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Heparan sulfate proteoglycans and human breast cancer epithelial cell tumorigenicity

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Abstract

Heparan sulfate proteoglycans (HSPGs) are key components of the extracellular matrix that mediate cell proliferation, invasion and cellular signalling. The biological functions of HSPGs are linked to their co-stimulatory effects on extracellular ligands (e.g., WNTs) and the resulting activation of transcription factors that control mammalian development but also associated with tumorigenesis. We examined the expression profile of HSPG core protein syndecans (SDC1-4) and glypicans (GPC1-6) along with the enzymes that initiate or modify their glycosaminoglycan chains in human breast cancer (HBC) epithelial cells. Gene expression in relation to cell proliferation was examined in the HBC cell lines MCF-7 and MDA-MB-231 following treatment with the HS agonist heparin. Heparin increased gene expression of chain initiation and modification enzymes including EXT1 and NDST1, as well as core proteins SDC2 and GPC6. With HS/Wnt interactions established, we next investigated WNT pathway components and observed that increased proliferation of the more invasive MDA-MB-231 cells is associated with activation of the Wnt signalling pathway. Specifically, there was substantial up-regulation (>5 fold) of AXIN1, WNT4A, and MYC in MDA-MB-231 but not in MCF-7 cells. The changes in gene expression observed for HSPG core proteins and related enzymes along with the associated Wnt signalling components suggest coordinated interactions. The influence of HSPGs on cellular proliferation and invasive potential of breast cancer epithelial cells are cell and niche specific. Further studies on the interactions between HSPGs and WNT ligands may yield clinically relevant molecular targets, as well as new biomarkers for characterization of breast cancer progression.

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Keywords

Heparan Sulfate Proteoglycans; Breast Cancer; MCF-7; MDA-MB-231; gene expression; Wnt signalling

INTRODUCTION

For breast cancers detected early, while still localised in the breast, chances of five-year survival are 90%. Patient survival declines after five years, and, after ten years is dependent on the stage at diagnosis [1]. The identification of the cellular origin of genetic and microenvironmental changes involved in breast tumor initiation, malignant conversion and the processes occurring during the establishment of secondary tumor sites are of great medical and social interest.

Within the mammary gland, the epithelial cells are embedded within an adipose and mesenchymal stroma separated by the basement membrane. This combination of the surrounding connective tissue and basement membrane is termed the extracellular matrix (ECM) [2,3]. Disruption of the carefully ordered ECM microenvironment (niche), important for tissue specific function [4,5], can alter cellular and tissue morphology. In breast tumors, abnormal epithelial cell growth, differentiation and invasion can aid in the development and expression of inherent genetic changes leading to tumorigenesis [5]. In addition, throughout the active process of metastasis, it is still unclear if the ability to degrade ECM constituents is mediated by factors produced by the tumor cells themselves or by the associated stromal cells [5-7].

Major constituents of the ECM, proteoglycans (PGs) structurally and functionally influence multiple cellular events including proliferation, differentiation and gene expression [8]. PGs are characteristically composed of a core protein to which one or more glycosaminoglycan (GAG) side-chains attach. Heparan sulfate proteoglycans (HSPGs) are ubiquitous to the cell surface and ECM, in particular the subendothelial and epithelial basement membranes, where HS co-ordinates ECM structural integrity and barrier function [9]. Moreover, the ECM is a reservoir for HSPGs that function to bind endogenous growth factors and support their bioactivity (<http://www.ncbi.nlm.nih.gov/pubmed/17682830>). HS sequences are not directly encoded by genes, but are created by an elaborate post-translational biosynthesis [10,11] with precursor HS chains synthesised in the Golgi as nonsulfated co-polymers attached to the HSPG core protein. The tissue specific complex HS sulfation pattern is produced following several co-ordinated temporal modification steps and determines ligand interactions including growth factors and morphogens (FGF, Wnts, BMPs), their receptors (FGFRs), and extracellular matrix structural molecules (collagen, fibronectin) [9,12,13].

