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DOI: <https://doi.org/10.1016/j.yexcr.2019.111503>

Posted at the Zurich Open Repository and Archive, University of Zurich

ZORA URL: <https://doi.org/10.5167/uzh-172353>

Journal Article

Published Version

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Originally published at:

Baumann, Julia; Huang, Sheng-Fu; Gassmann, Max; Tsao, Chih-Chieh; Ogunshola, Omolara O (2019). Furin inhibition prevents hypoxic and TGF -mediated blood-brain barrier disruption. *Experimental Cell Research*, 383(2):111503.

DOI: <https://doi.org/10.1016/j.yexcr.2019.111503>

Furin inhibition prevents hypoxic and TGF β -mediated blood-brain barrier disruption

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ARTICLE INFO

Keywords:

Furin
ALK-5
Barrier permeability
Cell migration
Primary endothelial cells

ABSTRACT

Hypoxic blood-brain barrier (BBB) dysfunction is a common feature of CNS diseases however mechanisms underlying barrier disturbance are still largely unknown. This study investigated the role of transforming growth factor β (TGF β), a cytokine known to induce expression of the proprotein convertase Furin, in hypoxia-mediated barrier compromise. We show that exposure of brain endothelial cells (ECs) to hypoxia (1% O₂) rapidly stimulates their migration. Additional exogenous TGF β (0.4 nM) exposure potentiated this effect and increased Furin expression in a TGF β type I receptor activin-like kinase 5 (ALK5) - dependent manner (prevented by 10 μ M SB431542). Furin inhibition prevented hypoxia-induced EC migration and blocked TGF β -induced potentiation suggesting existence of a feedback loop. TGF β and Furin were also critical for hypoxia-induced BBB dysfunction. TGF β treatment aggravated hypoxia-induced BBB permeability but ALK5 or Furin blockade reversed injury-induced permeability changes. Thus during insult Furin compromises endothelial integrity by mediating the effects of TGF β . Targeting the Furin or ALK5 pathway may offer novel therapeutic strategies for improving BBB stability and CNS function during disease.

1. Introduction

A stable well-functioning blood-brain barrier (BBB) is crucial to maintain and control cerebral homeostasis as well as ensure proper neuronal function. The BBB is formed by specialized brain endothelial cells (EC) that limit paracellular movement of substances to and from the brain parenchyma by their tight junctions (TJ) [1,2]. Pericytes and perivascular astrocytes present at the vascular wall also contribute to barrier maintenance [3]. BBB dysfunction allows the influx of damaging molecules, leading to neuronal cell loss and consequently cognitive decline and CNS disease [4].

Hypoxia is a key stress factor that induces BBB disturbance and increases vessel permeability through a variety of different mechanisms including EC cytoskeletal reorganization, TJ protein delocalization and altered molecular pathway signaling [5,6]. Hypoxic insults induce the expression of transforming growth factor- β 1 [7] and during CNS injury and disease levels of bioactive TGF- β 1 increase [7–9]. Transforming growth factor betas (TGF- β s) are known multifunctional growth factors that participate in regulation of key events during development, disease, and tissue repair. In the brain TGF- β 1, 2 and 3 are broadly expressed with TGF- β 1 widely recognized as an injury-related cytokine. Canonical TGF- β signaling is initiated by ligand binding to a high-affinity transmembrane TGF- β type II receptor (TGF β RII) that recruits

and phosphorylates the type 1 receptors activin-like kinase 1 and 5 (ALK1, ALK5). Subsequent phosphorylation of the downstream effector proteins Smad2 and Smad3 via ALK5 or Smad 1, 5 and 8 via ALK1 causes formation of a heteromeric complex with Smad4, nuclear translocation and transcriptional regulation [10]. At the level of the endothelium TGF- β can signal through both ALK1 and ALK5 and induce different molecular pathways depending on context or dosage [11].

The pleiotropic cytokine TGF β -1 (henceforth referred to as TGF β) is involved in many crucial physiological processes such as cell growth, cell differentiation, migration and apoptosis [11] depending on the cell type. For example, whilst the growth factor induced vessel formation in bovine capillary endothelial cells (BCEC), others showed it promoted apoptosis in BCECs and HUVECs and could inhibit the proliferation of bovine heart ECs [12]. Targeted deletion of various TGF β signaling pathway components in mice led to embryonic lethality due to impaired vascular development with leaky vessels [13] and/or angiogenesis defects [14]. In correlation dysfunctional TGF β signaling has been observed in several pathological conditions linked to vascular compromise such as cancer [15], fibrotic disease [16] and atherosclerosis [17]. The TGF β pathway is clearly a major regulator of blood vessel characteristics.

