CANCER ORIGINS — OPINION

Imaging innate immune responses at tumour initiation: new insights from fish and flies

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Abstract | Recent imaging studies in genetically tractable and translucent zebrafish and *Drosophila melanogaster* models have opened a window on the earliest stages of tumorigenesis, when pre-neoplastic cells first arise in tissues before they progress into full-blown cancers. Innate immune cells often find these cells soon after they develop, but this efficient surveillance is not always good for the host because although immune cells have phagocytic capacity, they can also nurture the growing clones of pre-neoplastic cells. We describe these newly observed early interactions between immune cells and cancer cells and speculate on their potential clinical implications.

Although intrinsic genetic lesions typically trigger the development of a cancer, extrinsic host-derived factors are now known to have a pivotal role in the development of malignant disease. The host immune system, which clearly evolved to protect tissue homeostasis by eliminating damaged cells or infectious agents, has the surveillance capacity to seek out cancer cells but unfortunately it can be subverted to enhance late-stage cancer promotion and progression[1,2]. Indeed, inflammation is considered one of the four emerging hallmarks of cancer[3], and infiltrating immune cell lineages make up a major part of the stromal compartment of any tumour. Moreover, there is strong evidence that the presence and activation state of various immune cell lineages — particularly of macrophages and neutrophils — can be prognostic of negative clinical outcome[2]. What is much less understood is the role of immune cells and the inflammatory response at the initial stages of cancer development, when early pre-neoplastic cells first arise in otherwise healthy tissues. Until recently, we had no information concerning when immune cells might first identify these aberrant cells that have the potential to seed a full-blown cancer. Our ignorance was largely because these key early moments are hidden from us, in patients and in all the standard animal models of cancer, because of the opacity of tissues. However, in recent years, improvements in imaging and the use of translucent model organisms, largely zebrafish, have allowed us to begin to visualize these early interactions between host cells and cancer cells, to uncover what it is about early pre-neoplastic cells that reveals them as 'abnormal', and to determine how immune cells may be subverted to nurture rather than remove potential cancer cells. New insights from such studies offer substantial potential for developing better cancer-preventive agents that might block any positive, trophic signals from inflammatory cells to pre-neoplastic cells, or trigger these cells to kill rather than nurture the abnormal cells before they become cancerous.

A rapid inflammatory response

It is now clear that healthy, normal cells can recognize aberrant neighbours and either kill or extrude them (BOX 1), and this can occur even without the involvement of the immune system. However, innate immune cells can rapidly detect and be drawn to any pre-neoplastic cell that might escape this first line of defence.

As *Drosophila melanogaster* embryos and zebrafish larvae are translucent, it is possible to observe the progress of any fluorescently tagged cell from the moment of its inception onwards (FIG. 1). Also, because of their genetic tractability, engineering of appropriate lines and combinatorial crossing of these various lines is considerably easier than for mouse models. In larval zebrafish studies, if any one of a variety of cell lineages (melanoblasts, goblet cells or keratinocytes) is forced to express transgenic constitutively active human mutant $HRAS^{G12V}$, then these mutant cells will be rapidly 'sensed' in an otherwise wild-type host epithelium[4,5]. Zebrafish larvae have a similar repertoire of innate immune cell lineages to mammals, including neutrophils and macrophages[6], and both of these can be drawn to, and interact with, pre-neoplastic cells while they are still only single cells, or at the latest when they have divided only once or twice[4]. Neutrophils arrive first; they glide beneath and around individual pre-neoplastic cells or a small clone of cells for several minutes before moving on to other nearby clones[4] (FIG. 2). These periods of attention may coincide with the duration of stochastic pulses of attractant (as discussed below). Just as in a wound repair scenario, macrophages arrive second but it may not be necessary for pre-neoplastic cells to have first been 'discovered' by neutrophils because blocking neutrophil recruitment does not substantially suppress recruitment of macrophages to clones of pre-neoplastic keratinocytes[5]. Once a clone of pre-neoplastic goblet or melanoblastic cells reaches >20 cells then inflammation becomes 'chronic' and neutrophils appear to remain[4] (FIG. 2). This failure of neutrophils to resolve from larger clones mirrors a chronic wound, in which inflammation persists, in contrast to a healing acute wound, in which inflammation resolves (the parallels between cancer and wound inflammation are discussed in more detail below). Expression of other transforming oncogenes (for example, *src*) can similarly lead to rapid detection of pre-neoplastic cells by the zebrafish innate immune response[4]; however, noticeably, in a *D. melanogaster* imaginal disc model of cancer (FIG. 1) it appears that detection may be a little less efficient. Simple expression of fly Ras^{G12V}, on its own, appears insufficient to rapidly draw in haemocytes — the single innate immune

cell lineage in *D. melanogaster*[7]. Rather, additional mutations are needed before an inflammatory response is observed[8].

