Transforming growth factor-β1 is resilient to heat-induced conformational change

Rui Tang,[a,b] Kaelyn Gasvoda,[a] Jacob Rabin,[b] Eben Alsberg*[a,b,c,d]

[a] Richard and Loan Hill Department of Biomedical Engineering, University of Illinois at Chicago, 909 S. Wolcott Ave., Chicago, IL 60612, USA

[b] Department of Biomedical Engineering, Case Western Reserve University, 10900 Euclid Avenue, Cleveland, OH 44106, USA

[c] Department of Orthopaedic Surgery, Case Western Reserve University, 10900 Euclid Avenue, Cleveland, OH 44106, USA.

[d] Departments of Mechanical & Industrial Engineering, Orthopaedics, and Pharmacology, University of Illinois at Chicago, 909 S. Wolcott Ave., Chicago, IL 60612, USA

* E-mail: ealsberg@uic.edu

Abstract

Transforming growth factor-β1 (TGF-β1) is a key signaling molecule in numerous biological pathways. Understanding its thermal stability helps advance the development of its production and storage, along with TGF-β1-based therapeutics. Herein, we report that the TGF-β1 molecule is thermally resilient as it gradually denatures during thermal treatment when the temperature increases to 90 °C but recovers native folding when the temperature decreases. Molecular dynamic simulation revealed that, although the protein's secondary structure is unstable under thermal stress, its confirmation is partially locked by intramolecular hydrogen bonding. Such intramolecular locks prevent the loss of TGF-β1 activity from misfolding and aggregation during recovery, as demonstrated by the capacity of the thermally treated protein to induce the differentiation of NIH/3T3 fibroblasts and human mesenchymal stem cells into

myofibroblasts and chondrocytes, respectively. These two bioassays confirmed the preservation of the functional activity of TGF-β1 after thermal stress. Given the importance and/or prevalence of TGF-β1 in biological processes, potential therapeutics and the human diet, our findings encourage the consideration of its thermostability for biomedical applications and nutrition.

Thermostability is a key factor for the production, storage, diagnostic, and therapeutic application of cytokines.[1-2] Since various cytokines are thermally sensitive, numerous methods have been employed to improve their resilience to thermal stress, such as de novo design[3] and predicted engineering.[4] Interestingly, however, while numerous proteins in the proteome are subjected to thermal-induced denaturation, recent findings have demonstrated that a subset are stable under thermal stress.[5] Therefore, discovering cytokines with high intrinsic thermal stability would be beneficial for furthering the understanding and applications of cytokines, with biomedical and nutritional applications.[2]

TGF-β1 is a secreted cytokine that is crucial for numerous essential cellular functions and biological processes. For example, TGF-β superfamily proteins, of which TGF-β1 is an important member, are essential signaling molecules the modulation of re-epithelialization, chemotaxis of leukocytes, and angiogenesis during wound healing.[6] Clinically, platelet-rich plasma, comprised of abundant TGF-β1,[7] has been cleared by the FDA to mix with bone graft materials to enhance bone graft handling properties.[8] Therapeutics using this protein must be produced, sterilized, transported, handled, and ultimately used, and the temperature during these steps can vary widely unless efforts are made to control it. In addition, members of the TGF-β protein family also widely exists in food, either freely, such as in milk, or bound with the extracellular matrix, such as in meat.[9] After oral ingestion, these proteins survive a harsh digestive environment and remain functional. For example, TGF-β1 from milk raises infant IgA production;[10] and orally administered TGF-β is biologically active in the intestinal mucosa and

elevates the serum TGF-β levels.[11] Notably, TGF-β1 may be exposed to different temperatures before consumption during food processing such as pasteurization and cooking. Therefore, understanding the thermostability of TGF-β1 is critical for biomedical and nutrition applications.

