Modeling colorectal cancer as a 3-dimensional disease in a dish: the case for drug screening using organoids, zebrafish, and fruit flies

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Abstract

This review discusses recent shifts in the understanding of colorectal cancer as a stem cell based disease, based on findings that tie patient prognosis to the presence of cancer stem cells in colorectal tumors. Currently no drugs specifically target CSCs in colorectal tumors. However, recent advances in the culturing of colorectal stem cells using mammalian organoids, zebrafish, and Drosophila offer promising avenues for anti-CSC drug discovery.

Introduction

This year over 600,000 people worldwide will die of colorectal cancer (CRC) (http://www.who.int). It is the third most frequently diagnosed and third most deadly cancer in the United States (http://www.cancer.org) [1]. Standard treatment for CRC shows some efficacy but falls short in terms of increasing long-term survival. The standard treatment combines surgery, radiation and a regime of three cytotoxic drugs: the DNA synthesis inhibitor 5-FU, the topoisomerase I inhibitor irinotecan and the platinum-based DNA crosslinking agent oxalaplatin. To increase long-term survival the standard treatment is sometimes combined with targeted drugs that block signaling pathways required by the tumor cells, such as bevacizumab, which targets the VEGFR pathway, and cetuximab and panitumumab, which target the epidermal growth factor receptor (EGFR) pathway [2]. On average these drugs extend survival on the order of months, not years. Although the success of these targeted drugs has been limited, the fact that they prolong life is encouraging and 20 new pathway specific drugs are in clinical trials [3]. There is mounting evidence, however, that advances in long-term survival will also require a new type of targeted drug, one that specifically kills tumor cells with stem cell properties, called ‘cancer stem cells’ (CSCs).

The search for anti-CSC drugs is a nascent field and a controversial one. CSCs have been identified in virtually every cancer studied, but there have been little data to back up their therapeutic relevance [4,5]. However, three papers now link CSC gene signatures in CRCs to patient survival [6–8]. While the biology underlying this relationship is not fully understood, the fact that CSC gene signatures predict patient outcome weighs heavily in favor of CSC therapeutic relevance. This review highlights recent papers that bring the discussion of CSCs closer to therapeutic applications, and discusses the potential of model systems – organoids, fish and flies – to identify anti-CSC drugs.
The CSC controversy
The CSC hypothesis posits that tumor growth and tumor recurrence are fueled by cells with stem cell characteristics, endowing them with the ability to make more copies of themselves and to give rise to all the differentiated cells of the tumor [9]. Support for this hypothesis has hinged on the xenograft assay, which tests the ability of human cells to initiate tumors when transplanted into mice. Typically, only a fraction of cells within a tumor can initiate tumors in this assay. These ‘tumor-initiating cells’ are also referred to as CSCs because they tend to give rise to tumors with the same cellular diversity and architecture of the parental tumor, reminiscent of the ability of normal stem cells to give rise to the diversity of cell types in a tissue. The xenograft assay thus distinguishes CSCs, the cells with the capacity to reinitiate tumors from the remaining ‘bulk cells’ of the tumor, which are by comparison inert.

The distinction of CSCs from bulk cells has generated widespread interest and scrutiny because of its radical therapeutic implication, namely that chemotherapeutics that shrink or ‘debulk’ tumors, will remain prone to the problem of tumor recurrence, whereas chemotherapeutics that target CSCs (typically a small population of cells), may be able to permanently eradicate tumors [10,11]. The controversy rests in whether the CSCs identified by the xenograft assay are meaningfully distinct from the bulk cells as to warrant a search for novel therapeutics that can target CSCs.

The debate about CSCs is really a debate over the reliability of the xenograft assay. The assay has been used to identify CSCs across many tumors including breast, brain and CRCs [12]. Indeed, it is the gold standard by which CSCs are defined [13]. However, the xenograft assay challenges cells to do much more than initiate tumors, and may therefore lead to the classification of cells as CSCs or bulk cells for reasons that have nothing to do with tumorigenicity. For example, in challenging human tumor cells to take root not merely at a different location from the parental tumor, but in a different species, the assay tests the ability of human cells to survive outside of humans. The ability of human tumor cells to thrive or not thrive when transplanted into a mouse might therefore reflect differences in their dependence on human-specific growth conditions rather than differences in their tumorigenicity [14].

