Preclinical Advances with Multiphoton Microscopy in Live Imaging of Skin Cancers

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Conventional, static analyses have historically been the bedrock and tool of choice for the study of skin cancers. Over the past several years, in vivo imaging of tumors using multiphoton microscopy has emerged as a powerful preclinical tool for revealing detailed cellular behaviors from the earliest moments of tumor development to the final steps of metastasis. Multiphoton microscopy allows for deep tissue penetration with relatively minor phototoxicity, rendering it an effective tool for the long-term observation of tumor evolution. This review highlights some of the recent preclinical insights gained using multiphoton microscopy and suggests future advances that could enhance its power in revealing the mysteries of skin tumor biology.

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INTRODUCTION

Static analyses of tissue specimens have long laid the groundwork for the study of skin cancers. In the past decade, significant preclinical advances have been made toward imaging of skin tumors in a live animal (intravital microscopy), where even highly dynamic cellular behaviors can be captured (Li et al., 2012; Pineda et al., 2015; Rompolas et al., 2012; Uchugonova et al., 2011). Moments from the earliest stages of tumor formation, starting with just a few cells, to rapid cell invasion into a blood vessel have been observed and have yielded novel insight into the temporal and spatial aspects of tumor biology.

IMAGING CANCER USING MULTIPHOTON MICROSCOPY

Live tumor imaging in preclinical animal research is most often accomplished with multiphoton laser scanning microscopy (MPM) because of deep tissue penetration and low phototoxicity (Condeelis and Weissweder, 2010). MPM relies on the simultaneous excitation of a fluorophore by two or more photons (Denk et al., 1990). This is achieved with tunable lasers that emit pulsed packets of photons of near-infrared wavelengths. As excitation is accomplished with more than one photon, the energy of each photon is lower (by a factor of N, where $N = 2$ for two-photon and $N = 3$ for three-photon excitation). The longer wavelength of the low-energy photons allows deeper tissue penetration. Although confocal microscopy can image effectively at 100–200 μm depending on the tissue, MPM can image at depths up to 1,000 μm (sufficiently covering epidermis and dermis) (Hoover et al., 2013). Because the excitation laser light is pulsed and focused by the objective, the density of photons sufficient for the simultaneous absorption of two photons by fluorophores is present only at the focal point. For these reasons fluorophore excitation is rare outside the plane of focus, and as a result there is considerably less background fluorescence and phototoxicity within tissue above or below. These features together allow for excellent optical sectioning, that is, the collection of fluorescent emissions emanating from only the focal plane.

This is in sharp contrast to confocal microscopy using conventional lasers. Here every photon along the entire excitation light path is sufficient for fluorescence, including above the plane of focus. Optical sectioning with confocal microscopy is instead achieved using a pinhole to exclude scattered light that is unlikely to derive from the plane of focus. However, confocal optical sectioning becomes compromised at greater tissue depths because the assignment of the position of fluorescent emissions becomes less accurate and the collection of emitted light inefficient. For these reasons, MPM is the preferred technique for intravital time lapse imaging, especially at deeper tissue levels where the quality of optical sectioning is superior.

Under MPM, tumor cells can be visualized using genetically encoded fluorescent reporters or endogenous fluorophores such as the cell metabolites reduced nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD) (Roberts et al., 2011). There are endogenous fluorophores found in the skin, including keratin and melanin (Breunig et al., 2012; Krasieva et al., 2013). However, most of these give off fluorescence that is too weak to be detectable with typical imaging parameters used in time lapse intravital imaging. The notable exception is the autofluorescence of hair, which is quite robust. In the tumor microenvironment, MPM can detect collagen via second harmonic signals. Highly organized noncentrosymmetric molecular arrays with a fibrillar structure, such as collagen bundles, can produce second harmonic generation, a type of combinatorial energy return that emits photons at double the

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frequency of the absorbed photons. Second harmonic generation is advantageous in that it does not require exogenous dyes or reporters, and it has been successfully used to image cancer remodeling of collagen in the extracellular matrix (Friedl et al., 2007).

Commercially available injectable fluorescent dyes (e.g., FITC-dextran or quantum dots) can also be used to label the lumen of blood vessels or mark phagocytic innate immune cells such as macrophages (Wyckoff et al., 2007). Genetically modified reporter mice can mark cells that transcribe particular genes, such as the CX3CR1-GFP strain that identifies monocyte lineage cells including macrophages and Langerhans cells (Figure 1a, and see Supplementary Movie S1 online). Thus, a rich combination of fluorescent tools is available for visualizing interactions between tumor cells, wild-type cells, and the tumor microenvironment. Furthermore, in the past several years, photoactivatable or photoswitchable fluorescent proteins have been engineered, whereby exciting a cell with a specific wavelength of light will induce its engineered proteins to fluoresce or change its emission wavelength (Rompolas et al., 2016; Welman et al., 2010). This enables the tracking of preselected cells over time.

