Glycosaminoglycan Synthesis for Embryoid Body Incorporation

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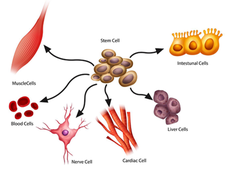
Abstract

Embryonic stem cells (ESCs) hold great promise for medical applications, but accurate, directed differentiation hinders its progress. Glycosaminoglycans (GAGs) on ESCs recruit growth factors, which direct their differentiation into the three germ layers: mesoderm, endoderm, and ectoderm. By using GAGS, we can influence ESC differentiation. Engineered stem cells lacking the gene Exostosin 1 (Ext 1), which extends the sugar chains in GAGS, were shown to be unable to differentiate and thus remained pluripotent. Past experiments in knockout stem cell cultures lacking native Heparan Sulfate GAGs showed that GAG mimetics were able to rescue differentiation into neural precursor cells.[1] While this was achieved in monolayer culture, looking at a 3D cell scaffold called an embryoid body (EB) is more representative of a developing embryo and allows for a greater variation in differentiation. Using a GAG mimetic of n=150 repeating units, a dose and time dependence for GAG mimetic uniform penetration throughout EBs has been established with confocal microscopy. Furthering work on the incorporation of GAG mimetics into EBs, we will be trying to establish gradients of GAG mimetics using various sized polymer. In our experiment, we used a shorter GAG mimetic of n=85 that should penetrate an EB more thoroughly and get into the center of an EB more quickly than a longer polymer.

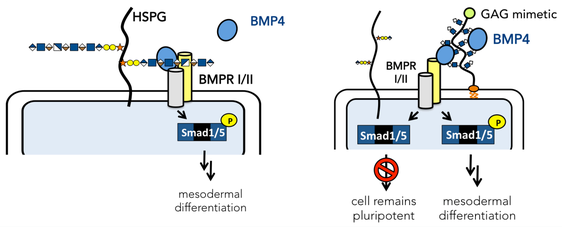
Background

Stem cells are pluripotent, meaning that they can differentiate into almost every cell type ranging from liver cells to heart cells.[2] Because of this ability, there has been extensive research on using stem cells for medical applications. However, the biggest hurdle in achieving this is being able to direct the stem cells effectively and precisely. Stem cell fate can be influenced based on the microenvironment surrounding the cell and also soluble ligands like growth factors. The use of glycans for growth factor recruitment shows great promise for stem cell differentiation.

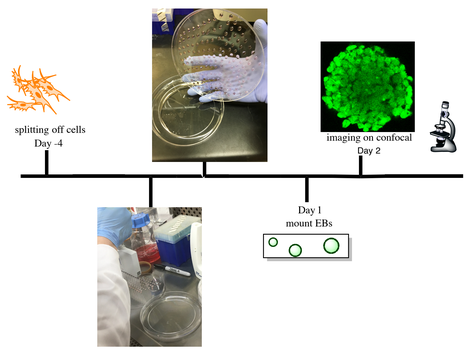


Glycans are carbohydrates, or sugars, found on the surface of and in cells that are crucial in interactions among cells. As secondary gene products, they are structurally complex and diverse, giving rise to a large array of functions within cells.[4] In the context of embryonic stem cells, GAGs found on ESC surfaces recruit stem cell growth factors. Fibroblast Growth Factor 2 (FGF2) and Bone Morphogenic Protein 4 (BMP4) are growth factors during early differentiation that lead to neuroectoderm and mesoderm formation respectively. Both have been shown to require interactions with heparan sulfate GAGs in order to carry out their signal and lead to differentiation. [4] Heparan sulfate is a long sugar chain composed of repeating dissacharide units. Along the chain are pockets of sulfated sugars, which utilize electrostatic interactions to recruit growth factors to the surface of the cells. Extostosin 1 (Ext 1) is the enzyme responsible for extending the disaccharides in the heparan sulfate chain. Cells lacking the Ext1 gene are unable to recruit FGF2 and BMP4 to the cells, and thus remain pluripotent.

Synthetic GAG mimetics created using a polymer backbone and ligated with heparan sulfate dissacharides have shown to rescue differentiation in Ext 1 knockout embryonic stem cells in monolayer cultures into neurons.[1] One caveat of this monolayer method is that cells are biased toward ectoderm differentiation because of the low cell density and minimal contact between cells. Embryoid bodies are shown to differentiate into the mesoderm and endoderm layers more readily because of higher cell density and contact. Therefore, research of ligated GAGs inserted into EBs could lead to the differentiation of more varied cell types and holds promise for medical applications.



Embryoid Body Formation



GAG Mimetic Synthesis

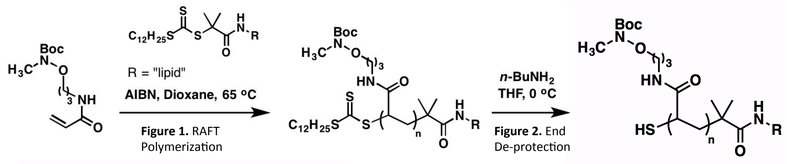


Figure 1. RAFT Polymerization

Figure 2. End De-protection

Figure 3. Fluorophore Ligation and Side Chain De-protection

Figure 4. Heparan Sulfate Ligation

A ratio of lipid chain transfer agent (CTA), Boc-protected N-methlyamino-oxypropylacrylamide monomer, and radical initiator produced poly(acrylamide) (AIBN) is measured out and put together in a Schlenk glass for RAFT polymerization (Fig 1). After the solution has run for 9 hours in an oil bath of 65°C to approximately 70% completion, the polymer is centrifuged out with hexanes. A n-butylamine in THF is added to cleave off the tri-thiol cabonate group in the end de-protection step (Fig 2). Then, Alexa Fluor 488 is attached to the polymer. Once fluorophore ligation is completed, the side protection BOC group is removed with a mixture of trimethylsilyl chloride and phenol (Fig 3). Next, the sugar Heparan Sulfate is ligated onto each repeating monomer unit in a urea and sodium acetate buffer of pH 4.5 (Fig 4). Finally, a solution of the GAG mimetics (Fig 5) surround solutions with EBs for a passive insertion of the polymer into the cell membranes (Fig 6).

