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Receptor mimicking TGF-β1 binding peptide for targeting TGF-β1 signaling

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Abstract

Prolonged and elevated transforming growth factor-β1 (TGF-β1) signaling can lead to undesired scar formation during tissue repair and fibrosis that is often a result of chronic inflammation in lung, kidney, liver, heart, skin and joints. We report new TGF-β1 binding peptides that interfere with TGF-β1 binding to the cognate receptors and thus attenuate its biological activity. We identified TGF-β1 binding peptides from the TGF-β1 binding domains of TGF-β receptors and engineered their sequences to facilitate chemical conjugation to biomaterials using molecular docking simulations. The *in vitro* binding studies and cell-based assays showed that RIPΔ, which was derived from TGF-β type I receptor, bound TGF-β1 in a sequence-specific manner and reduced the biological activity of TGF-β1 when the peptide was presented either in a soluble form or conjugated to a commonly used synthetic biomaterial. This approach may have implications for clinical applications such as treatment of various fibrotic diseases and soft tissue repair and offer a design strategy for peptide antibodies based on the biomimicry of ligand-receptor interactions.

Transforming growth factor-β1 (TGF-β1) is the most abundant TGF-β isoform in adult tissues and platelets,[1,2] and regulates a multitude of cellular processes including proliferation, differentiation, migration, apoptosis and extracellular matrix (ECM) production and remodeling.[3] This multifunctional growth factor is secreted mostly as an inactive latent complex and deposited in ECM.[4] Once released from ECM and activated, TGF-β1 exerts various biological functions in a spatiotemporally controlled manner. TGF-β1 mediates angiogenesis and vasculogenesis by modulating endothelial cell behavior in a context- and cell type-dependent fashion.[5,6] In addition, it promotes fibroblast proliferation,[7] maintains tissue homeostasis by controlling cellular behavior and immune response,[8] influences self-renewal and differentiation of stem cells,[9] and participates in the development and maintenance of articular cartilage.[10] However, aberrant TGF-β1 activity contributes to pathological conditions, such as autoimmune disorders,[11,12] cancer metastasis,[13] vascular disorders[14] and cartilage diseases.[15,16] In particular, prolonged and elevated TGF-β1 signaling can lead to undesired scar formation during tissue repair[17] and fibrotic diseases including pulmonary fibrosis, diabetic kidney disease, congestive heart failure, liver cirrhosis, systemic sclerosis, and skeletal muscle fibrosis.[18] Therefore, selective inhibition of TGF-β1 signaling has been a subject of interest for therapeutic use in clinical applications.

Therapeutic inhibition of TGF-β signaling has spanned numerous modalities including small molecular kinase inhibitors, neutralizing antibodies, and oligonucleotides. Small molecule inhibitors of TGF-β1 signaling pathway have been developed and explored clinically, but small molecule inhibitors of receptor kinases such as TGF-β type I (TβRI) and type II (TβRII) receptors are difficult to develop with high specificity owing to structural similarity between ATP-binding domains of kinases from the same family. For example, SB-431542 is a small molecule inhibitor of ALK5 (TβRI) with activity against activin receptors ALK4 and ALK7,[19] CK1δ, RIPK2, and

p38MAPK[20] and it was ultimately not explored clinically. Galunisertib, a small molecule inhibitor of TβRI currently being investigated in clinical trials for cancer, has strong activity against off-target kinases, representing plausible toxicity liabilities.[21] In addition, several molecules with activity against TGF-β1 have been explored preclinically as anti-fibrotic therapeutics including suramin,[22] decorin,[23] losartan,[24] relaxin,[25] and γ-interferon.[26] These molecules do not directly bind TGF-β1 or its receptors, and therefore unintended consequences due to off-target effects are unavoidable. On the other hand, neutralizing antibodies aiming to suppress pro-fibrotic TGF-β1 activity have been used to bind soluble TGF-β1 ligand and thus interfere ligand-receptor interaction and downstream signaling cascades. Humanized monoclonal TGF-β1 neutralizing antibodies have reduced scar formation during cutaneous wound healing[27] and skeletal muscle repair,[28] and have been tested in clinical trials for the treatment of sclerosis,[29] fibrosis[30] and metastatic cancer.[31] However, neutralizing antibodies have several limitations, including high cost and potential immunogenicity.[32] As an alternative, synthetic peptides having high affinity for TGF-β1 have emerged,[33,34] as they can be reproducibly produced at relatively low cost and chemically modified on demand. Initial approaches to design TGF-β1 inhibiting peptides have relied on phage display technology. Although phage display enables rapid screening of large peptide libraries for the ability to bind a target molecule, it can identify false positives and is not appropriate to identify peptides that bind a target molecule exclusively *via* a sequence-specific interaction.[35] Another strategy to design high affinity peptides is to mimic the way in which TGF-β1 binds to other biomolecules in nature. For example, peptides derived from the TGF-β1 binding domain of α2-macroglobulin (α2-M)[36] and TGF-β type III receptor (TβRIII)[37] have been shown to bind TGF-β1 with high affinity. However, since α2-M is a carrier protein that non-specifically binds numerous growth factors *via* solvent-exclusion interactions,[38–40] an α2-M-mimicking peptide may present

poor binding specificity to TGF-β1. Furthermore, a TβRIII-derived peptide contains nearly 60% hydrophobic amino acid residues and only one charged residue,[37] and therefore it is conceivable that its binding to TGF-β1 may occur *via* non-specific solvent-exclusion interactions.

