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TGFβ1 induces axonal outgrowth via ALK5/PKA/SMURF1-mediated degradation of RhoA and stabilization of PAR6

Abbr. Title: neurite outgrowth enhancing downstream signaling of TGFβ1

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Conflict of interest

None of the funding organizations had a role in the design and conduct of the study, collection, management, analysis, and interpretation of the data, preparation, review, or approval of the manuscript, or in the decision to submit the manuscript for publication. M.E.S. is the cofounder and president of the board of NovaGo Therapeutics Inc.

Abstract

TGFβ1 has repeatedly been associated with axonal regeneration and recovery after injury to the CNS. We found TGFβ1 up-regulated in the stroke-denervated mouse spinal cord after ischemic injury to the motor cortex as early as four days post injury (dpi) and persisting up to 28 dpi. Given the potential role of TGFβ1 in structural plasticity and functional recovery after stroke highlighted in several published studies, we investigated its downstream signaling in an *in vitro* model of neurite outgrowth. We found that in this model, TGFβ1 rescues neurite outgrowth under growth inhibitory conditions via the canonical TGFβR2/ALK5 signaling axis. Thereby, protein kinase A (PKA)-mediated phosphorylation of the E3 ubiquitin ligase SMURF1 induces a switch of its substrate preference from PAR6 to the Ras homologue A (RhoA), in this way enhancing outgrowth on the level of the cytoskeleton. This proposed mechanism of TGFβ1 signaling could underly the observed increase in structural plasticity after stroke *in vivo* as suggested by the temporal and spatial expression of TGFβ1. In accordance with previous publications, this study corroborates the potential of TGFβ1 and associated signaling cascades as a target for future therapeutic interventions to enhance structural plasticity and functional recovery for stroke patients.

Significance statement

This study addresses a mechanism for TGFβ1 to increase compensatory axonal sprouting and growth after cortical stroke, e.g. in the stroke-denervated cervical spinal cord, where it was previously implicated as a potential growth-inducer. The signaling pathway includes the canonical receptor components ALK5 and SMAD3 and a downstream modulation of the cytoskeleton via PKA/SMURF1 induced downregulation of RhoA and upregulation of PAR6. Defining the downstream signaling pathway through which TGFβ1 can induce neurite outgrowth may provide new clinical targets for future therapeutic interventions to increase compensatory sprouting, thus contributing to functional recovery.

Introduction

Interruption of the brain's blood supply, e.g. in case of ischemic stroke, can result in life-long disability due to the loss of neurons (Cramer, 2018; Murphy and Corbett, 2009). Most available therapeutics for stroke patients target the acute phase in the hope to protect neurons from the ischemic damage, while rehabilitation to date remains the only treatment option for chronic stroke patients (Krakauer et al., 2012; Wahl and Schwab, 2014; Zeiler and Krakauer, 2013). Both in human stroke patients as well as animal models of stroke, the spontaneous recovery observed in the weeks after the incident has been attributed in parts to structural plasticity of healthy cortical neurons and connections, e.g. horizontal neurons in the peri-infarct region or the contralateral corticospinal neurons (CSNs) in case of large strokes (Carmichael et al., 2017). In this regard, a better understanding of the molecular cues inducing, guiding and maintaining this compensatory sprouting and rewiring response is pivotal to develop novel therapeutic approaches to enhance recovery after stroke. Inflammation-derived cytokines and locally released growth factors may have a beneficial effect on the repair mechanisms by directly promoting axonal regeneration (Benowitz and Popovich, 2011; Vidal et al., 2013). In the cortex surrounding a focal stroke lesion the upregulated cytokine growth differentiation factor 10 (GDF10) was reported to enhance structural plasticity and motor recovery (Li et al., 2015). GDF10 is a member of the highly evolutionarily conserved transforming growth factor (TGF) superfamily, which also includes the TGFβ and bone morphogenic protein (BMP) cytokine families (Weiss and Attisano, 2013; Zhang et al., 2017). A recent study highlighted the involvement of TGFβ1 signaling in neurogenesis and axonal regeneration in the peri-infarct cortex by viral knockdown of the co-receptor ALK5, leading to decreased levels of the growth associated protein GAP43 within the first two weeks after stroke, concomitant with a decrease in functional recovery (Zhang et al., 2019). A recently published transcriptomic screen showed an upregulation of TGFβ1 in the stroke-denervated cervical hemicord of adult mice at 28 days after a large cortical stroke. This finding suggests that TGFβ1 could be a mediator of the observed compensatory

84 sprouting of the intact-side corticospinal tract (CST) and axon elongation/arborization in the spinal cord
85 (Kaiser et al., 2019).

