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Imatinib has deleterious effects on differentiating spermatogonia while sparing spermatogonial stem cell self renewal

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Abstract

Imatinib mesylate is among a growing number of effective cancer drugs that provide molecularly targeted therapy; however, imatinib causes reproductive defects in rodents. The availability of an *in vitro* system for screening the effect of drugs on spermatogenesis would be beneficial. The imatinib targets, KIT and platelet-derived growth factor receptor beta (PDGFRB), were shown here to be expressed in “germline stem” (GS) cell cultures that contain spermatogonia, including spermatogonial stem cells (SSCs). GS cell cultures were utilized to determine whether imatinib affects SSC self renewal or differentiation. GS cells grown in imatinib retained self renewal based on multiple assays, including transplantation. However, growth in imatinib led to decreased numbers of differentiated spermatogonia and reduced culture growth consistent with the known requirement for KIT in survival and proliferation of spermatogonia. These results build upon the *in vivo* studies and support the possibility of utilizing GS cell cultures for preclinical drug tests.

Keywords

Spermatogonial stem cells; self renewal; differentiation; imatinib mesylate; spermatogonia; fertility; drug test

1. Introduction

Imatinib mesylate (STI-571 or Gleevec; Novartis) is a tyrosine kinase inhibitor that selectively targets KIT, platelet derived growth factor receptor (PDGFR) and ABL among others [1–3]. Imatinib is the prototype of an ever-growing class of small molecule drugs that are designed to provide targeted therapy for cancer by inhibiting receptor tyrosine kinases, a class of enzymes whose activation is frequently associated with oncogenesis. For instance, chronic myeloid leukemia (CML) is caused by a translocation of chromosomes 9 and 22 that leads to the production of an aberrant BCR-ABL fusion protein, a constitutively active form of the ABL tyrosine kinase [4]. In a search for small molecule inhibitors of the cancer-causing BCR-ABL, imatinib was first identified on the basis of its ability to inhibit ABL and PDGFR autophosphorylation [1]. Imatinib received accelerated approval by the FDA for treatment of CML in 2001 based on the initial success of clinical trials. Upon the discovery that it also potently inhibits KIT, the proto-oncogene associated with gastrointestinal stromal tumors (GIST), its clinical use was quickly extended to the treatment of this disease as well

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[5,6]. It is now the first-line treatment for CML and GIST. Several other small molecule inhibitors targeting other families of receptor tyrosine kinases have been identified since the discovery of imatinib and these too have been approved for the treatment of various cancers. Furthermore, the use of imatinib extends beyond that of just CML and GIST as it has been shown to be effective for treating certain types of hypereosinophilic syndromes [7].

Imatinib has received FDA approval for treatment of certain pediatric cases of CML and other receptor tyrosine kinase inhibitors are also being investigated for use in pediatric patients [8]. Evaluating whether new drugs cause deleterious effects on the developing reproductive system is an important aspect of preclinical drug development, particularly if the drug is to be used in young patients. However, testing for a long-term effect on fertility in a mammalian model system *in vivo* is costly and complicated, even in rodents, very commonly used models for toxicity testing. For example, a drug that is detrimental to spermatogonial stem cell self renewal would only be expected to have a discernable effect on fertility after several months of treatment in a mouse or rat. Also, if *in vivo* drug treatment leads to reproductive defects then identifying the cellular target of the drug is extremely challenging because of the complexity of the reproductive system, which includes multiple types of somatic and germ cells as well as an endocrine component. Moreover, distinguishing between primary and secondary effects is difficult, at best.

The challenge of testing drug effects on the reproductive system in an animal model is illustrated by the myriad effects of imatinib treatment in mice and rats. Postnatal imatinib treatment in rats (five to seven days after birth) leads to increased germ cell apoptosis, reduced proliferation of peritubular myoid cells, increased plasma levels of luteinizing hormone and follicle-stimulating hormone, reduced sperm epididymal concentration and reduced litter size [9,10]. Given the known function of KIT and stem cell factor (SCF), the cognate ligand of KIT, in spermatogonial proliferation and survival, some of the effects of imatinib treatment are not surprising [11–13]. The other imatinib targets, PDGFRA and PDGFRB, are also expressed in the testes and inhibition of these factors by imatinib likely also contributes to the observed effects. For instance, PDGFRA is expressed in Leydig cells and PDGF signaling is important for the survival of these cells [14,15]. Finally, PDGFRB and a truncated cytosolic variant V1-PDGFRB are implicated in neonatal gonocyte development [16,17]. Inhibition of PDGFR signaling by treating mice with imatinib during the first five days after birth, before the onset of KIT expression, leads to defects in gonocyte proliferation and migration to the basement membrane. Consequently the onset of spermatogenesis is delayed and accompanied by a transient decrease in epididymal sperm counts and reduced testis weight; however, normal fertility is restored 120 days after treatment [16]. The rodent studies clearly point to the deleterious effects of imatinib on the reproductive system and are consistent with a report of oligospermia in a patient undergoing imatinib therapy [18]. The absence of detectable long-term effects of imatinib on fertility in rodents provides cautious optimism and numerous adult patients undergoing imatinib treatment have been able to have children [19]. Nonetheless, the possibility of indirect effects on spermatogonial stem cell self renewal as a consequence of treatment during the pubertal phase of development, for instance, is still formally possible.

