View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by RVC Research Online

RVC OPEN ACCESS REPOSITORY – COPYRIGHT NOTICE

This is the author's accepted manuscript of the following article:

Graham, K., Murphy, J. I. and Dhoot, G. K. (2016) 'SULF1/SULF2 reactivation during liver damage and tumour growth', *Histochemistry and Cell Biology*, 1-13.

The final publication is available at Springer via http://dx.doi.org/10.1007/s00418-016-1425-8.

The full details of the published version of the article are as follows:

TITLE: SULF1/SULF2 reactivation during liver damage and tumour growth

AUTHORS: Graham, K., Murphy, J. I. and Dhoot, G. K.

JOURNAL TITLE: Histochemistry and Cell Biology

PUBLICATION DATE: 25 March 2016 (online)

PUBLISHER: Springer Verlag, from Society for Histochemistry

DOI: 10.1007/s00418-016-1425-8

RVC Royal Veterinary College University of London

SULF1/SULF2 re-activation during liver damage and tumour growth.

Kurtis Graham, Joshua I Murphy, Gurtej K Dhoot*

Department of Comparative Biomedical sciences, The Royal Veterinary College, University of London, UK

* = the author for correspondence=tdhoot@rvc.ac.uk

ABSTRACT: Both SULF1 and SULF2 enzymes are undetectable in normal adult liver tissue despite their high level expression during fetal development. Most hepatocellular carcinomas unlike the normal adult liver, however, express variable levels of these enzmyes with a small proportion not expressing either SULF1 or SULF2. SULF1 expression, however, is not restricted to only fetal and tumour tissues but is also abundant in liver tissues undergoing injury-induced tissue regeneration as we observed during fatty liver degeneration, chronic hepatitis and cirrhosis. Unlike SULF1, the level of SULF2 activation during injury induced regeneration, however, is much lower when compared with fetal or tumour growth. Although a small fraction of liver tumours and some liver tumour cell lines can grow in the absence of Sulf1 and/or Sulf2, the *in vitro* overexpression of these genes further confirms their growth promoting effect while transient reduction in their levels by neutralisation antibodies reduces growth. Hedgehog signalling appeared to regulate the growth of both Hep3B and PRF5 cell lines since cyclopamine demonstrated a marked inhibitory effect while Sonic hedgehog (SHH) over- expression promoted growth. All Sulf isoforms promoted SHH-induced growth although the level of increase in PRF5 cell line was higher with both Sulf2 variants than Sulf1. In addition to promoting growth, the Sulf variants, particularly the shorter Sulf2 variant markedly promoted PRF5 cell migration in a scratch assay. The SULF1/SULF2 activation thus does not only promote regulated fetal growth and injury induced liver regeneration but also dysregulated tumour growth.

Keywords: Sulf1, Sulf2, hepatocellular carcinoma, hepatitis, cirrhosis, fatty degeneration

INTRODUCTION:

Liver tumours like most other cancers promote tumour growth by activating a number of cell signalling pathways (Giakoustidis A et al. 2015). Most growth promoting cell signalling pathways do not only require ligand receptor interaction but also heparan sulfate proteoglycan (HSPG) co-receptor participation to facilitate ligand/receptor interaction (Merry and Gallagher 2002). HSPGs constitute both cell surface and ECM located molecules. The precise role of HSPGs, however, is determined by their sulfation status initially specified intracellularly by sulfotransferases (Merry and Gallagher 2002; Ashikari-Hada S et al. 2004; Ford- Perriss M1 et al. 2002) that can be further edited extracellularly by two endosulfatases called Sulf1 and Sulf2 (Dhoot et al. 2001; Morimoto-Tomita et al. 2002; Rosen and Lemjabbar-Alaoui 2010). These enzymes hydrolyse 6-O sulfates of HSPGs that have differential effects on the activities of different cell signalling pathways since 6-O sulfation is required by certain signalling pathways such as receptor tyrosine kinase facilitated cell signalling (Lai et al. 2003; Uchimura et al. 2006; Wang et al. 2004). The activities of such signalling pathways are thus inhibited by these enzymes by the removal of 6-O sulfates (Dhoot 2012). The activities of some other cell signalling pathways such as Wnt or GDNF in contrast are promoted by these enzymes as HSPG de-sulfation facilitates their cell surface ligand/receptor interaction by ligand release from HSPG binding in ECM (Dhoot et al. 2001; Sahota and Dhoot 2009; Morimoto-Tomita et al. 2002; Ai et al. 2003; Dhoot 2012; Ai et al. 2007). Changes in Sulf1/Sulf2 expression in normal and pathological tissues could thus indicate their cell signalling activity in such tissues. The role of Sulf1/Sulf2 in HCC growth, however, is not clear due to some conflicting reports of Sulf1/Sulf2 upregulation (Bret et al. 2011; Gill et al. 2011; Gill et al. 2012; Gill et al. 2014; Nawroth et al. 2007; Lemjabbar-Alaoui et al. ; Rosen and Lemjabbar-Alaoui 2010) as well as the downregulation of such enzymes in HCC (Lai et al. 2008a; Lai et al. 2006; Xu G et al. 2014). This therefore raises the question as to whether Sulf1 or Sulf2 enzymes are preferentially expressed in normal adult or fetal liver or tumour tissues. This study was undertaken to determine if it is the normal or the tumour tissues that express high levels of Sulf1 or Sulf2 since a number of studies have reported the upregulation of Sulf1 and Sulf2 enzymes in tumours (Kroy DC et al. 2010; Gill et al. 2012; Gill et al. 2014; Bret et al. 2011; Nawroth et al. 2007) while others have reported the down-regulation of these enzymes in tumour tissues (Lai et al. 2006; Lai et al. 2008b; Xu G et al. 2014) compared with normal tissues thus implying that Sulfs and particularly Sulf1 is a tumour suppressor rather than a tumour growth enhancer. Since HCC develops over a long time period and is often preceded by additional steps such as hepatitis and cirrhosis, this analysis also included some other liver samples likely to activate cell signalling in response to liver damage-induced regeneration. It is critical to determine whether Sulf1 or Sulf2 are positively or negatively regulated in normal and regenerating or tumour tissues.

Hepatocellular carcinoma (HCC) is usually preceded by chronic hepatitis virus infection that includes both hepatitis virus B and C (Kwon YC et al. 2015; Tarocchi M et al. 2014). Such viral infection can change transcriptional activity to modify growth regulation by impacting cell signalling. In addition to HCC, other conditions such as fatty liver disease that leads to lipid accumulation in hepatocytes result in hepatocyte apoptosis/cell necrosis due to liver cell injury. This triggers inflammatory response implicating many cytokines/chemokines whose activities may be enhanced by Sulf1 and/or Sulf2 activation as for example has been shown for SDF1 (Uchimura et al. 2006). This could induce a transient change in gene expression or may even lead to HCC progression at later stages but such non-tumour tissues are not really normal controls for the purpose of Sulf expression in normal versus HCC. A de-regulated cytokine balance has been proposed to trigger the sequence from steatosis to non- alcoholic steatohepatitis, ultimately leading to liver cirrhosis and cancer (Kroy DC et al. 2010). This thus requires careful distinction of normal from HCC with several intermediate stages that can also activate Sulf1/Sulf2 enzymes in such cells but can neither be classified as tumour or normal liver.

Our earlier RT PCR analysis of human fetal and adult normal canine liver and some immunocytochemical analysis of some tumours demonstrated the increased expression of Sulf1/Sulf2 in a number of canine and some human tumours since normal adult liver unlike the fetal liver did not express detectable levels of Sulf1 or Sulf2 (Gill et al. 2012). In the present study, we undertook further analysis of both Sulf1 and Sulf2 in a larger number of normal adult (n=28), fetal (n=12) and HCC (n=105) human liver samples and 4 liver cell lines. We also carried out Sulf1/Sulf2 analysis of not only HCC and several normal livers but also a number of other liver samples with a potential to activate Sulf1/Sulf2 expression due to inflammatory response or a change in metabolism or activation of developmental programme due to regenerative response initiated by hepatocytic injury.

