Mechanosensitive expression of the mesenchymal subtype marker connective tissue growth factor in glioblastoma

Mechanical forces created by the extracellular environment regulate biochemical signals that modulate the inter-related cellular phenotypes of morphology, proliferation, and migration. A stiff microenvironment induces glioblastoma (GBM) cells to develop prominent actin stress fibres, take on a spread morphology and adopt trapezoid shapes, when cultured in 2D, which are phenotypes characteristic of a mesenchymal cell program. The mesenchymal subtype is the most aggressive among the molecular GBM subtypes. Recurrent GBM have been reported to transition to mesenchymal. We therefore sought to test the hypothesis that stiffer microenvironments—such as those found in different brain anatomical structures and induced following treatment—contribute to the expression of markers characterising the mesenchymal subtype. We cultured primary patient-derived cell lines that reflect the three common GBM subtypes (mesenchymal, proneural and classical) on polyacrylamide (PA) hydrogels with controlled stiffnesses spanning the healthy and pathological tissue range. We then assessed the canonical mesenchymal markers Connective Tissue Growth Factor (CTGF) and yes-associated protein (YAP)/transcriptional co-activator with PDZ-binding motif (TAZ) expression, via immunofluorescence. Replating techniques and drug-mediated manipulation of the actin cytoskeleton were utilised to ascertain the response of the cells to differing mechanical environments. We demonstrate that CTGF is induced rapidly following adhesion to a rigid substrate and is independent of actin filament formation. Collectively, our data suggest that microenvironmental rigidity can stimulate expression of mesenchymal-associated molecules in GBM.
softer than the surrounding brain\cite{15,16}, whereas others suggest they are stiffer\cite{17}. Further, some studies suggest that GBM may become more rigid after treatment\cite{18}.

The importance of mechanosignals derived from a tumour-secreted rigid, collagen-rich matrix is increasingly appreciated\cite{19}. Cells chiefly sense the mechanical properties of the surrounding environment (mechanosensing) through transmembrane integrin receptors located on the cell surface\cite{20}, which engage the extracellular matrix (ECM), then cluster forming focal adhesions and activating an internal signalling cascade. This promotes actin polymerization, where bundles of polymerized actin filaments (stress fibres) link to the integrin cytoplasmic tail. Myosin motor proteins on the stress fibres then exert contractile force through the focal adhesion onto the ECM\cite{21}. Consequently, in a rigid environment, increased internal contraction is generated in response and via this two-way force transmission, cells sense mechanical forces in the underlying tissue. Concomitantly, the outside-in mechanical signal received through the integrins is converted into biochemical signals.

Reports have demonstrated that GBM reacts to cues from a rigid microenvironment by spreading and forming prominent actin stress fibres\cite{22-24}. These phenotypes are reminiscent of mesenchymal transition, suggesting that a rigid tissue environment may contribute to the GBM mesenchymal program. The mesenchymal subtype is characterized by high level expression of connective tissue growth factor (CTGF)\cite{5}. CTGF expression is mechanosensitive\cite{25-28} and is one of the most upregulated genes in response to mechanical stress applied to fibroblasts\cite{29}. CTGF is a downstream target of well-known mechanotransductive transcriptional co-activators yes-associated protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ)\cite{25,30}. As YAP and its paralog TAZ share a distinctive WW domain that binds exclusively to Pro-Pro-X-Tyr binding motifs\cite{31}, and their individual roles in mechanosignalling have yet to be defined, they are often used either in conjunction or interchangeably. Experiments with drugs that variously induce actin polymerisation and depolymerisation, respectively, have led to the suggestion that mechanosensitive CTGF expression is inversely correlated with the ratio of monomer-to-filamentous actin\cite{32}, although there may be cell type specific differences\cite{33}.

The goal of the present study was to assess whether substrate rigidity induces CTGF in GBM, whether there are differences in rigidity-mediated CTGF expression between cell lines representing different GBM subtypes and whether CTGF regulation is inversely related to monomeric actin levels in GBM.