86 However, the mechanisms through which TGFβ1 may increase neurite outgrowth are still unclear.
87 TGFβ1 binding to its receptor TGFβR2 can result in the formation of heteromeric complexes with two
88 different type 1 receptors, ALK1 or ALK5, activating distinct downstream pathways often with opposing
89 functions (Curado et al., 2014; Finelli et al., 2013; Goumans et al., 2003; Hannila et al., 2013; König et al.,
90 2005; Li et al., 2015; Parikh et al., 2011; Saijilafu et al., 2013; Stegmuller et al., 2008; Zou et al., 2009).
91 TGFβ1 promotes axonal outgrowth *in vitro* in primary neurons, including cortical neurons (Abe et al.,
92 1996; Knöferle et al., 2010; Li et al., 2015) and blockage of the receptor TGFβR2 leads to shorter axons
93 (Yi et al., 2010). Intriguingly, direct activation of SMAD2, the canonical downstream target of TGFβ1
94 signaling, reduced axonal outgrowth in some instances (Knöferle et al., 2010; Stegmuller et al., 2008).
95 This discrepancy of axonal growth induction vs. inhibition may stem from canonical vs. non-canonical
96 signaling of TGFβ1. A better understanding of the downstream signaling pathway through which TGFβ1
97 can induce axonal outgrowth is, therefore, urgently needed; it may also provide interesting new clinical
98 targets for future therapeutic interventions.

99 In the present study, we show that TGFβ1 is transcriptionally upregulated in the region of the
100 axotomized cervical CST and in the premotor layers of the CST-deprived spinal grey matter as early as 4
101 days post injury (dpi). Using a simple but highly reproducible *in vitro* assay , we demonstrate that TGFβ1
102 treatment rescues neurite outgrowth in the growth inhibitory environment of crude spinal cord extract
103 through the canonical TGFβ1/ALK5 signaling axis. Further hypothesis driven pharmacological blockade
104 studies suggest an underlying signaling mechanism involving a PKA-mediated phosphorylation of the E3
105 ubiquitin ligase SMURF1, switching its substrate preference from PAR6 to Ras homologue A (RhoA). In
106 conclusion, we show that besides canonical transcriptional changes associated with TGFβ1, it also

enhances neurite outgrowth by downregulating RhoA - the downstream signaling mediator of many CNS associated growth inhibitory molecules. Thereby TGFβ1 has the potential to prime the neuronal cytoskeleton into a growth permissive state despite of the inhibitory CNS environment.

Materials and Methods

Animals.

All animal experiments were performed with the approval of and in strict accordance with the guidelines of the Zurich Cantonal Veterinary Office. A total of *n* = 21 adult C57BL/6J mice (2–3 months, 20–28 g, female, Charles River Laboratories) were used in this study. Only one sex was used to minimize lesion size variability and animal numbers. Animals were housed in groups of four to five under a constant 12 h light/dark cycle with food and water *ad libitum*.

Photothrombotic stroke.

For all surgeries, mice were initially anesthetized using 3–4% isoflurane, transferred to a stereotactic frame (Kopf Instruments) and kept at 1–2% isoflurane throughout the surgery. Body temperature was maintained at 37°C on a heating pad. All animals received a unilateral photothrombotic stroke to lesion the right side sensorimotor cortex as previously described (Kaiser et al., 2019; Watson et al., 1984). Briefly, the skull was exposed by a midline incision of the scalp. An opaque template with a defined opening (3 × 5 mm) was aligned to the midline over the right motor and premotor cortex (−2 to +3 mm A/P, 0–3 mm M/L related to Bregma). Five minutes after intraperitoneal injection of 0.1 ml Rose Bengal (10 mg/ml in 0.9% NaCl; Sigma-Aldrich), the skull was illuminated for 10.5 min with a cold light source (Olympus, KL1500LDC, 150W, 3000K) placed firmly on top of the skull. Control animals were given a sham operation without illumination of the skull. Postoperative care included recovery on a heating mat, sustained analgesia provided via drinking water (Novalgin, 2 mg/ml with 5% sucrose) and antibiotic treatment where necessary for 3 d.

Behavioral testing.

Behavioral tests were performed before surgery (baseline) as well as 4, 7, 14 and 28 days after photothrombotic stroke (dpi) of the right motor cortex. Post-stroke impairment and recovery of forelimb function was assessed using the cylinder test. Forelimb paw touches to the cylinder wall during spontaneous rearing behavior were recorded for 20 min or 30 rears in total (*n* = 12). Paw dragging of the affected limb was scored as the percentage of paw drags divided by total number of paw touches (Roome and Vanderluit, 2015). The horizontal ladder walk test was used as an additional, more sensitive assessment of skilled limb placement (Metz and Whishaw, 2009). Mice were habituated to the ladder run one day prior to test recordings; no further training was conducted. Three trials on a 40 cm long ladder with irregularly spaced rungs of 1–2 cm distance were recorded on each testing day (*n* = 12). The number of foot errors was measured as the number of total misplacements (slips) divided by the total number of steps taken. All recorded steps were analyzed and no videos were excluded for the analyses to avoid bias toward stroked groups (*n* = 3 videos per animal per test day).

Perfusion fixation and tissue processing.