A previous report showed that the conformation of TGF-β1 changes between the free state and bound state.[12] With affinity binding to cell receptors, the conformation of TGF-β1 switches from the closed-form to the open-form. Additionally, TGF-β1 protein remains inactive in complex with latent TGF binding proteins (LTBPs) or fibrillins.[13] However, a temperature increase to 80 °C from 25 °C results in conformational changes of the complex, unbinding of TGF-β1 from its inhibitors and subsequent activation.[13] These phenomena imply that the conformation of TGF-β1 is flexible and the cytokine may resist thermal stress, which is opposite to previous speculations of thermal instability.[2] This work investigates the conformational changes of TGF-β1 with thermal treatment and its impact on the protein's biological functions. The TGF-β1 protein is found to be resilient to thermal stress under experimental conditions.

To examine the stability of TGF-β1 under thermal stress, temperature-controlled circular dichroism (CD) spectrometry was employed to probe changes in its secondary structure directly. The CD spectra of the α-helix and β-sheet have been well established.[14] The α-helix has double negative peaks at 208 nm and 222 nm, and a positive peak at 191-193 nm. The β-sheet structure displays a negative peak at 215 nm and a positive peak at 198 nm. In contrast, the spectrum of random coil segments is opposite from those of α-helical and β-sheet structures. The CD spectrometry can be used to directly determine the deformation of the α-helix and β-sheet as well as the formation of random coil segments in TGF-β1. The native structure of TGF-β1 is comprised of 10% α-helices and 32% β-sheets.[15] At 25 °C, TGF-β1 displayed a negative peak at about 218 nm and a positive peak at about 200 nm (Figure 1a), which are believed to be contributed by the combination of the α-helix and β-sheet. When the temperature was increased to 90 °C at the rate of 5 °C per minute, the peak at 200 nm gradually became

negative, while the negative peak at 218 nm weakened, indicating the deformation of the α-helices and β-sheets and the formation of the random coils. After decreasing the temperature from 90 °C to 25 °C, both peaks progressively recovered (Figure 1b), suggesting the reformation of the α-helices and β-sheets.