**Results**

**CTGF expression is rigidity sensitive.** Mechanosensitive regulation of the mesenchymal marker CTGF was assessed in primary patient-derived GBM lines grown on Matrigel-coated poly-acrylamide hydrogels (PAGs) in a range of defined stiffnesses emulating brain tissue (0.2, 1 and 8 kPa)\cite{10,11}, and the upper stiffness limit was assessed in primary patient-derived GBM lines grown on Matrigel-coated poly-acrylamide hydrogels (PAGs) in a range of defined stiffnesses emulating brain tissue (0.2, 1 and 8 kPa)\cite{10,11}. Cells chiefly sense the mechanical properties of the surrounding environment (mechanosensing), then cluster forming focal adhesions and activating an internal signalling cascade. This promotes actin polymerization, where bundles of polymerized actin filaments (stress fibres) link to the integrin cytoplasmic tail. Myosin motor proteins on the stress fibres then exert contractile force through the focal adhesion onto the ECM\cite{21}. Consequently, in a rigid environment, increased internal contraction is generated in response and via this two-way force transmission, cells sense mechanical forces in the underlying tissue. Concomitantly, the outside-in mechanical signal received through the integrins is converted into biochemical signals.

Importantly, changes in CTGF expression were not simply a consequence of the replating process. This was demonstrated by analyses of cell lines that had been first incubated on 0.2 kPa and then been replated directly onto 0.2 kPa PAGs (Fig. 3c(ii)). Under these experimental conditions, CTGF expression in WK1 cells was reduced
even further and was unchanged in JK2 cells that had been replated onto 0.2 kPa PAGs. Collectively, these data suggest that increased CTGF is a consequence of cell growth on the rigid 50 kPa substrate environment.

Mechanosensitive CTGF expression precedes actin filament organization. To investigate the specific role of actin stress fibres in this stiffness-mediated effect on CTGF expression, cells were treated with the actin-depolymerising compound Latrunculin A (Lat A). Soluble/insoluble fractions extracted from cells grown on plastic dishes were first visualised and analysed via Western Blot and densitometry, respectively to determine the concentration of Lat A required (Fig. 4a). This revealed a dose dependent increase in the soluble fraction with increasing Lat A concentration, as expected (Fig. 4a), with 0.5 µM Lat A resulting in significantly increased
levels of soluble actin. Immunofluorescence of F-actin further confirmed the loss of polymerised actin (actin stress fibres) in cells treated with 0.5 µM Lat A (Fig. 4b).

Having established the conditions of Lat A for effective actin depolymerisation, these conditions were used to directly test whether CTGF expression precedes actin filament organization. WK1 cells were thus seeded onto 0.2 kPa gels overnight and subsequently detached, incubated with 0.5 µM Latrunculin A and replated in the presence of Latrunculin onto 50 kPa gels. Despite Latrunculin-mediated inhibition of stress fibre formation, CTGF expression was induced (Fig. 4cii,d). Similarly, CTGF expression was induced in JK2 cells despite actin disassembly with Latrunculin A prior to replating on 50 kPa gels (Fig. 4eii).

Mechanosensitive CTGF expression is independent of induced actin organization. We next investigated the reverse scenario to Lat A treatment by instead inducing actin stabilisation on soft gels with Jasplakinolide, an actin-binding cyclic peptide that increases actin nucleation. Optimisation of Jasplakinolide concentration in cells grown on tissue culture plastic, demonstrated that 100 nM Jasplakinolide stabilises actin in WK1 cells (Fig. 5a). Subsequently, cells were plated and incubated overnight on 0.2 kPa gels, then treated with 100 nM Jasplakinolide or vehicle for 1 h. This approach revealed that actin stabilisation with Jasplakinolide did not induce CTGF expression in WK1 cells on soft 0.2 kPa PAGs (Fig. 5b). These data suggest that actin filament stabilisation in WK1 cells on soft substrates does not stimulate CTGF expression. Thus, mechanosensitive CTGF expression in WK1 cells does not correlate with polymerisation of the actin cytoskeleton.

