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Embryonic Stem Cell Engineering with a Glycomimetic FGF2/BMP4 Co-Receptor Drives Mesodermal Differentiation in a Three-Dimensional Culture

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Abstract

Cell surface glycans, such as heparan sulfate (HS), are increasingly identified as co-regulators of growth factor signaling in early embryonic development; therefore, chemical tailoring of HS activity within the cellular glycocalyx of stem cells offers an opportunity to control their differentiation. The growth factors FGF2 and BMP4 are involved in mediating the exit of murine embryonic stem cells (mESCs) from their pluripotent state and their differentiation toward mesodermal cell types, respectively. Here, we report a method for remodeling the glycocalyx of mutant *Ext1*^{-/-} mESCs with defective biosynthesis of HS to drive their mesodermal differentiation in an embryoid body culture. Lipid-functionalized synthetic HS-mimetic glycopolymers with affinity for both FGF2 and BMP4 were introduced into the plasma membrane of *Ext1*^{-/-} mESCs, where they acted as functional co-receptors of these growth factors and facilitated signal transduction through associated MAPK and Smad signaling pathways. We demonstrate that these materials can be employed to remodel *Ext1*^{-/-} mESCs within three-dimensional embryoid body structures, providing enhanced association of BMP4 at the cell surface and driving mesodermal differentiation. As a more complete understanding of the function of HS in regulating development continues to emerge, this simple glycocalyx engineering method is poised to enable precise control over growth factor signaling activity and outcomes of differentiation in stem cells.

Graphical Abstract

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Notes

The authors declare no competing financial interest.

ASSOCIATED CONTENT

Supporting Information

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Experimental procedures and methods, spectral and chromatographic characterization of all polymers, biochemical and biological characterization of polymers **P1** and **P2**, and PCR primer sequences (PDF)

Embryonic stem cells (ESCs) isolated from developing embryos at the blastocyst stage are characterized by their pluripotency, an ability to undergo differentiation into all three primary germ layers: ectoderm, mesoderm, and definitive endoderm. Subsequently, these precursor cells can go on to produce the complete repertoire of cell types of the adult body.¹ As such, ESCs have been the focus of intense research to delineate the molecular mechanisms that govern their differentiation and to exploit these processes for therapeutic gain.² Murine ESCs (mESCs), which can be readily interrogated using genetic methods, have served as an important model for mapping the signaling pathways underlying early mammalian development and have provided useful tools for testing new approaches to harness the regenerative potential of stem cells.^{2,3}

The current model for germ layer specification in mESCs identifies members of the fibroblast growth factor (FGF), bone morphogenetic protein (BMP), and Wnt protein families as key regulators of this process (Figure 1).^{1,4,5} Activation of the MAPK pathway by FGF2 is required for differentiation toward nestin⁺ neural precursor cells (NPCs) and β -III-tubulin⁺ neurons.⁶ The FGF signaling pathway, in conjunction with the Wnt and BMP pathways, is also required to induce mesodermal differentiation.^{1,7,8} The emergence of nascent mesoderm is associated with the expression of the T-box transcription factor, Brachyury, which is subsequently downregulated as the cells migrate and differentiate further (Figure 1A). The reemergence of Brachyury expression⁷ together with fetal liver kinase (Flk1) at a later time point marks the formation of the hemangioblast, which subsequently produces endothelial and hematopoietic cells.⁹ In culture, BMP4, via the Smad protein signaling pathway, downregulates FGF and Wnt signaling, suppresses neuroectoderm formation, and drives mESCs toward mesoderm, pointing to the importance of spatial and temporal regulation of BMP4 activity during development.^{10,11}

While the coordinated expression of the FGF, Wnt, and BMP proteins provides one such control mechanism, the extracellular matrix and the cellular glycocalyx serve as coregulators in this process. In particular, heparan sulfate glycosaminoglycans (HS GAGs) have been identified as a class of glycans involved in spatial patterning of growth factors and facilitating signal transduction at the cell surface.^{12–14}

