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Glycosaminoglycans as regulators of stem cell differentiation

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Abstract

ES (embryonic stem) cell differentiation is dependent on the presence of HS (heparan sulfate). We have demonstrated that, during differentiation, the evolution of specific cell lineages is associated with particular patterns of GAG (glycosaminoglycan) expression. For example, different HS epitopes are synthesized during neural or mesodermal lineage formation. Cell lines mutant for various components of the HS biosynthetic pathway are selectively impaired in their differentiation, with lineage-specific effects observed for some lines. We have also observed that the addition of soluble GAG saccharides to cells, with or without cell-surface HS, can influence the pace and outcome of differentiation, again highlighting specific pattern requirements for particular lineages. We are combining this work with ongoing studies into the design of artificial cell environments where we have optimized three-dimensional scaffolds, generated by electrospinning or by the formation of hydrogels, for the culture of ES cells. By permeating these scaffolds with defined GAG oligosaccharides, we intend to control the mechanical environment of the cells (via the scaffold architecture) as well as their biological signalling environment (using the oligosaccharides). We predict that this will allow us to control ES cell pluripotency and differentiation in a three-dimensional setting, allowing the generation of differentiated cell types for use in drug discovery/testing or in therapeutics.

ES (embryonic stem) cells

Pluripotent self-renewing ES cells have significant potential applications in tissue and cell engineering. Their capacity to form any cell type of the adult body makes them excellent candidates for the treatment of diseases for which there is currently no cure available such as diabetes mellitus and Parkinson's disease [1,2]. However, there are significant hurdles which remain to be conquered before ES cells (or indeed the newly isolated induced pluripotent stem cells which share the potential of ES cells) can have large-scale application. Perhaps the most significant among these is the issue of defining and recreating a suitable culture environment for the cells. Indeed multiple environments are necessary as many groups have now demonstrated that the substrata and media requirements for the maintenance of a self-renewing pluripotent stem cell population are distinct from those for directed differentiation, and that each lineage is likely to have additional specific requirements. It appears likely that, as has been shown for adult stem cells, the behaviour of ES cells is influenced by both soluble (bio)chemical factors and by mechanotransduction via interaction of the cells with fibrous components of their substrata or with other cells [3].

ES cells have a characteristic phenotype with tight cell/cell junctions and a high nucleus/cytoplasm ratio leading to the growth of colonies with poorly defined cell/cell boundaries. This is particularly obvious when the cells are grown on a feeder cell layer (typically embryonic fibroblasts) which has traditionally been used as an optimal substratum for the derivation and culture of ES cells [4]. ES cells have previously been used as a model with which to understand early matrix protein deposition, and the importance of matrix proteins such as laminin, fibronectin and perlecan for early differentiation has also been demonstrated, perhaps most elegantly using the EB (embryoid body) method of differentiation [5–7].

Maintenance of pluripotency and self renewal of murine ES cells *in vitro* can be supported in feeder-free conditions through the use of serum (primarily fetal bovine serum) and the cytokine LIF (leukaemia inhibitory factor). LIF activates the JAK (Janus kinase)/STAT3 (signal transducer and activator of transcription 3) pathway by binding with the LIF and gpl30 receptors [8,9]. This maintains pluripotency and keeps ES cells in a state of constant self renewal by up-regulating pluripotent nuclear transcription factors such as Oct4, Sox2 and Nanog [8,10].

ES cells have a high proliferation rate, with mouse ES cells doubling approximately every 12 h [11]. The combination of high proliferation rate and self-renewal means that, under the right culture conditions, ES cells could represent an infinite stock of cells for tissue engineering applications. These combined characteristics also contribute to the clonogenic

Key words: cell scaffold, differentiation, embryonic stem cell, glycosaminoglycan, heparan sulfate.

Abbreviations used: 2OST, 2-O-sulfotransferase; 3OST, 3-O-sulfotransferase; 6OST, 6-O-sulfotransferase; BMP, bone morphogenetic protein; ECM, extracellular matrix; ERK, extracellular-signal-regulated kinase; ES, embryonic stem; FGF, fibroblast growth factor; Flk1, fetal liver kinase 1; GAG, glycosaminoglycan; GFP, green fluorescent protein; GlcA, D-glucuronic acid; GlcN, D-glucosamine; GlcNAc, *N*-acetylglucosamine; GlcNS, N-sulfoglucosamine; HS, heparan sulfate; HSPGs, heparan sulfate proteoglycans; IdoA, L-iduronic acid; JAK, Janus kinase; LIF, leukaemia inhibitory factor; MEFs, mouse embryonic fibroblasts; NDST, N-deacetylase N-sulfotransferase; NPCs, neural progenitor cells; PGs, proteoglycans; ScFv, single-chain variable fragment; STAT3, signal transducer and activator of transcription 3; VEGF, vascular endothelial growth factor.

