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Title: Nf1 loss and Extracellular Matrix stiffness: partner in crime
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INTRODUCTION

Neurofibromas are complex benign nerve sheath tumors, whose morbidity is mainly due to accumulation of fibrotic tissue in which $NF1^{-/-}$ Schwann cells (SCs) undergo uncontrolled proliferation, thus compressing nerves, organs and blood vessels. Plexiform neurofibromas (PNs) are a subset of neurofibromas for which there is no treatment, and complete surgical removal is often not feasible due to PN location, infiltration and size. PNs can progress to highly malignant sarcomas termed MPNSTs (malignant peripheral nerve sheath tumors), which are almost invariably lethal (1).

Neurofibromin (Nf1), the product of the *NF1* gene, is a Ras GAP controlling the GTPase activity of the p21Ras proto-oncogene. Thus, NF1 heterozygous cells display hyperactivation of Ras, which further increases when loss of heterozygosity (LOH) occurs at the NF1 locus (2). Thus, activation of Ras/Raf /ERK signalling in SCs is sufficient to make them more susceptible to proliferative signals provided by a $NF1^{+/-}$ niche (3). Nonetheless, the physiological response to Ras hyperactivation is cell-cycle arrest and/or senescence rather than transformation. Ras-mediated transformation of SCs probably relies on a step-wise process that integrates circuits of amplification signals from the local environment (4, 5).

The current view is that the tumorigenic proliferation of NF1 heterozygous SCs depends on an inflammatory milieu triggering a robust burst of mitogenic signals from both collagen-secreting fibroblasts and pericytes in which $NF1^{+/-}$ mast cells ($MCsNF1^{+/-}$) could play a pivotal role (6). $MCsNF1^{+/-}$ are present in large amount in neurofibroma microenvironment and have a central role

in sustaining chronic inflammation, cell proliferation and extracellular matrix (ECM) deposition leading to fibrotic tissue deposition (7). However, this model does not clarify the molecular networks required for providing SCs with the tumorigenic properties they have in PNs, such as the ability of growing in the absence of basal membrane binding, a high proliferative potential and a profound metabolic rewiring. To better understand the role of the $NF1^{+/-}$ niche in PN progression, we will put the scientific data collected in this research field in the wider conceptual framework emerging from the most recent studies in molecular oncology. Several lines of evidence mechanistically link fibrosis and cancer in the triad "wound healing/chronic fibrosis/ cancer" (WHFC) inspired by the old concept that "tumor is a wound that does not heal". This view is based on the observation that the same cell types, as well as the same soluble and matrix elements that drive wound healing also fuel chronic fibrosis and tumor progression via distinct signalling pathways (8). The final product of chronic fibrosis is an abnormal, fibrotic ECM, with specific settings of biochemical and bio-mechanical properties. The consequent dysregulation of mechanical homeostasis by new biomechanical forces generated by this anomalous ECM can drive tumor aggressiveness by inducing a mesenchymal-like switch in mutated cells so that they attain tumor-initiating or stem-like cell properties (9). Thus, while genetic modifications in tumor cells unquestionably initiate tumor growth, a dynamically evolving ECM might determine its progression by modulating the behaviour of both "initiated cells" and cancer-associated stromal cells (10). In this scenario, PNs may arise from the primary event of NF1 loss in SCs that become more sensitive both to mitogenic stimuli of growth factors provided by chronic inflammation and to the ECM-derived mechanical stress, potently committing cells to transformation through a complex orchestration of changes in the genomic landscape of SCs cells. Importantly, high mechanical stress in solid tumors can impede drug delivery and may drive tumor progression toward malignancy. Therefore, a better understanding of these processes might provide hints for the design of novel anti-neoplastic drugs and strategies.