HS GAGs are polysaccharides attached to core polypeptides of proteoglycans containing sulfated regions that harbor binding sites for various growth factors and their receptors (Figure 1B).¹⁵ The biosynthesis of HS is accomplished by the action of a heterodimeric complex of exostosin 1 and 2 (Ext1/2) glycotransferases, which promotes the addition of alternating *N*-acetylglucosamine (GlcNAc) and glucuronic acid (GlcA) residues to the growing polysaccharide chain. The GAG polymer is acted upon by a complex of *N*-

deacetylase and *N*-sulfotransferase (NDST) enzymes that initiate the formation of the sulfated protein binding regions. Further elaboration by epimerases (Hepi) converting GlcA residues into iduronic acids (IdoA) and 2-, 3-, and 6-*O*-sulfotransferases (Hs2st, Hs3st, and Hs6st, respectively) then fine-tunes the composition of the sulfated domains, thus providing a structural basis for selectivity in protein recognition.^{12,16}

Genetic manipulation of HS biosynthesis has illuminated the essential contributions of these biomolecules in embryonic development. Embryos produced from mice lacking the Ext1/2 glycotransferases fail to undergo gastrulation.^{17,18} This observation is mirrored *in vitro*, where Ext1^{-/-} mESCs are restricted in their ability to exit the pluripotent state and differentiate into nestin⁺ NPCs, indicating the requirement for HS structures for proper FGF signaling.^{19–21} BMP signaling and mesoderm differentiation are likewise impaired in these cells, showing delayed or absent expression of differentiation markers.^{22–24} In an embryoid body (EB) culture, under conditions that recapitulate the mesoderm differentiation process,²⁵ Ext1^{-/-} mESCs showed elevated Brachyury expression levels but did not express Flk1 and failed to form the hemangioblast.²³ Bypassing the requirement for HS in FGF signaling and lineage commitment using high concentrations of FGF2 partially restored differentiation of Ext1^{-/-} mESCs into endoderm and nestin⁺ NPCs; however, this treatment failed to produce mesodermal cell types.²² The mesodermal differentiation defect in Ext1^{-/-} mESCs was attributed to the disruption in FGF and BMP, but not Wnt, signaling due to the absence of functional HS.

Soluble HS and heparin can restore differentiation in Ext1^{-/-} mESCs. Experiments employing chemically and enzymatically altered heparinoids have provided insights into the structural requirements for HS in differentiation.^{20,23,26,27} For instance, high levels of sulfation (NS, 2S, 6S) generally support the activity of both FGF2 and BMP4; however, the latter was shown to require extended heparin oligosaccharide sequences (dp of 12–20),²³ while relatively short HS chains (dp of ~6) were sufficient to promote FGF2 signaling.²⁸ In addition, an increased stability of BMP4 was observed when soluble heparin was present during differentiation, suggesting a possible dual role for HS GAGs in co-reception of the signal at the cell surface and in protection against proteolytic degradation.²²

Engineering of the stem cell glycocalyx with synthetic HS co-receptors has emerged as a strategy to control this process,^{29–31} providing several advantages over the use of soluble heparin and heparinoid structures, which lack structurally well-defined growth factor binding sites and have to be introduced into a culture continuously throughout differentiation. In addition, exogenous heparinoids need to be applied over a narrow concentration range, above which they can inhibit signaling activity via sequestration of growth factors away from the cell surface.^{19,23,32}

In a recent example, chemically prefunctionalized heparinoids were covalently attached to proteins in the membranes of wild type mESCs via a genetically engineered halo tag.²⁹ This approach, in conjunction with the removal of endogenous HS structures through heparinase treatment, provided a long-term remodeling of the glycocalyx and enhanced differentiation of mESCs toward NPCs and β -III-tubulin⁺ neurons. We have shown that HS glycomimetic co-receptors with affinity for FGF2, and carrying phospholipid anchoring

units, could be introduced transiently ($t_{1/2} \sim 9$ h) into the glycocalyx of Ext1^{-/-} mESCs, where they reconstituted signaling through MAPK activation via the FGF2/FGF receptor (FGFR) complex and restored differentiation toward neuroectoderm and nestin⁺ NPCs.³⁰

In this study, we report that glycomimetic co-receptors with dual FGF2 and BMP4 activity allow for the exit of Ext1^{-/-} mESCs from their pluripotent state toward Brachyury⁺/Flk1⁺ mesoderm in an EB culture, via the Smad protein signaling cascade. This study provides new evidence of the utility of glycocalyx engineering in manipulating the outcomes of the stem cell differentiation process.