This study confirmed the up-regulation of Sulf1 and Sulf2 enzymes in a large proportion of the hepatocellular carcinomas. The Sulf1/Sulf2 up-regulation, however, was not restricted to only tumour growth but also

activated during liver damage induced-liver repair/regeneration during HCC-preceding stages. This study thus supports a hypothesis that Sulf1/Sulf2 activation relates to not only increased cell- signalling during normal development and regeneration (Gill et al. 2010; Hitchins et al. 2013) but also during abnormal *in vivo* and *in vitro* growth. This is further confirmed by Sulf1 and Sulf2 induced *in vitro* growth promotion of liver tumour cell lines.

Materials and methods:

Immunocytochemical procedures: Paraffin human fetal tissue sections were obtained from the US Biomax and MRC/Wellcome Human Developmental Biology Resource at ICH, UCL following ethical regulation approval. Other human tissues were obtained from US Biomax. The paraffin sections of all tissues were stained using immunofluorescence procedure as described previously (Gill et al. 2010). The binding of rabbit Sulf1 antibodies A and C diluted 1/200 and rabbit Sulf2 antibodies N (1/300) and D (1/100) were visualised using goat anti-rabbit biotinylated IgG followed by streptavidin-conjugated Alexa Fluor 594 or Alexa Fluor 488 fluorochrome. The α- feto protein expression was analysed using mouse monoclonal antibody 3H8 supplied by Cell signalling at 1/100 dilution following citrate buffer pH 6.00 pre- treatment and detection by goat anti mouse immunoglobulin linked Alexa Fluor 488. Sections treated with pre-immune rabbit sera were similarly incubated with fluorochrome-labelled secondary antibodies as controls (not shown). All fluorochrome-labelled secondary antibodies were diluted 1/400. All primary antibody reactions were incubated overnight at 4°C followed by secondary antibody incubations for 1 hour each at room temperature. Labelled tissue sections were mounted in fluorescent mounting medium (Sigma Aldrich) containing 2.5 µg/ml DAPI for nuclear visualisation and photographed using a Leica DM4000B fluorescent microscope.

For immunocytochemical quantification, the images were analysed using Volocity (version 6.3.1, PerkinElmer) software. For each fluorophore, all pixels with an intensity greater than a given intensity threshold were defined as positively labelled. From the resulting binarised image, any contiguous groups of pixels smaller than a given size threshold were excluded as noise. The total area of the remaining pixels was then determined per image. Both thresholds were defined empirically based on representative images and then held constant for each fluorophore. The intensity thresholds used, on 8 bit images, were 91 (for the red and blue fluorophores) and 103 (for the green fluorophore). The size thresholds were 5 pixels (for the red fluorophore) and 10 pixels (for the green and blue fluorophores).

The number of samples examined for immunohistochemical analysis of each group included two cores of each sample, the normal adult human (n=28), normal fetal (n=12), HCC (105), fatty liver degeneration (n=15), chronic hepatitis (n=22) and cirrhosis (n=29). Two regions of each sample were photographed for each tissue for immunohistochemical analysis.

RT-PCR analysis: Total RNA from Hep3B, HepG2, SkHep1 and PRF5 cells (supplied by ATCC) was prepared using Trizol to generate cDNA with reverse transcriptase using random primers. The PCR of such samples was carried out using Sulf1 primers P23 (5'-CGAGGTTCAGAGGACGGATA-3') and P24 (5'-GCCTCTCCACAGAATCATCC-3') and Sulf2 primers 186 (5'- CAACTGTGTTCTCCCTGCTGGGT-3') and 187 (5'- CTGGAGCATGTTGGTGAATTCC-3') to exons 5 and 9 generating 804bp Sulf1 and 802 bp Sulf2 PCR products. Sense and antisense primers 5′- CTATGAGCTGCCTGACGGTC-3′ and 5′-AGTTTCATGGATGCCACAGG-3′ were used to amplify 114 bp β-actin (nucleotides 798–912 bp). All PCR products following 40 amplification cycles were analysed in 2% agarose gels (Gill et al. 2014).

Cell culture and transfection: Human Sulf1 and Sulf2 cDNA constructs (full length and a variant with deletion of exons 6, 7 and 18) were cloned into pcDNA3. Human liver cell lines Hep3B and PRF5 were grown in Dulbecco′s modified Eagle′s (DMEM) medium with 10% foetal calf serum as described previously (Gill et al. 2014). Over- expression of Sulf1 or Sulf2 variants in both these cell lines was achieved by transfection with Biorad transfectin lipid reagent. The transfection of Sulf1 and Sulf2 variants also included the transfection with an EGFP expression vector while the control cells were transfected with EGFP/pcDNA3 alone. To enrich the mixed population of transfected cells, the normal growth medium was changed to DMEM/10%FCS with 800 µg/ml G418 following 48 h of growth in the normal medium. The Sulf1/Sulf2 over-expression by transfection was confirmed by the RT PCR analysis. Such transfected cells were used for *in vitro* and *in vivo* assays following 2–6 weeks of growth in G418 containing medium. For *in vitro* proliferation assays, 10,000 cells/well were seeded in multiple 24-well plates and the trypsinised cells counted after 1, 2, 3, 4 and 5 days of culture or only once after 5 days culture in certain assays (Gill et al. 2014). In addition to each sample consisting of a minimum of three replicates for each stage, each experiment was repeated three times.

For scratch assays, cells were grown until confluent before scratching with a pipette tip to obtain two perpendicular clear lines. Such scratches or clear lines were photographed at 0, 24, 48, 72 and 96 hours using an inverted Leica microscope. Distances between the edges of cleared lines were calculated by measuring the change in clear area at different time points following the change from time 0 by subtraction. A total of 10 such scratches were measured per group for each experiment repeated three times.

Statistical analysis: Statistical analysis was preformed using either a one-way ANOVA or a two-way ANOVA, where data depicting a P-value < 0.05 was considered statistically significant. To enable multiple comparisons, a Tukey's test was used to determine significant variation between Sulf transfects to their eGFP controls or between full length Sulf isoforms and their shorter variants.

RESULTS:

SULF1/SULF2 expression in normal adult, fetal and HCC tissues: This immunocytochemical analysis confirmed our earlier RT PCR study of little or no SULF1/SULF2 expression in normal adult liver using a number of different samples (n=28) but expression of both these enzymes in a number of fetal liver samples (n=12) (Figure 1). The immunohistochemical analysis of 105 human liver HCC samples showed the expression of either both SULF1 and SULF2 or only one of these enzymes in over 80% (± 5%) of the HCC samples. A proportion (15-25%) of the HCC samples, however, did not express either of these two enzymes (Figure 2). The composition of both SULF1 and SULF2 variants also varied in HCC samples with some samples expressing mainly full length catalytically active SULF1 or SULF2 while others expressing only the shorter variants without the catalytically active exon 6 (Figure 2) (Gill et al. 2012). The normal 4-5 month fetal livers in contrast only expressed the full length (Figure 1) and no shorter variants (not shown). These variants vary in their cell signalling activities as described in our earlier study (Gill et al. 2012; Gill et al. 2014). The relative proportions of full length (SULF1A & SULF2A) and shorter variants (SULF1B & SULF2B) of both SULF1 and SULF2 also varied amongst these samples (Figure 2). Unlike SULF1, SULF2 demonstrated a greater abundance of full length enzyme compared with the shorter variant (Figure 2G). RT PCR analysis of 4 different human liver cell lines demonstrated little or no expression of Sulf1 or Sulf2 (Figure 2H). A trace amount of Sulf1 was detected in only SkHep1 cells with no detectable expression in PRF5, Hep3B or HepG2 cells under normal culture conditions. Similarly, only a trace amount of Sulf2 was detectable in HepG2 with no expression being apparent in PRF5, Hep3B or SkHep1 cells.