All mice were terminally anesthetized with 3–5% isoflurane followed by injection of pentobarbital (300 mg/kg body weight, i.p.; Streuli Pharma AG). Animals were transcardially perfused with ice-cold Ringer's solution (containing 10^5 IU/L heparin, (Roche) and 0.25% $NaNO_2$). Brains and spinal cords were quickly dissected and snap-frozen on dry ice to preserve RNA quality. The brains were immersed in 4% PFA overnight before being transferred to 30% sucrose in phosphate buffer (PB) for cryoprotection. Samples were blinded from the point of tissue harvesting.

Analysis of lesion completeness.

For the accurate analysis of lesion size, brain cross-sections (40 µm) were stained on-slide with Cresyl violet solution for 1 min, dehydrated in a series of increasing ethanol concentrations and washed in Xylol before coverslipping with Eukitt (Sigma-Aldrich). Brain sections at four defined landmarks (1.98 mm, 0.98 mm, -0.22 mm, -1.34 mm, in relation to Bregma) were analyzed for stroke volume and depth of the

cortical lesion. Average cortical stroke depth was calculated as the average of lesion depth across these four landmarks.

In situ hybridization.

Sections (16 µm) of fresh frozen tissue were cut on a cryostat at −20°C and stored at −80°C. *In situ* gene expression was assessed using the RNAScope protocol (Advanced Cell Diagnostics, RNAscope Fluorescent Multiplex Assay) according to the manufacturer's protocol. Briefly, slices were fixed in 4% PFA for 20 min before being hybridized to the probe (RNAScope: TGF-β1, catalog #407751), which was further amplified using the branched DNA amplification methods. Sections were counterstained with DAPI and digitalized (Zeiss, Axio Scan.Z1, ×200). For analysis using ImageJ/FIJI, only slices with tears or folds were excluded; n = 3 sections per animal per group and spinal levels C5 and C6 were randomly selected. Quantification of mRNA expression was expressed as the percentage of white signal over black background in defined regions (CST in the dorsal funiculus and intermediate grey matter of the denervated hemicord) after applying a threshold that was manually defined for five randomly selected images of the dataset and averaged.

Neurite outgrowth assay.

Candidate factors were tested using a previously described neurite outgrowth assay (Maibach et al., 2020). Briefly, N1E-115 mouse neuroblastoma cells were plated at a density of 10’000 cells/cm2 in Neurobasal medium supplemented with 2% L-glutamine and 1% PenStrep to induce neuron-like differentiation. After 24h of differentiation the cells were supplemented with crude adult rat spinal cord CHAPS extract, and candidate factors or pharmacological agents were added. (Maibach et al., 2020)The following proteins and molecules were used at the indicated concentrations: 1 ng/ml TGFβ1 (R&D Systems), 1 nM TEW7197 (Selleckchem, S7530), 1 nM ML347 (Selleckchem, S7148), 10 nM SIS3 (Selleckchem, S7959), 10 nM A01 (Merck, SML1404), 10 nM Ht31 (Tocris Biotechne, 6286), 10 nM ATM (Merck, 157201). After an additional 24h outgrowth phase, the cells were fixed and counterstained with

Coomassie solution (0.25% Coomassie brilliant blue R250, 50% MeOH, 10% HoAC). Images were acquired randomly over the wells at 10x magnification. Mean neurite outgrowth per cell was quantified in ImageJ by applying a grid to the pictures and counting intersections of neurites with the grid lines and relating this number to the total number of cell bodies in the corresponding well (Ronn et al., 2000). Experiments were conducted in five biological replicates with three technical replicates per condition.

Immunoblotting

Cells were washed twice in PBS on ice and lysed in RIPA buffer (150 mM NaCl, 1% NP-40, 1% Sodium deoxycholate, 0.1% SDS, 50 mM Tris pH8) containing 2x HALT™ phosphatase inhibitor cocktail and 5 mM EDTA. The lysates were incubated on ice for 30 min and centrifuged at 13'000 g for 15 min at 4 °C. The supernatants were collected and stored at –80°C.

The samples were prepared in Laemmli buffer (Biorad) supplemented with 10% βMEtOH and denatured at 90°C for 3 min. The samples were separated on pre-cast 4-15% Mini PROTEAN R TXG TM gels (Biorad) at 250 V in Tris-Glycine running buffer (25 mM Tris, 192 mM Glycine, 0.1% SDS, pH 8.3). Proteins were transferred onto a 0.45 µm PVDF membrane in Tris-Glycine transfer buffer (25 mM Tris, 19 2mM Glycine, 20% MeOH) for 90 min with a constant current of 300 mA. Subsequently, membranes were blocked for 1 h with 5% BSA (Sigma) in TBS-T (10 mM Tris, 150 mM NaCl, 0.01% Tween-20, pH 7.5) and probed with primary antibodies overnight at 4°C. The membranes were washed 3 times in TBS-T, probed with secondary HRP-coupled antibodies (Thermo Fisher Scientific) at a concentration of 0.05 - 0.1 µg/ml for 1 h at RT and washed again 3 times in TBS-T. Detection was performed using SuperSignal™ West PICO (Thermo Scientific) or WesternBright™ Sirius (Advansta) chemiluminescent substrates, and images were acquired on the Gel Doc™ imager (Biorad). Densitometry analysis was performed with ImageJ/FIJI software, and values were normalized to the housekeeping gene GAPDH or total protein and the mean

value of the corresponding control group. For western blotting, the primary antibodies are summarized in Table 1.

