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Development of a targeting mechanism for regeneration of the intervertebral disc

A thesis submitted to the National University of Ireland, Galway for the degree of Doctor of Philosophy by:

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November 2014

Research Supervisors: Dr. J. Gerard Wall (Microbiology)
                Prof. Abhay Pandit (Network of Excellence for Functional Biomaterials)

Head of Discipline: Prof. James P’ O’Gara
Whether or not your efforts are smiled upon by fate, what really matters in the end is to be able to say: “I did what I was able”.

Louis Pasteur (1822–1895)
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Declaration regarding the work in this thesis

The work contained in this thesis is all my work. I have not obtained a degree in this University, or elsewhere based on this work.

_________________________________
Claire Cunningham
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Degeneration of the intervertebral discs (IVD) is a leading cause of neck and low back pain. While its aetiology is poorly understood, it is known to begin in the central nucleus pulposus region, leading to degradation of the extracellular matrix and loss of the osmotic, and ultimately mechanical, properties of the IVD. Many current treatments for lower back pain alleviate the pain temporarily but do not restore disc function. Biological regeneration approaches under investigation include administration of matrix-mimicking scaffolds, cells and/or therapeutic factors. Cell-targeting strategies are likely to improve the delivery of therapeutic moieties due to the low cell numbers in the IVD. Single-chain antibody fragments (scFvs) that bind IVD cells were isolated for potential delivery of therapeutics to restore disc function. Neural cell adhesion molecule 1 (NCAM1) was identified as a potential cell surface marker of IVD cells. The most cell-distal domain of NCAM1 was cloned and expressed in the Escherichia coli periplasm. Phage display technology was used to isolate a human scFv against the recombinant domain by panning a scFv library on the immobilised protein. Binding of the isolated B5 scFv was confirmed by ELISA and the scFv bound cultured rat astrocytes, as well as bovine nucleus pulposus and annulus fibrosus cells in immunocytochemical studies. The scFv also successfully labelled cells in bovine spinal cord and six-month and two-year old bovine IVD sections by immunohistochemistry. Taken together, these results illustrate the NCAM1-binding potential of B5 scFv and support a role for the scFv in targeting therapeutics to IVD cells for regeneration of the intervertebral disc. It is anticipated that the described scFv will be employed for targeted gene delivery to IVD cells using scFv-functionalised nanoparticles. Antibody fragments can provide cell-binding moieties at improved cost, time, yield and functionalisation potential over whole antibodies and the work demonstrates the feasibility of the combined protein expression/phage display approach in the isolation of cell-binding antibody fragments for use in a wide variety of targeting and delivery applications.
Chapter 1: Introduction

1.1 Introduction to the intervertebral disc (IVD)

Low back pain has a huge socioeconomic impact on today’s aging population. In industrialised nations, the lifetime incidence rate is in excess of 70% (Burton et al., 2006). The aetiology of lower back pain is unknown, however degeneration of the intervertebral disc (IVD) has been identified as a leading causative factor based on imaging studies (Iatridis et al., 2013). The human spine consists of 33 vertebral bodies which, with the exception of C1, C2 and the sacrum, are separated by an intervertebral disc (IVD; Silva-Correia et al., 2013). The intervertebral disc is composed of the nucleus pulposus, located in the centre of the disc, surrounded by the lamellae of the annulus fibrosis which are positioned between two cartilage endplates (CEP) that are integrated to the neighbouring vertebral bodies – Fig.1.1 (A).

The annulus fibrosus and nucleus pulposus are responsible for withstanding tensile and compressive stress. They resist spinal compression while allowing limited movements and dissipate loads evenly on the vertebral bodies when the spine is extended or flexed. Their role is mainly mechanical as they continuously transmit loads arising from body weight and muscle activity through the spinal column (Iatridis et al., 2013). They maintain flexibility,
support loads and dissipate energy in the spine. The cartilage endplate plays a vital role in maintaining the viability of nucleus pulposus cells and prevents bulging of the nucleus pulposus into the adjacent vertebral body.

1.2 Structure of the IVD

The lamellae of the annulus fibrosis consist mainly of collagen type I fibres that pass obliquely between vertebral bodies. The orientation of the fibres is reversed in successive lamellae. Collagen and proteoglycan in the AF comprise 60% and 25% of dry weight, respectively (Kepler et al., 2011). The nucleus pulposus is composed of a proteoglycan and water gel which is held together by a network of fine collagen type II and elastin fibres. Aggrecan is the most abundant protein in the disc and accounts for approximately 70% of the dry weight of the NP (Choi, 2009). The osmotic properties required to resist compression are attributable to the high anionic glycosaminoglycan content (chondroitin sulphate and keratin sulphate) of aggrecan. With increasing age, the water content of the disc decreases, especially in the nucleus pulposus, resulting in dehydration and inferior load-bearing ability (Colombier et al., 2014) - Fig.1.1(B).

The nucleus pulposus contains proteoglycan aggregates surrounded by a collagen fibre network. The biomechanical properties of a normal, healthy disc are dependent on its hydration. The negative molecular charge of the sulphated glycosaminoglycan chains of aggrecan draws and retains water molecules. The tissue swells until an equilibrium is reached where the swelling potential is balanced by the tensile forces in the collagen network (Whatley and Wen, 2012). The hydrated disc maintains a swelling pressure which is responsible for its resistance to compressive loads. When a compressive load is applied to the spine, some water is forced out of the disc, thereby increasing the aggrecan concentration and its swelling potential and resisting further compression. When the compressive load is removed, water is brought back into the tissue to restore the original equilibrium conditions and consequently, disc height is restored (Whatley and Wen, 2012).
The cells of the annulus fibrosis are elongated and fibroblast-like, running parallel to the collagen fibers. The initial population of cells in the nucleus pulposus are notochordal but are replaced when the human reaches 12 years of age by rounded cells, which are similar to chondrocytes of articular cartilage. During human growth, the cell density in the disc diminishes. Cell density is low in adults, especially in the nucleus (Iatridis et al., 2013). IVDs are typically avascular; in adult discs, blood vessels are confined to the outermost layers of the annulus. Metabolite transport is by diffusion and bulk fluid flow for small and large molecules, respectively. Nucleus cells become quiescent when there is a chronic lack of oxygen and die in the absence of glucose; therefore, the ability of disc cells to recover from metabolic or mechanical injury is restricted (Grunhagen et al., 2011). In the adult IVD, the cells located in the centre of the nucleus pulposus may be 7-8 mm from the nearest blood supply and therefore, may obtain limited or no diffusion of nutrients which affects cell viability and reduces the cell number in the disc (Grunhagen et al., 2011).

The process of disc degeneration is an aberrant, cell-mediated response to progressive, structural failure. A degenerate disc is one with structural failure combined with accelerated or advanced signs of aging (Adams and Roughley, 2006). Degeneration of the intervertebral disc is characterised by increased degradation of the extracellular matrix which accompanies an ingrowth of nerves and blood vessels into the normally aneural and avascular tissue (Silva-Correia et al., 2013). It has been proposed that degeneration starts in the NP and is strongly linked to the progressive loss of proteoglycans. The normal healthy IVD contains nerves only in the superficial outer layer of the annulus fibrosis, whereas the nucleus pulposus is devoid of nerves as aggrecan is reported to have an inhibitory effect on neurite outgrowth (Fields et al., 2014). During disc degeneration, the NP becomes dehydrated and fibrotic as degeneration progresses. Analysis of degenerated intervertebral discs from surgery revealed increased cellular apoptosis and surviving cells with a reduced capacity for proteoglycan synthesis, resulting in a decrease in proteoglycan content (Shankar et al., 2009). Degenerated discs have lower water content than normal age-matched discs and under load, they lose fluid and height rapidly and the discs tend to bulge (Shankar et al., 2009). Two characteristics of degeneration in the
NP include a reduction in both proteoglycan content and production of type II collagen. Simultaneously, type II collagen fibers are denatured and type I collagen production begins. The gelatinous, nucleus pulposus becomes fibrotic and dehydrated as degeneration progresses and ultimately, loses its hydrostatic properties, resulting in a disc that is unable to transmit intervertebral loads – Fig.1.1(B; Silva-Correia et al., 2013). The clinical symptoms of disc collapse include decreased disc height and decreased signal intensity on T2-weighted magnetic resonance imaging. The biomechanical symptoms include annulus fibrosis-like cracking and fissuring (Whatley and Wen, 2012).

There are three types of annulus fibrosis tears that can be identified: circumferential tears or ‘delaminations’ (caused by interlaminar shear stresses in older discs), peripheral rim tears (related to trauma and occur in the anterior annulus) and radial fissures that progress outward from the annulus. Radial fissures are associated with nucleus degeneration. They are increasingly common after the age of 10 years, especially in the lower lumbar spine (Adams and Roughley, 2006). A disc is described as ‘herniated’ or ‘prolapsed’ when radial fissures allow the nucleus pulposus to migrate relative to the annulus fibrosis, so the disc periphery is affected. The extent of nucleus migration determines if the disc herniation results in protrusion or extrusion of the nucleus material. When the end-plate is damaged, it decompresses the adjacent nucleus pulposus and transfers load onto the annulus fibrosis, resulting in a bulge in the nucleus cavity.

1.3 Current treatments for IVD degeneration

Back pain is not life-threatening but it results in high medical expenses for the patient and enormous loss in productivity in the workplace. Degeneration of the intervertebral disc is described using the Thompson grading system which grades the level of degeneration, from Thompson grade I (a healthy disc) to discs with advanced degeneration (Thompson Grade V; Thompson et al., 1990). Current treatments for patients of lower back pain are mainly symptomatic and alleviate the pain temporarily but do not restore disc function or prevent further degeneration (Bowles et al., 2011). Such treatments include physiotherapy, pain and anti-inflammatory medications and surgical intervention. Surgical intervention may include excision of the damaged tissue (discectomy), disc
arthroplasty (insertion of a mechanical device such as a cage or prosthesis to restore the intervertebral space after removal of damaged tissue) and fusion of adjoining vertebral bodies (spinal fusion). The types of surgical intervention available may offer temporary relief but do not provide a long-term solution. A discectomy may relieve pain but does not restore disc height or the original load bearing capacity. Spinal fusion, a routine surgical procedure as it provides symptomatic relief, is not an optimal treatment as it can result in limited flexibility (Putzier et al., 2005) and may lead to degenerative changes in adjacent vertebrae. Spine mechanics may also be affected by these procedures (Whatley and Wen, 2012; Iatridis et al., 2013).

In an attempt to preserve motion and reduce the incidences of degeneration in adjacent vertebrae, other approaches have been developed such as partial or total disc replacement (So et al., 2007; Zigler et al., 2007). Their effectiveness is questionable and ill-effects associated with their use include failure modes commonly associated with traditional metal/polyethylene arthroplasty, such as mechanical failure, dislodgement, polyethylene wear, and associated osteolysis and implant loosening (Bowles et al., 2011). There are two FDA approved artificial spinal discs in the US, the SB Charité® and ProDisc® with another undergoing clinical trials, the Maverick® (www.depuy.com; www.synthes.com). These implants allow spinal motion to be preserved and disc space height to be maintained but they cannot withstand compressive force due to their lack of elasticity. Other problems associated with current prosthetic devices include extrusion, infection, loosening and cytotoxicity (Whatley and Wen, 2012). Current artificial discs do not promote tissue remodelling; an alternative approach to spinal fusion and artificial disc replacement is required that will promote regeneration of natural IVD tissue. Therefore, a strategy using a biological approach that could regenerate the nucleus pulposus in the early stages of degeneration that would either halt and prevent further degeneration or reverse the current degeneration could offer a viable treatment for restoring disc function.
1.4 Potential regenerative strategies for IVD degeneration

The degree of degeneration would ultimately determine the strategy required to halt or reverse disc degeneration and alleviate the symptoms. Late stage degeneration might require complete replacement of the disc whereas repair of the disc itself might be possible during early degeneration. Regenerative approaches which could potentially offer treatment for IVD degeneration include the delivery of protein factors or proteinase inhibitors in the early stages of disc degeneration (grades II-III, Thompson scale); delivery of cells or gene therapy in the intermediate stages of disc degeneration (grade IV) and tissue engineering approaches in the advanced stage (grade V; Kepler et al., 2011; Whatley and Wen, 2012).

The normal disc has a low cell density which adds to the effects of increased cell senescence and decreased extracellular matrix production. Approaches that aim to improve cell viability and increase extracellular matrix production by targeting the disc population include in vitro expansion and re-implantation of disc tissue, mesenchymal stem cell implantation and delivery of platelet-rich plasma (Kepler et al., 2011). The avascular nature of the disc poses a threat to the success of these potential therapies due to the limited nutrient supply and clearance of waste products, thereby creating an acidic environment due to the build-up of lactic acid from anaerobic digestion. Rodent and canine models of disc degeneration have demonstrated the importance of cell re-implantation along with native disc extracellular matrix; Gruber and colleagues found that autologous cells were viable for ≤ 8 months post-implantation in the sand rat. The cells had integrated into the disc and were surrounded by normal matrix at time points up to 8 months post-engraftment (Gruber et al., 2002). Ganey and colleagues showed that transplanted disc chondrocytes survived for longer than 1 year with continued production of extracellular matrix that had a composition similar to normal intervertebral disc tissue in a canine model (Ganey et al., 2003). Hydrogels may provide a means to support cells in the disc post-implantation until they produce extracellular matrix (Calderon et al., 2010; Collin et al., 2011). This work has been translated to the clinic whereby a patient's cells are transplanted back into the disc region 12 weeks after a discectomy, in an autologous disc chondrocyte transplantation (ADCT). A large
scale clinical trial called EuroDisc assessed the effectiveness of ADCT as a treatment strategy for disc degeneration. Two years after ADCT, patients who had undergone ADCT showed a decrease in pain, with a significant increase in proteoglycan and fluid content as compared to patients who underwent discectomy alone (Meisel et al., 2007).

Intradiscal injection of mesenchymal stem cells (MSCs) is another potential treatment for IVD degeneration as MSCs can differentiate on a chondrogenic pathway and may have the ability to express IVD cell phenotypes. Risbud and colleagues demonstrated differentiation of rat MSCs to a nucleus pulposus phenotype in an alginate hydrogel after exposure to TGF-β in a hypoxic environment similar to the disc (Risbud et al., 2004). Steck and colleagues differentiated MSCs towards an IVD-like cell using TGF-β3, ascorbate and dexamethasone, and described the cells as IVD-like as their gene expression profile resembled native IVD tissue more closely than joint cartilage (Steck et al., 2005). Sakai and colleagues reported that after 24 weeks, degenerated discs of rabbit models regained a disc height value of ~91 % compared to the sham-operated group (disc degeneration induced, no MSC implantation) where disc height value was 67 %. The disc structure in the control group was indistinct compared to the characteristic central nucleus pulposus surrounded by annulus fibrosis in the MSC-treated group (Sakai et al., 2006). Feng and colleagues also made similar observations regarding disc structure after MSC-transplantation in a rabbit model (Feng et al., 2011). These studies indicate that MSCs may represent a valuable resource for cell therapy of the disc.

Gene therapy is another potential treatment strategy for IVD degeneration. The advantage of gene therapy over protein-based therapy is that the encoded protein is produced continuously and will therefore exceed the half-life(s) of injected proteins which will eventually become degraded. Adenovirus is the most common virus used for gene therapy studies but it is also the most immunogenic. Nishida and colleagues observed a five-fold increase in human transforming growth factor-β1 (TGF-β1) expression and a 100 % increase in proteoglycan synthesis in treated IVD compared to control discs in a rabbit model one week after transfection using adenovirus-mediated transfer of human TGF-β1 (Nishida et al., 1999). Moon and colleagues transfected human IVD cells using
recombinant adenovirus constructs bearing the TGF-β1, IGF-1 and BMP-2 genes; after 18 hours, there were 2.9-, 1.8- and 1.9-fold increases in proteoglycan synthesis, respectively. Human IVD cultures with double gene transfections had increases between 3.2- and 3.9-fold while transfection with the triple gene cocktail demonstrated a 4.7-fold increase in proteoglycan synthesis compared to the control (Moon et al., 2008). Zhang and colleagues reported that bovine NP cells cultured in monolayer and transduced with adenovirus expressing BMP-2 and -7 and sox9 accumulated the most proteoglycans (Zhang et al., 2006). Liu and colleagues showed an increase in proteoglycan and collagen II production after adeno-associated virus transfection (aav2) of NP cells in vitro with CTGF (connective tissue growth factor) and TIMP-1 (tissue inhibitor of metalloprotease-1) genes in rhesus monkey and rabbit models (Liu et al., 2010). Combined transduction of both CTGF and TIMP1 significantly promoted the expression of proteoglycan and collagen type II to levels greater than transduction of a single gene alone. Disc degeneration in the rabbit disc was reversed by re-implantation of the modified NP cells in another study (Liu et al., 2010; Liu et al., 2011). Transplantation of CTGF and TIMP1-transfected cells helped to maintain disc height and promoted synthesis of type II collagen and proteoglycan. The results of these studies demonstrate the potential of gene therapy as a treatment strategy for disc degeneration but issues surrounding choice of virus and vector safety must be first addressed for patient safety.

A tissue-engineering approach may offer a solution to the injured IVD where the emphasis shifts from disc excision to restoration of disc function using biological repair. There is currently no procedure established to achieve this outcome in the clinic. Recently, emphasis has shifted to the development of a composite tissue-engineered total disc replacement (TE-TDR) implant that contains both AF and NP tissue (Halloran et al., 2008; Bowles et al., 2010). This has the potential to be a crucial tool for clinical spine care due to the premise of restoration of disc function and spinal motion. Bonhassar and colleagues developed an implant of alginate and collagen seeded with NPs and AFs, respectively, that integrated with the rat caudal spine, generated functional tissue (collagen and proteoglycan-rich) in the disc space and produced a mechanically functional motion segment in the rat caudal spine (Bowles et al., 2011). This
study illustrates that a tissue engineered IVD implant can restore native IVD in the spine in a rat model.

Growth factors such as the bone morphogenetic proteins (BMPs) may also have potential in the treatment of IVD degeneration (Zhang et al., 2011). Proteoglycan accumulation was increased in NP cells in response to BMP-2 and BMP-7 whereas collagen accumulation increased in AF cells in response to BMP-2, BMP-13 and Sox9 (Zhang et al., 2006; Zhang et al., 2007). BMP-7 inhibits the inflammatory cytokine IL-1 and prevents matrix degradation while increasing its synthesis (Takegami et al., 2002). Based on promising results of in vitro and in vivo animal studies, the FDA approved Investigative New Drug clinical trials of recombinant human BMP-7 (also known as Osteogenic Protein 1, OP-1) and recombinant human BMP-14 (also known as GDF-5) in the USA (An et al., 2005; Chujo et al., 2006). Results from these studies are not yet available as it will take many years to evaluate the therapeutic benefits/efficacy of intradiscal injection of these proteins. Platelet-rich plasma (PRP) has also been investigated to treat disc degeneration as it is a rich source of growth factors. Obata and colleagues administered autologous platelet rich plasma releasate (obtained after clot formation) into a rabbit anular puncture model and observed an increase in the number of chondrocyte-like cells in the nucleus pulposus and the anterior annulus fibrosis (Obata et al., 2012). Work by Sawamura and colleagues using biodegradable gelatin hydrogel microspheres carrying PRP found that it suppressed disc degeneration in rabbit models compared to control groups (Sawamura et al., 2009).

1.5 Antibodies; monoclonal and polyclonal

A monoclonal antibody is produced from an immortalised B-cell clone. They are antigen-specific and homogeneous unlike polyclonal antibody mixtures. They therefore occupy an important role in the development of research, diagnostics and clinical therapies. The development of hybridoma technology in 1975 by Köhler and Milstein, for which they were awarded a Nobel Prize, allowed for the production of large quantities of murine monoclonal of predefined specificity (Köhler and Milstein, 1975). This was achieved by
immunising mice with a foreign antigen, removing its spleen to extract B-cells, followed by fusion with myeloma cells. This resulted in the formation of a hybridoma which produced large quantities of a single antibody molecule in an 'immortalised' B-cell line. The antibodies produced are directed at a specific epitope of the immunising antigen and are typically highly specific.

Polyclonal antibodies are obtained from the sera of immunised animals and consist of a heterogeneous mixture of antibodies directed against different epitopes on the antigen. The immunisation process can take several weeks but the serum obtained should be enriched with antibodies that bind the antigen used for immunisation. However, the serum will also contain antibodies against other, unrelated antigens to which the animal has been exposed. These antibodies can be removed by affinity purification as they potentially could interfere with downstream applications. The ability of polyclonal antibodies to recognise multiple epitopes means that they may cross-react with other proteins but they may also possess the ability to recognise proteins in their native and denatured states. Polyclonal antibodies often vary from batch to batch, causing problems with reproducibility in binding studies, whereas monoclonal antibodies are reproducible, highly specific due to single epitope binding and therefore, typically have low cross-reactivity with other structures (Sidhu, 2005).

1.6 Antibody structure

Antibody molecules belong to a family of globular proteins known as immunoglobulins. The classic antibody immunoglobulin structure is Y-shaped, as shown by the IgG class of antibodies. IgG molecules (approximately 150 kD) form homodimers consisting of two light chains and two heavy chains; each light chain pairs with a heavy chain and each heavy chain pairs with another heavy chain. The chains are linked by covalent interchain disulfide bonds and non-covalent interactions (Elgert, 1996). The two light chains (23 kD) and two heavy chains (50 kD) are identical. Each chain is divided into variable (V) and constant (C) regions – Fig.1.2. The amino-terminal end contains the V region while the carboxyl-terminal end contains the C region. There are areas of increased variability called hypervariable regions or complementarity-determining regions (CDRs) in the variable regions, three in each variable region. Intervening
sequences between the CDRs are more conserved and are termed the framework regions; these help to preserve the three-dimensional structure of the domains and the antigen-binding pocket. The variable region folds so that the CDRs are exposed on the solvent-exposed surface of the chain. When the light and heavy chains are joined, the CDRs of the chains form a pocket/cleft that serves as the antigen-binding site of an immunoglobulin (Elgert, 1996). The CDRs define the specificity of the antibody as the amino acid sequences of the CDRs determine the shape and ionic properties of the antigen binding site (Sela-Culang et al., 2013). The light chains are divided into two globular domains consisting of 214 amino acid residues. The light chain constant domain (C\textsubscript{L}) is predominantly conserved and consists of approximately 110 amino acids. The light chain variable domain (V\textsubscript{L}) has more amino acid sequence variation and contains approximately 108 amino acid residues. There are two types of light chains found in mammals, the kappa chain and the lambda chain (Weisser and Hall, 2009).

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Fig.1.2 The classic IgG molecule consists of four polypeptide chains; two identical heavy (red/purple) and light (yellow) chains. The Fab (fragment antigen binding; boxed in green) arms of the molecule carry out antigen binding. The Fv (variable fragment) consists of the variable regions of the heavy and light chains (circled in pink). Single-chain Fv fragments (scFvs) are constructed by joining the V\textsubscript{H} to the V\textsubscript{L} via a peptide linker. Figure is taken from http://homepage.usask.ca/~kmj127/antibody.jpg.

The IgG heavy chain contains four globular domains; an N-terminal variable domain (V\textsubscript{H}) and three constant domains (C\textsubscript{H1}, C\textsubscript{H2} and C\textsubscript{H3}) – Fig.1.2. Each domain has a characteristic tertiary structure consisting of two β-pleated
sheet structures, held together by an intra-molecular disulphide bond. The pleats are packed together to form a β-barrel-shaped structure (Elgert, 1996).

1.7 Antibody formats

Antibodies play a vital role in our defence against pathogenic organisms and toxins. The classic antibody structure is represented by the IgG class which is the main antibody found in serum (Fig.1.2). Enzymatic digestion of the whole antibody molecule produces smaller antibody fragments; the Fab and F(ab’)2 fragments are produced after digestion with papain or pepsin, respectively (Porter, 1959). Recombinant DNA technology has allowed engineering of antibody genes to produce various smaller formats of recombinant antibody fragments (Weisser and Hall, 2009). The single-chain variable fragment (scFv) of the antibody remains the most popular format where the V\textsubscript{H} and V\textsubscript{L} domains are joined with a flexible polypeptide linker to prevent dissociation of the domains. scFvs typically retain the target specificity and a similar affinity to the original antibody molecule (Bird et al., 1988). Examples of monovalent antibody fragments include the scFv, the Fv and the dsFv. The Fv fragment consists of non-covalently associated V\textsubscript{H} and V\textsubscript{L} regions and may be unstable at low concentrations due to the absence of inter-chain covalent bonds (Glockshuber et al., 1990). dsFvs consist of V\textsubscript{H} and V\textsubscript{L} chains connected by a disulphide bond that is engineered into the framework region. The limitations of scFvs include a short half-life in vivo due to their size and monovalency. However, they remain popular for therapeutic and diagnostic applications because of their low production costs and the genetic engineering approaches available to improve their stability, size and increase their valency (Girgis et al., 2011; Cyranka-Czaja et al., 2012; Srivastava et al., 2014b). Examples of multivalent antibody fragment formats include diabodies, triabodies and tetrabodies – Fig.1.3. The length of the inter-domain linker determines multimerisation of fragments; with linkers of 3-12 amino acids, scFvs associate to form dimers while trimers and tetrabodies are formed if the linker is 0-3 amino acids (Srivastava et al., 2014b).
Bi-specific antibodies and multi-specific antibodies are formed when two or more scFvs with different binding specificities are joined to form one molecule. They increase avidity and specificity by binding two epitopes on a single antigen (Holliger and Hudson, 2005). Examples include tandem scFv fragments (taFvs or Bis-scFv) and single chain diabodies (scDbs). Tandem scFv fragments consist of two scFv molecules joined by a linker (M) of varying length, usually 15-20 amino acids (scFvA-M-scFvB). The scDbs molecule is similar to taFvs, except for the length of the linker joining the V<sub>H</sub> and V<sub>L</sub> domains which is much smaller – 5 amino acids compared to 15-20 amino acids, hence the structure of this molecule is more compact than the taFv. The minibody is another multivalent scFv format which consists of bi- or multi-valent antibody fragments that have been modified by covalent linkage to other types of protein that tend to self-associate (Weisser and Hall, 2009). Bi-specific diabodies, tri-specific tribodies and tetra-specific tetrabodies can be formed by combining scFvs with different linker lengths and specificities to form these multi-specific fragments (Todorovska et al., 2001). Conversion to these multivalent formats results in increased functional affinity (termed avidity) and a slower dissociation rate for cell surface antigens (Ortega et al., 2013), though at the expense very often of reduced folding or assembly efficiency.
1.8 Filamentous phages

Filamentous phages constitute a large family of bacterial viruses that infect many Gram-negative bacteria. Their circular, ssDNA is encased in a long, flexible tube composed of thousands of copies of a single, major coat protein and a few minor coat proteins at the tip. Their genome is small, containing 12 or fewer genes and an intergenic region (IG) that contains sequences required for DNA replication and encapsidation. All filamentous phages characterised to date use pili, which are long and slender cell surface appendages, as receptors (Clackson and Lowman, 2004).

The mature pIII protein on the phage tip consists of three distinct domains, D1, D2 and D3, which are linked by the glycine rich tetra- and penta-peptide repeats L1 and L2, and a C-terminal membrane-anchoring hydrophobic segment. The interactions between D1, D2 and D3 contribute to a compact, stable arrangement of a ring of five pIII subunits at the tip of the particle (Chatellier et al., 1999).

Many types of phage have been used in phage display, including – filamentous phage, lambda phage and T7 phage (Levy et al., 2007; Tornetta et al., 2010; Van Dorst et al., 2011). Filamentous phages have a fixed diameter of about 6.5 nm and their length is determined by the size of the genome. They are produced and secreted from infected bacteria without cell killing or lysis, termed non-lytic phages. Lambda and T7 phage are lytic phage: the assembly of the phage particles occurs in the bacterial cytoplasm and they are released by lysis of the bacterial cells. Their genome is small (~ 6400 nucleotide ssDNA) and foreign DNA can be inserted into the non-essential region which creates longer phage, although susceptibility to breakage from vortexing increases with increasing phage length.

