UNIVERSITY OF LIVERPOOL

The Role of Chemically Modified Heparins on Embryonic Stem Cell Fate

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By

Adel Alghamdi

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Abstract

Embryonic stem cells have therapeutic potential in the treatment of different medical conditions, for example, tissue regeneration and wound repair. Current challenges affecting its clinical application include maintenance of pluripotency and directing lineage-specific differentiation. In that context, the extracellular matrix in the stem cell niche has been suggested to play an important role. Notably, the heparan sulfate proteoglycans (HSPGs) have been demonstrated to interact with and potentiate the effects of many classes of growth factors, including the family of FGFs. We have used selectively chemically modified heparins with different sulfation patterns as models of HS structure variations. Here we investigated their effects on mouse embryonic stem cells in terms of their lineage commitment. Broad structure-dependent effects on mouse ESC were noted on a range of markers of different lineage differentiation markers (by RT-PCR, and qPCR), and on various signalling pathway regulation (using Path scan signalling arrays). Using Western blotting, we also observed structure-dependent effects in neural differentiation assays, through influencing FGF signalling via Erk phosphorylation. receptor tyrosine kinases phosphorylation arrays added an extra evidence on the structure-dependent effects. Overall, we have discovered that HS structures can be used to modulate signalling pathways in mouse ESCs, leading to changes in lineage commitment of pluripotent cells. Such compounds have the potential for extensive exploitation in control of pluripotency and lineage differentiation of stem cells in biotechnology applications.

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List of Abbreviations

BM	Basement membrane
BMP	Bone morphogenic protein
cDNA	Complimentary deoxyribonucleic acid
CNS	Central nervous system
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonucleic acid enzyme
EBs	Embryoid bodies
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EG	Embryonic germ
EMT	Epithelia-mesenchymal transition
EGF	epidermal growth factor
Erk1/2	extracellular signal–regulated kinase 1/2
ESC	Embryonic stem cell
EXT1	Exostosin-1
EXT2	Exostosin-2
F	Feeder layer
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GAG	Glycosaminoglycan
HS	Heparan sulfate
HS2ST	2-O-sulfotransferase
HS3ST	3-O-sulfotransferase
HS6ST	6-O-sulfotransferase

HSPG	heparan sulfate proteoglycans
ICM	Inner cell mass
MAPK	mitogen activate protein kinase
MRF	myogenic regulatory factor
mRNA	messenger ribonucleic acid
LIF	Leukaemia inhibitory factor
LIFR	Leukaemia inhibitory factor receptor
MEF	Mouse Embryonic Fibroblasts
NA	N-Acetyl
NDST	N-Decetyl Sulftransferase
NPC	Neural progenitor cell
NS	N-Sulfatase
Pax7	paired box transcription factor 7
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Primitive endoderm
RT	Room temperature
RTKs	Receptor tyrosine kinases
RT-qPCR	Real-time quantitative polymerase chain reaction
RNA	Ribonucleic acid
STAT	Signal transducer and activator of transcription
Su1f1	Sulfatase enzyme 1
Sulf2	Sulfatase enzyme 2
Ser	serine
UA	uronic acid

Chapter 1

1 Introduction

1.1 Embryonic stem cells

1.1.1 Early Embryonic Development

Multi-cellular organism growth starts with a single-cell zygote that undergoes fast cell division to form the blastula. The first cell stage of embryogenesis is called cleavage. Cleavage is the repeated mitotic division of a zygote into blastomeres that cluster into a compact mass called morula. Cleavage continues until a blastula is formed as shown in (Figure 1.1a). The embryo is called a blastula after the cleavage has generated more than 100 cells. Usually, the blastula is a spherical cell layer (the blastoderm) surrounding a cavity filled with fluid or yolk (the blastocoel). At this point, mammals form a blastocyst structure defined by an inner cell mass that is separate from the surrounding blastula, as shown in (Figure 1.1b). The cells divide during cleavage without a mass rise; that is, one big single-celled zygote splits into several smaller cells. Each blastula cell is referred to as a blastomere.

Holoblastic (complete) cleavage or meroblastic (partial) cleavage can occur in two forms. The cleavage form relies on how much yolk there is in the eggs. The eggs have a tiny quantity of yolk and undergo holoblastic cleavage in placental mammals (including

humans), where the body of the mother supplies nourishment. Other species, such as birds, undergo meroblastic cleavage, with much yolk in the egg to feed the embryo during growth.

In the next phase of growth for mammals, the blastula forms the blastocyst. Here, the blastula cells are arranged in two parts: the internal mass of the cell, and the outer layer called the trophoblast. The inner cell mass is also known as an embryoblast, and this mass of cells will continue to form the embryo.

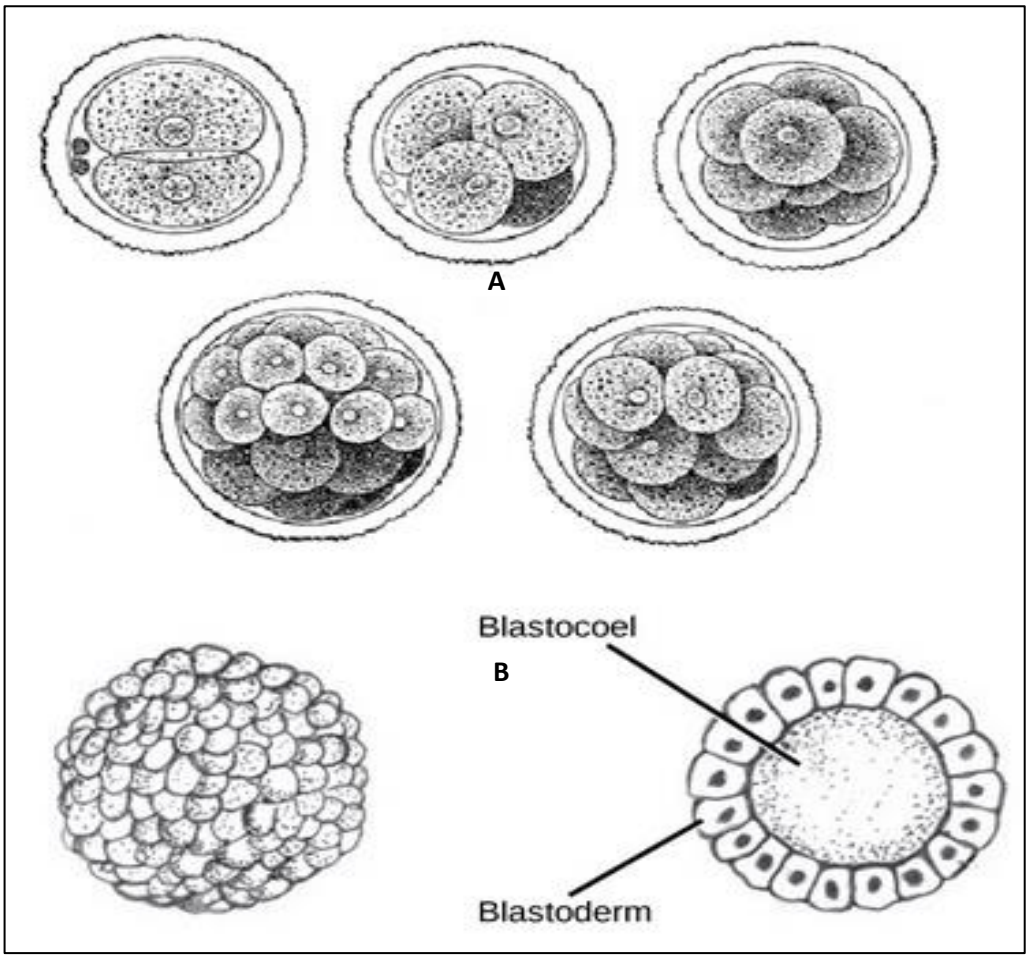

Figure 1.1. Repeated mitotic division of a zygote into blastomeres. (**A**) During cleavage, the zygote rapidly divides into multiple cells without increasing in size. (**B**) The cells rearrange themselves to form a hollow ball with a fluid-filled or yolk-filled cavity called the blastula. (Credit a: modification of work by Gray's Anatomy; credit b: modification of work by Pearson Scott Foresman, donated to the Wikimedia Foundation) (Courses.lumenlearning.com, 2019).

Embryonic stem cells are produced from cells found in the embryo when it is only a few days old. At this stage, the embryo is a ball of about 100 cells in humans, mice, and other mammals. It is known as a blastocyst and has two parts:

1. An outer cell layer, or trophectoderm, will form the placenta that will support the embryo as it grows within the uterus.
2. An inner clump of cells, known as the inner cell mass, is a 10–20 cell ball. These cells are either undifferentiated or unspecialised. They will extensively multiply and differentiate to make the many types of cells needed to form the whole animal (Figure 1.2).

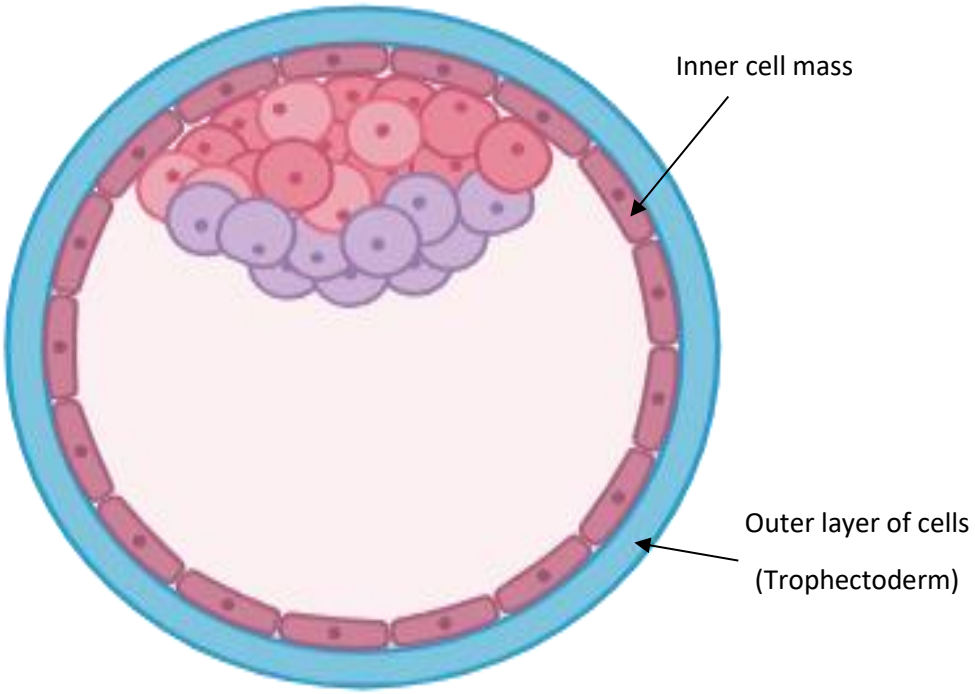

Figure 1.2. The rearrangement of the cells in the mammalian blastula. Two layers—the inner cell mass and the trophoblast—results in the formation of the blastocyst.

1.1.2 Totipotent, Pluripotent, and Multipotent

Totipotent cells can form all types of cells in a body, plus cells that are extraembryonic or placental. Embryonic cells are the only totipotent cells in the first pair of cell divisions after fertilisation. Pluripotent cells can produce all the types of cells that make up the body; pluripotent are regarded as embryonic stem cells. Multipotent cells may grow into more than one type that make up the body; pluripotent are regarded as embryonic stem cells. Multipotent cells may grow into more than one type of cell, but they are more restricted than pluripotent cells; multipotent are regarded as adult stem cells and cord blood stem cells (BioInformant, 2019).

1.1.3 Gastrulation

The characteristic blastula is a cell ball. The next phase of embryonic growth is body plan formation. The cells in the blastula are spatially rearranged to form three cell layers. This process is called gastrulation. The blastula folds in on itself during gastrulation to form the three layers of cells. Each of these layers is called a layer of germs, and each layer of germs distinguishes between distinct organs.

The endoderm, ectoderm, and mesoderm are the three germ layers, as shown in (Figure 1.3). The ectoderm gives rise to the epidermis and nervous system. The mesoderm creates the muscle cells and the body's connective tissue. The endoderm creates columnar cells discovered in the digestive system and many inner organs.

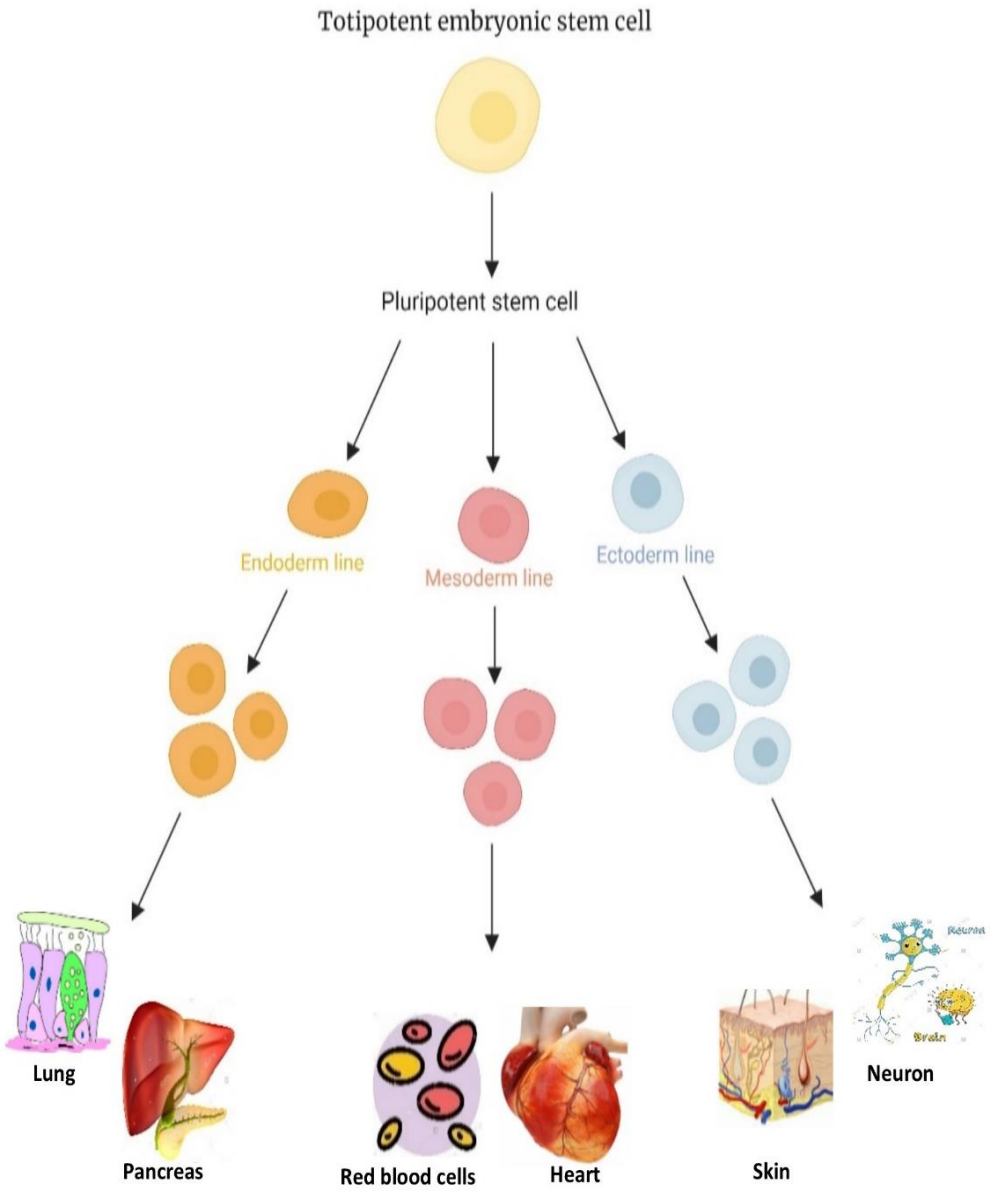

Figure 1.3: The steps of gastrulation, beginning from totipotent stem cell and ending with the formation of the organs.

1.1.4 Organogenesis

Organs are formed by the differentiation method from the germ layers. The embryonic stem cells express particular sets of genes during differentiation that will determine their ultimate type of cell. For instance, some ectoderm cells will express skin cell-specific genes. These cells will thus develop into epidermal cells. Cellular signalling cascades regulate the differentiation process.

In fruit flies (*Drosophila*) and the nematode *Caenorhabditis elegans*, scientists study organogenesis widely in the laboratory. *Drosophila* have sections along their bodies, and the patterning connected with segment formation has enabled researchers to explore which genes play significant roles in organogenesis at distinct points of time along the length of the embryo. The nematode *C. elegans* has about 1,000 somatic cells, and during their growth in the nematode life cycle, researchers can explore the destiny of each of these cells. Humans have little variation in cell lineage patterns compared to other mammals where embryo cell growth depends on cellular signals.

In vertebrates, the formation of the neural structure is one of the main steps during organogenesis. The ectoderm forms cells and tissues of the epithelium and neuronal tissues. Unique signalling molecules called growth factors cause specific neurons at the bottom of the ectoderm to become epidermis cells during the

formation of the neural structure. The rest of the core cells forms the neural plate. If growth factor signalling were interrupted, then the whole ectoderm would be distinguished into neural tissue. The neural plate undergoes a sequence of cell movements where it rolls up and forms a tube, as shown in (Figure 1.4), called the neural tube. The neural tube will give rise to the brain and spinal cord with further growth (Courses.lumenlearning.com, 2019).

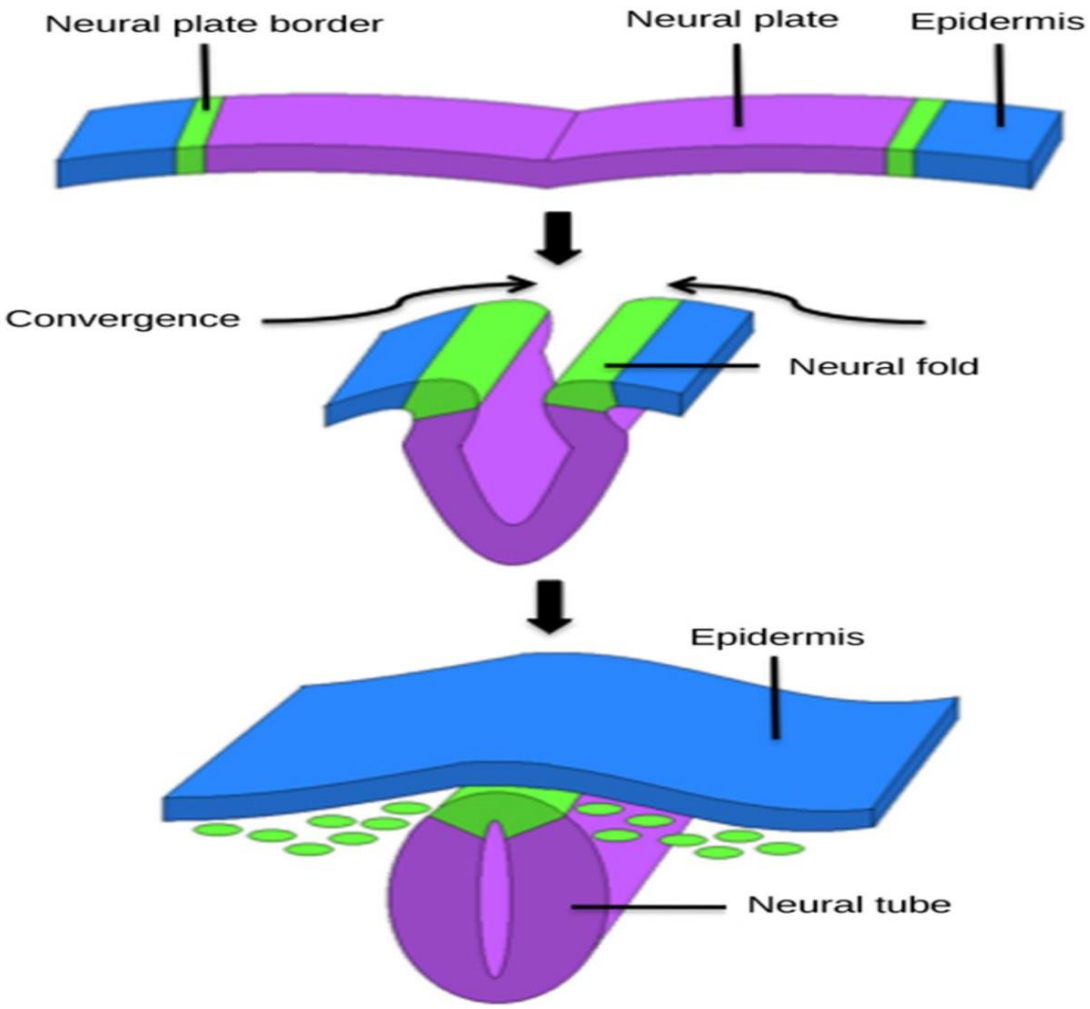

Figure 1.4. The central region of the ectoderm. Forms the neural tube, which gives rise to the brain and spinal cord.

1.1.5 Maintaining undifferentiated ES cells

The first factor that was used for maintaining undifferentiated ESC is the co-culture of ESCs with mouse embryonic feeder cells developed and preserved embryonic stem cells (ES) (Evans and Kaufman, 1981). Subsequent trials recognised cancer inhibitory factor (LIF) as one of the molecules derived from feeder cells that play a crucial role in maintaining these cells (Smith et al., 1988; Williams et al., 1988; Stewart et al., 1992). Recombinant LIF can substitute the feeder cell function and promote the development of undifferentiated ES cells in the presence of suitable batches of fetal calf serum (FCS) (Smith et al. 1988; Williams et al. 1988). Ying et al. (2003) revealed a function for BMP4 in the development of ES cells and showed that it could substitute the serum requirement in the presence of LIF. With these fresh advances, in the absence of serum or feeder cells, it is now feasible to develop ES cells with specified variables. Molecular analyses revealed that LIF functions through STAT3's gp130 activation (Niwa et al., 1998; Matsuda, 1999), whereas Smad activation and subsequent induction of helix–loop–helix Id variables mediate the impact of BMP4 on undifferentiated ES neurons.

Two other transcription factors, Oct3/4 (Niwa et al., 2000) and Nanog (Chambers et al., 2003; Mitsui et al., 2003), were shown to play a pivotal role in maintaining the undifferentiated state of ES

cells in addition to STAT3 and Id. The role of these transcription factors in ES cell renewal will not be discussed further here.

Regulating the growth of hES cells is less well understood and differs from that in mice because LIF and STAT3 seem to play no part in their self-renewal (Thomson, 1998; Reubinoff et al., 2000; Dahéron et al., 2004). With current protocols, hES cells in serum-free media supplemented with bFGF can be preserved on feeder cells (Amit et al., 2000). HES cells may also be cultivated in the lack of feeder cells if they are grown on Matrigel-or laminin-coated plates complemented by an embryonic fibroblast-conditioned medium in the mouse (MEF CM) (Xu et al., 2001). This protocol does provide for relatively easy maintenance of hES cell populations, although it is not as well defined as the circumstances for the development of mouse cells. Cells cultivated under these circumstances maintained normal karyotypes and stem cell features, including their pluripotent differentiation potential in vitro and in vivo, for > 100 population doublings.

Wnt pathway activation could replace the MEF CM requirement for short-term maintenance of undifferentiated hES cells (5–7 days) (Sato et al., 2003). It remains to be determined whether Wnt signalling has an effect on the self-renewal of hES cells over more extended periods through various passages. HES cells express both Oct4 and Nanog (Daheron et al., 2004; Ginis et al., 2004; Sato et al., 2004), indicating that this regulatory element may be

comparable to that found in mouse ES cells. There is no doubt that future studies will define specific molecules to maintain hES cells and will uncover the molecular processes that control their self-renewal.

1.1.5.1 Feeder cells

Feeder layer cells generally consist of cells that are arrested by adherent growth but are feasible and bioactive. These cells are used as a substratum for conditioning the medium on which other cells are cultivated, especially at low or clonal density. The feeder layer cells are often irradiated or otherwise treated to avoid proliferation. Faced with the absence of a method that enables large-scale colony development from single cells, Puck and Marcus first recorded the use of feeder cells in cell culture in 1955. Feeder cells have the ability to promote the *in vitro* survival and development of some fastidious cells that involve the existence of a variety of known or unknown soluble or membrane-bound growth factors and receptors. Although several types of cells depend entirely on physical contact with a feeder layer for survival and extension, some other feeder-dependent cells can be cultivated feeder-free provided that crop dishes are covered with extracellular matrix proteins such as laminin, collagen, fibronectin, or a combination of extracellular matrix elements (Matrigel) and supplemented with a medium that is conditioned by laminin.

1.1.5.2 Leukaemia inhibitory factor

The pathway of the Janus kinase-signal transducer and transcription activator (JAK-STAT) mediates the transmission of data from extracellular polypeptide signals via transmembrane receptors and directly targets gene promoters without the need for second messengers. Leukaemia inhibitory factor (LIF) belongs to the interleukin (IL)-6-type cytokine family, which signals the low-affinity LIF receptor (LIFR) through the prevalent receptor subunit gp130 in combination with a ligand-specific receptor subunit. LIF's binding to the LIFR induces its gp130 heterodimerisation. The formation of these complex outcomes is the activation of the receptor-associated Janus kinases (JAKs), the phosphorylation of receptor docking locations, and lastly the recruitment of the domain Src homology-2 (SH2) comprising enzymes such as STAT3 (signal transducer and transcription activator 3). STAT3 molecules are phosphorylated on residues of tyrosine 705 (Tyr705) when bound to the receptor and dimerise with another STAT3 phosphorylated. The dimers are then translocated in a controlled way to the nucleus, where they bind their target genes to promoters and enhancer areas (Figure 1.5). Cytokine signalling suppressor 3 (SOCS3) is a significant LIF/STAT3-pathway adverse regulator whose expression is

directly driven by STAT3. In many cell types and tissues in which the LIF-pathway is involved, SOCS3 performs a critical role.

Parallel to the activation of the STAT3-pathway, the binding of LIF to the LIFRβ/gp130 receptor results in the activation of the phosphatidylinositol-3 phosphate kinase (PI3K) pathways and the mitogen-activated protein kinase (MAPK). They are vital elements for regulating biological reactions in embryonic stem cells (ESCs) together with the JAK/STAT3 pathway. The active gp130 receptor may associate with the protein tyrosine phosphatase SHP-2 (Fukada et al., 1996), a MAPK signalling cascade beneficial effector, leading to Gab1 recruitment. The gp130/SHP-2/Gab1 complex initiates a cascade of phosphorylation culminating in the activation of kinases ERK1 and ERK2 (Takahashi-Tezuka et al., 1998) (Figure 1.5). Last, the activation of members of the PI3Ks class IA family by LIF binding to the LIFRβ/gp130 receptor. PI3Ks mediate signal transduction through downstream effector molecules including serine/threonine protein kinase B (PKB), which is involved in many cellular mechanisms such as cell cycle progression regulation, cell death, adhesion, migration, metabolism, and tumorigenesis (see for review Brazil, Yang, and Hemmings, 2004). GSK3β (glycogen synthase kinase 3β) is a prevalent PI3K/AKT-pathway target and Wnt-pathway target. These pathways regulate the inactivation of GSK3β phosphorylation.

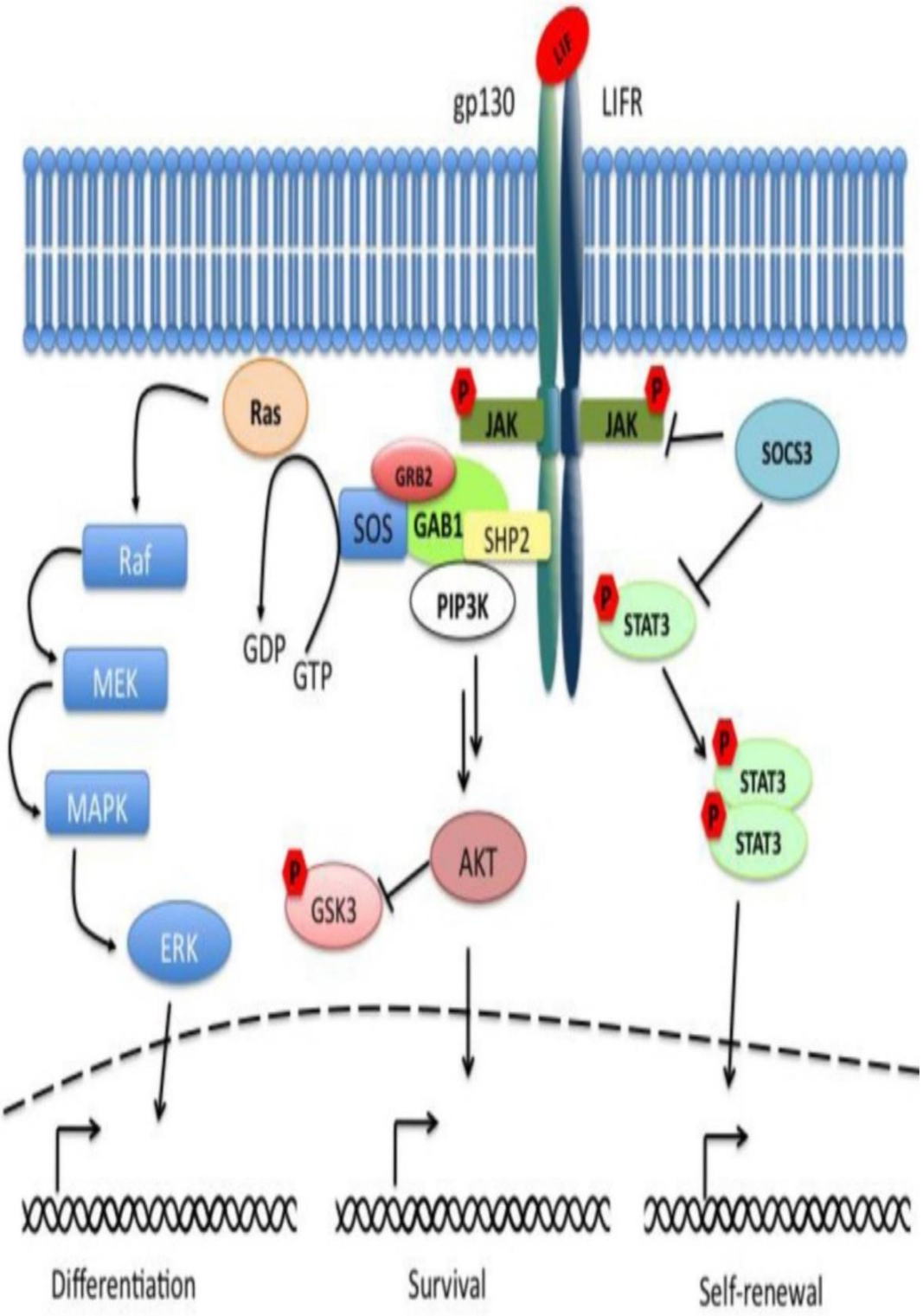

Figure 1.5. Schematic representation of the LIF-pathway. LIF acts through the LIFR/gp130 receptor and activates STAT3, an important regulator of mouse embryonic stem cell self-renewal. STAT3 is known to inhibit differentiation into both mesoderm and endoderm lineages by preventing the activation of lineage-specific differentiation programs. However, LIF activates also parallel circuitries like the PI3K-pathway and the MEK/ERK-pathway, but its mechanisms of action remain to be better elucidated. This review article aims at summarizing the actual knowledge on the importance of LIF in the maintenance of pluripotency and self-renewal in embryonic and induced pluripotent stem cells. (Graf, Casanova and Cinelli, 2011).

1.1.6 Lineage Commitment of mouse ESCs

Embryonic stem cells (ES) are pluripotent cells that can differentiate in both in vitro and in vivo into all adult cell lines. Pluripotent cells undergo symmetrical self-renewal and can be preserved and extended indefinitely in cell culture without losing their functional attributes (Smith, 2001). Therefore, these notable cells are regarded as an infinite and renewable source of types of adult cells with a broad spectrum of biotechnology and biomedicine applications (Murry and Keller, 2008).

Three differentiation strategies are typically used to differentiate ES cells: aggregation of cells into embryoid bodies (EBs) in suspension; plating cells as monolayers on extracellular matrix; or the strategy that have been used in this research study, which is co-culture with feeder cell lines that have differentiation-promoting activity (Smith, 2001). Placing pluripotent cells on feeder cells promotes a systematic assessment of the role of signalling variables in cell differentiation. ESC differentiation depends on silencing the self-renewing, extremely complicated signalling cascade and ESC reaction to particular environmental indications for making destiny choices. In vitro, ESCs may be directed to differentiate or undergo self-renewal and remain pluripotent.

1.1.6.1 Mesoderm differentiation

The mesoderm forms the middle layer of the early trilaminar embryo germ layers (ectoderm, mesoderm, and endoderm) formed by gastrulation. The middle germ layer produces connective tissues and muscle throughout the body, except in the head region, where some of these structures have a neural crest (ectoderm) origin. Connective tissue—cartilage, bone, blood, endothelium of the blood vessel, dermis, body—heart, skeleton, smooth muscle. As described briefly, mesoderm forms all adult human tissues except the nervous system, skin epidermis, and epithelia and can be classified as paraxial, intermediate, and lateral plate. According to the patterning phase, a vast range of transcription factors and activated signalling cascades occur, some of which are used in this project. Initially, the epithelial to mesenchymal transition (EMT), a crucial point during gastrulation and the formation of germ layers (Lehembre et al., 2008), was considered to be associated with the failure of E-27 cadherin expression (Frame and Inman, 2008). Brachyury, a T-box transcription factor (Inman and Downs, 2006), marks the mesoderm specification at the moment of gastrulation and is transiently expressed in the intermediate and axial mesoderm of the mouse from E7 to 8.5, making it a helpful marker for mesoderm differentiation in vivo. Mutations in the Bry gene result in

eventual embryonic lethality due to inadequate mesoderm and a lack of notochord, although growth up to the primitive streak is normal (Wilkinson, Bhatt, and Herrmann, 1990; Kispert and Herrmann, 1994). Because of somite divisions into sclerotomas, dermatomas, and myotomas, paraxial, intermediate and lateral plate, and mesoderm differentiation happen because of effective patterning. In the mouse, the first genes known to be asymmetrically recognised at this point are members of the TGFβ family member Nodal, around the node (Beddington and Robertson, 1999), followed by Lefty 2 and Pitx 2 in the left LPM and Lefty1 in the left-floor neural tube plate (Brennan, 2002; Saijoh). Through the creation of two cylinders of tissue on either side of the notochord, the paraxial Mesoderm grows, and after weeks 4 and 5, blocks of this tissue called somites bud off to the cranial end to start governing segmentation. It is known that the t-box transcription factor Tbx-6 works upstream of Nodal and has significant roles in presomitic mesoderm and somite border formation (Chapman et al, 1996; Hadjantonakis, Pisano, and Papaioannou, 2008). It is thought that Scleratom is initiated and retained by sonic hedgehog (Shh) and Noggin, molecules generated by the neural tube that antagonise BMP4. It is thought that the epithelial–mesenchymal transformation of the dermatome, which adds to the skin on the dorsal side of the body, and the myotome, which grows to form the skeletal musculature

of the neck, trunk, and limbs, depends on signals from the neural pipe 28. The intermediate mesoderm provides rise to the gonads, kidneys, and adrenal cortex (Evseenko et al., 2010) Pax-2 and Osr 1 are significant transcription factors first seen in the mediolateral mesoderm after gastrulation (James and Schultheiss, 2005) and Foxc1 and Foxc 2 forkhead transcription factors are essential (Wilm et al., 2004), as illustrated by Foxc1-deficient mice lacking mesoderm differentiation. Despite the complexity, mesoderm differentiation is the most well studied and well classified of the three germ lineages, perhaps underpinned by some default mechanism, considering the ease of differentiating mouse EMCs (mESCs) into hematopoietic, vascular, and pulmonary lineages. Differentiation of ESCs to mesoderm in the EB model is often accomplished using combinations of exogenous proteins (Liu et al., 2011) and offers little data on cell type (Era) positional data; therefore, much research is still required to optimise the use of this model for mesoderm-derived cells.

1.1.6.2 Endoderm-differentiation

The first germ layer is produced by gastrulation in the early stages of the trilaminar embryo germ (ectoderm, mesoderm, and endoderm). Note that the endoderm's historical name was ‘entoderm’. The endoderm adds the gastrointestinal tract,

breathing tract, renal bladder epithelia, and glands. This layer also adds to the growth of the gastrointestinal organ (liver and pancreas) connected with it. Initially, the overlying notochord, and consequently, a variety of growth factors governing growth and differentiation, appear to influence the layer. Note that this layer also lines the extra-embryonic yolk sac and allantois that are originally continuous with the intra-embryonic endoderm.

The endoderm is classically described as the embryo's inner layer, the first derivative of which is the digestive tract epithelia, from which organs such as the liver and pancreas are formed. Analogous to the growth of endoderm in the EB model, endoderm grows in vitro in close connection with mesoderm in vertebrates, and most endoderm cells derive from the primitive streak (Wells and Melton, 1999).

The extraembryonic endodermic structures referred to in this project are defined as the primitive endoderm, parietal endoderm, and visceral endoderm; they differ from the definitive endoderm, although they share many transcriptional markers. Endoderm differentiation persists during the growth of EB through the differentiation of cells on the EB periphery to extraembryonic primitive endoderm (PE), as described previously. GATA factors were recognised by regulating primitive endoderm differentiation as primary regulators of both extraembryonic and definitive

endoderm differentiation (Murakami, Okumura, and Uchiyama, 2005; Okumura et al., 2005). GATA variables are evolutionarily retained transcription regulators consisting of six members (GATA 1–6). GATA 1–3 are usually expressed in hematopoietic lines; for example, it was suggested that GATA2 and GATA3 play a part in early embryonic patterning in *Xenopus* and zebrafish (Zon et al., 1991), which was specifically identified in the chick's pre-primitive streak phase (Sheng and Stern, 1999). In comparison, GATA 4-6 are discovered primarily in mesoderm and endoderm lines (Ralston and Rossant, 2005), which are essential in early growth (Simon, 1995). Gata6 is used in this project. GATA 6 is a primary endoderm differentiation regulator, proven as GATA 6-null embryos lack primitive endoderm differentiation and subsequently have an absence of the visceral and parietal endoderm (Cai et al., 2008). Ectopic GATA 6 expression may bypass the necessary Grb2, which is essential in Nanog repression and primitive endoderm differentiation (Schrode et al., 2014), further emphasising GATA 6's significance in primitive endoderm differentiation. Alpha-fetoprotein (AFP), Sall 4, Sox 7, Sox 17, and HNF-4 are also crucial factors in the differentiation of extraembryonic endoderms, many of which interact with GATA variables to boost endoderm differentiation. AFP is the mammalian embryo's most abundant 30 protein recognised in the embryonic yolk sac and fetal liver (Spear and Tilghman, 1990),

although expression after birth has been shown to decline significantly.

The visceral endoderm is believed to secrete AFP, although it is suggested that synthesis and expression may be dependent on visceral endoderm interactions with the underlying ectoderm tissue. Nuclear hepatocyte factor 4 (HNF-4) is a transcription factor recognised as a DNA binding protein in liver extracts (Sladek, 1994). Primitive endoderm differentiation is believed to rely on HNF-4 because of its interaction with GATA6. Sall4, a component of the spalt like zinc finger family of transcription factors, has also been shown to be essential in differentiating primitive endoderm (Elling et al., 2006) and has been suggested to be specifically essential for the primitive endoderm differentiation Nanog repression phase (Frankenberg et al., 2011). Sox7 and Sox17 are believed to regulate the differentiation of parietal endoderms through relationships with GATA variables (Futaki et al., 2004), and one model suggested is that Sox 7 competes with GaATA 4 for binding FGF3 (Murakami et al., 2004). Interestingly, Sox 7 and Sox 17, specifically ascribed to Laminin alpha 1 (LamA1) interactions, are believed to rely on and influence BM formation (Zhou et al., 2015).

1.1.6.3 Ectoderm differentiations

The top layer of the first trilaminar embryo germ layers is created by gastrulation (ectoderm, mesoderm, and endoderm). The ectoderm, however, may be four prime areas: neural plate, neural crest, ectoderm surface, and placodes. Note that neural (central nervous system; brain and spinal cord) and neural crest (peripheral nervous system; sensory and sympathetic ganglia) are described in other pages. Because of the epidermis's (integumentary, skin contribution) ectodermic origin, its growth will be briefly stated.

Ectoderm is dissected into the outer or (surface) ectoderm, neural crest, and neural tube in vertebrates. Ectoderm-derived cell lines distinguish the epidermis to shape: skin, hair, nails, brain, and nervous systems. This neural tissue formation begins when the mesoderm-derived notochord induces overlapping ectoderm regions to form the neural plate. Subsequently, the neural plate folds to form the neural tube and dorsal/lateral polarity is formed. Neuroepithelial cells proliferate and differentiate within the neural tube into a multitude of ectodermal cell lines. The adult nervous system will shape 31 of these cells. Specific ectodermal lines include oligodendrocytes, astrocytes type-1 and-2, and progenitors of neurons. It is believed that the connection and interactions between BMP4, Noggin, and Chordin mainly underpin ectoderm differentiation; specifically, inhibiting BMP4–Noggin–Chordin interactions is considered necessary for neural plate folding

(Ambrosio et al., 2008). Pax2 and Pax6 are transcription factors containing paired box DNA binding domains and are evolutionarily retained in the growth of ectoderms. For instance, eye development is a classic demonstration of Pax6's significance; Pax6 mutants lose eye functionality in *Drosophila*, mice, and humans (Quiring et al., 1994). The expression of Pax6 is identified in a number of areas of the developing central nervous system of the mouse, including the presumptive retina from head to head (Turque, 1994; Kozmik, 2005), and thus constitutes a helpful marker of ectoderm differentiation in the EB model.

1.2 Heparan Sulfate

Heparan sulfate proteoglycans (HSPG)s are extracellular matrix (ECM) and cell surface macromolecules comprising a pivotal protein to which glycosaminoglycan (GAG) heparan sulfate (HS) chains are attached (Lin, 2004; Esko and Selleck, 2002).

1.2.1 Carbohydrates

Carbohydrates are a broad class of biomolecules, including monosaccharides and their derivatives, oligosaccharides, and polysaccharides. The generation of a polysaccharide chain begins with two monosaccharides being linked together by a glycosylation reaction, resulting in either (1) equatorial (β) bond

formation, where the bond makes a small angle compared to the ring plane, or (2) axial bond (α) formation, where the bond makes an angle of approximately 90° to the ring plane (Figure1.6). At least one anomeric carbon (carbon 1) is involved in the glycosidic bond, which is the monosaccharide stereocenter. The resulting disaccharide includes a free anomeric carbon that may be involved in additional glycosylation responses that increase the polysaccharide chain length. The anomeric end of sugar engaged in the response of glycosylation is called the 'reducing end'. The polysaccharide's non-anomeric side is called the 'non-reducing end'. Polysaccharides contain large numbers of hydroxyl groups

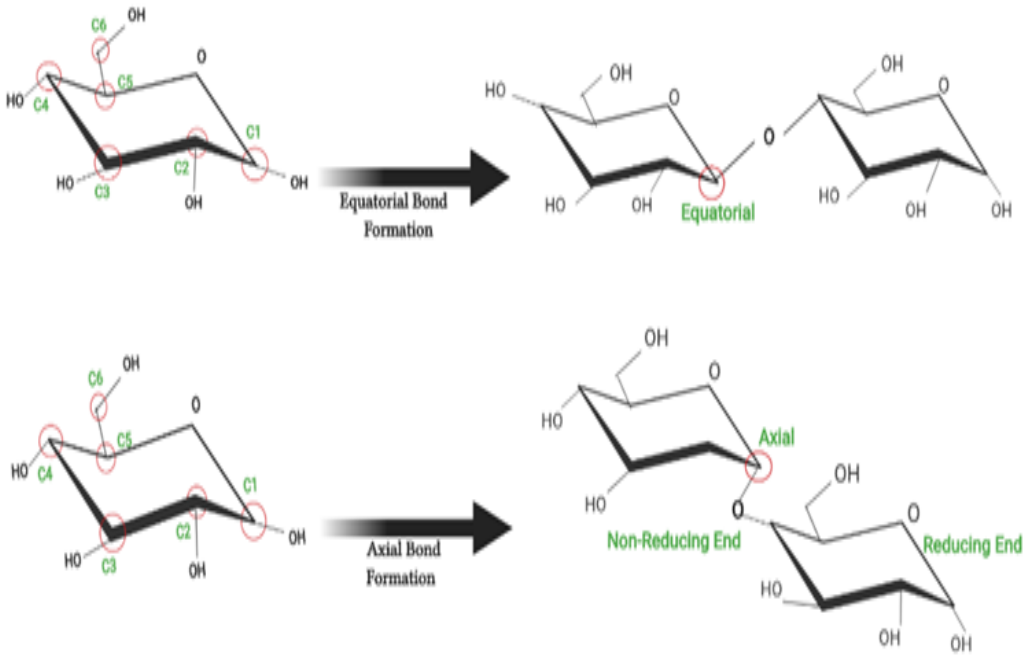

Figure 1.6. Scheme showing the glycosylation reaction. A general scheme showing the glycosylation reaction linking two monosaccharides of glucose together at the anomeric carbon (carbon 1) and carbon 4 to form a) equatorial (β-) glycosidic linkage and c) axial (α-) glycosidic linkages between the disaccharide maltose.

HS is a member of the glycosaminoglycan (GAG) family, consisting of abundant linear polysaccharides with a repeating disaccharide unit: (a) amino sugar, N-acetyl glucosamine (GlcN) for HS; and (b) uronic acid residue, either iduronic acid (IdoA) or glucuronic acid (GlcA). The GAG is described by the particular disaccharide unit and the O-glycosidic linkage stereochemistry. The structural complexity of the GAG chains is determined by the reality that they contain large numbers of hydroxyl groups that are functionalised differently with sulfate groups, resulting in a heavily negatively charged macromolecule capable of numerous electrostatic and other interactions. Depending on the GAG species, the position and sulfation rate are incredibly diverse. The sulfation location is determined by the carbon to which the sulfate group is connected, beginning from anomeric carbon, carbon 1. HS is present on the cell surfaces of nearly all mammalian cells and is characterised by a linear chain of 10–200 disaccharide units of N-acetyl-D-glucosamine (GlcNAc) linked to D-glucuronic acid (GlcA) with an average molecular weight of 25–100 kDa. It has the most variable structure in the GAG family (Turnbull, Powell, and Guimond, 2001) and thus has an enormous functional diversity potential within the GAG family (Turnbull et al., 2001). Indeed, the distinct biological roles HS plays mostly rely on its basic structure, which arises from the distinct sulfation concentrations and patterns and is called the 'heparanome' (Turnbull, Powell, and Guimond, 2001). Structural HS analyses show compositions of cells and tissue-specific HS, highlighting the significance of specific structure–

function operations (Lawrence et al., 2008; Ledin et al., 2004; Maccarana et al., 1996).

1.2.2 Classification of Glycosaminoglycans

Glycosaminoglycans can be classified into five primary groups as follows.

1- Hyaluronan

2- Chondroitin sulfate

3- **Dermatan sulfate**

4- Keratan sulfate

5- Heparan sulfate

Heparan sulfate

Heparan sulfate belongs to the macromolecule family of GAGs, which also involves chondroitin sulfate, dermatan sulfate, and keratan sulfate. These molecules are linear polysaccharides composed of repeating backbones of the disaccharide unit onto which modification patterns are superimposed, most particularly sulfate groups. HS stands out as the most variable family member because of the polymorphic nature of the extremely sulfated sequences within its chains (Turnbull, Powell and Guimond, 2001). HS proteoglycans (HSPGs) are macromolecules of a broad spectrum of vertebrate and invertebrate tissue cells connected

with the cell surface and ECM (Kjellén and Lindahl, 1991; Iozzo and San Antonio, 2001). They are involved in many developmental and pathological procedures through relationships with various ECM ligands such as growth factors and molecules of adhesion (Tumova, Woods and Couchman, 2000). It is usually thought that the interactions between HS and ligands rely on the amount and relative positions of carboxyl and especially sulfate groups within the HS chains.

