The generation of monoclonal antibodies to investigate perlecan turnover in cells and tissues

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The generation of monoclonal antibodies to investigate perlecan turnover in cells and tissues

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Abstract

Perlecan is an important basement membrane heparan sulfate (HS) proteoglycan that is essential for various cell signaling events involved in tissue development. Heparanase is a lysosomal enzyme involved in the turnover of HS. This project aimed to assist in researching the structure of HS on perlecan and how this structure changes with tissue development. This will be achieved by generating monoclonal antibodies that have an altered affinity for perlecan after heparanase treatment.

Recombinant perlecan domain I was characterized by ELISA and western blotting and used as the antigen for two fusions. The first fusion was focused on the production of IgM the common subtype of anti-glycosaminoglycans antibodies. However, no clones were produced, which may have been due to the lack of feeder layers. In order to address this problem, the fibroblast cell line MRC-5 was used as a feeder layer in the second fusion. From this fusion, we obtained 216 positive cultures, which were screened against full length perlecan from endothelial cells. Of these, 26 cultures were tested against heparanase treated perlecan, and then 2 cultures were chosen for subcloning based on the different immunoreactivity between enzyme treated and non-treated perlecan. From the 2 chosen cultures, 13 sub clones were derived and 10 of them were adapted into a serum free culture environment. The 10 monoclonal antibodies displayed strong immunoreactivity with full length perlecan in ELISA and Western Blotting. When they were used as primary antibodies in Immunocytochemistry, they were able to recognize the native perlecan deposited by human chondrocytes. When the cells were incubated with heparanase, antibody 5D7-2E4 and 13E9-3G5 showed an increase in immunoreactivity while antibody 13E9-3B3 gave a decrease. These three

antibodies will be the potential tools used in the future to study perlecan turnover in different cells and tissue. The remaining seven antibodies will also be very useful in the research of perlecan as they have been shown to bind to the protein core. In the future, it will be worth subcloning some of the frozen stored stocks of uncloned hybridomas, where there are potential opportunities to select antibodies, which will react with the carbohydrate chains on perlecan.

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1 Introduction

1.1 From proteoglycan to perlecan

1.1.1 Glycosaminoglycan

Glycosaminoglycans (GAG) are long unbranched carbohydrate chain consisting of repeating disaccharide units [1]. Four common types of GAGs are heparan sulfate (HS), chondroitin sulfate (CS)/dermatan sulfate (DS), keratan sulfate (KS) and hyaluronic acid (HA) [1]. HS and HA consist of N-acetylglucosamine and uronic acid; CS and DS consist of N-acetylgalactosamine and uronic acid, and KS consists N-acetylglucosamine and galactose [1]. Except for HA which is not sulfated and exists as a free GAG in the extracellular matrix, most GAGs are sulfated and attached covalently to proteins [1]. This report concentrates on HS and its turnover in association with perlecan a major HS proteoglycan that interests us most. The structures of low-sulfated and high-sulfated heparan sulfate disaccharides are shown in Figure 1-1. N-Acetylglucosamine and glucuronate are always paired and so do N-sulfaminoglucosamine and iduronate [2]. While the high-sulfated disaccharides are a major proportion of disaccharides in heparin, the low-sulfated disaccharides predominate in HS [2].

In extracellular matrix (ECM), GAGs occupy a large amount of space forming a hydrated gel. Other than space filling, they also bind to various macromolecules including many growth factors through charge interaction [3]. The distribution of the charge density extensively influences the activity and function of the GAG [4]. Furthermore, some specific interactions are derived from definite structural features, such as the pentasaccharide sequence that contains a 3-sulfated 2-deoxy-2-sulfaminogulcose and is essential for

enhancing the thrombin-antithrombin reaction of heparin [5].

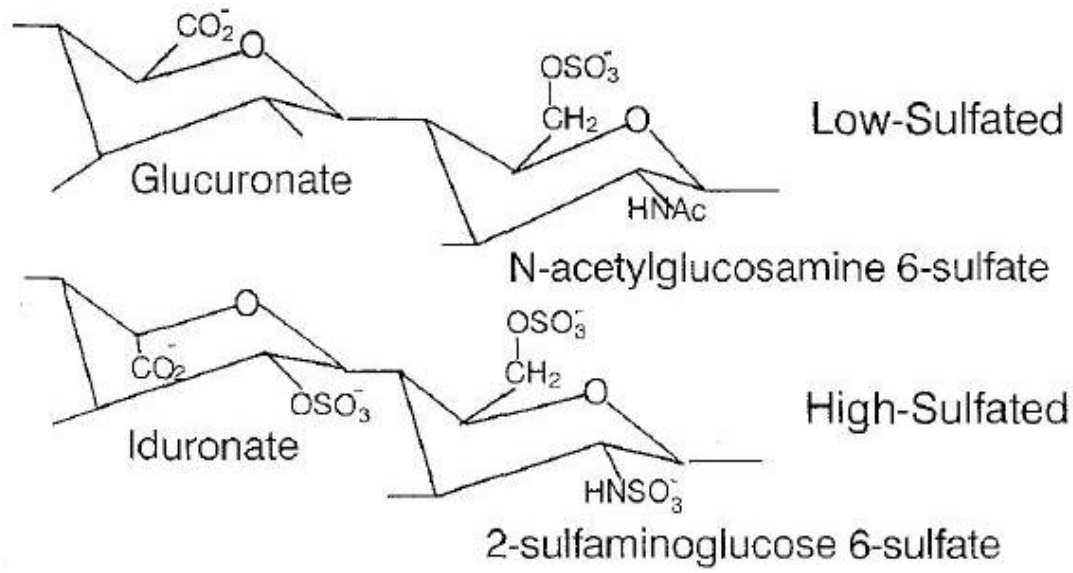

Figure 1-1 the structure of HS/heparin disaccharide (Sourced from [2])

1.1.2 Proteoglycan

A proteoglycan (PG) carries one or more covalently bound GAGs [6]. The size of the core proteins varies from 11 kDa to approximately 700 kDa and the number of GAGs varies from one to more than 100 [7-9]. The side chains may be CS, DS, KS, or HS depending on the xylosyltranserase receptors on the core protein, which are essential for the synthesis of most GAGs [6]. The protein glycosylation such as the decoration of proteoglycan with different GAGs is an enzymatic process occurring inside the lumen of endoplasmic reticulum and the Golgi apparatus. Based on the nature of chemical linkage between specific acceptor residues in the protein and carbohydrates, mammalian glycosylation can be divided into four types: N-linked and O-linked glycosylation, C-mannosylation, and glycosylphosphatidylinositol (GPI) anchors [10-13].

Since GAGs, with highly charged sulfate and carboxylate groups, are much larger than the

protein to which they are attached, they usually dominate the physical properties of the entire molecule and these charged GAGs give the PG the abilities to play multiple roles in many cellular events such as construction of ECM, cell adhesion and control of cell proliferation [6, 14].

Many different proteins can bind ionically to specific sugar sequences in the GAG chains on PG [15, 16]. The strength of the binding with these proteins is primarily determined by the degree of sulfation of the GAG chain [6]. For instance, heparin, the highly sulfated HS, can bind much more strongly than HS, CS or DS to ligands [17, 18]. The binding between PG and other molecules is also affected by the size and the number of GAG chains, for PGs with multiple GAG chains or longer GAG chains have more opportunities to access to multiple interactions with other molecules [19]. Besides the GAGs carried on PGs, core proteins are also capable of inducing the proteoglycan interaction with other macromolecules. For example, core proteins of cartilage proteoglycans such as aggrecan, not only bind to the HA but also can bind to simple sugars such as fucose and galactose [20].

All these interactions are critical for proteoglycan to function properly in cellular event. By their interactions with ECM molecules, proteoglycans are capable of influencing cell adhesion [6]. On one hand, some proteoglycans complement the integrin-mediated adhesion by promoting the organization of actin filaments in the attaching cells [21]. On the other hand, soluble proteoglycans can inhibit the cell from adhering to fibronectin and collagen by competing for GAG binding sites with cells surface proteoglycans and prevent the binding between integrin and cell attachment site by steric hindrance [22-25].

Studies carried out with heparin indicated a role of GAGs in the growth control [26]. The growth inhibition mediated by heparin was first described by Clowes and Karnovsky in 1977 [26]. Although some molecular mechanisms are still unknown, the available evidence suggests that the interaction with growth factors and the influence on cell cycle events may be involved [27]. However, in another situation cell proliferation can also be stimulated by heparin, where heparin serves as a reservoir of growth factors preventing them from degradation and enhancing the activity [28, 29].

1.1.3 Heparan Sulfate Proteoglycan

Heparan Sulfate Proteoglycans (HSPGs) are one class of PG and are found widely expressed on cell surfaces and in the extracellular matrix (ECM) where they have been suggested to be involved in cell adhesion, migration, proliferation, and differentiation [30]. The carbohydrate side chains on HSPG distinguished from other PG by having repeated disaccharide units composed of a hexosamine and uronic acid [31].

The degree of sulfation of the HS chains is variable and responsible for ligand binding specificity and subsequent biological functions of HSPG [32, 33]. Different cell type can decorate a protein core with HS chains in different ways inducing distinct biological function [34]. The GAG structures have been observed to change during the pathogenesis, during the development or in response to extracellular signals such as growth factors [35]. Many studies have been carried out on the interactions between HS and extracellular signalling molecules including growth factors, chemokines, adhesions proteins, and certain proteases, esterases, as well as HS degrading enzymes [36-38]. The HS plays an essential role in the regulation of inflammatory processed, cell growth and differentiation, lipid

transport and clearance, cell-cell and cell-matrix interactions and blood coagulation processes [39, 40]. The binding of chemokines to HS stabilizes their concentration gradients across the endothelial surface, which directs the migration of leukocytes [41]. HS also protects chemokines from proteolytic degradation and mediates their oligomerization promoting local concentrations of the chemokines surrounding their G-coupled signalling receptors [41]. Another example of the interaction between bio-molecule and HS structure is the binding to basic fibroblast growth factor (FGF2) and FGF receptor-1 (FGFR1). In this case, the association of this growth factor with HS not only prevents FGF2 from proteolysis and thermal denaturation, but also improves the activation of FGFR1 by largely enhancing the FGF2-FGFR binding [42, 43]. Although most interactions with ECM molecules are induced by HS chains, the protein cores are also able to interact with cytoskeletal components [44, 45]. In fact, some domains of these protein cores are highly conserved throughout evolution, which indicates that those domains may perform important roles [46].

1.1.4 Perlecan

Perlecan is present in the ECM as a large HSPG with a protein core (~470 kDa in human) consisting of five distinct structural domains [47]. Human perlecan is encoded by the gene HSPG2 which was mapped to chromosome 1 and was first described and characterized by Cohen et al. in 1993 [48, 49]. Figure 1-2 illuminates the structure of human perlecan. Perlecan domain I (N-terminal domain) consists of 172 amino acids with three Seri-Gly-Asp triplets which are potential GAG attachment sites [48]. CS, KS, or DS has also been found on Perlecan, although the predominant GAG is HS. The GAG substitution is tissue-specific depending on cell type and environment since certain combinations of GAGs are capable of affecting the interactions with surrounding ligands [50, 51]. Domain I is the only

domain that is unique to perlecan while the remaining four domains exhibit a high level of sequence similarity with other ECM and cell surface proteins [52]. Domain II of perlecan shares homology with the binding domain of the low density lipoprotein receptors and has an immunoglobulin-like repeat [53]. Domain III consists of four repeats which are homologous to the short arm of the laminin α chain [50]. With a 200 kDa molecular weight, Domain IV is the largest of the five domains consisting of 21 immunoglobulin-like repeats [50]. The C-terminal portion of perlecan, domain five (also known as endorepellin) contains two potential GAG attachment sites and three globular regions that are homologous to the G sub-domains of the laminin α chain and are separated by two sets of epidermal EGF-like repeats [50, 51]. Murine perlecan has a significantly smaller domain IV compared with human perlecan and contains in a cell-binding RGD sequence in domain III [47].

Perlecan can be found in nearly all basement membranes, mesenchymal organs and connective tissues, and plays a crucial role in tissue development and organogenesis by binding a variety of cell surface receptors, growth factor, ECM molecules and basement membrane proteins [39, 54]. For instance, HS (the main GAG chain present in perlecan) is essential for FGF2 signalling through FGFR1, thereby inducing receptor phosphorylation and receptor-mediated signal transduction [51]. In studies using perlecan knockout mice, nearly half of perlecan null mice died at embryonic day 10.5 and others died after birth suffering from severe defects such as abnormal basement membrane formation, defective developments of cephalic and long bone, including achondroplasia, suggesting that perlecan is important to many tissues in embryonic development [55, 56]. Perlecan has also been shown to interfere with the proliferation of smooth muscle cells by inhibiting the attachment of vascular smooth muscle cells to fibronectin [55]. Perlecan also exhibits an ability to prevent thrombosis and blood coagulation after injury by binding to the protease

inhibitor antithrombin III. This binding process relies on several essential sulfate groups on perlecan's HS chains, and results in conformational changes which increase the activity of antithrombin III [57]. Moreover, perlecan has previously been shown to play an important role in cartilage formation by supporting chondrocyte differentiation and is essential for normal functioning of acetylcholine esterase at the vertebrate neuromuscular junction [58].

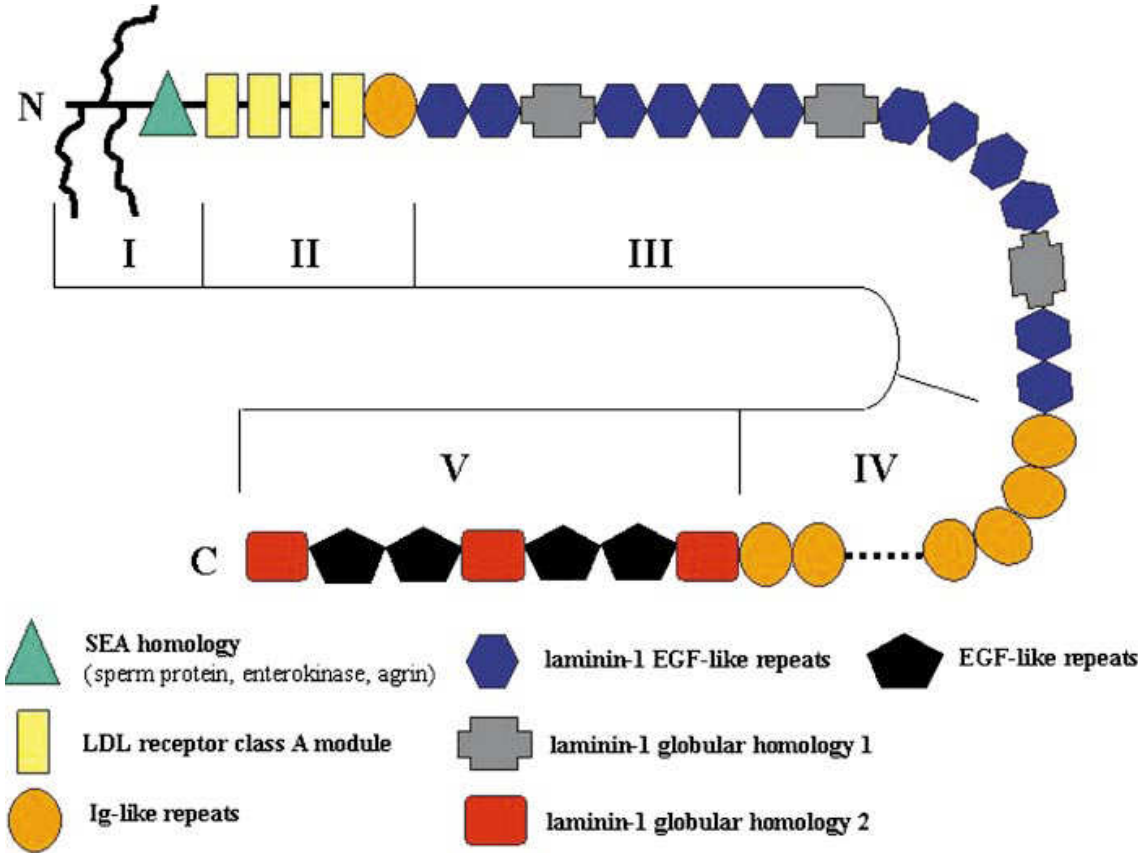

Figure 1-2 The structure of perlecan (sourced from [46])
The entire molecule is organized into five domains based on their distinct protein modules. Domain I is the N-terminal domain consisting of three GAG chains.

1.2 Heparanase and HSPG turnover

Mammalian heparanases are endoglycosidases that are able to degrade HS/heparin chains into shorter chain length oligosaccharides. Heparanase was first reported by Ögren and Lindahl in 1975 as an endoglucuronidase derived from mouse mast cells, capable of cleaving macromolecular heparin at a limited number of sites [59]. The single gene encoding a functional heparanase was cloned in 1999 by four groups [60-63], and since

then identical, or highly homologous cDNA sequences, were isolated from different cells of different species. The human heparanase gene, has a length of approximately 50kb and is mapped to chromosome 4q22 [64]. To secrete a mature heparanase, an inactivated 65-kDa precursor is first expressed in the Golgi [65]. This pro-heparanase is transferred into lysosomes and proteolytically processed into its active form, a heterodimer where the 50-kDa subunit is associated non-covalently with an 8-kDa peptide derived from the N-terminus of the 65-kDa pro-heparanase [65]. It is essential that the subunits are correctly folded within a cellular environment since individually expressed subunits does not have enzyme activity [66].

The activity of human heparanase is maximal between pH 5.5 and 5.8 and is abolished below pH 4.0 and above pH7.5 [67-69]. The hypothesis to explain this character is that the pH at sites of inflammation or matrix damage is lower than the surrounding physiological environment and the enzyme activated by the low pH environment stimulates cell migration and proliferation [67, 70, 71]. The restricted cleavage of HS/heparin chains by heparanase results in sugar units of 5-20 kDa [72]. Although all the substrate specificity studies have indicated that heparanase is an Endo-beta-D-glucuronidase, the HS/heparin sequence processed by the enzyme is not clear [69, 73-75]. Studies of a heparanase-GFP fusion protein together with immunostaining illustrated that the enzyme resided predominantly within late endosomes/lysosomes, both in transfected normal cells and in cells that over express heparanase [76, 77]. The synthesized heparanase –GFP in the lysosomes presented no sign of degranulation or trafficking, indicating that this enzyme is stored within these acidic cellular organs in a stable form [76]. The half-life of the newly produced heparanase was approximately 30 hours [78].

In normal development, heparanase contributes to the processes of tissue morphogenesis, regeneration and repair during embryonic development and in the adult, by assisting ECM remodelling, cell migration, adhesion and proliferation [79, 80]. Heparanase is also involved during the differentiation of many cells by processing nuclear functional HSPGs and therefore affecting HS-bound growth factors [81-83].

A crucial function of heparanase is to cleave the basement membrane HSPGs at the injured or inflammatory sites, encouraging the migration of leucocytes and releasing growth factors and chemokines which provoke the migration and proliferation of endothelial cells and fibroblasts [60, 61, 71]. In numerous other haematopoietic cells such as neutrophils, monocytes, B-lymphocytes, macrophages and dendritic cells, the active heparanase is also expressed [84-86]. The activity of heparanase is boosted by chemoattractants and inflammatory stimuli, leading to a relocation of the active enzyme from specific intracellular granules to the surface of the cell and liberation by degranulation [70, 87-89].

Tumour metastasis is also associated with heparanase. Cancer invasion and metastasis largely depends on the degradation of the various constituents of the ECM and the metastatic potential of cancer cells is associated with their ability to degrade HS [90, 91]. This is supported by an increased expression of active heparanase in cancer cells, which correlates with the poor prognosis in the patients [60, 61, 92, 93]. The conversion of non-metastatic murine T-lymphoma and melanoma cells into highly metastatic cells inducted by the transfection with human heparanase cDNA provided convincing and direct support for the importance of heparanase in tumour metastasises [60].

Moreover, heparanase contributes to the growth of tumours through angiogenesis. HSPGs

are prominent components of blood vessel walls [94]. HS and HS-degrading enzyme have been involved in a number of angiogenesis-related cellular events such as cell invasion, migration, adhesion and differentiation [44, 94, 95]. Heparanase facilitates the invasion and migration of endothelial cells, by degrading the HS chains of the subendothelial capillary basement membrane and therefore disrupting the physical barrier. Heparanase activity also results in the release of HS-bound angiogenic factors which largely promote the process of angiogenesis. For instance, the precise HS-fragments produced by heparanase are the key for FGF2-FGFR1-HS ternary complexes that raise biological consequences and endothelial cell proliferation [96-98].

Recently, the emerging evidences have been revealed that heparanase enhances the syndecan-1 shedding on cell surfaces [99, 100]. The shed ectodomain of sydecan-1 increases the growth, metastasis and angiogenesis of tumours [101]. The syndecan-1 shedding requires activated heparanase and also can be induced by bacterial heparitinase, indicating that this shedding might be a result of the enzyme mediated HS chains turnover [99, 100].

Besides the degradation through the action of heparanase, another way to regulate the HS turnover and its interaction with protein ligands is to regulate its synthesis. HS biosynthesis is a very specific and sequential event occurring mainly in the Golgi apparatus and endoplasmic reticulum, and involves a complex set of enzymes, reviewed by Whitelock Iozzo *et* al., 2005 [102]. Due to incomplete modification reactions in Golgi apparatus, the final HS molecule shows a typical domain structure: NS domains and NA domains, which are bridged by a “mixed sequences” [103]. NS domains cluster N- and O-sulfate groups to which protein ligands bind [33]. Therefore, the interaction of a ligand with ECM or cell

surface HSPG is depended on the number of NS domain synthesis.

1.3 Monoclonal antibodies (mAb) and Hybridomas

1.3.1 Antibodies

Antibodies (Abs), also known as immunoglobulins (Igs), are glycoproteins secreted by B lymphocytes to recognize and neutralize soluble foreign objects (antigens) [104]. Antigen binding is the primary function of Abs and each Ab actually binds only to one specific antigenic determinant which is called an epitope. The binding to antigen recruits inflammatory cells and mediators potentially leading to cell lysis resulting in the protection of the host.

The Ig monomer is a Y-shaped molecule that consists of four polypeptide chains, where two identical heavy chains and two identical light chains were connected by disulfide bonds [105]. Figure 1-3 shows the structure of an Ig monomer. Each chain can be divided into two structural domains (variable or V domain and constant or C domain) based on variability in the amino acid sequences [106]. Digestion of Ig monomer with papain results in three fragments: two identical antigen binding fragments (Fab) and a crystallisable fragment (Fc) [107]. The Fab region contains the antigen binding sites of the Ig and composed of one V and C domain from each heavy and light chain (V_L, V_H, C_L and C_{H1}) [107]. The Fc region contains the remainder of the two heavy chains each containing a C_{H2} and C_{H3} domain [107]. By binding to specific molecules such as various cell receptors and complement proteins, the Fc region mediates appropriate immune responses for a given antigen including opsonization, cell lysis, and degranulation of mast cells, basophils and eosinophils [105, 108, 109].

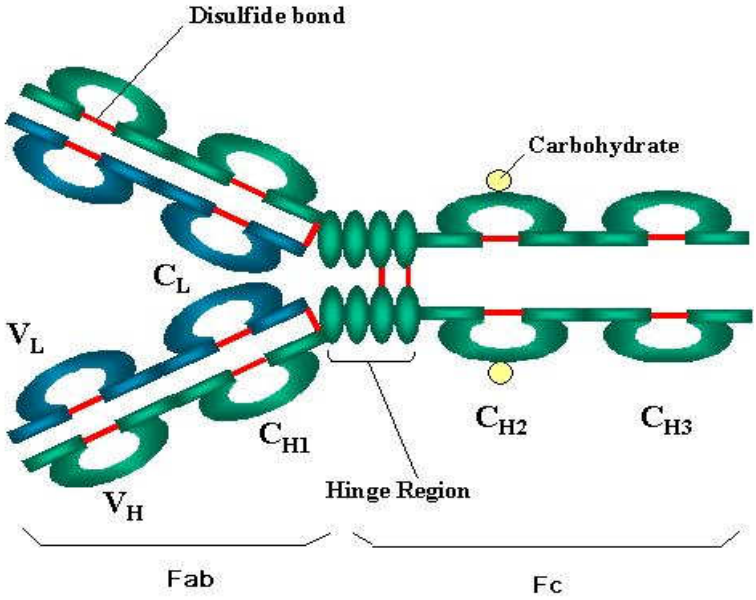

Figure 1-3 the structure of Ig monomer

Antibodies can be divided into different classes called isotype, based on the primary sequence of amino acids in the constant region of the heavy chain. The five isotypes in placental mammals are IgA, IgD, IgE, IgG and IgM with different biological functions. IgG is a monomer and it is the most crucial and abundant class providing the majority of antibody-based immunity against invading pathogens. IgG is the only antibody capable of crossing the placenta to give passive immunity to the fetus [110]. IgG can be further classified into four subclasses: IgG1, IgG2, IgG3 and IgG4. IgM normally presents as a pentamer but also can be a hexamer or a monomer. It is the first Ab appearing in the immune response with very high avidity for eliminating pathogens in the early stages of B cell mediated immunity before there is sufficient IgG [110, 111]. The high avidity of IgM allows it to bind to weakly cross-reacting antigens including some carbohydrate antigens. The IgM on the surface of B cells functions as a receptor for antigens and induces B cells to differentiate into antibody secreting plasma cells [110]. IgA is known as a monomer or

dimer and the major class of Ig in secretions such as tears, saliva, colostrums and mucus [112]. IgD exists as a monomer, functioning as a receptor for antigens mainly on B cell surfaces which have not been exposed to antigens [111]. IgE is monomer and has an extra domain in the constant region. IgE is responsible for allergies by binding to allergens and triggering histamine release from mast cells and basophils [110]. IgD and IgE do not bind to complement.