Phages infect strains of E. coli that have the conjugative F episome which encodes a thread-like appendage termed the F pilus which mediates the infection process between the phage g3p protein and the E. coli cell. This allows the phage ssDNA to enter the cell where it is converted to the double-stranded plasmid-like replicative form (RF) which undergoes rolling circle replication to make ssDNA and serves as a template for expression of coat proteins. The phage progeny are
assembled by packaging of ssDNA into protein coats and forced out through the bacterial membrane into the extracellular environment (Azzazy and Highsmith, 2002).

1.9 Phage display

Phage display technology was first described by George P. Smith in 1985 (Smith, 1985). Display of antibody fragments on the surface of bacteriophages was first described by McCafferty and co-workers in 1990 which allows for the production of recombinant antibodies with high specificity and affinity for a specific antigen (McCafferty et al., 1990). It is an in vitro selection technique in which polypeptides with desired properties are isolated from a large collection of polypeptides. Fusion of the gene of interest to a minor phage coat protein (pIII, encoded by gIII) results in phage particles that display the encoded protein and also, contain its gene, thus providing a direct link between the genotype and phenotype (Scott and Smith, 1990). Large repertoires of antibodies can be constructed from an immune or naïve host and displayed on the surface of filamentous phages in this manner, followed by isolation of recombinant antibodies with high specificity and affinity for a specific antigen by binding to an immobilised ligand and re-infection of E. coli cells in a cyclical process – Fig.1.4.

Antibody libraries are initially generated by injection of an antigen into a relevant host to elicit an immune response. The spleen is removed and the mRNA of the rearranged immunoglobulin genes is extracted from B-cells and used for cDNA synthesis. The heavy and light chain gene segments are amplified by polymerase chain reaction (PCR), combined in random V_H-V_L pairs, and cloned into a plasmid vector, e.g. phagemid, resulting in a huge phagemid library. In many studies, as in the present work, pre-prepared libraries, frequently from naïve rather than immunised donors, are used rather than generation of a library de novo. Infective phage particles are formed when these plasmids are co-transfected with helper phage into E. coli. Expression of an antibody fragment on the surface of the phage particle is achieved by in-frame fusion of its encoding gene to one of the phage coat protein genes. The scFv displayed phage libraries are then incubated with specific antigen in order to bind the scFv/phage of
interest in a process called biopanning. Unbound scFv/phages are removed by washing and the bound scFv/phage with specificities of interest are eluted. The eluted scFv/phage are then used to infect *E. coli* cultures for further propagation. The stringency of the selection process can be modified during panning for enrichment of the polyclonal phage population and the clones of interest are identified by ELISA analysis. The display of scFvs using phagemids (plasmids with a phage origin of replication and a bacterial origin of replication) is the most commonly used approach.

1.10 Phagemids

Phagemids share many elements with plasmids including multiple cloning sites, promoter, recombinant tags for purification and immunodetection of the recombinant protein, signal sequences, selection markers and a plasmid origin of replication. Phagemids are filamentous phage-derived vectors containing replication origins of a plasmid and a phage. They encode one kind of coat protein. The helper phage provide the structural and functional proteins required by the phagemid to generate a complete phage particle (Qi *et al.*, 2012). Phagemids can be converted to filamentous phage particles by co-infection with
helper phage such as R408, M13KO7, VCSM13 and KM13. Phagemids are preferred to phage for phage display for the following reasons. Firstly, genomes of phagemids are smaller and can accommodate a larger foreign DNA fragment. Secondly, the phagemids are more efficient in transformation which allows a phage display library with high diversity to be obtained. Thirdly, there are restriction enzyme recognition sites available in the genome of phagemids which allows for DNA recombination and gene manipulation. Fourthly, the expression level of fusion proteins can be controlled easily by a recombinant promoter. Finally, phagemids are usually more genetically stable than recombinant phages under multiple propagations (Qi et al., 2012).

Phagemid vectors contain only the fusion gene (coat gene fused to scFv) and no other phage genes, unlike phage vectors – Fig.1.5. Phage vectors with natural phage promoters generally have all copies of the coat protein displaying the heterologous protein, termed polyvalent display. Phagemid vectors which drive the display of protein on pIII under the control of an uninduced promoter will lead to monovalent display. Monovalent display permits selection based on pure affinity and allows high-binding clones to be identified whereas polyvalent display may prevent discrimination between high-binding and weak-binding clones (Clackson and Lowman, 2004). The type of coat proteins used to display foreign proteins are type III and type VIII. Type III phagemids are the most commonly used vectors in phage display (Qi et al., 2012). An amber stop codon is typically inserted between the foreign gene and its fused coat protein gene, thus allowing expression of the foreign protein as a fusion protein on the surface of the phage in suppressor E. coli strains, such as TG1, whereas in non-suppressor E. coli strains such as HB2151, the TAG amber codon is recognised as a stop codon and the scFv is produced in a non-fused, soluble form.
Fig. 1.5 In phagemids, the genes encoding wild type coat protein (gIII) and foreign fusion (gIII/scFv fusion) are carried by helper phage and phagemid, respectively. After infection with helper phage, the bacteria contain phagemid and helper phage DNA. pIII minor coat protein and pIII/scFv fusion protein are expressed on the tip of the phage (Willats, 2002).

There are two origins of replication present in the phagemid, a plasmid origin of replication that allows replication to a high copy number in an *E. coli* host and a filamentous phage replication of origin to allow production of single-stranded DNA vector and subsequent encapsidation into virus particles (virions) (Sidhu, 2005). *E. coli* cells carrying the phagemid must be infected with helper phage for production of such particles. Helper phage is a filamentous phage with a defective origin of replication that prevents efficient packaging. The infected cells then express all the phage wild-type proteins from the helper phage genome and a small amount of the fusion protein encoded by the phagemid – Fig. 1.5. Because the helper phage genome is poorly packaged, the majority of the phage particles contain the phagemid genome, thereby preserving the linkage between the displayed protein and its gene. The resulting phage particles may incorporate wild-type coat proteins from the helper phage or fusion coat protein from the phagemid. These phagemids that are secreted as virions are described as being ‘rescued’ by the helper phage (Clackson and Lowman, 2004; Sidhu, 2005).
1.11 pMod1

The pMod1 vector used in this work was modified from pHage 3.2 (Pansri et al., 2009). The vector (4541 bp) contains an ampicillin resistance gene for selection and maintenance of the phagemid – Fig.1.6. Transcription of genes is under control of the lac promoter. The phagemid contains two origins of replication: a FI origin and a ColEI origin for replication in phage and E. coli, respectively. Secretion of the scFv fragment is directed by Gene III leader peptide. The scFv gene contains hexa-histidine and myc tags to facilitate affinity purification and immunodetection. An amber stop codon between the recombinant tags and the gene III sequence allows production of the scFv in soluble, non-fused form in a non-suppressor strain such as E. coli HB2151 and as a fused scFv in a suppressor strain such as E. coli TG1 (Pansri et al., 2009).

Fig.1.6 Map of pMOD1 phagemid used in phage display in this work (Pansri et al., 2009)

1.12 Selection

Panning of a scFv library against an antigen of interest involves exposing the library to an immobilised target antigen to allow antigen-specific phage scFvs to bind to their target, removal of unbound phage by washing and eluted of the bound phage either by low or high pH treatment, proteolytic cleavage of scFvs from their host phage particles (using trypsin), or a combination of both (Clackson and Lowman, 2004; Pansri et al., 2009) – (Fig.1.4). Extremes of pH, denaturants, ionic strength, limited proteolysis or sonication can also be used for
non-specific elution of the bound phage (Pande et al., 2010) and multiple rounds of panning are normally required to remove non-target-specific phage from the eluted population.

The most widely used phage library screening approach is to immobilise the target on a solid phase support and expose the phage to the immobilised target. Immobilisation of most targets can be achieved by passive adsorption onto plastic tubes or plates, or biosensor chips. Targets may also be immobilised using affinity tags such as strepavidin capture of biotinylated targets, his-tagged targets immobilised to nickel-adsorbed plates or using antibodies to coat the support and capture the target if its conformation is altered by adsorption onto the plastic (Clackson and Lowman, 2004; Takakusagi et al., 2007; Cyranka-Czaja et al., 2012). Selection may also be performed using antigens in solution which allows lower concentrations of the antigen to be used and may promote selection of high-affinity phage binders. This method of selection can reduce avidity effects associated with solid surfaces while direct selection of antibodies against markers on cell surfaces may also be carried out on either monolayers of adherent cells or on cells in suspension.

The level of stringency used in selection is important, especially in the first panning round when library diversity is at its highest. Therefore, in panning round 1, efforts to capture all potential binders are made by relaxing the stringency level so that the highest number of the ‘fittest’ phage-binders are recovered (Smith and Petrenko, 1997). As the phage population becomes enriched for the antigen in later rounds, the stringency is increased to identify antigen-specific clones of high affinity that are able to withstand harsh washing conditions (Sidhu, 2005).

Washing stringency can be increased by increasing the number of washes, washing times or the concentrations of non-ionic detergent to remove poor- or weakly-bound phage. The washing stringency can influence the properties of the isolated phage binders. If washing is too stringent, low affinity but highly specific binders may be lost. However, if the washing step is not sufficiently stringent, populations of strong binders with low specificities will be captured (Willats, 2002). Negative subtraction is another method used to
discriminate binders and non-specific binders from the phage population, in addition to varying the blocking agent throughout the process and reducing the concentration of immobilised target in later rounds to increase stringency. Blocking agents typically used to block the sites not occupied by the target antigen on the support include bovine serum albumin (BSA), ovalbumin, casein or powdered skimmed milk.

### 1.13 Phage antibody libraries

Four different types of libraries can be constructed based on the source of V-genes. Non-immune or naïve libraries are produced using the V-genes from IgM-encoding mRNA of B-cells of non-immunised human donors that have been isolated from peripheral blood lymphocytes, bone marrow or spleen cells, or from similar animal sources. These libraries are unbiased and advantageous due to the ability to isolate an antibody to self, nonimmunogenic or toxic antigens. In addition, a shorter time is required for antibody generation and higher affinity binders can be isolated when a larger antibody repertoire is used (Pansri et al., 2009; Wang et al., 2012). The quality of the library is influenced by the unequal expression of the $V_H$ and $V_L$ V-gene repertoire, as well as potential limited diversity of the IgM repertoire of the donor(s) (Pande et al., 2010).

An immune library is constructed from V-genes derived from IgG-encoding mRNA of B-cells from an immunised animal or an infected human (Ayat et al., 2013). An immune antibody library will be enriched in antigen-specific antibodies, some of which will have undergone affinity maturation. The disadvantages of these libraries include the time required for construction due to immunisations, the lack of response to self or toxic antigens and the fact that a new library must be prepared for each antigen of interest (Weisser and Hall, 2009). Synthetic libraries contain antibodies that are built artificially by *in vitro* assembly of V, D and J gene segments (Harel Inbar and Benhar, 2012). The complementarity determining regions (CDRs) define the specificity of the antibody. CDR3 of the heavy chain ($V_H$-CDR3) has been engineered to introduce diversity in some synthetic libraries. $V_H$-CDR3 is the most diverse loop in composition and length, while the other five CDRs have varying but more limited structural variation (Pande et al., 2010). Semi-synthetic libraries are
constructed using a combination of natural and synthetic antibody diversity, accompanied by engineering of the CDR regions (Chan et al., 2011).

1.14 Production of recombinant antibodies

Hybridoma technology allows the production of monoclonal antibodies of defined antigen specificities in mammalian cell systems (Köhler and Milstein, 1975). Recombinant antibody technology, however, involves cloning of antibody variable region genes and expression as recombinant proteins, for example in the periplasmic space of Escherichia coli (Skerra and Plückthun, 1988; McCafferty et al., 1996; Chen et al., 2004). These fragments typically maintain the binding specificity of the parent monoclonal antibody (Bird et al., 1988). Bacteria, and particularly E. coli, are favoured for fragment expression as they can be expressed at low cost and are easily amenable to commercial scale up (Ferrer-Miralles et al., 2009; Spadiut et al., 2014). Examples of eukaryotic systems used to express antibody fragments include Saccharomyces cerevisiae, Pichia pastoris, insect cells, plant cells and mammalian cells (Weisser and Hall, 2009). One of the advantages of recombinant antibody technology is that immunisation can be avoided and the DNA of the antibody fragment may be manipulated to enhance antibody properties such as affinity (Thakkar et al., 2014), specificity (Sixholo et al., 2011) or stability (Ewert et al., 2004) while coupling to enzymes such as alkaline phosphatase (Hu et al., 2013) or green fluorescent protein (Sakamoto et al., 2011) to generate reporter antibodies.

1.15 Expression in E. coli

Recombinant DNA technologies led to the birth of the biopharmaceutical industry in the late 1970s. The US Food and Drug Administration (FDA) approved the commercialisation of humulin, the human insulin analog which was recombinantly produced in E. coli in 1982 and which represented a milestone in the biopharmaceutical industry. Currently almost 40 % of biopharmaceuticals on the market are produced in E. coli (Walsh, 2010, 2012). The market turnover of the industry was estimated at 100–120 billion US dollars per year in 2012, with more than 200 biopharmaceutical proteins on the market (Butler and Meneses-
Acosta, 2012; Berlec and Strukelj, 2013). *E. coli* is the preferred expression system for the production of small, non-glycosylated recombinant antibody fragments such as scFvs as production is faster and easier compared to expression systems like mammalian cell lines (Weisser and Hall, 2009). Recombinant expression in *E. coli* is controlled by inducible promoters such as the lac repressor-regulated *lac*, *trp* and *tac* promoters, while autoinduction is an emerging method of recombinant protein production using *lac* operon-controlled expression systems which does not need growth phase-dependent addition of inducer (Studier, 2005a). Instead, protein expression is induced following glucose depletion in the medium and studies by Rinas and colleagues have demonstrated target protein yields of up to four times higher compared to IPTG induction due to a higher biomass being obtained using this method (Li et al., 2011). Recombinant antibodies can be expressed in *E. coli* in the periplasmic space, the cytoplasm or extracellularly. Expression in the cytoplasm may result in inclusion bodies containing aggregates of reduced and unfolded antibodies due to the reducing environment and the inability to form stable disulphide bridges in this compartment (Spadiut et al., 2014). Approaches to reduce inclusion body formation include the use of modified *E. coli* strains for cytoplasmic disulphide bond formation (Levy et al., 2001) and co-expression with chaperones or secretion of the target protein to the periplasmic space by fusion of a leader peptide at the N terminus (Kolaj et al., 2009; Sonoda et al., 2011). Recombinant antibodies can also be secreted extracellularly using the α-hemolysin system of *E. coli* (Low et al., 2010). This system specifically secretes the target protein from the bacterial cytoplasm into the extracellular medium without a periplasmic intermediate. The more common approach for disulphide-linked recombinant proteins, however, is transport to the periplasmic space where disulphide bonds are introduced by the Dsb protein family in the oxidising environment to facilitate proper folding (Kolaj et al., 2009).

### 1.16 scFvs in ELISAs and other biotechnology applications

Antibodies high specificity and affinity for their target underlie their development for diagnostic and therapeutic applications. Antibodies are used as research tools for immunodetection of antigens in cells or tissue sections by
fluorescence microscopy or flow cytometry using fluorophore-labelled antibodies (Qian et al., 2013), detection of antigen in immunoblots using enzymes such as horseradish peroxidase (Moricoli et al., 2014), quantification and detection of antigen in enzyme-linked immunosorbent assay (ELISA; Johdi et al., 2013), and purification of ligands by immuno-affinity chromatography or immunoprecipitation (Esch et al., 2012). In these types of biotechnological applications, the antibody can be modified by addition of a fluorescent or radioactive label, an enzyme or biotin label for detection, depending on the intended application (Abuknesha et al., 2005; González-González et al., 2012; Johdi et al., 2013; Parker et al., 2013; Bu et al., 2014).

Immunoassays exploit the specific binding of an antibody to its antigen for detection and quantification of an analyte in an aqueous sample. They provide a sensitive and cost-effective manner to analyse samples. The well-established ELISA technique involves direct, sandwich or competitive formats on a solid-phase microtitre plate with multiple washing steps and a colourimetric read-out. Antibody fragments are a valuable alternative to full length monoclonal antibodies for immunoassay – and biosensor – applications as they provide small, stable, highly specific reagents against the target antigen (Srivastava et al., 2014b). scFvs have been validated in ELISA for application in agriculture, food safety, environmental and human health (Das et al., 2004; Shaw et al., 2008; Meyer et al., 2011; Nimmagadda et al., 2012; Wang et al., 2012; Ayat et al., 2013; Hu et al., 2013; Ribeiro et al., 2013). Variations of the ELISA include the use of functionalised scFv gold nanoparticles as demonstrated by Liu and colleagues for development of a more sensitive colourimetric immunoassay (Liu et al., 2009).

Radiolabelled monoclonal antibodies and recombinant antibody fragments are used in tumour imaging and therapy. Targeted delivery of small-molecule drugs can potentially improve the selective killing of tumor cells. Lu and colleagues showed that the use of anti-c-Met scFv-conjugated liposome improved chemotherapeutic drug delivery into the tumor cells (Lu et al., 2011). Girgis and colleagues engineered a functional diabody against CA19-9 which is a tumour antigen present on the majority of pancreatic cancers. The radiolabelled diabody produced high contrast antigen specific PET imaging (ratio 5:2,
tumour:blood) of pancreas cancer in xenograft models (Girgis et al., 2011). The same group further engineered this diabody and conjugated it to polymerised liposomal nanoparticles and demonstrated target-specific binding to human pancreatic cancer cells in vitro, indicating their potential to target treatment to these cells (Girgis et al., 2013). Full length monoclonal antibodies penetrate solid tumours slowly with a non-uniform distribution and exhibit slow clearance from the blood, hence, they are not optimal carriers of radionuclides (Weisser and Hall, 2009). Diabodies are preferred for in vivo imaging as they demonstrate rapid uptake by the tumour due to their size (~55 kDa) and they are eliminated through the kidneys, thereby reducing non-specific exposure to non-target tissues (Ortega et al., 2013). Applications in radiotherapy include the administration of radiolabelled scFv dimers attached to magnetic iron oxide nanoparticles which can result in tumour cell death if an external magnetic field is applied (Natarajan et al., 2008a; Natarajan et al., 2008b).

A tumour-targeting immunotoxin consists of an anti-tumour antibody linked to a tumour cell-killing moiety such as a cytotoxin. Peptide toxins include *Pseudomonas* exotoxin, Diphtheria toxin and Ricin which have been genetically coupled to monoclonal antibodies or antibody fragments in order to generate specific anti-cancer drugs which can kill dormant cells and overcome drug resistance (Zhu et al., 2013). The basic function of an immunotoxin is to bind the tumour cell followed by entry of the toxin into the cell cytoplasm which inhibits some cellular function and ultimately, results in cell death. Kreitman and colleagues observed complete remission in 61 % of patients suffering from Hairy–cell leukaemia using an anti-CD22 dsFv immunotoxin carrying *Pseudomonas* exotoxin A (Kreitman et al., 2001). Zhu and colleagues recently constructed a new recombinant immunotoxin for possible clinical use in patients with chronic myeloid leukemia (CML) - humanised scFv-ETA. Treatment of leukaemia cells with this immunotoxin resulted in 37 % cell death as measured by flow cytometry. This may represent a therapeutic strategy for treatment of CML (Zhu et al., 2013). These examples illustrate the therapeutic and diagnostic potential of recombinant antibody fragments which are increasingly preferred in these types of applications in vivo due to their specificity, size, compatibility with
E. coli expression platforms and ease of genetic manipulation (Srivastava et al., 2014b).

1.17 Neural cell adhesion molecule (NCAM1)

The neural cell adhesion molecule (NCAM1) is a member of the immunoglobulin superfamily. It is strongly expressed in the nervous system on the cell surface of astrocytes, oligodendrocytes, glial cells and neurons (Rønn et al., 1998; Krushel et al., 1999; Fewou et al., 2007), as well as on natural killer cells, in the neuroendocrine glands, and in the central and peripheral nervous system and cardiomyocytes (Jensen and Berthold, 2007). It is also a tumour-associated antigen and is expressed in small cell lung cancer, neuroblastoma, rhabdomyosarkoma, brain tumours, multiple myelomas and acute myeloid leukaemia (Zecchini and Cavallaro, 2010). It is a cell surface glycoprotein and mediates cell-cell adhesion and signalling in the nervous system and in non-neural tissues through homophilic and heterophilic interactions and it plays an important role in embryogenesis and development (Cunningham et al., 1987; Rønn et al., 1998). Its cell-cell adhesion capability allows it to perform as a receptor that is responsible for intracellular downstream signaling.

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Fig.1.7 (A) Structure of neural cell adhesion molecule (NCAM1). It consists of seven extracellular domains extending from the cell membrane. Ig1-Ig5 represent immunoglobulin-like modules and Fn3I and Fn3II are fibronectin type III-like moieties. N1-N6 represent sites of glycosylation of the protein (Nielsen et al., 2010). (B) The C-terminus is located in the cytoplasm (NCAM-180 and NCAM-140) or buried in the cytoplasmic membrane (NCAM-120) while the N-terminus protrudes from the cell surface (Rønn et al., 1998).
There are three major forms of NCAM1 as a result of alternative splicing, of which NCAM-180 and NCAM-140 are transmembrane and contain both transmembrane and cytoplasmic domains, while NCAM-120 is attached to the membrane via a glycosylphosphatidylinositol (GPI) anchor – Fig. 1.7. The extracellular portion of NCAM1 consists of five Ig-like domains and two fibronectin type III-like domains and it is post-translationally modified by the addition of glycosylation of the third, fourth and fifth Ig domains – Fig. 1.7.

1.18 Project objectives

Microarray and real-time RT-PCR studies from a previously study of canine IVD degeneration identified NCAM1 as a putative IVD cell surface marker due to its approximately 10-fold higher expression levels in NP than AF or articular cartilage cells (Sakai et al., 2009). This result was validated and confirmed by real time-PCR using IVD and cartilage cells, albeit with low level expression of NCAM1 (Rutges et al., 2010). The overall goal of this work was to develop a targeting mechanism, based on NCAM1, for regeneration of the intervertebral disc. The first objective was to express and purify a recombinant NCAM1 molecule to be used for antibody development against this potential NP-specific marker. The second objective was to isolate an NCAM1-binding human scFv and validate its binding of the target protein *in vitro*. The third objective was to develop a cell assay and demonstrate *ex vivo* binding of the scFv to NCAM1-expressing cell lines. The ultimate goal of the overall research programme is the development of a targeting mechanism for IVD cells by which therapeutic gene delivery, possibly mediated by scFv-displaying nanocapsules, could be effected for the purpose of regeneration of the degenerated IVD.
Chapter 2: Materials and Methods

2.1 Materials

The reagents and plasticware used throughout this work were purchased from the companies listed below.

Abcam, 330 Cambridge Science Park, Cambridge, CB4 0FL, United Kingdom.

Alere Ltd (formerly Inverness Medical UK Ltd), Pepper Road Hazel Grove, Stockport, SK7 5BW, United Kingdom.

Bio-Sciences Ltd, 3 Charlemont Terrace, Crofton Road, Dun Laoghaire, Co Dublin, Ireland.

Cruinn Diagnostics Ltd., 5b/6b Hume Centre, Park West Industrial Estate, Nangor Road, Dublin 12, Ireland.

Eurofins MWG Operon, Anzinger Str. 7a, 85560 Ebersberg, Germany.

Fannin Ltd, Fannin House, South County Business Park, Leopardstown, Dublin 18, Ireland.


GE Healthcare, Little Chalfont, Buckinghamshire HP7 9NA, United Kingdom.

LABPLAN, Allenwood Business Park, Naas, Kildare, Republic of Ireland.

Medical Supply Company Ltd., Damastown, Mulhuddart, Dublin 15, Ireland.

Melford Laboratories Ltd., Bildeston Road, Chelsworth, Ipswich, Suffolk, IP7 7LE, United Kingdom.
MERCK MILLIPORE Ltd., Tullagreen, Carrigtwohill, Co Cork, Ireland.

MyBio Ltd, Hebron Business Park, Kilkenny, Ireland.

New England Biolabs (UK) Ltd., 75/77 Knowl Piece, Wilbury Way Hitchin, Herts SG4 0TY, United Kingdom.

QIAGEN Ltd., Skelton House, Lloyd Street North, Manchester M15 6SH, United Kingdom.

Roche Diagnostics Ltd, Charles Avenue, Burgess Hill, West Sussex, RH159RY, United Kingdom.

Santa Cruz Biotechnology, Inc., Bergheimer Str. 89-2, 69115 Heidelberg, Germany.

Sarstedt Ltd., Sinnottstown Lane, Drinagh, Wexford, Ireland.

Sigma-Aldrich Ireland Ltd., Arklow, Ireland.

Sparks Lab Supplies, Unit 7, Block J, Greenogue Business Park, Grants Rd, Rathcoole, Co. Dublin, Ireland.

VWR International Ltd., Orion Business Campus, Northwest Business Park, Ballycoolin, Dublin 15, Ireland.

Plasticware and consumables were supplied by the following companies: Biosciences Ltd. (96 well flat-bottom MaxiSorp™ plates); Cruinn Diagnostics Ltd. (filter and non-filter tips); Fisher Scientific Ireland (30 ml sterile tubes, pipets, disposable cuvettes, MaxiSorp™ immuno tubes and stoppers, 2 ml cryovials, Superfrost® Plus and Lab-tek™ slides); Medical Supply Company Ltd. (electroporation cuvettes); Merck Millipore Ltd. (Stericup® filter units 0.22µm); Sarstedt Ltd., (15 ml and 50 ml sterile tubes); Sigma-Aldrich Ireland Ltd., (25 cm²/75 cm² tissue culture flasks); Sparks Lab Supplies Ltd (disposable syringes, 0.22 µm and 0.45 µm filters, 0.5 ml PCR tubes, spreaders, loops) and VWR International Ltd. (5 ml tips, polyester non-sterile films for 96-well plates and petri-dishes).
2.2 Equipment

The model number and manufacturer details of equipment used throughout this project are listed below.

Autoclave: HICLAVE™, HV-85L, HMC.

Benchtop Centrifuge: Sigma 1-15, Sigma Centrifuges.

High Performance Centrifuge: AVANTI™ J-20 XP Centrifuge, Beckman Coulter

Flow cytometer: Becton Dickinson FACSCanto™

Distilled water system: ELIX 35, Millipore.

Milli-Q water system: Milli-Q Advantage A10, Millipore.

SDS-PAGE electrophoresis unit: dual mini slab chamber with a discontinuous buffer system, AE-6450, ATTO.

Agarose gel electrophoresis unit: horizontal electrophoresis tank, 75.1214, Apollo Instrumentation.

Electroporator: 2510, Eppendorf.

Haemocytometer: Neubauer Counting Chamber, Hawksley.


Incubator (37° C CO₂): NUAIRE TS-Autoflow

Laminar flow hood: SterilGARD® III Advance, the Baker Company.

Laminar flow hood: GELAIRE Flow Laboratories

Microscope (inverted): Motic AE30, Wilovert.

Microscope (fluorescence): Olympus IX81 inverted epifluorescent microscope.

PCR thermal cycler: Flexigene thermal cycler, Techne.

Plate reader: Tecan, Genios.

Spectrophotometer: UV mini 1240, Shimadzu.
Protein transfer: Horizblot semi-dry transfer apparatus, ATTO.

Agarose gel visualisation: G:BOX, Syngene.

Pump for protein purification: HL-2, Shhuxi.

2.3 Reagents

2.3.1 Plasmids, cells and biological reagents

pIG6 was a kind gift previously received from Prof. Andreas Plückthun, Department of Biochemistry, University of Zurich, Switzerland. Rat astrocytes were provided by Benjamin Newland, NFB, NUI Galway. Bovine nucleus pulposus and annulus fibrosis cells, intervertebral disc sections and spinal cord sections were provided by Estelle Collin & Dr. Eugene See, NFB, NUI Galway. Human cDNA was provided by Dr. Sibylle Grad, AO Foundation, Davos, Switzerland for amplification of the human NCAM1-encoding gene. The RNA used to produce the cDNA was from NP cells of a 32 year old male patient (disc degeneration grade 1-2) that had a significantly higher NCAM1 gene expression compared to the corresponding sample from AF cells. The Yamo 1 human scFv library (Pansri et al., 2009) was a kind gift from Dr. Montarop Yamabhai, School of Biotechnology, Suranaree University of Technology, Thailand.