SULF1/SULF2 expression is also activated in response to fatty liver degeneration, chronic hepatitis and cirrhosis. To determine if SULF1/SULF2 expression in liver tissue was related to only fetal development and the onset of tumorigenesis or also related to tissue repair/regeneration in response to liver damage, we examined the expression of both these enzymes in a number of different human liver samples including not only normal and HCC samples but also samples with fatty liver degeneration (n=15), chronic hepatitis (n=22) and cirrhosis (n=29) (Figures 3 & 4). High levels of SULF1 expression are apparent in all individuals with fatty liver degeneration showing not only very large but also a number of quite small hepatocytes expressing varied levels of SULF1 (Figure 3.C1- C2). Different liver samples with chronic hepatitis (Figure 3.D1-D2) also expressed high levels of SULF1 in all samples although the distribution of SULF1 varied in different samples. For example, SULF1 expression level in some samples is much higher around the nucleus as well as extending into some nuclei as is indicated by some white arrows (Figure 3.D2). Similarly, high SULF1 expression is apparent in all cirrhotic livers (Figure 3.E1-E2). Although SULF1 expression is high in all such liver samples, their precise level of expression varied in different regions. SULF2 expression was also observed in some liver samples with fatty liver degeneration, chronic hepatitis and cirrhosis although its expression was apparent in only a small fraction of the samples and the SULF2 levels were much lower when compared with SULF1 levels (Figure 4). While SULF1 activation was detected in all such samples, a large proportion of liver samples with fatty liver degeneration, chronic hepatitis and cirrhosis thus did not show any SULF2 expression using immunocytochemical staining procedure in this study (Figure 4).

SULF1/SULF2 expression in some adult livers could be related to tumour cell growth or hepatic damage-induced regeneration as indicated by the activation of α-fetoprotein. Liver retains the potential to repair itself in response to injury. We therefore analysed the expression of α-fetoprotein associated with cell proliferation although the precise mechanism of its function is not known (Wang S1 et al. 2012). We examined if SULF1/SULF2 expression is related to liver regeneration in samples with fatty liver degeneration, chronic hepatitis and cirrhosis using normal adult and HCC tumour tissues as negative and positive controls (Figure

5). Immunocytochemical analysis showed no α-fetoprotein expression in normal adult liver but variable level of expression in different HCC tumours. This is indicated by only background staining of normal but much darker staining of not only HCC, but also liver samples representing fatty degeneration, chronic hepatitis and cirrhosis.

All tissue samples with HCC, fatty liver degeneration, chronic hepatitis and cirrhosis showed variable levels of α-fetoprotein expression showing regional variation (Figure 5).

Sulf1/Sulf2 expression levels modulate the growth of some liver tumour cells. The Sulf1/Sulf2 expression in a large majority of the liver tumours indicates their possible stimulatory role in promoting tumour growth even though a small proportion of primary tumours and a number of cell lines did not express any Sulf1 or Sulf2 and thus can grow in the absence of Sulf1/Sulf2. We transfected the Hep3B and PRF5 cells with some shorter and full length variants of both Sulf1 and Sulf2 to determine if Sulf over-expression promoted or inhibited growth using EGFP transfection as a control. Once the transfected cell population had been selected by growth in G418 containing cell culture medium, the growth of such cells was compared with EGFP controls over 5 day time period. Compared with PRF5, relative growth rate of Hep3B cells was slower (Figure 6). Nevertheless, both full length and shorter variant of Sulf1lacking exons 6 and 7 (HS1-67) increased growth of Hep3B cells although level of increase was higher with HS1-67 than HS1. Both shorter and full length Sulf2 also promoted the growth of Hep3B cells with HS2 showing higher increase than HS2-67 (lacking exons 6 & 7) although the relative increase with Sulf2 over- expression was lower than Sulf1 over-expression (Figure 6). As was the case with Hep3B, transfection of PRF5 cells with both full length (HS1) and shorter variant (HS1-67) of Sulf1 also demonstrated increased growth. Over-expression of both shorter and full length Sulf2 also promoted growth of PRF5 cells (Figure 6). While the level of increased growth was slightly higher with Sulf1 than Sulf2 in Hep3b cells this was reversed in the PRF5 cells showing higher increase with Sulf2 than Sulf1 (Figure 6). To further examine the role of Sulf1/Sulf2 in such tumour cell growth, we also added some SULF1 and SULF2 neutralisation antibodies to decrease the levels of these enzymes in transfected cell cultures. The addition of antibody C that reacts with all Sulf1 variants and antibody D that reacts with all SULF2 variants at the onset of the experiment demonstrated decreased levels of growth in both Sulf1 and Sulf2 transfected cells only during the first 3-4 days as no further antibody was added at a later stage (Figure 6).

Hedgehog signalling regulates hepatocyte tumour growth that is further modulated by Sulfs: To examine if Hedgehog signalling plays a role in the growth of Hep3B and PRF5 cells, we initially examined the effect of 5μM, 10μM and 20μM cyclopamine on their growth to rule out any off target effects described for certain cell lines (Meyers-Needham M1 et al. 2012). All three cyclopamine concentrations showed a linear dose response although the effect was much lower at 5μM concentration and increased effect was observed at 20 μM concentration that we used in all subsequent experiments in this study. The addition of cyclopamine to both Hep3B and PRF5 cells for 5 days markedly inhibited the growth of these cells highlighting the role of Hedgehog signalling in their growth (Figure 7). The addition of SHH at 10ng/ml in contrast promoted growth in all PRF5 and Hep3B control and Sulf1/Sulf2 transfected cells (Figure 7). The level of SHH-induced growth in PRF5 cells was greatest in both Sulf2 variant transfected cells when compared with the increase in Sulf1 transfected cells (Figure 7).

Some Sulf1/Sulf2 splice variants markedly enhance PRF5 cell motility: Further to the effect of Sulf1/Sulf2 over-expression on growth, we also examined if the Sulf1/Sulf2 overexpression affected the cell migration. The transfection of PRF5 cells with full length Sulf1 showed little effect on cell migration during 48 hours although a small increase in migration was apparent during later stages of 72 and 96 hours. Compared with the full length Sulf1 (HS1), the migration of HS1-67 transfected cells showed considerably greater level of migration. The highest increase in cell motility, however, was observed with PRF5 transfection with shorter variant of Sulf2 i.e. HS2-67 with the increase becoming apparent even after one day but with marked effects during subsequent stages of 2-4 days.

DISCUSSION:

The onset and progression of tumour growth is affected by a multitude of factors unique to each individual's genomic composition and environmental exposure. The key question, however, remains what drives such growth and how effectively the growth can be targeted using appropriate inhibitors. The activities of tumour growth drivers may be only marginally or highly dysregulated during early or late stages of tumour growth that often reflects the re-activation of a number of signalling pathways characteristic of fetal and neonatal growth.

Sulf1 and Sulf2 enzymes regulate the activities of a large number of signalling molecules in a negative or positive manner and therefore changes in their activities during development and tumour growth would indicate whether these enzymes act as tumour suppressors or tumour enhancers. The present study demonstrated the down-regulation of both Sulf1 and Sulf2 in normal adult liver following high levels of their expression during fetal development promoting growth-related active cell signalling. This is compatible with increased expression of Sulfs during fetal development of many tissues (Gill et al. 2010; Hitchins et al. 2013) and during the development of different tumour types (Gill et al. 2012; Gill et al. 2014; Lemjabbar-Alaoui et al.). It, however, differs from some other studies reporting Sulf1 to be a tumour suppressor in liver (Lai et al. 2006; Lai et al. 2008a; Xu G et al. 2014) thus implying higher level of expression in normal adult liver although some other adult tissues such as neuronal and skeletal tissues have been reported to continue expressing Sulf1/Sulf2 throughout life (Joy et al. 2015; Zaman et al. 2016). Sulf1 activation, however is not unique to fetal or tumour growth since a number of other conditions including injury-induced repair can also activate these enzymes. Such activation thus could be related to an inflammatory response but also to hepatocyte degeneration that could promote both inflammation and regenerative response since the liver has a remarkable ability to heal itself upon injury. Regenerative response has a lot of similarities to fetal growth but is often additionally associated with injury-related inflammation. The inflammatory cells themselves produce a number of cytokines and chemokines whose activities are regulated by the sulfation status of the HSPGs as for example Sulf1/Sulf2 have been shown to promote SDF1 activity (Uchimura et al. 2006). The degenerative process has also been shown to activate some cell signalling pathways as for example Wnt signalling has been shown to promote age-related macular degeneration (Tuo J 2015). While Sulf1 plays an active role not only in fetal and tumour growth but also in liver damage induced regeneration, Sulf2 activity in the latter was considerably lower compared with Sulf1.