We herein developed new TGF-β1 binding peptides *via* biomimicry of the TGF-β1-receptor complex, and assessed their ability to bind TGF-β1 and inhibit associated TGF-β1 biological activity when the peptides were either presented in a soluble form or conjugated to a commonly used synthetic biomaterial. We hypothesized that a peptide derived from the TGF-β1-binding domains of TβRI and TβRII would bind to TGF-β1 via molecular recognition. From the crystal structure of the complex of TGF-β1/TβRI/TβRII (PDB ID 3KFD),[41] we identified regions of TβRI and TβRII whose side chains appeared to localize near TGF-β1 as putative TGF-β1 binding peptides (Fig. 1a, Table 1). A previous mutagenesis study confirmed that the regions that we identified from the crystal structure of TβRII were strongly implicated in TGF-β1 binding.[42]

TGF-β1 binding peptides may be an attractive new therapeutic modality by themselves but also present several opportunities for the design of biomaterials capable of locally regulating TGF-β1 activity. Toward this goal, TGF-β1 binding peptides derived from TGF-β receptors were engineered to enable facile immobilization to the biomaterials and further optimized by molecular docking simulations. The peptides tested in this study were designed to have a cysteine residue at the N-terminus in order to enable covalent conjugation of the peptides to poly(ethylene glycol) (PEG) biomaterials *via* a thiol-ene reaction. For this reason, a cysteine residue was added to the N-terminus of TβRII-derived peptide-1 (RIIP-1). The sequences chosen for the other peptides, TβRI-derived peptide (RIP) and RIIP-2, each have an N-terminal cysteine in their native receptor sequence. Since the thiol group of cysteine can form intermolecular disulfide bonds and thereby complicate the thiol-ene reaction, cysteine residues other than the N-terminal cysteine (i.e.,

positions 5 and 8 in RIIP-1 and position 7 in RIIP-2) were substituted. Molecular docking simulations showed the highest predicted binding affinity between RIIP-1 and TGF-β1 when RIIP-1's cysteine residues at positions 5 and 8 were substituted with glycine, alanine, leucine, or serine. Glycine substitution was predicted to promote the highest TGF-β1 binding affinity (Fig. 1b), so we substituted cysteine residues at positions 5 and 8 of RIIP-1 with glycine, and we followed the same approach to substitute glycine for cysteine at position 7 of RIIP-2. We also designed corresponding negative control peptides, each containing the same amino acids as the parent peptide, with scrambled sequences to control for the effects of peptide hydropathy profile[43] on TGF-β1 sequestering (Fig. S1).

Molecular docking simulations were performed on folded peptide structures[44] of RIP, RIIP-1, and RIIP-2 and the crystal structure of TGF-β1 (PDB ID 3KFD)[41] to determine relative binding affinity.[45] The result showed that RIP exhibited the highest predicted TGF-β1 binding affinity to TGF-β1 (most negative value in kcal/mole) relative to RIIP-1 and RIIP-2 (Fig. 1c). We further interrogated the binding of RIP to TGF-β1 by iteratively truncating amino acids from the N- and C-termini of RIP and assessing binding *via* molecular docking simulations. Both RIP and its variant with the C-terminal valine truncated (RIPΔ) bound to TGF-β1 with high affinity. However, neither a variant of RIP with two C-terminal amino acids truncated nor variants of RIP with one or two truncated amino acids from the N-terminus bound TGF-β1 with appreciable affinity when compared with the scrambled RIP control (Fig. 1d). The native crystal structure of TGF-β1/TβRI shows that the side chain of the C-terminal valine of RIP, in the context of the full-length receptor, is orientated away from the TGF-β1 binding pocket (Fig. S2), providing a plausible explanation for the similar predicted binding affinity of RIP and RIPΔ. These data indicate that the terminal valine on RIP provides no enhancement of binding affinity to TGF-β1

and motivates the further characterization of RIP and RIPΔ for binding and inhibition of TGF-β1 activity.

Soluble peptides were examined for their influence on TGF-β1 activity using a T-lymphocyte proliferation assay.[46] We hypothesized that soluble TβR-derived peptides would bind TGF-β1 and attenuate inhibitory effect of TGF-β1 on interleukin 4 (IL-4) dependent proliferation of mouse T-lymphocyte HT-2 cells. As expected, soluble TGF-β1 inhibited IL-4 dependent proliferation in a dose dependent manner in a fluorescence-based cell viability assay (Fig. 2a). The IC_{50} of TGF-β1 in the HT-2 proliferation assay was 0.05 ng/mL (3.9 pM) is in good accordance with the published IC_{50} value of 0.04 to 0.2 ng/mL.[46] Initial screening of peptides was performed at a single concentration of peptides (10 μM) and TGF-β1 (0.1 ng/mL, 7.8 pM), with which the assay was anticipated to retain sensitivity to disrupted TGF-β1 signaling. RIP elicited significantly higher fluorescence intensity relative to the no peptide control and showed indistinguishable fluorescence compared with the no TGF-β1 control. This result suggests that RIP bound to TGF-β1 and effectively attenuated its biological activity (Fig. 2b). In the same assay, a scrambled version of RIP exhibited no difference in fluorescence compared with the no peptide control, reflecting no effect on TGF-β1 biological activity. While RIIP-1 significantly increased fluorescence relative to the no peptide control, the fluorescence intensity was not statistically different between RIIP-1 and its scrambled control. Neither RIIP-2 nor its scrambled control elicited any change in fluorescence compared to no peptide control. These results suggest that RIIP-1 and RIIP-2 did not affect TGF-β1 activity in a sequence-dependent fashion.

We further assessed the sequence specificity of RIP binding to TGF-β1 by employing: i) D-amino acid substituted RIP (D-RIP), consisting of all D-amino acids with reverse side chain orientations of RIP; ii) and retro-inverso RIP (RI-RIP), a peptide with the reversed sequence of D-

RIP and similar side chain orientation to RIP in its extended conformation. Due to the similar side chain topology, biological activity of RI-RIP was expected to emulate that of its parent peptide, RIP, whereas D-RIP having reverse side chain orientation was expected to have reduced biological activity. Soluble RI-RIP significantly suppressed TGF-β1 activity of inhibiting HT-2 cell proliferation, while the D-RIP peptide did not (Fig. 2c), suggesting that RIP attenuates TGF-β1 activity through sequence-specific binding. Taken together, these results provide a clearer demonstration of sequence-specific TGF-β1 binding and inhibition, relative to prior studies of TGF-β1 binding peptides.[33,34,37,47] Both molecular docking simulations and biological activity assays show that RIP outperformed the RIIP-1 and RIIP-2 peptides and together constitute a proof-of-concept that structural biochemistry together with computational modeling can aid in the initial identification and engineering of peptides that bind a specific macromolecule.

