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Corresponding Author: Dr. Karina Wright,

Corresponding Author's Institution: Keele University

First Author: Karina Wright

Order of Authors: Karina Wright; Kenzo Uchida; Jennifer Bara; Sally Roberts; Wagih El Masri; William Johnson

Abstract: BACKGROUND CONTEXT: Transplantation of bone marrow cells into spinal cord lesions promotes functional recovery in animal models and recent clinical trials suggest possible recovery also in humans. The mechanisms responsible for these improvements are still unclear.
PURPOSE: To characterise spinal cord motor neurite interactions with human bone marrow stromal cells (MSC) in an in vitro model of spinal cord injury (SCI).
STUDY DESIGN/SETTING: Previously we have reported that human MSC promote the growth of extending sensory neurites from dorsal root ganglia (DRG), in the presence of some of the molecules present in the glial scar which are attributed with inhibiting axonal regeneration following SCI. We have adapted and optimized this system replacing the DRG with a spinal cord culture to produce a central nervous system (CNS) model which is more relevant to the SCI situation.
METHODS: We have developed and characterised a novel spinal cord culture system. Human MSC were co-cultured with spinal motor neurites in substrate choice assays containing glial scar associated inhibitors of nerve growth. In separate experiments MSC conditioned media was analysed and added to spinal motor neurites in substrate choice assays.
RESULTS: As has been reported previously with DRG, substrate-bound neurocan and Nogo-A repelled spinal neuronal body adhesion and neurite outgrowth, but these inhibitory effects were abrogated in MSC/ spinal cord co-cultures. However, unlike DRG, spinal neuronal bodies and neurites showed no inhibition to substrates of myelin associated glycoprotein. In addition, the MSC secretome contained numerous neurotrophic factors which stimulated spinal neurite outgrowth, but these were not sufficient stimuli to promote spinal neurite extension over inhibitory concentrations of neurocan or Nogo-A.
CONCLUSIONS: These findings provide novel insight into how MSC transplantation may promote regeneration and functional recovery in animal models of SCI and in the clinic, especially in the chronic situation where glial scars (and associated neural inhibitors) are well established. In addition, we have confirmed that this CNS model predominantly comprises of motor neurons via immunocytochemical characterisation. We hope that this model may be used in future research to test various other potential interventions for spinal injury or disease states.

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3 **Authors names and affiliations:** Karina T. Wright[a,e], Kenzo Uchida[c], Jennifer J.
4 Bara [a,e], Sally Roberts[a,e], Wagih El Masri[b,e] and William E. B. Johnson[d].
5 [a]Centre for Spinal Studies; [b]Midlands Centre for Spinal Injuries: Robert Jones
6 and Agnes Hunt Orthopaedic Hospital, Oswestry, Shropshire, UK, SY10 7AG.
7 [c]University of Fukui, Department of Orthopaedics and Rehabilitation Medicine,
8 Fukui, Japan. [d]Life and Health Sciences, Aston University, Aston Triangle,
9 Birmingham, UK, B4 7ET. [e]Institute for Science and Technology in Medicine,
10 Keele University, Keele, Staffordshire, ST5 5BG, UK.
11 **Corresponding author:** Dr Karina T. Wright, Centre for Spinal Studies, Robert
12 Jones and Agnes Hunt Orthopaedic Hospital, Oswestry, Shropshire, UK SY10
13 7AG. Tel: +44 (1691) 404699; Fax: +44 (1691) 404170; Email:
14 Karina.Wright@rjah.nhs.uk.

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Introduction

Injury to the central nervous system (CNS) usually initiates a poor intrinsic regenerative response for a number of reasons. Immune reactions, which in other tissues may help to recruit reparative cells, often have a devastating effect on CNS tissue function. Inflammation and ensuing secondary cascades can cause extensive neuronal and glial cell death, as well as glial cell activation and hypertrophy [1]. In an effort to restore the blood brain barrier, astrocytes at the site of injury become reactive and synthesise a proteoglycan rich matrix [2]. Myelin debris associated molecules, including Nogo-A and myelin associated glycoprotein (MAG), are also released from damaged neural tissues [3]. These events combine to produce a hostile environment for nerve re-growth [2-6].

There has been extensive interest world-wide in the development of cell transplantation strategies for the treatment of CNS damage, in particular spinal cord injury (SCI). Many diverse potential cell therapies have been tested, each targeting different distinct stages of SCI and mechanisms of spinal cord repair [7-10]. Allogeneic embryonic stem cells (ES cells) and umbilical cord-derived cells, as well as possible autologous cell sources, including adult neural stem cells, Schwann cells and olfactory ensheathing cells have been shown to promote axonal regeneration and restore function in animal models of SCI [11-17]. These types of cell are thought to act in a number of ways depending on the cell type transplanted, including replacing dead or damaged neurons and glia, re-establishing neural networks, remyelinating demyelinated axons and reducing the hostile nature of the SCI lesion.

Autologous cell therapies derived from bone marrow have also been shown to enhance functional recovery in animal models of SCI and possibly in the clinic [10], but the repair mechanisms responsible are still largely unclear. Some controversial evidence exists which suggests that bone marrow cells, including marrow stromal cell (MSC) and hematopoietic stem cell fractions, may trans-differentiate to replace lost neurons and glia, in a manner similar to that proposed for ES cells and neural stem cells [18-22]. However, the consensus of opinion seems to be that for MSC transplantation at least, the most likely mode of activity is an induction of a diverse myriad of paracrine anti-inflammatory pathways and directly restorative cell-matrix and cell-cell interactions [23-29].

Previously we have used growth substrate choice assays to examine how human MSC influence neurite outgrowths from explants of chick dorsal root ganglia (DRG). We have demonstrated that MSC help neurites to overcome the effects of some of the major nerve inhibitory molecules found in SCI lesions, including neural proteoglycans, Nogo-A and MAG [30]. This established model of sensory nerve growth provided an excellent platform to examine in real-time possible cell-matrix and cell-cell interactions that may occur in the SCI milieu. In the current study, we have adapted and refined our system by replacing DRG explants with spinal cord cultures to provide a more relevant model of CNS nerve growth. We envisage that the establishment of a novel spinal nerve growth substrate assay, which comprises characterized motor neurons and relevant neural matrix molecules, will provide an invaluable research tool for testing SCI

84 therapeutics, which will have further applications in the broader fields of CNS

85 tissue engineering and repair.

86

Materials and methods

Ethics Statement

All research involving human participants was completed with written informed consent and Local Research Ethics Committee (LREC) approval: Shropshire & Staffordshire Strategic Health Authority, Reference Number: 04/02/RJH. Ethical approval and a Home Office project license for the study were not required under the United Kingdom Animal (Scientific Procedures) Act of 1986 because chicks were killed by decapitation (which is an appropriate method under Schedule 1 of the Act).

Human bone marrow stromal cell (MSC) culture

Bone marrow aspirates or bone chips were harvested from the iliac crest of individuals undergoing spinal fusion in the treatment for lumbar degenerative disorders (n=5; ages 29-53). Bone marrow aspirates and bone chips were kindly collected by spinal surgeons from the Centre for Spinal Disorders and sent to the Spinal Studies research laboratories for processing (both based at the RJAH Orthopaedic Hospital, Oswestry, UK).