Discussion

Data in this study have established that expression of the mesenchymal marker CTGF is regulated by substrate stiffness. Although it is widely suggested that CTGF expression occurs downstream of actin polymerisation, we show that CTGF expression occurs prior to spreading and in the presence of actin depolymerising drugs and, further, is not induced by actin polymerising agents. Our study reveals that mechanosensitive CTGF proceeds...
independently of actin polymerisation in the GBM cells under investigation. Instead, CTGF may be a crucial mediator for F-actin formation likely via the mechanoregulated pathways that precede it, such as YAP/TAZ. According to some studies, CTGF acts as a critical promoting factor for cell motility via F-actin stabilisation, large focal adhesion formation and lamellipodia/filopodia induction. The exact mechanisms underlying CTGF regulation by cytoskeletal and mechanotransduction pathways remain elusive. However, published studies suggest that RhoA seems to play an integral role. Studies support the induction of YAP/TAZ by mechanotransduction molecules such as RhoA, independent of their canonical role in Hippo signalling pathways. However, the exact mechanism of this has yet to be revealed. Neither microtubule disassembly, nor Rho kinase inhibition altered CTGF expression in WK1 cells on stiff environments (Supplementary Fig. 3), thus the causative signalling pathway still requires elucidation. Furthermore, interactions between Rho and Hippo family proteins in YAP/TAZ signalling are crucial future research targets. This is of particular interest as aberrant expression of YAP/TAZ is associated with the invasive mesenchymal type in GBM.

While the focus of the present study has been on cellular response to the elastic qualities of the underlying PAGs, most natural ECMs and tissues have both elastic and viscous qualities. Previous studies have suggested that the morphology of LN229 primary patient-derived GBM cells differ when exposed to purely elastic versus viscoelastic matrices. Magnetic Resonance Elastography measurements of GBM in vivo suggest that GBM are less viscous than the surrounding tissue. This has led to the intriguing proposition that GBM invade via viscous fingering, where a more viscous fluid is displaced by a less viscous fluid, potentially explaining the highly invasive