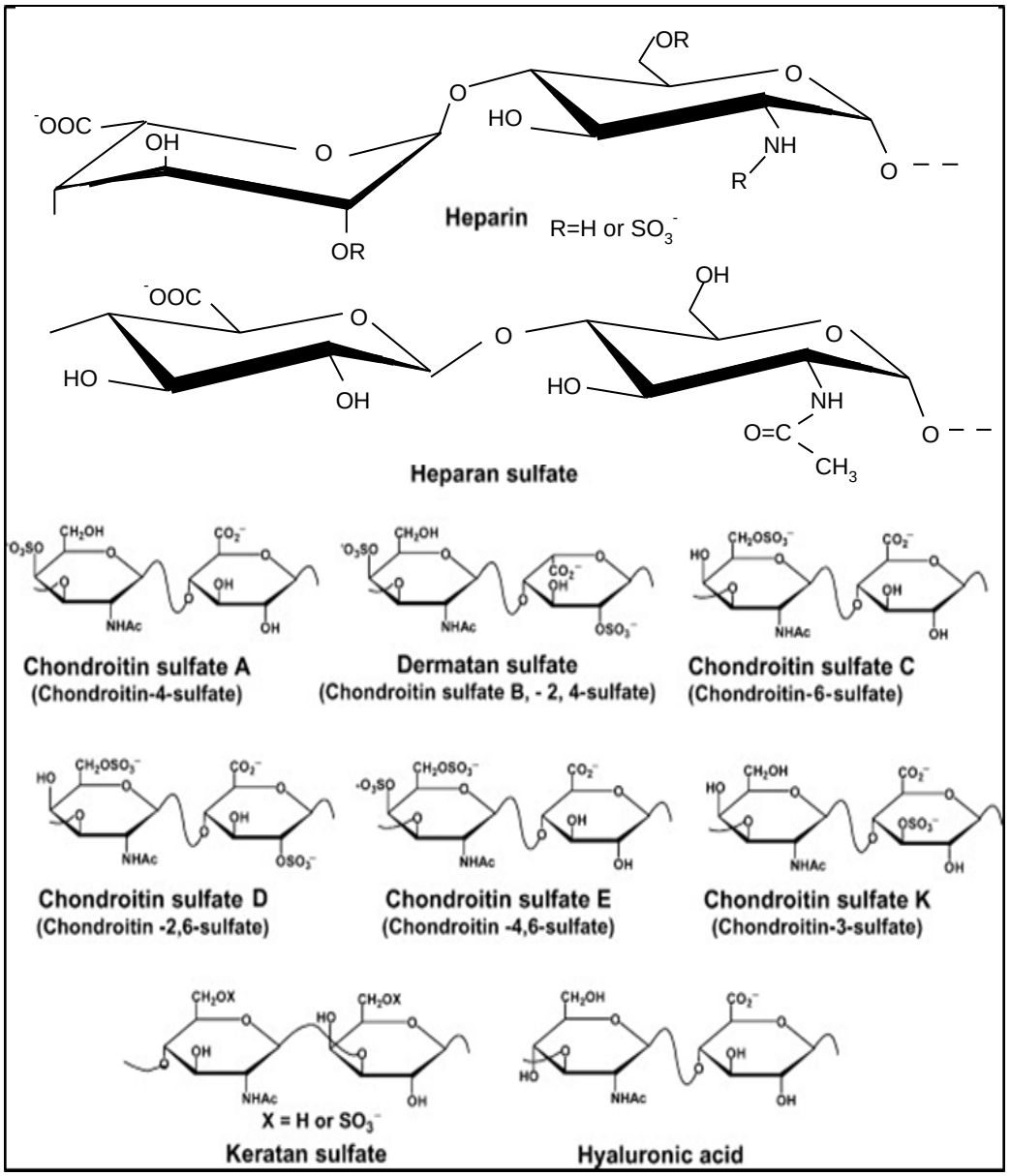

Figure 1.7. Major and minor disaccharide sequences of heparin. (HP)/heparan sulfate (HS) and major disaccharide sequences of chondroitin sulfate (CS)/dermatan sulfate (DS), keratan sulfate (KS), and hyaluronan (HA) (Cummings and Pierce, n.d.).

1.2.3 HS Structure

HS is not a single molecule but instead a varied family of associated molecules, composed of repeated subunits of uronic acid-glucosamine disaccharide arranged in sulfated (NS) and unsulfated (NA) areas with spacers between them; see (Figure 1.8). Variability in the replacement of N-sulfate, N-acetyl, and O-sulfate groups of the disaccharide subunits implies that theoretically there could be 48 disaccharides, although only 24 have been recognised to date. In most tissues, there is generally the same set of disaccharides, but their relative content differs quantitatively (Esko and Lindahl, 2001); HS and heparin are an example. HS and heparin show identical construction blocks, but the disaccharide subunits are present in distinct proportions; SO4 content in HS is smaller than heparin, leading in an excellent structure capable of encoding for greater data diversity (Rabenstein, 2002).

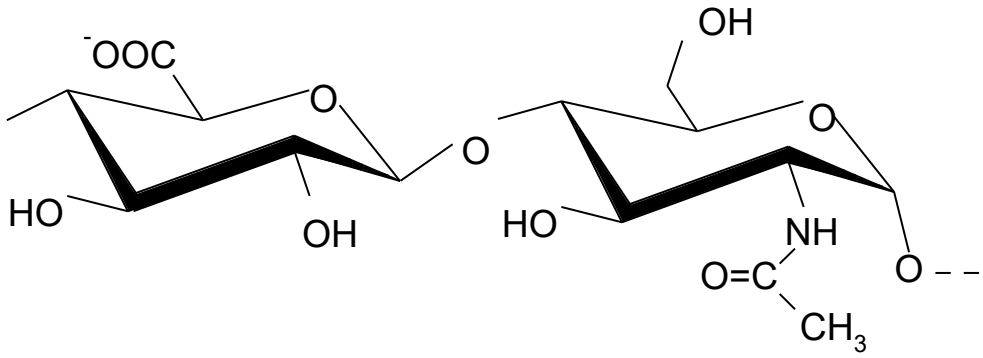

Figure 1.8. "Common N-acetylated disaccharide found in HS". Other structures include N-sulfation of glucosamine, and O-sulfation at positions C3 and C6 on glucosamine and C2 on uronic acids (see Fig 1.7).

1.2.4 HS Classification

HSPG is composed of a core protein to which several linear HS chains are covalently O-linked (Rabenstein, 2010). According to the distribution, HSPGs are divided into three classes: intracellular heparin PGs (serglycin); cell surface associated HSPGs (syndecan and glypican); and ECM HSPGs (perlecan, agrin, and collagen XVIII) (Rodgers, San Antonio, and Jacenko, 2008) (Figure 1.9).

Syndecans are plasma cell membrane elements. The syndecan family consists of four different members. Every syndecan has a brief cytoplasmic domain, a transmembrane domain, and an extracellular domain with attachment locations near the N-terminus for three to five HS or CS chains (Tkachenko, Rhodes, and Simons, 2005). Because of the separate extracellular domains, the molecular size of syndecan key proteins varies from 20 to 45 kDa (Cheng et al., 2016). In addition to the functions of cell–cell and cell–ECM interactions, syndecan interacts within cells, such as cytoskeletal organisations engaged in cell adhesion and signal transduction (Kwon et al., 2012). Many cell types convey more than one type of syndecan, and during growth and differentiation, the expression rates may alter (Jalkanen et al., 1991).

Glypicans are connected to the plasma membrane by anchoring glycosylphosphatidylinositol (GPI) (Figure 1.9) and bring three to four HS chains in general (International review of cell and molecular

biology volume 325 2016). Glypican, with a molecular size of approximately 60 kDa, is a family of six members, all highly expressed during embryonic growth (Veugelers et al., 1999).

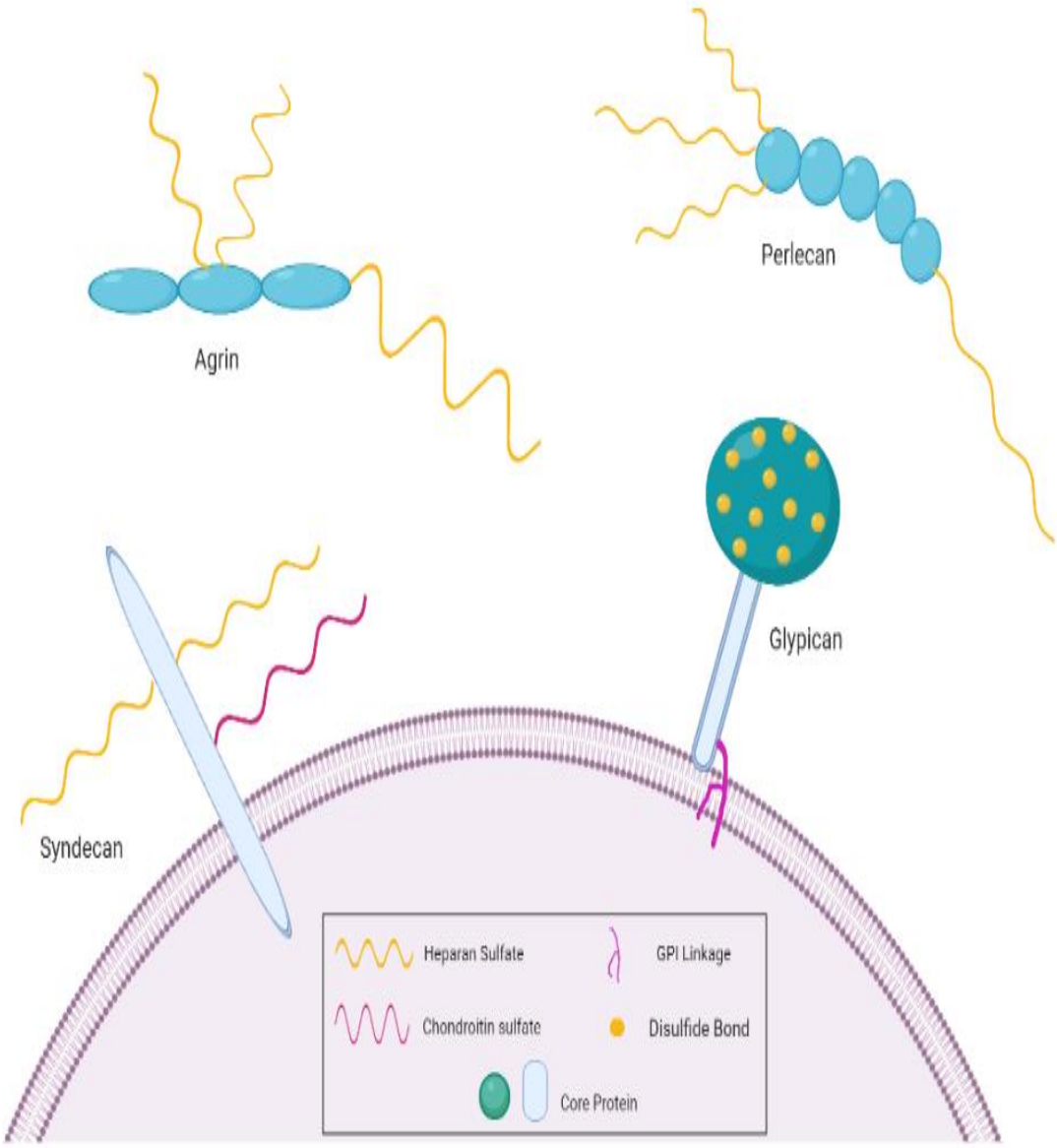

Figure 1.9. The three main classes of cell-surface heparan sulfate proteoglycans (HSPGs). (A) Syndecan core proteins are transmembrane proteins that contain a highly conserved C-terminal cytoplasmic domain. Heparan sulfate (HS) chains attach to serine residues distal from the plasma membrane. Some syndecans also contain a chondroitin sulfate (CS) chain(s) that attaches to a serine residue(s) near the membrane. (B) The glypican core proteins are disulphide-stabilized globular core proteins that are linked to the plasma membrane by a glycosylphosphatidylinositol (GPI) linkage. HS chains link to serine residues adjacent to the plasma membrane. (C) Perlecans are secreted HSPGs that carry HS chains.

1.2.5 HS Biosynthesis

HS is dynamically synthesised with comprehensive structural changes throughout, and thus, HS should be regarded as a heterogeneous family of structurally associated molecules with a wide variety of different functions. HS biosynthetic enzyme families are accountable for the HS synthesis that occurs after a non-templated system in the Golgi apparatus. Each enzyme's action does not complete the entire HS chain (Powell, 2004), leading to a high degree of structural diversity. Although the biosynthetic pathway remains a controversial subject, it is considered that the conventional pathway follows a standard order in which the biosynthetic enzymes of HS operate consistently and irreversibly (Lander and Selleck, 2000; Razi and Lindahl, 1995). In a three-step method involving chain initiation, polymerisation, and alteration, HS GAG chains are synthesised on a core protein by the sequential action of individual glycosyltransferases and modification enzymes. HS chain synthesis starts with the installation in the key polypeptide of a connection tetrasaccharide on serine residues. Four enzymes (Xyl transferase, Gal transferase I and II, and GlcA transferase I) catalyse this process, which sequentially adds individual sugar residues to the non-reducing end of the increasing chain. After the

connection region has been assembled, one or more α-GlcNAc transfers add a single α1,4-linked GlcNAc unit to the chain, initiating the polymerisation process of HS. HS chain polymerisation then occurs by adding alternating residues of GlcA and GlcNAc, catalysed by the EXT family of proteins. As the chain polymerises, it undergoes a series of modifications including GlcNAc N-deacetylation and N-sulfation, GlcA to IdoA C5 epimerisation, IdoA and GlcA C2 variable O-sulfation, GlcNAc and GlcNS C6 variable, and sometimes GlcN residue C3 variable. The HS GAG chains are about 100 or more sugar units long and have many heterogeneities of structure. (Figure 1.10).

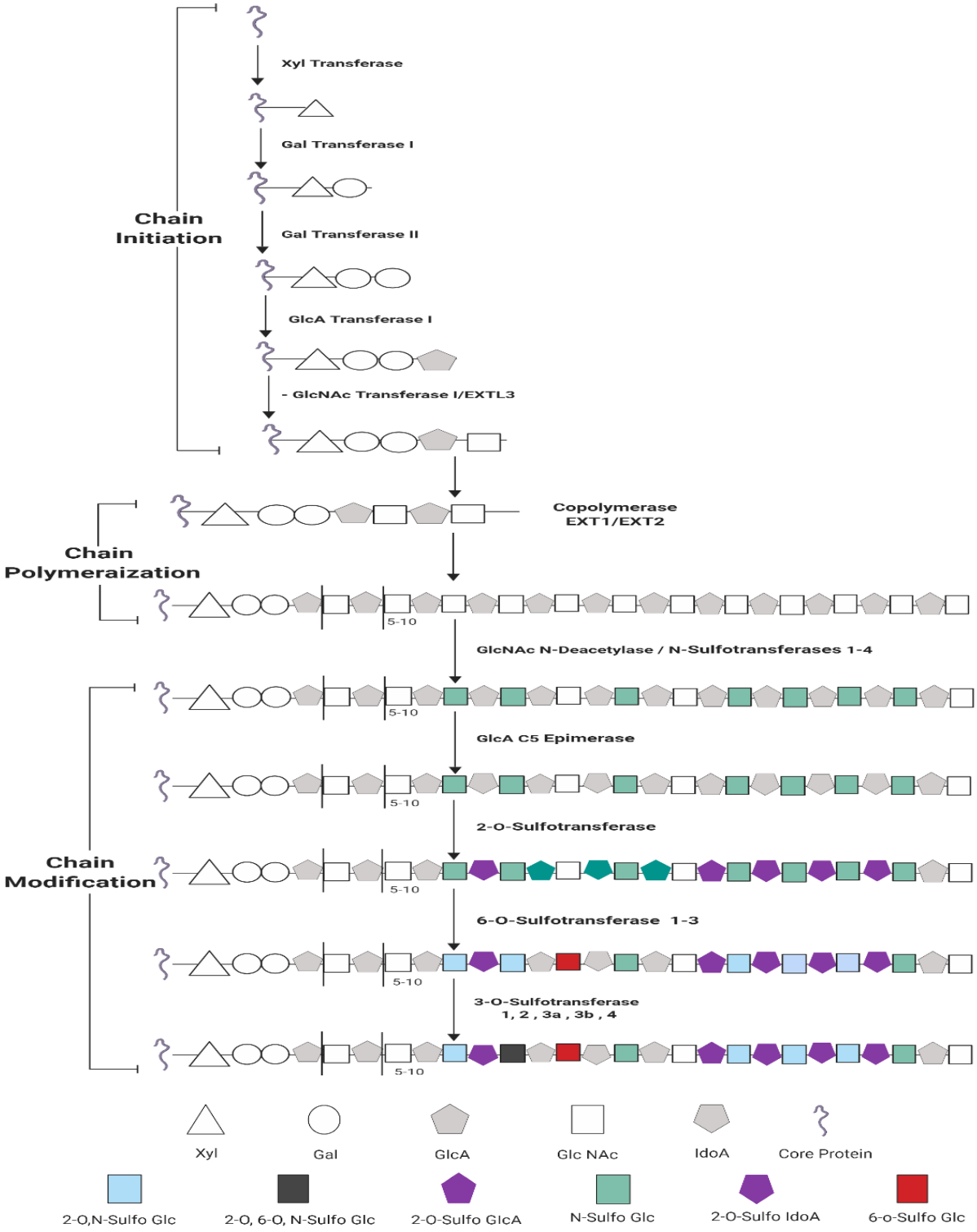

Figure 1.10. Schematic showing conventional view of the HS biosynthetic pathway. Schematic of HS biosynthetic pathways showing the activity of the HS biosynthetic enzymes depicted following a standard order. HS synthesis takes place in the Golgi apparatus following formation of the tetra saccharide linker on the core protein of xylose-galactose-galactose glucuronic acid. Chain elongation is initiated with EXTL3 which catalyses the addition of a glucosamine, followed by EXT1/2 which catalyse the addition of glucosamine and glucuronic acid units alternatively. This occurs concomitantly with chain modification starting with the NDSTS, which are dual action enzymes, catalysing the removal of an acetyl group from the amino sugar and the addition of a sulfate group. Chain modification continues with the 2-O-sulfotransferases, catalysing the addition of a sulfate group of the uronic acid residue and the 6-O- and 3Osulfotransferases catalysing the addition of sulfate groups on the amino sugar. These structural modifications lead to regions along the chain of high sulfation, termed S-domains interspersed with regions of lower sulfation, termed NA-domains. Intermediate domains (NA/NS) are also present flanking the NS domains.

1.2.6 Core proteins

HS GAGs are commonly connected to a core protein, of which there are three basic types, each representing a different mode of cell interaction and therefore determining whether that particular HSPG is expressed on the cell surface or in the ECM (Gandhi and Mancera, 2008):

. Syndecans

. Glypicans

. Basement membrane proteins

Syndecans are classified into four subfamilies; they include a preserved transmembrane domain and a typically tiny cytoplasmic domain (Lopes, Dietrich et al. 2006). Syndecans are identified in the morula phase on blastomere surfaces but are limited in the cavity of blasteol (Jokimaa et al., 1998; San Martin, Soto-Suazo, and Zorn, 2004).

Glypicans are classified into three subfamilies: Gpc1 and 2, Gpc3 and 4, and Gpc5 and 6. All of these are separate gene products with 14 highly conserved cysteine residues forming an expanded area (2 or 3 Ser-Gly sequences) near the plasma membrane and with a glycosylphosphatidylinositol (GPI) anchor (Lin, 2004). ECM proteins, as the name indicates, are ECM constituents, as

these HSPGs are secreted from the cell into the ECM, unlike syndecans and glypicans. They are believed to have a multi-domain framework (Lindblom, Carlstedt and Fransson, 1989; Kallunki, 1992), and there are three predominant sub-types (Iozzo et al., 1994).

. Agrin

. Perlecan

. Collagen XVIII

Agrin is accountable for the creation, maintenance and regeneration of the neuromuscular intersection, commonly expressed in the central nervous scheme (CNS) perlecan is expressed more widely throughout the early growth of embryos, recognised in the underlying uterine epithelia and trophoblast. In the pre-implanted embryo of the mouse, the expression is transient before the morula phase, and perlecan expression is improved through the blastocyst phase (Rohde et al., 1998; Abraham, Abraham et al., 2010). It is also reflected in cartilage and bone interstitial matrix tissue (Farach-Carson, Hecht, and Carson, 2005). Collagen XVIII's C-terminal domain, endostatin, has recorded angiogenesis characteristics and encourages tumour development through the induction of endothelial tissue apoptosis (Seppinen and Pihlajaniemi, 2011). Proteoglycans are various molecules based on the number of GAG chains, core protein type, and GAG side chain class (Turnbull and Gallagher,

1991; Maccarana et al., 1996). Unlike the HS biosynthetic enzyme knockout, HSPG core protein mutations usually induce mild defects, indicating that key proteins are more dispensable and/or can be compensated for by 44. Syndecan4 knockout mice show the deficient formation of fetal blood vessels (Ishiguro et al., 2000) and are more vulnerable to kidney harm (Ishiguro et al., 2001). Syndecan-3-null mice show deficiencies in muscle development (Cornelison, 2004), digestive system development (Westphal et al., 2000) and impaired development of the nervous system (Kaksonen et al., 2002). Syndecan-1-null mice show defects in the growth of the respiratory system hypo-responsiveness (Xu et al., 2005) and deficiency in the function of the immune scheme (Park et al., 2001; Gotte, Joussen et al. 2002). Gpc3-null mice give rise to overgrowth (Cano-Gauci, 1999; Chiao et al., 2002) and show skeletal issues due to an absence of BMP communication (Paine-Saunders et al., 2000). Perlecan-null mice show deficiencies in the growth of fetal tissues (Costell et al., 1999).

1.2.7 The Biological Role of HS

The complexity of the HS biosynthetic pathway leads to an extremely varied macromolecule capable of interacting with a multitude of proteins such as plasma proteins, cell surface proteins, enzymes, and growth factors called the HS 'interactome'

(Figure 1.11). There is growing evidence that HS structure is critical to proper protein interaction and therefore plays an essential role in regulating biological function (Lin, 2004; Pérez, Sawmiller, and Tan, 2016; Tambura and Nakato, 2017; Thacker et al., 2016; Zhao et al., 2015). To date, hundreds of HS binding proteins have been recognised (Meneghetti et al., 2015; Ori, 2008; Ori, Wilkinson, and Fernig, 2011). As a result, HSPGs play various roles in neurons and tissues, particularly in cell adhesion, which relies on the heparin-binding domain of matrix proteins such as fibronectin and laminin (Bernfield et al., 1999; Woods et al., 2000), and cell migration involving the recruitment of growth variables through interactions with HS (Arrington and Yost, 2009). (Figure 1.11) highlights some of HSPG's main biological features. Because HS is involved in multiple cellular processes, HS is crucial for regulating many biological processes, such as development and differentiation of stem cells and pathogenesis of diseases such as cancer (Knelson, Nee and Blobe, 2014), Alzheimer's disease (Zhang et al., 2014), HIV, and influenza (Connell and Lortat-Jacob, 2013; Patel et al., 1993). Several studies involving mutations in core proteins or HS biosynthetic enzymes have highlighted the critical role played by HPSGs as regulators of multiple biological functions in an attempt to understand the role of HS in biological regulatory processes (Sarrazin, Lamanna, and Esko, 2011). Furthermore, mouse mutants for the HS biosynthetic enzymes

show more serious phenotypic impacts than for proteoglycan mutants, with many of the knockouts studied to date being embryonic or perinatally deadly, highlighting the significance of the HS skeletal structure as discussed in Sarrazin, Lamanna, and Esko (2011).

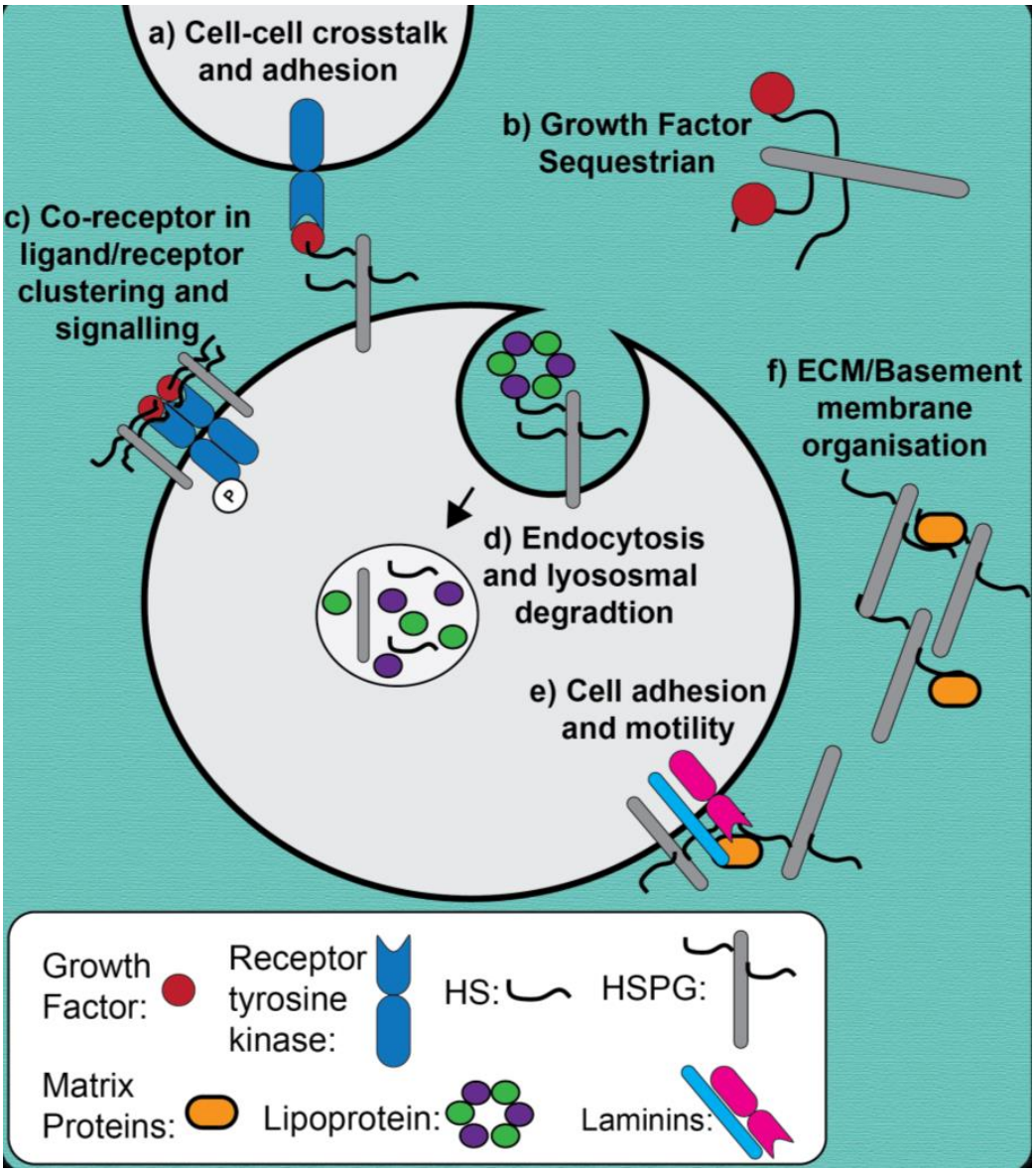

Figure 1.11. HSPGS participate in key biological functions. HS/HSPGs are engaged in regulating various biological procedures due to the high structural complexity of HS determined by chain length, the number of chains, sulfation pattern/level, and location or the protein nucleus to which it is connected. Some of the main procedures in which HS is engaged are shown in the diagram; (a) cell–cell cross-discussion and cell adhesion; (b) growth factor sequestration in ECM; (c) as a co-receptor in ligand/receptor clustering and signalling on the cell surfaced, endocytosis, and lysosomal degradation; (e) cell adhesion and motility based on their interaction with laminins, integrins, and matrix proteins; (f) basement membrane organisation (adapted from Bishop et al., 2007).

HS upon engagement to cell fate. Approximately 80% of GAGs generated in ESCs are HS, with a lower quantity of chondroitin sulfate (CS) generated (Lin et al., 2000; Nairn et al., 2007). General trends such as an increase in overall HS content and sulfation along differentiation were noted regardless of lineage destiny.

Interestingly, the increase in general HS content is followed by only small increases in EXT family gene transcript concentration, indicating that other enzyme regulation mechanisms account for the increase in HS content. In addition to increase in HS content, it is striking that the production of other GAGs, including hyaluronic acid and CS/dermatan sulfate (DS), is caused during ESC differentiation and is parallel to increases in most HS and core proteins CS/DS (Nairn et al., 2007). During EB formation, ECM elements are progressively articulated and discovered in EB microenvironments (Shukla et al., 2009). Increased manufacturing of GAG during differentiation can thus accommodate an increase in ECM deposition.

Analysis of the HS structure of undifferentiated ESCs disclosed that their HS chains bear remarkably little phosphate, with only about 30% N-sulfation. The potential function of this low-sulfated type of HS is uncertain, but it can serve to shield ESCs from signalling differentiation-induction, as discussed in the next chapter of this review. As ESCs move into engaged cell types, their HS chains become more and more sulfated (Johnson et al., 2007;

Nairn et al., 2007; Hirano et al., 2012). Changes in lineage engagement sulfation derive mainly from enhanced N-, 3-O-, and 6-O-sulfation. Lineage-specific upregulation of sulfotransferase mRNAs appears to be a significant contributor to enhanced sulfation. For example, the transition from undifferentiated ESCs to a neural Sox1+ population is followed by sharp increases in Ndst-4, 3-HSst-3a, and 3-HSst-5, mostly expressed in fetal brain tissue (Mochizuki et al., 2003; Yabe et al., 2005; Johnson et al., 2007). In comparison, increases in Ndst1, Ndst2, HS6st2, and Hs3sta1 (Nairn et al., 2007) are parallel to the enrichment of extraembryonic cell types. Such lineage-specific expression of HS biosynthetic enzymes will result in cell-specific HS displaying selectivity to growth variables and directly affecting the cell's ability to differentiate, as shown by both hematopoietic and neural differentiation mechanisms. It is possible to achieve hematopoietic differentiation of ESCs by forming EBs. Transient populations of brachyury-positive mesodermal cells will result in endothelial progeny in reaction to VEGF during EB differentiation. Using HS-specific phage-display antibodies, a specific HS4C3 epitope that requires N-and 6-O-sulfate and a single critical 3-O-sulfate for high-affinity binding are identified in mesodermal subpopulations. The HS4C3+ subpopulation shows increased hematopoietic capacity relative to HS4C3-mesodermal

cells, indicating that this epitope is extremely functional in hematopoietic growth (Baldwin et al., 2008).

Differences in cell differentiation potential occur from the remodelling of HS structures and will eventually result from modifications in HS affinity to particular cell signalling molecules and enable selective cell fate choices to be made. For instance, neural progenitor cell (NPC) HS demonstrates a reduction in FGF2 binding relative to ESC's HS binding and is associated with the expression of Sox1 (Johnson et al., 2007). Increases in N-and 6-O-sulfation and, to a lesser extent, 2-O-sulfation accompany the differentiation step of ESCs into NPCs. As neuro epithelial precursors move from proliferation to neuronal differentiation, their HS will subsequently undergo modifications in the pattern of 6-O-sulfation as well as the complete duration of the HS chain. This switch coincides with a switch from FGF2 to FGF1 signalling potentiation required to differentiate NPCs into terminally differentiated types of neural cells (Brickman, 1998). It appears that modifications in HS structure promote a continuous shift in FGF isoform affinity. Remodelling HS structures during the formation of NPCs reduces the affinity for FGF2, which may prime NPCs for differentiation by enabling affinity rises for other FGFs such as FGF1. Gradually, an FGF2-FGF1 affinity switch will follow and eventually drive neural distinction. Binding in situ assays with embryonic tissues disclosed an extra specificity layer in HS

affinity with FGFs. Even various complicated combinations of FGF-FGFR show differential affinities to developmental HS motifs and emphasise the critical position of HS-FGF-FGFR ternary complexes in the regulation of embryogenesis (Allen and Rapraeger, 2003).

In short, several trials have shown that HS is renovated during ESC differentiation and that changes in composition appear to drive lineage decisions. ESCs provide an attractive in vitro scheme from which to comparatively readily enrich, propagate, and analyse cells of particular developmental phases for their GAG content and composition. Stage-specific cell lines of reporters that enable smooth cell sorting coupled with differentiation protocols that direct lineage fate decisions are perfect instruments for studying HS structure–function relationships that pertain during vertebrate growth and will undoubtedly advance our knowledge of complicated modifications in HS structure and its growth modulation as well as ESC differentiation.

1.2.8 Heparan sulfate in development

HS biosynthetic enzyme expression is orchestrated in a spatiotemporal fashion, and distinct enzyme family members isozymes are expressed. As a result, as stem and progenitor cells advance through different lineage phases, HS chains are

continually redeveloped. Direct assessment of HS structure and content in undifferentiated and distinguished ESCs along with expression assays of HS biosynthetic genes has resulted in a stronger knowledge of developmental HS remodelling. The purification of HS from ESCs subject to different differentiation regimens shows both qualitative and quantitative changes in HS upon engagement to cell fate. Approximately 80% of GAGs generated in ESCs are HS, with a lower quantity of chondroitin sulfate (CS) generated (Lin et al., 2000; Nairn et al., 2007). General trends such as an increase in overall HS content and sulfation along differentiation were noted regardless of lineage destiny. Interestingly, the increase in general HS content is followed by only small increases in EXT family gene transcript concentration, indicating that other enzyme regulation mechanisms account for the increase in HS content. In addition to increase in HS content, it is striking that the production of other GAGs, including hyaluronic acid and CS/dermatan sulfate (DS), is caused during ESC differentiation and is parallel to increases in most HS and core proteins CS/DS (Nairn et al., 2007). During EB formation, ECM elements are progressively articulated and discovered in EB microenvironments (Shukla et al., 2009). Increased manufacturing of GAG during differentiation can thus accommodate an increase in ECM deposition.

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1.2.9 HS is required for ESC lineage commitment

In recent years, the role of HS in ESC self-renewal has been researched using different ES cell lines that are either deficient in HS or carry under-sulfated HS. These include EXT1 null (EXT1−/−); EXT1cn/cn, a mESC line produced by in vitro ablation of a conditional EXT1 allele; Ndst1/2−/−, and stably transfected si-EXT1 (EXT1-KD) ESCs. Phenotypes of self-renewal vary significantly between mutant lines of HS. EXT1−/−, EXT1cn/cn, Ndst1/2−/−, and stable siRNA EXT1 ESCs maintain their ability for self-renewal over prolonged periods of culture (Holmborn et al., 2004; Johnson et al., 2007; Kraushaar, Yamaguchi, and Wang, 2015; Pickford et al., 2011). On the other hand, siRNA-EXT1-KD ESCs transiently transfected display impaired self-renewal (Sasaki et al., 2007). Differences in the effectiveness of HS depletion can cause discrepancies between cell lines. The siRNA-EXT1-KD ESCs maintain about 20% of residual HS, exhibiting altered sulfation patterns compared to wild-type HS ESCs (Sasaki et al., 2007). In EXT1−/− and EXT1cn/cn ESCs, residual HS of the siRNA-EXT1-KD ESCs may influence pro-and anti-differentiation signals differently from the full absence of HS. However, EXT1's transient knockdown may show an instant impact, resulting in more easily compromised self-renewal than stable cell lines subject to choose over multiple passages. However, most trials to date have shown that endogenous HS is dispensable for self-renewal but becomes critical when ESCs are questioned to distinguish

withdrawal from LIF. Spontaneous adherent differentiation, EB suspension culture, and serum-free differentiation into neural precursors have shown that full loss of HS or under-sulfated HS results in lineage engagement failure. HS mutant ESCs, including EXT1–/–, EXT1cn/cn, and Ndst1/2–/–ESCs, do not distinguish into ecto-, meso-, and endoderm representative cell types but still maintain the expression of ESC markers (Johnson et al., 2007; Kraushaar, Yamaguchi, and Wang, 2015; Lanner et al., 2009; Holley et al., 2010; Forsberg et al., 2012).

Several HS-binding signalling molecules, including BMP, Wnt, and FGFs, have been demonstrated to critically control mESC self-renewal. BMP4 has anti-differentiation impacts mainly by activating the transcription factor Id1, which in turn is needed during self-renewal to maintain elevated concentrations of Nanog (Ying et al., 2003). By upregulating STAT3 expression, Wnt signalling converges with the LIF/STAT3 pathway and thus adds to pluripotency maintenance (Hao et al., 2006). Wnt's extra integration into the self-renewal circuit is accomplished by activating the T-cell factor 3 (Tcf3), which co-occupies several promoters in conjunction with extra pluripotency variables, including Nanog and Oct-4 (Cole et al., 2008). FGF signalling and activation of its downstream mediator MAP kinase, on the other hand, are necessary conditions for lineage engagement, as demonstrated by the research of ESCs treated with FGFR-and MAPK inhibitors (Hamazaki et al., 2006; Kunath et al., 2007; Ying et al., 2008). Closer

examination of signalling pathways engaged in ESC self-renewal has shown that HS is needed for ordinary FGF binding on the cell surface and subsequent intracellular signalling, in line with the role of HS as a co-receptor in the creation of the ternary HS-FGF-FGFR signalling complex. Therefore, promoting exit from ESC self-renewal by enabling FGF signalling is a significant role of HS. EXT1cn/cn ESCs also convey higher concentrations of Nanog in their undifferentiated states in the presence of LIF shows their inability to downregulate pluripotency genes, including Nanog, upon the abolition of LIF (Kraushaar, Yamaguchi, and Wang, 2015). Hence, loss of HS leads to a more naïve ground state in ESCs and further shows that HS works to tune ESCs to be in a less naïve state.

Although perhaps counterintuitive at first, this function of HS in regulating the cell fate of ESCs may be best described in the context of FGF signalling in the early embryo. The blastocyst's ICM exhibits heterogeneity in a non-overlapping fashion, as demonstrated by Nanog's expression and the primitive endoderm (PE) marker GATA6 (Yamanaka, Lanner, and Rossant, 2010). The ICM subpopulations of Nanog+ and GATA6+ will result in epiblast and PE, respectively. In this manner, ESCs imitate ICM cells in the presence of LIF under serum-containing conditions. In ESC societies, where the two lineage markers are expressed in a non-uniform way, similar heterogeneity is discovered. Two subpopulations of ESCs exist; one displays an

expression profile of Nanog+Gata6-epiblast-like cells, which are primed to differentiate into cell types of all germ layers. The other subpopulation is Nanog-Gata6+ and solely differentiates into extraembryonic endodermal cell types (Singh et al., 2007). If either subpopulation is isolated, the initial equilibrium between Nanog+ and Nanog-ESCs is re-established, indicating the presence of a metastable ESC state in which cells are not predetermined to assume either cell destiny but are capable of assuming alternative cell fates spontaneously and reversibly. FGF4 is the predominant FGF isoform expressed in the 8–16 cell morula and is gradually confined to ICM epiblast cells (Yuan et al., 1995). Experiments in vitro and ex vivo have shown that epiblast and PE segregation depends on FGF4 signals (Yamanaka, Lanner, and Rossant, 2010). In addition, therapy of ESCs with FGFR inhibitors results in less heterogeneity and a higher proportion of Nanog+GATA6-cells, indicating that inhibition of FGF signalling outcomes in the inability to create a PE population (Hamazaki et al., 2006). As learned from EXT1cn/cn and Ndst1/2-/-ESCs, HS and its N-sulfation are critical to FGF signalling (Kraushaar, Yamaguchi, and Wang, 2015; Lanner et al., 2009). In line with the suggested role of FGF signalling and its induction of PE-like cells, EXT1cn/cn ESCs express high concentrations of Nanog mRNA in their undifferentiated state and Ndst1/2–/– cells show higher homogeneity among ESCs with a more uniform amount of Nanog+ cells. In addition, blastocyst therapy with NaClO3, a sulfation inhibitor, avoids the development of PE, further proving that HS is engaged in

lineage alignment between epiblast and PE (Lanner et al., 2009). Overall, therefore, HS promotes FGF signalling and induces MAPK activity within a metastable ESC population needed for downregulation of Nanog and PE formation. A 'low-sulfated' HS species may shield ESCs from excessive FGF signalling that would otherwise lead to premature loss of 'stemming' and pluripotency. As proposed by EXT1cn/cn ESCs that fail to distinguish into ecto-, meso-, and endodermal cell types in the lack of LIF, HS further promotes germ layer differentiation of epiblast cells through Nanog downregulation (Kraushaar, Yamaguchi and Wang, 2015). In short, for an original epistable ESC cell state and segregation of epiblast and PE, HS appears to be needed and consequently it is needed for differentiation of the germ layer (Figure 1.12 A).

Examination of extra signalling pathways engaged in ESC self-renewal revealed deficiencies in EXT1–/–ESC signals of BMP4 (Holley et al., 2010; Kraushaar et al., 2012). Despite being more reduced than ordinary phospho-SMAD and ID1 concentrations, EXT1–/–ESCs display improved rather than impaired self-renewal. The observed phenotype can be clarified by BMP4 and FGF's relative contributions to self-renewal maintenance. Studies have shown that although MAPK activity is suppressed with chemical inhibitors, ESCs can be maintained in a "naïve ground state" in the lack of extrinsic signals such as BMP4 (Ying et al., 2008). Inhibition of MAPK activity is thus regarded as essential to self-renewal and does not

require BMP4 activity. Indeed, when BMP4 signalling is active, its capacity to inhibit ERK signalling is an essential mechanism for encouraging self-renewal (Qi et al., 2004). The instructive role of ERK self-renewal inhibition can explain why EXT1–/– and EXT1cn/cn ESCs display an enhanced self-renewal phenotype due to the dominant effect of FGF signalling inhibition and the associated net effect of MAPK levels regardless of impaired BMP4 activity. In short, HS performs dual roles in self-renewal through two distinct mechanisms: by modulating BMP4 and FGF signalling, respectively, it encourages and inhibits self-renewal. However, it appears that the regulatory function on FGF signals is dominant (Figure 1.12 B).

As some studies report HS as an adverse regulator and some as a favourable regulator of Wnt signalling, the role of HS in Wnt signalling, another significant pathway engaged in ESC self-renewal, is less evident. In EXT1cn/cn ESCs, there was an increase in Wnt signalling activity, compared to a decrease in Wnt activity recorded from EXT1 knockdown research (Sasaki et al., 2007; Kraushaar et al., 2012). HS may be a favourable self-renewal regulator, at least in the context of glypican 4, an abundant glypican expressed by ESCs, although it has not been shown whether HS chains or essential proteins are the real Wnt signalling mediators (Fico et al., 2012). Fas signalling has recently been revealed as being involved in encouraging ESC self-renewal and has been shown to rely on 3-O-sulfation of HS to activate this pathway (Hirano et al., 2012). Despite the need for further characterise the role of

Fas signalling in ESC self-renewal, this and other research have combined HS's rigid structure–function relationships into ESC self-renewal modulation.

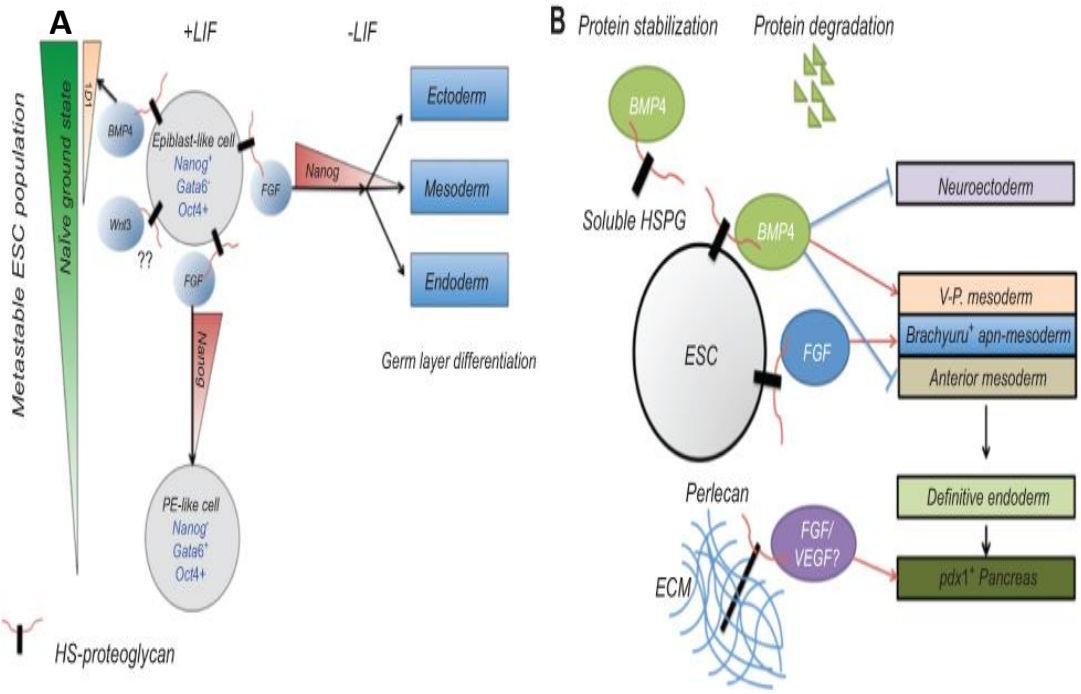


Figure 1.12. Role of HS in mESC self-renewal and differentiation.

(**A**) HS facilitates FGF signalling to downregulate Nanog and establish a PE-like cell type. HS modulates BMP4 signalling and is required for normal Id1 expression. Upon LIF withdrawal, HS facilitates FGF signalling and downregulation of Nanog to allow for multi-lineage differentiation. (**B**) HS facilitates FGF and BMP signalling to promote mesoderm induction and dorso-ventral mesoderm patterning, respectively. HS, through enhancement of BMP4 signalling, has inhibitory effects on neural differentiation. Secreted HS modulates BMP4 signalling by increasing ligand stability. Perlecan might facilitate FGF and/or VEGF signalling to mediate ESC differentiation into Pdx1+ pancreatic cells (Kraushaar, Dalton and Wang, 2013).

1.2.10 HS modulates ESC pluripotency

Numerous growth factors and morphogens regulate the embryonic lineage fates along with the three germ layers and subsequent differentiation steps. Many of them have heparin-binding domains, and HS may modulate them. The increase in HS sulfation and the cell-

specific appearance of selected HS motifs during different differentiation phases show that lineage specification may depend on HS. Ndst1/2–/– and EXT1–/– ESCs investigated this option. These trials indicate that HS is needed initially to cause gene expression connected with the three germ layers and to distinguish between mid and terminally distinguished cell types such as endothelial cells, osteoblasts, adipocytes, hemangioblast-type cells, and neurons (Jakobsson et al., 2006; Johnson et al., 2007; Holley et al., 2010; Forsberg et al., 2012). Considering the retention of pluripotency gene expression and alkaline phosphatase activity recorded from the cell populations examined in this research, it appears likely that the inability of EXT1–/– and Ndst1/2–/–ESCs to distinguish into the cell types as mentioned earlier may be attributable to the block in the original cell destiny engagement. During the initial and critical phase of self-renewal, we recently applied a modified adherent cell differentiation system that overcomes the EXT1cn/cn cell commitment block by adding high doses of FGF2 to the culture medium. The most striking features of aberrant EXT1cn/cn differentiation into germ layers were their failure to induce Brachyury's pan-mesoderm marker and abnormal gene expression involving dorso-ventral patterning, showing the critical significance of HS in ESC mesoderm differentiation (Kraushaar, Yamaguchi, and Wang, 2015).

Several developmental signalling pathways, including FGF/MAPK, BMP4, and Wnt (Lindsley, 2006; Lengerke et al., 2008; Willems and

Leyns, 2008; Hansson et al., 2009), control mesoderm differentiation in ESCs as well as early embryonic development. BMP signals have well-defined roles in mesoderm dorso-ventral patterns during gastrulation and ESCs. BMP4 signalling induces ventral-posterior mesoderm and inhibits anterior mesoderm, resulting in definitive endoderm (Hemmati-Brivanlou and Thomsen, 1995). Furthermore, BMP4 powerfully inhibits neuroectodermal distinction (Wilson and Hemmati-Brivanlou, 1995; Finley, Devata and Huettner, 1999; Kawasaki et al., 2000). In line with BMP4 roles, EXT1cn / cn ESCs show decreased expression of posterior mesoderm genes such as Evx1 and Mesp1 and endodermal gene overexpression such as Sox17 and Foxa2. BMP signalling restoration saves aberrant mesoderm differentiation and demonstrates that HS promotes BMP signalling to mediate mesoderm distinction (Kraushaar et al., 2012). Chlorate treatment that inhibits HS chains sulfation also results in BMP4 signalling deficiencies and induces accelerated neural differentiation at the expense of mesodermal differentiation (Sasaki et al., 2010). Such improved neural differentiation was also observed in knockdown cells PAPST1 and PAPST2, which perform decreased sulfation concentrations in their HS and CS chains (Sasaki et al., 2010).