Bone chips were perfused with Dulbecco's Modified Eagle's Medium (DMEM/F12), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin (P/S) (Invitrogen Life Technologies, Paisley, UK). Mononuclear cells isolated by density gradient centrifugation at 900g for 20 minutes over Lymphoprep™ (Fresenius Kabi Norge, AS) were plated out in DMEM/ 20% FBS + P/S medium (Invitrogen Life Technologies) at a seeding density of 20 x 10^6 cells per flask. After 24 hours, non-adherent cells were removed and the

adherent cell populations were cultured in monolayer and were maintained in a humidified atmosphere of 5% CO_2 at 37^0C through to passage II-III in DMEM/ 10% FBS + P/S medium. MSC cultures used in this study were characterised according to the MSC CD immunoprofile criteria published by the International Society for Cellular Therapy [31].

Embryonic chick neuronal cultures

Spinal cords were dissected from E4.5 hybrid brown chicks as described previously [32] and cut into 10-20 pieces per cord, such that all were of approximately equal size. These were then digested in 20μl of trypsin (2.5% w/v; final concentration 0.05%) in PBS (Invitrogen Life Technologies) for 15 minutes at 37^0C whilst agitating frequently. The trypsin supernatant was removed and replaced by 900μl of neuronal culture medium (NCM; L-15 culture medium supplemented with 1% (v/v) insulin, transferrin and selenium, 1% (v/v) P/S (L-15/ ITS-X/ P/S medium, Invitrogen Life Technologies), 1% (v/v) horse serum and 1.5mg/ml glucose) (Sigma-Aldrich, Poole, UK) and 100μl of 4% (w/v) bovine serum albumin (BSA; Sigma-Aldrich). The spinal cord tissue was homogenized using a pipette and spinal cord neuronal cells were isolated by density gradient centrifugation at 500g for 15 minutes over a warmed 1.5ml cushion of 6.8% (v/v) Nycodenz® (Serva, Heidleberg, Germany). Dissociated cells were seeded into 24-well tissue culture plates (Co-star, Corning Inc, NY) pre-coated with nerve-permissive and nerve-inhibitory molecules (see below) in NCM supplemented with 0.4% (v/v) N2-supplement and 10ng/ml basic fibroblast growth factor (bFGF)

(Invitrogen Life Technologies) at a seeding density of 3 x 10^5 cells per well and were maintained in a humidified atmosphere of 5% CO_2 at 37^0C for 72 hours.

Optimisation of chick neuronal culture growth substrata

Briefly, some wells were pre-coated with a thin layer of protein-binding nitrocellulose (BA85, Schleicher & Schuell, Dassel, Germany). Pre-coated and uncoated plates were then further incubated with either PBS or 25µg/ml laminin (derived from Engelbreth-Holm-Swarm mouse tumour, BD Biosciences, Oxford, UK) in PBS. After coating, all wells were washed repeatedly with PBS prior to seeding with neuronal cultures. Control DRG plates for SC1 immunostaining were established using embryonic chick day 10 DRG as described previously [30].

MSC/ neuronal co-cultures

MSC were labelled with Cell Tracker™ Red Fluorescent Probe (Cambrex Bioscience, Wokingham, UK) following the manufacturer's protocol. Labelled cells were seeded (at a density of 5 x 10^3 cells/ cm^2) in DMEM/ 10% FBS + P/S into plates coated with nerve-permissive and nerve-inhibitory substrata (see below). After 24 hours, any non-adherent cells were removed and wells washed repeatedly before adding N2 and bFGF-supplemented NCM. Neuronal cultures were then immediately seeded into each well and the MSC/ neuronal co-cultures maintained in a humidified atmosphere of 5% CO_2 at 37^0C for 72 hours. Control plates of neuronal cultures seeded alone, i.e., without pre-seeded MSC, were established at the same time in N2 and bFGF-supplemented NCM.

Mixed substrate preparation

Neurocan, isolated from embryonic chick brains and purified with a monoclonal antibody (Millipore, Billerica, MA), was used to coat tissue culture plates in restricted localities, as described previously [30, 33,34]. Briefly, wells were pre-coated with a thin layer of protein-binding nitrocellulose (see above), which was then blotted with 350μm wide strips of filter paper (Whatman No.1, GE Healthcare, Maidstone, Kent, UK) that had been soaked in neurocan at concentrations ranging from 1μg-50μg/ml (in PBS). After the filter strips had dried and been removed, the plates were then washed with PBS. The restricted localisation of the neurocan on the culture plates was visualized by inclusion of a marker dye (5% v/v rhodamine B, Sigma-Aldrich) in the neurocan solution. The same technique was used to prepare culture plates with substrates of 10-400μg/ml recombinant Nogo-A on nitrocellulose or 10-400μg/ml recombinant MAG on nitrocellulose (both R&D Systems, Abbingdon, UK). After coating, all wells were washed repeatedly with PBS prior to seeding with neuronal cultures and/ or MSC.

SC1 and neurofilament (NF) 200kD immunostaining

SC1 is a cell surface adhesion molecule expressed on motor neuron cell bodies and axons [35], which can be used to purify motor neurons from spinal cord tissues [36]. Neuronal cell cultures were immunolabelled with SC1 for motor neuron characterisation, whereas NF immunolabelling was used for neurite quantitation because the NF immunofluorescence was much stronger and hence better for the counting of fine neurite extensions.

Neuronal cell cultures were fixed by gently adding an equal volume of 4% (w/v) buffered paraformaldehyde (BDH Biosciences) to the culture medium in each well for 10 minutes. Wells were washed with PBS twice for 10 minutes. Cells were then incubated for 1 hour with a blocking buffer of 10% goat serum (Vector Laboratories, Burlingame, CA) in PBS at room temperature. Mouse monoclonal anti-SC1 (neat) (kindly donated by Prof Hideaki Tanaka, Kumamoto University, Japan) or anti-NF (1:200) (clone NE14, Sigma-Aldrich) were used as the primary antibodies, and goat anti-mouse Alexa Fluor 488 (1:100) (Invitrogen Life Technologies) was used as a secondary antibody. Cells were incubated with the primary antibody for 1 hour and the secondary antibody for 40 minutes at room temperature to stain neuronal bodies and their neurites fluorescent green.

Fibronectin and laminin immunostaining

MSC cultures were fixed by gently adding an equal volume of 4% (w/v) buffered paraformaldehyde to the culture medium in each well for 10 minutes. Wells were washed with PBS twice for 10 minutes. Cells were then incubated for 20 minutes with a blocking buffer of 15% horse serum (Vector Laboratories) in PBS at room temperature. Rabbit polyclonal anti-fibronectin (250μg/ml) or anti-laminin (25μg/ml) (both, Sigma-Aldrich) were used as the primary antibodies, and biotinylated goat anti-rabbit (50μg/ml, Vector Labs) was used as a secondary antibody followed by a fluorescein-streptavidin complex (20μg/ml, Vector Labs). Cells were incubated with the primary antibody overnight, the secondary antibody for 40 minutes and the fluorescein-streptavidin complex for 20 minutes at room temperature.

