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THE ROOTS OF NEUROFIBROMAS IN NEUROFIBROMATOSIS 1

A Question of Multipotency, Differentiation and Hiding

by

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ABSTRACT

Neurofibromatosis type 1 (NF1) is an autosomal dominant cancer predisposition syndrome that affects about 1 in 3500 individuals worldwide. NF1 is caused by mutations in the *NF1* gene that encodes the tumor suppressor protein neurofibromin, an inactivator of the Ras oncogene. The hallmarks of NF1 include pigmentary lesions of the skin, Lisch nodules of the iris and cutaneous neurofibromas. Cutaneous neurofibromas are benign tumors composed of all the cell types of normal peripheral nerve. The traditional view of neurofibroma development has been that cutaneous neurofibromas arise from the disruption of the small nerve tributaries of the skin and subsequent proliferation of the resident cells. The second hit mutation in the *NF1* gene has been considered as a prerequisite for neurofibroma development. The second hit is detectable in a subpopulation of primary Schwann cells cultured from neurofibromas.

This thesis challenges the traditional concept of neurofibroma development. The results show that cutaneous neurofibromas are intimately associated with hair follicular structures and contain multipotent precursor cells (NFPs), suggesting that neurofibromas may arise from the multipotent cells which reside in hair follicles. Furthermore, this study presents that neurofibroma-derived Schwann cells that harbor bi-allelic inactivation in the *NF1* gene express HLA class II genes and may act as non-professional antigen presenting cells. The CD4- and FoxP3-positive cells detected in cutaneous neurofibromas suggest that these cells may represent regulatory T cells (Tregs) which interact with HLA II –positive cells and aid the tumor cells in hiding from the immune system and are thus mediators of immune tolerance.

This thesis also investigated neurofibroma development in the oral cavity and the use of different biomarkers to characterize cellular differentiation in neurofibromas. The results revealed that oral neurofibromas are not rare, but they usually appear as solitary lesions contrary to multiple cutaneous neurofibromas and present high heterogeneity within and between tumors. The use of class III β-tubulin as a marker for neuronal differentiation led to an unexpected finding showing that multiple cell types express class III β-tubulin during mitosis.

The increased understanding of the multipotency of tumor cells, cellular differentiation and ability to hide from immune system will aid in the development of future treatments. Specifically, targeting Tregs in NF1 patients could provide a novel therapeutic approach to interfere with the development of neurofibromas.

Keywords: Neurofibromatosis, neurofibroma, Schwann cell, stem cell

Eeva-Mari Jouhilahti

NEUROFIBROOMAN JUURILLA — Solujen monikykyisyys, erilaistuminen ja piiloutuminen

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TIIVISTELMÄ

Neurofibromatoosi 1 (NF1) on vallitsevasti periytyvä, *NF1*-geenin mutaatiosta aiheutuva syövälle altistava syndrooma, jonka esiintyvyys on noin 1:3500. Tuumorinsuppressorina toimivan *NF1*-geenin tuote, neurofibromiini, muuttaa aktiivisen Ras-GTP:n inaktiiviseksi Ras-GDP:ksi. *Ras* puolestaan tunnetaan onkogeeninä eli syöpägeeninä. Neurofibromatoosin tunnusmerkkeihin kuuluvat ihon maitokahviläiskät, Lischin kyhmyt iiriksessä sekä ihon neurofibroomat. Ihon neurofibroomat ovat hyvänlaatuisia kasvaimia, jotka koostuvat kaikista normaalin ääreishermon soluista. Perinteinen näkemys ihon neurofibrooman synnystä onkin, että kasvaimet syntyvät ihon pienten hermojen hajoamisen ja sitä seuraavan solujen jakautumisen seurauksena. Keskeistä neurofibrooman synnyn kannalta on *NF1* geenissä tapahtuva second-hit mutaatio, joka on paikannettu osasta neurofibroomasta viljeltyjä Schwannin soluja.

Tämän väitöskirjatyön tulokset kyseenalaistavat perinteisen näkemyksen ihon neurofibrooman synnystä. Tulokset osoittavat, että neurofibroomat esiintyvät iholla karvatupen välittömässä läheisyydessä ja että kasvaimista voidaan eristää monikykyisiä alkusoluja (NFP). Nämä seikat yhdessä johtavat päätelmään, että neurofibroomat voivat saada alkunsa karvatupen alueella sijaitsevista monikykyisistä soluista. Tämä väitöskirjatyö osoittaa myös, että neurofibroomasta viljellyt Schwannin solut, joissa molemmat *NF1* alleelit ovat inaktivoituneet, ilmentävät HLA II -geenejä. Tätä voidaan pitää merkkinä solujen mahdollisesta roolista antigeenejä esittelevinä soluina. Neurofibroomasta paikannetut CD4- ja FoxP3-positiiviset solut voivat edustaa regulatorisia T-soluja, jotka kommunikoidessaan HLA II – positiivisten solujen kanssa auttavat kasvaimen soluja piiloutumaan immuunijärjestelmältä. Tuloksena on immunotoleranssi.

Tässä väitöskirjassa tutkittiin myös suussa esiintyviä neurofibroomakasvaimia sekä biomarkkereiden käyttöä tutkittaessa neurofibrooman solujen erilaistumista. Tulokset osoittivat, että suun neurofibroomat ovat yleisiä, mutta esiintyvät yksittäisinä toisin kuin lukuisat ihon neurofibroomat. βIII-tubuliinin käyttö biomarkkerina tunnistamaan neuronaalista erilaistumista johti yllättävään löydökseen. Neuroni-spesifisenä pidetty proteiini paikallistuikin mitoottisten solujen tumasukkulaan eineuronaalisissa soluissa kuten ihon fibroblasteissa ja keratinosyyteissä.

Lisääntynyt ymmärrys kasvainsolujen monikykyissyydestä, erilaistumisesta ja kyvystä piiloutua immuunisysteemiltä tulee auttamaan tulevaisuuden hoitomuotojen kehittämisessä. NF1:n kohdalla erityisesti regulatorisiin T-soluihin kohdistettavat hoitomuodot voivat mahdollisesti auttaa tulevaisuudessa neurofibroomien hoidossa.

Avainsanat: Neurofibromatoosi, neurofibrooma, Schwannin solu, kantasolu

TABLE OF CONTENTS

ABBREVIATIONS

AA	amino acid
AC	adenylate cyclase
ADD	attention deficit disorder
ADHD	attention deficit hyperactivity disorder
AMP	adenosine mono phosphate
ATP	adenosine tri phosphate
bFGF	fibroblast growth factor, basic
bp	base pair
BSA	bovine serum albumin
cAMP	cyclic AMP
CD	cluster of differentiation
cDNA	complementary DNA
CFC	Cardio-Facio-Cutaneous syndrome
CK15	cytokeratin-15
CSRD	cysteine/serine-rich domain
CSC	cancer stem cell
CTLA	cytotoxic T lymphocyte antigen
Dhh	*Desert Hedgehog*
DMEM	Dulbecco's Modified Eagle Medium
ECM	extracellular matrix
EGF	epidermal growth factor
EVI2A/B	ecotropic viral integration site
FAK	focal adhesion kinase
FBS	fetal bovine serum
FDR	false discovery rate
FDG-PET	2-[^{18}F]-fluoro-2-deoxy-D-glucose-PET
FoxP3	forkhead box P3
GAP	GTPase activating protein
GAP43	growth-associated protein 43
GFAP	glial fibrillary acidic protein
GIST	gastrointestinal stromal tumor
GO	Gene Ontology
GPCR	G-protein coupled receptor
GRD	GAP related domain
HLA	human leukocyte antigen
HRP	horse radish peroxide
HSC	hematopoietic stem cells
IBMX	3-isobutyl-1-methylxanthine
IL	interleukin
JMML	juvenile myelomonocytic leukemia
kb	kilo base
kDa	kiloDalton
KGM	keratinocyte growth medium
LPA	lysophosphatidic acid
MAG	myelin associated glycoprotein
MAL	myelin and lymphocyte protein
MAPK	mitogen-activated protein kinase
MBP	myelin basic protein
MDSC	myeloid-derived suppressor cell
MHC	major histocompatibility complex
MPNST	malignant peripheral nerve sheath tumor
mRNA	messenger ribonucleic acid
NF1	human neurofibromatosis 1
NF1	human *NF1* gene
Nf1	mouse neurofibromatosis 1
Nf1	mouse *Nf1* gene
NF1$^{-/-}$	cells with the *NF1* second hit
NF1$^{+/-}$	cells carrying the constitutional *NF1* mutation only
NF2	human neurofibromatosis 2
NFP	neurofibroma-derived precursor cell
NIH	National Institutes of Health
NLS	nuclear localization signal
NRG-1	neuregulin 1
OMG	oligodendrocyte myelin glycoprotein
P0	myelin protein 0
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PET	positron emission tomography
PH	pleckstrin homology
PKA	protein kinase A
PMP22	peripheral myelin protein 22
SC	Schwann cell
SFM	serum free medium
SKP	skin derived precursor cells
SPRED1	sprouty-related, EVH1 domain containing 1
TAM	tumor-associated macrophage
TBD	tubulin-binding domain
TGD	total gene deletion
TGF	transforming growth factor
TNF	tumor necrosis factor
Treg	regulatory T cell
UTR	untranslated region

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by the Roman numerals I-V:

I Eeva-Mari Jouhilahti, Sirkku Peltonen and Juha Peltonen. Class III β-tubulin is a component of the mitotic spindle in multiple cell types, *J Histochem Cytochem* 56(12):1113-9. (2008)

II Eeva-Mari Jouhilahti, Vivian Visnapuu, Tero Soukka, Heikki Aho, Sirkku Peltonen, Risto-Pekka Happonen and Juha Peltonen. Oral soft tissue alterations in patients with neurofibromatosis, *Clin Oral Investig* 16(2):551-558. (2012)

III Eeva-Mari Jouhilahti, Sirkku Peltonen, Tom Callens, Elina Jokinen, Anthony M. Heape, Ludwine Messiaen and Juha Peltonen. The development of cutaneous neurofibromas, *Am J Pathol* 178(2):500-505. (2011)

IV Eeva-Mari Jouhilahti, Sirkku Peltonen, Elina P. Jokinen, Tom Callens, Heikki Aho, Eric Legius, Olli Lassila, Ludwine Messiaen and Juha Peltonen. A second hit in the *NF1* tumor suppressor gene leads to the up-regulation of HLA class II genes and recruitment of regulatory T cells, Submitted

In addition, some unpublished data are presented in this thesis.

1. INTRODUCTION

Neurofibromatosis 1 (NF1), previously referred to as von Recklinghausen's disease, is an autosomal dominant cancer predisposition syndrome that affects about 1 in 3500 individuals worldwide without bias for sex or ethnicity (Huson et al., 1989; Lammert et al., 2005). NF1 is caused by mutations in the *NF1* gene that encodes for the tumor suppressor protein neurofibromin, an inactivator of Ras oncogene (Bollag et al., 1993; Legius et al., 1993). Clinical diagnosis of the disease is based on the presence of *café-au-lait* macules, axillary freckling, hamartomas of the iris (Lisch nodules), optic pathway gliomas, distinctive osseous lesions such as sphenoid wing dysplasia or pseudarthrosis, and neurofibromas (Stumpf et al., 1988). Cutaneous neurofibromas are the hallmarks of NF1. They are mixed cell tumors composed of Schwann cells, fibroblasts, perineurial cells, mast cells, and axonal processes all embedded in collagenous extracellular matrix (Peltonen et al., 1988). A key finding in the context of this view has been that a subpopulation of Schwann cells in human neurofibromas carries a second hit in the *NF1* gene (Serra et al., 2000). In skin tissue, Schwann cells are typically found in association with small nerves. Thus, the traditional view of neurofibroma development has been that cutaneous neurofibromas arise as a result of the disruption of small nerve tributaries of the skin and subsequent proliferation of the resident cells.

The aim of the present study was to further elucidate the pathogenesis of cutaneous neurofibromas by challenging the traditional concept of neurofibroma development. The present study investigates cutaneous neurofibromas in the context of multipotent precursor cells, cellular differentiation, and gene expression changes in neurofibroma-derived Schwann cells.

2. REVIEW OF THE LITERATURE

2.1 Neurofibromatosis 1 (NF1)

2.1.1 Clinical features of NF1

Neurofibromatosis 1 is a fully penetrant disorder with variable expressivity (reviewed by Huson, 2008). It affects multiple tissues, anatomical structures and functions. The complications vary between individuals, even within the same family, and the clinical severity of NF1 increases with age. The clinical diagnosis is based on the presence of two or more of the findings shown in **Figure 1**. The list of the seven diagnostic criteria was established by the National Institutes of Health (NIH) in 1987 (Stumpf et al., 1988), and ever since the criteria have been shown to be both highly specific and sensitive for adults with NF1 (Boyd et al., 2009). However, not all children manifest these criteria. A comprehensive *NF1* mutation screen can detect gene mutations in over 92% of tested patients fulfilling the NIH diagnostic criteria (Messiaen et al., 2000; Messiaen and Wimmer, 2008). To confirm the molecular NF1 diagnosis, mutation analysis is especially important in very young children with a negative family history, and only partially fulfilling the NIH diagnostic criteria. It has been suggested that a pathogenic mutation in the *NF1* gene be added to the list of diagnostic criteria (Huson, 2008).

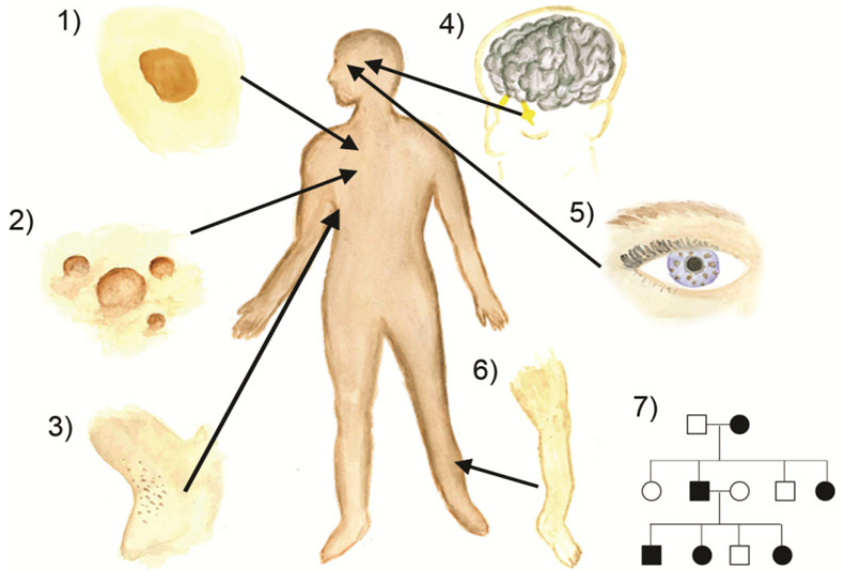


Figure 1. The diagnostic criteria of the National Institutes of Health for NF1. Two or more must be present. 1) six or more *café-au-lait* macules with diameters of more than 5 mm in pre-pubertal patients, and more than 15 mm in post-pubertal patients; 2) two or more neurofibromas of any type, or one plexiform neurofibroma; 3) axillary or inguinal freckling; 4) optic glioma; 5) two or more Lisch nodules of the iris; 6) a distinctive osseous lesion, such as pseudarthrosis or sphenoid wing dysplasia; or 7) a first-degree relative diagnosed with NF1 according to the preceding criteria (Stumpf et al., 1988).

Cutaneous manifestations are the most common signs of NF1(Huson, 2008). These manifestations include *café-au-lait* macules, skin fold freckling and neurofibromas. The *café-au-lait* macules are well demarcated lesions and get their name from the color of the skin lesion. They can be present at birth, and they occur in almost all individuals with NF1 within the first year of life. Skin fold freckling of axillae or groin regions generally occurs between 3 and 5 years of age, and majority of adult patients have this sign (Huson, 2008).

Cutaneous or dermal neurofibromas appear as small skin colored or slightly hyper-pigmented nodules on the skin. Their size typically varies from clinically invisible up to 2 to 3 centimeters diameter. The cutaneous neurofibromas start to grow at puberty and their number increases and may reach thousands with age (Evans et al., 2010). Although the cutaneous neurofibromas have not been shown to undergo malignant transformation, their high number causes the main disease burden of adult NF1 patients (Kodra et al., 2009). The other main type of neurofibromas, plexiform neurofibroma is a congenital lesion which can form a large disfiguring mass in association with nerves. The plexiform neurofibroma can appear on the skin as hyper-pigmented region. While cutaneous neurofibromas never undergo malignant transformation, 10-13% of NF1 patients develop malignant peripheral nerve sheath tumors (MPNSTs) which usually, but not always arise from plexiform neurofibromas (Evans et al., 2002). In those rare cases, MPNSTs arise directly from nerves. Besides neurofibromas, NF1 patients are prone to develop other tumors. The optic glioma, which is included in the diagnostic criteria, is a slowly growing tumor of the optic nerve and it is present in 15% to 20% of patients (Listernick et al., 2007). Another common ophthalmologic problem includes the hyper-pigmented nodules of the iris called Lisch nodules. These nodules are present in >95% of adult NF1 patients (Ferner, 2007).

In addition to skin manifestations, NF1 is characterized by numerous skeletal abnormalities which can be categorized as either generalized or focal manifestations (Elefteriou et al., 2009). The generalized skeletal abnormalities include osteopenia, osteoporosis and shortness of stature. These manifestations occur frequently in association with NF1, but they are usually mild. Decreased bone mineral density has been reported in up to 50% of NF1 patients (Elefteriou et al., 2009). The focal abnormalities including dystrophic scoliosis and sphenoid wing or long bone dysplasia are relatively rare.

The additional features of NF1 include lytic bone lesions, headache, speech disorders, ADD/ADHD and learning disabilities (Mautner and Boltshauser, 2008). Furthermore, rare complications, affecting less than 5% of patients, include epilepsy, hydrocephalus and cardiovascular problems (Ferner, 2010).

Several developmental syndromes caused by mutations in genes encoding proteins of the Ras signaling pathway and thus called RASopathies have phenotypes overlapping with those of NF1 (Denayer et al., 2008; Tidyman and Rauen, 2009).

Neurofibromatosis 2 (NF2) is caused by the mutations in the *NF2* gene on chromosome 22. The NF2 gene encodes for merlin protein which interacts with cytoskeleton (Muranen et al., 2007). The hallmark of the disease include bilateral schwannomas of the eight cranial nerve (Evans, 2009). The estimated incidence of NF2 is 1 in 25 000 (Evans, 2009).

Other syndromes with the involvement of Ras signaling pathway include Noonan syndrome, LEOPARD syndrome, Cardio-Facio-Cutaneous (CFC) syndrome, Costello syndrome, and Legius syndrome. These conditions all share a variable degree of learning difficulty, cardiac defects, facial dysmorphism, short stature, macrocephaly and skin abnormalities (Tidyman and Rauen, 2009). Confusing with the NF1 diagnosis, the clinical features of the Legius syndrome resemble and partially overlap with those of NF1, including multiple *café-au-lait* spots, axillary freckling and macrocephaly (Brems et al., 2007; Spurlock et al., 2009). Legius syndrome is also associated with cognitive abnormalities (Brems et al., 2007). However, Legius syndrome does not present with Lisch nodules in the iris, neurofibromas or central nervous system tumors. The syndrome is caused by mutations in the *SPRED1* gene at 15q14, and the gene product is part of the Sprouty/SPRED proteins, which are negative regulators of Ras (Brems et al., 2007). Molecular testing for NF1 is thus useful for diagnostic confirmation in the case where an adult patient with *café-au-lait* macules and axillary freckles has no neurofibromas. Approximately 1 - 2% of patients meeting NIH clinical diagnostic criteria for NF1 in fact harbor *SPRED1* mutations (Messiaen et al., 2009).

NF1 can also be classified as neurocristopathy or phakomatosis (MacCollin and Kwiatkowski, 2001; Qualman et al., 1986). The former refers to the embryological origin of the disease and the fact that many of the lesions characteristic for NF1 may arise from the cells derived from embryonic neural crest cell lineage. The term phakomatosis refers to a neurocutaneous syndrome which is a disorder involving nervous system and result in lesions on the skin and eye.