In the present study we sought to directly test the effect of imatinib on spermatogonia using a recently developed culture system for propagating spermatogonial stem cells *in vitro*, termed germline stem (GS) cells [20]. GS cells can be propagated for years in culture as a result of spermatogonial stem cell (SSC) self renewal, which is promoted in culture media by the growth factors glial cell derived neurotrophic factor (GDNF) and fibroblast growth factor 2(FGF2) [21]. GS cells also require “feeder cells,” mitotically inactivated mouse embryonic fibroblasts (MEFs). The cultures comprise SSCs in addition to their differentiating progeny. Interestingly, long-term time lapse imaging of rat GS cell cultures

reveals that the SSCs generate new SSCs and differentiating cells with constant probability [22]. That is, the proportions of SSCs and differentiating cells are essentially invariant with SSCs representing an estimated 17% of the germ cell population; however, as the lifespan of differentiated cells is limited in culture, the differentiated cell population is constantly being replenished by the SSCs [22].

In this study we examine the expression pattern of imatinib targets PDGFRB and KIT in GS cell cultures and then test the effect of imatinib on SSC self renewal and differentiation. We show that PDGFRB and KIT are both expressed in the germ cell fraction of GS cell cultures, although KIT expression is much more substantial. In contrast, MEF feeder cells exhibit significant PDGFRB expression. Our data also indicate that imatinib treatment of GS cells results in a reduction in the number of differentiated spermatogonia. SSC self renewal is unaffected. The data are consistent with rodent studies and begin to validate the GS cell culture system as a preclinical model for testing the effect of drugs on spermatogonial development, the foundation of spermatogenesis and male fertility.

2.1 Materials and Methods

2.1. Cell culture

GS cell lines, DGC1 (wild-type) and MAF4 (GFP) were described by Dann et al. [23]. DR4 MEFs (SCRC-1045, ATCC, Manassas, VA, USA) mitotically inactivated by mitomycin C or irradiation (12,000 Rad) were used as feeder cells [20,24]. Experiments with imatinib were performed in F12GFB medium except for the cell death imaging experiments (see section 2.6). F12GFB contained Dulbecco's Modified Eagle's Media Nutrient Mixture **F-12** Ham (Sigma-Aldrich, St. Louis, MO, USA) with 10 ng/mL recombinant rat **G**DNF (R & D Systems, Minneapolis, MN, USA), 10 ng/mL recombinant human basic **F**GF (Sigma-Aldrich, St. Louis, MO, USA), 1X **B**27 without Vitamin A (Invitrogen, Carlsbad, CA, USA), 1% fetal bovine serum (Hyclone, Rockford, IL, USA), 2 mM glutamine, 1X antibiotic-antimycotic (Invitrogen, Carlsbad, CA, USA) and 10 μ M 2-Mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA). Imatinib (generous gift of Novartis, Basel, Switzerland) was resuspended in water (10 mM), stored at -20°C , and diluted in media (1 μ M final concentration) just prior to addition to the culture. Cultures were split or media replaced every two to four days with imatinib treated wells receiving media freshly mixed with imatinib. 5 mM all-trans-RA (Sigma-Aldrich, St. Louis, MO, USA) in ethanol was stored at -80 (less than two weeks) and diluted to 1 μ M in media prior to applying to cells; vehicle (0.1% ethanol) was the control for RA experiments.

2.2. Cell staining

For KIT immunostaining, trypsinized cells were washed in PBS/FBS (phosphate buffered saline with 1% fetal bovine serum), incubated for ~ 45 min on ice in PE-conjugated anti-Kit antibody (2B8, Abcam, Cambridge, MA, USA) or PE-conjugated IgG isotype control (RTK4530, Abcam, Cambridge, MA, USA) at 1 mg/ml in PBS/FBS and washed in PBS/FBS prior to analysis. For PDGFRB immunostaining, trypsinized cells were incubated in PE-conjugated anti-PDGFRB (APB5, eBioscience, San Diego, CA, USA) at 4 μ g/ml in PBS/FBS and washed in PBS/FBS prior to analysis. For SOHLH1 immunostaining, trypsinized cells were washed in PBS, fixed in 4% paraformaldehyde (10 min on ice) permeabilized in PBT (PBS with 0.1% triton) and incubated overnight at 4°C with rabbit anti-SOHLH1 (1.5 μ g/mL, Abcam, Cambridge, MA, USA) or rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Following three washes in PBT the cells were incubated with Dylite 649-conjugated donkey anti-rabbit (~ 1.3 μ g/mL, Jackson ImmunoResearch, West Grove, PA, USA) and then washed again three times in PBT prior to analysis. FACSscan or FACSCalibur (Becton Dickinson, San Jose, CA, USA) was utilized