RESULTS

Considering the role of HS in promoting FGF and BMP signaling during mesodermal differentiation, we reasoned that a functional synthetic co-receptor for these proteins should include HS fragments capable of binding to both FGF2 and BMP4. The structural requirements for HS function in FGF2 binding and signaling have now been reasonably established,^{33,34} with relatively short (dp of ~ 6) fragments with high levels of *N*-sulfation and 2-*O*-sulfation having been identified as being necessary for FGF2 binding with an additional 6-*O*-sulfate required for signaling. The structural requirements for HS in BMP4 signal transduction are still rather poorly defined.^{35,36} While no HS binding site has been identified within the heterodimeric BMP receptor I and II (BMPRI/II) complex, the BMP4 protein is retained on a heparin affinity column indicating the presence of an HS recognition domain.^{37–39} The HS binding domain is thought to reside in the disordered N-terminal region of BMP4,⁴⁰ complicating the crystallographic analysis of HS–BMP4 interactions. However, unlike the case for FGF2, evidence points to the requirement for longer (dp of >12) fragments of HS in BMP4 signaling.²³

We set out to delineate the fine HS structure responsible for high-avidity binding of BMP4; therefore, we analyzed the interactions of this protein toward an array of chemoenzymatically derived HS oligosaccharides [HS 1–24 (Figure 2)]. The array contained oligosaccharides ranging from tetrasaccharides to nonasaccharides with well-defined sulfation patterns. While we observed minimal binding of BMP4 to oligosaccharides devoid of sulfation (HS 1–6) or carrying only *N*-sulfated motifs (HS 7–12), the introduction of additional 2-*O*- and 6-*O*-sulfation (HS 13–16 and HS 17–19, respectively) resulted in enhanced binding of BMP4 to the HS structures. Octasaccharides containing a combination of *N*-, 2-*O*-, and 6-*O*-sulfation motifs (HS 20–22) exhibited the strongest association with BMP4 according to their increasing level of sulfation. Interestingly, inclusion of a 3-*O*-sulfation modification (HS 24) led to weakened BMP4 binding despite the octasaccharide's higher negative charge density.

With a clearer picture of the structural requirements for binding of HS to BMP4, we set out to generate a synthetic HSmimetic glycopolymer co-receptor with dual FGF2 and BMP4 activity capable of supporting mesodermal differentiation in Ext1^{-/-} mESCs. Previously, we have produced a library of glycopolymers using an HS fragment-based strategy, where differentially sulfated HS disaccharides derived by heparinase depolymerization of HS polysaccharides were conjugated to polyacrylamide scaffolds.³⁰ These HS fragments were

conjugated to polymer side chains containing *N*-methyaminooxy functionality reactive toward the disaccharides' reducing end. While GAG disaccharides alone do not show a high affinity for growth factors, their multivalent presentation achieved by glycopolymers can reconstitute the activity of their parent GAGs.^{41,42} From our initial library, two polymer structures containing disaccharides sulfated in both 2-*O*- and 6-*O*-positions with additional *N*-acetamido or *N*-sulfo modifications emerged (D2A6 and D2S6,⁴³ respectively) that exhibited high affinity for FGF2.³⁰ Both polymers also showed the ability to enhance MAPK signaling via the FGF2/FGFR complex and initiate neuroectodermal differentiation when introduced into the glycocalyx of Ext1^{-/-} mESCs using passive membrane insertion via a phospholipid anchor. While both D2A6 and D2S6 disaccharides exhibited overlapping FGF2 binding profiles, the former afforded higher-valency conjugates with improved biological activities.