MSC-CM neuronal culture assays

MSC-CM (n=6) were generated as described previously [30] and stored at -20^{o}C prior to use. Neuronal cell cultures were seeded in MSC-CM in culture plates that had either been uniformly coated in nitrocellulose or coated with nitrocellulose and strips of neurocan (50μg/ml) or Nogo-A (400μg/ml), as described above. Control neuronal cultures were maintained in non-conditioned media under the same conditions. Neurite outgrowth was measured after 72 hours in culture.

MSC-CM neurotrophic protein arrays

MSC-CM were screened for a panel of 23 neurotrophic proteins using custom designed antibody arrays (RayBiotech Inc, Norcross, GA) according to the manufacturer's instructions. In brief, array membranes with protein antibodies spotted in duplicate were incubated with blocking buffer for 30 minutes at room temperature. MSC-CM were thawed and incubated with the membranes overnight at 4°C. Membranes were washed and then incubated with a Biotin-conjugated antibody for 1 hour. Wash steps were repeated as before and membranes incubated with HRP-conjugated streptavidin for 2 hours. Following another series of wash steps, membranes were incubated with a chemiluminescent detection reagent provided in the kit for 2 minutes. Positive signals were visualised with a chemiluminescence imaging system (ChemiDoc™ EQ, Bio-Rad Laboratories Srl, Italy). Array data was semi-quantified by measuring the sum of the intensities of the pixels within each spot boundary x pixel area, with image analysis software (Quantity One® version 4.6.3, Bio-Rad, Italy). A signal from a clear part of the array was subtracted from all data to

account for background signal. A mean was taken from the two duplicate spots for each factor. Levels of neurotrophic factors were normalised to positive controls (provided in the kit) and to the number of MSC that had generated a standard volume of conditioned media.

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 black and white Hamamatsu digital camera (C4742-95) and examined using IPLab software (Version 3.6, Nikon). For determination of the optimal substrate for growth of embryonic chick spinal neuronal cultures, cell aggregates and neuronal body adhesion, and neurite outgrowth were counted using phase contrast and fluorescence images. A cell aggregate was determined as a cluster of more than one adhered cell visible under phase microscopy. NF immunolabelling, visible under fluorescence microscopy was used to stain neurites and to determine those cell aggregates that were of a neuronal phenotype. Hence, those cell aggregates that were immunopositive for NF and possessed neurites (that is, if a neurite ≥25μm in length were in contact with a neuronal body) were then described as ‘neuronal bodies’. For substrate choice assays, the number of neuronal bodies with neurites that had adhered onto substrates of plastic, nitrocellulose, laminin, neurocan, Nogo-A or MAG were quantified using fluorescent images. For all analysis, adhered neuronal bodies with neurites were counted after 72 hours in culture. The number of red fluorescent MSC that were present on the nitrocellulose, neurocan, Nogo-A or

MAG substrates in each digitized image was also scored. For substrate choice assay quantitation, results from at least 5 separate cultures and 5 separate images per culture were pooled and combined.

MSC-CM neurite outgrowth assays were viewed and quantified using phase contrast microscopy and digitized images captured and examined using the Cell IQ® Imagen system and Analyser software (Chip-Man Technologies, Tampere, Finland). In brief, phase contrast images of cultures (n=12 controls and n=24 MSC-CM) were captured using a fully automated system every 2-3h over a period of 72 hours. From these images, the Cell-IQ® Analyser software automated search tool 'neurite finder' generated temporal neurite length data for each culture condition.

Statistical analysis

The Mann-Whitney *U* test was used to assess significant differences: (i) between the frequency of neuronal bodies adhered with extending neurites onto uniform substrates of plastic, nitrocellulose and laminin, (ii) between the frequency of neuronal bodies adhered with extending neurites onto each of the adjacent substrates in substrate choice assays of nitrocellulose versus neurocan, Nogo-A or MAG in neuronal and MSC co-cultures, compared to control neuronal cultures alone, (iii) between the frequency of MSC adhered onto each of the adjacent substrates in substrate choice assays of nitrocellulose versus neurocan, Nogo-A or MAG. The relationship between the relative amounts of each neurotrophic protein and the total neurite outgrowth in each MSC-CM was determined using the Spearman ranked correlation coefficient *rs*.

Results

Nitrocellulose substrates promote optimal growth of embryonic chick spinal neuronal cultures.

Embryonic chick spinal cells formed aggregates which adhered to both plastic and nitrocellulose substrates with or without laminin coating to varying degrees (Fig 1A). A number of fibroblastic cells adhered to substrates of plastic alone; a small proportion of these cells extended neurites but these were difficult to distinguish from neighbouring aggregates in close proximately. Cells seeded onto substrates of plastic coated with laminin or nitrocellulose formed discrete cell aggregates and the majority of these aggregates possessed neurites. The frequency of neurites was increased on substrates of plastic coated with nitrocellulose compared to plastic (with or without laminin) (Fig 1B). For substrates of nitrocellulose coated plastic with laminin, fibroblastic cell aggregates were so confluent they could not be reliably separated for quantitation.

Embryonic chick spinal cell aggregates and neurites were then fixed and immunostained for neurofilament (NF) (Fig 1C). A large proportion of those cell aggregates which had adhered to plastic alone were lost following fixation and immunostaining. The discrete cell aggregates which had adhered to the laminin or nitrocellulose coated plastic were identified as NF immunoreactive neuronal bodies. In addition, the extended cell processes that were seen emanating from these cell aggregates were identified as NF immunoreactive neurites. The frequency of neurites was increased on substrates of nitrocellulose coated plastic

compared to plastic (with or without laminin) (Fig 1D). For substrates of nitrocellulose coated plastic with laminin, there was no clear aggregation of NF immunoreactive cells to form discrete neuronal bodies, with a confluence of cells growing across the substrate instead; hence the distribution of discrete NF immunoreactive neuronal bodies could not be measured.

Embryonic chick spinal neuronal cultures are immunopositive for the motor neuron marker SC1

Embryonic chick dorsal root ganglion (DRG) cultures were negative for SC1 immunocytochemical staining as were isotype matched control wells (Figs 2A and 2B). In contrast, a large proportion of spinal neuronal cultures were immunopositive for SC1 (with corresponding negative staining of isotype matched control wells) (Figs 2C-E).

Embryonic chick spinal neuronal cultures are inhibited by neurocan and Nogo-A but not MAG

Neuronal bodies with neurites were repelled by neurocan and Nogo-A in a concentration-dependent manner (Fig 3A and 3B). At high neurocan and Nogo-A concentrations (50µg/ml and 400µg/ml respectively), neuronal body adhesion and neurite outgrowth was almost completely inhibited (<1 neuronal body with at least one neurite per image). At lower neurocan and Nogo-A concentrations (1-10µg/ml and 10-200µg/ml respectively), increasing numbers of neuronal bodies and neurites adhered to neurocan and Nogo-A substrates and extended neurites. In contrast, neuronal cultures seeded onto nitrocellulose: MAG substrate assays showed no preference for either substrate, i.e. neuronal bodies and neurites were

not inhibited by MAG at any concentration (5-400μg/ml) (Fig 3C). No evidence of neuronal cell death (as delineated by cell detachment) was observed in any of the cultures tested.

