UNIVERSITY OF CALIFORNIA, SAN DIEGO

# Establishing a cellular microarray to visualize glycan mediated signaling in mouse embryonic stem cells

A Thesis submitted in partial satisfaction of the requirements for the degree

Master of Science

in

Chemistry

by

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2016

The Thesis of Austen Larson Michalak is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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University of California, San Diego

2016

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# Abbreviations used:

Ab- antibody

Akt- protein kinase B

A-PEG- amino-polyethylene glycol

BMP- bone morphogenetic protein

BMPR- BMP receptor

CHO- Chinese hamster ovary

ECM- extracellular matrix

EXT1- exotosin 1

ERK-extracellularly regulated kinase

FGF- fibroblast growth factor

FGFR- fibroblast growth factor receptor

GAG- glycosaminoglycan

Glc- glucose

GlcA- glucuronic acid

GlcN- glucosamine

GlcNAc- N-acetyl-glucosamine

GlcNS- N-sulfate-glucosamine

Gp130- glycoprotein 130

GRB2- growth factor bound protein 2

GSK3- glycogen synthase kinase 3

HS-heparan sulfate

HSPG- heparan sulfate proteoglycan

IdoA- Iduronic acid

JAK- Janus kinase

LIF-leukemia inhibitory factor

MAPK-mitogen activated protein kinase

MEK- mitogen/extracellular signal-regulated kinase

mESC- mouse embryonic stem cell

NDST- N-deacetylase-N-sulfotransferase

OST- O-sulfotransferase

PAPS- 3'-phosphoadenosine-5'-phosphosulfate

PI3K- phosphoinositide 3-kinase

SHP- Src homeodomain phosphatase

SOS- son of the sevenless

STAT- signal transducer and activator of transcription

TBST- tris buffered saline with tween 20

TF- transcription factor

TGF-β- transforming growth factor β

UDP- uridine di-phosphate

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# Acknowledgements

I would like to whole heartedly thank my PI, Dr. Kamil Godula for his ongoing support throughout this work, for believing in me and giving me a chance to succeed. Without his help, none of this would be possible. I would also like to deeply thank Dr. Mia Huang, for her collaboration and many helpful conversations. Finally, my colleagues Greg Triger and Kelsey Krug provided assistance in developing microarray preparation and optimization.

My previous mentors Dr. Antje Almeida and Dr. Ann O'Neil provided early inspiration and kept me on course to pursue higher academic studies. They believed in me before I did.

I would also like to thank Dr. Cathy Merry for her kind gift of $EXT1^{-/-}$ cell lines to the Godula lab.

The support of my family was essential, my mother provided her love and cookies; and my brother means the world to me. Also, I could not have done this work without my friends Brooks Venters, Stephen Brown, Matthew Cook and Philippe Broom as they provide grounding and relief in the form of successful surfing expeditions and jam sessions.

Finally, I would like to thank my recently departed father, Gerald Michalak. He taught me to love science, sail fast, and love the taste of salt water on the wind. He set an example of how to live life well. Fair winds Dad.

ABSTRACT OF THE THESIS

Establishing a cellular microarray to visualize glycan mediated signaling in mouse embryonic stem cells

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Master of Science in Chemistry

University of California, San Diego, 2016

Professor Kamil Godula, Chair

Glycosaminoglycan (GAG) mediated growth factor signaling initiates differentiation in embryonic stem cells (ESCs). Manipulation of the GAG interactome affords a novel tool to understand and direct stem cell differentiation. In this study, a high-throughput miniaturized cellular microarray platform, consisting of gelatin robotically spotted onto passivated glass slides is established to assess the effects of cellular glycan microenvironment on growth factor signaling in mouse embryonic stem cells (mESCs). Heparan sulfate (HS) acts as co-receptor for fibroblast growth factor 2 (FGF2) and the subsequent activation of the mitogen activated protein kinase (MAPK) pathway is measured by extracellular regulated kinase (ERK)

phosphorylation. Using HS deficient EXT1$^{-/-}$ ESCs, FGF2 signaling is rescued using soluble and immobilized heparin. Immobilization of glycans on cellular microarrays offer potential as a powerful tool for systematically evaluating and directing cellular differentiation.

Then, using conventional culture conditions, the interactions of HS and FGF2 are screened using *bis*-2-methyl-4-amino-quinolyl-6-carbamide, or surfen, and compared to the commercial FGFR inhibitor PD173074. Surfen reversibly antagonizes heparan sulfate in the μm range and can HS mediated interactions can be rescued using soluble heparin, a highly sulfated HS analog.

# I. Introduction

## Overview and objectives

Embryonic stem cells (ESCs) offer great potential for therapeutic applications, as well as understanding development, and thus have been a topic of great interest in past decades. However, before stem cells can be therapeutically useful for regenerative medicine, it is important to understand and control the process of cellular differentiation. One can imagine the problems a patient might experience if stem cells were introduced to the body and then differentiated into the wrong cell type, leaving the patient with a potentially malignant teranoma. Ideally, the process of differentiation can be directed to yield mature cells of well-defined phenotypes.

The extracellular glycans of mammalian cells can encode functional information to rival that of nucleic acids (42). Extracellular proteoglycans (PGs) are high affinity co-receptors for growth factors involved in differentiation, such as fibroblast growth factor 2 (FGF2). Growth factors initiate signaling events which trigger differentiation and subsequent lineage commitment, and thus glycans exert potent effects on differentiation. This glycan based approach offers a unique method to modulate FGF2 signaling in ESCs. The goals of this project are to ultimately manipulate FGF2 signaling in cells lacking endogenous PGs by presenting covalently

immobilized glycans onto growth substrates. To meet the latter goal, cells were cultured in a cellular microarray format, which offers a medium to high throughput platform for assaying FGF2 signaling as a function of the ESC "glyco-enviroment".

Another aim is to probe the roles of glycans in FGF2 mediated signaling activation using small molecule antagonists of glycans. In this context, glycans offer a novel approach to epi-genetically program stem cell differentiation.

## 1. Stem Cells

The first pluripotent cell lines were derived from mouse blastocysts in 1981 by two groups: Martin Evans, Matthew Kaufman of Cambridge University as well as Gail Martin at University of California, San Francisco (1). Since their discovery, stem cells have become an increasingly promising area of research with great potential to improve understanding for organismal development and to enable therapeutic applications in regenerative medicine. The primary properties of ESCs are: 1) continuous self-renewal, and 2) pluripotency, which is the ability to differentiate into any germ line (15). Chemical signaling, as well as cell-cell contact and environmental cues together determine whether ESCs will differentiate into any of the three primary germlines: the outer germ layer, *ectoderm*, which gives rise to nervous system and skin organs; the middle layer; *mesoderm*, which develops into cardiac tissues and skeletal muscle, and the inner germ layer; *endoderm*, which gives rise to the tissues of gut, liver, and pancreas (17). An intricate web of balanced signaling pathways determines the state of ESCs and can initiate differentiation or restrict them in a

continuous self-renewal cycle. Here, I will discuss how a balance of the LIF, MAPK and BMP signaling pathways regulates pluripotency and differentiation.

When first mESC lines were established, it was found that mESCs could only maintain pluripotency when co-cultured with a layer of mitotically quiescent mouse embryonic fibroblasts (MEFs), termed feeder cells. These feeder cells were known to secrete factors which allow ESCs to thrive and proliferate (1). The protein responsible for maintaince of mESC pluripotency was identified as the leukemia inhibitory factor (LIF). Later, another essential component was identified, the bone morphogenetic protein 4 (BMP4) (15, Fig 1.1A). It was discovered that media supplemented with serum containing BMP4 and LIF could sustain ESC populations in the absence of feeder cells and, furthermore, MEFs lacking a functional LIF gene could not sustain healthy populations of pluripotent ESCs (1).

In mESCs, the balance between pluripotency and differentiation is carefully regulated by alternate cell signaling pathways. The condition of pluripotency and self-renewal is maintained by a combination of BMP4 and LIF signaling. However, the addition of FGFs, such as FGF2, will instruct the cell to switch from a state of pluripotent self-renewal to differentiation. Once differentiation is initiated, BMP4 signaling will direct mESCs in mesodermal lineages, while sustained FGF signaling will promote differentiation into neuroectodermal lineages (Fig. 1.1B).

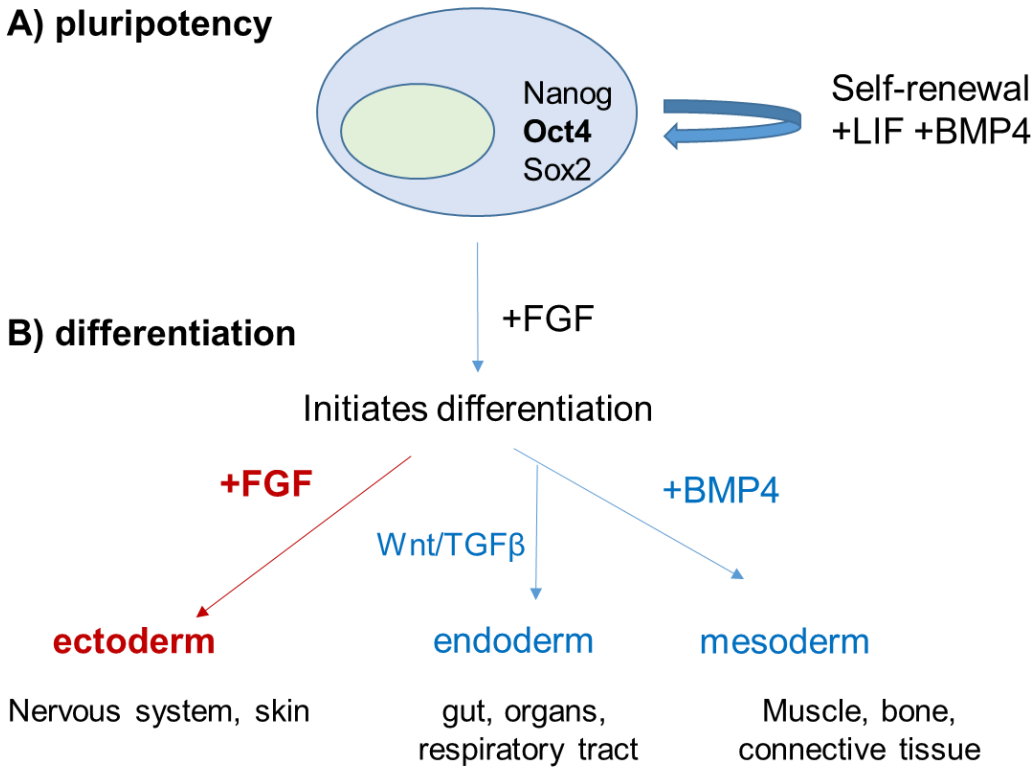



**Fig 1.1 A simplified mESC overview.** A) A balance of signaling pathways maintains the cell in a pluripotent state of continuous self-renewal. B) FGF mediated MAPK activation halts self-renewal and initiates differentiation. Cues such as growth factor gradients determine the outcome of differentiation, with BMP4 promoting meso/endodermal lineages while sustained FGF signaling directs mESCs to a ectodermal fate.

## LIF maintains pluripotency via the core transcription factors Nanog, Sox2, and Oct4

LIF is one of eight members of the interleukin-6 (IL-6) cytokine family (16). As the LIF signaling pathway was elucidated, it was found that LIF upregulated the expression of the core pluripotency transcription factors Nanog, Oct4, and Sox2. This trio of transcription factors are termed master regulators of pluripotency, as they are

essential for maintaince of a pluripotent state (16). It was found that LIF signaling activates several pathways, the LIF/JAK/STAT3 pathway, LIF/PI3K/AKT pathway, and LIF/GRB2/MAPK pathway (see figure 1.2 below). LIF, and all members of the IL-6 cytokine family signal through the transducer element glycoprotein 130 (gp130).

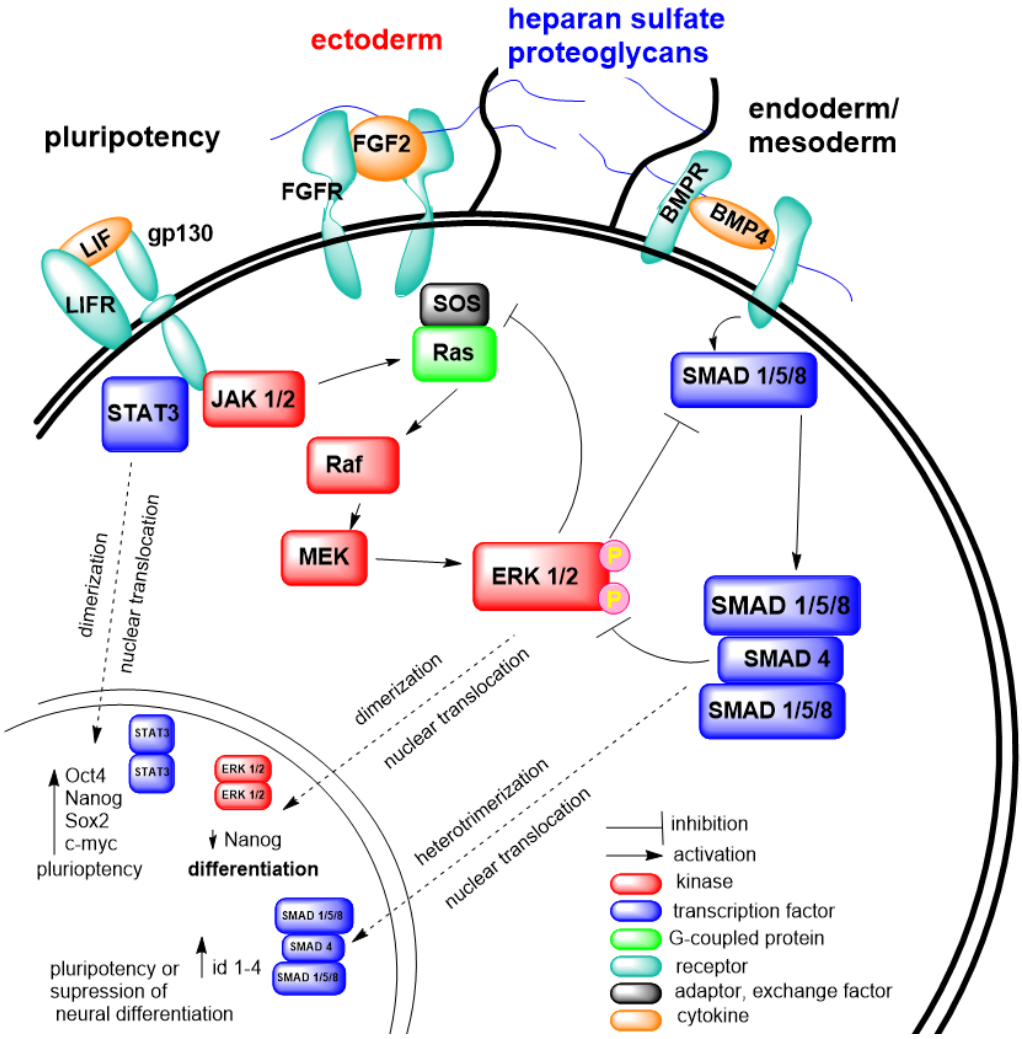



**Fig 1.2 Signaling pathways which regulate mESC fate.** Interactions between pathways which regulate mESC fate. LIF is the primary pathway which maintains pluripotency, however it mildly activates the MAPK/ERK pathway. BMP4 signaling directly opposes this by suppressing ERK signaling via DUSP9. Together LIF and BMP4 maintain a pluripotent state in mESCs.

