

Role of Eph/ephrin Molecules in Mediating Cross-talk Between Stromal and Neural Populations in the Regulation of Neural Differentiation, Adhesion and Migration

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Abstract

There is substantial evidence from the literature that suggests a close association between the skeletal and nervous systems at the anatomical and physiological level, and this cellular and molecular cross-interaction has been termed the neuro-osteogenic network, critical for skeletal growth, homeostasis, and repair.

The Eph and ephrin molecules are the largest family of receptor tyrosine kinases. These membrane-bound molecules are contact-dependent and predominantly function through cell-to-cell interactions. Forward signalling occurs when the ephrin ligand activates signalling in the Eph expressing cell while reverse signalling can also occur where Eph receptors activate signalling through the ephrin ligand expressing cells. The Eph/ ephrin interactions have known roles independently in the skeletal system regulating bone homeostasis, and skeletal growth and repair, and within the nervous system influencing axon guidance and patterning. Therefore, the roles of Eph/ ephrin mediated cross talk between neural and bone marrow stromal cell (BMSC) populations in regulating neural differentiation, adhesion and migration were investigated.

An *in vitro* neural differentiation assay was established for the human neuroblastoma line SH-SY5Y which showed upregulated expression of *EPHRINA2*, *EPHRINA5* and *EPRHINB1* with the progression of neuronal differentiation. Previous studies in our laboratory have shown that human BMSC highly express *EPHA3*, *EPHA4*, *EPHB2* and *EPHB4* at the gene and protein levels. Therefore, the roles of Eph/ ephrin interacting pairs in mediating cross -communication between BMSC and neurons, respectively, were assessed. These included the stromal expressing Eph receptors with neural expressing ephrin ligands (1) EphA3/A4 with ephrinA2 (2) EphA3/A4 or EphB2 with ephrinA5 and (3) EphB2/B4 with ephrinB1, respectively. To examine the importance of ephrin reverse signalling on neural differentiation, adhesion and migration, soluble EphA3-Fc, EphA4-Fc, EphB2-Fc, EphB4-Fc fusion molecules, were used to activate reverse signalling of the ephrinA2, ephrinA5 and ephrinB1 ligands, respectively. The gene expression of early and mature neuronal and sensory markers in the SH-SY5Y line

showed a differential response following activation of the ephrinA and ephrinB ligands during neural induction. However, there was no significant difference in neuronal morphology. The activation of both ephrinA and ephrinB ligands inhibited SH-SY5Y neural adhesion, with reduced neural adhesion in response to EphA4-Fc, EphB2-Fc and EphB4-Fc for two hours, and in response to EphA4-Fc and EphB4-Fc for twenty-four hours. EphB2-Fc and EphB4-Fc stimulation did not exert an effect on neural transwell migration where these molecules were not in direct contact with the neural-like cells. There was no significant difference in neural adhesion, or neural and stromal migration, in response to blocking ephrinB1 on the neural population. To determine functional relevance of the neuro-osteogenic network, the avian embryo dorsal root ganglia (DRG) that innervate the hind limbs, were cultured *in vitro* in the presence of a range of Eph-Fc molecules. DRG neurite cell bodies within the Eph-Fc occupied spaces was significantly decreased for DRG pooled from the lumbar region while a strong trend of differential DRG neurite cell body number and neurite invasion was observed within the Eph-Fc occupied spaces for DRG from specific lumbar compartments. Future studies would investigate the importance of ephrinA2, ephrinA5 and ephrinB1 mediated cross-communication between the neural and BMSC populations during skeletal and neural development and fracture repair.

Declaration

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Signed

Nethmi Abeynayake

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Abbreviations

ANOVA	Analysis of variance
APS	Ammonium persulphate
BDNF	Brain-derived neurotrophic factor
BMP	Bone morphogenetic protein
BMPR-I, BMPR-II	BMP type I or type II receptor
BMSC	Bone marrow stromal/ mesenchymal stem cells
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
CGRP	Calcitonin gene related peptide
CNS	Central nervous system
CRLR	Calcitonin receptor-like receptor
CT	Threshold cycle
CXCL12	C-X-C motif chemokine ligand 12
CXCR4	C-X-C chemokine receptor type 4
DAPI	4',6-diamidino-2-phenylindole
DCC	Deleted in colorectal cancer
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DPSC	Dental pulp stem cells
DRG	Dorsal root ganglia
DRG11	Dorsal root ganglia homeobox11
EDTA	Ethylenediaminetetraacetic acid

Eph	Erythropoietin-producing hepatocellular tyrosine kinase receptor
Ephrin	Eph receptor-interacting proteins
FACS	Fluorescence-activated cell sorting
FCS	Foetal calf serum
FN1, FN2	Fibronectin type III domains
GFP	Green fluorescent protein
GPI	glycosylphosphatidylinositol
HBSS	Hanks' balanced salt solution
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
hESC	Human embryonic stem cells
HO	Heterotopic ossification
LBD	Ligand binding domain
mRNA	Messenger ribonucleic acid
MSC	Mesenchymal stem/stromal cells
NF-H	Neurofilament heavy chain
NF-L	Neurofilament light chain
NF-M	Neurofilament medium chain
NGF	Nerve growth factor
NP40	Nonidet P40 substitute
NSC	Neural stem cells
NSE	Neuron-specific enolase
NT	Neurotrophins
OPG	Osteoprotegerin
P	Passage

PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline + 0.1% Tween-20
PDGF	Platelet-derived growth factor
PDZ	PSD-95, Dlg and ZO-1
PFA	Paraformaldehyde
PNS	Peripheral nervous system
PVDF	Polyvinylidene difluoride
RAMP1	Receptor activity-modifying protein 1
RANKL	Receptor activator of nuclear factor kappa-B ligand
RIPA	Radioimmunoprecipitation assay
RT	Room temperature
RT-PCR	Real time - polymerase chain reaction
SAM	Sterile alpha motif
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate - polyacrylamide gel electrophoresis
Sema3A	Semaphorin3A
SFK	Src-family kinases
SH2	SRC homology 2
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline with 1% Tween-20
TEMED	N,N,N,N-tetramethylethylenediamine
α -MEM	Minimum essential medium Eagle with alpha-Modification

Chapter 1 Introduction

Chapter 1 incorporates the following published review publication with the permission of the publisher Elsevier:

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1.1 Structure of the skeletal system

The skeleton refers to the framework of the body, which provides a variety of functions including structural support for the rest of the body, capacity for movement, protection of vital internal organs, and maintenance of mineral homeostasis and acid-base balance (Clarke, 2008). The adult human skeleton has a total of 213 bones excluding the sesamoid bones (Clarke, 2008). Bones can be categorised into long bones, short bones, flat bones, and irregular bones (Clarke, 2008).

The long bones are composed of a hollow shaft or diaphysis; cone-shaped metaphyses found below the growth plates; and the rounded epiphyses above the growth plates (Figure 1.1A). The diaphysis is composed of dense compact/ cortical bone while the metaphysis and epiphyses consist of cancellous/ trabecular bone surrounded by a relatively thinner shell of compact bone (Figure 1.1A). A layer of fibrous connective tissue called the periosteum surrounds the outer shell of compact bone. Thick collagenous fibres called Sharpeys' fibres attach the periosteum tightly to the compact bone (Figure 1.1B). In contrast, the cancellous bone consists of a meshwork of trabecular plates and rods that are interspersed within the bone marrow compartment.

Both compact and cancellous bone are composed of structural units termed osteons that are cylindrical structures. Haversian canals are located within the centre of each osteon where each Haversian canal contains blood vessels and nerve fibres surrounded by 20 to 30 concentric rings of bone known as lamellae (Figure 1.1B) (Patterson-Kane and Firth, 2014). The Haversian canals run parallel to the surface lengthwise through the long bone and are linked to each other by transverse Volkmann's canals that also link to the periosteum and bone marrow (Figure 1.1B) (Patterson-Kane and Firth, 2014).

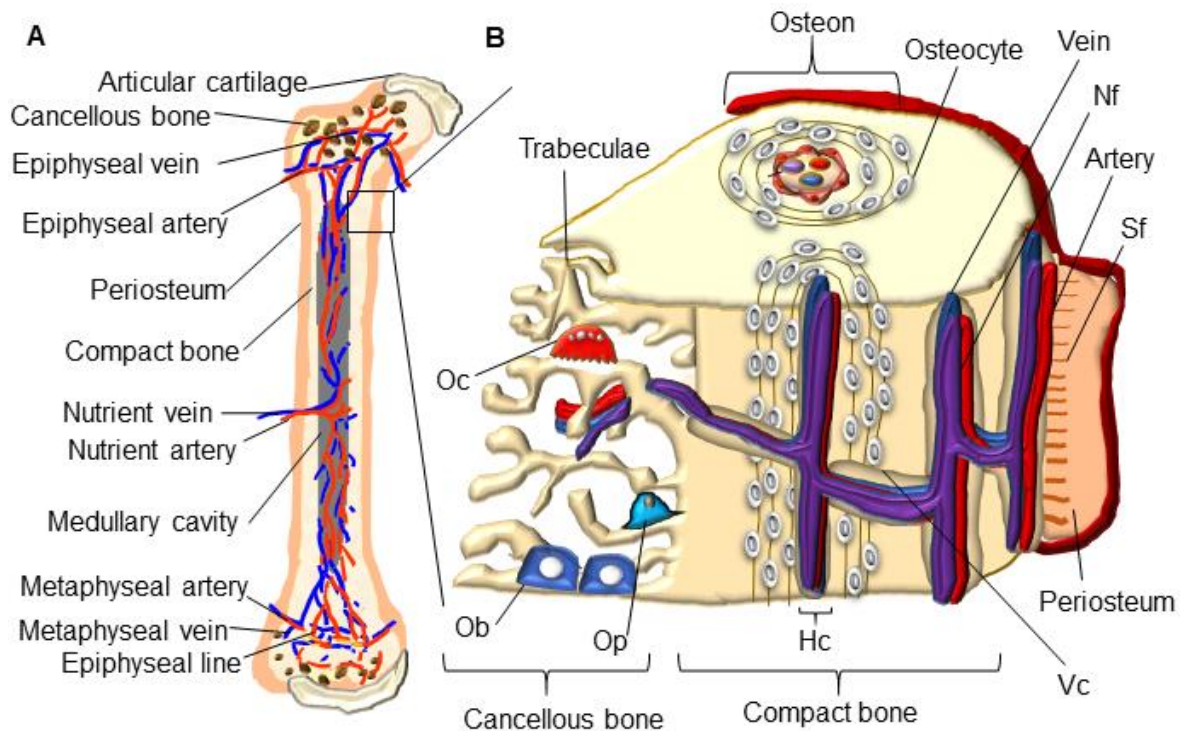


Figure 1.1: Bone and its associated vasculature and neural network. At a macroscopic scale, bone is categorized into compact and cancellous bone. Compact bone is the outer shell covered by a layer of connective tissue known as the periosteum containing Sharpeys' fibers (Sf). Cancellous bone comprises of trabecula, which are a network of lamellar bone plates and rods. Bone consists of structural units known as osteons. Each osteon is a cylindrical structure with a central Haversian canal (Hc) that contains blood vessels and is surrounded by 20 to 30 concentric rings of bone known as lamellae, created by osteoblasts (Ob) that become entrapped osteocytes that communicate with the Haversian canals through canaliculi. The Haversian canals run parallel to the surface lengthwise down the axis of long bone and are linked to each other by transverse Volkmann's canals (Vc). The skeleton is highly innervated with sensory and autonomic nerve fibres (Nf), which reside within the perichondrium, periosteum, bone marrow, growth plate and mineralized trabecular and cortical bone traversing through the nutrient canals, Volkmann's canals, and Haversian systems parallel to the vasculature. The cell types involved in the skeletal system include the osteoprogenitor cells (Op), osteoblasts (Ob), osteoclasts (Oc) and osteocytes. Obtained from Abeynayake et al. (2021) and re-printed with permission from the publisher Elsevier.

1.2 Development of the skeletal system

During embryonic development, bone formation occurs by two alternative mechanisms, endochondral ossification or intramembranous ossification.

1.2.1 Endochondral ossification

Endochondral ossification refers to the process of bone formation where the cartilage matrix is initially laid down and systematically replaced by bone (Mackie et al., 2008). Endochondral ossification forms all long bones of the appendicular skeleton (limbs) and the axial skeleton (vertebrae and ribs) (Maes and Kronenberg, 2016).

The process of endochondral ossification begins with the condensation of undifferentiated mesenchymal stem/stromal cells (MSC) or bone marrow stromal cells (BMSC) (Hallett et al., 2019). MSC have the potential to differentiate into different cell types, including stromal cells, fibroblasts, osteoblasts, chondrocytes, myocytes, adipocytes and neural cells (Pittenger et al., 1999, Gronthos et al., 2003, Shi and Gronthos, 2003, Urrutia et al., 2019). The outer layer of the MSC condensation forms the perichondrium, a dense layer of irregular connective tissue that surrounds the cartilage template of developing bone (Mackie et al., 2008), while the inner cells of the condensation proliferate and differentiate into chondrocytes (Mackie et al., 2008). The chondrocytes at the centre of this matrix stop proliferating and increase in volume to become hypertrophic (Mackie et al., 2008). These hypertrophic chondrocytes instruct the adjacent perichondrial cells to undergo osteoblast differentiation, also known as osteogenic differentiation. Osteoblasts are cuboidal cells that are located along the bone surface that secrete an extracellular organic collagen-containing matrix called osteoid (Hallett et al., 2019). Osteogenic differentiation is regulated by key transcription factors that include RUNX2 (Otto et al., 1997), OSTERIX (Nakashima et al., 2002) and WNT/ β -CATENIN (Day et al., 2005) which in turn are regulated by other transcription factors (Komori, 2006). The hypertrophic chondrocytes secrete growth factors to promote the invasion of blood vessels into the perichondrium, known as vascularization, which forms the periosteum. This process of systematically replacing the cartilage with bone begins in the middle of the cartilage template, referred to as the primary ossification centre (Hallett et al., 2019). These hypertrophic

chondrocytes also express molecular signals, which include the signalling protein Indian hedgehog that directs the mineralisation (ossification) of the surrounding osteoid (Hallett et al., 2019). This mineralization occurs when the collagen-containing osteoid accrues hydroxyapatite crystals (Boivin et al., 2009). The process occurs in two phases with the first caused by a rapid initiation referred to as primary mineralization followed by a slower mineral accrual referred to as secondary mineralization. This secondary mineralisation continues until optimal mineralisation is achieved (Boivin et al., 2009). At birth, a secondary ossification centre forms in the epiphyses of long bones, through successive events of chondrocytes undergoing hypertrophy, vascular invasion, and osteoblast differentiation. The process of endochondral ossification is illustrated in Figure 1.2.

The process of bone elongation is dependent on the rate at which the chondrocytes undergo hypertrophy (Hallett et al., 2019). The growth that occurs at the epiphyseal plate called interstitial growth, results in the lengthening of bone and contributes to the increasing height of the body. (Ortega et al., 2004, Mackie et al., 2008). Bones also increase in diameter, which can continue even after longitudinal growth ceases. This appositional growth is mediated by osteoclasts resorbing old bone that lines the medullary cavity, while osteoblasts produce new bone tissue, which is added to the free surface of cortical bone (Ortega et al., 2004, Mackie et al., 2008). The coordinated process of endochondral ossification is essential to the development and growth of long bones of the body, but also contributes to fracture repair, as is discussed in section 1.4.

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Figure 1.2: Schematic diagram showing the initial stages of endochondral ossification.

Bone development begins with mesenchymal condensation to form a cartilage model. Following chondrocyte hypertrophy and cartilage matrix mineralization, the primary, and then secondary ossification centres form, and the cartilage becomes systematically replaced by bone. Adapted from Mescher (2013).

1.2.2 Intramembranous ossification

Intramembranous ossification refers to the form of bone formation that is driven by the direct differentiation of MSC into osteoblasts and occurs in the flat bones of the skull and clavicles (Erickson and Payne, 2019). Once the osteoblasts become embedded within the mineralised matrix, they reside in small pockets called lacunae and have the potential to terminally differentiate into osteocytes (McGee-Lawrence et al., 2013). Osteocytes establish a connective network within the bone by communicating with surface osteoblasts and bone-resorbing cells termed osteoclasts via neuron-like processes called canaliculi that contact each other and other

cells by means of gap junctions (Capulli et al., 2014). This newly mineralised tissue is known as a bone spicule and several spicules fuse together to form trabeculae, known as primary spongiosa, which in turn fuse to form woven bone (McNamara, 2011). Over time, this woven bone is further modelled to become lamellar bone, with concentric lamellae surrounding the Haversian canals within each osteon (McNamara, 2011).

1.3 Bone homeostasis

Bone undergoes a restructuring process known as bone remodelling where old, micro-damaged bone is removed and replaced with new, mechanically stronger bone (Clarke, 2008). Bone remodelling is critical to preserve bone mass and strength, and maintain mineral homeostasis (Clarke, 2008). Bone constantly renews by the balanced action of osteoblastic bone formation and osteoclastic bone resorption which maintains bone homeostasis in order to control skeletal mass (Lerner et al., 2019, Lerner, 2012). Furthermore, bone is highly innervated with sensory and autonomic nerve fibres (Chartier et al., 2018, Bjurholm et al., 1988) and this innervation of bone has been shown to regulate bone homeostasis and skeletal repair.

The osteoclasts are specialized multinucleated giant cells that are capable of resorbing bone (Roodman, 1999). These osteoclasts are of hematopoietic origin, specifically the monocyte-macrophage lineage (Parvizi and Kim, 2010, Roodman, 1999). Osteoclast formation, or osteoclastogenesis is regulated by cytokines including macrophage colony stimulating factor and receptor activator of nuclear factor kappa-B ligand (RANKL) (Tanaka et al., 1993). Macrophage colony stimulating factor is required for the proliferation, survival, and differentiation of osteoclast precursors and the survival of mature osteoclasts (Tanaka et al., 1993). RANKL belongs to the tumour necrosis factor superfamily and is critical for osteoclast formation. Osteoprotegerin (OPG) is a membrane-bound and secreted protein that binds RANKL with high affinity and therefore inhibits RANKL activity at the RANK receptor (Tsuda et al., 1997). Therefore, osteoclast activation and function are also mediated by the RANK/RANKL/OPG axis. Furthermore, the c-Fos transcription factor is critical for osteoclast differentiation (Wang et al., 1992) because it is required for the upregulation of RANKL in osteoclast precursors and therefore, osteoclastogenesis (Arai et al., 2012). During bone

remodelling, osteoblasts respond to signals generated by osteocytes or direct endocrine activation signals and recruit osteoclast precursors which resorb bone (Raggatt and Partridge, 2010).

Osteocytes also regulate bone remodelling by secreting the glycoprotein sclerostin that suppresses osteoblast differentiation and activity by inhibiting the wnt/beta-catenin signalling pathway (Winkler et al., 2003). Furthermore, osteocytes influence bone resorption in an indirect manner by producing RANKL which promotes osteoclastogenesis (Nakashima et al., 2011), and in a direct manner by initiating local osteolysis which is the destruction of the osteocyte lacunar bone matrix (Tsourdi et al., 2018).

As described previously, the bone vasculature is a critical component of bone growth, where intramembranous ossification and endochondral ossification occur in association with blood capillaries. The endothelial cells on the lining of the blood vessels regulate bone remodelling via the secretion of angiocrine factors which include platelet-derived growth factor, transforming growth factor and fibroblast growth factor which promote osteoprogenitor survival (Kusumbe et al., 2014), OPG which inhibits osteoclastogenesis (Ciriza, 2015), and notch which promotes osteogenesis (Ramasamy et al., 2014).

In addition, bone marrow, soft tissue that resides within the bone cavity, comprises of critical cellular and molecular components within the bone marrow microenvironment that maintain skeletal integrity and bone homeostasis (Arthur and Gronthos, 2021). Cellular components include endothelial cells, perivascular cells, neural cells, Schwann cells, and cells of the mesenchymal and hematopoietic lineages. The bone marrow is the source of the mesenchymal stem/stromal cells which are multipotent stem cells that give rise to cells of the chondrogenic lineage (chondroprogenitors, proliferating, resting, pre-hypertrophic and hypertrophic chondrocytes), osteogenic lineage (osteoprogenitors, osteoblasts, bone lining cells, osteocytes), as well as other cell types that include the stromal cells, adipocytes, reticular cells and smooth muscle cells (Arthur and Gronthos, 2021).

1.4 Fracture repair

A skeletal fracture is characterised as a breach in the structural continuity of the bone cortex that occurs as a result of trauma which is also associated with injury to the surrounding soft tissues (Sheen and Garla, 2020). During fracture healing, the process of bone regeneration recapitulates the process of endochondral ossification that occurs during bone development (Sheen and Garla, 2020). Post-fracture, the blood vessels that supply the bone rupture forming a hematoma around the fracture site which clots (Marsell and Einhorn, 2011). There is an increase in pro-inflammatory cytokines such as the interleukins and bone morphogenetic proteins (BMP) at the site of skeletal injury which attract macrophages and monocytes which remove the damaged tissue (Marsell and Einhorn, 2011). Recruited MSC undergo BMP-driven differentiation into chondroblasts and osteoblasts. Chondrogenesis occurs where collagen-rich fibrocartilaginous callus is laid down. This process is followed by endochondral ossification where the soft callus becomes replaced by a hard bony callus (Marsell and Einhorn, 2011). This immature woven bone is further remodelled by osteoblasts and osteoclasts into mature lamella bone to restore the normal biomechanical properties of bone (Marsell and Einhorn, 2011).

1.5 The association between the skeletal and nervous systems

The skeleton is highly innervated with both sensory and autonomic nerve fibres, which reside within the perichondrium, periosteum, bone marrow, Volkmann's canals, growth plate and mineralized trabecular and cortical bone (Nencini and Ivanusic, 2016, Bjurholm et al., 1988). Sensory, and to a lesser extent, autonomic nerves form meshwork patterns within the periosteum (Hill and Elde, 1991). These sensory and autonomic nerve fibres also penetrate cortical bone traversing through the nutrient canals, Volkmann's canals and Haversian systems parallel to the vasculature (Chartier et al., 2018).

During early limb development, the sensory nerves are present in the perichondrium and periosteum as sensory innervation occurs prior to birth (mouse, embryonic day 15) (Sisask et al., 2013). The autonomic peripheral nerves appear only at the later developmental stages in these areas since autonomic innervation is established after birth (mouse, post-natal day 4)

(Sisask et al., 2013). The two processes that control topographic sensory and motor innervation of the limbs during embryonic development are (1) axon guidance - to steer extending axons in the correct direction, and (2) axon fasciculation - to ensure the growth of sets of co-extending axons within tight bundles (Luxey et al., 2013).

The sensory nerves originate from the dorsal root ganglia (DRG) of the spinal cord and target the periphery where they innervate mineralized bone, the periosteum and the bone marrow (Bjurholm et al., 1988). Therefore, these neurons connect the respective fields in the periphery to the dorsal horn to enable the central processing of various external stimuli such as pain and pressure (Brazill et al., 2019). The cell bodies of the sensory neurons are localized within the DRG. The patterning of the dorsal peripheral sensory axon projections occurs along the pathway of dorsal motor axon projections that are pre-formed in response to cues expressed by BMSC (Bonanomi and Pfaff, 2010, Wang et al., 2011, Honig et al., 1986). During normal hind limb innervation, the motor axons initially converge at the base of the limb bud and then adopt dorsal and ventral trajectories within the limb (Luria and Laufer, 2007).

The primary afferent sensory, autonomic and sympathetic nerve fibres of the peripheral nervous system also associate with the arterioles and sinusoids within the bone marrow, which are adjacent to the bone trabeculae and osteoblasts (Chenu, 2004, Takeda et al., 2002). The primary afferent neurons that innervate the bone marrow and periosteum are nociceptive, having the ability to sense and transmit information about potentially damaging stimuli such as pain, determined by their expression of the nociceptive markers CGRP, substance P and the tyrosine receptor kinase A (Grässel and Muschter, 2017, Nencini and Ivanusic, 2016, Grässel, 2014).

The nervous system also contributes to bone homeostasis by regulating MSC, where sensory nerves secrete neurotransmitters that include substance P, calcitonin gene related peptide (CGRP), nerve growth factor (NGF) and semaphorin3A (Sema3A). These factors promote the migration of BMSC to the front line of osteogenesis, and promote osteoblast differentiation and therefore, bone formation and repair (Wang et al., 2009, Tomlinson et al., 2017, Hayashi

et al., 2012, Wang et al., 2010). Moreover, the sympathetic nerves secrete norepinephrine, which suppresses osteogenesis and stimulates osteoclast formation to promote bone resorption (Moore et al., 1993). In addition, the sympathetic and parasympathetic nerve fibres that innervate the walls of the blood vessels regulate the contraction and tension of these walls (Amiya et al., 2014). As there is evidence of a close association between the skeletal and neural projections, the gap of knowledge is the molecular mechanisms that mediate cross-communication between the stromal/bone cells and the neural cells where this neuro-osteogenic network has already been established.

1.6 The functional importance of cross-talk between the skeletal and nervous systems

The importance of cross-talk between the skeletal and nervous systems for the function of the individual systems has been demonstrated, where disorders such as osteoarthritis, spinal cord injury, neurogenic heterotopic ossification (HO), and osteoporosis arise because of defects in this cross-talk, as outlined in Table 1.1. There are also numerous studies of animal models that have demonstrated the functional consequences of damage to this skeletal-neural cross-talk on bone (Aro, 1985, Edoff et al., 1997, Madsen et al., 1998, Hamdan et al., 2014, Nordsletten et al., 1994) and the nervous system (Burt-Pichat et al., 2005, Williams and Ivanusic, 2008, Cherruau et al., 1999, Hill et al., 1991, Sandhu et al., 1987, Sandhu et al., 1990).

Defective neural innervation has been demonstrated to impact fracture repair and skeletal growth (Aro, 1985, Nordsletten et al., 1994, Madsen et al., 1998, Hamdan et al., 2014, Edoff et al., 1997). In rat studies, the sciatic denervation of the tibia following a tibial fracture significantly reduced the size of the callus formed during the initial stages of fracture healing (Aro, 1985). Since the callus stabilizes the fractured bone and serves as the location of active bone remodelling, this denervation clearly affects the skeletal aspect of fracture healing (Aro, 1985). This finding is consistent with the observation that CGRP-containing sensory nerves such as the sciatic nerve contributes to fracture vascular control, angiogenesis and osteogenesis following injury, and therefore bone regeneration (Hukkanen et al., 1993). Furthermore, rats subjected to a tibial fracture followed by one-centimetre resections of their femoral and sciatic

nerves had mechanically weaker fracture calluses when compared to the fracture only control animals that received sham operations, which left their nerves undamaged (Madsen et al., 1998). The nerve resection did not provide complete denervation and instead only changed the innervation pattern of the healing fracture suggesting that intact innervation is necessary for normal fracture healing (Madsen et al., 1998). Impaired fracture healing upon denervation has also been demonstrated in rabbits (Hamdan et al., 2014). Likewise correct neural innervation was found to be important for correct skeletal development as demonstrated by the denervation of the saphenous and sciatic nerves during development, which perturbed the skeletal development of the rat foot (Edoff et al., 1997). Bone loss was also observed within de-nerved bone in adults (Edoff et al., 1997). These studies collectively demonstrate a functional relationship between the skeletal and nervous systems but the mechanisms underlying the cross-communication between the systems are still unclear.

Furthermore, disruptions to the skeletal system exert adverse effects on the nervous system (Burt-Pichat et al., 2005, Williams and Ivanusic, 2008, Cherruau et al., 1999, Hill et al., 1991, Sandhu et al., 1987, Sandhu et al., 1990). One study of oestrogen deficiency induced bone loss using an ovariectomy rat model which resulted in a significant reduction in the nerve fibre density within the tibia (Burt-Pichat et al., 2005). In addition, other reports used a mechanical insult such as bone drilling and demonstrated a resultant activation of specific ascending spinal pathways that relayed information to the superficial dorsal horn of the spinal cord (Williams and Ivanusic, 2008). In response to the trauma, the osteoclast regulator c-Fos was upregulated within the nuclei of neural cells innervating the bone. These studies demonstrate that altered bone homeostasis influences innervation and nerve stimulation. The consequences that arise from damage to either the skeletal or the neural system, outlined in this section, are underpinned by disrupted communication at the cellular level detailed in section 1.7.

Table 1.1: Examples of skeletal disorders associated with damage to the nervous system. Adapted from Abeynayake et al. (2021) with permission from the publisher Elsevier.

Skeletal disorders	Impact on skeletal system	Association with nervous system
Major skeletal fractures	Partial or complete break in the bone leading to pain and disability (Gardner et al., 2006, Einhorn and Gerstenfeld, 2015, Marsell and Einhorn, 2011, Lim and Sirichai, 2016, Grässel, 2014).	Correct innervation is required for proper fracture healing (Madsen et al., 1998, Aro, 1985, Hamdan et al., 2014, Long et al., 2010, Grässel, 2014, Nordsletten et al., 1994).
Osteoporosis	Reduced bone mass and strength and gradual deterioration of bone microarchitecture (Gardner et al., 2006, Akkawi and Zmerly, 2018, Dobbs et al., 1999, Lane et al., 2000, Pouresmaeili et al., 2018, Grässel and Muschter, 2017).	Sympathetic denervation increases bone resorption (Hill et al., 1991, Sandhu et al., 1987, Cherruau et al., 1999, Sandhu et al., 1990). Sensory and sympathetic neurotransmitters are involved in osteoporosis pathology (Grässel and Muschter, 2017). Efficacy of β-blockers in the treatment of osteoporosis is attributed to increased sympathetic nervous activity (Graham et al., 2008). Bone loss is associated with reduced nerve fibre density (Burt-Pichat et al., 2005).

		Mechanical insult of bone drilling activates specific ascending spinal pathways that relay information to the superficial dorsal horn of the spinal cord (Williams and Ivanusic, 2008).
Osteoarthritis	Chronic abnormal remodelling of synovial joint leading to progressive loss of cartilage, bone marrow lesions and synovial inflammation (Hunter, 2018, Chen et al., 2017, Loeser et al., 2012, Man and Mologhianu, 2014, Ohtori et al., 2012, Schaible, 2012, Suri et al., 2007, Zhu et al., 2019).	Associated bone pain is mediated by inflammatory neural mediators such as NGF (Sofat et al., 2011). Monoclonal antibodies that target NGF effectively treat osteoarthritic pain (Schmelz et al., 2019).
Neurogenic HO	Ectopic formation of bone in abnormal anatomical locations (Salisbury et al., 2010, Jensen et al., 1987, Salga et al., 2019, Tuzmen et al., 2018).	Neurogenic HO is induced by injury to the spinal cord with pathogenesis accelerated by CGRP (Sang et al., 2019). Neuropeptide substance P is increased in the lesions of patients with HO and mouse models of HO. HO phenotype lost with deletion of the gene that encodes substance P (Kan et al., 2011).

		Substance P was elevated in the serum of patients with neurogenic HO. Serum from neurogenic HO mice induces osteogenic differentiation <i>in vitro</i> (Genet et al., 2015).
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1.7 Cellular and molecular interactions between the skeletal and nervous systems

The term “neuro-osteogenic network” coined by Togari and colleagues describes the cell-cell and molecular interactions between the neural populations and the osteoblast/osteoclast populations that underpin the cross-communication between the skeletal and neural tissues (Togari et al., 2012, Brazill et al., 2019, Abeynayake et al., 2021). At the cellular level, neuronal axons and osteoblasts form membrane-membrane contacts (Takeda et al., 2002). Direct communication between nerve cells and osteoblasts has also been demonstrated *in vitro* (Obata et al., 2007). A co-culture study showed that the activation of neurites from murine superior cervical ganglia, induced by the addition of scorpion venom, in turn activated differentiated osteoblasts that were in close proximity (Obata et al., 2007). Both neurite and osteoblast activation were measured by intracellular Ca^{2+} mobilization. The communication between the neurites and osteoblasts was required for osteoblast activation, as osteoblast activation was not observed in the presence of scorpion venom alone (Obata et al., 2007). The proposed mechanism was that venom-induced depolarization of the neurite membrane triggered the release of neurotransmitters that bound to their receptors on osteoblasts to induce the activation of Ca^{2+} mobilization within the osteoblasts (Obata et al., 2007).

Direct communication between nerve cells and osteoclasts has also been shown using a similar approach, where the activation of the neurites in turn activated differentiated murine osteoclasts which were in close proximity, based on assessment of intracellular Ca^{2+} mobilization (Suga et al., 2010). In other reports, direct communication between the sensory neurons and osteoblasts was reported to be mediated by factors such as glutamate, substance P and adenosine triphosphate (Kodama et al., 2017). Subsequently, silk protein-based folding scaffolds that support the 3D co-culture of neurons and human MSC have been developed which promote the osteogenic differentiation of MSC and directional axonal growth, and therefore have potential in the fields of tissue regeneration and engineering (Huang et al., 2020).

The cellular interactions between the skeletal and nervous systems are in turn underpinned by underlying molecular mechanisms that mediate cellular interactions between the bone cells and neurons. One of the first observations that the nervous system regulates skeletal metabolism was based on a study that used the Ob/ob mouse model that possessed a recessive mutation in leptin, an adipocyte-produced hormone which acts on the arcuate nucleus of the hypothalamus (Takeda et al., 2002). A phenotype of increased bone mass restricted to the trabecular compartment was reported in the mice with leptin deficiency compared to the wild-type mice. This phenotype was corrected with intra-cerebroventricular infusion of Leptin (Takeda et al., 2002). These findings suggest that bone formation via osteoblast function is regulated by central leptin-mediated signals that activate the sympathetic nervous system and act on $\beta 2$ adrenergic receptors on osteoblasts.

Following this discovery, a cohort of molecules that regulate both skeletal and neural development (Table 1.2) and post-natal bone/ neural tissue repair (Table 1.3) have been identified including: BMP, CGRP, C-X-C receptor type 4 (CXCR4)/CXCL12 ligand, Eph/ephrin, Netrins, Neurotrophins, Semaphorins and Slit/Robo. Some of these molecules act via chemoattraction/chemorepulsion mechanisms such as CXCR4/CXCL12, Netrins, BMP and CGRP while the Eph/ephrin interactions occur via contact-dependent mechanisms (Figure 1.3). These molecules mediate communication between the osteoclasts and osteoblasts to regulate skeletal development and maintain bone homeostasis; while some of these molecules also mediate cross-talk between bone cells (osteogenic and osteoclastic) and neurons (Figure 1.3). For example, it has been proposed that the Bmp molecules (Bmp-2 and Bmp-6) secreted by the sympathetic neurons bind to their respective receptors (Bmp Type I or Type II receptor, BMPR-I or BMPR-II, respectively) expressed on osteoblasts to promote osteoblast differentiation (He et al., 2013). Furthermore, it has been reported that osteoclast-secreted Netrin-1 binds to the Unc5/Dcc receptors on the sensory axons to induce sensory axon growth within subchondral bone (Zhu et al., 2019).

Both chemokine-dependent and contact-dependent signalling have been reported to be important for correct morphogenesis (Ellison et al., 2016, Haas and Gilmour, 2006) and skeletal

and neural functions (Brylka and Schinke, 2019, Civitelli, 2008, Holland et al., 1998, Miller, 2010). Eph/ephrin molecules are expressed on neurite projections and stromal populations, and Eph/ephrin interactions are a contact-dependent means of cross-communication between sensory and motor neurons, and the bone/stromal cells. As the Eph/ephrin interactions have known roles in independently regulating skeletal/stromal and neuronal populations, the Eph/ephrin interactions may mediate cross-communication between the neural and stromal populations to regulate neural function.

Table 1.2: Molecules involved in the development and maturation of skeletal and nervous systems. Adapted from Abeynayake et al. (2021) with permission from the publisher Elsevier.

Molecule	Role in the skeletal system	Role in neural system
BMP	Bmp-2 signalling upregulates osteoblast differentiation and osteogenesis (Lee et al., 2000, Pederson et al., 2008). BMP-7 is required for skeletal development (Luo et al., 1995).	Sympathetic neurons promote osteoblast differentiation via Bmp-2 and Bmp-6 signalling (He et al., 2013).
CGRP	CGRP stimulates osteoblast differentiation, proliferation, and mineralization (Wang et al., 2010, Wang et al., 2011). CGRP inhibits osteoclast differentiation and activity (Ding et al., 2010, Ishizuka et al., 2005).	CGRP is necessary for nociception and peripheral and central sensitization (Nencini and Ivanusic, 2016, Iyengar et al., 2017).
CXCR4/CXCL12	CXCR4/CXCL12 signalling promotes osteogenic differentiation (Hosogane et al., 2010, Zhu et al., 2011).	CXCR4/CXCL12 signalling regulates neuronal migration and axon guidance (Li et al., 2011, Arthur et al., 2009).

		CXCR4/CXCL12 signalling establishes the trajectory of mouse spinal motor neurons (Lieberam et al., 2005, Li et al., 2005, Miyasaka et al., 2007). CXCR4/CXCL12 signalling regulates migration and final terminations of trigeminal and dorsal root ganglion cells (Knaut et al., 2005, Odemis et al., 2005). Mutant mice with a global homozygous deletion of <i>Cxcr4</i> exhibit decreased innervation (Odemis et al., 2005).
Eph/ephrin	EphA2/ephrinA2 interactions induce osteoclast differentiation and suppress osteoblast differentiation (Irie et al., 2009). EphA4 expressed by osteoclasts negatively regulates osteoclast activity (Stiffel et al., 2014). EphB2/ephrinB1 reverse signalling enhances osteoblast differentiation (Xing et al., 2010).	Mediates patterning of the dorsal peripheral sensory axon projections along pre-formed dorsal motor axon projections (Wang et al., 2011, Bonanomi and Pfaff, 2010, Honig et al., 1986). EphB2/ephrinB1 signalling controls axon fasciculation (Luxey et al., 2013).

	ephrinB1 signalling is required for correct skeletal development (Nguyen et al., 2016, Cheng et al., 2013). EphB4/ephrinB2 signalling between osteoblasts and osteoclasts suppresses osteoclast differentiation and enhances osteogenic differentiation (Zhao et al., 2006). EphB4-activated ephrinB2 signalling within the osteoblast lineage is critical for the late stages of osteoblast differentiation (Takyar et al., 2013). Increased autophagy in ephrinB2-deficient osteocytes is associated with elevated secondary mineralization and brittle bone (Vrahnas et al., 2019). ephrinB2 signalling in osteoblasts promotes bone mineralization by preventing apoptosis (Tonna et al., 2014). Chondrocyte-expressed ephrinB2 promotes cartilage destruction by osteoclasts during endochondral ossification (Tonna et al., 2016).	Eph/ephrin interactions regulate axon guidance in the limb (Helmbacher et al., 2000, Eberhart, 2004, Croteau et al., 2019, Munoz et al., 2005, Jayasena et al., 2005).
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	EphB4/ephrinB2 bidirectional signalling promotes chondrogenesis and endochondral bone growth (Wang et al., 2014).	
Netrins	Netrin-1 downregulates the expression of osteogenic differentiation genes (Sato et al., 2017). Osteoclast-secreted Netrin-1 induces sensory axon growth in subchondral bone (Zhu et al., 2019).	Netrin-1 and Netrin-2 proteins guide commissural axons within the embryonic spinal cord (Kennedy et al., 1994).
Neurotrophins	Increased mRNA expression of <i>Ngf</i> , <i>Bdnf</i> , and <i>Neurotrophin-3</i> observed during osteoblast proliferation and differentiation (Nakanishi et al., 1994).	Neurotrophins regulate neuron survival, axon growth, synaptic plasticity and neurotransmission (McAllister et al., 1999, Reichardt and Isabel, 1998, Paves and Saarma, 1997). Neurotrophins mediate axon guidance (Gundersen and Barrett, 1979, Letourneau, 1978), axon outgrowth and fasciculation of sensory neurons (Lentz et al., 1999).
Semaphorins	Sema3A suppresses bone resorption and promotes bone formation (Hayashi et al., 2012).	Sema3A induces growth cone collapse (Luo et al., 1993, Kobayashi et al., 1997).

	Osteoblast-derived Sema3B stimulates osteoclast formation (Sutton et al., 2008). Sema4D produced by osteoclasts induces contact repulsion of osteoblasts (Deb Roy et al., 2017) and inhibits osteoblast activity and bone formation (Negishi-Koga et al., 2011).	
Slit/Robo	Slit2 inhibits osteoblast differentiation (Sun et al., 2009). Osteoclast and osteoblast secreted Slit3 is required for skeletal development (Kim et al., 2018, Xu et al., 2018).	Slit and Robo proteins regulate development and patterning of midline glia and commissural axons (Rothberg et al., 1990, Long et al., 2004). Robo/Slit signalling is required for the correct morphology and migration of sensory neurons (Gonsior and Ismat, 2019). Slit proteins stimulate the branching and elongation of sensory axons including the DRG neurites (Zhang et al., 2010, Wang et al., 1999) and mediate appropriate assembly of the nervous system (Brose et al., 1999).

Table 1.3: Molecules that regulate the repair of the skeletal and/ or nervous systems. Adapted from Abeynayake et al. (2021) with permission from the publisher Elsevier.

Molecule	Repair role in skeletal system	Repair role in neural system
BMP	Recombinant human BMP-2 and BMP-7 promote bone formation and enhance bone union in spine-fusion surgeries (Burkus et al., 2004). Recombinant human BMP-2 is used in the reconstruction of diaphyseal tibial fractures (Jones et al., 2006).	Recombinant human BMP-2 induces peripheral nerve inflammation (Nguyen et al., 2017).
CGRP	CGRP promotes fracture healing (Li et al., 2007).	CGRP promotes neuroinflammation following injury (Nguyen et al., 2017). Increased expression of spinal Cgrp contributes to fracture-induced pain in rats (Shi et al., 2015).
Eph/ephrin	EphB4 enhances the process of endochondral ossification and inhibits remodelling during fracture repair (Arthur et al., 2013).	Blocking EphA4 promotes axon regeneration and functional recovery following spinal cord injury (Goldshmit et al., 2011, Fabes et al., 2007).

	Targeted deletion of <i>ephrinB1</i> within osteoprogenitor cells results in an osteoporotic phenotype (Arthur et al., 2018). Conditional knockout of <i>ephrinB1</i> in osteogenic progenitors delays endochondral ossification during fracture repair (Arthur et al., 2020). Cartilage-specific deletion of <i>ephrinB2</i> in mice results in an osteoarthritis-like phenotype during aging <i>in vivo</i> (Valverde-Franco et al., 2016). Treatment with ephrinB2 positively impacts the abnormal metabolism of human osteoarthritic chondrocytes (Kwan Tat et al., 2009). Ablation of <i>ephrinB2</i> in Type II Collagen expressing cells delays fracture repair (Wang et al., 2020).	ephrinB2 on astrocytes inhibits axon growth and impedes recovery following spinal cord injury (Ren et al., 2013).
Neurotrophins	NGF-targeting therapy alleviates bone pain induced by fractures without disrupting healing (Koewler et al., 2007).	Increased expression of spinal NGF contributes to fracture-induced pain in rats (Shi et al., 2015).

		NGF has potential in the treatment of neurological disorders (Chiaretti et al., 2017).
Semaphorins	Sema3A improves fracture healing (Kenan et al., 2019).	Sema3A inhibitor promotes peripheral nerve regeneration in murine model of corneal transplantation (Omoto et al., 2012).
Slit/Robo	Slit3/Robo signalling increases the bone mass of mice with ovariectomy-induced bone loss (Kim et al., 2018). Recombinant Slit3 enhances fracture healing and counteracts bone loss in a murine model of post-menopausal osteoporosis (Kim et al., 2018).	Slit3/Robo signalling is important in the repair of the peripheral nervous system after injury (Chen et al., 2020).