TGF β has been shown to induce expression of the proprotein convertase Furin in synovial cells and fibroblasts [18]. Furin, a calcium-

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<https://doi.org/10.1016/j.yexcr.2019.111503>

Received 5 April 2019; Received in revised form 25 June 2019; Accepted 15 July 2019

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dependent ubiquitously expressed cellular endoprotease [19], is known to process several protein precursors associated with vascular remodeling and cell movement such as vascular endothelial growth factor C (VEGF-C) [20], platelet derived growth factor (PDGF-BB) [21] and membrane-type 1 matrix metalloprotease 1 (MT1-MMP) [22]. Very recently it was demonstrated that Furin deficiency in myeloid cells attenuated revascularization after oxygen-induced retinopathy [23]. Overall a crucial role for Furin in modulating various vascular endothelia possibly via existence of feedback loops between the enzyme and its substrates has been suggested. In this regard it is important to note that a number of studies using cells of other origin have also convincingly shown Furin to be a TGF β converting enzyme [24–26]. Whether signaling between these two factors occurs at the BBB and/or modulates barrier dysfunction is unknown.

The aim of this study was to investigate the effect of TGF β on barrier stability during injury and the potential involvement of Furin in the process. We demonstrate that TGF β potentiates hypoxia-induced brain EC migration and BBB permeability, and that Furin plays a key role in mediating these effects.

2. Materials and methods

2.1. Cell culture

The rat brain endothelial cell line RBE4 [27] was used for experiments at passages between 36 and 49 at 80–90% confluency. Cells were maintained on rat-tail collagen coated petri dishes in 1:1 α MEM/Ham's F-10 medium mixture supplemented with 10% FBS, 300 μ g/mL Geneticin purchased from Gibco® (Thermo Fisher Scientific, MA, USA) and 1 ng/mL basic fibroblast growth factor (PeproTech, NJ, USA). Rat-tail collagen was isolated as previously described [28] and diluted to 250 μ g/mL in 70% Ethanol.

2.2. Primary endothelial cell isolation

Primary rat brain endothelial cells (primary ECs) were isolated from 8 to 10 week old male Wistar rats according to Coisne et al. [29], with slight modifications. Cortices were homogenized after removal of meninges in a Dounce homogenizer. Microvessels were isolated by adding an equal volume of 30% dextran solution to the homogenate and subsequent centrifugation at 3000g for 25 min at 4 °C, then filtered through a 60 μ m nylon mesh to remove larger vessels. After digestion in HBSS buffer supplemented with 10 mM HEPES, 0.1% BSA, 2 mg/mL collagenase-dispase, 10 μ g/mL DNase I and 147 ng/mL TLCK, for 45 min at 37 °C, microvessels were re-suspended in endothelial media (DMEM Glutamax II supplemented with 10% calf serum, BME amino acids, vitamin solution, 2 mM L-glutamine, 1 ng/mL bFGF, 59 μ g/mL gentamycin sulfate). After plating on collagen IV coated culture dishes pericyte contamination was prevented by exposing cultures to 3 μ g/mL puromycin-containing media for 24 h. Cells were used in experiments on day 5 without passaging.

2.3. Hypoxic and substance exposures

Hypoxic exposures were performed in a hypoxic glove box chamber at 37 °C, 5% CO₂ and 1% O₂ (InVivoO₂ 400, Ruskinn Technologies, Pencoe UK). An internal O₂ sensor monitored O₂ concentrations constantly. The following substances were added exogenously to the cell media: 0.4 nM human TGF β -1 (PeproTech) dissolved in 10 mM citric acid, 10 μ M ALK5 inhibitor SB431542 (Tocris, Bristol, UK) in DMSO, 20 μ M Naphthofluorescein (Furin inhibitor, Santa Cruz, TX, USA) in DMSO, 20 μ M Decanoyl-Arg-Val-Lys-Arg-chloromethylketone (CMK, Furin inhibitor, Santa Cruz) in H₂O. Cells were pretreated with inhibitors for 1 h prior to hypoxic and TGF β exposures.

