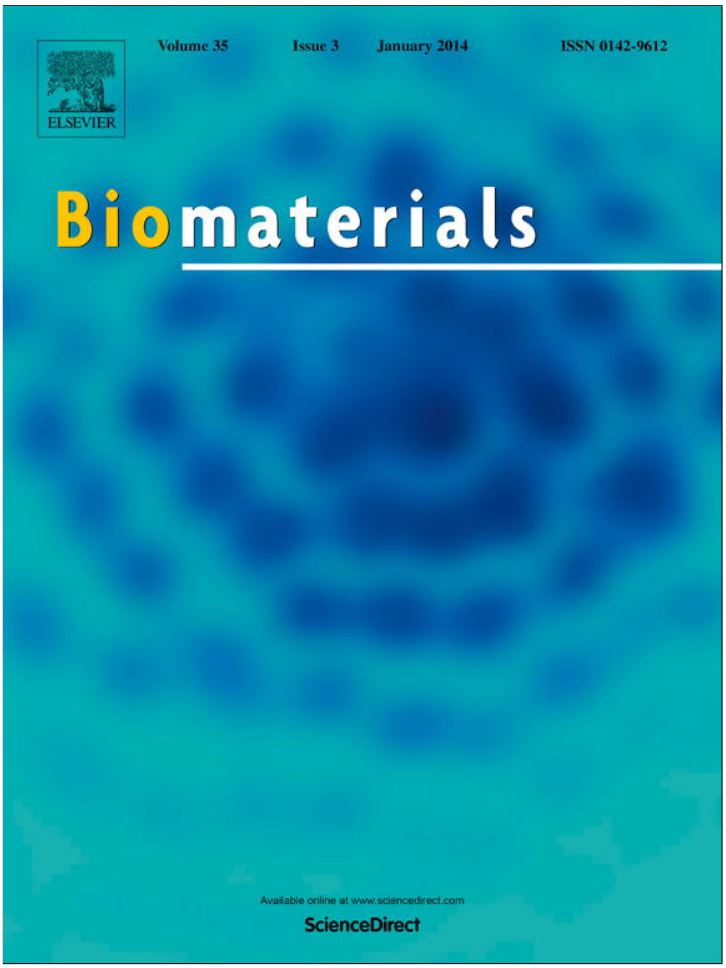

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The effect of multivalent Sonic hedgehog on differentiation of human embryonic stem cells into dopaminergic and GABAergic neurons

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ABSTRACT

Stem cell differentiation is regulated by complex repertoires of signaling ligands which often use multivalent interactions, where multiple ligands tethered to one entity interact with multiple cellular receptors to yield oligomeric complexes. One such ligand is Sonic hedgehog (Shh), whose post-translational lipid modifications and assembly into multimers enhance its biological potency, potentially through receptor clustering. Investigations of Shh typically utilize recombinant, monomeric protein, and thus the impact of multivalency on ligand potency is unexplored. Among its many activities, Shh is required for ventralization of the midbrain and forebrain and is therefore critical for the development of midbrain dopaminergic (mDA) and forebrain gamma-aminobutyric acid (GABA) inhibitory neurons. We have designed multivalent biomaterials presenting Shh in defined spatial arrangements and investigated the role of Shh valency in ventral specification of human embryonic stem cells (hESCs) into these therapeutically relevant cell types. Multivalent Shh conjugates with optimal valencies, compared to the monomeric Shh, increased the percentages of neurons belonging to mDA or forebrain GABAergic fates from 33% to 60% or 52% to 86%, respectively. Thus, multivalent Shh bioconjugates can enhance neuronal lineage commitment of pluripotent stem cells and thereby facilitate efficient derivation of neurons that could be used to treat Parkinson's and epilepsy patients.

1. Introduction

Biochemical cues within the stem cell niche that instruct cell fate decisions are often incorporated into larger structures, for example via self-assembly or by immobilization to the extracellular matrix. Understanding the importance of this nanoscale spatial organization in controlling cell behavior both advances our basic knowledge of stem cell and developmental biology and enables applications in tissue engineering and regenerative medicine. Multivalent interactions – where multiple ligands on one entity bind multiple receptors on another – are one such class of nanoscale organization that naturally occur in many biological processes ranging from growth factor and morphogen signaling to the attachment of a virus to a cell surface [1]. Multivalent ligands are often collectively more potent than corresponding monovalent interactions due to an enhanced ability to occupy and/or cluster their receptors [2–4]. Engineered biomaterials are increasingly being employed to design systems that emulate important biological features of natural niches [5,6], and synthetic biomimetic multivalent ligands may be more potent than monovalent ligands in regulating cell fate decisions, such as stem cell differentiation.

Shh, a potent morphogen that specifies cell fate choices in tissues throughout the developing embryo is one factor that has been suggested to function as a multimeric form [7–9]. Within the neural tube – the germinal origin of the central nervous system (CNS) – a gradient of Shh initially emanating from ventral mesenchyme tissue and subsequently the floor plate cells within

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the tube patterns differentiation of ventral progenitor domains in a concentration dependent manner [10]. The resulting ventral progenitors give rise to many neuronal cell types along the neuraxis including midbrain dopaminergic (mDA) [11] and GABA producing inhibitory neurons [12], which undergo degeneration in Parkinson's disease (PD) [13] or are impaired in epileptic disorders [14–16], respectively. Efforts to develop cell replacement therapies for these intractable neurodegenerative diseases routinely manipulate Shh signaling to ventralize neurally differentiating human pluripotent stem cells (hPSCs) and thereby generate mDA [17] or GABAergic [15] progenitors.

Small molecule agonists of Shh signaling have been used to induce the dopaminergic and GABAergic differentiation of hPSCs. Recombinant Shh, however, is 1–2 orders of magnitude more potent on a molar basis in patterning neural cell fate [18,19], and when used in combination with saturating levels of Shh agonist purmorphamine, recombinant Shh can further increase the efficacy of lineage-specific neuronal differentiation protocols [17]. Therefore, it appears that activation of Shh signaling with the protein ligand, which binds to the cell membrane receptor Patched, offers potential advantages compared to small molecule agonists, which regulate the downstream effector Smoothened (SMO).