Another confounding problem with xenografting human tumor cells to mouse hosts is that the ability of a cell to initiate a tumor is contingent not only on the cell, but also on the mouse. This ‘mouse effect’ can have a huge impact as demonstrated by Quintana et al. [15] who tested human melanoma cells in two mouse strains: the sick NOD/SCID strain (non-obese diabetic/severe combined immunodeficiency), and the even sicker NOD/SCID IL2rg−/− strain (interleukin-2 receptor gamma chain null). In the NOD/SCID strain only 1 in a million melanoma cells were found to reinitiate tumors, leading to the conclusion that melanoma CSCs are rare. However, in the NOD/SCID IL2rg−/− strain as many as one in three cells reinitiated tumors, revealing that melanoma CSCs are actually quite common. These results demonstrate that in some instances the mouse effect obscures the ability to detect CSCs, leading to the conclusion that CSCs may not be as rare as originally argued. However, this possibility does not negate the existence of CSCs as a class of cancer cells worthy of therapeutic consideration.

While studies such as the one by Quintana et al. [15] clearly demonstrate that the xenograft assay is flawed, this does not mean that the CSC hypothesis itself is flawed. Indeed the fact that human tumor cells can initiate tumors in xenograft assays confirms that tumor cells with stem cell properties exist. The critical question should not revolve around the strengths and flaws of the xenograft assay, but instead should ask what relation, if any, do CSCs have on outcome of human cancers?

Until recently, this has been surprisingly difficult to answer because CSCs are hard to isolate. For example, the CSCs identified by xenograft assays are not typically isolated as pure populations of cells. Instead, CSCs have been identified as cells that become enriched within populations of tumors cells sorted by their expression of cell surface proteins. For example, colorectal CSCs have been enriched by sorting tumor cells on the basis of expression of CD133 [16,17] and CD44 [18]. In some instances, individual colorectal CSCs have been identified in CD44 populations based on their ability to initiate tumors as single cells in xenograft assays [19]. However, no definitive CSC-specific markers were identified by these experiments, thus precluding further purification and study of CSCs. Interestingly, the breakthrough linking stemness to cancer cells has come from a better understanding of normal stem cells rather than the CSCs identified by xenograft assays. This has been pioneered in the understanding of CRCs, the subject of this review, and is likely to apply to cancers generally.

Gene expression signatures of intestinal stem cells bulk up the CSC hypothesis
In 2007 and 2008, a variety of intestinal stem cell (ISC) specific markers were identified [20], leading to the unequivocal identification of two distinct classes of stem cells: a rapidly cycling population of Lgr5-expressing cells located at the crypt base [21] and a quiescent population of Bmi1-expressing cells located at the +4 position relative to the crypt base cells [22] reviewed by Li and Clevers [23] (Fig. 1). Lgr5 encodes a G-coupled protein receptor that promotes Wnt signaling by binding the Wnt agonist R-spondin [24]. It is uniquely expressed by the rapidly cycling stem cells, but is dispensable for Lgr5 stem cell self-renewal and differentiation [24]. Bmi1 is a member of the Polycomb Repressive Complex 1 (PRC1); it is expressed in stem cells
in several tissues [25] and within the gut it is expressed in the quiescent +4 stem cells.

By in vivo lineage tracing, using the promoters of Lgr5 and Bmi1 to drive the expression of stable reporters, the Lgr5 and Bmi1 cell populations were shown to give rise to all the differentiated cell types in the intestine: the absorptive enterocytes and the three secretory lineages: goblet cells which secrete mucous, enteroendocrine cells which secrete hormones; and paneth cells which secrete innate immunity proteins such as defensins, as well as ligands for the Wnt, Notch, and EGFR pathways [26,27] (Fig. 1). Additionally, recent experiments have shown that Lgr5 and Bmi1 cells can each directly give rise to all of the differentiated cell lineages independently of one another [28–30].