What are the early attractants?

In zebrafish larvae, early detection of aberrant cells is, at least in part, a consequence of stochastic release of reactive oxygen species (ROS) in the form of H_2O_2. In *HRAS*^{G12V}-transformed melanoblasts and goblet cell-containing skin tissue (a wet epithelium), H_2O_2 is synthesized by the enzyme dual oxidase (DUOX)[4]. This is precisely the same mechanism previously shown to cause early tissue damage detection by innate immune cells, leading to wound inflammation in zebrafish larvae and *D. melanogaster* embryos[9,10]; indeed, blocking DUOX activity in either wounded[9,10] or transformed tissues 'blinds' immune cells to either of these lesions. However, it is still not clear whether immune cells detect H_2O_2 directly, possibly via SRC-like kinases[11], or indirectly through other attractants that are activated or released by a cascade of events initiated by ROS exposure, such as chemokine (CXC motif) ligand 8 (CXCL8; also known as interleukin-8 (IL-8))[12], or whether a combination of both direct and indirect mechanisms are involved in H_2O_2 detection. Extrapolating from what is known about the cues that drive attractant release after wounding may offer hints as to what might be the upstream triggers leading to DUOX activation and ROS release, including, for example, intracellular calcium flashes or spikes[13] that can lead to activation of DUOX[14] or extracellular ATP release. Similarly, comparisons between the immediate-early wound damage signal and initiating cancers may also reveal alternative potential attractants for immune cells; for example, osmotic changes at wound sites can draw in leukocytes[15].

In mammalian tissues, DUOX is only expressed by wet epithelia and so it might not be the NADPH oxidase that is responsible for releasing ROS either after skin wounding or in cancer cells arising within the skin, but it might be functioning in the human gut epithelium and airway as a mechanism to signal the presence of aberrant cells in these organs. Increased ROS release seems to be a common feature of several oncogenic pathways, although operating through different mechanisms[16–19], and so it is likely to be a universal attractant released by pre-neoplastic cells with different oncogenic mutations.

It is well known that several damage-associated molecular pattern molecules (DAMPs) can attract immune cells to wound sites[20,21], and one such potential signal released by early-stage cancers could be extracellular matrix fragments at sites of disrupted basement membrane. For example, fly *Ras*^{G12V} and Scribbled-null (*scrib*$^{-}$) mutant pre-neoplastic clones arising within the *D. melanogaster* imaginal disc epithelium appear to attract haemocytes primarily to regions where the basement membrane is partially degraded[8,22], as revealed by loss of green fluorescent protein (GFP) in flies that express an enhanced GFP (eGFP)–collagen IV fusion protein[8,23]; *D. melanogaster* matrix metalloproteinase 1 (Mmp1) is required to degrade the basement membrane and is expressed by fly tumours when haemocyte-derived tumour necrosis factor (TNF) is present, suggesting that the haemocytes might be regulating when and how the basement membrane is broken down[24]. However, almost nothing is currently known about when basement membrane disruption first occurs after the development of an initiating pre-neoplastic cell, or whether basement membrane damage is as much a consequence of immune cell recruitment as it is a cause.

Other DAMPs are likely to recruit immune cells to aberrant cells within otherwise healthy tissue. Indeed, recent studies of ultraviolet-treated late-stage melanomas in mice have shown that high mobility group box 1 (HMGB1) can be released by radiation-damaged cancer cells[25], indicating another potential leukocyte attractant that may also function at early stages of cancer initiation. Furthermore, expression profiling studies in various late-stage human cancers have revealed induction of chemokines and cytokines[26], and these might similarly be upregulated in early-stage pre-neoplastic cells. Indeed, a recent study in zebrafish larvae shows that CXCL8 is expressed by *HRAS*^{G12V}-expressing epidermal cells, and this must be a major component of the immune cell attractant signal because global knockdown of its receptor, CXC chemokine receptor type 2 (CXCR2), leads to a significant disruption of neutrophil recruitment to these pre-neoplastic cell clones[5]. A similar oncogenic lesion in cells from a different tissue origin might release distinct immune cell attractants. For example, in an oncogenic human *KRAS*^{G12V}-driven hepatic cancer model in zebrafish, transforming growth factor-β (TGFβ) appears to be the main factor that draws neutrophils into the liver[27].

