

Cell Surface Thiol Engineering Mechanoregulates Myogenic Differentiation via the FAK–PI3K–AKT Axis

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Skeletal muscle regeneration is essential in conditions like muscle injury and muscular dystrophy, primarily relying on the activation and differentiation of myogenic progenitors. This study explores the role of redox-modulated surface thiol modification in myogenesis, using tris(2-carboxyethyl)phosphine hydrochloride (TCEP) to reduce disulfide bonds and generate free thiol groups on the cell surface. The findings show that TCEP treatment significantly increases cell surface thiols, activating the focal adhesion kinase (FAK)-phosphoinositide 3-kinase (PI3K)-protein kinase B (AKT) signaling pathway, and promoting cell spreading, adhesion, and actin cytoskeleton remodeling. This biochemical modulation upregulates myogenic and fusion-related markers, facilitating multinucleated myotube formation. Biophysical analysis using traction force microscopy (TFM), intracellular force microscopy (IFM), and monolayer stress microscopy (MSM) demonstrates that TCEP-treated cells, particularly at muscle-relevant stiffness (20 kPa), exhibit increased traction, intracellular, and monolayer stresses compared to untreated cells. These results suggest that redox-modulated surface thiol modification, combined with specific stiffness conditions, enhances myogenic differentiation and myotube maturation through biomechanical signaling. This proof-of-concept study advances the understanding of how redox-modulated surface engineering and substrate stiffness regulate myogenesis, offering novel therapeutic strategies for muscle regeneration and optimization of stem cell-based therapies for muscle tissue repair

1. Introduction

Muscle stem cells, located between the basal lamina, constitute less than 5% of the mononuclear cells in skeletal muscle tissue, yet possess remarkable regenerative capabilities.^[1] These cells not only maintain their self-renewal capacity but also differentiate into myogenic progenitor myoblasts, crucial for the development, growth, maintenance, and repair of skeletal muscles.^[2] Upon muscle injury, activated skeletal muscle stem cells undergo self-renewal and proliferation, differentiating into myotubes or myofibers through the fusion of mononuclear myoblasts into multinucleated myotubes.^[3] This process is driven by the fusion of myoblasts, which activates muscle progenitors through cell–cell and cell–matrix interactions.^[4]

Muscle-wasting diseases such as cachexia, muscular dystrophy, and age-related sarcopenia are characterized by impaired skeletal muscle regeneration and progressive muscle loss.^[5] In addition to these diseases, biological processes such as cellular senescence and external factors

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like severe muscle damage have also been shown to impair muscle regeneration, further contribute to muscle wasting.^[6] Therefore, stem cell transplantation has emerged as a promising therapeutic approach, offering a renewable cell source for treating muscle wasting.^[7] Beyond the intrinsic properties of skeletal muscle stem cells, the local and systemic environments critically influence the fate of skeletal muscle stem cells within the native skeletal muscle tissues.^[8] The extracellular matrix (ECM) within the stem cell niche is pivotal for the regeneration of skeletal muscle stem cells.^[9] When activated, skeletal muscle stem cells are regulated through interactions within the microenvironment, including cell–cell and cell–matrix interactions.^[10] For example, fibro-adipogenic progenitor cells regulate the self-renewal, proliferation, and differentiation of skeletal muscle stem cells.^[9,11] Differentiation is regulated through cell–cell interactions and cell–matrix adhesion, promoting cellular fusion. Skeletal muscle stem cells recognize the ECM via integrins (α - and β -subunits) on the cell membrane, where integrin activity leads to the phosphorylation of focal adhesion kinase (pFAK), controlling muscle remodeling and intracellular tension.^[4a] Furthermore, FAK-mediated cell adhesion promotes cytoskeletal remodeling through phosphoinositide (PI3K)/protein kinase B (AKT) signaling.^[12]

Recent advances in redox biology suggest that extracellular thiol–disulfide exchange can dynamically modulate the structure and function of cell surface proteins, including integrins and adhesion molecules.^[13] However, the interplay between redox-mediated surface remodeling and cellular mechanotransduction remains poorly understood, particularly in the context of skeletal muscle stem cell behavior. Here, we introduce a redox-modulated cell surface engineering strategy using tris(2-carboxyethyl)phosphine hydrochloride (TCEP), a membrane-impermeable reducing agent, to selectively convert surface disulfide bonds into free thiols on myoblasts.^[14] We hypothesize that this thiol induction enhances cell–matrix adhesion and intracellular tension in a stiffness-dependent manner, thereby promoting myogenic differentiation through FAK–PI3K–AKT signaling.

This proof-of-concept study is, to our knowledge, the first to systematically integrate redox modulation with stiffness-tunable hydrogel platforms to investigate how redox cues influence mechanosensitive behaviors during myogenic fusion. We combine immunocytochemistry, gene expression analysis, and quantitative force microscopy, including traction force microscopy (TFM) and intracellular force microscopy (IFM), and monolayer stress microscopy (MSM), to establish a mechanistic link between redox state, adhesion dynamics, cytoskeletal contractility, and myogenic potential. By elucidating this novel redox–mechanotransduction axis, our findings provide insight into the combinatorial regulation of myogenesis and suggest a poten-

tial strategy for optimizing preconditioning protocols in stem cell-based muscle regeneration therapies. Furthermore, these insights may inform future in vivo approaches targeting redox pathways to restore muscle function in fibrotic or degenerative conditions.^[15]

2. Results and Discussion

2.1. Cell Surface Thiol Engineering Using TCEP, a Reducing Agent, and Assessment of Its Cell Cytotoxicity

To dissect the role of cell surface redox state in regulating myogenic behavior, we employed a redox-modulated surface engineering strategy using TCEP, as shown in the schematics in **Figure 1a**. Proteins on the plasma membrane are stabilized by disulfide bonds, many of which participate in structural integrity and receptor-mediated adhesion.^[13] By selectively reducing these extracellular disulfide bonds, TCEP induces the exposure of free thiol groups (–SH) on cysteine residues, thereby enabling modulation of the redox environment at the cell–material interface.^[14,16]

This approach was chosen based on the need for a biocompatible, extracellularly restricted, and transient surface engineering method to investigate redox–mechanosensitive coupling in muscle stem cell differentiation. Moreover, by adjusting the pH, the reduction rate and degree could be precisely controlled, ensuring that TCEP remained impermeable to the phospholipid bilayer of the cell membrane and thus did not penetrate the intracellular space.^[14b,17] For example, Baronas et al. directly compared the membrane permeability of TCEP and dithiothreitol (DTT) by measuring the redox states of both extracellular and intracellular environments after treatment.^[18] While DTT, a membrane-permeable reducing agent, altered the redox states both outside and inside the cell, TCEP treatment for 5 min resulted in similar extracellular reduction but had negligible effects on intracellular redox status. This evidence strongly suggests that TCEP acts exclusively at the cell surface and does not enter the cytoplasm. To this end, C2C12 murine myoblasts were treated with TCEP under optimized conditions (1 mM for 5 min) to ensure efficient thiol formation with minimal perturbation to cell physiology.

TCEP-induced thiol presentation was confirmed by staining with Alexa Fluor 488-conjugated maleimide (mFluor 488), a thiol-reactive fluorescent probe. Cells were labeled in suspension to minimize integrin engagement and ensure uniform labeling across the membrane, as visualized by 2D and 3D confocal microscopy (**Figure 1b** and **Figure S1a**, Supporting Information). In the control group, maleimide-488 fluorescence on the cell surface was barely detectable, whereas cells treated with TCEP at concentrations ranging from 0.5 to 2 mM exhibited widespread fluorescence across the entire cell surface, demonstrating enhanced maleimide-488 labeling of the cell surface. The 3D images obtained by confocal microscopy confirmed the uniform distribution of free thiol groups across the cell surface. This indicates that maleimide can stably form covalent bonds with cysteine residues in thiol-containing proteins on the cell membrane, confirming the successful formation of free thiol groups on the cell surface, as evidenced by the observed location and intensity of maleimide-488 fluorescence.^[19] Additionally, the amount of exposed free thiols was quantified using Ellman's assay (**Figure 1c**). There was a

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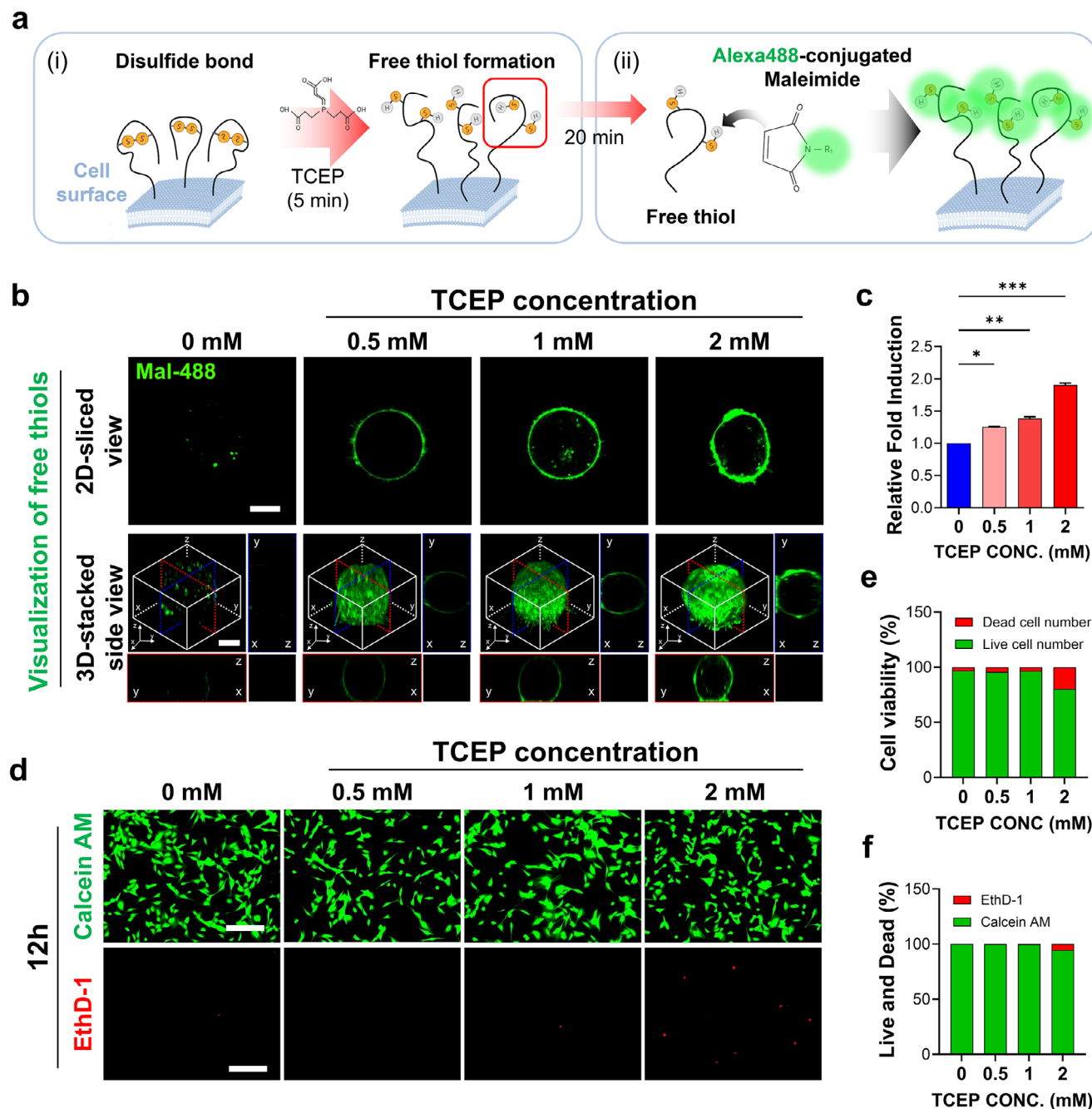


Figure 1. Surface modification and viability of C2C12 cells with free thiols through cell surface reduction. C2C12 cells were treated with TCEP under suspension conditions for 5 min at RT and visualized using maleimide-based mFluor dyes and Ellman assays. Cell viability was assessed 12 h post-treatment. (a) Schematic illustration of (i) a disulfide bond reduced to a free thiol group and (ii) Alexa 488-conjugated maleimide (green) binding to the free thiol group. (b) Fluorescent visualization of free thiol group by mFluor (maleimide 488 staining) in a TCEP concentration-dependent manner. Scale bar = 10 μ m. (c) Quantification of free thiol groups using Ellman's assay ($n = 3$). (d) Fluorescence visualization of live and dead by calcein AM-stained (green) and ethidium homodimer-1-stained (EthD-1) (red) C2C12 cells in a TCEP concentration-dependent manner (replication = 3). Scale bar = 200 μ m. Calcein AM was used to visualize live cells and EthD-1 was used to visualize dead cells. (e) Cell viability was assessed by the trypan blue exclusion assay. (f) quantification of live and dead cell percentages based on Calcein AM (live cells, green) and EthD-1 (dead cells, red) staining. (0 mM, $n = 10$; 0.5 mM, $n = 11$; 1 mM, $n = 9$; 2 mM, $n = 9$). Data were analyzed using the Kruskal–Wallis test followed by Dunn's post-hoc test. Data are presented as the mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

significant increase in the free thiol content in the TCEP-treated groups (compared to the control), which was in line with the visualization results obtained with maleimide-488.