MSC promote embryonic chick spinal neuronal adhesion and neurite extension over substrata of neurocan, Nogo-A and MAG

In MSC/ neuronal co-cultures, neuronal bodies were able to adhere and extend neurites over high neurocan and Nogo-A concentrations, such that ~5 neuronal bodies with neurites per image were present on 50μg/ml neurocan and ~3 neuronal bodies with neurites per image were present on 400μg/ml Nogo-A. However, the inhibitory effects of neurocan and Nogo-A on neuronal adhesion and neurite outgrowth were only partially abrogated when compared to substrates of nitrocellulose. Some of the pre-seeded MSC appeared to align at the borders of nitrocellulose with neurocan or Nogo-A, suggesting that these cells were also inhibited by the nerve-inhibitory matrix molecules. Nonetheless, it was apparent that even at high neurocan and Nogo-A concentrations, some MSC were still able to adhere to the neurocan and Nogo-A substrates and it was to these MSC that the adherent neuronal bodies and neurites were often co-localised (Figs 4A and 4B). MSC, neuronal bodies and neurites were not inhibited by MAG at any concentration (5-400μg/ml) (Fig 4C). Nonetheless, neuronal body adhesion and neurite extension was increased on all substrates, including neurocan, Nogo-A, MAG and nitrocellulose when in co-culture with MSC in comparison to the absence of MSC. In addition, MSC traversing inhibitory

substrata were immunopositive for the nerve permissive matrix molecules laminin and fibronectin (Fig 4D).

MSC conditioned media (MSC-CM) promotes spinal neurite outgrowth over nitrocellulose but not neurocan or Nogo-A inhibitory substrata

MSC-CM significantly increased spinal neurite extension over nitrocellulose substrates compared to control cultures in non-conditioned media (Fig 5A). We have detected several neurotrophic proteins in MSC-CM which may be important in stimulating spinal neurite outgrowth (Fig 5B). Of the neurotrophic factors identified, the levels of granulocyte colony stimulating factor (GCSF), fibroblast growth factor-4 (FGF-4) and matrix metalloproteinase-8 (MMP-8) correlated significantly to the quantity of neurite outgrowth detected (Spearman Rank *rs* 0.57, p=*0.014, *rs* 0.57, p=***<0.0001 and *rs* 0.66, p=**0.0032 respectively). However, MSC-CM alone was not sufficient stimulus to promote neurite outgrowth over inhibitory concentrations of neurocan or Nogo-A (Fig 5C).

Discussion

MSC transplantation for the treatment of SCI has proven efficacious in terms of promoting axonal regeneration and functional recovery in animal models and possibly in the clinic [10]. However, few definitive experiments have addressed the mechanisms involved in this process. We have developed a substrate choice assay to examine how spinal nerves interact in co-culture with MSC, specifically in the context of molecules that are present at the site of SCI and that are considered to form major inhibitors to axonal regeneration. Using this model we have shown that spinal neuronal bodies and neurites are inhibited by neurocan and Nogo-A in a concentration dependent manner, akin to DRG sensory neurites, which we have reported previously [30]. Increased concentrations of these extracellular inhibitors, however, were required to observe a similarly 'complete' inhibition, e.g. 50μg/ml of neurocan and 400μg/ml Nogo-A completely inhibited spinal neurites, compared to 10μg/ml of neural proteoglycans (which includes neurocan) and 200μg/ml Nogo-A for the complete inhibition of DRG neurite outgrowth. However, unlike DRG sensory neurites, spinal cultures were not inhibited by MAG substrates at any of the concentrations tested (up to 400μg/ml). Hence, using this CNS system we have shown that one of the proposed inhibitors in the glial scar (MAG) may not be as potent in CNS systems as it is in DRG systems, which may have important implications for our understanding of nerve growth inhibition in the SCI setting. There is some supportive evidence in the literature for these findings which suggest that MAG may not be a crucial inhibitor of axonal regeneration in the CNS. For example,

Bartsch et al. [37] have shown that MAG deficient mice exhibit poor axonal regrowth following either optic nerve or corticospinal tract transection *in vivo*, although MAG has been shown by others to repel both peripheral nervous system (PNS) and CNS nerve growth [30, 38, 39].

There are a number of distinctions between these neuronal cultures which might account for the differences we have observed in their response to substrate choice assays, compared to those results previously reported. The most obvious is the developmental stage of each tissue source; in the current study, spinal cultures were isolated 4.5 days after fertilization, compared to our previous work using DRG explants from day 10 embryos [30]. The expression of axonal guidance ligands and receptors, including myelin receptors are known to change throughout CNS and PNS development [40-44], which may explain why spinal and DRG cultures exhibit different sensitivities to MAG. In addition, both our current and previous methods of primary neuronal culture isolation included few (if any) purification steps and hence, these cultures are composed of mixed cell populations. We are in the process of characterising those 'fibroblast-like' cells visible in CNS and PNS cultures, which are likely to have influenced the sensitivity of neuronal cultures in substrate 'choice' assays. There is a possibility that other CNS cell types may have reduced the sensitivity of spinal neurites to MAG substrates, perhaps by physically masking or blocking inhibitory epitopes, or by secreting growth factors that blocked the inhibitory effects of MAG, e.g. brain derived neurotrophic factor (BDNF) [45]. In contrast, Schwann cells, which may be present in mixed PNS cultures, could exacerbate sensory nerve reactivity

to MAG via an additive effect, as Schwann cells themselves express nerve inhibitory MAG [46]. Furthermore, each culture environment varies greatly in media composition and growth factor supplementation which may also impact directly on the sensitivity of neurites to inhibitory substrates, including MAG [45, 47]. For example, the exposure to neurotrophins has been shown to upregulate chimaerin (one of the Rho-GTPase activating proteins) in cerebellar neurons [48]. The expression of chimaerin in the cerebellum is correlated with abolishment of the inhibitory effects of MAG in development and ectopic expression of chimaerin in cerebellar neurons *in vitro* results in resistance to MAG induced neurite inhibition [48].

We have demonstrated that MSC co-culture reduces the inhibitory effects of neurocan and Nogo-A on spinal neuronal body adhesion and neurite outgrowth and enhances spinal neurite outgrowth over all of the substrates tested (neurocan, Nogo-A, MAG and nitrocellulose). We have also shown that MSC were repelled by high concentrations of neurocan and Nogo-A (but not MAG substrates). Hence, at high concentrations MSC could clearly be seen to align along inhibitory neurocan and Nogo-A borders. Although MSC were inhibited to a much lesser extent than spinal neuronal bodies and their associated neurites. This is not too surprising as we already know that MSC may have an increased capacity to adhere to and migrate over neural proteoglycans, Nogo-A and MAG compared to other cell types [30]. The exact mechanisms responsible for the abrogation of spinal nerve inhibition to neurocan and Nogo-A in MSC co-cultures may involve a number of complex paracrine, cell-matrix and cell contact-

mediated interactions. We and others have previously reported that MSC-CM promotes neurite outgrowth from DRG explants and that and that MSC synthesise a number of soluble cytokines and other growth factors that are known to stimulate nerve extension including NGF, BDNF and vascular endothelial growth factor [26, 30, 49]. In this study we have shown that MSC-CM promotes spinal neurite outgrowth and contains several neurotrophic proteins, including GCSF, FGF-4 and MMP-8 which significantly correlated to the level of spinal neurite stimulation observed. However, we show that MSC-CM alone was insufficient stimuli to promote spinal neurite extension over inhibitory concentrations of neurocan or Nogo-A.