2.1.2 Genetic background of neurofibromatosis 1

The mutations in the *NF1* gene that encodes for the tumor suppressor protein neurofibromin cause the NF1 syndrome (Bollag et al., 1993; Legius et al., 1993). The inheritance of NF1 follows an autosomal dominant trait. All affected individuals studied thus far have shown to be heterozygous for an *NF1* mutation, and persons with constitutive inactivation of both alleles of the *NF1* gene have not been characterized. Homozygous *Nf1*$^{-/-}$ mice die *in utero*, indicating that neurofibromin is essential for development (Brannan et al., 1994; Jacks et al., 1994). Approximately half of the NF1 patients have inherited their first *NF1* mutation, and the other half has the disease caused by a *de novo NF1* mutation. The penetrance of NF1 is 100% by age 20 although the expressivity is highly variable, even within the same family (Viskochil, 2002). Mild forms of NF1 can be caused by mosaicism, which refers to an individual with two genetically distinct cell lines resulted from a post-zygotic gene mutation (Boyd et al., 2009). Three different forms of mosaicism occur in association with NF1: generalized,

segmental and gonadal (Ruggieri and Huson, 2001). Somatic mutations occurring early during embryonic development produce generalized mosaicism, which is characterized by a mild disease indistinguishable from classical NF1. Mutations occurring at a later stage of embryonic development result in segmental NF1, where manifestations are restricted to one segment of the body. Gonadal mosaicism is a rare form of mosaicism; the mutation is present only in the gonads, and it can be suspected if two or more children of unaffected parents have NF1 (De Raedt et al., 2008).

The *NF1* gene was identified by positional cloning in 1990, and it was located on chromosome 17, band q 11.2 (**Figure 2**) (Cawthon et al., 1990; Viskochil et al., 1990; Wallace et al., 1990). The *NF1* gene is one of the largest genes in the human genome, spanning ~280 kb of genomic DNA, and being composed of 57 constitutive exons and 4 alternatively spliced exons (9br, 10a2, 23a, 48a) (Upadhyaya, 2008). The *NF1* gene contains two large introns, 1 and 27b, spanning more than 60 kb each. The intron 27b contains three small genes, *EVI2A*, *EVI2B* (ecotropic viral integration site) and *OMG* (oligodendrocyte myelin glycoprotein) which are transcribed in the reverse orientation to the *NF1* gene (Buchberg et al., 1990; O'Connell et al., 1990; Viskochil et al., 1991). *EVI2A* and *EVI2B* are human homologues of the murine *Evi-2A* and *Evi-2B*, which are involved in the development of leukemia in those animals. *OMG* is a membrane glycoprotein expressed in the human central nervous system during myelination.

The rate of *de novo* mutations in the *NF1* gene locus is one of the highest observed in any single gene disorder being approximately 1×10^{-4} per gamete per generation (Huson et al., 1989). The large size of the *NF1* gene alone does not explain the high frequency of *de novo* mutations. The *NF1* mutation spectrum is highly variable and only little is known about the genotype-phenotype correlations in NF1. Total gene deletion (TGD), which is also called as *NF1* microdeletion, affects about 5% of NF1 patients (Kluwe et al., 2004). The deletion covers the entire *NF1* gene and its flanking sequences containing a total of 17 genes as described in **Figure 2** (De Raedt et al., 2004). The *NF1* microdeletion leads to a severe form of NF1 with a higher burden of cutaneous neurofibromas, dysmorphic features, and learning disabilities (Descheemaeker et al., 2004; Venturin et al., 2004).

The second genotype-phenotype association involves a specific, 3-bp, in-frame deletion in exon 17 (c.2970–2972 delAAT) of the *NF1* gene, a condition which is characterized by a mild phenotype in many patients (Upadhyaya et al., 2007). These patients show typical pigmentary features of NF1 and Lisch nodules, but do not develop cutaneous, or clinically detectable plexiform neurofibromas.

In general, mutations in the *NF1* gene are spread over the entire coding region and the most often encountered *NF1* mutations represent nonsense mutations, missense mutations, frameshift mutations or mutations affecting mRNA splicing (Messiaen and Wimmer, 2008). The majority of the *NF1* mutations lead to truncation of the protein (Messiaen et al., 2000; Park and Pivnick, 1998). Sporadic germline *NF1* gene mutations exhibit a sex bias, with the vast majority (> 80%) of mutations being of

paternal origin (Jadayel et al., 1990). However, most of the microdeletions appear to be of maternal origin (Upadhyaya et al., 1998).

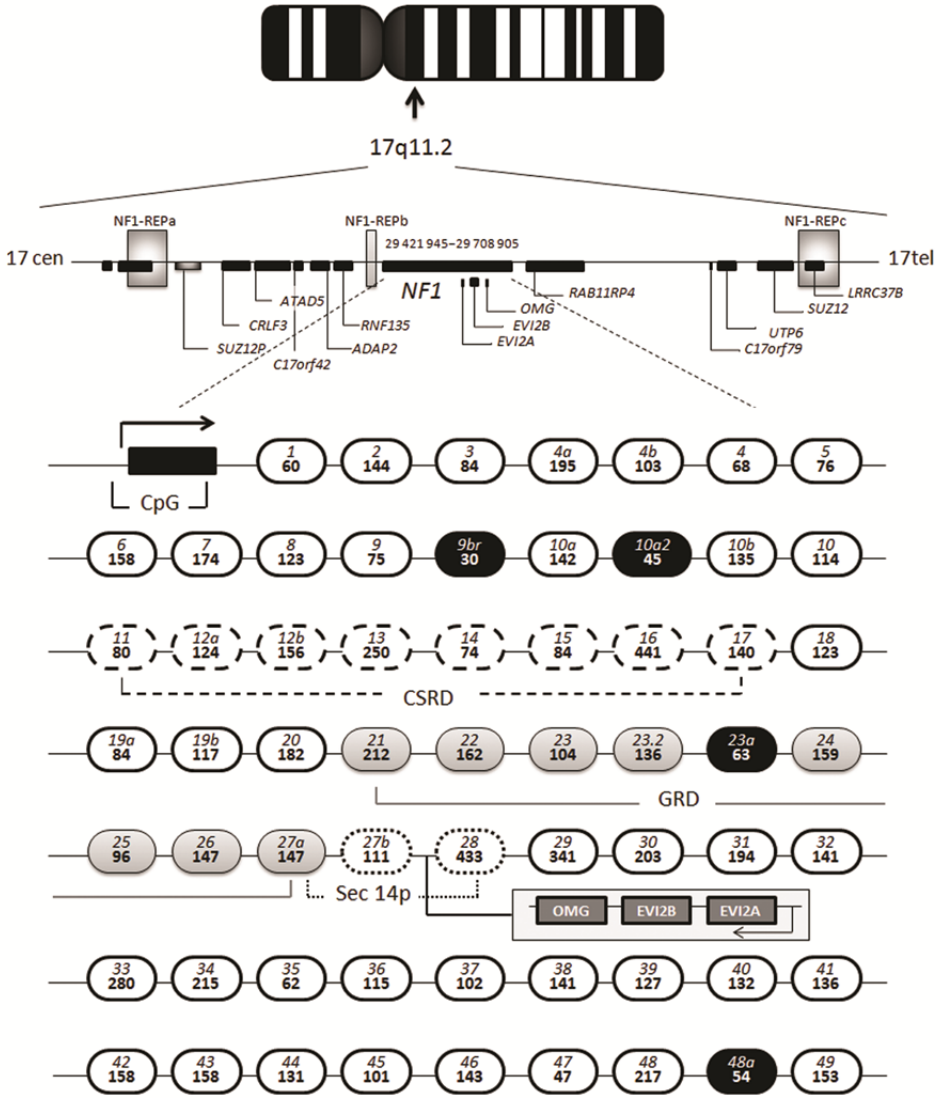


Figure 2. A schematic representation of *NF1* gene region and the structure of the *NF1* gene located on chromosome 17. The relative positions of the three NF1-REPs corresponding to the break-point positions for NF1 microdeletions are indicated together with the genes located within the region. The illustration of the *NF1* gene structure presents all the exons of the *NF1* gene with alternatively spliced exons colored black (not drawn to scale). The core promoter of the gene is shown as a rectangular box. The three principal protein domains in neurofibromin are indicated: CSRD, the cysteine/serine-rich domain; GRD, the GTPase activating protein (GAP)-related domain; and Sec14p, a region with homology to lipid-binding domain. The three embedded genes in intron 27b (*OMG*, *EVI2A*, *EVI2B*) that are transcribed in the opposite orientation to the *NF1* gene are indicated. Modified from (Upadhyaya, 2010; Zickler et al., 2012).

The *NF1* gene encodes an 11—13-kb mRNA with a 3.5-kb 3'-untranslated region and a ~9-kb open reading frame (Bernards et al., 1992; Marchuk et al., 1991; Wallace et al., 1990). The *NF1* mRNA is ubiquitously expressed in various cell types but most abundant in the nervous system (Daston et al., 1992; Wallace et al., 1990). The observed differences in the size of *NF1* mRNA can be explained by the alternative splicing of the transcript (Bernards et al., 1992). The alternatively spliced transcripts are differentially expressed depending on the tissue (reviewed by Skuse and Cappione, 1997). It has also been speculated that other gene loci are involved in modulating the expression of the NF1 phenotype (Metheny et al., 1995).

The expression of *NF1* is regulated at multiple levels, resulting in rapidly alterating levels of *NF1* mRNA and protein (**Table 2.1**.) (Cichowski et al., 2003; Peltonen and Peltonen, 2008). Proteins interacting with either *NF1* mRNA, or the protein, or causing their degradation, can be considered as modifiers of *NF1* expression. In the cytoplasm of migrating and cell junction-forming cells, some of the *NF1* mRNA is targeted to the cell periphery via actin microfilaments (Ylä-Outinen et al., 2002).

Furthermore, the silencing of *NF1* mRNA expression and the rapid oscillation of *NF1* mRNA and protein levels highlight the complexity of the regulation of the *NF1* gene (Koivunen et al., 2000; Pummi et al., 2000). The decay of *NF1* mRNA is poorly understood. It has been, however, shown that the disruption of actin filaments facilitates *NF1* mRNA degradation (Ylä-Outinen et al., 2002).

MicroRNAs (miRNAs), which hybridize to the 3' UTR of target mRNAs, and can repress mRNA translation or mediate its cleavage, have gained a lot of attention in recent years. The topic has also been addressed in context of NF1 by showing that a miRNA, miR-10b, may regulate NF1 tumorigenesis by targeting the *NF1* mRNA 3'UTR and modulating the Ras signaling pathway (Chai et al., 2010).

Table 2.1. Regulation of *NF1* expression

Target	Effects	Refs.
Promoter methylation	*NF1* gene silencing.	(Fishbein et al., 2005; Harder et al., 2004)
Transcriptional control	bFGF-, PDGF- and EGF-induced up-regulated transcription of *NF1* gene.	(Atit et al., 1999; Cichowski et al., 2003; Griesser et al., 1997; Hirvonen et al., 1998; Kuorilehto et al., 2006; Kuorilehto et al., 2004; Lee and Friedman, 2005; Wrabetz et al., 1995; Ylä-Outinen et al., 1998)
RNA processing	Alternative splicing of exons 9br, 10a-2, 23a, and 48a. Exon 10a-2 is spliced in between exons 10a and 10b.	(Baralle et al., 2006; Gutmann et al., 1995; Kaufmann et al., 2002; Nishi et al., 1991; Vandenbroucke et al., 2002)
mRNA transport	*NF1* mRNA targeting to cell periphery via actin microfilaments in migrating and cell junction-forming cells; possible mediator, HuR.	(Haeussler et al., 2000; Ylä-Outinen et al., 2002)
mRNA level	Rapidly oscillating steady-state levels of *NF1* mRNA. Down-regulation of *NF1* mRNA leading to the terminal differentiation of epithelial cells.	(Hermonen et al., 1995; Kaufmann et al., 1999; Pummi et al., 2000; Ylä-Outinen et al., 2002)
Translational control	Increased translational efficiency, or protein stabilization. Increased neurofibromin levels after nerve injury.	(Wrabetz et al., 1995)
Post-translational modifications	Phosphorylation at the cysteine/serine-rich domains of neurofibromin, possibly by PKA and PKC-α.	(De Schepper et al., 2006; Hirvonen et al., 1998; Izawa et al., 1996; Mangoura et al., 2006; Welti et al., 2008)
Protein targeting	Targeting of neurofibromin near the plasma membrane via the cytoskeleton, in order to enable the interaction with Ras-GTP and syndecan. Targeting to the nucleus or nucleoli.	(Bollag et al., 1993; De Schepper et al., 2006; Gregory et al., 1993; Hsueh et al., 2001; Kaufmann et al., 2002; Koivunen et al., 2000; Li et al., 2001)
Protein degradation	Regulation by the ubiquitin-proteasome pathway triggered by PDGF, EGF and lysophosphatidic acid (LPA); requires sequences adjacent to the catalytic GAP-related domain of neurofibromin.	(Cichowski et al., 2003)

Modified from (Jouhilahti et al., 2011)

2.1.3 The structure and functions of neurofibromin

The protein product of the *NF1* gene is called neurofibromin, and it was first identified as a cytosolic protein composed of 2818 amino acids (AA) with a molecular weight of ~280 kDa (DeClue et al., 1991; Gutmann et al., 1991). Later studies have shown that different cell types display a variable subcellular localization, and that neurofibromin can also be detected in nucleus (Koivunen et al., 2000; Li et al., 2001; Vandenbroucke et al., 2004). Neurofibromin is ubiquitously expressed but the protein levels differ in different tissues and in different developmental and functional stages (Daston and Ratner, 1992; Daston et al., 1992; Gutmann et al., 1991; Hermonen et al., 1995; Hirvonen et al., 1998). The protein is highly conserved among vertebrates showing 98% identity with the mouse homolog (Bernards et al., 1993). Furthermore, the neurofibromin of Drosophila is 60% identical to the human protein (The et al., 1997).

Neurofibromin interacts with several signaling pathways. For instance, it has been shown to participate in the activation of p21ras (Ras), modulation of adenylate cyclase (AC), and interaction with other signaling molecules such as kinesin-1, protein kinase A and C, syndecan, caveolin and the amyloid precursor protein (**Figure 3**) (Trovó-Marqui and Tajara, 2006; Welti et al., 2008). Neurofibromin has also been shown to interact with microtubules, which is a common feature of many tumor suppressor genes such as the NF2 protein merlin (Bollag et al., 1993; Muranen et al., 2007). However, very little is known about the functions of neurofibromin, and none of the known interactions alone can explain the different symptoms related to NF1.

The only well-characterized function of neurofibromin is its RasGAP activity. Neurofibromin contains a GAP (GTPase activating protein) related domain (GRD) (Xu et al., 1990b), encompassing amino acids 1203-1549 (GenBank sequence NP_000258.1) corresponding to exons 20-27a (**Figure 2**). The domain is highly homologous to the yeast RasGAP proteins IRA1 and IRA2 (Xu et al., 1990a). Via its GRD sequence, neurofibromin accelerates the conversion of active Ras-GTP to inactive Ras-GDP in various cell types, and acts as a negative regulator of the p21ras-signaling pathway (**Figure 3**) (Le and Parada, 2007). In general, the Ras GTPases interact with multiple pathways including the MAPK and PI3K cascades, which regulate cellular growth and differentiation. However, the NF1-GRD domain represents only about 10% of the protein sequence and it is not enough to explain all interactions of neurofibromin.

Neurofibromin also contains a bipartite lipid-binding module (NF1-Sec-PH, AA 1560-1698 and 1715-1816) composed of a segment homologous to the yeast Sec14p protein and the pleckstrin homology (PH)-like domain (Aravind et al., 1999; D'Angelo et al., 2006; Welti et al., 2007). This domain locates C-terminal to GRD. The Sec14 portion can potentially mediate the exchange of phospholipids between membrane bilayers, affect the subcellular location of the protein, and affect protein-protein interactions of NF1 (Phillips et al., 2006; Welti et al., 2011). Furthermore, neurofibromin contains cysteine/serine-rich (CSRD, AA 543-909) and tubulin-binding (TBD) domains that are located N-terminal to the GRD (Bollag et al., 1993; Fahsold et al., 2000). These two

domains can act in opposite ways: tubulin binding to the TBD inhibits the GAP activity, whereas phosphorylation of the CSRD by PKC-α enhances the same activity (reviewed by Carroll, 2012). The C-terminal end of neurofibromin contains a focal adhesion kinase (FAK)-interacting domain suggesting the role of neurofibromin in cell adhesion, cell growth and also in cell signaling via this domain (Kweh et al., 2009). The FAK-binding domain of neurofibromin contains a nuclear localization signal (NLS) encoded by exon 43 (Vandenbroucke et al., 2004).

In addition to the functional domains characterized in human neurofibromin, the studies on *Drosophila* have shown that a part of the C-terminal region (AA 1748-2818) is involved in *Nf1*-mediated signaling through adenylate cyclase (Guo et al., 1997; Hannan et al., 2006). Neurofibromin can participate in the activation of AC via two different pathways one of which is dependent on G-protein subunit alpha, and the other involves the RasGAP activity of neurofibromin (Hannan et al., 2006). Typically, the activation of AC is mediated by the Gα subunit of G-protein coupled receptors (GPCRs) and upon ligand binding the AC synthesizes cyclic AMP (cAMP) from ATP (Beavo and Brunton, 2002). Cyclic AMP can activate the cAMP-dependent protein kinase A (PKA), which in turn transduces the signal by phosphorylating its target proteins. The *Nf1*-regulated AC/cAMP pathway is involved in many biological processes including the control of body size and learning as exemplified in flies (Guo et al., 2000; The et al., 1997).

It has also been shown that neurofibromin alters cAMP levels in astrocytes and Schwann cells (Dasgupta et al., 2003; Kim et al., 2001). In Schwann cells, high intracellular cAMP levels promote cell proliferation, and at least one function of *Nf1* gene is to antagonize the accumulation of cAMP (Kim et al., 2001). The question "How *Nf1* loss in Schwann cells leads to the increase of cAMP" has not been answered. However, the increased cAMP levels correlate to elevated levels of cyclin D1 in cultured Schwann cells. High expression of cyclin D1 leads to the progression of cell cycle through G_1 phase and results in cell proliferation. On contrary to Schwann cells, high intracellular cAMP levels in astrocytes provide a strong growth inhibitory signal. *Nf1* deficient astrocytes show reduced generation of cAMP, and thus increased proliferation (Dasgupta et al., 2003). The results suggest that neurofibromin functions at the level of AC possibly facilitating the AC activation (Dasgupta et al., 2003).

cAMP generation in many cell types results in enhanced calcium influx (Kamp and Hell, 2000). In the context of neurofibromatosis 1, *Nf1* loss in murine astrocytes followed by low cAMP levels correlates to the reduced calcium influx (Dasgupta et al., 2003), and defective calcium signaling has also been demonstrated in keratinocytes cultured from NF1 patients (Korkiamäki et al., 2002; Korkiamäki et al., 2005).

Neurofibromin acts as a classical tumor suppressor gene following the Knudson two-hit hypothesis of tumor formation (Knudson, 1971). This function is supported by the facts that some distinct lesions characteristic of NF1 are associated with the loss of both functional *NF1* alleles in certain cell types and that a somatic second-hit mutation has been demonstrated in several benign and malignant tumors in NF1 (De Raedt et al.,

2008; Legius et al., 1993; Maertens et al., 2006). Increased activity of Ras and its downstream effectors has been observed in NF1-associated tumors and NF1-deficient cells and thus the tumor suppressor function of neurofibromin is believed to occur by its actions as a RasGAP (Bollag et al., 1996; Guha et al., 1996; Kim et al., 1995; Lau et al., 2000).

