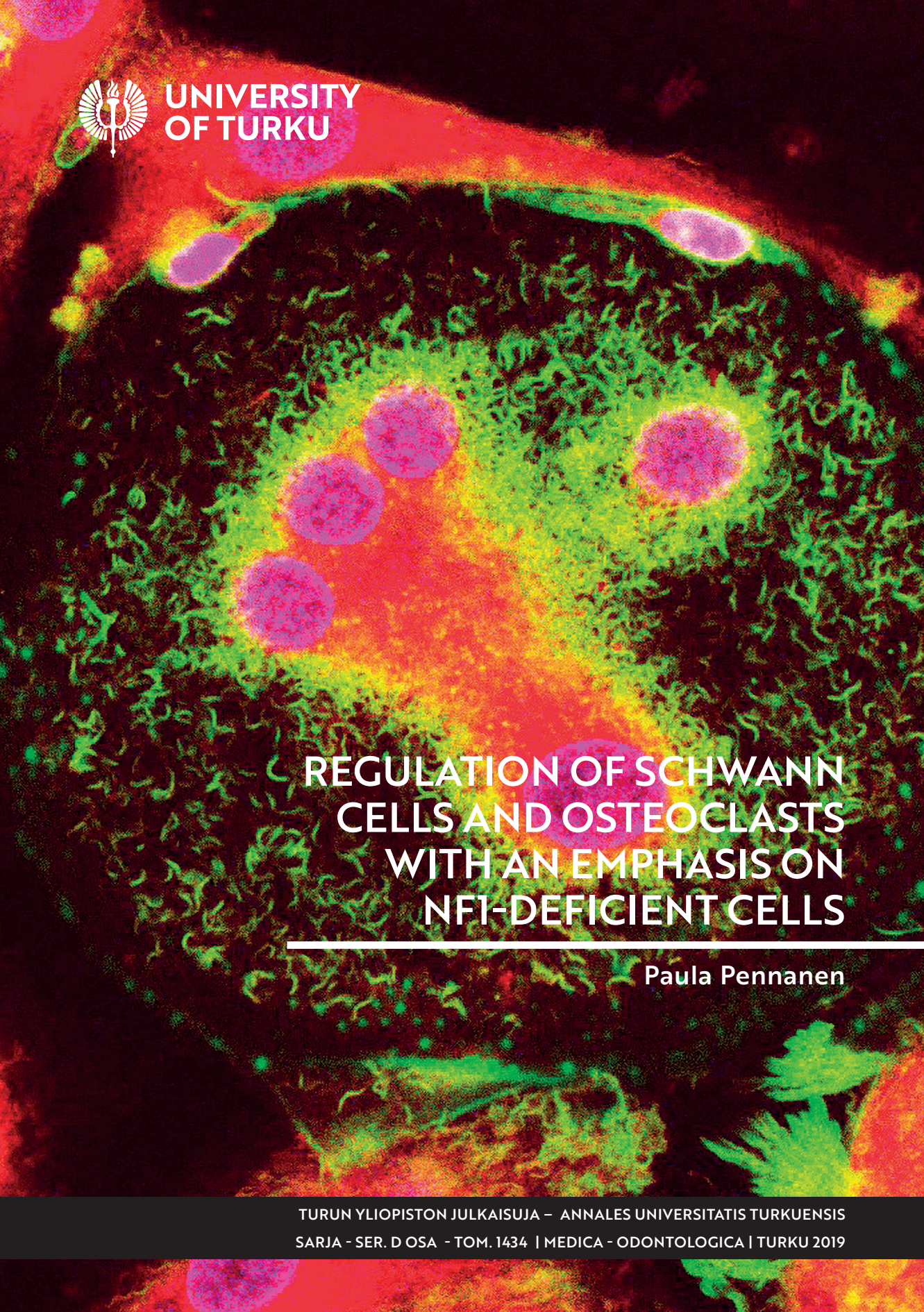

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To my Family

ABSTRACT

Paula Pennanen: REGULATION OF SCHWANN CELLS AND OSTEOCLASTS WITH AN EMPHASIS ON NF1-DEFICIENT CELLS University of Turku, Faculty of Medicine, Institute of Biomedicine, Cell Biology and Anatomy & Dermatology and Venereal Diseases, Turku Doctoral Programme of Molecular Medicine (TuDMM), Annales Universitatis Turkuensis, Medical-Odontologica, Turku, Finland, 2019

Neurofibromatosis type 1 (NF1) is a dominantly inherited cancer predisposition syndrome, which affects one in 2000 individuals worldwide. NF1 exhibits a variety of clinical symptoms. Neurofibromas, the hallmarks of NF1, carry two different genotypes of Schwann cells: cells with a germline mutation and a healthy *NF1* allele ($NF1^{+/-}$) and those with an inactivation of both alleles ($NF1^{-/-}$). These tumors typically start to appear during puberty, and their number and size grow during pregnancy indicating hormone responsiveness. The present study showed that cultured $NF1^{-/-}$ Schwann cells were more sensitive to the effects of estradiol, testosterone, and human chorionic gonadotropin (hCG) than $NF1^{+/-}$ cells.

Multinucleated osteoclasts are responsible for bone resorption and actin ring formation is essential to bone resorption. The dynamic actin structure undergoes rapid changes and plays several roles including cellular movement and maintenance of the cell shape. The osteoclast actin structure consists of a podosome belt with a loose actin "cloud" on its top. STED and confocal microscopy revealed a new insight into osteoclast actin structure. This was a curved and branched actin structure extended from the substratum level to the top of the cell co-localizing with membrane lipids. In addition, thick actin containing micrometer-level tubes were bridging adjacent osteoclasts above the level of the substratum.

The roles of the Ras/PI3K/Akt/mTOR, Ras/Raf/MEK1/2/ERK1/2, calcium-PKC, and p38 signaling pathways in human osteoclast differentiation and bone resorption were evaluated. Down-regulation of most of these pathways by chemical inhibitors decreased the number of osteoclasts from NF1 patients and controls. However, inhibition of p38 increased osteoclastogenesis but not the function of the cells. In addition, inhibition of Ras increased the number of control osteoclasts but decreased the number of NF1 cells. We found that the number of osteoclasts appeared to correlate with the phosphorylation of ERK1/2, which may act as a key driver of human osteoclast differentiation.

The aim of this in vitro study was to further develop, characterize, and test culture conditions for cell types involved in the pathogenesis of two distinct hallmark lesions of NF1. Specifically, these lesions include the neurofibroma tumors largely composed of Schwann cells; and the skeletal lesions such as osteoporosis and false joints, where osteoclasts apparently play a major role.

Keywords: Neurofibromatosis 1, Schwann cell, osteoclast, actin, signaling pathway

TIIVISTELMÄ

Paula Pennanen: Schwannin solut neurofibroomien ja osteoklastit luun hajotuksen säätelijöinä. Esimerkkinä solut, joilla on NF1 geenin mutaatio. Turun yliopisto, Lääketieteellinen tiedekunta, Biolääketieteen laitos, Solubiologia ja anatomia & Iho- ja sukupuolitautioppi, Turun molekyylilääketieteen tohtoriohjelma (TuDMM), Annales Universitatis Turkuensis, Medica-Odontologica, 2019.

Neurofibromatoosi tyyppi 1 (NF1) on vallitsevasti periytyvä syöpätyypeille altistava oireyhtymä, jonka esiintyvyys on maailmanlaajuisesti 1/2000. NF1 sairauteen liittyy useita erilaisia kliinisiä löydöksiä. Neurofibroomat ovat NF1 sairauden keskeisimpiä tunnusmerkkejä, jotka sisältävät kahden eri genotyypin Schwannin soluja: osassa soluista on heterotsygoottinen $NF1^{+/-}$ genotyyppi, jossa toinen alleeli on toimiva, kun taas osalle soluista on tapahtunut toisenkin alleelin katoaminen $NF1^{-/-}$. Neurofibroomilla on taipumus ilmestyä murrosiän aikana ja niiden koko sekä määrä kasvavat raskauden aikana. Kasvainten ajatellaan reagoivan hormoneihin. Tässä tutkimuksessa osoitettiin, että erityisesti $NF1^{-/-}$ genotyypin Schwannin solut ovat erityisen herkkiä estradiolille, testosteronille ja koriongonadotropiinille.

Monitumaisten osteoklastien tehtävä on hajottaa luuta. Aktiinirenkaan muodostuminen on välttämätöntä luun hajotukselle. Aktiinin dynaaminen rakenne muuttuu nopeasti ja aktiinilla on useita rooleja solujen liikkumisesta solun tukirangan muodostumiseen. Osteoklastien aktiinirakenne muodostuu niin kutsutusta podosomivyöstä ja sen päällä olevasta löyhästä aktiini pilvestä. Väitöskirjassa tutkittiin STED- ja konfokaalimikroskopian avulla osteoklastien aktiinirakennetta. Tutkimuksessa havaittiin, että taipuisa ja haarautuva aktiinirakenne ulottui osteoklastin kasvualustalta solun yläosaan asti. Tutkimuksessa löydettiin mikrometrin kokoisia tunneleita kahden eri osteoklastin välillä, jotka kulkeutuivat yhdestä solusta toiseen.

Väitöskirjassa tutkittiin Ras/PI3K/Akt/mTOR, Ras/Raf/MEK1/2/ERK1/2, kalsium-PKC, and p38 signalointiteiden roolia osteoklastien erilaistumisessa. Useimpien signalointiteiden todettiin vähentävän osteoklastien erilaistumista sekä kontrolli että NF1-potilaiden soluilla. Tutkimuksessa havaittiin, että p38 signaloinnin estäminen lisäsi osteoklastien erilaistumista molemmilla tutkituilla solutyypeillä. Lisäksi havaittiin, että Ras-signaloinnin estäminen lisäsi kontrolli solujen määrää, kun taas NF1 potilaiden osteoklastien määrä väheni. Väitöskirjatutkimuksessa todettiin osteoklastien määrän korreloivan ERK1/2 fosforylaation kanssa, joka saattaa olla osteoklastien erilaistumisen avaintekijä. Tämän *in vitro* tutkimuksen tarkoituksena oli tutkia soluja, jotka ovat osallisina NF1-sairauden patogeneesissä ja erityisesti sairauden keskeisimmissä tunnusmerkeissä. Näihin kuuluvat ihon neurofibroomat, jotka koostuvat suurelta osin Schwannin soluista sekä luuston leesiot, kuten osteoporoosi tai valenivelen muodostuminen, joissa osteoklasteilla on ilmeisesti merkittävä rooli.

Avainsanat: Neurofibromatoosi 1, Schwannin solu, osteoklasti, aktiini, signalointitie

TABLE OF CONTENTS

ABBREVIATIONS

Akt	protein kinase B
ALP	alkaline phosphatase
AR	androgen receptor
ARE	androgen response element
Arp2/3	actin-related protein 2/3
bFGF	basic fibroblast growth factor
BMD	bone mineral density
BMU	basic multicellular unit
BrDU	5-bromo-2-deoxyuridine
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CTX	carboxy-terminal cross-linking telopeptide of type 1 collagen
CYP17A1	cytochrome P450
DHEA	dehydroepiandrosterone
DHT	dihydrotestosterone
D-MEM	Dulbecco's modified eagle medium
DXA	dual-energy X-ray absorptiometry
E_1	estrone
E_2	17β-Estradiol
E_3	estriol
E_4	estetrol
ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal regulated kinase
ERα/β	estrogen receptor alpha/beta
FBS	fetal bovine serum
FSD	functional secretory domain
FSH	follicle-stimulating hormone
FTS	farnesyl thiosalisylic acid
GAP	GTPase activating protein
GEF	guanine nucleotide exchange factor
GDP	guanosine diphosphate
GnRH	gonadotropin-releasing hormone
GTP	guanosine triphosphate
GTPases	guanosine triphosphatases
hCG	human chorionic gonadotropin
HDL	high density lipoproteins
HSD3B1	type 1 3β-hydroxysteroid dehydrogenase
HSD17B3	type 3 17β-hydroxysteroid dehydrogenase
LH	luteinizing hormone
LH/CGR	luteinizing hormone/chorionic gonadotropin receptor
MAPK	mitogen-activated protein kinase
M-CSF	macrophage colony-stimulating factor
MEK	mitogen activated protein kinase
MLT	micrometer level tube
MPNST	malignant peripheral nerve sheath tumor
MRI	magnetic resonance imaging
mTOR	mammalian target of rapamycin, mechanistic target of rapamycin
NF1$^{-/-}$	cells with the *NF1* second hit
NF1$^{+/-}$	cells carrying the constitutional *NF1* mutation only
NF1	neurofibromatosis 1
NF2	neurofibromatosis 2
NF1	human *NF1* gene
NIH	National Institutes of Health
OPG	osteoprotegerin
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PI3K	phosphoinositide 3-kinase
PIP2	phosphatidylinositol-4,5,bisphosphate
PIP3	phosphatidylinositol-3,4,5-trisphosphate
PKA	cyclic adenosine monophosphate-dependent protein kinase A
PKC	protein kinase C
PLC	phospholipase C
p38 MAPK	mitogen-activated protein kinase p38
QoL	quality of life
Raf	rapidly accelerated fibrosarcoma
Ras	rat sarcoma protein
RANK	receptor activator of nuclear factor kappa-
RANKL	ligand for receptor activator of nuclear factor kappa-B
STAR	steroidigenic acute regulatory protein
STED	stimulated emission depletion microscopy
STX3451	2-(3-bromo-4,5-dimethoxybenzyl)-7-methoxy-6-sulfamoyloxy-1,2,3,4-tetrahydroisoquinoline
SZ	sealing zone
TNTs	tunneling nanotubes
TRACP	tartrate-resistant acid phosphatase 5B
TRAF	tumor necrosis factor-α receptor-associated protein
WASP	Wiskott-Aldrich Syndrome protein
2ME2	2-methoxyestradiol
α-MEM	minimum essential medium, alpha modification

LIST OF ORIGINAL PUBLICATIONS

I Pennanen P, Peltonen S, Kallionpää RA, Peltonen J. The effect of estradiol, testosterone, and human chorionic gonadotropin on the proliferation of Schwann cells with *NF1*$^{+/-}$ or *NF1*$^{-/-}$ genotype derived from human cutaneous neurofibromas. Mol Cell Biochem., 2017, 444(1-2):27-33

II Pennanen P*, Alanne MH*, Fazeli E, Deguchi T, Näreoja T, Peltonen S, Peltonen J. Diversity of actin architecture in human osteoclasts: Network of curved and branched actin supporting cell shape and intercellular micrometer level tubes. Mol Cell Biochem., 2017, 432(1-2):131-139

III Pennanen P, Kallionpää RA, Peltonen S, Nissinen L, Kähäri VM, Heervä E, Peltonen J. Signaling pathways in human osteoclasts differentiation: ERK1/2 as a key player. *Submitted manuscript 2019*

*Equal contribution

1 INTRODUCTION

Neurofibromatosis 1 (NF1) is a fully penetrant neurocutaneous and skeletal syndrome caused by mutations in the NF1 gene. If one parent carries the NF1 mutation, then half of the children inherit the disease from their parent. The other half inherits the disease from the healthy parents through spontaneous mutation, which has occurred during formation of gametes. A NF1 patient germline mutation of the NF1 gene ($NF1^{+/-}$) is in each cell. NF1 is as common in women as in men. However, gender influences symptoms of the disease. NF1 affects multiple organs, and symptoms vary between individuals even in the same family. There is no cure for NF1. Treatment of the disease focuses on treatment of the symptoms.

Neurofibromas are the hallmarks of NF1, and neurofibromas are present in half of the 10-year–old patients. During the age of 20, 80% of the patients have neurofibromas. Neurofibromas are benign skin tumors, which cause severe esthetic or social burden. These tumors arise during early puberty, and increase in amount and size during pregnancy. There is no medical treatment against these tumors.