There are other explanations which might account for spinal neurites extending over inhibitory substrates in MSC co-cultures. MSC are known to synthesise numerous extracellular matrices that support neuronal cells and provide an optimal surface for nerve growth [50]. We have shown using our model that migrating MSC provide permissive matrix ‘bridges’ of laminin and fibronectin over nerve inhibitory substrates. In addition, in many sequences and on all inhibitory substrata tested, MSC and spinal neurites co-localised. Whereby MSC appeared to act as adhesive ‘stepping stones’ for neurite extension. Alternatively, nerve-inhibitory molecules, particularly neural proteoglycans such as neurocan, may have been degraded by matrix metalloproteinases (MMPs), e.g. MMP-1, MMP-2, MMP-13, which MSC are known to synthesise [51]. We have previously demonstrated that cell contact-mediated events, such as towing of neurites and bridging of inhibitory substrata, may play an important role in

MSC abrogating the DRG nerve-inhibitory effects of neural proteoglycans, Nogo-A and MAG [30]. Further experimentation using this system will aim to elucidate which of these mechanisms contribute to MSC stimulation of spinal neurite outgrowth over neurocan and Nogo-A, and to what extent. This may help to identify molecular targets to further enhance nerve growth in SCI environments.

There are few primary motor neuron culture protocols available for scientists to examine new therapies for CNS repair, particularly in the context of the injured spinal cord. We have modified an existing protocol [32] to test embryonic motor neurons, as characterised by SC1 staining, cultured on substrate choice assays. We suggest that the development of this assay and its refinement for the testing of adult spinal motor neurite outgrowth over different growth surfaces will provide a valuable tool to examine motor neuron and glial cell-matrix and cell-cell interactions, not readily achieved when using complex *in vivo* models. We anticipate that this novel system may help to further elucidate some of the mechanisms of increased axonal regeneration that has been noted following MSC transplantation for the treatment of SCI, as well as having wider application in the field of spinal therapeutics.

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Figure Legends

Figure 1. Nitrocellulose promotes optimal growth of embryonic chick spinal neuronal cultures. A: Spinal cord cells formed many small fibroblastic aggregates with few neurites on plastic alone, larger cell aggregates with some neurites were formed on plastic pre-coated with laminin. Similar large cell aggregates were formed on nitrocellulose alone but with many more associated neurites. Cells seeded onto nitrocellulose pre-coated with laminin formed an almost confluent fibroblastic culture with few neurites. Examples of neurites are indicated by black arrows. Digitized images under phase contrast microscopy (calibration bar = 100μm). B: The number of cell aggregates with neurites was significantly increased on nitrocellulose substrates alone compared to plastic (with or without laminin) (*p=0.0042 and *p=0.0062 respectively, Mann Whitney *U* test: data shown are from at least 5 separate cultures and 5 separate images per culture combined ± SEM). C: Most cell aggregates on plastic substrates were lost following fixation and immunostaining, some neurofilament staining of neuronal bodies and neurites was observed on plastic pre-coated with laminin. On nitrocellulose alone many neuronal bodies were immunopostive for neurofilament as were a complex network of associated neurites. The nuclei of cells seeded onto nitrocellulose pre-coated with laminin were stained but few neurites were visible. Digitized images under fluorescence microscopy (calibration bar = 100μm). D: After immunostaining for neurofilament the number of neuronal bodies with neurites was significantly increased on nitrocellulose substrates alone compared to plastic (with or without laminin) (*p=0.0034 and *p=0.0067

653 respectively, Mann Whitney *U* test: data shown are from at least 5 separate
654 cultures and 5 separate images per culture combined ± SEM).
655 Figure 2. Embryonic chick spinal neuronal cultures are immunopositive for SC1,
656 a motor neuron marker. A-E: Representative digitized images of identical fields
657 are shown from left to right. Left panels show phase images and right panels
658 show immunolocalisation for SC1 (A and C) and isotype matched controls (B and
659 D). A and B are DRG explants (negative for SC1 staining), C and D are spinal
660 neuronal cultures (positive for SC1 staining). E: At low magnification
661 encompassing a wide field of view, many if not all neuronal bodies and neurite
662 networks in spinal neuronal cultures were immunopositive for SC1. Calibration
663 bars = 100μm.
664 Figure 3. Neurocan, Nogo-A and MAG spinal neuronal body adhesion and
665 neurite outgrowth assays. A-C Digitized images of identical fields are shown from
666 left to right under phase contrast and fluorescence microscopy (middle panels
667 illustrate the location of the neurocan, Nogo-A or MAG, right panels show NF
668 immunolabelled neuronal bodies and neurites, calibration bars = 100μm). A:
669 Neurocan substrates repelled neuronal body adhesion and neurite outgrowth in a
670 dose dependant manner. The difference in the frequency of neuronal bodies with
671 neurites which had adhered to neurocan substrates compared to nitrocellulose
672 was significant at concentrations of 1, 5, 10 and 50μg/ml (**p=0.059 and
673 ***p<0.0001 Mann Whitney *U* test). B: Nogo-A substrates repelled neuronal body
674 adhesion and neurite outgrowth in a dose dependant manner. The difference in
675 the frequency of neuronal bodies with neurites which had adhered to Nogo-A

substrates compared to nitrocellulose was significant at concentrations of 50, 100, 200 and 400μg/ml (***p<0.0001 Mann Whitney *U* test). C: There was no difference in the frequency of neuronal bodies with neurites which had adhered to MAG substrates compared to nitrocellulose at any of the concentrations tested (10, 50, 100, 200 or 400μg/ml). Data shown are from at least 5 separate cultures and 5 separate images per culture combined +/-SEM.

Figure 4. In MSC co-cultures, the inhibitory effects of neurocan and Nogo-A substrates on neuronal body adhesion and neurite outgrowth were reduced. MSC co-cultures also enhanced neuronal body adhesion and neurite outgrowth over MAG. A-C: Digitized images of identical fields are shown from left to right under phase contrast and fluorescence microscopy (middle panels illustrate the location of the neurocan, Nogo-A or MAG and fluorescently labelled MSC, right panels show NF immunolabelled neuronal bodies and neurites, calibration bars = 100μm). A: MSC adhesion was reduced on high concentrations of neurocan (10 and 50μg/ml) compared to nitrocellulose (*p=0.0217 and ***p<0.0001 Mann Whitney *U* test). Neuronal body adhesion and neurite extension was only inhibited at the highest concentration of neurocan (50μg/ml) in MSC co-cultures (***p<0.0001 Mann Whitney *U* test). B: MSC adhesion was reduced on 400μg/ml Nogo-A substrates compared to nitrocellulose (***p<0.0001 Mann Whitney U test). Neuronal body adhesion and neurite extension was only inhibited at the highest concentration of Nogo-A (400μg/ml) in MSC co-cultures (***p<0.0001 Mann Whitney *U* test). C: There was no difference in the frequency of MSC or neuronal bodies with neurites which had adhered to MAG substrates compared