We next quantitatively examined the affinity of RIP and its derivatives to TGF-β1 via surface plasmon resonance spectroscopy (SPR), where the peptide affinity to TGF-β1 was determined by monitoring SPR signal change upon TGF-β1 binding to a peptide-immobilized substrate. Since molecular docking simulation predicted that RIPΔ with a truncated C-terminal valine binds TGF-β1 with similar affinity as full-length RIP, we measured association rate constants (k_a), dissociation rate constants (k_d) and equilibrium dissociation constants (K_D; k_d/k_a) of RIP, RIPΔ, and scrambled RIP from SPR sensorgrams. RIP, RIPΔ, and scrambled RIP exhibited equilibrium K_D values of 98.3, 183, and 745 nM, respectively, suggesting that both RIP and RIPΔ bound TGF-β1 with higher affinity than scrambled RIP by at least four-fold (Table 2). Given that RIPΔ presents a shorter, less hydrophobic sequence than RIP with predicted or experimental equilibrium binding affinity comparable to RIP, we further explored the ability of RIPΔ to bind and inhibit TGF-β1 activity in soluble form and as a component of PEG-based biomaterials.

We next quantified TGF-β1 binding to RIPΔ by immobilizing a BSA-RIPΔ conjugate onto a 96-well microplate for use as a "capture antibody" in a TGF-β1 ELISA. An assay titrating the concentration of BSA-conjugated RIPΔ and scrambled RIP at a fixed concentration of TGF-β1 revealed greater magnitude of binding of TGF-β1 to BSA-RIPΔ relative to BSA-scrambled RIP (Fig. S3). Further, the immobilized BSA-RIPΔ conjugate bound a significantly larger amount of TGF-β1 at equilibrium when compared with a BSA-scrambled RIP conjugate at a fixed concentration of immobilized BSA conjugates and TGF-β1 concentrations at or above 5 ng/mL (Fig. 3a). BSA-RIPΔ binding to TGF-β1 was significantly higher than that of a BSA-only control at TGF-β1 concentrations over 2.5 ng/mL, suggesting that binding of TGF-β1 to RIPΔ was sequence-specific. Although BSA-RIIP-1 and BSA-RIIP-2 bound significantly more TGF-β1 than the BSA only control at 40 ng/mL TGF-β1, neither of them showed superior TGF-β1 binding when compared to each respective scrambled control peptide at any concentration of TGF-β1 (Fig. S4).

Next, the inhibitory effect of RIPΔ on TGF-β1 signaling was evaluated in multiple cell-based biological activity assays, using both human umbilical vein endothelial cells (HUVECs) and HT-2 cells. HUVEC proliferation was suppressed by TGF-β1 at concentration range of 10 to 1000 ng/mL with the highest level of inhibition at 41 pg/mL (3.2 pM) (Fig. S5).[48,49] Using this model, we hypothesized that soluble RIPΔ would increase HUVEC proliferation by binding and blocking the TβRI-binding domain on TGF-β1. Addition of 10 μM RIPΔ increased HUVEC proliferation in the presence of 41 pg/mL TGF-β1, relative to the scrambled RIP control and the no peptide control (Fig. 3b). These data indicate that RIPΔ bound TGF-β1 and thereby blocked downstream signaling of TGF-β1. In contrast, no statistical differences were observed between TβRII-derived peptides (i.e., RIIP-1 and RIIP-2) and their controls in the HUVEC-based assay. We performed an HT-2 proliferation assay described above with 1 ng/mL TGF-β1 (78 pM), which is ten-fold higher

than the IC_{50} (0.1 ng/mL, 7.8 pM), to rigorously assess activity of engineered peptides. Similar results were obtained in an HT-2 proliferation assay, where only RIPΔ reduced the inhibitory effect of TGF-β1 on HT-2 proliferation, whereas RIIP-1 and RIIP-2 and their scrambled controls failed to increase fluorescence intensity suggesting no measurable activity in the more rigorous assay format (Fig. 3c). Taken together, these data demonstrate that RIPΔ successfully bound TGF-β1 in a sequence-specific manner and inhibited its biological activity in general agreement with molecular docking simulations. RIIP-1 significantly increased the fluorescence intensity in the more sensitive HT-2 assay with 0.1 ng/mL TGF-β1 (Fig. 2b) but failed to show any effect in the more rigorous assay with 1 ng/mL TGF-β1 (Fig. 3c), suggesting RIIP-1 has limited activity as an inhibitor of TGF-β1. The failure of RIIP-1 and RIIP-2 to bind and inhibit TGF-β1 remains an open research question. One possibility is that the glycine substitutions abrogated interaction at the binding interface with the cysteine in the native receptor sequence. Another possibility is that essential amino acids mediating TβRII binding to TGF-β1 lie outside of the sequences employed here. Preliminary molecular docking simulations with a peptide extending the sequence of RIIP-1 by 10 additional N-terminal residues and 3 additional C-terminal residues relative to the TβRII sequence showed enhanced TGF-β1 binding to nearly the level predicted of RIP (data not shown). Further exploration and optimization of RIIP-1 and RIIP-2 binding to TGF-β1 remains as future work.