When LIF binds to its extracellular receptor, gp130 is recruited to the membrane to form a heterodimer (16). This heterodimer actives janus kinase (JAK) which subsequently phosphorylates four tyrosine residues on gp130, creating a docking site for the transcription factor signal transducer and activator of transcription 3 (STAT3) (16). Two units of STAT3 are then recruited to the activated gp130 complex where they are phosphorylated on $Tyr^{705}$, to form an activated STAT3 homodimer. The activated homodimer is then imported into the nucleus where it can bind its consensus sequence to activate target transcription factors Oct4, nanog, and Sox2.

LIF also maintains pluripotency through a separate pathway, the LIF/PI3K/Akt pathway. In this pathway, activated JAK leads to upregulation of the pro-pluripotency genes nanog and c-myc via phosphorylation of phosphoinositide 3-kinase (PI3K), which activates the kinase Akt, also known as protein kinase B. Activated Akt then inactivates glycogen synthase kinase 3 (GSK3), which inhibits expression of c-myc and nanog (16). While the TFs Nanog, Oct4, and Sox2 recruit RNA polymerase to initiate transcription adjacent to promoters, c-myc regulates these genes by triggering promoter proximal pause release of RNA polymerase II, affording cells an additional level of gene regulation (15,18).

Finally, one of the less well characterized effects of LIF signaling is the mild activation of the mitogen activated protein kinase (MAPK) pathway, which occurs when activated JAK phosphorylates the tyrosine phosphatase Src homeodomain phosphatase (SHP2), which will then activate the MAPK pathway via growth factor

bound protein 2 (GRB2) (19). The MAPK pathway initiates differentiation, which opposes the pro-pluripotent actions of LIF (16,19). However, LIF activation of MAPK does not trigger a departure from pluripotency. LIF driven MAPK activation is actively mitigated by BMP4 signaling, which suppresses MAPK signaling via the Erk specific phosphatase, Dusp9. Dusp9 deactivates the key MAPK kinase, extracellularly regulated kinase (ERK).

## The role of BMP4 signaling in pluripotency and meso/endoderm differentiation in mESCs

Although it was known that factors present in serum or secreted by feeder cells were indispensable for mESC pluripotency in addition to LIF, it was not until 2003 that the cytokine bone morphogenetic protein 4 (BMP4), was identified as a key regulator of pluripotency. BMP4 signaling activates an ERK specific phosphatase, Dusp9, which directly inhibits ERK signaling (28). A combination of both BMP4 and LIF bypasses the requirement for serum/feeder cells (44).

BMP4 is a member of the transforming growth factor β (TGF-β) family, an ancient and highly conserved cytokine family involved in embryonic development, adult tissue homeostasis, and differentiation (44,45). BMP4 is intimately connected to both mESC differentiation and self-renewal.

In addition to maintaince of pluripotency, BMP4 promotes differentiation into mesodermal and endodermal lineages once differentiation has been initiated. Upon LIF withdrawal, BMP4 signaling upregulates meso/endodermal markers such as

brachyury, Flk1, and Hoxb1, and BMP4 signaling is abundant in the primitive streak, an area of mesodermal tissue differentiation during embryonic development (44).

## The BMP4/SMAD signaling cascade

To initiate signaling, BMP4 dimerizes and binds its receptor, BMPR. It is known that heparan sulfate acts as a co-receptor to promote formation of the activated receptor complex, however, the exact mechanism is yet to be characterized (46). The activated complex then recruits and phosphorylates/activates SMAD1/5/8 proteins, a family of receptor regulated transcription factors. The activated SMAD 1/5/8 can then dissociate and bind SMAD 4 in a 2:1 ratio, and the active SMAD1/5/8: SMAD4 heterotrimer is imported into the nucleus where it regulates transcription of target DNA. One of the main targets of BMP4/SMAD signaling is the inhibitor of differentiation/DNA binding (Id) proteins, which prevent premature differentiation of stem cells (47). BMP is regulated by other pathways, and has also been shown to work synergistically with the Wnt/β-catenin pathway to regulate gene expression.

## The role of FGF signaling in mESC differentiation

It is well known that the cytokines fibroblast growth factors 2 (FGF2), and 4 (FGF4), are linked to the departure from pluripotency and initiate differentiation in mESCs. FGF2 signaling regulates differentiation in pluripotent mESCs via the MAPK pathway. FGF2 also activates the PI3K pathway and PLCγ pathway, however, these pathways ultimately lead to activation of the MAPK pathway. It is known that the MAPK pathway is the primary driving force behind FGF2 induced differentiation,

as MEK and fibroblast growth factor receptor (FGFR) inhibitors exhibit similar effects on the pluripotency regulator, nanog. In absence of adequate FGF2 signaling cells are unable to exit pluripotency (23). Wild type mESCs secrete FGF4 in an autocrine/paracrine manner, and it is reported that $FGF4^{-/-}$ mESCs show reduced potential to differentiate upon LIF withdrawal, highlighting the essentiality of FGF driven MAPK activation for differentiation (6). FGF signaling is shown to suppress the pluripotency regulator, nanog, and attenuates BMP4 signaling via ERK's phosphorylation of the SMAD1 linker region, through regulatory kinase, Smurf1. Phosphorylation of SMAD1's linker region decreases SMAD1 activity and thus downstream signaling (6, 22).

The classical MAPK pathway consists of a cascade of kinases which begins with an extracellular signaling event in which FGF2 dimerizes and binds a dimer of its receptor, FGFR. This activated complex can be termed a "dimer of dimers" and its formation is highly dependent on adjacent glycosaminoglycan (GAG) chains which will be described in detail in subsequent sections. The formation of this activated complex triggers auto-phosphorylation of tyrosine residues on the intracellular domain of FGFR. To initiate the MAPK cascade, activated FGFR recruits the adaptor proteins Grb2, FRS2, and SOS, which results in phosphorylation and thus activation of each member of the kinase cascade in the following order: Ras→Raf→MEK→ERK. Upon activation, a significant population of ERK will dimerize and translocate the nucleus, where ERK will suppress nanog inhibition of pro-differentiation genes.

ERK is a central signal transducer and is implicated in cell proliferation, migration, and homeostasis (43). In addition to nuclear targets, ERK also has over 50 cytoplasmic targets, and must therefore be carefully regulated to maintain proper signaling. To carefully direct ERK signaling, each step in the Ras-Raf-Mek-Erk cascade affords multiple levels of regulation and signal amplification. When Erk is activated, a negative feedback loop deactivates the upstream guanine nucleotide exchange factor, sons of the sevenless (SOS), which suppresses Ras activation and thus Erk. Erk also regulates its own activation by inhibitory phosphorylation of the directly upstream component, MEK (26). MAPK signaling must be carefully regulated because MAPK drives cell proliferation and has profound implications in cancer. For example, 90% of pancreatic cancers involve a Ras mutation (27).

The reciprocal relationship of ERK and BMP4 is an example of how opposing pathways must be carefully balanced to maintain proper biological function. One effect of MAPK/ERK signaling is the negative regulation of BMP4 signaling by activation the regulatory kinase Smurf1, which attenuates SMAD1 activity by phosphorylating its linker region (24,25). Active BMP4 signaling will actively suppress Erk signaling by activation of the Erk specific phosphatase, Dusp9 (28). The relationships between BMP4, FGF2 and LIF signaling are depicted above in figure 1.2.

# 2. Glycosaminoglycans, and their roles in differentiation and signaling

## Glycan overview

Glycosylation is one of most common forms of post-translational modifications, however, due to the nature of glycosylation, it remains poorly characterized. It is estimated that at least 50% of all known proteins are glycosylated in some form, yet we still have much to learn about glycans and their biological roles (2). Glycosylation encodes a vast amount of biologically relevant information that is not revealed in DNA/RNA sequencing. In crystallography, native glycans are often removed when obtaining a crystal structure due to their highly flexible nature, which makes them difficult to resolve. Yet, glycosylation is a tightly regulated process that has great effects on the nature of proteins and binding interactions, protein folding and maturation, as well as development and other biological processes. The collection of glycans and their interactions may be collectively termed the glycome. In this thesis, a particular class of glycans, the glycosaminoglycans, are relevant to FGF mediated MAPK activation.

## Glycosaminoglycans, and their relevance to biological processes

Glycosaminoglycans (GAGs) are a ubiquitous class of long unbranched heteropolysaccharides tethered to a proteoglycan (PG) core protein which are expressed virtually in all mammalian cells. GAGs facilitate a vast number of

biological processes in early development and throughout the mature life of an organism. In early stages of development, GAGs carefully direct tissue specification, and in a mature organism, they regulate and mediate growth factor binding, cell-matrix interactions, cell-cell interactions and they often function as a co-receptor for cytokines, as well as viral recognition elements which control infection (2,9). GAGs represent a major component of the extra cellular matrix (ECM), and they may be membrane bound, or secreted, and may be stored for future secretion (12). There are six principal types of GAGs, divided by the repeating saccharides which comprise them: heparan sulfate (HS) and its highly sulfated form, heparin, chondroitin sulfate, dermatan sulfate, keratan sulfate, and the non-sulfated hyaluronic acid or hyaluronan (13). This work will focus on HS and its highly sulfated analog, heparin.

## Structure of heparan sulfate

Heparan sulfate is an ancient and ubiquitous GAG which has been highly conserved throughout evolution (5). HS is a long and unbranched heteropolysaccharide which is covalently linked to a serine residue by a conserved tetrasaccharide linker consisting of xylose, galactose, galactose and glucuronic acid (GlcA). The linker region is followed by alternating units of glucuronic acid (glcA), or its C5 epimer, idouronic acid (IdoA) and N-glucosamine which is usually N-acetylated (GlcNAc) or N-sulfated (GlcNS). HS has a highly heterogeneous structure with long N-acetylated (NA) regions containing high amounts of GlcNAc, punctuated by shorter regions where GlcNAc has been N-deacetylated/N-sulfated to generate GlcNS (NS). Each NS region is flanked on either side by a boundary region of intermediate

sulfation, NA/NS regions (5, 8). The long NA regions of HS may provide increased flexibility so the bioactive NS regions can interact without conformational hindrance, and furthermore this flexibility may allow multiple interactions along the same HS chain which are independent of each other (14).

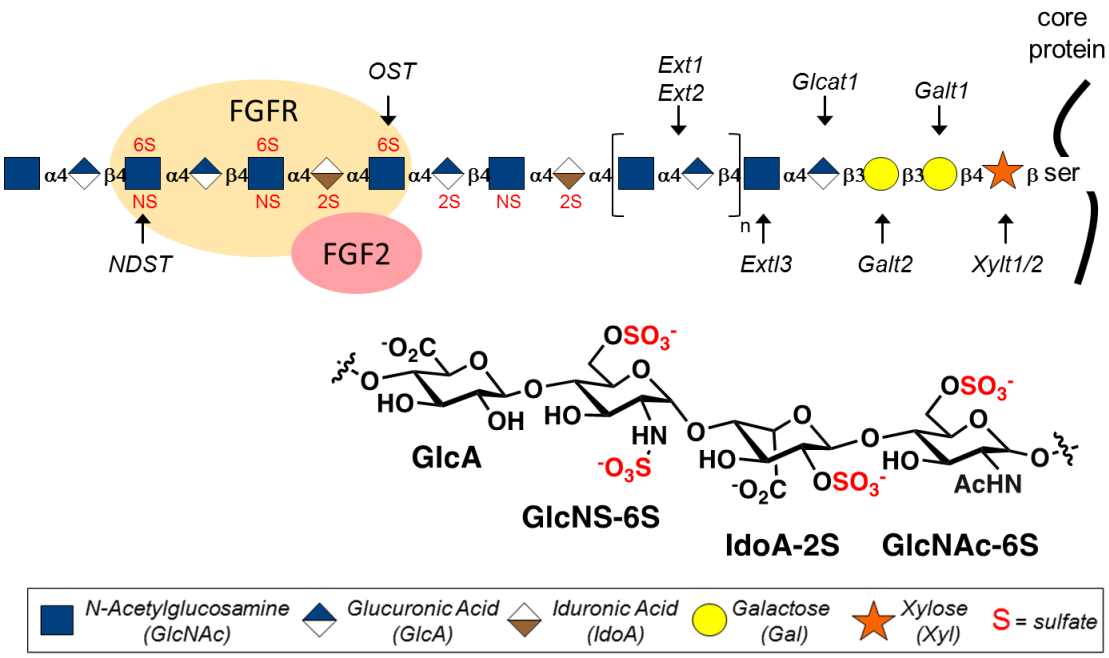



**Fig. 1.3 Structure and modification of HS.** Unmodified HS consists of alternating GlcNAc and GlcA saccharides. In the golgi, HS undergoes 2-O-sulfation and epimerization of GlcA to 2-O-IdoA, and 6-O sulfation N-sulfation of GlcNAc. The sulfation pattern of HS can preferentially bind growth factors and thus encode functional information. Figure adapted from (55).

## Biosynthesis of heparan sulfate

Biosynthesis of HS takes place primarily in the Golgi and is a concerted effort of over 20 HS-specific enzymes which initiate, extend and modify the growing HS chain (9). The biosynthetic enzymes utilize the uridine diphosphate (UDP) conjugated

monosaccharides to elongate the chain. All biosynthetic proteins are type II transmembrane proteins, with the exception of OST-1(9). HS chain linkage consists of a conserved tetrasaccharide linker to a serine residue of the peptide backbone, usually flanked by one or more acidic residues (11). Initiation begins when a xylosyltransferase covalently attaches UDP-xylose to a serine residue, resulting in an O-linkage (5,9,11). Once the polysaccharide chain is initiated by xylose, galactose transferases I, II and GlcA transferase add two UDP-Gal, followed by a UDP-GlcA. Once the linker region is complete, a commitment of this intermediate to HS occurs when GlcNAc is added by exotosin glycosyl transferases I and II (EXT1 and EXT2) (11). EXT1 and EXT2 elongate the HS chain by adding alternating residues of GlcA and GlcNAc to the non-reducing end of the chain (11,5).