1.3.2 Monoclonal antibodies and Hybridomas

A monoclonal antibody is the antibody secreted by one single clone of B lymphoid cell and all the antibody molecules have the same amino acid sequence and the same binding properties. Although genetic approaches are applied to an increasing degree, the most common way to generate monoclonal antibodies is still to establish cell lines called hybridomas. The classical method to construct hybridomas was developed by Kohler, G. and C. Milstein in 1975 [113]. In their technique, B cells from an immunized animal making useful antibodies are fused with a transfected or mutated myeloma cell line called a fusion partner to obtain a new hybrid cell line, hybridoma [113]. The myeloma cell lines usually are selected variants which have lost or partially lost their own immunoglobulin expressions and are sensitive to hypoxanthine, aminopterin and thymidine (HAT). Selected fusion partners are shown in Table 1-1.

Species	Identification	Ig produced	Reference
Mouse	NS1	Light chain only	[113]
	Sp2/0-Ag14	None	[114]
	F0	None	[115]
	X63-Ag8	None	[116]
Rat	Y3	Light chain only	[117]
Human	SK0-007	IgE	[118]
	GM1500 6TG-A12	IgG	[119]
	LICR LON/HMy2	IgG	[120]

Table 1-1 Selected myeloma cell lines used as fusion partner

Special growth supports are required during selection directly after fusion and each following sub cloning steps [115]. Such a supporting environment is usually provided by different cell populations called feeder cells (feeder layers), or by many different conditioned media or growth factors added to hybridoma cultures [121-124]. A proper feeder layer can largely enhance the fusion efficiencies especially for weak immunogens and reduce the spleen cell input approximately 50 times [115]. Cell populations most frequently used as feeder cells for murine hybridomas are peritoneal macrophages, fibroblasts or contact inhibited 3T3 cells [125-127]. Table 1-2 shows the efficiency of the fusion in the presence of various feeder layers.

The type of feeder layer	Established Hybridomas/planted wells (%)
Medium only	1.5
Peritoneal macrophages	1.7
Spleen cells	2.1
NIH-3T3	0
HeLa R-19	2.6
Vero	9.5
IMR-90	9.9
MRC-5	17.6
W1-38	18.1

Table 1-2 comparisons of some feeder layers (sourced from [128])

1.3.3 Other approaches to obtain monoclonal antibodies

The fact that monoclonal antibodies are derived from animals limits their use as systemic therapeutic agents in humans. Recently, the understanding of antibody structure and function and advances of recombinant technologies gives researchers many alternative methods to produce antibodies, such as phage display, yeast display and ribosomal display. Among those, phage display is the most common [129]. Following the immunization of animals, recombinant antibody technology involves some basic steps [130-133]:

1. To recover the antibody genes from the source cells;
2. To amplify and clone the genes into an appropriate vector;
3. To introduce the vector carrying the antibody genes into a host;
4. To screen for interested antibodies and achieve expression of adequate amounts of functional antibody.

Due to the difficulties to manipulate and express large genes encoding entire antibody, the recombinant antibodies are usually expressed as a smallest functional unit of an antibody (scFV or Fab) [134]. This smaller sized antibody-like peptide has better penetration of solid tissue and rapid clearance of unbound antibody from the circulation, which make it superior for medical imaging and other applications [135]. Recombinant antibodies can be expressed in either eukaryotic or prokaryotic system. Prokaryotic hosts eliminate the risk of adventitious mammalian viruses in the antibody preparation, but may produce endotoxins or other contaminants [136]. Eukaryotic systems have superiority in retain normal post-translational modifications but eukaryotic cells are more difficult and expensive to culture, and give lower yields [137-139].

However those methods also have several drawbacks. The affinities of these antibodies were relatively low, as might be expected in a primary immune response [140]. Due to the complex structural requirements, many misfolded proteins unable to bind the epitope are expressed especially in prokaryotic system [141]. Although recombinant antibody has many advantages, this technology can not replace conventional antibody methods, until those limitations are overcame.

1.4 The aims and rationale of this project

One of the challenges of current studies on HSPG is the lack of molecular tools to monitor the turnover of HS chains during normal development or pathologic processes. These difficulties have partially been alleviated by the use of antibodies which recognized the epitopes with in particular core protein or GAG chains. Both sets of antibodies are essential in the study of this important group of molecules. Even though a few antibodies against

GAGs have been described in past years, the non-immunogenic nature of GAG chains have given a relatively smaller pool of antibodies against the structure of HS chain. In this project, our aim was to generate monoclonal antibodies which could be either sensitive to structural change in HS chains or against the protein core of human perlecan. The newly produced antibodies would be used in Immunocytochemistry or Immunohistochemisty, where the cells or tissues had been processed in different ways, to test the sensitivity of the antibodies. They also can be used in Western Blotting of extracts from tissues or cells at different stages of development to determine the proportion changes of different PG.

The generated antibodies could be divided in to three groups, according to the epitopes they against. The three situations are illustrated in Figure 1-4. Antibody A is against the native GAG chains attached to the protein core. Once the GAG chains are cleaved off, A will lose its epitope. Antibody B is against the residual GAGs' stub generated by the treatment with mammalian heparanase (not bacteria heparinase). Antibody B's epitope only occurs where the GAG chains are digested by mammalian heparanase. The epitope of antibody C is on the protein core which will not be affected by any glycosidases. The MAbs specifically against epitopes located within carbohydrate chains are of major interest as they can detect the subtle changes in the length or sulfation patterns of the GAG chains, which are always related to developmental or pathological processes. Even though it is possible that no GAG antibodies are generated due to the weak immunogenicity of carbohydrates and the limitation of time, there is still a good opportunity to obtain antibodies against the protein core. The protein core antibodies are also very valuable in researching PGs including detection, characterization and purification.

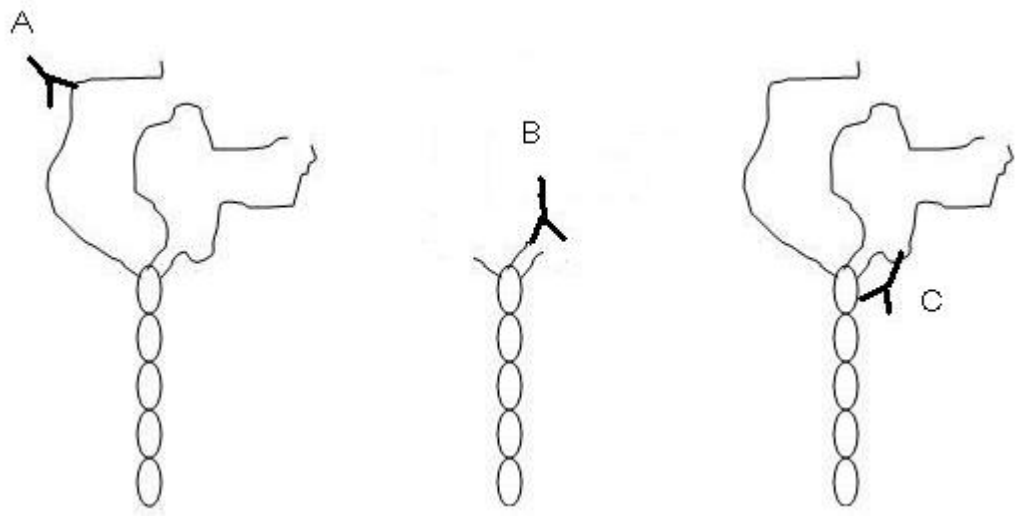

Figure 1-4 the antibodies that could be raised in this project

The specific aims of this project include:

- To collect approximately 10 litres of media conditioned by recombinant human embryonic kidney cells which are known to secrete perlecan consistently;
- To isolate milligrams of perlecan from the collected media for animal immunization and hybridomas screening;
- To generate monoclonal antibodies against isolated perlecan by hybridoma technology;
- To characterize the antibodies by ELISA, Western blotting and immunocytochemistry;
- To use the monoclonal antibodies in the study of distribution and turnover of perlecan in immunocytochemistry where human chondrocytes were used as the model.

2 Materials and Methods

2.1 Materials

DMEM and M199 were purchased from Sigma. Penicillin/streptomycin and trypsin were purchased from Invitrogen. The protein free medium UltraDOMA-PF came from Lonza Group Ltd Switzerland. Tissue culture flasks and plates (T75, T25, 96-well plate, 24-well plate) were from Greiner Bio-One. FBS, Penicillin/streptomycin and trypsin were purchased from Invitrogen. The purifications were carried on using the Bio-RAD Biologic LP system. Trizma base, Bis-Tris and Tween 20 came from Sigma. The plate reader was Infinite F200 from Tecan. The fluorescent microscopy was Axioskop2 MAT microscope from Carl Zeiss Micromaging Inc.. Electrophoresis Apparatus, Novex Mini-cell, Novex semi-dry blotter, 3-8% bis-tris gels, NuPAGE LDS 4X sample buffer, FBS, Penicillin/streptomycin and trypsin were purchased from Invitrogen. The Transfer membrane (Immobilon-PSQ: pore size 0.2um) were from Millipore in USA. Coomassie blue came from Sigma. Blotting filter paper was from Bio-Rad Developer (ILFORD Phenisol high contrast film developer) and fixer (ILFORD Nypam rapid fixer) for western blot using chemiluminescence were purchased from ILFORD, UK. Biotinylated anti-mouse Ig, Streptavidin Fluorescin and Streptavidin conjugated to horseradish peroxide were products of GE. 4’ 6’-diamidino-2-phenylindole (DAPI) was from Molecular Probes, USA. Sheep anti-mouse HRP-conjugated were purchased from Chemicon. The sources of all the antibodies used in this project were detailed in section 2.2.

2.2 Antibodies used in this project

Perlecan domain I protein core antibodies, CSI 001-71 and CSI 001-76 were raised by the fusion of Sp2/0 myeloma and B cells from the spleens of the mice immunized with whole bovine corneal endothelial cell ECM and purified using Protein A-Sepharose column [142, 143]. 7B5 MAb was purchased from Abcam. It was generated using recombinant human perlecan containing domain II, III and V and mapped to domain III by Western blotting and ELISA [144]. 10E4 and 3G10 are two MAbs specifically against heparan sulfate-related epitopes. 10E4 reacts with native heparan sulfate chains and the reactivity is fully abolished by the treatment with heparanase or heparitinase but not heparanase I or heparanase II [145]. The epitope for 3G10, in contrast, only occurs after heparitinase but not heparanase treatment [145]. MAb 5D4 is able to recognize the determinant in keratan sulfate in native proteoglycan of wide variety of species including human [146]. Chondroitin sulfate antibodies, 1B5, 2B6 and 3B3 were produced against chondroitinase ABC-treated cartilage pro heparitinase teoglycan, where 1B5 reacts with unsulfated Chondroitin; 2B6 recognizes Chondroitin 4-sulfate while 3B3 detects Chondroitin 6-sulfate [146, 147]. HepSS-1 was produced by the immunization/transplantation with a murine methylcholanthrene-induced fibrosarcoma, Meth-A [148]. HepSS-1 reacts with an epitope present in heparan sulfate glycosaminoglycans in a wide range of species including human. HepSS-1, 10E4, 3G10, 5D4, 1B5, 2B6 and 3B3 are commercially available from Seikegaku, Tokyo, Japan. Anti-EGFP antibody is a rabbit polyclonal antibody that was purchased from Sigma. CS56 was purchased from Sigma and is a MAb against Chondroitin sulfate whereas 5D5 was a gift from Dr F. Rahemtulla, University of Alabama, Birmingham, Alabama, USA. Various antibodies involved in this project and their specificity were listed in Table 2 1.

Name	Isotype	Epitope
1B5	IgG	Unsulfated Chondroitin
10E4	IgM	Native HS chain
2B6	IgG	Chondroitin 4-sulfate
3B3	IgM	Chondroitin 6-sulfate
3G10	IgG2b	heparitinase treated HS proteoglycan
5D4	IgG	Native Keratan sulfate
5D5	IgG	Native versican protein core
7B5	IgG	Perlecan domain III protein core
CSI 100-71	IgG	Perlecan domain I protein core
CSI 100-76	IgG	Perlecan domain I protein core
Anti-EGFP	Rabbit Polyclone	EGFP
CS56	IgM	Chondroitin sulfate
HepSS-1	IgM	Native HS chain

Table 2-1 list of antibodies used in this project

2.3 Methods

2.3.1 Cell culture

2.3.1.1 Recombinant human embryonic kidney (HEK 293) cell

HEK293 cells were transfected with domain I of perlecan either as a fusion protein with EGFP on C-terminus (DI6) or alone (DI2), as described by Graham, et al. in 1999. DI2 and DI6 cells were already in the lab when the project started. These cells were used as a source of perlecan/antigen for most of the studies performed in this thesis. Cells were grown in T75 tissue culture flasks or TripleFlasks in the presence of M199 supplemented with 10% FBS and 50μg/ml penicillin/streptomycin at 37℃ in an atmosphere of 5% CO_2. Both DI2 and DI6 were selected in Geneticin (G418 Sulfate) to ensure that cells were secreting a significant amount of the recombinant products. The medium was changed every 3 days and the conditioned medium was collected, pooled and stored at -20℃ for the future use.

2.3.1.2 C11-STH vascular endothelial cells

C11-STH cell line was isolated from a human umbilical venous endothelial cell culture and was spontaneously transformed [50]. The cell line has been used as a research tool for endothelial cell biology. In this project, the ECM developed by C11-STH served as a potential screening tool for hybridomas.

The medium used for C11-STH contained M199, 20% FBS, 1mM $NaHCO_3$, 10mM HEPES, 50U/ml PS and 1mM Sodium pyruvate and was supplemented with 75μg/ml ECGS and 3μg/ml heparin. Cells were initially grown in T75 flasks that had been coated

with 10μg/ml fibronectin for one hour at 37℃, prior to being seeded, in order for the cells to adhere. When cells were confluent, they were passaged by treating with trypsin for 3 minutes at 37 ℃. Cell suspensions were centrifuged at 1500RMP for 5 minutes and resuspended in the medium. Cell suspensions of 50μl at the density of 5×10^4 cells/ml were transferred to each well on half of a 96-well tissue culture plate which was pre-coated with 10μg/ml fibronectin.

The cells on the plates were grown for an additional week after reaching confluency to ensure that enough ECM was synthesized. ECM was then harvested by treatment with 0.03% Ammonia solution for five minutes. The treatment was performed more than once until all the cells were removed, which could be observed under a phase contrast microscope. The harvested ECM was washed twice with 100μl of DPBS per well. The harvested ECM could either be used immediately or stored under 100μl of DPBS at -20℃ for the future use.

2.3.1.3 Human Coronary Artery Endothelial Cell (HCAEC)

HCAECs were derived from normal human coronary arterial tissue by the Heart Research Institute Australia. HCAEC was cultured using the same method as C11 STH described in 2.2.1.2

2.3.1.4 Chondrocyte

Chondrocytes are highly differentiated cells that are found in cartilage and produce ECM molecule residing in the cartilage [149]. Chondrocytes derived from normal articular

cartilage were used in the section 4.1.2. The isolation of Chondrocytes was carried out by another PhD student (Christine Chuang) in the lab.

Chondrocytes were grown in DMEM supplemented with 10% FBS, 50μg/ml penicillin/streptomycin and 50μg/ml Ascorbic Acid. Slides sterilized in 70% ethanol at 37℃ for ten minutes were placed in one Petri dish and seeded with 1 ml of cell suspension at the density of $5x10^4$ cells. Before flooding the Petri dish with 15 ml of media, the seeded slide was incubated at 37℃ for two hours for the cells to attach to the surface. The incubation of the slide was prolonged for another two or three days after confluency to maximize the deposition of ECM

2.3.1.5 Hybridoma production

2.3.1.5.1 Sp2/0-Ag14---the fusion partner

Sp2/0-Ag14 was developed from Sp2/HL-Ag, which itself was isolated by several steps from Sp2-HLGK, a hybrid of Balb/c spleen cell and myeloma cell line X63-Ag8 [114]. The characteristics of resistance to 8-azaguamine and sensitivity to Hypoxanthine Aminopterin Thymidine (HAT) can be used to kill the unfused Sp2/0-Ag14 cells. No synthesis of Ig by this fusion partner is a great advantage for monoclonal antibody production [114]. Sp2/0-Ag14 was prepared in the presence of DMEM supplemented with 10% FBS and 50μg/ml penicillin/streptomycin at 37℃ in an atmosphere of 5% CO_2.

2.3.1.5.2 MRC-5--- the feeder layer

The human MRC5 fibroblast cell line was developed in September 1966 from lung tissue taken from an aborted 14 week fetus [150]. MRC-5 was initially grown in complete α-MEM and then switched to complete DMEM, the medium used to grow hybridomas. 96-well plate were seeded with 100 µl of MRC-5 at the density of 1×10^6 cells/ml and grow for 3-4 days post confluency before being used as feeder layers for hybridomas.

2.3.1.5.3 Immunization

Five Balb/c mice were divided into two groups. The protocol for group one was aimed at the production of IgM, where an aliquot of 100µg DI2 perlecan in 100µl DPBS emulsified with Freund's complete adjuvant (FCA) was injected to the Balb/c mouse followed by one booster injection with 200µg DI2 perlecan in 200µl DPBS without any adjuvant 10 days after primary injection. The primary injection was intraperitoneal injection while the booster injection was intravenous injection. Seven days after the primary injection (day 7), blood collected from the tails of the mice was tested for the presence of antibodies against perlecan by ELISA as described in section 2.2.3.1.

The protocol for group two was performed for the production of IgG. The primary injection was performed in the same way as performed for the group one. Three additional intraperitoneal booster injections were given every 10 days after the primary injection using 100µg DI2 perlecan well mixed with Freund's incomplete adjuvant (FIA). Seven days after the second booster injection (day 27), blood collected from the tails of the mice was tested for the presence of antibodies against perlecan by ELISA as described in section 2.2.3.1. An aliquot of 200µg DI2 perlecan in DPBS without any adjuvant was injected intravenously 10 days after the third booster injection as the final booster injection. The immunization schedules are briefly elucidated in Figure 2-1

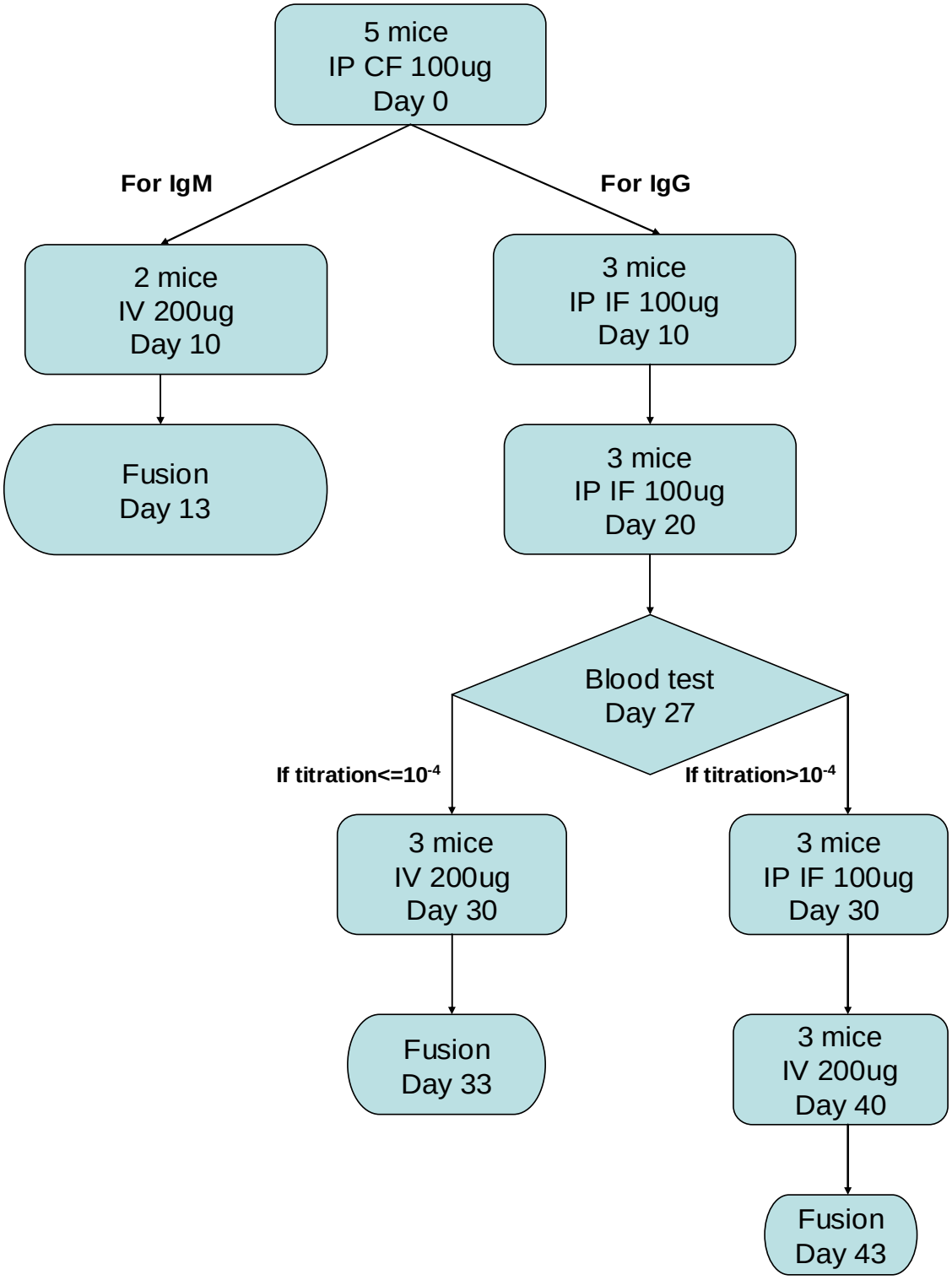

Figure 2-1 the immunization schedule

2.3.1.5.4 Fusion

Three days after the final booster injection, spleens of the sacrificed mice were dissected aseptically and placed in a sterile Petri dish containing 3 mls serum-free DMEM to keep the spleens moist. The spleen cells were dissociated by firmly grinding the spleen against a sterile sieve in a Petri dish containing 10ml of serum-free DMEM until there was only fat and connective tissue left on the mesh. The spleen cells were mixed with SP2/0 at a ratio of 5:1. The cell mixtures were centrifuged and the supernatants were discarded. After breaking up the cell pellet by tapping the bottom of the centrifuge tube, fusion between the two types of cells was induced with the addition of 50% PEG 2000. PEG was diluted by adding serum-free DMEM and centrifuging. Cells were then resuspended in 50ml DMEM supplied with 1×HAT, 20% FBS and 50μg/ml penicillin/streptomycin. The cell suspension was distributed to wells (200μl/well) on the 96-well plates previously seeded with MRC-5. To feed the cells, on day 10, 100μl media from each well were carefully aspirated and 100μl fresh HAT contained DMEM were added. The media were tested by ELISA as described below for positive clones when the cells occupied more than 50% of the bottom surface of the well. Selected positive clones were transferred to 24-well plates and fed with 1×HT, 20% FBS and 50μg/ml penicillin/streptomycin.

2.3.1.5.5 Sub cloning

Selected hybridomas were suspended in DMEM supplemented with 20% FBS and 50μg/ml penicillin/streptomycin at the concentration of 1 cell/100μl and the suspensions were dispensed 100μl per well on the 96-well plates previously seeded with MRC-5. The plates were incubated for one week without changing the medium and checked under phase contrast microscope to ensure that each well had only one single isolated clone. The

conditioned media from the clones were assayed by ELISA against antigen. The cloned cells were chosen to be expanded in 24-well plates.

2.3.2 Chromatography

2.3.2.1 Anion-Exchange Chromatography (Diethylaminoethyl) (DEAE-Sepharose)

The column (100ml) was equilibrated using two column volumes of 250mM NaC1, 20mM Tris, 10mM EDTA, pH 7.5 (DEAE running buffer) at the flow rate of 2ml/min. Filtered conditioned media (filtered using Whatman 150 mm filter paper, #1001-150) were loaded at 4℃ and then the column was washed with 3 bed volumes of running buffer or until an A_{280} baseline was achieved. Bound proteins were eluted with 1M NaC1, 20mM Tris, 10mM EDTA, pH 7.5 at the flow rate of 1ml/min and collected in an ice-cold container. The column was regenerated and stored in 2M NaC1, 20mM Tris, 10mM EDTA, pH 7.5.

