Manipulating the hypoxic tumour microenvironment to study therapy resistance

Summary

Cancer is the second leading cause of death globally according to the World Health Organisation. While survival rates are increasing there is still a high unmet need in many cancers for new treatment modalities and personalised treatment to further increase survival. New methods to overcome treatment-resistant tumours are vitally important in instances where the tumour becomes resistant to a particular therapy.

In **chapter 2** we review the current knowledge on the role of Notch in breast cancer. Notch is a cell-cell communication system that, depending on the context, can act as both an oncogene and a tumour suppressor. In breast cancer, there is overwhelming evidence that Notch plays a role in both development and progression with high Notch activity being associated with a more aggressive disease and poor patient outcome. While mutational changes in Notch are limited, expression, activity and cross-talk with other oncogenic pathways are found in many breast cancers. Furthermore, Notch has been shown to play an important role in the response to radiotherapy, chemotherapy, hormonal and targeted therapies. Critically, there is strong evidence that treatment-resistant breast cancers can be re-sensitised through inhibition of Notch, providing a rationale for combining Notch inhibition with current therapies.

Hypoxia is a common feature found in solid tumours arising from an imbalance between oxygen consumption and delivery in the tumour. The high proliferation and metabolic activity of tumours coupled with the inefficient tumour vasculature leads to areas of hypoxia within the tumour. Hypoxia is associated with worse outcomes in many different cancers regardless of treatment type. Notch and hypoxia have also been shown to influence several of the same pathways including parts of the metastatic cascade and neoangiogenesis. The Notch target gene DLL4 and the hypoxia-inducible factor (HIF) target gene VEGF are critical for the formation and maturation of new blood vessels. Previous research in our lab has shown a larger hypoxic fraction in Notch overexpressing tumours suggesting reduced functional vasculature or increased survival and adaptation of hypoxic cancers cells with increased Notch activity.

In **chapter 3** we extend this work to investigate how hypoxia influences Notch activity in this model and how this can affect Notch activity in distant cells. We use conditioned medium from these cells exposed to different oxygen conditions to see whether Notch signalling could be induced in reporter cells independent of cell contact. We find that hypoxic cells are able to upregulate Notch activity in recipient cells. Importantly this upregulation in activity can be abrogated through the use of Notch inhibitors. This points to a possible application of Notch inhibitors to reduce the hypoxic fraction of tumours, however, whether this holds true *in vivo* is unknown. Future research into the effect of Notch inhibitors on the hypoxic fraction of Notch expressing tumour cells and their microenvironment is therefore needed.

In **chapter 4** we develop a novel strategy to label hypoxic cells in a temporally controlled manner. We created an 'oxygen sensing' HIF1α-GFP-CreER^{T2} fusion protein which also incorporates temporal control through the ER^{T2} domain which translocates the protein

to the nucleus when 4-hydroxytamoxifen (4OHT) is present. We coupled this with a fluorescent reporter and transduced them into a H1299 non-small cell lung cancer model. We characterise this model both *in vitro* and *in vivo* showing permanent labelling of hypoxic cells only when 4OHT is added. We show that hypoxic cells can be visualised at single-cell resolution *in vivo* via intravital microscopy, and *ex vivo* via immunohistochemistry. Using this model we find a proliferative advantage for post-hypoxic cells *in* vivo when compared with unlabelled cells. This implies that hypoxia permanently alters the cellular phenotype and behaviour, but what causes this increased proliferation is still unknown. Future research should be aimed at finding the (epi)genetic and proteomic changes that cause this phenotype.

In **chapter 5** we refine this system further with the substitution of the CMV promoter for EF1α in the oxygen sensing construct to facilitate its use in cell lines where CMV silencing occurs. We coupled this with a reporter construct that also contains the Diphtheria toxin receptor protein allowing labelled cells to be selectively ablated upon administration of Diphtheria toxin. We characterised this system *in vitro* using the 4T1 murine metastatic breast cancer model, showing oxygen and 4OHT dependent labelling of cells which can then be selectively ablated with Diphtheria toxin. Next, we optimised the 4T1 cell line *in vivo* in an immunocompetent orthotopic model. We determined the hypoxic fraction at different tumour volumes to determine the timing and dosing of tamoxifen. Next, we optimised the dose of diphtheria toxin needed to kill all labelled cells within days after tamoxifen administration in tumours and determined the growth response of 4T1 tumours to single and fractionated radiotherapy. We show that hypoxic cells can be labelled through the administration of tamoxifen and can then be subsequently ablated with diphtheria toxin. Our hypothesis is that Diphtheria toxin will function as the 'perfect' drug or hypoxic cytotoxin and will sensitize tumours to RT treatment. Ongoing studies are investigating the benefits of hypoxic cell depletion with Diphtheria toxin and tumour control after radiotherapy.

In **chapter 6** we describe the generation of a novel knock-in mouse strain which can be used to lineage trace hypoxic cells. At present, there are no mouse strains that can identify hypoxic cells in vivo at the single-cell level. Such systems would be invaluable in our further understanding of the role of hypoxia in normal tissue development, homeostasis, tissue regeneration and in pathological processes such as cancer inflammation, tissue ischemia including cardiovascular and neurodegenerative diseases. To enable lineage tracing of HIF-1 activated cells throughout development and in adult mouse tissues we fused the c-terminal oxygen dependent degradation domain of HIF1a HIF-1α (aa 1-603)- to a eGFP-CreER^{T2} into theHIF1 locus in C57Bl/6 embryonic stem cells. We show that the ES cells that were used to generate the mice are able to report on hypoxia when a reporter was introduced. We obtained germline transmission of the HIF1-Cre-ER allele and obtained hemizygous mice that are viable and fertile. The generated mouse strain should provide a valuable tool to study hypoxic cell behaviour *in vivo* and in the assessment of hypoxia modification and targeting strategies.