## Modification of heparan sulfate: de-acetylation, sulfation, and epimerization

After initiation and elongation of the HS chain, HS undergoes extensive remodeling and modification which results in a heterogeneous distribution of NS domains interspersed among the NA regions. Also, the fine structure of HS varies depending upon which tissue the HS is produced in. For example, the rare moiety GlcA-GlcNS3S is critical for binding anti-thrombin and it occurs predominately in endothelial cells and mast cells, and is seldom found elsewhere (11).

Once the core saccharide chain of HS is elongated, remodeling begins with N-deacetylase/ N-sulphotransferase (NDST), which deacetylates GlcNAc and replaces

the acetyl group with N linked sulfate groups, generating GlcNS (5,11). The sulfate donor molecule used by NSDT is 3'-phosphoadenosine-5'-phosphosulfate (PAPS). After deacetylation and sulfation, a C-5-epimerase may epimerize the C5 hydroxy group to generate iduronic acid, (IdoA). Finally, the highly variable sulfation patterns of HS arise from O-sulfotransferases (OST) consuming PAPS to add sulfate groups to various carbons on saccharide residues. Modifications are made at the C2 hydroxy group of IdoA or GlcA, at C6 of GlcNAc and GlcNS residues, and less frequently at C3 of GlcN residues (5). There are at least four NDSTs, five 3OSTs and 6OSTs known, all of which catalyze the same reaction, but with different affinities and kinetics. The subtle differences in isoforms and UDP-monosaccharide availability may account for the diversity of sulfation patterns found in HS, and the incomplete modification gives rise to the NA NA/NS and NS regions. For example, the four NDSTs catalyze the same reaction but differ in the amount of N-deacetylase and N-sulfotransferase activity, and furthermore, the expression of these genes follows a tissue specific and developmentally regulated GAG modifications (11).

## Heparan sulfate is essential for BMP and FGF signaling

Both FGF and BMP signaling pathways require extracellular glycosaminoglycans for significant signaling events to occur, and it has been shown that HS is essential for mESCs to exit self-renewal and initiate differentiation (21). It is estimated 80% of GAGs produced in mESCs are HS, and as differentiation progresses in mESCs, their GAGs become increasingly sulfated (8,9).

Heparan sulfate is necessary for effective FGF and BMP signaling. The interactions between FGF, FGFR and HS are relatively well characterized. While it is known that HS is required for effective BMP signaling events, this interaction is not as well characterized (29,30).

Heparan Sulfate, specifically sulfated/modified regions of HS, are required for activation of FGFR by many different FGFs, however the nature and specific sulfate moieties required for may differ between FGFs (31). FGF2 is implicated in commitment to differentiation, and binds FGFR with high affinity when HS chains containing 2-O sulfated idouronic acid/glucuronic acid and 6-O sulfated GlcNAc residues are present (32,33). These sulfate groups of HS facilitate binding by promoting electrostatic and hydrogen boning interactions within the basic cleft of the FGF2/FGFR complex. The interaction strength of HS is only moderately sensitive to salt concentration, suggesting that non-ionic interactions contribute significantly to binding (49). It should also be noted that sulfation is not the only effector of HS/FGF/FGFR interactions, the epimerization of glucuronic acid to idouronic acid influences the stereo chemical presentation of the carboxylic acid which is critical in binding events. For example, it has been shown that knockout of the epimerase responsible for this transformation leads to aberrant and delayed FGF2 binding and MAPK activation (34). In the active complex, two molecules of FGF2 dimerize with two FGFR proteins on the cell surface to create a “dimer of dimers”. Heparan sulfate

chains from adjacent proteoglycans lie in-between these proteins to form an activated heteropentamer (29), as shown in figure 1.4.

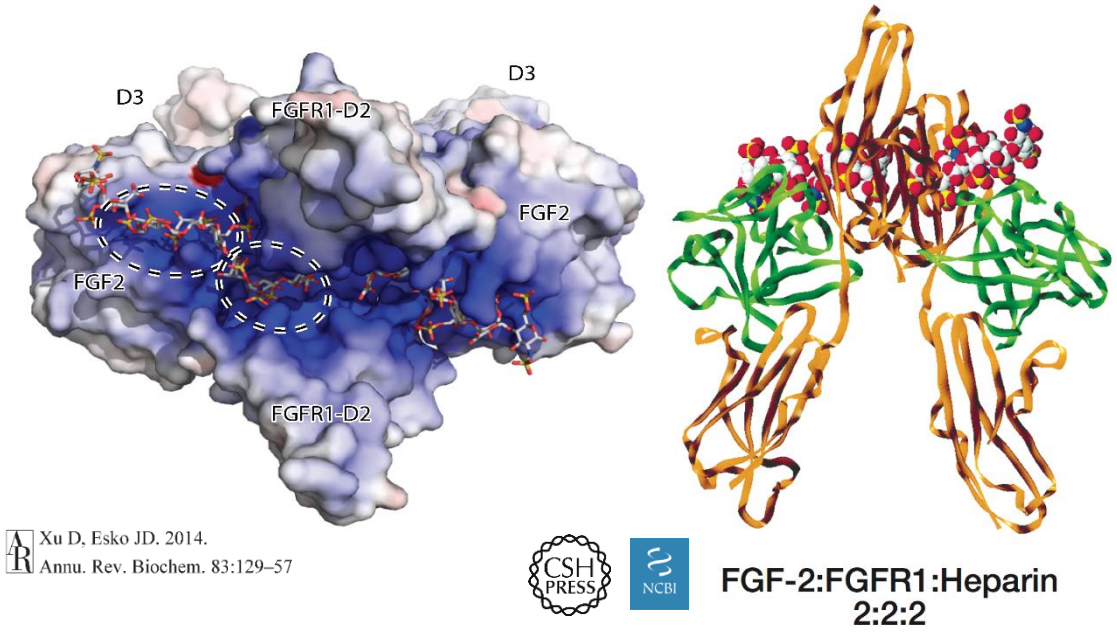



**Figure 1.4 Crystal structure of HS/FGF2/FGFR complex**. HS is a high affinity co-receptor for FGF2/FGFR interactions. A) A basic surface in FGF2/FGFR complex accommodates HS interactions. High affinity HS binding sites are circled. B) HS facilitates FGF2/FGFR complex formation, shown in green and orange, respectively. as well as hydrogen binding contribute to formation of "dimer of dimers" and subsequent MAPK activation.

Since HS GAGs are essential for FGF2 driven MAPK activation which regulates the process of differentiation, it is feasible to imagine the possibility of influencing or manipulating differentiation by removal or manipulation of native GAGs. A powerful strategy for influencing differentiation is the removal of native glycans and remodeling the glycocalyx, which has been shown to direct stem cell fate (3, 54).

## 3. Methods of GAG manipulation

The role glycans in ESC differentiation is currently a topic of increasing interest, as they hold potential to influence the differentiation process. Being able to control or influence the outcome of differentiation is essential for realization of ESC based therapeutics. To understand the effects of glycans on differentiation, the native glycans of mESCs must first be selectively removed or inactivated so they cannot participate in cellular signaling processes. There several chemical and biological methods available to the modern scientist to accomplish this.

### 3.1 Genetic methods: EXT1$^{-/-}$ cells are devoid of heparan sulfate

The presentation of GAGs can be manipulated genetically, by knocking out any of the concerted biosynthetic enzymes that produce or modify GAG chains. Recent developments in CRISPR/CAS9 technology currently enables researchers to edit the genome with unprecedented ease and precision. This affords a powerful tool for researchers, as interference with native proteins involved in elongation or modification of GAGs produces dramatic effects on the biological processes they regulate. The EXT1$^{-/-}$ mutation knocks out a key enzyme responsible for elongating the linear HS chain, and thus would give rise to cells which are severely deficient of functioning HS. The EXT1$^{-/-}$ mutation is lethal in embryonic mice by day 8.5, underlining the importance of HSPGs in development (10). However, a line of mESCs was isolated and stabilized in culture from EXT1$^{-/-}$ mice prior to lethality. The advantage of this cell line is that HSPGs, and the interactions they facilitate are

abolished, giving researchers a “HS-free slate” to work with. Notably, making genomic alterations which abolish HS production differ in enzymatic and chemical methods of inhibition in the fact that they permanently abolish cellular HS, while many enzymatic or chemical methods may transiently or incompletely abolish functional HS.

## 3.2 Enzymatic methods: heparinase treatment

Another way to alter GAGs on the cell surface is to enzymatically degrade them using heparinase, also known as heparin lyase. Three isoforms exist, which are known as heparinase I, II and III, the function of which is to enzymatically cleave heparin/ high sulfated HS, HS, or both, respectively (56). Enzymatic treatment of cells will transiently and incompletely remove HS chains, which may be inadequate if complete abolition of HS is required.

Enzymatic treatment with heparinases is commonly employed to yield biologically active fractions from HS preparations. Following digestion, heparan sulfate oligosaccharides are subjected to column purification to fractionate HS fragments of similar size or sulfation. One drawback to this approach is the inherent heterogeneity of HS prepared in this manner.

## 3.3 Chemical methods: HS antagonists and biosynthesis inhibitors

Today, there are several proteins and small molecules available to transiently antagonize heparan sulfate interactions in biological systems. Proteins or protein

mixtures, such as lactoferrin and cationic peptide mixture, protamine, bind HS and heparin with μM to nM affinities, and depending on the source, may display heterogeneity. Today, protamine is administered therapeutically to patients suffering from heparin overdose, but the high concentration required is less than ideal, some patients may be at risk off target effects (35). Surfen, a small molecule which antagonizes HS in the μM range, has been shown to reversibly antagonize HS. Surfen holds promise as a tool to influence cellular differentiation by modulating HS dependent signaling.

Additionally, small molecules which disrupt the biosynthesis and modifications of HS are useful tools to remove or reduce the functional HS produced by cells. It has been known for several decades that sodium chlorate in the mM range inhibits the of production of high energy sulfate donor molecule, PAPS. Chlorate achieves this by inactivating ATP-sulfurylase, the protein responsible for PAPS synthesis (50). However, to achieve inhibition of sulfation, a high concentration of chlorate may be required, up to 30mM in cell media. The high concentration of chlorate required for sulfurylase inhibition makes chlorate treatment unsuitable for in vivo and many in vitro applications.

There are several small molecules which interfere with enzymes responsible for HS or GAG chain elongation. Fluoroxyloside decoys can target the xylotransferase which is responsible for xylose linkages. Xylose is the first saccharide added to the critical tetrasaccharide linker region which precedes elongation of all GAG chains (51). Using this decoy approach, a moderate reduction in cellular GAGs can be

achieved with mild cytotoxic effects. A possible method to overcome incomplete HS inhibition by Fluoroxyloside decoys is the development of a small molecule which covalently inhibits specific proteins responsible for GAG biosynthesis.

## 4. High-throughput analytical tools to dissect glycan interactions and function

Given the nature and complexity of glycan interactions, there are several challenges to thoroughly understand these interactions and their biological relevance. Ideally, a high throughput screening platform would allow researchers to systematically untangle this intricate web of interactions while simultaneously determining their biological relevance. In this project, I aim to dissect the effects of glycan interactions directly on MAPK activation using a microarray screening platform. Considering the precedent established by others, immobilized glycans can influence FGF2 induced MAPK signaling, it could be possible that differentiation can be carried out on a microarray platform containing immobilized glycans (36).

Glycan Microarrays have previously been used to measure interactions of glycans, such as binding affinities with a variety of substrates (4). While this provides a relevant tool to profile glycan binding, a cellular microarray is required to directly asses the biological outcomes of glycan mediated interactions in mESCs. A cellular microarray consists materials suited for cellular growth robotically printed in a defined pattern, usually on a non-adherent surface. Biological changes can be assessed utilizing immunocytochemistry or via fluorescent reporter proteins. There are special

considerations when fabricating cellular microarrays such as substrate stiffness, three dimensional character of colonies, seeding density optimization to avoid colony overgrowth, and passivation of the areas of the array which are not spotted with adherent cellular substrate (37,38). In these ways microarrays differ significantly from traditional monolayer culture. Special consideration must be given for mESC applications, as ESC cell lines are generally more sensitive to environmental conditions.

As a strategy to direct stem cell signaling and thus fate, glycans may be immobilized to specific spots in the array, where the effects of those glycans on signaling process may be monitored. It may very well be possible to direct stem cell fate by glycan presentation to cell lines which lack functioning native GAGs. In this work, I will develop an approach to assay the MAPK signaling which leads to differentiation in mESCs.

## Cellular growth on microarrays and passivation strategies

There are several strategies to passivate the background areas of a cellular microarray, such as covalently bound amino-polyethylene glycol (A-PEG), BSA passivation, and coating the slide with highly non-adherent acrylamide coating (38,39). For this work acrylamide was chosen due to superior resistance to cellular growth, and using an approach pioneered by Karl Willert's group and optimized by my fellow graduate student Greg Trieger and undergraduate Kelsey Krug, microarray slides were activated and then coated with an acrylamide gel (40). Chemical etching

with dilute base, followed by glutaraldehyde activation makes the glass surface bind tightly to the acrylamide gel. However, the one caveat of acrylamide coating is the thickness of the coating must be as uniform as possible to produce colonies which are of equivalent heights from the glass slide for optimum imaging.

Once slides are passivated with acrylamide, adherent surfaces are then spotted onto the slide. Gelatin, which is denatured animal collagen, provides an excellent substrate for cell growth. The stiffness, or bloom factor, of the gelatin and host animal must be optimized for mESC adhesion. In this work, Pig gelatin of bloom factor 180 was printed on acrylamide coated slides. The combination of acrylamide passivation with gelatin printing affording equal diameter spots with little off target cell growth.

It has been shown that if the stiffness of the substrate is increased, stem cells are more likely to differentiate into primitive osteoblasts, which would be found growing in a stiff substrate such as bone in vivo. On the opposite end of the spectrum, a softer substrate promotes neural differentiation outcomes, as these characteristics may mimic soft tissues found in the brain (48). Perhaps this could be exploited to influence the outcome of differentiation.

Another factor which differentiates microarray culture from traditional culture is the potential for 3D growth in microarrays. On plastic cell culture plates, cells will often spread out and “lie down” across the plate as they grow. In microarrays, cells are unable to spread out in an unrestricted manner, and as colonies grow on printed

substrate, cells will begin to grow upwards on top of each other. It is yet to be determined how this plays a role of visualizing glycan interactions in microarrays.

For this work, an approximately monolayer colony of mESCs is optimal, this enhances imaging fidelity by keeping the fluorescent reporters in the same focal plane. This enables the laser scanning, or fluorescence microscopy to simultaneously focus on all fluorophores for proper imaging. It is worth noting that confocal microscopy or similar methods may be able to circumvent this issue if the need arises.

