratio of EdU$^+$ CySCs to total number of CySCs was calculated (Figure 9E and 9F, Table 1). In control testes, after 8 days of aging the fraction of EdU-positive CySCs was 0.4, while in PVR RNAi testes the fraction of EdU-positive CySCs was 0.34 (Figure 9E, Table 1). This experiment was repeated for aging progeny for 12 days and the results agreed with our observations for day 8 timepoint (Figure 9B, 9D, and 9F). Therefore, this ratio suggests that loss of PVR in the CySCs resulted in a significant decrease in the cell division rate in the CySCs. In contrast, the number of GSCs and EdU$^+$ GSCs and the fraction of EdU$^+$ GSCs over total number of GSCs were quantified after 8 and 12 days of aging, and there was no significant difference between the experimental group and the control suggesting that inhibiting PVR in the cyst lineage cells has no non-autonomous effect on the population of GSCs and proliferating GSCs (Figure 10A-F).
Figure 9. PVR inhibition in the cyst cells caused significant reduction of CySCs and cycling CySCs and CySCs cell division rate. A) The number of Zfh1+ CySCs after 8 days of aging. B) The number of Zfh1+ CySCs after 12 days of aging. C) The number of Zfh1+ EdU+ CySCs after 8 days of aging. D) The number of Zfh1+ EdU+ CySCs after 12 days of aging. E) Fraction of Zfh1+ EdU+ CySCs over total number of Zfh1+ CySCs after 8 days of aging. F) Fraction of Zfh1+ EdU+ CySCs over total number of Zfh1+ CySCs after 12 days of aging. Images were analyzed and the P value was calculated using the Student t-test. * shows P value of lower than 0.05, ** shows P value of lower than 0.001, *** shows P value of lower than 0.0001.
### Table 1.

**Average and sample number of CySCs, EdU⁺ CySCs, ration of EdU⁺ CySCs/ CySCs, GSCs, EdU⁺ GSCs, EdU⁺ GSCs/ GSCs**

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<th>EdU⁺ CySCs/ CySCs (n)</th>
<th>GSCs (n)</th>
<th>EdU⁺ GSCs (n)</th>
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### Table 2.

**The P value of CySCs, EdU⁺ CySCs, ration of EdU⁺ CySCs/ CySCs, GSCs, EdU⁺ GSCs, EdU⁺ GSCs/ GSCs compared to the control. P value was calculated by unpaired Students’ t-test.**

<table>
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<th>P Value compare to the control</th>
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<th>EdU⁺ CySCs</th>
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Figure 10. PVR inhibition in the cyst cells had no effect on the GSCs numbers around the hub and division rate. A) The number of GSCs after 8 of aging. B) The number of GSCs after 12 days of aging. C) The number of EdU+ GSCs after 8 days of aging. D) The number of EdU+ GSCs after 12 days of aging. E) Fraction of EdU+ GSCs over total number of GSCs after 8 days of aging. F) Fraction of EdU+ GSCs over total number of GSCs after 12 days of aging. Images were analyzed and the P value was calculated using the Student t-test. There was no significant difference between the control and experimental groups.

The EGFR pathway was also inhibited by expression of an RNAi transgene to compare the results of inhibiting this RTK to PVR inhibition (Figure 8E and 8F). A double mutant of PVR RNAi and EGFR RNAi transgene line was created to see the effect of eliminating these RTKs in the cyst lineage cells at the same time (Figure 8G and 8H). In testes that EGFR was inhibited, the number of CySCs and EdU+ CySCs was significantly reduced, similar to what we observed with PVR RNAi (Figure 9A-D, Table
We also calculated the fraction of EdU\(^+\) CySCs over total number of CySCs and it showed significant decrease and thus significant reduction of CySCs cell division rate like PVR RNAi testes even after 8 days of aging the flies in 29°C (Figure 9E and 9F, Table 1, Table 2). As with our PVR inhibition results, there was no significant difference in the population of GSCs, EdU\(^+\) GSCs and their fraction between the EGFR RNAi testes and the wildtype testes (Figure 10A-F, Table 2). The PVR EGFR double mutant testes also showed a significant decrease in total population of CySCs and EdU\(^+\) CySCs and their fraction after aging for 8 and 12 days (Figure 9A-F, Table 1, Table 2). As with PVR and EGFR knock downs, there was no significant difference in GSCs, EdU\(^+\) GSCs and their fraction for the double PVR EGFR knock downs compare to the wildtype testes (Figure 10A-F, Table 1, Table 2). Interestingly, when both PVR and EGFR were knocked down in the testes, the loss of CySCs and EdU\(^+\) CySCs was not significantly different from each single RTK being inhibited by themselves. For these experiments, similar results were obtained with both eight and twelve days of aging.

The EGFR RNAi testes showed the accumulation of Transit Amplifying (TA) spermatogonia and cysts of germ cells with more than 16 TA spermatogonia was observed, as has been previously described (Amoyel et al., 2016; Gupta et al., 2018). Interestingly, in PVR RNAi testes, the differentiation of germline cells also seemed disrupted and in some of the testes no spermatocytes were observed and testes were filled with early differentiating germline cells and a cyst of germline cells dividing more than 16 times was observed (Figure 11).
Figure 11. PVR inhibition in the cyst cells disrupted the differentiation of germline cells. DNA is stained in blue, Hub in white, germ cells in magenta, and cyst cells in green. The hub is shown by a white star. The dashed line shows a cyst of germ cells with more than 16 TA spermatogonia. Scales bar = 10 µm.
Dominant Negative Inhibition of PVR Transgenes Expression Confirms that PVR is Required for Regulating Cell Division Frequency in the Cyst Stem Cells

To confirm the results of PVR inhibition using RNA interference, dominant negative transgenes for both PVR and EGFR were expressed. The C587 Gal4; Tubulin Gal80ts virgin female flies were used as a driver and it was crossed to wildtype male flies as the control group and to PVR DN or EGFR DN male flies (Figure 12A-F). The crosses were raised in 18°C and after the F1 generation flies eclosed, they were shifted to 29°C. Flies were aged for 8 or 12 days after eclosion.