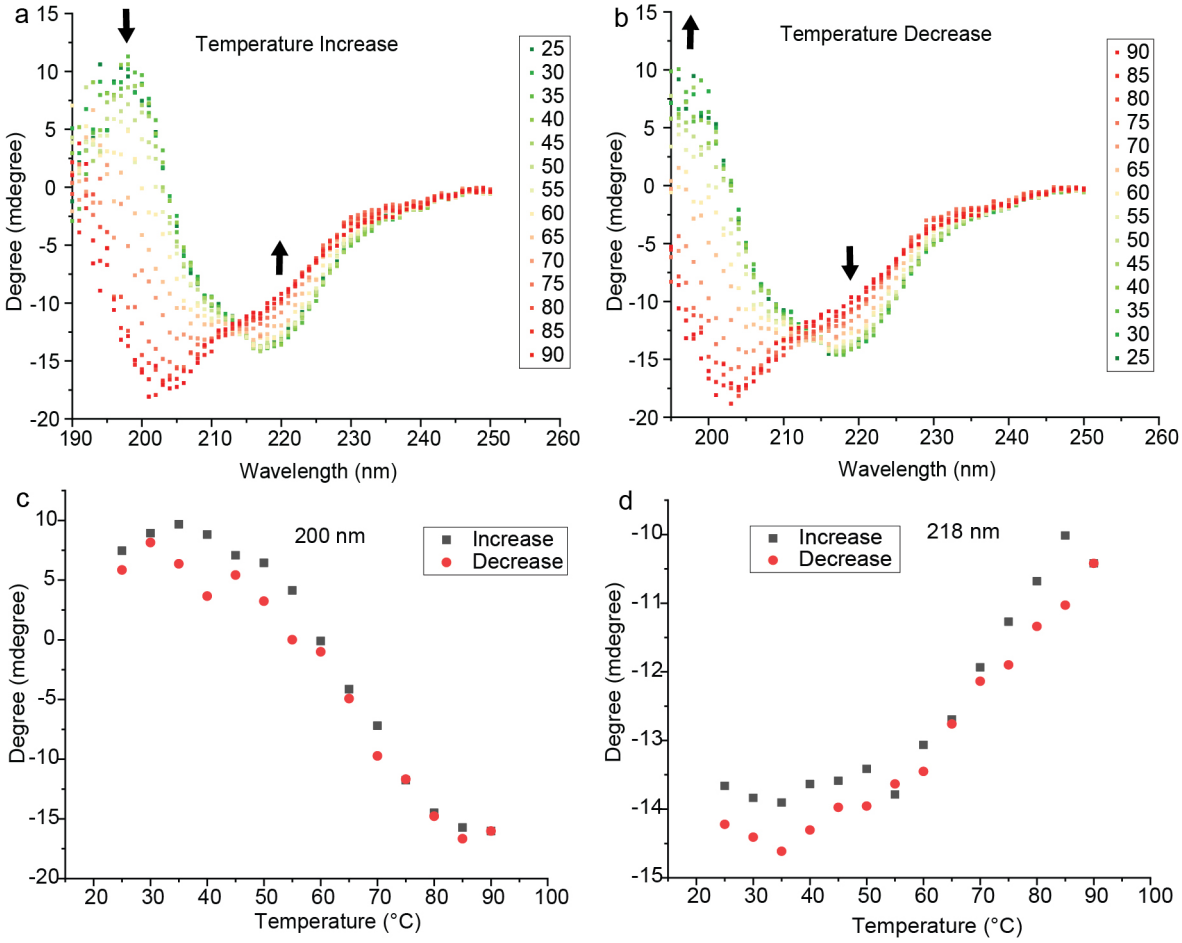

Figure 1. TGF-β1 unfolds at high temperatures but recovers when the temperature decreases. a) CD spectra of TGF-β1 with the temperature increased from 25 °C to 90 °C. Arrows indicate peak shift directions. b) CD spectra of TGF-β1 with the temperature decreased from 90 °C to 25 °C. Arrows indicate peak shift directions. c) CD spectral values of TGF-β1 at 200 nm with temperature changes. c) CD spectral values of TGF-β1 at 218 nm with temperature changes.

Values of the CD spectra at 200 nm (Figure 1c) and 218 nm (Figure 1d) versus temperature were plotted to further analyze the conformational changes. The curvatures of these values as the temperature increased and decreased were similar, suggesting a conformational change of TGF-β1 with temperature increase and full recovery with temperature decrease. Additionally, the values of both peaks at 200 nm and 218 nm remained relatively consistent between 25 °C and 45 °C. Further increase in the temperature resulted in gradual changes in the peak values which suggests progressive denaturation. The negative peak at 200 nm is an indicator of the deformation of the α-helix and β-sheets and the formation of random coils. At approximately 85 °C, random coil formation was almost complete, shown in Figure 1c. Increasing the temperature to 90 °C did not change the negative peak value, whereas temperature decrease resulted in reduced random coil structure. The reduction of the negative peak at 218 nm also indicates the decomposition of the α-helices and β-sheets. With the temperature decrease to 25 °C, both peaks restored to their original status, indicating the recovery of the conformation. Together, the temperature-controlled CD spectra results confirmed that the conformation of TGF-β1 is resilient against thermal-induced denaturation. Although the protein is deformed at high temperatures, the native conformation recovers when the temperature is reduced.

To further understand how the protein denatures, the molecular dynamics of TGF-β1 (Protein Data Bank ID: 1KLA[15]) was simulated at 25 °C and 500 °C for 10 ns, and 100 °C for 100 ns, using the software VMD with QwikMD plugin (Figure 2).[16] At 100 °C, the conformation of the protein experienced a gradual and partial unfolding (Figure 2a). Comparing the heatmaps for the secondary structure change at 25 °C and 100 °C (Figure 2b), both β-sheets (yellow) and α-helices (pink) were denatured at 100 °C in a time course. However, both of them partially withstood the thermal stress throughout the simulation. Increasing the simulation temperature to 500 °C demolished all α-helices and β-sheets instantly as expected (Figure S1). The extent of similarity of the protein structure at different time points was calculated as the Root Mean