2.3.2.2 Q Sepharose Fast Flow column

The column (10ml) was equilibrated using two column volumes of 250mM NaC1, 20mM Tris, 10mM EDTA, pH 7.5 (Q running buffer) at the flow rate of 1ml/min. DEAE eluting peak diluted 1 in four in Milli-Q water, were loaded at 4℃ and then the column was washed with 3 bed volumes of running buffer or until an A_{280} baseline was achieved. Bound proteins were eluted with 1M NaC1, 20mM Tris, 10mM EDTA, pH 7.5 at the flow rate of 1ml/min and collected in an ice-cold container. The column was regenerated and stored in 2M NaC1, 20mM Tris, 10mM EDTA, pH 7.5.

2.3.2.3 Immunoaffinity chromatography (IAC) columns

Monoclonal antibody CSI 001-76 has been immobilized on a 1ml IAC column by a previous student in the lab. After the equilibration of the IAC column with five bed volumes of 1M NaC1, 20mM Tris, 10mM EDTA, pH 7.5 (IAC running buffer), the column was loaded by recirculating the eluting peak from Q Sepharose overnight at the flow rate of 1ml/min at 4℃. Before eluting the column with 6M urea, in DPBS, pH 7.5, an A_{280} baseline was obtained by flowing the IAC running buffer through the column. The eluted proteins was collected in an ice-cold container and then dialyzed against DPBS to eliminate the traces of urea. The purified Perlecan was aliquoted, labeled and frozen at -20℃ for the future use. The column was regenerated with 5 bed volumes IAC running buffer and stored at 4℃.

2.3.2.4 Antibody purification---Protein A chromatography

The column (1ml) was equilibrated with 5 bed volumes of DPBS. Ascites or the conditioned media from hybridoma cultures were filtered through 0.45μm membrane and applied to the column at the flow rate of 1ml/min at 4℃. The column was washed with 5 bed volumes of DPBS or until an A_{280} baseline was obtained. The column was eluted with 0.1M glycine, pH3.0 at the flow rate of 1ml/min. The eluted protein was collected in an ice-cold container, neutralized immediately with 1M Tris, pH9.0 and then dialyzed against DPBS over night at 4℃. Sodium Azide was added into the purified antibodies at a final concentration of 0.02%. Antibodies were aliquoted, labeled and frozen at -20℃ for future

use. The column was regenerated with 5 bed volumes binding buffer and stored in 20% ethanol at 4℃for long term.

2.3.3 Enzyme-Linked ImmunoSorbent Assay (ELISA)

2.3.3.1 Indirect ELISA

High-binding ELISA plates were coated with 50 μl of antigens (the concentration was varied from 1μg/ml to 10μg/ml depending on the purity and activity of antigen.) in DPBS at 4℃ overnight. The wells were washed three times with washing buffer (0.1% Tween 20 in DPBS) and blocked for two hours in DPBS with 100μl/well of 0.02% W/V casein at room temperature. The wells were washed three times with washing buffer and 50 μl of primary antibodies in suitable dilution in blocking buffer were added to the wells. After one hour of incubation at room temperature, the plates were washed three times with washing buffer and loaded with 50 μl/well of biotinylated anti-mouse IgG diluted 1/1000 in blocking buffer as secondary antibody. After one hour of incubation followed by three washes, 50 μl/well of HRP-streptavidin complex diluted 1/500 in blocking buffer was pipetted into wells and incubated for half an hour at room temperature. Plates were washed four times with washing buffer and developed with 1mM ABTS (2,2'-azino-di-(3 ethylbenzthiazoline sulfonic acid)) in 70mM citrate-phosphate buffer, pH4.2. Plates were left in room temperature for 10 to 30 minutes and the absorbance was measured at the wave length of 405nm.

2.3.3.2 Sandwich ELISA

High-binding ELISA plates were coated by 50 µl of rabbit anti-mouse IgG diluted 1/1000 in DPBS at 4℃ overnight. The wells were washed three times with washing buffer (0.1% Tween 20 in DPBS) and blocked for two hours in DPBS with 100µl/well of 0.02% W/V casein at room temperature. The wells were washed three times with washing buffer and 50 µl of conditioned media from hybridomas were added to the wells. After one hour of incubation at room temperature, the plates were washed three times with washing buffer and loaded with 50 µl/well of anti-mouse-HRP IgG diluted 1/1000 in blocking buffer. After one hour of incubation, plates were washed well for 4 times with washing buffer and developed with 1mM ABTS (2,2'-azino-di-(3 ethylbenzthiazoline sulfonic acid)) in 70mM citrate-phosphate buffer, pH4.2. Plates were left in room temperature for 10 to 30 minutes and the absorbance was measured at the wave length of 405nm.

2.3.4 The Bicinchoninic Acid (BCA) assay

BSA standards were diluted in DPBS at a series of concentration from 0µg/ml to 25µg/ml to create a standard curve. 25 µl of standard and neat or diluted samples were added in triplicates to wells of a 96-well plate. The plate was incubated for 30 minutes at 37 ℃ following the addition of working reagent (Pierce BCA kit). Subsequent absorbance was read at 562 nm.

2.3.5 Western Blotting

2.3.5.1 SDS-PAGE (NuPAGE)

A NuPAGE™ 3-8% Tris-Acetate was set up on the Invitrogen Novex system. The running buffer consisted 50 mM Tricine, 50 mM Tris base and 0.1% SDS at pH 8.24. Samples were diluted 3 in 4 using 4 time sample buffer bought from Invitrogen and loaded 5-15 μg/well into the gel. The Gel was run at a constant voltage of 160V until the blue dye had migrated approximately to the bottom of the gel, which took around one hour. The gel could be either fixed for 10 minutes with 40% methanol, 10% acetic acid and stained with Coomassie Brilliant Blue or transferred to a membrane for Western blotting.

2.3.5.2 Western Blotting

The blotting was performed as described by the manufacturer (NOVEX) on NOVEX® semi-dry blotter. Following SDS-PAGE, the gels were equilibrated in NuPAGE transfer buffer (50 mM Bicine, 50mM Bis-Tris, 0.058% EDTA, 0.05% SDS) for 10 minutes. Because of the hydrophobicity of PVDF membranes, they need to be wet in methanol for several seconds first before equilibrated with the transfer buffer for 10 minutes. The 4x 2.5mm thick blotting filter paper was also soaked in transfer buffer before use. A “sandwich” structure was set up as shown in Figure 2-2.

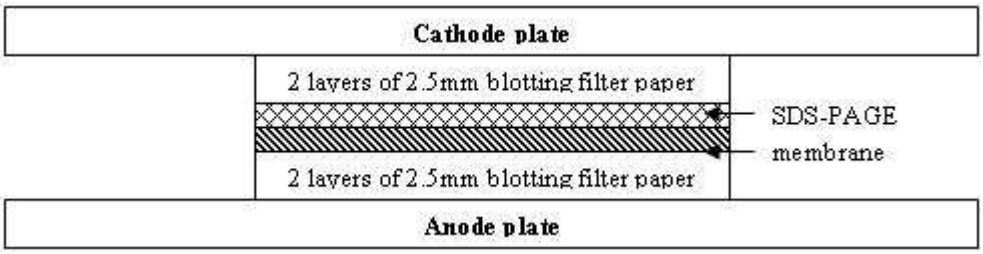

Figure 2-2 the sandwich structure for western blotting

There should be no bubbles between each layer. The knobs were evenly tightened to ensure even pressure across the whole membrane. Too little compression can allow proteins to migrate between the gel and membrane resulting in smearing band whereas too much compression can distort the gel. It was important that the pressure of the gel slabs was even and gels were not moved after placed on the membrane.

The transfer was carried for one hour at a constant voltage of 20 V/gel. After the electrophoretic transfer of protein was complete, the membrane was removed form the "sandwich" and washed in TBS to remove SDS. The gel could be stained with Coomassie Brilliant Blue for the protein that might remain in the gel.

2.3.5.3 Immunostaining of Western blots

The PVDF membrane was blocked in 1%BSA/TBST for one hour. The membrane was cut into strips and the individual strips were incubated with proper primary antibodies diluted in 1%BSA/TBST at 4℃ overnight. The strips were washed twice with TBST and incubated for one hour in room temperature with HRP-conjugated rabbit anti-mouse IgG diluted in 1%BSA/TBST. The strips were washed with TBST for six times over a period of one hour and rinsed twice in TBS before chemiluminescence development. The chemiluminescent reagents prepared in a tray were added on the PVDF membranes for a few minutes. The

strips were placed between two transparency papers. In darkroom, an X-ray film was exposed by placing onto the membrane for few seconds to minutes. The x-ray film was submerged in developer solution for a few seconds or minutes then transferred in fixing solution and rinsed with water.

2.3.6 Immunocytochemistry

Cells were cultured on tissue culture slides for one week post confluency (see section 2.1.4). The cells were fixed with ice cold 100% acetone and washed with TBS. The cell layer on the slide was divided into several sections by lines drawn using wax pen. The slide was blocked with 1%BSA/TBST for one hour at room temperature. Primary antibodies of interest were pipetted on the sections and incubated at 4℃ overnight. The slide was washed twice with TBST (5 minutes for each) and rinsed once with TBS. The slide was incubated with biotinylated rabbit anti-mouse IgG diluted 1/1000 in 1%BSA/TBST for one hour at room temperature. The slide was washed twice with TBST (5 minutes for each) and rinsed once with TBS. Development reagent (SA-FITC) at 1/500 dilution in TBS was added on the slide and incubated for 30 minutes in the dark. Slides were washed four times with TPBS (five minutes for each). DAPI (1/1000 diluted in TBS) was added and incubated for 10 minutes in the dark as counterstain. The slide was washed with TBST for four times and rinsed twice with TBS. Slides were mounted in TBS on fluorescent microscopy and photos were taken in 24 hours after staining.

3 Monoclonal antibody production

3.1 Introduction

Experiments were primarily set up to generate monoclonal antibodies against mammalian heparanase cleavage sits. This has not been achieved before by any other researchers. Antigen was isolated from collected conditioned media by DEAE chromatography followed by an IAC. Antibodies were produced by traditional hybridoma technology. Two fusions were performed aiming for different antibody isotypes. The first fusion was designed for IgM production, while the second fusion was designed for IgG production using a longer term immunization schedule compared to the first one. The screening procedure was divided into three steps:

1. Screening against Rabbit anti-mouse whole Ig for whole Ig production;
2. Screening against full length perlecan;
3. Tertiary screening against heparanase treated perlecan.

Sub clones were adapted into a serum free environment and further characterized by Western blotting.

3.2 Results

3.2.1 Purification of antigens

The DI2 and DI6 cell lines were cultured for a period of over six months to obtain enough conditioned media, from which the majority of antigen used in this project was purified. Recombinant perlecan domain I (DI) and recombinant perlecan domain I with EGFP tag (DI-EGFP) were purified from the media conditioned by DI2 cell line and DI6 cell line, respectively. Media were applied to a DEAE column and the protein was eluted by

increasing the concentration of NaCl in the solution (Figure 3-1). Regeneration with 2M salt solution did not elute any further protein. The eluted protein from DEAE was concentrated by Q Sepharose Fast Flow chromatography which gave a similar eluting pattern to DEAE column (Figure 3-2). CSI 001-76 IAC was used to bind the perlecan present in the Q sepharose elute (Figure 3-3). The bound proteins were eluted off when the conductivity dropped from 120mS/cm to 100mS/cm. The purified perlecan was desalted by dialysis against DPBS over night and then it was concentrated after the dialysis bag was buried in a bed of sucrose to draw off the liquid.

The BCA assay was utilized for protein quantification, whereby albumin standards were used to create the standard curve of known protein concentration which was plotted absorbance (Figure 3-4). The standard curve and corresponding protein concentration are displayed in Figure 3-4. The protein concentrations according to the BCA assay were 1 mg/ml and 0.83 mg/ml for DI and DI-EGFP respectively. The total amount of DI obtained from 9 liters of DI2 conditioned media was approximately 2mg and 1mg for DI-EGFP from 6 liters of media conditioned by DI6 cell line.

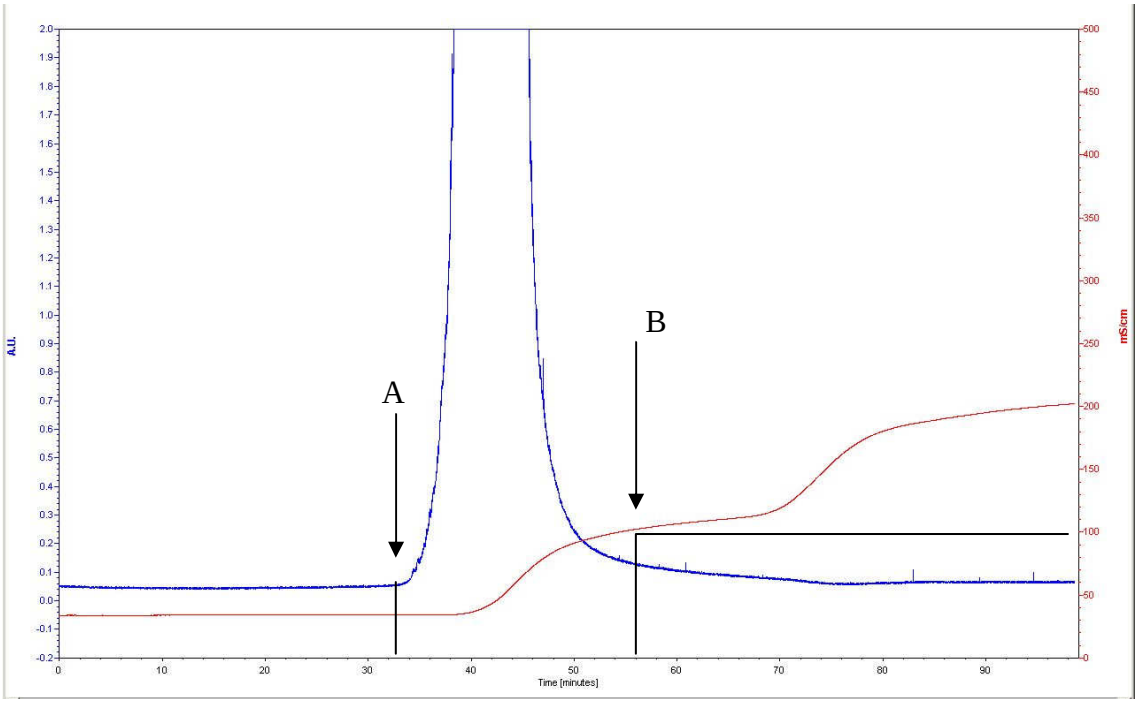

Figure 3-1Elution of DEAE column

The blue line indicates the UV absorbance at A_{280}, while the red line shows the conductivity. The column was loaded at 4℃ overnight and eluted at room temperature with 1M NaC1, 20mM Tris, 10mM EDTA, pH 7.5 at a flow rate of 2ml/min. The regeneration was performed using with 2M NaC1, 20mM Tris, 10mM EDTA, pH 7.5. Point A is the time when collection started, whereas Point B indicates the time when collection was stopped.

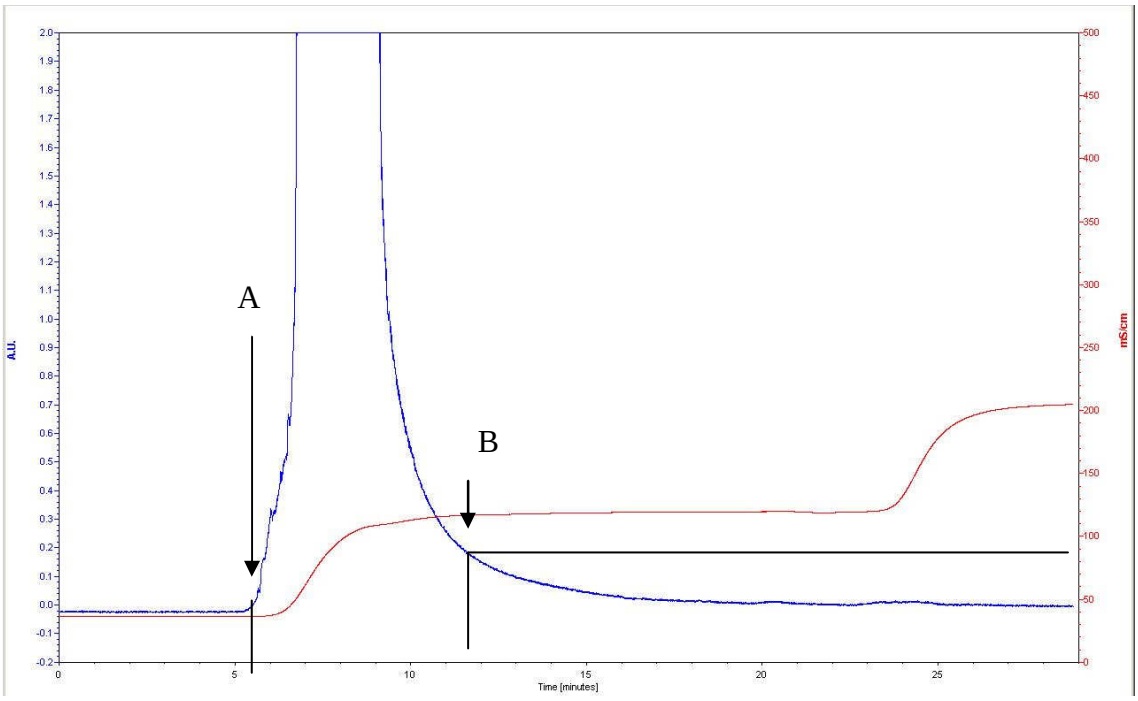

Figure 3-2 Elution of Q Sepharose column

The blue line indicates the UV absorbance at A_{280}, while the red line shows the conductivity. The column was loaded at 4℃ overnight and eluted at room temperature with 1M NaC1, 20mM Tris, 10mM EDTA, pH 7.5 at a flow rate of 2ml/min. The regeneration was performed using with 2M NaC1, 20mM Tris, 10mM EDTA, pH 7.5. Point A is the time when collection started, whereas Point B indicates the time when collection was stopped.

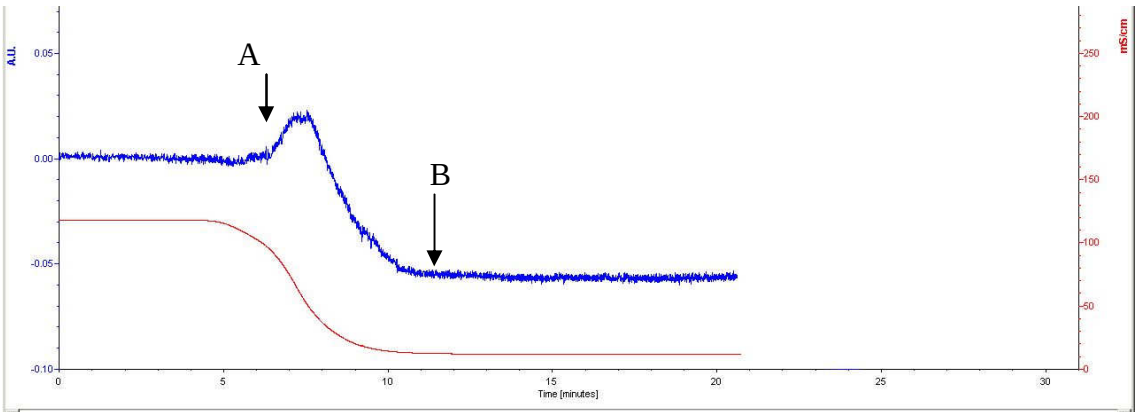

Figure 3-3 Elution of IAC

The blue line indicates the UV absorbance at A_{280}, while the red line shows the conductivity. The column was loaded by recycling the sample at 4℃ overnight and eluted at room temperature with 6 M urea at a flow rate of 1ml/min. The regeneration was carried out using 1M NaC1, 20mM Tris, 10mM EDTA, pH 7.5. Point A is the time when collection started, whereas Point B indicates the time when collection was stopped.

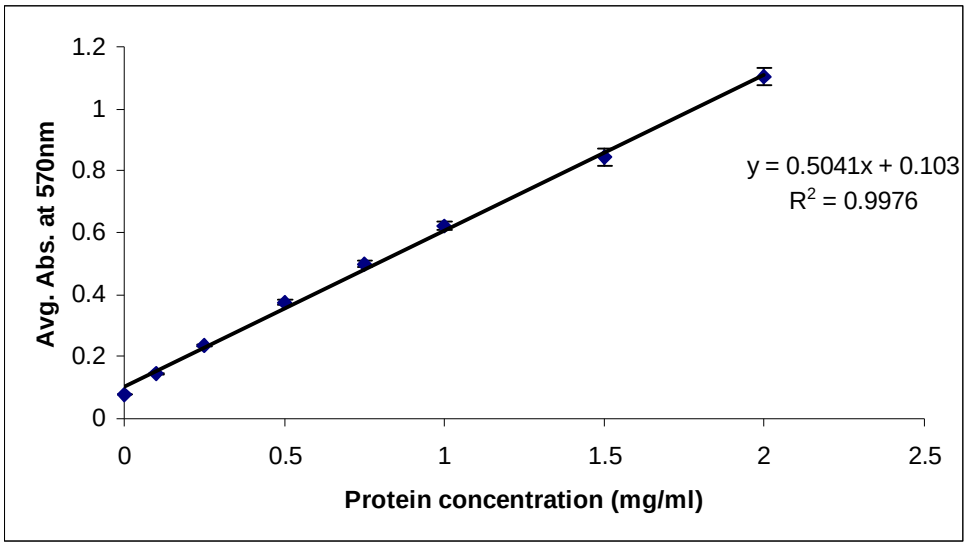

Figure 3-4 Determination of protein concentration in purified DI and DI-EGFP proteins using BCA Assay

The assay was carried out using commercial kit from Pierce. Albumin standard was added in triplicates with the concentration from 0-2.5 mg/ml and samples were coated neat in triplicates. The volume of the samples and standards in each well was 10 µl. Following the addition of 200 µl of working reagent, the plate was incubated for 30 mins in 37℃ and absorbance was read at 570nm. The standard curve and corresponding equations were created using Microsoft Excel.

3.2.2 Characterization of antigens

Purified DI and DI-EGFP were run on a 3-8% SDS-PAGE gel together with the protein standards. They displayed bands that were smears instead of the sharp bands usually observed for protein samples. These results are shown in Figure 3-5. According to the protein standards, the molecular weight of DI-EGFP was estimated to be between 70-180 kDa, while DI was estimated to be between 30-80 kDa. Another smear around 450 kDa in the lane containing the DI sample was observed, which could possibly be full length material also secreted by the same cells.

Before fixation, the gel was observed under UV light and a fluorescent smear could be seen at the same position as the DI-EGFP. The image is shown in Figure 3-6. The smear had a similar pattern as the Coomassie blue stained DI-EGFP sample on SDS-PAGE.

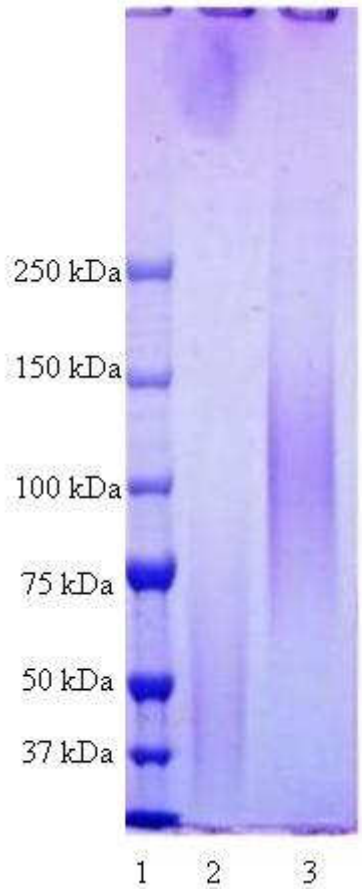

Figure 3-5 Tris-Acetate 3-8% SDS-PAGE (NuPAGE) of purified DI2 and DI6 materials

Lane 1 contained the pre-stained protein standards. Lane 2 was loaded with 10µg of purified DI in 20µl sample buffer. Lane 3 contained 10µg of purified DI-EGFP in 20ul sample buffer. The gel was stained by Coomassie Brilliant Blue. The marker used was Pre-stained High Molecular Weight protein standard (HiMark^{TM} LC5699) Due to the 50 kDa of EGFP tag, the domain I secreted by DI6 is slightly bigger than domain I from DI2.

Figure 3-6 Fluorescence of perlecan domain I with EGFP

Lane 1 contained the pre-stained protein standards. Lane 2 was loaded with 10µg of purified DI in 20µl sample buffer. Lane 3 contained 10µg of purified DI-EGFP in 20ul sample buffer. The marker used was Pre-stained High Molecular Weight protein standard (HiMark^{TM} LC5699). The sample in lane 2 was not visible under UV light due to the lack of EGFP.

Following the purification of antigen, an ELISA was carried out where antibodies were used to probe for the presence of CS, KS, HS, EGFP, perlecan domain III and perlecan domain I. Full length perlecan secreted by HCAEC was used as the positive control. Results can be seen in Figure 3-7. Compared to HCAEC, no significant amount of perlecan domain III was contained in the samples isolated from DI2 or DI6 conditioned media, while the existence of perlecan domain I could be detected by CSI 001-76 although the signals were not as strong as the HCAEC perlecan. KS, CS and HS were equally produced by DI6, whereas in the DI2 sample HS was more highly expressed. EGFP could only be detected in sample containing DI-EGFP.