To evaluate whether reduction of the cell surface by TCEP affected cell viability, we conducted a live/dead assay and performed cell counting. Live/dead assays were used to determine the cytotoxicity of the free thiol groups (at working concentration) on the reduced cell surface membrane. The assays revealed that cell viability did not decrease (compared to the negative control group), and cells treated with <2 mM TCEP remained in a live state, whereas 2 mM TCEP resulted in a 5% increase in dead cells (Figure 1d,f). Additionally, analysis of live and dead cells confirmed by hemocytometer cell counting showed that <2 mM TCEP did not affect cell viability, but 2 mM TCEP-treated cells showed a significant 19.6% decrease in viability (Figure 1e). Furthermore, the quantification of the free thiol groups on the cell surface revealed a concentration-dependent increase. Viability assays confirmed that a concentration of <2 mM had no adverse effect on cell viability. Therefore, 1 mM was chosen as the optimal concentration in subsequent experiments to provide the efficient formation of free thiol groups without compromising cell viability. We further characterized the temporal stability of the surface thiol groups. Following TCEP treatment, cells were incubated in suspension and analyzed at 0, 3, and 6 h post-treatment. Maleimide-488 staining intensity declined markedly within 3 h (Figure S2a, Supporting Information), indicating partial reoxidation of thiol groups and highlighting the reversible and transient nature of the redox modification.

Stem cell engineering, focused on regulating the stem cell microenvironment, has expanded into cell therapy to enhance cell survival and differentiation.^[20] In particular, engineering cell surfaces using chemical, biological, and material approaches, induces cellular functions and activities.^[21] Muscle stem cells represent a critical focus, as their regenerative ability can be improved by modulating the extracellular environment.^[22] Studies suggest that the cysteine residues present on the cell surface are the main source of free thiol groups that are exposed to the extracellular environment.^[23] In cellular and animal models, this exposure has been reported to enhance the cellular uptake of various synthetic compounds, thereby strengthening cell adhesion. Additionally, Kim et al. demonstrate the feasibility of using fluorescent mesoporous silica nanoparticles for localized drug delivery by modifying the surface of TCEP-treated cells through maleimide-thiol coupling.^[16] During this covalent binding process, TCEP treatment reduced disulfide bonds on the cell surface, leading to the formation of free thiol groups, as confirmed using a fluorescent dye with a maleimide functional group (mFluor). The process successfully facilitated the formation of free thiol groups in various human cancer cell lines (HeLa cells), human mesenchymal stem cells (MSCs) derived from bone marrow, and mouse myoblasts (C2C12 cells) without compromising cell viability or altering metabolic and functional properties following transplantation into mice.

Taken together, these results demonstrate that TCEP enables precise, surface-localized, and temporally tunable modulation of cell surface thiol content without compromising cell viability or intracellular homeostasis. This redox-engineering strategy establishes a robust and biocompatible platform for surface-specific chemical modification, enabling downstream investigations into

how extracellular redox cues may influence stem cell behavior. Importantly, these findings address a foundational gap by validating a method for selectively remodeling the cell surface redox state without perturbing intracellular physiology, a prerequisite for exploring redox-mechanical coupling in muscle progenitor systems.

2.2. Enhanced Cell-ECM Adhesion Through Redox-Induced FA Assembly

We investigated the effect of cellular thiols on ECM to determine whether thiol groups on the cell surface contribute to the formation of focal adhesions (FAs). TCEP-treated C2C12 cells were cultured on tissue culture plates (plastic or glass-bottom dishes) for 12 h, and cell adhesion was assessed. TCEP-treated C2C12 cells showed significantly increased transcript-level expression of FAK, vinculin, and paxillin, which are downstream components of focal adhesion (FA) signaling (Figure 2a). The transcript-level expression of PI3K and AKT, which are related to cytoskeletal remodeling, was also increased.

To confirm the increase in cell adhesion and cytoskeletal rearrangement induced by the presence of thiol groups on the cell surface 12 h after single-cell seeding, we utilized a custom MATLAB program to define cell boundaries based on F-actin fluorescence images and compare cell area. Within each identified cell area, the fluorescence intensities of F-actin, pFAK, and vinculin were quantified. An increase in cell spreading was observed following TCEP treatment, as indicated by the enlargement of cell area (Figure 2b, and Figure S3a,b Supporting Information). Similarly, it was confirmed that the cell intensity of F-actin, which represents the cytoskeleton of cells, and the cell size increased with TCEP treatment. Similarly, TCEP-treated cells exhibited enhanced F-actin intensity and increased cell size, suggesting reinforced cytoskeletal organization. The normalized F-actin intensity relative to cell area also increased, further confirming cytoskeletal rearrangement (Figure 2c). pFAK and vinculin were assessed by immunofluorescence staining, which revealed their expression at the cell edge (Figure 2d-h).

In general, vinculin resides in the cytoplasm in an autoinhibited, inactive conformation, maintained by intramolecular interactions between its head and tail domains.^[24] Upon integrin engagement with the ECM, Talin is activated and subjected to mechanical tension, resulting in a conformational change that exposes vinculin-binding sites.^[25] This interaction leads to vinculin activation, promoting its recruitment to FAs at the cell periphery.^[26] Activated vinculin can further bind to F-actin, reinforcing adhesion sites and facilitating the transmission of mechanical signals into the cell interior. Therefore, vinculin observed at the cell edge and cytoplasm was considered as potentially activated and included in our analysis.

To quantitatively analyze FA features, we restricted the detection area to subcellular regions ranging from 1.5 to 30 μm^2 within the total cell area, thereby isolating discrete adhesion sites. Using this approach, FA area, number per cell, and fluorescence intensity were quantified. Consistent with visual observations, the intensity, area, and number of FAs at the cell edge, including pFAK and vinculin, were approximately twofold higher in the TCEP-treated group than in the control group. Additionally, vin-

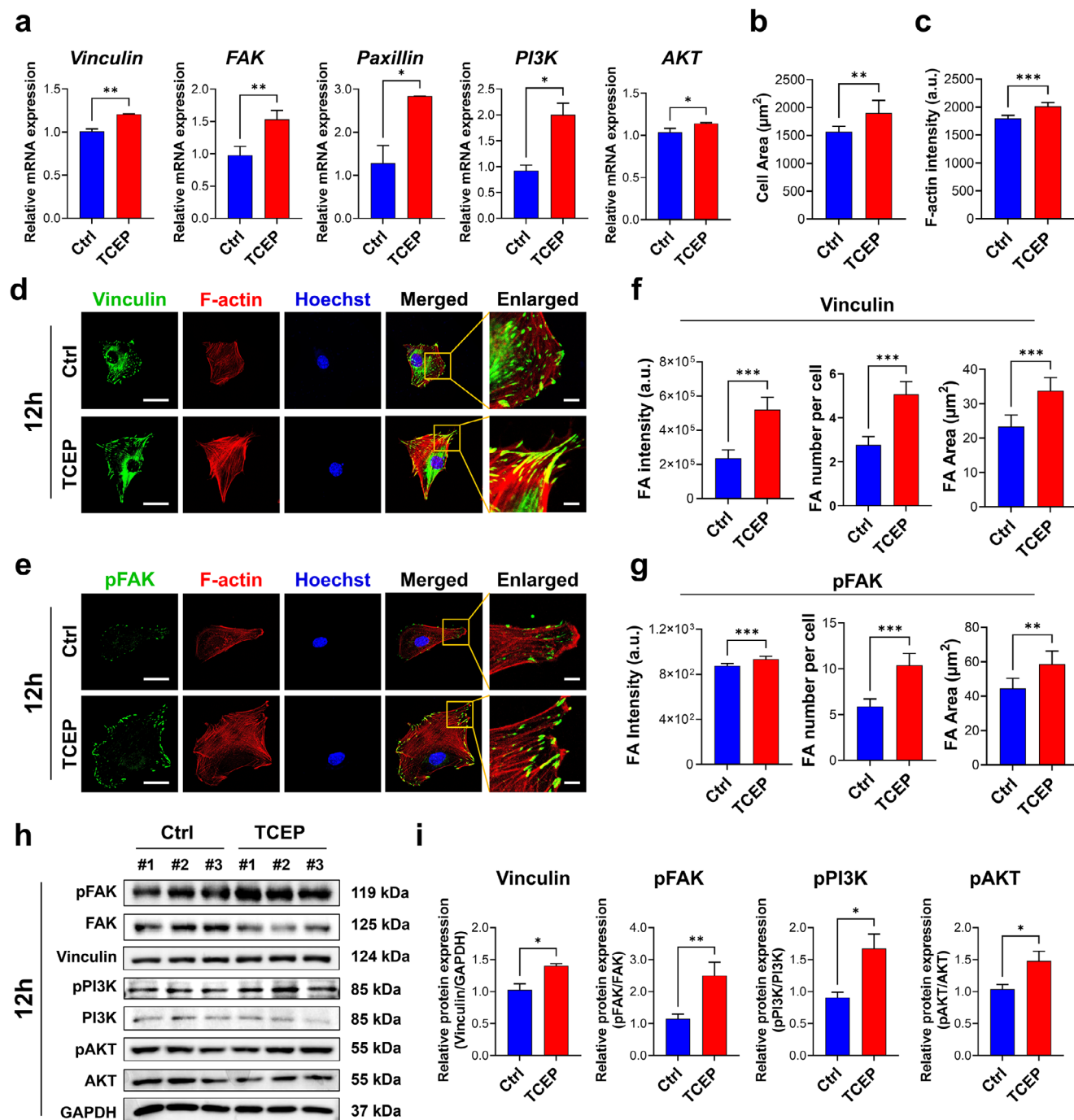


Figure 2. Enhanced cell adhesion and cell cytoskeleton rearrangement through cell surface modification. Cells were collected 12 h after 1 mM TCEP treatment for subsequent qPCR, immunofluorescence (IF) staining, and Western blot analyses. (a) Transcript-level expression of FA-related markers assessed by qPCR. (b, c) Quantification of cell area and F-actin intensity based on F-actin-defined cell boundaries (for ctrl, $n = 194$; for TCEP, $n = 169$). F-actin intensity was normalized to the corresponding cell area. (d, e) Representative IF images showing FA-related markers vinculin (green) and phosphorylated FAK (pFAK, green), along with F-actin (red) and nuclei (blue; Hoechst). Scale bar = 25 μm (cropped image = 5 μm). (f, g) Quantification of vinculin and pFAK-positive FA area and intensity (for vinculin, $n = 216$ (ctrl), $n = 325$ (TCEP); for pFAK, $n = 180$ (ctrl), $n = 143$ (TCEP)). (h, i) FA-related protein expression assessed by western blotting of TCEP-treated C2C12 cells ($n = 3$, respectively). Data were analyzed using the Kruskal–Wallis test followed by Dunn’s post-hoc test. Data are presented as the mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

culin was predominantly expressed between the nucleus and cell edges, with a notable increase observed in the C2C12 cells treated with TCEP to induce surface thiol groups. Similarly, pFAK was primarily localized at the cell base and showed increased intensity and number in the TCEP-treated group compared to the control group.

The role of TCEP in promoting cell adhesion through thiol group formation has been extensively studied.^[27] Cha et al. demonstrated that reducing the surface area of human glioblastoma cancer cells promotes the formation of free thiol groups, which enhances the expression of FA markers such as FAK and vinculin. This process also reorganizes the cytoskeletal arrangement of F-actin in the ECM while reducing invasiveness, suggesting a novel therapeutic approach for treating invading malignant cancers.^[28] Our study also confirmed that the formation of free thiol groups induced by TCEP treatment enhanced cell adhesion, as evidenced by increased cell area, F-actin intensity, and FAs. This suggests that TCEP promoted cytoskeletal organization, thereby influencing cellular morphology.

Additionally, the levels of phosphorylated FAK and vinculin were increased in the TCEP-treated group. Phosphorylation of PI3K and AKT and downregulation of FA signaling intermediaries were also increased, as confirmed by western blotting (Figure 2h,i). Similarly, Kim et al. reported that the reduction of MSC surface markers suppresses transforming growth factor- β (TGF- β) signaling while enhancing FAK activation, which collectively downregulates Mothers against decapentaplegic homolog 2 (SMAD2)—a downstream marker of the osteogenic potential marker CD105—and promotes early osteogenic differentiation.^[29] Taken together, these findings suggest that a reduction in the cell surface can enhance FAK activation, subsequently altering intracellular signaling pathways and modulating cellular responses.

Tamura et al. investigated the effects of phosphatase and tensin homolog (PTEN) inhibition on the interactions between FAK, PI3K, and AKT in glioblastoma and breast cancer cells.^[30] They demonstrated that FAK, PI3K, and AKT, which were suppressed by PTEN, were reactivated by PTEN dephosphorylation (PTEN-trapping mutant, D92A). This occurs through an interaction with the phosphorylation site of FAK at Tyr397, leading to the sustained phosphorylation of PI3K and AKT. This is because the previously restricted interaction with the ECM is restored, enabling intracellular signal transduction. Similarly, our findings suggest that the slight reduction in muscle progenitor cells induced by TCEP treatment leads to an increase in cell adhesion markers, such as pFAK and vinculin, as well as cytoskeletal remodeling through the enhanced activation of PI3K, AKT, and F-actin. This implies that TCEP may enhance interactions and binding to the external environment.