THE DARK SIDE OF TISSUE REPAIR: FIBROSIS

Fibrosis is defined as the excessive accumulation of fibrous connective tissue (components of the ECM such as collagen and fibronectin) in and around inflamed or damaged tissue, which can lead to permanent scarring and organ malfunction (11). In physiological conditions ECM is a complex network of macromolecules that assemble into three-dimensional supramolecular structures (12, 13). This molecular meshwork maintains the hydration and pH of the local microenvironment and

regulates the availability of growth factors and cytokines, thus controlling growth, survival, motility and differentiation of cells by tuning the ligation to specific cell receptors (14-16). After tissue injury**,** inflammation prompts a reparative response called wound healing (WH) through the potent activation of macrophages and mast cells (MCs) (17, 18). WH substitutes damaged cells with a collagen-enriched ECM, thus creating a scar endowed with strong, elastic fibers aligned in a parallel fashion (immature ECM) whose contraction permits the closure of the wound. Repetitive or persistent injurious stimuli determine chronic fibrosis, thus prolonging inflammation and indefinitely triggering an aberrant WH that leads to the formation of mature fibrotic ECM known as a scar. Under these dyshomeostatic conditions, the equilibrium between the population of M2 type macrophages that promote fibrosis (and are pro-neoplastic in the tumor microenvironment) and M1 type macrophages that drive the inflammatory response and are anti-fibrotic (and tumor suppressive in a neoplastic mass) is unbalanced, with M2 macrophages prevailing (19, 20). Recent data indicate that also MCs are involved in all phases of WH/fibrotic cascade. MCs are chemoattracted by anaphylotoxins C3a and C5a of the complement system. Depending on the WH stage, MCs produce and secrete both tissue plasminogen activator (tPA) in its free and enzymatically active form and plasminogen, key components of the plasminogen/fibrinolytic system involved in fibrin homeostasis (*i.e.*, resorption of blood clots) and tissue remodelling (21, 22) (20). This suggests that MCs cooperate with macrophages in this process. Once a clot is created, MCs take avoid further fibrin accumulation by prolonging the bleeding time (23) and by secreting the tryptase–heparin complexes that degrade fibrinogen in order to avoid its excessive conversion into fibrin by thrombin (24). Mouse MCs express the thrombin receptor Par-1 (22) that triggers the release of fibrinogen-destroying tryptase–heparin complexes when the local concentration of thrombin is unusually high. In support of these data, some pediatric mastocytosis patients who have an excess of hTryptase-β+/ heparin+ MCs in their tissues have excessive bleeding of their skin and gastrointestinal tract (25, 26). These finding suggest an explanation of the concurrent association of neurofibromatosis type I and ulcerative colitis reported in still few but significant clinical cases being mast cells, implicated in the pathogenesis of both diseases (28). MCs act as scavengers of cell debris and, in conjunction with macrophages, help to generate new tissue through growth factors secretion (proliferation phase). In addition, MCs secrete the serine protease chymase that promotes both secretion and activation of TGF-beta, the main factor triggering ECM deposition, in different cell types (ECM deposition phase) (29, 30).

Under the potent stimulation of TGF-beta and of growth factors such as platelet-derived growth factor (PDGF), which are secreted by both macrophages and mast cells, and of tensional forces

exerted by the fibrotic ECM, fibroblast proliferate and differentiate into myofibroblasts. This population of stromal cells displays a high capacity to synthesize ECM components that exert further tensile forces (31-33). Therefore, myofibroblasts can promote the formation of large, rigid collagen bundles that mechanically strengthen and stiffen the tissue after their crosslinking by lysyl oxidases (LOX) enzymes, and eventually contribute to wound closure. Hyperproliferation of these stromal cells and of other cell types is a hallmark of aberrant wound healing sustained by chronic inflammation (34). In experimental models of renal, pulmonary and cardiac fibrosis, endothelial cells can switch to a mesenchymal phenotype, a process known as endothelial-mesenchymal transition (EndMT). It is possible to envision that also in PN microenvironment EndMT, together with epithelial-mesenchymal transition (EMT), can contribute to fibroblast accumulation, which in turn can generate mesenchymal cells that express the myofibroblast markers αSMA (smooth muscle protein A) and smooth muscle protein 22α. However, it is unclear whether functional myofibroblasts seen in fibrosis or cancer derive from epithelial or endothelial cells. Certainly, vascular endothelial cells have demonstrated considerable plasticity to generate other cell types during embryonic development and disease progression (35).

