Evaluating the effect of Dovitinib, a potent non-selective FGFR inhibitor, on oligodendrocytes *in vitro*

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1. Introduction

1.1. Multiple sclerosis

Multiple sclerosis (MS; encephalomyelitis disseminata) is a chronic inflammatory disease of the central nervous system (CNS). Affecting more than 2 million people worldwide (Thompson, Baranzini, Geurts, Hemmer & Ciccarelli, 2018) it represents one of the most common causes for neurological disabilities especially in young patients (Browne P et al., 2014). In 2015 around 240.000 patients were treated for multiple sclerosis in Germany (Holstiege, Steffen, Goffrier, Bätzing, 2017). Furthermore, recent studies indicated a rising prevalence of MS around the globe also indicating a much larger estimated number of unknown cases owing to limited access to medical diagnostics such as MRI (Thompson et al., 2018).

First signs of the diseases pathology were depicted in 1838 by British pathologist Robert Carswell as “a remarkable lesion of the spinal cord accompanied with atrophy” (Carswell, 1838). It however took until the late 19th century that it was recognized as independent disease by French neurologist Jean-Martin Charcot (Hacke, 2016). Initial diagnosis is usually made at an age between 20 and 40 years affecting more female than male patients (ratio f:m 2-3:1) (Compston & Coles, 2008). Distribution is not equal around the globe showing a higher incidence in northern parts of the northern hemisphere (Hacke, 2016) and thus indicating a role of environmental factors in disease aetiology.

1.1.1. Aetiology

There is, until now, no specific and definite cause of MS known in medical research (Compston & Coles, 2008). Instead, its aetiology is considered to be multifactorial taking into account various aspects of environment and genetic susceptibility promoting the development of the disease (Høglund, 2014). The genetic component is supported by family studies showing an increased risk in relatives of diagnosed MS patients (Høglund, 2014). Out of many discovered genetic predispositions the most important one so far is the HLA-DRB1*15:01 allele (Dobson & Giovannoni, 2019). These family studies

however do not explain all the aspects of disease development leaving an important impact of environmental factors which can explain the inhomogeneous distribution around the globe with higher incidence in the northern hemisphere (Hacke, 2016). Studies evaluating the risk after migration at different ages also suggest an important role of environment especially in early childhood (Hacke, 2016). For example Vitamin D levels are considered one of these environmental aspects as they are lower in northern areas due to less exposure to sunlight corresponding with the prevalence of MS (Dobson & Giovannoni, 2019; Høglund, 2014). Another risk factor often associated with MS is Epstein-Barr virus as a systemic infection (Compston & Coles, 2008).

Several other factors are currently being discussed for their implication in MS such as cigarette smoking, saturated fat diet, sex hormones, obesity and other viral infections (Høglund, 2014; Thompson et al., 2018). Overall current reviews favour the environmental factors over the genetic ones as more important regarding the susceptibility for MS (Dobson & Giovannoni, 2019; Thompson et al., 2018).

1.1.2. Clinical Subtypes

The clinical appearance of MS can be divided into different subtypes. The first manifestation often occurs as clinically isolated syndrome (CIS). The CIS shows first symptoms of a demyelinating disease without fulfilling criteria for MS diagnosis (Compston & Coles, 2008). Regarding the clinical course of the disease there are different subtypes describing the progress. The relapsing remitting MS (RRMS) features defined relapses as attacks with acute clinical worsening of symptoms followed by full or partial remission between attacks. Upon first diagnosis, more than 80% of patients show the relapsing remitting subtype of which around 50% later convert into secondary progressive MS (SPMS) showing a continuous increase of disease severity (Hacke, 2016). Approximately 20% of all MS patients present a primary progressive type (PPMS) with an uninterrupted disease progression from the onset. Beside these forms there also is an intermediate form showing a continuous increase of disease severity with attacks on top (Compston & Coles, 2008). Figure 1 shows a graphical visualization of clinical MS subtypes.

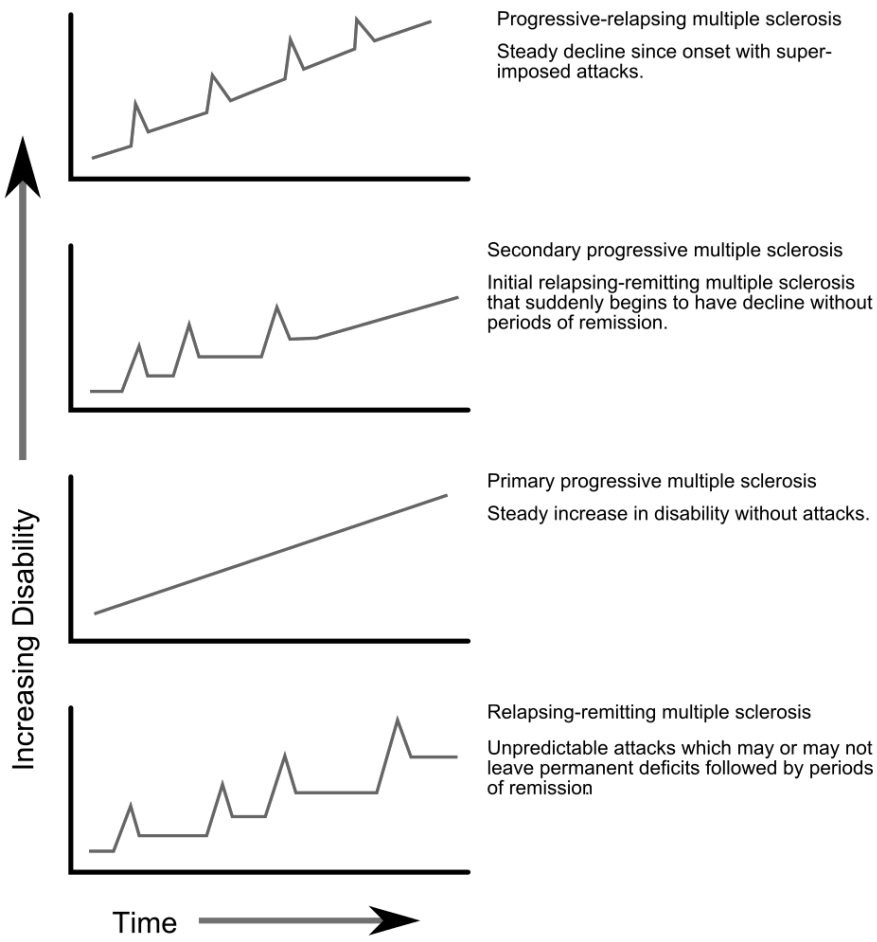

Figure 1 MS progression forms showing the state of disability depending on the time of disease progression for the clinical subtypes described above. The most common clinical progression type, the relapsing remitting multiple sclerosis, is displayed in the lowest graph. The acute relapses shown by the peaks are followed by phases of clinical remission in between.
From: https://commons.wikimedia.org/wiki/File:Ms_progression_types.svg, accessed on 03.12.2021, © public domain.

Even though these definitions of subtypes are simply descriptive they are still important for choosing an appropriate disease-modifying therapy.

1.1.3. Pathophysiology

Multiple sclerosis is considered an autoimmune disease featuring two major aspects of pathophysiology: on the one hand chronic inflammation within the CNS and on the other hand neurodegeneration describing the loss of neurons or axons (Compston & Coles, 2008). Important actors in these autoimmune processes are the adaptive immune system

featuring T- and B-cells as well as the innate immune system with phagocytic cells such as macrophages (Thompson et al., 2018).

As already mentioned regarding the aetiology, the processes that lead to an initiation of the autoinflammatory processes are still subject of a controverse discussion. The subsequent central inflammatory reactions are mainly caused by autoreactive lymphocytes, which are activated in the periphery, crossing the blood-brain-barrier (BBB) (Compston & Coles, 2008). Presence of these cells, to a certain degree even in the CNS, also occurs in healthy individuals but at a lower level than observed in MS patients. Increased numbers of immune cells in the CNS are ascribed to a failed regulation for example through regulative T-cells (Viglietta, Baecher-Allan, Weiner & Hafler, 2004). After crossing the BBB, especially T-lymphocytes recognize structures within the CNS such as myelin proteins as antigens. One possible explanation for this failed autoimmune reaction is a molecular mimicry with viral structures resembling myelin proteins (Compston & Coles, 2008) while other sources suggest that the triggering event for the autoimmunity may take place either within the CNS or outside of it. An event outside of the CNS might for example be a systemic viral infection (Thompson et al., 2018).

The T-cells activity against myelin structures causes phagocytosis of myelin sheaths and oligodendrocytes by macrophages and thus demyelination of axons in the CNS which explains subsequent symptoms by impaired signal conduction. The role of the innate immune system includes the activation of lymphocytes as well as microglial activation with subsequent secretion of cytokines and other immune mediators (Thompson et al., 2018).

Further pathologic processes lead to depletion of oligodendrocytes and axonal degeneration as well as remyelination as compensatory mechanism which plays a key role in damage repair but is considered impaired in MS (Lindner et al., 2015). All these processes contribute to the formation of so-called plaques which can be seen in MRI diagnostic or biopsies. Common sites for plaques are the areas around the corpus callosum and the lateral ventricles, the optic nerve, brain stem, spinal cord and brain tissue in close relation to the subarachnoid space (Stadelmann, 2011). Alongside these more common areas, plaques can arise anywhere in the CNS explaining the variety of symptoms caused by this disease.

Figure 2 shows a representative MRI scan of a patient suffering from MS, it shows plaques at typical periventricular sites (Hemond & Bakshi, 2018).

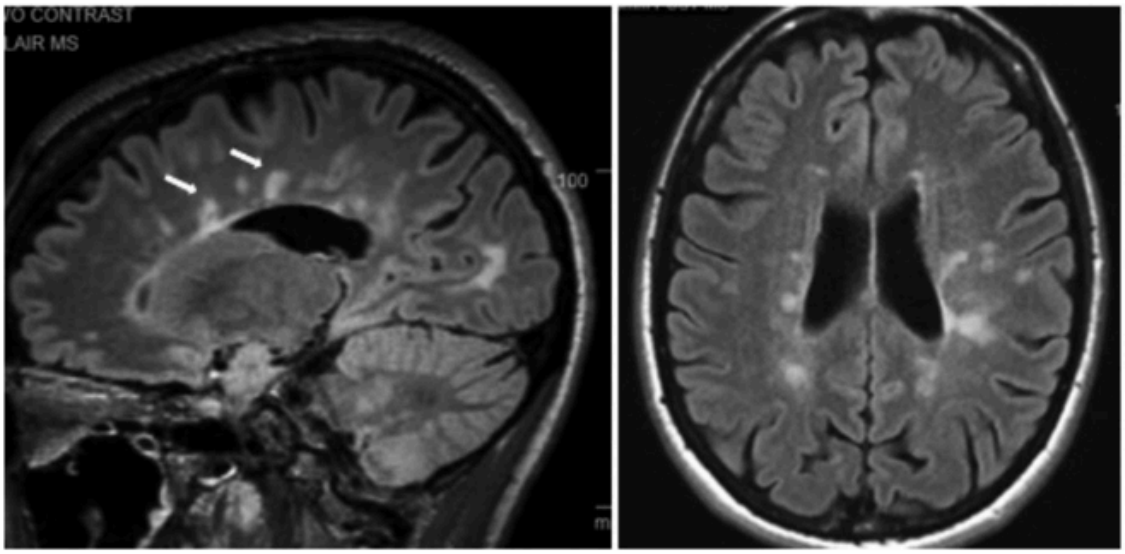

Figure 2 MRI scan of a MS patient showing typical lesions from Hemond et al 2018. The lefts picture shows a sagittal image, the arrows point to characteristic periventricular lesions forming so called Dawson's fingers. On the right side there is an axial scan of the same patient. (Hemond & Bakshi, 2018) (© to Cold Spring Harbor Laboratory Press)

Lesions mainly appear in two different states: chronic and acute lesions. While chronic lesions display a significant loss of myelin with only few infiltrating cells but obvious fibrous gliosis (Stadelmann, 2011), early active lesions show more signs of acute inflammation and myelin destruction. In these active lesions a lot of activated lymphocytes can be found showing phagocyted myelin proteins including MOG (myelin oligodendrocyte glycoprotein), CNPase (cyclic nucleotide phosphodiesterase) and MAG (myelin-associated glycoprotein) (Stadelmann, 2011). Also B-lymphocytes infiltrate the CNS contributing to follicles in the meninges. These cells act as part of the humoral immune response by producing intrathecal antibodies that can be used for diagnostic of cerebrospinal fluid (CSF) and appear as the oligoclonal bands (Compston & Coles, 2008). Beside these antibodies in the cerebrospinal fluid, other pathophysiological features can be used for diagnostic purposes such as imaging of neural/axonal loss or astrocyte activation (Thompson et al., 2018).

Remyelination as a compensatory process in demyelinated areas takes place in all kinds of plaques at various degrees. It does however mostly fail to reach a sufficient level compared to the detrimental degenerative processes (Duncan et al., 2017). Oligodendrocytes and their progenitor cells are the main actors in the process of remyelination and can be found especially in the periphery of acute lesions (Compston & Coles, 2008). Their migration and differentiation depend on various signalling factors and can be easily interrupted by changes within the microenvironment as seen in MS and its models (Lindner et al., 2015). This role of oligodendrocytes proves their importance for the pathophysiology of MS and thus we have chosen these cells to further investigate signalling pathways and their effects.

1.1.4. Symptoms

Demyelination and axonal degeneration as described above impair the neuronal signal transmission by retarding the saltatory conduction. The retarded transmission may lead to a functional loss of the nerve conduction resulting in negative symptoms (e.g. loss of vision in optic neuritis). However, demyelinated nerves may also become hyperexcitable and thus cause positive symptoms including inappropriate sensations (Thompson et al., 2018). As these lesions may appear at almost any place within the CNS, their clinical presentation shows an immense diversity. Nevertheless, certain patterns can be observed in many patients: Affection of the optical nerve often leads to visual restrictions in patients, lesions of the spinal cord cause central paresis while cerebellar infestation may cause tremor and, in rare cases, so called Charcot triad (nystagmus, intention tremor and chanting language). Furthermore, many patients suffer from inappropriate sensations like tingles and other sensory distortions such as numbness or seldom pain (Hacke, 2016).

Most of the clinical features described above are not very specific for multiple sclerosis. There are however some characteristics for the disease. One of these rather characteristic symptoms is the Uhthoff phenomenon which describes a temporary increase in symptoms severity with increased body core temperature. This mechanism is presumably caused by temperature dependent conductivity in partially demyelinated axons which makes the MS symptoms vary depending on the body temperature (Hacke, 2016). The Lhermitte’s

symptom (transient paraesthesia in extremities or spine upon neck flexion) represents another characteristic of MS (Compston & Coles, 2008).

The scales to describe the patient's clinical status include the EDSS (Expanded Disability Status Scale) which mostly refers to motoric skills like independent walking and the MS-functional-composite-scale including other motoric aspects and cognitive skills (Hacke, 2016).

1.1.5. Diagnosis

Since there is no pathognomonic clinical or diagnostic feature of multiple sclerosis, diagnosis relies on combination of both clinical and paraclinical results (Thompson et al., 2018). Early diagnostic criteria like Schumacher's from 1965 (Schumacher et al., 1965) included only clinical features while later criteria like Poser criteria called for laboratory markers in combination with clinical appearances of relapses. The decisive part for all these diagnostics was the dissemination of time and location displaying affections in different parts of the CNS occurring at various stages of the disease (Karussis, 2014).
Today, the McDonald criteria combine clinical aspects with paraclinical diagnosis including analysis of cerebrospinal fluid (CSF) and MRI scans. The revised edition from 2010 allowed a MS diagnosis after only one attack if additional paraclinical conditions are met (Karussis, 2014). The current McDonald criteria from 2017 focus even more on the advanced paraclinical surveillance in order to allow a precise diagnosis as early as possible (Thompson et al., 2018). Table 1 shows the current diagnostic criteria for patients with relapsing remitting MS, the most common type of clinical progression.

For an appropriate treatment early diagnosis is substantial. Therefore, biomarkers play a crucial role in today's research. Discovery of various markers mainly including antibodies in serum or liquor raised hope for earlier discovery of disease. Despite the progress made especially in paraclinical diagnosis, there remains the need for even more sensitive markers since there is, until now, no reliable and definitive predictor for MS (Karussis, 2014).

Table 1 Table of McDonald criteria for diagnosis of relapsing remitting MS adapted from Thompson et al 2018. Depending on the objective clinical manifestations additional data is necessary to diagnose the most common subtype of multiple sclerosis. Criteria to diagnose other MS subtypes like primary progressive MS are slightly different (Thompson et al., 2018).

Objective clinical manifestations	Additional data needed to diagnose MS
≥ 2 clinical attacks ≥ 2 clinical objective lesions	none
≥ 2 clinical attacks 1 clinical objective lesion	Dissemination in space (MRI or CSF or further clinical attack indicating different site)
1 clinical attack ≥ 2 clinical objective lesions	Dissemination in time (MRI or second clinical attack)
1 clinical attack 1 clinical objective lesion	Dissemination in space and in time

Given the heterogeneity of MS symptoms there are many diseases showing a similar pattern of clinical impairment such as but not limited to neuromyelitis optica spectrum disorders (NMOSD), acute disseminated encephalomyelitis (ADEM) or infectious diseases of the CNS (Hacke, 2016).

1.1.6. Treatment

Treatment of MS can be divided into three main proportions: Handling of acute attacks with glucocorticoids, so-called disease modifying therapy (DMT) through immune modulation and a symptomatic therapy relieving disabling effects of the disease. There is however no known way to finally cure the disease even though DMT may reduce the frequency of relapses and decelerate the diseases progression (Gholamzad et al., 2019).
For acute attacks and relapses, high dose glucocorticoids represent the first line of therapy. In refractory cases, a dose escalation can be performed followed by plasmaphereses or immunoadsorption as ultima ratio (Hacke, 2016).

Disease modifying therapeutics aim on reducing the frequency of relapses and the progression of the disease in general. To do so, most available drugs target the immune system of patients to suppress the detrimental impacts of a dysregulated immune activity

(Gholamzad et al., 2019). Beside different targets within the immune system a main difference between the drugs, which also affects the patients' daily life, is their form of administration. While classic DMT like IFN-ß require regular self-injections, newer drugs like Fingolimod or Teriflunomide can be administered orally. Novel antibody therapeutics like Natalizumab or Ocrelizumab necessitate intravenous injections (Doshi & Chataway, 2017). Today, we have a continuously growing field of disease modifying therapeutics with various indications mostly depending on the MS subtype and other patient related circumstances. Figure 3 shows the current recommendations from the current German guideline for immunosuppressive therapy in patients with relapsing remitting multiple sclerosis (Hemmer, 2021).

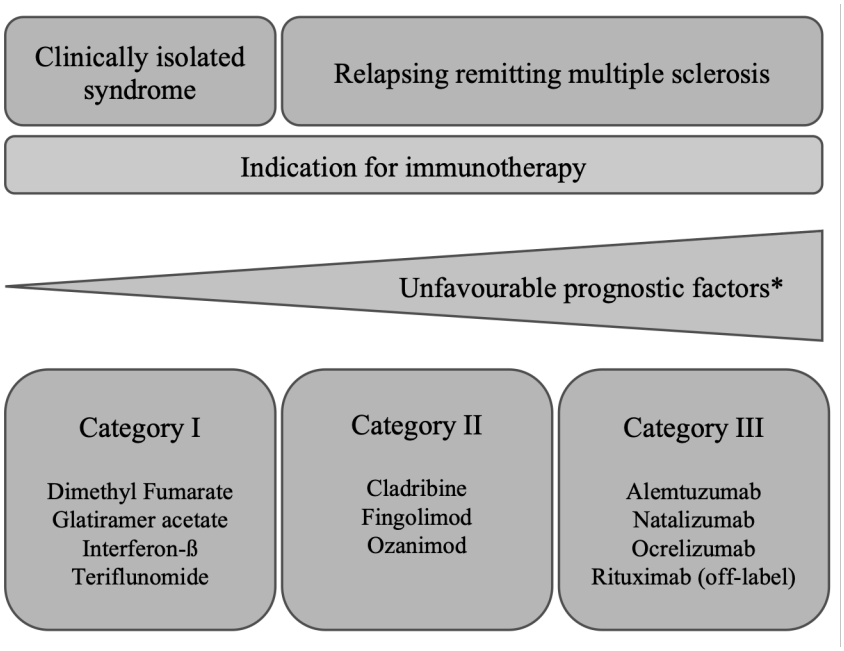

Figure 3 Therapeutic algorithm for patients suffering from relapsing remitting multiple sclerosis. Adapted from Hemmer B. et al., Diagnose und Therapie der Multiplen Sklerose, Neuromyelitis-optica-Spektrum- Erkrankungen und MOG-IgG-assoziierten Erkrankungen, S2k-Leitlinie, 2021, in: Deutsche Gesellschaft für Neurologie (Hrsg.), Leitlinien für Diagnostik und Therapie in der Neurologie. Online: www.dgn.org/leitlinien (accessed on 09.12.2021).
Substances are listed alphabetically.
*Young age, polysymptomatic onset, insufficient remission after relapse, high number of lesions, spinal lesions, high intrathecal immunoglobulin synthesis.