We found that the population of CySCs, dividing CySCs per testis, and the cell division rate of CySCs was significantly reduced when PVR and EGFR were inhibited in the testes after eight or twelve days of aging (Figure 12C-F). In control testes, after 8 days of aging, the normal structure of the niche in the testis was detected, with an average of 28 CySCs per testis (Figure 12A, Figure 13A, Table 3). In contrast, the number of CySCs in PVR DN testes averaged 20, which was significantly lower than that found in control testes after 8 days of aging in 29°C (Figure 13A, Table 3, Table 4). The number of CySCs per testis that were positive for EdU stain were also measured, and a significant decrease between EdU-positive cells in control (average of 11 CySCs per testis) and PVR DN (average of 7 CySCs per testis) was observed (Figure 13C). To determine whether this significant decrease in EdU+ CySCs was due to the decrease in total number of CySCs, or lower cell division rate of CySCs, the ratio of EdU+ CySCs to total number of CySCs was calculated (Figure 13E and 13F, Table 3). In control testes, the fraction of EdU-positive CySCs was 0.4, while in PVR RNAi testes the fraction of EdU-positive
CySCs was 0.3 (Figure 13E and 13F, Table 3). Thus, this ratio implies that loss of PVR in the CySCs resulted in a significant decrease in the cell division rate in the CySCs (Table 4). Dominant negative experiments were repeated for day12 and results agreed with day 8 time point results (Figure 13B, 13D, and 13F). Like PVR RNAi experiments, the population of GSCs, cycling GSCs, and the cell division rate of GSCs did not change (Figure 14A-F). As we observed with PVR RNA interference, the dominant negative inhibition of PVR resulted in accumulation of early staged differentiated germline cells throughout some testes and cysts of germ cells with more than 16 TA spermatogonia was observed (Figure 15).
Figure 12. PVR inhibition in the cyst cells significantly reduced the population of CySCs and cycling CySCs in the testes. A) Control testis niche for day 8. B) Control testis niche for day 12. C) PVR DN transgene stem cell niche for day 8. D) PVR DN transgene stem cell niche for day 12. E) EGFR DN transgene testis for day 8. F) EGFR DN transgene testis for day 12. DNA is stained in blue, Hub in white, germ cells in magenta, and cyst cells in green. The hub is shown by a white star. All samples were aged in restrictive temperature of 29°C for 8 and 12 days. Scales bar = 10 µm for all images.
Figure 13. PVR inhibition in the cyst cells caused significant reduction of CySCs and cycling CySCs and CySCs cell division rate. A) The number of Zfh1+ CySCs after 8 days of aging. B) The number of Zfh1+ CySCs after 12 days of aging. C) The number of Zfh1+ EdU+ CySCs after 8 days of aging. D) The number of Zfh1+ EdU+ CySCs after 12 days of aging. E) Fraction of Zfh1+ EdU+ CySCs over total number of Zfh1+ CySCs after 8 days of aging. F) Fraction of Zfh1+ EdU+ CySCs over total number of Zfh1+ CySCs after 12 days of aging. Images were analyzed and the P value was calculated using the Student t-test. * shows P value of lower than 0.05, ** shows P value of lower than 0.001, *** shows P value of lower than 0.0001.
Table 3.

*Average and sample number of CySCs, EdU\(^+\) CySCs, ration of EdU\(^+\) CySCs/ CySCs, GSCs, EdU\(^+\) GSCs, EdU\(^+\) GSCs/ GSCs*

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Table 4.

*The P value of CySCs, EdU\(^+\) CySCs, ration of EdU\(^+\) CySCs/ CySCs, GSCs, EdU\(^+\) GSCs, EdU\(^+\) GSCs/ GSCs compared to the control. P value was calculated by unpaired Students’ t-test*

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Figure 14. PVR inhibition in the cyst lineage cells had no non-autonomous effect on the GSCs numbers around the hub and division rate. A) The number of GSCs after 8 days of aging. B) The number of GSCs after 12 days of aging. C) The number of EdU+ GSCs after 8 days of aging. D) The number of EdU+ GSCs after 12 days of aging. E) Fraction of EdU+ GSCs over total number of GSCs after 8 days of aging. F) Fraction of EdU+ GSCs over total number of GSCs after 12 days of aging. Images were analyzed and the P value was calculated using the Student t-test. There was no significant difference between the control and experimental groups.
Figure 15. PVR inhibition using dominant negative transgenes in the cyst cells disrupted the differentiation of germline cells. DNA is stained in blue, Hub in white, germ cells in magenta, and cyst cells in green. The hub is shown by a white star. The dashed line shows a cyst of germ cells with more than 16 TA spermatogonia. Scales bar = 10 µm.
**Individual PVR Mutant Cyst Stem Cells Are not Maintained as well as Wild Type Cyst Stem Cells**

To gain further insights into the role of PVR in the cyst cells, next we examined inhibition of PVR using mitotic recombination. Clone induction was used with three different mutant alleles of PVR (PVR$_1$, PVR$_5$, and PVR$^{cl197}$). All clones were induced using a hs FLP in males at 37°C and then aged for 0, 2, 4, 8, 16, and 20 days in order to observe whether the PVR mutant cells could persist as stem cells. Mitotic recombination can only be induced in mitotically active cells, therefore only CySCs, GSCs, and transit amplifying spermatogonia are able to have clones induced. Mutant clones were negatively marked by lack of GFP (Figure 16A and 16B). Each testis was tallied as to whether it had a mutant CySC or GSC clone, or not. If testes did not have any cyst lineage cells or germline cells clones, they were removed from the calculations as it was assumed that the random clone induction, or immunostaining did not work in them. We hypothesized that if PVR is required for self-renewal, mutant clones would be observed at early time points, but would disappear at later time points after clone induction.

In control testes, there was a normal decrease in the percent of testes with a marked clone, and this is a consequence of aging and stem cell turnover. At early time points after clone induction, we found no difference in the rate of CySC clones between the control and PVR mutants. At later time points, however, we observed a modest but statistically significant decrease in the percent of testes with PVR mutant clones in the CySCs (Figure 17, Table 5). This significant difference was specifically shown in PVR$_1$ mutant clones for CySCs in eight and 16 days of aging after clone induction and P value for both time points were less than 0.05 and statistically significant from the control.
testes. Thus, PVR mutant CySC clones are not able to persist as well compared to wildtype CySCs. As a control, we also tallied the percent of testes with GSC clones, and found no difference between control and PVR mutant GSCs (Figure 18, Table 6).

**Figure 16.** CySCs mutant clones do not persist as stem cells as well as the wildtype CySCs in the niche. PVR mutant clones are shown with the lack of GFP. Hub is marked by dashed line, all cells are stained with GFP and germline cells are marked with anti-Vasa in magenta, cyst cells are marked with anti-Tj with red. A) colored testis. B) black and white testis. The white arrows show CySCs mutant clones. Green arrows show CySCs with GFP⁺ Tj⁺ stain. The yellow arrows show GSC mutant clones. Scales bar = 10 µm for all images.
Figure 17. PVR inhibition by mitotic recombination in the cyst cells with 3 PVR mutant alleles. Testes were aged and dissected for 5 time points. Stars indicate the significant difference between control and experimental groups with P value of lower than 0.05. Images were analyzed and the P value was calculated using the Chi-squared test.

Figure 18. GSCs clones were used as an internal control for the mitotic recombination. Images were analyzed and the P value was calculated using the Chi-squared test.
Table 5.

*PVR inhibition by mitotic recombination in the cyst cells with 3 PVR mutant alleles.*
The percentage of CySCs clones and the sample number for time points 2, 4, 8, 16, and 20 is showed in the table above.

<table>
<thead>
<tr>
<th>% of testes with CySCs clones</th>
<th>Day 2 (n)</th>
<th>Day 4 (n)</th>
<th>Day 8 (n)</th>
<th>Day 16 (n)</th>
<th>Day 20 (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>82.87 (200)</td>
<td>67.5 (94)</td>
<td>53.43 (131)</td>
<td>25.97 (154)</td>
<td>22.5 (40)</td>
</tr>
<tr>
<td>PVR1</td>
<td>75.55 (45)</td>
<td>73.52 (68)</td>
<td>20.22 (89)</td>
<td>17.88 (123)</td>
<td>15 (40)</td>
</tr>
<tr>
<td>PVR5</td>
<td>87.5 (72)</td>
<td>82.81 (64)</td>
<td>46.61 (118)</td>
<td>22.45 (96)</td>
<td>20.5 (40)</td>
</tr>
<tr>
<td>PVR C1197</td>
<td>68.42 (76)</td>
<td>66.66 (48)</td>
<td>40.90 (89)</td>
<td>17.83 (157)</td>
<td>15.5 (40)</td>
</tr>
</tbody>
</table>

Table 6.

*PVR inhibition by mitotic recombination in the cyst cells with 3 PVR mutant alleles.*
The percentage of GSCs clones and the sample number for time points 2, 4, 8, 16, and 20 is showed in the table above.