2.3.2 Bacterial strains

i. *E. coli* W3110 - F' λ′ rph-1 INV(rrnD, rrnE)

ii. *E. coli* TOP10F' - F'[lacI^q Tn10(tec^R)] mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 deoR nupG recA1 araD139 Δ(ara-leu)7697 galU galK rpsL(Str^R) endA1 λ'

iii. *E. coli* BL21 - *E. coli* B F- dcm ompT hsdS(rB^-mB^-) gal [malB^+K-12(λ^S)]

iv. *E. coli* TOP10 - F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(Str^R) endA1 λ'

v. *E. coli* TG1 - F' [traD36 proAB^- lacI^q lacZΔM15]supE thi-1 Δ(lac-proAB) Δ(mcrB-hsdS)Δ5, (rK^-mK^-)

vi. *E. coli* HB2151 - K12 ara Δ(lac proAB) thi/F' proA^-B lacIq lacZΔM15
2.3.3 Primers

Primers were made up to a stock concentration of 100 pmol/µl using molecular grade water. The working concentration used in PCR reactions was 10 pmol/µl.

Table 2.1 Primers for NCAM1-Ig1 generation by overlap PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>T&lt;sub&gt;m&lt;/sub&gt; (° C)</th>
<th>Sequence (5’ - 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pIG F</td>
<td>53.1</td>
<td>CAGGAAACAGCTATGACCATGATTACG</td>
</tr>
<tr>
<td>pIG R</td>
<td>59.1</td>
<td>CTTGCTGGGAAAACAATATCCACCATGACCATGATTACG</td>
</tr>
<tr>
<td>NCAM Ig1 2F</td>
<td>59.2</td>
<td>GGTATCCGGTACCGTAGCGCAGGGGCCCAGCTGAGGATATAGCACCCAGGAGGCGCTTGACTACACTG</td>
</tr>
<tr>
<td>NCAM Ig1 R</td>
<td>57.5</td>
<td>CCAGAGAATTCGTTGACGGTGCCTTGACTACACTG</td>
</tr>
</tbody>
</table>

Table 2.2 Primers for F4 mut scFv generation by overlap PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>T&lt;sub&gt;m&lt;/sub&gt; (° C)</th>
<th>Sequence (5’ - 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pIGF</td>
<td>53.1</td>
<td>CAGGAAACAGCTATGACCATGATTACG</td>
</tr>
<tr>
<td>ompA-F4rev</td>
<td>59.1</td>
<td>GCACCAGCTGCACCTGGGCCCAGCTGAGGATACGGTACG</td>
</tr>
<tr>
<td>F4mutrev</td>
<td>59</td>
<td>CTTGGAGGCTGCGGCCCAGGAGGCGCTTGAGGATACGGTACG</td>
</tr>
<tr>
<td>ompA-F4for</td>
<td>57.2</td>
<td>GGTATCCGGTACCGTAGCGCAGGGGCCCAGGAGGCGCTTGAGGATACGGTACG</td>
</tr>
<tr>
<td>F4topIG6rev</td>
<td>46.7</td>
<td>GGTCAAGCTATCATGATGCTGATGATGATGCTGAGGCGCTTGAGGATACGGTACG</td>
</tr>
<tr>
<td>F4mutfor</td>
<td>59</td>
<td>CTGGGTCCGCCAGGCTCCAGG</td>
</tr>
</tbody>
</table>

Table 2.3 Primers for scFv sequencing

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ - 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMOD1for</td>
<td>GTGAAAAAAATTTATTTCG</td>
</tr>
<tr>
<td>pMOD1rev</td>
<td>CAGAGATCAGTTTCTG</td>
</tr>
<tr>
<td>scFv rev</td>
<td>CAGAGATCAGTTTCTGTCG</td>
</tr>
</tbody>
</table>

2.3.4 Antibodies

i. Monoclonal mouse anti-polyHistidine peroxidase conjugate – Cat. No. A7058, Sigma.


iii. Polyclonal rabbit anti-<i>myc</i> - Cat. No. sc789, Santa Cruz biotechnologies.
iv. Alexa Fluor® 488 Goat Anti-Rabbit IgG (H+L) – Cat. No. A11008, Molecular Probes®Life Technologies.
vi. Alexa Fluor® 488 Goat Anti-Mouse IgG (H+L) – Cat. No. A11001, Molecular Probes®Life Technologies.
viii. Polyclonal rabbit anti-6X His tag® FITC-conjugate – Cat. No. Ab1206, Abcam.
ix. Monoclonal mouse anti-c-myc (clone 9E10) – Cat. No. M4439, Sigma.
x. Polyclonal rabbit anti-mouse immunoglobulins peroxidase conjugate – Cat. No. P0161, Dako.
xii. Polyclonal goat anti-fluorescein peroxidase conjugate – Cat. No. PA1-26804, Thermoscientific.

2.3.5 Antibiotics
i. Ampicillin – 100 µg/ml in dH₂O (filter sterilised). Cat. No. A9518, Sigma.

2.3.6 Protein molecular weight markers
i. Page Ruler Plus Prestained protein marker (Cat. No. 26619, Thermo Scientific).
ii. Pre-stained SDS-PAGE standards broad range (Cat. No. 161-0318, Biorad).

2.3.7 DNA molecular weight markers
i. DNA Molecular Weight Marker X (0.07–12.2 kbp) – Cat. No. 11 498 037 001, Roche Applied Science.
ii. DNA Molecular Weight Marker XIV (100–1500 bp) - Cat. No. 11 721 933 001, Roche Applied Science.
iii. GeneRuler™ 100 bp DNA Ladder – Cat. No. SMO241, Fermentas.

2.3.8 DNA-modifying enzymes and molecular reagents

i. Phusion High Fidelity DNA polymerase 2 U/µl – Cat. No. F530L, 500 U, Thermo Scientific.

ii. Deoxynucleotide Solution Mix (dNTP Mix, 10 mM concentration of each dNTP present) – Cat. No. N0477, New England Biolabs.

iii. T4 DNA Ligase (Cat. No.M0202S) and Reaction Buffer (10x) – New England Biolabs.


2.3.9 Commercial DNA and protein kits

- QIAquick gel extraction kit (Cat. No. 28706, Qiagen).
- Plasmid purification- QIAprep Spin Miniprep kit (Cat. No. 27106, Qiagen).
- Zero Blunt® TOPO® PCR Cloning kit (Cat. No. K2860-20, Invitrogen).
- DC™ protein assay kit (Cat. No. 500-0112, Biorad).

2.4 Buffers and solutions

All solutions were made up to the stated volume using Milli-Q or distilled water unless otherwise stated. Molecular biology grade water was used in solutions for use with DNA.

2.4.1 Buffers, media and reagents

- 10x TE buffer (1 l): 100 ml 1 M Tris-HCl pH 7.5, 20 ml 500 mM EDTA pH 8.
- 50x TAE(1 l): 242 g Tris base, 57.1 ml Glacial acetic acid, 18.6 g EDTA in 1 l of dH₂O.
- 10x DNA-loading buffer (100 ml): 20 ml 50x TAE, 40 g glycerol, 10 mg bromophenol blue.
• 6x DNA-loading buffer (100 ml): 60 g glycerol, 12 ml 0.5 M EDTA, pH 8, 10 mg bromophenol blue.
• SOC medium (1 l): 20 g tryptone, 5 g yeast extract, 0.5 g NaCl, 0.186 g KCl, 0.952 g MgCl₂, 20 ml of sterile 1 M glucose.
• 1 M glucose: 18 g/100 ml dH₂O.
• LB medium (1 l): 10 g tryptone, 5 g yeast extract, 10 g NaCl, pH 7.4.
• LB agar (1 l): as above, supplemented with 15 g agar prior to autoclaving.
  • ZY media: 10 g tryptone, 5 g yeast extract, 925 ml dH₂O.
  • 50x 5052 (100 ml): 25 g glycerol (weigh in beaker), 73 ml water, 2.5 g glucose, 10 g alpha-lactose (added in this sequence).
  • 20x NPS (100 ml): 90 ml water, 6.6 g (NH₄)₂SO₄, 13.6 g KH₂PO₄, 14.2 g Na₂HPO₄.
• 3M sodium acetate (100 ml): 40.8 g sodium acetate, pH 5.2.
• 75 % ethanol: 75 % made with molecular biology grade water (Sigma).
• 100 % ethanol – Cat. No. 1009832500, Merck Millipore.
• Agarose: 0.8–2 % agarose in 1x TAE buffer.
• Ethidium bromide – Cat. No. H5041, Promega.
• TMB for ELISA - Cat. No. T0440, Sigma.
• 0.5 M Imidazole (1 l): 34.04 g in 1 l dH₂O.
• 8x HisTrap buffer for protein purification (1 l): 233.8 g NaCl, 9.6 g NaH₂PO₄, 14.239 g Na₂HPO₄.2H₂O, pH 7.4.

2.4.2 Phage display reagents
• Yamo I human scFv library received from P. Pansri (Pansri et al., 2009) along with KM13 helper phage, E. coli TG1 for phage propagation and E. coli HB2151 for expression of soluble scFv.
• TYE agar (1 l): 10 g tryptone, 5 g yeast, 8 g NaCl, 15 g agar.
• 2x YT (1 l): 16 g tryptone, 10 g yeast, 5 g NaCl.
• H-Top agar (1 l): 10 g tryptone, 8 g NaCl, 7 g agar.
• Minimal media (100 ml): 1.5 g agar, 0.1 g (NH₄)₂SO₄, 0.05 g sodium citrate dihydrate, 98.5 ml of 1x M9 salts, 1 ml 1 M MgSO₄.7H₂O.
• 20% Polyethylene glycol 6000, 2.5 M NaCl (100 ml): 20 g PEG 6000, 14.6 g NaCl.
• 10x PBS (1 l): 80 g NaCl, 2 g KCl, 2.4 g KH$_2$PO$_4$, Na$_2$HPO$_4$·2H$_2$O, pH 7.4.
• Trypsin: 10 mg/ml stock in 50 mM Tris-HCl pH 7.4, 1 mM CaCl$_2$ (filter sterilised).
• Phage elution buffer (100 ml): 50 mM Glycine HCl pH 2 (0.3753 g/100 ml dH$_2$O).
• Phage neutralisation buffer (100 ml): 200 mM Na$_2$HPO$_4$ pH 7.5 (2.84 g/100 ml dH$_2$O).
• IPTG (100 ml, 1 M): 23.83 g in 100 ml dH$_2$O (filter sterilised). Cat. No. MB1008, Melford.

2.4.3 Protein extraction buffers
• Resuspension buffer (0.75 M Sucrose, 0.1 M Tris pH 8) 100 ml: 25.67 g sucrose, 10 ml 1 M Tris pH 8.
• Novagen® rLysozyme™ solution 30 KU/µl - Cat. No. 71110, EMD Millipore.
• Dialysis tubing for scFv, MWCO 12400 - Cat. No. D0655, Sigma.
• Dialysis tubing for NCAM1: Snakeskin® tubing, MWCO 7000 - Cat. No. 68700, Thermo Scientific.

2.4.4 SDS-PAGE and Western blotting reagents
• 10x SDS-PAGE running buffer (1 l): 10 g SDS, 30.3 g TRIS, 144.1 g glycine.
• Beta-mercaptoethanol - Cat. No. M7154, Sigma.
• 40% Bis-acrylamide - Cat. No. A7802, Sigma.
• 12.5% separating/resolving gel (2): 6.11 ml of H$_2$O, 4.69 ml of 40% Bis-acrylamide, 3.9 ml of 1.5 M Tris pH 8.8, 150 µl of 10% SDS, 150 µl of 10% APS and 6 µl of TEMED.
• 4.5% stacking gel (2): 3.6 ml of H$_2$O, 640 µl of 40% Bis-acrylamide, 630 µl of 1 M Tris pH 6.8, 50 µl of 10% SDS, 50 µl of 10% APS and 6 µl of TEMED.
- 2x Sample reducing buffer (10 ml): 1 ml of 1.5 M TRIS pH 6.8, 0.6 ml of 20 % SDS, 3 ml glycerol, 1.5 ml beta-mercaptoethanol, 1 mg bromophenol blue.
- InstantBlue™ for staining gels - Cat. No. ISB1L, Expeideon.
- Transfer buffer: 20 % methanol in 1x SDS-PAGE running buffer.
- Whatman® cellulose chromatography paper - Cat. No. Z691011, Sigma.
- Nitrocellulose membrane (0.45µm) - Cat. No. 88018, Thermoscientific.
- TMB for membranes - Cat. No. T0565, Sigma.
- 5x TBS (1 l): 30 g TRIS, 44 g NaCl, 14.7g CaCl$_2$ H$_2$O, adjust to pH 7.5.

2.4.5 Reagents for cell culture

- Fetal bovine serum (FBS) - Cat. No. F2442, Sigma.
- Penicillin-streptomycin (50x) - Cat. No. P4458, Sigma.
- L-glutamine - Cat. No. G3126, Sigma.
- DMSO - Cat. No. 276855-100 ml, Sigma.
- Dulbecco’s modified eagle’s medium/Nutrient Mixture F-12 Ham (Cat. No. D8437, Sigma) was supplemented with 10 % FBS and 1 % penicillin-streptomycin for primary rat astrocytes.
- Dulbecco’s modified eagle’s medium (Cat. No. D6429, Sigma) was supplemented with 10 % FBS and 1 % penicillin-streptomycin for bovine nucleus pulposus and annulus fibrosis cells.

2.4.6 Reagents for immunocytochemistry

- Paraformaldehyde - Cat. No. 158127, Sigma.
- Goat serum - Cat. No. G9023, Sigma.
- DAPI dihydrochloride: 10 mg in 2 ml dH$_2$O. Cat. No.D1306, Invitrogen.
- Prolong® Gold anti-fade reagent - Cat. No. P36934, Life Technologies.
2.4.7 Reagents for immunohistochemistry

- Proteinase K (30 U/mg) - Cat. No. P2308-5MG, Sigma.
- Chondroitinase ABC (20 U/µl) - Cat. No. C2905, Sigma.

2.5 Methods

2.5.1 Preparation of electrocompetent bacteria

Buffers were prepared in advance and stored on ice before use. All centrifugation steps were carried out at 4°C. A vial of non-competent cells was removed from the -80°C freezer and quickly thawed in the 37°C waterbath. The cells were removed with a sterile transfer pipette and transferred to a 50 ml Sarstedt containing 10 ml of growth medium (LB). The culture was grown overnight at 37°C and 250 rpm. On the following day, this culture was used to inoculate 500 ml of LB so that the starting OD$_{600}$ was 0.05. Using the same culture conditions as before, the cells were grown until an OD$_{600}$ of 0.5-0.6 was reached. The culture was left on ice for 30 min before being centrifuged for 20 min at 5000 rpm. The supernatant was discarded and the cells were washed with 1 mM HEPES pH 7 followed by centrifugation at 5000 rpm for 20 min. The cells were washed twice with 10% glycerol, each wash was followed by a 20 min centrifugation step. The pellet was resuspended in 1 ml of 10% glycerol and 50 µl of resuspended cells were aliquotted per tube. The cells were snap-frozen using liquid nitrogen and stored at -80°C freezer.

2.5.2 Preparation of chemically competent bacteria

Cells were treated as above until an OD$_{600}$ of 0.5-0.6 was reached. The culture was centrifuged at 3000 g for 5 min at 4°C and was resuspended in 200 ml of pre-chilled 100 mM CaCl$_2$. These cells were stored on ice for 15 min followed by another centrifugation step. This CaCl$_2$ incubation and centrifugation steps were repeated before resuspending the cells in 1 ml of pre-chilled 100 mM CaCl$_2$ with 20% glycerol. Aliquots of 50 µl were prepared on ice, snap frozen in liquid nitrogen and stored at -80°C.
2.5.3 Preparation of bacterial glycerol stocks

Overnight liquid cultures from freshly streaked plates were grown overnight in growth medium containing 100 µg/ml ampicillin at 37° C at 250 rpm. The next day, 500 µl of the overnight culture was transferred to a cryovial containing 500 µl of 40 % glycerol, mixed well and snap-frozen using liquid nitrogen. The tubes were stored at -80° C.

2.6 DNA manipulation techniques

2.6.1 PCR amplification and cloning of NCAM1-Ig1 construct

An overlap PCR approach was used to generate the NCAM1-Ig1 expression construct. The initial reactions amplified two fragments; the pIG6 plasmid and the cDNA served as templates for generation of fragments 1 and 2, respectively. For the former fragment, PCR reactions contained: 13 µl molecular grade water, 4 µl of 5x Phusion HF buffer, 0.4 µl of 10 mM dNTPs, 1 µl (10 pmol) of each oligonucleotide primer and 0.2 µl of Phusion DNA polymerase. Reaction condition were 98° C, 3 min; 30x (98° C, 10 sec; 56° C, 30 sec; 72 ° C, 15 sec); 72 ° C, 10 min and bands of interest were excised and purified using QIAquick gel extraction kit – section 2.3.9.

For the latter fragment, PCR reactions contained 11.4 µl molecular grade water, 2 µl of cDNA, 4 µl of 5x Phusion HF buffer, 0.4 µl of 10 mM dNTPs, 1 µl (10 pmol) of each oligonucleotide primer and 0.2 µl of Phusion DNA polymerase. Reaction conditions were 98° C, 3 min; 15x (98° C, 10 sec; 50° C, 30 sec; 72 ° C, 30 sec); 10x (98° C, 10 sec; 53° C, 30 sec; 72 ° C, 30 sec); 10x (98° C, 10 sec; 56° C, 30 sec; 72 ° C, 30 sec); 72 ° C, 10 min and bands of interest were excised and purified using a QIAquick gel extraction kit.

For the overlap PCR to combine the two initial products, reactions contained 12.6 µl molecular grade water, 0.4 µl of purified fragment 1, 0.4 µl of purified fragment 2, 4 µl of 5x Phusion HF buffer, 0.4 µl of 10 mM dNTPs, 1 µl (10 pmol) of each oligonucleotide primer (pIGF and NCAM Ig1R) and 0.2 µl of Phusion DNA polymerase. Reaction conditions were 98° C, 3 min; 15x (98° C, 10 sec; 50° C, 30 sec; 72 ° C, 30 sec); 10x (98° C, 10 sec; 53° C, 30 sec; 72 ° C, 30 sec); 10x (98° C, 10 sec; 56° C, 30 sec; 72 ° C, 30 sec); 72 ° C, 10 min and bands of the expected molecular weight were excised and purified using a QIAquick gel extraction kit.
The overlap product pIGNCAM1-Ig1 was cloned into pCR-Blunt® II-TOPO® plasmid using the Zero Blunt® TOPO® PCR Cloning kit as per the manual’s instructions – section 2.3.9.

2.6.2 Transformation

A vial of electro-competent cells (E. coli TOP10) was thawed on ice and the relevant plasmid DNA was added and mixed gently using the pipette. The cells/DNA were transferred to a pre-cooled electroporation cuvette and electroporated using 1800 V. An aliquot of 250 µl of pre-incubated SOC medium or LB was added to the cuvette containing the cells before being transferred to a 15 ml tube. The tube was incubated horizontally with shaking at 250 rpm for 1 hour at 37° C. Afterwards, the cells were spread on LB/kanamycin plates (50 µg/ml) for the pCR-Blunt® II-TOPO® plasmid or LB/ampicillin (100 µg/ml) plates in the case of pIG6 plasmid.

2.6.3 Plasmid purification

An overnight liquid culture was prepared by picking a colony from a freshly streaked plate (LB agar with ampicillin or kanamycin) and inoculating 5 ml of growth medium containing the same antibiotic. The culture was grown overnight at 37° C at 250 rpm. Plasmid DNA was purified using the QIAprep Spin Miniprep kit – section 2.3.9.

2.6.4 Subcloning of NCAM1-Ig1 gene into expression vector

EcoRI and XbaI were used to excise the plG/NCAM1-Ig1 construct from the pCR-Blunt® II-TOPO® vector. Restriction enzyme digests were performed in the manufacturer’s recommended digest buffer. Reactions were carried out in a waterbath at 37° C (unless otherwise specified) for 2 h or overnight for large-scale digestions in 20-25 µl volumes.

Meanwhile, pIG6 plasmid DNA supplied by Dr. Sylvain Robin was used to transform E. coli TOP10 cells, followed by extraction of plasmid DNA from overnight cultures. After digestion using EcoRI and XbaI and electrophoretic separation, bands of the expected size were excised and purified using the QIAquick Gel extraction kit.
For ligation into the pIG6 vector, a molar ratio of 10x insert: 1x vector was used. Ligation reactions were carried out at 16° C overnight in 20 µl volumes and using T4 DNA ligase.

2.6.5 Precipitation of DNA

Sodium acetate 3 M pH 5.2 was added to the DNA to a concentration of 300 mM sodium acetate. Pre-chilled 100 % ethanol (2.5 volumes) were added to the DNA and vortexed vigorously and stored at -20° C for at least 1 h followed by centrifugation at 14000 rpm for 30 min at 4° C. The supernatant was removed taking care not to disturb the pellet. The same volume of 70 % ethanol (pre-chilled) was added to the DNA and vortexed for 5 min followed by a 5 min centrifugation at 14000 rpm at 4° C. The supernatant was removed and the tube was left open on the bench for 20-30 min. Afterwards, the DNA was resuspended in molecular grade water and allowed to stand with the top closed for 15-20 min.

2.6.6 Electroporation of E. coli TOP10 cells

The ligated NCAM1-Ig1/pIG6 vector product was precipitated as described in section 2.6.5 and resuspended in 2.5 µl of molecular grade water. A transformation experiment was carried out as described previously in section 2.6.2 using 2.5 µl of the precipitated DNA. Single colonies from the transformation plates were grown overnight in LB/ampicillin (100 µg/ml) at 37° C and 250 rpm. After DNA extraction, 5.0 µl of DNA was digested using XbaI and EcoRI and the digested products were electrophoresed on a 0.8 % agarose gel to analyse the cloning reaction.

2.6.7 Generation and cloning of F4 mut construct

Following design of the F4 scFv mutagenesis procedure, an overlap PCR approach was used to generate the construct. For the initial three reactions, the pIG6 plasmid and F4 clone DNA served as templates for generation of fragment 1 and fragments 2 and 3, respectively. After each product was generated separately (P1, P2 and P3), the relevant bands were excised from agarose gels using a QIAquick Gel Extraction kit and the purified products served as template to P1+P2, P2+P3 and, ultimately, P1+P2+P3 combinations by overlap PCR. PCR reactions typically contained 16.25 µl molecular grade water, 5 µl of 5x Phusion
GC buffer, 0.5 µl of 10 mM dNTPs, 1 µl (10 pmol) of each oligonucleotide primer and 0.25 µl of Phusion DNA polymerase. Reactions were carried out as follows:

**Product P1:** Template: pIG6vegf121; Primers: pIGF and OmpAF4 rev; Reaction parameters: 98° C, 3 min; 30x (98° C, 10 sec; 51° C, 30 sec; 72 ° C, 30 sec); 72 ° C, 10 min.

**Product P2:** Template: F4 scFv; Primers: OmpAF4 for and F4 mut rev; Reaction parameters: 98° C, 3 min; 30x (98° C, 10 sec; 55° C, 30 sec; 72 ° C, 30 sec); 72 ° C, 10 min.

**Product P3:** Template: F4 scFv; Primers: F4 mut for and F4to pIG6 rev; Reaction conditions: 98° C, 3 min; 10x (98° C, 10 sec; 44° C, 30 sec; 72 ° C, 30 sec); 10x (98° C, 10 sec; 50° C, 30 sec; 72 ° C, 30 sec); 10x (98° C, 10 sec; 57° C, 30 sec; 72 ° C, 30 sec); 72 ° C, 10 min.

For P1+P2 and P2+P3, PCR reactions contained 12.6 µl molecular grade water, 0.4 µl of fragment A, 0.4 µl of fragment B, 4 µl of 5x Phusion HF buffer, 0.4 µl of 10 mM dNTPs, 1 µl (10 pmol) of each oligonucleotide primer (pIGF and NCAM Ig1R) and 0.2 µl of Phusion DNA polymerase. Reactions were carried out as follows:

**Product P1+P2:** Templates: purified products 1(neat) and 2 (1:100); Primers: pIG F and F4 mut rev; Reaction parameters: 98° C, 3 min; 30x (98° C, 10 sec; 51° C, 30 sec; 72 ° C, 30 sec); 72 ° C, 10 min.

**Product P2+P3:** Templates: Purified products 2(1:10) and 3(1:100); Primers: OmpAF4 for and F4topIG6 rev; Reaction conditions: 98° C, 3 min; 10x (98° C, 10 sec; 44° C, 30 sec; 72 ° C, 30 sec); 10x (98° C, 10 sec; 51° C, 30 sec; 72 ° C, 30 sec); 10x (98° C, 10 sec; 55° C, 30 sec; 72 ° C, 30 sec); 72 ° C. Where necessary, PCR reactions were repeated and scaled up in volume to generate sufficient product to proceed with the final linking reaction, which was carried out as follows:

**P1+2/P3 or P2+3/P1:** Templates: purified products 1+2 and 3 for combination 1 OR purified product 1 and 2+3 for combination 2; Primers: pIG F and F4to pIG6rev; Reaction conditions: 98° C, 3 min; 10x (98° C, 10 sec; 44° C, 30 sec; 72 ° C, 30 sec); 10x (98° C, 10 sec; 48° C, 30 sec; 72 ° C, 30 sec); 10x (98° C, 10 sec; 52° C, 30 sec; 72 ° C, 30 sec); 72 ° C, 10 min.
After purification of an amplification product of the expected size, the purified product was cloned into pCR-Blunt® II-TOPO® vector using the Zero Blunt® TOPO® PCR Cloning kit. The ligation reaction was carried out at 16°C overnight. Electrocompetent *E. coli* TOP10 cells were transformed using the ligated product as described previously. Transformants were screened by restriction analysis using *EcoR*I.

### 2.6.8 Subcloning of F4 mut construct into the expression vector, pIG6

pIG6 vector DNA and the F4 mut-pCR-Blunt® II-TOPO® vector DNA were prepared from overnight cultures, digested using *Xba*I and *Hind*III overnight at 37°C, electrophoresed on a 1% gel and the relevant bands of the linearised pIG6 vector (from pIG6vegf121) and the F4pIG construct were excised and purified using the QIAprep Spin Miniprep kit. Ligation of the F4 mut PCR product into the pIG6 vector was carried out as described previously (section 2.6.4). Transformants were screened as outlined in section 2.6.4. Plasmid DNA from selected clones was sequenced by Eurofins MWG Operon using vector-specific primers or M13for and M13rev universal primers for clones in the pCR-Blunt® II-TOPO® vector system (Invitrogen).

### 2.7 **Protein expression**

The methods described below were used in expression studies of NCAM1-Ig1 (in *E. coli* W3110) and the scFv(s); B5 scFv in *E. coli* HB2151 and F4 scFv in *E. coli* BL21.

#### 2.7.1 Expression using IPTG

A single colony from a freshly streaked plate was grown overnight at 37°C at 250 rpm in 10 ml LB containing the relevant antibiotic. This starter culture was then used to inoculate 50-500 ml LB media containing relevant antibiotic, to an OD	extsubscript{600} of 0.05. The culture was grown at 37°C with shaking at 250 rpm until the OD	extsubscript{600} reached between 0.5 and 0.6. The culture was induced by the addition of 0.5–1 mM IPTG and grown for 3-16 h at 225 rpm at 25°C C. Small-scale cultures were typically carried out in 50 ml volumes and a pre-induction sample and hourly, post-induction samples corresponding to OD	extsubscript{600} = 1 were collected for analysis.
2.7.2 Expression using autoinduction media

After inoculation to an OD$_{600}$ of 0.05 as described above, cultures were grown at 37° C and 250 rpm until an OD$_{600}$ of 0.9–1.0 was reached. This culture was used to inoculate 464 ml of ZYP-5052 autoinducing medium containing 0.5 ml 1 M MgSO$_4$, 10 ml 50x 5052, 25 ml 20x NPS and ampicillin (100 µg/ml) and incubated at 25° C and 225 rpm for 18-24 h (Studier, 2005b). Small-scale cultures were typically carried out in 50 ml volumes where a pre-induction sample and post-induction samples (18, 24 and 48 h) corresponding to OD$_{600}$ = 1 were collected for analysis.