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capacity of ES cells; under optimal conditions, a single ES cell can divide to form a homogeneous population of cells [12].

Human ES cells

As with mouse ES cells, human ES cells were originally derived on a feeder layer of MEFs (mouse embryonic fibroblasts), providing a surface for attachment and secreting an unknown combination of factors into the surrounding medium [4]. If human ES cells are to be applied therapeutically, it is necessary for a defined xenobiotic-free environment (free from feeder layers and animal derived products) to be determined. The necessity for an animal-product-free culture environment was highlighted by Martin et al. [13] who demonstrated the incorporation of non-human sialic acid Neu5Gc by human ES cells cultured on a MEF feeder layer. Many researchers have attempted to identify the essential components provided by the MEF feeder layer in the hope of using non-animal-derived alternatives to these to support fully defined culture of human ES cells. Among the various factors so far identified, HSPGs (heparan sulfate proteoglycans) are of particular interest to our group. A study by Levenstein et al. [7] found that MEFs secrete a range of HSPGs into the medium, including perlecan, agrin, syndecan-4 and glypicans 1 and 4. The presence of secreted HSPGs within MEF-conditioned medium is essential for stabilizing and assisting the binding of FGF2 (fibroblast growth factor-2), which is essential for human ES cell culture. The addition of HS (heparan sulfate) and heparin to unconditioned media enhanced the culture of human ES cells when supplemented with 40 ng/ml FGF2 [7]. However, cells were only sustained for three passages, indicating the necessity of other MEF-secreted factors in human ES cell self-renewal. The study also observed a dose-dependent increase in FGF2 binding with increasing concentrations of HS/heparin and the ability of conditioned medium to support human ES cell culture was undermined by heparinase treatment [7].

Heparan sulfate

HS is a ubiquitous GAG (glycosaminoglycan), highly conserved during evolution [14]. HS chains can exist as covalently bound components of cell-surface PGs (proteoglycans) such as syndecans and glypicans and as constituents of the ECM (extracellular matrix) [15]. The ability of HSPGs to bind a wide variety of protein ligands is primarily (although, importantly, not entirely) a function of their HS moieties. HS chains can modulate interactions between receptors and receptor ligands, including key factors in stem cell biology such as FGF (fibroblast growth factor) family members and other soluble ligands such as BMP (bone morphogenetic protein) [15,16]. The ability of HS chains to bind specific factors and to influence specific interactions is a direct result of the regulated complexity of HS structure [17]. HS chains are composed of disaccharide subunits, alternating between uronic acid [GlcA (D-glucuronic acid) or IdoA (L-iduronic acid)] and GlcN (D-glucosamine) subunits [14]. The structural composition of the HS chain is dependent on a number of factors and varies between tissue and cell types [14,18]. HS biosynthesis occurs in the Golgi, initiated by the assembly of four sugars into a tetrasaccharide O-linked to a serine residue of a protein core [14,19] (Figure 1). After formation of this region, a GlcNAc (*N*-acetylglucosamine) unit is added to the chain; this addition initiates the polymerization of the growing HS chain by the transferases Ext1 and Ext2, adding alternating units of GlcA and GlcNAc [14,19–21].

