Title: The nucleotide diphosphate kinase, *nm23H1*, is expressed by human bone marrow-derived mesenchymal stem cells and stimulates neurite outgrowth *in vitro* independently of its kinase activity.

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Abstract

We recently identified that human bone marrow-derived mesenchymal stem cells (MSC) stimulate neurite outgrowth *in vitro* via direct neuronal contact and also through the secretion of neurotrophic agents into conditioned medium. Here we show that human MSC express and synthesise the nucleoside diphosphate kinase, *nm23H1*. Nm23H1 protein was detectable in MSC cell extracts by Western blotting and on the cell surface of MSC via flow cytometry. To examine the role of MSC-synthesised nm23H1 in stimulating nerve growth we have adsorbed recombinant human nm23H1 proteins to nitrocellulose-coated tissue culture plates in defined locations versus its delivery in solution. This method of substrate-bound presentation to neurons mimicked its presence on the MSC cell surface. E10 chick and adult rat dorsal root ganglia (DRG) were used as models of nerve growth. We show that substrate-bound nm23H1 both promoted and directed DRG neurite outgrowth in a concentration-dependent manner. Neurotrophic effects were also seen, but to a much lesser extent when nm23H1 was presented in solution. When chick DRG explants settled directly onto relatively high concentrations of substrate-bound nm23H1, DRG neurite outgrowth was extensive, rapid and effectively limited to the nm23H1 substrate, i.e. the growth cones of extending neurites turned away from adjacent otherwise permissive extracellular matrix substrates to remain on the nm23H1. Nm23H1 significantly enhanced the adhesion of dissociated rat DRG neurons and stimulated rat and chick neurite outgrowth in the presence and absence of supplementary nerve growth factor. In other cell systems, intracellular nm23H1 has been shown to signal through the rho kinase family. However, we found that a mutant form of nm23H1 (H118F) which does not possess kinase activity also exhibited nerve-stimulatory activity to a comparable extent as the wild type protein. These findings demonstrate a

novel function for extracellular nm23H1 in stimulating and directing nerve growth and suggest that this may represent one of the mechanisms via which human MSC exhibit neurotrophic activity.

Key words: mesenchymal stem cell, nerve growth, nm23H1, extracellular activity.

Introduction

Mesenchymal stem cells (MSC) have received considerable attention as potential cell therapeutics for the damaged central nervous system, in particular spinal cord injury (SCI). There are several reasons for this. MSC can be isolated and culture expanded for autologous applications with relative ease (Dezawa et al., 2005; Wright et al., 2008). MSC synthesise known neurotrophic agents, including nerve growth factor and brain-derived neurotrophic factor (Neuhuber et al., 2005; Himes et al., 2006). When transplanted into animal models of SCI, MSC integrate into the damaged host tissue, where they have been reported to reduce cavity formation (Ankeny et al., 2004) and stimulate axonal regeneration through the lesion site (Himes at el., 2006). In addition to providing soluble trophic support following transplantation, MSC have also been suggested to act as "guiding strands" for regenerating axons to extend across SCI lesions (Hofstetter et al., 2002). In support of such a contact guidance role, we recently demonstrated that co-culture of MSC with neurons helps their extending neurites to bridge and hence overcome the repellent effects of extracellular neural chondroitin sulphated proteoglycans (CSPG), Nogo-A and myelin associated glycoprotein (MAG). These substrates represent elements present in the damaged spinal cord and are considered to form major blocks to nerve growth and axonal regeneration after injury [refs]. In this analysis, we found that the ability of dorsal root ganglia (DRG) neurites to extend across substrates of inhibitory CSPG, Nogo-A and MAG was associated with direct contact of the neurites with the MSC. Conversely, soluble MSC-synthesised factors which were present in MSC-conditioned medium were insufficient to promote DRG neurite outgrowth across such an inhibitory environment (Wright et al., 2007).