Among the signalling pathways that control transition to a mesenchymal phenotype, transforming growth factor-β (TGFβ) family signalling has a predominant role through the SMAD3/α-catenin axis. In response to TGFβ, SMAD3 induces expression and nuclear import of transcription factor MRTFs, and cooperates with MRTFs to induce myofibroblast differentiation, partly by inducing the expression of SNAIL2 (36, 37). In addition, β-catenin, which is released from cell-to-cell tight junctions, antagonizes the inhibitory effect of SMAD3 on the MRTF–SRF complex, which in turns increases αSMA expression. A variety of other cell types may be recruited and differentiate into myofibroblasts (38). For instance, in injured liver, both portal fibroblasts and hepatic stellate cells can generate myofibroblasts, depending on the site and type of damage. In the nerve, both interstitial pericytes and fibroblasts are supposed to be a major source of myofibroblast recruitment (39).

With the intent to explain the hyperproliferative phenotype of SCs in neurofibromas, Parrinello et al. proposed that $NF1^{-/-}$ SCs possess a lower ability to bind and wrap axons due to decreased expression of Sema 4F, one of the molecules involved in this process. In their mouse model, $NF^{+/-}$ nerves present a subtle axonal segregation defect that becomes evident in bundles constituted by C-fiber axons (unmyelinated axons) or Remak bundles. Postnatally, Remark bundles showed an increased number of unsorted axons, defined as unstable. According to this model, over time these

defects result both in disruption of the bundles and in inadequate Schwann cell–axonal interactions, leading to neuropathy. As expected, axonal damage triggers the rapid de-differentiation of Schwann cells, which revert to a progenitor-like state and enter cell cycle. Concomitantly, an inflammatory response initiates, with recruitment of macrophages and masts cells, which clear myelin and axonal debris and elicit a robust cytokine and growth factor stimulation of the stromal cells. The authors state that, while in normal tissues there is the activation of molecular mechanisms that silence the WH process, a self-sustaining WH loop is established in NF1 neurons, once the inflammatory response is elicited. It has been speculated that this "chronic inflammatory loop" depends on a continuous process of SC dissociation, de-differentiation and proliferation, together with infiltration of immune cells and ECM deposition (40, 6). $NF^{+/-}$ MCs fuel this "chronic inflammation", as Ras hyperactivation prompts multiple gain-of-function effects. $NF^{+/-}$ MCs over-express the c-Kit receptor, a key regulator of mast cell generation and bioactivity and therefore the number of MCs recruited at the inflammatory site in a c-Kit-dependent way is higher in NF1 patients compared to healthy individual (7). Moreover, c-Kit-dependent processes as cell proliferation, survival, migration and degranulation *in vitro* and *in vivo* are increased, as Ras is an effector of the c-Kit receptor (**Figure 1)**, (29, 41). Accordingly, knocking-down the c-kit gene in the hematopoietic differentiation lineage prevents tumorigenesis in a PN murine model (42). Transplantation experiments have shown that these aberrant MCs critically sustain tumour microenvironment through trophic support. Notably, the amount of secreted, pro-inflammatory cytokines is increased in $NF^{+/-}$ MCs (43). For instance, Yang et al. demonstrated that $NF1^{+/-}$ MCs secrete 2.5-fold higher levels of TGF-β than WT mast cells *in vitro* (42) (**Figure 2**). These data increasingly support the idea that $NF1^{+/-}$ MCs are critical effectors in the paracrine induction of neurofibroma pathogenesis.