for cytometry. MEFs and spermatogonia could be distinguished based on differences in forward and side scatter (MEFs exhibited greater scatter than spermatogonia) and gating was changed accordingly. All dot plots shown are representative examples from multiple (at least two) experiments. Analysis of synaptonemal complex protein 3 (SCP3) was performed on cells treated as described (imatinib/RA) for three days in a 96-well high-content imaging plate (BD Biosciences, San Jose, CA, USA) and then fixed and immunostained as described [23]. Analysis of SCP3 chromatin-associated staining was performed by observing fluorescence using a 40X objective on a BD435 high content imaging system (Becton Dickinson, Franklin Lakes, NJ, USA). Cells with punctate sub-nuclear staining were scored as SCP3 positive while cells containing diffuse staining were scored as negative. Bromodeoxyuridine (BrdU) immunostaining was performed using the BrdU Flow Kit (BD Pharmingen, San Jose, CA, USA). After 10 days of imatinib treatment, cells were pulsed with 10 μ M BrdU for ~1.5 hours prior to harvesting for fixation. PE-conjugated Annexin (BD Pharmingen, San Jose, CA, USA) was used to detect apoptosis in DGC1 cells after 9–10 days of imatinib treatment.

2.3. Quantitative RT-PCR

Germ cells were harvested by trypsinization and incubated on a gelatin-coated well to remove MEFs prior to RNA extraction. The MEFs evaluated in Figure 1 were naïve (never in interaction with germ cells). RNA extraction was performed from cell pellets washed in PBS using RNA Stat-60 (Tel-test, Friendswood, TX, USA) or Rneasy Plus Mini Kit (Qiagen, Valencia, CA, USA) and following the manufacturer's instructions. Reverse transcription was performed using Moloney Murine Leukemia Virus reverse transcriptase (MMLV-RT; Indiana University Cultivation and Bioprocessing Facility, Bloomington, IN, USA) or iScript (Biorad, Hercules, CA, USA). Quantitative PCR was performed on cDNA samples with primers to detect *Actin, beta (Actb)* or *Pdgfrb* (listed in Table 1) using Perfecta SYBR Fast Mix Low Rox (Quanta Biosciences, Gaithersburg, MD, USA) on a Stratagene MX3000P machine (Agilent Technologies, Santa Clara, CA, USA). Detection of *Actb* was used to normalize the quantity of input RNA in each cDNA reaction. The $\Delta\Delta C_t$ method or standard curve methods were employed for obtaining relative transcript quantities. At least two independent experiments were conducted for all relative quantities reported.

2.4. Transplantation

Transplantation procedures were carried out essentially as described in [25]. All animal procedures were performed in accordance with the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center. Six week old athymic nude mice (HSD:athymic nude - FOXN1nu; Harlan) were injected intraperitoneally with 38 mg/kg busulfan and used for transplantation four to six weeks later. Donor cells were MAF4 cells treated with or without 1 μ M imatinib for eight or nine days (data pooled from two experiments). To prepare the donor cells for transplantation, they were trypsinized together with MEFs, washed in media, filtered using a 40 μ M cell strainer (Falcon), counted with a hemocytometer to determine the germ cell concentration (MEFs and germ cells were distinguished based on size and morphology) and resuspended in media at between $3\text{--}5 \times 10^6$ germ cells/mL. Cells were stored on ice during the transplantation procedure and 3 μ L of trypan blue (0.25%) added to a 10 μ L aliquot of cells just prior to loading the needle. Mice were anesthetized by intraperitoneal injection of Nembutal (80 mg/kg) and donor cells injected through an efferent ductule. The efficiency of filling within the seminiferous tubules was visualized with trypan blue. Analyses were performed about eight weeks post-transplantation. Colonies, defined as a group (typically from ~20 to over 100 cells) of closely associated GFP+ spermatogonia, were counted and the data normalized to 10^5 cells injected, based on the concentration of cells injected.

2.5. In vitro cluster assay

MAF4 GS cells were treated for ten days with or without 1 μ M imatinib. Treated or untreated cells were trypsinized and used to seed multiple wells (six wells each) of a 96-well high-content imaging plate (Becton Dickinson, Franklin Lakes, NJ, USA) at a density of 500 cells per well. GS cells that were previously treated with imatinib were further treated with imatinib during the cluster assay time-frame. Clusters were counted six days after seeding and were defined as a group of six or more cells. The seeding density, six day time-frame and definition of a cluster were all in accordance with the original description of this assay [26]. The mean and standard error mean (SEM) of clusters per well for two experiments were presented.