As MS treatment varies for the different clinical subtypes, the correct anticipation of disease progression form (Fig. 1) is important for each patient. One aspect underlining this importance is the fact that for primary progressive MS there are currently only very

few therapeutic options including the anti-CD20 antibody Ocrelizumab (Dobson & Giovannoni, 2019).

Regarding the variety of MS associated symptoms like spasticity, fatigue or depression, there is a wide field of possible supportive therapy including drug-based treatments as well as physical- or psychotherapy. Symptomatic therapy is however limited and thus neurological symptoms such as fatigue or spasticity pose major restrictions in the daily life of multiple sclerosis patients (Hacke, 2016).

Despite multiple drugs available (as listed above) there is until today no efficient agent which goes beyond anti-inflammatory effects and instead targets pathophysiological mechanisms including demyelination. Even though the current therapeutics can significantly reduce relapse rates, there is no sufficient effect on disability or continuous neurodegeneration (Rajendran, Böttiger, Stadelmann, Karnati & Berghoff, 2021). To eventually cure the disease, it is therefore necessary to work on different approaches. These new approaches might include targeting signalling pathways to find drugs eliminating the diseases cause. Beside antibodies targeting for example inhibitors of myelination (VX15/2503 antibody against semaphorin 4D, LaGanke et al., 2017) the field of growth factors including FGF along with the associated receptors continues to gain attention.

1.2. Myelination in the central nervous system

For the proper function of the central as well as the peripheral nervous system, a rapid signal transmission is the fundamental requirement. Acceleration of conduction speed can be achieved by two major mechanisms: Increasing the nerves diameters or insulating the nerve fibres which is commonly attained by myelination. The lipid-rich myelin is wrapped around the nerves in multiple layers acting as an insulation leaving only small gaps (Nodes of Ranvier) where new action potentials can be formed (Hartline & Colman, 2007).

Nerves in the CNS as well as in the periphery can be found in myelinated or unmyelinated forms. Both types of nerve fibres can conduct electric signals (action potentials) through

changes of their membrane potential. Apart from the nerve diameter, myelination is an important factor for signal transmission velocity. Therefore signalling in myelinated nerves is significantly faster than in similar unmyelinated ones which becomes obvious in demyelinating diseases that can be diagnosed by a reduced nerve conduction velocity in electromyography (Hacke, 2016).

This myelination of nerves and thus a quick signal transmission is essential for an appropriate function of the whole organism which becomes obvious in demyelinating diseases such as multiple sclerosis when loss of myelin and thus impaired signalling causes severe symptoms throughout the whole organism. Beside the initial myelination of nerve fibres, remyelination after axonal damage or pathological demyelination also is essential to preserve and restore elaborate brain functions (Gruchot et al., 2019). This process requires a complex interaction of cell recruitment such as but not limited to oligodendrocytes and their progenitors as well as an appropriate microenvironment provided for instance by microglia (Gruchot et al., 2019). The importance of this process can be concluded from the consequences of its failure in demyelinating diseases where a faulty demyelination causes and maintains the neurological symptoms.

1.2.1. Role of oligodendrocytes

While in the peripheral nervous system myelination is sustained by Schwann cells (Hacke, 2016), oligodendrocytes fulfil this crucial task in the CNS. To do so, oligodendrocytes wrap myelin sheaths around the axons acting as a kind of insulation for the nerve fibres. Internodes between the myelinated regions, the so-called Nodes of Ranvier, feature voltage gated sodium channels which are essential for generating and conducting electric potentials (Chamberlain & Nanescu, 2016). In short, mature oligodendrocytes make contact to nearby axons by developing multiple membrane extensions. These extensions wrap around the axons and assemble several layers of myelin sheaths around them (Snaidero & Simons, 2017).
Loss of these myelin sheaths as observed in demyelinating diseases like multiple sclerosis can lead to severe symptoms through the loss or impairment of signal conduction as described in MS symptoms. Beside the myelination of CNS axons, recent studies indicated a positive effect of oligodendrocytes on axon integrity in general. While the

exact mechanisms remain unclear, metabolism, axonal transport or neurotrophic factors such as brain derived neurotrophic factor (BDNF) synthesized by oligodendrocytes possibly play key roles in cell interaction and thus underline the important role of oligodendrocytes for the whole CNS (Chamberlain & Nanescu, 2016).

1.2.2. Development of oligodendrocytes

The development of oligodendrocytes consists of several steps including migration, proliferation and maturation. Based on different protein expression, OL development can be divided into three distinct stages: first the oligodendrocyte progenitor cells (OPCs) further developing into pre-myelinating oligodendrocytes and ultimately mature myelinating oligodendrocytes (Rajendran, Böttiger, Stadelmann et al., 2021).

The CNS glial cells originally derive from neural stem cells (NSC) within the embryonic brain that further differentiate into oligodendrocyte precursor cells (OPC) under the influence of various transcription factors including FGF (Furusho, Ishii, Hebert & Bansal, 2020; van Tilborg et al., 2018). Similar circumstances considering distinct growth factors lead to a further differentiation into mature oligodendrocytes that can fulfil their roles in sustaining myelination and axonal integrity in the CNS as described above (Chamberlain & Nanescu, 2016). Bögler et al showed in 1990 that the maturation of oligodendrocytes is associated with a more complex phenotype featuring a multipolar morphology (Bögler, Wren, Barnett, Land & Noble, 1990). For a visual depiction of oligodendrocyte development see figure 4.

Apart from this regular oligodendrocyte development, the recruitment of oligodendrocyte progenitors in response to axonal damage plays a key role in repair processes within the CNS where once again growths factors including FGF play a crucial role (van Tilborg et al., 2018).

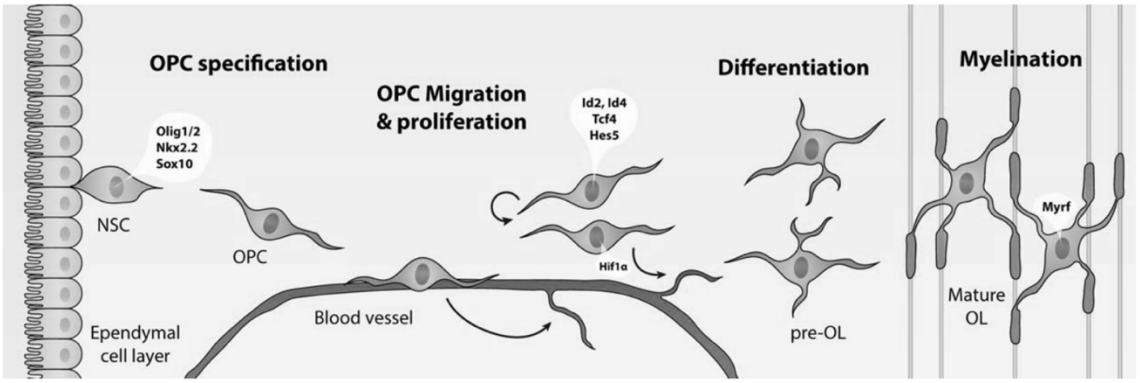

Figure 4 Schematic depiction of oligodendrocyte development from van Tilborg et al, 2018
Deriving from neural stem cells (NSC) oligodendrocyte progenitor cells (OPC) require distinct signalling molecules and transcription factors to migrate within the nervous system. After migration and proliferation, they develop premyelinating capacities (pre-OL) and eventually transform into mature myelinating oligodendrocytes (van Tilborg et al., 2018). (©2017 van Tilborg et al. GLIA Published by Wiley Periodicals, Inc. Glia.)

1.2.3. Oligodendrocytes in multiple sclerosis

Since multiple sclerosis is a demyelinating disease of the central nervous system, oligodendrocytes as the myelin producing cells of the CNS represent key actors of the disease pathophysiology. The importance of repair processes driven by oligodendrocytes within the CNS can be seen in multiple sclerosis lesions, where OPC keep on being recruited to demyelinated sites (Chamberlain & Nanescu, 2016). Demyelination and axonal damage mark key aspects of MS pathophysiology as described above and even though OPC are being attracted towards the lesions, their differentiation and thus functional role in remyelination often fails (Lindner et al., 2015). This underlines one more time the attractivity of oligodendrocytes and their signal pathways as potential targets for novel MS treatments. Considering the pathological conditions prevailing in MS there are a lot of factors, including FGFs, influencing oligodendrocytes and their capacity to migrate, proliferate, differentiate and ultimately exert remyelination (Gruchot et al., 2019).

1.3. Fibroblast growth factors and their receptors

The Fibroblast growths factors along with their receptors cover a vast field of functions ranging from early development to homeostasis and cell survival (Ornitz & Itoh, 2015). Apart from their role in cancer development (Turner & Grose, 2010) recent studies *in vitro* experiments and *in vivo* models indicated an important role of signals mediated through this complex system for demyelinating diseases such as multiple sclerosis (Lindner et al., 2015; Mohan et al., 2014).

1.3.1. FGFs and FGFRs

The group of mammalian fibroblast growth factors (FGFs) comprises 18 secreted glycoproteins mediating their signals through four different types of receptor tyrosine kinases (FGFR) (Ornitz & Itoh, 2015). They are accompanied by several other types of FGF and also another receptor subtype (FGFR5), which exert direct nuclear functions or modulate the downstream signals initiated by interactions of other FGF and FGFR (Turner & Grose, 2010).

The 18 secreted FGFs can be divided into different subgroups. Their various effects range from developmental regulation to control of basic cellular processes such as proliferation or survival through their cellular receptors. Taken together, recent studies in mice and human were able to identify up to 22 FGF ligands (Ornitz & Itoh, 2015). While these growth factors along with their receptors are essential at various stages of development, deregulation of the FGF/FGFR signalling has proven to be a driving force in several diseases reaching from cancer to neurodegenerative disorders including multiple sclerosis (Mohan et al., 2014; Turner & Grose, 2010).

1.3.2. FGFR signalling

Despite some endocrine FGFs, most of the ligands are secreted and act in either an autocrine or paracrine way (Rajendran, Böttiger, Stadelmann, et al., 2021). Upon binding of the FGF as ligand to the FGFR as receptor, a dimerization of two receptor molecules takes place. This dimerization leads to a shift in receptor conformation that activates an

intrinsic kinase domain. Further phosphorylation of adaptor proteins by this kinase domain induces several downstream pathways. Whilst the detailed interconnection of the signalling is not going to be described here, many of the signals run into three complex main pathways: The activation of RAS (Ras GTPase) leads to the expression of RAF (serine/threonine-specific protein kinases) and MAPK (mitogen activated protein kinase). The other important downstream pathways are the PI3-Akt pathway (Phosphoinositide 3-kinase), which acts anti-apoptotic, and the PLCy (phospholipase Cy) pathway further activating protein kinase C, which again contributes to RAS activation (Turner & Grose, 2010).

The effects of FGF / FGFR signalling cover a wide field of biological effects ranging from embryonal development to homeostasis and metabolism in adult organism. Other important aspects of FGF functions can be seen as a regulator of organogenesis in several organ systems or as a mediator of repair processes after tissue injury (Ornitz & Itoh, 2015). Considering the CNS and especially oligodendrocytes, FGF receptor signalling is essential during the initial development of oligodendrocyte progenitor cells as well as for the later growths of myelin sheaths in the process of myelination and remyelination (Furusho et al., 2020).

1.3.3. FGFRs in oligodendrocytes

Early studies found that the cells of the oligodendrocyte lineage express FGFR1, 2 and 3 (Bansal, Kumar, Murray, Morrison, & Pfeiffer, 1996). FGFs and their receptors were shown to play a role in the development of oligodendrocytes especially at early stages in the recruitment of OPCs (Farreny et al., 2018). In accordance with the different roles of FGFR during development the expression patterns of the distinct FGFR subtypes vary at different stages of oligodendrocyte maturation: While FGFR1 is evenly expressed throughout all developmental stages, FGFR2 shows the highest expression in mature oligodendrocytes and FGFR3 is especially found in progenitor cells (Bansal et al., 1996). Through observation of the effect of FGF-2 in oligodendrocytes at different developmental stages Bansal et al described a wide field of FGF/FGFR signalling impacts in oligodendrocytes: In OL progenitor cells FGF-2 enhances proliferation, migration and survival while in mature OLs it causes a downregulation of myelin proteins as well as a

re-entry into cell cycle and thus a trend towards less differentiated cell types (Bansal, 2002).

In vivo, the effects of FGFR signals in oligodendrocytes are accompanied by a multiplicity of other intra- and extracellular factors including inflammatory environment or other cell types (Gruchot et al., 2019). Nonetheless we investigated the role of FGFR signalling apart from other influences *in vitro* to gain a better understanding of the involved mechanisms. Figure 5 shows a graphic depiction of FGF receptor signalling in oligodendrocytes as well as some effects of FGFR inhibition by Dovitinib that will be described further on.

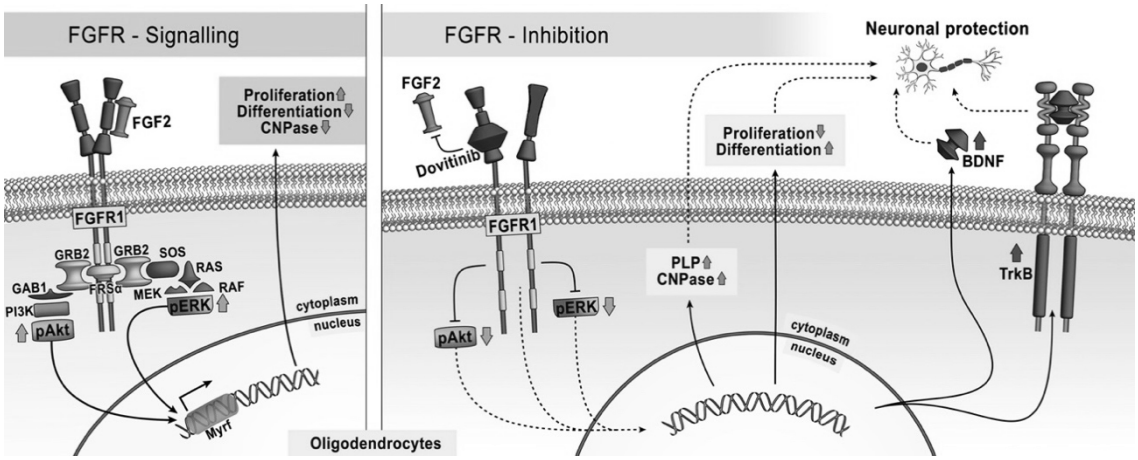

Figure 5 FGFR signalling and FGFR inhibition modified from Rajendran et al., 2021 The left picture shows the signalling following the binding of FGF2 to the FGF receptor 1. Dimerization of two receptor molecules leads to activation of several downstream signalling pathways and eventually upregulation of pAkt and pERK. The right-hand picture depicts the effects of FGF receptor 1 blocking by Dovitinib. Downstream molecules pAkt and pERK are downregulated. Along with other upregulated pathways such as TrkB and BDNF this leads to an increase of myelin proteins (PLP and CNPase) which contributes to neuronal protection. (Rajendran, Böttiger, Dentzien et al., 2021) (© 2021 by Rajendran et al. Licensee MDPI, Basel, Switzerland)

1.3.4. FGFR in multiple sclerosis

Several recent studies point towards an important role of FGF/FGFR signalling in multiple sclerosis and corresponding models representing demyelinating diseases. In samples from cerebrospinal fluid (CSF) as well as in the serum of patients suffering from MS there is an increase of FGF2 compared to healthy control probands (Sarchielli et al.,

2008). Going along with these findings, tissue samples from lesions in MS patients showed an increased FGF2 expression within areas of active demyelination and also in the periphery of chronic lesions (Clemente, Ortega, Arenzana & de Castro, 2011). Beside the growth factor itself, also the expression of its receptor FGFR1 is upregulated in oligodendrocytes (especially OPCs) occupying areas of actively demyelinating and chronic lesions (Clemente et al., 2011). Furthermore, oligodendrocytes in lesions that already show signs of remyelination exhibit an increase in FGFR1 (Mohan et al., 2014). Matching results were found in animal models of Cuprizone demyelination (model for toxic demyelination) where Armstrong et al. found an increase in FGF2 expression patterns after induction of demyelination (Armstrong, Le, Frost, Borke, & Vana, 2002). Besides the altered expression patterns in disease condition, direct administration of FGF2 into the CSF of rats and thus activation of FGFR caused a significant reduction of myelin (Butt & Dinsdale, 2005).

Further *in vivo* studies dealing with animal models revealed that a conditional knockout of FGFR1 following chronic demyelination increased the number of mature oligodendrocytes and thus the remyelinating potential within the lesions. This indicates that FGFR1 signalling might be an inhibitor of repair processes (Zhou, Pannu, Le, & Armstrong, 2012). Similar knockout studies with mice suffering from experimental autoimmune encephalomyelitis (EAE, common model of demyelinating disease) ameliorated the disease severity compared to placebo treatment: Rajendran et al. used a FGFR1 knockout in oligodendrocytes which significantly reduced the EAE severity score compared to control mice (Figure 6) (Rajendran, Giraldo-Velásquez, Stadelmann & Berghoff, 2018), while Kamali et al showed the same effect after a FGFR2 knockout in oligodendrocytes in EAE induced mice (Kamali et al., 2020). Both of these studies lay focus on another important signalling pathway that is affected by FGF/FGFR signalling: The brain-derived neurotrophic factor (BDNF) and its receptor Tropomyosin receptor kinase B (TrkB). These molecules represent another important regulatory system for oligodendrocytes, their differentiation and ultimately for myelination (Vondran, Singh, Honeywell & Dreyfus, 2011).

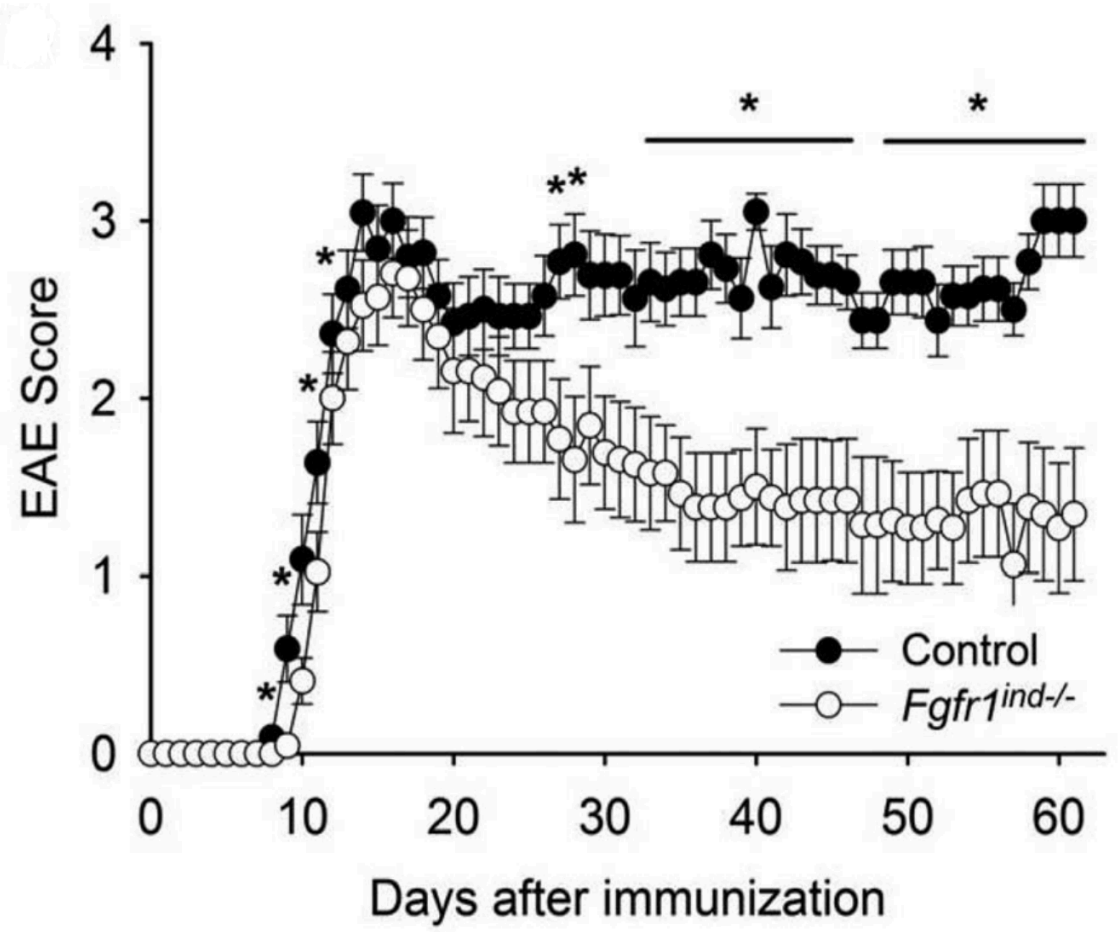

Figure 6 EAE score in oligodendrocyte specific FGFR1 knockout and control mice from Rajendran et al., 2018. EAE score after induction with MOG35-55 peptide. Mice with an oligodendrocyte specific FGFR1 knockout show a significantly lower EAE score in the chronic phase of the disease from day 33 indicating a protective role of FGFR inhibition (Rajendran et al., 2018). (© 2017 International society of Neuropathology)

Taken together, these expression patterns and other data under pathologic conditions argue for an importance of FGFs and their receptors and gave us reason to further evaluate the effects of these signals in demyelinating diseases. Targeting FGF / FGFR signalling might prove a promising approach to reduce demyelination, enhance remyelination and thereby reduce symptoms in MS and other demyelinating diseases.