The *NF1* gene plays a role in bone remodeling, and half of the patients have some kind of bone abnormalities. Bone loss affects everybody and is part of age-related bone loss. Bone remodeling is based on bone resorption by osteoclasts and bone formation by osteoblasts, and this process occurs throughout life. The osteoclast cytoskeleton is a dynamic structure that enables cell movement, invasion, cell shape, and positioning of the cell organelles. Actin is one of the main types of filaments of the osteoclasts, and it is different in osteoclasts according to their substratum, on glass, or on bone. On glass, actin forms a podosome belt on the substratum level with an actin cloud on its top. On bone, actin forms a sealing zone. Actin plays a role in intracellular membrane transport.

Multiple signaling pathways are involved in differentiation of osteoclasts. Excess differentiation of osteoclasts is involved in different bone diseases, such as osteoporosis. Therefore, a deeper understanding of osteoclast molecular mechanisms and cellular behaviors is important.

2 REVIEW OF LITERATURE

2.1 Neurofibromatosis 1 (NF1)

2.1.1 The clinical diagnosis of NF1

Neurofibromatosis 1 is an autosomal dominant disorder affecting 1:2000 people (Uusitalo et al., 2014). The clinical diagnosis of NF1 requires the meeting of two or more criteria of the disease, which has been established in 1987 by the National Institute of Health (NIH) (Stumpf et al., 1988). Diagnosis is based on the presence of two of the following: six or more café-au-lait spots (pigmented spots on the skin, >5 mm in pre-pubertal patients, >15 mm in post-pubertal patients); two or more neurofibromas of any type or one or more plexiform neurofibroma; axillary or inguinal freckling; an optic glioma; two or more Lisch nodules in the eyes (small dark spots on iris); a typical skeletal abnormality for NF1, such as pseudarthrosis or dysplasia of long bone; and a first degree relative with NF1 by the above criteria. Other findings related to NF1 are scoliosis, short stature, learning disabilities, and macrocephaly (Boyd et al., 2009).

The NF1 phenotype is 100% penetrant, and by the age of 8, nearly all NF1 patients have 2 or more of the symptoms included in the NIH Diagnostic criteria (DeBella et al., 2000; Duong et al., 2011). NF1 affects multiple organs, and the symptoms are highly variable and unpredictable even within the same family (Viskochil, 2002).

2.1.2 Genetic background of NF1

Neurofibromatosis 1 is a result of a germline mutation in the *NF1* tumor-suppressor gene, which is located on chromosome 17, band q11.2. The size of this large gene is 350 kilobases, which may partly explain the high number of mutations. Half of the NF1 patients have inherited their disease from their parents, and the other half has no family history due to a spontaneous de novo mutation (Hirbe and Gutmann, 2014; Parada, 2000).

The *NF1* gene encodes a tumor suppression protein called neurofibromin, which is one of the GTPase-activating proteins. It consist of 2818 amino acids, and its molecular weight is 280 kDa (DeClue et al., 1991). Neurofibromin acts as a Ras-GTPase-activating protein (Ras-GAP), which functions as a negative regulator of Ras by converting active Ras-GTP to inactive Ras-GDP (Johnson et al., 1994; Le and Parada, 2007). Mutation in the NF1 gene causes loss of functional neurofibromin. This, in turn,

leads to hyperactive cells growing and dividing in an uncontrolled way that can lead tumor formation (Ferner and Gutmann, 2013; Heervä et al., 2010; Parada, 2000). This is presented in Figure 1.

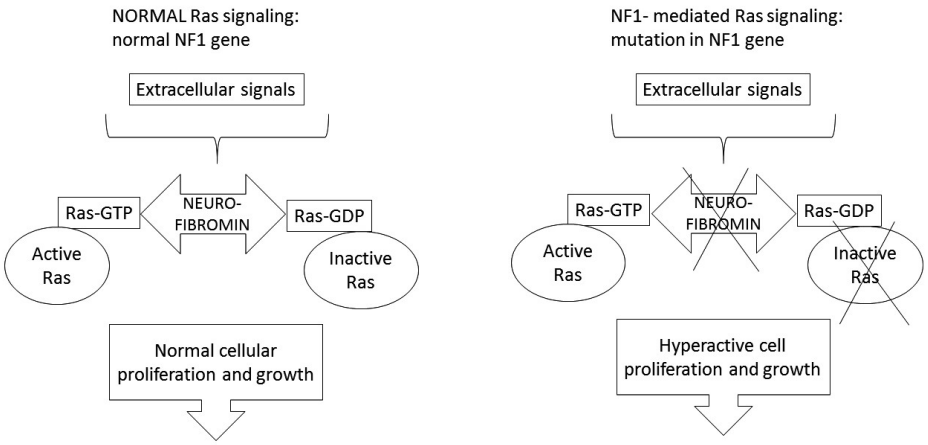

Figure 1. Neurofibromin as a negative regulator of Ras signaling.

The molecular diagnostics of this disease is still difficult, partly due to the large size of the *NF1* gene, because over 2800 different germline mutations have been reported (Koczkowska et al., 2018). All patients with NF1 seem to carry a heterozygous mutation of the disease, and a loss of both alleles of the *NF1* gene in the germline has not been shown (Jouhilahti et al., 2011b). The spectrum of the mutations in NF1 is very wide, but only a few phenotype-genotype correlations have been identified (Pasmant et al., 2012). Microdeletion of 17q11.2, meaning deletion of the entire *NF1* gene and surrounding genes, is known to cause a severe form of NF1 in 5 – 10% of patients (Kluwe et al., 2004; Pasmant et al., 2010). Mild NF1 disease occurs in a mosaic form, when an individual has two genetically unequal cell lines as a result of mutation occurring early during embryonic development (Boyd et al., 2009; Colman et al., 1996). According to Ars et al. (2000), the most common mutations of NF1 are those affecting mRNA splicing (Ars et al., 2000). Other mutations in the *NF1* gene include point mutations, insertions, and deletions (Koczkowska et al., 2018).

There are also other genetic diseases belonging to group of RASopathies, syndromes caused by germline mutations in Ras/mitogen-activated protein kinases (MAPK)-pathway genes. These syndromes include, inter alia, Noonan, Costello, cardio-facio-cutaneous, and Legius syndromes (Tidyman and Rauen, 2010). RASopathies, especially Legius syndrome that is caused by mutations in the *SPRED1* gene, may be mistaken for NF1 disease, because these syndromes share the same clinical features, such as axillary freckling, café-au-lait spots, and learning disabilities (Brems and Legius, 2013). Based

on the similarity of the clinical features of these diseases, it is important to understand the overlapping pathophysiology of NF1 and these other RASopathies.

Neurofibromatosis type 2 (NF2) is caused by mutations in the *NF2* gene located on chromosome 22, band q12.2. NF2 syndrome is much less common than NF1. The hallmark of NF2 are bilateral vestibular schwannomas (Evans, 2015).

2.1.3 Tumors in NF1

The benign cutaneous tumors called neurofibromas cause the main disease burden in adults and are the hallmarks of neurofibromatosis 1. Neurofibromas are present in half of 10-year-old patients and stand in 80 % of 20-year-old NF1 patients (DeBella et al., 2000; Duong et al., 2011; Kodra et al., 2009). The number of these tumors is highly variable among patients, and the number can be few, hundreds, or even thousands (Verma et al., 2018). Cutaneous neurofibromas are located in the dermis, and they rarely cause medical problems other than tenderness or itch but may cause severe esthetic or social burden. There is no cure or way to stop the growth of these tumors, although removal of neurofibromas with a carbon dioxide laser offers a feasible method to decrease the number of tumors (Chiang et al., 2012).

Internal neurofibromas are major disadvantages in NF1. Neurofibromas can be present underneath the dermis in the subcutis or in larger nerves within muscles. Compared to cutaneous neurofibromas, subcutaneous tumors cause more often pain and neurological symptoms (Ferner, 2010; Khosrotehrani et al., 2005).

Plexiform neurofibromas arise from multiple nerve fascicles, tend to grow along the nerve length, and extend into surrounding structures. They are often found in young children, can be present at a time of birth, and can continue growing during adolescence (Joshi et al., 2015; Nguyen et al., 2011). Over half of the NF1 patients have internal plexiform neurofibromas, which can be detected with whole body magnetic resonance imaging (MRI). Although these tumors are benign, they can cause severe symptoms such as functional disability, disfigurement, and a danger to life by becoming malignant (Jett et al., 2015; Korf, 1999; Mautner et al., 2008). Plexiform tumors are not radiosensitive, and their response to chemotherapy is low, so the most effective treatment is surgery (Wise et al., 2005).

Subcutaneous and plexiform neurofibromas can develop into malignant peripheral nerve sheath tumors (MPNSTs), but MPNSTs can also arise sporadically (Ferner and Gutmann, 2002). MPNSTs in NF1 affect mostly young and middle-aged adults. They tend to metastasize early, and MPNSTs are the leading cause of mortality in NF1. The

lifetime risk for MPNST is about 16%, and 5-year survival is only 30-50% (Evans et al., 2002; Uusitalo et al., 2016).

2.1.4 Skeletal lesions and osteoporosis in NF1

Half of the NF1 patients have some kind of bone abnormalities. Skeletal manifestations in NF1 can be divided into focal and generalized lesions. The focal lesions include, among others, scoliosis, pseudarthrosis of a long bone, macrocephaly, and sphenoid wing dysplasia. Generalized skeletal lesions include short stature and low bone mineral density (BMD) including osteopenia and osteoporosis (Elefteriou et al., 2009; Ferner, 2010).

Scoliosis affects over 20% of NF1 patients. This spinal deformity's severity may vary from mild (i.e., idiopathic scoliosis) to severe and can be life-threatening curvatures (i.e., dystrophic scoliosis) (Akbarnia et al., 1992; Tsirikos et al., 2005). The pathophysiology of pseudarthrosis is not known. However, double inactivation of the *NF1* gene in mesenchymal progenitors from the periosteum has been considered important, and it has been suggested that this lesion is due to altered neurofibromin-Ras signaling. Also markedly high numbers of osteoclasts have been found in histological analysis of NF1-related pseudarthrosis tissues. Pseudarthrosis of long bones affects 5% of NF1 patients, and 50 - 80% of patients with pseudarthrosis have NF1 (Heervä et al., 2010; Sant et al., 2015; Stevenson et al., 1999). Sphenoid wing dysplasia affects approximately 5% of NF1 patients. These patients suffer from partial or complete absence of the greater wing of the sphenoid, and the best treatment modality is a surgical procedure (Lotfy et al., 2010).

Macrocephaly (i.e., unusually large head size) is one of the clinical signs of NF1, and it occurs in at least half of the NF1 patients, especially in children (Karvonen et al., 2013; Morris et al., 2016). NF1 children are shorter compared to age-mates, which partly explains the fact that NF1 adults are shorter than unaffected counterparts. Short stature affects half of the NF1 patients (Soucy et al., 2013; Vassilopoulou-Sellin et al., 1995).

Many studies have shown that low bone mineral density (BMD) is common in NF1 (Heervä et al., 2012; Kuorilehto et al., 2005; Lammert et al., 2005a). The lowest BMD values have been obtained from load-bearing sections of the body, such as legs and spine (Kuorilehto et al., 2005). In addition to an age-related decrease of BMD, osteoporosis and osteopenia are common in NF1 patients, and osteopenia progresses often to osteoporosis (Brunetti-Pierri et al., 2008; Heervä et al., 2013).

Osteoporosis is a major public health problem in the world. Low BMD, low intake of vitamin D and calcium, hormonal factors, gender, family history for fractures, lifestyle

choices (i.e., tobacco, alcohol, spending a lot of time sitting), and certain diseases like celiac disease increase the risk of osteoporosis. Aging people and postmenopausal women are especially at a high risk for fractures in the hip, the spine, and other bones (Finkelstein et al., 2008; Lane, 2006). During the normal lifespan, the amount of bone tissue keeps growing and reaches its maximum strength and density at around the age of 30. Thereafter, bone mass begins to drop with age. The decrease in estrogen production after menopause is associated with reduced bone mass, which further increases the risk of osteoporosis in women (O'Flaherty, 2000). Osteoporosis is usually diagnosed clinically with dual-energy X-ray absorptiometry (DXA), when a person's BMD T-score is equal to or more than 2.5 standard deviations below the mean BMD of the population. Other methods that can be used to diagnose osteoporosis are quantitative computer tomography and quantitative ultrasound (Kanis, 2002).

2.1.5 Other manifestations of NF1

Optic pathway gliomas are the most common central nervous system benign tumors in NF1. Usually these tumors arise in young children, and they are present approximately in 20% of patients with NF1 (Rodriguez et al., 2008). Although most optic gliomas are low-grade tumors, they can cause vision disturbances, proptosis (e.g., bulging of the eye), pain, and disturbances in hormone production. Hormonal problems emerge, when the tumor spreads to the hypothalamus causing early puberty (Boyd et al., 2009; Listernick et al., 2007). There is no effective treatment available for symptomatic optic pathway glioma. Radiotherapy is not recommended, because it may lead to secondary malignancies later. Observation, especially for non-symptomatic tumors in children, is recommended. Also chemotherapy is accepted, if the severity of the symptoms can be decreased (Thomas et al., 2015). Another ophthalmic features of NF1 are small, dome-shaped, and light brown Lisch nodules, which are harmless and present in 95% of adults NF1 patients (Huson et al., 1987).

Café-au-lait spots are hyperpigmented macules and are observed in 95% of patients with NF1. They are usually the first signs of NF1, and present at birth, or develop within first years of life (DeBella et al., 2000). Another cutaneous manifestation is freckles of skinfolds. This appears at the age of 3 to 5, and practically all adults have them. They are present in the area of the armpit, groins, neck, or under breasts (Boyd et al., 2009). Nevus anemicus, a localized area of pale spots, is also a common skin finding and may be useful in supporting the diagnosis of NF1 especially in children (Vaassen and Rosenbaum, 2016).

A wide range of neurological problems is part of NF1 disease. Mild cognitive impairment, learning disabilities, and behavioral problems are seen in 50 to 80% of NF1

people. The intelligence of NF1 children is usually normal, although under the average value when compared to healthy children (Friedman, 2002; Lehtonen et al., 2013).

Overall, the vast majority of NF1 manifestations are not life threatening, but they affect the quality of life (QoL) in different ways, such as causing aesthetic problems. Unpredictability of NF1 may also cause stress. Questionnaires to measure the QoL in NF1 are helpful in individual patient's assessment (Ferner et al., 2017; Wolkenstein et al., 2001).

2.2 Hormones

Hormones may play a role in dermal neurofibromas of NF1 disease. These skin benign tumors start to grow during puberty, and their number and size increase during pregnancy (Posma et al., 2003; Roth et al., 2008a). Sex hormones mediate their effects by binding to their receptors. Cholesterol is the starting material for the biosynthesis of steroids (Figure 2). Cholesterol can be synthesized *de novo* from acetate by all tissues, especially the liver, or obtained from the diet. Low density lipoproteins (LDL) transfer cholesterol to the cells and high density lipoproteins (HDL) from the cells (Charlton-Menys and Durrington, 2008). Cholesterol is converted to pregnenolone in the inner mitochondrial membrane by the CYP11A1 enzyme (Nebert and Russell, 2002). The steroidogenic acute regulator (STAR) plays an important role in lipid metabolism by transferring cholesterol from the outer mitochondrial membranes to inner mitochondrial membranes (Lin et al., 1995). Pregnenolone can be converted into progesterone by type 1 3β- hydroxysteroid dehydrogenase (HSD3B1) enzyme, which is located either in the ovarian theca cells or testis Leydig cells, or into 17α-hydroxypregnenolone and further to dehydroepiandrosterone (DHEA) by CYP17A1 (Miyabayashi et al., 2015; Ye et al., 2011). Progesterone can be synthesized into 17-hydroxyprogesterone by CYP17A1 and further to androstenedione (Miller and Auchus, 2011).