The link between stemness and CRC was made by three independent groups who looked specifically at the gene expression signatures of Lgr5 stem cells. They found that gene expression signatures associated with Lgr5 stem cells were enriched in advanced human CRCs [6–8]. Each group arrived at Lgr5 stem cell expression signatures differently, using either healthy mouse ISC s [6], healthy human ISCs [8], or human adenoma (early tumor) ISCs [7,8]. Yet each came to the same conclusion: based on comparisons with hundreds of publicly available colorectal tumor expression profiles they found that patient prognosis could be predicted based on how well the patients’ tumors matched Lgr5 expression signatures. In each case, tumors with Lgr5-like expression signatures tended to be more deadly than those lacking the signature.

Merlos-Suárez et al. [6] found that tumors enriched with mouse ISC derived Lgr5 signatures were 10× more likely to recur than tumors lacking enrichment of this signature. In response to criticism that the signature was from a mouse, they ‘humanized’ the signature by keeping only those genes that had been previously shown to be expressed in human crypt tissue samples [31]. The humanized Lgr5 signature likewise had prognostic power; however, it was no greater than the complete mouse-derived signature, thus confirming the results and utility of the original mouse signature. Consistent with these results, de Sousa et al. [7] found that an Lgr5-like signature derived from primary cultures of human CSC spheroid cells could be used to predict the likelihood of tumor recurrence using the same dataset as Merlos-Suárez et al., as well as a set of 90 CRC stage II patients that they followed from their own institute. A slightly different approach was taken by Dalerbra et al. [8], in which they used single cell PCR to define ‘bottom of the crypt’ Lgr5 associated signatures of immature cells and ‘top of the crypt’ associated signatures of differentiated cells in human healthy colon and adenomas. Interestingly, they found that a two-gene classifier that specifically excludes Lgr5 associated signatures was sufficient to predict the likelihood of tumor recurrence in a set of over 1500 tumor samples. Each of the three studies arrived at the same conclusion: predictions based on the Lgr5 signature (or lack of it) provided better prognostics than the long-standing practice of staging tumors with the cell morphological standards set forth by the American Joint Committee on Cancer (http://www.cancerstaging.org).

The fact that three groups working with different sources of Lgr5 related signatures and hundreds of different tumor expression profiles found that Lgr5 gene signatures correlate with tumorigenicity, indicates that tumor cells with Lgr5 related signatures are therapeutically relevant. The next step will be to understand what it is about the biology of Lgr5 related cells that makes them especially tumorigenic. The most obvious explanation is that because Lgr5 related
signatures are derived from *bona fide* stem cells, tumor cells expressing Lgr5 related signatures have stem-like features, making them the most deadly cells within the tumor, as predicted by the CSC hypothesis.

However, another possibility has been raised by de Sousa *et al.* [7], who argue that the Lgr5 signatures within tumors are incomplete representations of the Lgr5 signatures of *bona fide* stem cells, thus raising the possibility that the deadly tumor cells might not be stem-like at all. They argue that the tumor Lgr5 signature has more in common with ‘de-differentiated cells’ than the stem cells from which the signature was derived. However, the possible identities of Lgr5 tumor cells, as either stem-like or ‘de-differentiated,’ are not mutually exclusive and will be resolved by a deeper understanding of how the genes in the Lgr5 signature relate to stemness.

As a case in point, two of the genes missing from the tumor Lgr5 signature are Lgr5 itself and Axin, both expressed downstream of the Wnt pathway [20]. Since the Wnt pathway is essential for stem cell maintenance, the lack of expression of some Wnt target genes in tumor Lgr5 cells might suggest that they are missing key elements of stemness. However, because the Wnt pathway drives the expression of several target genes in ISC [20], the lack of expression of some targets is not equivalent to the lack of Wnt signaling. For example, Lgr5, the first Wnt target gene found to definitively mark the crypt base stem cells, is not required for stem cell maintenance [24,32]. Thus, the lack of expression of Lgr5 in tumor ‘Lgr5 cell’ signatures is unlikely to impact the stemness of those cells. The crypt base stem cells are called Lgr5 cells for historical reasons, because they express Lgr5, not because they require Lgr5.

Moreover, some genes that are important for the stemness of normal stem cells may not be required in the context of the altered genetics of tumorigenic stem cells. Axin, another Wnt target missing from tumor Lgr5 signatures, is a perfect example of this: in normal ISCs Axin forms a complex with adenomatous polyposis coli (APC) to inhibit the Wnt pathway. The complex shuttles the Wnt transcriptional activator, β-catenin to the proteasome. However, in the majority of CRCs, APC is no longer present or active. Because Axin does not appear to be sufficient to suppress β-catenin alone, its absence or presence in the majority of CRCs is probably obsolete. Thus while Axin is an integral part of the normal ISC signature, it does not appear to be an essential component of the CSC signature.