699 to nitrocellulose at any of the concentrations tested (10, 50, 100, 200 or
700 400μg/ml). Black arrows indicate co-localisation of MSC and spinal neurites,
701 white arrows indicate independent binding of neurites to inhibitory substrates.
702 Data shown are from at least 5 separate cultures and 5 separate images per
703 culture combined +/-SEM. D: MSC shown bridging nerve inhibitory substrata
704 were immunopositive for laminin and fibronectin.
705 Figure 5. MSC conditioned media (MSC-CM) stimulates spinal neurite outgrowth,
706 but not over inhibitory neurocan or Nogo-A substrata. A: Representative digitized
707 images of neurite outgrowth over nitrocellulose in control media (top panel) and
708 MSC-CM (bottom panel) under phase contrast microscopy are shown with
709 digitized CellIQ® 'neurite finder' overlays, calibration bars = 100μm. Analysing
710 pooled data (n=6 MSC-CM) demonstrated a marked and significant increase in
711 neurite length following culture in MSC-CM compared to control medium
712 (*$p<0.0384$ Mann Whitney *U* test). B: MSC-CM contained several neurotrophic
713 proteins which were detected using custom designed antibody arrays. Arbitrary
714 signal intensity readings were normalised to MSC number, data shown are from
715 MSC-CM combined +/-SEM. C: MSC-CM was not sufficient stimiuli to promote
716 neurite extension over inhibitory substrata of neurocan (top panels) or Nogo-A
717 (bottom panels). Digitized images of identical fields are shown from left to right
718 under phase contrast and fluorescence microscopy (middle panels illustrate the
719 location of the neurocan or Nogo-A, right panels show NF immunolabelled
720 neuronal bodies and neurites, calibration bars = 100μm). There was no
721 difference in the frequency of neuronal bodies with neurites which had adhered to

722 nitrocellulose or inhibitory neurocan or Nogo-A substrata in neuronal growth
723 media compared MSC-CM. Data shown are from at least 5 separate cultures and
724 5 separate images per culture combined +/-SEM.

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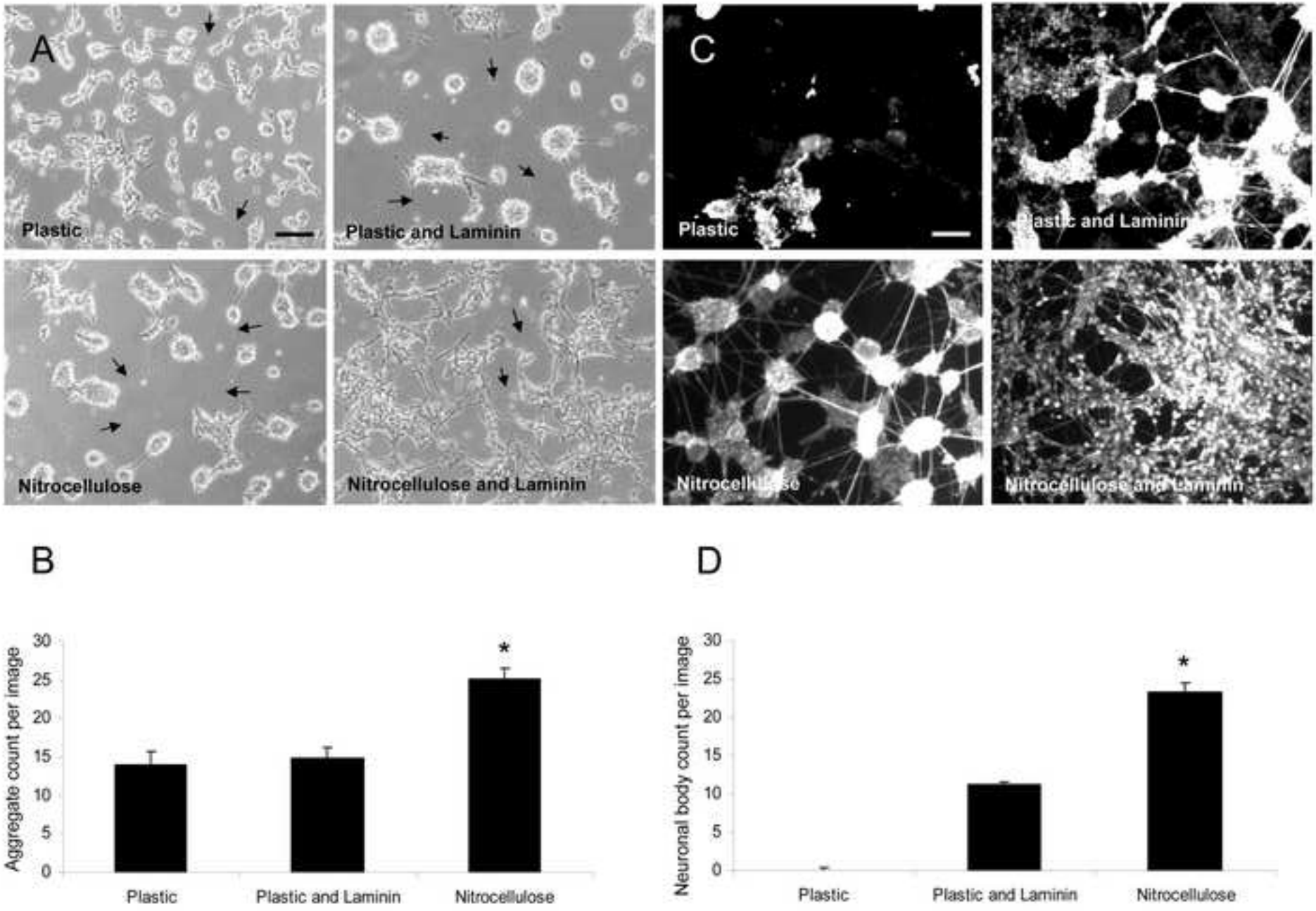