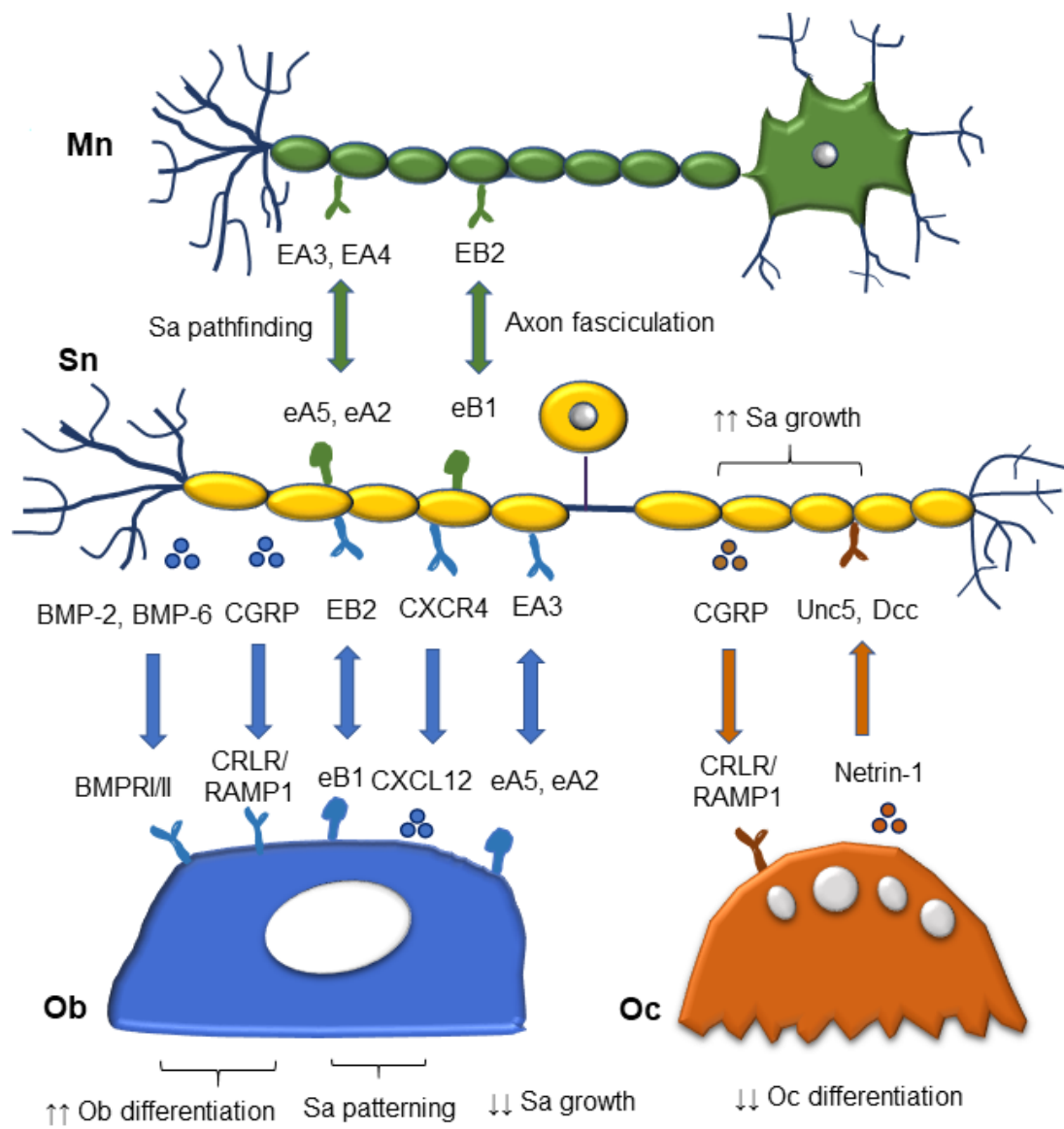


Figure 1.3: The molecular mechanisms mediating communication between sensory and motor neurons with osteoblasts and osteoclasts. EphA3 and EphA4 receptors (EA3, EA4) on motor neurons (Mn) interact with ephrinA2 and ephrinA5 (eA2, eA5) ligands on sensory neurons (Sn) to mediate sensory axon (Sa) pathfinding. The EphB2 (EB2) receptor on motor neurons interacts with ephrinB1 (eB1) on sensory neurons to regulate axon fasciculation. Sensory neurons interacting with osteoblasts (Ob), via interactions between: the Bone Morphogenetic Proteins (BMP-2 and BMP-6) and their corresponding receptors (Bmp Type I or Type II receptor, BMPR-I or BMPR-II), respectively; Calcitonin Gene Related Peptide (CGRP) with G protein-coupled calcitonin receptor-like receptor (CRLR) and G protein-coupled receptor activity-modifying protein 1 (Ramp1), CRLR/RAMP1, respectively. Sensory axon patterning is mediated by interactions between sensory neurons and osteoblasts: EphB2 (EB2) and ephrinB1 (eB1), respectively, and C-X-C motif chemokine 12 (CXCL12) and C-X-C chemokine receptor type 4 (CXCR4). Sensory axon growth is facilitated by interactions between EphA3 (EA3) on sensory neurons and ephrinA5 and ephrinA2 (eA5, eA2) on osteoblasts. The molecular interactions between sensory neurons and osteoclasts are CGRP: CRLR/RAMP1 to inhibit osteoclast differentiation, and Netrin-1 secreted by osteoclasts and their receptors (Deleted in colorectal cancer (Dcc) and Unc5) to promote sensory axon growth. Obtained from Abeynayake et al. (2021) and re-printed with permission from the publisher Elsevier.

1.8 Eph and ephrin molecules

1.8.1 Eph/ephrin structure

The Eph and ephrin molecules are the largest family of receptor tyrosine kinases. These membrane-bound molecules are contact dependent and predominantly function through cell to cell interactions (Pasquale, 1997). The Eph receptors were first identified by Hirai and colleagues within the erythropoietin-producing hepatocellular, or Eph, carcinoma cell line (Hirai et al., 1987). Their corresponding ligands, the **ep**h receptor **int**eracting proteins, were termed ephrin or Efn molecules (Flanagan and Vanderhaeghen, 1998, Pasquale, 1997). There are 14 Eph receptors expressed by humans (nine EphA receptors, EphA1 – A8 and EphA10 and five EphB receptors, EphB1 – B4 and EphB6) and eight ephrin ligands (five ephrinA

ligands, ephrin-A1 – A5, and three ephrinB ligands, ephrin-B1 – B3) (Pasquale, 1997, Nagano et al., 2013).

The ephrin ligands are sub-divided into two groups: ephrinA and ephrinB. Both subclasses of ephrin possess an extracellular Eph-receptor binding domain. However, there are key differences in their structure (Murai and Pasquale, 2003). The ephrinA molecules are tethered to the extracellular portion of the membrane via a glycosylphosphatidylinositol anchor (Pasquale, 2005) (Figure 1.4). This GPI anchor comprises a single molecule of phosphatidylinositol, which has a carbohydrate chain link through the C-6 hydroxyl of the inositol (Pasquale, 2005). In contrast, the ephrinB molecules are transmembrane proteins (Pasquale, 2008). The ephrinB ligands contain a transmembrane helix that is followed by a short cytoplasmic domain that contains several conserved tyrosine residues as sites for phosphorylation, followed by an intracellular PSD-95, Dlg and ZO-1 domain (PDZ)-binding motif at the C-terminus (Murai and Pasquale, 2003) (Figure 1.4).

Eph receptors consist of a globular ligand binding domain, a cysteine-rich region that contains a Sushi domain and an epidermal growth factor-like domain, and two fibronectin type III domains, FN1 and FN2 (Figure 1.4). FN2 is followed by a transmembrane helix, and an intracellular region that comprises a juxtamembrane region containing several conserved tyrosine residues, a tyrosine kinase domain, a sterile- α motif domain, and a C-terminal PDZ-binding motif (Pasquale, 1997) (Figure 1.4). The transmembrane Eph receptors are also subdivided into the EphA and EphB subclasses based on their binding affinity towards the ephrinA or ephrinB ligands (Pasquale, 1997, Flanagan and Vanderhaeghen, 1998). The receptors and ligands of both subclasses bind with varying affinity and promiscuity within the respective subclass (Figure 1.5). Limited binding occurs between subclasses with the exceptions of EphA4 which interacts with ephrinB1-3, and EphB2 which interacts with ephrinA5 (Figure 1.5) (Gale et al., 1996, Qin et al., 2010, Holland et al., 1998, Himanen et al., 2004).

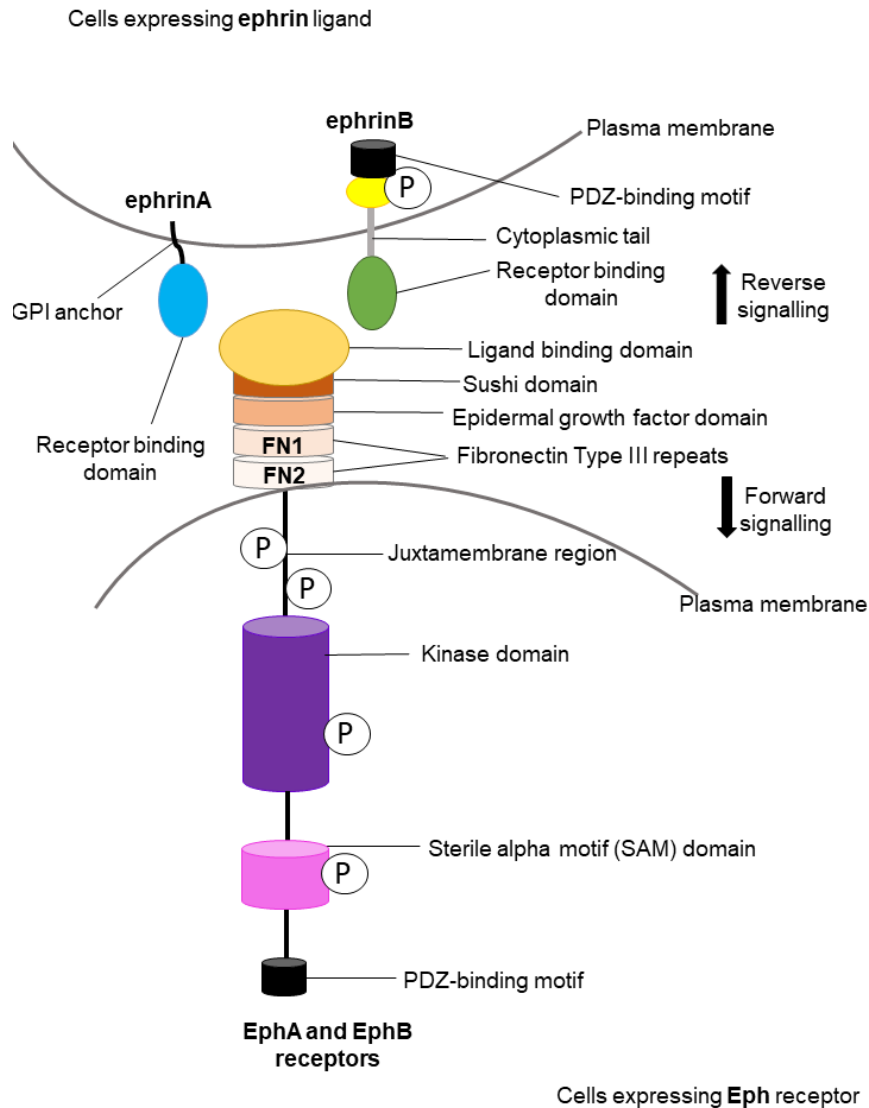


Figure 1.4: Structure of the Eph receptors and ephrin ligands. Both the A and B subclasses of ephrin possess an extracellular Eph-receptor binding domain. The ephrinA molecules are tethered to the extracellular portion of the membrane via a glycosylphosphatidylinositol (GPI) anchor. The ephrinB molecules are transmembrane proteins. The ephrinB ligands contain a transmembrane helix that is followed by a short cytoplasmic domain that contains several conserved tyrosine residues as sites for phosphorylation, followed by an intracellular PSD-95, DIg and ZO-1 domain (PDZ)-binding motif at the C-terminus. Eph receptors consist of a globular ligand binding domain, a cysteine-rich region that contains a Sushi domain and an epidermal growth factor-like domain, and two fibronectin type III domains, FN1 and FN2. FN2 is followed by a transmembrane helix, and an intracellular region that comprises a juxtamembrane region containing several conserved tyrosine (Y) residues, a tyrosine kinase domain, a sterile- α motif domain, and a C-terminal PDZ-binding motif.

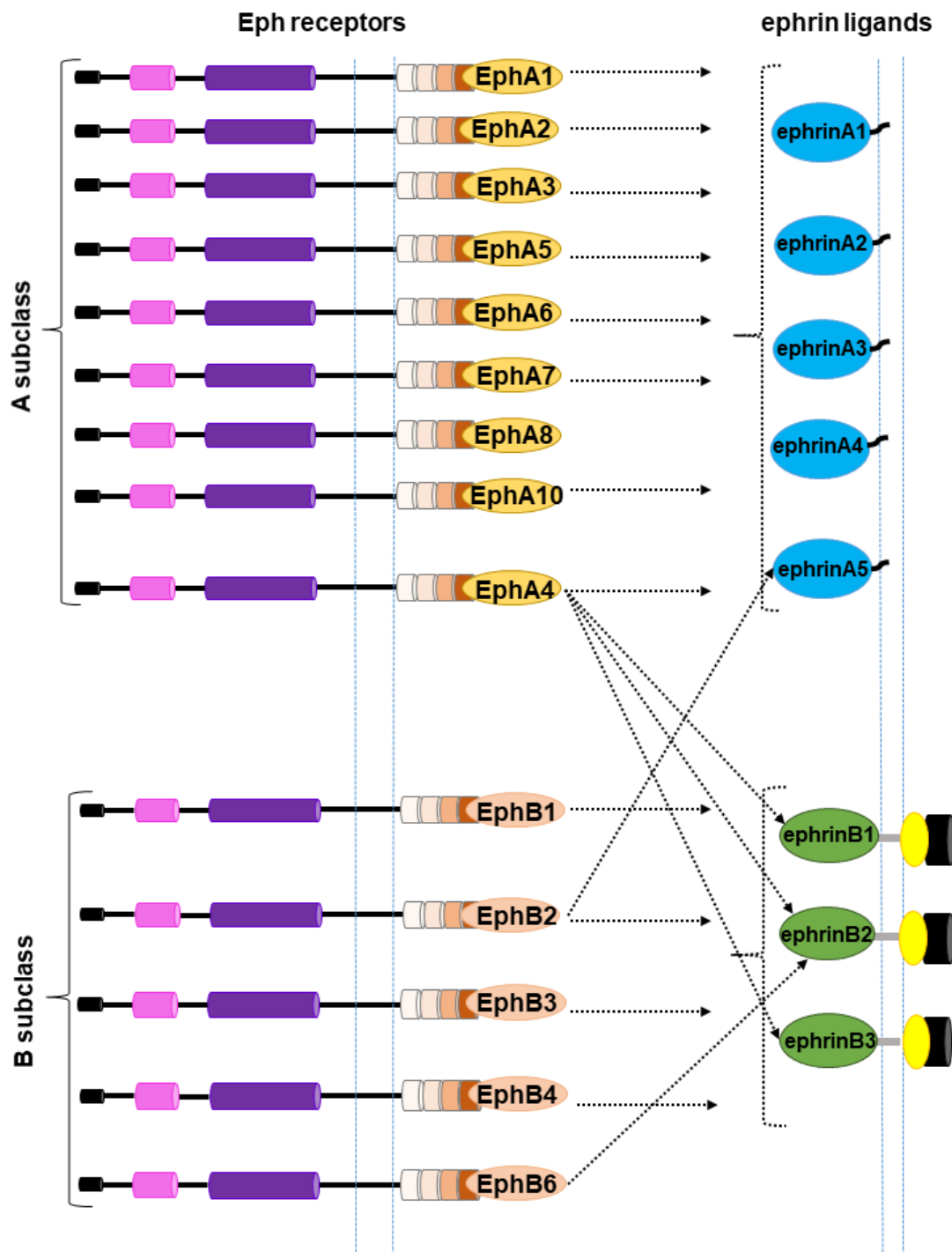


Figure 1.5: Binding between the Eph and ephrin molecules of the A and B subclasses. Promiscuous binding (black dashed lines) occurs between the human Eph receptors and ephrin ligands within each of the A and B subclasses. Limited binding occurs between the A and B subclasses, with the exception of EphA4 which binds to ephrinB1-3, and EphB2 which interacts with ephrinA5.

1.8.2 Eph/ephrin signalling

While the Eph and ephrin molecules are generally regarded as the receptors and ligands, respectively, Eph/ephrin signalling is made more complex by the fact that both the Eph and ephrin molecules can act as either the receptor or ligand (Pasquale, 2008, Pasquale, 2010, Himanen et al., 2007). The Eph and ephrin molecules form receptor-ligand pairings that can mediate signalling into either Eph or ephrin expressing cells. Forward signalling occurs when the ephrin ligand activates signalling in the Eph expressing cell. This forward signalling through the Eph receptor is mediated following the assembly of Eph/ephrin heterodimers into clusters that consist of four Eph receptors and four ephrin ligands to form a functional signalling unit. Prior to activation, the Eph receptors are maintained in an inactive conformation where the kinase domain interacts with its own juxtamembrane region resulting in the auto-inhibition of its kinase activity (Wybenga-Groot et al., 2001). Upon Eph activation by binding to an ephrin, there is autophosphorylation of two tyrosine residues, which subsequently induces a conformational change in the juxtamembrane domain that disrupts the auto-inhibition of the kinase domain. This leads to the alignment of adenosine triphosphate and subsequent activation of the tyrosine kinase domain (Pasquale, 2005). The active kinase domain initiates downstream intracellular signalling into the receptor-expressing cells, and this event is known as forward signalling.

To add to the complexity of these molecules, the Eph receptors also demonstrate non-catalytic functions by mediating a response through kinase-independent signalling (Liang et al., 2019). Two of the EphA receptors EphA10 and EphB6 are classed as pseudokinases because they lack key amino acids that are essential for kinase activity (Jacobsen and Murphy, 2017). Emerging evidence suggests that EphB6 can be phosphorylated by other Eph receptors, such as EphB1 and EphB4, potentially leading to a reciprocal regulation of EphB6 through direct interaction with its kinase-active counterparts (Freywald et al., 2002).

The Eph/ephrin interactions described above are known as *trans* interactions, where binding occurs between the Eph receptor and its cognate ephrin ligand, located on opposing cells (Boyd

et al., 2014, Pasquale, 2010). In addition to *trans* interactions, Eph forward signalling can also be mediated by a *cis* interaction which occurs between Eph and ephrin molecules residing on the same cell (Yin et al., 2004). Eph receptors have been proposed to interact with ephrin ligands in *cis* using their canonical ligand-binding domain (Yin et al., 2004) as well as their membrane-proximal fibronectin type III domain (Carvalho et al., 2006). This *cis* binding does not lead to active signalling, and instead has been reported to interfere with receptor activation by the ephrinA ligand presented on surrounding cells (Carvalho et al., 2006, Yin et al., 2004). By studying changes in ephrin expression in motor neurons, it was found that the balance between the relative contributions of *trans* signalling and *cis* attenuation to axon guidance were influenced by the subcellular distribution of the ephrin molecules to membrane patches that contain Eph receptors (Kao and Kania, 2011).

Eph receptors also activate signalling through the ephrin ligand expressing cells, and this event is referred to as reverse signalling (Klein and Kania, 2014). Forward signalling is accompanied by ephrin clustering on the ligand-expressing cells, which triggers a reverse signal activated by the Eph receptors through the ephrin-expressing cells (Wybenga-Groot et al., 2001, Janes et al., 2014, Pasquale, 2010). Since the ephrin molecules lack an enzymatic domain, reverse signalling is enabled by Eph receptor binding inducing ephrinB phosphorylation by Src-family kinases (SFK) which in turn creates binding sites for the Src homology 2 (SH2) domains of signalling proteins such as the adaptor Grb4 (Cowan and Henkemeyer, 2001, Palmer et al., 2002). The ephrinB molecules can also initiate reverse signalling via the recruitment of signalling proteins containing PDZ domains to the ephrinB PDZ-binding motifs (Lu et al., 2001). While the ephrinA molecules lack a cytoplasmic domain, they activate reverse signalling by triggering the downstream activation of SFK and phosphoinositide 3-kinase. This interaction might involve a signal-transducing co-receptor such as the neurotrophin receptors Trk/B and p75 (Marler et al., 2008) or the clustering of plasma membrane microdomains (Holen et al., 2008, Davy et al., 1999, Davy and Robbins, 2000).

Furthermore, bidirectional signalling can occur in certain situations where the Eph and ephrin function as both a receptor and ligand simultaneously. This involves the Eph and ephrin

molecules binding together and simultaneously activating downstream signalling pathways within the respective Eph and ephrin expressing cells (Irie et al., 2009, Zhao et al., 2006, Trinidad et al., 2010, Pasquale, 2010, Pasquale, 2008, McLaughlin et al., 2003). The mechanisms that determine the decision between unidirectional and bidirectional signalling are yet to be uncovered.

Collectively, Eph/ephrin signalling is highly complicated, giving rise to differential responses for processes such as cell adhesion and repulsion, proliferation, migration, apoptosis, and tumour promotion and suppression (Pasquale, 2010, Pasquale, 2008, Kania and Klein, 2016, Niethamer and Bush, 2019).

1.8.3 Role of Eph/ephrin signalling in the skeletal system

The Eph receptors and ephrin ligands have multiple roles in skeletal development, postnatal bone homeostasis and skeletal repair through their role on both osteogenic and monocytic/osteoclastogenic populations. (Xing et al., 2010, Vrahnas et al., 2019, Arthur and Gronthos, 2021, Arthur et al., 2011, Arthur et al., 2013, Nguyen et al., 2016, Arthur et al., 2018, Arthur et al., 2020, Shimizu et al., 2011, Wang et al., 2014, Stiffel et al., 2014, Takyar et al., 2013, Irie et al., 2009, Zhao et al., 2006).

The A subclass of the Eph/ephrin family have been implicated in cellular differentiation where EphA2/ephrinA2 interactions induce osteoclast differentiation and suppress osteoblast differentiation (Irie et al., 2009). Furthermore, forward signalling mediated by the EphA4 receptor on osteoclasts has been demonstrated to negatively regulate osteoclast activity (Stiffel et al., 2014).

Previous studies from our laboratory have illustrated that EphB/ephrinB molecules are expressed within human bone marrow trephine samples and by *ex vivo* expanded human BMSC, specifically EphB1, EphB2, EphB4, ephrinB1, ephrinB2 and ephrinB3 (Arthur et al., 2011). These studies demonstrated that ephrinB reverse signalling inhibited BMSC attachment

and spreading while EphB2 forward signalling promoted BMSC migration (Arthur et al., 2011). In addition, the activation of ephrinB1 and/or ephrinB2 molecules that were expressed on the MSC via the addition of EphB2-Fc and EphB4-Fc fusion proteins enhanced osteogenic differentiation (Arthur et al., 2011). These findings suggest roles for EphB/ephrinB signalling in the recruitment, migration, and differentiation of BMSC during bone homeostasis. However, the functional association between the neural cells and the bone/stromal cells, and the role of the Eph/ephrin interactions in mediating this cross-talk during the regulation of bone homeostasis was not investigated.

The B subclass of the Eph/ephrin family have been investigated in more detail during bone formation, particularly the communication between EphB4 and ephrinB2, driving cross-talk between osteoblasts and osteoclasts, to enhance osteogenic differentiation and suppress bone resorption, respectively (Zhao et al., 2006). Furthermore, *EphB4* overexpression within osteoblasts increased endochondral ossification while inhibiting osteoclast development during femoral fracture repair (Arthur et al., 2013). Importantly, both EphB4 and ephrinB2 are also expressed by osteoblasts, where EphB4-activated ephrinB2 signalling within the osteoblast lineage is critical for the gene expression of the proteins osteocalcin and sclerostin which are important regulators of osteoblast differentiation (Takyar et al., 2013). This EphB4/ephrinB2 signalling has been reported to be regulated through parathyroid hormone signalling (Allan et al., 2008). Moreover the targeted deletion of *ephrinB2* in osteocytes is associated with elevated secondary mineralization and subsequently, brittle bone (Vrahnas et al., 2019), while EphB4/ephrinB2 bidirectional signalling between growth plate chondrocytes, osteoblasts and osteoclasts promotes chondrogenesis and endochondral bone growth (Wang et al., 2014). The Eph/ephrin interactions have known roles independently regulating skeletal development, and postnatal bone homeostasis, and regulating neuronal processes such as axon guidance and neural patterning (Eberhart, 2004, Jayasena et al., 2005, Eberhart et al., 2000, Eberhart et al., 2002, Helmbacher et al., 2000, Kramer et al., 2006, Krull et al., 1997, Luxey et al., 2013, Munoz et al., 2005, Winning and Krull, 2011). Therefore, the Eph/ephrin interactions may mediate cross-communication between neurite projections and the stromal/bone cells. However, a limitation of these studies is that they did not investigate the effects of the

Eph/ephrin signalling on neural processes such as neuronal patterning and axon guidance and instead only addressed the effects on bone growth and homeostasis.

A previous study has implicated ephrinB2 as a critical regulator of fracture repair, where the deletion of *ephrinB2* in type II collagen expressing cells blocked the activation of murine periosteal stem cells, inhibited their proliferation and suppressed their differentiation into osteoblasts and chondrocytes (Wang et al., 2020). These changes led in turn to the decreased conversion of cartilage to bone within the callus, which suppressed new bone formation and impaired fracture repair. Type II collagen is a chondrogenic differentiation marker not expressed by periosteal stem cells. The effects of the local chondrogenic environment on periosteal stem cell activation and function were proposed by the authors to either relate directly to the deletion of *ephrinB2* in the chondrocytes, or the unexplored actions of *ephrinB2* within the Type II collagen expressing cells that affects periosteal stem cells and osteoblasts (Wang et al., 2020).

The related ligand ephrinB1 is also a critical player in skeletal development and postnatal bone homeostasis (Arthur et al., 2018, Arthur et al., 2019, Arthur et al., 2013, Arthur et al., 2020, Nguyen et al., 2016). Global deletion of the human ephrinB1 gene (*EPHRINB1*) results in craniofrontonasal syndrome in humans (van den Elzen et al., 2014, Wieland et al., 2004), with similar defects observed in mice with a global knockout of *ephrinB1* (Compagni et al., 2003, Davy et al., 2004). In the context of bone homeostasis and skeletal repair, the targeted deletion of *ephrinB1* within osteoprogenitor cells results in an osteoporotic phenotype (Arthur et al., 2018) and delayed endochondral ossification during the fracture repair process (Arthur et al., 2020). Another study found that changes in ephrinB1 expression during bone healing two weeks post-fracture suggested a role for ephrinB1 during endochondral ossification specifically in the development of the fracture callus tissue (Kaur et al., 2019). At the cellular level ephrinB1 reverse signalling, through activation by its cognate receptor EphB2, promotes both chondrogenic and osteogenic differentiation (Arthur et al., 2011, Cheng et al., 2013, Xing et al., 2010, Nguyen et al., 2016, Arthur et al., 2018, Arthur et al., 2020). In contrast, ephrinB1

activation of EphB2 forward signalling in human peripheral blood mononuclear cells inhibits osteoclast differentiation and function (Arthur et al., 2018).

Collectively, the Eph and ephrin molecules of both subclasses are key players within the skeletal system with critical roles in the regulation of skeletal development, bone homeostasis and fracture repair. As the Eph/ephrin interactions have known roles in independently regulating the skeletal and nervous systems, the Eph/ephrin interactions may mediate cross-communication between the neural and stromal/bone populations to regulate neural and skeletal function. However, this has yet to be investigated because research within the musculoskeletal field is required to address the effects of manipulating Eph/ephrin signalling on neural development, maintenance and repair.

1.8.4 Role of Eph/ephrin signalling in the nervous system

Eph/ephrin mediated interactions between the skeletal and nervous systems are also important for the correct patterning and localization of the sensory and motor axons during development (Eberhart, 2004, Jayasena et al., 2005, Eberhart et al., 2000, Eberhart et al., 2002, Helmbacher et al., 2000, Kramer et al., 2006, Krull et al., 1997, Luxey et al., 2013, Munoz et al., 2005, Winning and Krull, 2011). The sensory axons track the pre-formed motor axons via interactions between the EphA3 or EphA4 receptors on the motor axons and the corresponding ephrinA2 and ephrinA5 ligands on the sensory axons (Wang et al., 2011). During neuronal patterning of the peripheral tissues, the navigation of the axonal processes is restricted by signalling between Eph/ephrin interactions with the reciprocal ephrin ligand/Eph receptor on the surrounding tissue (Eberhart, 2004, Eberhart et al., 2002). For example, during limb bud development, communication between EphB2 expressed by motor and sensory axons, and ephrinB1 expressed by the sensory axons and limb bud mesenchyme controls axon fasciculation in the limb (Luxey et al., 2013).

During normal hind limb innervation, the motor axons within the lateral motor column that target the limb target tissues initially converge at the base of the limb bud and then adopt dorsal

and ventral trajectories within the limb (Luria and Laufer, 2007). This patterning is disrupted in mutant mice with a global deletion of the *EphA4* gene, where presumptive dorsal motor axons enter the ventral compartment of the limb instead (Helmbacher et al., 2000). The growth of EphA4-positive lateral motor column axons is inhibited within ephrinA5-positive regions of the hind limb mesoderm due to a repulsive interaction (Eberhart, 2004). Importantly, the interactions between ephrinA5 expressed within the mesenchyme and the EphA receptors on the growth cones of sensory axons inhibits the growth of sensory axons not only in the limbs but also within the facial tissue (Munoz et al., 2005). EphA3 which is highly expressed during the trigeminal sensory innervation within the avian embryo, mediates the guidance of the ophthalmic nerves via interactions with ephrinA2 or ephrinA5 (Jayasena et al., 2005). It has also been shown that the movement of spinal motor axons and neural crest cells are influenced by different guidance mechanisms despite traversing the same pathway (Koblar et al., 2000), where EphB:ephrinB1 interactions pattern the migration of trunk neural crest cells (Krull, 1998), but are not required for the patterning of spinal motor axons (Krull et al., 1997).

The Eph and ephrin molecules also have a function in the injury and recovery of the central nervous system (CNS), where blocking EphA4 promotes axon regeneration as well as functional recovery in mice and rats following injury to the spinal cord (Goldshmit et al., 2011, Fabes et al., 2007). The conditional deletion of *ephrinB2* within astrocytes, specialized glial cells, also improved axon regeneration in mice following spinal cord injury. This finding showed that astrocyte-derived ephrinB2 inhibits axon growth and impedes recovery following spinal cord injury (Ren et al., 2013). In addition, EphB1/ephrinB1 signalling was involved in the modulation of spinal inflammatory cytokines in a rat model of bone cancer pain, suggesting this interaction could be a potential therapeutic target in the treatment of this condition (Dong et al., 2011). Furthermore, the lack of functional EphB1 receptors in a variety of inflammatory, tissue and nerve injury models led to blunted sensitivity to pain and accelerated recovery (Cibert-Goton et al., 2013). Recently, ephrinB1 was reported to modulate glutamatergic inputs into a specific subset of neurons that express proopiomelanocortin and regulate glucose homeostasis and feeding (Gervais et al., 2020).

The role of Eph and ephrin interactions in cancer has also become more evident over the past two decades (Buckens et al., 2020). EphA2 plays a key role in the metastatic onset of a common bone malignancy Ewing sarcoma, where upregulation of this receptor is associated with increased metastatic potential, speculated to be due to the promotion of endothelial cell migration towards the Ewing sarcoma tumours favouring angiogenesis within these tumours (Sáinz-Jaspeado et al., 2013, Garcia-Monclús et al., 2018). There are several on-going clinical studies that target the EphA2 receptor in prostate, lung, and colorectal cancers (Xiao et al., 2020, Martini et al., 2019, Kurose et al., 2019, Tan et al., 2019). The EphA3 receptor and to a smaller extent, the EphA2 and EphB2 receptors, are also commonly over-expressed in glioblastomas, the most common and aggressive primary malignant brain tumours. An ephrinA5-based cytotoxin that targets tumour cells over-expressing these receptors has been developed to treat this malignancy (Thakkar et al., 2014, Ferluga et al., 2016). A recent study reported the development of novel peptide molecules that target EphA2 on glioblastoma stem cells, illustrating their future potential in the treatment of glioblastomas (Affinito et al., 2020).

The EphA4 receptor is currently being investigated as a therapeutic target in Alzheimer's disease, where blockade of EphA4 signalling via the administration of small-molecule inhibitors reverses impaired synaptic plasticity in mouse models to alleviate the disease phenotype, although the underlying mechanisms are yet to be properly elucidated (Fu et al., 2014, Gu et al., 2018, Vargas et al., 2018). More recently, the postnatal genetic ablation of EphA4 in the forebrain in a mouse model of Alzheimer's disease was reported to improve social memory associated with an increase in dendritic spine length (Poppe et al., 2019). EphA4 signalling has also been recently identified as a potential target in the treatment of trigeminal neuropathic pain. Daily treatment with an EphA4-antagonist EphA4-Fc which acted as a soluble decoy that mimicked the EphA4 receptor, and blocked the activation of the endogenous membrane-bound EphA4, limited the development of neuropathic pain in response to nerve injury in a rat model (Kim et al., 2020). Similarly, ephrinB2/EphB1 signalling within the spinal cord has been recently reported to be a major player in post-inflammatory and stress-induced visceral hypersensitivity, the major mechanism underlying abdominal pain experienced by patients with irritable bowel syndrome and inflammatory bowel diseases. Therefore, these interactions are a potential target in the treatment of these disorders (Theofanous et al., 2020).

Other studies have shown that the stimulation of ephrinB2/EphB4 signalling exerts neuroprotective effects during the post-ischemic stroke recovery phase, by enhancing vascular repair mechanisms and reducing brain swelling after mild cerebral ischemia (Ghori et al., 2017).

In summary, Eph/ephrin signalling is required for proper skeletal development, bone homeostasis and fracture repair. Eph/ephrin interactions also influences the CNS and PNS by regulating early axonal patterning and promoting axon regeneration and recovery following injury. The Eph/ephrin molecules are known to be expressed by the neural and skeletal/stromal populations. As the Eph/ephrin molecules have known roles in independently regulating the skeletal and nervous systems, the Eph/ephrin interactions may mediate cross-communication between the neural cells and the stromal/bone cells, where this neuro-osteogenic network has already been established, to regulate development, maintenance, and repair of the skeletal and neural systems. This gap of knowledge is created because research into the effect of manipulating Eph/ephrin signalling within the nervous system has yet to examine the effects on bone growth and repair, and conversely, further research within the musculoskeletal field is required to address the neuronal system. Greater collaboration between the two research fields would lead to enhanced investigation into the effects on the skeletal system in response to changes in the nervous system mediated by the Eph/ephrin interactions and vice versa, which would broaden our understanding of the neuro-osteogenic network and the molecular mechanisms that regulate this network.

1.9 Thesis Objectives

Emerging evidence in the literature describes a physical and functional association between the neural and skeletal systems. There are several studies that have provided substantial evidence of the Eph/ephrin interactions independently regulating the skeletal system by affecting osteogenic and osteoclastic populations and regulating the CNS and PNS by influencing early axonal patterning. Therefore, these contact-dependent Eph/ephrin interactions may mediate cross-communication between the neuronal processes and the bone/stromal populations to regulate the development, maintenance, and repair of the skeletal and nervous systems.

However, this has not been investigated in detail. Eph/ephrin signalling may have potential in the treatment of skeletal diseases and conditions, as this signalling has already been established in clinical trials as a pharmacological target in cancer (Xiao et al., 2020, Martini et al., 2019, Kurose et al., 2019, Tan et al., 2019), Alzheimer's disease (Fu et al., 2014, Gu et al., 2018, Vargas et al., 2018) and neuropathic pain (Kim et al., 2020). The present thesis investigated the roles of the Eph/ephrin interactions in mediating cross-communication between the BMSC and neural populations.

1.9.1 Hypothesis

Eph-ephrin mediated cross-communication between the neural cells and the human BMSC regulates neural differentiation, adhesion and migration.

1.9.2 Aims

1. Identify the Eph/ephrin expression profile of neural cells that corresponds to the Eph/ephrin expression profile of human BMSC populations.
2. Identify the role of Eph/ephrin interactions in mediating communication between the neural populations and the human BMSC populations to regulate neural differentiation, adhesion, and migration.
3. Identify the role of Eph/ephrin interactions in mediating communication between the neural populations and the human BMSC populations to regulate neurite cell body number and neurite invasion from avian embryo dorsal root ganglia.

Chapter 2 Materials and methods

2.1 Materials

2.1.1 Reagents

Table 2.1: Reagents

Reagents	Catalogue Number	Supplier
10% Neutral-buffered formalin	AJA2518-5L	Thermofisher Scientific
4% Paraformaldehyde solution	SANTSC-281692	Bio Strategy
4',6-diamidino-2-phenylindole (DAPI)	D9542	Sigma-Aldrich
Acrylamide (30% Bis solution)	161-0158	BioRad
Agarose	A9539	Sigma-Aldrich
All-trans Retinoic Acid	R2625	Sigma-Aldrich
Alpha-modified Minimum Essential Medium Eagle (α -MEM)	M4526	Sigma-Aldrich
Ammonium persulphate (APS)	215589	Sigma-Aldrich
Animal-Free Human CXCL12	AF-30028A	PeptoTech
Bovine Serum Albumin (BSA) Protein Standard	23209	Pierce
Chloroform	10077.6B	BDH Solvents
Dexamethasone sodium phosphate	N/A	Mayne Pharma
Dimethyl sulfoxide (DMSO)	DA013-2.5L-P	Chem Supply
dNTP mix (10 mM)	FISBIODN-10M	Adelab Scientific
Dulbecco's modified Eagle's medium (DMEM) High Glucose (4500 mg/L)	D5671	SAFC
Ethanol (analytical grade)	EA043	Chem Supply

Ethylenediaminetetraacetic acid (EDTA)	EL023	Chem Supply
Foetal Calf Serum (FCS)	12003C	SAFC
Glycerol	GA010	Chem Supply
Glycine	G8898	Sigma-Aldrich
Glycogen	10901393001	Sigma-Aldrich
Ham's F-12 Nutrient Mix	11765054	Gibco
Hanks' balanced salt solution (HBSS)	H9269	Sigma-Aldrich
Hydrochloric acid (HCl)	HT020	Chem Supply
Isopropanol	PA013	Chem Supply
Laminin Mouse Protein, Natural	23017015	Thermofisher Scientific
L-ascorbate-2-phosphate	013-12061	Wako Pure chemical
L-glutamine	G7513	Sigma-Aldrich
Magnesium chloride hexahydrate	M2670	Sigma-Aldrich
Methanol	MA004-P	Chem. Supply
N,N,N,N-tetramethylethylenediamine (TEMED)	T9281	Sigma-Aldrich
N.N-dimethylformamide	33120	Sigma-Aldrich
N2 Supplement	17502001	Gibco
N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)	H0887	Sigma-Aldrich
Naphthol AS-BI Phosphate	N2125	Sigma-Aldrich
Nonidet P40 Substitute (NP40)	74385	FLUKA
OligodT	C1101	Promega
Penicillin 5000 U/mL, streptomycin 5mg/mL	P4333	Sigma-Aldrich

Peroxidase Substrate Solution (3, 3' – diamino benzidine, DAB)	D-5637	Sigma-Aldrich
Phosphate Buffered Saline (PBS)	D8537	Sigma-Aldrich
Poly-L-Lysine	P4707	Sigma-Aldrich
Potassium chloride (KCl)	PO02000500	Chem Supply
Potassium dihydrogen orthophosphate (KH ₂ PO ₄)	10203	BDH
Primers for RT-PCR		Sigma-Aldrich
Protease Inhibitor tablets, EDTA-Free	04693132 001	Roche
Protein ladder for Western blots (Precision Plus Protein Dual Colour Standards)	1610374	Bio-Rad
Random Hexamer Primers (500 ng/μL)	RP-6	Geneworks
Recombinant human/murine/rat Bone Derived Neurotrophic Factor (BDNF)	450-02	PeproTech
Sodium Bicarbonate (NaHCO ₃)	S6014	Sigma-Aldrich
Sodium Chloride (NaCl)	SL046	Chem Supply
Sodium Dodecyl Sulphate (SDS)	444464T	Merck
Sodium Pyruvate	S8636	Sigma-Aldrich
Superscript IV reverse transcriptase	1809050	Life Tech Supply Centre
SYBR Green ROX RT-PCR master mix	330523	Qiagen
Tris	TRIS01	Chem Supply
Triton-X-100	X100	Sigma Aldrich
TRIzol	15596-018	Invitrogen
Trypan blue (0.4%)	T8154	Sigma-Aldrich
TrypLE™ Express Enzyme, phenol red	12605028	Gibco
Trypsin	59418C	SAFC Biosciences

Trypsin-EDTA	T4174	Sigma-Aldrich
Tween-20	P1379	Sigma-Aldrich
Vybrant DiD Cell-Labelling Solution	V22887	Thermofisher Scientific
White Leghorn Chicken Eggs (Fertilised)	N/A	HiChick Breeding Company

2.1.2 Antibodies

Table 2.2: Primary antibodies

Name	Catalogue Number	Raised against	Supplier	Concentration
anti-ephrinA2	AF603	Goat	R&D systems	200 µg/mL
anti-ephrinA5	38-0400	Rabbit	Invitrogen	250 µg/mL
anti-ephrinB1	AF473	Goat	R&D systems	200 µg/mL
anti-Neurofilament-Medium chain	13-0700	Mouse	Invitrogen	500 µg/mL
anti-Neurofilament-Medium chain	34-1000	Mouse	Invitrogen	500 µg/mL
anti-Neurofilament-Heavy chain (NE14)	N5389	Mouse	Sigma	N/A
anti- α -Tubulin (YL1/2)	ab6160	Rat	Abcam	1 mg/mL
anti- β -III Tubulin (Tuj1)	801201	Mouse	Biolegend	1 mg/mL

Table 2.3: Negative controls

Name	Catalogue Number	Raised against	Supplier	Concentration
1D4.5	N/A	Mouse IgG _{2a}	MSC Laboratory	1 mg/mL
Rabbit IgG	02-6102	Rabbit	Invitrogen	5 mg/mL
Goat IgG	02-6202	Goat	Invitrogen	5 mg/mL

Table 2.4: Secondary and tertiary antibodies

Name	Catalogue number	Supplier	Concentration
Conjugated			
TRITC-Phalloidin	P1951	Sigma	500 µg/mL
Mouse secondary			
Goat anti Mouse Biotin	1030-09	Southern Biotech	500 µg/mL
Goat anti Mouse IgG-FITC	1030-02	Southern Biotech	1 mg/mL
Horse anti Mouse IgG Biotin, Rat adsorbed	BA-2001	VECTOR	500 µg/mL
Rabbit secondary			
Goat anti Rabbit Biotin	BA-1000	VECTOR	1.5 mg/mL
Goat anti Rabbit Dylight 680	35568	Life Technologies	1 mg/mL
Goat anti Rabbit IgG DyLight 800	SA5-35571	Life Technologies	1 mg/mL
Goat anti Rabbit IgG-FITC	4054-02	Southern Biotech	500 µg/mL
Goat secondary			
Rabbit anti Goat Biotin	BA-5000	VECTOR	1.5 mg/mL
Donkey anti Goat IgG DyLight 680	SA5-10090	Life Technologies	500 µg/mL
Rabbit anti Goat IgG FITC	6026-02	Southern Biotech	500 µg/mL
Rat secondary			
Goat anti Rat IgG DyLight 680	SA5-10022	Life Technologies	500 µg/mL
Tertiary antibodies			
Streptavidin Alexa 488	S-11223	Molecular Probes	1 mg/mL
Streptavidin, Peroxidase	SA-5004-1	VECTOR	1 mg/mL

2.1.3 Fusion proteins and blocking peptides

Table 2.5: Fusion proteins

Name	Catalogue number	Supplier	Concentration
EphA3-Fc	640-A3-200	R & D Systems	200 µg/mL
EphA4-Fc	641-A4-200	R & D Systems	200 µg/mL
EphB2-Fc	467-B2-200	R & D Systems	200 µg/mL
EphB4-Fc	446-B4-200	R & D Systems	200 µg/mL
Human anti-Goat IgG clustering agent	109-005-008	Jackson ImmunoResearch	2 mg/mL
Human-IgG ₁ -Fc	N/A	CSIRO	1 mg/mL

Table 2.6: Blocking peptides

Name	Peptide ID	Peptide sequence (N terminus to C terminus)	Supplier
ephrinB1 blocking peptide	PTD-EFNB1-C	GRKKRRQRRRPPQGGGVQEMPP QSPANIYYKV	Mimotopes
Scramble peptide	PTD-Scram	GRKKRRQRRRPPQGGGEISKPMY PQVQVYPNA	Mimotopes

2.1.4 Kits

Table 2.7: Kits

Name	Catalogue Number	Supplier
RC/DC protein assay kit	500-0121	Bio-Rad Laboratories
Pierce Detergent Compatible Bradford Assay Kit	23246	Thermofisher Scientific
Protein Sample Loading Buffer and Polyvinylidene difluoride (PVDF) Membrane Kit	926-31097	LI-COR

2.1.5 Buffers, media and solutions

Table 2.8: Buffers, media and solutions

10% Separating gel (10% polyacrylamide gel)	RO water (7.9 mL), 30% bis-acrylamide (6.7 mL), Tris (5 mL; 1.5 M; pH 8.8), SDS (200 µL; 10% w/v), APS (200 µL; 10% w/v) were mixed. Immediately before use, TEMED (8 µL) was added and the solution was mixed.
Basal media for SH-SY5Y cells	DMEM with 4500 mg/L glucose containing 10% (v/v) FCS, 2 mM L-Glutamine, 1 mM Sodium Pyruvate and Penicillin (25 iu/mL) and Streptomycin Sulphate (25 µg/mL)
Blocking buffer for staining cells for flow cytometry analysis	HBSS containing 5% (v/v) FCS, 5% human serum, 10% BSA, and penicillin (50 i.u./mL) and streptomycin sulphate (50 µg/mL)
DMEM/F12 media	1:1 ratio of DMEM with 4500 mg/L glucose and Ham's F12, containing 100 units/ml penicillin, 0.1 mg/ml streptomycin
FACS Fix for flow cytometry analysis	1% (v/v) Formalin and 2% (w/v) glucose in 1x PBS

F12HS10 media	Ham's F12, 100 units/ml penicillin, 0.1 mg/ml streptomycin, 10% (v/v) FCS and 1% (v/v) of the 100x concentrate of N2 supplement
Membrane blocking solution for Western blotting	5% Skim Milk in TBS-Tween made fresh on the day of use by dissolving 2 g of skim milk in 40 mL TBS-Tween at room temperature with agitation. 200 μ L of (10% w/v) Sodium Azide was added to prevent bacterial contamination
Modified Radioimmunoprecipitation Assay (RIPA) lysis buffer	Tris-HCl (200 μ L; 1 M; pH 8), EDTA (40 μ L; 0.5 M), Sodium Chloride (750 μ L; 2 M), Nonidet P40 (1 mL; 10% v/v), SDS (100 μ L; 10% w/v), Sodium Deoxycholate (500 μ L; 10%), Roche inhibitor cocktail (5 mL; 2x stock) were mixed thoroughly and RO water was added to reach final volume of 10 mL, which was stored in 1 mL aliquots at -20°C for up to three months
Osteogenic differentiation media for BMSC	α -MEM supplemented with 5% FCS, 2 mM L-Glutamine, 1 mM Sodium Pyruvate, Penicillin (50 i.u/mL) and Streptomycin sulphate (50 μ g/mL), 100 μ M of L-ascorbate-2-phosphate, 0.1 μ M Dexamethasone sodium phosphate and 2.6 mM Potassium dihydrogen orthophosphate (KH_2PO_4)
Growth media for BMSC	α -MEM supplemented with 10% (v/v) FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 μ M of L-ascorbate-2-phosphate, 50 U/mL penicillin and 50 μ g/mL streptomycin
Peroxidase substrate (DAB) solution for immunohistochemistry	Stock solution was prepared of 75 mg/mL by dissolving 25 g of the DAB solution in 333.3 mL distilled water, and stock solution was stored in 300 μ L aliquots at -20°C. The working solution was prepared as follows: 13 mL Part A (1.7 mL 0.2 M HCl in 100 mL distilled water) and 13 mL Part B (6 g TRIS in 250 mL distilled water) were mixed with 24 mL distilled water. The pH was adjusted to 7.65 – 7.70. 50 μ L hydrogen peroxide and 1 aliquot of the DAB stock solution was added to this immediately before use
PFE solution for Flow analysis	PBS + 2% FCS + 2mM EDTA
Phase I Neural-inductive Media for SH-SY5Y cells (Days 0 to 3)	DMEM with 4500 mg/L glucose, supplemented with 4 mM L-Glutamine, Penicillin (50 iu/mL) and Streptomycin Sulphate (50 μ g/mL), 5% (v/v) FCS and 10 μ M All-trans retinoic acid