Naturally produced Shh is covalently modified by cholesterol and palmitate [20,21]. These lipid moieties were initially believed to tether the protein to the cellular plasma membrane, yet in the neural tube Shh secreted from the notochord and floor plate act in long range to organize the pattern of ventral neurogenesis [7,22]. Shh's long-range signaling effects observed during organismal development have recently been attributed to its nanoscale clustering and multimerization within the secreting cell's membrane prior to release as a diffusible and multivalent molecule [7–9,23,24]. Furthermore, recent observations that Shh can be assembled into a soluble multimeric protein complex with a hydrophobic core of lipids help explain this transport [25]. The resulting multimeric form of Shh is also reported *in vitro* to be even more potent than monovalent recombinant Shh [7,24]; however, Shh multimerization and secretion rely on complex mammalian posttranslational modifications and secretory mechanisms that are not fully understood [26] and that render the production of natural, multivalent Shh for regenerative medicine applications problematic. We previously demonstrated that a bio-inspired, multivalent, conjugate form of Shh was more active in a murine fibroblast bioassay [27], raising the possibility that multivalent Shh bioconjugates may potentially serve as valuable materials to more effectively direct the differentiation of hPSCs into therapeutically valuable cell types compared to recombinant Shh or small molecule agonists of Shh signaling.

In this work we generated multivalent Shh with defined spatial distribution by conjugating recombinant Shh to linear Hyaluronic acid (HyA) polymers at various stoichiometric ratios and investigated the putative role of multivalency in Shh signaling during neuronal differentiation of hPSCs. We evaluated whether biomimetic Shh conjugates can be used as a bioactive material to enhance ventralization of neural progenitors and lineage commitment to therapeutically relevant neuronal phenotypes including mDA and GABAergic neurons in direct comparison with equimolar amounts of monomeric recombinant Shh, and ten-fold higher levels of Shh pathway small molecule agonist (SAG).

2. Materials and methods

2.1. Recombinant protein production, purification, and bioconjugation

A bacterial expression vector encoding Shh with a C-terminal hexahistidine tag and cysteine (pBAD–Shh) was transformed into chemically competent BL21 *Escherichia coli*. In addition, valine and isoleucine residues were introduced to the Shh N-terminus to increase potency by mimicking the hydrophobic palmitic acid modification of endogenous Shh. Protein expression was induced by the addition of 0.1% (w/v) L-arabinose in TB media for 5 h at 30 °C. Cells were lysed, and Shh was purified via immobilized metal–ion affinity chromatography (IMAC) on a Biologic DuoFlow System. The purified protein was dialyzed into pH 6.5 PBS containing 10% glycerol and EDTA. SDS-PAGE revealed a single band of the predicted size. Shh was conjugated to 800 kDa HyA through a two-step reaction using carbodiimide chemistry at the HyA carboxylate group and a maleimide reaction at the protein C-terminal cysteine. In the first step, EMCH, Sulfo-NHS, and EDC were added to a solution of HyA in MES buffer and allowed to react at 4 °C for 4 h, followed by dialysis. Recombinant Shh was reduced with a 200-fold molar excess of TCEP at 4 °C for 5 min. The Shh was added to HyA–EMCH at the desired molar ratios and allowed to react at 4 °C overnight. The Shh-conjugated HyA was dialyzed to remove unreacted Shh. Shh concentrations were measured using a BCA assay. Also, the degrees of substitution and valency of bioconjugates were analyzed by size-exclusion chromatography with multiangle laser light scattering paired with refractive index detection and ultraviolet spectroscopy (SEC-MALS-RI-UV) as described [28].

2.2. Dopaminergic and GABAergic differentiation of human embryonic stem cells

The H1 (WiCell) hESC line was cultured on Matrigel-coated cell culture plates (BD) in X-Vivo medium (Lonza) supplemented with 80 ng/ml FGF2 (PeproTech) and 0.5 ng/ml TGF-β1 (R&D Systems) or in mTeSR1 maintenance medium (Stem Cell Technologies). HESCs were removed from the tissue culture plates using a sterile cell scraper and partially dissociated by gentle pipetting. The cell clusters were resuspended in hESC culture medium lacking the mTeSR1 supplement, or lacking FGF2 and TGF-β1 for X-Vivo culture, and transferred to ultra low-attachment plates (Corning Incorporated) for embryoid body (EB) formation. HESCs were aggregated for 5 days and then seeded on Matrigel-coated plates, and the culture medium was then supplemented with N2 and B27 (Invitrogen) and treated with 100 ng/ml FGF-8 and 200 ng/ml Shh (R&D-Shh), SAG, m-Shh, or HyA–Shh (1:5, 1:10, 1:20, 1:40). After 9 days, cells were mechanically passaged onto poly-L-ornithine (Sigma Aldrich) and laminin (Invitrogen, 20 μg/ml) coated plates and cultured with FGF8 and Shh for an additional 5 days. Thereafter, FGF8 and Shh were withdrawn, and cells were matured for 16 days with BDNF (10 ng/ml) and GDNF (10 ng/ml, Peprotech).

To further increase neural progenitor yield, neural rosettes were isolated. EBs were seeded onto Matrigel coated plates for 14 days under high density conditions ($100 \times 10^3/cm^2$) with FGF8 and Shh conjugates or controls. At day 14, structures with a rosette-like morphology were mechanically isolated and plated on poly-L-ornithine and laminin coated plates or 8-well chamber slides at a lower density ($20 \times 10^3/cm^2$) in the presence of Shh for an additional week. Next, BDNF (10 ng/ml) and GDNF (10 ng/ml) were added to achieve neuronal maturation for an additional 2–3 weeks. As a control, corresponding amounts of HyA polymer without Shh conjugation were added. For GABAergic differentiation, cells were differentiated similarly, except FGF8 was excluded during neural patterning, and GDNF was excluded during neuronal maturation.

2.3. Gene expression analysis by RT-PCR

Using random primers and MultiScribe Reverse Transcriptase (Applied Biosystems) in a 20 μl reaction, complementary DNA was synthesized from 1 μg total RNA isolated from undifferentiated hESCs, EBs after 5 days of culture in suspension, and at days 9, 14, and 30 of neural differentiation following EB formation. The PCR analysis was carried out with Taq DNA polymerase (New England Biolabs). Equal amounts of RNA were tested in PCR reactions under the same conditions to verify the absence of genomic DNA amplification. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified as an internal control in gene expression analysis. Primer sequences (see Table. S1 in Supplementary data) were obtained from the PrimerBank website (http://pga.mgh.harvard.edu/primerbank/) and synthesized by Life Technologies.