The role of HSPG core proteins has been examined in a number of studies in different cancer types using *in vitro* and *in vivo* human and murine models with specific core proteins shown to have tissue and disease specific associations. Of the core proteins, several studies examining the modulation of growth factor interactions and cellular proliferation have identified concomitant alterations in glypican expression. Glypican-3 (GPC3) one of the most highly investigated members of the HSPG family, was identified as a novel tumor

marker of melanoma [14], and its expression associated with neuroblastoma and Wilms' tumor [15]. GPC3 is highly expressed in hepatocellular carcinoma (HCC), and is currently being evaluated as a target for antibody and cell-based therapies of HCC [16,17]. In a murine breast cancer model, GPC3 appears to have a dual role in mediating Wnt signalling and apoptosis [18]. In addition, glypican-1 (GPC1) was shown to be induced in pancreatic cancers and to be strongly expressed in breast cancers with the remaining glypicans (2, and 4-6) relatively undetected in these tumors [19,20]. Members of the syndecan family of HSPG core proteins are also postulated to have a role in breast tumors with overexpression of syndecan-1 (SDC1) and an absence of syndecan-4 (SDC4) previously correlated with poor prognosis and aggressive phenotype [21].

HSPG core protein and growth factor interactions are well documented, with promotion or inhibition of cell proliferation shown to be dependant on HS type, expression pattern and accessibility to ligands (reviewed in [13]). As HSPGs not only provide a storage depot for heparin-binding molecules in the cell microenvironment, but also decisively regulate their accessibility, function and mode of action [22], including growth factor interactions, a role for HS chains in the localised invasion and metastases in breast tumors is feasible. What has yet to be fully investigated is the role of the side chain initiation and modification enzymes, which determine the final niche specific sulfation profile, have in breast tumor initiation, progression and metastases. In this study we have examined the gene expression profiles of key HSPG genes including side chain initiation and modification enzymes, along with the sulfation enzymes and core proteins (syndecans, glypicans) in two human breast cancer cell culture models. This expression profile was assessed in response to the HS agonist heparin along with the Wnt pathway for insight into potential roles for HSPGs in breast cancer epithelial cell proliferation and migration.

METHODS

Cell Culture

The human breast cancer lines MCF-7 and MDA-MB-231 were obtained from ATCC and grown routinely as a monolayer in culture. MCF-7 cells were maintained in 50% DMEM, 50% RPMI medium containing 10% foetal calf serum and 100 U/mL penicillin/streptomycin. MDA-MB-231 cells were maintained in RPMI medium containing 10% foetal calf serum and 100 U/mL penicillin/streptomycin. Cells were grown in a 5% CO₂ humidified atmosphere at 37°C. Cells were plated at $1 \times 10^4/\text{cm}^2$ in 100mm culture dishes in maintenance or dosed media. Dosed conditions were maintained for one, three or five days with media supplemented with appropriate concentrations of heparin. Media was changed and cells re-dosed every 2 days. Experiments were conducted in triplicate. Cell culture medium was obtained from Lonza (Australia). Heparin and sodium chlorate were obtained from Sigma Aldrich (Australia).

RNA Isolation and Reverse Transcription

For RNA isolation, cells were washed twice in 1X phosphate buffered saline (PBS) and placed on ice. To each culture dish, 1mL of TRIzol (Invitrogen) was added onto the cell monolayer, the monolayer homogenised, and samples stored at -80°C for at least 24 hours.

RNA was isolated using the Invitrogen TRIzol Extraction protocol and RNeasy MiniKit (Qiagen, Australia) for RNA clean up. The quality and quantity of isolated RNA was assessed using a Nanodrop (Thermo Scientific, Australia). For conversion to cDNA, 250ng of RNA was reverse transcribed using 250U Superscript III, 2mM DTT, 1× First Strand buffer (Invitrogen, Australia), 300ng random hexamers (Promega, Australia), 500μM dNTPs (NEB, Australia), and 100U RNaseOUT (Promega) in a 50μL reaction. Resulting cDNA was diluted to a working concentration of 40ng/μL.

Q-PCR

We investigated the gene expression changes of 30 genes involved within the biosynthesis and modification pathway of heparan sulfate proteoglycans as well as their core proteins and associated enzymes. For the genes of interest, mRNA levels were quantified at Days 1, 3 and 5 (D1, D3, D5) using Q-PCR. Briefly, 120ng cDNA was amplified using the ABI7900 (Life Technologies). 100uM primers were used with a 2X supermix (BioRad/Promega) in a 10μL final reaction volume. Standard (50°C × 2 minutes, 95°C × 10 minutes, 50 cycles at 95°C × 1 min, 60°C × 30 sec) or fast cycling conditions (50°C × 2 minutes, 95°C × 3 minutes, 50 cycles at 95°C × 1s, 60°C × 3s) were applied. Gene expression was normalised against expression of *18S* and calculated using 2^{-CT} . Specific primer sequences for the genes investigated are summarised in Table 1. Variation between dosed and control (undosed), at each timepoint, was assessed using a Student's T-test. Mean gene expression (2^{-CT}) was calculated between 3 independent experiments and graphed with SEM. All experiments were performed in quadruplicate. Significance was set at $\alpha < 0.05$.