One of the long term goals of this project is to establish a cellular microarray in which the effects of glycan mediated signaling on differentiation can be fluorometrically investigated in a high-throughput manner.

# II. Materials and Methods

### Cell culture

All mouse embryonic cell lines were cultured feeder free in treated plastic well plates (Corning Costar) at 5% $CO_2$ and 37°C. Cells were cultured in mESC maintaince media consisting of KO-DMEM (Gibco, Cat. #10829-018) media supplemented with 10% fetal bovine serum, non-essential amino acids (NEAA, Gibco, Cat. #11140-050), L-glutamine (1% of 200x solution, Gibco, Cat. #25030-081), 2-mercaptoethanol (Gibco, Cat. #21985-023) and LIF (Millipore ESGRO, 1000U/mL). Serum free media is of identical composition to mESC maintaince media except for the exclusion of FBS. Cells were passaged every other day and spilt at a ratio of 1:10 ($10^5$ cells). When experiments required large number of cells, cells were expanded under similar conditions using T25 or T75 flasks (Corning, treated).

### Growth factor stimulation

Cells were seeded either 6 well plates at a density of $10^5$ cells/$cm^2$ or onto microarray wells at a density of $4x10^4$ cells/$cm^2$ and allowed to adhere for 24 hours in mESC maintaince media. Cells were then subjected to serum free conditions overnight. Following serum starvation, cells were washed with DPBS (Corning cellgro, Cat. #21-031) and treated with serum free media containing various amounts of FGF2 (Gibco, Cat. #PHG0264) with or without 5µg/mL heparin (Acros Organics, Cat. #411212500). Immediately following starvation, cells were returned to the

incubator for 15 mins. After this incubation period, cells were placed directly onto ice and either subjected to western blotting or immunocytochemical protocols. (see below)

**Western blotting**

Following incubation with FGF2, media was aspirated and cells were washed with 4°C PBS. Cells were lysed with cell lysis buffer (cell signaling, Cat. #9803) containing 1mM PMSF (cell signaling, Cat. #8553) and 1x protease inhibitor cocktail (cell signaling, Cat. #5872) and allowed to sit for 10 minutes prior to scraping with cell scraper (Corning, Cat. #353085). Lysates were centrifuged to remove cell debris and protein in supernatant was quantitated using a BCA assay kit (Thermo, Cat. #23225).

5µg of protein was then resolved on a 10% SDS-PAGE gel and blotted onto pre-wetted immobilon-FL 0.45µm PVDF membranes. Prior to immunostaining, membranes were blocked for 1 hour at room temperature in 5% BSA (Spectrum, Cat. #A3611) in tris-buffered saline with 0.1% (v/v) tween-20 (TBST) (Fisher, Cat. #BP337). Primary antibody incubations were conducted overnight at 4°C. Membranes were then washed 3x with TBST and incubated with the appropriate HRP conjugated secondary antibodies for 1 hour at room temperature. Membranes were then washed 3x with TBST, and Luminata forte HRP detection reagent was applied. Membranes were exposed to ECL amershaw hyperfilm.

**Table 2.1 Antibodies used in western blotting.** All antibodies were purchased from Cell Signaling Technology.

| Epitope | Clone # | Lot # | dilutions | Cat. # |
|---|---|---|---|---|
| Mouse α tubulin | DM1A | 8 | 1:10,000-1:35,000 | 3837 |
| Rabbit Phospho-ERK | D13.14.4E | 15 | 1:4000-1:5,000 | 4370 |
| Rabbit Total-ERK | 137F5 | 14 | 1:4000-1:5000 | 4695 |
| Anti-Rabbit-HRP | N/A | 26 | 1:10,000 | 7074 |
| Anti-mouse-HRP | N/A | 29,31 | 1:10,000 | 7076 |

For sequential antibody labeling, membranes were stripped of antibodies using restore PLUS Western blot stripping buffer (Thermo, Cat. #46430) for 25 minutes at room temperature. Membranes were then washed 3x with TBST and blocked with 5% BSA in TBST for 1 hour at room temperature prior to antibody staining.

Following membrane visualization, densitometry was performed using ImageJ software (National Institute of Health). A box tool was used to select individual lanes and dark bands were plotted as peaks against a white background using a white to black standard curve. Peaks were then differentiated from background using a line tool and half peak area was taken for the peak of interest. and darkness of bands was quantitated. The pERK and tERK densities were each normalized to alpha tubulin and then the ratio of (pERK/ α tubulin)/(tERK/ α tubulin) was calculated for each lane. Experiments were then normalized to negative controls to determine the relative pERK fold increase of each lane to that of negative control, usually unstimulated cells.

**Immunocytochemistry**

After stimulation cells were immediately washed with cold DPBS and fixed for 10 minutes at room temperature in 4% paraformaldehyde. Then cells were washed 3x with cold PBS and cellular membranes were permeabilized using cold methanol for 20 minutes. Cells were then washed 3x with PBS and blocked for 1 hour at room temperature with ICC blocking buffer (3% (w/v) BSA, 2% (v/v) goat serum. The appropriate primary antibody was applied overnight in ICC blocking buffer at 4°C. Cells were washed 3x with PBS and corresponding secondary antibodies were applied for 1 hour at room temperature. Cells were washed 3x with PBS and nuclei were stained with 4',6'-diamindino-2-phenylindole (DAPI) for 15 minutes at room temperature. Cells were then washed 3x with PBS and mounted overnight at room temperature using ProLong Gold antifade (cell signaling, Cat. #9071). The next day, cells were subjected to ICC imaging using a Zeiss Axio optical microscope equipped with a Lumen Dynamics X-cite fluorescent lamp.

**Table 2.2 Antibodies used for immunocytochemistry in microarrays.** All antibodies were purchased from Cell Signaling Technology.

| Epitope | Clone # | Lot# | dilutions | Cat. # |
|---|---|---|---|---|
| Rabbit Phospho-ERK | D13.14.4E | 15 | 1:200-1:300 | 4370 |
| Mouse Total-ERK | L34F12 | 16 | 1:200-1:500 | 4696 |
| Anti-mouse-AF555 | N/A | 13 | 1:1000 | 13 |
| Anti-Rabbit AF647 | N/A | 8 | 1:1000 | 8 |

**Microarray experiments**

Microarrays were prepared as previously described elsewhere (40,35). In brief, Schott Nexterion uncoated glass slides were obtained and washed with milliQ $H_2O$, acetone, and the methanol before being cleaning in 0.05 M NaOH for 1 hour. Slides were then subjected to methacrylate silanization in 98% toluene,2% 3-(Trimethoxysilyl)propyl methacrylate solution for 1 hour, and rinsed and dried. After silanization, slides were activated with a 0.5% glutaraldehyde solution for 1 hour and washed thoroughly. An 10% acrylamide gel coating was fabricated on top of the activated glutaraldehyde surface a covered with a coverslip. Gelatin was printed using a sonoplot GIX microplotter desktop non-contact printer equipped with a piezo dispenser tip, or an ArrayIt printer, courtesy of Varki lab, biomedical facility at UCSD. NHS heparin was synthesized by first creating an activated ester using heparin (Carbosynth, OH09354) and EDC-HCl (Aldrich, cat#03449). Then NHS-heparin was made using N-hydroxysuccinimide (Aldrich, cat#130672) and coupled activated EDC-heparin. NHS-heparin was then coupled to primary amines present in gelatin to covalently immobilize heparin. Microarrays were analyzed using an Axon GenePix 4000B microarray scanner (molecular devices), equipped with a Cy3 and Cy5 filter (which correspond to the fluorophores AF555 and AF647).

# III. Experimental Results

The goals of this project are:

1. **Visualizing effects of GAG micro-environment on MAPK signaling in mESC model in microarrays**
2. **Modulating GAG interactions in wild type mESCs with small molecules**

## 1.1) Construction and design of cellular microarrays with immobilized glycans

Careful consideration must be given to design, construction, and experimental conditions when constructing cellular microarrays, which will be optimized for each cell line. As previously stated, microarrays were fabricated using a modified procedure developed by Karl Willert's group, and optimized by my colleague Greg Trieger (35). Briefly, glass microarray slides were etched with sodium hydroxide, and then coated with methacrylate, which is then converted into a reactive glutaraldehyde surface which can be covered with a layer of acrylamide hydrogel, which is an non-adhesive surface to prevent off target cell growth. Before gelatin is robotically spotted onto acrylamide, acrylamide is "snap dried" at 40°C to partially dehydrate the hydrogel. When gelatin is printed on top of a dehydrated hydrogels, adhesive forces which "pull" the gelatin into the hydrogel matrix of the acrylamide.

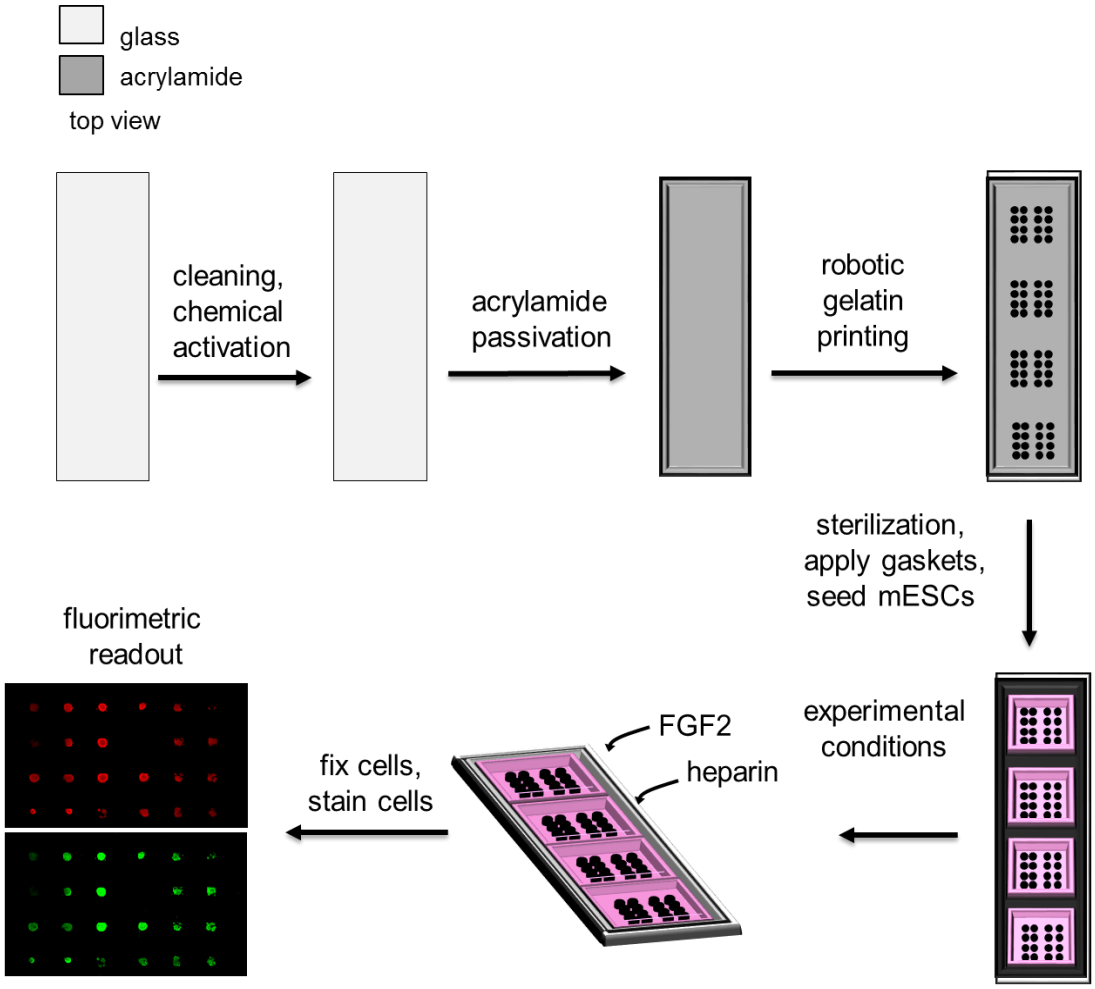



**Figure 3.1 Microarray construction and workflow.** Slides are prepared by chemical washing and activation, followed by acrylamide passivation of the surface to inhibit cell growth. Gelatin is then arrayed onto the acrylamide surface, over which cells can be seeded and cultured on top of well-defined glycan microenvironments.

## Immobilized vs. soluble heparin

It has been previously shown that immobilized glycans or GAGs can affect the GAG mediated processes of cells cultured on top of them. In an approach developed by Jeremy Turnbull's group, fibroblasts lacking sulfated HS were seeded on top of covalently immobilized heparin oligosaccharides (36). An increase in phospho-ERK

was observed via immunostaining, showing that immobilized glycans can affect the signaling processes of cells seeded on top of them.

This approach shows great promise and establishes precedent for influencing HS mediated signaling. However, in previous work, the digested and fractionated heparin oligosaccharides lack a well-defined structure and sulfation motif, which has been shown the be essential for HS to act as an FGF2/FGFR co-receptor (36). Synthetic GAG mimetics of well-defined sulfation and structure have been shown to promote FGF2 signaling in ESCs, and these two approaches may be combined to yield a high-throughput platform for dissecting GAG mediated interactions in mESCs (54).

In this work, to determine the effects of immobilized GAGs on FGF2 induced ERK activation, heparin was covalently appended to primary amines in pig gelatin via an EDC activated ester coupling intermediate. $EXT1^{-/-}$ cells with abolished or functionally diminished GAGs can then be seeded onto the gelatin microarray, which may contain immobilized GAGs or GAG mimetics. The effects rendered by immobilized heparin can be assayed either fluorometrically or by western blot. These immobilized GAGs can be compared to soluble GAGs, which have been shown to robustly affect FGF2 signaling in HS deficient cells (5,20).

## 1.2) Optimization of FGF2 stimulation via western blotting

A primary aim of this project is to fluorometrically visualize FGF2 stimulation assays in a microarray format. However, initial efforts were unsuccessful, as the difference in fluorescently stained phospho-ERK was not detectable between stimulated and control conditions. In order to establish stimulation conditions which would could be successfully implemented on a microarray with detectable differences in signaling, the relative increase in ERK phosphorylation upon stimulation was assessed for several cell lines. Also, the concentrations and time for optimal phospho-ERK stimulation were assessed to maximize the difference between stimulated and unstimulated conditions.

Western blotting is a low throughput technique that is an accepted standard for observing protein levels in biological systems, so to optimize stimulation conditions, I used this technique to observe phospho-ERK induction upon stimulation in traditional culture conditions. Western blotting is also likely to be more sensitive than a higher throughput fluorescent microarray platform, so subtle differences in ERK signaling can be observed with blots which may be difficult to distinguish in a fluorescent microarray. To normalize phospho-ERK levels to the total protein content collected from cell lysates, phospho-ERK was normalized to the house keeping protein alpha tubulin, blots were stripped of antibodies and re-probed for total ERK, which was also normalized to alpha tubulin. The ratio of (phospho-ERK/ α tubulin)/(total-ERK/ α tubulin) could then be quantitated via densitometry and normalized to negative control to compared stimulation conditions.