Consequently, impaired mesoderm differentiation and increased neural differentiation phenotypes of several HS mutant ESCs support HS's role in enabling BMP4 signalling. Just as HS and its FGF signalling modulation are critical to original cell fate engagement,

other cell fate choices that depend on FGF signalling involve HS during differentiation. For example, we discovered that HS is required for normal expression concentrations of the Brachyury T-box transcription factor, which is essential for mesoderm establishment, by enabling FGF signalling (Kraushaar et al., 2012). In short, the strong proof was gathered to demonstrate that HS is a critical element for differentiating ESC into germ-layer type cells by facilitating both FGF and BMP signalling to encourage mesoderm induction and patterning (Figure 1.12 B).

HSPGs such as perlecan and cell surface syndecans and glypicans are abundantly expressed by ESCs. The perlecan knockout significantly decreases the potential for differentiation into Pdx1+ pancreatic cells of ESC-derived mesoderm progenitors (Higuchi, Shiraki, and Kume, 2011). The precise molecular mechanism by which HS modulates pancreatic differentiation remains unknown but may require interactions between growth factors such as VEGF and/or FGF that cause perlecan and pancreas (Higuchi, Shiraki, and Kume, 2011).

1.3 Research aims

There is clear evidence showing that structurally diverse HS molecule will interact with many proteins, including growth factors, morphogens, and adhesion molecules, controlling critical processes of development in invertebrates and vertebrates to regulate various

developmental processes. Their self-renewal and pluripotency characteristics characterise embryonic stem cells (ESCs). Self-renewal enables ESCs to proliferate indefinitely in their undifferentiated state, whereas pluripotency implies their ability to differentiate into the adult body's three germ layers and ultimately all cell types. Several cell signalling pathways regulate both traits tightly. Recent studies have highlighted the importance of HS in modulating ESC functions, in particular, its lineage destiny. Here we study and test the effect of the structural changes of HS during ESC differentiation, and the molecular mechanisms by which modified HS modulates cell fate.

1.4 Research objectives

1.4.1 ESCs differentiation and fate commitment

1. Comparison of altered pluripotent stem cell fate decisions in response to treatment with chemically modified heparins after two different incubation periods 2-days, and 7-days.
2. Pluripotent stem cells under control (standard) conditions can differentiate or proliferate according to typical stem cell behaviour, generating a range of cell types. This work involved a comparison between the three experiment controls zero-time, 2-days, and 7-days.

3. Using q PCR, compare gene profiling of the two-control used zero-time, and 7-days.
4. That different chemically modified heparins can differentially alter embryonic stem cell fate and lineage commitment decisions. This work involves comparisons between different compound treatments for 7-days with control conditions 7-days, measured by qPCR.

1.4.2 Chemically modified heparins mechanism effect on ESCs and cell signalling.

1. The direct effect of over-sulfated heparin on ERK-phosphorylation, using Western blotting.
2. The effects of different chemically modified heparins on embryonic stem cell fate decisions, focusing on the activation of specific tyrosine kinase receptors measured by a membrane-based sandwich immunoassay, and compared to the controls.
3. The effects of 7 different chemically modified heparins at two different concentration 100 and 1000 ng/ml on ESC signalling pathways, after an incubation period of 2 days, measured by sandwich *ELISA assay*.
4. The effect of selected chemically modified heparins (compounds 4, 5, and 6), on HS biosynthesis, measured by a qPCR *assay*.

Chapter 2

2. Material and Methods

Introduction

This chapter will be indicating all different kind of materials and will be describing general experiments and procedures that were performed during the research with the function and reasons of choosing and using that specific experiment.

2.1 Equipment

2.1.1 Cell culture equipment's

Equipment's	Manufacturer
Cell culture dishes (3cm², 10cm², 145cm²)	Corning incorporated life scince
Cell culture Flask (75cm², 175cm²)	Corning incorporated life scince
cell culture multiwell plates (96-WELL)	Cyto One
cover slips (10 mm in diameter)	Corning incorporated life scince
Neubauer improved counting chamber	Corning incorporated life scince
Sterile syringe filters, (0.22 µm)	Corning incorporated life scince
Sterile needle (0.9*25mm)	Corning incorporated life scince
Vacuum bottle filter (500ml, 250ml)	BIOFIL

2.1.2 Protein biochemistry equipment's

Equipment's	Manufacturer
Amersham Protran 0.45um. Nitrocellulose	GE Healthcare
blotting paper	GE Healthcare/Whatman
Clarity western ECL	BIO-RAD
Glass plates	BIO-RAD
Comb 10-well	BIO-RAD
Gel releasers	BIO-RAD
Casting stand	BIO-RAD
4-Gel system. SDS-Page chamber	BIO-RAD
Pierce BCA protein assay kit	Thermo Fisher
PH reader	GE Healthcare

2.1.3 Molecular biology equipment

Equipments	Manufacturer
Agarose gel chamber	BIO-RAD
Agarose gel documentation system	BIO-RAD
Nano drop-BioPhotometer	BIO-RAD
Centrifuges refrigerated bench-top centrifuge 5804 R rotors: A 4-44, F 34-6-38 refrigerated microcentrifuge 5415 R rotor: F 45-24-11 ultracentrifuge Optima XL-90 Rotor: SW 41-Ti	GE Healthcare
GeneAmp PCR System 2400	Thermo Fisher
microplate reader PHOmo	Thermo Fisher
pH meter SevenEasy	Thermo Fisher

2.2 Chemicals

2.2.1 Cell culture chemicals

Chemical	Manufacturer
Penicillin-Streptomycin (10,000 U/mL)	Thermo Fisher
DMEM	Thermo Fisher
Neurobasal Plus Medium	Thermo Fisher
B-27 Plus Supplement (50X)	Thermo Fisher
N-2 Supplement (100X)	Thermo Fisher
KnockOut Serum Replacement Medium	Thermo Fisher
ESGRO Leukemia Inhibitory Factor	Sigma-Aldrich
dimethyl sulfoxide (DMSO)	Sigma-Aldrich
fetal bovine serum (FBS)	Thermo Fisher
GlutaMAX Supplement	Thermo Fisher
Trypsin-EDTA (0.5%), no phenol red	Thermo Fisher
TrypLE Express Enzyme (1X)	Thermo Fisher
Accutase - Cell Detachment Medium	Thermo Fisher
ß-mercaptoethanol	Sigma

2.2.2 Protein biochemistry chemicals

Chemical	Manufacturer
Acrylamide/bis solution (30 %)	Sigma
Ammonium persulfate (APS)	Sigma
BCA Protein assay reagent A 500 ml	Sigma
Sample Buffer, Laemmli 2× Concentrate	Sigma
N,N,N′,N′-Tetramethylethylenediamine	Sigma
Clarity Western ECL Substrate	BIO-RAD
TWEEN 20	Sigma
Restore Western blot stripping buffer	Thermo Scientific
Ponceau S red staining solution	Sigma
Sodium dodecyl sulfate (SDS)	Thermo Scientific
Tris	Thermo Scientific
Glysin	Thermo Scientific

2.2.3 Molecular biology chemicals

Chemical	Manufacturer
Bovine Serum Albumin solution (BSA)	Sigma
DNA Gel Loading Dye (6X)	Thermo Scientific
d NTP Mix 500 ul	Thermo Scientific
My Taq DNA Polymerase (500 Units)	Thermo Scientific
DNase I, RNase-free (1 U/µL)	Thermo Scientific
Oligo(dT)12-18 Primer	Thermo Scientific
SuperScript II Reverse Transcriptase	Thermo Scientific
Taq DNA Polymerase (5 U/µL)	Thermo Scientific
Agarose (Moleculare Grade)	BIOLINE
M-MLV Reverse Transcriptase Buffer	Thermo Scientific
Taq Buffer (10X)	Thermo Scientific
Autoclaved deionized water (dH_2O)	Thermo Scientific
DNA Ladder 100BP 50UG	Thermo Scientific
DNA Ladder 1KB 100UG	Thermo Scientific
TBE Buffer (Tris-borate-EDTA) (10X)	Thermo Scientific
TAE (10X)	Thermo Scientific

2.3 Cell Culture

2.3.1 Preparation of gelatinised culture dishes

0.2 - 0.1 % (w/v) gelatin solution was added to tissue culture dishes and incubated at room temperature for approximately 30 min. Then all gelatine solution was aspirated and replaced with media when necessary.

2.3.2 Medium

MEF Feeder cells medium: High glucose DMEM (Gibco, Invitrogen, UK) was supplemented with 10 % fetal bovine serum (PAA), 2 mM L-glutamine (Gibco Invitrogen, UK), 1 % NEAA (Gibco, Invitrogen, UK), 1 mM 2-βmercaptoethanol (Gibco Invitrogen, UK).

ESC medium: high glucose DMEM (Gibco, Invitrogen, UK) was supplemented with 1 mM β-mercaptoethanol (Gibco, Invitrogen, UK), 2 mM L-glutamine (Gibco, Invitrogen, UK), 1000 U / mL leukaemia inhibitory factor (LIF) (Millipore, UK) and 2% fetal bovine serum (ThermoFisher).

Serum free medium: KnockOut D-MEM (ThermoFisher), KnockOut Serum Replacement (ThermoFisher), 1000 U / mL leukaemia inhibitory factor (LIF) (Millipore, UK), Recombinant

mouse leukemia inhibitory factor (LIF) (ThermoFisher), 1 mM β-mercaptoethanol (SIGMA)

Neuron medium: CTS Neurobasal™ Medium (ThermoFisher), CTS B-27 Supplement, XenoFree (ThermoFisher), N-2 Supplement (100X) (ThermoFisher).

2.3.3 Routine culture of R1 mESC

R1 mESCs were cultured on 3.5 cm tissue culture dishes, coated with 0.1 % gelatine in mESC medium. mESC medium was removed from confluent dish of R1 mESCs and cells were washed once with PBS. 1 x trypsin/EDTA was added for 3-5 min, and subsequently neutralised with STO medium, before centrifugation at 64g for 2.5 min. STO medium was removed and R1 mESCs resuspended in mESC medium depending on culture condition. Typically, R1 mESCs were sub-cultured every second day at a ratio of 1:3 and incubated at 37°C and 5 % CO2, unless otherwise stated.

2.3.4 Serum-free R1 mESCs culture

R1 mESCs were cultured in KnockOut DMEM, and KnockOut Serum Replacement, and MEM Non-essential Amino Acids Solution, 10 mM 1 mLGlutaMAX-I, 100X 1 mL 2-mercaptoethanol, and 1000X 100 µLLIF, 10 µg/mL, under these conditions sub-

culture technique is exactly as described previously, except R1 mESCs were passaged every 3 days.

2.3.5 Routine culture of MEF feeders

MEF cells were cultured in 10 cm tissue culture dishes (Corning) and coated with 0.1-0.2 % gelatine in MEF medium. MEF medium was removed from a confluent dish of MEF cells and cells were washed once with PBS. 1 x trypsin/EDTA was added for 3-5 min, and subsequently neutralised with MEF medium, before centrifugation at 64g for 2.5 min. MEF medium was removed and cells re-suspended in fresh MEF medium. MEF cells were sub cultured every second day at a ratio of 1:4 and incubated at 37°C and 5 % CO2.

2.3.6 Preparation of MEF feeders

One confluent 10 cm dish of MEF cells were inactivated with mitomycin-C (ThermoFisher). 5 mL of total 10 mL of MEF medium was removed from a confluent dish and 100 µL of 1 mg / mL stock of mitomycin-C (final concentration = 20 µg/mL) added. Cells were incubated for 2 h in 37 ° C and 5 % CO2. The medium was removed and then cells washed thoroughly three to four times with PBS, then 5 mL 1x trypsin/EDTA was added for 3-5 min. The trypsin was neutralised by transferring to 5 mL MEF medium before centrifugation at 64g for 2.5 min. MEF medium was removed and

cells re-suspended in 9 mL MEF medium. 0.5 mL of cell suspension was added to 12 x 3.5 cm gelatinised tissue culture dishes. The volume was made up to 1.5 mL with MEF medium and incubated at 37°C and 5 % CO2.

2.3.7 Freezing cells

Medium was removed from the culture dish and cells were washed once with PBS, followed by trypsinisation with TrypLE™ Express Enzyme (1X), no phenol red for 3-5 min, which was neutralised with equal volumes of MEF medium, before centrifuging at 64g for 2.5 min. Medium was again removed and cells were re-suspended in cell culture freezing medium (ThermoFisher), typically in 0.5 mL volume in a cryovial. The cryovial was placed in a freezing chamber containing isopropanol overnight at - 80°C. The cells were transferred to liquid nitrogen if long term storage was necessary.

2.3.8 Thawing of cells

The cryovial containing the desired cells was removed from liquid nitrogen and thawed rapidly in a water bath at 37°C. The cells were then transferred to MEF medium and centrifuged at 64g for 2.5 min. Medium was removed and replaced with appropriate medium (depending on cells i.e. MEF medium for MEF cells), and

cells were finally transferred to culture dish and incubated at 37°C 5 % CO2.

2.4 Western blot

Following separation of proteins by SDS-PAGE, proteins were transferred onto nitrocellulose membrane using the following method. Transfer cassettes contained the following layers: a sponge, Whatman 3MM blotting paper, nitrocellulose membrane, SDSPAGE gel, Whatman blotting paper and a sponge (all equipment was pre-immersed in transfer buffer containing 20% v/v methanol, 0.19M glycine and 0.05M Tris). The transfer cassette was then placed in a transfer tank filled with transfer buffer for 6 hours at 280mA. Following the transfer, nitrocellulose membranes were stained to visualise proteins and subsequently destained in deionised water and TBST. Nitrocellulose membranes were kept in 5% skimmed dried milk in TBST for 30 minutes in order to block non-specific binding sites. Following blocking, primary antibodies (Table 2.4) were diluted in 5% skimmed dried milk in TBST and incubated with the nitrocellulose membrane overnight at 4°C on a rocking table. Following incubation with primary antibodies, nitrocellulose membranes were washed in TBST 6 times for 5 minutes each time. Nitrocellulose membranes were then incubated with secondary antibodies conjugated to horse-radish peroxidase (HRP) in 5% skimmed dried milk in TBST for 2 hours at room

temperature. Following incubation, nitrocellulose membranes were again washed in TBST 6 times for 5 minutes each time. Proteins were then visualised by washing nitrocellulose membranes in enhanced chemiluminescence (ECL) reagent for 1 minute, sealed in saran wrap and exposed to autoradiography film (Kodak) for the required amount of time.

2.5 Polymerase chain reaction PCR

RT-PCR / q PCR

DNase Treatment

Treatment of total RNA with RNase-free DNase is required to remove contaminating genomic DNA. Follow the procedure according to the manufacturer's instructions.

Reverse Transcription Reaction

all reagents will be thawed completely on ice. All reactions should be assembled on ice. Into a nuclease-free tube we mix : 1 - 5 µg of DNase treated total RNA, 1 µl of random primers X µl of nuclease-free dH2O for a final volume of 12 µl, the we will Mix and incubate at 70° C for 10 minutes, tube will be placed on ice immediately, briefly we centrifuge the tube, and add the following to each tube: 4 µl of 5X Reverse Transcription Buffer ,2 µl of 0.1 M DTT, 1 µl of 10 mM dNTPs, mix and incubate at room temperature for 10

minutes. Then we incubate at 42° C for 2 minutes, we Add 1 µl RNase H- Reverse Transcriptase (200 units/ µl). pipetting Mix, then we incubate at 42° C for 50 minutes, followed by incubation at 70° C for 15 minutes, after that we dilute reactions 5-fold by adding 80 µl of nuclease-free dH2O.

PCR Reaction

In a thermal cycler we perform the following program: 94° C for 4 minutes, (30-35 cycles) ,94° C for 45 seconds, 55° C for 45 seconds, 72° C for 45 seconds, 72° C for 10 minutes.

The PCR products can be analysed by 1.5 - 2% agarose gel electrophoresis.

2.6 Intracellular Signalling (Paths can, Sandwich ELISA):

Preparing Cell Lysates

Thaw 1X Cell Lysis Buffer, and mix thoroughly. Supplement Cell Lysis Buffer with a cocktail of protease inhibitors. Keep lysis buffer on ice, remove media and wash cells once with ice-cold 1X PBS, Remove PBS and add ice-cold Cell Lysis Buffer. For adherent cells, use 0.5 ml cell lysis buffer for each plate (10 cm in diameter). Incubate on ice for 2 minutes, Tilt the plate and collect the lysate into a clean micro tube, Optional step: micro centrifuge the lysate at maximum speed for 3 minutes at4°C and transfer the supernatant to a new tube. This step is

usually not required but can help remove any particles or large cell debris, if present. Lysate may be used immediately or stored at - 80°C in single-use aliquots, immediately before performing the assay, dilute lysates to 0.2 – 1.0 mg/ml in Array Diluent Buffer. Set aside on ice.

Procedure

1. After the microwell strips have reached room temperature, break off the required number of microwells. Place the microwells in the strip holder. Unused microwells must be resealed in the storage bag and stored at 4°C immediately.
2. Cell lysates can be used undiluted or diluted with sample diluent (supplied in each PathScan Sandwich ELISA Kit, blue color). Individual datasheets or product webpage for each kit provide information regarding an appropriate dilution factor for lysates and kit assay results.
3. Add 50 µl of each undiluted or diluted cell lysate to the appropriate well. Seal with tape and press firmly onto top of microwells. Incubate the plate for 2 hr at room temperature. Alternatively, the plate can be incubated overnight at 4°C.
4. Gently remove the tape and wash wells:

4.1 Discard plate contents into a receptacle.

4.2 Wash 4 times with 1X Wash Buffer, 150 µl each time per well.

4.3 For each wash, strike plates on fresh paper towels hard enough to remove the residual solution in each well, but do not allow wells to dry completely at any time.

4.4 Clean the underside of all wells with a lint-free tissue.

5 Add 50 µl of detection antibody (green color) to each well. Seal with tape and incubate the plate at room temperature for 1 hr.

6 Repeat wash procedure (Section C, Step 4).

7 Add 50 µl of HRP-linked secondary antibody (red color) to each well. Seal with tape and incubate the plate at room temperature for 30 min.

8 Repeat wash procedure (Section C, Step 4).

9 Prepare detection reagent working solution by mixing equal parts 2X LumiGLO Reagent and 2X Peroxide.

10 Add 50 µl of the detection reagent working solution to each well.

11 Use a plate-based luminometer set at 425 nm to measure Relative Light Units (RLU) within 1–10 min following addition of the substrate.

2.7 Mouse Phospho-RTK Array

Cell Lysates - Rinse cells with PBS and remove any remaining PBS before adding lysis buffer. Solubilize the cells at 1 x 107 cells/mL in Lysis Buffer 17 prepared with protease inhibitors. Pipette up and down to resuspend and rock the lysates gently at 2-8 °C for 30 minutes.

Microcentrifuge at 14,000 x g for 5 minutes, and transfer the supernate into a clean test tube. Quantitation of sample protein concentration using a total protein assay is recommended. The maximum allowable lysate volume is 250 µL/array. Cell lysates should be used immediately or aliquoted and stored at ≤ -70 °C. Thawed lysates should be kept on ice prior to use.

Procedure

Bring all reagents to room temperature before use. Keep samples on ice.

1. Prepare all reagents and samples as directed in the previous sections.

2. Pipette 2.0 mL of Array Buffer 1 into each well of the 4-Well Multi-dish that will be used. Array Buffer 1 is used as a block buffer.

3. Using flat-tip tweezers, remove each array to be used from between the protective sheets.

4. Place one array into each well of the 4-Well Multi-dish. The array number should be facing upward.

5. Incubate for 1 hour at room temperature on a rocking platform shaker. Orient the tray so that each array rocks from end to end in its well.

6. While the arrays are blocking, prepare samples by diluting the desired quantity of cell lysate in 1.25 mL of Array Buffer 1. Adjust to a

final volume of 1.5 mL with Lysis Buffer 17 as necessary. The maximum allowable cell lysate volume is 250 µl/array.

7. Aspirate Array Buffer 1 from the 4-Well Multi-dish. Add the prepared samples and place the lid on the 4-Well Multi-dish.

8. Incubate overnight at 2-8° C on a rocking platform shaker.

9. Carefully remove each array and place into individual plastic containers with 20 mL of 1X Wash Buffer. Rinse the 4-Well Multi-dish with deionized or distilled water and dry thoroughly.

10. Wash each array with 1X Wash Buffer for 10 minutes on a rocking platform shaker. Repeat two times for a total of three washes.

11. Dilute the Anti-Phospho-Tyrosine-HRP Detection Antibody in 1X Array Buffer 2 using the dilution factor on the vial label. Pipette 2.0 mL into each well of the 4-Well Multi-dish.

12. Carefully remove each array from its wash container. Allow excess buffer to drain from the array. Return the array to the 4-Well Multi-dish containing the Anti-Phospho-Tyrosine-HRP and cover with the lid.

13. Incubate for 2 hours at room temperature on a rocking platform shaker.

14. Wash each array as described in steps 9 and 10.

15. Carefully remove each membrane from its wash container. Allow excess Wash Buffer to drain from the membrane by blotting the lower

edge onto paper towels. Place each membrane on the bottom sheet of the plastic sheet protector with the identification number facing up.

16. Pipette 1 mL of the prepared Chemi Reagent Mix evenly onto each membrane.

17. Carefully cover with the top sheet of the plastic sheet protector. Gently smooth out any air bubbles and ensure Chemi Reagent Mix is spread evenly to all corners of each membrane. Incubate for 1 minute.

18. Position paper towels on the top and sides of the plastic sheet protector containing the membranes and carefully squeeze out excess Chemi Reagent Mix.

19. Remove the top plastic sheet protector and carefully lay an absorbent lab wipe on top of the membranes to blot off any remaining Chemi Reagent Mix.

20. Leaving membranes on the bottom plastic sheet protector, cover the membranes with plastic wrap taking care to gently smooth out any air bubbles. Wrap the excess plastic wrap around the back of the sheet protector so that the membranes and sheet protector are completely wrapped.

21. Place the membranes with the identification numbers facing up in an autoradiography film cassette.

22. Expose membranes to X-ray film for 1-10 minutes.

Chapter 3

The effect of chemically modified heparins on embryonic stem cell lineage commitment.

3.1 Introduction

Proteoglycans of heparan sulfate (HS) are found on all cell basement membranes where they interact with a large number of physiologically essential macromolecules and thus influence biological processes (Esko and Selleck, 2002; Bülow and Hobert, 2006). The proteoglycans ' HS polysaccharide chains, covalently attached to various core proteins, carry negatively charged groups of sulfates and carbohydrate. The positioning of these sulfate groups on the two monosaccharide (N-acetyl glucosamine and hexuronic acid) building blocks contributes to the specificity of the interactions. Many growth factors and cytokines bind to HS proteoglycans on the cell surface, acting as co-receptors for signalling molecules (Grobe et al., 2002). Furthermore, HS is essential during embryonic development for creating and maintaining morphogens and cytokine gradients (Kreuger et al., 2004). Pluripotent stem cell differentiation provides a model system for analysing changes that occur during embryogenesis

and development of mammals. The addition of chemically-modified heparin components allows researchers to produce defined differentiated cell populations from pluripotent embryonic stem cells (Jaenisch and Young, 2008). A series of mechanisms that include, but are not limited to, the maintenance of morphogen gradients and co-receptor functions regulate developmental signalling (Lin, 2004; Kreuger et al., 2006; Bishop, Schuksz and Esko, 2007). Different chemical and genetic approaches have recently addressed the function and structure-function relationships of the HS in stem cell self-renewal and differentiation. This research project will aim to provide a clearer picture of the chemically-modified heparins and their role in stem cell self-renewal and differentiation with a significant focus on ESCs. This chapter will focus on presenting laboratory experiments addressing the effects of the exogenous modified heparins on mouse embryonic stem cell differentiation, or proliferation, and also help in understanding the specific roles of each selected chemically modified heparins on the fate of ES cells. We investigated the ability of chemically modified heparins on ES cells to develop into different lineages. Based on the expression of differentiation markers, we show that ES cells can take on a specific lineage fate.

3.2 Research aim

There is clear evidence showing that structurally diverse HS molecule will interact with many proteins, including growth factors, morphogens, and adhesion molecules, controlling critical processes of development in invertebrates and vertebrates to regulate various developmental processes. Their self-renewal and pluripotency characteristics characterise ESCs embryonic stem cells. Self-renewal enables ESCs to proliferate indefinitely in their undifferentiated state, whereas pluripotency implies their ability to differentiate into the adult body's three germ layers and ultimately all cell types. Several cell signalling pathways regulate both traits tightly. Recent studies have highlighted the importance of HS in modulating ESC functions, in particular, their lineage destiny. Here we study and test the effect of the structural changes of HS during ESC differentiation, and the molecular mechanisms by which modified HS modulates cell fate.

3.3 General Hypothesis

Different structures of HS will promote differentiation of ESCs from the pluripotent state to alternative lineages and proliferation. Moreover, they may play an essential role in the maintenance of the pluripotent state of stem cell.

In this chapter I will show:

1. Comparison of altered pluripotent stem cell fate decisions in response to treatment with chemically modified heparins after two different incubation periods 2-days, and 7-days.
2. Pluripotent stem cells under control (standard) conditions can differentiate or proliferate according to typical stem cell behaviour, generating a range of cell types. This work involved a comparison between the three experiment controls zero-time, 2-days, and 7-days.
3. Using q PCR, compare gene profiling of the two-controls used, zero-time, and 7-days.
4. That different chemically modified heparins can differentially alter embryonic stem cell fate and lineage commitment decisions. This work involves comparisons between different compound treatments for 7-days with control conditions 7-days, measured by qPCR.

3.4 Culture Conditions using Chemically Modified Heparins on Mouse Embryonic Stem Cell.

Embryonic stem cells were cultured to confluency on feeder cell culture conditions, using pluripotent stem cell medium, supplemented with LIF (Leukemia Inhibitory Factor) and KOSR

(Knockout Serum Replacement), after confluence, pluripotent stem cells medium was sucked out and cells were washed using PBS, followed by TrypLE cell treatment for 3-5 minutes in 37°C incubator, then centrifuged and re-cultured for mechanical Feeder cell depletion from Pluripotent Stem Cells. Before beginning the differentiation experiments, pluripotent stem cells were counted and cultured in 35 mm gelatine-coated plates, using the pluripotent stem cell medium supplemented with LIF (Leukemia Inhibitory Factor) and KOSR (Knockout Serum Replacement), and for maintaining and stimulating adhesion of stem cell after removing feeder cells, then differentiation protocols will begin using LIF-free medium and (FBS) within two different incubation periods 2-days and 7-days for control and treated conditions, and with the addition of 1000ng/ml of different chosen chemically modified heparins 2, 4, 5, and 6 (see Table 3.2). To allow pluripotent stem cells to freely differentiate to their preferred lineage fate under these treatment conditions, cell culture medium was changed daily along with fresh modified heparins at the same concentration.

3.5 Methods used

3.5.1 Reverse transcription polymerase chain reaction RT-PCR with agarose gel electrophoresis:

Methods used in this chapter were carried out as described in methods chapter 2 (section 2.5). The Pluripotent Stem Cell Assessment Primer Pair Panel describes the mRNA transcripts of 14 genes commonly used as markers for the Assessment of Pluripotency and lineage-committed of ESCs. GAPDH and positive control were used as controls for the effective synthesis of cDNA (see Figure 3.1).

A

Pluripotent SC	DNA Size (bp)	Ectodermal	DNA Size (bp)	Endodermal	DNA Size (bp)	Mesodermal	DNA Size (bp)	germ Cells	DNA Size (bp)
Nanog	547	Nestin	388	AFP	451	Brachyury	216	Stella	218
Oct-3/4	536	Otx2	449	GATA-4	479				
SOX2	406	TP63	587	PDX-1	421				
DPPA5		SOX2		SOX17					
				FoxA2					

B

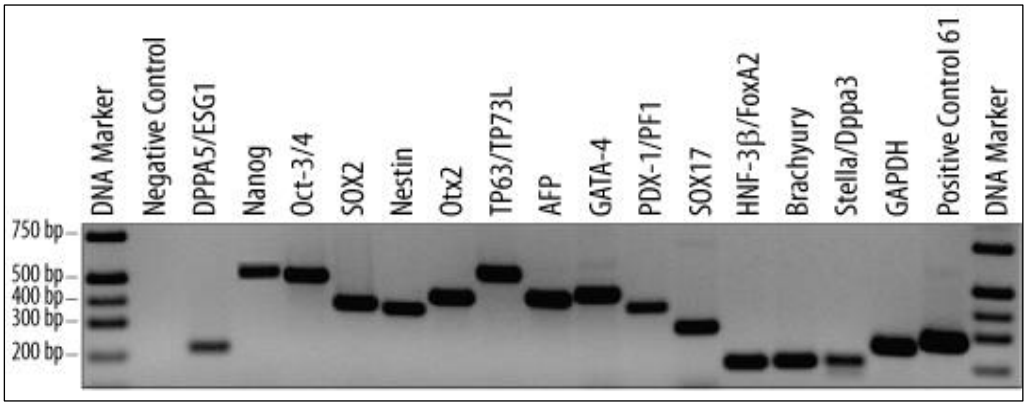


Figure 3.1: **Details of genes studied by RT-PCR to monitor pluripotency and lineage-commitment of embryonic stem cells. A**. List of 15 genes commonly used as markers for the assessment of ESCs, and the expected sizes of PCR products. **B**. Expected PCR products after separation of standards (R&D Systems) by 2% agarose gel electrophoresis according to the description in Chapter 2, Methods.

3.5.2 Quantitative RT-PCR qPCR.

Quantitative PCR-based essay is the most popular technique for characterizing or confirming gene expression patterns in separate sample populations. The most common genes generally used as pluripotency and differentiation fate detectors of early stem cell diffrentiation are as follows (see Table 3.1).

Pluripotent SC	Ectoderm	Endoderm	Mesoderm
Dppa5a	Ascl1	Afp	Gata1
Nanog	Bmp4	Cxcr4	Kdr
Pou5f1	En2	Foxa2	Pdgfrb
Shh	Foxd3	Gata4	T
Sox2	Foxg1	Gsc	Tbx5
Stat3	Gbx2	Pdx1	
Tcf3	Lmx1a	Smad2	
Utf1	Ncam1	Smad3	
	Nes	Sox17	
	Neurog2	Trp63	
	Ngfr		
	Otx2		
	Pax2		
	Pax6		
	Pitx3		
	Sox1		
	Tubb3		
	Wnt1		

Table 3.1: List of 41 genes measured by qPCR to indicate stem cell lineage or pluripotency. qPCR was performed according to the description in Chapter 2, Methods.

3.6 Results

3.6.1 Experimental control conditions used measured by RT-PCR.

The first important step in the investigation of the effect of specific chemically-modified heparins on stem cell fate using RT-PCR, was to establish gene expression levels in pluripotent stem cells under the standard control conditions, without any treatment addition. These results will then represent a sound base for judging changes in gene expression. These conditions, designated the zero-time control, involve pluripotent stem cells cultured in the presence of LIF and feeder-free conditions. On the other hand, the research has two more control conditions which are essential for showing changes in the lineage commitment of embryonic stem cells, using medium free of all chemically modified heparins but also after LIF-withdrawal. These conditions will establish changes in gene expression that occur normally after LIF-withdrawal, due to the effects of any growth factors or other medium supplements during the culture period. Thus they will show the natural lineage differentiation of untreated pluripotent stem cells; these two control conditions are designated the 2-day and 7-day controls. In the 2and 7-day controls, stem cells are cultured in LIF minus and feeder free conditions, with chemically modified heparins added at a concentration of 1000 ng/ml for two or seven days (Figure 3.2).

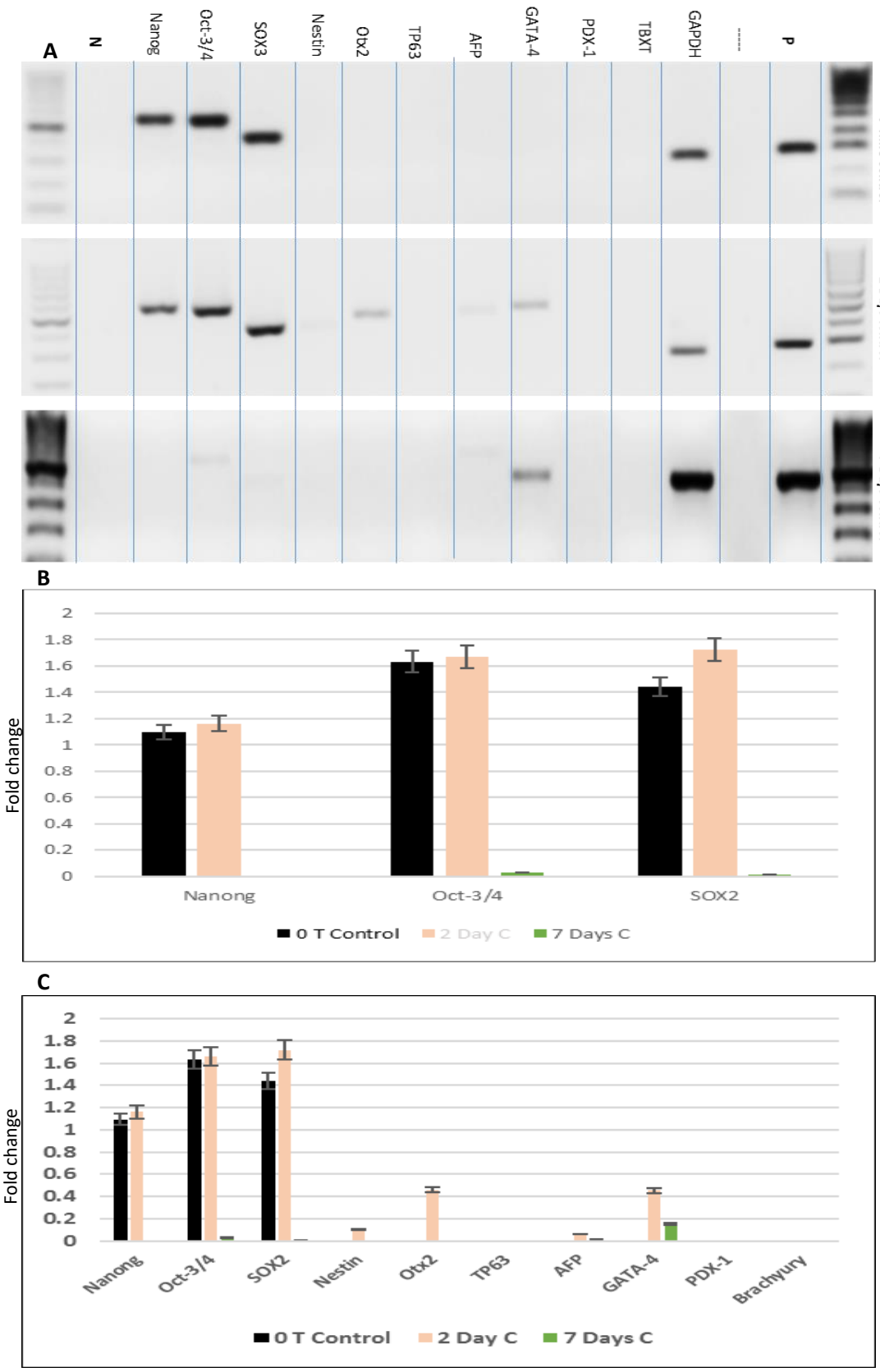


Figure 3.2. RT-PCR product for R1 embryonic stem cells, showing gene expression of the three controls used in RT-PCR zero-time control, 2-days control, and 7-days control, for 2, expressed using agarose gel electrophoresis. A: The RT-PCR products were obtained from control condition samples zero-time, 2-days, and 7-days, from three replicates each, and run on a 2% agarose gel to determine their profile list. Products were recognised by their size compared to known positive control and internal control GAPDH, N: Negative control, P: Positive control. **B: Pluripotent gene expression changes by time and compared to zero-time control pluripotent R1 stem cells. C: Gene expression of lineage markers.** Nestin, Otx2, AFP, and GATA-4, all are expressed at the 2-day control condition. The 7-days control condition showed entirely negative expression of all genes except a low expression of AFP, and GATA-4 endoderm markers.

The zero-time control conditions represents the pluripotent state of stem cells, cultured in a pluripotency maintenance medium with LIF and feeder cells, and serum-free. Under these conditions expression of key markers of undifferentiated stem cells were evident, including Nanog, Oct-3/4, and Sox2. There was also an apparent absence of all other lineage markers (Fig 3.2). The 2 day control showed continued strong expression of pluripotency markers, and complete absence of the markers PDX-1 and TBXT, with some expression of Nestin, Otx2, Afp, and GATA4. Thus differentiation was underway at this early stage of the culture conditions. In contrast in the 7-day control conditions weak expression of endoderm lineage markers AFP and GATA-4 were noted, along with very weak expression of the pluripotency markers Oct-3/4 and Sox2 were noted.

As expected, the stem cells after the removal of LIF gradually downregulated pluripotency genes, with an initial very weak lineage decision toward both ectoderm and endoderm lineages in the 2-day control, then towards the endoderm lineage in the 7-day control. Thus under control culture conditions after LIF-withdrawal, the ESCs differentiate, losing expression of pluripotency markers, and mainly shift their commitment to the endodermal lineage by 7-day.

3.6.2 Chemically modified heparins affect embryonic stem cell fate as measured by RT-PCR.

General considerations for experimental design: Stem cell fate commitment was measured using RT-PCR in two different treatment time conditions (2 and 7-days) compared to pluripotent state stem cell zero-time control and the 2-day and 7-day control conditions. Based on screening experiments focussed on effects on signalling pathways see Chapter 4, three chemically modified heparins were chosen for the initial RT-PCR experiments, namely compounds 4, 5 and 6 (see Table 3.2). These chemicahlly modified heparins were tested for their affects on ESCs lineage commitment, after the withdrawal of LIF and depletion of feeder cells from the culture plates. The chemically modified heparins were added continuously to the culture medium, allowing them to affect embryonic stem cell fate choices. The experiments were designed to evaluate the effect of the compounds, and also the length of incubation, based on two periods of culture conditions 2-days, and 7-days, allowing monitoring of the lineage commitment fate of the embryonic stem cell after the withdrawal of LIF. All chemically modified heparins were used at 1000 ng/ml concentration and mixed with stem cell medium after the withdrawal of LIF and feeder cell depletion, as mentioned. Cells were maintained in 5% CO2 at 37ºC; after the end of each

incubation period, cells were collected for mRNA purification and RT-PCR, with PCR product separation performed by agarose gel electrophoresis.

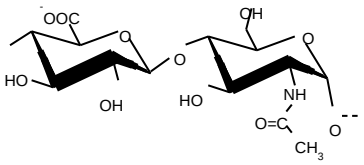
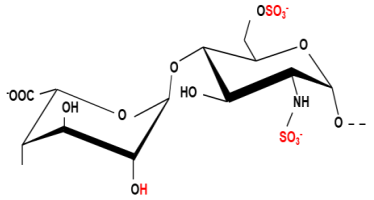
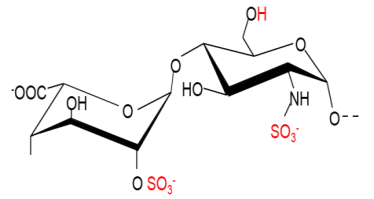
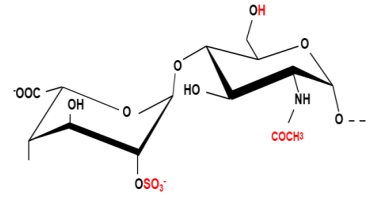
Compound number	Modified Heparin Compound	Text description	Schematic of structure
2	Over-sulfated heparin	R1 =SO3-, R2 =SO3-, R3=SO3- + Additional sulfates at the C3 of glucosamine and C3 of uronic acid	
4	2de-Sulfated-N-Sulfated-Heparin	R1 =H, R2 =SO3-, R3 =SO3	
5	6de-Sulfated-N-Sulfated-Heparin	R1 =SO3- , R2 =H, R3 =SO3-	
6	2de-Sulfated-N-Acetylated Heparin	R1 =H, R2 =SO3-, R3 =COCH3	

Table.3.2: General disaccharide repeating structures of chemically modified heparin derivatives tested. The table shows the 4 modified heparins that were used in the experiments. Modified heparins compounds were prepared and supplied by Dr.Ed Yates. R1, C2 on uronic acid; R2, C6 on glucosamine; R3, N2 on glucosamine.

3.6.2.1 Effects of chemically modified heparin 4 (2de-S-NS,6S) on ESC differentiation

The effects on gene expression of addition of chemically modified heparin 4 to R1 ESCs in culture for either 2-days or 7-days was measured by RT-PCR (Fig 3.3).

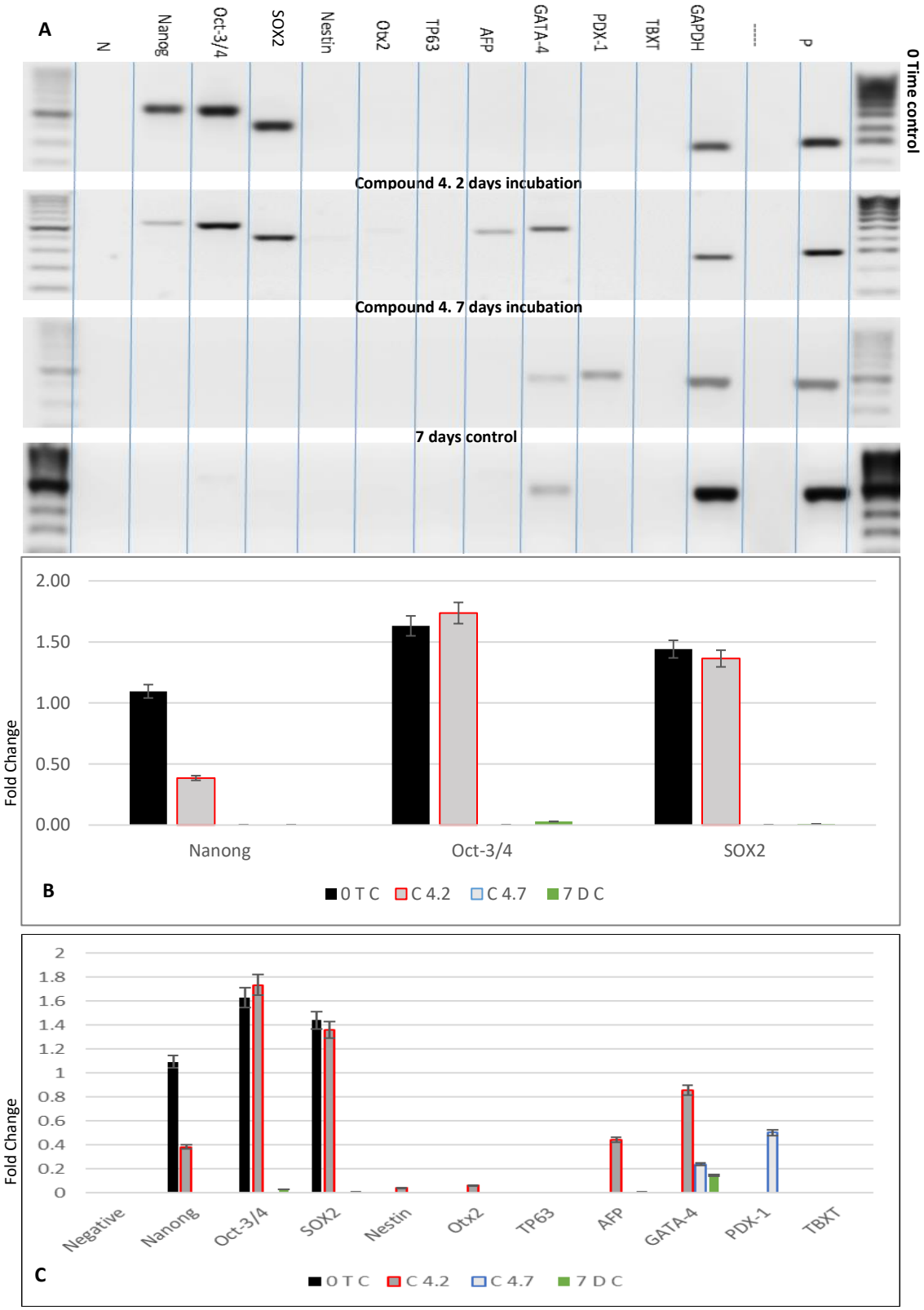


Figure 3.3. RT-PCR of R1 embryonic stem cells, after treatment and incubation with compound 4, for 2 or 7-days, in LIF free conditions. A: The RT-PCR products were obtained from control condition samples zero-time, 2-days, and 7-days, from three replicates each, and run on a 2% agarose gel to determine their profile list. Products were recognised by their size compared to known positive control and internal control GAPDH, N: Negative control, P: Positive control. **B:** Pluripotent gene expression changes by time and compared to zero-time control pluripotent R1 stem cells and 7-days control conditions. **C**: bar chart showing all lineage markers expression, comparing all R1 stem cell culture conditions.

At 2-days treatment a reduction in the level of Nanog was notable compared to the 2-day control (see Fig 3.2). Expression of Otx2 and Nestin was absent compared to weak expression in the 2-days control. Expression levels of AFP and GATA-4 were elevated compared to weaker expression in the 2-day control. These results indicate that more rapid loss of pluripotency and enhanced endodermal differentiation are promoted by chemical modified heparin 4. After 7-day of treatment, all pluripotency genes were absent as also seen in the 7-day control. In contrast, both GATA4 and PDX-1 were expressed after 7-day, compared to only GATA-4 in the 7-day control. This data indicates increased endodermal differentiation is induced by exposure of R1 ESCs to chemically modified heparin 4.

3.6.2.2 Effects of chemically modified heparin 5 (2S, 6de-S, NS) on ESC differentiation

The effects on gene expression of addition of chemically modified heparin 5 to R1 ESCs in culture for either 2-days or 7-days was measured by RT-PCR (Fig 3.4).

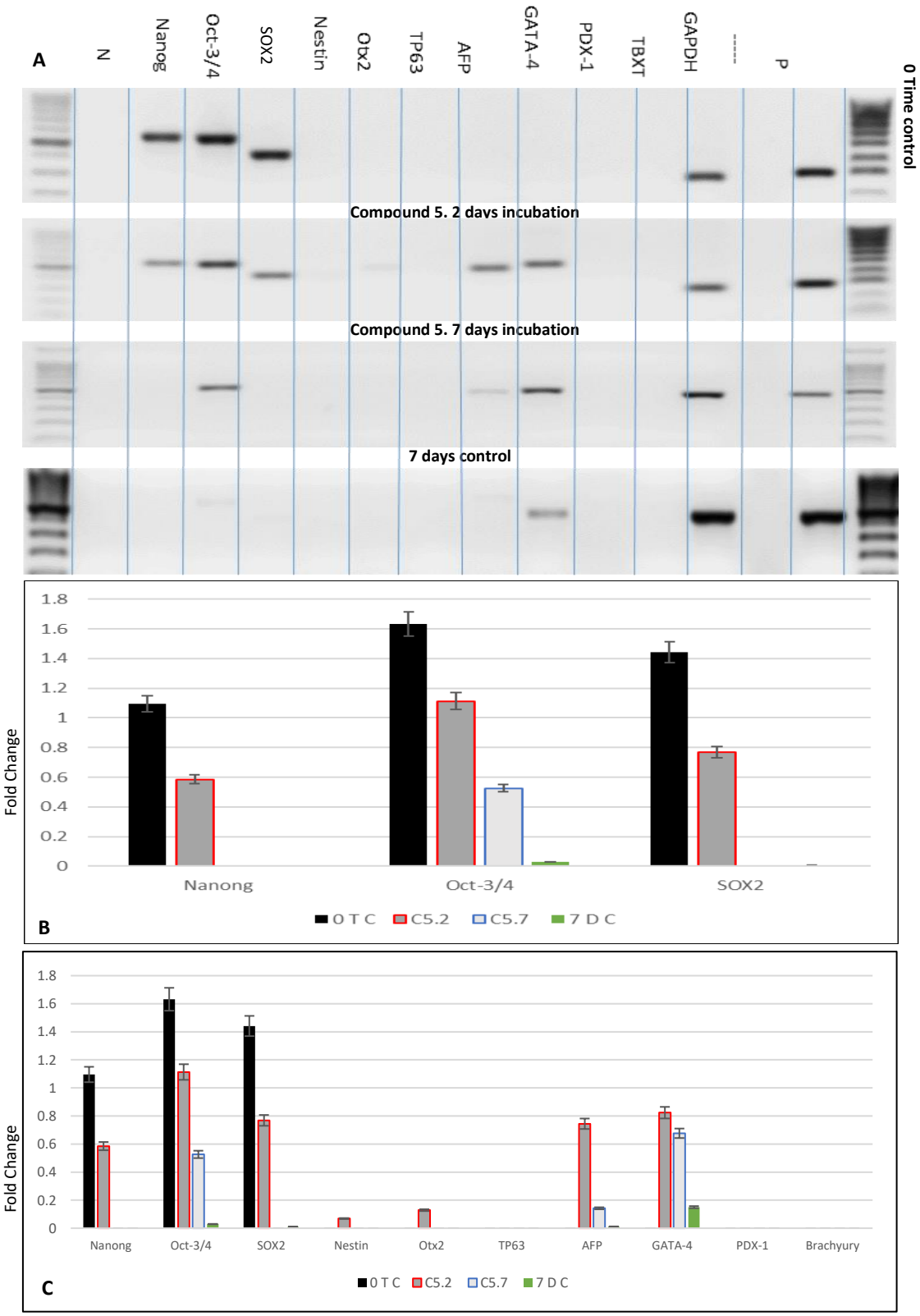


Figure 3.4. RT-PCR OF R1 embryonic stem cells, after treatment and incubation with compound 5, for 2 or 7-days, in LIF free conditions. A: The RT-PCR products were obtained from control condition samples zero-time, 2-days, and 7-days, from three replicates each, and run on a 2% agarose gel to determine their profile list. Products were recognised by their size compared to known positive control and internal control GAPDH, N: Negative control, P: Positive control**. B:** Pluripotent gene expression changes by time and compared to zero-time control pluripotent R1 stem cells and 7-days control conditions. **C:** bar chart showing all lineage markers expression, comparing all R1 stem cell culture conditions to zero-time control, and 7-days control.