Nm23H1 belongs to a family of multifunctional, broad spectrum nucleoside diphosphate (NDP) kinases with roles relating to signal transduction, gene expression, cell proliferation and differentiation, embryonic development, tumour progression and cell migration (reviewed in de S Otoro 2000; Hartsough & Steeg 2000; Fournier et al, 2003; Outas et al., 2003; Postel 2003). In particular, both *in vivo* and *in vitro* studies have suggested that there is a clear association of nm23H1 function with the regulation of nerve growth. During mouse embryogenesis, *nm23M1* (the murine homologue of *nm23H1*) is specifically expressed first in central and peripheral neural tissues, including the brain, spinal cord and spinal and cranial ganglia (Arnaud-Dabunat 2003). Stable transfection of the PC12 cell line, a frequently used model of neuronal differentiation, with *nm23M1* has been shown to result in accelerated cell cycle arrest, increased levels of neurofilament expression and polymerised β-tubulin and increased neurite outgrowth (Gervasi 1996). Conversely, reduced levels of nm23M1 protein in PC12 cells following anti-sense *nm23M1* transfection inhibited neurite outgrowth in response to treatment with nerve growth factor. Transfection of human DR-nm23, another member of the NDPK family with 70% homology to nm23H1, was similarly shown to enhance neurite outgrowth in murine N1E-115 neuroblastoma cells (Amendole 1997).

In this study, we have demonstrated that human bone marrow-derived MSC express and synthesise nm23H1, with nm23H1 protein detected in cell extracts via Western blotting and on the cell surface of MSC by flow cytometry. Furthermore, we have performed *in vitro* experiments with recombinant nm23H1 proteins in order to examine the potential influence of nm23H1 on nerve growth. These experiments were designed to mimic how nm23H1 may be presented by MSC either following extracellular transport or on their cell surface. We demonstrate that substrate-bound

extracellular nm23H1 both stimulated neurite outgrowth and provided directional cues for growth cone pathfinding. Hence, we report an entirely novel activity for extracellular nm23H1 and suggest that this activity may account in part for the neurotrophic effects of MSC on neurons.

Materials and Methods

Mesenchymal stem cells (MSC): Following informed consent and with local research ethical committee approval, human bone marrow-derived mesenchymal stem cells (MSC) were isolated from iliac crest biopsies and culture-expanded as described previously (Wright et al., 2007). We have previously reported that these culture-expanded stromal cell populations differentiate along osteogenic, adipogenic and chondrogenic lineages, and are immunonegative for CD34 and CD45, whilst immunopositive for CD105 (Wright et al., 2008). Hence, they exhibit the MSC phenotype as classified by the International Society for Stem Cell Research (ref). All MSC cultures used for nm23H1 analysis were at passages II-III.

Embryonic chick dorsal root ganglia (DRG) explant cultures: E10 chick DRG explants were prepared according to previously described methods (Johnson *et al*., 2002; Wright *et al*., 2007). Briefly, chick DRG were dissected from E10 hybrid brown chicks, cut into 2 or 3 explants such that all explants were approximately of equal size and these then seeded into culture plates of substrate choice assays, pre-prepared as described below.

Dissociated cultures of adult rat DRG. Dissociated DRG neurons were prepared from adult male Sprague Dawley rats, as described previously [Ahmed et al., 2005]. Briefly, L4-L7 DRG pairs were treated with 0.125% (w/v) collagenase type XI

(Sigma, Poole, Dorset, UK) and 200U/ ml DNase I (Worthington Biochem, New Jersey, USA) at 37^{o}C, 5% (v/v) CO_2 for 2 hours, and triturated several times to dissociate cells from tissue. Following centrifugation through 15% FCS to remove debris and cell pelleting, the resultant cell pellets