It is now well established that MCs correlate with NF1-associated PNs, and several evidences suggest that they take part in the process of neurofibromagenesis. Tucker et al. examined the density and distribution of MCs within NF1- associated neurofibromas classified histologically as encapsulated or diffuse based on the presence or absence of the perineurium. They observed that mast cell density and distribution differentiate the two basic types of NF1-associated neurofibromas, and correlated with the invasiveness behaviour of PN (44). Coherently, $NF1^{-/-}$ Schwann cells over-express the ligand of tyrosine kinase receptor c-Kit, the stem cell factor (SCF), which is responsible of increased attraction of mast cells. Remarkably, Vincent Riccardi reported a

successful therapy after a long treatment with Ketotifen, a potent MC stabilizer. He described amelioration of neurofibroma-associated itching, pain and tenderness, and patients reported that there was a consistent improvement in a general sense of well-being, considering the decrease in the rates of appearance and growth of cutaneous neurofibromas (45). This evident benefit is consistent with recent reports accounting for a functional cooperation between MCs and axons in pain control. The proximity between MCs and neurons potentiates critical molecular cross talks that result in a synergistic contribution to the initiation and propagation of long-term changes in pain responses to chronic inflammation via intricate signal networks of neurotransmitters, cytokines and adhesion molecules (46). Sensory as well as autonomic (essentially sympathetic) nerves are present within the skin and influence a variety of physiological and pathological cutaneous functions. During inflammation or trauma, and particularly during WH, a significant increase in levels of neuromediators occurs (47). This effect is MC dependent and plays an important regulatory role; however, further studies are needed to mechanistically elucidate this functional connection.
In light of these observations, a therapeutic strategy based on MC stabilizers could be an advisable choice to tackle several NF1-associated symptoms, such as persistent itching, hypertrophic scars, congenital melanocytic nevi or autoimmune diseases, only citing few of them that might rely on MC deregulation (26). Vincent Riccardi was the first to propose important connections between Nf1 loss and excessive WH (48). Hence, knockout mice have shown fibroblast hyperplasia and increased collagen accumulation (49). In 2007, Miyawaki T et al. investigated whether wounds produced in the patients with NF1 produce keloid or hypertrophic scars. This study showed that the patients with NF-1 and PN, form scars without keloid or hypertrophic changes, whereas those with a solitary neurofibroma after surgery had a higher risk to develop hypertrophic scars (50). The following studies do not do not retrace the same path: Jussi Koivunen et al. found an equally effective epidermal WH between NF1 patients and healthy controls (49). In addition, dermal WH appeared to function normally in NF1 patients based on retrospective and follow-up study of biopsy scars, suggesting that neurofibromin is not a crucial WH regulator. Given these different report data, more evidences are required to connect the aetiology of PN and scar in a WH milieu.

Salgado et al. have functionally connected the Large/Giant Congenital Melanocytic Nevi, one of the NF1 hallmarks, with increased MC number and activity (51, 52). In addition, histological analyses of fractures in mouse models that recapitulate features of the clinical pseudarthrosis of tibia (CPT) showed invasion by fibrous and highly proliferative tissue; mean amounts of fibrous tissue were

increased upward of 10-fold in fractures of $Nf1^{-/-}$ mice compared to their wild type counterpart. In healed fractures, the increased proliferating, active ERK-positive cells were not osteoclasts but probably stromal cells, indicating that in CPT chronic inflammation stimulates their proliferation and that they seriously affect bone reconstitution in the fracture callus (53, 54). CPT is also accompanied by proliferative disorders under inflammatory conditions in arteries and veins. Two reports (55, 56) describe thick-walled arteries and veins with a small lumen within the fibrotic tissue near the pseudarthrosis site. The proliferation rate of the periarteriolar tissue underneath the wound was increased and proliferative cells appeared positive for active ERK, suggesting that Nf1 loss, chronic fibrosis and WH form a signalling system that alters tissue homeostasis independently of the kick-off stimulus. Yet, vasculopathies are frequent in NF1, first observed by Vincent Riccardi, vasculopathy is now well accepted as NF1 hallmark (57-60). Further work is required to mechanistically dissect and integrate these clinical and experimental observations. The most recent research on chronic fibrosis paves the way to reach this goal. Nancy Ratner's group published a computational analysis of the gene signature characteristics of the MC/SC network (61). This work represents a step forward in understanding the major pathways involved in the interplay between these cell types and suggested a model by which decreased type-I interferon signalling plays a central role in neurofibroma growth.