Wnt Gene Expression Arrays

Expression analysis of the Wnt pathway was conducted using the 384-well format of the Lonza StellarArray qPCR arrays (Lonza, Australia) and their validated gene primer sets focused on the Wnt pathway. Expression was determined in MCF-7 and MDA-MB-231 cells following heparin treatment, using four biological replicates of dose and control samples at D1, D3 and D5. cDNA was synthesised as described and amplified using StellarArray protocols. Analysis of the resulting gene expression changes was conducted as described for Q-PCR. Gene expression was analysed as per the manufacturer's formula with expression normalised relative to *18S* using their online Global Pattern Recognition (GPR)™ Analysis tool (version 2.0).

RESULTS

Heparin increases the proliferation of more invasive HBC cells

An initial dose response curve was conducted by the addition of the HS analogue, heparin, to the cell cultures (1 μg/mL to 25 μg/mL), resulting in a dose of 10 μg/mL identified for the remainder of the heparin treatments. At this dose (10 μg/mL) the MCF-7 cells had a 33% reduction in cell number between control and dosed cells at D1. This was followed by a 20% increase in cells in the dosed cultures above controls by D3 then another phase of slow cell growth in the dosed cells with a 2.7% decrease in cell number compared to controls by D5 (Figure 1A). Under the same conditions, MDA-MB-231 cells demonstrated a 59% increase in cell number above controls at D1, and 24% at D3. There was a marked increase in cell

proliferation and difference in cell number between D3 and D5 in the MDA-MB-231 treated cells, with a 75% increase in cell number compared to the control cultures (Figure 1B). Under normal cell maintenance media conditions, both cell populations showed, as expected, an increase in cell number from D1 to D3. Interestingly, the MCF-7 cells show little increase in cell number between D3 and D5, while the MDA-MB-231 cells demonstrated a significant increase in cell number between these two time points. The ER +ve and poorly invasive MCF-7 cells grew as distinct cobblestone colonies retaining an epithelial appearance. In contrast, the ER-ve, invasive and metastatic MDA-MB-231 cells maintained a more 'fibroblast-like' morphology. These differences between the two cell populations, along with their ER status and invasive potential are also likely to influence the demonstrated proliferation rate in response to heparin treatment. Clearly the addition of the HS agonist to the cell cultures elicited a strong proliferative response in the MDA-MB-231 cells, with little observed difference and potentially a slight inhibition of proliferation evident in the MCF-7 cultures between D3 and D5. Having established these proliferation differences between the HBC cells both in control cells and in cultures treated with heparin, we next examined the expression of genes associated with HSPG production.

HS chain initiation and N-sulfation expression is altered following heparin treatment

HS chain initiation is catalysed by the action of EXT enzymes (EXT1 and EXT2) and C5-epimerase. Following the co-ordinated action of these enzymes, the commitment to a HS chain is determined by NDSTs, through the introduction of critical N-sulfation at points along the side chain. Of the four NDSTs, NDST1 and 2 are more widely physiologically expressed, with NDST3 and 4 primarily expressed during development and in the brain. Following treatment with heparin, the gene expression profile of all the HS chain initiation enzymes (EXT1/2 and C5-Epimerase) in MCF-7 cells increased at D1 (EXT1: $p=0.00016$; EXT2: $p=0.055$; C5-Ep: $p=0.00062$), followed by decreased expression by D3 (EXT1: $p=9.92 \times 10^{-5}$; EXT2: $p=3.75 \times 10^{-5}$; C5-Ep: $p=0.23$) and increased expression levels by D5 (EXT1: $p=0.081$; EXT2: $p=0.019$; C5-Ep: $p=0.203$). This expression profile was also observed for the NDST1 and 2 genes in these cells with expression significantly increased at D1 (NDST1: $p=0.0163$; NDST2: $p=0.0016$), reduced by D3 (NDST1: $p=2.66 \times 10^{-9}$; NDST2: $p=6.08 \times 10^{-6}$), and increased by D5 (NDST1: $p=0.0153$; NDST2: $p=0.0051$) (Figure 2A and 2B). In the MDAMB-231 cells, gene expression followed a similar trend in terms of their expression profile following treatment with heparin. The chain initiation enzymes EXT1 and 2 and C5-epimerase all increased between D1 and D3 (D1: EXT1: $p=0.202$; EXT2: $p=0.0071$; C5-Ep: $p=0.190$; D3: EXT1: $p=1.77 \times 10^{-8}$; EXT2: $p=6.63 \times 10^{-8}$; C5-Ep: $p=6.66 \times 10^{-5}$) followed by reduced gene expression by D5 (EXT1: $p=4.82 \times 10^{-5}$; EXT2: $p=0.00069$; C5-Ep: $p=0.00013$). This same trend was observed for NDST1 gene expression (D1: $p=0.0147$; D3: $p=3.31 \times 10^{-5}$; D5: $p=0.0144$). In contrast, the gene expression profile of NDST2 at D1 and D3, although at a significantly lower level when compared to MCF-7 cells, continued to gradually increase at each time point (D1: $p=0.944$, D3: $p=0.409$) (Figure 2C and 2D). In MCF-7 cells, the observed gene expression level of the HS chain initiation and commitment enzymes was well above the level observed in MDA-MB-231 cells in both control and heparin treated cultures. In addition, most of these genes, with the exception of NDST2 in the MDA-MB-231 cultures, had their highest level of