were responded in . After this tissue digestion, the DRGs were removed and placed into 10ml of warmed NBA media and centrifuged for 1 minute at 120g (Henderson Biomedical). The supernatant was removed and 1ml of warmed NBA media supplemented with 20□g/ ml B27X 50 supplement (Invitrogen), 2.5□g/ ml L-glutamine and antibiotics was added to each pellet. Pellets were then titurated repeatedly with a cut off 1ml pipette filtered tip (Starstedt). This process was repeated using progressively finer pipette tips until a milky suspension of dissociated cells was achieved. Five hundred microlitres of cell suspension was then layered over 2ml of a 15% (w/v) BSA column consisting of 1ml of unsupplemented NBA media and 1ml of 30% (w/v) BSA fraction V (Sigma-Aldrich) and cells were separated by density gradient centrifugation for 10 minutes at 120g (Henderson Biomedical). This centrifugation step separates the neurons from debris and most of the smaller glial cells. The neuronal enriched isolate formed a loose pellet from which the supernatant debris was removed. One or two pellets were then re-suspended in 100µl of trypsin inhibitor DNAse solution (TID), which consisted of NBA supplemented with 3mg/ ml BSA, 50□g/ ml DNase I (M6101; Promega, Southampton, UK), 250□g/ ml soybean typsin II inhibitor (Sigma-Aldrich), 10□g/ ml magnesium sulphate (Sigma-Aldrich) and antibiotics in PBS. Pellets were pooled, titurated thoroughly to prevent clumping and a viable cell count performed. Finally 2 x 10^3 viable neurons per well were seeded into 6 well culture plates, which had been prepared with the substrate choice

assay of nm23H1, nm23H1 (H118F) or the irrelevant His-tag control recombinant AKR. Fresh supplemented NBA media with 50ng/ ml NGF was added daily to induce neurite extension.

2.5.3.3: Immunolocalisation of □-III tubulin on adult rat DRG neuron cultures.

Dissociated DRG neurons seeded on the substrate choice assays were fixed for 15 minutes with 4% (v/v) formalin in PBS. Plates were washed in PBS, followed by incubation with a blocking solution of PBS containing 5% (v/v) normal goat serum (NGS) (Sigma-Aldrich) and 0.1% Triton-X100 (Sigma-Aldrich) for 15 minutes. Excess blocking buffer was removed; then the slides were incubated overnight at 4^{o}C with a polyclonal rabbit anti rat antibody specific for □-III tubulin at 18□g/ ml (Sigma-Aldrich). The primary antibody was diluted in the same blocking buffer (5% NGS in PBS). Samples were then washed repeatedly in PBS and incubated for 1 hour with a secondary anti-rabbit IgG Alexa 488 green fluorescent antibody (50μg/ ml in PBS- Invitrogen). After final washes in PBS, fresh PBS was added to each well and the plates stored at 4^{o}C for microscopy.

Preparation of recombinant wild type (WT) nm23H1 and mutant nm23H1 (H118F).

The nm23H1 his-tagged clone was kindly provided by Dr Patricia Steeg (Bethesda, MD). The clone was transformed into *E. coli* BL21 (DE3) cells (Novagen, CN Biosciences UK Ltd, Nottingham, UK) according to the manufacturer's instructions. Bacterial cultures for inductions were prepared by inoculation of fresh L-Broth media, containing 100μg/mL Ampicillin (Sigma), with an overnight starter culture to achieve a dilution of 1 in 50. This culture was grown to an OD_{600} of 0.6 and recombinant

nm23H1 protein expression was induced with 1mM IPTG (Sigma) for 2 hours. Recombinant protein was prepared by the Bugbuster Ni-NTA His•Bind Purification Kit (Novagen), according to manufacturer's instructions. To generate nm23H1 (H118F), complementary oligonucleotides containing the histidine to phenylalanine mutation at position 118 were synthesised where:

3'-TTGGCAGGAACATTATACATGGCAGTGATTCTGTGGAGAGTGC-5'

3'-TTGGCAGGAATTTTATACATGGCAGTGATTCTGTGGAGAGTGC-5'

These oligoneucleotides were used with the Qiagen Quikchange XL site-directed mutagenesis kit (Qiagen), following the manufacturer's instructions. The mutated plasmid was transformed into XL10-Gold ultra competent cells (Strategene) and the recombinant mutant protein produced as for the WT nm23H1.