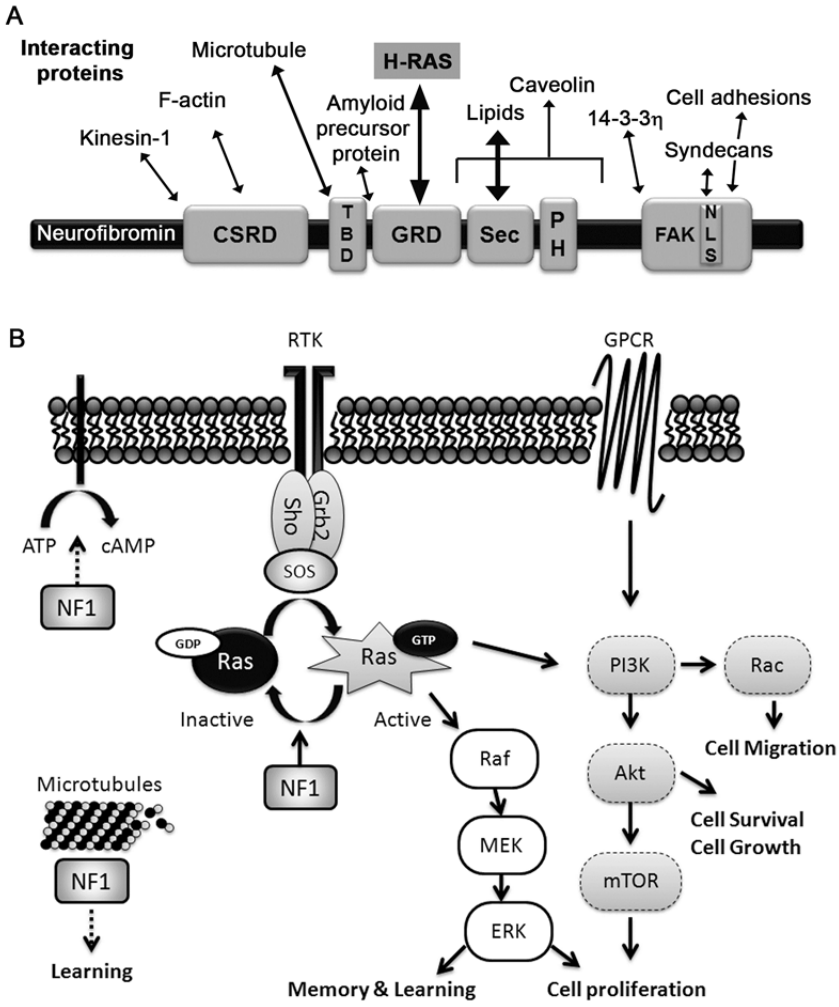


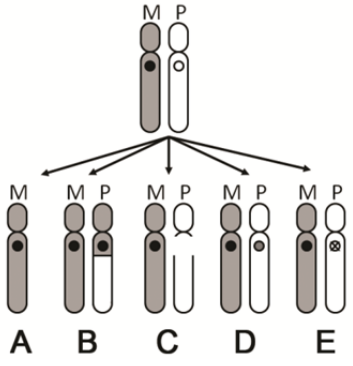
Figure 3. Functional domains and interactions of neurofibromin **A**) Schematic illustration of the functional domains present in neurofibromin and interacting proteins. The domains include CSRD: Cystein and serine rich domain; TBD: Tubulin binding domain; GRD: GAP-related domain; Sec: Sec14-like domain; PH: PH-like domain; FAK: focal adhesion kinase-interacting domain; NLS: nuclear localization sequence. **B**) Representation of neurofibromin's interactions with the Ras and PI3K pathway. Loss of neurofibromin expression leads to elevated Ras activity, dysregulated cell growth and tumorigenesis. Neurofibromin may also associate with microtubules and modulate the cAMP-PKA signaling pathway. Modified from Carroll, 2012; Le and Parada, 2007; Welti et al., 2008.

2.1.4 NF1 second hit mutations and related lesions

A somatic second hit mutation leading to bi-allelic inactivation of the *NF1* gene (See **Box 1.** for mechanisms of somatic inactivation) has been demonstrated in selected lesions related to NF1. Particularly, the *NF1* second hit mutation is a pertinent finding in a subpopulation of cultured neurofibroma Schwann cells (Maertens et al., 2006; Serra et al., 2000). In analogy, the presence of the *NF1* second hit has been shown in melanocytes of *café-au-lait* macules, and in alpha-actin-positive glomus cells of NF1-related glomus tumors (Brems et al., 2009; De Schepper et al., 2008). In addition, *NF1* second hit mutation has been shown in two NF1 patients with pseudarthrosis, but in the fibrous tissue of the pseudarthrosis tissue (Stevenson et al., 2006). Double inactivation of the *NF1* gene has also been observed in certain NF1-related malignancies, such as MPNSTs, pheochromocytomas, astrocytomas and juvenile myelomonocytic leukemia (JMML) (reviewed by De Raedt et al., 2008). Furthermore, somatic *NF1* gene mutations also occur in association with a number of common cancers unrelated to NF1 (Laycock-van Spyk et al., 2011). These observations raise the questions as to what symptoms and signs are caused by haploinsufficiency as such, and what is the role played by the inactivation of both *NF1* alleles. Haploinsufficiency refers to a state in which one functional allele of a tumor suppressor gene is not sufficient to maintain the normal function (Berger et al., 2011; Berger and Pandolfi, 2011). Even partial inactivation of a tumor suppressor gene can critically contribute to tumorigenesis.

Box 1. Somatic Inactivation of a Tumor Suppressor Gene

Somatic inactivation of a tumor suppressor gene can occur via five principal mechanisms: loss of a whole chromosome e.g. via nondisjunction (A), somatic recombination (B), interstitial deletions (C), somatic intragenic mutation (D) or epigenetic change (E). Somatic intragenic mutations typically include point mutations and small intragenic deletions. Epigenetic changes such as methylation and transcription repression can lead to the silencing of the target gene without mutation. (Aittomäki and Peltomäki, 2006; Gottfried et al., 2006)



M= maternal chromosome, P= paternal chromosome. White circle= normal allele, black dot= mutated allele, grey dot= separate mutation in the 2nd allele, crossed circle= allele is inactivated due to the epigenetic reason.

The mutational spectrum and the relative frequencies of different mutation types differ between *NF1* germline mutations and somatic second-hit mutations (Maertens et al., 2006; Thomas et al., 2011). The patient-associated germline mutations and their spectrum are well characterized, whereas the somatic mutations described thus far are relatively few (Thomas et al., 2011). However, among the lesions caused by the *NF1* second hit, the mutations have been best characterized in cutaneous neurofibromas (De Raedt et al., 2008; Maertens et al., 2006; Thomas et al., 2011). The intragenic *NF1* mutations, particularly frameshift mutations have been shown to be the most prevalent

somatic/ second hit mutations (Maertens et al., 2006). Furthermore, the nonsense mutations and deletions comprising ≥4 nucleotides are remarkably more commonly detected as second-hit mutations than as germline mutations, while the frequency of somatic missense mutations, splicing mutations and small insertions is lower than that observed in the *NF1* germline mutation spectrum (Maertens et al., 2006). Total gene deletion (TGD) has been reported to be responsible for the somatic inactivation in 20—40% of neurofibromas sampled from non-microdeletion patients (De Raedt et al., 2008; Laycock-van Spyk et al., 2011) whereas TGD is never the second-hit in patients with *NF1* microdeletions (De Raedt et al., 2006). The germline mutation in *NF1* microdeletion patients covers the entire *NF1* gene and its flanking sequences (see 2.1.2).

2.2 Tumorigenesis

Tumorigenesis refers to the development of tumors, which can be either benign or malignant. These abnormal tissue masses are also referred to as neoplasms, and the malignant neoplasms are called cancer. Tumors are typically composed of multiple cell types with heterotypic interactions with one another, but their initial growth is generally caused by a single population of neoplastic cells.

Tumorigenesis is a multistep process during which the normal cells change their behavior, evade proliferative control and start to form disfigured masses. This process requires many changes to take place both in tumorigenic cells themselves and in their microenvironment. In addition to neoplastic cells, tumors contain a variety of normal cells that contribute to the tumor development.

In 2000, Hanahan and Weinberg described the six hallmarks of cancer that are needed when the normal cell progresses to neoplastic tumor cell (Hanahan and Weinberg, 2000). These hallmarks include: self-sufficiency of growth signals, evasion of programmed cell death, insensitivity to growth inhibitory signals, limitless replicative potential, sustained angiogenesis, and tissue-invasive and metastatic capacity. These capabilities are, however, not sufficient to explain the complexity of the tumorigenesis. In 2011, Hanahan and Weinberg supplemented the list of hallmarks with two enabling characteristics “Genome instability and mutation” and “Tumor-promoting inflammation” and two emerging hallmarks “Deregulating cellular energetics” and “Avoiding immune destruction”.

2.2.1 Tumorigenesis and immunity

The link between inflammation and tumor development was established already in 1863, when German pathologist Rudolf Virchow hypothesized that there is a connection between tumor initiation and the sites of chronic inflammation (Balkwill and Mantovani, 2001). During the past decade, it has been accepted that the immune system plays an important role in tumorigenesis: its task is to identify and eliminate cancerous or foreign cells in the body, and the emergence of a tumor is an evidence of its failure. The role of immune system in cancer progression has also been noted in the updated list of the hallmarks of cancer (Hanahan and Weinberg, 2011). The novel

hallmarks of cancer progression include tumor-promoting inflammation and the avoidance of immune destruction. The latter involves active evasion by cancer cells from the attack and elimination of immune cells; these capabilities highlight the dichotomous roles of the immune system that both antagonizes and enhances tumor development and progression.

Balkwill and Mantovani (2001) also suggested that the inflammatory cells and cytokines found in tumors contribute to tumor growth, progression and immunosuppression rather than hinder these processes. The inflammatory cells in the tumor microenvironment and in the infiltrating tumors include tumor-associated macrophages (TAMs), dendritic cells, myeloid-derived suppressor cells (MDSC) and lymphocytes such as T regulatory cells or Tregs (Balkwill and Mantovani, 2001; Vasievich and Huang, 2011). These cells can protect the tumor from discovery and elimination (Vasievich and Huang, 2011). TAMs are attracted to tumors and maintained by chemo attractant and colony-stimulating cytokines produced by tumor cells. TAMs can promote cell proliferation, angiogenesis and metastasis by producing growth and angiogenic factors and proteases that degrade the extracellular matrix (Balkwill and Mantovani, 2010).

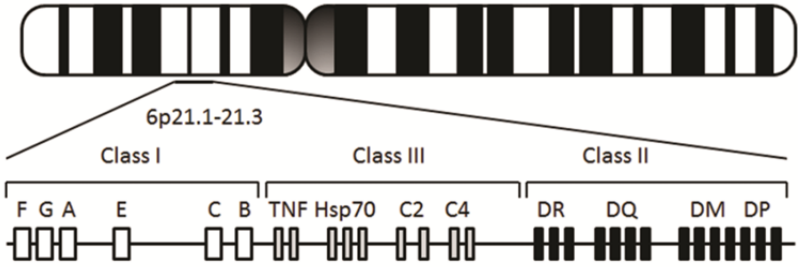
The dendritic cells are typically bone marrow derived cells that are seeded in all tissues (reviewed by Palucka and Banchereau, 2012). They act as mediators between innate and adaptive immune system by detecting the environment through their molecular sensors and transmitting the acquired information to lymphocytes by presenting the captured antigen via major histocompatibility complex (MHC) molecules (See **Box 2.** for HLA class II). Activated (mature) dendritic cells, which are loaded with antigen, can launch the differentiation of antigen-specific T cells into effector T cells. Non-activated (immature) dendritic cells can present self-antigens to T cells which can differentiate into Tregs. Tregs down-regulate the functions of other lymphocytes leading to immune tolerance. Dendritic cells thus have a dichotomous role: they can activate the antigen-specific immunity, but they can also participate in the maintenance of tolerance. In tumors, dendritic cells usually have immature phenotype and their ability to stimulate antigen-specific T cell immunity is compromised (Balkwill and Mantovani, 2001).

The current understanding is that virtually all neoplastic lesions contain immune cells which can form either subtle infiltration detectable only with cell-type specific antibodies, or gross inflammations that are apparent even by standard histochemical staining techniques (Hanahan and Weinberg, 2011). Furthermore, the inflammatory tumor microenvironment can supply bioactive molecules including growth factors that sustain proliferative signaling, survival factors that limit cell death, and pro-angiogenic factors and ECM-modifying enzymes that facilitate angiogenesis, invasion and metastasis. In addition, inflammatory cells can secrete reactive oxygen species that are mutagenic for nearby cancer cells (reviewed by Hanahan and Weinberg, 2011). The pro-inflammatory cytokines including tumor necrosis factor (TNF), and interleukins IL-1 and IL-6 are commonly detected in different tumors (Balkwill and Mantovani, 2001). Different cytokines can affect various cellular processes such as survival,

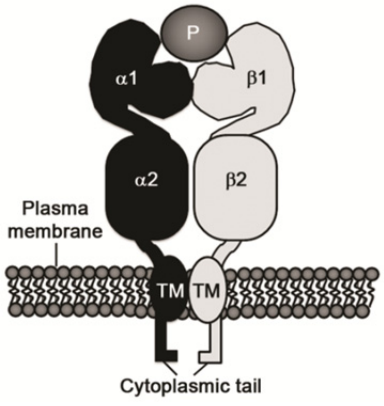
growth, mutation, proliferation, differentiation and cellular movement of tumor and stromal cells.

Box 2. HLA Class II

In humans, MHC is also called human leukocyte antigen (HLA) system. The best known function of MHC/HLA molecules is to present short, pathogen derived peptides to T cells in order to initiate the adaptive immune response (Klein and Sato, 2000). Furthermore, MHC class II molecules have been shown to act as signaling receptors and signal transducers (Al-Daccak et al., 2004). The human HLA complex locates on chromosome 6, and it is divided into three regions as shown below (I, II and III). The HLA region contains over 200 genes, but many of these genes have nothing to do with immunity. The HLA genes that are involved in immune response fall into two functionally and structurally different classes, I and II. Classical class I genes include HLA-A, -B and -C. Class I genes are expressed by most somatic cells whereas the class II genes are typically expressed by a subgroup of immune cells, the professional antigen presenting cells, that include B cells, activated T cells, macrophages, dendritic cells, and thymic epithelial cells (Klein and Sato, 2000). Class III HLA genes are not related to class I and class II genes structurally or functionally, but are encoded between them and include several secreted proteins with immune functions such as components of complement system (C2 and C4).



The HLA class II genes code for the α and β polypeptide chains of the class II molecules. The designation of their loci consists of three letters: the first (D) indicates the class, the second (M, O, P, Q or R) the family and the third (A or B) the chain.



Structurally, HLA II molecules, as shown on the right, are heterodimers composed of an α-chain and a β-chain which both have four domains. HLA II molecules are type I integral membrane proteins with transmembrane domains (TM), short cytoplasmic tails and four large extracellular domains: the peptide-binding domains (α1 and β1), and the immunoglobulin-like domains (α2 and β2). (Klein and Sato, 2000)

The body's own tissues are protected against immune-mediated damages by self-tolerance (Milojevic et al., 2008). Tolerance is generally divided into two categories: central tolerance and peripheral tolerance (Töpfer et al., 2011; Zou, 2006). Central tolerance is established when T cells with high affinity receptors for MHC/self-peptides are eliminated during lymphocyte development in thymus (Töpfer et al., 2011). Peripheral tolerance refers to T cells which are specific for self-peptides in the periphery, but which display a low affinity T cell receptor (Töpfer et al., 2011). T cells comprising the peripheral tolerance are under normal circumstances in an ignorant state and need to be activated by professional antigen presenting cells, usually dendritic cells.

The existence of peripheral T cells exerting a suppressor function has been a matter of debate for many years (Töpfer et al., 2011). Nowadays it is accepted that the immunologic self-tolerance in periphery is maintained by a subpopulation of activated T cells, called Tregs (Sakaguchi et al., 1995). Tregs can functionally suppress self-antigen-reactive T cells and immune response by influencing the activity of another cell type (Töpfer et al., 2011; Zou, 2006). Two main groups of Tregs exist: natural Tregs (nTregs), which represent 5% to 10% of peripheral CD4-positive T cells, and induced or adaptive Tregs (iTregs) (Ménétrier-Caux et al., 2012). However, several other regulatory T cells have also been described. Classically, nTregs are thymus-derived T cells characterized by the expression of the cell surface antigens CD4 and IL-2Rα chain (CD25) (Sakaguchi et al., 1995). Furthermore, the transcription factor forkhead box P3 (FoxP3) is crucial for the development and suppressive function of Tregs and it is stably expressed by these cells (Fontenot et al., 2003; Hori et al., 2003). Antigenic stimulation under tolerogenic conditions, defined by high concentration of transforming growth factor -β (TGF-β), induces iTregs (Ménétrier-Caux et al., 2012; Töpfer et al., 2011). iTregs can secrete TGF-β and IL-10, and contribute to the development of an antigen-specific immunosuppressive response (Ménétrier-Caux et al., 2012). Both types of Tregs are able to inhibit proliferation and cytokine production in the effector T cells (Sakaguchi et al., 2009; Töpfer et al., 2011). Furthermore, upon activation the Tregs can also express HLA-DR (Milojevic et al., 2008; Yi et al., 2006) (See **Box 2.** for HLA class II).

The key molecules contributing to immunosuppression by Tregs include IL-2 and cytotoxic T lymphocyte antigen 4 (CTLA4) (Sakaguchi et al., 2009). IL-2 has ability to activate Tregs and it is crucial for the differentiation of effector T cells (Boyman and Sprent, 2012). IL-2 is produced mainly by CD4-positive T helper cells, but also by activated dendritic cells and mast cells (Boyman and Sprent, 2012). The Tregs are unresponsive to the proliferative signal from T cell receptor, and they do not produce IL-2 (Thornton and Shevach, 1998). Tregs can suppress immune responses by contact-dependent manner via CTLA4 signaling (Sakaguchi et al., 2009), and by secreting or inducing dendritic cells to produce the immunosuppressive cytokines, IL-10 and TGF-β (Janikashvili et al., 2011).

Since the immune rejection of established cancer is rare, the immunosuppressive tumor microenvironment and self-tolerance seems to play an important role in cancer by hindering the attack against cancer cells. Tregs are one of the main obstacles of successful immunotherapy (Mellman et al., 2011; Ménétrier-Caux et al., 2012; Vasievich and Huang, 2011). Thus the mechanisms that hinder the Treg trafficking, differentiation and function are potential therapeutic options for cancer treatment (Zou, 2006).

2.2.2 Stem cells in tumor development

Stem cells can be defined as cells that are capable of proliferation, self-renewal, and conversion to multiple differentiated cell types that can regenerate tissues (Reya et al., 2001; Tuch, 2006). More specifically, stem cells are able to generate both self-renewing and differentiating daughter cells through symmetric and asymmetric cell divisions (Knoblich, 2010). While symmetric cell division leads to generation of two stem cells, asymmetric division results in one stem cell and one cell that start to differentiate. An important characteristic of a stem cell thus includes the ability to control the balance between self-renewal and differentiation. Stem cells are classified according to their differentiation capacity into pluripotent, multipotent and unipotent stem cells. Pluripotent stem cells are capable to differentiate into all three somatic germ layers, whereas multipotent stem cells can differentiate only into cells of one tissue or germ layer (Ulloa-Montoya et al., 2007). Unipotent stem cells include adult tissue-specific stem cells which have capacity to differentiate into only one cell type. On the other hand, stem cells can be grouped into embryonic and non-embryonic or adult stem cells. The embryonic stem cells represent pluripotent cells whereas adult stem cells have more restricted differentiation potential, and are thus multipotent or unipotent.

Stem cells and tumor cells have similarities such as self-renewal and indefinite proliferative potential which have raised the question whether tumors contain or even arise from stem cells (Reya et al., 2001). Early studies using hematopoietic stem cells (HSC) provided evidence that leukemias arise from mutations that accumulate in HSCs (Bonnet and Dick, 1997; Lapidot et al., 1994). These studies also demonstrated that only a small subpopulation of tumor cells was capable of extensive proliferation. Later studies on solid tumors have also shown that only a proportion of cells are clonogenic *in vitro* and *in vivo*, reflecting the phenotypic heterogeneity (Pantic, 2011; Reya et al., 2001). Today, it is accepted that many cancers contain cancer stem cells (CSCs) which have the ability of unlimited growth, self-renewal, and differentiation. These cells are potentially responsible for the tumor growth and metastasis.

The molecular and cellular basis of the cellular heterogeneity within tumors has been a central question in cancer biology (Visvader, 2011). The key issue is whether the different cell types originate from different cells of origin, and what the role of cancer stem cells is. It is well evidenced that tumor development requires several alterations in the normal cells for neoplastic progression (Hanahan and Weinberg, 2011). These include genome instability and accumulation of mutations. Due to their self-renewal capacity, stem cells persist longer periods of time than mature cells and thus they have

much higher opportunity for mutations to accumulate. Although the terms cell of origin and cancer stem cell have been used interchangeably, the CSCs are not necessarily the tumor initiating cells that acquire the tumor-promoting mutations (Visvader, 2011). However, growing evidence supports the fact that either stem or progenitor cells can act as targets for tumor initiation in various solid tumors (Visvader, 2011). The characterization of the cells responsible for tumor growth and metastasis promotes the development of new therapeutics.