First leukocyte contacts

Movies of the earliest interactions between innate immune cells and pre-neoplastic cells are intriguing. When a neutrophil moves off after its first interactions with a pre-neoplastic cell, a tethering link may be retained between the two cells (FIG. 2d). These tethers resemble the nanotubes previously observed to link interacting T cells, which are known to mediate signalling and virus transfer between cells[28]. Just as previously described for T cells, these tethers can be

Box 1 | Cancer cell extrusion

Potential cancer cells can often be extruded by their healthy neighbours without assistance from the immune system. *In vitro* studies in which transforming oncogenes (together with fluorescent reporter transgenes) are expressed in individual cells within an otherwise wild-type sheet of epithelial cells, result in substantial numbers of these pre-neoplastic cells 'popping' out from among their wild-type neighbours in ways that mirror the extrusion of apoptotic cells from epithelial sheets[59–61]. The same is seen *in vivo*, with *src*-expressing cells often extruded from the epithelium of early zebrafish embryos at a stage before the development of immune cells, indicating that host tissues have some capacity to recognize aberrant cells and remove them without need of an inflammatory response[59]. Extrusion occurs while the pre-neoplastic cell is still alive, and is dependent on Rho signalling and filamin accumulation in neighbouring wild-type cells[60]. Presumably, this process continually occurs within adult tissues, and thus may function as a checkpoint to prevent the progression of many potential cancer precursor cells without triggering an inflammatory response. However, it clearly does not rid tissues of all potentially malignant cells; indeed, the polarity of the extrusion can reverse, leading to basal extrusion, which can initiate invasion[62]. Cell competition studies, largely in the *Drosophila melanogaster* imaginal disc, have revealed another mechanism whereby aberrant cells can be detected and deleted without the need for immune cell involvement. Several interacting signalling pathways, including Myc, Wnt and Hippo pathways, feed into an alternative splicing regulatory mechanism that, in turn, regulates the balance of the calcium channel Flower isoforms expressed by a cell, and this enables cells to determine their neighbours' relative fitness. 'Loser cells' then activate apoptotic pathways and are deleted, whereas 'winner cells' proliferate more rapidly (reviewed in REF. 63). It seems likely that similar cell competition episodes occur in vertebrate tissues but this has been poorly investigated to date. One study in vertebrate haematopoietic tissue indicated that relative levels of p53 might be used to determine 'winner' and 'loser' status between neighbours and drive the most aberrant cells towards senescence and/or death[64].

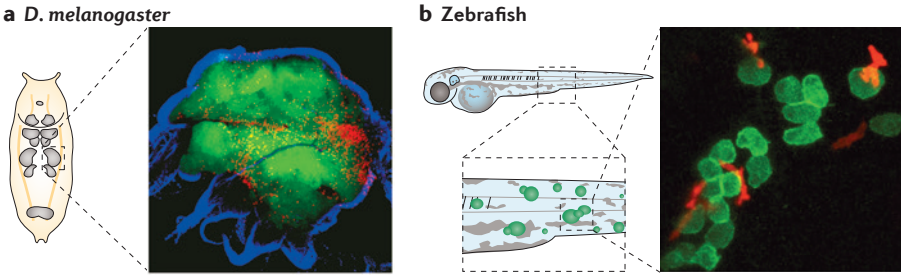


Figure 1 | **Translucent models of immune cell–cancer cell interactions.** *Drosophila melanogaster* and zebrafish are both genetically tractable and so offer opportunities for insights into cancer that are not available in mice or humans. *D. melanogaster* has been instrumental in identifying several cancer genes and dissecting signalling pathways upstream and downstream of these genes[68], and zebrafish is providing insight in large part because of its capacity for large-scale genetic and small-molecule screens[69]. Moreover, zebrafish tumours resemble human cancers at histological, transcriptomic and epigenetic levels[69]. Perhaps of equal importance, both of these models are also translucent at various stages in their development, which enables imaging of the early stages of cancer initiation. **a** | In *D. melanogaster*, most of these studies involve clones of transformed cells (green) within the epithelium of the larval imaginal discs, which will become the body parts of the adult fly. The imaginal discs are only amenable for imaging after removal from the larva and so live imaging of the haemocyte (red) response is only possible for brief periods, and these studies are generally of fixed whole-mount preparations. **b** | Zebrafish embryos and larvae are accessible and translucent, and thus very amenable for imaging throughout development (although less so in the adult zebrafish without mutations for deleting pigment cells), and so interactions between immune cells (red) and pre-neoplastic cells (green) can be live imaged from when these cells first develop, and for long periods of up to 24 hours or more. *D. melanogaster* imaginal disc image courtesy of M. Vidal, Beatson Institute for Cancer Research, Glasgow, UK. The zebrafish image is from the authors' own library and is representative of images in REF. 4.

derived from the plasma membrane from either or both participating cells. Whether the innate immune cell–pre-neoplastic cell tethers serve any signalling function or are merely mechanical links remains to be determined but at minimum they appear to be a means to draw immune cells back to the same pre-neoplastic cell, sometimes on several occasions[4]. To have made sufficient contact to form linking tethers, immune cells must somehow have breached the basement membrane that separates them from the pre-neoplastic cells within the host epithelium, but when or how this happens has not been investigated yet.