2.4. Dopamine analysis

Media conditioned for 48 h by DA neuron cultures (differentiated for 30 days) were collected. Alternatively, dopamine release was induced by first conditioning cultured cells in Hanks' balanced salt solution (HBSS) for 15 min and then replacing it with HBSS containing 56 mM KCl for 15 min at 37 °C. 20 μl of dopamine stabilization buffer, consisting of 2.4 mM EGTA and 2.3 mM gluthatione in 10 ml of 0.1 M NaOH, were added. A HPLC kit (Chromsystems) was used to extract monoamines, whose levels were then determined by HPLC coupled to an electrochemical detector using MD-TM mobile phase.

2.5. Immunocytochemistry

Cultures were fixed with 4% paraformaldehyde for 10–15 min. The primary antibodies used were: mouse anti-Oct4 (1:100, Santa Cruz Biotechnology), mouse anti-SSEA-4 (1:500, Millipore), rabbit anti-Pax6 (1:200, Covance), mouse anti-Otx2 (1:50, R&D Systems), mouse anti-Msx1/2 (1:100, Developmental Studies Hybridoma Bank), mouse anti-βIII-Tubulin (Tuj-1) (1:250, Sigma), mouse anti-Microtubule-Associated Protein 2 (MAP2) (1:500, BD Biosciences), rabbit anti-TH (1:1000, Pel-

Freez), and rabbit anti-GABA (1:2000, Sigma Aldrich). Cultures were incubated with secondary antibodies conjugated Cy3 or Cy5 (1:500, Jackson ImmunoResearch Laboratories), or Alexa 594-conjugated anti-rabbit and Alexa 488-conjugated anti-mouse antibodies (1:1000, Invitrogen), in PBS containing 1% BSA for 2 h. Cultures were counter-stained with DAPI (Molecular probes) and imaged using a Zeiss Axio Observer A1 inverted microscope or a Zeiss LSM 710 confocal microscope.

2.6. Cell quantification and statistics

The numbers of neurons were quantified manually. The numbers of cells stained with DAPI were counted automatically with ImageJ software. For cell quantifications, images of 9 different fields per well from three wells were acquired using a 20× objective. For neuronal cell counts, fields where Tuj-1 and MAP2 positive neurons could clearly be seen and distinguished from each other were selected without consideration of number of neurons present in the field or neurotransmitter expression in neurons. Neuronal cell morphology was carefully examined to confirm expression of markers Tuj-1, MAP2, TH, and GABA. Differences in percentages of cells were tested by analysis of variance followed by pair-wise comparisons of group means using the Tukey–Kramer method for multiple comparisons generated by the GraphPad InStat software (GraphPad Software Inc.). Levels of significance are indicated by asterisks on the line graphs and figure legends. Differences were considered significant at $p < .05$. Data represent the mean values (±SD) of cell counts from triplicate samples from three independent experiments.

3. Results

3.1. Multivalent Sonic hedgehog bioconjugate synthesis

To investigate whether valency plays a role in Shh bioactivity during neuronal patterning of hESC differentiation, we synthesized multivalent bioactive Shh conjugates by grafting recombinantly produced, cysteine-modified, N-terminal Shh to a biological polymer, high molecular weight HyA, using a hydrazide–maleimide hetereobifunctional cross-linker (EMCH) [28] (Fig. 1A). To enable investigation of whether ligand valency impacts neuronal fate restriction, we generated Shh–HyA conjugates at various stoichiometric ratios ranging from 4 to 27 Shh molecules per HyA polymer chain. Conjugate valencies were determined by a protein assay as