On the basis of our array binding data indicating the requirement of 2-*O*- and 6-*O*-sulfation for high-affinity binding to BMP4, we assessed the ability of the D2A6-modified polymer to engage this protein. Using RAFT polymerization, we have synthesized a biotinylated D2A6 glycopolymer, **P1-D2A6** (Figure 3A; DP = 200; <MI>D = 1.19; valency = 155). In addition, we prepared a control polymer containing the 6-*O*-sulfated monosaccharide *N*-acetylglucosamine, **P1-GlcNAc6S** (Figure 3A; DP = 200; <MI> = 1.19; valency = 177), which was not expected to engage BMP4 despite its highly anionic character. We tested both polymers for their binding to BMP4 using a capture enzyme-linked immunosorbent assay (ELISA). In this assay, BMP4 was immobilized via its antibody to the plate surface and the binding of soluble biotinylated polymers **P1** was detected using horseradish peroxidase (HRP) conjugated to streptavidin and the chromogenic reagent 3,3',5,5'-tetramethylbenzidine (TMB). As anticipated, the **P1-D2A6**, but not **P1-GlcNAc6S**, polymer showed strong association with the immobilized BMP4 protein (EC₅₀ = 14 nM), which was comparable to that of a biotinylated heparin control (EC₅₀ = 13 nM) (Figure 3B). We used a similar assay to also confirm the binding of the polymers to FGF2 (Figure S15), with both **P1-D2A6** and heparin showing similar binding affinities (EC₅₀ values of 7 and 6 nM, respectively). These data demonstrate that the synthetic glycopolymer **P1-D2A6** successfully recapitulates the binding interactions between the natural HS polysaccharide and both BMP4 and FGF2 proteins in a glycan- and sulfation-dependent manner, while **P1-GlcNAc6S** does not engage either protein to an appreciable degree.

We have previously demonstrated the ability of the D2A6carrying glycopolymers to act as functional co-receptors for FGF2 in the activation of the MAPK pathway in Ext1^{-/-} mESCs.³⁰ To test whether the binding of BMP4 to **P1-D2A6** may similarly translate to signaling activity in these cells, we synthesized membrane-targeting polymer analogues **P2-D2A6** and **P2-GlcNAc6S** containing phospholipid anchors (Figure 3A; DP = 130; <MI> = 1.17; valencies of 69 and 64, respectively) and monitored effects on Smad protein phosphorylation downstream of BMP4 receptors I and II after glycocalyx remodeling. Ext1^{-/-} mESCs in a monolayer culture were first remodeled with polymers **P2** by incubation (3 μM) for 1 h at 37 °C. The cells were washed to removed unbound polymers and stimulated with BMP4 (2 ng) for 2 h. Conditions containing untreated cells and cells stimulated in the presence of heparin (1 μg) were included as negative and positive controls, respectively. After

stimulation, cell lysates were collected and changes in levels of Smad1/5 phosphorylation (normalized to the Smad1 total protein concentration) were assessed using an ELISA (Figure 3B). The Ext1^{-/-} mESCs remodeled with polymer **P2-D2A6** or in the presence of soluble heparin (1 μ g) showed a 2-fold enhancement of Smad1/5 phosphorylation compared to cells remodeled with **P2-GlcNAc6S** and in an untreated control (Figure 3C), indicating that polymer **P2-D2A6** can act as a co-receptor for BMP4 at the surface of Ext1^{-/-} mESCs and allow for signal transduction via the Smad signaling pathway. Thus, **P2-D2A6** should be able to support mesodermal formation in an Ext1^{-/-} EB culture by, first, facilitating activation of FGF2 signaling and exit from the pluripotent state and by mediation of the BMP4 response to drive further mesodermal differentiation.

We set out to establish a protocol for glycocalyx remodeling with polymers **P2** in the three-dimensional EB structures⁴⁴ derived from Ext1^{-/-} mESCs to test this hypothesis. During EB formation, the outer layer cells form close contacts and deposit extracellular matrix proteins creating a dense shell, which can pose a challenge for the delivery of materials into the inner core of the structures.^{45,46} To ensure full incorporation of the glycopolymers throughout the EB structures, we optimized the remodeling conditions with respect to polymer concentration and incubation time and used confocal microscopy to monitor the distribution of the fluorescently labeled (AF488) polymers (Figure 4A). We observed a concentration-dependent polymer distribution across the EB structure (Figure S19), with full penetration achieved for both polymers (3 μ M) after 2 h at 37 °C. The outer EB regions showed increased brightness, likely due to both the higher number of compacted cells forming the EB shell and the increase in the level of ECM protein deposition leading to polymer retention and accumulation. To ensure that remodeling occurred through membrane insertion of the lipidated glycopolymers rather than their physical retention within the EB structure, EBs were dissociated with Accutase and the cells were analyzed via flow cytometry for the presence of the fluorescent polymer marker (AF488). A robust and equivalent increase in cell fluorescence was observed for both polymers **P2** (Figure 4C), indicating similar levels of incorporation of the polymers into the EBs regardless of their glycan structure.