The reason for variation in Sulf1/Sulf2 expression in different studies of HCC is not clear but could relate to many other conditions inducing Sulf1/Sulf2 changes in non- tumour liver that is not strictly normal as the liver has a great injury-induced regenerative potential. The Sulf1/Sulf2 expression in “normal” non-tumour liver is thus more complex due to its frequent fat accumulating hepatocytic degeneration leading to liver damage-induced repair/regeneration. The present study demonstrates high SULF1 expression during fatty liver degeneration that may constitute only a small or large part of an apparently “normal” liver. Any liver damage would promote its repair/regeneration by activating growth promoting signalling pathways similar to fetal liver development that clearly deploys Sulf1/Sulf2 activity. Such liver regeneration is not restricted to only fatty liver degeneration but is also a response to any other damage leading to cirrhosis. Cirrhosis is a complication of liver disease involving loss of liver cells and irreversible scarring of the liver often associated with alcohol consumption and persistent viral hepatitis B and C infections. Cell signalling plays a critical role in development and regeneration of many tissues but the regeneration is often intimately associated with inflammation in response to tissue damage. The inflammatory cells themselves produce a number of cytokines and chemokines whose activities are further regulated by the sulfation status of the HSPGs.

Sulfs have been described to play a major role in normal growth as well as tumour growth but less is known about their role in virus uptake by the cell during many viral infections. Heparan sulfate (HS) acts as a co-receptor for the cellular entry of viruses including not only hepatitis B and C viruses (Barth H et al. 2003; Schulze A et al. 2007) but also many other viruses such as herpes simplex virus 1 (Shukla D et al.). Viral uptake thus is particularly relevant to liver that is prone to many viral infections including hepatitis that often precedes the development of hepatocellular carcinoma. Virus entry into hepatocytes is a multi-step process in which many factors are involved including HSPGs whose sulfation levels play a critical role in this process (Tátrai P1 et al. 2010; Xu Y et al. 2015). Interactions between HSPGs and its ligands are fine-tuned by the sulfation pattern of its heparan sulfate (Ashikari- Hada S et al. 2004). Hepatitis-related inflammation and HSPG-sulfation regulated virus uptake by hepatocytes can thus further modulate Sulf1/Sulf2 expression in such cells since many of these processes are likely to activate cell signalling pathways requiring Sulf1/Sulf2 modulation. Immunochemical staining with SULF1 and SULF2 antibodies in the present study detected raised levels of particularly SULF1 but only occasionally of SULF2 in many such samples that were not tumorigenic. Although Sulf1/Sulf2 up-regulation could relate to a number of different conditions, some of these changes appeared to be related to regenerative growth process as indicated by the activation of α-fetoprotein in such samples that was not detectable in the normal adult liver. We did not, however, quantify the precise correlation of Sulf1/Sulf2 activation with α-fetoprotein expression in this study.

The expression of Sulf1/Sulf2, however, is not essential for growth as is indicated by the observations that a number of liver cell lines *in vitro* and a small proportion of the primary tumours *in vivo* did not express any SULFs. We therefore further analysed the role of Sulf1/Sulf2 in tumour growth by over-expressing these enzymes by transfection of Hep3B and PRF5 liver cell lines. Both these cell lines grew well in culture but the

over-expression of individual full length as well as shorter variants of both Sulf1 and Sulf2 promoted their growth further with some quantitative differences in their rate of growth. Both Sulf1 and Sulf2 thus have the potential to modify hepatocyte cell growth if and when required *in vivo* conditions. The positive stimulatory growth of both liver cell lines by Sulf1 as well as Sulf2 was further confirmed by their reduced growth upon treatment with neutralisation antibodies.

The similarity of growth promotion by both full length and shorter SULF1 and SULF2 variants in these cultures may relate to the presence of a number of growth factors in FCS containing medium. It could be more informative to analyse possible functional differences using more defined media in futher studies. This study also showed the shorter Sulf1 and Sulf2 variants particularly HS2-67 to markedly promote cell motility. It is therefore possible that HS2-67 expression could promote tumour metastasis by enhancing specific cell signalling pathways.

Any number of cell signalling pathways can promote tumour growth. The Hedgehog signalling appeared to play a major role in the growth of both Hep3B and PRF5 cells since their growth was markedly inhibited by cyclopamine but promoted by SHH protein exposure. It was not, however, clear which variants preferentially promoted SHH signalling since all isoforms appeared to promote growth except with a much higher rate of growth in the presence of both Sulf2 variants in comparison with Sulf1.

This study thus did not show any evidence of Sulf1 being a tumour suppressor although Sulf1 and Sulf2 can demonstrate differential activation during tumour growth and particularly during liver damage-induced tissue regeneration.

FIGURE LEGENDS:

Figure 1: Immunocytochemical staining of normal adult and 4 & 5 month fetal liver stained for total Sulf1 and Sulf2 expression using immunofluorescence procedure. Scale bar = 100μM.

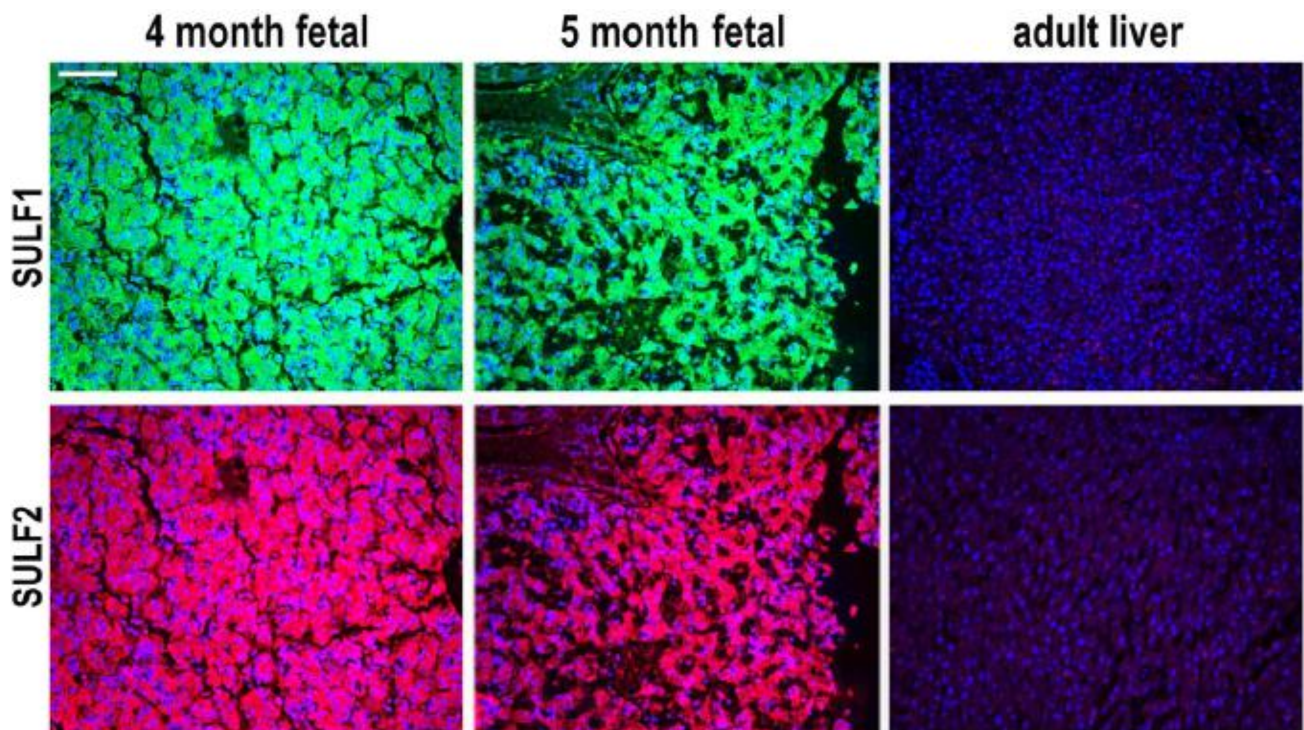


Figure 2: Immunocytochemical staining of liver samples from normal and two different HCC patients each stained for catalytically active Sulf1(A, B, C) & Sulf2 (, D, E, F) labelled green, and for total Sulf1 (A1, B1, C1) and Sulf2 (D1, E1, F1) labelled red using immunofluorescence procedure. G demonstrates the quantitative differences (relative proportions:%age) in the expression patterns of both Sulf1 and Sulf2 in normal versus HCC tumours, ***p*<0.001, ****p*<0.0001. G shows the Sulf1 and Sulf2 mRNA expression in 4 liver cell lines and a positive control using RT PCR analysis. Scale bar = 100μM.