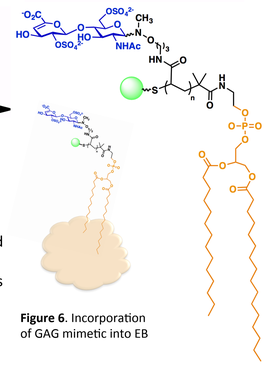
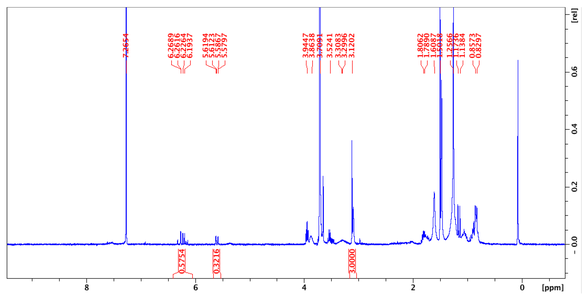


Figure 6. Incorporation of GAG mimetic into EB

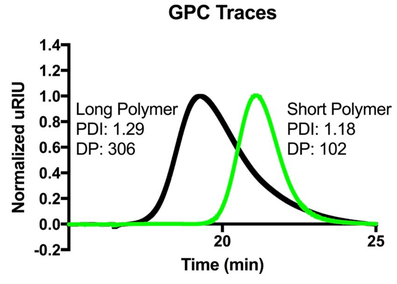
Figure 5. Complete GAG Mimetic with lipid tail

NMR Spectroscopy



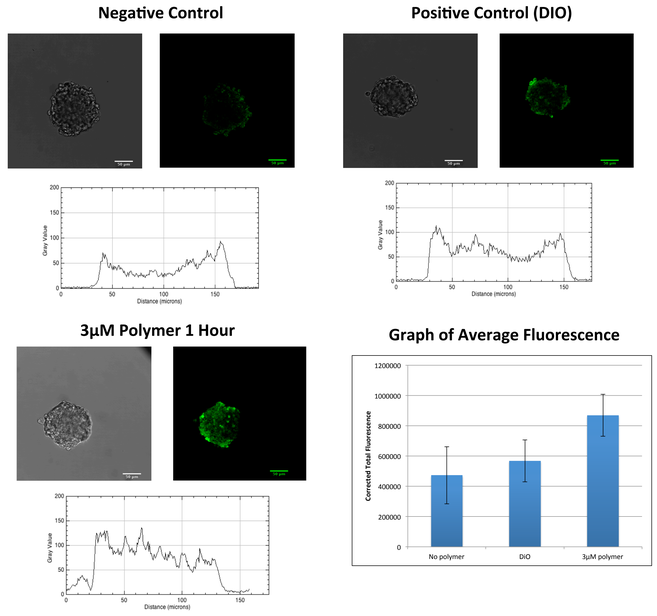
The nuclear magnetic resonance graph shows a way to find the DP of the polymers through the integration of the polymer and monomer peaks. By comparing the areas, we can find the percent completion of the polymerization and therefore the length of the polymer.

Gel Permeation Chromatography



Gel Permeation Chromatography (GPC) separates analytes on the basis of size. Within the chromatography column, a porous gel retains smaller molecules in the pores for a longer time than larger molecules that are able to slip through more easily. So, the longer GAG mimetics come out first. The PDI is a ratio of the Weight Average Molecular Weight over the Number Average Molecular Weight that quantifies width of the peak that correlates to the dispersity of the different lengths of polymers. A PDI of < 1.3 indicates a narrow chain length distribution and is ideal.

EB Imaging



Discussion

In past work with EBs and GAG mimetics, a time and concentration dependence was established. In our experiment, The negative control shows a low average fluorescence of EBs in comparison to that of the DIO and polymer sample. The DIO stain EBs, the positive control, is supposed to visualize even penetration of the EB to compare to the samples with polymer. Our sample likely had differences in fluorescence because the lipophilic dye was not left in for an adequate amount of time. As expected and in accordance with past work, the fluorescence of the concentrated 3µM polymer incorporation was greater than the negative control. There was a greater fluorescence around the edges of the EB, meaning that there was a greater concentration of GAG mimetic found in those areas. Even though there were differences in concentration, they were relatively small differences in fluorescence; this shows great promise for more even incorporation. Though we also made slides of 0.3µM incorporated EBs, we were unable to screen and analyze them.

In the future, we could test three different lengths of polymers side by side to look for the affect of polymer size on EB incorporation. If there is a difference, multiple cell layers within EBs could be formed. Short polymers and long polymers together in solution ligated with different types of sugars could be able to incorporate into the EBs at different rates to enable multiple layers of cells.

References

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Acknowledgements

Thank you so much to Professor Komives and Academic Connections for providing me with this opportunity to work in the Godula Lab, to Professor Godula for allowing me to work in his lab, Logan for mentoring me and teaching me about organic chemistry and everything else in the lab, and to Matt for teaching me about cell cultures and western blotting and mentoring throughout.