In summary, although Lgr5 and Axin are expressed in *bona fide* ISCs, the absence of these genes in the Lgr5 tumor signature likely has no impact on the stemness or biology of these cells. Thus, while the exact ‘stemness’ of Lgr5 related tumor cells remains to be determined, the fact that the signature is from Lgr5 stem cells suggests that studying the biology of Lgr5 stem cells will contribute to the development of effective cancer therapeutics.

**Two stem cell populations in the intestine may give rise to different classes of CSCs**

In addition to the fast cycling Lgr5 stem cells, the intestine is also maintained by a population of slow cycling cells at the +4 position of the crypt, marked by the expression of Bmi1 [22,30,33], telomerase reverse transcriptase (mTERT) [29], and Hopx [28]. Since the cells at the +4 position are rarely seen co-expressing all three of these markers at the same time, it is unclear if slow cycling stem cells at the +4 position are functionally distinct from one another [34]. However, because there are no data indicating that the +4 cells are functionally different from each other, they are often referred to by the founding marker of the +4 population, Bmi1.

The slow cycling cells at the +4 position may prove even more informative than the Lgr5 cell population because they exhibit hallmarks of CSCs that are specifically associated with the increased tumorigenicity and drug resistance seen in advanced stages of cancer [5]. For example, they are largely quiescent [22], a feature associated with the drug resistance of CSCs. Consistent with this possibility, the slow cycling cells, but not the faster cycling Lgr5 cells, are radio-resistant [29,33]. In addition, they express high levels of mTERT, an enzyme associated with the long-term survival of stem cells, and presumably the indefinite growth of CSCs. Given these similarities with CSCs, it will be interesting to compare the gene expression signature(s) of slow cycling stem cells with that of the Lgr5 stem cells and to determine their respective prognostic power.

Just as the normal intestine is maintained by two (or more) distinct populations of stem cells, CRCs are likely to be maintained by multiple types of CSCs, each constituting different fractions of the population and each providing different prognostic power.

**Models to identify anti-CSC drugs for CRC**

Based on biology of stem cells, it should be possible to eliminate CSCs by two distinct approaches: one is to block stem cell maintenance and the other is to induce stem cell differentiation. Stem cell maintenance can be blocked in the intestine by interfering with either the Wnt or Notch signaling pathways [35,36]. Of these pathways, the Wnt pathway has been a major focus for colorectal therapeutics because it is hyperactivated in over 85% of colorectal tumors [37]. Although mutations in the Notch pathway are not commonly associated with CRCs, blocking the Notch pathway is also effective at stopping the progression Wnt-induced adenoma models [38]. Moreover, inhibition of the Notch pathway not only blocks stem cell proliferation but also converts all the progenitor cells (called transient amplifying cells) into terminally differentiated goblet cells.

Thus, one method with which to identify CSC therapeutics is to design drug screens to target the Wnt and Notch signaling pathways. Indeed two recent studies independently
identified small molecules that suppress activating mutations in the Wnt pathway by stabilizing Axin, an endogenous repressor of the Wnt pathway [39,40]. Likewise a class of inhibitors of the Notch pathway called gamma-secretase inhibitors have been shown to effectively block stem cell maintenance and to force the differentiation of progenitor cells in murine models of intestinal adenoma [38] and a related cancer, metaplastic Barrett’s esophagus [41].

While the effects of chemically blocking the Wnt and Notch pathways are exciting in their own right, they also point to the feasibility of conducting more ambitious ‘unbiased screens’ to capture any small molecule, regardless of the specific pathway it might affect, for its ability to impact stem cell maintenance and/or differentiation. However, thus far there have been only a handful of unbiased anti-CSC screens reported for the entire CSC field [42,43] and none involve intestinal CSCs. The major hurdle to developing such screens has been finding methods to culture stem cells. This is a challenge because stem cells, perhaps more than any other cell type, depend on interactions with their microenvironment or niche [44]. One method to overcome this problem is to develop culturing conditions that can mimic the complexity of the niche. Yet another method is to circumvent the niche problem altogether by screening in whole animals.