Phase II Neural-inductive media for SH-SY5Y cells (Days 3 to 12)	DMEM with 4500 mg/L glucose and Ham's F12 Nutrient mixture, supplemented with 2 mM L-Glutamine, Penicillin (50 iu/mL) and Streptomycin Sulphate (50 µg/mL), 1% (v/v) of the 100x concentrate of N2 supplement and 50 ng/mL BDNF
PUCKS-EDTA	Potassium chloride (250 µL; 1 M), Sodium chloride (1.625 mL; 4 M), Sodium Bicarbonate (150 µL; 1 M), D-glucose (250 µL; 1 M), HEPES (1 mL; 0.5M; pH 7.3), EDTA (100 µL; 0.5 M) and 46.625 mL RO Water were mixed
Roche complete mini protease inhibitor cocktail	Protease inhibitor (one tablet) was dissolved in RO water (25 mL) to make a 2x stock and this was stored in 5 mL aliquots at -20 °C
SDS Running buffer for SDS-PAGE	Tris (30.3 g), glycine (144 g) and SDS (10 g) were dissolved in RO water. The pH was adjusted to 8.3 and the volume was made up to 1 L. The 10x solution was stored at room temperature and was diluted at 1:9 in RO water prior to use for a final concentration of 0.3% (w/v) Tris-HCl, 1.44% (w/v) glycine and 0.1% (w/v) SDS
Sodium citrate (10 mM) for antigen retrieval in immunohistochemistry	Citric acid (10.5 g) and 5 M sodium hydroxide (25 mL) were dissolved in 5 L distilled water, and the pH was adjusted to 6.0
Stacking gel (5% polyacrylamide gel)	RO water (2.5 mL), 30% Bis-acrylamide (0.75 mL), Tris (1.125 mL; 0.5 M; pH 6.8), SDS (45 µL; 10%) and APS (45 µL; 10%) were mixed by inversion. Immediately before use, TEMED (4.5 µL) was added and the solution was mixed
TBS/1% Tween-20 (TBS-T)	Tween 20 (1 mL) was added to 100 mL TBS (10x) and 899 mL RO water. The solution was gently mixed to avoid bubbles and was stored at room temperature
Transfer buffer for SDS-PAGE	Tris (12.104 g) and Glycine (57.66 g) were dissolved in 3 L RO water. Methanol (0.6 L) was added and the volume was made up to 4 L. The solution was stored at 4°C and was used up to 2 times before discarding
Tris-buffered saline (TBS), 10x	Tris (24.2 g) and sodium chloride (80 g) were dissolved in RO water. The pH was adjusted to 7.6 and the volume was made up to 1 L. The solution was stored at room temperature and was diluted 1:9 in RO water before use

2.1.6 Primers for real-time PCR

Table 2.9: Primer sequences for real-time PCR

Gene name	Accession No.	Forward sequence (5' - 3')	Reverse sequence (5' - 3')
Hs <i>DRG11</i>	NM_001276451.1	TTGATGACGGGTTTCTGCGT	TCTGGAACCAAACCTGCACTC
Hs <i>EPHA1</i>	NM_005232	CAAGGACGCAGAGACACTGA	TCTCGAATGGTGAAGCTCTG
Hs <i>EPHA2</i>	NM_004431	TGGTACTGCTGGACTTTGCTG	GTCGCCAGACATCACGTTG
Hs <i>EPHA3</i>	NM_005233	TTGCCAAGGAATTGGATGC	AGCCAACTTTTCAGGGTCTTAATG
Hs <i>EPHA4</i>	NM_004438.3	GGCTCAGAGGGTGTATATTGAGA	TGAAACGCTCTTTGTCGTTGT
Hs <i>EPHA5</i>	NM_182472.2	TGGGAAGAGATTGGTGAAGTG	CATCGGCAGCAATGGTATC
Hs <i>EPHA6</i>	NM_001080448.2	AGGTGTGGTAAGGAAGGACTGG	GCTTTGGACCTTGTGGAAGAGT
Hs <i>EPHA7</i>	NM_004440.3	TACCCCGATACGAACATAACCAG	AGGAAGACTGTTACAATCCCTCA
Hs <i>EPHA8</i>	NM_020526.3	CCTGGCAATGATTGTGACTG	CATAGCCCAGGTCTGAGAGGTA
Hs <i>EPHB1</i>	AF037331	GTGGCTACGATGAAAACCTGAAC	CTGGTTGGGCTCGAAGACAT
Hs <i>EPHB2</i>	AF025304	ATGAACACGATCCGCACGTA	TTGGTCCGTAGCCAGTTGTTCT
Hs <i>EPHB3</i>	NM_004443	CACTGAGTTCATGGAAACTGC	GTAGGATCGGAGGGGTCATC
Hs <i>EPHB4</i>	NM_004444	GCCGCAGCTTTGGAAGAG	CATCCAGGCCGCTCAGTT
Hs <i>EPHB6</i>	NM_004445	TGGGACGAGGTGAGTGTCTCTG	CCACATGACATGCCTCAAAGG
Hs <i>EPHRINA1</i>	NM_004428.02	CCCCAGTCCAAGGACCAA	GATTTTGCCACTGACAGTCACC
Hs <i>EPHRINA2</i>	NM_001405.2	AGAAGTTCCAGCTCTTCACGC	CGGGCTGCTACACGAGTTAT
Hs <i>EPHRINA3</i>	NM_004952.4	TCTCTGGGCTACGAGTTCCAC	CAGCACGTTGATCTTCACATTG
Hs <i>EPHRINA4</i>	NM_005227.2	GAGTTCTGGCCAGTGCTTGA	AATTCGCAGAAGACGAAGAATC
Hs <i>EPHRINA5</i>	NM_001962.2	GTTGACGCTGGTGTCTCTGG	ATGGTAGTCACCCCTCTGGAAT
Hs <i>EPHRINB1</i>	NM_004429	AGCTCCCTCAACCCCAAGTT	GGCAGATGATGTCCAGCTTGT
Hs <i>EPHRINB2</i>	NM_004093	CCTCTCCTCAACTGTGCCAAA	CCCAGAGGTTAGGGCTGAATT

Hs <i>EPHRINB3</i>	NM_001406	AGGTGCTTCTCCGAGTGG	GGAGGTTGCATTGCTGGT
Hs <i>NESTIN</i>	NM_006617.1	CGCACCTCAAGATGTCCCTC	CAGCTTGGGGTCCTGAAAGC
Hs <i>NF-L</i>	NM_006158.4	CTGGAAATCGAAGCATGCCG	TGATCGTGTCTGCATAGCG
Hs <i>NF-M</i>	NM_005382.2	CAGCTCGAAAGCCACTCAGA	GGATGGTGTCTGGTAGCTG
Hs <i>NF-H</i>	NM_021076	CAGCCAAGGTGAACACAGAC	GCTGCTGAATGGCTTCCT
Hs <i>NSE</i>	NM_001975.2	TGCACAGGCCAGATCAAGAC	CCAGGCAAGCAGAGGAATCA
Hs <i>B-ACTIN</i>	NM_001101	GATCATTGCTCCTCCTGAGC	GTCATAGTCCGCCTAGAAGCAT
Hs <i>BIII-TUBULIN</i>	NM_006086.3	AACCAGATCGGGGCCAAGTT	AGGCACGTACTTGTGAGAAGAG
Hs <i>PERIPHERIN</i>	NM_006262.4	ATAAAGACGACTGTGCCTGAGGT	GGGCAGAAGACTTGTCCAGC

2.1.7 Equipment

Table 2.10: Equipment

Equipment	Catalogue Number	Supplier
10% Mini-PROTEAN TGX Stain-free protein gels	4568036	Bio-Rad Laboratories
CFX Connect™ Real-Time PCR detection system	1855201	Bio-Rad Laboratories
Class II laminar flow hood	N/A	Topsafe 1.2, Bio Air
Confocal Microscope	TCS SP8 X	Leica Microsystems
Corning 6.5 mm Transwell with 8 µm polycarbonate membrane cell culture inserts	CLS3422	Sigma-Aldrich
Dumont Tweezers, style 5	T05-822	ProSciTech
Flow Cytometry – Cell analyser	BD LSRFortessa X-20	BD Biosciences
Flow Cytometry – Cell analyser	BD FACS Canto	BD Biosciences

GENESYS 10S UV-Vis Spectrophotometer	840-208100	Thermofisher Scientific
Glass coverslips (No. 0, 5 mm)	G400-05	ProSciTech
Glass coverslips (No. 0, 8 mm)	G400-08	ProSciTech
Hemocytometer for cell counts	Weber Scientific	3048-12
Incubators for cell culture	MCO-18A1C	Sanyo Oceania
Infrared imaging system for Western blotting	Odyssey CLx	LI-COR Biosciences
Inverted Microscope	IX53	Olympus
Inverted Microscope	CKX31	Olympus
Lab-Tek Chamber slides	NUN178599	Thermofisher Scientific
Mini-gel (1.5 mm) combs	165-3365	Bio-Rad Laboratories
Mini-gel (1.5 mm) glass plate	165-3312	Bio-Rad Laboratories
Mini-PROTEAN 3 System Glass Plates	165-3308	Bio-Rad Laboratories
Mini-PROTEAN TETRA Vertical electrophoresis cell	165-8005	Bio-Rad Laboratories
Mini-Trans Blot Electrophoretic Transfer Cell	170-3930	Bio-Rad Laboratories
NanoDrop 8000 Spectrophotometer	ND-8000-GL	Thermofisher Scientific
Powerpac Basic Power Supply	1645050	Bio-Rad Laboratories
Silicone culture-inserts, 4-well for self-insertion (ibidi)	80469	DKSH
Stereo Microscope	SZX7	Olympus
Veriti 96 well Thermal Cycler	4375786	Thermofisher Scientific

2.1.8 Software

CFX Manager Software version 3.0 (Bio-Rad Laboratories)

FACSDiva software (BD Biosciences) version 8.0

FlowJo™ Software version 10.7.1 (Becton, Dickinson and Company)

GNU Image Manipulation Program (GIMP) version 2.10.22

GraphPad Prism version 9.0. (GraphPad Software)

Image Studio Lite Quantification Software version 5.2 (LI-COR Biosciences)

ImageJ version 1.53 (NIH and LOCI)

Leica Application Suite X (LAS X) Software version (Leica Microsystems) Version 3.0.13

NanoDrop 8000 Operating Software, version 2.3.3 (Thermofisher Scientific)

2.2 Methods

2.2.1 Cell culture techniques

2.2.1.1 Culture of cells

All cell culture procedures were conducted in a sterile environment of Class II laminar flow hood. SH-SY5Y cells were cultured at 50,000 cells/cm² in appropriate culture media in a 37°C humidified atmosphere in the presence of 5% CO₂. BMSC were obtained from human subjects with approval from the Royal Adelaide Hospital ethics committee (940911a) and cultured at 8000 cells/cm² in appropriate culture media in a 37°C humidified atmosphere in the presence of 5% CO₂.

2.2.1.2 Liberation of cells

On achieving 80-90% confluence, cells were detached with Trypsin. To do this, the culture media was aspirated, and cells were washed once with pre-warmed HBSS and then detached from the flasks by treatment with 2.5 mL Trypsin-EDTA (0.25%) for 3-5 minutes at 37°C. Cells were washed with HBSS supplemented with 5% FCS and centrifuged at 1400 rpm for 8

min at 4°C. The supernatant was aspirated, and the cell pellet was resuspended in 1 mL of appropriate culture medium, and a fraction of this cell suspension was either transferred for re-seeding in further experiments, or cryopreservation.

2.2.1.3 Cryopreservation of cells

Cells that required extended storage were cryopreserved at -196°C. Cells were cryopreserved in FCS containing 10% (v/v) of DMSO (1 mL/ ampoule). Cells (~1 x 10⁶ cells/ ampoule) were placed in 0.5 mL/ ampoule of 100% cold FCS, immediately prior to freezing, 0.5 mL/ ampoule of pre-prepared, cold FCS containing 20% (v/v) DMSO solution was added drop wise to cells in 100% cold FCS on ice. The 1 mL/ ampoule cell suspensions were transferred to 1.5 mL ampoules and positioned in a Mr Frosty containing isopropanol. The Mr Frosty was placed at -80°C to allow cooling of 1°C per minute. Once at -80°C, the ampoules were transferred to the vapour phase of liquid nitrogen for extended storage at -196°C.

2.2.1.4 Thawing of cryopreserved cells

Cells removed from liquid nitrogen (-196°C) were immediately thawed at room temperature (RT). The cell suspension was immediately transferred dropwise to a 14 mL polypropylene tube containing cold HBSS supplemented with 5% FCS and centrifuged at 1400 rpm for 8 min at 4°C. The media was aspirated, and the cell pellet was resuspended in 1 mL of appropriate media and cell counts were performed.

2.2.2 Functional Assays

2.2.2.1 Neural differentiation assay

The wells of plates (6-well, 12-well, 24-well, 48-well and 96-well plates) were pre-coated with 0.01% Poly-L-Lysine solution for five minutes at room temperature and then washed twice with PBS. The SH-SY5Y cells were seeded in the wells at a density of 50,000 cells/ cm² in basal media (Table 2.8) and cultured at 37°C humidified atmosphere in the presence of 5% CO₂. This was referred to as Day -1. Twenty-four hours later (Day 0), the basal media was replaced with Phase I neural-inductive medium (Table 2.8). The Phase I media was replaced

with Phase II neural-inductive media (Table 2.8) on day 3 and subsequently replaced with fresh Phase II medium on days 6 and 9 until day 12 which was considered the endpoint of neural induction (day 12) (Table 2.11). Samples were then processed for RNA or protein analysis.

Alternatively, on day 0, the Human-IgG-Fc, EphA3-Fc, EphA4-Fc, EphB2-Fc or EphB4-Fc fusion proteins (1, 5 and 10 µg/mL) were pre-clustered with Human anti-Goat IgG clustering agent (10, 50 and 100 µg/mL, respectively), in neural-inductive media for 1 hour at room temperature (Table 2.11). Cells were exposed to these molecules during each media change of the neural differentiation assay. Bright field images were obtained for each condition on days 0, 3, 6, 9 and 12 of the neural differentiation assay, and analysed as described in section 2.2.3.6. Samples were processed for RNA or protein analysis on day 12.

Table 2.11: Protocol for the neural differentiation assays established for the SH-SY5Y human neuroblastoma line

Assay	Day -1	Day 0	Day 3	Day 6	Day 9	Day 12
Neural differentiation assay	Seeding of SH-SY5Y cells in Basal media	Change to Phase I neural-inductive media	Change to Phase II neural-inductive media	Change to Phase II neural-inductive media	Change to Phase II neural-inductive media	Endpoint of neural induction reached.
Neural differentiation assay including Eph-Fc	Seeding of SH-SY5Y cells in Basal media	Addition of clustered Eph-Fc in Phase I neural-inductive media	Addition of clustered Eph-Fc in Phase II neural-inductive media	Addition of clustered Eph-Fc in Phase II neural-inductive media	Addition of clustered Eph-Fc in Phase II neural-inductive media	Endpoint of neural induction reached

2.2.2.2 Testing different methods of cell detachment for the adhesion assay

SH-SY5Y cells were cultured under neural-inductive conditions and liberated on day 12, and detached by three different methods using Trypsin, TrypLE Express Enzyme or PUCKS-EDTA as outlined in Table 2.12.

Table 2.12: Protocol for detachment methods tested to liberate SH-SY5Y cells

Detachment method	Protocol
Trypsin	Cells were washed with HBSS and incubated with Trypsin (2 mL per 75 cm ² flask) for 1-2 minutes. Samples were washed with HBSS supplemented with 5% FCS.
TrypLE	Cells were washed once with PBS, and TrypLE was added (2 mL per 75 cm ² flask) for 1-2 minutes. To quench this, HBSS supplemented with 5% FCS was added to form a single-cell suspension.
PUCKS-EDTA	The cells were prepared as a single cell suspension in PUCKS-EDTA for 20 minutes, washed in HBSS supplemented with 5% FCS, and resuspended in basal media.

Percentage viability was determined for cells detached by each method. Differentiated SH-SY5Y cells detached with each of the three methods were re-seeded at 50,000 cells/cm² for twenty-four hours and fixed in 4% PFA for 30 minutes and stained with DAPI (section 2.2.3.7.i) and imaged under fluorescence microscopy (Olympus IX53). Neural adhesion was assessed by quantifying the DAPI-stained nuclei re-adhered to the wells under each condition using ImageJ software.

2.2.2.3 Adhesion assay to measure neural adhesion in response to Eph-Fc

Differentiated SH-SY5Y cells were liberated with TrypLE and re-adhered to pre-bound EphA3-Fc, EphA4-Fc, EphB2-Fc or EphB4-Fc molecules or human IgG-Fc control proteins for two and twenty-four hours, and neural adhesion was measured by quantifying the DAPI-stained nuclei re-adhered to the wells under each condition. The wells of ninety-six well flat-bottomed plates were coated with 0.01% Poly-L-lysine solution for 5 minutes at room temperature and washed twice with PBS. The Human-IgG-Fc, EphA3-Fc, EphA4-Fc, EphB2-

Fc or EphB4-Fc fusion proteins at 5 µg/mL were pre-clustered with 50 µg/mL of Human anti-Goat IgG clustering agent in PBS for 1 hour at room temperature. The pre-clustered Fc-fusion proteins were added to the pre-coated wells and left to adhere for 2 hours at 37°C in a 5% CO₂ incubator followed by one PBS wash. Differentiated SH-SY5Y cells were liberated with TrypLE on day 12 and re-seeded at 50,000 cells/cm² in Phase II neural-inductive media in the presence of the Fc molecules. After 2 and 24 hours, cells were fixed in 4% PFA for 30 minutes, and stained with Phalloidin and DAPI (section 2.2.3.7.i) and imaged under fluorescence microscopy (Olympus IX53). Neural adhesion was assessed by quantifying the DAPI-stained nuclei re-adhered to the wells under each condition using ImageJ software.

2.2.2.4 Adhesion assay to assess the effect of ephrinB1-mediated signalling between neurons and BMSC on neural adhesion

Green fluorescent protein (GFP) expressing human BMSC obtained from two donors at passage 4 were cultured in growth media in separate culture flasks for 4 days. BMSC from each of the flasks were detached with Trypsin and seeded at 20,000 cells/cm², the concentration determined to generate a confluent stromal monolayer, in growth media overnight. The next day, the neural-induced SH-SY5Y cells were liberated with TrypLE. The cells were stained with the Vybrant DiD-labelling dye (as outlined in section 2.2.3.7.v), and either left untreated as a control, or incubated with the ephrinB1 blocking peptide or scramble peptide (100 µM) in Phase II neural-inductive media in Eppendorf tubes at 37°C in 5% CO₂ for 45 minutes, with manual rotation every 5 minutes for adequate mixing. The growth media on the BMSC were aspirated, and fresh growth media of half the well volume was added to each well. Phase II neural-inductive media containing the neural-induced SH-SY5Y cells (50,000 cells/cm²) were added in half the well volume to each well and allowed to adhere for 24 hours. The cells were fixed in 10% neutral buffered formalin. The cells were imaged under confocal microscopy (TCS SP8 X) and neural adhesion was assessed by quantifying the DiD-labelled SH-SY5Y cells on the stromal monolayer under each condition using ImageJ software.

2.2.2.5 Transwell migration assay to measure neural migration in response to Eph-Fc

Firstly, 600 μL of Phase II neural-inductive media, either alone or with 100 ng/mL human CXCL12 was pipetted into the bottom chambers of 8 μm polycarbonate membrane transwell inserts. SH-SY5Y cells were liberated with TrypLE on day 12 of neural induction, spun down and resuspended in a single-cell suspension in 1 mL of Phase II neural-inductive media. SH-SY5Y cells were seeded into the upper chambers of the transwell inserts at 1×10^5 cells/insert or 2×10^5 cells/insert in 100 μL /insert of Phase II neural inductive media. The samples were incubated for 24 hours at 37°C in 5% CO_2 to allow migration. Following the 24-hour incubation, each insert was transferred to a new 24-well plate containing PBS. The media in the upper chamber of each transwell insert was aspirated. Cotton tips were used to gently remove a layer of cells from the upper surface of the insert membrane. The cells on the lower surface of the transwell inserts were fixed by placing the insert in 100% methanol for 15 minutes. The methanol solution was replaced with two washes with PBS. The transwell inserts were then placed in wells containing 5 $\mu\text{g/mL}$ DAPI to stain for 3 minutes and rinsed in two washes of PBS and imaged in PBS under fluorescence microscopy (Olympus IX53). The DAPI-stained nuclei were quantified per condition using ImageJ software. Alternatively, SH-SY5Y cells were seeded into the upper chambers at 1×10^5 cells/insert, while the Human-IgG-Fc, EphB2-Fc or EphB4-Fc fusion proteins (1, 5 and 10 $\mu\text{g/mL}$) were pre-clustered with Human anti-Goat IgG clustering agent (10, 50 and 100 $\mu\text{g/mL}$, respectively) for 1 hour at room temperature. These Fc molecules or CXCL12 (100 ng/mL) in neural-inductive media were added into the bottom chambers.

2.2.2.6 Culture-insert migration assay to assess the effect of ephrinB1-mediated signalling between neurons and BMSC on neural and stromal migration

Human GFP-positive BMSC obtained from three different donors at passage 4 were cultured in growth media for 4 days in separate culture flasks. Wells were coated with Poly-L-Lysine, washed once with PBS and allowed to dry. Nine 4-well silicone culture inserts, where each insert comprised of four defined cell-free gaps, was inserted into the respective wells of a 12-well plate. A scalpel blade was used to outline the inserts on the bottom of the plate. BMSC from each of the flasks were detached with Trypsin and seeded at 50,000 cells/ cm^2 in growth

media in the two opposite quadrants of the 4-well insert (Figure 2.1). In addition, SH-SY5Y cells at day 12 of neural differentiation were liberated with TrypLE, and either left untreated as a control, or incubated with ephrinB1 blocking peptide or the scramble peptide (100 μ M) in Phase II neural-inductive media for 45 minutes at 37°C in Eppendorf tubes. These samples were manually rotated every 5 minutes to ensure adequate mixing. The SH-SY5Y cells were seeded at 50,000 cells/cm² in Phase II neural-inductive media in the two opposite quadrants (Figure 2.1). The plate was incubated at 37°C overnight to allow the BMSC and the neural SH-SY5Y cells to adhere. The next day, the media within each well of the insert was removed. The insert was removed and 2 mL of the 1:1 growth media: Phase II neural-inductive media was added to each well of the 12-well plate. The plate was incubated at 37°C in 5% CO₂ to allow migration. Images were obtained immediately after insert removal and at time intervals for a maximum of 32 hours after insert removal. The distance travelled by the cells can be assessed in this assay because the distance defined for each of the four cell-free gaps created by the insert is 500 μ m. The number of GFP-positive stromal cells that invaded into the neural-containing quadrants, and the number of neural cells present with a defined area aligning the boundary of the neural-stromal quadrants (500 μ m by 1500 μ m) were quantified per condition (Figure 2.1) using ImageJ software.

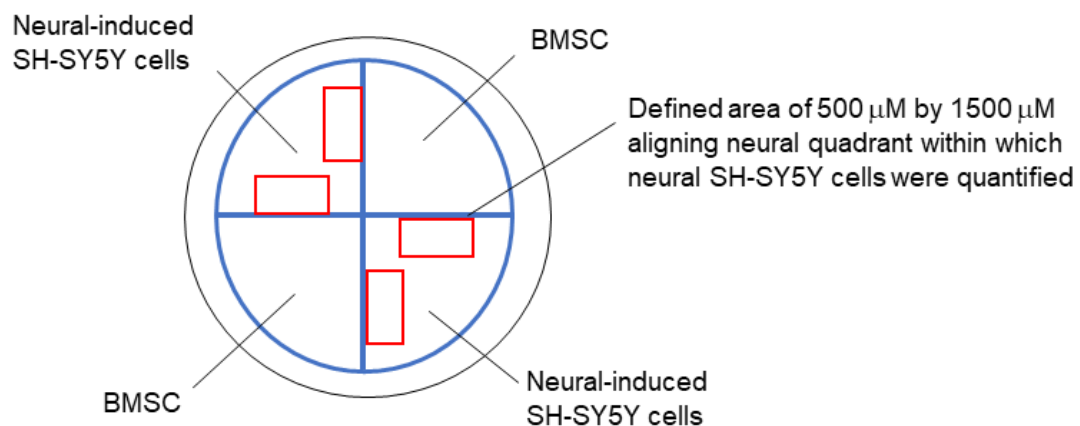


Figure 2.1: Timeline schematic for 4-well insert co-culture migration assay. Human BMSC were seeded in opposing quadrants, while neural-induced SH-SY5Y cells (untreated or pre-treated with the ephrinB1 blocking peptide or scramble peptide) were seeded in remaining quadrants. Inserts were removed, and images were obtained for a maximum of 32 hours to assess migration.

2.2.2.7 Dorsal root ganglion substratum assays

2.2.2.7.i DRG neurite cell body assay

The wells of 16-well glass chamber slides were coated with 0.01% Poly-L-Lysine solution for five minutes and then washed twice with PBS. Next, 5 µg/mL of Human-IgG-Fc, EphA3-Fc, EphA4-Fc, EphB2-Fc and EphB4-Fc were independently pre-clustered with 50 µg/mL Human anti-Goat IgG clustering agent in PBS for 1 hour at room temperature. The pre-clustered Fc fusion proteins coated the floor of each respective well and the chamber slides were incubated at 37°C for two hours. The wells were rinsed twice with PBS to remove any unbound protein. The coverslips were coated with 7 µg/mL of laminin in DMEM/F12 and incubated for 2 hours at 37°C. The unbound laminin was aspirated and each dish was replaced with fresh F12HS10 media (Table 2.8). Fertilised white leghorn chicken embryos grown at 37°C were harvested on embryonic day 7 (Ethics approval SAHMRI SAM126). The embryos were placed in warm F12HS10 media and decapitated. The embryonic tissues were removed. Under a stereo microscope, the organs were removed with fine forceps and the DRG pooled from both sides of the lumbar vertebral column or from the individual lumbar positions (L) 4-8 were isolated. The DRG explants were kept on ice, and individually aspirated and seeded in the middle of the Eph-Fc occupied wells. DRG explants were incubated at 37°C for 48 hours, imaged and fixed in 4% PFA for 30 minutes, and stained with anti-NF-M and DAPI (outlined in 2.2.3.7.vi). The DAPI-stained neurite cell bodies extending from the DRG body were quantified per DRG using ImageJ software.

2.2.2.7.ii DRG neurite invasion assay

The wells of the 16-well glass chamber slides were coated with Poly-L-Lysine, and the Fc molecules were pre-clustered as described in section 2.2.2.7.i. Anti-mouse IgG-FITC antibody was added to the pre-clustered Fc molecules at a final 20 µg/mL. The pre-clustered Fc along with the IgG-FITC was pipetted to form a small spot in the corner of each respective well. A permanent pen was used on the bottom of the well to outline the Eph-Fc occupied area of each well. Each dish was incubated at 37°C for two hours. The wells were rinsed twice with PBS to remove any unbound protein. The coverslips were coated with laminin and the DRG explants were isolated as described in section 2.2.2.7.i, kept on ice, individually aspirated and seeded

near the Eph-Fc occupied spots. Once the DRG were allowed to settle for 30 minutes at room temperature, they were incubated for 48 hours at 37°C. The DRG cultures were fixed in 4% PFA for 30 minutes and stained with Phalloidin (outlined in 2.2.3.7.i). The average lengths of the Phalloidin-stained axons extending from the Eph-Fc boundary inwards were quantified per DRG using ImageJ software.

2.2.3 Analysis

2.2.3.1 Total RNA isolation and quantification

At the conclusion of the assay, the media was aspirated and the cells were washed with ice-cold PBS. TRIzol (1 mL per $5\text{--}10 \times 10^6$ cells) was pipetted into the wells for 2 minutes to lyse RNA from cells. TRIzol was collected from replicate wells per condition into a cold Eppendorf tube, and stored at -20°C for further analysis by RT-PCR. These tubes containing TRIzol were thawed, and chloroform was added to this solution (200 μL per mL TRIzol) and the tubes were shaken vigorously for 15 seconds before incubation at room temperature for 3 minutes. The samples were centrifuged at 12,000 g for 15 min at 4°C, which resulted in phase separation. The upper aqueous phase was transferred to a new Eppendorf tube and total RNA was precipitated via the addition of isopropanol (500 μL per mL TRIzol) and 1-2 μL of glycogen. This preparation was incubated for 10 minutes at RT. The preparation was centrifuged at 12,000 g for 10 minutes at 4°C. The supernatant was aspirated and the pellet was washed in ice-cold 75% ethanol (500 μL per tube) and centrifuged at 7500 g for 5 minutes at 4°C. Residual ethanol was removed, and the RNA was air-dried for 10 min. The dried RNA was dissolved in RNase free water at RT for 10 min. The NanoDrop spectrophotometer was used to quantify and analyse the purity of the RNA samples. Samples were stored at -20°C or processed immediately to generate cDNA.

2.2.3.2 Complementary DNA (cDNA) synthesis

cDNA was generated using the SuperScript IV Reverse Transcriptase kit. To do this, 1 μg of total RNA was added to 0.2 mL tubes followed by 1 μL oligo(dT) (0.5 $\mu\text{g}/\text{mL}$), 1 μL random primers (0.1 $\mu\text{g}/\text{mL}$), 1 μL dNTP mix (10 mM) and RNase free water to reach a total volume

of 14 μL per tube. This mixture was heated to 65°C for 5 minutes and then incubated on ice for at least 1 minute. Next, a reaction mixture containing 5x first-strand buffer, 0.1M DTT and SuperScript IV reverse transcriptase (200 U / μL) was generated and 6 μL of this mixture was added per reaction tube. The samples were incubated at 23°C for 10 minutes followed by further incubation at 55°C for ten minutes, and finally, incubation at 80°C for 10 minutes. The newly synthesised cDNA at a volume of 20 μL was diluted to 100 μL with RNase free water and stored at -20°C to be used as a template in RT-PCR.

2.2.3.3 Real-time PCR (RT-PCR)

A reaction mixture containing 2 μL of sample cDNA, 7.5 μL of 2x SYBR green, 0.75 μL (10 μM) mixed primer pairs (Table 2.9) and 4.75 μL RNase free water was mixed to generate a 15 μL reaction for each PCR reaction/ tube in the Bio-Rad Real-Time PCR machine using the cyclic conditions outlined in Table 2.13. Gene expression was calculated using the delta-CT method, where threshold cycle (CT) values from each triplicate reaction were averaged and calibrated relative to control beta-actin CT values.

Table 2.13: PCR cycling parameters

Activation	Hold 15 minutes - 95°C 15 seconds - 95°C
Cycling (40 cycles)	25 seconds - 60°C 20 seconds - 72°C
Final extension	Hold 3 minutes - 72°C
Melt Curve	90 second pre melt, 5 second steps 1 degree per step, 72°C - 99°C

2.2.3.4 Protein isolation and quantification

The cells were washed once with 1 mL of ice-cold PBS, and 300 μL of modified RIPA lysis buffer (outlined in Table 2.8) was pipetted in to lyse protein from the cells. The adherent SH-SY5Y cells were liberated from the wells using a cell scraper. The cell lysates were transferred

to an Eppendorf tube which was kept on ice for 20 minutes and stored at -20°C for future protein analysis. The cell lysate was thawed and centrifuged for 30 minutes at 16,000 g at 4°C. The supernatant was transferred to a new tube from which an aliquot was taken. Protein concentration was quantitated using the Detergent Compatible Bradford Assay kit and the UV Spectrophotometer according to the manufacturer's instructions.

2.2.3.5 Western Blotting

Once protein concentrations were determined, 50 µg of protein per lane was loaded onto a 10% polyacrylamide gel (outlined in Table 2.8). Sodium dodecyl sulphate - Polyacrylamide gel electrophoresis (SDS-PAGE) was performed. The proteins were transferred to a 0.45 µm PVDF membrane via electrophoretic transfer. The polyacrylamide gel was placed in contact with the PVDF membrane inserted between two electrodes submerged in a conducting solution within a transfer tank. The transfer was run at 100 V for 1 hour at 4°C.

The membrane was blocked with 5 mL of blocking buffer (Table 2.8) for 1 hour at RT. The membrane was probed with the goat anti-ephrinA2, rabbit anti-ephrinA5 and goat anti-ephrinB1 primary antibodies (2 µg/mL) in blocking buffer overnight at 4°C on a rocker. The membrane was washed four times, 5 minutes per wash, in TBS-T. The membrane was probed with either 0.025 µg/mL of anti-Goat IgG DyLight 680 or 800, or 0.05 µg/mL of goat anti-rabbit DyLight 680, in blocking buffer for 1 hour at RT on a rocker. The membrane was washed a further four times with TBS-T with five minutes per wash. The membranes were imaged and analysed using Image Studio Lite software to check if EPHRINA2, EPHRINA5 and EPHRINB1 could be detected. The membranes were blocked with blocking buffer for 1 hour at RT and re-probed with 0.2 µg/mL of rat anti- α -tubulin antibody in blocking buffer overnight at 4°C on a rocker, washed in TBS-T and probed with 0.05 µg/mL Goat anti Rat IgG DyLight 680 in blocking buffer for 1 hour at RT, and re-analysed to check if the housekeeping α -TUBULIN protein could be detected.

2.2.3.6 Quantitative analysis of neuronal morphology in response to Eph-Fc

The bright field images obtained (Olympus IX53) on day 12 of the assay were quantitatively analysed using ImageJ software. The number of unipolar neurons, bipolar neurons and total neurite number per single neural cell was quantified per field of view, for four fields of view per well and triplicate wells per condition.

2.2.3.7 Staining

2.2.3.7.i Phalloidin and DAPI staining of cultured cells and DRG explants

Cells were permeabilized in 0.1% Tween-20 in PBS and stained with 1 µg/mL Phalloidin for two hours at RT in the dark, washed thrice with PBS and then stained with 5 µg/mL DAPI for 15 minutes at RT in the dark, and washed once with PBS and then viewed and imaged under fluorescence microscopy (Olympus IX53). The DRG explants were stained with 2.5 µg/mL Phalloidin and 5 µg/mL DAPI using the method previously described.

2.2.3.7.ii Immunocytochemistry on neural SH-SY5Y cells to test neural marker and ephrin expression

SH-SY5Y cells were fixed at the endpoint of the neural differentiation assay in 4% PFA for 30 minutes and washed thrice in PBS-T. Cells were blocked with blocking solution (10% horse serum and 5% normal serum that the primary antibody was raised in, either goat, rabbit or mouse and diluted in PBS-T), for 30 minutes at room temperature. Samples were incubated with Primary antibodies or Ig negative controls (concentrations outlined in Table 2.14) goat-anti-ephrinA2, goat-anti-ephrinB1, rabbit-anti-ephrinA5, mouse-anti-NFM, mouse-anti-β-III Tubulin, Goat IgG, Rabbit IgG, 1D4.5 in blocking solution overnight at 4°C. The samples were washed thrice with PBS-T and incubated with the secondary antibodies (rabbit anti-goat IgG biotin, goat anti-rabbit IgG biotin, goat anti-mouse biotin) in blocking solution for 2 hours at room temperature. Cells were washed thrice with PBS and incubated with tertiary antibody Streptavidin Alexa 488 and Phalloidin in blocking solution for 2 hours at room temperature in the dark (Table 2.14). Cells were washed thrice in PBS and incubated with DAPI (5 µg/mL)

for 15 minutes at room temperature, washed in PBS and imaged under fluorescence microscopy (Olympus IX53).

Table 2.14: Concentrations of antibodies used in immunocytochemistry to stain SH-SY5Y cells

Primary antibodies	Stock concentration	Final concentration
anti-ephrinA2	200 µg/mL	2.5 µg/mL
anti-ephrinA5	250 µg/mL	2.5 µg/mL
anti-ephrinB1	200 µg/mL	2.5 µg/mL
anti-Neurofilament-Medium chain	500 µg/mL	2.5 µg/mL
anti-β-III Tubulin (Tuj1)	1 mg/mL	4 µg/mL
Negative controls		
1D4.5	1 mg/mL	4 µg/mL
Rabbit IgG	5 mg/mL	2.5 µg/mL
Goat IgG	5 mg/mL	2.5 µg/mL
Conjugated		
TRITC-Phalloidin	500 µg/mL	1 µg/mL
Secondary		
Goat anti Mouse Biotin	500 µg/mL	2.5 µg/mL
Goat anti Rabbit Biotin	1.5 mg/mL	5 µg/mL
Rabbit anti Goat Biotin	1.5 mg/mL	3 µg/mL
Tertiary antibodies		
Streptavidin Alexa 488	1 mg/mL	4 µg/mL

2.2.3.7.iii Testing ephrin expression on neural SH-SY5Y cells by flow cytometry

The SH-SY5Y cells were liberated with TrypLE at the endpoint of the neural differentiation assay and washed in HBSS supplemented with 5% FCS. The media was aspirated and the cell pellet was washed once in 1 mL PBS. The samples were centrifuged (1400 rpm), the media was aspirated and the cells were washed once in 1 mL of HBSS supplemented with 5% FCS. The cells were blocked for one hour on ice in blocking buffer (Table 2.8), 50,000 cells were transferred per flow stain to each of the flow tubes. The cells were washed twice in HBSS supplemented with 5% FCS. The primary antibodies or IgG controls (anti-ephrinA2, anti-ephrinA5, anti-ephrinB1, Rabbit IgG, Goat IgG) were added to the cells (10 µg/mL) in blocking buffer and incubated on ice for 1 hour. Cells were washed twice with 1 mL of HBSS supplemented with 5% FCS and incubated with the secondary antibodies (anti-goat IgG FITC and anti-rabbit IgG FITC, 10 µg/mL in blocking buffer) on ice for 30 minutes and washed twice with 1 mL of HBSS supplemented with 5% FCS. The cells were fixed with FACS FIX (500 µL, Table 2.8). Cell surface expression of the ephrinA2, ephrinA5 and ephrinB1 was assessed by flow cytometry analysis of the stained cells and the corresponding negative controls (BD FACS-CANTO). Viable, single cells were gated based on the characteristics of forward and side scatter. FITC expression was assessed within this gated population and the percentage of neural SH-SY5Y cells that expressed FITC were quantified for the experimental and control cell populations.

2.2.3.7.iv Immunohistochemistry to stain fracture sections and avian embryo sections for ephrin and neural markers

A previous study generated femoral fractures in eight-week old mice with ethics approval (SA Pathology Animal Ethics committee #56/09) (Arthur et al. 2013). The fractured femora that were harvested at 2 weeks post fracture from wildtype mice were previously embedded. In the current study, serial sections were obtained from the paraffin-embedded samples, and the sections were mounted on silane-coated slides and dried until the wax melted. The sections were dewaxed and rinsed in absolute alcohol and blocked with 0.5% hydrogen peroxide in methanol at room temperature for 30 minutes. The sections were rinsed twice in PBS and

heated in citrate retrieval solution (Table 2.8) in the microwave oven for 10 minutes until the solution started to boil. The sections were removed and allowed to cool below 50 °C for 30 minutes and were rinsed twice in PBS. The tissue section on each slide was circled with a PAP pen and incubated in 3% NHS for 30 minutes. The NHS was drained, and the sections were incubated with the primary antibody (goat anti-ephrinA2, goat anti-ephrinB1, rabbit anti-ephrinA5, mouse anti-NF-H, concentrations outlined in Table 2.15) at room temperature overnight. The sections were rinsed twice in PBS and incubated with the secondary antibody (anti-goat, anti-rabbit and anti-mouse, concentrations outlined in Table 2.15) for 30 minutes, and then rinsed twice in PBS for 5 minutes each. The sections were incubated with streptavidin peroxidase (2 µg/mL) for 15 minutes, rinsed in PBS, and peroxidase substrate solution (Table 2.8) was applied for 7 minutes. The sections were rinsed in running tap water and counterstained with Mayer's haematoxylin. The sections were dehydrated, cleared, mounted and imaged.

Fertilised white leghorn chicken embryos grown at 37°C were harvested on embryonic day 10 (stage 35) (Ethics approval SAHMRI SAM126), decapitated, and fixed in 10% neutral buffered formalin, decalcified in 10% EDTA and embedded in paraffin. Serial transverse sections through the spinal cord and DRG at the level of the limb were obtained from the paraffin-embedded samples, and stained with goat anti-ephrinA2, goat anti-ephrinB1, rabbit anti-ephrinA5, mouse anti-NFH and mouse anti-NFM (concentrations outlined in Table 2.15) using the method previously described.

Table 2.15: Concentrations of antibodies used in immunohistochemistry to stain fracture and avian embryo sections

Primary antibodies	Stock concentrations	Final concentration
anti-ephrinA2	200 µg/mL	1 µg/mL
anti-ephrinA5	250 µg/mL	5 µg/mL
anti-ephrinB1	200 µg/mL	2.67 µg/mL
anti-Neurofilament-Heavy chain	N/A	1 : 100,000
anti-Neurofilament-Medium chain	500 µg/mL	0.25 µg/mL
Secondary		
Horse anti Mouse Biotin	500 µg/mL	2 µg/mL
Goat anti Rabbit Biotin	1.5 mg/mL	6 µg/mL
Rabbit anti Goat Biotin	1.5 mg/mL	6 µg/mL
Tertiary antibodies		
Streptavidin peroxidase	1 mg/mL	2.5 µg/mL

2.2.3.7.v DiD labelling of neural SH-SY5Y cells for confocal imaging and flow cytometry

Neural-induced SH-SY5Y cells were liberated with TrypLE on day 12 and resuspended into a single-cell suspension in HBSS supplemented with 5% FCS. This cell suspension was centrifuged at 1400 rpm for five minutes, media was aspirated, and the cell pellet was resuspended in 10 mL of PBS. The cells were centrifuged at 1400 rpm for five minutes, media was aspirated, and the cell pellet was resuspended in 1 mL of PBS. 1.6×10^6 cells were transferred into a separate flow cytometry tube to set up the DiD-negative gate and 100 µL of PFE solution (Table 2.8) was added. Cells (1.6×10^6 cells/ tube) were transferred into a 12 mL polypropylene tube containing PBS (1×10^6 cells per mL of PBS). Vybrant DiD cell-labelling solution (Table 2.1) was added directly to this cell suspension (5 µL of supplied solution was added per 1 mL of cell suspension) and the tube was mixed and incubated at 37°C for 20

minutes. PBS was added to achieve a final volume of 10 mL, the suspension was centrifuged at 1400 rpm for 5 minutes, and the PBS was gently aspirated leaving a blue cell pellet which was resuspended in 1 mL PBS. Next, 100 μ L of the cells were transferred to a flow cytometry tube and the cells were washed in 3 mL PBS. The PBS was aspirated and the flow cytometry tubes were vortexed, resuspending the stained cells. 100 μ L of PFE solution was added to the cells. The cells were analysed by flow cytometry (BD LSRFortessa X-20) to assess cell surface expression of the DiD fluorophore. The percentage of neural SH-SY5Y cells that expressed the DiD fluorophore was quantified for the experimental and control cell populations. The remaining cells were seeded into the wells of a 24-well plate for confocal imaging using the following method. 9 mL of Phase II neural-inductive media was added to resuspend the cells. This suspension was centrifuged at 1400 rpm for 5 minutes, the media was aspirated, and the cell pellet was resuspended in the remaining 1 mL of Phase II neural-inductive media. The stained cells were seeded at 50,000 cells/cm² for two hours and imaged under confocal microscopy (TCS SP8 X) and the images were analysed using ImageJ software.

2.2.3.7.vi Staining of DRG explants - DRG neurite cell body assay

The DRG explants were permeabilised in 0.1% Triton-X for 15 minutes, incubated with blocking solution (5% goat serum in PBS) for one hour at RT and incubated with the primary antibody or IgG control (anti-NFM, 1D4.5) at 2.5 μ g/mL in blocking solution overnight at 4°C. Samples were washed thrice in PBS and incubated with goat anti-mouse biotin antibody (3 μ g/mL) in blocking solution for two hours at RT. The cultures were washed thrice in PBS and incubated with 4 μ g/mL Streptavidin Alexa 488 and 2.5 μ g/mL of Phalloidin for two hours at RT in the dark, washed thrice in PBS and then viewed and imaged under fluorescence microscopy (Olympus IX53).

2.2.3.8 Statistical analysis

Statistical analysis was performed using GraphPad Prism version 9.0.0 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com”.