2.3. Enhanced Myogenic Differentiation Following Cell Surface Thiol-Mediated FAK Phosphorylation

We observed that TCEP treatment enhances cell–matrix adhesion via the formation of cell surface thiol groups. This effect is accompanied by increased phosphorylation of focal adhesion kinase (pFAK), cytoskeletal remodeling, and cell spreading. Based on these findings, we hypothesize that surface thiol formation

indirectly promotes myoblast fusion by strengthening focal adhesions and generating intracellular tension. These mechanical conditions are known to facilitate closer cell–cell contact, which is a prerequisite for efficient myoblast fusion.^[31] While direct evidence linking surface thiols to native cell–cell adhesion is limited, prior studies offer supporting insight. For instance, engineered thiol groups have been shown to mediate artificial intercellular conjugation via maleimide-functional ligands, suggesting their potential to promote intercellular interactions.^[32] Similarly, in glioma spheroids, TCEP-mediated surface reduction enhanced vinculin expression and reduced spheroid invasion, suggesting improved intercellular cohesion via redox modulation.^[28] Although the precise molecular mechanism remains to be fully defined, these data collectively support a model in which TCEP-induced thiol engineering enhances cell–matrix adhesion and mechanotransduction via the FAK–PI3K–AKT axis, thereby indirectly promoting cell–cell contact and myogenic fusion. Thus, to test this hypothesis, myogenic differentiation was induced by switching to differentiation medium 12 h after TCEP treatment, and the cells were cultured in differentiation medium for 4 d in a plastic tissue culture plate (plastic) (Figures 3a and S4a, Supporting Information). After 4 d of myogenic differentiation, C2C12 cells treated with TCEP showed increased transcript-level expression of the differentiation markers MyHC, desmin, MyoD, and Myf5 (Figure 3b). Additionally, the expression of the fusion-related marker caveolin-3 (Cav3) increased, whereas that of markers related to cell membrane remodeling, namely, Tmem8cf, M-chd15, and Myomerger, showed no significant changes in the TCEP-treated group (Figure 3c). Additionally, the transcript-level expression of vinculin; Fibronectin, which are cell adhesion markers that regulate cell–cell interactions during differentiation, increased (Figure 3d). The expression of genes involved in PI3K/AKT signaling, which mediate the downstream cytoskeletal remodeling of FAK signaling, was also upregulated in the TCEP-treated group (Figure 3e). At the protein level, increased expression of myogenic differentiation markers MyHC, desmin, and MyoD, the fusion marker Cav3, as well as, vinculin, phosphorylated FAK, and phosphorylated PI3K/AKT signaling components was observed (Figures 3f–h).

The process of myoblast fusion is influenced by extracellular cues that transmit mechanical signals to the intracellular environment, modulating signaling pathways and cellular tension.^[33] Inhibition of FAK, essential for fusion during differentiation, leads to reduced muscle regeneration by preventing the formation of large muscle fibers (>40 μ m) in damaged mouse muscle.^[31a,34] FAK suppression of adhesion markers during early differentiation impairs differentiation by inhibiting Cav3 expression and reducing actin levels. Additionally, integrin inhibition suppresses muscle differentiation and fusion, highlighting the importance of extracellular recognition and adhesion in muscle differentiation.^[31a] In line with these findings, vinculin, a mechanosensitive adhesion protein, has also been implicated in stiffness-dependent myogenic differentiation. Although vinculin knockdown in hMSCs did not impair FA formation or traction force as determined by TFM, it significantly reduced MyoD expression, suggesting its function as a force-sensitive regulator of myogenic gene expression through MAPK1 interaction.^[35]

Myoblast fusion involves the merging of mononucleated cells into multinucleated myotubes via the actions of fusion-related

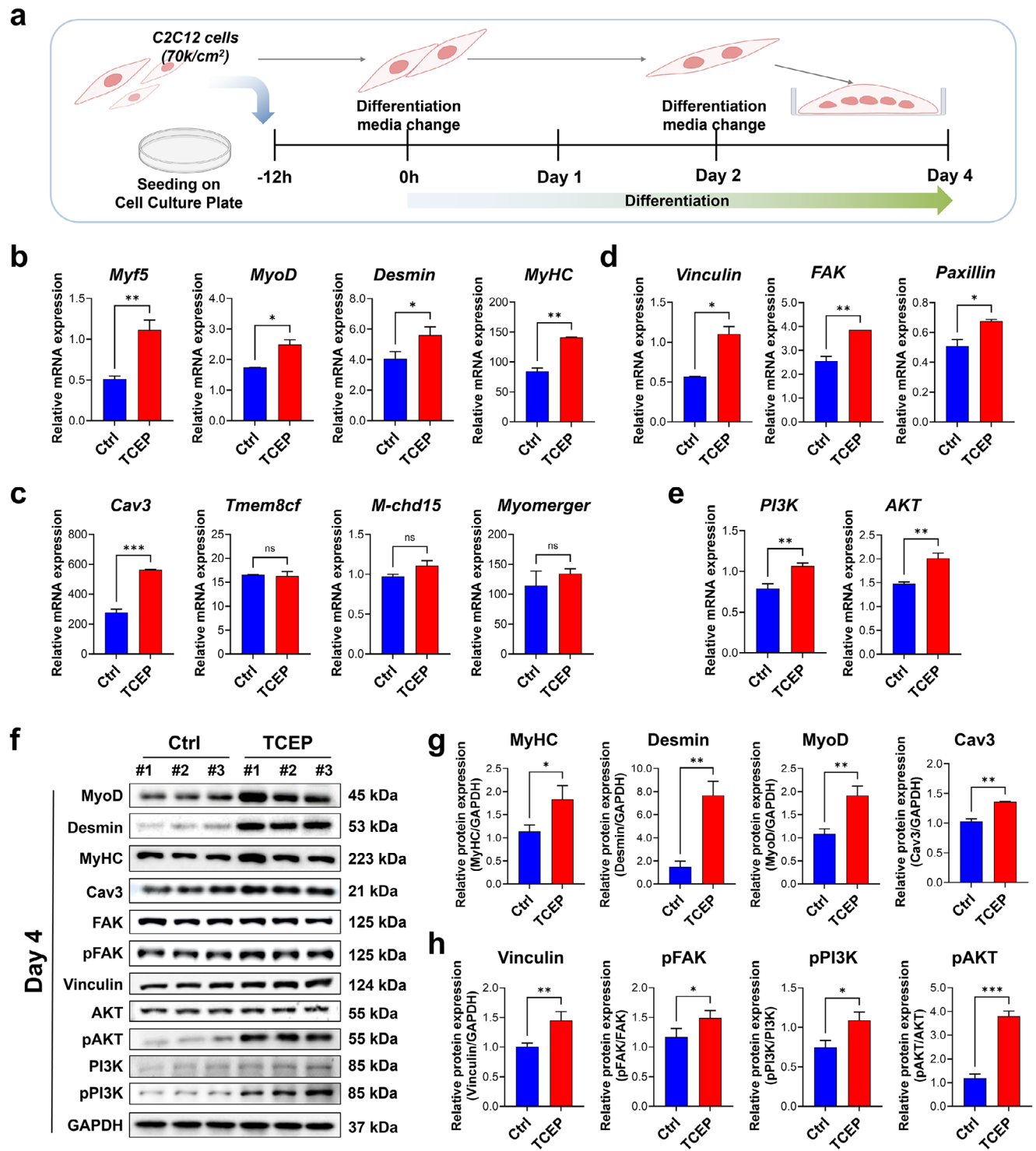


Figure 3. Enhanced C2C12 cell fusion, focal adhesion dynamics, and differentiation through cell surface reduction. (a) Schematic overview of the experimental timeline: C2C12 cells were treated with 1 mM TCEP for 5 min, seeded, and allowed to adhere for 12 h before induction of myogenic differentiation for 4 d. (b) Transcript levels of differentiation-related markers (Myf5, MyoD, desmin, and MyHC) assessed by qPCR ($n = 3$). (c) Transcript levels of fusion-related markers (Cav3, Tmem8cf, M-chd15, and myomerger) assessed by qPCR ($n = 3$). (d) Transcript levels of adhesion-related markers (Vinculin, fibronectin). (e) Transcript levels of cytoskeleton remodeling-related markers (PI3K, AKT) assessed by qPCR ($n = 3$, respectively). (f-h) Western blot analyses of differentiation-related markers, focal adhesion markers, and cytoskeletal remodeling-related proteins in TCEP-treated C2C12 cells ($n = 3$ per group). Data were analyzed using the Kruskal–Wallis test followed by Dunn’s post-hoc test. Data are presented as the mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

proteins such as Cav3, myomaker, M-cdh15, and myomerger.^[36] In contrast, inhibition of integrin $\alpha 7$ has been shown to reduce the expression of these proteins and impair membrane remodeling and fusion, along with decreased MyoD, MyoG, and MyHC expression.^[37] These observations highlight the importance of ECM recognition and adhesion in regulating fusion competency. In our study, although TCEP treatment led to a significant increase in differentiation markers, the expression of membrane remodeling proteins such as myomaker, M-cdh15, and myomerger was not altered. These proteins facilitate membrane curvature and fusion pore formation between adjacent cells and are sensitive to the physical microenvironment.^[38] Given that tissue culture plastic possesses high stiffness,^[39] we hypothesize that the rigidity of the substrate diminished the relative impact of TCEP on membrane remodeling, despite its enhancement of adhesion and cytoskeletal organization.

To further assess myogenic outcomes, immunofluorescence staining was performed for MyHC (MF20) and α -actinin, along with vinculin and pFAK. TCEP-treated cells showed a higher proportion of MyHC-positive cells, indicating enhanced differentiation (Figures 4a, S4b, Supporting Information). These cells also exhibited stronger cytoplasmic vinculin expression and increased pFAK localization at the periphery, suggesting enhanced FA formation during differentiation. α -Actinin staining revealed more organized sarcomeric structures in TCEP-treated cells, indicating advanced maturation of myotubes.

Quantification of myogenic differentiation based on the percentage of MyHC-positive cells revealed a 23.8% increase in the TCEP group compared to controls (Figure 4b). Fusion index analysis, stratified by the number of nuclei per MyHC-positive cell, showed that TCEP-treated cells had a 34.3% decrease in mononucleated cells and increases in 2–5 nuclei (15.6%), 6–9 nuclei (58.1%), and ≥ 10 nuclei (76.3%) categories (Figure 4c, Figure S4c, Supporting Information). Since cell seeding densities were identical, the relative decrease in mononuclear cells supports a greater extent of myogenic fusion, consistent with previous literature.^[40]

Furthermore, α -actinin, which localizes to the Z-line of sarcomeres, plays a pivotal role in organizing sarcomeric structure and serves as a key indicator of myofibril maturation during myogenesis.^[41] By anchoring sarcomeres to the cell membrane, α -actinin enables the proper alignment and visualization of striated muscle architecture. It also interacts with membrane-associated proteins such as talin and vinculin, which together act as scaffolds linking the cytoskeleton to mechanotransductive signaling pathways.^[42] Immature myoblasts typically display disorganized α -actinin patterns, whereas mature myotubes exhibit periodic striations. Line intensity profiling in ImageJ revealed that TCEP-treated cells showed highly regular, periodic peaks indicative of organized sarcomeres (Figure 4d), further supporting enhanced cytoskeletal and myofibrillar maturation. Taken together, these findings suggest that surface redox modulation via TCEP enhances focal adhesion formation and mechanotransduction through FAK signaling, leading to increased cytoskeletal organization, myoblast fusion, and myotube maturation.

2.4. Enhanced Cell Adhesion and Cell Cytoskeleton Rearrangement via Modulated Cell Surface Reduction on Three Different Polyacrylamide (PAA) Hydrogel Stiffness

We investigated the effects of cell surface thiol generation via redox modulation (TCEP treatment) on C2C12 cell adhesion and cytoskeletal remodeling across three physiologically relevant substrate stiffness conditions: 11, 20, and 49 kPa (Figure 5a). To confirm enhanced adhesion through redox-mediated surface engineering, we performed immunofluorescence staining for pFAK and vinculin 12 h post-TCEP treatment, focusing on their distribution at the cell edge and perinuclear regions (Figure 5b and c). Additionally, F-actin intensity analysis revealed that cytoskeletal remodeling was enhanced, with the intensity increasing progressively with increasing stiffness levels and TCEP treatment, except TCEP-treated cells at 20 kPa. Furthermore, on all three PAA gels of different stiffnesses, both vinculin and pFAK were predominantly expressed between the nucleus and the cell edges, with their intensities at the cell edges increasing with increasing stiffness levels.

Fluorescence images were analyzed using MATLAB to quantify the cell, F-actin intensity (Figure 5d,e), and focal adhesion parameters including the intensity, area, and number of pFAK and vinculin signals (Figure 5f,g, and Figure S5a,b, Supporting Information). At 11 and 49 kPa, no significant differences in cell area were observed between TCEP-treated and control groups. However, at 20 kPa, cell spreading was substantially enhanced in the TCEP group, showing a 16% increase in area compared to control.

Consistent with these trends, F-actin intensity progressively increased with stiffness in control cells, while TCEP treatment further amplified F-actin levels specifically at 20 kPa. A similar trend was observed in pFAK intensity, number, and area (Figure S5a, Supporting Information). Vinculin intensity also increased at 20 kPa with TCEP, but the number of focal adhesions decreased, likely due to the enlargement and merging of adhesion areas, suggesting enhanced maturation of focal adhesions (Figure S5b, Supporting Information).