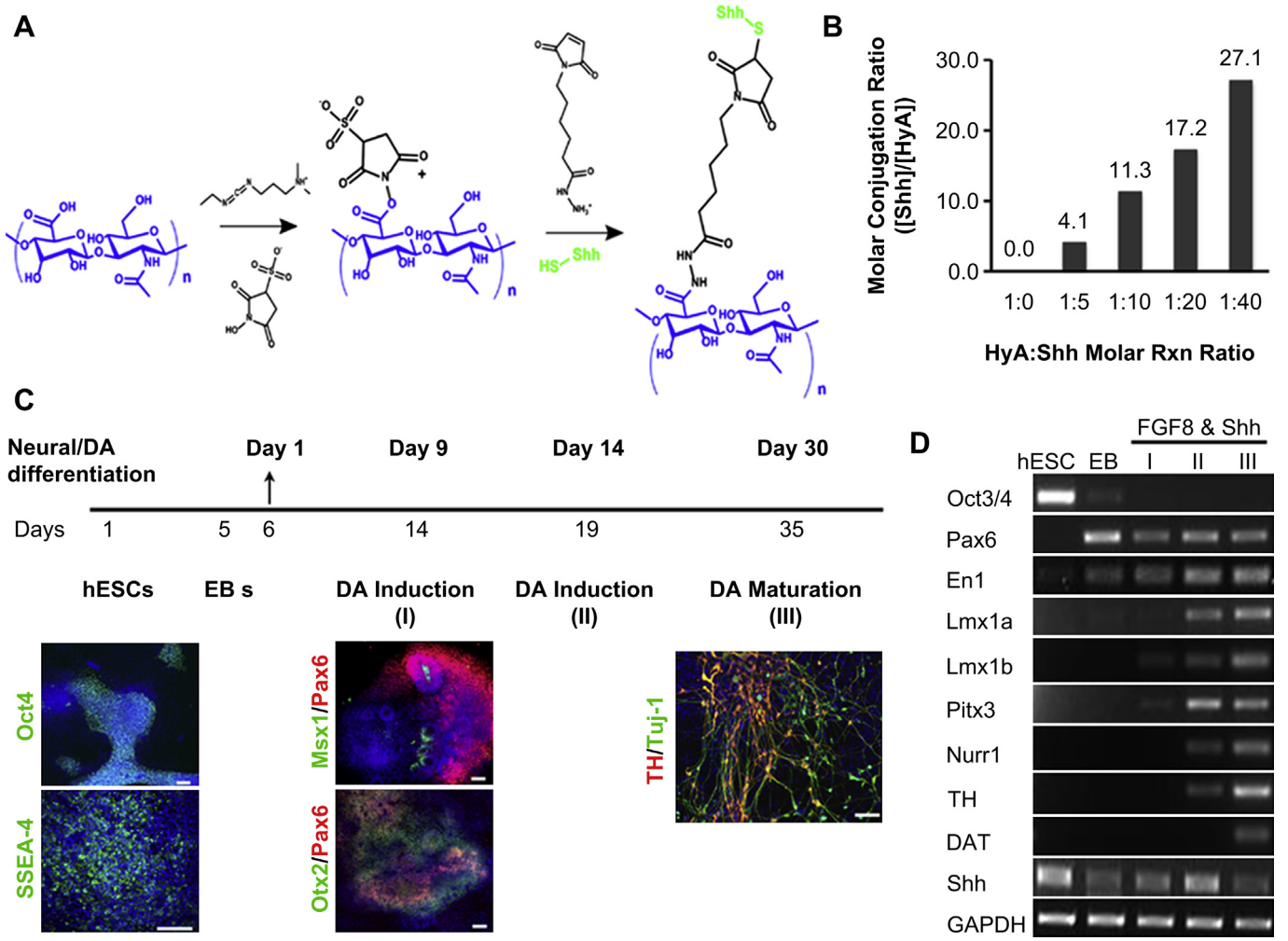

Fig. 1. (A) Representation of the chemical synthesis of hyaluronic acid (HyA)–Sonic hedgehog (Shh) bioconjugates using a hydrazide-maleimide hetereobifunctional cross-linker (EMCH) to anchor recombinantly produced cysteine-modified Shh to the carboxylic acid groups of the linear HyA polymers. (B) Bioconjugate valency characterization of HyA–Shh products by SEC-MALS. (C) Schematic representation of the paradigm for differentiating hESCs into midbrain dopaminergic (mDA) neurons with the aid of the midbrain instructive factors Shh and FGF8. Cultures stained with antibodies against pluripotent stem cell markers Oct3/4 and SSEA4 at day 1, the early neural progenitor marker Pax6 and midbrain progenitor markers Otx2 and Msx1 at day 14, and the neuronal and DA marker Tuj-1 and TH at day 35 confirmed the DA differentiation of hESCs. Scale bar = 100 μm (D) Complementary DNA from undifferentiated cultures, embryoid bodies (EBs), and cells at various stages of differentiation as illustrated in the differentiation scheme in panel (A) were analyzed by RT-PCR for a number of midbrain specific transcription factors including Engrailed 1 (En1), Lmx1a, Lmx1b, Pitx3, and Nurr1, and TH and the Dopamine transporter (DAT) expressed in mature DA neurons. The levels of Shh expression were assessed in all cultures. Amplification was performed for 35 cycles, and GAPDH was amplified simultaneously as an internal control under the same conditions.

well as refractive index detection, ultraviolet spectroscopy, and size exclusion chromatography coupled to multi-angle light scattering (SEC-MALS) [28] (Fig. 1B).

3.2. Effect of multivalent Shh on dopaminergic differentiation

To establish a timeline for mDA neuronal differentiation, hESCs were first cultured in suspension for 5 days to form embryoid bodies (EBs). Midbrain DA differentiation was then achieved by differentiating the resulting EBs in adherent conditions for 9 days in the presence of the established mDA specifying factors Shh (monomeric) and FGF8 [11]. After 9 days of differentiation in adherent cultures (Fig. 1C, DA Induction I), expression of the neural progenitor marker Pax6 [29] and midbrain progenitor markers Otx2 [30] and Msx1 [31] was detected in the majority of colonies. At this time, cultures were passaged and exposed to Shh and FGF8 for an additional 5 days (DA Induction II), followed by exposure to glial cell line-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF) to promote DA maturation [32] for an additional 16 days (Fig. 1C, DA Maturation). RT-PCR analysis of the pluripotency marker Oct3/4; the midbrain specific transcription factors Engrailed 1 (EN1), Lmx1a, Lmx1b, Pitx3, and Nurr1; and the mature DA neuronal dopamine transporter (DAT) protein confirmed the developmental progression of hESCs to a mature mDA phenotype [33] (Fig. 1D).

As the specification of midbrain neural progenitors is predominantly mediated by Shh [11]which has been suggested to function as a soluble multimeric complex during development [7–10,23,24], we next investigated whether this factor's multivalency impacts midbrain differentiation of hESCs *in vitro*. EBs were again formed for 5 days and transferred to adherent conditions for neural induction. On the first day of adherent culture differentiation, cells were exposed to the mDA-inducing factor FGF8 in combination with a Shh signaling activator – either the small molecule SMO agonist SAG, a commonly used commercial form of Shh (R&D), our recombinantly produced monomeric Shh (termed m-Shh), or HyA–Shh bioconjugates with polymer:Shh ratios of 1:4, 1:11, 1:17, or 1:27. Cells were cultured for 14 days (Fig. 1C, DA Induction I and II)