expression at D3, with the lowest level of expression observed in MCF-7 cells also occurring at D3.

O-sulfation enzyme gene expression changes following heparin treatment

Following the initiation and commitment to HS side chains, further modifications include the addition of *O*-sulfation, primarily by 2-*O*-sulfotransferases followed by glucosamine 6-*O*-sulfation. *O*-sulfation of HS side chains is critical for interactions with ligands and downstream signalling including FGF2 where the binding of the FGF2/FGFR complex is dependent on the presence of 2-*O*- and 6-*O*-sulfation in the HS chain [12]. Once again, the overall level of gene expression of the sulfation enzymes examined was markedly higher in the MCF-7 (approx. 20 fold) than the MDA-MB-231 cells. HS2ST1 and HS6ST1 gene expression was significantly increased in MCF-7 cells at D1 (HS2ST1: $p=2.088\times 10^{-5}$; HS6ST1: $p=0.0122$), decreased at D3 (HS2ST1: $p=0.716$; HS6ST1: $p=5.82\times 10^{-6}$), and increased again by D5 (HS2ST1: $p=0.00012$; HS6ST1: $p=1.54\times 10^{-6}$). Gene expression of HS6ST2 peaked at D1 ($p=5.09\times 10^{-5}$) followed by a decrease in expression level that remained steady relative to control cultures from D3 to D5 (D3: $p=0.747$; D5: $p=0.484$). In the MDA-MB-231 cells, a significant increase in HS2ST1 gene expression was observed at D3 when compared to control cultures ($p=3.82\times 10^{-5}$). The gene expression changes observed at D1 showed little variation between control and heparin treated cells, while at D5 there was a significant increase for HS6ST1 (Day 5; HS2ST1: $p=0.0087$; HS6ST1: $p=0.00128$) (Figure 2E).

GPC5 and 6 may have a role in invasive potential

The glypicans are a family of six (GPC1-6) HSPG core proteins bound to the cell surface through a glycosylphosphatidylinositol (GPI) anchor with extensive interactions during cell signalling. Expression of glypicans has been associated with both the stromal and epithelial cellular compartments in human breast cancers [18]. Following heparin treatment of MCF-7 cells, only expression of GPC3, 4 and 6 were observed at all timepoints. Significantly increased gene expression was observed for GPC3 at D1 and D5 (D1: $p=0.0393$, D5: $p=7.68\times 10^{-5}$) and GPC4 at D3 ($p=2.03\times 10^{-5}$) and GPC6 at D3 ($p=1.66\times 10^{-7}$). In the MDA-MB-231 cells, GPC1, 5 and 6 were observed at all timepoints and following treatment of the cultures with heparin, no significant changes in gene expression of these glypicans were observed at D1. Significantly increased expression at D3 was observed for all three of these glypicans (GPC1: $p=4.39\times 10^{-5}$; GPC5: $p=0.0271$; GPC6: $p=2.94\times 10^{-9}$). This increased expression continued at D5 with a significant increase in expression of GPC1 ($p=0.00867$) and GPC5 observed ($p=0.00217$) (Figure 3C, 3D). These data demonstrate a potential role for GPC3 in tempering MCF-7 proliferation *in vitro* and GPC5 and GPC6 in the significant proliferation and potentially increased migratory potential observed in the MDAMB-231 cells at the D3 and D5 timepoints.