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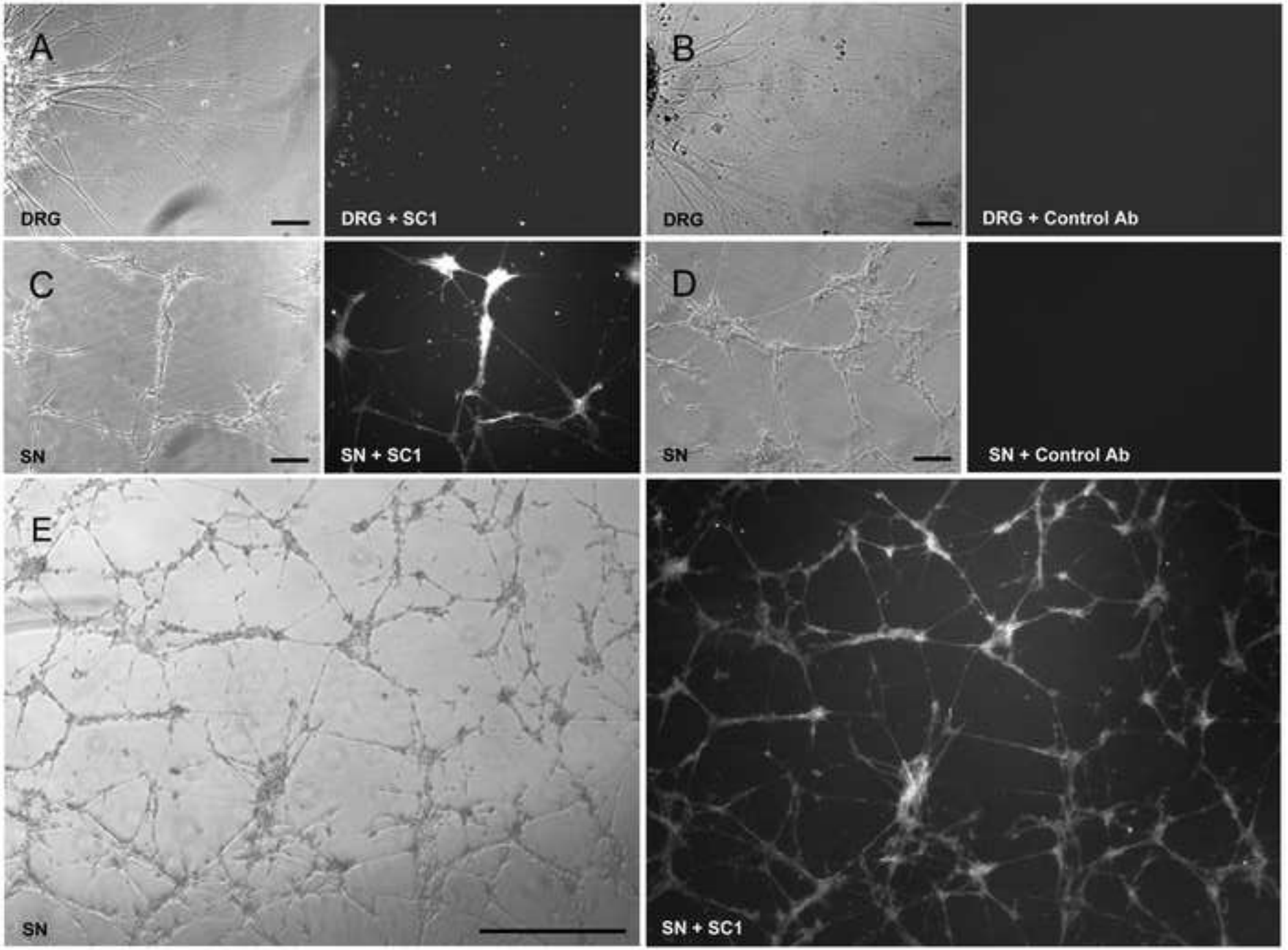

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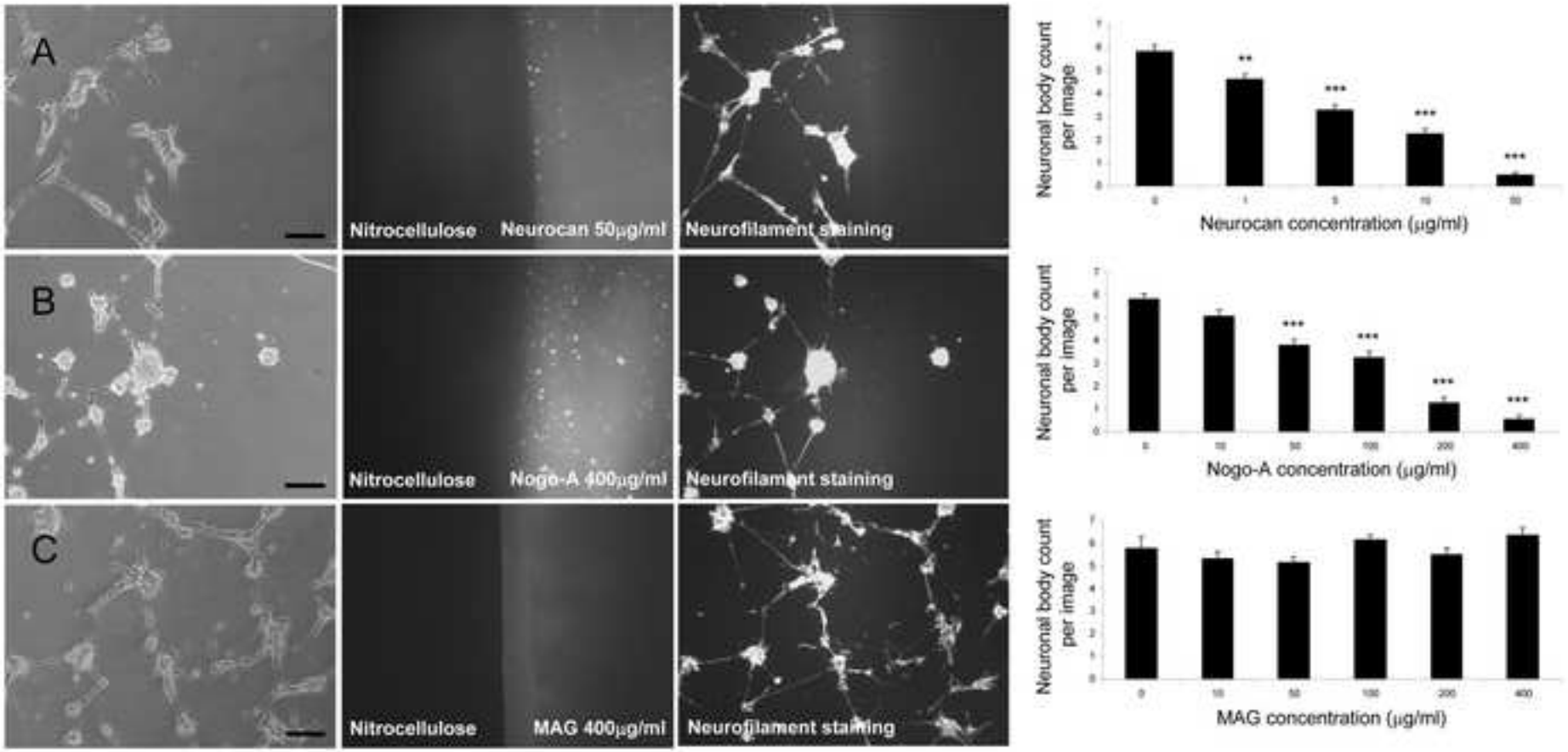