In spite of their interest, these data need a note of caution. In fact, in other tumour models leukocyte subsets other than MCs do infiltrate, including both myeloid- and lymphoid-lineage cells that do not meet the classical definition of cells involved in an inflammatory immune response. For instance, in pancreatic carcinoma cellular immunity is greatly influenced by cytokines and chemokines that derive from tumour cells themselves (for example, IL-6, IL-1, IL-10 etc.) or by immune cells different from MCs (IL-4, IL-5, IL-13, IFN-γ etc.), further enriching the complexity of the tumor microenvironment. The scenario is made more multifaceted by the discovery of new fibrogenic mesenchymal progenitor cells (MPCs) in the lungs of patients with idiopathic pulmonary fibrosis (IPF) suggesting the existence of fibrotic mechanisms that do not relate to inflammation (31, 62). Taken together, these evidences suggest the possibility that molecular mechanisms alternative to MC-dependent inflammation might sustain fibrosis also in PNs. Therefore, while there are substantial evidences that early inflammation is a risk factor for PNs development, the molecular mechanisms accounting for the profound genomic changes should be placed in a progression model initiated by the biological changes triggered by the first genetic lesion, the LOH of the Nf1 gene, toward malignant transformation, under the biomechanical pressure elicited by fibrosis.

THE FIBROTIC TISSUE AS A TUMOR INCUBATOR: NEW HINTs TO ENVISION PN DEVELOPMENT

Numerous clinical and pathological observations have established a clear relationship between inflammation, fibrosis, and cancer (8, 63). A first observation highlighted that tumours are likened to wounds that fail to heal (64, 65). In fact, tumour stroma exhibits some of the characteristics found in an unresolved wound (66) such as a 'stiffened' microenvironment. In the present paragraph, we will report new findings dissecting the role of ECM stiffness on cancer development following a rational tread linking fibrosis and PN onset.

Abnormal ECM deposition, remodelling and post-translational modifications of ECM components are today well recognized incubators for cancer development. For instance, skin fibrosis associated with recessive dystrophic epidermolysis bullosa leads to highly metastatic skin carcinomas (67); progressive lung scarring associated with IPF is a risk factor for lung cancer development (68). In liver, persistence of chronic inflammation has been associated with progressive hepatic fibrosis and the development of cirrhosis histologically characterized by destruction of liver tissue structure following a marked ECM accumulation. ECM stiffness is a prognostic factor of cirrhosis and correlates with hepatic adenocarcinoma onset (69). Coherently, YAP transcription factor expression and activity, one of the hallmark of the stiff ECM derived from the Hyppo pathway, has been identified in fibrosis of the lung (70), liver (71).

Tissue measurement of the elastic modulus (stiffness) using atomic force microscopy (AFM) has confirmed the observation that local ECM of developing solid tumors is generally enriched of thick collagen fibres with respect of their normal counterparts. Although tumors are a gathering of stiff and compliant regions, tumors harboring the stiffest regions were overall the most aggressive (72). In particular, patients with breast tumors containing the highest number of stiff regions within the stroma have the worst prognosis. Up to now, no study has been carried out to figure out whether ECM stiffness could represent a prognostic marker for growth or malignant transformation of PNs, despite the presence of a high number of stiff nodules in rapidly growing neurofibromas.