1.3.5. FGFRs as therapeutic targets

Beside its role in neurodegenerative diseases as described above, FGF / FGFR interaction and especially aberrant signalling is a crucial step in tumorigenesis of several

malignancies like bladder cancer, breast cancer etc. (Turner & Grose, 2010). Given the important role of FGF/FGFR signalling in cell cycle regulation, mutations in FGF or FGFR genes can lead to an uncontrolled signalling which stimulates cells and thus leads to malignant degeneration (Ornitz & Itoh, 2015). Thus, blocking of the pathways appears to be a logical attempt in preventing aberrant cell proliferation.

In this context there are many novel treatments successfully targeting this FGFR network in anti-cancer treatment while in other disorders this approach has not yet been sufficiently explored (Katoh, 2016).

1.4. Dovitinib

Dovitinib (Fig 7) (TKI-258, CHIR-258) is a benzimidazole–quinolinone compound lactate salt (Porta, Giglione, Liguigli & Paglino, 2015) and acts as a non-selective inhibitor of various receptor tyrosine kinases. While our study mainly lays focus on the inhibition of FGFR it also targets various other receptors including vascular endothelial growths factor receptor (VEGFR), platelet derived growths factor receptor (PDGFR), c-Kit and others (Fumarola et al., 2017). *In vitro* the compound inhibits the proliferation as well as the expression of downstream signals such as pAkt and pERK in several tested cell lines (Gaur et al., 2014; Lee et al., 2015). *In vivo*, this antiproliferative effect is currently being used in anti-cancer treatment (Fumarola et al., 2017). Recent clinical trials using Dovitinib comprise targeting several malignancies such as but not limited to gastric cancer (National Library of Medicine [NLM] NCT01576380), prostate cancer (National Library of Medicine [NLM] NCT00831792) and breast cancer (National Library of Medicine [NLM] NCT01262027) (https://clinicaltrials.gov, accessed on 02.02.2022).

Regarding the variety of FGF / FGFR functions and their currently discovered roles in other diseases including demyelinating conditions as described above, there remain further areas of application for FGFR inhibitors as investigated in this study.

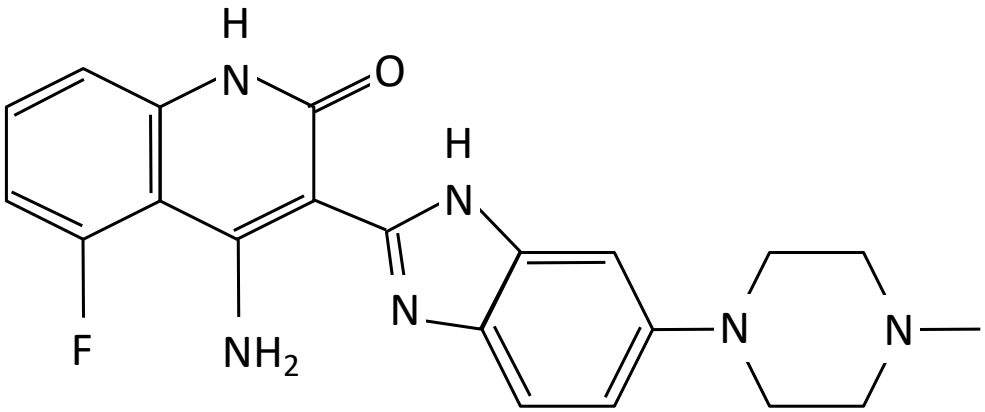

Figure 7 Molecular structure of Dovitinib (TKI-258, 1-amino-5-fluoro-3-(6-(4-methylpiperazin-1-yl)-1H-benzo[d]imidazol-2-yl)quinolin-2(1H)-one, C21H21FN6O). Own depiction of the molecular structure of Dovitinib, adapted from Selleckchem technical data Dovitinib, (www.selleckchem.com, 2018).

2. Aims

Recent studies in demyelinating disease models such as EAE suggest an important role of FGFs and their receptors for the pathogenesis as well as for the compensatory mechanisms of multiple sclerosis and other demyelinating diseases. It is especially claimed that FGFR signalling exerts detrimental effects considering neuroinflammation and demyelination (Kamali et al., 2020; Rajendran, Böttiger, Stadelmann et al., 2021).
Despite the current interest in these signalling factors the exact mechanisms and effects exerted within the complex field of MS pathophysiology remain unclear.
Given the new findings on the role of FGF/FGFR signalling in demyelinating diseases it was hypothesized that *in vitro* blocking of FGFR receptors in oligodendrocytes would increase the cells neuroprotective and myelinating capacities.
Aim of this study was to characterize the effects of Dovitinib, a non-selective FGFR inhibitor, on OLN-93 oligodendrocytes *in vitro*.

The objectives of the study are

to evaluate effects of Dovitinib on cell proliferation and cytotoxicity *in vitro*,

to assess the expression of FGFR1 and its downstream signals after FGFR blocking,

to assess the expression of myelin proteins and other neuroprotective agents including BDNF and TrkB to draw conclusions on the cells capacity to prevent demyelination and enhance remyelination in disease conditions.

3. Materials and methods

3.1. Materials

3.1.1. Cells

The OLN-93 oligodendrocytes were originally provided by Markus Kipp from Aachen University. As permanent rat cell line, OLN-93 cells resemble primary oligodendrocytes. This cell line was first established by Richter-Landsberg and Heinrich (Richter-Landsberg & Heinrich, 1996).

3.1.2. Buffers

Buffer	Components	Volume
Running Buffer for SDS PAGE 1X (1 litre)	-Rotiphorese® 10X Running Buffer -H_2O	100 ml 900 ml
Transfer Buffer for transblotting (1 litre)	-Rotiphorese® 10X Running Buffer -Methanol -H_2O	100 ml 200 ml 700 ml
5% BSA (bovine serum albumin) as blocking buffer for WB (1 litre)	-BSA fraction V -TBST	5 g 100 ml
5% BSA (bovine serum albumin) as blocking buffer for ICC (1 litre)	-BSA fraction V -PBS	5 g 100 ml
TBS 20X (1 litre) pH 7.2 to 7.6	-Tris -NaCl -H_2O	48.4 g 175.4 g 1000 ml
TBS-Tween (TBST) 1X washing buffer (1 litre)	-20X TBS -H_2O -0.1% Tween®20	50 ml 950 ml 1 ml
6x SDS-PAGE Loading Buffer (300 ml)	-60 mM Tris-HCl (pH 6.8) -2% SDS -0.01% Bromophenol blue -10% Glycerol -ddH_2O -β-Mercaptoethanol	36 ml 60 ml 60 mg 60 ml 144 ml 65 µl/ml

10% Ammonium Persulfate (APS) (10 ml)	-APS -H_2O	1 g 10 ml
10% SDS (Sodiumdodecylsulfate)	-SDS -H_2O	1 g 10 ml

3.1.3. Primary Antibodies

Name	Company	Article No.	Host	Method	Molecular weight (kDa)	Dilution
Anti-Flg (C-15) (FGFR1)	Santa Cruz Biotech, CA, USA	sc-121	Rabbit	WB	110	1:500
Anti-pERK p-44/42	Cell Signaling Tech, MA, USA	4370s	Rabbit	WB/IF	42/ 44	1:1000
Anti-pAkt (Ser473)	Cell Signaling Tech, MA, USA	4060s	Rabbit	WB/IF	60	1:1000
Anti-BDNF	Santa Cruz Biotech, CA, USA	sc-546	Rabbit	WB	13/ 17	1:500
Anti-proBDNF	Santa Cruz Biotech, CA, USA	Sc65514	Mouse	WB/IF	13/ 34	1:200
Anti-Trk B (F-1)	Santa Cruz Biotech, CA, USA	Sc377218	Mouse	WB/IF	95	1:100
Anti-PLP (G-17)	Santa Cruz Biotech, CA, USA	Sc23570	Goat	WB/IF	30	1:1000
Anti-CNPase (B-1)	Santa Cruz Biotech, CA, USA	Sc166019	Mouse	WB/IF	46/ 48	1:500

Anti-Flg (M2F12) (FGFR1)	Santa Cruz Biotech, CA, USA	Sc57132	Mouse	IF	120/ 145	1:50
Anti-GAPDH (G9)	Santa Cruz Biotech, CA, USA	Sc-365062	Mouse	WB	38	1:2000

3.1.4. Secondary Antibodies

Name	**Company**	**Article No.**	**Host**	**Method**	**Dilution**
Anti-Rabbit	Santa Cruz Biotech, CA, USA	SC2004	Goat	WB	1:1000
Anti-Mouse	Santa Cruz Biotech, CA, USA	SC2096	Donkey	WB	1:1000
Anti-Goat	Santa Cruz Biotech, CA, USA	SC2020	Donkey	WB	1:2500
Anti-Rabbit, Alexa Fluor 488	Invitrogen, Carlsbad, USA	A-11070	Goat	IF	1:500
Anti-Mouse, Alexa Fluor 488	Invitrogen, Carlsbad, USA	A-21121	Goat	IF	1:500
Anti-Goat, Alexa Fluor 594	Invitrogen, Carlsbad, USA	A-11080	Rabbit	IF	1:500

3.1.5. Ladders for Western Blot

PageRuler™ Plus Prestained Protein Ladder	#26619	Thermo Scientific, Il, USA
PageRuler™ Plus Prestained Protein Ladder	#26616	Thermo Scientific, Il, USA

3.1.6. Kits

Kit	Manufacturer	Cat. No.	Method
Cell Proliferation Reagent WST-1	Roche Applied Science, Mannheim, Germany	11 644 807 001	Proliferation assay
peqGOLD Total RNA Kit	Peqlab, Erlangen, Germany	12-6834-02	RNA extraction
SuperSignal™ West Pico Chemiluminescent Substrate	Thermo Scientific, Il, USA	34080	Western Blot
Cytotoxicity detection Kit (LDH)	Roche Diagnostics, Mannheim, Germany	11 644 793 001	Cytotoxicity assay
iTaqTM Universal SYBR® Green qPCR Supermix	Bio-Rad, CA, USA	L001752	PCR
QuantiTect® Reverse Transcription Kit	Qiagen GmbH, Hilden, Germany	205313	Reverse Transcription

3.1.7. Treatment for experiments

	Name	Company	Cat. No.	Dilution
FGF	rhFGF basic	R&D Systems, MN, USA	233-FB	25 µg in 2.5 ml DPBS -> 10 µg/ml
Dovitinib	Dovitinib (TKI-258, CHIR-258)	Selleckchem, TX, USA	S1018	10 mg in 5.0965 ml DMSO -> 5 mM
DMSO	Dimethylsulfoxid	Carl Roth, Karlsruhe, Germany	A994.1	25 µg in 2.5 ml DPBS

3.1.8. Primers for PCR

Primers were bought from Eurofins Genomics, Ebersberg, Germany

Primer	Sequence (5' → 3')
FGFR1	Forward: CGTGCCTGTGGAAGAACTTT
	Reverse: CCGCATCATCATGTACAGCTC
FGFR3	Forward: GCTTAAGCGACAGCAGGT
	Reverse: GACCTTCTCCTGAGGACAGC
FGF2	Forward: CGACCCACACGTCAAACTAC
	Reverse: TTCGCACACACTCCCTTGAT
CNPase	Forward: CCAACAGGATGTGGTGAGGA
	Reverse: AGCTGTCTTGGGTGTCACAA
NGF	Forward: CGCATCGCTCTCCTTCACA
	Reverse: CACTGAGGTGAGCTTGGGTC
TGF-ß	Forward: CTGCTGACCCCCACTGATAC
	Reverse: AGCCCTGTATTCCGTCTCCT
SEMA3A	Forward: TCTGTAGGTAGCCACATTCGAT
	Reverse: TCTGTAGGTAGCCACATTCGAT
IL4	Forward: TCCTTACGGCAACAAGGAACA
	Reverse: AAATTTGCGAAGCACCCTGG
IL6	Forward: CACTTCACAAGTCGGAGGCTT
	Reverse: GCCATTGCACAACTCTTTTCTCA
TrkB	Forward: CACACACAGGGCTCCTTA
	Reverse: AGTGGTGGTCTGAGGTTGG
BDNF	Forward: CTACGTGTCCAAGTGCAATCC
	Reverse: AATCGCCAGCCAATTCTCTTT
PLP	Forward: GTGTTCTCCCATGGAATGCT
	Reverse: TGAAGGTGAGCAGGGAAACT
GAPDH	Forward: AGTGCCAGCCTCGTCTCATA
	Reverse: GGTAACCAGGCGTCCGATAC

3.1.9. Chemicals

Name	Manufacturer
Triton X-100 solution	Sigma-Aldrich, Steinheim, Germany
DMEM 1X Medium for Cell culture	Gibco, Invitrogen, Carlsbad, USA
Penicillin Streptomycin as medium supplement	Gibco, Invitrogen, Carlsbad, USA
Trypan blue	Carl Roth, Karlsruhe, Germany

RNAse free H_2O	Millipore, CA; USA
Tween 20	Merck, Darmstadt, Germany
Prolong® Gold Antifade Reagent mounting medium for IF	Cell Signaling Tech, MA, USA
Fetal bovine serum	Gibco, Invitrogen, Carlsbad, USA
0.5 % Trypsin-EDTA (10X)	Gibco, Invitrogen, Carlsbad, USA
DAPI staining solution	Carl Roth, Karlsruhe, Germany
Fluorescence Mounting Medium	DAKO Agilent, CA, USA
PBS (10x)	PAN Biotech, Aidenbach, Germany
DMSO	Carl Roth, Karlsruhe, Germany
Rotiphorese® 30 % acryl-bysacrylamid mix	Carl Roth, Karlsruhe, Germany
10% SDS (Sodiumdodecylsulfate)	Neolab, Heidelberg, Germany
Ammonium persulfate	Carl Roth, Karlsruhe, Germany
Tetramethylethylendiamin	Carl Roth, Karlsruhe, Germany
BSA (bovine serum albumin)	Merck, Darmstadt, Germany
PFA (Paraformaldehyde)	Sigma Aldrich, Taufkirchen, Germany
Protease/Phosphatase Inhibitor cocktail	Roche, Manheim, Germany
Methanol	Merck, Darmstadt, Germany
Sodium chloride (NaCl)	Carl Roth, Karlsruhe, Germany
Trishdroxymethyl aminomethan (Tris)	Carl Roth, Karlsruhe, Germany
Rotiphorese® 10x SDS-PAGE	Carl Roth, Karlsruhe, Germany
Resolving Gel Buffer pH 8.8	BioRad, München, Germany
Stacking Gel Buffer pH 6.3	BioRad, München, Germany

3.1.10. Laboratory instruments

Instrument	Manufacturer
Microscope for cell culture	A.KRÜSS Optronic GmbH, Hamburg, Germany
ELISA-Reader Multiscan EX	Thermo electron corporation, Langenselbold, Germany

StepOne® Real-Time PCR system	Applied Biosystems, Darmstadt, Germany
Trans-Blot® SD Semi-dry transfer cell	BioRad, München, Germany
Water bath	Memmert GmbH + Co.KG, Germany
Sanyo Incu-safe incubator for cell culture	Ewald Innovationstechnik GmbH, Bad Nenndorf, Germany
Mini-PROTEAN® Tetra Cell for SDS-PAGE	BioRad, München, Germany
Consort EV231 Power Supply for electrophoresis	Sigma-Aldrich, Steinheim, Germany
ECL Chemocam Imager	Intas-Science-Imaging Instruments GmbH, Göttingen, Germany
Nanophotometer P330	Implen GmbH, München, Germany
Neubauer improved chamber	Karl Hecht "Assistent", Altnau TG, Schweiz
Cell culture cabinet	NuAire, Plymouth, MN USA
Thermomix	Eppendorf Vertrieb Deutschland GmbH, Wesseling-Berzdorf, Germany
Hettich centrifuge (cooling)	Hettich GmbH, Kirchlengen, Germany
Centrifuge Universal 32 R	Hettich GmbH, Kirchlengen, Germany
Magnetic stirrer	IKA Werke GmbH, Staufen, Germany
Casting frame for SDS gels	BioRad, München, Germany
Pipette boy	INTEGRA Biosciences GmbH, Fernwald, Germany
Centrifuge micro 120	Hettich GmbH, Kirchlengen, Germany
Vortexer vortex-Genie2	Heidolph Instruments GmbH & Co. KG, Schwabach, Germany
Thermocycler MyCycler	BioRad, München, Germany
Fridges and freezers (+4 °C, -20 °C, -80 °C)	Different companies
Axioplan 2 Fluorescence Microscope	Carl Zeiss, Jena, Germany

3.1.11. Laboratory Consumables

Consumables	Manufacturer
Cell scraper	GreinerBioOne, Frickenhausen, Germany
Cellstar® 6 Well and 24 well Cell Culture Plate	GreinerBioOne, Frickenhausen, Germany
Cellstar® 75 cm^2 Cell culture flasks	GreinerBioOne, Frickenhausen, Germany
Cellstar® Flat bottom with Lid, TC-Plate, 96 well, sterile	GreinerBioOne, Frickenhausen, Germany
Cellstar® U-shape with Lid, TC-Plate, 96 well, sterile	GreinerBioOne, Frickenhausen, Germany
Cryo TubeTM vials (1,8 ml; 4,5 ml)	Nunc A/S, Roskilde, Denmark
Cryobox	Ratiolab GmbH, Dreieich, Germany
Falcon tubes (15 ml & 20 ml)	GreinerBioOne, Frickenhausen, Germany
Syringe (20 ml)	B. Braun, Melsungen, Germany
Coverslips (18 mm)	R. Langenbrinck, Emmendingen, Germany
Filter paper for transblotting	BioRad, München, Germany
Filter paper for ICC	Medite, Burgdorf, Germany
Microscope slide	R. Langenbrinck, Emmendingen, Germany
Amersham™ Protran™ Nitrocellulose membrane	GE Healthcare, Buckinghamshire, UK
MicroAmp® Fast Reaction Tubes	Applied Biosystems, Darmstadt, Germany
MicroAmp® Optical Cap Strips	Applied Biosystems, Darmstadt, Germany
Glass plates for SDS gels	BioRad, München, Germany
Combs for SDS gels	BioRad, München, Germany
Pipette tips sterile (10 µl, 20 µl, 100 µl, 200 µl, 1000 µl)	Nerbe Plus GmbH, Winsen (Luhe), Germany
Cellstar® sterile glass pipettes (5 ml, 10 ml, 25 ml)	GreinerBioOne, Frickenhausen, Germany

Ministart® syringe filter (0.2 µm)	Sartorius Stedim Biotech GmbH, Göttingen, Germany
Glass Pasteur pipettes 150 mm	Brand, Wertheim, Germany
Eppendorf tubes 1.5 ml, 2 ml	Eppendorf Vertrieb Deutschland GmbH, Wesseling-Berzdorf, Germany
Nonsterile pipette tips (10 µl, 100 µl, 1000 µl, 5 ml)	Sarstedt, Nürnbrecht, Germany

3.1.12. Software

- Graph Pad Prism Software Version 5.01 (GraphPad Software, Inc. CA, USA)
- ZEN software for microscope (Zen 2.3, ZEISS, Jena, Germany)
- StepOne RealTime PCR Software v2.1 (Applied Biosystems, Darmstadt, Germany)
- Image J software (Image J 1.52a, National Institute of Health, USA)
- ChemoStar Imager (0.4.18.0, 2016, Intas-Science-Imaging Instruments GmbH, Göttingen, Germany)

3.2. Methods

3.2.1. Cell culture

In the present study, OLN-93 oligodendrocytes were used. Initially, these cells were kindly provided by Markus Kipp, Aachen University. OLN-93 is a permanent oligodendroglial cell line which primarily derives from spontaneously transformed rat brain glial cells and was established by Richter-Landsberg and Heinrich. Regarding their antigenic structures, these cells are similar to primary oligodendrocytes (Richter-Landsberg & Heinrich, 1996). They express several oligodendrocyte lineage marker proteins such as NG2, CNP, MBP and PLP (Buckinx et al., 2009). The cells were received from the stock of the MS-workgroup (Department of Neurology, Experimental Neurology, Justus Liebig University Giessen). Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % fetal bovine serum and 1 % Penicillin and Streptomycin was used as growth medium. Cells were seeded in 12-15 ml growth medium in 75 cm^2 adherent cell culture flasks. The cells were incubated in a humidified incubator at 37 °C, with 5 % CO_2 and 95 % humidity, cells that were more than 80 % confluent were used for sub-culturing and for experiments. Sub-culturing and seeding of cells for experiments were performed in a biological safety cabinet (NuAire, Plymouth, MN USA) under sterile conditions.