MECHANISMS OF TUMOR GROWTH

The initial events in the development of human cancers remain a mystery and a ripe area for investigation, especially given the recent discovery that physiologically normal skin harbors surprisingly high numbers of mutations including oncogenic drivers (Martincorena et al., 2015). What, in addition to mutational burden, is required to drive the birth of tumors from normal skin? To answer this question, the ability to observe tumors from their earliest stages when they were just one or a few cells large would be essential.

Through continuous live imaging of BRAF and p53 mutated zebrafish, Kaufman et al. (2016) accomplished this feat and found that the key step to initiating melanoma was transformation into an embryonic progenitor state marked by Crestin activity. This kind of embryonic reprogramming has also been shown to be important for basal cell carcinoma growth (Youssef et al., 2012). Because Crestin is found only in zebrafish, it remains to be seen whether a similar phenomenon holds true in mice and humans. To conduct these imaging experiments in mice, however, would most likely require MPM as opposed to the confocal microscopy that was used. Although confocal microscopy is highly suited for imaging zebrafish because of their small size and translucent skin, it often does not achieve sufficient depth penetration into mammalian skin while avoiding phototoxic damage.

Once a cell becomes tumorigenic, it will give rise to clones that will inevitably encounter neighboring wild-type cells. An intriguing question is *What results from this interaction?* The classic paradigm is that tumor-promoting genes convert tumor cells into "supercompetitors" that can induce apoptosis in wild-type cells, as seen in overactivation of the Wnt/β-catenin pathway (Wagstaff et al., 2013). The Wnt/β-catenin pathway is a common cause of pilomatricoma, colon and liver cancer, and a growth pathway for basal cell carcinoma (Kajino et al., 2001; Youssef et al., 2012). Using MPM to observe Wnt-induced tumors in the hair follicle, Deschene et al. (2014) found that surprisingly, mutant cells caused their wild-type neighbors to proliferate by secreting Wnt ligands, thus fueling collective tumor growth (Figure 1b and Supplementary Movie S2 online). It remains to be seen what implications this has for cancer treatment, because drugs designed to target mutant cells may not be effective against wild-type cells that can form parts of a tumor. This would be necessary if these wild-type cells could continue to spread Wnt signaling non-cell autonomously in the absence of mutant cells.

MECHANISMS OF INVASION AND METASTASIS

Most cancer patients who die succumb to complications of metastasis (Fein et al., 2013; Patel et al., 1978). A thorough understanding of the mechanisms by which tumor cells escape from their site of origin is thus critical. Traditionally, studies are done by injecting mice with tumor cells and harvesting organ tissue to look for metastases. What dynamic behaviors occur in between—how tumor cells invade tissue and disseminate—is largely unknown. For example, why do only 0.01% of melanoma cells released into the circulation form metastases (Luzzi et al., 1998)? With the advent of live imaging technologies, it may be possible to capture the cell movements and interactions that are critical to tumor dissemination.

Cancer cells have been known to be slow moving, requiring focal adhesions to collagen fibers for their movement, and they secrete metalloproteinases to invade the extracellular matrix. However, when metalloproteinase inhibitors were found to be clinically ineffective (Overall and Kleifeld, 2006), it was discovered via MPM studies that tumor cells could convert to a fast, amoeboid-like movement pattern without reduction in migration rates (Wyckoff et al., 2006). In fact, most melanoma cells adopt this movement pattern in vivo, preferring to migrate along existing open tracks in the extracellular matrix rather than remodel it via protease activity (Clark and Vignjevic, 2015). When both types of movement patterns were blocked with their respective inhibitors, there was synergy in reducing cancer cell motility and invasion (Sahai et al., 2003). Thus, MPM microscopy not only provides sufficient resolution to detect key differences in cell movement behaviors but can also be used to evaluate the impact of preclinical drugs.

Live imaging has also shown the intricate steps involved when a cancer cell attempts to disseminate to distant organs. Melanoma cells, within minutes upon arrival at lung capillaries, will shed parts of their cytoplasm that can independently migrate along vessel walls, instituting a metastatic niche (Headley et al., 2016). In contrast, in the brain, they will first extravasate at a vascular branching point and then develop lamellipodia-like fingers to wrap around endothelial cells, creating capillary loops to fuel their proliferation (Kienast et al., 2010). Through imaging revisits over days, it was found that micrometastases that ultimately regressed were ones in poorly vascularized areas, where few available brain vessels could be co-opted. Melanoma metastases were not observed to induce angiogenesis in the brain, helping to explain why anti-vascular endothelial growth factor inhibitors were ineffective in controlling their growth. It is remarkable how live imaging has been able to show the different mechanisms melanoma cells use to colonize