Interestingly, at 49 kPa, TCEP treatment did not significantly alter focal adhesion number, area, cell spreading, or cytoskeletal intensity compared to controls. This apparent plateau effect may reflect mechanotransductive saturation, a state in which supra-physiological stiffness levels (>30 – 40 kPa) fail to further promote cell contractility, spreading, or adhesion signaling due to maximal activation of mechanosensing pathways.^[43] These findings support that redox-induced surface remodeling via TCEP enhances cell adhesion and cytoskeletal organization in a stiffness-dependent manner, with maximal effects occurring at muscle-mimetic stiffness (20 kPa) and diminishing at excessively stiff conditions.

Diseased tissues with impaired mechanical properties contribute to functional decline, e.g., diminished regeneration and promotion of pathological alterations.^[44] This emphasizes the critical role of ECM mechanical stiffness in tissue function, highlighting the need for further pathological research to better understand its implications in disease and regeneration. Previously, Gilbert et al. reported that muscle stem cells grown on a substrate

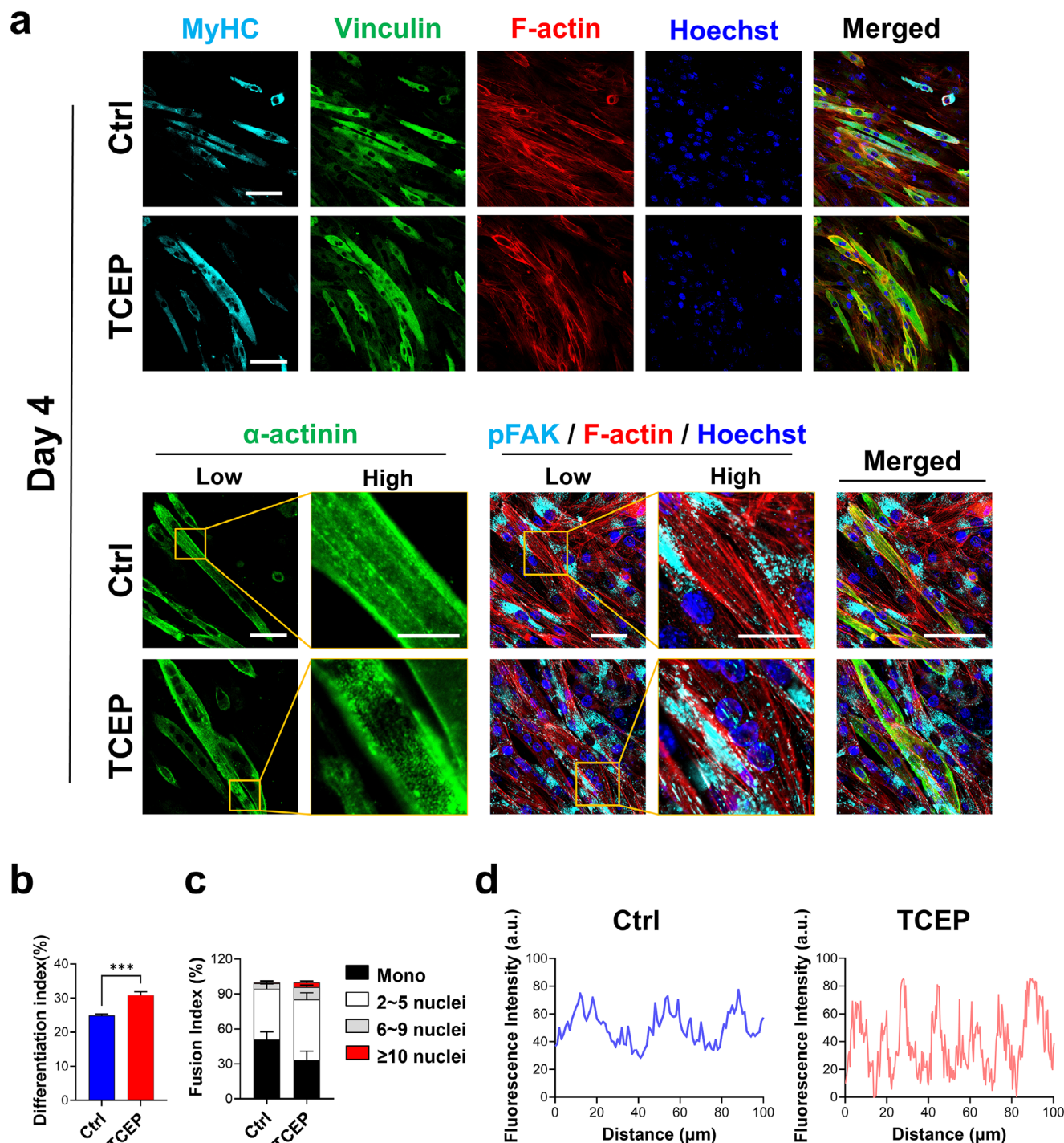


Figure 4. Enhanced C2C12 cell adhesion and fusion promote sarcomeric maturation following cell surface reduction. (a) Representative immunofluorescence images of C2C12 cells after 4 d in myogenic induction medium, stained for MyHC (Cy5), vinculin (green), F-actin (red), and nuclei (blue; Hoechst). In parallel, a separate set of cells was stained for α -actinin (green), pFAK (Cy5), F-actin (red), and nuclei (blue) to assess sarcomeric organization and focal adhesion signaling. Scale bar = 50 μ m (cropped images = 12.25 μ m). (b) Differentiation index calculated as the percentage of MyHC-positive nuclei relative to the total nuclei. (c) Fusion index calculated as the proportion of MyHC-positive cells containing 1, 2–4, 5–9, or ≥ 10 nuclei (ctrl: $n = 41299$; TCEP: $n = 45353$). (d) Quantification of sarcomeric maturation based on α -actinin organization, categorized into punctate, partially striated, and fully striated patterns, analyzed using ImageJ ($n = 3$). Data were analyzed using the Kruskal–Wallis test followed by Dunn’s post-hoc test. Data are presented as the mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

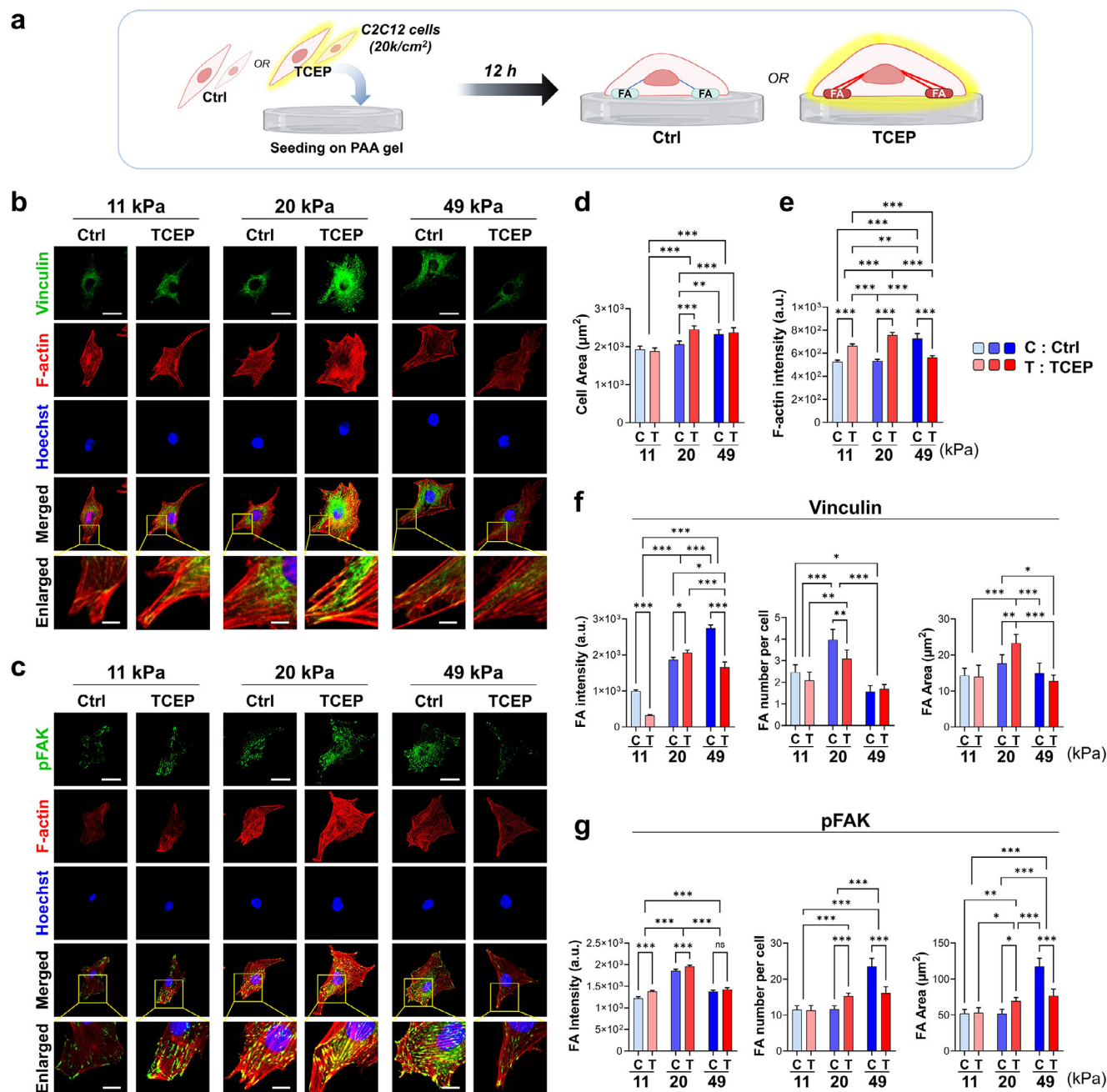


Figure 5. Effect of cell surface reduction on focal adhesion dynamics of C2C12 cells cultured on polyacrylamide (PAA) gels of different stiffness. C2C12 cells were treated with 1 mM TCEP for 5 min and then seeded onto PAA gels with elastic moduli of 11, 20, and 49 kPa cultured in growth medium. (a) Schematic of the experimental workflow showing TCEP-treated cells seeded on PAA substrates. (b,c) Representative immunofluorescence images of focal adhesion-related markers vinculin and phosphorylated FAK (pFAK) in TCEP-treated and control cells. Scale bar = 25 μm (Scale bar of crop image = 10 μm). (d) Quantification of single-cell area [for 10 kPa, $n = 196$ (ctrl) and $n = 202$ (TCEP); for 20 kPa, $n = 160$ (ctrl) and $n = 184$ (TCEP); for 50 kPa, $n = 197$ (ctrl) and $n = 237$ (TCEP)]. (e) The intensity of F-actin, a cell cytoskeletal-related marker, was assessed using cell boundaries defined by F-actin staining [for 10 kPa, $n = 342$ (ctrl) and $n = 313$ (TCEP); for 20 kPa, $n = 400$ (ctrl) and $n = 597$ (TCEP); for 50 kPa, $n = 380$ (ctrl) and $n = 347$ (TCEP)]. The F-actin intensity was normalized to the corresponding cell area defined by the boundary. (f) Vinculin area and intensity analysis [for 10 kPa, $n = 137$ (ctrl) and $n = 97$ (TCEP); for 20 kPa, $n = 175$ (ctrl) and $n = 205$ (TCEP); for 50 kPa, $n = 103$ (ctrl) and $n = 337$ (TCEP)]. (g) pFAK area and intensity analysis [for 10 kPa, $n = 173$ (ctrl) and $n = 166$ (TCEP); for 20 kPa, $n = 198$ (ctrl) and $n = 381$ (TCEP); for 50 kPa, $n = 214$ (ctrl) and $n = 180$ (TCEP)]. Data were analyzed using the Kruskal–Wallis test, followed by Dunn’s post-hoc test (statistical significance was removed for clarity). Data are presented as the mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