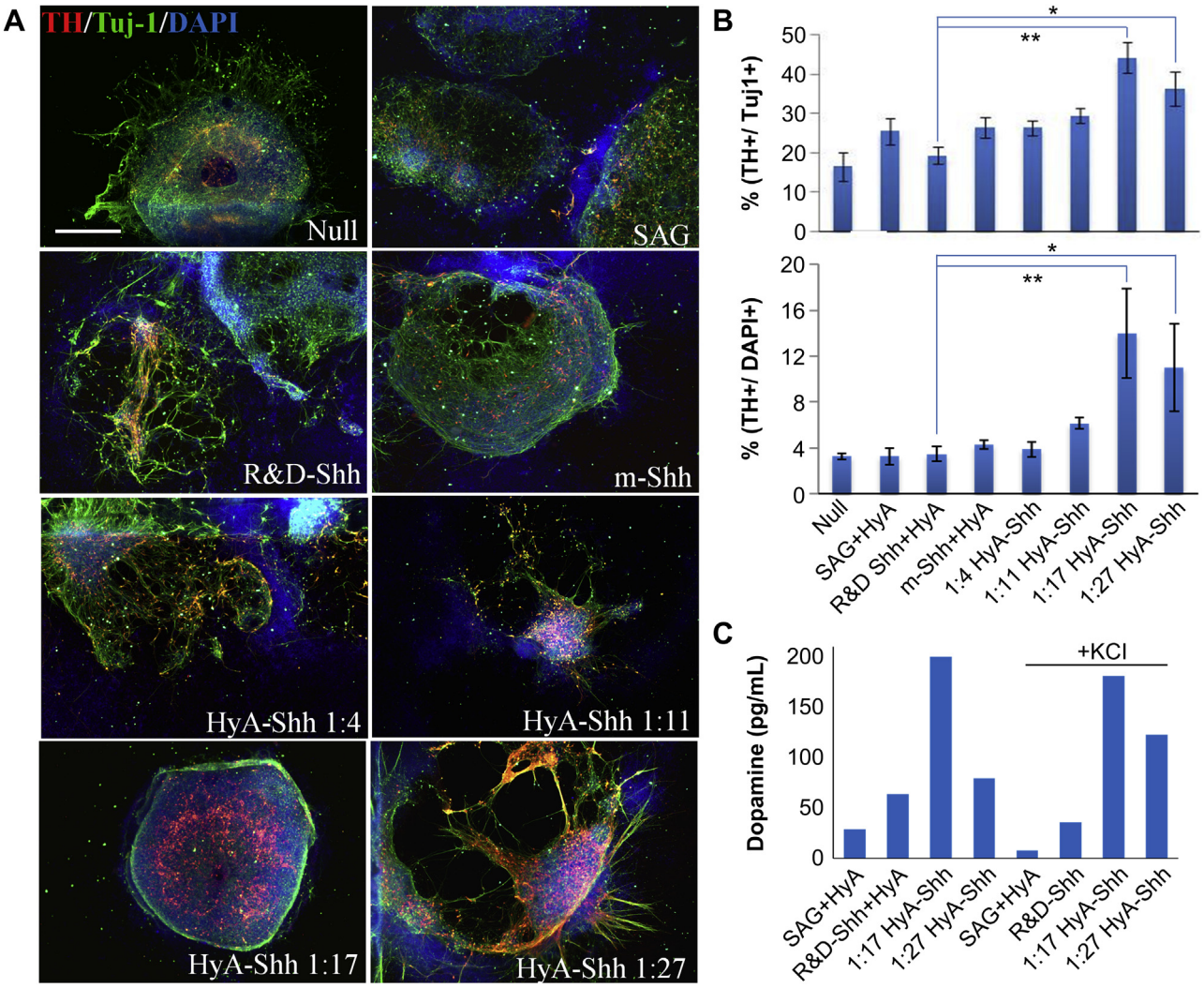

Fig. 2. (A) Immunocytochemical expression analysis of the neuronal marker Tuj-1 and the DA marker TH at 35 days of differentiation in untreated cultures (Null), and in the presence of a Shh pathway activator SAG, our recombinantly produced monomeric Shh (m-Shh), monomeric Shh obtained from R&D (R&D-Shh), and HyA–Shh bioconjugates with polymer:Shh ratios of 1:4, 1:11, 1:17, or 1:27 in combination with FGF8. Nuclei are counterstained with DAPI. (B) Quantitative analysis of the percentage of Tuj1+ neurons, as well as the percentage of Tuj1+ cells belonging to a DA phenotype (TH+) in the different conditions, illustrating the progressive increase in TH+ cells from SAG or monomeric forms of Shh to the conjugates of higher valencies. Error bars indicate standard deviations. (*$p < .05$, **$p < .001$). Scale bar = 100 μm. (C) Direct measurement of dopamine in media conditioned by differentiated cultures treated with HyA–Shh conjugates, and controls for 48 h, or media collected after 15 min of KCl-induced depolarization by high-performance liquid chromatography (HPLC).

with a passage on day 9. FGF8 and the Shh signal activators were then withdrawn, and GDNF and BDNF were added to promote DA neuronal survival and maturation. After 30 total days of neural differentiation, immunocytochemical analysis of the DA marker tyrosine hydroxylase (TH) and neuronal marker Tuj-1 revealed that Shh with a valency of 1:17 increased the overall number of TH + neurons approximately 3-fold and the fraction of neurons expressing TH more than 2-fold (Fig. 2A,B) compared to monomeric Shh. Results with a higher valency, 1:27, were similar (Fig. 2A,B). Since the ability to synthesize and release the neurotransmitter dopamine is the characteristic feature of dopaminergic neurons, we used high-performance liquid chromatography (HPLC) to compare the ability of the cells generated under conditions with the Shh pathway agonist SAG, monomeric R&D-Shh, or the higher valency Shh conjugates (1:17 and 1:27) to release dopamine. Media conditioned for 48 h by differentiated cultures treated with HyA–Shh conjugates or controls showed higher levels of dopamine in conditions with Shh bioconjugates with polymer:Shh ratio of 1:17 (Fig. 2C). Analogously, media collected after 15 min of KCl-induced depolarization also exhibited increased levels of dopamine from cultures patterned with higher valencies of Shh (1:17, 1:27) compared to SAG or monomeric Shh (R&D-Shh) (Fig. 2C). These results establish that morphogen valency impacts neuronal specification and maturation of mDA neurons.

To further increase the fraction of cells that belong to a mDA fate following EB formation, we increased the cell density and initial differentiation time in the presence of FGF8 and various forms of Shh (Fig. 1C, DA Induction I), isolated rosette-like structures comprised mainly of neural progenitor cells, and further differentiated them in adherent conditions at lower densities to enhance neuronal differentiation (Fig. 1C, DA Induction II). Also, to assess reproducibility within this study, we used newly synthesized bioconjugates at valencies close to those that previously exhibited maximal potencies (~1:17 in Fig. 2B). After the 14 day incubation in FGF8 and Shh, DA induction of neural progenitors was significantly higher in conditions with high HyA:Shh ratio conjugates (1:12 and 1:16) as indicated by the expression of mDA progenitor markers Lmx1a and Msx1 (Fig. 3A) and the DA marker TH (Fig. 3B).

Cultures under the influence of HyA:Shh conjugates with ratios of 1:16 induced expression of Lmx1a and Msx1 in 61% of colonies as compared to 36% in cultures treated with the monomeric form of Shh (R&D-Shh) (Fig. 3A). Colonies containing 30 or more TH-expressing neurons comprised about 71% of colonies in cultures influenced by Shh bioconjugates with polymer:Shh ratios of 1:16, versus 34% of colonies in cultures treated with the monomeric Shh (Fig. 3B).

