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Glycocalyx Remodeling with Proteoglycan Mimetics Promotes Neural Specification in Embryonic Stem Cells

Mia L. Huang,[†] Raymond A. A. Smith,[†] Greg W. Trieger, and Kamil Godula*

Department of Chemistry and Biochemistry, University of California, San Diego, California 92093-0358, United States

Supporting Information

ABSTRACT: Growth factor (GF) signaling is a key determinant of stem cell fate. Interactions of GFs with their receptors are often mediated by heparan sulfate proteoglycans (HSPGs). Here, we report a cell surface engineering strategy that exploits the function of HSPGs to promote differentiation in embryonic stem cells (ESCs). We have generated synthetic neoproteoglycans (neoPGs) with affinity for the fibroblast growth factor 2 (FGF2) and introduced them into plasma membranes of ESCs deficient in HS biosynthesis. There, the neoPGs assumed the function of native HSPGs, rescued FGF2-mediated kinase activity, and promoted neural specification. This glycocalyx remodeling strategy is versatile and may be applicable to other types of differentiation.

Embryogenesis requires a delicate balance of external biochemical cues that instruct the formation of organismal complexity. Among these are growth factors (GFs), which activate key signaling pathways involved in gene regulation. The glycocalyx of stem cells, a complex ensemble of membrane-associated glycoproteins and glycolipids, is an important intermediary in GF signaling.[1] For instance, proteoglycans (PGs), through their polysaccharide appendages called glycosaminoglycans (GAGs), recruit members of many GF families to the cell surface and present them to their receptors.[2] Harnessing glycocalyx interactions to regulate GF signaling and define the outcome of stem cell differentiation may open new opportunities for generating medically useful cell lineages and advancing cell-based therapies.

In mouse embryonic stem cells (mESCs), PGs with heparan sulfate (HS) GAGs composed of alternating units of variously sulfated glucosamine and uronic acid orchestrate the formation of complexes between fibroblast growth factors (FGFs) and their receptors, FGFRs (Figure 1A).[3] Subsequent phosphorylation of the Extracellular Signal-regulated Kinases 1 and 2 (Erk1/2) and downstream signaling events that ensue result in differentiation of mESCs into neural precursor cells (NPCs).[4] In mESCs lacking exostosin 1 (Ext1), an enzyme responsible for the biosynthesis of HS, FGFs fail to form functional complexes with FGFRs leading to cell arrest in an embryonic state (Figure 1B).[4c,5] The sulfation patterns of GAGs are believed to be responsible for PG activity[6] and neural differentiation of mESCs is accompanied by changes in HS sulfation.[4c] Taken together, these observations suggest a regulatory function for PGs, which may be key to determining stem cell fate.

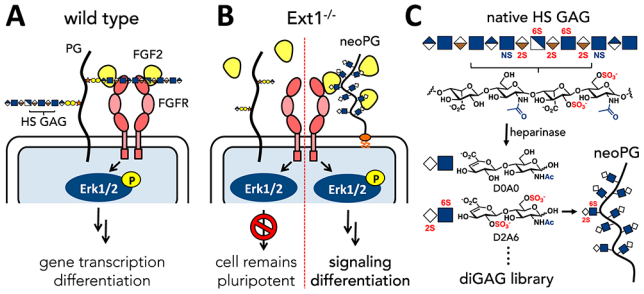

Figure 1. Glycocalyx remodeling strategy for influencing stem cell specification. HSPGs are required for FGF signaling during development (A). Synthetic neoproteoglycans (neoPGs) are introduced to surfaces of ESCs deficient in HS biosynthesis to rescue FGF signaling and enable differentiation (B). Glycan building blocks derived from native HS serve as growth factor recognition elements for neoPGs (C).

Herein, we report a cell-surface engineering strategy that targets GAG-mediated growth factor signaling to influence stem cell specification.

Glycocalyx remodeling has emerged as a powerful strategy for introducing specific glycan epitopes to the cell surface, where they can mediate a range of biological processes. This can be achieved through manipulation of metabolic pathways responsible for glycan biosynthesis,[7] by covalent grafting of glycans to surface proteins,[8] or through passive insertion of lipid-functionalized glycoconjugates into the cell membrane.[9] The latter approach is particularly appealing, as it has minimal impact on existing membrane structures. For instance, elegant studies by Bertozzi and co-workers using synthetic lipid-functionalized glycopolymers have revealed the roles of various glycocalyx components in receptor oligomerization and immunomodulation.[10] Most recently, lipid-terminated chondroitin sulfate GAGs from natural sources were introduced onto rat cortical neurons, where they enhanced nerve growth factor-mediated signaling and promoted neural outgrowth.[11] Unfortunately, native HS is highly structurally heterogeneous and not amenable to the targeting of specific growth factor interactions.