3.2.1.1. Treatments

To determine the effect of the drug on OLN-93 oligodendrocytes, cells were treated with either FGF, Dovitinib, or a combination of both. We included a control group which was not treated with Dovitinib or FGF for comparison and quantification of results as well as another internal DMSO (Dimethyl sulfoxide) control (in which Dovitinib was diluted) to rule out possible effects of the solvent. For all experiments the following treatment setup was used in minimum of triplicates:

Table 2 Treatment scheme for experimental setups

Control	FGF	Dovitinib	2+ (Dov + FGF)	DMSO
• No treatment	• 25 ng/ml bFGF	• 1 µM Dovitinib	• 25 ng/ml bFGF + 1 µM Dovitinib	• Same amount as Dovitinib for each setup

3.2.1.2. Thawing frozen OLN-93 cells

Cells were stored at -80 °C in 1.8 ml cryotubes. For seeding from the frozen stock, cells in cryotube were thawed at room temperature and then suspended in 6 ml prewarmed PBS. After centrifugation (4 minutes at 12000 rpm) the cell-pellet was resuspended in 15 ml prewarmed growth medium and seeded in an appropriate cell culture flask. Thawed cells were split at least two times before being used for experiments.

3.2.1.3. Passaging OLN-93 cells

When cells reached at least 80 % confluency, they were sub-cultured. After washing the cells with 5 ml of prewarmed PBS, cells were loosened from the bottom of the flask using 2 ml of prewarmed 0.5 % Trypsin-EDTA which detaches cells from the surface by its proteolytic activity. Growth medium was used to stop the trypsinization after 2 minutes of incubation at 37 °C. Suspension containing the cells was centrifuged (4 minutes at 12000 rpm) and cell pellets were resuspended in desired volume of prewarmed growth medium. Additional medium was added to this medium-cell-suspension in order to reach the desired dilution (splits ranging from 1:2 to 1:8).

3.2.1.4. Freezing OLN-93 cells

Freezing medium is necessary to prevent the formation of ice crystals which may destroy the cells while freezing. Freezing medium was prepared by mixing 90 % fetal bovine serum and 10 % DMSO. Cells were collected from the flasks as described in section

3.2.1.2 and resuspended in 1 ml freezing medium. Cryotubes with 1 ml of this cell suspension were stored at -80 °C and then in liquid nitrogen for long term storage.

3.2.1.5. Cell counting

For all of the following experiments a predetermined cell concentration was required for seeding to get reproducible results. For facilitated counting, cells were collected from the flasks as described in section 3.2.1.2 and 10 µl of the cell suspension was mixed with an equal amount of Trypan blue. This compound allows the differentiation of viable and dead cells, as it only enters cells with a disrupted plasma membrane during the process of cell death. Viable cells were counted in a Neubauer improved chamber in four large squares and added (Gstraunthaler, Gerhard, Lindl, 2013). For counting, the smaller squares were used for orientation by following a scheme as indicated by the arrow in Figure 8.

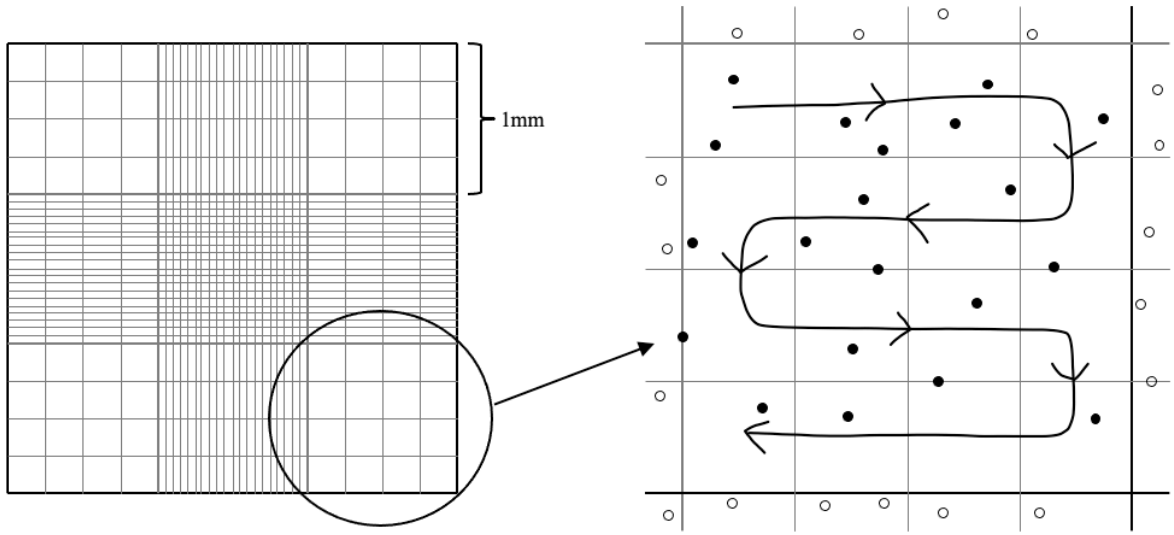

Figure 8 Cell counting in a Neubauer improved counting chamber. Own depiction of an improved Neubauer counting chamber on the left with a magnification of one large square including some cells for counting on the right-hand side. Cells were counted in a total of 16 small squares following the depicted scheme for orientation. Only cells inside the large square (indicated as black dots) were counted. This procedure was repeated for each of the four large squares (Adapted from Gstraunthaler & Lindl, Zell- und Gewebekultur, page 122, 7th ed., Springer Spektrum, 2013).

By the following formula cell concentration was calculated including a chamber specific coefficient and the dilution factor.

"Counted cells x 2 x 10000 / 4 = cells / ml"

3.2.2. Proliferation

3.2.2.1. WST-1 assay

The WST-1 (water-soluble tetrazolium salt-1) assay (Roche Diagnostics, Mannheim, Germany) is based on the mitochondrial conversion of tetrazolium salts contained in the reagent to formazan which shows a different absorbance spectrum in ELISA reading. The amount of formazan is regulated by the enzyme activity in the assay which correlates with the number of viable cells. Thus the measured differences in absorbance reflect the different number of cells in each well (Roche, Cell Proliferation Reagent WST-1).

One flask of 80-90 % confluent cells was used to seed for each assay. The cells were collected from the flask as described in section 3.2.1.2 and resuspended in 1 ml prewarmed growth medium before counting as in section 3.2.1.4. According to the calculated cell concentration the suspension was diluted with prewarmed growth medium to reach the desired amount of 10 ml at a concentration of 2 x 10^5 cells/ml. These 10 ml were split to 5 x 2 ml and the following treatment was added:

Table 3 Experimental groups and treatment of OLN-93 cells in WST-1 assay

Control	**2ml medium-cell-suspension +**	No treatment
FGF		5 µl bFGF stock (10 µg/ml) for 25 ng/ml
Dovitinib		2 µl Dovitinib stock (1 mM) for 1 µM
FGF + Dovitinib		5 µl bFGF stock + 2 µl Dovitinib stock
DMSO		2 µl DMSO
Blank	1 ml growth medium only, no cells, no treatment	

The cells were seeded in a 96-well F-bottom plate at a final volume of 100 µl/well and a concentration of $2x10^4$ cells/well in 5 triplicates of each treatment and incubated for 24 hours at 37 °C with 5 % CO_2. A blank control containing only medium was included to obtain base values for calculation.

For analysis of proliferation 10 µl of WST-1 reagent were added to each well. The plate was again incubated at 37 °C and 5 % CO_2. The absorbance was measured after 30 minutes, 60 minutes and 2 hours using ELISA-Reader (Multiscan EX, measured wavelength according to manufacturer: 405nm, reference wavelength: 492 nm). Calculation of relative proliferation was carried out as described in manufacturer´s data sheet (Roche, Cell Proliferation Reagent WST-1, content version: Feb. 2011).

3.2.2.2. Haemocytometer cell counting

For manual cell counting, cells were collected as described in section 3.2.1.2. One flask of 80-90 % confluent cells was used per assay. Cells were counted, diluted in prewarmed medium to 0.5 x 10^5 cells/ml and seeded in six well plates at a final concentration of 1.5 x 10^5 cells in 3 ml/well. Plates were incubated at 37 °C and 5 % CO_2 for 24 hours before cells were counted using a Neubauer improved chamber (see section 3.2.1.4). Triplicates were used for each treatment. Relative cell numbers were calculated and normalized to 100 %.

3.2.2.3. DAPI staining

DAPI (4′,6-diamidino-2-phenylindole) is a fluorescent stain that preferentially binds to adenine-thymine rich regions in the cell's nucleus. DAPI staining was performed as final step after specific antibodies were used for immunofluorescence (IF) staining to quantify the total number of cells for various experimental setups in IF staining.

3.2.3. Cytotoxicity - LDH assay

To assess possible cytotoxic effects of the Dovitinib treatment (or the FGF + Dovitinib combined treatment) on OLN-93 cells, cytotoxicity detection kit (Roche Diagnostics, Mannheim, Germany) was used. Lactate dehydrogenase (LDH) is an enzyme which can be found in different isoforms in the cytoplasm of all cells (Holmes & Goldberg, 2009). Damaged or dead cells release this enzyme into the supernatant where it catalyses its usual reaction: the formation of pyruvate from lactate as well as the other way around. The reduction of the cofactor NAD^+ to $NADH+H^+$ is used for the conversion of tetrazolium salt INT (2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyltetrazolium) to the slightly red coloured formazan salt (Holmes & Goldberg, 2009) which shows a significant absorption at around 500 nm that cannot be seen for the tetrazolium salt. The measured absorbance is directly correlated to the amount of active enzyme in the supernatant and thereby to the number of damaged or dead cells (Roche Diagnostics, Cytotoxicity Detection Kit, version March 2016).

Cells were used from one flask, collected and treated as described in section 3.2.2.1 (WST-1 assay). After counting as described in section 3.2.1.4, cells were diluted to a concentration of 0.5 x 10^5 cells/ml and seeded in 96-well U-bottom plates at a final volume of 100 µl/well and a concentration of 0.5 x 10^4 cells/well. Medium without cells was used for blank values and cell suspension with Triton X100, which lyses the cells, as a high control. The plates were incubated at 37 °C with 5 % CO_2 for 24 hours. After incubation the plates were centrifuged at 250 G for 10 minutes, supernatant was taken out and pipetted into a new 96-well F-bottom plate according to the same scheme and 85 µl of LDH reagent were added to each well. For 84 filled wells in the plate, a total amount of 8,6 ml LDH reagent was prepared as described in the manufacturer's data sheet. Plates were incubated for 20 minutes at room temperature protected from light. For analysis of relative cytotoxicity, absorbance was measured at 492 nm and 620 nm in ELISA-Reader (Multiscan EX) and calculated as described in data sheet (Roche Diagnostics, Cytotoxicity Detection Kit, version March 2016).

3.2.4. Protein biochemistry

3.2.4.1. Protein extraction

For protein extraction OLN-93 cells were collected from three flasks at 80-90 % confluence as described in section 3.2.1.2. The cell pellet was resuspended in 5 ml prewarmed growth medium and diluted (1:12) for treatment.
Treatment was added as follows:

Table 4 Experimental groups and treatment of OLN-93 cells for protein extraction

Control	**12 ml medium-cell-suspension +**	No treatment
FGF		30 µl bFGF stock (10 µg/ml) for 25 ng/ml
Dovitinib		12 µl Dovitinib stock (1 mM) for 1 µM
FGF + Dovitinib		30 µl bFGF stock + 12 µl Dovitinib stock
DMSO		12 µl DMSO

After application of the respective treatment, OLN-93 cells were incubated in T-75 flasks at 37 °C with 5 % CO_2 for 24 hours. For protein extraction, cells were washed with PBS after incubation and loosened from the flask using a cell scraper. For the following steps, cell lysate was cooled in ice. Cells were centrifuged (1 min, 14.000 rpm, +4 °C) and 300 µl lysis buffer (150 mM NaCl, 20 mM Tris HCl, 1 mM EDTA, 10 % glycerol, 1 % NP40, 0.01 % sodium azide, supplemented with protease inhibitors) was added to each approach. The lysate was incubated for one hour, vortexed every 10 minutes and afterwards centrifuged for 20 minutes at 14.000 rpm at 4 °C. Supernatant was used for protein quantification and further analysis. Concentration of protein was determined using a Nanophotometer. Lysis buffer was used for blank value. Protein samples were normalized to a concentration of 1 µg/µl and stored at -20 °C for following experiments.

3.2.4.2. SDS-PAGE, Transblotting and Western Blot

Western Blot principle: SDS-PAGE (sodium dodecyl sulphate–polyacrylamide gel electrophoresis) is a biochemical method that separates protein molecules ranging from

5 kDa to about 250 kDa (Laemmli, 1970) by their molecular weight using an electric field. Briefly, the SDS binds to the proteins and thus neutralizes their intrinsic charges. Proteins, which are now all charged equally negative, migrate towards the anode in the electric field applied to the gel with a speed depending on the mass of each protein. In a process called transblotting separated proteins are transferred to a nitrocellulose membrane by applying an electric field that makes the charged proteins migrate from the gel to the membrane. The membranes are then incubated with a primary antibody which binds to the protein of interest. In the next step these primary antibodies are bound by a secondary antibody which is coupled to the enzyme horseradish peroxidase (HRP) that can transform a given substrate into a chemiluminescent product which subsequently can be detected by a camera.

Gels for SDS-PAGE were prepared in advance using the following components for one gel:

Table 5 SDS-PAGE gels components and preparation

10 % resolving gel for SDS-PAGE	5 % stacking gel for SDS-PAGE
4.0 ml - H_2O 3.3 ml – 30 % acryl-bysacrylamid mix 2.5 ml – 1.5 M Tris (pH 8.8) 0.1 ml – 10 % SDS 0.1 ml – 10 % ammonium persulfate 0.006 ml - TEMED	2.1 ml - H_2O 0.5 ml – 30 % acryl-bysacrylamid mix 0.38 ml – 1.5 M Tris (pH 6.8) 0.03 ml – 10 % SDS 0.03 ml – 10 % ammonium persulfate 0.003 ml - TEMED
Gels were made at a thickness of 1,5 mm with 10 wells and stored at +4 °C for a maximum of 2 days before use.	

Procedure: An equal amount of protein sample volume of 30 µl per gel (containing 30 µg of protein) was used for SDS-PAGE after mixing each sample with 10 µl of loading dye. Protein samples were denaturized by boiling in thermomixer at 95 °C for 5 minutes. Samples were centrifuged for a few seconds after boiling and loaded to the gel for separation by SDS-PAGE. For running the gel, the following power supply program was used: Step 1: 90 V, 300 mA, 150 W for 30 minutes, step 2: 120 V, 1000 mA, 150 W for 2:30 hours. After separation, proteins were transferred (Trans Blot, semi dry transfer cell,

BioRad) to a Nitrocellulose membrane (power supply program: 25 V, 250 mA, 150 W for 1:10 hours) and incubated in 5 % BSA for 1 hour to block nonspecific binding sites on the samples. Membranes were then incubated with primary antibody diluted in 5 % BSA overnight at 4 °C and washed with 1xTBST for 3 times at 5 minutes each. Secondary antibody was added (diluted in 5 % BSA) and incubated for 2 hours at 4 °C before using the same washing procedure.

SuperSignal™ West Pico chemiluminescent substrate (Thermo Scientific, Il, USA) (1 ml for half membrane, 2 ml for whole membrane at a 1:1 ratio) was added to membrane for 3 minutes in order to visualize protein bands before development in ECL Chemocam imager (INTAS Science Imaging Instruments GmbH, Göttingen, Germany). GAPDH was used on every membrane as a loading control and for later analysis. Quantification was performed with ImageJ software (NIH, USA) using the untreated control samples and the GAPDH control as references.

3.2.5. Molecular biology

3.2.5.1. RNA extraction and quantification

OLN-93 cells for molecular biology were seeded and incubated as done for protein extraction (section 3.2.4.1). After 24 hours of incubation, RNA extraction for each treatment was done with peqGOLD Total RNA Kit as described in the manufacturer's data sheet (peqGOLD Total RNA Kit, PEQLAB_v0815_E, VWR international GmbH, Darmstadt, Germany).
RNA was quantified using a Nanophotometer with RNAse free H_2O as blank value and normalized to a final concentration of 1 µg / 20 µl in RNAse free H_2O before being stored at -20 °C for further use.

3.2.5.2. cDNA synthesis

To obtain cDNA from our RNA for the PCR we had to perform reversed transcription. An enzyme called reverse transcriptase resembles free DNA nucleotides using RNA as

template and thereby creates a complementary DNA strand (cDNA). QuantiTect® Reverse Transcription Kit (Qiagen GmbH, Hilden, Germany) was used for cDNA synthesis. Steps were done as described in the corresponding data sheet (see figure 9 for steps from manufacturer's data sheet). In short, the RNA was cleaned and diluted, mixed with a buffer, DNA primers and the reverse transcriptase. To allow the enzymes activity, the whole mix was incubated at 42 °C for 15 minutes and then heated up to 95 °C to stop the reaction by inactivation of the enzyme.

Incubation took place in a temperature regulating incubator, samples were kept in ice between the steps. After reverse transcription process cDNA was filled up to 200 µl with PCR grade H_2O and stored at -20 °C until being used for qRT-PCR.

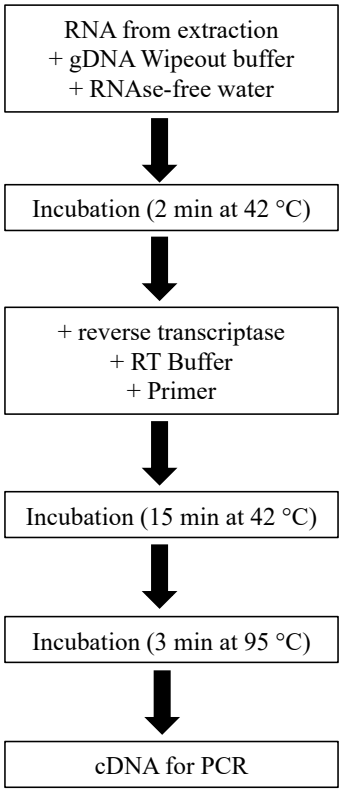

Figure 9 Reverse transcription procedure. Flowchart of necessary steps for synthesis of cDNA from RNA. Adapted from QuantiTect Reverse Transcription Handbook 03/2009 (Qiagen GmbH, Hilden, Germany).

3.2.5.3. Quantitative real time PCR

We performed quantitative real time PCR to analyse gene expression of the treated OLN-93 cells. Therefore, 1 µl of the diluted cDNA was added to 7 µl of H_2O, 1 µl of forward and reverse primer (FP and RP, respectively) and 10 µl of SYBR® Green qPCR Supermix (Bio-Rad, CA, USA). Primers were used for FGFR1, BDNF, TrkB, PLP, CNPase, IL4, IL6, SEMA3a and TGF-ß. Reactions took place in MicroAmp fast reaction tubes loaded to StepOne® Real-Time PCR system (Applied Biosystems). In a precycling stage, samples were heated up to 95 °C for 10 minutes to denature the cDNA. Amplification of the DNA occurred in 40 repetitive cycles: Denaturation at 95 °C for 15 seconds followed by annealing at 60 °C for 1 minute. The amplified DNA was slowly heated up to 95 °C while the absorbance was measured by the system. SYBR Green as an intercalating agent shows a higher fluorescence when bound to the small fold of double-stranded DNA and thus allows a quantification of the amount of amplified DNA. A negative control without any cDNA template was included for every setup and each gene was investigated at least 4 times in duplicates. For quantification of the results, the 2-ΔΔCt method was used (Livak & Schmittgen, 2001) setting the average values of untreated cells as 100 %.

3.2.6. Immunocytochemistry

Treated cells and untreated controls were stained with respective primary and conjugated secondary antibodies for quantification of positive and negative cells regarding the respective protein. Furthermore, stained cells were examined for their morphology and quantified dividing complex cells from rather immature cells (Bögler et al., 1990).