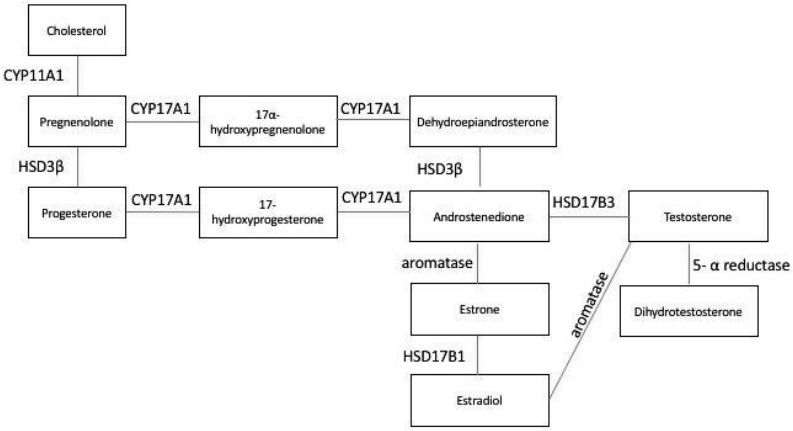

Figure 2. Sex hormone synthesis.

2.2.1 Gonadotropins

The biosynthesis of sex hormones is controlled through the feedback system of hypothalamus-pituitary-target tissue axis (Figure 3). Follicle stimulating hormone (FSH) and luteinizing hormone (LH) control ovarian steroid hormone biosynthesis and the development of the ovarian follicles in females. In males, LH and FSH stimulate the aromatization of estrogens and control the synthesis of testosterone.When more sex hormones are needed, the hypothalamus increases secretion of gonadotropin-releasing hormone (GnRH), which further stimulates the secretion of LH or FSH into general circulation. (Bain, 2007; Plant, 2015; Velarde, 2014).

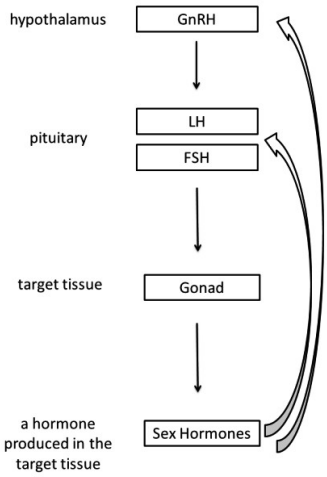

Figure 3. The feedback system of hypothalamus-pituitary-target tissue axis.

Human chorionic gonadotropin (hCG) and LH share the same receptor (LH/CGR) and their molecular structures are similar. However, they play different roles in reproductive physiology (Choi and Smitz, 2014). The receptor is found in the gonads, ovary, testis, skin, breast, adrenals, and neuroendocrine cells (Theofanakis et al., 2017). LH is responsible for regulating gonadal steroidogenesis and ovulation through the hypothalamic-pituitary-gonadal axis (Liu et al., 2007; Takahashi et al., 2016). hCG is a glycoprotein, which is present in four different isoforms and has a crucial role in gaining and maintaining pregnancy. It is produced by syncytiotrophoblast cells, and its mission is to stimulate the secretion of progesterone in the corpus luteum, stimulate angiogenesis within a pregnant uterus, and differentiate cytotrophoblasts into syncytiotrophoblasts (Cole, 2010). Pituitary hCG is present in men and women, thus, it is produced by gonadotrophic cells of the anterior pituitary. Pituitary hCG mimics LH function and may initiate the ovulation (Choi and Smitz, 2014).

LH cannot support pregnancy, however, hCG is also used clinically to induce ovulation in assisted fertilization (Stenman et al., 2006). Free β-hCG is found at low levels during normal pregnancy, but it is also known to promote cancer cell growth (Stenman et al., 2006). hCG is also expressed in malignant tumors, and it seems to play a role in the formation of tumors microvascular channels reminding of small blood vessels, which provide nutrients to tumor (Gao et al., 2016). In cancer predisposition syndrome NF1, an increase in the size and number of skin neurofibromas during pregnancy has been reported (Xiong et al., 2015). The connection between growing neurofibromas and pregnancy is unknown. However, it maybe that hormones influence on the growth of cells, especially Schwann cells, inside of the neurofibromas (Roth et al., 2008b). The influence of hCG seems to promote the cell signaling of cAMP, but also the extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation (Riccetti et al.,

2017) and it is known that the phosphorylation of ERK1/2 promotes the dedifferentiation of Schwann cells (Napoli et al., 2012).

2.2.2 17-β-Estradiol

Estrogens are responsible for pubertal development, regulation of the menstrual cycle, increasing bone mass and muscle strength, and improving cardiovascular systems (Hall et al., 2001; Messinis et al., 2014; Shirtcliff et al., 2009; van den Beld et al., 2000). The activation of estrogen is mediated through the estrogen receptors ERα and ERβ, which translocate to the nucleus (Marin-Castaño et al., 2003). The classical ligand-dependent pathway requires estradiol attachment to the ER following a conformational change of the complex, which leads to translocation of the estradiol/ER complex to the nucleus (Hall et al., 2001). There are several signaling pathways activating under the including of estradiol, which are Ras/Raf/mitogen-activated protein kinase (MEK)/ERK and phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) (Driggers and Segars, 2002). Estrogen is mainly produced in the ovaries in addition to testis, adipose tissue, brain, vascular endothelium, bone cells, and aortic smooth muscle cells (Simpson, 2003). Estrogen is the major female hormone and plays an important role also in males. There are four naturally occurring estrogens: estrone (E1), estradiol (E2), estriol (E3), and estetrol (E4). The most active form of estrogen is estradiol (17β-estradiol, E_2) (Kuiper et al., 1997; Thomas and Potter, 2013). Estrogen is synthesized from androgens in many tissues through aromatase, which is found in many tissues and cells. Estradiol synthesis takes place, when aromatase converts androstenedione to estrone, and type 3 17β- hydroxysteroid dehydrogenase (HSD17B1) finalizes the synthesis (Figure 2) (Hilborn et al., 2017; Stocco, 2012)

The main burden of NF1 disease is neurofibromas affecting the quality of life (QoL) (Wolkenstein et al., 2001). The main cell type of neurofibromas are Schwann cells (Viskochil, 2003). It has been shown that neurofibromas, neurofibroma-derived Schwann cells and Schwann cells isolated from sciatic nerve contain ERs (Fishbein et al., 2007; Geller et al., 2008; Gu et al., 2018; McLaughlin and Jacks, 2003). Interestingly, E_2 increases the proliferation and differentiation of Schwann cells (Chen et al., 2016). In addition, the influence of E_2 were reported to increase the size of mouse xenograft model of malignant peripheral nerve sheat tumors (MPNSTs), which develop from Schwann cells (Li et al., 2010; Perrin et al., 2007). However, there are only few studies related to estrogen action on Schwann cells, although clinical findings show that neurofibromas appear during puberty.

2.2.3 Testosterone

Testosterone plays many biological roles. It is needed for pubertal development, development of male reproductive tissues, increasing muscle and bone mass, has roles in hematopoietic and neural systems, and is necessary in the immune system (Davey and Grossmann, 2016). The effects of testosterone and other androgens are mediated through the androgen receptor (AR), which is expressed in many cells and tissues (Gao et al., 2005). When testosterone binds to AR, it induces a conformational change, dissociates proteins from the AR, which leads to translocation of the testosterone/AR complex to the nucleus, where it binds to androgen response elements (AREs) of DNA. This procedure leads to gene expression and recruitment of cofactor proteins and other signaling pathways (Gao et al., 2005; Tan et al., 2015). The testosterone/AR complex can also activate 2nd messenger pathways without binding to DNA, such as Ras/raf/MEK/ERK or PI3K/Akt (Liao et al., 2013). AR can even activate without androgen attachment as in hormone-resistant prostate cancer (Dehm and Tindall, 2006; Saraon et al., 2014). Testosterone is the major androgen in men, where it is mainly produced in testes Leydig cells (Shima et al., 2013). In women, testosterone is produced in ovaries and adrenal glands. It is synthesized from androstenedione by HSD17B3 or through DHEA conversion by HSD17B3 into androstenediol and further by HSD3B1 to testosterone (Burger, 2002; Roy et al., 2014). Testosterone can be further converted to dihydrotestosterone (DHT) by 5α-reduction in the prostate. It is responsible for the most of testosterone's biological action (Luu-The et al., 2008). Testosterone (not DHT) can be synthesized to estradiol via aromatase, p450aro, in certain tissues, such as ovary, testis, bone, prostate, placenta, brain, adipose tissue, and breast carcinoma tissue (Stocco, 2012).

Testosterone levels increase during pregnancy (O'Leary et al., 1991). Neurofibroma growth is related to hormonal influence. Androgen receptors are expressed in neurofibroma tumors and neurofibroma-derived Schwann cell cultures (Fishbein et al., 2007). Androgen synthesis is few steps from progesterone synthesis, and it has been shown that 75% of the neurofibromas express progesterone receptors and progesterone increases the proliferation of Schwann cells (McLaughlin and Jacks, 2003; Overdiek et al., 2008). However, the role of androgens in Schwann cells is still unknown.

2.3 Pathogenesis of neurofibromas

2.3.1 Structure and supporting cells of peripheral nerve

The human nervous system can be divided into two sections: the central nervous system and the peripheral nervous system. Shortly, the central nervous system consists of the brain and the spinal cord, and peripheral nervous system consists of ganglia, cranial, and spinal nerves. The tissue of the peripheral nerve consists of several types of cells, e.g., Schwann cells, satellite cells, fibroblasts, adipocytes, perineurial cells, macrophages, and nerve cells, also called as neurons. Neurons (Figure 4) provide a fast pathway for electrochemical nerve impulses (Fu and Gordon, 1997; Richard et al., 2014).

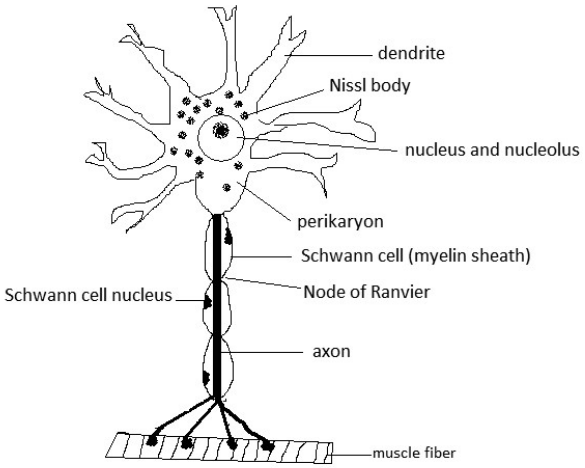

Figure 4. Overview of the structure of a neuron. Dendrites branch from the soma of a neuron and allow the neuron to receive information. The information conducts down the axon and passes the information to an effector cell, e.g., a muscle cell. Schwann cells cover the axon to form a protective myelin sheath. The gaps between the Schwann cells are called the nodes of Ranvier.

The outer layer of peripheral nerve is covered by a connective tissue called epineurium, which surrounds blood vessels and several axon bundles, also called as nerve fascicles. These axon bundles are enclosed by the perineurium. All axons, except those of the smallest sensory nerve endings, are coated by Schwann cells. Schwann cells may form the myelin layer or surround several axons without forming myelin. Axon-Schwann cell units are buried in connective tissue called endoneurium. Together, the epineurium, perineurium, and endoneurium form a protective covering of a peripheral nerve. The

collagen structure provides the strength of peripheral nerve, and adipocytes protect the nerve from pressure (Campbell et al., 2013; Peltonen et al., 2013).

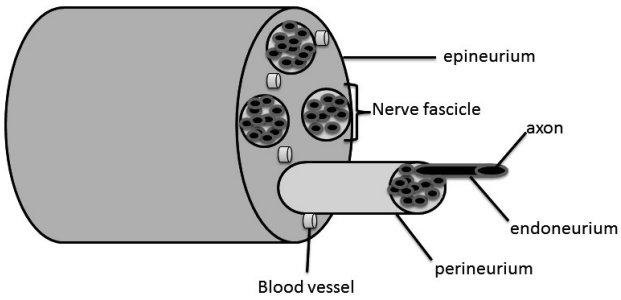

Figure 5. Structure of a peripheral nerve. The outer layer of the peripheral nerve is enclosed by connective tissue called epineurium. Inside of the epineurium there are multiple nerve fascicles, which are covered with perineurium. Nerve fascicles consist of bundles of axons, which are enclosed in the endoneurium. Blood vessels supply nutrients to peripheral nerve cells and are located between and inside the fascicles.

2.3.2 Cellular structure of cutaneous neurofibromas

Benign cutaneous neurofibromas are the hallmarks of NF1 disease. The rubbery consistency of neurofibromas is due to collagenous extracellular matrix (ECM), where Schwann cells, fibroblasts, perineurial cells, endothelial cells, and mast cells are embedded (Peltonen et al., 1988). In neurofibromas of NF1 patients, all the cells carry a constitutional mutation in the *NF1* gene ($NF1^{+/-}$). In addition, a subpopulation of Schwann cells also carry a second hit in the gene ($NF1^{-/-}$) (Serra et al., 1997). Histological and *in vitro* studies have shown that the majority of the cell population in neurofibromas consists of Schwann cells and fibroblasts (Peltonen et al., 1988; Pummi et al., 2006). The fibroblasts' main function is to support the structure of connective tissue. Fibroblasts are specialized for the secretion of ECM components such as Type I, III, and VI collagens, glycosaminoglycans, and other important glycoproteins such as fibronectin. Fibroblasts have the ability to migrate and secrete collagenous material into the wound further promoting wound healing. They also have the ability to synthesize laminin. Fibroblasts differ according to their tissue location; they are different, e.g., in skin or on bone matrix. It has been suggested that components of ECM can influence differentiation of immature fibroblasts (Ivey, 2017; Jaakkola et al., 1989a; Nomura et al., 1994; Olsen et al., 1989). Fibroblasts can be detected by the expression of CD34,

and it seems that neurofibroma fibroblasts originate from endoneurial fibroblasts (Hirose et al., 2003).

The mission of perineurial cells is to form the perineurium surrounding a nerve fascicle, which consists of bundles of Schwann cells covering the axons. Perineurial cells produce laminin and secrete fibronectin and type I, III, and VI collagen. Claudin-1 can be used as a marker for perineurial cells (Jaakkola et al., 1989b; Peltonen et al., 1987; Pummi et al., 2006).

Neurofibromas are dependent on blood supply, because blood vessels provide nutrients to the tumor. The linings of the blood vessels are formed by endothelial cells. Endothelial cells have the capacity to proliferate and form new vessels when necessary. Friedrich et al. (2015) showed that vascular invasion in NF1 neurofibromas was highly variable and presented a higher mean compared to vascular invasion of healthy control skin biopsies. However, there was no statistical difference between these two groups. NF1 neurofibromas may bleed when removed, and this may be because of alterations in the structure of the vascular wall (Friedrich et al., 2015).

Collagen production is necessary for tumor formation. Mast cells of neurofibromas induce proinflammatory growth factors such as basic fibroblast growth factor (bFGF) and transforming growth factor-*beta* (TGF-*beta*), which in turn accelerates collagen production by fibroblasts (Yang et al., 2006). In addition, masts cells secrete platelet-derived growth factor (PDGF) promoting vascular invasion of tumors (Bhowmick et al., 2004; Yamabe et al., 2000). Masts cells are located in endo-, peri-, and epineurial structures of the peripheral nerves (Bienenstock et al., 1991). Histological studies have shown that mast cells are located differentially in NF1-related cutaneous and plexiform types of neurofibromas. In cutaneous neurofibromas, mast cells were located densely and throughout the tumor, while in plexiform tumors, the number of mast cells was lower, and they were located in the periphery of the tumor (Tucker et al., 2011).

