Investigating the use of marine-derived glycosaminoglycans as mimetics for heparan sulphate in fibroblast growth factor signalling

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Abbreviations

Ala - Alanine

Arg - Arginine

Asn – Asparagine

CBL – Casitas B-cell Lymphoma

COOH – Carboxylic Acid

CRKL – Crk-like Protein

CS – Chondroitin Sulphate

D1 – Immunoglobulin-like I domain

D2 – Immunoglobulin-like II domain

D3 – Immunoglobulin-like III domain

DAG – Diacylglycerol

DS – Dermatan Sulphate

ECM – Extracellular Matrix

ER – Endoplasmic Reticulum

Etv4 – ETS Translocation Variant 4

Etv5 – ETS Translocation Variant 5

Extl1 – Exotosin-like Glycosyltransferase 1

Extl2 – Exotosin-like Glycosyltransferase 2

Extl3 – Exotosin-like Glycosyltransferase 3

FGF – Fibroblast Growth Factor

FGFR – Fibroblast Growth Factor Receptor

FRS2 – Fibroblast Growth Factor Substrate 2

FRS2α – Fibroblast Growth Factor Receptor Substrate 2 Alpha

GAB1 – GRB2-associated-binding Protein 1

GAG – Glycosaminoglycan

Gal – Galactose

GalT-I – Galactosyltransferases 1

GalT-II – Galactosyltransferases 2

GlcA – Glucuronic Acid

GlcAT-I – Glucuronyltransferase 1

GlcN – D-glucosamine

GlcNAc – N-acetyl D-glucosamine

$GlcNH_3$ - N-unsubstituted Glucosamine

GlcNS – N-sulphated Glucosamine

Gln - Glutamine

GRB2 – Growth Factor Receptor-bound Protein 2

HA – Hyaluronic Acid

HCL – Hydrochloric Acid

His - Histidine

HS – Heparan Sulphate

HSPG – Heparan Sulphate Proteoglycans

IB2 – Islet-brain 2

IdoA – Iduronic Acid

Ig – Immunoglobulin

IL-3 – Interleukin 3

IP_3 – Inositol Triphosphate

kDa – Kilodalton

KS – Keratan Sulphate

Lys – lysine

MAPK – Mitogen-activated Protein Kinase

MAP – Mitogen-activated Protein

MTT - 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide

mTOR – Mammalian Target of Rapamycin

NA domains – N-acetylated Disaccharide Units

NS domains – N-sulphated Disaccharide Units

PAPS - 3'-phosphoadenosine 5'-phosphosulfate

PI3K – Phosphoinositide-3 Kinase

PIP_2 – Phosphatidylinositol 3,4-bisphosphate

PKC – Protein Kinase C

PLC-γ - Phospholipase Gamma

S domains – Sulphated Disaccharide Units

SDS – Sodium Dodecyl Sulphate

Ser - Serine

SOS – Son of Sevenless

Thr - Threonine

TM – Transmembrane

TSC2 – Tuberous Sclerosis Protein 2

UDP – Uridine Diphosphate

Val - Valine

Xyl – Xylose

XylT – Xylosyltransferase

Tables

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Abstract

Fibroblast growth factors (FGFs) are polypeptide growth factors and are potent regulators of cell proliferation, cell differentiation, cell migration and angiogenesis (Ornitz and Itoh, 2001). FGFs can bind with fibroblast growth factor receptors with the aid of heparan sulphate. As mammalian heparin was known to bind with many FGFs and FGFRs. Marine glycosaminoglycans were tested to see if they could bind with FGF and FGFR to gain greater understanding on the binding between glycosaminoglycans with FGF and FGFR. Marine glycosaminoglycans were tested due to their unique and diverse structures and to test for the biological activity involving with FGF signalling, MTT assay were used in the experiment.

Different variants of polysaccharides derived from Salmon, Cod and Hake were tested. It was shown that all the marine polysaccharide variants were able to promote FGF2 signalling in cells that were transfected in FGFR1c. At a high concentration, all the Cod variants were able to promote FGF2/FGFR2c signalling, and some Salmon variants were not able to promote FGF2 signalling whilst some were able to show some cell proliferation. All the Hake variants were not able to promote FGF2/FGFR2c signalling except for Hake F5. All variants of marine polysaccharides derived from Salmon, Cod and Hake were not able to promote FGF1 signalling in cells transfected with FGFR1c or FGFR2c with the exemption of variant Hake F3.

Introduction

Wound healing is a complex biological process, as cells encounter a variety of growth factors that can alter cell behaviour (Grazul-Bilska et al., 2003). Cells can migrate, proliferate, or differentiate to other cell types depending on their response to growth factors (Sweeney et al., 2001). The growth factors promote various intracellular signalling pathways responsible for different cellular outcomes. Many growth factors are regulated by carbohydrates, such as heparan sulphate and heparin, to support the promotion of intracellular signalling acting as a co-receptor (Forsten-Williams et al., 2008).

Fibroblast growth factors (FGFs) are cell signalling proteins that can bind with fibroblast growth factor receptors (FGFRs) which regulate a broad spectrum of biological functions in both *in vivo* and *in vitro*, including mitogenesis, cellular migration, differentiation, and angiogenesis (Yun et al., 2010). FGFs play a fundamental role in early development, such as mesoderm patterning in the early embryo and organogenesis. In adults, they are important in angiogenesis and wound repair (Turner and Grose, 2010). Although FGFs are essential in normal and embryonic development, FGFs have been involved in several disease mechanisms, mainly expressed as oncogenes or tumour products, to promote the survival of tumours and angiogenesis (Krufka et al., 1996). FGFs are expressed in almost all tissues, and the mammalian FGF family contains 22 genes categorised into seven subfamilies. There are 18 fibroblast growth factor ligands where FGFs are known to bind (Ornitz and Itoh, 2015). Activation of FGFR requires heparan sulphate proteoglycans to induce pleiotropic responses leading to numerous cellular processes (Eswarakumar et al.,

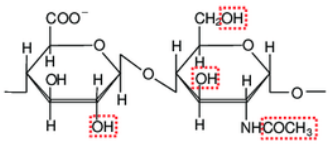
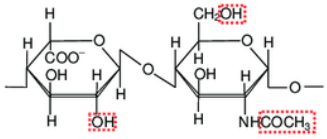
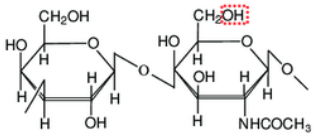
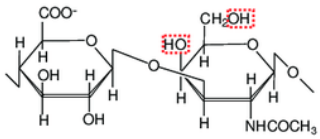
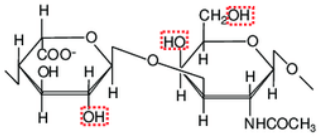
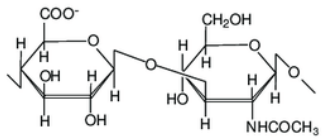
2005). Activated FGFRs trigger multiple signalling pathways, including the RAS/MAPK pathway, PI3K/AKT pathway, and PLC-γ pathway, which initiate a variety of cellular outcomes (Ornitz and Itoh, 2015).

FGFRs are essential in crucial cell behaviours such as proliferation, differentiation, and cell survival. FGFRs are present in many different cell types. Evidence shows that deregulation of FGF signalling can contribute to the pathogenesis of many cancers in different tissue types. Tumour development can be caused by abnormal FGF signalling by promoting cancer cell proliferation and survival. Aberrant FGF signalling can also support tumour angiogenesis (Turner and Grose, 2010). Many conditions such as congenital syndromes, skeletal dysplasias and deafness can be caused by faulty FGF signalling. These pathological conditions are most likely caused by gain or loss of function mutations within the ligands or the receptors (Teven et al., 2014).

Glycosaminoglycans

Glycosaminoglycans (GAGs) are linear and negatively charged polysaccharides with a molecular weight of approximately 10-100 kDa (Miller et al., 2014). There are usually two main types of GAGs non-sulphated and sulphated. The sulphated GAGs include chondroitin sulphate (CS), keratan sulphate (KS), dermatan sulphate (DS), heparan sulphate (HS) and heparin, whereas non-sulphated GAGs include hyaluronic acid (HA) (Gandhi and Mancera, 2008).

Table 1. List of different types of glycosaminoglycans and their respective monosaccharide and disaccharide units. *The structure of repeating disaccharide units in their respective glycosaminoglycan are shown without the sulphation. The red dotted boxes are displayed to mark the potential of sulphation occurring. Obtained from (Prydz and Dalen, 2000).*

GAG	Hexuronic or Iduronic acid	Galactose	Hexosamine	Disaccharide composition
Heparan sulphate/ Heparin	D-glucuronic acid **(GlcA)** L-iduronic acid **(IdoA)**	-	D-glucosamine **(GlcNAc)**	 **GlcA β(1→4) GlcNAc α((1→4)**  **IdoA α(1→4) GlcNAc α(1→4)**
Keratan sulphate	-	Galactose **(Gal)**	D-glucosamine **(GlcNAc)**	 **Gal β(1→4) GlcNAc β(1→3)**
Chondroitin sulphate	D-glucuronic acid **(GlcA)**	-	D-galactosamine **(GalNAc)**	 **GlcA β(1→3) GalNAc β(1→4)**
Dermatan sulphate	D-glucuronic acid **(GlcA)** L-iduronic acid **(IdoA)**	-	D-galactosamine **(GalNAc)**	 **IdoA β(1→3) GalNAc β(1→4)**
Hyaluronic acid	D-glucuronic acid **(GlcA)**	-	D-glucosamine **(GlcNAc)**	 **GlcA β(1→3) GlcNAc β(1→4)**

In mammalian cells, GAGs are usually found linked to various core proteins known as proteoglycans, where they are ubiquitous and occur in many tissues (Yung and Chan., 2007). Proteoglycans are present on the cell membrane's outer surface or the extracellular matrix (ECM) (Rabenstein, 2002). Proteoglycans are sometimes known as mucopolysaccharides as they are viscous and contain lubricating properties showing that they are present in mucous secretion (Gandhi and Mancera, 2008). Proteoglycans consist of a core protein with one or multiple GAGs chains are covalently attached (Sarrazin et al., 2011). The GAGs chains are large complex carbohydrate molecules that can interact with various proteins involving multiple physiological functions and pathological processes (Raman et al., 2005). The proteins such as chemokines, cytokines, growth factors, morphogen enzymes and adhesion molecules can bind with GAGs chains via electrostatic interaction (Morla, 2019) (Gandhi and Mancera, 2008). The electrostatic interaction is caused by the negatively charged uronic acid or the sulphate group and the positively charged amino acid in the protein. Furthermore, the non-ionic interactions between GAGs and their binding partners caused the binding's specificity and selectivity (Morla, 2019). The binding of the GAGs chain and proteins is involved in cellular responses such as cell adhesion, growth and differentiation, cell signalling and anticoagulation (Morla, 2019) (Yamada et al., 2011).

Heparan sulphate proteoglycans

Heparan sulphate proteoglycans (HSPGs) can be classified into three classes: the syndecans, the glypicans and secreted proteoglycans.

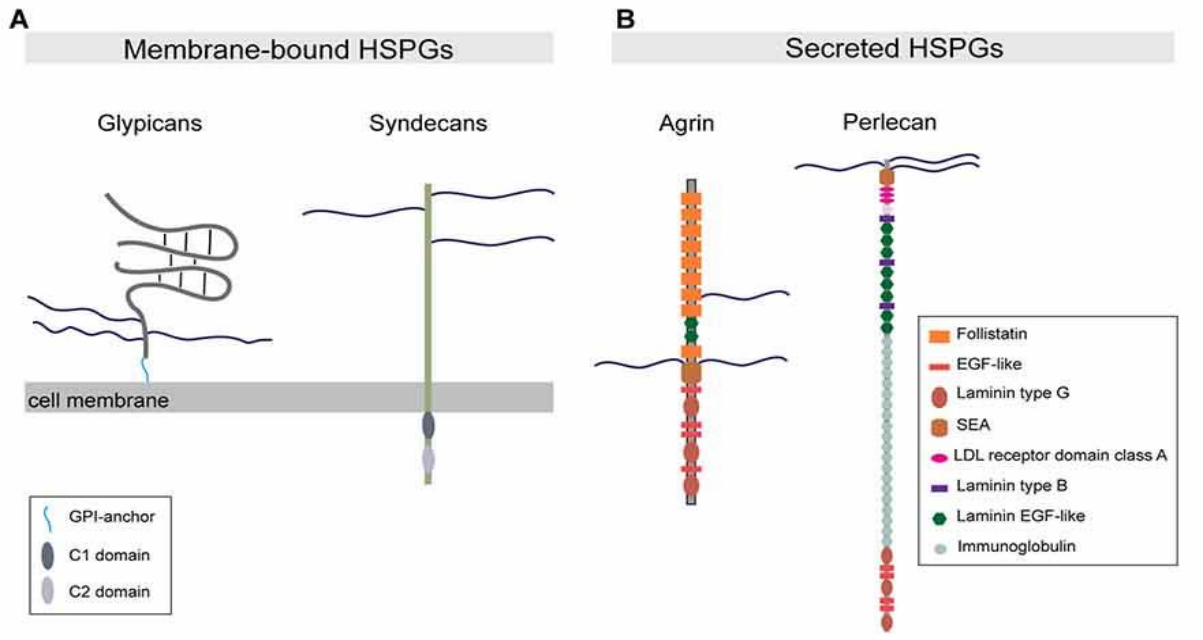


Figure 1. The heparan sulphate proteoglycan protein's family. *Figure A shows the structure of membrane bound HSPG. Figure b shows the composition of secreted HSPGs. Adapted from (Condomitti and de Wit, 2018).*

Four syndecans families are known to contain a transmembrane region with two constant regions (C1 and C2) in the cytoplasmic domain (Tumova et al., 2000) (Perrimon and Bernfield, 2000). The transmembrane regions consist of motifs of alanines and glycines (van Horssen et al., 2003). They are responsible for the dimerisation of the protein core, producing a homodimer, resulting in a specific motif in the domain that forms an SDS-resistant complex (Afratis et al., 2016). The C-terminal cytosolic region is responsible for binding cytosolic regulatory factors and is known to interact with several intracellular kinases, which promote various crucial cell

functions (van Horssen et al., 2003) (Afratis et al., 2016). The syndecans can have multiple GAGs chains attached to the serine residue of the core protein, making them N-terminal polypeptides (Afratis et al., 2016). Most cell types express at least one syndecan; however, some cells and most tissues express multiple syndecans (van Horssen et al., 2003). Syndecan-1 can be found mainly in the epithelial and plasma cells, while syndecan-2 is widely present in the mesenchymal cells such as fibroblasts and smooth muscle. Syndecan-3 is usually found in neural tissues and developing musculoskeletal tissues. On the other hand, syndecan-4 is abundant in most cell types (Afratis et al., 2016). Glypicans are covalently linked to the outer plasma membrane by glycosylphosphatidylinositol anchors but lack the cytoplasmic connection (Tumova et al., 2000) (Kirkpatrick and Selleck, 2007). There are six families of the glypicans gene where all the family members share limited sequence homology. All members of glypicans contain 14 conserved cysteine residues. The glypicans have 2-3 HS chains attached to the core protein near the C-terminus, close to the cell membrane (Häcker et al., 2005). Both syndecans and glypicans can be cleaved under certain conditions acting as soluble ectodomains. The syndecans core proteins can be proteolytically cleaved at the cell surface and displayed in wound fluids. These soluble ectodomains cleaved from the extracellular domain of HSPGs could be involved in the regulation of growth factors within the extracellular space (Perrimon and Bernfield, 2000). The HSPGs can be cleaved by proteinase and heparinase, allowing the HSPGs to be more diffusible (Matsuo and Kimura-Yoshida, 2013).

Different cell types have a specific core protein with distinct heparan sulphate chains with specific ligand properties (Tumova et al., 2000). The cell types or the physiological

state of the cells determines the sulphation patterns on the GAGs chain and not by the core protein. However, it remains unclear if the same cells carry the same core protein with the same GAGs chain (Simon and Parish, 2013).