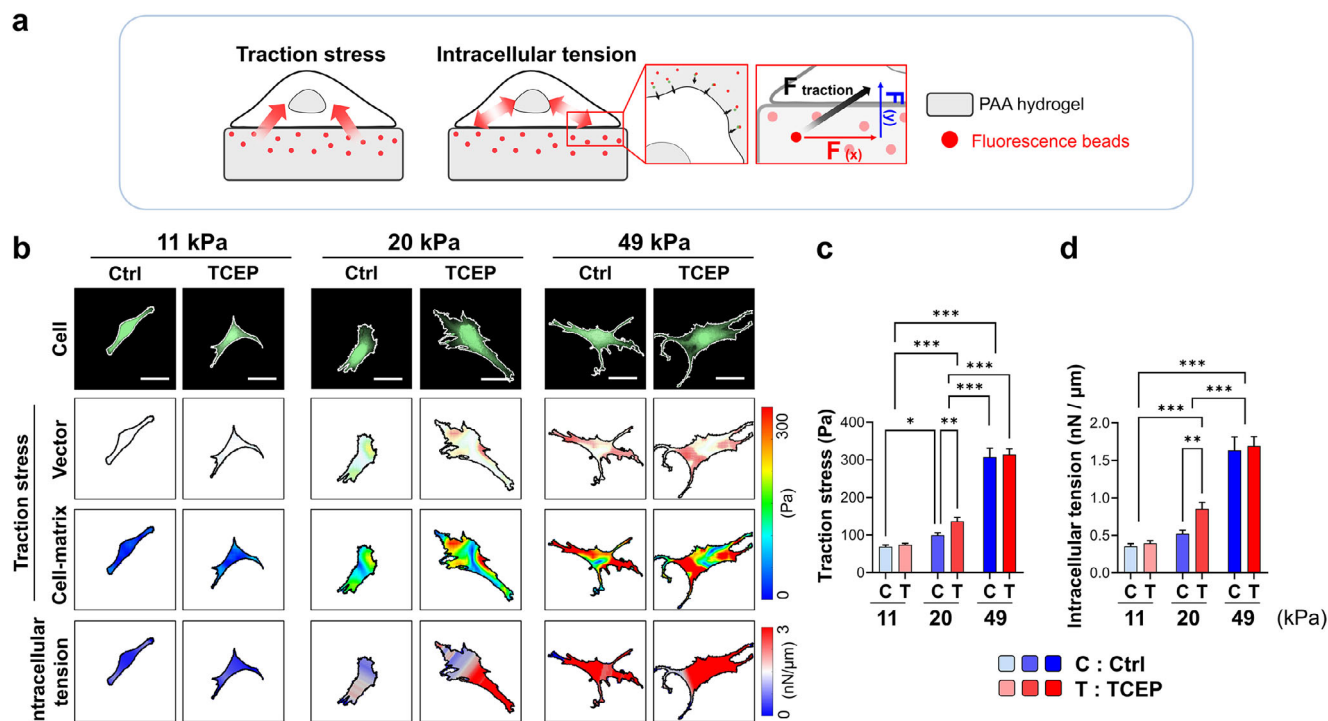


Figure 6. Effect of cell surface reduction on traction stress and intracellular tension of C2C12 cells cultured on PAA gels with varying stiffness. C2C12 cells were treated with 1 mM TCEP for 5 min, seeded onto PAA gels with elastic moduli of 11, 20, and 49 kPa, and allowed to adhere for 12 h prior to biophysical measurements. (a) Schematic illustration of traction force microscopy (TFM) and intracellular force microscopy (IFM) for analyzing cellular mechanics. (b) Cell tracker-stained image, traction stress, and intracellular tension range analysis on PAA gels at three different stiffnesses. Scale bar = 100 μm. (c) Mean cell stress, and (d) intracellular tension of C2C12 cells [for 11 kPa, $n = 196$ (ctrl) and $n = 202$ (TCEP); for 20 kPa, $n = 160$ (ctrl) and $n = 184$ (TCEP); for 49 kPa, $n = 197$ (ctrl) and $n = 237$ (TCEP)]. Data were analyzed using the Kruskal–Wallis test, followed by Dunn's post-hoc test (statistical significance was removed for clarity). Data are presented as the mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

with a stiffness of 12 kPa showed 10% engraftment efficiency in mice, which was similar to the expression of freshly isolated cells (16%) and muscle fibers expressing the activation differentiation marker MyHC.^[45] They also found that these cells exhibited better regenerative potential than cells cultured on substrates with varying stiffness levels. Similarly, Madl et al. suggested that muscle stem cells in aged muscle tissue remain quiescent and lose regenerative potential compared to those in healthy stiffness (approximately 12 kPa using a hydrogel with light-sensitive ortho-nitrobenzyl ester crosslinks, before transitioning to a soft gel) and demonstrated that cells cultured on a stiff matrix for 1 d maintained the expression of proliferation markers (MyoD, Pax7, EDU, and MyoG), whereas 3-day cultures exhibited reduced marker expression and retained mechanical properties of the stiff matrix.^[46]

Furthermore, transitioning from soft to stiff gels reduces proliferation marker expression and alters tissue stiffness, suggesting that changes in mechanical memory and stiffness hinder muscle regeneration. Collectively, our studies highlight the importance of ECM stiffness and mechanical memory in the regulation of muscle stem cells. They suggest that regenerative processes can be disrupted under conditions such as aging or fibrosis and that changes in the mechanical microenvironment may influence regeneration. Based on this, we selected stiffness conditions of 11 kPa (soft), 20 kPa (muscle-specific stiffness), and 49 kPa (stiff, resembling a fibrotic environment).

2.5. Traction Stress and Intracellular Tension in Single Cells with Modulated Surface Reduction

To further verify cell adhesion through protein analysis through immunofluorescence staining and to confirm it mechanically, we analyzed the traction stress, and intracellular tension of single cells at muscle-specific stiffnesses of 11, 20, and 49 kPa (Figure 6a). As the stiffness increased, the cell traction stress increased significantly, with values that were 44% and 67.6% higher at 20 and 49 kPa, respectively, than at 11 kPa (Figure 6b,c). Similarly, intracellular tension (stress) showed increases of 31.8% at 20 kPa and 68.2% at 49 kPa compared to the control group (Figure 6b,d).

Interestingly, TCEP-treated cells exhibited the most pronounced effects at 20 kPa, showing a 27.3% increase in traction stress and a 39.2% increase in intracellular tension compared to untreated controls. This enhancement was not observed at 11 kPa or 49 kPa (Figure 6c,d). Notably, in TCEP-treated cells at 49 kPa, focal adhesion number and vinculin intensity did not significantly increase relative to control, despite traction forces remaining unchanged. Previous studies have shown that traction force correlates more strongly with substrate stiffness and cell spreading than with focal adhesion number alone,^[47] and our data confirmed that cell area remained constant across groups at 49 kPa. Furthermore, vinculin is known to undergo force-dependent stabilization through conformational changes within vinculin–talin–actin linkages, without necessarily altering total

signal intensity,^[48] supporting the notion that mechanical output may remain stable even in the absence of focal adhesion expansion. Together, this may be attributed to why TCEP's effects on adhesion and tension were pronounced at 20 kPa but diminished at 49 kPa despite stable force output. Together, these findings suggest that TCEP-mediated surface thiol formation selectively enhances adhesion and tension at muscle-mimetic stiffness (20 kPa), but this effect is diminished at supraphysiological stiffness (49 kPa), likely due to saturation of mechanosensing pathways.

Notably, during early-stage (96 h) differentiation of C2C12 myoblasts, the real-time expression of Myomerger, a membrane fusion marker, increased up to 72 h, mirroring a rise in cell tension and highlighting the tight coupling between cytoskeletal tension and myogenic fusion.^[49] Therefore, these results suggest the importance of tensile forces and redox-modulated cell–cell and cell–matrix interactions in facilitating myogenic differentiation. We propose that TCEP-induced thiol generation on the cell surface enhances both adhesion and intracellular tension, thereby supporting the mechanical requirements for efficient early-stage myoblast fusion and differentiation.

2.6. Stiffness-Dependent Myogenic Differentiation Regulated by Redox-Mediated Cell Surface Thiol Modulation

Stem cells exhibit varying proliferation and differentiation abilities, depending on the stiffness of the tissue or matrix microenvironment.^[50] Engler et al. reported that matrix stiffness regulates lineage determination of MSCs, with muscle-associated marker clustering within the range of 10–17 kPa.^[39b] Moreover, inhibiting myosin with blebbistatin reduced muscle gene expression, implicating matrix stiffness as a regulator of differentiation through cytoskeletal dynamics.

To evaluate whether cell surface thiol formation induced by TCEP modulates stiffness-sensitive myogenic differentiation, C2C12 cells were cultured on PAA gels of 11, 20, and 49 kPa and induced to differentiate for 5 d (Figures 7a and S6a, Supporting Information). TCEP-treated C2C12 cells showed significantly elevated expression of the differentiation-related markers Myf5, MyoD, desmin, and MyHC at 20 kPa (Figure 7b). At 11 kPa, only Myf5 and MyoD, markers of the initiation of early differentiation, were increased, whereas the later expressed markers desmin and MyHC did not show any changes in TCEP-treated cells. In contrast, at 49 kPa, no differences were observed in the expression of most differentiation markers.

We also confirmed that the transcript-level expression of fusion-related markers, including Cav3, Tmem8cf, M-cdh15, and Myomerger, was consistently upregulated in the TCEP-treated group at 20 kPa (Figure 7c). At 11 kPa, only Myomaker, a protein that facilitates the initial membrane juxtaposition during fusion, was increased, while other fusion markers remained unchanged. At 49 kPa, expression of all fusion markers was markedly reduced. Notably, Myomaker and Myomerger play non-redundant roles in membrane remodeling and successful myoblast fusion: Myomaker promotes membrane apposition, while Myomerger completes the fusion process.^[51] Furthermore, at 11 kPa, early-

stage differentiation gene expression was activated, leading to slower differentiation. In contrast, at 49 kPa, differentiation was minimal. However, at 20 kPa, the TCEP treatment significantly enhanced muscle differentiation, suggesting that mechanical stiffness plays a crucial role in muscle regeneration.

In our study, the expression of fusion-related membrane remodeling markers such as Tmem8cf, M-cdh15, and Myomerger was markedly upregulated by TCEP in cells on PAA gels but not on tissue culture plastic (TCP). This observation suggests that intracellular tension, modulated by substrate stiffness, may play a critical role in regulating these membrane fusion genes. However, it is important to note that PAA gels were conjugated with collagen I to enable cell adhesion, whereas TCP lacks this defined ECM interface. As such, biochemical signals from the collagen coating may also contribute to the observed gene expression patterns, independently or synergistically with stiffness cues. Consequently, the differential expression of fusion genes observed between hydrogel and plastic substrates must be interpreted cautiously, considering the combined influence of substrate stiffness and biochemical cues. Further research is warranted to elucidate how gel-based ECM systems control membrane remodeling and fusion machinery during muscle differentiation.

FA signaling also demonstrated stiffness-specific sensitivity. Mechanistically, FAs, which connect the cytoskeleton to the extracellular matrix (ECM) and neighboring cells, mediate mechanotransductive signals essential for both adhesion and fusion.^[52] The cytoskeleton, acting as a conduit between the membrane and the nucleus, facilitates cellular force transmission and fusion dynamics.^[53] Extracellular environmental changes sensed at the ECM–cell or cell–cell interfaces modulate adhesion, cytoskeletal remodeling, and ultimately fate decisions.^[54] In our study, vinculin expression exhibited an increasing trend at 10 and 20 kPa substrates after 5 days of differentiation, while no significant changes were observed at 50 kPa (Figure 7d). This stiffness-dependent response suggests that vinculin-mediated focal adhesion (FA) signaling is most active within the physiological range of muscle stiffness. Notably, TCEP-treated groups exhibited significant upregulation of FAK and paxillin at 20 kPa, but not at 11 or 49 kPa. These findings suggest that surface thiol engineering enhances adhesion signaling selectively under optimal mechanical conditions. Supporting this, Holle et al. previously identified vinculin as a key mechanosensor in MSCs, where its knock-down impaired MyoD expression without affecting FA morphology or traction force generation, supporting a biochemical signaling role in mechanotransduction.^[35]

Finally, TCEP treatment led to increased expression of PI3K and AKT at 20 kPa (Figure 7e), suggesting enhanced cytoskeletal remodeling aligned with elevated adhesion and fusion gene expression. Taken together, our findings indicate that redox-induced surface thiol modulation augments stiffness-specific mechanotransduction via enhanced focal adhesion signaling and cytoskeletal remodeling to drive myogenic differentiation. However, due to the limited understanding of gel-specific regulation of membrane fusion markers, our results should be interpreted as an important step toward unraveling the complex crosstalk between redox signaling and mechanical cues in engineered ECM environments.