2.6 Microscopic analysis of cluster size and cell death

MAF4 GS cells were used to seed multiple wells of a 96-well high-content imaging plate (Becton Dickinson, Franklin Lakes, NJ, USA) at a density of 12,000 cells per well. The original formulation of GS cell culture media described by Kanatsu-Shinohara et al. was used because it provides superior adherence of germ cells to feeder cells compared to simpler formulations such as F12GFB [20,27]. Cells were treated for nine days with or without 1 μ M imatinib. In one trial, duplicate wells were analyzed and in the other trial three of four quadruplicate wells were analyzed with the remaining wells being omitted based on the observation that they had considerably reduced cell numbers compared to the other replicates. Ethidium homodimer-2 (Invitrogen, Carlsbad, CA, USA) was added at a final concentration of 1 μ M just prior to imaging. Imaging was performed using a BD Pathway 435 High-Content Bioimager equipped with a 10X Olympus objective. Attovision software (Becton Dickinson, Franklin Lakes, NJ, USA) was used to obtain 36 images each of red (ethidium homodimer-2) and green (GFP) fluorescence to form montages covering a major portion of each well. The same software was then used to perform segmentation analysis of each montage to define areas of green and red fluorescence. The number of total dead cells or total dead cells within GFP clusters was determined for each well. An unpaired t test was used to evaluate whether there was a statistically significant difference between numbers obtained for control and imatinib treated wells.

3. Results

3.1. KIT and PDGFRB expression in GS cell cultures

The two major components of the GS cell culture system are MEF feeder cells and spermatogonia, which include the SSCs. We first sought to analyze expression of the imatinib targets, KIT and PDGFRB in both of those components. A significant portion (~60%) of the spermatogonia in GS cell cultures have previously been shown to express KIT protein and we confirmed this observation (Fig. 3A and 6F) [20,23]. In addition to KIT, PDGFRB mRNA and protein were also found to be detectable in GS cell cultures, although the frequency of PDGFRB positive spermatogonia (0.4%) was much lower than KIT positive spermatogonia (Fig. 1A). To confirm that the rare PDGFRB expressing cells were germ cells, as opposed to contaminating MEFs, we used a GS cell line (MAF4) in which the germ cells were transgenically modified to express GFP under control of the Ubiquitin C promoter [23]. PDGFRB positive cells could be detected in the GFP positive fraction indicating that PDGFRB was indeed expressed in a fraction of spermatogonial cells (Fig. 1A). PDGFRB was also found to be strongly expressed in MEFs. MEFs expressed 76-fold greater levels of *Pdgfrb* mRNA than GS cells based on quantitative RT-PCR analysis (mean fold-change in two independent experiments, data not shown). Furthermore, over 30% of MEFs expressed PDGFRB protein (Fig. 1B). In contrast, MEFs expressing KIT were very rare (Fig. 1C). From these data, we concluded that within GS cell cultures KIT was predominantly expressed in the germ cells while PDGFRB was predominantly expressed in

the MEFs. Nonetheless, a small but reproducible fraction of germ cells also expressed PDGFRB.

Previous studies of PDGFRB expression during postnatal germ cell development have shown that it is expressed in gonocytes during the first few days after birth; however, spermatogonia, the direct cellular descendants of the gonocytes are thought to not express PDGFRB [15,16]. Therefore the PDGFRB expression in spermatogonia that we observed was surprising. Our GS cell cultures were derived from ~9 day-old mice testes. At this age the only germ cells present are spermatogonia. Therefore, the GS cell cultures would not be expected to contain any gonocytes. To investigate further the identity of the rare PDGFRB positive germ cells, we utilized an *in vitro* differentiation model. We have previously shown that retinoic acid treatment of GS cells leads to spermatogonial differentiation in a manner that is highly analogous to the *in vivo* process of differentiation [23,28]. Upon RA treatment of GS cells, markers of differentiated spermatogonia are induced while markers of undifferentiated spermatogonia/stem cells are greatly reduced. Furthermore, RA treatment results in the loss of stem cell activity in a transplantation assay [23]. If PDGFRB expression in GS cells were associated with the stem cell fraction, then PDGFRB expression would be expected to decrease following RA treatment. Alternatively, if PDGFRB expression were associated with spermatogonial differentiation, then its expression would be expected to increase following RA treatment. We analyzed *Pdgfrb* mRNA expression at one and three days following RA exposure using quantitative RT-PCR. Both the full length and variant forms of *Pdgfrb* mRNA increased after three days of RA exposure, although no change was detected following only one day of exposure (Fig. 2A). An increase in the percentage of PDGFRB expressing cells was also observed following three days of RA exposure based on fluorescence-activated cell sorting (FACS) analysis (Fig. 2C). These data showed that PDGFRB expression was associated with spermatogonial differentiation. As GS cell cultures are known to contain SSCs as well as their differentiating progeny, the rare PDGFRB cells observed under normal culture conditions are likely to represent a subset of spermatogonia undergoing differentiation.