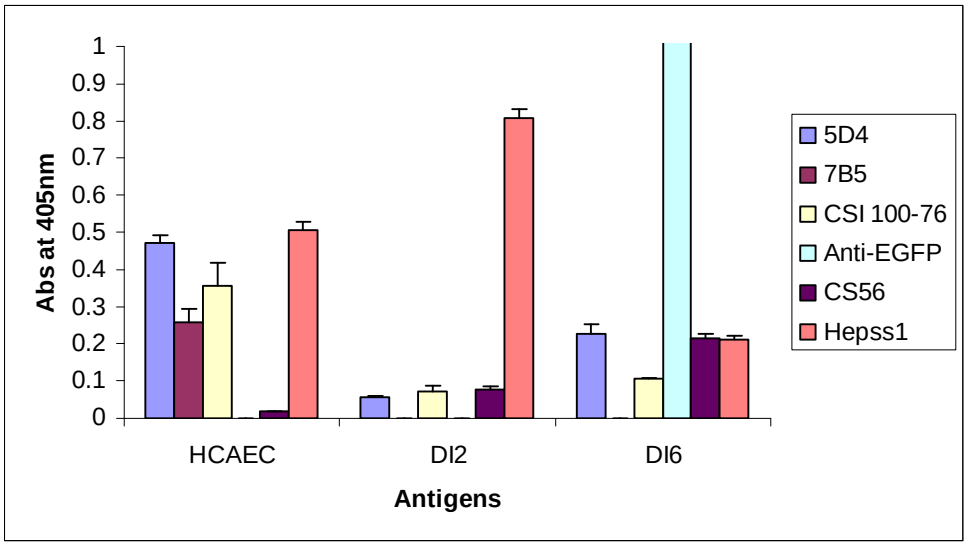

Figure 3-7 The ELISA of purified DI2 and DI6 materials
HCAEC perlecan was coated on the plate with the concentration of 3.0µg/ml while DI and DI-EGFP perlecan were coated with the concentration of 5.0µg/ml. All the samples were tested in duplicates. The specificity of each antibody can be found in Table 2-1 list of antibodies used in this project
Because of the high activity of EGFP, when the colours of other wells were fully developed, the signal of anti-EGFP was over scale. All the values shown were corrected by background.

3.2.3 ECM ELISA

A feasibility study was carried out to check whether the ECM ELISA could be used as a screening method for the hybridoma production. Cells on the plate were either removed by treatment with 20mM NH_4OH as described in section 2.2.1.2 or fixed with formalin. Results were given in Figure 3-8. Generally, the two treatments gave the same pattern in the ELISA result, while all signals obtained were greater in the formalin treated wells. The perlecan present in the ECM was recognized by protein core antibodies 7B5 and CSI 001-76. The reactivity with CS56, Hepss1 and 5D4 indicated that significant amounts of CS, HS and KS were expressed in the ECM. DI2 produced more CS compared to HCAEC and DI6 while there were more KS and HS in the ECM deposited by HCAEC and DI6. Considering

the differences in the affinity for different antibodies, the equal signal strength might not necessarily mean that the three GAGs were equally expressed in the ECM synthesized by DI6. Since the data had relatively high standard deviations and backgrounds (see Figure 3-8), ECM ELISA was not used anywhere else in this thesis.

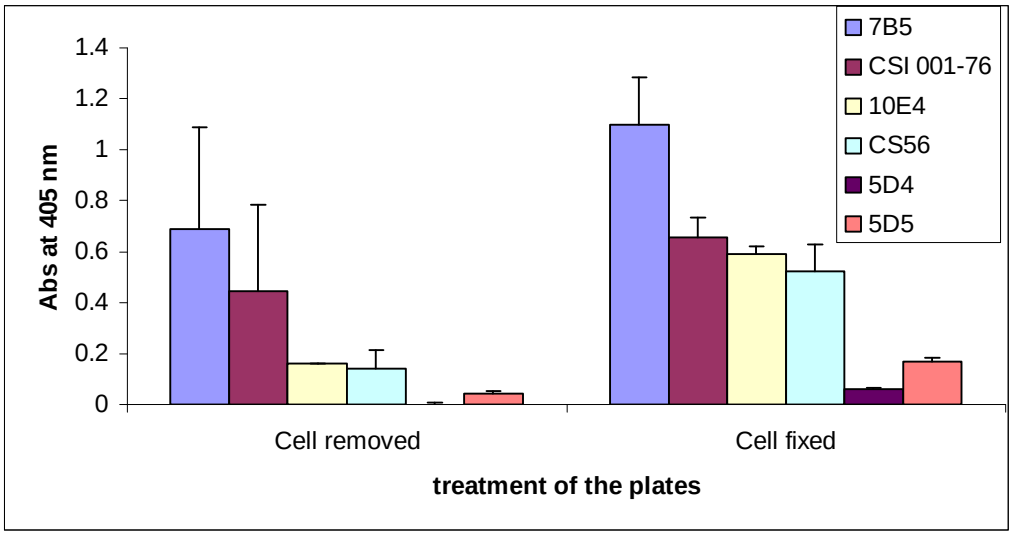

Figure 3-8 ECM ELISA

The specificity of each antibody can be found in Table 1-1. The cells grown on the plate were C11-STH cells. The methods of the cell culture and treatment referred to section 2.2.1.2. All the samples were tested in duplicates. All the values shown were corrected by background

3.2.4 Two fusions

3.2.4.1 Titrations of antibodies in tail blood

Five mice were numbered as one through five. To decide which of the mice should be used for IgM production, an ELISA three days before the scheduled last injection was performed to test the level of the antibodies against appropriate antigen present in the mice. Accordingly seven days after the primary injection (day 7), blood samples were taken from the tails of five mice and a titration ELISA was performed against DI-EGFP. Figure 3-9

indicates the results of the blood test. All five mice responded very well to the immunization and produced a significant amount of antibodies which could still be detected at 1/10,000 dilution. Of these injected mice, two were chosen for the IgM production. Mouse No.5 was chosen for its highest whole Ig production. Since the secondary antibody used to detect the bound primary antibody was anti-whole-mouse-Ig, mouse No.4 was also selected, in case the IgM content of the whole Ig from mouse No.5 was low. Mouse No.1, No.2 and No.3 were left to use in the IgG production and hence were boosted a further three times to maximize the production of IgG.

Another blood test was performed on mouse No.1, No.2 and No.3 seven days after the second booster injection (day 27). The results are shown in Figure 3-10. All three mice were generating significant amounts of antibodies which could still be detected at a 1/32000 dilution. As was shown in the first blood test, mouse No.3 was producing relatively more antibodies than mouse No.2 and mouse No.1. Blood from the non-immunized mouse gave a false positive result at the lowest dilution (1/500), which could be caused by some non-specific binding between the blood sample and the coated ELISA plate. It is interesting to note that this false positive did not occur in previous blood tests where the plate was coated with DI perlecan. Some information about the two groups of mice is listed in Table 3-1.

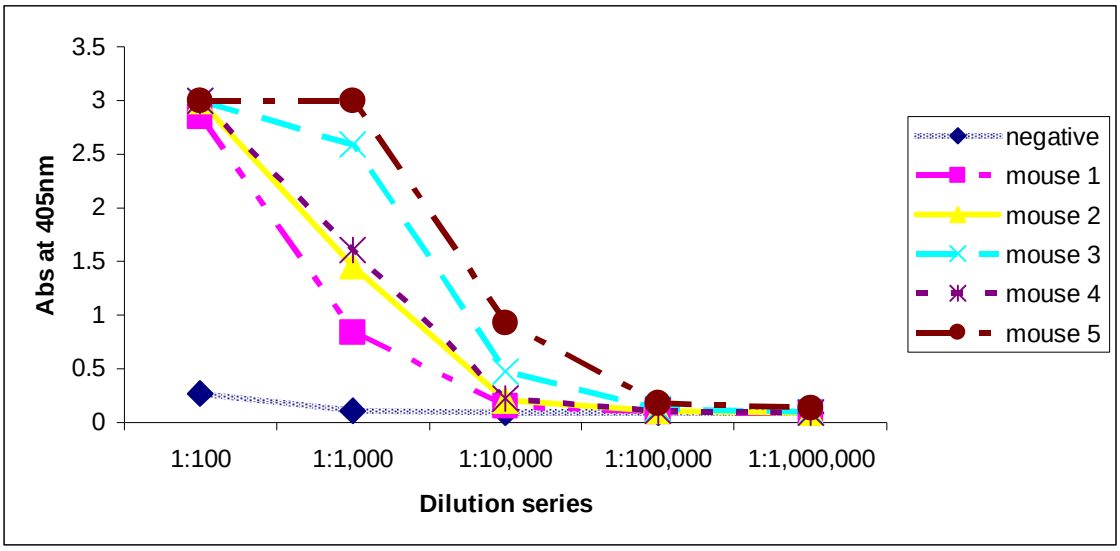

Figure 3-9 Blood titration seven days after primary injection

The high binding ELISA plate was coated with perlecan domain I isolated from DI6 conditioned media at the concentration of 5.0µg/ml. The negative control was the blood collected from a non-immunized mouse. The blood samples were diluted in DPBS. Due to the small amount of blood taken from tails, replicates were not performed and therefore no standard deviation was calculated.

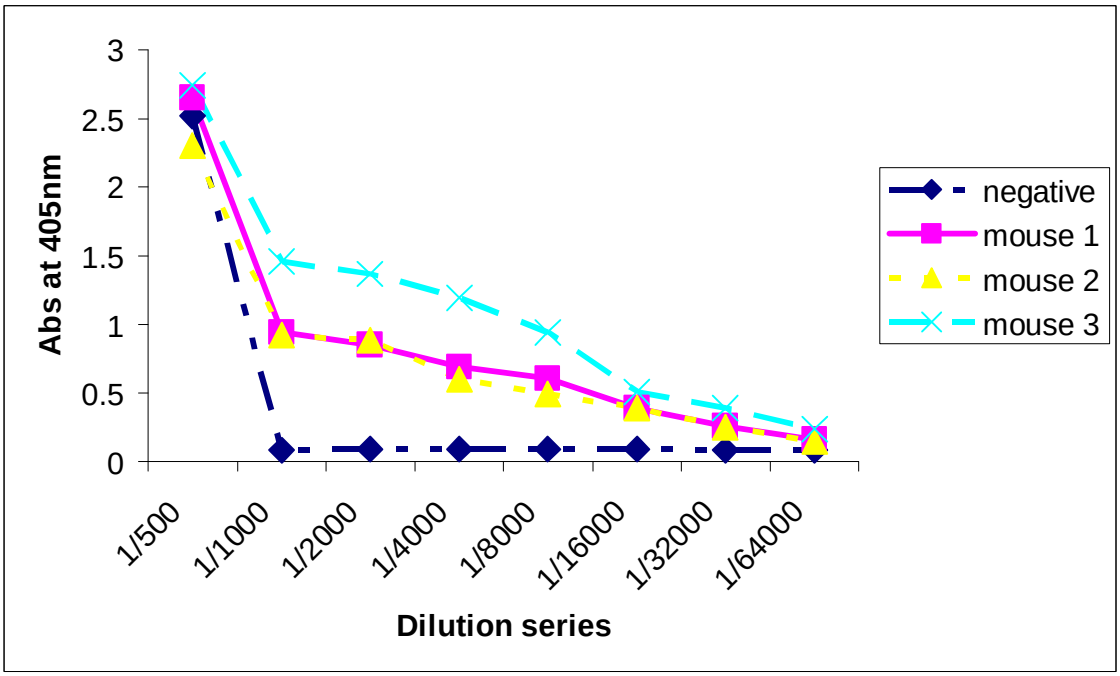

Figure 3-10 Blood titration seven days after the second injection

The high binding ELISA plate was coated with full length perlecan isolated from HCAEC at the concentration of 3.0µg/ml. The negative control was the blood collected from a non-immunized mouse. The test was performed in DPBS system. Due to the small amount of blood taken from tails, replicates were not performed and therefore no standard deviation was calculated.

Group No.	Aim	Mouse No.	Times of antigen injection	time of sacrifice
1	IgM production	No.5 No.4	One primary injection plus one booster injection	Day 13
2	IgG production	No. 1 No. 2 No.3	One primary injection plus three booster injection	Day 30

Table 3-1The two groups of mice for different isotypes of MAb production
Five mice were divided into two groups based on the ELISA on tail blood. Mouse No.5 and No.4 were included in the group for IgM production and injected 2 times in total, while mouse No.1, N.o2 and No.3 were in the IgG production group where the injection was performed 4 times in total. The period of immunization for group one lasted for 13 days whereas 30 days for group two.

3.2.4.2 First fusion for IgM production

At day 13, mice in group one (mouse No.4 and No5) were sacrificed and spleens were taken for the fusion. The details of cell numbers used in this fusion can be found in Table 3-2. Ten 96-well plates were set up and maintained for two months. No hybridoma was raised and all the cells died. Reasons for the failure of this experiment are discussed in 3.3

Mouse	The number of isolated spleen cells used	The number of SP2/0 used	Plate number
No.5	1.21×10^{8}	2.4×10^{7}	1-6
No.4	9.7×10^{7}	1.9×10^{7}	7-10

Table 3-2 Details of cells in the fusion for IgM production
From the spleen of mouse No.5, 1.21×10^{8} cells were isolated to fuse with 2.4×10^{7} SP2/0, whereas from the spleen of mouse No.4, 9.7×10^{7} cells were isolated to fuse with 1.9×10^{7} SP2/0. Cell mixture from mouse No.5 and No.4 were added into 6 and 4 96-well tissue culture plates, respectively.

3.2.4.3 Second fusion for IgG production

In this fusion and all future sub cloning procedures, MRC-5 cells were used as a feeder layer to give the new raised hybridomas essential growth support. At day 30, mice in group two (mouse No.1, No.2 and No.3) were sacrificed and the spleens were used to isolate B cells. The details of cell numbers used in this fusion can be found in Table 3-3. Theoretically, fused cells were those that survived in HT medium for more than seven days. One week after fusion there were 800 wells in which cells were growing. Many of the clones occupied more than 50% of the bottom surface of the well. These cells represented successful fused hybridomas and the following screening procedures were used to select for appropriate antibodies.

Mouse	Isolated spleen cells	SP2/0 used	Plates number
No.1	1.32×10^{8}	2.5×10^{7}	1-4
No.2	2.1×10^{8}	4.2×10^{7}	5-11
No.3	2.9×10^{8}	5.8×10^{7}	12-20

Table 3-3 Details of cells in the fusion for IgG production

From the spleens of mouse No.1, No2 and No.3, 1.32×10^{8}, 2.1×10^{8} and 2.9×10^{8} cells were obtained to fuse with 2.5×10^{7}, 4.2×10^{7} and 5.8×10^{7} SP2/0 cells, respectively. Plates No.1-4 were set up with the cells from mouse No1, plates No.5-11 were set up with the cells from mouse No.2 and plates No.12-20 were set up with the cells from mouse No.3.

3.2.5 Screening

3.2.5.1 Screening for whole Ig production

A sandwich ELISA as described in section 2.2.3.2 was used to test the conditioned media taken from the 800 wells where cells were growing (see section 3.2.4.3). This step was used to screen for whole Ig production, which was expected to help us eliminate the cultures that did not produce Ig and hence reducing the number of samples that needed to be screened for the production of anti-perlecan antibody. However, all were strongly positive (data not shown), which indicated cells growing in the wells were all secreting significant amounts of mouse Ig. Since this step did not help reducing the number of cultures, we moved onto specific screen against human perlecan.

3.2.5.2 Screening against full length perlecan

Following the sandwich ELISA, the conditioned media of all the selected cultures were tested against full length perlecan secreted by HCAEC. Of the 800, 216 displayed strong positive signals (data not shown) and were chosen to transfer into 24-well plates pre-seeded with MRC-5 for cell expansion. When the cells on 24-well plate reached about 80% confluency, the conditioned media were tested (data not shown). Of these, 26 cultures with the highest antibody production were chosen for tertiary screening against heparanase treated perlecan. The identities of the 26 cultures could be found in Table 3-4. While the 26 selected cultures were transferred to new 24-well plates for the further screening procedures, all 216 cultures were pooled and stocked in 12 cryovials as potential candidates to be cloned in the future. Due to our primary goal to raise antibodies against the residual GAGs' stubs generated by the treatment with heparanase, 19 negative cultures from the 800

culture wells were randomly chosen and tested against heparanase treated perlecan. The results were shown in Figure 3-11. 10E4 and 7B5 were used as controls of the effectiveness of heparanase. After enzyme treatment, the reactivity between 10E4 and the coated ELISA plate dropped dramatically, since the heparanase digestion removed the 10E4 epitopes on HS chains of the coated perlecan. The heparanase treatment did not influence the ability of 7B5 to recognize perlecan, as the antigenic determinant for 7B5 is on the protein core which should not be affected by heparanase. The 10E4 and 7B5 controls demonstrated that the enzyme digestion was successful and the protein core was not degraded by the heparanase. The MRC-5 conditioned medium was also be used as a negative control. None of the 19 chosen cultures showed a significant increase in reactivity after the treatment. These results revealed that the tested antibodies could not recognize the new epitopes generated by heparanase treatment. The fact that culture 6 became positive might be the result of slow growth of the cells secreting antibodies. In previous ELISAs, the numbers of the cells secreting antibodies may have been too small to generate detectable amounts of antibodies.

Resource	Identities
Mouse No.1	2A5, 2B6, 2B8, 3A5, 3E1, 4G2
Mouse No.2	5D7, 6A10, 7A8, 7C9, 9C11, 10C11, 11F3
Mouse No.3	12D7, 12H6, 13E9, 14E5, 15A8, 16B8,17H4, 18H5, 19F11, 20D11, 20F12, 20G5

Table 3-4 The identities of the 26 selected culture chosen for further propagation. The two boxed clones were those chosen to sub clone and used to probe the cultures of human chondrocytes.

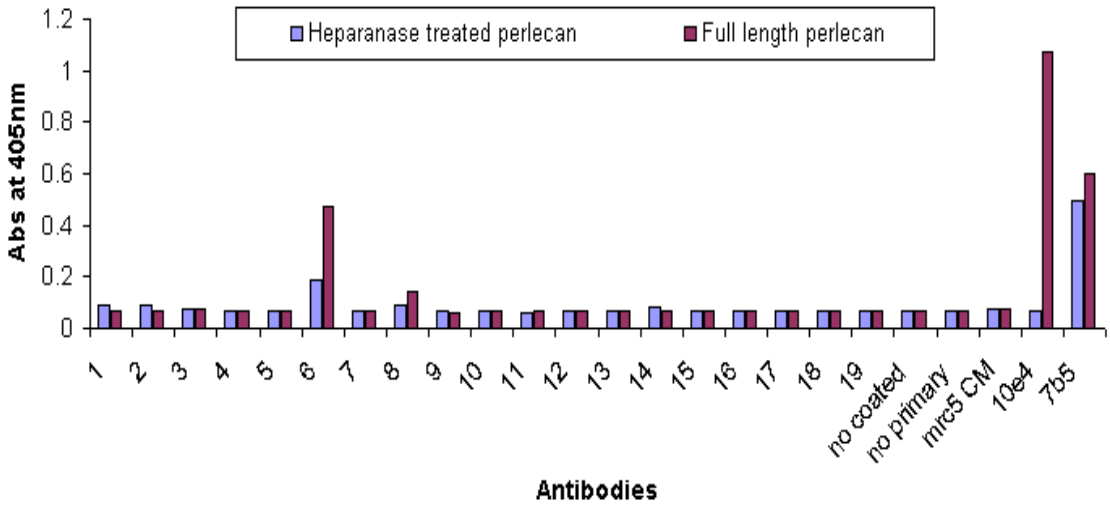

Figure 3-11 ELISA results of 19 negative cultures

The ELISA plate was coated with 3.0µg/ml of full length perlecan purified from HCAEC conditioned medium. The enzyme digestion had been performed at 37℃ for 4 hours with 0.6 µg/ml of heparanase in DPBS. Due to the small volume of conditioned media that could be collected from tissue culture plates, the ELISA was not done in replicates and therefore no standard deviation was calculated.

3.2.5.3 Tertiary screen against heparanase treated perlecan and domain I perlecan

The selected 26 cultures were transferred to new 24-well tissue culture plates and grown. The conditioned media were tested by ELISA against HCAEC perlecan and heparanase digested HCAEC perlecan when the cells occupied about 80% of the bottom surface of the well. Data are shown in Figure 3-12. As the ELISA in section 3.2.2.2, 10E4 and 7B5 were used as controls for the enzyme and antigen. More than 60% of the new generated antibodies reacted to the enzyme treatment in the similar way as 7B5. Antibodies 2B8, 3E1 and 7C9 were noted to have slightly increased signals after the treatment with heparanase, which might be the result that those media contained the antibodies which epitopes exposed more or even occurred only after the heparanase process. Antibodies 2B6, 4G2, 5D7, 11F3, 16B8, 20D11 and 20F12 showed greater and significant decrease in comparison with the others in reactivity with perlecan after enzyme treatment, indicating the media conditioned

by those cells might contain the antibodies which epitopes were sensitive to heparanase. However these inferences might not be conclusive, as all tests were done in single well format with no standard deviation.

The 26 antibodies were also tested for reactivity against domain I perlecan. Results were shown in Figure 3-13. Since most of GAGs on perlecan are on domain one, 10E4 was used here as the positive control for the presence of DI. 7B5 was the positive control for the full length perlecan and negative control for DI. While most of the antibodies had low affinity to DI, 5D7 and 13E9 gave significant reactivity. These results indicated that there were hybridomas produced DI antibodies in the cultures of 5D7 and 13E9.

5D7 was chosen as the first culture to be sub cloned for its sensitivity to heparanase treatment and the greatest reactivity with DI. 13E9 was also chosen to be cloned together with 5D7. 13E9 gave the second strongest reactivity with DI and represented the majority of the 26 selected cultures in the ELISA against heparanase digested HCAEC perlecan. While 5D7 and 13E9 were transferred to 25 cm^2 tissue culture flasks to expand for sub cloning, all 26 were frozen for future sub cloning and characterization.

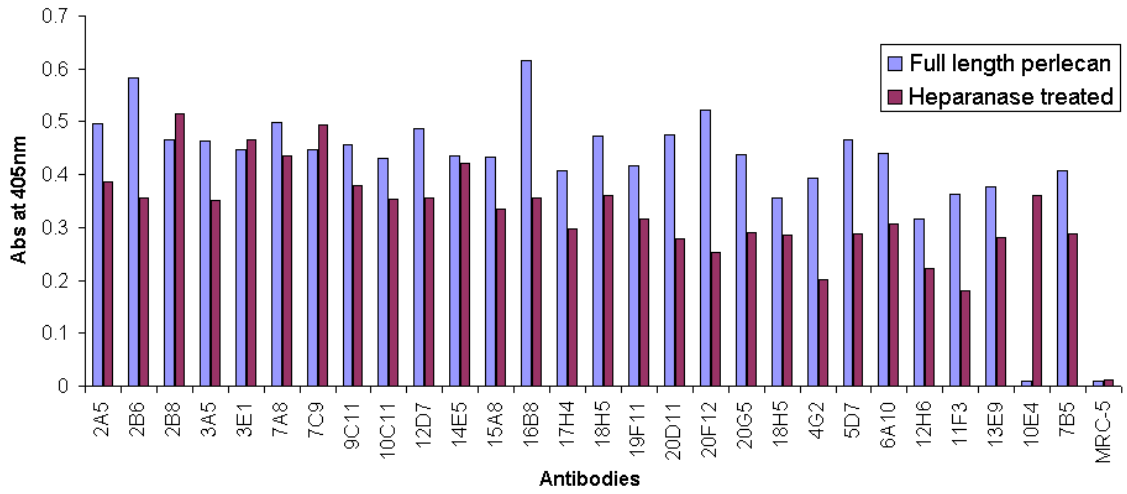

Figure 3-12 Tertiary screen against heparanase treated perlecan

The ELISA plate was coated with 3.0 µg/ml of full length perlecan purified from HCAEC conditioned medium. The enzyme treatment had been performed at 37℃ for 4 hours with 0.60µg/ml of heparanase in DPBS. All the values shown were corrected with background. Antibody 10E4 was control for the effect of enzyme treatment. MRC-5 conditioned medium was used as a negative control to ensure that there is nothing secreted by MRC-5 cells that could bind to the coating material. Due to the small volume of conditioned media that could be collected from tissue culture plates, the ELISA was not done in replicates and therefore no standard deviation was calculated.