Local regulation of TGF-β1 activity via biomaterials was motivated by the incidence of off-target toxicities of systemically administered TβRI inhibitors. For example, galunisertib, an orally bioavailable TβRI inhibitor currently being evaluated in clinical trials for the treatment of solid tumors, required a cautious clinical approach to avoid cardiac toxicities identified during preclinical development and deemed to be target-related owing partly to incidence of similar

cardiac toxicities with other TβRI inhibitors in the clinic.[21,50] Hydrogel microspheres are an injectable biomaterial amenable to local regulation of target activity *via* sequestering as previously demonstrated using PEG microspheres containing VEGFR2-derived peptides.[51,52] Here we hypothesized that RIPΔ-conjugated PEG microspheres could specifically sequester TGF-β1 from biological solution. RIPΔ was covalently conjugated to PEG microspheres via a UV-initiated thiol-ene reaction between thiol groups of the N-terminal cysteine residue of peptides and the norbornene groups of the PEG chains (Fig. 4a).[51] The density of peptide incorporated was systematically changed by varying the ratio of peptide to norbornene functional groups. The peptide-conjugated PEG microspheres were then incubated in 10% fetal bovine serum (FBS) medium containing ^{125}I-radiolabeled TGF-β1. RIPΔ-conjugated microspheres having higher peptide density (4.2% and 12.5%) bound significantly more TGF-β1 compared to blank microspheres with no peptide, while RIPΔ-conjugated microspheres bound more TGF-β1 than scrambled RIP-conjugated microspheres at the peptide density of 12.5% (Fig. 4b). An effective equilibrium dissociation constant ($K_{D,eff}$) was calculated to compare the relative binding affinity of peptide-conjugated microspheres to TGF-β1 in 10% serum, revealing that RIPΔ bound with 2-2.5 fold higher binding affinity than scrambled RIP (Fig. S6). When microspheres presenting 12.5% peptide were included in the HT-2 cell proliferation assay, RIPΔ-conjugated microspheres attenuated the inhibitory effects of 1 ng/mL TGF-β1 (78 pM) on HT-2 cell proliferation in culture when compared to both blank microspheres and microspheres with scrambled RIP (Fig. 4c). These data suggest that RIPΔ-conjugated microspheres sequestered and thereby reduced the biological activity of soluble TGF-β1 activity.

Finally, we measured TGF-β1 binding by peptide-conjugated microspheres incubated in platelet-rich plasma (PRP), which has been clinically used to treat various musculoskeletal soft

tissue injuries. Reduction of TGF-β1 activity in PRP could ultimately reduce fibrosis in applications where PRP is applied to repair skeletal muscle, tendon and ligament tissues. Local regulation of TGF-β1 activity via sequestering to RIPΔ-conjugated microspheres can address the clinical need for localized musculoskeletal wound healing. RIPΔ-conjugated microspheres captured TGF-β1 from PRP, as they reduced TGF-β1 concentration by up to 26% (Fig. 4d). Each of the microsphere concentrations tested in PRP (2-32 mg/mL) achieved over 15% TGF-β1 reduction. The $K_{D,effective}$ was calculated for each microsphere concentration, revealing that 2 mg/mL RIPΔ microspheres sequestered endogenous TGF-β1 from PRP ($K_{D,effective}$ ~ 215 nM) with comparable binding affinity compared to sequestering of supplemented TGF-β1 in 10% serum ($K_{D,effective}$ ~ 211 nM) and compared to SPR (equilibrium K_D ~183 nM). These results demonstrate RIPΔ microspheres sequester TGF-β1 with high affinity and specificity and suggest a potential therapeutic application of RIPΔ-containing microspheres to locally reduce TGF-β1 activity during soft tissue healing. Sequestering and modulation of TGF-β1 activity in serum-containing solutions and PRP highlights the specificity of sequestering to RIPΔ-conjugated microspheres, as serum and PRP contain hundreds of unique proteins at high abundance.

In summary, we demonstrated the feasibility of using existing structural data to design growth factor-binding peptides based on biomimicry of the growth factor-receptor interaction. We designed TGF-β1 binding peptides from the TGF-β1 binding domains of TβRs identified by published crystallographic data. In agreement with molecular docking simulations, experimental data revealed that RIP and RIPΔ, whose sequences were derived from TβRI, bound to TGF-β1 in a sequence-specific manner and inhibited its biological activity. RIPΔ, a truncated variant of RIP, was shown theoretically and experimentally to retain its ability to bind TGF-β1, but with reduced hydrophobicity relative to the parent peptide. Future embodiments of this TGF-β1 sequestering

approach may benefit from further optimization of RIP and RIPΔ by introducing non-canonical amino acids, which can enhance the affinity of a particular peptide-growth factor interaction and enhance peptide serum stability.[53] This approach may have implications for clinical applications such as treatment of various fibrotic diseases and soft tissue repair. It is noteworthy that a similar, previous approach has been used to identify VEGF-binding peptides, and to fabricate biomaterials that reduce VEGF-dependent angiogenesis.[54] Therefore, biomimetic design of growth factor receptor-mimicking biomaterials could potentially become a broader approach for local modulation of growth factor activity on demand.

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References

1 G. S. Ashcroft, *Microbes Infect.*, 1999, **1**, 1275–1282.

2 R. K. Assoian, A. Komoriya, C. A. Meyers, D. M. Miller and M. B. Sporn, *J. Biol. Chem.*, 1983, **258**, 7155–7160.

3 S. Barrientos, O. Stojadinovic, M. S. Golinko, H. Brem and M. Tomic-Canic, *Wound Repair Regen.*, 2008, **16**, 585–601.

4 J. P. Annes, J. S. Munger and D. B. Rifkin, *J. Cell Sci.*, 2003, **116**, 217–224.

5 M. J. Goumans, G. Valdimarsdottir, S. Itoh, A. Rosendahl, P. Sideras and P. ten Dijke, *EMBO J.*, 2002, **21**, 1743–1753.