After these first interactions, both neutrophils and macrophages are observed to phagocytose some of the contacted pre-neoplastic cells, but the two leukocytic lineages do this in very different ways: neutrophils engulf small patches of plasma membrane from pre-neoplastic cells (FIG. 2e) in a behaviour previously described (in observations of membrane transfer between dendritic cells and T cells) as 'trogocytosis', which has been reported to lead to antigen presentation[29]. This uptake of fragments of interacting cells may be reciprocal, as particles of neutrophil membrane can sometimes be seen within pre-neoplastic cells too (FIG. 2h). By contrast, macrophages seem capable of phagocytosing whole pre-neoplastic cells (FIG. 2f). Most pre-neoplastic cells experience some degree of direct cell–cell contact with leukocytes, but some pre-neoplastic cells appear not to experience this contact, suggesting heterogeneity among pre-neoplastic cells in their ability to attract innate immune cells. Indeed, some pre-neoplastic cells might be gaining the potentially beneficial influences of immune cells by their local proximity to pre-neoplastic cells that have interacted with leukocytes, without revealing their existence and thus risking being phagocytosed themselves.

Trophic interactions

Despite the fact that innate immune cells can be phagocytic, overall it appears that early interactions with innate immune cells are largely beneficial to the growth of pre-neoplastic cells. For example, transient depletion of neutrophils, macrophages, or both, at the time when early clones of transformed cells first arise in tissues, dramatically reduces the proliferative index of these clones of cells, while having very little effect on the number of clones[4,30]. Therefore, innate immune cells must function as a source or trigger for the production of trophic factors for the pre-neoplastic cells. Recent studies in zebrafish show that exogenous addition of the stable prostaglandin E2 (PGE2) analogue, dimethyl-PGE2, can partly rescue the growth of *HRAS*^{G12V}-transformed cells in the absence of immune cells[30], suggesting that prostaglandins might be at least one component of the trophic signal (or signals) released by innate immune cells to promote transformed cell growth. The PGE2 rescue effect is considerably less potent if neutrophils alone, rather than macrophages alone, are depleted, suggesting that the neutrophil trophic support comes from other factors in addition to PGE2.

It may be that these trophic signals target specific cells within a growing pre-neoplastic clone. Indeed, PGE2 is a trophic factor for stem cell and progenitor cell proliferation in several tissues[31–34]. It would be of interest to know whether PGE2 is a key trophic factor that is specific for promoting cancer stem cell expansion at these early stages or soon afterwards, during tumour initiation.

There is considerable literature indicating that low-dose non-steroidal anti-inflammatory drugs (NSAIDs), including aspirin, which blocks cyclooxygenase (COX)-mediated PGE2 synthesis, can prevent the onset of gut and other cancers[35], and so it is tempting to speculate that one mechanism of action for these drugs is their inhibition of this early source of prostaglandin trophic support when pre-neoplastic cells are first establishing themselves in tissues. As described above, prostaglandin supplements cannot entirely rescue the growth of pre-neoplastic cells in zebrafish larvae depleted of leukocytes[30], suggesting that there are other, as yet undiscovered, trophic signals from these early visiting leukocytes. Identifying any additional immune-derived trophic signals at these early stages might reveal potential targets for future cancer preventatives either alone or in combination with aspirin or other NSAIDs.

Clearly, genetic context is also important. A study in a *D. melanogaster* imaginal disc cancer model provides evidence that TNF released from activated haemocytes promotes the growth of *Ras*^{G12V};*scrib*$^{-}$ clones, but the same factor drives patches of *scrib*$^{-}$; discs large-null (*dlg*$^{-}$) transformed cells to undergo apoptotic cell death and subsequent extrusion from the host epithelium (BOX 1). Here, the genetic composition of pre-neoplastic cells has a strong influence on how they interact with host immune cells. So far, all of the studies suggesting that host innate immune cells are trophic to pre-neoplastic cells have used oncogenic RAS-induced tumours as a model, but it would be of interest to establish whether host innate immune cells also promote the proliferation of pre-neoplastic cells with other genetic lesions.

Other influences

The effects of early immune cell recruitment may not only encourage pre-neoplastic cells to divide; signals delivered by recruited immune cells can also direct cells towards the initial steps of partial epithelial to mesenchymal transition (EMT) that are pivotal for progression in some cancers. Evidence for this comes from recent studies in zebrafish larvae showing that *HRAS*^{G12V}-transformed cells in the epidermis begin expressing markers of EMT, including slug and vimentin, only after making contact with recruited neutrophils[5]. Of course, it is likely that these early immune cell interactions with pre-neoplastic cells lead to other transcriptional and epigenetic changes and potentially also to increased genetic instability that may, in turn, be early events in progression towards malignancy[36]. All the studies described so far involve pre-neoplastic cells arising from within the host larval tissue, but zebrafish are also used as a model for xenografting murine and human cancer cells, and several of these studies have shown how host immune cells can aid migration of the xenografted cells (BOX 2).