First, the relative phospho-ERK increase was characterized for three cell lines: Oct4-GFP, EXT1$^{-/-}$ and E14 mESCs. E14 cells are wild type mESCs, while Oct4-GFP cells have a GFP gene co-expressed with master pluripotency regulator Oct4. Both OCT4-GFP and E14 cell lines display wild type HS, and thus should respond similarly to FGF2 stimulation. In contrast to E14 and OCT4-GFP cells, EXT1$^{-/-}$ cells lack functional EXT1 gene and correspondingly, functional HS.

Initially, wild type E14 cells displaying were stimulated with FGF2 to assess the phospho-ERK levels upon stimulation. E14 cells were stimulated with various concentrations of FGF2 to determine which elicits greatest stimulation of ERK (fig. 3.2). Although somewhat counter-intuitive, sub ng/mL doses of FGF2 appear to elicit greater ERK responses, which has been previously reported in literature (41).

It should be noted that in western blots, error bars are generally omitted because it is difficult to average semi-quantitative data from separate blots. Alternatively, total ERK and alpha tubulin levels are used to normalize phospho-ERK for varying levels of protein, and each blot is considered individually. It is curious that 5ng/mL seems to illicit less stimulation than 25 or 0.5 ng/mL FGF2, and this may be due to the semi-quantitative nature of western blotting.

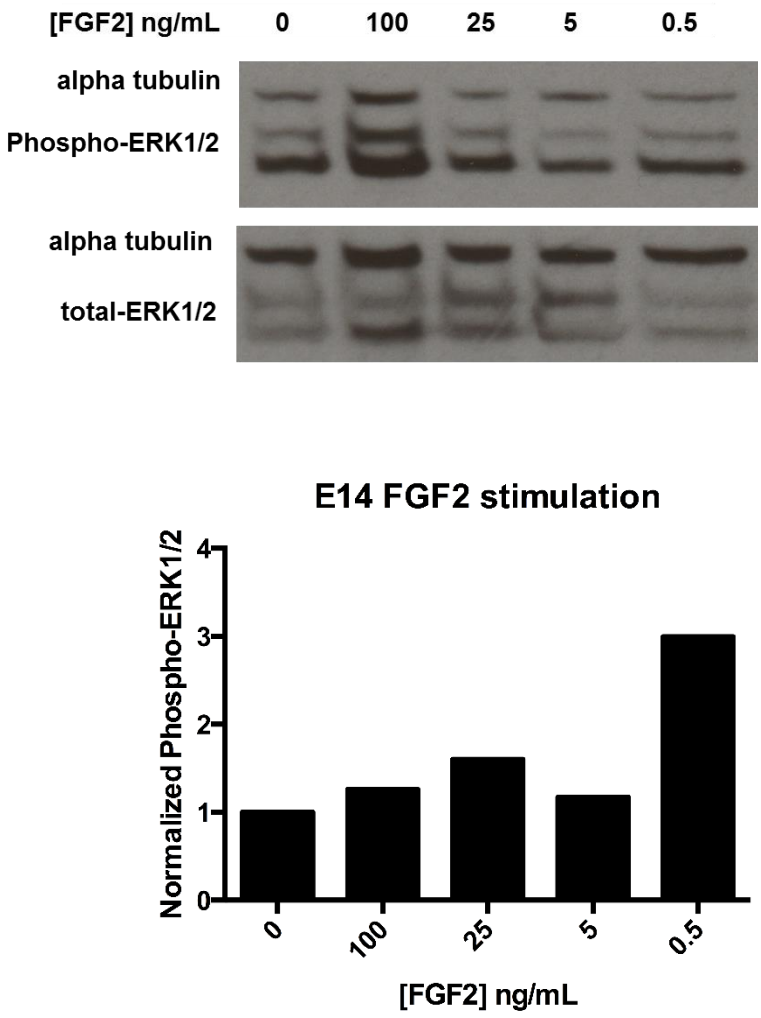
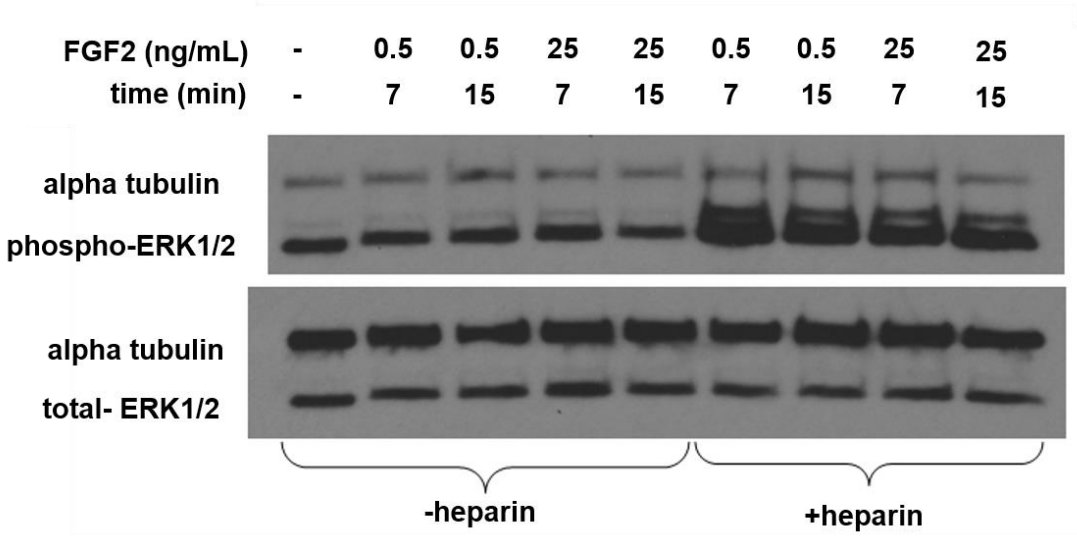



**Fig. 3.2 E14 stimulation with FGF2.** Blots with corresponding densitometry data. Lower concentrations of FGF2 are elicit greater response and MAPK activation. Higher concentrations of FGF2 are sub-optimal for stimulation and at extremely high concentrations of FGF2, an inhibitory effect was previously observed (41).

Considering the observation that 0.5 ng/mL FGF2 appeared to illicit superior phospho-ERK induction in wild type mESCs, EXT1$^{-/-}$ cells were then examined for phospho-ERK activation following FGF2 stimulation. Since EXT1$^{-/-}$ cells do not possess functional HSPGs, FGF2 and FGFR are expected to interact with greatly

reduced affinity- and thus MAPK activation should be greatly reduced. The addition of a highly sulfated HS analog, heparin, mimics the role of HSPGs as a co receptor, thus facilitating high affinity growth factor/receptor interactions. Notably, it was observed that the addition of exogenous heparin in the absence of FGF2 also activates MAPK pathway, however there is strong literature precedent for this observation (20). Exogenous soluble heparin likely activates MAPK synergistically with FGF4, a growth factor which is naturally secreted by mESCs.

ERK activation in $EXT1^{-/-}$ cells was assessed as a function of FGF2 concentration and stimulation time. In agreement with E14 cells, $EXT1^{-/-}$ cells show greater ERK activation in response to lower doses of growth factors. A relatively larger fraction of ERK is phosphorylated at 7-minute time point, likely due to time dependent attenuation of ERK signaling. Negative feedback is an essential feature of MAPK signaling cascades, and additionally phosphatases may decrease the levels of post-stimulation phospho-ERK over time (52).



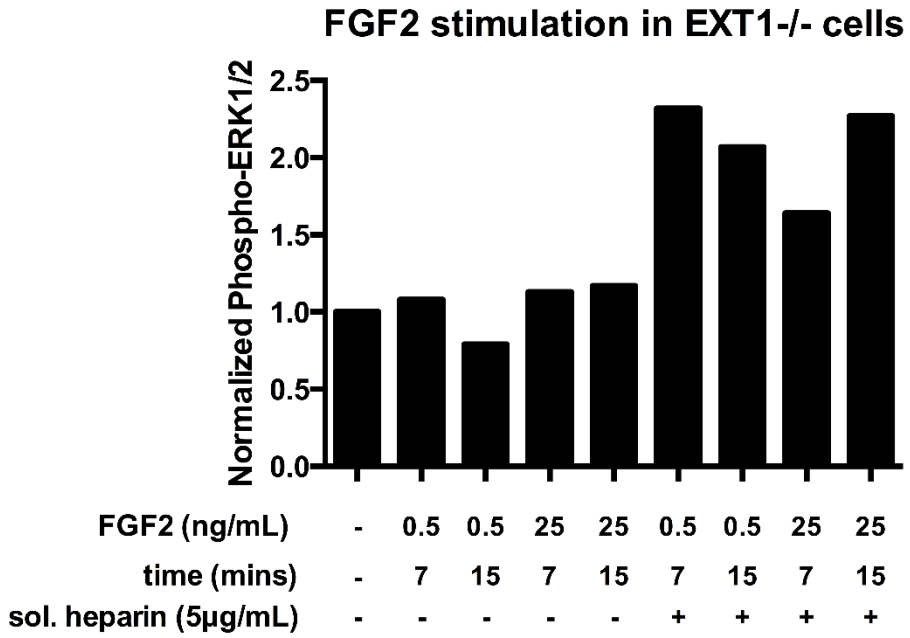




**Fig. 3.3 Stimulation of EXT1$^{-/-}$ cells as a function of [FGF2] and time.** Cells were stimulated with 0.5 ng/mL and 25 ng/mL FGF2 and lysates were collected at 7 and 15 minute time points. The addition of 5µg/mL soluble heparin is essential for a phospho-ERK induction, due to the absence of cell surface HSPGs.

Due to the lack of HS, EXT1$^{-/-}$ cells may have lower basal levels of phospho-ERK prior to stimulation than wild type E14 cells, perhaps the lack of HS reduces phospho-ERK activation due to autocrine signaling by secreted FGF4. For this reason, EXT1$^{-/-}$ cells are better suited for fluorometric analysis in microarrays, as the relative increase for phospho-ERK upon stimulation may be greater than that observed in wild type mESCs.

## 1.3) Fluorometric detection of FGF2 signaling in microarrays

Under optimized conditions, EXT1$^{-/-}$ cells were stimulated with FGF2 in microarrays. Initially, to validate the microarray platform against traditional culture, EXT1$^{-/-}$ cells in microarrays were lysed and then blotted to compare phospho-ERK levels observed in microarrays to traditional culture methods. When preparing microarrays for western blot readout, all gelatin spots in a well must be of identical composition, since all spots on the microarray will be pooled together when cells are lysed. This approach can be used to validate the microarray technology against traditional culture, but the identical spot composition and low throughput make western blots unsuitable as a standard microarray readout. Also, western blots require several µg of protein, increasing the number of cells and regents to carry out biological experiments. Microarrays hold potential as a fast and cost effective platform for screening GAG dependent signaling, but first must be validated using traditional cell culture and standard analysis methods. Here, EXT1$^{-/-}$ cells were scrapped from a microarray and blotted to visualize phospho-ERK levels.

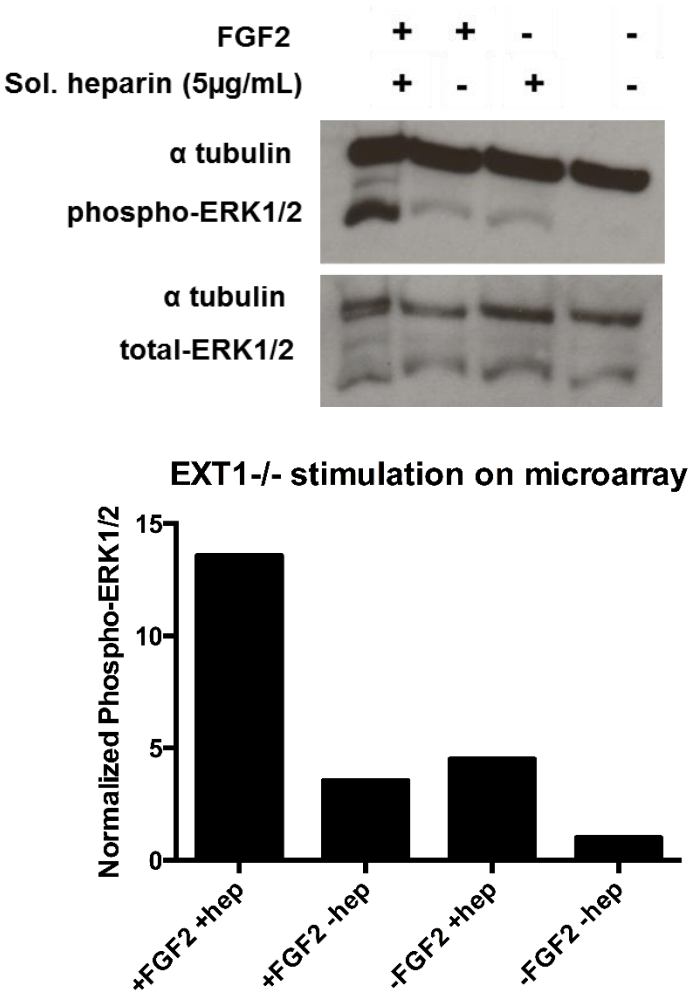
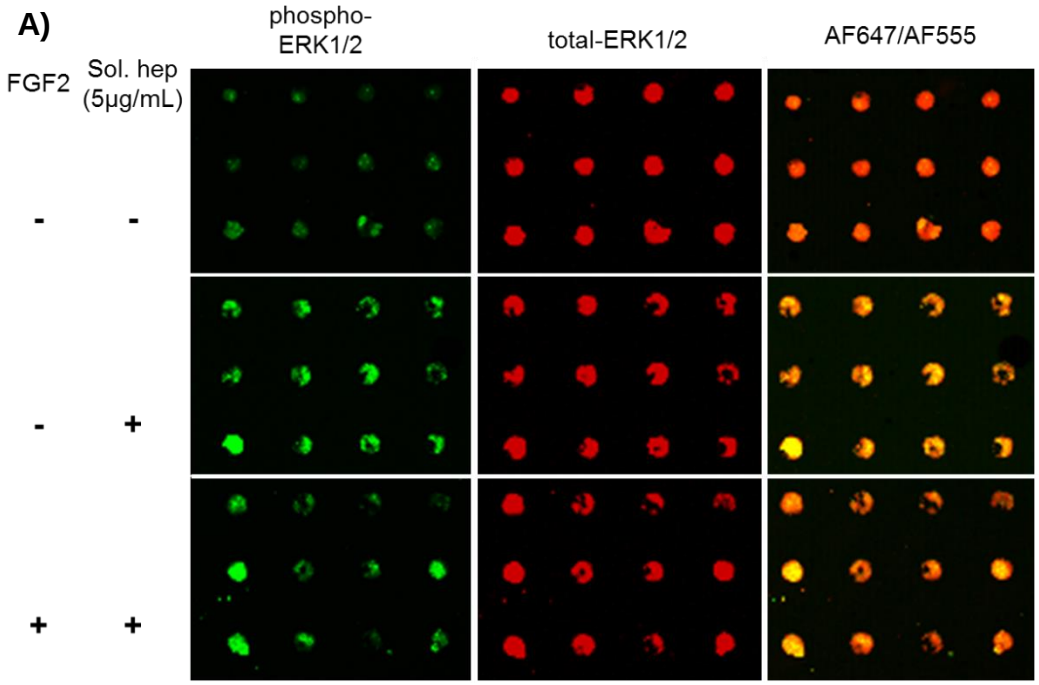



**Figure 3.4 Western blot of EXT1$^{-/-}$ cells stimulated with FGF2 on microarray.** Western blot shows that normalized phospho-ERK levels correspond to those observed under traditional culture conditions. Interestingly, the addition of soluble heparin alone did illicit a robust increase in phospho-ERK1/2.