6 E. Y. Yang and H. L. Moses, *J. Cell Biol.*, 1990, **111**, 731–741.

7 A. Leask and D. J. Abraham, *FASEB J.*, 2004, **18**, 816–827.

8 M. Pakyari, A. Farrokhi, M. K. Maharlooei and A. Ghahary, *Adv. Wound Care*, 2013, **2**, 215–224.

9 T. Watabe and K. Miyazono, *Cell Res.*, 2009, **19**, 103–115.

10 W. Wang, D. Rigueur and K. M. Lyons, *Birth Defects Res. Part C - Embryo Today Rev.*, 2014, **102**, 37–51.

11 G. J. Prud'homme, *Lab. Investig.*, 2007, **87**, 1077–1091.

12 S. Sanjabi, S. A. Oh and M. O. Li, *Cold Spring Harb. Perspect. Biol.*, 2017, **9**, a022236.

13 C. Neuzillet, A. Tijeras-Raballand, R. Cohen, J. Cros, S. Faivre, E. Raymond and A. De Gramont, *Pharmacol. Ther.*, 2015, **147**, 22–31.

14 P. Bertolino, M. Deckers, F. Lebrin and P. Ten Dijke, *Chest*, 2005, **128**, 585S-590S.

15 J. Shen, S. Li and D. Chen, *Bone Res.*, 2014, **2**, 1–7.

16 W. Finnson, Kenneth, *Front. Biosci.*, 2012, **S4**, 251.

17 S. R. Beanes, C. Dang, C. Soo and K. Ting, *Expert Rev. Mol. Med.*, 2003, **5**, 1–22.

18 K. J. Gordon and G. C. Blobe, *Biochim. Biophys. Acta - Mol. Basis Dis.*, 2008, **1782**, 197–228.

19 S. K. Halder, R. D. Beauchamp and P. K. Datta, *Neoplasia*, 2005, **7**, 509–521.

20 J. Vogt, R. Traynor and G. P. Sapkota, *Cell. Signal.*, 2011, **23**, 1831–1842.

21 J. M. Yingling, W. T. McMillen, L. Yan, H. Huang, J. S. Sawyer, J. Graff, D. K. Clawson, K. S. Britt, B. D. Anderson, D. W. Beight, D. Desaiah, M. M. Lahn, K. A. Benhadji, M. J. Lallena, R. B. Holmgaard, X. Xu, F. Zhang, J. R. Manro, P. W. Iversen, C. V. Iyer, R. A.

Brekken, M. D. Kalos and K. E. Driscoll, *Oncotarget*, 2018, **9**, 6659–6677.

22 Y. S. Chan, Y. Li, W. Foster, F. H. Fu and J. Huard, *Am. J. Sports Med.*, 2005, **33**, 43–51.

23 J. Zhu, Y. Li, W. Shen, C. Qiao, F. Ambrosio, M. Lavasani, M. Nozaki, M. F. Branca and J. Huard, *J. Biol. Chem.*, 2007, **282**, 25852–25863.

24 T. Kobayashi, K. Uehara, S. Ota, K. Tobita, F. Ambrosio, J. H. Cummins, S. Terada, F. H. Fu and J. Huard, *J. Appl. Physiol.*, 2013, **114**, 262–273.

25 S. Negishi, Y. Li, A. Usas, F. H. Fu and J. Huard, *Am. J. Sports Med.*, 2005, **33**, 1816–1824.

26 W. Foster, Y. Li, A. Usas, G. Somogyi and J. Huard, *J. Orthop. Res.*, 2003, **21**, 798–804.

27 M. Shah, D. M. Foreman and M. W. Ferguson, *J. Cell Sci.*, 1995, **108**, 985–1002.

28 M. Zimowska, A. Duchesnay, P. Dragun, A. Oberbek, J. Moraczewski and I. Martelly, *Int. J. Cell Biol.*, 2009, **2009**, 659372.

29 C. P. Denton, P. A. Merkel, D. E. Furst, D. Khanna, P. Emery, V. M. Hsu, N. Silliman, J. Streisand, J. Powell, A. Åkesson, J. Coppock, F. Van Den Hoogen, A. Herrick, M. D. Mayes, D. Veale, J. Haas, S. Ledbetter, J. H. Korn, C. M. Black and J. R. Seibold, *Arthritis Rheum.*, 2007, **56**, 323–333.

30 J. Voelker, P. H. Berg, M. Sheetz, K. Duffin, T. Shen, B. Moser, T. Greene, S. S. Blumenthal, I. Rychlik, Y. Yagil, P. Zaoui and J. B. Lewis, *J. Am. Soc. Nephrol.*, 2017, **28**, 953–962.

31 J. C. Morris, A. R. Tan, T. E. Olencki, G. I. Shapiro, B. J. Dezube, M. Reiss, F. J. Hsu, J. A. Berzofsky and D. P. Lawrence, *PLoS One*, 2014, **9**, e90353.

32 K. D. Ratanji, J. P. Derrick, R. J. Dearman and I. Kimber, *J. Immunotoxicol.*, 2014, **11**, 99–109.

33 S. Serratì, F. Margheri, M. Pucci, A. R. Cantelmo, R. Cammarota, J. Dotor, F. Borràs-Cuesta, G. Fibbi, A. Albini and M. Del Rosso, *Biochem. Pharmacol.*, 2009, **77**, 813–825.

34 R. N. Shah, N. A. Shah, M. M. D. R. Lim, C. Hsieh, G. Nuber and S. I. Stupp, *Proc. Natl. Acad. Sci. U. S. A.*, 2010, **107**, 3293–3298.

