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AUTHOR'S VIEWS

Connect-four: genomic analyses of regenerating stem cells identifies zygotic Dux factors as tumor initiators

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ABSTRACT

How, if and in which cell types embryonic gene expression programs are elicited to induce tumor formation remains poorly understood. Through genomic analyses of regenerating, p53 deficient muscle stem cells we identified various oncogenomic amplifications, including but not limited to, the zygotic transcription factor *Duxbl*/*DUXB* to initiate tumorigenic transformation.

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A central challenge in cancer biology is the characterization of the 'cancer-cell-of-origin', i.e. the first cell that transforms and acquires sufficient capabilities to propagate into a tumor. Notably, it is commonly thought that progressive accumulation of genomic alterations and/or mutations are needed for neoplastic transformation.[1] The hypothesis has been put forward that tissue-resident stem cells (SC) are more susceptible, due to their extended lifespan, to acquire oncogenomic lesions that would transform them to cancerous SCs. The inherent capacity of regenerative SCs to self-renew and proliferate, together with the observation that cancer often occurs in tissues with high regenerative capacity, supports this hypothesis.[2]

The adult skeletal muscle provides a paradigmatic example of tissue regeneration which is mediated by and dependent on rare *Pax7* expressing muscle SCs (also known as satellite cells).[3] Under physiological resting conditions, muscle SCs are predominantly quiescent but become activated upon regenerative cues such as an inflicting injury or during chronic regeneration in certain muscle diseases. Mice harboring an inactivating mutation in the gene *dmd* encoding for Dystrophin (known as mdx mice) mimic certain features of Duchenne muscular dystrophy including persistent and progressive muscle degeneration of mature myofibers. This elicits a constant regenerative pressure on the skeletal muscle and results in continuous activation of muscle SCs contributing to *de novo* myofiber formation.[4] Recently, it was shown that germline inactivation of the tumor suppressor protein *Trp53* (*TP53* in humans, best known and hereafter referred to as *p53*) in chronically regenerating mdx mice develop fusion-negative rhabdomyosarcoma (RMS),[5,6] a rare and aggressive childhood cancer and the most common soft-tissue sarcoma in children and adolescents.[7] The cancer cell-of-origin under these settings is still unknown but we reasoned that muscle SCs could be a cellular origin of RMS due to the constant regenerative pressure on the muscle SC compartment in mdx mice. To test this we set up an inducible strategy coupling fluorescent lineage tracing and p53 deletion specifically in muscle SC in the mdx background (these mice are referred to as $SC^{p53/mdx}$). In our model, all $SC^{p53/mdx}$ mice developed lineage-traced embryonic rhabdomyosarcomas (eRMS) in, or in immediate proximity to, the musculature of extremities clearly indicating the cancer cell of origin to be Pax7 expressing muscle SCs in these animals. Contrary, wild-type, mdx or SC^{p53} mice never developed tumors, which led us to conclude that muscle SC-specific loss of *p53* in a regenerative environment is sufficient to generate RMS, or conversely, that a regenerative environment enables RMS formation upon muscle SC-specific loss of *p53*. Importantly, fluorescent lineage tracing of muscle SC through activation of a $Rosa26^{Tomato}$ reporter enabled FACS-based separation of tumor propagating cells (TPC) from the primary tumors, i.e.: lineage traced muscle SC successors that had transformed and were *p53* deficient (TPC^{TOMpos}) and non-lineage traced cells (TPC^{TOMneg}) that essentially contained non-recombined, intact *p53*. Only TPC^{TOMpos} but not TPC^{TOMneg} developed secondary tumors when injected into immunocompromised mdx-nude mice further confirming that "genuine" RMS-forming cells originate from muscle SCs. These data additionally disclosed that the (non-traced) stromal cells in fact are not responsible for tumor initiation nor are they capable of forming tumors in secondary recipients (Figure 1(a)).

We found that *p53*-deficient muscle SCs show increasing rates of DNA double-strand breaks which prompted us to search for recurrent genomic alterations in purified TPC^{TOMpos} that would point to causal mutations playing a role in tumor formation. Surprisingly however, the mutational load in TPC^{TOMpos} was astonishingly low indicating that a progressive accumulation of mutations in muscle SCs is likely not the cause of tumorigenic