These neural progenitors were then matured into neurons (Fig. 1C, DA Maturation). Consistent with the increase in mDA neural progenitor marker expression in colonies after the initial

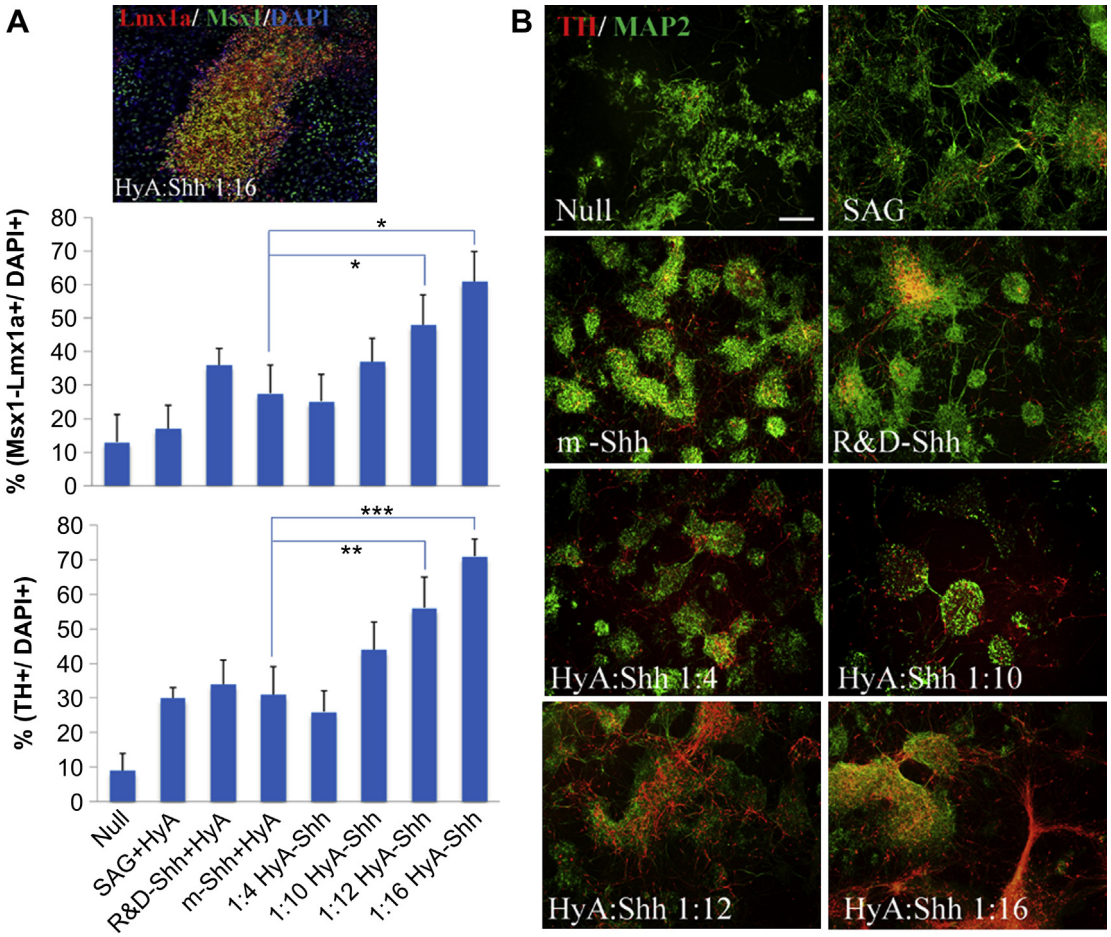

Fig. 3. (A) Quantitative analysis of the co-expression of midbrain progenitor transcription factors Msx1 and Lmx1a or TH in colonies treated with FGF8 and SAG, monomeric forms of Shh (R&D-Shh and m-Shh), or the Shh multimers with valencies of 1:4, 1:10, 1:12, or 1:16 after 14 days of neural induction. The image insert in the top bar graph shows an example of an immunostained colony expressing Msx1 and Lmx1a in conditions treated with FGF8 and HyA polymers carrying 16 Shh ligands. $^{*}p < .05$, $^{**}p < .001$, $^{**}p < .001$. (B) Representative images of colonies expressing TH and MAP2 in the different conditions indicate an increase in the generation of TH + neurons in cultures exposed to multivalent Shh in relation to monomeric form of Shh or SAG. Scale bar = 100 μm.

