Microenvironmental cues in cancer stemness

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Intra-tumour heterogeneity manifests at the level of mutational burden, but also at a functional level within genetically homogenous populations. An innovative modelling approach suggests that stemness within colorectal tumours is defined by microenvironmental cues secreted from cancer associated fibroblasts rather than cell intrinsic properties.

It is well accepted that tumours display extensive cellular and molecular heterogeneity, making it cumbersome to identify cancer stem cells (CSCs) that sustain tumour growth[1,2]. Adult stem cells and CSCs often share molecular and growth features, which has permitted better characterisation of CSCs in multiple malignancies[3]. Previous studies have adopted an *Lgr5*$^+$ stem cell-centric model to describe the growth dynamics of colorectal cancer (CRC)[4,5], and have demonstrated that intestinal adenomas retain cellular hierarchy[6,7]. Furthermore, several studies have postulated the potential utility in targeting the *Lgr5*$^+$ CSCs in colorectal cancer (CRC) patients[8,9]. In this issue of *Nature Cell Biology*, Lenos et al take an unbiased approach to model growth of colorectal tumours and determine that markers of stem cells, such as *Lgr5*, and stemness are not one and the same[10]. The authors further define that the tumour microenvironment is able to dictate stemness in CRC models and that tumours grow from the edge rather than via ubiquitous expansion of the tumour bulk.

Through the combination of mouse models and organoid xenografts, targeted ablation of *Lgr5*$^+$ cells in CRC has been shown to be sufficient to perturb tumour growth[4,5]. However, following cessation of *Lgr5*$^+$ cell depletion, tumour expansion is immediately reinstated, which reveals *Lgr5*$^+$ cells as a major contributor to tumour growth and demonstrates the impressive plasticity within CRC. The latter phenomenon indicates that in addition to cell intrinsic mutational burden, cell extrinsic cues dictate tumour cell fate and stemness. The findings in the current study indicate that cells positive for “stem cell markers” take a back seat, and instead the environmental cues provided by surrounding stromal cells confer stemness and drive tumour cell plasticity.

Lenos et al use a marker-free tamoxifen inducible labelling system to stochastically label tumour cells in mouse xenograft models, employing both a traditional two-dimensional cell line and two patient derived spheroid lines. The sizes of individual labelled clones were evaluated at various time points post induction, and the distributions of clone sizes over time were integrated into a stochastic model of tumour growth. This model was designed to infer whether tumour growth is inherently driven by cells at the extreme of an intrinsic hierarchical structure consistent with a CSC-centric model, or dictated by extrinsic factors consistent with a microenvironmental niche model.

These data indicated that all tumour lines when implanted subcutaneously into mice did not contain evidence of an intrinsic hierarchical organisation, and thus clonal out-growth was defined by environmental factors rather than cell intrinsic factors. The authors continued by approximating a cellular division rate of between 0.15 and 0.35 effective divisions per day for clonogenic cells and defining that the vast majority (>98%) of all clonogenic cells were housed toward the edges of the tumour. This fundamental growth dynamic of CRC was mimicked by orthotopic implantation of xenografts into the caecum, indicating the subcutaneous microenvironment is not driving a unique growth dynamic of CRC xenografts.

In these models, the majority of proliferative cells (marked by Ki67) were located in the outermost 300µm of the tumour; a feature that the authors demonstrated was consistent with that of primary human CRC. This characteristic tumour proliferation at a holistic level is consistent with a model of surface tumour growth, and accurately predicted the rate of macroscopic tumour expansion. The authors conclude that CRC grows from surface expansion while the central bulk of the tumour contributes little to tumour growth (Figure 1), which is supported by another recent publication[11].

It has previously been reported that environmental cues are able to dictate CRC cell fate in xenograft models[12]. The authors employed an innovative enzymatic digestion technique to separate the outermost areas of the tumour bulk from the core region. RNA sequencing comparing the two populations revealed, as expected, enrichment for genes associated with proliferation at the tumour edge. Notably, no enrichment for stem cell signatures or difference in the proportion of Lgr5+ cells was detected in either population, and this was confirmed by RNAish and immunohistochemistry for stem cell markers. These data highlight the discrepancy between CSC marker expression and functionality.

The importance of the microenvironment was again highlighted by complete plasticity within the tumour bulk. The authors transplanted the tumour centre following enzymatic digestion back into mice and observed a rapid reinstatement of surface growth features. Furthermore, the centre tumour cells were functionally indistinguishable from the edge cells when compared using an *in vitro* clonogenicity assay. Thus, when the environmental cues are identical, tumour cell plasticity enables a proliferative state regardless of their origin. It appears that conclusions cannot be drawn between clonogenicity assays and contribution towards tumour growth rates. The authors demonstrated that time to tumour establishment (tumour volume >100mm^3) correlates with the measured clonogenicity of a cell population, where expansion following tumour establishment does not.