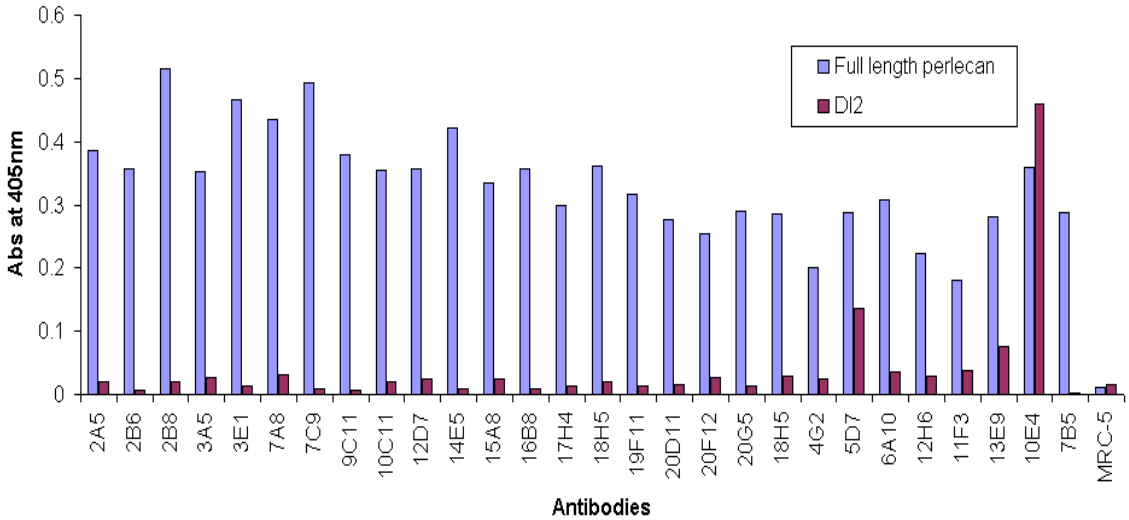

Figure 3-13 Tertiary screens against DI

The ELISA plate was coated with 3.0µg/ml of full length perlecan purified from HCAEC conditioned medium and 5.0 µg/ml DI. All the values shown were corrected with background. MRC-5 conditioned medium was used as a negative control to ensure that there is nothing secreted by MRC-5 cells that could bind to the coating material. Due to the small volume of conditioned media that could be collected from tissue culture plates, the ELISA was not done in replicates and therefore no standard deviation was calculated.

3.2.6 Sub clones

3.2.6.1 Sub clones grown in FBS containing medium

Following the expansion of 5D7 and 13E9 in 25 cm^2 flasks, they were cloned by limiting dilution (see section 2.3.1.5.5). The cloning of 5D7 resulted in 5 hybridomas while 8 hybridomas were derived from 13E9. The identities of these sub clones could be found in Table 3-5.

The sub clones were seeded on a 24-well plate and the conditioned media were collected for ELISAs against DI, HCEAC perlecan and heparanase treated perlecan. Data were shown in Figure 3-14 and Figure 3-15. CSI 001-76 was used as a positive control for the presence of the domain I of perlecan and 10E4 was used as the control for heparanase treatment. 7B5 was the positive control for full length perlecan and the negative control for DI. The sub clones from 5D7 were all capable to recognize the full length perlecan from HCEAC, but none of them showed significant binding to DI or was sensitive to the heparanase treatment. The explanation for this could be that the hybridomas responsible for the decrease signal or the reactivity to DI in the ELISA shown in Figure 3-12, were lost during the cloning procedures. The same thing might have happened to the 13E9 sub clones. However, 13E9-3C5, 13E9-3D5 and 13E9-3E9 were noted for their slightly greater affinity to DI. While all the sub clones were adapted into the serum free environment, they were also stocked.

Parent culture	Identities of the sub cloned
5D7	5D7-2D2 , 5D7-2D6, 5D7-2E4, 5D7-2F4, 5D7-2G7
13E9	13E9-3B3, 13E9-3B9, 13E9-3C5, 13E9-3D5, 13E9-3D7, 13E9-3E7, 13E9-3E9, 13E9-3G5

Table 3-5 The identities of the sub clones isolated from 5D7 and 13E9

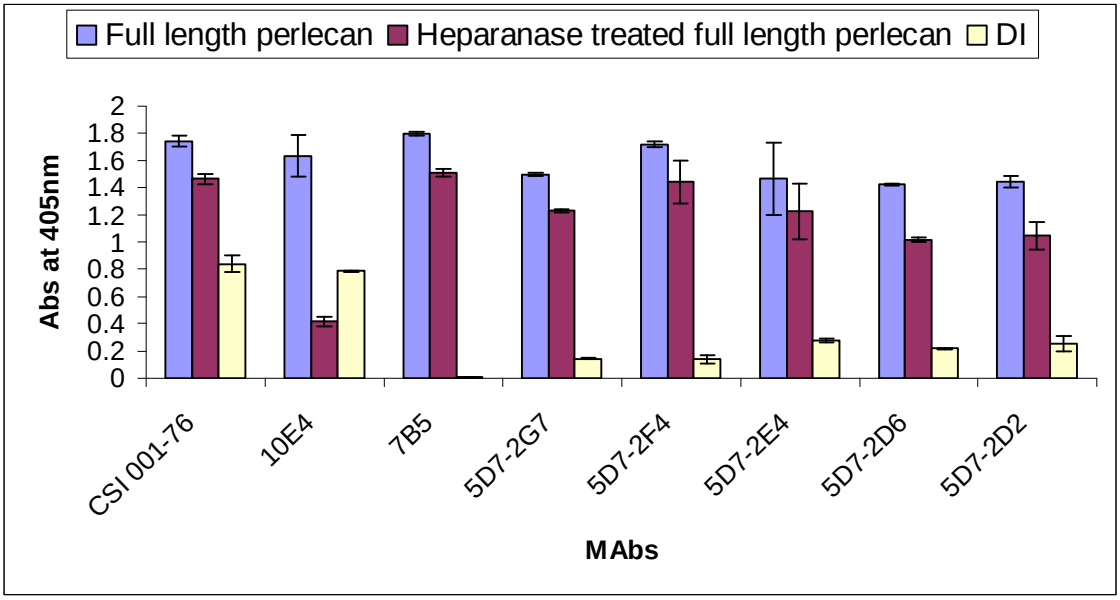

Figure 3-14 ELISA on the serum containing media conditioned by sub clones of 5D7

The ELISA plate was coated with 3.0µg/ml of full length perlecan purified from HCAEC conditioned medium and 5.0 µg/ml DI. . The enzyme digestion had been performed at 37℃ for 4 hours with 0.6 µg/ml of heparanase in DPBS. All the samples were tested in duplicates. All the values shown were corrected for background.

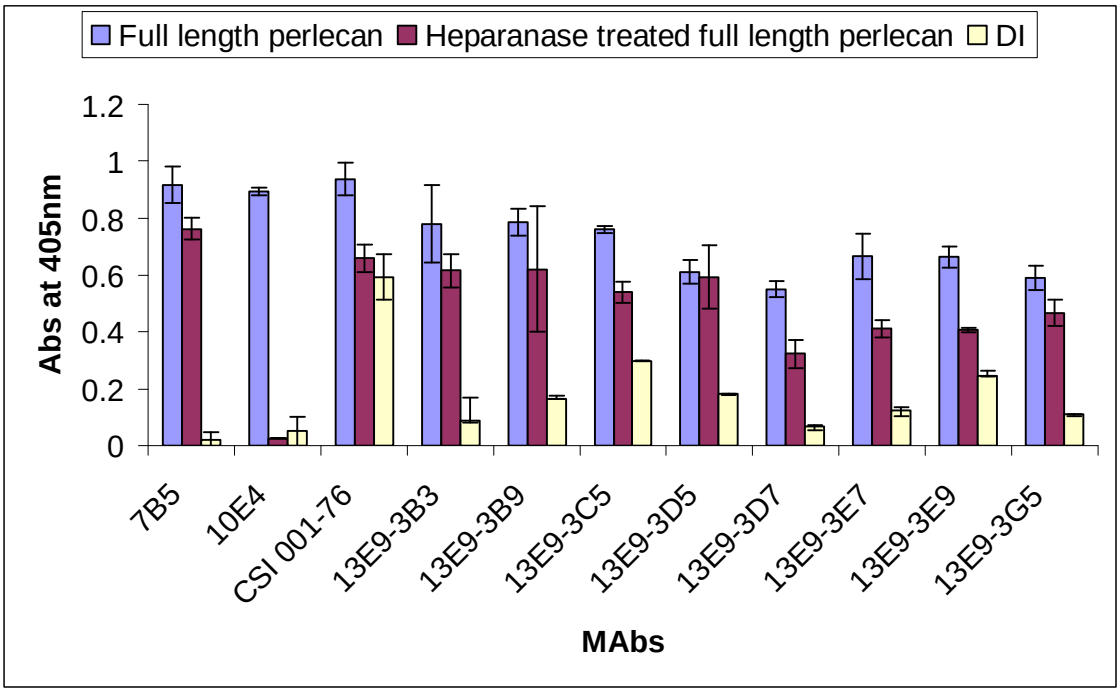

Figure 3-15 ELISA on the serum containing media conditioned by sub clones of 13E9

The ELISA plate was coated with 3.0µg/ml of full length perlecan purified from HCAEC conditioned medium and 5.0 µg/ml DI. . The enzyme digestion had been performed at 37℃ for 4 hours with 0.6 µg/ml of heparanase in DPBS. All the samples were tested in duplicates. All the values shown were corrected for background.

3.2.6.2 Sub clones grown in serum free medium (SFM)

Growing hybridomas in SFM benefits further characterization and downstream purification greatly, therefore we have tried to adapt all of the sub clones into a serum free environment. Due to the limitation of time, instead of sequential weaning, it was achieved by direct adaptation where cells were transferred into 100% serum free medium directly at a higher density than usual. Three sub clones 5D7-2D2, 5D7-2D6 and 5D7-2F4 did not survive from this adaption process. The remaining ten survived and were grown on 24-well plates and the conditioned media were collected.

SFM conditioned by the ten sub clones were tested by ELISA against full length perlecan, DI and DI-EGFP. The results are shown in Figure 3-16. Anti-EGFP together with 7B5 and CSI 001-76 were controls for the three coated antigens. All of the ten of the newly generated monoclonal antibodies were capable of recognizing full length perlecan secreted by HCAEC but do not bind to either DI or DI-EGFP (see Figure 3-16). Their immunoreactivity were very similar to domain III antibody 7B5, which might indicate that these antibodies are not domain I antibodies.

The conditioned SFM were also used in western blotting to probe native HCAEC perlecan, where chemiluminescent development was used to increase the sensitivity (see section 2.3.5). In the positive control where CSI 001-76 was used as the primary antibody, perlecan was stained with a molecular weight over 460kDa. All of the blots probed with conditioned SFM gave the bands at the same position, suggesting that all of the ten antibodies were able to recognize HCAEC perlecan in western blotting. As was the positive control CSI 001-76, 8 out of 10 newly generated antibodies bound to some large molecules left at the bottom of the loading well, which was also transferred to the membrane. Both 5D7-2G7 and 13E9-3D7 did not react with this large molecular weight material. This band was particularly strong on the blots probed with 13E9-3E7, 13E9-3E9 and 13E9-3G5. 5D7-2G7 and 13E9-3D7, especially 13E9-3D7, were noted for the very neat bands they gave.

The antibodies were also used to probe recombinant perlecan domain I from DI2 cell line. However, due to the low concentration of new batch of DI, no staining was observed even in the blot for positive control, where A76 was used as primary antibody (data not shown).

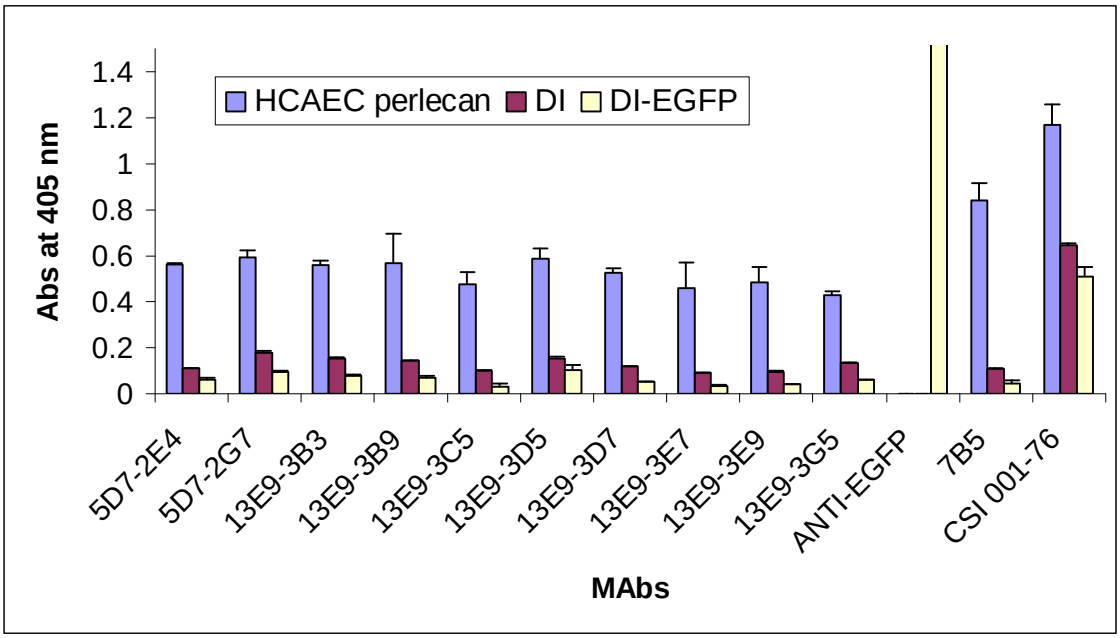

Figure 3-16 ELISA using the SFM conditioned by sub clones

The ELISA plate was coated with 3.0µg/ml of full length perlecan purified from HCAEC conditioned medium and 5.0 µg/ml of domain I isolated from DI 2 or DI6 conditioned medium. The ELISA was done in duplicate. All the values shown were corrected for background.

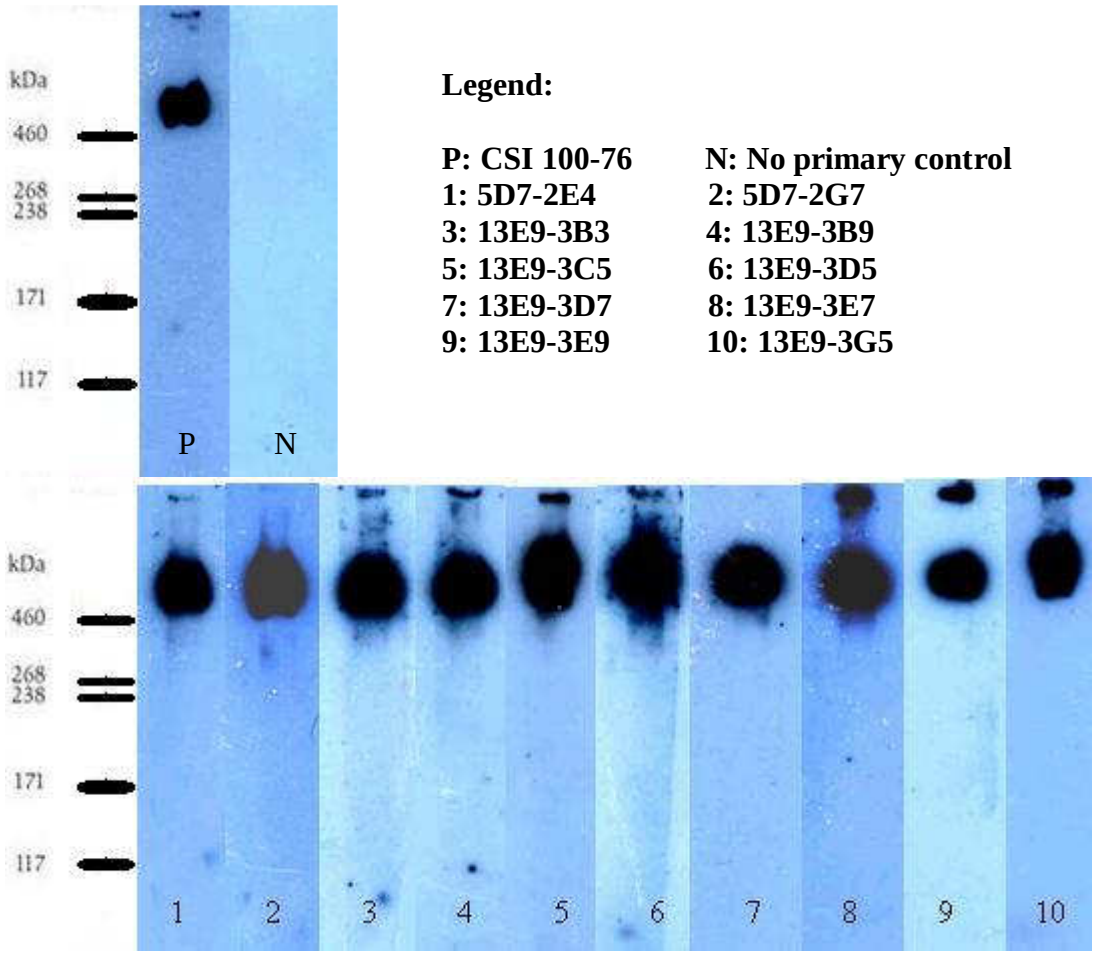

Figure 3-17 western blotting of the SFM conditioned by sub clones

Native HCAEC perlecan 7µg/well was separated in Tris-Acetate 3-8% SDS-PAGE (NuPAGE) gels and then transferred on immuno-G membranes. The blot probed with CSI 100-76 diluted 1/5000 was used the positive control. The blots were probed with 1/100 SFMs diluted conditioned by hybridomas and developed with chemiluminescent detection system.

3.3 Discussion

3.3.1 Antigen purification and characterization

Basement membrane HSPGs have been isolated from a wide range of sources such as kidney glomerulus, aorta, cultured cells and tumour cell lines [151-153]. In this study, we used transfected HEK 293 cell lines (DI2 and DI6) as the main sources of antigens [48]. The reason for this will be discussed in detail in section 3.3.2. Briefly, the PGs give much greater immunogenicity than free GAGs and anti-protein core antibodies as products of this project are also very useful research tools.

Perlecan is an extremely difficult macromolecule to isolate in pure form, especially in substantial quantities, due in part to its inherent ability to interact with a number of different proteins and macromolecules. Previous methods of perlecan isolation involved cesium chloride density gradient centrifuge, DEAE chromatography and gel filtration chromatography [154-156]. However, there were some major problems with those methods such as contaminations by other PGs and free GAG chains and degradation of the perlecan core protein. In this project, purifications were carried out by an IAC following two DEAE chromatography runs. The introduction of IAC not only gave a purified perlecan fraction of high purity but it also reduced the duration of the procedure, and therefore largely protected perlecan from degradation. However, the use of Q Sepharose column may not be necessary. Since it is a stronger anion-exchange column than DEAE, all of the molecules bound to DEAE can bind to Q Sepharose column. It did not help removing the unwanted molecules but prolonged the procedure which might cause the degradation of perlecan core protein.

In SDS-PAGE (see Figure 3-5), perlecan domain I was visualized as a broad smear since the degradation and variability of GAGs attached to the protein core. DI decorated with three GAG chains, migrated as a 30- to 80-kDa protein, which roughly matched the reported value of 20 kDa expected for DI protein core and average 20 kDa for each GAG chain [157, 158]. In fact, the majority of the GAG chains on DI are smaller than 20 kDa possibly due to some GAG chain degradation that occurred during the purification procedure or shorter GAG chains were synthesized in the DI2 cells. Due to the 27 kDa EGFP tag, DI-EGFP protein core was expected to have a molecular mass of 70 kDa. In the SDS-PAGE, DI-EGFP gave a smear which ranged from 70 kDa to 180 kDa suggesting an average size of 20 kDa for each GAG chain. The explanations of the longer GAG chains on DI-EGFP may either be that the complex of EGFP and core protein provided more stability to the entire molecule or the DI6 cells naturally synthesized larger GAG chains.

In the SDS-PAGE, another band in the lane of DI was located at the spot of the protein with a molecular weight more than 400 kDa. This large sized molecule was possibly full length perlecan, which was actually confirmed by the existence of many anti full length perlecan antibodies produced in this work. A western blotting should have been done to clarify the contamination prior to the immunization. Frequent selection of the cells with Geneticin could be a solution to this problem. In case that even transfected cells with neo gene still make full length perlecan, this problem can also be easily addressed by an additional IAC such as 7B5 column, targeted non-domain I moiety of perlecan to remove the full length molecules.

3.3.2 Antibody production

3.3.2.1 The outcomes from various stages of the production of the antibodies

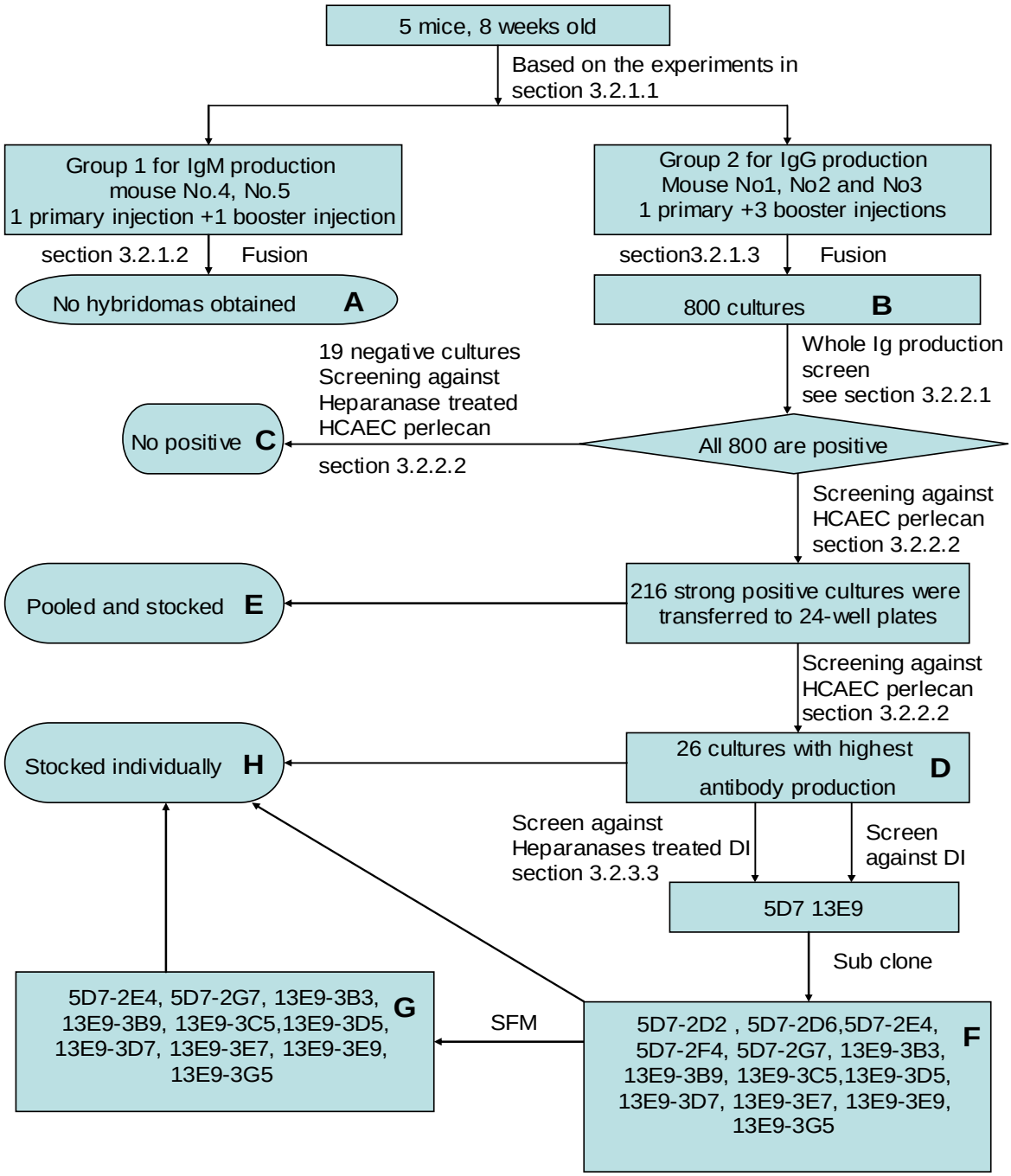

Figure 3-18 the flowchart about the antibody production

In Figure 3-18, the whole procedure and outcomes of the antibody production part in this project were briefly elucidated. Box A and B were the results of two fusions, respectively. The completely different outcomes indicated that there were some major improvements in the immunization or fusion that had be done for the second group of mice. The reason could be the longer term of immunization which gave the hosts enough time to active sufficient amount of B cells, or most likely the use of MRC-5 feeder layers. This will be discussed further in following sections. We put a lot of effort into trying to select for the antibodies which could only recognize the GAG stubs generated by heparanase treatment. Nevertheless, due to the expensive price of heparanase and limited amount of perlecan, it was impossible for us to test every single negative fusion cultures against the enzyme treated perlecan. Therefore, nineteen negatives were randomly selected on the basics of strong growth and screened with heparanase processed perlecan, but none of them were sensitive to this treatment, as shown in box C. After two specific screens against full length perlecan, 26 cultures with the highest antibody production were chosen for tertiary screen against perlecan domain I and heparanase processed perlecan, as shown in box D. Additionally, the 216 positive cultures obtained from the first specific screens with full length perlecan were pooled and stocked as recovery backups (Box E). Based on the tertiary screen, 5D7 and 13E9 were selected and sub cloned. From these two, thirteen sub clones were isolated (Box F). Three of the isolated sub clones were lost, when they were adapted to SFM for future up-scaling of MAb production using bioreactors (Box G). After the stage of box D shown in the flowchart, cells were stocked every step to ensure none of them could be lost accidentally (box H). Based on the test results during the screening process, it was proven that the new established hybridomas were secreting MAbs against full length perlecan but not domain I or GAG chain.