Square Deviation (RMSD) by the software (Figure 2c). The curves demonstrated that TGF-β1 maintained its confirmation at 25 °C and rapidly deformed at 500 °C. At 100 °C, the protein experienced rapid conformational change within the first nanosecond, and then a slower gradual conformational change occurred. The protein became static after 70ns, evidenced by the almost flattened curve (Figure 2c). Since hydrogen bonding is one of the most dominant forces to secure the secondary structure of proteins, the number of hydrogen bonds throughout the simulation was also calculated (Figure 2d and Video S1). Compared to 25 °C (Figure 2d and Video S2), there are a substantial amount of hydrogen bonds remaining throughout the simulation at 100 °C. The hydrogen bond number versus time under different simulation temperatures was also quantitatively plotted (Figure 2e). Hydrogen bond number remained stable between approximately 40 and 60 at 25 °C. At 100 °C, after an initial drop, the number of the hydrogen bonds fluctuated between approximately 20 and 40 for the rest of the simulation. In contrast, increasing the simulation temperature to 500 °C quickly broke almost all intramolecular hydrogen bonds (Figure S2) and destroyed the secondary structure (Video S3). Therefore, it can be speculated that intramolecular hydrogen bonds contributed to the protein's conformational stability under thermal stress. Hydrogen bonds play a critical role in locking protein structures at an intermediate state under thermal stress, usually between 40 – 60 °C, which can be recovered to the native state instead of full denaturation.[17] For TGF-β1, temperatures up to 100 °C are not high enough to fully break all of the protein's intramolecular hydrogen bonds. Under these conditions, the conformation of the protein is locked to the intermediate state, offering the possibility of full recovery after the removal of the thermal stress.