3.2. Spermatogonial differentiation in imatinib treated GS cell cultures

KIT is required for survival of differentiated spermatogonia [13,29]. Therefore, we hypothesized that inhibition of KIT activity via imatinib would result in a reduction in the fraction of differentiated spermatogonia present in GS cell cultures. To test this hypothesis we evaluated the levels of differentiation in GS cell cultures following imatinib treatment using immunostaining and flow cytometry to quantify two markers of differentiation: spermatogenesis and oogenesis specific basic helix-loop-helix 1 (SOHLH1) and KIT. SOHLH1 is a germ cell specific transcription factor. Similar to KIT, the onset of SOHLH1 expression coincides with the transition from undifferentiated spermatogonia to differentiated spermatogonia [30]. We found that the number of differentiated cells in GS cell cultures was reduced by imatinib treatment (9–10 days). That is, the percentages of KIT-positive and SOHLH1-positive cells were both reduced by ~40% (Fig. 3A,B). The percentage of KIT-positive cells was also evaluated after long-term culture in imatinib and a similar degree of reduction was observed independent of the number of times the cells were passaged in media containing imatinib. Finally, reduction of KIT-positive cells following imatinib treatment was observed in multiple cell lines (Fig. 3C).

To distinguish between the possibility that imatinib inhibited the survival of differentiated cells versus inhibited the generation of differentiated spermatogonia, we tested the effect of imatinib on RA induced differentiation. While GS cell cultures contain a steady-state level of spermatogonial differentiation, evidenced by the presence of KIT-positive and SOHLH1-positive cells, GS cells can be stimulated to differentiate to a more mature developmental stage by RA. That is, early meiotic cells containing chromatin-associated SCP3 become

widely prevalent in RA treated GS cells although they are not normally observed in standard culture conditions [23]. To test whether imatinib inhibits RA induced differentiation, we quantified chromatin-associated SCP3 immunostaining in GS cells treated with imatinib, retinoic acid, or both reagents for three days. Imatinib had no effect on RA induced SCP3 immunostaining (Fig 4). These results suggested that imatinib does not inhibit the process of spermatogonial differentiation. Instead, the reduction in numbers of KIT-positive and SOHLH1-positive cells observed following imatinib treatment may reflect a reduction in the survival of differentiating spermatogonia.

3.3. Imatinib does not effect spermatogonial stem cell self renewal

The prevailing model is that KIT is expressed in differentiated spermatogonia but not the SSCs [12,31]. PDGFRB has also not been detected in SSCs. Therefore, we hypothesized that inhibition of KIT and PDGFRB activity via imatinib would have no direct effect on the SSCs. To quantitate relative stem cell numbers in GS cell cultures treated with imatinib, we used the spermatogonial transplantation assay, the gold standard for quantification of SSCs [32]. In a transplantation assay germ cells are injected via the rete testes into the seminiferous tubules of a mouse that has been pre-treated to ablate the endogenous germ cells. SSCs in the donor cell population migrate to the basement membrane of the seminiferous tubules and proliferate, forming a colony. The relative number of SSCs present in injected cell populations is determined based on the number of colonies observed eight weeks after transplantation. Imatinib treated or untreated GFP expressing GS cells were transplanted into the testes of busulfan treated host mice. Imatinib treated and untreated cells yielded similar numbers of colonies indicating that imatinib does not effect the numbers of SSCs in GS cell cultures (Fig. 5A). We also performed an *in vitro* cluster formation assay that has recently been shown to provide a faithful assessment of SSC numbers similar to the transplantation assay [26]. GS cells normally proliferate in clusters and each cluster has been shown to be derived from a single cell. The relative number of clusters formed after six days *in vitro* is highly correlated to the relative number of SSCs measured by *in vivo* colonization following transplantation [26]. We subjected imatinib treated or untreated GS cells to the *in vitro* cluster formation assay. Imatinib had no effect on *in vitro* cluster formation, a result that further corroborated the results of the transplantation assay (Fig. 5B). Long term maintenance of GS cell cultures depends on SSC self renewal. Consistent with the idea that SSC self renewal is not effected by imatinib, we could maintain GS cells in the presence of imatinib for several passages over a span of 41 days. Altogether these data showed that imatinib had no effect on SSC self renewal *in vitro*. Furthermore, the reduction of differentiated cell numbers in GS cell cultures did not lead to a detectable enrichment in SSCs, possibly because the SSCs represent a minority of cells in GS cell cultures and subtle effects would not be detectable with the available assays.

