UNIVERSITÀ DEGLI STUDI DI PADOVA

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Searching for molecular mechanisms sustaining tumor formation and progression in Neurofibromatosis type 1

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To my Mom, Dad

Azadeh and Ali

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SUMMARY

Neurofibromatosis type 1 (NF1, OMIM # 162200), also known as von Recklinghausen, is an autosomal dominant disease caused by mutations of the *NF1* gene coding a 2818 amino acid protein, neurofibromin (*Nf1*). More than 900 different mutations in the *NF1* gene have been identified (HGMD, Human Genetic Mutation Database). Mutations of *NF1* gene cause a variety of clinical manifestations such as the optic gliomas, neoplasms of the haematopoietic system and learning disabilities. However, the hallmark of NF1 is the development of multiple benign peripheral nerve sheath tumors called neurofibromas. Neurofibromas are complex tumors arising from peripheral nerve sheaths and mainly composed of Schwann Cells (SCs) homozygous for mutated *NF1*, mast cells (MCs) and fibroblasts (FBRs) both heterozygous for the same mutation. The plexiform variety can progress to highly malignant sarcomas termed Malignant Peripheral Nerve Sheath Tumors (MPNSTs), which are almost invariably lethal.

Up to now, any effective therapy able to either reduces neurofibroma size and its incidence or to counteract its formation, has not been developed yet. The main feature of neurofibroma is a rigid structure due to massive deposition of collagen of different types by activated FBRs. These cells, named myofibroblasts (mFBRs), are massively stimulated by mast cell-secreting Transcription Growth Factor-Beta (TGF-Beta) to produce growth factors such as Platelet Growth Factor (PDGF), Fibroblast Growth Factor (FGF) and collagen. This leads to both potent SCs proliferation and deposition of rigid extracellular matrix (ECM).

Cells' haploinsufficient for *Nf1* display hyper-activation of Rat Sarcoma (Ras), which further increases when Loss of Heterozigozyty (LOH) of *NF1* occurs. Thus, the activation of Ras/ Rapidly Accelerated Fibrosarcoma (Raf) /Extracellular signal-regulated Kinase (ERK) signaling in SCs is sufficient to make them more susceptible to proliferative signals provided by a $NF1^{+/-}$ niche. However, the physiological response to Ras hyper-activation 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 niche. A major component of the niche is the ECM, a complex network of macromolecules whose the elasticity (ranging from soft to stiff and rigid),

contributes to development and cancer. ECM elasticity determines how a cell senses and perceives external forces and thus provides a major environmental cue that determines cell behavior. Indeed, the focal adhesion complex, which consists of integrins, multicomplex of adaptors and signaling proteins, can be viewed as a mechanosensor linking the actomyosin cytoskeleton with the ECM. How lack of *Nf1* may impact on the complexity of ECM-cell dynamic and how the great rigidity of the ECM in neurofibromas influences SCs' behavior, is still unknown. Among the three functional domains described in the *Nf1* protein, a Focal Adhesion Kinas (FAK) binding domain has been identified and *Nf1* has been shown to interact with FAK, paving the way for the enunciation of new hypothesis aimed to explain the route of SCs transformation toward cancer.

Rational: as in other tumors {Lu, 2012 #289}, {Yu, 2011 #292}), also in the plexiform neurofibromas, the tumorigenic phenotype of SCs is fostered by the amplification of integrated signaling pathways triggered by loss of *Nf1*, Ras hyper activation and deregulated ECM. Changes of the mechanical properties of ECM due to increased collagen secretion by mFBRs might actively contribute to tumor progression by influencing gene expression profile of the cells through the enhancement of Ras signaling pathway triggered by FAK. The deep investigation of these biological changes triggered in SCs by ECM formation is the goal of the present project.

Project Goals: To shed light into this issue, we intend 1) to generate a novel three-dimensional experimental model *in vitro* reproducing the multicellular complexity of neurofibromas with primary cells. Immortalized cells, indeed, are not suitable for our aims since the molecular oncologists consider the immortalization process as the first hit leading to tumorigenic phenotype, because of the changes which made for cell cycle control in gene expression 2) to assess the requirement ECM for SCs transformation in this new *in vitro* system identifying the proper ECM composition and stiffness in matrigel (structural and non structural components) required for neurofibroma's formation.

Results:

Isolation of primary SCs and FBRs from Neurofibromas and their biological characterization:

1) We have already isolated and cultured in 2D our SCs and FBRs $NF1^{+/-}$ according to Serra methodology {Serra, 2000 #210}. These cells have been isolated from plexiform neurofibroma biopsies after informed consent of patients by our Milan and Rome University collaborators.

To get two populations of SCs and FBRs we have cultured cells in selective Medium (according to {Serra, 2000 #210}, and our new unpublished protocol) and characterized them biochemically by: S100B {Tucker, 2011 #321} and p75 markers specifically recognizing SCs and collagen I secretion, alpha smooth muscle actin (α-SMA) expression, Smad2/3 activation, Abl kinase activation characterizing mFBR activity {Kojima, 2010 #150}.

2) We have already obtained colonies of SCs growing in 3D *in vitro* system as described in step 1 and 2 (in transwell-like chambers to permit autocrine stimulation between mFBRs and SCs). Our preliminary data show that primary SCs generate colonies only when plated in an ECM/reconstituted basement membrane Collagen I-Matrigel of at list 3 mg/ml. However, we have still to set up the culture conditions to keep cells in highly proliferating state.

Preliminary indications in immortalized Mouse Embrionic Fibroblasts (MEFs)

In other cellular models as in mouse FBRs $NF1^{-/-}$, we have found that the absence of Neurofibromin correlates with deregulation of FAK Y397 and Y925 phosphorylation both in absence of integrin clustering and after ligand stimulation. Further, the tumorigenesis assay showed that MEF$NF1^{-/-}$ability to form colonies is affected by both MECK inhibitor and FAK inhibitor (Y15) indicating the cooperative role of FAK and PDGFBB growth factor in the tumorigenesis process mediated by *Nf1*. Consistently, immunoprecipitation experiments showed that in *Nf1* null cells, Growth factor receptor-bound protein2 (Grb-2), the RAS pathway initiator, interacts with FAK also in absence of collagen in a PDGFBB ligand-dependent way, thus suggesting that FAK and growth factor receptors can cooperate to increase the Ras activity to a threshold required to induce tumorigenesis.

SOMMARIO

Neurofibromatosi tipo 1 (NF1, OMIM # 162200), nota anche come di von Recklinghausen, è una malattia autosomica dominante causata da mutazioni del gene *NF1* che codifica una proteina coi 2818 aminoacidi , detta neurofibromina (Nf). Più di 900 diverse mutazioni nel gene *NF1* sono state identificate (HGMD, Database di mutazione genetica umana). Mutazioni del gene *NF1* causano una varietà di manifestazioni cliniche quali il glioma ottico, neoplasie del sistema ematopoietico e disabilità dell'apprendimento. Tuttavia, il segno distintivo della NF1 è lo sviluppo dei tumori benigni nella guaina dei nervi periferici, chiamati neurofibromi. I neurofibromi sono tumori complessi originati da guaine nervose periferiche e costituiti prevalentemente da cellule di Schwann omozigote mutate per *NF1*, mastociti e fibroblasti entrambi eterozigoti per la stessa mutazione. I plessiformi possono progredire a sarcomi altamente maligni denominati MPNSTs (schwannomi maligni), che sono quasi sempre letali.

Ad oggi non e' ancora stata sviluppata alcuna terapia efficace in grado di ridurre la dimensione e incidenza dei neurofibromi, o atta a contrastarne la formazione. La caratteristica principale dei neurofibromi è la loro struttura rigida conseguente alla massiccia deposizione di collagene prodotto dai fibroblasti attivati. Queste cellule, denominate miofibroblasti, sono fortemente stimolate da mastociti che producono fattore di crescita trascrizionale-Beta (TGF-Beta) per produrre poi fattori di crescita, come fattore di crescita piastrinico, fattore di crescita dei fibroblasti e collagene. Ciò comporta sia la potente proliferazione di cellule di Schwann che la deposizione di matrice extracellulare rigida.

Cellule aploinsufficienti per *Nf1* comportano iperattivazione di Ras, che aumenta ulteriormente con LOH. L'attivazione di vie di segnale di Ras/Raf/ERK in cellule di Schwann rende le cellule più suscettibili ai segnali proliferativi forniti dalla nicchia $NF1^{+/-}$. Tuttavia, la risposta fisiologica a Ras iperattivato è l'arresto del ciclo cellulare e/o senescenza piuttosto che trasformazione. La trasformazione Ras-mediata di cellule di Schwann probabilmente si basa su un procedimento che integra diversi segnali dipendenti da circuiti di amplificazione della nicchia stessa. Uno dei più importanti componenti della nicchia è la matrice extracellulare (ECM), una rete complessa di macromolecole con plasticità variabile che contribuisce alla progressione tumorale. L'elasticità di

ECM determina la modalità con cui una cellula percepisce le forze esterne e quindi fornisce un importante spunto ambientale che determina il comportamento cellulare. In effetti le adesioni focali, che consistono di integrine, adattatori multicomplesi e proteine di segnale, possono essere visti come meccano-sensori che collegano il citoscheletro con la ECM. Come la mancanza di *Nf1* possa avere un impatto significativo sulla complessità di dinamismo di ECM-cellula o come la grande rigidezza dell'ECM in neurofibroma influenzi il comportamento delle cellule di Schwann, è ancora sconosciuto. Tra i tre domini funzionali descritti nella proteina, un dominio di legame, FAK, sulla proteina è stato identificato e *Nf1* ha mostrato di interagire con FAK, spianando la strada per l'enunciazione di una nuova ipotesi per spiegare il percorso trasformazionale delle cellule di Schwann verso il cancro.

Razionale: come in altri tumori {Lu, 2012 #289}, {Yu, 2011 #292}), anche nei neurofibromi plessiformi il fenotipo trasformato di SCs è favorito dall'amplificazione della segnalazione di percorsi integrati attivati sia da perdita di *Nf1*, Ras iperattivazione che deregolamentato di matrice extracellulare (ECM). Le modifiche delle proprietà meccaniche di ECM a causa dell'aumento di secrezione di collagene dai miofibroblasti potrebbe contribuire attivamente alla progressione del tumore, influenzando profili di espressione genica delle cellule attraverso la valorizzazione di segnale di Ras pathway generato dall'adesione focale (FAK). L'indagine in profondità di queste modificazioni biologiche attivate in SCs dalla formazione di ECM è l'obiettivo del presente progetto.

Al fine di far luce su questo argomento, abbiamo intenzione di 1) generare un nuovo modello sperimentale tridimensionale in vitro che riproduce la complessità di neurofibroma pluricellulari con le cellule primarie. Cellule immortalizzate, infatti, non sono adatte per i nostri scopi poiché gli oncologi molecolari consideranno il processo di immortalizzazione come il primo colpo che conduce al fenotipo oncogenico, a causa dei cambiamenti che sono stati fatti per il controllo del ciclo cellulare di espressione genica; 2) valutare l'esigenza di ECM nella trasformazione di cellule di Schwann cell (SCs) in questo nuovo sistema in vitro identificando la corretta composizione dell'ECM e rigidità in matrigel (strutturali e non strutturali) per formazione di neurofibroma.

Risultati:

Isolamento delle cellule di Schwann e Fibroblasti primarie da Neurofibromi e la loro caratterizzazione biologica:

1) Abbiamo già isolato e coltivato in 2D le nostre cellule di Schwann e Fibroblasti $NF1^{+/-}$ secondo la metodologia di Serra {Serra, 2000 #210}. Queste cellule sono state isolate da biopsie di neurofibromi plessiformi dopo aver consenso informato dei pazienti mediante i nostri collaboratori presso Università di Milano e di Roma.
Per ottenere due popolazioni delle cellule di Schwann e Fibroblasti abbiamo coltivato le cellule in terreno selettivo (secondo {Serra, 2000 #210}, e il nostro nuovo protocollo inedito) e caratterizzato dal punto di vista biochimico: S100B {Tucker, 2011 #321} e p75 marcatori che riconoscono specificamente le cellule di Schwann e secrezione del collagene di tipo I, espressione dell'actìna alfa del muscolo liscio (α-SMA), attivazione di Smad2/3, attivazione di abl chinasi e caratterizzare l'attività di myo-fibroblasti {Kojima, 2010 #150}.

2) abbiamo già ottenuto le colonie di cellule di Schwann cresciute nel sistema 3D in vitro come descritto nella fase 1 e 2 (in transwell-like chamber per permettere la stimolazione autocrina tra myofibroblasti e le cellule di Schwann). I nostri dati preliminari mostrano che le cellule primarie di Schwann generano delle colonie solo quando piastrate in una ECM/ membrana basale ricostituita del collagene di tipo I di Matrigel di almeno 3 mg/ml. Tuttavia, dobbiamo ancora impostare le condizioni migliori di cultura per mantenere le cellule altamente proliferanti.

Indicazioni preliminari in fibroblasti Embrionali immortalati Murini (MEFs)

In altri modelli cellulari come nel fibroblasti *NF1*$^{-/-}$ murini (MEFs), abbiamo trovato che l'assenza di neurofibromina scorrela con la deregolata di fosforilazione del FAK in Y397 e Y925 sia in assenza di raggruppamento di integrine che dopo la stimolazione con ligando. Inoltre, il saggio di tumorigenesi mostrava che la capacità di cellule di MEF*Nf1*$^{-/-}$ di formare colonie è influenzata sia da inibitore di MECK che inibitore FAK Y15 indicante il ruolo di cooperatzione di FAK e PDGFBB, fattore di crescita, nel processo di tumorigenesi mediato da *NF1*. Coerentemente, gli esperimenti di immunoprecipitazione hanno mostrato che in cellule null *NF1*, il recettore del fattore di crescita di proteina legata2 (Grb-2), l'iniziatore di via di segnale di RAS, interagisce con FAK anche in assenza di collagene in un modo PDGFBB ligando-dipendente, suggerendo così che FAK e recettori di fattori di crescita possono cooperare per aumentare l'attività di Ras con un valore di soglia necessario per indurre la tumorigenesi.

1. INTRODUCTION

1. INTRODUCTION

1.1 Neurofibromatosis Type 1

Neurofibromatosis type 1 (NF1, OMIM # 162200), known as von Recklinghausen, an autosomal dominant disease, is also sometimes referred to as 'peripheral neurofibromatosis' caused by mutations of the *NF1* gene coding a 2818 amino acid protein, neurofibromin (*Nf1*). More than 900 different mutations in the *NF1* gene have been identified (HGMD, Human Genetic Mutation Database, http://www.hgmd.cf.ac.uk/ac/index.php) [(Clementi, M., et al., 1990)(Wallace MR, et al.,1990)]. Spontaneous mutations occur in 50% of cases; therefore, the *NF1* locus may represent a mutational hotspot in the human genome. All affected individuals are heterozygous for loss-of-function mutations in *NF1* gene; because homozygosity in murine models has been shown to be lethal to embryos [Jacks et al., 1994], it is believed that one functional *NF1* allele is necessary for survival. Some individuals demonstrate NF1 features in a localized pattern; this syndrome is termed segmental neurofibromatosis, and this phenotype is likely owing to a post zygotic, somatic mutation of the *NF1* gene in an early stage of fetal development (somatic mosaicism). Most of these mutations cause protein truncation, possibly resulting in the absence of a mature protein, but missense point mutations are also found. A clear genotype/phenotype correlation is lacking, and extensive studies on mutated *Nf1* proteins are missing. It is unknown if a common functional paradigm embeds all mutations, or if subtle changes in the defects caused by the different mutations explain the degrees of *NF1* expressivity. For instance, it is unclear whether mutations affect expression, stability, sub cellular localization, post-translational modifications, or the panel of the *Nf1* protein's interactors.

Mutation of *NF1* gene causes a variety of clinical manifestations such as the optic gliomas, neoplasias of the hematopoietic system and learning disabilities. However, the hallmark of NF1 is the development of multiple benign peripheral nerve sheath tumors called neurofibromas. Neurofibromas are complex tumors arising from peripheral nerve sheaths and mainly composed of Schwann Cells (SCs) homozygous for mutated *NF1*, Mast Cells (MCs) and Fibroblasts (FBRs) both heterozygous for the same mutation [Gottfried et. al,2010]. The plexiform variety can progress to highly malignant sarcomas termed Malignant Peripheral Nerve Sheet Tumors (MPNSTs) which are almost invariably lethal.

Up to now, any effective therapy able to either reduce neurofibroma's size and incidence or to counteract its formation has not been developed yet [Rubin, J.B. and Gutmann, D.H. 2005]. The main feature of neurofibromas is a rigid structure due to massive deposition of collagen of different types by activated fibroblasts. These cells, named myofibroblasts (mFBRs) are massively stimulated by mast cell-secreting Transcription Growth Factor-Beta (TGF-Beta) [(Yang FC et al.,2006)(Parrinello S et al.,2008)] to produce growth factors such as Platelet Growth Factor (PGF), Fibroblast Growth Factor (FGF) and collagen. This leads to both potent SCs proliferation and deposition of rigid Extra Cellular Matrix (ECM).