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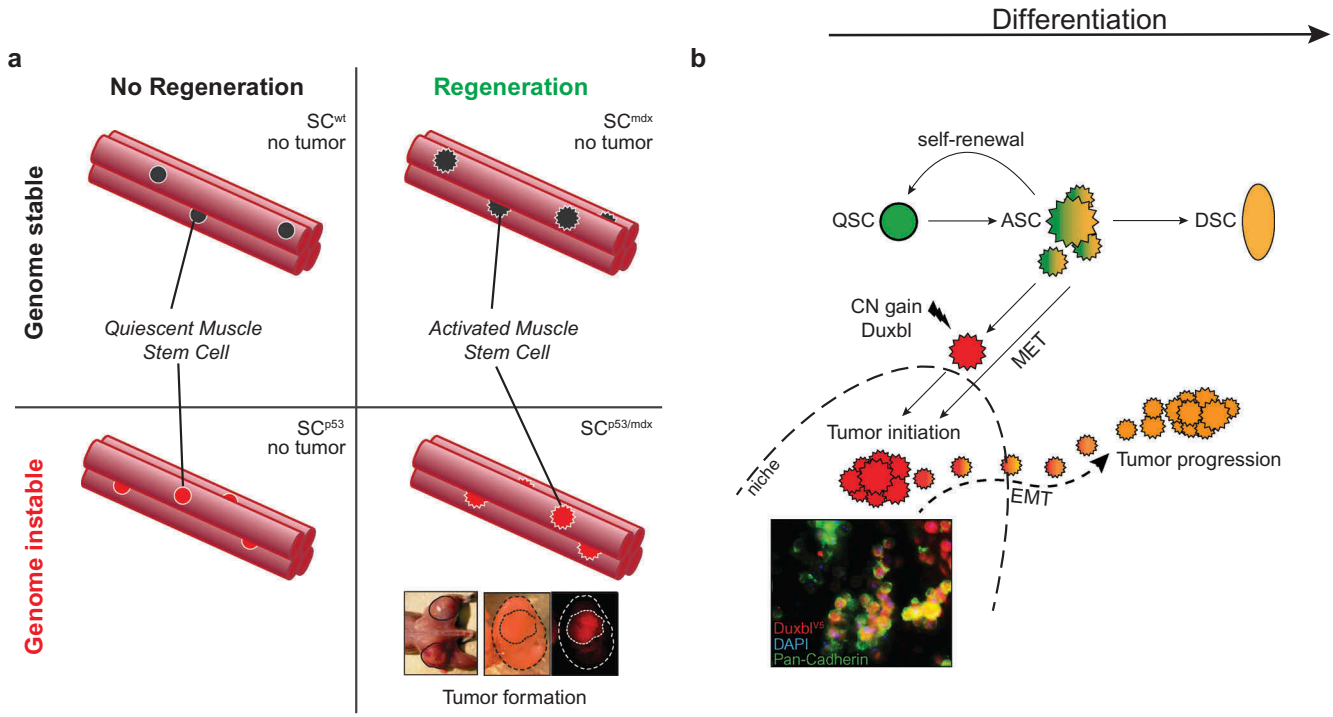

Figure 1. Interplay of stem-cell dependent tissue regeneration and genomic instability in tumor formation. (a) Relationship of regeneration and genomic instability in rhabdomyosarcoma (RMS) formation from muscle stem cells (SC). Both muscle regeneration and genomic instability are necessary to induce RMS tumors originating from muscle SCs. (b) Model of *Duxbl/DUXB*-mediated tumorigenesis. Healthy SCs contribute to muscle regeneration by differentiation of activated SCs upon injury. Copy number gain or expression of *Duxbl/DUXB* in activated SCs suppresses differentiation and promotes a gain of plasticity accompanied by epithelialization and initiation of tumorigenic colonies (inset). A secondary event likely involving EMT enables outgrowth of tumor cells from the tumor colony. ASC, activated stem cell; CN, copy number; DSC, differentiated stem cell; EMT, epithelial-to-mesenchymal transition; MET, mesenchymal-to-epithelial transition; QSC, quiescent stem cell.

transformation. Instead, and in almost every individually analyzed specimen, we identified discrete genomic copy number (CN) amplifications many of which harbor genes of known mutational targets of eRMS including *Yap1, Cdk4, Met, Jun* and others. A subset of tumors revealed a more subtle but recurrent amplification of the poorly described genomic locus 14qA3. This region harbors the *Duxbl* gene which is synteny to its putative human ortholog *DUXB. Duxbl/DUXB* belongs to the homeobox-containing Dux family of transcription factors with human DUX4 as its founding member. Notably, DUX4, or the murine ortholog Dux, was recently shown to be responsible for driving gene expression signatures known as zygotic gene activation (ZGA) at the cleavage stage of totipotent zygotes.[8,9] These observations led us to test whether Dux transcription factors might act at a putative interface of stem cell potency and tumor formation. To this end, we overexpressed *Duxbl* in wildtype muscle SCs. Strikingly, this impaired myogenic differentiation and resulted in the emergence of immortalized and morphologically rounded clones (referred to as SC^{Duxbl}) prone to spontaneously form epithelial-like spherical aggregates, which is reminiscent of mesenchymal-to-epithelial (MET) transition. Intriguingly, we found that SC^{Duxbl} expressed the pluripotency factors *Sox2* and *Klf4* both of which are required for inducing the essential process of MET during reprogramming of somatic cells to induced pluripotent stem cells (iPSCs).[10] Transplantation of SC^{Duxbl} led to tumor formation upon subcutaneous transplantation in immunocompromised mice. Interestingly however, SC^{Duxbl} contributed to myofiber formation when injected directly into the strongly pro-differentiating environment of skeletal muscle. Thus, depending on the local microenvironment SC^{Duxbl} can adopt different cell fates. We concluded that overexpression of *Duxbl* confers a cellular plasticity via a MET-like process but a niche with constant low differentiation pressure is required to facilitate tumor initiation and colonization which could be provided in conditions of chronic injury (Figure 1(b)). The Dux-factor/ZGA axis appears to play an important role beyond RMS since we found this axis to be reactivated in over different 30 types of human cancer. From our study a unifying theme emerges indicating how genomic instability can trigger reactivation of zygotic gene networks through a low number of oncogenomic lesions in regenerating SCs, which in turn can elicit (re-)acquisition of SC plasticity features acting at a three-sided interface of SC potency, cancer formation and regenerative potential.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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