3.3.2.2 The discussions on immunogenicity

It is difficult to raise antibodies against HS due to the low immunogenicity of these polysaccharide structures [159-161]. In most cases, the immunogenicity of weak immunogens such as haptens, could be improved by conjugating or cross-linking the molecule with an immunogenic carrier protein which provides T cells epitopes promoting the B-cell response. The widely used carriers are keyhole limpet hemacyanin (KLH) and bovine serum albumin (BSA). However, GAGs, even when coupled experimentally to proteins, can not elicit a sufficient immune response for antibody production [162]. In contrast, PGs isolated from cartilage were reported to have the ability to elicit antibody production in various animals even though the antigen determinants tend to be located within the protein moiety [163-165]. Moreover, the protein core left by enzymic removal of GAGs is strongly immunogenic [166]. 5D4, 1B5, 2B6, 3B3 and stub antibody 3G10 were generated by immunization with enzyme-digested PG [145, 146]. In fact, the protein core here served as a carrier for GAG chains. This is the reason why the PG was chosen by us as the immunogen over HS alone, although we were most interested in anti-HS MAbs. However, we did not do any enzyme treatment of the antigens, because heparanase exists in the body and once the antigen was injected, the host would process it with this enzyme present in the lysosomes. However, it has been reported that the enzyme was less than 0.0001% of total cellular proteins and the expression and activation were strictly restricted [69]. Accordingly, in the future if anti-stub antibodies are required, the enzyme treatment will be performed prior to the immunization.

Another reason that PG antibodies are difficult to raise is that PGs are ubiquitous components and highly conserved throughout evolution. The host body does not recognize the injected PG as a “foreigner”. In order to evoke an immune response, a molecule must be

recognized as non-self by the host immune system. When an antigen is introduced into an organism, the greater the phylogenetic distance between the animal species providing the antigen and the animal species to be immunized, the better the immune response will be evoked [167]. The specific unresponsiveness to self antigens is critical to prevent the immune system from attacking the host itself but become an obstruction in antibody production. This problem can be addressed by choosing a host evolutionarily far away from the species providing the immunogen [167]. For example, member of the avian family can be used as the hosts to produce antibodies against mammalian PG but if the PG is isolated from an avian source, a mammalian host is a better choice. Mouse anti-native chondroitin sulfate antibody CS-56 was prepared through the immunization with ventral membranes of cultured chicken fibroblasts [168]. There is actually additional advantage of using the chicken (avian) as hosts. After the immunization of chickens, antibodies pass from the blood to the egg yolk and the chicken egg antibodies (IgY) can be extracted continuously with a yield of approximately 100 to 250 mg of IgY/egg [169]. Although the IgYs from eggs are polyclonal antibodies, they are still good tools for detection and purification.

Use of liposomes is also an approach to enhancing the immunogenicity. 10E4 was raised by immunizing mice with liposome-incorporated membrane HSPG from humans [145]. The antigens are either entrapped within the aqueous compartments of the liposome or linked covalently to the surface bilayer of the liposome. Studies revealed that the titration of antibody obtained with liposome entrapped protein were much higher than those obtained with the free protein [170]. Liposomes give several properties, such as low toxicity, low immunogenicity, and biodegradability, making them a better in-vivo carrier of proteins. As we have discussed in the previous paragraph, the immunogenicity of weak immunogens can be largely enhanced by conjugation with a protein carrier. However, carrier proteins may

their own problems bring problem as well, because the host organisms tend to produce antibodies against the carrier protein preferentially. Once the anti-carrier-protein antibodies are produced, the immune system clear the host body of the target antigen-carrier protein complex before the initiation of an immunological response to the target antigen. The low immunogenicity of liposomes decreases the possibility to induce anti-carrier antibodies. We extrapolate this as particularly the case in the production of anti-GAG antibody using PG, as antigens, since we did generate a large pool of anti-protein core antibodies. If liposomes are used alternatively to Freund's adjuvant, the hydrophobic protein moiety may be encapsulated inside of the bilayer membrane and isolated from the host immune system, while the hydrophilic GAG chains were displayed on the surface exposed to the host immune system. In fact, this was confirmed by a study where two anti-HA antibody, 10G6 and 5F11 were raised [171]. In this study, while the mice immunized with HA-liposomes produced serum antibodies to HA, the mice immunized with HA- Freund's adjuvant did not develop any antibody to HA measurable by ELISA [171]. Liposomes could not only improve the immunogenicity but could also switch a T-cell-independent antigen to a T-cell-dependent one [172, 173]. It will benefit the antibody production if the transient IgM production can be converted into a persisted IgG production which is usually elicited by T-dependent antigens. This will be discussed further in the following section. In addition, the liposome entrapped immunogens would give a continued stimulus to these immune cells, as the layers of the liposome were degraded and therefore further antigen molecules would become exposed [174].

3.3.2.3 Why IgM, why two groups?

Antigens can be divided into T-cell-dependent or T-independent antigens based on whether they are able to induce helper activity from T cells [110]. Carbohydrate or polysaccharide based antigens including GAGs, are T-cell-independent antigens. T-cell-independent antigens generally induce the production of IgM antibodies, which is the reason that many anti-GAG antibodies are IgM isotype. Different from IgGs, the secretion of IgM antibodies is a primary and transient response in laboratory animals and humans, and therefore we need two set of immunization schedules with different durations.

The immunization schedules for the two groups were originally set up according to the antibody response curve in the body during the period of immunization shown in Figure 3-19. IgM is generally viewed as an isotype expressed on naive B cells and secreted after primary activation without immunological memory persisting [167]. Because of its high valency, pentameric IgM is more efficient than other isotypes in binding antigens with many repeating epitopes such as GAG chains [167]. This is probably the other explanation of why many of anti-GAGs antibodies are IgM. For these reasons, we had a special short term immunization schedule aimed on IgM production. However, this shorter term immunization might not be necessary. Previous studies showed that even though the generated antibodies belong to IgM isotype, none of them were prepared by such a special short term immunization. 10E4 were produced after four immunizations in about four weeks [145]. CS-56 was raised after three injections in a period of more than three weeks [168]. The immunization with three injections in more than six weeks resulted in the generation of HepSS1 [148]. Another IgM anti-HS antibody was named JM403 which was not used in this project. JM403 was the result of an immunization with five injections during a period of 63 days [175]. Furthermore, although T-cell-independent antigen elicits

primarily IgM, some subclasses of IgG (IgG2 and IgG3) also respond to it [176]. In fact, 1B5, 2B6, 3G10 and 5D4 all belong to IgG isotype [145, 146].

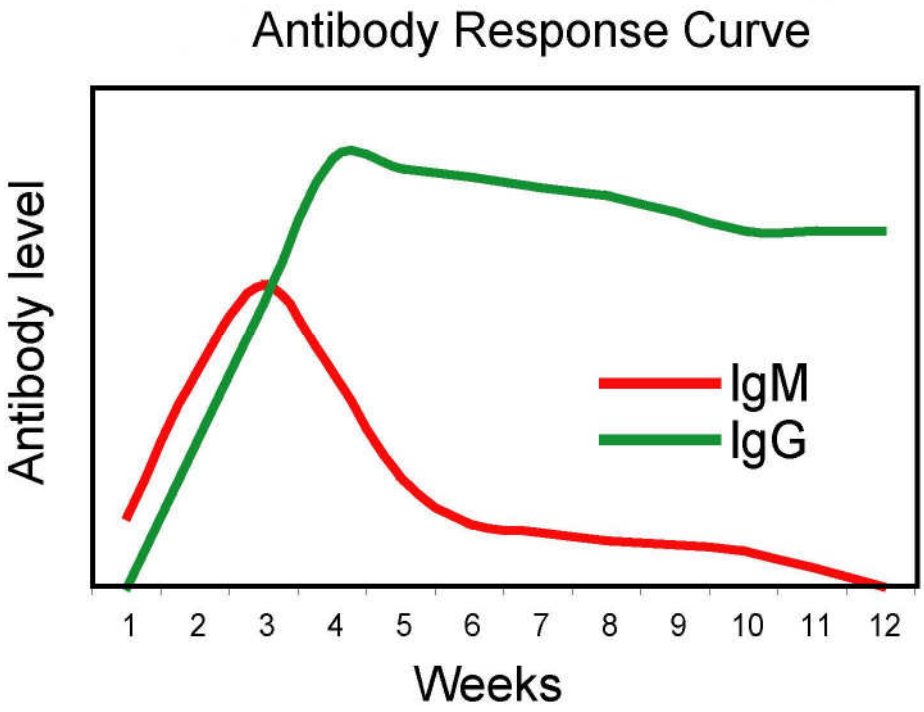

Figure 3-19 Antibody response curve during the immunization
The serum antibody response shows that IgM expression/secretion is followed by switching to IgG three weeks after primary injection.

3.3.2.4 Simpler immunization, more sophisticated screen

In this project, more than six months were spent on large scale antigen preparation including medium collection, purification and characterization. Most of the purified antigens were consumed in the immunization. However, the immunization may not require highly purified antigen since the ability to detect a monoclonal antibody of interest is basically dictated by the selection strategy. Moreover, we think that the complex formed by impurity may even improve the immune response. Therefore, instead of using purified perlecan, cells or ECM can be harvested and used as immunogens. The time and antigens

saved could be used to develop a more sophisticated screening procedure. In fact, CS-56 was isolated from mice immunized with ventral membrane and HepSS-1 was raised from mice immunized directly with fibrosarcoma cells [148, 168].

Since a lot of the antigens (perlecan domain I) were used for the immunization, we were facing a shortage of domain I material for screening and had to start screening with full length perlecan. Screening with full length perlecan gave the positive results for not only domain I antibodies but also the antibodies against other domains in perlecan, which increased the number of positive clones and therefore increased the following workload severely. The ideal selection strategy should be the one able to eliminate the unwanted cultures and find the cultures of interest instantly. In this project, if we had enough DI and DI-EGFP, the best way would have been to screen against the domain I materials with and without enzyme treatment directly, which would made the work a lot more efficient and much less laborious.

3.3.2.5 The importance of feeder layers

Except for the times of antigen injection, another major difference of two groups was the use of feeder layer. Based on Table 1-2, MRC-5 (the second best and easiest one for us to obtain) was chosen as the feeder layer for the second group, which may have caused the very positive outcome. Weak immunogens are generally accompanied by low fusion efficiencies. Such was the case in the report. The use of MRC-5 feeder layers increased the opportunity for a successful fusion by increasing the total number of new growing hybrid cells as well as supporting their proliferation. Since screening processes were based on the antibodies secreted into the culture media, improved proliferation gave a larger number of

cells which allowed for earlier and more sensitive detection. Consequently, more positive hybridomas may be found and stabilized during the screening procedures. In addition, the MRC-5 supplied an environment for maintaining some important but particularly fastidious hybridoma cultures. Although a large number of feeder layer were used for hybridoma culture, superiority of the human fetal lung fibroblasts over other cells has already been reported in a coculture model investigating the cloning efficiency of hybridoma cells [128].

3.3.2.6 Other things to consider

The immunization route is one of the critical steps in the antibody production. The most frequently used routes of injection for antibody productions are subcutaneous (s.c.), intradermal (i.d.), intramuscular (i.m.), intraperitoneal (i.p.), and intravenous (i.v.). I.m. and i.d. injections are the most effective ways to evoke host immune response, but cause a lot of pain and serious side effects such as sciatic nerve damage [177]. The production of CS-56 used footpad injections (one of i.d.) [168]. However, considering animal welfare and ethics, we were trying to eliminate discomfort to animals as much as we could so i.m. and i.d. were not chosen. We did not use s.c due to the slow antigen absorption [177]. In this project, primary immunization was i.p injection with FCA. Depending on the antibody titration achieved in tail blood, animals were immunized one or two further times by i.p injections with FIA. Final booster injection was administrated by i.v injection without any adjuvant. The antibodies titration in the blood after the introduction of antigen revealed that the immunization protocol was very successful (see Figure 3-9 and Figure 3-10).

The up-scaling of MAb production is traditionally accomplished by collecting ascites that is produced by injecting the hybridoma into the abdominal cavity of the mouse. With a

constant temperature, an optimal nutrient and oxygen supply, and the efficient removal of waste, the in vivo environment makes an ideal incubator for hybridomas [178]. In the abdominal cavity, hybridomas grow to high densities and secreted antibody accumulates to high concentration. Nevertheless, a few reasons limited the use of MAb produced by ascites such as pain and distress to the animals and the contaminations with viruses and other microorganisms [179]. Therefore, considering animal ethics, instead of the traditional method of producing large amount of antibodies by ascites we chose to produce MAbs via growth of hybridomas in serum free medium. Most cloned hybridomas were already adapted to a serum free environment and ready for up-scaling in bioreactors. Actually, the conditioned SFM without any purification and concentration process can be used directly in most lab based assays.

The phage display technology presents another way to generate antibodies that cannot be easily isolated by the conventional hybridoma technology. This technology passes the immune system and the immunization procedure and consequently overcomes the problem raised by weak immunogenicity. The antibodies of interest can be selected from libraries where numerous antibodies present on the surfaces of phages [129]. However, while this technology is under rapid development in recent years, there are few recombinant antibodies with high affinity as is the case in conventional hybridoma technology [140]. This is not a first choice for most labs involved in antibody production.

3.4 Conclusion

In this part of work, recombinant perlecan domain I (DI and DI-EGFP) were successfully purified and characterized. Tree milligrams of recombinant materials were obtained from 9 litres of conditioned media. In ELISA, the purified protein did not react with domain III antibody. The results of SDS-PAGE indicated the molecular mass of the purified material matched the previously reported data of perlecan domain I.

The purified antigens were used for animal immunization and hybridoma screening Hybridoma cell lines secreting anti-perlecan antibodies were successfully raised from the second fusions. 10 sub clones ware adapted into serum free medium. In ELISA and Western Blotting, the cloned antibodies can recognize the full length perlecan extracted from HCAEC.

4 Cell study using human chondrocyte

4.1 Introduction

Apart from vascularised tissues, perlecan is also a major pericellular HS-proteoglycan in cartilaginous tissues [180]. It is indicated by in vitro studies that perlecan can induce early stages of cartilage differentiation and cooperate with chondrogenic growth factors [181]. In permanent cartilages including the meniscus, tibial plateau and femoralcondyle cartilages, perlecan displays a prominent localisation pattern which lasts from new born to the mature adult [182, 183]. The tibial and femoral growth plates of the long bones display an extracellular as well as a prominent pericellular distribution in the hypertrophic chondrocytes of these tissues[182].

Human chondrocytes were used as a model to characterise the antibodies further. The antibody sensitivity to heparanase treatment was examined. In this study fixation techniques have also been explored. The serum free media conditioned by the hybridomas were used to detect the presence of perlecan on tissue culture slides where human chondrocytes had been grown for one week.

4.2 Results

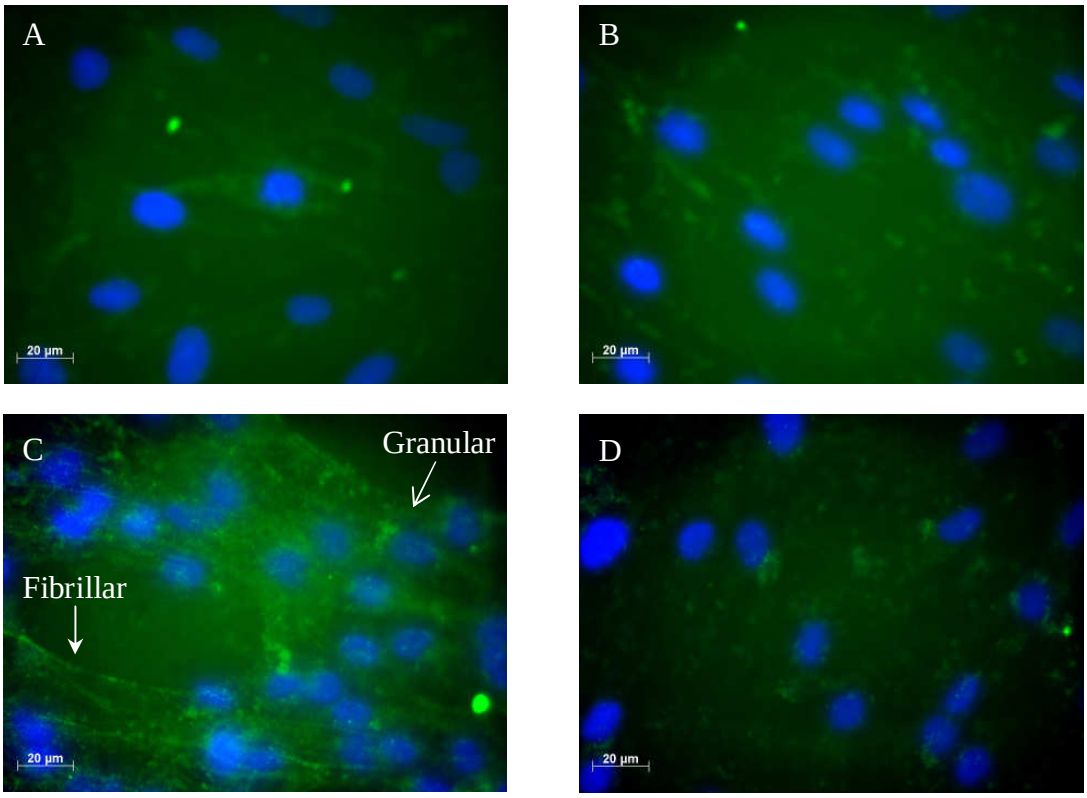

Figure 4-1 The expression of HS in human cells
Human chondrocytes ($5x10^4$ cells in 1 ml of medium) were seeded onto glass slides and grown for seven days to allow enough time for the cells to lay down ECM. Prior to fixation, cells shown in panels B and D were treated with 1 µg/ml heparanase for 4 hours at 37℃. Panels A and C were incubated with PBS, when panels B and D were incubated with the heparanase. Panels A and B were fixed with 4% paraformaldehyde for 15 minutes whilst C and D were fixed with 100% ice-cold acetone for 1 minute. All panels were probed with anti-HS MAb, 10E4 at a concentration of 2µg/ml and the fluorescence was detected on a fluorescence microscope. The nuclei were stained with DAPI which is blue under fluorescence.

10E4 MAb was used to probe for HS expressed by human chondrocytes at day 7 of culture (Figure 4-1 C). The immunoreactivity was largely reduced when the cells were incubated with heparanase, indicating the incubation resulted in a removal of HS (compare Figure 4-1 C to Figure 4-1 D). The intensity of 10E4 immunoreactivity was reduced when the cells were fixed with paraformaldehyde (compare Figure 4-1 C with Figure 4-1 A). The expression of HS by human chondrocyte was seen largely diffusive but there were regions of microfibrillar and granular staining.

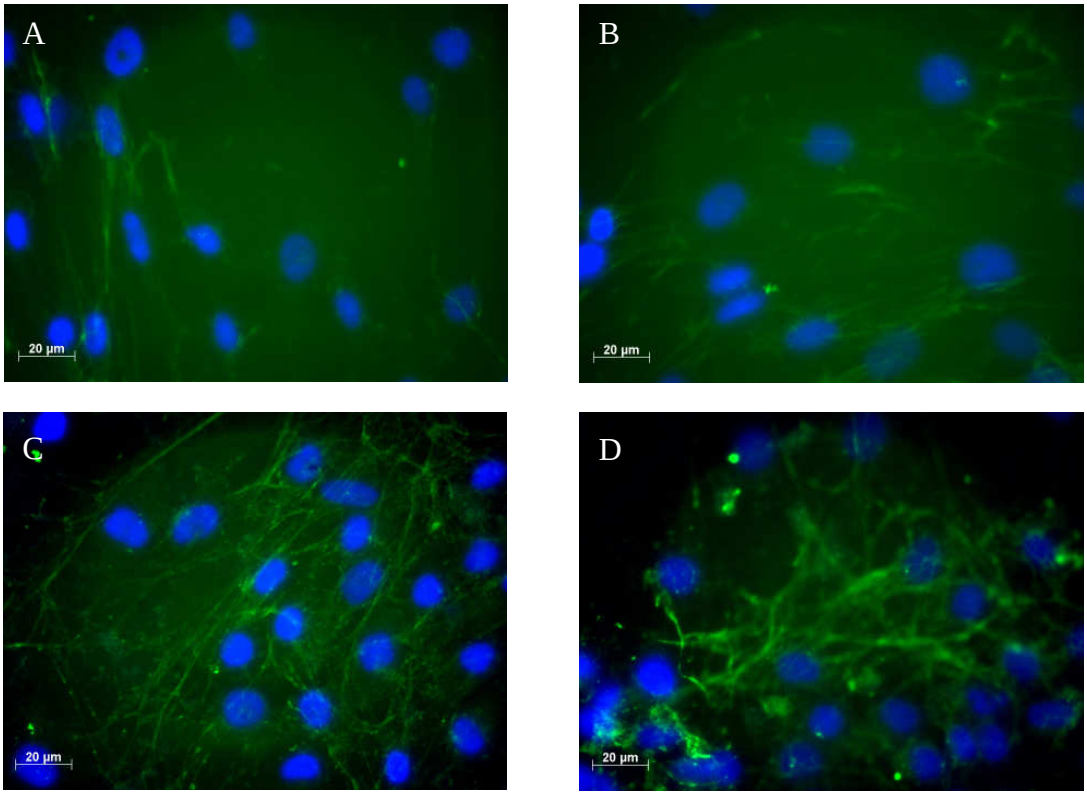

Figure 4-2 The expression of perlecan in human cells
Human chondrocytes ($5x10^4$ cells in 1 ml of medium) were seeded onto glass slides and grown for seven days to allow enough time for the cells to lay down ECM. Prior to fixation, cells shown in panels B and D were treated with 1 µg/ml heparanase for 4 hours at 37℃. Panels A and C were incubated with PBS, when panels B and D were incubated with the heparanase. Panels A and B were fixed with 4% paraformaldehyde for 15 minutes whilst C and D were fixed with 100% ice-cold acetone for 1 minute. All panels were probed with anti-perlecan MAb, CSI-076 at a concentration of 2µg/ml and the fluorescence was detected on a fluorescence microscope. The nuclei were stained with DAPI which is blue under fluorescence.

The anti-perlecan MAb (CSI-076) was used to probe for the presence of perlecan and the reactivity would be expected to be unaffected by removal of HS chains. The localization of perlecan was identified as a fabrillar structure surrounding the chondrocyte cells (Figure 4-2 C). The level of immunoreactivity increased when the HS chains were removed by the heparanase, suggesting that CSI-076 reacted with domain I of perlecan (compare Figure 4-2 C with Figure 4-2 D). This result supports the data shown previously in this thesis (Figure 3-7). Fixation with paraformaldehyde reduced the immunoreativity of CSI-076 for domain I of perlecan (compare Figure 4-2 C and D with Figure 4-2 A and B)

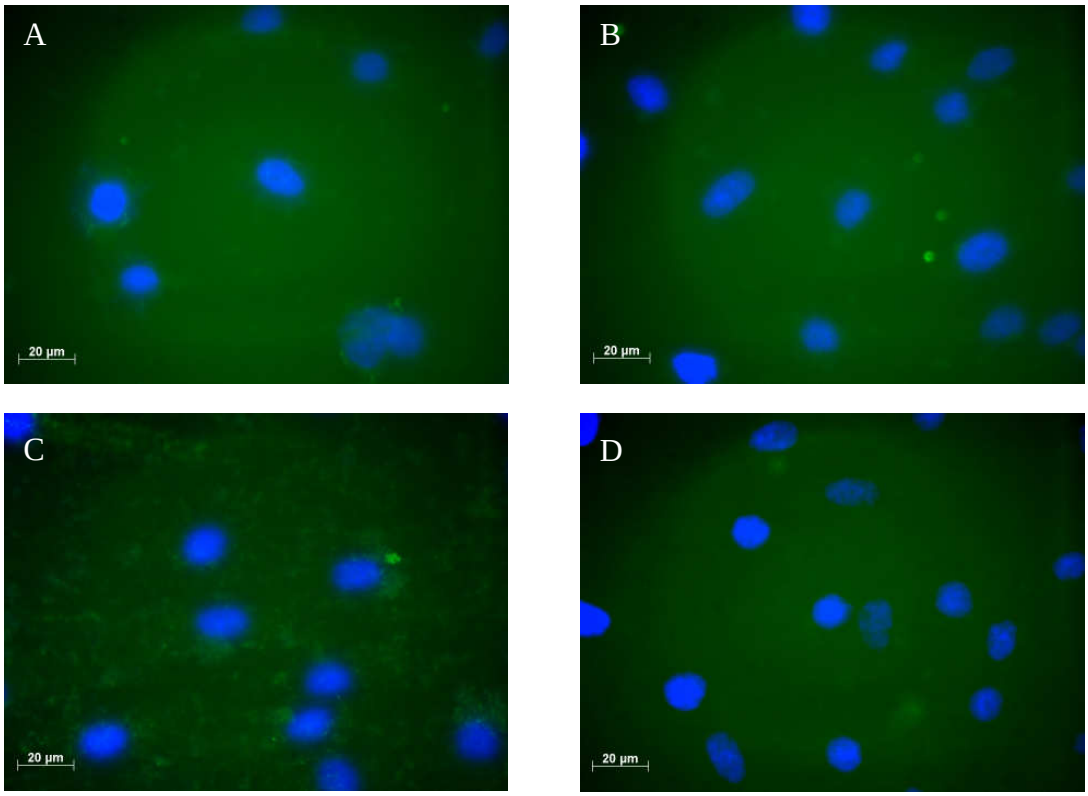

Figure 4-3 No primary control
Human chondrocytes ($5x10^4$ cells in 1 ml of medium) were seeded onto glass slides and grown for seven days to allow enough time for the cells to lay down ECM. Prior to fixation, cells shown in panels B and D were treated with 1 µg/ml heparanase for 4 hours at 37℃. Panels A and C were incubated with PBS, when panels B and D were incubated with the heparanase. Panels A and B were fixed with 4% paraformaldehyde for 15 minutes whilst C and D were fixed with 100% ice-cold acetone for 1 minute. While other slides were incubated with the primary antibody, the control slides were incubated with PBS. The fluorescence was detected on a fluorescence microscope. The nuclei were stained with DAPI which is blue under fluorescence.