Commercially available purified neural PG isolated from embryonic chick brains, the major types in this mixed preparation including neurocan, phosphacan, versican and aggrecan (Chemicon; CC117, Temecula, CA), and type I collagen (isolated from calf tail; C8919, Sigma-Aldrich) were used to coat tissue culture plates in restricted localities. This technique provides for a substrate "choice" assay and was used to model the interaction of neurons to extracellular matrix molecules present within the developing and injured CNS (Snow *et al*., 1990; Snow *et al*., 2001). The culture plates were prepared as follows:

(i) plates were pre-coated with a thin layer of protein-binding nitrocellulose in methanol (1mm^2/ ml) (BA85, Schleicher & Schuell, Dassel, Germany) by adding an excess of the solution, then removing it to leave a very thin film deposited as the methanol evaporated.

(ii) this thin layer was then blotted with 350μm wide strips of filter paper (Whatman No.1, cut with a McIlwain tissue chopper, Campden Instruments, Loughborough, UK)

which had been pre-soaked in neural PG at concentrations ranging from 0.1μg - 100μg/ ml in PBS. The restricted localisation of the neural PG on the culture plates was visualized by inclusion of 10% (v/v) marker dye rhodamine B (Sigma-Aldrich) in the neural PG solutions.

(iii) after the filter strips had dried such that they could be tapped off and removed, the plates were further coated with a solution of 100μg/ ml of type I collagen (Sigma-Aldrich) (in PBS) for 5 minutes.

(iv) after collagen-coating, all wells were washed repeatedly with PBS prior to seeding with explants of embryonic day 10 (E10) chick DRG in the presence or absence of MSCs (Figure 2.4- see section 2.4.2 for details of culture/ seeding protocols).

The binding of neural PG in defined areas on the nitrocellulose substratum that had both chondroitin sulphate (CS) and keratin sulphate (KS) GAG side chains was confirmed by immunolabelling. After blotting the neural PG to the nitrocellulose coated plates, the culture substrata were blocked for 1 hour with 5% (w/v) FCS in PBS. Excess blocking buffer was removed; then the wells were treated with 0.25 units/ ml chondroitinase ABC (Sigma-Aldrich) in PBS for 1 hour in order to expose the CS and KS epitopes. Wells were washed repeatedly in PBS, then incubated with monoclonal primary antibodies specific for CS and KS disaccharide epitopes at 1 in 1000 and 1 in 500 respectively, in PBS (kindly provided by Dr. B. Caterson, Cardiff University). Parallel wells were incubated with irrelevant isotype-matched antibodies (Dako) as negative controls. After incubation with the primary antibodies all wells were washed in PBS before immunopositivity was revealed using the Vector ABC System described in section 2.2.4.3.

The same technique was used to prepare culture plates with substrates choices of 100μg/ ml collagen I and strips of MAG and Nogo-A at concentrations ranging from 10μg - 200μg/ ml in PBS (R&D systems, Abbingdon, UK), recombinant human nm23H1 (wild type nm23H1 and a mutant form that does not have nucleoside diphosphate kinase (NDPK) activity - H118F), or aldo-keto-reductase (AKR), all kindly provided by Dr. C. Bunce) at various concentrations.

2.5.1: MSC expression of *nm23H1*.

2.5.1.1: Total RNA isolation.

Approximately 5 x 10^5 cells were trypsinised, re-suspended in PBS and centrifuged in a microfuge for 10 minutes at 500g (Henderson Biomedical) to produce a pellet. Total RNA isolation was then performed using an RNeasy® Mini Kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. Briefly, the pelleted cells were lysed by re-suspending in 350□l of lysis buffer, which contains highly denaturing guanidine-thiocyanate. This immediately inactivates RNAses to ensure purification of intact RNA. The resultant suspension was homogenised by repeatedly titurating through a 19 gauge needle and syringe. Samples were then mixed with 70% (v/v) ethanol (Sigma-Aldrich) and this mixture applied to an RNeasy Mini spin column and centrifuged in a microfuge for 15 seconds at 8000g (Henderson Biomedical). The total RNA was now bound to the membrane in the tube and any contaminants were washed through. The RNA was then eluted from the membrane by washing through with 30□l of RNAse free water into a new collection tube (Qiagen).