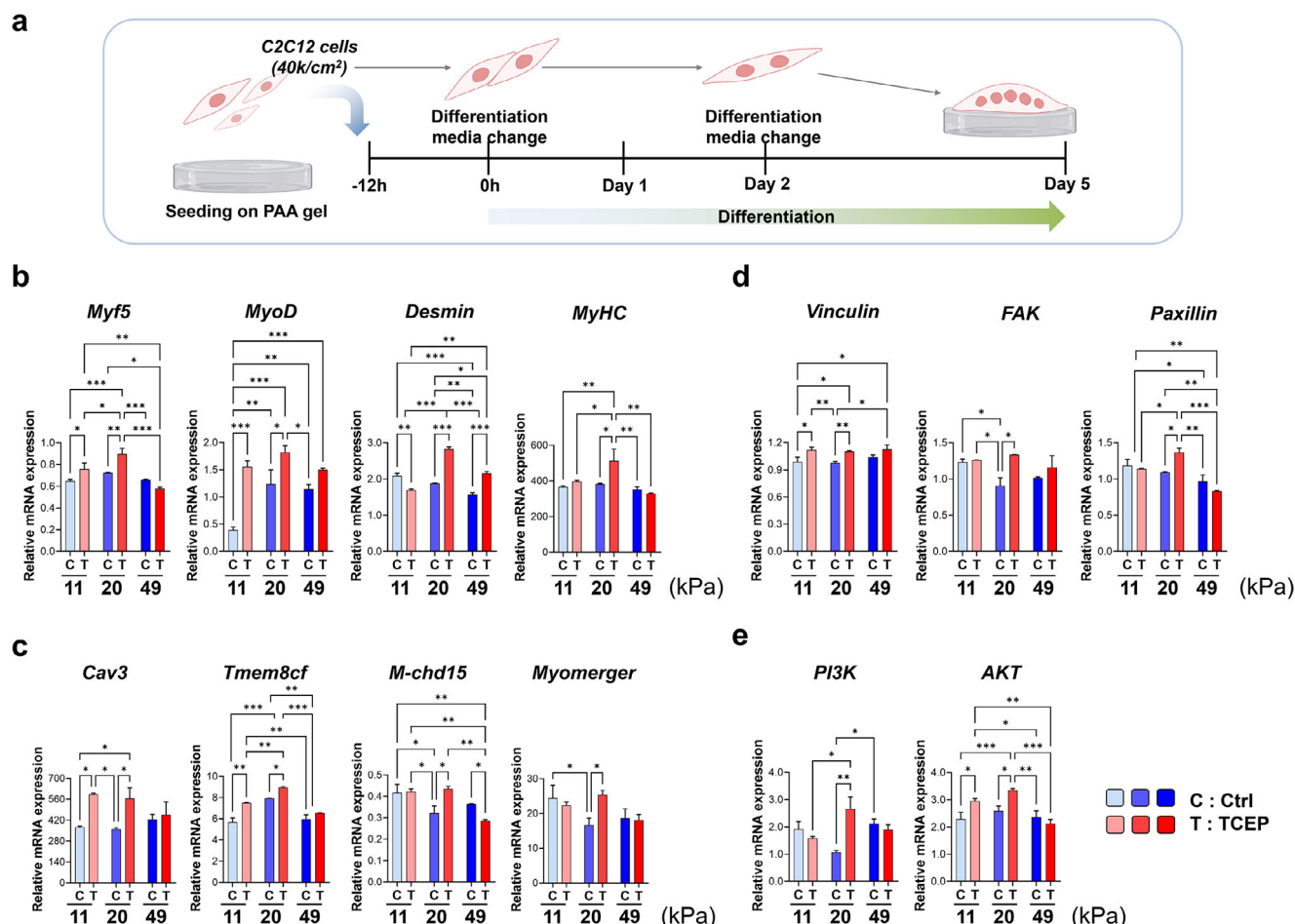


Figure 7. Enhanced C2C12 cell fusion and differentiation through thiol-mediated surface reduction on PAA gels. (a) Schematic illustration of the experimental timeline. C2C12 cells were treated with 1 mM TCEP for 5 min and seeded onto PAA gels of defined stiffness. After 12 h of adhesion, the medium was replaced with myogenic induction medium (DM), and cells were cultured for 5 d. (b) Transcript-level expression of differentiation-related markers (Myf5, MyoD, desmin, and MyHC), (c) Expression of fusion-related markers (Cav3, Tmem8cf, M-chd15, and myomerger), (d) Expression of adhesion-related markers (vinculin, fibronectin), and (e) Expression of cytoskeleton remodeling-related markers (PI3K, AKT) assessed using qPCR ($n = 3$). Data were analyzed using the Kruskal–Wallis test followed by Dunn’s post-hoc test (statistical significance was removed for clarity). Data are presented as the mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

2.7. Stiffness-Dependent Myogenic Fusion and Sarcomeric Maturation Through Redox-Mediated Mechanotransduction

To further assess the impact of stiffness on morphological myogenic outcomes, C2C12 cells cultured on PAA gels of defined stiffness were immunostained for MyHC, Desmin, α -actinin, and FA markers following 5 days of differentiation (Figures 8a,d and S6b, Supporting Information). Immunofluorescence staining for MyHC, Desmin, and α -actinin revealed an increased number of multinucleated myotubes in the TCEP-treated group specifically at 20 kPa, suggesting improved differentiation under muscle-mimetic stiffness conditions. The differentiation index, calculated as the percentage of MyHC-positive cells relative to the total cell count, was significantly higher in the TCEP-treated group at 20 kPa, whereas no significant differences were noted at 10 or 50 kPa (Figure 8b).

To assess the extent of myogenic fusion, we categorized MyHC-positive cells by the number of nuclei per myotube

(mono-, 2–5, 6–9, and ≥ 10 nuclei) and calculated their proportions (Figure 8c and Figure S6c, Supporting Information). At 20 kPa, TCEP treatment notably reduced the proportion of mononucleated cells and increased the fraction of multinucleated myotubes—particularly those with ≥ 10 nuclei—indicating enhanced fusion. A milder effect was also observed at 10 kPa, while at 50 kPa, TCEP treatment resulted in fewer multinucleated and more mononucleated cells, suggesting fusion impairment under excessive stiffness.

Furthermore, differences in focal adhesion formation in differentiated cells were evaluated using Vinculin and pFAK staining. Vinculin expression was predominantly higher in differentiated cells compared to surrounding undifferentiated (MyHC-negative) cells. While Vinculin expression showed minimal variation between 10 and 50 kPa conditions, a marked increase was observed specifically in the TCEP-treated group at 20 kPa. Similarly, pFAK expression followed the same trend, indicating that TCEP-induced surface thiol modulation enhanced focal adhesion

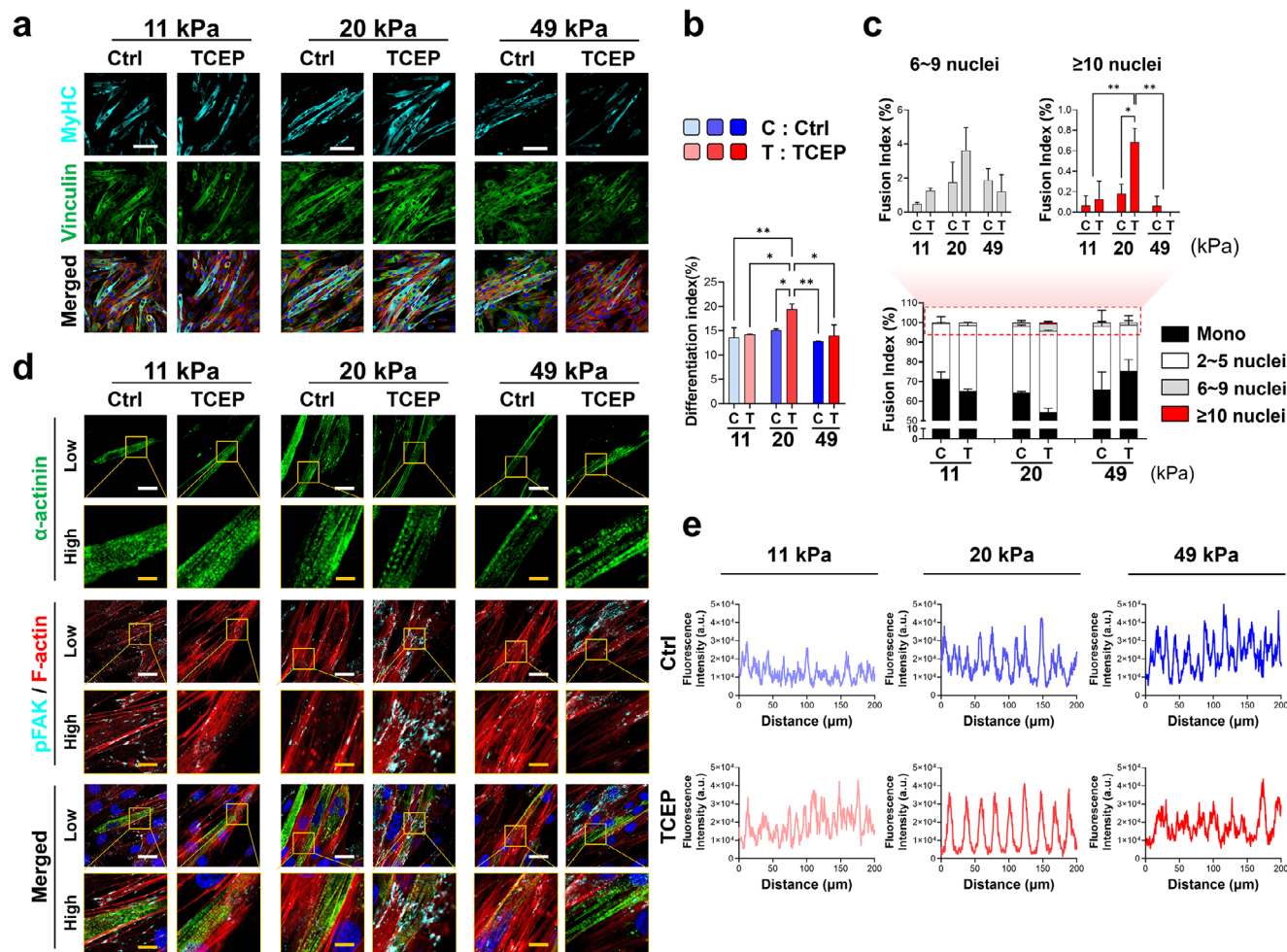


Figure 8. Enhanced C2C12 cell fusion and differentiation through thiol-mediated cell surface reduction on PAA gels. (a) Immunofluorescence visualization of C2C12 cells stained for myosin heavy chain (MyHC, Cy5), vinculin (green), F-actin (red), and nuclei (Hoechst, blue) after 5 d of differentiation on PAA gels with different stiffnesses, with or without TCEP treatment. Scale bar = 25 μm . (Scale bar of crop image = 12.25 μm .) (b) Differentiation index, calculated as the percentage of MyHC-positive cells relative to the total number of cells. (c) Fusion index, determined by categorizing MyHC-positive cells based on the number of nuclei per myotube (mono-, 2–5, 6–9, and ≥ 10 nuclei) and calculating their relative proportions. Red dashed boxes indicate magnified views of representative myotubes with 6–9 and ≥ 10 nuclei. [for 11 kPa, $n = 17\,352$ (ctrl) and $n = 18\,965$ (TCEP); for 20 kPa, $n = 27\,832$ (ctrl) and $n = 19\,330$ (TCEP); for 49 kPa, $n = 19\,809$ (ctrl) and $n = 17\,436$ (TCEP)]. (d) Immunofluorescence images showing α -actinin (green), phosphorylated FAK (pFAK, Cy5), F-actin (red), and nuclei (blue; Hoechst). Representative α -actinin staining patterns were classified as punctate, partially striated, or fully striated. Scale bar = 25 μm . (Scale bar of crop image = 12.25 μm) (e) Quantitative analysis of α -actinin pattern organization using ImageJ, followed by classification of myotubes into each pattern category ($n = 3$). Data are presented as the mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

formation and myogenic differentiation preferentially at muscle-mimetic stiffness.

To assess sarcomeric organization, α -actinin staining was analyzed by intensity profiling using ImageJ. Regularly spaced intensity peaks were used to classify striation into punctate, partially striated, or fully striated architectures (Figure 8d,e). Fully striated sarcomeric structures were most prevalent in the TCEP-treated group at 20 kPa, while disorganized or punctate patterns were dominant at 10 and 50 kPa, regardless of treatment. These findings align with the increased fusion index and focal adhesion activation observed at 20 kPa, supporting that this stiffness condition promotes optimal cytoskeletal maturation. In agreement with our results, Denes et al. demonstrated that C2C12 cells cultured on micropatterned gelatin hydrogels formed more mature myotubes with well-organized sar-

comeric α -actinin compared to non-patterned substrates, emphasizing the role of ECM architecture in guiding cytoskeletal assembly and myogenic maturation.^[55] Consistent with this, our observation of differential α -actinin patterns across stiffness conditions further highlights the interplay between substrate mechanics and cytoskeletal organization during myogenic differentiation.

Importantly, our study introduces a novel paradigm by integrating redox-mediated surface thiol engineering with defined mechanical environments to enhance myogenesis. To our knowledge, this is the first report that systematically investigates how surface redox modifications can modulate stiffness-dependent mechanotransduction and fusion in muscle stem cells. This work addresses a critical knowledge gap in stem cell mechanobiology by illustrating the combinatorial regulation of cell adhesion, cy-

toskeletal architecture, and myogenic potential through redox-mechanical coupling.

2.8. Redox-Mediated Enhancement of Traction and Intracellular Tension at Muscle-Relevant Stiffness

The differentiation of muscle stem cells occurs in a monolayer at 90–100% confluence to facilitate cell–cell interactions. To investigate how these interactions are influenced by stiffness and redox state, traction force microscopy (TFM) and monolayer stress microscopy (MSM) were performed on C2C12 cells after 5 days of differentiation on PAA gels of 11, 20, and 49 kPa (Figure 9a,b).

Cell traction stress increased with increasing substrate stiffness but plateaued at 11 kPa and declined at 49 kPa. Notably, TCEP treatment significantly elevated both cell traction stress and intracellular tension by 22.7% and 32%, respectively, specifically at 20 kPa (Figure 9c,d). This mechanical activation was not observed at 11 kPa and was attenuated at 49 kPa. These data suggest that TCEP-induced cell surface thiol modulation reinforces intracellular contractility in a stiffness-dependent manner, with optimal mechanotransduction signaling occurring at muscle-mimetic stiffness (20 kPa).

Intracellular tension maps derived from monolayer stress microscopy (MSM) revealed spatial heterogeneity, with localized regions of elevated (red) and reduced (blue) (Figure 9b). While this pattern may initially appear atypical for uniformly confluent monolayers, such spatial variability is consistent with prior reports on collective force transmission in contractile cell sheets. In particular, Tambe et al. demonstrated that intracellular stress distributions in epithelial monolayers exhibit pronounced heterogeneity due to long-range mechanical coupling mediated by cadherin-based cell–cell adhesions and coordinated cytoskeletal networks across the monolayer.^[56] Thus, in the context of C2C12 myoblasts, which rely heavily on cell fusion and cytoskeletal remodeling during differentiation stress, these spatial gradients likely reflect regional differences in cytoskeletal contractility and cell–cell force integration.