Figure 3: Sulf1 expression pattern examined by immunofluorescence using antibody C that detects total Sulf1 in 2 liver samples from different individuals to highlight any individual differences in each case of fatty liver degeneration (C1-C2), chronic hepatitis (D1-D2) and cirrhosis (E1-E2) alongside HCC (B1-B2) and normal (A1-A2) livers as positive and negative controls. D2 also includes a magnified region of thissection to highlight the presence of SULF1 in some nuclei but not others as indicated by the arrows. F summarises the quantitative differences in Sulf1 expression in normal versus different pathological conditions, ***p<0.0001. The quantitative differences for each group represent a number of samples while some of the images shown in this figure represent minimal as well as maximal staining. Scale bar = 100µM.

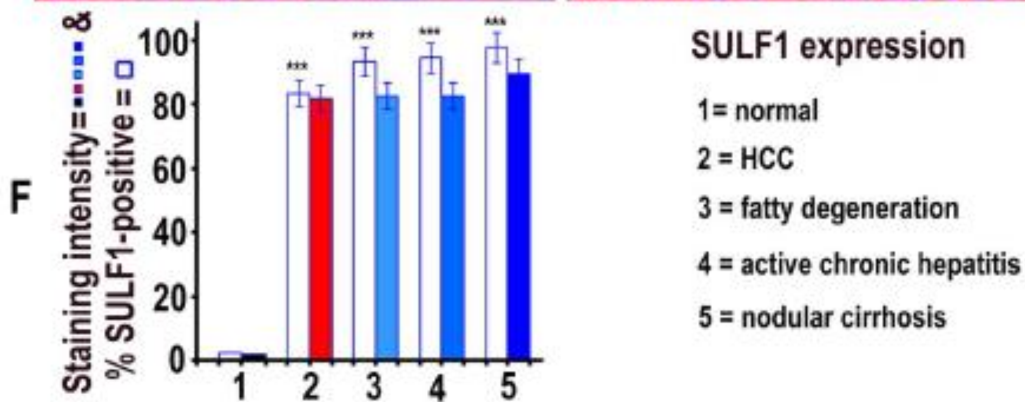


Figure 4: Sulf2 expression pattern by immunofluorescence using antibody that detects total Sulf2 in 2 liver samples from different individuals to highlight any individual differences in each case of fatty liver degeneration (C1-C2), chronic hepatitis (D1-D2) and cirrhosis (E1-E2) alongside HCC (B1-B2) and normal (A1-A2) livers as positive and negative controls. F summarises the quantitative differences in Sulf2 expression in normal versus different pathological conditions, *p<0.05, ***p<0.0001. Scale bar = 100μM.

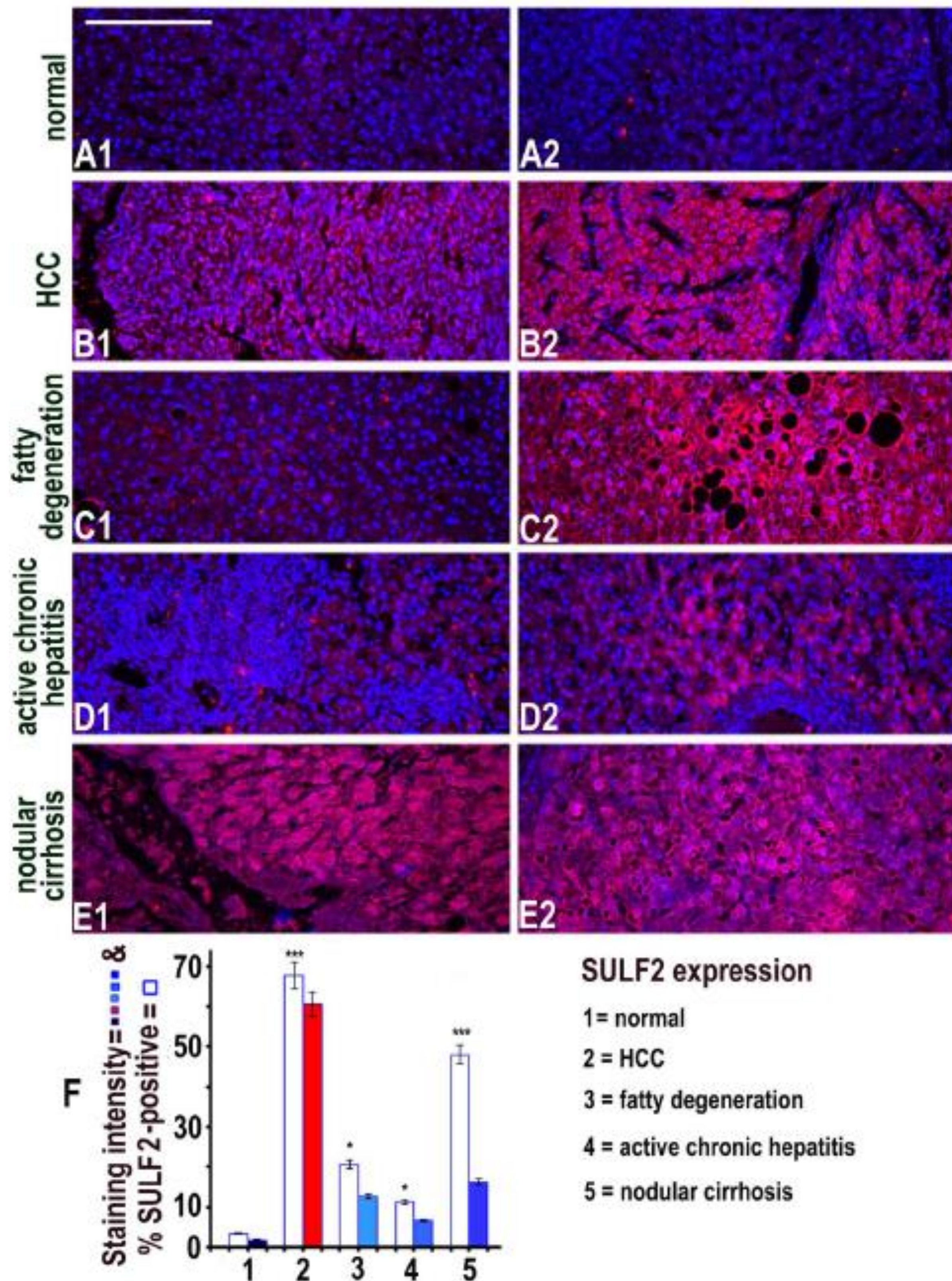


Figure 5: The immunocytochemical expression pattern of α-feto protein using immunofluorescence procedure in 2 liver samples from different individuals in each case of fatty liver degeneration (C1-C2), chronic hepatitis (D1-D4) and cirrhosis (E1- E4) alongside HCC (B1-B4) and normal (A1-A4) livers as positive and negative controls. Scale bar = 100μM.