Structure

Heparan Sulphate

Heparan sulphates (HS) are sulphated GAGs that are unbranched chains of disaccharide repeats. They are heavily sulphated at numerous points on their sugar residues. HS can display as conjugated to amino acids known as heparan sulphate proteoglycans (HSPGs) or unconjugated chains (Nagarajan et al., 2018). Heparan sulphate consists of alternating repeating disaccharide units in a linear sequence (Rabenstein, 2002). The disaccharide units consist of uronic acid and a derivative of glucosamine shown in figure 2 (Esko and Selleck, 2002). The uronic acid is usually D-glucuronic acid (GlcA) or L-iduronic acid (IdoA), and some uronic acid is sulphated at the Carbon-2 position (GlcA2S or IdoA2S) (Rabenstein, 2002) (Esko and Selleck, 2002). A derivative of glucosamine in the heparan sulphate chain can be either N-sulphated (GlcNS) or N-acetylated (GlcNAc) or unsubstituted (GlcNH3) (Esko and Selleck, 2002). The derivative of glucosamine can be variably sulphated at multiple positions where they can be sulphated at 6-O (GlcNS(6S) or GlcNAc(6S)) or 3-O (GlcNS(3S)) (Rabenstein, 2002).

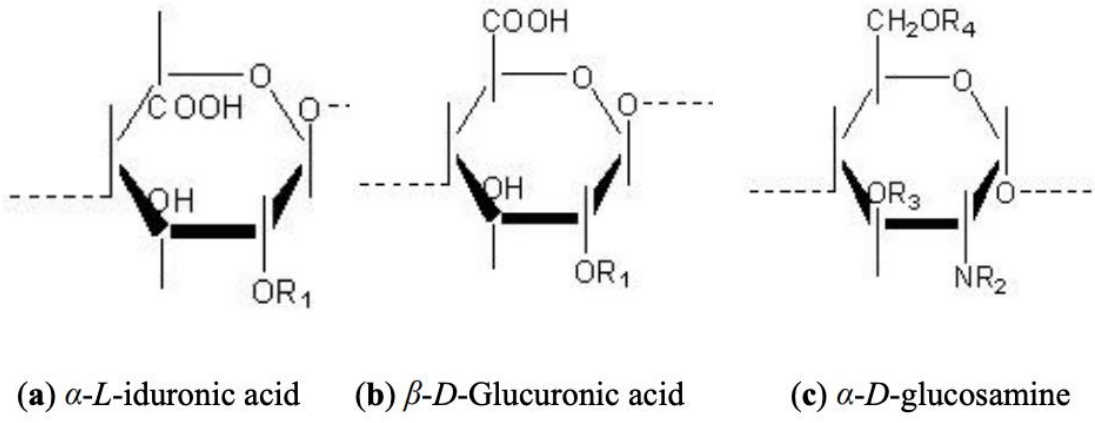


Figure 2. Monosaccharide units of heparan sulphate. *The repeating disaccharide units are composed of uronic acid usually, L-iduronic acid (a) or D-Glucuronic acid (b) and a derivative of glucosamine (c). Variety of modification can occur on the R positions of the monosaccharide units during chain modification in the biosynthesis of Heparan Sulphate. The R positions can have certain functional groups such as hydroxyl, acetyl, and sulphate. R1=H or SO3−, R2 =H2COCH3 or SO3−, R3= H or SO3−, R4= H or SO3−. Adapted from (Puvirajesinghe and Turnbull, 2012).*

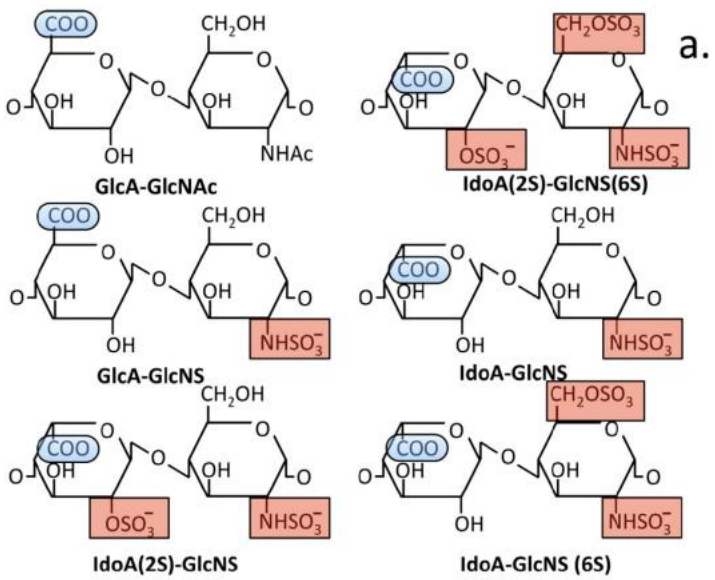


Figure 3. Disaccharide units of heparan sulphate. *These disaccharides are composed of monosaccharide units shown in figure 2. The carboxylate group are shown in blue, and the red shading indicates that the disaccharide unit can be modified by sulphation. Adapted from (Hayes and Melrose, 2020).*

Heparan sulphate chains consist of 50 to 150 repeating disaccharide units. The disaccharide units are N-acetylated or N-sulphated glucosamine linking to a uronic acid. The N-acetylated disaccharide unit consists of GlcA relating to the Carbon-1 of GlcNAc, whereas N-sulphated disaccharide is composed of GlcNS linking with IdoA, a Carbon-5 epimer of GlcA (Schenauer et al., 2009). The linkage between the two monosaccharides is the alpha linkage between the carbon-1 and carbon-4 of each unit (Lortat-Jacob, 2009). HS chains exhibit different types of domains, the NA domains showing little or no sulphation consisting of GlcA-GlcNAc repeating disaccharide unit or NS domains showing more highly sulphated regions consisting of IdoA-GlcNS repeating unit and mix domains showing a mixture of NA and NS domains (Power et al., 2004).

Heparin

Heparin is a highly sulphated and linear polysaccharide with a structure that is closely related to heparan sulphate. Currently, heparin is widely used as an anticoagulant drug. However, heparin is also vital in multiple biological activities such as blood coagulation, cell growth, and wound healing (Lee et al., 2004). Heparin can be found and produced in the connective tissue-type mast cells, stored in the cytoplasmic granules (Hagner-McWhirter et al., 2000). Heparin chains are produced mainly in mast cells, where they are attached to various serine residues of the core protein, serglycin. During mast cell degranulation, the heparin chains are obtained by proteases digesting the heparin core protein to release peptidoglycan heparin which is then modified into GAG heparin beta-endoglucuronidase (Linhardt, 2003). Mast cell heparins contain a pentasaccharide structure with high-affinity binding to antithrombin (Mulloy et al., 2016). The pentasaccharide within heparin binds with antithrombin, which induces a conformational change in the reactive centre loop of the serpin. This increases the rate of antithrombin, inhibiting its target serine proteases. (Weitz., 2003). Heparin repeating unit contain L-iduronic acid (IdoA) and D-glucosamine (GlcN) interacting by an alpha 1-4 linkage. Heparins contain typically three sulphate groups attached to the 2-hydroxyl group of iduronic acid, and the other two are bonded with 6-hydroxyl and two amino groups of the glucosamine group (Faham et al., 1996).

Biosynthesis of Heparan sulphate and Heparin

The biosynthesis of heparan sulphate occurs in the Golgi apparatus, where numerous enzymes are involved in producing a non-sulphated polysaccharide chain which then undergoes a sequential non-template driven series of modifications (Turnbull et al., 2001). Usually, two to four chains are attached to the core proteins. However, the number of side chains attachment sites depends on the core proteins (Perrimon and Bernfield, 2000). The activation of monosaccharides to nucleoside diphosphate sugars (usually UDP-sugar) and the activation of sulphate to 3'-phosphoadenosine 5'-phosphosulphate (PAPS), which is a universal sulphur donor, is essential for the synthesis of HS chains (Sasarman et al. 2016). The PAPS supplies the sulphate groups attached to the HS side chains by sulphotransferase (van Horssen et al., 2003). Glycosyltransferase has one or two DXD sugar-binding motifs for urine diphosphate (UDP)-sugar. The binding utilises the nucleotide sugars as donor substrates, and the nucleotide sugars that bind with UDP are xylose (UDP-xyl), galactose (UPD-Gal), D-glucosamine (UDP-GlcA) and N-acetylglucosamine (UPD-GlcNAc), which are required for GAG synthesis (Sugahara and Kitagawa., 2002). These nucleotides sugars are transported from the cytoplasm to assemble the heparan sulphate chains (Bishop et al., 2007).

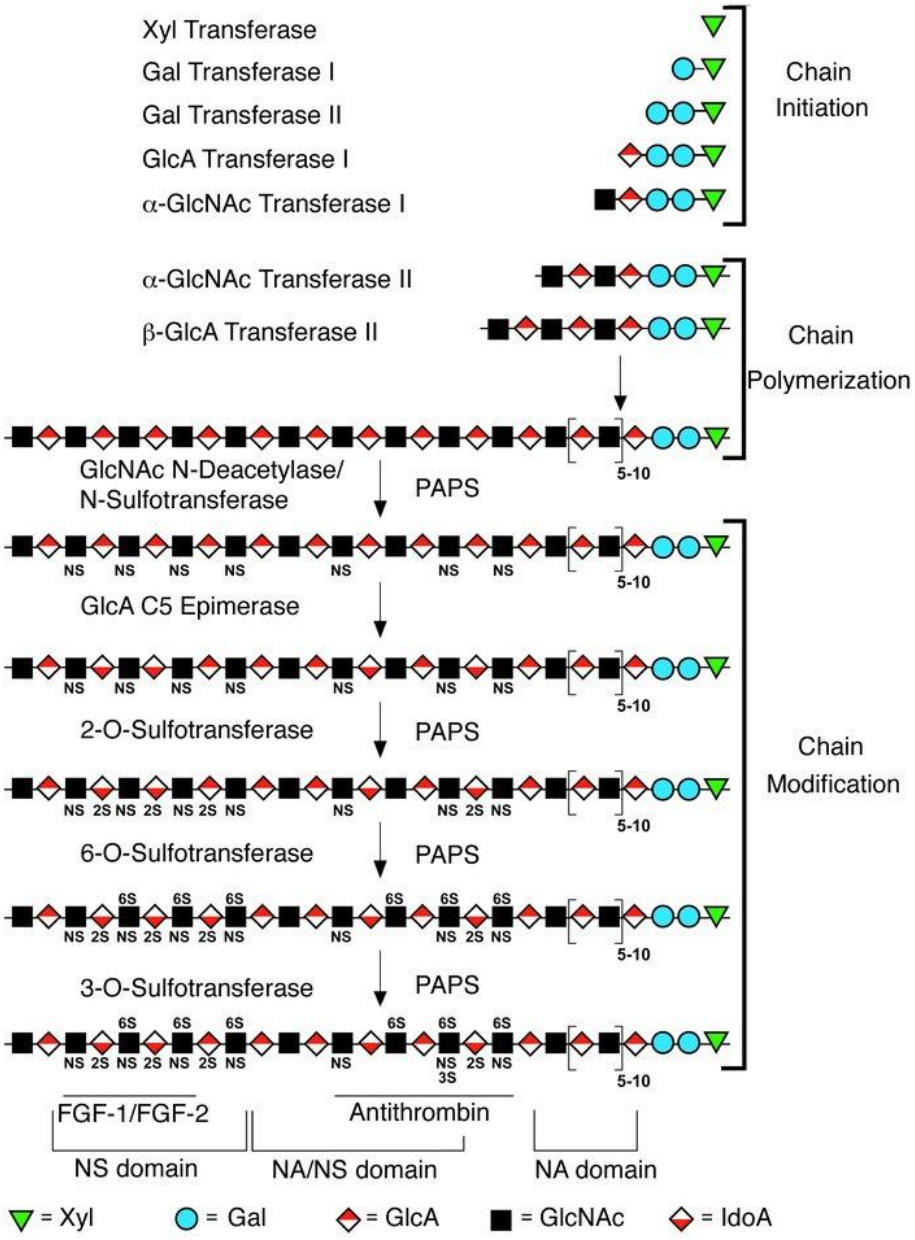


Figure 4. The biosynthesis of Heparan Sulphate chain. *Heparan sulphate glycosaminoglycan chains are synthesised, attaching to a core protein, and undergoing numerous chain modifications. The production of heparan sulphate chain occurs in a three-step process: chain initiation, chain polymerisation, and chain modification. The identification of the recognition sites for FGF1/2 and antithrombin are displayed on this figure. Obtained from (Esko and Lindahl, 2001).*

Heparan sulphate chains contain the GAG-protein linkage tetrasaccharide (GlcAβ1-3Galβ1-3Galβ1-4xylβ1-O-Ser), which is the same for chondroitin sulphate, where the GAGs chains are covalently bound to the serine residue of the core protein (Ueno et al., 2001). A core protein and the O-linked oligosaccharide production occurs in the rough endoplasmic reticulum (van Horssen et al., 2003). In the endoplasmic reticulum and the cis-Golgi, xylosylation of a serine residue from the core protein is occurred by xylosyltransferase (XylT) after translation. The Xylose residue is transferred through a beta-linkage from UDP-xyl to a serine residue in the core protein, catalysed by XylT (Esko and Selleck, 2002). The xylosylation causes the hydroxyl group of serine residue attached to the core protein (van Horssen et al., 2003). After that, galactosyltransferase I and II (GalT-I, GalT-II) allow two galactose attachments to the xylose residue (Huang et al., 2011). Completion of the linkage region is caused by GlcA being transferred through β1-3 linkage from UDP-GlcA to Galβ1-3Galβ1-3Galb1-4xylβ1-O-Ser catalysed by glucuronyltransferase (GlcAT-I) (Kitagawa et al., 1999).

After completing the tetrasaccharide linkage region, N-acetylated glucosamine is added to the linkage region by a unique transferase called Extl3 (Kreuger and Kjellén, 2012). After that, chain polymerisation occurs, allowing the attachment of GlcA and GlcNAc (Carlsson et al., 2008). They are added alternatively by a heparan sulphate polymerases called Extl1 and Extl2, where both enzymes form a heterodimeric complex. This includes a repeated 1,4-linked disaccharide polymer and can form up to 50 disaccharides or more (Multhaupt and Couchman, 2012). After polymerisation, it undergoes chain modification causing the heparan sulphate to go through sequential enzymatic modifications (OLczyk et al., 2015). The first modification removes the N-

acetyl group from GlcNAc and replacing with sulphate. The enzyme responsible for the first modification is the bifunctional N-deacetylase/N-sulfotransferase (NDST) enzyme. This enzyme cause N-deacetylation and N-sulphation of GlcNAc residues in heparan sulphate chains (Sugahara and Kitagawa, 2002). The second modification is epimerisation of many but not all D-GlcA residue to L-iduronic acid (IdoA) by glucuronyl C-5 epimerase (Kreuger and Kjellén, 2012). Some of the iduronates undergo 2-O sulphation after the epimerisation by 2-O sulfotransferase. The C-5 epimerase and the 2-O sulfotransferase forms a complex of each other (Multhaupt and Couchman, 2012). IdoA residues in heparan sulphate are responsible for providing conformational flexibility to the chain allowing the interaction with specific proteins. The HS chain undergoes a considerable amount of O-sulphation by multiple enzymes (Dreyfuss et al., 2009). These O-sulphations cause the uronic acids (GlcA or IdoA) to be sulphated at 2-O-position and the GlcNAc to be sulphated at the 3-O and 6-O-positions. The enzymes responsible for the sulphation are 3-O and 6-O-sulfotransferases (Liu and Linhardt, 2014) (Carlsson et al., 2008).

As a result of the non-template driven aspect of its biosynthesis, heparan sulphate is very diverse structurally This causes variability in the heparan sulphate sequences. It was shown that different tissues or cells produce differences in heparan sulphate structure. Different cell types express different heparan sulphate sequences even though they have the same core proteins (Turnbull et al., 2001). Heparan sulphate contains the same set of disaccharide units in most tissues, but the relative content varies quantitatively. For example, in endothelial cells and connective tissue mast cells, heparan sulphate contains more GlcA-GlcNS3S disaccharide unit as this unit is critical

in binding with antithrombin hence why the pentasaccharide includes this disaccharide unit. On the other hand, heparan sulphate in the kidney contains more IdoA2S-GlcNS3S disaccharide units (Esko and Lindahl., 2001). Cells can change the structure of heparan sulphate by being exposed to extracellular signals such as growth factor signalling (Turnbull et al., 2001). The biosynthetic enzymes cause variation during the biosynthesis process of heparan sulphate. The biosynthetic enzymes can work independently or sequentially, allowing the heparan sulphate to have a diverse structure. Additionally, studies have shown that many different biosynthetic enzyme isoforms are possible, suggesting that the tissue or cell specific heparan sulphate is caused by specific enzyme isoforms allowing them to have different heparan sulphate sequences (Sasisekharan, 2000).