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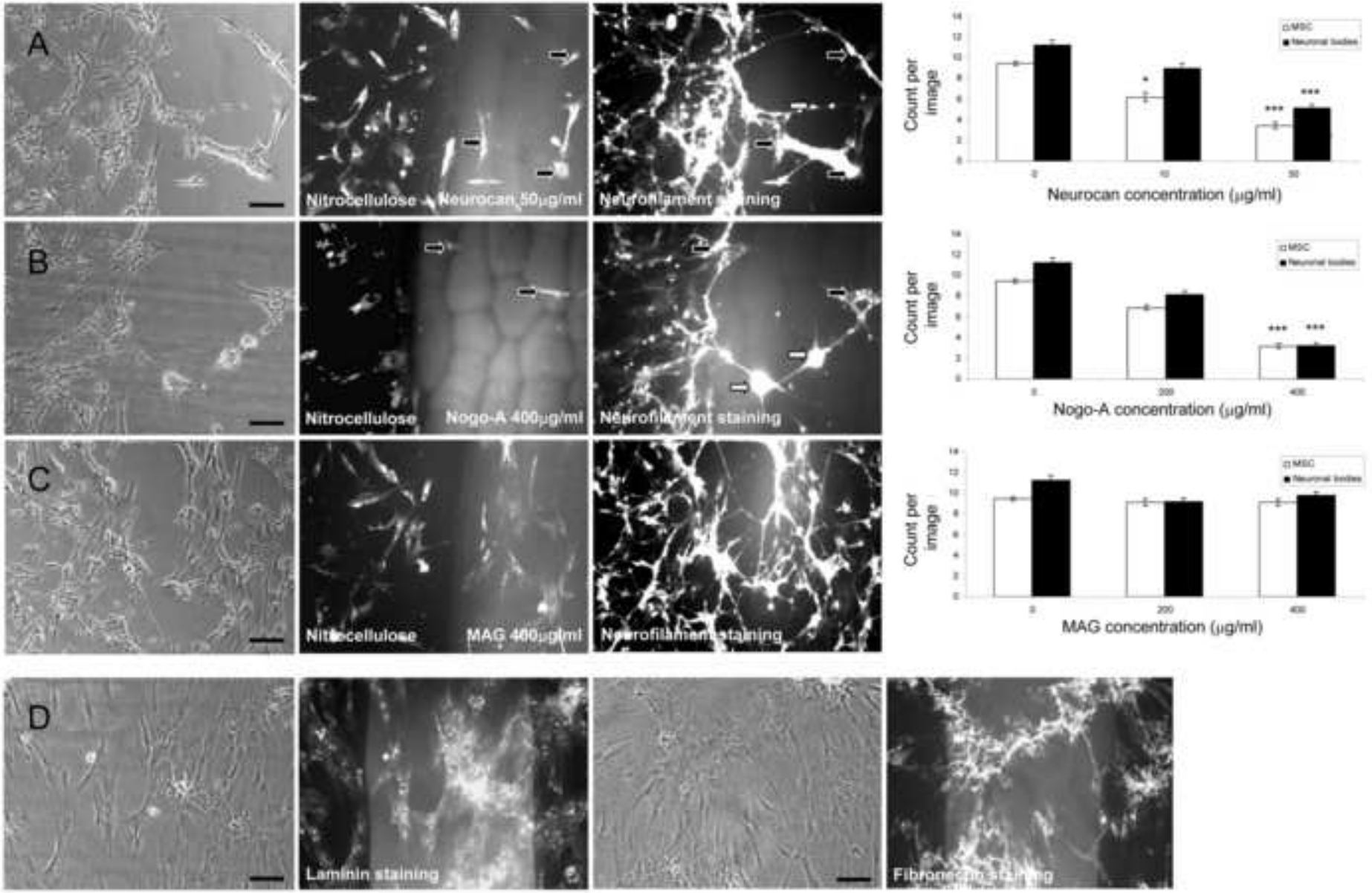

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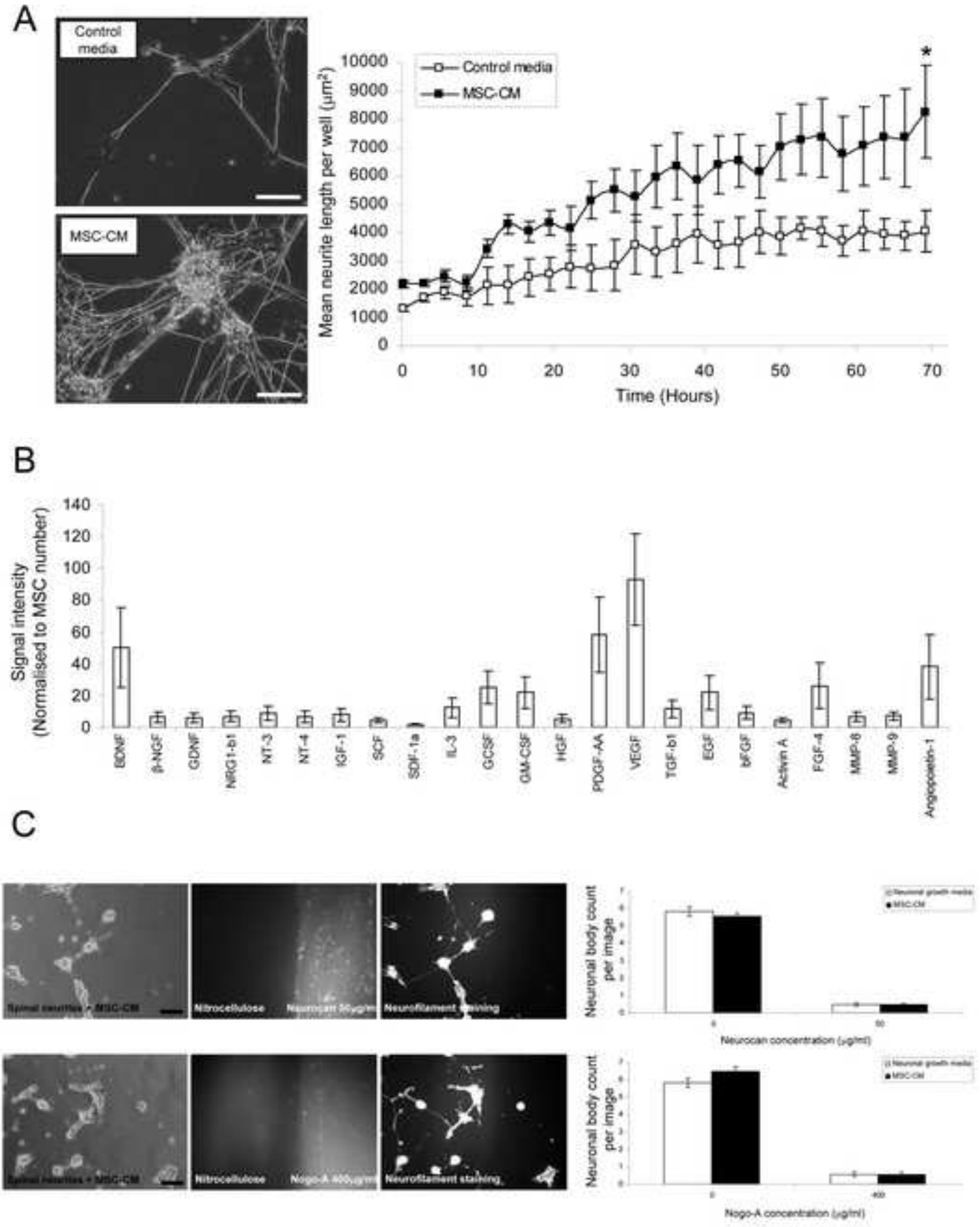

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