Western blot analysis confirms that FGF2 stimulations with soluble heparin can restore signaling to EXT1$^{-/-}$ cells on microarrays. When heparin and FGF2 were added to culture media, a robust increase in phospho-ERK was observed. Interestingly, the addition of soluble heparin alone did not induce a large increase in phospho-ERK.

Next, EXT1$^{-/-}$ cells were stimulated on microarrays using a fluorometric readout. The stimulation conditions were identical to the previous western blotting microarray experiments, however for analysis, cells on microarrays were fixed, permeabilized, and stained with primary and secondary antibodies for phospho and total ERK.

EXT1$^{-/-}$ mESCs were seeded on arrays as in western blotting experiment (fig 3.4) but stained for phospho-ERK and total-ERK using AF555 and AF647 labeled secondary antibodies, respectively. Once stained, arrays were scanned with a dual laser scanner to rapidly image them (fig 3.5). After scanning, colonies were individually imaged on a light microscope with a fluorescent excitation lamp. Prior to microscopy, slides were stained with the nuclear stain 4',6'-diamindino-2-phenylindole (DAPI) mounted in an anti-fade mounting agent. Indeed, results did appear to confirm scanning analysis of microarray slides, with +heparin conditions showing a robust increase of phospho-ERK fluorescence compared to -heparin conditions.



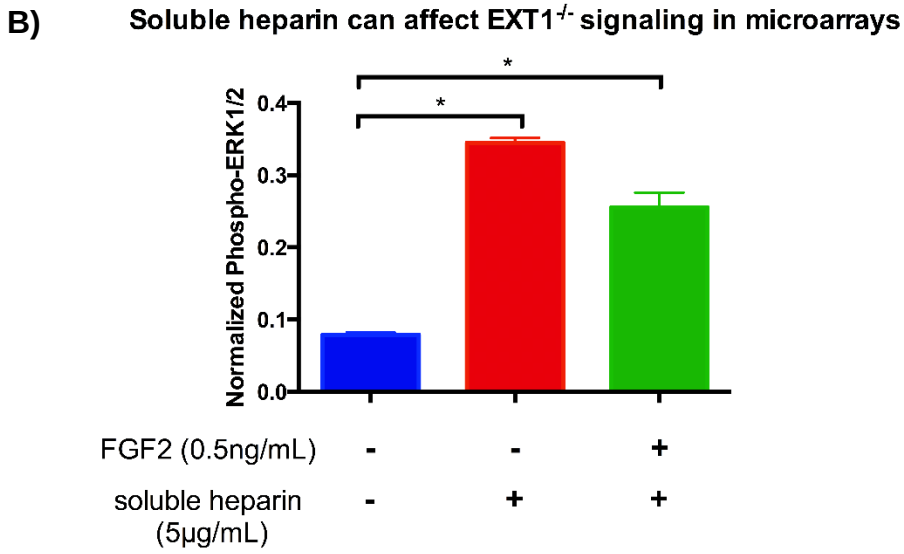




**Figure 3.5 Soluble heparin restores FGF2 signaling of EXT1$^{-/-}$ cells in microarrays** A) Microarray scan showing soluble heparin can affect EXT1$^{-/-}$ signaling in microarrays. Phospho-ERK1/2 stained with AF-555(green) and total ERK1/2 stained with AF-647 (red). B) fluorometric readout of microarray, the ratio of average mean fluorescence of each colony was calculated individually and then the pERK/tERK ratios for each colony were averaged. * indicates $p<0.01$.

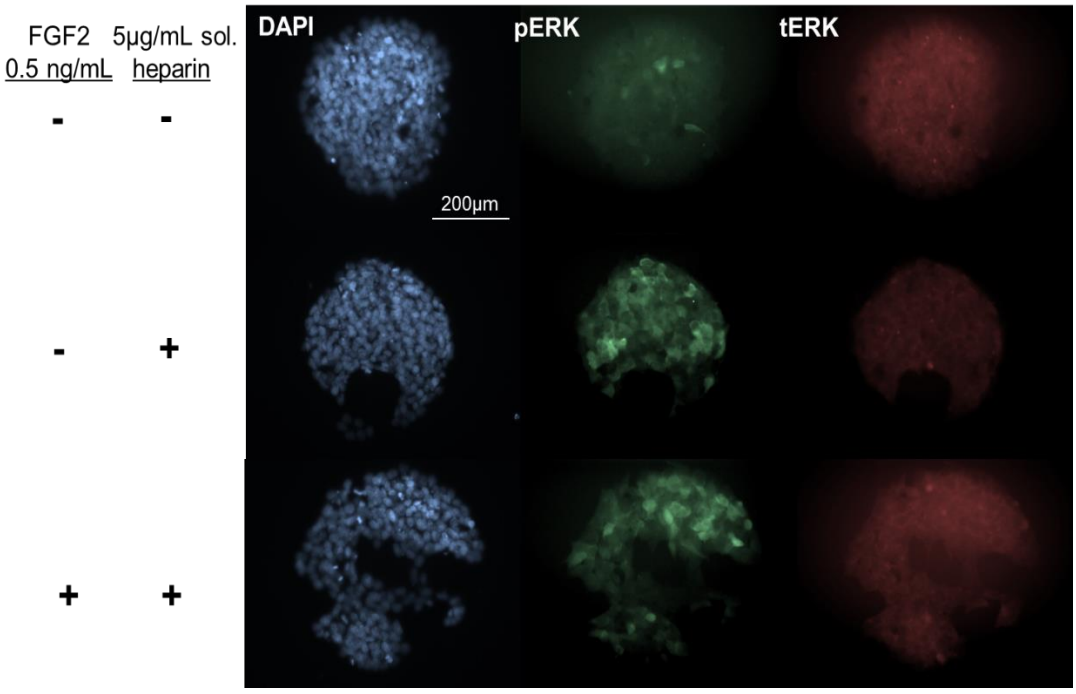



**Figure 3.6 Immunocytochemical staining of microarray**. (same array as shown in figure 3.5). EXT1$^{-/-}$ cells were stimulated with or without 5µg/mL soluble heparin. A robust increase in phosphorylated ERK is observed after stimulation in the presence of 5µg/mL soluble heparin.

EXT1$^{-/-}$ mESCs responded to FGF2 stimulation in the presence of soluble heparin, showing promise for the fluorometric readout of ERK signaling in presence of immobilized glycans. Ideally, mESCs could be seeded onto immobilized GAGs or GAG mimetics to induce effects of HS mediated signaling. To test this approach, heparin was covalently linked to gelatin via a method optimized by my colleague Greg Trieger, and then immobilized heparin could be incorporated into gelatin spots where mESCs are cultured. Preliminary data suggests immobilized heparin can significantly influence FGF signaling.

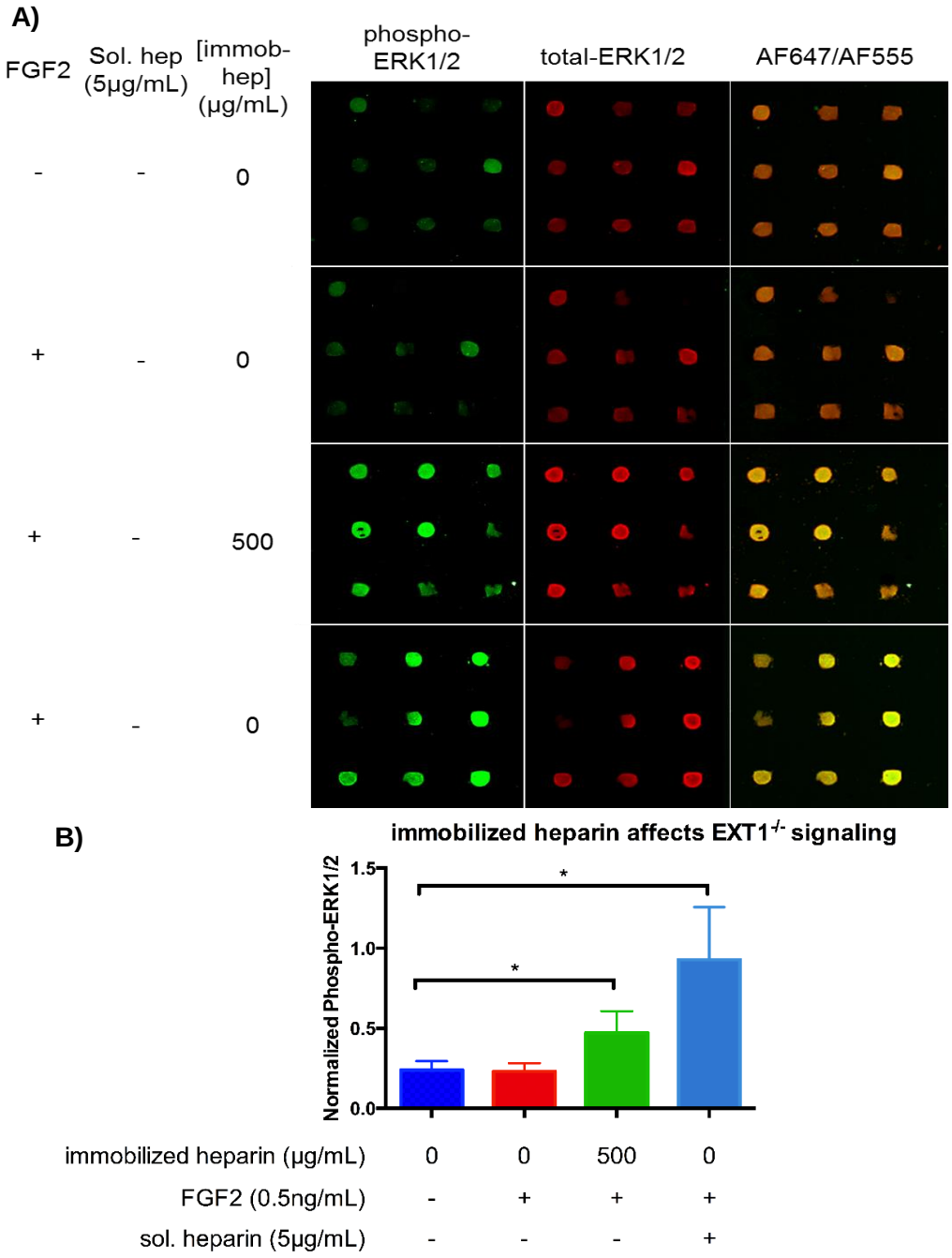



**Fig. 3.7 Immobilized heparin can affect FGF2 stimulation in EXT1$^{-/-}$ cells.** EXT1$^{-/-}$ cells were seeded onto either 0 or 500µg/mL immobilized heparin gelatin and stimulated with 0.5ng/mL FGF2. A) Scan of EXT1$^{-/-}$ cells showing increased phospho-ERK(green) in presence of immobilized heparin. pERK levels were normalized to corresponding tERK levels. B) fluorometric readout shows statistical significance. * indicates $p<0.01$.

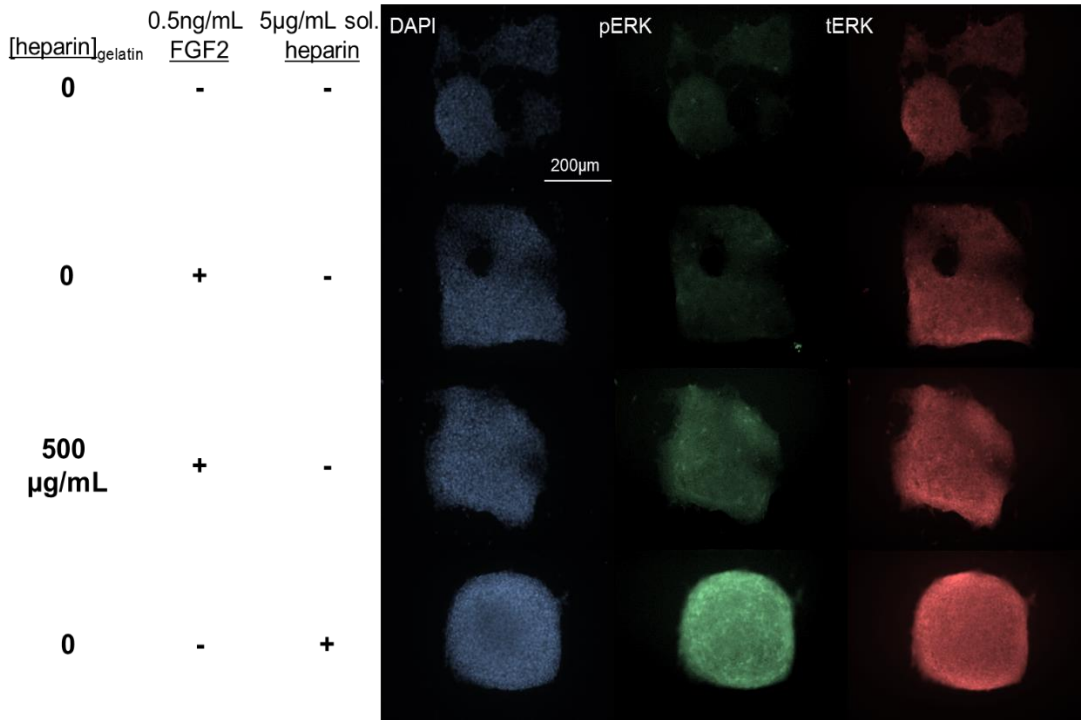



**Fig. 3.8 Immunocytochemical imaging of phospho-ERK in mESCs cultured on immobilized heparin.** $EXT1^{-/-}$ cells were cultured on immobilized heparin and then stained for phospho and total ERK. DAPI was included to visualize total numbers of cells.