Chapter 3 Investigating the role of Eph/ephrin interactions on neural differentiation

3.1 Introduction

Contact dependent Eph/ephrin interactions contribute to skeletal homeostasis and repair by influencing the adhesion, migration and differentiation capacity of osteogenic and osteoclastic populations (Xing et al., 2010, Takyar et al., 2013, Zhao et al., 2006, Irie et al., 2009, Arthur et al., 2011, Arthur et al., 2013, Nguyen et al., 2016, Arthur et al., 2018). In addition, these molecules are involved in neural development, maintenance and regeneration (Eberhart, 2004, Klein, 2004, Jayasena et al., 2005, Helmbacher et al., 2000, Luxey et al., 2013, Munoz et al., 2005, Winning and Krull, 2011). As the Eph/ephrin molecules have known roles in independently regulating the skeletal and nervous systems, the Eph/ephrin interactions may mediate cross-communication between the neural and bone/stromal cells.

There is evidence of Eph/ ephrin interactions regulating neural differentiation, however different members within the family have contrasting roles. Within the A subclass, EphA4 expressed by the neural stem cells (NSC) acting through forward signalling, has been reported to maintain postnatal and adult NSC within adult neurogenic niches in an undifferentiated state (Khodosevich et al., 2011). In contrast, EphA4 forward signalling has a functional role in neurogenesis (Chen et al., 2020). The interaction of the EphA4 receptor with platelet-derived growth factor receptor β (PDGFR β) promoted neuronal differentiation of neural progenitor cells *in vitro*, which was inhibited by the dominant-negative EphA4 mutant which lacked the juxtamembrane and kinase domains (Chen et al., 2020). Similarly, ephrinA2 has been reported to repress cell proliferation and promote excitatory neuron differentiation in neural progenitors within the developing neocortex. Where this response was suppressed by the shRNA-induced knockdown of ephrinA2 (Homman-Ludiye et al., 2017). Collectively, these studies suggest roles for EphA4, ephrinA2 and ephrinA5 in regulating neural differentiation, where EphA forward and ephrinA reverse signalling pathways are mediated by various signal molecules such as the corresponding Eph/ephrin molecules or PDGF.

In addition, mice carrying a global null mutation in *EphrinA5* displayed impaired neural differentiation of neural progenitors (Hara et al., 2010). The astrocyte-expressed ephrinA5 was reported to communicate with EphA receptors to mediate interactions between NSC and blood vessels, where angiogenesis regulates neurogenesis (Hara et al., 2010). The gaps in knowledge surround the members of the EphA family involved and the mechanism by which the ephrinA5-expressing astrocytes facilitate an interaction between the NSC and blood vessels to regulate neurogenesis (Hara et al., 2010). Furthermore, the other factors involved in NSC-endothelial communication, the contribution of this signalling to neurogenesis, and the nature of the link between neurogenesis and angiogenesis is still unclear. However, these findings demonstrate that Eph/ephrin interactions mediate cross-communication between different cellular populations (astrocytes and vascular cells) which supports the hypothesis of this thesis that the Eph/ephrin interactions may mediate cross-communication between the neural and stromal populations.

Within the B subclass of the Eph/ ephrin family members, EphB2 forward signalling promotes a neuronal fate in adult subventricular neural precursors which was proposed to occur via the regulation of downstream factors such as Notch1 and Zic1 (Katakowski et al., 2005). Additionally, forward EphB4-ephrinB2 signalling suppresses neural differentiation of dental pulp stem cells (DPSC), and this effect was reversed by suppressing EphB4 forward signalling by using an EphB4-specific peptide inhibitor (Heng et al., 2018). EphB4 also regulates neuronal differentiation of human embryonic NSC *in vitro* which is hypothesised to be achieved via its effects on downstream signalling pathways such as Notch signalling (Liu et al., 2017). Moreover, the addition of ephrinB1 in combination with the factors CXCL12, pleiotrophin and insulin-like growth factor 2 promoted the dopaminergic neuron differentiation of human embryonic stem cells (hESC) *in vitro*. The limitations of this study were that the molecular mechanism by which ephrinB1-mediated signalling occurred was not determined. However, it was reported that the EphB2 and EphB4 receptors known to bind to ephrinB1 with high affinity were detected in undifferentiated hESC, and *EPHB2* expression increased with the progression of dopaminergic differentiation (Vazin et al., 2009). In contrast, other studies have shown that ephrinB1 expression in neural progenitors within the developing neocortex is necessary to maintain the progenitor fate *in vivo*, where the targeted knock down of *Ephrinb1*

in murine cortical neural progenitors induced neuronal differentiation (Qiu et al., 2008). This ephrinB1-reverse signalling was proposed to regulate the downstream PDZ-RGS3 signalling pathway. However, there are gaps in knowledge concerning which of the EphB receptors are involved in activating the ephrin-RGS pathway and the mechanisms of action (Qiu et al., 2008). Collectively, these findings suggest contrasting roles for EphB and ephrinB signalling in regulating neural differentiation which may depend on the cell type, species, and type of neural differentiation. There are limitations in our knowledge about the nature of the signalling pathways that are activated downstream upon the activation of EphB forward and ephrinB reverse signalling pathways. It is unclear whether this signalling is mediated by neural-neural interactions or interactions between neurons and a different cell population, where the contributions of these interactions during neural differentiation are yet to be determined.

The Eph/ephrin molecules have known roles in independently regulating the stromal/skeletal populations during skeletal development, homeostasis and repair, and the neural cells during neural development, maintenance, regeneration and repair. Therefore, the Eph/ephrin interactions may mediate cross-talk between the neural and bone/stromal populations via the formation of a neuro-osteogenic network to regulate neural differentiation. This chapter investigates *in vitro* the role of Eph/ephrin interactions in mediating cross-communication between BMSC and neural cells to regulate neural differentiation using the human neuroblastoma cell-line SH-SY5Y. The SH-SY5Y cells are a subclone of the SK-N-SH cell line which was originally established from a bone marrow biopsy obtained from a four year old patient with neuroblastoma (Biedler et al., 1973). This cell line is a suitable *in vitro* model for studying neural differentiation as several neural differentiation protocols have been established in the literature for this cell line which has been utilised as a suitable neuronal cell culture model in neuroscience research into disorders such as Parkinson's disease (Encinas et al., 2000, Constantinescu et al., 2007, Forster et al., 2016, Shipley et al., 2016). It should be noted that Eph/ephrin signalling has been studied for its role in regulating neural differentiation of the SH-SY5Y line, where increased expression levels of ephrinA3 with decreased expression levels of ephrinA5 contributed to dopaminergic differentiation of human SH-SY5Y cells (Wang et al., 2016).

3.2 Results

3.2.1 Differential neural marker expression profile following neural induction

The neural differentiation assay that was used for the SH-SY5Y line was modified from a protocol previously described by Forster and colleagues (section 2.2.2.1) (Forster et al., 2016). Bright field images were obtained on days 0, 3, 6, 9 and 12 of the neural differentiation assay under basal conditions and neural-inductive conditions (Figure 3.1). There was a distinct difference in the morphology of the SH-SY5Y cells under neural-inductive conditions compared to the cells under basal conditions, and this difference was seen from day 3 until the endpoint of the neural differentiation assay on day 12 (Figure 3.1). Under neural-inductive conditions, the cells displayed bipolar neuron-like morphology and long axonal processes. This morphology was more pronounced as neural differentiation progressed, with clusters of neuronal cells that have formed larger neuronal networks with nearby neuronal cells via the formation and extension of neurite processes. Under basal conditions, the SH-SY5Y cells did not display the described neural morphologies rather the cells appeared rounded and flat.

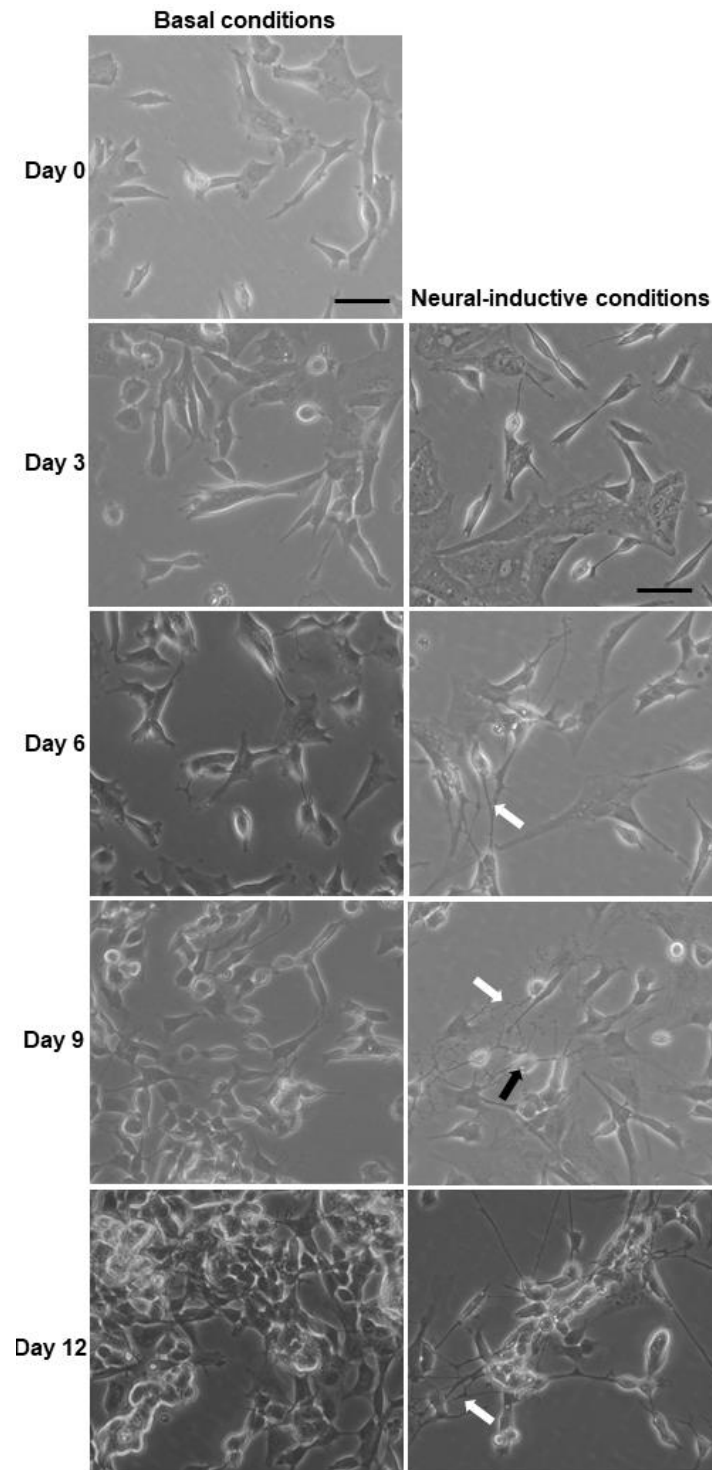


Figure 3.1: Neuronal morphology of the SH-SY5Y cells following neural induction. Bright field images obtained on days 0, 3, 6, 9 and 12 of the neural differentiation assay where SH-SY5Y cells were cultured under basal conditions or neural-inductive conditions ($n = 3$ wells per culture condition with bright field images obtained across 4 fields of view per well). Neurite processes (white arrows). Bipolar neuron (black arrow). Scale bar = 100 μm .

The neurofilament proteins are class IV intermediate filament proteins, comprised of neurofilament light (NF-L), neurofilament medium (NF-M), neurofilament heavy (NF-H) and α -internexin which are expressed as the neuron matures. Nestin is a class VI intermediate filament protein that is present primarily within developing neurons (Yuan et al., 2017). During neuron maturation, Nestin filaments are gradually replaced by Class IV subunits which includes NF-L, NF-M and NF-H eventually forming a highly stable axonal NF cytoskeleton (Nixon and Shea, 1992). In addition, β -III Tubulin is a microtubule element of the tubulin family found almost exclusively in neurons and in testis cells (De Gendt et al., 2011). Dorsal root ganglia homeobox protein (DRG11) is a transcription factor that is expressed in the CNS neurons in the dorsal horn of the spinal cord and in the PNS sensory neurons, and expression of this gene is consistent with neurons of a sensory-like disposition (Rebelo et al., 2007). The gene expression levels of the neural markers *NESTIN*, *β -III-TUBULIN*, *NF-M* and *DRG11* were measured on days 0, 3, 6, 9 and 12 of the neural differentiation assay under basal conditions and neural-inductive conditions (section 2.2.3.3) (Figure 3.2).

Overall, there was an increase in the expression of *NESTIN* from day 0 to 12 following neural induction (Figure 3.2A). The *NESTIN* expression was significantly higher under neural-inductive conditions compared to basal conditions by days 3, 9 and 12 of the neural differentiation assay, yet *NESTIN* expression was significantly less under neural-inductive conditions compared to basal conditions on day 6 (Figure 3.2A). The absolute expression levels were still low at each time point, which is consistent with the finding that *NESTIN* is an early neuronal marker which is mainly expressed by developing neurons (Yuan et al., 2017). Additionally, there was an increase in *β -III TUBULIN* expression following neural induction (Figure 3.2B). *β -III TUBULIN* expression was significantly higher under neural-inductive conditions than basal conditions on days 6, 9 and 12 of the neural differentiation assay (Figure 3.2B). Consistent with a role as a late neuronal marker, overall, the expression of *NF-M* showed an increase from days 0 to 12 under neural-inductive conditions (Figure 3.2C). The expression of *NF-M* was significantly higher under neural-inductive conditions compared to basal conditions on days 3, 6, 9 and 12 (Figure 3.2C). Overall, the expression of the sensory marker *DRG11* showed an increase from days 0 to 12 under neural-inductive conditions (Figure 3.2D).

Furthermore, expression of *DRG11* was significantly higher under neural-inductive conditions compared to basal conditions on days 9 and 12 (Figure 3.2D) suggesting these neural-inductive conditions may induce neurons towards a more sensory disposition.

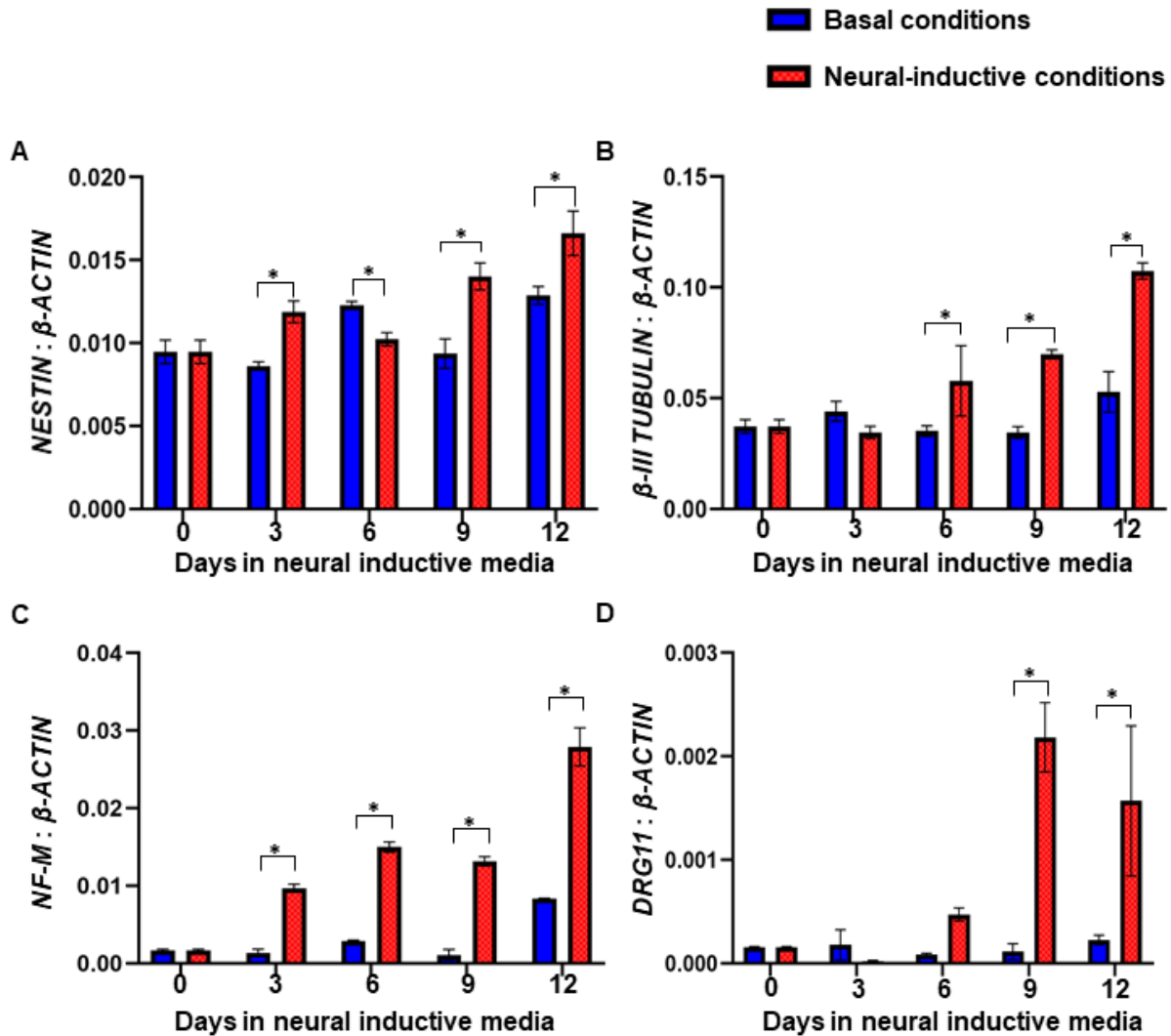


Figure 3.2: Neural genes are upregulated following neural induction. The gene expression levels of the genes (A) *NESTIN* (B) *β -III TUBULIN* (C) *NF-M* and (D) *DRG11* were characterised in the SH-SY5Y line on days 0, 3, 6, 9 and 12 under basal and neural-inductive conditions by RT-PCR analysis. Data represents mean $\pm$ SD ($n=3$ wells of a PCR from 3 pooled wells per culture condition). Statistical analysis: Ordinary two-way ANOVA with Sidak's multiple comparisons between basal conditions and neural-inductive conditions for each time point, $*p < 0.05$.

3.2.2 Differential Eph/ephrin expression profile following neural induction

The gene expression levels of the A and B subclass Eph receptors (*EPHA1-8*, *EPHB1-4*, *EPHB6*) and ephrin ligands (*EPHRINA1-5*; *EPHRINB1-3*) were characterised under basal and neural inductive conditions on days 0, 3, 6, 9 and 12 of the neural differentiation assay by RT-PCR analysis (Figures 3.3 to 3.6). The statistical analyses conducted on these data sets was ordinary two-way ANOVA with Sidak's multiple comparisons between basal conditions and neural-inductive conditions for each time point, * $p < 0.05$.

3.2.2.1 EphA gene expression profile following neural induction

The EphA receptors were differentially expressed during neural differentiation. It was observed that *EPHA1* was only weakly detected on day 3, and only under neural-inductive conditions (Figure 3.3A). The expression of *EPHA2* was significantly higher under neural-inductive conditions compared to basal conditions on days 3 and 12. *EPHA2* was not detected under either condition on day 6. On day 9, *EPHA2* expression was significantly higher under basal conditions than neural-inductive conditions (Figure 3.3B). It was found that *EPHA2* displayed the highest level of expression within the EphA subclass. Furthermore, *EPHA3* expression by the SH-SY5Y cells was not detected under either neural inductive or basal conditions at any of the time points during the neural differentiation assay suggesting the cell line may not express EphA3 (Figure 3.3C). It is important to note that the primer sets against EphA1-8 and ephrinA1-5 that were used in these RT-PCR experiments, where low or no expression was seen for some molecules, have been previously used on the hematopoietic lineages and these results have been published, and the efficiency of these primers have been confirmed (Nguyen et al., 2017). In addition, *EPHA4* expression was significantly higher under basal conditions compared to neural inductive conditions on day 3, although this expression decreased again by days 9 and 12 under both conditions (Figure 3.3D). The absolute expression of this receptor remained extremely low at every time point under both conditions.

EPHA5 expression showed a significant upregulation under basal conditions, compared to neural inductive conditions on days 3 and 9 (Figure 3.3E). However, the absolute expression of this receptor was low at every time point under both conditions. There was a significant increase in the expression of *EPHA6* under neural inductive conditions compared to basal conditions on days 6, 9 and 12 (Figure 3.3F). Overall, there was an increase in *EPHA6* expression on days 6, 9 and 12 under neural-inductive conditions when compared to basal conditions at the corresponding time point (Figure 3.3F). *EPHA7* expression was significantly higher under basal conditions compared to neural inductive conditions on day 9 while a significant increase in *EPHA7* expression under neural-inductive conditions was observed on day 12 (Figure 3.3G). A significant upregulation in *EPHA8* expression was observed at day 9 under neural-inductive conditions compared to basal conditions (Figure 3.3H).

In summary, *EPHA2*, *EPHA6* and *EPHA7* were the most differentially expressed *EPHA* receptors at the endpoint of the neural differentiation assay, with significantly increased expression under neural inductive conditions compared to basal conditions by day 12. *EPHA2* was the highest expressed receptor following neural induction.

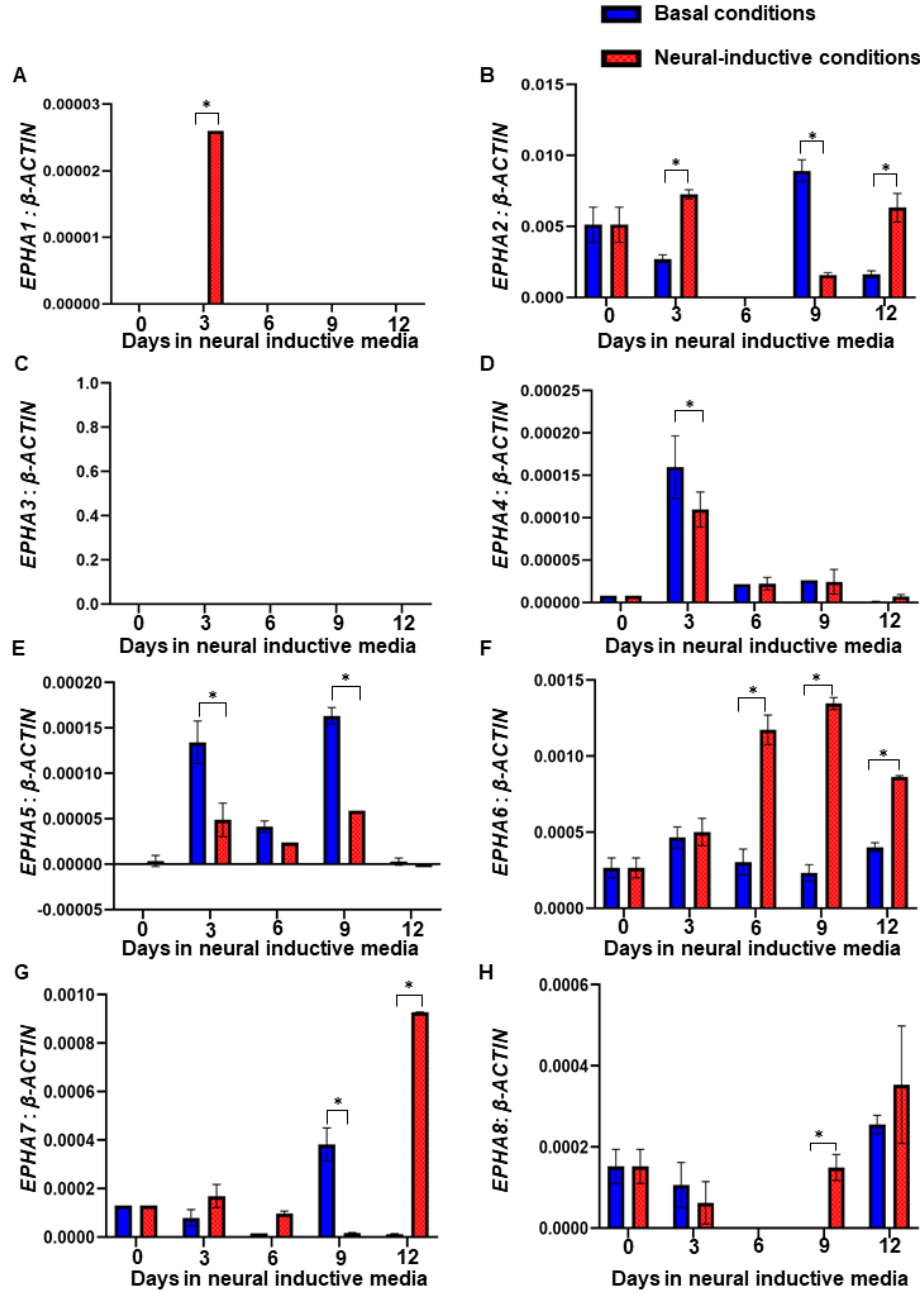


Figure 3.3: The characterisation of the EphA receptor gene expression profile following neural induction of SH-SY5Y cells. RT-PCR analysis of (A) *EPHA1*, (B) *EPHA2*, (C) *EPHA3*, (D) *EPHA4*, (E) *EPHA5*, (F) *EPHA6*, (G) *EPHA7* and (H) *EPHA8*. *EPHA* gene expression profile by SH-SY5Y cells was characterized on days 0, 3, 6, 9 and 12 of the neural differentiation assay under basal and neural-inductive conditions by RT-PCR analysis. Data represents mean $\pm$ SD (n=3 wells of a PCR from n = 3 pooled wells per culture condition). Statistical analysis: Ordinary two-way ANOVA with Sidak's multiple comparisons between basal conditions and neural-inductive conditions for each time point, *p < 0.05.

3.2.2.2 EphB gene expression profile following neural induction

The *EPHB1* and *EPHB6* receptors were differentially expressed during neural differentiation of the SH-SY5Y cells (Figure 3.4A, E). Overall, there was an increase in gene expression under neural-inductive conditions compared to basal conditions although the absolute expression was relatively low in comparison to the expression levels of the other EphB receptors. By days 3 and 9, the gene expression levels of *EPHB1* and *B6* were significantly higher under basal conditions compared to neural-inductive conditions. Conversely, by days 6 and 12, the gene expression levels for *EPHB1* and *B6* were significantly higher under neural-inductive conditions compared to basal conditions. It was observed that the expression of *EPHB2* was significantly higher under neural-inductive conditions compared to basal conditions by days 3 and 6 (Figure 3.4B). However, *EPHB2* expression significantly increased by days 9 and 12 under basal conditions compared to neural-inductive conditions. A significant increase in the expression of the *EPHB3* receptor under basal conditions compared to neural-inductive conditions on days 3 and 12 was shown (Figure 3.4C). However, by day 9, *EPHB3* expression was significantly higher under neural-inductive conditions than basal conditions. It was found that *EPHB4* expression was significantly upregulated under basal conditions on days 3 and 12, while expression was significantly lower under neural-inductive conditions compared to basal conditions by day 9 (Figure 3.4D). Interestingly, the expression levels between *EPHB2* and *EPHB4* were comparable except under basal conditions at days 3 and 9 for EphB4 (Figure 3.4B, D).

In summary, the *EPHB1* and *EPHB6* receptors showed significantly higher gene expression levels under neural-inductive conditions compared to basal conditions. The gene expression levels of the *EPHB2*, *EPHB3* and *EPHB4* receptors were significantly lower under neural-inductive conditions compared to basal conditions on day 12, and on day 3 for *EPHB3* and *EPHB4*. Day 3 corresponds to the start of Phase I neural differentiation where a significant increase in *NF-M* expression was observed (Figure 3.2C); while day 12 corresponds to the endpoint of Phase II neural differentiation where significant upregulation in both *NF-M* and *B-III TUBULIN* expression was observed (Figure 3.2B, 3.3).

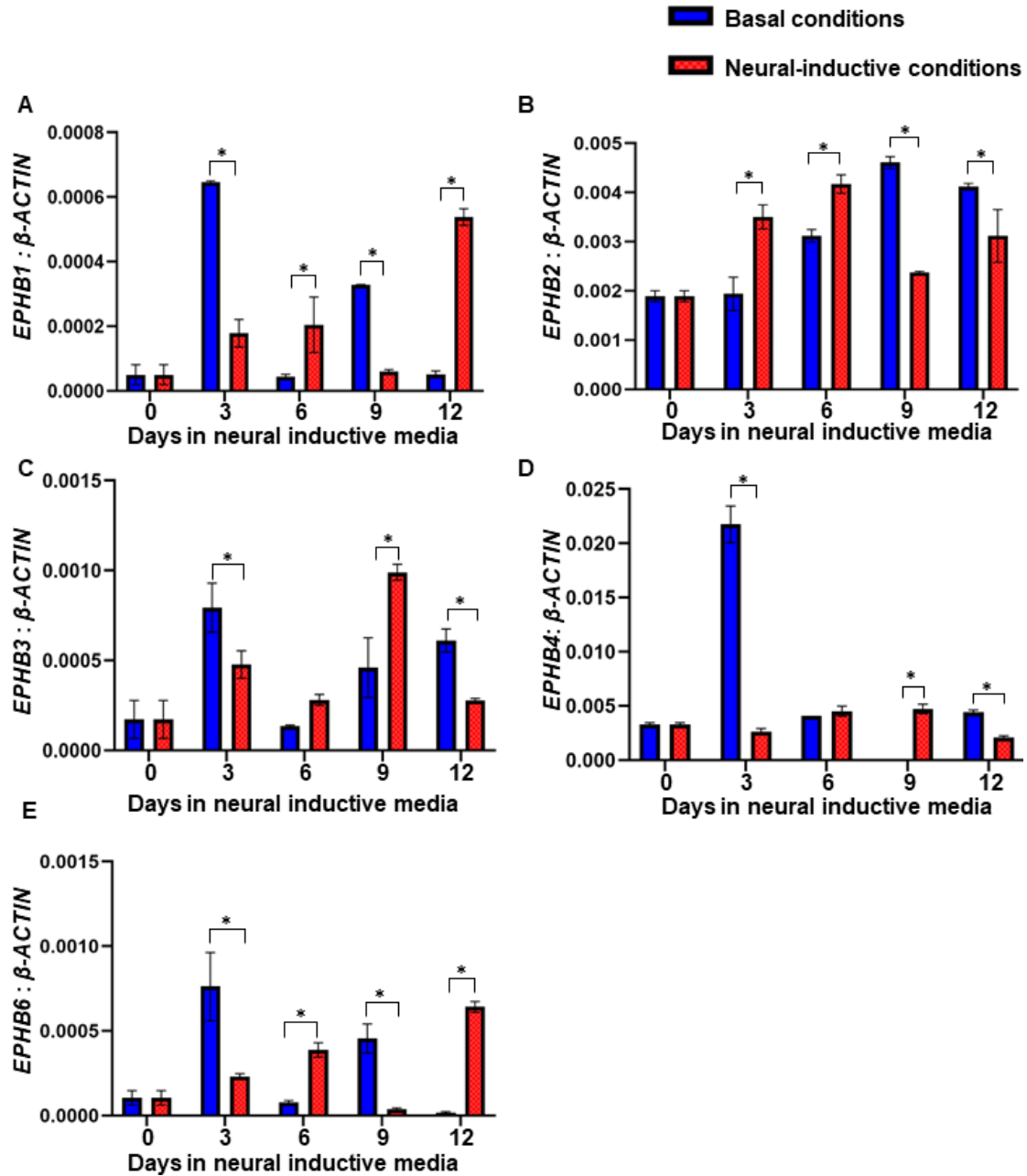


Figure 3.4: The characterisation of the EphB receptor gene expression profile following neural induction of SH-SY5Y cells. RT-PCR analysis of (A) *EPHB1*, (B) *EPHB2*, (C) *EPHB3* (D) *EPHB4* and (E) *EPHB6*. EphB gene expression profile by SH-SY5Y cells was characterized on days 0, 3, 6, 9 and 12 of the neural differentiation assay under basal and neural-inductive conditions by RT-PCR analysis. Data represents mean $\pm$ SD (n=3 wells of a PCR from n=3 pooled wells per culture condition). Statistical analysis: Ordinary two-way ANOVA with Sidak's multiple comparisons between basal conditions and neural-inductive conditions for each time point, *p < 0.05.

3.2.2.3 ephrinA gene expression profile following neural induction

The expression of *EPHRINA1* was significantly higher under basal conditions compared to neural-inductive conditions on day 3, with no significant difference in expression observed at the other time points (Figure 3.5A). While a significant increase in *EPHRINA2* expression was observed under basal conditions on day 6, overall, there was increase in the expression of *EPHRINA2* following neural-induction, with a significant upregulation in *EPHRINA2* expression under neural-inductive conditions on day 12 (Figure 3.5B). However, the expression of *EPHRINA2* was relatively low in comparison to the other *EPHRINA* molecules. A significant increase in the expression of *EPHRINA3* was observed under neural inductive conditions compared to basal conditions on days 3 and 9 (Figure 3.5C). It was observed that the *EPHRINA4* expression remained quite low under both conditions, where a significant increase in expression was observed on day 12 under basal conditions compared to neural-inductive conditions (Figure 3.5D). Conversely, an increase in *EPHRINA5* expression following neural induction was demonstrated on days 9 and 12, while the expression of *EPHRINA5* was significantly lower on day 3, under neural inductive conditions compared to basal conditions (Figure 3.5E). However, the expression of this ligand was upregulated significantly under neural-inductive conditions compared to basal conditions on days 9 and 12. *EPHRINA5* was the highest expressed *EPHRINA* ligand (Figure 3.5E).

In summary, differential expression of the *EPHRINA2*, *EPHRINA4* and *EPHRINA5* ligands was observed at the endpoint of the neural differentiation assay where the relative expression levels of *EPHRINA5* was higher than the other molecules while the expression level of *EPHRINA4* was the lowest (Figure 3.5D, E). Specifically, the expression of *EPHRINA2* and *EPHRINA5* was significantly higher under neural-inductive conditions compared to basal conditions.

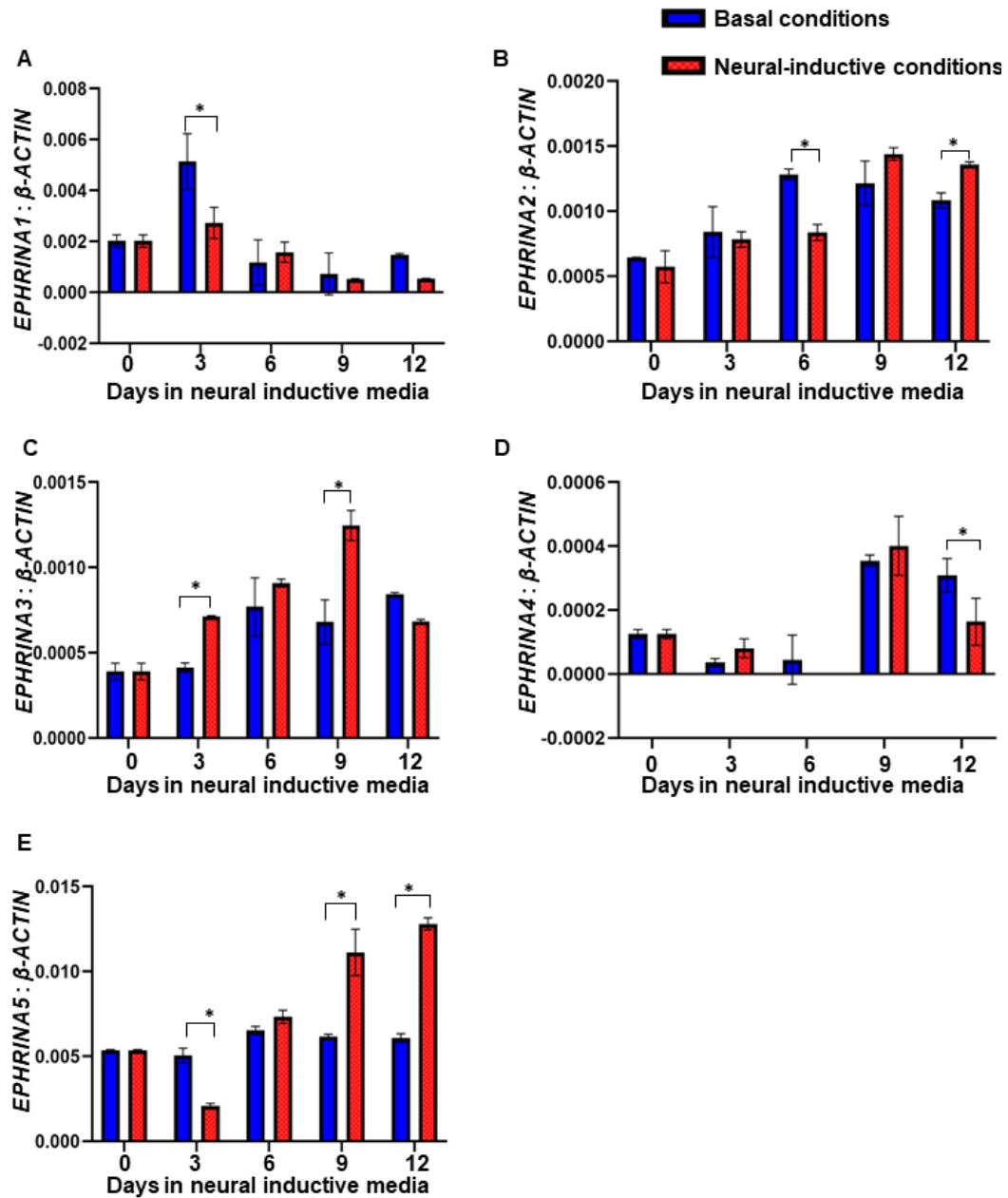


Figure 3.5: The characterisation of the ephrinA ligand gene expression profile following neural induction of SH-SY5Y cells. RT-PCR analysis of (A) *EPHRINA1*, (B) *EPHRINA2*, (C) *EPHRINA3*, (D) *EPHRINA4*, (E) *EPHRINA5*. EPHRINA gene expression profile by SH-SY5Y cells was characterized on days 0, 3, 6, 9 and 12 of the neural differentiation assay under basal and neural-inductive conditions by RT-PCR analysis. Data represents mean $\pm$ SD (n=3 wells of a PCR from n=3 pooled wells per culture condition). Statistical analysis: Ordinary two-way ANOVA with Sidak's multiple comparisons between basal conditions and neural-inductive conditions for each time point, * $p < 0.05$.

3.2.2.4 ephrinB gene expression profile following neural induction

EPHRINB1 expression was significantly downregulated under neural inductive conditions compared to basal conditions by day 3. However, *EPHRINB1* expression was significantly higher under neural-inductive conditions compared to basal conditions on days 9 and 12 (Figure 3.6A). Overall, *EPHRINB2* expression increased following neural induction, this increase was observed on days 3, 6 and 9, while no significant difference in expression was observed on day 12 (Figure 3.6B). The expression of *EPHRINB3* remained extremely low under neural induction, where *EPHRINB3* expression was significantly higher under basal conditions compared to neural-inductive conditions on days 3, 6 and 9 (Figure 3.6C). In summary, only *EPHRINB1* showed differential expression at the endpoint of the neural differentiation assay, where expression was significantly higher under neural-inductive conditions compared to basal conditions. However, it was observed that *EPHRINB2* was expressed at almost twice the level of *EPHRINB1*.

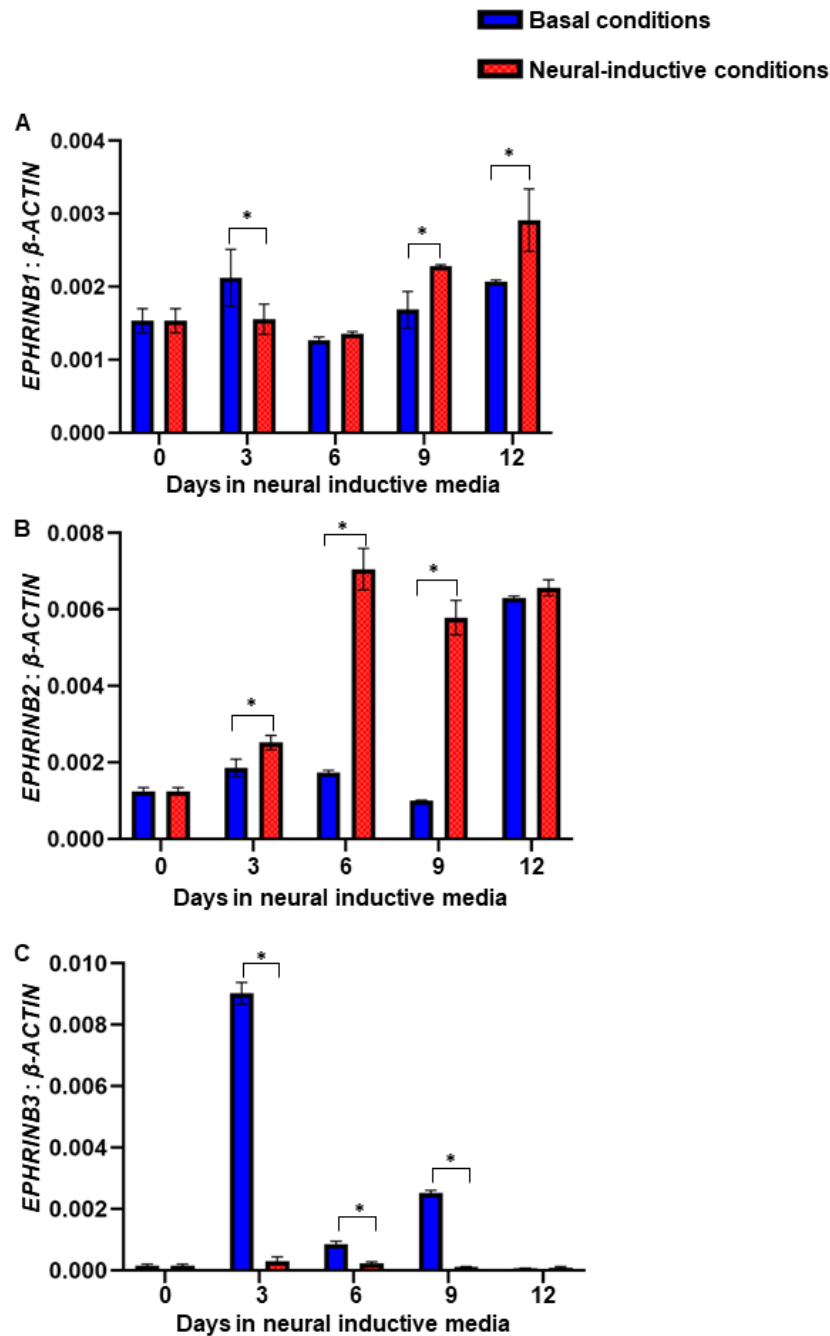


Figure 3.6: The characterisation of the ephrinB ligand gene expression profile following neural induction of SH-SY5Y cells. RT-PCR analysis of (A) *EPHRINB1*, (B) *EPHRINB2* and (C) *EPHRINB3*. The EPHRINB gene expression profile by SH-SY5Y cells was characterized on days 0, 3, 6, 9 and 12 of the neural differentiation assay under basal and neural-inductive conditions by RT-PCR analysis. Data represents mean $\pm$ SD (n=3 wells of a PCR from n=3 pooled wells per culture condition). Statistical analysis: Ordinary two-way ANOVA with Sidak's multiple comparisons between basal conditions and neural-inductive conditions for each time point, *p < 0.05.

The difference in expression of the Eph/ephrin family characterized under neural-inductive conditions compared to basal conditions during the neural differentiation assay is summarized in Table 3.1. Based on these observations, we investigated the functions of ephrinA2, ephrinA5 and ephrinB1 on neural differentiation.

Table 3.1: Eph and ephrin characterisation in SH-SY5Y cells during the neural differentiation assay. (+/-) indicates $p < 0.05$ increase/decrease, respectively, under neural-inductive conditions compared to basal conditions. A blank box indicates there was no significant difference in expression under either condition.

Gene	Day 3	Day 6	Day 9	Day 12
<i>EPHA1</i>	+			
<i>EPHA2</i>	+		-	+
<i>EPHA3</i>				
<i>EPHA4</i>	-			
<i>EPHA5</i>	-		-	
<i>EPHA6</i>		+	+	+
<i>EPHA7</i>			-	+
<i>EPHA8</i>			+	
<i>EPHB1</i>	-	+	-	+
<i>EPHB2</i>	+	+	-	-
<i>EPHB3</i>	-		+	-
<i>EPHB4</i>	-		+	-
<i>EPHB6</i>	-	+	-	+
<i>EPHRINA1</i>	-			
<i>EPHRINA2</i>		-		+
<i>EPHRINA3</i>	+		+	
<i>EPHRINA4</i>				-
<i>EPHRINA5</i>	-		+	+
<i>EPHRINB1</i>	-		+	+
<i>EPHRINB2</i>	+	+	+	
<i>EPHRINB3</i>	-	-	-	

3.2.2.5 Assessing neural and EPHRIN protein expression following neural induction

Protein was isolated on days 0, 3, 6, 9 and 12 of the neural differentiation assay under basal and neural inductive conditions (section 2.2.3.4). Western blot analysis was performed to detect ephrinA2, ephrinA5 and ephrinB1 (section 2.2.3.5). The housekeeping protein α -TUBULIN was detected, with a band of the expected size of approximately 50-52 KDa observed in each sample (Figure 3.7). However, when the membrane was probed for EPHRINB1 (expected size 40-45 KDa, Figure 3.7A) and EPHRINA5, (expected size 33 KDa, Figure 3.7C), bands of these sizes were not observed. Furthermore, when the membrane was probed for EPHRINA2 (expected size of approximately 24 KDa), a weak band of this size and other non-specific bands were observed (Figure 3.7B).