2.4. Cell viability

Viability of confluent cells was assessed using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). MTT solution (Sigma-Aldrich, MO, USA) was added to the media (final concentration 0.5 mg/mL) and incubated for 1 h at 37 °C. The media was removed and formazan crystals dissolved in DMSO. Optical density was measured at 570 nm on a spectrophotometer (Thermo Labsystems, Multiskan RC Model 351) with reference filter at 670 nm.

2.5. Cell migration

RBE4 cells were grown in 6-well plates and a scratch was introduced vertically with a 200 μ L pipette tip. Three pictures were taken per well before and after the exposures with an inverted microscope coupled to an 8-bit CCD camera (Axiocam HR, Carl Zeiss, Switzerland). Image analysis using ImageJ software (NIH, USA) was used to determine scratch width. Mean migration (μ m/h) was calculated by evaluating the difference in gap size before and after the exposure.

2.6. Permeability assay

ECs were grown to confluency on 0.4 μ m Transwells (Corning Incorporated, NY, USA). After exposures fresh medium containing 1 mg/mL Lucifer yellow CH, lithium salt (Life technologies, OR, USA) was added to the upper compartment. At 9, 15, 30, 45 and 60 min aliquots were taken from the bottom compartment and tracer flux was determined with a fluorescence plate reader (FLx800, Biotek Instruments, Winooski, VT). A clearance slope established from the measurements obtained at the different time points was used to calculate the permeability coefficient (Pe) [30].

2.7. Western blotting

Cells were lysed in whole cell lysis buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 1% NP-40) supplemented with protease inhibitor cocktail (Calbiochem, Darmstadt, Germany), 1 mM sodium orthovanadate and 0.5 mM phenylmethanesulfonyl fluoride. Protein concentrations were determined with a Pierce BCA protein assay (Thermo Fischer Scientific). Proteins (50 μ g) were separated by SDS-PAGE then transferred to nitrocellulose membranes and blocked with 5% non-fat dried milk or 5% BSA in TBS. After incubation in primary antibodies over night at 4 °C membranes were washed then incubated with the corresponding HRP-conjugated secondary antibody. Bands were visualized with a luminescent image analyzer (Fujifilm, LAS-3000, Switzerland) and quantification was performed with ImageJ software (NIH, USA) using β -actin as loading control. The following antibodies were used: *anti-Furin* (SC-20801, Santa Cruz), *anti-HIF-1 α* (NB100-479 W-1, NovusBio), *anti-pSmad2* (138D4, Cell Signaling), *anti-Smad2* (D43B4, Cell Signaling), *anti-Smad1/5* (D5B10, Cell Signaling), *anti-Smad1* (D59D7, Cell Signaling) and β -actin (A5441, Sigma-Aldrich).

2.8. Quantitative RT-PCR

RNA was isolated from cells using TRIzol® reagent (Thermo Fisher). Total RNA (1 μ g per sample) was reverse transcribed with oligo-dT primers using the ImProm-II Reverse Transcriptase kit (Promega, Switzerland) according to the manufacturers protocol. Real-time quantitative PCR (qPCR) was performed with an ABI 7500 Fast Real-Time PCR System using Power SYBR® Green Master Mix (Applied Biosystems, Switzerland). The following primers were employed: Furin 5'-CAG AAG CAT GGC TTC CAC AAC-3' and 5'-TGT CAC TGC TCT GTG CCA GAA-3' and β -actin 5'-CTG GCT CCT AGC ACC ATG AAG-3' and 5'-GCC ACC GAT CCA CAC AGA GT-3'. Serial cDNA dilution series were used to establish optimal primer conditions resulting in > 90% efficiency. To exclude primer dimer and off-target amplifications melting

curves were performed and confirmed by gel electrophoresis. Data was normalized to β -actin and fold changes calculated using the $\Delta\Delta Ct$ method.

2.9. Statistical analysis

Statistical Analyses were performed with Prism GraphPad 6. Results are shown as mean values $\pm$ standard deviation with a minimum of 3 independent experiments. Statistical significance was determined by two-way ANOVA when comparing more than two different groups with different exposures or unpaired Student's t-test with homoscedasticity. A p value less than 0.05 was considered significant.