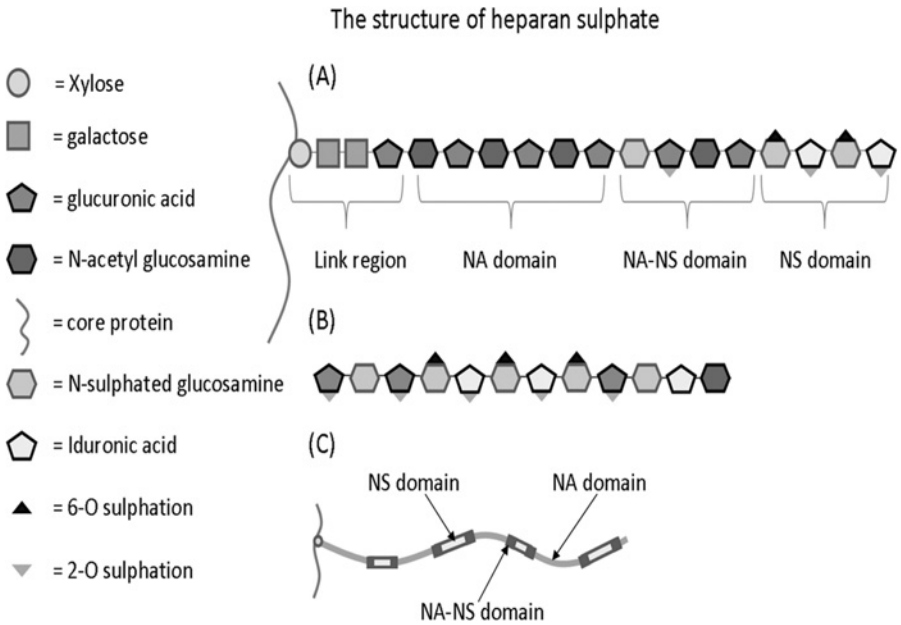
During, or immediately following, polymerization, the nascent HS chain undergoes a number of modifications involving the enzymes NDST (N-deacetylase N-sulfotransferase), epimerase and 2OST, 6OST and 3OST (2-O, 6-O and 3-O-sulfotransferase respectively) [14,21]. NDST, 6OST and 3OST all belong to multi-isomer families, with different isoforms of the enzymes catalysing modifications to different sequences within the nascent HS chain. NDST removes the N-acetyl group from a subset of GlcNAc residues and replaces it with a sulfate group forming GlcNS (*N*-sulfoglucosamine) [22,23]. This event is followed by the epimerization of GlcA at the C-5-hydroxy group to IdoA via the action of C-5-epimerase. Finally, 2OST and 6OST initiate variable O-sulfation of GlcA and IdoA at the C-2-hydroxy group and of GlcNAc and GlcNS at the C-6-hydroxy group respectively [14,18,19,23]. In certain cases the C-3-hydroxy group moiety of GlcN may also be sulfated via 3OST [14,23]. The incomplete yet interdependent action of this bank of enzymes (which have been suggested to form a 'GAGosome') leads to an organized domain structure in the mature HS chain with regions of high modification (and sulfation) termed NS domains spaced between regions of low modification called NA domains [14,18,19,24,25]. Alternating GlcNS- and GlcNAc-containing regions (NA/NS domains) form boundaries between these domains. The organization of these domains varies widely from one tissue to another owing to tissue-specific expression of enzyme isoforms such as the NDSTs, sulfotransferases and sulfatases [14,19]. Further complexity comes from the action of cell-membrane-displayed sulfatases which 'edit' the sulfation pattern, removing selected 6OST [26].

HS in ES cells and their differentiated progeny

Mouse ES cells express an unusually low-sulfated form of HS with low levels of contiguous N-sulfation [27]. Interestingly, the exit from self-renewal and subsequent commitment to differentiation appears to be dependent on the generation of specific sulfated epitopes within the HS chains. We previously monitored neural differentiation of mouse ES cells expressing a GFP (green fluorescent protein) reporter gene under the control of Sox1 (a transcription factor specifically up-regulated during neural differentiation). Sox1–GFP-expressing NPCs (neural progenitor cells) had increased

Figure 1 | **The structure of HS**

(**A**) HS represented as repeating disaccharide units joined to a protein core via a link region. (**B**) Six disaccharides forming a highly sulfated motif, typical of an NS region. (**C**) Overall structure of the chain; highly sulfated regions of variable length are flanked by small cross-over regions, which are in turn linked by long non-sulfated regions [14,25].



expression of HS biosynthesis enzymes including NDST4, 3OST and 6OST-2/-3 which correlated with increases in N-, 6-O and 2-O sulfation observed within the NPC HS when compared with that of ES cells. As part of this study, we were also fortunate to have access to a panel of phage-display-derived anti-HS antibodies which had been used extensively to demonstrate the exquisite cell- and tissue-type-specificity of HS sulfation patterning. By careful analysis of cell populations using flow cytometry and the ScFv (single-chain variable fragment) panel, we were able to demonstrate that, in addition to the shift in HS composition, there were changes in the presentation of selected sulfated epitopes displayed by the cells as they differentiated. For example, we detected increased binding of the ScFv antibody RB4EA12, which preferentially binds HS rich in GlcA/IdoA-GlcNS(6S) to the NPCs compared with the ES cells. Interestingly, this correlated with a decrease in binding of FGF2 to the cells [27].

An overall increase in HS chain sulfation following loss of pluripotency and lineage commitment was also observed during mesodermal differentiation from ES cells, this time using conditions optimized for formation of haemangioblast-type cells. In this study, we used the HS-epitope-specific ScFv antibodies to demonstrate that, although sulfation increased during both neural and mesodermal differentiation, the patterns found within HS from specific lineages were distinct. Progressive mesodermal differentiation of mouse ES cells was characterized by the transient cell-surface presentation of HS with high-affinity binding to ScFv antibody HS4C3 [28]. Weak binding of HS4C3 has been observed to oligosaccharides with N-, 2-O and 6-O-sulfation, with high-affinity binding characterized by additional 3-O-sulfation [29]. It is therefore possible that the subpopulation of cells expressing high levels of the HS4C3 epitope might express HS with increased 3-O-sulfation, although we were not able to demonstrate this. Interestingly, when the subpopulation of cells expressing high levels of the HS4C3 epitope [together with the mesodermal marker brachyury (Bry) and the VEGF (vascular endothelial growth factor) receptor Flk1 (fetal liver kinase 1)] were isolated and cultured alongside the HS4C3-negative population, the cells with high levels of the HS4C3 epitope were found to have an increased ability to generate haemangioblast progenitors and endothelial colonies [28].