At 2-day treatment, a reduction in the level of Nanog and SOX2 was notable compared to the 2-day control (see Fig 3.2). Expression of Otx2 and Nestin was absent at 2-day control compared to weak expression in the 2-day control. Expression levels of AFP and GATA-4 were elevated compared to weaker expression in the 2-day control. These results indicate that more rapid loss of pluripotency and enhanced endodermal differentiation are promoted by chemical modified heparin 5. After 7-day of treatment, all pluripotency genes were absent, except the Oct-3/4 gene remains in moderately elevated level compared to a complete absence of all pluripotency genes in the 7-day control. In contrast, both GATA4 and AFP were expressed after 7-days, compared to the only low level of GATA-4 in the 7-day control. These data indicate increased endodermal differentiation is induced by exposure of R1 ESCs to chemically modified heparin 5.

3.6.2.3 Effects of chemically modified heparin 6 (2de-S, 6S, NAc) on ESC differentiation

The effects on gene expression of addition of chemically modified heparin 6 to R1 ESCs in culture for either 2-days or 7-days was measured by RT-PCR (Fig 3.5).

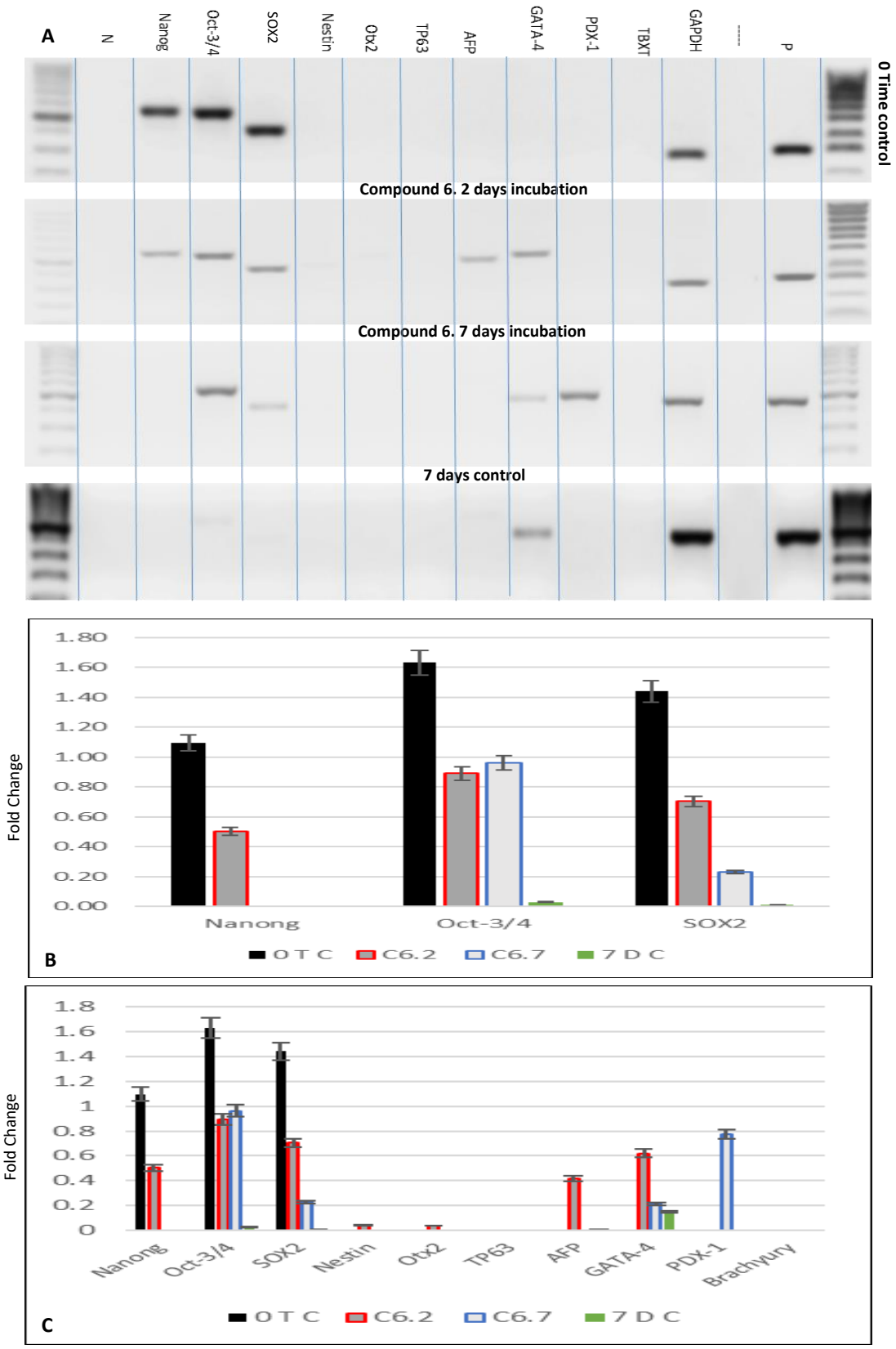


Figure 3.5. RT-PCR of R1 embryonic stem cells, after treatment and incubation with compound 6, for 2 or 7 days, in LIF free conditions. A: The RT-PCR products were obtained from control condition samples zero-time, 2-days, and 7-days, from three replicates each, and run on a 2% agarose gel to determine their profile list. Products were recognised by their size compared to known positive control and internal control GAPDH**,** N: Negative control, P: Positive control**. B:** Pluripotent gene expression changes by time and compared to zero-time control pluripotent R1 stem cells and 7-days control conditions. **C:** bar chart showing all lineage markers expression, comparing all R1 stem cell culture conditions to zero-time control, and 7-days control.

At 2-day treatment, a reduction in the level of Nanog and SOX2 was notable compared to the 2-day control (see Fig 3.2). Expression of Otx2 and Nestin was absent at 2-day control compared to weak expression in the 2-day control. Expression levels of AFP and GATA-4 were elevated compared to weaker expression in the 2-day control. These results indicate that more rapid loss of pluripotency and enhanced endodermal differentiation are promoted by chemically modified heparin 6. After 7-day of treatment, only one pluripotency gene was absent, and the other two Oct-3/4 and SOX2 gene remain in moderate levels compared to a complete absence of all pluripotency genes in the 7-day control. In contrast, both GATA4 and PDX-1 were expressed after 7-day, compared to the only low level of GATA-4 in the 7-day control. This data indicates increased endodermal differentiation is induced by exposure of R1 ESCs to chemically modified heparin 6.

3.6.2.4 Comparison between different chemically modified heparins of differential effects on ESC differentiation

Treatment for 2-days: These experiments were designed to investigate whether chemically modified heparins differ in their effects on ESC differentiation, taking due note of standard changes which occur in untreated control cells. When comparing

all the data on the compounds to the zero-time control, and the 2-day control, the results showed that the endodermal markers AFP, and GATA-4 were expressed in all the three compound treatment conditions (Fig 3.6). The ectodermal marker otx2 was also more weakly expressed after treatment with all three compounds, especially 4 and 6, compared to the control. In general, the expression of pluripotency markers Nanog, Oct3/4, and SOX2 was maintained at 2-days, even in the presence of the compounds. However, compound 4 was probably the most active in decreasing pluripotency since Nanog levels were apparently decreased (Figure 3.6). For a semi quantitative summary (see Table 3.3 for semiquantitative summary).

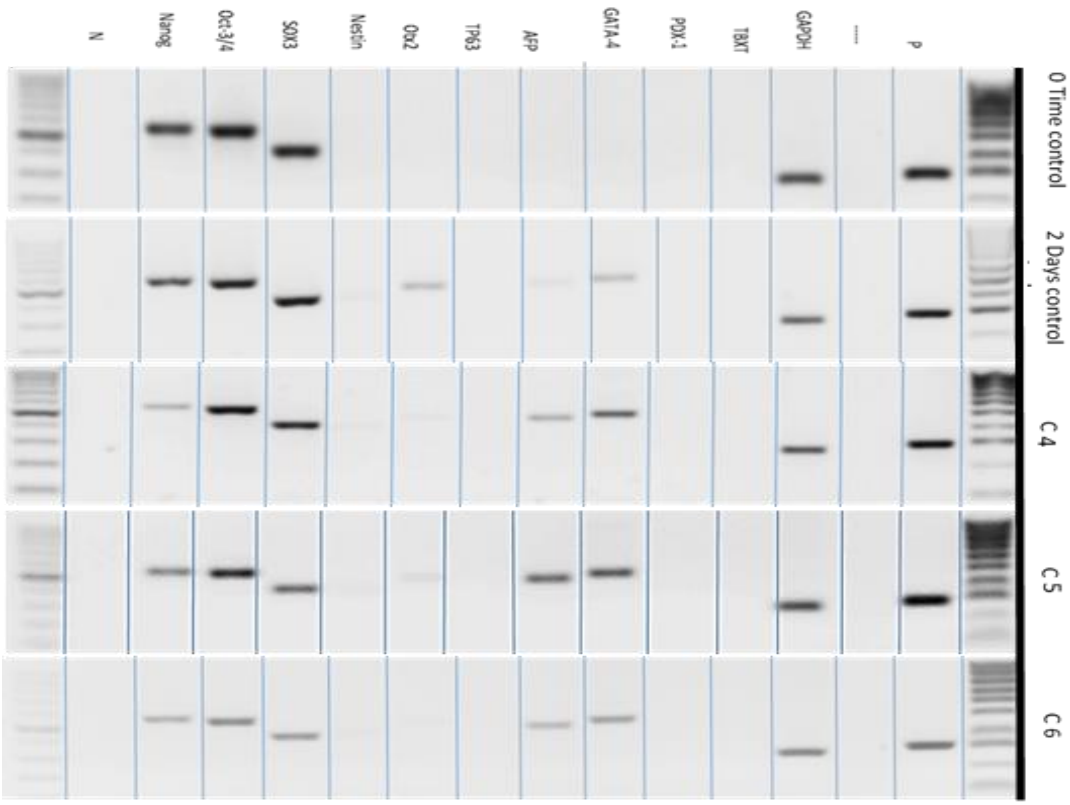


Figure 3.6: Summary of RT-PCR data for 2-day treatments of R1 ESCs with chemically modified heparins. Agarose gel electrophoresis data for PCR products was performed, allowing comparison of treatment conditions with compounds (4, 5 and 6), relative to the 2-day and zero-time controls.

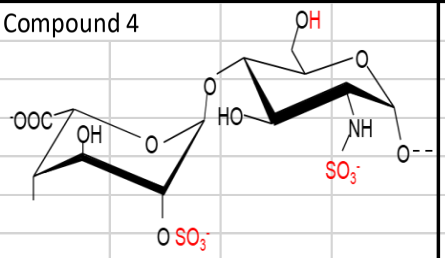
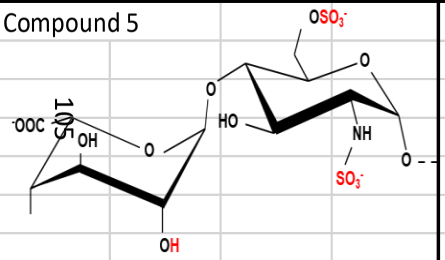
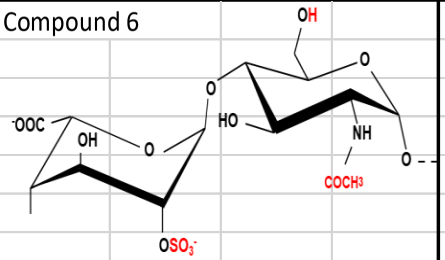
2 D: 2-Days incubation period	Undefrentiated ESC						Ectoderm						Endoderm						Mesoderm	
	Nanog		Oct-3/4		SOX2		Nestin		Otx2		TP63		AFP		GATA 4		PDX-1		TBXT	
	2 D	2 C	2 D	2 C	2 D	2 C	2 D	2 C	2 D	2 C	2 D	2 C	2 D	2 C	2 D	2 C	2 D	2 C	2 D	2 C
Compound 4 	+	+	+	+	+	+	+	+	+	+			+	+	+	+				
Compound 5 	+	+	+	+	+	+	+	+	+	+			+	+	+	+				
Compound 6 	+	+	+	+	+	+	+	+	+	+			+	+	+	+				

Table 3.3. Data collected from Figures (3.2, 3.3, 3.4, 3.5) showing overall comparisons of gene expression from R1 stem cells incubated with the compounds for 2-Days, and compared to the 2-Day control conditions. RT-PCR gel-electrophoresis data collection and analysis. N=3.

Treatment for 7-days: When comparing all 7-day treatments with the zero-time control, and 7-day control, the results (Fig 3.7) clearly showed the apparent decrease in pluripotency gene expression compared to the 2-day treatment conditions (Fig 3.6). Nanog was absent in the case of all 3 compound treatments, as observed in the 7-day control. Oct-3/4 was the only pluripotency gene that remained clearly expressed, but only with compound 5 and 6. With compound 4, Oct3/4 was lost entirely, as observed for the 7-day control. Thus compounds 4 and 5 interestingly maintained a degree of pluripotency.

We observed that the ectoderm lineage genes were completely absent at day-7 since no expression of the two ectoderm lineage markers (Nestin, Otx2) was observed in any compound treatment conditions at day-7, as also seen in the control cells (Fig 3.7). AFP, an endodermal marker, was also negative, except with compound 5 where its expression was induced compared to no expression in control cells. The other endodermal marker GATA-4 was expressed at 7-days in control cells but also all the compound treatment conditions; especially compound 5showed the strongest effect on increasing GATA-4 gene expression. Thus compound 5 seems to most strongly promote endoderm lineage differentiation. However, the PDX-1 endodermal gene was expressed at 7-days in stem cells incubated in medium containing

compounds 4 and 6, but not 5 and was absent in the 7-day control. In general, compound 4 was the only compound that after 7 days of stem cell treatment, ultimately decreased the expression of all pluripotency markers to zero and thus equivalent to the day-7 control. No expression of ectoderm genes was noted after the two-day incubation period. After 7-days of treatment with compounds, the gene expression patterns were different between the 3 compounds; the endoderm marker AFP was negative with all compounds except compound 5. GATA-4 continued to be expressed in all compound conditions, the same as the two-day treatment condition; again, compound 5 was the most active compound affecting the expression of GATA-4. (Figure 3.7 A, B). For a semi quantitative summary (see Table 3.4).

Finally, the comparison between the two incubation periods used 2-day, and 7-day, and the affect of the chemically modified heparins, show that the incubation time and the variation in the compounds structure and sulfation patterns are both significant factors that must be considered when studying the fate commitment of ESCs under the effect of any chemically modified compounds (see Table 3.5).

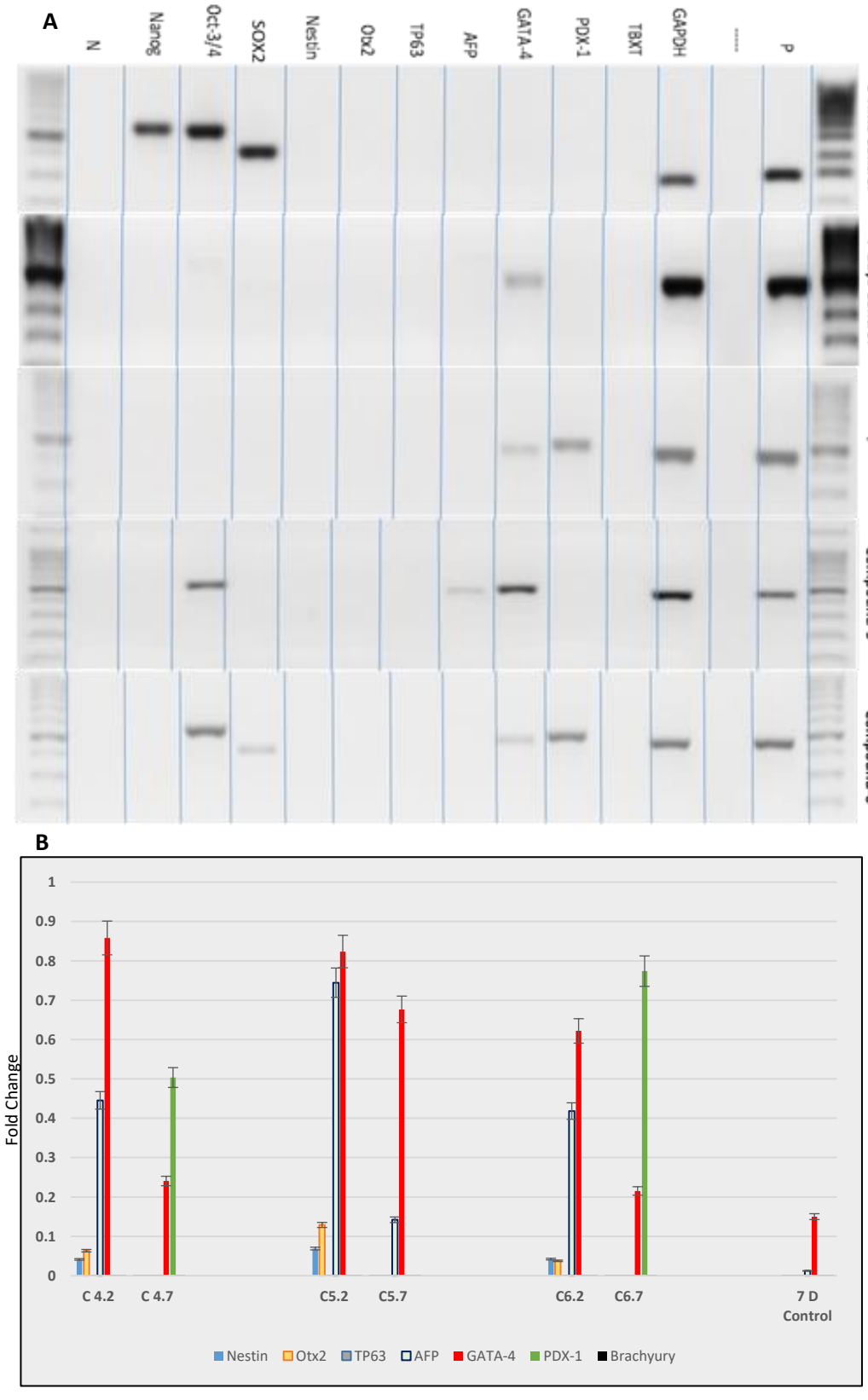


Figure 3.7. Summary of RT-PCR data for 7-day treatments of R1 ESCs with chemically modified heparins. A: Agarose gel electrophoresis data for PCR products was performed, allowing comparison of treatment conditions with compounds 4, 5 and 6, relative to the 7-day and zero-time controls. **B:** Bar chart showing the relative changes in gene expression of different lineage markers. Markers for the three embryonic germ layers were detected by RT-PCR gel-electrophoresis, in three biological replicates of R1 stem cells, maintained for 7-days with compounds 4, 5, or 6, compared to the 2- and 7-day controls.

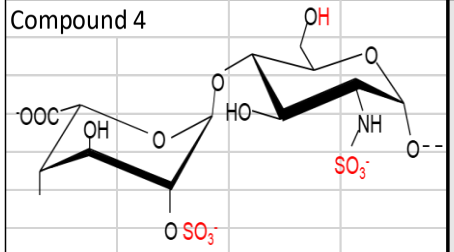
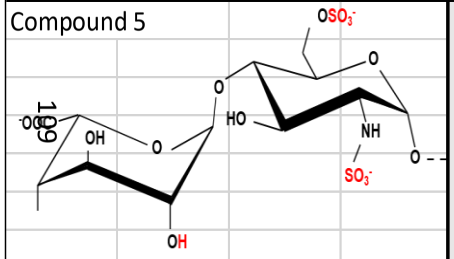
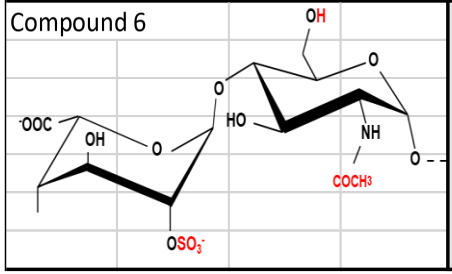
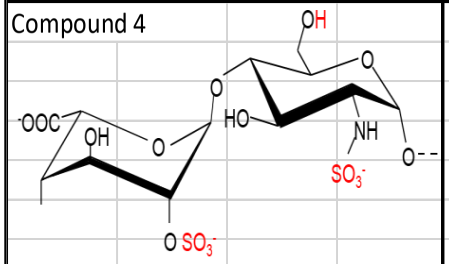
7 D: 7-Days incubation period	Undefrentiated ESC						Ectoderm						Endoderm						Mesoderm	
	Nanog		Oct-3/4		SOX2		Nestin		Otx2		TP63		AFP		GATA 4		PDX-1		TBXT	
	7 D	7 C	7 D	7 C	7 D	7 C	7 D	7 C	7 D	7 C	7 D	7 C	7 D	7 C	7 D	7 C	7 D	7 C	7 D	7 C
Compound 4 			+												+		+			
Compound 5 			+	+									+		+					
Compound 6 			+		+										+	+	+			

Table 3.4. Data collected from Figures (3.2, 3.3, 3.4, 3.5) showing overall comparisons of gene expression from R1 stem cells incubated with the compounds for 7-Days, and compared to the 7-Day control conditions. RT-PCR gel-electrophoresis data collection and analysis. N=3.

2 D: 2-Days incubation period. 7 D: 7-Dyas incubation period.	Undefrentiated ESC						Ectoderm						Endoderm						Mesoderm	
	Nanog		Oct-3/4		SOX2		Nestin		Otx2		TP63		AFP		GATA 4		PDX-1		TBXT	
	2 D	7 D	2 D	7 D	2 D	7 D	2 D	7 D	2 D	7 D	2 D	7 D	2 D	7 D	2 D	7 D	2 D	7 D	2 D	7 D
Compound 4 	+		+		+		+		+				+		+			+		
	+		+		+								+		+			+		
			+		+										+					
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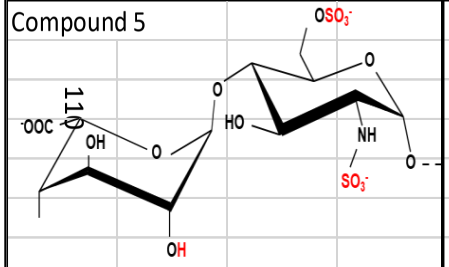
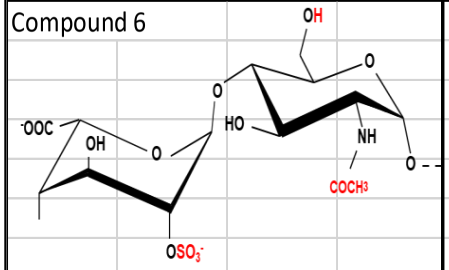
Compound 5 	+		+	+	+		+		+				+	+	+	+				
	+		+	+	+								+		+	+				
			+												+					
Compound 6 	+		+	+	+		+		+				+		+	+		+		
	+		+	+	+								+		+			+		
			+	+	+	+												+		

Table 3.5. Data collected from Figures (3.2, 3.3, 3.4, 3.5) showing over all comparisons between the DNA collected from stem cells incubated with the compounds, for (2, and 7) days, and after the RT-PCR gel-electrophoresis data collection and analysis. N=3.

Overall it was clear that the addition of soluble modified heparins to the mESC cultures could influence the timing and direction of differentiation pathways induced by withdrawal of LIF in the standard culture conditions. In addition, the effects of the compounds showed some variation from each other, confirming the original hypothesis, that different chemically modified heparins having different sulfation patterns would differentially alter cell responses and effect stem cell fate decisions.

3.6.3 Chemically modified heparins affect embryonic stem cell fate as measured by qRT-PCR.

The initial observation obtained by RT-PCR experiments using gel-electrophoresis of RT-PCR products showed the direct effect of the length of incubation, and the effect of adding different chemically modified heparin compounds, on stem cell lineage commitment (Section 3.6.1 and 3.6.2).

Experiments to further assess and confirm the effects of modified heparins on embryonic stem cell differentiation were undertaken using qRT-PCR. Over-sulfated heparin was also tested in these further experiments, along with the three compounds that were selected previously with variations of sulfation modification (4,5 and 6). Data from qPCR is quantitative data from RT-PCR, compared to only semiquantitative, and should thus give a better

understanding of the effect of the compounds on embryonic stem cell differentiation. Results were compared to the two control conditions zero-time control which is the primary undifferentiated state of the stem cells, and the 7-day control. To investigate the effects of the chemically modified heparin compounds on stem cell lineage commitment, R1 ESCs were treated with each of the four modified heparins by addition to the stem cell differentiation medium (compounds 2, 4, 5, and 6) for 7-day, followed by mRNA extraction and qPCR measurement using the Taqman array system, as described in Methods Chapter 2. Comparisons of 7-day control data vs zero-time control and 7-day treatment data vs zero-time control are described in (Appendix 1). The data below cover the key comparisons of day 7 compound treatments vs 7-day controls; all were cultured in quadruplicate to provide n=4 biological replicates.

3.6.3.1 Overview comparison of effects of compound treatments for 7-day vs 7-day control

The comparison to 7-day control will show only the effect of the compounds, removing any other possible effect of the medium or any supplements in the medium, since the 7-day control were cultured in the same conditions and medium as the other

compound treatment conditions, except that the 7-day control is free from any compounds (Figure 3.8). This overview shows that a significant number of genes are either upregulated 2-fold, or downregulated to lower than 0.5-fold. The overview also shows that the compounds differ in their effects on gene expression.

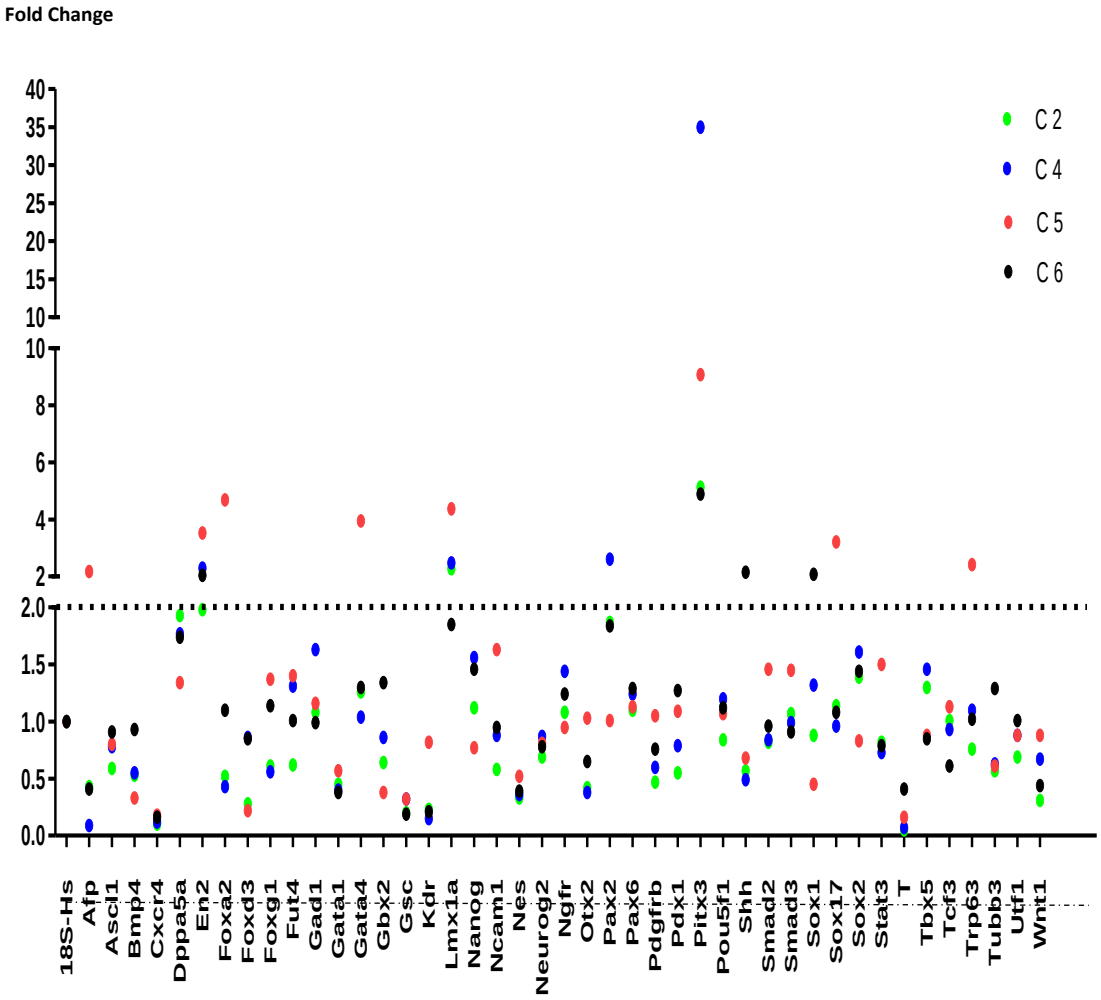


Figure 3.8. Summary of qPCR data obtained from R1 ESCs cultured with all compounds for 7-days. The dot chart shows all genes with significant downregulated or upregulated expression compared to 7-day control. After the 7-days of the incubation period, any qPCR result of the expression of any gene under or equal to a 0.5-fold change was considered as a significantly decreased result. And above 2-fold change was considered as a significant increase.

Significantly upregulated gene expression data:

After obtaining all the gene expression data, fold change analysis was performed using the 7-day control data as the biological control of the experiment. Any data of all conditions shows ≥ 2-fold change was considered as upregulated gene expression data. (Table3.3, and Figure 3.9).

	Compound 2	Compound 4	Compound 5	Compound 6
Afp	0.43	0.09	**2.17**	0.41
En2	1.98	**2.29**	**3.53**	**2.04**
Foxa2	0.52	0.43	**4.68**	1.10
Gata4	1.26	1.04	**3.95**	1.30
Lmx1a	**2.27**	**2.48**	**4.37**	1.85
Pax2	1.87	2.61	1.01	1.84
Pitx3	**5.13**	**35.00**	**9.08**	**4.89**
Shh	0.57	0.49	0.68	**2.15**
Sox1	0.88	1.32	0.45	**2.08**
Sox17	1.14	0.96	**3.21**	1.08
Tbx5	1.30	1.46	0.88	0.85
Trp63	0.76	1.10	**2.42**	1.02

Table 3.3. Summary of the qPCR data, obtained from R1 ESCs and analysed to show significantly upregulated expression of all genes compared to the day-7 control. After the 7-day of the incubation period, with the 4 modified compounds any qPCR result of the expression of any gene over or equal to a 2-fold change (red), was considered as a significantly increased

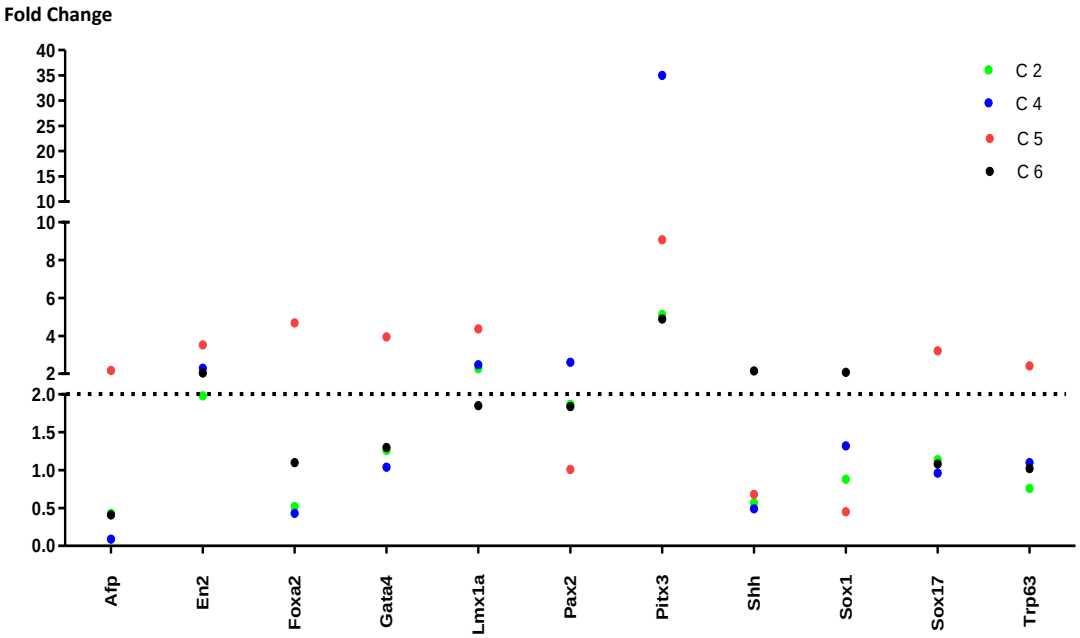


Figure 3.9: Summary of qPCR data obtained from R1 ESCs cultured with all compounds for 7-days. The dot chart shows all genes with significant upregulated expression compared to 7-day control. After the 7-days of incubation period, any qPCR result of the expression of any gene above or equal to a 2-fold change was considered as a significant increase.

After 7-days of incubation with the chemically modified heparins, ESC gene expression analysis by qPCR showed that each compound had its unique effect on upregulation of genes associated with the lineage commitment and fate of the stem cells.

Compound 5 was the most active compound by stimulating 7 different genes to a higher level of expression than all other compounds but across a broad range of image markers. In contrast, compound 2 was the least effective compound on ESCs in terms of activation of the expression of all genes compared to all other compounds. In a very strong effect on one of the most essential Ectoderm lineage genes, compound 4 had the most active and strong effect noted by the high expression of the Pitx3 gene, at 35-fold almost three times higher than the nearest compound 5. This high fold change indicates the potency of compound 4 to stimulate the ESCs towards the Ectoderm lineage.

Significant downregulated gene expression data:

After obtaining all the gene expression data, fold change analysis was performed using the 7-day control data as a biological control of the experiment. Any data of all conditions showing ≤ 0.5-Fold change was considered as downregulated gene expression (Table 3.4, and Figure 3.10).

	Compound 2	Compound 4	Compound 5	Compound 6
Afp	0.43	0.09	2.17	0.41
Bmp4	0.53	0.55	0.33	0.93
Cxcr4	0.10	0.12	0.18	0.16
Foxa2	0.52	0.43	4.68	1.10
Foxd3	0.28	0.86	0.22	0.85
Gata1	0.45	0.40	0.57	0.38
Gbx2	0.64	0.86	0.38	1.34
Gsc	0.20	0.32	0.32	0.19
Kdr	0.23	0.15	0.82	0.21
Nes	0.33	0.36	0.52	0.39
Otx2	0.42	0.38	1.03	0.65
Pdgfrb	0.47	0.60	1.05	0.76
Shh	0.57	0.49	0.68	2.15
Sox1	0.88	1.32	0.45	2.08
T	0.05	0.07	0.16	0.41
Wnt1	0.31	0.67	0.88	0.44

Table 3.4: Summary of the qPCR data, obtained from R1 ESCs and analysed to show Significantly downregulated expression of all genes compared to the 7-day control. After the 7-day of the incubation period, with the 4 modified compounds, any qPCR result of the expression of any gene under or equal to a 0.5-fold change, was considered as a significantly decreased result.

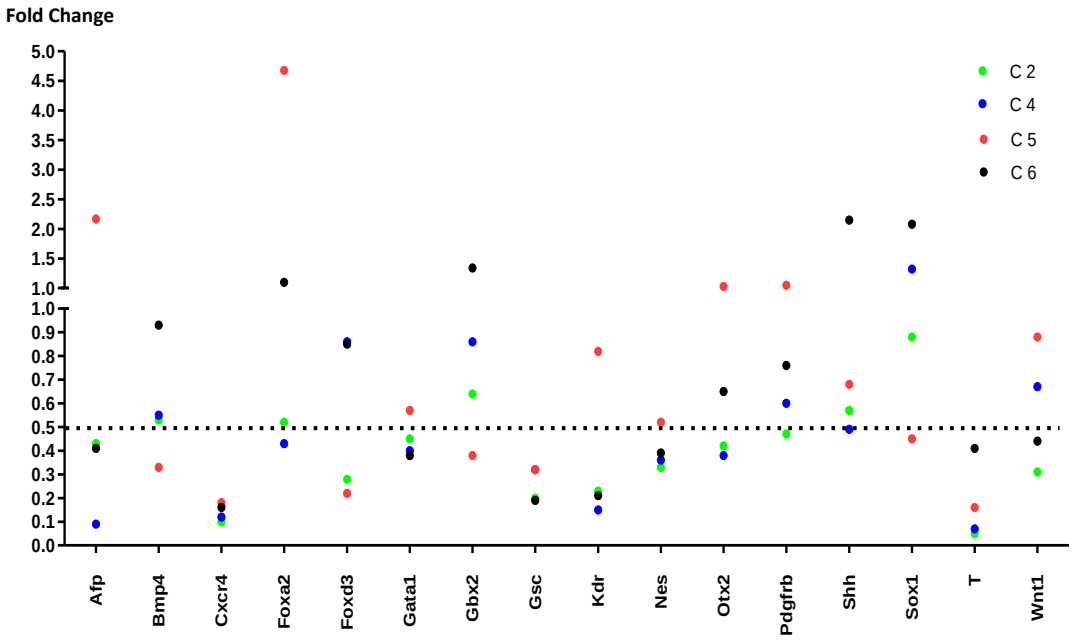


Figure 3.10. Summary of qPCR data obtained from R1 ESCs cultured with all compounds for 7-days. The dot chart shows fold change data analysis, showing 16 significantly downregulated lineage gene markers, based on the comparison to the 7-days Control. All results were analysed and compared to the 7-day control, and any result exceeded or equalled to a 0.5-Fold Change was considered as a significantly decreased.

After 7-days of incubation with the chemically modified heparins, ESC gene expression analysis by qPCR showed that each compound had its unique effect on downregulation of genes associated with the lineage commitment and fate of the stem cells.

Compound 5 remained the most active chemically modified compound, not only because it was the most effective compound for upregulating genes, but because it was the least effective compound on ESCs to downregulate genes compared to all other compounds. Compound 2 and 4 had almost similar effects on downregulation of the same number of genes, indicating that compound 2 and 4 share similar properties. Compound 6 had a moderate affecting gene less than compound 4, but was more active than compounds 2 and 4.

In general, and after data analysis based on the 7-day control condition, the results obtained showed that chemically modified heparins had differential effects on the fate of stem cells after LIF withdrawal, observed by significant up- and down-regulation of specific lineage genes.

As mentioned in the introduction, the 7-day control are stem cells that are cultured for 7-days, incubated and maintained otherwise in the same culture conditions.

These general comparisons, focused on the overall behaviour of the chemically modified heparins, confirmed the original hypothesis, that different chemical modifications of heparin (and their different sulfation patterns) would differentially alter cell responses and effect stem cell fate decisions.

Specific and sensitive results obtained by qPCR and analysed as a fold change data, based on the gene expression of the 7-days control were important to show the direct effect of each chemically modified heparin. In the next section of data analysis, data will show the individual effect of each chemically modified heparin on stem cell fate commitment, in terms of increased and decreased expression effects on relevent lineage genes.

3.6.3.2 Individual chemically modified heparins differ in their effects on stem cell fate commitment

In this section, the results of gene expression for 7-day treatment of R1 ESCs are described for each individual chemically modified heparin, compared to the 7-day control. The analysis is summarised in terms of the effects on either pluripotency genes, or genes involved in specific lineage commitments (ectodermal, endodermal or mesodermal).

3.6.3.2.1 Over-Sulfated heparin (Compound 2)

Pluripotency: the effect was not significant; there were no upregulated or downregulated pluripotency genes. Ectodermal markers: per-Sulfated heparin resulted in downregulation of 4 genes, namely Foxd3, Nes, Otx2, and Wnt1; but in contrast also upregulated 2 genes Lmx1a, Pitx3. Endodermal markers: 3 genes were significantly downregulated, namely Afp, Cxcr4 and Gsc, and there were no upregulated endoderm genes. Mesodermal markers: 4 downregulated mesodermal genes were observed, namely GATA1, Kdr, Pdgfrb and T. There were no upregulated mesodermal genes.Overall it appeared that a 7-day treatment with compound 2 compared to the 7-day control did not affect loss of pluripotency but resulted largely in suppression of ectodermal and mesodermal differentiation, but promoted endodermal differentiation. Data are summarised in Table 3.5 and Figure 3.11.

Downregulated	≤ 0.5	Upregulated	≥ 2
Pluripotent	0	Pluripotent	0
Ectoderm	4	Ectoderm	2
Foxd3	0.28	Lmx1a	2.27
Nes	0.33	Pitx3	5.13
Otx2	0.42	Endoderm	0
Wnt1	0.31	Mesoderm	0
Endoderm	3		
Afp	0.43		
Cxcr4	0.1		
Gsc	0.2		
Mesoderm	4		
Gata1	0.45		
Kdr	0.23		
Pdgfrb	0.47		
T	0.05		

Table 3.5. Summary of qPCR product obtained from R1 ESCs cultured with compound 2 for 7-days. The table shows fold change data analysis, showing all genes with significant downregulated or upregulated expression with over-sulfated heparin compared to 7-dayscontrol. After the 7-days of the incubation period, any qPCR result of the expression of any gene under or equal to a 0.5-fold change, was considered as a significantly decreased result. And above or equal to a 2-fold change was considered as a significant increase.

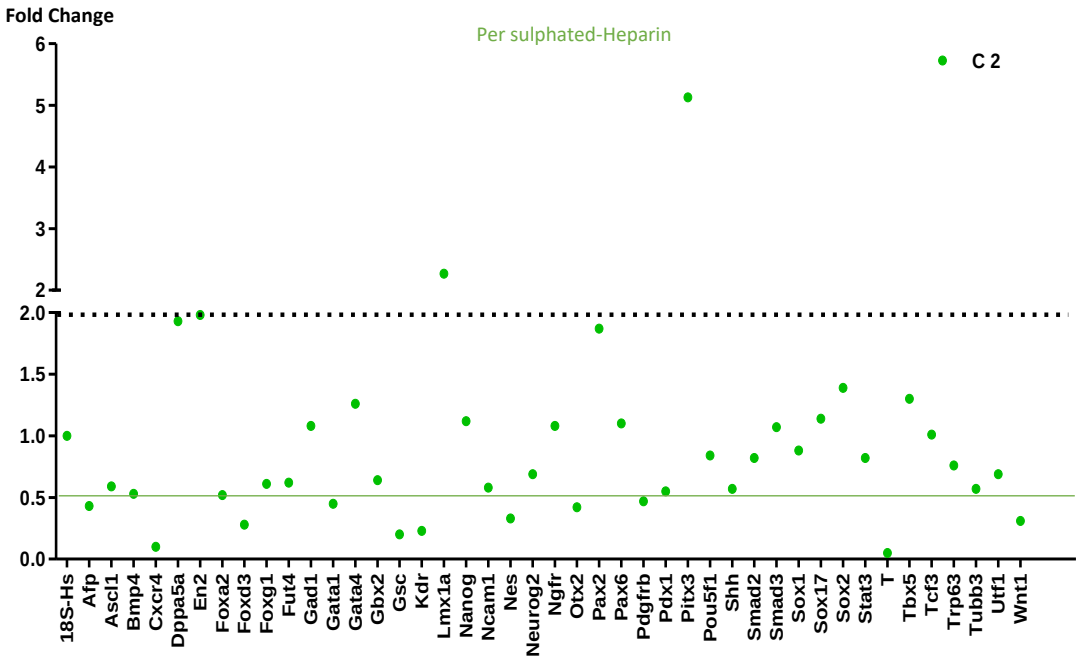


Fig 3.11. Summary of qPCR product obtained from R1 ESCs cultured with compound 2 for 7-days. The dot chart Shows fold change data analysis, showing all genes with significant downregulated or upregulated expression with over-sulfated heparin compared to 7-day control. After the 7-days of the incubation period, any qPCR result of the expression of any gene under or equal to a 0.5-fold change, was considered as a significantly decreased result. And above or equal to a 2-fold change was considered as a significant increase.

3.6.3.2.2 2de-Sulfated-N-Sulfated-Heparin (Compound 4)

Pluripotency: only one pluripotent gene was downregulated (Shh). The expression level by fold change was 0.49-fold change, just on the arbitrary border line of a 0.5-fold change. Ectodermal markers: Only two downregulated genes (Nes, Otx2) were detected, and there were 4 upregulated genes (En2, Lmx1a, Pax2, Pitx3), Pitx3 had very high gene expression 35-fold change. Endodermal markers: 4 ectoderm lineage markers were detected and analysed to be significantly downregulated (Afp, Cxcr4, Foxa2, Gsc), with no upregulated genes. Mesodermal markers: 3 downregulated mesoderm genes (GATA1, Kdr, T) were obsarved, and there were no upregulated mesoderm genes detected.

Overall it appeared that a 7-day treatment with compound 4 compared to the day-7 control resulted in only minor reduction in one pluripotency gene, supressed both mesodermal and endodermal genes, but enhanced ectodermal gene expression. Data are summarised in (Table 3.6 and Figure 3.12).

Downregulated	≤ 0.5	Upregulated	≥ 2
Pluripotent	**1**	**Pluripotent**	**0**
Shh	0.49	**Ectoderm**	**4**
Ectoderm	**2**	En2	2.29
Nes	0.36	Lmx1a	2.48
Otx2	0.38	Pax2	2.61
Endoderm	**4**	Pitx3	35
Afp	0.09	**Endoderm**	**0**
Cxcr4	0.12	**Mesoderm**	**0**
Foxa2	0.43		
Gsc	0.32		
Mesoderm	**1**		
Gata1	0.4		
Kdr	0.15		
T	0.07		

Table 3.6 Summary of qPCR product obtained from R1 ESCs cultured with compound 4 for 7-days. The table shows fold change data analysis, showing all genes with significant downregulated or upregulated expression with compound 4 compared to 7-day control. After the 7-days of the incubation period, any qPCR result of the expression of any gene under or equal to a 0.5-fold change, was considered as a significantly decreased result. And above or equal to a 2-fold change was considered as a significant increase.