The synthesis for heparin is very similar to heparan sulphate; however, the structure of the disaccharide repeats, and the sulphation pattern is quite different. Usually, heparin disaccharides contain 2.7 sulphate groups per disaccharide, whereas heparan sulphate contains one or fewer sulphate groups per disaccharide (Shriver et al., 2011). Therefore, HS has less N- and O-sulphation than heparin, but HS also contains less IdoA compared to heparin (Hagner-McWhirter et al., 2000). Heparan sulphate chains include multiple sulphate rich-S- domains (Harmer, 2006). Heparan sulphate chains contain more saccharide unit (50kDa) compared to heparin fragments (20kDa), where native heparin chains are cleaved by heparinase in mast cells (Lindahl and Kjellén, 2013). This explains the difference in binding properties in certain growth factors (Harmer, 2006) (Meneghetti et al., 2015).

Fibroblast Growth Factors

FGF is expressed in most mammalian tissues, and it was first discovered as a mitogen for cultured fibroblast in 1973 from a pituitary extract (Eswarakumar et al., 2005) (Yun et al., 2010). It was initially identified as a protein promoting fibroblast proliferation (Yun et al., 2010). Currently, FGF is one of the largest families of polypeptide growth factors showing 22 known mammalian FGFs (Olsen et al., 2003). FGFs are signalling proteins and have a molecular weight ranging from 17 to 34kDa (Ornitz and Itoh, 2015) (Eswarakumar et al., 2005). All FGFs families share a conserved sequence of approximately 120-130 amino acids showing up to 16-65% sequence identity (Eswarakumar et al., 2005). The core region showing the conserved sequence is a β-trefoil fold consisting of 12 β-strands arranged in three sets of four-stranded β-sheets (Olsen et al., 2003). The 12 antiparallel strands form a cylindrical barrel closed by various amino- and carboxy-terminal stretches. FGF structure is topologically identical to interleukin 1β (Powers et al., 2000). Most FGFs contain an internal core domain with 28 highly conserved residues and six similar amino acids. Ten out of the 28 highly conserved residues interact with the FGF receptor (Ornitz and Itoh, 2001).

Due to the biochemical function, sequence similarities and evolutionary relationship, they are grouped into seven subfamilies and currently, 5 of them are paracrine FGFs, one subfamily of endocrine FGFs and one subfamily intracellular FGFs (Ornitz and Itoh 2015). The paracrine and endocrine FGFs are classified as canonical FGFs where they are expressed on the cell surface or in the extracellular matrix where HSPGs acts as a coreceptor. On the other hand, endocrine FGFs, also known as hormone-like FGFs

(FGF19, 21 and 23), functions as an endocrine behaviour, but they bind to FGFRs in Klotho proteins (Perez-Garcia et al., 2018).

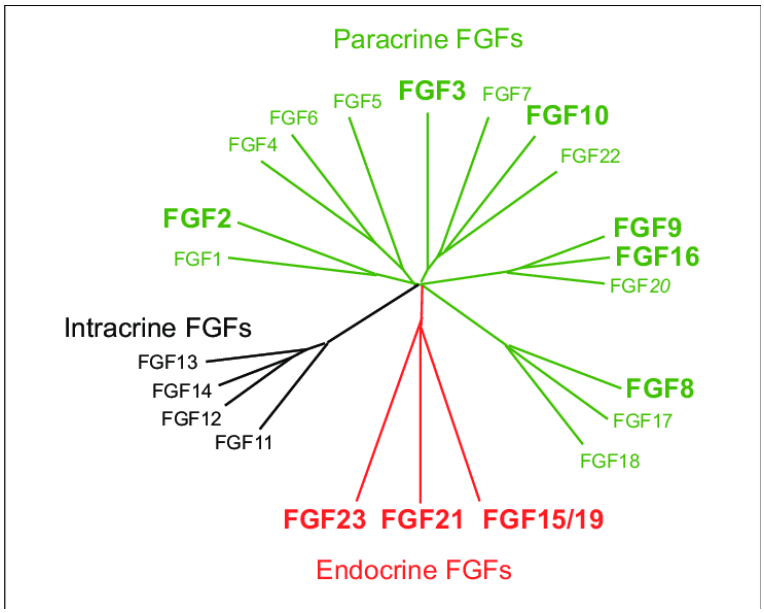


Figure 5. A representation of 22 members of FGF and the classification of their subfamilies. *Subfamilies FGF1, FGF4, FGF7, FGF8 and FGF9 are classified as paracrine FGFs (green), whereas subfamily FGF15/19 is an endocrine FGF (red). Subfamily FGF11 is classified as intracrine FGFs (black). Obtained from (Itoh et al., 2016).*

FGF1 Subfamily

FGF1 subfamily consists of FGF1 and FGF2, where these FGFs lack secretory signal peptides. They are readily exported from cells by translocation across the cell membrane. In some cases, FGF1 and FGF2 can be found in the nucleus of some cells. It was shown in some studies that extracellular FGF1 is transported across the plasma membrane through the cytosol and then enters the nucleus (Ornitz and Itoh 2015). Both FGF1 and FGF2 do not have a signal peptide (Prudovsky et al., 2008). FGF1 and

FGF2 were widely studied on their structure and it was identified that FGF1 and 2 have 12 antiparallel β-strands in the conserved core domain. It also has a β-trefoil structure consisting of four-stranded β-sheets arranged in a triangular array. Two of the β-strands (β10 and β11) consist of numerous basic amino acids sugars that can bind with heparin forming a heparin-binding site on FGF2 (Ornitz and Itoh, 2001). Although the FGF1 subfamily was the first FGFs to be discovered, their physiological roles remain unclear. However, it was shown likely to affect the vascular tone or reduce blood pressure. FGF2 has been shown to display angiogenic properties and promote cellular responses such as proliferation, migration, and inhibiting apoptosis of endothelial cells (Laestander and Engström, 2013). FGF1 is the only fibroblast growth factor that can activate all the FGFR splice variants, while FGF2 can only activate the IIIc splice variant of FGFR1-3, FGFR4 and IIIb splice variant of FGFR1 (Ornitz and Itoh 2015).

FGF4 Subfamily

FGF4 subfamily comprises FGF4, FGF5 and FGF6, where all members are secreted proteins with cleavable N-terminal signal peptides to help them mediate biological processes. The FGF4 subfamily proteins act as extracellular proteins, enabling them to bind and activate the tyrosine kinases receptors (Ornitz and Itoh, 2015). The FGFR that the FGF4 subfamily proteins that can activate are the FGFR IIIc splice variants (1-3) and FGFR4 (Tiong et al., 2013). FGF4 is vital for organ development as it affects the trophoblast proliferation process. FGF4 is also responsible for limb and heart development, while FGF5 is responsible for hair growth cycle regulation (Laestander and Engström, 2013).

FGF7 Subfamily

FGF7 subfamily consists of FGF3, FGF7, FGF10 and FGF22 (Ornitz and Itoh, 2015). FGF3, 7 and 10 could activate the IIIb splice variant of FGFR2, while FGF3 and FGF10 can activate the IIIb splice variant of FGFR1 (Tiong et al., 2013). FGF7 subfamily is involved in many organ developments showing FGF3 is responsible for the inner ear structure development and FGF7 is vital for kidney development (Laestander and Engström, 2013). FGF7 sometimes can be referred to as keratinocyte growth factor (KGF) as it is responsible for activation of early differentiation in keratinocytes which allow the undifferentiated cells to be differentiated (Belleudi et al., 2014). In addition, FGF7 and FGF10 are involved in vesicle clustering and neurite branching in the brain and are known to be presynaptic organisers (Laestander and Engström, 2013).

FGF8 Subfamily

FGF8, FGF17 and FGF18 are grouped into the FGF8 subfamily, which are growth factors containing N-terminal cleaved signal peptides responsible for activating IIIc splice variant of FGFR1-3 and FGFR4 (Ornitz and Itoh, 2015) (Tiong et al., 2013). FGF8 plays a significant role in the development of many body parts. FGF17 contributes to the development of many organs such as the limb, ear, eye, and brain, affecting forebrain development. In addition, FGF18 is critical for the development of the bone (Laestander and Engström, 2013).

FGF9 Subfamily

The growth factors in the FGF9 subfamily does not contain a classical N-terminal signal peptide but include an internal hydrophobic region allowing the growth factor to act as a non-cleaved signal. This allows the transportation of the growth factors into the endoplasmic reticulum (ER) and the secretion from the cells (Ornitz and Itoh, 2015). The FGF9 subfamily consists of FGF9, FGF16 and FGF20, and these growth factors activate the IIIb splice variant of FGFR3, FGFR4 and the IIIc splice variant FGFR1-3 (Tiong et al., 2013). In addition, FGF9 can initiate the secretion of several ligands for the growth factors in the FGF7 subfamily. FGF9 can also upregulate the proliferation of mesenchymal tissues as many studies show FGF9 knockout leads to reduced production of specific ligands and reduced rate of mesenchymal-epithelial signalling (Laestander and Engström, 2013).

FGF15/19 Subfamily

The 15/19 subfamily consists of FGF15/19, FGF21 and FGF23 and these FGFs are known to be endocrine FGFs. These growth factors can bind with HS or heparin at low affinity, and the reduced heparin-binding affinity facilitates the release from the ECM (Ornitz and Itoh, 2015). Although it can bind to HS or heparin, the endocrine FGFs prefer to bind with proteins from the Klotho family to mediate their biological response in an FGFR-dependent manner showing that the klotho proteins are the

preferred co-factors. FGF19 can stimulate bile acid synthesis and initiate the oxidation of fatty acids. In the fasting state, the FGF21 stimulates glucose uptake in the adipocytes, leading to reduced glucose levels in the bloodstream. This was demonstrated by injecting FGF21 into diabetic mice, causing a reduction in the concentration of insulin, glucagon, glucose, and triglycerides. FGF23 is vital for vitamin D regulation (Laestander and Engström, 2013).

FGF11 Subfamily

FGF11 subfamily is also known as intracellular FGFs (iFGFs), where they are not secreted and have no interaction with FGFR (Olsen et al., 2003). However, the intracellular FGFs can interact with voltage-gated sodium channels, mainly the cytosolic carboxy-terminal tail section. The interaction with the carboxy-terminal tail can help with the regulation of subcellular localisation of voltage-gated sodium channels that can be found at the axon initial segment. This regulation occurs during the development and ion-gating properties of the channel in mature neurons and other cells such as cardiomyocytes (Ornitz and Itoh, 2015). Furthermore, some intracellular FGFs have been identified to interact with many different proteins. For example, FGF12 can interact with MAP kinase scaffolding proteins, and IB2 whilst FGF13 can interact with the microtubules (Olsen et al., 2003).

Fibroblast Growth Factor Receptors

There are five FGFR families where four families are receptor tyrosine kinases designated as the high-affinity FGF-receptors FGFR1-FGFR4 (Eswarakumar et al. 2005). The fifth member is FGFR5 which lacks the FGF signalling tyrosine kinase domain, but it was shown to bind with FGFs (Dieci et al., 2013). Although the structure is like other FGFRs, the tyrosine kinase domain is replaced with an intracellular tail with a histine-rich motif causing FGFR5 to be unable to produce a signal by transphosphorylation (Tiong et al., 2013).

The transmembrane tyrosine kinase of FGF receptors (FGFRs) is the primary agent of signal transduction, and it is vital for the binding of FGF. The activation of intracellular signalling transduction is caused by the activation of tyrosine kinase activity (Wilkie et al., 1995). The FGFRs contain three essential domains: extracellular ligand binding, a single transmembrane domain, an intracellular tyrosine kinase domain (Tiong et al., 2013). The extracellular part of the receptor contains a hydrophobic signal peptide region and two or three immunoglobulins (Ig) like subunits (D1, D2 and D3) where D2 and D3 are the main areas where the FGF binds to (Dai et al. 2019) (Tiong et al. 2013). A linker region between D1 and D2, called the acidic box, is responsible for receptor autoinhibition (Dai et al. 2019) (Dieci et al., 2013). The acidic box contains a highly conserved motif rich in aspartate acid (Dai et al. 2019). Ig-like III domain (D3) has different spliced variants of each receptor caused by alternative splicing. This shows that different FGFRs would have different selectivity and affinities for specific FGFs and the transduced signal (Venkataraman et al. 1999). The alternative splicing of Ig-like III fragment of FGFR1, 2 and 3 caused to generate different isoforms leading to different

ligand-binding specificity, most commonly on the alternate exon on the second half of the Ig-III domain (Guimond et al., 2009). The isoforms are Ig-IIIb and Ig-IIIc present in epithelium and mesenchyme, respectively (Dieci et al., 2013). Other splice variants could eliminate the first immunoglobulin loop, but the biological consequences remain unknown (Guimond et al., 2009). After the binding from the extracellular ligand-binding domain with FGF, the transmembrane domain allows the transportation of signal transduction from the extracellular region to the cytoplasmic region, which is the juxtamembrane region. A split tyrosine kinase domain is connected with the juxtamembrane region, and the end COOH tail is attached to the tyrosine kinase domain (Tiong et al., 2013). The immunoglobulin-like II and III of the extracellular ligand-binding domains are responsible for binding the ligand, mainly FGF and HSPGs (Dieci et al., 2013).

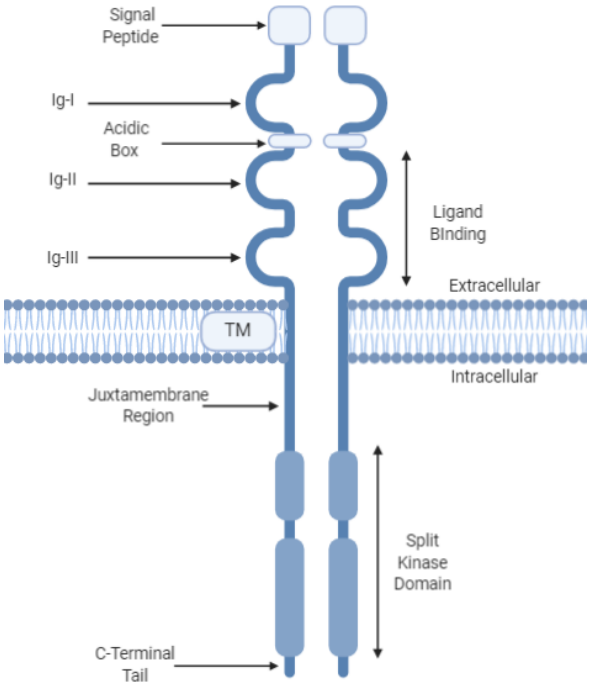


Figure 6. Structure of FGFR family of receptor tyrosine kinase showing a schematic representation of the different domains. *The extracellular part of the receptor consists of 3 immunoglobulin-like domains where D2 and D3 are responsible for ligand binding. It is followed by a transmembrane region (TM), juxtamembrane region, the split tyrosine kinase domain and C-terminal tail. The split kinase domain is responsible for the receptor activation by the tyrosine phosphorylation. Adapted from (Knights and Cook, 2010).*

The determination of the ligand-binding specificity in the FGFR is caused by the alternate exon usage of the Ig-like III domain (D3) of the FGFR, creating three different isoforms, IIIa, IIIb and IIIc. The isoform IIIa is enCoded from exon seven only, whereas IIIb and IIIc are enCoded by the exon 7/8 and 7/9, respectively (Tiong et al., 2013). Therefore, the IIIa splice variant is not observed in the FGFR. However, the other two isoforms, IIIb and IIIc, are displayed in FGFR1-3, where IIIb is commonly expressed in

the epithelial tissue and IIIc is expressed in the mesenchymal tissue (Zhang et al., 2006). The FGFR4 is only displayed in one isoform IIIc as the FGFR4 gene is unique (Tiong et al., 2013).