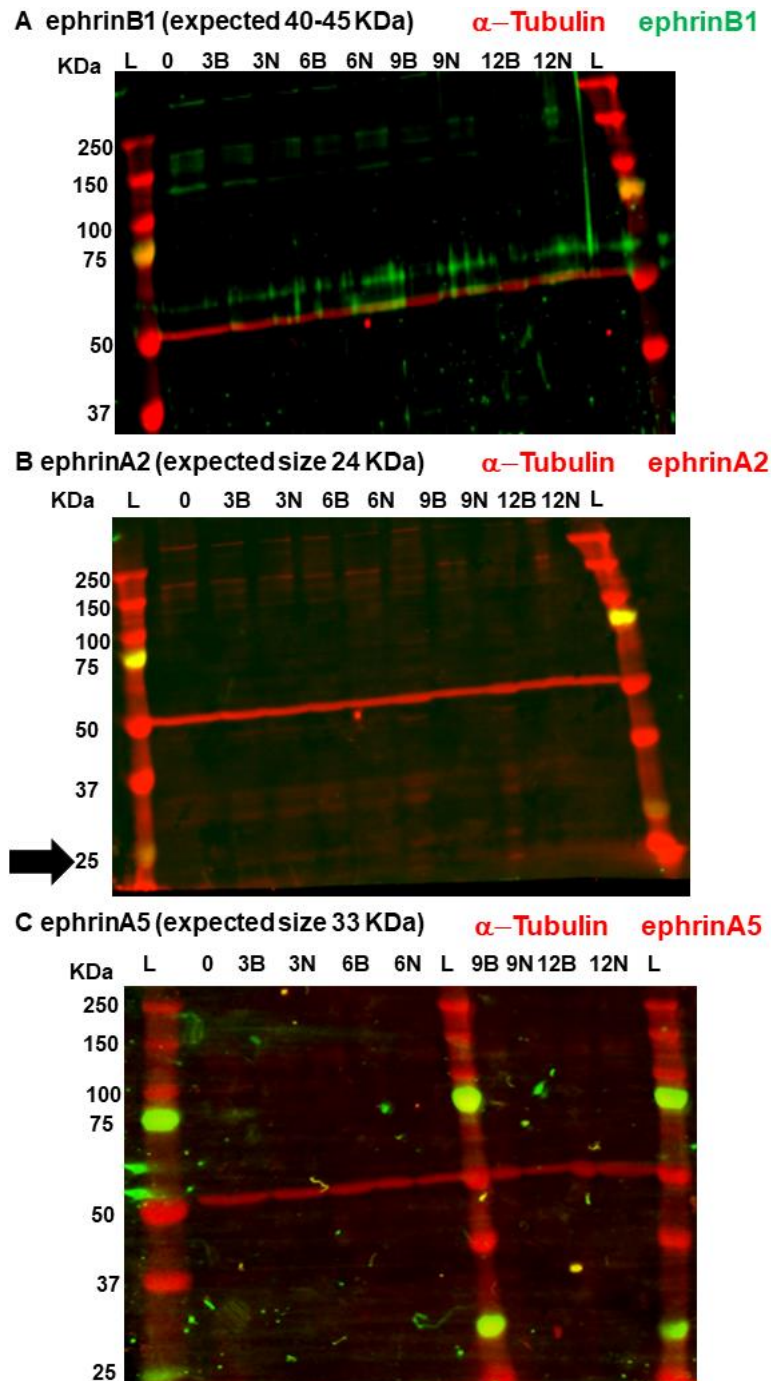


Figure 3.7: Testing for EPHRINA2, EPHRINA5 and EPHRINB1 by Western blot analysis under basal or neural-inductive conditions. Western blot analysis of protein isolated on days 0 (0), 3, 6, 9 and 12 of neural differentiation assay under basal (3B, 6B, 9B, 12B) and neural-inductive (3N, 6N, 9N and 12N) conditions. Protein isolated from n=6 pooled wells per culture condition. Samples were probed with anti-rat α -TUBULIN antibody (red) and (A) goat anti-ephrinB1 antibody (green) (B) goat anti-ephrinA2 (red) and rabbit anti-EPHRINA5 antibody (red). L = protein ladder. Specific staining corresponding to EPHRINA2 (black arrow).

At the endpoint of the neural differentiation assay, the SH-SY5Y cells were fixed and stained for neural markers with mouse anti-B-III TUBULIN (Tuj1 clone) and mouse anti-NF-M (Figure 3.8) and stained with the goat anti-ephrinA2, rabbit anti-ephrinA5, goat anti-ephrinB1 antibodies (Figure 3.9) (section 2.2.3.7.ii). The SH-SY5Y cells stained positive for NF-M and B-III TUBULIN compared to the mouse IgG2A control, which confirmed protein expression of these neural markers (Figure 3.8 A, B, C), consistent with the gene expression profiles. However, when compared to the goat IgG control, the cells showed very weak staining positive for EPHRINA2 or EPHRINB1 (Figure 3.9 A, C, D). Furthermore, when compared to the rabbit IgG control, the cells showed very weak staining positive for EPHRINA5 (Figure 3.9 B,E).

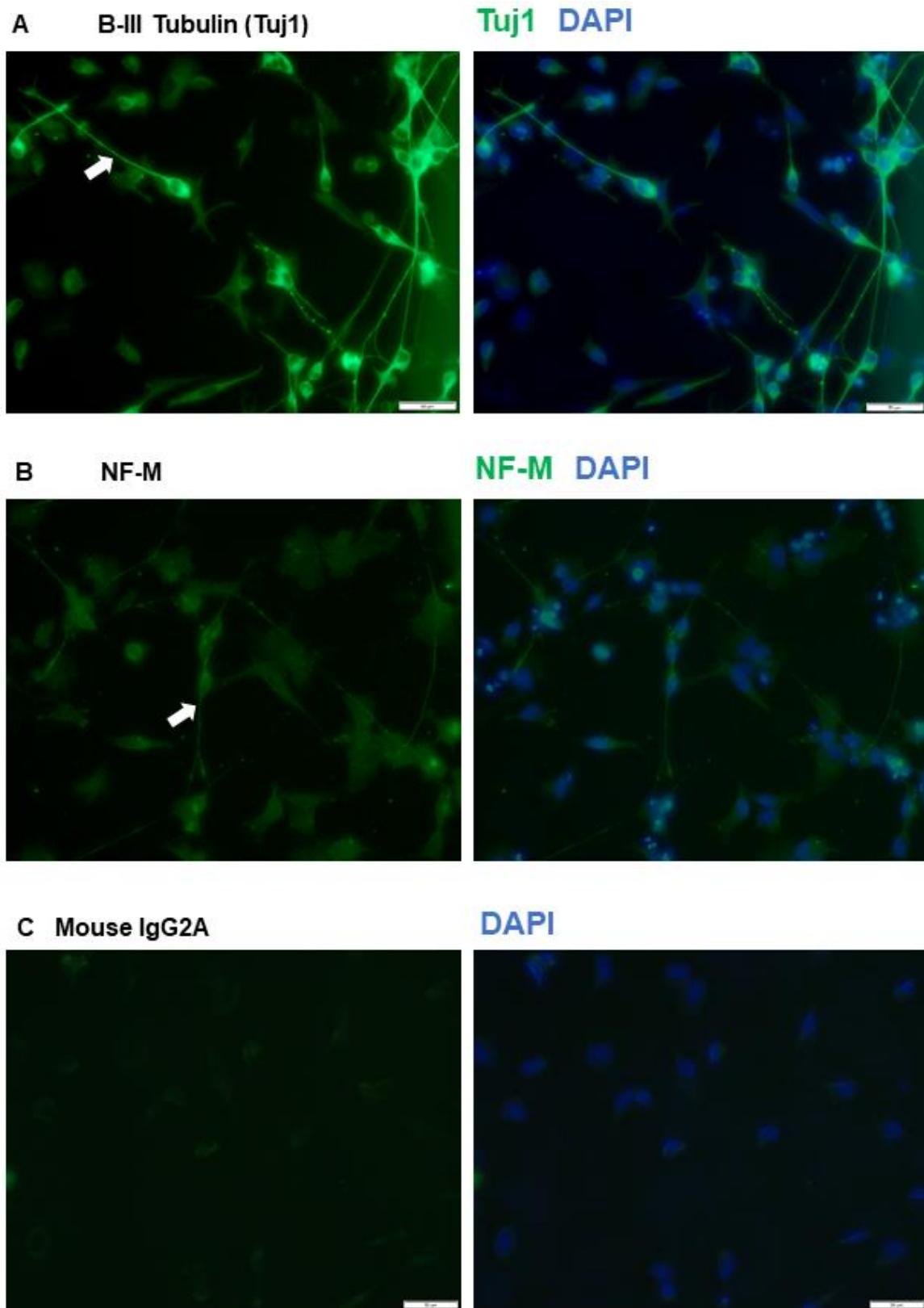


Figure 3.8: Immunocytochemistry on SH-SY5Y cells to detect B-III TUBULIN and NF-M. SH-SY5Y cells were fixed at the endpoint of the neural differentiation assay and stained with mouse anti-Tuj1 and mouse anti-NFM (green) and DAPI (blue). N=3 wells per culture condition. Scale bar = 50 μ m.

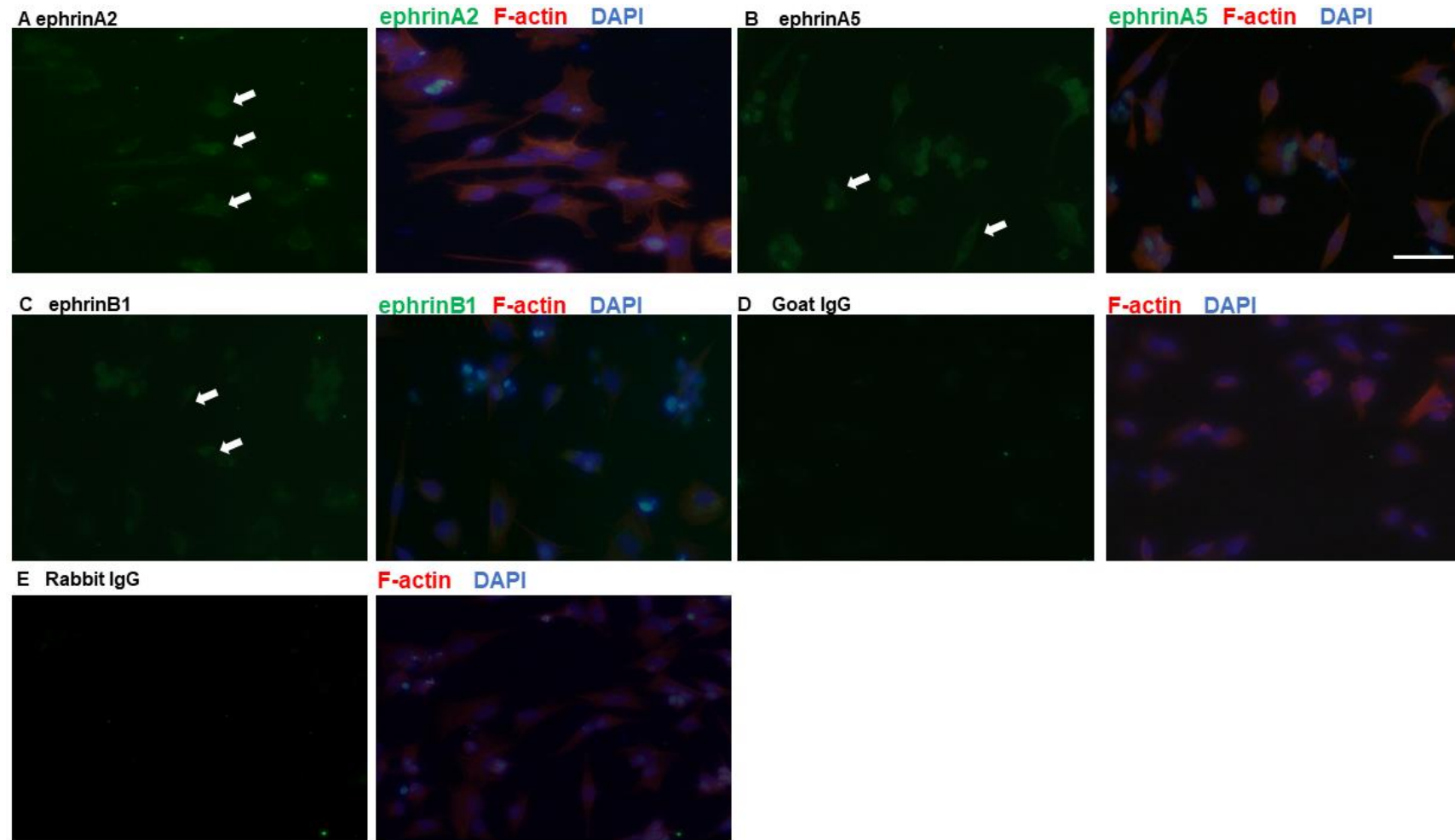


Figure 3.9: Immunocytochemistry on SH-SY5Y cells to detect EPHRINA2, EPHRINA5 and EPHRINB1. SH-SY5Y cells were fixed at the endpoint of the neural differentiation assay and stained with goat anti-ephrinA2, rabbit anti-ephrinA5 and goat anti-ephrinB1 (green), DAPI (blue) and Phalloidin (red). N=3 wells per culture condition. Scale bar = 50 μ m. Specific staining is indicated by white arrows.

Since the anti-EPHRINA2, anti-EPHRINA5 and anti-EPHRINB1 antibodies did not work in our hands during Western blot analysis and produced very weak staining in immunocytochemistry, these antibodies were tested in flow cytometry analysis where SH-SY5Y cells at the endpoint of the neural differentiation assay were liberated and stained with the primary antibodies goat anti-ephrinA2 and goat anti-ephrinB1 and rabbit anti-ephrinA5 antibodies followed by the secondary antibodies anti-goat IgG FITC and anti-rabbit IgG-FITC, and flow cytometry analysis was performed to determine the percentage of SH-SY5Y cells that stained positive for FITC (Figure 3.10) (section 2.2.3.7.iii). While some staining was detected with flow cytometry analysis, expression was very low with 1.77% of the neural SH-SY5Y cells staining positive for ephrinA2, 3.57% for ephrinB1 (Figure 3.10B) and 1.95% for ephrinA5 (Figure 3.10C).

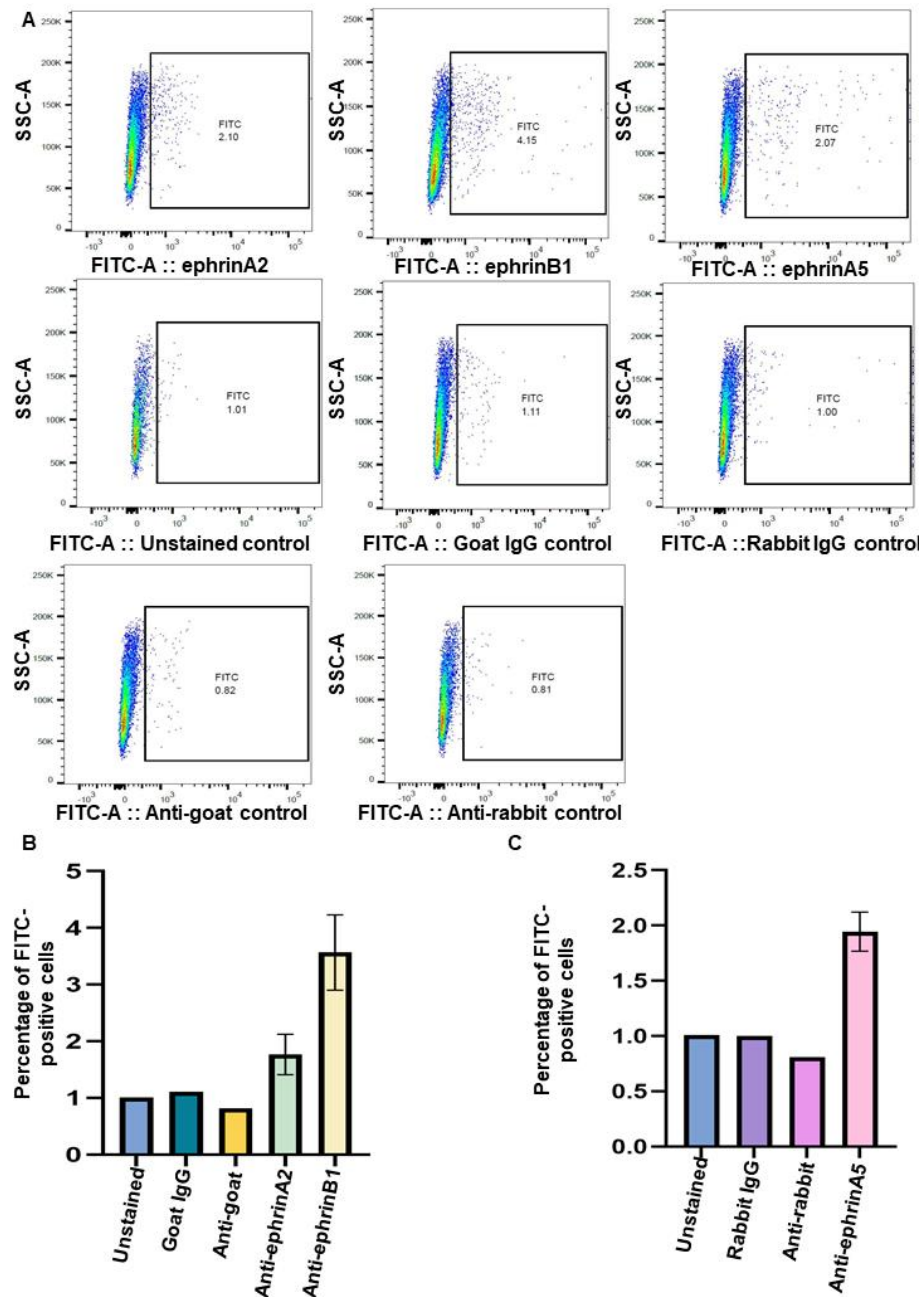


Figure 3.10: Flow cytometry analysis to assess ephrinA2/A5 and ephrinB1 expression in neural-induced SH-SY5Y cells. (A) At the endpoint of the neural differentiation assay, SH-SY5Y cells were liberated and stained with goat anti-ephrinA2, goat anti-ephrinB1 and rabbit anti-ephrinA5 followed by anti-goat IgG-FITC and anti-rabbit IgG FITC. Unstained, goat IgG, anti-goat secondary-only, rabbit IgG and anti-rabbit secondary-only controls were included. Percentage of FITC-positive SH-SY5Y cells after staining for (A) ephrinA2 and ephrinB1 and (B) ephrinA5 in flow cytometry. Data represents mean $\pm$ SD ($n = 3$ tubes for ephrinA2, A5 and ephrinB1 staining).

3.2.3 Effect of Eph/ephrin signalling on neural differentiation

3.2.3.1 The activation of ephrinA and ephrinB ligands did not affect neuronal morphology

As presented in this chapter, the human neuroblastoma line SH-SY5Y expressed *EPHRINA2*, *EPHRINA5* and *EPHRINB1* molecules with increasing neural differentiation. Our laboratory has previously characterised the gene and protein Eph/ephrin expression profile of *ex vivo* expanded human BMSC. EphA3, EphA4, EphB2 and EphB4 receptors were expressed by human BMSC and their expression was upregulated compared to the other Eph/ephrin molecules (Arthur et al., 2011). Therefore, functional molecules that can interact with the ephrin ligands expressed by the SY-SY5Y cells were used in the following studies. Fc-fusion proteins are composed of an immunoglobulin Fc domain of IgG that is directly linked to a peptide or protein of interest to stimulate this protein of interest (Czajkowski et al., 2012). The soluble EphA3-Fc, EphA4-Fc, EphB2-Fc and EphB4-Fc fusion proteins were used to mimic the EphA3, EphA4, EphB2 and EphB4 receptors that are known to be expressed by the stromal population, which would in turn potentially activate the corresponding ephrinA2, ephrinA5 and ephrinB1 ligands, respectively, which were found to be expressed by the neural population. The role of the Eph/ephrin interactions in mediating cross-talk between neural and stromal populations in the regulation of neural differentiation will be investigated.

Preliminary experiments were performed to determine whether Eph/ephrin interactions alter neuronal differentiation and the optimal concentration of Eph-Fc at which this occurs (section 2.2.2.1). A biologically and physiologically relevant range of concentrations of clustered Eph-Fc were studied, specifically 1, 5 and 10 µg/mL (Stokowski et al., 2007). The analysis of the bright field images obtained at the day 12 endpoint of neural differentiation assay indicated that 5 µg/mL of Eph-Fc was sufficient to observe a gross morphological difference in the SH-SY5Y cells (Figure 3.11).

The bright field images obtained at the endpoint of this neural differentiation assay were analysed, and the number of unipolar neurons and bipolar neurons, and neurite number per cell were quantified in response to 5 µg/mL of Eph-Fc and compared to the Human-Fc control

(Figure 3.12) (section 2.2.3.6). Comparison to Human-Fc is the most appropriate control as this ensures that any differences in the response to the Eph-Fc are not due to the extracellular portion of the IgG domain of the Fc fusion protein. There was no significant difference in the neuronal morphology of the SH-SY5Y cells in response to exposure to EphA3-Fc, EphA4-Fc, EphB2-Fc and EphB4-Fc during neural induction when compared to the Human-Fc control (Figure 3.12). Specifically, there was no difference in the average percentage of unipolar neurons and bipolar neurons observed per field of view (Figure 3.12A-B) and average axon number per neural cell quantitated (Figure 3.12C) between the Human-Fc control and the Eph-Fc treatment groups. However, there was a trend toward an increase in the percentage of unipolar neurons and a decrease in the percentage of bipolar neurons observed (Figure 3.12A-B) in response to exposure to EphB2-Fc, although this did not reach statistical significance. There was an additional trend towards a decrease in the percentage of unipolar neurons and an increase in the percentage of bipolar neurons in response to EphB4-Fc exposure, which again did not reach statistical significance (Figure 3.12A-B).

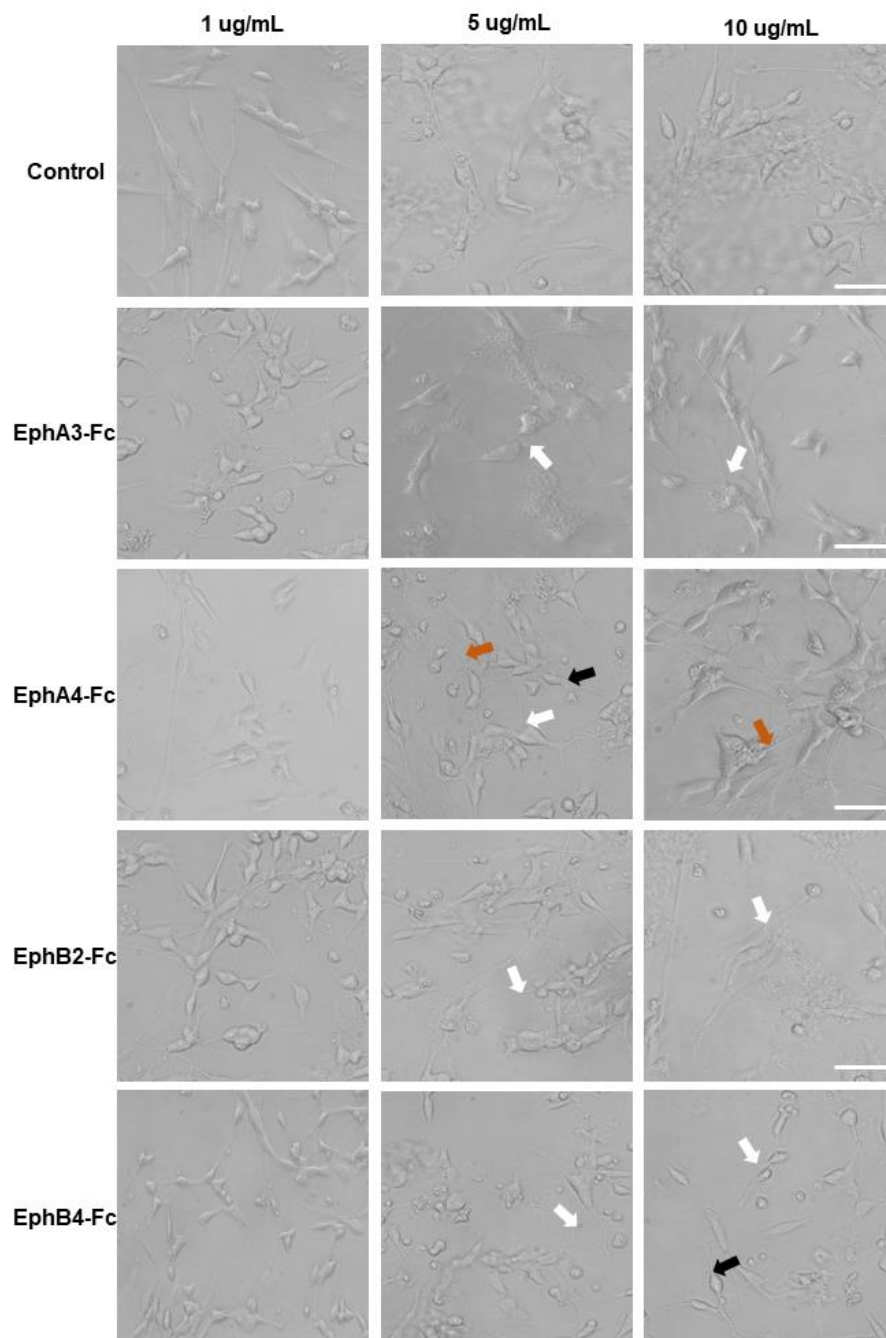


Figure 3.11: Neuronal morphology of SH-SY5Y cells in response to Eph-Fc during neural differentiation. Bright field images were obtained at the end of the neural differentiation assay, where 1, 5 and 10 $\mu\text{g/mL}$ of Human-IgG-Fc, EphA3-Fc, EphA4-Fc, EphB2-Fc and EphB4-Fc were included in the neural-inductive media at each media change on days 3, 6 and 9. ($n = 3$ wells per culture condition with bright field images obtained across four fields of view per well). Scale bar = 50 μm . Neurite processes (white arrow), bipolar neurons (black arrow) and unipolar neurons (brown arrow).

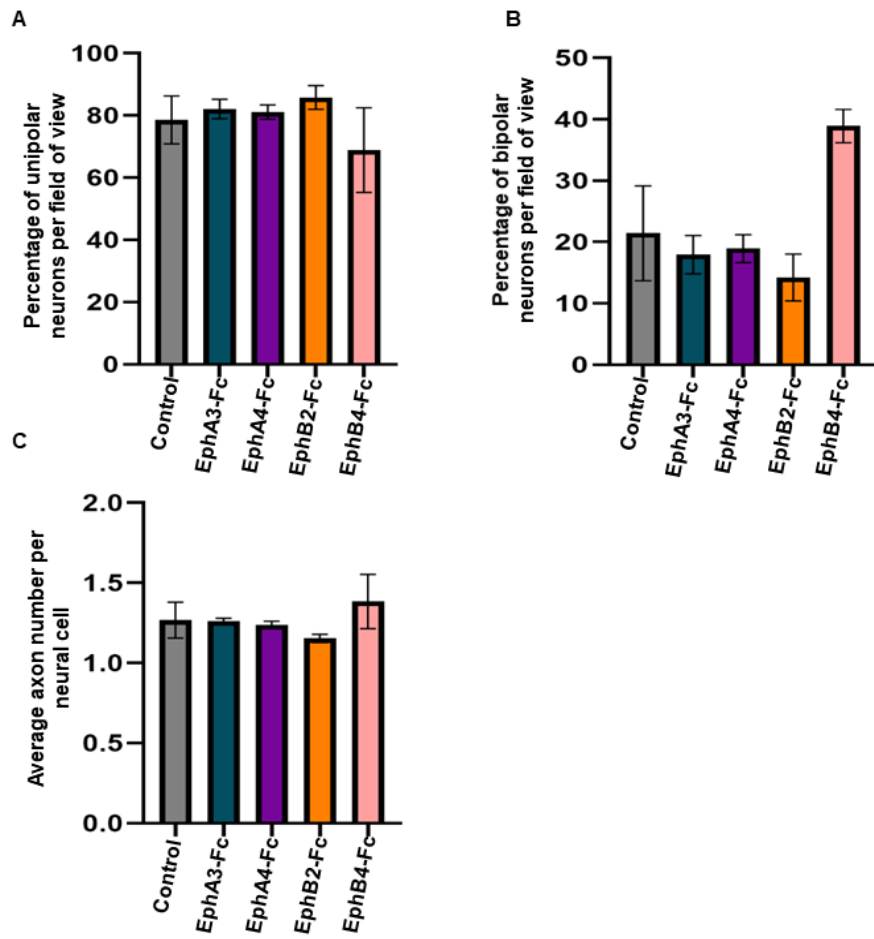


Figure 3.12: No significant differences in neural morphology of SH-SY5Y cells were observed in response to Eph-Fc during neural differentiation. Bright field images were obtained on Day 12 of the neural differentiation, in the presence of 5 $\mu\text{g/mL}$ Human-IgG-Fc, EphA3-Fc, EphA4-Fc, EphB2-Fc and EphB4-Fc. The number of unipolar neurons and bipolar neurons, and axon number per neural cell were quantified per field of view. Data represents mean $\pm$ SD ($n = 3$ wells per culture condition with 4 fields of view per well). Statistical analysis: Paired Student t-test comparing the control and the Eph-Fc treatment groups.

3.2.3.2 The activation of ephrinA and ephrinB ligands affects neural marker gene expression

The gene expression levels of the neural markers *NESTIN*, *β -III TUBULIN*, *NSE*, *NF-L*, *NF-M*, *NF-H*, *PERIPHERIN* and *DRG11* were assessed at day 12 of the neural differentiation assay when exposed to 5 μ g/mL of pre-clustered Human-IgG-Fc, EphA3-Fc, EphA4-Fc, EphB2-Fc and EphB4-Fc in each media change (Figure 3.13). *NESTIN*, *NF-L* and *PERIPHERIN* gene expression levels decreased in response to EphA4-Fc (Figure 3.13 A, D, G). *NESTIN*, *NF-M* and *NF-H* gene expression levels were significantly upregulated in response to EphB4-Fc (Figure 3.13 A, E, F). *PERIPHERIN*, *NF-L* and *NF-M* gene expression levels decreased in response to EphB2-Fc (Figure 3.13 D, E, G). Neuron-specific enolase (NSE) is a neuronal form of the glycolytic enzyme enolase localised within the central and peripheral neurons and the neuroendocrine cells (Kirino et al., 1983). There was no significant change in *NSE* and *β -III TUBULIN* gene expression levels in response to any of the Eph-Fc molecules (Figure 3.13 B, C). The gene expression level of *DRG11* was significantly upregulated in response to EphB4-Fc (Figure 3.13 H).

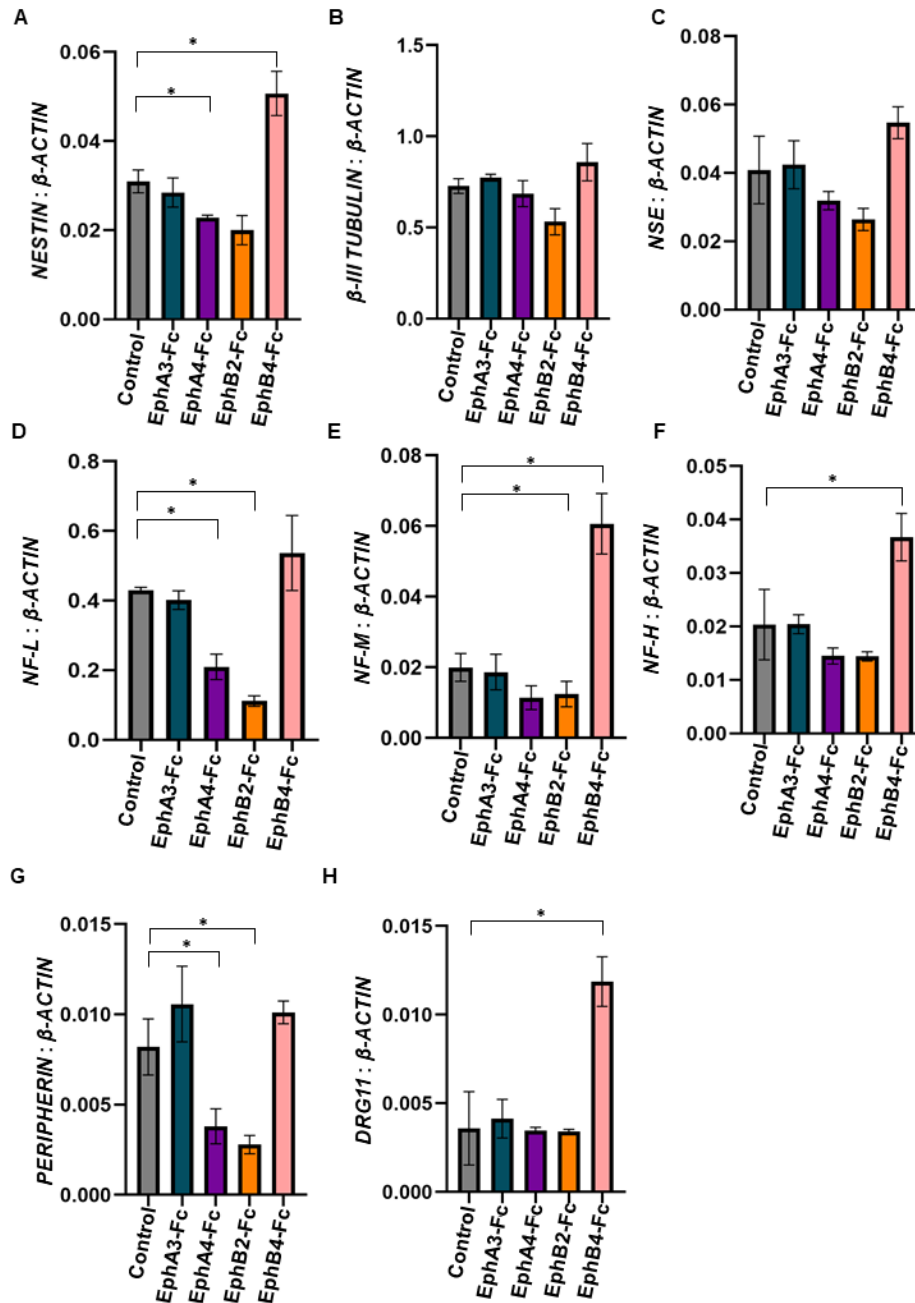


Figure 3.13: Differential gene expression of neural markers in response to Eph-Fc during neural differentiation. RT-PCR analysis of the gene expression levels of *NESTIN*, *β -III-TUBULIN*, *NEURON SPECIFIC ENOLASE (NSE)*, *NEUROFILAMENT-LIGHT (NF-L)*, *NEUROFILAMENT-MEDIUM (NF-M)*, *NEUROFILAMENT-HEAVY (NF-H)*, *PERIPHERIN* and *DRG11*. These genes were characterised in the SH-SY5Y line on day 12 of the neural differentiation assay in the presence of either 5 μ g/mL of Human-Fc, EphA3-Fc, EphA4-Fc, EphB2-Fc and EphB4-Fc. Control = Human-Fc. Data represents mean $\pm$ SD (n = 3 wells of a PCR from n=3 pooled wells per culture condition). Statistical analysis: Paired Student t-test comparing the control to each of the Eph-Fc treatment groups, *p < 0.05.

3.3 Discussion

The neural differentiation assay that we established for the SH-SY5Y line differed from the Forster et al. protocol (Forster et al., 2016) as it was carried out for 12 days instead of 10 days because the neuronal morphology was more pronounced at this end timepoint. The expression of the neural markers *NESTIN*, *B-III TUBULIN*, *NF-M* and *DRG11* were characterised following neural induction with an overall increase in the expression of *NESTIN* from day 0 to 12 under neural-inductive conditions. This is consistent with evidence in the literature that mature neurons within the adult brain express Nestin, which is proposed to be important for the remodelling of the cytoskeleton to support neural plasticity (Hendrickson et al., 2011).

In our study, the human neuroblastoma line SH-SY5Y showed upregulated expression of *EPHRINA2*, *EPHRINA5* and *EPHRINB1* with the progression of neural differentiation. *EPHRINA2*, *EPHRINA5* and *EPHRINB1* were found to be the most differentially expressed, with a significant increase in expression under neural-inductive conditions compared to basal conditions at the endpoint of the neural differentiation assay. This is consistent with the literature where certain studies have identified positive roles for ephrinA2, ephrinA5 and ephrinB1 in regulating neural differentiation (Vazin et al., 2009, Hara et al., 2010, Homman-Ludiye et al., 2017). Contradictory roles have been reported for ephrinA5 in the SH-SY5Y cell line, however, this was a study of dopaminergic neural differentiation conducted under different neural-inductive conditions which may explain the contrasting findings for the same cell line (Wang et al., 2016). A contradictory role for ephrinB1 in regulating neural differentiation has also been reported, however it should be noted that this was observed upon the *in vivo* knockdown of ephrinB1 in murine cortical neural progenitors (Qiu et al., 2008).

The change in the Eph/ephrin gene expression profiles between timepoints observed during the neural differentiation assays may reflect the heterogeneity of the SH-SY5Y cells within the population (Forster et al., 2016) or the different proportions of early and mature neuronal SH-SY5Y cells per experimental replicate at any given time point. Previous reports have shown that some markers such as alkaline phosphatase in the osteogenic lineage may be highly expressed in immature cell types such as MSC but show downregulated expression within

mature cells such as functional mineral-producing osteoblasts which provides an example of the same marker demonstrating different functional roles within the same cell lineage between different stages of the cell development program (Golub and Boesze-Battaglia, 2007), and it is possible that the Eph/ephrin molecules function in a similar manner.

A limitation of this study is that while the increase in expression of *EPHRINA2*, *EPHRINA5* and *EPHRINB1* compared to basal conditions at the corresponding timepoints based on RT-PCR is suggestive of a higher abundance of these molecules, consideration must be given to differences in the efficiency of the primer pairs that detect specific Eph and ephrin molecules as well as differences in the dynamics of each PCR reaction. These differences are partially addressed with the use of the delta-CT method in the analysis of this RT-PCR data, where threshold cycle (CT) values from each triplicate PCR reaction were averaged and calibrated relative to the reference control beta-actin CT values. In addition, the primer pairs against the Eph/ephrin A molecules of the A subclass have been used on the haematopoietic lineage and their primer efficiency have been confirmed (Nguyen et al., 2017).

Another limitation is that the Eph/ephrin gene expression profile could not be confirmed at the protein level by the anti-ephrinB1 antibody which has been previously shown to work for Western blot analysis and immunocytochemistry (Kayser et al., 2006, Wu et al., 2012). Similarly, anti-ephrinA2 and anti-ephrinA5 previously shown to work for Western blotting (Worku et al., 2018, Irie et al., 2009) did not work in our hands during these applications. It should be noted that the anti-ephrin antibodies showed some reactivity with flow cytometry although expression was relatively low. The weak protein expression profiles of these EPHRIN of interest are consistent with the low absolute RNA levels of expression that were observed in the gene expression profiles of these molecules during the neural differentiation assay. Furthermore, our Western blotting detection system may not have been sensitive enough to detect the staining, as the band corresponding to EPHRINA5 expression in the SH-SY5Y line appeared weak in other studies (Wang et al., 2016). If time permitted, western blot, immunocytochemistry and flow cytometry would have been repeated while using positive controls of either purified EPHRINA2, EPHRINA5 and EPHRINB1 protein or SH-SY5Y cells

that overexpressed these proteins to assess whether these anti-ephrin antibodies worked as previously demonstrated in other studies.

The role of the Eph/ephrin mediated cross-communication between the stromal and neural populations, in regulating neural differentiation was assessed. Our laboratory has previously characterised the gene and protein Eph/ephrin expression profile of *ex vivo* expanded human BMSC. EphA3, EphA4, EphB2 and EphB4 receptors were expressed by human BMSC and their expression was upregulated compared to the other Eph/ephrin molecules (Arthur et al., 2011). It is known that EphA3 binds with affinity to both ephrinA2 and ephrinA5 while EphA4 interacts with ephrinA2 and ephrinA5, but can also interact with ephrinB1 and ephrinB2 (Pasquale, 1997). Furthermore, EphB4 binds with high affinity to ephrinB2 and can also interact with ephrinB1 (Pasquale, 1997) whereas EphB2 binds with affinity to ephrinA5 and ephrinB1. In addition, ephrinB1 interacts predominantly with EphB2, but also binds to EphB4 (Pasquale, 2008). The expression profile data identified ephrinA2, ephrinA5 and ephrinB1 as the most differentially expressed ligands following neural induction. Based on the binding affinity of these ligands with their respective Eph receptors, the following three Eph/ephrin interacting pairs were investigated for their involvement in the cross-talk between BMSC and neurons, respectively, to regulate neural differentiation. These included: (1) EphA3 or EphA4 with ephrinA2 (2) EphA3, EphA4 or EphB2 with ephrinA5 and (3) EphB2 or EphB4 with ephrinB1 (Figure 3.14). To assess this, ephrin reverse signalling within the SH-SY5Y cells was activated through stimulation with soluble EphA3-Fc, EphA4-Fc, EphB2-Fc and EphB4-Fc, respectively, which mimic the EphA3, EphA4, EphB2 and EphB4 receptors that are known to be expressed by the stromal population.

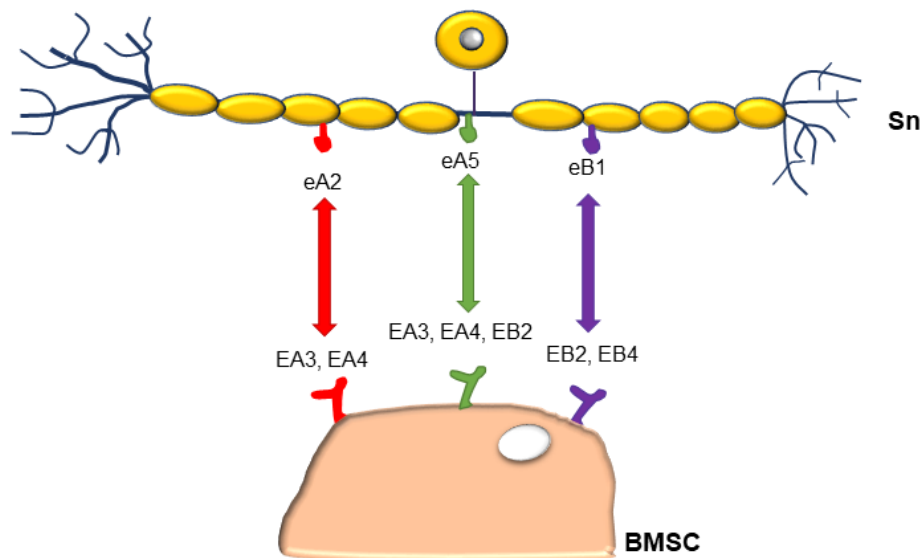


Figure 3.14: The Eph/ephrin interacting pairs that were investigated for their involvement in the cross-talk between BMSC and neurons, respectively. These interacting pairs included: (1) EphA3 or EphA4 with ephrinA2 (2) EphA3, EphA4 or EphB2 with ephrinA5 and (3) EphB2 or EphB4 with ephrinB1. Sensory neuron (Sn), ephrinA (eA), ephrinB (eB), EphA (EA) and EphB (EB).

In contrast to DPSC which exhibited changes in morphology only in response to 10 $\mu\text{g/mL}$ Eph-Fc (Stokowski et al., 2007) but consistent with the findings for BMSC (Arthur et al., 2011), 5 $\mu\text{g/mL}$ of Eph-Fc was sufficient to see a gross morphological difference in the SH-SY5Y cells. The activation of the ephrinA and ephrinB ligands via the stimulation of the corresponding EphA and EphB receptors, respectively, differentially affected neural marker gene expression. *NESTIN*, *NF-L* and *PERIPHERIN* gene expression levels decreased in response to EphA4-Fc. *NESTIN*, *NF-M* and *NF-H* gene expression levels were significantly upregulated in response to EphB4-Fc. *PERIPHERIN*, *NF-L* and *NF-M* gene expression levels decreased in response to EphB2-Fc. There was no significant change in *NSE* and β -III *TUBULIN* gene expression levels in response to any of the Eph-Fc molecules. The gene expression level of *DRG11* was significantly upregulated in response to EphB4-Fc. The differential response in neural marker expression in response to Eph-Fc suggests that different ephrinA and ephrinB ligands on the neuronal population have varied influence on neural maturation and differentiation. This is

consistent with the literature where ephrinA2 has been reported to promote neural differentiation of neural progenitors (Homman-Ludiye et al., 2017, Wang et al., 2016) while contradictory roles in regulating the neural differentiation of neural progenitors have been identified for ephrinA5 (Hara et al., 2010, Wang et al., 2016) and ephrinB1 (Qiu et al., 2008, Vazin et al., 2009), suggesting contrasting roles for the Eph/ephrin family in regulating neural differentiation which may depend on the cell type, species, and type of neural differentiation.

Differential expression in *DRG11* was observed under neural-inductive conditions suggesting that the neural-inductive media may induce neurons towards a more sensory-like disposition. In addition, the gene expression level of *DRG11* was significantly increased in response to EphB4-Fc. DRG11 is a sensory-like marker, and sensory neurons are involved in sensing pain. Therefore, future research into the role of Eph/ephrin mediated cross-communication between bone/ stromal populations and sensory neurons in regulating bone pain may be required. EphB1/ephrinB1 signalling has already been established as a potential therapeutic target in the treatment of bone cancer pain through its involvement in the modulation of spinal inflammatory cytokines in a rat model of bone cancer pain (Dong et al., 2011).

Collectively, the findings from this chapter suggest that changes in Eph/ephrin signalling mediated changes in the gene expression profile of selective neural markers, but this change in genotype was not reflected by a significant change in neuronal phenotype. Therefore, it was proposed that these Eph and ephrin molecules may influence other neural functions such as neural adhesion and migration instead, which will be investigated next.