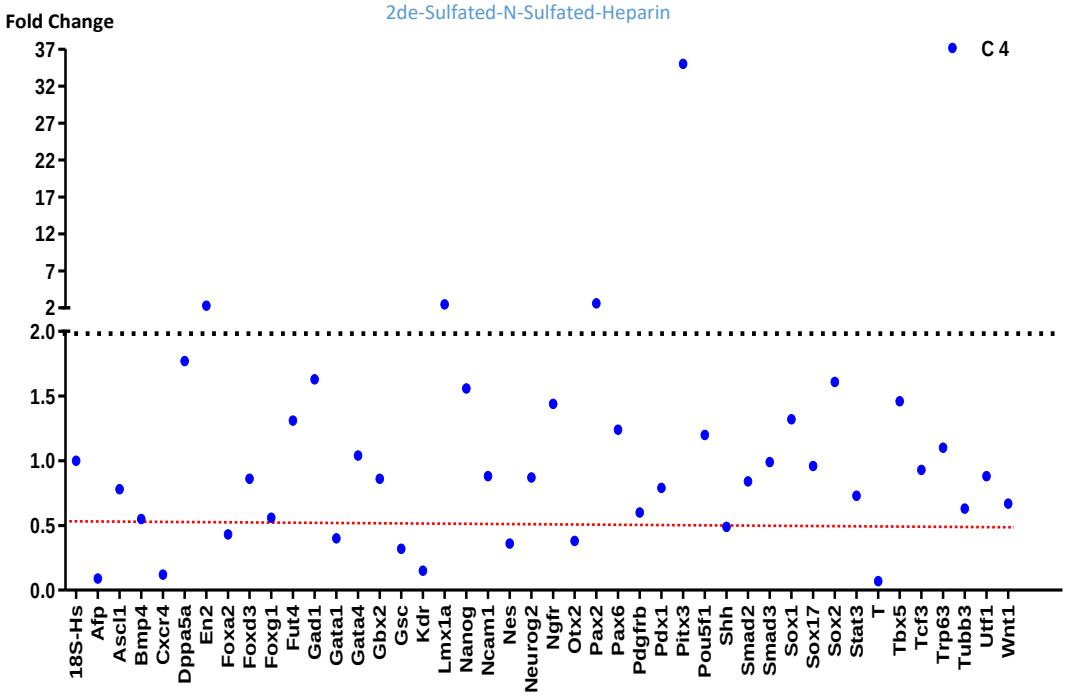


Figure 3.12. Summary of qPCR data obtained from R1 ESCs cultured with compound 4 for 7-days. The dot chart shows fold change data analysis, showing all genes with significant downregulated or upregulated expression with compound 4 compared to 7-days control. After the 7-days of the incubation period, any qPCR result of the expression of any gene under or equal to a 0.5-fold change was considered as a significantly decreased result. And above or equal to a 2-fold change was considered as a significant increase.

3.6.3.2.3 6de-Sulfated-N-Sulfated-Heparin Compound 5

Pluripotency: no significant changes in gene expression. Ectodermal markers: 4 genes were downregulated (Bmp4, Foxd3, Gbx2, Sox1), and there were 3 significantly upregulated genes (En2, Lmx1a, Pitx3), with compound 5. Pitx had very high fold increase in expression (9.8-fold change). Endodermal markers: endoderm lineage gene expression was differentially affected by compound 5, and was the only compound with the minimum number of downregulated genes. It was also the only compound that significantly upregulated 5 endoderm genes, different than all other compounds. The significantly downregulated genes were Cxcr4 and Gsc, and significantly increased genes were Afp, Foxa2, GATA4, Sox17, and Trp63. Mesoderm markers: uniquely compared to all other compounds, compound 5 treated stem cells, showed downregulation of only one mesoderm lineage gene, and no upregulated genes were apparent.

Overall it appeared that a 7-day treatment with compound 5 compared to the day-7 control resulted in no significant effect on pluripotency genes, and largely enhanced endodermal gene expression. Only a single mesodermal gene was reduced, and mixed effects on ectodermal gene expression were observed. Data are summarised in (Table 3.9 and Figure 3.12).

Downregulated	≤ 0.5	Upregulated	≥ 2
Pluripotent	**0**	**Pluripotent**	**0**
Ectoderm	**4**	**Ectoderm**	**3**
Bmp4	0.33	En2	3.53
Foxd3	0.22	Lmx1a	4.37
Gbx2	0.38	Pitx3	9.08
Sox1	0.45	**Endoderm**	**5**
Endoderm	**2**	Afp	2.17
Cxcr4	0.18	Foxa2	4.68
Gsc	0.32	Gata4	3.95
Mesoderm	**1**	Sox17	3.21
T	0.16	Trp63	2.42
		Mesoderm	**0**

Table 3.7. Summary of qPCR data obtained from R1 ESCs cultured with compound 5 for 7-days, the table shows fold change data analysis. This shows all genes with significant downregulated or upregulated expression with compound 5 compared to 7-day control. After the 7-days of the incubation period, any qPCR result of the expression of any gene under or equal to a 0.5-fold change, was considered as a significantly decreased result. And above or equal to a 2-fold change was considered as a significant increase.

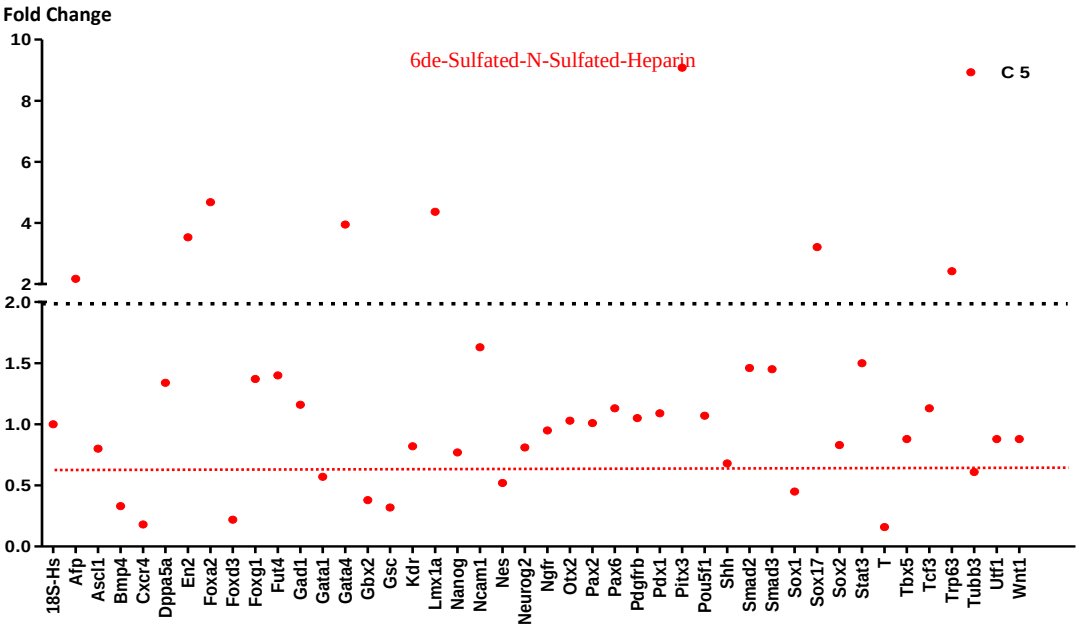


Figure 3.13. Summary of qPCR data obtained from R1 ESCs cultured with compound 5 for 7-days. The dot chart shows fold change data analysis, showing all genes with significant downregulated or upregulated expression with compound 5 compared to 7-day control. After the 7-days of the incubation period, any qPCR result of the expression of any gene under or equal to 0.5-fold change, was considered as a significantly decreased result. And above or equal to a 2-fold change was considered as a significant increase.

3.6.3.2.4 2de-Sulfated-N-Acetylated Heparin Compound 6

Pluripotency: all compounds except compound 6 failed to upregulate any of the pluripotent lineage marker genes, whereas compound 6 was the only compound that affected the stem cell after 7-day treatment to upregulate the pluripotency gene Shh. Shh marker was significantly downregulated with compound 4 treatment. Ectodermal markers: 2 genes were downregulated (Nes, Wnt), and 3 ectodermal markers were significantly upregulated (En2, Sox1, Pitx3). Endodermal markers: 3 significantly downregulated genes were noted (Afp, Cxcr4, Gsc), and there were no upregulated genes with compound 6. Mesodermal markers: 3 significantly downregulated genes were noted (GATA1, Kdr, T), and no significantly upregulated genes were observed. Overall it appeared that a 7-day treatment with compound 6 compared to the 7-day control resulted in increased expression of pluripotency genes, and supressed both mesodermal and endodermal genes. A mixed effect on ectodermal gene expression was noted. Data are summarised in Table 3.8 and Figure 3.14.

Downregulated	≤ 0.5	Upregulated	≥ 2
Pluripotent	**0**	**Pluripotent**	**1**
Ectoderm	**2**	Shh	2.15
Nes	0.39	**Ectoderm**	**2**
Wnt1	0.44	En2	2.04
Endoderm	**3**	Sox1	2.08
Afp	0.41	Pitx3	4.89
Cxcr4	0.16	**Endoderm**	**0**
Gsc	0.19	**Mesoderm**	**0**
Mesoderm	**3**		
Gata1	0.38		
Kdr	0.21		
T	0.41		

Table 3.8. Summary of qPCR product obtained from R1 ESCs cultured with compound 5 for 7-days, the table is Showing fold change data analysis. The table shows all genes with significant downregulated or upregulated expression with compound 5 compared to 7-days control. After the 7-days of the incubation period, any qPCR result of the expression of any gene under or equal 0.5-fold change, was considered as significantly decreased result. And above or equal 2-fold change was considered as a significant increase.

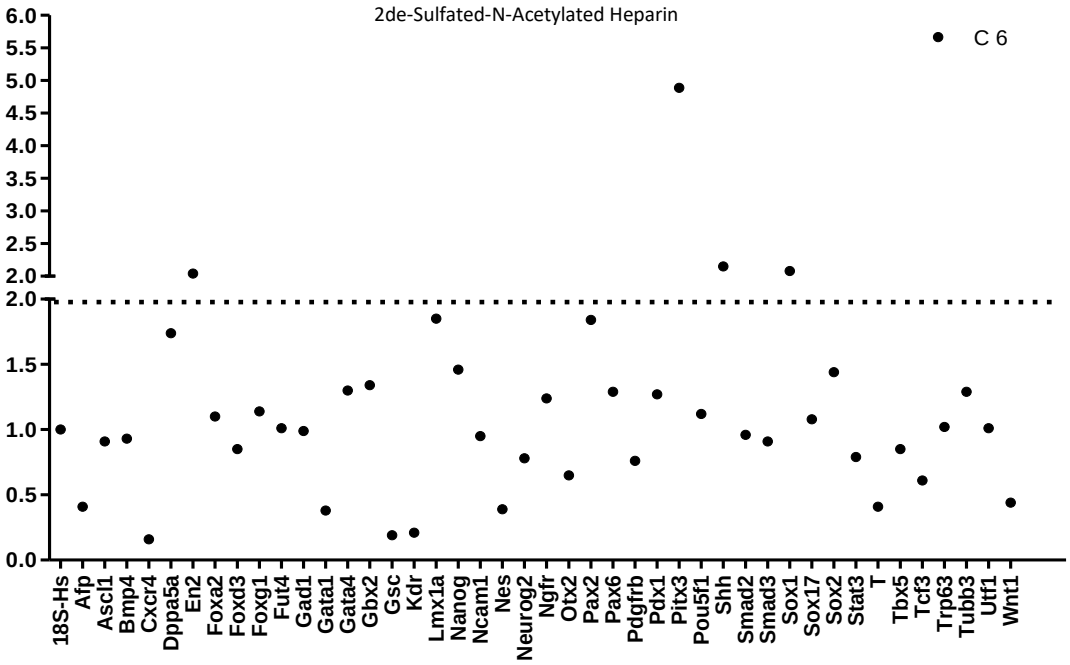


Figure 3.14. Summary of qPCR data obtained from R1 ESCs cultured with compound 6 for 7-days. The dot chart shows fold change data analysis, showing all genes with significant downregulated or upregulated expression with compound 6 compared to 7-day control. After the 7-days of the incubation period, any qPCR result of the expression of any gene under or equal to a 0.5-fold change, was considered as a significantly decreased result. And above or equal to a 2-fold change was considered as a significant increase.

3.6.3.3 Similarities in the effect of chemically modified heparins on stem cell gene expression.

Here are the results of gene expression for 7-day treatment of R1 ESCs are described in the context of similarities of effects. It is noteworthy to look at similar effects of all used compounds on the embryonic stem cell fate at the end of the 7-day incubation period. The qPCR data analysis, compared to the 7-day control, showed the similarity of effect in increasing three genes representing the Ectoderm lineage, and decreasing three genes representing two germ layer mesodermal, and endodermal lineages. Data are summarised in (Table 3.9 and Figure 3.15).

A	C 2	C 4	C 5	C 6
Cxcr4	0.10	0.12	0.18	0.16
Gsc	0.20	0.32	0.32	0.19
T	0.05	0.07	0.16	0.41

B	C 2	C 4	C 5	C 6
En2	1.98	2.29	3.53	2.04
Lmx1a	2.27	2.48	4.37	1.85
Pitx3	5.13	35.00	9.08	4.89

Table 3.9: Summary of the similar effects of modified heparins on R1 stem cell lineage commitment markers measured by qPCR compared to 7-day control. This table summarises the effects of compounds 2, 4, 5 and 6 which were similar at the day-7 time point. **A**, significant decreases under or equal to a 0.5-fold change (green) in specific lineage genes. **B**, significant increases above or equal to a 2-fold change (red) in specific lineages.

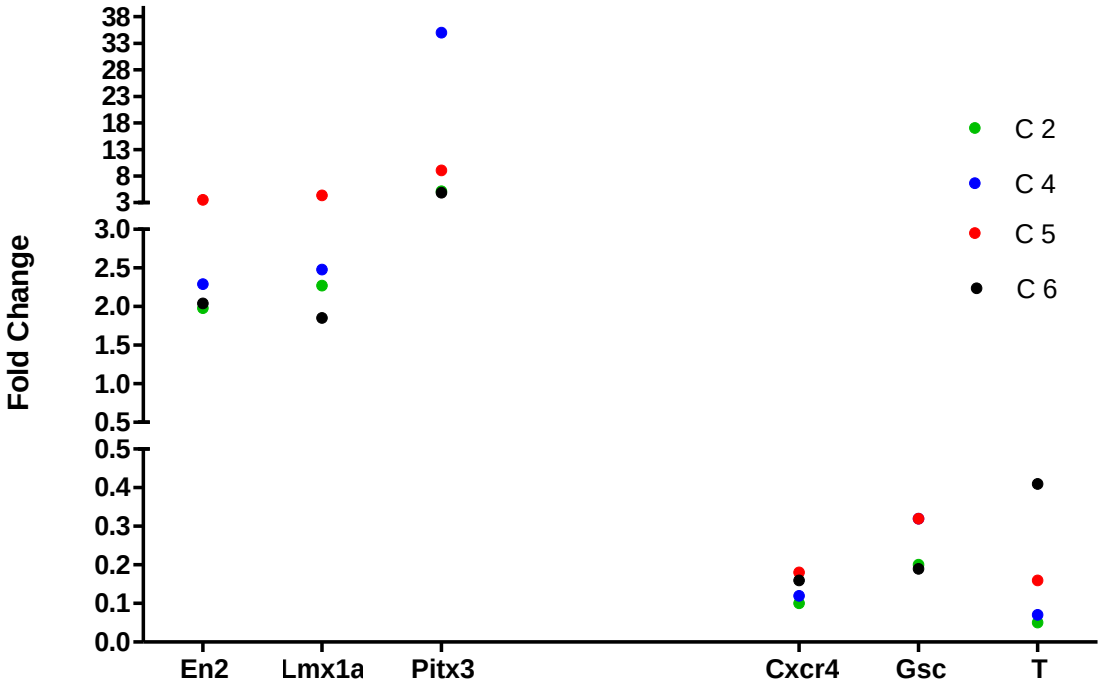


Figure 3.15. Summary of the similar effects of all compounds on R1 ESCs after 7-days of incubation. The dot chart shows fold change data analysis, showing all genes with significant downregulated or upregulated expression. Any qPCR result of the expression of any gene under or equal to a 0.5-fold change was considered as significantly decreased result. And above or equal to a 2-fold change was considered as a significant increase.

The similarity in the effect of chemically modified heparins used in the research on ESCs fate and lineage commitment is an important consideration. The selected compounds that have been used as a treatment added to the ESCs for 7-days of incubation were each shown to have significant and unique effects as shown previously (in section 3.6.3.2).

After further analysis and comparisons performed on all compound effects on all genes indicating different lineages, it is evident and significant as a general view, that all selected chemically modified compounds are in general ectoderm lineage stimulators, but with variations in the strength of the effect.

On the other hand, all chemically modified heparins did not show any significant mesodermal lineage gene activation, but rather, all the compounds downregulated all mesodermal genes, and they all acted similarly on significantly downregulating the T mesodermal gene.

The only compound that stimulated endodermal markers individually was compound 5; compound 5 stimulated significantly 5 endodermal genes that are important in the ESCs lineage commitment decisions. When analysing data to determine the similar activity of the chemically modified heparins, we can see that all compounds significantly downregulated two endodermal markers, Cxcr4 and Gsc.

3.6.3.4 Comparisons between the effects of chemically modified heparins on ESC differentiation from the perspective of different germ lines ectoderm, endoderm, mesoderm and pluripotency genes.

3.6.3.4.1 Pluripotency genes:

In general, all compounds after 7-days of incubation, and after qPCR gene expression analysis, showed that stem cells are gradually exiting the pluripotent state to any chosen lineage commitment.

We confirmed in all previous results in this chapter that the different chemically modified compounds will cause a different effect on stem cell behaviour. This was also the case when evaluating the effect on pluripotent markers. All compounds had a similar effect on downregulating pluripotent genes. The exception was compound 6 which showed less potency and less rapid effect on the pluripotency genes than all other compounds. This was clear in the gene expression of the shh pluripotency gene, showing upregulated expression even after 7-days of incubation. Data are summarised in Table 3.10 and Figure 3.16.

	C2	C4	C5	C6
Dppa5a	1.93	1.77	1.34	1.74
Nanog	1.12	1.56	0.77	1.46
Pou5f1	0.84	1.20	1.07	1.12
Shh	0.57	0.49	0.68	**2.15**
Sox2	1.39	1.61	0.83	1.44
Stat3	0.82	0.73	1.50	0.79
Tcf3	1.01	0.93	1.13	0.61
Utf1	0.69	0.88	0.88	1.01

Table 3.10. Summary of the effects of modified heparins on R1 stem cell lineage commitment on pluripotency genes measured by qPCR compared to 7-day control. This table summarises the effects of all compounds (2, 4, 5 and 6) on the expression of pluripotency genes at the 7-day time point after the treatment with each compound for 7-days.

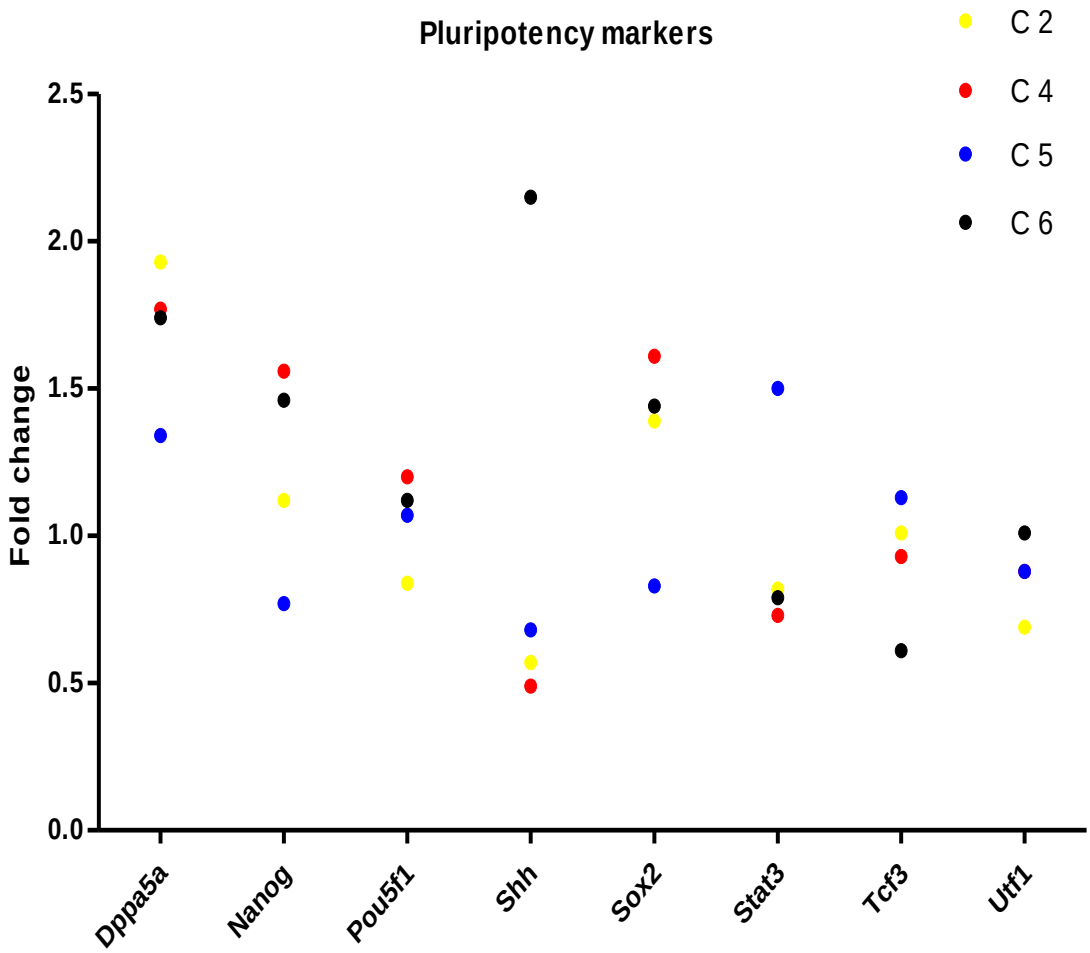


Figure 3.16: Summary of qPCR data obtained from R1 ESCs cultured with all compounds for 7-days shows pluripotency genes. The dot chart shows fold change data analysis, showing all genes with significant downregulation or upregulation expression with all compounds compared to 7-day control. Any qPCR result of the expression of any gene under or equal to a 0.5-fold change, was considered as a significantly decreased result. And above or equal to a 2-fold change was considered as a significant increase.

3.6.3.4.2 Endodermal markers

In a general view of gene expression after the 7-days incubation, and under the effect of compound treatments, all compounds had a similar effect on the stem cell toward endodermal markers, except an apparent different effect of genes analysed from cells treated with compound 5.

Lineage-specific gene expression of the endodermal markers Afp, Foxa2, Gata4, Sox17, Trp63 were all upregulated only in compound 5 treated stem cells. Data are summarised in Table 3.11 and Figure 3.17.

	C2	C4	C5	C6
Afp	0.43	0.09	2.17	0.41
Cxcr4	0.10	0.12	0.18	0.16
Foxa2	0.52	0.43	4.68	1.10
Gata4	1.26	1.04	3.95	1.30
Gsc	0.20	0.32	0.32	0.19
Pdx1	0.55	0.79	1.09	1.27
Smad2	0.82	0.84	1.46	0.96
Smad3	1.07	0.99	1.45	0.91
Sox17	1.14	0.96	3.21	1.08
Trp63	0.76	1.10	2.42	1.02

Table 3.11. Summary of the effects of modified heparins on R1 stem cell lineage commitment on endodermal lineage markers measured by qPCR compared to 7-day control. This table summarises the effects of all compounds (2, 4, 5 and 6) on the expression of endodermal markers at the 7-day time point after the treatment with each compound for 7-days. 2-fold change was considered as a significant increase (red), and 0.5-fold change was considered as a significantly decreased result (green)

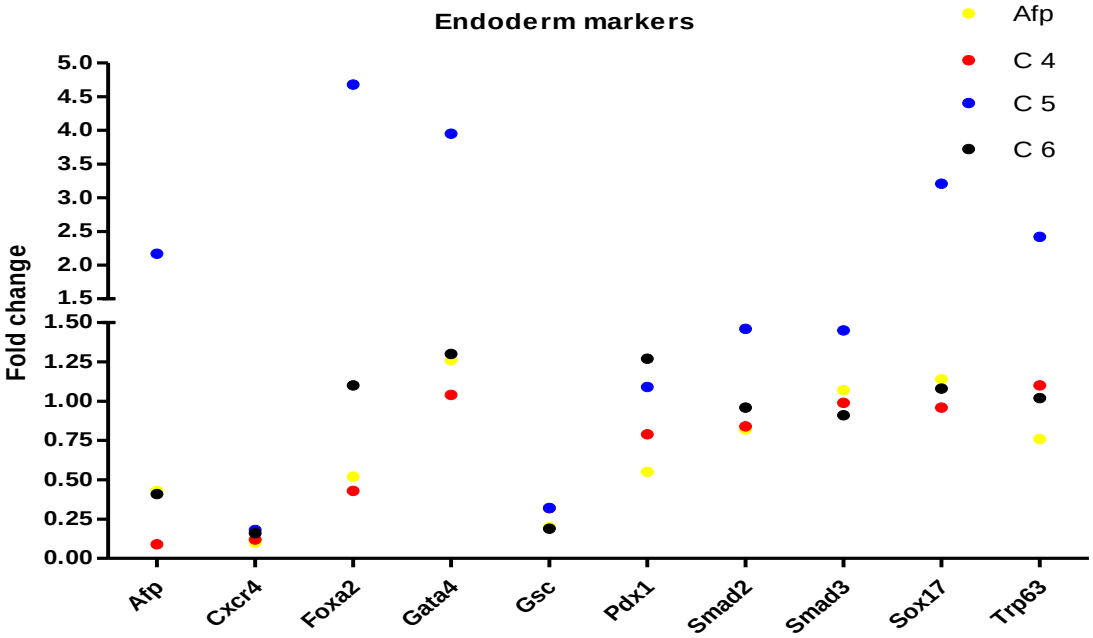


Figure 3.17: Summary of qPCR data obtained from R1 ESCs cultured with all compounds for 7-days. The dot chart shows fold change data analysis for ectodermal markers, showing all genes with significant downregulation or upregulation expression with all compounds compared to 7-day control. Any qPCR result of the expression of any gene under or equal to a 0.5-fold change, was considered as a significantly decreased result. And above or equal to a 2-fold change was considered as a significant increase.

3.6.3.4.3 Mesodermal markers

Mesoderm lineage markers gene expression after qPCR gene analysis again showed similar action of all compounds with the exception of compound 5. All compounds in general downregulated mesoderm lineage markers, whereas compound 5 demonstrated the least downregulation effect compared to all other compounds after 7-days of incubation. Data are summarised in Table 3.15 and Figure 3.18.

	C2	C4	C5	C6
Gata1	0.45	0.40	0.57	0.38
Kdr	0.23	0.15	0.82	0.21
Pdgfrb	0.47	0.60	1.05	0.76
T	0.05	0.07	0.16	0.41
Tbx5	1.30	1.46	0.88	0.85

Table 3.12. Summary of the effects of modified heparins on R1 stem cell lineage commitment on mesodermal lineage markers measured by qPCR compared to 7-day control. This table summarises the effects of all compounds (2, 4, 5 and 6) on the expression of mesodermal markers at the 7-day time point after the treatment with each compound for 7-days. 0.5-fold change was considered as a significantly decreased result (green)

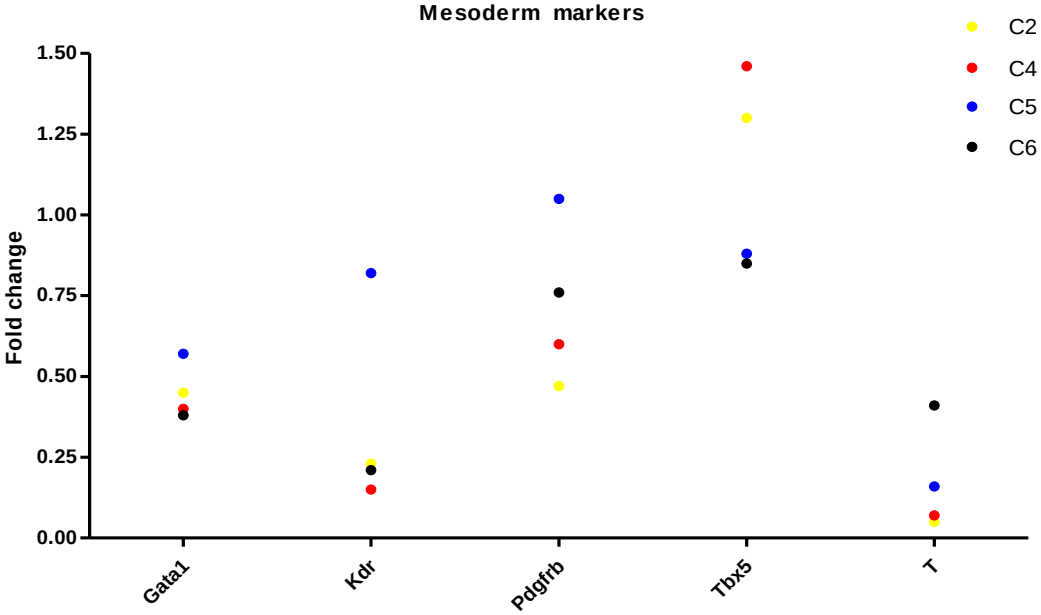


Figure 3.18: **Summary of qPCR data obtained from R1 ESCs cultured with all compounds for 7-days**. The dot chart shows fold change data analysis for mesodermal lineage markers, showing all genes with significant downregulation or upregulation expression with each compound compared to 7-day control. Any qPCR result of the expression of any gene under or equal to a 0.5-fold change, was considered as a significantly decreased result. And above or equal to a 2-fold change was considered as a significant increase.

3.6.3.4.4 Ectodermal markers

In general, all compounds had a similar effect on the stem cell behaviour toward the Ectoderm lineage. Three ectodermal markers were upregulated in all stem cells incubated with all compounds; the three genes were En2, Lmx1a, and Pitx3. Data are summarised in Table 3.16 and Figure 3.19.

Ectoderm	C2	C4	C5	C6
Ascl1	0.59	0.78	0.80	0.91
Bmp4	0.53	0.55	0.33	0.93
En2	1.98	2.29	3.53	2.04
Foxd3	0.28	0.86	0.22	0.85
Foxg1	0.61	0.56	1.37	1.14
Gbx2	0.64	0.86	0.38	1.34
Lmx1a	2.27	2.48	4.37	185
Ncam1	0.58	0.88	1.63	0.95
Nes	0.33	0.36	0.52	0.39
Neurog2	0.69	0.87	0.81	0.78
Ngfr	1.08	1.44	0.95	1.24
Otx2	0.42	0.38	1.03	0.65
Pax2	1.87	2.61	1.01	1.84
Pax6	1.10	1.24	1.13	1.29
Pitx3	5.13	35.00	9.08	4.89
Sox1	0.88	1.32	0.45	2.08
Tubb3	0.57	0.63	0.61	1.29
Wnt1	0.31	0.67	0.88	0.44

Table 3.13 Summary of the effects of modified heparins on R1 stem cell lineage commitment on ectodermal lineage markers measured by qPCR compared to 7-day control. This table summarises the effects of all compounds (2, 4, 5 and 6) on the expression of ectodermal markers at the 7-day time point after the treatment with each compound for 7-days. 2-fold change was considered as a significant increase (red), and 0.5-fold change was considered as a significantly decreased result (green)

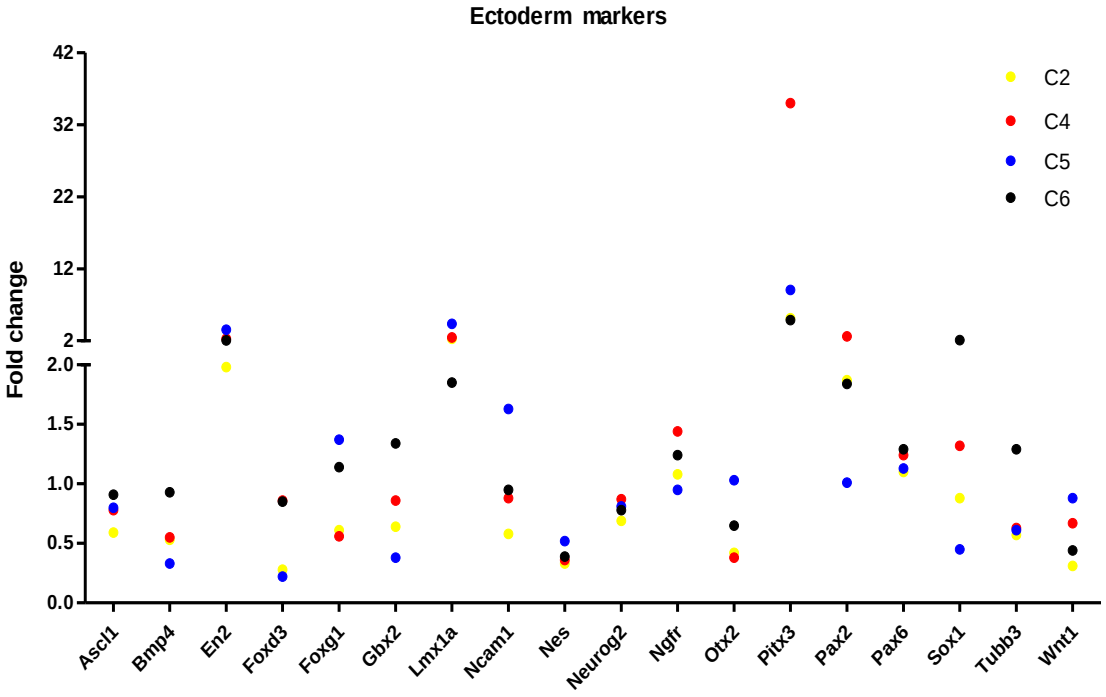


Figure 3.19: Summary of qPCR data obtained from R1 ESCs cultured with all compounds for 7-days. The dot chart shows fold change data analysis for ectodermal markers, showing all genes with significant downregulation or upregulation expression with all compounds compared to 7-day control. Any qPCR result of the expression of any gene under or equal to a 0.5-fold change, was considered as a significantly decreased result. And above or equal to a 2-fold change was considered as a significant increase.

Finally, an overall comparison between all compounds showed that all compounds had different effects on the lineage fate decisions of ESCs after 7-days of incubation in a LIF and feeder free condition. All compounds had a similar general effect on the ESC differentiation to the ectoderm lineage, with different potency depending on the specific chemical modification for each compound. The strongest effect on the ESCs toward ectoderm lineage was compound 4, due to higher Pitx3 gene expression, combined with the highest decrease of the pluripotency genes. In contrast compound 2 (over-sulfated heparin) had the same effect as compound 4 on the ectoderm lineage but with less Pitx3 expression and without any significant pluripotency gene

downregulation. (Table 3.17). Compound 5, had a unique and different effect on the ESCs by stimulating both ectoderm and endoderm lineages with more effect towards the endoderm lineage, combined with no significant down or up regulation of the pluripotency markers. Compound 6 had the most effect on the pluripotent genes by significantly upregulating shh pluripotency markers at the 7-day incubation point, with a moderate effect on ectoderm lineage (see Table 3.17).

Compound Number	Pluripotent	Ectoderm		Endoderm		Mesoderm		Result
2	NS	↓ 4 ↑ 2	Pitx3 5.13	↓ 3 ↑ 0	Cxcr4 0.1 Gsc 0.2	↓ 4 ↑ 0	Kdr 0.23 T 0.05	Promote ectoderm differentiation
4	↓ 1	↓ 2 ↑ 4	Pitx3 35	↓ 4 ↑ 0	Afp 0.09 Cxcr4 0.12	↓ 3 ↑ 0		Enhance Ectoderm differentiation
5	NS	↓ 4 ↑ 3	Foxd3 0.22 Lmx1a 4.37 Pitx3 9.08	↓ 2 ↑ 5	Cxcr4 0.18 Foxa2 4.68	↓ 1 ↑ 0	T 0.16	- Enhance Ectoderm differentiation. - Promote Endoderm differentiation
6	↑ 1	↓ 2 ↑ 3	Pitx3 4.89	↓ 3 ↑ 0	Cxcr4 0.16 Gsc 0.19	↓ 3 ↑ 0	Kdr 0.21	Promote ectoderm differentiation Promote pluripotent stage.

Table 3.14: Summary of the different effects of modified heparins on R1 stem cell lineage commitment markers measured by qPCR compared to 7-day control. This table summarises the effects of compounds (2, 4, 5 and 6) at the 7-day time point, any qPCR result of the expression of any gene under or equal to a 0.5-fold change in specific lineage genes was considered as a significantly decreased result (Green), and any significant increases above or equal to a 2-fold change in specific lineages, was considered as a significantly increased result (Red).

3.7 Discussion

HS chains control developmental signals by acting as a co-factor through a multitude of processes involving, but not restricted to, maintaining co-receptor functions and morphogen gradients (Lin, 2004; Kreuger et al., 2006; Bishop et al., 2007). As ESCs undergo differentiation, HS is expressed in ESCs and is further modified in a cell-specific behaviour (Johnson et al., 2007; Nairn et al., 2007). Given the critical significance of HS during vertebrate development in modulating cytokines, growth factors, and matrix biology, it is not unexpected, and it is well known that HS is a significant stem cell fate regulator. However, little is known about how sulfation patterns influence this function. Recent attempts have used multiple chemical and genetic methods in stem cell self-renewal and differentiation to tackle the function and structure-function relationship of HS. In this research, we concentrated on the direct effects of different HS structures, exemplified by selectively modified heparins, on the behaviour of embryonic stem cells in the maintenance of pluripotency and the early differentiation stages.

The capacity of ESC for self-renewal and their ability to differentiate into ectoderm, mesoderm, and endoderm, makes these cells attractive for researchers to investigate the effect of

external factors on their behaviour. However, the multiple derivations of ESCs and their ability to generate multiple lineages requires characterizing these ESC sub-populations by their unique genetic profiles.

This has been achieved here by using two well established PCR techniques; although the two techniques analyse the same material, which is the mRNA, they have different outcomes in terms of their sensitivity and quantitation.

Pluripotency gene expression – the overall view from RT-PCR data showed that the gene expression of different pluripotency stem cell markers is a significant indicator of the exit of pluripotent stem cell from self-renewal and proliferation to differentiation and lineage commitment. As mentioned in the Introduction and Experimental Aims, we are investigating stem cell behaviour and the effects of treatment with different chemically modified heparins for different amounts of time. Pluripotency was tested in all culture conditions in the previous experiment, including the three control conditions, and the incubation with chemically modified heparins for 2 days or 7 days.

The pluripotency markers decreased clearly from the highest expression in the 0-time control (as expected in the initial pluripotent state) until negative expression on two different treatment conditions namely compound 4 treatment, (7 days), and the 7-day control condition. Compound 5 treatment condition at the 7-day incubation period had the

same decreased expression of the pluripotent genes, except for remaining low expression of Oct-3/4. Compound 6 treatment condition had almost the same behaviour as compound 5 in expressing lower pluripotency genes than 0-time condition and the 2-day incubation periods, but with detection of low levels of SOX2. Taking a general view, the pluripotency gene expression decreased, as expected, with more incubation time, both with and without compounds. The 7-day control shows the importance of time in stem cell differentiation, after withdrawal of LIF stimulates initial differentiation, as seen in (Figure 3.6). Compound 6 had the most substantial effect on lowering all pluripotency markers (Nanog, Oct3-4, and SOX2), while compound 4 had the most substantial effect on maintaining pluripotent markers at a higher level than the control for all pluripotency markers (Figure 3.20).

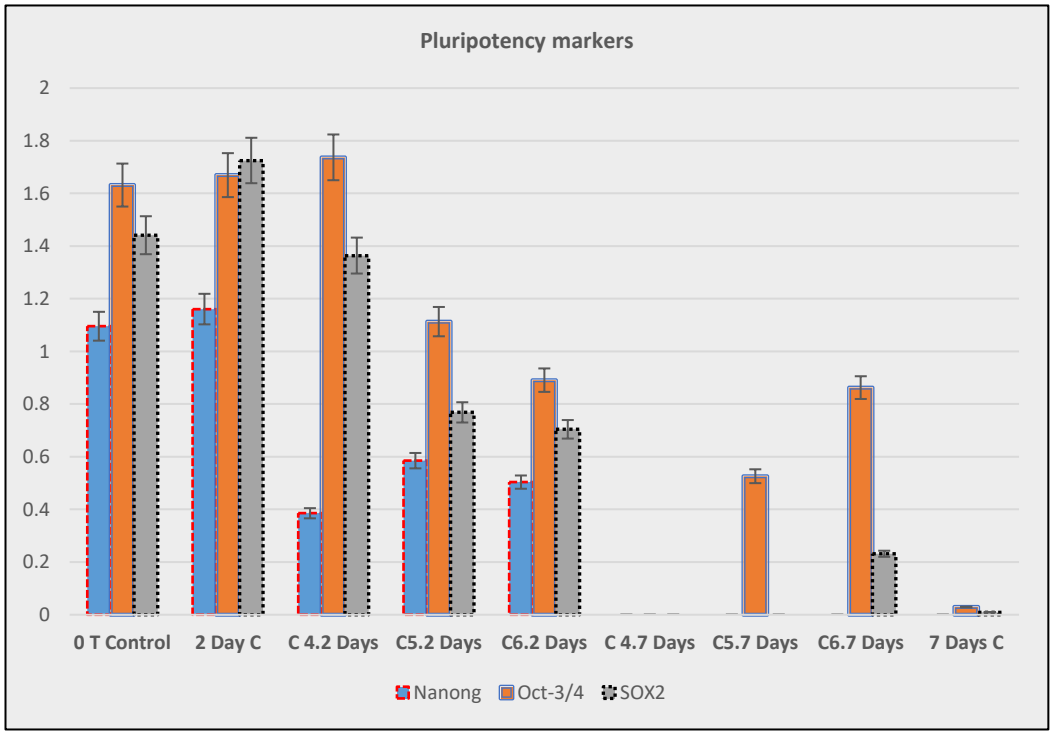


Figure 3.20: **Bar chart showing an overall view of relative change in pluripotent gene expression in all incubation conditions.** The three pluripotent markers gene expression in all condition after the withdrawal of LIF except in 0-time control. It is showing an apparent decrease in the expression by time from the 0-time control to the 7-day control condition.

Experimental controls measured by RT-PCR shows three types of controls were used within the research; each one of the controls had a specific purpose and was very useful in showing the baseline conditions with which to compare the degree of effect of the chemically modified heparins on ESC behaviour.

0 Time control: This is the baseline condition for the primary cells; they were cultured and stored according to ATCC recommendations, as mentioned previously in the Methods section, and maintained using LIF treatment. LIF is regularly added to the mESC culture medium and the removal of LIF results in rapid initiation of mESC differentiation; because of this critical necessity to maintain pluripotency, LIF intracellular signalling was a primary focus of researchers studying the regulatory processes of self-renewal and pluripotency in mESCs (Hirai, Karian and Kikyo, 2011). Serum-free particular medium from (Fisher), with specific combinations of hormones, nutrients and purified serum proteins, can be used instead of the traditional serum. Such media are provided by many companies and are similar to unique medium used by Johansson and Wiles (1995); mESC survived in this serum-free medium for at least 6 days in a pluripotent state (Johansson and Wiles, 1995). Pluripotent stem cells were also maintained by culturing on feeder cells, PSCs generally grow best when connected to other cells or an

extracellular matrix and traditionally grow in cultures with feeder layers (usually, mouse embryonic fibroblasts (MEFs) that have been irradiated or treated with mitomycin C (MMC). The reason is to prevent them from dividing. Induced pluripotent stem (iPS) or Embryonic stem (ES) were treated with feeder cells in an undifferentiated state without losing their pluripotency (Evans and Kaufman, 1981). The primary reason behind why Mitomycin C-treated MEFs maintain undifferentiated cells remains unknown. The most common explanation is that MEFs secrete molecules necessary to keep PSCs in their undifferentiated state (Lee et al., 2009). All three factors mentioned are well known in the literature to maintain and control stem cell pluripotency. The main reason for using the zero-time control was to compare the different behaviour of the pluripotent stem cells with the original state of the stem cells before the treatment culture period.

2 Day control: These controls are stem cells that were cultured in LIF-, and feeder-free conditions, without the addition of the chemically modified heparins, for two days.

7 Day control: This control represented stem cells that were cultured in LIF-free, and feeder-free conditions, without the addition of any of the chemically modified heparins, for seven days.

The main reason for using the 7-day and 2-day controls, was to provide a base line for the stem cell behaviour during the incubation period without the effect of chemically modified heparins. The outcome from the comparison between the treated stem cells and the 2 or 7-day controls was the specific direct effect of the modified heparins. By comparing with the analysis of the experimental control conditions by RT-PCR (Figure 3.2), it is very clear that zero-time control were maintained by the three factors supporting pluripotency (LIF, feeder cells, and serum-free conditions) with clear expression of the primary pluripotency genes Nanog, Oct3/4, and Sox3. On the other hand, the 2 and 7-day controls are cultured without the addition of the chemically modified heparins for 2 and 7-days. The 2-day control condition cells expressed the pluripotency genes with the same (or little more) level as the zero-time control, indicating that the two-day time point is too early for significant changes in stem cell fate and behaviour to be detected; however, there was observed mixed expression pattern between the still positive pluripotent genes markers, but also weak gene expression of Nestin and Otx2 representing ectoderm lineage, and AFP and GATA-4 representing endodermal lineage. In contrast, at 7-day control conditions, almost zero levels of the pluripotent genes were expressed, indicating that with more time in the standard LIF- culture conditions lead stem cells differentiate towards a number of their

preferred fates. In this condition and after 7-days, the differentiation gene expression was decreased and almost equalled the zero-time condition for the ectodermal and mesodermal lineages, with low level expression of the two endodermal lineage markers AFP and GATA-4, indicating that this was the default lineage cell fate under the control conditions used (Figure 3.2).

The screening assay using RT-PCR was important to give a wide view on the affect of the chemically modified heparins on the ESCs fate commitment decision, and to focus on the structure variations between each compound to understand and compare the direct relation between the structure and effects on lineage commitment. In LIF free and feeder free conditions, the RT-PCR in general showed that all pluripotency genes were reduced by incubation time with or without the addition of the compounds. After longer incubation time the pluripotency genes reduced completely with compound 4, moderately with compound 5, and compound 6 was the least effective in reducing the pluripotency genes. The chemically modified heparins effects on the pluripotency genes were confirmed when using the quantitative technique qPCR, the data analysis showed that compound 6 was the most effective compound on significantly upregulating pluripotency genes.

The variation in the modification of each chemically modified heparin, resulted in different effects in the direction of ESCs lineage commitment fate decisions, when using the semiquantitative RT-PCR assay there was clearly similarity in the effect of compound 4, and compound 6, when affecting ESC expression of GATA4 and PDX-1 representing endoderm lineage. On the other hand, compound 5 had the same affect on the ESCs, but with upregulation of GATA-4 and AFP. In general, after using LIF free and feeder free conditions in a medium containing each chemically modified heparin, the gene expression analysis by RT-PCR of all expressed endoderm genes was similar with compound 4 and 6, whereas a unique behaviour of compound 5 on ESCs was noted by the expression of different GATA-4 and AFP.

On the other hand, the qPCR as a quantitative technique, is more specific and sensitive, and confirmed the RT-PCR results by showing the completely different behaviour of compound 5, and the similarity of the affect of all other compounds on the ESC gene expression after the incubation period with all compounds.

The qRT-PCR data clearly confirmed that addition of soluble modified heparins to the mESC cultures could influence their differentiation pathways induced by withdrawal of LIF in the standard culture conditions. In addition, the qRT-PCR data also confirmed that the effects of the compounds varied from each

other, confirming our hypothesis that their differential sulfation patterns would differentially alter cell responses.

Both compounds 4 and 5 contain N-sulfation, but differ only in the presence of 6-O-sulfates (in compound 4) and 2-O-sulfates (in compound 5). The fact that differences were observed between their effects suggests that these 2 types of O-sulfate are mediating different biological mechanisms. Similarly, over-sulfation in over-sulfated heparin (compound 2) clearly changes its properties compared to the lower sulphated forms in (compounds 4,5 and 6). Compound 6, which contains N-acetyl groups instead of N-sulfates, and only contains 6-O-sulfate groups, can be usefully compared with compound 4, to conclude that the additional presence of N-sulfate groups clearly creates a compound with a different ability to affect stem cell differentiation.