3. Results

3.1. TGF β promotes EC migration

Till now the role of TGF β signaling has been mainly studied in peripheral ECs whereas its effects on the brain endothelium remain largely debated. In the first instance, we aimed to establish how increased TGF β levels impact brain EC quiescence during hypoxic injury using a classic scratch migration assay and the rat brain microvascular endothelial (RBE4) cell line. Notably, our studies have shown this cell line exhibits the same responses as primary cells but with increased sensitivity to oxygen deprivation [6]. Scratch assays were performed during 6 h normoxic and hypoxic exposure with exogenous TGF β and ALK5 inhibitor treatment (Fig. 1A&B). Quantification showed no significant changes under normoxic conditions with any of the treatments (Fig. 1C). In contrast hypoxia significantly increased EC migration and TGF β potentiated this effect. Notably, both hypoxic and TGF β -mediated migration were completely blocked when ECs were pretreated with the TGF β receptor I inhibitor (SB431542). Thus TGF β potentiates hypoxia-mediated cell migration via a TGF β R1 dependent mechanism.

3.2. Hypoxia and TGF β modulate furin protein and expression and activation

Since existence of a feedback loop between TGF β and Furin has been implicated in various other (non-vascular) cells [24–26], we asked if a similar mechanism could underlie the movement we observed. As Furin mRNA levels were unaffected in our treatment conditions (Supp.

Fig. 1) we evaluated whether Furin activation correlated with the TGF β -induced migratory effects. Hypoxic HIF-1 α stabilisation confirmed the endogenous endothelial cell response was as expected and unaffected by TGF β treatment (Fig. 2A). Corresponding Furin immunoblots showed two prominent bands corresponding to the inactive precursor form at 96 kDa and the mature active form at 90 kDa (Fig. 2A). Quantitative analysis of the individual bands revealed a significant decrease of the Furin precursor under hypoxia. Notably, TGF β had no impact on normoxic precursor levels but induced hypoxic precursor levels by 50% to reach the baseline normoxic levels (Fig. 2B). Mature Furin levels remained unchanged by hypoxia compared to the normoxic baseline (Fig. 2C) but significantly increased during hypoxic TGF β exposure, with a trend to also increase in normoxia. This data suggests that during stress (hypoxic) conditions high TGF β levels might potentiate EC Furin activation by elevating precursor levels and inducing protein maturation.

3.3. Furin activation is dependent on ALK5 signaling

In ECs, canonical binding of TGF β to ALK5 causes downstream activation and phosphorylation of Smad2 resulting in stimulation of angiogenesis [31], whereas signaling via ALK1 induces phosphorylation of Smad1, 5 and 8 and subsequent endothelial quiescence [32]. Exposure of brain ECs to TGF β but not hypoxia increased phosphorylated Smad2 levels whereas the opposite effect was observed on phospho-Smad1/5 levels (Supp. Fig. 2&3). In line with suppressing cell migration (Fig. 1), ALK5 blockade also prevented TGF β -mediated Smad2 phosphorylation but not the hypoxia-induced Smad1/5 activation. As Furin levels were also strongly potentiated by both hypoxia and TGF β (Fig. 2) we asked if ALK5 blockade would suppress these effects (Fig. 3). Indeed immunoblotting showed SB431542 abrogated expression of the Furin precursor and mature forms by more than 50% during hypoxia and TGF β exposure (Fig. 3). Interestingly, this pattern was also observed during normoxia (Supp. Fig. 4) suggesting Furin activity is highly dependent on ALK5 activation in both injury and physiological conditions.

3.4. Furin inhibition prevents EC migration

We next evaluated if Furin is the mediator of hypoxia- and TGF β -induced EC migration. A scratch assay was performed over 6 h hypoxia

Fig. 1. TGF β promotes EC migration

Representative micrographs of scratched confluent RBE4 monolayers exposed to 0.4 nM TGF β , the ALK5 inhibitor SB431542 (10 μM) or a combination of both prior to Nx (A) or Hx (B) incubation for 6 h. Scale bar = 100 μm . Images were quantified and mean migration in $\mu\text{m/h}$ is presented (C). Mean $\pm$ SD (n = 3), 2way ANOVA, **p < 0.01 compared to Nx UNT, #p < 0.05 ##p < 0.01 ###p < 0.001 compared to Hx TGF β .

Fig. 2. Both hypoxia and TGFβ modulate Furin.