<table>
<thead>
<tr>
<th>% of testes with GSCs clones</th>
<th>Day 2 (n)</th>
<th>Day 4 (n)</th>
<th>Day 8 (n)</th>
<th>Day 16 (n)</th>
<th>Day 20 (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>73.28 (200)</td>
<td>71.5 (94)</td>
<td>70.99 (131)</td>
<td>51.29 (154)</td>
<td>45 (40)</td>
</tr>
<tr>
<td>PVR1</td>
<td>88.88 (45)</td>
<td>88.23 (68)</td>
<td>85.39 (89)</td>
<td>61.78 (123)</td>
<td>52.5 (40)</td>
</tr>
<tr>
<td>PVR5</td>
<td>88.88 (72)</td>
<td>86.87 (64)</td>
<td>83.89 (118)</td>
<td>42.70 (96)</td>
<td>41.05 (40)</td>
</tr>
<tr>
<td>PVR C1197</td>
<td>89.47 (76)</td>
<td>83.33 (48)</td>
<td>80.68 (89)</td>
<td>61.14 (157)</td>
<td>42.5 (40)</td>
</tr>
</tbody>
</table>

**Pvf₁ Misexpression in the Cyst Cells**  
**Led to Tumorous Accumulation of Cyst Cells in the Testis**

The presence of Pvf₁ ligand was previously reported in the testes (Terry et al., 2006) and its accumulation in the hub was confirmed with antibody stain and enhancer trap in our experiments. With the purpose of determining the role PVR signaling pathway
in the *Drosophila* testis stem cell niche. The expression pattern results showed that Pvf₁ is only being expressed in the hub cells. We used the UAS Gal4 system to misexpress Pvf₁ in the cyst lineage cells beside hub cells using two different drivers, C587 Gal4, and Tj Gal4, and limiting the misexpression to adults with the inclusion of Tubulin Gal80ts.

The C597 Gal4 and Tj Gal4 crosses were grown at room temperature (25°C) and after the F₁ generation eclosed they were shifted and aged at 29°C for 3 and 5 days. The cross with C587 Gal4; Tubulin Gal80ts was raised at 18°C and then shifted to 29°C to age for 3 and 5 days. We found that when Pvf₁ was misexpressed in the cyst lineage cells beside hub cells where it is normally expressed, an over-proliferation of cyst cells in the testes was observed (Figure 19B). This tumorous accumulation of cyst cells in the testes almost eliminated all the germ cells. Cyst cells also were capable of dividing away from the hub, as shown by white arrows (Figure 19B). In wildtype testis, CySCs can only divide next to the hub (Figure 19A).
Figure 19. Pvf₁ over-expression in the cyst cells cause over-proliferation of them and elimination of germ cells. A) On the left a C587 Gal4 control testis is shown. B) Cyst cells over-expressing Pvf₁ filled the testis. EdU⁺ cyst cells can be observed away from the hub (arrows). DNA is stained in blue, Hub in white, germ cells in magenta, and cyst cells in green. The hub is shown by a star. Scales bar = 10 µm for all images.

**Constitutive PVR Triggers Hyper Proliferation of Cyst Lineage Cells in the Testis**

To further investigate the role of PVR signaling pathway in the cyst cells of the *Drosophila* testis, we used a constitutively activated PVR transgene (λ PVR) and expressed it in the cyst cells using the C587 Gal4 driver with Tubulin Gal80ts. Crosses were raised at 18°C and then shifted to 29°C and were aged for 3 days. We found that constitutively activated PVR was also able to induce cyst cells to divide away from the hub. In Wildtype testis, CySCs can only divide next to the hub (Figure 20A). Cyst cells accumulated as a large tumor, and the population of germline cells were almost completely lost (Figure 20B). These results are consistent with the Pvf₁ misexpression in the cyst lineage cells.
**Figure 20.** PVR gain-of-function in CySCs causes neoplastic over-proliferation of CySCs and germ line cells are lost in the testis. A) normal testis with Vasa-positive Germ cells and Tj positive cyst cells and the hub stain in Fas3 in the C587-Gal4; Tub Gal80ts control testis. B) Cyst cells over-expressing constitutively activated PVR (λPVR) occupied the testis and germ cells are almost lost in the testis after 3 days of aging in restrictive temperature of 29°C. DNA is stained in blue, Hub in white, germ cells in magenta, and cyst cells in green. The hub is shown by a star. Scales bar = 10 µm for all images.

**Tumor Suppressor Merlin Overexpression Inhibits Cyst Stem Cells Over-Proliferation Caused by Constitutively Activated PVR in the Cyst Lineage Cells**

A previous study showed that the tumor suppressor Merlin is being expressed on the cell membrane of the cyst lineage cells in the *Drosophila* testis and if you inhibit Merlin while expressing the mitogenic ligand Dpp, cyst cells over-proliferate suggesting that Merlin is a potent tumor suppressor in the cyst lineage cells. Thus, we decided to investigate the role of Merlin in inhibiting the PVR pathway in the cyst cells. We hypothesized that Merlin might be able to inhibit the tumorous accumulation of cyst cells caused by constitutively activated PVR. We created a line with both a Merlin
overexpression transgene and the constitutive PVR transgene, and crossed it to the cyst lineage drive C587 Gal4 with tubulin Gal80\(^{ts}\). Flies were grown in 18°C and then shifted to 29°C and were aged for 3 and 8 days. We found that overexpression of Merlin was able to prevent the tumor formation caused by constitutive PVR (Figure 21).

**Figure 21.** Merlin gain-of-function in CySCs halted the neoplastic over-proliferate of the CySCs caused by PVR overexpression in the cyst cells. A) Control testis aged for 8 days. B) Double mutants of PVR constitutive active and Merlin overexpression in the CySCs after aging for 8 days C) Constitutively activated PVR (\(\lambda\) PVR) in the CySCs after aging for 3 days. DNA is stained in blue, Hub in white, germ cells in magenta, and cyst cells in green. The hub is shown by a white star. Scales bar = 10 \(\mu\)m for all images.
Conclusions

PVR Regulates the Cell Division Frequency of the Cyst Stem Cells

The data presented here show that PVR plays a role in the cell division rate of the CySCs in the *Drosophila* testis stem cell niche. This was demonstrated via loss of function experiments, as inhibiting PVR in the CySCs by RNA interference and dominant negative transgene expression resulted in significant reduction of CySCs at the niche and the cycling CySCs (Figure 9A-D and Figure 13A-D). It was also demonstrated by constitutive PVR expression, or ectopic Pvf₁ ligand expression, which resulted in tumorous accumulation cyst lineage cells throughout the testis, and misregulated cycling of these cells outside of the niche (Figure 19B and Figure 20B). Although the EGFR loss of function phenotype was similar in that the cycling rate and CySC numbers were decreased, constitutive EGFR did not promote tumor formation. These results suggest that PVR and EGFR have at least partially distinct roles in the cyst lineage of the testis.

A previous study indicated that the tumor suppressor Merlin accumulates on the cell membrane of the cyst lineage cells in the *Drosophila* testis, and loss of *merlin* led to excess CySCs (Inaba et al., 2017). Another study has shown that Merlin inhibits EGFR, PDGF, VEGF, and other RTKs in mammalian cells and inhibit proliferation (Larsson et al., 2008). Our result show that when constitutively activate PVR is combined with Merlin overexpression in the cyst cells lineage, Merlin suppresses PVR signaling and is able to prevent any tumor formation due to hyperproliferation of cyst cells in the testis (Figure 21B).
Non-autonomous Effects of PVR on Germline Differentiation

PVR non-autonomously affects the differentiation of germline cells in the testis. As described previously, inhibition of EGFR signaling pathway in the CySCs results in a significant reduction in the population of CySCs, and these testes were filled with early differentiated germline cells and the transit amplifying spermatogonia mitotic division was not synchronous meaning that cyst of germline cells dividing more than four times was observed (Amoyel et al., 2016). Our data from inhibiting EGFR agreed with the previous study and suggested that inhibiting PVR can result in germline cells differentiation disruption (Figure 11 and Figure 15). This differentiation disturbance can be due to significant reduction of cyst cells in the testis. Essentially, there are not sufficient cyst cells present in the testis to support the differentiation of germline cells and like EGFR, PVR might be involved in the correct encasement of germline cells.