2.3.3 Origin of cutaneous neurofibromas

Neurofibromas contain the same cell types as peripheral nerves allowing for speculation that neurofibromas arise from the small nerves or nerve branches (Jo and Fletcher, 2014; Jouhilahti et al., 2011a). In normal peripheral nerve, axon Schwann cell units are shielded and sheltered by perineurial cells of perineurium (Piña-Oviedo and Ortiz-Hidalgo, 2008). The most abundant cells in neurofibromas are Schwann cells, which are dissociated from nerves (Peltonen et al., 1988). The hyper-proliferation of the Schwann cells has been shown to be the initiator of these benign tumors. Part of the neurofibroma Schwann cells carry heterozygous *NF1* mutation ($NF1^{+/-}$), and the other part of Schwann cells carry also a second hit, leading to the inactivation of both alleles of the

NF1 gene (*NF1*$^{-/-}$) (Serra et al., 1997). This is supported by the mouse studies showing that mice carrying only *Nf1*$^{+/-}$ mutation did not develop neurofibromas (Jacks et al., 1994). However, mice (chimeric mice) injected with *Nf1*$^{-/-}$ embryonic stem cells into the blastocysts developed *Nf1*$^{-/-}$ cells and also high number of neurofibromas. This approach resembles the human condition, where part of the somatic cells carry a second hit of the *NF1* gene (Cichowski et al., 1999). One view of neurofibroma development suggests that a second hit in part of the Schwann cells of the nerve initiates perineurium disruption and starts uncontrolled cell proliferation towards tumor growth (Colman et al., 1995; Serra et al., 1997; Zhu et al., 2002).

Different studies with mice knockout models have shown that formation of neurofibromas require both *Nf1*$^{+/-}$ cells, such as mast cells and fibroblasts, and *Nf1*$^{-/-}$ Schwann cells in the microenvironment for tumor. Another study related to the stem cell niche, approaches the initiation of skin benign neurofibromas with alternative explanation (Jouhilahti et al., 2011a). Neurofibromas tend to locate nearby hair follicles, which are the source of multipotent stem cells. These multipotent neurofibroma-derived precursor cells had the potential to differentiate to Schwann cells, neurons, epithelial cells, and adipocytes which suggests that multipotent stem cells can be involved in the development of neurofibromas.

2.3.4 Schwann cells in vivo and in vitro

Schwann cells play a role in maintaining the peripheral nerve. Schwann cells originate from neural crest cells from where they differentiate first into Schwann cell precursors and further into immature Schwann cells, and finally to either myelinating or non-myelinating Schwann cells (Jessen and Mirsky, 1997). Many growth factors regulate differentiation and proliferation of Schwann cells. Schwann cells express insulin receptors, and it is known that insulin activates MAPK and PI3K/Akt pathways, and further Schwann cell differentiation, survival, and myelination (Le Roith and Zick, 2001; Russell et al., 2000; Shetter et al., 2011; Syroid et al., 1999).

Neuregulins, such as heregulin can activate epidermal growth factor receptor (EGFR), because it contains a homology domain to epidermal growth factor (EGF), which binds to EGF receptors of erbB2 and erbB3 in Schwann cells. Long-term exposure with heregulin to gain change in gene expression stimulates Schwann cell proliferation together with forskolin. Heregulin activates MAPK and forskolin raises the cyclic nucleotide cyclic adenosine monophosphate (cAMP) and its principal target, the cAMP-dependent protein kinase A (PKA) (Jessen and Mirsky, 1997; Rahmatullah et al., 1998; Seamon and Daly, 1981). It is known that cAMP is an essential route for many Schwann cell growth factors. The effect of cAMP to Schwann cell differentiation and Schwann cell proliferation acts through the cAMP-PKA pathway (Monje, 2015). The

NF1 gene plays a role in maintaining a balance between Ras and the level of cAMP (Kim et al., 2001). Loss of *Nf1* leads to abnormal regulation of the cAMP-PKA pathway. A study showed that cAMP levels were multiple in Schwann cells cultured from *Nf1*-null mice and levels of cyclin D1 were increased compared to wild-type mice Schwann cell cultures. Loss-of-function of *Nf1* seemed to promote the formation of cAMP (Kim et al., 2001). During the G1 phase of the cell cycle, cyclin D1 promotes the cell cycle independently. However, the activation of Ras is critical to promote the synthesis of cyclin D1 to continue the proliferation of the cells (Hitomi and Stacey, 1999). Perhaps because of the hyperactive Ras in NF1, the level of cyclin D1 is elevated and the proliferation of Schwann cells is increased. Heregulin together with forskolin maintain high levels of cyclin D1, and therefore, maximal proliferation of Schwann cells in a cell culture (Kim et al., 1997; Rahmatullah et al., 1998).

To detect the mature Schwann cells from the other cell types, a biomarker of S100B has been widely used. Schwann cells are also identified by their bipolar shape (Peltonen et al., 1988; Scarpini et al., 1988). Different mouse models have been developed to study the phenotypes and pathogenesis of NF1. Mouse models with NF1 patient-specific mutations showed that the frame and severity of the disease is depends on the mutation, and symptoms can vary from disorganized non-myelinating axons and neurofibromas to generation of non-viable mice (Li et al., 2016).

2.4 Bone dynamics

2.4.1 Bone structure and function

The skeleton of an adult is composed of over 200 bones and cartilage. The skeleton provides structural support of the body, facilitates movements, protects internal organs, stores and releases minerals (e.g., calcium and phosphorus), and produces blood cells (i.e., hematopoiesis). The morphology of the bone can be viewed macroscopically and microscopically as seen in Figure 6. The outer layer of bone is coated by the highly innervated periosteum, which explains the pain-sensitivity of the tissue. Periosteum covers cortical bone, which constitutes about 80% of the skeletal mass. The structure of cortical bone is very dense and hard. Osteons are basic structural units of compact bone. They consist of concentric layers of calcified bone made by osteocytes, and a central canal, called the Haversian canal, that contains blood vessels, nerves, and lymphatics. The blood vessels of Haversian canal are interconnected with blood vessels on the surface of the bone. The lamellar structure of the osteons is due to arrangement of collagen fibrils. The endosteum membrane layer covers trabecular bone. This faces the medullary canal containing yellow bone marrow in long bones (Clarke, 2008;

Weatherholt et al., 2012). The volume of hematopoietic red bone marrow in bone cavities decreases with age and its replaced with yellow bone marrow filled with adipocytes (Takeshita et al., 2014). Trabecular bone is spongious, higher in surface area, less dense, and softer compared to cortical bone. It constitutes of about 20% of the bone mass, and it is found at both ends of long bones. The spongious structure called trabeculae, is due to a network of irregularly-shaped thin bars and broad plates. (Clarke, 2008; Weatherholt et al., 2012).

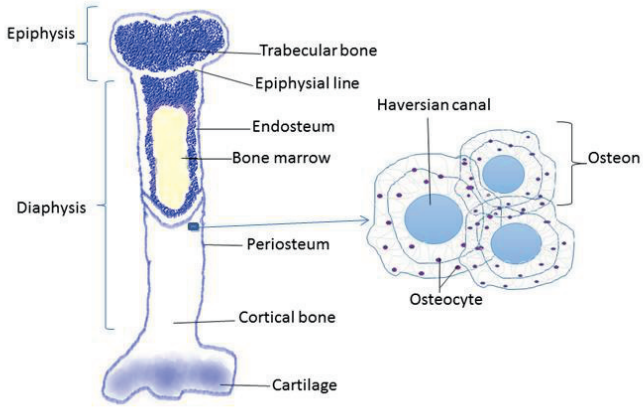

Figure 6. The macroscopic- and microscopic structures of long bone.

Bone is constituted of minerals (50-70%), an organic matrix (20-40%), water (5-10%), and lipids (2-3%). The composition may vary according to the state of maturation and turnover, age, anatomic location, diet, and health (Boskey, 2013; Rey et al., 2009). The organic matrix contains only two to five percent of cells and five percent of water. Collagen, primarily type I collagen, constitutes 90% of the organic matrix of the bone. Osteocalcin, osteonectin, and osteopontin are non-collagenous proteins that promote mineralization and bone formation. Bone contains also different cytokines and growth factors, which are important for bone growth, turnover, cell activation, and differentiation. Hydroxyapatite crystals, which are calcium phosphate minerals, are the main inorganic mineral component of the bone. (Buckwalter et al., 1996; Florencio-Silva et al., 2015; Wagermaier et al., 2006).

2.4.2 Bone cells

The main cell types of bone are osteoblasts, osteocytes, and osteoclasts. Osteoblasts and osteocytes are of mesenchymal origin, while osteoclasts are of hematopoietic origin

(Méndez-Ferrer et al., 2010). Macrophages are also hematopoietic in origin, and considered as a new class of bone cells interdependent with bone (Kaur et al., 2017).

Osteoblasts have a single nuclei. They form bone and make up approximately five percent of all bone cells. Osteoblasts act in groups. They synthesize collagens, non-collagenous proteins, such as osteonectin, osteopontin, and proteoglycans. Osteoblasts also express alkaline phosphatase (ALP). Osteoblasts take care of the bone mineralization by settling the hydroxyapatite crystals into collagen fibers (Caetano-Lopes et al., 2007; Florencio-Silva et al., 2015). To contribute to the differentiation, activity, and survival of other cells and their own activity, osteoblasts produce different soluble factors, such as receptor activator of nuclear factor kappa β ligand (RANKL), the RANKL inhibitor osteoprotegerin (OPG), and macrophage colony stimulating factor (M-CSF). When osteoblasts are at the end of the bone formation cycle, they can go to apoptosis, become bone lining cells, or osteocytes (Crockett et al., 2011). The lifespan of osteoblasts is three months (Manolagas, 2000).

Osteocytes are mature and permanent bone cells comprising most of the total amount of bone cells. Osteocytes have a lifespan of over 20 years. They are derived from bone-forming osteoblast cells trapped in the bone matrix. However, they are different in morphology and function, because they lose a large part of their organelles (Aarden et al., 1994; Rochefort et al., 2010). They turn into dendritic-like cells with their long appendages, which they use as a net for communication with each other, and also with osteoblasts and bone lining cells (Bonewald, 2011). Osteocytes secrete RANKL, M-CSF, and OPG, which further regulate the generation of osteoclasts (Bellido, 2014).

Bone lining cells are also differentiated from osteoblasts, which have completed their bone-forming function. The morphology of these cells is flat and elongated, and they have flat nuclei. Bone lining cells cover the non-remodeled surface of the bone. The function of these cells is still unknown. However, it has been suggested that perhaps they regulate the osteoclast bone resorption by secreting collagenase to matrix, before osteoclasts can attach to the bone. These lining cells act through messages from osteocytes, which sense the need of bone remodeling. Thus, they are a major source of osteoblasts during adulthood. Especially, quiescent bone lining cells are suggested to differentiate into osteoblasts (Matic et al., 2016; Parfitt, 2001).

Osteoclasts are large, highly polarized multinucleated cells with specialized morphological features (Teitelbaum, 2007). Multinucleated osteoclasts are the only cells that resorb the bone. They derive from a monocyte/macrophage lineage, differentiate towards an osteoclastic phenotype, and then fuse. Interestingly, mononuclear osteoclasts have been reported to have the ability to resorb the bone *in vitro* (Teitelbaum, 2007). Bone resorption needs a lot of energy, and osteoclasts have a high number of mitochondria (Williams et al., 1997). The lifespan of osteoclasts is two weeks (Manolagas, 2000). Numerous hormones and cytokines regulate osteoclast

differentiation and activity. However, the main regulator is RANKL//RANK/OPG signaling, where RANK binds to RANKL promoting osteoclast formation, activation, survival, and bone resorption. OPG inhibits osteoclast bone resorption by binding to RANKL (Boyce and Xing, 2008). Tartrate-resistant acid phosphatase 5b (TRACP) is an enzyme, which is a significant marker of osteoclasts. The biological function of TRACP is still unclear. However, it has been suggested that TRACP plays a role in bone resorption. Osteoclast transports degraded bone components through the cell with the help of transcytotic vesicles, where TRACP has been located. There are two forms of TRACP that circulate in human blood. TRACP 5a is derived from macrophages and dendritic cells, and TRACP 5b is derived from osteoclasts. TRACP 5b can be used for quantifying the number of osteoclasts (Halleen et al., 2006). C-terminal-crosslinked telopeptide of type I collagen (CTX) is a degradation product of type I collagen, which is released into the serum *in vivo* or medium *in vitro* after osteoclast bone resorption. CTX correlates with osteoclasts activity (Rissanen et al., 2008). *In vitro*, there are two important criteria for the definition of mature osteoclasts, such as the number of nuclei per cell (at least three) and TRACP activity (Kylmäoja et al., 2018; Minkin, 1982).

2.4.3 Bone remodeling

Bone remodeling is based on bone resorption by osteoclasts and bone formation by osteoblasts, and this process occurs throughout life. Most of the adult bone formation occurs after bone resorption. However, about three percent of bone forming occurs without resorption event and this is called bone modeling (Hattner et al., 1965; Ominsky et al., 2015). In addition, it has been shown that bone loading increases bone modeling (Ducher et al., 2005). The bone modeling and remodeling is regulated by local and systemic factors, and an imbalance in these processes could lead to bone loss and further to severe diseases, such as osteoporosis (Eriksen, 2010) or osteopetrosis. During the bone remodeling process, osteoclasts and osteoblasts form a basic multicellular unit (BMU), the organization of which differs in cortical and trabecular bone. On trabecular bone, osteoclasts travel approximately 25 μm each day, and they resorb the bone by making a trench with a depth of 40-60 μm. Trabecular bone is remodeled more dynamically than cortical bone. Almost five percent of cortical bone is remodeled every year. In cortical bone, osteoclasts resorb a cylindrical tunnel with a size of 2000 μm long and 200 μm wide in the bone with a speed of 20-40 μm per day. Thus, the BMU unit maintains its size for many months despite of short-lived osteoclasts, which are replaced then with new ones. Finally, the tunnel is fulfilled by thousands of osteoblasts that rebuild the bone (Parfitt, 1994).

The resorption process starts, when mononuclear preosteoclasts migrate to the bone surface, and they fuse to a group of multinucleated osteoclasts. Before that, osteocytes

send signals through osteoblasts and bone lining cells for the need of resorption of old bone. Mononuclear preosteoclasts fuse and activate to mature osteoclasts with the help of RANKL/RANK signaling by these other bone cells (Boyce et al., 2012). Bone lining cells prepare the bone surface for osteoclast resorption by secreting collagenases (Parfitt, 2001). Bone resorption can start, when an osteoclast attaches directly and indirectly to bone surface with the help of transmembrane molecules CD44 and $a_V\beta_3$ integrin and intracellular proteins, such as talin, vinculin, and F-actin (Deguchi et al., 2016). After attachment to the bone, the osteoclast polarizes and forms four different membrane domains: the sealing zone and a ruffled border underneath the osteoclast, a functional secretary domain (FSD) on top of the cell, and a basolateral membrane. Osteoclast degrades mineralized bone matrix by dissolving crystalline hydroxyapatite and proteolytic cleavage of the organic matrix, which includes collagen. The organic matrix is degraded with several proteolytic enzymes. The degraded products are removed via osteoclasts by transcytotic vesicles and released into extracellular space. Osteoclast bone resorption releases bone minerals, such as calcium into the bloodstream, which is very important for calcium regulation balance of the body. Finally, osteoclast returns to the non-resorbing area, or undergoes an apoptosis (Väänänen et al., 2000).