35 M. Vodnik, U. Zager, B. Strukelj and M. Lunder, *Molecules*, 2011, **16**, 790–817.

36 D. J. Webb, D. W. Roadcap, A. Dhakephalkar and S. L. Gonias, *Protein Sci.*, 2000, **9**, 1986–1992.

37 I. J. Ezquerro, J. J. Lasarte, J. Dotor, I. Castilla-Cortázar, M. Bustos, I. Peñuelas, G. Blanco, C. Rodríguez, M. D. C. G. Lechuga, P. Greenwel, M. Rojkind, J. Prieto and F. Borrás-Cuesta, *Cytokine*, 2003, **22**, 12–20.

38 G. Bhattacharjee, I. R. Asplin, S. M. Wu, G. Gawdi and S. V. Pizzo, *J. Biol. Chem.*, 2000, **275**, 26806–26811.

39 S. Mathew, S. Arandjelovic, W. F. Beyer, S. L. Gonias and S. V. Pizzo, *Biochem. J.*,

2003, **374**, 123–129.

40 K. P. Crookston, D. J. Webb, J. Lamarre and S. L. Gonias, *Biochem. J.*, 1993, **293**, 443–450.

41 S. Radaev, Z. Zou, T. Huang, E. M. Lafer, A. P. Hinck and P. D. Sun, *J. Biol. Chem.*, 2010, **285**, 14806–14814.

42 G. De Crescenzo, C. S. Hinck, Z. Shu, J. Zúñiga, J. Yang, Y. Tang, J. Baardsnes, V. Mendoza, L. Sun, F. López-Casillas, M. O'Connor-Mccourt and A. P. Hinck, *J. Mol. Biol.*, 2006, **355**, 47–62.

43 J. Kyte and R. F. Doolittle, *J. Mol. Biol.*, 1982, **157**, 105–132.

44 J. Maupetit, P. Derreumaux and P. Tufféry, *J. Comput. Chem.*, 2009, **31**, 726–738.

45 O. Trott and A. J. Olson, *J. Comput. Chem.*, 2010, **31**, 455–461.

46 M. L. S. Tsang, L. Zhou, B. L. Zheng, J. Wenker, G. Fransen, J. Humphrey, J. M. Smith, M. O'Connor-McCourt, R. Lucas and J. A. Weatherbee, *Cytokine*, 1995, **7**, 389–397.

47 J. Dotor, A. B. López-Vázquez, J. J. Lasarte, P. Sarobe, M. García-Granero, J. I. Riezu-Boj, A. Martínez, E. Feijoó, J. López-Sagaseta, J. Hermida, J. Prieto and F. Borrás-Cuesta, *Cytokine*, 2007, **39**, 106–115.

48 R. Hirai and K. Kaji, *Exp. Cell Res.*, 1992, **201**, 119–125.

49 T. Tsukada, K. Eguchi, K. Migita, Y. Kawabe, A. Kawakami, N. Matsuoka, H. Takashima, A. Mizokami and S. Nagataki, *Biochem. Biophys. Res. Commun.*, 1995, **210**, 1076–1082.

50 A. J. Stauber and K. M. Credill, *J. Clin. Toxicol.*, 2014, **4**, 1000196.

51 D. G. Belair and W. L. Murphy, *Acta Biomater.*, 2013, **9**, 8823–8831.

52 N. A. Impellitteri, M. W. Toepke, S. K. Lan Levengood and W. L. Murphy, *Biomaterials*, 2012, **33**, 3475–3484.

53 C. Piossek, K. H. Thierauch, J. Schneider-Mergener, R. Volkmer-Engert, M. F. Bachmann, T. Korff, H. G. Augustin and L. Germeroth, *Thromb. Haemost.*, 2003, **90**, 501–510.

54 D. G. Belair, M. J. Miller, S. Wang, S. R. Darjatmoko, B. Y. K. Binder, N. Sheibani and W. L. Murphy, *Biomaterials*, 2016, **93**, 27–37.

Table 1. TGF-β1 binding peptides derived from TGF-β receptors.

Peptide ID	Origin	Amino acid sequence [a]	Scrambled sequence
RIP	TGF-β type I receptor	CIAEIDLIPRDRPFV	CLIDFRIPADREVPI
RIPΔ	TGF-β type I receptor	CIAEIDLIPRDRPF	-
RIIP-1	TGF-β type II receptor	CPQLGKFGDVRF	CQGFPDVGRFKL
RIIP-2	TGF-β type II receptor	CSITSIGEKP	CGTIPISKES

[a] The substituted glycine residues are shown with underbars.

Table 2. Average association and dissociation rate constants (k_a and k_d), dissociation constants (K_D), and equilibrium K_D determined by surface plasmon resonance (SPR).

Peptide	Conc. (μM)	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_D (M)	Equilibrium K_D (M)
RIP	10-20	1.40×10^5	8.36×10^{-3}	6.00×10^{-8}	9.83×10^{-8}
RIPΔ	5-20	1.92×10^5	1.99×10^{-2}	1.06×10^{-7}	1.83×10^{-7}
Scrambled RIP	10-20	6.85×10^4	3.52×10^{-2}	7.65×10^{-7}	7.45×10^{-7}