Chapter 4

Mechanisms involved in the effects of HS compounds on ES cell differentiation

4.1 Introduction

Heparan sulfate (HS) is a linear, and highly sulfated polysaccharide present in virtually all mammalian tissues on the cell surface and the extracellular matrix (ECM) (Lin, 2004). Proteins that interact with HS include many signalling molecules, growth factors and ECM elements that are of significant developmental importance. HS chains control developmental signals by acting as co-factors through a multitude of processes involving, but not restricted to maintaining morphogen gradients and co-receptor functions (Lin, 2004; Kraushaar, Dalton and Wang, 2013). HS modulates other physiological tasks in mammals beyond development (Bishop et al., 2007; Fuster and Wang, 2010; Sarrazin et al., 2011). As ESCs undergo differentiation, HS is abundantly expressed in ESCs and is further altered in a cell-specific way (Nairn et al., 2007). Given the critical significance of HS during vertebrate development in modulating growth factors, cytokines, and matrix biology, it is not surprising that HS is viewed as a significant stem cell fate regulator. Recent attempts have used multiple chemical and genetic methods in stem

cell self-renewal and differentiation to tackle the function and structure-function relationship of HS (Romito and Cobellis, 2016). Based on multiple experimental strategies used to study HS, it is not simple to draw findings and interpretations from current research. During biosynthesis, which takes place in the cells Golgi compartment, the structure of the generated HS chains is defined. N-deacetylase / N-sulfotransferases (NDSTs), which initiate the modification responses, play an essential central part in the design of the sulfation pattern as subsequent changes, such as O-sulfation in different positions, happen in the vicinity of N-sulfate groups (Esko and Lindahl, 2001). A better understanding of the role of sulfation pattern requires systematic studies on the effects of HS and modified heparins in defined cell systems such as ESC differentiation.

4.2 Chapter Aim:

In this chapter, research will focus on the study of the mechanisms involved in the effects of HS compounds on ESC differentiation demonstrated in Chapter 3. The experiments will investigate the mechanisms underlying the effect of chemically modified heparins on the maintenance of pluripotency or altered differentiation behaviour of stem cells. The results supported a role for HS and its modifications on embryonic stem cell function and fate during cell culture.

4.3 General Hypotheses

Three hypotheses will be investigated:

Firstly, that different modifications of the structure of the HS will not only promote differentiation of embryonic stem cell to specific lineages or maintain pluripotency, but can also change HS biosynthesis in the stem cells. This could potentially occur by regulating in a specific way receptor tyrosine kinase and cell signalling pathways.

Secondly, that these effects will be different from one chemically modified heparin to another. (i.e. will be structure-dependent).

Thirdly, that over-sulfated heparin acts to enhance Dopaminergic neuronal differentiation initiation by inhibiting the Erk signalling pathway.

In this chapter I will show:

1. The direct effect of over-sulfated heparin on Erk-phosphorylation, using Western blotting.

2. The effects of different chemically modified heparins on embryonic stem cell fate decisions, focusing on the activation of specific tyrosine kinase receptors measured by a membrane-based sandwich immunoassay, and compared to the controls.

3. The effects of 7 different chemically modified heparins at two different concentrations 100 and 1000 ng/ml on ESC signalling pathways, after an incubation period of 2 days, will be measured by sandwich *ELISA assay*.

4. The effect of selected chemically modified heparins (compounds 4, 5, and 6), on HS biosynthesis, will be measured by a qPCR *assay*.

4.4 Cell Culture Conditions for studying Mouse Embryonic Stem Cells

Embryonic stem cells were cultured to confluence in feeder cell culture conditions, using pluripotent stem cell medium, and supplemented with LIF and Knockout Serum Replacement. After confluence, pluripotent stem cell medium was aspirated, and cells were washed using PBS, followed by TrypLE cell treatment for 3-5 minutes at 37°C. Cells were then centrifuged and re-cultured for mechanical feeder cell depletion from the pluripotent stem cells. Before beginning the differentiation experiments, pluripotent stem cells were counted and cultured in 35 mm gelatin coated plates, using the pluripotent stem cell medium supplemented with LIF and Knockout Serum Re-placement. The LIF-free medium was used at the beginning of the differentiation protocols for the two different incubation periods 2 and 7-days for control and treated

conditions, and with different chosen chemically modified heparins. Pluripotent stem cells could freely differentiate to their preferred lineage fates, under the selective external addition of the modified heparins. Cell culture medium was changed daily containing the same modified heparin concentration.

4.5 Results

4.5.1 The direct effect of over-sulfated heparin on Erk-phosphorylation.

The experiment was performed to study the effect of over-sulfated heparin on ERK-phosphorylation. After the withdrawal of LIF and feeder cell depletion, stem cells were cultured in neuron medium for three different periods (5, 25, and 45 min). The N2B27 neuron medium was supplemented with 1000 ng/ml over-sulfated heparin. After each period, stem cells were lysed using RIPA buffer, and a Western blot was run to obtain the results to show the effect of the over-sulfated heparin on the phosphorylation of Erk (Figure 4.1). Each period of over-sulfated heparin medium treatment had its matching untreated control; the control for each period was ESC in N2B27 medium without the addition of over-sulfated heparin. All conditions were compared to the primary state of the stem cell (t = 0), which represents the ESC in the

pluripotent medium without any unique additions. It was observed that ERK phosphorylation was decreased at each time point compared to the untreated controls due to the effect of over-sulfated heparin. The decrease was most notable (40-50 % reduction) at the longer the points (25 and 45 min), indicating that sustained signalling via ERK was particularly affected by the presence of over-sulfated heparin.

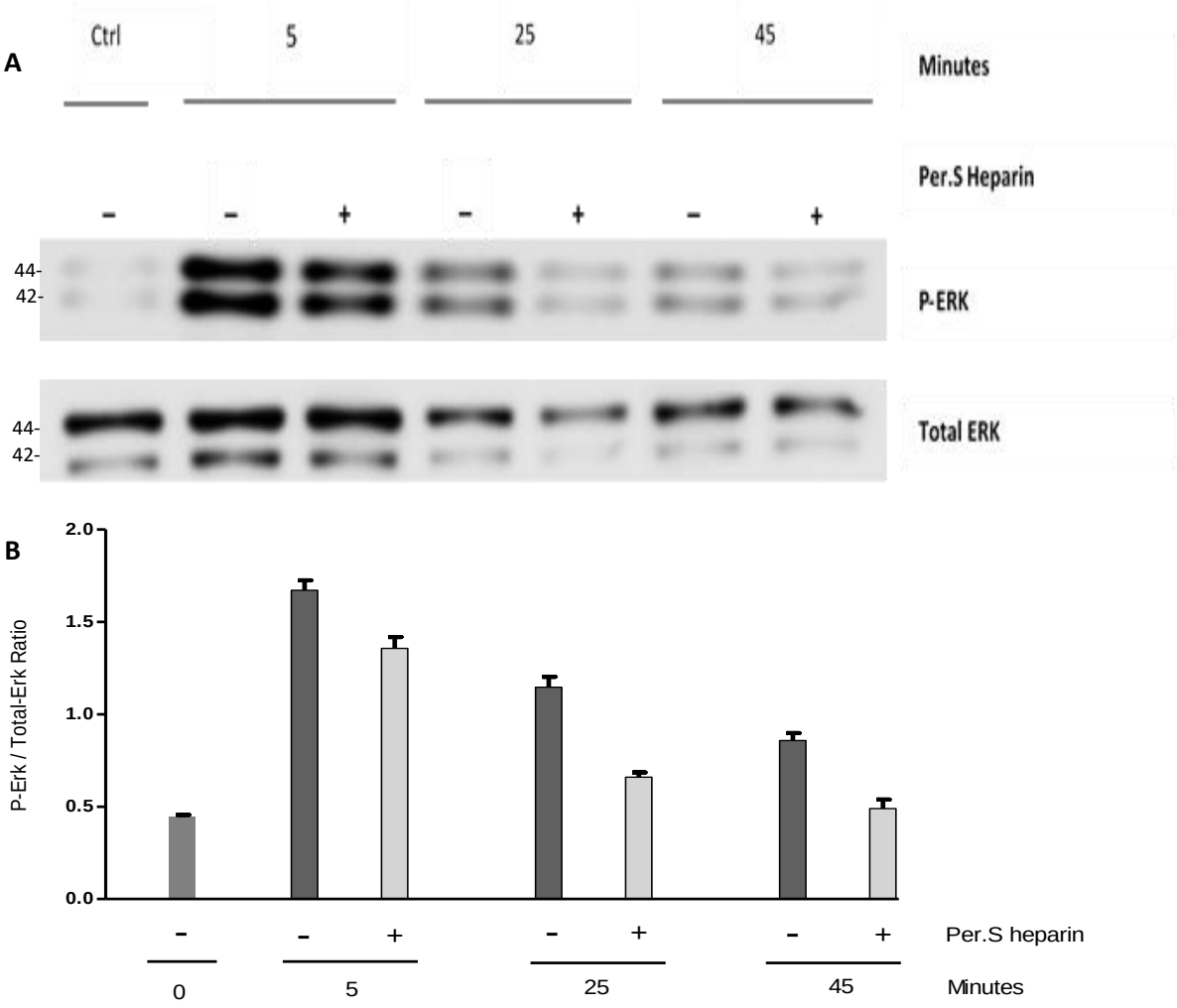


Figure 4.1: **Over-sulfated heparin supresses ERK phosphorylation in R1 cells**. R1 ES cells were switched from routine culture conditions to N2B27 neural differentiation media, in either the absence (control) or the presence of over-sulfated heparin at 1000 ng/ml (treated). After incubation for the indicated times (5, 25 or 45 min), cells were lysed using RIPA buffer and analysed by Western blotting to measure levels of P-ERK and total ERK. **A**, Western blotting data using antibodies specific for P-ERK and total ERK. **B**, quantitation of ratio of P-ERK to total ERK. (+, SD, n = 3).

4.5.2 Effect of modified heparins on signalling by receptor tyrosine kinases (RTK)

A mouse phospho-receptor tyrosine kinase slide array was used to identify potential changes in RTK phosphorylation. Protein array technology development facilitates 39 distinct phosphorylated mouse RTKs to be screened. Each capture antibody on the slide was chosen using samples of lysate prepared from ligand-treated cell lines known to express the target receptor or cell lines transfected with a cDNA encoding a specific RTK. When ligand-treated lysates were not accessible, recombinant tyrosine-phosphorylated RTK proteins were used to select capture antibodies.

It is essential and adequate for stem cells to signal through RTKs to drive control of several developmental processes in the central nervous system and other biological activities. Within each of the four RTK families, receptors are activated by ligand sets that do not cross-activate receptors from the other three families, and thus their activation can be regulated independently by the availability of ligand. In these experiments, we investigated the activation of the RTKs collectively to determine the changes in signalling related to stem cell lineage fate, or pluripotency maintenance. The RTK activation experiments were performed by the lysate collection of stem cells treated initially in N2B27 neuron medium

and after the withdrawal of LIF. In the first part of this experiment, one of the chemically modified heparins (over-sulfated heparin) was used at a concentration of 1000 ng/ml to match the earlier experiment and to test the immediate effect of the compounds on the RTK receptor responses at 5 and 25 minutes. In the other RTK experiments, the modified heparins 2, 4, 5, and 6 were incubated with the stem cells for 7 days in standard media after LIF withdrawal.

In all experiments, samples and controls were run on the nitrocellulose membrane assays in duplicate. The cell lysate was diluted and incubated on the mouse phospho-RTK array. All unbound material was washed away after binding the extracellular domain of both non-phosphorylated and phosphorylated RTKs. Anti-phospho-tyrosine antibody combined with horseradish peroxidase (HRP) was used for chemiluminescence detection of phosphorylated tyrosine on stimulated receptors.

4.5.2.1 The effect of over-sulfated heparin on RTK-signalling in ES cells

ESCs were cultured with over-sulfated heparin at a concentration of 1000 ng/ml to match the earlier experiment in (section 4.5.1) and to test the immediate effect of the compounds on the RTK receptor responses at 5 and 25 min (Figure 4.2).

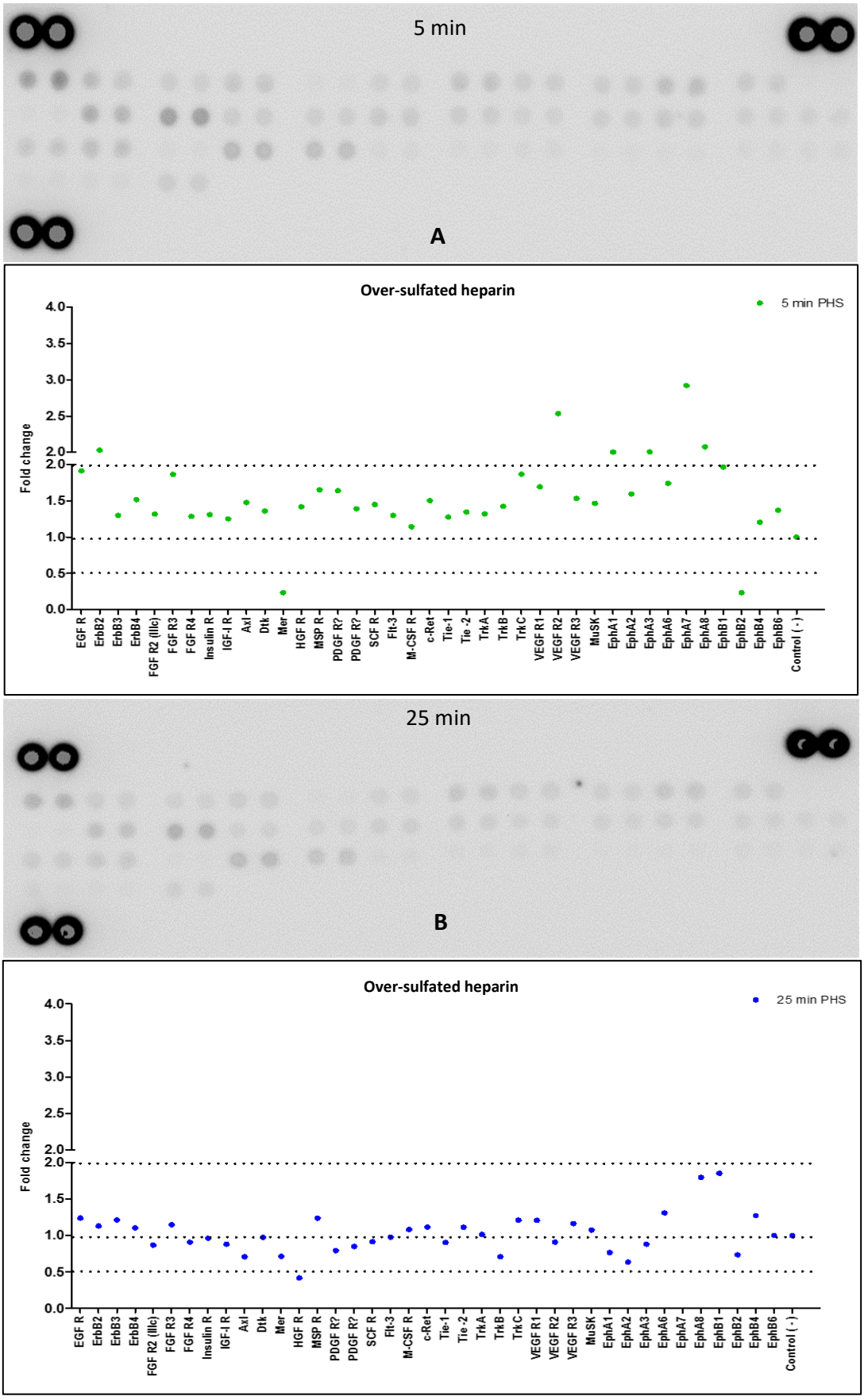


Figure 4.2. The mouse phospho-RTK array detects changes in phosphorylation of RTKs in over-sulfated heparin treated cell lysates. Detection was performed using a nitrocellulose P-RTK array (R&D systems) as described in methods. **A**. R1 mouse stem cells were treated with 1000 ng/mL OVER-sulfated heparin for 5 minutes. **B**. R1 mouse stem cells were treated with 1000 ng/mL over-sulfated heparin for 25 minutes. Data is shown for a single representative experiment.

In the previous experiment, over-sulfated heparin was tested in two different conditions and the outcome of the experiment demonstrated that over-sulfated heparin would suppress ERK phosphorylation at the 5, 25, and 45 min time points, demonstrated by Western blotting results (section 4.5.1 Figure 4.1).

The treatment of ESCs using over-sulfated heparin also affected the phosphorylation of RTKs, tested using the mouse phosphor-RTK array to detect the changes in the phosphorylation after 5 and 25 minutes. The short incubation period with over-sulfated heparin (5 minutes) showed that the RTKs phosphorylation was initially slightly high in most cases, with over 1.5-2-fold increase for almost all RTKs receptors. In contrast at the 25 minutes incubation time with over-sulfated heparin, the RTKs were mostly downregulated, or similar to control level. The exception was potential activation of some of the Ephrins such as Eph AB and Eph B1.

Further investigation was then needed with a longer incubation period, and using a wider range of modified compounds, to compare the effects of the chemically modified heparins with over-sulfated heparin and to evaluate the individual effects of each compound.

4.5.2.2 The effect of treatment with chemically modified heparins for 7 days.

Stem cells were grown in a pluripotent growth medium supporting pluripotency and supplemented with LIF and maintained on feeder cells. LIF was withdrawn, and feeder cells were depleted, and in a standard media, all stem cells were treated with four compounds (over-sulfated heparin 2, 4, 5, and 6) at a concentration of 1000ng/ml, for 7-days. The final quantitated data is shown in Figures 4.3, and 4.4 (when comparing the results with the zero-time control), and Figures 4.6, and 4.7 (when comparing the results with the 7-day control).

4.5.2.2 Comparing all compounds to zero-time control

The zero-time control condition is one of the two controls that were used in this research. First the findings will be compared to the zero-time control. In this part of the RTKs experiment, the incubation period with compounds was 7-days in ESCs standard medium. After maintaining ESC in a pluripotent medium, feeder cells were removed, and LIF is withdrawn. All 4 compounds were added at a concentration of 1000 ng/ml, 3 biological samples were used for each condition on three RTK films (n=3 biological replicates) (see Figure 4.3, and 4.4).

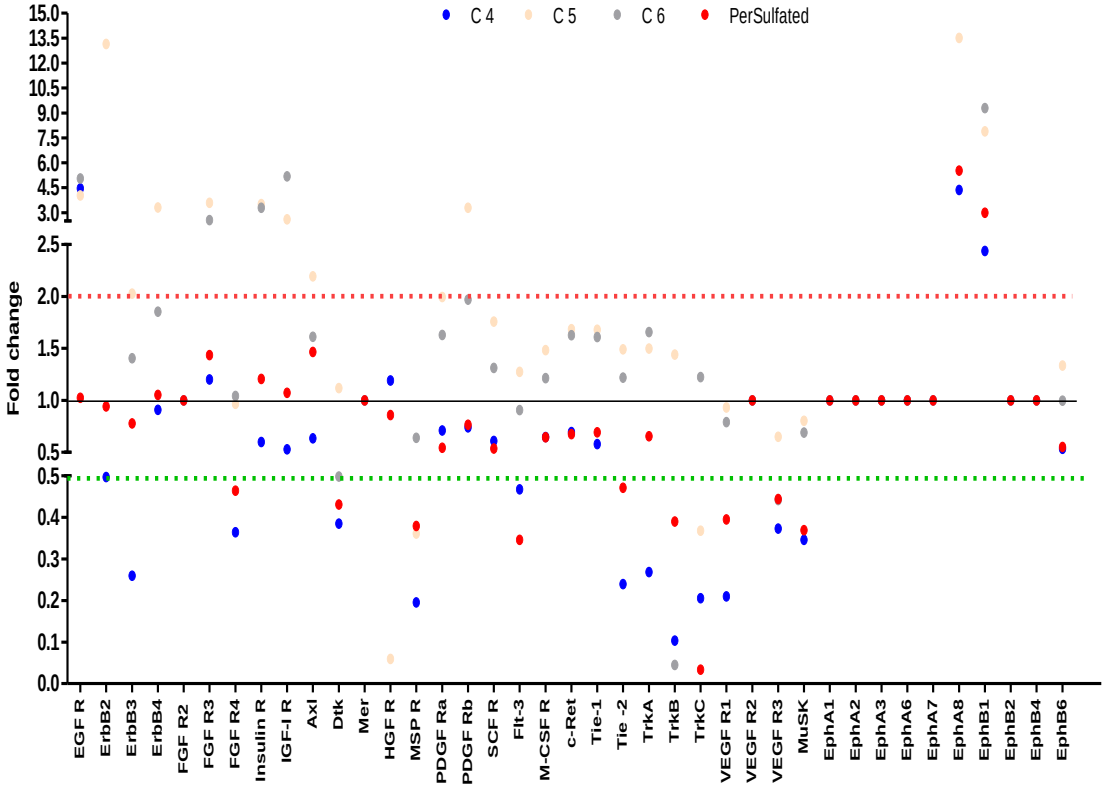


Figure 4.3. Mouse phospho-RTK array data overall analysis for R1 ESCs treated with different modified heparins and compared to Zero-time control. Data show the total phosphorylated receptors after each compound treatment in ESCs standard media, all compounds were cultured for 7-days in standard media.

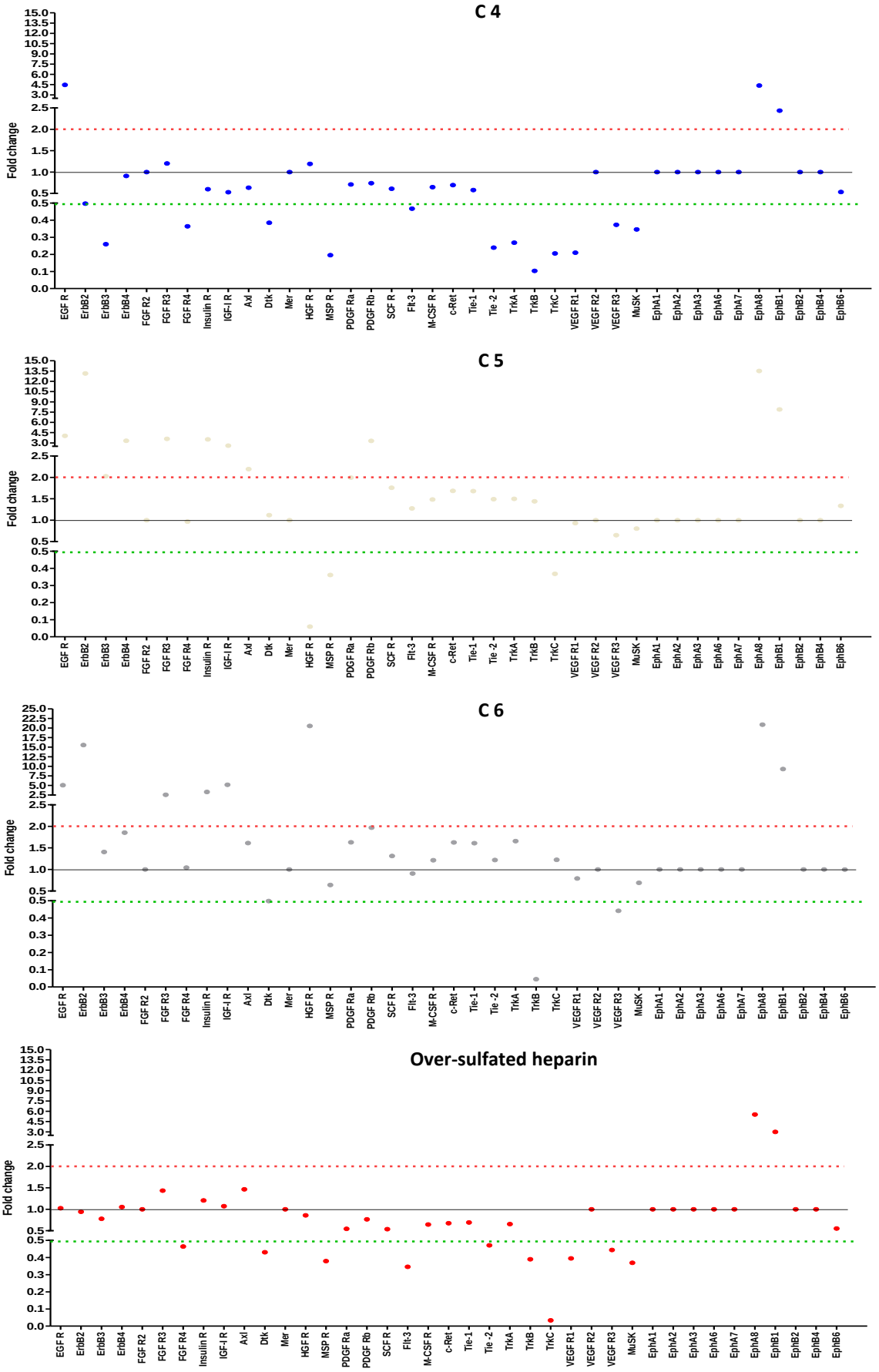


Figure 4.4. Individual dot chart showing mouse phospho-RTK array data overall analysis for R1 ESCs treated with different modified heparins and compared to zero-time control. The dot charts show the total phosphorylated receptors after each compound treatment in ESCs standard media, all compounds were cultured for 7-days in standard media.

The data analysis compared to the zero-time control intrestingly showed that EphA8, EphB1 are significantly increased (3-20 fold) in all stem cell treated conditions with all compounds over-sulfated, 4, 5,and 6. Over-sulfated heparins 2 and 4 had the lowest overall effect on increasing RTK phosphorylation, and 5 had the greatest level of significantly increased activation of RTKs phosphorylation. High similarity was detected with compounds 5 and 6 (Table 4.1).

	Compound 4	Compound 5	Compound 6	Over-Sulfated
EGF R	4.46242	4.0424	5.05541	1.02392
ErbB2	0.497455	13.15127	15.54033	0.94274
ErbB3	0.259776	2.027083	1.406571	0.77820
ErbB4	0.909405	3.303135	1.852697	1.05209
FGF R3	1.201039	3.592728	2.562481	1.43599
FGF R4	0.36451	0.967908	1.043399	0.46411
Insulin R	0.600465	3.514574	3.30186	1.20573
IGF-I R	0.529052	2.600837	5.188476	1.07242
Axl	0.634133	2.194165	1.613269	1.46474
Dtk	0.384937	1.119436	0.498737	0.43109
HGF R	1.192593	0.059259	20.53333	0.85925
MSP R	0.195331	0.360894	0.640412	0.37941
PDGF Rα	0.709506	1.995696	1.629886	0.54283
PDGF Rβ	0.741062	3.288696	1.969132	0.76504
SCF R	0.610291	1.759175	1.312334	0.53575
Flt-3	0.467264	1.274671	0.907646	0.34623
M-CSF R	0.648072	1.483988	1.213326	0.64583
c-Ret	0.695245	1.686343	1.627313	0.67603
Tie-1	0.580274	1.680183	1.610411	0.69333
Tie -2	0.239689	1.491384	1.218503	0.47161
TrkA	0.268806	1.498663	1.657807	0.65613
TrkB	0.103415	1.44112	0.045082	0.39016
TrkC	0.205456	0.367775	1.225405	0.03393
VEGF R1	0.209799	0.930796	0.790639	0.39545
VEGF R3	0.373083	0.650587	0.441588	0.44444
MuSK	0.345916	0.804581	0.68968	0.36910
EphA8	4.357895	13.50877	20.87719	5.53333
EphB1	2.43758	7.88417	9.288288	2.99356
EphB6	0.534397	1.335428	0.996617	0.55131

Table 4.1. RTKs data analysis for R1 ESC treated with different chemichally modified heparins compared to zero-time control. The table Shows all phosphorylated receptors, with significantly upregulated (Red) or dowregulated (green).

4.5.2.2.2 Comparing all compounds to the 7-day control.

The other and most important control that was used to evaluate the effect of the compounds was the 7-day control, which has the same conditions but without the addition of chemically modified heparins. The treatment culture medium contained the 4 compounds (over-sulfated, 4, 5, and 6) at 1000ng/ml. At the end of the incubation period, cells were lysed and the lysate subjected to the RTK phosphorylation assay. Data for all compounds were then compared to the 7-day control. An overview of the data is shown in (Figure 4.5), and a breakdown of the data for each individual compound is shown in (Figure 4.6).

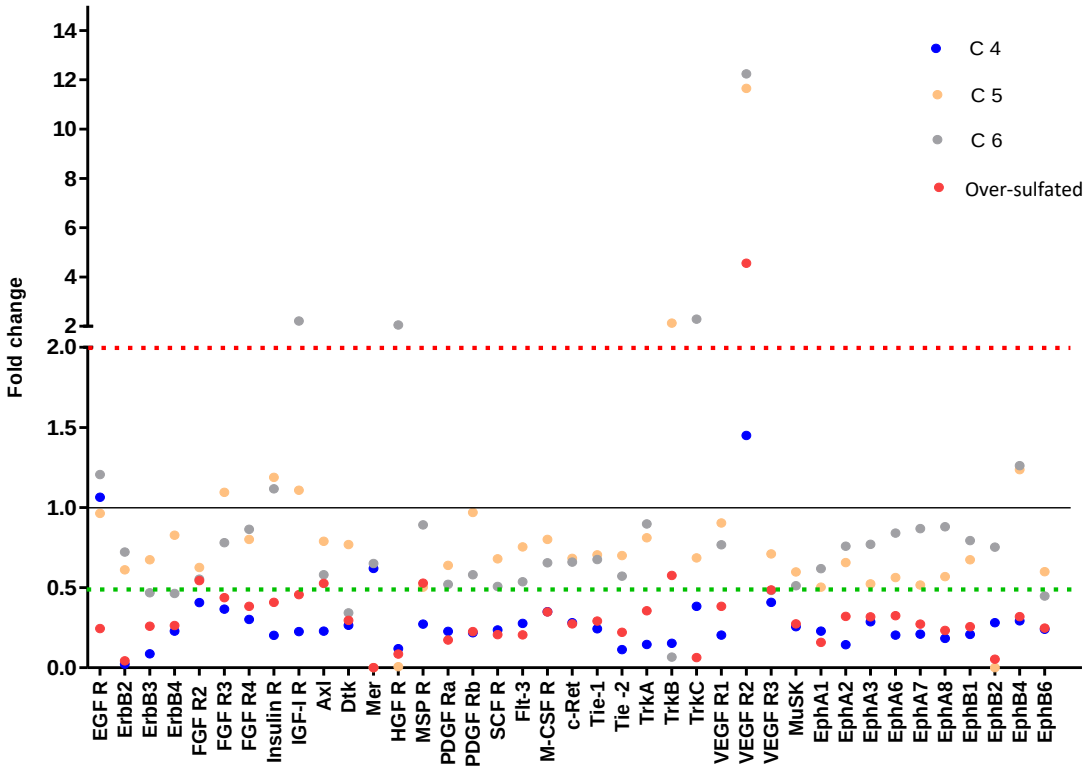


Figure 4.5. Mouse phospho-RTK array data overall analysis for R1 ESCs treated with different modified heparins and compared to 7-day control. Data shows the total phosphorylated receptors after each compound treatment in ESCs standard media, all compounds were cultured for 7-days in standard media.

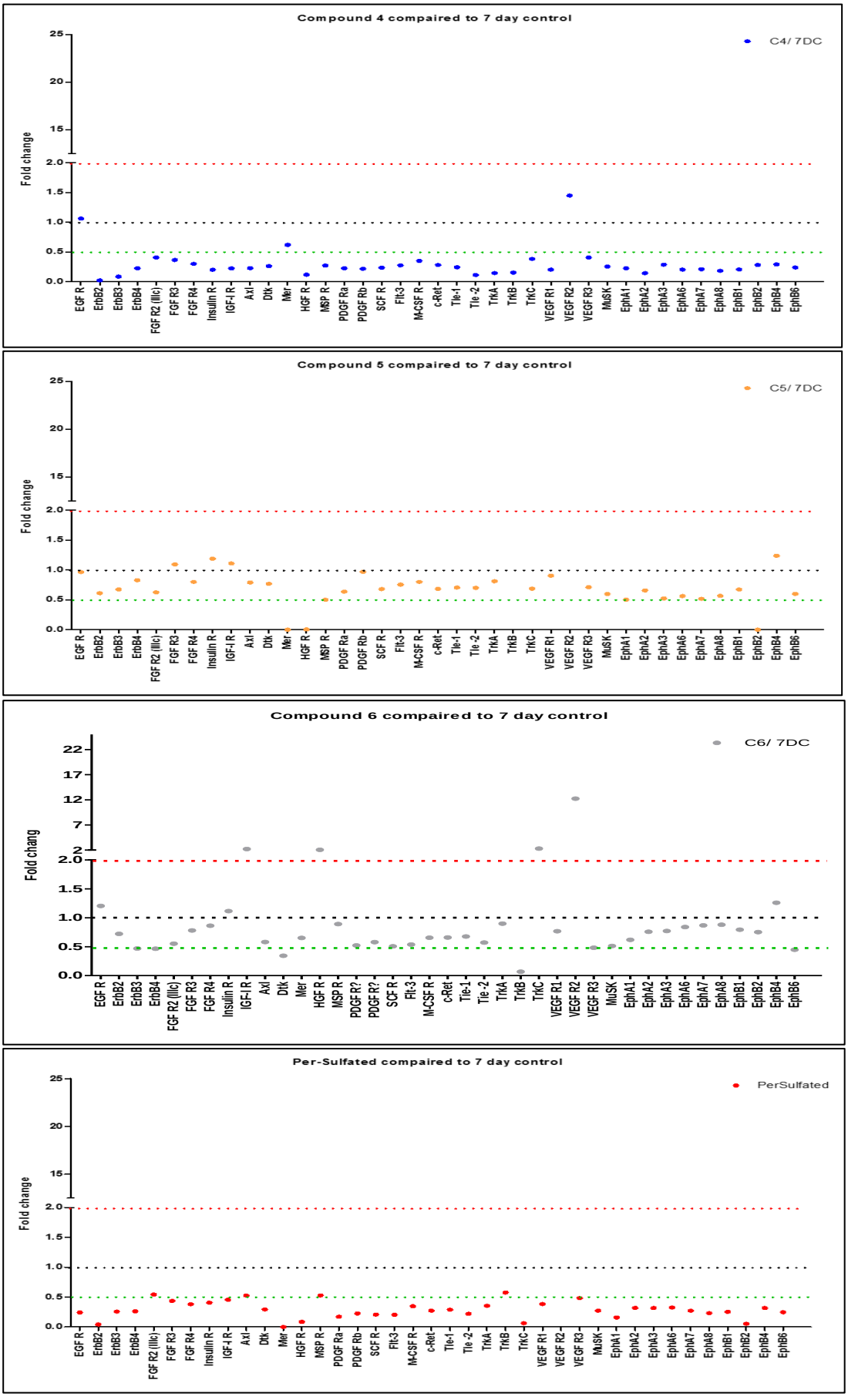


Figure 4.6. Individual dot chart showing mouse phospho-RTK array data overall analysis for R1 ESCs treated with different modified heparins and compared to 7-day control. The dot charts show the total phosphorylated receptors after each compound treatment in ESCs standard media, all compounds were cultured for 7-days in standard media.

The overall data analysis (Table 4.2) compared to the 7-day control showed that VEGF R2 phosphorelation was significantly increased by compounds 5 and 6, and also over-sulfated heparin. Interestingly over-sulfated heparin compound had an almost similar affect as compound 4. In contrast compounds 5 and 6 had relatively neutral effects on most RTKs, but an upregulation effect on four RTK receptors (Insulin R, IGF-I R, VEGF R2 and EphB4). Despite some similarities being noted, each compound produced a specific altered pattern of RTK phosphorylation.

	C4/ 7 D C	C5/ 7 D C	C6/ 7 D C	Over-Sulfated
EGF R	1.064257	0.964085	1.205681	0.24420044
ErbB2	0.023118	0.61117	0.722195	0.043811675
ErbB3	0.086486	0.674865	0.468281	0.259083391
ErbB4	0.227995	0.828122	0.464486	0.263769289
FGF R2 (IIIc)	0.407501	0.625403	0.552232	0.544408652
FGF R3	0.365913	1.094573	0.780694	0.437494182
FGF R4	0.301737	0.801223	0.863713	0.384190878
Insulin R	0.203062	1.188539	1.116605	0.407749174
IGF-I R	0.225554	1.108831	2.212036	0.457215398
Axl	0.228268	0.789831	0.580727	0.527263833
Dtk	0.26472	0.769832	0.342979	0.296464613
Mer	0.620332	0	0.651452	0
HGF R	0.119392	0.005933	2.055617	0.086021505
MSP R	0.272041	0.502622	0.891911	0.528420611
PDGF Rα	0.226975	0.638433	0.521409	0.17365711
PDGF Rβ	0.218347	0.968985	0.580187	0.225413859
SCF R	0.236112	0.680597	0.507722	0.207275123
Flt-3	0.276728	0.7549	0.537536	0.205052006
M-CSF R	0.349717	0.800799	0.654743	0.34850878
c-Ret	0.281514	0.682823	0.658921	0.273736128
Tie-1	0.243579	0.705283	0.675995	0.291037338
Tie -2	0.112511	0.70006	0.571968	0.221375058
TrkA	0.145679	0.812194	0.898442	0.355589781
TrkB	0.152806	2.12939	0.066613	0.576503835
TrkC	0.38327	0.686069	2.285941	0.063295165
VEGF R1	0.203686	0.903678	0.767604	0.38392933
VEGF R2	1.449383	11.64938	12.23704	4.555555556
VEGF R3	0.407905	0.71131	0.482804	0.48592694
MuSK	0.256959	0.597671	0.512319	0.274218013
EphA1	0.227968	0.503242	0.619307	0.159209157
EphA2	0.144578	0.656591	0.758613	0.320439653
EphA3	0.286547	0.523115	0.77079	0.318339581
EphA6	0.203662	0.56326	0.841063	0.325078793
EphA7	0.210065	0.515918	0.868344	0.272209092
EphA8	0.183646	0.569274	0.879787	0.233180541
EphB1	0.208292	0.673705	0.793687	0.255801166
EphB2	0.281407	0	0.752827	0.05339196
EphB4	0.292563	1.237071	1.261419	0.319177048
EphB6	0.240136	0.600087	0.447839	0.247737638

Table 4.2. RTKs data analysis for R1 ESCs treated with different chemically modified heparins compared to the 7-day control. Shows all phosphorylated receptors, with significantly upregulated (Red) or downregulated (Green). RTKs as noted.

4.5.3 Measuring effects of chemically modified heparins on intracellular signalling pathways by Pathscan ELISA.

As a first step to study the effects of the exogenous modified heparins on mouse embryonic stem cell differentiation, and also to help us select modified heparins most likely to influence ES cell differentiation, Pathscan ELISA methodology was used to determine the effects of the full library of modified heparins on ES cells. The Pathscan method is a sandwich ELISA using a slide-based antibody array, founded upon the sandwich immunoassay principle. The array kit allows for the simultaneous detection of 18 essential and well-characterized signalling molecules when phosphorylated or cleaved (Figure 4.7, and 4.8). Target-specific capture antibodies have been spotted in duplicate onto nitrocellulose-coated glass slides. In terms of specificity and sensitivity, the Pathscan detects the indicated cellular proteins and signalling nodes only when phosphorylated or cleaved at the specified residues. No significant cross-reactivity has been observed between targets, and the experiment was optimized for cell lysates diluted to a total protein concentration between 0.2 and 1 mg/ml. The experiment was designed to investigate and find the best concentration of treatment compounds to use in the differentiation experiments, and the most significant stimulators of the embryonic stem cells from the chemically modified heparin library of 7 compounds used. For each condition and incubation period (3) biological replicates were used with an incubation period of 2-days, and two controls a zero-time control which indicates the primary state of the stem cells before any treatment, and a 2-day control without any compound treatment and within the same conditions.

Intracellular Signaling

	Target	Phosphorylation Site	Modification
1	Positive Control	N/A	N/A
2	Negative Control	N/A	N/A
3	ERK1/2	Thr202/Tyr204	Phosphorylation
4	Stat1	Tyr701	Phosphorylation
5	Stat3	Tyr705	Phosphorylation
6	Akt	Thr308	Phosphorylation
7	Akt	Ser473	Phosphorylation
8	AMPKα	Thr172	Phosphorylation
9	S6 Ribosomal Protein	Ser235/236	Phosphorylation
10	mTOR	Ser2448	Phosphorylation
11	HSP27	Ser78	Phosphorylation
12	Bad	Ser112	Phosphorylation
13	p70 S6 Kinase	Thr389	Phosphorylation
14	PRAS40	Thr246	Phosphorylation
15	p53	Ser15	Phosphorylation
16	p38	Thr180/Tyr182	Phosphorylation
17	SAPK/JNK	Thr183/Tyr185	Phosphorylation
18	PARP	Asp214	Cleavage
19	Caspase-3	Asp175	Cleavage
20	GSK-3β	Ser9	Phosphorylation

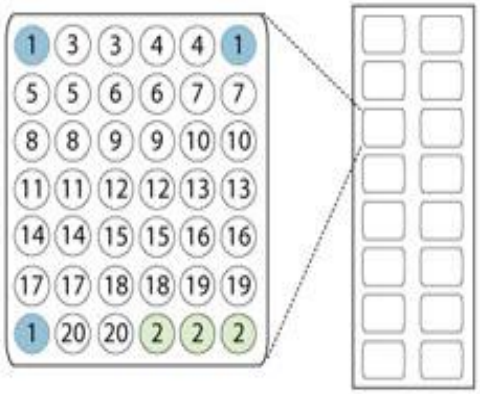


Figure 4.7: Target map of the Pathscan Intracellular Signalling Array Kit (Chemiluminescent). (Readout) #7323 and Kits, 2019).

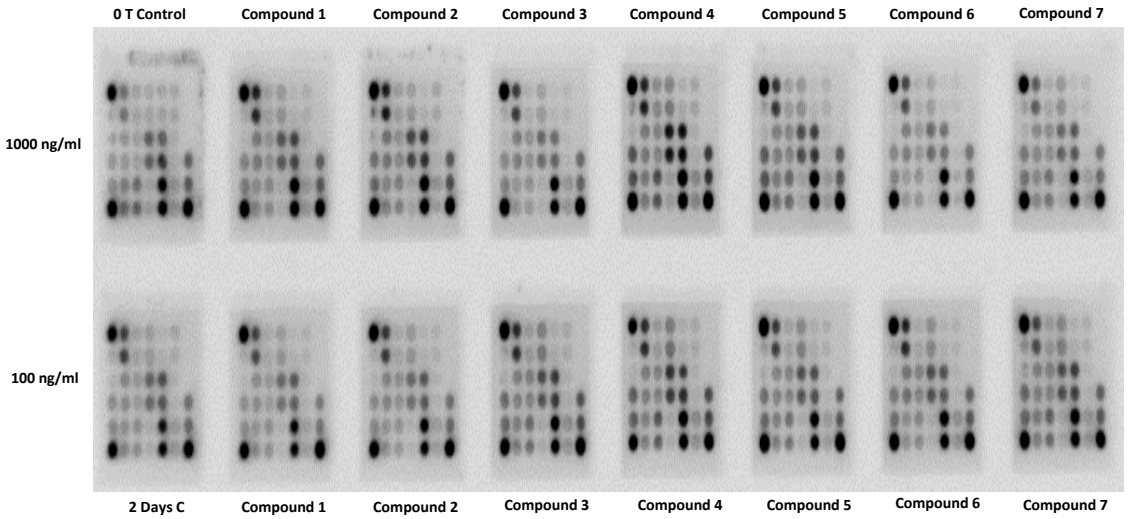


Figure 4.8: overall data of Pathscan Elisa assay slide, showing the result of triplicate samples of stem cells cultured in LIF free medium in 16 different treatment conditions. Data shows the effect of chemically modified heparins 1-7 on stem cell culture after 48 hours, testing at two chosen concentrations (1000 or 100 ng/ml). The top left corner panel is the zero-time control, and the lower left corner panel is the 48h-control (treatment in standard R1 stem cell media without the addition of heparins).

Summary of the conditions used:

- Zero-time control (pluripotent stem cell medium with LIF).

- 2-day control (medium free from LIF, no compounds added).

- treatment conditions (compounds 1 to 7, free from LIF, incubated with ESC for 2-days), the details of the compounds are summarised in (Table 4.3). An example of the overall data is shown in Figure 4.9

	Modified Heparin Compound	**Abbreviation**	**R Group Assignment**
1	Heparin	2S, 6S, NS	R1 =SO3-, R2=SO3-, R3=SO3-
2	Over-sulphated heparin	2S, 6S, NS	R1 =SO3-, R2 =SO3-, R3=SO3- + Additional sulfates at the C3 of glucosamine and C3 of uronic acid
3	N-Acetylated-Heparin	2S, 6S, NAc	R1 =SO3-, R2 = SO3-, R3 =COCH3
4	2de-Sulfated-N-Sulfated-Heparin	2de-S, 6S, NS	R1 =H, R2 =SO3-, R3 =SO3
5	6de-Sulfated-N-Sulfated-Heparin	2S, 6de-S, NS	R1 =SO3- , R2 =H, R3 =SO3-
6	2de-Sulfated-N-Acetylated Heparin	2de-S, 6S, NAc	R1 =H, R2 =SO3-, R3 =COCH3
7	6de-Sulfated-N-Acetylated Heparin	2S, 6de-S, NAc	R1 =SO3- , R2 =H, R3 =COCH3

Table 4.3: Structures of chemically modified heparin derivatives screened in Pathscan Elisa assays. This table shows the 7 modified heparins that were used in the experiment, with details of their general disaccharide repeat structures.

4.5.3.1 Comparison between the two controls conditions 2-day and zero-time control.

The comparison between the two controls used in the experiment using Pathscan Elisa assay, shows that the two controls (zero-time and 2-day controls) share the same pattern of effect on ESCs. Namely the effect of the two conditions of the phosphorylation events in the Pathscan Elisa decreased in general, with only one significant increase in Akt/Thr308 after 2-days of incubation. The zero-time control shows higher signalling than the 2-day control for Stat3, AMPKa and Bad (see Figure 4.9).

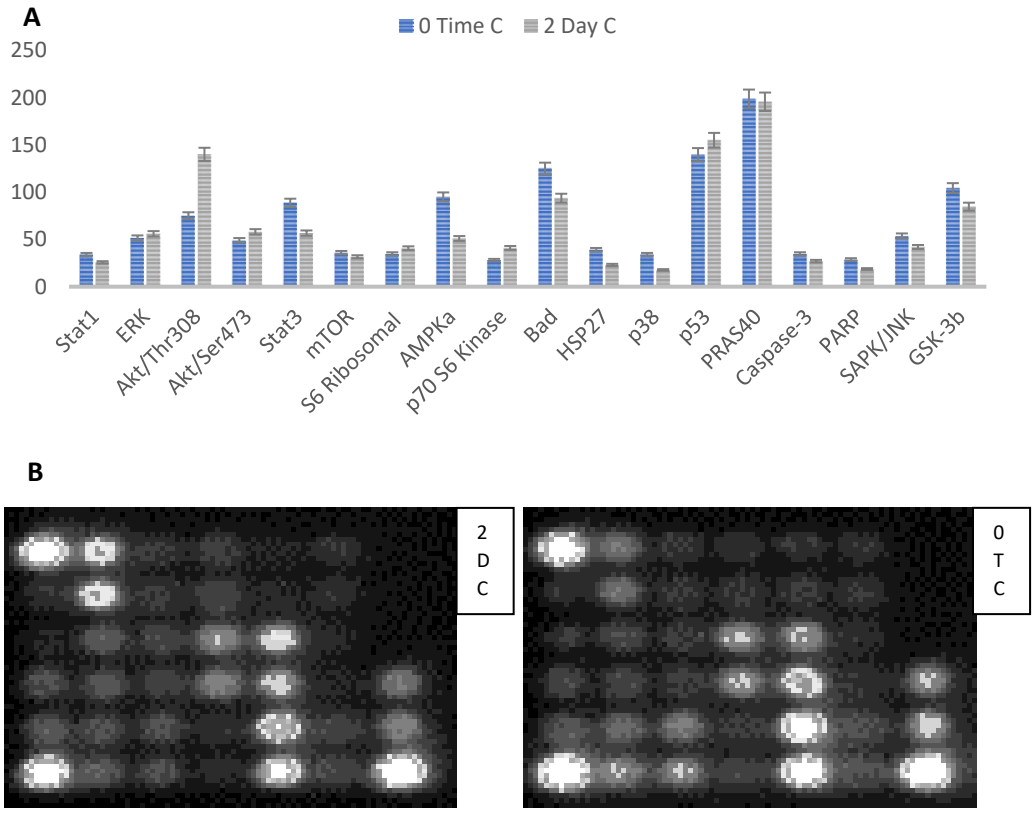


Figure 4.9: Pathscan sandwich ELISA experiment, showing comparison between the two-experiment controls. Showing the effect of varying medium and treatment period, zero-time control containing pluripotent stem cells in pluripotent maintaining medium, and 2-day control containing stem cell in medium without LIF for 2-days.(**A**) bar chart graph showing the comparison between the two controls and showing error bars from the mean and SD of the three triplicates used.(**B**) Image of the full pathscan card showing the phosphorylation levels of each control with variation in the level of phosphorylation.