Next, we assessed the effects of incorporation of glycopolymers **P2** within the EB structures on BMP4 association. The EBs were fixed and then incubated with BMP4 (100 nM, 18 h, 4 °C), and the localization of the protein was visualized after immunofluorescence staining (Figure 4A,B). Confocal microscopy indicated co-localization of the BMP4 and **P2-D2A6** signals. No enhancement in BMP4 binding was observed for EBs remodeled with polymer **P2-GlcNAc6S** compared to that of polymer-untreated EBs, which is in agreement with the BMP4 binding profiles of polymers **P1** determined by an ELISA (Figure 3B). Thus, glycopolymer **P2-D2A6** can be effectively diffused throughout the three-dimensional EB structure and provide binding sites for BMP4 within the Ext1^{-/-} mESC glycocalyx.

Having established that polymer **P2-D2A6** can act as a functional co-receptor for both FGF2 and BMP4 proteins and facilitate signal transduction through their associated MAPK and Smad signaling pathways, respectively, we next tested its ability to support mesodermal differentiation in Ext1^{-/-} mESCs. Mesodermal differentiation in wild type mESCs in an EB culture is characterized by a rapid loss of pluripotency markers (i.e., SSEA-1, Oct4, or Noggin) and the emergence of mesodermal markers, such as Brachyury, at approximately

day 2–3 of differentiation.⁸ The Brachyury message is then transiently attenuated, until it reappears together with the expression of the kinase, Flk1, and they together mark the emergence of the hemangioblast.^{7,23}

To test whether the remodeling of Ext1^{-/-} EBs with synthetic FGF2/BMP4 co-receptor **P2-D2A6** may drive mesodermal commitment, we remodeled EBs with both polymers **P2** at day 0 of differentiation, after removing leukemia inhibitory factor from the medium and allowing EBs to form for 2 days (days -2 to 0). Conditions including polymer-untreated EBs and EBs differentiated in the presence of soluble heparin (1 μ g), which has been demonstrated to restore HS-dependent signaling in Ext1^{-/-} mESCs,²³ were included as negative and positive controls, respectively.

Differentiation was monitored in glycocalyx-remodeled EBs by collecting lysates over 10 days and assessing the mRNA expression patterns of embryonic, mesodermal, and neuroectodermal markers using reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis (Figure 5). As expected, we observed rapid loss of the expression of embryonic markers Oct4, Sox2, and Noggin under all conditions (Figure 5 and Figure S20) and the emergence of the Brachyury marker by day 3 of differentiation, which was somewhat delayed in EBs treated with glycopolymer **P2-GlcNAc6S** (day 6, Figure 5). Brachyury expression continued unabated (3–5-fold enhancement) in untreated EBs and EBs remodeled with P2-GlcNAc6S, which is characteristic for Ext1^{-/-} mESCs lacking functional cell surface HS structures (Figure 5 and Figure S20). This message was downregulated by days 6 and 8 under conditions including soluble heparin (1 μ g) and in EBs treated with polymer **P2-D2A6**, respectively, to reemerge again by day 10 of differentiation under both conditions concomitant with the expression of Flk1. Successful progress toward the formation of mesoderm under these conditions was corroborated by the progressive transcriptional upregulation of the inhibitor of differentiation 1 (Id1) factor, which is activated through BMP signaling and blocks neural differentiation.¹¹ In untreated EBs and EBs remodeled with **P2-GlcNAc6S**, expression of Flk1 and Id1 was absent throughout the course of differentiation, indicating their failure to successfully advance toward mesoderm. Closer examination of levels of transcripts for the neural markers Sox1, nestin, and FGF5 indicated some neural commitment^{22,23,47} in EBs remodeled with polymers **P2-D2A6** and under conditions supplemented with heparin compared to EBs treated with **P2-GlcNAc6S** or in untreated control EBs. While the employed differentiation conditions are optimized to promote mesodermal differentiation in EBs, the formation of cell types associated with another germ layer is typically observed.⁴⁸