(A) Representative Western blot of Furin precursor (96 KDa) and mature (90 KDa) forms and HIF-1α stabilisation at 6 h hypoxia with and without TGFβ treatment. Blot quantification and graphical representation shows changes of the Furin forms under hypoxia and TGFβ treatment (B&C). β-actin was used as loading control. Data are expressed as mean ± SD of at least n = 3 independent experiments. Students t-test and 2way ANOVA, *p < 0.05 compared to Nx UNT, \$p < 0.05 compared to Hx UNT.

Fig. 3. Furin activation is dependent on TGFβ receptor-1 ALK5 signaling.

(A) Representative blot showing Furin protein expression after TGFβ or SB431542 treatment. Quantitative graphs show comparative levels of both precursor (B) and mature (C) Furin. Mean ± SD (n = 3), Students t-test, \$p < 0.05, §§p < 0.01 compared to Hx UNT, ###p < 0.001 to Hx TGFβ.

Fig. 4. Furin inhibition prevents EC migration

Representative micrographs of scratched RBE4 monolayers incubated with TGFβ, the Furin inhibitors Naphthofluorescein (Naph, 20 μM) or CMK (20 μM) or a combination of both prior to Hx exposure for 6 h (A). Scale bar = 100 μm. Quantified data (B) shows changes in EC migration under all conditions. Mean ± SD of at least n = 3 independent experiments. 2way ANOVA, ***p < 0.001 compared to Nx UNT, ###p < 0.01, ##p < 0.01 compared to Hx TGFβ, §§p < 0.01 to Hx UNT.

as previously in the presence and absence of TGFβ and the Furin inhibitors Naphthofluorescein [33] and CMK. Clear differences were apparent after visual inspection of the samples (Fig. 4A). Quantification showed hypoxia-induced cell migration was significantly potentiated when combined with exogenous TGFβ as expected however treating cells with Furin inhibitors completely abrogated this response even in the presence of TGFβ (Fig. 4B). Notably, both inhibitors also blocked normoxic EC migration by up to 60% (Supp. Fig. 5). These observations highlight that TGFβ-induced EC migration is mediated by Furin and again underline an important physiological role for Furin.

3.5. TGFβ potentiates hypoxia-mediated EC permeability

To understand whether injury-increased TGFβ levels contribute to altered barrier integrity we performed permeability assays on primary microvascular ECs isolated from adult rat brain and RBE4 (data not shown) in presence and absence of exogenous TGFβ (Fig. 5).

Experiments were performed for 24 h (Figs. 5A) and 48 h (Fig. 5B) under normoxic and hypoxic conditions. Hypoxia significantly increased permeability of EC monolayers as frequently observed in many studies including our own [3]. Exposure to TGFβ further potentiated hypoxia-induced barrier dysfunction although no effects were observed under normoxia (Fig. 5A). Pretreatment with SB431542 completely abrogated TGFβ and hypoxic driven effects to baseline normoxic levels. Indeed at both time points a similar outcome was seen although the effects at 48 h were clearly more enhanced (Fig. 5A&B). Thus sustained injury-induced activation of the endothelial TGFβ-ALK5 pathway culminates in barrier disturbance.

3.6. Furin inhibition prevents hypoxia-induced barrier permeability

Finally, due to the observed feedback between the proteins, we investigated if Furin inhibition would also suppress the barrier changes induced by hypoxic and/or TGFβ exposure. Again we employed the

Fig. 5. TGFβ potentiates hypoxia-mediated EC permeability via ALK5

Permeability assay was performed on primary rat endothelial cells grown to confluency on transwells then exposed to 0.4 nM TGFβ or 10 μM SB431542 during 24 h (A) and 48 h (B) hypoxia. Dashed line indicates normoxic baseline measurements. 2way ANOVA and Students t-test, §§p < 0.01, §§§p < 0.001 compared to Hx CTRL, ##p < 0.01 compared to Hx TGFβ treatment, Students t-test.