4.5.3.2 Effects of Compound 1 on the intracellular signalling pathways of stem cells.

The two concentration 1000 and 100 ng/ml of compound 1 when added to the ESCs culture for a period of 2-days did not show any significant effect on the phosphorylation on the Pathscan Elisa cards, except that 1000 ng/ml concentration showed a slightly higher and clearer signalling as a general pattern (see Figure 4.10).

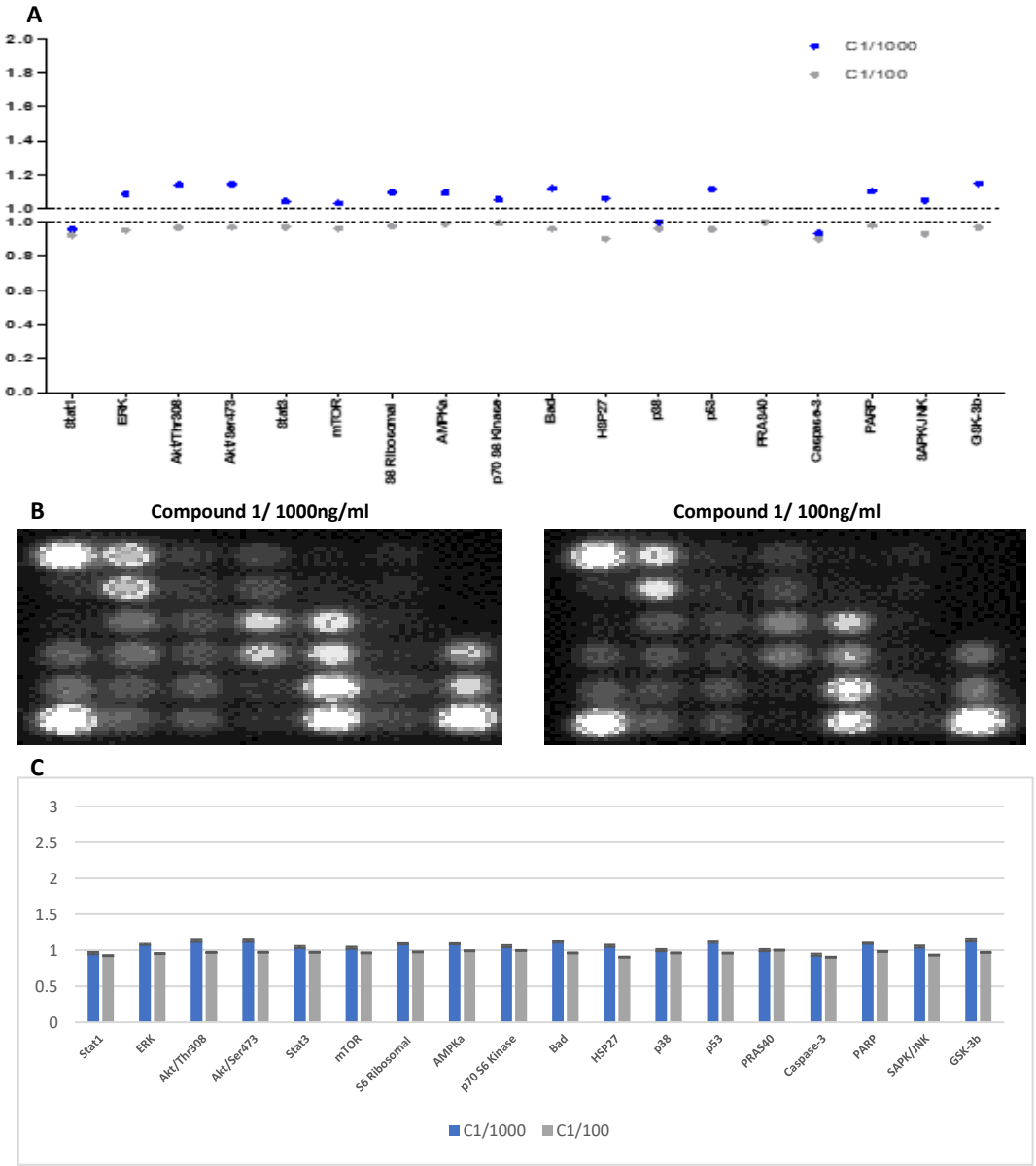


Figure 4.10. Pathscan sandwich ELISA experiment, showing the effect of varying concentration of chemically modified heparin (1) and treatment period of two days. The compound was added at different concentration of 100 and 1000 ng/ml in general stem cell medium without LIF. (**A**) Dot chart graph showing the full dataset, with the comparison between the two concentrations and showing error bars from the mean and SD of the three triplicates used. (**B**) Pathscan card showing the image of the phosphorylation at each concentration with variation in the strength of the phosphorylation. (**C**) Bar chart showing the comparison between the two concentrations and showing error bars from the mean and SD of the three triplicates used.

4.5.3.3 Effects of Compound 2 on the intracellular signalling pathways of stem cells.

The two concentration 1000 and 100 ng/ml of compound 2 (the over-sulfated heparin) when added to the ESCs culture for a period of 2-days showed clear higher phosphorylation with 1000 ng/ml compound especially with ERK, AMPKA, BAD, and P53 signalling proteins (see Figure 4.11).

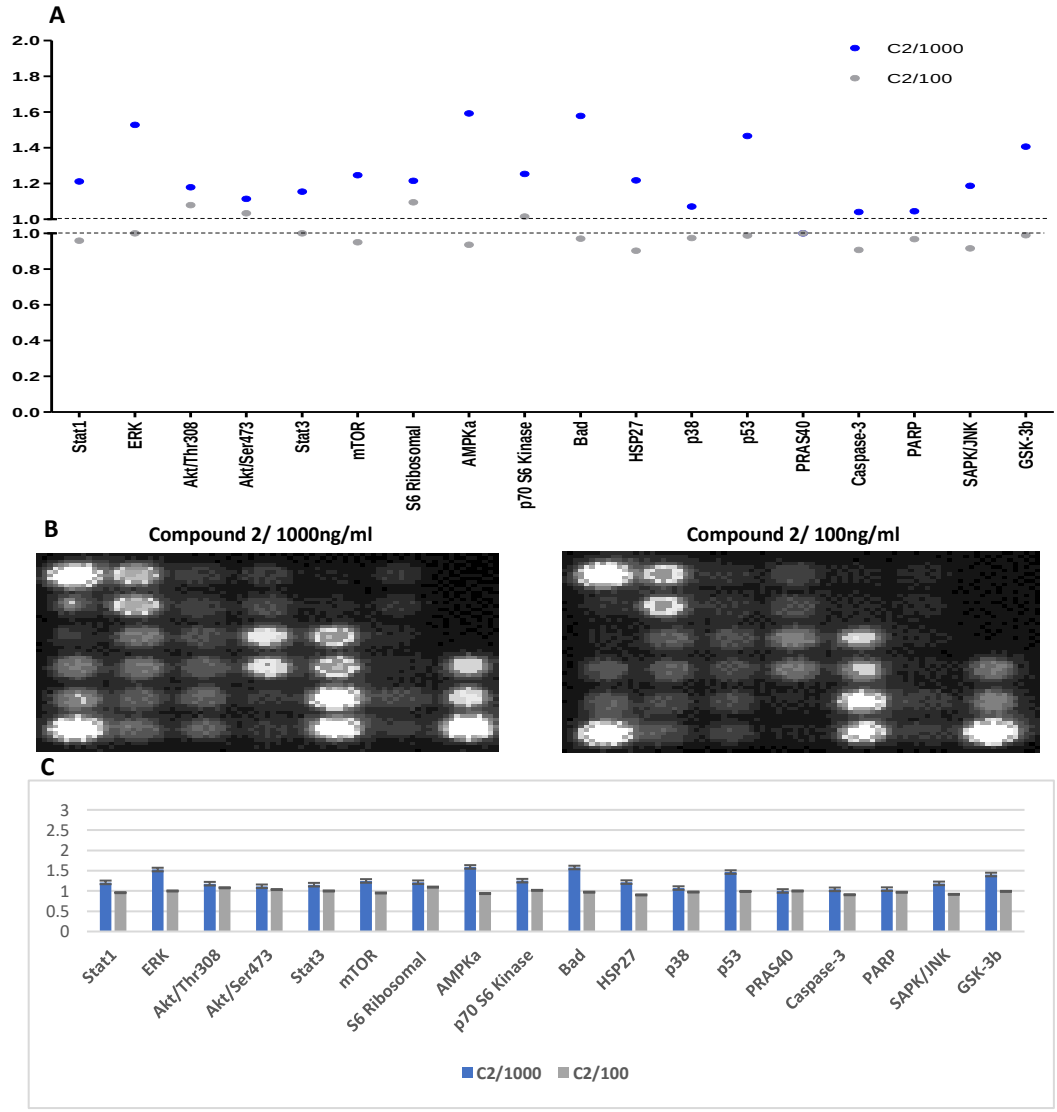


Figure 4.11. Pathscan sandwich ELISA experiment, showing the effect of varying concentration of chemically modified heparin (2) and treatment period of two days. The compound was added at different concentration of 100 and 1000 ng/ml in general stem cell medium without LIF. (**A**) Dot chart graph showing the full dataset with the comparison between the two concentrations and showing error bars from the mean and SD of the three triplicates used. (**B**) Pathscan card showing the image of the phosphorylation at each concentration with variation in the strength of the phosphorylation. (**C**) Bar chart showing the comparison between the two concentrations and showing error bars from the mean and SD of the three triplicates used.

4.5.3.4 Effect of Compound 3 on the intracellular signalling pathways of stem cells.

The two concentrations 1000 and 100 ng/ml of compound 3 when added to the ESCs culture for a period of 2-days shows a clear difference in the phosphorylation of all proteins on the Pathscan Elisa card. The 100 ng/ml concentration of compound 3 activated the phosphorylation of the proteins on the Pathscan card higher than the 1000 ng/ml concentration, especially with AMPKA and p70 kinase, which showed no activity at the 1000 ng/ml concentration (see Figure 4.12).

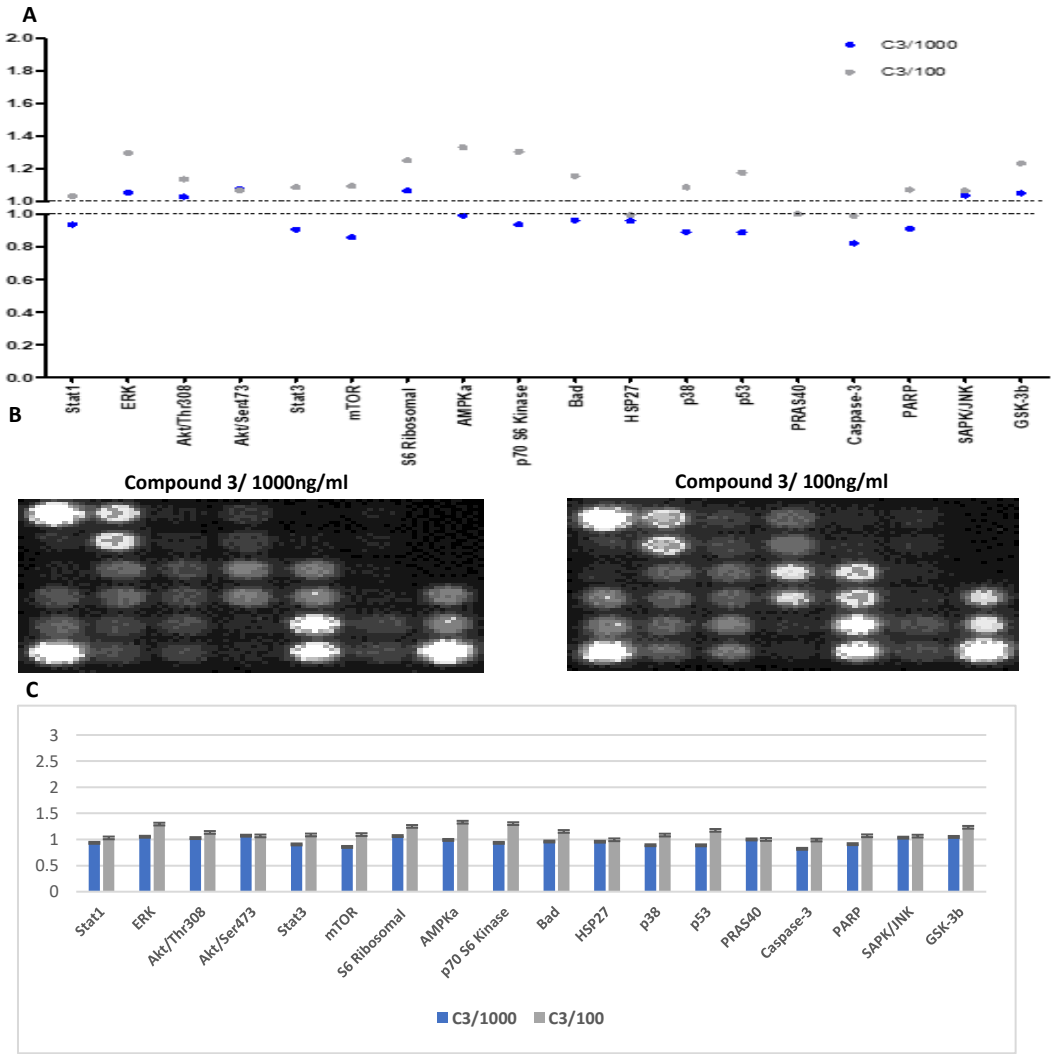


Figure 4.12. Pathscan sandwich ELISA experiment, showing the effect of varying concentration of chemically modified heparin (3) and treatment period of two days. The compound was added at different concentration of 100 and 1000 ng/ml in general stem cell medium without LIF. (**A**) Dot chart graph showing the full data set, with the comparison between the two concentrations and showing error bars from the mean and SD of the three triplicates used. (**B**) Apart from the full path scan card showing the image of the phosphorylation of each concentration with variation in the strength at the phosphorylation. (**C**) Bar chart showing the comparison between the two concentrations and showing error bars from the mean and SD of the three triplicates used.

4.5.3.3 Effect of Compound 4 on the intracellular signalling pathways of stem cells.

The two concentrations 1000 and 100 ng/ml of compound 4 when added to the ESCs culture for a period of 2-days showed an active reaction at both compound concentrations. Compound 4 was the most active compound from the set of compounds used in the assay. The 1000 ng/ml treatment showed higher activity in 4 protein phosphorylation (Stat1, Erk, p70 kinase, and p38) p38 was the highest signal (see Figure 4.13).

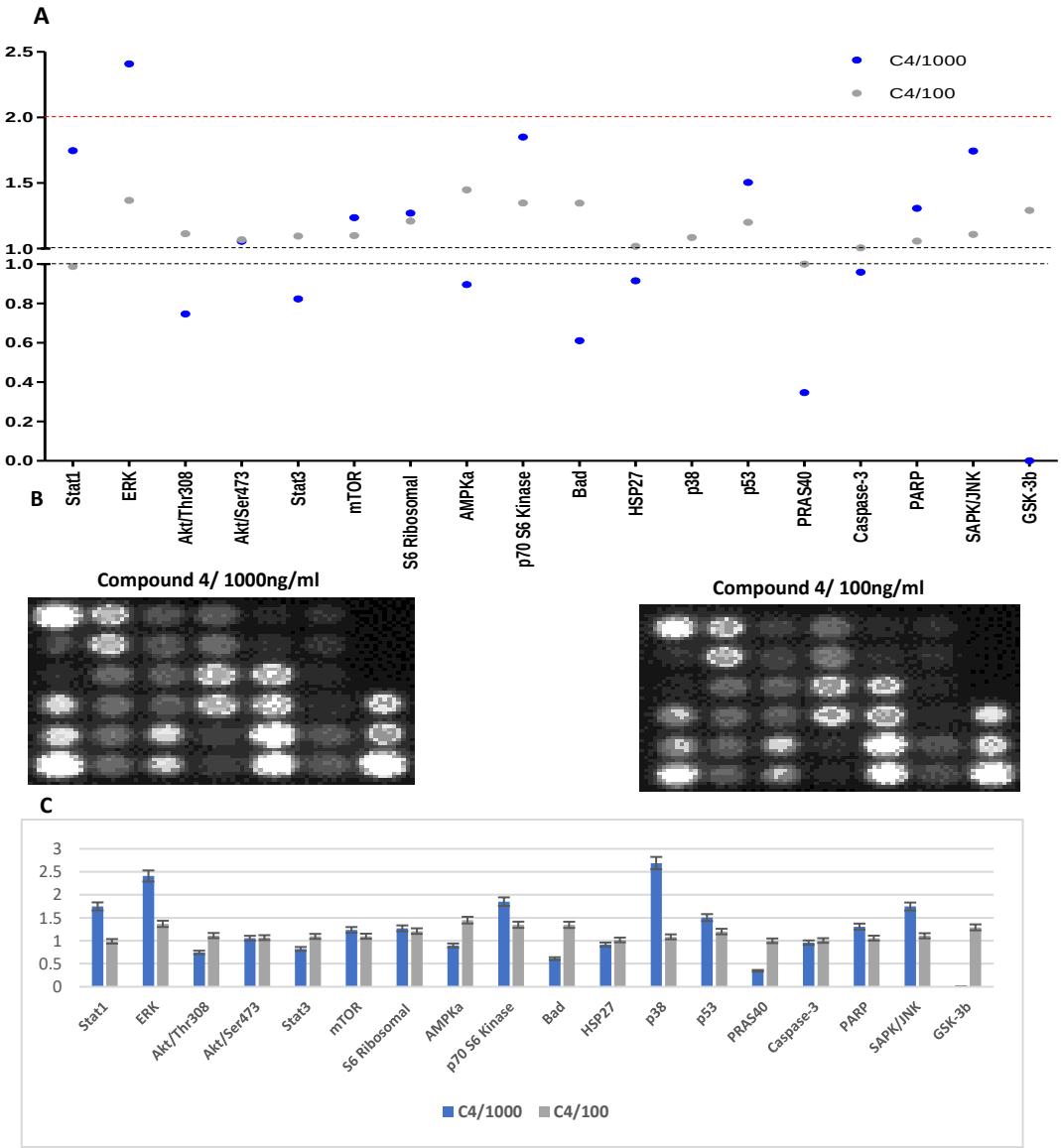


Figure 4.13. Pathscan sandwich ELISA experiment, showing the effect of varying concentration of chemically modified heparin (4) and treatment period of two days. The compound was added at different concentration of 100 and 1000 ng/ml in general stem cell medium without LIF. (**A**) Dot chart graph showing the full data set, with the comparison between the two concentrations and showing error bars from the mean and SD of the three triplicates used. (**B**) Apart from the full path scan card showing the image of the phosphorylation of each concentration with variation in the strength at the phosphorylation. (**C**) Bar chart showing the comparison between the two concentrations and showing error bars from the mean and SD of the three triplicates used.

4.5.3.6 Effect of Compound 5 on the intracellular signalling pathways of stem cells.

The two concentrations 1000 and 100 ng/ml of compound 5 when added to the ESCs culture for a period of 2-days did not show any significant effect on the phosphorylation events on the Pathscan Elisa cards, except that 1000 ng/ml concentration shows a slightly higher and more clearer signalling as a general view. ERK showed the highest phosphorylation activity with compound 5 (see Figure 4.14).

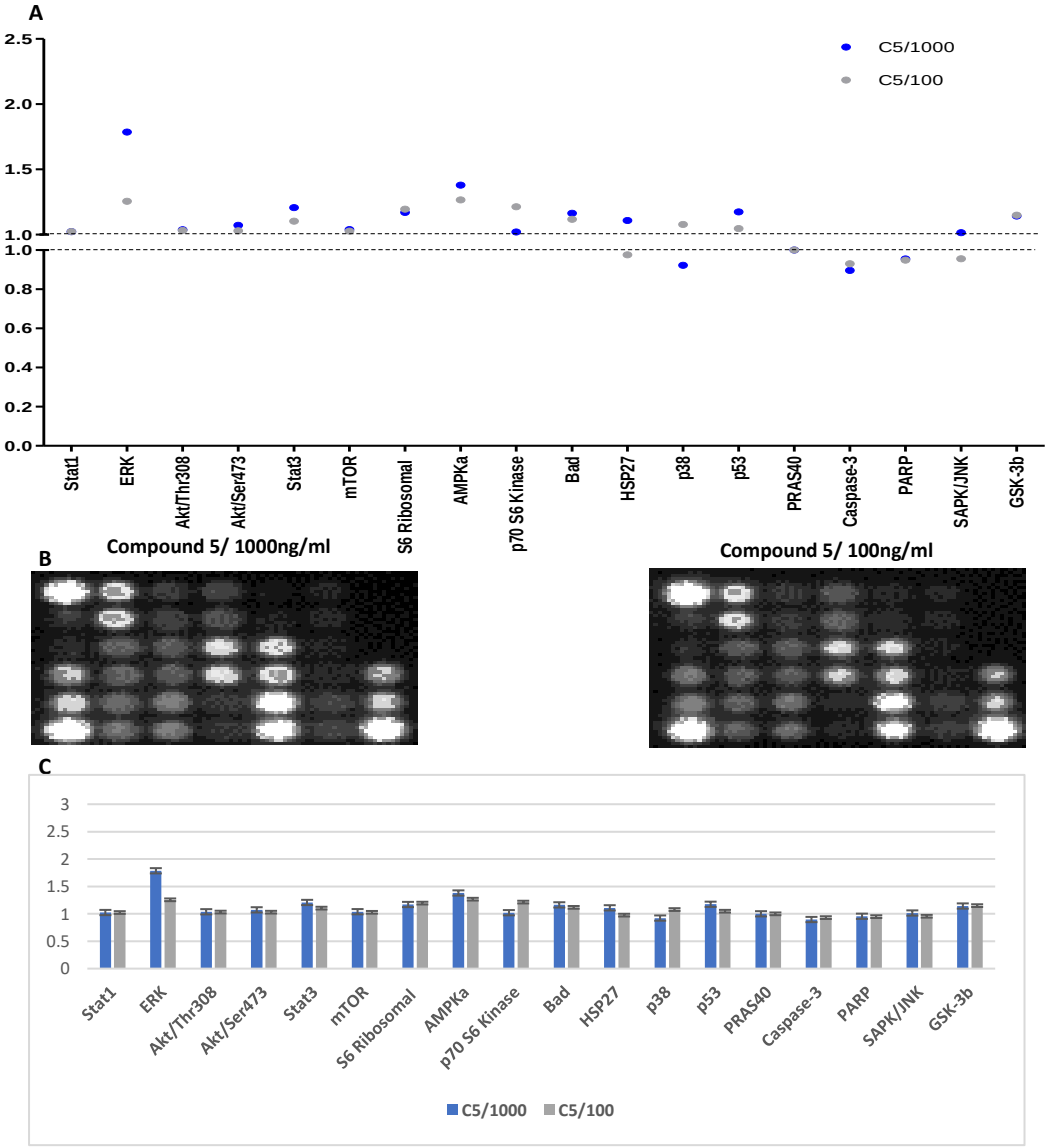


Figure 4.14. Pathscan sandwich ELISA experiment, showing the effect of varying concentration of chemically modified heparin (5) and treatment period of two days. The compound was added at different concentration of 100 and 1000 ng/ml in general stem cell medium without LIF. (**A**) Dot chart graph showing the full data set, with the comparison between the two concentrations and showing error bars from the mean and SD of the three triplicates used. (**B**) Apart from the full path scan card showing the image of the phosphorylation of each concentration with variation in the strength at the phosphorylation. (**C**) Bar chart showing the comparison between the two concentrations and showing error bars from the mean and SD of the three triplicates used.

4.5.3.7 Comparison between Compound 6 on the intracellular signalling pathways of stem cells.

The two concentrations 1000 and 100 ng/ml of compound 6 when added to the ESCs culture for a period of 2-days showed an active reaction at both compound concentrations. The 100 ng/ml shows higher activity in general (see Figure 4.15).

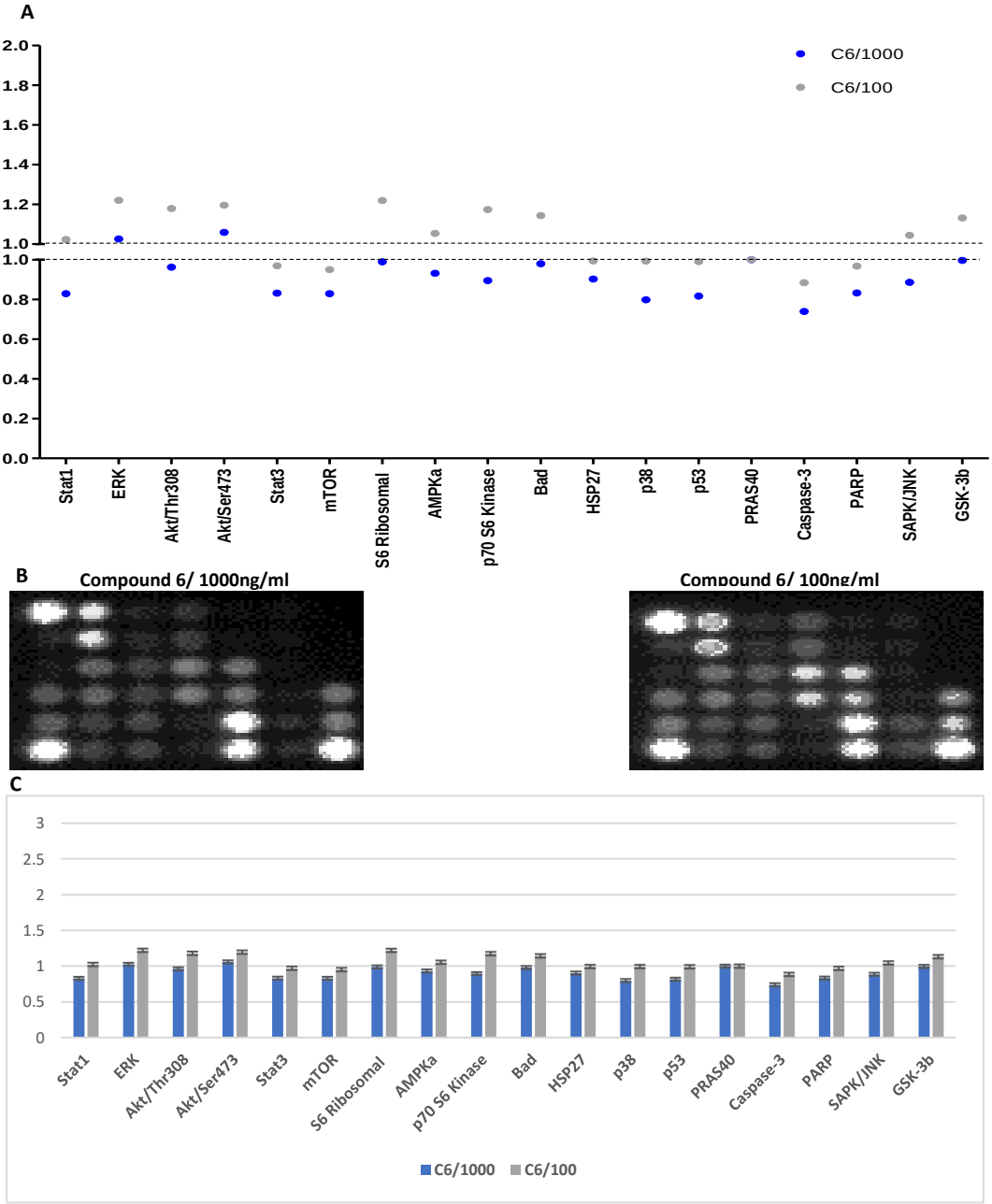


Figure 4.15. Pathscan sandwich ELISA experiment, showing the effect of varying concentration of chemically modified heparin (6) and treatment period of two days. The compound was added at different concentration of 100 and 1000 ng/ml in general stem cell medium without LIF. (**A**) Dot chart graph showing the full data set, with the comparison between the two concentrations and showing error bars from the mean and SD of the three triplicates used. (**B**) Apart from the full path scan card showing the image of the phosphorylation of each concentration with variation in the strength at the phosphorylation. (**C**) Bar chart showing the comparison between the two concentrations and showing error bars from the mean and SD of the three triplicates used.

3.5.3.8 Effect of Compound 7 on the intracellular signalling pathways of stem cells.

The two concentrations 1000 and 100 ng/ml of compound 3 when added to the ESCs culture for a period of 2-days showed a clear difference in the phosphorylation of all proteins on the Pathscan Elisa card. The 100 ng/ml concentration of compound 3 activated the phosphorylation of the proteins on the Pathscan card higher than the 1000 ng/ml concentration, especially with ERK and AMPKa, which showed less activity at the 1000 ng/ml concentration (Figure 4.16)

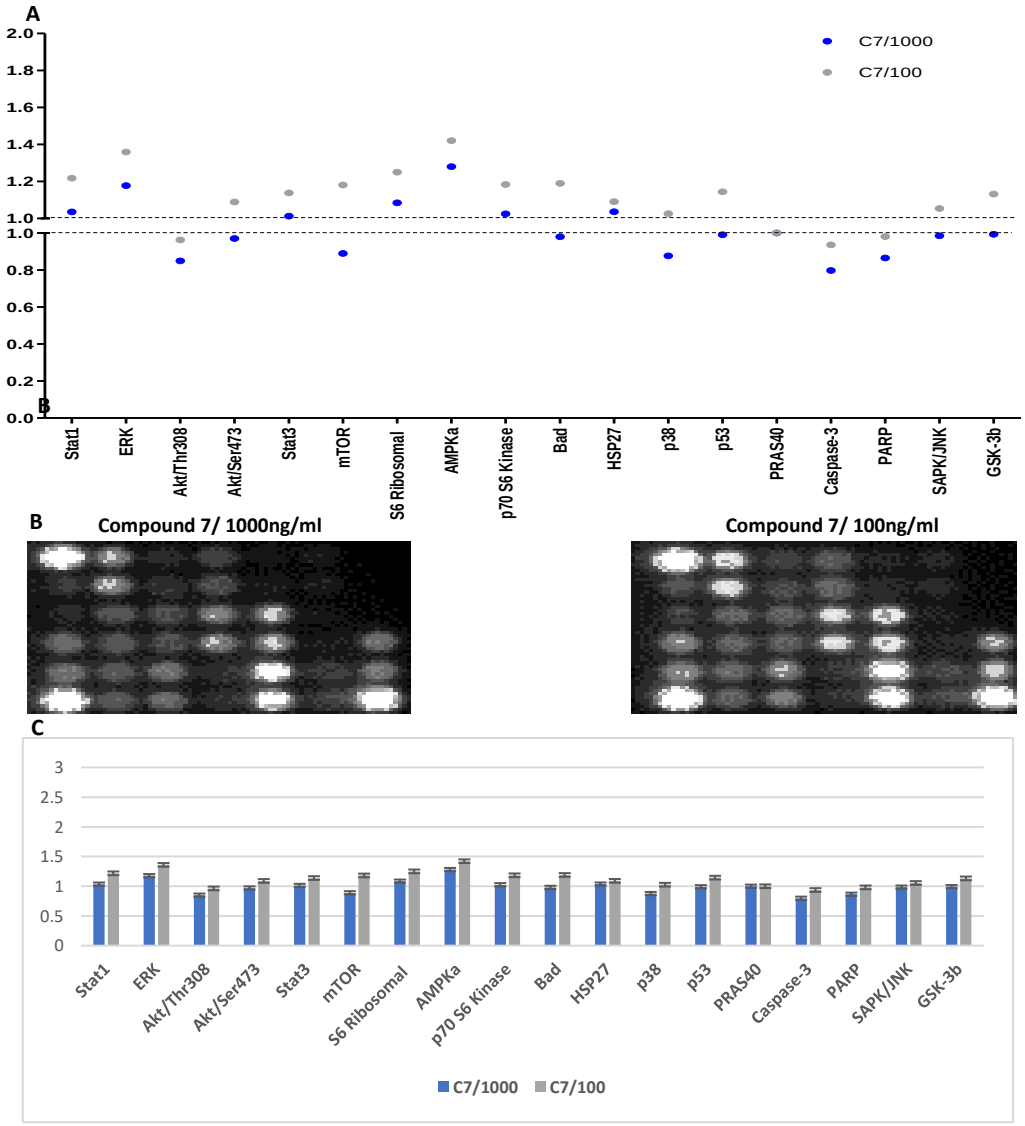


Figure 4.16: Pathscan sandwich ELISA experiment, showing the effect of varying concentration of chemically modified heparin (7) and treatment period of two days. The compound was added at different concentration of 100 and 1000 ng/ml in general stem cell medium without LIF. (**A**) Dot chart graph showing the full data set, with the comparison between the two concentrations and showing error bars from the mean and SD of the three triplicates used. (**B**) Apart from the full path scan card showing the image of the phosphorylation of each concentration with variation in the strength at the phosphorylation. (**C**) Bar chart showing the comparison between the two concentrations and showing error bars from the mean and SD of the three triplicates used.

A summary table of the Pathscan data was prepared (Table 4.4). These highlights key changes in stem cell signalling after the treatment with compounds 1 to 7, at the two concentrations used (1000 and 100 ng/ml). In overall view we can see that the concentration of 1000 ng/ml had the most significant effects in all stem cells treated with the chosen seven compounds, except with compounds 3,6 and 7, for which the data clearly showed that phosphorylation was more active at the 100 ng/ml concentration. Compounds 1 (normal heparin), and 2 were the only compounds that stimulated the phosphorylation of almost all markers with 1000 ng/ml concentration and, on the other hand, almost all markers did not show any activity when using 100 ng/ml concentration. At the two days incubation period, almost all compounds showed different levels of stimulation on stem cells, with higher phosphorylation stimulation of stem cell differentiation markers when using 1000 ng/ml, as mentioned previously. The most active and strongest effect on phosphorylation in stem cells was in the case of treatment with Compound 4 at the concentration of 1000 ng/ml, in this case, Erk and p38 markers were upregulated and stimulated significantly at ~ 2-fold higher levels than all markers in all conditions, PRAS40 and GSK-3b markers were downregulated and stimulated significantly at ~ 0.5-fold lower than all markers under all conditions. PRAS40 marker was the only marker that did not show any activation and all PCR readings except with Compound4 1000 ng/ml were around the control level, with Compound 4 the expression of PRAS40 was significantly downregulated by 0.5-fold lower than all other markers.

	C1 1000	C1 100	C2 1000	C2 100	C3 1000	C3 100	C4 1000	C4 100	C5 1000	C5 100	C6 1000	C6 100	C7 1000	C7 100
Stat1	0.95	0.92	1.21	0.95	0.93	1.02	1.74	0.98	1.02	1.02	0.82	1.02	1.03	1.21
ERK	1.08	0.95	1.52	1.00	1.05	1.29	2.40	1.36	1.78	1.25	1.02	1.22	1.17	1.35
Akt/Thr308	1.14	0.96	1.17	1.07	1.02	1.13	0.74	1.11	1.03	1.03	0.96	1.17	0.85	0.96
Akt/Ser473	1.14	0.97	1.11	1.03	1.07	1.06	1.05	1.06	1.07	1.03	1.06	1.19	0.97	1.08
Stat3	1.04	0.97	1.15	1.00	0.90	1.08	0.82	1.09	1.20	1.10	0.83	0.97	1.01	1.13
mTOR	1.03	0.96	1.24	0.95	0.85	1.09	1.23	1.09	1.03	1.02	0.83	0.95	0.89	1.18
S6 Ribosomal	1.09	0.97	1.21	1.09	1.06	1.25	1.27	1.21	1.17	1.19	0.99	1.22	1.08	1.25
AMPKa	1.09	0.99	1.59	0.93	0.99	1.33	0.89	1.44	1.38	1.26	0.93	1.05	1.28	1.42
p70 S6 Kinase	1.05	0.99	1.25	1.01	0.93	1.30	1.85	1.34	1.02	1.21	0.89	1.17	1.02	1.18
Bad	1.12	0.96	1.57	0.97	0.96	1.15	0.61	1.34	1.16	1.11	0.98	1.14	0.98	1.19
HSP27	1.06	0.90	1.21	0.90	0.95	0.99	0.91	1.01	1.10	0.97	0.90	0.99	1.03	1.09
p38	1.00	0.96	1.07	0.97	0.89	1.08	2.68	1.08	0.92	1.07	0.79	0.99	0.87	1.02
p53	1.11	0.95	1.46	0.98	0.88	1.17	1.50	1.20	1.17	1.04	0.81	0.99	0.99	1.14
PRAS40	1.00	1.00	1.00	1.00	1.00	1.00	0.34	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Caspase-3	0.93	0.90	1.04	0.90	0.82	0.98	0.96	1.00	0.89	0.93	0.74	0.88	0.79	0.93
PARP	1.10	0.98	1.04	0.96	0.91	1.07	1.30	1.05	0.95	0.94	0.83	0.96	0.86	0.98
SAPK/JNK	1.04	0.93	1.18	0.91	1.03	1.06	1.74	1.10	1.01	0.95	0.88	1.04	0.98	1.05
GSK-3b	1.14	0.96	1.40	0.99	1.04	1.23	0	1.29	1.14	1.14	0.99	1.13	0.99	1.13

Table 4.4. Pathscan data analysis for R1 ESCs treated with all 7-chemically modified heparins compared to the 2-day control. Shows all phosphorylated receptors, with significantly upregulated (red) or downregulated (green) RTKs as shown, and (gray) colours represents non-activated data near to the 2-day control phosphorelation.

4.5.4 Effect of chemically modified heparins on heparan sulfate biosynthesis gene expression analysis by qPCR.

During embryonic development, HSPGs have essential functions on the cell surface and in the extracellular matrix. They function as vital co-receptors and allow the generation and maintenance of morphogen gradients through their interaction with multiple exogenous variables. During biosynthesis, the sulfation pattern of the side chains of heparan sulfate HS is established, determining the binding variables and the affinity of its interaction with protein targets. Furthermore, after biosynthesis, endo-6-O-sulfatases (Sulf 1 and 2, see below) or heparanase, an endo-glycosidase that produces HS fragments, may modify the HS chains. In the Golgi compartment, biosynthesis includes the action of several distinct enzymes, some of which involve more than one isoform. The enzymes mentioned most here are Ext1, which together with Ext2 forms the functional HS polymerase, and glucosaminyl N-deacetylase / sulfotransferases (NDSTs), which are accountable for the HS N-sulfation that regulates later enzymatic responses, thus influencing the general composition of HS chains (Tamm, Kjellén and Li, 2012; Multhaupt and Couchman, 2012). Then we also studied the expression of HS biosynthesis genes, to address the hypothesis that the modified heparins might also induce changes in their expression levels in stem cell culture in the lab. Initial experiments were conducted with pluripotent stem cells

followed by culture of stem cells in a medium containing selected chemically modified heparins, and appropriate control conditions with the same medium without any HS compounds.

4.5.4.1 The effect of the chemically modified heparins and their concentration in culture medium.

The treatment of stem cell cultures using the chemically modified heparins is the primary aim of this research. To ensure the best result and the most evident effects and outcomes, the compounds were added to the stem cell medium at two concentrations (100 and 1000 ng/ml). On the other hand, there were 7 chemically modified heparins used; each of the 7 compounds used has different chemical modifications (Table 4.3). In initial experiments all compounds were screened. In subsequent experiments the work focussed on the compounds which had the most significant effects on stem cell signalling measured by pathscan ELISA (section 4.5.3). All modified heparins were tested. Pluripotent stem cells were grown in a pluripotent maintaining medium with LIF, and feeder cells. LIF was withdrawn, and cells treated with 100 or 1000 ng/ml of the compounds for 2 days, Cells were lysed using Buffer RLT, and mRNA extracted using RNeasy kit. Real Time-quantitative PCR (qPCR) was performed using custom-designed TaqMan arrays cards containing probes against genes involved in HS biosynthesis. A summary of the resulting data is shown in (Table 4.5).

	C 7.1000	C 7.100	C 6.1000	C 6.100	C 5.1000	C 5.100	C 4.1000	C 4.100	C 3.1000	C 3.100	C 2.1000	C 2.100	C 1.1000	C 1.100
Adam15	1.16	1.18	2.11	2.62	1.95	1.09	1.61	1.04	1.24	1.89	1.40	1.06	3.81	1.87
Adamts1	1.30	1.62	3.30	2.42	3.33	2.01	2.53	1.27	1.57	2.16	2.20	1.44	7.68	3.20
Adamts4	1.09	1.42	1.82	2.40	1.94	1.37	1.95	1.53	0.51	1.85	1.70	1.47	4.14	1.71
Agrn	1.20	1.53	2.69	2.04	2.42	1.46	2.14	1.28	0.70	2.00	3.02	1.23	5.85	3.26
Chst11	1.19	0.78	1.73	2.39	1.56	0.89	1.16	1.11	2.17	2.30	1.93	1.08	2.72	3.65
Chst15	1.71	2.36	5.07	4.18	4.73	2.56	5.44	2.78	2.67	4.74	2.70	2.89	11.10	3.00
Ext1	1.90	2.48	5.07	4.30	6.09	2.96	3.48	2.87	3.16	3.93	8.68	2.03	23.30	4.55
Ext2	2.04	2.34	4.33	2.99	4.15	3.27	3.66	2.14	1.78	3.16	4.74	2.74	10.85	5.72
Extl1	0.52	0.29	0.67	1.00	0.75	0.41	1.22	0.76	1.37	0.65	4.11	0.52	1.26	4.66
Extl2	1.67	1.94	2.97	2.23	4.00	2.31	3.12	1.92	1.44	3.23	4.00	1.98	9.27	2.75
Extl3	1.59	0.95	2.51	1.66	2.23	1.28	2.53	1.44	1.97	1.77	2.45	1.31	5.26	2.05
Glce	1.57	1.82	3.59	3.62	3.71	2.16	3.85	2.14	1.27	3.10	5.78	1.98	6.82	9.33
Gpc1	1.30	1.36	3.23	2.71	2.50	1.72	1.59	1.42	1.41	1.87	3.41	1.29	6.03	5.95
Gpc2	0.33	1.00	0.43	0.84	0.20	0.20	0.19	0.09	0.45	0.30	7.87	0.09	3.90	1.88
Gpc3	1.00	1.61	1.00	1.69	1.00	1.22	1.00	0.66	1.00	1.00	1.00	1.00	1.00	1.00
Gpc4	1.69	2.38	4.12	3.81	3.75	3.13	3.44	2.05	2.05	3.22	3.30	2.76	12.70	4.37
Gpc5	1.00	0.73	2.28	0.07	1.00	0.24	0.32	2.10	4.00	2.79	19.12	0.38	0.56	1.15
Gpc6	2.35	3.76	5.30	5.39	5.25	3.45	5.13	3.88	1.54	6.84	3.86	2.95	9.95	7.87
Hpse2	0.03	0.03	0.07	0.30	0.00	0.04	0.12	0.01	17.11	0.24	1.04	0.26	0.40	1.34
Hpse	0.12	0.13	0.26	0.22	0.34	0.18	0.32	0.07	0.25	0.17	0.47	0.23	0.53	0.39
Hs2st1	2.40	2.61	4.97	3.73	4.92	3.02	3.91	2.78	2.05	3.44	4.87	2.94	9.15	3.94
Hs3st1	1.92	2.61	4.95	1.24	5.40	2.84	3.83	2.55	1.94	2.68	2.12	3.06	11.76	1.65
Hs3st2	0.01	1.00	0.01	0.96	1.00	0.01	0.02	1.00	15.38	0.09	0.26	0.09	2.51	5.03
Hs3st3a1	1.00	1.00	1.00	33.37	1.00	5.11	1.00	0.29	1.00	0.00	1.00	11.12	1.00	1.00
Hs3st3b1	2.00	2.46	5.51	3.68	4.33	1.51	4.00	2.05	1.28	5.12	5.01	2.84	9.63	3.16
Hs3st4	0.18	0.06	0.00	1.00	0.05	0.32	0.01	0.01	1.30	0.09	0.79	0.30	0.30	0.77
Hs3st5	1.00	16.55	1.00	10.44	1.00	1.13	1.00	1.00	1.00	0.53	1.00	0.58	1.00	6.11
Hs3st6	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.90	1.00	0.02	1.00	1.13	1.00	0.13
Hs6st1	0.51	0.42	0.92	0.57	0.90	0.44	1.03	0.60	1.60	2.23	0.82	0.28	1.57	0.97
Hs6st2	1.13	1.77	3.44	2.07	1.59	1.45	2.17	1.19	1.98	2.30	2.90	1.99	5.42	3.43
Hs6st3	0.35	0.88	1.27	0.89	1.33	0.98	1.05	0.68	0.98	0.82	0.63	0.90	1.48	0.84
Hspg2	1.60	2.00	4.02	4.80	3.87	2.09	2.06	1.95	3.05	5.00	5.14	1.79	10.91	8.46
Lrp1	1.31	2.25	4.20	4.31	3.36	2.30	2.79	1.93	2.69	4.57	3.10	1.95	10.63	7.08
Ndst1	1.37	2.42	4.41	2.25	3.62	1.86	4.13	2.07	1.15	3.13	2.83	2.44	6.90	4.10
Ndst2	2.35	1.97	3.76	2.63	3.36	1.83	2.43	2.31	1.17	2.45	2.89	2.84	5.23	3.05
Ndst3	3.13	0.45	1.24	0.98	1.02	0.86	1.01	0.51	15.08	0.79	9.63	1.36	1.21	1.92
Ndst4	0.01	1.00	0.01	0.09	0.01	0.00	0.75	1.00	1.28	0.07	2.92	0.09	0.75	0.64
Sdc1	0.56	0.57	0.91	1.56	1.15	0.67	1.24	0.53	0.02	0.93	1.56	1.02	1.92	1.23
Sdc2	0.78	1.80	1.49	1.32	2.44	1.46	1.13	1.29	0.68	1.14	1.80	1.49	5.71	1.80
Sdc3	2.39	2.64	5.31	3.70	4.85	3.36	4.43	3.79	2.86	5.33	5.03	2.94	12.40	8.08
Sdc4	1.63	1.69	2.41	2.02	3.55	2.10	2.63	2.27	0.87	1.40	2.39	2.29	6.09	2.57
Sulf1	1.15	2.29	4.20	3.27	3.04	2.00	2.87	1.77	3.14	3.86	21.32	2.45	7.16	20.15
Sulf2	2.30	3.04	4.79	4.71	4.05	2.61	3.40	2.31	1.69	3.79	5.27	2.09	9.63	6.44
Tgfbr3	1.17	1.61	2.16	2.04	2.47	1.92	2.88	1.12	0.75	1.29	2.02	1.43	7.02	2.60
Timp3	1.60	1.78	3.87	3.22	3.49	2.22	2.51	1.88	1.15	2.05	2.28	1.96	7.25	3.31

Significant Upregulation	Upregulation	Significant Downregulation	Downregulation	Negative

Table 4.5. qPCR data analysis showing the result of treatment of R1 stem cells with 7 different chemically modified heparins. Two different concentrations (100 and 1000 ng/ml) were used for each of the compounds for two days, all data were compared to the 2-day control and normalised by 18s rRNA. (light red-upregulation) 1-2 fold change and (red-significant upregulation) 2+ fold; (light green-downregulation) 0.5-1 and (green-significant downregulation) <0.5.

As observed in Chapter 3, chemically modified heparins can play a role in the fate of stem cells, and the effects vary from one compound to another, according to the number and position of the sulfate groups. The concentration of the compounds can also affect the strength or specific action of each compound. Based on the data analysis summarised in (Table 4.5) Figure 4.18 shows the dot graphs for gene expression levels detected by qPCR at each concentration. All modified heparins were tested, pluripotent stem cells were grown in a pluripotent maintaining medium with LIF, and feeder cells. LIF was withdrawn, and ES cells were treated with 100 or 1000 ng/ml with all 7 chemically modified heparins for 2 days, Cells were lysed using Buffer RLT, and mRNA extracted using RNeasy kit. Real Time-Quantitative PCR (qPCR) was performed using custom-designed TaqMan arrays cards containing probes against genes involved in HS biosynthesis. A summary of the resulting data is shown in (Table 4.5). At the two days incubation period, almost all compounds showed different levels of stimulation on stem cells, with higher HS biosynthesis genes expression when using 1000 ng/ml, as mentioned previously (Figure 4.17).