When EGFR and PVR were over expressed in the cyst cells, differentiating TA spermatogonia and spermatocytes were lost in the testis (Hudson et al., 2013). However, in constitutively activated PVR testes an over-proliferation and neoplastic expansion of cyst cells was observed and cyst cells were dividing away from the hub. In contrast, in constitutively activated EGFR testes, no over-proliferation of the cyst cells was observed and cyst cells were only dividing next to the hub as in wildtype which shows that function of PVR is different from function of EGFR in CySCs (Hudson et al., 2013).

Role of PVR in Stem Cell Competition at the Niche

Furthermore, our results demonstrated that the PVR mutant CySCs clones do not persist as stem cells in the testis. Since mitotic recombination technique tests how mutant
cells function in the setting of a normal niche, with the other cells non-mutant, this outcome might be due to the lack of competence of PVR mutant clones to compete with the wildtype CySCs. Previous studies argued that the RTK signaling pathway EGFR is regulating the completion of CySCs for a spot at the niche with other CySCs (Amoyel et al., 2016; Amoyel et al., 2014). It is possible that this decrease in the percentage of the testes with CySCs mutant clones is due to the requirement of PVR signaling pathway in the CySCs to keep them fit to be able to compete and remain in the stem cell niche.
CHAPTER III

EXAMINING THE ROLE OF BONE MORPHOGENIC PROTEIN SIGNALING PATHWAY IN THE MAINTENANCE OF THE STEM CELLS IN THE DROSOPHILA TESTIS STEM CELL NICHE

Introduction

The adult tissues maintain their homeostasis by sustaining a population of stem cells. These stem cells are distinguished by their ability to self-renew to maintain their population and differentiation to different cell types in the tissue. The majority if not all the stem cells are located in a distinct microenvironment, called the stem cell niche. Stemness molecular signals from the niche need to be strongly controlled to be active only inside this microenvironment to sustain the proper balance of stem cell self-renewal vs. differentiation.

The Drosophila testis stem cell niche provides an ideal model system to study different signaling pathways involved in the maintenance, proliferation, and competition between stem cells (de Cuevas and Matunis, 2011). The stem cell niche in the testis is called the hub, which supports two populations of stem cells. The first population of stem cells are the germline stem cells (GSCs) which undergo several transit amplifying divisions followed by meiosis and gives rise to sperms, and the second population of stem cells are the cyst stem cells (CySCs) which gives rise to cyst cells after differentiation.
There are several signaling pathways that their ligand is secreted from the hub that are responsible for regulating the behavior of the stem cells. Bone morphogenic protein (BMP) signaling pathway is one of these pathways that its ligands Decapentaplegic (Dpp) and Glass Bottom Boat (Gbb) are secreted from the hub. gbb is also expressed in the CySCs (Inaba et al., 2017; Kawase et al., 2004; Michel et al., 2011; Shivdasani and Ingham, 2003). This pathway has three type-I cytokine receptors called thick veins (tkv), saxophone (sax), and baboon (babo) and two type-II cytokine receptors called punt (put) and wishful thinking (wit) (Leatherman and DiNardo, 2008; Schulz et al., 2004; Shivdasani and Ingham, 2003).

The BMP signaling pathway is involved in regulating GSC self-renewal (Kawase et al., 2004; Shivdasani and Ingham, 2003). The bag-of-marbles (bam) gene is repressed by the BMP signaling pathway in the GSCs, and this inhibition is essential for GSC self-renewal. It was shown that expressing excess Bam in GSCs forced their differentiation and the population of GSCs were lost in the testis and in bam mutant testis, TA spermatogonia created cysts of germ cells with more than 16 spermatogonia in them (Kawase et al., 2004). Previous studies indicated the role of the BMP signaling pathway in GSCs by over-activating different components of the BMP signaling pathway genes in germline lineage cells, they observed a mild increase in the number of GSCs and the differentiation of spermatogonia into spermatocytes fails and clusters of spermatogonial cysts were observed (Insco et al., 2012; Matunis et al., 1997; Schulz et al., 2004; Shivdasani and Ingham, 2003). Furthermore, by ablating different components of BMP pathway genes, they lost the population of the GSCs in the testis (Bunt and Hime, 2004; Kawase et al., 2004; Michel et al., 2011; Schulz et al., 2004; Shivdasani and Ingham,
In *gbb* mutant testes, the population of the GSCs is lost rapidly in the niche but the gonialblast daughter cells are still able to undergo TA divisions and create 16 spermatogonia which suggests that *gbb* expression is required for the maintenance of the GSCs in the testis (Kawase et al., 2004). In contrast, *dpp* mutant testis had little to no effect on the population of GSCs in the niche. When *dpp* mutant testis had one mutated copy of *gbb*, the loss of GSCs were significant compared to heterozygous testis for *gbb* that had normal testis architecture and GSCs population (Kawase et al., 2004). Kawase et al. results indicated that *dpp* and *gbb* act together to regulate GSCs maintenance in the niche (Kawase et al., 2004). Moreover, when *dpp* was overexpressed in the cyst lineage cells, there was a moderate increase in the number of spermatogonia which is due to its known role of Dpp in suppressing differentiation (Inaba et al., 2017).

The fact that CySCs are exposed to the BMP signaling pathway ligands in the niche raised different questions about the role of this pathway in the CySCs. We know that CySCs are exposed to the BMP ligands in the niche (Kawase et al., 2004; Shivdasani and Ingham, 2003). We first became interested in whether BMP signaling may also play a role in CySCs, since the CySCs maintenance factor Zfh-1 is a homolog of the mammalian ZEB genes, which are known to interact with receptor activated Smads, the intracellular transducers of BMP signaling (Postigo, 2003; Verschueren et al., 1999). Recent work on Merlin revealed for the first time that BMP signaling may be mitogenic for the cyst lineage cells, as constitutively activated Tkv receptor (a type I BMP signaling pathway receptor) caused cyst cell tumors in the testes (Inaba et al., 2017). A recent study has suggested that Tkv is inhibiting the BMP signaling pathway outside of the stem cell niche by acting as a trap for the ligand Dpp to confine this ligand to the niche (Xu et al., 2003).
2018). This study showed that by depleting Tkv in the cyst lineage cells, an accumulation of early staged differentiated germ cells was observed in the testes. The CySCs tkv mutant clones also showed an accumulation of GSCs and gonialblasts and there was a significant increase in the population of dividing GSCs in the testes. The number of Zfh1 positive CySCs was measured in Tkv RNAi testes and Xu et al. reported a significant increase in the population of the CySCs in the niche. Also, an increased accumulation of phosphorylated Mad (receptor-activated intracellular transducers of BMP signaling pathway) in GSCs and gonialblasts was shown in Tkv RNAi testes which demonstrates increased Dpp-dependent BMP signaling activity in the germ cells. Xu et al. concluded that Tkv receptor functions as a sink for the ligand Dpp and restrain the BMP signaling pathway to the stem cell niche (Xu et al., 2018).