Cells haploinsufficient for *Nf1* display hyper activation of Rat sarcoma (Ras) [Cichowski K and Jacks T, 2001], which further increases when Loss Of Heterozygozity (LOH) of *NF1* occurs [Le LQ and ParadaLF,2007]. Thus, the activation of Ras/Rapidly Accelerated Fibrosarcoma (Raf)/ Extracellular Regulated Kinase (ERK) signaling in SCs is sufficient to make them more susceptible to proliferative signals provided by a $NF1^{+/-}$ niche [Harrisingh MC and Lloyd AC, 2004]. However, the physiological response to Ras hyper activation 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 niche. A major component of the niche is the ECM, a complex network of macromolecules whose the elasticity (ranging from soft and compliant to stiff and rigid), contributes to development and cancer [Aragona M et al, 2013].

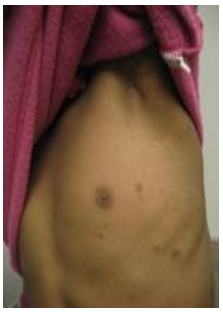
ECM elasticity determines how a cell senses and perceives external forces and thus provides a major environmental cue that determines cell behavior. Indeed, the focal adhesion complex, which consists of integrins, multicomplex of adaptors and signaling proteins, can be viewed as a mechanosensor linking the actomyosin cytoskeleton with the ECM [Pengfei Lu et al,2012].

How lack of *Nf1* may impact on the complexity of ECM-cell dynamic and how the great rigidity of the ECM in neurofibroma influences SCs' behavior, is still unknown. Among the three functional domains described in the *Nf1* protein, a Focal Adhesion Kinase (FAK) binding domain has been identified and *Nf1* has been shown to interact

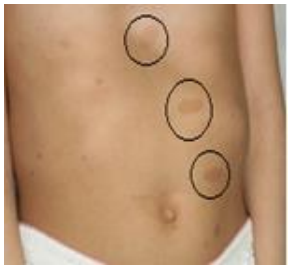
with FAK [Kweh F. et al, 2009], paving the way for the enunciation of new hypothesis aimed to explain the route of SCs' transformation toward cancer.

The NF1 symptoms

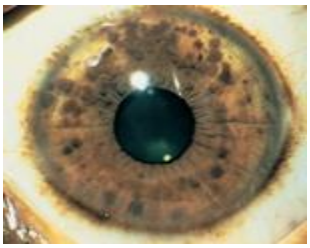
NF1 displays a 100% penetrance, but expressivity is extremely variable. Patients affected by this disease show defects in tissues derived from the neural crest and for this reason NF1 has been described as a neurocristopathy. However, the symptoms are not restricted to this tissues [Cichowski and Jacks, 2001].



Skin Fold Freckling

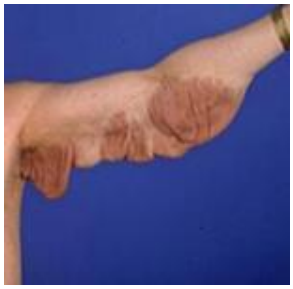


Cafè-au-laits spots

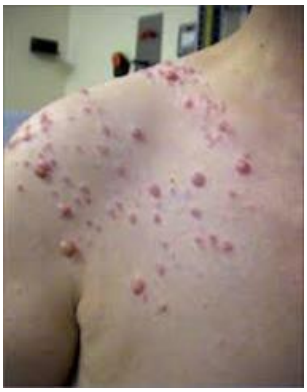


Lisch nodule of urface of iris

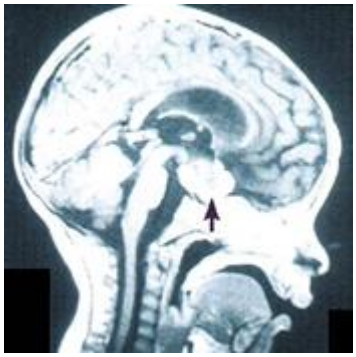
Clinical features include iris hamartomas, *café au lait* spots in melanocytes, freckling in the axillary or inguinal regions, learning deficiencies caused by defects in GABAergic neuron transmission, osseous lesions due to altered osteoclast-osteoblast interactions, defects in endothelial cells leading to vascular infarcts [Hersh, 2008].



Plexiform Neurofibromas



Dermal Neurofibromas



Optic Pathway Glioma

Neoplastic transformation mainly affects neural crest-derived cells. Most tumors originate in the nervous system: astrocytomas and gliomas of the optic pathway in the Central Nervous System (CNS); neuroblastomas and neurofibromas, which constitute a hallmark of the syndrome, in the Peripheral Nervous System (PNS). Neurofibromas are benign tumors arising from peripheral nerve sheaths, and display a distinctive degree of complexity because of their extensive cellular heterogeneity. They are composed of SCs, FBRs, MCs, perineural and endothelial cells, pericytes, and of a large amount of ECM surrounding axons [(Gottfried, 2010) (Staser et al, 2010)]. Neurofibromas can be either dermal superficial or deep plexiform tumors. Plexiform neurofibromas can progress to highly malignant sarcomas, MPNSTs, which are endowed with a very poor prognosis [Parrinello and Lloyd, 2009]. NF1 patients may also develop non-nervous system tumors, as gastrointestinal stromal tumor, somatostatinoma, pheocromocytoma, rhabdomyosarcoma, breast cancer and juvenile myelomonocytic leukemia [(Brems et al, 2009)(Cutts et al, 2009)].

Treatment

Just as mentioned there is no any therapeutic product or even effective therapy to reduce the incidence and size of neurofibromas as well as the formation of malignant tumors. The current therapies for cutaneous neurofibromas that cause disfigurements include surgery and, in some instances, CO_2 laser treatment or electrodessication [Rasmussen SA, et al., 2000].

Plexiform neurofibromas are largely bulked when clinically indicated, although their infiltrative nature presents significant challenges during surgery, and some patients will experience nerve damage or significant hemorrhage [Evans, D. G. *et al.*2010]. Malignant transformation of plexiform neurofibromas into MPNSTs is a significant problem in individuals with NF1 and is a leading cause of death in Individuals with *NF1*-associated plexiform neurofibromas. These patients must be monitored for a change in tumor growth and for signs and symptoms of transformation, including the development of pain, neurological deficit (weakness), or constitutional symptoms (weight loss, night sweats). While Magnetic resonance imaging (MRI), can define the anatomic location and extent of a Peripheral Nervous System Tumors (PNST), it does not provide accurate information regarding malignant transformation [De Luca, A. *et al.* 2004].

Treatment of MPNSTs involves surgical resection with subsequent to be effective; however, there is currently no standard care for these deadly cancers [Stumpf, D. A. *et al* 1987].

Unfortunately, long-term survival is rare when MPNST occurs in patients with NF1 because of lung and bone metastases as well as local tumor recurrence [Rasmussen, S. A., et al.,2001].

The NF1 Gene

The NF1 gene structure and functions

In 1987, genetic linkage analysis of a large number of independent families was used to map the *NF1* locus close to the centromer on the long arm of chromosome 17 [(Hersh JH; et al., 2008)(Gottfried ON, et al.)]. In 1990, the *NF1* gene was identified by positional cloning and it was located at 17q11.2 [(Ballester et al.,1990)(Xu et al., 1990)(Parrinello S,et al.,)]. The *NF1* gene is complex, spans more than 350 kb of genomic DeoxyRibonucleic Acid (DNA), and contains 60 exons [Brems H, et al., 2009]. The *NF1* gene produces an 11- to 13-kb Messenger Ribonucleic Acid (mRNA) [(Wallace et al., 1990)(Cutts BA, et l,.2009)]. This is expressed in almost all tissues [Parrinello S, et al., 2009]. But it is most highly expressed in the brain, spinal cord, and the peripheral nervous system [(Levine, S. M., et al.,2008)(Prada, C. E. *et al.*2012)].

The NF1 gene product, Neurofibromin

Neurofibromin is a large peptide (about 250 kDa) composed by 2818 amino acids (a.a) in humans and 2820 a.a in mice [Gutmann et al., 1991]. It is ubiquitously expressed during embryonic development and it is also present in a variety of cell types in adults [Gutmann et al., 1995], but it is most abundant in the nervous system, where it is found in neurons, oligodendrocytes, and SCs [Daston et al., 1992].

Sequence analysis of neurofibromin reveals a region of homology with the catalytic domain of the mammalian p120-Ras-GAP, a GTPase-activating protein (GAP) for the Ras family of proto-oncogenes [Xu et al., 1990]. Moreover, neurofibromin shows an extended similarity with the *Saccharomyces cerevisiae* Ras-GAP proteins IRA1 and IRA2 [Ballester et al., 1990]. Exons 20-27a of neurofibromin encodes this “GAP related domain” (GRD), which

induces the conversion of Ras from its active Guanosine TriPhosphate (GTP)-bound conformation to its inactive Guanosine DiPhosphate (GDP)-bound form.

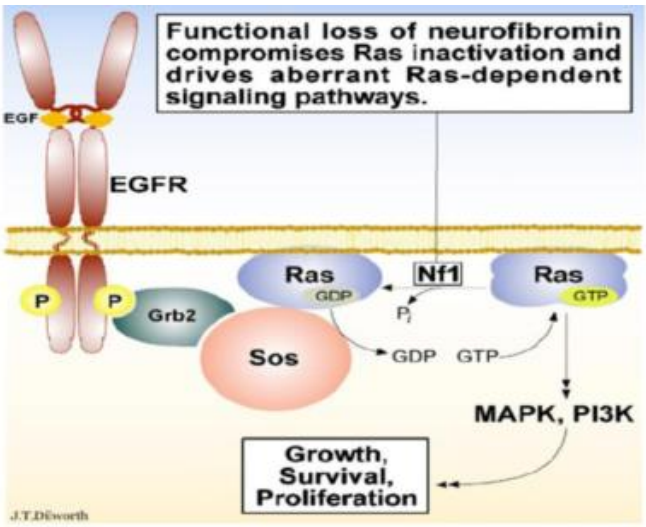


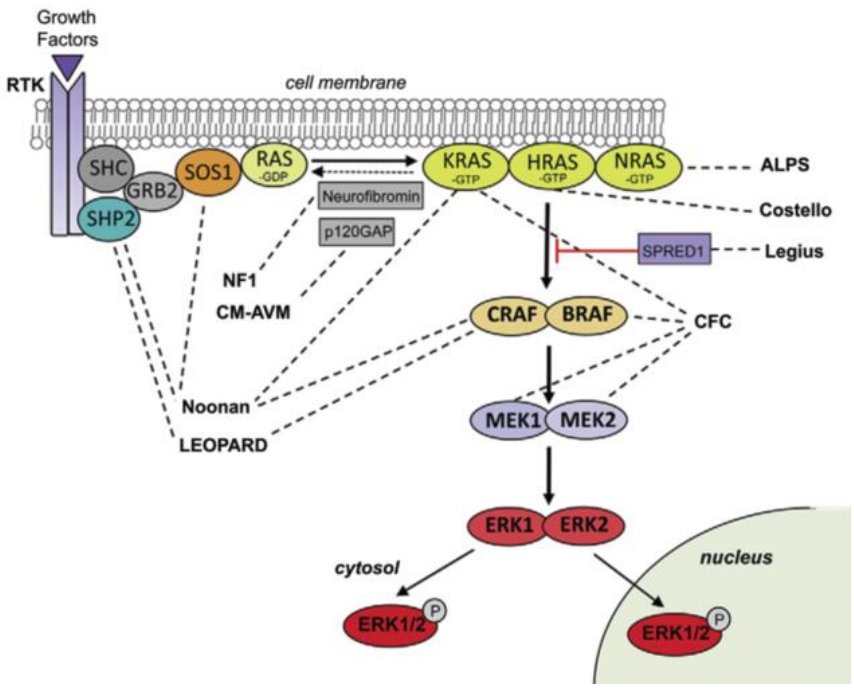
Figure. Loss of neurofibromin deregulates Ras signaling. In response to growth factors, receptor tyrosine kinases (in the example Extracellular Growth Factor Receptor (EGFR)) recruit a guanine nucleotide exchange factor Sos, which activates Ras. In its active, GTP-bound form, Ras initiates several signaling cascades, including the Mitogen Activated Protein Kinase (MAPK) and PI3K pathways, in order to regulate cell growth, survival, and proliferation. Functional loss of neurofibromin compromises Ras inactivation and drives aberrant Ras dependent signaling, which contributes to tumor formation and progression [Dilworth et al., 2006].

Ras is activated at the plasma membrane upon binding of growth factor receptors to specific ligands, triggering the recruitment of a complex containing the adapter protein growth factor receptor bound protein 2 (Grb2) and the Ras guanine nucleotide exchange factor Sos, which catalyzes Ras switch to its GTP-bound state. This active form of Ras then binds and activates the kinas Raf and phosphatidylinositol 3-kinase (PI3K), which are the apical inducers of two kinas signaling cascades [Le and Parada, 2007].

Neurofibromin, via its GRD, exerts a reverse effect on Ras by increasing its GTP hydrolysis rate; as an unrestrained Ras activity is a major oncogenic determinant neurofibromin acts as a tumor suppressor protein. Many *NF1*-deficient tumors exhibit elevated levels of Ras-GTP, supporting the observation that neurofibromin is a key regulator of Ras signaling. The functional domain of neurofibromin, Ras, acts as part of a signal transduction pathway that is activated by growth factors and their receptors [Bernards A. 1995]. Increased Ras-GTP leads to increased signaling through Raf kinase, which activates a kinase cascade involving MEK kinase and the Erk1 and

Erk2 isoforms of MAPK resulting in cell proliferation [(Bollag G, et al1996)(Downward J: 1998)(Sternberg PW,et,al.1998)]. Increased Ras-GTP also protects cells from apoptosis by activating mammalian Target Of Rapamycin (mTOR) [(Bhola P, et.al,2009)(Johannessen CM, et al.,2005)]. Studies confirm that neurofibromin negatively regulates this mTOR pathway with loss of neurofibromin expression in established human neurofibroma cell lines associated with high levels of mTOR activity [(Bhola P, et. al,2009)(Johannessen CM, et al.,2005)]. The mTOR pathway is constitutively activated in both *NF1*-deficient primary cells and human tumors, is dependent on Ras and PI3K activation, and is mediated by the phosphorylation and inactivation of the Tuberous Sclerosis Complex 2 (TSC2)-encoded protein tuberin by Protein Kinase B (PKB or AKT) [Johannessen CM, et al., 2005]. Overall, Ras is a key component of many growth factor signaling pathways, and in the absence of neurofibromin it is constitutively activated, resulting in increased cell proliferation and survival [Viskochil DH. 1999]. Moreover, direct inhibition of Ras (with farnesyltransferase inhibitors) or of Ras pathway molecules (with MEK and AKT inhibitors) as well as replacement of the *NF1*-GRD reverses the proliferative phenotype of *NF1* deficient cells [(Hiatt et al., 2001)(Upadhyaya M. et al.,1997)]. These results strongly suggest that the tumorigenic potential conferred by loss of neurofibromin at least partially results from the growth advantage provided by deregulated Ras activity.

The Ras/MAPK pathway is critical to normal development by its regulation of cell proliferation, differentiation, motility, growth, apoptosis, and cell senescence [Tidyman WE, Rauen KA, 2009]. Interestingly, there are multiple developmental syndromes in addition to NF1 that form tumors by abnormalities in the Ras pathway, and they are referred to as the "RASopathies" or "neuro-cardio-facial-cutaneous syndromes" [(Denayer E, de Ravel T, Legius E., 2008)(Tidyman WE, Rauen KA,2009)]. These diseases include Noonan syndrome, LEOPARD syndrome, Costello syndrome, capillary malformation–arteriovenous malformation, and cardio-facio-cutaneous syndrome [Tidyman WE, Rauen KA., 2009].



Tumorigenesis in NF1 and therapeutic implications

Figure. Ras/MAPK pathways responsible for multiple developmental syndromes, including NF1, Noonan syndrome, LEOPARD syndrome, hereditary gingival fibromatosis 1, capillary malformation–arteriovenous malformation (CM-AVM) syndrome, Costello syndrome, autoimmune lymphoproliferative syndrome (ALPS), cardio-facio-cutaneous (CFC) syndrome, and Legius syndrome, have considerable phenotypic overlap. Thus, targeted molecular treatment may be effective for multiple diseases. Figure reproduced with permission from Tidyman and Rauen [Tidyman WE, Rauen KA: The RASopathies: developmental syndromes of Ras/MAPK pathway deregulation. Curr Opin Genet Dev 19:230–236, 2009].

They are related by germline mutations in genes that encode protein components of the Ras/MAPK pathway, and these mutations may result in increased signal transduction [Tidyman WE, Rauen KA., 2009]. Each syndrome exhibits unique phenotypic features; however, there are overlapping clinical features including characteristic facial features, cardiac defects, skin abnormalities, developmental delay, and a risk of malignancy [Tidyman WE, Rauen KA., 2009]. As just mentioned, there are several *Nf1*-related pathways that are significant in the pathogenesis of other developmental syndromes, including mTOR, which contributes to tuberous sclerosis [(Ehninger D, et. al., 2009)(Huang J, et,al.,2009)(Mi R, et al., 2009)(Mozaffari M, et al., 2009)(Denayer and colleagues,2008)] noted that these syndromes with common molecular pathways demonstrated the important roles for evolutionarily conserved pathways not only in oncogenesis, but also in cognition, growth, and development.