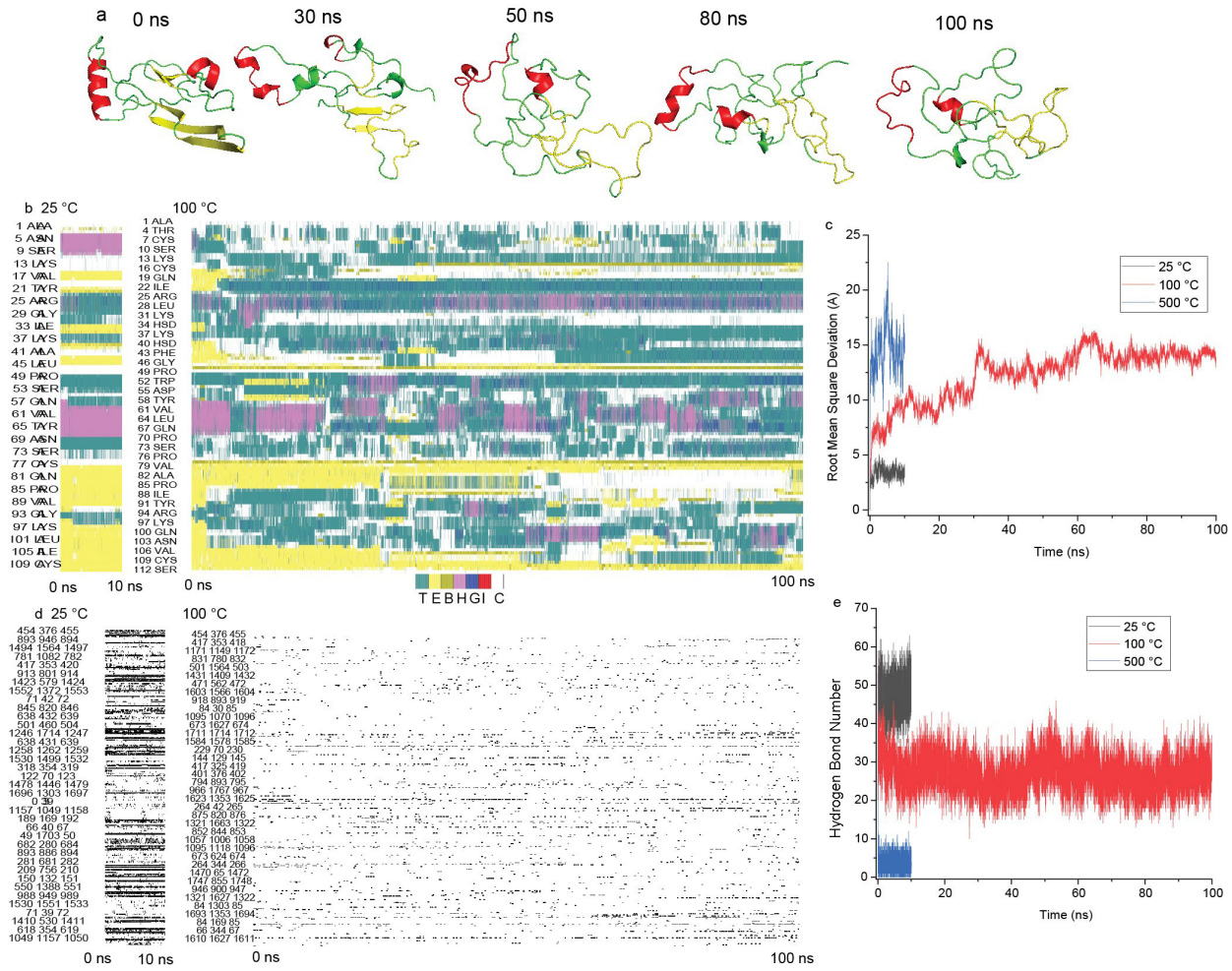

Figure 2. Molecular dynamics simulation of TGF-β1. a) Confirmation changes of TGF-β1 at 100 °C at different time points. Colors indicate secondary structures at the beginning: Green: coil; Red: α-helix; Yellow: β-sheet. b) Heatmaps of conformation changes of TGF-β1 at different time points simulated at 25 °C and 100 °C. Color symbols: T: turn; E: β-sheet extended; B: β-bridge; H: α-helix; G: 3-10 Helix; I: π-helix; C: coil. Y-axis represents the order of the amino acid residues. c) RMSD plots at 25, 100 and 500 °C during molecular dynamics simulation. d) Heatmaps of hydrogen bond presence inside TGF-β1 at different time points simulated at 25 °C and 100 °C. Black indicates hydrogen bond formation. Numbers on Y-axis indicate atom IDs during simulation. e) Molecular dynamics simulation plots of hydrogen bond number over time at

25, 100 and 500 °C. Simulations at 25 and 500 °C were performed for 10 ns. Simulation at 100 °C was performed for 100 ns.

Next, the bioactivity of TGF-β1 after thermal treatment was investigated. Since TGF-β1 is a versatile chemokine involved in multiple signaling pathways,[18] two functional tests were employed to independently validate its activity. As TGF-β1 induces the differentiation of fibroblast into myofibroblasts,[19] in the first test, the capacity of thermally treated TGF-β1 to promote this process was investigated. TGF-β1 solution was placed in boiling water for 5 minutes and cooled down to room temperature before introducing to the cells. Native TGF-β1 without thermal treatment was used as the positive control. NIH/3T3 cells, a model fibroblast cell line, was employed for the myofibroblast differentiation. Naturally, NIH/3T3 cells express a low level of α-smooth muscle actin (α-SMA), a myofibroblast marker. After induction to myofibroblasts with TGF-β1, the expression level of α-SMA increases. NIH/3T3 cells were first plated onto cell culture plates with 10% fetal bovine serum (FBS) for 6 h, followed by reducing the FBS concentration to 2.5% for another 24 h to sensitize the cells to TGF-β1. For myofibroblast induction, NIH/3T3 cells were cultured in media containing TGF-β1 and 2.5% FBS for another 48 h. The expression of α-SMA in the induced cells was then determined by Western blot (Figure 3a and Figure S3). Quantification of the blots indicated that both untreated and boiled TGF-β1 elevated the expression of the α-SMA, compared to β-actin expression (Figure 3b). In contrast, the control group without the addition of TGF-β1 only weakly expressed α-SMA. This experiment demonstrated that TGF-β1 maintained its bioactivity following recovery from thermal treatment.

