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Sensitization of Transforming Growth Factor-β Signaling by Multiple Peptides Patterned on DNA Nanostructures

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Abstract

We report sensitization of a cellular signaling pathway by addition of functionalized DNA nanostructures. Signaling by transforming growth factor β (TGFβ) has been shown to be dependent on receptor clustering. By patterning a DNA nanostructure with closely spaced peptides that bind to TGFβ we observe increased sensitivity of NMuMG cells to TGFβ ligand. This is evidenced by translocation of secondary messenger proteins to the nucleus and stimulation of an inducible luciferase reporter at lower concentrations of TGFβ ligand. We believe this represents an important initial step towards realization of DNA as a self assembling and biologically compatible material for use in tissue engineering and drug delivery.

Keywords

DNA; Nanostructure; TGFβ; Nanotechnology

Structural DNA nanotechnology has made great advances in the creation of self-assembled addressable nano structures by taking advantage of the predictable interactions of DNA base pairing.[1-3] Especially the method of scaffolded DNA origami, which relies on an excess of many short “staple” strands to fold a long, single-stranded scaffold into almost any desired shape, has proven successful as a robust means of patterning inorganic, organic and biological molecules with nanoscale precision.[4,5] The biological and biocompatible nature of DNA allows for these versatile structures to interface with cellular systems without prohibitive cytotoxicity. Recent research has demonstrated that DNA nanostructures enjoy increased resistance to enzymatic digestion,[6] and they retain their structural integrity for extended periods of time in cell lysate,[7] intracellular cytosol,[8,9] and blood plasma.[10] Such

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Supporting Information. Experimental procedures, DNA sequences and supporting data. This material is available free of charge via the Internet at http://pubs.acs.org.

resistance to degradation can be further improved by various covalent modifications of DNA oligonucleotides.[11] The stability of DNA nanostructures in biological environments has allowed for their application in the targeting of living cells,[12,13] and it has been shown that DNA icosahedra and origami structures can function as drug carriers for doxorubicin.[14,15] In addition, both DNA wireframe tetrahedra and origami structures have been coated with CpG oligonucleotides and used as potent vehicles for immunostimulation.[16,17] One of the advantages of using DNA nanostructures is the ease with which they can be modified as illustrated by the creation of a tunable, artificial extracellular matrix (ECM) constructed from DNA motifs decorated with fibronectin domains.[18] Since many cell surface receptors activate through ligand mediated oligomerization followed by phosphorylation of intracellular receptor domains, we hypothesized that extracellular DNA nanostructures might confer even greater control over cellular behavior through the pre-clustering and thus activating or sensitizing of cell surface receptors. To test this hypothesis we set out to test pre-clustering of transforming growth factor-β (TGFβ) receptors using multiple copies of a peptide ligand patterned on DNA nanostructures. TGFβ plays an important role in the feedback control system between ECM and cells and, as such, is an attractive target with significant implications in tissue engineering.[19] TGFβ activation occurs through TGFβ binding to two TGFβ type I receptors and two TGFβ type II receptors (TβRI and TβRII, respectively). TGFβ binding activates TβRII, causing phosphorylation of TβRI and initiation of a signaling cascade.

Lingyin Li et. al. demonstrated pre-clustering of TβRI and TβRII by functionalizing a gold surface with a peptide that binds to both TβRI and TβRII without blocking the TGFβ binding site.[20] A major shortcoming of such surface-based systems is that, although the overall concentration of peptide bound to the surface can be varied, the specific nanoscale density or clustering of peptide cannot be known or controlled. In the study reported herein, we control the nanoscale patterning of the TβR binding peptide of Lingyin Li *et. al.* upon DNA origami by sandwiching tetravalent streptavidin molecules between biotinylated peptide and biotinylated origami. Tall rectangular origami of Rothemund's original design[21] was modified by leaving out 12 centrally located staple strands (strands denoted as: t-1r14f, t-1r18f, t-1r16f, t-1r20f, t-1r14e, t1r14f, t-1r16e, t1r16f, t-1r18e, t1r18f, t-1r20e, t1r20f). New strands were designed with a different crossover scheme allowing for 5′ modifications to be displayed on one face of the structure and 10 strands were biotinylated creating a central patch of biotin moieties. Figure 1a illustrates a schematic of the streptavidin as blue dots located on orange rectangular origami, and Figure 1b illustrates a sideview of the nano-assembly.