To date, neurofibromin is known to have an additional functional domain, the yeast phosphatidylinositol-transfer protein (Sec14)p-like domain [Aravind et al., 1999] which is located between amino acids 1545-1816 and is homologous to the yeast Sec14p, a protein involved in the regulation of intracellular proteins and lipid trafficking. This domain belongs to a bipartite structural module that includes also a pleckstrin homology (PH)-like domain which is considered responsible for the regulation of the neighboring *NF1*-Sec domain. Even though the biological role of Sec14p domain in *Nf1* is currently unknown, prediction of a Sec14p-like lipid binding domain in neurofibromin opens new lines of investigation of its Ras-GAP function in terms of regulation by lipids, and suggests that neurofibromin can be involved in other transduction pathways.

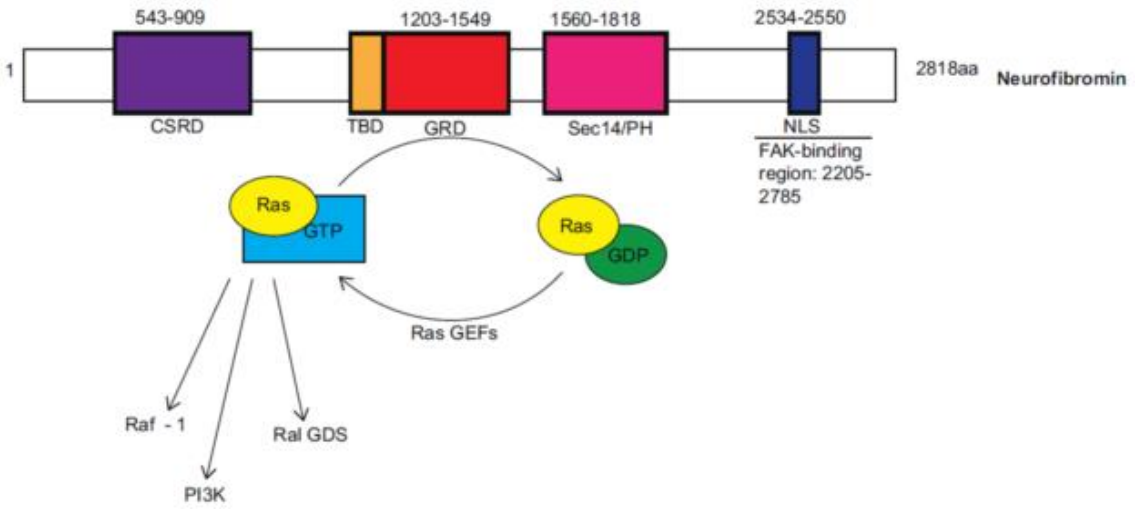


Figure: Diagram illustrating known functional domains within neurofibromin**.** Domains are indicated as follows: CSRD, cysteine/serine-rich domain; TBD, tubulin-binding domain; GRD, GAP-related domain; Sec14/PH, Sec14- homologous domain and pleckstrin homology domain; NLS, nuclear localization sequence. Numbers above each sub domain indicate the positions of the corresponding amino acids within the 2818 amino acid length of the human neurofibromin protein [Brossier and Carroll, 2012].

Neurofibromin localization

Even though the Ras-GAP activity currently appears to represent the only clearly defined biochemical function of this giant protein, a number of interaction partners of neurofibromin have been reported: Protein Kinase A (PKA) or cAMP-dependent protein kinas, protein kinas C (PKC), caveolin-1, FAK, tubulin, amyloid precursor protein, syndecan, kinesin-1, nuclear PMLbodies (are punctate structures found in the nuclei of certain cells), the UBX-UBD protein (Ubiquitin regulatory X-D proteins) Protein, Expressed in T-cells and Eosinophils in Atopic Dermatitis (ETEA) and p97/valosin-containing protein (p97/VCP). Neurofibromin could interact with different partners also

depending on its dynamic intracellular movement which regulates not only protein localization, but also protein function. Neurofibromin appears to be predominantly cytoplasmic, with different cell types displaying a variable sub cellular localization. Some studies have also observed neurofibromin in the nucleus as it contains a nuclear localization sequence (NLS) at the C-terminus [Vandenbroucke et al., 2004]. The possible function of neurofibromin within the nucleus remains unknown. Translocation to the nuclear compartment could be a mechanism to regulate the GAP function of neurofibromin by sequestration in the nucleus, as Ras is located at the plasma membrane. It is possible that during development, the sub cellular targeting of neurofibromin depends on posttranslational modifications, such as phosphorylation, regulates the targeting of proteins. Indeed, neurofibromin contains multiple PKA and PKC serine or threonine phosphorylation consensus sites which are located within the N-terminus of neurofibromin and, in particular, in the cysteine-rich domain (CSRD). EGF stimulation was shown to increase the association of neurofibromin with actin through a PKC-dependent phosphorylation of neurofibromin. Moreover, this modification increases the Ras-GAP activity of neurofibromin [Mangoura et al., 2006].

1.2 The benign tumors in Neurofibromatosis Type 1

Neurofibromas

The hallmark feature of the disease is the formation of multiple peripheral nerve sheath tumors, neurofibromas, which can arise as dermal lesions or grow internally along the plexus of major peripheral nerves. While these lesions are benign, they can be debilitating and deforming. In addition, a subset of these tumors can progress to malignant neurofibrosarcomas. Neurofibromas are rarely present in childhood, but develop during puberty and pregnancy, suggesting a hormonal influence on tumor growth.

Dermal Neurofibromas: The most common type of neurofibroma can arise from small nerve radicals or larger nerve branches and grow as discrete lesions in the dermis or epidermis ranging from 0.1 to several centimeters in diameter. They are collectively called dermal neurofibromas and patients can develop thousands of them which, depending on location, can be painful and disfiguring. One of the key features of neurofibromas is their heterogeneity as they are composed of all cell types normally found in the peripheral nerve .However, the

structure of the nerve fascicle seems to be highly disorganized, with increased number of SCs and FBRs, increased collagen deposition, extensive MCs infiltration and disruption of the perineurium. Moreover, unlike in the normal nerve, the majority of SCs in neurofibromas are found dissociated from axons and axonal degeneration is present.

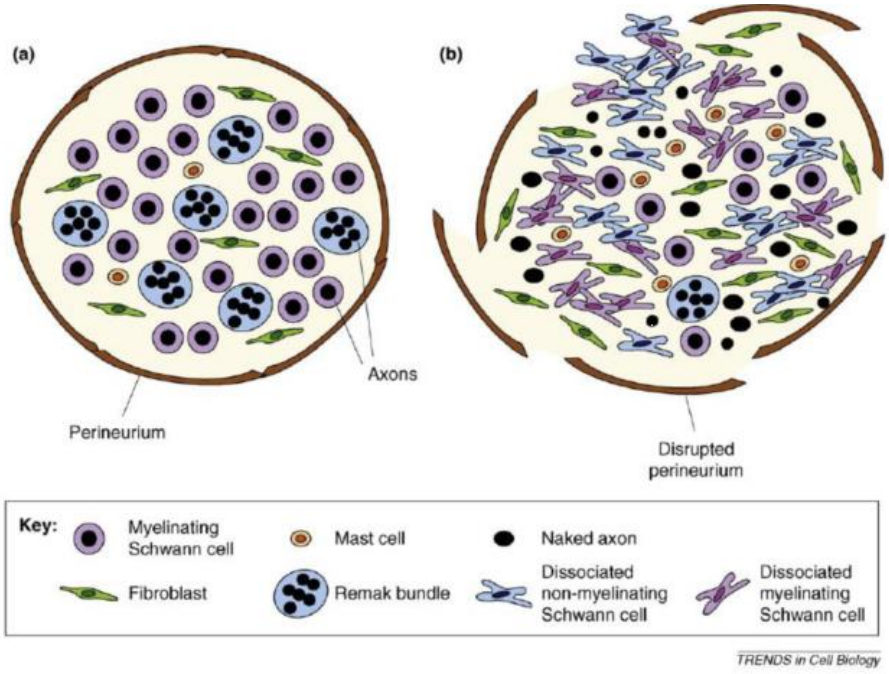


Figure: Structure of normal nerves and neurofibromas. Diagram showing a cross-section of (a) a normal nerve fascicle and (b) the aberrant structure of nerve fascicles in neurofibromas [Parrinello and Lloyd, 2009].

Plexiform neurofibromas: In contrast to dermal neurofibromas, which are typically small, plexiform neurofibromas can develop internally along the plexus of major peripheral nerves and become quite large, involving an entire limb or body region. They occur in about 30% of NF1 individuals and are considered as congenital lesions. While these are benign tumors, they can be debilitating and can progress to malignancy. Indeed, plexiform neurofibromas harbor a 5% lifetime risk of transformation into MPNSTs. These are highly malignant and metastatic cancers with a high mortality and a poor response to chemotherapy and radiation. The difference in the mechanism of tumorigenesis between cutaneous neurofibromas and plexiform is unclear; however, the timing of their development as well as their growth properties may indicate differences in the mechanism of tumor initiation.

Origin of neurofibromas

The molecular mechanisms underlying tumor development in NF1 have been obscure. Although second hit mutations affecting the inherited wild-type *NF1* allele have been clearly identified in the myeloid leukemias and pheochromocytomas in NF1 patients, such mutations have been reported for only a small number of neurofibromas. The difficulty in detecting mutations may be due in part to the complex nature of these lesions, which are composed of multiple cell types, not all of which are expected to develop a second mutation. Moreover, it has also been suggested that *NF1* heterozygosis may be sufficient for development of benign neurofibromas (haplo-insufficiency), with full loss of *NF1* function being restricted to the progression to MPNSTs.

Research over the past decade using mouse models has greatly enhanced our knowledge of neurofibroma development and malignant progression in NF1. The mouse and human *NF1* genes are highly related, the amino acid sequence of neurofibromin is 98% identical, and there is also significant similarity between the non-coding regions of the mRNA. Mice homozygous for null *Nf1* mutation in exon 31, a region that is often mutated in human NF1 patients, are embryonic lethal at E13.5 secondary to defective heart and malformation of the major cardiac outflow tracts. Defects in renal, hepatic and skeletal muscle development were also observed; however, nervous system pathology was limited to enlargement of sympathetic ganglia secondary to neuronal hyperplasia [(Brannan et al., 1994)(Gottfried et al., 2006)(Jacks et al., 1994)]. Heterozygous *Nf1* mutant mice are viable and only have increased incidence of pheochromocytomas and myeloid leukemias beyond 10- to 12-month old; however, they do not develop PNSTs or other characteristic symptoms of human NF1. One possible explanation for this observation is that LOH necessary for neurofibroma development is impaired in mice. Perhaps, given the shorter time of gestation and lifespan compared with humans, the $Nf1^{+/-}$ mice do not have the necessary window of opportunity to undergo effective LOH in target cells to initiate neurofibroma formation. Cichowski in 1999 addressed this issue elegantly when they created chimeric mice by injecting $Nf1^{-/-}$ ES cells into wild type blastocysts. These mice developed microscopic plexiform neurofibromas derived from the injected ES cells, demonstrating the requirement of *Nf1* homozygosis for tumor formation. However, the degree of chimerism in

these mice occurs randomly and cannot be controlled genetically. As a result, it was difficult to establish the target cell or whether other cell types contribute to the tumorigenesis.

As in human neurofibromas SCs were the most common cell type present in neurofibromas it was hypothesized that this cell type, or their precursors, could be the initiating cell for tumor development. SC-specific *Nf1*-deficient mice were derived by crossing *Nf1flox/flox* mice with the *Krox20-cre* transgenic mice, an embryonic SC-specific promoter [Zhu et al., 2002]. Mice with a conditional knock-out of *Nf1* only in embryonic SCs, but wild-type in all other cell lineages (*Nf1flox/flox; Krox20-Cre*), exhibit microscopic hyperplasia in sensory ganglia but do not develop neurofibromas. However, when mice homozygous for *Nf1* mutation ($Nf1^{-/-}$) in SCs but heterozygous for *Nf1* ($Nf1^{+/-}$) in all other somatic cells (*Nf1flox/-; Krox20-Cre*) were generated, these mice developed multiple classic plexiform neurofibromas with a massive degranulating MCs infiltration, modeling human neurofibroma. These genetic studies implied the SC origin for neurofibroma. Nevertheless, in addition to nullizygosity at *Nf1* locus in SCs, haploinsufficiency of *Nf1* in the tumor microenvironment is also required for the tumorigenesis.

Genetically engineered mouse models of PNS neoplasia.

	Transgenic mouse model	Phenotype	Limitations
Early models	$Nf1^{\Delta 33/\Delta 33}$	Die by E13.5 due to cardiac failure	Early death prevents observation of tumorigenic effects of *Nf1* loss
	$Nf1^{\Delta 33/+}$	Develop pheochromocytomas (15% incidence); show accelerated development of other non-NF1 tumors as compared to wild-type mice	No neurofibromas or MPNSTs observed
	$Nf1^{-/-}$;$Nf1^{+/-}$ chimeras	Multiple plexiform neurofibromas present in animals with intermediate level of chimerism	Cannot control which cell types are $Nf1^{+/-}$ and which are $Nf1^{-/-}$
	$Nf1^{flox/flox}$;Krox20-Cre	Schwann cell hyperplasia	No neurofibromas or MPNSTs observed
	$Nf1^{flox/-}$;Krox20-Cre	Plexiform neurofibroma development by 1yr of age (demonstrating importance of both $Nf1^{+/-}$ and $Nf1^{-/-}$ cells in neurofibroma formation)	Krox20 promoter is expressed in Schwann cells and boundary cap cells, making it hard to identify a clear progenitor

Table: Genetically engineered mouse models of neurofibroma formation. Both nullizygosity at *Nf1* locus in SCs and haploinsufficiency of *NF1* in the somatic tissue are required for neurofibroma (Brossier and Carroll,2012).

The loss of both *NF1* alleles, which lacks functional neurofibromin, has a substantial growth advantage [Muir D, et al., 2001] and a loss of negative autocrine growth control [Muir D., 1995]. Neurofibromin- deficient SCs secrete

increased kit ligand, which serves as a chemo attractant for MCs expressing c-kit receptor. Also, heterozygous inactivation of *NF1*$^{+/-}$ promotes migration of MCs on α4β1 integrins (MC surface proteins), in response to the kit ligand. Overall, the loss of *NF1* in SCs results in an increase in growth factor production that initiates a paracrine loop, and it is important for tumor initiation and progression [Yang FC, et al., 2003]. Normally, growth factors cooperate to suppress cell death in SC precursors [Gavrilovic Jet al.,1995], but growth factor deregulation is thought to be involved in tumorigenesis [Ratner N, et al., 1990]. Changes in growth factor expression may be the direct result of *NF1* gene loss or from secondary genetic events [Mashour GA, et al., 2001]. Abnormal growth factor receptor expression also has a role in tumorigenesis, progression, and malignant transformation.

Timing in Nf1 loss

Even though it is well established that neurofibromas arise from SCs that undergo LOH at the *NF1* locus, the specific cell type within the SC lineage in which this occurs has been the topic of much debate. The SC lineage is well characterized and specific markers for the different cell types within the lineage have been identified. SCs originate from migrating neural crest stem cells (NCSCs) and SC precursors and immature SCs [Mirsky et al., 2008].

Around birth, immature SCs differentiate into the two main mature SC types of the peripheral nerve, myelinating and non-myelinating SCs. The former myelinate single large-caliber axons and the latter wrap multiple small-caliber axons in structures known as Remak bundles. Importantly, SC progenitors differentiate by late gestation and do not persist in the adult peripheral nervous system. Instead, upon nerve injury, the requirement for new cells is met by the regenerative ability of mature differentiated SCs to transiently de-differentiate to a progenitor-like state [Parrinello and Lloyd, 2009].

Three independent groups have recently tested whether neurofibromas originate from embryonic stem cells or differentiated adult cells by generating conditional mouse models in which *Nf1* LOH was induced in the SC lineage at different times during embryonic development [(Joseph et al., 2008)(Wu et al., 2008)(Zheng et al., 2008)]. Elimination of *Nf1* expression in neural crest cells, the earliest stage in Schwannian differentiation, was achieved by mating *Nf1flox/−* mice with *Wnt1-Cre*, *Mpz-Cre*, and *Pax3-Cre* animals. Although these mice had abnormal

sympathetic ganglia and adrenal glands and died at birth, they did not develop neurofibromas. Given the early death of these animals, the observation of the tumorigenic effects of *Nf1* loss is prevented. However, the authors observed that *Nf1* loss significantly increased the frequency of stem cells at early embryonic stages but they were no more detectable by late gestation [Joseph et al., 2008] indicating that early progenitors with stem cell properties do not persist in the adult where they could give rise to neurofibroma. This observation together with the fact that *Nf1flox/–; Krox20-Cre* mice develop neurofibromas, and Krox20 is not expressed in neural crest cells, argues that *Nf1* loss in neural crest cells is not required for neurofibroma pathogenesis.