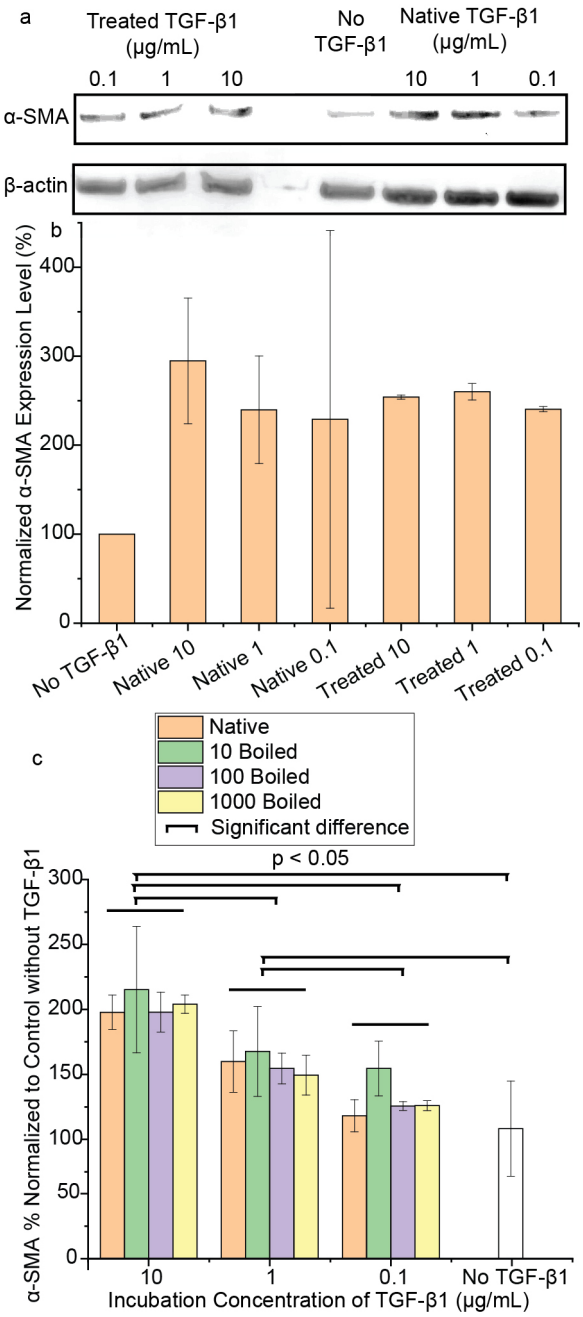

Figure 3. Heated TGF-β1 maintains its ability to induce fibroblast differentiation to myofibroblasts after 48 h treatment. a) Western blots of α-SMA expressed by NIH/3T3 cells after myofibroblast induction in the presence of untreated or thermally treated TGF-β1 at different incubation concentrations from 0.1 to 10 ng/mL. β-actin served as the loading control. b) Semi-quantitative analysis of two independent replicates of western blots of α-SMA levels normalized

to β-actin levels. Note that the error bars represent the standard deviation of two replicates. c) ELISA of α-SMA expressed by NIH/3T3 cells after myofibroblast induction in the presence of untreated or thermally treated TGF-β1 at different boiling concentrations from 10 to 1000 μg/mL (labeled as 10/100/1000 Boiled) and incubation concentrations from 0.1 to 10 ng/mL. The α-SMA level without induction was normalized to 100%. Groups within the same incubation concentration of TGF-β1 have no significant difference ($p > 0.05$; one-way ANOVA); Groups among different incubation concentrations exhibiting significant differences ($p < 0.05$; one-way ANOVA) have been labeled.