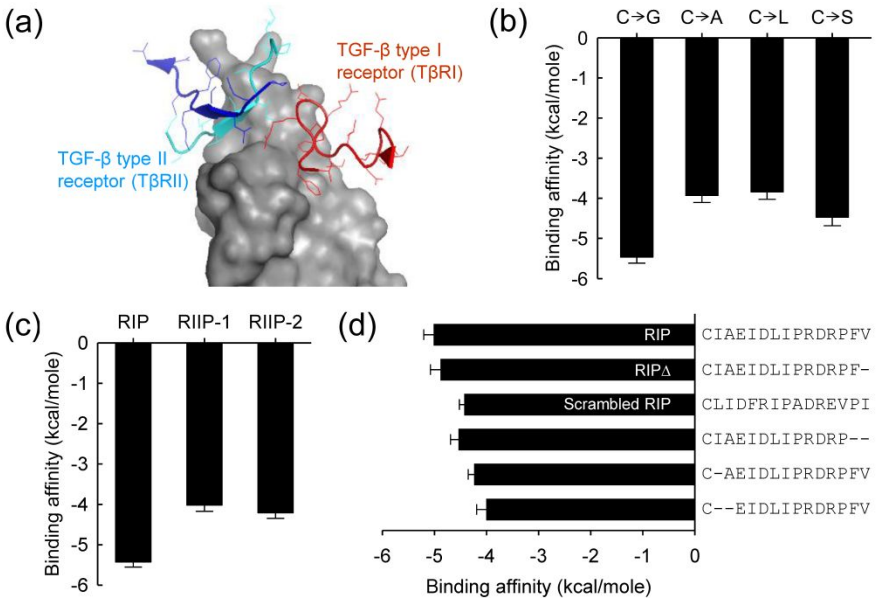

Figure 1. Design of putative TGF-β1 binding peptides. (a) Interface of TGF-β receptors and TGF-β1 in complex of TGF-β1/TβRI/TβRII. TGF-β1 and the TGF-β receptor domains from which RIP, RIIP-1 and RIIP-2 were derived are shown in gray, red, blue and cyan, respectively. Note that only receptor residues that TGF-β1 binding peptides were derived from are displayed. (b) Partial blind docking analysis of TGF-β1 to RIIP-1 variants where cysteine residues were substituted with glycine (C→G), alanine (C→A), leucine (C→L) or serine (C→S). (c) Fully blind docking analysis of TGF-β1 with RIP, RIIP-1, and RIIP-2. (d) Fully blind docking analysis of TGF-β1 with truncated RIP variants.

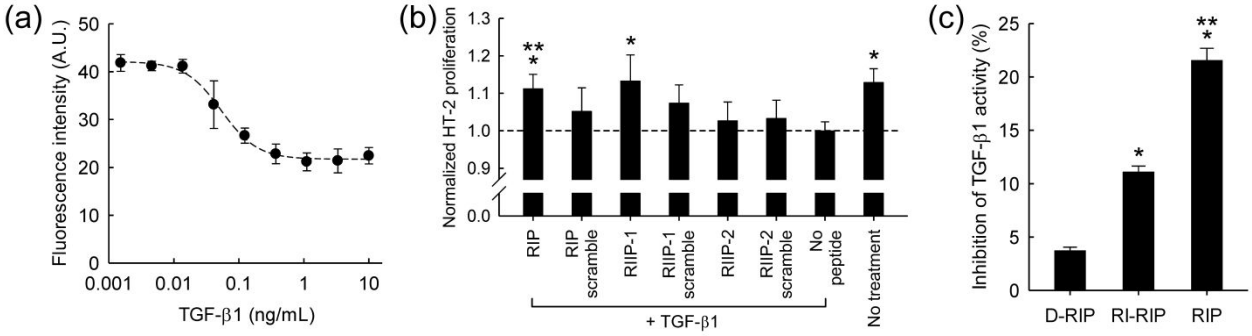

Figure 2. Influence of soluble TGF-β1 binding peptides on TGF-β1-mediated cell function. (a) Inhibitory effect of TGF-β1 on HT-2 cell proliferation in culture. (b) Effect of soluble TGF-β1 binding peptides (10 μM) on HT-2 proliferation in presence of 0.1 ng/mL TGF-β1. Fluorescence intensity of each condition was normalized to the no peptide + TGF-β1 condition. * $p<0.05$ compared to no peptide + TGF-β1 control by two-way ANOVA and Bonferroni post-hoc test, and ** $p<0.05$ compared to RIP scramble control by two-way ANOVA and Bonferroni post-hoc test. Data were aggregated from two independent experiments. (c) Effect of soluble RIP isomers, D-substituted version of RIP (D-RIP) and retro-inverso RIP (RI-RIP) (100 μM) on HT-2 proliferation in presence of 1 ng/mL TGF-β1. * $p<0.05$ compared to D-RIP, and ** $p<0.05$ compared to RI-RIP by Student's t-test.

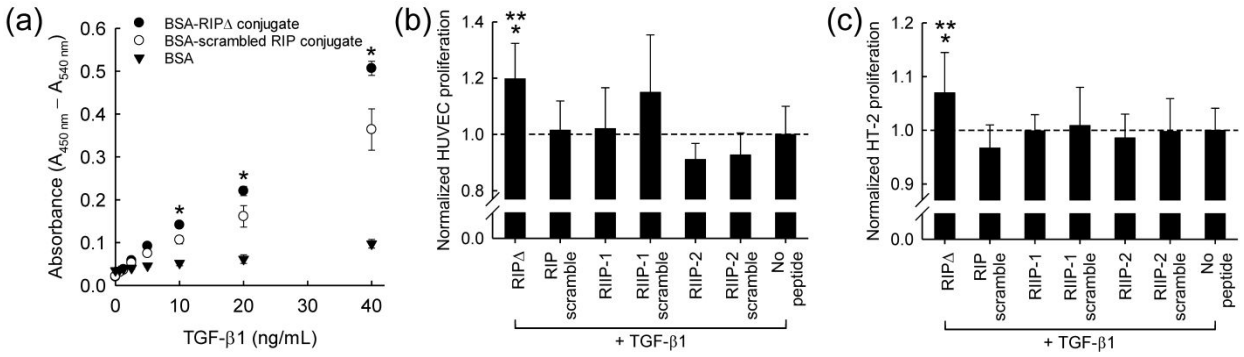

Figure 3. (a) TGF-β1 binding to immobilized BSA-conjugated TβRI-derived peptides. Absorbance was measured by modified ELISA, where BSA-conjugated peptides were used as a capture antibody. * $p<0.05$ compared to BSA-scrambled RIP conjugate for a given concentration by two-way ANOVA with Bonferroni post-hoc test. (b) Effect of soluble TGF-β1 binding peptides (10 μM) on HUVEC proliferation in presence of 41 pg/mL TGF-β1. Data is presented by normalizing HUVEC number after 48 hours in culture to that of no peptide condition. * $p<0.05$ compared to no peptide control, and ** $p<0.05$ compared to respective scrambled peptide by two-way ANOVA with Bonferroni post-hoc test. (c) Effect of soluble TGF-β1 binding peptides (10 μM) on HT-2 proliferation in presence of 1 ng/mL TGF-β1. Data is presented as normalized fluorescence intensity of each condition relative to that of no peptide condition. * $p<0.05$ compared to no peptide control, and ** $p<0.05$ compared to respective scrambled peptide by two-way ANOVA with Bonferroni post-hoc test.