Cells were taken for immunofluorescence staining from 80-90 % confluent flasks, extracted and counted as described in section 3.2.1.2, 3.2.1.4 respectively, before seeding on sterilized coverslips (18mm) in 12 well-plates at a final concentration of 6 x 10^4 cells/well. After incubation and washing with PBS, cells were fixed in 4 % PFA for 10 minutes at room temperature. Triton X-100 (0,5 % in PBS) was used to permeabilize the cells during 5 minutes of incubation at room temperature. Before application of the primary antibodies, unspecific binding sites were blocked by an incubation in 5 % BSA (in PBS) for 1 hour. The primary antibodies were used diluted in 1 % BSA in PBS (for antibodies and dilutions see 3.1.3). Cells were incubated in the primary antibody

overnight at 4 °C and afterwards washed three times in PBS followed by the application of secondary fluorescent antibodies (all diluted in 1 % BSA in PBS, for secondary antibodies and dilutions see 3.1.4). After three more washing cycles, a counterstaining with DAPI was performed by incubating the cells in 300 µl of DAPI solution (1 µg/ml) for 20 minutes protected from light. Cells were washed three more times and afterwards mounted on microscope slides by taking the coverslips out of the well plate and carefully inverting them on a drop of antifade mounting medium. Slides were kept at room temperature protected from light for 5 minutes and then sealed with clear nail polish to prevent air from getting between slide and coverslip. Samples were stored at +4 °C before and after analysis. Images of fluorescence were acquired with a Zeiss microscope operated by Zen software with an appropriate filter for each channel (488 nm and 555 nm) at a magnification of up to 40x. To quantify positive cells for each antibody, 10 fields at 5x magnification across the whole slide were randomly selected and 30 cells in each field were counted. Cells with a high intensity and appropriate location of signal were considered as positive. The number of positive cells in untreated control cells was considered 100 % and results are shown as percent of positive cells compared to control cells. The same counting protocol was used to quantify the differences in cell morphology. Here, cells with more than two appendices were counted as matured cells whereas cells with only two or less appendices were considered immature regarding their morphology. To rule out any unspecific binding of the secondary antibodies each setup included a negative control omitting the primary antibody. The primary antibodies used for IF staining were identical with the ones used in western blot so their specificity could be confirmed by examination of the molecular weight in the western blot results.

3.2.7. Statistics

All experiments were carried out in triplicates and performed at least three to six times for each setup. Statistical analysis was performed using Graph Pad Prism Software Version 5.01 (GraphPad Software Inc., CA, USA). Results were compared using one way ANOVA and Bonferroni's Multiple Comparison test. $P < 0.05$ was considered statistically significant (* $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$). Results of RT-PCR for myelin inhibitors were calculated by Mann-Whitney-U-test (# $P < 0.05$, ## $P < 0.005$, ### $P < 0.001$) Values are shown as mean ± standard error of the mean (SEM).

4. Results

4.1 FGFR 1 expression is decreased in Dovitinib treated OLN-93 cells

The expression of FGFR1 was investigated as it represents one major target for Dovitinib and plays an important role in demyelinating diseases (Kamali et al., 2020; Rajendran et al., 2018). We therefore performed IF staining to check the FGFR1 expression on a cellular level (Fig. 10 A+B) and RT-PCR for RNA levels (Fig. 10 C).

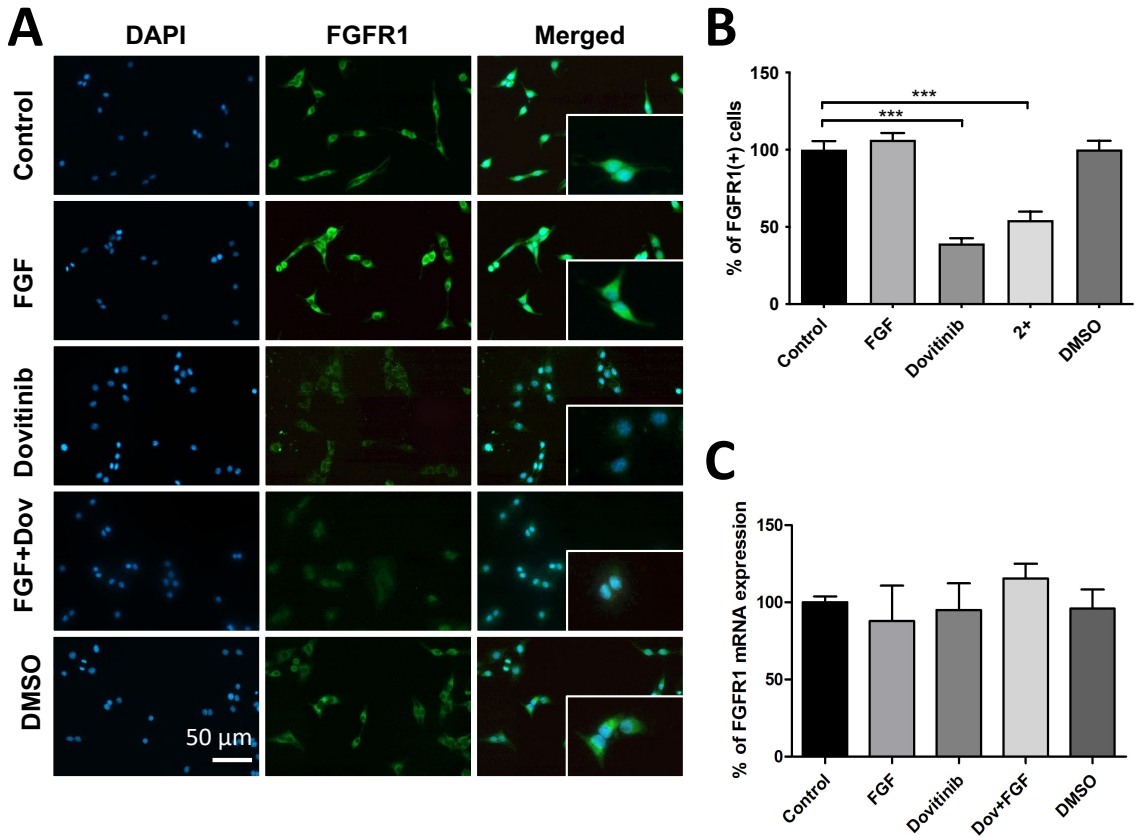

Figure 10 FGF receptor 1 expression (A-C) of OLN-93 cells incubated with Dovitinib, FGF, Dovitinib and FGF (2+), or DMSO for 24h. Representative sections of immunofluorescence staining (A). Quantitative analysis of immunofluorescence staining revealed reduced numbers of FGFR1+ cells after dovitinib treatment (B). FGF2 and DMSO did not affect the number of FGFR1+ cells. qRT-PCR showed no regulation of FGFR (FGFR1) mRNA (C). Data are presented as mean ± SEM. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$. Values for control cells were considered 100 %, all other results were put in relation to control values.

Quantification of the staining revealed a significant reduction of FGFR1 expression on cellular level after Dovitinib treatment compared to untreated control cells ($P < 0.001$; Fig. 10 B). The combination of Dovitinib and FGF induced the same effects ($P < 0.001$;

Fig. 10 B) while treatment with FGF only, just like DMSO, did not seem to regulate the FGFR1 expression on the cellular level (Fig. 10 B). Despite these effects observed in IF staining, RT-PCR for FGFR1 gene did not show any significant regulation at all (Fig. 10 C).

4.2 Dovitinib inhibits the proliferation of OLN-93 cells *in vitro*

Given the importance of FGF/FGFR signals for cell development and growth (Ornitz & Itoh, 2015) as well as the discrepancy between cell proliferation and maturation as described above, we examined the effect of Dovitinib on OLN-93 proliferation by WST1 assay. Cells were grown in 10 % FBS supplemented DMEM culture medium at 37 °C under humidified conditions. Examination of the proliferation was performed by WST-1 assays and manual cell counting each after 24 hours. The WST-1 assay after 24 hours of incubation showed significantly less proliferation in cells treated with Dovitinib compared to untreated cells ($P < 0.001$; Fig. 11 A). A combination of Dovitinib with FGF showed the same effect ($P < 0.001$; Fig. 11 A) while FGF alone enhanced the proliferation compared to control ($P < 0.05$; Fig. 11 A). DMSO treated cells showed the same proliferation as untreated control cells (Fig. 11 A). The manual cell counting in similar experimental setups confirmed the results from WST-1 assays by showing the same tendencies. There were significantly less cells after Dovitinib treatment ($P < 0.001$; Fig. 11 B) or Dovitinib + FGF treatment ($P < 0.005$; Fig. 11 B) compared to untreated control cells or FGF treatment without Dovitinib ($P < 0.001$, Fig. 11 B).

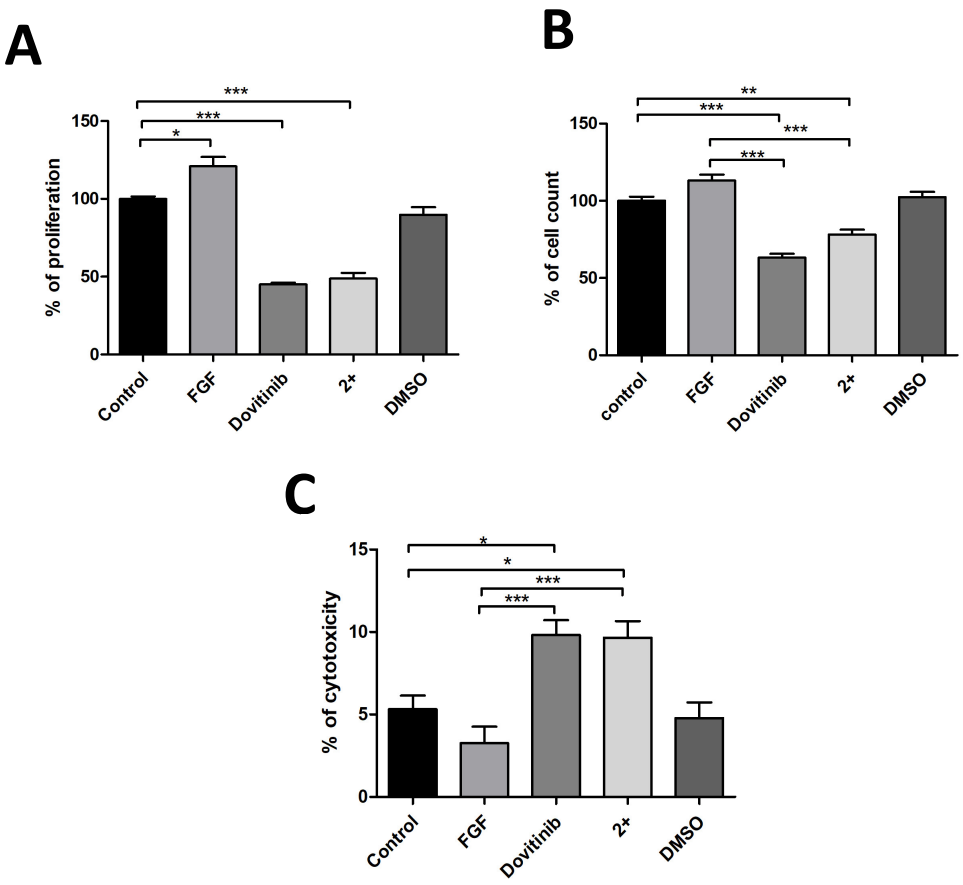

Figure 11 Cell viability (A-C) of OLN-93 cells incubated with Dovitinib, FGF, Dovitinib and FGF (2+), or DMSO for 24h. Dovitinib as well as the combination with FGF2 caused a significant antiproliferative effect (A+B) and showed increased cytotoxicity to these cells (C). Data are presented as mean ± SEM. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$. For (A) and (B) values for control cells were normalized to 100 %, all other results were considered in relation to control values.

4.3 Dovitinib shows cytotoxic effects on OLN-93 cells *in vitro*

As Dovitinib inhibits receptors for essential growth factors such as but not limited to FGFR1, we investigated possible cytotoxic effects of the compound in OLN-93 cells. Using the LDH cytotoxicity assay after 24 hours we found that cells treated with Dovitinib or Dovitinib combined with FGF showed significantly more cytotoxicity than untreated control cells after 24 hours ($P < 0.05$; Fig. 11 C). In contrast to that, FGF as only treatment slightly reduced the cytotoxicity without reaching significance while DMSO as treatment for cells did not cause any differences regarding cytotoxicity compared to control cells (Fig. 11 C).

4.4 Cells treated with Dovitinib show a higher complexity

The morphology of oligodendrocytes correlates with their grade of maturation and thus their capability of myelination as this can only be performed by mature oligodendrocytes featuring a complex morphology (Bögler et al., 1990). After adhering to growth vessel surface OLN-93 cells mainly show a bipolar shape (Fig. 12 A). To investigate the cells morphology after treatment, we took pictures of cells stained with PLP fluorescent antibody after 24 hours of incubation on glass coverslips. Cells with more than two appendices were considered as maturated (Fig. 12 B).

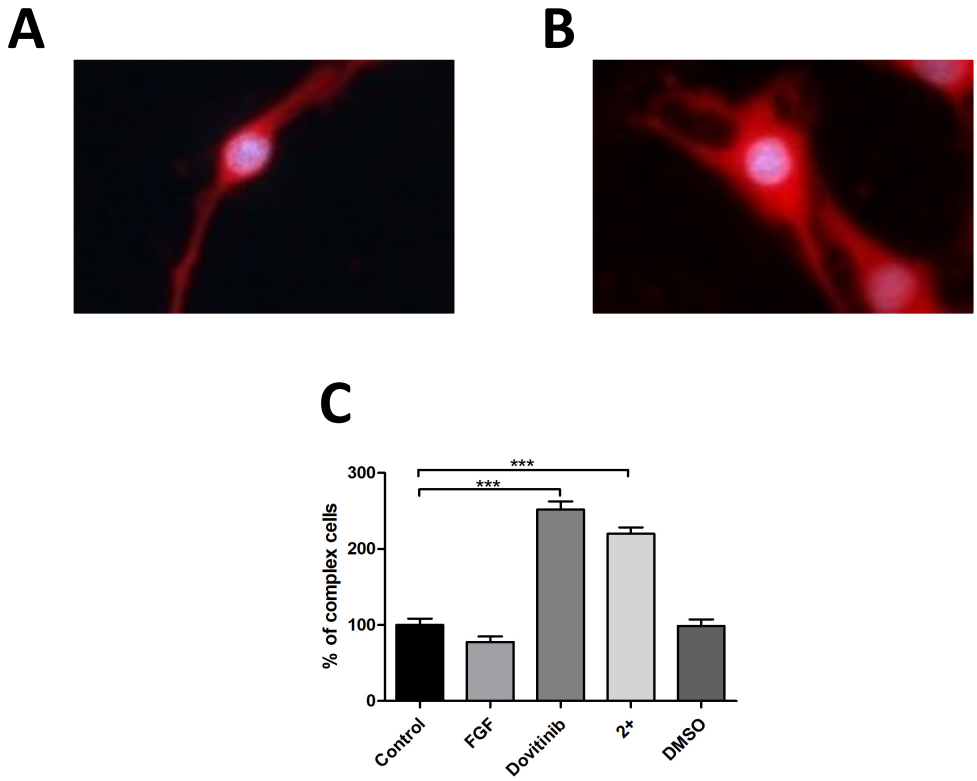

Figure 12 Complexity (A-C) of OLN-93 cells incubated with Dovitinib, FGF, Dovitinib and FGF, or DMSO for 24h. Dovitinib and Dovitinib + FGF treated cells showed a significantly higher proportion of complex cells (C). (A) shows a representative basic OLN93 cell with only two appendices while (B) shows a more complex cell featuring several appendices. Data are presented as mean ± SEM. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$. Values for control cells were normalized to 100 %, all other results were considered in relation to control values.

In cells treated with Dovitinib we observed a significantly higher degree of complex cells compared to control ($P < 0.001$; Fig. 12 C) similar to the cells treated with a combination

of Dovitinib and FGF ($P < 0.001$; Fig. 12 C) whereas treatment with FGF only showed slightly fewer complex cells compared to control without this trend reaching significance. DMSO did not alter the cells morphology (Fig. 12 C).

4.5 Dovitinib decreases the expression of FGFR downstream signals

ERK (extracellular signal-regulated kinases) and Akt (also known as protein kinase B) are key parts of the FGFR downstream signalling pathways (Katoh, 2016). Upon ligand binding, several mechanisms lead to phosphorylation and thus activation of these molecules. To quantify the amount of the phosphorylated and thus activated Akt (pAkt) and ERK (pERK) we performed IF staining (Fig. 13 A for pAkt and 18 A for pERK) and WB with the suitable anti-pAkt (Fig. 13 C) and anti-pERK (Fig. 14 C) antibodies.

After 24 hours of experimental incubation, expression of pAkt was significantly lower in cells treated with Dovitinib, as well as in those treated with Dovitinib and FGF combined compared to control cells. This trend could be observed on the cellular level ($P < 0.001$; Fig. 13 B) as well as on the protein level ($P < 0.005$; Fig. 13 D). On the protein level Dovitinib alone had a stronger effect than the combination with FGF ($P < 0.001$ vs $P < 0.005$) while FGF alone as well as DMS did not significantly affect the expression of pAkt (Fig. 13 B and D).

Analysis of pERK expression after 24 hours incubation revealed a similar pattern of regulation. Treatment with Dovitinib caused a significantly lower expression after 24 hours compared to untreated cells on cellular level ($P < 0.001$; Fig. 14 B) and on protein level ($P < 0.05$; Fig. 14 D). Cells treated with the combination of Dovitinib and FGF showed only a slight decrease in pERK expression which reached significance on cellular level ($P < 0.001$; Fig. 14 B) but not on protein level (Fig. 14 D). As observed in pAkt expression, neither treatment with FGF alone nor DMSO caused any significant changes in pERK levels on cellular or protein level (Fig. 14 B and D).

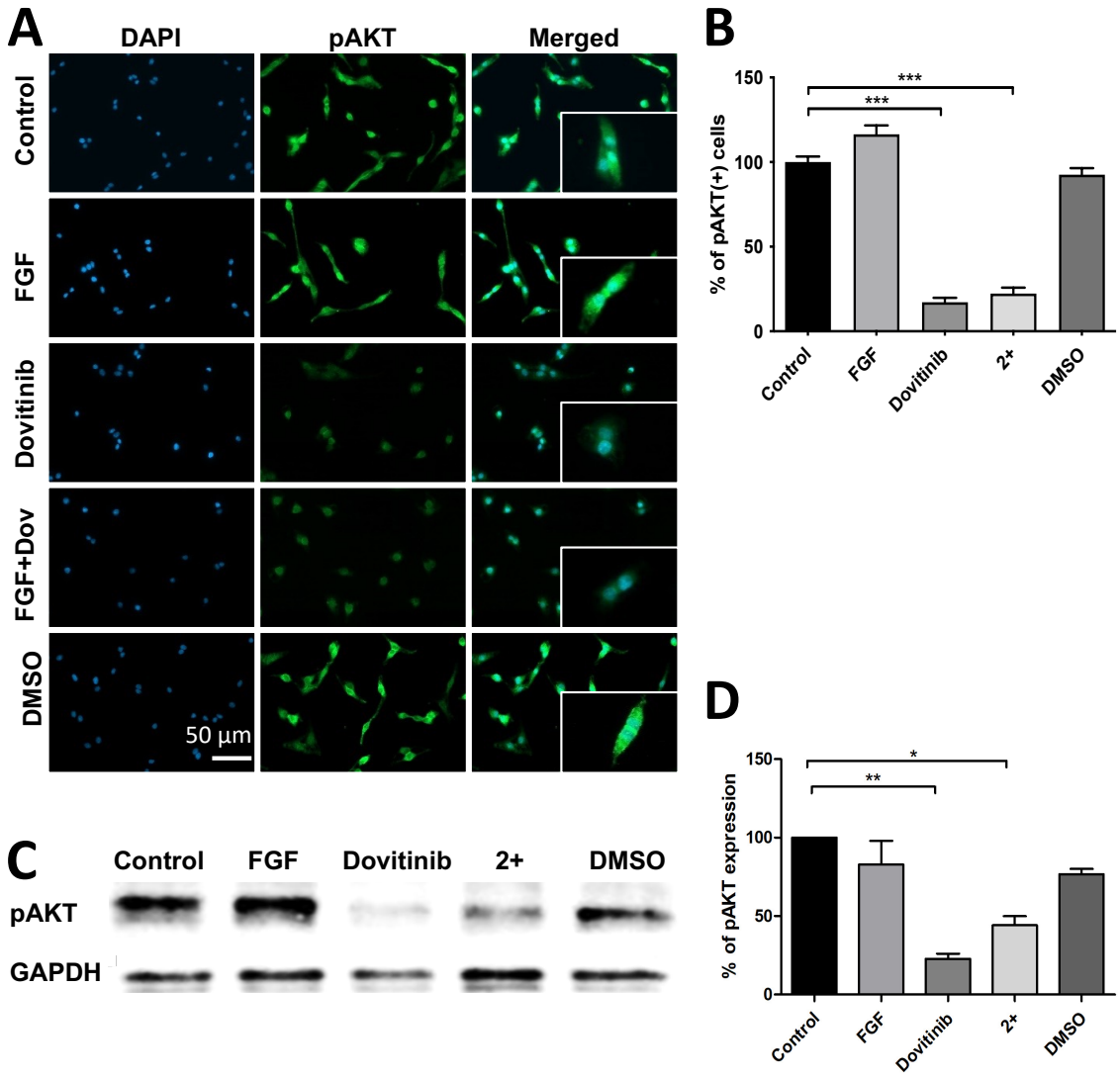

Figure 13 Cellular distribution (A-B) and protein expression (C-D) of pAkt in OLN-93 cells incubated with Dovitinib, FGF, Dovitinib and FGF (2+), or DMSO for 24h. Representative sections of immunofluorescence staining (A). Quantification of staining showed a reduction in pAkt+ cell count for Dovitinib and Dovitinib + FGF2 treated cells (B). Representative Western Blot image of pAkt analysis (C). Quantification of Western Blots showed less pAkt expression in Dovitinib and Dovitinib + FGF2 treated cells (D). Data are presented as mean ± SEM. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$. Values for control cells were normalized to 100 %, all other results were considered in relation to control values.