In context of NF1, the potential role of stem cells in tumor formation has also been studied. In 2007 Roth et al. demonstrated that mouse embryonic stem cells with $Nf1^{+/+}$, $Nf1^{+/-}$ and $Nf1^{-/-}$ genotypes can be differentiated into Schwann cell –like cells (Roth et al., 2007). Later studies by Parada and coworkers have shown that skin derived precursor cells (SKPs) with $Nf1^{-/-}$ genotype can give rise to dermal neurofibromas in *Nf1* heterozygous mice (Le et al., 2009). SKPs were previously characterized as multipotent adult stem cells derived from dermis of mammalian skin (Fernandes et al., 2004; Toma et al., 2001). SKPs can proliferate and differentiate in culture to produce neurons, glia, smooth muscle cells, and adipocytes. These cells are supposed to reside in the dermal papilla of hair follicles (Biernaskie et al., 2009) (See **Box 3.** for skin structure). The dermal papilla resides at the base of the hair follicle and regulates the hair follicle growth and development (Driskell et al., 2011).

Another source of stem cells in the hair follicle area have been characterized in mice and man by the expression of neural stem cell marker nestin, but negativity for cytokeratin-15 (CK15) (Amoh et al., 2010; Yu et al., 2006). Specifically, mouse studies have shown that these cells reside in the upper hair follicle immediately below the sebaceous glands and above the hair follicle bulge area. The nestin-positive and CK15-negative cells are able to differentiate into neurons, glial cells, keratinocytes, melanocytes, and smooth muscle cells (Amoh et al., 2005b). In addition, novel blood vessels in the skin arise from nestin-positive cells (Amoh et al., 2004). From the therapeutic point of view, the hair follicle stem cells have been noted as a promising source of easily accessible multipotent stem cells, particularly for neurologic diseases (Amoh et al., 2010; Driskell et al., 2011).

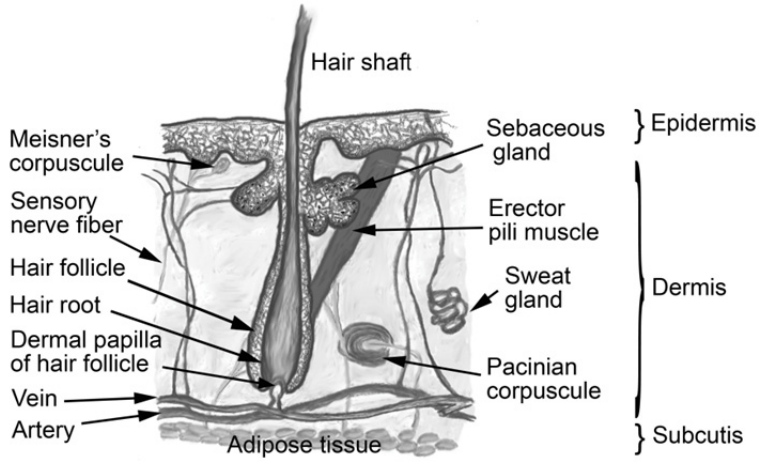
Box 3. The Skin

Skin is the largest organ in the body accounting for about 7% of total body weight with a surface area of 1.2—2.2 square meters and thickness varying from 1 to 5 mm. Structurally, skin is composed of three main layers: epidermis, dermis, and hypodermis or subcutis.

Epidermis is the outermost, protective layer of the body. The keratin producing epithelial cells, keratinocytes, form the main cell type in epidermis and they are organized in stratified layers. Other cell types residing in epidermis include melanin producing melanocytes, Langerhans' cells which recognize external antigens, and Merkel cells which are closely associated with disc-like sensory nerve endings. Keratinocytes arise from rapidly dividing cells residing in the basal layer of epidermis which is tightly bound to the underlying dermis by the basement membrane. The cells differentiate while moving upwards and forming epidermal layers: the basal, the spinous, the granular and the cornified layer. The uppermost cornified layer is composed of 20—30 layers of dead cells.

Dermis residing underneath the epidermis is composed of strong and flexible fibrous connective tissue with collagen and elastic fibers. The cell types within the dermis include fibroblasts, macrophages and occasional mast cells and white blood cells. The majority of the skin appendixes including hair follicles and oil and sweat glands are situated in the dermis. Dermis is also rich with blood vessels, free nerve endings and Meisner's corpuscules which function as touch receptors. The layers of the dermis are the superficial papillary layer and deeper reticular layer.

Subcutis is the deepest layer of the skin and it is mainly composed of adipose tissue with varying degree of compartmentalization by fibrous septa.



Sources: (Marieb and Hoehn, 2008; Young et al., 2006b)

2.3 Tumorigenesis in NF1

Neurofibromas are the hallmark of type 1 neurofibromatosis. In addition to neurofibromas, NF1 patients are susceptible to the development of other tumor types. These tumors include gastrointestinal stromal tumors (GISTs), juvenile myelomonocytic leukemia (JMML), astrocytomas, phaeochromocytomas and glomus tumors.

2.3.1 Classification of neurofibromas

Neurofibromas are benign hamartoma tumors traditionally considered to arise from the supporting and connective tissue cells of peripheral nerves. Several different classifications for neurofibromas exist, and they are based on either the neurofibroma location or involvement with different compartments of nerve (Evans et al., 2004; Ferner, 2010; Friedman, 2002; Riccardi, 2007). The distinction between the different types of neurofibromas have been a topic of debate among "neurofibromatologists" (Ferner, 2010). Neurofibromas can be classified into different subgroups such as cutaneous, subcutaneous, nodular plexiform, and diffuse plexiform (Friedman, 2002), or localized cutaneous, diffuse cutaneous, localized intraneural, plexiform, massive soft tissue and visceral neurofibromas (Evans et al., 2004). The neurofibroma classification by Riccardi is based on the involvement and contribution of different proportions of the nerve sheath (Riccardi, 2007). Riccardi classifies neurofibromas into endoneurial, perineurial or intra fascicular, and epineurial subtypes.

To simplify the classification, in the context of the present thesis, neurofibromas are divided into two main categories: dermal or cutaneous and plexiform neurofibromas. Both tumor types share the same cellular characteristics; however, they also display marked differences: their maximal growth rates follow different time-tables, their average size is quite different, and the plexiform neurofibromas have a marked potential for malignant transformation, while the cutaneous neurofibromas never turn malignant (Peltonen et al., 2009). Cutaneous neurofibromas are usually soft, skin-colored or slightly reddish lesions of the skin being non-encapsulated and slowly growing tumors which start to arise at puberty. They are intimately associated with the dermis and their size usually varies between a few millimeters and ~2 cm, rarely exceeding 3 cm. On the contrary, the maximal growth of plexiform neurofibromas takes place during early childhood. Furthermore, the plexiform neurofibromas may form large disfiguring masses involving nerve trunks and infiltrating the surrounding tissues (Peltonen et al., 2009). The hyperpigmentation of the skin can be a sign of underlying plexiform neurofibroma.

2.3.2 Composition of neurofibromas

Neurofibromas are composed of nerve-derived cellular elements including Schwann cells, fibroblasts, perineurial cells, axonal processes and mast cells which are all embedded in an abundant collagenous extracellular matrix (Peltonen et al., 1988) (See **Box 4.** for nerve structure). However, the cells that form organized structures in normal peripheral nerve are organized in a haphazard manner in neurofibromas.

The closer evaluation of tumors by histopathologic, electron microscopic and immunohistochemical techniques has specified cellular differentiation characteristics of this heterogenous cell population. Schwann cells are the most numerous cells typically consisting 60-80% of the cells in neurofibromas (Peltonen et al., 1988). Histologically, Schwann cells within neurofibroma tissue are seen with elongated, wavy nuclei and branched cytoplasmic processes covered by a continuous basement membrane (Hirose et al., 1986; Lassmann et al., 1976; Peltonen et al., 1988). A subpopulation of tumor Schwann cells have been shown to harbor a bi-allelic inactivation of the *NF1* (Serra et al., 2000). Thus they are considered to represent a key cell type when considering the pathogenesis of neurofibromas.

Fibroblasts are another major component of neurofibromas. They have abundant cytoplasm with numerous cell organelles and they have been considered to be of endoneurial origin (Erlandson, 1985; Lassmann et al., 1976).

Clusters of perineurial cells have been shown to be distributed around the rudimentary nerves within cutaneous neurofibromas and also at the periphery of some neurofibromas (Pummi et al., 2006). Perineurial cells are covered by a continuous basement membrane and they have long slender cell processes with numerous intracellular vesicles (Erlandson and Woodruff, 1982; Lassmann et al., 1976). Unlike fibroblasts, perineurial cells are covered by basement membrane and connected by cell-cell junctions.

Most of the volume of a neurofibroma is composed extracellular matrix including collagen types I, III, IV, V and VI, and non-collagenous glycoproteins, fibronectin, laminin and nidogen (reviewed by Jaakkola et al., 1989a). The question of which cell types contribute to the accumulation of extracellular matrix in neurofibromas has been addressed in culture (Jaakkola et al., 1989a; Jaakkola et al., 1989b). Schwann cells, perineurial cells and fibroblasts all express genes for collagens I, III and VI. Furthermore, they all produce laminin. Basement membrane-specific type IV collagen is expressed by Schwann cells and perineurial cells. Perineurial cells and fibroblasts are the sources of fibronectin in neurofibromas.

In addition to nerve-derived and haphazardly organized cell types, neurofibromas also host epithelial, endothelial and muscle cells (Peltonen et al., 1988). Within the neurofibroma, epithelial cells are seen in appendages of skin, such as elements of hair follicles and glandular structures, endothelial cells are associated with abundant vasculature and muscle cells can be detected in large-sized blood vessels as well as in a small erector pili muscle attached to a hair follicle.

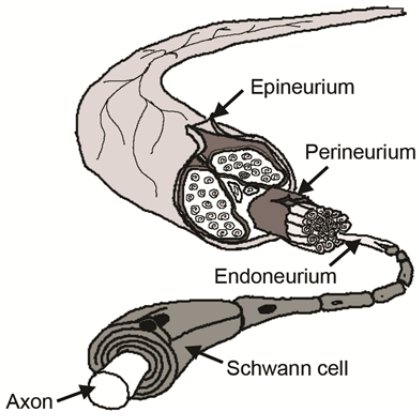
Box 4. Structure and Cellular Composition of the Peripheral Nerve

The peripheral nerve is composed of axonal processes, which are associated with Schwann cells, and the supporting connective tissue consisting of several different cell types. These cell types include fibroblasts, perineurial cells, adipocytes, macrophages and mast cells. Furthermore, small lymphatics and blood vessels transverse the connective tissue and nourish the entire nerve. Schwann cells cover the axons and either myelinate or sheath them. Schwann cells themselves are surrounded by a continuous basement membrane.

Structurally, the peripheral nerve is organized as one or more bundles of axonal processes which are tied up together by the connective tissue. The bundles of axons are called nerve fascicles. The connective tissue of the peripheral nerve is composed of three layers: endoneurium, perineurium and epineurium.

Endoneurium is the innermost connective tissue layer surrounding the axon-Schwann cell units and capillary blood vessels. It contains sparse fibroblasts, macrophages and mast cells embedded in a collagenous extracellular matrix.

Perineurium surrounds the endoneurium to form fascicles. It is composed of perineurial cell layers separated by layers of collagenous extracellular matrix. Perineurial cells are covered by basement membrane and they are connected by cell-cell junctions. The number of perineurial cell layers varies with the size of the nerve trunk decreasing towards the distal end of the nerve.



Epineurium forms the outer sheath of the nerve providing mechanical strength. It is composed of fibrocollagenous connective tissue with fibroblasts, mast cells and adipocytes. Epineurium encloses the nerve fascicles, small lymphatics and blood vessels.

Sources: (Young et al., 2006a)

2.3.3 Pathogenesis of neurofibromas

Historically, neurofibromas have been thought to arise as a consequence of a disruption of a normal nerve (**Figure 4**). The hypothesis was supported by the fact that neurofibromas contain all the cell types of normal peripheral nerve organized in a haphazard manner; in neurofibromas, Schwann cells are found dissociated from axons and the perineurium is disrupted (Peltonen et al., 1988).

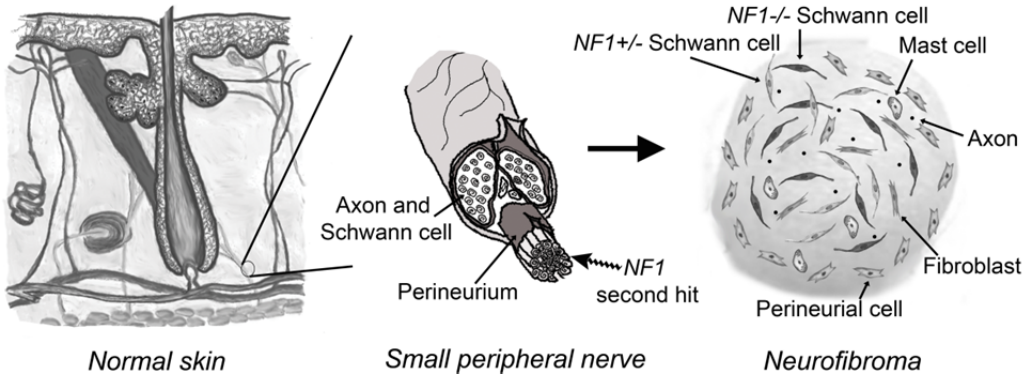


Figure 4. Schematic presentation of the development and composition of cutaneous neurofibroma. The traditional view of neurofibroma development suggests that the *NF1* second hit takes place in Schwann cells of the small nerve tributaries of the skin. As a consequence, the perineurium disrupts and the haphazardly organized tumor starts to grow.

The tumor suppressor hypothesis with double inactivation in the *NF1* gene in a subpopulation of tumor cells has been shown to be relevant to neurofibroma development (Colman et al., 1995; Serra et al., 1997). Already prior to the characterization of a cell type harboring the *NF1* second hit, several observations supported that Schwann cell is a primary cell type for neurofibroma formation (reviewed by Gottfried et al., 2006). The supporting facts included the notion that Schwann cells were the most numerous cells in neurofibromas (Peltonen et al., 1988), neurofibroma-derived Schwann cells presented highly reduced levels of *NF1* mRNA (Rutkowski et al., 2000), and Schwan cells isolated from *Nf1* knockout mice appeared biologically abnormal (Kim et al., 1997b; Muir, 1995; Sheela et al., 1990). Furthermore, cytogenetic abnormalities were identified from Schwann cells cultured from plexiform neurofibromas (Wallace et al., 2000).

The establishment of the cell culture conditions that allowed the culturing of Schwann cells and fibroblasts separately and the enrichment of two genetically different Schwann cell populations, $NF1^{+/-}$ and $NF1^{-/-}$, enabled the characterization of a specific cell type carrying the second hit on the *NF1* gene (Kluwe et al., 1999; Rutkowski et al., 2000; Serra et al., 2000). As a consequence, a second hit on the *NF1* gene has been detected in a subpopulation of tumor Schwann cells, but not in other cell types in human neurofibromas. It should also be noted that a unique second hit is found in each tumor indicating that Schwann cell population with *NF1* second-hit is clonal (Maertens et al., 2006). Thus, separate neurofibromas of the same patient carry individual *NF1* second-hits.

It has been recognized that the haploinsufficient supporting Schwann cells, perineurial cells, fibroblasts and mast cells that form an *NF1* heterozygous background for $NF1^{-/-}$ Schwann cells are a prerequisite for the neurofibroma development (McLaughlin and Jacks, 2002; Zhu et al., 2002). For instance, $NF1^{+/-}$ fibroblasts show an abnormal response to cytokines, increased collagen deposition together with increased

proliferation, and *NF1*$^{+/-}$ Schwann cells can model the tumor microenvironment by inducing angiogenesis and invasion (reviewed by Gottfried et al., 2006). Furthermore, *in vitro* studies on mice have demonstrated that the *Nf1*$^{-/-}$ Schwann cells secrete five times more kit ligand than the *Nf1*$^{+/-}$ or *Nf1*$^{+/+}$ Schwann cells (Yang et al., 2003). The kit ligand serves as a chemo attractant for mast cells expressing c-kit receptor. Further studies on neurofibroma development in mice emphasized the role of *Nf1* heterozygous bone marrow containing tumor infiltrating mast cells in neurofibroma formation (Yang et al., 2008). In general, it has been shown that inflammatory cells including mast cells participate in tumor progression and angiogenesis (Coussens and Werb, 2002; Hanahan and Weinberg, 2011).

As described above, the cutaneous and plexiform neurofibromas display marked differences in developmental timing, maximal size and malignant transformation. Thus the pathogenesis of these different tumor types should be considered separately. Several mouse models, discussed below, have been generated to elucidate the pathogenesis of neurofibromas. However, the large majority of mouse studies have focused exclusively on plexiform neurofibromas. Furthermore, it should be noted that these models only partially reflect the human disease.

About one-third of NF1 patients develop clinically detectable plexiform neurofibromas. Plexiform neurofibromas are thought to be congenital lesions indicating that the bi-allelic inactivation leading to the development of plexiform neurofibromas apparently takes place in embryonic cells (Wu et al., 2008; Zhu et al., 2002). These cells have a potential to generate large populations of cells carrying a single *NF1* second-hit mutation. The growth of plexiform neurofibromas during early childhood further distinguishes these tumors from cutaneous neurofibromas, which do not become detectable before puberty (Ferner, 2010). In contrast to cutaneous neurofibromas, plexiform neurofibromas may turn malignant, highlighting their clinical importance. However, the malignant transformation requires additional genetic changes in the tumor cells genome. In addition to bi-allelic inactivation of the *NF1* gene, mutations in other regulatory genes have been described in MPNSTs. These include, for example, the homozygous deletion of *CDKN2A*, which encodes *p16*^{INK4A}, and *p14ARF* and *TP53* loss (Gottfried et al., 2006; Peltonen and Peltonen, 2008). Furthermore, karyotypes in many MPNSTs are complex. It remains to be elucidated whether NF1-related MPNSTs are always derived from plexiform neurofibromas, or whether they can arise directly from peripheral nerves.

The fact that cutaneous neurofibromas start to grow during puberty may reflect the developmentally late onset of the *NF1* second-hit mutation in these tumors. In *Nf1* heterozygous mice cutaneous neurofibromas can be generated from *Nf1* deficient SKPs suggesting another possibility for the origin of these tumors (Le et al., 2009). The *Nf1*$^{-/-}$ genotype obviously provides the tumor cells with a growth advantage, but it is, however, not known what proportion of the Schwann cells within a cutaneous neurofibroma carries an *NF1*$^{-/-}$ genotype and how much of the tumor growth can be explained by the increase in the number of other cells with an *NF1*$^{+/-}$ genotype (Serra et al., 2000).

To conclude, neurofibromas are lesions characterized by a bi-allelic inactivation of the *NF1* gene in a subpopulation of tumor Schwann cells (Serra et al., 2000). The current understanding of the key role of Schwann cells is based on studies with conditional knock-out mice, and the genetic analysis of cells cultured from human neurofibromas (Maertens et al., 2006; Serra et al., 2000; Zhu et al., 2002). These studies have collectively shown that the bi-allelic inactivation of the *NF1* gene is a prerequisite for neurofibroma formation, but that tumorigenesis can only occur in an $NF1^{+/-}$ background.

2.3.4 Nf1 mouse models for neurofibroma development

The first Nf1 mouse models ($Nf1^{+/n31}$) described by Jacks et al. (1994) and Brannan et al. (1994) harbored a disruptive neomycin cassette in the exon 31 of the mouse *Nf1* gene. In this model, the *Nf1* heterozygous mice did not develop neurofibromas or *café-au-lait* spots but they had a shortened life span and they occasionally developed pheochromocytomas and leukemia. The homozygosity in these mice led to embryonic lethality caused by heart malformations at embryonic day 12.5—13.5. Later studies have revealed that *Nf1* heterozygous mice also show neuronal deficiencies (Cui et al., 2008), increased risk for vascular pathology such as neointima formation after injury and vascular inflammation (Lasater et al., 2010), and increased risk for susceptibility to osteoporosis (Yang et al., 2006).