SDC2 and SDC4 expression may correlate with tumorigenicity

The HSPG core protein family of syndecans carry a number of HS side chains at their distal ends. Syndecans play roles in cell-cell adhesion, cell motility and cell-ECM interactions in a tissue specific manner. Interestingly, the level of gene expression of the syndecan genes was

similar between the two cell lines examined. In the MCF-7 cultures, following heparin treatment, cells displayed significantly increased gene expression in SDC1 and SDC4 at each of the timepoints examined (D1: $p=0.02038$, $p=0.04545$, D3: $p=0.0119$, $p=2.20 \times 10^{-6}$ and D5: $p=0.01617$, $p=0.04776$). In addition, both SDC2 and SDC3 demonstrated significant increases in gene expression at D3 ($p=9.21 \times 10^{-7}$, $p=0.03896$) and D5 ($p=0.00176$, $p=0.00307$). Following heparin treatment of the MDA-MB-231 cells, gene expression of SDC1 remained relatively unchanged when compared to control cultures at all timepoints. In contrast, the remaining syndecans (SDC2, 3, and 4) all demonstrated a significant increase in gene expression at D3 (SDC2: $p=1.56 \times 10^{-5}$, SDC3: $p=3.24 \times 10^{-5}$, SDC4: $p=0.00029$), with SDC2 expression levels highest at D5 ($p=0.00179$) (Figure 3). Although the syndecan gene expression profile in both cell lines examined did not show the distinctive D3 up/downregulation, distinct differences in the gene expression profile were observed. SDC4 was observed to be the dominant syndecan expressed in MCF-7 cells, and SDC2 the dominantly expressed syndecan in the MDA-MB-231 cells.

Wnt Pathway analysis

The interaction of HSPGs with several growth factor families, including the Wnts, is well established. We utilised a Wnt pathway gene expression array to examine the two HBC lines under our culture conditions. In the MCF-7 cells, significantly increased gene expression at D1 following the addition of heparin to the cultures was observed in genes generally associated with the canonical pathway (APC2, DISDC1, DKK1, FRAT1, FZD5, WNT2, WNT4), as well as the cell cycle (APC2, RHOA). Perhaps most interesting however, was the down regulation of gene expression at D3 of genes associated with the canonical Wnt pathway (APC, DIXDC1, NKD2, TCF7L2) but also those associated with cell migration (APC, RHOA) and the cell cycle (APC, JUN, RHOA, TCF7L2). By D5 there was clear down regulation of all genes within the array, in particular Wnt5a (Figure 5). This gene was the first Wnt gene found to be overexpressed in human breast cancers [23], where it has been shown to have elevated expression in both benign and primary tumors, and low expression in cell lines and normal breast tissue [23].

In contrast to the results seen in MCF-7 cells, MDA-MB-231 cells showed a variety of significant changes at D1 and widespread changes throughout the Wnt pathway at D5. Significant downregulation of several canonical (DVL1, FRAT2, WNT3A) and growth and proliferation genes (KREMEN1) was observed at D1 in the MDA-MB-231 cells following the addition of heparin to the cultures. The only gene to demonstrate significant upregulation was CTNNBIP1, which plays a role in canonical Wnt signalling and process of cell proliferation with a three-fold increase ($p=0.002$) in gene expression observed. The most striking difference between the two cell types however, was the almost universal significant increase in gene expression at the D5 timepoint in the MDA-MB-231 cultures. The gene with the greatest increase in gene expression at D5 was WNT4, with a 15-fold increase ($p=0.006$). This data suggests that MDA-MB-231 cells are more susceptible to the influence of heparin, resulting in increased proliferation mediated by the Wnt pathway.

DISCUSSION

HSPGs and proliferation vs. migration

The two HBC cell lines examined in this study represent different ends of the spectrum of human breast cancers, one highly invasive, highly metastatic (MDA-MB-231) and one lowly invasive, poorly metastatic (MCF-7). By examining the differences between these two cell types, we hoped to identify some significant gene expression changes associated with the postulated role of the matrix or localised niche in the regulation of breast epithelial cell tumorigenicity. The HSPGs are central to a number of cellular processes and it is feasible that these proteins may play a role in breast tumor initiation and progression. Tissue or cell specific regulation of HS biosynthesis (reviewed in: [22,24,25]) dictates subtle and selective changes in the interactions between HS and its ligands [22,26,27]. HSPGs can play a role in both the inhibition and the promotion of all aspects of cell functioning including proliferation and migration. HSPGs have been shown to inhibit cellular invasion by promoting tight cell-cell and cell-ECM interactions, and by maintaining the structural integrity and self-assembly of the ECM [22,28,29]. Indeed, a characteristic of malignant transformation is down-regulation of GAG biosynthesis, especially HS chains [22,28,29]. Previous studies clearly demonstrate a role for HSPGs in promoting cell proliferation and migration that is both cell and niche specific [22,30,31]. In this study, with the exception of the HSPG core proteins (syndecans and glypicans), the level of gene expression of HSPG genes examined associated with chain initiation, *N*-sulfation and *O*-sulfation enzymes was greater in MCF-7 cells when compared to MDA-MB-231 cells. Although the expression level was significantly higher in the MCF-7 cultures, addition of the HS analogue heparin to the cultures decreased the gene expression of these chain initiation and sulfation enzymes in the MCF-7 cells but stimulated their expression in the MDA-MB-231 cells.