Table 2. Interaction between the FGF ligands and the FGFR isoforms. *The FGF19 subfamily (FGF19, FGF21 and FGF23) enCode for paracrine FGFs, which bind to their receptor using Klotho protein as a cofactor while the other FGFs are paracrine and activate the receptor with heparin or Heparan sulphate as a cofactor. The two major splice variants, IIIb and IIIc, are essential determinants of ligand binding specificity (Tiong et al., 2013).*

Interaction with Receptor and ligand	
FGFR Isoform	**FGF Ligands specificity**
FGFR1 IIIb	1, 2, 3, 10 and 22
FGFR1 IIIc	1, 2, 4, 5, 6, 19, 20 and 21
FGFR2 IIIb	1, 3, 4, 6, 7, 10 and 22
FGFR2 IIIc	1, 2, 4, 5, 6, 8, 9, 17, 18, 19, 21 and 23
FGFR3 IIIb	1 and 9
FGFR3 IIIc	1, 2, 4, 8, 9, 17, 18, 19, 21 and 23
FGFR4	1, 2, 4, 6, 8, 9, 16, 17, 18 and 19

Fibroblast growth factor signalling

FGFs are secreted glycoproteins that are readily released from the extracellular matrix (ECM) by proteases, heparinases or specific FGF binding-proteins (Grose and Turner 2010). Secreted FGFs induce a biological response by binding and activating cell surface tyrosine kinase FGFR. The binding of FGFR and FGF are unique as each FGFR ligand are specific to certain FGFs, and the specificity is further regulated by the alternative splicing of FGFRs (Itoh and Ornitz, 2004). The binding of FGF and FGFR requires heparan sulphate to form a ternary complex of FGF, FGFR and HS, suggesting that HS act as a co-receptor and allows the stabilisation of the FGF: FGFR interaction (Xu et al., 2012) (Grose and Turner 2010). The formation of the FGF: FGFR complex caused a decrease in FGF concentration for the initiation of signalling through its receptor and extended the time of the response (Bishop et al., 2007). The heparan sulphate chains from HSPGs can regulate receptor dimerisation by interacting with FGF and FGFR, causing transphosphorylation of intracellular tyrosine kinase domains and triggering multiple signalling pathways (Guimond and Turnbull, 1999). The main intracellular cascades mediated by the FGF signal are the RAS-MAPK, the PLC-γ and the PI3K-AKT pathways, which lead to numerous cellular outcomes (Diez Del Corral and Morales, 2017).

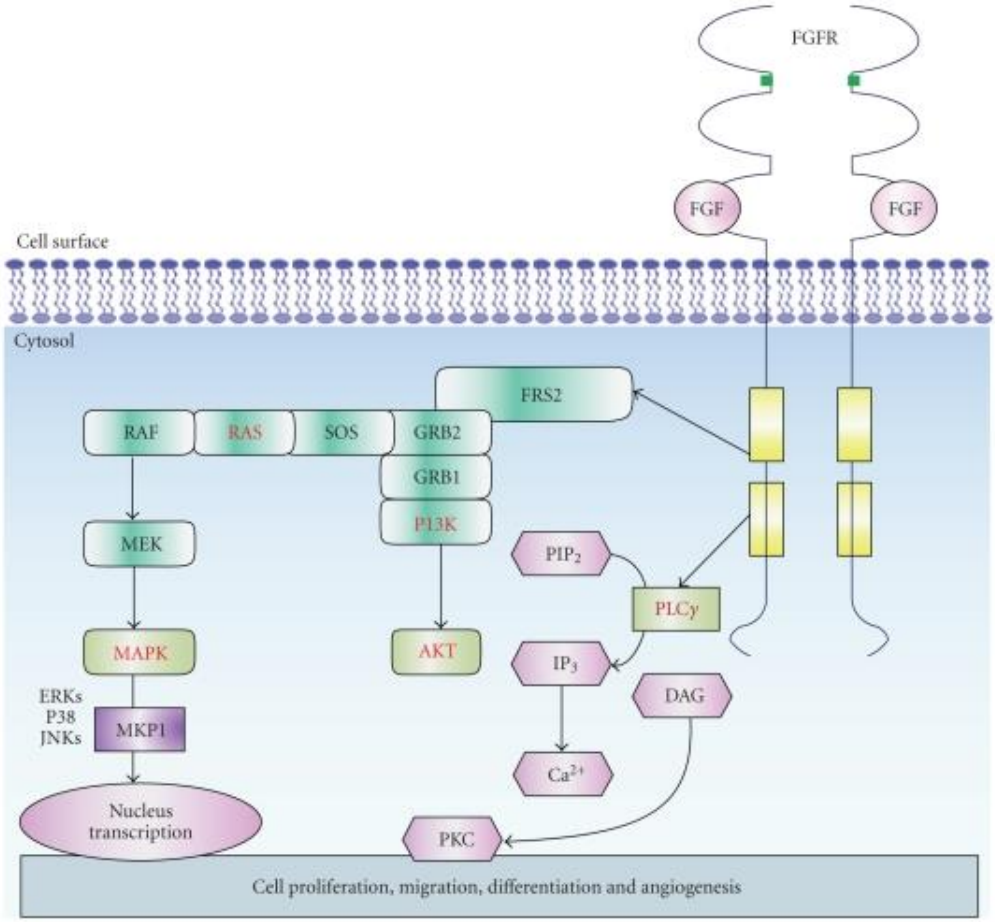


Figure 7. FGF signalling pathway. *The binding of FGF, heparan sulphate and FGFR cause tyrosine phosphorylation, which activates multiple pathways such as the RAS/MAPK pathway, PI3K/AKT pathway and PLC-γ pathway. The pathways are responsible for numerous cellular processes. Obtained from (Yun et al., 2010).*

RAS-MAP kinase pathway is associated with cell proliferation and differentiation (Teven et al., 2014). The phosphorylation of FRS2 activates the RAS-MAPK pathway a (Dieci et al., 2013). However, the phosphorylation of FRS2a can be partially dependent on the phosphorylation of Y463 in the presence of CRKL. After phosphorylation, the FRS2a interacts with the growth factor receptor-bound 2 (GRB2), which further activates the RAS-MAPK pathway through the recruitment of SOS (Brewer et al.,

2016). The SOS activates the RAS GTPase causing the activation of the MAPK pathway leading to the activation of various Ets transcription factors such as Etv4 and Etv5, and negative regulators of the FGF signalling pathways, including SPRY, CBL and SEF (Ornitz and Itoh, 2015).

PI3K-AKT pathway is like the RAS-MAPK pathway where the phosphorylation of FRS2 activates it, which interacts with GRB2 (Dieci et al., 2013). The difference between the two pathways is that the PI3K-AKT pathway is activated through the recruitment of GAB1, leading to the activation of other signalling complexes. The adaptor protein GAB1 activates the enzyme PI3K, which then phosphorylate the enzyme AKT where AKT is responsible for multiple activities (Schlessinger, 2004). This pathway is responsible for stimulating cell growth and proliferation by activating mTOR complex 1. The AKT is activated to phosphorylate and inhibit the TSC2 (Yu and Cui, 2016). PI3K-AKT pathway is involved with cell survival, cell fate determination and can also impact cell polarity (Teven et al., 2014).

Activated FGFR tyrosine kinase activates the enzyme PLC-γ by binding to the phosphotyrosine at the COOH tail (Dieci et al., 2013). This causes the phosphorylation of PLC-γ, which then hydrolyse the phosphatidylinositol 4,5-bisphosphate (PIP_2) (Teven et al., 2014). This hydrolysation causes the production of inositol triphosphate (IP_3) and diacylglycerol (DAG), where IP_3 is responsible for the increase in intracellular calcium ions and DAG is accountable for the activation of protein kinase C (PKC) (Dorey and Amaya, 2010). This pathway is responsible for cell morphology, cell migration and cell adhesion (Teven et al., 2014).

FGF-HS-FGFR complex

Currently, the importance of heparan sulphate in FGF signalling has been well established; however, the significance of the role of heparan sulphate in FGF signalling has been poorly explained (Wu et al., 2003). It is known that the heparan sulphate proteoglycans can bind to specific growth factors caused by the electrostatic interaction between the negatively charged sulphate group from the side chain and the basic amino acid from the proteins. This caused some growth factors to require HSPGs as a co-receptor to bind with their respective cell-surface receptor (van Horssen et al., 2003). This proposed that the functionality and the formation of the FGF, FGFR and HS complexes may depend on the distribution of the negatively charged function group rather than the specific sequence of the sulphated residues in the heparan sulphate chains (Bishop et al., 2007). The length of the chain, the sulphation pattern of the chain and the stoichiometry of FGF: FGFR: HS complexes can determine the interaction of FGF, FGFR and heparan sulphate (Ostrovsky et al., 2001).

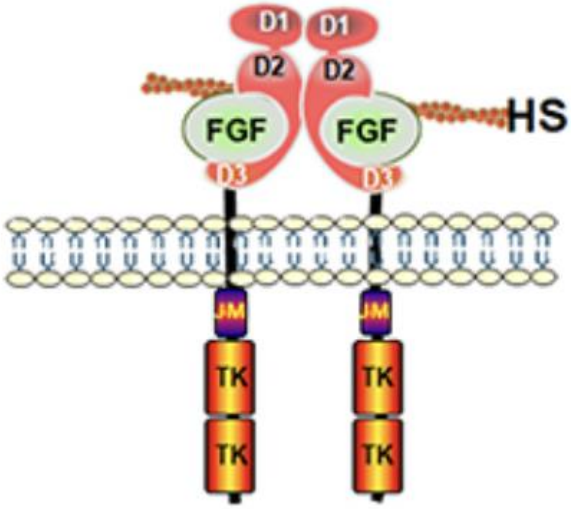


Figure 8. A representation of the FGF-FGF-HS complex. *Obtained from (Deng et al., 2021).*

The interaction of HS, FGF and FGFR is determined by the size of the HS chain, which hugely reflects in the spatial arrangement of FGF and FGFR. It was proposed that there are three modes where HS chains can interact with the proteins. They are cis, trans and mix modes, where mix mode contains both cis and trans modes (Wu et al., 2003). These proposed modes require a different length of the heparan sulphate chain to interact with the proteins. In the trans mode, hexasaccharide was able to interact with two FGF1 while a dodecasaccharide was able to bind with one FGF2 and one FGFR1 in the cis mode. For the mix mode, hexadecasaccharide could fully span a heparin-binding site and form a 2:2 FGF1: FGFR2 complex (Harmer, 2006). The binding of FGF requires 4-6 mer oligosaccharides but needs 8-10mer oligosaccharides to activate signalling (Powell, 2004). However, shorter biological active heparin oligosaccharide has been found to promote FGF signalling, suggesting that an accurate method to determine the size of HS in FGFR signalling was still necessary (Wu et al., 2003). Heparan sulphate has a different structure in different tissues or developmental stages, allowing the heparan sulphate to activate or inhibit FGF signalling. It was believed that the functional groups in HS chains during the chain modification are responsible for interacting with FGFs (Wu et al., 2003). Cellular studies have identified that different sulfo groups in the GAGs chains are needed to initiate the FGF signalling (Zhang et al., 2009). The 2-O-sulphation from the iduronic sugar was essential for the binding of FGF2, while the 6-O-sulphation was necessary for binding FGFR1 (Wu et al., 2003). FGF2 can recognise a pentasaccharide containing 2-O-sulphation iduronic acid residue with a glucosamine residue attached where glucosamine does not need to hold a 6-O-sulphation (Zhang et al., 2009). However, a massive amount of 6-O sulfo

groups was required to activate FGF signalling, and 6-O-sulphation from heparin was essential for forming the ternary complex of FGF, FGFR and HS (Gandhi and Mancera., 2008) (Zhang et al., 2009). This explains why a high level of sulphation in HS chains is required to facilitate FGF signalling and aid the formation of a ternary complex with FGF and FGFR (Xu et al., 2013).

The stoichiometry of HS in FGF: FGFR signalling complex can affect the initiation of intracellular signalling by receptor dimerisation and transphosphorylation. It was displayed that a particular HS chain can bind to one FGF and two FGFRs while another specific HS chain can bind to two FGFs and one FGFR (Wu et al., 2003). Heparin was heavily used to study the thermodynamics and kinetics of the interaction with FGF1 or FGF2. The FGFs can promote signalling by interacting with specific cell-surface FGF receptors with heparin. Crystallography techniques show that the stoichiometry of heparin in FGF: FGFR complexes and the technique display that the FGF2 interacts with FGFR1 and decasaccharide heparin fragment forming a 2:2:2 dimeric ternary complex. For FGF1, decasaccharide heparin could interact with two FGF1 ligands and two FGFR2, forming an asymmetric complex 2:2:1 (Gandhi and Mancera., 2008).

Binding of FGF and FGFR

Heparin is the main GAGs used to study the interaction between FGF and FGFR. Highly sulphated octa- or decasaccharide heparin fragments are required for FGF to bind with FGFR (Ornitz et al., 1995). Furthermore, 2-O and 6-O desulphated heparins were inactive and did not promote intracellular signalling, suggesting that the sulfo groups are essential in binding FGF and FGFR, meaning that 2-O and 6-O-sulphation in the

GAG chains are essential. 6-O-desulphated heparin could bind to the FGF but did not initiate intracellular signalling and make it unavailable to the receptor (Esko and Selleck., 2002). FGF1 and FGF2 are also widely used to study the interaction with HS, mainly heparin and FGFRs, primarily to find the binding of specific sulphate groups in heparin oligosaccharides. FGF1 recognised a certain octasaccharide containing the sequence of IdoA2SGlcNS6SIdoA2S trisaccharide motif. FGF1 also requires 6-O sulfo groups for FGF signalling (Harmer, 2006).

Identifying FGF-HS-FGFR binding specificities is vital to understanding the interaction and mechanism involved in normal development and pathogenesis (Zhang et al., 2006). Nevertheless, finding the binding properties between HS and FGF is vital as it could be used as a therapeutic drug to reduce the angiogenic activity of FGF (Presta et al., 2003). This explains that modulating the FGF signalling could be a desirable area to investigate for cancer treatment (Lima et al., 2017). The 6-O-desulphated heparin was able to bind with FGF2 but could not form a ternary complex with FGFR and FGF. Furthermore, the small oligosaccharide such as tetrasaccharides could interact with FGF2, but for mitogenic activity, it requires 18mer or higher oligosaccharides to interact (Presta et al., 2003). Understanding and identifying the patterns of GAGs chains that bind with high-affinity proteins can show whether specific GAGs sequences can activate or inhibit activity. Furthermore, this allows further understanding of the role of GAGs in biological processes, including cancer in humans which can provide information to control numerous disease processes like cancer progression and metastasis (Sasisekharan et al., 2002).

Marine-derived GAGs

Marine-derived glycosaminoglycans have attracted considerable attention towards therapeutic development and the interaction with other molecules. Marine habitats present a vast diversity where more organisms are yet to be discovered (estimates of between 33 and 66% of species) and could have the potential to be crucial bioactive compound producers due to their physical and structural characteristics (Mycroft-West et al., 2018). Marine species can survive and adapt to their extreme environment in terms of physical and chemical conditions. Due to the extreme conditions, marine organisms can develop unique biosynthetic pathways to help them grow and survive. As marine species have different evolutionary pathways than mammalian species, glycosaminoglycans from the marine resource are unique in structure and functions (Valcarcel et al., 2017).

The marine-derived GAGs have a homogeneous structure showing no alteration in their structure, whereas mammalian derived GAGs show high structural variability according to the cell and tissue types (Zainudin et al., 2014). Marine organisms produce rare disaccharide units like CS-K, where the disaccharide units are linked to form together unusual sequences (Valcarcel et al., 2017). Marine-derived GAGs consist of sulphated sugar residue, mainly iduronic acid, glucuronic acid, galactose, fucose and rhamnose (Hachim et al., 2019). Due to the high abundance of sulphate groups in the GAGs chains, this alters the charge density of the GAGs chain (Valcarcel et al., 2017). Like mammalian derived GAGs, marine-derived GAGs can bind with growth factors and other proteins (Pomin., 2014). The similarity of the two GAGs is the biological activity,

and the interaction with proteins depends on the sulphation pattern, conformation of the complex, and the sugar residue sequence (Hachim et al., 2019). However, studying modified heparin shows that in many cases, the interaction between GAGs and proteins is not specific, and the charge density and the presence of specific sulphated units cause the interaction between GAGs and proteins. This explains the importance of using marine-derived GAGs to show in-depth knowledge of the GAGs interaction with proteins (Valcarcel et al., 2017).

Marine polysaccharide has shown many beneficial characteristics of using marine organism rather than mammalian derived GAGs. Marine GAGs are shown to have less anticoagulant activity compared to heparin extracted from porcine and bovine as there is always a risk of mammalian animal disease that could affect humans, such as mad cow disease (Kirchen et al., 2018). Currently, no toxic effect has been associated with the use of marine-derived GAGs explaining why extracting GAGs from marine resources is a safer option (Pavao, 2014). An advantage of using marine GAGs is that they can diminish the off-target effect and show no contamination with mammalian pathogens and prions (Mycroft-West et al., 2018). Extraction and isolation of GAGs polysaccharide from the marine organism is easier to extract than mammalian GAGs showing a more effortless procedure for extraction. Marine glycans have a higher concentration in the tissue, around 0.5% of the dry weight compared to heparin extracted from pig intestinal mucosa (0.022%) (Pavao., 2014).