There is evidence that the refolding capacity of certain proteins may be concentration-dependent.[20-21] With higher concentration, denatured proteins tend to interact and aggregate during refolding, which can result in a reduced yield of active proteins.[21] Simulation results (Video S1 and Figure 2c) suggest that intramolecular hydrogen bonds locked the conformation of TGF-β1 at 100 °C. CD tests also revealed that the conformation of TGF-β1 at the concentration of 200 μg/mL, 20,000 times higher than the usual working concentration,[22] could recover (Figure 1). Therefore, it can be postulated that the interactions of denatured TGF-β1 molecules at high concentrations are minimal. To test this hypothesis, TGF-β1 solution was placed into boiling water at different concentrations (i.e., 10, 100, and 1000 μg/mL) for 5 minutes before myofibroblast induction. If the recovery of TGF-β1 is concentration-dependent, the activity of TGF-β1 at higher boiling concentrations should be reduced after dilution to the induction concentrations (0.1 - 10 ng/mL) in culture media as described earlier. Otherwise, the activity of TGF-β1 at all boiling concentrations should be similar. After induction for 48 h, α-SMA expression was quantitatively detected by enzyme-linked immunosorbent assay (ELISA; Figure 3c). The results revealed that the response of NIH/3T3 cells to TGF-β1 for myofibroblast induction is incubation concentration-dependent rather than boiling concentration-dependent. At the same incubation concentration, TGF-β1 thermally treated at different concentrations showed

similar induction activity. However, at higher incubation concentrations of TGF-β1, cells expressed significantly higher levels of α-SMA regardless of boiling concentration. These results suggest that TGF-β1 may not have aggregated and fully recovered from thermal stress. Along with CD and simulation results, these findings indicate that the structure of TGF-β1 under thermal stress at 100 °C is locked by intramolecular hydrogen bonds, which leads to conformational and functional recovery once the thermal stress is removed.

TGF-β1 also induces the chondrogenesis of human mesenchymal stem cells (hMSCs). Therefore, the second method used to independently confirm TGF-β1 bioactivity with and without thermal treatment was to induce chondrogenic differentiation of hMSCs. To perform the test, hMSCs were cultured in a V-bottom low attachment 96-well plate in high cell density aggregates. Untreated or boiled TGF-β1 was supplemented to basal pellet media (see Supporting Information for composition) at 10 ng/mL for three weeks to examine their effect on hMSC chondrogenic differentiation. After harvesting the aggregates, glycosaminoglycan (GAG), an extracellular matrix molecule marker of neocartilage formation,[23-24] and DNA content were quantified, and GAG/DNA ratio of each sample was calculated as a measure of cell chondrogenic activity. With the addition of TGF-β1 with or without thermal treatment, a substantial amount of GAG per aggregate was detected, although cell aggregates with thermally treated TGF-β1 generated approximately 20% less GAG (Figure 4a). These results were within the range previously observed under similar conditions with untreated TGF- β1.[22-23] As average DNA amount per aggregate was similar, after normalization, the GAG/DNA ratios similarly demonstrate that both untreated and thermally treated TGF-β1 drive hMSCs in aggregates to produce this important cartilage ECM molecule at levels previously reported in the literature.[22-23] The tissues were also stained with Safranin O, a dye for GAG (Figure S4). The positively stained tissues confirmed GAG production in both groups that were similarly distributed, supporting the biochemical assay findings. This experiment was repeated with the same donor

3 additional times (Figure S5), and all resulted in recovery after thermal treatment of TGF-β1 (~32% – 95%, calculated from GAG production normalized by DNA amount). In comparison, no GAG was detected (Table S1) without the addition of TGF-β1, indicating the necessity of TGF-β1 supplementation for promoting chondrogenesis of hMSCs in this system. Together, these results confirmed that TGF-β1 maintained a high level of its chondrogenic induction capacity after thermal stress.