Chapter 4 Investigating the role of Eph/ephrin interactions on neural adhesion and migration

4.1 Introduction

The findings from Chapter 3 indicated that the Eph/ephrin interactions did not significantly influence neural differentiation. Based on the Eph/ephrin gene expression profiles, the ephrin molecules that showed an increase in expression during neural induction when compared to basal conditions were further studied for their role in regulating neural adhesion and migration. Eph/ephrin signalling is known to influence neural adhesion and migration by acting as guidance cues during development to mediate the correct formation of synapses within many different areas of the developing nervous system (Dottori et al., 1998, Eberhart, 2004, Jiao et al., 2008, Kao and Kania, 2011) and during axon regeneration and repair (Goldshmit et al., 2011, Chen et al., 2021, Duffy et al., 2012, Parrinello et al., 2010, Overman et al., 2012). Differentiation, adhesion and migration are important processes involving many different cell lineages such as osteogenic, osteoclastic and neural lineages that regulate limb development and skeletal repair (Tomlinson et al., 2020, Thiel et al., 2018, Su et al., 2018, Egawa et al., 2014). The study of Eph/ephrin interactions is complex and must be considered between both the neural and stromal populations due to the bi-directional nature of the Eph/ ephrin interactions (Pasquale, 2008, Pasquale, 2010). Therefore, the role of the Eph/ephrin molecules that showed an increase in expression during neural induction when compared to basal conditions was investigated further for their role in mediating cross-communication between the stromal and neural populations to regulate neural adhesion and migration.

There is evidence in the literature of Eph/ephrin interactions regulating neural adhesion, where ephrinB1 maintains apical adhesion of neural progenitors (Arvanitis et al., 2013). In addition, ephrinA5 has been shown to change the adhesive property of cortical neurons by inducing the formation of cell aggregates (Zimmer et al., 2007). The role of Eph/ephrin signalling in regulating neural migration is well documented. Within the A subclass, EphA4/ephrinA2 reverse signalling has been shown to promote the migration of cortical interneurons (Steinecke et al., 2014) while ephrinA5 mediated reverse signalling enhances the motility of cortical neurons (Zimmer et al., 2007). Within the B subclass, EphB1 and ephrinB3 cooperatively

regulate the migration of neural progenitors in the hippocampus where mice lacking EphB1 and EphB2 receptors have significantly fewer neural progenitors in the hippocampus (Chumley et al., 2007). Furthermore, the disruption of EphB/ephrinB signalling disrupts neuronal migration of neuroblasts within the adult subventricular zone (Conover et al., 2000).

Furthermore, Eph/ephrin communication between the skeletal and nervous system mediates patterning and localization of the sensory and motor axons during development. This was demonstrated using avian and mouse models (Eberhart, 2004, Eberhart et al., 2000, Jayasena et al., 2005, Eberhart et al., 2002, Helmbacher et al., 2000, Kramer et al., 2006, Krull et al., 1997, Luxey et al., 2013, Munoz et al., 2005, Winning and Krull, 2011). During normal hind limb innervation, the motor axons within the lateral motor column that target the limb target tissues initially converge at the base of the limb bud and then adopt dorsal and ventral trajectories within the limb (Luria and Laufer, 2007). This patterning is disrupted in mutant mice with a global deletion of the *EphA4* gene, where presumptive dorsal motor axons enter the ventral compartment of the limb instead (Helmbacher et al., 2000). The importance of EphA4 in commissural axon guidance has also been demonstrated, where mice with a null mutation in the *EphA4* gene display a loss of coordination of limb movement and a resultant hopping, kangaroo-like gait due to disrupted commissural axon patterning along the corticospinal tract (Dottori et al., 1998).

The avian model is a well-established developmental model especially in the study of limb bud development (Tickle, 2004) and neural patterning and axon guidance (Landmesser and Morris, 1975). Within the chick embryo, the DRG located in the lumbar region (L) of the spinal cord, specifically DRG L4-8 form the ischiadic plexus which forms the sciatic nerve that innervates the hindlimbs (Landmesser and Morris, 1975). The Eph/ephrin expression profile has been characterised during the first 48 hours of chick embryogenesis (Baker and Antin, 2003, Baker et al., 2001, Iwamasa et al., 1999). The avian model is a suitable system for investigating axonal guidance, and neural migration and patterning, where the role of Eph/ephrin interactions in regulating neuronal processes has been previously reported (Eberhart et al., 2000, Koblar et al., 2000, Krull et al., 1997, Krull, 1998, Winning and Krull, 2011, Araujo et al., 1998). The studies

showed that sensory axons track pre-formed motor axons via interactions between the EphA3 or EphA4 receptors on the motor axons and the corresponding ephrinA2 and ephrinA5 ligands on the sensory axons (Wang et al., 2011). During neuronal patterning of the peripheral tissues, the navigation of the axonal processes is restricted by signalling between Eph/ephrin interactions with the reciprocal ephrin ligand/Eph receptor on the surrounding tissue (Eberhart, 2004, Eberhart et al., 2002). Similar studies have been carried out where the axon guidance of the neuronal projections extending from the trigeminal ganglia was measured in response to ephrinA5-Fc (Jayasena et al., 2005). This study identified that ephrinA5 interacts with EphA3 in order to mediate axon guidance of the trigeminal neuronal projections (Jayasena et al., 2005).

During limb bud development, communication between EphB2 expressed by motor and sensory axons, and ephrinB1 expressed by the sensory axons and limb bud mesenchyme controls axon fasciculation in the limb (Luxey et al., 2013). The growth of EphA4-positive lateral motor column axons is inhibited within ephrinA5-positive regions of the hind limb mesoderm due to a repulsive interaction (Eberhart, 2004). Importantly, the interactions between ephrinA5 expressed within the mesenchyme and the EphA receptors on the growth cones of sensory axons inhibits the growth of sensory axons not only in the limbs but also within the facial tissue (Munoz et al., 2005). EphA3 which is highly expressed during the trigeminal sensory innervation within the avian embryo, mediates the guidance of the ophthalmic nerves via interactions with ephrinA2 or ephrinA5 (Jayasena et al., 2005). It has also been shown that the movement of spinal motor axons and neural crest cells are influenced by different guidance mechanisms despite traversing the same pathway (Koblar et al., 2000), where EphB:ephrinB1 interactions pattern the migration of trunk neural crest cells (Krull, 1998), but are not required for the patterning of spinal motor axons (Krull et al., 1997).

The human neuroblastoma line SH-SY5Y showed upregulated expression of *EPHRINA2*, *EPHRINA5* and *EPHRINB1* with the progression of neural differentiation. These *EPHRINA2*, *EPHRINA5* and *EPHRINB1* ligands were the most differentially expressed, with a significant increase in expression under neural-inductive conditions compared to basal conditions at the endpoint of the neural differentiation assay. This Eph/ephrin gene expression profile

characterised *in vitro* is also consistent with the avian embryo expression profile, where sensory axons express ephrinA2 and ephrinA5 which interact with EphA3/A4 receptors on the motor axons (Wang et al., 2011). Sensory axons also express ephrinB1 which interacts with the EphB2 expressed by the sensory and motor axons (Luxey et al., 2013). Therefore, the avian embryo is an appropriate *ex vivo* model to investigate the role of Eph/ephrin mediated cross-communication between the neural and stromal populations in regulating DRG neurite cell body number and DRG neurite invasion. The avian embryo DRG that innervate the hind limbs were cultured *in vitro* in the presence of a range of Eph-Fc molecules.

Eph-ephrin communication is vital for early axonal patterning (Eberhart et al., 2000, Cramer et al., 2004, Jayasena et al., 2005, Munoz et al., 2005). As the Eph/ephrin molecules have known roles in independently regulating the skeletal and nervous systems, the Eph/ephrin interactions may mediate cross-talk between the neural and bone/stromal cells. There is limited knowledge about whether the Eph/ephrin interactions mediate cross-talk between the neural and stromal populations to regulate neural and stromal function, and whether these interactions occur via direct (contact-dependent) or indirect (chemoattractant/chemo-repellant) mechanisms for the neural population. A further gap of knowledge is whether this Eph/ephrin mediated cross-communication regulates neural adhesion and migration. It is known that Eph/ephrin signalling within the nervous system regulates neural adhesion and migration. Furthermore, EphB/ephrinB signalling has been reported to mediate human MSC attachment and migration (Arthur et al., 2011). Therefore, we investigated whether activating reverse signalling within the neural population via the stimulation of the Eph receptors expressed on the stromal population influenced neural adhesion and migration, and DRG neurite cell body number and DRG neurite invasion.

4.2 Results

4.2.1 Effect of Eph/ephrin interactions on neural adhesion

4.2.1.1 Testing the different methods of cell detachment

Trypsin cleaves surface antigens (Tsuji et al., 2017) which may include the extracellular portion of the Eph/ephrin. Eph/ephrin proteins are re-established after a short period of time of 24-48 hours after cell detachment and therefore, Trypsin is an acceptable detachment method to use in long term functional studies. Two well established approaches include PUCKS-EDTA (Stokowski et al., 2007) and TrypLE (Shaw et al., 2014) which have been developed to liberate cells when performing short-term functional assays that investigate Eph/ephrin communication. These three cell detachment methods were tested to determine the optimal method for differentiated SH-SY5Y cells that did not disrupt Eph/ephrin function during the adhesion assays where the cells would be detached and re-adhered to pre-bound pre-clustered Eph-Fc for two and 24 hours (section 2.2.2.2). Detachment of the differentiated SH-SY5Y cells with TrypLE gave a viability of 88% (Figure 4.1), while 88.8% viability was obtained by detachment with Trypsin. There was a trend observed of Trypsin producing a higher number of cells compared to PUCKS-EDTA and TrypLE (Figure 4.1). Since the cell viability obtained using both TrypLE and Trypsin were similar, TrypLE was deemed the suitable method of liberating differentiated SH-SY5Y neural cells, considering Trypsin could not be used for these studies. Therefore, TrypLE was used for all subsequent short-term functional studies such as adhesion and migration assays.

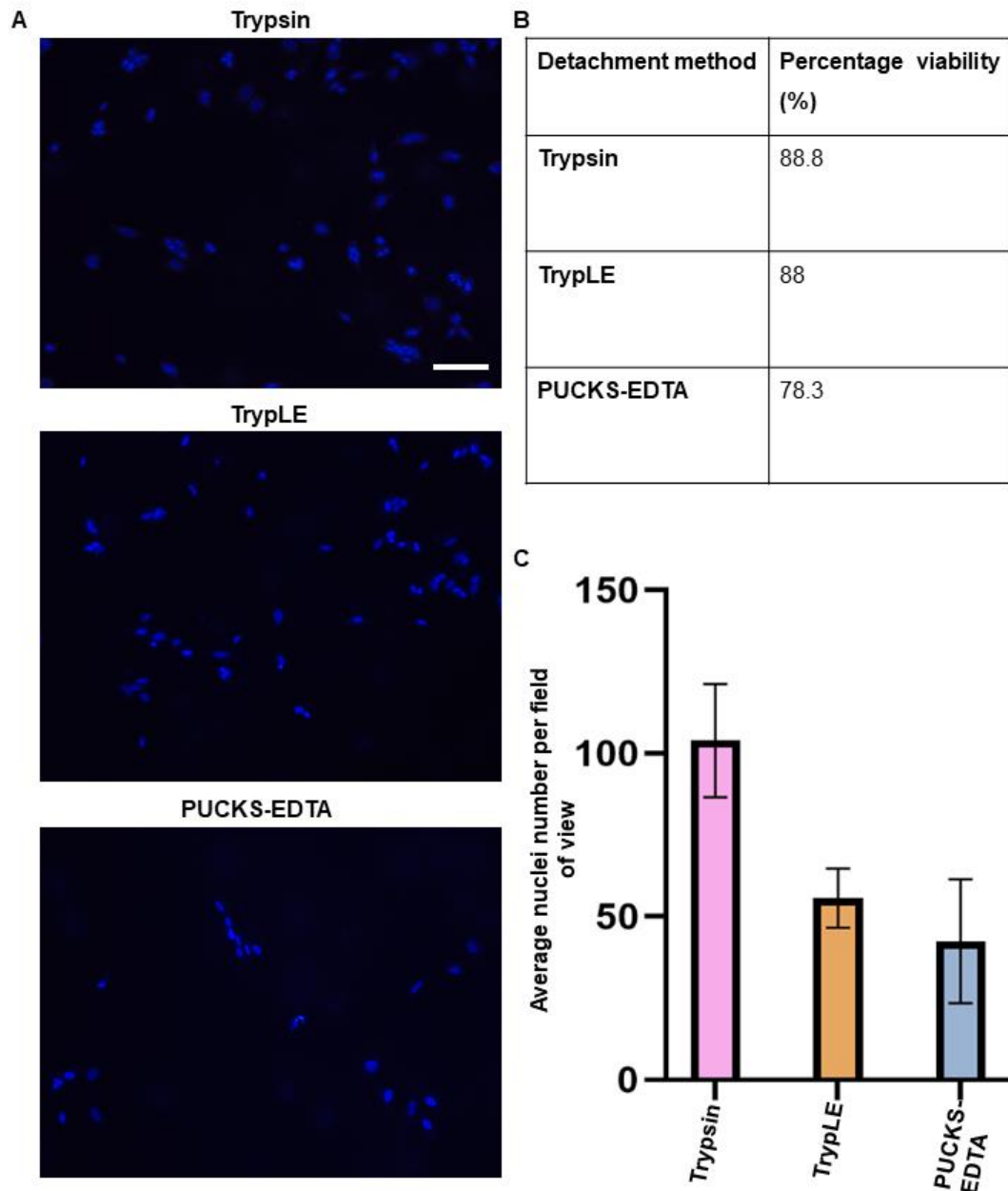


Figure 4.1: Neural adhesion of differentiated SH-SY5Y cells determined after detachment using Trypsin, TrypLE and PUCKS-EDTA. (A) Differentiated SH-SY5Y cells were liberated using the three methods and re-adhered for 24 hours and cells were stained with DAPI (blue). (B) Percentage viability was calculated and (C) DAPI positive nuclei were quantified per field of view. Data represents mean $\pm$ SD (cells were counted in 4 fields of view per well with $n=3$ wells per culture condition). Scale bar = 100 μ m. Statistical analysis: Paired Student t-test comparing the detachment groups.

4.2.1.2 Effect of ephrinA and ephrinB activation on neural adhesion

Adhesion assays were performed on differentiated SH-SY5Y cells that were liberated with TrypLE at the endpoint of neural induction (day 12) and re-adhered to pre-bound 5 µg/mL Human-Fc, EphA3-Fc, EphA4-Fc, EphB2-Fc and EphB4-Fc, respectively (section 2.2.2.3). The neural adhesion at two and twenty hours was quantified. Images were obtained and the average number of nuclei per field of view were quantitated across triplicate wells per condition. For each treatment group, the DAPI-positive nuclei were quantitated across four fields of view per well with triplicate wells per condition. Neural adhesion was inhibited in response to exposure to EphA4-Fc, EphB2-Fc and EphB4-Fc for two hours when compared to Human-Fc (Figure 4.2). In addition, neural adhesion was inhibited in response to exposure to 5 µg/mL of EphA4-Fc and EphB4-Fc for twenty-four hours compared to Human-Fc (Figure 4.3).

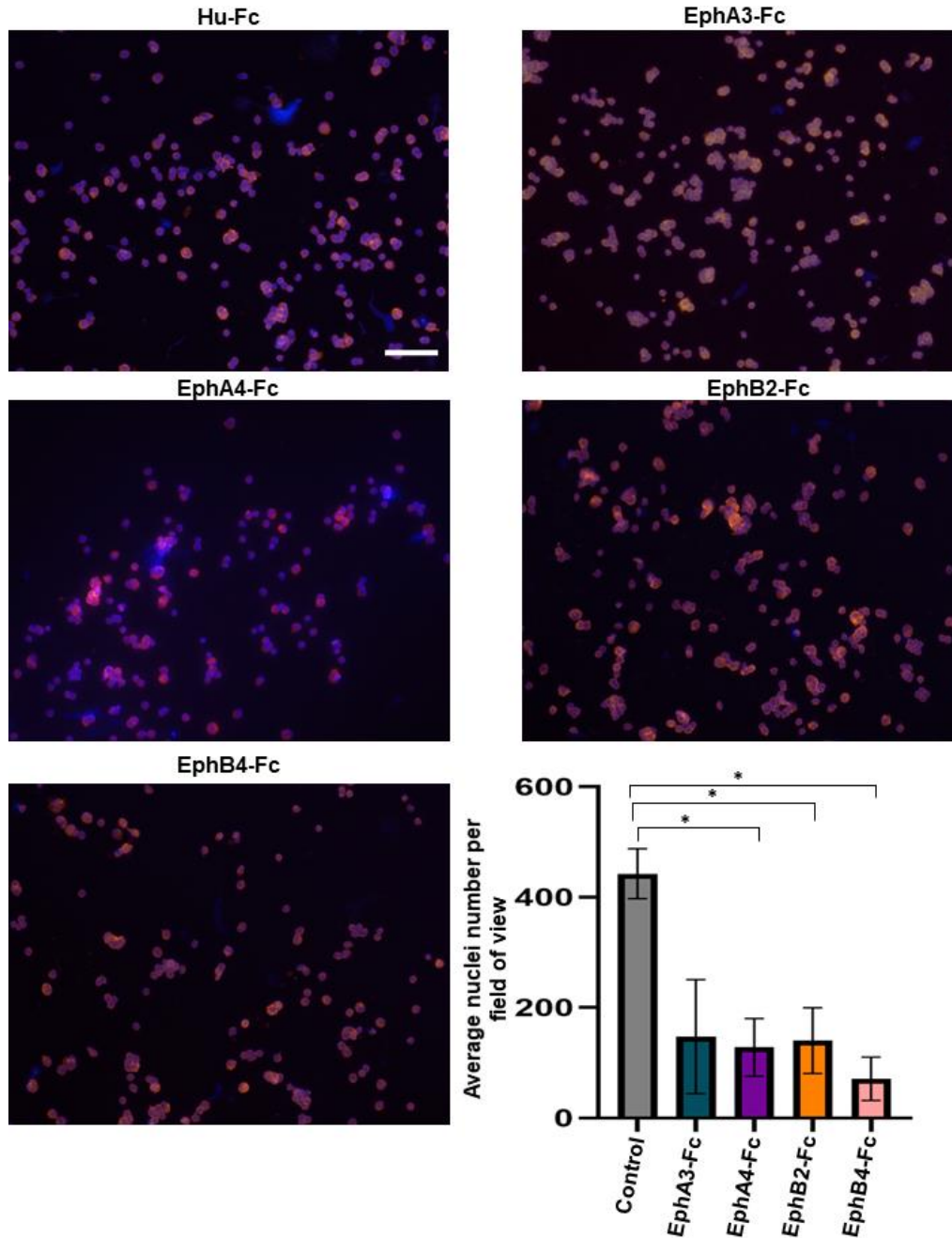


Figure 4.2: Neural adhesion was inhibited in the presence of EphA4-Fc, EphB2-Fc and EphB4-Fc. Differentiated SH-SY5Y cells were liberated with TrypLE on day 12 of the neural differentiation assay and re-adhered to 5 $\mu\text{g/mL}$ of pre-bound pre-clustered Human-IgG-Fc, EphA3-Fc, EphA4-Fc, EphB2-Fc and EphB4-Fc for two hours. Cells were stained with Phalloidin (red) and DAPI (blue). Control = Human-Fc. Data represents mean $\pm$ SD (cells were counted in 4 fields of view per well with $n=3$ wells per culture condition). Scale bar = 100 μm . Statistical analysis: Paired Student t-test comparing the Human-Fc control group and the Eph-Fc treatment groups, $*p < 0.05$.

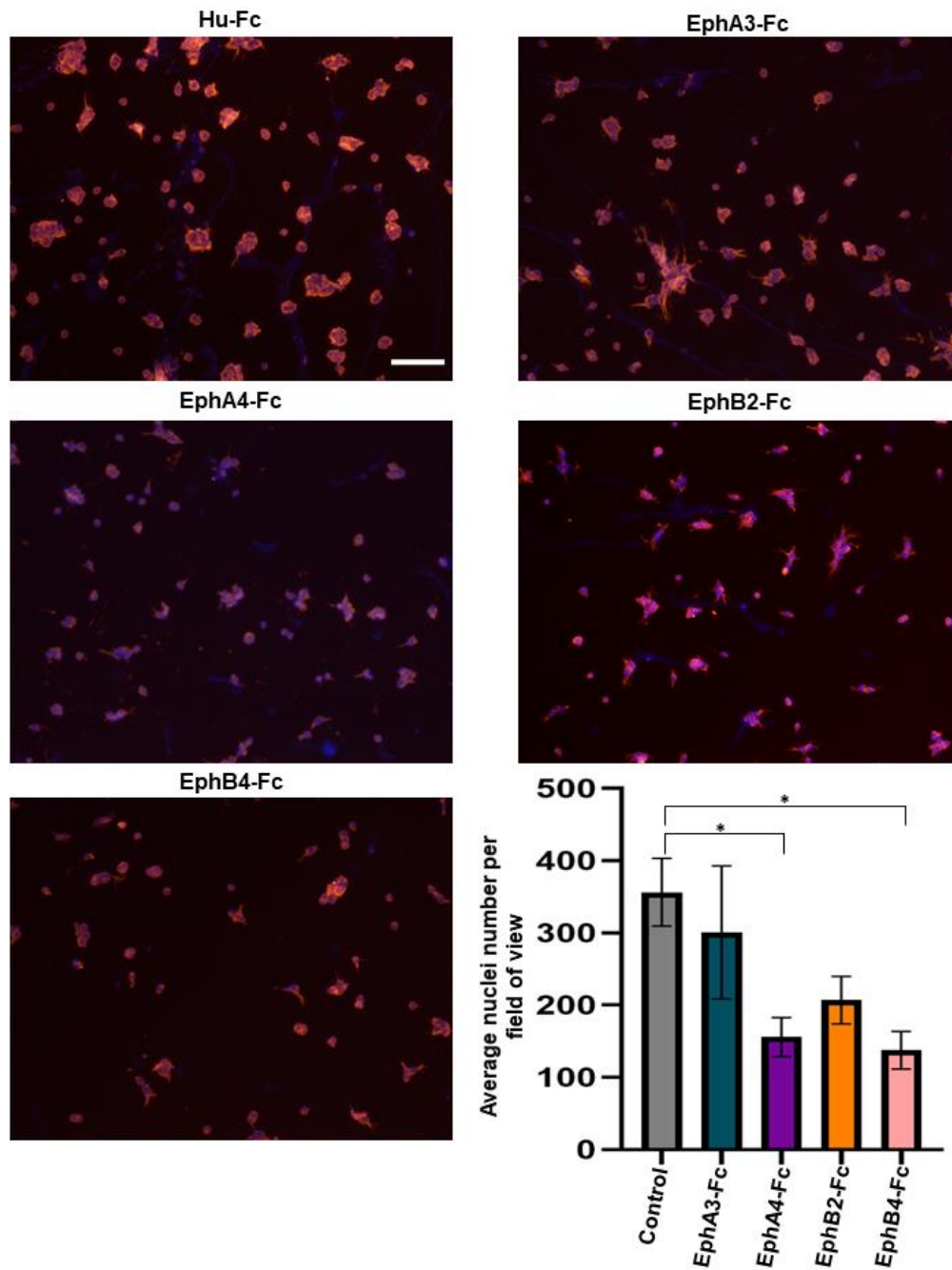


Figure 4.3: Neural adhesion was inhibited in the presence of EphA4-Fc and EphB4-Fc. Differentiated SH-SY5Y cells were liberated with TrypLE on day 12 of the neural differentiation assay and re-adhered to 5 $\mu\text{g/mL}$ of pre-bound pre-clustered Human-IgG-Fc, EphA3-Fc, EphA4-Fc, EphB2-Fc and EphB4-Fc for 24 hours. Cells were stained with Phalloidin (red) and DAPI (blue). DAPI-positive nuclei were quantified per field of view. Data represents mean $\pm$ SD. Cells were counted in 4 fields of view per well with $n=3$ wells per culture condition. Scale bar = 100 μm . Statistical analysis: Paired Student t-test comparing the Human-Fc control group and the Eph-Fc treatment groups, $*p < 0.05$.

4.2.2 Role of Eph/ephrin interactions in mediating cross-communication between neurons and BMSC to regulate neural adhesion

To determine whether the Eph/ephrin interactions facilitate cross-communication between BMSC and neurons to regulate neural adhesion, co-culture assays were utilised. Due to the Eph/ephrin expression pattern on BMSC and neural SH-SY5Y cells, the EphB2/ephrinB1 and EphB4/ephrinB1 signalling between BMSC and neural cells, respectively, was investigated. A co-culture assay allowing direct co-culture of neurons and BMSC was established (section 2.2.2.4). The protocol for this was developed based on previous studies that have established co-culture assays for neurons and osteoblasts (He et al., 2013, Tomoda et al., 2020). The neural SH-SY5Y cells were stained with the lipophilic DiD-labelling dye and then co-cultured with GFP-positive human BMSC for 24 hours. Therefore, the DiD-labelling of the SH-SY5Y cells was tested in a preliminary experiment to ensure the DiD-labelling of the cells was confirmed by flow cytometry analysis, and to confirm the labelled cells were visible under confocal microscopy.

4.2.2.1 Testing DiD labelling on differentiated SH-SY5Y cells

Differentiated SH-SY5Y cells were liberated on Day 12 and stained with the Vybrant DiD cell-labelling solution (section 2.2.3.7.v). Flow analysis was used to compare the percentage of DiD-positive cells in the unstained control SH-SY5Y cells (Fig. 4.4A) and the experimental sample of DiD-stained SH-SY5Y cells (Figure 4.4B) to test that the dye works by flow detection. The results showed that 61.9% of the SH-SY5Y cells stained DiD-positive (Figure 4.4B). Differentiated SH-SY5Y cells liberated at the endpoint of neural-induction, stained with the Vybrant DiD cell-labelling solution and replated for two hours, appeared red in colour under confocal microscopy suggesting that DiD labelling was successful in the staining of the SH-SY5Y cells (Figure 4.4C-D).

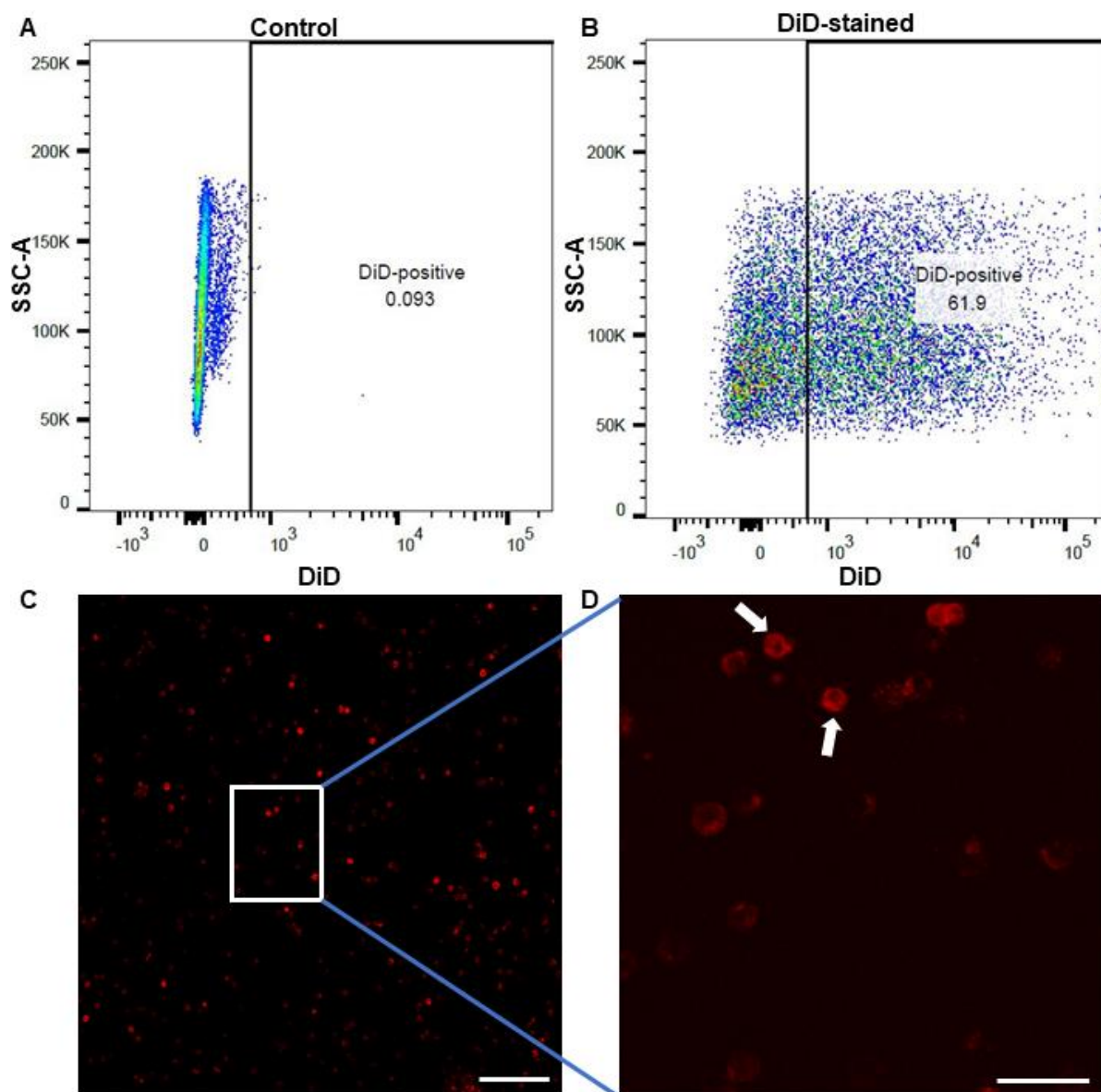


Figure 4.4: Flow cytometry analysis and confocal imaging of DiD-labelled SH-SY5Y cells. Differentiated SH-SY5Y cells at the endpoint of neural induction were liberated and stained with the Vybrant DiD labelling dye and flow cytometry analysis was performed on the (A) unstained (control) SH-SY5Y cells and the (B) DiD-stained SH-SY5Y cells. Differentiated SH-SY5Y cells stained with the Vybrant DiD cell-labelling solution were replated two hours before imaging under confocal microscopy. (C) Scale bar = 500 μm and (D) 100 μm .

4.2.2.2 Assessing neural adhesion of neural SH-SY5Y cells pre-treated with ephrinB1 blocking peptide, on a stromal monolayer

Genetically modified human BMSC have been previously generated by our laboratory where these cells were transfected with the PRUF-IRES-GFP retroviral plasmid to express GFP (Isenmann et al., 2009). To investigate the role of EphB/ephrinB1 mediated cross-talk between the stromal and neural populations, respectively, in regulating neural adhesion, the DiD-stained neural SH-SY5Y cells were liberated and left to re-adhere on top of a GFP-positive stromal monolayer for 24 hours (section 2.2.2.4). Neural adhesion was assessed in untreated neural cells and neural cells previously incubated with the ephrinB1 blocking peptide or the scramble peptide. This ephrinB1 blocking peptide functions intracellularly and becomes distributed within the cytoplasm and binds to the downstream effector proteins that interact with the C-terminus of ephrinB1 (Tanaka et al., 2010). The cells were fixed after 24 hours and imaged under confocal microscopy to visualise the DiD-stained neural SH-SY5Y cells and the GFP-positive stromal monolayer simultaneously. (Figure 4.5 A-C). The DiD-labelled cells on top of the GFP-positive stromal monolayer were quantified per field of view (Figure 4.5D). No significant difference in neural adhesion was observed in response to ephrinB1 blocking when compared to the scrambled control (Figure 4.5D).

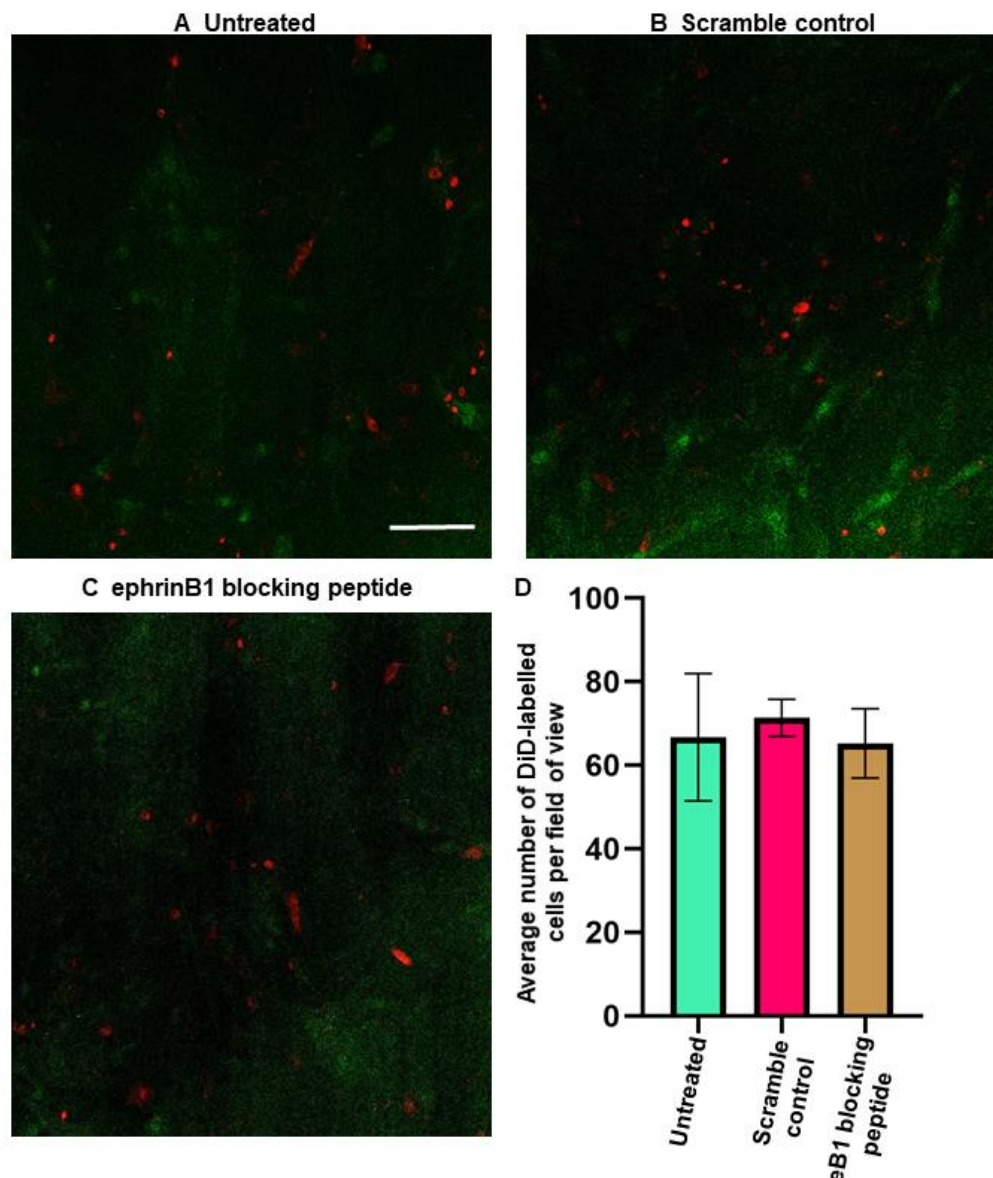


Figure 4.5: Co-culture experiment where neural-induced SH-SY5Y cells pre-treated with the ephrinB1 blocking peptide were adhered on a GFP-positive stromal monolayer. Differentiated SH-SY5Y cells were liberated, stained with DiD-labelling dye and re-adhered on top of a GFP positive stromal monolayer after pre-treatment with ephrinB1 (eB1) blocking peptide or scramble peptide, and fixed after 24 hours to assess neural adhesion. Data represents mean $\pm$ SD (cells were counted in 4 fields of view per well in 2 wells per donor and n=2 donors per culture condition). Statistical analysis: Paired Student t-test comparing the control (untreated neural cells) group and each of the treatment groups (scramble and ephrinB1 blocking peptide treatment groups). (A-C) Scale bar = 100 μ m.

4.2.3 Indirect effect of Eph/ephrin interactions on neural migration

The current study showed changes in neural marker gene expression and inhibited neural adhesion in response to the EphB2-Fc and EphB4-Fc molecules. Both EphB2 and EphB4 bind with affinity to ephrinB1 (Pasquale, 1997), where *EPHRINB1* showed upregulated expression in the SH-SY5Y cell line following neural induction. To determine whether Eph-ephrin communication can influence migration of SH-SY5Y cells indirectly via a chemoattractant/chemorepellent mechanism, a transwell migration assay was utilised (section 2.2.2.5). Neural-differentiated SH-SY5Y cell migration was measured in response to indirect contact with the EphB2-Fc and EphB4-Fc molecules. To confirm that SH-SY5Y neural cells were able to migrate, the transwell migration of these cells was measured in response to a known chemoattractant CXCL12. Previous studies have shown that CXCR4-CXCL12 signalling is necessary for the migration of neuronal precursors and regulation of axon guidance (Li and Ransohoff, 2008, Arthur et al., 2009). CXCL12 (100 ng/mL) was placed in the media in the lower chamber of the transwell insert. Transwell migration was significantly increased in response to CXCL12 compared to the media only negative control (Figure 4.6). This protocol was developed from previous transwell assays that have used SH-SY5Y cells (Ding et al., 2014, Joshi et al., 2007) and shown a positive response to CXCL12 (Ding et al., 2014).

Subsequently, replicate wells were set up to assess the transwell migration of the differentiated SH-SY5Y cells in response to EphB2-Fc and EphB4-Fc, compared to Human-Fc, where three concentrations of Fc were tested: 1, 5 and 10 µg/mL. There was no significant difference in transwell migration in response to either EphB2-Fc or EphB4-Fc when compared to Human-Fc at any of the concentrations tested (Figures 4.7 to 4.9)

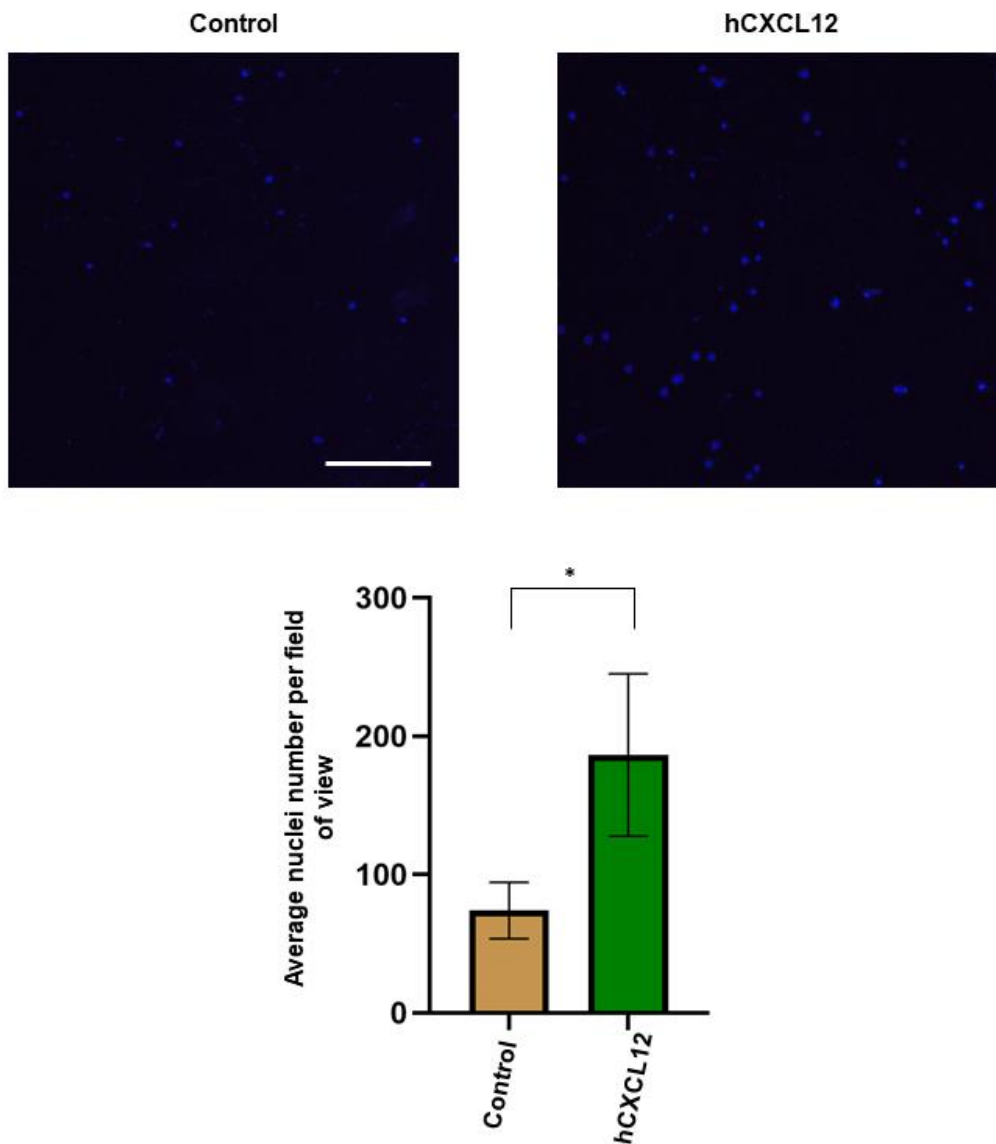


Figure 4.6: Differentiated SH-SY5Y migrated in response to indirect exposure to CXCL12. Differentiated SH-SY5Y cells were seeded into the upper chambers of transwell inserts with media alone or in the presence of 100 ng/mL human CXCL12 in the bottom chambers and incubated for 32 hours. The cells on the lower surface of the membrane were stained with DAPI (blue). Images were obtained (Scale bar = 200 μ m). Data represents mean $\pm$ SD (number of cells counted in three fields of view per well, with n=3 wells per condition). Statistical analysis: Paired Student t-test comparing the control and the CXCL12 treatment groups, *p < 0.05.

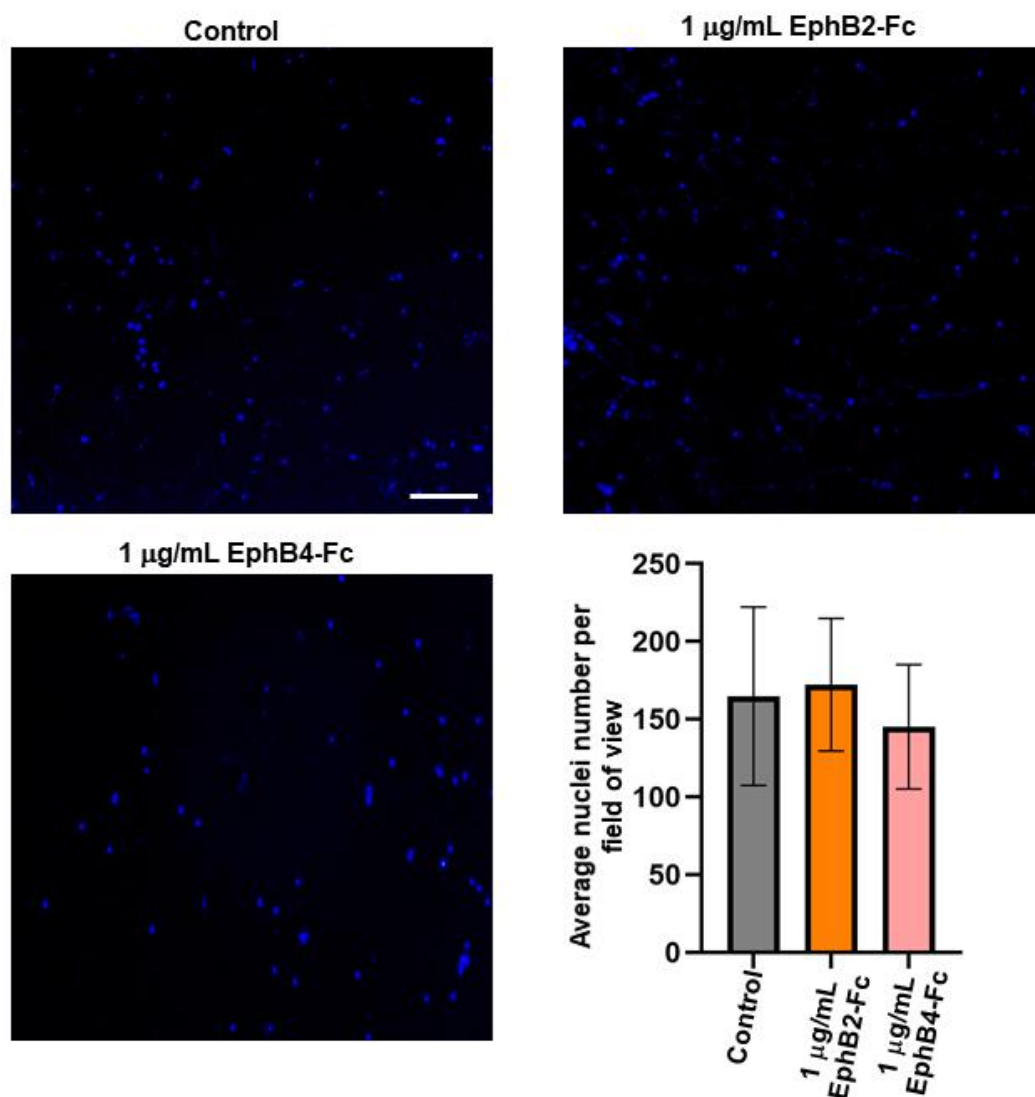


Figure 4.7: Differentiated SH-SY5Y cells did not migrate in response to indirect exposure to EphB2-Fc or EphB4-Fc. Differentiated SH-SY5Y cells were seeded into the upper chambers of transwell inserts with media alone or in the presence of 1 $\mu\text{g/mL}$ Hu-Fc, EphB2-Fc or EphB4-Fc in the bottom chambers and incubated for 32 hours. The cells on the lower surface of the membrane were stained with DAPI (blue). Images were obtained (Scale bar = 200 μm). Control = Human-Fc. Data represents mean $\pm$ SD (number of cells counted in three fields of view per well, with $n=3$ wells per condition). Statistical analysis: Paired Student t-test comparing the control and the Eph-Fc treatment groups.