Figure 3. Connective tissue growth factor expression is rapidly induced by a stiff tissue environment. (a) WK1 cells grown on 0.2 kPa gels (ctrl) or grown on 0.2 kPa gels, then detached and replated onto 50 kPa gels as per the schematic and for the indicated times. Cells were immunostained with anti-CTGF antibodies, DAPI to detect nuclei and phalloidin to detect F-actin. Scale bar = 20 µm. (b) Mean surface area of cells cultured as shown in (a). Each data point represents the average from 3 biological repetitions (n = 35 for each respective group). Error bars indicate ± SEM. Symbols denote statistical significance: **p < 0.01; *p < 0.05; NS = not significant (p < 0.05); One-way ANOVA with Tukey’s post-comparison test. (c) Plots show fluorescence intensity of CTGF. WK1 and JK2 cells were treated as follows: (i) incubated on 0.2 kPa PAGs, (ii) incubated for 24 h on 0.2 kPa PAGs then transferred to fresh 0.2 kPa PAGs for 1 h, (iii) incubated on 50 kPa PAGs and (iv) incubated for 24 h on 0.2 kPa PAGs then transferred to 50 kPa PAGs for 1 h. Graphs show data pooled from ≥ 13 fields of view. Error bars indicate ± SEM. Symbols denote statistical significance: ****p < 0.0001; ***p < 0.001; *p < 0.05; Ns = not significant (p < 0.05); One-way ANOVA with Tukey’s post-comparison test.
Figure 4. Mechanosensitive CTGF expression precedes actin filament organization. (a) The concentration of Latrunculin A required to disassemble actin filaments was determined by western blot analysis of the insoluble versus soluble β-actin fraction following treatment of cells grown in plastic tissue culture dishes with the indicated Latrunculin A concentrations. Data points in graph show mean proportion of insoluble actin in WK1 cells as a percentage of the total β-actin (sum of insoluble plus soluble fraction) from three independent biological repeats. Error bars indicate ± SEM. Symbols denote statistical significance: **p < 0.01; ***p < 0.001; N = not significant (p < 0.05); One-way ANOVA with Tukey’s post-test. (b) Control untreated cells and cells treated with 0.5 µM Latrunculin A for 1 h, as indicated, grown on glass coverslips. Cells were fixed and stained with DAPI to show nuclei and phalloidin to detect F-actin. (c) WK1 cells cultured on 0.2 kPa gels were detached and replated onto 50 kPa gels either untreated (i, top row, ctrl) or in the presence of 0.5 µM Latrunculin A (ii Lat A, bottom row) as indicated in the schematic. Cells were then fixed and immunostained with anti-CTGF, DAPI to detect cell nuclei and phalloidin to detect F-actin—shown individually in grey scale and as a colour overlay in the final column. (d) Quantification of percentage of CTGF positive cells from the conditions described in (c). Data points represent three independent biological repeats. Error bars indicate ± SEM. Symbols denote statistical significance: N = not significant (p < 0.05); Students’ t-test. (e) JK2 cells. Scale bars = 50 µm.
phenotype that typifies GBM. Beyond these material properties of tissue, the biochemical composition is also key. The present study has been restricted to analysis of cells cultured on Matrigel-coupled PAGs, chosen because the primary patient-derived cell lines used in this study are cultured and maintained in Matrigel-coated vessels. However, studies suggest that Hyaluronic Acid (HA)—a major component of the brain extracellular matrix—can significantly influence GBM malignancy. It has been reported that the presence of HA in a soft environment can phenocopy the effects of stiffness on GBM biology. Thus, in future it will be important to understand how the intersection of elasticity, viscosity and biochemical composition of the matrix contribute more broadly to GBM cell biology.

Ultimately, it could be inferred that the mechanosenstive pathways that drive CTGF expression in GBM may be dependent on upstream RhoA-mediated YAP/TAZ activity. It is important to note, however, that there was not a consistent linear correlation between YAP/TAZ nuclear localisation and CTGF expression between 8 and 50 kPa surfaces, thus the elucidation of this pathway requires further analysis. Given the mechanosensitive status of CTGF, mechanical stimuli may be capable of suppressing deregulated or aberrant signalling initiated by other activation pathways. These findings are novel in the context of GBM research and highlight the importance of mechanoregulation in cancer malignancy, as well as the need for continued research and investigation into the regulatory role of the extracellular environment in malignant cell behaviour. Not only is the stiffness of GBM not yet unequivocally established, there is also a lack of understanding of the entire range of tissue forces encountered by GBM cells as they invade the brain, for example invasion along blood vessels. Potentially, microregional mechanical heterogeneities in the brain may alter cellular transcription programs, pushing cells

*Figure 5.* Mechanosensitive CTGF expression is independent of actin organization. (a) The concentration of Jasplakinolide required to stabilise actin filaments was determined by western blot analysis of the insoluble versus soluble β-actin fraction following incubation with the indicated concentrations of Jasplakinolide in cells grown on tissue culture plastic. Data points on the graph represent the insoluble actin as a percentage of total actin, collated from 3 biological repetitions. Error bars indicate ± SEM. Symbols denote statistical significance: *p < 0.05; NS = not significant (p < 0.05); One-way ANOVA with Tukey’s post-comparison test. (b) WK1 cells cultured on 0.2 kPa PAGs and grown in the presence of vehicle (first column of images) or 100 nM Jasplakinolide (second column of images) for 1 h, as per the schematic. Cells were fixed and immunostained with anti-CTGF, DAPI to detect cell nuclei and phalloidin to detect F-actin—shown individually in grey scale and as a colour overlay in the final row. Data points on the graph show the percentage of CTGF positive WK1 cells after an hour of culture on 0.2 kPa PAGs in the presence of vehicle (control) or 100 nM Jasplakinolide from 3 independent replicates. Error bars indicate ± SEM. Symbols denote statistical significance: N = not significant (p < 0.05); unpaired T-test.
into more aggressive mesenchymal phenotypes. It may therefore be prudent to consider the entire biomechanical life cycle of GBM as they encounter microregional mechanical heterogeneities in the brain or when treated with anti-cancer therapies.