Since these marine polysaccharide shows unique structure and there have been few disadvantages displayed when using heparin derived from porcine or bovine. There

are growing demand in using alternative sources for therapeutic uses. (Mycroft-West et al., 2018). Commercial heparin is currently obtained from porcine commonly in found in intestinal mucosa and obtained from bovine lung and intestine. These sources are considered as waste so currently they have economic and environmental advantages but due to the safety concern, it has shown greater interest to explore alternative sources which can diminish those safety concerns that currently present in the commercial heparin (Valcarel et al., 2017). It is shown that some marine polysaccharide shows mixture of anticoagulant activity. Many heparins and heparan sulphate derived from ascidians, crabs and sea urchins displays low anticoagulant activity compared to mammalian heparin (Valcarel et al., 2017). For example, heparin derived from crab (*G. cruentata*) show less anticoagulant activity than heparin due to its low levels of trisulphated disaccharide which is a key component when defining the pentasaccharide structure. The pentasaccharide structure is vital when binding with antithrombin. Due to its low anticoagulant activity and low bleeding effect, heparin derived from crab is a suitable candidate when developing therapeutic agents (Andrade et al., 2013). However, some marine heparin shows higher anticoagulant activity than mammalian heparin. For example, heparin derived from molluscs displays high anticoagulant activity and shows high affinity binding to antithrombin III (Valcarel et al., 2017). Since there are mixture response in term of anticoagulant, the potential of marine polysaccharide is dependent on the anticoagulant factor. If the polysaccharide displays higher anticoagulant activity, replacing the commercial heparin as a safer alternative. If the polysaccharide displays a lower anticoagulant activity, investigating other potential benefits could be a solution due to its low

bleeding effect. Furthermore, it has the potential in tissue regeneration or the development of antiviral and anti-tumour drugs (Mycroft-West et al., 2018).

Aims

There has been a great interest in FGF and FGFR as it has shown involvement in the biology of diseases including cancer, cardiovascular diseases, metabolic diseases and even orphan diseases. Currently, 18 different FGFs can bind to 4 FGFR genes resulting from alternative splicing and have shown that association in vitro signalling, cellular responses and progression to a range of diseases (Herbert et al., 2014). In addition, FGF and FGFR are known to bind with heparan sulphate, and due to that, many studies show that many oligosaccharides can bind with FGF and FGFR at the cell membrane to promote FGF signalling. However, it was revealed that some oligosaccharides could attach to the FGF but could not initiate the signalling, suggesting that some oligosaccharides can act as a competitive inhibitor.

Due to greater interest in FGF signalling with glycosaminoglycans, the use of marine-derived GAGs was used in this experiment to determine whether HS derived from marine organisms can be used to activate various intracellular signalling pathways in proliferative phenotypes of fibroblasts in response to members of the FGF family of growth factors. In addition, the marine-derived GAGs have no toxic effect and have more glycans concentration during the extraction process. Currently, porcine and bovine-derived heparin is used to detect the binding with FGF and FGFR and has been used for future therapeutic development, but it has more significant anticoagulant activity and could produce diseases like mad cow disease.

This study aims to isolate the polysaccharides from marine organisms and selectively desulphated the marine polysaccharides. The parental and desulphated heparan sulphate is then tested to see any biological activity involving FGF signalling and assess the parental and desulphated marine polysaccharides for off-target effects such as anticoagulation. The marine polysaccharides are also tested to see any occurrence of inhibition of FGF signalling if the marine polysaccharides were not able to promote FGF signalling.

Methods

Materials

The marine glycosaminoglycans used in the experiment was isolated from Salmon, Hake and Cod. Marine glycosaminoglycans was provided by Dr Courtney Mycroft-West from Keele University (Keele, UK).

BaF3 Cell culture

BaF3 cells obtained from murine were transfected with FGFR1c or FGFR2c isoform and needed IL-3 for growth and survival as BaF3 cells lack heparan sulphate and FGFRs. The BaF3 cells were kept in a -80°c freezer. To thaw the cells, 14ml of BaF3 medium containing RPMI-1640 with 2mML-glutamine, 100U/mL penicillin, 100μg/mL streptomycin sulphate and 10% foetal calf serum was added to 1ml of cells and then centrifuged. After centrifugation, 1ml of cells was added to a TC25 flask with 4ml of BaF3 medium and 5μl of IL-3 (2ng/ml). Then, the cells are split to maintain the growth of the cells every 2-3 days in a 1:10 ratio by removing 4.5ml of the cells and adding 4.5ml of BaF3 medium with 5μl of IL-3.

MTT Proliferation assay

Approximately 10,000 cells were plated onto a 96 well plate with 100μl of BaF3 medium and then incubated with and without IL-3 used as a positive and negative control, respectively, FGF (1nM) and heparin derivatives or fish GAGs at a range of

concentrations (0.001 to 10μg/ml) for 72 hours at 37°C. After 72 hours, 5μl of tetrazolium salt MTT solution (5mg/ml) was added to each well to measure cell proliferation by forming formazan crystals and then incubated for 4 hours at 37°C. Next, 50μl of 10% SDS with 0.1% HCL was added to each well to dissolve the purple formazan crystals if present and then incubated overnight. The proliferation assay was measured by reading the absorbance at 570nm and 630nm using a plate reader.

Inhibition assay (MTT proliferation)

Approximately 10,000 cells were placed onto a 96 well plate with 100μl of BaF3 medium and then incubated with and without IL-3 as positive and negative control respectively for 72 hours at 37°C. Fish GAGs at a range of concentration (0.001 to 10ug/ml) are incubated with cells, BaF3 medium, FGF (1nM) and heparin (200ng/ml) to display whether the fish GAGs are competitive inhibitors or not. After 72 hours, 5μl of tetrazolium salt MTT solution (5mg/ml) was added to each well to measure the cell proliferation by forming purple formazan crystals and then incubated for 4 hours at 37°C. Next, 50ul of 10% SDS with 0.1% of HCl was added to each well to dissolve the purple formazan crystals if present and then incubated overnight. The proliferation assay was measured using a plate reader by reading the absorbance value at 570nm and 630nm.

Statistical Analysis

Statistical analysis was performed using GraphPad software. Two-way ANOVA and Tukey's multiple comparison test were used to determine the significant difference between the control and each variant of fish GAGs.

Results

Proliferation Assays on BaF3-FGFR1c cells.

The ability of various marine GAGs to promote FGF signalling was examined using the BaF3 cells. These BaF3 cells are mouse B-lymphocytes, which lack endogenous heparan sulphate, and the transfection of FGFR is needed to examine the FGF signalling. In this assay, the isoform FGFR1c is transfected into the BaF3 cells. The exogenous test FGF and a marine GAG were added into the culture medium to see if the marine GAG supports the FGF signalling. The cells will multiply if the components support FGF signalling, and the multiplication of the cells is detected by means of MTT. If no cell proliferation occurs, this shows that the component is unable to support the FGF signalling. Parental heparin was used as a comparison data as it was revealed that heparin with FGF2 could support the FGF signalling causing the cells to proliferate. IL-3 was used as a positive control to see if the cells are working as BaF3 cells are IL-3 dependent cells that fail to respond to FGF2 without heparin.

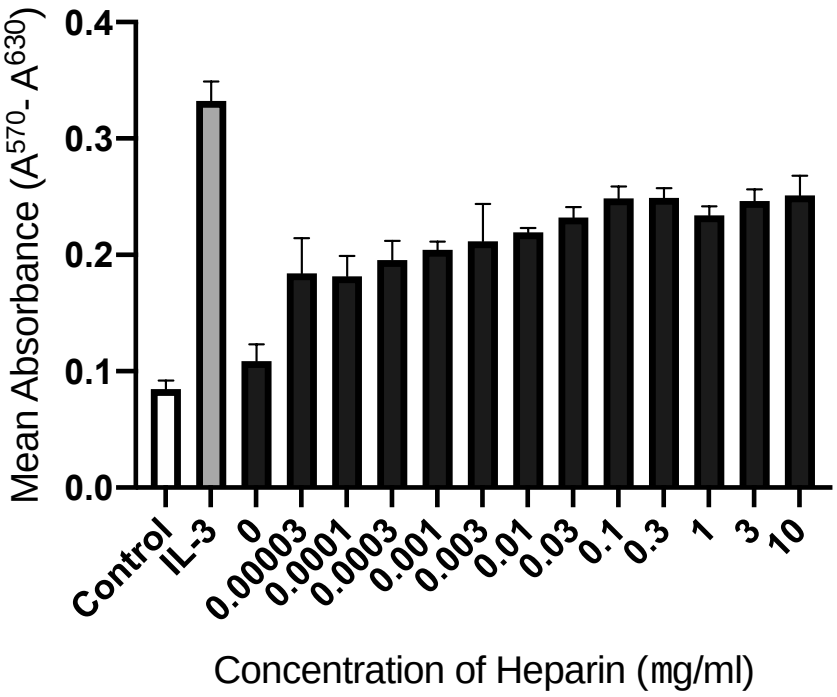
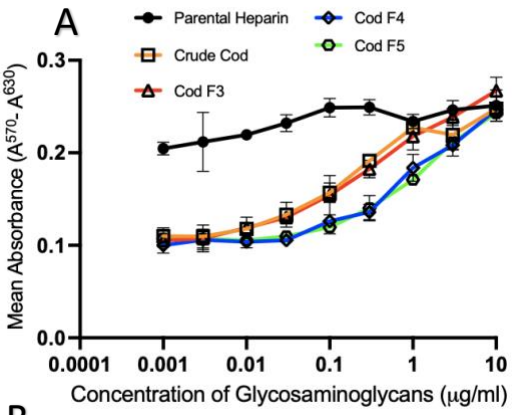


Figure 9. MTT Proliferation Assay on BaF3-FGFR1c cells with Parental Heparin and FGF2. *FGF2 (1nM) was added with an increasing heparin concentration to the BaF3 cells transfected with FGFR1c. The absorbance values were measured after five days.*

BaF3 cells transfected with FGFR1c cultured with FGF2 and parental heparin shows increased binding of FGF2 and increased proliferation response as the concentration of heparin increases. According to previous studies, the growth in proliferation response with FGF2 and heparin was expected, and it is used as comparison data for the marine polysaccharides (Salmon, Cod, and Hake).

A



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Crude	***		N.S	*	*
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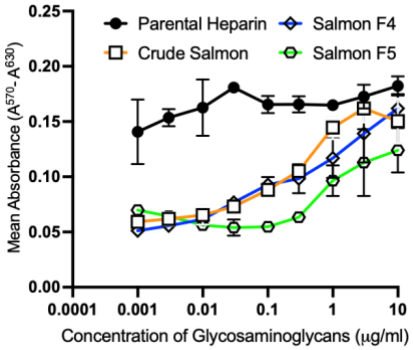
F4	***	*	*		N.S.
F5	***	*	*	N.S.	

B



	Parental	Crude	F4	F5
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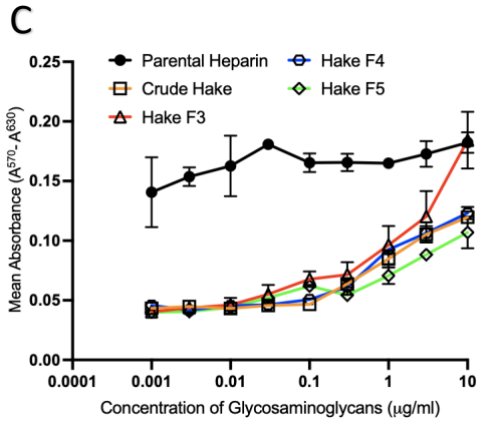
Crude	***		N.S.	**
F4	***	N.S.		*
F5	***	**	*	

C



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Parental		***	***	***	***
Crude	***		N.S.	N.S.	N.S.
F3	***	N.S.		N.S.	*
F4	***	N.S.	N.S.		N.S.
F5	***	N.S.	*	N.S.	

Figure 10. MTT proliferation assays on BaF3-FGFR1c cells with various marine polysaccharides and FGF2. *FGF2 (1nM) was added with increasing concentration of heparin or various marine polysaccharides (A Cod, B Salmon, C Hake) to the BaF3 cells transfected with FGFR1c. After five days, the absorbance values were measured at 570nm and 630nm. The table show results of statistical comparison between the heparin and the marine polysaccharides using Two-way ANOVA and Tukey's test. *P<0.05; **P<0.01; ***P<0.001; N.S., not significant.*

All the Cod compounds show similar stimulation to the parental heparin at 10μg/ml. Crude Cod and variant Cod F3 show better FGF2/FGFR1c signalling stimulation than variant Cod F4 and F5. Crude Cod and variant Cod F3 start to promote signalling at 0.01ug/ml, whilst the other two variants of Cod start to show mitogenic activity at 0.1μg/ml.

All the variants of Salmon polysaccharides were able to support FGF2 signalling but promote less FGF signalling compared to the parental heparin. Salmon F5 shows less proliferation response than the other two variants of Salmon polysaccharides. Crude Salmon showed maximal stimulation at 3μg/ml, whilst the different two variants needed higher concentration to show the maximal stimulation due to increased proliferation from 1-10μg/ml. Crude Salmon and variant Salmon F4 start to promote FGF signalling at 0.03μg/ml, whilst Salmon F5 start to show mitogenic activity at 1μg/ml.

All the Hake GAGs with FGF2 used in the MTT proliferation assay can support the FGF signalling. However, all the Hake compounds except variant Hake F3 show less cell proliferation than the parental heparin, suggesting that it is ineffective compared to parental heparin. Furthermore, variant Hake F3 and F5 start to show binding at 0.03μg/ml, while crude Hake and variant Hake F4 promote FGF2 signalling at 0.3μg/ml.

The absorbance value for parental heparin in figure 10A is different to the absorbance value in figure 10B and 10C. This is because the tests were conducted on a different day so the result might differ due to the condition of the Baf3 cells. Parental heparin was needed to be done each time the MTT proliferation assay was performed for the accuracy of the experiment but also determine if the BaF3 cells that was transfected with FGFR1c was working.

The same isoform FGFR1c is transfected into the BaF3 cells. The exogenous test FGF1 and marine GAGs were added into the culture medium to see if the marine GAGs support FGF1 signalling. Parental heparin was used as a comparison data as it was shown that heparin with FGF1 could support the FGF signalling causing the cells to proliferate. IL-3 was used as a positive control to see if the cells are working as BaF3 cells are IL-3 dependent cells that fail to respond to FGF2 without heparin.

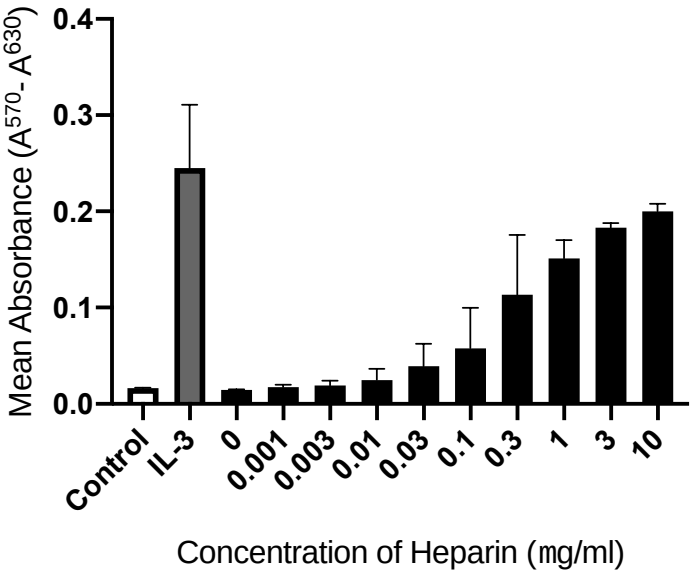
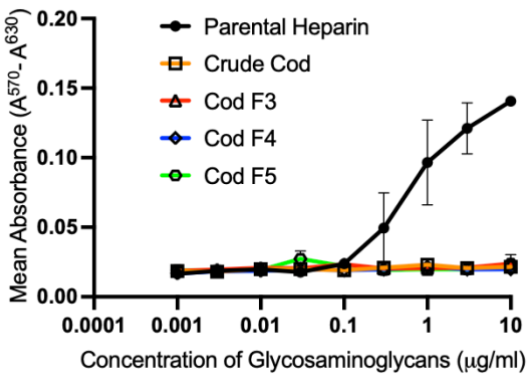


Figure 11. MTT Proliferation Assay on BaF3-FGFR1c cells with Parental Heparin and FGF1. *FGF1 (1nM) was added with increasing concentration of heparin to the BaF3 cells transfected with FGFR1c. The absorbance values were measured after five days.*

BaF3 cells transfected with FGFR1c cultured with FGF1 and parental heparin shows increased binding of FGF1 and increased proliferation response as the concentration of heparin increases. According to previous studies, the increase in proliferation response with FGF1 and heparin was expected, and it is used as comparison data for the marine polysaccharides (Salmon, Cod and Hake). Parental heparin starts to promote FGF signalling at 0.03μg/ml.