It is well known that a crucial event for cancer progression is the transition to a mesenchymal phenotype, forcing cells to undergo a global reprogramming toward the acquisition of structural and functional changes, including loss of cell polarity and tight cell-cell junctions. Very recent experimental studies provided the proof of concept that stiff ECM orchestrates a complex program for activation of a pro-wound repair state through continuous instructions to resident and invading immune and inflammatory cells and normal cell populations (i.e pericytes, resident stem cells,

fibroblasts) (31). The physiological counterpart of this program is activated in cells during WH and is responsible of a gene expression rewiring in the sense of an epithelial-mesenchymal transition (EMT), initiated and controlled by signalling pathways that respond to extracellular cues.
EMT is a biological routine that play essential roles during specific conditions, such as embryonic development. Its aberrant reactivation promotes cancer cell plasticity and fuels both tumour initiation and metastatic spread through a panel of transcription factors (EMT-TF) as Snail, SNAI226, ZEB1 and ZEB2, E47 (also known as TCF3), Krüppel- like factor 8 (KLF8) and Brachyury (73). Emerging data highlight the complexity of the process and pinpoint aspects that have been poorly explored (74, 75). Indeed, a high level of plasticity between epithelial and mesenchymal phenotypes has lately been proposed when cells displaying transient "hybrid" states were described (75). This program is kept under control by a feedback process. An epithelial cell must be permissive to undergo a full EMT: the mesenchymal end-stage can be achieved when different regulatory networks are fundamentally disturbed; this probably requires the disruption of more than one molecular circuitry. Indeed, the activation of SNAI1 or ZEB2 transcription factors disturbs homeostasis in some tissues, but if the cell is not fully permissive, EMT is unable to occur. EMT in cells is often observed when the expression of an EMT transcription factor is combined with an additional acquired oncogenic activity. For instance, in pancreatic ductal adenocarcinoma (PDAC) concomitant activation of TWIST combined with a mutant K-Ras may set off EMT through different signalling cascades. The tumor progression model of the pancreatic adenocarcinoma indicates that ductal cells are first sensitized to EMT by K-Ras hyperactivation (76, 77). The first evidence about a possible connection between Ras activation caused by neurofibromin loss and EMT dates back to 2010, when Arima et al. observed an increase in the mRNA and protein expression levels of EMT-related transcription factors in neurofibroma specimens and NF1-derived Schwann cells (78). In 2012 Beak et al. identified neurofibromin as a key mediator of epicardial EMT and in the generation epicardial-derived cells (EPDCs). Mutant epicardial cells transitioned more readily to mesenchymal cells *in vitro* and *in vivo* and loss of Nf1 caused increased EPDC proliferation and resulted in more cardiac fibroblasts (79). These data may explain the recurrence of cardiac hypertrophy, a phenotypic trait that is part of the spectrum of NF1-related diseases and in general of several other RASopathies, as Noonan Syndrome (80).
Although a mechanistic explanation functionally linking loss of neurofibromin and EMT has to be elucidated, inspired by PDAC model, it is possible to speculate that the double allelic mutation of *NF1* makes cells permissive to EMT, as it elicits Ras over-activation. Nonetheless, it has to be considered that loss of a RAS-GAP as neurofibromin compromises Ras inactivation only by