The gene expression changes demonstrated in this study emphasise the role of HSPGs in mediating cell proliferation and migration through alterations in matrix interactions resulting in changes in cell growth. Increased gene expression of EXT1 and 2 along with C5-epimerase was clearly observed as expected, in the early growth phase of both the MCF-7 and MDA-MB-231 cells. Interestingly, the addition of HS to the cultures significantly downregulated expression of all three genes in the MCF-7 cells at D3. Conversely, the addition of the HS agonist to the MDA-MB-231 cells increased gene expression at all timepoints in all three genes. The EXT genes are responsible for side-chain initiation, followed by *N*-sulfation, catalysed by the NDSTs, resulting in commitment of the side-chains to the HS rather than the CS family of proteins. Reduced NDST1 and EXT gene expression at the D3 timepoint in the MCF-7 cells may reflect decreased migratory potential, suggested previously to occur through reduced EXT/ERK interaction and decreased collagen I binding (reviewed in [32]). In contrast, the increased expression of these genes seen in MDA-MB-231 cells may reflect the increased migratory potential of these HBC cells. Importantly these diverse cellular mechanisms are clearly mediated by the addition of HS to the cultures. This converse pattern of gene expression between the two cell lines is also apparent in the gene expression profile of the 2-*O* and 6-*O* HS sulfation enzymes. It is now commonly accepted that FGF2-FGFR-mediated signalling is dependent on specific HS fine structure, particularly *N*- or 6-*O*-sulfation of the glucosamine units and

2-*O*-sulfation of the iduronic acid units (reviewed in: [33-37]). HSPGs are integral in localising FGF2 near its receptor and stabilising the ligand-receptor complex allowing signal transduction [33,38,39]. The increased gene expression profile of the N-sulfation, 2-*O*- and 6-*O*-sulfation enzymes (NDST, HS2ST, HS6ST) associated with the addition of heparin to the MDA-MB-231 cultures support the increased activation of FGF2 signalling. In addition, along with increased growth, the binding of FGF2 to FGFR has been shown to stimulate migration and differentiation in several cancers [33,40,41], and specific FGF2 isoforms have been demonstrated to influence tumorigenicity of MDA-MB-231 cells [42]. Interestingly, previous data from our group has demonstrated that *N*-sulfation is more crucial than *O*-sulfation for heparin-Wnt3a interactions in the osteogenic model, although both forms of sulfation contribute to both binding affinity and biological activity [43].

HSPG core proteins and breast cancer epithelial cell proliferation and migration

The glypicans are GPI-linked membrane bound proteins carrying several HS chains near the tip of their ectodomain [22,44], which through growth factor and ligand interactions, mediate cellular differentiation and proliferation. Overexpression of glypicans has been reported in a number of cancers with their expression profile often correlated with the syndecans. Previous work by Matsuda *et al* (2001), demonstrated significantly increased gene expression of GPC1 in human breast cancer, with GPC3 and GPC4 moderately increased [19]. In the same study, the overexpression of GPC1 correlated with increased SDC1 expression and GPC2 and GPC5 were undetected [19]. This was reflected in our study with GPC1 and GPC6 strongly expressed in both cell lines, although expression in MCF-7 cells was higher for both genes than in MDA-MB-231. In the highly invasive/highly metastatic MDA-MB-231 cells, expression of GPC2, GPC3, GPC4 and GPC5 was too low to detect. However, in the MCF-7 cells, we observed very low expression of GPC3 and moderate expression of GPC4.