Parallels with wounds and infection

Virchow was one of the first to talk of parallels between wound healing and cancer growth[37], and Dvorak is famously quoted as saying "tumours are wounds that do not heal"[38]. In large part, these comments were made because tumour stroma and wound granulation tissue histology look alike, but in several regards it seems that the inflammatory response to an early initiating cancer may also be much the same as to a wound. Several studies in *D. melanogaster* show that the genes that are upregulated following overexpression of oncogenic *Ras*^{G12V} are involved in the same pathways that are triggered following wounding and/or infection[8,39]. We already know that some of the damage attractants are the same in *D. melanogaster* and vertebrate models, and we can speculate that neutrophil and macrophage phenotypic switching occurs in similar ways, and may drive similar consequences. We know, for example, that angiogenesis — which is an essential feature of both the healing wound and of growing cancers — is highly dependent on signalling from macrophages[40]. It is also well established that wounding can trigger cancer initiation and/or provide the ideal niche for cancer growth[41]. A new study of ours in zebrafish larvae shows how small wounds, made in the vicinity of growing clones of pre-neoplastic cells, lead to rapid re-direction of

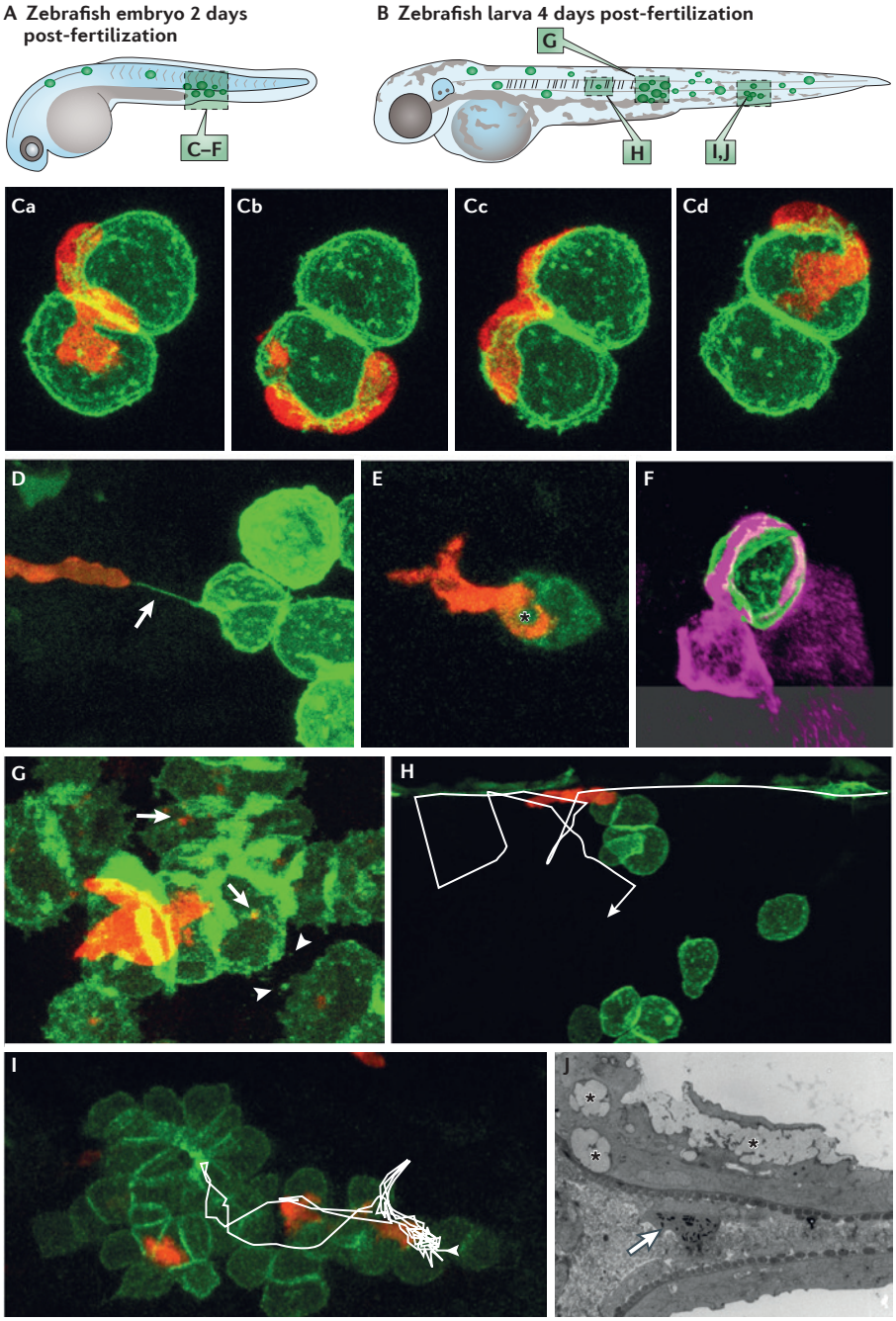


Figure 2 | **Using zebrafish to investigate immune cell–cancer cell interactions.** The schematics illustrate the sites for imaging immune cell–cancer cell interactions 2 and 4 days post fertilization in zebrafish embryos (part **A**) and larvae (part **B**), as the clones of pre-neoplastic cells grow. Four time-lapse stills at 2-minute intervals (part **C**; starting from left) to illustrate gliding of a neutrophil (red) over a pre-neoplastic cell (green) as it first divides (see Supplementary information S1 (movie)). A neutrophil (part **D**; red) moves away from a small clone but retains a tether (arrow) linking it to the pre-neoplastic clone. A neutrophil captured as it 'engulfs' a patch of membrane (part **E**; asterisk) from a pre-neoplastic cell. Supplementary information S2 (movie) shows this 'nibbling' phenomenon in real time. A single macrophage (part **F**; mauve) attempting to engulf a whole pre-neoplastic cell. A neutrophil (part **G**; red) in the vicinity of a clone of pre-neoplastic cells, with clear red and green particles, suggesting that pre-neoplastic cells may take up fragments of immune cells (arrows), and pre-neoplastic cells may release membrane