2.7.3 Periplasmic extraction of protein

Cells were harvested by centrifugation (9605 g, 20 min) and washed by resuspension in phosphate-buffered saline (PBS). Periplasmic protein was extracted from re-centrifuged cells by resuspension of the cell pellet in 15 ml of 750 mM sucrose/100 mM Tris (pH 8), followed by addition of 30 ml of 1 mM EDTA in a dropwise manner. After incubation for 10 min at room temperature, 30 kU of r-lysozyme was added and cell lysis was allowed to proceed for 30 min at room temperature with gentle rocking. Addition of 4.3 ml of 20 mM MgCl$_2$ was followed by incubation for 10 min at room temperature, addition of 50 µg/ml DNase I for 10 min on ice, and centrifugation at 8000 g for 10 min to yield soluble (supernatant) periplasmic protein fractions. The soluble extract was passed through a 0.45 µm filter and dialysed overnight against 5 l pellet of immobilised metal affinity chromatography (IMAC) binding buffer (3.98 M NaCl, 80 mM NaH$_2$PO$_4$, 80 mM Na$_2$HPO$_4$2H$_2$O) at 4° C. The remaining pellet (‘insoluble fraction’) was resuspended in the same volume of 1x PBS as that of the periplasmic extract and retained for analysis alongside the soluble and whole cell extracts.

2.7.4 SDS-PAGE and Western blotting

These techniques were carried out as described by Sambrook (Sambrook and Russell, 2001). SDS-PAGE gels were prepared and electrophoresed at 20–25 mA per gel for approximately 80 min. Proteins were visualised by staining gels for 1-2 h in 5 ml of InstantBlue™ (Expedeon). The gels were washed with
distilled water for 5 min before being imaged. Proteins separated on SDS-PAGE gels were electrophoretically transferred onto a nitrocellulose membrane at 60 mA for 45 min. Membranes were blocked using 5 % SMP/PBS for 1 h at room temperature and proteins were detected using anti-polyhistidine HRP-conjugated antibody in 2 % BSA/PBS (1:1000) for 1 h with gentle agitation and the addition of 1-2 ml of TMB substrate for membranes. Reactions were allowed to develop for 5–10 min.

2.7.5 Purification of NCAM1-Ig1 and scFv(s)

A 1 ml HisTrap affinity column (GE Healthcare, UK) was stripped and charged according to the supplier’s instructions before purification of proteins. A 10 ml volume of 1x HisTrap buffer (3.98 M NaCl, 80 mM NaH$_2$PO$_4$, 80 mM Na$_2$HPO$_4$·2H$_2$O) was first passed through the column followed by 10 ml of water. The column was stripped using 10 ml of stripping buffer (50 mM EDTA in 1x HisTrap buffer) and washed with 10 ml of water after each solution was applied. The column was charged using 2 ml of 0.1 M NiSO$_4$, washed and equilibrated with binding buffer containing 10 mM Imidazole before purification. Tween 20 (2 % v/v) and 10 mM imidazole were added to bacterial cell extracts before passing through at a flow rate of 1 ml/min. The column was washed with 10 ml, 3 ml and 3 ml of binding buffer containing 20 mM, 50 mM and 80 mM imidazole, respectively, before elution using binding buffer containing 100 mM (for scFvs) or 300 mM (for NCAM1-Ig1) imidazole. Eluted fractions were dialysed overnight in 1 l of sterile 1x PBS (scFv) or in 4 l of sterile 1x PBS (NCAM1) at 4° C to remove the imidazole. Purified proteins were analysed by SDS-PAGE and Western blotting.

2.7.6 DC™ Protein Assay

After purification, the purified samples were pooled and the protein concentration was determined using the DC™ (detergent compatible) protein assay from Biorad, according to the manufacturer’s instructions. Protein standards were prepared using BSA/PBS and were read at 600 nm using the Tecan plate reader. Protein concentrations were calculated from a standard curve.
2.8 Phage display techniques

_E. coli_ cells were cultured and electroporated as described in section 2.6.6. Transformation of chemically competent cells was carried out by heat shock as described by Sambrook (Sambrook and Russell, 2001).

2.8.1 Preparation of _E. coli_ TG1 stocks

A minimal medium plate was streaked with a glycerol stock of _E. coli_ TG1 cells. This plate was incubated for 24 h at 37° C. A single colony was used to inoculate 5 ml of 2xYT media and grown with shaking at 37° C until OD<sub>600</sub> was 0.4–0.6. Glycerol was added to the culture to 20 % and it was mixed by gentle swirling. Stocks of _E. coli_ TG1 were prepared by aliquotting 50 µl of cells per tube, snap-freezing and storage at -80° C.

2.8.2 Preparation of helper phage KM13

For efficient infection of phage, _E. coli_ TG1 must be in log phase (OD<sub>600</sub> = 0.4-0.5). An overnight culture of _E. coli_ TG1 was grown as described in section 2.8.1, diluted 1:100 into fresh 2xYT medium and grown for approximately 2 hours until the OD<sub>600</sub> was 0.4-0.5. An aliquot of 200 µl of TG1 were infected with 10 µl of 10<sup>-3</sup> to 10<sup>-10</sup> dilutions of KM13 helper phage in a 37° C water bath (without shaking) for 30 min. These dilutions were added to 3 ml of molten H-top agar (~45° C) and mixed by inverting the tube 2-3 times before pouring onto pre-warmed TYE plates (no antibiotics). The plates were allowed to set at room temperature before overnight incubation at 37° C.

A small well-isolated plaque was added to 5 ml of fresh _E. coli_ TG1 at an OD<sub>600</sub> = 0.4-0.5. This was grown for 2 h with shaking at 37° C before being added to 500 ml of 2xYT in a 2 l flask and grown for a further hour at 37° C. Kanamycin was added to a final concentration of 50 µg/ml and grown overnight at 30° C in a shaking incubator. The overnight culture was centrifuged at 10800 g for 15 min. A volume of 100 ml PEG/NaCl (20 % polyethylene glycol 6000, 2.5 M NaCl) was added to 400 ml of supernatant and stored on ice for 1 h. This was followed by centrifugation at 10800 g for 30 min and the pellet was resuspended in 8 ml of PBS and 2 ml of PEG/NaCl was added. This was mixed by swirling and stored on ice for 20 min before being centrifuged at 13000 g for 30 min to remove the PEG/NaCl. The pellet was resuspended in 5 ml of PBS.
and passed through a 0.22 µm filter to remove bacterial debris. Aliquots of the helper phage were stored at 4° C (storage up to 1 month) and the remainder was stored at -80° C (with 15 % glycerol) for long term storage.

2.8.3 Titering of helper phage KM13

KM13 is the protease cleavable helper phage described in Kristensen and Winter (Kristensen and Winter, 1998). A volume of 5 µl of trypsin stock solution (10 mg/ml) was added to 45 µl of helper phage and this was incubated in a non-shaking waterbath at 37° C. A volume of 1 µl of trypsin-treated phage was diluted in 1 ml of PBS and five 100-fold serial dilutions were made in 1 ml aliquots of PBS. 50 µl of the six dilutions were added to six separate tubes containing 1 ml of E. coli TG1 at an OD$_{600}$ of 0.4-0.5 and mixed gently before being added to tubes containing 3 ml of molten (~45° C) H-Top agar. After mixing by gentle inversion, they were poured onto TYE plates (no antibiotic). The same series of dilutions was performed using non-trypsin treated phage. If the titre of the trypsin treated phage was $10^5$–$10^8$ lower than that for non-trypsin treated phage, the procedure was repeated using another plaque.

2.8.4 Preparation and resurrection of bacterial glycerol stocks

Bacterial glycerol stocks were prepared for stable storage of both individual clones and aliquots of phage libraries in E. coli by adding 500 µl of 40 % glycerol to either 500 µl bacteria scraped from agar plates or 500 µl of bacteria grown to an OD$_{600}$ of 0.8-0.9. Stocks were resurrected by incubation of a stock scraping on TYE agar containing 1 % glucose and 100 µg/ml ampicillin overnight at 37° C (individual clones) or by thawing at 37° C and growth in 2xYT containing 1 % glucose and 100 µg/ml ampicillin (phage library).

2.8.5 Replication of the Yamo I library (human scFv library)

An aliquot of 5 µl of the library was used to infect 20 ml of E. coli TG1 at OD$_{600}$ of 0.4-0.5. The infected E. coli TG1 was incubated without shaking 37° C for 30 min and swirled gently at 10 min intervals. This was followed by centrifugation for 5 min at 4000 rpm at 4° C to pellet the bacteria. A 10-ml volume of the supernatant was removed and the remaining supernatant was used to resuspend the pellet by gentle inversion. After resuspension, 100 µl of cells
were spread on TYE plates containing 100 µg/ml ampicillin and 1 % glucose. The plates were incubated at 37° C overnight. The colonies were harvested in 500 µl of 2xYT per plate using a spreader. This was combined in a conical flask to ensure mixing and the library was stored in aliquots in 10 % glycerol at -80° C.

2.8.6 Determination of library size

A series of 10-fold dilutions of unpanned library was prepared in sterile PBS. A 100-µl aliquot of each dilution was used to infect 900 µl of *E. coli* TG1 at OD<sub>600</sub> = 0.4-0.5. The cells were incubated in a non-shaking waterbath at 37° C for 30 min. Afterwards, 100 µl of infected cells from each dilution were spread on TYE plates containing 100 µg/ml ampicillin and 1 % glucose and incubated overnight at 37° C.

2.8.7 Rescuing the phage library

An aliquot of 50 µl of unpanned library was allowed to thaw on ice and used to inoculate 100 ml of 2xYT containing 100 µg/ml ampicillin and 1 % glucose. The culture was grown until an OD<sub>600</sub> of 0.4-0.5 was reached. At this point, 2 x 10<sup>11</sup> of helper phage were added to 50 ml of culture followed by incubation in a non-shaking waterbath at 37° C for 30 min. After centrifugation for 12 min at 3250 g, the pelleted cells were resuspended in 100 ml of 2xYT containing 0.1 % glucose, 100 µg/ml ampicillin and 50 µg/ml kanamycin and grown overnight at 30° C and 250 rpm. The culture was centrifuged for 35 min at 3250 g and the resulting supernatant was mixed with 20 % PEG 6000/2.5 M NaCl (1 part PEG/NaCl to 4 parts supernatant) in a conical flask. This was incubated on ice for 1 h during which the flask was swirled gently every 15 min to ensure complete mixing. Afterwards, the phage/PEG mixture was centrifuged for 30 min at 15000 g, the resulting supernatant was discarded and the white film was resuspended in 4 ml of sterile PBS and filtered through a 0.22 µm filter to remove bacterial debris. Some of the rescued unpanned library was maintained at 4° C (up to 1 month) and the remainder was stored at -80° C (with 15 % glycerol) for long term storage.
2.8.8 Determination of phage titre

The phage input for panning and the eluted phage (output) were titered by making a series of 10-fold dilutions of phage in sterile 1X PBS and using 100 µl of each dilution to infect 900 µl of _E. coli_ TG1 at OD$_{600}$ of 0.4-0.5 and incubation in a non-shaking water bath at 37° C for 30 min. A 100-µl volume of infected cells from each phage dilution was spread on TYE plates containing 100 µg/ml ampicillin and 1 % glucose and incubated overnight at 37° C. The colonies were counted and used to calculate the phage titer.

2.8.9 Antigen immobilisation test

An immunotube was coated with 100 µg/ml NCAM1-Ig1 overnight at 4° C. After two PBS washes, tubes were blocked with 1 % BSA/PBS for 2 h at room temperature. The wash step was repeated before incubation with 1 ml of anti-polyhistidine HRP-conjugated antibody (1:1000 in 1 % BSA) for 1 h at room temperature standing upright. The tubes were washed with 0.1 % Tween/PBS twice followed by two washes with 1x PBS. A 1-ml volume of TMB substrate was added to each tube and the reaction was allowed to proceed for 2-3 min before being stopped with an equal volume of 1 M H$_2$SO$_4$. Values were corrected for similar tubes coated with 100 µg/ml BSA.

2.8.10 Biopanning

Phage libraries were panned against recombinant NCAM1-Ig1 using a solid phase protocol. Two different panning experiments were carried out using the Yamo I library (Pansri et al., 2009).

i. Panning experiment 1

Two maxisorp tubes (Nunc) were coated overnight with 50 µg/ml and 500 µg/ml NCAM1-Ig1 in 1 % BSA/PBS at 4° C. The immunotubes were washed three times with 1x PBS followed by blocking with 3 % BSA/PBS. The tubes were filled completely with blocking solution and left to stand upright for 2 h at room temperature. The previous wash step was repeated and 10$^{12}$ of the prepared phage were incubated in the tubes in 4 ml of 2 % BSA/PBS for two h at room temperature. For the first hour, the tubes were placed on a rotating table and for the second hour, they were left upright. Afterwards, the supernatant was discarded and the tubes were washed 20 times (eight washes with 0.1 %
Tween/PBS, seven washes with 0.2 % Tween/PBS and five washes with 0.5 % Tween/PBS). Eighteen of the washes were pour in/pour out; the last two washes were each of 5 min duration. This was followed by 20 washes with sterile 1x PBS (pour in/pour out). Bound phage were eluted by adding 500 µl of trypsin-PBS (1 mg/ml trypsin in 1x PBS) and placed on a rotating table for 10 min at room temperature. An aliquot of 500 µl of 50 mM glycine-HCl pH 2.0 was added to the tubes and they were incubated for a further 10 min on the rotating table. This was neutralised by adding 500 µl of 200 mM Na₂HPO₄ pH 7.5. The eluted phages were titered using the protocol described in section 2.8.8.

A 250-µl volume of the eluted phages was added to 1.75 ml of *E. coli* TG1 at OD₆₀₀ 0.4-0.5 and incubated at 37° C in a non-shaking water bath. Afterwards, 100 µl of the infected *E. coli* TG1 were spread per plate on TYE plates containing 1 % glucose and 100 µg/ml ampicillin and the plates were incubated at 37° C overnight. Colonies were harvested in 1 ml 2xYT per plate and mixed, aliquotted and stored in 10 % glycerol at -80° C. Eluted phages from panning round 1 (P1) were rescued as previously described in section 2.8.7. For panning round 2, the immunotube was coated with 50 µg/ml of NCAM1-Ig1 and equal volumes of rescued phages from panning against 50 and 500 µg NCAM1-Ig1 were used (1.1 ml of each rescued phage population). For panning round 3, 1 ml of phage in 1 % BSA/PBS was used for the phage incubation step. Panning round 4 was carried out in the same manner as previously described.

ii. Panning experiment 2

Panning was carried out as described above until panning round 2, with the exception that NCAM1-Ig1 was coated on immunotubes at 100 µg/ml. For panning round 2, immunotubes were coated with 100 µg/ml of NCAM1-Ig1 and blocked using 3 % BSA/PBS. 1 ml of rescued phages from panning round 1 in 3 ml of 2 % BSA/PBS was used for the phage incubation step. For panning round 3, the tube was blocked with 2 % ovalbumin and incubated with 1 ml of phage in 3 ml of 2 % ovalbumin for the phage incubation step. Panning round 4 was carried out by blocking with 2 % Marvel and incubating 1 ml of phage in 3 ml of 2 % Marvel for the phage incubation step. Panning round 5 was carried out by reducing the NCAM1-Ig1 coating to 25 µg/ml and blocking with 2 % BSA.
2.8.11 ELISA analysis of polyclonal phage pools

The phage from each panning round were screened by ELISA for binding to the target NCAM1-Ig1. The wells of a microtitre plate were coated with 50-100 µg/ml NCAM1-Ig1 in PBS overnight at 4°C. Wells were washed four times with PBS before being blocked with 200 µl of blocking agent for 2 h at room temperature. The washing step was repeated as before. Input phage titers were normalised for each well and phage were added in 2 % blocking agent and incubated at room temperature for 1 h. The wells were washed with 0.1 % Tween 20/PBS four times followed by four washings with PBS. The excess PBS was removed by gently patting the plate onto tissue paper. A volume of 100 µl of anti-M13 HRP-conjugated antibody (1:5000) was added to each well and incubated at room temperature for 1 h. The previous washing step was repeated. A volume of 100 µl of TMB substrate was added to each well and upon development of colour, the reaction was stopped using 1 M H₂SO₄ and the absorbance (OD₄₅₀) measured.

For panning experiment 1, the wells were blocked with 2 % SMP/PBS and the phages were incubated in 2 % BSA/PBS. For panning experiment 2, the wells were blocked with 2 % Marvel and the phages were incubated in 2 % Marvel/PBS.

2.8.12 Monoclonal phage-scFv ELISA

Individual colonies from titration plates with the enriched population were used to inoculate 100 µl of 2xYT containing 100 µg/ml ampicillin and 1 % glucose in a 96-well plate. Cultures were grown overnight with shaking (250 rpm) at 37°C. A volume of 2 µl of each culture was used to inoculate 200 µl of 2xYT containing 100 µg/ml ampicillin and 1 % glucose in a 96-well plate and grown at 37°C for 2 h in a shaking incubator (250 rpm). Glycerol was added to the original plate (‘masterplate’) to a final concentration of 15 % and it was stored at -80°C. After 2 h incubation at 37°C, 25 µl of 2xYT containing 100 µg/ml ampicillin, 1 % glucose and 10⁹ helper phage was added to each well and incubated with shaking (250 rpm) for 1 h at 37°C. The plate was centrifuged at 1800 g at 4°C for 10 min and supernatants were removed. The pellets were resuspended in 200 µl of 2xYT containing 100 µg/ml ampicillin and 50 µg/ml kanamycin and grown overnight with shaking (250 rpm) at 30°C. The plate was
centrifuged for 10 min at 1800 g at 4°C and the supernatants were transferred to a fresh 96-well plate. Phage-containing culture supernatants (50 µl) were assayed for antigen binding by ELISA. The monoclonal phage ELISA was carried out as described in section 2.8.11. The phage containing supernatants were added in the appropriate blocking agent to a final concentration of 2 % blocking agent.

2.8.13 ScFv analysis techniques

i. **Expression of soluble scFv from 96 well plate**

   Individual colonies of *E. coli* TOP10F’cells transformed with phagemids of interest were used to produce scFvs as described in section 2.8.12. Briefly, cells were grown to an OD$_{600}$ of 0.9, 25 µl of 2xYT containing 1 mM IPTG and 100 µg/ml ampicillin was added to each well and plates were incubated overnight at 30°C with shaking at 250 rpm. Plates were centrifuged for 10 mins at 1800 g and supernatants transferred to a fresh plate. A volume of 50 µl of supernatant was assayed as described in section 2.8.13 (iii) using 100 µl of antic-myc produced in mouse (clone 9E10) in 2 % Marvel (1:5000) per well for 1 h at room temperature followed by 100 µl of anti-mouse HRP-conjugated antibody (1:1000) in 2 % Marvel.

ii. **Small-scale expression of scFv by IPTG induction**

   Small-scale cultures of *E. coli* TOP10F’ cells were grown overnight in 5 ml of 2xYT containing 100 µg/ml ampicillin and 1.0 % glucose. A 25 ml culture of 2x YT containing 100 µg/ml ampicillin and 1.0 % glucose was set up from this culture with a starting OD$_{600}$ of 0.05. This culture was grown at 37°C at 250 rpm until the OD$_{600}$ reached 0.9–1.0 and centrifuged to remove the glucose. The cells were resuspended in 25 ml of 2x YT containing 100 µg/ml ampicillin and 0.5 mM IPTG and grown for 4-16 h at 25°C with shaking (225 rpm). Cells were harvested and a periplasmic extraction carried out as described in section 2.7.3. Both the supernatant and the periplasmic extract were analysed on SDS-PAGE gels.
iii. **ELISA analysis of scFvs**

Wells of a microtitre plate were coated with NCAM1-Ig1 as described in Section 2.8.11. After blocking and washing, 50 µl of periplasmic extract in 2 % Marvel/PBS was added to wells and incubated at room temperature for 1 h. The wells were washed with 200 µl of 0.1 % Tween 20/PBS four times followed by four washes with 200 µl of 1x PBS. A 100-µl volume of anti-c-myc produced in mouse (clone 9E10) in 2 % Marvel (1:5000) was added per well was incubated for 1 h at room temperature. The wells were washed as described previously and 100 µl of anti-mouse HRP-conjugated antibody (1:1000) in 2 % Marvel was added to each well for 1 h at room temperature. After incubation, the wells were washed as described previously and 100 µl of TMB substrate was added to each well. Reactions were stopped using 1 M H₂SO₄ and absorbance (OD₄₅₀) was measured.

For inhibition ELISAs, the periplasmic extract or purified scFvs were pre-incubated with varying concentrations of NCAM1-Ig1 at room temperature while plate wells were blocked. The remainder of the procedure is described in section 2.8.13(iii).

For purified scFvs, the wells of a microtitre plate were coated with NCAM1-Ig1 in PBS and stored overnight at 4° C. The ELISA was carried out as above with the exception that 50 µl of purified B5 scFv were added to the wells and incubated at room temperature for 1 h. Washes, antibody incubation and reaction development were as described above.

For inhibition ELISAs, plate wells were coated with 100 µg/ml NCAM1-Ig1 in PBS while scFvs were incubated with varying concentrations of NCAM1-Ig1 prior to addition to the wells. Antibody detection and reaction development were as described above.

### 2.9 Cell culture

#### 2.9.1 Culture of mammalian cells

All cell culture was performed aseptically in a class II laminar flow hood and the cell lines were grown as horizontal cultures in 25 cm²/75 cm² tissue culture flasks in a 5 % CO₂ incubator at 37° C. Cell lines were fed every 2-3 days. Once cultures reached 80 % confluency, the cells were passaged. The media was removed and the cells were washed with Hanks Balanced salt
solution before 2 ml of trypsin was added to the cells. The flask was placed in the incubator for 5-10 min to aid trypsinisation. The trypsinised cells were neutralised on addition of 10 ml of media; 2 ml of these cells were added to a 75 cm² tissue culture flask containing 10 ml of media. Astrocytes were fed with Dulbecco's Modified Eagle Medium/Ham’s F-12 supplemented with 10 % FBS and 1 % penicillin/streptomycin. Nucleus pulposus and annulus fibrosis cells received Dulbecco's Modified Eagle Medium supplemented with 1 % penicillin/streptomycin.

2.9.2 Cell handling protocol

For thawing, a vial of cells was removed from liquid nitrogen storage and quickly thawed in the 37°C water bath. Cells were removed with a sterile transfer pipette and transferred to a 25 cm² flask containing 5 ml growth medium. The culture was grown overnight at 37°C in 5 % CO₂. Media was carefully removed from the flask and fresh media was added.

Cells were counted using a Neubauer haemocytometer and trypan blue. An aliquot of the cell suspension was diluted in an equal volume of trypan blue and 20 µl was pipetted onto the counting chamber of the haemocytometer. Cells were counted in multiple squares and the total number of cells was calculated, taking into account the relevant dilutions. Viable cells formed clear bright cells while the dead cells were stained blue.

To freeze cells, the trypsinised cells with added medium were transferred to a sterile 15 ml tube and centrifuged for 5 min at 1200 g. The supernatant was discarded and the pellet was resuspended using freezing media (DMSO:FBS, 1:9; v/v), slowly added to the pellet. The suspension was mixed and aliquotted into pre-cooled cryovials (1 ml/vial). The vials were stored for 2-3 days at -80°C prior to being stored in liquid nitrogen.

2.10 Cell binding studies

2.10.1 Conjugation of scFv to FITC

Conjugation of the scFv to FITC was carried out as described by Kantor (Kantor and Roederer, 2004). Purified scFv (1 ml) was dialysed in 500 ml of reaction buffer overnight at 4°C in 7000 MWCO dialysis tubing. A volume of
50 µl of fluorescein (1 mg/ml in DMSO) was added to 950 µl of the dialysed scFv, adding 10 µl every 10 min with constant stirring. After stirring for 1 h, the tube was covered in aluminium foil to prevent photo bleaching. Unreacted FITC was removed by dialysis for 24 h in storage buffer with two buffer changes. Aliquots of storage buffer were retained during the coupling reaction and the fluorescence was read at 480 nm excitation and 520 nm emission to monitor coupling.

2.10.2 ELISA analysis using NCAM1-Ig1 and conjugated scFv-FITC

The wells of a microtitre plate were coated with dilutions of NCAM1-Ig1 in PBS (starting concentration of 338 µg/ml) and stored overnight at 4° C. Wells were washed four times with 200 µl of sterile 1x PBS before being blocked with 200 µl of 2 % SMP/PBS for 2 h at room temperature. The washing step was repeated as before, following which 100 µl of a 1:10 dilution of FITC-conjugated B5 scFv was added to wells and plates were incubated at room temperature for 1 h. The wells were washed with 200 µl of 0.1 % Tween 20/PBS four times before reading the fluorescence. A 100-µl volume of polyclonal anti-fluorescein HRP-conjugated antibody (1:5000) was added to each well and incubated at room temperature for 1 h, before repeating the previous washing step. Colour development and measurement were as described in section 2.8.11.

2.10.3 Immunocytochemical assay using rat astrocytes

The wells of an eight-well chamber slide (Lab-tek™ slides, Thermo Scientific) were washed with Hanks Buffer salt solution before coating with poly-L-lysine solution and incubating at 37° C for 1 h. After removal of the poly-L-lysine, wells were washed with Hank’s Buffer salt solution followed by media before seeding with 20,000 astrocytes per well and incubating for 1-2 days at 37° C. The media was removed and the cells were washed twice with 1x PBS (pre-cooled). The cells were fixed using 4 % paraformaldehyde and incubated at room temperature for 15 min. After removal of the paraformaldehyde, the wells were washed twice with pre-cooled 1x PBS before blocking with 1 % BSA/PBS for 30 min at room-temperature. Afterwards, the wells were washed three times for 5 min each and 2.8-5 µg of the scFv (a
control scFv or the FITC-conjugated scFv) in 0.1 % BSA was added to each well, followed by incubation overnight at 4° C. Wells with the conjugated scFv–FITC were blocked with 1 % BSA/PBS and the antibody was incubated in 0.1 % BSA/PBS overnight at 4° C. Wells were washed three times for 5 min each with 0.1 % Tween 20/1x PBS on a rocking apparatus. A 100-µl volume of primary antibody (mouse monoclonal anti-polyhistidine, 1:200) was added in 0.1 % BSA/PBS followed by incubation for 1 h at room temperature. Afterwards, wells were washed three times for 5 min each with 0.1 % Tween 20/1x PBS with rocking. A goat anti-mouse Alexa Fluor® 488-conjugated antibody (100 µl of 1:500 dilution) was added and incubated for 1 h at room temperature (covered in aluminium foil). The washing step was repeated and 100 µl of DAPI (1:1000) in PBS was added to each well and incubated at room temperature for 10 min followed by two washes of 5 min each with rocking. Excess liquid was removed from the chamber slide before adding Prolong® Gold anti-fade reagent to preserve fluorescence. Analyses were carried out in parallel with a commercial mouse monoclonal anti-NCAM1 (“ERIC1”) antibody and the goat anti-mouse reporter antibody.

2.10.4 Immunocytochemical assay using bovine nucleus pulposus and annulus fibrosis cells

The procedure was carried out as described above for rat astrocytes, with the following modifications: wells were blocked using 1 % goat serum; 100 µl of primary antibody (mouse monoclonal anti-polyhistidine, 1:50) in 0.1 % goat serum was incubated in wells for 1 h at room temperature; with a 1:500 dilution of goat anti-mouse Alexa Fluor® 488-conjugated antibody was used; and a parallel antibody combination of polyclonal rabbit anti-myс (1:10) and goat anti-rabbit Alexa Fluor® 488-conjugated antibody (1:500) in 0.1 % BSA/PBS was used for detection.

2.10.5 Immunohistochemistry of bovine spinal and IVD sections

Sections were collected on Superfrost® Plus slides (Fisher Scientific Inc., Dublin, Ireland) and stored at -20° C until use. Sections were washed three times (5 min each) with PBS prior to treating with proteinase K at 20 µg/ml (Sigma) at 37°C for 5 min, followed by three further PBS washes.
blocking with 2 % BSA in PBS at room temperature for 1 h, tissues were washed three times with PBS and incubated with 5 µg scFv in 0.2 % BSA/PBS overnight. After three 5 min washes with PBS containing 0.1% Tween 20, tissues were incubated with a 1:50 dilution of a mouse monoclonal anti-polyhistidine IgG (Sigma, H1029) or a 1:15 dilution of a rabbit polyclonal anti-myc IgG (Santa Cruz Biotechnologies, SC789) in 0.2 % BSA/PBS for 1 h at room temperature. Three PBS/0.1% Tween 20 washes (5 min each) were followed by antibody detection using goat anti-rabbit Alexa Fluor® 488-conjugated antibody (Invitrogen Technologies, A11008) or goat anti-mouse Alexa Fluor® 488-conjugated antibody (Invitrogen Technologies, A11001) as appropriate, diluted 1:500 in 0.2 % BSA/PBS, for 1 h at room temperature. Afterwards, tissues were washed three times with PBS/0.1 % Tween 20 for 5 min each. Nuclei were stained using a 1:1000 dilution of DAPI in PBS. After incubating for 10 min at room temperature, the slides were washed twice with PBS. The fluorescence was preserved using Prolong® Gold anti-fade reagent.

