

Inflammation at the inception of cancer: using fish and fly to tell the story

Yi Feng and Lisa Kelly

(University of Edinburgh, UK)

The use of fruit fly (*Drosophila melanogaster*) and zebrafish (*Danio rerio*) as models in cancer biology has recently led to the discovery that the earliest pre-cancerous cell induces an inflammatory response, which was later found to be important for pre-neoplastic cell (PNC) early progression by providing trophic support for PNC growth. Therefore, an understanding of the mechanisms that regulate the trophic inflammatory response toward PNCs might help us develop cancer prevention strategies. The use of fly and fish models for live imaging studies of the initiation of cancer and what we know so far about the signalling mechanisms involved during the earliest interactions between PNC and its host is discussed here, as well as some future perspectives.

The development of cancer is a multi-step process whereby a single cell changes and gains a growth advantage over its neighbours to become a pre-neoplastic cell (PNC). These changes arise as a result of genetic mutations in key genes and lead to uncontrolled growth of the mutant PNC, ultimately giving rise to a mass of transformed cells referred to as a tumour. While tumour progression is dependent on the accumulation of these changes over time, extrinsic factors derived from the host tissue also play an important role in tumour development. Cells within the tumour microenvironment including fibroblasts, endothelial cells, adipocytes and various immune cells, provide a vast array of signals to the developing transformed cell. Interestingly, these signals can have conflicting roles, as both pro- and anti-tumour signals are present in the tumour microenvironment. The balance of these conflicting extrinsic signals plays a vital role in determining the eventual progression or elimination of the tumour.

Immune cells such as lymphocytes and natural killer cells can sense a mutant, 'non-self' cell and generate an anti-tumour immune response, which eradicates transformed cells, preventing the development of tumours. However, transformed cells are capable of evading this anti-tumour response by exploiting immune-suppressive pro-inflammatory signals for tumour promotion. These pro-inflammatory signals establish a non-resolving inflammatory environment similar to that seen in chronic inflammatory conditions such as inflammatory bowel disease. For this reason, cancer has been referred to as 'a wound that does not heal'. This chronic inflammatory environment is thought to contribute to cancer progression in a number of ways. In particular, tumour-associated

macrophages and neutrophils that are conditioned in the inflammatory tumour microenvironment can suppress the anti-tumour immunity of the host. In addition, tumour-associated macrophages and neutrophils provide growth factors and inflammatory mediators to stimulate proliferation of transformed cells, angiogenesis and tumour spread. Neutrophils can also provide reactive oxygen and nitrogen species that can contribute to DNA damage and genomic instability. Recent data suggest that the inflammatory response toward tumours is elicited during the earliest initiation stages of tumourigenesis, namely the pre-neoplastic stage and its role in tumour development has become an exciting area of research.

New animal models for studying tumour initiation

Whilst uncontrolled chronic inflammation is considered to be a hallmark of cancer and the role of the inflammatory microenvironment in tumour promotion has been well established, there is very little empirical data on how the inflammatory response is initiated during tumourigenesis or the cells and signals involved in igniting the chronic inflammatory environment in cancer. This is largely due to difficulties in visualizing the initiation of a tumour from the pre-neoplastic stage *in vivo* using traditional murine cancer models. Recently, new cancer models have been developed using other model organisms, namely drosophila (*Drosophila melanogaster*) and zebrafish (*Danio rerio*). In contrast to mice, these organisms can be genetically manipulated with ease, allowing the generation of complex transgenic strains. This, combined with the optical transparency of these organisms, provides

new opportunities to visualize tumour initiation from its inception so as to monitor the development of the tumour microenvironment at the cellular and molecular level using advanced imaging techniques.

The zebrafish is an attractive model organism for studies of inflammatory responses during tumour initiation. It has a similar repertoire of innate inflammatory cells as humans, and in zebrafish the functional innate immune cells are present from very early developmental stages, from 22 hours post-fertilization (hpf) onward. However, in zebrafish, cells of the adaptive immune system are functional much later, at 2 weeks post-fertilization. This provides a time-window whereby the influence of inflammatory responses during tumour initiation can be investigated without the functional complexity of adaptive immunity.

Drosophila has been established as a model organism for decades. While it shows less genomic conservation with humans than the zebrafish, it provides a further *in vivo* system for modelling human disease conditions. Combining these established models with modern imaging techniques can provide important insights into the role of inflammatory responses in the developing tumour microenvironment.