The remaining sections of this review discuss three emerging model systems that are likely to provide new opportunities to screen for drugs that can block the growth of colorectal CSCs: organoids, zebrafish and fruit flies. Organoids are ex vivo ‘mini-guts’ that can be cultured long-term directly from mice [45] or grown from individual ISC [46] using culture conditions that mimic ISC niche signals. Zebrafish larvae and fruit flies by contrast provide in vivo ‘mini-guts,’ which exhibit many features in common with their mammalian counterparts. To date none of these systems have been used for anti-CSC drug screens. However, recent advances indicate that each system could evolve into new screening paradigms for the discovery of anti-CSC drugs for CRC.

**Organoids: 3-dimensional mini-guts in a dish**

In 2009 two groundbreaking papers were published showing that ISCs can be maintained for long periods lasting from months to a year within ex vivo mini-gut or organoid structures [45,46]. Ootani *et al.* cultured minced pieces of gut with myofibroblasts, cells that constitute part of the niche, whereas Sato *et al.* cultured single Lgr5 stem cells with growth factors they had identified in the niche. Both methods led to the development of self-renewing organoids that contained the full complement of cells of the intestinal tract.

The resulting organoids were found to resemble both the morphology and functionality of the normal intestine. For example, organoids grow as a single layer of cells that folds into the basic crypt-villus-architecture of the normal intestine (Fig. 2). Additionally, recent work by the Watanabe laboratory (2012) [47] showed that organoids retain the functionality of the intestine when transplanted back into test animals. This was demonstrated by elegant transplantation experiments showing that GFP-labeled organoids can replace damaged colonic tissue when transplanted into colons of mice that had been treated with the colitis inducing chemical dextran sodium sulfate (DSS) [47]. Together these experiments demonstrate that stem cells cultured within organoids retain the capacity for stem cell self-renewal and differentiation.

The initial establishment of organoids quickly led to a series of papers describing methods to culture organoids from a variety of intestinal samples. A slew of ligands, small molecules, and extracellular matrices have been used, including:
EGF, WNT3A, R-SPONDIN, NOGGIN, hepatocyte growth factor, nicotinamide, gastrin, a p38 inhibitor, an Alk4/5/7 inhibitor, as well as laminin-enriched matrigel, BSA and collagen (Fig. 2). Using combinations of these factors, organoids can be cultured from mouse stomach small intestine and large intestine [45–49], as well as from healthy human colon cells and CRCs [47,49,50]. Moreover, organoids can be initiated not only from rapidly cycling Lgr5 stem cells but also from slowly cycling Bmi1 stem cells [28,30,33]. The ability to create organoids from so many different sources of stem cells opens the possibility of a wide range of chemical screens to address specific biology of different kinds of intestinal cancers.

Given that stem cells both self-renew and differentiate within organoid cultures, it is conceivable to use these cultures to screen drugs that affect maintenance and/or differentiation. Indeed, it has been found that the stem cells in organoids respond to Wnt and Notch inhibitors in the same way as they do in vivo. For example, interfering with the Wnt pathway by addition of the secreted inhibitor Dickkopf-1 results in loss of stem cell maintenance, resulting in loss of the organoids. Conversely augmenting Wnt signaling with high doses of either the Wnt agonist R-spondin, or Wnt3A itself, leads to hyper-proliferation of stem cells [45,49]. Stem cell differentiation in organoids is also sensitive to chemical perturbations. For example, treating organoids with a drug that blocks the Notch pathway, the gamma-secretase inhibitor dibenzazepine, drives the differentiation of progenitor cells into goblet cells [45,49]. These experiments thus establish the ‘drugability’ of organoids.

Translating the druggability of organoids into large-scale screens is clearly on the horizon but will require optimization of organoid culturing conditions. For example, the frequency at which organoids are formed in each culture, called the ‘plating efficiency,’ tends to be under 10%, although there has been recent improvement by co-culturing Lgr5 cells with paneth cells [51]. In addition, the types of organoids that form have not been standardized, making it difficult to develop standardized assays and comparisons between assays [52]. Thus increasing the frequency and consistency of organoids will greatly increase the feasibility of chemical screens. Given the pace with which organoid technology has grown, it will not be long before organoids are employed in large-scale screens for anti-CSC drugs.