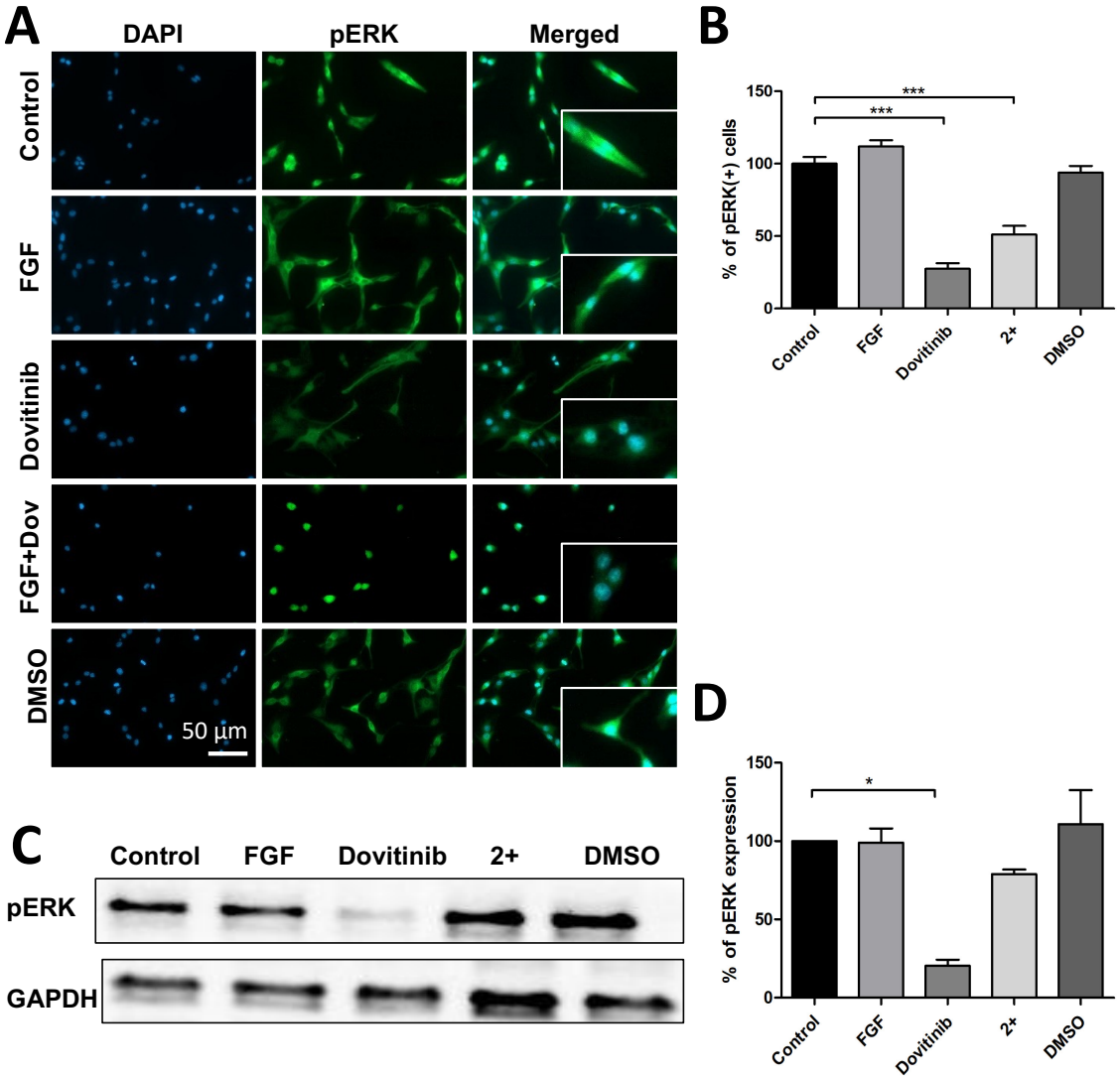

Figure 14 Cellular distribution (A-B) and protein expression (C-D) of pERK in OLN-93 cells incubated with Dovitinib, FGF, Dovitinib and FGF (2+), or DMSO for 24h. Representative sections of immunofluorescence staining (A). Quantification of staining showed a reduction in pERK+ cell count for Dovitinib and Dovitinib + FGF2 treated cells (B). Representative Western Blot image of pERK analysis (C). Quantification of Western Blots showed significantly less pERK expression in Dovitinib treated cells (D). Data are presented as mean ± SEM. * $p < 0.05$, *** $p < 0.001$. Values for control cells were normalized to 100 %, all other results were considered in relation to control values.

4.6 Dovitinib increases the expression of neuroprotective factors

Signalling via the neurotrophin BDNF and its receptor TrkB has recently been proven a positive regulator of myelination *in vitro* and in animal models (Kamali et al., 2020; Rajendran et al., 2018). To investigate any possible regulations of their expression in our FGFR inhibited OLN93 cells, we used anti TrkB and anti BDNF antibodies for Western Blot (Fig. 15 D; 16 D) and IF staining Fig. 15 A; 16 A). Besides we carried out RT-PCR for BDNF (Fig. 15 C) and TrkB (Fig. 16 C) genes. On the cellular level IF staining revealed a significantly higher expression of BDNF in Dovitinib treated cells compared to control cells ($P < 0.005$; Fig. 15 B). A similar result was observed for TrkB: here we found a significant increase of TrkB expression in Dovitinib ($P < 0.001$; Fig. 16 B) and Dovitinib + FGF treated cells ($P < 0.05$; Fig. 16 B). FGF alone and DMSO as treatment did not change the expression of either BDNF or TrkB in IF staining (Fig. 15 B, 16 B).

BDNF gene expression was significantly higher in cells treated with Dovitinib ($P < 0.001$; Fig. 15 C) or Dovitinib + FGF ($P < 0.005$; Fig. 15 B) than in control cells while FGF and DMSO did not show significant differences compared to untreated cells (Fig. 15 B). In protein analysis by Western Blot, we observed a significant increase for BDNF in Dovitinib treated cells ($P < 0.05$; Fig. 15 E) and a slight increase in cells treated with Dovitinib + FGF which failed to reach significance (Fig. 15 E).

TrkB expression on protein level was significantly higher in Dovitinib treated cells ($P < 0.005$; Fig. 16 E) as well as in Dovitinib + FGF treated cells ($P < 0.05$; Fig. 16 E) compared to control cells. In contrast to that, no significant regulation of TrkB gene expression could be observed in RT PCR after compound treatment (Fig. 16 C).

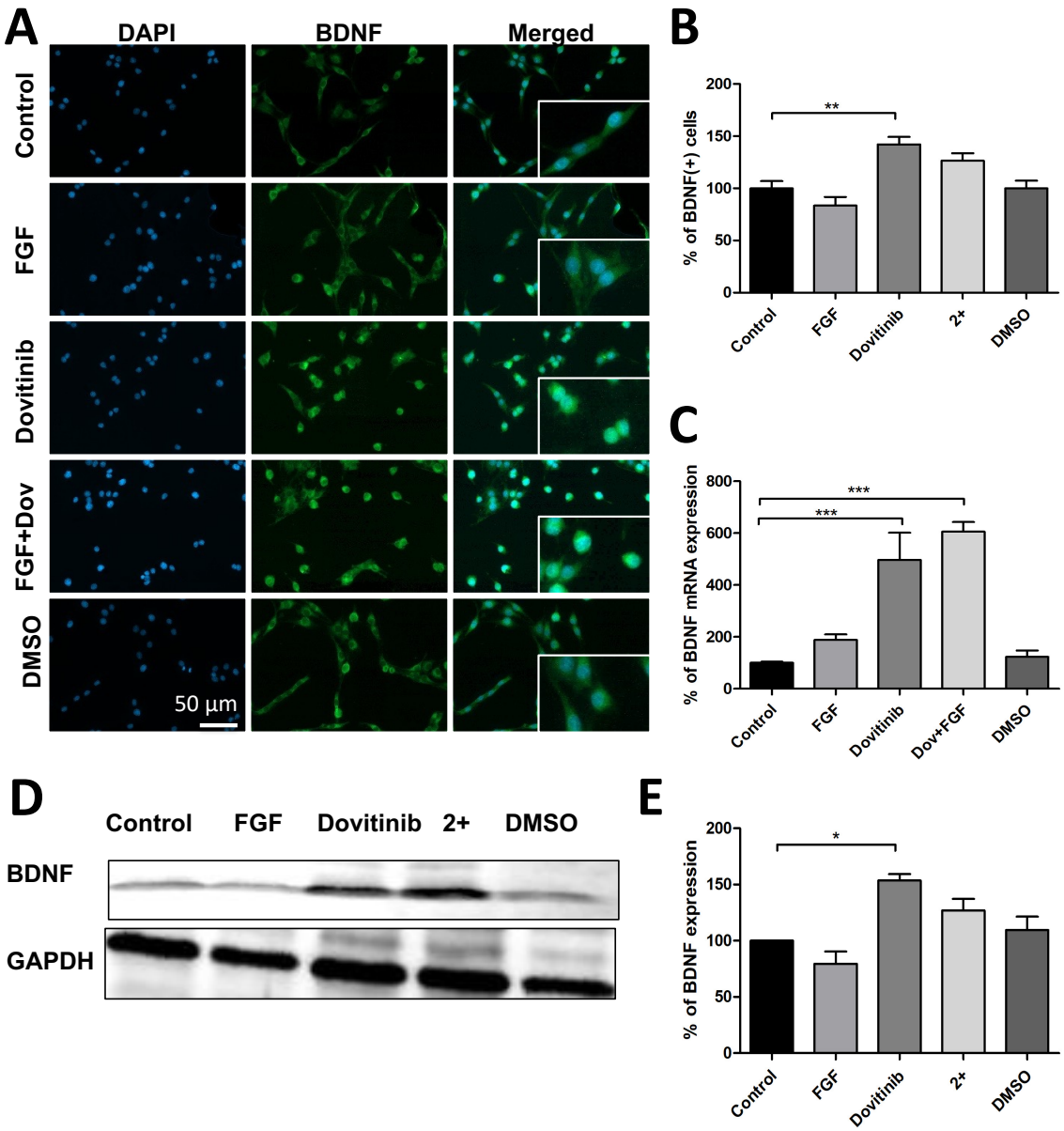

Figure 15 Cellular distribution (A-B), gene expression (C) and protein expression (D-E) of BDNF in OLN-93 cells incubated with Dovitinib, FGF, Dovitinib and FGF (2+), or DMSO for 24h. Representative sections of immunofluorescence staining (A). Quantification of staining showed an increase in BDNF+ cell count for Dovitinib treated cells (B). PCR analysis of BDNF genes revealed higher BDNF mRNA expression in Dovitinib and Dovitinib + FGF2 treated cells (C). Representative Western Blot image of BDNF analysis (D). Quantification of Western Blots showed higher BDNF expression in Dovitinib treated cells (E). Data are presented as mean ± SEM. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$. Values for control cells were normalized to 100 %, all other results were considered in relation to control values.

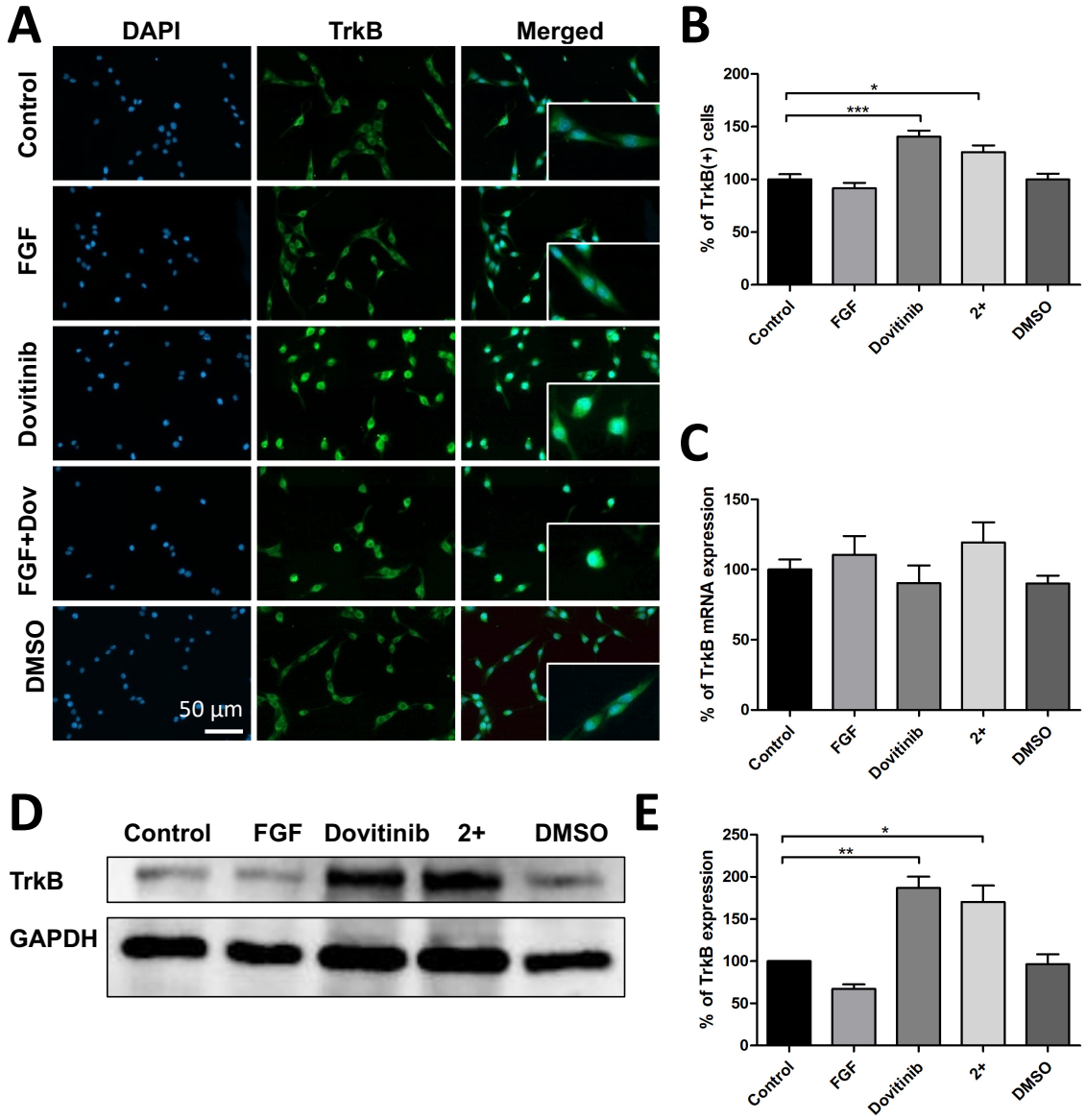

Figure 16 Cellular distribution (A-B), gene expression (C) and protein expression (D-E) of TrkB in OLN-93 cells incubated with Dovitinib, FGF, Dovitinib and FGF (2+), or DMSO for 24h. Representative sections of immunofluorescence staining (A). Quantification of staining showed an increase in TrkB+ cell count for Dovitinib and Dovitinib + FGF2 treated cells (B). PCR analysis of TrkB genes (C). Representative Western Blot image of TrkB analysis (D). Quantification of Western Blots showed higher TrkB expression in Dovitinib and Dovitinib + FGF2 treated cells (E). Data are presented as mean ± SEM. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$. Values for control cells were normalized to 100 %, all other results were considered in relation to control values.

4.7 Expression of myelin proteins is increased by Dovitinib

PLP and CNPase both are myelin proteins and represent an important part of the myelin produced by oligodendrocytes within the CNS (Snaidero & Simons, 2017). We therefore examined the expression of these proteins on cellular level by IF staining (Fig. 17 A and 18 A), protein level by Western Blot (Fig. 17 D and 18 D) and RNA level by RT-PCR (Fig. 17 C and 18 C) to draw conclusions to a possible change in myelinating capacities of the OLN-93 cells.

IF staining with anti-PLP and anti-CNPase antibodies after 24 hours revealed a significantly higher expression of these proteins in Dovitinib and Dovitinib + FGF treated cells in comparison with untreated cells (PLP: $P < 0.001$; Fig. 17 B; CNPase: $P < 0.001$; Fig. 18 B). FGF alone did not cause significant changes in PLP or CNPase expression on cellular level even though we could observe a slight decrease for CNPase which did not reach a significant level (Fig. 18 B). The trends observed in Western Blot analysis were quite similar to the ones from IF staining despite the fact that for both proteins it only reached a significant level for Dovitinib compared to control (PLP: $P < 0.005$; Fig. 17 E; CNPase: $P < 0.005$; Fig. 18 E). The trend towards a lower CNPase expression after FGF treatment observed on cellular level was significant on the protein level ($P < 0.005$; Fig. 18 E) while other treatments did not cause significant changes in myelin protein expression.

Analysis of RNA levels by RT-PCR for CNPase genes showed a significantly higher gene expression after Dovitinib treatment compared to control cells ($P < 0.05$; Fig. 18 C) and a significantly lower expression after FGF treatment ($P < 0.05$; Fig. 18 C) while the combination of both, just like DMSO treatment, did not alter the CNPase gene expression after 24 hours. PLP gene expression was significantly higher in Dovitinib ($P < 0.001$; Fig. 17 C) and Dovitinib + FGF ($P < 0.001$; Fig. 17 C) treated cells compared to untreated cells. The trend towards less PLP gene expression after FGF treatment compared to control failed to reach significance (Fig. 17 C) and DMSO did not alter the PLP gene expression at all (Fig. 17 C).