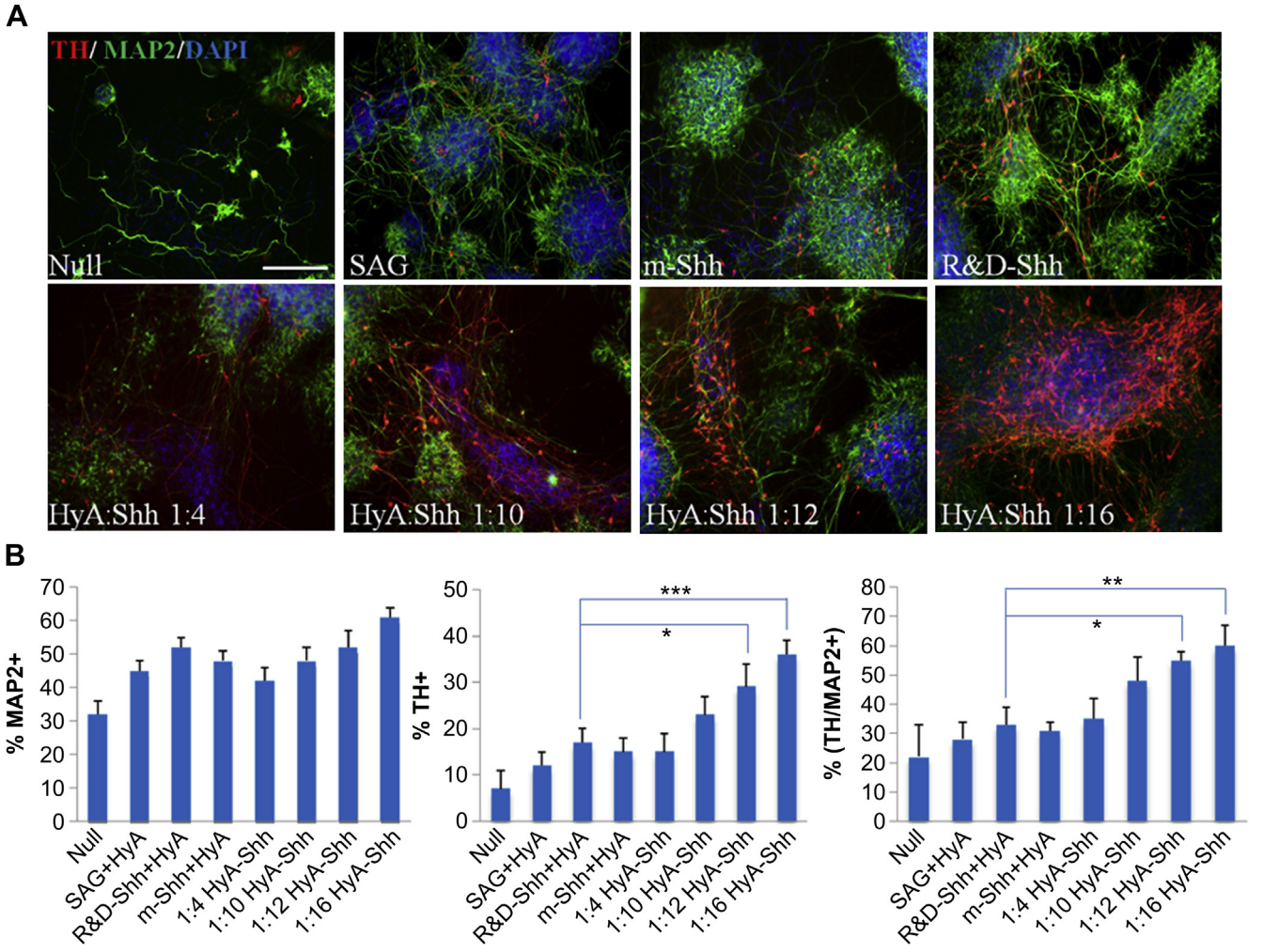

Fig. 4. (A) Midbrain neuronal lineage specification analysis of isolated neural rosettes after neuronal maturation by TH and MAP2 expression. Cultures were differentiated in the absence of any instructive cues, continued exposure to SAG, or conjugates bearing various Shh valencies for 7 days and further matured in the presence of neurotrophic factors. Scale bar = 100 μm. (B) Quantification results of percentages of cell expressing TH and MAP2 are presented in bar graphs, revealing that the percentages of TH expressing neurons and the fraction of the mature neuronal population restricted to a midbrain DA phenotype can be nearly doubled by increasing the valency of Shh during the course of neuronal differentiation*$p < .05$, **$p < .001$, **$p < .001$.

neural induction stage (Fig. 3A), immunocytochemical analysis after a total of 35 days of differentiation post-EB formation established that DA differentiation was enhanced with the multimeric forms of Shh (Fig. 4A,B).

Specifically, the fraction of TH + neurons in the culture increased from 17% for monomeric Shh (R&D-Shh) to 36% in cultures treated with Shh with a valency of 16 (Fig. 4A,B), and the fraction of neurons that were committed to a mDA phenotype increased from 33% to 60% (Fig. 4B).

3.3. Effect of multivalent Shh on GABAergic differentiation

During development, Shh also acts as a cell fate specifying factor in the forebrain, giving rise to ventral forebrain neural progenitors that subsequently develop into GABAergic interneurons [12,34,35], which may offer promise for the treatment of epilepsy and seizure [14–16]. It has been shown that exposure to Shh can increase the GABAergic differentiation of hPSCs [36]. To demonstrate broader application for multivalent Shh in directed stem cell differentiation, we investigated whether Shh valency impacts differentiation of hESCs to forebrain GABAergic neurons. Similar to the optimized DA differentiation paradigm, forebrain differentiation was carried out via 5 day EB-formation followed by 14 days of differentiation in the presence of the small molecule agonist SAG, multivalent Shh conjugates, or monomeric forms of Shh. At this stage, rosettes were manually isolated and further exposed to Shh conjugates or controls for 7 days. Isolated neural progenitor cells were matured for an additional 14 days in the presence of BDNF, previously shown to play a crucial role in the development and functional maturation of forebrain neurons [37,38]. Immunocytochemical staining of the GABA neurotransmitter and MAP2, a marker for mature neurons, demonstrated that multivalent conjugates substantially increased the commitment of neural progenitors to a GABAergic lineage compared to monomeric Shh from 30% to 56% of all cells, and from 52% to 86% of neurons (Fig. 5), resulting in a relatively pure GABAergic neuronal population.

4. Discussion

While the signaling events that govern stem cell fate decisions are intricate, chemistry and materials synthesis can enable the engineering of biomimetic systems and structure that progressively emulate and restore the complexity of the stem cell niche signals. In this study, we addressed the question of whether the valency of Shh