Table 1– Antibodies used for Western Blotting.

Target	Host Species	Dilution	Clone	Company	Catalogue Number
p-SMAD3 S423/425	rabbit	1:1000	C25A9	Cell Signaling Technology	9520
SMAD2/3	rabbit	1:1000	D7G7	Cell Signaling Technology	8685
RhoA	rabbit	1:1000	67B9	Cell Signaling Technology	2117
PAR6	rabbit	1:500		Abcam	ab111823
p-ERK1/2 T202/Y204	rabbit	1:2000		Cell Signaling Technology	9101
ERK1/2	rabbit	1:1000	137F5	Cell Signaling Technology	4695
p-S6P S235/236	rabbit	1:1000	D57.2.2E	Cell Signaling Technology	4858
S6P	mouse	1:1000	54D2	Cell Signaling Technology	2317
TGFβR2	goat	1:500		R&D Systems	AF-241
GAPDH	mouse	1:20'000	6C5	Abcam	ab8245

RhoA assessment in growth cones.

Cells were fixed for 15min in 4% PFA and subsequently blocked and permeablized in blocking buffer (0.1 % Triton-X, 10 % BSA in PBS) for 1 h. Primary antibody (mouse anti-RhoA (Santa Cruz, SC-418), 1:500) was applied in 1 % BSA in PBS and incubated over night at 4° C. The samples were washed three times in PBS, followed by incubation with secondary antibodies (1 µg/ml anti-mouse Cy3-coupled antibodies (Invitrogen, A10521, A10520)) in 1 % BSA in PBS for 1 h at RT. After washing three times in PBS, cells were counterstained with 1:100 Phalloidin Alexa Fluor 488 (Invitrogen, A12379) to visualize the actin cytoskeleton and DAPI (50 nM, Invitrogen, D3571) as a nuclear counterstain. The samples were

coverslipped and imaged at 200x. For image acquisition, exposure times were kept constant and below grey scale saturation. For immunofluorescence normalization in ImageJ/FIJI, the signal in the phalloidin channel was thresholded and an area mask was created around the fluorescent object. This mask was then applied onto the RhoA pictures and the total pixel intensity within the area was measured. This value was normalized to the area of the growth cone or cell body. Similarly, a nuclear counter stain was used to define the area of the nucleus.

Statistical analysis.

Statistical analysis was performed with Prism 7.0 (GraphPad Software). To detect differences between groups and within groups over time , two-way ANOVA followed by Dunnett's multiple-comparisons (MC) was used. Other symbols might be used to indicate two comparisons in one graph. In bar graphs, all data are plotted as mean standard error of the mean (± SEM), while data were normalized to solvent controls in the neurite outgrowth assays. Dots in the behavioral graphs represent individual animals. For all neurite outgrowth assays, n=5 biological replicates with n=3 technical replicates per condition. 200-400 cells per technical replicate were counted. Throughout the manuscript, *=$p \leq 0.05$, **=$p \leq 0.01$, and ***=$p \leq 0.001$.

Results

TGFβ1 is Endogenously Upregulated at Early Time Points in the Stroke Denervated Hemicord

In mice, a significant degree of spontaneous re-innervation of the stroke denervated cervical spinal cord by sprouting of contralesional corticospinal neurons (CSNs) can be detected 28 days after large lesions to the motor cortex (Bachmann et al., 2014; Kaiser et al., 2019; Ueno et al., 2012). At this late time point after stroke, TGFβ1 expression was found to be up-regulated in the cortex (Ata et al., 1999; Doyle et al., 2010; Knuckey et al., 1996; Lehrmann et al., 1995; Zhu et al., 2001) as well as in the stroke-denervated hemicord (Kaiser et al., 2019). However, sprouting of contralateral cortical axons may be initiated much earlier in the stroke-denervated hemicord. To address whether TGFβ1 could serve as a trigger and modulator of structural plasticity in the stroke-denervated hemicord, we evaluated the expression pattern of TGFβ1 within the dorsal funiculus and grey matter at selected, early time points after stroke (2 dpi, 4 dpi, 7 dpi, 28 dpi) (Figure 1A, B). We ensured that stroke lesions were consistent in size and location at all time points. Strokes were primarily localized to the sensory-motor cortex including the premotor regions and successfully ablated all cortical layers with little to no impact on the corpus callosum or deeper structures (Figure 1C) .This stroke model induced a behavioral deficit in the forelimbs which recovered partially over the course of four weeks as assessed by paw dragging and foot faults in the horizontal ladder task (Figure 1D). *In situ* hybridization for TGFβ1 in the stroke-denervated spinal cord showed an increase of TGFβ1 mRNA at 4 dpi in both analyzed regions, the CST domain of the dorsal funiculus as well as the intermediate laminae of the grey matter (iGM) (Figure 1E, F). This increased expression is transient in the iGM and persists up to 28 dpi in the CST, suggesting that TGFβ1 is present at time points when growth initiation and axon elongation and arborization of contralesional CSNs and other tracts occur.