2.5.1.2: Quantification of RNA by fluorescence spectrometry.

RNA was quantified by binding Syber Green II fluorescent dye according to the manufacturer's instruction (Invitrogen). Briefly, purified RNA samples were diluted 1 in 100 in Syber Green II solution and the fluorescence intensity measured (at excitation wavelengths of 485nm and emission wavelengths of 528nm using a FLx800 fluorescence microplate reader (Biotech Instruments, Newton, Bedfordshire, UK). These readings were compared to a standard curve of known RNA standards (kindly provided by Dr. Mike Marshall, Charles Salt Laboratories, RJAH Orthopaedic Hospital).

2.5.1.3: Reverse transcription (RT) of RNA.

Reaction mixtures were prepared in PCR tubes for each sample by combining 2□l of Mg-free buffer, 1.6□l of 25mM $MgCl_2$ (catalyst), 1.6□l of 2.5mM dNTP (deoxynucleosides- the monomers that form the DNA), 1□l of RNase inhibitor, 1□l of reverse transcriptase (the enzyme which transcribes single stranded RNA into double stranded DNA), 1□l of Oligo dTs (the primer for first strand DNA synthesis), with a final addition of the required volume of RNA sample that equated to 50ng of RNA plus RNAse free water to a volume of 11.8µl. All reagents apart from the RNA samples were provided in kit form (Applied PE Biosystems, Foster City, CA, US). Negative controls were also prepared replacing the test sample in the reaction mix with RNAse free water (Qiagen). Tubes were then placed in the thermal cycler, the thermocycling programme consisted of one cycle of 42^0C for 30 minutes and 99^0C for 5 minutes followed by a temperature hold for 30 seconds at 4^0C to produce RT product.

2.5.1.4: Conventional polymerase chain reaction (PCR) for *nm23H1* expression.

The reaction mixtures were prepared in PCR tubes for each sample by combining 1□l of RT product to 2□l of Mg Taq buffer, 1.8□l of 25mM $MgCl_2$ (catalyst), 1.6□l of 2.5mM dNTPs, 0.5□l of DNA polymerase (the enzyme that catalyzes the polymerization of deoxyribonucleotides), 2.5ml of forward primers and 2.5ml of reverse primers with 14.5□l of RNAse free water (Applied PE Biosystems). The primer sequences were as follows: *nm23H1* forward 5' primer GGCCTGGTGAAATACATGCA and nm23H1 reverse 3' primer GGCCCGTCTTCACCACAT (ALTA Bioscience, University of Birmingham, UK). Negative controls were also prepared replacing the test sample in the reaction mix with the RT product after RT with RNAse free water. Tubes were then placed in the thermal cycler, the thermocycling programme consisted of a 94^0C temperature hold for 30 seconds followed by 36 cycles, where each cycle had three steps; denaturation at 94^0C for 30 seconds, annealing at 56^0C for 2 minutes, and elongation at 72^0C for 1 minute. The last cycle ended with a final elongation temperature hold at 72^0C for 5 minutes.

The PCR products were then separated by gel electrophoresis to compare their size with DNA standards, using a 1kb ladder from 50 to 800 bp with 50 bp increments. PCR products and standards were loaded into wells of 2% (w/v) agarose gels containing 1□l/ ml SYBR safe DNA gel stain and made up in 0.5 x TBE buffer. The DNA ladder was diluted to 100□l/ ml in RNAse free water containing 100□l/ ml of tracking dye (blue juice). Five microlitres of tracking dye was also added to PCR products. The agarose gels were then completely covered with 0.5 X TBE buffer and run at 100V for 1 hour. Gels were then visualised under a UV light and digitised images collected using image analysis software (from PD Quest for Windows).