The multivalency of streptavidin results in the potential for up to three peptides to be displayed at each origami biotinylation site assuming a one to one binding between biotins on the origami and streptavidin. This assumption is reasonable when we consider the geometry of biotinylation sites on the origami compared to the geometry of streptavidin. The protein is a tetramer with 4 biotin binding sites spaced no more than 3.8 nm apart (PDB entry 3RY1).[22] On the other hand the closest spacing of biotin on the modified origami is two helices over. With a 2 nm helix diameter and 1 nm interhelix spacing this results in a distance of approximately 5 nm. It is thus unlikely for a streptavidin tetramer to bridge

between two origami bound biotins. Therefore it is reasonable to assume that each origami biotinylation site up will result in the binding of up to three biotinylated peptides.

Nano-platforms were assembled stepwise. In order to purify assemblies from excess component building blocks, multiple spin-filtration steps were performed using filters selected to retain the nano-assemblies but to allow unassembled, individual components to flow through. To investigate structural integrity of the nano-assemblies after spin-filtration steps, we performed atomic force microscopy (AFM). Figure 1c shows an AFM image of the annealed origami containing the modified staple strands and 1d shows the final nano-assemblies following spin filtration; the peptide-streptavidin complex is visible as a centrally located patch on the origami rectangles.

During activation of the TGFβ pathway, phosphorylation of TβRI exposes a Smad protein binding site which results in Smad2 and Smad3 phosphorylation. Phosphorylated Smad2/3 forms a complex with Smad4 and are then translocated to the nucleus where they act as transcription factors in association with other proteins[23]. Initiation of the signaling pathway can thus be monitored by examining the translocation of Smad proteins into the nucleus. This is accomplished by staining fixed cells with a primary Smad antibody and secondary Fluorescent antibody. Li *et. al.* reported that growing NMuMG cells on surfaces presenting TβR-binding peptides sensitize the cells by preclustering TβR and causing endogenous TGFβ in the culture medium to trigger a signaling event leading to a translocation of the Smad2/3 proteins to the cell nucleus.[20] In our initial experiments in which cells were treated with TGFβ and peptide-bearing nano-assemblies, we found that endogenous levels of TGFβ in NMuMG cell cultures were not sufficient to elicit a detectable response. Titrating TGFβ we also found reduced sensitivity in cells compared to that reported by Li *et. al* with no Smad translocation observed using concentrations below 50 pM of added TGFβ. This prompted us to add a background level of 40 pM TGFβ along with nano-assemblies in order to detect a positive perturbation of the signaling pathway. Figure 2 shows representative micrographs of NMuMG cells cultured for 18 hours with or without nano-assemblies.

In cells exposed to 40 pM TGFβ, no translocation of the Smad proteins to the cell nucleus was observed. This contrasts with cells exposed to 200 pM TGFβ where the Smad2/3 stain predominantly overlaps with a DAPI stain of the nuclear DNA. To test the efficacy of our nano-assemblies, cells were exposed to 300 pM nanostructure and 40 pM background TGFβ. This resulted in Smad2/3 translocation into the nucleus as evidenced by co-localization of Smad2/3 stain and DAPI DNA stain. Controls were also done with streptavidin and peptide premixed before addition to the cell culture as well as samples containing peptide alone. The multivalency of streptavidin could conceivably lead to limited clustering when the tetrameric protein binds up to four biotinylated peptides. However as the micrographs in Figure 2 show, no Smad2/3 translocation is observed when premixed streptavidin and peptide is added to the cell culture. Neither of these control conditions activated Smad translocation.

The above observations can be quantified by measuring average micrograph intensity of the nucleus and comparing it to that of the cytosol. This results in a nuclear/cytosolic ratio below 2 for the control conditions with only 40 pM background TGF-β1, peptide and

streptavidin+peptide. This contrasts with a ratio above 3 for the conditions with TGF-β1 and nano-assembly (see the Supplemental Information for details – Fig. S3).

In order to achieve a statistically significant measure of the biological pathway induction we employed a luciferase assay responsive to Smad mediated TβR stimulation. Cells were transfected and then exposed to either 200 pM TGFβ or 40 pM TGFβ and nano-assemblies. Concluding from Figure 2 that no Smad2/3 translocation takes place when treating cells with peptide or STV+peptide these conditions were included as negative controls. Figure 3 shows relative luminescence values.

Samples treated with nano-assemblies showed significant activation of the Smad pathway compared to the negative control samples treated with peptide alone or peptide and streptavidin pre-incubated. The highest level of pathway activation was seen in the positive control sample exposed to 200 pM TGFβ.

This indicates that our nano-assemblies are sensitizing NMuMG cells to TGFβ. We hypothesize that this occurs because preclustering of the receptors by the nano-assemblies results in decreased entropic cost compared to the normal pathway where receptor clustering occurs after TGFβ binding.