To overcome the problem with embryonic lethality and the fact that tumorigenesis seems to require double inactivation of the *Nf1* gene, chimeric Nf1 mice were created (Cichowski et al., 1999). *Nf1*-deficient embryonic stem cells were injected into wild type C57BL/6 blastocysts. In this model, animals exhibiting a moderate degree of chimerism developed neurofibromas and several other hallmark symptoms of NF1. A high degree of $Nf1^{-/-}$ chimerism led to the death of the animals by 1 month of age. The contribution of $Nf1^{-/-}$ cells in the neurofibromas was assessed by using embryonic stem cells with $Nf1^{-/-}$ genotype containing β-galactosidase expressing transgene. This model suggested that neurofibromas arise from the cells derived from $Nf1^{-/-}$ embryonic stem cells (Cichowski et al., 1999).

To further address the identity of the cell type that undergoes tumor initiation, the conditional knockout mice with Cre-loxP-site-specific-recombination-construct flanking exons 31 and 32 were established. This model allowed the tissue specific inactivation of the *Nf1* gene. Zhu et al. (2002) described $Nf1^{flox/flox}$ mice with Cre-placed downstream of the promoter element for Krox20, which is a peripheral nerve myelination-related gene expressed in approximately 10% of Schwann cells and Schwann cell precursors. These $Nf1^{flox/flox}$*;Krox20cre* mice, in which only a portion of Schwann cells carried the $Nf1^{-/-}$ genotype while all the other cells were of wild type, failed to develop neurofibromas. However, the $Nf1^{flox/-}$*;Krox20cre* mouse model, in which minority of Schwann cells carried the $Nf1^{-/-}$ genotype in an otherwise *Nf1* heterozygous background developed dorsal root ganglia tumors with histology resembling that of plexiform neurofibromas. Thus, Zhu et al. (2002) demonstrated that

neurofibroma formation requires *Nf1* nullizygous Schwann cells and that tumorigenesis can only occur in an *Nf1* heterozygous microenvironment.

In order to investigate the importance of the timing of the *Nf1* second hit mutation during Schwann cell development, Wu et al. (2008) described a mouse model in which loxP sequences were inserted to flank the exon 31 of *Nf1* gene and the Cre recombinase was expressed from *Desert Hedgehog* (*Dhh*) regulatory sequences. *Dhh* is expressed in the developing glial cells, but not in neural crest cells or neurons. These mice developed plexiform neurofibromas and dermal neurofibromas growing under dermal muscle layer, thus differing from human cutaneous neurofibromas. The authors suggest that the progenitor cell for these dermal tumors may be the neural crest-derived cell of the hair follicle which presents stem cell features. The results on $Nf1^{flox/flox}$; *DhhCre* mice suggested that the need for a *Nf1* heterozygous background could be overcome with early and widespread bi-allelic inactivation of the *Nf1* gene.

Further experiments with $Nf1^{flox/-}$;*Krox20cre* and $Nf1^{flox/flox}$;*Krox20cre* mice demonstrated that *Nf1* haploinsufficiency in bone marrow-derived cells form a tumor microenvironment sufficient for tumor formation in the context of the $Nf1^{-/-}$ genotype in Schwann cells (Yang et al. 2008). $Nf1^{flox/flox}$; *Krox20cre* mice with an $Nf1^{+/-}$ bone marrow transplant developed plexiform neurofibromas in dorsal root ganglia. They also showed decrease in one-year survival rate and exhibited motor paralysis and weight loss. In contrast, irradiated $Nf1^{flox/-}$; *Krox20cre* with a wild-type bone marrow transplant did not develop tumors. In the same study, the effect of c-kit receptor tyrosine kinase, which controls mast cell development and function, was tested by intercrossing the $Nf1^{+/-}$ mice with two strains of mice with point mutations in c-kit receptor. The bone marrow transplant experiment repeated with $Nf1^{+/-}$ bone marrow with the c-kit receptor mutation did not lead to tumor formation. These experiments thus demonstrated that plexiform neurofibroma formation requires an *Nf1* haploinsufficient and c-kit competent hematopoietic system.

As a consequence of this notion, imatinib mesylate (Gleevec/ Glivec), a potential inhibitor of c-kit was used to treat the tumorigenic $Nf1^{flox/-}$; *Krox20cre* mice. Volumetric analysis performed with FDG-PET showed that imatinib mesylate reduced tumor volume and metabolic activity in treated mice approximately 50%. This led to the first experimental therapy in which a 3-year-old girl with a highly vascularized, nonresectable, and progressively growing neurofibroma was treated with imatinib mesylate. After 3 months treatment MRI showed a 70% reduction in tumor volume. However, it is still unclear whether these results can be replicated in a wider population of patients with NF1 of varying ages and tumor burdens.

In 2009, Parada's group published the first Nf1 mouse study modeling the cellular origin of dermal or cutaneous neurofibromas (Le et al. 2009). The authors crossed two mouse strains; CMV-$CreER^{T2}$ mice, which had a tamoxifen-inducible Cre transgene driven by the broadly expressed cytomegalovirus (CMV) promoter, and $Nf1^{flox/-}$; *ROSA26* mice to obtain *CMV-*$CreER^{T2}$;$Nf1^{flox/-}$;*ROSA26* mice. The *ROSA26* disruption leads to widespread expression of beta-galactosidase, which allowed tracing of the

targeted cells. SKPs were harvested from these mice, exposed to 4-OH-tamoxifen and implanted back into the same animal. The mice failed to develop dermal neurofibromas, but did develop plexiform neurofibromas when the SKPs were re-implanted into the sciatic nerves. However, topical application of tamoxifen to the skin of *CMV-CreER^{T2}; Nf1$^{flox/-}$; ROSA26* mice induced delayed formation of dermal neurofibromas. The delay in tumor development was considered to reflect the effect of the tumor microenvironment. Furthermore, the *Nf1$^{-/-}$* SKPs induced dermal neurofibromas at the graft sites in the pregnant female but not male *CMV-CreER^{T2}; Nf1flox^{-}; ROSA26* mice implicating the role of steroid hormones in the dermal neurofibroma development.

2.3.5 Oral mucosal neurofibromas

Neurofibromas are most commonly lesions of the skin, and in general population they rarely affect the oral cavity (Depprich et al., 2009; do Nascimento et al., 2010). Clinically, solitary oral neurofibromas have been described as submucosal, non-tender, discrete masses which may occur at any age (Wright and Jackson, 1980). Histologically, they are well demarcated from the surrounding connective tissue and are composed of elongated, fusiform tumor cells with comma-shaped nuclei (Wright and Jackson, 1980). The tumor matrix is myxomatous and microvacuolated with wavy collagen fibers and numerous mast cells. Immunohistochemistry has proved to be a useful tool in the diagnosis of different neural connective tissue tumors (Chrysomali et al., 1997).

NF1-related oral soft tissue and radiographic manifestations have been reported in a limited number of full-length papers (Baden et al., 1984; D'Ambrosio et al., 1988; Friedrich et al., 2003; Shapiro et al., 1984). In addition to those, several case studies have reported NF1-related oral tumors. The oral alterations have been shown to be present in 72–92% of persons with NF1 (D'Ambrosio et al., 1988; Shapiro et al., 1984). The most common findings include oral neurofibromas, enlarged fungiform papillae, intrabony lesions, wide inferior alveolar canals, and enlarged mandibular foramina (Shapiro et al., 1984; Visnapuu et al., 2007). The intraoral tumors have been detected in ~ 25% of NF1-patients, and thus the presence of neurofibromas within the oral cavity is not uncommon in association with NF1. The tongue, the buccal mucosa, the alveolar ridge, the gingiva, the lips, the palate, the floor of the mouth and the pharyngomaxillary space have been reported to be affected with tumors in association with NF1, the tongue being the most common location (D'Ambrosio et al., 1988; Powell et al., 2006; Shapiro et al., 1984). Other reported findings concerning oral soft tissue in NF1 patients include macroglossia and enlarged papillae of the tongue (Baden et al., 1984; Shapiro et al., 1984).

2.4 Schwann cells in health and disease

Schwann cells are the supporting cells of the peripheral nervous system, and their classical role is to ensheathe the axons in peripheral nerves. Based on their morphology, biochemical properties and type of axon contact, Schwann cells can be divided into four classes: myelinating Schwann cells, non-myelinating Schwann cells,

perisynaptic Schwann cells, and satellite cells of the peripheral ganglia (Corfas et al., 2004). Myelinating and non-myelinating Schwann cells form the two best characterized classes. Each myelinating Schwann cell covers a single, large diameter axon and creates a myelin sheath. They express myelin proteins such as myelin basic protein (MBP), peripheral myelin protein 22 (PMP22), myelin protein 0 (P0), myelin associated glycoprotein (MAG), and myelin and lymphocyte protein (MAL). Non-myelinating Schwann cells can cover several small-diameter axons of C-fibers and form Remak bundles in which afferent fibers with slow conduction velocity are separated by cytoplasm of a Schwann cell. Compared to myelinating Schwann cells, the non-myelinating Schwann cells express higher levels of glial fibrillary acidic protein (GFAP), the low-affinity neurotrophin receptor p75 and the cell adhesion molecule L1 (Corfas et al., 2004).

2.4.1 The origin of Schwann cells

All different types of Schwann cells are primarily derived from a single precursor cell type, the neural crest progenitor cell (reviewed by Jessen and Mirsky, 2005). These precursor cells give rise to Schwann cell precursors, which develop into immature Schwann cells and finally, to one of the mature Schwann cell types. The differentiation of these cells can be followed by screening the expression of differentiation marker proteins. As exemplified in mice, Schwann cell precursors start to express P0, growth-associated protein 43 (GAP43) and F-spondin, and further differentiated immature Schwann cells express S100beta and low levels of P0 (reviewed by Corfas et al., 2004). The Schwann cell precursors are best characterized from mice embryos between embryonic days E12-13, whereas immature Schwann cell stage is described between E13-15 (Jessen and Mirsky, 2005). Postnatal fate of immature Schwann cells is determined by axonal contact. The association with a single large diameter axon (>~1µm) leads to the myelinating phenotype whereas the smaller axons are covered by non-myelinating Schwann cells (**Figure 5**). Both of these mature phenotypes are reversible. In response to nerve injury, myelinating and non-myelinating Schwann cells can take the phenotype of immature Schwann cells (Jessen and Mirsky, 2005). Also the Schwann cell precursors can be diverted to other neural crest derivatives, at least *in vitro*. Furthermore, the neural crest progenitor cells can undergo multilineage differentiation. In addition to Schwann cells, they have potential to differentiate into fibroblasts (Joseph et al., 2004).

Communication between axons and Schwann cells is essential for Schwann cell survival, myelination and normal nerve function. The transmembrane glycoprotein MAG is expressed by Schwann cells and plays a role in the formation and maintenance of myelin, but it also functions as a ligand for an axonal receptor (Quarles, 2007). Furthermore, the small-MAG isoform has been characterized as a microtubule-associated protein which possibly links the axons and myelinating glial cell cytoskeleton (Kursula et al., 2001). Especially, the Schwann cell precursors are dependent on survival signals from axons whereas the mature Schwann cells have also own autocrine survival circuits (reviewed by Jessen and Mirsky, 2005). The growth factor neuregulin 1 (NRG-1) and its receptors, the ErbB receptor tyrosine kinases have

been shown to act as key regulators of axon-Schwann cell interactions and Schwann cell development (Corfas et al., 2004). The neurons express NRG-1 while the Schwann cells express ErbB2 and ErbB3 receptors. The receptor-ligand interaction can induce the differentiation of neural crest cells into Schwann cell phenotype, induce Schwann cell proliferation in culture and regulate Schwann cell motility and migration.

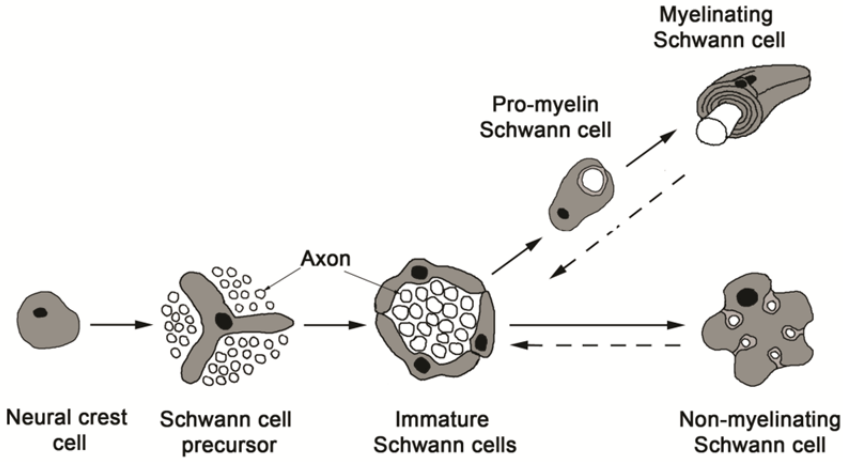


Figure 5. The development of Schwann cells. The illustration summarizes the phases and transitions during Schwann cell development. Dashed lines indicate the reversible phases towards a less differentiated stage. Modified from (Jessen and Mirsky, 2005).

2.4.2 Schwann cells in culture

Adult Schwann cells are typically quiescent cells, proliferating only in association with nerve injury or when a Schwann cell tumor arises. Also *in vitro*, Schwann cells normally proliferate very slowly (reviewed by Stewart et al., 1991). Schwann cell proliferation *in vitro* requires stimulation with a combination of different growth factors. These growth factors include basic fibroblast growth factor (bFGF), platelet derived growth factor (PDGF), glial growth factor (GGF) and insulin-like growth factor I (IGF-I) (Schumacher et al., 1993; Stewart et al., 1991). The above mentioned growth factors stimulate DNA synthesis also in a serum-free culture medium whereas TGF-β1 can act as a Schwann cell mitogen only in association with other serum factors (Stewart et al., 1991). It has been shown that the co-stimulation with growth factors and an agent that elevates intracellular cAMP levels is required to enhance Schwann cell proliferation. In culture, Schwann cell proliferation can be further enhanced by insulin which binds to the IGF-I receptor, and thus induces the mitogenic effect (Hanemann et al., 1998; Schumacher et al., 1993). Moreover, insulin affects the shape of Schwann cells by increasing the proportion of spindle-shaped Schwann cells (Hanemann et al., 1998).

The activation of the intracellular messenger pathway acting via cAMP is essential for Schwann cell proliferation. In Schwann cells, the cAMP enhances the action of neuregulin 1 (NRG-1), the most potent Schwann cell mitogen by increasing the

activation of ERK and Akt signaling pathways which both are required for cell cycle progression (Monje et al., 2008) (**Figure 6**). NRG-1, which can support Schwann cell maturation, survival and motility, is a member of the heregulin growth factor family (Rahmatullah et al., 1998). In general, heregulins stimulate cell proliferation by binding to and activating ErbB receptors, which are transmembrane receptor tyrosine kinases with homology to the epidermal growth factor (EGF) receptor. Ligand-dependent activation of ErbB receptors leads to activation of the mitogen-activated protein kinase (MAPK) pathway, and finally to cell proliferation (Rahmatullah et al., 1998). cAMP- dependent regulation is mediated by protein kinase A (PKA) (Kim et al., 1997a). Intracellular cAMP directly activates PKA, which in turn enhances the NRG-1-induced phosphorylation and activation of ErbB2 and ErbB3 (Monje et al., 2008). The activation leads to the enhanced ERK and Akt signaling. In these cells, the MAPK cascade seems to represent an intersection of cross talk between two signaling pathways initiated by receptor tyrosine kinases and cAMP (Kim et al., 1997a). However, the mechanisms that regulate adenylate cyclase activity in Schwann cells are poorly understood. In many other cell types, such as fibroblasts, the proliferation is inhibited by cAMP (reviewed by Rahmatullah et al., 1998).

Forskolin, a diterpene derived from roots of *Coleus forskohli* (**Figure 6**), is a direct activator of adenylate cyclase causing reversible enzyme activation (Seamon et al., 1981), and it is commonly used in Schwann cell culture media. Furthermore, in culture conditions, the degradation of cAMP can be inhibited by 3-isobutyl-1-methylxanthine (IBMX), which is a potent phosphodiesterase inhibitor (Seamon et al., 1981). The combination of 0.5 µM IBMX together with 0.5 µM forskolin have been shown to produce the most potent inhibitory effect on fibroblast growth without negative effect on Schwann cells (Rutkowski et al., 1995). Furthermore, together with forskolin, heregulin induces the maximal proliferation of Schwann cells by sustaining high levels of cyclin D1 expression and hyper phosphorylation of pRb as Schwann cells progress through the G_1 phase of the cell cycle (Rahmatullah et al., 1998). Heregulin also stimulates transient phosphorylation of cAMP response element binding protein (CREB), which is one of the targets of PKA-mediated phosphorylation and which, in its phosphorylated form, functions as transcription factor activating several target genes (Rahmatullah et al., 1998).

In addition to growth factors and cAMP, the components of the ECM affect Schwann cell proliferation and survival (Chernousov et al., 2008). Typical protein components of the ECM include collagens, laminin heterodimers, nidogen and proteoglycans. As an example of collagens, collagen type IV, which is discussed in more detail in the biomarker section (**2.5**), is a component of the basement membrane that surrounds Schwann cells. It helps Schwann cells to attach and spread by an integrin $\alpha1\beta1$- and $\alpha2\beta1$- mediated manner (Chernousov et al., 2008). The interactions promote Schwann cell differentiation. Laminins are another important component of the ECM. They give structure to the basement membrane and provide attachment sites for cells via cell surface proteins (Chernousov et al., 2008). Furthermore, laminins act as ligands for receptors on cells and thus initiate signaling cascades that influence cell behavior and survival. *In vitro*, Schwann cell survival and proliferation can be enhanced by coating

the culture dishes with laminin and poly-L-Lysine, which further enhances the cell attachment (Hanemann et al., 1998).

As discussed above, the key factor supporting Schwann cell growth *in vitro* is forskolin, which in combination with differentiation growth factors, especially heregulin, leads to Schwann cell proliferation (Rahmatullah et al., 1998). In the context of *NF1* deficiency, two genetically different Schwann cell populations, $NF1^{+/-}$ and $NF1^{-/-}$, can be enriched from neurofibromas using different culture conditions (Maertens et al., 2006; Rosenbaum et al., 2000; Serra et al., 2000), the main difference being the presence of forskolin in the culture medium of the $NF1^{+/-}$ cells and the absence of forskolin in the culture medium of the $NF1^{-/-}$ cells. Serra and coworkers (2000) showed that neurofibroma-derived Schwann cells carrying the $NF1^{-/-}$ genotype were absent in passage 6 if cells were grown in forskolin-containing medium. These culture conditions gave growth disadvantage to $NF1^{-/-}$ Schwann cells. The removal of forskolin produced an enrichment of $NF1^{-/-}$ Schwann cells. This can be explained by the fact that at least one function of neurofibromin is to antagonize the accumulation of cAMP and the expression of cyclin D1 in Schwann cells (Kim et al., 2001). Thus the $NF1^{-/-}$ Schwann cells can sustain high cAMP levels without the forskolin-mediated stimulation of adenylate cyclase.

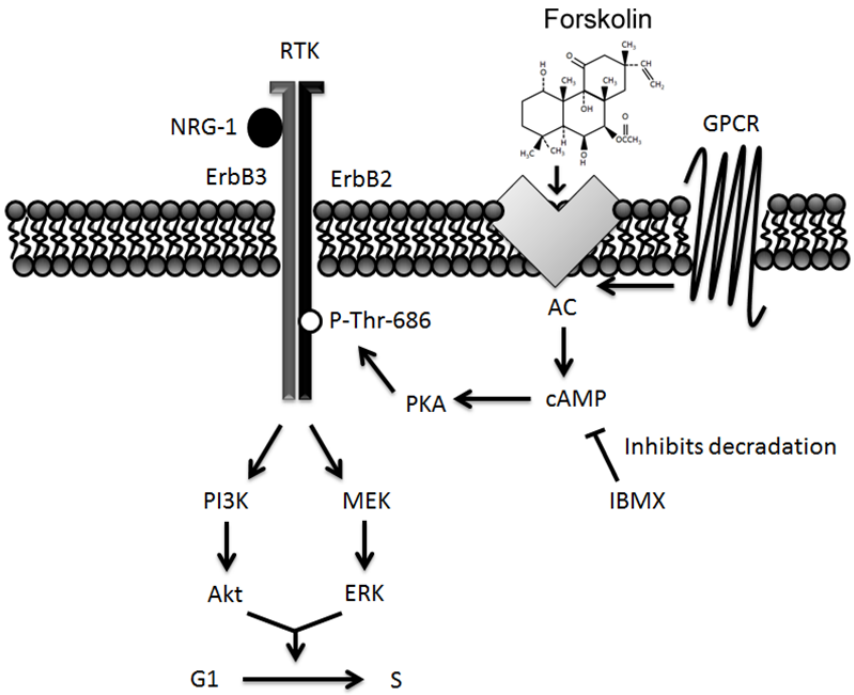


Figure 6. Cross talk between signaling pathways via receptor tyrosine kinase (RTK) and adenylate cyclase (AC) is illustrated. Forskolin acts directly at the AC-enzyme complex leading to the accumulation of intracellular cAMP and phosphorylation of ErbB2/ErbB3 receptor via PKA. The phosphorylation enhances the action of NRG-1. Modified from (Monje et al., 2008).