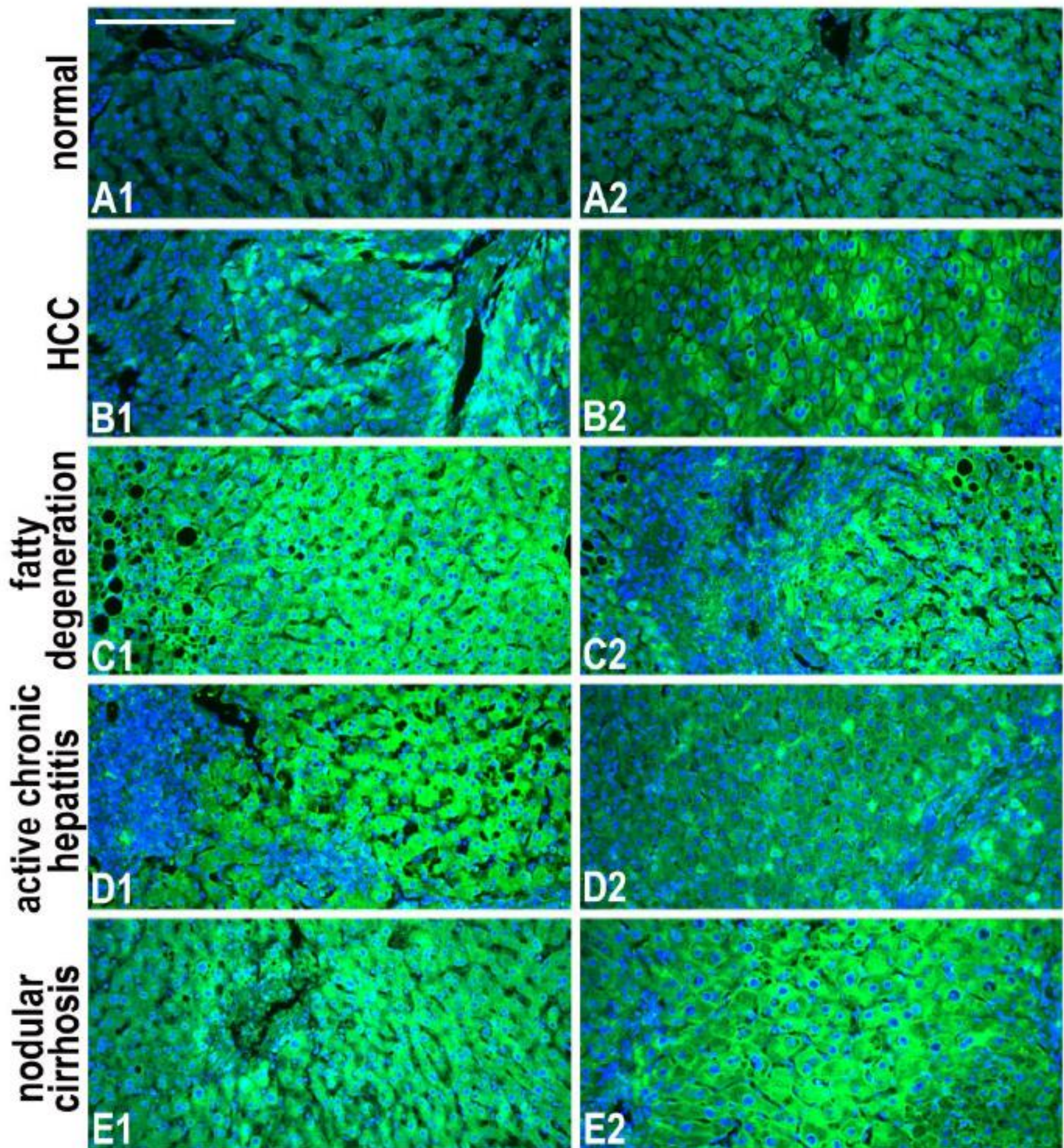


Figure 6: The effect of over-expression of full length (HS1 and HS2) and Shorter Sulf1 (HS1-67) and Sulf2 (HS2-67) variants on the growth of Hep3B (A, B) and PRF5 (C, D) cells over 5 days of culture. Compared with the control (EGFP), over-expression of all Sulf1 and Sulf2 variants showed increased growth. The growth rate of PRF5 cells with both full length and shorter Sulf1 (HS1, HS1-67: E & G) and Sulf2 (HS2, HS2-67: F, H) expression was significantly reduced by 200ng/ml Sulf1 and 200ng/ml Sulf2 neutralisation antibodies C and D at days 3 and 4 respectively, *p<0.05, **p<0.001,***p<0.0001.