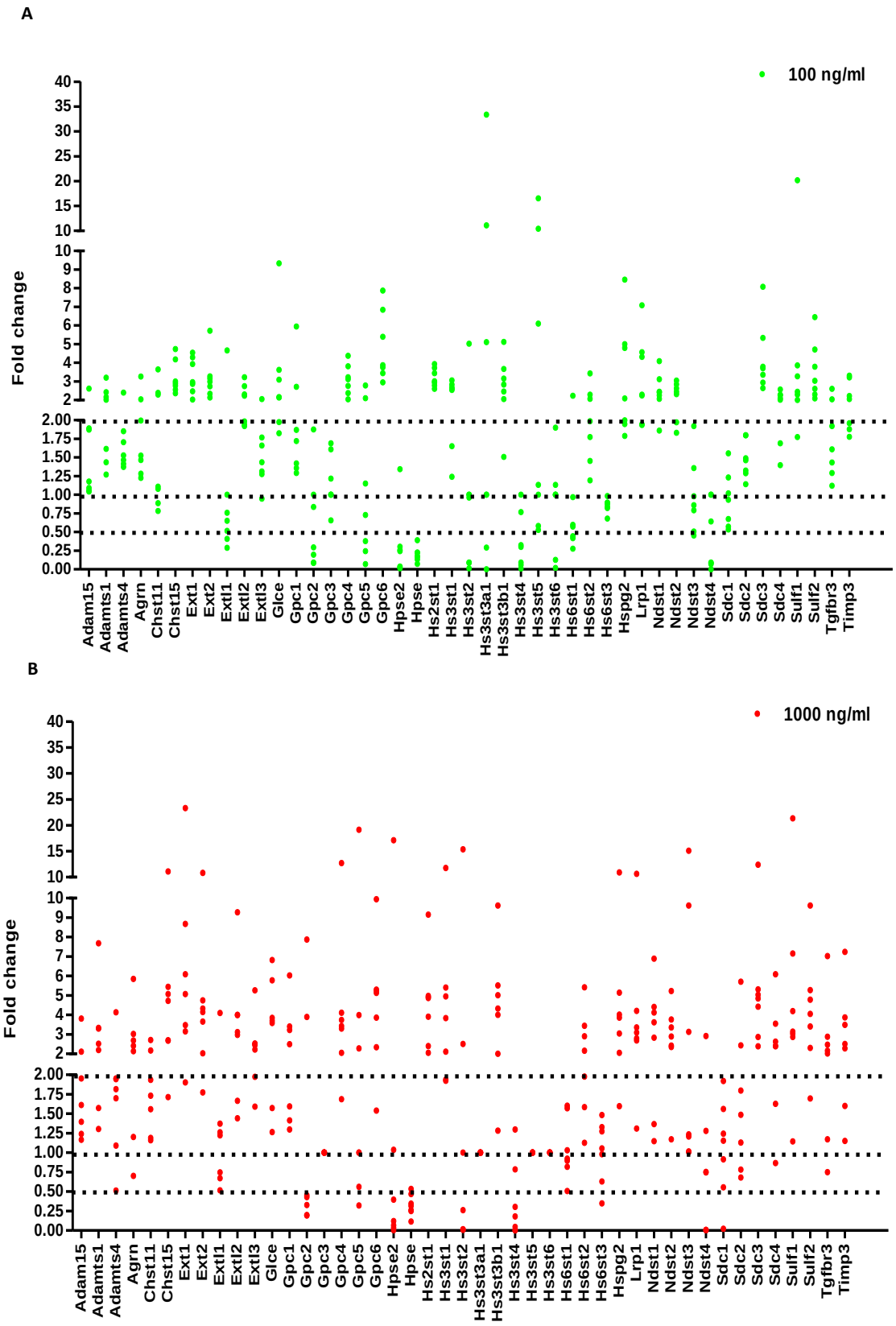


Figure 4.17. Dot chart qPCR data analysis of R1 ESC cultured with 7 chemically modified heparins, at two different concentrations. Duplicate stem cells were cultured for two days, then lysed and analysed for HS gene biosynthesis by q RT-PCR to evaluate the effect of compounds and their concentration. **A**; showing the gene expression at 100ng/ml treatment concentration. **B**. showing data from 1000ng/ml treatment concentration. Each dot indicates a different compound. all data were compared to the 2-day control and normalised by 18s rRNA.

4.5.4.2 The affect of selected chemically modified heparins (4, 5, and 6) on the heparan sulfate biosynthesis during the incubation period.

After testing all 7 different modified heparin compounds at two different concentrations, and based on the experiment findings, compounds 4, 5, 6 were further investigated.On the next qPCR experiments, with an incubation period of 7 days, and at only one concentration 1000ng/ml. R1 treated cells with compound 4,5, or 6 were analysed by qPCR. The experiment was run using two biological control conditions (zero-time and 7-day control). With the zero-time control, there were no significant changes in all HS biosynthesis markers, all data were fluctuating between 0.5 and 2-fold change (Figure 4.18).

4.5.4.2.1 Comparison of compounds 4, 5, and 6 to zero-day control.

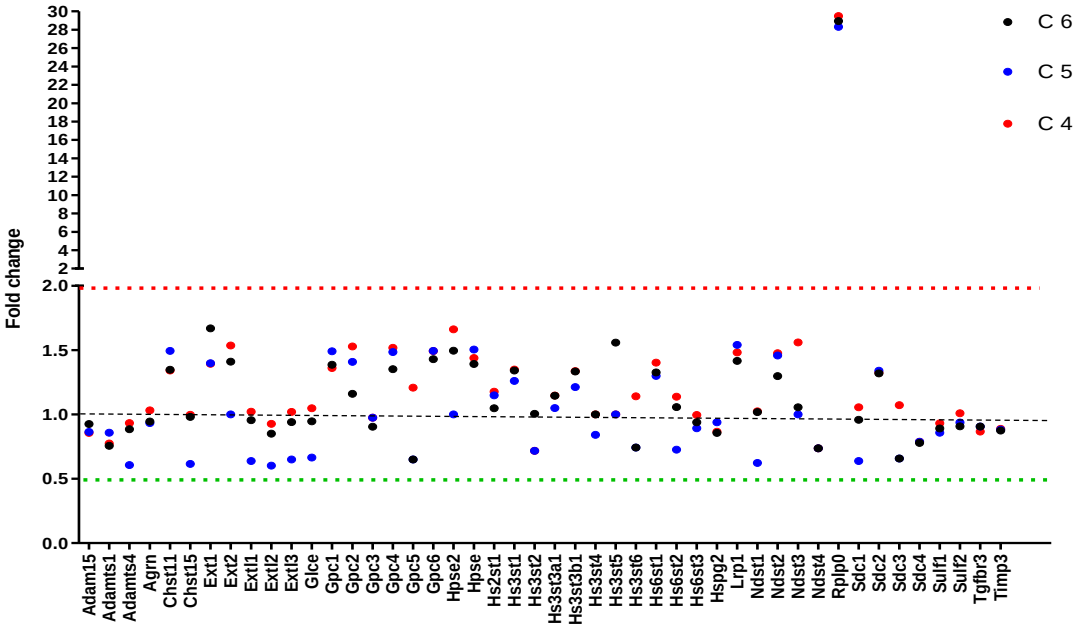


Figure 4.18. Dot chart qPCR data analysis of R1 ESC cultured with 7 chemically modified heparins and compared to zero-time control. Triplicate R1 stem cells were cultured for 7 days, then lysed and analysed for HS gene biosynthesis by q RT-PCR to evaluate the effect of compounds 4,5, and 6 at 1000 ng/ml treatment concentration. All data were compared to the zero-time control and normalised by 18s rRNA.

Table 4.6 gives a quantitative summary of the qPCR data. The data show that Compound 4 was the most effective compound due to the higher number of upregulated HS biosynthesis markers detected in ESC treated by Compound 4, and the data analysis shows that Compound 4 had the least number of downregulated markers, but C5 had the highest number of downregulated HS biosynthesis genes. Compounds 5 and 6 had almost the same effect on R1 ESCs in the treatemnt time by sharing the upregulating effect of 12 markers and the downregulating effect of 3 markers. Other genes like (Gpc3) had no change with any compounds (Table 4.6).

Gene	C4	C5	C6
Ext1	1.395159	1.399316	1.66971
Ext2	1.536058	1	1.411621
Extl1	1.021402	0.637793	1
Extl3	1.020605	0.650668	1
Glce	1.048192	0.665789	1
Gpc1	1.361069	1.491533	1.386555
Gpc2	1.529056	1.410022	1.161285
Gpc3	1	1	1
Gpc4	1.518055	1.485557	1.352953
Gpc5	1.208605	0.650517	0.650517
Gpc6	1.49255	1.495361	1.430844
Hpse2	1.66243	1	1.496152
Hpse	1.438629	1.505584	1.392667
Hs2st1	1.176756	1.14942	1
Hs3st1	1.348967	1.261223	1.342131
Hs3st2	0.71809	0.71809	1
Hs3st3a1	1.147621	1.050885	1.145452
Hs3st3b1	1.336939	1.213086	1.334486
Hs3st4	1	0.84203	1
Hs3st5	1	1	1.559591
Hs3st6	1.14148	0.743397	0.743397
Hs6st1	1.403719	1.300408	1.327432
Hs6st2	1.138921	0.726947	1.057638
Hs6st3	1	0.892817	1
Hspg2	0.86644	1	0.856375
Lrp1	1.483154	1.541773	1.417547
Ndst1	1.025295	0.623336	1.018566
Ndst2	1.477219	1.459202	1.298864
Ndst3	1.560228	1	1.05649
Ndst4	0.736831	0.736831	0.736831
Sdc1	1.055456	0.638717	1
Sdc2	1.326307	1.341168	1.318929
Sdc3	1.07283	0.658143	0.658143
Sdc4	0.787794	0.788797	0.778509
Sulf1	1	0.859183	0.891359
Sulf2	1.009431	0.937814	0.907524
Timp3	0.89344	0.887219	0.879207

Table 4.6. qPCR data of HS biosynthesis gene expression. Showing the data for all compounds compared to zero-time control. Red represents upregulated genes, green represents downregulated genes. All data were compared to the zero-time control and normalised by 18s rRNA.

4.5.4.2.2 Comparison of compounds 4, 5, and 6 to 7-day control.

This section of the HS biosynthesis experiment presents data for gene expression of the HS biosynthesis markers and compared to the 7-day control. R1 ESC were cultured in LIF and feeder conditions, then switched to the differentiation conditions after the withdrawal of LIF and feeder cell depletion. The experiment was performed for 7 days. The calculation has been done by choosing the most stable internal control 18s and the preferred biological control based on the fold change equation, and we obtained these results.

The data analysis of qPCR compared to the 7-day control continued to show the same general findings as the comparison with the zero-time control. Compound 4 was the most active compound showing the highest number of upregulated genes and the lowest number of downregulated genes. Compound 5 was downregulating the highest number of HS biosynthesis markers then comes compound 6 with a moderate effect. (Figure 4.19).

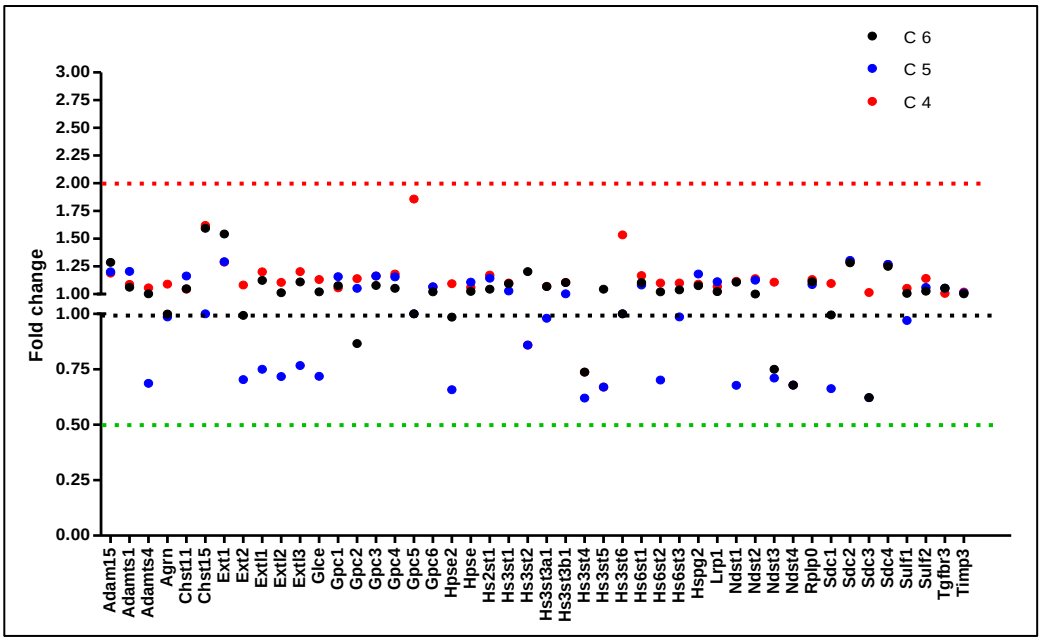


Figure 4.19: Dot chart qPCR data analysis of R1 ESC cultured with 7 chemically modified heparins and compared to 7-day control. Triplicate R1 stem cells were cultured for 7 days, then lysed and analysed for HS gene biosynthesis by q RT-PCR to evaluate the effect of compounds 4,5, and 6 at 1000 ng/ml treatment concentration. All data were compared to the 7-day control and normalised by 18s rRNA.

Table 4.7 gives a quantitative summary of the qPCR data. The data show that Compound 4 was the most effective compound due to the higher number of upregulated HS biosynthesis markers detected in ESC treated by Compound 4, and the data analysis shows that Compound 4 had the least downregulated markers with only 4 markers downregulated compared to 19 markers by Compound 5 and 7 markers by Compound 6. Compounds 5 and 6 had almost the same effect on R1 ESCs in the treatemnt time by sharing the upregulating effect of 19 markers and the downregulating effect of 6 markers. Hs3st4 and Ndst4 markers were both dowregulated in all three conditions of compounds treatment by the three compounds 4, 5 and 6. Overall, data analysis after the comparisons using zero-time control and 7-day control shows the same general effect of all compound tretment conditions, the data in general shows that Compounds 4, and 5 were the most active compounds, and there was a similar effect in both treatments with Compounds 5, and 6. The number of markers that were upregulated in R1 stem cell with all the three compound increased when data analyses wher compared to the 7-day control (Table 4.7).

Gene	C 4	C 5	C 6
Agrn	1.09	0.99	1.00
Ext1	1.29	1.29	1.54
Ext2	1.08	0.70	0.99
Extl1	1.20	0.75	1.12
Extl2	1.11	0.72	1.01
Extl3	1.20	0.77	1.11
Glce	1.13	0.72	1.02
Gpc1	1.06	1.16	1.08
Gpc2	1.14	1.05	0.87
Gpc3	1.16	1.16	1.08
Gpc4	1.18	1.16	1.05
Gpc5	1.86	1.00	1.00
Gpc6	1.06	1.07	1.02
Hpse2	1.09	0.66	0.99
Hpse	1.06	1.11	1.03
Hs2st1	1.17	1.14	1.04
Hs3st1	1.10	1.03	1.09
Hs3st2	0.86	0.86	1.20
Hs3st3a1	1.07	0.98	1.07
Hs3st3b1	1.11	1.00	1.10
Hs3st4	0.74	0.62	0.74
Hs3st5	0.67	0.67	1.05
Hs3st6	1.54	1.00	1.00
Hs6st1	1.17	1.08	1.10
Hs6st2	1.10	0.70	1.02
Hs6st3	1.10	0.99	1.04
Hspg2	1.09	1.18	1.08
Lrp1	1.07	1.11	1.02
Ndst1	1.12	0.68	1.11
Ndst2	1.14	1.13	1.00
Ndst3	1.11	0.71	0.75
Ndst4	0.68	0.68	0.68
Rplp0	1.13	1.09	1.11
Sdc1	1.10	0.66	1.00
Sdc2	1.29	1.30	1.28
Sdc3	1.02	0.62	0.62
Sdc4	1.27	1.27	1.25
Sulf1	1.05	0.97	1.01
Sulf2	1.14	1.06	1.03

Table 4.7: qPCR data obtained from stem cells cultured with chemically modified heparins 4, 5 and 6 compared to the 7-day control. Showing the upregulated and downregulated result of HS biosynthesis genes. Red is representing upregulated genes, green is representing the downregulated genes. All data were compared to the zero-time control and normalised by 18s rRNA.

4.6 Discussion

1. The effect of over-sulfated heparin on ERK phosphorylation.

Following the stem cell differentiation experiments and the detection of the direct effects of chemically modified heparins compounds on stem cell fate, further investigation was needed to understand the underlying mechanisms. It was hypothesized that altered signalling from the surface receptors of the stem cell to the downstream signalling pathways was involved. The first step in the logical research investigation was to study well-known markers or cell signalling pathways to guide further investigation. One well-studied signalling pathway related to cell cycle development, proliferation, differentiation, and carcinogenesis is the extracellular signal-regulated kinase (ERK)/mitogen activated protein kinase (MAPK) signal transduction cascade. The ERK / MAPK pathway transforms the signal of growth factors across the cell membrane by sequentially activating Ras-like GTPase, Raf, Mek, and ERK. (Shaul and Seger, 2007, Pearson et al., 2001). Activated ERK subsequently phosphorylates its downstream targets, such as Elk1, c-Fos, p53, and c-Jun, to modulate transcriptional output (Gille, Sharrocks and Shaw, 1992). ERK signalling is needed in differentiated somatic cells for the

advancement of the cell cycle and cell proliferation, as well as for normal growth (Pearson et al., 2001).

We also sought to examine the effects of adding over-sulfated heparin to mouse ES cells in neural differentiation medium. Previous work in the Turnbull lab has shown that over-sulfated heparin can promote enhanced differentiation of ES cells towards a dopaminergic neuron lineage when the cells are cultured and treated specifically in the first phase of the differentiation protocol in N2B27 media. The working hypothesis was that this compound might be inhibiting FGF signalling and that this would be observed as a decrease in ERK phosphorylation. To address this hypothesis, we planned a western blot experiment. In this ERK-phosphorylation experiment R1 ES cells were exposed to N2B27 media either without the addition of over-sulfated heparin (control plate) or in the presence of over-sulfated heparin, with cells being treated for 5, 25 or 45 minutes. The experiment was undertaken, and results indicated that ERK was phosphorylated in ES cells grown in N2B27 (Fig 4.1 A). The level of ERK and P-ERK were quantitated by scanning the ERK and P-ERK bands, and this data are shown in Fig 4.1 B. In the presence of N2B27 media it was apparent that there was an initial increase in ERK phosphorylation at 5 minutes and then a decrease from this peak level at the 25- and 45-minute time points (Fig 4.1, panel A). However, based on the ratio of P-ERK to ERK, the levels of

phosphorylation were lower at all times points in the present of over-sulfated heparin (Fig 4.1, panel B). Thus, it appears that over S heparin suppresses the peak level of ERK phosphorylation significantly, and perhaps most notably also reduces the level of sustained phosphorylation at the 25- and 45-minute time points. The data supported the hypothesis that over-sulfated heparin acts to inhibit growth factor signalling, at least by FGFs which are known to act via the ERK pathway and are regulated by HS. This inhibitory effect (ie. suppression of FGF signalling) could result in altered priming of the ESCs such that enhanced dopaminergic neural differentiation then occurs downstream. This would be consistent with the fact that FGF signalling is well known to be critical for early stages of neural differentiation (Kunath et al, 2007; Stavridis et al, 2007). Furthermore, our data is also consistent with the work of Li et al. (2011) in which FGFR and ERK inhibitors were able to promote enhanced dopaminergic neuron differentiation when used in similar early-stage cultures for such neuronal cell production.

2. Effect of modified heparins on receptor tyrosine kinase (RTK) signalling

The initial studies showing that over-sulfated heparin could inhibit ERK pathway suggested that a likely mechanism involved

inhibition of cell surface RTKs upstream of ERK. RTKs are commonly expressed transmembrane proteins that act as receptors for growth factors, neurotrophic factors, and other molecules which provide extracellular signalling inputs to cells. At residues in the cytoplasmic tail, they undergo tyrosine phosphorylation after ligand binding. This contributes to the binding of protein substrates and the docking sites for adaptor proteins engaged in the transduction of RTK-mediated signals. In several developmental processes, RTKs have critical functions, including controlling cell survival, proliferation, and motility.

RTK activation was initially tested in R1 stem cells that were cultured in neuron medium with and without the addition of over-sulfated heparin. The experiment was performed for two time periods (5 and 25 minutes), and it was clear from the results obtained (Figure 4.3) and by the detection of phosphorylated tyrosine on activated receptors that over-sulfated heparin downregulated phosphorylation of a broad range of tyrosine kinase receptors, including FGF receptors (Table 4.8).

This supports the western blot data on suppression of ERK phosphorylation obtained in section (4.5.1) and indicates a plausible mechanism of action for over-sulfated heparin. It is also consistent with the Li et al. (2011) report that chemical FGF inhibitors can induce enhanced dopaminergic neural differentiation, and suggests that over-sulfated heparin acts to enhance dopaminergic neuron differentiation at least in part by inhibiting FGF signalling. However, based on the present

results we cannot exclude the possibility that inhibitory effects on other pathways (as shown here) are also involved in this altered priming response of the ESCs. Indeed, it is also possible that the FGFR inhibitor (PD173074) used by Li et al (2011), which targets the FGFR 1 RTK, might also be affecting other RTKs, since it is known to have cross-specificity for inhibition of other RTKs such as VEGFR, PDGFR and others (Mohammadi et al, 1998).

Our data are also consistent with the fact that intracellular inhibitors of RTK signalling are widely involved in the regulation of developmental signalling in NSPC and often determine developmental outcomes of RTK activation (Annenkov, 2013), including neuron lineage commitment.

	5 min incubation	25 min incubation
EGF R	1.913007703	1.239333667
FGF R2	1.318225957	0.870145885
FGF R3	1.864428054	1.149473684
FGF R4	1.285264869	0.908535382
PDGF R α	1.639825283	0.792952435
PDGF R β	1.390677735	0.850097014

Table 4.8: Receptor Tyrosine Kinase (RTK) data analysis of R1 stem cells incubated with over-sulfated heparin for 5 or 25 minutes. The table shows that over-sulfated heparin downregulated the phosphorylation of the RTK with time. All R1 stem cells in the 25-minute incubation with over-sulfated heparain showed lower phosphorylation than in the 5-minute incubation condition. Over-sulfated heparin concentration, 1000 ng/ml; n=3. Control level is 1.

3. Comparing selected modified heparin compounds to zero- and 7-day controls

In comparing the effects of all the modified heparin compounds to the zero and day 7 controls we found here the incubation time and type of chemically modified heparin used for stem cell treatment all affected RTK phosphorylation.

The results obtained from the incubation of all compounds with stem cells for 7days showed that compound 4 and over-sulfated heparin had almost the same effect on the downregulation of many the tyrosine kinase receptors. On the other hand, and in contrast, compounds 5 and 6 upregulated the RTKs and had similar effects to each other.

Over-sulfated heparin continued to downregulate the same receptors that were shown in the 5- and 25-minute time point experiments (Figure 4.3). Compound 4, as mentioned, shared the same behaviour as the over-sulfated heparin except that it upregulated (EGFR) when compared to the 0 time and 7-day control. The over-expression and mutation of the epidermal growth factor receptor (EGFR), along with other cellular pathway mutations, plays a significant role in GBM maintenance and progression (Ayuso-Sacido et al., 2009). Effects on EGFR however were unexpected since EGF is not a heparin-binding growth factor, so the potential mechanism of this effect is not yet clear. Overall, due to the high similarity of compound 4 behaviour on RTKs

to the effects of over-sulfated heparin, they may share similar effects on ERK phosphorylation and other signalling events, resulting in similar biological behaviour of treated stem cells. Compound 5 and compound 6 upregulated most of the RTKs and they shared the same effect of upregulation on 4 receptor types (Insulin R, IGF-I R, VEGF R2, and EphB4). Significantly, all compound shared the upregulation of RTK phosphorylation of VEGF R2. The higher increase in this receptor phosphorylation is unexpected due to the inhibition of ERK pathway; for example, inhibition of the ERK pathway markedly promoted the differentiation of smooth muscle cells from early vascular progenitors (VPs) from hiPSC-derived mesodermal cells (Harding et al., 2017). Overall our results are interesting as they demonstrate that differential regulation of RTK phosphorylation events can be achieved by modified heparins in a structure-dependent manner.

4. Pathscan ELISA experiments

Phosphorylation and proteolysis are two frequent post-translational covalent changes that constitute important biological regulatory processes. Detecting these changes on a collection of cellular proteins that play a well-understood part in cell biology can provide a useful view of the status of intracellular signalling.

Experiments were designed to investigate and find the chemically modified heparin compounds that had the most significant effects, and

the best concentrations for applying them, in the differentiation experiments. For each condition and incubation period we cultured triplicate biological samples for a period of 2 days, and two controls; a zero-time control (which indicates the primary state of the stem cell before any treatment), and a 2-day control (without any compound treatment and with the same conditions of all other conditions for 2 days).

Comparison between the two control conditions (2 day and zero-time controls):

The phosphorylation of the zero-time control was with all receptors higher than the 2-day control. This is likely due to the stem cell in the primary state having limited external stimulating factors; the medium was maintained with LIF to retain cell pluripotency. The 2-day control condition after withdrawal of LIF and depletion of feeder cell represents the effect of the changed medium only, without compounds.

Comparison between all modified heparin compounds and their different effects on mESCs:

ERK: The phosphorylation of ERK was with all tested compounds nearly the same as in the 2-day control, except that with compound 4 it was significantly elevated (at both concentrations, 1000 and 100 ng/ml). However, compound 5 at 1000 ng/ml also caused upregulation

of P-ERK. Phosphorylation of ERK is important because its activation is required for exiting from pluripotency to neural differentiation. ERK activity suppresses pluripotency gene expression to induce endoderm specification, and ERK signalling duration determines the distinction between priming and differentiation. Nanog blocks Gata6 induction but not the inhibition of pluripotency by ERK (Hamilton and Brickman, 2014). It has been suggested that ERK activity is necessary for differentiation of all three germ layers in vitro (Kunath et al., 2007). Moreover, *in vivo* research on multiple FGF-ERK mutants show that this pathway is necessary for early endoderm differentiation (Chazaud et al., 2006).

Stat1: The phosphorylation of Stat 1 was highly upregulated only with compound 4, whereas other compounds produced regular phosphorylation nearly the same as the phosphorylation of the 2-day control.

p38 and p53: Compound 4 when added to the medium for 2-days induced higher phosphorylation compared to all other compounds. Elevation of p38 phosphorylation was significant at 1000ng/ml, but not at 100 ng/ml concentration. A similar effect was noted on p53 but the upregulation was not significant. p53 functions at multiple stages of embryonic development (Jain and Barton, 2018), as well as in ageing (Tyner et al., 2002). Normal differentiation, development and ageing require p53 levels to be precisely regulated spatially and temporally

(Jain and Barton, 2018). P53 can cause ESC differentiation by immediately suppressing the expression of Nanog, one of the ESC self-renewal master transcription factors (Lin et al., 2004). The p38 protein kinase-activated protein pathway (p38MAPK) inhibits neurogenesis and is required for mesodermal formation (Hadjal et al., 2013).

PRAS40 (AKT1): The phosphorylation of PRAS40 was not affected with most compounds, being equal to the control phosphorylation with no statistically significant differences. Surprisingly, the exception was compound 4 which significantly downregulated PRAS40 phosphorylation. PRAS40 is an essential regulatory component that can determine cell survival through apoptotic caspase 3 activation. Akt pathway controls cell survival and mTOR activity through the inhibitory post-translational phosphorylation of PRAS40 that leads to subcellular binding of PRAS40 to the cytoplasmic docking protein 14-3-3 (Chong et al., 2012).

SAPK/JNK: Compound 4 showed interesting activities on protein phosphorylation in the Path scan ELISA experiment. Its unique behaviour was notable with most of the proteins being different than with all other chemically modified heparins. Again compound 4 was the most active compound on stem cells, causing upregulated phosphorylation of SAPK/JNK when incubated for two days. In contrast, other compounds demonstrated similar SAPK/JNK phosphorylation similar to the two-day control. Compound 5 was also

interesting due to its similar effects to over-sulfated heparin and compound 4. Compound 6 was the least active compound and shared similar effects with compound 1 (standard unmodified heparin). These selected compounds were used in all experiments after the general screening undertaken in initial experiments, which provided an overview on the effect of all compounds. At the final step of this experiment, we chose to work also with over-sulfated heparin due to its higher sulfation and the need to investigate the effects of that compound in the neural differentiation and the underlying signalling mechanisms.

Effect of modified heparins on HS biosynthesis gene expression-analysis by qPCR.

1. **The effect of concentration of 7 chemically modified heparins in culture medium.**

   The first step in this experiment was to investigate the effect of different chemically modified heparins using two different concentrations (100 and 1000 ng/ml). The data obtained from the qPCR and after analysis of data listed in Table 2, showed that the chemically modified heparins showed the strongest trend towards effects at 1000 ng/ml (Figure4.2), but did not reach statistically significant levels. In most cases both concentrations had almost the same effect when there was an increase or decrease in the gene expression. There were some

significantly downregulated HS biosynthesis genes, and other genes were significantly upregulated.

2. **The effect of selected chemically modified heparins (4, 5, 6) on HS biosynthesis during the incubation period.**

   Comparing the gene expression of (4, 5, and 6) chemically modified heparins to zero-time control.

   After data analysis and the deletion of the primary stem cell condition by comparing the compound with zero-time control, this comparison aimed to show the apparent change of the stem cell gene expression after the 7-day incubation with the compounds. However, for this comparison it must be noted that the day-zero control did not have the same conditions of incubation and medium and other external factors may change or play a role in the result. Comparison with the 7-day control is discussed separately below. The strongest downregulation effects on HS biosynthesis gene expression were by compound 5; compound 4 on the other hand was the most active regulator of stem cells for expression of upregulated genes, while compound 6 had the highest number of negatively expressed genes (Extl1, Extl3, Glce, Hs2st1, Hs6st2) compared to the pluripotent stem cell zero-time control.

   It was notable that the Gpc3 (glypican-3) gene was downregulated in all conditions. Cell surface heparan sulfate proteoglycans are composed of

a membrane-associated protein core substituted with a variable number of HS chains. Members of the glypican-related integral membrane proteoglycan family (GRIPS) contain a core protein anchored to the cytoplasmic membrane via a glycosylphosphatidylinositol linkage. These proteins may play a role in the control of cell division and growth regulation (Sarrazin, Lamanna and Esko, 2011). Also notable was that Hs3st5 HS biosynthesis gene was downregulated by compounds 4 and 5 but upregulated by compound 6. Hs3st5 belongs to a group of heparan sulfate 3-O-sulfotransferases that transfer sulfate from 3-prime-phosphoadenosine 5-prime phosphosulfate (PAPS) to heparan sulfate and heparin (Xia et al., 2002). The Ndst4 gene was also downregulated in all stem cells with all compounds, and this could potentially result in altered patterns of N-sulfation in HS.

Comparing the gene expression of chemically modified heparins (4, 5, and 6) to 7-day control.

The 7-day control is when stem cells are cultured in a medium containing the same conditions as treatment conditions but lacking the chemically modified heparins. This condition, after the data analysis, will take account of any other effects other than the specific effect of the compounds. From data shown in Table 5, compound 5 continued to be the least active compound in stimulating the HS biosynthesis genes, and compound 4 had the same effect on the biosynthesis gene expression. The change in the primary analysis from 0-time control and 7-day

control did not alter the results in general, possibly indicating that the behaviour of the stem cells was relatively stable in the culture conditions used. Ext2, Hpse2, Ndst3, and Sdc3 were all downregulated in in response to both compounds 4 and 6. Ndst4 was downregulated in all treatment conditions compared to the 7-day control. Overall this suggest that one response to exogenous treatment with modified heparins is altered expression of HS biosynthesis genes, and related genes for core proteins and processing enzymes. Such effects have not been previously described in the literature, though one report has indicated that heparin treatment of smooth muscle cells can alter endogenous sulfation of HS (Skaletz-Rorowski et al, 1996), which would be consistent with this idea. Although specific effects of each compound on expression of genes related to HS biosynthesis gene may be significant for lineage commitment of stem cells, it may be that these lineage effects were earlier and more apparent than the changes in the HS biosynthesis, which occurred later. The research aim was to focus directly on the affect of the chemically modified heparins on the fate of stem cells and understand the mechanism behind stem cell behaviour or commitment toward specific or multilple lineages directions, with in the incubation periods chosen when culturing ESCs under different conditions. The model in (Figure 4.20) describes and shows the working model that the research were built on when performing lab experiments and planning all lab techniques to record and proof any answers towards the research hypothesis. In chapter 3 we performed (qPCR, and RT-PCR) to

investigate ESCs gene expression and fate commitment in different periods of time incubations. Chapter 4 was the mechanism investigating chapter, the research explained the mechanisms of external chemically modified heparins effects on the intracellular signalling and HS biosynthesis by testing the effect on RTKs receptors, testing downstream signalling pathways and testing HS biosynthesis genes expression.

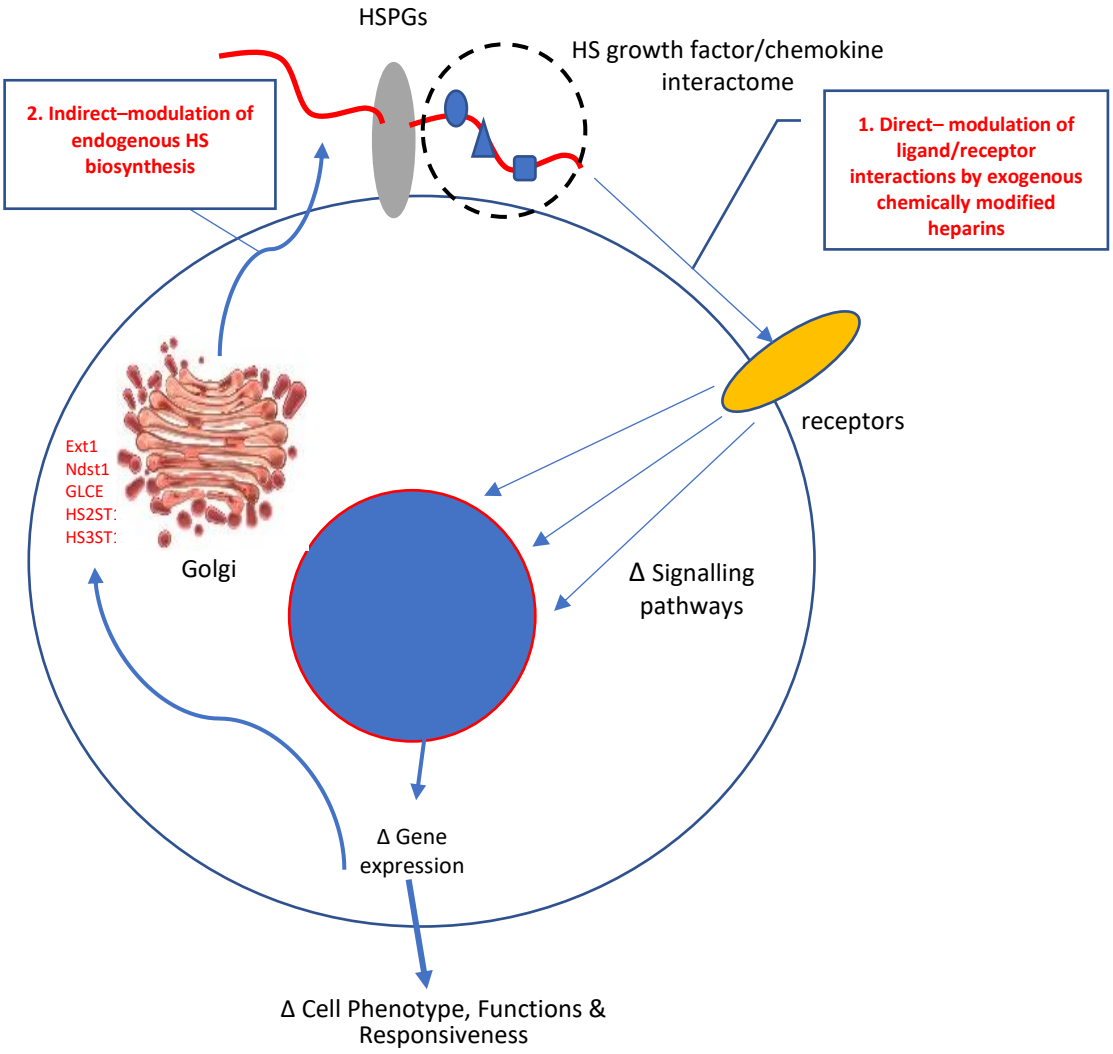


Figure 4.20. Model for the effects of chemically modified heparins on stem cells. The regulation of cell signalling pathways by HSPGs and other growth factors shows the process of gene expression and stem cell fate commitment, and the modulation of endogenous HS by 1- direct and indirect modulations.

Chapter 5

General Discussion and Future Directions

Heparan sulfate is a dynamic, structurally complex biomolecule which regulates multiple biological processes because of its ability to bind numerous proteins and to facilitate many signalling pathways for growth factors (Turnbull, Powell and Guimond, 2001). HS chains control developmental signals by acting as co-factors within a multitude of processes involving but not restricted to maintaining co-receptor functions and morphogen gradients (Lin, 2004; Kreuger et al., 2006) and furthermore HS is a significant stem cell fate regulator. Recent attempts have used multiple chemical and genetic methods in stem cell self-renewal and differentiation to tackle the function and structure-function relationship of HS. HS is abundantly present on the cell surface of undifferentiated and differentiated ESCs (Holmborn et al., 2004). Recent studies have demonstrated changes in HS structure as ESCs undergo differentiation, and that particular HS epitopes appear on subpopulations of differentiated ESCs, suggesting that HS regulates the growth of ESC differentiation and cell lineage (Baldwin et al., 2008, Nairn et al., 2007).

R1 mouse stem cells from the ATCC were the type of stem cells used here in testing the effect of the chemically modified heparins on stem cell fate from self-renewal differentiation. The maintenance of the R1 stem cells (before the differentiation or signalling mechanism culturing protocols) was by using LIF and feeder cells and following standard stem cell

culturing protocols to maintain healthy and pluripotent stem cells for experimental use.

Leukaemia inhibitory factor (LIF) is a cytokine class 6 interleukin that controls a wide range of features of development. LIF receptor (LIFR) associates with gp130 and activates JAK / STAT and MAPK signals (Auernhammer and Melmed, 2000) after LIF binds to the LIF receptor (LIFR). To maintain the mouse embryonic stem cell phenotype, LIFR activation of STAT3 is crucial, by controlling the proliferation, invasion and differentiation of trophoblasts after blastocyst attachment (Auernhammer and Melmed, 2000). We found that using feeder cells that secrete LIF, and provide a suitable stem cell culture environment, with additional external recombinant LIF, was effective in maintaining the pluripotency of the stem cells; this was clear in RT-PCR gel-electrophoresis data described in Chapter 3.

In feeder-free LIF free conditions we found that over-sulfated heparin has a unique effect on ERK phosphorylation, where our results indicated a significant downregulation of phosphorylation (Figure 4.1). This experiment used over-sulfated heparin in a neural stem cell medium for extended times, with ERK phosphorylation decreasing with longer times of incubation. The data supported the hypothesis that over-sulfated heparin acts to inhibit signalling, possibly by FGFs, and this effect is known to result in enhanced dopaminergic neural differentiation (Li et al. 2011).

From all 7 different chemically modified heparins we chose 4 to establish the differentiation experiments using qPCR and RT-PCR. The selection of these compound was based on mechanism experiments to evaluate effects on signalling pathways and the downstream signalling pathways likely to be involved in different stem cell biological process supporting pluripotency, proliferation or differentiation.

A list of chemically modified heparins were chosen as described in Chapter 4, and we found that phosphorylation of cellular proteins in pluripotent stem cells was higher than the day-2 control; this is due to the fact that the stem cells after two days without any pluripotency maintenance conditions undergo differentiation even without the effect of the compounds. The 2-day incubation of the 7 chemically modified heparins showed the significant effects of compound 4 (2de-Sulfated-N-Sulfated-Heparin) on cells; the compound 4 effects were notably in a completely different direction to all other compounds, especially at the 1000ng/ml concentration. Other research supports these findings; for example, in MSC aggregates, a de-sulfated heparin cell coating promotes more chondrogenic distinction than an over-sulfated heparin coating (Jennifer and Johnna, 2016). The variation in the sulfation patterns if over-sulfated or de-sulfated on the other hand, may cause more specific biological effects on other kinds of cell, especially as shown here for embryonic stem cells (Table 4.8). Over-sulfated heparin and compound 4, 5, and 6 were chosen due to their effects on protein phosphorylation and

signalling mechanisms, and thus provided a wide range of differently modified heparin structures for the evaluation of effects on stem cell proliferation and differentiation.

The protein tyrosine kinases (RTKs) are a significant and varied family of multiple genes found in metazoans. Their main functions are to regulate the organism's multicellular aspects. Signals of development, differentiation, adhesion, motility, and death from cells to cells are frequently transmitted through tyrosine kinases (Robinson, Wu and Lin, 2000). To continue our investigation on the chemically modified heparins using the most active concentration option (1000 ng/ml was selected), Pathscan ELISA phosphorylation data was used to select 4 compounds (2, 4, 5, and 6). As a first investigation we used over-sulfated heparin, incubated for a period the same as that used in the western blot experiment. The findings supported our western blot finding, namely downregulation of P-RTK (including potentially P-FGFR) and then downstream reduction in P-ERK. It was clear that phosphorylation decreased from 5 min to 25 min, supporting the fact that over-sulfated heparin mediates its effect on ERK phosphorylation at least partly by downregulating phosphorylation of RTK receptors (Figure 4.2).

We also performed a more extensive experiment by using a 7-day incubation period, and analysed the data compared with 0-time control and 7-day control. We found that the time period of incubation and the type of chemically modified heparin significantly affected RTK phosphorylation and stem cell fate commitment. The results obtained

from the incubation of all compounds with stem cells for 7-days showed that compound 4 and over-sulfated heparin had almost the same effect on the downregulation of many RTKs. On the other hand, compounds 5 and 6 had similar effects to each other by upregulating the RTKs.

Over-sulfated heparin continued to downregulate the same receptors that were shown in the 5- and 25-min experiments (Figure 4.3). Compound 4, as mentioned, shared the same behaviour as the over-sulfated heparin except that interestingly it upregulated EGFR when compared to the 0 time and 7-day control. The over-expression and mutation of the epidermal growth factor receptor (EGFR), along with other cellular pathway mutations, plays a significant role in GBM maintenance progression (Ayuso-Sacido et al., 2009). The high similarity of compound 4 behaviour on RTKs as over-sulfated heparin, suggests that they may share the same effect on ERK phosphorylation and other biological behaviours on stem cells.

Compound 5 and Compound 6 upregulated most of the RTKs, and they shared the same effect of upregulation on 4 receptors (Insulin R, IGF-I R, VEGF R2, EphB4). Significantly, all compounds shared the regulation of RTK phosphorylation of VEGFR2. The high increase in this receptor phosphorylation is expected due to the inhibition of ERK pathway, which results in induced early vascular progenitors (VPs) from hiPSC-derived mesodermal cells, and the inhibition of the ERK pathway markedly promoted the differentiation of smooth muscle cells (Harding et al., 2017). PCR was performed to investigate the role of HS biosynthesis as an

essential biological process, and to continue the investigation on the mechanism of the effect of chemically modified heparin compounds on stem cell fate decision. The data obtained from Table 4.5 in Chapter 4, indicated that compound 5 was the least active compound in stimulating expression of HS biosynthesis genes in both data comparisons with the two controls, and compound 4 had the same effect (as a compound highly active on stem cells) that stimulates the altered expression of biosynthesis genes. The change in the primary analysis from zero-time control and 7-day control did not change the result in general, indicating that the behaviour of the stem cells was stable in this respect under control conditions. This does not negate the specific effect of each compound on the expression of HS biosynthesis genes but means that other experiments investigating the effect of the compounds on the lineage commitment within the maximum incubation period of 7 days were earlier and more apparent than the changes in the HS biosynthesis.

To investigate mRNA expression in stimulated stem cells for the effect of the chemically modified heparins compounds, two experiments were used; RT-PCR and qPCR. In the first stem cell differentiation experiment, the pluripotent stem cells incubated under pluripotency maintenance condition showed higher expression of three pluripotent genes indicating the pluripotent primary state, and this result is supported by most of the research that indicates that LIF and feeder cells are pluripotency maintenance factors. The zero-time control cells did not express any differentiation genes as expected (Figure 3.2). The 2-day

control was incubated in differentiation medium and under feeder-free and LIF-free conditions without any modified heparins. After two days we observed a gradual decrease in pluripotent gene expression on the gel electrophoresis RT-PCR products, and the appearance of some of the differentiation markers. In the 7-day control cells pluripotent markers were completely downregulated, but the differentiation markers also decreased in number and level of expression (Figure 3.2). The main aim from the experiment was to test the effect of the chemically modified heparins and the differences in effects between them. Further experiments were to examine the specific effects of each compound.

When evaluating the results obtained from the two incubation periods and from each compound individually compared to the three control conditions (Figure 3.7) we could clearly see that the Ectoderm lineage genes were completely absent (no expression of the two Ectoderm lineage markers, Nestin and Otx2, expressed with all compound conditions at both two incubation periods). And the Endoderm marker AFP was essentially negative, except with compound 5 where a minimal amount of gene was expressed. GATA-4 was continually expressed in the 7-day compound treated conditions, and compound 5 again was the compound with the greatest effect on this gene, representing the Endoderm lineage. The Endoderm gene PDX-1 was expressed at 7 days of treatment; this gene was expressed in stem cells incubated in medium treated by compounds 4 and 6, but not 5. There was a clear change of gene expression between different incubation times, and there was a

difference in levels of gene expression from one compound to another in each time condition (Figure 3.7).

The qPCR results thus confirmed the RT-PCR results that each compound had a unique effect on the behaviour of stem cells after considering any changes noted in the control cells.

- **Future Directions**

Overall this study provides a bridge between chemically modified heparins and control of stem cell fate decisions. The data highlight the importance of the structure of the HS component in stem cell regulation. This study has also provided novel findings on regulation of signalling pathways for stem cell control. Further applications can be investigated concerning exogenous use of modified heparins on stem cells. However, this project has also opened up avenues for further research questions such as:

1- What regulates the structural changes of HS during stem cell pluripotent state, at the proliferation state, and within the differentiation state?

2- What other signalling pathways are affected by changes in HS during the stem cell pluripotency state or after the shift from pluripotency towards differentiation and fate commitment?

3- Can we precisely direct stem cells to a range of specific lineage fate decisions using any of the chemically modified heparins?

4- Understanding how and why the structure of HS is regulated remains a significant challenge in the HS field. In this project, we investigated different chemically modified heparins, including regular heparin and over-sulfated heparin. The results confirmed that there is a specific effect of each compound on signalling pathways during differentiation, resulting in altered gene expression by the stem cells treated by the modified compounds. Evidence was also found that HS biosynthesis was changing and regulated by time and linked to stem cell fate decisions. Therefore, further experiments to evaluate the biosynthetic protein levels and to better understand the activity of HS biosynthesis enzymes, and their regulation, is required.

5- Future experiments might involve investigating the effects of modified heparins on FGF signalling and ERK phosphorylation, and possibly other signalling pathways, and may lead to new knowledge in the field of stem cell fate commitment.

6- Due to the critical effect of the incubation time and different chemically modified heparins and their concentrations, further research could investigate the effect of multiple additional concentrations and other culture conditions, and timing and length of incubations. For example, the use of different unique mediums (including modified heparins) to change the environment to achieve specific stem cell responses such as lineage commitment could be investigated.

7- Working in stem cell research is very critical and sensitive, because of mechanical or handling effects on stem cells, and due to the sensitivity of changing the standard conditions for stem cells. Future stem cell research could be carried out under continuous feeder-free serum-free conditions to avoid potential stresses to cells when being switched from feeder cell support to feeder-free experimental conditions. It might also be possible to develop better feeder-free conditions exploiting modified heparins to maintain pluripotency.

Concluding Remarks

1- Over-sulfated heparin downregulated ERK phosphorylation, directing stem cellS to a dopaminergic neuronal lineage. Downregulation of multiple P-RTKs (including potentially P-FGFR) was evident and this could explain the downstream reduction in P-ERK.

2- Clear results and findings indicated that differently modified heparin compounds had specific effects on stem cell fate decisions.

3- The modified heparins differentially affect cell signalling processes, providing a plausible mechanism for their differential effects on stem cell fate.

4- The incubation period and concentration of extracellular factors such as heparins added to the stem cell medium or culture conditions, have specific effects on stem cell fate.

5- Further studies on the effects of modified heparins on stem cell fate might lead to useful applications in biomaterials and tissue engineering

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