In this study, we asked the question whether BMP signaling pathway is playing a role in the maintenance and division rate of the CySCs in the Drosophila testis stem cell niche prior to the Xu et al. publication on the role of Tkv receptor in the niche. To answer this question, we used two different Gal4 drivers to inhibit the BMP signaling pathway type I receptor Tkv in the testis via RNAi.

**Materials and Methods**

**Fly Stocks and Husbandry**

The following stocks was used as they are described in Flybase and they were grown in room temperature 25°C: C587 Gal4, C587 Gal4; Tub Gal80ts/CyO, yw; Tj Gal4/CyO, w; UAS Tkv RNAi. For the control the canton S flies were crossed to the Gal4 drivers.
**thickvein Inhibition in the Cyst Lineage Cells**

To inhibit tkv in the CySCs the UAS Gal4 system and RNA interference was employed that is described in Chapter II. In summary, UAS Gal4 system is a tissue specific transgene activation system that activates the gene transcription of target genes in a specific tissue using tissue specific promoters (Brand and Perrimon, 1993). Two different drivers of C587 Gal4 and Tj Gal4 drivers were used to activate the Tkv RNAi transgenes in the CySCs. RNA interference is an endogenous mechanism that degrades the mRNA transcripts of the target gene in the cell and as a result inhibits gene expression of that gene (Clemens et al., 2000). In addition, transgene expression was limited to adults using C587 Gal4; Gal 80<sup>ts</sup>. Flies were raised in room temperature (25°C), except for the crosses with the C587 Gal4 Tubulin Gal80<sup>ts</sup> that were raised in 18°C. All F<sub>1</sub> generation male progeny were aged at 29°C for 8 or 12 days.

**Tissue Fixation and Staining**

All samples were dissected and immunostained with the same methodology as mentioned in chapter II. Male flies were dissected in Ringer’s solution and the testes were fixed in 4% formaldehyde for 20 minutes. After multiple washes with PBTx, samples were blocked in 4% donkey serum in PBTx for at least an hour. Samples were incubated in primary antibody diluted in blocking solution overnight at 4°C, then washed multiple times with PBTx. Next, secondary antibody was applied for at least an hour, followed by several PBTx washes. Tissues were stained with Hoechst 33342 dye at 1ug/mL in PBTx to stain the DNA in the cells. Finally, testes were equilibrated in 50% glycerol solution for at least an hour before fine dissection and mounting. Testes were
mounted on slides in 90% glycerol with N-propyl gallate as an anti-bleaching agent. Slides were stored in the -20°C freezer to preserve the fluorescence stain.

The mouse anti-Fas3 antibody (Developmental Studies Hybridoma Bank, used at 1:50 dilution) was used to stain the hub cells, goat anti-Vasa (Santa Cruz, 1:300 dilution) was used to stain the germline cells, rabbit anti-Zfh1 (a kind gift from Ruth Lehmann, 1:5000 dilution) was used to stain CySCs, guinea pig anti-Tj (a kind gift from Dorothea Godt, 1:10,000 dilution) was used to stain the cyst lineage cells. Testes were mounted on slides, and imaged on a Zeiss 700 confocal microscope. Testis images were quantified for the number of CySCs (Zfh-1 positive cell) and the number of CySCs in S phase (EdU+ Zfh-1 positive cells) in the testis for all time points. CySCs were defined by staining positive for Zfh1 and not being more than a two germ cell distance away from the niche. The ICY software was used to visualize and analyze the images using the spot detector option.

To verify the cycling rate of cells in the testis, an EdU staining was performed to mark cells that are undergoing S phase by integration of the thymidine analog 5-ethynyl-2'-deoxyuridine (EdU) in to the DNA while it is replicating.

**Statistical Analysis**

All the quantifications for Tkv loss of function experiments using RNA interference were statistically analyzed with unpaired Students’ t-test for the experimental group vs the control in Excel.
Results

Bone Morphogenic Protein Signaling is Required for the Maintenance of Cyst Stem Cells in the Niche

To investigate the role of BMP signaling pathway in the CySCs, we inhibited the type I BMP pathway receptor Tkv by RNA interference using the tissue specific driver C587 Gal4. To assess possible changes in the maintenance and cell division rate of CySCs and GSCs, Vasa, Zfh1, and EdU stains were used (Figure 22A-D).

In control testes, after aging the testes for 8 days, the normal architecture of the niche was viewed, with an average of 28 CySCs per testis (Figure 22A, Figure 23A). In contrast, the number of CySCs in Tkv RNAi testes averaged 15, which was significantly lower than that found in control testes after 8 days of aging in 29°C (Figure 23A, Table 7, Table 8). The number of cycling CySCs were assessed and it also showed a significant decrease, from an average of 11 cells in the control to an average of 6 in the Tkv RNAi testes after 8 days of aging (Figure 23C, Table 7, Table 8). To understand whether this significant decrease in EdU+ CySCs was due to the decrease in total number of CySCs or lower cell division frequency of CySCs, the fraction of EdU+ CySCs over total number of CySCs was calculated (Figure 23E and 23F). Interestingly, this ratio demonstrated that loss of Tkv in the CySCs showed no significant effect on the cell division rate of the CySC meaning that although there were less CySCs overall, but these cells were cycling at a similar rate as those in control testes (Table 8). In order to test whether RNAi knock down of tkv in the cyst lineage had a non-autonomous effect on the germline cells, the number of GSCs was quantified. We found a significant decrease in the number of GSCs compare to the control testes, with an average of 7 in the control testes compare to an
average of 5 in the Tkv RNAi testes after 8 days of aging (Figure 23G, Table 7, Table 8). Also, an accumulation of early stage germline cells were observed in the manipulated testes (Figure 22B and 22D).

Figure 22. Tkv inhibition in the cyst cells caused significant reduction of CySCs and GSCs after 8 days of aging. A) Control testis niche after aging for 8 days in 29°C. B) Control testis after aging for 12 days. C) Tkv RNAi testis after 8 days aging in restrictive temperature of 29°C. D) Tkv RNAi testis after 12 days aging in restrictive temperature of 29°C. DNA is stained in blue, Hub in white, germ cells in magenta, and cyst cells in green. The hub is shown by a white star. Scales bar = 10 µm for all images.
Figure 23. Tkv inhibition in the cyst cells resulted in significant reduction of CySCs, GSCs and cycling CySCs but had no effect on their cell division rate. A) Number of Zfh<sup>1</sup> CySCs after aging for 8 days in 29°C. B) Number of Zfh<sup>1</sup> CySCs after aging for 12 days. C) The number of Zfh<sup>1</sup> EdU<sup>+</sup> CySCs after aging for 8 days. D) The number of Zfh<sup>1</sup> EdU<sup>+</sup> CySCs after aging for 12 days. E) Fraction of Zfh<sup>1</sup> EdU<sup>+</sup> CySCs over total number of Zfh<sup>1</sup> CySCs after aging for 8 days. F) Fraction of Zfh<sup>1</sup> EdU<sup>+</sup> CySCs over total number of Zfh<sup>1</sup> CySCs after aging for 12 days. G) Number of GSCs after aging for 8 days. H) Number of GSCs after aging for 12 days. *** shows the two-tailed P value of less than 0.0001 between the experimental group and their control. Images were analyzed and the P value was calculated using the student t-test.
Table 7.

_Tkv inhibition RNA interference in the cyst cells._ Average and sample number of CySCs, EdU⁺ CySCs, ration of EdU⁺ CySCs/ CySCs, and GSCs is demonstrated in this table.