## 3. Inhibitors of GAG mediated signaling

The small molecule surfen was recently shown to antagonize FGF2 signaling in Chinese hamster ovary (CHO) cells by the Jeff Esko and Yitzak Tor (35). A talented postdoc in my group, Mia Huang, was screening surfen for its effects on mESC differentiation in traditional culture. I collaborated with her to investigate the effects of surfen on FGF2 induced MAPK activation in mESCs using western blotting analysis.

To begin screening the effects of surfen in FGF2 stimulation, the effects of surfen was compared to a well-known inhibitor of FGFR, PD173074. Both surfen and PD173074 are stored in DMSO, which can be known affect stem cell differentiation (53), so a small amount of DMSO was included to ensure the added DMSO did not affect FGF2 stimulation of ERK1/2.

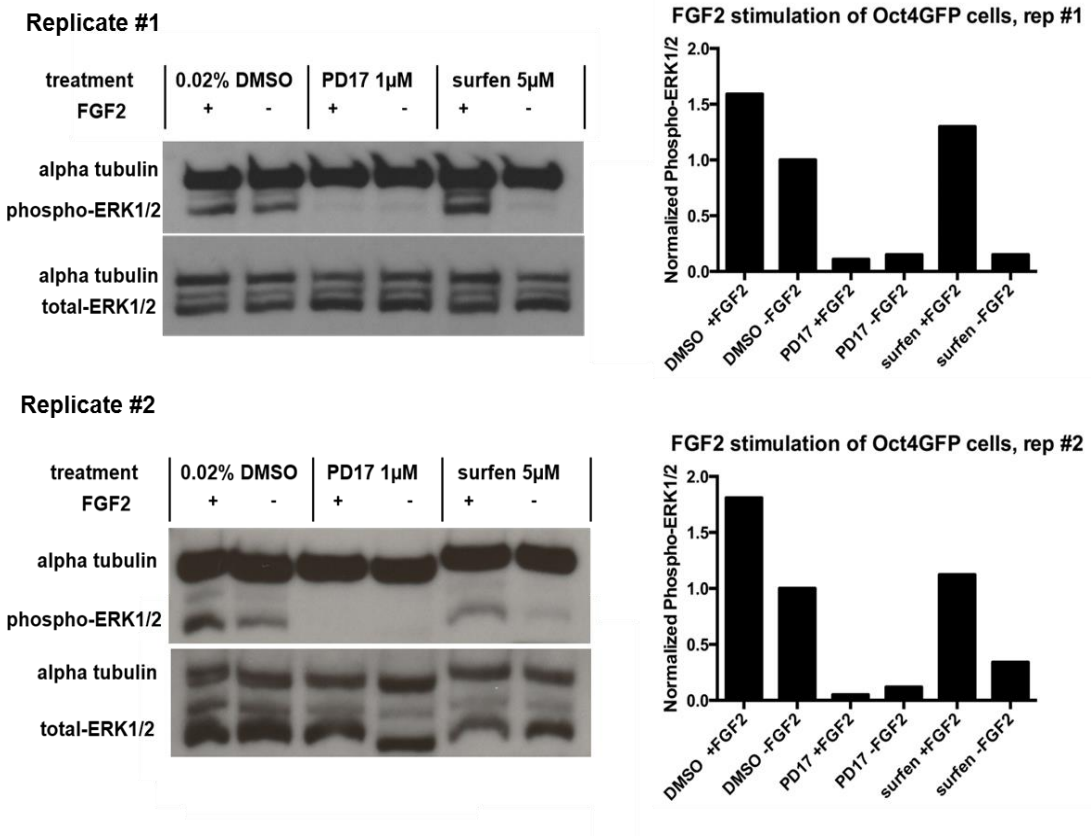



**Fig. 3.9 Oct4-GFP stimulation with FGF2.** Oct4-GFP cells were stimulated in presence of 1.0μM PD173074 and 5.0μM surfen. Cells were stimulated in the presence of 0.02% DMSO as a control, as inhibitors of MAPK signaling are stored in DMSO.

In agreement with E14 mESCs, OCT4-GFP cells respond similarly to FGF2 in the presence of 0.02% DMSO (lane 1, fig 3.9). When the FGFR inhibitor PD173074 was

added to culture, phospho-ERK was abolished with or without the addition of FGF2 (lanes 3 and 4, fig 3.9). This result is not unexpected, however, as the $IC_{50}$ for PD173074 is in the 25nM range, and 1.0μM PD173074 greatly exceeds this. The addition of surfen to media rendered a modest decrease in normalized phospho-ERK at 5.0 μM, as the $IC_{50}$ of surfen for HS is approximately 1.5μM (35). Although replicates vary slightly, trends were conserved.

Since 5.0μM surfen exhibited a modest inhibition of MAPK activation, the response of MAPK activation was assessed as a function of surfen concentration to investigate whether the effects on HS signaling could be dose dependent. At concentrations greater than 5.0 μM, a significant reduction in FGF2 driven MAPK activation is observed, while concentrations of 1.25 and 2.5 μM surfen have little to no effect.

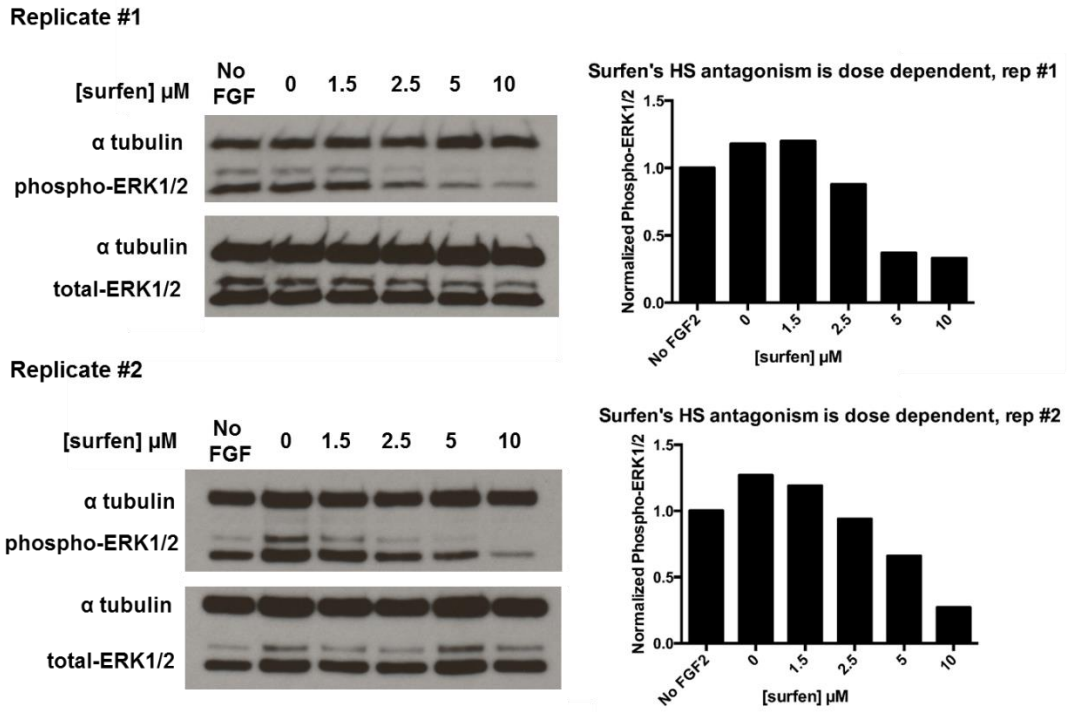



**Fig. 3.10 Dosage titration of surfen for Oct4-GFP mESCs**. Surfen's antagonism of HS is dose dependent, allowing researchers to finely tune the antagonism of GAGs. At concentrations greater than 5µM, FGF2 stimulation is abolished, while 1.5 and 2.5 µM surfen show little inhibition of FGF2 induced MAPK signaling.

Next, I show that the effects of surfen on FGF2 stimulation is reversible, that is, the addition of exogenous soluble heparin can overcome the antagonistic effects of surfen to restore HS mediated FGF2 signaling in mESCs. The mechanism of this rescue is likely heparin's competition with endogenous HS for surfen binding. Furthermore, it is important to note that since heparin displays greater sulfation than HS, it binds surfen with greater affinity, which may aid in the rescue of HS mediated signaling (35). Indeed, when 5µg/mL heparin was added to surfen treated Oct4GFP cells, a robust rescue of MAPK activation was observed, as shown in figure 3.11.

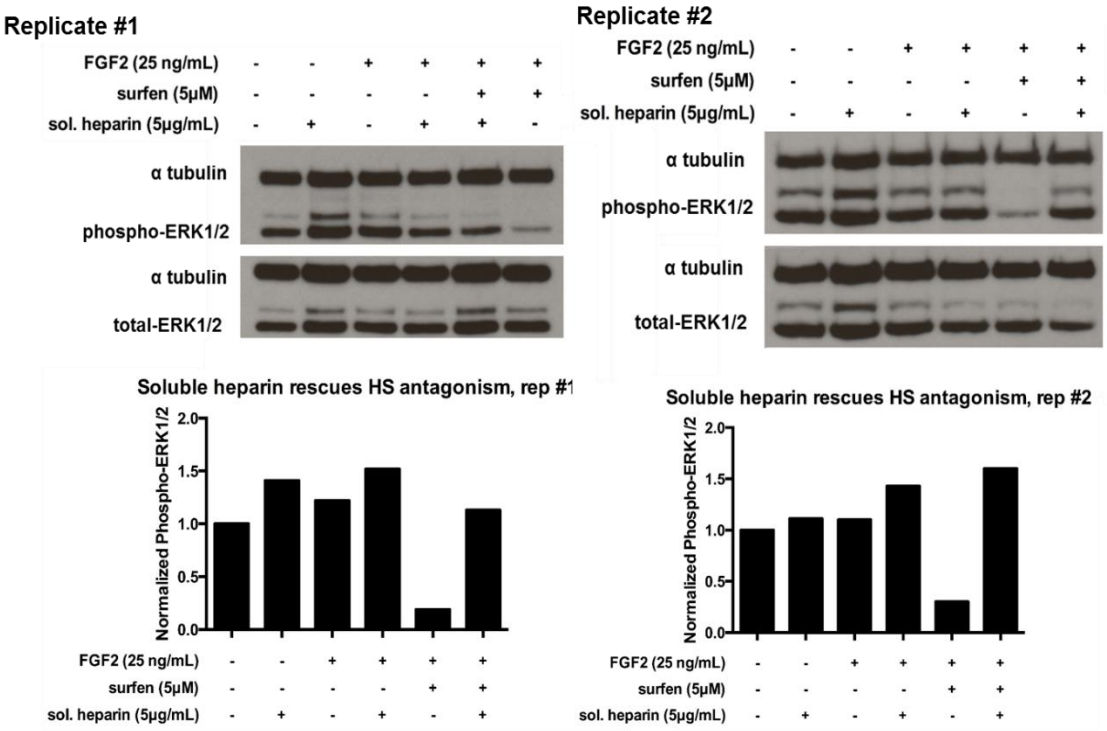



**Fig. 3.11 Rescue of FGF2 signaling via soluble heparin.** The addition of 5μg/mL soluble heparin can overcome surfen antagonism of heparan sulfate, restoring MAPK activation. This finding suggests that surfen inhibition of HS mediated signaling is reversible.

The ability of soluble heparin to displace surfen and rescue MAPK activity is a significant finding, implicating that surfen could be used to temporarily inhibit or delay MAPK signaling and thus mESC differentiation, and this inhibition could be removed at the desired time to restore HS mediated signaling. Mia, who was working on mESC differentiation, observed that surfen's maintaince of pluripotency via HS antagonism was reversible, and she titrated in increasing amounts of heparin to reverse the effects of surfen on differentiation of mESCs.

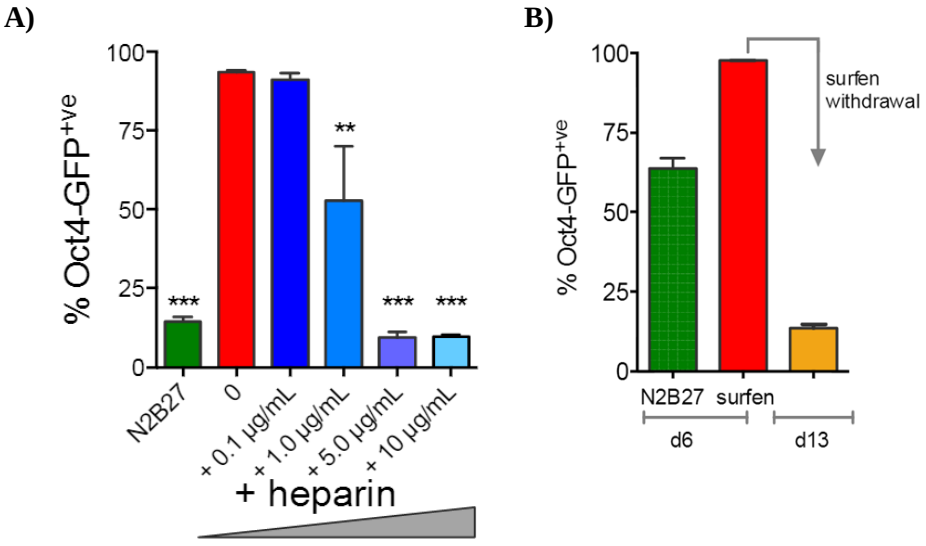



**Fig. 3.12 Effects of surfen on mESC differentiation are reversible.** Work performed by Mia Huang, A) Here Mia shows that surfen's maintaince of pluripotency of can reversed by addition of soluble heparin, resulting in a decrease in OCT4 and thus pluripotency. B) Mia's data shows that the effects of surfen are reversible, and upon surfen withdrawal OCT4 expression decreases.

Taken together, these results show that surfen can be used to influence *in vitro* mESC differentiation in reversible, dose dependent manner. Surfen may hold potential as a tool to manipulate ESC differentiation in the future.

# IV. Discussion/closing statements

The glycome has become a subject of increasing interest in recent years, as it regulates a myriad of biological processes, yet still is largely uncharacterized. While proteins are templated, glycosylation is a heterogeneous and un-templated process. This makes glycans difficult to study, yet it is suspected the functional information encoded in glycans can rival that of DNA or RNA (42). Researchers will need to understand glycans and their functions in highly organized systems to increase understanding of biological systems.

In this work, my colleagues and I develop a microarray platform where the GAG microenvironment can directly affect cellular processes, such as MAPK signaling which triggers the departure from pluripotency, and potentially differentiation. Soluble heparin, even in the absence of FGF2, is able to elicit dramatic biological responses from $EXT1^{-/-}$ cells, which highlights the power glycans yield over biological processes.