Furthermore, we propose that the intracellular stress at each stiffness plays a significant role in muscle stem cell differentiation, as indicated by mechanical activation coinciding with elevated expression of fusion markers such as Cav3, Tmem8cf, and Myomerger at 20 kPa, which were unresponsive at 49 kPa. This suggests that myogenic fusion is not simply stiffness-promoted but occurs in a narrow stiffness-specific window that aligns with native muscle mechanics. Furthermore, this response was potentiated by redox-mediated surface thiol modulation, indicating a novel interaction between mechanical and redox cues.

Similarly, Camman et al. observed that in muscular dystrophies, the ECM transitions to a fibrotic state, increasing the stiffness from between 12 and 15 kPa to between 50 and 70 kPa.^[57] Using 3D printing, collagen hydrogels were modified by adjusting the EDC/NHS concentration to control the pore size, allowing a stiffness range of 15–25 kPa for healthy tissues, and 45–75 kPa for fibrotic tissues. When C2C12 myoblasts were embedded in the fibrotic matrix, no multinucleated myotubes formed after 5 d of differentiation, and the cells remained mononucleated. In contrast, key differentiation markers (dystrophin, myosin, and actin) were upregulated in the healthy matrix. However,

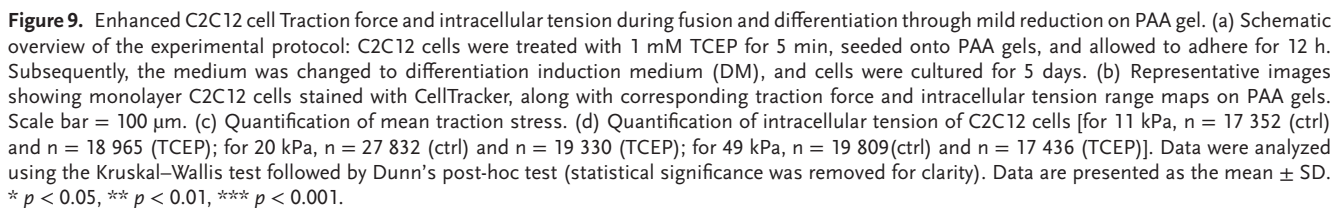
in the fibrotic matrix, the upregulation of collagen1 and Loxl4, along with the downregulation of metalloproteinases (MMP 2, 9, 11, and 13), suggested a reduced potential for matrix remodeling and muscle regeneration. Taken together, these results suggest that regulating Young's modulus of the extracellular environment is crucial for enhancing the efficiency of muscle progenitor, thereby promoting muscle tissue regeneration.

3. Conclusion

The differentiation of skeletal muscle stem cells is critical for muscle regeneration and is intricately regulated by both biochemical and biomechanical cues in the extracellular environment. In this study, we introduced a redox-modulated surface engineering approach using TCEP, a cell-compatible reducing agent, to selectively convert cell surface disulfide bonds into free thiol groups. This thiol induction enhanced cell–matrix adhesion and cell–cell interactions, leading to increased traction stress and intracellular tension within the actin cytoskeleton. These mechanical changes, in turn, promoted myogenic differentiation and fusion into multinucleated myotubes in a substrate stiffness-dependent manner, with the most pronounced effects observed at muscle-mimetic stiffness (20 kPa) (Figure 10).

To our knowledge, this is the first study to demonstrate that redox-induced cell surface remodeling can synergize with defined matrix stiffness to modulate focal adhesion signaling and myogenic fusion gene expression in a hydrogel-based context. This combinatorial approach addresses a significant gap in the field of stem cell mechanobiology, where redox signaling and cell culture substrate mechanics have traditionally been studied in isolation. By linking these two regulatory dimensions, our findings reveal a previously unappreciated mechanism of mechanotransduction modulation and suggest that redox status may serve as a tunable switch for optimizing stem cell responsiveness to physical microenvironmental cues.

While this work lays a mechanistic foundation for redox-mechanosensitive regulation of myogenesis, we acknowledge two key limitations. First, our experiments were conducted using the murine C2C12 myoblast cell line, which, although well-established and reproducible, lacks the full regenerative and phenotypic complexity of primary skeletal muscle stem cells (satellite cells). The use of C2C12 cells was a strategic choice to establish proof-of-concept under controlled and reproducible conditions. However, extrapolation of these findings to native stem cell behavior requires cautious interpretation. To address this, extending our approach to patient-derived skeletal muscle stem cells (satellite cells) and assessing its translational relevance is necessary. Second, our study was conducted in a 2D culture system, which does not fully recapitulate the spatial and mechanical complexity of native muscle tissue. Future studies incorporating 3D culture platforms or in vivo models are necessary to confirm whether the observed redox-mechanotransductive effects are preserved in more physiologically relevant settings. Nonetheless, this work contributes to a broader understanding of how extracellular redox and mechanical cues can be tuned to regulate muscle progenitor behavior in both pathological and engineered conditions.



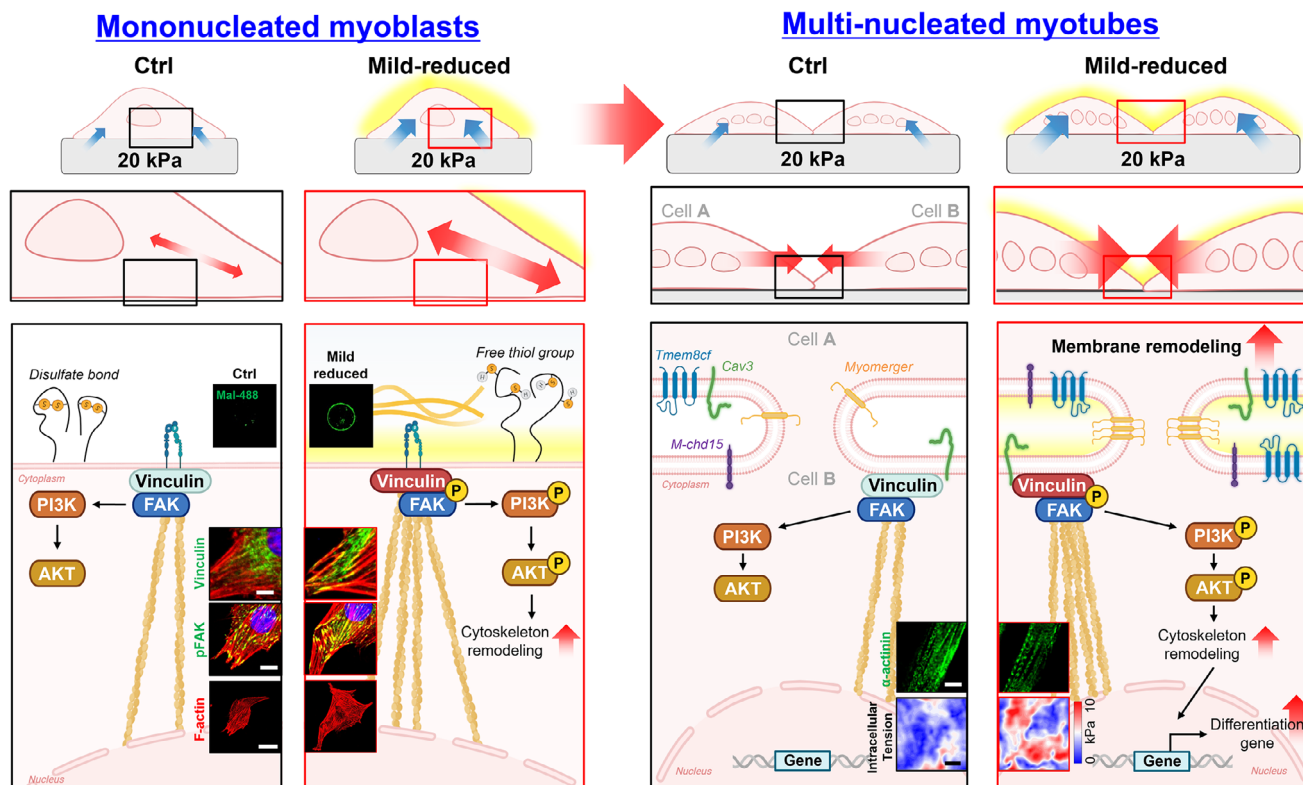


Figure 10. Proposed mechanism of how cell surface reduction promotes myogenic differentiation of myoblasts through a FAK–PI3K–AKT axis, which was corroborated by TFM, IFM, and MSM evaluation.

4. Experimental Section

Cell Culture and Differentiation: The murine skeletal myoblast cell line C2C12 was cultured in growth medium consisting of high-glucose Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL, Waltham, MA, USA) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco-BRL) and 1% (v/v) penicillin–streptomycin (PS; Gibco-BRL) at 37 °C in humid air with 5% CO₂. For myogenic induction on tissue culture plastic (TCP), C2C12 cells were seeded at a density of 7×10^4 cells/cm². Once the cells reached 80–90% confluency (~12 h post-seeding), the growth medium was replaced with myogenic induction medium consisting of high-glucose DMEM supplemented with 2% v/v horse serum (Gibco-BRL) and 1% v/v PS. Cells were cultured for 4 d, with daily medium changes. For myogenic induction on polyacrylamide (PAA) gels with stiffnesses of 11, 20, and 49 kPa, C2C12 cells were seeded at a density of 4×10^4 cells/cm². After cell attachment, the growth medium was replaced with myogenic induction medium composed of a 1:1 mixture of Ham's F-12 Nutrient Mix (Gibco-BRL) and low-glucose DMEM (HyClone, Cytiva, Marlborough, MA, USA) supplemented with 2% v/v horse serum and 1% v/v PS. Cells were cultured for 5 d, with medium changes every 2 d.

TCEP-Induced Cytotoxicity in C2C12 Cells: Cells at a density of 4×10^6 cells/mL were collected by centrifugation at 1,200 rpm and suspended in 2 mL of PBS containing 0, 0.5, 1, or 2 mM tris (2-carboxyethyl) phosphine hydrochloride (TCEP, C4706, Sigma-Aldrich, St. Louis, MO, USA). The samples were incubated at room temperature (RT) for 5 min, washed with PBS, and mixed with 0.4% trypan blue solution (Gibco, Waltham, MA, USA) in a 1:1 ratio. The number of live (unstained) and dead (blue-stained) cells was counted using a hemocytometer under a light microscope. For cytotoxicity visualization, TCEP-treated cells were seeded at 2×10^4 /cm² cells in 24 plates in a growth medium. After seeding control and TCEP-treated cells for 12 h, the cells were stained using a Live/Dead viability/cytotoxicity kit (L3224, Thermo Fisher Scientific, Waltham, MA, USA)

containing calcein AM (4 mM; 1:2000 diluted) and ethidium homodimer solution (2 mM, 1:1000 diluted) in growth medium at 37 °C for 30 min, followed by washing in PBS. The cells were then immediately imaged through fluorescence microscopy (EVOS Cell Imaging Systems, Thermo Fisher Scientific). The number of live and dead cells was analyzed in equal fields of view (2048 × 1536 pixels) for each sample, and the number of dead and live cells was calculated as a percentage of the total cells.

Synthesis of Maleimide-488 and Quantification of Surface Thiols in C2C12 Cells: For the quantification of free thiols, 5,5-dithiol-bis-(2-nitrobenzoic acid) (Ellman's reagent; 4 mg/mL, 22 582, Thermo Fisher Scientific) was used following the manufacturer's instructions. A stock solution of Ellman's reagent was prepared by dissolving 4 mg in 1 mL of reaction buffer. The reaction buffer contained 1 mM EDTA and 0.1 M sodium phosphate buffer (pH 8), containing 1 M Na₂HPO₄ and 1 M NaH₂PO₄ in deionized water. Thiol groups were quantified using a cysteine standard (C7352; 2 mM, Sigma-Aldrich). C2C12 cells (2×10^6) were resuspended in reaction buffer, mixed with Ellman's working solution, and incubated for 15 min, after which the absorbance was measured at 412 nm using a 96-well microplate reader. The thiol content was normalized to cell numbers. For visualization of free thiols, TCEP-treated C2C12 cells were washed with PBS and then stained using Alexa Fluor 488 C5 maleimide (mFluor; 1 mg/mL, Thermo Fisher Scientific) diluted in PBS solution (final concentration: 3 µg/mL) for 20 min at 37 °C. During the incubation, the tubes were tapped every 5 min. After staining, cells were washed with PBS and imaged using a laser scanning confocal microscope (LSM-780; Carl Zeiss) equipped with a Plan-Apochromat 63 × oil immersion objective lens (NA = 1.4) at the Soonchunhyang Biomedical Research Core Facility, Korea Basic Science Institute (KBSI). Images were acquired at a pixel size of 0.05 µm/pixel. Z-stack images of TCEP-treated C2C12 cells were captured at 30 slices with 0.6 µm intervals, covering the entire cell volume. The images were reconstructed as 2D cross-sections and 3D projections using ImageJ (version 1.53c).