2.5 Osteoclasts

2.5.1 Osteoclasts domains

An osteoclast is an effective machine to resorb the bone. To enable this process, the osteoclast attaches to the bone with a specialized actin-rich structure known as the sealing zone (SZ). Attachment of osteoclasts to the bone surface is mediated via integrin receptors involved in cell-cell and cell-matrix adhesions: $\alpha_V\beta_3$ (vitronectin receptor), $\alpha_2\beta_1$ (collagen receptor), $\alpha_V\beta_1$, and $\alpha_V\beta_5$. Specially, $\alpha_V\beta_3$ is highly expressed in osteoclasts, and it plays a key role in the formation of the SZ. The inhibition of $\alpha_V\beta_3$ reduces bone resorption without changing the number of osteoclasts (Lakkakorpi et al., 1993; Nakamura et al., 1999; Nesbitt et al., 1993). The SZ forms a tightly isolated area between the ECM and osteoclast cell membrane. Formation of SZ is followed by osteoclast polarization resulting in three different membrane domains. One of the domains of polarized osteoclast is the basolateral membrane, which is not in contact with mineralized bone matrix. It is important in cell-cell contacts, because different proteins and receptors needed in cellular contacts are seen there, such as vitronectin receptors and connexin-43. Connexin forms gap junctions between osteoclasts, and gap junctions are needed for diffusion of small metabolites, growth factors, ions, other molecules, and nutrients (Giepmans and van Ijzendoorn, 2009; Ilvesaro and Tuukkanen,

2003). The osteoclast forms a long finger-like structure, which is a highly ruffled membrane structure underneath the cell and which faces the bone. The space between the ruffle border and bone is called Howship's lacuna. The bone mineral is resorbed at low pH, created by secretion of hydrochloric acid by the vacuolar H^+-ATPase. Cathepsin K is sectered from osteoclasts to the resorption space under the cell, and it secretes the bone organic matrix, mainly type I collagenase (Troen, 2004; Väänänen et al., 1990). The ruffled border is responsible for a bidirectional pathway, which includes substances that resorb the bone, and uptake and transcytosis of bone degradation products. The degraded bone matrix is transferred inside the cell, which are endocytosed in transcytotic vesicles and are moved to the top of the osteoclast toward the plasma membrane's functional secretory domain. The degraded bone matrix is released there into the extracellular base (Itzstein et al., 2011; Mulari et al., 2003). These domains will disappear, when osteoclasts have done the resorption and detach from the bone.

2.5.2 The cytoskeleton of osteoclasts

The cytoskeleton of the eukaryotic cell is composed of proteins cables, which are located all over the cell cytoplasm organized into a network. The cytoskeleton of osteoclasts undergoes highly dynamic changes to enable the changes in cell shape, invasion, migration, correct positioning of the organelles, cellular contacts and intracellular membrane transport. There are three main types of filaments of cytoskeleton: intermediate filaments, microtubules, and microfilaments (Fletcher and Mullins, 2010).

Intermediate filaments are composed of a variety of proteins, such as vimentin. The main function of intermediate filaments is to provide mechanical stability and structural support to the cell (Watanabe et al., 1995; Wilson, 2011). Microtubules are highly dynamic structures, whose main functions are to support the cell structure, but they are also involved in the transport of material within cells (Fletcher and Mullins, 2010; Goldstein and Yang, 2000). It has been shown that inhibition of microtubules interfere with osteoclast podosome belt formation (Ti et al., 2015).

Actin is the main component of microfilaments. Bone resorption and the actin ring are the hallmarks of osteoclasts. The basic structure of dynamic actin is monomeric globular actin (G-actin), which polymerizes to helical F-actin. The structure of two-stranded helical polymer F-actin filaments is formed, when the two polymerized F-actin chains are bound side-by-side with an equivalent binding between monomers. Actin filaments are abundant components of the cytoskeleton and composition for many cellular structures, and actin determines the shape and movement of the cell (Gurel et al., 2014). Highly branched actin filaments support and build up the forces, which are needed in either motility or interaction with other cells like osteoclasts (Pollard and

Borisy, 2003). The existence of many actin binding or actin-related proteins, which can cross-link to actin filaments, controls a highly organized, bundled, and branched composition of the actin network (Fletcher and Mullins, 2010). The actin network is continuously remodeled, assembled, and disassembled, e.g., by severing, capping, or depolymerizing the filaments (Zalli et al., 2016). Tyrosine kinase c-Src controls actin filament rearrangement and remodeling, thus, it plays multiple roles in integrin signaling (Destaing et al., 2008). The disruption of c-Src gene in mice results in osteopetrosis suggesting a role of the c-Src in osteoclast function, especially for cell attachment to the bone (Miyazaki et al., 2004). Cortactin is a substrate of c-Src, and it plays a role in actin polymerization and rearrangement. Numerous proteins, such as cortactin, activate actin-related protein 2/3 (Arp2/3) complex into actin filaments stabilizing actin branching. The Arp2/3 complex forms branched actin filaments with a 70^{O} angle to existing actin filament, so that new filaments can grow to old ones (Mullins et al., 1998). To save the actin network dynamic structure, cofilin severs and triggers the twist of actin filaments (McGough et al., 1997; Pavlov et al., 2007).

In osteoclasts, the actin cytoskeleton is different according to their substratum, such as on glass, or on bone. On glass, actin forms a podosome belt, which is surrounded by a loose actin cloud, on the substratum level of the cell. The actin cloud is organized by $\alpha_V\beta_3$ integrin among other proteins. Podosomes are short-lived actin-rich structures found usually in the periphery of the cell that contain other proteins, such as WASP, Arp2/3, CD44, cortactin, and gelsolin. They are needed for cellular adhesion, movement, and invasion. Actin-rich podosome structures change during osteoclast differentiation and activation. First, podosomes are organized as clusters and then into short-lived rings. When maturation occurs, these structures turn into a podosome belt on the cell periphery, and finally into a sealing zone to ensure the bone resorption. Podosomes are packed densely in the SZ. Formation of the SZ requires also the interaction of microtubules, cofilin, and cortactin (Saltel et al., 2008; Zalli et al., 2016).

Multiple nuclei and specific actin structures are associated with osteoclasts. Interestingly, actin filaments play a role in nuclei movement. It has been shown that both tubulin and actin play also a role in nuclei positioning. Nuclei are located in special regions or evenly inside of the cell. The location of the nuclei are carefully arranged, especially when there are syncytial nuclei inside the cell (i.e.,multinucleated cells) (Starr and Han, 2003). Therefore, actin may have an important role in nuclear positioning. Actin filaments are also needed to anchor the nuclear envelope with different proteins, e.g., Anc-1. Nuclear positioning is poorly understood. However, impaired nuclear positioning is known to lead to various diseases such as progeria or myopathies (Gundersen and Worman, 2013).

2.5.3 Fusion and contacts of osteoclasts in vitro

Cell fusion is an important but exceptional process in the body. Cell fusion is needed during fertilization, muscle differentiation, and fusion of monocyte progenitors into osteoclasts. The first step of cell fusion is to find the fusion partner and to have cell-to-cell contact, which leads to local membrane deformations and further to dynamic changes of lipid bilayers (Chernomordik and Kozlov, 2008). Effective bone resorption needs multinucleated osteoclasts, however, the mechanism in the fusion process is still unknown. Recent studies have shown that osteoclast fusion needs a fusion acceptor cell and fusion donor cell. Four different kinds of cell fusion have been shown. Prior to fusion, cells can make on broad membrane contact with each other, one cell can take the other cell in a phagocytic cup, cells can contact each other with a narrow tube, or one cell goes on top of the other cell just before fusion (Søe et al., 2015). In addition, mononucleated cells can fuse with multinucleated cells, or two multinucleated cells can fuse together. More mature osteoclasts prefer to fuse with less mature osteoclasts (Søe et al., 2015). Osteoclast fusion can also happen through two different types of cell contacts. When cells are in broad membrane contact, small preosteoclasts fusion is mediated by the membrane protein CD47. In addition, the membrane protein CD43 and syncytin-1 promote the fusion of multinucleated osteoclasts (Hobolt-Pedersen et al., 2014; Møller et al., 2017).

Cellular crosstalk is essential for osteoclast fusion. Gap junctions are hydrophilic intercellular channels formed by connexin proteins. These channels are needed for osteoclast differentiation, diffusion of ions, exchange of nutrients, metabolites, and small soluble molecules (Schilling et al., 2008). Inhibition of gap junctions in mouse bone marrow cultures decreases the differentiation of osteoclasts and bone resorption (Kylmäoja et al., 2013). Membrane tunneling nanotubes (TNTs) are F-actin rich structures that bridge from cell to cell without touching the substratum. TNTs are 50-200 nm in diameter and can connect to cells even over long distance. These tunnels enable rapid transport for membrane vesicles, organelles, and small molecules (Gerdes and Carvalho, 2008; Rustom et al., 2004). TNTs have a role in cell metabolism by transferring calcium ions from cell to cell. Many different cell types form TNTs enabling cellular contact and exchange of signals even from distant cells (Dupont et al., 2018). Osteoclast precursors form TNTs while searching a fusion partner, and TNTs disappear prior to fusion. Inhibition of the formation of TNTs with latrunculin B decreases the fusion of mononuclear cells into osteoclasts (Takahashi et al., 2013). Two different methods have been observed to lead to TNT formation. A protrusion of a cell elongates, until it reaches a neighboring cell, which may lead to membrane fusion. TNT may also form when two cells in contact move apart and leave an intercellular nanotube between the cells (Davis and Sowinski, 2008).

2.5.4 Signaling in osteoclastogenesis

There are several studies exploring osteoclastogenesis-related signaling pathways. However, they have mostly been explored with rodent cells. Osteoclastogenesis is stimulated by two different molecules called M-CSF and RANKL that originate from osteoblasts and other bone cells. M-CSF binds to c-Fms receptor and RANKL binds to RANK receptor on the cell membrane, and they stimulate crucial signaling pathways in osteoclastogenesis involved in differentiation, activation, function, and survival of osteoclasts (Kim and Kim, 2016). Osteoclast signaling is a highly complex system that is not fully understood. Many skeletal diseases are due to an increased number of osteoclasts, or impaired osteoclast functions. Therefore, there is a need to increase the understanding of osteoclast signaling. This study concentrates to understand the role of Ras/Raf/MEK/ERK, PKC, PI3K/Akt/mTOR, and p38 MAPK signaling pathways in osteoclast differentiation (Figure 7).

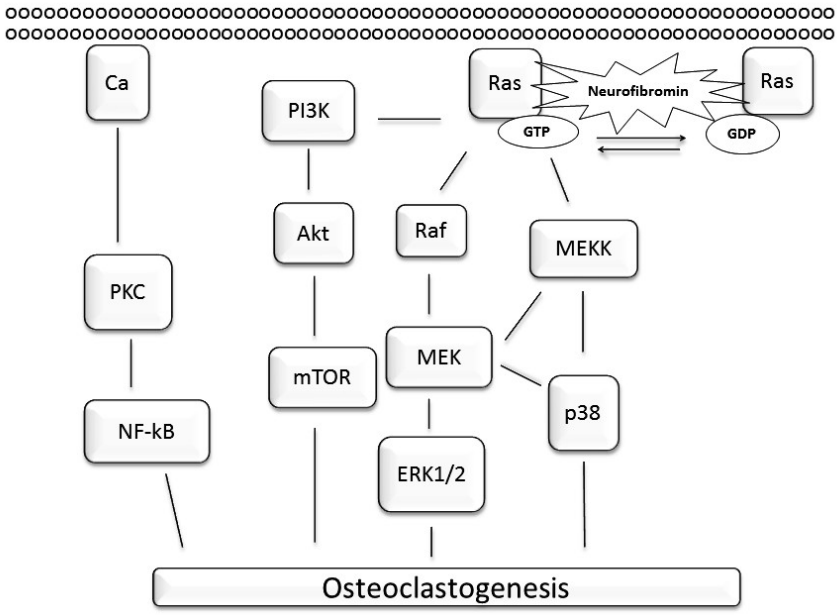

Figure 7. Signaling pathways in osteoclastogenesis.

M-CSF/c-Fms complex is known to activate downstream signals such as MEK/ERK and PI3K/Akt. RANKL binding to RANK receptor activates tumor necrosis factor receptor-associated proteins (TRAFs), especially TRAF6, which transmits the signal to several different downstream targets such as MEK/ERK, PI3K/Akt, and mitogen-activated protein kinase p38 (p38 MAPK) among other pathways (Kim and Kim, 2016).

Ras plays a central role in cell signaling, and its activity is regulated with more than 170 Ras-related proteins. Monomeric small guanosine triphosphatases (GTPases) belong to

the proteins of a Ras superfamily, and they can hydrolyze active GTP into inactive GDP, which further influences on the Ras activity (Colicelli, 2004). Guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) regulate the activity of GTPases, as GEFs catalyze GDP into GTP and GAPs hydrolyze GTP into GDP (Bos et al., 2007). The osteoclast adhesion to bone is dependent on the activity of the GTPases and, therefore, the signaling of Ras (Itzstein et al., 2011). NF1 tumor-suppressor gene protein neurofibromin down-regulates the activity of Ras. Mutation in the *NF1* gene is known to result in hyperactive osteoclasts (Heervä et al., 2010). A gain-in-function of NF1 osteoclasts leads to increased bone resorption (Heervä et al., 2010; Rauen et al., 2015). Ras has many downstream targets, and the main pathways are Ras/Raf/MEK/ERK (MAPK pathway) and the Ras/PI3K/Akt/mTOR (Chappell et al., 2011). Ras activity is needed in every phase of the cell cycle (Castellano and Downward, 2011). Farnesyl thiosalisylic acid (FTS) is a well known inhibitor of Ras inhibiting all isoforms of it (Laheru et al., 2012). The few available studies related to inhibition of Ras on osteoclastogenesis show that inhibition of Ras decreases the number of mononuclear cells and rodent osteoclast survival (Aizman et al., 2014; Bradley et al., 2008).

MEK/ERK signaling belongs in the cascade of the MAPK family. MEK/ERK signaling can be activated by M-CSF and RANKL to promote osteoclast survival (Bradley et al., 2008; Kim and Kim, 2016). Phosphorylation of ERK1 and ERK2 (also known as p42/p44 MAPK) by MEK increases their enzymatic activity (Zhang and Liu, 2002). MEK1/2 is the only known activator of ERK1/2 (Favata et al., 1998). Therefore, specific MEK inhibitors can be used to understand the basic mechanism behind MEK/ERK signaling in cells. PD98059 and UO126 are inhibitors affecting the activation of MEK1/2 (Favata et al., 1998). The effect of these inhibitors toward rodent osteoclast differentiation is contradictory. However, most studies have shown that inhibition of MEK decreases osteoclast differentiation (Amano et al., 2015; Breitkreutz et al., 2007; Hotokezaka et al., 2002).

In addition to MEK/ERK1/2, osteoclast cell differentiation and function is regulated through another MAPK called p38 MAPK (Thouverey and Caverzasio, 2015). There are four isoforms of p38 MAPK (α, β, γ and δ). It has been shown that osteoclasts and their precursors express at least p38α (Böhm et al., 2009). BIRB796 inhibits all isoforms of p38, whereas, SB203580 inhibits α and β isoforms of p38 MAPK (Kuma et al., 2005; Yong et al., 2009). Studies have reported that inhibition of p38 MAPK decreases the number of rodent osteoclasts (Li et al., 2002; Matsumoto et al., 2000).

The lipid PI3K is involved in cellular growth, proliferation, and survival. It is composed of two different subunits. The first one is the lipid kinase catalyzing subunit p110, and the other one is p85, which regulates the p110 activity (Ponzetto et al., 1993). PI3K phosphorylates lipid phosphatidylinositol-4,5-bisphosphate (PIP2) and generates it into

the lipid second messenger phosphatidylinositol-3,4,5-trisphosphate (PIP3), which can activate several downstream targets (Luo et al., 2003; Miao et al., 2010). In the M-CSF/c-Fms complex, the tyrosine residue of Y-559 activates c-Src that further activates PI3K and Akt pathway (Kim and Kim, 2016) Both Ras and PI3K are needed in the cell cycle to activate the cells from the G0 into the G1 phase (Castellano and Downward, 2011). Osteoclasts originate from hematopoietic cells. Zebrafish studies have shown that Ras/PI3K/Akt signaling is a key regulator of hematopoiesis (Liu et al., 2008). Interestingly, studies with hematopoietic progenitors suggest crosstalk between the PI3K and MAPK pathway. PI3K can activate Raf and further the downstream signaling Raf/MEK/ERK through Ras by an unknown mechanism (Wandzioch et al., 2004). PI3K is known to associate with $\alpha_V\beta_3$ and Src in osteoclasts (Hruska et al., 1995). Inhibition of PI3K inhibits formation of F-actin structure and further osteoclast attachment on bone and plastic, suggesting that PI3K plays a role in osteoclast actin cytoskeleton formation (Lakkakorpi et al., 1997). Inhibition of PI3K with the inhibitor LY294002 decreases osteoclast number in rodent cells (Bradley et al., 2008).

mTOR kinase is a member of PI3K-related kinase family, and it regulates cell growth and metabolism. There are two different mTOR complexes: mTOR1 and mTOR2. Regulatory-associated protein of mTOR1 is called raptor, and it is essential for the activity and regulation of mTOR kinase. The regulatory-associated protein in mTOR2 is called rictor (Laplante and Sabatini, 2009). The mTOR interacts with MAPK and PI3K/Akt pathways, and inhibition of mTOR in patients with metastatic disease activates Ras/MAPK pathway (Carracedo et al., 2008). Inhibition of mTOR with an inhibitor of rapamycin decreased the amount of TRACP-positive rodent osteoclasts and TRACP activity of the cells, suggesting that mTOR1 plays a crucial role in osteoclast differentiation (Dai et al., 2017).