Due to the largely uncharacterized nature of glycosylation, researchers in the future will need tools to rapidly unveil glycan mediated interactions and their complexities. While glycan microarrays can quickly identify lectins or binding partners for specific glycans, an alternate approach to directly screen for biological effects of glycan mediated interactions would be useful. Here, I describe a fabricated cellular microarray which directly analyzes protein signaling as a function of glycan dependent extracellular signaling events.

There are several important implications of this work, such as increasing our understanding of glycan mediated interactions and nascent potential for therapeutic applications. Understanding the processes affecting ESC differentiation hold great potential for regenerative medicine, and small molecule antagonists, such as surfen, offer unique tools to direct differentiation. One can imagine a situation where targeted stem cell therapy could be delivered to a patient, and cells could be held in a pluripotent state by the delivery of surfen to the site of therapy. Of course, one major hurdle of this proposition is that small molecule effectors of glycans would need to be delivered to patients in a highly targeted and controlled manner, because GAGs facilitate a multitude of biological functions.

The microarray platform devised and described here holds potential as a nascent high throughput screening technique which could be applied to understand a multitude of glycan mediated interactions. One can image a situation where each spot of the microarray could have a different glycan immobilized prior to cell seeding. Then one could directly assess biological changes inside the cell as a function of glycans presented to cells. For these purposes, it is also invaluable for researchers to have several tools to remove native glycan/GAG functions so these relationships may be systematically investigated in a controlled manner, and scientists need a high throughput platform to do so. Biological reagents such as inhibitors, growth factors, media may be costly or difficult to obtain and miniaturization of biological experiments can decrease operating cost- another advantage offered by the microarray platform. Hopefully, in the near future, cellular microarrays will provide a valuable

tool for investigating and elucidating the effects of glycans on a variety of cellular processes.

# V. References


1) Smith, A. (2001) Embryo-derived stem cells: Of Mice and Men. *Annual Review of Cell Biology*, 17, 435-462

2) Laurent N., Voglmeir J., and S. Flitsch. (2008) Glycoarrays- tools of determining protein-carbohydrate interactions and glycoenzyme specificity. *Journal of RSC: chemical. Communications*, 4400-4412

3) Huang M., Godula K. (2014) Priming the Glycocalyx for Neural Development. *ACS Chemical Neuroscience*, 5, 873-875

4) Park S., Gildersleeve, J., Blixt, O., and Shin, I., (2013) Carbohydrate Microarrays. *RSC Chem. Soc. Rev.*, 42, 4310-4326

5) Smith, R., Meade, K., Pickford, C., Holley, R., and Merry, C., (2011) Glycosaminoglycans as regulators of stem cell differentiation. *Biochem. Soc. Trans.*, 39, 383-387

6) Kunath, T., Saba-El-Leil, M., Almousailleakh, M., Wray, J., Meloche, S., and Smith, A. (2007) FGF stimulation of the ERK 1/2 signaling cascade triggers transition of pluripotent embryonic stem cells from self-renewal to lineage commitment. *Development.* 134, 2895-2902

7) Rossant, J., and Lanner, F. (2010) The role of FGF/Erk signaling in pluripotent cells. *Development*. 137, 3351-3360

8) Pickford, C., Holley, R., Rushton, G., Stavridis, M., Ward, C., and Merry, C. (2011) Specific Glycosaminoglycans Modulate Neural Specification of Mouse Embryonic Stem Cells. *Stem Cells.* 29, 629-640

9) Kraushaar, D., Dalton, S., and Wang, L. (2013) Heparan sulfate: a key regulator of embryonic stem cell fate. *Biol. Chem.*, 394(6), 741-751

10) Österholm, C., Barczyk, M., Busse, M., Grønning, Reed. R., and Kusche-Gullberg, M. (2009) Mutation in the Heparan Sulfate Biosynthesis Enzyme EXT1 influences Growth Factor Signaling and Fibroblast Interactions with the Extracellular Matrix. *The Jou. of Biolog. Chem.*284(50), 34925-34943

11) Esko, J., and Lindahl, U. (2001) Molecular diversity of Heparan sulfate. *J. Clin. Invest.* 108, 169-173

12) Varki, Ajit, Jeffery Esko, Koji Kimata, and Ulf Lindahl. "Ch 16, Proteoglycans and Sulfated Glycosaminoglycans." *Essentials of Glycobiology, 2nd ed*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1999. N. pag. Print.

13) Afratis, N., Gialeli, C., Nikitovic, D., Tsegenidis, T., Karousou, E., Theocharis, A., Pavao, M., Tazanakakis, G., and Karamanos, N. (2012) Glycosaminoglycans: key players in cancer cell biology and treatment. *FEBS J.* 279, 1177-1197

14) Mobli, M., Nilsson, M., Almond, A. (2007) The structural plasticity of heparan sulfate NA-domains and hence their role in mediating multivalent interactions is confirmed by high accuracy $^{15}$N-NMR relaxation studies. *Glycoconj. J.* 25, 401-414

15) Yeo, J. and Ng, H. (2013) The transcriptional regulation of pluripotency. *Cell Research.* 23, 20-32

16) Hirai, H., Karian, P., and Kikyo N. (2011) Regulation of embryonic stem cell self-renewal and pluripotency by leukemia inhibitory factor. *Biochem J.* 438(1), 11-23

17) Weissman, I. (2000) Stem Cells: Units of Development, Units of Regeneration, and Units in Evolution. *Cell.* 100, 157-168

18) Rahl, P., Lin, C., Seila, A., Flynn, R., McCuine, S., Burge, C., Sharp, P. and Young, R. c-Myc regulates transcriptional pause release (2010) *Cell.* 141(3), 432-445

19) Dance, M., Montagner, A., Salles, J., Yart, A., and Raynal, P. (2007) The molecular functions of Shp2 in the Ras/Mitogen-activated protein kinase (ERK1/2) pathway. *Cellular signaling*. 20, 453-459

20) Delehedde, M., Seve, M., Sergeant, N., Wartelle, I., Lyon, M., Rudland, P., and Fernig, D. (2000) Fibroblast Growth Factor-2 Stimulation of $p42/44^{MAPK}$ Phosphorylation and IκB Degradation Is Regulated by Heparan Sulfate/Heparin in Rat Mammary Fibroblasts. *J. of Biological Chemistry.* 275:43, 33905-33910

21) Kraushaar, D., Yamaguchi, Y., Wang, L. (2010) Heparan Sulfate is Required for Embryonic Stem Cells to Exit from Self-renewal. *J. of Biological Chemistry.* 285:8, 5907-5916

22) Hamazaki, T., Kehoe, S., Nakano, T., Terada, N. (2006) The Grb2/Mek Pathway Represses Nanog in Murine Embryonic Stem Cells. *Molecular and cellular biology*. 26:20 7539-7549.

23) Stavridis, M., Lunn, S., Collins, B., Storey, K. (2007) A discrete period of FGF-induced Erk1/2 signaling is required for vertebrate neural specification. *Development.* 134, 2889-2894

24) Roskoski, R. (2012) ERK1/2 MAP kinases: Structure, function, and regulation. *Pharmacological Research.* 66, 105-143

25) Sapkota, G., Alarcón, C., Spagnoli, F., Brivaniou, A., Massague, J. (2007) Balancing BMP Signaling through Integrated Inputs into the Smad1 Linker. *Cell Press*. 25:441-454

26) Shaul, Y., and Seger, R. (2007) The MEK/ERK cascade: From signaling specificity to diverse functions. *Biochimica et biophysica acta.* 1773:1213-1226

27) Dhillon, A. Hagan, S., Rath, O., Kolch, W. (2007) MAP kinase signaling pathways in cancer. *Oncogene.* 26:3279-3290.

28) Li, Z., Fei, T., Zhang, J., Zhu, G., Wang, L., Lu, D., Chi, X., Teng, Y., Hou, N., Yang, X., Zhang, H., Han, J., Chen, Y. (2012) BMP4 Signaling Acts via Dual-Specificity Phosphatase 9 to Control ERK Activity in Mouse Embryonic Stem Cells. *Cell Stem Cell.* 10:171-182

29) Mohammadi, M., Olsen, S., and Goetz, R. (2005) A protein canyon in the FGF-FGFR receptor dimer selects from an á la carte menu of heparan sulfate motifs. *Current Op. in Structural Biology.* 15:506-516

30) Kraushaar, D., Rai, S., Condac, E., Nairn, A., Zhang, S., Yamaguchi, Y., Moremen, K., Dalton, S., and Wang, L. (2012) Heparan Sulfate Facilitates FGF and BMP Signaling to Drive Mesoderm Differentiation of Mouse Embryonic Stem Cells. *J. of Biological Chemistry.* 287,27:22691-22700.

31) Powell, A., Fernig, D., and Turnbull, J. (2002) Fibroblast Growth Factor Receptors 1 and 2 Interact Differently with Heparin/Heparan Sulfate. *J of Biological Chemistry.* 277,32: 28554-28563.

32) Guimond, S., Maccarana, M., Olwin, B., Lindahl, U., and Rapraeger, A. (1993) Activating and Inhibitory Heparin Sequences for FGF-2 (Basic FGF). *J. of Biological Chemistry.* 268,32:23906-23914.

33) Miao, H., Ishai-Michaeli, R., Atzmon, R., Peretz, T., and Vlodavsky, I. (1995) Sulfate Moieties in the Subendothelial Extracellular Matrix Are Involved in Basic Fibroblast Growth Factor Sequestration, Dimerization, and Stimulation of Cell Proliferation. *J. of Biological Chemistry.* 271,9:4879-4886.

34) Jia, J., Maccarana, M, Zhang, X., Bespalov, M., Lindahl, U., and Li, J. (2009) Lack of L-Idouronic Acid in Heparan Sulfate Affects Interactions with Growth Factors and Cell Signaling. *J. of Biological Chemistry.* 284,23:15942-15950

35) Schuksz, M., Fuster, M., Brown, J., Crawford, B., Ditto, D., Lawrence, R., Glass, C., Wang, L, Tor, Y., and Esko, J. (2008) Surfen, a small molecule antagonist of heparan sulfate. *PNAS.* 105,35:10375-13080

36) Puvirajesinghe, T., Ahmed, Y., Powell, A., Fernig, D., and Turnbull, J. (2012) Array-Based Functional Screening of Heparin Glycans. *Cell Press, Chemistry and Biology.* 19:553-558

37) Ranga, A., Gobaa, S., Okawa, Y., Mosiewicz, K., Negro, A., and Lutolf, M. (2014) 3D niche microarrays for systems-level analyses of cell fate. *Nature comm.* 5,4324:1-10

38) Ghaemi, S., Harding, F., Delalat, B., Vasani, R., and Voelcker, N. (2013) Surface Engineering for Long-Term Culturing of Mesenchymal Stem Cell Microarrays. *ACS Biomacromolecules.* 14:2675-2683

39) Fernandes, T., Kwon, S., Bale, S., Lee, M., Diogo, M., Clark, D., Cabral, J., and Dordick, J. (2010) Three-Dimensional Cell Culture Microarray for High-Throughput Studies of Stem Cell Fate. *Biotechnology and Bioengineering.* 106,1:106-118

40) Brafman, D., Chien, S., and Willert, K. (2012) Arrayed cellular microenvironments for identifying culture and differentiation conditions for stem, primary, and rare cell populations. *Nature protocols.* 7,4:703-716

41) Zhu, H., Duchesne, L., Rudland, P., and Fernig, D. (2010) The heparan sulfate co-receptor and the concentration of fibroblast growth factor-2 independently elicit different signaling patterns from the fibroblast growth factor receptor. *Cell communication and signaling.* 8,14:1-10

42) Gama, C., Tully, S., Sotogaku, N., Clark, P., Rawat, M., Vaidehi, N., Goddard, W., Nishi, A., and Hsieh-Wilson, L. (2006) Sulfation patterns of glycosaminoglycans encode molecular recognition and activity. *Nature chem. Bio.* 2,9:467-473

43) Roux, P. and Blenis, J. (2004) ERK and p38 MAPK-Activated Protein Kinases: a Family of Protein Kinases with Diverse Biological Functions. *Microbiology and molecular biology reviews.* 68,2:320-344

44) Li, L., and Chen, Y. (2013) Functions of BMP signaling in embryonic stem cell fate determination. *Experimental Cell Research.* 319:113-119

45) Sieber, C., Kopf, J., Hiepen, C., and Knaus, P. (2009) Recent advances in BMP receptor signaling. *Elsevier cytokine & growth factor reviews.* 20:343-355

46) Kuo, W., Digman, M., and Lander, A. (2010) Heparan Sulfate Acts as a Bone Morphogenetic Protein Coreceptor by Facilitating Ligand-induced Receptor Hetero-oligomerization. *Molec. Bio. of the Cell.* 21:4028-4041

47) Rahman, S., Akhtar, N., Jamil, H., Banik, R., and Asaduzzaman, S. (2015) TGF-β/BMP signaling and other molecular events: regulation of osteoblastogenesis and bone formation. *Bone research.* 3:1-20

48) Engler, A., Sen, S., Sweeney, H., and Discher, D. (2006) Matrix Elasticity Directs Stem Cell Lineage Specification. *Cell* 126:677-689

49) Xu, D., and Esko, J. (2014) Demystifying Heparan Sulfate-Protein Interactions. *Annu. Rev. Biochem*. 83:129-157

50) Rapraeger, A., Guimond, S., Krufka, A., and Olwin, B. (1994) Regulation by Heparan Sulfate in Fibroblast Growth Factor Signaling. *Methods in enzymology.* 245:219-232

51) Garud, D., Tran, V., Victor, X., Koketsu, M., and Kuberan, B. (2008) Inhibition of Heparan Sulfate and Chondroitin Sulfate Proteoglycan Biosynthesis. *J. of biological chemistry.* 283,43:28881-28887

52) Huang, C., and Ferrell, J. (1996) Ultrasensitivity in the mitogen-activated protein kinase cascade. *PNAS biochemistry.* 93:10078-10083

53) Chetty, S., Pagliuca, F., Honore, C., Kweudjeu, A., and Melton, D. (2013) A simple tool to improve pluripotent stem cell differentiation. *Nature methods.* 10:553-556

54) Huang, M., Smith, R., Trieger, G., and Godula, K. (2014) Glycocalyx Remodeling with Proteoglycan Mimetics Promotes Neural Specification in Embryonic Stem Cells. *J. Am. Chem. Soc.* 136: 10565-10568

55) Sarrazin, S., Lamanna, W. and Esko, J. (2011) Heparan Sulfate Proteoglycans. *Cold Spring Harb Perspect Biol.* 3:a004952

56) Desai, U., Wang, H., and Linhardt, R. (1993) Specificity Studies on the Heparin Lyases from *Flavobacterium heparinum*. *Am. Chem. Soc.* 32,32:8140-8145