Potential role of HS in ES cell biology

The findings described above led us to consider the role played by HS in ES cell biology and in early differentiation. We were interested to note that a medium designed for the serum-free culture of mouse ES cells which contained inhibitors of key signalling pathways led to cells evolving a very similar phenotype to that of HS-deficient Ext1$^{-/-}$ cells. Ext1$^{-/-}$ cells grow on gelatin in very tight colonies with well-defined borders and little evidence of spontaneous differentiation. High levels of the pluripotency markers Nanog and Oct4 are found in these cells, demonstrating that HS is not required for the maintenance of the ES cell phenotype [30]. Work by Wang et al. [31] has demonstrated that FGF2 signalling is impaired in these cells, and a similar study by Lanner et al. [32] using a chemical inhibitor of sulfation to disrupt HS patterning

also found FGF2 and FGF4 signalling to be impaired. We, and others, have demonstrated subsequently that HS is however required for progression from self-renewal to a differentiated state [27,30]. Ext1$^{-/-}$ cells are unable to form embryoid bodies, to down-regulate Oct4 on removal of LIF or to generate neural precursors when cultured in a neurogenic assay [27]. Similarly, ES cells lacking two key enzymes involved in N-sulfation of HS, or cells treated to disrupt sulfation, were also impaired in differentiation [32]. It therefore appears that the low-sulfated HS displayed by mouse ES cells may function to assist maintenance of pluripotency, perhaps acting to suppress signalling via HS-dependent factors such as FGFs and BMPs. Owing to the particular ability of HS to interact simultaneously with multiple pathways, this would retain the balance of signalling required to maintain the 'ground state' of pluripotency as proposed by Ying et al. [33].

The up-regulation of HS biosynthetic enzymes observed when comparing pluripotent and differentiated cell populations suggests that, at least in part, the increased sulfation detected on differentiated cells is due to the changing composition of the HS biosynthetic machinery [27,28]. The increased sulfation is likely to alter the ability of the cell population to interact with soluble factors such as members of the FGF and BMP families, which are known to be critical for early lineage selection during exit from self-renewal [34]. It is well established that the process of differentiation of ES cells to a specific lineage requires those cells to respond to a sequence of factors driving the cells to exit self-renewal, to commit to differentiation and then to progress further towards a mature differentiated cell type [35,36]. Echoing early embryonic development, different lineages are influenced by distinct cohorts of factors. For example, neural differentiation has been shown to require a tightly regulated response to FGF4, signalling via the ERK (extracellular-signal-regulated kinase)/MAPK (mitogen-activated protein kinase) pathway. A study by Stavridis et al. [37] found that a discrete period of high ERK phosphorylation was necessary to enable cells to exit self-renewal and commit to neural differentiation. Similarly, studies investigating haemangioblast formation have uncovered a role for a sequence of factors including VEGF and members of the BMP family [36]. It is known that factors which interact with HS as part of a co-receptor complex can also influence HS biosynthesis. Signalling via these factors might therefore establish a positive-feedback loop whereby, once a cell has received a signal in response to a factor requiring sulfated HS, that cell is then triggered to produce more highly sulfated HS, enabling more efficient signalling. Because there is likely to be some degree of selectivity in the ability of HS-dependent factors to interact with the HS chains displayed by the cell population, depending on the exact patterning of the chains, this might be a mechanism whereby multiple cell types are able to be generated from an (apparently) relatively homogeneous cell population. It also suggests that it might be possible to selectively activate particular pathways and therefore drive differentiation to specific cell lineages by providing HS oligosaccharides of defined pattern and known ligand activity. Although much of the preceding discussion is speculation, we have initial data to support this hypothesis and have been able to demonstrate differential HS requirements to support the acquisition of haemangioblast and neuroectodermal precursor cell types from mouse ES cells.

This intriguing finding has led us to propose that GAGs (either purified from biological sources or, more likely generated by chemo-enzymatic methods [38,39]) may prove to be useful future tools to drive efficient differentiation of ES cells. The requirement for expensive recombinant factors in differentiation regimens may therefore be reduced.

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