All the panels gave clear background, indicating that there was no non-specific. No difference was shown when the cells were incubated with the heparanase (compare Figure 4-3 A and C with Figure 4-3 B and D, respectively). The different fixation did not gave any change either (compare Figure 4-3 A and B with Figure 4-3 C and D, respectively).

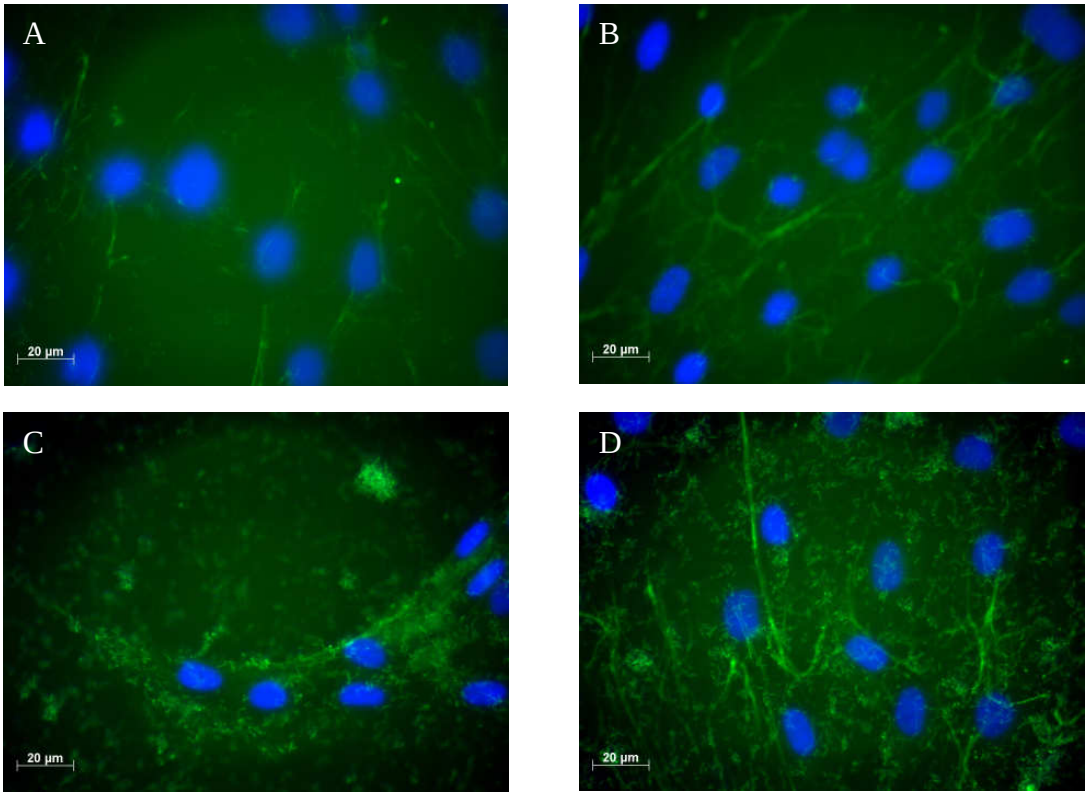

Figure 4-4 The expression of perlecan in human cells probed with 5D7-2E4
Human chondrocytes ($5x10^4$ cells in 1 ml of medium) were seeded onto glass slides and grown for seven days to allow enough time for the cells to lay down ECM. Prior to fixation, cells shown in panels B and D were treated with 1 µg/ml heparanase for 4 hours at 37℃. Panels A and C were incubated with PBS, when panels B and D were incubated with the heparanase. Panels A and B were fixed with 4% paraformaldehyde for 15 minutes whilst C and D were fixed with 100% ice-cold acetone for 1 minute. All panels were probed with SFM conditioned by 5D7-2E4 and the fluorescence was detected on a fluorescence microscope. The nuclei were stained with DAPI which is blue under fluorescence.

Perlecan expressed by human chondrocytes was detected by 5D7-2E4. In panel C and D the distribution of perlecan was shown as granular and fibrillar structures while in panel A and B, the distribution was shown as fibrillar structures only. The intensity of immunostaining was reduced by the fixation with paraformaldehyde, but the staining was still significant. (compare Figure 4-4 C and D with Figure 4-4 A and C). The immunoreactivity was increased when the cells were incubated with heparanase, suggesting the removal of HS exposed the epitope (compare Figure 4-4 A and C with Figure 4-4 B and D, respectively).

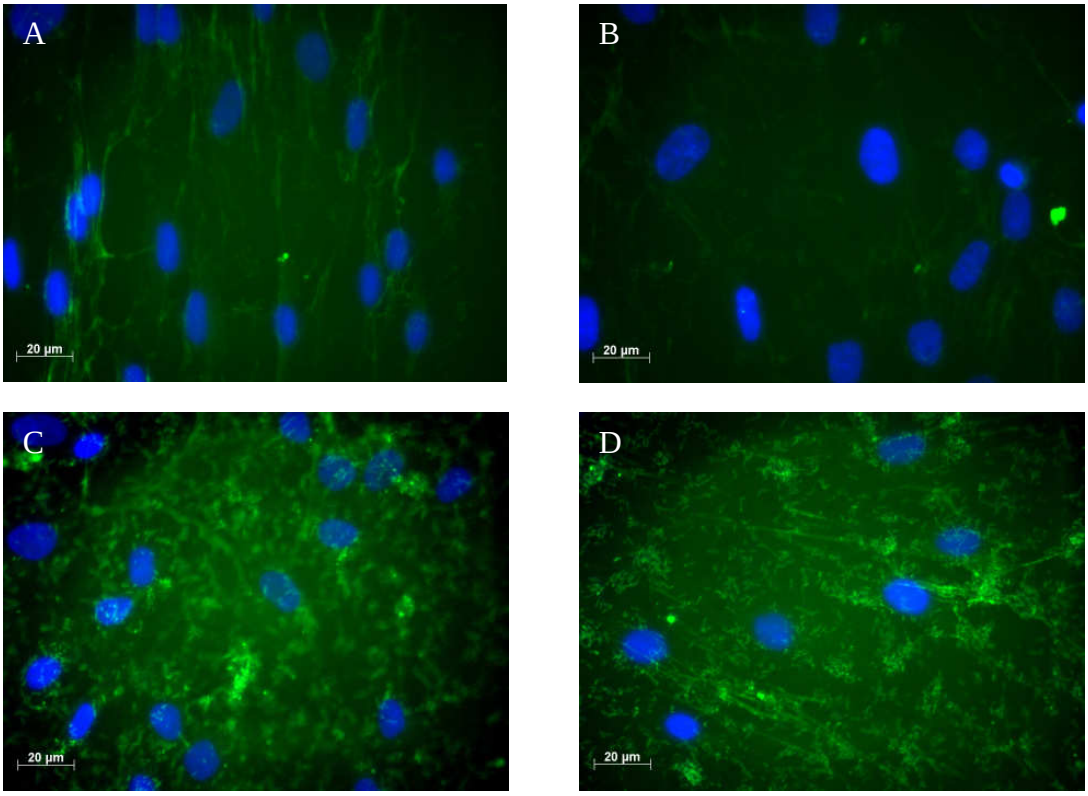

Figure 4-5 expression of perlecan in human cells probed with 5D7-2G7
Human chondrocytes ($5x10^4$ cells in 1 ml of medium) were seeded onto glass slides and grown for seven days to allow enough time for the cells to lay down ECM. Prior to fixation, cells shown in panels B and D were treated with 1 µg/ml heparanase for 4 hours at 37℃. Panels A and C were incubated with PBS, when panels B and D were incubated with the heparanase. Panels A and B were fixed with 4% paraformaldehyde for 15 minutes whilst C and D were fixed with 100% ice-cold acetone for 1 minute. All panels were probed with SFM conditioned by 5D7-2G7 and the fluorescence was detected on a fluorescence microscope. The nuclei were stained with DAPI which is blue under fluorescence.

Perlecan expressed by human chondrocytes was detected by MAb 5D7-2G7. In panel C and D of Figure 4-5, the distribution of perlecan was granular in ECM while in panel A and B of Figure 4-5, perlecan were seen as fibrillar structures deposited in the ECM. The immunoreactivity was not affected by the removal of HS chains (compare Figure 4-5 A and C with Figure 4-5 B and D, respectively). The level of immunoreactivity was largely reduced when the cells were fixed with paraformaldehyde (compare Figure 4-5 C and D with Figure 4-5 A and B, respectively).

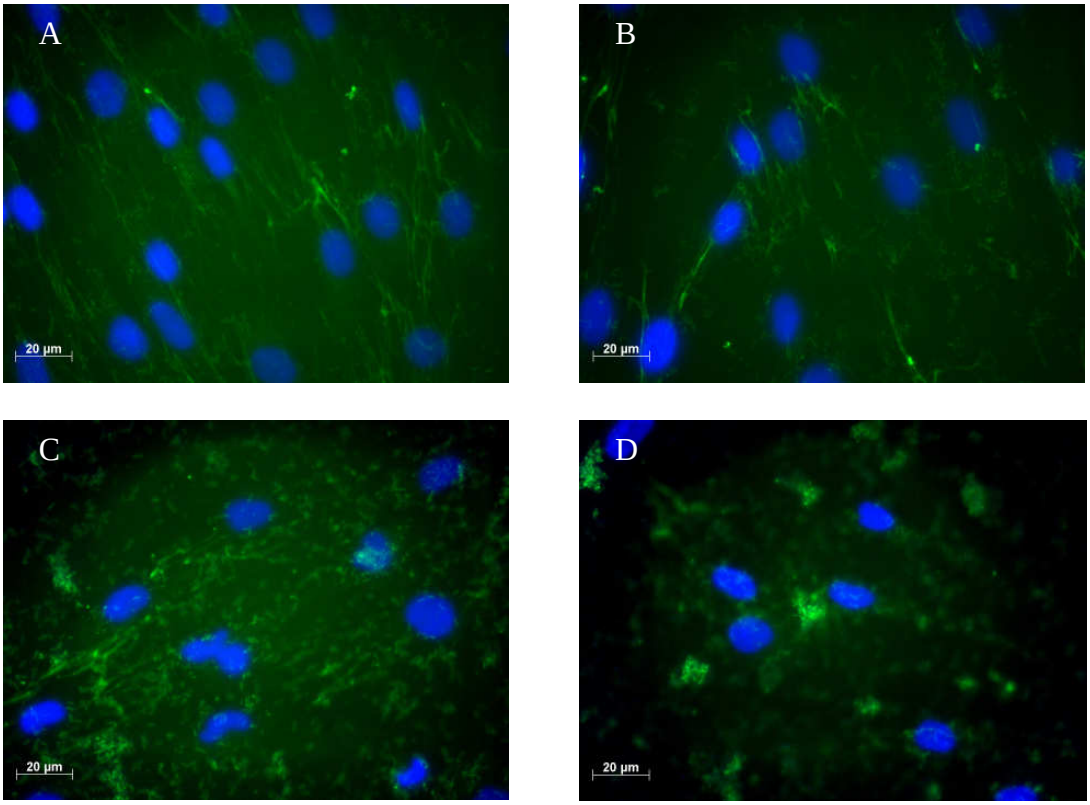

Figure 4-6 the expression of perlecan in human cells probed with 13E9-3B3
Human chondrocytes ($5x10^4$ cells in 1 ml of medium) were seeded onto glass slides and grown for seven days to allow enough time for the cells to lay down ECM. Prior to fixation, cells shown in panels B and D were treated with 1 µg/ml heparanase for 4 hours at 37℃. Panels A and C were incubated with PBS, when panels B and D were incubated with the heparanase. Panels A and B were fixed with 4% paraformaldehyde for 15 minutes whilst C and D were fixed with 100% ice-cold acetone for 1 minute. All panels were probed with SFM conditioned by 13E9-3B3 and the fluorescence was detected on a fluorescence microscope. The nuclei were stained with DAPI which is blue under fluorescence.

Perlecan expressed by human chondrocytes was detected by MAb 13E9-3B3. In the panels fixed with ice-cold acetone (Figure 4-6 C and D) perlecan staining was shown as granular while in the panels fixed with paraformaldehyde the staining of perlecan was fibrillar (Figure 4-6 A and B). The fixation with paraformaldehyde reduced the staining intensity. The level of immunoreactivity was also reduced when the cells were incubated with heparanase, suggesting that 13E9-3B3 might be sensitive to conformational changes caused by the degradation of HS (compare Figure 4-6 C with Figure 4-6 D).

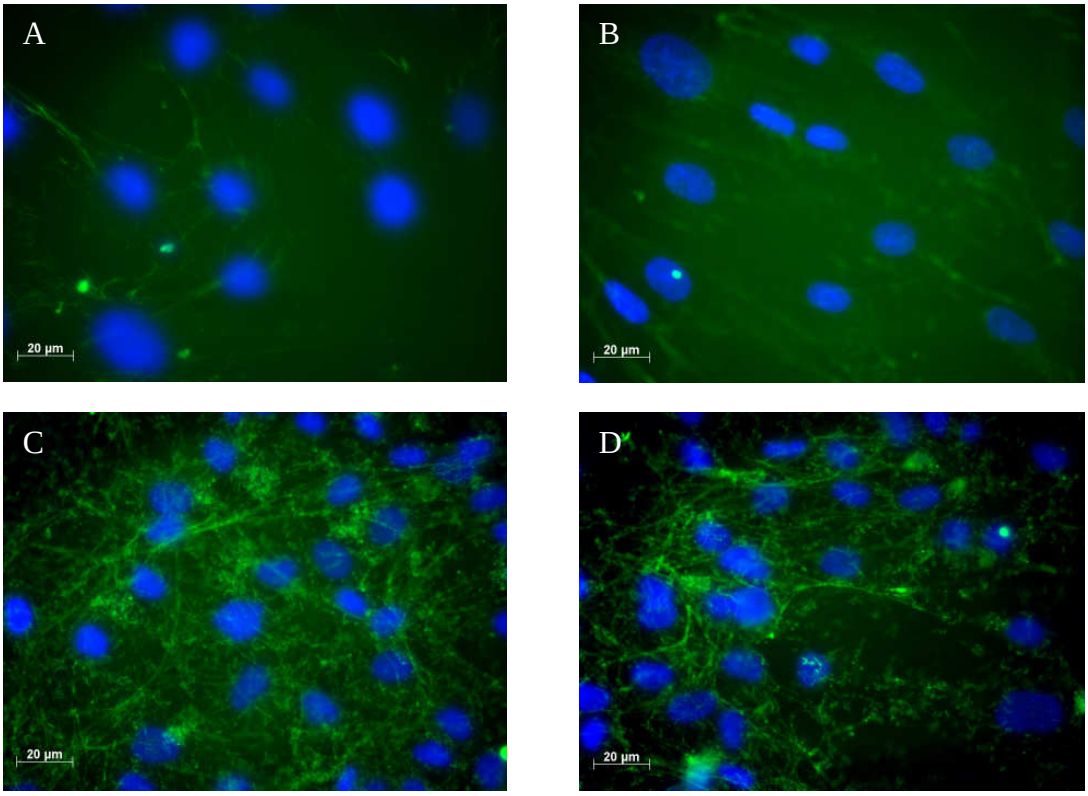

Figure 4-7 the expression of perlecan in human cells probed with 13E9-3B9
Human chondrocytes ($5x10^4$ cells in 1 ml of medium) were seeded onto glass slides and grown for seven days to allow enough time for the cells to lay down ECM. Prior to fixation, cells shown in panels B and D were treated with 1 µg/ml heparanase for 4 hours at 37℃. Panels A and C were incubated with PBS, when panels B and D were incubated with the heparanase. Panels A and B were fixed with 4% paraformaldehyde for 15 minutes whilst C and D were fixed with 100% ice-cold acetone for 1 minute. All panels were probed with SFM conditioned by 13E9-3B9 and the fluorescence was detected on a fluorescence microscope. The nuclei were stained with DAPI which is blue under fluorescence.

Perlecan expressed by human chondrocytes was detected by MAb 13E9-3B9. In the panels fixed with ice-cold acetone (Figure 4-7 C and D), the distribution of perlecan was seen to be fibrillar and granular pattern while in the panels fixed by paraformaldehyde the staining was seen to be fibrillar only (Figure 4-7 A and B). The intensity of staining was largely reduced by the fixation with paraformaldehyde (compare Figure 4-7 C and D with Figure 4-7 A and B).The amount of immunoreactivity was not affected by the removal of HS (compare Figure 4-7 A and C with Figure 4-7 B and D, respectively).

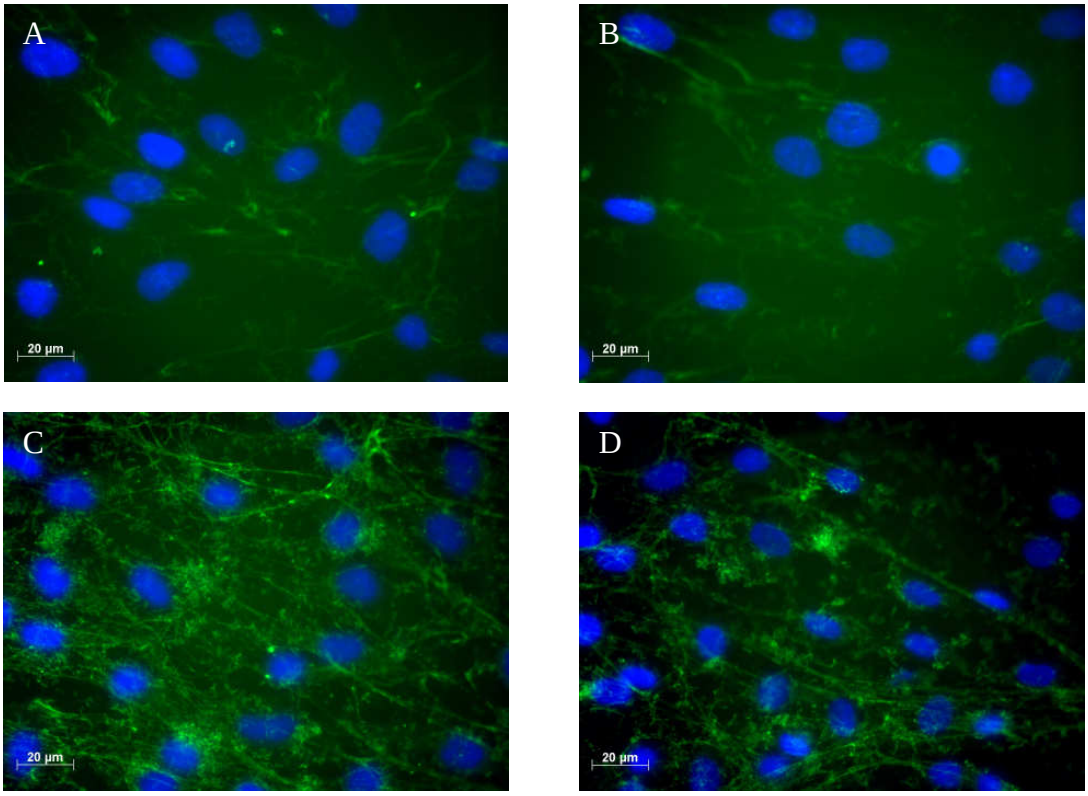

Figure 4-8 the expression of perlecan in human cells probed with 13E9-3C5
Human chondrocytes ($5x10^4$ cells in 1 ml of medium) were seeded onto glass slides and grown for seven days to allow enough time for the cells to lay down ECM. Prior to fixation, cells shown in panels B and D were treated with 1 µg/ml heparanase for 4 hours at 37℃. Panels A and C were incubated with PBS, when panels B and D were incubated with the heparanase. Panels A and B were fixed with 4% paraformaldehyde for 15 minutes whilst C and D were fixed with 100% ice-cold acetone for 1 minute. All panels were probed with SFM conditioned by 13E9-3C5 and the fluorescence was detected on a fluorescence microscope. The nuclei were stained with DAPI which is blue under fluorescence.

Perlecan expressed by human chondrocytes was detected by MAb 13E9-3C5. The staining pattern for this MAb was identical to 13E9-3B9, and also to the staining for the following MAbs: 13E9-3D5, 3E9-3D7, 13E9-3E7 and 13E9-3E9.

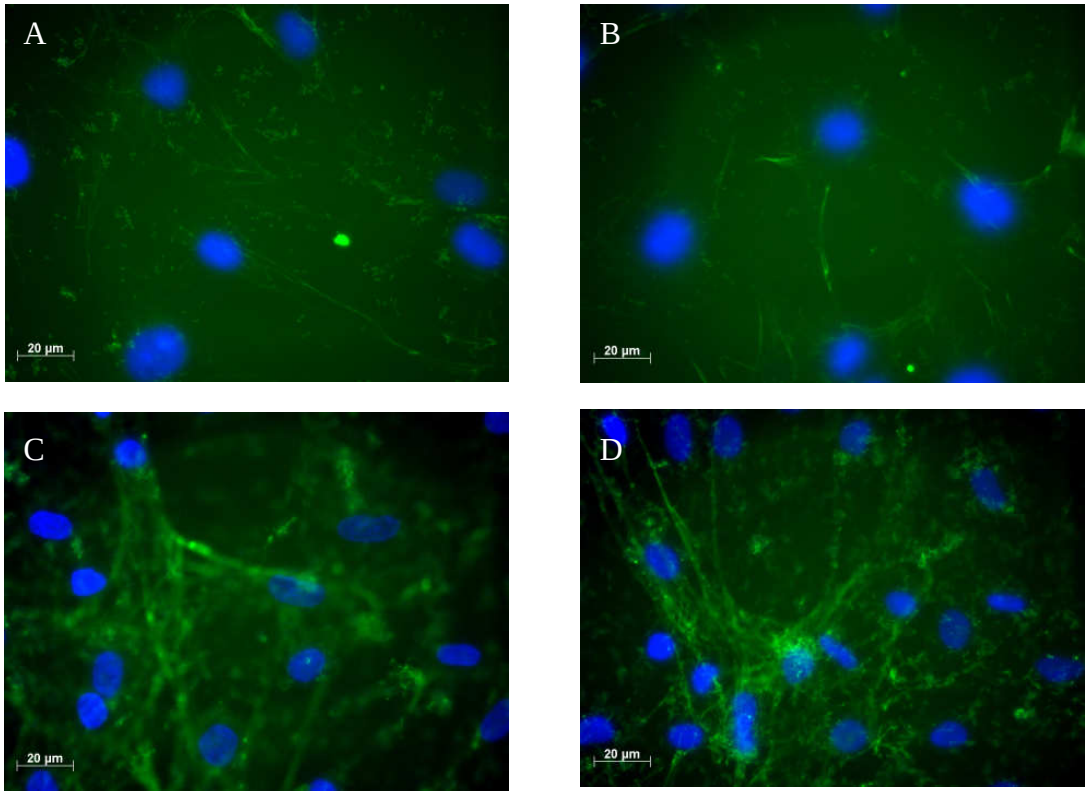

Figure 4-9 the expression of perlecan in human cells probed with 13E9-3D5
Human chondrocytes ($5x10^4$ cells in 1 ml of medium) were seeded onto glass slides and grown for seven days to allow enough time for the cells to lay down ECM. Prior to fixation, cells shown in panels B and D were treated with 1 µg/ml heparanase for 4 hours at 37℃. Panels A and C were incubated with PBS, when panels B and D were incubated with the heparanase. Panels A and B were fixed with 4% paraformaldehyde for 15 minutes whilst C and D were fixed with 100% ice-cold acetone for 1 minute. All panels were probed with SFM conditioned by 13E9-3D5 and the fluorescence was detected on a fluorescence microscope. The nuclei were stained with DAPI which is blue under fluorescence.

Perlecan expressed by human chondrocytes was detected by MAb 13E9-3D5.