It is conceivable that the efficacy of the nano-assembly could be increased by replacing the non-covalent biotin-streptavidin linkage with a direct covalent coupling between the peptides and the DNA origami. This would also allow for tighter control of peptide position and multiplicity than provided by the tetravalent streptavidin. Additionally, a covalent attachment would make it easier to eliminate free peptide in the assembly samples which might further improve pre-clustering of receptors. TGFβ's role in the extracellular matrix has led to it being used frequently in tissue engineering along with other members of the TGFβ superfamily, such as bone morphogenic protein (BMP). The most common method for delivering these growth factors to the site of action has been by way of a carrier such as a collagen sponge, as in the FDA approved INFUSE® Bone Graft.[24] The goal of the carrier is to deliver a localized dose of cytokine and a scaffold for cellular attachment. Limitations include diffusion of cytokine out of the carriers at undesired sites or with undesired timing, as well as cytokine denaturation within the carrier. With our current demonstration and DNA's proven efficacy as an extracellular material we believe DNA nanostructures might find greater use in tissue engineering by allowing for highly localized control of cytokine activation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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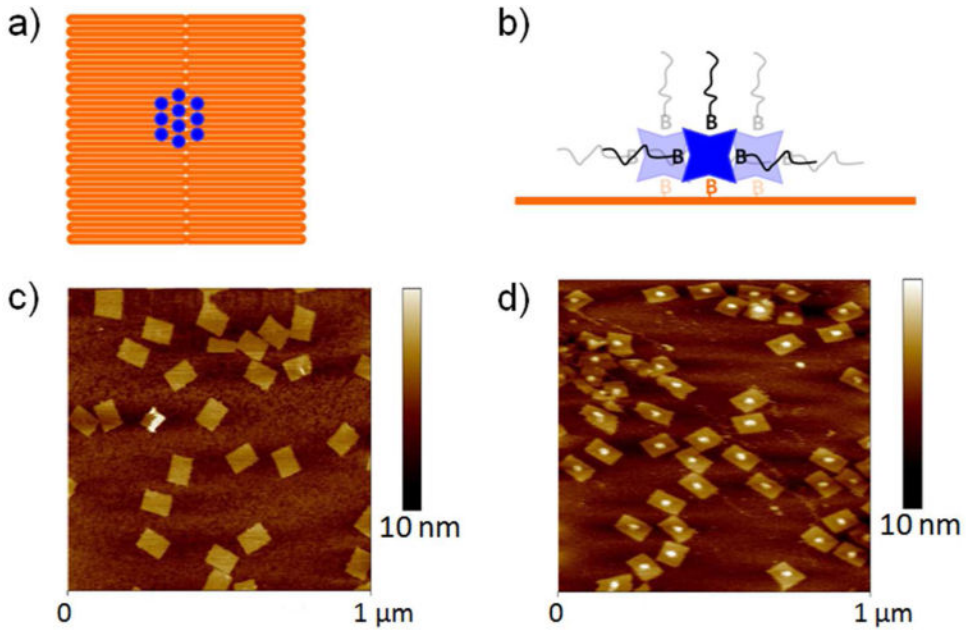

Figure 1.

a) Schematic of the DNA origami with biotin/streptavidin sites marked in blue. b) side schematic of the nano-assembly. c) Origami structures before assembly with streptavidin and peptide. d) Nano-assembly after spin purification. Pep-streptavidin complex is visible in the center of the origami structure.