Prior to staining, tissue sections were treated using proteinase K and/or chondroitinase ABC (Sigma) or with neither treatment. Chondroitinase ABC (20 U/ml) was made up to 0.250 U/ml in 0.1 M TRIS-acetate buffer pH 8. After addition of chondroitinase ABC, the section was incubated at 37° C for 30 min followed by three 5 min washes with PBS. Proteinase K treatment was carried out after chondroitinase ABC, as described above. PBS was added to the sections receiving no treatment to prevent drying out during the incubations.

2.10.6 Immunohistochemistry – inhibition study using scFv and bovine IVDs

Samples for inhibition studies were prepared by incubating 2-5 µg of the scFv with different concentrations of NCAM1-Ig1 (8-32 µg) at room temperature for 1 h prior to addition to cells or tissues. NCAM1-Ig1 (5 µg) was also incubated with a range of concentrations of B5 scFv (2.5-10 µg). The volume was kept constant for each reaction by the addition of PBS. After blocking, the ‘out-competed samples’ were incubated with the tissue section and the protocol was followed as described in section 2.10.5. NCAM1-Ig1 (5-10 µg) was also incubated with the tissue section as a control.
2.10.7 Flow cytometry

After harvesting bovine nucleus pulposus and annulus fibrosus cells, the cells were washed with PBS and centrifuged for 5 min at 700 g. All centrifugation steps were carried out at 4°C. Cells were resuspended in PBS and strained using a sterile cell strainer (70 µm nylon mesh, Fisherbrand). After counting (section 2.9.2), 10^6 cells were added to each tube, centrifuged for 5 min at 1000 g and resuspended in 200 µl of PBS. The scFv (5 µg) was added to the cells and incubated at 4°C for 1 h. The suspension was washed with 3 ml of PBS/0.1 % Tween 20 and centrifuged for 5 min at 700 g. A volume of 200 µl of mouse monoclonal anti-polyhistidine IgG (1:200) in 1x PBS was added to the relevant wells and incubated at 4°C for 1 h. Afterwards, the wash step was repeated using 0.2 % Tween/PBS. Goat anti-mouse Alexa Fluor® 488-conjugated antibody (200 µl of a 1:500 dilution) was added to the cells and incubated, covered in foil, for 1 h at 4°C. After washing with 0.1 % Tween, the cells were resuspended in 1 ml of PBS and analysed by flow cytometry.

Subsequently, the following changes were made to improve the above protocol; 2 % goat serum/PBS was used for blocking for 30 min at 4°C before the cells were incubated with 5 µg of the scFv; antibodies were incubated in 0.2 % goat serum/PBS; 5 µg of rabbit polyclonal anti-6X His tag® FITC-conjugated antibody was used for single staining; and a 1:200 dilution of goat anti-mouse Alexa Fluor® 488-conjugated antibody was used in the double staining procedure.

scFv binding was also detected using a rabbit polyclonal anti-myc IgG (200 µl of 5 µg/ml) followed by goat anti-rabbit Alexa Fluor® 488-conjugated antibody (200 µl of 5 µg/ml). Cells were incubated with 2 % goat serum and subsequent antibody incubations were carried out in 0.2 % goat serum/PBS. Controls carried out to ensure specificity of the signal included: 200 µl of 5 µg/ml of commercial anti-NCAM1 ERIC1 monoclonal antibody followed by goat anti-mouse Alexa Fluor® 488-conjugated antibody (200 µl of 5 µg/ml); goat anti-mouse Alexa Fluor® 488-conjugated antibody alone (200 µl of 5 µg/ml); mouse monoclonal anti-polyhistidine IgG (200 µl of 5 µg/ml) followed by goat anti-mouse Alexa Fluor® 488-conjugated antibody (200 µl of 5 µg/ml); and 5 µg scFv followed by goat anti-mouse Alexa Fluor® 488-conjugated antibody (200 µl of 5 µg/ml).
In an effort to reduce non-specific binding observed with the anti-polyhistidine reporter antibody, cells were blocked with 7 % human serum for 15 min on ice before addition of 5 µg of scFv in 3 ml of 0.2 % Tween/PBS and centrifugation of cells at 350 g for 5 min. The pellet was resuspended in 200 µl of 7 % mouse serum and incubated on ice for 15 min, 5 µg of mouse anti-polyhistidine antibody was added to the cells and incubated for 1 h on ice. After the addition of 3 ml of 0.2 % Tween/PBS, cells were centrifuged at 350 g for 5 min. The pellet was resuspended in 200 µl of 7 % goat serum and incubated on ice for 15 min. An aliquot corresponding to 5 µg of goat anti-mouse Alexa Fluor® 488-conjugated antibody was added to the cells and incubated for 1 h on ice. The cells were washed with 3 ml of 0.2 % Tween/PBS at 350 g for 5 min, centrifuged and the resulting pellet was resuspended in 500 µl of 1x PBS and analysed by flow cytometry using a BD FACSCanto A.
Chapter 3: Results

3.1 Generation, production and purification of NCAM1-Ig1

3.1.1 Choice of target: NCAM1 as the candidate marker

Candidate target genes were chosen based on previously published microarray and real-time RT-PCR analysis of patients with varying degrees of degeneration as defined by the Thompson scale. The criteria used for choosing a candidate target gene were (i) upregulation on nucleus pulposus (NP) compared to annulus fibrosus (AF) or articular cartilage cells and (ii) expression of the protein on the cell surface for availability for targeting with antibodies. NCAM1 was first identified as a potential nucleus pulposus marker in canine studies incorporating microarray analysis and evaluation of molecules of interest using quantitative real-time RT-PCR and immunohistochemistry (Gajghate et al., 2009). The beagle dog was used in this study as this breed is chondrodystrophoid which means that the notochordal cell population is absent in the nucleus pulposus of adult dogs and their pattern of disc degeneration is comparable to that seen in humans. In addition, in a human IVD study carried out by Grad et al (2010), NCAM1 was more (10 times) highly expressed in nucleus pulposus cells than in annulus fibrosis (AF) or articular cartilage (AC) cells as determined by real-time RT-PCR (Rutges et al., 2010). Investigation of the properties of NCAM1 identified that it is expressed at the cell surface with a large, characteristic seven-domain structure (Fig.3.1) exposed at the external cell surface (Rønn et al., 1998; Nielsen et al., 2008). These two studies and the previous characterisation of NCAM1 properties and subcellular location led to the protein being chosen as the candidate marker for nucleus pulposus targeting in this work.

NCAM1 is an integral membrane glycoprotein that can regulate both cell-cell (homophilic) and cell-substrate (heterophilic) interactions (Rønn et al., 1998; Atkins et al., 2004). It is expressed on neural cell types including neurons, astrocytes and oligodendrocytes (Krushel et al., 1999; Fewou et al., 2007). It belongs to the cell adhesion molecules of the immunoglobulin superfamily that are characterised by the presence of an Ig-like domain. The extracellular part of
NCAM1 is composed of five Ig-like domains and two fibronectin type III-like domains (Fig. 3.1). The construct designed and generated in this project consisted of the outermost Ig domain of NCAM1, denoted NCAM1-Ig1.

Fig. 3.1 Schematic representation of the structure of the NCAM1 protein. The C-terminus is buried in the cytoplasm and the N-terminus protrudes from the cell surface (Nielsen et al., 2008).

Fig. 3.2 Partial amino acid sequence of human NCAM1 (111 amino acids): amino acids 1-19 (red) are the signal sequence and the first domain (Ig1) consists of amino acids 20-111 (green) - UniProtKB/Swiss-Prot P13591 (NCAM1_HUMAN).

3.1.2 Cloning of NCAM1-Ig1 domain for recombinant expression

After retrieval of the human NCAM1 sequence from GenBank (BCO47244), an overlap PCR experiment and primers were designed to generate and clone the outermost NCAM1-Ig1 domain (Figs. 3.3 and 3.4) into the pIG6 expression vector in use in our research group (Ge et al., 1995). The domain sequence for NCAM1 was obtained from UniProtKB/Swiss-Prot P13591 (Fig. 3.2) – NCAM1 sequence is provided in Appendix I. An N-terminal, ompA signal peptide sequence was added to the gene in cloning to direct transport of the translated protein to the periplasm and a C-terminal hexa-histidine tag was introduced for purification of the mature protein and detection by Western blotting.
The individual polymerase chain reactions (PCRs) to generate the intermediate fragments were optimised firstly by using different concentrations of template DNA; altering the annealing temperature; using a combination of different annealing temperatures (gradient PCR) and using the initial amplicon to serve as a template for subsequent PCRs. In the case of the pIG6 fragment containing the *ompA* leader sequence, an amplicon was readily generated at an annealing temperature of 53°C and following excision from an agarose gel, it
was purified using an extraction kit and re-electrophoresed on a 2 % agarose gel (Fig. 3.4(A)).

Fig. 3.4 Analysis by agarose gel electrophoresis of generation of NCAM-Ig1 construct. (A). Purified pIG6-ompA PCR product. Lane 1: Molecular weight marker X (Roche); lane 2: purified PCR product. The expected size of product is 143 bp. (B) Result of optimised amplification of NCAM1-Ig1 domain-encoding gene. Lane 1: No-template control; lane 2: Molecular weight marker X (Roche); lane 3: PCR product. The expected size of the PCR product is 311 bp (circled). The circled band was purified to homogeneity and re-evaluated on an agarose gel prior to overlap PCR. (C) and (D) Overlap PCR products generated using templates from (A) and (B). Lane 1: pIG6 vector template DNA; lane 2: Molecular weight marker XIV (Roche); lane 3: NCAM1-Ig1 template DNA; lanes 4-6: overlap PCR products obtained using different molar ratios of the initial products. As the expected size of product was 402 bp, the overlap PCR product corresponding to this size (brightest band in (C)) was excised and purified (lane 7) for cloning.

No product was amplified in the initial PCRs of the NCAM1-Ig1 domain. Upon modification of template concentration, lowering of annealing temperature, use of graded annealing temperatures, increasing the number of cycles and addition of MgCl₂ and DMSO to act as a chaotropic agent, a mixed product containing the expected sized product (311 bp) and a number of additional bands
was obtained, suggesting mispriming/non-specific binding of the primers. Although additional bands were present, the band of interest was also present, indicating that the PCR required optimisation. Upon repeating the PCR reaction with an annealing temperature ($T_a$) of 50°C for 15 cycles, 53°C for 10 cycles and 56°C for 10 cycles, a more intense PCR product of the expected size and with fewer additional bands due to the reduced mispriming was obtained (Fig.3.4(B)). Following excision of this band, it was purified and electrophoresed on a 2% agarose gel.

Following amplification of the individual NCAM1-Ig1 fragments, overlap PCR reactions were carried out using a number of concentrations and relative ratios of the initial products. The pIG6 vector fragment and the NCAM1-Ig1 fragment were allowed to hybridise, followed by amplification of the desired full length product using pIG forward primer and NCAM Ig1 reverse primer (Heckman and Pease, 2007). After 30 cycles of PCR with an annealing temperature of 50°C, a product of the expected size of 402 bp was visible upon gel electrophoresis, thus suggesting that the full-length construct had been produced (Fig.3.4(C)). The product of interest was excised and purified (Fig.3.4(D)). Due to a high $A_{260}/A_{280}$ ratio, the purified product was cleaned using a PCR Clean-up kit and quantified at 9 ng/µl before cloning.

Following clean-up of the overlap PCR product, it was cloned using the Zero Blunt® TOPO® PCR cloning kit which allows for the direct insertion of blunt-end PCR products into a plasmid vector. The linearised plasmid vector (pCR®-Blunt II-TOPO®; 3519 bp) has Vaccinia virus DNA topoisomerase I covalently bound to the 3’ end of each DNA strand and accepts PCR products with compatible ends as inserts, with topoisomerase facilitating their ligation. The transformation efficiency was increased by the addition of salt to the cloning reaction and increasing the incubation time from 5 minutes to 30 minutes. The salt allows for longer incubation times, preventing topoisomerase I from rebinding and possibly nicking the DNA after ligation. Electrocompetent E. coli cells were transformed with the resultant recombinant plasmid (pCR®-Blunt II-TOPO® construct) using an insert:vector ratio of 9:1 and plated onto LB/kanamycin plates.

The three resulting transformants were screened by EcoRI restriction digestion. Two of the three yielded appropriately sized inserts (Fig.3.5) with
sequences identical to each other (Fig.3.6(A)) and to the expected NCAM1-Ig1 domain sequence. The annotated sequence is shown in Fig.3.6(B). The third clone wasn’t further analysed as it did not contain an insert. After sequence confirmation, the inserts of the expected size were subcloned into the pIG6 expression vector using EcoRI and XbaI for recombinant protein production. Successful clones were identified by restriction digestion.

Fig.3.5 Restriction analysis of NCAM1-Ig1 clones. Two transformants were screened using EcoRI enzyme. Lane 1: Molecular weight marker X (Roche); lanes 2 and 3: plasmid DNA from clones 1 and 3 digested with EcoRI, respectively.

Fig.3.6(A) Sequence alignment of NCAM1-Ig1 clones 1 and 3. The signal sequence (ompA) is coloured red; the database-derived sequence of NCAM-Ig1 is coloured blue; the EcoRI and XbaI restriction sites are coloured green and orange, respectively.
Fig.3.6(B) Annotated predicted amino acid sequence of the NCAM1-Ig1 clones generated using ExPASy translate tool. The nucleotides or amino acids corresponding to the features of interest are coloured using the scheme in Figure 3.6(A).

3.1.3 Recombinant production of NCAM1-Ig1

Small-scale expressions of the two NCAM1-Ig1 clones were set up in *E. coli* TOP10 cells. Growth of cultures after induction using 0.5 mM IPTG at 25°C is shown in Fig.3.7(A). Culture aliquots equivalent to 1.0 OD\textsubscript{600} unit of cells were taken hourly for preparation of whole cell extracts. From the immunoblot, it can be seen that the highest yield of NCAM1-Ig1 protein was obtained after 3 h induction (Fig.3.7(B)). Samples for analysis were normalised per cell density. *E. coli* W3110 cells were transformed with the pIG6/NCAM1-Ig1 vector to test this strain in further NCAM1-Ig1 expression experiments.

Fig.3.7 Growth of *E. coli* TOP10 cells during NCAM1-Ig1 expression by IPTG induction (A) and immunoblot analysis of NCAM1-Ig1 produced in *E. coli* TOP10F' cells (B). Whole cell extracts from two NCAM1-Ig1 clones were collected at different time points post-IPTG induction. Lanes 1 and 2: pre-induction samples for clones 1 and 3, respectively; lane 3: PageRuler Plus Prestained marker; lanes 4-6: +1 h, +3 h and +24 h.
h post-induction, respectively (clone 1); lanes 7-9: +1 h, +3 h and +24 h post-induction, respectively (clone 3). Lane 10 contains a control protein for the immunoblot.

_E. coli_ W3110 cells were transformed with the pIG6/NCAM1-Ig1 (clone 3) vector to test this strain for NCAM1-Ig1 production using IPTG induction and autoinduction. Firstly, small-scale expressions were carried out as previously described using the transformed cells and induction by 0.5 mM IPTG. Whole cell extracts for the pre-induction and post-induction samples were analysed by immunoblotting (Fig.3.8(B)), with the corresponding OD$_{600}$ values presented in Fig.3.9. No noticeable difference in NCAM1-Ig1 cellular yield was noted after 2 h of induction.

**Fig.3.8** Analysis of NCAM1-Ig1 expression upon IPTG induction in _E. coli_ W3110 by SDS-PAGE followed by Coomassie blue staining (A) or immunoblotting (B). Recombinant NCAM1-Ig1 is estimated to be 11.3 kDa (red arrowed). Lane 1: PageRuler Plus Prestained marker; lane 2: Whole cell extracts from _E. coli_ cells pre-induction; lane 3: 1 h post-induction; lane 4: 2 h post-induction; lane 5: 3 h post-induction; lane 6: 4 h post-induction; lane 7: 5 h post-induction; lane 8: 6 h post-induction.

**Fig.3.9** Growth of _E. coli_ W3110 cells during NCAM1-Ig1 expression using IPTG induction.
NCAM1-Ig1 was also expressed overnight in E. coli W3110 using autoinducing media where the lac promoter is induced by the presence of lactose after glucose has been depleted from the medium. Protein expression was induced by auto-induction for 18, 24 and 48 h after which OD$_{600}$ values of 5.03, 5.63 and 4.86, respectively, were measured. Cell samples were harvested before induction for analysis (whole cell extract). After extraction from the periplasm, soluble and insoluble fractions and whole cell extracts were analysed; these revealed that the highest protein yields were reached after 24 h of production. Therefore, this expression was repeated in 500 ml culture volumes for increased yield to facilitate protein purification. No recombinant protein was detected in the insoluble protein fractions in either expression experiment (Fig.3.10).

Fig.3.10 Analysis of auto-induction expression of NCAM1-Ig1 in E. coli W3110 strain for 18, 24 and 48 h by immunoblot. Recombinant NCAM1-Ig1 is estimated to be 11.3 kDa (red arrowed). Lane 1: PageRuler Plus Prestained marker; E. coli W3110 cells harvested: lane 2: before induction (whole cell extract); lanes 3-5: after 18 h induction; lanes 6-8: after 24 h induction; lanes 9-11: after 48 h induction. Whole cell extracts are in lanes 3, 6 and 9; soluble fractions are in lanes 4, 7 and 10 and insoluble fractions are in lanes 5, 8 and 11. Lane 12 contains a control protein for the immunoblot.

3.1.4 Purification of NCAM1-Ig1

After extraction of soluble protein from the periplasm of expressing E. coli cells and overnight dialysis to remove salts used during extraction, the resulting dialysate was purified by immobilised metal affinity chromatography (IMAC). This is based on a high-affinity interaction between the hexa-histidine tag in the recombinant protein and immobilised Ni$^{2+}$ ions that enables specific
purification of appropriately tagged proteins. Contaminant proteins are removed by incubation with increasing concentrations of imidazole which competes with proteins for binding to the immobilised Ni$^{2+}$.

![Fig.3.11](image)

**Fig.3.11** Analysis of IMAC purification of NCAM1-Ig1 by (A) Coomassie blue stained SDS-PAGE and (B) Immunoblot. Lane 1: Biorad broad range molecular weight marker; lane 2: pre-column sample; lane 3: column flow-through; lanes 4-6: 20 mM, 50 mM and 80 mM imidazole column washes, respectively; lanes 7–14: NCAM1-Ig1 eluted using 100 mM imidazole; lanes 15-19: protein eluted using 300 mM imidazole; lane 20: protein eluted using 500 mM imidazole; lane 12: control protein for immunoblot.

After washing with increasing concentrations of imidazole to remove *E. coli* proteins, the recombinant protein was eluted using 100 mM and 300 mM imidazole (arrowed in Fig.3.11). The IMAC protocol was optimised by introducing more washing steps and washing with higher concentrations of imidazole, resulting in a clean, apparently pure protein sample (by Coomassie blue staining and visual inspection) that was suitable for phage display work. After purification, the eluted fractions containing purified protein were pooled and dialysed in PBS at 4°C overnight to remove imidazole; the pooled sample was analysed by SDS-PAGE after dialysis to confirm the protein was intact (Fig.3.12).
Fig. 3.12 Analysis of purified NCAM1-Ig1 for phage display work; Coomassie blue stained SDS-PAGE. Lane 1: PageRuler Plus Prestained marker; lane 2: pooled fractions of IMAC-purified and dialysed NCAM1-Ig1 protein.

The Biorad DC protein assay was utilised to measure the concentration of the purified protein. This is a colourimetric assay for determining protein concentration based on the Lowry assay. Typical total protein yields from a 1 l culture of NCAM1-Ig1 expressed in *E. coli* W3110 strain were 1-1.5 mg.
3.2 Identification of NCAM1-Ig1 binders using phage display

3.2.1 Biopanning of NCAM1-Ig1 using the Yamo I library

Prior to panning, immobilisation of the recombinant NCAM1-Ig1 on immunotubes was investigated using an anti-polyhistidine HRP-conjugated antibody. A rapid colour change was observed upon addition of substrate to NCAM1-Ig1 coated immunotubes (Fig.3.13), indicating successful immobilisation of the antigen. For panning, a large, non-immunised human scFv library of over $10^8$ scFv clones (Pansri et al., 2009) was used. The high diversity of this library results from the rearrangement of variable region genes from one hundred and forty non-immunised donors. The titer of repropagated phage for panning round 1 was determined to be $6 \times 10^8$/ml while the KM13 helper phage titre was $4.8 \times 10^{12}$/ml.

![OD450 vs Samples]

Fig.3.13 Immobilisation test demonstrating detection of hexahistidine tag on immunotubes coated with NCAM1-Ig1 or BSA (each 100 µg/ml). Bars represent the mean of three replicate wells for each condition and the error bars refer to the standard deviation value.

For panning, immunotubes were coated overnight with 500 µg/ml (round 1 only) or 50 µg/ml NCAM1-Ig1. Phage enrichment was investigated by titering phage and calculating input/output ratios for each panning round (Table 3.1).
Table 3.1 Titors of input and output phage for each panning round

<table>
<thead>
<tr>
<th>Panning round</th>
<th>NCAM1-Ig1 conc (µg/ml)</th>
<th>Input phage (pfu/ml)</th>
<th>Eluted phage (pfu/ml)</th>
<th>Blocking agent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>50</td>
<td>9 x 10^{11}</td>
<td>1.28 x 10^{7}</td>
<td>BSA</td>
</tr>
<tr>
<td>1b</td>
<td>500</td>
<td>9 x 10^{11}</td>
<td>3.9 x 10^{6}</td>
<td>BSA</td>
</tr>
<tr>
<td>2</td>
<td>50</td>
<td>2.43 x 10^{11}</td>
<td>1.2 x 10^{6}</td>
<td>BSA</td>
</tr>
<tr>
<td>3</td>
<td>50</td>
<td>6.2 x 10^{10}</td>
<td>7.4 x 10^{6}</td>
<td>BSA</td>
</tr>
<tr>
<td>4</td>
<td>50</td>
<td>3.9 x 10^{10}</td>
<td>3.9 x 10^{7}</td>
<td>BSA</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>2 x 10^{11}</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ELISA analysis of the polyclonal phage population after each round of panning demonstrated an increase in binding after round 3 (R3), thus indicating that the population had become enriched (Fig. 3.14). A ‘no phage’ control was used to compare to background binding of the anti-M13 HRP-conjugated antibody.

![Phage pools after rounds of panning](image)

Fig. 3.14 ELISA analysis of NCAM1-Ig1 binding of eluted polyclonal phage-scFv populations. P0: Unpanned library. P1-P4: Polyclonal phage preparations eluted after rounds 1 to 4 of library panning against NCAM1-Ig1. NCAM1-Ig1 was coated on plates at 100 µg/ml. ‘No antigen’ wells were also included for analysis.

3.2.1.1 Screening of the polyclonal phage pool after R3

Ninety-two randomly selected colonies from round 3 were screened for NCAM1-Ig1 binding. From these, 4 consistently high NCAM1-Ig1 binders were identified (Fig. 3.15). After transformation of the non-suppressor strain *E. coli* TOP10F’ to allow expression of the scFvs in a soluble (unfused) format, scFvs
were expressed using 1 mM IPTG induction for 20 h at 30°C. No binding of NCAM1-Ig1 was detected using the periplasmic extracts in an ELISA.

Fig. 3.15 Screening of the polyclonal phage population after panning R3. NCAM1-Ig1 binders D8, F3, F4 and H3 were consistently positive in repeated experiments. Bars represent the mean of three replicate wells for each condition and the error bars refer to the standard deviation value.

3.2.1.2 Further analysis of putative NCAM1-Ig1 binders

E. coli supernatants containing scFvS produced by IPTG induction of the positive clones were used in an ELISA but no NCAM1-Ig1 binding was detected. Western blot analysis of culture supernatants, whole cell pellets and periplasmic extracts also failed to detect scFv in any of these clones (data not shown).

A second small-scale expression of the four clones with highest signals in phage ELISA (D8, F3, F4 and H3) and four randomly selected clones from the unpanned library was carried out but no scFvs were again detected in any of the clones even though restriction digest analysis confirmed the presence of a gene of the expected size in a number of these clones.
Sequencing of three of the previously positive anti-NCAM1-Ig1 clones (D8, F3 and H3) revealed point mutations resulting in a stop codon in the scFv linker region, with an additional stop codon found in V_H FW4 in clone H3 (Fig.3.16). As a result, the V_L is not expected to be translated in the host E. coli cells, explaining the absence of detectable scFv in immunoblots targeting the C-terminal myc and hexahistidine tags. In order to determine if this point mutation was introduced during panning or was present in the original library, phagemid DNA from two randomly selected, unpanned library colonies was sequenced and these did not contain the mutation, indicating that the point mutation was introduced during panning (not shown).

![Fig.3.16 Predicted amino acid sequences of scFv genes from clones D8, F3 and H3. Framework regions are highlighted in green, CDRs in pink, the gIIIp leader sequence in light blue. The hexahistidine and myc tags are highlighted in blue and yellow, respectively. The stop codons arising from the point mutations are highlighted in red.](image)

Sequencing of the F4 clone, however, revealed an amber stop codon in V_H FW2 at position 40 (Fig.3.16) which explains the apparently contradictory results of its high binding in phage-displayed form – when it was produced in the amber-suppressing E. coli TG1 strain (Fig.3.15) – but absence of binding or
detectable scFv when expressed in soluble format in the non-suppressor E. coli TOP10F’. To facilitate expression and purification of the F4 scFv for further characterisation, the amber stop codon was changed to glutamine by site-directed mutagenesis.

3.2.2 Generation of F4 mut scFv

3.2.2.1 Generation of F4 mut scFv by overlapping extension PCR

Overlapping extension PCR was used to convert the stop codon in V\textsubscript{H} FW2 of the F4 scFv to a ‘CAG’ (glutamine) followed by cloning into the pIG6 expression vector (Fig.3.17). ‘CAG’ was selected as it is the most abundant
glutamine codon in *E. coli*. An N-terminal, *ompA* signal peptide sequence was also added to direct transport of the translated protein to the periplasm and a C-terminal hexahistidine tag for purification and detection of the protein by Western blotting (Fig. 3.18).

![Fig. 3.18 Design of F4 mut scFv construct (936 bp). The construct contains an N-terminal, *ompA* signal peptide sequence and a C-terminal hexahistidine to direct transport of the translated protein to the periplasm and to facilitate its purification and detection, respectively. Excision of the insert using *Xba*I and *Hind*III results in a product of 895 bp. Details of primer sets used in reactions are outlined in Table 3.2.](image)

Three sets of primers were used to generate the F4 mut scFv fragment (Table 3.2). The overlap PCR reactions were optimised to produce an amplification product for each target region and the final overlap product was cloned into TOPO® cloning vector (Fig. 3.18). After sequence confirmation, the constructs were excised from the TOPO® vector and subcloned into the pIG6 expression vector for recombinant protein production. Restriction digest analysis confirmed that subcloning had been successful. The sequences of F4 and F4 mut scFv are provided in Appendix II.