3.4. Imatinib inhibits GS cell culture expansion

Although GS cells could be maintained for multiple passages in imatinib, we noticed that the total number of germ cells present following imatinib treatment (9–10 days) was modestly reduced (~30%, Fig. 6A). A similar reduction in germ cell numbers following imatinib treatment was observed when an imaging assay was used to determine germ cell cluster size by virtue of ubiquitous GFP expression in the MAF4 GS cell line (Fig. 6A, B). Imatinib has been reported to cause reductions in proliferation or increases in apoptosis, depending on the cell type examined. We sought to determine whether the reduction in GS cell culture expansion was a consequence of either reduced proliferation or increased apoptosis. We found that bromodeoxyuridine incorporation was similar in imatinib treated and untreated cultures (day 10) suggesting that proliferation rates were not affected (Fig. 6C). Somewhat surprisingly, Annexin V, a marker for apoptotic cells, was also not affected (Fig. 6D). To further investigate the possibility that imatinib treatment led to increased germ cell death we

analyzed imatinib treated and untreated (day 9) MAF4 cells with the cell-impermeant red fluorescent nucleic acid stain, ethidium homodimer-2, which labels dead cells because of their compromised membrane structure. The number of total dead cells was determined using software-assisted counting of images spanning major areas from each culture well; additionally, the number of germ cell cluster-associated dead cells was determined by analyzing red fluorescence within areas of GFP expression (germ cell clusters). We found that the total cell death as well as death associated with germ cell clusters was subtly increased following imatinib treatment (Fig 6E). The increase was not statistically significant but a consistent trend was observed in multiple experiments. Wells containing only MEFs, but no germ cells exhibited no change in the quantity of dead cells in the presence of imatinib (data not shown). The fact that we were only able to discern a modest increase in germ cell death may be because imatinib treatment resulted in a continual low-level depletion of germ cells over a prolonged period of time, during which dead cells may have been lost while exchanging the culture media.

In our experiments imatinib treatment was initiated immediately following cell passaging. The reduction in germ cell expansion was most pronounced after prolonged growth (9–10 days) in imatinib: that is, once cluster sizes of untreated cells were large and the culture was ready for passaging. This prompted us to investigate whether normal cultures containing larger clusters exhibit increased KIT expression, thereby leading to a period of heightened imatinib sensitivity during the later phase of culture growth. We tested the effect of cluster size/germ cell density on KIT expression using flow cytometry and found that KIT expression increased as cluster size/germ cell density increased (Fig. 6F). These results may explain why the effect of imatinib on cell growth was most pronounced in mature cultures (9–10 days after passaging), when the cluster sizes are larger.

4. Discussion

In the present study we show that imatinib reduces differentiated cell numbers and overall GS cell culture growth. Importantly, spermatogonial stem cell self renewal is unaffected by imatinib treatment. The effect on GS cell cultures is likely mediated by inhibition of KIT activity in differentiating spermatogonia, which represent a significant proportion of the heterogeneous GS cell population. The fact that our results are consistent with *in vivo* studies evaluating the effect of imatinib on reproductive physiology in rodents supports the possibility of including the GS cell culture system in preclinical *in vitro* drug testing models for fertility.

Imatinib therapy is the first-line treatment for CML and GIST. Other types of cancer are also likely to be treated with imatinib if activation of KIT or PDGFR is shown to be the molecular cause. Furthermore, while imatinib treatment is predominantly used in adult patients, the pediatric patient population is ever-expanding. Nonetheless, the effect of imatinib directly on spermatogonia has not been evaluated despite the importance of spermatogonia in the maintenance of fertility. Three studies, all focusing on the prenatal period, evaluated the effect of imatinib on the male reproductive system in rodents. Basciani et al. [16] injected imatinib into newborn mice every eight hours during the first five days of life. Nurmio et al. [9,10] injected imatinib into rats on either postnatal days five-seven, or only day five. In rodents, a dominant feature of germ cell development during this postnatal period is the migration of gonocytes from the center of the seminiferous tubule to the basement membrane and imatinib treatment substantially inhibited this process. As gonocyte migration is an essential antecedent to the establishment of the undifferentiated spermatogonia/SSC pool and to spermatogonial differentiation, it is possible that later effects that were also observed, such as a reduction in the number of spermatogonia, were secondary to the effect of incomplete gonocyte migration. Considering that in humans

gonocyte migration is mostly completed by birth, the effect of imatinib on SSCs and spermatogonia, the pertinent cellular targets for younger males undergoing imatinib therapy, has remained an important and outstanding question.

We have provided conclusive evidence that imatinib does not hinder self renewal of SSCs. First, GS cells could be maintained for over a month with multiple passages in the presence of 1 μ M imatinib. Second, imatinib treated GS cells were capable of *in vivo* colonization following transplantation at a rate similar to untreated GS cells. Third, imatinib treated and untreated GS cells yielded similar cluster numbers in an *in vitro* cluster formation assay. These results are consistent with existing data that links KIT expression and function with differentiated spermatogonia rather than with SSCs. Mouse studies were also suggestive of the idea that imatinib treatment does not adversely affect self renewal because long-term (120 days) effects on seminiferous tubule histology and sperm production were not observed [16]. Nonetheless, as discussed above, the imatinib treatments were during the postnatal period prior to the formation of spermatogonia and may not be comparable to imatinib treatment of a pubertal male patient. In sum, our data are in agreement with the *in vivo* data suggesting that SSC self renewal is not affected by imatinib and build upon the previous studies by directly demonstrating the imperviousness of SSC self renewal to imatinib.