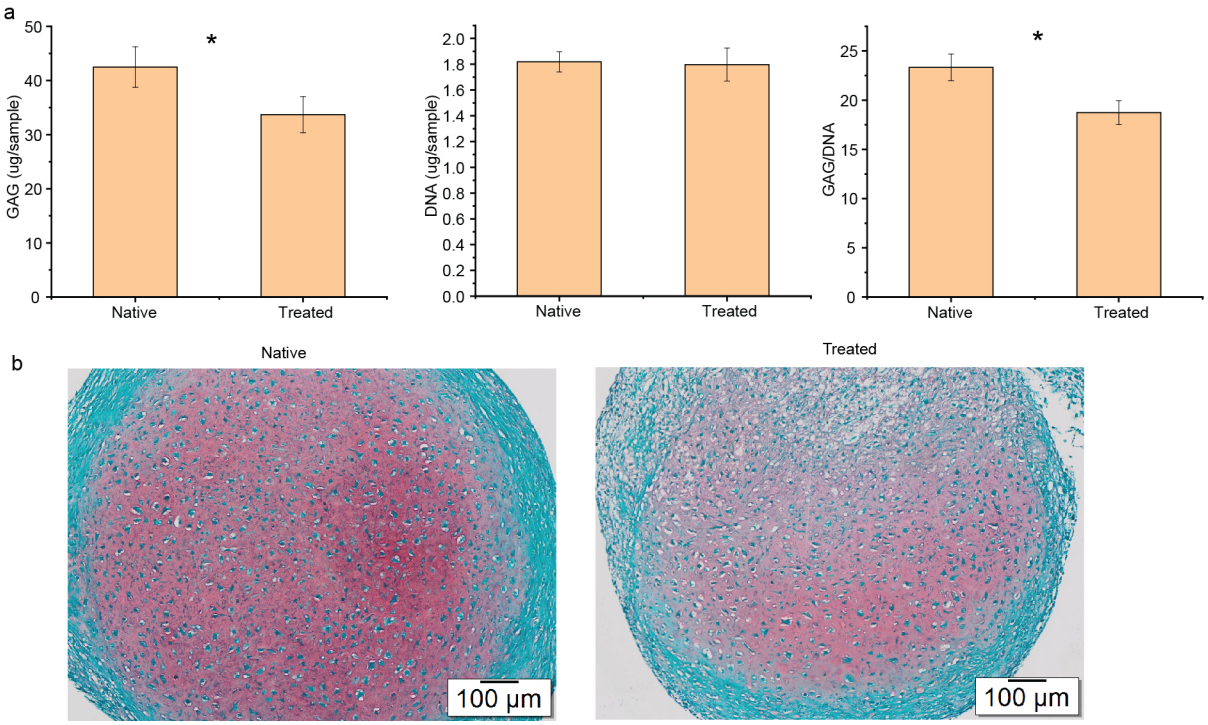

Figure 4. GAG and DNA analysis of hMSC aggregates with untreated TGF-β1 or thermally treated TGF-β1 after 3 weeks culture. a) Results of biochemical assays. * indicates significant difference analyzed by Student's t-test ($p < 0.05$). b) Histological images of tissue sections stained with Safranin O, a dye for GAG (red), and counterstained with Fast Green (green).

From our findings, although TGF-β1 partially unfolds at high temperature, both its conformation and functionality exhibit recovery after the temperature reduces to 25 °C. These results contradict the previous speculation that TGF-β1 is thermally unstable[2] and provide

strong evidence indicating that this protein is resilient to thermal stress. This research suggests that less stringent temperature conditions may be considered for the production, storage, and use of this protein. In addition, given the positive or negative roles of TGF-β in certain diseases such as autoimmune disease[25] and cancer,[26] the diets for certain patients may need further investigation and optimization to boost or suppress the activities of ingested TGF-β1.

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