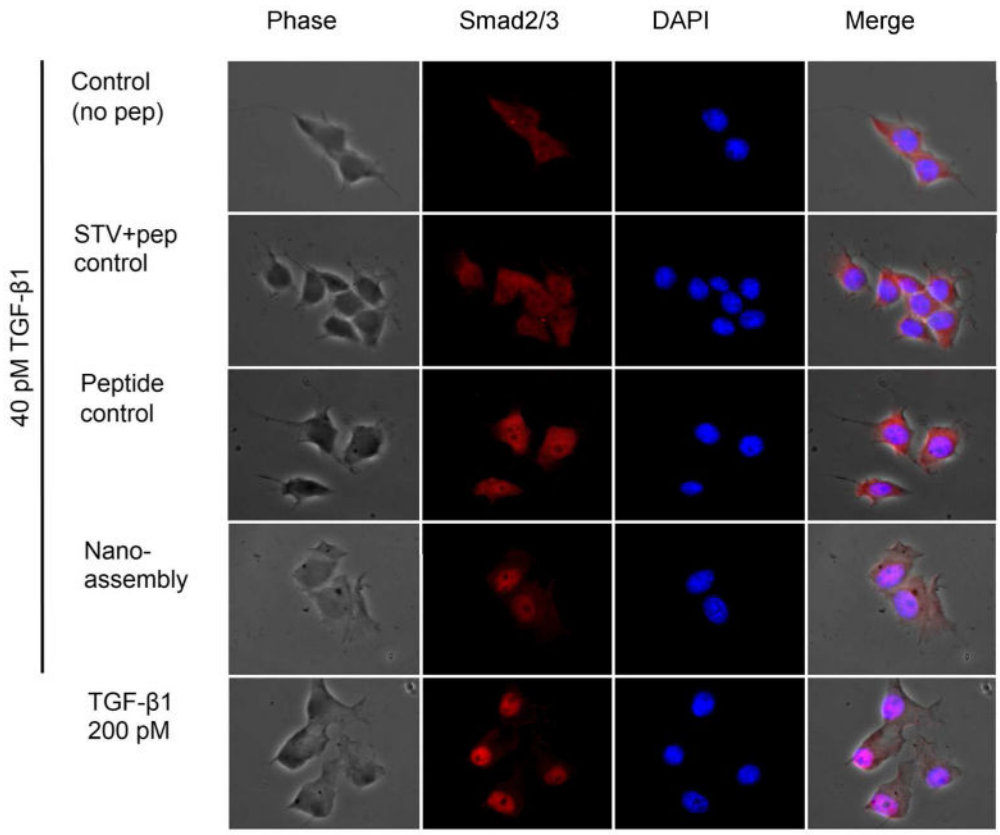

Figure 2.

Representative micrographs of NmuMG cells cultured for 18 hours and then fixed and stained with DAPI and antibody against Smad2/3 (red stain). Top row is a control culture with a background level of 40 pM TGFβ added. As can be seen the red stain is diffuse throughout the cell body with no apparent localization in the cell nucleus. Similar results are seen in the second and third rows showing controls with added peptide at 9 nM and and premixed peptide and streptavidin in a 4 to 1 ratio with streptavidin at 3 nM. The fourth row from the top shows cells treated with nano-assemblies. As can be seen in the Smad2/3 column this treatment results in translocation of Smad2/3 into the nucleus. This mimics the effect observed when cells are treated with a high dose (200 pM) of TGFβ, as in the fifth row.

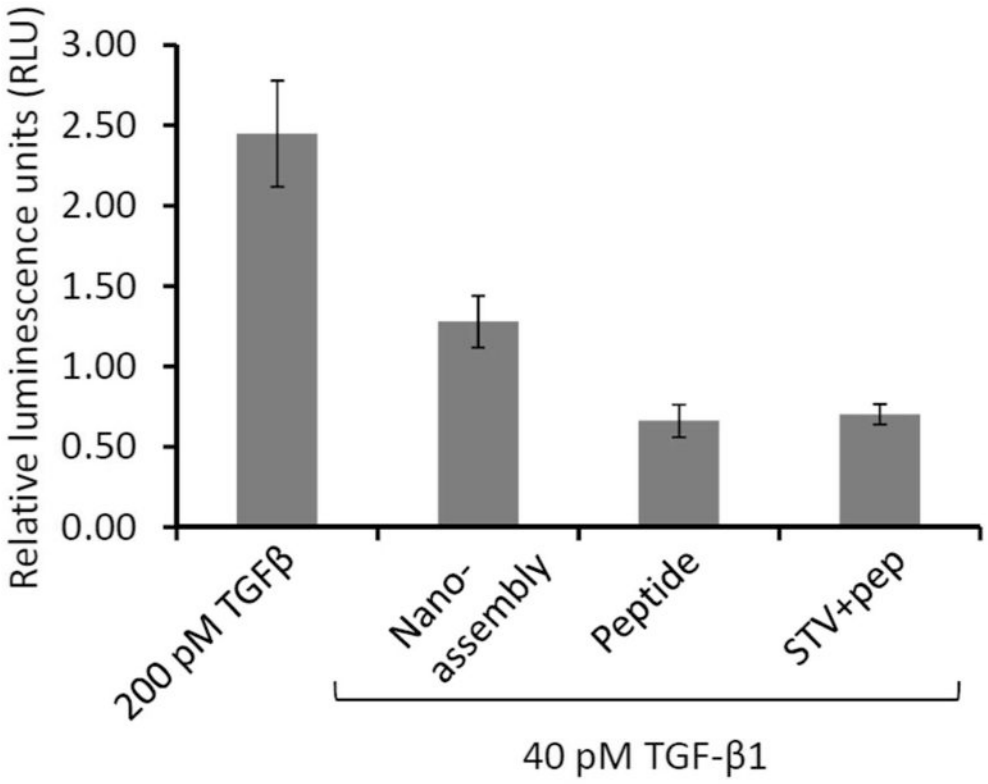

Figure 3.

Luminescence from cell samples treated with 200 pM TGFβ, Nano-assembly and 40 pM TGFβ, peptide and 40 pM TGFβ and pep-streptavidin complex and 40 pM TGFβ. Error bars represent standard deviation.