TGFβ1 Rescues Neurite Outgrowth in Inhibitory Environment via Canonical ALK5/SMAD3 Signaling

We first investigated the potential of TGFβ1 to rescue neurite outgrowth in a growth inhibitory environment. To model the inhibitory *in vivo* CNS environment, we used our previously established neurite outgrowth assay (Maibach et al., 2020). In this assay, application of crude spinal cord CHAPS extract (SCE) to the differentiated neuron-like cell line N1E-115 over a 24h process formation phase, inhibits outgrowth in a dose dependent manner, without affecting cell survival. As TGFβ1 was shown to increase axon length in neurons, including primary cortical neurons (Abe et al., 1996; Knöferle et al., 2010; Li et al., 2015), we first established an effect of TGFβ1 in our neurite outgrowth model. We found that already nano molar concentrations of TGFβ1 were able to restore neurite outgrowth of IC50 SCE treated N1E-115 cells to approximately 80% (Figure 2A, B).

We investigated the specific receptor complex through which the neurite outgrowth promoting TGFβ1 signaling might be transduced (Figure 2C). While pharmacological inhibition of ALK1 had no effect on the TGFβ1 mediated rescue of neurite outgrowth in the inhibitory SCE treatment conditions, inhibition of ALK5 prevented the TGFβ1 mediated rescue (Figure 2D, E). This suggests that the TGFβ1 signal is propagated via the canonical TGFβR2/ALK5 signaling receptor complex. Phospho-profiling of the ALK5 downstream effector SMAD3 revealed an increased phosphorylation/activation of SMAD3 in the TGFβ1 treated conditions (Figure 2F, G). Accordingly, pharmacological inhibition of SMAD3 prevented the TGFβ1 mediated rescue effect (Figure 2H). Taken together, these results demonstrate that TGFβ1 signals via the canonical TGFβR2/ALK5/SMAD3 axis to rescue neurite outgrowth under CNS growth inhibitory conditions.

TGFβ1 Induces SMURF1-Mediated Downregulation of RhoA and Stabilization of PAR6

We did not observe an increase in neurite outgrowth in the absence of SCE after 24 h of TGFβ1 treatment alone (Figure 1B) with the extremely low TGFβ1 concentration used in this study. This lack of a general growth promoting effect suggests that TGFβ1 signaling specifically interferes with and cancels

the SCE-induced growth inhibitory signaling cascades. We therefore hypothesized that TGFβ1 could negatively influence the RhoA/Rho-associated, coiled-coil containing protein kinase (ROCK) pathway, a key downstream signaling effector of many growth inhibitory molecules (Thiede-Stan and Schwab, 2015). One possible mechanism for such crosstalk could be the SMURF1 mediated RhoA ubiquitination which targets RhoA for degradation and thereby enhances neurite outgrowth (Narimatsu et al., 2009; Sahai et al., 2007; Smith et al., 2005; Vohra et al., 2007; Wang et al., 2003). The substrate preference of SMURF1 is modulated by PKA dependent phosphorylation, which increases its binding affinity for RhoA relative to PAR6 (Cheng et al., 2011). In parallel to enhanced RhoA degradation, the membrane associated PAR6/PKC complex is stabilized. Both PKA activation (via association with SMAD3) and induction of a PAR6/protein kinase C (PKC) complex at the membrane have been reported as downstream effectors of TGFβ1 signaling in endothelial cells (Ozdamar et al., 2005; Wang et al., 1998; Yang et al., 2013), making it a possible mechanism for the observed rescue of neurite outgrowth by TGFβ1.

To test this hypothesis, we inhibited selected key molecules of the proposed non-canonical signaling cascade (Figure 3A). Inhibition of SMURF1 by the blocker A01 (Cao et al., 2014) prevented the TGFβ1 elicited rescue of neurite outgrowth from SCE- induced growth inhibition (Figure 3 B). Likewise, inhibition of substrate recruiting PKA scaffolding protein A kinase anchoring protein (AKAP) by Ht31 (Kennedy and Scott, 2015) as well as pharmacological inhibition of PKC and PAR6 interaction ATM (Butler et al., 2015; Erdogan et al., 2006; Stallings-Mann et al., 2006) resulted in the loss of the TGFβ1 mediated rescue effect (Figure 3 C, D). Furthermore, TGFβ1 co-treatment with SCE resulted in a significant downregulation of RhoA levels both globally (Figure 3 E, F) and locally in growth cones (Figure 3 H, I). This reduction of RhoA was associated with increased PAR6 levels (Figure 3 E, G), supporting the hypothesis of a TGFβ1 induced switch in SMURF1 substrate preference leading to lower levels of RhoA and higher levels of PAR6 as an underlying mechanism for the observed neurite outgrowth recovery.