**Materials and methods**

**Cell lines.** The patient-derived GBM cell lines JK2, SJH1, RN1 and WK1 were kindly provided by the Brain Cancer Research Unit, QIMR Berghofer Medical Research Institute, Brisbane, Australia, from patient-derived surgical aspirates. Patient tissue was collected by the team at QIMR following written informed consent and with human ethics approval from the Royal Brisbane and Women’s Hospital, Brisbane and the QIMR Berghofer Institute and in accordance with approved guidelines. All lines were independently Short Tandem Repeat (STR) profiled by CellBank Australia by our lab.

**Cell culture maintenance and media.** All cell lines were cultured as mono-layers and maintained in filter-capped tissue culture flasks coated with 1% (v/v) Matrigel (Matrigel Matrigel Basement Membrane Matrix LDEV-Free; BD Biosciences, Mississauga, Canada) in Dulbecco’s Modified Eagle Medium (DMEM; ThermoFisher, MA USA). Culture vessels were coated with Matrigel by covering the internal surface with 1% Matrigel in DMEM High Glucose Pyruvate media and incubation for 1 h at 37 °C or overnight at 4 °C. Cells were maintained at 37 °C and 5% CO₂ in KnockOut® DMEM/F-12 (ThermoFisher) supplemented with recombinant Human EGF (20 ng/mL; ThermoFisher), recombinant Human FGFb (10 ng/mL; ThermoFisher), glutatione (20 mM/mL; ThermoFisher), penicilin/streptomycin (100 U/mL; ThermoFisher), StemPro Neural Supplement (20 ng/mL; ThermoFisher) and heparin (20 ng/mL; Sigma-Aldrich, MO USA). Cells were sub-cultured for a maximum of 35 passages, with cells detached for passaging with StemPro Accutase (ThermoFisher).

**Polyacrylamide (PA) hydrogels.** PA 35 mm Petrisoft™ (plastic-bottomed) and Softview™ (glass-bottomed) products (Matrigen, CA USA) of defined stiffness (0.5, 1, 8 and 50 kPa) were used for bright field microscopy and confocal microscopy, respectively. Stiffness calibrations are performed by Matrigen on each batch of solution of a targeted stiffness for quality assurance. All PAGs were coated with 1% Matrigel solution for 1 h and rinsed with phosphate buffered saline (PBS, ThermoFisher) before cells were seeded.

**Immunofluorescence.** Cells were seeded onto Matrigel-coated PAGs and glass coverslips at a density of 7–9 × 10⁶ in supplemented media. Media was aspirated and adherent cells were washed with 1 × PBS, fixed with 4% paraformaldehyde (PFA, Sigma-Aldrich) (v/v) for 10 min at room temperature, then washed three times with 1 × PBS and permeabilised for 5 min in permeabilising solution—0.2% Triton X-100 (v/v) (Sigma-Aldrich) in wash buffer (0.5% bovine serum albumin (BSA, ThermoFisher, (w/v) in PBS). Permeabilising solution was aspirated, and the cells were washed three times with wash buffer. For TAZ staining, samples were first blocked for 1 h at room temperature with 5% BSA in normal serum (10% Donkey serum (sigma-Aldrich) (v/v) in 1 × PBS). Cells were then incubated with a primary antibody targeting CTGF (1:1000, Ab6692, Abcam) or TAZ (1:200; Ab110239; Abcam) for 1 h at room temperature followed by washing three times with wash buffer and incubation with fluorescently-tagged secondary antibody (Abcam) diluted at 1:1000 in normal serum for 1 h at room temperature. Cells were counter-stained with DAPI (Sigma-Aldrich) and fluorescein (ThermoFisher) using standard protocols and washed a final three times with wash buffer and twice with distilled water before mounting onto microscope slides.