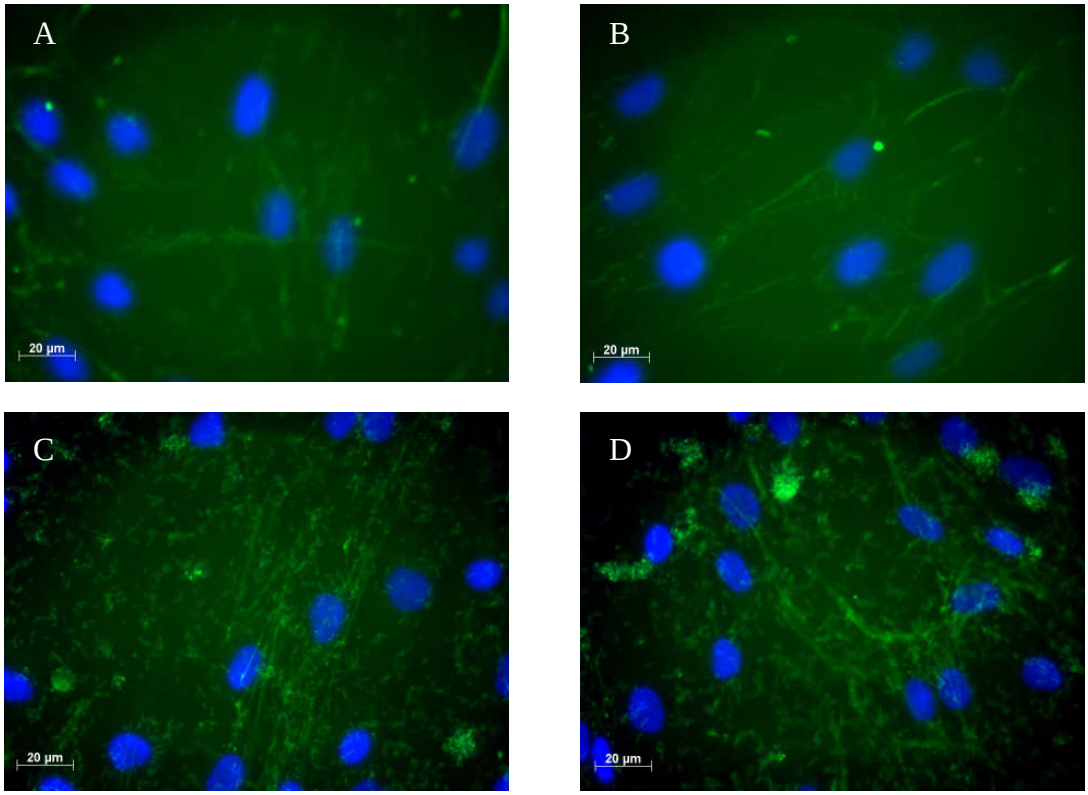

Figure 4-10 the expression of perlecan in human cells probed with 13E9-3D7
Human chondrocytes ($5x10^4$ cells in 1 ml of medium) were seeded onto glass slides and grown for seven days to allow enough time for the cells to lay down ECM. Prior to fixation, cells shown in panels B and D were treated with 1 µg/ml heparanase for 4 hours at 37℃. Panels A and C were incubated with PBS, when panels B and D were incubated with the heparanase. Panels A and B were fixed with 4% paraformaldehyde for 15 minutes whilst C and D were fixed with 100% ice-cold acetone for 1 minute. All panels were probed with SFM conditioned by 13E9-3D7 and the fluorescence was detected on a fluorescence microscope. The nuclei were stained with DAPI which is blue under fluorescence.

Perlecan expressed by human chondrocytes was detected by MAb 13E9-3D7.

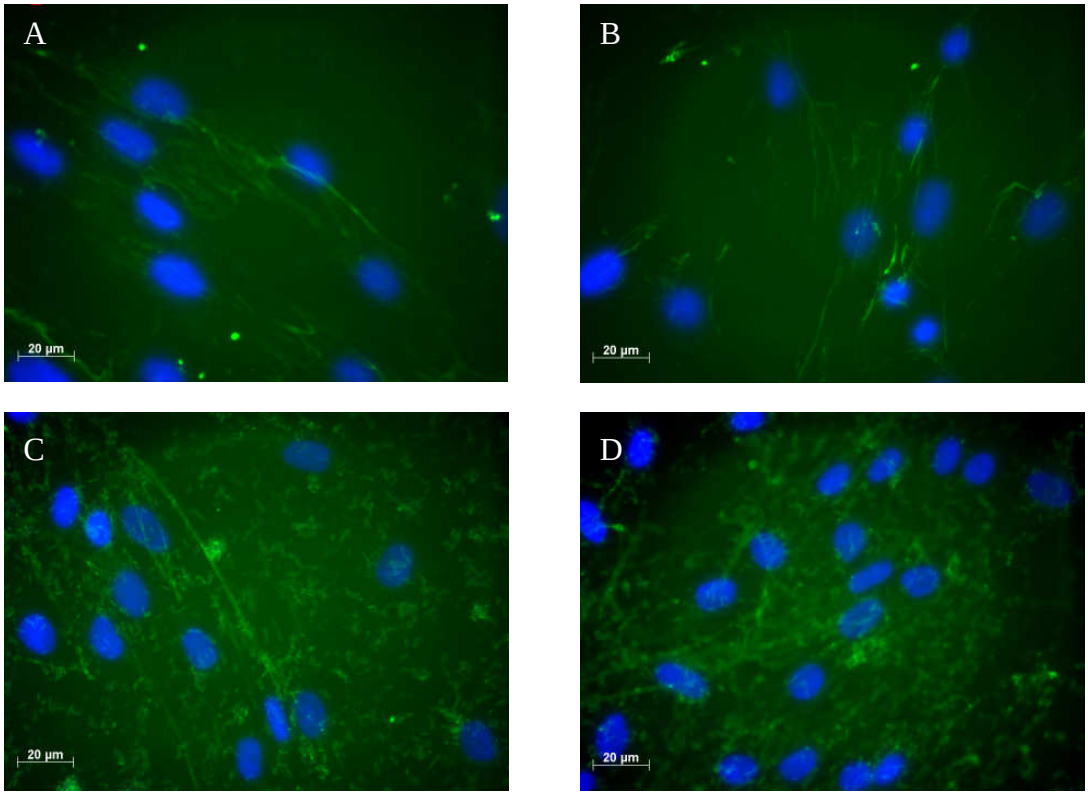

Figure 4-11 the expression of perlecan in human cells probed with 13E9-3E7
Human chondrocytes ($5x10^4$ cells in 1 ml of medium) were seeded onto glass slides and grown for seven days to allow enough time for the cells to lay down ECM. Prior to fixation, cells shown in panels B and D were treated with 1 µg/ml heparanase for 4 hours at 37℃. Panels A and C were incubated with PBS, when panels B and D were incubated with the heparanase. Panels A and B were fixed with 4% paraformaldehyde for 15 minutes whilst C and D were fixed with 100% ice-cold acetone for 1 minute. All panels were probed with SFM conditioned by 13E9-3E7 and the fluorescence was detected on a fluorescence microscope. The nuclei were stained with DAPI which is blue under fluorescence.

Perlecan expressed by human chondrocytes was detected by MAb 13E9-3E7.

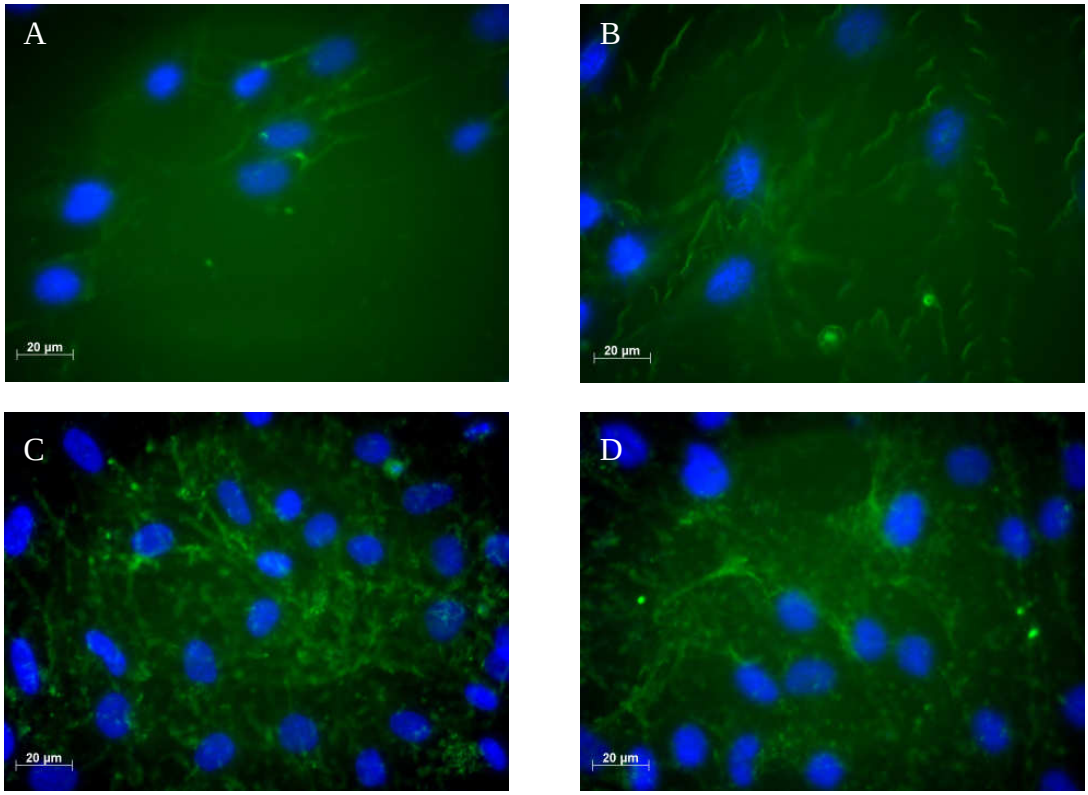

Figure 4-12 the expression of perlecan in human cells probed with 13E9-3E9
Human chondrocytes ($5x10^4$ cells in 1 ml of medium) were seeded onto glass slides and grown for seven days to allow enough time for the cells to lay down ECM. Prior to fixation, cells shown in panels B and D were treated with 1 µg/ml heparanase for 4 hours at 37℃. Panels A and C were incubated with PBS, when panels B and D were incubated with the heparanase. Panels A and B were fixed with 4% paraformaldehyde for 15 minutes whilst C and D were fixed with 100% ice-cold acetone for 1 minute. All panels were probed with SFM conditioned by 13E9-3E9 and the fluorescence was detected on a fluorescence microscope. The nuclei were stained with DAPI which is blue under fluorescence.

Perlecan expressed by human chondrocytes was detected by MAb 13E9-3E9.

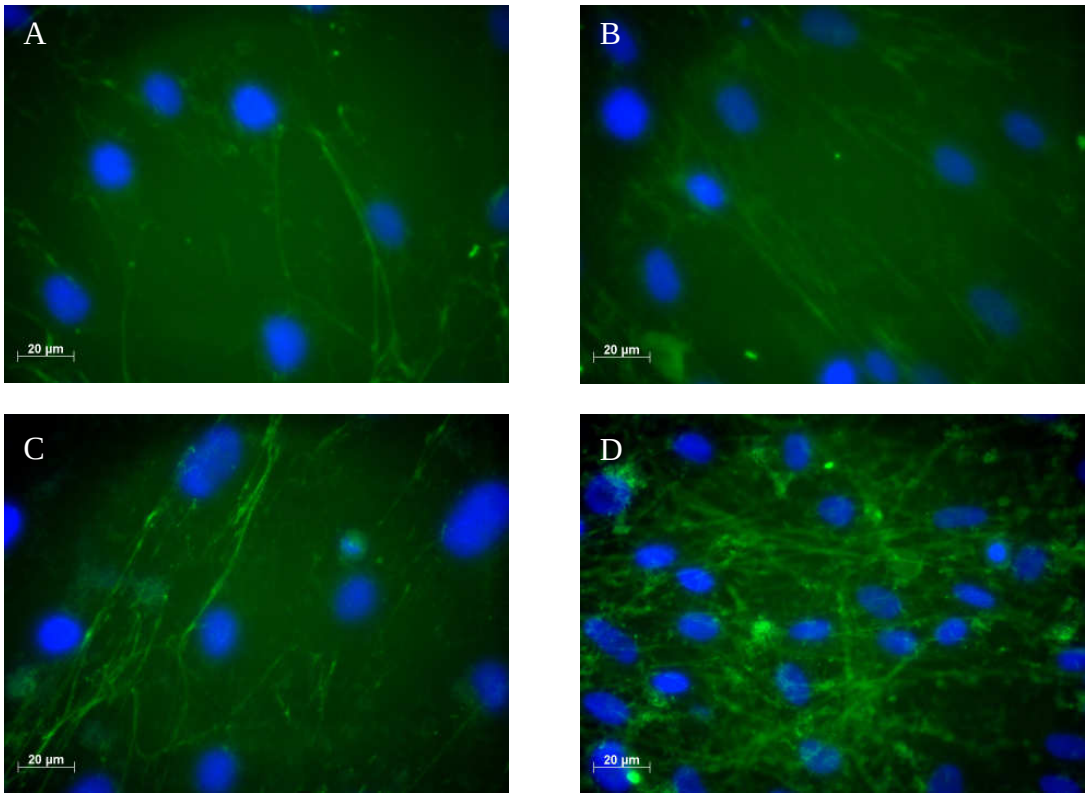

Figure 4-13 the expression of perlecan in human cells probed with 13E9-3G5
Human chondrocytes ($5x10^4$ cells in 1 ml of medium) were seeded onto glass slides and grown for seven days to allow enough time for the cells to lay down ECM. Prior to fixation, cells shown in panels B and D were treated with 1 µg/ml heparanase for 4 hours at 37℃. Panels A and C were incubated with PBS, when panels B and D were incubated with the heparanase. Panels A and B were fixed with 4% paraformaldehyde for 15 minutes whilst C and D were fixed with 100% ice-cold acetone for 1 minute. All panels were probed with SFM conditioned by 13E9-3G5 and the fluorescence was detected on a fluorescence microscope. The nuclei were stained with DAPI which is blue under fluorescence.

When the human chondrocytes were probed with 13E9-3G5 the distribution of perlecan was demonstrated to be microfibrillar in ECM (Figure 4-13 C and D). An increase in immunoreactivity was detected when the cells were incubated with heparanase (compare Figure 4-13 C with B), suggesting that this antibody is react with the epitope that was exposed by the removal of HS. Fixating the cells with paraformaldehyde reduced the amount of immunoreactivity, but the significant fibrillar staining could still be seen (Figure 4-13 A and B).

4.3 Discussion

Previous studies on the turnover of proteoglycans have been carried out using [^{35}S] sulfate-labeled glycosaminoglycan [184, 185]. In the substratum adhesion sites, approximate 80% radiolabeled HSPG turned over during the first 12 hour of the chasing period [185]. A portion of them was degraded while the majority was released form the adhesion sites [185]. This was further detailed by the study using human colon carcinoma cells, where 55% HSPG was released into the medium ($t_{1/2}$ ~2.5) and was neither endocytosed nor degraded [184]. The remaining 45% was internalized and degraded into some fragments [184]. It was also note that the degradation of HS was manifestly inhibited by chloroquine [184]. The explanation would be that the addition of chloroquine changes the pH in the environment and decreases heparanase activity which has a pH for optimized activity between pH5.5 and 5.8 [67].

In the early 1990s, Hughes et al developed the use of monoclonal antibody technologies to indentify new epitope in protein created as specific terminal to demonstrate enzymatic cleavage products [186, 187]. They named the specific cleavage sites as catabolic neoepitopes. In these studies, catabolic neoepitopes were generated by the action of matrix proteinases such as aggrecanases or MMPs on the IGD of aggrecan. These antibodies have been used to study the proteolysis of aggrecan and correlative enzymes and thereby investigate cartilage proteoglycan aggregate catabolism in the pathogenesis of arthritis. Similar approaches have been undertaken widely in many fields of biomedical research [188, 189]. Indeed, the generation of antibody 3G10 was base on this idea but the epitope was a stub produced by endoglycosidase (heparitinase) not protease [145]. With these in

mind, this project aimed primarily at producing monoclonal antibodies against the cleavage site raised by mammalian heparanase process.

The results presented in this study provided experimental evidence for the ability of the newly generated antibodies to recognize the native perlecan expressed by human chondrocytes in vitro. Although most antibodies were not sensitive to the removal of the HS chains, the exceptions were 5D7-2E4, 13E9-3B3 and 13E9-3G5. The immunoreactivity of 5D7-2E4 and 13E9-3G5 to perlecan was enhanced when cells were incubated with heparanase, while the intensity of 13E9-3B3 staining was decreased with the removal of HS. In chapter three of this thesis, it was demonstrated by ELISA that our new antibodies are neither against HS nor against DI. Therefore, 5D7-2E4 and 13E9-3G5 are possibly against perlecan domain V where there are potential sites for GAG attachment and when it is removed by heparanase, the antibodies (5D7-2E4 and 13E9-3G5) can access their epitopes more easily and consequently give higher levels of immunoreactivity with perlecan. For 13E9-3B3, the epitope may be determined by the three dimensional shape (tertiary structure) of the protein, rather than the amino acid sequence (the primary structure). When the HS chains are removed from perlecan, the conformation of perlecan may change resulting in the loss of epitope for 13E9-3B3 and decreased immunofluorescence. Our primary aim was to use these antibodies to study the turnover of HSPGs which depends on the action of heparanase. If the antibodies (5D7-2E4, 13E9-3B3 and 13E9-3G5) are able to recognize the differences on perlecan induced by heparanase treatment in vitro, they are potentially capable of monitoring the turnover of HS on perlecan in vivo.

The immunochemical data clearly demonstrated that a significant amount of perlecan was produced and deposited into ECM by human chondrocytes in vitro. The intensity of the immunostaining was not seen as a gradient, decreasing as one moved further from the cell, although it was previously reported that perlecan was stained more intensely in the pericellular region [190]. Actually, a study using growth plate cartilage proved perlecan was not exclusively distributed pericellularly [182]. The distribution of perlecan in cartilage changed throughout the stages of development, but at present it is not clear under what conditions the cell alters its GAG substitution pattern [180, 182]. Using the new generated MAbs (5D4-2E4 and 13E9-3B3) may be helpful to clarify the turnover of GAG chains.

The turnover of proteoglycan is not limited to the enzymatic removal of GAG chains and it also involves the enzymatic processing of protein core [191]. Although all our antibodies are likely to be protein core antibodies, there are still possibilities for them to be used in the study of HS chains on perlecan. There is a potential mechanism that perlecan protein core antibodies can be used to monitor the changes of GAGs. The sites of GAG substitution on perlecan are located in domain I and V, both of which are termini of the molecule [48, 50, 51]. Proteolytic processing at either end results in not only variations of the size of the protein core but also the removal of GAGs. If the antibodies are against domain I or domain V, the enzymatic removal of their epitopes is expected to associate with the removal of some GAG chains on the molecule. In fact, using a number of anti-perlecan protein core antibodies Whitelock et al demonstrated that stromelysin, collagenase, and plasmin reduced the immunoreactivity toward the domain I antibodies and were also effective at liberating bound growth factors [191]. Their research also showed that the serine protease thrombin had only minimal effects on the immuno-purified perlecan protein core and the immunoreactivity of domain I and III of perlecan in matrix. It also released very little bFGF

from perlecan [191]. Therefore, thrombin may have been liberating the growth factor in other indirect ways, such as degradation of the surrounding ECM, activating ECM-bound proteases, and stimulating the heparanase activity [192].

There were some very fine granular structures stained by the protein core antibodies and most of them were in the experiments using ice-cold acetone as a fixative. There were two possible explanations. For one thing, since the nature of acetone is to precipitate proteins (discussed in the following paragraph), the granular structures are actually the precipitated perlecan. For another thing, the very fine granular signal could also be some molecular forms in the course of biosynthesis [193]. My hypothesis is that the molecular forms may be the precursor of perlecan, which have not been decorated by any GAG chain yet, and therefore could not be stained by the anti-HS MAb. They did not occur when cells were fixed with paraformaldehyde, since the fixation with paraformaldehyde could not penetrate the cells efficiently.

Moreover, to study GAG substitution turnover in cartilage development, the antibodies against aggrecan may be also very useful. Aggrecan is another major proteoglycan in ECM which is primarily and extensively substituted with CS [194]. Unlike perlecan which only has a limited number of GAG attachment sites, aggrecan has over 100 predicted GAG attachment sites [195]. The removal of GAGs may give the molecule more significant changes in conformation, which can increase the sensitivity of the tests. Indeed, it has been demonstrated that the nature of CS on aggrecan is modulated throughout tissue development and pathological process [196].

It was a general phenomenon in this project that ice-cold acetone fixation gave the better Immunostaining than paraformaldehyde fixation. For immunological studies, the fixation protocol is essential for the preservation of target antigens. Cells may response to a fixative in different ways due to the different protein content and structural arrangement. Incorrect preparation can block or impede target epitopes in cells. The methods that are best for the preservation of tissue structure also vary depending upon the protein under examination [197]. Fixatives may be classified into three groups: formation of cross-linkages (e.g., aldehydes such as glutaraldehyde or formalin); protein denaturation by coagulation (e.g., acetone and methanol); or a combination of the above. Paraformaldehyde induces cross-links between a various reactive end-groups of protein and consequently changes the secondary and tertiary structure of protein in a degree depending on the concentration and pH of the solution. Acetone, on another hand, has the ability to penetrate membranes and dehydrate the molecules [197]. Methanol/Acetone precipitates proteins and permeabilizes the cells at the same time while in the paraformaldehyde fixation permeabilization has to be done afterwards. Cross-linking agents, such as formalin and paraformaldehyde, have been reported to affect antigenicity adversely. Therefore methanol and acetone agents are advised for preserving immunohistochemical staining intensity for various antigens [198, 199]. Given that optimization is always needed to achieve a good and reliable immnuostaining result, there was no universal standard protocol that I could follow and it required some background experiments to work out the best conditions for the various antibodies. To optimize any fixation method for tissues and antibodies, several aspects need to be evaluated. In the present study, we have performed experiments to determine the best fixation that optimizes preservation of the epitopes on perlecan. Ice-cold acetone seems to be more appropriate fixatives for the antibodies used in this study.

4.4 Conclusions

Human chondrocytes were successfully stained by all newly generated MAbs. In the term of fixation technique, Ice-cold acetone fixed slides gave the better results in the experiments compared to Paraformaldehyde fixed ones. The intensity of the immunostaining is summarized briefly in Table 4-1 and Figure 4-1 to Figure 4-13 show the immunostaining obtained for each antibody.

Identities of antibodies used	Paraformaldehyde fixation		ice-cold acetone fixation	
	Heparanase treated	Non treated	Heparanase treated	Non treated
No primary	-	-	-	-
10E4	+	-	+ + +	-
CSI-076	+	+ +	+ +	+ + +
5D7-2E4	+	+ +	+ +	+ + +
5D7-2G7	+	+	+ + +	+ + +
13E9-3B3	+ +	+	+ + +	+ +
13E9-3B9	+	+	+ + +	+ + +
13E9-3C5	+	+	+ + +	+ + +
13E9-3D5	+	+	+ +	+ +
13E9-3D7	+	+	+ + +	+ + +
13E9-3E7	+	+	+ + +	+ + +
13E9-3E9	+	+	+ + +	+ + +
13E9-3G5	+	+	+ +	+ + +

Table 4-1 the immunostaining intensity of human chondrocytes probe with anti-perlecan antibodies.

The intensity of immunofluorence increases when the number of "+" increases. "-" indicates negative staining. These antibodies underlined demonstrate a change in immunoreactivity when the cells were incubated with heparanase.

5 Overall conclusion and future works

In this project, 3 mg of recombinant perlecan domain I (DI and DI-EGFP) were successfully isolated from 9 litres of conditioned media. In ELISA, the purified protein did not react with domain III antibody. The results of SDS-PAGE indicated the molecular mass of the purified material matched the previously reported data of perlecan domain I. The purification strategy can be optimized further by using an ICA targeting full length perlecan (but not domain I) instead of Q Sepharose column.

Hybridoma cell lines secreting anti-perlecan antibodies were successfully established. 10 sub clones ware adapted into serum free medium. In ELISA and Western Blotting, the cloned antibodies can recognize the full length perlecan extracted from HCAEC. To save purified domain I material, full length perlecan, ECM or cell can be used directly as immunogen for the immunization. To optimize the screening process is one of the major challenges that need to be done if other fusions are required in the future. The selection strategy should focus more on the desired moiety instead of a broad range of epitopes throughout the entire molecule.

The perlecan deposited in the ECM by human chondrocytes was successfully probed by the newly generated MAb. Antibody 5D7-2E4, 13E9-3B3 and 13E9-3G5 were noted for their sensitivity to heparanase treatment.When the cells were incubated with heparanase, immunoreactivity of antibody 5D7-2E4 and 13E9-3G5 showed an increase while a decreased immunoreactivity was observed for antibody 13E9-3B3. The antibodies will be further characterized and used in Immunocytochemistry of other cells or tissues to determine perlecan turnover over the time. They can also be used in Western Blotting of extracts from different cells and tissues at different stage of development to see the changes of the proportion of perlecan at each time point. Some antibodies such as 13E9-3B3 and 5D7-2E4 will be used to stain paraformaldehyde fixed tissues for their relativity stronger immunoreactivity than other antibodies with paraformaldehyde fixed cells.

To reselect the stocked cultures that have not been cloned is also worth doing in the future. The uncloned cultures are large pools of many antibodies, where there are potentially antibodies against perlecan domain I, HS chains and even HS stub produced by heparanase digestion.

6 References

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