Mouse models in which *Nf1* was ablated in SC precursors (SCPs; also known as neural crest stem cells) were obtained using *3.9Periostin-Cre* (which is active in SCPs by E11) and *P0a-Cre* (expressed in SCPs beginning at E12.5) driver lines. While the majority of *Nf1flox/–; 3.9Periosin-Cre* animals died by the 4th postnatal week [Joseph et al., 2008], *Nf1flox/–; P0a-Cre* animals survived and formed neurofibromas in adult limb nerve [Zheng et al., 2008]. Interestingly, the proliferating cells in these neurofibromas were p75+, GFAP+ and BLBP−, suggesting that mature non-myelinating SCs rather than SCPs were the cell type giving rise to neurofibromas in this model. In keeping with this idea, hyper-proliferative non-myelinating SCs were found in the postnatal sciatic nerves of *Nf1flox/–; P0a-Cre* mice prior to neurofibroma development. The time window of tumor development is consistent with findings from Wu and colleagues, who showed that recombination of *Nf1* driven by the Desert Hedgehog (Dhh) promoter at E12.5 in SC precursors resulted in neurofibroma development in peripheral nerve roots, but not in nerve trunks [Wu et al., 2008]. Unlike the tumors arising in *Nf1flox/–; P0a-Cre* mice, neurofibromas developing in *Nf1flox/flox; Dhh-Cre* mice contained numerous BLBP+ cells, suggesting that immature SCs were the progenitors for these tumors. Interestingly, the development of neurofibromas in *Nf1flox/flox; Dhh-Cre* mice occurred despite the presence of a wild-type *Nf1* microenvironment; no evidence was found for Cre-mediated recombination in mast cells, endothelial cells or endoneurial fibroblasts, despite the fact that Dhh-expressing progenitors capable of differentiating into both SCs and endoneurial fibroblasts have been found in peripheral nerve [Joseph et al., 2004]. Therefore, neoplastic transformation of NCSCs or no neuronal restricted nerve progenitors could potentially yield clonal tumors containing both SCs and FBRs.

Given these contradictory results, it is not yet clear whether the neoplastic SCs within plexiform neurofibromas are derived from mature non-myelinating SCs, immature SCs or both cell types. The possibility that these neoplastic SCs arise from another source such as boundary cap cells also has not yet been ruled out. Intriguingly, these findings could have implications for our understanding of the enormous variability in the severity of the human disease, even among members of the same family.

Given the marked differences in the clinical behavior of dermal and plexiform neurofibromas, it is possible that the neoplastic cells in these neurofibroma subtypes are derived from distinct progenitors. Neural-crest derived precursor cells capable of both Schwann and melanocytic differentiation, termed skin-derived precursors (SKPs), are presented in the dermis of adult mice.

Consistent with the hypothesis that SKPs give rise to dermal neurofibromas, topical administration of tamoxifen to neonatal *Nf1flox/–; CMV-CreERT2; Rosa26-LacZ(stop)* mice results in dermal neurofibroma formation at the site of tamoxifen administration [Le et al., 200]. Further, SKPs isolated from these animals and treated *ex vivo* with tamoxifen to inactivate *Nf1* were also capable of generating neurofibromas upon autologous subcutaneous transplantation into pregnant mice, indicating that these progenitors (and not other cell types residing in the dermis) were the cell of origin of the dermal neurofibromas. Interestingly, $Nf1^{-/-}$ SKPs were also capable of forming plexiform neurofibromas when auto-grafted into sciatic nerves. Thus, while SKPs residing in the dermis may be the cell of origin of dermal neurofibromas, these cells are apparently highly similar to the neurofibroma-initiating cells in peripheral nerve. The distinct clinical behavior of these tumors may primarily reflect differences in their microenvironment rather than their cell of origin [Brossier and Carroll, 2012].

Mechanisms of early tumorigenesis

SC's behavior is strictly controlled by axonal contact [Corfas, G. et al. 2004]. Axons provide survival, proliferative and differentiation signals during embryogenesis and, in adulthood, are thought to maintain SCs in a differentiated state to ensure the correct functioning of the nerve [(Chan, J.R. et al. 2006)(Michailov, G.V. et al. 2004)]. Surprisingly, very little is known about the molecules that mediate axon–glial interactions. It is accepted; however,

that loss of proper SC–axonal interactions can lead to neuropathy, and the pathology of human and mouse neurofibromas has consistently shown that the majority of SCs are found without axonal contact [Corfas, G. et al. 2004]. However, the significance of this lack of interaction in tumor development and the mechanisms involved remained unclear. The new studies described earlier add credence to these observations and, by demonstrating that dissociation from axons is a crucial early event in SC tumourigenesis, underlie the importance of this process. Work from Parrinello's laboratory also supports these findings and provides a mechanistic explanation for this important event [Parrinello, S. et al.2008]. Using primary co-culture systems from the Nf1fl/fl mouse model, showed that acute loss of *Nf1* in SCs was sufficient to prevent their association to axons and induced axonal dissociation of SCs in established co-cultures [Simona Parrinello and Alison C. Lloyd]. Mechanistically, it is found that this disruption to SC–axonal interactions was the result of Ras–Raf–ERKdependent down regulation of a protein expressed on the surface of SCs, semaphorin 4F (Sema4F) [Simona Parrinello and Alison C. Lloyd]. Sema4F is a poorly studied transmembrane member of the semaphorin family of guidance molecules, the expression of which is retained postnatal in the peripheral nervous system [Encinas, J.A. et al. 1999)(Kruger, R.P. et al.2005)]. Importantly, Sema4F was readily detectable both in freshly isolated SCs and in vivo in nerves but was strongly down regulated in neurofibromas. Three main conclusions are suggested by these findings: first, that Sema4F is normally expressed in adult nerves in which it probably has an important role in maintaining peripheral nerve structure and function; second, that ERK-mediated Sema4F down regulation is a key mechanism of neurofibroma initiation, responsible, at least in part, for the early dissociation of non-myelinated SCs observed in mouse models; and third, the continued down regulation of Sema4F is probably required for neurofibroma progression, possibly by preventing reassociation of SCs to axons. How might Sema4F-mediated loss of axonal contact lead to neurofibroma development? In both the P0A-Cre Nf1fl/_ and the DhhCre;Nf1fl/fl mouse models, dissociation of non-myelinated SCs is rapidly followed by proliferation, suggesting that, in adult nerves, loss of axonal contact somehow renders SCs more proliferative. In the Parrinello's work, they provided experimental evidence supporting this idea. They showed that when SCs were seeded onto axons and cultured in the absence of external mitogens, SCs initially proliferated rapidly in response to axonal signals as expected. By contrast, once SCs fully occupied the axonal network, the cultures became quiescent and could not be stimulated to enter the cell cycle by addition of

serum mitogens. If Sema4F was down regulated in these established cultures before stimulation, however, dissociated SCs responded to external mitogens by undergoing proliferation. Importantly, cells lacking Sema4F expression did not proliferate in the absence of serum, indicating that proliferation was not triggered by a direct effect of Sema4F on the cell cycle, but rather by an indirect effect of loss of axonal contact. These results demonstrate that axonal signals are anti-proliferative and dominant over environmental mitogenic cues. Conversely, disruption of SC–axonal interactions is pro-tumourigenic because it renders SCs more responsive to environmental proliferative stimuli. This is consistent with a previous study showing that loss of axonal contact through loss of ErbB receptor signaling resulted, surprisingly; in the proliferation of non-myelinating SCs [Chen, S. et al. 2003)]. Thus, Parrinello's laboratory could begin to delineate a model of early neurofibroma formation in which the increase in Ras–Raf– ERK signaling that results from *Nf1* loss leads to Sema4F down regulation and dissociation of non-myelinating SCs. Next, the dissociated cells, freed from the growth-suppressive signals of the axon, are able to respond to external mitogenic cues and undergo unscheduled proliferation.

Neurofibromas progression and inflammation

The NF1 nerve microenvironment is pro-tumourigenic in nature (i.e. it provides mitogens and growth factors capable of promoting the proliferation of dissociated non-myelinating SCs). A large body of evidence supports this hypothesis and indicates that the $Nf1^{+/-}$ microenvironment is a key contributor to tumor development and that MCs in particular have a fundamental role in this cooperation [(Zhu, Y. et al.2002)(Le, L.Q. and Parada, L.F.2007)(Wu, M. et al.2005)]. Indeed, the presence of extensive MC infiltration in pathological specimens of both mouse and human neurofibromas is a common observation. Remarkably, recent exciting work on the Krox20- Cre Nf1fl/_ model from the Clapp's laboratory has taken these observations further and convincingly demonstrated that neurofibroma development is contingent upon infiltration of $Nf1^{fl/-}$ MCs in the tumour mass [Yang, F.C. et al.2008].

Consistent with this important study, in the P0A-Cre $Nf1^{fl/-}$ mouse model [Zheng, H. et al.2008] SC proliferation was accompanied by the development of an inflammatory response with extensive MC infiltration at the onset of

tumourigenesis. The trigger for this response seemed to be degeneration of the axons left unprotected after SC dissociation. Importantly, as tumourigenesis progressed, inflammation and nerve degeneration became even more extensive in these animals and eventually led to demyelination of myelinating SCs and further axonal loss. Thus, early degeneration of Remak bundles triggers a series of events that mimics the normal response to nerve injury. However, although under physiological conditions these events promote regeneration, in the context of NF1 they are instead ultimately conducive to neurofibroma progression. This phenomenon might be more clearly illustrated by drawing a comparison with normal nerve regeneration.

In normal nerve repair, inflammatory cells are recruited to clear axonal and myelin debris and promote the proliferation of de-differentiated SCs through secretion of growth factors and cytokines [(Fawcett, J.W. and Keynes, R.J.1990)(Scherer, S. a.S., J. L.2001)(Chen, Z.L. et al. 2007)(Hirata, K. and Kawabuchi, M.2002)]. This is normally a transient and self-contained process and, upon axonal regeneration, inflammation subsides and SC re-differentiate into myelinating and non-myelinating phenotypes [(Fawcett, J.W. and Keynes, R.J. 1990)(Scherer, S. a.S., J. L.2001)]. The Parrinello's laboratory has previously shown that elevated Ras signaling inhibits SC myelination [(Harrisingh, M.C. and Lloyd, A.C.2004)(Harrisingh, M.C. et al. 2004)] suggesting that, once de-differentiated in response to inflammatory signals, SCs would remain in a progenitor-like proliferative state and, as such, further contribute to tumor progression. This model would explain the longstanding clinical observation that neurofibroma formation is fostered by local trauma and injury because generation of a wound is likely to initiate with this pro-tumourigenic process more rapidly [(Riccardi, V.M.1992)(Cichowski, K. and Jacks, T.2001)(Riccardi, V.M. (1981)].

2. MATERIALS AND METHODS

2. MATERIALS AND METHODS

Materials:

Murine Embryonic Fibroblasts (MEFs) & Primary Cells isolated from Plexiform Neurofibromas

Cells used as the early experimental model were MEF, isolating from mice wild-type MEF $Nf1^{+/+}$, heterozygous MEF for $Nf1^{+/-}$ and K. O. MEF $Nf1^{-/-}$ (for the protein neurofibromin) and immortalized with virus SV40 kindly provided by Prof. Stein from Telaviv University.

And other cell types which were used recently in our 3D *in vitro* experimental model are primary SCs $Nf1^{-/-}$, mFBRs and MCs both heterozygote for $Nf1^{+/-}$ isolated from human neurofibromas, according to Serra methodology {Serra, 2000 #210}.These cells have been isolated from plexiform neurofibroma biopsies after informed consent of patients by our Milan and Rome University' collaborators.

Cell Cultures

Our MEF cells were normally cultured and grown in adhesion with the flasks' membrane with 75 cm^2 BD Falcon or in normal petri plates 58 cm^2 in D-MEM medium (Dulbecco's Modified Eagle's Medium, High Glucose with Sodium Pyruvate and L-glutamine, Euroclone), with addition of: fetal bovine serum, FBS (10% v/v, Fetal Bovine Serum, Euroclone) which provides the essential growth factors; 5 mM L-glutamine (L-glutamine solution 200 mM, Euroclone), essential amino acids; penicillin (2mg/ml) and streptomycin (2mg/ml) (Penicillin Streptomycin solution 100X, Euroclone) useful to prevent any bacterial contamination. Flasks/ Petri are then kept in an incubator at 37 °C in an atmosphere of enriched air with 5 % of CO_2.

Matrigel Culture for growing our Primary SCs in 2D and 3D experimental methods: To the DMEM contained of 10% of Bovine Serum added: Heregulin, Insulin, Foskoline, IBMX, PDGF-BB, EGF, Laminine, Fibronectine, and Collagen and at the very end point added also cold matrigel.

Primary Human Fibroblasts' culture medium: To the DMEM contained of 10% of Bovine serum added 10ng/ml of PDGF-BB (40ug/ul), EGF and Insulin.

Antibodies and Growth Factors:

Primary antibodies: used in this study are: anti-pErk polyclonal rabbit, specific against the two isoforms Erk1 and 2 when phosphorylated individually or both phosphorylation sites: Thr202/Tyr204 of ERK1, Thr185/Tyr187 of Erk2 (Cell Signaling); anti-ERK which detects the levels of total ERK in the cell (Cell Signaling); anti-pAkt polyclonal rabbit, specific for the phosphorylation of Akt1 at the level of Ser473 (Cell Signaling); anti-Akt, which detects the levels of total Akt in the cell (Cell Signaling); anti-GAPDH (Millipore), primary antibody for glyceraldehydes 3-phosphate dehydrogenize (GAPDH), the enzyme that catalyzes the sixth step of glycolysis, used as a control of protein loading, as the expression of this protein is not influenced by processes that have been investigated in this thesis;GSK-3alpha/beta (001-A): c-7291 from Santa Cruz. Phospho-GSK-3alpha/beta (Ser21/9) antibody from Cell Signaling. Anti-pNF1 polyclonal rabbit (Cell Signaling) specific for the sequence containing the serine 2515 phosphorylated; anti-NF1 mouse monoclonal (Novus Biologicals); anti-pFAK polyclonal rabbit, recognizes a sequence phosphorylated on tyrosine 387 (Thr397); anti-FAK rabbit monoclonal. Phospho-FAK (Tyr925) from Cell Signaling. Anti-phospho-Src (Tyr416) clone 9A6 Monoclonal antibody (Millipore). YAP (63.7): sc-101199 mouse monoclonal antibody. Purified Mouse Anti S100B from BD Transduction Laboratories. Anti-Beta Catenin, clone 7F7.2 monoclonal antibody (Millipore). PARP-1 (H-250): sc-7150 from Santa Cruz. P53 (Pab 246): sc-100 from Santa Cruz. Alpha- Smooth Muscle Actin antibody from Cell Signaling.

Secondary antibodies: Amersham ECL Anti-Mouse IgG, Horseradish Peroxides- Linked Species- Specific Whole Antibody (from sheep) from GE Healthcare. Amersham ECL-Anti-rabbit IgG, Horseradish Peroxides- Linked Species-pecific Whole Antibody (from donkey) from GE Healthcare.

Inhibitors, growth factors and others: Purified Mouse Anti-Growth Factor Receptor Bound Protein-2 (GRB2) from BD Transduction Laboratories. Recombinant Human Heregulin- Bet1from PeproTech. FAK inhibitor (1, 2, 4, 5.Benzenetetraamine tetra-hydrochloride) from Sigma-Aldrich. Beta-Gal (14B7) Mouse mAb from cell signaling.

Matrigel/ Matrix from CORNING. Human PDGF-BB from PeproTech. Fibronectin Serum Bovine from SIGMA ALDRICH. Fibronectin Solution from Human Fibroblasts from Sigma Aldrich. MEK inhibitor(Selumetinib), PD-98059 CALBIOCHEM. Rh EGF and FGF from R&D Systems. 3-IsoButyl-1-MethylXanthine (IBMX) Sigma.

Methods:

Immunoprecipitation (IP) and western blotting (WB)

Protein immunoprecipitations were carried out on total cellular extracts. Lysates were pre-cleared by incubating them with protein A-Sepharose beads (Sigma) for 1 hour at 4°C; they were then incubated in agitation for 18 hours at 4°C with the antibody conjugated to fresh protein A-Sepharose beads. Where indicated, an unrelated antibody was added as a negative isotype control. Beads were then washed several times in lysis buffer.

Proteins extracted from total cell lysates or from immunoprecipitations were then boiled for 5 min in Laemmli sample buffer, separated in reducing conditions on SDS-polyacrylamide gels and transferred onto Hybond-C Extra membranes (Amersham) following standard methods. Primary antibodies were incubated over night at 4°C.