A



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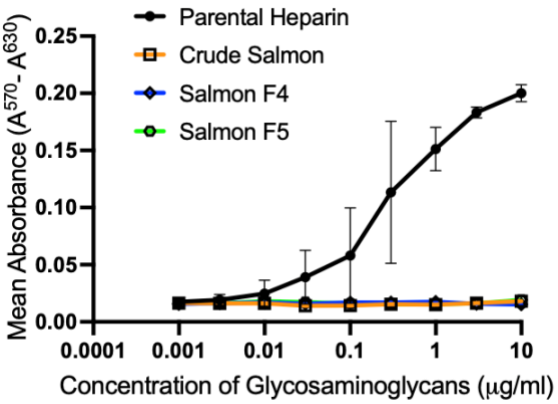
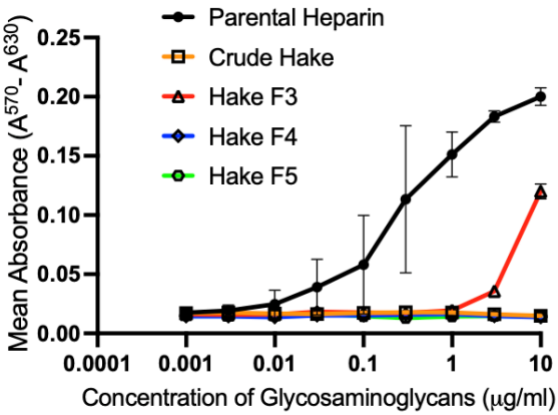
Parental		***	***	***	***
Crude	***		N.S.	N.S.	N.S.
F3	***	N.S.		N.S.	N.S.
F4	***	N.S.	N.S.		N.S.
F5	***	N.S.	N.S.	N.S.	

B



	Parental	Crude	F4	F5
Parental		***	***	***
Crude	***		N.S.	N.S.
F4	***	N.S.		N.S.
F5	***	N.S.	N.S.	

C



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Parental		***	***	***	***
Crude	***		N.S.	N.S.	N.S.
F3	***	N.S.		N.S.	N.S.
F4	***	N.S.	N.S.		N.S.
F5	***	N.S.	N.S.	N.S.	

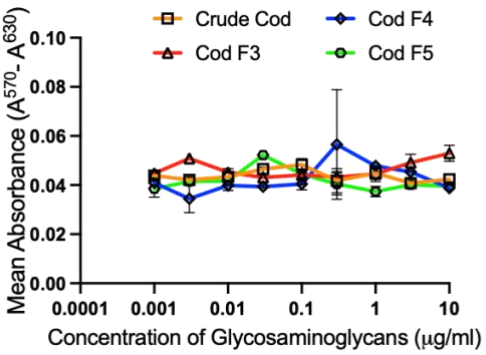
Figure 12. MTT proliferation assays on BaF3-FGFR1c cells with various marine polysaccharides and FGF1. *FGF1 (1nM) was added with increasing concentration of heparin or various marine polysaccharides (A Cod, B Salmon, C Hake) to the BaF3 cells transfected with FGFR1c. After five days, the absorbance values were measured at 570nm and 630nm. The table show results of statistical comparison between the heparin and the marine polysaccharides using Two-way ANOVA and Tukey's test. *P<0.05; **P<0.01; ***P<0.001; N.S., not significant.*

All the marine GAGs, except Hake F3, with FGF1, could not support the FGF signalling due to no cell proliferation occurring during the MTT assay. However, the Hake F3 supported FGF signalling showing cell proliferation but not as much as parental heparin as most of the fish compounds could not support the FGF signalling. In addition, the Hake F3 could not activate FGF signalling at a low concentration but start to show some cell proliferation at 3μg/ml, suggesting that a higher dose of Hake F3 is required to activate FGF signalling.

The absorbance value for parental heparin in figure 12A is different to the absorbance value in figure 12B and 12C. This is because the tests were conducted on a different day so the result might differ due to the condition of the Baf3 cells. Parental heparin was needed to be done each time the MTT proliferation assay was performed for the accuracy of the experiment but also determine if the BaF3 cells that was transfected with FGFR1c was working.

Further experiments were conducted to see if the fish compounds can bind with FGF1 and prevent FGF signalling from occurring by using the same cell proliferation (MTT) assay. The cell culture medium contains exogenous FGF, parental heparin and marine GAGs. In addition, parental heparin was added to the cell culture medium to see if the marine GAGs could act as a competitive inhibitor, as shown in figure 11 that parental heparin can activate FGF1 signalling.

A



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Crude		N.S.	N.S.	*
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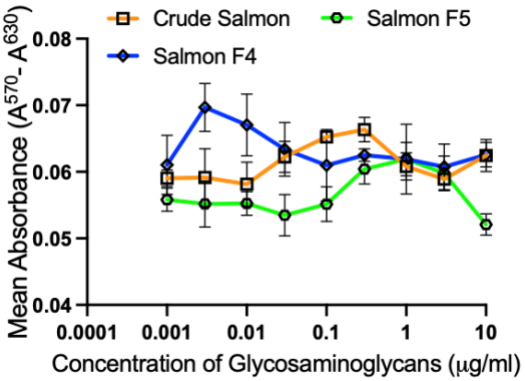
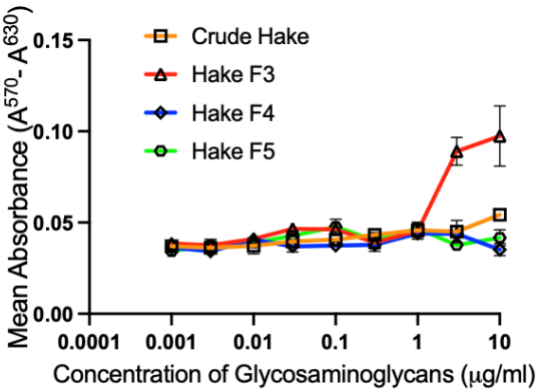
F4	N.S.	N.S.		N.S.
F5	*	**	N.S.	

B



	Crude	F4	F5
Crude		N.S.	***
F4	N.S.		***
F5	***	***	

C



	Crude	F3	F4	F5
Crude		*	N.S.	N.S.
F3	*		*	*
F4	N.S.	*		N.S.
F5	N.S.	*	N.S.	

Figure 13. MTT Inhibition assays on BaF3-FGFR1c cells with various marine polysaccharides and FGF1. *FGF1 (1nM) and heparin (200ng/ml) were added with increasing concentrations of various marine polysaccharides (A Cod, B Salmon, C Hake) to the BaF3 cells transfected with FGFR1c. After five days, the absorbance values were measured at 570nm and 630nm. The table show results of statistical comparison between the heparin and the marine polysaccharides using Two-way ANOVA and Tukey's test. *P<0.05; **P<0.01; ***P<0.001; N.S., not significant.*

All the variant of Cod and Hake compounds shows no inhibitory effect on FGF1/FGFR1c signalling. However, the inhibition assay proves in figure 8 that variant Hake F3 can support FGF1 signalling due to the increased cell proliferation at 3μg/ml. For crude Salmon and variant Salmon F4 show no inhibitory effect on the FGF1 signalling during variant Salmon F5 show potential inhibition on FGF1 signalling at 3μg/ml.

Since there were no control to assess the inhibitory effect on the FGF1/FGFR1c signalling and to see the comparison with a substance that can inhibit the FGF1/FGFR1c signalling. Repeating this assay with a control to compare the inhibitory effect is needed. Using chlorate as a control can be used to see if the marine glycosaminoglycan has an inhibitory effect. It is shown that using chlorate can block the production of endogenous heparan sulphate which prevent the binding of FGF. This caused the prevention of the FGF signalling since the heparan sulphate cannot bind to the FGF and FGFR. (Guimond et al., 1993)

Proliferation Assays on BaF3-FGFR2c cells.

The ability of various marine GAGs to promote FGF signalling was examined using the BaF3 cells. The isoform FGFR2c is transfected into the BaF3 cells. The exogenous test FGF and a marine GAG were added into the culture medium to see if the marine GAG supports the FGF signalling. The cells will multiply if the components support FGF signalling, and the multiplication of the cells is detected by means of MTT. If no cell proliferation occurs, this shows that the component is unable to support the FGF signalling. Parental heparin was used as a comparison data as it was revealed that heparin with FGF2 could support the FGF signalling causing the cells to proliferate. IL-3 was used as a positive control to see if the cells are working as BaF3 cells are IL-3 dependent cells that fail to respond to FGF2 without heparin.

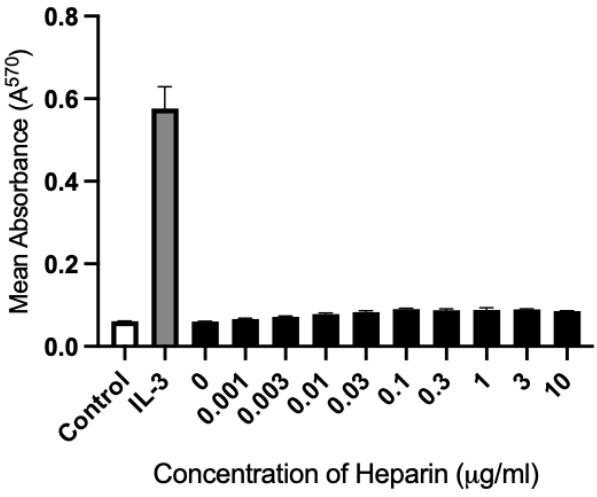
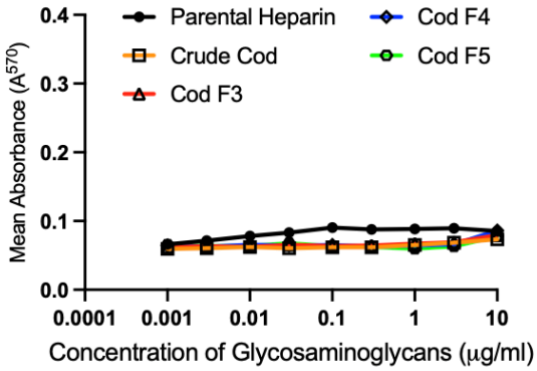


Figure 14. MTT Proliferation Assay on BaF3-FGFR2c cells with Parental Heparin and FGF2. *FGF2 (1nM) was added with increasing concentration of heparin to the BaF3 cells transfected with FGFR2c. The absorbance values were measured after five days.*

BaF3 cells transfected with FGFR2c cultured with FGF2 and parental heparin show no cell proliferation compared to the IL-3 shown in figure 14 suggests that parental heparin cannot activate FGF signalling.

A



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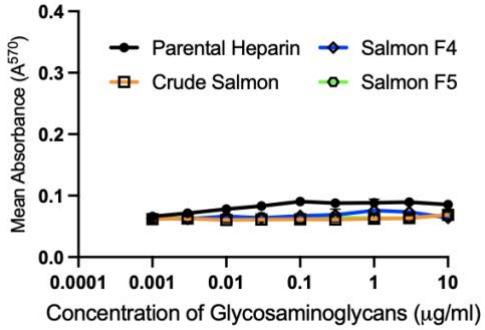
Crude	***		N.S.	N.S.	N.S.
F3	***	N.S.		N.S.	N.S.
F4	***	N.S.	N.S.		N.S.
F5	***	N.S.	N.S.	N.S.	

B



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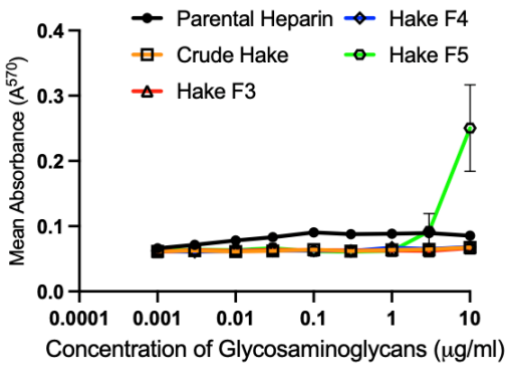
F5	***	N.S.	N.S.	

C



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Crude	N.S.		N.S.	N.S.	*
F3	N.S.	N.S.		N.S.	*
F4	N.S.	N.S.	N.S.		*
F5	N.S.	*	*	*	

Figure 15. MTT proliferation assays on BaF3-FGFR2c cells with various marine polysaccharides and FGF2. *FGF2 (1nM) was added with increasing concentration of heparin or various marine polysaccharides (A Cod, B Salmon, C Hake) to the BaF3 cells transfected with FGFR2c. The absorbance values were measured at 570nm after five days. The table show results of statistical comparison between the heparin and the marine polysaccharides using two-way ANOVA and Tukey's test. *P<0.05; **P<0.01; ***P<0.001; N.S., not significant.*

All the variants of Cod heparan sulphate could not support FGF2 signalling at a low concentration (<1μg/ml) due to low cell proliferation. However, the activation of FGF2 signalling starts to occur after 1μg/ml. All the variants of Salmon polysaccharides were able to show some cell proliferation but not as much compared to heparin. Figure 15b shows that crude Salmon and variant Salmon F5 show some cell proliferation at 10μg/ml, suggesting that they could activate FGF2 signalling whilst variant Salmon F4 show maximal stimulation at 1μg/ml. On the other hand, all the variants of Hake GAGs, except for variant Hake F5, could not activate FGF2 signalling, whilst variant Hake F5 could proliferate the cells at 3μg/ml. Figure 15c also shows that the parental heparin could not activate FGF2 signalling compared to Hake F5. When comparing to figure 15, heparin in figure 15c has a similar mean absorbance value compared to heparin in heparin figure 15a and 15b. This might suggest that the heparin could support a little FGF2 signalling when BaF3 cells were transfected with FGFR2c.

Although previous studies have shown that heparin can induce FGF2/FGFR2c signalling, there could be many reasons why heparin couldn't work during the MTT assay. This could be cross-contamination during the procedure of MTT assay, or the cells were not fully transfected with the FGFR2c receptor. Another possible explanation is that the Hake F5 might promote cell proliferation different rather than using FGF2 signalling. BaF3 cells require IL-3 to maintain the growth and the multiplication of the BaF3 cells as glycosaminoglycans are known to bind many different proteins. Hake F5 might have been able to phosphorylate the tyrosine residues in the beta-subunit of the IL-3 receptor (Pyarajan et al., 2008), or it can bind with the FGFR2c without FGF2, which then promote FGF signalling pathway by transphosphorylation. However, further investigation needs to be conducted for confirmation if heparin can promote FGF2 signalling or not and see if Hake F5 can promote cell proliferation without adding the FGF2.

The same isoform FGFR2c is transfected into the BaF3 cells. The exogenous test FGF1 and marine GAGs were added into the culture medium to see if the marine GAGs support FGF1 signalling. Parental heparin was used as a comparison data as it was shown that heparin with FGF1 could support the FGF signalling causing the cells to proliferate. IL-3 was used as a positive control to see if the cells are working as BaF3 cells are IL-3 dependent cells that fail to respond to FGF2 without heparin.

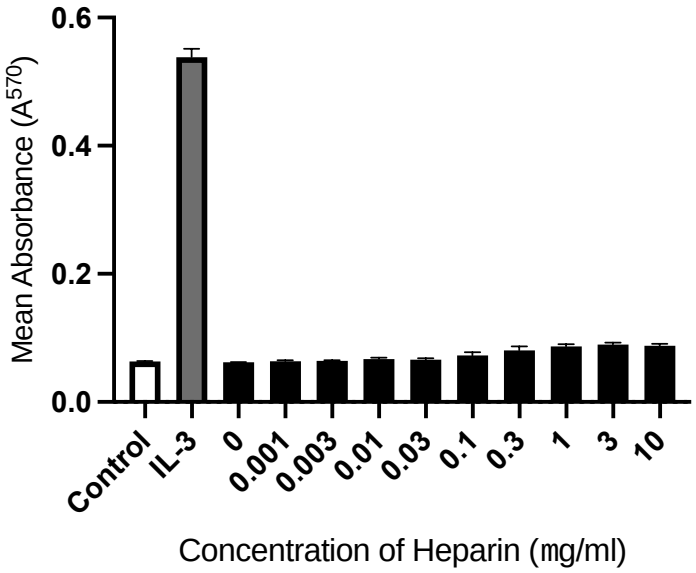
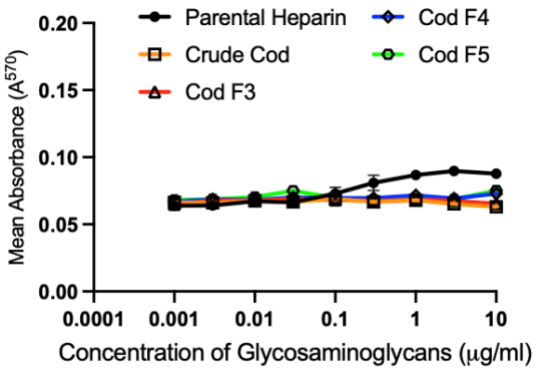


Figure 16. MTT Proliferation Assay on BaF3-FGFR2c cells with Parental Heparin and FGF1. *FGF1 (1nM) was added with increasing concentration of heparin to the BaF3 cells transfected with FGFR2c. The absorbance values were measured after five days.*

BaF3 cells transfected with FGFR2c cultured with FGF1 and parental heparin show no cell proliferation compared to the IL-3 shown in figure 12 suggests that parental heparin cannot activate FGF signalling.