2.5.2: MSC synthesis of nm23H1 protein.

2.5.2.1: Protein Extraction and quantification.

Cells were trypsinised, re-suspended in PBS and centrifuged in a microfuge for 10 minutes at 500g (Henderson Biomedical) to produce a pellet. Cell pellets of 10^6 cells were then re-suspended in 20µl of RIPA buffer, which contained 1% (v/v) nonidet P40 (Sigma-Aldrich), 0.5% (w/v) sodium deoxycholate (Sigma-Aldrich) and 0.1% (w/v) sodium deoxysulphate (Sigma-Aldrich) in distilled water, on ice for 30 minutes. This lyses the cell membrane and releases the intracellular proteins. The resultant cell extract was then centrifuged in a microfuge for 10 minutes at 1500g (Henderson Biomedical) to pellet debris and the total protein fraction obtained by removing the supernatant. The total protein concentration was quantified by measuring the optical density (OD) at 280nm using an ND-100 spectrophotometer (Labtech instruments, Laval, Canada), where cell extract readings were initially blanked with RIPA buffer alone. The breast carcinoma cell line, MCF7, which is known to synthesise nm23H1 (Lin *et al*., 2002), provided a positive control for this extraction procedure.

2.5.2.2: Gel electrophoresis and Western blotting for nm23H1.

Cell extracts, MSC CM and unconditioned media (negative control), and recombinant human nm23H1 (kindly supplied by Dr. C. Bunce) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 4-12% NuPAGE ® Bis--Tris gels and NuPAGE® running buffer (Invitrogen), then transferred to Immobilon-P membranes (Millipore Corp, Bedford, MA, US) using NuPAGE® transfer buffer (Invitrogen) containing 10% methanol and an XCell II™ Blot Module according to manufacturers instructions (Invitrogen). Membranes were then stained for 2-3 minutes with 0.1% (v/v) Ponceau (Sigma-Aldrich) in 5% (v/v) acetic acid

(BDH) in distilled water in order to confirm protein transfer and then rinsed with distilled water.

For immunolocalisation of nm23H1, membranes were blocked for 1 hour with 10% (w/v) non-fat milk powder (Marvel, Premier Brands, UK) in PBS and probed with rabbit polyclonal anti-nm23H1 antibodies (Santa Cruz Biotechnology CA, US) overnight at 4^0C. Membranes were then washed three times in PBS and probed with a secondary goat anti-rabbit IgG-horse radish peroxidase (Amersham) for 30 minutes. Membranes were washed again in PBS and the presence of nm23H1 was revealed using a commercially available kit for enhanced chemiluminescence (ECL-SuperSignal® West Pico Chemiluminescent western blotting analysis system Pierce Biotechnology, Rockford, IL, US). According to the manufacturer's instructions membranes were incubated with the ECL substrate for 15 minutes before being visualised under chemiluminescent light. Digitised images of the membranes were collected using image analysis software from PD Quest for Windows.

2.5.2.3: Flow cytometry of MSCs for the presence of cell surface nm23H1.

Flow cytometry was used to assess the cell surface nm23H1 immunoreactivity of MSCs as follows: after trypsinisation, cells were re-suspended in PBS containing 2% (w/v) BSA and 10% (v/v) normal human Ig for 60 minutes to block non-specific binding. MSCs were then incubated at 4^0C for 30 minutes with monoclonal anti-nm23H1 antibody (clone 37.6, Santa Cruz) in 2% (w/v) BSA in PBS. MSCs were incubated also with isotype-matched IgG2a (Dako) as a negative control. MSCs were then washed with 10ml of 2% (w/v) BSA in PBS and centrifuged for 10 minutes at 179g. Immunoreactivity was determined using a FACScan flow cytometer and analysed using Cell Quest software.

2.5.2.4: Nm23H1 immunolocalisation of MSC cytospins and monolayers.