The gene expression changes in EBs observed by RT-qPCR analysis were confirmed by the detection of cell surface markers of mesodermal formation (Flk1) and pluripotency (SSEA1) at day 10 of differentiation. EBs were dissociated with Accutase, and the cells were immunostained with AlexaFluor 647 (AF647)-conjugated primary antibodies against either Flk1 or the embryonic marker, SSEA1, and analyzed using flow cytometry (Figure 6). In agreement with embryonic marker expression profiles detected by RT-qPCR analysis, we observed a significant decrease in the level of SSEA1 under all conditions compared to that of cells maintained in an embryonic culture (Figure 6, left), while only EBs differentiated in

the presence of **P2-D2A6** or heparin produced Flk1⁺ cell populations [27 or 34%, respectively (Figure 6, right)].

DISCUSSION

Cell surface and extracellular matrix glycans, such as HS GAGs, have been increasingly identified as co-regulators of growth factor signaling during early development.^{14,36,49}

While current *in vitro* approaches for recapitulating the developmental process to derive specialized cells for therapeutic applications from isolated or induced pluripotent cells focus primarily on mapping the combinations of factors and their associated signaling pathways needed to guide the differentiation process, the contributions of glycans often remain unexplored and underutilized.^{50,51}

The ubiquitous and tightly regulated expression of cell surface HS GAGs accompanies embryonic development and is characterized by alterations in the fine structures of their sulfated regions,^{13,19} which serve as recognition elements for a large number of signaling proteins and their receptors. As such, these glycans offer a unique target for manipulating the cell differentiation process. While in limited instances the HS GAG structure–activity relationships have been delineated (e.g., in the organization of the FGF2/FGFR signaling complex),³³ in many cases molecular level details of interactions of HS with proteins, including BMP4, are still lacking. Despite the yet incomplete understanding of selectivity in HS–protein interactions, chemical methods for manipulating the stem cell glycocalyx to harness HS functions and gain control over cellular differentiation have begun to emerge.^{29–31} Early examples include the introduction of heparinoids or HS-mimetic glycopolymers onto the cell surface of enzymatically deglycosylated wild type mESCs or Ext1^{−/−} mESC mutants with defects in HS biosynthesis, respectively, to promote FGF2 signaling and neural differentiation.

In the study presented here, we sought to expand this strategy to produce mesodermal cell lineages, which requires inputs from both FGF2 and BMP4 signaling pathways. First, we delineated the composition of sulfated regions in HS needed for BMP4 binding using an oligosaccharide microarray binding assay and then developed synthetic HS-mimetic coreceptors capable of promoting both BMP4 and FGF2 activity in the glycocalyx of Ext1^{−/−} mESCs.

The microarray screen revealed a general positive correlation between the increasing overall negative charge of the HS fragments and the level of BMP4 association, with the strongest binding observed for octasaccharides containing a combination of N-, 2-*O*-, and 6-*O*-sulfation (Figure 2). Notably, BMP4 binding was attenuated when the negative charge density was increased further by the introduction of 3*O*-sulfate groups. This observation suggests that while BMP4 is known to bind to heparin, which is a highly sulfated variant of HS, it may not interact with its most negatively charged regions containing the 3-*O*-sulfate modification, responsible for heparin's anticoagulant activity.

The enhanced association of BMP4 with HS oligosaccharides containing both 2-*O*- and 6-*O*-sulfation indicates overlapping binding preferences with FGF2, which requires both

modifications for signaling.³⁰ While differences in selectivity between FGF2 and BMP4 with respect to the sulfation patterns they recognize are not clear, prior studies identified a requirement for longer oligosaccharides (dp of >12) to allow for signaling through BMP4²³ compared to the relatively short (dp of ~6) fragments that are sufficient for FGF2 activity.²⁸