### Table 3.2 Primers used to generate F4 mut scFv construct

<table>
<thead>
<tr>
<th>Product</th>
<th>Primer</th>
<th>Template</th>
</tr>
</thead>
<tbody>
<tr>
<td>Product 1</td>
<td>1F - pIG Forward</td>
<td>pIG6 vector</td>
</tr>
<tr>
<td><em>(ompA – F4 VₜH FW1)</em></td>
<td>1R - ompAF4 Reverse</td>
<td></td>
</tr>
<tr>
<td>Product 2</td>
<td>2F - ompAF4 Forward</td>
<td>F4 scFv</td>
</tr>
<tr>
<td><em>(VₜH FW1 – VₜH FW2)</em></td>
<td>2R - F4mut Reverse</td>
<td></td>
</tr>
<tr>
<td>Product 3</td>
<td>3F - F4mut Forward</td>
<td>F4 scFv</td>
</tr>
<tr>
<td><em>(VₜH FW2 – VₜL FW4, 6His)</em></td>
<td>3R - F4topIG6 Reverse</td>
<td></td>
</tr>
<tr>
<td>Product 1+2 (intermediate)</td>
<td>1F - pIG Forward</td>
<td>Product 1 and Product 2</td>
</tr>
<tr>
<td></td>
<td>2R - F4mut Reverse</td>
<td></td>
</tr>
<tr>
<td>Product 2+3 (intermediate)</td>
<td>2F - ompAF4 Forward</td>
<td>Product 2 and Product 3</td>
</tr>
</tbody>
</table>
Fig. 3.19 Generation of F4 mut scFv construct. (A) Amplification of three separate regions yielded Products 1 (137 bp), 2 (155 bp) and 3 (711 bp) using primers outlined in Table 4.2. These products were joined by overlap PCR to generate intermediate products using respective primers (B) which was later linked to Product 1 to generate the final product (C). Lane 1: 100 bp molecular weight marker; lane 2: Product 1; lane 3: Product 2; lane 4: Product 3; lane 5: Product 1+2; lane 6: Product 1; lane 7: Product 2; lane 8: Product 3; lanes 9 and 10: Product 2+3; lanes 11 and 12: Product 1+2+3.

### 3.2.2.2 Expression of F4 mut scFv

The F4 mut scFv was expressed in the non-suppressor *E. coli* strain BL21 using both auto-induction and IPTG induction approaches and yielded low levels of scFv of the predicted size of 28.1 kDa (Fig. 3.20). Yields of purified F4 scFv were 160 µg from 1 l (total protein yield). Work with the F4 mut scFv clone and optimisation of expression was continued by another researcher in the lab for continued evaluation of the potential usefulness of this scFv in IVD targeting and no further work was carried out with the clone in this project.
Fig. 3.20 Western blot analysis of F4 mut scFv expression following (A) 0.5 mM IPTG induction and (B) autoinduction in E. coli BL21. The estimated size of the protein as determined using Uniprot is 28.1 kDa – red arrowed. For (A); Lane 1: molecular weight marker; lane 2: pre-induction; lanes 3, 4, 5, 6, 7, 8 and 9: post-induction samples at 1 h, 2 h, 3 h, 4 h, 5 h, 6 h and 24 h, respectively. Lane 10: purified NCAM1-Ig1. For (B); lanes 2, 5 and 8: whole cell extract following 18 h, 24 h and 48 h induction, respectively. Lanes 3, 6 and 9: soluble fractions following 18 h, 24 h and 48 h induction, respectively. Lanes 4, 7 and 10: insoluble fractions following 18 h, 24 h and 48 h induction, respectively. Lane 11: purified NCAM1-Ig1.

3.2.3: Panning #2 of NCAM1-Ig1 using the Yamo I library

3.2.3.1 Second biopanning of NCAM1-Ig1 using the Yamo I library

During analysis and mutagenesis of the F4 scFv clone, panning of the Yamo I library was repeated to identify additional NCAM1-Ig1 binding scFvs. In order to reduce non-specific binding, the blocking agent was alternated for each round of panning (P1: Marvel; P2: BSA; P3: Ovalbumin; P4: Marvel and P5: BSA). The titer of repropagated phage for panning round 1 was $6 \times 10^{11}$/ml, thereby exceeding the original library size. The titer of KM13 helper phage was $6.4 \times 10^{12}$/ml. Immunotubes were coated with 100 µg/ml of NCAM1-Ig1 in PBS and bound phages were eluted using trypsin and glycine-HCl (pH 2.0) Enrichment of the phage population was observed after each successive round of panning (Table 3.3).

<table>
<thead>
<tr>
<th>Round</th>
<th>NCAM1-Ig1 conc (µg/ml)</th>
<th>Input phage (pfu/ml)</th>
<th>Eluted phage (pfu/ml)</th>
<th>Blocking agent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100</td>
<td>$6 \times 10^{11}$</td>
<td>$2.25 \times 10^{7}$</td>
<td>Marvel</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>$4 \times 10^{11}$</td>
<td>$3.2 \times 10^{6}$</td>
<td>BSA</td>
</tr>
<tr>
<td>3</td>
<td>100</td>
<td>$1.2 \times 10^{12}$</td>
<td>$1.5 \times 10^{4}$</td>
<td>Ovalbumin</td>
</tr>
<tr>
<td>4</td>
<td>100</td>
<td>$1.9 \times 10^{11}$</td>
<td>$2.1 \times 10^{7}$</td>
<td>Marvel</td>
</tr>
</tbody>
</table>
3.2.3.2 Polyclonal phage ELISA

After four rounds of panning, an anti-NCAM1-Ig1 polyclonal phage ELISA was carried out and demonstrated an increase in binding after round 4 in repeated experiments (Fig.3.21).

![Fig.3.21 ELISA analysis of NCAM1-Ig1 binding of eluted polyclonal phage-scFv populations. P0: Unpanned library. P1-P4: Polyclonal phage preparations eluted after rounds 1 to 4 of library panning against NCAM1-Ig1. NCAM1-Ig1 was coated on plates at 100 µg/ml. Bars represent the mean of three replicate wells for each condition and the error bars refer to the standard deviation value.](image)

3.2.3.3 Screening of polyclonal phage pools by monoclonal phage ELISA

After the fourth round of panning, 160 individual clones were screened in monoclonal phage ELISA, from which potential NCAM1-Ig1 binders were identified. Additional panning was continued with the NCAM1-Ig1 coating concentration reduced from 100 µg/ml to 25 µg/ml in the fifth round and 3 % BSA used as the blocking agent (Table 4.3). While an increase in binding was observed after the additional round, a population of high affinity BSA binders was subsequently detected in the eluted phage, indicating that phage had non-specifically bound to BSA during this final panning round.
3.2.3.4 Sequencing of the putative NCAM1-Ig1 binders

scFv-encoding genes of clones that demonstrated NCAM1-Ig1 binding during monoclonal phage ELISA were sequenced. Sequence analysis of 6 of the clones using IgBlast revealed intact, full-length scFv antibody fragments. Of these, clones B5 and E3 were identical (Fig.4.22) as were A7, G3, H2 and H3 (Fig.3.23). Partial scFv sequences obtained from clones A6 and H4 were identical to the latter group. This group was found to be 87% identical and 90% homologous to an anti-EBV (Epstein Barr virus) LMP1 (latent membrane protein)-binding scFv A4 (synthetic construct) by BLAST analysis (accession number, ABA55014.1). The former group was 77% identical and 84% homologous to anti-EBV LMP1 scFv B8 (synthetic construct) - accession number, ABA55013.1.

![Fig.3.22 Alignment of predicted amino acid sequences of clones B5 and E3. The framework regions are highlighted in pink, CDRs in green and the linker in blue.](image-url)
3.2.3.5 Phage inhibition ELISA

Inhibition ELISAs were carried out to determine if the phage/NCAM1-Ig1 binding could be inhibited by pre-incubating the phages of interest with NCAM1-Ig1 in solution. Pre-incubation is expected to result in reduced antigen binding as the NCAM1-Ig1 in solution will compete for phage binding with the immobilised molecules. B5 and A6 phages were used as representatives of the two groups of antibody fragments isolated from the panning. The signal decreased with increasing NCAM1-Ig1 concentrations used for inhibition in the case of B5 phage (Fig.3.24) whereas pre-incubation of A6 phage with different concentrations of NCAM1-Ig1 did not reduce its binding to the immobilised NCAM1-Ig1 (not shown). Therefore B5 scFv was chosen for further studies as it consistently produced desired results in ELISA analysis.
Fig. 3.24 Inhibition ELISA with B5 scFv (phage) using different concentrations (300 µg/ml, 100 µg/ml and 25 µg/ml) of NCAM1-Ig1 for pre-incubation with scFv prior to binding to immobilised antigen. Bars represent the mean of three replicate wells for each condition and the error bars refer to the standard deviation value.

3.2.4 Expression of putative NCAM1-Ig1 binder

3.2.4.1 Expression of clone B5 in non-suppressor E. coli TOP10F’ cells

The periplasmic extracts and cell supernatants generated from non-suppressor E. coli TOP10F’ cells were analysed by ELISA and Western blotting; no scFv(s) were detected by anti-polyhistidine HRP-conjugated antibody in either case.

3.2.4.2 Expression of clone B5 in non-suppressor E. coli HB2151 cells

Phagemid DNA from clone B5 was used to transform non-suppressor strain E. coli HB2151 cells followed by protein expression using 1 mM IPTG or auto-induction. Whole cell extracts, soluble and insoluble protein fractions were prepared and analysed by SDS-PAGE (Figs. 3.25 and 3.26).
Fig. 3.25 SDS-PAGE analysis by (A) Coomassie staining and (B) western blotting of B5 scFv expression in E. coli HB2151 cells using 1 mM IPTG for overnight induction. Lane 1: molecular weight marker; lanes 2 and 3: positive control for anti-polyhistidine HRP-conjugated antibody; lanes 4, 5, 6 and 7: whole cell extract, insoluble fraction, soluble fraction (fresh) and soluble fraction (stored at -20°C) for clone B5, respectively; the expected size of B5 scFv product is 28.3 kDa (red arrowed).

Fig. 3.26 SDS-PAGE analysis of B5 scFv by (A) Coomassie staining and (B) western blotting following autoinduction in E. coli HB2151. Lane 1: molecular weight marker; lanes 2, 5 and 8: whole cell extract following 18 h, 24 h and 48 h induction, respectively. Lanes 3, 6 and 9: insoluble fractions following 18 h, 24 h and 48 h induction, respectively. Lanes 4, 7 and 10: soluble fractions following 18 h, 24 h and 48 h induction, respectively. Lane 11: purified NCAM1-Ig1. The estimated size of the protein as determined using Uniprot is 28.3 kDa (red arrowed).

3.2.4.3 Purification of B5 scFv

Purification of B5 scFv was carried out using IMAC (Fig. 3.27). The predicted $M_r$ of the scFv is 28.3 kDa which was consistent with western blotting analysis. Denatured B5 scFv was detected by anti-polyhistidine and anti-c-myc HRP-conjugated antibodies in immunoblots (data not shown). Detection of the
scFv with two different antibodies allows its binding to be validated which can be important in immunocytochemistry and immunohistology for confirmation of binding. However, it must be noted that the scFv is used in its native state for these detection strategies.

Fig. 3.27 (A) Coomassie staining and (B) western blot analysis of purification of B5 scFv (arrowed). Lane 1: molecular weight marker; lane 2: pre-column sample; lane 3: sample flow-through; lanes 4–6: washing with 20 mM, 50 mM and 80 mM imidazole, respectively; lanes 7–12: eluted fractions of B5 scFv using 100 mM imidazole. Total protein yield from this purification from a 1 l culture was 720 µg. The predicted size of the protein is 28.3 kDa (red arrowed).

3.2.5 Assessing putative NCAM1-Ig1 binders in ELISA using purified scFv

As the phage-displayed B5 scFv exhibited NCAM1-Ig1 binding in standard ELISA and inhibition ELISA experiments, soluble B5 scFv was also screened for NCAM1-Ig1 binding in ELISA (Figs. 3.28 and 3.29). B5 scFv bound NCAM1-Ig1, from neat (267 µg/ml) to 0.0267 µg/ml (Fig. 3.28) while its binding (at 67 µg/ml scFv) could be clearly inhibited by pre-incubation with soluble NCAM1-Ig1 domain at concentrations up to 267 µg/ml (Fig. 3.29).
Fig. 3.28 ELISA analysis binding of purified B5 scFv to NCAM1-Ig1 (267 µg/ml to 0.0267 µg/ml). ‘SMP’ represents skimmed milk powder. ‘NCAM1-Ig1 - no B5’ received PBS instead of scFv. Bars represent the mean of three replicate wells for each condition and the error bars refer to the standard deviation value.

These results indicated that the B5 scFv bound the most external domain (NCAM1-Ig1) of NCAM1 and had potential for use in cell targeting and drug delivery in the IVD region.

Fig. 3.29 Analysis of NCAM1-Ig1 binding of B5 scFv by inhibition ELISA. Binding to immobilised NCAM1 (100 µg/ml) was inhibited by pre-incubation of the purified scFv with varying concentrations of soluble NCAM1-Ig1. SMP: binding to skimmed milk powder control. Bars represent the mean of three replicate wells for each condition and the error bars refer to the standard deviation value.
3.2.6 Coupling of B5 scFv to fluorescein for fluorescent labelling

The fluorescent dye fluorescein isothiocyanate (FITC) reacts with free amino groups (primarily the amine groups of lysine residues) of proteins or peptides to form a stable thiourea bond and thus produce the desired dye–protein conjugates. B5 scFv was coupled to FITC for immunofluorescence studies to visualise binding of the scFv to the candidate marker protein ex vivo. After coupling of B5 scFv, unbound FITC was removed by dialysis. Some protein loss was incurred during the coupling and multiple dialysis steps, from 63.4 µg/ml to 26 µg/ml. The procedure was monitored by measuring the fluorescence at each stage (Fig.3.30). The increase in fluorescence after the first dialysis was due to unbound FITC in the storage buffer while the low fluorescence after the second dialysis indicates that most of the unbound FITC was removed in the initial dialysis.

Fig.3.30 Coupling of B5 scFv to FITC. The reaction was monitored by measuring the fluorescence before and after each dialysis step.

The ability of the coupled B5 to bind NCAM1-Ig1 antigen was confirmed by ELISA (Fig.3.31). The concentration-dependent binding indicated that the coupled B5 scFv had potential as a visualisation tool to detect NCAM1 in immunofluorescence studies in vitro and ex vivo.
Fig. 3.31 ELISA binding analysis of detection of immobilised NCAM1-Ig1 using coupled B5 scFv-FITC. Detection of the coupled scFv was carried out using anti-FITC HRP-conjugated antibody. Controls included included a ‘no scFv’ well and a ‘no antigen well’. Bars represent the mean of three replicate wells for each condition and the error bars refer to the standard deviation value.
3.3: From identification of NCAM1 as a cell marker to demonstration of *ex vivo* binding of B5 scFv

3.3.1 Demonstration of *ex vivo* binding of B5 scFv to rat astrocytes

A cell assay for NCAM1-Ig1 binding by the purified B5 scFv was established using rat astrocytes as they have been reported to have high levels of NCAM1 expression (Rønn *et al.*, 1998; Gajghate *et al.*, 2009). After expression and IMAC-based purification of the B5 scFv, specific staining of astrocytes was visible using the scFv, detected using mouse anti-polyhistidine and FITC-labelled goat anti-mouse antibodies. No staining was detectable using a control scFv (2H12) with an irrelevant binding specificity (Fig. 3.32). Optimisation of the immunocytochemical detection was achieved by increasing the incubation time of the scFv with cells from 1 h to overnight, the use of goat serum instead of bovine serum albumin for blocking as the secondary antibody was of goat origin, and removal of scFv aggregates by filtration or brief centrifugation prior to use. The most effective optimisation was achieved by increasing the concentration of Tween 20 in the washes (from 0.05 % to 0.2 %) and increasing the number of washes. Initial experiments with a FITC-conjugated B5 scFv yielded a low cell-specific signal in repeated analyses, which may be due to a reduced active scFv concentration after the coupling reaction. Subsequent staining experiments were carried out using the uncoupled scFv and a sandwich format to amplify the signal for improved detection.
Fig. 3.32 Immunocytochemical analysis of rat astrocytes using B5 scFv (5 µg), followed by anti-histidine (mouse) and anti-mouse-FITC (goat) reporter antibodies – image (A). Detection of the scFv on the cell surface is indicated by the FITC-labelling, represented by the green staining. Cell nuclei were stained using DAPI, represented by the blue staining. A control scFv was included to demonstrate the specificity of B5 scFv – image (B). The ‘no anti-histidine’ reporter antibody control is shown in image (C) and binding of a commercial anti-NCAM1 antibody in image (D).

3.3.2 Demonstration of ex vivo binding of B5 scFv – bovine nucleus and annulus fibrosis cells

Binding of the B5 scFv to bovine nucleus pulposus and annulus fibrosis cells was also carried out as these cell types are the intended target for drug delivery in vivo. After optimisation of antibody concentrations, clear binding of the B5 scFv to both AF and NP cells was observed (Fig. 3.33), albeit with a slightly higher degree of non-specific binding than had been observed with the rat astrocytes.

NCAM1 was initially identified in microarray studies as having considerably higher expression in nucleus pulposus than annulus fibrosus (AF) cells (Sakai et al., 2009). However, similar levels of binding to NP and AF cells were observed in this investigation (Fig. 3.33). As NCAM1 was previously characterised in human- and canine-derived microarray data which were later
validated by real-time PCR experiments using human cells, it is possible that the difference (up to 10-fold) in NCAM1 gene expression between these two cell types is not found in bovine cells. The conjugated secondary antibody (goat anti-mouse FITC) exhibited no detectable binding to the NP or AF cells – data not shown.

3.3.3 Immunohistochemistry using B5 scFv and bovine spinal cord sections

A commercial anti-NCAM1 monoclonal antibody was used in immunohistochemistry experiments to demonstrate that NCAM1 was expressed in the tissue sections. Bovine spinal cord sections were subjected to
chondroitinase ABC and proteinase K treatments, in combination and separately, before staining. No binding was evident when the tissues were treated with chondroitinase ABC whereas the scFv exhibited clear binding to proteinase K-treated bovine sections that could be detected using either anti-polyhistidine or anti-myc antibodies and their respective secondary antibodies (Fig.3.34).

3.3.4 Immunohistochemistry using B5 scFv and bovine IVD sections

The commercial anti-NCAM1 monoclonal antibody also detected NCAM1 expression in bovine IVD tissue sections – 6 month and 2 year old samples (data not shown). The scFv demonstrated binding to both the NP and the AF of the 6 month old samples and to a lesser extent in the 2 year old samples using a rabbit polyhistidine antibody and an anti-rabbit Dylight 568-conjugated reporter antibody (Fig.3.35 (A) - (D)). Pre-incubation of B5 scFv with an excess of soluble NCAM1-Ig1 before staining inhibited the B5 scFv binding by occupying the scFv binding pocket before incubation to cells. These experiments clearly demonstrated the ex vivo binding capacity of B5 scFv to bovine IVD tissues and supported its potential as a targeting tool for delivery to human IVD cells in vivo also.
Fig.3.35 Immunohistochemical analysis of B5 scFv binding to bovine IVD tissues using a rabbit polyhistidine antibody and an anti-rabbit Dylight 568-conjugated reporter antibody. Images (A) and (E): six months old NP tissue; images (B) and (F): six months old AF tissue; Images (C) and (G): two years old NP tissue; Images (D) and (H): two years old AF tissue. Images (A)-(D) demonstrated binding of B5 scFv to bovine IVD tissue. The signal was inhibited by pre-incubating B5 scFv with soluble NCAM1 before staining as shown in the corresponding images (E)-(H). Cell nuclei are stained blue (DAPI) while scFvs attached to the cell surface are stained red (Image courtesy of Dr. Akshay Srivastava).

Attempts to detect bovine IVD cell binding by B5 scFv using flow cytometry was consistently unsuccessful. Passaged and non-passaged cells were used in experiments as it was thought that there may be a loss of cell surface marker expression following cell passage. A FITC-conjugated anti-hexahistidine antibody was employed for direct detection to avoid extensive cell loss during the extensive washing required in the staining procedure but no specific binding signal could be obtained. Similarly, a sandwich detection format using an unconjugated anti-myc and an anti-polyhistidine antibody was utilised but the anti-polyhistidine antibody exhibited a high level of non-specific binding that did not allow further investigation.
Chapter 4: Discussion

4.1 Discussion

The goal of this project was to develop a targeting mechanism to facilitate regeneration of the intervertebral disc. The three specific objectives towards achieving this goal were: recombinant production of a candidate protein marker for nucleus pulposus and/or annulus fibrosis cells; isolation of a single chain antibody fragment (scFv) that bound the marker; and \textit{in vitro} characterisation and validation of binding of the scFv to IVD cells \textit{ex vivo}. This body of work describes the isolation of a recombinant antibody fragment (scFv) that binds the most external domain of NCAM1, NCAM1-Ig1; it is anticipated that further development of a cell-targeting, nanoparticle-mediated delivery mechanism to NCAM1-expressing intervertebral disc cells, while outside the scope of the present study, will continue upon its completion.

The candidate target protein was selected based on data from collaborators’ microarray and real-time PCR studies of IVD cells (Gajghate \textit{et al.}, 2009; Rutges \textit{et al.}, 2010). These data were generated in studies of chondrodystrophic dogs and comparative studies of humans with varying degrees of degeneration, as defined by the Thompson scale. Potential biomarkers that allowed nucleus pulposus cells to be distinguished from annulus fibrosis cells in the former approach were further evaluated by real-time RT-PCR analysis of gene expression in human cells. The reason for using beagles is that they, like some other breeds of dog, are chondrodystrophic, meaning that the notochordal cell population gradually disappears as the young adult ages, in common with human intervertebral discs. Neural cell adhesion molecule (NCAM1) was identified from these studies as significantly more highly expressed (up to 10-fold) in NP than AF or cartilage cells, despite having a low expression level overall in the study cells (Rutges \textit{et al.}, 2010).

NCAM1 was chosen as a protein target for drug delivery due to its higher expression in NP than other co-IVD-resident cell types as disc degeneration is proposed to begin in the nucleus pulposus (Freemont \textit{et al.}, 2001; Anderson and Tannoury, 2005). The presence of a large extracellular module in the protein was
also expected to facilitate antibody recognition and binding of NCAM1-expressing cells. Nevertheless, a fundamental question throughout much of this project remained whether it is actually possible to identify a genuinely NP-specific marker, given the reported similarities between NP and AF cells (Gruber et al., 2010; Minogue et al., 2010; Power et al., 2011). Other candidate markers reported during the course of the project included periostin, which is expressed in the IVD of the sand rat and humans; in this case the outer annulus region has been reported to contain the highest proportion of periostin-positive cells (88.8 %) compared to just 18.5 % periostin-positive cells in NP cells in humans, as determined by immunohistochemical localisation and microarray analysis (Gruber et al., 2010). Further evaluation of periostin, an extra-cellular matrix protein involved in fibrosis, identified an upregulation in degenerated discs and an expression pattern of significant induction in response to stress in degenerated but not non-degenerated NP cells (Tsai et al., 2013).

Recombinant protein production (RPP) is a well-developed technology of expression of heterologous proteins in recombinant expression hosts. One of the most commonly used hosts is E. coli, which is frequently preferred over eukaryotic or other prokaryotic options based on its low cost, high product yields, simplicity and extensive development of the necessary cloning and procedures (Ferrer-Miralles et al., 2009). Initial expression of a foreign gene in E. coli often results in low protein yields, however, leading to a requirement for optimisation of the expression protocol. Strategies typically employed for optimisation include: screening of E. coli strains with different molecular or metabolic properties; modification of the induction procedure, particularly with respect to duration or temperature of induction; and supplementation of molecular chaperone-encoding genes to improve folding or secretion of the recombinant polypeptide. In this work, successful cloning of the NCAM1-Ig1 gene into the expression vector was followed by a pilot expression using 0.5 mM IPTG in E. coli TOP10 cells. An autoinduction approach was also investigated in E. coli W3110 strain. Autoinduction of recombinant proteins in E. coli is based on diauxic growth (glucose-lactose shift) of the host cells on lactose after exhaustion of more preferred carbon sources in the medium. It was developed for high-level protein expression with pET vectors and other IPTG inducible
expression systems and has gained significantly in popularity in the RPP field in recent years (Studier, 2005a). It combines a period of high-density growth on preferred nutrients, such as glucose, leading to high cell densities, with automatic induction of protein expression from \( lac \)-based promoters by lactose in the medium upon depletion of glucose and removal of its \( lac \) promoter inhibitory effect. The glucose concentration in the medium is established so that induction is initiated in the mid to late log phase of growth (Studier, 2005b), which is traditionally determined in IPTG induction procedures by continuous sampling and monitoring of cultures. Using this autoinduction approach, Li and colleagues reported a final yield of three target proteins – GFP, glutathione-S-transferase tagged GFP and human fibroblast growth factor – of approximately 4 times higher than with IPTG induction, which was due to the higher final biomass achieved in the expressing cultures. The proteins were produced using a T7 promoter and pET vectors in \( E. coli \) DE3 lysogens such as BL21 (DE3) and Rosetta 2 (DE3) strains (Li et al., 2011). In the present study, while no difference in protein yield was observed for induction periods of longer than 2 h using IPTG, higher protein yields were obtained using autoinduction, as determined by immunoblotting. This observation is consistent with numerous reports of higher protein yields with autoinduction than IPTG-based induction approaches (Attrill et al., 2009; Ukkonen et al., 2013). Further investigation of expression of the NCAM1-Ig1 gene could involve cloning into a pET vector with a T7\( lac \) promoter and expression in \( E. coli \) BL21 (DE3) cells but for the purpose of the current study, yields of purified protein that were sufficient for antibody isolation were obtained without further modification of the expression protocol.

Protein can be extracted from the \( E. coli \) periplasm using a variety of methods, including chloroform treatment, sonication, freeze-thaw methods or osmotic shock treatment. NCAM1-Ig1 was extracted using a modified osmotic shock treatment in this work (Hu et al., 2005; Gunnarsen et al., 2010), incorporating a pretreatment of cells with 5 mM \( Ca^{2+} \) prior to osmotic shock, which has been reported to improve recovery of a recombinant creatinise enzyme from 60 % to 75 % (Chen et al., 2004). Purification of the expressed protein was carried out using immobilised metal affinity chromatography (IMAC), in which a high-affinity interaction between a recombinant hexa-histidine tag on the target
protein and immobilised nickel (Ni\(^{2+}\)) or cobalt (Co\(^{2+}\)) ions on commercial resins or beads is exploited to yield simple and rapid single-step purification of a tagged recombinant protein to almost homogeneity. Immobilised nickel was used in this work though the efficiency of cobalt-linked carboxymethyl aspartate supports has been reported to be superior by some authors (Chaga et al., 1999; Pina et al., 2013). A high purity and yield of the purified NCAM1 domain was evident using a nickel nitriloacetic acid resin and so the use of a Co\(^{2+}\)-based strategy was not investigated.

Phage display is an *in vitro* library screening technique that allows polypeptides or proteins with desired binding properties to be isolated from a large combinatorial collection of molecules. A human scFv library developed by Pansri and colleagues was employed in this study to identify a scFv ligand for the NCAM1-Ig1 domain. This naïve library has a repertoire of 1.5 x 10\(^8\) clones and was constructed using antibody genes from non-immunised donors, which effectively increases its antibody diversity by avoiding the immunologic bias associated with antigen-selected collections (Pansri et al., 2009). The availability of such a human scFv library is highly advantageous as the longterm goal of this work is to translate the research into a clinical drug delivery application. The human origin of the Yamo I library (Pansri et al., 2009) is such that no additional engineering of isolated antibodies is required for humanisation or to avoid a human anti-mouse antibody (HAMA) response *in vivo* (Presta, 2006; Kim et al., 2010; Lee et al., 2013). Other researchers have previously raised a murine-derived monoclonal antibody (“ERIC1”) against NCAM1 and humanised the \(V_H\) and \(V_L\) regions to reduce their immunogenicity *in vivo* (Whittington et al., 2001), though humanisation of antigen-binding pockets in this manner can be labour-intensive, expensive, may not completely remove immunogenicity (Harding et al., 2010) and can affect the affinity for antigen or expression properties of the relevant antibody (Safdari et al., 2013). This emphasises the importance of using a human scFv library when the application is patient/therapy-related. The advantages of the use of the scFv format over whole antibody molecules or other fragment formats, meanwhile, include their improved ability to penetrate solid tissues such as tumours, the fact that they typically retain the specificity and affinity for antigen of the parent antibody, the ease with which they can be
genetically manipulated by mutagenesis to improve their immunogenicity, stability, specificity and affinity, and their improved biodistribution and clearance properties due to their small size (Weisser and Hall, 2009; Hu et al., 2011; Srivastava et al., 2014b).