In these organisms, cancer models have been generated by the overexpression of oncogenes such as oncogenic HRAS or vSRC in a variety of cell lineages and/or clonal mutation of tumour suppressor genes; these give rise to PNCs, which have the potential to establish a tumour. Inducible systems have been used to give greater control over oncogene overexpression. Such systems grant spatial and temporal control of PNC induction, which, when combined with transgenic inflammatory cell reporter lines, allows developing PNCs and cells within their microenvironment to be monitored from the time of initiation throughout tumour development.

Inflammatory responses during tumour initiation

Recent work has shown that PNCs induce an inflammatory response early on during initiation. In a larval zebrafish model where oncogenic forms of human HRAS or vSRC are constitutively expressed in larval skin, neutrophils and macrophages are recruited to the developing PNC at very early stages. The innate immune cells dynamically interact with the PNCs, actively making physical contact and investigating individual PNCs before moving on. As PNCs progress and the number of PNCs increases, these pro-inflammatory interactions fail to resolve and the innate immune cells remain in the developing tumour microenvironment, establishing a chronic inflammatory environment. Recruitment of these early immune cells occurs as a result of attractants released from PNCs. One of the cues required for innate

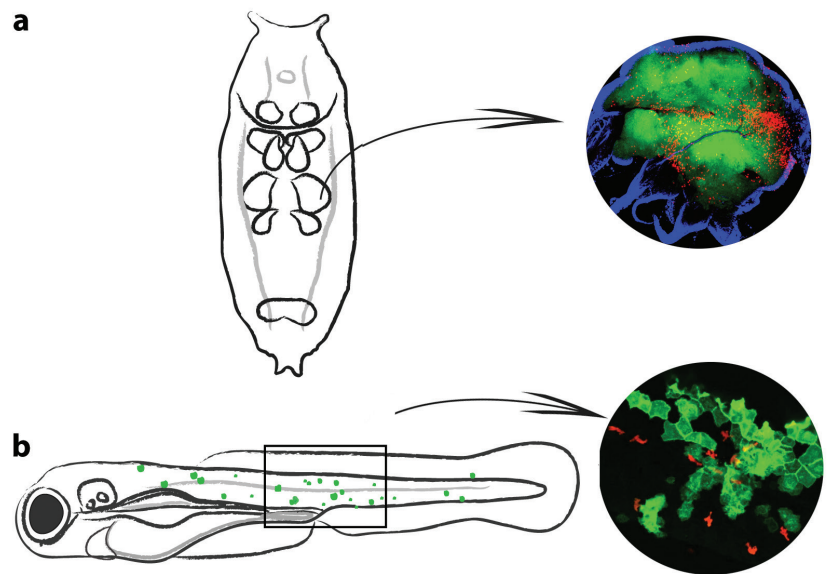


Figure 1: (a) In fruit fly larvae, tumours often grow in the epithelium of the larval imago disc which needs to be dissected out, so the imaging time for live interaction between haemocytes (red) and tumour cells (green) is short or the sample is often imaged after fixing. (b) Zebrafish larvae are translucent and accessible, pre-neoplastic cells (PNC, green) can be induced in skin epithelium, the *in vivo* live imaging of immune cell (red) and PNC interaction can last for hours or even days.

immune cell recruitment is hydrogen peroxide (H_2O_2), which is both directly chemotactic to innate immune cells and modifies the extracellular environment thereby indirectly enhancing innate recruitment. Cytokines such as CXCL8 (also referred to as IL-8) and TGF β are also involved in the recruitment of neutrophils to the developing PNCs. We envisage more factors will be uncovered that promote the early innate immune sensing of PNCs.

Drosophila, on the other hand, has a single lineage of immune cells called haemocytes. These cells are macrophage-like in nature and are the main cellular mediators of innate inflammation. Interestingly, expression of mutant HRAS alone induces activation and recruitment of haemocytes toward PNCs, but this seems to be stronger in specific tissues. In addition, multiple mutations can induce a stronger inflammatory response in this model, which suggests that various mutations in PNCs have distinct contributions toward the host inflammatory response and tissue-specific signals may be involved in modulating the inflammatory response induced by PNCs.