Fig. 6. Furin inhibition prevents hypoxia-induced barrier permeability Primary rat endothelial cells were grown to confluency on transwells or culture dishes before hypoxic exposure to 0.4

nM TGFβ, CMK (20 μM) or CMK combined with TGFβ for 48 h. Permeability assays (A) and MTT assays (B) were subsequently performed. Mean ± SD (n = 4), 2way ANOVA and Students t-test, **p < 0.01, ****p < 0.0001 compared to Normoxia, ##p < 0.01 to Hx TGFβ, \$\$p < 0.01, \$\$\$p < 0.001 to Hx UNT.

transwell permeability assay using primary rat ECs (Fig. 6A) and RBE4 (data not shown). Blocking Furin activation with CMK was highly efficient at preventing hypoxia-mediated barrier disruption after 48 h. Similar to observations in the migration assay, the CMK inhibitor resulted in a tighter monolayer than observed under normoxic conditions (Fig. 6A). Surprisingly however the combination of CMK and exogenous TGFβ dramatically increased EC barrier permeability by more than 5 fold. This led us to use MTT assays to assess if the observed permeability changes were due to compromised cell viability. Data showed that when combined with hypoxia TGFβ treatment already compromised cell function up to 20%. Whilst CMK alone had no effect, the combination treatment reduced mitochondrial activity by 50% (Fig. 6B). Thus Furin blockade in the presence of elevated TGFβ reduces cell viability and strongly impairs BBB functionality.

4. Discussion

Maintaining BBB integrity is crucial for CNS homeostasis. Hypoxia and ischemia are well known to compromise barrier stability via TJ and extracellular matrix disruption leading to permeability changes [5]. Such alterations ultimately contribute to disease progression [4]. This study shows that both hypoxia and exogenous TGFβ promote cell migration and barrier dysfunction via activation of the proprotein convertase Furin. Blocking Furin abrogates hypoxia and TGFβ-induced endothelial migration and permeability indicating a potential future target for vascular-related interventions.

Stimulatory effects of TGFβ on cell migration have been reported in intestinal [34] and renal epithelial cells [35], renal microvascular EC [36] and cancer cells [37] under normoxic conditions although high doses of TGF-β may inhibit non-brain EC migration [11,36]. In contrast TGFβ did not induce migration of brain ECs in normoxia but only during hypoxic insult in correlation with a study performed on fibroblasts [38]. Our observations thus highlight that TGFβ signaling activates angiogenic processes in brain EC only during injury conditions, a poignant difference between peripheral cell types and brain EC.

TGFβ induces the expression of Furin, a proprotein convertase whose up regulation consistently correlates with tumor cell activation and invasiveness [39]. In synovial cells [40] and bovine aortic endothelial cells [41] TGFβ was shown to increase Furin transcription. Despite no clear effects on mRNA levels, TGFβ treatment clearly induced Furin protein levels in brain EC. Endothelial TGFβ can signal through two different TGFβ type I receptors, ALK5 and ALK1, which

either promote angiogenesis or induce vascular quiescence respectively [11,32]. TGFβ signaling through ALK5 activates Smad2 and 3 whilst signaling through ALK1 activates Smad1, 5 and 8 [8,42]. In brain ECs differential Smad signaling was clearly stimuli-related with Smad1/5 phosphorylation being hypoxia-dependent and Smad2 activation being TGFβ-dependent. It seems likely that a balance between activation of ALK1 versus ALK5 determines whether ECs remain quiescent or initiate an angiogenic response, an observation previously described by Goumans et al., in 2002 [11]. In HUVEC and bovine aortic EC ALK5 majorly contributed to angiogenic processes [31,43]. Similarly ALK5 blockade prevented TGFβ-induced activation of Smad2 (without affecting pSmad1/5 levels) and suppressed brain EC migration in agreement with those findings. Notably, ALK5 activation was also linked to BBB dysfunction in mouse brain endothelial cell (bEnd.3) monolayers wherein TGFβ-induced permeability, via down regulation of the TJ protein Claudin-5, was mediated by Smad3 [44]. Overall our data supports the notion that suppressing ALK5 activation could be key for maintaining BBB function. Our data also suggests Furin modulation is strongly dependent on TGFβ/ALK5 signaling and mediated by Smad2 in brain EC in line with other work [18,40,45]. Whether Smad2 directly or indirectly regulates the effects of TGFβ on Furin levels is currently under investigation.

A curious observation from this work is that TGFβ induced mature Furin levels under both normoxic and hypoxic conditions but enhanced migration only occurred during hypoxia. Since Furin is a bona fide HIF-1 target [46] it seems plausible that the HIF-1 pathway could somehow contribute to this effect. HIF-1 targeted knockdown studies will further clarify this involvement.