**Whole-animal screens**

A complementary approach to using cultured cells for drug screening is to use whole animal models. Whole animal models are appealing because they retain the physiology of the stem cell niche, which is essential to the maintenance and differentiation of stem cells. Because the niche exists within a milieu of many cell types and signals, both local and systemic, it is virtually impossible to fully recapitulate it in vitro. For example the ingredients list for growing organoid cultures lacks instructive ligands that are present in the in vivo niche, but are hard to supplement exogenously, such as tumor necrosis growth factor-alpha and the Notch ligand Delta-like 4, DLL4 [27] (Fig. 2). However, even if it were possible to provide all the niche ligands exogenously the approach would remain problematic because it would preclude the ability to discover drugs that interfere with niche signal production. These drawbacks to culturing stem cells in vitro can be avoided by working with whole animal models, such as zebrafish, distinguished for its adaptability to whole animal chemical screens, and Drosophila, distinguished for its amenability to genetic dissection of pathways and cell lineages. Both of these organisms possess excellent models of the mammalian intestine (as described below) and are therefore excellent candidates for the development of unbiased whole animal chemical screens.

On a gross anatomical level the intestinal architecture differs between mammals, fish and fruit flies: in the mouse small intestine, the epithelium folds into the characteristic crypt-villus architecture, in zebrafish it folds into villus structures but not crypts, and in Drosophila the epithelium exists as a tube with no villi or crypts. However, despite their structural differences, the intestines of Drosophila, zebrafish and mammals are composed of similar cell types [53–57]. Broadly, the intestines in each are maintained by proliferative cells that give rise to two types of post-mitotic cell types: (1) absorptive cells called enterocytes (ECs) that have a characteristic brush border conserved from flies to mammals and (2) secretory cells which include paneth cells (mammalian specific), goblet cells (vertebrate specific), and enteroendocrine cells (conserved from flies to mammals) (Fig. 1). Thus, the Drosophila and zebrafish intestines represent stripped down versions of the mammalian gut, but as detailed below, they retain essential features of the stem cell biology and niche.

Currently, much more is known about the ISC cells in Drosophila than in zebrafish, in part because of the more facile genetics of Drosophila. These studies reveal striking parallels between Drosophila and mammalian intestines. The ISCs in Drosophila, like in mammals, were identified by lineage tracing [58], and were found to possess features in common with both Lgr5 and Bmi1 stem cells. Like mammalian Lgr5 stem cells they divide once a day to give rise to daughter cells that either remain as stem cells or that differentiate into evolutionarily conserved intestinal cell types, ECs and secretory cells [58,59]. Recent papers indicate that in both flies [60] and mice [51] neutral competition underlies the fate of stem cell daughter cells to either remain a stem cell or to adopt a differentiated cell fate. Drosophila ISCs also resemble Bmi1 cells in that they exhibit resistance to cytotoxic agents. This was demonstrated in Bmi1 cells using gamma-irradiation [28,29,33] and in Drosophila ISCs with the DNA damaging agent bleomycin [61]. These studies show that Bmi1 cells and Drosophila ISCs are not only resistant, but also exhibit
compensatory growth to replace injured cells in the epithelium, such as Lgr5 cells in mammals and ECs in flies [61,62]. Thus, the Drosophila ISCs resemble behaviors of both the Lgr5 ‘everyday cycling stem cells’ as well as Bmi1 ‘reserve stem cells’ of the mammalian intestine.

Drosophila ISCs are similar to their mammalian counterparts, not just on the cellular level but also molecularly. For example, Apc functions as a tumor suppressor in both flies and mammals. In flies, Apc loss drives stem cell hyper-proliferation [63], reminiscent of the growth of early adenomas due to loss of Apc heterozygosity in mammals [37]. Likewise, as in mammals, Notch is required for the specification of ECs. In the absence of Notch, differentiation of daughter cells is diverted from the EC fate to secretory fates: goblet cells in mammals and enteroendocrine cells in Drosophila [58,59]. Interestingly, the role of Notch as a cell fate determinant may be easier to track in Drosophila than in mammals because it is required specifically for differentiation, whereas in mammals, Notch is required not only for differentiation but also for stem cell maintenance. Thus, in the absence of Notch, the stem cell lineage in mammals is lost altogether, whereas in Drosophila the stem cells continue dividing without differentiating, resulting in stem cell ‘tumors.’ These parallels, both molecular and cellular, indicate that the results of screens in Drosophila can have direct bearing on our understanding of the physiology of mammalian ISCs.