Preparation for Immunofluorescence staining of 3D model of cells embedded in Matrigel for confocal microscopy:

Reagents:

10X Phosphate Buffered Saline (PBS): To prepare 1L add 80g sodium chloride (NaCl), 2 g potassium chloride (KCl), 14.4g sodium phosphate, dibasic (Na2HPO4) and 2.4g potassium phosphate, monobasic (KH2PO4) to 1L dH2O. Adjust pH to 8.0. Methanol, 100X. Permeeabilization Buffer: 0.1% Triton X-100 in PBS. Blocking Buffer (1X PBS/ 1% BA). Fluorochrome-conjugated secondary antibody in PBS.

Procedure:

Aspirate Medium. Rinse cells 1X with PBS to remove serum. Cover cells to a depth of 2-3 mm with ice-cold 100% methanol for 15 min at -20°C. Rinse 3X, 5min each. Permeabilize using 0.1% Triton X-100 in NH4Cl 50mM for 5 min

in Room Temperature (RT). Rinse 3X, 5min each. Blocking (1X PBS/ 3% normal FBS, 1% BSA) 1 h RT. Rinse 2X rapidly and place the primary antibody 1h RT. (Beta catenin and S100B). Rinse 3X, 10 min each. Secondary antibody in 2% FBS in PBS, dilution 1:500 for 45 min in RT. Rinse 3X with PBS 10 min each.

Matrigel protocol:

The day before the experiment put an aliquot of matrigel in the fridge and put in the refrigerator, even the plates and pipette tips. At the day of the experiment purr 300ul of matrigel into the 24 wells. Incubate at 37 degrees for at least half an hour.

Remove cells, centrifugate them, purr them in DMEM 4% serum and counting them. 50000 Cells must be brought to 0.5 ml of DMEM 4% serum (such an amount of cells is for 2 wells) add to the cells 0.5 ml of assay medium (or always in 1-1 ratio with the volume of cells.) plate in each well 0.5 ml final solution of cells thus obtained composition of assay medium DMEM 10 ng/ml PDGF (the stock solution is 40 ng/ul) Matrigel 4% (so that the final solution containing Matrigel 2 %).

Isolation of SCs from neurofibromas:

Frozen tumor pieces were thawed, mechanically dissociated and further digested in DMEM, 10% serum, 500 U/ml penicillin/streptomycin, 160 U/ml collagenase type 1 and 0.8 U/ml dispase grade1. After an incubation period of 18-20 h at 37° C and 10% CO_2 in the medium, tissue pieces were completely dissolved by triturating with a narrowed Pasteur pipette. The resulting cell suspensions were transferred to a 50 ml Falcon tube containing DMEM with 10% FBS, centrifuged at 3080 g for 10 min and resuspended in Schwann Cells Medium (SCM) composed of DMEM, 10% FBS, 500 U/ml penicillin/treptomycin, 0.5mM 3-iso-butyl-L-methylxanthine, 10 nM beta-heregulin, 0.5 uM forskolin and 2.5 ug/ml insulin. SCM was freshly prepared for each feeding. Cells were seeded at a density of 25000 cells/cm^2 onto six-well plate or onto eight-well plastic labtek slides; both coated with 1 mg/ml poly-L-lysine and 4 ug/ml natural laminin. Cultures were incubated in a humidified atmosphere at 37°C and 10% CO_2. SCM was changed twice a week and cells are passaged when were confluent (usually after 5-7 days).

3. RESULTS

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At our very early studies we worked on the protein neurofibromin (*Nf1*) which was conducted *in vitro* using an experimental model, murine embryonic cells derived from mice wild type (MEF $Nf1^{+/+}$), heterozygous (MEF $Nf1^{+/-)}$ and homozygous (MEF $Nf1^{-/-}$) for *NF1* gene, kindly granted by Prof. Stein from University of Telaviv. We believed these cells were a valuable model in that they are FBRs, as stated previously, which represent one of the cellular components that contribute to formation of neurofibromas. In addition, the MEF cells expressing native levels of neurofibromin may be a starting model to explore the physiological role of *Nf1*.

The first aim was to validate the experimental system, i.e. to verify that the MEF cells $Nf1^{-/-}$ were really missing the *Nf1*, either directly by controlling the expression of the protein, and indirectly by exploring the status of ERK the Ser/Thr kinase downstream of Ras.

As shown in figure 1 which shows an experiment Western blotting with commercial antibody directed against the *Nf1*, the protein was clearly recognized in sharp bands and in agreement with the genotype cell (a band charge in native cells, a band poorer for the heterozygous cells, the absence of a band in cells lacking neurofibromin).

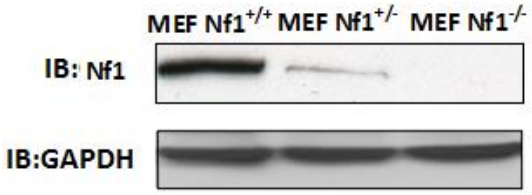


Figure1: Western blotting anti-*Nf1* and anti-GAPDH on cells MEF $Nf1^{-/-}$, $Nf1^{+/-}$ e $Nf1^{+/+}$

In the next experiment, described in Figure 2 we have tested in western blotting the phosphorylation levels and therefore the basal activation of the kinesis ERK in only two cell types, on which we focused our research: MEF $Nf1^{+/+}$ and MEF $Nf1^{-/-}$. Previous studies have in fact shown that *Nf1* has the function of Ras-Gap or negatively controls the activity of Ras. It is therefore expected, that in cells where *Nf1* was missing, Ras would have higher activity than to the cells where *Nf1* was expressed in a physiological manner. The obtained data, together, confirm that in the MEF $Nf1^{-/-}$, the *Nf1* is not expressed and, consequently, Ras, is more active.

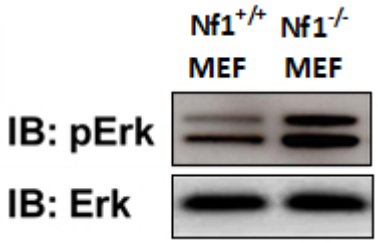


Figure2: *Western blotting* anti-pERK and anti-ERK on cells MEF $Nf1^{-/-}$, $Nf1^{+/+}$

Focus forming assay:

In order to verify whether the cells which are not expressing *Nf1* had acquired transforming capacity, was conducted the assay of Focus Forming. This assay allows observing the cell growth in the absence of adhesion to the substrate which is another typical "hallmark" of transformed cells. The non-transformed cells, in fact, survive only in adherence to the basal membrane that contributes to the orderly architecture of the tissue. Before cellular seeding, plastic plates were treated (coating) 12 hours with 0.3 µg/ml poly-lysine per well. The use of this synthetic molecule creates an adhesion surface that mimics the basal membrane. Once plated, the cells are kept for 21 days in low serum (0.5 %) and are treated every three days with PDGFBB (2ng/ml and 10ng/ml) in order to observe a dose-response to growth factor. After five days we observed the appearance of foci in MEF cells $Nf1^{-/-}$ while all cells MEF $Nf1^{+/+}$ were detached. The "foci" are cellular aggregates composed of living cells, proliferating in the absence of nutrient substrates. In the following days, the foci increased in number and magnitude, parameters that direct to the transforming power of some cells. The foci measurement indicates that the MEF $Nf1^{-/-}$ cells display transforming potential increased in presence of the PDGFBB ligand. As shown by Figure 3, while in absence of the growth factor PDGFBB we observe only three foci, these increase to 21 in cells treated with 2ng/ml and 46 with 10ng/ml as measured by using a program specially designed for the purpose.

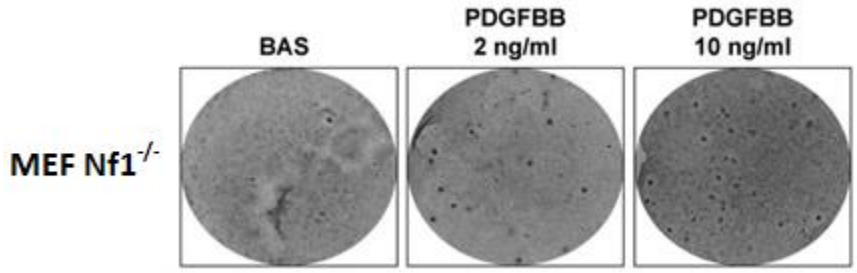


Figure3: *Focus forming assay* to show the potential of developing tumors by MEF $Nf1^{-/-}$ cells via stimulation with growth factor PDGFBB

Analysis of molecular cascades downstream of Ras:

Our aim was to investigate the activation state of "transient" effectors downstream of Ras in both cell types MEF $Nf1^{+/+}$ and MEF $Nf1^{-/-}$ cells. The serine/threonine kinases ERK and Akt are involved respectively in the transcription downstream of the factors of anti apoptotic pathways. Loss of control of the process of cell replication and insensibility to stimuli of death represent two peculiar characteristics of the tumor cells. In the next experiment which is compared, in kinetics, the activation of molecular effectors downstream of Ras, proteins with activities serine-threonine kinesis Erk and Akt in presence and absence of neurofibromin are shown. For this purpose, the MEF $Nf1^{+/+}$ and MEF $Nf1^{-/-}$ were stimulated with the growth factor PDGFBB since it is known that its receptor tyrosine kinase PDGFBBR expressed in MEFs, powerfully stimulates Ras and plays an important role during the formation of the neurofibromas. The experimental conditions were the following: 5 min of stimulation with the ligand PDGFBB are able to make the receptor PDGFBBR's levels in maximum activation that progressively decreases in times of longer stimulation. The cells were stimulated in kinetics, with 10ng/ml of PDGFBB, as a result of a depletion of nutrients for 48h. Depletion of serum has a dual function: to synchronize all of the cells at the same point of the cell cycle (G1) and to "turn off" any other signal transduction pathway due to the presence of growth factors and then to zero the contribution of other stimulations. The latter aspect is very important since kinase as ERK are downstream of many molecular cascades, our need instead was to observe the effect on the kinase activity of ERK mediated purely from the receptor PDGFBBR and the Ras-GAP that control Ras. Differences

between enzymatic activity of ERK in native cells and gene *NF1* missing cells, determine the more suitable time windows for studying the enzymatic activity in these cells.

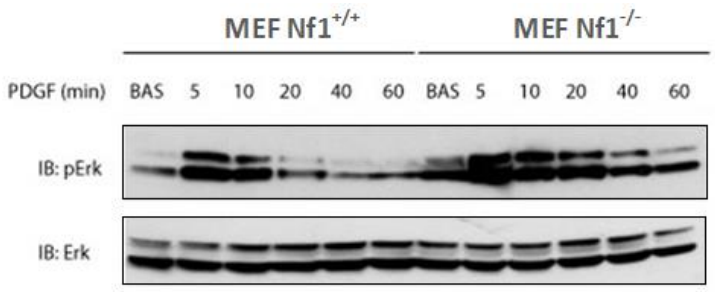


Figure4: *Western blotting* with primary antibodies against pERK and total ERK on the cell lyses of MEF $Nf1^{+/+}$ e $Nf1^{-/-}$ and stimulated with PDGFBB.

The analysis of the experiment highlights a difference in total protein between the two cell types; therefore, it will not be possible to compare the absolute values of activation of the kinases but only the pattern of their activation curve in kinetics.

The kinetics analysis evidences that, as expected, ERK has the greatest activation in cells native already at 5 min of stimulation. As previously noted, there is difference in the levels of activation of ERK (pERK) already in the two basal of MEF $Nf1^{+/+}$ and MEF $Nf1^{-/-}$ not stimulated. In fact, in cells lacking of neurofibromin the balance between the activated form of ERK is the inactive moves toward the active, even without PDGFBB indicating that the effect of the Ras-GAP is significant even in the absence of stimulation. Noticeable, kinetics of activation of ERK between the two cell types following stimulation with the growth factor is different: in native cells, the protein kinase shows a peak of activation already after 5 min of stimulation, similarly to what happens in cells lacking of *Nf1*. It is interesting to note that while in latter the activation levels of ERK continue to be supported up to 20 minutes of stimulation in the MEF $Nf1^{+/+}$, levels of pERK decrease already after 10 minutes, until returning to basal levels at 20 minutes.

It was subsequently investigated also the transient activation of serine/threonine kinase Akt. The Akt is activated by phosphatidyl-inositol 3-kinas PI3K as a result of direct activation of PI3K by the receptor PDGFBB or indirect by

Ras. Therefore, in our experimental model Akt should be very informative because its activity is mainly due to the activation of the receptor and secondly by Ras.

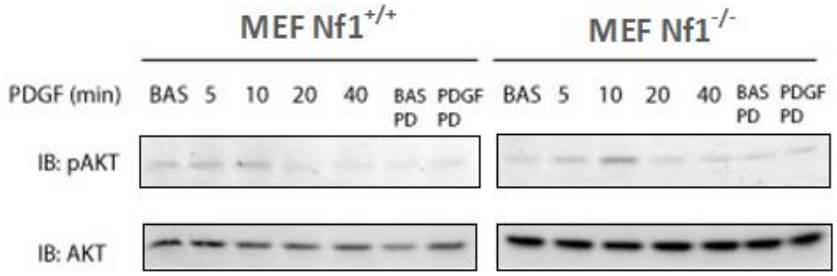


Figure 5:. Western blotting anti pAkt and anti-Akt on cell lysates MEF $Nf1^{+/+}$ and MEF $Nf1^{-/-}$ treated in kinetics with PDGFBB.

In this case, comparing the two basal cells of MEF $Nf1^{+/+}$ and MEF $Nf1^{-/-}$, there seem to be no differences in the levels of pAkt. A careful analysis, however, aided by quantification of signal density carried out with NIH program, reveals a significant and widely reproduced difference between the kinetics of activation of Akt in MEF $Nf1^{+/+}$ and MEF $Nf1^{-/-}$ following stimulation with the PDGFBB. In MEF $Nf1^{+/+}$ the difference between the basal levels of pAkt and those 10 minutes of treatment is shorter than the increase seen in 10 minutes in MEF $Nf1^{-/-}$, indicating that Akt also suffers from the activity of *Nf1* when present, but unlike ERK, for which the presence or absence of *Nf1* influence the time of inactivation, for Akt instead the opposite seems true. This could be explained by the fact that the phosphorylation of Akt is a contribution of Ras .Then if Ras is more active, Akt also is more active.

The next question that we posed was whether the transforming potential of the cells following stimulation with PDGFBB is depended on other signaling pathways or not. During the Focus forming, for example, we have noticed that the MEF $Nf1^{+/+}$ cells are detached from the plate as a result of depletion of serum while the MEF $Nf1^{-/-}$were still attached. This was an opportunity to hypothesize that *Nf1* could somehow influence the ability of the cell to attach the substrate. In the literature it is reported that *Nf1* interacts and regulates FAK, a key protein in the network receptor of growth factors-integrins. Of the few existing jobs on *Nf1*, one in particular shows the importance of the functional interaction *Nf1*-FAK for the adjustment of focal adhesions, structures that are crucial to the accession and the cell movement. To formulate a suitable case we have investigated whether FAK could contribute to the neoplastic phenotype.

We explored the phosphorylation site on amino acid tyrosine of FAK, following stimulation with PDGFBB in the presence and absence of *Nf1*.

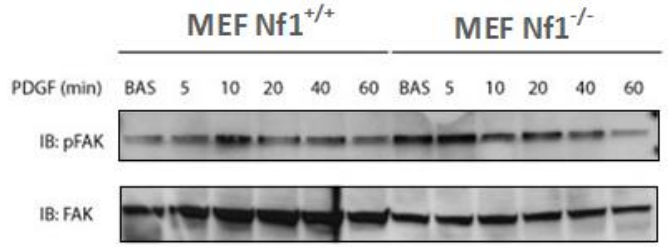


Figure6: Western blotting anti pFAK and anti-FAK on cell lysates MEF $Nf1^{+/+}$ and MEF $Nf1^{-/-}$ treated in kinetics with PDGFBB.

In this experiment is evident that the FAK phosphorylation at the y397 site is already present in the $NF1^{-/-}$ MEF cells in absence of PDGFBB stimulation indicating that the absence of *Nf1* may affects this phosphorylation. Further, FAK phosphorylation kinetics is different between the two cell types following stimulation with PDGFBB.

To investigate the reason why the FAK is more active in MEF $Nf1^{-/-}$, we explored the possibility that these cells started to produce them self growth factors and thus basically stimulate FAK. We collected the MEF $Nf1^{-/-}$ supernatant from confluent flasks. Then MEF $Nf1^{-/-}$ and MEF $Nf1^{+/+}$ were starved for 48 hours were stimulated in different minutes by MEF $Nf1^{-/-}$ supernatant and PDGFBB to also make a comparison between the potential of these two stimulators. We observed that MEF $Nf1^{-/-}$ supernatant can also stimulate the cells, confirming the presence of growth factors in the medium.