hampering the Ras-associated GTPase function, whereas the oncogenic mutations of Ras keep it into a constitutive and growth factor independent activity and therefore have a wider impact on the enzyme and on the transduction pathway that stems from it. Hence, it is reasonable to envisage that $NF1^{-/-}$ cells need an additional stimulus that cooperates with deregulation of Ras signalling to drive the gene reprogramming required for EMT; such a stimulus could come from a stiff matrix.
Cells sense and convert exogenous forces into signaling pathways through a mechanism termed mechanotransduction. The best understood mechanotransduction mechanism is initiated through ECM-dependent integrin activation and clustering, resulting in vinculin activation that promotes focal adhesion assembly, focal adhesion kinase (FAK) and paxillin phosphorylation, subsequent Rho–ROCK-dependent actin remodelling, and reciprocal actomyosin-mediated cell contractility. ECM stiffness and elevated epithelial mechanosignaling enhance growth factor receptor-dependent PI3K signalling to foster malignant cell behaviour. Meanwhile, tumor cells also generate high traction forces that disrupt cell–cell junctions, compromise tissue polarity, promote anchorage-independent survival, and enhance invasion through EMT (73).
A hallmark of EMT is the downregulation of E-cadherin to reinforce the destabilization of adherent's junctions. The EMT transitioning cells lose their association with epithelial cells and acquire an affinity for mesenchymal cells through homotypic N-cadherin interactions; these interactions are weaker than homotypic E-cadherin interactions and facilitate cell migration and invasion (74, 81). EMT also activates the expression of neural cell adhesion molecule (NCAM), another adhesion molecule that interacts with N-cadherin to modulate the activity of RTKs that are associated with it (82). NCAM interacts with the SRC family tyrosine kinase FYN to facilitate the assembly of focal adhesions, migration and invasion. This is interesting in the NF1 perspective, as NCAM has been found in non-myelinating SCs from normal nerves and overexpressed by SCs from patients with chronic axonal neuropathies and Schwannomas. By contrast, its expression was lower in MPNST, probably because SC identity is lost in favour of dedifferentiated, highly invasive cells. Many of the pathological conditions described up to now, express mesenchymal markers, as well as a stem-like molecular signature that has been associated with stiff ECM, to treatment resistance (83). The therapeutic experience on PDAC clearly teaches that fibrotic tissue is a real barrier for several therapeutic molecules. The picture is made more complicated by the EMT that already has profoundly changed the gene expression pattern of the cells toward pluripotency and this is the probably the reason why the trial with pirfenidone did not succeeded on patients with NF1. Indeed, the Weaver group clearly showed that TGF-β and other pro-fibrogenic growth factor

inhibition is ineffective on PDAC since the thick and mature Collagen fibers are already formed and biomechanically control gene expression of both tumour and stromal cells (76). However, new therapeutic strategies are under investigation, one of these has been suggested by Jian et al. (84). The authors show that in PDAC tumors immunotherapy associated to Focal adhesion kinases (FAK inhibitor) could be a successful strategy.

One of the hallmarks of cancer development is rewiring of cellular metabolism (85). It has long been postulated that cancer cells upregulate aerobic glycolysis in order to provide the building blocks necessary to rapidly proliferate (86). Recently, the role of the mitochondria as a biosynthetic "factory" for cancer cell proliferation has become more apparent (87), while accumulating evidences have shown a new interplay between biophysical forces and metabolism. According to this new model, Ras-dependent activation of PI3K/AKT signalling regulates the expression of glucose transporters, GLUT1 and GLUT4 (88), via hexokinase, thereby stimulating phosphofructokinase activity and increasing glucose uptake (89, 90), an action critical for enhanced aerobic glycolysis.
In parallel, matrix stiffness further potentiates this loop mediating PI3K activation of AKT via Fak kinase and Myc-dependent matrix stiffness-induced expression of miR18a, which inhibits PTEN expression and metabolism switch to glycolysis. In addition, both increased thickness and compromised vasculature, change tumor microenvironment and trigger a poor outcome predictor: hypoxia (91). Through activation of HIF-1, a potent oncogenic transcription factor, hypoxia acts to reinforce the Warburg effect, as HIF1α induces the expression of genes encoding glycolytic enzymes and glucose transporters. Metabolic reprogramming by Ras oncogene is carried out predominantly through the upregulation of hypoxia-inducible factor 1α (HIF1α), which forms the HIF transcription factor when bound to HIF1β and is well recognized for its ability to stimulate a glycolytic shift. Ras-induced concurrent activation of MAPK and PI3K effector pathways triggers the stimulation of mTOR activity and mTOR-mediated cap-dependent translation of HIF1α. RAS dependent upregulation of HIF1α has been implicated in enhancing both the transport and the glycolytic capture of glucose, as well as its processing to biosynthetic intermediates. Oncogenic RAS increases the transcription of the glucose transporter GLUT1 thus providing cells the increased ability to take up glucose. The oncogene regulates also the expression of glycolytic enzymes, such as hexokinase, phosphorfructokinase and lactate dehydrogenase. Given this multiplicity of metabolic changes governed by oncogenic Ras, it is possible to envisage the participation of other Ras-targets other than HIF1α, still unknown.