On the basis of the similar binding preferences of FGF2 and BMP4, we identified a glycopolymer structure with the HS disaccharide motif, D2A6, containing both 2-*O*- and 6-*O*-sulfation (Figure 3A), which could act as a common coreceptor for both proteins. We have previously identified the ability of this polymer to bind to FGF2 and facilitate signal transduction via the FGF receptor.³⁰ We have now demonstrated that this polymer can also engage BMP4 with an avidity similar to that of heparin (Figure 3B) and, when embedded within the cellular glycocalyx of Ext1^{-/-} ECS via a lipid anchor, allowed for the activation of the Smad signaling pathway after BMP4 stimulation (Figure 3C). The D2A6 disaccharide motif was required for the biological activity of the glycopolymer, because an analogous polymer containing the 6-*O*-sulfated monosaccharide, GlcNAc6S, showed no significant binding to either FGF2 or BMP4 despite its highly polyanionic character.

The binding activity of the D2A6 glycopolymers toward FGF2 and BMP4 is notable and likely stems from the architecture of the synthetic mimetic materials, which is distinct from that of HS structures in several key respects. Mainly, *N*-deacetylation and *N*-sulfation of glucosamine residues are required for priming of nascent HS chains for further elaboration of growth factor binding domains by HS epimerases and *O*-sulfotransferases,¹⁵ and numerous studies have pointed to the requirement of *N*-sulfation in HS for FGF2 binding and signaling.^{33,34} Therefore, the D2A6 disaccharide is present only in minute quantities in heparinase digests of isolated HS polysaccharides and may not contribute to growth factor binding in native HS but provide sufficient affinity when presented in a multivalent fashion on polymer scaffolds.

Despite their macromolecular architecture (Mw ~ 50 kDa), the polymers diffused freely into EB structures formed from Ext1^{-/-} mESCs and anchored via their lipid modifications to the outer leaflet of the cellular plasma membranes. Levels of polymer incorporation into the inner core of the EB structure were influenced by the concentration of the materials during remodeling. This behavior potentially affords the opportunity to pattern EBs and spatially control growth factor activity. Once embedded within the cellular glycocalyx, the polymers promoted association of BMP4 with the cell surface based on the structure of their glycan appendages and in agreement with their binding profiles established by an *in vitro* ELISA (Figure 4). After induction of differentiation, BMP4 binding polymer P2-D2A6 was able to support the exit of Ext1^{-/-} mESCs in an EB culture toward mesoderm and, eventually, the formation of Brachyury⁺/Flk1⁺ mesodermal populations (Figures 5 and 6). Untreated EBs and EBs treated with control polymer P2GlcNAc6S were able to exit from their pluripotent state toward premesoderm (Brachyury⁺) but failed to transiently attenuate Brachyury expression and successfully establish Flk1⁺ mesodermal cell types. The fact that the onset of Brachyury and Flk1 expression was somewhat delayed in Ext1^{-/-} mESCs remodeled with **P2-D2A6** compared to those differentiated in the presence of heparin is worth noting. However, we observed an overall high abundance of the Flk1 message and lower levels of expression of the neural markers, Sox1, nestin, and FGF5, at day 10 of

differentiation under both conditions. The observed differences in timing and outcomes of differentiation between glycocalyx-engineered cells and cells cultured in the presence of soluble heparin may stem from the larger structural and functional heterogeneity of heparin compared to that of **P2-D2A6** as well as the timing of their activities. While the presence of soluble heparin is required throughout the entire course of differentiation, **P2-D2A6** is introduced into the cellular glycocalyx only once at the induction of differentiation and has a cell surface residence half-life of ~9 h.³⁰

In conclusion, we have demonstrated that synthetic HS-mimetic co-receptors for FGF2 and BMP4 can act as functional surrogates for native HS GAG structures at the surfaces of mESCs and drive their mesodermal differentiation. The ease of chemical cell surface glycocalyx engineering with glycomaterials, together with the new information regarding HS specificity emerging from modern glycomics platforms, may enable, in the future, selective activation of signaling pathways in response to endogenous biochemical cues to direct stem cell differentiation.

METHODS

Detailed experimental methods and additional supporting data are provided in the Supporting Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

FGF2 and BMP4 growth factor signaling mediates mesodermal differentiation in mESCs.