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<tr>
<th>Average #</th>
<th>CySCs (n)</th>
<th>EdU⁺ CySCs (n)</th>
<th>EdU⁺ CySCs/ CySCs</th>
<th>GSCs (n)</th>
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<tbody>
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<tr>
<td>TJ Gal4 tkv RNAi D8</td>
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<td>6.1 (16)</td>
<td>0.39</td>
<td>5 (32)</td>
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<td>C587 Gal4 tkv RNAi D12</td>
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<td>5.1 (18)</td>
<td>0.35</td>
<td>4.8 (32)</td>
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</table>
The P value of CySCs, $EdU^+\text{ CySCs}$, ration of $EdU^+\text{ CySCs}/\text{ CySCs}$, and GSCs compared to the control. P value was calculated by unpaired Students’ t-test

<table>
<thead>
<tr>
<th>P Value compare to the control</th>
<th>CySCs</th>
<th>$EdU^+\text{ CySCs}$</th>
<th>$EdU^+\text{ CySCs}/\text{ CySCs}$</th>
<th>GSCs</th>
</tr>
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<td>0.2976</td>
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</tbody>
</table>

In order to test whether the phenotype might have arisen from defects in niche formation, or other effects during development, we inhibited $tkv$ knockdown to adults using C587 Gal4; Tub Gal80$^{ts}$. Crosses were raised in 18°C and the progeny were shifted to 29°C and aged for 8 or 12 days (Figure 24A-D). Agreeing with the previous experiment, $tkv$ inhibition resulted in lower number of stem cells in the niche, with control testes having a mean of 27 CySCs, and Tkv RNAi testes having a mean of 15 and an average of 7 GSCs in the control testes, and an average of 5 GSCs in the Tkv RNAi testes after aging for 8 days (Figure 25 A-H, Table 7, Table 8). The average of Edu positive CySCs were also significantly reduced from 11 in the control testes to 6 in the Tkv RNAi testes after 8 days of aging (Figure 25C, Table 7, Table 8). The ratio of $EdU^+\text{ Zfh1}^{+}\text{ CySCs}$ over total number of CySCs was calculated and it showed no significant difference with the wildtype testes, as we found previously with the C587 Gal4 driver alone (Figure 25E and 25F, Table 7, Table 8). As mentioned above, a differentiation
defect of the germ line cells was observed in the Tkv RNAi testes as an accumulation of early stage germ cells was shown in the manipulated testes (Figure 24B and 24D).

Figure 24. Tkv inhibition in the cyst cells caused significant reduction of CySCs and GSCs after 8 days of aging. A) Control testis niche after aging for 8 days in 29°C. B) A) Control testis after aging for 12 days. C) Tkv RNAi testis after 8 days aging in restrictive temperature of 29°C. D) Tkv RNAi testis after 12 days aging in restrictive temperature of 29°C. DNA is stained in blue, Hub in white, germ cells in magenta, and cyst cells in green. The hub is shown by a white star. Scales bar = 10 µm for all images.
Figure 25. Tkv inhibition in the cyst cells resulted in significant reduction of CySCs, GSCs and cycling CySCs but had no effect on their cell division rate. A) Number of Zfh\textsuperscript{1}+ CySCs after aging for 8 days in 29°C. B) Number of Zfh\textsuperscript{1}+ CySCs after aging for 12 days. C) The number of Zfh\textsuperscript{1}+ EdU\textsuperscript{+} CySCs after aging for 8 days. D) The number of Zfh\textsuperscript{1}+ EdU\textsuperscript{+} CySCs after aging for 12 days. E) Fraction of Zfh\textsuperscript{1}+ EdU\textsuperscript{+} CySCs over total number of Zfh\textsuperscript{1}+ CySCs after aging for 8 days. F) Fraction of Zfh\textsuperscript{1}+ EdU\textsuperscript{+} CySCs over total number of Zfh\textsuperscript{1}+ CySCs after aging for 12 days. G) Number of GSCs after aging for 8 days. H) Number of GSCs after aging for 12 days. *** shows the two-tailed P value of less than 0.0001 between the experimental group and their control. Images were analyzed and the P value was calculated using the student t-test.
A third Gal4 driver, Tj Gal4, was also used to inhibit the BMP pathway receptor *tkv* by RNA interference. We performed the same analysis in this experiment, and our data agreed with the results of previous experiments. To assess possible changes in the maintenance and cell division rate of CySCs and GSCs, Vasa, Zfh1, and EdU stains were used (Figure 26A-D). The population of CySCs and GSCs around the hub was significantly decreased with an average of 28 CySCs in control and 15 CySCs in Tkv RNAi testes and an average of 8 GSC in control testes and 5 GSCs in Tkv RNAi testes (Figure 27A-B and 27 G-H, Table 7, Table 8). Likewise, the number of EdU+ Zfh1+ CySCs was significantly lower in the Tkv RNAi testes with an average of 6 compared to the control with an average of 11, but the fraction of EdU+ Zfh1+ CySCs over total average number of CySCs showed no difference compared to the wildtype testes after 8 days of aging (Figure 27C-D and Figure 27E-F, Table 8). Similarly, an accumulation of early stage germ cells was observed in Tkv RNAi testes agreeing with previous experiments (Figure 26B and 26D).
Figure 26. Tkv inhibition in the cyst cells caused significant reduction of CySCs and GSCs after 8 days of aging. A) Control testis niche after aging for 8 days in 29°C. B) A) Control testis after aging for 12 days. C) Tkv RNAi testis after 8 days aging in restrictive temperature of 29°C. D) Tkv RNAi testis after 12 days aging in restrictive temperature of 29°C. DNA is stained in blue, Hub in white, germ cells in magenta, and cyst cells in green. The hub is shown by a white star. Scales bar = 10 µm for all images.
Figure 27. Tkv inhibition in the cyst cells resulted in significant reduction of CySCs, GSCs and cycling CySCs but had no effect on their cell division rate. A) Number of Zfh\textsuperscript{1} CySCs after aging for 8 days in 29°C. B) Number of Zfh\textsuperscript{1} CySCs after aging for 12 days. C) The number of Zfh\textsuperscript{1} EdU\textsuperscript{+} CySCs after aging for 8 days. D) The number of Zfh\textsuperscript{1} EdU\textsuperscript{+} CySCs after aging for 12 days. E) Fraction of Zfh\textsuperscript{1} EdU\textsuperscript{+} CySCs over total number of Zfh\textsuperscript{1} CySCs after aging for 8 days. F) Fraction of Zfh\textsuperscript{1} EdU\textsuperscript{+} CySCs over total number of Zfh\textsuperscript{1} CySCs after aging for 12 days. G) Number of GSCs after aging for 8 days. H) Number of GSCs after aging for 12 days. *** shows the two-tailed P value of less than 0.0005 between the experimental group and their control. Images were analyzed and the P value was calculated using the student t-test.
Conclusions

The data in this chapter revealed that the BMP signaling pathway is not only the main self-renewal regulator in GSCs in the niche but also is required for the maintenance of the CySCs in the *Drosophila* testis stem cell niche (Kawase et al., 2004; Shivdasani and Ingham, 2003). Inhibiting BMP signaling pathway by inhibiting *tkv* in the cyst lineage cells resulted in significant decrease in the number of CySCs and cycling CySCs. Interestingly, when the fraction of cycling CySCs over total number of CySCs in the niche was calculated for the control and Tkv RNAi testes, the results showed no significant difference between the two groups (Table 8). These results suggest that although BMP signaling is required for the maintenance of the stem cells in the niche, it appears to have no effect on the cell division rate of the CySCs in the testis.