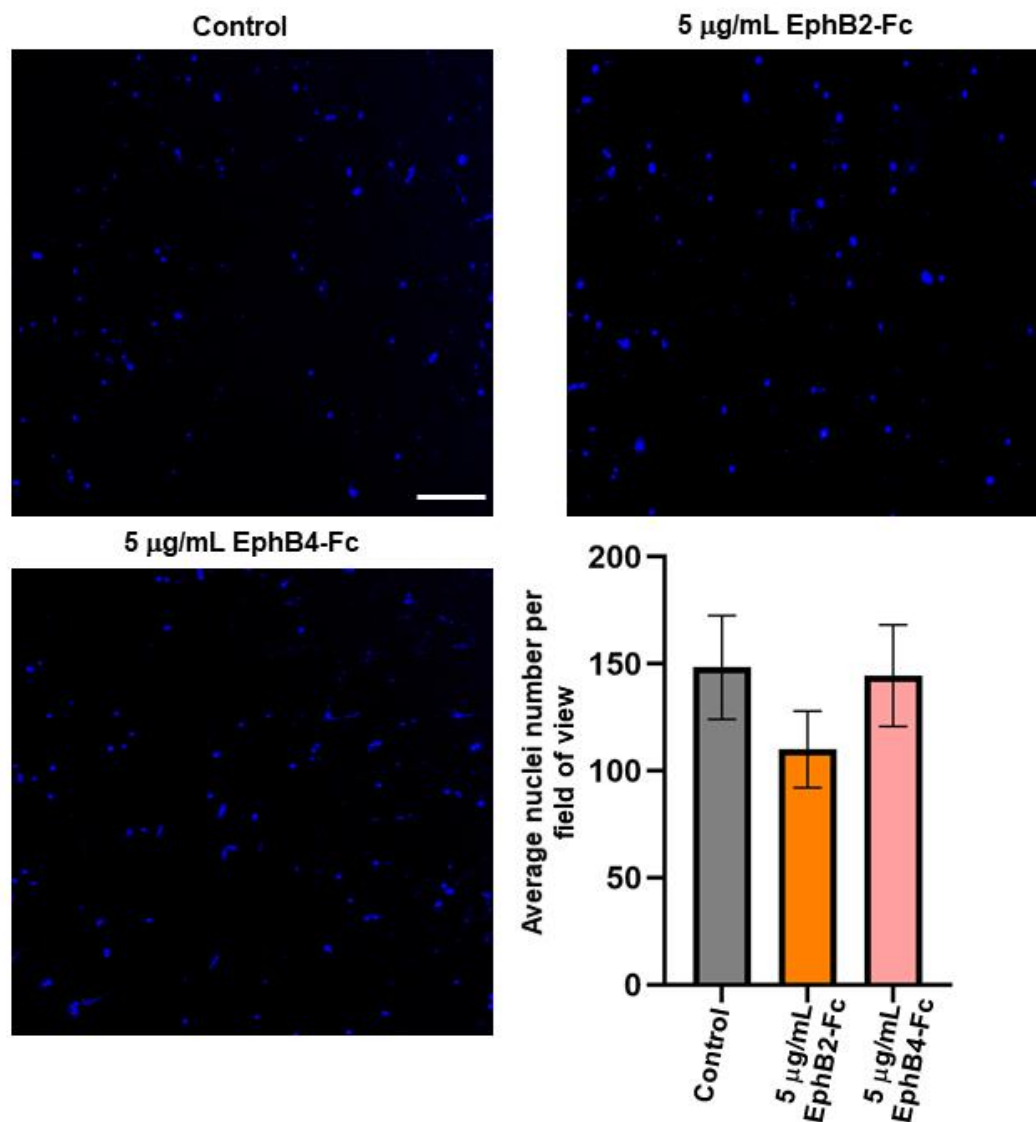


Figure 4.8: Differentiated SH-SY5Y cells did not migrate in response to indirect exposure to EphB2-Fc or EphB4-Fc. Differentiated SH-SY5Y cells were seeded into the upper chambers of transwell inserts with media alone or in the presence of 5 µg/mL Hu-Fc, EphB2-Fc or EphB4-Fc in the bottom chambers and incubated for 32 hours. The cells on the lower surface of the membrane were stained with DAPI (blue). Images were obtained (Scale bar = 200 µm). Control = Human-Fc. Data represents mean ± SD (number of cells counted in three fields of view per well, with n=3 wells per condition). Statistical analysis: Paired Student t-test comparing the control and the Eph-Fc treatment groups.

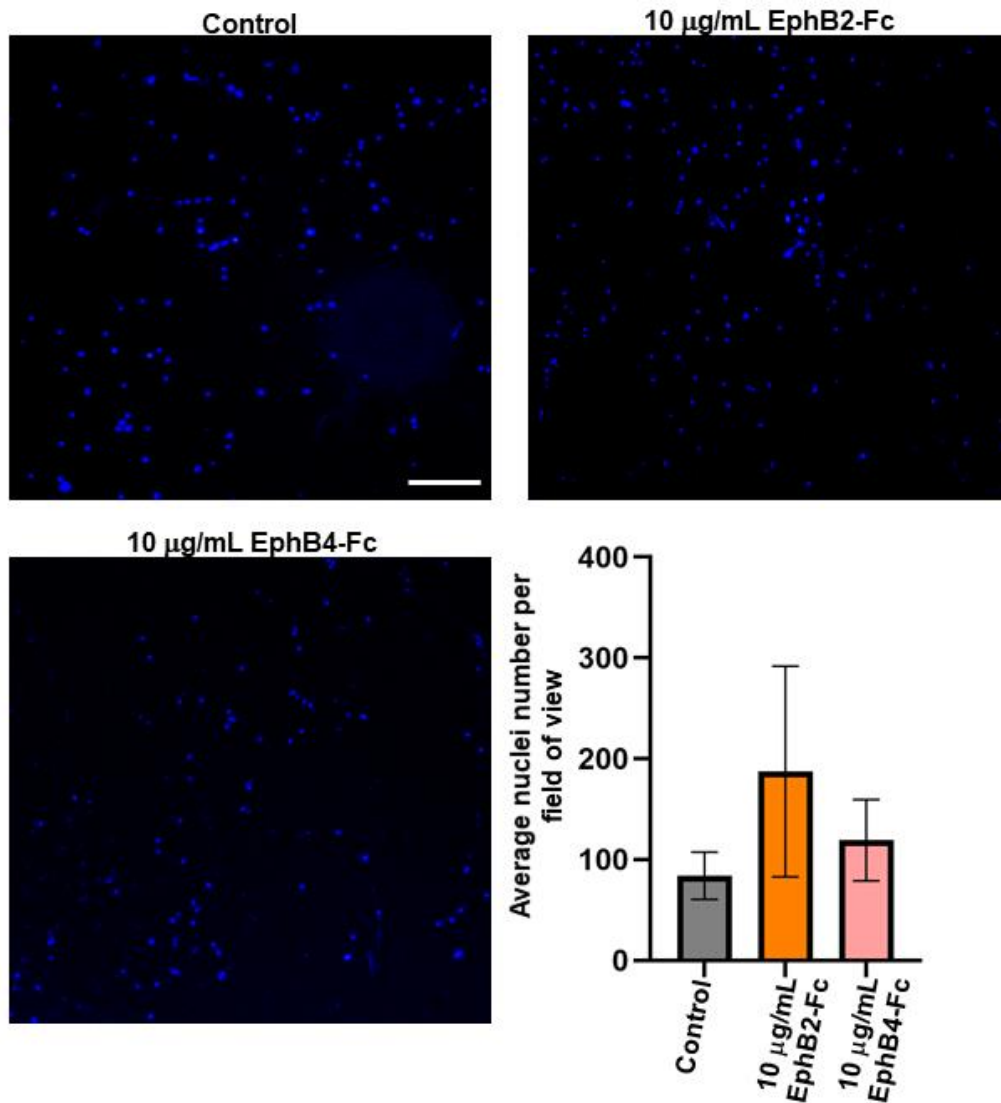


Figure 4.9: Differentiated SH-SY5Y cells did not migrate in response to indirect exposure to EphB2-Fc or EphB4-Fc. Differentiated SH-SY5Y cells were seeded into the upper chambers of transwell inserts with media alone or in the presence of 10 µg/mL Hu-Fc, EphB2-Fc or EphB4-Fc in the bottom chambers and incubated for 32 hours. The cells on the lower surface of the membrane were stained with DAPI (blue). Images were obtained (Scale bar = 200 µm). Control = Human-Fc. Data represents mean ± SD (number of cells counted in three fields of view per well, with n=3 wells per condition). Statistical analysis: Paired Student t-test comparing the control and the Eph-Fc treatment groups.

4.2.4 Direct effect of Eph/ephrin interactions in mediating cross-communication between neurons and BMSC to regulate neural and stromal migration

To determine whether the Eph/ephrin interactions facilitate cross-communication between BMSC and neurons to regulate neural and stromal migration, co-culture assays were utilised (section 2.2.2.6). Due to the Eph/ephrin expression pattern on BMSC and neural SH-SY5Y cells, the EphB2/ephrinB1 and EphB4/ephrinB1 signalling between BMSC and neural cells, respectively, was investigated. A 4-well migration assay was employed to assess the migration of the BMSC and neural cells following pre-treatment of the neural cells with ephrinB1 blocking peptide. This protocol was developed based on previous studies in the literature that have established co-culture assays for neurons and osteoblasts (He et al., 2013, Tomoda et al., 2020) and co-culture assays for osteoblasts and osteoclasts (Deb Roy et al., 2017). In this assay, undifferentiated BMSC were seeded in opposing quadrants and neural-induced SH-SY5Y cells were seeded in the remaining opposing quadrants. Three conditions were tested where the BMSC were cultured with neural-induced SH-SY5Y cells that were either non-treated or pre-treated with 100 μ M of the ephrinB1 blocking peptide or scramble control peptide. Images were obtained over 32 hours to assess the migration of the stromal and neural cells across the boundary and into the neighbouring quadrants (Figure 4.10A).

The neural SH-SY5Y cells did not cross the boundary or migrate into the neighbouring quadrants across the three conditions tested, for the 32-hour duration of the experiment (Figure 4.10A). In contrast, the BMSC crossed the 500 μ m boundary and migrated into the neighbouring quadrants within 24 hours after insert removal in the three conditions tested (Figure 4.10A). There was no significant difference in the number of stromal cells that migrated into the neighbouring neural quadrant between the scramble control and the ephrinB1 blocking peptide treatment group, although a trend was observed towards stromal migration being increased in response to ephrinB1 blocking peptide treatment on the neural cells (Figure 4.10B). There was a trend towards a reduced number of neural cells found along the boundary of the neural quadrant, within a defined area of 500 μ m by 1500 μ m in the ephrinB1 blocking peptide treatment group compared to the scramble control, however, this did not reach statistical significance (Figure 4.10C).

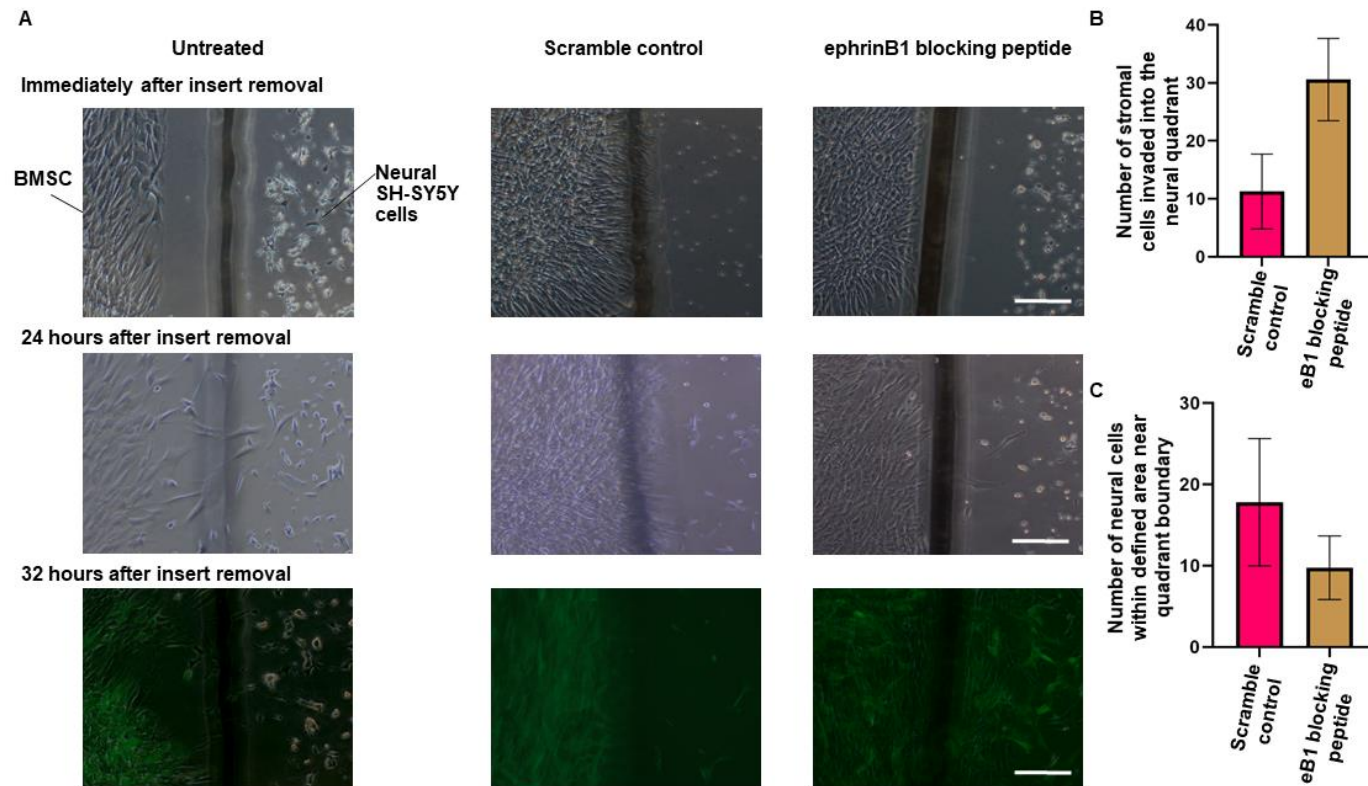


Figure 4.10: GFP-positive BMSC co-cultured with neural-induced SH-SY5Y cells pre-treated with the ephrinB1 blocking peptide. (A) Neural SH-SY5Y cells pre-treated with ephrinB1 blocking peptide (RHS of image) and GFP-positive BMSC (LHS of image) were seeded in 4-well culture inserts and images were obtained at time intervals for a maximum of 32 hours after insert removal. Scale bar = 200 μ m. (B) GFP-positive stromal cells that migrated into the neural quadrants were quantified. Data represents mean $\pm$ SD (n=3 donors per condition with n=2 samples per donor). Statistical analysis: Unpaired Student t-test comparing the control (scramble-treated) and the ephrinB1 blocking peptide treatment group and C) the number of neural cells within a defined area aligned against neural quadrant (500 μ m by 1500 μ m) were quantified. Data represents mean $\pm$ SD (n = 3 donors). Statistical analysis: Paired Student t-test comparing the control (scramble-treated) and the ephrinB1 blocking peptide treatment group.

4.2.5 Effect of Eph/ephrin interactions on neurite cell body number and neurite invasion

4.2.5.1 Effect of Eph/ephrin interactions on neurite cell body number and neurite invasion for DRG pooled from the lumbar region

To determine the functional relevance of the neuro-osteogenic network, the avian embryo DRG that innervate the hind limbs were cultured *in vitro* in the presence of a range of substratum-bound Eph-Fc molecules (section 2.2.2.7). During the *in vitro* characterisation studies, we found that *EPHRINA2*, *EPHRINA5* and *EPHRINB1* expression were upregulated in the SH-SY5Y human neuroblastoma cell line following neural induction, consistent with the avian embryo expression profile where sensory axons express ephrinA2 and ephrinA5 which interact with EphA3/A4 receptors on the motor axons (Wang et al., 2011), and ephrinB1 which interacts with EphB2 expressed by the sensory and motor axons (Luxey et al., 2013). These findings show conservation of mechanism between species. Therefore, the avian embryo is an appropriate *ex vivo* model to investigate the role of Eph/ephrin interactions on DRG neurite cell body number and DRG neurite invasion.

DRG pooled from the lumbar region of the avian embryo were cultured on wells completely coated with 5 µg/mL of substratum-bound Human-Fc, EphA3-Fc, EphA4-Fc, EphB2-Fc and EphB4-Fc (section 2.2.2.7.i). This assay assessed whether the DRG survived on the substratum-bound Eph-Fc molecules and quantified the DAPI-positive neurite cell bodies around the DRG body which were compared between the Eph-Fc treatment groups and the Human-Fc control (Figure 4.11). The DRG survived on all the substratum-bound Eph-Fc molecules and extended processes (Figure 4.11C). However, the neurite cell body number around the DRG body was significantly decreased in response to EphA3-Fc, EphA4-Fc, EphB2-Fc and EphB4-Fc when compared to the Human-Fc control (Figure 4.11C).

Next, the DRG pooled from the lumbar region were cultured near a defined spot of pre-bound 5 µg/mL of Human-Fc, EphA3-Fc, EphA4-Fc, EphB2-Fc and EphB4-Fc molecules (section 2.2.2.7.ii). This assay assessed whether the behaviour of the DRG neurites changed in response to contact with the Eph-Fc molecules, specifically whether the DRG neurites were non-

responsive and invaded the Eph-Fc occupied space as normal or whether the Eph-Fc molecules exerted a repulsive effect. Images were obtained under fluorescence microscopy and the lengths of the Phalloidin-stained axons from the start of the Eph-Fc boundary inwards were measured, as illustrated in Figure 4.12A. There was a trend towards the substratum-bound EphA4-Fc and EphB2-Fc being repulsive to the DRG neurites when compared to the Human-Fc control although this did not reach statistical significance (Figure 4.12B). The DRG neurites appeared non-responsive to the EphA3-Fc and EphA4-Fc and invaded these Eph-Fc occupied spaces as normal when compared to the Human-Fc control (Figure 4.12B).

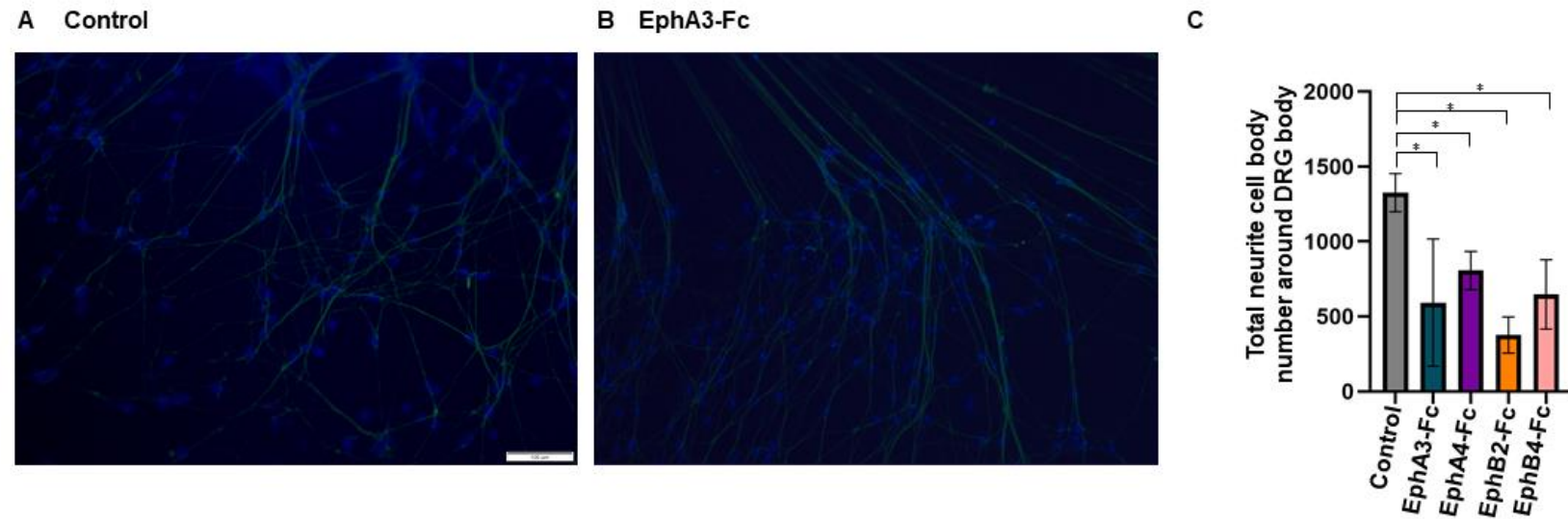


Figure 4.11: Neurite cell body number from DRG pooled from the lumbar region in response to Eph-Fc. DRG were isolated from avian embryos (harvested on embryonic day 7) and cultured on pre-bound Human-Fc (Hu-Fc), EphA3-Fc, EphA4-Fc, EphB2-Fc and EphB4-Fc for 48 hours, ex vivo cultures were stained with anti-NFM (green) and DAPI (blue). (A) Neurite cell body number around DRG pooled from the lumbar region in response to Human-Fc and EphA3-Fc. Scale bar = 100 μ m (B) DAPI-positive neurite cell bodies around the DRG body were quantified per DRG. Control = Human-Fc. Data represents mean $\pm$ SEM (n = 6 animals per treatment group). Statistical analysis: Unpaired Student t-test comparing the control and the Eph-Fc treatment groups, *p < 0.05.

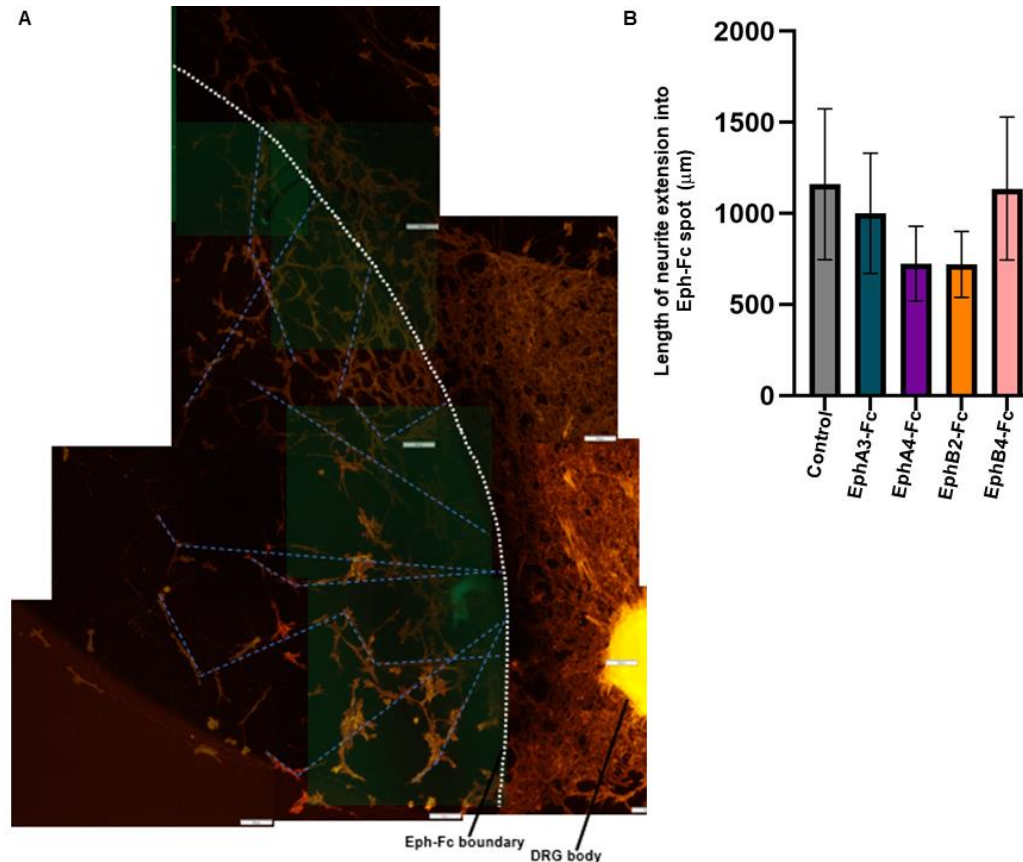


Figure 4.12: Neurite invasion from DRG pooled from the lumbar region in response to Eph-Fc. DRG pooled from the lumbar region were isolated from avian embryos (harvested on embryonic day 7) and cultured near spots of Human-Fc, EphA3-Fc, EphA4-Fc, EphB2-Fc and EphB4-Fc for 48 hours. A) Schematic for invasion analysis showing the lengths of the Phalloidin-stained fibres (examples illustrated as blue dotted lines) from the start of the Eph-Fc boundary (white dotted line) inwards were measured for each DRG. (Anti-mouse IgG-FITC antibody (green – Fc boundary), Phalloidin (red - axonal processes) (n = 6 animals per condition). Scale bar = 100 μm. Control = Human-Fc. Data represents mean ± SEM (n = 6 animals per treatment group). Statistical analysis: Unpaired Student t-test comparing the control and the Eph-Fc treatment groups.

4.2.5.2 Effect of Eph/ephrin interactions on neurite cell body number and neurite invasion for DRG pooled from the lumbar regions (L) 4-6 and (L) 7-8

The DRG at positions lumbar (L) 4-8 within the avian embryo form the ischiadic plexus which in turn forms the sciatic nerve which provides the main sensory innervation of the hindlimbs of the chick embryo (Landmesser and Morris, 1975). The sciatic nerve is also composed of the common peroneal nerve and the tibial nerve which originate from DRG (L) 3-4 and the sural nerve which originates from DRG (L) 4-5 (Laedermann et al., 2014). In addition, DRG (L) 4-6 provides innervation into the dorsal shank component of the hindlimb (Lance-Jones and Landmesser, 1981). Therefore, this assay was repeated to measure the individual responses in neurite cell body number from DRG from positions (L) 4, 5, 6, 7 and 8 on pre-bound 5 µg/mL of Human-IgG-Fc, EphA3-Fc, EphA4-Fc, EphB2-Fc or EphB4-Fc and presented as pooled data from (L) 4-6 and (L) 7-8 (Figure 4.13). A trend towards a decrease in neurite cell body number from DRG (L) 4-6 in response to substratum-bound EphA4-Fc compared to the Human-Fc control was observed although this did not reach statistical significance (Figure 4.13B). The DRG neurites appeared non-responsive to the EphA3-Fc, EphB2-Fc and EphB4-Fc and invaded these Eph-Fc occupied spaces as normal when compared to the Human-Fc control (Figure 4.13B). A trend towards increased DRG neurite cell body number in response to EphB4-Fc compared to the Human-Fc control was observed but this did not reach statistical significance (Figure 4.13B). In addition, a strong trend towards neurites from DRG (L) 7-8 being repelled in response to the EphA3-Fc, EphA4-Fc EphB2-Fc and EphB4-Fc compared to the Human-Fc control was observed, although this did not reach statistical significance (Figure 4.14B). In summary, DRG obtained from lumbar regions (L) 4-6 and (L) 7-8 showed a differential response in neurite cell body number within each of the Eph-Fc-occupied spaces (Figure 4.13B, 4.14B).

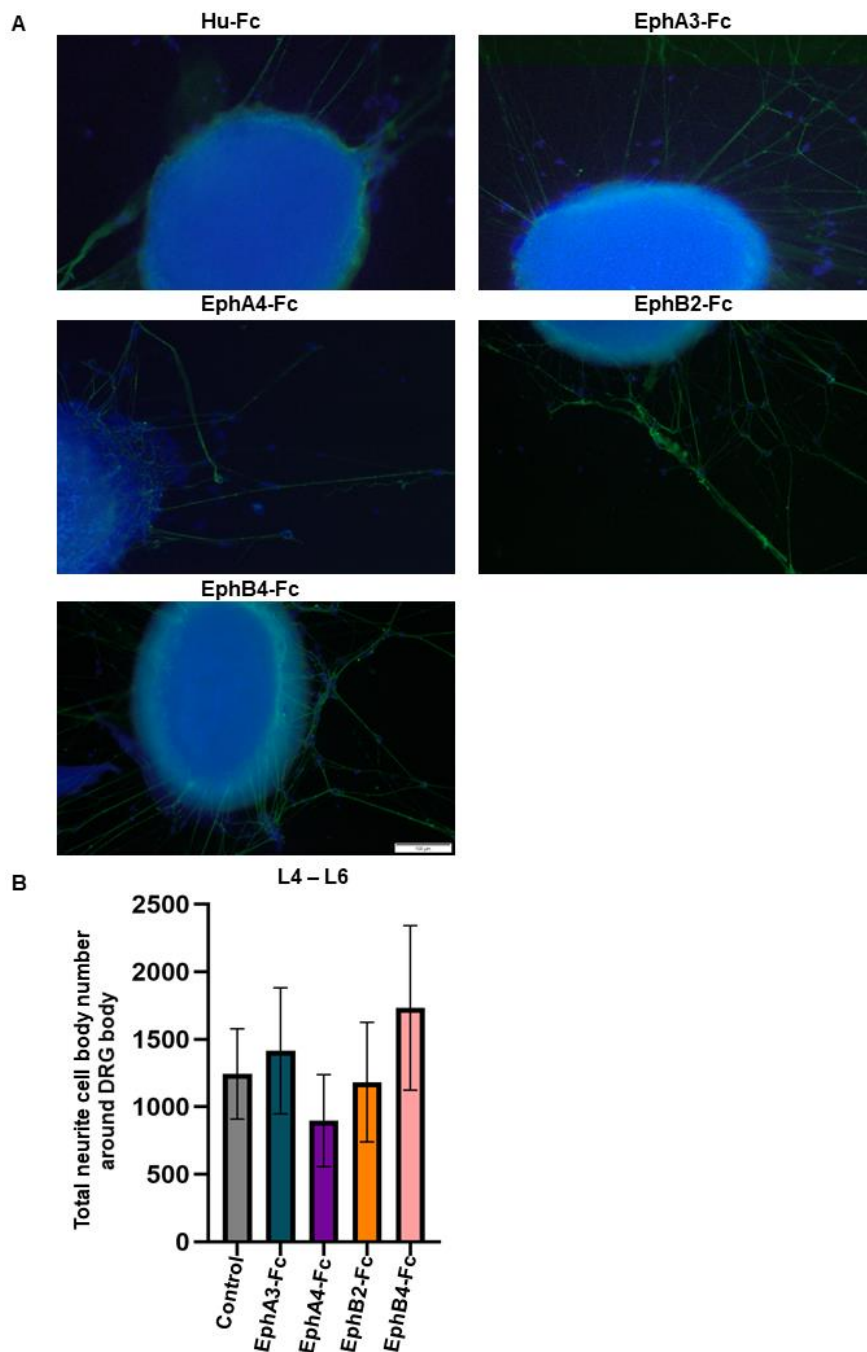


Figure 4.13: Neurite cell body number from DRG from lumbar regions (L) 4-6 in response to Eph-Fc. (A) DRG were isolated from the lumbar regions L4-L6 of avian embryos (harvested on embryonic day 7) and cultured on pre-bound Human-Fc, EphA3-Fc, EphA4-Fc, EphB2-Fc and EphB4-Fc for 48 hours. Cells were stained with anti-NF-M (green) and DAPI (blue). Scale bar = 100 μ M. (B) DAPI-positive nuclei around the DRG body were quantified for each DRG. Data represents mean $\pm$ SEM (n = 5 animals per treatment group). Control = Human-Fc. Statistical analysis: Unpaired Student t-test comparing the control and the Eph-Fc treatment groups.

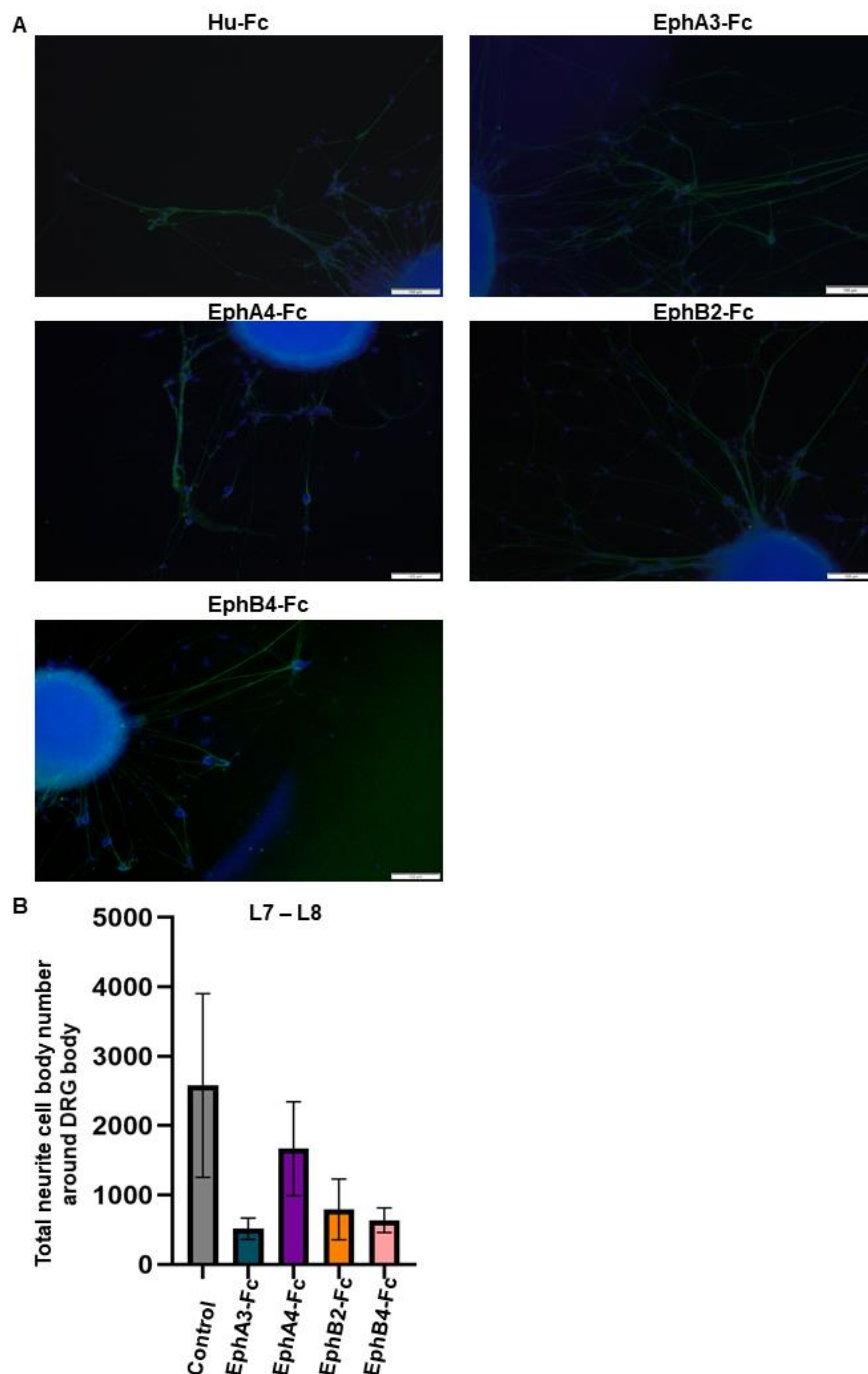


Figure 4.14: Neurite cell body number from DRG from lumbar regions (L) 7-8 in response to Eph-Fc. (A) DRG were isolated from the lumbar regions L7-8 of avian embryos (harvested on embryonic day 7) and cultured on pre-bound Human-Fc, EphA3-Fc, EphA4-Fc, EphB2-Fc and EphB4-Fc for 48 hours. Cells were stained with anti-NF-M (green) and DAPI (blue). Scale bar = 100 μ M. (B) DAPI-positive cell bodies around the DRG body were quantified for each DRG. Data represents mean $\pm$ SEM (n = 5 animals per treatment group). Control = Human-Fc. Statistical analysis: Unpaired Student t-test comparing the control and the Eph-Fc treatment groups.

In addition, the individual responses in neurite invasion from DRG isolated from positions (L) 4, 5, 6, 7 and 8 were assessed by culturing the DRG near defined spots of pre-bound 5 μ g/mL of Human-IgG-Fc, EphA3-Fc, EphA4-Fc, EphB2-Fc or EphB4-Fc (Figure 4.15A). This data was presented as pooled data from lumbar positions (L) 4-6 and (L) 7-8 (Figure 4.15B). Neurite invasion from DRG from lumbar regions (L) 4-8 were measured in response to Eph-Fc by quantifying the length of the individual neurite fibres from the beginning of the spot, extending inwards into the spot (Figure 4.15B) using the method previously illustrated in Figure 4.12. Neurites from DRG (L) 4-6 appeared non-responsive to EphA3-Fc, EphA4-Fc, EphB2-Fc and EphB4-Fc and invaded these Eph-Fc occupied spaces as normal when compared to the Human-Fc control. There was a trend towards increased neurite invasion from DRG (L) 4-6 in response to EphA3-Fc when compared to the Human-Fc control, although this did not reach statistical significance (Figure 4.15B). There was a trend towards neurites from DRG (L) 7-8 being repelled in response to EphA4-Fc when compared to the Human-Fc control (Figure 4.15C). Neurites from DRG (L) 7-8 appeared non-responsive to EphA3-Fc, EphB2-Fc and EphB4-Fc and invaded these Eph-Fc occupied space as normal, when compared to the Human-Fc control (Figure 4.15C). A trend towards increased neurite invasion in response to EphB2-Fc and EphB4-Fc was observed, however, this did not reach statistical significance (Figure 4.15C).

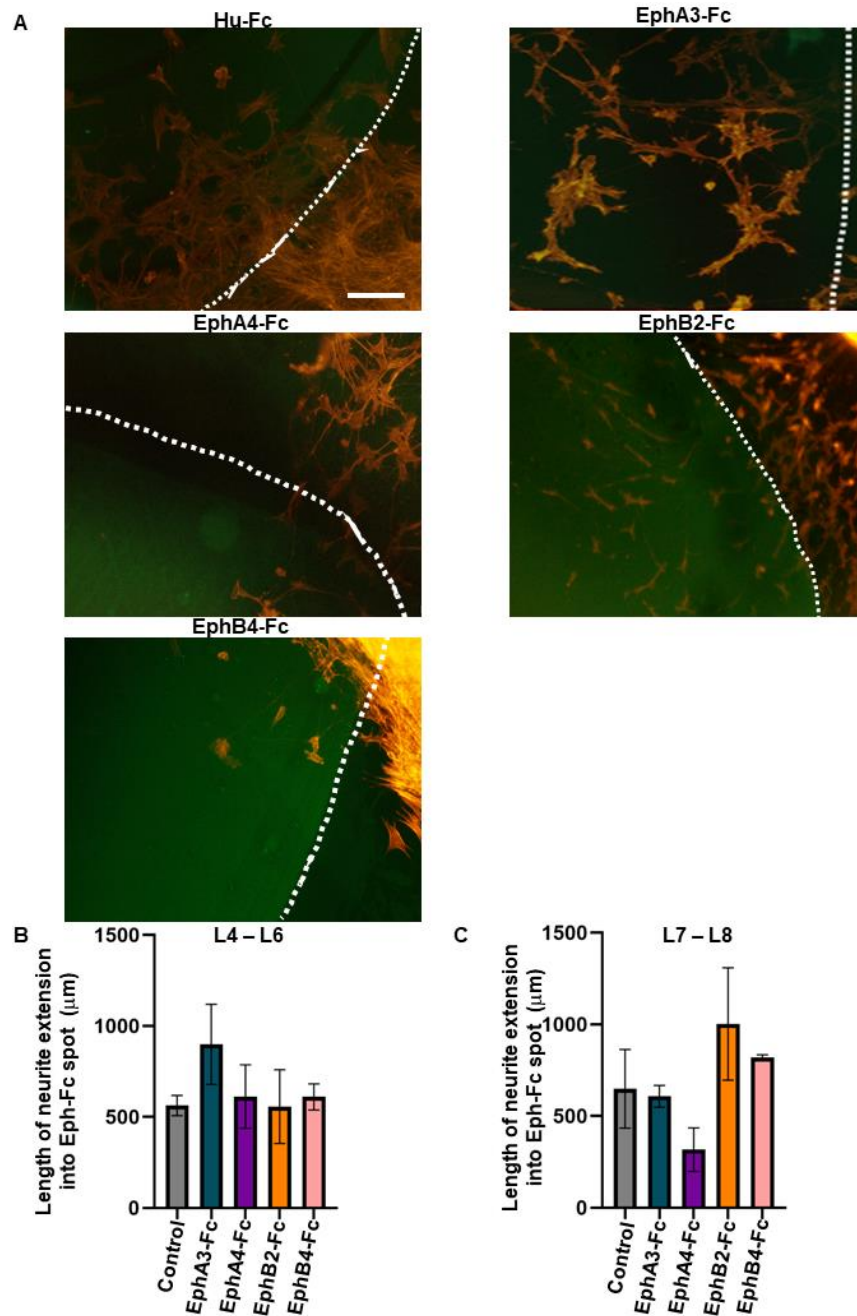


Figure 4.15: Neurite invasion from DRG pooled from lumbar regions (L) 4-6 and (L) 7-8 in response to Eph-Fc. (A) DRG from the lumbar regions L4-6 and L7-8 were isolated from avian embryos (harvested on embryonic day 7) and cultured near spots of pre-bound Human-Fc, EphA3-Fc, EphA4-Fc, EphB2-Fc and EphB4-Fc for 48 hours (Anti-mouse IgG-FITC antibody (green – Fc boundary), Phalloidin (red - axonal processes) Scale bar = 200 μm. (B,C) Representing the measurement of Phalloidin stained axons from the start of the Eph-Fc boundary inwards for DRG pooled from L4-6 and L7-8. Control = Human-Fc. Data represents mean ± SEM (n = 5 animals per treatment group). Statistical analysis: Unpaired Student t-test comparing the control and the Eph-Fc treatment groups for each lumbar region.

4.2.5.3 EPHRIN and neural marker protein expression within the chick embryo spinal cord and DRG

Serial sections of avian embryos harvested on embryonic day 10 (stage 35) were stained with anti-ephrinA2, anti-ephrinB1 and anti-ephrinA5 and stained for the neural markers NF-H and NF-M (Figure 4.6) (section 2.2.3.7.iv). These serial sections show the morphology of the chick embryo, as cross-sections through the spinal cord and DRG at the level of the limb. In these serial sections, staining for NF-M and NF-H revealed that the DRG are closely associated with the growth plates from where bone extends (Figure 4.16A). The cartilage, chondrocytes and emerging cortical bone are apparent within the growth plate (Figure 4.16B). In addition, the serial sections showed that the DRG which stained positive for NF-M and NF-H also appeared to stain positive for ephrinA2, ephrinA5 and ephrinB1 (Figure 4.16C) consistent with previous studies that have shown the avian ephrin expression profile of sensory axons (Wang et al., 2011, Luxey et al., 2013).

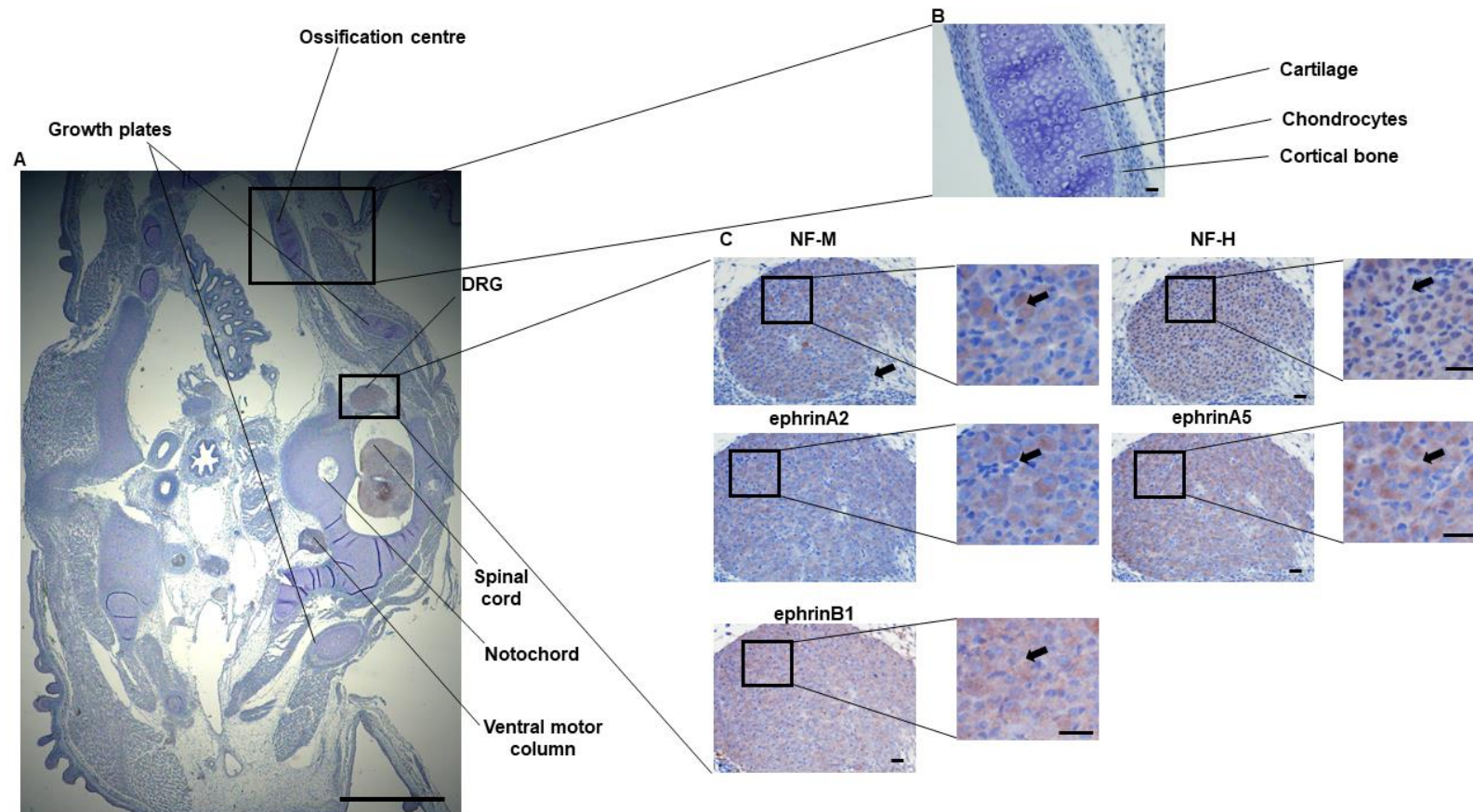


Figure 4.16: EPHRIN and neural marker protein expression within a cross-section through the spinal cord of an avian embryo. Avian embryos were harvested on day 10, embedded in paraffin and transverse serial sections were obtained through the spinal cord at the level of the limb. DRG and nerve bundles stained positive for the neural markers NF-M and NF-H (brown) and stained positive for ephrinA2, ephrinA5 and ephrinB1 (brown). (A) Section stained with NF-H. Scale bar = 500 μ m (B) Ossification centre stained with NF-H. Scale bar = 20 μ m and (C) Serial sections of the DRG stained with antibodies against NF-M, NF-H, ephrinA2, ephrinA5 and ephrinB1. Scale bar = 20 μ m. Specific staining denoted by black arrows.