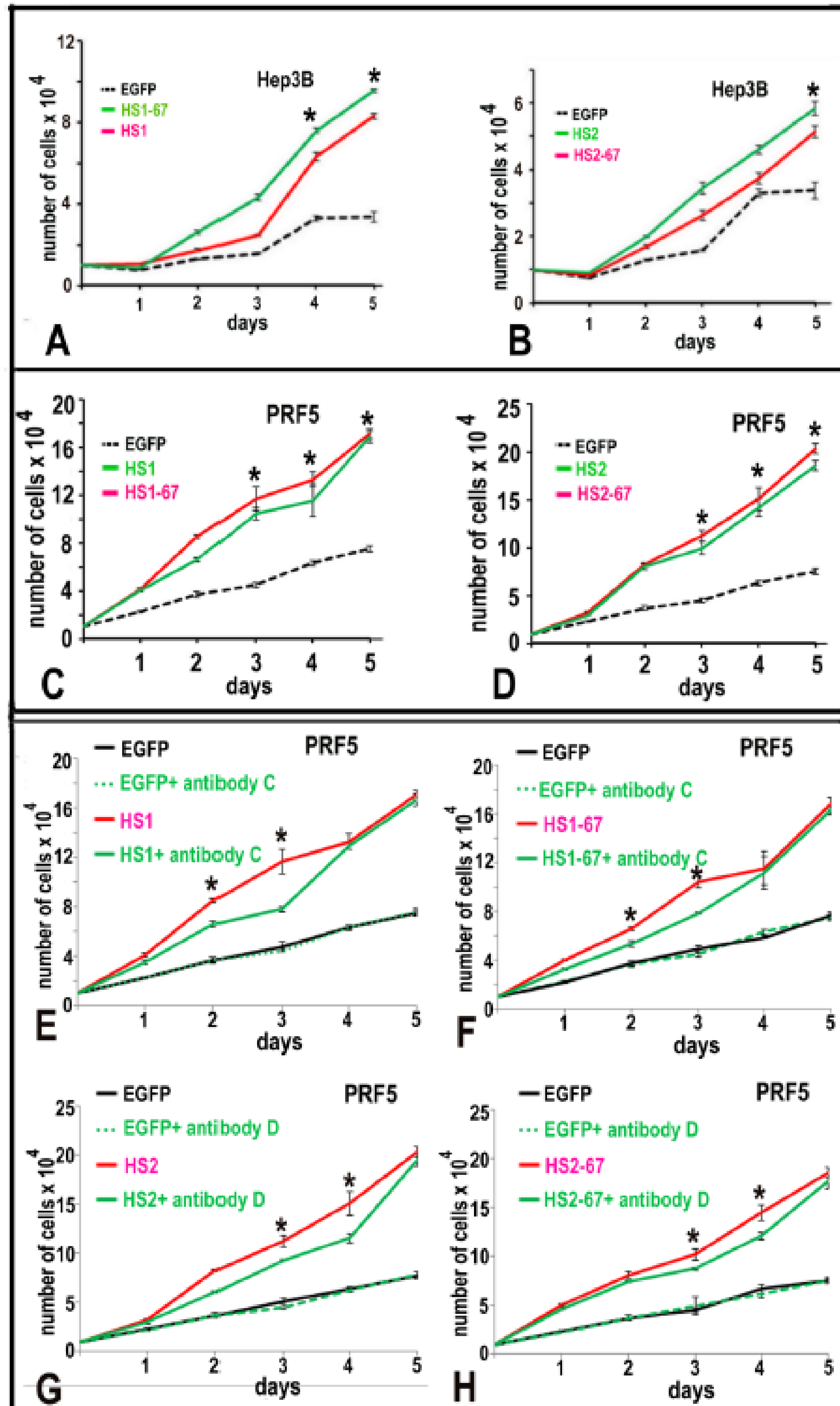
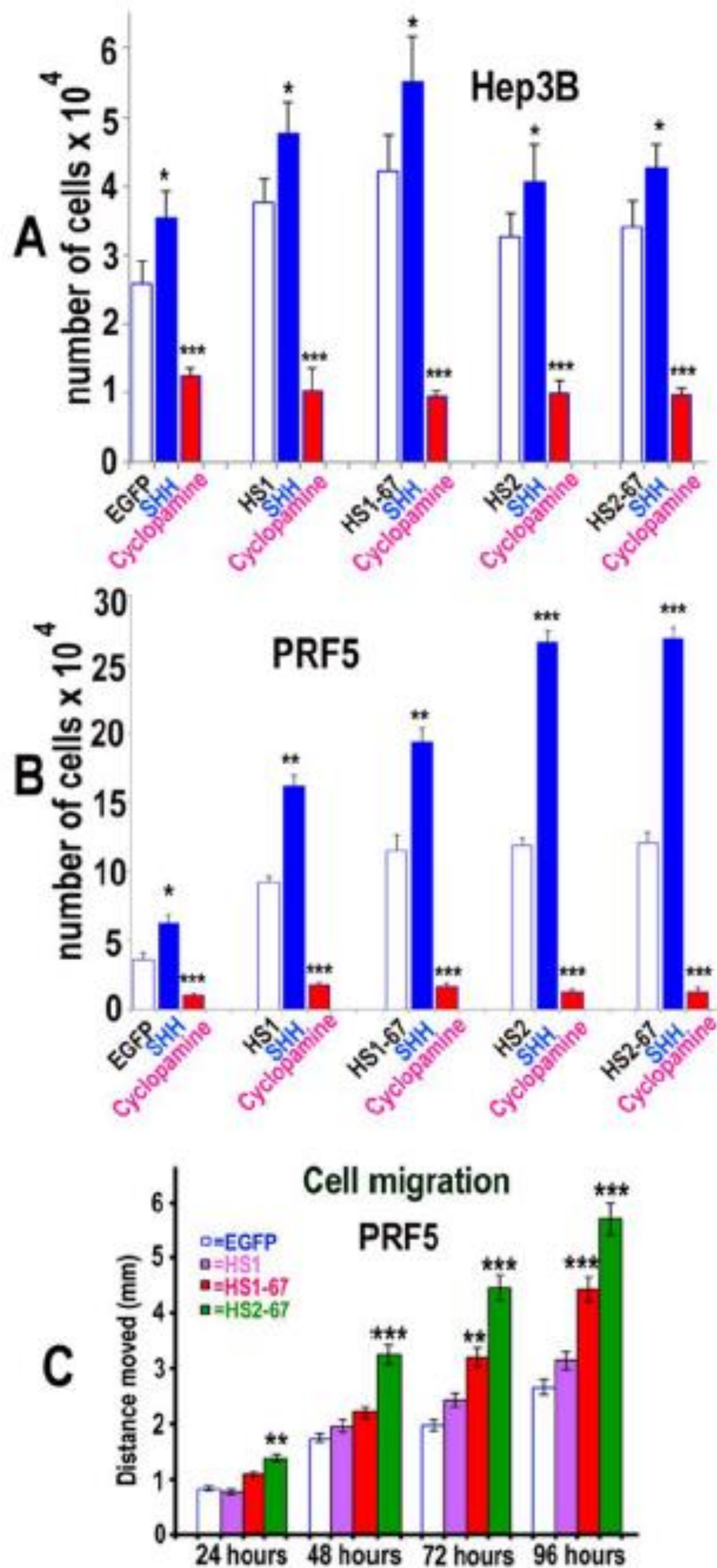


Figure 7: The effect of 10ng/ml SHH and 20μM cyclopamine on the growth of Hep3B (A) and PRF5 (B) cells transfected with different Sulf1 and Sulf2 variants over 5 days in culture. C: the migration of PRF5 cells transfected with different Sulf1 and Sulf2 variants over 4 day time period, *p<0.05, **p<0.001,***p<0.0001.



Acknowledgments: This work was supported by RVC research studentships.

References:

Ai X, Do AT, Lozynska O, Kusche-Gullberg M, Lindahl U, Emerson CP, Jr. (2003) QSulf1 remodels the 6-O sulfation states of cell surface heparan sulfate proteoglycans to promote Wnt signaling. J Cell Biol 162 (2):341-351. doi:10.1083/jcb.200212083

Ai X, Kitazawa T, Do AT, Kusche-Gullberg M, Labosky PA, Emerson CP, Jr. (2007) SULF1 and SULF2 regulate heparan sulfate-mediated GDNF signaling for esophageal innervation. Development 134 (18):3327-3338. doi:134/18/3327 [pii]Ashikari-Hada S, Habuchi H, Kariya Y, Itoh N, Reddi AH, K. K (2004) Characterization of growth factor- binding structures in heparin/heparan sulfate using an octasaccharide library. J Biol Chem 279 (13):12346-12354

Barth H, Schafer C, Adah MI, Zhang F, Linhardt RJ, Toyoda H, Kinoshita-Toyoda A, Toida T, Van Kuppevelt TH, Depla E, Von Weizsacker F, Blum HE, TF. B (2003) Cellular binding of hepatitis C virus envelope glycoprotein E2 requires cell surface heparan sulfate. J Biol Chem 278 (42):41003-41012

Bret C, Moreaux J, Schved JF, Hose D, Klein B (2011) SULFs in human neoplasia: implication as progression and prognosis factors. J Transl Med 9:72. doi:1479-5876-9-72 [pii]

Dhoot GK (2012) Recent Progress and Related Patents on the Applications of SULF1/SULF2 Enzymes in Regenerative Medicine and Cancer Therapies. . Recent Patents on Regenerative Medicine 2: 137-145

Dhoot GK, Gustafsson MK, Ai X, Sun W, Standiford DM, Emerson CP, Jr. (2001) Regulation of Wnt signaling and embryo patterning by an extracellular sulfatase. Science 293 (5535):1663-1666. doi:10.1126/science.293.5535.1663

Ford-Perriss M1, Guimond SE, Greferath U, Kita M, Grobe K, Habuchi H, Kimata K, Esko JD, Murphy M, JE. T (2002) Variant heparan sulfates synthesized in developing mouse brain differentially regulate FGF signaling. Glycobiology 12 (11):721-727

Giakoustidis A, Giakoustidis D, Mudan S, Sklavos A, R. W (2015) Molecular signalling in hepatocellular carcinoma: Role of and crosstalk among WNT/ß-catenin, Sonic Hedgehog, Notch and Dickkopf-1. Can J Gastroenterol Hepatol 29 (4):209-217

Gill R, Hitchins L, Fletcher F, Dhoot GK (2010) Sulf1A and HGF regulate satellite-cell growth. J Cell Sci 123 (Pt 11):1873-1883. doi:10.1242/jcs.061242

Gill RB, Day A, Barstow A, Liu H, Zaman G, Dhoot GK (2011) Sulf2 gene is alternatively spliced in mammalian developing and tumour tissues with functional implications. Biochem Biophys Res Commun 414 (3):468-473. doi:10.1016/j.bbrc.2011.09.088

Gill RB, Day A, Barstow A, Zaman G, Chenu C, Dhoot GK (2012) Mammalian Sulf1 RNA alternative splicing and its significance to tumour growth regulation. Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine 33 (5):1669-1680. doi:10.1007/s13277-012-0423-2

Gill RM, Michael A, Westley L, Kocher HM, Murphy JI, Dhoot GK (2014) SULF1/SULF2 splice variants differentially regulate pancreatic tumour growth progression. Exp Cell Res 324 (2):157-171. doi:10.1016/j.yexcr.2014.04.001

Hitchins L, Fletcher F, Allen S, Dhoot GK (2013) Role of Sulf1A in Wnt1- and Wnt6-induced growth regulation and myoblast hyper-elongation. FEBS Open Bio 3:30-34. doi:10.1016/j.fob.2012.11.007

Joy MT, Vrbova G, Dhoot GK, Anderson PN (2015) Sulf1 and Sulf2 expression in the nervous system and its role in limiting neurite outgrowth in vitro. Experimental neurology 263:150-160. doi:10.1016/j.expneurol.2014.10.011

Kroy DC, Beraza N, Tschaharganeh DF, Sander LE, Erschfeld S, Giebeler A, Liedtke C, Wasmuth HE, Trautwein C, KL. S (2010) Lack of interleukin-6/glycoprotein 130/signal transducers and activators of transcription-3 signaling in hepatocytes predisposes to liver steatosis and injury in mice. Hepatology 51 (2):463-473. doi:doi: 10.1002/hep.23322.