The syndecans regulate cell adhesion, proliferation and differentiation, key aspects of tumor growth and associated metastases, through sequential adhesion, motility and proliferation of the tumor cells [45,46]. SDC1 is the best characterised of the syndecans and is mainly expressed in epithelial cells [47,48] with *in vitro* models suggesting SDC1 promotes tumorigenesis through the regulation of tumor cell adhesion [49-51], proliferation [52], and angiogenesis [53]. In breast cancer, upregulation of SDC1 has been previously observed to correlate with poor prognosis and aggressive phenotype [21,54,55], while expression of SDC4 has been associated with better prognosis in breast cancer patients [21]. SDC4 is widely expressed, though usually at low levels in normal tissue, and is a key adhesion molecule [48,56], unique among the syndecan family members to localise at sites of cell-matrix adhesions, including focal adhesions [57,58]. When overexpressed, SDC4 promotes excess focal adhesion formation resulting in reduced cell migration [59]. Often, SDC1 is associated with an absence of SDC4 and vice versa, consistent with the distinct expression, localisation, and regulation of the two syndecans [21]. Both SDC1 and SDC4 expression levels have been demonstrated to correlate with FGF2/FGFR1 receptor signalling, suggesting elevated co-receptor activity in breast carcinomas [60]. In our study we observed higher SDC4 gene expression in MCF-7 cells than in the MDA-MB-231 cells, supporting previous observations that syndecans are required for increased cell proliferation in breast

cancers. The increased expression level of SDC2 in the MDA-MB-231 cells, upregulated by the addition of heparin to the cultures at all timepoints, supports previous studies demonstrating the importance of SDC2 in mediating cell proliferation, adhesion and tumorigenicity in colon, lung and breast cancer cells [50,61-64]. In contrast SDC4 has been shown to have an anti-migratory/ anti-invasive effect [63], supported here by the increased expression level of SDC4 in MCF-7 cells and the increase in the expression level of this gene following the addition of heparin to the cultures.

HSPGs and Wnt signalling in breast cancer epithelial cell proliferation and migration

The WNT family of secreted growth factors regulates developmental processes of cell fate and polarity, as well as general cell maintenance processes including homeostasis and cell cycle regulation. Currently, there are 19 WNT ligands in humans, which bind to the Frizzled (FZD) family of receptors and the co-receptors LRP5 and LRP6. WNT signalling comprises three pathways: the canonical pathway and two non-canonical pathways: planar cell polarity (PCP) and a calcium ion-dependent pathway. The well-studied canonical WNT pathway signals through β -catenin and regulates the cell cycle, cell growth, and proliferation. Both non-canonical pathways mediate their cellular events independent of β -catenin, with the PCP pathway regulating cytoskeletal dynamics and cell motility, and the WNT/calcium pathway promoting NFAT transcription [65]. HSPGs have been demonstrated to mediate both canonical and non-canonical Wnt signalling events including through *N*- and *O*-sulfation by mediating the physical binding and optimal co-stimulation of Wnt3a, promoting osteoblast differentiation [43]. The manifestation of cancer by aberrant Wnt signalling most likely results in degradation of β -catenin prior to nuclear translocation and Wnt signalling activation [66]. Several target genes of β -catenin signalling have now been identified and some of their functions are consistent with control of cellular growth, differentiation and survival [66]. With extensive and varied roles of HSPGs in activation and inhibition of many signalling pathways responsible for cellular growth and differentiation it is reasonable to assume the deregulation of some of these critical pathways is due to changes in HSPG gene expression. For instance, GPC3 is able to inhibit both canonical (morphology and migration) and non-canonical (morphogenesis and polarity) Wnt signals [67,68]. In addition, previous work by Yiu *et al* (2011) demonstrated GPC6 to have a key role in promoting the invasive migration of MDA-MB-231 cells through inhibition of canonical- β -catenin and Wnt signalling, and up regulation of non-canonical Wnt5a signalling through the activation of JNK (c-Jun-N-terminal kinase) and p38 MAPK (mitogen-activated protein kinase) [65]. The data presented supports this with increased GPC6 in MDA-MB-231 cells following treatment with heparin along with decreased expression of Wnt5a in MCF-7 cells and increased non-canonical expression in the MDA-MB-231 cultures. Here, we provide evidence supporting differing roles for specific HSPGs on breast cancer epithelial cell tumorigenic potential. In addition, this role utilises the Wnt pathway and is dependent upon the HSPG and the epithelial cell characteristics. Hence, we suggest that the influence of HSPGs on breast cancer epithelial cell proliferation and invasive potential is cell and niche specific.

In conclusion, the observed gene expression differences between the two breast cancer epithelial cell models demonstrated clear differences in the ability of HSPG genes to

influence cell proliferation, viability and potentially motility and tumorigenicity in response to the HS analogue heparin. In addition, these effects are mediated through interactions with specific members of the Wnt pathway to differentially mediate breast cancer epithelial cell proliferation and migration. Further understanding of these key interactions between specific HSPGs and Wnts may provide new biomarkers and perhaps an opportunity to target abnormal cells during breast cancer progression.