A



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Crude	*		N.S.	***	***
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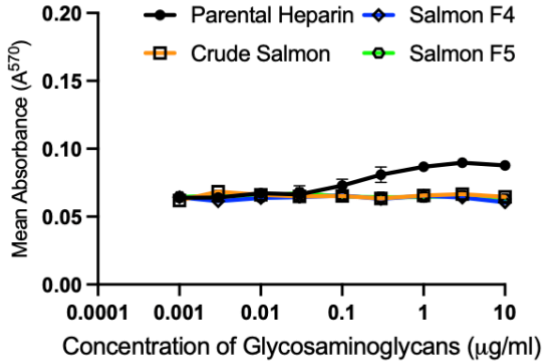
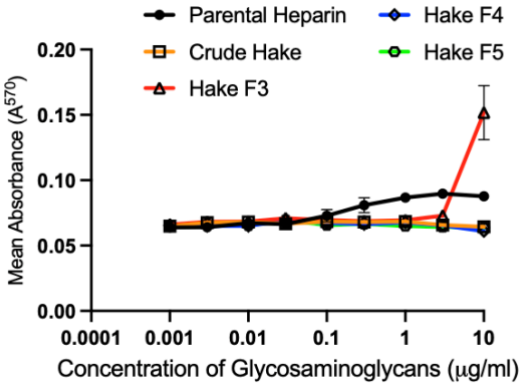
F4	N.S.	***	**		N.S.
F5	N.S.	***	**	N.S.	

B



	Parental	Crude	F4	F5
Parental		***	***	***
Crude	***		N.S.	N.S.
F4	***	N.S.		N.S.
F5	***	N.S.	N.S.	

C



	Parental	Crude	F3	F4	F5
Parental		*	N.S.	**	**
Crude	*		N.S.	N.S.	*.
F3	N.S.	N.S.		N.S.	N.S.
F4	**	N.S.	N.S.		N.S.
F5	**	*	N.S.	N.S.	

Figure 17. MTT proliferation assays on BaF3-FGFR2c cells with various marine polysaccharides and FGF1. *FGF1 (1nM) was added with increasing concentration of heparin or various marine polysaccharides (A Cod, B Salmon, C Hake) to the BaF3 cells transfected with FGFR2c. The absorbance values were measured at 570nm after five days. The table show results of statistical comparison between the heparin and the marine polysaccharides using one-way ANOVA and Tukey's test. *P<0.05; **P<0.01; ***P<0.001; N.S., not significant.*

Figure 17a showed no mitogenic activity when crude Cod and variant Cod F3 were added with FGF1. However, variant Cod F4 and F5 show the potential of FGF1 signalling at 10μg/ml. Figure 17b shows no mitogenic activity on all the Salmon polysaccharides. Figure 17c shows no cell proliferation occurring for crude Hake and variant Hake F4 and F5, while variant Hake F3 can activate FGF1 signalling at 10ug/ml and show better stimulation than parental heparin.

Discussion

Detection of FGF2 signalling in BaF3-FGFR1c/2c cells

Previous studies show that mammalian heparin can bind with FGF2 and FGFR1/FGFR2, allowing intracellular signalling to occur. However, studying modified heparin shows that in many cases, the interaction between GAGs and proteins is not specific, and the charge density and the presence of specific sulphated units cause the interaction between GAGs and proteins (Valcarcel et al., 2017). Glycosaminoglycans derived from marine organisms were tested in the experiment to see if they have a similar ability to mammalian heparin interacting with FGF. Marine glycosaminoglycans possess a unique and diverse structure that cannot be found in heparan sulphate or heparin derived from terrestrial animals (Valcarcel et al., 2017). Marine polysaccharides have a homogeneous structure showing no alteration in their structure, whereas mammalian derived GAGs show high structural variability according to the cell and tissue types (Zainudin et al., 2014). These marine glycosaminoglycans also contain unusual sulphation patterns, which provide a greater understanding of the requirement for the binding of FGF and FGFR. This explains the importance of using marine-derived GAGs to show in-depth knowledge of the GAGs interaction with proteins (Valcarcel et al., 2017). Different variants of polysaccharides derived from Salmon, Cod and Hake were used. The result shows that all variants of marine polysaccharides used in the experiment can bind with FGF2 and promote FGF signalling with the BaF3 cells transfected with FGFR1c. For the FGF2/FGFR2c signalling, the result shows that the Cod variants could not promote the signalling due to low cell proliferation. For the

Hake polysaccharides, all the Hake variants could not promote FGF2/FGFR2c signalling except for variant Hake F5. All the Salmon variants were also able to promote some FGF2 signalling.

Heparan sulphate can bind to FGF2 due to its residues 2-O-sulphated L-iduronate and N-sulphated D-glucosamine (Goodger et al., 2008). Pentasaccharides contain identical residues that bind to FGF2 (Zhang et al., 2009). However, for FGF signalling to occur, heparan sulphate needs to bind with FGFR and FGF. FGF2 signalling requires 6-O-sulphated residues in heparan sulphate to bind with FGFR1. 2-O-sulphation is vital for the binding of FGF2 (Zhang et al., 2009), whilst 6-O-sulphation is essential for the binding with FGFR1 allowing signal transduction to occur (Wu et al., 2003). However, 6-O-sulphation is not needed to bind FGF2 (Zhang et al., 2009). The two residues, 2-O-sulphated iduronate and N-sulphated glucosamine present in the saccharide chain, is vital in FGF signalling. As shown in hexasaccharide interacting with FGF2, one disaccharide from the hexasaccharide was mainly interacting with the heparin-binding site of FGF2, offering a potential of hydrogen bond-forming and salt bridge interaction (Faham et al., 1998). The residue 2-O-sulphated iduronate formed six out of the nine hydrogen bonds, and the other three came from the N-sulphated glucosamine. Polysaccharides derived from Cod, Hake, and Salmon could promote FGF2 signalling. This could suggest that the marine polysaccharide could have the residues 2-O-sulphated iduronate and N-sulphated glucosamine. The marine polysaccharide could have residues forming nine hydrogen bonds, enabling them to bind with FGF. The marine polysaccharide could also contain 6-O-sulphated residue as it can bind with FGFR allowing mitogenic activity to occur.

Crystallography of the FGF2:FGFR1 complexes shows that the heparin oligosaccharide can bind with two FGF and two FGFR (Schlessinger et al., 2000). D2 domain of the FGFR is positively charged, allowing the heparin oligosaccharide to form a bond with the positively charged D2 domain (Plotnikov et al., 1999). This allows two FGF and two FGFR to be connected, forming a 2:2:1 FGF-FGFR-HS complex (Harmer et al., 2006). This suggests that the marine polysaccharide can interact with a positively charged domain which seems reasonable as marine polysaccharide is highly sulphated.

Previous studies show that decasaccharides can form 30 hydrogen bonds with two FGF and FGFR (Schlessinger et al., 2000). This suggests that some variants of the fish compound that can activate FGF signalling can also form 30 hydrogen bonds with the FGF and FGFR. Twenty-five hydrogen bonds are included in the presence of heparin in the first FGF: FGFR complex, whilst the other five hydrogen bonds are made in the second FGF: FGFR complex showing a 1:2:2 ratio of heparin, FGF and FGFR (Schlessinger et al., 2000). Nine hydrogen bonds are formed on the heparin-binding site of immunoglobin-like subunit II (D2) of FGFR where residue lysines 160, 163, 172, 175 and 177 are responsible for forming hydrogen bonds (Schlessinger et al., 2000). Interaction with FGF and heparin shows 16 hydrogen bonds present. Ten hydrogen bonds are sulphated mediated, whilst the other six hydrogen bonds could be involved with the carboxylate, linker, or ring oxygens of heparin (Schlessinger et al., 2000). Previously hexasaccharide heparin can interact with FGF2 and show that particular surface residue forms the heparin-binding site on FGF (Faham et al., 1996). These surface residues are found in numerous locations of the β-strands of FGF. The surface

residues forming the heparin-binding sites are Asn-27, Arg-120, Thr-121, Lys-125, Lys-129, Gln-134, Lys-135 and Ala-136. These residues are in various β-strands where Asn-27 is situated at the β1-β2 loop, and Arg-120 and Thr-121 are located in the β9-β10 loop. The rest of the residues are located at the β11-β12 loop (Schlessinger et al., 2000). This study could imply that the marine polysaccharide that activates FGF signalling by binding with FGF contains a sequence that allows them to bind with the surface residues of the heparin-binding site in FGF2.

With FGF2, two hydrogen bonds are linked with the 6-O-sulphate group of heparins whilst the other remaining hydrogen bonds are involved with the N-sulphate and 2-O-sulphate groups of heparins. The two hydrogen bonds involving the 6-O-sulphate group of heparins are formed by interacting with the residue Lys-135 and Thr-121 of FGF2 (Schlessinger et al., 2000). This could prove that the marine GAGs that can activate FGF2 signalling could have these sulphation patterns allowing them to form hydrogen bonds and interact with the residues promoting FGF2-heparin interaction.

Detection of FGF1 signalling in BaF3-FGFR1c/2c cells

Mammalian heparin was known to promote FGF1 signalling by binding with FGF1 and FGFR. However, the marine polysaccharide used in the experiment could not promote FGF1 signalling showing that the marine polysaccharide cannot bind to FGFR. Therefore, the marine polysaccharide could not bind with FGF1.

Polysaccharides derived from Cod, Salmon and Hake cannot activate FGF1 signalling and cannot bind with FGF1 proven with the inhibition assay, except for Hake F3. However, earlier studies showed that FGF1 could recognise octasaccharide containing the sequence of IdoA2SGlcNS6SIdoA2S trisaccharide motif (Harmer, 2006). This explains that the fish GAGs might not have the IdoA2SGlcNS6SIdoA2S trisaccharide motif in their polysaccharide except for Hake F3 suggests that the variant Hake F3 may contain the exact or similar sequence of IdoA2SGlcNS6SIdoA2S trisaccharide in it.

A study shows that many 6-O-sulphate groups in GlcNS residue are needed to activate FGF1 signalling, but it is not required for FGF2 signalling (Ishihara et al., 1997). FGF1 signalling requires N, 2-O and 6-O sulphate groups for binding and activation (Robinson et al., 2005). 6-O-sulphation is necessary for the binding of FGF1 but not for FGF2. However, at least one 6-O-sulphate group is required to form ternary complexes. For FGF1/FGFR1 signalling, the three sulphate groups play a critical role during receptor dimerisation. 2-O sulphation is essential for FGF1 binding, whilst both 2-O and 6-O sulphation is critical for FGFR1 binding. In addition, N-sulphation is critical for forming the ternary FGF-FGFR-HS complex (Wu et al., 2003).

The heparin-binding region of FGF1 consists of three surface loops, and the sulphate groups cause the interaction with heparin from the heparin. Earlier studies show that decasaccharide can dimerise FGF1 in a trans configuration by binding with two FGF1 (Brown et al., 2013). The oligosaccharide sequences are N-sulphated glucosamine, and 2-O-sulphated iduronate (i.e., GlcN-IdoA-GlcN) required to bind with FGF1 while adding 6-O-sulphation on the third glucosamine strengthen the dimerisation by

interaction with another FGF (Canales et al., 2006). These residues form the heparin-binding site; Asn18, Lys113, Lys118, Gln127 and Lys128. The three-hydroxyl group from the iduronate residue is responsible for forming the hydrogen bond with residues Asn18, and Lys113 whilst the 3-O-sulphate group in the disaccharide shows electrostatic interaction with the residue Lys118, which enhances the binding affinity (Hu et al., 2012). With the interaction of FGF1 and heparin, the 6-O sulphation of the glucosamine unit can interact with residue Asn114 of FGF1 (Kreuger et al., 1999). 2-O sulphated iduronate interacts closely with residues Lys126 and Lys127 of FGF1 and the N-sulphate groups from the glucosamine were found to interact with residues Lys142 and Gln141. (Pellegrini, 2001).

The requirement for oligosaccharides is particular when binding to FGF and FGFR as the electrostatic and topological characteristics of the canyon are unique for each FGF signalling, i.e. the canyon of FGF1 with FGFR1c should be distinct from FGF2-FGFR1c (Schultz et al., 2017). The sulphation on the disaccharide units of the glycosaminoglycan is very specific when interacting with the heparin-binding region of FGF and FGFR. Studies show that the 6-O sulphation is critical for FGF1 binding but not for FGF2. However, 6-O sulphation is very important for interaction with FGFR in FGF2 signalling (Wu et al., 2003). The sequence of the glycosaminoglycans is also vital for the activation of FGF signalling especially FGF1 signalling. The reducing end of disaccharide units is mainly important as they can interact with the heparin-binding domain of FGF and FGFR, which allow them to form hydrogen bonds and electrostatic bonding. Electrostatic interaction occurs between the FGF1 and the hexasaccharide caused by the positively charged amino acid residues, Lys127, Ser130, Arg133, Lys142

and the negatively charged sulphate groups from the hexasaccharide. In addition, some other residues Asn32, Gln141, ala143 and lys132 from FGF1, were also involved with the interaction with the hexasaccharide, especially in the region of GlcN6-GlcN4, whilst the residues Arg136, Lys126 and Arg133 show close contact with the iduronate units in the hexasaccharide (Canales et al., 2006)

Interaction with FGFR

Studies have shown that the single disaccharide at the nonreducing end of the heparin is responsible for the interaction with FGFR. The 6-O-sulphate and the N-sulphate from the disaccharide interact with lys160, lys163, lys175 and lys177 from the heparin-binding loop of FGFR1. In addition, 2-O-sulphated iduronic acid interacts with Lys172 of FGFR1 (Pellegrini, 2001). The residues from the heparin-binding loop of FGFR2 that interact with the 6-O-sulphate saccharide are lys161 and lys164 whilst N-sulphate of the disaccharide interacts with the residue Lys176 and Arg178 from FGFR2 (Pellegrini, 2001). 6-O-sulphation is important for the binding of FGFR as it is required for mitogenicity but not for FGF2 binding (Guimond et al., 1993). 6-O-sulphated glucosamine is involved with the residues lys164 and His167 from FGFR, which allow them to form hydrogen bonds. In addition, the 6-O-sulphation interacts with Thr174 and Val175 via a Van Der Waals bond. The residues lys164 is important when interacting with 6-O-sulphated glucosamine as it can neutralize the 6-O-sulphation allowing the sulphate group to be partially buried (Pellegrini et al., 2000).

Several studies suggest that heparan sulphate proteoglycan can bind to two FGF and two FGFR, causing receptor dimerization, allowing FGF signalling to occur (Goetz and Mohammadi, 2013). The binding of heparin causes the dimeric assembly to the sulfate ion sites through the electropositive channel. In addition, the C termini of FGFR located in the immunoglobulin-like subunit III (D3) is responsible for receptor dimerization in the presence of heparan sulphate proteoglycan (Stauber et al., 2000). This may suggest that the fish GAGs could also bind to two FGF and two FGFR, causing receptor dimerization.

Marine Glycosaminoglycans

Polysaccharides isolated from marine species express unique, diverse structures while showing a low anticoagulant potential (Mycroft-West et al., 2018). Due to the diverse structure, this ultimately leads to rare GAGs in marine organisms that cannot be found in terrestrial animals (Valcarcel et al., 2017). The marine ecosystem shows approximately 33% to 66% of marine species yet to be discovered, which can provide a potential of novel GAG-like compounds for future therapeutic applications (Mycroft-West et al., 2018). Marine glycosaminoglycans show a distinct sulphation pattern allowing them to produce a change in charge density in the GAG chains. Marine GAGs also offer unusual sulphation patterns and sequences of saccharides showing greater interest in their composition and ability to bind with proteins.