The synthesis of uniform HS polysaccharides poses a significant challenge, whereas shorter HS oligomers that are more synthetically manageable[12] typically exhibit limited biological activity. This shortcoming can be remedied by taking advantage of multivalency effects.[13] Seeberger and co-workers first demonstrated that dendrimers functionalized with

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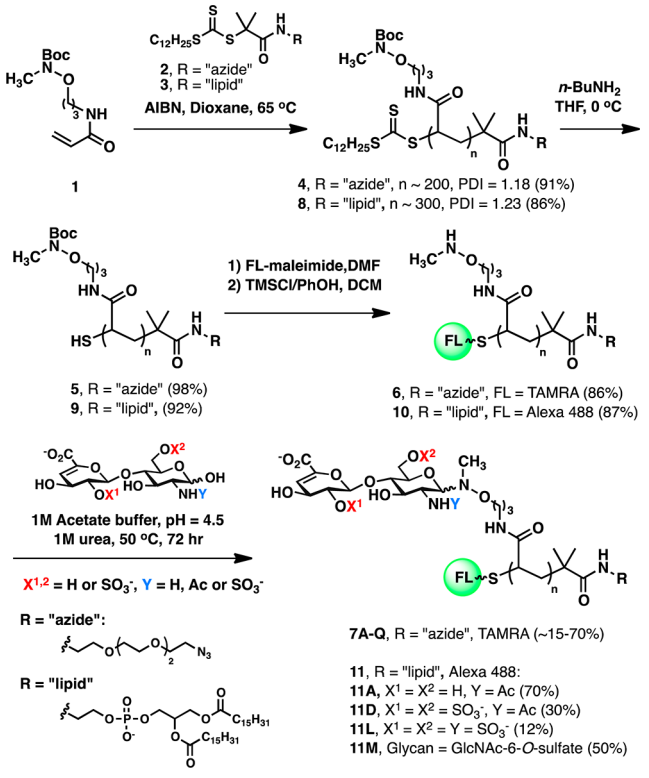
synthetic HS hexasaccharides were able to potentiate Erk1/2 signaling.[14] A major breakthrough followed when Hsieh-Wilson and her co-workers showed that soluble linear polymers decorated with synthetic GAG disaccharides were able to inhibit neural outgrowth and alter chemokine activity.[15]

Inspired by this minimalistic approach, we designed a strategy for generating mimetics of HSPGs—neoproteoglycans or neoPGs—that completely obviates the need for GAG synthesis (Figure 1C). For the recognition element in our glycopolymers, we chose disaccharides (diGAGs) generated by depolymerization of HS by bacterial heparinases and available in pure form from commercial sources. The diGAG structures were incorporated into a poly(acrylamide) scaffold decorated with pendant *N*-methylaminooxy groups, which are reactive toward the hemiacetal functionality of the reducing glycans.[16] We developed a microarray platform to identify neoPG candidates with specificity toward FGF2 that can be introduced into the glycocalyx of Ext1$^{-/-}$ mESCs to promote their differentiation into NPCs.

First, we prepared a key polymer intermediate **6** primed for diGAG conjugation and terminated with an azido group for covalent immobilization on cyclooctyne-coated glass via the strain-promoted azide−alkyne cycloaddition (Scheme 1).[17]

Scheme 1. Synthesis of Neoproteoglycans



RAFT polymerization[18] of Boc-protected *N*-methlyamino-oxypropylacrylamide monomer (**1**) in the presence of an azide-terminated chain transfer agent **2** and a radical initiator produced poly(acrylamide) **4** with good control over molecular weight (DP ≈ 200) and with narrow chain length distribution (PDI = 1.18). The trithiocarbonate end group in **4** was removed by treatment with *n*-butylamine in THF, and the liberated sulfhydryl group was conjugated to a tetramethyl-rhodamine (TAMRA) maleimide to provide a fluorescent label for quantification. The side chains in **4** were deprotected using trimethylsilyl chloride and phenol to give the desired intermediate **6**.

Conjugation of diGAGs as well as several glucosamine derivatives to polymer **6** proceeded smoothly under acidic conditions (1 M acetate buffer, pH = 4.5) at 50 °C to afford a library of neoPG structures **7A**−**Q** (Figure 2). Using ^{1}H NMR

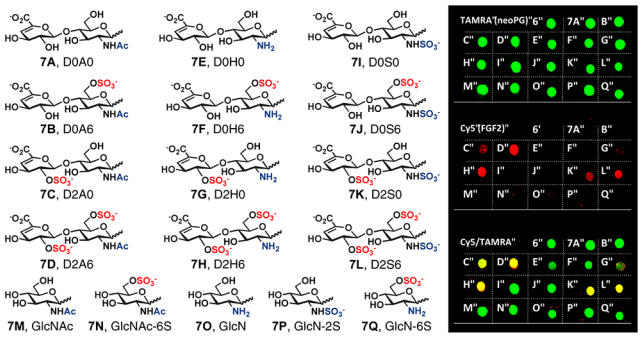


Figure 2. Microarray screen of a library of TAMRA-labeled neoPGs representing most naturally occurring HS sulfation motifs identified neoPGs with specificity for FGF2.