A correlation between Ki67$^+$ tumour cells and the distance to the nearest cancer-associated fibroblast (CAF) was observed, which prompted the authors to investigate the functional significance of CAFs and tumour epithelial growth dynamics. To test this, co-cultures were established, which revealed increased epithelial cell growth when cultured with CAFs. These findings were corroborated using conditioned medium from CAFs, indicating the importance of CAF secreted factors in regulating epithelial cell growth. To identify factors which may influence tumour growth distribution, the authors interrogated their RNA sequencing and identified Osteopontin (OPN) as the most highly expressed secreted factor in CAFs. Levels of OPN have previously been shown to be increased in solid malignancies including CRC[13,14]. Clonal analysis of OPN-overexpressing tumour cells after subcutaneous implantation, revealed that ubiquitous expression of OPN afforded increased proliferation to clones in the central tumour region and a decreased variation in clone sizes. Functionally,

OPN overexpression also resulted in increased Ki67 positivity in the central tumour regions which was not associated with a change in CAF distribution throughout the tumour (Figure 2).

Using the stochastic clone system, the authors determined that chemotherapy with a combination of oxaliplatin and 5-fluorouracil suppressed rate of tumour growth, but did not impact fundamental tumour growth dynamics. Treated tumours contained a higher proportion of cells containing “stem cell markers”, such as *Lgr5*, but nevertheless retained a surface growth dynamic. These analyses provide evidence that even though “stem cell markers” are increased during chemotherapeutic intervention, the mode of surface tumour growth is unperturbed. This surface growth characteristic is dictated by microenvironmental influences that are not targeted by current chemotherapeutics.

The authors provide compelling evidence that challenges the promise of directly targeting CSCs in primary CRC. This observation is partially supported by previous work demonstrating that *Lgr5*$^{+}$ cells have an enhanced ability to establish tumours in the liver and subcutaneous environments but are not required for primary tumour survival[5]. Combining these data with those of the current study advocates that the ability of a cell to establish a tumour and to drive tumour growth should be considered as independent cellular traits. Furthermore, an important follow-up study will be to determine whether, in a different tumour microenvironment, CAFs also influence metastatic seeding and tumour growth dynamics.

Lenos et al have elegantly described the growth kinetics and dynamics of colorectal tumours, and identified OPN as a secreted factor by which CAFs can dictate tumour cell fate. These data illuminate a previously underappreciated concept that stromal cues can influence tumour cell hierarchy and subsequently have profound effects upon the tumour mass as a whole. Importantly, this newly emphasised critical role for molecular determinants of stemness in established tumours shifts the focus from targeting tumour cell populations that express canonical stem cell markers more towards targeting the niche that provides tumours with their much-needed CSC lifeline.

1. Sottoriva, A. *et al.* A Big Bang model of human colorectal tumor growth. *Nat Genet* **47,** ng.3214 (2015).
2. Roerink, S. *et al.* Intra-tumour diversification in colorectal cancer at the single-cell level. *Nature* 1–6 (2018). doi:10.1038/s41586-018-0024-3
3. White, A. C. & Lowry, W. E. Defining the role for adult stem cells as cancer cells of origin. *Trends in Cell Biology* (2015). doi:10.1016/j.tcb.2014.08.008
4. Shimokawa, M. *et al.* Visualization and targeting of LGR5+ human colon cancer stem cells. *Nature* **545,** nature22081 (2017).
5. Melo, F. *et al.* A distinct role for Lgr5+ stem cells in primary and metastatic colon cancer. *Nature* **543,** nature21713 (2017).
6. Kozar, S. *et al.* Continuous clonal labeling reveals small numbers of functional stem cells in intestinal crypts and adenomas. *Cell Stem Cell* **13,** 626–33 (2013).
7. Schepers, A. G. *et al.* Lineage tracing reveals Lgr5+ stem cell activity in mouse intestinal adenomas. *Science* **337,** 730–5 (2012).
8. Junttila, M. *et al.* Targeting LGR5^{+} cells with an antibody-drug conjugate for the treatment of colon cancer. *Sci Transl Med* **7,** 314ra186–314ra186 (2015).
9. Gong, X. *et al.* LGR5-Targeted Antibody–Drug Conjugate Eradicates Gastrointestinal Tumors and Prevents Recurrence. *Mol Cancer Ther* **15,** 1580–1590 (2016).