The structure of the marine compounds used in the experiments remains unknown, but sources from Valcarcel et al., 2017 shows us the percentage of disaccharide units

in Cod and Salmon which were isolated from the bone and Mycroft-West et al., 2018 provided the molecular weight of the Salmon and the Cod polysaccharide. Both sources state that the Cod and Salmon glycosaminoglycans are chondroitin sulphate, containing different disaccharide units than heparan sulphate (Valcarcel et al., 2017) (Mycroft-West et al., 2018). Chondroitin sulphate is composed of alternating disaccharide units of glucuronic acid and N-acetylgalactosamine, where the hydroxyl group can be sulphated at variable positions (Volpi, 2011). Chondroitin sulphate can also be found in terrestrial animal sources where they mainly express non-sulphated disaccharide (CS-0) and monosulphated disaccharide, which is sulphated at position 4 or 6 of the N-acetylgalactosamine (CS-A and CS-C, respectively) (López-Álvarez et al., 2019). While marine chondroitin sulphate shows disulphated (CS-B, CS-D, CS-E, CS-K and CS-L) or trisulphated disaccharide units (CS-M, CS-S and CS-T) (Vessella et al., 2021). The CS-D unit expresses 2-O sulphated glucuronic acid and 6-O sulphated GalNAc, whilst the CS-E unit shows di-4,6-O sulphated GalNac and glucuronic acid as their repeating unit. CS-B, also known as dermatan sulphate, shows 2-O sulphated iduronic acid and 4-O sulphated GalNAc as their disaccharide unit (Sugahara et al., 2003).

The chondroitin sulphate extracted from Cod possesses the molecular mass of 18.12kDa with mainly composed of a C-unit (27.3%), A-unit (60%) and D-unit (10%). The chondroitin sulphate from Cod possesses more D-unit than Salmon chondroitin sulphate (4%) by 6%. Salmon chondroitin sulphate is comprised of A-unit (51%), C-unit (37%) and D-unit and have a molecular mass of 20.07kDa (Valcarcel et al., 2017) (Mycroft-West et al., 2018). The Cod compounds show more binding affinity of FGF2

and FGFR1/2 than other fish compounds shown in figure 2 due to more D units. D-unit disaccharide consists of 2 sulphation (2S and 6S), vital for binding FGF2, allowing intracellular signalling. However, the fish compounds could not activate FGF1 signalling suggesting the importance of N-sulphation in the glucosamine from the disaccharide units, which chondroitin sulphate does not have.

The structure of polysaccharides derived from Hake remains unknown. However, from looking at previous studies and the result obtained from this experiment, an assumption can be made that the polysaccharide derived from Hake could have a similar sulphation pattern to the polysaccharide derived from Salmon and Cod. This is because they all can activate FGF2-FGFR1c signalling. However, variant Hake F3 could also activate FGF1-FGFR1c and FGF1-FGFR2c signalling, suggesting that the variant Hake F3 could have a different sulphation pattern than the rest of the marine polysaccharide, which allows the activation of both FGF signalling.

Heparin promotes FGF2 signalling at a very low concentration, and FGF1 signalling for BaF3 cell transfected with FGFR1c whilst the fish chondroitin sulphate used in the experiment was able to promote FGF2 signalling at a high concentration but did not promote FGF1 signalling. The marine polysaccharides could not prevent FGF1 signalling showing that the marine polysaccharide cannot bind to FGF1. Another study used different types of chondroitin sulphate (CS-E), indicating that FGF2 could bind CS-E with similar binding affinity to heparin but interacted weakly with FGF1 showing a similar reaction with some of the marine polysaccharides used in the experiment (Wu et al., 2003). CS-E and dermatan sulphate show a greater binding affinity for FGF2 due

to the 4-O-sulphation from the N-acetyl galactosamine sugar (Hachim et al., 2019). Using alternative marine chondroitin sulphate or dermatan sulphate containing high disulphated disaccharide units could promote FGF signalling due to its high sulphation pattern in their sequence. Those high sulphation patterns could be susceptible to binding with FGF and FGFR, causing receptor dimerization, leading to intracellular signalling.

Using marine GAGs has shown greater interest in the interaction between FGF/FGFR with GAGs, as some marine polysaccharides were able to promote FGF signalling. An example demonstrated that fucoidans obtained from Saccharina japonica (Brown Algae) activated FGF2/FGFR1c signalling. However, the greater interest came from the sulphation pattern that the marine polysaccharide provided. This is because the sulphate content influences the binding with FGF and FGFR than the molecular weight of the saccharide (Geng et al., 2018). For example, fucosylated chondroitin sulphated derived from sea cucumber can bind with FGF1 and FGF2. Fucosylated chondroitin sulphate is a distinct sulphated polysaccharide made of alternating beta-1-4-linked glucuronic acid and beta-1-3-linked N-acetyl-galactosamine disaccharide units with alpha fucose linking to GlcA residue at O-3 position acting as a branch (Li et al., 2018).

The properties of GAGs have shown greater interest, and currently trying to utilize GAGs for the delivery of proteins such as growth factors and cytokines. It is still ongoing clinical goals in drug delivery to the therapeutic sites. To achieve this clinical goal is the use of GAG-based biomaterial when delivering cytokines and growth factors. The use of GAG-based biomaterial has been widely studied and has shown

potential to travel to the target cells and regulate tissue remodelling process or normal healing processes. Delivery of FGF2 is currently the focal point when researching GAG-based biomaterials. Numerous GAGs have shown high binding affinity to FGF2 and could travel to its target cells (Hachim et al., 2019). For example, CS hydrogel has the potential for neural tissue repair and was abled self-renewal of neural stem cells (Karumbaiah et al., 2015). Another example is DS-based biomaterial which can promote cell proliferation during wound healing (Taylor et al., 2005). Heparin is currently the most commonly used for protein delivery as it is readily available and has a high level of sulphation. It is also used in clinic as an anticoagulant (Hachim et al., 2019). However, there are some disadvantages of using heparin due to its high variability in structure, making it very hard to mimic the structure and sulphation degree due to its complex biosynthesis process. Testing out different GAGs from various resources and seeing if they bind with proteins would be ideal as the marine polysaccharides could bind with FGF2 as an example. Further exploiting the marine polysaccharides would be useful and see if they can bind with other proteins. The use of GAGs derived from marine organisms would be an excellent alternative as it is a homogenous structure, so it does not show any alteration in the structure (Zainudin et al., 2014). Marine GAGs are easier to extract and obtain a high yield of compounds during the extraction process compared to mammalian GAGs (Pavao., 2014).

Competitor inhibitor

MTT inhibition assay was conducted to see the potential inhibitory effect on FGF signalling. MTT inhibition assay was mainly tested on FGF1/FGFR1c signalling as it was shown in figure 12 that the marine polysaccharides were not able to promote FGF1 signalling. Several tissues in vivo have shown that many FGFs, especially FGF1 and FGF2, are crucial for angiogenesis, essential for tumour growth and progression. There is an overproduction of autocrine FGF in many tumour types; for example, upregulation of FGF2 and FGF8 initiates cell survival and neoangiogenesis in hepatocellular carcinomas (Brooks et al., 2012). It has seemed that increased plasma levels of FGFs are found in multiple cancer types and shown that the release of FGFs cause to degrade of the extracellular matrix and acts as tumours invade. The first instance occurred in human melanomas where high levels of FGFR1 and FGF2 were observed, and the overproduction of autocrine FGF was produced by the cancer cells, leading to tumorigenesis (Presta et al., 2017).

A model was suggested to reduce tumour growth and angiogenesis. An anti-angiogenic effect could be achieved if heparin or heparin-like compounds at high concentration sequester the angiogenic factor, i.e., FGF, by competing with the heparan sulphate proteoglycan at the cell surface (Ghiselli, 2019). Heparin or heparin-like compounds will bind with the FGF, which prevent the interaction of FGF with target cells acting as an FGFR decoy, and this interaction leads to the development of FGF traps (Presta et al., 2017). Studies show that heparin exhibit antitumor behaviour by inhibiting growth factor signalling, reducing the growth factor stimulation of

tumour cells, and suppressing tumour angiogenesis (Borgenström et al., 2003). Furthermore, deselecting certain sulphation from heparin can inhibit FGF2 signalling as Guimond et al., 1993 show that 6-O desulphated heparin could bind with FGF2 and block the mitogenic activity suggesting that it may block the ternary form of the FGF2-HS-FGFR complex (Guimond et al., 1993). The use of heparin was a possibility for cancer therapy, but there were limitations of using heparin due to its anticoagulant activity and treatment of thrombosis was required if a higher dose of heparin is used (Borgenström et al., 2003).

Variant Salmon F5 got the potential of inhibiting FGF1 signalling in BaF3 cell transfected with FGFR1c (figure), but further investigation is required with high concentration to see if the cell proliferation has decreased when both heparin and variant Salmon F5 is added with exogenous FGF1. There has been greater interest in glycosaminoglycans in growth factor stimulation, as many natural glycosaminoglycans have shown an antitumor effect. The diet based anti-angiogenesis approach have shown greater interest due to its no to minor adverse side effects to humans and shown few plant-based molecules such as carotenoids that can inhibit angiogenesis. It was demonstrated that marine algal carotenoids could inhibit FGF2 signalling, which leads to a decrease in endothelial proliferation (Ganesan et al., 2013).

Limitations

Figures 6 and 8 show that heparin produces no or little mitogenic activity on the BaF3 cells transfected with FGFR2c when added FGF1 or FGF2. However, earlier studies show that heparin can bind FGF1 and FGF2 with FGFR2c and promote signal transduction shown in table 1. However, the figure shows limited activation of FGF1 and FGF2 signalling with the parental heparin due to low absorbance value. Due to the low absorbance value for cell proliferation when FGF1 and FGF2 are added with parental heparin shown in Figures 14 and 16, repeating the MTT proliferation assay is needed with the parental heparin and the fish compounds. The low absorbance value for the parental heparin could result in the cells not responding well with the parental heparin, and the FGF as the positive control (IL-3) showed a good amount of mitogenic activity. This means there could be an issue with the cells or the exogenous fibroblast growth factors. However, some of the Hake compounds could promote signalling despite having low mitogenic activity from the parental heparin, suggesting that there could be a problem with the parental heparin.

Conclusion

Based upon the MTT proliferation assay, it was shown that the marine polysaccharide derived from Cod, Salmon, and Hake was able to facilitate the FGF2/FGFR1c signalling at higher concentration. However, compared with the parental heparin, it was not able to promote as much FGF signalling at low concentration. For the FGF1/FGFR1c signalling, it was shown that the marine polysaccharide was not able to promote the signalling except for the Hake F3. Hake F3 was able to promote the signalling at a high concentration. But compared to the parental heparin, Hake F3 did not promote as much signalling at 10μg/ml. The MTT inhibition assay confirms that some of the marine compounds could not promote FGF1/FGFR1c as they could not bind to FGF1, preventing the formation of FGF1/HS/FGFR1 complex. However, there could be a potential of variant Salmon F5 that can inhibit FGF1/FGFR1 signalling as it can compete with heparin to bind FGF1. For the BaF3 cells transfected with FGFR2c, the Cod and Salmon compounds promoted FGF signalling when added exogenous FGF2, whilst the Hake compounds show no mitogenic activity except for Hake F5. All the marine compounds except for variant Hake F3 could not promote FGF1 signalling. However, heparin shows little cell proliferation, which was unexpected as it was known that heparin promotes FGF2 and FGF1 signalling with FGFR2c as a receptor.

By observing the result for the BaF3 cells that were transfected with FGFR1c cells, it was shown that marine polysaccharide was able to facilitate FGF2/FGFR1c signalling and Hake F3 was able to promote FGF1/FGFR1c signalling. For the cells that were transfected with FGFR2c, Hake F5 was able to facilitate FGF2 signalling and Hake F3

was able to promote signalling. Since it was shown that some of the marine polysaccharide can promote FGF signalling. This means that they can be used as memetic of heparin at a higher concentration. However, it requires more marine polysaccharide to promote FGF signalling than heparin. So economically, they are insufficient at the current time but by investigating other marine polysaccharide. There could be a potential for marine polysaccharide to be used as a mimetic for heparin. Investigating other polysaccharide from different marine species could be potential candidate in providing more information on the structure of FGF and FGFR and how the marine polysaccharide interacts with FGF and FGFR and identify the key component when binding with FGF and FGFR.

As it was confirmed that some marine polysaccharide was able to promote FGF signalling, it means that these marine polysaccharides could have similar or same binding properties to heparin since they can both binds to FGF and FGFR which allow them to promote FGF signalling causing the mitogenic activity in the MTT proliferation assay. Further investigations on the structure of marine polysaccharide can allow us to determine the similarities between the heparin and marine polysaccharide and to identify which binding properties allows them to bind with FGF and FGFR. Since it is known that heparin can bind to FGF1 and FGF2 due to sulphation pattern in the structure (Xu et al., 2013). Investigating the structure of the marine polysaccharide can allow us to determine whether these marine polysaccharides exhibit a similar sulphation pattern to heparin or determine whether FGF binding attracts different binding properties.

Marine polysaccharides have shown many beneficial characteristics when compared to mammalian derived GAGs. They can offer great diversity to its structure showing interesting biological properties (Senni et al., 2011) as marine glycosaminoglycan can bind to growth factors and potentially inhibit growth factor signalling. The marine glycosaminoglycans could play an essential role in developing cell therapy and regenerative medicine when binding with growth factors and future cancer therapy development due to the ability to inhibit growth factor signalling (Valcarcel et al., 2017) (Senni et al., 2011). Although marine glycosaminoglycan could be the future of treatment for many diseases, it could hold possible adverse side effects as some marine polysaccharides show similar risks like heparin by causing bleeding and thrombocytopenia due to its high anticoagulant activity. Therefore, conducting a safety assessment on the marine GAGs would be required by examining the anticoagulant activity to ensure the safety of the unique marine GAGs (Valcarcel et al., 2017). However, studies show that some marine GAGs have less anticoagulant activity than heparin extracted from porcine and bovine as there is always a risk of mammalian animal disease that could affect humans, such as mad cow disease (Kirchen et al., 2018). Currently, no toxic effect has been associated with the use of marine-derived GAGs explaining why extracting GAGs from marine resources is a safer option (Pavao, 2014). An advantage of using marine GAGs is that they can diminish the off-target effect and show no contamination with mammalian pathogens and prions (Mycroft-West et al., 2018). Not only it is safer to use, but it is also easily extracted from marine resources showing cheaper production and extraction process. Extraction and isolation of GAGs polysaccharide from the marine organism is easier to extract than mammalian GAGs showing a more effortless procedure for extraction. In

addition, marine glycans have a higher concentration in the tissue, around 0.5% of the dry weight compared to heparin extracted from pig intestinal mucosa (0.022%) (Pavao., 2014). Nevertheless, marine glycosaminoglycans could be the new heparin analogs and have the potential of becoming the new natural drugs with little or no side effects to treat various diseases (Pavao., 2014).

Further Investigations

Further investigation is required on the structure of the fish GAGs to see how they interact with FGF and FGFR, especially in FGF2 signalling, as it can promote FGF2 signalling with cells transfected with FGFR1c isoform. Analysing the sulphate group concentration can be done using the barium chloride gelatin method. In addition, structural analysis of the fish GAGs can be done using the Fourier transform infrared spectroscopy, which can provide molecular weight and the functional groups in the fish GAGs (Hou et al., 2017).

An inhibition assay could be conducted to see if the fish polysaccharide can inhibit the FGF1 signalling as some of the variants of fish compounds could not activate FGF1-FGFR2c signalling. Suggesting the fish polysaccharide can inhibit FGF signalling by binding with FGF and prevent the formation of the FGF-FGFR-HS complex. Assessing the marine polysaccharides for off-target effects (anticoagulation) would be beneficial as they could be used as a treatment for reducing the stimulation of growth factors for tumour growth and angiogenesis.

Heparin can bind with numerous FGF with their respective FGFR shown in table 1, so using different FGFs to see if the marine polysaccharide could be used as mimetic for heparin and understand more about the requirement for the interaction of each FGFs and FGFRs. Using different polysaccharides derived from various marine organisms to see if they can activate or inhibit FGF1 or FGF2 signalling, as different marine organisms have different sulphation patterns, which will help to know the importance of sulphation when interacting with FGF and FGFR. When isolating glycosaminoglycans from marine organisms, rare chondroitin sulphate could be found, which has not been discovered. These rare chondroitin sulphates could potentially display a strong binding affinity to FGF and FGFR, which could be a perfect heparin memetic.

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