Protein kinase C (PKC) is a lipid-sensitive enzyme that has many roles in cell proliferation, differentiation, and survival in cells (Dekker and Parker, 1994). PKC is activated by growth factor receptors. PKC activates phospholipase C (PLC), which further hydrolyzes PIP2 (Steinberg, 2008). Ca^{2+}-dependent PKC represents a classical group of isoforms of PKC: α, β, and γ isoforms (Kohout et al., 2002). PKC has been linked to osteoclast differentiation and formation. Inhibition of PKCβ decreased the number of TRACP positive mouse osteoclasts and suppressed ERK/MEK activation suggesting a role of PKCβ in osteoclast differentiation and MEK/ERK signaling pathway (Lee et al., 2003). The PKC-specific inhibitor GÖ6976 inhibits isoforms of α and β, and is an effective small molecule drug in cell cultures (Bailey et al., 2014; Koivunen et al., 2004).

3 AIMS OF THE STUDY

1. To investigate the effect of sex hormones on Schwann cells cultured from cutaneous neurofibromas and having one or two hits of the *NF1* gene.

2. To investigate the actin structure of peripheral blood-derived human osteoclasts on glass surfaces with confocal and STED microscopies.

3. To study the human osteoclast differentiation pathways in mononuclear cell progenitors from healthy persons and from patients with NF1.

4 MATERIALS AND METHODS

This chapter summarizes materials and methods used in this study. More detailed descriptions are available in the original publications (I-III).

4.1 Ethical approval, permissions, and samples

This study has been performed in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Southwest Finland Hospital District, Turku, Finland. Participants gave their informed written consents to osteoclasts and Schwann cell cultures. Blood samples from healthy persons were obtained from the personnel of Institute of Biomedicine, University of Turku. Blood samples and fresh neurofibroma tissues were obtained from the Department of Dermatology from Turku University Hospital. The study was carried out at Turku University Hospital and the University of Turku.

4.2 Schwann cell cultures from human neurofibromas (I)

NF1 patient's cutaneous neurofibromas were removed with a CO_2 laser and immersed immediately into RPMI medium (Gibco, Grand Island, NY, Cat. No. 21870-076). Neurofibromas were cut into small pieces and immersed for 3-7 days in preincubation medium shown in Table 1.

Table 1. Preincubation medium of the neurofibromas.

Reagent name	Concentration	Source	Cat. No.
DMEM, high glucose (4.5g/l)	89%	Gibco, Grand Island, NY	41966-029
FBS, USA origin	10%	Gibco, Grand Island, NY	16000-044)
Penicillin-Streptomycin	1% (100 µg/ml)	Gibco, Grand Island, NY	15140-122
Amphoterecin B	1.25 µg/ml	Gibco, Grand Island, NY	15290-018
Forskolin	2 µmol/l	Merck, Darmstadt, Germany	344270

Thereafter, a neurofibroma was dissociated enzymatically with preincubation medium shown in Table 2 and mechanically with pipette tips.

Table 2. Enzymatic dissociation medium of the neurofibromas.

Reagent name	Concentration	Source	Cat. No.
DMEM, high glucose (4.5g/l)	89%	Gibco, Grand Island, NY	41966-029
FBS, USA origin	10%	Gibco, Grand Island, NY	16000-044)
Penicillin-Streptomycin	1% (100 µg/ml)	Gibco, Grand Island, NY	15140-122
Collagenase	160 U/ml	Merck, Darmstadt, Germany	234153
Dispase I	0.8 U/ml	Roche, Indianapolis, Indiana, USA	04942086001

Cell culture plastics were coated for Schwann cell cultures with poly-L-lysine and laminin, which is shown in Table 3.

Table 3. Poly-L-lysine and laminin coating.

Reagent name	Concentration	Source	Cat. No.
Poly-L-lysine	1 mg/ml	Sigma-Aldrich, ST Louis, MO	P-1524
Laminin	1 mg/ml	Gibco, Invitrogen, Grand Island, NY	23017-015

Half of the gained cell suspension from neurofibroma was plated in $NF1^{+/-}$ medium to enrich $NF1^{+/-}$ Schwann cells. The other half was treated for 24 hours with medium containing forskolin ($NF1^{+/-}$ medium, Table 4) followed by serum-free medium (Table 5) for 24 hours, and finally in proliferation medium without forskolin ($NF1^{-/-}$ medium as shown in Table 4 to gain $NF1^{-/-}$ Schwann cells).

Table 4. $NF1^{+/-}$ and $NF1^{-/-}$ Schwann cell proliferation medium.

Reagent name	Concentration	Source	Cat. No.
DMEM, high glucose (4.5g/l)	89%	Gibco, Grand Island, NY	41966-029
FBS, USA origin	10%	Gibco, Grand Island, NY	16000-044)
Penicillin-Streptomycin	1% (100 µg/ml)	Gibco, Grand Island, NY	15140-122
3-isobutyl-1-methyl xanthine (IBMX)	0.5 mM	Sigma-Aldrich, St.Louis, MO	I-5879
Beta 1-heregulin	10 nM	Peprotech, Rocky Hill, NJ	100-03
Insulin	2.5 µg/ml	Sigma-Aldrich, St.Louis, MO	I-0516
Forskolin*	0.5 mM	Merck, Darmstadt, Germany	344270

*$NF1^{-/-}$ Schwann cells were cultured in medium without forskolin with the exception when $NF1^{-/-}$ Schwann cells were looking unhealthy or cells were divided, forskolin was added to the medium for the first day.

Table 5. N2-supplement medium to gain $NF1^{-/-}$ Schwann cells.

Reagent name	Concentration	Source	Cat. No.
DMEM, high glucose (4.5g/l)	73%	Gibco, Grand Island, NY	41966-029
F12-medium	25%	Gibco, Grand Island, NY	11765-054
Penicillin-Streptomycin	1% (100 µg/ml)	Gibco, Grand Island, NY	15140-122
N2-supplement	1%	Gibco, Grand Island, NY	17502-048
IBMX	0.5 mM	Sigma-Aldrich, St. Louis, MO	I-5879

4.3 Incubation of Schwann cells with sex hormones

The effect of sex hormones on human $NF1^{+/-}$ and $NF1^{-/-}$ Schwann cell proliferation was quantified with a 5-bromo-2-deoxyuridine (BrDU) colorimetric enzyme-linked immunosorbent assay (ELISA). 6000 cells per well of passage 5 or greater Schwann cells were plated on coated 96-well plates in proliferation medium without forskolin and in colorless DMEM (Gibco, Cat. No 31053-028). Sex hormones shown in Table 6 were added at day 0 at concentrations of 0.001-100 nM for 48 or 96 hours. Absorbance at 450 nm was measured to determine BrDU corporation of the cells.

Table 6. Reagents and equipment of Schwann cell proliferation study with sex hormones.

Reagent name	**Source**	**Cat. No**
Estradiol	Sigma-Aldrich, St. Louis, MO	E2758
Testosterone	Sigma-Aldrich, St. Louis, MO	86500
hCG	Sigma-Aldrich, St. Louis, MO	C0684
BrDU	Roche Diagnostics, Mannheim, Germany	11647229001
Blocking reagent for BrDU Assay	Roche Diagnostics, Mannheim, Germany	11647229001
Hidex microplate reader, The Plate Chameleon Multilabel Counter, Hidex Oy, Turku, Finland		

4.4 Imaging and immunohistochemistry (I-III)

4.4.1 Microscopy

Table 7. Microscopes used in cell imaging

Microscope	Used in study
Carl Zeiss AxioImager M1 microscope equipped with AxioCam ICc3 camera, Carl Zeiss Zen 2012 (blue edition) software and Image J 1.49 software	I, III
Leica TCS SP5 Confocal microscope equipped with photomultiplier tubes as detectors, LAS AF software and Image J 1.49 software	II, III
Leica TCS SP5 STED equipped with MaiTai HP, LAS AF software and Image J 1.49 software	II

In addition, images were further processed into collages with CorelDraw X7.

4.4.2 Antibodies and fluorescent compounds

Table 8. List of antibodies and fluorescent compounds.

Reagent name	Source	Cat. No	Used in study
Atto-647N Phalloidin	Sigma-Aldrich, St. Louis, MO	65906	II
Alexa-Fluor 488 Phalloidin	Molecular Probes, Eugene, OR	A12379	II
Hoechst 33342	Molecular Probes, Eugene, OR	H3570	II, III
Rabbit mab to human/mouse c-Src	Cell Signaling Technology, Danvers, MA	2109	II
Mouse mab to human/mouse cortactin	Santa Cruz, Biotechnology, Santa Cruz, CA	55579	II
Rabbit pab to human/mouse anti-arp2	Abcam, Cambridge, UK	ab47654	II
Mouse mab to human/mouse cofilin	Santa Cruz, Biotechnology, Santa Cruz, CA	53934	II
Alexa-Fluor 488-conjugated goat anti-mouse IgG	Abcam, Cambridge, UK	ab150113	II
Alexa-Fluor 488-conjugated goat anti-rabbit IgG	Molecular Probes, Eugene, OR	A11008	II
DiI, 1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate	Life Technologies, Carlsbad, CA	V22889	II
Quant-iT™ PicoGreen® dsDNA Reagent	Life Technologies, Carlsbad, CA	P7581	II
Abberior Phalloidin conjugated STAR635	Abberior, Göttingen, Germany	2-0205-002-5	II, III

4.5 Human peripheral blood mononuclear cell differentiation into osteoclasts (II, III)

Briefly, the mononuclear cells were collected with Ficoll-Paque PLUS (GE HealthCare Bio-Sciences, Uppsala, Sweden) centrifugation from fresh blood samples of healthy persons and NF1 patients. Then cells were counted with Bürker-Türk counting chamber, half million cells were seeded on glass coverslips (12 mm round, thickness of 0.17 mm Marienfeld GmbH & Co.kG, Germany) or bone slices on 96-well plate (Purchased from Pharmatest Services Ltd., Turku, Finland) and differentiated to multinuclear osteoclasts in the presence of medium given in Table 9. Half of the medium was replaced with fresh medium every 3-4 days. Cells were cultured on glass coverslips for 8-10 days (II) or 4-5 days (III) and on bone slices for 16 days (III).

Table 9. Medium composition for human osteoclasts differentiation.

Reagent name	Concentration	Source	Cat. No.
AlphaMEM	89%	Gibco, Grand Island, NY	41061-029
Inactivated Fetal bovine serum, USA origin, iFBS	10%	Gibco, Grand Island, NY	16000-044)
Penicillin-Streptomycin	1% (100 µg/ml)	Gibco, Grand Island, NY	15140-122
Recombinant Human sRANK Ligand, RANKL	20 ng/ml	Peprotech, Rocky Hill, NJ	310-01
Macrophage Colony Stimulating Factor, M-CSF	10 ng/ml	Peprotech, Rocky Hill, NJ	300-25

4.6 Human peripheral blood mononuclear cell differentiation into macrophages (II)

To prepare macrophage culture, the mononuclear cells were collected with Ficoll-Paque PLUS centrifugation from fresh blood samples of healthy persons. Cells were counted and half million cells were seeded on glass coverslips in a serum-free medium, allowed to settle for two hours, and then washed to remove non-adherent cells. Thereafter, cells were cultured for 7 days with macrophage medium shown in Table 10.

Table 10. Macrophage culture medium.

Reagent name	Concentration	Source	Cat. No.
AlphaMEM	89%	Gibco, Grand Island, NY	41061-029
Inactivated Fetal bovine serum, USA origin, iFBS	10%	Gibco, Grand Island, NY	16000-044)
Penicillin-Streptomycin	1% (100 µg/ml)	Gibco, Grand Island, NY	15140-122
Macrophage Colony Stimulating Factor, M-CSF	10 ng/ml	Peprotech, Rocky Hill, NJ	300-25

4.7 Inhibitors used to study osteoclast differentiation pathways (III)

Table 11. Summary of inhibitors. (Modified from the original publication III.)

Inhibitor	Source	Target of the inhibitor	Concentration	Cat. No.
Vehicle +DMSO	-	Control	0.05%	-
FTS	Cayman chemical company, Ann Arbor, MI, USA	All isoforms of Ras	10 µM	1001051
GÖ6976	Calbiochem, San Diego, CA, USA	PKC α, β	0.01 µM	365250
U0126	Cell Signaling, Danvers, MA, USA	MEK	0.1 µM	9903S
PD98059	Calbiochem, San Diego, CA, USA	MEK	1 µM	513000
LY294002	Cell Signaling, Danvers, MA, USA	PI3K α, δ, β	1 µM	9901S
Rapamycin	Cell Signaling, Danvers, MA, USA	mTOR	0.001 µM	9904S
BIRB796	Axon, Medchem BV, Croningen, the Netherlands	p38 α, β, γ, δ	10 µM	1358
SB203580	Calbiochem, San Diego, CA, USA	P38 α, β	1 µM	559389

4.8 Other used methods (I-III)

Table 12 summarizes other methods used in the original publications, where they have been described in detail.

Table 12. The methods used in the original publications (I-III).

Method	Used in study
Specific TRACP protein staining	II, III
Immunohistochemical labeling	I-III
Western blotting	III
Carboxy-terminal collagen crosslinks (CTX) measurement	III
Image analysis	I-III

4.9 Statistical analysis

Table 13. The statistical methods used in the original publications (I-III).

Statistical method	Used in study
Linear-mixed effects regression with random intercepts	I, III
Statistical software R version 3.3.2 with ImerTest package version 2.0-33	I
Statistical software R version 3.3.0 with ImerTest package version 2.0-32 and Ime4 (Version 1.1-12)	III
Linear mixed effects models with Poisson distribution with nested random intercepts	III

5 RESULTS AND DISCUSSION

5.1 Culturing of NF1$^{+/-}$ and NF1$^{-/-}$ Schwann cells from human neurofibroma

The method for culturing NF1$^{+/-}$ and NF1$^{-/-}$ Schwann cells from human neurofibromas was established by Serra et al. (Serra et al., 2000). Our results showed that relatively small (Ø 5-15 mm) neurofibromas were optimal sources for cultures when considering the growth potential of Schwann cells. These cells were cultured in DMEM containing phenol red, knowing that phenol red is similar to estrogen and binds ERs of the cells (Berthois et al., 1986; Welshons et al., 1988). When NF1$^{+/-}$ and NF1$^{-/-}$ Schwann cells were cultured in colorless DMEM, the cells proliferated slower, and the viability of the Schwann cells decreased. Thereafter, the Schwann cells were decided to let proliferate in red DMEM, but the hormone studies were carried out in colorless DMEM. Our results showed that culturing Schwann cells from neurofibromas is a very delicate process. Culturing the cells without serum, which always contains small amounts of hormones, was not feasible because the cells did not survive. The used culturing protocol for Schwann cell cultures from human neurofibromas separated two genetically different Schwann cells populations, which were even shown by examining the morphology of the cells. *NF1*$^{+/-}$ Schwann cells were dense, formed linear bundles, and swirls, while *NF1*$^{-/-}$ Schwann cells were homogenous and were a typical spindle-shaped cell.