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Figure 1. Cell growth curves in the presence or absence of heparin (10µg/mL)
Solid line: Control, Dashed line: 10µg/mL Heparin (A) MCF-7, (B) MDA-MB-231

Figure 2. Expression of glycosaminoglycan chain initiation and modification enzymes following heparin treatment

Solid bars: Control, Open bars: MCF-7 Heparin (10 μ g/mL) Grey Bars: MDA-MB-231 Heparin (10 μ g/mL) (A) MCF-7 NDST1 and NDST2 (B) MDA-MB-231 NDST1 and NDST2 (C) MCF-7 EXT1, EXT2 and C5-Epimerase (D) MDA-MB-231 EXT1, EXT2 and C5-Epimerase (E) MCF-7 HS2ST1 and HS6ST1 (F) MDA-MB-231 HS2ST1 and HS6ST1.

Figure 3. Expression of core proteins following treatment with heparin
Solid bars: Control, Open bars: MCF-7 Heparin (10µg/mL), Grey bars: MDA-MB-231 Heparin (10µg/mL) (A) SDC2 (B) SDC4 (C) GPC5 (D) GPC6

Figure 4. Heat map representation of gene expression changes of genes involved in the Wnt pathway
Panels (A) and (B) significance; red: significant *p*-values, green: non-significant *p*-values. The darker red, the more significant the *p*-value. Panels (C) and (D) fold change; blue: decreased expression, white: no change, red: increased expression. The darker the red the greater the fold change. (A) MCF-7 *p*-values (B) MDA-MB-231 *p*-values (C) MCF-7 fold change (D) MDA-MB-231 fold change

Table 1

Primer Sequences for Q-PCR.

Gene	Forward Primer	Reverse Primer	Amplicon Size	Ref Seq (GenBank)
NDST1	TGGTCTTGGATGGCAAACG	CGCCAAGGTTTGTGGTAGTC	107	NM_001543
NDST2	CCTATTTGAAAAAAGTGCCACCTACT	GCAGGGTTGGTGAGCACTGT	105	NM_003635
NDST3	ACCCCTTCAGACCGAGCATACTC	CCCGGGACCAAACATCTCTT	151	NM_004784
NDST4	ATAAAG CCAATGAGAACAGCTTACC	GGTAATATGCAGCAAAGGAGATTGA	122	NM_022569
EXT1	TGACAGAGACAACACCGAGTATGA	GCAAAGCCTCCAGGAATCTGAAG	119	NM_000127.2
EXT2	CAGTCAATTAAAGCCATTGCCCTG	GGGATCAGCGGGAGGAAGAG	149	NM_000401
C5-Epimerase	AGCTGTCAAGCCAACCAAAATAA	CTTACTAGCCAATCACTAGCAGCAA	138	AY635582
HST2ST1	TCCCG CTCGAAGCTAGAAAAG	CGAGGGCCATCCATTGTATG	80	NM_012262
HS6ST1	AGCGGACGTTCAACCTCAAGT	GCGTAGTCGTACAGCTGCATGT	139	NM_004807
HS6ST2	TCTGGAAAGTGCCAAGTCAAATC	ATGGCGAAATAAAGTTCATGTT GAA	116	NM_147175
SDC1	CTGGGCTGGAATCAGGAATATTT	CCCATTGGATTAAGTAGAGTTTTC	76	BC008765.2
SDC2	AGCTGACAACATCTCGACCACTT	GCGTCGTGGTTTCCACTTTT	72	NM_002998.3
SDC3	CTTGGTCACACTGCTCATCTATCG	GCATAGAACTCCTCCTGCTTGTC	118	AF248634
SDC4	CCACG TTTCTAGAGGCGTCACT	CTGTCCAACAGATGGACATGCT	76	BC030805.1
GPC1	GGACATCACCAAGCCGGACAT	GTCCACGTCGTTGCCGTTGT	100	NM_002081
GPC2	TGATCAGCCCCAACAGAGAAA	CCACTTCCAACCTTCCTTCAAACC	75	NM_152742
GPC3	GATACAGCCAAAAGGCAGCAA	GCCCTTCATTTTCAGCTCATG	71	NM_004484.
GPC4	GGTGAACCTCCAGTACCACTTTACA	GCTTCAGCTGCTCCGTATACTTG	71	NM_001448
GPC5	GCTCACCTCAATGGACAAAATT	GTTGGCAAGCGTCTCTTCACT	159	NM_004466
GPC6	CAGCCTGTGTTAAGCTGAGGTTT	GATGTGTGTGCGTGGAGGTATGT	71	NM_005708.