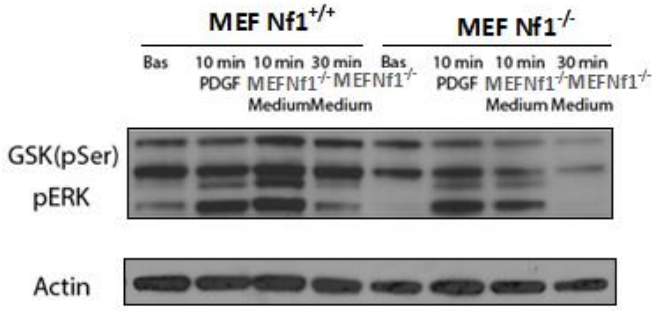


Figure11: Stimulation of MEF $Nf1^{+/+}$ and MEF $Nf1^{-/-}$ with two different stimulators; 1- PDGF-BB and 2- Medium conditioned of $MEFNf1^{-/-}$

Next, we asked if *Nf1* interacts with FAK. We immuonoprecipitated neurofibromin and we detected the co-immunoprecipitation of FAK with neurofibromin.

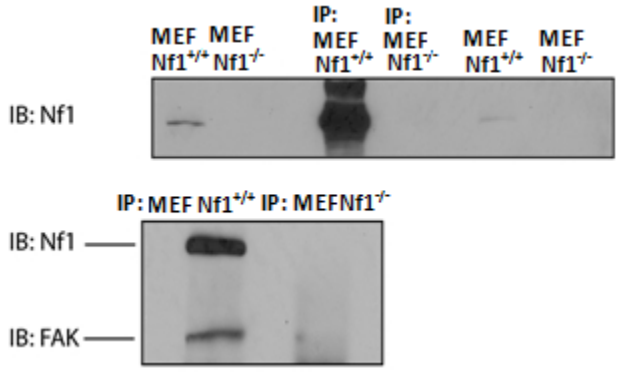


Figure12: Immunoprecipitation of *Nf1* with FAK

We also investigated the possible influence of the PDGFBBR on the phosphorylation of serine 2515 of *Nf1*. This site of phosphorylation is one of the sites identified in work of phosphoproteomic as activated by growth factors and whose biological meaning is still unknown. This study reveals that the serine residue 2515 possesses a regulatory activity of *Nf1* by negatively regulating its function. The experiment was conducted in western blotting with a new phospho-specific antibody directed against the Ser2515 of the human protein homologous to the murine on MEFs stimulated or not with PDGFBB for 5 minutes.

The results, unfortunately, gave a weak indication about a possible phosphorylation in Ser of *Nf1*.

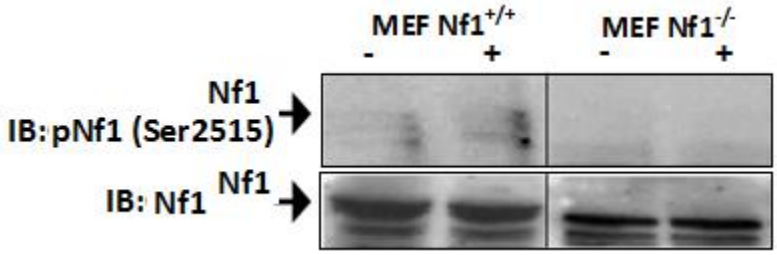


Figure7: *Western blotting* anti-p*Nf1* and anti-*Nf1* on the cell lysates of MEF $Nf1^{+/+}$ e MEF $Nf1^{-/-}$ with and without stimulation with PDGFBB.

In order to understand the different level of phosphorylation of Src and GSK, we performed dose response experiments on the MEF $Nf1^{-/-}$ and MEF $Nf1^{+/+}$ stimulated or not with PDGFBB. The activities of the Src family of non- tyrosine kinase receptors have been implicated in tumor cell invasion [reviewed by Irby and Yeatman, 2000].

Src activity is significantly higher in metastatic tissues than in normal tissues [Talamonti et al., 1993]. Furthermore, v- src is constitutively activated form of c- src is more potent than activated Ras in generating highly metastatic cells [Tatsuka et al., 1996]. We found that Src kinase activity is higher in MEF $Nf1^{-/-}$ in comparison with MEF wild type.

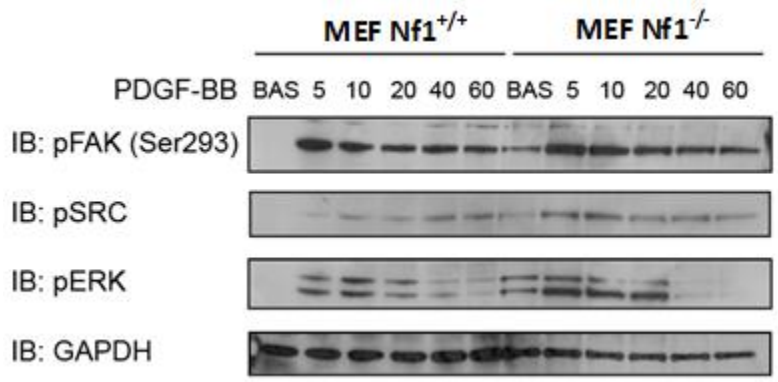


Figure8: PDGF-BB 10ng/ml used for kinetic stimulation of MEF $Nf1^{+/+}$ and MEF $Nf1^{-/-}$ cells after 48 h in serum deprivation

Next, we started a preliminary analysis of the pathways known to be involved in the Cell/ECM route. We have observed that in MEF $Nf1^{-/-}$, FAK is more activated even in absence of growth factors or integrin stimulation, and cell treatment with PDGFBB further increases FAK phosphorylation through phosphospecific antibody against the Tyr 397 and cell proliferation.

To test the importance of FAK in tunmorigenesis we performed several tumorigenesis assays. These experiments were done in matrigel 2% of serum in presence and absence of Collagen and PDGFBB treated with Selumetinib (Sel) 10uMm or Y15 (FAK inhibitor) 50 uM during 3 days. As shown in figure 9, the absence of *Nf1* in immortalized cells is a sufficient stimulus to promote in vitro tummorigenesis. ERK1/2 and FAK inhibitors counteract this tumorigenic phenotype suggesting the involvement of FAK in this process.

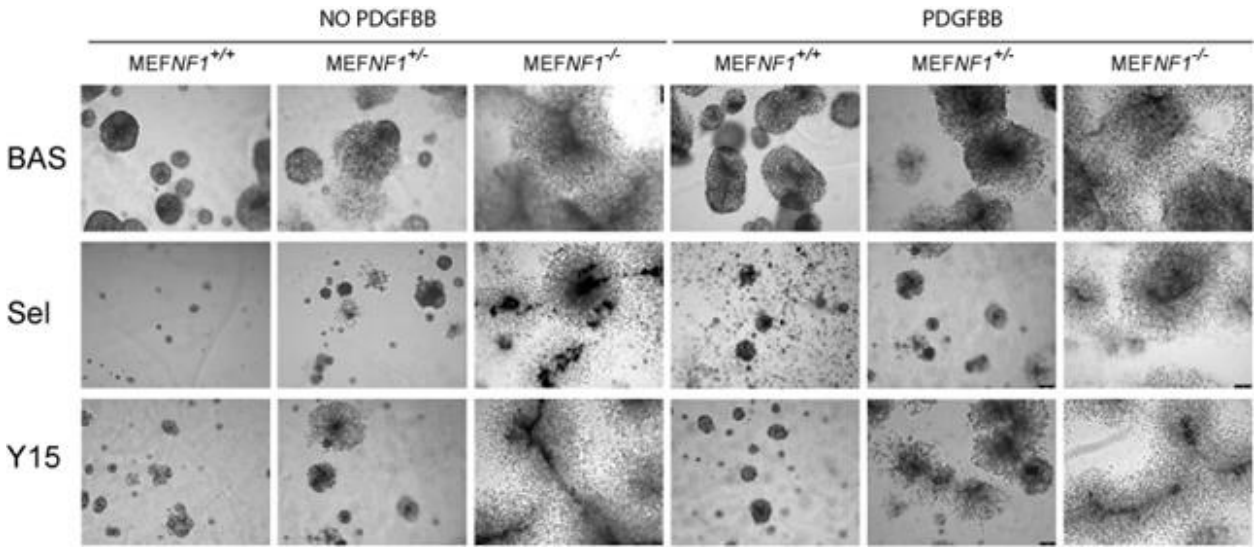
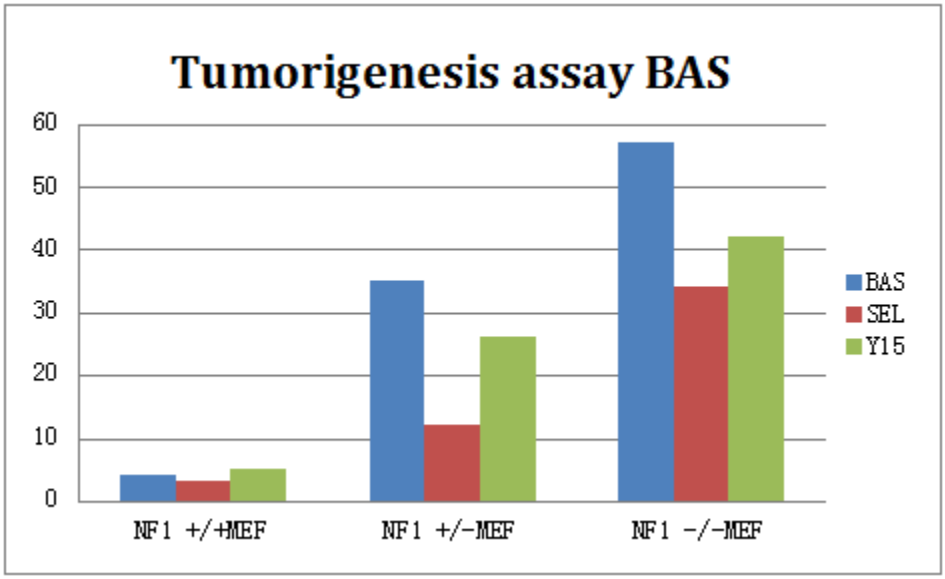
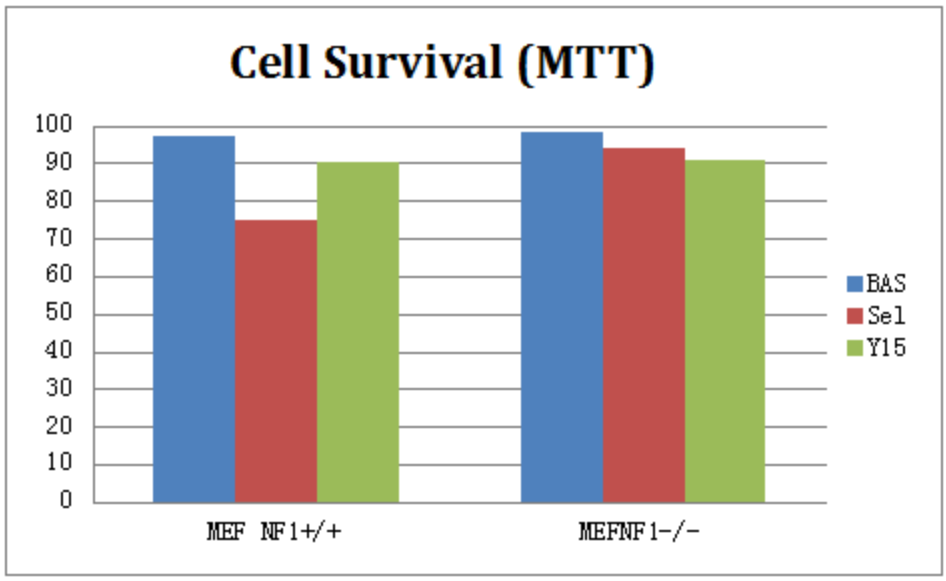


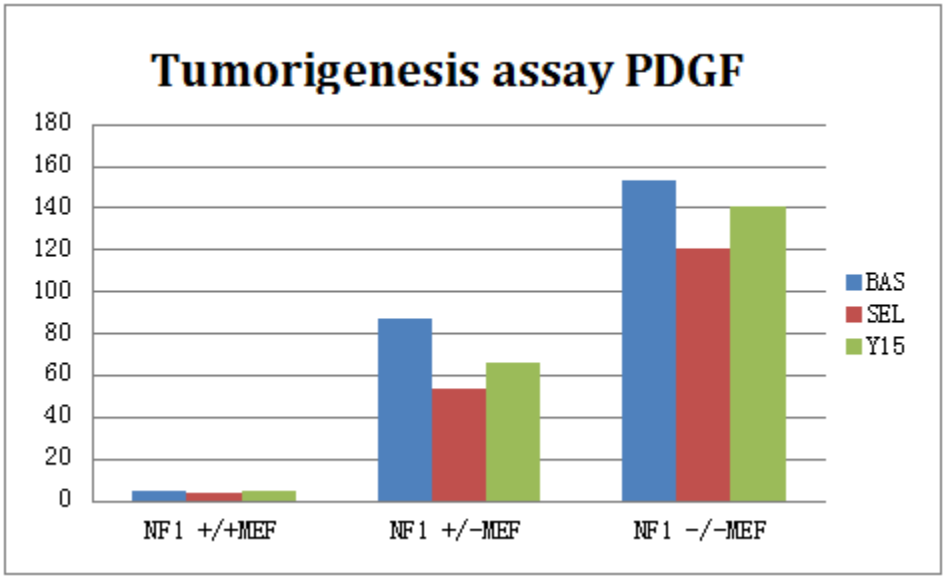
Figure9: Cell treatment with 40uM of MAPK inhibitor and 50uM of FAK inhibitor in presence/ absence of PDGFBB stimulation.



a) In this histograms we observe that FAK inhibitor Y15 decreases the number of colonies (this is not toxic in 2D as it shown in histogram bellow) showing that FAK has a role in tumorigenesis of MEF immortalized cells.



b) This histogram shows that Y15 and Sel inhibitors are not toxic.



c) Sel inhibitor decreases the formation of colonies.

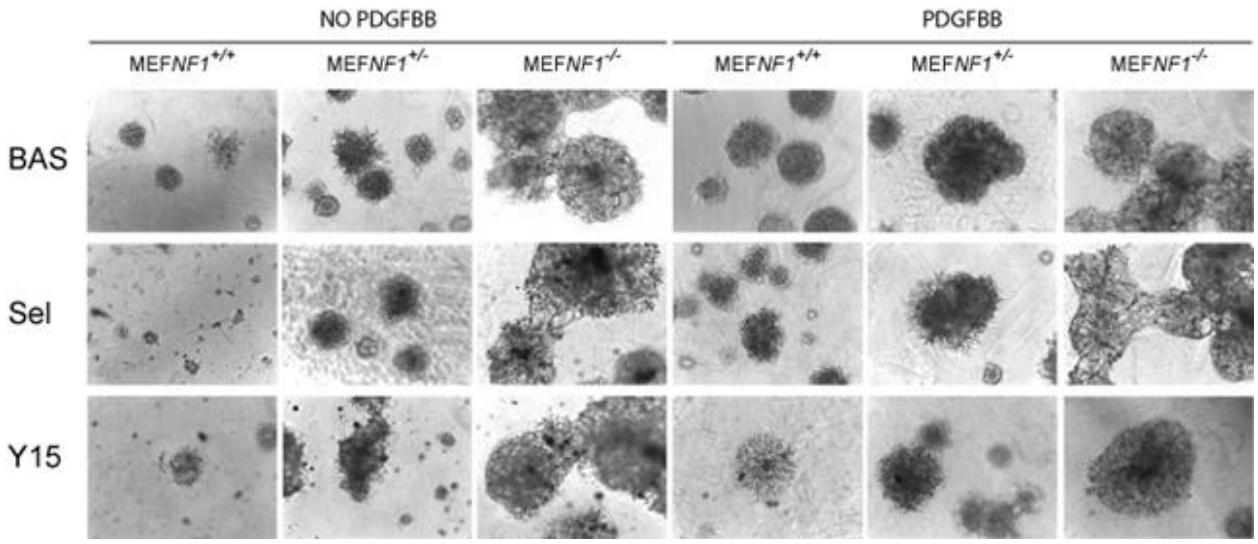
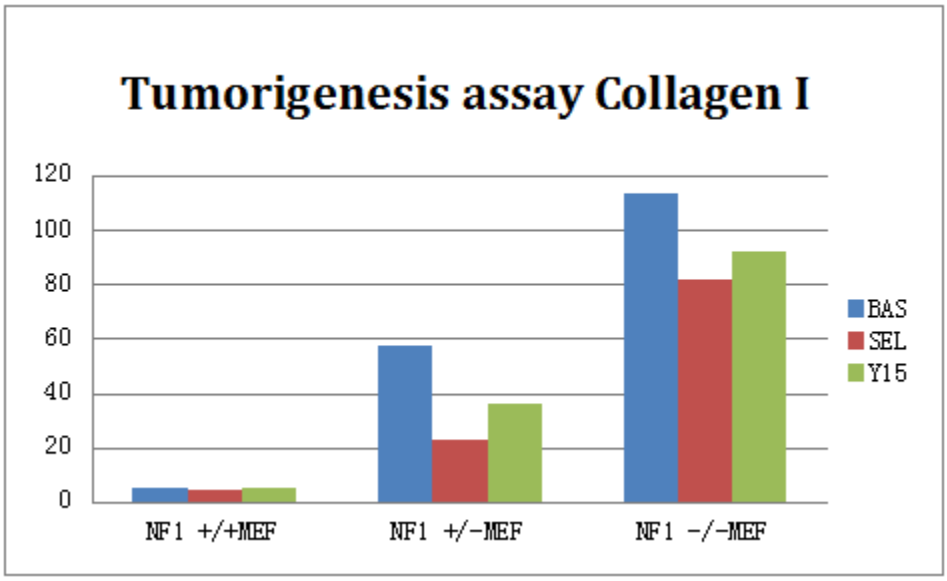
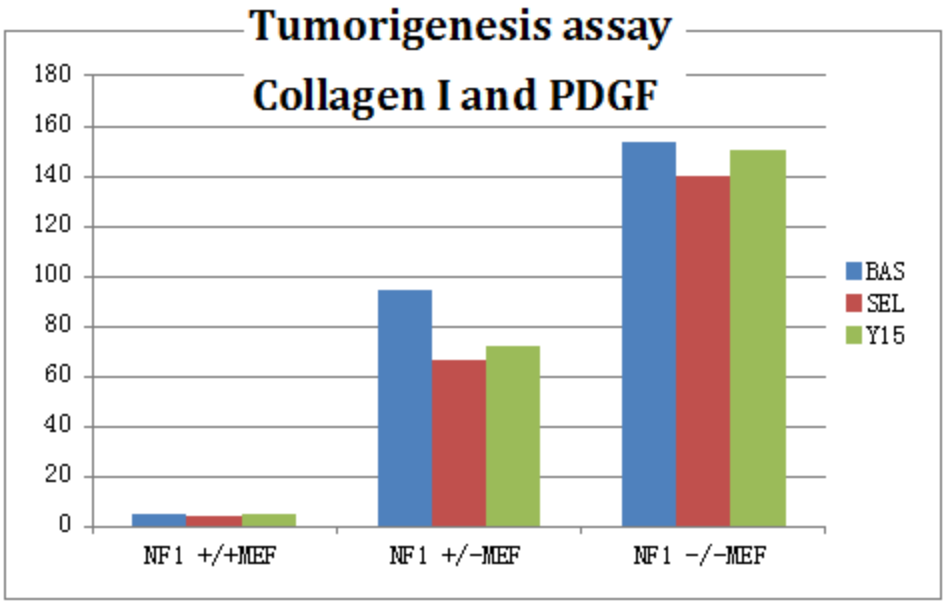