vesicles (arrowheads). Tracks (part **H**; white) of a neutrophil as it encounters and then moves away from a small clone of pre-neoplastic cells. As clone size increases, the tracks of each neutrophil appear confined to within a single clone (part **I**). Transmission electron microscopy (TEM) view of the zebrafish fin in the region of a pre-neoplastic clone (part **J**; asterisks) reveals a neutrophil (arrow) possibly en route to these cells. Images in parts **D**–**F** are from the authors' own library and are representative of data from REF. 4. Images in parts **H** and **I** are adapted from REF. 4, Public Library of Science.

the wound-recruited neutrophils towards neighbouring pre-neoplastic cells. This consequently leads to the proliferative growth of those pre-neoplastic cells as they now receive more contact with innate immune cells that are drawn to the nearby wound than if there had been no wound[42]. Such observations, if confirmed in humans, clearly have clinical implications for taking biopsy samples in the vicinity of cancerous or pre-neoplastic tissues, and for cancer surgeries.

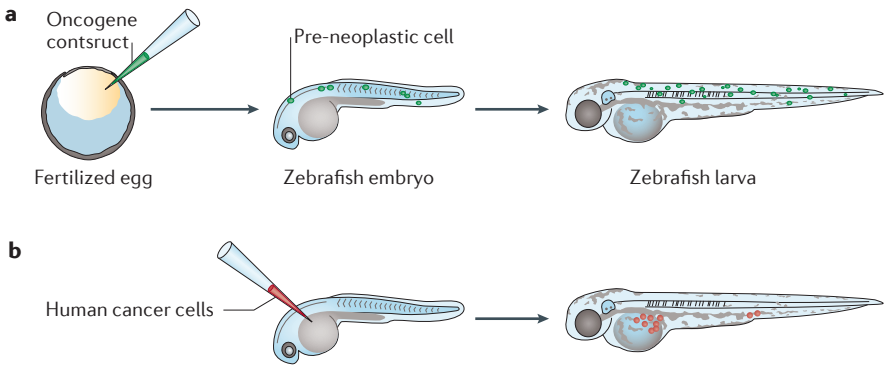
Implications for patients

Several interventions might have an impact on the number and/or activity state of immune cells at sites of cancer initiation: one of these, as described above, is local wounding, or other tissue damage, which occurs at sites of biopsy or surgery, and which could also occur following radiotherapy. Clearly, it will be important to determine how acute tissue damage of this sort and the consequent acute (but resolving) wound inflammatory response might affect any nearby pre-neoplastic cells and/or cancer cells left behind after local planned or unplanned tissue damage. This is a very understudied field but there are considerable clinical anecdotes suggesting that surgery can either exacerbate or improve different types of cancer outcome[43], and it is very likely that some of these effects will be through the wound inflammatory response. A recent retrospective analysis suggested that the administration of anti-inflammatory agents, such as the NSAID ketorolac, to patients before and after mastectomy led to a lower recurrence of their breast cancer[44], but alternative therapeutic approaches will be to dampen the downstream trophic support signals derived from these inflammatory cells (rather than the inflammatory response per se), when these factors are known. Similarly, preventive treatments at the time of investigative biopsy procedures may be beneficial in tissue in which pre-neoplastic cells are potentially present.