Importantly, the interactions between developing PNCs and innate immune cells appear to be trophic in nature, aiding survival and proliferation of PNCs. Zebrafish models suggest that prostaglandins, including prostaglandin E2 (PGE $_2$), released by inflammatory cells

are important for aiding PNC growth, while *drosophila* models indicate that cytokines, such as TNF α , are involved. To date, efforts are continuing to uncover additional trophic factors produced by innate immune cells during tumour initiation.

Signalling pathways involved in regulating tumour initiation

How the trophic inflammatory response is regulated during tumour initiation has become a burning question, and answers to this question may pave the way to the development of novel cancer prevention therapies. Studies in flies have revealed the importance of Janus kinase/Signal Transducer and Activator of Transcription (JAK/STAT) and c-Jun N-terminal kinases (JNK) signals in regulating PNC and host cell interaction. PNC intrinsic JAK/STAT appears to determine the outcome of PNC and host cell competition. JNK activation in PNC leads to their elimination whilst JNK activation in host cells is required for their growth-promoting function. The cell-type-specific functions make these pathways difficult to target without finding cell-type-specific effectors for their activation. Using various transgenic signalling reporters one could start screening for effector molecules that are specifically required in certain cell types. In flies, various signalling reporters such as JAK/STAT, JNK, ERK and Imd (NF- κ B) have been used to study PNC–host interaction and inflammatory response during tissue damage.

In zebrafish models, several signalling reporters have recently been generated, which have enabled live imaging of the TGF β , Notch, Bmp and Shh activities during zebrafish cancer development. Again, cell-

specific activation of these signals was observed either in PNCs or their neighbours. Interestingly, in zebrafish models, most transformed cells up-regulate TGF β signalling in the developed tumour, suggesting an immune-suppressive environment.

One key regulatory pathway that has been implicated in regulating both tumour cell survival and host inflammatory response is the NF- κ B signalling pathway. NF- κ B is aberrantly activated in a wide range of human cancers. As a consequence, it promotes cell survival through up-regulation of anti-apoptosis proteins and growth factors; it induces innate immune cell activation in the tumour microenvironment through up-regulation of pro-inflammatory cytokines; and NF- κ B is key to propagating a chronic inflammatory state, which promotes metastasis and tumour spread. Using a NF- κ B reporter fish we have seen up-regulation of the pathway within PNCs as well as recruited immune cells suggesting its involvement in the inflammatory response during tumour initiation.

Given its extensive role in a wide variety of cellular processes, it is a challenge to pharmacologically target NF- κ B for cancer treatment. Therefore, it is important to further dissect more cell-specific effectors of the pathway so as to guide the design of targeted therapy and prevention.

Future Directions

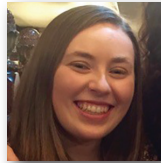
We have come a long way in our understanding of the inflammatory microenvironment during tumour initiation and development. New models in fish and fly have allowed unprecedented *in vivo* imaging capabilities, allowing us to observe transformed cells from their inception and to monitor their interactions with surrounding cells, particularly inflammatory



cells. However, much remains to be done. The intricate signalling interactions between developing transformed cells and early visiting inflammatory cells can now be imaged *in vivo* thanks to genetic engineering of models for signalling activities in pathways of interest. Elucidation of these complex signalling interactions will help us further understand the trophic inflammatory response that appears to be vital for tumour initiation and progression. This could lead to the identification of novel therapeutic targets for cancer prevention. Methodologies for high-throughput compound screening in *Drosophila* and zebrafish continue to be improved. Therefore, these models could be used not only to characterize the early interactions in the tumour microenvironment, but also for identification of pharmacological compounds which may be suitable as therapeutic agents. Taking a forward look, these models will continue to evolve and will provide deeper insight into cellular interactions and signalling changes that occur in the tumour microenvironment. ■



Dr Yi Feng is a Wellcome Trust Sir Henry Dale Fellow and Wellcome Trust-Beit Prize Fellow. Her research focus is to understand the mechanisms that regulate tumour initiation, in order to prevent the development of cancer. She has successfully established a zebrafish model for live imaging studies of the earliest events of tumourigenesis in vivo, and her research has led to the discovery that the host inflammatory response toward pre-neoplastic cells can promote tumour initiation. Her research group use an in vivo live imaging approach and a range of transgenic zebrafish strains to study mechanisms that regulate the pre-neoplastic cell induced inflammatory response during tumour initiation. Email: yi.feng@ed.ac.uk.



