Fibroblasts keep melanoma safe from harm

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The history of intravital microscopy is rooted in the study of cellular interactions in specific tissue environments. Imaging cells on glass coverslips is useful for mapping out basic features of signal transduction pathways, such as the regulation of the actin cytoskeleton by Rho-family GTPases. But the answers to many biological questions lie in the fine spatial and temporal details of signal transduction: “where” and “when” questions which can only be investigated in situ. This applies to diverse processes from T-cell / B-cell interactions within lymph nodes, to cancer-stromal cell interactions within tumors, to virtually all of embryonic development. More recently, intravital microscopy has found an important role to play in drug discovery, answering questions of where, when, and for how long drugs hit their targets at the tissue, cellular, and sub-cellular levels. This trend has been accelerated by the development of FRET biosensors which allow signal transduction to be imaged with high spatial and temporal resolution in pre-clinical cancer models.1

The development of B-Raf inhibitors provides an example of the promise and peril of targeted therapies, i.e. drugs designed to specifically interfere with only cancer cells.2 Around 50% of melanoma patients carry a mutation at V600, with the majority of these being V600E.3 Early clinical trials showed unprecedented improvements in overall and progression free survival of B-Raf V600E metastatic melanoma patients treated with the B-Raf inhibitor vemurafenib.4 These trials were accompanied by astonishing images of cancer patients riddled by metastatic melanoma being apparently cleared of their disease.4 Unfortunately, the benefits were short-lived and in most cases a form of melanoma returned which was completely resistant to the effects of the inhibitor. Intensive research has since uncovered several different mechanisms of acquired vemurafenib resistance, which generally involve B-Raf independent re-activation of the MAP-kinase pathway.

Now, the Sahai group have used a combination of intravital microscopy and 3-dimensional culture systems to uncover a new type of drug resistance which emerges through tumor-stroma interaction. They used a FRET biosensor for ERK kinase, the terminal kinase of the MAP kinase cascade (Fig. 1) to study the response of both cancer and stromal cells to inhibition of B-Raf, the first kinase of the cascade. Surprisingly, their work shows that an off-target effect of B-Raf inhibition is activation of melanoma-associated fibroblasts (MAFs), which maintain ERK activation within the melanoma cells despite B-Raf inhibition. The MAFs do this by increasing production of extra-cellular matrix, especially fibronectin, which re-activates Erk though melanoma signaling pathways downstream of β1 integrin.

The study begins with the simple observation that the B-Raf inhibitor PLX4720 impaired the growth of 2 mouse melanoma cell lines in vitro (5555 and 4434 cells), but did not retard the growth of the same cells grown as subcutaneous tumors. These melanoma cell lines were subsequently transfected with a nuclear version of the EKAREV FRET reporter,6 and the authors used intravital microscopy to assess the time course of Erk activation in subcutaneous tumors following daily PLX4720 treatment. They could show that Erk was effectively inhibited at 4 hours following the first treatment, however by 24 hours Erk activity had returned to pre-treatment levels and these “re-activated” cells were no longer responsive to drug treatment. Interestingly, small

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clusters of tumor cells which remained responsive to PLX4720 treatment were found, which co-localized with regions of low host-cell density. This suggested the possibility that resistance to Erk inactivation might result through interaction between tumor and stromal cells.

The authors next made use of 3-dimensional cultures (spheroids and tumor explants) grown in collagen gel. Such systems are highly useful for bridging the gap between simplistic but tractable cell-culture models and complex model organisms. Melanoma tumor explants grown in collagen, containing both tumor and stromal cells, remained refractory to PLX4720 treatment in contrast to spheroid monocultures, which responded by nuclear fragmentation. The function of stromal fibroblasts in conferring drug resistance was confirmed by culturing melanoma cell lines with either of 2 human melanoma associated fibroblast (MAF) cell lines (although normal fibroblasts could not fulfil this role). FRET imaging of EKAREV revealed that melanoma cells co-cultured with MAFs in the presence of PLX4720 regained Erk activity after 12 hours whereas monoculture spheroids did not. The targeted localization of the EKAREV probe to the nucleus facilitated elegant single-cell analysis, which captured the heterogeneity of Erk activity and drug response within strands of invasive cells. Interestingly, the regained Erk activity was sensitive to MEK inhibition, suggesting that re-activation of the MAP kinase pathway occurred downstream of Raf. Erk activity in the melanoma cells had thus become B-Raf independent within 12 hours of MAF co-culture.