analysis, we established the extent of incorporation for the individual glycans (Table S1). Not surprisingly, we observed a decrease in glycan incorporation with increasing negative charge. Typical ligation efficiencies ranged from ~50%−70% for nonsulfated glycans to ~15% for the trisulfated diGAG, D2S6[19] (Table S1). Nonetheless, we anticipated that even the lowest ligation efficiency should provide sufficient valency (~30 diGAGs) to support FGF2 binding.

Azido-terminated neoPGs **7A**−**Q** were microarrayed on cyclooctyne-functionalized glass and evaluated for binding of FGF2. Only a subset of our neoPG structures effectively engaged FGF2 (Figure 2). We observed that 2-*O*-sulfation on the uronic acid residue was required for FGF2 binding with additional affinity derived from 6-*O*-sulfation of the glucosamine unit, consistent with known FGF2 specificities for GAG motifs.[6] Neither neoPG **7A** carrying the nonsulfated diGAG (D0A0) nor neoPG **7N** decorated with *N*-acetylglucosamine-6-*O*-sulfate (GlcNAc-6S) showed any appreciable binding of the growth factor, indicating a requirement for both the disaccharide motif and a specific sulfation pattern for recognition (Figure 2). FGF2 binding across the entire library was abolished in the presence of soluble heparin (1 μg/mL), further confirming that FGF2 binding to neoPGs was glycan-specific (Figure S39). Normalizing the fluorescence intensities of Alexa Fluor 647 used for the detection of FGF2 in the microarray to the TAMRA signal associated with the underlying glycopolymers provided a semiquantitative means to rank the neoPGs according to their relative affinity toward FGF2 (Figure S39). The best FGF2 binder that emerged from our screen was neoPG **7D** carrying the 2,6-*O*-disulfated diGAG, D2A6 (Figure 2).

To evaluate whether the ability of neoPGs to bind FGF2 established in our microarray screen can be recapitulated on the surface of mESCs, we synthesized analogs of neoPGs **7A**, **D**, and **N** functionalized with a phospholid tail for membrane insertion and an Alexa Fluor 488 (AF488) tag for imaging (neoPGs **11**, Scheme 1). Incubation of Ext1$^{-/-}$ mESCs in solutions of neoPGs **11** in base media at 37 °C for 1 h led to a successful introduction of the polymers to the cell surface (Figure 3A). The amount of neoPG delivered to the cell surface

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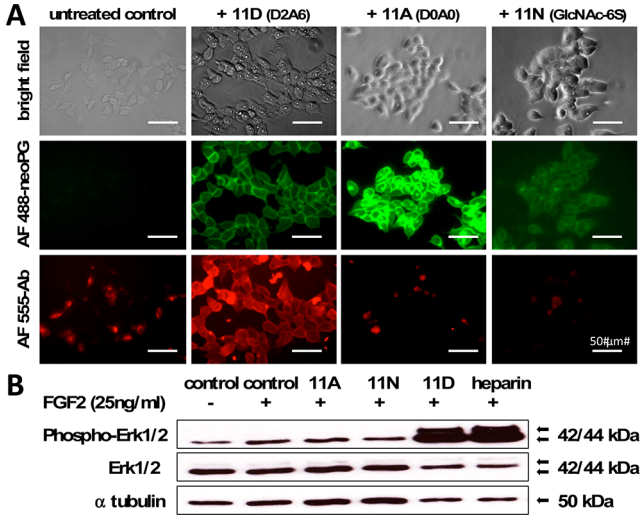

Figure 3. Glycocalyx remodeling rescued FGF2-mediated signaling in Ext1$^{-/-}$ mESCs. NeoPGs **11A**, **D**, and **N** (1 μM) inserted into membranes of Ext1$^{-/-}$ mESCs (*green*) and promoted FGF2 binding according to the structure of their glycans (*red*) (A). FGFs binding neoPG **11D** enhanced Erk1/2 phosphorylation (B).