(A) BMP4 attenuates FGF2 signaling after initial exit of mESCs from the pluripotent state and drives differentiation toward early Brachyury⁺ mesodermal precursors. Further mesodermal differentiation produces hemangio-blast co-expressing both Brachyury and Flk1 markers. (B) Cell surface HS polysaccharides are required as co-receptors for both FGF2 and BMP4 to activate signal transduction. The enzymatic assembly of sulfated regions within HS provides a structural basis for selectivity in growth factor interactions.

Figure 2.

Analysis of interactions of BMP4 with chemically defined HS oligosaccharides. BMP4 exhibits a preference for binding to HS oligosaccharides with high-charge density regions containing combinations of *N*-, 2-*O*-, and 6-*O*-sulfation modifications. Addition of 3-*O*-sulfate attenuates BMP4 binding.

Figure 3.

Synthetic HS-mimetic glycopolymers act as functional coreceptors for BMP4. (A) Glycopolymers with low dispersity indices ($D<MI> < 1.20$) containing biotin (**P1**; DP = 200) or phospholipid (**P2**; DP = 130) modifications and bearing 2-*O*- and 6-*O*-sulfated HS disaccharides (D2A6) or *N*-acetylglucosamine 6-*O*-sulfate monosaccharides (GlcNAc6S) were generated via the RAFT technique. (B) In an enzyme-linked immunosorbent assay, HS-mimetic glycopolymer **P1-D2A6** exhibited binding to surface-immobilized BMP4 comparable to that of heparin (EC_{50} values of 14 and 13 nM, respectively). Glycopolymer **P1-GlcNAc6S** showed a low affinity for BMP4. (C) Remodeling of *Ext1*^{-/-} mESCs with lipid-conjugated glycopolymer **P2-D2A6**, but not **P2-GlcNAc6S**, enhances phosphorylation of Smad1/5 proteins in response to stimulation with BMP4. Reactivation of the Smad signaling pathway is also observed in the presence of soluble heparin (1 μ g).

Figure 4.

Glycocalyx engineering of *Ext1*^{-/-} mESC embryoid bodies (EBs) with HS-mimetic glycopolymers **P2**. (A) Micrographs of day 0 EBs treated with AF488-labeled polymers **P2** show extensive remodeling with both polymers (3 μ M, 2 h, 37 $^{\circ}$ C) independent of glycan structure (green). Only remodeling with polymer **P2-D2A6** provided an enhanced affinity of BMP4 for the cell surface, as visualized by immunostaining with the AF350-labeled anti-BMP4 antibody (teal). (B) Total mean fluorescence intensity of polymers **P2** (AF488) and the anti-BMP4 antibody (AF350) measured at the midpoint of EB structures ($n = 10$) by confocal microscopy. (C) Total mean fluorescence intensity of cells remodeled with AF488-labeled polymers **P2** measured by flow cytometry after EB dissociation.

Figure 5.

Mesodermal differentiation of *Ext1*^{-/-} mESCs in an EB culture after glyocalyx remodeling with HS-mimetic glycopolymers **P2**. Relative transcriptional abundances of pluripotency (Oct4), mesodermal (Brachyury, Flk1, and Id1), and neuroectodermal (nestin) markers over 10 days of differentiation in an EB culture were determined by reverse transcription quantitative polymerase chain reaction (RT-qPCR; GAPDH and mESC). A rapid loss of pluripotency markers and the appearance of Brachyury expression were observed under all conditions. Only EBs remodeled with polymer **P2-D2A6** (3 μ M) or cultured in the presence of soluble heparin (1 μ g) showed attenuation of Brachyury expression after day 6 or 3, respectively. Under these conditions, the Brachyury message reemerged together with the onset of mesodermal Flk1 marker expression by day 10 of differentiation.

Figure 6.

Expression of embryonic and mesodermal cell surface markers in glyocalyx-remodeled EBs on day 10 of differentiation. All conditions saw a significant decrease in the level of cell surface pluripotency marker SSEA1 as detected after immunostaining by flow cytometry in cells dissociated from day 10 EBs. Elevated levels of Flk1 protein expression were detected in cells from day 10 EBs only after remodeling with **P2-D2A6** (3 μ M) or cultured in the presence of soluble heparin (1 μ g).