Lisa Kelly is an inflammation PhD student working with Dr Yi Feng in the Centre for Inflammation Research at the University of Edinburgh. She holds a BA Science (Mod. Molecular Medicine) from The University of Dublin, Trinity College, and a master's degree in Biomedical Sciences from The University of Edinburgh. In 2015 she received a Principle's Career Development Scholarship from The University of Edinburgh to undertake her PhD. Her work currently focuses on the links between inflammatory and metabolic changes in the tumour microenvironment during cancer initiation and development in a zebrafish model system. Email: s1464592@sms.ed.ac.uk.

Further Reading

References

- Feng, Y. and Martin, P. (2015) Imaging innate immune responses at tumour initiation: new insights from fish and flies. *Nat. Rev. Cancer* **15**, 556–562
- Hanahan, D. and Weinberg, R.A. (2011) Hallmarks of cancer: the next generation. *Cell* **144**(5), 646–674
- Feng, Y., Renshaw, S. and Martin, P. (2012) Live imaging of tumor initiation in zebrafish larvae reveals a trophic role for leukocyte-derived PGE₂. *Curr. Biol.* **22**, 1253–1259
- Feng, Y., Santoriello, C., Mione, M., Hurlstone, A. and Martin, P. (2010) Live imaging of innate immune cell sensing of transformed cells in zebrafish larvae: parallels between tumor initiation and wound inflammation. *PLoS Biol.* **8**, e1000562
- Yan, C., Huo, X., Wang, S., Feng, Y. and Gong, Z. (2015) Stimulation of hepatocarcinogenesis by neutrophils upon induction of oncogenic kras expression in transgenic zebrafish. *J. Hepatol.* **63**(2), 420–428
- Freisinger, C.M. and Huttenlocher, A. (2014) Live imaging and gene expression analysis in zebrafish identifies a link between neutrophils and epithelial to mesenchymal transition. *PLoS One* **9**, e112183
- Wu, M., Pastor-Pareja, J.C. and Xu, T. (2010) Interaction between Ras(V12) and scribbled clones induces tumour growth and invasion. *Nature* **28**, 463(7280), 545–548
- Pastor-Pareja, J.C., Wu, M. and Xu, T. (2008) An innate immune response of blood cells to tumors and tissue damage in *Drosophila*. *Dis. Model. Mech.* **1**(1), 144–154
- Takino, K., Ohsawa, S. and Igaki, T. (2014) Loss of Rab5 drives non-autonomous cell proliferation through TNF and Ras signaling in *Drosophila*. *Dev Biol.* **395**(1), 19–28
- Cordero, J.B., Macagno, J.P., Stefanatos, R.K., Strathdee, K.E., Cagan, R.L. and Vidal, M. (2010) Oncogenic Ras diverts a host TNF tumor suppressor activity into tumor promoter. *Dev. Cell.* **18**(6), 999–1011
- Tamori, Y., Suzuki, E. and Deng, W.M. (2016) Epithelial tumors originate in tumor hotspots, a tissue-intrinsic microenvironment. *PLoS Biol.* **14**(9)
- Schiavone, M., Rampazzo, E., Casari, A., et al. (2014) Zebrafish reporter lines reveal *in vivo* signaling pathway activities involved in pancreatic cancer. *Dis. Model. Mech.* **7**(7), 883–894
- Baud, V. and Karin, M. (2009) Is NF-κB a good target for cancer therapy? Hopes and pitfalls. *Nat. Rev. Drug Discov.* **8**(1), 33–40

Additional reading

- Grivennikov, S.I., Greten, F.R. and Karin, M. (2010) Immunity, inflammation, and cancer. *Cell* **140**, 883–899
- Evans, R.I. and Wood, W. (2011) *Drosophila* embryonic hemocytes. *Curr. Biol.* **21**, R173–R174
- Hoesel, B. and Schmid, J.A. (2013) The complexity of NF-κB signaling in inflammation and cancer. *Mol. Cancer* **12**, 86
- Amoyel, M., Anderson, A.M. and Bach, E.A. (2014) JAK/STAT pathway dysregulation in tumors: a *Drosophila* perspective. *Semin. Cell. Dev. Biol.* **28**, 96–103
- Vidal, M. (2010) The dark side of fly TNF: an ancient developmental proof reading mechanism turned into tumor promoter. *Cell Cycle.* **9**(19), 3851–3856