Discussion

We observed an upregulation of TGFβ1 mRNA at 4 dpi in the stroke-affected CST and the intermediate grey matter (laminae 5-7) in the cervical spinal cord after a large stroke to the motor cortex, a time point when compensatory axonal sprouting of the CST may be triggered. This increased expression persisted in the CST area in the dorsal funiculus up to 28 dpi. *In vitro* neurite outgrowth analysis showed that TGFβ1 can rescue neurite outgrowth through the canonical ALK5/SMAD3 signaling axis in a growth inhibitory environment. Downstream of the TGFβR2/ALK5 receptor, TGFβ1 led to the accumulation of PAR6, and negatively impacted on the RhoA/ROCK pathway by reducing the SCE induced accumulation of RhoA. We propose an underlying signaling mechanism involving PKA dependent SMURF1 regulation, leading to a substrate preference switch of SMURF1 from PAR6 to RhoA, thereby decreasing RhoA and increasing the growth enhancing state of the cytoskeleton. Such a mechanism could be the basis on which TGFβ1 mediates compensatory sprouting of re-innervating CSN axons in the stroke-denervated spinal cord.

Following stroke, TGFβ1 has been reported to exert neuroprotective effects: Administration of TGFβ1 led to reduced infarct sizes (Gross et al., 1993; Prehn et al., 1993), while blocking TGFβ1 signaling led to increased ischemic damage (Ruocco et al., 1999). Direct neuronal or glial effects could not be distinguished from anti-inflammatory effects, however. Evidence for TGFβ1 as a regulator of axonal growth is sparse and contradictory. Several *in vitro* studies correlated TGFβ1 treatment with axonal growth promotion in several subtypes of neurons (Abe et al., 1996; Knöferle et al., 2010; Li et al., 2015; Stegmuller et al., 2008; Walshe et al., 2011), while others reported an inhibitory or no effect at all (Do et al., 2013; Ho et al., 2000; Jaskova et al., 2014). These varying effects may be driven by context- and cell-specific signaling of TGFβ (Massagué, 2012); our present finding that TGFβ1 alone did not affect neurite outgrowth, but reversed the growth inhibitory effect of a crude spinal cord protein extract are in line with such a concept. The importance of studying the effects of TGFβ1 on axonal growth *in vivo* is thus

evident, however, interpretation of the results may be complex owing to the pleiotropic roles of TGFβ in the control of cell proliferation, differentiation, wound healing and immune system (Kang et al., 2009; Morikawa et al., 2016).

Previous studies have reported indirect effects of TGFβ1 on modulation of axonal growth (Li et al., 2017), e.g. by induction of astrocyte proliferation (Kohta et al., 2009). Endogenous TGFβ1 may also serve as a trophic factor as TGFβ1 deficiency leads to an exacerbated neuronal cell death after facial nerve axotomy (Makwana et al., 2007). TGFβ1 can be secreted by astrocytes and by immune cells, thereby often functioning as an immunosuppressant (Liddelow et al., 2017; Tripathi et al., 2017; Vidal et al., 2013). Recently, TGFβ1/ALK5 signaling was associated with increased dendritic plasticity of cortical neurons, axonal sprouting of corticorubral projections and increased motor recovery after ischemic reperfusion injury (Zhang et al., 2019).

A previous study reported an upregulation of TGFβ1 mRNA in the stroke-denervated spinal cord at 28dpi (Kaiser et al., 2019), suggesting that TGFβ1 might serve as a local tissue-derived pro-regenerative cue for re-innervating axons after cortical stroke. Here, we observed an increase in TGFβ1 mRNA as early as 4 days post injury. While we found a transient increased expression of TGFβ1 in the intermediate grey matter, this increase persisted in the dorsal funiculus in the CST area. Interestingly, this temporal and spatial expression profile matches the previously described activation of microglia and infiltration of macrophages (Kaiser et al., 2019), suggesting that these cells may be the predominant source of TGFβ1 in the stroke-denervated spinal cord. This interpretation is in line with a previous finding of increased TGFβ1 expression in microglia/macrophages in the stroke penumbra early after middle cerebral artery occlusion (Doyle et al., 2010).