The crucial difference between *NF1*$^{+/-}$ and *NF1*$^{-/-}$ Schwann cell cultures was the presence or absence of forskolin in the proliferation medium. Forskolin was used continuously in *NF1*$^{+/-}$ Schwann cell cultures. However, *NF1*$^{-/-}$ Schwann cells needed forskolin momentarily to stimulate the cell proliferation after passaging the cells by trypsinization or when cells looked non-viable. It seems that neurofibromin reduces cAMP and cyclin D1 in Schwann cells (Kim et al., 2001), and in turn, Ras-deficient *NF1*$^{-/-}$ Schwann cells have increased levels of cAMP and cyclin D1, which it maintains by itself with an unknown mechanism and without the continuous need of forskolin.

Schwann cell cultures, especially *NF1*$^{+/-}$ cultures, contain a lot of fibroblasts. To achieve pure Schwann cell cultures free of fibroblasts is possible by using different growth factors and passaging the cells repeatedly. It is known that fibroblast proliferation is inhibited by cAMP. Thus, IBMX together with forskolin has become a useful tool to inhibit the growth of fibroblasts (Espinoza and Wharton, 1986; Roberts et al., 2018).

5.2 The effect of estradiol on the proliferation of *NF1*$^{+/-}$ and *NF1*$^{-/-}$ Schwann cells

NF1 is a hormone-dependent disorder. Cutaneous neurofibromas arise during puberty and increase in number and size during pregnancy (Dugoff and Sujansky, 1996; Xiong et al., 2015). Estradiol is needed for pubertal growth. Before puberty begins, estradiol levels are low. The release of GnRH from GnRH neurons in pulses leads to activation of the pituitary ovarian axis and secretion of LH and FSH hormones and further to estrogen stimulation in ovaries, which initiates puberty in girls around the age of 8 years old (Herman-Giddens et al., 1997; Ojeda et al., 2010). Estrogen concentrations can vary widely during the menstrual cycle (Veldhuijzen et al., 2013). The levels of estradiol increase during early pregnancy and continue to play an important role in maintaining the pregnancy (Albrecht and Pepe, 2010). Since the estrogen levels and neurofibromas increase during the timespan of puberty and pregnancy, it leads to a hypothesis that estradiol influence on neurofibroma tumor formation. Since neurofibromas consist mainly of Schwann cells, we characterized the effect of estradiol on the proliferation of *NF1*$^{+/-}$ and *NF1*$^{-/-}$ Schwann cells.

The results showed that genotype influences the response of Schwann cells to steroid hormones. The effect of estradiol on the proliferation of human *NF1*$^{+/-}$ and *NF1*$^{-/-}$ Schwann cells after two days with concentrations of 0.001-10nM had no consistent effect compared to the vehicle control. However, estradiol increased significantly, to 86-99% *NF1*$^{-/-}$ Schwann cell proliferation on day four at concentrations 0.001-10 nM. Even the exclusion of one outlier yielded a significant increase in *NF1*$^{-/-}$ Schwann cell proliferation up to 40-56%.

New treatments are needed to diminish the tumor burden of NF1 patients. The conventional treatments include surgical or laser removal of the tumor. A naturally occurring metabolite, 2-methoxyestradiol (2ME2), is found at low levels in human plasma and at a ten times higher levels during pregnancy (Berg and Kuss, 1992). An analogue of 2ME2, STX3451 [(2-(3-bromo-4,5-dimethoxybenzyl)-7-methoxy-6-sulfamoyloxy-1,2,3,4-tetrahydroisoquinoline)] was found to induce apoptosis in MPNST cell lines, in cells derived from benign plexiform neurofibromas, and other tumor-deriver NF1 cell lines, and it was shown to disrupt actin- and microtubule-based cytoskeletal structures and to inhibit PI3 and mTOR signaling pathways (Shen et al., 2015). Treatment with 2ME2 reduced even hormone-responsive cancer tumor growth (Qadan et al., 2001). This treatment could be speculated to help to decrease neurofibroma growth in a topical formula.

5.3 The effect of testosterone on the proliferation of *NF1*$^{+/-}$ and *NF1*$^{-/-}$ Schwann cells

Neurofibroma-derived Schwann cells, whose both NF1 alleles were mutated, responded to testosterone by increased proliferation. Our results elucidate the proliferation mechanism of *NF1*$^{-/-}$ Schwann cells and may pave the way for future therapeutic approaches. Specifically, the effect of testosterone on the proliferation of human *NF1*$^{+/-}$ Schwann cells after two or four days of treatment with concentrations 0.001-10 nm had no consistent effect. However, testosterone increased *NF1*$^{-/-}$ Schwann cell proliferation after two days by 15 – 22%. In addition, after four days of treatment, the proliferation of *NF1*$^{-/-}$ Schwann cell increased up to 110%. There was some variation on day four results, which showed one cell line reacting more strongly to hCG compared to the other cell lines. Yet, exclusion of it on this cell line yielded a significant increase in *NF1*$^{-/-}$ cell proliferation, which was still up to 49-60% compared to vehicle control.

The neck, head, back, shoulders, chest, abdomen, limbs, and face are common locations of benign neurofibromas (Bongiorno et al., 2010; Cannon et al., 2018). It has been shown that the number and size of neurofibromas increased especially in the back and abdominal area in an 8-year follow-up study (Cannon et al., 2018). Puberty- and sex hormone-related diseases, such as acne, locate to the same areas of skin. Even if acne and neurofibromas are two totally different skin-related diseases, androgens play an important role in both of them. Hormones stimulate the sebaceous glands of the hair follicles, which are androgen target tissues, and this further increases the production of the sebum leading to acne (Lai et al., 2012). The circulating testosterone, DHEA, and androstenedione can be converted into testosterone, which is catalyzed further into DHT with 5α-reductase in skin sebocytes, excretory duct cells of sweat glands, dermal papilla cells, endothelial cells of small vessels, and Schwann cells of cutaneous myelinated nerves (Asada et al., 2001; Eicheler et al., 1995; Luu-The et al., 1994; Zouboulis et al., 2007). The mechanism between AR and acne is still unclear, and it has been suggested that AR enhances growth factors of certain cells. The origin of neurofibromas from hair follicles (Jouhilahti et al., 2011a) and skin poses the question that the growth of neurofibromas could be inhibited by preventing the action of androgens in hair follicles, because it has been shown that inhibiting AR from follicles at least decreases sebocyte activity (Lai et al., 2012). Estrogen-containing oral contraceptives decrease the levels of free testosterone by increasing the hormone binding globulin that has a positive effect in the treatment of acne disease in women (Trivedi et al., 2017). A study related to contraceptives and NF1 disease has indicated that estrogen-containing oral contraceptive has no influence on the size of neurofibromas (Lammert et al., 2005b). It can be speculated that this is also due to the ability of estrogen-containing contraceptives to increase the hormone-binding globulin, and that decrease the amount of free testosterone further.

NF1-related neurofibromas increase in size and amount during puberty, and this might be explained by the fact that before puberty, the testosterone levels are low and at the beginning of puberty, GnRH stimulates LH and FSH in the pituitary to produce testosterone in testis, and the level of testosterone continues to grow until the age of 40 (Kelsey et al., 2014). In addition, aromatase enzyme can convert testosterone into estradiol in different tissues and cells, such as neuronal cell bodies and presynaptic terminals, astrocytes, and adipose tissue (Balthazart and Ball, 2006; Garcia-Segura et al., 1999; Lee et al., 2013).

5.4 The effect of human chorionic gonadotropin (hCG) on the proliferation of *NF1*$^{+/-}$ and *NF1*$^{-/-}$ Schwann cells

The size and amount of neurofibromas has been reported to increase during pregnancy (Dugoff and Sujansky, 1996; Xiong et al., 2015). The level of different sex hormones changes during pregnancy, but the pregnancy hormone called hCG has not been studied in this context. hCG is secreted from the placenta syncytiotrophoblast cell layer and is responsible for embryo implantation through maintenance of the corpus luteum and its progesterone stimulation, fetal growth, angiogenesis and continuation of the pregnancy (Cole, 2010; Strott et al., 1969). The level of hCG reaches its peak during the first trimester.

Our results revealed that two- and four-day treatment of *NF1*$^{+/-}$ Schwann cells with hCG did not have an effect on the proliferation of the cells. Interestingly, hCG increased the proliferation of *NF1*$^{-/-}$Schwann cells already after two days by 29 - 51%. On day four, the highest concentration of hCG, 100 nm, had no such effect on the cell proliferation. However, hCG concentrations of 0.001-10 nM increased significantly the proliferation of *NF1*$^{-/-}$ Schwann cells up to 170%.

This is the first study to address the influence of hCG on Schwann cells, and more specifically on *NF1*$^{+/-}$ and *NF1*$^{-/-}$ Schwann cells cultured from neurofibromas of NF1 patients. The influence of hCG should be studied also in normal Schwann cells. There are many studies on hCG concentrations during normal pregnancy. It would be interesting to study the hCG concentrations during a NF1 patient's pregnancy and determine whether there is any difference compared to normal pregnancy.

5.5 Complexity of Schwann cell development in NF1 neurofibroma formation

A complete loss of neurofibromin leads to uncontrollable Ras signaling and further to vulnerable responses to different growth factors, such as sex hormones. This effect was

seen in increased proliferation of *NF1*$^{-/-}$ Schwann cells, but also in unresponsiveness of *NF1*$^{+/-}$ Schwann cells towards the tested sex hormones. Estradiol, testosterone, and hCG are known to interact with the Ras pathway (Driggers and Segars, 2002; Fan et al., 2008; Liao et al., 2013), but also through several different signaling pathways, such as Ras/Raf/MEKK/ERK, p38 MAPK, PI3K/Akt, PKC, and cAMP (Basualto-Alarcón et al., 2013; Choi and Smitz, 2014; Liu et al., 2006; Marino et al., 2006; Pike et al., 2008; Walker, 2011). Neurofibromin plays a role in these pathways (Helfferich et al., 2016), which raises the possibility whether we could decrease *NF1*$^{+/-}$ and *NF1*$^{-/-}$ Schwann cell proliferation with different inhibitors as a purpose to find therapeutic targets to decrease the amount and size of neurofibromas. The Ras pathway and downstream signals of Ras have been studied in a variety of human tumors. Especially, the Ras/Raf/MEK/ERK pathway is involved in cell proliferation and further in the development of tumors into MPNSTs (Farid et al., 2014; McCubrey et al., 2007). Investigating the Ras-deficient pathways may produce critical information, but also lead to knowledge how to inhibit of hyperactive cells and different lesions of the NF1 disease. Promising results have been already gained with a MEK inhibitor, which inhibited the growth of the tumors, reduced the number of blood vessels, and the volume of tumors (Dombi et al., 2016; Jessen et al., 2013).

It is also important to understand the overlapping pathophysiology of sex hormones and other NF1-related tumors based on similarity of the cell composition, e.g., Schwann cells of the tumors. It has been shown that MPNST responded to the estrogen antagonist tamoxifen treatment in an ER-independent manner (Byer et al., 2011). This raises the possibility that a hormone antagonist treatment could decrease the size of neurofibromas. After all, NF1 is a hormone-related disease.

The amount of neurofibromas and the severity of the disease vary even among the members of the same family. Together with other major life transitions of puberty, manifestation of the neurofibromas make early puberty a difficult time. Stress exposure in puberty may cause responses to gonadal hormones and modify ER density especially in brain areas (Holder and Blaustein, 2014). Emotional and physiological responses go hand-in-hand. This should be considered as a very significant aspect in this disease, and luckily there are studies that highlight the importance QoL in patients with NF1 (Wolkenstein et al., 2001).

5.6 Curved and branched actin architecture in human osteoclasts

The function of osteoclasts culminates in the dynamic structure of actin. The familiar osteoclast actin structure podosome belt consists of F-actin cores called podosomes and loose F-actin cloud instantly on top of the podosomes (Saltel et al., 2008). The actin structure is different on glass or on bone. This is because osteoclast forms a bone-

anchored adhesion structure called the SZ between bone and osteoclast to enable bone resorption (Lakkakorpi and Väänänen, 1991; Takito et al., 2018). However, osteoclasts have also been studied also on glass, which enables a deeper insight to the inside of the cell. In the present study, we used confocal and STED microscopy to investigate the osteoclast actin structure on glass. The STED microscopy images revealed that osteoclast actin was curved and branched from bottom to top of the cell instantly in the vicinity of membrane ruffles in the cell periphery. The comprehensive curved and branched actin structure may allow the cell to remodel itself rapidly in response to cell migration and fusion. STED imaging results in super-resolution images that have bypassed other light microscopic imaging techniques. Because of this, we find it tempting to speculate that the actin cloud was first named as a cloud instead of seeing the whole actin structure from the bottom to the top of the cell.

5.7 Macrophages and keratinocytes branched actin structure

The findings in osteoclasts of curved and branched actin structures raised the question whether this kind of actin structure is specific to osteoclasts. To study other cell types, we differentiated macrophages from human peripheral blood and cultured keratinocytes from human skin samples. Both of these cell types showed curved and branched actin structure. The cytoskeleton plays several roles in the cells by organizing cell contents, connecting the cell to external environment and allows cell movement and changes in cell shape (Fletcher and Mullins, 2010). The cell cytoskeleton is studied usually *in vitro*, because it is very difficult to study in living organisms and tissues. Confocal and STED microscopy enable a more precise study of the cell. Thus, they are important techniques to expand our knowledge about the cytoskeleton and understanding cellular behavior.

5.8 The expression of c-Src, cortactin, cofilin, and ARP2/3 in osteoclast actin structure

The osteoclast actin cytoskeleton undergoes dynamic changes during cell migration, movement, fusion, and resorption (Teitelbaum, 2011). We studied the actin related proteins, which modify the structure of actin. c-Src rearranges and remodels actin filaments, cortactin acts with c-Src and promotes actin polymerization, cofilin severs actin filaments, and ARP2/3 branches actin (Daly, 2004; Destaing et al., 2008; Pavlov et al., 2007; Smith et al., 2013). Our results were in accordance with previous studies on the function of the actin-related proteins. Double labeling for actin and c-Src showed that they colocalized in the periphery of the cell, and it gathered around the nuclei. Actin and cortactin colocalized also in the cell periphery and around the nuclei. Cofilin and actin did not colocalize, but cofilin was found in the middle of the cell and nearby

nuclei. We suggest that the location of c-Src and cortactin enables changes in cell actin structure, when the cell forms its shape and contacts with other cells. ARP2/3 appeared abundantly in the substratum level of the osteoclast and in the branching points of the actin network. We suggest that ARP2/3 is needed, especially when the osteoclast detaches from the substratum and during cell movement. In addition, the abundant occurrence of c-Src, cortactin and cofilin in the vicinity of nuclei may suggest that they are involved in the movement of nuclei together with actin (Dupin et al., 2011; Gundersen and Worman, 2013). It would be interesting to study osteoclast nuclei positioning and movement in live cell imaging with osteoclasts, which are multinucleated cells and fuse with each other.

5.9 Micrometer level actin-rich tubes between two osteoclasts

Tunneling nanotubes (TNTs) are actin-rich structures, which transport small molecules, proteins, vesicles, and different cellular components from one cell to another through a nanometer-level intracellular tunnel (Dupont et al., 2018). Our findings revealed actin-rich micrometer level tubes (MLTs), which bridged between two osteoclasts above the substratum level and were enclosed with membranes of the connected cells. Interestingly, nuclei were seen in the vicinity of the MLTs, and they were surrounded by curved and branching actin. Compared to several TNTs per cell, only one MLT was seen at a time. The size of the MLTs was about 1–5 µm and the length was up to 40 µm, which is greater than the usual size 50-200 nm size of TNTs (Gerdes et al., 2013). Because TNTs transport small structures of the cell, we speculated that MLTs may transport bigger structures, such as nuclei.