Kwon YC, Bose SK, Steele R, Meyer K, Di Bisceglie AM, Ray RB, R. R (2015) Promotion of Cancer Stem-Like Cell Properties in Hepatitis C Virus-Infected Hepatocytes. J Virol 89 (22):11549-11556. doi:doi: 10.1128/JVI.01946-15.

Lai J, Chien J, Staub J, Avula R, Greene EL, Matthews TA, Smith DI, Kaufmann SH, Roberts LR, Shridhar V (2003) Loss of HSulf-1 up-regulates heparin-binding growth factor signaling in cancer. J Biol Chem 278 (25):23107-23117. doi:10.1074/jbc.M302203200 [pii]

Lai JP, Sandhu DS, Shire AM, Roberts LR (2008a) The tumor suppressor function of human sulfatase 1 (SULF1) in carcinogenesis. J Gastrointest Cancer 39 (1-4):149-158. doi:10.1007/s12029-009-9058-y

Lai JP, Thompson JR, Sandhu DS, Roberts LR (2008b) Heparin-degrading sulfatases in hepatocellular carcinoma: roles in pathogenesis and therapy targets. Future Oncol 4 (6):803-814. doi:10.2217/14796694.4.6.803

Lai JP, Yu C, Moser CD, Aderca I, Han T, Garvey TD, Murphy LM, Garrity-Park MM, Shridhar V, Adjei AA, Roberts LR (2006) SULF1 inhibits tumor growth and potentiates the effects of histone deacetylase inhibitors in hepatocellular carcinoma. Gastroenterology 130 (7):2130-2144. doi:S0016-5085(06)00734-7 [pii]

Lemjabbar-Alaoui H, van Zante A, Singer MS, Xue Q, Wang YQ, Tsay D, He B, Jablons DM, Rosen SD Sulf-2, a heparan sulfate endosulfatase, promotes human lung carcinogenesis. Oncogene 29 (5):635-646. doi:onc2009.365 [pii]

Merry CL, Gallagher JT (2002) New insights into heparan sulphate biosynthesis from the study of mutant mice. Biochemical Society symposium 69:47-57

Meyers-Needham M1, Lewis JA, Gencer S, Sentelle RD, Saddoughi SA, Clarke CJ, Hannun YA, Norell H, da Palma TM, Nishimura M, Kraveka JM, Khavandgar Z, Murshed M, Cevik MO, B. O (2012) Off-target function of the Sonic hedgehog inhibitor cyclopamine in mediating apoptosis via nitric oxide-dependent neutral sphingomyelinase 2/ceramide induction. Mol Cancer Ther 11 (5):1092-1102 doi:doi: 10.1158/1535-7163.MCT-11-0705

Morimoto-Tomita M, Uchimura K, Werb Z, Hemmerich S, Rosen SD (2002) Cloning and characterization of two extracellular heparin-degrading endosulfatases in mice and humans. J Biol Chem 277 (51):49175-49185. doi:10.1074/jbc.M205131200

Nawroth R, van Zante A, Cervantes S, McManus M, Hebrok M, Rosen SD (2007) Extracellular sulfatases, elements of the Wnt signaling pathway, positively regulate growth and tumorigenicity of human pancreatic cancer cells. PLoS One 2 (4):e392. doi:10.1371/journal.pone.0000392

Rosen SD, Lemjabbar-Alaoui H (2010) Sulf-2: an extracellular modulator of cell signaling and a cancer target candidate. Expert Opin Ther Targets 14 (9):935-949. doi:10.1517/14728222.2010.504718

Sahota AP, Dhoot GK (2009) A novel SULF1 splice variant inhibits Wnt signalling but enhances angiogenesis by opposing SULF1 activity. Exp Cell Res 315 (16):2752-2764. doi:10.1016/j.yexcr.2009.06.029

Schulze A, Gripon P, S. U (2007) Hepatitis B virus infection initiates with a large surface protein- dependent binding to heparan sulfate proteoglycans. Hepatology 46 (6):1759-1768

Shukla D, Liu J, Blaiklock P, Shworak NW, Bai X EJ, Cohen GH, Eisenberg RJ, Rosenberg RD, PG. S A novel role for 3-O-sulfated heparan sulfate in herpes simplex virus 1 entry. Cell 99 (1):13-22

Tarocchi M, Polvani S, Marroncini G, A. G (2014) Molecular mechanism of hepatitis B virus-induced hepatocarcinogenesis. World J Gastroenterol 20 (33): 11630-11640. doi: 10.3748/wjg.v20.i33.11630.

Tátrai P1, Egedi K, Somorácz A, van Kuppevelt TH, Ten Dam G, Lyon M, Deakin JA, Kiss A, Schaff Z, I. K (2010) Quantitative and qualitative alterations of heparan sulfate in fibrogenic liver diseases and hepatocellular cancer. J Histochem Cytochem 58 (5):429-441. doi: 10.1369/jhc.2010.955161.

Tuo J WY, Cheng R, Li Y, Chen M, Qiu F, Qian H, Shen D, Penalva R, Xu H, Ma JX, Chan CC. (2015) Wnt signaling in age-related macular degeneration: human macular tissue and mouse model. J Transl Med 13 (1):330-342. doi: 10.1186/s12967-015-0683-x.

Uchimura K, Morimoto-Tomita M, Bistrup A, Li J, Lyon M, Gallagher J, Werb Z, Rosen SD (2006) HSulf-2, an extracellular endoglucosamine-6-sulfatase, selectively mobilizes heparin-bound growth factors and chemokines: effects on VEGF, FGF-1, and SDF-1. BMC Biochem 7:2. doi:10.1186/1471-2091-7-2

Wang S1, Jiang W, Chen X, Zhang C, Li H, Hou W, Liu Z, McNutt MA, Lu F, G. L (2012) Alpha-fetoprotein acts as a novel signal molecule and mediates transcription of Fn14 in human hepatocellular carcinoma. J Hepatol 57 (2):322-329. doi: 10.1016/j.jhep.2012.03.029.

Wang S, Ai X, Freeman SD, Pownall ME, Lu Q, Kessler DS, Emerson CP, Jr. (2004) QSulf1, a heparansulfate 6-O-endosulfatase, inhibits fibroblast growth factor signaling in mesoderm induction and angiogenesis. Proc Natl Acad Sci U S A 101 (14):4833-4838. doi:10.1073/pnas.0401028101

Xu G, Ji W, Su Y, Xu Y, Yan Y, Shen S, Li X, Sun B, Qian H, Chen L, Fu X, Wu M, C. S (2014) Sulfatase 1 (hSulf-1) reverses basic fibroblast growth factor-stimulated signaling and inhibits growth of hepatocellular carcinoma in animal model. Oncotarget 5 (13):5029-5039

Xu Y, Martinez P, Séron K, Luo G, Allain F, Dubuisson J, S. B (2015) Characterization of hepatitis C virus interaction with heparan sulfate proteoglycans. J Virol 89 (7):3846-3858. doi: 10.1128/JVI.03647-14

Zaman G, Staines K, Farquharson C, Newton PT, Dudhia J, Chenu C, Pitsillides A, Dhoot GK (2016) Expression of Sulf1 and Sulf2 in cartilage, bone and endochondral fracture healing. Histochem Cell Biol 145 (1):67-79