Immunofluorescence Staining: Cells cultured in 24-well plates and confocal dishes were fixed with 4% paraformaldehyde (PFA) for 15 min at RT and washed three times with PBS. The fixed cells were permeabilized with 0.1% (v/v) Triton X-100 for 10 min at RT and washed three times with PBS. The cells were blocked using 1% (w/v) bovine serum albumin (BSA) and 0.05% (v/v) Tween 20 (P1379; Sigma-Aldrich) in PBS for 30 min at RT. Subsequently, the samples were incubated overnight at 4 °C with the appropriate primary antibodies (diluted in 1% BSA and Tween 20 in PBS). After incubation, the cells were washed three times with fresh PBS and then incubated for 1 h at RT with the corresponding secondary antibodies, also diluted in 1% w/v BSA and 0.05% v/v Tween 20 in PBS. Detailed information on all antibodies used is provided in Table S1 (Supporting Information). For cytoskeletal and nuclear staining, F-actin was labeled using phalloidin, and nuclei were stained with Hoechst 33 342 (stock concentration: 2 mg/mL; working concentration: 2 µg/mL, corresponding to a 1:1000 dilution of stock) for 1 h, followed by washing with PBS. All fluorescence images were acquired using a laser confocal microscope (LSM-710; Carl Zeiss) equipped with a Plan-Apochromat 40 × water immersion objective lens (NA = 1.2; pixel size: 0.1 µm/pixel).

Western Blotting Analysis: Cells were lysed in a protein extraction solution (RIPA, Elpis Biotech, Daejeon, Korea) containing protease (5 892 970 001, Sigma-Aldrich) and phosphatase inhibitors (4 906 837 001, Sigma-Aldrich). Samples were centrifuged at 12,000 g and 4 °C for 10 min. Protein concentration was determined using the Bradford assay (5 000 205, Bio-Rad, Hercules, CA, USA). A standard curve was constructed using a Quick Start bovine serum albumin standard kit (0.125–2.0 mg/mL, 5 000 207, Bio-Rad). Gel electrophoresis was performed using 6% or 12% (w/v) polyacrylamide gels, based on the molecular weight of the proteins. Proteins were transferred to polyvinylidene fluoride blotting membrane (GE Healthcare, Chicago, IL, USA), blocked with 5% BSA in 1 × Tris-Buffered Saline containing Tween 20 (TBST, pH 7.5), and probed with the following primary antibodies, overnight at 4 °C with gentle agitation and washing three times with TBST. The membranes were then incubated with horseradish peroxidase-conjugated (HRP) secondary antibodies (1:2000; Bio-Rad) for 1 h at RT, followed by three times TBST washes. Detailed information on all primary and secondary antibodies is provided in Table S2 (Supporting Information). Protein bands were visualized using the Enhanced Chemiluminescence (ECL) Prime Western Blotting Detection Reagent (cat# RPN2235, Cytiva, IL, USA) and imaged using an Amersham Imager 600 (GE Healthcare, IL, USA).

RNA Isolation and Real-Time PCR: Total RNA was extracted using TRIzol reagent (15 596 018, Invitrogen, Carlsbad, CA, USA) and collected in a 1.5 mL tube. cDNA was synthesized using 1 µg RNA. Reverse transcription was performed according to the manufacturer's instructions using the ReverTra Ace qPCR RT master mix (FSQ-301; Toyobo, Osaka, Japan). qPCR was performed using the SYBR Green real-time PCR master mix (QPK-201, Toyobo, Osaka, Japan). All experiments were performed with at least three or four biological replicates per group, and the expression levels of the genes of interest were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primer sequences used are listed in Table S3 (Supporting Information).

Myogenic Differentiation Image Analysis of C2C12 Cells: To quantify the degree of myogenic differentiation of murine myoblast C2C12 cells, the myosin heavy chain-positive cellular area, differentiation, and fusion indices were calculated using NIH ImageJ software, following established methodologies reported previously.^[3c,6b] The differentiation index was determined as the ratio of MyHC-positive cells to the total number of cells.

$$\text{Differentiation index (\%)} = \frac{\text{number of MyHC positive nuclei}}{\text{Total number of nuclei}} \times 100\% \quad (1)$$

Additionally, the fusion index was calculated as the ratio of myotubes with 1, 2–4, 5–9, or > 10 nuclei to the total number of MyHC-positive cells. At least 50 000 nuclei per group were analyzed from five randomly selected fields of view at each time point, using images generated by stitching nine

10 × magnification images.

Fusion index (%) =

$$\frac{\text{number of mono or multi (2 – 4, 5 – 9, or > 10) nuclei}}{\text{total number of MyHC positive nuclei}} \times 100\% \quad (2)$$

Image Analyses for Cell Morphology, FAs, and α -Actinin Organization: To investigate the effects of surface thiol modulation on C2C12 cell morphology, adhesion, and differentiation, cells were treated with 1 mM TCEP and allowed to adhere to culture substrates for 12 h prior to fixation and immunostaining. Cells were stained with CellTracker Green (C7025, Invitrogen, Carlsbad, CA, USA) to visualize overall morphology.

F-actin organization was visualized using fluorescence microscopy (EVOS M7000, Thermo Fisher Scientific) equipped with a 20 × objective lens (NA = 0.45, pixel size: 0.31 µm/pixel). Focal adhesion markers, including phosphorylated FAK (pFAK) and vinculin, were imaged using the LSM-710 confocal microscope with a Plan-Apochromat 40 × water immersion lens (NA = 1.2; pixel size = 0.1 µm/pixel). α -Actinin-stained sarcomeric structures were imaged with digital zoom (pixel size = 0.3 µm/pixel) to enhance resolution.

Morphological parameters and focal adhesion features were analyzed using custom MATLAB-coded programs (MathWorks, Natick, MA, USA), as previously described.^[31,32] Briefly, cell images were binarized using a histogram-based threshold algorithm to minimize subjective bias in threshold selection.^[32] Cell area was computed by counting segmented pixels and converting to µm² using the pixel scale. Focal adhesion analysis was performed using a custom MATLAB-coded program to minimize the subjective determination of threshold values, thereby reducing variability and experimental errors, as detailed in a previous study.^[36b] The image processing pipeline involved background noise removal by excluding the lowest 0.5% of pixel intensities, followed by region of interest (ROI) selection and image binarization using a threshold set at approximately 5% of the maximum intensity. Size filtering was applied to retain FA regions between 1.5 and 30 µm², thereby excluding small noise artifacts and large aggregates. From the binarized images, FA parameters, including number and area per cell, were quantified. Total FA area was calculated by summing the pixel area of all segmented adhesions per cell, while mean FA area was defined as the average size of individual adhesions within each cell.

Additionally, total and mean focal adhesion (FA) intensities were calculated by summing or averaging the pixel intensities within segmented FA regions, respectively. F-actin intensity was quantified using two approaches: first, within manually defined cell boundaries, and second, within cell boundaries segmented from binarized images using the MATLAB-coded algorithm. In both cases, the total F-actin intensity of each cell was normalized to its corresponding cell area (µm²) to account for variations in cell size across treatment groups.

For analysis of sarcomeric striation, α -actinin-stained myotubes were evaluated using the straight-line tool in Fiji (ImageJ, NIH). Fluorescence intensity profiles were generated along the longitudinal axis of each myotube, and regularly spaced intensity peaks corresponding to Z-line localization of α -actinin were identified. Peak-to-peak distances were measured and averaged to estimate sarcomere length and assess the periodicity and regularity of sarcomeric organization.

PAA Gel Making: O mimic the mechanical properties of skeletal muscle tissue, polyacrylamide (PAA) hydrogels of varying stiffness were prepared: soft (11 kPa, acrylamide/bis-acrylamide [AA/Bis] = 10%/0.1%), medium (20 kPa, AA/Bis = 8%/0.26%), and stiff (49 kPa, AA/Bis = 10%/0.30%), following established protocols.^[58] The elastic moduli and formulations of these gels were based on previously published atomic force microscopy (AFM) measurements and preparation methods, as summarized in Table S4 (Supporting Information). For clarity, these conditions are referred to as 11, 20, and 49 kPa gels throughout the main text; Figures.^[59] Briefly, glass-bottom dishes (cat# 101 350, SPL, Pocheon, Korea) were rendered hydrophilic by briefly passing the glass surface through a Bunsen burner flame, followed by treatment with 0.1 M sodium hydroxide (39155S0350, Junsei, Tokyo, Japan). The surface was then acti-

vated with (3-aminopropyl)trimethoxysilane (281 778, Sigma–Aldrich) and 0.5% glutaraldehyde (G5882, Sigma–Aldrich). Polymerization was initiated by adding 0.6% ammonium persulfate (AMP001, BioShop, Burlington, ON, Canada) and 0.4% *N,N,N',N'*-tetramethylethylenediamine (TEMED) (TEM001, BioShop). Gels were formed by sandwiching the activated glass with an untreated coverslip (diameter 18 mm; 011 1580, Marienfeld, Königshofen, Germany). After 1 h of polymerization, the top coverslip was carefully removed, and the gel surface was functionalized with type I collagen (150 µg/mL; 354 236, Corning, NY, USA) using the photoactivatable cross-linker Sulfo-SANPAH (22 589, Thermo Scientific, West Etobicoke, ON, Canada). The collagen conjugation was carried out under UV light (365 nm; Blak-Ray XX-15L, UVP, Upland, USA) to promote stable coating and ensure cell adhesion. Gels were subsequently sterilized under short-wave UV light (254 nm; Mineralight XX-15S, UVP) for 10 min and then equilibrated with complete cell culture medium at 37 °C and 5% CO₂ for at least 10 min before cell seeding.

Cell–ECM Forces Analysis by TFM: To visualize C2C12 cell morphology, cells were stained with Cell Tracker Green (C7025, Invitrogen, Carlsbad, CA, USA). To measure cellular forces, red fluorescent beads (0.01%, excitation/emission = 580/605 nm; dia. = 0.5 µm, F8812, Invitrogen) were vortexed and added to the PAA monomer solutions for single-cell and collective cell (differentiated), respectively. Fluorescent beads, green fluorescent cell membranes, and differential interference contrast images were acquired using a laser confocal microscope (LSM-710; Carl Zeiss) with a Plan-Apochromat 20 × water immersion objective lens (NA = 1.2) for collective cell and single-cell imaging, respectively. The image pixel size was 0.35 µm/pixel, respectively. Bead displacements were calculated using the particle image velocimetry method in MATLAB (MathWorks), comparing images from the null-force state (absence of cells) and force state (presence of cells).^[60] The null-force state was determined by adding 10% SDS (196-08675; FUJIFILM Wako Chemicals, Japan) to remove the cells. The finite element method was used to solve the partial differential equations using Abaqus (Dassault Systèmes, Paris, France). The traction stress vector ($TS = [TS_x, TS_y]$) in the *x*- and *y*-directions were determined from the stress tensor acquired through the finite element method analysis. The magnitude of traction stress ($TS_{mag} = \sqrt{TS_x^2 + TS_y^2}$) was determined as the mean stress magnitude exerted under the cell.

Cell–Cell and Intracellular Force Analysis by IFM and MSM: After the measurement of cell–ECM (traction) stresses, IFM^[61] was used to determine intracellular tensions of isolated single cells, while MSM^[62] was used to determine intracellular stresses of collective cells. In brief, intracellular tensions within a single cell were determined by averaging the tension along the major and minor axes ($IT = [IT_x, IT_y]$).^[61] The tension along each axis was calculated by dividing the cells into two domains along the intracellular section line and applying Newton's law to each domain. The major and minor axis lines were determined using eigenvalue analysis to compute the contractile moment.^[63] Negative *IT* values indicated extensional tension, whereas positive values indicated compressive tension. The magnitude of the intracellular tension ($IT_{mag} = \sqrt{IT_x^2 + IT_y^2}$) is defined as the vector sum of the intracellular tensions.

Intracellular stress in collective cells was determined using MSM, as previously described.^[62b,64] Briefly, maximum (IS_{max}) and minimum (IS_{min}) intracellular stresses were calculated using eigenvalue analysis of the intracellular stress tensor. Intracellular stress magnitude was defined as $IS_{mag} = \sqrt{IS_{max}^2 + IS_{min}^2}$.

Statistical Analysis: Data were statistically analyzed using GraphPad Prism version 10 (GraphPad Software Inc., USA). Bar graphs were constructed to represent mean values, with error bars indicating the standard deviation (SD). The statistical significance of the differences observed in bar graphs was determined using Student's *t*-test for comparisons between two groups and one-way analysis of variance, followed by Tukey's post-hoc analysis for comparisons among multiple groups. For mechanical stress data, including TFM and MSM, bar graphs were generated to depict the mean and SD, and statistical comparisons were performed using the non-parametric Kruskal–Wallis test, followed by Dunn's test for post-hoc analysis (Figures 6 and 7). Statistical analysis was performed using

the Kruskal–Wallis test followed by Dunn's post-hoc test. No significant differences were observed between the groups, and the asterisks used for statistical significance in the original analysis have been removed to better reflect the data without emphasizing any specific differences. Statistical significance was indicated by asterisks as follows: **p* < 0.05, ***p* < 0.01, and ****p* < 0.001.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

J.K., S.S.H., and J.H.P. contributed equally to the work. as co-first authors. J.K. contributed to the conceptualization, methodology, investigation, formal analysis, writing of the original draft, and writing—review and editing. S.S.H. contributed to the conceptualization, methodology, investigation, formal analysis, and writing of the original draft. J.H.P. contributed to the methodology, formal analysis, and writing of the original draft. M.J.B., J.K., J.H.K., J.H.C., and H.W.K. contributed to the formal analysis. J.H.L. contributed to the investigation, formal analysis, and writing of the original draft. M.R.L. contributed to the investigation, formal analysis, and writing of the original draft. Y.H. contributed to the conceptualization, methodology, investigation, formal analysis, writing of the original draft, writing—review and editing, and funding acquisition.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

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