2.4.3 The immunocompetence of Schwann cells

The progress in Schwann cell research during recent years has increased knowledge and broadened the functions of Schwann cells beyond myelination. The role of Schwann cells in association with the immune response has been recognized. In inflammatory neuropathies, such as Guillain-Barré syndrome and chronic inflammatory demyelinating polyneuropathy, Schwann cells become targets of an autoimmune response (Meyer zu Hörste et al., 2008). However, they can also modulate the local inflammation by humoral mechanisms such as production and release of nitric oxide, a mediator with immunosuppressive properties (Meyer zu Hörste et al., 2008). Overall, Schwann cells have a potential to display the entire spectrum of immune response which includes antigen presentation and recognition, regulation of immune response by secreting soluble factors, and termination of the immune response via the interaction of Fas and FasL, the mediators of apoptosis (Meyer zu Hörste et al., 2008). Fas and FasL are the death receptors on the surface of the target cell and its ligand expressed by the killer cell, respectively. Furthermore, Schwann cells have also potential to express major MHC class II genes (Scarpini et al., 1990). Schwann cells contain the elements required to process both endogenous and exogenous peptides and present them to T lymphocytes (Meyer Zu Horste et al., 2010). The endogenous and exogenous antigens can be presented on MHC class II molecules.

2.5 Neurofibroma biomarkers

In cell biology, biomarker refers to a molecule that allows the detection and identification of a particular cell type. In the context of the present thesis, biomarker also refers to an antigenic marker the expression of which is linked to a certain stage of cellular differentiation. In neurofibroma tissue containing cells dissociated from organized structures, the identification of different cell types is impossible without biomarkers. The different cell types characterized in cutaneous neurofibromas and the molecular markers used in their identification are listed in **Table 2.2**. The biomarkers essential for this thesis are discussed in more detail below. More attention is paid to the class III β-tubulin which is in central role in the first subproject of this thesis.

Table 2.2. Cellular components of neurofibromas and their biomarkers

Cell type	Molecular marker
Schwann cell	S100, Collagen IV
Perineurial cell	Claudin 1, Collagen IV
Fibroblast	CD34
Neuron[1]	Neurofilament
Phagocytic cell	α1-antitrypsin
Mast cell	Factor VIII
Epithelial cell	Cytokeratin Epithelial membrane antigen (EMA)
Endothelial cell	Factor VIII
Muscle cell	Desmin

[1]Only axonal processes, not nerve cell bodies are seen in neurofibromas
References: (Peltonen et al., 1988; Pummi et al., 2006; Tardío, 2009)

S100 proteins are a family of calcium binding proteins which are soluble in 100% saturated ammonium sulfate in neutral pH (Moore, 1965; Zimmer et al., 1995). S100 proteins are localized in the cytoplasm or nucleus and they are homo- or heterodimeric proteins composed of two polypeptide subunits with molecular mass of ~10 kDa (Isobe et al., 1977; Zimmer et al., 1995). The *S100A1* (S100A protein) and *S100B* (S100B protein) genes are the main S100 genes expressed in nervous tissue, and they are combined as homo or heterodimers of A- and B-subunits to form S100a0 (AA), S100a (AB) and S100b (BB) (Gonzalez-Martinez et al., 2003). S100 proteins are mainly expressed by Schwann cells and Schwann-related cells of the peripheral nerves, but they have also been detected in a subpopulation of peripheral neurons and certain non-neuronal cell types (Gonzalez-Martinez et al., 2003). Specifically, *S100B* is predominantly expressed in Schwann cells and astrocytes and can thus be used as a biomarker when identifying the cell type. Additionally, in cutaneous tissue also melanocytes and Langerhans cells show positivity for S100B protein (Park and Min, 2003). Peltonen et al. 1988 showed that 60—80% of the cells in cutaneous neurofibromas are positive for S100 as detected by antibody against S100A and S100B, and these cells were considered to represent Schwann-like cells. The staining reaction was most intense in Schwann cells locating within nerve structures with axons. In culture, Schwann cells are often characterized by their bipolar shape. Scarpini and coworkers showed that the antibody directed against mixture of bovine brain S100 proteins specifically labeled cultured Schwann cells but not fibroblasts providing evidence of its usefulness as a Schwann cell marker *in vitro* (Scarpini et al., 1986).

Type IV collagen is one of the major constituents of the basement membranes. The protein consists of three ~180 kDa polypeptide chains (Paulsson, 1992). Two molecules of collagen IV can interact in a head-to-head fashion, and four molecules of collagen IV are frequently connected to form a spider-shaped structure (Paulsson, 1992). These two types of interactions allow the formation of a network that serves as a scaffold for the basement membrane. Most cells within neurofibromas have been shown to be positive for collagen IV reflecting that most cells of the tumor are covered by basement membranes (Peltonen et al., 1988). Schwann cells and the perineurial cells are covered by basement membranes and can thus be detected by immunolabeling for type IV collagen.

CD34 is a transmembrane glycoprotein with molecular mass of 115 kDa (Chaubal et al., 1994; Tardío, 2009). It is expressed by human hematopoietic progenitor cells and myeloid lineage cells as well as in endothelial and certain fibroblast-related mesenchymal cells (Chaubal et al., 1994). In normal skin, CD34 expression has been characterized in endothelial cells, interstitial and perivascular spindle-shaped and dendritic cells, in elongated cells around the midportion of the hair follicles, and in spindle cells around the secretory coils of the eccrine sweat glands (Nickoloff, 1991; Tardío, 2009). A growing number of CD34-reactive cutaneous tumors have been reported (Tardío, 2009). In peripheral nerve sheath tumors including neurofibromas, CD34-positive cells have been suggested to represent endoneurial fibroblasts based on their triple negativity to Schwann cell marker S100, epithelial and perineurial cell

marker EMA and perineurial cell marker glucose transporter protein 1 (Glut1) (Hirose et al., 2003). The CD34-positive cells are typically more numerous at the periphery of neurofibromas than in the central areas (Tardío, 2009). A majority of MPNSTs are, however, negative for CD34 and in schwannomas only a few slender cells in the peripheral zones and Antoni B areas show positivity for CD34 (Tardío, 2009).

Mast cells are intrinsic defense cells that are functionally related to basophils (Young et al., 2006c). Mast cells are found in all supporting tissue but their prevalence is high in the skin and around blood vessels. Mast cells are degranulating cells that release histamine and other vasoactive mediators. Staining with certain blue basic dyes such as toluidine blue can reveal the cytoplasmic granules within mast cells and the dyes can be used for the detection of mast cells. Isaacson (1975) demonstrated that a high concentration of mast cells is characteristic of neurofibromas and can be a distinction criterion between neurofibromas and MPNSTs. In that study mast cells were detected using buffered thionin (Isaacson, 1976). Peltonen et al. (1988) showed that cutaneous neurofibromas contain individual scattered cells positive for Factor VIII-related antigens. These cells were also suggested to represent mast cells. To support the observation mast cells were further recognized by the Alcian blue stain at pH1.0, and the density of positive cells corresponded to that observed with Factor VIII (Peltonen et al., 1988).

Nestin is a large (~200kDa) intermediate filament protein (see **Box 5.** The Cytoskeleton) which is predominantly expressed in rapidly dividing progenitor cells of developing and regenerating tissues, and it is down-regulated and replaced by tissue specific intermediate filament proteins upon differentiation (Michalczyk and Ziman, 2005). Nestin often reappears transiently after injury. More specifically, nestin is found to be expressed by a majority of mitotically active central and peripheral nervous system progenitors that give rise to both neurons and glia. Furthermore, hair follicles have been recognized as a niche for nestin-positive stem cells as discussed in section 2.2.2 (Amoh et al., 2005a; Amoh et al., 2005b). Nestin is broadly used as a marker for stem cells characterized by multipotency, self-renewal and regeneration.

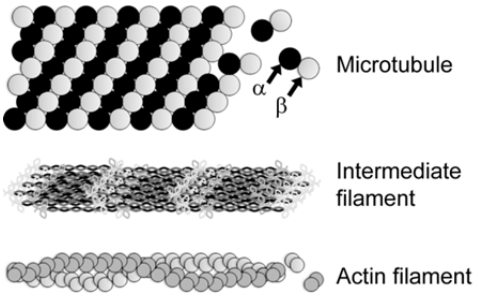
Box 5. The Cytoskeleton

The cytoskeleton is dynamic system composed of intracellular filaments. The filamentous network is not only essential for cell shape, but also plays a crucial role in many cellular processes such as intracellular transport and cell division. The cytoskeletal filaments are divided into three major types: microtubules, intermediate filaments and microfilaments. The latter are also called actin filaments or F-actin.

Microtubules are hollow tubes with the diameter of ~25 nm and they are constructed from 13 protofilaments that interact laterally with each other. The protofilaments are polymerized from two structural tubulin subunit proteins, α- and β-tubulin, which form heterodimers. Microtubules rise from a microtubule-organizing center called a centrosome. They take part in various modes of cellular movement and in the maintenance and modulation of cell morphology. The cellular movements include molecular trafficking along microtubules via dynein and kinesin-type ATPases, movement of cilia and flagella, and translocation of chromosomes during the formation and disassembly of the mitotic spindle. (Honore et al., 2005; Walczak and Heald, 2008)

Intermediate filaments are a superfamily of 10 nm fibers exhibiting cell-type specific patterns of expression. Intermediate filaments include acidic and basic keratins, vimentin-like proteins, neurofilament proteins, nuclear lamins and nestin. Two protein monomers with a central helical domain and globular domains at both ends are coiled around a common axis to form a dimer. The two dimers then link to each other to form a tetramer and eight tetramers finally join together to form an intermediate filament. Intermediate filaments increase the mechanical strength and motility of cells and they also participate in cell signaling. (Eriksson et al., 2009)

Microfilaments are composed of ubiquitously expressed actin proteins that exist as two protofilament polymers wound together in a right-handed helix. The diameter of an actin filament is 5-9 nm. In addition to regulating cell shape together with other cytoskeletal components, microfilaments have a central role in many cellular processes including muscle contraction, cell motility, cell division and cytokinesis, cell signaling and cell junctions. Actin was first described in muscle where it forms the contractile bundles together with the motor protein myosin. The association with myosin causes the sliding of actin filaments closer to each other leading to muscle contraction or cytokinesis in dividing cells. (Wickstead and Gull, 2011)



2.5.1 βIII-tubulin

In human, seven β-tubulin isotypes exist with different tissue distribution (Ludueña, 1998). These isotypes differ primarily in their C-terminal variable domain comprised of 15 amino acids (McKean et al., 2001; Sullivan, 1988). The heterogeneity in tubulin is pronounced in the central nervous system, specifically during development when tubulin isoforms are differently expressed compared to mature neurons (Farina et al., 1999). Class III β-tubulin has been considered to be a neuron-specific marker molecule, and its presence is considered as an indicator of dynamic instability (Katsetos et al., 2003b). Dynamic instability refers to rapid transitions between growing and shrinking states of individual microtubules and to the reservoir of readily available pool of unstable monomers that can be rapidly recruited to different cellular compartments (Burbank and Mitchison, 2006). *In vitro* studies have shown that class III β-tubulin decays more slowly than class II β-tubulin and assembles to form more dynamic α-β-tubulin heterodimers than other isotypes. Furthermore, class III β-tubulin is the only β-tubulin subtype regulated by phosphorylation, which may reflect its role in the regulation of microtubule assembly and disassembly *in vivo* (Katsetos et al., 2003a). Expression of class III β-tubulin has also been shown to represent a useful tool to investigate early phases of neuronal differentiation in human embryonic development and when differentiating neurons from stem cells (Easter et al., 1993; Katsetos et al., 1993; Katsetos et al., 2003b; Kukharskyy et al., 2004; Svendsen et al., 2001).

The expression of class III β-tubulin has also been investigated in the context of tumorigenesis (Katsetos et al., 2003b). Altered patterns of expression have been noted in brain tumors. It is important to note that class III β-tubulin is not present in normal differentiated glial cells. In addition to neurons and neuronal tumors, class III β-tubulin has been detected in selected malignancies, such as in breast cancers and other malignant epithelial tumors (Hasegawa et al., 2003; Jirásek et al., 2007). Some studies have associated expression of class III β-tubulin with histologically high grade of malignancy in non-neuronal tumors (Katsetos et al., 2003a), and described class III β-tubulin as a marker of cancer cells' resistance to taxanes (Hasegawa et al., 2003; Lee et al., 2007). In general, the central role of microtubules during mitosis has emphasized tubulins as an important target for cancer drugs (Kavallaris, 2010).

The mouse monoclonal antibody TuJ1 is commonly used to detect class III β-tubulin expression. The antibody reacts with an epitope located within the last seven amino acids of the extreme C-terminal, isotype-defining domain of class III β-tubulin (Katsetos et al., 2003b; Lee et al., 2007). TuJ1 recognizes both the phosphorylated and unphosphorylated forms of class III β-tubulin.

3. AIMS OF THE STUDY

The purpose of present study was to investigate the pathogenesis of cutaneous neurofibroma tumors from a novel perspective. The specific aims of the study were:

1. To evaluate the use of class III β-tubulin as a biomarker in NF1-associated tumors and tumor-derived cells

2. To characterize the type and frequency of oral soft tissue alterations in neurofibromatosis, specifically focusing on oral tumors

3. To investigate the role of multipotent progenitor cells to the development of cutaneous neurofibromas

4. To investigate the effect of the *NF1* second hit mutation comparing the transcriptomes of the neurofibroma-derived Schwann cell cultures enriched towards $NF1^{+/-}$ or $NF1^{-/-}$ genotype

4. MATERIALS AND METHODS

4.1 Materials

4.1.1 Tissue samples (I-IV)

All human tissue material was obtained from Turku University Central Hospital, Turku, Finland, with the approval of the Ethical Committee of the Hospital District of Southwest Finland and with appropriate written consent from the patients. Normal human skin samples were obtained from plastic surgeries of healthy persons, the MPNSTs and neurofibromas were obtained from the Department of Pathology or Department of Oral Diseases, fresh neurofibroma tissue for culturing of Schwann cells was from the Department of Dermatology, and the great auricular nerve was provided by the Department of Otorhinolaryngology-Head and Neck Surgery. Red blood cells were obtained from a voluntary healthy female.

4.1.2 Cell lines (I)

5637 and T24 human urinary bladder cancer cell lines were purchased from American Type Culture Collection (ATCC; Rockville, MD). Cell line 5637 represents Grade 2 and T24 represents Grade 3 carcinomas.

4.1.3 Antibodies

Table 4.1. List of primary antibodies

Antibody	Cat no	Source	Used in:
FITC-conjugated anti-human CD4	88-8999-40	eBioscience Ltd, Hatfield, UK	IV
Mouse mab to *Xenopus laevis* beta Actin	ab6276	Abcam, Cambridge, UK	I, IV
Mouse mab to (QBEnd/10) to human CD34	790-2927	Ventana Medical Systems, Inc, Tucson, AZ	II
Mouse mab to CD9	sc009 kit	R&D systems, Minneapolis, MN	III
Mouse mab to cow S100 beta	ab11179	Abcam, Cambridge, UK	III, IV
Mouse mab to E-cadherin	sc009 kit	R&D systems, Minneapolis, MN	III
Mouse mab to human collagen type IV	C1926	Sigma-Aldrich, St. Louis, MO	II-IV
Mouse mab to human cytokeratin 14	C8791	Sigma-Aldrich, St. Louis, MO	III
Mouse mab to human HLA-DQ [SPV-L3]	ab23632	Abcam, Cambridge, UK	IV
Mouse mab to human HLA-DR [TAL 1B5]	ab20181	Abcam, Cambridge, UK	IV
Mouse mab to human integrin α–4	ab220	Abcam, Cambridge, UK	III
Mouse mab to human nestin	ab22035	Abcam, Cambridge, UK	III
Mouse mab to human neurofilament	ab8970	Abcam, Cambridge, UK	II, IV

Table 4.1 List of primary antibodies continues

Antibody	Cat no	Source	Used in:
Mouse mab to human/mouse FoxP3	14-7979	eBioscience Ltd, Hatfield, UK	IV
Mouse mab to SSEA-1	sc009 kit	R&D systems, Minneapolis, MN	III
Mouse mab Tuj-1 to rat Class III beta-tubulin	MMS-435P	Covance, Princeton, NJ	I, II, III
Mouse mab, Tuj-1, to human Class III beta-tubulin	ab53234	Abcam, Cambridge, UK	I
PE-conjugated anti-human Foxp3	88-8999-40	eBioscience Ltd, Hatfield, UK	IV
Rabbit mab to human alpha-tubulin	ab52866	Abcam, Cambridge, UK	I
Rabbit mab to human CD3 [2GV6]	790-4341	Ventana Medical Systems, Inc, Tucson, AZ	IV
Rabbit mab to human CD4 [SP35]	790-4423	Ventana Medical Systems, Inc, Tucson, AZ	IV
Rabbit mab to human CD43 [L60]	760-2511	Ventana Medical Systems, Inc, Tucson, AZ	IV
Rabbit mab to human CD5 [SP19]	790-4451	Ventana Medical Systems, Inc, Tucson, AZ	IV
Rabbit mab to human CD8 [SP57]	790-4460	Ventana Medical Systems, Inc, Tucson, AZ	IV
Rabbit mab to human HLA-DR [EPR3692]	ab92511	Abcam, Cambridge, UK	IV
Rabbit mab to human S100 beta	ab52642	Abcam, Cambridge, UK	IV
Rabbit mab to rat p44/42 MAPK (ERK1/2)	4695	Cell Signaling Technology, Danvers, MA	IV
Rabbit pab to cow S100	18-0046	Invitrogen, Eugene, OR	II-IV
Rabbit pab to human fibronectin	A-245	Dako, Glostrup, Denmark	III
Rabbit pab to human FoxP3	ab10563	Abcam, Cambridge, UK	IV
Rabbit pab to human nestin	ab5968	Abcam, Cambridge, UK	III
Rabbit pab to rat phosphor-p44/42 MAPK (ERK1/2, Thr202/Tyr204)	9101	Cell Signaling Technology, Danvers, MA	IV
Rabbit pab, sc-67, to human neurofibromin	sc-67	Santa Cruz Biotechnology, Santa Cruz, CA	III
Rat mab [YL1/2] to S.cerevisiae tubulin	ab6160	Abcam, Cambridge, UK	I

mab, monoclonal antibody; pab, polyclonal antibody

Table 4.2. List of secondary antibodies and nuclear stain

Antibody	Cat no	Source	Used in:
Alexa-Fluor 488-conjugated goat anti-mouse IgG	A11029	Molecular Probes, Eugene, OR	I, III, IV
Alexa-Fluor 568-conjugated goat anti-rabbit IgG	A11011	Molecular Probes, Eugene, OR	I, III, IV
Cy3 conjugated goat anti-rat IgG	112-165-167	Jackson Immuno Research Europe, Suffolk, UK	I
Hoechst 33342	H3570	Invitrogen, Eugene, OR	I, III, IV
Horse anti-mouse IgG, HRP-linked	7076	Cell Signaling Technology, Danvers, MA	I, III, IV
Goat anti-rabbit IgG, HRP-linked	7074	Cell Signaling Technology, Danvers, MA	I, III, IV

HRP, horse radish peroxide

4.2 Methods

4.2.1 Cell culture media (I, III, IV)

Table 4.3. Medium composition for human normal keratinocytes (I, IV)

Ingredient	Concentration	Source	Cat no
KGM-SFM	100%	Gibco/ Invitrogen, Eugene, OR	10744

KGM, keratinocyte growth medium; SFM, serum free medium

Table 4.4. Medium composition for human normal skin fibroblasts and cancer cell lines 5637 and T24 (I)