Although SSC self renewal was not affected by imatinib, transient reductions in GS cell culture growth were observed. That is, fewer cells and smaller clusters of cells were visible compared to controls after GS cells were grown in media containing imatinib for more than a week. We also observed a reduction in the frequency of SOHLH1-positive and KIT-positive differentiated spermatogonia. However this reduction is not likely to be caused by an inhibition in the differentiation process, as imatinib did not effect RA induced differentiation. Rather, the reduction in differentiated spermatogonia in GS cell cultures was likely a consequence of the reduced survival of differentiated spermatogonia that arise as GS cells are maintained in culture. This idea is in agreement with the known requirement for KIT signaling for survival of differentiated spermatogonia [13,29]. Interestingly, we found that in normal GS cell cultures, higher levels of KIT expression were correlated with larger cluster sizes and greater cell density, conditions normally found in cultures that have been maintained for longer time periods after passaging. A correlation between KIT expression and cell density in GS cells was also recently reported by others, although in the context of the feeder-free laminin culture system, KIT-positive cells also retained stem cell activity [33]. Our data suggest the following model to describe the dynamic and heterogeneous nature of GS cells. SSC proliferation leads to the production of new SSCs (KIT negative, imatinib resistant) and differentiating spermatogonia (KIT positive, imatinib sensitive). As cultures are maintained without passaging and cluster size increases, the levels of differentiation (KIT expression) increase; however, in the presence of imatinib the differentiated cells that arise have a reduced ability to survive, limiting the overall growth and differentiation state of cultures. Nonetheless, GS cell cultures can be maintained continually in the presence of imatinib because SSC self renewal is unaffected by imatinib. We favor the idea that the observed effects of imatinib are mediated by KIT, but have not ruled out a role for PDGFR signaling in GS cell cultures. However, in the testes, PDGFRB activity in gonocytes is stimulated by the secretion of PDGF ligand from Sertoli cells; in contrast, in GS cell cultures PDGFRB expressing germ cells are extremely rare.

In recent years the National Research Council outlined its vision for more refined approaches to toxicity testing that should make greater use of cellular model systems and take advantage of the latest technologies such as high throughput screening [34]. Also, reduced dependence on animals for toxicity and drug testing would clearly be beneficial. An ideal *in vitro* cell system captures numerous characteristics of the *in vivo* developmental process that it models. Indeed, GS cells exhibit multiple characteristics of spermatogonia,

their *in vivo* counterpart: expression of numerous protein markers, cell divisions with incomplete cytokinesis, and differentiation response to retinoic acid, among others. Importantly, GS cells are proven to contain functional SSCs based on their ability to colonize, self renew, and generate functional sperm *in vivo* following transplantation, a property that distinguishes GS cells from other spermatogonial culture systems. Furthermore, GS cells are not artificially immortalized by the addition of transforming transgenes. Nonetheless, the utility of GS cells as a model system for the preclinical evaluation of the effect of drugs on germ cells has only begun to be evaluated [35]. With the continuous arrival of new drugs that target molecular pathways that are not only deregulated in cancerous cells but also required for physiological function in normal cells, the likelihood of ‘off-target’ effects on spermatogenesis is very real.

We illustrate here the value of the GS cell culture system for examining the effect of the widely used cancer drug, imatinib, on the founder cells of spermatogenesis. Using this *in vitro* system we could evaluate both spermatogonial stem cell self renewal and differentiation. Our results are consistent with *in vivo* data that indicate a transient but significant deleterious effect on fertility, sperm production and testes weight. Additionally, Marcon et al. [35] very recently reported an imaging based method for evaluating cytotoxicity in rat GS cells that would be amenable to high through-put screening. Their study, which evaluates the effect of chemotherapeutic drugs such as cisplatin, together with our study begin to establish the GS cell culture system as an important alternative to animals for testing the effect of drugs on early stages of spermatogenesis.

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Fig. 1. PDGFRB and KIT expression in GS cell cultures. Forward/side scatter dot plot (left) and fluorescence dot plot analyses of immunostained GS cells and MEFs. (A) MAF4 GS cells (>95% GFP positive) were immunostained with PE-conjugated anti-PDGFRB (right) or PE-conjugated isotype control (middle). (B) MEFs never previously exposed to germ cells were immunostained with PE-conjugated anti-PDGFRB (right) or PE-conjugated isotype control (middle). (C) MEFs never previously exposed to germ cells were immunostained with PE-conjugated anti-KIT (right) or PE-conjugated isotype control (middle).

Fig. 2.

PDGFRB expression following RA induced differentiation of GS cells. (A) Histogram depicting relative expression levels of total (full length and V1) *Pdgfrb* mRNA or *V1-Pdgfrb* mRNA by quantitative real time RT-PCR analysis in retinoic acid (RA) or vehicle treated DGC1 cells (3 days). Expression is shown relative to vehicle treated cells using *Actb* primers as an internal control for normalization. Forward/side scatter dot plot (B) and fluorescence dot plot (C) analyses of immunostained GS cells treated with retinoic acid (RA) or vehicle control (3 days).