As described above, it seems that almost all oncogene-transformed cells interact with leukocytes soon after pre-neoplastic cells develop within a host tissue. Although this should be the ideal opportunity to eliminate precursor cancer cells, it appears that too frequently this is not the outcome. Are there ways in which we could increase opportunities for the elimination of pre-neoplastic cells by forcing even more interactions between innate immune cells and transformed cells and by encouraging immune cells to suppress rather than nurture early pre-neoplastic cells? Several classic interventions appear to act on the immune system to enhance the killing of cancer cells, including Coley's toxins — a crude cocktail of heat-killed Gram-negative and Gram-positive bacteria that was the first example of a cancer immunotherapy treatment[45]. Local or systemic infection of patients with microbial concoctions in the late 1800s led to 'dissolving' of a series of inoperable sarcomas, in ways that were not understood then but now appear to be dependent on the activation of an innate immune response[45]. A modern vestige of Coley's toxins is the treatment of bladder cancer with attenuated Bacillus Calmette–Guérin (BCG)[46]. This encouragement of 'host innate immunity' in late-stage cancers indicates that it may be possible to modify a cancer inflammatory response and to direct it towards killing cancer cells rather than supporting their growth; if such approaches also operated at cancer initiation stages then this might lead the way towards the development of new, effective, early-stage cancer preventives, just as aspirin is already proving to be.

Box 2 | Zebrafish can also be used to model cancer by xenografting

Zebrafish are used both as *de novo* cancer models, and as translucent *in vivo* hosts for xenografted human cancers. Expression of oncogenic transgenes (alongside fluorescent reporter genes) that are under the regulation of specific lineage promoters drives the formation of *de novo* pre-neoplastic cells (green) in zebrafish embryos or larvae, and subsequently leads to cancers derived from these tissues, in ways that resemble how a cancer arises in a human with a somatic oncogenic mutation (see the figure, part **a**). An alternative strategy for studying cancer growth in zebrafish is to xenograft murine or human cancer cells into the host fish (see the figure, part **b**). This is done by injection of these cells (green) either locally, into the perivitelline space, or into the circulation via the Duct of Cuvier. Such approaches do not directly recapitulate the cancer initiation stages but may model steps in the metastatic spread of cancer, before or after cancer cells have entered the circulation. They offer an unprecedented opportunity to live image the behaviour of human cancer cells *in vivo* and their interaction with the innate immune system. A recent systematic comparison of many highly migratory cancer cell lines showed that the degree of spread of these cells from the site of perivitelline injection mirrors their invasion potential *in vitro*[65]. A more recent study co-injecting both cancer cells and macrophages derived from tumours showed that macrophages previously exposed to interleukin-6 (IL-6) and tumour necrosis factor (TNF) enhance metastatic spread[66]. All of these studies utilized the embryos and early larval stages as the xenograft hosts because at these stages zebrafish do not yet have an adaptive immune system, and therefore immune rejection is avoided. But now the adult zebrafish is also available for such studies because of a recently developed immunocompromised *Rag2* mutant line, which has reduced numbers of T cells and B cells analogous to Rag-deficient mice[67].



Future directions

Imaging signalling interactions. Understanding the signalling interactions between pre-neoplastic cells and the first immune cells that they encounter will be fundamental to finding novel therapeutic targets for cancer prevention at these earliest stages. Already, using transgenic reporters in a zebrafish *KRAS*^{G12D} pancreatic model of cancer, it is proving possible to live image the signalling activities — for example TGFβ, Notch, bone morphogenetic protein (BMP) and sonic hedgehog (SHH) signalling — of transformed cells[47]. By crossing lines expressing the right combinations of reporters in both immune cells and pre-neoplastic cells[48], it will be possible to observe both sides of the signalling interaction as immune cells first encounter cancer cells. It will be essential to understand how the trophic inflammatory phenotype is first triggered in immune cells. In the near future we will be in a position to glean considerably more information as we combine

the newly established imaging opportunities available in zebrafish and *D. melanogaster* to report, in real time, these molecular signalling interactions and link them to the downstream behavioural and metabolomic outputs of both pre-neoplastic and immune cells.

Similarities to metastatic seeding? Could the observations of how newly developed pre-neoplastic cells are nurtured by innate immune cells be replicated when individual metastatic cells seed to secondary sites? Recent studies in mice have shown that metastasis can occur at sites of microclots in vessels and that this depends on macrophage guidance[49]. Other cancers that spread by shedding individual cells or small clones of cells onto adjacent host tissues, such as ovarian cancer[50], may similarly be dependent on innate immune cell support for initial integration and growth at secondary sites. If immune cells do indeed regard a newly metastasized cell in the same way as a recently developed pre-neoplastic cell, then studies of these early interactions in translucent model organisms become doubly important and may reveal preventive targets that function at both of these rate-limiting stages of cancer progression.