MSC cytospins and monolayers were fixed for 10 minutes with 4% (v/v) formalin in PBS. Endogenous peroxidase activity was blocked by incubation with 3% (v/v) H_2O_2 in methanol for 30 minutes. Slides and plates were washed in PBS, followed by incubation with a blocking buffer of PBS containing 1.5% (v/v) normal horse serum for 20 minutes. Excess blocking buffer was removed and the cytospins or monolayers then incubated with saturating amounts of mouse monoclonal anti nm23H1 antibody (clone 37.6) in PBS. Parallel slides or wells were incubated with isotype-matched IgG2a (Dako) as negative controls. The samples were washed in PBS again, followed by a 1 hour incubation with secondary biotinylated anti-mouse IgG antibodies at 50□g/ ml (Vector Labs) in PBS. The samples were then PBS washed and incubated with a fluorescein-streptavidin complex (Vector Labs) for 30 minutes. Following final washes in PBS, cytospins were coverslipped in mountant containing DAPI (Vecta Labs) to counterstain nuclei for cytospins, whilst monolayers were immersed in PBS.

Real time PCR for nm23 H1 gene expression by MSC. Reactions were performed using an ABI Prism 7700 sequence detector (Applied Biosystems, CA, USA) with 18S ribosomal RNA as internal standard. Gene specific primers were synthesized by Alta Bioscience (University of Birmingham, UK) and probes by PE Applied Biosystems (Roche, USA). Each reaction contained 900 nM of gene specific 5' and 3' primers, 1X Mastermix (containing pre-optimized dNTPs, $MgCl_2$ and buffer concentrations; Applied Biosystems), 125-175 nM gene specific probe (5'-6-FAM, 3'-TAMRA labeled), 50 nM 18S 5' and 3' primers and 200 nM 18S probe (5'-VIC,

3’-TAMRA labeled) and cDNA (equivalent to 12.5 ng of reverse transcribed RNA), in a total volume of 25 μl. Reactions were replicated 4 times on 2 separate runs.

Nm23 H1 reactions:

5’ primer GGCCTGGTGAAATACATGCA;

3’ primer GGCCCGTCTTCACCACAT;

probe 6-FAM-CTCCCAGACCATGGCAACTACCGG-TAMRA.

Cycle threshold (Ct) values were obtained graphically for test genes and 18S internal standard. Gene expression was normalized to the 18S and represented as ΔCt values. Comparison of gene expression between control and treated samples was derived from subtraction of control ΔCt values from treatment ΔCt values to give a ΔΔCt value and relative gene expression was calculated as $2^{-\Delta\Delta Ct}$ and normalized to controls.

2.4.1: Preparation of cell culture substrate choice assays.

Commercially available purified neural PG isolated from embryonic chick brains, the major types in this mixed preparation including neurocan, phosphacan, versican and aggrecan (Chemicon; CC117, Temecula, CA), and type I collagen (isolated from calf tail; C8919, Sigma-Aldrich) were used to coat tissue culture plates in restricted localities. This technique provides for a substrate “choice” assay and was used to model the interaction of neurons to extracellular matrix molecules present within the developing and injured CNS (Snow *et al*., 1990; Snow *et al*., 2001). The culture plates were prepared as follows:

(i) plates were pre-coated with a thin layer of protein-binding nitrocellulose in methanol ($1mm^2$/ ml) (BA85, Schleicher & Schuell, Dassel, Germany) by adding an excess of the solution, then removing it to leave a very thin film deposited as the methanol evaporated.

(ii) this thin layer was then blotted with 350μm wide strips of filter paper (Whatman No.1, cut with a McIlwain tissue chopper, Campden Instruments, Loughborough, UK) which had been pre-soaked in neural PG at concentrations ranging from 0.1μg - 100μg/ ml in PBS. The restricted localisation of the neural PG on the culture plates was visualized by inclusion of 10% (v/v) marker dye rhodamine B (Sigma-Aldrich) in the neural PG solutions.

(iii) after the filter strips had dried such that they could be tapped off and removed, the plates were further coated with a solution of 100μg/ ml of type I collagen (Sigma-Aldrich) (in PBS) for 5 minutes.