Convertase enzymes exist in an immature preform with a prosegment still attached. Several studies have demonstrated the use of convertase prosegments as inhibitors of their parent enzymes *in vitro* and *ex vivo* [47,48]. During the maturation of Furin, the prosegment acts as a chaperone in the auto-activation pathway as well as a potent auto-inhibitor [49]. Indeed profurin overexpression in vascular smooth muscle cells decreased endogenous Furin protein levels and reduced cell proliferation and migration [50]. Further hepatic overexpression of the Furin prosegment reduced atherosclerosis progression and decreased vascular remodeling after injury *in vivo* [50]. In analogy we observed downregulation of the Furin precursor during hypoxia indicating that potential inhibition of maturation is lifted, resulting in increased Furin activity in line with our migration assay results. This data supports the conclusion that increased Furin activity is ultimately responsible for

hypoxic EC migration especially since Furin inhibition not only prevented hypoxic EC migration but also abrogated TGF β -induced effects. In agreement Furin inhibition also attenuated TGF β -enhanced migration of cardiac fibroblasts [51], reduced invasiveness/migration and tumorigenicity of human cancer cells *in vivo* [52] and *in vitro* [47]. Hence similar mechanisms underlie brain EC migration and likely contribute to increased barrier permeability during injury conditions. Although not elucidated here, a possible mechanism of Furin-mediated cell movement is likely linked to processing of matrix metalloproteinases (MMPs). Synthesized as inactive propeptides, MMPs undergo endoproteolytic cleavage to attain their active forms [53] resulting in extracellular matrix degradation and barrier disruption [54]. Accordingly hypoxia is known to increase Furin-mediated MT1-MMP processing [22,46]. Since Furin inhibition in human cancer cells and cardiac fibroblasts decreased processing and activation of MT1-MMP/MMP-2 [51,55] it is very tempting to speculate that TGF β -induced brain EC migration is driven by Furin-mediated activation of MMPs.

At the BBB, a TGF β -induced migratory (activated) EC phenotype implies barrier compromise as cytoskeletal alterations lead to a consequential loss of tight junction organization. Indeed TGF β induced brain EC migration and permeability and aggravated injury-induced barrier compromise via an ALK5-dependent mechanism in contrast with a study showing that activation of ALK5 by TGF β inhibits migration in non-brain ECs [13]. The unique qualities of BBB ECs including lack of fenestrations, more extensive tight junctions, and reduced pinocytic vesicular transport is clearly significant [6]. Although decreased viability of brain EC observed during TGF β treatment was unexpected our data further underscores that negative EC responses to the growth factor can be reversed by inhibiting either ALK5 or Furin activity. Indeed blocking of either abrogated hypoxic barrier disruption agreeing with the notion that these pathways are tightly entwined and induce a migratory/activated phenotype. It is now very evident that Furin activation also plays an important role in injury-induced BBB disturbance. However an intriguing and unexpected finding, given Furin inhibition prevented short-term hypoxic and TGF β -induced migration, was that combined prolonged TGF β treatment and Furin blockade caused more disruption than TGF β stimulus alone in correlation with dramatically compromised viability. The reason behind this is unclear but disruptive and negative time-dependent effects of sustained TGF β signaling at the BBB and potential confounding effects of modulating multiple downstream pathways must be better understood.

Overall our data suggests both hypoxia- and TGF β -mediated EC permeability can be prevented by blocking Furin and/or ALK5 activation. Notably, a large source of native active TGF β that can be released upon different injury stimuli *in vivo* lies within the perivascular cells and/or the extracellular matrix. Thus further studies using more complex BBB models such as co-culture of ECs with perivascular cells will be needed to provide further insight into the highly complex molecular mechanisms and cellular cross-talk that take place during injury *in vivo*. In summary, controlling Furin or ALK5 activation at the BBB could offer a novel therapeutic option for improving barrier functionality and CNS function during disease.

Acknowledgements

The authors are grateful to Dr. Michele Bernasconi for his scientific input and discussion and Maurizio Roveri for his technical contribution. Part of the experimental work was performed at the Center for Clinical Studies at the Vetsuisse Faculty of the University of Zurich. This work was supported by SNF grant numbers 31003A_150062 and 31003A_170129 to O.O.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.yexcr.2019.111503>.

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