Figure10: Treating cells in presence of Collagen with 40uM of MAPK inhibitor and 50uM of FAK inhibitor. PDGFBB increases the number and size of colonies that look much dense in presence of collagen.



a) The y15 is less able to affect tumorigenesis in presence of collagen in MEF Nf1$^{-/-}$.

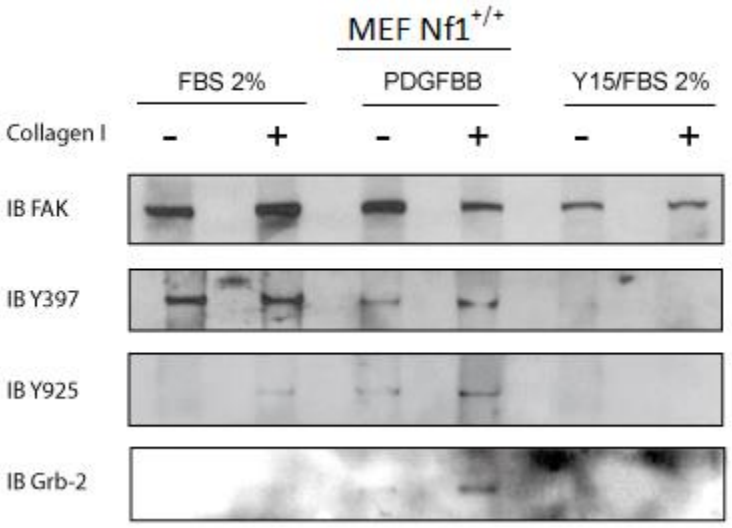


b) Y15 and Sel are even less effective in presence of both Collagen and PDGF in MEF $Nf1^{-/-}$.

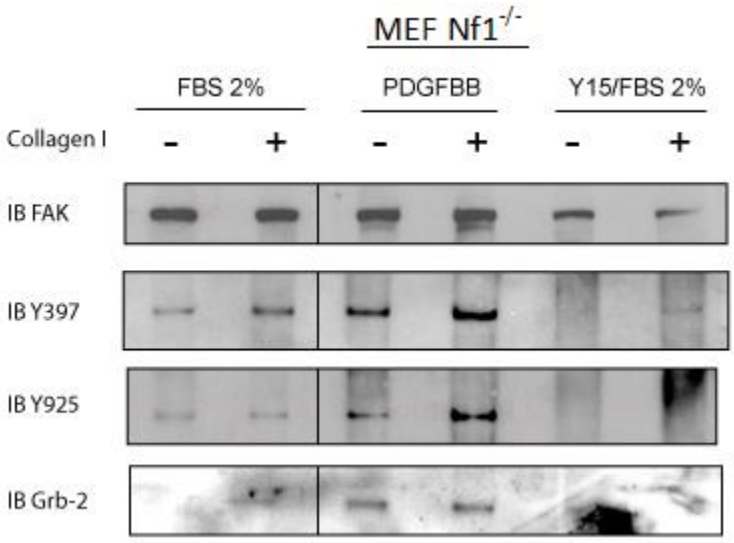
Taken together, our data indicate that PDGFBB and the absence of *Nf1* cooperate to induce a tumorigenic phenotype through FAK activity. Consistently our previous data showing that in $Nf1^{-/-}$ FAK is more phosphorylated, the FAK inhibitor Y15 in these cells affected tumorigenesis whereas the collagen stimulation markedly enhanced this phenotype.

Next, we asked whether FAK contribuites to tumorigenesis. Our hypothesis was that FAK increases Ras activity through Grb-2. To assess this hypothesis, we performed an immuonoprecipitation of FAK in the same culture condition of the tumorigenic assay and we monitored the phosphorylation of Tyr397 and Tyr925. Phosphorylated Tyr 397 site of FAK binds to and phosphorylates Src that in turn phosphorylates Y925 triggering Grb2 binding (see figure 13).

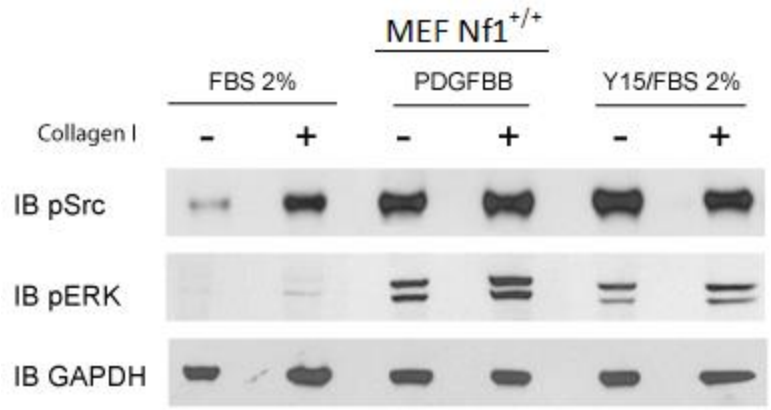
Figure13: FAK increases Ras activity through Grb-2 binding



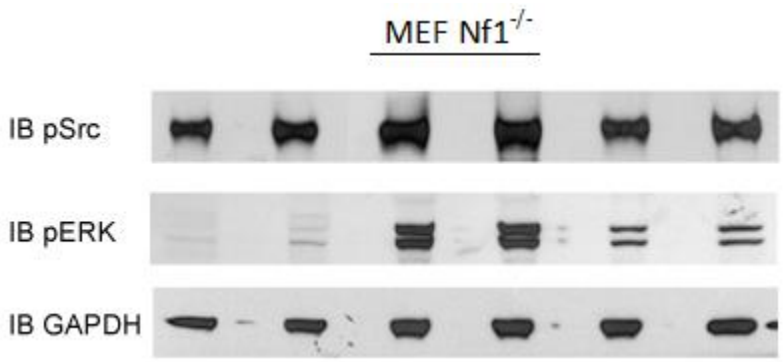
a) Different phosphoryation sites of FAK in MEF Nf1$^{+/+}$.



b) Here we observe that following phosphorylation of FAK at Tyr 397 site after PDGFBB and /or collagen stimulation, Src binds to and phosphorylates Y925 which in turn binds Grb2 in MEF Nf1$^{-/-}$. We can also observe that in MEF Nf1$^{-/-}$ Grb2 co-precipitates independently from collagen in presence of PDGFBB.



c) We observe the phosphorylation of Src and ERK in MEF $Nf1^{+/+}$ and make a comparison with MEF $Nf1^{-/-}$.



d) As depicted in this experiment we notice the phosphorylation of Src in MEF $Nf1^{-/-}$.

In accordance to our results, other studies have shown that loss of FAK in SCs results in decreasing of SCs' proliferation but does not affect process extension [Grove et., al 2007].

Similarly to our data, in NF2 tumors FAK has been found over-phosphorylated, suggesting similar molecular mechanisms at the tumor onset in NF1 and NF2 [Poulikakos et. Al, 2006]. In NF2 the ECM-mediated signalling is transduced by two co-transcription factors: YAP and TAZ. We therefore decided to explore also their potential involvement in the $Nf1^{-/-}$MEF tumorigenesis.

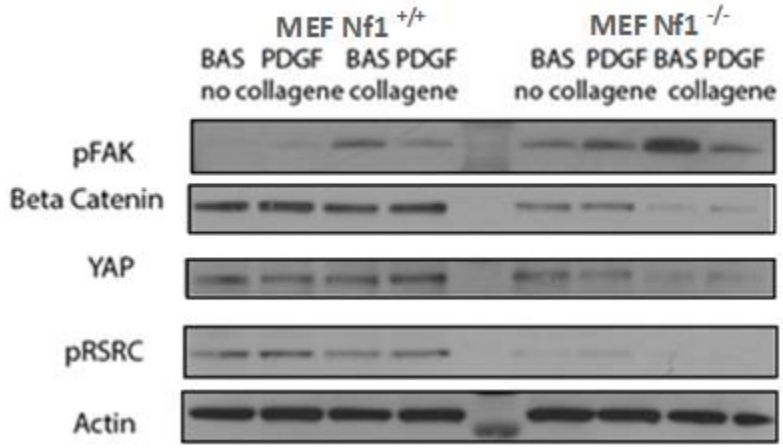


Figure14: Exploration of pFAK and protein levels of YAP and Beta Catenin by western blotting with specific antibodies on total lysates of MEF Nf1$^{-/-}$ & MEF Nf1 $^{+/+}$ cultured in different conditions: stimulated or not with PDGFBB in presence/absence of Collagen As depicted in this figure the protein levels do not significantly decrease in MEF *NF1*$^{-/-}$.

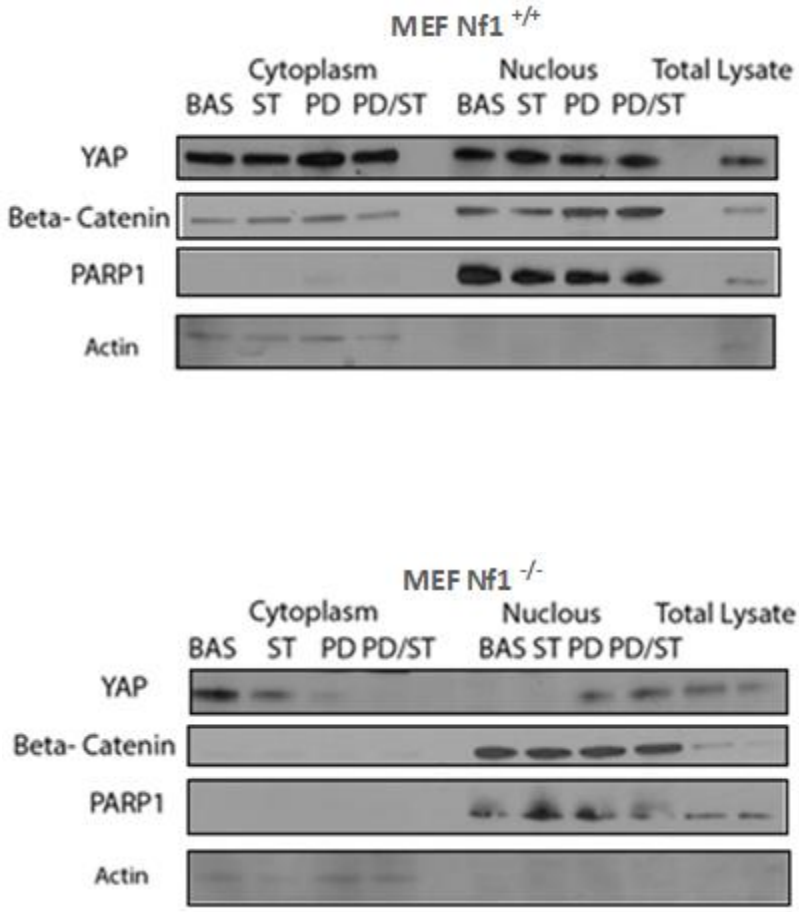


Figure15: Absence of *Nf1* favors translocation of Beta-catenin but not YAP and TAZ into the nucleus.

A conclusion of our very early experiments:

Neurofibromas are mainly composed of SCs, FBRs and strong collagen I deposition due to inflammatory cells infiltration. These tumors arise from SCs that undergo LOH at the *NF1* locus, which results in loss of functional neurofibromin in these cells and RAS hyper activation. Our hypothesis is that transformed phenotype is fostered by the amplification of integrated signaling pathways triggered by ECM, Ras hyper activation and loss of *Nf1*. We have preliminary data showing that *Nf1* interacts with and regulates FAK and affects Beta -catenin and YAP/TAZ activity. Further, we have found that lack of *Nf1* is sufficient to activate the key mediator of ECM signaling also in absence of external structural proteins stimuli. As next experiments we want to connect Ras or FAK with Beta -catenin and YAP/TAZ and tumorigenesis in 3D experiments at different collagen 1 concentrations in order to reinforce the hypothesis that *Nf1* loss might potentiate Ras hyper activation through FAK kinase that in turn receives and amplifies the ECM signal leading to neoplastic transformation.

Results and conclusion of the 3rd, last year:

How lack of *Nf1* may impact on the complexity of ECM-cell dynamic and how the great rigidity of the ECM in neurofibroma influences SCs behavior, is still unknown.

In the plexiform neurofibromas, the tumorigenic phenotype of SCs could be fostered by the amplification of integrated signaling pathways triggered by loss of *Nf1*, Ras hyper activation and deregulated ECM. Changes of the mechanical properties of ECM due to increased collagen secretion by mFBRs recognized in the *NF1* disease {Robert, 2014 #300} might actively contribute to tumor progression by influencing gene expression profile of the cells.

Preliminary results:

Primary SCs and FBRs isolation from Neurofibroma and their biological characterization:

We have already isolated and cultured in 2D SCs and Nf1 $^{+/-}$ FBRs according to Serra methodology {Serra, 2000 #210}. These cells have been isolated from plexiform neurofibroma biopsies after informed consent of patients by our Milan and Rome University collaborators. To get two populations of SCs and FBRs we have cultured cells in selective Medium ({Serra, 2000 #210}, and our new unpublished protocol) and biochemically characterized: S100B [Tucker, 2011 #263] and p75 markers specifically recognizing SCs and collagen I secretion, alpha smooth muscle actin (α-SMA) expression, Smad2/3 activation, Abl kinas activation characterizing mFBR activity [Kojima, 2010 #150].