Two panning experiments were carried out to identify a NCAM1-Ig1 binder. In the first experiment, immunotubes were coated with two different concentrations of NCAM1-Ig1 (50 µg/ml and 500 µg/ml) while the antigen concentration remained the same for successive rounds at 50 µg/ml. The reason for initial coating with a higher antigen concentration was to recover as many binders as possible in the initial stages rather than just a small number of high-affinity molecules as any non-specific, weakly bound phage would be removed during the subsequent washing steps. The level of stringency was increased throughout the panning through increasing the concentration of Tween 20 in the wash solution and the duration of washes. Four strong NCAM1-Ig1 binders were identified using this panning procedure despite its relatively low level of stringency. Of these, clone F4 was the outstanding candidate and demonstrated high binding to immobilised NCAM1-Ig1 repeatedly in monoclonal phage ELISAs. In the second panning experiment, the NCAM1-Ig1 concentration used in coating was increased to 100 µg/ml for all rounds except the last round, in which 25 µg/ml NCAM1-Ig1 was used in order to restrict the population to high affinity binders. The blocking agent was also alternated for each round of panning to avoid selection of block-specific antibodies, though this still occurred in the final panning round, resulting in the isolation of numerous BSA-binding phages.

The panning experiments identified two scFvs that bound to NCAM1-Ig1 with different levels of affinity. The first clone, F4, contained a stop codon in VH FW2 that prevented its full-length expression in a non-suppressor E. coli strain. The occurrence of stop codons in library clones arises from the use of PCR to artificially construct scFvs from randomly combined VH and VL genes derived from naïve B cells (Pokorny et al., 2008). The amber stop codon was changed to glutamine by site-directed mutagenesis, allowing expression of the F4 scFv in soluble format for purification and characterisation. The resultant F4 mut scFv gene was cloned for expression with an ompA leader sequence to transport the
scFv to the periplasmic space for disulphide bond formation and correct folding. High yields of numerous antibody fragments expressed in the periplasmic space have been reported by various groups but in this work the yield of purified F4 scFv remained low, which can result from properties such as plasmid copy number, characteristics of the expression host, or the leader sequence or promoter. Another possible reason for low expression levels of recombinant proteins can be the presence of rare codons that are favoured in the source organism of the cloned gene. Rare codons can lead to growth arrest of cells and premature translational termination in recombinant protein production, with the likelihood of high protein expression level correlated to a codon adaption index (CAI; www.genscript.com). In silico optimisation of the F4 mut scFv sequence revealed the possibility to increase the CAI by codon optimisation from its current value of 0.62 to 0.79 – just under the 0.8 value generally rated as compatible for expression - with a concomitant increase in usage of high frequency codons from 37% to 60% (expressed as percentage of the total gene codons) and reduction in the usage of low frequency codons from 8% to 3% in the newly designed gene. While this investigation of the F4 scFv gene does not necessarily indicate an incompatibility between the component human V\textsubscript{H} and V\textsubscript{L} genes and the E. coli translation machinery, it appears possible from the analysis that codon optimisation of the scFv gene for expression in E. coli might lead to higher yields of translated product. Other researchers have successfully used this codon optimisation approach to improve periplasmic expression of a humanised scFv (anti-Hepatitis B surface antigen) in E. coli, resulting in a 100-fold increase in soluble periplasmic expression (Tiwari et al., 2010). A less labour intensive strategy to initially investigate whether codon bias is limiting translation efficiency is by using an E. coli strain such as Rosetta (DE3) which encodes additional copies of tRNA molecules for codons that are commonly used in eukaryotes but rarely used in E. coli. This approach has found considerable success with Tegel and colleagues who reported increased yields in 35 of 68 proteins tested in their extensive study (Tegel et al., 2010; Huang et al., 2012).

The second panning procedure led to the isolation of clone B5, which ultimately became the focus of the remaining work, and seven other NCAM1-Ig1 binders. Six of the clones were identical to each other and the remaining two
clones were also identical, indicating a strong selection pressure favouring scFv antibody fragments that bound the immobilised NCAM1-Ig1. The B5 clone group performed better in inhibition ELISAs and so soluble B5 scFv was expressed in E. coli HB2151 amber suppressor cells and purified using IMAC. Protein yields were limited somewhat by leakage of scFv from cells into the extracellular environment during the overnight autoinduction expression and so purification of additional scFv molecules from the medium, scale-up of expression or other optimisation approaches to increase the yield of purified protein might have benefitted subsequent FITC-conjugation of the scFv through the availability of higher scFv concentrations. This conjugation approach was designed to provide labelled anti-NCAM1-Ig1 scFv for ease of detection of cell targeting in immunohistochemical studies but provisional analyses indicated that the use of a signal-amplifying reporter antibody yielded better results than this direct detection approach.

Demonstration of the \textit{ex vivo} binding of the isolated scFv is important because of its intended application in targeting of cells expressing the full length NCAM1 molecule. NCAM1 consists of five Ig-like domains followed by two Fn-like domains that are closest to the membrane. The Ig-like domains exhibit the classical immunoglobulin domain conformation of two anti-parallel beta sheets, packed together face to face and stabilised by a hydrophobic core which is formed by the side chains of amino acids from both beta sheets (Soroka \textit{et al.}, 2010). As the conformation of the Ig-like domains in NCAM1 is not expected to be influenced by their neighbouring domains, the scFv library was panned against the most external Ig-like domain of NCAM1 that had been produced recombinantly in \textit{E. coli}. Binding to the NCAM-Ig1 domain was validated \textit{in vitro} using standard and inhibition ELISAs, while \textit{ex vivo} binding was confirmed in a cell assay using rat astrocytes which express NCAM1, as confirmed using a commercial anti-human NCAM1 mouse IgG (Krushel \textit{et al.}, 1999). The advantages of setting up the assay using astrocytes rather than IVD cells include the ease of availability of the primary astrocyte cell line compared to the NP/AF cells, which were difficult to obtain and required specialised personnel for harvesting.
Successful labelling of the rat astrocytes using the B5 scFv led to the assay being extended to bovine nucleus pulposus and annulus fibrosis cells as these are the intended target for the organ culture model and gene delivery in vivo. The ability of the scFv to bind NCAM1 in both the astrocyte assay and in NP/AF cells in bovine spinal cord and IVD sections demonstrates that the B5 scFv also recognises its target epitope when presented in the form of the complete NCAM1 molecule. This approach of expression of component domains derived from complex, often membrane-bound proteins has previously been employed in studies such as the isolation of scFvs against extra-domain B fibronectin (EDB-Fn) from tumours (Johdi et al., 2013), tumour endothelial marker 1 (TEM-1) from newly forming blood vessels in tumours (Marty et al., 2006) and carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) from aggressive melanomas and carcinomas including thyroid and pancreatic cancer (Moricoli et al., 2014). Nevertheless, this successful binding of NCAM1 was essential to confirm that recombinant production of the isolated NCAM1-Ig1 domain in the E. coli periplasm generated a correctly folded domain protein structure. Failure of the scFv to bind cells or tissues expressing NCAM1 could indicate an inability of the recombinant expression host to express – or, more likely, correctly fold – the domain independently of the rest of the protein.

The affinity and specificity for antigen are important properties of a cell-targeting antibody or antibody fragment intended for use in in vivo delivery applications. Strategies for demonstrating specificity and/or affinity include immunofluorescence using cells and tissue sections, flow cytometry and surface plasmon resonance. The availability of a monoclonal antibody allows for a comparison of binding between the scFv and the monoclonal antibody which validates the specificity of the scFv, but not direct comparison of their affinities due to the different signal-reporting mechanisms utilised. In this work, the commercial murine anti-human NCAM1 mouse IgG confirmed expression of NCAM1 in the target tissues and the scFv exhibited a similar binding to the control monoclonal antibody in binding and inhibition studies with bovine spinal cord and intervertebral discs. Furthermore, the commercial monoclonal anti-NCAM1 antibody exhibited a higher signal intensity in binding studies than the scFv although the fluorescence intensity wasn’t quantified. This lower signal
could be due to the lower number of binding sites in the scFv due to its monovalency as observed in previous comparison of a scFv and mAb against hepatoma and normal liver tissues (Tungpradabkul et al., 2005). Parker and colleagues also reported similar findings with their scFv against prostate specific membrane antigen (PSMA) but they attributed the lower intensity binding of the scFv to a lower degree of Alexa Fluor 488 labelling of the smaller protein molecule (Parker et al., 2013). The binding strength of scFvs can be increased by dimerisation of the fragment (Cyranka-Czaja et al., 2012) or by the use of site-directed random mutagenesis of CDRs to increase affinity (Dong et al., 2003), amongst other approaches.

The advantages of the scFv format chosen for this project include its ease of and lower cost of production compared with whole antibodies, while scFvs can also be engineered to improve their affinity or introduce novel properties. The importance of the inhibition study results and the lack of binding from a control scFv clearly indicated the potential for cell specific targeting of the isolated scFv. In the absence of flow cytometry data on antigen binding by the B5 scFv, the inhibition IHC results with six-month and two-year old bovine discs is an important demonstration of the specificity of binding of the antibody fragment. The bovine discs from the two-year old animal are expected to exhibit more degeneration than the 6-month old discs as disc degeneration progresses with age though in this study, no clear pattern of change in NCAM1 expression with age could be determined.

Two techniques commonly employed in investigating gene expression differences between cells or tissues, as in the case of NP and AF cells in this work, are real-time RT-PCR and immunohistochemistry. The former provides data regarding gene upregulation or downregulation in the cell, culminating in a gene expression profile of the cell, and the latter allows for the expression level of a protein product of these genes to be determined. Ideally, there should be a correlation between the data obtained from both techniques though it was noticeable in this study that the pattern of differential expression of NCAM1 in NP in comparison with AF cells reported in previous microarray analyses were not observed in our immunohistochemistry-based investigation of whole cells (Gajghate et al., 2009). Possible reasons for conflicts in data from mRNA and
protein analyses of the same cells include differences in the detection limits of mRNA and protein, instability of the mRNA in vivo leading to inefficient or incomplete translation of transcripts, short half-life of proteins or variations between biological samples. As a precursor to this study, NCAM1 was initially identified as more highly expressed in normal discs from a beagle dog using microarray, real-time reverse transcriptase PCR (qRT-PCR) and immunohistochemistry approaches (Sakai et al., 2009). In their work, NCAM1 had a NP/AF signal log ratio of approximately 1.4 in microarray and real-time PCR results, while its higher protein expression in the NP than in the AF was confirmed by immunohistochemistry. It is important to note that the overall NCAM1 expression was low, however, though numerous other biomolecules with much higher expression levels and higher signal log ratios of NP to AF were discarded in our investigation in favour of NCAM1 based on the latter’s expression on the cell surface and, therefore, potential use in cell targeting.

While one of the stumbling blocks in IVD research is the identification of a suitable animal model for disc degeneration due to differences in gene expression profiles between species, mature caprine and canine discs are often preferred as they are similar to human discs as the notochordal cell population is absent from the invertebral disc in adult animals (Hoogenboom et al., 1998; Sakai et al., 2009). Therefore, this upregulation was validated in human IVD cells when Rutges and colleagues used real time RT-PCR to demonstrate that NCAM1 expression is increased in NP compared to AF cells. While this analysis was based on discs from 11 patients aged 22-81 with no known history of IVD degeneration and mostly assessed at Thompson scores from 1-3 (on the 5 point scale), with just one disc rated as a stage 4, no correlation was observed between age or stage of degeneration and the level of NCAM expression observed (Rutges et al., 2010). Nevertheless, low levels of expression of NCAM1 notwithstanding, it remains to be explained that immunostudies in this work did not identify a similar difference in signal between bovine nucleus pulposus and annulus fibrosis cells. In an independent study, Minogue and colleagues also reported a 13.4-fold change value of NCAM1 between NP and AF cells which was calculated based on the mean fluorescence intensity expression for each cell type from microarray data. They used IVD tissue from young bovine animals (18-36 months) though NCAM1 was not selected in their investigation for
subsequent validation of expression using quantitative real-time PCR (Minogue et al., 2010). As bovine cells were also used in IHC experiments in our work, species differences do not explain the apparent discrepancy in our data compared to other published NCAM1 gene expression profiles. Other possible reasons for this difference include a change in cell surface marker expression due to the extraction and culturing process, or as a result of the collagenase treatment used after the cells are isolated. This has previously been observed in the case of adipose derived stem cells (ADSCs) which lose their stemness after passaging (Guercio et al., 2013). Other researchers have also demonstrated, in comparing procedures for removal of ADSCs from fat tissue, that the extraction method can influence the expression level of differentiation markers (Keck et al., 2014). For this study, one possible approach to investigating the basis for the difference between previous gene expression data and our immunodetection results would be to carry out real-time RT-PCR analysis of NCAM1 gene expression in parallel with the cell stainings to determine whether the apparent discrepancy in expression occurred between the NCAM1 mRNA and protein levels in our samples also.

Targeted scFv delivery is employed to increase cellular uptake of payload-bearing nanoparticles. Targeted delivery of small-molecule drugs can potentially improve the selective killing of tumor cells, as demonstrated when the use of anti-c-Met scFv-conjugated liposomes improved chemotherapeutic drug delivery into tumor cells (Lu et al., 2011). The in vivo success of nanoparticle delivery relies on the availability of suitable targeting molecules that will permit rapid receptor internalisation (Loomis et al., 2010). Qian and colleagues demonstrated increased cellular uptake using anti-CD44v6 scFv-targeted nanoparticles. CD44v6 is a cell surface protein that is overexpressed in pancreatic and gastric cancers. The researchers developed a scFvCD44v6-conjugated PEG-PDLLA nanoparticle (scFv-As-NP) in this work for targeted delivery of As (arsenite ion) to CD44v6-positive tumour cells (PANC-1). Inhibition studies were carried out by pre-incubating the cells with free scFv prior to incubation with the conjugated nanoparticles (scFv-RhB-NPs) which blocked the scFvCD44v6 mediated endocytosis and conjugation of the scFv with arsenite-containing nanoparticles enabled more efficient delivery of As and
exhibited higher cytotoxic activity than non-targeted particles (As-NPs) in human pancreatic cancer cells PANC-1 (Qian et al., 2013). These types of studies demonstrate why scFvs are employed for targeting applications: a combination of their high degree of antigen specificity with their small size and the ease with which they can be genetically manipulated to improve their affinity. Their small size allows more target-binding molecules to be functionalised to the nanoparticle compared to a full length antibody which increases their avidity, while nanoparticles with encapsulated therapeutic moieties or radiotracers can be functionalised with scFvs for targeting or imaging and detection, respectively. Iyer and colleagues reported that their M1 scFv-conjugated immunoliposomes (containing $^{111}$In) exhibited selective tumor targeting and rapid internalisation into both epithelioid (M28) and sarcomatoid (VAMT-1) subtypes of human mesothelioma in vitro and in vivo, demonstrating its potential for targeting of liposomal drugs and radionuclides for imaging and therapy of this malignant disease (Iyer et al., 2011).

It is hypothesised that the development of scFv-functionalised nanoparticles in this study will lead to increased specificity and enhanced uptake of drug-carrying vectors into target cells. Targeted delivery of nanoparticles via scFv molecules has previously been reported to improve not only cell-specific delivery to cells but also to increase cellular uptake of the particles and their payloads. The in vivo success of such a targeting approach relies on the identification of suitable surface molecules that will permit receptor internalisation upon scFv binding (Loomis et al., 2010). Conjugation of c-Met-binding scFvs to liposomes has been demonstrated to improve the delivery of chemotherapeutic drugs into c-Met-expressing tumor cells (Lu et al., 2011) while CD44v6-targeted scFvs increased cellular uptake of arsenate ion from scFv-linked nanoparticles into CD44v6-positive pancreatic and gastric tumour cells (Qian et al., 2013). These and numerous other studies demonstrate the potential power of scFvs in targeting applications, in addition to the advantages associated with their small size, such as enabling more target-binding ligands to be conjugated to a nanoparticle, and the ease with which they can be engineered to improve their clinically relevant properties in vivo. Cell- or tissue-specific scFvs also find application in targeting radiotracers to cells or tissues for imaging and
detection, as in the case of the internalisation of scFv-conjugated immunoliposomes containing $^{111}$In into malignant human mesothelioma cells in vitro and in vivo (Iyer et al., 2011). Current disc restoration strategies emphasise biological approaches to treatment, such as delivery of proteins or proteinase inhibitors, administration of cells or therapeutic genes and tissue engineering approaches (reviewed in chapter 1). While the IVD region is avascular, aneural and has no lymphatic drainage, this potential limitation in terms of access nevertheless provides a contained environment that is ideal for intra-discal injection of genes or growth factors as a potential therapy as the normally short circulatory half-life of scFvs is not a drawback in this setting compared to systemic administration. Coupling of scFvs to drug carriers will also typically increase the half-life of the former due to an increase in size and so reduced clearance of conjugates, while increasing their hydrodynamic volume by PEGylation or albumin fusions have also been demonstrated to extend the half-life of scFvs (Evans et al., 2010; Kontermann, 2011). This can allow increased accumulation in tissues and improved internalisation by receptor endocytosis upon scFv binding. A therapeutic strategy that could regenerate the nucleus pulposus and/or annulus fibrosus in the early stages of degeneration (Thompson, grades I, II or III) and that could slow or prevent further degeneration, or regenerate degenerated tissue, could offer a treatment for restoring disc function. This clinical approach could only be undertaken if expression profiles were identified and validated in all stages of disc degeneration, this would allow degeneration stage-specific targeting. For this study, further analysis of NCAM1 expression in discs of patients with different levels of degeneration is crucial.

Targeted delivery of genes for early-stage IVD regeneration has not yet been reported in the literature and the present findings could contribute to the development of a new therapeutic strategy for early stage disc degeneration. Continued work towards this goal with the isolated B5 scFv in the host group after the completion of this project has involved conjugation of the scFv to polyamidoamine (PAMAM)-coated hyaluronic acid nanoparticles via surface amine groups in order to deliver genes to NP and AF cells in culture and in a bovine organ culture model (Srivastava et al., 2014a).

NCAM1 is also a tumour-associated antigen expressed on many types of
cancers, including small cell lung cancers and neuroblastomas (Jensen and Berthold, 2007; Zecchini and Cavallaro, 2010). The scFv antibody fragment identified in this study could therefore also be employed in either tumour diagnosis or in a cancer-targeting therapeutic approach. Its potential usefulness might be investigated initially in vitro in a cancer cell line such as B104 neuroblastoma which is known to express NCAM1 (Luo and Miller, 1999; Miller et al., 2006). A correlation between upregulation of NCAM1 expression and poor prognosis has also been observed in certain cancers. For example, increased NCAM1 upregulation correlates with cancer progression in neuroblastoma (Zecchini and Cavallaro, 2010). Therefore, NCAM1-binding scFvs such as B5 from this work might, with further study and validation, find both diagnostic (and imaging) and prognostic application in such cancers in vivo, as well as a role in delivery of cytotoxic moieties to treat relevant cancers. Based on the present results, binding of the B5 scFv to NCAM1-expressing cancer cells could be established using a similar immunofluorescence-based approach to that described in this work, followed by validation using patient sera or cerebrospinal fluids as these contain high levels of soluble NCAM1 in many brain tumours. This type of approach might also lend itself to monitoring of a patient’s response to therapy due to the observed fluctuation in NCAM1 levels with prognosis. Other possible developments could include changing the format of the B5 scFv to a diabody, such as by replacing the current fifteen amino acid, Gly-Ser-rich intrapopolypeptide linker with a shorter, five amino acid linker which prevents intramolecular V\textsubscript{H}-V\textsubscript{L} association (Desplancq et al., 1994). This increased valency, and therefore avidity, could improve uptake by multi-epitopic cancer cells, as reported with a human antibody fragment (A11) specific for cancer-associated psoriasin (Cyranka-Czaja et al., 2012). Here, changing the scFv to a diabody format significantly reduced antibody-antigen dissociation in a breast cancer cell line, leading to the potential for a more efficient cell targeting and drug delivery to the target cells. The B104 neuroblastoma cell line would be an ideal cell line to investigate this approach as the cells can grow in a serum-free medium and NCAM1 expression can be stimulated up to four-fold by treating the cells with TGF\beta1 (Luo and Miller, 1999).
The objective of this project was to isolate IVD cell-binding scFv(s) using phage display technology and validate their use in delivery of nanocapsules to IVD cells for disc regeneration. The data presented suggest that the B5 scFv isolated in this work could be employed as a targeting moiety to deliver nanoparticle-encapsulated genes as a potential therapy for early stage IVD regeneration. In vitro and organ culture studies using nucleus pulposus and annulus fibrosus cells with the scFv-tethered nanocapsules will now be used to determine if targeting is feasible and ultimately, to assess the potential of the B5 scFv in development of such a gene delivery approach.

4.2 Conclusion

The goal of this project was to develop a targeting mechanism for regenerative molecules within the intervertebral disc, as part of a broader programme of targeted therapy of the degenerated IVD. IVD degeneration is a leading causative factor of lower back pain. The intervertebral disc is composed of the central nucleus pulposus, located in the centre of the disc, surrounded by the lamellae of the annulus fibrosis. Disc degeneration is characterised by increased degradation of the extracellular matrix and an ingrowth of nerves and blood vessels into the normally aneural and avascular tissue. Current treatments typically alleviate pain temporarily but do not restore disc function or prevent further degeneration (Whatley and Wen, 2012). In this work, a human scFv antibody fragment that binds neural cell adhesion molecule (NCAM1) was isolated for use in delivery of therapeutic genes to NCAM1-expressing IVD cells.

NCAM1 was identified as a potential NP cell marker in previously published studies of canine and human IVD (Sakai et al., 2009; Rutges et al., 2010). NCAM1 was more highly expressed in NP than AF cells in both studies though the overall expression level was low. Recombinant expression of the most cell-distal domain of NCAM1 (NCAM1-Ig1) was carried out in E. coli and yielded a purified antigen for scFv library panning experiments.

Panning led to the isolation of two scFvs of interest, termed F4 and B5. The former contained a stop codon in V_H FW2, necessitating site-directed mutagenesis to facilitate its expression in soluble scFv format in a non-
suppressor *E. coli* strain, albeit with a continued low expression level. In the case of the B5 scFv, its binding of the NCAM1-Ig1 domain was confirmed by ELISA, while immunocytochemistry experiments using rat astrocytes and bovine NP and AF cells demonstrated that the scFv also recognised membrane-bound, full-length NCAM1 protein in its native cellular environment. Specificity of the NCAM1 binding of the scFv was confirmed by inhibiting the signal using competing antigen. ScFv binding to both NP and AF cells was similar, indicating a similar NCAM1 expression level in the two bovine IVD cell types. While this appears inconsistent with previous reports of significantly higher expression in NP compared with AF cells, it raises the possibility that the isolated scFv can be used to deliver therapeutic moieties to both NP cells, where disc degeneration is proposed to begin, and AF cells.

It is anticipated that the scFv will be employed for targeted gene delivery using scFv-functionalised nanoparticles. To date, the scFv has been successfully conjugated to polyamidoamine (PAMAM)-coated hyaluronic acid nanoparticles and the activity of the tethered scFv has been confirmed by FACS (not presented in this thesis). The targeting potential of these scFv-conjugated nanoparticles and particle uptake will be investigated initially using a bovine IVD organ culture model, which allows mechanical loads to be applied to a bovine disc *in vitro* to mimic the physiological and mechanical environment. It is anticipated from similar studies of scFvs that the presence of the fragment will improve cellular uptake of the nanoparticle and its therapeutic payload, leading to a potential for a highly specific delivery mechanism to target degeneration in the IVD region. The isolated B5 scFv may also find application in tumour targeting as NCAM1 is associated with many types of cancers, including small cell lung cancers and neuroblastomas (*Zecchini and Cavallaro, 2010*). It is expected that this investigation will proceed in parallel with the IVD targeting analysis due to the compatibility of techniques and expertise involved in the two approaches.

Overall, this work has designed and expressed in recombinant form in *E. coli* a conformationally intact domain of the neural cell adhesion molecule 1 membrane protein; isolated a human scFv that binds the domain and the whole protein in its natural cellular environment; demonstrated the potential usefulness of the scFv in IVD regeneration; and established the validity of the combined
protein expression/phage display approach described to isolation of cell-binding antibody fragments for use in a wide variety of targeting and delivery applications.
References


total disc replacement versus circumferential fusion for the treatment of 1-level degenerative disc disease. Spine 32, 1155-1162; discussion 1163.
Appendix I

Reviewed, UniProtKB/Swiss-Prot P13591 (NCAM1_HUMAN) – the complete amino acid sequence of the neural cell adhesion molecule (858 amino acids).