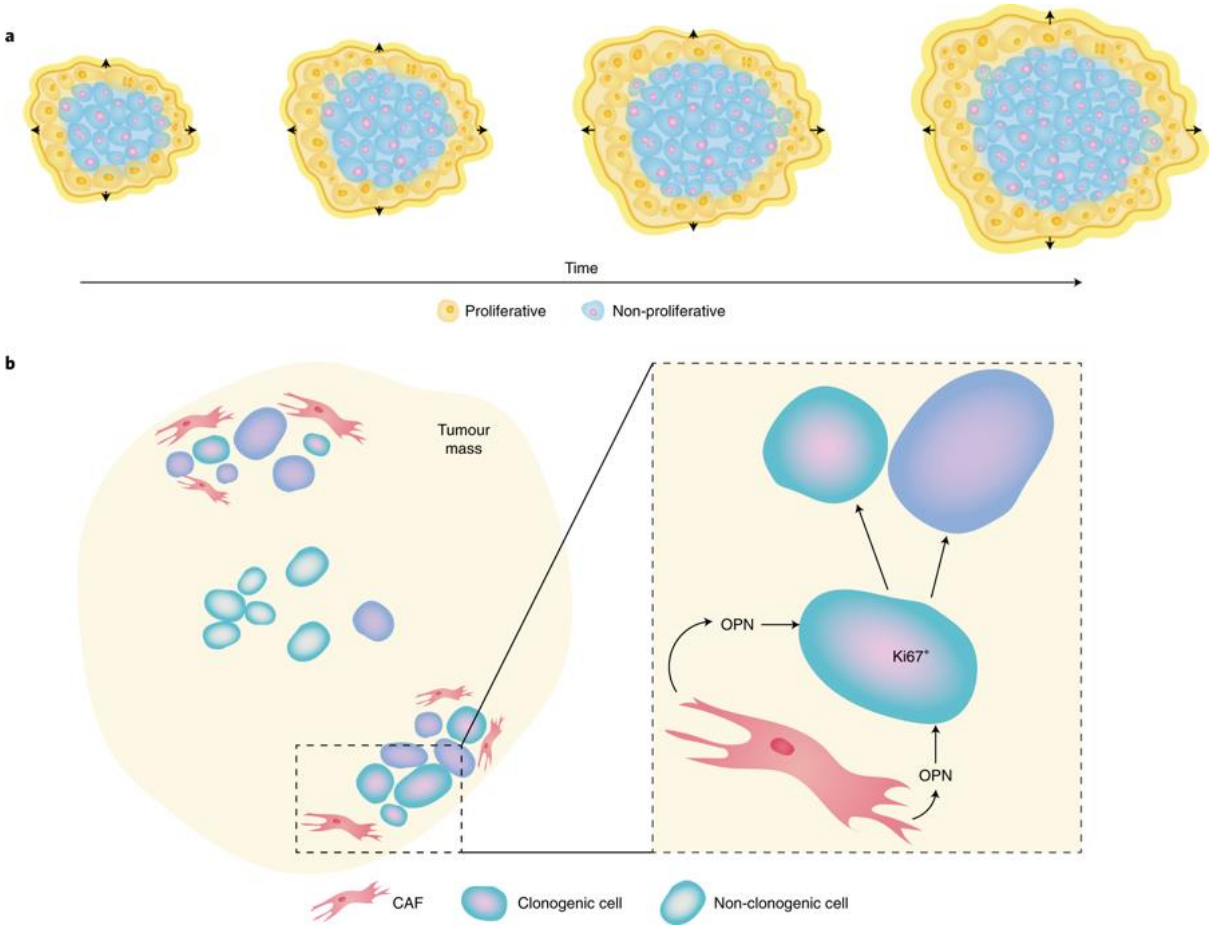
10. Lenos, K. et al. Stem cell functionality is microenvironmentally defined during tumor expansion and therapy response in colon cancer. *Nature Cell Biology* (2018).
11. Lamprecht, S. *et al.* Multicolor lineage tracing reveals clonal architecture and dynamics in colon cancer. *Nat Commun* **8,** 1406 (2017).
12. Vermeulen, L. *et al.* Wnt activity defines colon cancer stem cells and is regulated by the microenvironment. *Nat Cell Biol* **12,** 468–476 (2010).
13. Likui, W., Hong, W., Shuwen, Z., Yuangang, Y. & Yan, W. The Potential of Osteopontin as a Therapeutic Target for Human Colorectal Cancer. *J Gastrointest Surg* **15,** 652–659 (2011).
14. Wei, R., Wong, J. & Kwok, H. Osteopontin -- a promising biomarker for cancer therapy. *J Cancer* **8,** 2173–2183 (2017).

Figure legend

Figure 1. Cancer stem cells drive CRC expansion from the tumour edge.

(A) Schematic of CRC growth dynamics over time showing tumour expansion is driven from the tumour edge (black arrows) and not from the centre (blue circle).

(B) Schematic illustrating the relationship between cancer-associated fibroblasts (CAFs) and clonogenic cells (strawberry). Tumour cells located at the tumour edge are optimally placed to drive clonogenicity and tumour expansion, which is aided by secreted factors, such as osteopontin (OPN), from CAFs. Clonogenic cells in the tumour centre do not give rise to large clonal patches like the clonal cells situated at the tumour edge. The ability of a cell to give rise to progeny is promoted by its proximity to CAFs which are found mostly at the edges of the tumour. Inset shows CAF secreted OPN enhancing proliferation of Ki67+ of cancer cells.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