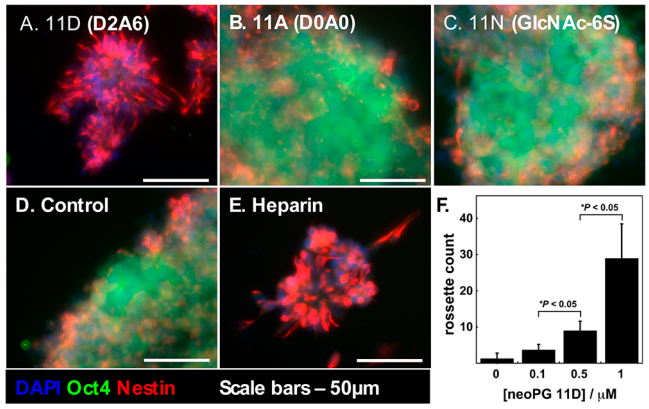

Figure 4. Neural differentiation of neoPG-remodeled Ext1$^{-/-}$ mESCs. NeoPG **11D** with affinity toward FGF2 promoted differentiation into neural rosettes (A), while cells remodeled with neoPGs **11A** and **N** (B and C) or left untreated (D) retained their embryonic characteristics. Soluble heparin also promoted differentiation (E). The ability to produce rosettes improved in cells treated at increasing concentrations of neoPG **11D** (F).

is proportional to the polymer concentration in the media and the incubation time, offering control over the extent of cell-surface remodeling (for optimization of these variables for neoPG **11N**, see Figure S40). The degree of remodeling by the different neoPGs was assessed by fluorescence microscopy (Figure 3A). Interestingly, the nonsulfated neoPG **11A** exhibited higher levels of membrane incorporation relative to **11D** and **11N**, presumably due to its lower overall negative charge. To sustain differentiation, the neoPGs need to remain active on the cell surface for a period of several hours.[4b] To establish the membrane residence time for our polymers, we cultured Ext1$^{-/-}$ mESCs remodeled with neoPG **11D** and monitored its clearance from the cell surface using an anti-AF488 antibody. Satisfyingly, >50% of the neoPG still remained localized to the cell surface after 8 h (Figure S42).

In agreement with our microarray data, we observed enhanced FGF2 binding to Ext1$^{-/-}$ mESCs remodeled with neoPG **11D** carrying the sulfated diGAG, D2A6, while **11A** and **N**, which have undetectable affinity for FGF2, failed to recruit the growth factor to the cell surface (Figure 3A). To determine whether enhanced FGF2 binding also translated into induction of Erk1/2 phosphorylation required to initiate differentiation, we conducted a growth factor stimulation assay. Ext1$^{-/-}$ mESCs remodeled with neoPGs **11** were stimulated with exogenous FGF2 for 15 min. Changes in phosphorylation were assessed by Western blot analysis of protein isolated from cell lysates. As expected, induction of Erk1/2 signaling was observed only for neoPG **11D** (Figure 3B) or in the presence of soluble heparin as reported previously.[5]

To assess whether neoPGs can induce neural specification in Ext1$^{-/-}$ mESCs, we performed differentiation in monolayer culture.[20] Concordant with previous work, Ext1$^{-/-}$ mESCs failed to undergo neural specification after 6 days, as evidenced by expression of the pluripotency marker, Oct4.[4c] Gratifyingly, mESCs remodeled with neoPG **11D**, which promotes FGF2 recruitment to the cells' surface and stimulates the associated Erk1/2 signaling, successfully exited from their pluripotent state and formed characteristic nestin-positive neural rosettes with decreased Oct4 expression (Figure 4A).[21] In comparison, neoPGs **11A** and **N**, which do not engage FGF2, had no effect on differentiation and colonies expressing high levels of Oct4 similar to those in untreated Ext1$^{-/-}$ mESCs remained abundant (Figure 4B–D).

A dose-dependent reduction in the number of neural rosettes was observable upon decreasing the surface density of **11D** (Figure 4F), which correlated with increased Oct4 expression as well as attenuated Erk1/2 phosphorylation in our stimulation assay (Figure S49). It should be noted that addition of soluble heparin at 5 μg/mL to the culture medium also rescued neural differentiation in Ext1$^{-/-}$ mESCs (Figure 4E). However, titers of heparin need to be established to prevent sequestering of FGF2 away from the cell surface and introduced continuously over a period of at least 4 days.[4d] This contrasts with the ability of neoPG **11D** to sustain neural specification in Ext1$^{-/-}$ mESCs after only one initial treatment prior to differentiation. These results demonstrate the power of glycocalyx engineering as a strategy to influence cellular responses that ultimately determine the outcome of stem cell differentiation. We anticipate that this technology, powered by the ease of neoPG synthesis and the versatility of the microarray platform, can be rapidly extended to differentiation of other cell types, including therapeutically useful human pluripotent cells.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Glycopolymer synthesis and characterization, microarray fabrication and FGF2-binding assay. Cell membrane incorporation of neoPGs, FGF2-binding to remodeled cells, Erk1/2 stimulation assay and neural differentiation procedures for Ext1$^{-/-}$ and wild type mES cell lines. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

kgodula@ucsd.edu

Author Contributions

†M.L.H. and R.A.A.S. contributed equally.

Notes

The authors declare no competing financial interest.

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