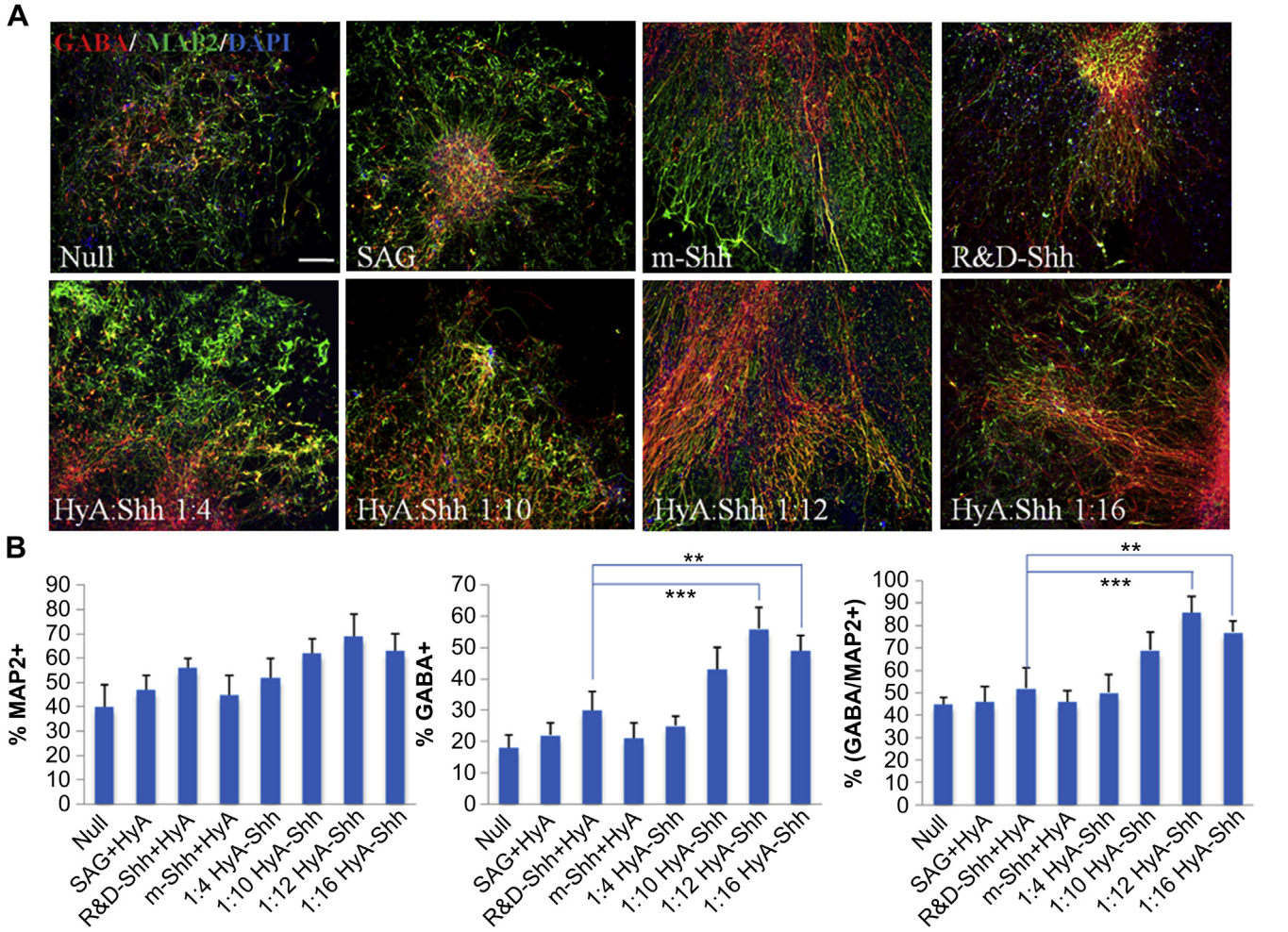

Fig. 5. (A) Representative immunolabeling images of cortical cultures after 14 days of differentiation of dissected rosettes. Cultures were under the control of monomeric Shh, Shh of higher valencies, SAG, or control conditions, demonstrating differential expression of the neurotransmitter GABA by MAP2+ neuronal populations. Scale bar: 100 μm. (B) Qualitative analysis of the proportion of GABA + neurons and MAP2+ neurons co-expressing GABA, revealing that the ventralization effect of forebrain neural progenitors by Shh can be modulated by valency. $^{**}p < .001$, $^{**}p < .001$.

plays an instructive role in the patterning of stem cells. Shh is naturally assembled into multivalent structures whose signaling and transport properties are different from monomers [7–9]. However, it is difficult to emulate this natural assembly process to either generate potent ligands or to gain potential insights into these structure–function relationships. We demonstrated that engineered multivalent ligands that imitate the oligomerization of natural Shh ligand are more potent in inducing the differentiation of two types of therapeutically relevant cells from hESCs.

In particular, Shh and FGF8 signaling promote mDA derivation of stem cells [11], and replacement of defective dopamine producing neurons with healthy new mDA neurons derived from hESCs is a promising clinical treatment for Parkinson's disease [39]. Additionally, Shh acts as a ventralizing factor in the forebrain to specify the GABAergic phenotype [12,34–36]. Such GABAergic inhibitory neurons are integral in regulating neuronal excitability throughout the nervous system [19], and reductions in GABAergic neuronal density and abnormalities in the forebrain have been associated with a number of neurological diseases including epilepsy and schizophrenia [14,15]. Although hESC-based replacement therapy for treatment of these and other disorders is a promising approach, there are challenges in cell production. First, to ensure safety of the transplanted cells for clinical applications and reduce the risk of adverse events, it is imperative to obtain pure cultures committed to a particular cellular phenotype. For example, contamination of DA neurons with other cells was associated with dyskinesia adverse side effects in Parkinson's clinical trials [40]. In addition, scaling up biological and especially stem cell processes to treat a large patient population is challenging, and any increases in the yield of a desired cell type could reduce the size of a costly bioprocess. Finally, improved control over the composition of a culture may enhance efforts to use hPSCs as more accurate models of human development and disease.

Naturally produced Shh is covalently modified by cholesterol and palmitate [20,21]. These lipid modifications were initially believed to tether the protein to the cellular plasma membrane, yet in the neural tube, Shh secreted from the notochord and floor plate act in long range to organize the pattern of ventral neurogenesis [7,22]. Recent observations that Shh can be assembled into a soluble multimeric protein complex with a hydrophobic core of lipids help explain this transport [25], and the multimer may also be higher in potency. To investigate how Shh multimerization impacts potency, we designed multivalent Shh conjugates by attaching multiple copies of recombinant Shh to a naturally occurring biopolymer, HyA [28]. This study demonstrates that Shh's ability to specify midbrain or forebrain neuronal fates from hESCs can be regulated by altering Shh ligand valency. Based on the initial observation that bioconjugates comprised of ~17 Shh molecules per chain were

superior for mDA differentiation (Fig. 2), we optimized our protocol to increase the yield of mDA and GABAergic neurons by isolating neural rosettes and increasing the exposure time to these Shh conjugates. As a result, lineage commitment of hESCs to mDA and forebrain GABAergic neurons increased approximately 2-fold relative to either monomeric Shh or the small molecule agonist. Future work may explore how the binding of these flexible polymer–ligand complexes to the receptor may compare to the binding of natural Shh oligomers, what role receptor clustering may play in signaling, and whether cells can vary the valency to tune the bioactivity of the ligand.

5. Conclusions

We demonstrate that engineered multivalent ligands that mimic the oligomerization of natural Shh ligand are more potent in enhancing fate specification of hESCs into mDA and GABAergic neurons, for strategies to treat Parkinson's disease and epilepsy and seizure, respectively. Patterning factors with increased potency could improve the purity of a desired cell type and thereby potentially reduce deleterious outcomes. These results can both improve the ability to generate therapeutically relevant cell types, as well as lend future insights into the mechanisms of cell patterning.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2013.10.025.

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