5.10 Nucleus inside of the micrometer tubes

The larger size of MLTs compared to TNTs raised the question about the function of these tubes. Expectedly, we observed nuclei inside of these MLTs in stained cell images. Nuclei were elongated inside the tubes and round inside the osteoclasts. The nuclei seen inside of the curved and branched actin nest in the vicinity of MLTs suggest the possibility that they transport nuclei. We speculate that these MLTs are used in cell fusion and communication among neighboring cells.

5.11 Signaling pathway studies of osteoclast differentiation

Studying of signaling pathways is complicated because of the multiple crosstalk with other signaling pathways. However, it helps us to understand how we could control

cellular behavior. Osteoclasts are responsible for bone resorption and increased bone resorption may lead to osteoporosis (Kling et al., 2014). Most studies related to osteoclast signaling pathways have been carried out using cultured rodent osteoclasts. Understanding the differences between rodent and human studies allows us to carry out more accurate studies. Mononuclear cell progenitors from human peripheral blood were cultured on glass and bovine bone slices in the presence of RANKL and M-CSF to differentiate osteoclasts in order to understand the role of Ras/Raf/MEK/ERK, PKC, PI3K/Akt/mTOR, and p38 signaling pathways in osteoclast differentiation (Figure 8). Furthermore, abnormalities in signaling caused by the NF1 mutations were elucidated by comparing osteoclasts of control persons and NF1 patients under the influence of certain signaling pathway inhibitors. The purpose of this study was to obtain detailed information and a framework, which we can use to study cellular function and disease mechanisms. Our results showed that viable osteoclasts were seen in all of the tested conditions, suggesting low levels of toxicity. Inhibition of the most signaling pathways affected the differentiation of osteoclasts. The specific results about each pathway are explained below.

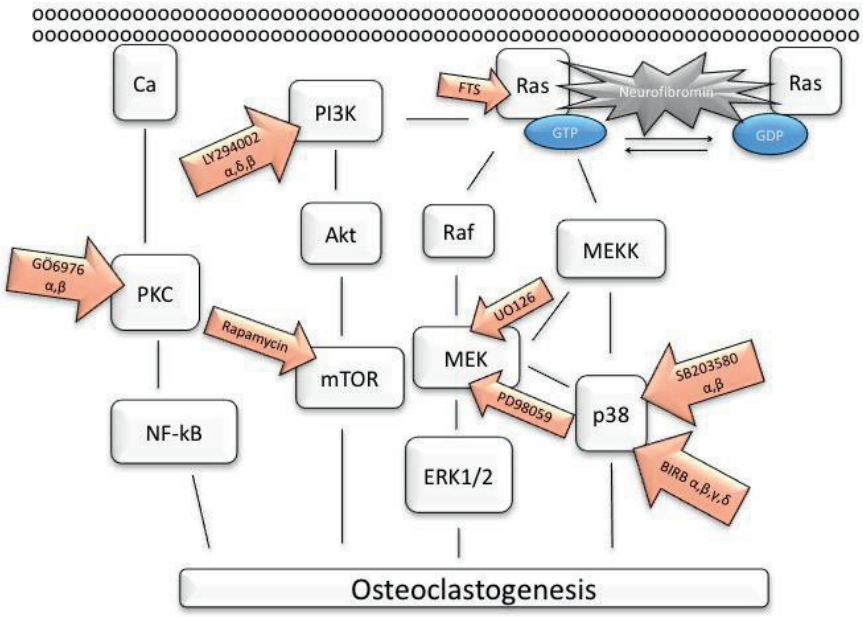

Figure 8. The studied pathways and inhibitors of osteoclastogenesis. (Modified from the original publication III.)

5.12 The effects of inhibitors of PKC, MEK, PI3K, and mTOR on osteoclast differentiation

Osteoclast differentiation is mediated through many signaling pathways (Figure 7) (Kim and Kim, 2016). Previous studies on murine osteoclasts have shown that inhibition of MEK affects ERK1/2 phosphorylation (Chappell et al., 2011) and decreases osteoclastogenesis (Amano et al., 2015; Breitkreutz et al., 2007). The inhibition of PKC decreased the number of osteoclasts and phosphorylation of ERK1/2 in murine cells (Shin et al., 2014). Parallel results have been gained from rodent studies, where PI3K was inhibited, and the number of osteoclasts decreased (Moon et al., 2012; Xing et al., 2016). In addition, studies using rodent osteoclasts showed that the inhibition of mTOR decreased osteoclast differentiation (Dai et al., 2017; Zhang et al., 2017). To evaluate the role of the same signaling pathways in human osteoclasts, we examined the effects of inhibition of PKC (GÖ6976), MEK (PD98059 or U0126), PI3K (LY294002), and mTOR (Rapamycin) on osteoclast differentiation of control persons and NF1 osteoclasts. Therefore, we differentiated osteoclasts on glass for 4-5 days. Visual inspection and counting of the control cells revealed that the number of osteoclasts decreased, when PKC and MEK pathways were inhibited. However, inhibition of PI3K and mTOR did not significantly affect the number of osteoclasts. The inhibition of PKC, MEK, PI3K, and mTOR in Ras-deficient cells of NF1 patients decreased significantly the number of osteoclasts and induced a distinct decrease in phosphorylation of ERK1/2 with all of the used inhibitors.

The Ras/Raf/MEK/ERK pathway is strongly related with the NF1 disease, because loss of the neurofibromin leads to up-regulation of the Ras pathway (Boyd et al., 2009). Therefore, we speculate that the inhibition of PKC, MEK, PI3K, and mTOR in NF1-deficient cells functions as a feedback system through hyperactive Ras, and manifests as decreased phosphorylation of ERK1/2 occurs.

To investigate whether inhibition of PKC, MEK, PI3K, and mTOR has an effect on the phosphorylation of ERK1/2, Akt, or p38 MAPK, western blot studies were carried out. These results showed that inhibition of MEK, PI3K, and mTOR had no effect on the phosphorylation of ERK1/2 or p38 MAPK despite the decreased number of healthy osteoclasts. However, inhibition of MEK and mTOR increased the phosphorylation of Akt in cells from control persons. It can be speculated that increased phosphorylation of Akt is due to interaction of Ras/PI3K/Akt and Ras/Raf/MEK/ERK1/2 pathways and also the ability of mTOR to regulate the activity of Akt (McCubrey et al., 2012). The combination of inhibitors of mTOR and MEK decreased the number and phosphorylation of Akt of control osteoclasts even more. We suggest that the inhibition of MEK activates the Akt/mTOR pathway during healthy osteoclast differentiation. This phenomenon has also been seen in a hematopoietic IL-3/GM-CSF-dependent murine myeloid FDC-P1 cell line (Shelton et al., 2003).

Inhibition of PKC decreased the number of control cells and had no effect on the phosphorylation of ERK1/2, Akt, or p38, which led us to speculate about an independent role of PKC in healthy osteoclast differentiation.

Monitoring the morphology of the cells is essential for successful cell culture experiments to learn about the actual morphology of the cells, and, moreover, to confirm the well-being of the cells. Monitoring of the inhibitor-treated cells revealed viable and healthy-looking cells in all cases. Unexpectedly, actin staining revealed that treatment of the cells with inhibitors prevented the actin ring formation. We draw the conclusion that inhibitors were not toxic to the cells, but the genuine mission of osteoclasts, which is bone resorption, is infeasible. Previous studies related to osteoclast signaling and inhibition of PKC, MEK, PI3K, and mTOR have been performed using rodent osteoclasts. Our data shows a similar regulation in human osteoclasts.

5.13 The effects of inhibition of p38 MAPK on osteoclast differentiation

A following subproject of this thesis was to examine the effects of inhibition of p38 MAPK with inhibitors of SB203580 and BIRB796 on control and NF1 osteoclast differentiation. p38 MAPK signaling plays an important role in osteoclast formation and maturation (Cong et al., 2017). RANKL attachment into RANK interacts with an adapter protein TRAF6, which phosphorylates p38 MAPK in osteoclasts precursors (Kim and Kim, 2016). It has been shown that inhibition of p38 MAPK blocks osteoclast differentiation (Choi et al., 2013; Li et al., 2002; Matsumoto et al., 2000) while its activation increases survival of osteoclasts (Yamashita et al., 2008). However, these experiments have been carried out with rodent cells or *in vivo* experiments with mice. We were interested in comparing monocytes isolated from peripheral blood of control persons and NF1 patients, once the cells were differentiated into osteoclasts and p38 MAPK signaling of differentiated cells was interfered by chemical inhibitors.

The number of osteoclasts increased significantly when control and NF1 cells were treated with p38 MAPK inhibitors. Visual inspection of the cells revealed that p38 MAPK treatment produced osteoclasts, which were larger in size compared to the cells without treatment. When osteoclast differentiation was delayed apparently because of donor variability, only precursor cells were observed in the vehicle wells. However, p38 MAPK inhibition promoted osteoclast differentiation in these cases, and led to the development of mature osteoclasts.

Firm actin ring formation was revealed with actin phalloidin staining and it was still visible when cells were inhibited with combination of p38 MAPK and PKC, MEK or mTOR inhibitors. The combined inhibitor treatments kept osteoclast numbers at the

vehicle level. Western blot results confirmed that inhibition of p38 MAPK decreased its phosphorylation. In addition, a modest decrease in Akt phosphorylation was observed in both healthy and NF1 cells. Interestingly, p38 MAPK inhibition increased ERK1/2 phosphorylation in control, but not in NF1 cells.

After we showed that inhibition of p38 MAPK increased the number of osteoclasts and did not have an effect on actin ring formation in healthy and NF1 cells, we studied the function of osteoclasts on bone. The cells were cultured on bone slices for 16 days. The osteoclast activity was measured from cell culture media with a CTX assay, which showed that the inhibition of p38 MAPK blocked osteoclast activity in control and NF1 osteoclasts. Our results showed that inhibition of p38 MAPK regulates osteoclast differentiation, but not the activity of the cells.

Because there was no difference in the effects of the p38 MAPK inhibitors SB203580 and BIRB796 and that the α isoform of p38 MAPK regulates murine osteoclasts (Böhm et al., 2009), it led us to speculate that human osteoclasts express p38 MAPK isoforms α and/or β. Western blot results confirmed that inhibition of p38 MAPK decreased the phosphorylation of p38 MAPK. In addition, there was a decrease in the phosphorylation of Akt in control and NF1 cells. However, we noticed a strong interaction between inhibition of p38 MAPK and phosphorylation of ERK1/2, which was increased in control but not in NF1 patient cells. It seemed that an increased cell number correlated with an increased phosphorylation of ERK1/2. Based on these results, we suggest that inhibition of p38 MAPK and its decreased phosphorylation activated the phosphorylation of ERK1/2 in control cells. This result led us speculate that p38 MAPK interacts strongly with ERK1/2. Both p38 MAPK and ERK1/2 are responsible for osteoclast differentiation (He et al., 2011; Li et al., 2002). It seems that these two signaling pathways interact together to rescue the cell. When the other signaling pathway is blocked, the second one activates. Because the inhibitors were constantly in the culture, not even the increased phosphorylation of ERK1/2 could save the function of the osteoclasts. Our results revealed that p38 MAPK is an important signaling pathway in osteoclast differentiation. Another major finding of this study was that the osteoclast number correlated with the phosphorylation of ERK1/2. These observations lead us to speculate that ERK1/2 is a key driver of human osteoclast differentiation.

5.14 The effects of inhibition of Ras on osteoclast differentiation

Ras proteins belong to the small GTPases, which are the key regulators of different cellular events. NF1 protein neurofibromin stimulates the conversion of active Ras-GTP into inactive Ras-GDP (Kiuru and Busam, 2017). Many signaling pathways mediate through Ras, which is often upregulated in human cancer (Campbell and Der, 2004). A study using mouse osteoclasts showed that inhibition of Ras decreased the activation of

ERK, PI3K, and osteoclast survival (Bradley et al., 2008). There are very few studies about the inhibition of Ras and osteoclast differentiation. However, there are several studies of Ras downstream signals. Therefore, we studied Ras inhibition on osteoclast differentiation of control persons and NF1 patient cells, where the Ras pathway is up-regulated. In this experiment, we observed that healthy cells behaved differently compared to Ras-deficient cells of NF1. The number and phosphorylation of ERK increased in control osteoclasts and decreased in NF1 cells, when cells were treated with a Ras inhibitor FTS. This difference may be due to the *NF1*-deficient and hyperactive Ras signaling, where the limit of the activity has already been reached because of *NF1*-deficiency. Furthermore, the inhibition of Ras increased the phosphorylation of Akt in both cell lines.

This study demonstrates that Ras is an important regulator of osteoclast differentiation. Inhibition of Ras affects downstream signals of Ras/Raf/MEK/ERK1/2 and Ras/PI3K/Akt but not of p38 MAPK. This let us to speculate that there is no straight connection from Ras to p38 MAPK signaling, but it functions through MEK/ERK1/2. Again we noticed a connection between the number of osteoclasts and the phosphorylation of ERK1/2 as an increased number of osteoclasts increased the phosphorylation of ERK1/2, and it was totally opposite in NF1 cells compared to vehicle. Inhibition of Ras decreased the number of NF1 cells and the phosphorylation of ERK1/2. In addition, we found that signaling in NF1 osteoclasts differed from healthy cells. The *NF1* gene plays a role in osteoclast signaling and differentiation. Hyperactive Ras can lead to excess cell growth and hyperactive cells. However, our results showed that the role of ERK1/2 increases, when Ras is regulated with chemical inhibitors. There are direct and indirect ways to target Ras, where cross-talk and feedback loops challenge the shutting down of the Ras signaling (Simanshu et al., 2017). One way to influence Ras-related diseases is to pass the Ras and influence Ras downstream signals. There are promising results from a phase-1 study using an inhibitor of MAPK kinase (MEK1/2) called selumetinib in children with NF1, where symptoms caused by hyperactive Ras were improved because of the treatment (Dombi et al., 2016). If we do not find a way to affect the main cause of the disease, we can try to find a cure for the symptoms. This lets us to speculate that inhibition of ERK1/2 can decrease the amount of hyperactive osteoclasts in NF1 patients.

6 CONCLUSIONS

The aim of this study was to clarify the effects of sex hormones on the proliferation of *NF1*$^{+/-}$ and *NF1*$^{-/-}$ Schwann cells. We also studied the role of the *NF1* gene in the signaling and differentiation of osteoclast. In addition, the actin structure of osteoclast was of interest. The following conclusions were made:

- The proliferation of *NF1*$^{-/-}$ Schwann cells increased by up to 99-170% in response to estradiol, testosterone or hCG treatment, while there was no consistent effect on the *NF1*$^{+/-}$ genotype cells. These results highlight the significance of sex hormones in the regulation of neurofibroma growth. This is also the first study to point out the effect of hCG on the proliferation of Schwann cells *in vitro*.

- Curved and branched actin located under the membrane of the osteoclast extending from podosomes at the substratum level to the top of the cell. It was also seen inside of macrophages and keratinocytes.

- Actin plays a role in grouping and perhaps transportation of nuclei from one osteoclast to another. Nuclei were observed inside of micrometer level tubes, MLTs, which bridged between two osteoclasts above the substratum level.

- Of the signaling pathways explored in this study: Ras/PI3K/Akt/mTOR, Ras/Raf/MEK1/2/ERK1/2, calcium-PKC, and p38 MAPK affected the differentiation of human osteoclasts.

- Inhibition of PKC, MEK, PI3K, and mTOR decreased the number of human osteoclasts. Inhibition of PKC, MEK, PI3K, and mTOR decreased the phosphorylation of ERK in NF1 cells.

- The inhibition of p38 MAPK promoted differentiation but not the activity of human osteoclasts in samples derived from control persons and NF1 patients. Inhibition of p38 MAPK increased the phosphorylation of ERK in control osteoclasts.

- Inhibition of Ras increased the number of healthy osteoclasts and the phosphorylation of ERK, whereas inhibition of Ras decreased the number of NF1 osteoclasts and decreased the phosphorylation of ERK.

- The role of p38 MAPK and ERK1/2 phosphorylation as key drivers of osteoclast differentiation was assessed. In addition, the number of osteoclasts appeared to correlate with the phosphorylation of ERK1/2.

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