Ingredient	Concentration	Source	Cat no
DMEM, high glucose (4,5 g/l)	89%	Gibco/ Invitrogen, Eugene, OR	41966-029
FBS	10%	Variable sources	
Penicillin/ Streptomycin	100 U/ml (1%) 100µg/ml	Gibco/ Invitrogen, Eugene, OR	15140-122

DMEM, Dulbecco's Modified Eagle Medium; FBS, Fetal bovine serum

Table 4.5. Medium composition for human neurofibroma-derived Schwann cells (I, III, IV)

Ingredient	Concentration	Source	Cat no
DMEM, high glucose (4,5 g/l)	89%	Gibco/ Invitrogen, Eugene, OR	41966-029
FBS, US origin	10%	Gibco/ Invitrogen, Eugene, OR	16000-044
Penicillin/ Streptomycin	100 U/ml (1%) 100µg/ml	Gibco/ Invitrogen, Eugene, OR	15140-122
IBMX	0.5 mM	Sigma-Aldrich, St. Louis, MO	I-5879
Beta 1-heregulin	10 mM	Sigma-Aldrich, St. Louis, MO	H7660
Insulin	2.5 µg/ml	Sigma-Aldrich, St. Louis, MO	I-6634
Forskolin[1]	0.5 µM	Sigma-Aldrich, St. Louis, MO	F-6886

[1] *NF1*$^{-/-}$ Schwann cells were cultured in a medium without forskolin; DMEM, Dulbecco's Modified Eagle Medium; FBS, Fetal bovine serum; IBMX, 3-isobutyl-1-methylxanthine

Table 4.6. Medium composition for human neurofibroma-derived precursor cells (NFPs) (III, IV)

Ingredient	Concentration	Source	Cat no
DMEM, high glucose (4,5 g/l)	45%	Gibco/ Invitrogen, Eugene, OR	41966-029
Ham's F-12 Nutrient Mix	45%	Gibco/ Invitrogen, Eugene, OR	21765-029
Penicillin/ Streptomycin	100 U/ml (1%) 100µg/ml	Gibco/ Invitrogen, Eugene, OR	15140-122
B27 supplement	1X (2%)	Gibco/ Invitrogen, Eugene, OR	17504
L-glutamine	2 mM	Gibco/ Invitrogen, Eugene, OR	35050-038
Hepes	15 mM	Lonza, Cologne, Germany	17-737E
EGF	20 ng/ml	Gibco/ Invitrogen, Eugene, OR	PHG0314
bFGF	10 ng/ml	Gibco/ Invitrogen, Eugene, OR	PHG0264

DMEM, Dulbecco's Modified Eagle Medium; FBS, Fetal bovine serum; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGF, epidermal growth factor; bFGF, fibroblast growth factor-basic

4.2.2 Differentiation of cells (III, IV)

Culturing of human neurofibroma Schwann cells and enrichment towards $NF1^{+/-}$ and $NF1^{-/-}$ genotypes was performed as described (Rosenbaum et al., 2000; Serra et al., 2000). To induce differentiation of the NFP cells into Schwann cells, the medium was replaced with the Schwann cell proliferation medium (**Table 4.5**). For adipogenesis, cells were incubated in adipocyte differentiation medium (**Table 4.7**). The adipogenic differentiation was detected by Oil Red O stain (Cambrex Bio Science, Walkersville, MD). Differentiation into neuronal phenotype occurred spontaneously in NFP medium.

Table 4.7. Medium composition for adipocytes (III)

Ingredient	Concentration	Source	Cat no
NFP medium		See **Table 4.6.**	See **Table 4.6.**
IBMX	0.5 mM	Sigma-Aldrich, St. Louis, MO	I-5879
Insulin	1.7 µM	Sigma-Aldrich, St. Louis, MO	I-6634
Indomethacin	100 µM	Sigma-Aldrich, St. Louis, MO	I-7378
Dexamethasone	1 µM	Sigma-Aldrich, St. Louis, MO	D-4902

IBMX, 3-isobutyl-1-methylxanthine

4.2.3 siRNA transfection (IV)

$NF1^{+/-}$ Schwann cells, NFPs, normal human keratinocytes and keratinocytes from a patient with NF1 were transfected with three different NF1 siRNAs: SASI_Hs01_00129006 (si1), SASI_Hs01_00129008 (si2) and SASI_Hs02_00302218 (si3) (Sigma-Aldrich, St. Louis, MO) and negative control siRNA (cat no 1027310; Qiagen, Venlo, Netherlands). In controls, siRNA and transfection reagents were omitted. The transfection was done using SiLentFect Lipid reagent (Bio-Rad, Hercules, CA), and the transfection medium contained siRNA at concentration of 75 nM.

4.2.4 NF1 mutation analysis (III, IV)

Mutation analysis of the *NF1* gene was carried out in Ludwine Messiaen's lab (University of Alabama at Birmingham) as described (Messiaen et al., 2000; Messiaen and Wimmer, 2008). Briefly, two parallel cell cultures per Schwann cell line were established, one for RNA and one for DNA isolation. Cultures subjected to RNA isolation were treated with 200 µg/ml of puromycin (Sigma-Aldrich) for 2-4 hours before isolation, to circumvent the nonsense-mediated RNA decay. The entire coding region was amplified in three overlapping cDNA fragments of ~4kb, which were sequenced using BigDye Terminator Cycle Sequencing Kit (4337456 v3.1; Applied Biosystems, Foster City, CA). Multiplex ligation-dependent probe amplification analysis was performed using the P081 and P082 assay (MRCHolland, Amsterdam, The Netherlands) to search for any copy-number changes that might have escaped detection by direct sequencing at the cDNA level. All alterations found at the cDNA level were confirmed at the genomic DNA (gDNA) level.

4.2.5 Histology and ultrastructure (II-III)

Table 4.8. Histological staining methods

Method:	Used in:
Van Gieson (VG) staining	III
Hematoxylin-eosin (HE) staining	II
Masson's trichrome staining	II
Toluidine blue staining	II, III
Congo red staining	II

4.2.6 Three-dimensional reconstruction of cutaneous neurofibromas (III)

The pictures from sequential neurofibroma sections were adjusted and rotated using Adobe Photoshop Version 8.0, and the three-dimensional models were created using 3D-DOCTOR software (Able Software Corp., Lexington, MA).

4.2.7 Protein analyses

Table 4.9. Methods of protein analyses

Method:	Used in:
Indirect immunofluorescence labeling	I, III, IV
Avidin-biotin labeling	I-IV
Tissue microarray	IV
Western transfer analysis	I, III, IV
Immunoprecipitation-Western transfer analysis	IV

4.2.8 Microscopy (I-IV)

Table 4.10. Microscopes used in cell and tissue imaging

Microscope:	Used in:
Leica DMRB fluorescence microscope	III
Zeiss AxioImager M1 microscope equipped with AxioCam ICc3 camera and AxioVision Release 4.8 software	II-IV
Zeiss LSM 510 META confocal microscope and LSM 3.0 software	I-III
Olympus BX51 virtual microscope equipped with an Olympus U-CMAD3 camera and dotSlide2.1 software	I-IV
Olympus IX71 microscope equipped with Cell^R 2.6 software	III

4.2.9 RNA analyses (I,II,IV)

Table 4.11. RNA-based methods

Method:	Used in:
Isolation of RNA	I, III, IV
RT-PCR	I, III
Quantitative RT-PCR	IV
Agilent whole human genome microarray	I
Illumina Sentrix® Human HT-12 v.3 Epression BeadChips	IV

4.2.10 Bioinformatics analyses (IV)

Table 4.12. Methods of bioinformatics analyses

Method:	Reference	Used in:
Gene Ontology (GO)	http://www.geneontology.org/	IV
GOrilla tool	http://cbl-gorilla.cs.technion.ac.il/ (Ashburner et al., 2000; Eden et al., 2009)	IV
DAVID Bioinfomatics Resourses 6.7	http://david.abcc.ncifcrf.gov/gene2gene.jsp (Dennis et al., 2003)	IV
BioCarta database	http://www.biocarta.com/	IV
Kyoto Encyclopedia of Genes and Genomes (KEGG) database	http://www.genome.jp/kegg/	IV
Ingenuity Pathway Analysis (IPA)	http://www.ingenuity.com	IV

4.2.11 Statistical analysis (IV)

In microarray data analysis, R package *limma* was used for the statistical testing between the studied groups (Smyth, 2005). The paired experiment design was taken into account in the statistical testing. A moderated *t*-test was applied to test differential expression, and a false discovery rate (FDR) adjustment of the P-values was performed to correct for multiple testing. The statistical analysis for western blots was performed using two-tailed, paired Student's *t*-test. P-values of 0.05 or less were considered as statistically significant. All tests and calculations were made with SPSS for Windows 14.0.1 software (SPSS Inc.).

5. RESULTS AND DISCUSSION

5.1 The expression of Class III β-tubulin in neurofibromas and in the mitotic spindle of multiple cell types (I-IV)

Class III β-tubulin has been previously considered as a neuron-specific marker (Katsetos et al., 2003b). Furthermore, the expression of class III β-tubulin has been investigated in context of tumorigenesis and it has been described as a marker of cancer cells' resistance to taxanes (Hasegawa et al., 2003; Katsetos et al., 2003b; Lee et al., 2007). In the present study, the Tuj-1 antibody directed against class III β-tubulin was first tested as a marker for neuronal differentiation in neurofibromas and in cultured neurofibroma-derived precursor cells (NFPs).

5.1.1 Class III β-tubulin is localized to mitotic spindles of multiple cell types in vitro (I)

Unexpectedly, the expression of class III β-tubulin was detected in spindle microtubules of mitotically active NFPs *in vitro*. The finding raised a question whether the class III β-tubulin, was also expressed in other cell types than neurons during mitosis. To answer this question, human normal keratinocytes and fibroblasts were studied *in vitro* for their class III β-tubulin expression. In both cell types, class III β-tubulin expression was detected, and it was localized to mitotic spindles. More specifically, class III β-tubulin became detectable in early prophase, the immunoreaction was most intense during metaphase and anaphase, and it lasted to the point when the midbody of cytokinesis became detectable. Detection of the ~50kDa band by western transfer analysis and corresponding mRNA by RT-PCR and Agilent whole human genome microarray further confirmed the expression of class III β-tubulin in the cells studied.

5.1.2 Class III β-tubulin is expressed in MPNST (I)

The presence of class III β-tubulin in mitotic cells *in vivo* was studied by immunolabeling of an MPNST removed from a patient with NF1. Class III β-tubulin expression was detected in a subpopulation of tumor cells undergoing mitoses. In cutaneous neurofibromas with hardly any mitotic cells, the Tuj-1 antibody exclusively detected axons. Collectively, the results *in vitro* and *in vivo* suggest that class III β-tubulin may be needed for the formation of mitotic spindle, and may serve as a marker for mitotic cells. Previous studies have further shown that class III β-tubulin is the only β-tubulin subtype regulated by phosphorylation, and it has been speculated to reflect class III β-tubulin's role in the regulation of microtubule assembly and disassembly *in vivo* (Katsetos et al., 2003a). Regulation of microtubule assembly is essential during mitosis, and may thus explain the presence of class III β-tubulin in mitotic cells. Furthermore, the finding leads to the speculation that the presence of class III β-tubulin may relate to the intense movement of chromosomes along microtubules taking place

during mitosis, analogous to the situation operative in the fast axonal trafficking for very long distances in a cellular scale.

5.1.3 Class III β-tubulin serves as a marker for neuronal differentiation and mitosis (II-IV)

Although the expression of class III β-tubulin was evident in mitotic cells representing multiple cell types, in tissues with hardly any mitotic cells, such as typical cutaneous neurofibromas class III β-tubulin expression is seen only in axons, and it can thus be applied as neuronal marker. However, based on the current study it should be recalled that in mitotically active tissues, class III β-tubulin expression does not necessarily indicate neuronal differentiation. In those cases, cells positive for class III β-tubulin may represent neurons but also non-neuronal mitotic cells. Other neuronal markers such as neurofilament are recommended to confirm the presence of neurons.

Later on during the thesis project, class III β-tubulin was used as a biomarker for neuronal differentiation when characterizing the cellular differentiation of multipotent NFPs *in vitro* (III) and oral mucosal neurofibromas *in vivo* (II). The positivity for class III β-tubulin combined to cell morphology typical for neurons, or more specifically, to slender axonal processes, together provided a strong evidence of the presence of neurons. Furthermore, the gene expression profiling of neurofibroma-derived Schwann cells enriched towards $NF1^{+/-}$ or $NF1^{-/-}$ genotype revealed high class III β-tubulin (*TUBB3*) expression in both cell types (IV), which may attest to the mitotic activity of these cells.

5.2 Type and frequency of oral soft tissue alterations in patients with neurofibromatosis (II)

The second subproject of this thesis is a story about unexpectedly common oral soft tissue growths associated with neurofibromatosis. Specifically, the study focused on the cellular differentiation in oral tumors, and the comparison of neurofibromas growing in the oral cavity to those found in other body locations. The type and frequency of oral soft tissue alterations was studied by clinical examination of 103 patients with NF1 and three patients with NF2. This was also a subproject in a larger study the purpose of which was to evaluate the craniofacial and dental manifestations of NF1 in Finland (Heervä et al., 2011; Visnapuu et al., 2007; Visnapuu et al., 2011a; Visnapuu et al., 2011b). The current study included the highest number of NF1 patients systematically subjected to the clinical oral investigation to date, and also contained histopathological and immunohistochemical data of the samples removed. Most of the previous studies describing oral manifestation in neurofibromatosis were case studies and only a limited number represented full-length papers (Baden et al., 1984; D'Ambrosio et al., 1988; Friedrich et al., 2003; Shapiro et al., 1984).

5.2.1 The oral soft tissue alterations are common findings in NF1 (II)

The study began with ten soft tissue samples removed from the oral cavity of patients with neurofibromatosis and with the question whether these samples represent

neurofibromas. They were first considered to represent all oral tumors in the patients studied. Since the literature searches dating back to 1980's revealed that different soft tissue alterations were shown to affect 72—92% of persons with NF1 (D'Ambrosio et al., 1988; Shapiro et al., 1984), the patient files of all patients included in the current study were carefully analyzed. The results revealed that 76 out of 103 patients (74%) with NF1 presented one or several abnormal growths in their oral soft tissue. The most common soft tissue alterations included oral tumors, overgrowths of gingival soft tissue and enlarged lingual papillae, classified based on their location and according to earlier studies. The findings were equally common in both sexes. Although the findings of the present study corresponded to the figures in earlier studies, the tumors were still unexpectedly common.

5.2.2 Clinical classification of oral tumors associated with NF1 (II)

The study focused on the oral tumors which were discovered in 37% (38 patients) of patients with NF1, and removal of disturbing growth was recommended to 15 patients. Although oral mucosal tumors were common findings in patients with NF1 and the tumors started to grow during puberty, most of the tumors were solitary, discrete oral mucosal neurofibromas, which differed markedly from the clinical presentation of numerous cutaneous neurofibromas. The tumors growing in the oral cavity of NF1 patients were first categorized as plexiform or discrete oral mucosal tumors according to clinical inspection. The cellular characteristics were evaluated from nine tumors removed from NF1 patients.

5.2.3 Oral plexiform neurofibromas show immunohistology typical of neurofibromas (II)

Based on clinical inspection, three of these tumors represented plexiform neurofibromas. They displayed the histology and immunohistology consistent with that of neurofibromas (Peltonen et al., 1988). These characteristics included positivity for biomarkers S100, collagen IV, and CD34, and the presence of neural elements within an abundant collagenous matrix. Also consistent with the histology of neurofibroma, the tumors contained numerous mast cells, as visualized by toluidine blue staining. The identity of these three samples was thus confirmed as plexiform neurofibroma. Clinically, it is important to note that the plexiform neurofibromas affecting the oral cavity of NF1 patients are comparable with plexiform neurofibromas growing in other body locations. Thus, they may bear a risk for malignant transformation.

5.2.4 Discrete oral mucosal neurofibromas show both similarities and differences compared to cutaneous neurofibromas (II)

The tumors categorized as discrete oral mucosal neurofibromas were more heterogeneous group than the plexiform neurofibromas. Five out of six tumors displayed S100-positive cells within collagenous matrix, presence of neuronal elements and positivity for CD34, and were thus classified as neurofibromas. However, they presented marked heterogeneity in the number of S100-positive cells between tumors

and even within a single tumor. In analogy, the number of mast cells was highly variable.

One out of six discrete NF1-related mucosal tumors did not contain neural elements. Instead, the histology of this highly collagenous lesion resembled that of a scar. Furthermore, one of the discrete lesions removed from oral soft tissue located at the maxillary tuberosity and could have been classified as overgrowth of gingival soft tissue instead of oral tumor. The immunohistochemistry confirmed the presence of S100-positive cells in the lesion and it was thus classified as neurofibroma. The finding was consistent with the gingival neurofibromas described previously (Cunha et al., 2004; García de Marcos et al., 2007). It, however, raises a question whether the oral soft tissue alterations other than those classified as oral tumors may be caused by the hyper proliferation of Schwann cells and may thus represent neurofibromas based on their immunohistochemical characteristics.

The results showed that oral soft tissue alterations are common findings in the patients with NF1. The heterogeneity in discrete oral mucosal neurofibromas emphasizes the importance of immunohistochemical characterization of these lesions. Clinical inspection is thus not sufficient for the diagnosis of neurofibroma. The discrete oral mucosal tumors may cause discomfort and they can be removed but most of them do not require any treatment.

Table 5.1. Comparison of the discrete oral and cutaneous neurofibromas

	Discrete oral neurofibroma	**Cutaneous neurofibroma**
Prevalence	50% of adult patients	99% of adult patients
Clinical presentation	Solitary	Multiple
Typical age of onset	Puberty	Puberty
Concern to a patient	Patients often unaware of tumor, sometimes debilitating	Often the main cosmetic and debilitating problem
Biomarkers and cellular composition		
S100/ Schwann cells	Marked inter- and intratumoral variability	60—80% of cells positive
Collagen IV	Most cells positive	Most cells positive
CD34	Marked positivity	Marked positivity
Neuronal elements	Present	Present
Mast cells	Marked inter- and intratumoral variability	Present

5.3 Pathogenesis of cutaneous neurofibromas (III-IV)

Previous knowledge has suggested that cutaneous neurofibromas arise from small peripheral nerves during adolescence and adulthood. This view was supported by the fact that neurofibromas contain all the cell types of normal peripheral nerve organized in a haphazard manner (Peltonen et al., 1988). The third and fourth subprojects of this thesis addressed the questions about the initial phases of the neurofibroma's pathogenesis: "Are peripheral nerves and their Schwann cells the initial site of cutaneous neurofibroma development?", and "How does the *NF1* second hit mutation

change the expression of all other genes?" The answers were sought by analyzing the cells cultured from cutaneous neurofibromas and by confirming the findings by staining and labeling tissue sections. In this thesis project, cutaneous neurofibromas served as a source for not only the Schwann cells but also for multipotent precursor cells named NFPs.

5.3.1 Culturing of neurofibroma-derived precursor cells (NFPs) (III)

Since the Schwann cells of the peripheral nerves represent a rather quiescent cell population and were thus considered to be unlikely hosts for somatic *NF1* second hits, the question of other sources arose. These thoughts and the concurrent developments in the cancer stem cell field led to the development of novel culturing method and characterization of multipotent NFP cell population from cutaneous neurofibromas (III). To create these NFP cultures, fresh neurofibromas were dissociated and subjected to culture protocols which enabled the establishment of Schwann cell cultures and their enrichment towards $NF1^{+/-}$ or $NF1^{-/-}$ genotypes (Serra et al., 2000). The unattached cells were collected at the first medium change of Schwann cell cultures and further subjected to culture conditions which had previously been shown to favor stem cell growth (Castrén et al., 2005). These culture conditions produced NFP cultures with stem cell-like characteristics. The cultures started from only a few cells produced highly proliferative cell colonies growing on the surface of the culture dish or forming cell spheres. Time-lapse imaging showed that while dividing, the attached NFPs first acquired spherical morphology and then underwent rapid division. NFPs expressed stem cell and early differentiation markers, specifically nestin.