Although the genetics of the intestinal lineage are not as well defined in zebrafish, the zebrafish intestine clearly exhibits features in common with the mammalian intestine including reliance on Wnt and Notch signaling. For example, loss of Apc heterozygosity leads to spontaneously forming adenomas [64], and loss of Notch signaling drives daughter cells to differentiate into goblet and other secretory cell fates [65].

While much of the biology of the ISCs is conserved between Drosophila, zebrafish and mammals, the paneth cells that constitute the mammalian niche [27] are absent in Drosophila and zebrafish. However, although the paneth cells are missing, key ligands that are expressed from the paneth niche in mammals have been identified in the microenvironment of Drosophila ISCs (Fig. 2). For example, in mammals the paneth cells secrete WNT, DELTA and EGF, and in Drosophila these same three signals exist in the niche milieu: WNT and EGF ligands are supplied by the surrounding visceral muscle [66,67] and DELTA is expressed on the stem cell itself [68]. Thus, screens in Drosophila and by extension zebrafish, have the ability to identify drugs that interfere with niche signal production and reception.

Similarities between the cellular make up and genetics of Drosophila and zebrafish ISCs with mammalian ISCs indicate that both would be good models for whole animal chemical screens. In the case of zebrafish, screening conditions are already well established for embryos and larvae [69]. However, to take full advantage of these features it will be necessary to develop ISC tumor models with higher penetrance. For example, the zebrafish equivalent of Apc<sup>min</sup> mice acquires spontaneous adenomas in only 11% of the fish examined [64]. Similarly conditional over-expression of a transgenic human KRAS oncogene exhibited low penetrance, leading to tumors in only one-third of the examined fish. These technical problems will likely be overcome with improvements in transgene expression, leading to robust, highly screenable in vivo models of colorectal CSCs.

To date no large-scale chemical screens have been reported using adult Drosophila. However, in theory it should be possible to perform such screens because drugs can be mixed with fly food and fed to flies by a variety of techniques [70]. Indeed, some small molecules have been shown to affect the proliferation and differentiation of Drosophila ISCs. For example, Ohlstein and Spradling [58] showed that in feeding adults the Notch gamma-secretase inhibitor (DAPT) difluorophenylacetyl-alanyl-phenylglycine-t-buty-l-ester phenocopied the genetic loss of Notch in ISCs, resulting in an increase of enteroendocrine cells at the expense of maintaining ECs, as well as the development of ISC ‘tumours’. Similarly, Amcheslavsky et al. [61] showed that feeding adult flies bleomycin and DSS produced drastic effects in the intestinal epithelium, leading to damage of the ECs and compensatory proliferation of the ISCs. These results are exciting because they demonstrate that the fly intestine can be used to identify and study the effects of drugs on both ISCs as well the ECs, a major constituent of the stem cell microenvironment.

Screening for drugs that block colorectal CSCs may soon be possible using organoids, zebrafish and Drosophila. Each system contributes unique advantages that complement the others. Organoids can be derived from human intestine and will probably be the model of choice for large-scale screens, as well as for personalized medicine using tissue directly from patients. However, because organoids rely on an artificial niche, screens with zebrafish and Drosophila will provide an opportunity to screen both stem cells and niche cells under more physiologically balanced conditions. Zebrafish embryos and larvae are already primed for large-scale screening, whereas screens in Drosophila adults will require a number of innovations.

**Concluding remarks**

Recent papers [6–8] establish the therapeutic relevance of CSC, showing that stem cell gene signatures can be used as a prognostic to predict the outcome of different CRCs. These findings will hopefully steer the discussion of colorectal CSCs away from arguments about the semantics of stemness to the biology of stemness that underlies the prognostic power of stem cell gene signatures. New advances in the study of ISCs in organoids, zebrafish and fruit flies are greatly advancing
our understanding of the biology of CSCs and should pave the way to novel CSC drug discovery.

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