We addressed the potential downstream pathways of TGFβ1 leading to increased neurite outgrowth under growth inhibitory conditions. We conducted our studies using aa *in vitro* model the

neuroblastoma-derived cell line N1E-115 treated with crude spinal cord extract (SCE) (Maibach et al., 2020). While this model lacks the direct translational impact of primary cell cultures, it ensures a high reproducibility between studies that primary cultures fail to provide owing to an often large batch-to-batch variability and contamination with non-neuronal cells. High sensitivity and reproducibility is a crucial prerequisite for a routine assay that is used for therapeutic compound screening. By using the well characterized neuron-like N1E cells, we were also able to avoid non-neuronal cell contamination by e.g. astrocytes, which might serve as a primary source for TGFβ1 and thus interfere with our results in this study. An additional advantage of this assay is the ability to distinguish effects of the treatment on neurite outgrowth directly vs. neuronal cell death as counted by number of cells. Treatment of N1E cells with SCE did not lead to an increase in cell death, but to a stunted neurite outgrowth with cells portraying a "panning" like phenotype. Co-treatment with TGFβ1 led to a robust increase in neurite outgrowth under these inhibitory conditions. Moreover, the neurite growth inhibitory effects of SCE in the N1E cultures were highly comparable to those previously published in primary cortical or hippocampal neurons, and the TGFβ1 mediated rescue of neurite outgrowth from SCE induced growth inhibition in our *in vitro* model of N1E-115 cells was dependent on ALK5 and SMAD3, as was shown to be the case for cortical neurons *in vivo* (Zhang et al., 2019).

We then went on to pharmacologically block selected downstream targets of TGFβ1/ALK5 signaling to identify potential effects on neurite outgrowth in this model. By applying the candidate factors in a well defined time frame after initial neurite outgrowth and in combination with inhibitory SCE, we were able to address post-translational signaling mechanisms, including phosphorylation and ubiquitination. While these studies allow us to address the direct effects of pharmacological interventions, we did not address effects on a transcriptional level in this study.

Application of SCE was shown to increase RhoA levels both in the cell body as well as in the growth cone. RhoA activity is associated with neurite retraction in response to growth cone collapsing agents (Feltrin and Pertz, 2012; Jeon et al., 2012). Pharmacological blockade of RhoA or the downstream effector Rho-kinase (ROCK) abolished the inhibitory effects of SCE, specifically also of its active ingredients Nogo-A and MAG (Niederöst et al., 2002). TGFβ1 treatment was associated with a downregulation of RhoA back to baseline levels (control group without SCE treatment), indicating that TGFβ1 signaling might have led to a decreased response to the inhibitory molecules of SCE such as Nogo-A or MAG.

We further saw an accumulation of PAR6 protein in the TGFβ1 treated N1E-115 cells, suggesting increased protein stability or synthesis resulting from the TGFβ1 treatment. Previous studies have described TGFβ1 mediated regulation of proteasomal processes in neuronal cells (Knöferle et al., 2010; Tadlock et al., 2003). Along these lines, we found that the TGFβ1 mediated rescue of neurite outgrowth depended on the E3 ubiquitin ligase SMURF1 as well as PKC/PAR6 interaction. In various cell types, TGFβ1 signaling induces cellular polarity by recruitment and activation of the PAR6/PKC complex to TGFβR2 (Ozdamar et al., 2005). Other studies suggested that this complex relocates SMURF1 to the membrane, where it induces RhoA degradation (Narimatsu et al., 2009; Sahai et al., 2007; Wang et al., 2003) and neurite outgrowth (Bryan et al., 2005; Vohra et al., 2007).

Notably, in our study the TGFβ1 induced neurite outgrowth was also dependent on PKA activation. Previous studies have shown that PKA is a downstream target of TGFβ1 and is associated with SMAD proteins (Wang et al., 1998; Yang et al., 2013). PKA mediated phosphorylation of the ubiquitin ligase SMURF1 in the RhoA interaction domain was shown to increase SMURF1 affinity for RhoA relative to PAR6, thereby contributing to axon formation *in vitro* and neuronal polarization *in vivo* (Cheng et al., 2011). It is possible that TGFβ1 signaling may also influence additional Rho-like GTPases such as Rac and Cdc42 andeffectively regulate neurite outgrowth through additional signaling pathways (Zhang, 2009).

Based on the present data, we propose that TGFβ1 enhances axonal outgrowth via stabilization of PAR6 and degradation of RhoA via a PKA/SMURF1 dependent mechanism. These results can guide future experiments to analyse these mechanisms *in vivo* and in particular in corticospinal neurons after a cortical stroke.

Endogenous TGFβ1 levels were shown to be increased in early phases after stroke, within the critical period of plasticity (Doyle et al., 2010). While the present and other studies suggest that prolonged treatment with TGFβ1 or a downstream effector of the TGFβ1/ALK5 signaling pathway may be used to enhance plasticity and extend its critical window after stroke (Li et al., 2015; Zhang et al., 2019), a systemic delivery of TGFβ1 would bring about unwanted negative side-effects owing to the plethora of functions of TGFβ1 as a inflammation related molecule (Akhurst and Hata, 2012). It is thus necessary to understand the specific signaling mechanisms by which TGFβ1 mediates structural plasticity and axonal outgrowth, both *in vitro* and *in vivo*, to develop a successful therapeutic agent.