Fig. 3.

Imatinib treated GS cells comprise a reduced fraction of differentiated cells. (A) Forward/side scatter dot plot (left) and fluorescence dot plot analyses of imatinib treated or untreated (10 days) DGC1 cells immunostained with PE-conjugated anti-KIT or PEconjugated isotype control. (B) Forward/side scatter dot plot (left) and fluorescence dot plot analyses of imatinib treated or untreated (10 days) DGC1 cells fixed, permeabilized and immunostained with anti-SOHLH1 or isotype control. Dylite 649-conjugated secondary antibody was used for detection. (C) The histograms depict the percentage of KIT-positive cells in imatinib treated cells relative to untreated cells (mean $\pm$ SEM from at least two experiments). In the left histogram the cumulative total number of days of imatinib treatment is shown (left

histogram; 7–10 days, not passaged in imatinib; 17–22 days, passaged once; 39–49 days, passaged twice). Data are pooled from multiple experiments. In the histogram on the right the results are presented as a function of cell line (10 days of treatment, not passaged)

Fig. 4. Imatinib does not inhibit RA induced differentiation (A) The percentage of germ cells containing chromatin associated SCP3 immunostaining after three days of growth in four conditions: ethanol (vehicle), ethanol + 1 μ M imatinib, 1 μ M RA, 1 μ M RA + imatinib. Data are shown as the mean $\pm$ standard error mean (SEM) from duplicate wells. (B) Representative images for each condition. SCP3 immunostaining is pseudo-colored green. DAPI staining, for visualizing DNA, is pseudo-colored blue. The images shown have the blue and green channels merged. Arrows indicate examples of cells that were counted positive for chromatin associated SCP3 immunostaining.

Fig. 5. SSC quantitation following imatinib treatment of GS cells. (A) Colony number normalized to 10^5 cells injected eight weeks following transplantation of imatinib treated (8–9 days) or untreated DGC1 cells. Data are shown as means $\pm$ standard error mean (SEM) for 13 recipient testes (control) or 16 recipient testes (imatinib) from three experiments. (B) Cluster number per well six days following seeding of wells with 500 imatinib treated (10 days) or untreated MAF4 cells. Data are shown as means $\pm$ standard error mean (SEM) for 10 wells (control) or 12 wells (imatinib) from two experiments.

Fig. 6.

GS cell culture growth and cell death in the presence of imatinib. (A) The histogram on the left depicts a ratio of the fold-cell expansion (cells recovered/cells plated following 8–10 days of growth in imatinib) in imatinib treated cells relative to untreated cells. The means $\pm$ standard error mean (SEM) from three experiments are shown for each of two cell lines (DGC1 and MAF4). The histogram on the right depicts a ratio of the mean green fluorescent (GFP) area per cluster on day 9 in imatinib treated MAF4 cells relative to untreated cells. The means $\pm$ standard error mean (SEM) from two experiments are shown. (B) Examples of a quarter of 6×6 montages of images of GFP used for quantifying germ cell cluster size in Fig. 6A. (C and D) Forward/side scatter dot plot (top, left) and fluorescence dot plot

analysis of imatinib treated (“Imatinib”) or untreated (“Control”) DGC1 cells. Representative plots from multiple experiments are shown. (C) Cells treated with BrdU for 1.5 hours or untreated (“no BrdU”), fixed, permeabilized and immunostained with APC conjugated anti-BrdU. (D) Cells stained with PE-conjugated Annexin or unstained (“Neg.”). (E) The histograms on the top depict the mean $\pm$ standard error mean (SEM) of total dead cells per image from two trials with two or three images per trial. The histograms on the bottom depict the mean $\pm$ standard error mean (SEM) of total dead cells colocalized with green fluorescence (GFP) per image in two trials (left and right) with two or three images per trial. (F) Mean percentage of KIT positive cells $\pm$ standard error mean (SEM) for duplicate wells seeded at 1.1×10^5 cells per cm^2 (low), 2.1×10^5 per cm^2 (medium) or 4.2×10^5 per cm^2 (high).

Table 1

Primers used for quantitative RT-PCR

mRNA	Sequence
<i>Actb</i> - forward	TGGAATCCTGTGGCATCCA
<i>Actb</i> - reverse	TTACGGATGTCAACGTCACACT
<i>Actb</i> - forward	GGCTGTATTCCCCTCCATC
<i>Actb</i> - reverse	TGCCAGATCTTCTCCATGTC
<i>Pdgfrb</i> (V1)- forward*	ATGCCTTCTGTCTGTGGCT
<i>Pdgfrb</i> (V1)- reverse*	GCCGGAGTCACCCAAGGTA
<i>Pdgfrb</i> (total)- forward	AGCGACACTCCAACAAGCATT
<i>Pdgfrb</i> (total)- reverse	GCAGGGCGTTGCTGTAGAG

* As described in Wang and Culty (2007)