Additionally, inhibiting *tkv* in the cyst lineage cells had a non-autonomous effect on the population of the GSCs the way that they were not able to maintain their population and a significant decrease of the GSCs population around the hub was observed. Our data also showed an accumulation of early staged germ cells in the manipulated testes when Tkv was inhibited in the cyst lineage cells. We suggest that when *tkv* was ablated in the cyst lineage cells, the partial loss of CySCs and differentiated cyst cells might be the reason for the defect in germ line cells differentiation and accumulation of early stage germ cells because there are not enough cyst cells in the testis to encyst the germ line cells and support and guide their differentiation. Another hypothesis could be that inhibiting BMP pathway in the cyst lineage cells could result in inappropriate encapsulation of the germ line cells with cyst cells and resulting in germ cells differentiation defect.
CHAPTER IV

CONCLUSIONS

Adult stem cells play an important role in tissue homeostasis and regeneration by replacing lost, old, or damaged cells. Maintenance of stem cells depends on intrinsic and extrinsic signals from their microenvironment in the stem cell niche. Paracrine Signals received from the niche and adjacent stem cells regulates stem cell maintenance, division rate, and competition.

**PVR Signaling Regulates Cyst Stem Cell Competition and Cell Division Rate in the Niche**

The data presented in this dissertation on PVR signaling pathway suggests a role for this RTK in proliferation and survival of CySCs. PVR-deficient CySCs showed a significant decrease in their population and cell division rate. PVR signaling seems to be a potent regulator of the cell division rate in CySCs since constitutively activated PVR resulted in neoplastic expansion of the CySCs and led to cyst cells dividing away from the hub (Figure 20B). Moreover, CySCs with reduced PVR signaling activity divided less frequently and the population of the CySCs were significantly reduced. These results agree with the reported role of PVR in regulating the cell division frequency of the *Drosophila* intestine stem cells, where inhibition of PVR in the intestine stem cells resulted in significant reduction of stem cells and constitutively activating PVR in the intestine stem cells resulted in over-proliferation of these cells suggesting the role of PVR
as a regulator of the cell division in *Drosophila* intestine stem cells (Bond and Foley, 2009).

Our mutant clones analysis with PVR showed that PVR mutant CySCs in a wildtype environment were not maintained over time as well as control cells. The difference in maintenance rates was small, but statistically significant. We suggest this result indicates that the CySCs that were mutant for *pvr* could not persist in the niche and were outcompeted and replaced by the wildtype CySCs. The idea of PVR involvement in regulating the stem cell competition is favored over PVR regulating self-renewal of the CySCs, since the PVR\textsubscript{1} and PVR\textsuperscript{c1197} mutant clones showed a modest decrease in persisting next to the hub due to lack of PVR signaling in CySCs and PVR mutant clones and inhibition of PVR in the cyst lineage cells did not result in complete loss of CySCs population as might be expected if self-renewal was impacted by ablation of PVR in the cyst lineage cells.

The PVR mutant clone conclusions agree with previous studies on the role of another RTK pathway, EGFR signaling in the CySCs, which demonstrated that EGFR pathway is signaling through MAPK signaling pathway and is operating by regulating CySCs competition in the niche, while Jak-Stat signaling pathway is the main regulator of CySCs self-renewal in the niche (Amoyel et al., 2016; Kiger et al., 2001; Leatherman and DiNardo, 2008, 2010). The indication of the PVR signaling pathway regulating CySCs cell division rate and stem cell competition in the niche suggests that PVR is a compelling CySCs regulator in the *Drosophila* testis stem cell niche and functions by multiple inputs controlling CySCs cell division rate and their ability to compete with other stem cells (including other CySCs and GSCs) in the niche. Interestingly, although
reduced PVR activity in the CySCs has significantly lowered their population in the niche by lower division rate and being outcompeted from the niche, this alteration did not affect the population of GSCs around the hub which means that inhibiting PVR in the CySCs has no non-autonomous effect on population of GSCs around the hub.

Overall, PVR is not required for the maintenance of the CySCs in the niche since inhibiting PVR did not result in deletion of CySCs from the niche but according to our data, PVR plays a role in regulating mitosis and cell division of the CySCs in the testis.

Germline Cells Differentiation is Compromised by Inhibiting PVR in the Cyst Stem Cells

When PVR was inhibited in the entire cyst cell population, the usual and appropriate germ cell differentiation was disrupted and small early germ cells were accumulated in some testes. Moreover, in some testes, cysts of transit amplifying germ cells with more than 16 dividing spermatogonia were observed, which shows that TA spermatogonia are undergoing more than four synchronous mitosis division and are dividing inappropriately. Taken together, experiments that inhibit PVR in the cyst lineage cells indicate that PVR performs autonomously in the CySCs and regulates their cell division rate, but also non-autonomously compromises germ cells differentiation within the testes, as it might be due to fewer Zfh1<sup>+</sup> CySCs in the niche. These results might be simply due to the fact that the cell division frequency of the CySCs are lower and there are not enough daughter cyst cells remaining in the testis to support the differentiation of the TA spermatogonia. This phenotype has some similarities to that observed when CySCs were completely ablated from the niche; in this experiment, early staged germ cell accumulation was often observed in the testis, presumably due to the lack of cyst cells to guide their differentiation program (Lim and Fuller, 2012). Another possibility would be
that the early stage germ cells are encysted, but they are unable to promote appropriate differentiation because of the lack of PVR signaling as was observed with EGFR loss function in cyst lineage cells (Gupta et al., 2018; Hudson et al., 2013).

In the future, investigating the total number of cyst cells in the testes would be an appropriate next step to confirm our interpretation about having less cyst cells in the testis resulted in inappropriate differentiation of TA germ cells. Also, it would be interesting to look at encasement of the germ cells with cyst cells where PVR is knocked down to see if PVR ablation in the cyst lineage cells can disrupt correct encapsulation of germ cells in the testis.

**PVR Has a Separate Function than Epidermal Growth Factor Receptor in the Cyst Stem Cells of the Drosophila Testis Stem Cell Niche**

In female *Drosophila* ovaries during border cell migration to the oocytes, it is thought that PVR is redundant with EGFR function (Duchek et al., 2001). Here, we reject the possibility that PVR and EGFR signaling act redundantly since, when PVR or EGFR were knocked down in the CySCs, they both significantly reduced the population of the CySCs with similar potency. However, when PVR and EGFR simultaneously were knocked down in the cyst lineage cells, data demonstrated that although the population and cell division frequency of the CySCs were significantly lower in these testes compared to the control testes, it was not significantly different from inhibiting PVR or EGFR alone. If PVR and EGFR were functioning redundantly with each other, we would expect a more drastic change in the population of CySCs when we knock down PVR and EGFR simultaneously compared to single knock downs of PVR or EGFR.
The fact that constitutively activated PVR results in neoplastic expansion of the cyst cells in the testis and constitutively activated EGFR results in differentiation of the GSCs and no cell over-proliferation is observed indicates that there is something different in the intracellular signaling elicited by PVR compared to EGFR. It has previously shown than EGFR signals downstream and functions by activating the MAPK/ERK pathway and promotes the differentiation of the cyst cells and correct encapsulation of germ cells in the testis (Kiger et al., 2000; Sarkar et al., 2007; Tran et al., 2000).