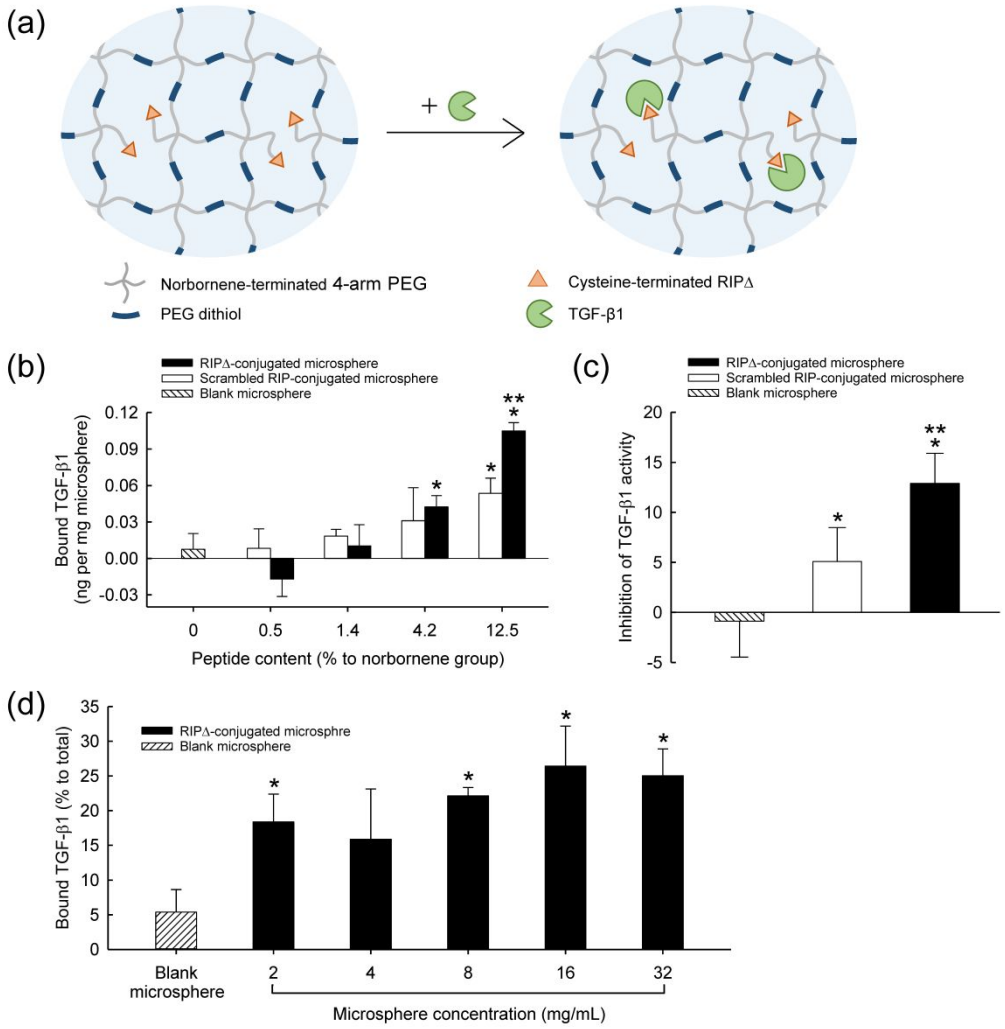

Figure 4. RIPΔ-conjugated microspheres for TGF-β1 sequestering. (a) Schematic illustration of RIPΔ-conjugated PEG microsphere and its action upon exposure to TGF-β1. (b) TGF-β1 binding to RIPΔ-conjugated microspheres having different peptide densities when microspheres were incubated in the medium supplemented with 10% FBS and 1 ng/mL TGF-β1. * p<0.05 compared to blank microsphere, and ** p<0.05 compared to scrambled RIP-conjugated microsphere by two-way ANOVA with Bonferroni post-hoc test. (c) Influence of TGF-β1 sequestering of RIPΔ-conjugated microspheres on HT-2 proliferation in the presence of 1 ng/mL TGF-β1. * p<0.05 compared to blank microsphere and ** p<0.05 compared to scrambled RIP-conjugated microsphere by Student's t-test. (d) TGF-β1 binding to RIPΔ-conjugated microspheres in the PRP. * p<0.05 compared to blank microsphere by Student's t-test.

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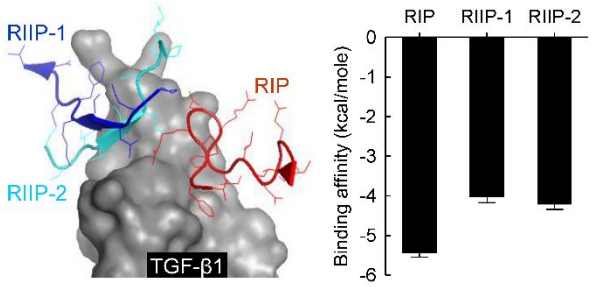

Transforming growth factor-β1 (TGF-β1) binding peptides were developed *via* biomimicry of TGF-β1/TGF-β receptors complex to attenuate biological activity of TGF-β1 when presented either in a soluble form or conjugated to synthetic biomaterials.