Early roles for adaptive immunity. *D. melanogaster* only have an innate cellular immune response and so can offer very little insight into the role of adaptive immune cells in cancer detection and killing. Furthermore, to date, all the zebrafish studies of immune cell interactions with cancer cells have been performed at early larval stages before the adaptive immune system becomes functional (which occurs from about week 2 of development). Yet, we know from studies in mice and humans that the adaptive immune system is heavily involved in surveillance and potential killing of cancer cells, so perhaps zebrafish could offer insight here too. There are now good reporter lines for T and B cells[51,52] and, just as later-stage larval and adult fish are now being used to image skin healing[53], it will also be possible to live image immune cell interactions with pre-neoplastic cells at these later stages, perhaps with the additional aid of translucent adult lines such as casper[54]. This should help determine when adaptive immune cells first interact with a growing clone of pre-neoplastic cells and what the involvement of innate immune cells might be in highlighting their presence.

Next step: mammals? The reason why studies in fish and flies can be so revealing is the translucency of these models, which makes peering through tissues a possibility. The opacity of most mammalian tissues is due to light absorption and scattering because of the differing refractive indexes of mammalian cells and tissues, but they can be made more translucent. Indeed, 'optical clearing', largely by means of intradermal injection of glycerol, is being developed to allow better clinical imaging and treatment of diseased tissues, including skin cancers[55]. Intravital imaging, using multiphoton and spinning disc microscopy, is also providing us with new opportunities to step beyond the current complex *in vitro* three-dimensional human autologous or tumour slice models[56], in which any immune response is likely to be considerably altered, and to make high-resolution *in vivo* movies of immune cell–cancer cell interactions in mammalian tissues. When this is in relatively accessible locations — for example, within mouse mammary carcinomas — such new imaging strategies are enabling the viewing of macrophage involvement in metastatic spread and of altered immune cell–cancer cell interactions following chemotherapy and other treatments[57,58]. But for now, the tiny size of zebrafish larvae and *D. melanogaster* imaginal discs, and their unmatched translucency, means that the earliest moments of cancer initiation, as pre-neoplastic cells first develop, remain best studied in fish and flies.

Conclusions

Now that translucent model organisms have allowed us an *in vivo* window into the earliest stages of cancer initiation, we can observe how transformed clones of pre-neoplastic cells grow and interact with host tissue and cells, and in particular with the host immune system. We can live image signalling events during cancer initiation, which may lead to a better understanding of the development and evolution of pre-neoplastic cells in the host. Zebrafish, in particular, provide opportunities to both genetically and pharmacologically perturb signalling events during cancer that might block the development of pre-neoplastic cells. These first opportunities to observe and modulate how innate immune cells interact with newly developed pre-neoplastic cells are already revealing details that may offer clues for cancer-preventive treatments and other potential therapeutics. More insights from these model organisms are expected soon, and in the future there is potential to use them for fast-throughput genetic and small-molecule screens to identify therapeutic targets and for discovering inhibitors of trophic signals from immune cells, which could be both preventive and therapeutic for cancer.

Glossary

Adaptive immune cells
T cells and B cells that promote cell-mediated immunity (T cells) and humoral antibody-mediated immunity (B cells). Adaptive immunity is an antigen-specific response.

Damage-associated molecular pattern molecules
(DAMPs). Molecules that are released at sites of cell damage, and can be full proteins, such as high mobility group box 1 (HMGB1), cleaved extracellular matrix components, DNA or RNA, or even small molecules, including ATP.

Dual oxidase
(DUOX). A member of the family of reactive oxygen species (ROS)-generating NADPH oxidases. In humans, DUOX1 and DUOX2 are largely expressed by wet epithelia. Zebrafish has only one DUOX homologue and it is mainly localized to wet epithelia.

Granulation tissue
Formed during the tissue repair process; composed of mixed cell and tissue types, including new blood vessels, fibroblasts, newly deposited extracellular matrix, and immune cells.

Innate immune cells
Primarily neutrophils and macrophages that contribute to the inflammatory response.

Matrix metalloproteinase 1
(MMP1). One of the members of the MMP family, which are key players in matrix remodelling — for example, during wound healing — and operate by clipping various extracellular matrix and other molecules at key motifs.

Neutrophil and macrophage phenotypic switching
Neutrophils and macrophages are two leukocyte lineages known to switch from a pro-inflammatory (N1 or M1) phenotype to a pro-healing/tumorigenic (N2 or M2) phenotype, according to the signals that they receive.

Wet epithelium
Epithelial tissues that contain mucus-secreting cells, which provide a mucus layer that retains water; wet epithelial tissues include lung epithelium and gut epithelium, and in zebrafish, the skin as well.

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Competing interests statement

The authors declare no competing interests.

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