**Replating.** For experiments involving replating, 3.2 × 10⁵ cells were seeded onto 0.2 kPa PA 35 mm plates and incubated overnight. The following day, cells were detached and replated onto fresh PAs as indicated. Fluorescent images were captured using an IX81 inverted microscope (Olympus) with an ORCA-AG ERG cooled CCD camera (Hamamatsu Photonics) at 20× magnification.

**Actin polymerisation and depolymerisation.** Cells were fractionated into detergent–soluble and detergent-insoluble components as previously described. Briefly, Soluble and insoluble fractions were isolated following detergent extraction with CSK (cytoskeleton stabilization buffer: 0.3 M sucrose, 0.5% Triton X-100, 10 mM PIPES, pH 6.8, 100 mM KCl, 1 mM CaCl₂, 2.5 mM MgCl₂, 1% aprotinin, 50 mM NaF, 0.1 mM Na₃VO₄) and 0.1% SDS-RIPA (50 mM Tris–Cl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 0.1% SDS, 1% sodium deoxycholate) respectively, as previously described. Soluble and insoluble volumes were equalized and equal volumes of each loaded onto SDS-PAGE gels and separated by electrophoresis. Percentage insoluble actin is determined from densitometric measurement of insoluble actin/(soluble actin + insoluble actin). Latrunculin A (Cayman Chemical, MI USA) was prepared as a solution in Phosphate Buffered Saline (PBS) and control cells treated with matched PBS vehicle control. Jasplakinolide (Cayman Chemical, MI USA) was prepared as a solution in ethanol, and control cells treated with matched ethanol vehicle control. Separated proteins were electrophoretically blotted to PVDF membranes and membranes probed and developed with Western Lightening Chemiluminescence reagent (PerkinElmer). To determine the effect of Latrunculin A treatment on mechanosensitive CTGF expression cells were first plated on 0.2 kPa gels, detached with Acutase and then plated onto 50 kPa gels in the presence of 0.5 μM Latrunculin A for 1 h. To determine the effect of Jasplakinolide, cells grown on 0.2 kPa gels were exposed to 100 nM Jasplakinolide for 1 h.

**Imaging, image processing, measurement and statistical analysis.** Cells images were captured using an IX81 inverted microscope (Olympus) with ORCA-AG ERG cooled CCD camera (Hamamatsu Pho-
tonics). CTGF fluorescence intensity was analysed in ImageJ with a custom-designed analysis plugin, ‘imagej-interactive-thresholding’ (https://github.com/christophmark/imagej-active-thresholding), and with Metamorph image analysis software (Molecular Devices, CA USA). Cell areas were masked using the extent of their stained actin cytoskeletons, then the average fluorescence intensity of CTGF signal within these segmented areas was calculated (Supplementary Fig. 4). Background signal was calculated using negative control slides exposed to secondary antibody only, and subtracted from all slides. The data were normalised to cell number in each region of interest via concurrent nuclei counts. Statistical analysis was undertaken using Prism 8 (Graphpad). Nuclear localisation of YAP/TAZ immunostaining was scored by 3 independent, blinded scorers, assessing nuclear localisation of cells co-stained with DAPI to detect nuclei. Blinding was achieved using Blinder software35. Cells that were negative for YAP/TAZ immunostaining were excluded from the analysis.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Competing interests
The authors declare no competing interests.

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