The present study investigates a potential signaling mechanism through which TGFβ1 induces neurite outgrowth in a growth inhibitory environment *in vitro* and possibly compensatory axonal sprouting *in vivo*. This signaling pathway includes the canonical receptor components ALK5 and SMAD3 and a downstream activation of the cytoskeleton via PKA/SMURF1 induced downregulation of RhoA and upregulation of PAR6.

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Figure Legends

Figure 1 – TGFβ1 is upregulated in the stroke-denervated cervical spinal cord at early time-points, transiently in the intermediate grey matter and persistently in the degenerating CST in the dorsal funiculus. (A, B) Experimental study timeline: Adult female C57BL/6 mice received a large unilateral cortical stroke (Sham: n=6; 2dpi: n=3; 4dpi: n=3; 7dpi: n=4; 28dpi: n=5; total: n=21). All animals were tested behaviorally and euthanized at 2 dpi, 4 dpi, 7 dpi or 28 dpi, respectively. (C) Average cortical stroke depth showed no significant differences among the experimental groups with strokes reaching into deep cortical layers to the corpus callosum (100%). Estimated location of corticospinal neurons in layer V is indicated by a red dotted line. The sensorimotor area was specifically injured (Nissl staining at 7 dpi, representative picture). Scale bar = 500 µm. (D) Behavioral analysis for paw drags in the cylinder test and number of missteps on the irregular horizontal ladder shows that stroke induction resulted in a deficit in motor behavior with a subsequent, partial functional recovery within 28 dpi. # is used for statistical comparison to baseline levels, * for comparison to 4 dpi. (E) Representative pictures of TGFβ1 mRNA expression in the degenerating CST and intermediate grey matter (iGM) of the spinal levels C5/6 in Sham animals as well as in mice at 2, 4, 7 and 28 dpi. Scale bar = 50 µm. (F) Quantification of the percentage of area signal in the CST shows an upregulation of TGFβ1 mRNA starting at 4 dpi and persisting until 28dpi. In the iGM, TGFβ1 mRNA is transiently up-regulated at 2 and 4 dpi. Means +/-SEM of 5 independent experiments are shown; each experimemnt has 3 wells with 200-400 cells per well per condition. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Figure 2 – TGFβ1 rescues neurite outgrowth in inhibitory environment via canonical TGFβR2/ALK5/SMAD3 signaling axis. (A) Timeline for neurite outgrowth assay: N1E-115 neuron-like cells were plated and differentiated for 24h by serum deprivation. Candidate factors with/without SCE were supplemented for a 24h outgrowth period, after which neurite outgrowth was assessed. Neurite outgrowth from N1E-115 cells (left panel) is inhibited by SCE (middle panel) and rescued by SCE in combination with TGFβ1 (right panel). Scale bar = 50 µm. (B) Quantification of mean outgrowth per cell in presence or absence of SCE or 1ng/ml TGFβ1 normalized to solvent control condition. (C) Schematic representation of the pathways of TGFβ1 signaling axis. (D) Quantification of mean outgrowth per cell of SCE treated N1E-115 cells in presence of either ALK5 inhibitor (TEW-7197) or TGFβ1 or combined treatment. (E) Quantification of mean outgrowth per cell of SCE treated N1E-115 cells in presence of either ALK1 inhibitor (ML348) or TGFβ1 or combined treatment. (F, G) Representative western blots for p-SMAD3, total SMAD3 or GAPDH of cells treated with SCE and TGFβ1 or both in combination after 15min (F) and quantification thereof (G). (H) Quantification of mean outgrowth per cell of SCE treated N1E-115 cells with either SMAD3 inhibitor (SIS3) or TGFβ1 or combined treatment. Means +/-SEM of 5 independent experiments are shown; each experimemnt has 3 wells with 200-400 cells per well per condition. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Figure 3 – TGFβ1 induces SMURF1 mediated downregulation of RhoA. (A) Schematic representation of the downstream signaling pathway proposed in this study whereby activated SMAD3 activates PKA which turns P-SMURF1 to RhoA for ubiquitination; in turn, the PAR6/PKC complex is stabilized. (B) Quantification of mean outgrowth per cell of SCE treated N1E-115 cells with either SMURF1 inhibitor (A01) or TGFβ1 or combined treatment. (C) Quantification of mean outgrowth per cell of SCE treated N1E-115 cells with either AKAP inhibitor (Ht31) or TGFβ1 or combined treatment. (D) Quantification of mean outgrowth per cell of SCE treated N1E-115 cells with either PAR6-PKC binding inhibitor (ATM) or TGFβ1 or combined treatment. (E - G) Representative western blots for RhoA and PAR6 in N1E-115 cells +/- SCE and/or TGFβ1 (E) and quantification thereof (F, G). (H) Representative pictures of growth cones stained for RhoA (red) and f-actin cytoskeleton (green). Scale bar = 10 µm (I) Quantification of RhoA in growth cones (GC) and cell bodies (CB), respectively. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.