(iv) after collagen-coating, all wells were washed repeatedly with PBS prior to seeding with explants of embryonic day 10 (E10) chick DRG in the presence or absence of MSCs (Figure 2.4- see section 2.4.2 for details of culture/ seeding protocols).

The binding of neural PG in defined areas on the nitrocellulose substratum that had both chondroitin sulphate (CS) and keratin sulphate (KS) GAG side chains was confirmed by immunolabelling. After blotting the neural PG to the nitrocellulose coated plates, the culture substrata were blocked for 1 hour with 5% (w/v) FCS in PBS. Excess blocking buffer was removed; then the wells were treated with 0.25 units/ ml chondroitinase ABC (Sigma-Aldrich) in PBS for 1 hour in order to expose the CS and KS epitopes. Wells were washed repeatedly in PBS, then incubated with monoclonal primary antibodies specific for CS and KS disaccharide epitopes at 1 in 1000 and 1 in 500 respectively, in PBS (kindly provided by Dr. B. Caterson, Cardiff University). Parallel wells were incubated with irrelevant isotype-matched antibodies (Dako) as negative controls. After incubation with the primary

antibodies all wells were washed in PBS before immunopositivity was revealed using the Vector ABC System described in section 2.2.4.3.

The same technique was used to prepare culture plates with substrates choices of 100µg/ ml collagen I and strips of MAG and Nogo-A at concentrations ranging from 10µg - 200µg/ ml in PBS (R&D systems, Abbingdon, UK), recombinant human nm23H1 (wild type nm23H1 and a mutant form that does not have nucleoside diphosphate kinase (NDPK) activity - H118F), or aldo-keto-reductase (AKR), all kindly provided by Dr. C. Bunce) at various concentrations.

2.4.3: Microscopy, image capture and analysis.

Cultures were viewed using phase contrast and fluorescence microscopy (Nikon Eclipse TS100, Nikon, Kingston-upon-Thames, UK). Digitized images were captured with a Hamamatsu digital camera (C4742-95, Hamamatsu photonics, Welwyn Garden City, Hertfordshire, UK) at various time points and examined using IPLab software (Version 3.6, Becton Dickinson) adapting methods previously described (Johnson *et al*., 2002). The number of neurites that had extended from chick E10 chick DRG explants located on collagen onto substrates of neural PG, MAG or Nogo-A were quantified. Such that neurite outgrowth was scored only where the explant itself was not in contact with the inhibitory substrate and where a minimum of 10 neurites/ explant had reached the inhibitory substrate. A "crossing" event was defined as a neurite extending from the collagen-only borderline at least 5□m onto the neural PG, MAG or Nogo-A. Only a single crossing event was scored if a neurite was observed on the inhibitory substrate within 25□m of any other neurite crossing along the collagen borderline. This was to minimize the possibility of outgrowth over the inhibitory substrates arising through fasciculation but by definition limited the number

of neurite crossings to a maximum of 24 per image, due to the size of each image captured at a magnification of x 100. The number of MSCs where the majority of the cell area was adhered to the neural PG, MAG or Nogo-A substrates in each digitized image was also scored.

2.4.3.1: Time-lapse Video Microscopy.

Digitized images were captured over extended periods using phase contrast microscopy and a digital video camera (TK-1280E; JVC, London, UK) which was kept within a tissue culture incubator. The digitised images were converted into video files using Media Studio Video Editor (version 3.5; Ulead Systems, Karst, Germany).

2.6: Statistical analysis.

All data was analysed using non parametric tests as the data was either not normally distributed (tested for using the Anderson-Darling test for normality) or was not continuous, e.g. when there was an artificial upper limit to values which occurred when counting DRG neurite crossing over neural PG substrates (see section 4.1). Non parametric tests employed include the Spearman ranked correlation coefficient, the Kruskal-Wallis non parametric analysis of variance (ANOVA) and the Mann-Whitney *U* test.