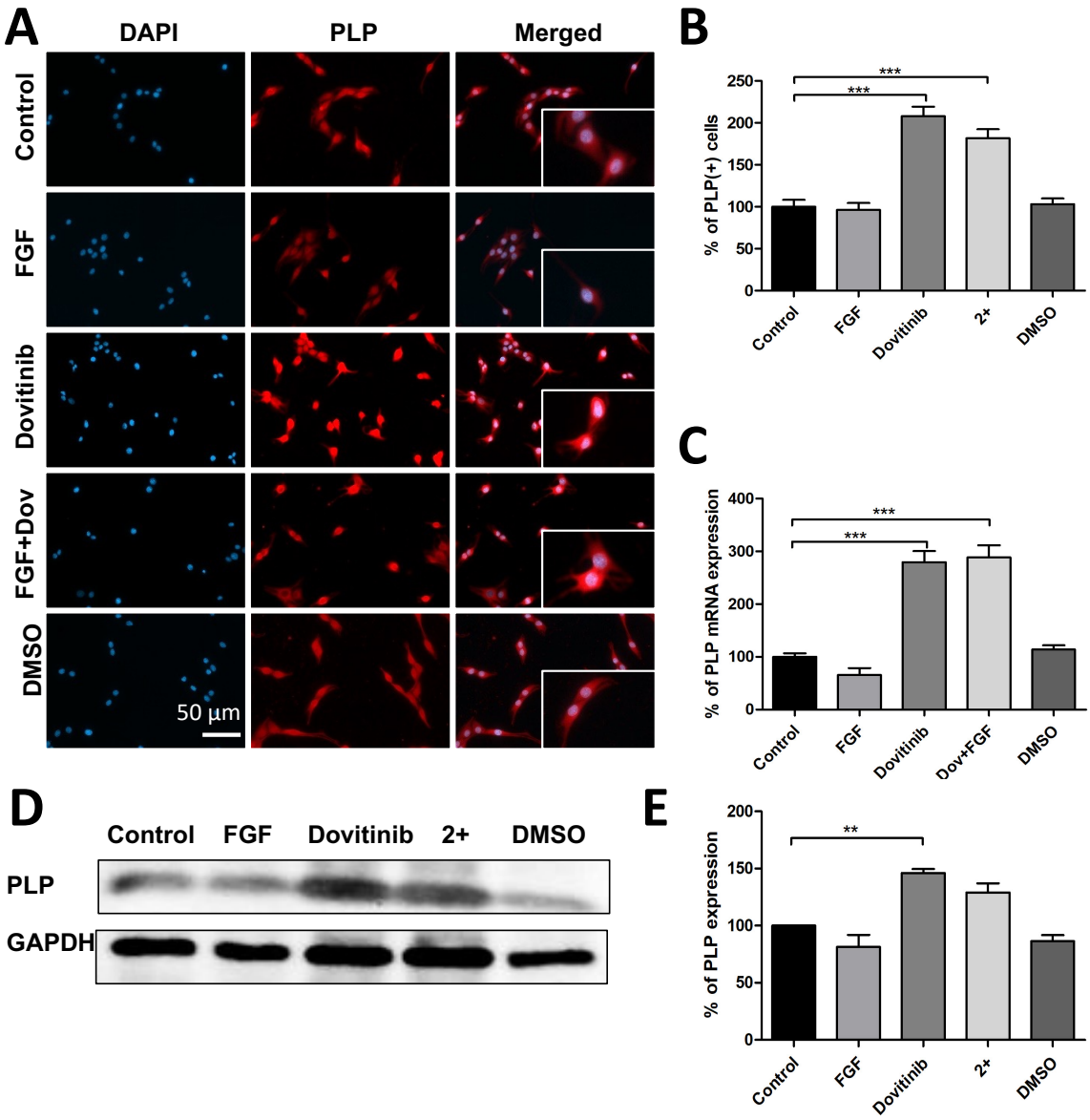

Figure 17 Cellular distribution (A-B), gene expression (C) and protein expression (D-E) of PLP in OLN-93 cells incubated with Dovitinib, FGF, Dovitinib and FGF (2+), or DMSO for 24h**.** Representative sections of immunofluorescence staining (A). Quantification of staining showed an increase in PLP+ cell count for Dovitinib and Dovitinib + FGF2 treated cells (B). PCR analysis of BDNF genes revealed higher PLP mRNA expression in Dovitinib and Dovitinib + FGF2 treated cells (C). Representative Western Blot image of PLP analysis (D). Quantification of Western Blots showed higher PLP expression in Dovitinib treated cells (E). Data are presented as mean ± SEM. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$. Values for control cells were normalized to 100 %, all other results were considered in relation to control values.

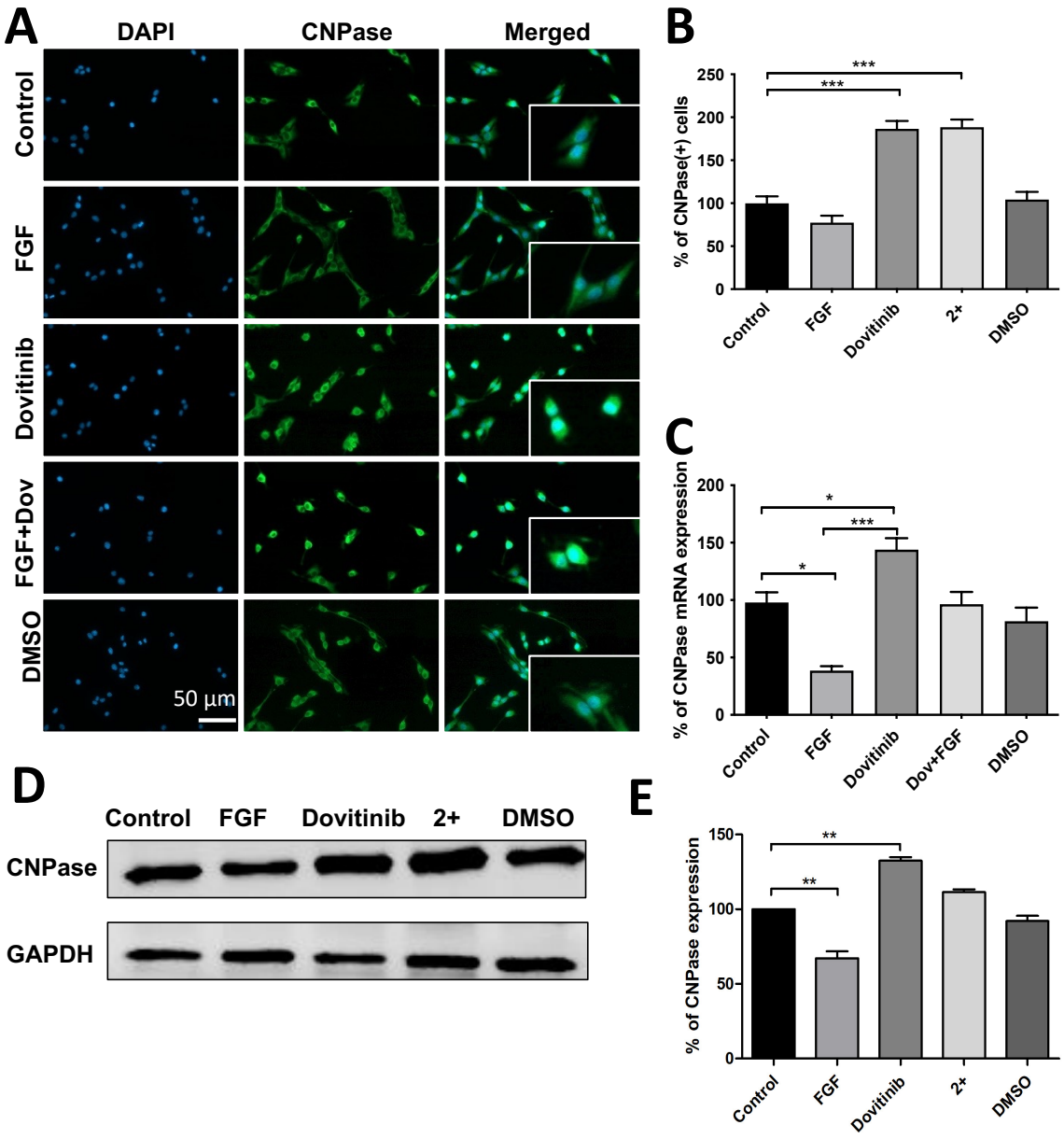

Figure 18 Cellular distribution (A-B), gene expression (C) and protein expression (D-E) of CNPase in OLN-93 cells incubated with Dovitinib, FGF, Dovitinib and FGF (2+), or DMSO for 24h. Representative sections of immunofluorescence staining (A). Quantification of staining showed an increase in CNPase+ cell count for Dovitinib and Dovitinib + FGF2 treated cells (B). PCR analysis of CNPase genes revealed higher CNPase mRNA expression in Dovitinib treated cells (C). Representative Western Blot image of CNPase analysis (D). Quantification of Western Blots showed higher CNPase expression in Dovitinib treated cells (E). Data are presented as mean ± SEM. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$. Values for control cells were normalized to 100 %, all other results were considered in relation to control values.

4.8 Treatment with Dovitinib decreases gene expression of myelin inhibitor

Semaphorin 3A (SEMA3A) and transforming growth factor ß (TGF-ß) are known to suppress (re-)myelination by impeding OPCs migration and differentiation (Eixarch, Gutiérrez-Franco, Montalban & Espejo, 2013). We therefore assessed their expression on mRNA level in our FGFR inhibited oligodendrocytes (Fig. 19 A and 19 B).

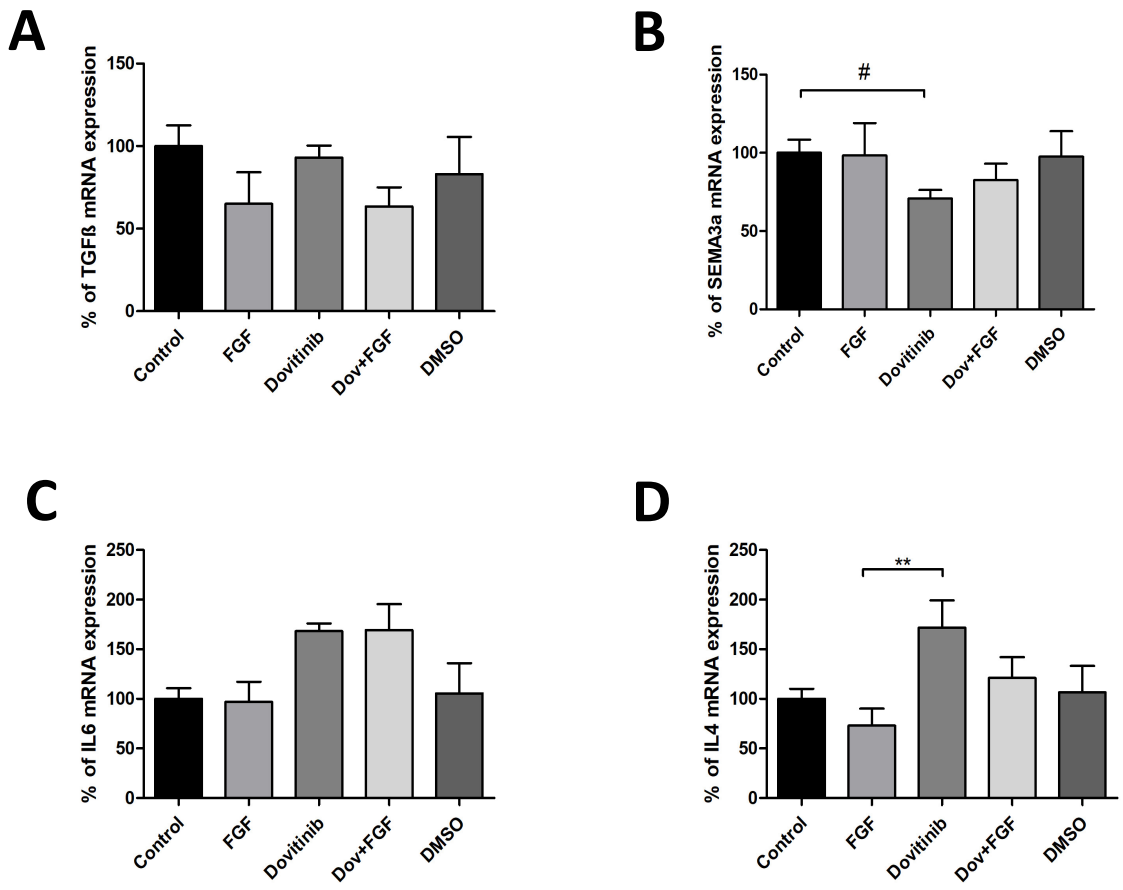

Figure 19 mRNA levels of TGFβ1 (A), SEMA3A (B), IL6 (C) and IL4 (D) in OLN-93 cells incubated with Dovitinib, FGF, Dovitinib and FGF (2+), or DMSO for 24h. Expression of SEMA3A (B) mRNA was reduced by Dovitinib (B). Dovitinib and FGF2 did not significantly regulate TGFβ (TGFB1) mRNA (A). Dovitinib significantly increased IL4 mRNA expression (D). Data are presented as mean ± SEM. * $p < 0,05$, ** $p < 0.005$, # $p < 0,05$ Mann Whitney test. Values for control cells were normalized to 100 %, all other results were considered in relation to control values.

For SEMA3A we observed a significantly lower expression in Dovitinib treated cells compared to control cells after 24 hours ($P < 0.05$; Fig. 19 B) while Dovitinib in combination with FGF caused only a slight decrease that failed to reach a significant level

(Fig. 19 B). FGF alone as well as DMSO did not show any differences in SEMA3A gene expression compared to control (Fig. 19 B).
Regarding the TGF-ß gene expression we could not observe any significant regulations linked to Dovitinib treatment in RT-PCR (Fig. 19 A).

4.9 Higher gene expression of interleukins 4 and 6 after Dovitinib treatment in OLN-93 cells

The interleukins 4 and 6 (IL-4 and IL-6) both represent important elements in mammalian immune reactions. While IL-6 mostly acts in a pro-inflammatory manner and plays a key role in autoimmune diseases (Tanaka, Narazaki & Kishimoto, 2014), it may also decrease immune reactions through various mechanisms (Ho & Miaw, 2016). Interleukin 4 is generally considered an anti-inflammatory cytokine (Ho & Miaw, 2016). We examined the gene expression of these exemplary two cytokines to gather information about general inflammatory conditions after Dovitinib treatment in OLN-93 cells (Fig. 19 C and 19 D).

Evaluation of the gene expression revealed a significantly higher IL-4 mRNA expression in cells treated with Dovitinib compared to FGF treated cells after 24 hours of incubation ($P < 0.005$; Fig. 19 D). In combination with FGF, the same trend could be observed without reaching the significant levels (Fig. 19 D) whereas cells incubated in FGF trend to a lower IL-4 gene expression compared to untreated cells, this trend did however fail to reach significance (Fig. 19 D).

For IL-6 there occurred no significant changes in gene expression between Dovitinib treated and control cells even though there was a trend towards a slightly higher gene expression (Fig. 19 C). Once again DMSO as solvent for treatments did not alter gene expression in cells compared to untreated cells (Fig. 19 C).

5. Discussion

The crucial importance of signals mediated through the FGF/FGFR system for the organism's development and homeostasis is well known (Ornitz & Itoh, 2015). Despite these physiological functions, current investigations of FGFR signalling in neurodegenerative models suggested that these receptors along with their ligands play a key role in pathophysiology of neurodegenerative diseases such as multiple sclerosis (Kamali et al., 2020; Rajendran, Böttiger, Stadelmann et al., 2021). In spite of these new findings the exact pathophysiological mechanisms induced by FGF/FGFR signals remain unclear and leave room for further investigation.

In the present study we investigated the effect of non-selective FGFR blocking using Dovitinib in oligodendrocytes *in vitro*. Our findings suggest that inhibition of FGF signalling in these cells raises their neuroprotective capacities by activation of BDNF/TrkB signals and subsequent increase of myelin proteins *in vitro*. *In vivo* this approach could be used in the treatment of demyelinating diseases including multiple sclerosis. Here the effects of FGFR blocking might target the neurodegeneration. Furthermore it might inhibit the initial demyelination of axons and enhance remyelination within the CNS for all these processes represent major hallmarks of MS pathology and are not yet sufficiently targeted in therapy (Compston & Coles, 2008).

5.1. FGFR1 protein and gene expression

Investigations of biopsies from MS patients in accordance with animal models showed that the expression of FGF receptor 1 (FGFR1), as one of the essential parts in FGF signalling pathway, is upregulated under pathological conditions in MS lesions (Clemente et al., 2011). *In vitro* results from our OLN-93 oligodendrocytes showed a decreased expression of this receptor on the cellular level after FGFR1 inhibiting treatment with Dovitinib (Fig. 10 A and 10 B). These results go along with other trials, where Dovitinib or similar drugs were used on different, mostly cancer, cell lines. Fumarola et al. showed a decrease of FGFR1 expression after Dovitinib treatment in squamous cell lung cancer cells (Fumarola et al., 2017), while Zhao et al. used AZD4547, a more selective FGFR

inhibitor, which decreased the FGFR1 expression in SKBR3 breast cancer cell lines (Zhao et al., 2017).

On the gene level in PCR tests we could not observe the same results. Here the treatment with Dovitinib did not seem to affect the FGFR1 gene expression after 24 hours of incubation (Fig. 10 C). Since Dovitinib is a non-selective TKI inhibitor, which also targets various other growth factor receptors besides FGFR1 (Fumarola et al., 2017), it is possible that the effect on FGFR1 gene expression might be influenced by signals mediated through other pathways. Another factor that might affect the results of PCR test is our time of observation. In our study we examined a period of 24 hours, which might be too short to see subsequent regulation of FGFR1 mRNA expression after receptor blockade. Furthermore, differences between the cellular expression of FGFR1, which is decreased in Dovitinib treated cells, and the genetic level, which does not seem to be affected by Dovitinib treatment within the duration of our experiment (Fig. 10), can be due to a compensatory upregulation on the mRNA level after cellular downregulation of FGFR1. It might, however, also be explained by a posttranscriptional degradation of FGFR1 leading to a decrease in cellular expression without affecting the FGFR1 mRNA levels.

Besides our findings *in vitro* there are other aspects of FGF receptor effects supporting their proinflammatory role in demyelinating diseases. Rajendran et al. showed that a cell specific knockout of FGFR1 in oligodendrocytes led to a decrease of immune cell infiltration into the cerebellum and less inflammatory activity in an EAE animal model of MS (Rajendran, R., Rajendran, V. et al., 2021). This supports our notion of a neuroprotective and anti-inflammatory effect of oligodendrocyte specific FGF receptor inhibition even beyond the regulations on protein levels. Despite the unquestionably important role of FGF signals for early myelination and other developmental processes (Fortin, Rom, Sun, Yayon & Bansal, 2005) our results along with earlier EAE studies (Kamali et al., 2020; Rajendran et al., 2018) suggest a different, rather detrimental, effect in other microenvironments such as chronic neuroinflammation as seen in EAE or MS.

5.2. Dovitinib exerts cytotoxic effects on oligodendrocytes *in vitro*

Treatment of the OLN93 cells with Dovitinib exerted a significant cytotoxic effect *in vitro* (Fig. 11 C). Similar effects were observed in other studies using the same compound on different cell lines (Gaur et al., 2014; Li & Yao, 2013; Liang, Liu, Wu, Cai & Li, 2012). Like the proliferation of cells, their survival also depends on growth factor signals like FGF (Turner & Grose, 2010) so that blocking of these signals can explain the increase of cytotoxicity under treatment. Given the broad spectrum of receptor inhibition exerted by Dovitinib, a certain cytotoxicity can be expected after administration of the drug. This possible side effect must be considered for further investigation of this compound for example in animal studies.

We do, however, have to keep in mind that our experiment took place *in vitro* while *in vivo* there are various other effectors present modulating cytotoxic effects on the cells so that we cannot transfer the cytotoxic situation from our cells to *in vivo* without further studies.

5.3. Proliferation and maturation of oligodendrocytes treated with Dovitinib

The ultimate differentiation of immature oligodendrocytes and their progenitors into mature OLs is an important requirement for the process of myelination. The maturation is therefore crucial during remyelination as an answer to pathological demyelination in multiple sclerosis. It has further been shown that this differentiation and thus the remyelination as repair process is impaired in MS (Franklin & Kotter, 2008).

Bögler et al. showed that the differentiation of oligodendrocyte progenitor cells to myelin producing mature OLs is associated with a multipolar morphology. In this context we considered more complex cells featuring two or more cellular appendices as more differentiated and mature (Bögler et al., 1990) (Fig. 12 A and 12 B). Initiation of proliferation is a major function of FGF/FGFR signalling in general (Ornitz & Itoh, 2015). Following the blockade of FGFR by Dovitinib we observed a decrease in cell proliferation independent of additional application of FGF2 as growth factor compared to untreated cells (Fig. 11 A and 11 B). This reduced proliferation in cells treated with

Dovitinib was also observed in other, mostly cancer, cell lines using the same compound (Gaur et al., 2014; Lee et al., 2015). Impaired proliferation can be considered as an effect of the blocking of vital cellular functions mediated through the FGF signalling such as survival and proliferation (Turner & Grose, 2010). Also the blocking of other growth factor receptors like PDGFR or VEGFR by the non-selective inhibitor Dovitinib may enhance the negative effects on the cell proliferation. Supporting the antiproliferative effect of our FGFR inhibiting compound, FGF treatment alone exerted a significant proliferative effect (Fig. 11 A).

Unlike the cited publications with cancer cell lines, where a suppression of cell growth is a desirable outcome, this decrease of proliferation is not the primary intention of our experiment. When considering myelination within the CNS, not only the number of oligodendrocytes but also their stage of development is important. Thus, in the context of neurodegenerative diseases, maturation of oligodendrocytes is at least equally important as their proliferation. In view of this we observed a significantly higher proportion of complex mature cells when treated with Dovitinib compared to untreated control cells (Fig. 12 C). Other studies dealing with oligodendrocyte development also found a negative correlation between proliferation and migration on the one hand and maturation as well as differentiation on the other hand showing that a reduced proliferation of these cells can be accompanied by an increase in their maturation (Liu et al., 2018).