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MLQTKDLIWT LFFLGTAVSL QVDIVP5QGE ISVGESKIFFL CQVAGDAKDK DISWFSPNGE
KLTPNQGRIS VWMDSSSST LTIYANNMDD AGIYKCVVTG EDGSESEAVTV NVKIPQKLMP
KNAPTPQUEF EGEDAVIVCD VVSSLPTII NWHKGADVIL KKDVFIVLVS NNYLQIEGIK
KTEGTYRCE GRILARGEIN FKDIQVIVNV FPFIQARQGNI VNATANLQGS VTLVCDAEQF
PEPTMSWTKD GEQIEQEDD EKYIFSDGG QLTIIKVDKN DAEYICIAE NKAGEQDATI
HLKVFAPKIK TYVENQTAKE LEEQVTIECE AGSDLPSIT WRTSNRINSS EKEASWTRPE
KQETLDGHMV VRSHARVSSL TLKSTQYTTA GEYICTASNT IGQDSQSMYL EVYQAPKLQG
PVAVVTWEGV QVINITCEVFA YPSATISWFR DGQLLPSSNY SNIKYNTPS ASYLEVTPDG
ENDFGNYNCT AVNRIGQESL EFILVQADTP SSPSIDQVEP YSSTAQVQFD EPEATGGVPI
LKYKAERRG GEEVWHSWFY DAKEASMEGI VTIVGLKPET TYAVRCLAAN KGLGGEISAA
SEFKTQPQGS EPSAPKLEGQ MGEDGNSIKV NLKQDDGGS PIRHYLVRIR ALSREWKPEI
RLPSGDHVM LKSLDMNAYE EYVYVAEQQ GKSAAHFVF RTSAQPTAIP ANGSPTSGHS
TGAIVGILIV IFVILLVVDV ITCYFLMKCG LFMCIAVNLG KKAGPGAGKG DMEGKAASS
KDESKPIVE VRTEERETPN HDGGKHTEPN ETTPLTEFEK GPVEAKPFCQ ETETKPAE
VKTVPNDAQ TKENESKA
```
Appendix II

Whole length sequence of clone F4 scFv mut - pIG forward primer is highlighted.

F4 mut scFv
TCTATAGGGCGATTGGCCCTCTAGATGTGCTCAGGACAGCTGAGTATGATAT
CTGCGAACATTGCGCCTTTCTAGAAGACATAATGAAAAATATAT
GGATATGGATATCTGACGCTACGCTACGCTACGCTACGCTACGCTACGCTACGCT
CTGACGCTACGCTACGCTACGCTACGCTACGCTACGCTACGCTACGCTACGCT
CTGACGCTACGCTACGCTACGCTACGCTACGCTACGCTACGCTACGCTACGCT
CTGACGCTACGCTACGCTACGCTACGCTACGCTACGCTACGCTACGCTACGCT
CTGACGCTACGCTACGCTACGCTACGCTACGCTACGCTACGCTACGCTACGCT
CTGACGCTACGCTACGCTACGCTACGCTACGCTACGCTACGCTACGCTACGCT
CTGACGCTACGCTACGCTACGCTACGCTACGCTACGCTACGCTACGCTACGCT
CTGACGCTACGCTACGCTACGCTACGCTACGCTACGCTACGCTACGCTACGCT
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CTGACGCTACGCTACGCTACGCTACGCTACGCTACGCTACGCTACGCTACGCT
CTGACGCTACGCTACGCTACGCTACGCTACGCTACGCTACGCTACGCTACGCT
CTGACGCTACGCTACGCTACGCTACGCTACGCTACGCTACGCTACGCTACGCT
CTGACGCTACGCTACGCTACGCTACGCTACGCTACGCTACGCTACGCTACGCT
CTGACGCTACGCTACGCTACGCTACGCTACGCTACGCTACGCTACGCTACGCT

Comparison of the amino acid sequence before and after site-directed mutagenesis to change the stop codon to a glutamine (highlighted in green)

| F4stop | MAQVQLVQSGGGLQGGLSRLSASAGFTPSSYAHWVR-APGKGEYVSAISSNGST 59 |
| F4mut | MAQVQLVQSGGGLQGGLSRLSASAGFTPSSYAHWVR-APGKGEYVSAISSNGST 60 |

| F4stop | YYADSVKGRFTISRDNSKNTLYLQMSLAEDTAVYYCVKPGRLSIFGVVIAAYRNYYY 119 |
| F4mut | YYADSVKGRFTISRDNSKNTLYLQMSLAEDTAVYYCVKPGRLSIFGVVIAAYRNYYY 120 |

| F4stop | GLDVMQGTTTVSAGGGGGSSSSGGGSSSLQDAFVPSVGAQVTRCQNNLRRY 179 |
| F4mut | GLDVMQGTTTVSAGGGGGSSSSGGGSSSLQDAFVPSVGAQVTRCQNNLRRY 180 |

| F4stop | YASNYKPPQAPVLSIKKNNRPPGIDRPFSGSGSDSTSGTQVIAQRAEAYDYSRSS 239 |
| F4mut | YASNYKPPQAPVLSIKKNNRPPGIDRPFSGSGSDSTSGTQVIAQRAEAYDYSRSS 240 |

| F4stop | DSGGNRVLGKVTGTVLGAHHHHHHH 267 |
| F4mut | DSGGNRVLGKVTGTVLGAHHHHHHH 268 |
Isolation and Characterisation of a Recombinant Antibody Fragment That Binds NCAM1-Expressing Intervertebral Disc Cells

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Abstract

Degeneration of the intervertebral discs (IVD) is a leading cause of neck and low back pain. Degeneration begins in the central nucleus pulposus region, leading to loss of IVD osmotic properties. Regeneration approaches include administration of matrix-mimicking scaffolds, cells and/or therapeutic factors. Cell-targeting strategies are likely to improve delivery due to the low cell numbers in the IVD. Single-chain antibody fragments (scFvs) that bind IVD cells were isolated for potential delivery of therapeutics to degenerated IVD. The most cell-distal domain of neural cell adhesion molecule 1 (NCAM1) was cloned and expressed in Escherichia coli. Phage display technology was used to isolate a human scFv against the recombinant domain by phanning a scFv library on the immobilised protein. The isolated scFv bound cultured rat astrocytes, as well as bovine nucleus pulposus and annulus fibrosus cells in immunocytochemical studies. The scFv also labelled cells in bovine spinal cord and six-month- and two-year-old bovine IVD sections by immunohistochemistry. Antibody fragments can provide cell-binding moieties at improved cost, time, yield and functionalisation potential over whole antibodies. The described scFv has potential application in delivery of therapeutics to NCAM1-expressing cells in degenerated IVD.

Introduction

Degeneration of the intervertebral discs (IVD) is a leading causative factor of neck and low back pain [1]. It has a lifetime incidence in excess of 70% [2] and is a main source of disability and lost workdays in industrialised nations. While it is particularly linked to aging, mild disc degeneration has been noted in as many as 20% of teenagers [3,4].

The intervertebral discs are located between the vertebral bodies in the spinal column and transmit loads arising from body weight and muscle activity. The IVD consists of a central gelatinous and highly hydrated nucleus pulposus (NP), bound peripherally by the annulus fibrosus (AF) and flanking cartilaginous end plates [5]. The NP is rich in extracellular matrix (ECM) proteins and is composed mainly of type II collagen and proteoglycans, predominantly aggregan, whereas the fibrous AF consists of organised lamella composed mainly of type I collagen [6]. The ECM is produced and maintained by the IVD cells, which are markedly depleted in aged or degenerated tissue [7].

Disc degeneration begins in the NP with the progressive loss of proteoglycans, coupled with synthesis of type I collagen [1]. As a result, the NP becomes fibrotic and dehydrated, leading to loss of osmotic properties, reduction in disc height and, ultimately, painful and disabling pathophysiologicals in the spinal column [8,9]. Current treatments are non-curative, typically alleviate pain only temporarily and many are highly invasive. Of these, non-surgical treatments include physiotherapy and the use of anti-inflammatory drugs. Surgical interventions, such as disc fusion or replacement, may affect spine biomechanics, can be suboptimal in delaying degeneration [10] and can even increase degeneration in neighbouring spinal segments [11].

Recent advances in IVD tissue engineering include the development of ECM-mimicking scaffolds [12-14] containing stem or disc-derived cells to replenish cell numbers [15,16] and/or protein- and DNA-based drugs to slow degeneration and/or stimulate resident cells [17]. Given the low density of
resident IVD cells, cell-specific targeting of therapies is likely to enhance the efficacy of such treatment approaches.

Cell targeting typically exploits antibodies to target specific cells and to improve uptake of attached payloads [18, 19]. Recombinant antibody fragments are antibody-derived molecules that retain the binding properties of their parent antibodies. They exhibit better tissue penetration and more rapid clearance than whole antibodies due to their reduced size and can be easily engineered to add novel functions such as imaging or therapeutic moieties [20]. Single-chain antibody fragments (scFv's) consist of antibody heavy and light chain variable domains, joined by a peptide linker [21]. ScFv's specific for ligands of interest can be isolated from large, diverse scFv collections using a bacteriophage-based display technology that mimics the humoral response in vitro [21]. Importantly, this approach can provide entirely human isolated scFv's for in vivo applications. Recombinant fragments are also typically produced in bacterial hosts which are much more rapid and less expensive than animal or hybridoma technologies used in polyclonal or monoclonal antibody production.

Neural cell adhesion molecule (NCAM1; CD56) is an immunoglobulin superfamily member that acts as a receptor for intracellular signaling and plays an important role in embryogenesis and development [22]. It has been found to be upregulated in NP compared to AF cells and articular chondrocytes in canine [23] and human [24] tissues, indicating its potential usefulness as a target for delivery of therapeutics to IVD cells.

This study utilized a phage display and protein expression approaches to successfully isolate a NCAM1-binding scFv in vitro. The isolated scFv bound NCAM1 expressing IVD cells and may be of interest for application in drug delivery systems specifically targeting IVD cells, envisaging disc regeneration.

Materials and Methods

Ethics Statement

Five-month-old and two-year-old cat bovine fresh tails were collected directly with permission after sacrifice of animals at Galway City Abattoir. Soft tissues surrounding IVDs (muscles and ligaments) were removed and cells and tissues were prepared as previously described [19]. All work was performed on explanted tissue and cells and, as biological material was harvested from practices undertaken for the purposes of recognised animal husbandry, did not require ethical approval under relevant Irish legislation (Statutory Instrument No. 543 of 2012). The NieuW rat astrocyte cell line was a kind gift from Professor J. Fawcett, University of Cambridge and was cultured as previously outlined [25].

Materials

Materials were purchased from Sigma-Aldrich (Ireland) unless otherwise stated. Escherichia coli strains TOP10 and W3110 were used to express the IgG1 domain of NCAM1 (NCAM1-IgG1), T69 to propagate phage and HB2151 for scFv expression. The modified KM13 helper phage (MRC HGMP Resource Centre, Cambridge, UK) provided phage proteins for phagenoid replication. The Yarnol human scFv library was from

Montarop Yamanbhai, Suranaree University of Technology, Thailand [25]. The pGEX vector for protein expression was from Andreas Frickenh, University of Zürich, Switzerland.

Bioinformatic analysis and cloning

Human NCAM1 nucleotide sequence was retrieved from GenBank (NCBI/BM44). The NCAM1-IgG1 domain structure (PDB ID 2WCM) [26] was analysed using DeepView Swiss-PdbViewer [27] (www.expasy.org/spdbv). The gene encoding NCAM1-IgG1 was amplified from human cDNA using omna_NCAM1_F and NCAM1_R primers (Table S1), to add a C-terminal hexahistidine tag for protein detection and purification. The amplification product was combined by overlap PCR with an E. coli ompA leader sequence, for secretion to the E. coli periplasm, and FLAG motif for protein detection [26], which were amplified from pGEX using ompA_F and ompA_NCAM1_R primers (Table S1). The combined product was cloned into the pGEX vector and sequenced prior to expression. IgGELAST (www.ncbi.nlm.nih.gov/blast2) was used to identify antibody variable genes homologous to isolated scFv.

Recombinant protein expression

NCAM1-IgG1 was expressed in E. coli W3110 cells containing pGEX6P-NCAM1-IgG1 using an auto-induction approach [26, 30]. A 500-ml culture in ZYP-5052 medium was grown at 25°C with shaking for 24 h prior to harvesting of cells by centrifugation. After resuspension of cells in phosphate-buffered saline (PBS) and re-centrifugation, periplasmic proteins were extracted [31, 32] and dialysed overnight against 51 of immobilised metal affinity chromatography (IMAC) binding buffer (3.98 M NaCl, 80 mM NaH2PO4, 80 mM Na2HPO4, 2M C) at 4°C. For expression of scFv, phagomorph DNA was extracted from overnight cultures and used to transform non-amber-suppressor E. coli HB2151 cells. A freshly transformed colony from TYE agar plates containing 1% glucose and 100 µg/ml ampicillin was used to inoculate 5 ml of LB containing glucose and ampicillin, followed by protein expression and extraction as described above.

Protein purification

Protein purification was carried out by IMAC [33]. Tween-20 (2% v/v) and 10 mM imidazole were added to protein extracts before passing through a 1-ml HiTrap affinity column (GE Healthcare, UK) at 1 ml/min. The column was washed with 10 ml, 5 ml and 5 ml of binding buffer containing 20 mM, 50 mM and 80 mM imidazole, respectively, before elution using binding buffer containing 100 mM, 500 mM and 800 mM NCAM1-IgG1 imidazole. Eluted fractions were dialysed as above and purified proteins were analysed by SDS-PAGE, immunoblotting [33] and enzyme immunoassay (ELISA; below).

Isolation of NCAM1-IgG1-specific scFv

3.4 Procedures for phage propagation and titration were as described previously [25, 34]. Immunobots were coated with 100 µg/ml NCAM1-IgG1 in PBS for 16 h at 4°C and blocked with 2% blocking agent (Round 1, 4: Marvel skinned milk powder
Rat astrocytes and bovine NP and AF cells were washed twice with PBS before fixing with 4% paraformaldehyde in PBS for 15 min and blocking with 1% goat serum in PBS for 30 min at room temperature. After incubation with 5 μg BSA or control 2H12.2 (20 μg/ml), scFvs were detected using a murine anti-polystyrene monoclonal antibody (1:200 in PBS/0.1% goat serum), followed by a goat anti-mouse FITC-conjugated antibody (Molecular Probes, USA) diluted 1:200 in PBS/0.1% goat serum and detected using a goat anti-mouse FITC-labeled antibody (Molecular Probes), diluted 1:250 as above. Antibodies were incubated for 1 h at room temperature and three 5-min washes were carried out with PBS/0.1% Tween-20 after each incubation. Cell nuclei were stained using DAPI (1:10,000 in PBS) for 15 min at room temperature, followed by two 5-min washes with PBS. Imaging was performed using an Olympus XG2 inverted epifluorescent microscope.

Immunohistochemistry

Bovine caudal spinal cord tissue was extracted from the spinal canal and harvested from two-year-old bovine tail. Tissues were fixed with 4% paraformaldehyde in PBS. After three washes with PBS, tissues were infiltrated overnight with 20% sucrose. Flash-frozen in liquid nitrogen-cooled isopentane and cut into 8 μm frozen sections were cut on a Leica CM 1850 cryostat (Laboratory Instruments & Supplies Ltd., Ireland). Sections were collected on Superfrost® Plus slides (Fisher Scientific Inc., Ireland) and stored at -20°C. Sections were washed three times with PBS prior to treating with 20 μg/ml (30 U/ml) protease K at 37°C for 5 min, followed by three further PBS washes. After blocking with PBS/0.2% BSA at room temperature for 1 h and three PBS washes, tissues were incubated with 5 μg BSA or 0.5 μg of a commercial murine anti-NCAM monoclonal antibody (Sigma, 1:200 in PBS/0.2% BSA overnight at 4°C. After three washes with PBS/0.1% Tween-20, a mouse monoclonal anti-polystyrene IgG (Fischer Scientific Inc., Ireland) diluted 1:50 in 0.2% SSA, was added to cells containing scFv for 1 h at room temperature. Three PBS/1% Tween-20 washes were followed by incubation with an anti-mouse FITC-conjugated antibody (Invitrogen Technologies), diluted 1:100 in 0.2% BSA/PBS, for 1 h at room temperature. After three washes with PBS/0.1% Tween-20, cell nuclei were counterstained using DAPI (1:10,000 in PBS, Invitrogen Technologies) for 10 min at room temperature, followed by two washes with PBS. Fluorescence was preserved using Prolong™ Gold Antifade reagent (Invitrogen Technologies).

For IVD immunohistochemistry, soft tissues surrounding IVDs were removed from six-month and two-year-old animals and IVD tissues were harvested. After collection of 10-μm thick transverse cryosections in glass slides as described above, sections were dried for 15 min at room temperature. Bovine scFvs (500 μg of 10 μg/ml) diluted in PBS/1% goat serum, were incubated with sections overnight at 4°C. After five washes in 0.05% Tween-20, slides were incubated for 1 h at room temperature.
Figure 1. Schematic representation of the closed NCAM1-Ig1 domain. A. Schematic of human NCAM1 structure. Immunoglobulin-like domains (Ig1-Ig6) are represented by oval: tenascin type III-like domains (1F13, 2F13) are shown as rounded rectangles. The most cell-distal, N-terminal Ig1 domain (arrowed), was cloned and expressed in E. coli. B. Crystal structure of NCAM1-Ig1 domain (PDB ID 2NGC) [20]. The C-terminal end, to which a hexahistidine peptide tag was attached for purification, is circled. Residues Glu176, Lys181 and Phe182 which are involved in the formation of the Ig1-Ig2 dimer interface are shown in green. C. Structure of the recombinant NCAM1-Ig1 domain expressed in E. coli, ompA leader peptide for secretion to bacterial periplasm, this is cleaved (arrowed site) during translocation of the cytoplasmic membrane. FLAG: DYKDDDDK amino acid motif for protein detection. NCAM1-Ig1: gene encoding Ig1 domain of human NCAM1. 6His: hexahistidine tag for detection and purification of the recombinant NCAM1 domain.

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In turn, the rabbit anti-polyhistidine IgG and an anti-rabbit Dylight 588-conjugated antibody. Slides were counterstained with DAPI, diluted 1:5000 in methanol for 10 min at room temperature and washed three times with PBS/0.05% Tween-20 before preservation of fluorescence. In parallel analyses, the 85 scFv was pre-incubated with purified NCAM1-Ig1 at 50 μg/ml prior to investigation with sections.

Results

Cloning of recombinant NCAM1-Ig1

NCAM1 contains five Ig-like modules and two tenascin-like modules connected via a membrane-spanning domain to a signal-transducing cytoplasmic region (Figure 1). Based on GenBank sequence BC047244, the sequence encoding the most cell-distal Ig-like domain, NCAM1-Ig1, was amplified and cloned into the pGEX expression vector. The resultant construct also contained an N-terminal ompA leader sequence for secretion of the polypeptide product to the E. coli periplasm, an adjacent FLAG tag for its detection [20], a C-terminal hexahistidine motif for IMAC-based purification and detection of the translated product, and flanking restriction enzyme sites. The confirmed sequence of the recombinant NCAM1-Ig1 domain is shown in Figure S1.

Expression and purification

Expression of recombinant NCAM1-Ig1 in E. coli TOP10 using IPTG induction generated low yields of predominantly insoluble protein (data not shown). Functional yields were increased by using E. coli W3110 and adopting an auto-induction protocol [29], whereby expression was induced following glucose depletion in the medium. This resulted in higher yields of NCAM1-Ig1 polypeptide and reduced proportions of insoluble protein. After isolation of soluble periplasmic proteins, 2.3 mg (determined using a Nanodrop 2000c Thermo Scientific) of the 11.3-kDa hexahistidine-tagged NCAM1-Ig1 domain was purified to near-homogeneity by IMAC per litre of bacterial culture (Figure 2). Elute fractions
Figure 2. IMAC purification of recombinant NCAM1-lg1 expressed in E. coli. (A) Coomassie stain and (B) western blot analysis of purification. Lane 1: molecular weight markers; lane 2: soluble periplasmic proteins from host bacterial cells; lane 3: IMAC column flow through; lane 4: column washes with 20 mM, 50 mM and 100 mM imidazole; lanes 5, 6: proteins eluted using 100 mM imidazole. An anti-human IgG antibody was used to detect the target protein in (B). Arrows indicate proteins of the expected molecular weight (the predicted size of the NCAM1-lg1 domain is 11 kDa).

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isolating the highest protein concentrations were dialysed against PBS prior to use.

Isolation of NCAM1-lg1-specific scFv

Five rounds of panning of the Yarrei human scFv phage library were carried out on immobilized NCAM1-lg1. Washing stringency and antigen concentration were manipulated to enhance phage enrichment, which was identified after 4.5 panning rounds (Figure 3A). This correlated with a significant increase in NCAM1-lg1 binding in eluted polyclonal phage populations after the fourth panning round (Figure 3B).

ELISA screening of phage-displayed scFv's from 100 randomly selected E. coli clones from panning round 4 identified eight NCAM1-binding molecules. DNA sequencing revealed two unimutated, full-length scFv gene sequences between six clones and eight scFv's in two scFv's. Based on these results, clones F4 and B5 from the former group were investigated by ELISA and B5 was selected for further analysis as it exhibited more efficient binding to free NCAM1-lg1 domain in competitive ELISA (Figure 3C). The DNA sequence of the B5 scFv V4 domain exhibited highest homology to human IG-7/2-gammain V gene using IqBLAST and nucleotide identities in excess of 90% to V4 domains from human anti-HIV and anti-Epstein Barr virus LMP1 (latent membrane protein) antibodies using BLAST [37]. The B5 V4, meanwhile, exhibited highest homologies to germinal YLIX YLIX J gene and 13L12 and 13L13 J genes, and sequence identities of 95% or greater to human V4 genes from a number of anti-HIV virus antibodies.

In vitro activity of B5 scFv

The B5 scFv was expressed in soluble, non-fused form using the non-suppressor E. coli HB2181 strain to terminate translation at the amber stop codon between the scFv and the phage PIII protein [35]. After purification using IMAC, its binding, and inhibition by the purified NCAM1-lg1 domain was observed by ELISA (Figure 3C).

The ability of B5 scFv to bind fulllength NCAM1 protein on the cell surface was initially investigated using rat astrocytes, which have been reported to have high NCAM1 expression levels [22,23]. Cells were clearly labelled using the B5 scFv and not a control, non-immuno-biding scFv [31,33], while a similar labelling pattern was observed using a commercial mouse anti-human NCAM1 IgG (Figure 4A).

The ability of B5 scFv to bind bovine spinal cord and IVD-derived cell NP and AF cells was also confirmed. In immunocytochemical studies, signal intensities were similar for AF and NP cells in repeated analyses (Figure 4B). Similarly, the B5 scFv demonstrated extensive labelling of cells in bovine spinal cord sections (Figure 5). Binding could be successfully inhibited using the encoded heparin-binding or mAb (not shown) recombinant caps [35] and the scFv again exhibited a similar labelling pattern to the commercial anti-NCAM1 murine antibody.

In the case of IVD tissues, the commercial anti-NCAM1 antibody and B5 scFv demonstrated similar binding patterns (not shown). Meanwhile, the B5 scFv exhibited significant binding to both six-month old and two-year old bovine tissues, and to both NP and AF cells (Figure 6). Higher signal intensities, and a higher proportion of labelled cells was observed for NP tissue at two years compared to those at six months, while the opposite was the case for AF tissues. In all samples, binding could be inhibited almost completely by the soluble NCAM1-lg1 domain, indicating the specificity of scFv binding to NCAM1 in the two tissues.

Discussion

In this study, we describe the isolation of a recombinant antibody fragment that binds neural cell adhesion molecule NCAM1-lg1 domain for potential application in targeting therapeutics to NCAM1-expressing IVD cells.

Tissue engineering approaches envisaged for the degenerative IVD typically incorporate the addition of healthy cells, reconstitution of the physical and osmotic properties of the disc region via a scaffold and the delivery of anabolic, anti-catabolic or anti-inflammatory agents to promote regeneration or delay further degeneration. Progress towards improved therapies has been hampered by an enhanced understanding of the pathophysiology of degeneration [38]. Demonstration of the restorative properties of growth factors such as BMP-2 [39], the
Figure 3. Isolation of NCAM1-binding scFvs by phage display. (A) Titer of input and output phage populations throughout library panning. Phu = phage-forming units. (B) ELISA analysis of NCAM1-Ig1 binding activity of eluted polyclonal phage-scFv populations. P0: Unpanned library. P1-P4: Pooled phage preparations eluted after rounds 1 to 4 of library panning against immobilised NCAM1-Ig1. NCAM1-Ig1 was coated in ELISA wells at 100 μg/ml. Bars represent the mean of three replicate wells and error bars indicate the standard deviation of the mean. (C) Competitive ELISA analysis of NCAM1-Ig1 binding of B5 scFv. Binding of the purified scFv to immobilised NCAM1-Ig1 (103 μg/ml) was out-competed by pre-incubating the scFv with soluble NCAM1-Ig1 (267 μg/ml) prior to ELISA analysis. SMP: skimmed milk powder control. Bars represent the mean of three replicate wells and error bars indicate the standard deviation of the mean.

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Figure 4. Immunocytochemical analysis of antibody binding to rat astrocytes and bovine IVD cells. A, Rat astrocytes were incubated with a. Anti-NCAM1 85 scFv; b. Control 2H4 IgG; or c. Commercial anti-NCAM1 mouse IgG, followed by detection using mouse monoclonal anti-histidine and goat anti-mouse-FITC reporter antibodies, respectively. B. Analysis of 85 scFv binding to bovine nucleus pulposus (a,c) and annulus fibrosus (b,d) cells. a,b,c Cells labelled with 85 scFv, followed by appropriate reporter antibodies. c,d. As for a,b but no 85 scFv. Cell nuclei are stained blue using DAPI, and scFvs attached to the cell surface are stained in green. Scale bar is 100 μm in all images.

development of reservoir systems to deliver cells and simulate the resident tissue [15,40], and the advent of whole organ culture bioreactors to reproduce the mechanical and physiological conditions of the IVD [1,41].

We hypothesize that targeting delivery of therapeutic factors to cells will enhance their efficiencies in regenerating or slowing degeneration of naturally resident and newly administered cells in the subchondral disc environment. This approach takes cognizance of the low cell numbers in the IVD, even before further depletion occurs upon degeneration, which begins as early as their second decade in many individuals. While the IVD region is avascular and contains limited cell types and numbers, cell-specific targeting of therapeutic moieties may clarify also be necessary in more diverse, less contained environments to avoid misplacement of the therapeutic approach in vivo as well as to enhance uptake.

The exquisite target specificity of antibodies is commonly exploited to deliver payloads to specific cell types. Traditional monoclonal antibody isolation and production processes, however, are protracted, labor-intensive and expensive. Recombinant antibody-derived fragments, conversely, retain the antigen-binding pocket and, thus, binding ability of whole immunoglobulins but offer advantages of cost, speed and ease of production in expression platforms such as E. coli. In addition, they have a greatly reduced size [1,42] (important in, e.g., tissue penetration) and can be readily functionalized with novel properties (e.g. therapeutic or imaging moieties) using readily accessible protein engineering techniques [36,32]. While the increased functionalization capacity and reduced
production costs of scFv, compared with full-length monovalent antibodies will be advantageous in targeting delivery of biomolecules in the IVD, the avascular microenvironment means that the faster clearance of scFv is unlikely to be a significant factor, unlike in the case of systemic delivery of therapeutics. Importantly for in vivo applications, library approaches such as phage display allow entirely human molecules to be isolated in vitro, a breakthrough not yet achieved with mainstream monoclonal antibody technologies.

While NCAM1 is also widely expressed in non-IVD cells, it was selected as an IVD cell marker in this work due to its elevated expression on NP and AF cells compared to articular cartilage cells in the contained IVD micro-environment in canine [23] and human [24] tissues. The cell-distal NCAM1-Ig-like domain was used for antibody isolation to maximize accessibility to cell-targeting scFvs while the previous expression of numerous immunoglobulin superfamily domains in E. coli [22,32,44,45] and the distinct modular structure of NCAM1 (Figure 1) increased the likelihood that the Ig-like domain could be expressed in folded, structurally intact form in the bacterial host. Following some expression optimization, a soluble, recombinant product of the expected molecular weight was purified to near-homogeneity for scFv isolation.

Phage display technology identifies high-affinity antibody fragments against immobilised ligands by mimicking the human B cell response, utilising bacteriophage particles to display antibody fragments [21]. Library panning yielded a phage-scFv with NCAM1-Ig-like binding that could be out-competed by the soluble domain. Fragments with integenic stop codons were also isolated due to use of an amber-suppressing E. coli strain for display of scFvs [25]; these were not investigated further due to their incompatibility with the subsequent expression system.

The ability of the isolated B5 scFv to bind full-length cell-bound NCAM1 was investigated using rat astrocytes and bovine NP and AF cells. Comparison of NCAM1-Ig-like sequences identified 87% amino acid identity between the human and bovine, and 94% between the human and rat domains (not shown). Immunocytochemical studies confirmed binding of the cell-tethered NCAM1 protein by the recombinant scFv with similar labelling of rat and bovine cells by the scFv and a commercial anti-NCAM1 antibody. The scFv exhibited higher signal intensity than the monoclonal antibody, albeit using different reporting systems that precluded quantitative comparison of their affinities. Typically, however, antibody fragments from phage libraries can exhibit affinities in the nanomolar range and further affinity improvement is a relatively routine in vitro task [46], unlike mutagenesis and isolation of improved monoclonal antibodies [47].

A NP/AF gene expression ratio of around 10 has been reported in beagle tissue [23], beagles proving a good model of human disc degeneration as they lose their notochordal cell population as the young adult ages; conversely, a microradiography study noted no difference in NCAM1 expression between human NP and AF tissues [48]. No difference in signal intensity was noted between bovine NP and AF cells upon scFv binding in this work. This labelling pattern indicates that B5 scFv-mediated delivery approaches would target both NP and AF cells in the IVD. As degeneration is hypothesised to begin in the central NP region but with early degenerative processes also evident in the surrounding AF [46,20], this labelling profile is consistent with current treatment practices.
which target both NP and AF cells affected by the degenerative process to promote production of new ECM or growth factors.

The expression of numerous proteins has been demonstrated to vary in the IVD as natural ageing or accelerated degeneration occur [23,30,40]. The B5 scFv exhibited binding to both six-month and two-year-old NP and AF tissues (Figure 6), with higher signals at two years in NP and six months in AF tissues. More detailed investigation of human IVD tissues of different ages and disease grades would be required to determine the ability of the scFv to bind cells throughout the degenerative cycle. The demonstrated ease of isolation and expression of cell-binding scFvs in this study,
however, suggests that scFvS against multiple antigens could be co-administered to maximise delivery of therapeutics to IVD cells at distinct degenerative states.

We have demonstrated in this work that a combined image display and recombinant protein expression approach can be used to isolate scFv antibody fragments that bind surface markers on IVD cells. The observed BC scFv has potential application in targeting of therapeutic moieties to NCAM1 expressing NP and AF cells in aged or diseased IVD. Furthermore, the use of recombinant antibody fragments provides a source of cell-binding molecules for use in tissue engineering applications at significantly lower cost, in reduced times, at higher yields and with greater potential for functionalisation than whole antibodies.

Supporting Information

Figures

Figure S1. DNA and predicted amino acid sequence of NCAM1 expression construct. In the DNA sequence, the XbaI and FokI restriction sites used for cloning are shown in italic and underline. The Shine-Dalgarno sequence is underlined and the translation start and stop codons are boxed. In the amino acid sequence, the mEPK leader is highlighted in yellow, the FLA3 sequence in teal and the hexahistidine tag in grey.

Table S1. Oligonucleotides used in the study and predicted sizes of products.

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Author Contributions

Concepted and designed the experiments: CC AS EC SG MA AP GW. Performed the experiments: CC AS EC. Analyzed the data: CC AS EC SG MA AP GW. Contributed reagents/materials/analysis tools: SG MA AP GW. Wrote the manuscript: CC AS EC SG MA.