Despite the absence of known Schwann cell growth factors or serum, a number of NFP cells showed differentiation characteristics consistent with Schwann cell identity. These characteristics included spindle shaped morphology and S100-positivity. The finding suggests that the *NF1* mutation in the NFPs directs their differentiation towards Schwann cell phenotype independent of axonal contact. NFPs were also able to differentiate into fibroblasts, epithelial cells, adipocytes, and neurons attesting to their multilineage differentiation potential. Our unpublished data suggests that these cells can also be differentiated into osteoclasts. Further investigation will show whether these cells can differentiate into other cell types. To conclude, neurofibromas served as a source for cells with high proliferation capacity in previously described stem cell medium and potential to generate cells with multiple differentiation characteristics. These characteristics are consistent with multipotent progenitor cells (Reya et al., 2001; Tuch, 2006).

5.3.2 Neurofibroma Development – Alternative Explanation

The *NF1* mutation analysis revealed that the NFPs characterized in the present study represented $NF1^{+/-}$ genotype (see section **5.4**). Recent findings on the Nf1 mouse model have revealed that multipotent precursor cells with $Nf1^{-/-}$ genotype are a potential cell of origin of cutaneous neurofibromas (Le et al., 2009). Regardless of the *NF1* genotype, the results of the present study also suggest that human cutaneous neurofibromas contain multipotent precursor cells, NFPs. The finding leads to

speculation that the *NF1*$^{-/-}$ Schwann cells in human cutaneous neurofibromas may also arise from multipotent progenitor cells which gain a second *NF1* mutation and consequently differentiate into Schwann-like cells. Considering the fact that a distinct second hit is found from each tumor, a single cell gives rise to a clonal Schwann cell population with *NF1*$^{-/-}$ genotype. Assuming that a stem cell gains the *NF1* second hit mutation after which it undergoes asymmetric cell division giving rise to one progenitor cell and one cell that starts to proliferate and differentiate towards Schwann cell phenotype, each tumor may host only one progenitor cell with *NF1*$^{-/-}$ genotype. With human tissue material, it is challenging to restore the initial stage of tumor formation and isolate a single multipotent cell with *NF1*$^{-/-}$ genotype. The developments in higher resolution technologies may in the future allow the isolation of a progenitor cell with the *NF1*$^{-/-}$ genotype.

Since the cultured NFPs showed positivity for nestin and since hair follicles have been recognized as a niche for nestin-positive stem cells which have potential to differentiate into different cell types including Schwann cells and neurons (Amoh et al., 2005a; Amoh et al., 2005b), the presence of nestin expressing cells in neurofibromas *in vivo* was studied. The immunolabeling for nestin showed that neurofibromas host spindle-shaped tumor cells and small blood vessels which are nestin-positive. The finding was consistent with previous results showing the differentiation potential of nestin positive cells (Amoh et al., 2005a; Amoh et al., 2005b; Amoh et al., 2004). The nestin expression in Schwann cell-like cells in neurofibromas may reflect that neurofibroma Schwann cells are not mature Schwann cells. The presence of multipotent precursor cells in neurofibromas was further studied by using a panel of other biomarkers for multipotency and cell differentiation.

To further elucidate the potential association of cutaneous neurofibromas and hair follicles, the structure of cutaneous neurofibromas was studied by creating three dimensional histology models of six typical cutaneous neurofibromas. These models showed the presence of elements of the hair follicular apparatus in all samples studied. The structures included the hair and its follicle, erector pili muscle and sebaceous gland, and these structures were often separated from each other by typical neurofibroma tissue. Further investigation of the developmentally early phases of cutaneous neurofibromas, and the earlier result published by Peltonen group (Karvonen et al., 2000) revealed the close contact of the hair follicle apparatus and clinically invisible neurofibroma. Even the healthy-looking area of skin removed from NF1 patient revealed minor S100-positive tumor growths around hair follicles. These results suggest that the intimate association of hair follicular structures and microscopic neurofibromas is not coincidental. The immunohistochemical characteristics of a clinically invisible cutaneous neurofibroma were confirmed by immunolabelings for S100 and collagen IV. Both markers showed positivity and localization consistent with that seen in larger cutaneous neurofibromas. The presence of mast cells was also demonstrated by toluidine blue staining.

Earlier studies had characterized multipotent skin derived precursor cells (SKPs) which potentially reside in hair roots of mice and have the potential to generate

subpopulations of cells expressing neuronal, glial, smooth muscle and adipocyte markers (Fernandes et al., 2004; Fernandes et al., 2008; Toma et al., 2005). Together these results suggest that the multipotent cells present in the hair follicles may contribute to the development of cutaneous neurofibromas.

5.3.3 HLA class II genes are expressed by $NF1^{-/-}$ cells (IV)

In the fourth subproject of this thesis, the Schwann cell cultures established from nine cutaneous neurofibromas and enriched towards $NF1^{+/-}$ or $NF1^{-/-}$ genotype, were subjected to the investigation of the effect of the *NF1* second hit mutation to the transcriptomes of these cell types. The mRNA-based gene expression profiling of these cell cultures yielded unexpected findings; in comparison of the $NF1^{-/-}$ and $NF1^{+/-}$ cells, inflammatory response, and antigen presentation and processing were highlighted. More specifically, the $NF1^{-/-}$ genotype was intimately associated with the up-regulation of HLA class II genes. Although HLA II expression is generally related to professional antigen presenting cells, also Schwann cells have the potential to express HLA class II genes (Scarpini et al., 1990). Furthermore, previous studies have also shown that Schwann cells have the potential to display the entire spectrum of immune responses including antigen presentation and recognition, regulation of immune response by secreting soluble factors, and termination of immune response via the interaction of Fas and FasL, the mediators of apoptosis (Meyer zu Hörste et al., 2008).

The expression of HLA class II was further studied at the protein level in the cultured cells. The results showed consistently that HLA-DR protein levels were higher in $NF1^{-/-}$ Schwann cells. Silencing of *NF1* gene expression in $NF1^{+/-}$ Schwann cells, human normal keratinocytes and NFPs showed that the HLA class II expression was induced in Schwann cells only. The results of the current study suggest that tumor Schwann cells with $NF1^{-/-}$ genotype may act as dendritic or non-professional antigen presenting cells and they do not act like typical Schwann cells. This finding may also reflect the plasticity of Schwann cells.

5.3.4 Schwann cell identity of neurofibroma-derived cells (IV)

Further notions from the cell cultures showed that, the Schwann cells cultured under conditions that favor $NF1^{+/-}$ or $NF1^{-/-}$ genotype were phenotypically different. Schwann cells enriched towards $NF1^{+/-}$ genotype had wider cellular processes still retaining spindle-shaped morphology, and they were tightly attached to the surface of the culture dish. On the contrary, $NF1^{-/-}$ cells showed slender processes, and the cells got easily detached upon trypsinization. The gene expression analysis revealed that myelination associated genes were expressed both by $NF1^{-/-}$ and by $NF1^{+/-}$ cells which is consistent with their identity as Schwann cells. However, these genes were down-regulated in $NF1^{-/-}$ cells compared to $NF1^{+/-}$ cells which may attest to the lower state of differentiation of $NF1^{-/-}$ cells. This finding also supports the concept that neurofibromas arise from multipotent progenitor cells: the neurofibroma Schwann cells have never been true Schwann cells or in contact with axons but they are rather differentiating towards Schwann cell phenotype. The finding raises the questions “Is *NF1* mutation sufficient to induce cellular differentiation towards Schwann cell

phenotype" and "What is the effect of the cellular microenvironment". Further cell culture assays would be needed to show whether *NF1*$^{-/-}$ Schwann cells can form myelin and thus represent myelinating Schwann cells and is their myelination potential different from that of *NF1*$^{+/-}$ Schwann cells. The Schwann cell identity and myelination potential of neurofibroma-derived Schwann cells are interesting topics of further investigation.

5.3.5 Inflammation and neurofibroma development (IV)

In cancer biology, the role of inflammatory cells in tumor development has been recognized (Hanahan and Weinberg, 2011). In the context of neurofibroma development, the presence of mast cells within tumor is an established fact evidenced by both human and mice studies (Peltonen et al., 1988; Yang et al., 2008; Zhu et al., 2002). The data presented in the subprojects II and III of the current thesis is consistent with the previous results concerning mast cells. Mast cells, however, represent the only previously described inflammatory component of neurofibromas. The results of the subproject IV showed the HLA class II expression in *NF1*$^{-/-}$ Schwann cells, and HLA-DR expression was also seen in a subpopulation of cells in neurofibroma *in vivo*. These results raised the question of the presence and role of other inflammatory cells in cutaneous neurofibromas.

A sophisticated guess was that HLA class II expression might reflect the presentation of tumor antigens by *NF1*$^{-/-}$ cells to regulatory T cells which further mediate the immune tolerance. The immunolabelings of neurofibroma tissues for lymphocyte markers revealed scattered lymphocytes. Furthermore, the results showed that CD4-positive lymphocytes were more numerous than CD8-positive cells. Since a small subset (5% to 10%) of CD4-positive cells in human peripheral blood has been shown to simultaneously express FoxP3 and are thus classified as regulatory T cells, the CD4-positive cells in neurofibromas may represent Tregs (Loser and Beissert, 2012). Further immunolabeling of neurofibroma tissue for transcription factor FoxP3 revealed positive nuclei, and double immunofluorescence labeling revealed cells which simultaneously express CD4 and FoxP3. FoxP3 was also detected from neurofibroma tissue by western blot following immunoprecipitation. Since the previous literature describes regulatory T cells based on their expression of CD4 and FoxP3 (Fontenot et al., 2003; Hori et al., 2003; Sakaguchi et al., 1995), the cells characterized in the present study may represent Tregs.

To conclude, the Schwann cells expressing HLA class II may present tumors as self-antigens, and as such they may be involved in immune tolerance which is mediated by regulatory T cells. The presence of Tregs allows the tumor growth by hiding the tumor from the immune system's attack. Considering the treatments against tumors, Tregs have been one of the main obstacles of successful immunotherapy (Mellman et al., 2011; Ménétrier-Caux et al., 2012; Vasievich and Huang, 2011). However, human cancer immunotherapy has recently become applicable, and the results of the current study renders to speculation that targeting regulatory T cells in NF1 patients could

provide a novel therapeutic approach to interfere with the development of neurofibromas and alleviate the tumor burden in this disease.

5.4 *NF1* mutations (III-IV)

NF1 mutation analysis was performed on NFP cultures established from three patients and on Schwann cells cultured from nine tumors from three patients. The NFPs and Schwann cells were cultured and Schwann cells were enriched towards $NF1^{+/-}$ or $NF1^{-/-}$ genotype using previously described culture conditions (Serra et al., 2000), and RNAs and DNAs were isolated from cultured cells at the Department of Cell Biology and Anatomy, University of Turku by the author of this thesis. The mutation analysis was performed at Professor Ludwine Messiaen's laboratory at the Department of Genetics, University of Alabama at Birmingham. Against all expectations and after careful analysis, only one *NF1* mutation was characterized from each NFP culture (III). In Schwann cells cultured in the absence of growth factor forskolin, two *NF1* mutations were detectable in each cell line. The detected mutations are shown in **Table 5.2**.

Two out of the four germline mutations detected in this study were previously described whereas the other two represented novel mutations. The germline mutation in patient 1/A was a mutation that creates a novel exonic splice donor site, and by doing so, it causes missplicing of exon 10b (Messiaen et al., 1999). The missplicing leads to the skipping of the last 62 nucleotides of exon 10b and in addition to an immediate stop-codon at the mRNA level. At the gDNA level, the mutation resembles missense mutation. The mutation has been previously described to affect at least 6 patients (Messiaen et al., 1999; Pros et al., 2008). Patient 2 presented a point mutation in the exon 7. The mutation causes a disruption of exonic splicing enhancer element and to some extend leads to the skipping of the exon itself (Zatkova et al., 2004). Otherwise, the mutation leads to a premature stop-codon. The mutation has been previously characterized in at least seven patients (Laycock-van Spyk et al., 2011; Pros et al., 2008). Patient 3/B showed the presence of a previously unpublished nonsense mutation affecting exon 22. The mutation thus causes a stop-codon at the exon 22 which locates within the sequence encoding for the GRD of neurofibromin (see **Figure 2**). The germline mutation in patient C was a novel 13 base pair deletion leading to a frameshift in exon 3. A different two base pair deletion representing frameshift mutation but affecting the same nucleotide has been previously described (Pros et al., 2008).

Somatic *NF1* second hits represented novel mutations in 3/9 tumors. In patient 1, the somatic second hit detected in Tu1 was a previously described nonsense mutation in exon 23.2 (Laycock-van Spyk et al., 2011). Exon 23.2 encodes sequences within the GRD region of neurofibromin. Furthermore, patient 1 presented 2 different-sized total gene deletions in Tu2 and Tu3: one deletion was the typical Type 1 1.4 Mb deletions; the other was a larger atypical deletion. Tu1 from patient 2 represented a previously described frameshift mutation in exon 22 affecting the GRD region (Pros et al., 2008). The second hit mutations detected in Tu2 and Tu3 were novel mutations in exons 16 and 26, respectively. Somatic second hit mutation detected in the Tu1 of patient 3

represents a previously described nonsense mutation in exon 41 (Fahsold et al., 2000). The mutation characterized in the Tu2 of patient 3 was a novel intronic mutation locating in intron 14. The same mutation in intron 10a as detected in Tu3 of patient 3 has been reported to affect splicing or to cause in frame deletion (De Luca et al., 2004).

The characterization of the *NF1* mutations from the cultured cells had a great importance to the reliability and interpretation of the results in subprojects III and IV. The information with concern to whether two different *NF1* mutations or only a single *NF1* mutation were detectable in each cell line was especially instrumental.

Table 5.2. *NF1* mutations characterized from cultured NFPs and Schwann cells (SC).

Patient	Tumor	Cell type	*NF1* 1st mutation	*NF1* 2nd mutation	Described in:
Patient A	Tu1	NFP	c.1466A>G, Y489C, p.Tyr489*	not found	III
Patient B	Tu4	NFP	c.3868A>T, p.Lys1290*	not found	III
Patient C	Tu1	NFP	c.232_244del	not found	III
Patient 1/A	Tu1	SC+/-	c.1466A>G, Y489C, p.Tyr489*		III-IV
Patient 1/A	Tu1	SC-/-	c.1466A>G, Y489C, p.Tyr489*	c.4084C>T, p.Arg1362*	III-IV
Patient 1/A	Tu2	SC-/-	c.1466A>G, Y489C, p.Tyr489*	TGD, Type1	III-IV
Patient 1/A	Tu3	SC-/-	1466A>G, Y489C, p.Tyr489*	TGD, Atypical, >1.4Mb	III-IV
Patient 2	Tu1	SC+/-	c.910C>T, p.Arg304*		IV
Patient 2	Tu1	SC-/-	c.910C>T, p.Arg304*	c.3758_3762delTCTAC, p.Leu1253Profs*9	IV
Patient 2	Tu2	SC-/-	c.910C>T, p.Arg304*	c.2556_2557delinsTT, p.GLN853*	IV
Patient 2	Tu3	SC-/-	c.910C>T, p.Arg304*	c.[4478A>T; 4480C>T], p.[Asn1493Ile;Gln1494*]	IV
Patient 3/B	Tu1	SC+/-	c.3868A>T, p.Lys1290*		III-IV
Patient 3/B	Tu1	SC-/-	c.3868A>T, p.Lys1290*	c.7285C>T, p.Arg2429*	III-IV
Patient 3/B	Tu2	SC-/-	c.3868A>T, p.Lys1290*	c.2325+1G>A	III-IV
Patient 3/B	Tu3	SC-/-	c.3868A>T, p.Lys1290*	c.1392+1G>A	III-IV

TGD, total gene deletion

6. SUMMARY AND CONCLUSIONS

The present study investigated the development and immunohistochemical characteristics of cutaneous and oral mucosal neurofibromas associated with neurofibromatosis 1. Furthermore, the use of different biomarkers in neurofibromas and neurofibroma-derived cultured cells was evaluated. The following conclusions were made on the basis of the results of the present study:

1. Class III β-tubulin is a component of the mitotic spindle in cultured, non-neuronal cells as exemplified by human normal keratinocytes and fibroblasts. Class III β-tubulin is also expressed by neurofibroma-derived progenitor cells with high mitotic activity. Furthermore, class III β-tubulin expression in association with mitotic spindles is detected in a malignant peripheral nerve sheath tumor (MPNST).

2. Oral soft tissue growths including oral tumors, overgrowths of gingival soft tissue, and enlarged papillae of the tongue are common findings in NF1. The characterization of oral soft tissue growths requires the use of immunohistochemical analyses. Collagen IV, S100, and CD34 are useful biomarkers in the analysis of NF1-related oral soft tissue tumors.

3. Neurofibromas host multipotent precursor cells, NFPs, with $NF1^{+/-}$ genotype. These cells have high proliferation capacity, and they can differentiate into multiple cell types including Schwann cells, fibroblasts, epithelial cells, adipocytes and neurons. The suggested niche of NFPs includes nestin positive cells in the hair follicle. Hair follicles are usually found to be associated with cutaneous neurofibromas of different developmental stages.

4. HLA class II genes are over expressed in $NF1^{-/-}$ Schwann cells cultured from human cutaneous neurofibromas while myelination-related genes are down-regulated possibly suggesting the lower differentiation stage of these cells. HLA-DR expression is also seen in a subpopulation of cells in neurofibroma tissue suggesting that these cells may act as non-professional antigen presenting cells. The tumors also host scattered CD4/ FoxP3-positive cells, consistent with regulatory T cell identity. Together these findings suggest that immune tolerance or evasion by Tregs may allow the tumor growth. Targeting regulatory T cells in NF1 patients could provide a novel therapeutic approach to interfere with the development of neurofibromas.

The novel concept for neurofibroma development is summarized in **Figure 7**. The findings of the current study are in conformity with the existing concepts regarding the role of cancer stem cells and the role of immune cells in tumor development (Hanahan and Weinberg, 2011; Reya et al., 2001). These finding increase the understanding of the previously poorly understood properties and phases of neurofibroma development.

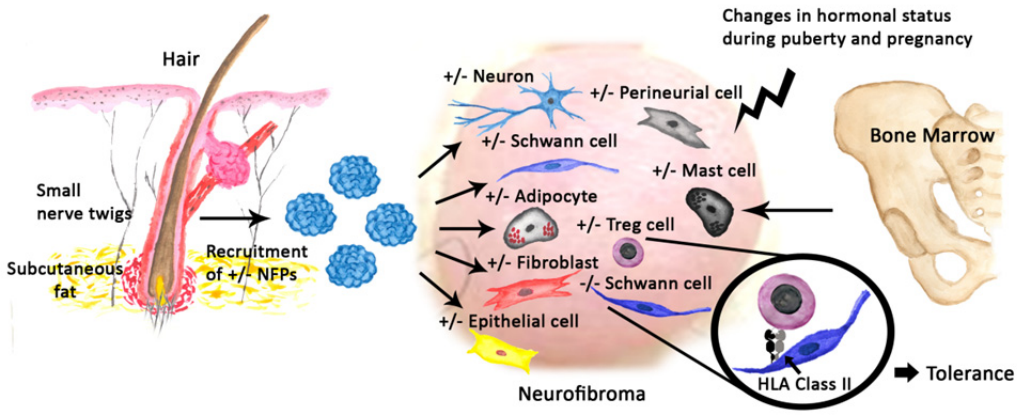


Figure 7. Novel model for the development of cutaneous neurofibromas. As opposed to the traditional understanding of neurofibroma development originating from dissociated peripheral nerve components, the proposed model views neurofibromas as a result of divergent cellular differentiation of multipotent precursor cells. The close proximity of hair and incipient neurofibromas suggests that the multipotent *NF1*$^{+/-}$ cells residing in this area are the major source of neurofibroma-derived precursor cells (NFPs). NFPs can give rise to the different cell types found in the neurofibromas. Previous studies have evidenced that the development of neurofibromas includes the presence of a clonal population of *NF1*$^{-/-}$ Schwann cells in a microenvironment harboring other cell types with an *NF1*$^{+/-}$ genotype. The present study showed that *NF1*$^{-/-}$ Schwann cells express HLA class II molecules suggesting that these cells may act as non-professional antigen presenting cells which present tumors as self-antigens to T cells. Furthermore, the presence of scattered CD4/ FoxP3-positive cells suggests that regulatory T (Treg) cells act as mediators of immune tolerance. The tolerance allows the tumor growth. As suggested by studies on mice the neurofibroma mast cells may be derived from bone marrow. In addition to these cellular components and interactions, the development of neurofibromas apparently requires changes in the hormonal status. The *NF1* genotypes of the cells are indicated as +/- or -/-. Modified from (Jouhilahti et al., 2011).

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