Mohan et al. showed that FGF1 application despite promoting the cells proliferation and migration impaired the differentiation of primary oligodendrocytes (Mohan et al., 2014). Going along with these findings our oligodendrocytes treated solely with FGF also showed a trend towards less complexity (Fig. 12). In accordance with this assumption our FGFR inhibited cells with a reduced rate of proliferation showed a more complex structure and featured a higher grade of maturation (Fig. 12 C). This is an important finding as the maturation of oligodendrocytes is essential for producing myelin sheaths and thus perform myelination within the CNS (Snaidero & Simons, 2017). Considering this correlation, a trend towards more mature cells might, despite a multitude of other factors affecting the myelin formation, theoretically prevent demyelination and enhance remyelination under pathological circumstances *in vivo*. This idea is sustained by the increase of myelin proteins in cells treated with Dovitinib as further explained below (Fig. 17 and 18).

Given the findings of our study including the ameliorated differentiation of OLN-93 cells treated with Dovitinib compared to control cells (Fig. 12 C), FGF/FGFR signalling can be considered a negative key factor for the process of OL differentiation and subsequent remyelination. Therefore, inhibition of proliferation is another finding suggesting that FGFR may be an interesting target for further therapy of demyelinating diseases including MS.

5.4. Reduced pERK and pAkt expression after Dovitinib treatment

The proteins Akt and ERK are two of the main actors of downstream cascades activated by FGF/FGFR signalling. Upon ligand binding, two receptor molecules perform a dimerization, which activates kinase domains and subsequently phosphorylates, and thus activates, downstream signals including ERK and Akt (Ornitz & Itoh, 2015). Our study showed a lower pAkt and pERK expression in cells treated with Dovitinib compared to control cells (Fig. 13 and 14), which matches similar *in vitro* experiments using Dovitinib along with other FGFR inhibiting compounds on various cell lines (Gaur et al., 2014; Liang et al., 2012). Taking pERK and pAkt as key downstream signalling components of the FGF / FGFR pathway, their downregulation after FGFR inhibition is an appropriate reaction to be expected. However, in recent *in vivo* studies a selective FGF receptor knockout in mice caused an increased phosphorylation of ERK and Akt that was correlated to a positive outcome in models of neurodegenerative diseases with an increase of myelination (Flores et al., 2008; Rajendran et al., 2018). In contrast to this, our findings show a lower pAkt (Fig. 13 B and 13 D) and pERK (Fig. 14 B and 14 D) expression after FGFR inhibition suggesting a link between the decrease of ERK and Akt phosphorylation and an increase in myelin proteins as positive marker for myelination.

These differences between the *in vivo* models and our *in vitro* experiments considering the trend of downstream signal phosphorylation could be differently explained. On the one hand *in vivo* models include many extracellular factors that cannot all be reproduced in cell culture but still affect cells in their natural environment. On the other hand, we used a rather unspecific inhibitor which does, unlike a specific knockout *in vivo*, also affect various other receptor tyrosine kinases and thus their downstream signalling. As

ERK and Akt are also part of other receptors signalling pathways, the non-selective blocking of many of these receptors could affect these pathways in a stronger way than targeting one specific receptor by an induced knockout (Rajendran et al., 2018).

It is known that ERK and Akt, along with their activated phosphorylated forms, are essential for oligodendrocyte cell cycle including proliferation (Goetz & Mohammadi, 2013). Nevertheless, our results in combination with other recent studies suggest other pathways to be crucial for differentiation and ultimately myelination exerted by OLs. As described below, the BDNF/TrkB signalling pathway seems to represent another important regulator of myelination as it is, in contrast to pERK and pAkt, upregulated like the increased myelin proteins.

5.5. Increased expression of BDNF and TrkB in oligodendrocytes treated with Dovitinib

The neurotrophic factor BDNF and its receptor TrkB play key roles in the differentiation and migration of oligodendrocytes along with the myelin protein expression *in vitro* and *in vivo* (Vondran et al., 2011). Other studies investigating models of neurodegenerative diseases associated an increased expression of BDNF and TrkB with a positive clinical outcome (Rajendran et al., 2018). Additionally, the activation of TrkB through different agonists also reduced clinical symptoms in similar models (Makar et al., 2016). In agreement with these findings our results after Dovitinib treatment showed increased expression of both BNDF (Fig. 15) and TrkB (Fig. 16) in combination with a higher maturation of oligodendrocytes and increased myelin proteins compared to untreated cells. Taken together, all these findings suggest that BDNF/TrkB signalling may influence the development and function of oligodendrocytes and subsequently also the role of mature oligodendrocytes in maintaining axonal integrity within the CNS. Furthermore, the higher expression of BDNF and TrkB in our FGFR blocked cells indicates a link between the signalling via FGF/FGFR and the expression of BDNF and TrkB which has also been observed in previous studies targeting FGFR1 *in vivo* (Rajendran et al., 2018). This is especially interesting in combination with the decreased pERK and pAkt levels in our treated cells as it indicates BDNF/TrkB pathway as an independent and potentially compensatory pathway.

Another role of oligodendrocyte produced BDNF in the CNS is a supportive role for axons as this substance enhances the function of neurons *in vitro* (Chamberlain & Nanescu, 2016). Thus the elevated levels of BDNF and TrkB after FGFR inhibition might be another neuroprotective way of FGFR inhibition. As already discussed considering the FGFR1 expression, differences between cellular level and genetic level in the TrkB expression might be due to a compensatory regulation on the mRNA level following the cellular alteration of TrkB expression. The difference might however again be explained by the experimental duration being too short to observe further changes in mRNA level.

5.6. Increased PLP and CNPase expression after Dovitinib treatment

The proteins PLP and CNPase both represent major components of mammalian myelin within the CNS (Snaidero & Simons, 2017). Given their important role for the initial myelination and for sustaining existing myelin sheaths under physiological and pathological circumstances (Snaidero & Simons, 2017), we considered them to be markers for (re-)myelination *in vivo*. The examination of these myelin proteins under Dovitinib treatment showed a higher expression in treated cells compared to control cells (Fig. 17 and 18). These results go along with those from earlier studies where the stimulation of FGFR by application of FGF-2 to oligodendrocytes caused a downregulation of myelin proteins CNPase, PLP and MBP (Bansal, 2002). This downregulation of myelin proteins after application of FGF could also be observed in our cells for CNPase expression after FGF2 treatment (Fig. 18 C and 18 E). All these findings indicate a detrimental role of FGF/FGFR signalling for the expression of myelin proteins.

Other studies dealing with demyelinating models *in vivo* showed a correlation between the expression patterns of myelin proteins and disease severity. Rajendran et al. as well as Kamali et al. showed in EAE models that an upregulation of myelin proteins after certain FGFR knockouts was associated with a milder disease course (Kamali et al., 2020; Rajendran et al., 2018). For human disease it is also known that patients who feature remyelination on a larger scale show less severe symptoms and disability (Skaper, 2019). Regarding this correlation between myelin protein expression and disease severity in MS or animal models, we considered the increased expression of myelin proteins in our

Dovitinib treated cells a positive predictor for disease outcome *in vivo*. If further studies *in vivo* can prove that cells or organisms treated with Dovitinib also increase their myelin protein expression, this might inhibit demyelination and improve remyelination in animal models and ultimately also in human disease.

For some time, the process of remyelination has been a target for MS therapy strategies but while earlier approaches like neural stem cell transplantation have already been abandoned (Franklin & Kotter, 2008) the idea to target growths factors and their receptors in order to enhance the intrinsic remyelination might be a more promising therapeutic option.

5.7. Expression of myelin inhibitors SEMA3A and TGF-ß

Class 3 semaphorins including SEMA3A are known to suppress myelination *in vivo* (Eixarch et al., 2013). The mechanisms behind this suppression are at least partially similar to the ones postulated for FGF / FGFR signalling described above. They are said to inhibit the later differentiation of oligodendrocytes into mature myelinating cells. Along with other suppressors of myelination like TGF-ß, SEMA3A by this means negatively regulates the extent of myelination within the CNS (Eixarch et al., 2013).

Matching these recent findings, our present study showed an increased expression of myelin proteins accompanied by a lower expression of SEMA3A in cells treated with Dovitinib compared to control cells (Fig. 19 B). Similar trends regarding potential inhibitors of myelination like SEMA3A were observed in other studies *in vitro* as well as *in vivo*. Rajendran et al. and Kamali et al. both showed that a selective FGFR 1 or FGFR 2 knockout in oligodendrocytes in demyelinating models as well as FGFR inhibition *in vitro* can reduce the expression of myelin inhibiting proteins (Kamali et al., 2020; Rajendran et al., 2018). Again, differences between our *in vitro* results and other studies *in vivo*, especially considering the TGF-ß expression that was not altered in our study (Fig. 19 A), can partly be explained by the *in vitro* setting that cannot imitate all the crucial factors of *in vivo* myelination. Considering SEMA3A as an inhibitor of myelination these results nonetheless support the positive effects of Dovitinib treatment on myelination capacities of oligodendrocytes *in vitro*.

5.8. Interleukin expression after Dovitinib treatment

Interleukins play crucial roles in the regulation of the mammalian immune system and thus also in autoimmune diseases such as multiple sclerosis (Wang et al., 2018). The interleukins 4 and 6 are both said to possibly exert immunosuppressive effects among other functions (Ho & Miaw, 2016) and might therefore be helpful in prevention of the autoimmune processes in the pathogenesis of MS. Especially IL-4 is responsible for several anti-inflammatory mechanisms (Dong, Fu, Ji, Li & Gu, 2018) while IL-6 is considered to also act in a rather proinflammatory way (Tanaka et al., 2014). Given the anti-inflammatory functions of IL-4 an upregulation, as observed in Dovitinib treated cells compared to untreated cells (Fig. 19 D), might act immunosuppressive and thus supports a possible neuroprotective effect of FGFR blockade. It therefore provides another explanation of the observed positive effects of FGFR blocking *in vivo* (Kamali et al., 2020; Rajendran, Böttiger, Stadelmann et al., 2021; Rajendran et al., 2018).

In our study we measured the expression of interleukins by OLN-93 cells; *in vivo* there are, however, many different cell types such as primary immune cells like T-cells that contribute to the level of cytokines and interleukins forming a complex system of pro- and anti-inflammatory signals. Taking these complex interactions into consideration, our observations in isolated oligodendrocytes cannot represent all aspects that have to be considered *in vivo*. Furthermore, we must bear in mind that the expression of interleukins can also be affected by cell death caused by cytotoxic effects of our drug. All in all, the expression of interleukins in OLN-93 cells treated with Dovitinib can point out another possible positive effect regarding autoimmune diseases including MS. Meanwhile, these findings must be considered as part of a highly complex pathophysiological system with countless other influences *in vivo*.

5.9. Conclusions

In vitro, the unselective FGFR inhibitor Dovitinib showed interesting effects on OLN-93 oligodendrocytes including a more complex phenotype as well as a higher expression of myelin proteins. It did, however, impair the proliferation of OLN-93 cells and furthermore exerted cytotoxic effects (Fig. 11 A and B) possibly by blocking crucial cell

signalling mechanisms. Despite these rather negative effects concerning proliferation other recent studies showed that for the process of myelination not only the cell number but also their grade of maturation is essential to sufficiently perform myelination (Skaper, 2019). As already described above, it is further claimed that there is a negative correlation between proliferation and maturation of oligodendrocytes and their precursors (Skaper, 2019). In accordance with these hypotheses our OLN-93 cells, even though they were impaired in proliferation, showed a higher grade of maturation (Fig. 12) along with more myelin protein (Fig. 17 and 18) after Dovitinib treatment. We interpret these changes in our oligodendrocyte cell line as indicator for higher myelinating capacities *in vitro* and ultimately *in vivo*.

To gain better understanding of the underlying mechanisms of these alternations in myelin protein expression we examined several downstream signalling pathways linked to FGFR signalling. ERK and Akt along with their activated forms (pERK and pAkt) are known to be important parts of FGFR signalling (Turner & Grose, 2010). Following FGFR blockade in our OLN-93 cells, the expression of pERK (Fig. 14) and pAkt (Fig. 13) was reduced compared to control cells. The findings considering the regulation of pERK and pAkt after FGFR blocking are however inconsistent between several *in vitro* and *in vivo* studies. Rajendran et al. and Kamali et al. found an increase of downstream signalling molecules after FGFR blocking (Kamali et al., 2020; Rajendran et al., 2018). Several *in vitro* studies found similar results as we did (Gaur et al., 2014; Liang et al., 2012). The discrepancies of these results can be ascribed to different circumstances *in vivo* and *in vitro*, it might yet also provide information on other pathways regulating the differentiation and subsequent myelination. One of these pathways relevant for myelination might contain BDNF along with its receptor TrkB. This complex is substantial for oligodendrocyte migration differentiation and ultimately myelin protein expression (Vondran et al., 2011). Along with our results (Fig. 15 and 16) the studies mentioned above all showed an increase in BDNF / TrkB signalling independent of the differences in pERK and pAkt expression.

After pathological demyelination within the CNS a complex repair process is initiated to perform compensatory remyelination (Franklin & Kotter, 2008). For some time it has been known that this repairing process is insufficient in patients suffering from MS which can explain progressive neurodegeneration and thus irreversible disability (Gruchot et al.,

2019). *In vivo* studies targeting various FGF receptors in demyelinating models showed an increase of myelin proteins associated with a milder disease course (Kamali et al., 2020; Rajendran et al., 2018). This indicates a potentially ameliorated remyelination after FGFR inhibition. In accordance with these findings our FGF receptor inhibited oligodendrocytes expressed more myelin proteins than untreated cells (Fig. 17 and 18) referring to a possibly enhanced remyelination *in vivo*.

After FGFR blockade by Dovitinib our OLN93 cells showed an increase in myelin proteins (Fig. 17 and 18) combined with a more complex phenotype (Fig. 12). Despite the decreased proliferation and cytotoxic effects of the treatment (Fig. 11) these findings indicate higher myelinating capacities of the treated cells as initially hypothesized. In conclusion, even though we only studied a permanent cell line *in vitro*, the idea to selectively inhibit FGF receptor signalling in oligodendrocytes represents a promising approach to target the neurodegeneration in demyelinating diseases like multiple sclerosis.

Summary

Several recent studies indicate a crucial and most likely detrimental role of fibroblast growth factors (FGF) and their receptors (FGFR) in the field of demyelinating diseases including multiple sclerosis. Despite the large field of physiological FGFR functions in development and homeostasis, knockout of these receptors ameliorated the clinical course of animals in demyelinating disease models. Given these new insights into pathophysiology of demyelinating diseases we claimed that medicated FGFR blocking *in vitro* would positively influence myelination and neuroprotection.

While the mentioned animal models show the importance of FGF/FGFR signals for demyelinating diseases, the distinct mechanisms, especially after their inhibition, remain unclear. To evaluate these mechanisms on a cellular level in our study we treated OLN-93 oligodendrocytes with the unselective FGFR inhibitor Dovitinib and examined the expression of downstream signals, myelin proteins and neuroprotective agents as well as the morphological development. In accordance with our hypothesis, OLN-93 cells treated with Dovitinib showed a more complex phenotype accompanied by a higher expression of myelin proteins as well as BDNF and TrkB. Further changes could be seen for FGF/FGFR downstream signals where the expression of pAkt and pERK was reduced in cells treated with Dovitinib. Despite a lower proliferation and some cytotoxic effects of Dovitinib treatment we still assessed these changes of protein expression patterns as beneficial considering overall aspects of myelination and neuroprotection.

Given the complexity of multiple sclerosis as an autoimmune disease, there is a variety of factors to be considered when discussing the influence of FGF/FGFR as a circumscribed signalling pathway. To further evaluate the effects of these *in vivo* influences on myelination there is a need for additional *in vivo* studies. Regarding our *in vitro* results with increased myelin proteins as well as neuroprotective agents and recent studies we do, however, consider the inhibition of FGF receptors a promising approach in future therapy of multiple sclerosis.

Zusammenfassung

Aktuelle Studien weisen auf eine wichtige Rolle der Fibroblasten-Wachstumsfaktoren und ihrer Rezeptoren im Feld der demyelinisierenden Erkrankungen hin. Zu diesen Erkrankungen gehört unter anderem die Multiple Sklerose (MS). Ungeachtet der vielen wichtigen Funktionen dieser Wachstumsfaktoren und ihrer Signalwege in der normalen Entwicklung und Homöostase, führt ein selektiver Knockout der Rezeptoren in Mäusen in einem demyelinisierenden Modell zu einem milderen Krankheitsverlauf. In Anbetracht dieser aktuellen Erkenntnisse gingen wir davon aus, dass eine medikamentöse Blockade von Fibroblasten-Wachstumsfaktor-Rezeptoren *in vitro* sich positiv auf die Myelinisierung und Neuroprotektion auswirken würde.

Während *In-vivo*-Modelle die wichtige Rolle der FGF / FGFR-Signale für demyelinisierende Erkrankungen zeigen, sind die genauen Effekte weiterhin unklar. Um eben diesen Mechanismus auf zellulärer Ebene besser zu verstehen, wurden in unserer Studie OLN-93-Oligodendrozyten mit dem unselektiven FGFR-Inhibitor Dovitinib behandelt und auf die Expression von Signalproteinen, Myelin-Proteinen sowie die morphologische Entwicklung untersucht. In Übereinstimmung mit unserer Hypothese, zeigten die mit Dovitinib behandelten Zellen einen komplexeren Phänotyp mit einer höheren Expression von Myelin-Proteinen sowie einer Erhöhung der neuroprotektiven Proteine BDNF und TrkB. Weitere Unterschiede im Vergleich zu Kontrollzellen fanden sich bei den Signalmolekülen Akt und ERK, hier zeigte sich eine geringere Expression der aktivierten Formen der Signalmoleküle (pAkt, pERK) in behandelten Zellen. Trotz gewisser zytotoxischer Effekte und einer niedrigeren Proliferation der mit Dovitinib behandelten Zellen, werteten wir die Veränderungen im Hinblick auf Myelin-Bildung und Neuroprotektion als positiv.

Unter Berücksichtigung der Komplexität der MS als Autoimmunerkrankung gibt es eine Vielzahl weiterer Faktoren, die berücksichtigt werden müssen, um die Effekte der FGFR Signale zu beurteilen. Um das Zusammenspiel aller Faktoren zu verstehen, bedarf es weiterer Studien *in vivo*. Nichtsdestotrotz stellt die Inhibition von Fibroblasten-Wachstumsfaktor-Rezeptoren mittels Dovitinib in Zusammenschau unserer Befunde einen vielversprechenden neuen Therapieansatz für die Multiple Sklerose und andere demyelinisierende Erkrankungen dar.

Abbreviations

2+	Refers to treatment with Dovitinib and FGF in our experimental setup
ADEM	Acute disseminated encephalomyelitis
AKT	Refers to protein kinase B
BBB	Blood brain barrier
BDNF	Brain-derived neurotrophic factor
CIS	Clinically isolated syndrome
CNPase	Cyclic nucleotide phosphodiesterase
CNS	Central nervous system
CSF	Cerebrospinal fluid
DAPI	4′,6-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
DMT	Disease modifying therapy
DNA	Desoxyribonucleic acid
EAE	Experimental autoimmune encephalomyelitis
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinases
FGF	Fibroblast growths factor
FGFR	Fibroblast growths factor receptor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HRP	Horseradish peroxidase
IF	Immunofluorescence
IL	Interleukin
LDH	Lactate dehydrogenase
MAG	Myelin-associated glycoprotein
MNOSP	Neuromyelitis optica spectrum disease
MOG	Myelin oligodendrocyte glycoprotein
MRI	Magnetic resonance imaging
mRNA	Messenger-ribonucleic acid
MS	Multiple sclerosis
NSC	Neural stem cells
OL	Oligodendrocyte
OPC	Oligodendrocyte progenitor cell

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGFR	Platelet derived growths factor receptor
PLP	Myelin proteolipid protein
PPMS	Primary progressive multiple sclerosis
RRMS	Relapsing remitting multiple sclerosis
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphfate–polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SEMA3A	Semaphorin 3A
SPMS	Secondary progressive multiple sclerosis
TGF-ß	Transforming growths factor beta
TrkB	Tropomyosin receptor kinase B
VEGFR	Vascular endothelial growths factor receptor
WB	Western Blot
WST-1	Water-soluble tetrazolium salt-1

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Publications

Publication derived from this study:

Rajendran,R.; Böttiger,G.; Dentzien, N.; Rajendran, V.; Sharifi, B.; Ergün, S.; Stadelmann, C.; Karnati, S.; Berghoff, M. (2021) Effects of FGFR Tyrosine Kinase Inhibition in OLN-93 Oligodendrocytes. Cells 2021, 10, 1318. https://doi.org/10.3390/cells10061318

Poster presentation:

Science-Day 2019 JLU Giessen

Effects of the non-selective FGFR inhibitor Dovitinib on oligodendrocytes.

Dentzien, N., Rajendran, R., Berghoff, M.

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