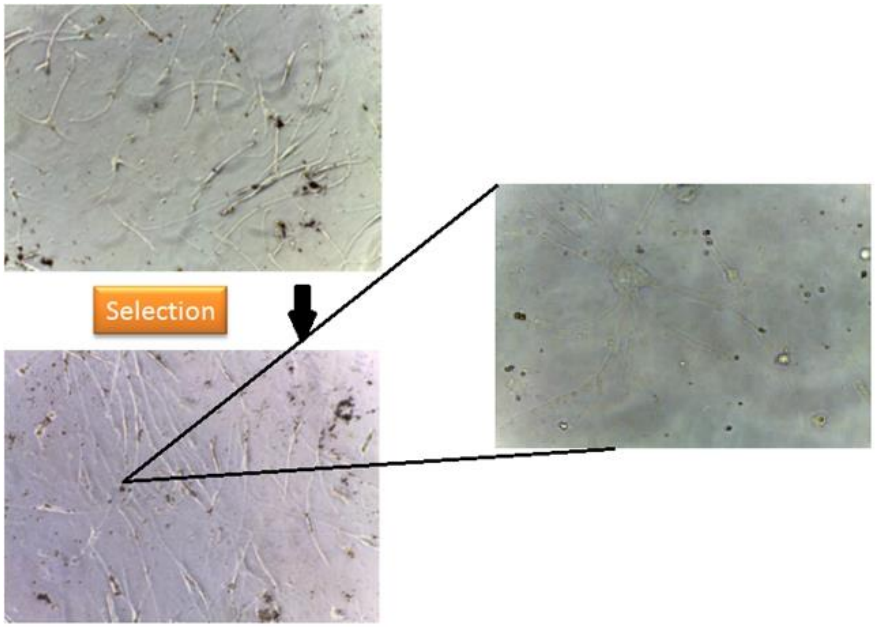


Figure16: The main cellular model: SCs and mFBRs in a Novel 3D Multicellular Model

Steps: 1) Cell isolation and cell culture in 2D on Fibronectin and Laminin 2) Cell election in two different cell lines: SCs and mFBRs

Immunofluorecence analysis of SCs and mFBRs isolated from neurofibroma

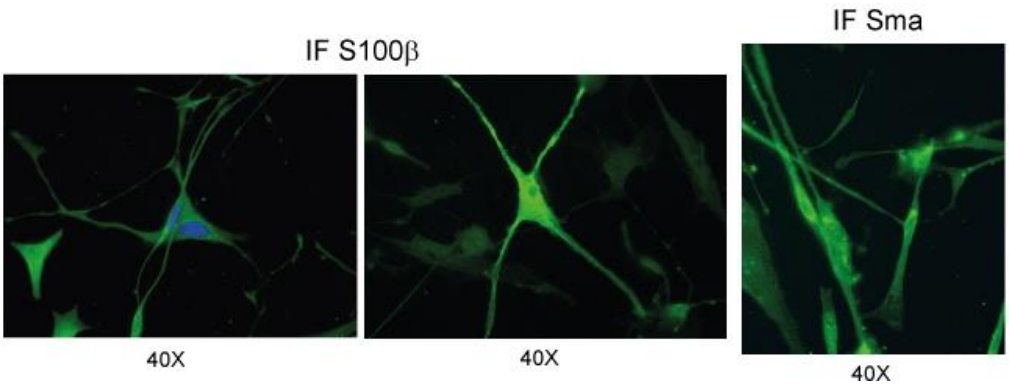


Figure17: SCs detection in vitro by immunoflouresence

Generation of the multicellular in vitro system 3D with soft ECM.

The neurofibroma-derived FRBs and SCs co-culture are performed in a transwell-like system: SCs are seeded within the upper chamber whereas the FBRs in the lower chamber. This system is suitable to perform the biochemical analysis keeping physically separated SCs and FBRs thus allowing the paracrine stimulation such as in the tumor.

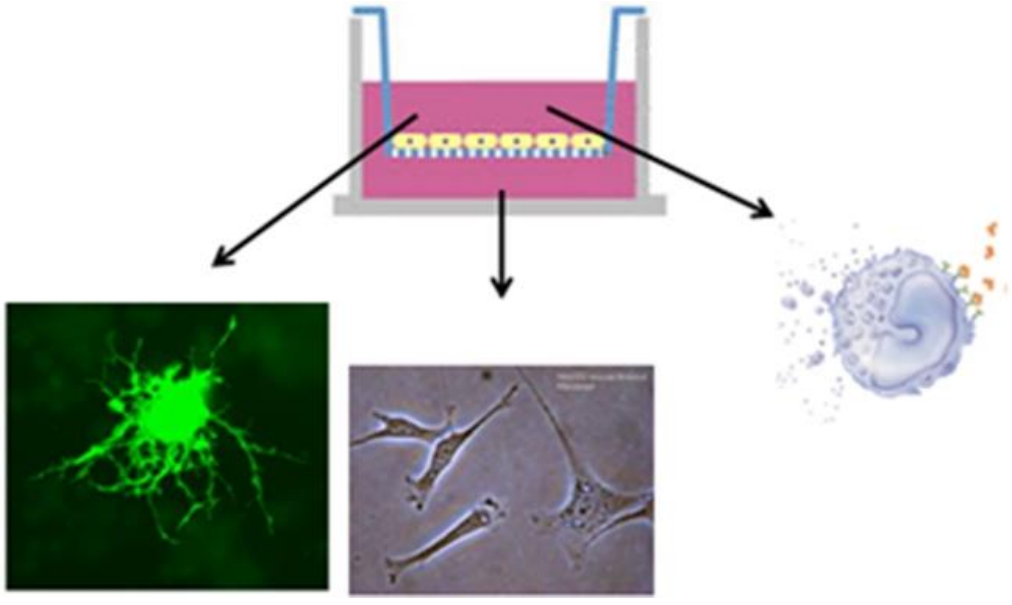


Figure 18: Plating cells separately in the transwell/like system. This permits the paracrine stimulation and biochemical investigation.

Cells are grown in Matrigel with exogenous 3D soft ECM/reconstituted basement membrane matrigel (soft gel: 0.7 Kpa). ECM composition: as in the plexiform neurofibroma 40 % of collagens (type I, III and IV) and adhesive ECM proteins as fibronectine and laminin; to keep the system closer to the *in vivo* model, we culture the mFBRs in conditioned medium by activated MCs and 15 mM L-ribose. As MCs we use LAD-2 cell line (kindly provided by collaborators of Naple University) that will be activated by Tumor necrosis factor-alpha(TNF-α) stimulation.

To characterize the SCs behavior in this experimental system, we will perform the canonical proliferation, apoptosis and senescence assays in Matrigel [Gomez-Sanchez, 2013 #302].

Generation of the multicellular in vitro system 3D with stiff ECM.

We will explore the effect of increased stiffness on SCs and FRBs neurofibroma-derived cells embedded in a 3D stiff ECM/reconstituted basement membrane matrigel. To get this goal we will investigate precisely the stiffness range of the plexiform neurofibroma determined by rheology assessment by our Padua University collaborators on 20 samples histological and genetically characterized from different patients. In general stroma stiffness in tumors ranges from 5000 to 40000 Pa [Levental, 2009 #303]. Subsequently, we will reproduce *in vitro* different pressure values measured *ex vivo* by increasing collagen I and collagen IV concentration in presence of non metabolizable linkers such as L-ribose and we will observe the SCs growth ability.

Our preliminary data show that primary SCs cells generate small colonies only when plated in a ECM/reconstituted basement membrane matrigel of at list 700 Pa.

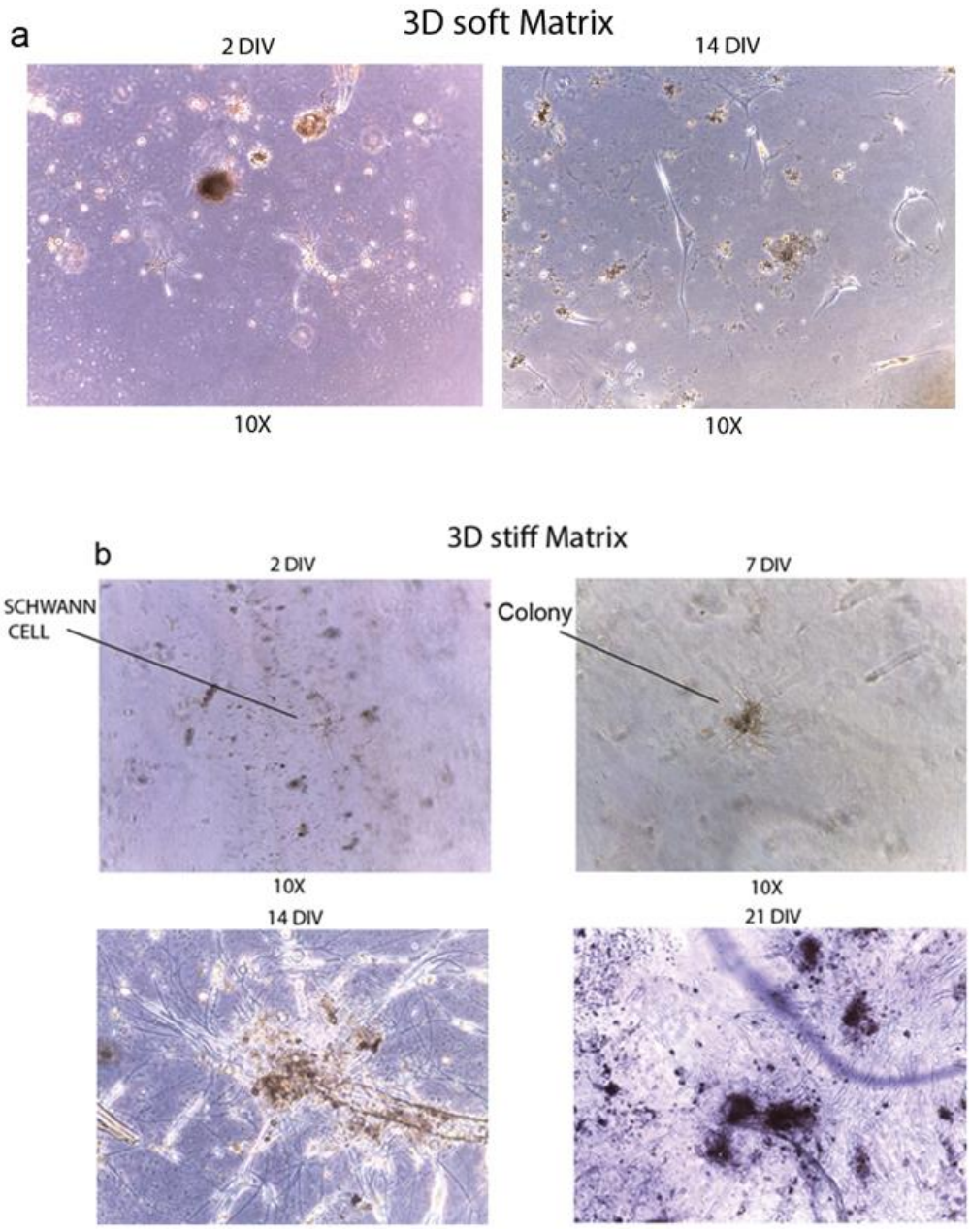


Figure 19: SCs isolated from Plexiform Neurofibroma selectively separated from FBRs and plated in 3D collagenI Matrigel. a) Primary SCs cultured in soft/ standard Collagen I Matrix in presence of mFBRs and MCs-conditioned medium growing in soft/ standard Collagen I Matrix. NOTE: CELLS SURVIVE BUT DO NOT PROLIFERATE. **b)** Primary SCs cultured in stiff Collagen I Matrix in presence of mFBRs and MCs-conditioned medium.NOTE: CELLS PROLIFERATE AND FORM A COLONY.

4. CONCLUSION

4. CONCLUSION

As in other tumors, also in the plexiform neurofibroma, the tumorigenic phenotype of SCs is fostered by the amplification of integrated signaling pathways triggered by loss of *Nf1*, Ras hyper activation and deregulated ECM. Changes of the mechanical properties of ECM due to increased collagen secretion by mFBRs might actively contribute to tumor progression by influencing gene expression profile of the cells through the enhancement of Ras signaling pathway triggered by FAK. We asked ourselves how FAK possibly could contribute to tumorigenesis"? In mouse *NF1*$^{-/-}$ FBRs, we have found that the absence of Neurofibromin correlates with deregulation of FAK Y397 and Y925 phosphorylations both in absence of integrin clustering and after ligand stimulation. Further, the tumorigenesis assay showed that MEFNf1$^{-/-}$ ability to form colonies is affected by both MECK inhibitor and FAK inhibitor Y15 indicating the cooperative role of FAK and PDGFBB growth factor in tumorigenic process mediated by *Nf1*. Consistently, immunoprecipitation experiments showed that in *Nf1* null cells, Grb-2, the RAS pathway initiator, interacts with FAK also in absence of collagen in a PDGFBB ligand-dependent way, thus suggesting that FAK and growth factor receptors can cooperate to increase the Ras activity to a threshold required to induce tumorigenesis.

Our novel three-dimensional experimental model *in vitro* reproduces the multicellular complexity of neurofibroma with primary cells. We are figuring up proper ECM composition and stiffness in matrigel for SCs transformation and neurofibroma's formation. We have already obtained colonies of SCs growing in 3D *in vitro* system. Our preliminary data shows that primary SCs generate colonies only when plated in an ECM/reconstituted basement membrane Collagen I-Matrigel of at list 3 mg/ml. However, we have still to set up the culture conditions to keep cells in highly proliferating state.

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ABBREVIATIONS

(2D)	Two- dimensional
(3D)	Three- Dimensional
(a.a)	amino acids
(ALPS)	Autoimmune Lymphoproliferative Syndrome
(AMPK)	AMP-activated protein kinas
(CAFs)	Cancer Associated Fibroblasts
(CFC)	Cardio-Facio-Cutaneous
(CM-AVM)	Capillary Malformation–Arteriovenous Malformation Syndrome
(CNS)	Central Nervous System
(CRD)	Cysteine-Rich Domain
(CSRD)	Cysteine/Serine-Rich Domain
(Dhh)	Desert Hedgehog
(D-MEM)	Dulbecco's Modified Eagle's Medium
(DNA)	Deoxyribonucleic Acid
(ECM)	Extra Cellular Matrix
(EGFR)	Extracellular Growth Factor Receptor
(ERK)	Extracellular signal-Regulated Kinase
(ETEA)	Expressed in T-cells and Eosinophils in Atopic Dermatitis
(FAK)	Focal Adhesion Complex
(FBS)	Fetal Bovine Serum
(FGF)	Fibroblast Growth Factor
(FGF-2)	Fibroblast Growth Factor 2

(GABA)	neurotransmitter Gamma-AminoButyric Acid
(GAPDH)	Glycerldehydes 3-phosphate dehydrogenize
(GDP)	Guanosine DiPhosphate
(GFP)	Green Fluorescence Protein [An efficient method for screening effective siRNAs using dual-luciferase reporter assay system]
(Grb2)	Growth Factor Receptor Bound Protein-2
(GRD)	GAP related domain
(GTP)	Guanosine TriPhosphate
(HGMD)	Human Genetic Mutation Database
(Ink4a/Arf expression)	INhibitors of CDK4/Alternate Reading Frame
(IP)	Immuno-Precipitation
(IRA1 and IRA2)	*Saccharomyces cerevisiae* Ras-GAP proteins
(KCl)	Potassium chloride
(Ki-67)	or MKI67 is a cellular marker for proliferation
(LOH)	Loss of Heterozigozyty
(MAPK/ MEK)	Mitogen Activated Protein Kinase
(MCs)	mast cells
(MEF)	Murine Embryonic Fibroblasts
(mFBRs)	myofibroblasts or activated fibroblsts
(MPNSTs)	Malignant Peripheral Nerve Sheath Tumors
(MRI)	Magnetic resonance imaging
(mRNA)	Messenger Ribonucleic Acid
(mTOR)	mammalian Target Of Rapamycin

(NaCl)	sodium chloride
(NCSCs)	Neural crest stem cells
(*NF1*)	Neurofibromatosis type 1
(Nf1)	Protein Neurofibromin
(NLS)	Nuclear localization sequence
(Oct6)	Octamer-binding transcription factor 6
(OMIM)	Online Mendelian Inheritance in Man
(p120-Ras-GAP)	a GTPase-activating protein (GAP) for the Ras family of proto-oncogenes
(p97/VCP)	p97/valosin-containing protein
(PAR2)	Protease activated receptor 2
(PBS)	Phosphate Buffered Saline
(PDAC)	Pancreatic Ductal Adino Carcinoma
(PDGF)	Platelet Growth Factor
(PH)	pleckstrin homology
(PI3K)	phosphatidylinositol 3-kinase
(PKA)	Protein Kinase A or cAMP-dependent protein kinas
(PKB or AKT)	Protein Kinase B
(PKC)	protein kinas C
(PML bodies)	Nuclear dots (also known as nuclear bodies, nuclear domains, or PML bodies) are punctate structures found in the nuclei of certain cells
(PN)	pseudo-neurofibroma in vitro
(PNS)	Peripheral Nervous System
(PNST)	Peripheral Nervous System Tumors

(POU domain)	is derived from the names of three transcription factors: Pit-1, Oct-1 and Oct-2, the neural **U**nc- 86 transcription factor from Caenorhabditis elegans
(Raf)	Rapidly accelerated fibrosarcoma
(Ras)	Rat Sarcoma
(RT)	Room Temperature
(SCM)	Schwann Cells Medium
(SCPs)	SC precursors; also known as neural crest stem cells
(SCs)	Schwann Cells
(Sec14)	yeast phosphatidylinositol-transfer protein
(Sel)	Selumetinib inhibit of ERK1/2
(Sema4F)	semaphorin 4F
(siRNA)	Small interfering RNA
(SKPs)	skin-derived precursors
(SMA)	Smal Muscle actin
(SMAD)	Mothers Against DPP Homolog 4
(Sos)	Ras guanine nucleotide exchange factor
(TBD)	tubulin-binding domain
(TCF**/**LEF)	T-cell factor/lymphoid enhancer factor
(TGFBeta)	Transcription Growth Factor-Beta
(TNF-α)	Tumor necrosis factor**-**alpha
(TSC2)	Tuberous Sclerosis Complex 2
(UBX-UB protein)	Ubiquitin regulatory X-D proteins
(WB)	Western Blotting

(WT)	wild type
(Y15)	FAK inhibitor