A fascinating interplay between Ras activity, HIF-α and metabolic changes in NF1 has been very recently unrevealed by Andrea Rasola's group. They found that the lack of neurofibromin induces a glycolytic phenotype and decreased respiration in a Ras/ERK-dependent way (92). The authors provide and interesting mechanistic model by which in the mitochondrial matrix a fraction of active ERK1/2 binds to and activates the mitochondrial chaperone TRAP1 that in turn inhibits succinate dehydrogenase (SDH), the respiratory complex II (93). Consequently, the resulting increase in intra-cellular oncometabolite succinate levels inhibits the prolyl hydroxylases responsible for dispatching HIF-1α to the proteasome for degradation (94).

All together, these findings indicate the existence of a crosstalk among NF1 loss-dependent signalling, ECM stiffening, EMT and metabolic interconnections, having a profound impact on tumor progression. Although additional studies are required to fully characterize the complexity of this signalling network, this new axis could represent a novel avenue to pursue in the search of the molecular cascades responsible for neurofibroma onset and development.

Figure legend

Figure 1

Kit tyrosine kinase receptor activation is essential for mast cell growth, differentiation and survival and for inducing mast cell migration/homing through chemotaxis. KIT catalytic activity and downstream signaling is initiated upon dimerization induced by binding of its specific ligand stem cell factor (SCF) or random collisions favored by receptor over-expression. The consensus of studies on mast cells suggests that downstream signaling generated by activated KIT alone are insufficient to induce degranulation but it cooperates with the high affinity for IgE receptor, FcεRI via Pi3K signaling that ultimately elicits PKC activation. In addition, following aggregation with FcεRI, the adaptor molecule LAT (linker for activation of T cells) becomes phosphorylated in a LYN- and SYK (spleen tyrosine kinase)-dependent manner. This results in direct or indirect binding to LAT of the cytosolic adaptor molecules PLC-g triggering degranulation. Kit receptor activation induces cytokine production and chemotaxis through Ras/Erk axis.

DAG, diacylglycerol; IP3, inositol-1,4,5-trisphosphate; PIP2 phosphatidylinositol-4,5-bisphosphate.

Figure 2

MCs Mast cells during wound healing govern both inflammatory and reparative pathway. MCs secreted histamine acts through its various receptors to induce smooth muscle contraction or relaxation, enhanced permeability across vascular endothelial cells, and itching or pain by activating sensory neurons. Production of a wide range of cytokines and chemokines promote adaptive immune responses (Th1 and Th2) and immunosuppression. The figure shows that in $NF1^{-/+}$ MCs both growth factors and cytokine secretion is increased under the control of an over-activated Kit tyrosine kinase receptor due to both increased ligand stimulation and random collisions.

Figure 3

Tumorigenesis is a complex program induced by the aberrant activation of different cellular processes. Extracellular Matrix (ECM) stiffness and oncogenic activation of Ras GTP-ase represent two hits able to induce a radical change in the gene landscape of the cells. Cells bearing oncogenic mutations, under ECM pressure respond with a profound gene expression renewal activating programs fundamental to orchestrate either the metabolic switch toward glycolysis or cell identity such as Epithelial-Mesenchymal transition (EMT) and Endothelial-Mesenchymal (End-MT) transition.

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