In the future, it would be interesting to determine the PVR signaling downstream pathway, and how it might differ from that induced by EGFR. Inhibiting different components of MAPK like raf, and compare the manipulated testes with those testes that PVR is inhibited in them might shed some light on whether PVR is functioning through MAPK pathway. Also, it would be interesting to look at the concentration of dpErk (active form of Erk) in the cyst cells since accumulation of dpErk indicated mediation of MAPK signaling in the cell. Thus, if PVR pathway is signaling through MAPK signaling pathway, when we inhibit PVR in the cyst lineage cells we expect to see less concentration of dpErk in these cells and when PVR is constitutively activated, we expect to observe high abundance of dpErk in the cyst lineage cells.
Tumor Suppressor Merlin is Able to Hinder the Mitogenic Effect of Constitutively Activated PVR in the Cyst Stem Cells

The present study showed that constitutively activated PVR resulted in neoplastic over-proliferation and expansion of the cyst cells in the testis. A recent study demonstrated the role of tumor suppressor Merlin in regulating CySCs division through contact dependent inhibition of proliferation (Inaba et al., 2017). By constitutively activating PVR and over-expressing *merlin* in the cyst lineage cells simultaneously, we confirmed that Merlin is capable of inhibiting any tumor formation caused by constitutively activated PVR. This result agrees with previous studies that were discussing the role of Merlin (NF2) in controlling and regulating cell proliferation by restricting the PDGF, VEGF, EGFR, and other RTKs to access to the cell membrane by endocytosing and degrading these receptors and thus, inhibit their function in mammalian cells (Ammoun et al., 2008; Chiasson-MacKenzie et al., 2015; Curto et al., 2007; Lallemand et al., 2008).

In the future, it would be interesting to perform further experiments to determine the mechanisms in which Merlin inhibits PVR in the cell and whether Merlin is limiting PVR access to the membrane or facilitate PVR endocytosis and degradation in the cyst lineage cells. For this experiment an anti-PVR antibody stain can be performed to determine whether Merlin is able to reduce the concentration of PVR at the membrane when we simultaneously over-express Merlin and constitutively activating PVR in the cyst lineage cells. We expect to see high abundance of PVR on the membrane when PVR is constitutively activated and thus, if Merlin is sequestering PVR from the membrane,
we expect to see less abundance of PVR on the membrane when Merlin is over-expressed in the cyst lineage cells.

**Bone Morphogenic Protein Signaling Pathway Regulates Stem Cell Maintenance in the Drosophila Testis Stem Cell Niche**

A recent study suggested that Tkv, the BMP signaling pathway type I receptor, acts as a sink/trap and sequesters the BMP ligand Dpp. The authors suggested that sequestration of Dpp ligand blocks activation of the BMP signaling pathway outside of the testes stem cell niche. Xu et al. showed that in the testes where tkv was inhibited in the cyst lineage cells, the population of the Zfh₁⁺ CySCs were significantly higher compare to the control testes as a result of BMP activation outside of the stem cell niche (Xu et al., 2018). In contrast, our results showed the opposite, that when Tkv is inhibited within the cyst lineage cells, the number of CySCs were significantly lower compared to the control. We tested our results using C587 Gal4, C587 Gal4; Tubulin Gal80turtle, and Tj Gal4 genetic drivers and all our results agreed with each other and confirmed our primary results. This difference in results could involve the way that the CySCs were counted. We limit the number of Zfh₁⁺ CySCs to those in close proximity to the niche of the stem cells. Also, we used rabbit anti-Zfh₁, a kind gift from Ruth Lehmann, and Xu et al. raised and generated the rabbit anti-Zfh₁. Using the same antibody with different sources also might be the reason of this difference in our result and Xu et al. results. Different techniques in analyzing and staining CySCs might be the rationale that we observed different results and different conclusions from Xu et al. but we do not fully understand why we obtained different results than they did. Although different results were obtained on the number of Zfh₁⁺ CySCs in our experiments from Xu et al. experiments, our results agreed with Xu et
al. results on the accumulation of early stage germ cells throughout the testis and interestingly the phenotype and structure of the testes where Tkv was inhibited in our experiments seemed similar to the Xu et al. testes expressing Tkv RNAi (in the cyst lineage cells Xu et al., 2018). One way to understand this conflicting results better is to do an anti-Dpp antibody stain and examine whether there is a difference in concentration of Dpp ligand in the testis when Tkv is inhibited or constitutively activated. According to Xu et al. results if Tkv acts as a trap for the Dpp ligand, when we inhibit Tkv we should observe a higher abundance of Dpp ligand in the testis and when Tkv is constitutively activated we should see a lower concentration of Dpp ligand in the testis since Xu et al. suggested that Tkv acts as a sink/trap for the ligand Dpp.

We propose that BMP signaling pathway has an autonomous effect on CySCs and is required for maintaining normal numbers of CySCs in the niche. We also observed that when tkv was ablated by RNAi in the cyst lineage, the GSC population was significantly decreased compared to the control testes and disrupted the differentiation of the germ cells and an accumulation of early stage germ cells were observed. Although BMP signaling is regulating the maintenance of CySCs and GSCs in the niche, it had no effect on the cell division rate of the CySCs. Our results contradict with previous published paper that showed inhibition of Tkv in the CySCs resulted in a significant increase in the number of CySCs (Xu et al., 2018). Here we showed that Tkv ablation results in a significant decrease in the population of CySCs and GSCs in the niche.

In the future, it would be interesting to look at the differentiation of cyst cells in Tkv RNAi testes and stain for the differentiation marker Eya that only marks differentiated cyst cells and see whether inhibiting Tkv in the cyst lineage cells can result
in early differentiation of the CySCs in the niche. It also would be interesting to look at expression of Bam in the testis that Tkv is inhibited and see whether Bam is being misexpressed in the testis. Also, defect in differentiation of germ cells and accumulation of early stage germ cells could be another phenotype that needs to be examined more carefully. It would be interesting to look at the encystment of these early stage germ cells with cyst cells and overall number of cyst cells in the testis to confirm whether this phenotype is due to lack of enough cyst cells in the testis to support the germ cell differentiation or it is due to lack of germ line encystment by the cyst cells.

Summary

Overall, this dissertation highlights the role of different signaling pathways in regulation of stem cells and the value of studying the Drosophila testis stem cell niche as a model system to study different signaling pathways and their mechanisms in stem cell biology. In this research, first we discussed the role of PVR in regulating the CySCs cell division frequency in the testis for the first time. We observed that although inhibiting PVR in the cyst lineage cells had no effect on the population of the GSCs but non-autonomously disrupted the differentiation of the germ cells. Second, we investigated the role of BMP signaling pathway in CySCs and we observed that inhibiting BMP signaling pathway in cyst cells can affect the population of GSCs and their differentiation. This understanding underlines the need to study the stem cell behavior in the tissue to grasp its overall biological importance. This research showed that the Drosophila testis architecture can change drastically when one single signaling pathway is ablated or activated in one type of cells and this shows the importance of regulation of different signaling pathways in the stem cells and the implication and significance of studying signaling pathways in a
simple structured stem cell niche in the *Drosophila* testis for the hope of grasping a better understanding of the more complex stem cell niches in higher organisms.
References


Larsson, J., Ohishi, M., Garrison, B., Aspling, M., Janzen, V., Adams, G.B., Curto, M.,
Hematopoietic Stem Cell Behavior by Altering Microenvironmental Architecture.
Cell Stem Cell 3, 221-227.


Leatherman, J. (2013). Stem Cells Supporting Other Stem Cells. Frontiers in Genetics 4,
257, 1-6.

Leatherman, J.L., and DiNardo, S. (2008). Zfh-1 Controls Somatic Stem Cell Self-
renewal in the Drosophila Testis and Nonautonomously Influences Germline

Biology 12, 806-811.

Regulates Germline Proliferation in a Smox/dSmad2-Dependent Manner in

Cell and Developmental Biology 21, 605-631.