4.3 Discussion

A functional association between the skeletal system and the neural processes has been reported, where defective neural innervation has been demonstrated to impact fracture repair and skeletal growth (Aro, 1985, Nordsletten et al., 1994, Madsen et al., 1998, Hamdan et al., 2014, Edoff et al., 1997). Furthermore, denervation affects the skeletal aspect of fracture healing, and innervation by CGRP-containing sensory nerves such as the sciatic nerve contributes to fracture healing and bone regeneration (Hukkanen et al., 1993). In addition, correct neural innervation is reported to be important for correct skeletal development (Edoff et al., 1997), and bone loss has been observed within de-nerved bone in adults (Edoff et al., 1997). Therefore, neural adhesion, migration and invasion in response to Eph-ephrin signalling between the neural and stromal populations were investigated.

The ephrinA2, ephrinA5 and ephrinB1 ligands on the neural population were activated via the stimulation of the corresponding EphA3, EphA4, EphB2 and EphB4 receptors on the stromal population using Fc fusion proteins. The activation of both the ephrinA and ephrinB ligands inhibited SH-SY5Y neural adhesion. This was evident with the reduced neural adhesion in response to EphA4-Fc, EphB2-Fc and EphB4-Fc for two hours, and in response to EphA4-Fc, and EphB4-Fc for twenty-four hours. It has been reported that EphB2 binds to ephrinB1, while EphB4 binds with high affinity to ephrinB1 and some affinity to ephrinB2 (Pasquale, 1997). *EPHRINB1* expression was significantly upregulated in the SH-SY5Y line under neural-inductive conditions compared to basal conditions at the endpoint of the neural differentiation assay. In contrast, *EPHRINB2* did not show differential expression under neural-inductive conditions compared to basal conditions. Therefore, these results may suggest that if the EphB2-Fc or EphB4-Fc acts to stimulate the ephrinB1 ligand on the neural population, this ephrinB1 mediated reverse signalling may inhibit neural adhesion. There is evidence in the literature of ephrinB1 maintaining apical adhesion of murine primary neural progenitor cells to the extracellular matrix (Arvanitis et al., 2013). However, in the present study, the adhesion of the neural-differentiated SH-SY5Y human cell line was studied which may explain the difference in findings. Previous studies have shown that EphA4 binds with affinity to ephrinA2 and ephrinA5 (Pasquale, 1997). These results therefore suggest that the EphA4-Fc stimulation may have activated the neural-expressed ephrinA2 or ephrinA5 ligands, which may be

consistent with previous findings that ephrinA5 changed the adhesive property of cortical neurons by inducing the formation of cell aggregates (Zimmer et al., 2007). However, it should be noted that there is no evidence currently in the literature that suggests that ephrinA2 regulates neural adhesion.

The findings of inhibited neural adhesion in response to Eph-Fc molecules are consistent with previous findings in the literature that the Eph/ephrin molecules mediate contact-dependent repulsion of the neurons to regulate neural patterning during development. Where, EphA4 has been reported to induce repulsive ephrinB3 signalling to segregate and therefore, regulate the patterning of specific subsets of interneurons (Zimmer et al., 2011). EphA4 has also been demonstrated to be critical for axon repulsion in the spinal circuits that control locomotion (Wegmeyer et al., 2007). This is consistent with the findings of the current study where neural adhesion of the neural SH-SY5Y cells was inhibited in response to exposure to EphA4-Fc.

Changes in neural marker gene expression and inhibited neural adhesion were observed in the neural differentiated SH-SY5Y cells in response to exposure to the EphB2-Fc and EphB4-Fc molecules. Both EphB2 and EphB4 bind with affinity to ephrinB1 (Pasquale, 1997). The effect of EphB2-Fc and EphB4-Fc mediated cross-communication between the stromal populations and neural cells in regulating neural adhesion and migration was investigated. The indirect effect of the EphB2-Fc and EphB4-Fc via a chemoattractant/ chemorepellent mechanism on the migration of differentiated SH-SY5Y cells was investigated. Increased migration of the differentiated SH-SY5Y cells was observed in response to exposure to the known positive control CXCL12. This observation was consistent with previous findings (Ding et al., 2014), however, there was no significant difference in the migration of the differentiated SH-SY5Y cells in response to EphB2-Fc or EphB4-Fc stimulation at any of the concentrations tested. Therefore, neither EphB2-Fc nor EphB4-Fc showed a significant effect on transwell migration of differentiated SH-SY5Y cells. This observation may be explained by the fact that these Eph/ephrin molecules are contact dependent, and although the SH-SY5Y cells adhere to the transwell membranes, the cells are not in direct contact with the soluble Eph-Fc on the basal surface. When juxtaposed with the findings that DPSC and BMSC are responsive to indirect

exposure to Eph/ephrin molecules (Arthur et al., 2009), it is possible that neural migration does not respond in a similar manner. Therefore, if time had permitted, a co-culture insert migration assay would have been investigated to test the direct effect of the Eph-Fc on neural migration where two of the quadrants were coated with EphB2-Fc or EphB4-Fc while the remaining quadrants were seeded with neural SH-SY5Y cells, and images would have been obtained to assess neural migration in response to EphB2-Fc and EphB4-Fc.

The role of ephrinB1-mediated reverse signalling within the neural population on regulating neural adhesion, and neural and stromal migration was investigated. BMSC were co-cultured with neural SH-SY5Y cells that were pre-treated with the ephrinB1 blocking peptide. The ephrinB1 blocking peptide (PTD-EFNB1-C) is a synthetic peptide that was generated from fusing a fragment of the ephrinB1 C-terminus (amino acids 331-346) to the PTD peptide from the HIV-TAT protein (Tanaka et al., 2010). This ephrinB1 blocking peptide functions intracellularly and becomes distributed within the cytoplasm and binds to the downstream effector proteins that interact with the C-terminus of ephrinB1 (Tanaka et al., 2010). No significant differences in SH-SY5Y neural adhesion or neural/stromal migration were observed when ephrinB1 signalling was blocked in the neural SH-SY5Y cells. Previous findings have shown that EphB2 forward signalling stimulated by ephrinB1 in the stromal population promotes stromal migration (Arthur et al., 2011). These results suggest that the signalling mediated by the C-terminus of the ephrinB1 ligand (including the PDZ binding motif) does not appear to facilitate the ephrinB1/EphB2 or ephrinB1/EphB4 mediated cross-communication between the neural and stromal populations. However, ephrinB1 can also mediate signalling through tyrosine phosphorylation of its cytoplasmic domain mediated by Src family kinases (Palmer et al., 2002) which is unaffected by the ephrinB1 blocking peptide, and may therefore, mediate this cross-communication. If time had permitted, this would have been tested by transducing neural SH-SY5Y cells with full length ephrinB1 constructs or dominant-negative over-expressing constructs and repeating the co-culture adhesion and migration assays. These Eph and ephrin dominant-negative overexpressing constructs have been used in other studies (Yang et al., 2006, Haldimann et al., 2009). These constructs would have been used to identify the mode of Eph/ephrin signalling that occurs between the neural and stromal populations. The dominant negative constructs for the Eph/ephrin molecules overexpress the functional

extracellular domain of the molecule but lack the specific intracellular regions required for forward signalling, specifically the (1) entire cytoplasmic portion, (2) tyrosine kinase domain or (3) amino acid sequence at the C-terminus which includes the PDZ-binding motif (Yang et al., 2006). Therefore, these experiments will confirm if ephrinB1-mediated signalling occurs through tyrosine kinase signalling or the PDZ-binding motif to regulate neural adhesion and neural and stromal migration.

If time permitted, further co-culture adhesion and migration assays would have been repeated with siRNA knockdown of the Eph receptors (EphA3, EphA4, EphB2 and EphB4) on the stromal population or the ephrin ligands (ephrinA2, ephrinA5 and ephrinB1) on the neural population as described in chapter 5. Longer-term studies could be performed using stromal lines overexpressing the Eph receptors outlined above, or stromal cells transduced with dominant negative constructs against these Eph receptors to elucidate which Eph receptor mediates cross-communication with the neural population, as described in chapter 5. In addition, blocking peptides against the EphA4, EphB2 and EphB4 receptors expressed on the stromal population, and blocking peptides against the ephrinB1 ligand on the neural population could have been utilised in these assays (Riedl and Pasquale, 2015). It should be noted that there are no blocking peptides developed against the receptor binding domain of the ephrinA ligands. Furthermore, the co-culture insert migration assay would have been repeated with live imaging under confocal microscopy of DiD-stained neural SH-SY5Y cells and GFP-positive stromal cells to allow a separate investigation into active cell movement and cellular changes in real time as described in chapter 5.

While a significant role for the Eph and ephrin interactions in regulating neural differentiation and neural migration could not be established, the findings from this study suggested that Eph/ephrin interactions influence neural adhesion. However, manipulating EphB2/ephrinB1 or EphB4/ephrinB1 mediated cross-communication between the stromal and neural populations, respectively, by blocking the c-terminal of the ephrinB1 ligand on the neural population did not affect neural adhesion or neural/stromal migration. The functional importance of Eph/ephrin signalling in regulating DRG neurite growth and invasion was investigated. The

Eph/ephrin gene expression profile characterised *in vitro* in the human SH-SY5Y cell line following neural induction (*EPHRINA2*, *EPHRINA5* and *EPHRINB1*) is consistent with the avian Eph/ephrin expression profile, where sensory axons have been shown to express ephrinA2, ephrinA5 and ephrinB1 (Wang et al., 2011, Luxey et al., 2013). Therefore, the present study utilised the avian embryo DRG that innervate the hindlimbs, which were cultured in the presence of a range of substratum-bound Eph-Fc molecules. The avian embryo system is an appropriate *ex vivo* model to investigate the role of the Eph/ephrin interactions in mediating cross-communication between the neural and stromal populations in regulating neurite cell body number and neurite invasion. The protocol that we used in the DRG substratum assays was based on a previous study by Jayasena and colleagues (Jayasena et al., 2005) that investigated the effect of ephrinA5-Fc on axon guidance from the trigeminal ganglia. As the DRG are the source of innervation for the hindlimbs, in this study, the protocol was developed to measure neurite cell body number and neurite invasion from the DRG body in response to Eph-Fc.

In these experiments, the DRG were isolated and cultured using a protocol developed by Powell and colleagues for the culture of DRG from chick embryos (Powell et al., 2014). The DRG were isolated from avian embryos on embryonic day 7 (Powell et al., 2014). In the first instance, the DRG within the lumbar region were pooled together and cultured on wells completely coated with substratum-bound Eph-Fc. While the DRG survived in all conditions and extended neurites, the neurite cell body number around the DRG body was significantly decreased in response to EphA3-Fc, EphA4-Fc, EphB2-Fc and EphB4-Fc when compared to the Human-Fc control. When the DRG pooled from the lumbar region were cultured near a defined spot of substratum-bound Eph-Fc, there was a trend towards the substratum-bound EphA4-Fc and EphB2-Fc being repulsive to the neurites when compared to the Human-Fc control although this did not reach statistical significance. The neurites appeared non-responsive and invaded the EphA3-Fc and EphB4-Fc occupied space as normal when compared to the Human-Fc control.

As a range of DRG contribute to multiple nerves that innervate the hindlimb, we further assessed the individual responses in neurite cell body number and neurite invasion of DRG from specific lumbar compartments in response to exposure to the Eph-Fc molecules. The DRG at positions lumbar (L) 4-8 within the avian embryo form the ischiadic plexus which in turn forms the sciatic nerve which provides the main sensory innervation of the hindlimbs of the chick embryo (Landmesser and Morris, 1975). The sciatic nerve is also composed of the common peroneal nerve and the tibial nerve which originate from DRG (L) 3-4 and the sural nerve which originates from DRG (L) 4-5 (Laedermann et al., 2014). In addition, DRG (L) 4-6 innervate the dorsal shank within the hindlimbs (Lance-Jones and Landmesser, 1981). It was observed that the DRG obtained from lumbar regions (L) 4-6 and (L) 7-8 showed a differential response in neurite cell body number and neurite invasion to each of the Eph-Fc molecules, with a strong trend of inhibited neurite invasion of DRG from (L) 7-8 into Eph-Fc occupied spaces, although statistical significance was not observed between the conditions. These findings are a consistent response for the function of the Eph/ephrin family because the contact-dependent Eph/ephrin molecules mediate a repulsive response in neural innervation (Wegmeyer et al., 2007, Zimmer et al., 2011). Furthermore, transverse serial sections of avian embryos harvested on day 10 (stage 35) were obtained, which showed the cross-section through the chick embryo DRG and spinal cord at the level of the limb. Staining of these serial sections showed that the nerve bundles and DRG showed overlapping expression of the neural markers and the EPHRINA2, EPHRINA5 and EPHRINB1, consistent with previous studies that have characterised the avian ephrin expression profile of the sensory axons (Luxey et al., 2013, Wang et al., 2011). These findings also showed that the anti-ephrin antibodies worked in immunohistochemistry.

In summary, while a significant role for the Eph and ephrin interactions in regulating neural differentiation and neural migration could not be established, the findings from this study suggested that Eph/ephrin interactions influence neural adhesion. However, manipulating EphB2/ephrinB1 or EphB4/ephrinB1 mediated cross-communication between the stromal and neural populations, respectively, by blocking the c-terminal of the ephrinB1 ligand on the neural population did not affect neural adhesion or neural and stromal migration. Furthermore, the neurites from DRG pooled from the lumbar region were significantly repelled when

cultured on substratum-bound Eph-Fc molecules. When DRG from specific lumbar compartments was cultured, it was found that the neurites from the DRG obtained from lumbar regions (L) 4-6 and (L) 7-8 showed differential responses in neurite cell body number and neurite invasion to each of the Eph-Fc molecules although statistical significance was not observed between the conditions. While each of the DRG experiments was performed twice such that each Eph-Fc treatment was tested on five animals, this lack of statistical significance may be the result of a lack of statistical power in these assays. Therefore, if time permitted, these DRG neurite assays would have been repeated such that each Eph-Fc treatment was tested on more animals. A power calculation was performed using statistical software to determine the number of animals needed per condition to obtain statistical power and it was determined that n=13 animals per treatment group would be appropriate to achieve a 20% difference between the Human-Fc and Eph-Fc treatment groups with statistical power of 0.8 and $p < 0.05$ statistical significance in a t-test (Brant).

Chapter 5 General discussion and future directions

5.1 Thesis summary

There is substantial evidence from the literature that suggests a close association and cross-communication between the osteoblasts and osteoclasts of the skeletal system and the neural cells of the nervous system at the anatomical and physiological levels (Obata et al., 2007, Nencini and Ivanusic, 2016, Kodama et al., 2017, Chenu, 2004). The cellular and molecular interaction between the neural and skeletal systems has been termed the neuro-osteogenic network, and this cross-communication is critical for the correct function of both systems (Lerner and Persson, 2008, Togari et al., 2012, Abeynayake et al., 2021). Importantly, skeletal growth and repair are dependent on correct neural innervation (Aro, 1985, Nordsletten et al., 1994, Madsen et al., 1998, Hamdan et al., 2014, Edoff et al., 1997) while disruptions to the skeletal system exert adverse effects on the nervous system (Burt-Pichat et al., 2005, Williams and Ivanusic, 2008). To date, different molecular mechanisms have been reported to mediate communication between bone and neural populations, including BMP, CGRP, CXCR4/CXCL12 signalling, Eph/ephrin interactions, Netrins, Neurotrophins, Semaphorins and Slit/Robo (Abeynayake et al., 2021, He et al., 2013, Zhu et al., 2019). These molecules mediate communication between the osteoclasts and osteoblasts to regulate skeletal development and maintain bone homeostasis, while some of these molecules also mediate cross-talk between bone cells (osteogenic and osteoclastic) and neurons to regulate the development of the skeletal and neural systems and post-natal bone/ neural tissue repair as previously described.

The Eph and ephrin family of receptor tyrosine kinases predominantly function through cell-to-cell interactions (Pasquale, 1997). The Eph/ ephrin interactions have known roles independently in the skeletal system regulating bone homeostasis, and skeletal growth and repair (Xing et al., 2010, Vrahnas et al., 2019, Arthur et al., 2011, Arthur et al., 2013, Nguyen et al., 2016, Arthur et al., 2018, Arthur et al., 2020, Shimizu et al., 2011, Wang et al., 2014, Stiffel et al., 2014, Takyar et al., 2013, Irie et al., 2009, Zhao et al., 2006). Eph/ephrin signalling also influences early axon guidance and patterning within the central and peripheral nervous systems (Eberhart, 2004, Jayasena et al., 2005, Eberhart et al., 2000, Eberhart et al., 2002,

Helmbacher et al., 2000, Kramer et al., 2006, Krull et al., 1997, Luxey et al., 2013, Munoz et al., 2005, Winning and Krull, 2011). Therefore, the roles of the Eph/ ephrin interactions in mediating cross-talk between the neural and stromal populations to regulate neural and stromal function were investigated.

An *in vitro* neural differentiation assay was established for the human neuroblastoma line SH-SY5Y, using a protocol modified from earlier studies (Forster et al., 2016). The Eph/ephrin expression profile was characterised following neural induction using the SH-SY5Y human neuroblastoma line. The SH-SY5Y line showed upregulated expression of *EPHRINA2*, *EPHRINA5* and *EPHRINB1* with the progression of neural differentiation. *EPHRINA2*, *EPHRINA5* and *EPHRINB1* were the most differentially expressed with a significant increase in expression under neural-inductive conditions compared to basal conditions at the endpoint of the neural differentiation assay. This is consistent with the literature where positive roles for ephrinA2, ephrinA5 and ephrinB1 in regulating neural differentiation have been identified (Vazin et al., 2009, Hara et al., 2010, Homman-Ludiye et al., 2017). However, contradictory roles have been identified for ephrinA5 (Wang et al., 2016) and ephrinB1 (Qiu et al., 2008). A limitation of this study is that the Eph/ephrin gene expression profile could not be confirmed at the protein level by immunocytochemistry and Western blotting as these antibodies did not work in our hands for these assays. While the antibodies showed some reactivity in flow cytometry, the ephrin expression levels were low. The weak protein expression profiles of these EPHRIN of interest are consistent with the low absolute RNA levels of expression that were observed in the gene expression profiles of these molecules during the neural differentiation assay. Furthermore, our Western blotting detection system may not have been sensitive enough to detect the staining.

Studies examined the roles of three Eph/ ephrin interacting pairs mediating cross-talk between BMSC and neurons, respectively, in regulating neural function. These included the stromal expressing Eph receptors with neural expressing ephrin ligands (1) EphA3 or EphA4 with ephrinA2 (2) EphA3, EphA4 or EphB2 with ephrinA5 and (3) EphB2 or EphB4 with ephrinB1, respectively (Pasquale, 1997, Pasquale, 2008). To assess this, reverse signalling mediated

through EphA3-Fc, EphA4-Fc, EphB2-Fc and EphB4-Fc activation was used to induce the neural-expressed ephrinA2, ephrinA5 and ephrinB1 ligands. In response, the gene expression levels of early and mature neuronal and sensory-like markers were differentially expressed in the SH-SY5Y line following neural induction in response to specific Eph-Fc. This suggests that different ephrinA and ephrinB ligands on the neuronal population have varied influence on neural maturation and differentiation. This is consistent with other findings that have shown that ephrinA2 promotes neural differentiation of neural progenitors (Homman-Ludiye et al., 2017, Wang et al., 2016) while conflicting roles in regulating the neural differentiation of neural progenitors have been identified for ephrinA5 (Hara et al., 2010, Wang et al., 2016) and ephrinB1 (Qiu et al., 2008, Vazin et al., 2009). However, there was no significant difference in neuronal morphology.

The activation of both the ephrinA and ephrinB ligands inhibited SH-SY5Y neural adhesion. This was evident with the reduced neural adhesion in response to EphA4-Fc, EphB2-Fc and EphB4-Fc for two hours, and in response to EphA4-Fc and EphB4-Fc for twenty-four hours. Therefore, these results may suggest that if the EphB2-Fc or EphB4-Fc acts to stimulate the ephrinB1 ligand on the neural population, this ephrinB1 mediated reverse signalling may inhibit neural adhesion. These findings of inhibited neural adhesion in response to Eph-Fc molecules are consistent with previous studies showing that Eph/ephrin molecules provide contact-mediated repulsive cues that regulate the navigation of neuronal axons (Zimmer et al., 2011, Wegmeyer et al., 2007).

The effect of EphB2-Fc and EphB4-Fc mediated cross-communication between the stromal populations and neural cells in regulating neural migration was also investigated. The data showed that neither EphB2-Fc nor EphB4-Fc exerted a significant effect on neural migration through indirect exposure via transwell inserts, which suggests that neural migration does not respond indirectly to Eph/ephrin molecules unlike DPSC and BMSC (Arthur et al., 2009). No significant difference between the neural adhesion and neural/stromal migration was observed in response to blocking the PDZ-binding motif of ephrinB1 ligand on the neural population. However, it is possible ephrinB1 signalling is mediated by tyrosine phosphorylation of the

cytoplasmic domain. Therefore, if time permitted, the co-culture adhesion and migration assays would have been repeated with siRNA knockdown of the Eph receptors (EphA3, EphA4, EphB2 and EphB4) on the stromal population or the ephrin ligands (ephrinA2, ephrinA5 and ephrinB1) on the neural population. Longer-term studies could be performed using stromal lines overexpressing the Eph receptors outlined above, or stromal cells transduced with dominant negative constructs against these Eph receptors to elucidate which Eph receptor mediates cross-communication with the neural population. Similarly, neural SH-SY5Y cells could have been transduced with ephrinB1 overexpressing dominant negative constructs. In addition, blocking peptides against the EphA4, EphB2 and EphB4 receptors expressed on the stromal population, and blocking peptides against the ephrinB1 ligand on the neural population could have been utilised in these assays (Riedl and Pasquale, 2015).

The Eph/ephrin gene expression profile characterised *in vitro* in the human neuroblastoma cell line SH-SY5Y is consistent with the avian embryo expression profile, where sensory axons express ephrinA2, ephrinA5 and ephrinB1 (Wang et al., 2011, Luxey et al., 2013). Since conservation of mechanism between species has been identified, it was investigated whether substratum-bound Eph-Fc molecules were repulsive to specific subsets of DRG neurites. In the first instance, the DRG pooled from the lumbar region were cultured on wells coated completely with substratum-bound Eph-Fc. While the DRG survived in all conditions, the neurite cell body number around the DRG body was significantly decreased in response to EphA3-Fc, EphA4-Fc, EphB2-Fc and EphB4-Fc when compared to the Human-Fc control. When the DRG pooled from the lumbar region were cultured near a defined spot of Human-Fc, EphA3-Fc, EphA4-Fc, EphB2-Fc and EphB4-Fc molecules, there was a trend towards the substratum-bound EphA4-Fc and EphB2-Fc being repulsive to the DRG neurites when compared to the Human-Fc control although this did not reach statistical significance. As a range of DRG contribute to multiple nerves that innervate the hindlimb, DRG from specific lumbar compartments were cultured on or near the substratum-bound Eph-Fc molecules. The DRG at positions lumbar (L) 4-8 within the avian embryo form the sciatic nerve which provides the main sensory innervation of the hindlimbs of the chick embryo (Landmesser and Morris, 1975). In addition, DRG (L) 4-6 provides innervation into the dorsal shank component of the hindlimbs (Lance-Jones and Landmesser, 1981). DRG obtained from lumbar regions (L) 4-6

and (L) 7-8 showed a differential response in neurite cell body number and neurite invasion to each of the Eph-Fc although statistical significance was not observed between the conditions. This lack of statistical significance may be due to a lack of statistical power in these assays. Therefore, if time permitted, these DRG assays would have been repeated to produce greater statistical power. A power calculation was performed using statistical software (Brant) which yielded a sample size of $n=13$ animals per treatment group which would be needed to achieve a 20% difference in neurite response between the Human-Fc control and the Eph-Fc treatment groups with a power level of 0.8 and statistical significance of $p < 0.05$ in a t-test. The key findings from this thesis have been summarised in Figure 5.1.

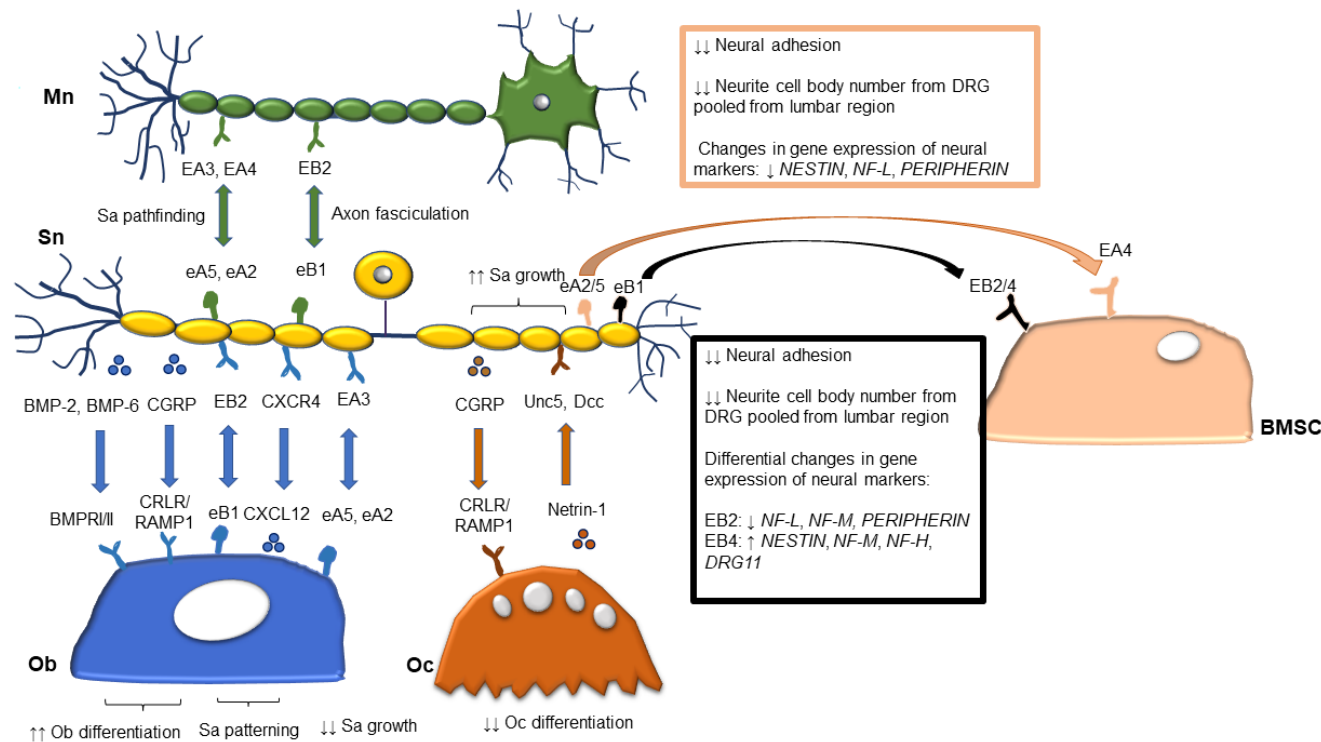


Figure 5.1: The molecular mechanisms mediating communication between sensory and motor neurons with osteoblasts and osteoclasts, and key findings from this project of potential molecular mechanisms that mediate communication between neurons and BMSC. Cross-talk between neurons and BMSC may occur between the ephrinA2/A5 (eA2/5) ligands on the neural population and the EphA4 (EA4) receptor on BMSC, and neural-expressed ephrinB1 (eB1) and stromal expressed EphB2/4 (EB2/4) to regulate DRG neurite cell body number, neural adhesion, and neural marker gene expression. Sn = Sensory neuron, Mn = Motor neuron, Ob = Osteoblast, Oc = Osteoclast. Modified from Abeynayake et al (2021).

The clinical significance of these findings is that there is potential for Eph/ephrin mediated cross-talk to be targeted in the treatment of pathologies such as osteoporosis, or the acceleration of fracture repair. Osteoporosis is a major contributor to debilitating skeletal fractures associated with increased mortality rates (Nazrun et al., 2014). Bisphosphonates are the first line of pharmacotherapy that are administered in the treatment of osteoporosis and are potent anti-resorptive agents (Ma et al., 2017). However, the long-term use of bisphosphonates can result in atypical femoral fractures characterized by absent or delayed fracture healing (Lenart et al., 2009). These observations suggest that the increased inhibition of bone remodelling induced by bisphosphonates leads to the accumulation of microdamage, which compromises the mechanical strength of bone and increases the risk of fractures (Ma et al., 2017). Due to the limitations of bisphosphonates, newer anti-resorptive agents such as Cathepsin K inhibitor, and anabolic therapies that promote bone formation have been developed in the treatment of osteoporosis (Russow et al., 2018, Mukherjee and Chattopadhyay, 2016). Furthermore, both osteoporosis and trauma-induced fracture are associated with chronic and persistent skeletal pain which requires prolonged pain management strategies (Mantyh, 2014). These pain management strategies include continued treatment with non-steroidal anti-inflammatory drugs or opioids that are associated with severe long-term side effects such as gastrointestinal damage, renal toxicity and addiction (Marras and Leali, 2016). The extended use of opioids is also associated with a lower bone mineral density and higher incidence of osteoporosis and osteopenia (Grey et al., 2011, Duarte et al., 2013).

The limitations of the current treatment strategies highlight the necessity for novel strategies that treat osteoporosis and accelerate fracture repair that target not only the bone cells but also address the associated neural issues. Eph/ephrin interactions have known involvement during fracture repair, skeletal development and progression of osteoporosis (Arthur et al., 2013, Nguyen et al., 2016, Arthur et al., 2018, Arthur et al., 2020). Targeting and manipulating the Eph/ephrin mediated communication between neural and bone populations may be a potential novel strategy to treat osteoporosis and accelerate fracture repair. There is fundamental evidence that the molecular mechanisms mediating cross-talk between the neural and stromal/bone cells contribute to skeletal health. The findings from this study may contribute towards further studies that investigate the importance of Eph/ephrin mediated communication

during skeletal and neural development and fracture repair. Eph/ephrin signalling already has great potential in the treatment of skeletal health and has already been established as a pharmacological target in cancer and neurological disorders (Xiao et al., 2020, Fu et al., 2014, Vargas et al., 2018, Martini et al., 2019, Kurose et al., 2019, Tan et al., 2019). In addition, there is potential for Eph/ephrin signalling to be targeted in the treatment of osteoarthritis and bone pain, where EphB1/ephrinB1 signalling has already been established as a potential therapeutic target in the treatment of the pain associated with bone cancer (Dong et al., 2011) while EphB4 has been implicated in osteoarthritis and treatment with ephrinB2 has been shown to alleviate the abnormal metabolism of human osteoarthritic chondrocytes (Kwan Tat et al., 2009, Kwan Tat et al., 2008). Following any promising results from these studies, inhibitors and agonists could be developed in the future that target these Eph/ephrin interactions, which could be assessed for their effects on skeletal development and facilitating fracture repair and the treatment of skeletal pathologies such as osteoporosis and osteoarthritis.

5.2 Future directions

If time permitted, further *in vitro* and *in vivo* experiments would have been performed to investigate the roles of Eph/ephrin mediated communication between the neurons and BMSC in regulating neural adhesion and migration, and whether this was mediated by the molecules of interest: ephrinA2, ephrinA5 and ephrinB1. The importance of Eph/ephrin mediated communication during skeletal development and fracture repair would have also been investigated.

5.2.1 Investigate the role of EphB2/ephrinB1 and EphB4/ephrinB1 signalling in regulating neural adhesion

If time had permitted, the role of EphB2/ephrinB1 signalling and EphB4/ephrinB1 signalling in mediating cross-communication between the stromal and neural populations would have been investigated. An adhesion assay would have been utilised, where neural SH-SY5Y cells would be liberated with TrypLE on day 12, and left untreated, or incubated with either 100 μ M of the ephrinB1 blocking peptide or the scramble peptide, and re-plated in wells pre-coated

with Human-IgG-Fc, EphB2-Fc or EphB4-Fc fusion proteins, and neural adhesion would have been assessed at two and 24 hours. This would determine whether the inhibition of neural adhesion observed in response to EphB2-Fc at two hours, and in response to EphB4-Fc at two and 24 hours, would be reversed by blocking ephrinB1 signalling within the neural population.

5.2.2 Investigate the role of EphA/ephrinA and EphB/ephrinB signalling in mediating cross-communication between BMSC and neurons to regulate neural adhesion and neural/stromal migration

If time permitted, the co-culture adhesion and migration assays would have been repeated after siRNA knockdown of an Eph receptor of interest (EphA3, EphA4, EphB2 or EphB4) on the stromal population or an ephrin ligand of interest (ephrinA2, ephrinA5 or ephrinB1) on the neural population. siRNA knockdown of the ephrinB1 ligand would have been performed on the neural population, and these neural cells would have been stimulated with either EphB2-Fc or EphB4-Fc to investigate the functional response. *EPHRINB2* was highly expressed on neural SH-SY5Y cells, although it should be noted that differential gene expression of this molecule was not observed under neural-inductive conditions compared to basal conditions at the endpoint of the neural differentiation assay. The ephrinB2 ligand binds with high affinity to EphB4 and some affinity to EphB2 and EphA4 (Pasquale, 1997, Qin et al., 2009). Therefore, the assays would have been repeated to investigate whether the ephrinB2 or ephrinB1 ligand expressed on the neural population influences neural adhesion and neural/stromal migration. Where, siRNA knockdown of the ephrinB1 ligand would have been performed on the neural population, and these neural cells would have been stimulated with either EphB2-Fc or EphB4-Fc to investigate the functional response. The neural population could have also been treated with the blocking peptide available against ephrinB2.

Longer-term studies would have been performed using stromal lines overexpressing the Eph receptors outlined above, or stromal cells transduced with dominant negative constructs against these Eph receptors to elucidate which stromal expressed Eph receptor mediates cross-communication with the neural population. Similarly, neural SH-SY5Y cells would have been transduced with ephrinA2/A5 or ephrinB1/B2 dominant negative constructs and co-cultured

with BMSC. This would confirm the EphB (EphB1/B2 or EphB4) receptors on the stromal population that interact with the ephrinB (ephrinB1 or ephrinB2) ligands on the neural population to regulate neural adhesion, and whether this interaction is mediated through tyrosine kinase signalling or the PDZ-binding motif. In addition, blocking peptides against the EphA4, EphB2 and EphB4 receptors expressed on the stromal population would have been utilised in these assays, as used in other studies (Riedl and Pasquale, 2015). Collectively, these experiments would determine the mechanism of cross-communication between the BMSC and neurons that regulates neural adhesion and migration.

Furthermore, the co-culture insert migration assay would have been repeated with live imaging under confocal microscopy of DiD-stained neural SH-SY5Y cells and GFP-positive stromal cells to allow a separate investigation into active cell movement and cellular changes in real time. Time lapse and live cell imaging would have been performed to record active cell movement of the stromal and neural cells in real-time during the migration assays. Based on the context of the spatial and temporal changes observed during live-cell imaging of osteoclasts (McDonald et al., 2021), this data may provide more information about the migration behaviour of the stromal and neural cells.

5.2.3 Investigate the role of Eph/ephrin mediated cross-communication in skeletal and neural development

If time had permitted, *in ovo* studies would have been carried out to investigate the role of EphB2/ephrinB1 and EphB4/ephrinB1 mediated cross-communication in skeletal and neural development (Ethics approval SAHMRI SAM126). The EphB2-Fc or EphB4-Fc fusion proteins or EphB2/EphB4/ephrinB1 blocking peptides would have been injected into the right hind limb bud while the control would have been injected into the left hind limb bud of the developing avian embryo (stage 16, condensation of mesoderm in the limb bud, and stage 18/19, established limb buds (Hamburger and Hamilton, 1951). Embryos would have been harvested at stage 28/29 (Day 5-6) and stage 35 (Day 9) and embedded in paraffin. These paraffin sections would have been stained for EphB2, EphB4, ephrinA2, ephrinA5 and ephrinB1 and counterstained for the neural marker β -III Tubulin (Tuj1). Masson's trichrome

stains would have been generated for these slides to identify newly mineralized bone. Histomorphometric analysis would have been performed using the semi-automated image analysis system Osteomeasure to detect changes in neural and skeletal development. Co-localisation of neural marker and ephrin staining would have been investigated to determine whether EPHRIN expression coincides with the nerve bundles. Concurrently, micro-computed tomography analysis would have been used to show this osseous-neural relationship within a 3D setting, where the developing bones could be visualised, and specialised contrasting agents would have been developed to visualise the neural tissue in relation to the bone. This non-invasive system would have allowed functional and anatomical changes in bone and the neural processes to be measured as changes in trabecular and cortical bone volume and thickness, and changes in number, density, diameter and length of nerve fibres, respectively.

If time had permitted, further studies would have been carried out which would have involved the generation of mutant avian embryos with a conditional deletion of *EPHRINA2*, *EPHRINA5* and *EPHRINB1* under a sensory-like promoter such as the transcription factor DRG11 (Rebelo et al., 2007). The DRG neurite assays would then be repeated where DRG explants would be isolated from avian embryos and cultured on pre-bound Eph-Fc molecules to investigate the specific role of these Eph/ephrin interactions within the desired sensory neuronal cell type in regulating neurite cell body number and neurite invasion. A CRISPR/Cas9 gene editing approach could also be taken to introduce precise mutations in the functional sites of the *EPHRINA2*, *EPHRINA5* and *EPHRINB1* ligands or to delete the respective genes in the sensory neurons to investigate the role of these molecules, similar to other studies that have used this precise gene editing method to knockout genes in avian models (Oishi et al., 2016, Koslová et al., 2020).

5.2.4 Investigate the role of Eph/ephrin mediated cross-communication in skeletal repair

The current thesis showed that Eph/ephrin cross-communication between the neural and stromal populations appears to influence neural adhesion and neurite cell body number and neurite invasion. These findings warrant further investigation into the importance of

Eph/ephrin cross-communication during skeletal repair. This investigation was started by staining for EPHRIN expression within mouse fracture sections (section 2.2.3.7.iv). A previous study generated femoral fractures in eight-week old mice with ethics approval (SA Pathology Animal Ethics committee #56/09) (Arthur et al. 2013). The femur forms via endochondral ossification which begins in mice between E12.5 and E13.5 and proceeds through development until the bones have matured (Kaufman and Kaufman, 1992). The fractured femora that were harvested at 2 weeks post-fracture from wildtype mice were previously embedded. In the current study, sections obtained from these samples were stained with anti-ephrinA2, anti-ephrinB1 and anti-ephrinA5, where the chondrocytes within the cartilage stained positive for ephrinB1 but did not stain positive for ephrinA2 or ephrinA5 (Figure 5.2C).

If time had permitted, further studies would have been carried out which would have involved the generation of mutant mice with a conditional deletion of *EphrinB1* under a sensory neuron specific promoter such as the transcription factor DRG11 which is expressed in small DRG neurons and within the developing dorsal horn (Rebelo et al., 2007). Femoral fractures would have been generated in these mice. The fractured and non-fractured femora would have been harvested at 2-, 4-, 6-, 8- and 12-weeks post-fracture from the wildtype and ephrinB1 knock-out mice. These femora would have been fixed, decalcified and embedded in paraffin and stained for ephrinA2, ephrinA5 and ephrinB1 and counterstained for NF-M. Innervation/denervation patterns would have been compared between fractured and non-fractured femora by histomorphometric analysis using the Osteomeasure System. Co-localisation of neural marker and ephrin staining would have been assessed to determine whether EPHRIN expression coincides with the nerve bundle. Furthermore, EPHRIN distribution within the nerves that innervate the bone at the fracture site would have been compared between the wildtype and ephrinB1 knockout mice to assess the response to skeletal trauma. Concurrently, micro-computed tomography analysis would have been used to analyse any functional and anatomical changes in bone and the nerve processes as previously described.

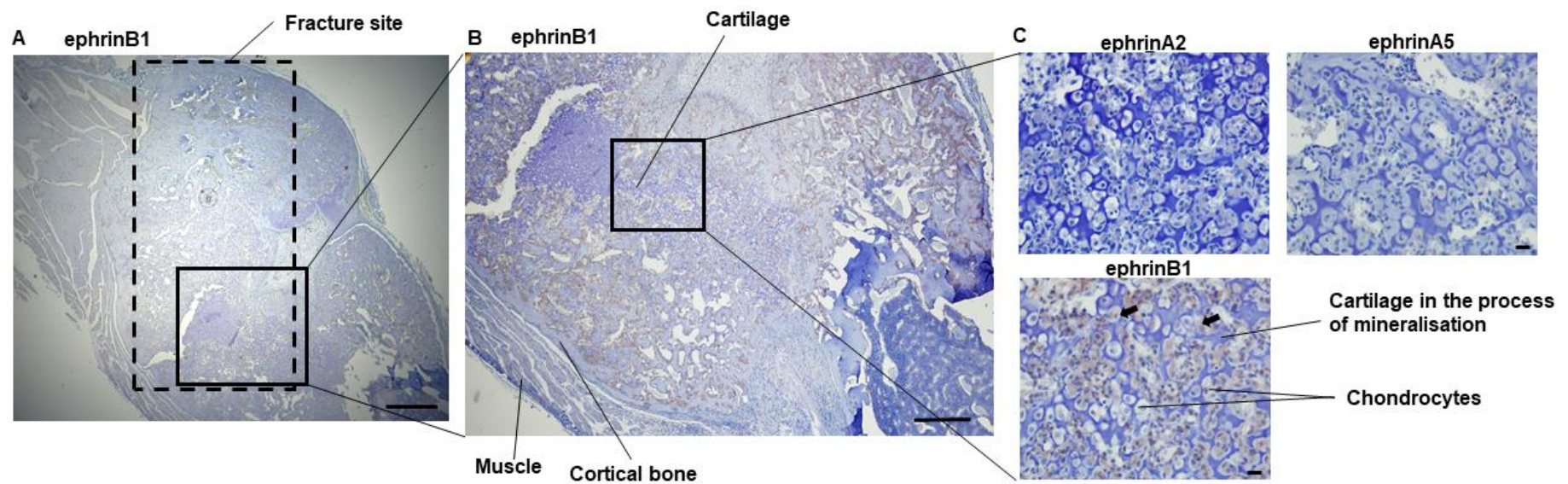


Figure 5.2: EPHRIN protein expression within serial femoral sections harvested from wildtype mice two-weeks post-fracture. Serial sections were obtained from previously acquired paraffin-embedded femora harvested two weeks post-fracture from wildtype mice. Serial sections were stained with antibodies against ephrinA2, ephrinA5 and ephrinB1 (brown). (A, B) Fracture callus stained with anti-ephrinB1. (A) Scale bar = 500 μ m (B) Scale bar = 200 μ m and (C) Chondrocytes within the fracture callus stained with antibodies against ephrinA2, ephrinA5 and ephrinB1. Scale bar = 20 μ m. Specific staining within chondrocytes (black arrows).

5.3 Concluding remarks

In the last few decades, there has been increasing interest in the neuro-osteogenic network and its potential for therapeutic targeting in the treatment of skeletal and neural pathologies. However, limited information has been available about the molecular mechanisms responsible for mediating this cross-communication between the neural and skeletal populations. The present research has presented, for the first time, that the Eph/ephrin interactions and specifically the activation of the ephrinA2, ephrinA5 and ephrinB1 ligands expressed on the neural population via the stimulation of the corresponding Eph receptors did not significantly influence neural differentiation or neural migration but did inhibit neural adhesion, and neurite cell body number from avian embryo DRG pooled from the lumbar region. Manipulating Eph/ephrin mediated cross-communication between the neural and stromal populations by blocking ephrinB1 signalling within the neural population did not affect neural adhesion or neural and stromal migration.

These are original and novel findings that contribute to our current understanding of the neuro-osteogenic network and of the underlying Eph/ephrin mediated mechanisms that may facilitate cross-communication between the stromal and neural populations to regulate neural and stromal function. The work in this thesis has generated several relevant questions and future research is warranted into the exact molecular mechanisms of Eph/ephrin mediated cross-talk in regulating neural and stromal function, and its importance during skeletal and neural development and fracture repair. These findings may contribute towards further studies that investigate the potential for the development of agonists and antagonists as therapeutic agents that target Eph/ephrin signalling to facilitate fracture repair and treatment of conditions such as osteoporosis and osteoarthritis that are significantly influenced by innervation. The impact of these novel molecules on skeletal pathologies, and any underlying side effects, should be carefully assessed to determine the clinical potential of these therapies.

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