In a critical experiment, the authors next introduced EKAREV into the MAFs, and unexpectedly found that Erk became activated in these cells following PLX4720 treatment (Fig. 1). This “paradoxical” activation was associated with increased contractility and collagen fibril production in organotypic cultures. Stiff polyacrylamide gels coated with fibronectin or a mixture of extra-cellular matrix components were also able to protect against death by PLX4720 treatment, demonstrating that the MAFs themselves were not required and ruling out protection through conventional paracrine signaling. Importantly, the protective effect of MAF co-cultures on PLX4720 treated melanoma cells was lost when β1 integrin or FAK were knocked-down in the melanoma cells. Similarly, FAK and Src inhibitors were effective at blocking re-activation of Erk signaling in spheroid co-cultures treated with PLX4720. FAK and Src are important mediators of β1 integrin signaling, which have not previously been implicated in B-Raf resistance and did not effect Erk activity on their own. Translation of this work back into mouse showed that the combination of B-Raf and FAK inhibitors slowed the growth of subcutaneous melanoma tumors much more effectively than either drug alone, suggesting future opportunities for clinical therapy.

The evolution of drug resistance is a major issue in cancer therapy. Resistance is thought to occur through a wide variety of mechanisms, but is underpinned by a process of continuous mutation arising through genetic instability. In this regard it is important that tumor cells not only survive, but proliferate in order to accumulate sufficient mutations to develop autonomous drug resistance. Could stromal protection contribute to the development of melanoma-autonomous resistance to B-Raf inhibition? To investigate this question Hirata et al. used 2 human melanoma cell lines (A375 and WM2666.4) which, unlike the original 5555 and 4434 melanoma lines, responded by growth inhibition to PLX4720 both in vitro and in vivo. Subcutaneous tumors grown from either of these 2 melanoma lines maintained a constant size or shrank somewhat during 10 – 15 d of PLX treatment. This led the authors to postulate that survival of cells within the residual tumor would be supported by signals from the stroma. Intravital imaging revealed that surviving tumor cells retained high levels of Erk activation despite PLX4720 treatment, and were indeed surrounded by a dense network of collagen fibrils. Furthermore, immunofluorescence of tissue sections revealed increased levels of fibronectin, tenasin-C, and active β1 integrin; all pointing toward higher levels of matrix-dependent signaling. As with 5555 and 4434 subcutaneous tumors, the combination of BRAF and FAK inhibitors more effectively inhibited the growth of subcutaneous A375 tumors than either drug alone. Crucially, when melanoma cells were isolated from the drug-resistant tumors and grown as monolayers they regained sensitivity to PLX4720 treatment, demonstrating that drug resistance had not yet become cell autonomous.

Figure 1. The MAP kinase cascade promotes proliferation within melanoma cells. PLX4720 inhibits signaling from B-RAF, but paradoxically activates production of extra-cellular matrix (especially fibronectin) in melanoma-associated fibroblasts. This leads to re-activation of the MAP kinase pathway in melanoma cells downstream of RAF via activation of β1 integrin. The activity of Erk, the terminal kinase of the pathway, is read out using the FRET reporter EKAREV.
Interestingly, however, Ki67 staining showed that PLX4720 treatment did not inhibit proliferation in the residual tumor mass. This suggests that the residual drug resistant tumor cells remained proliferative, and therefore capable of generating and accumulating mutations over time.

In summary, this work highlights the power of FRET reporters in combination with intravital microscopy to interrogate pharmacodynamic responses in pre-clinical disease models. This approach can reveal unexpected, even paradoxical, side effects such as the opposite response of signaling pathways in different cell types to the same drug. This work again highlights the importance of tumor-stroma interactions, which have been shown to impact many areas of cancer progression and therapy. Previously the ECM has been postulated to play a more passive role in limiting therapeutic efficacy, for example by limiting the perfusion of drugs into pancreatic tumors. Now, however, we have an example of the ECM playing a more active role in preventing drug response. Time will tell whether this is a unique example or the paradigm for a new type of drug resistance.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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