

Phylogenetic profiling of genes involved in axonal regeneration

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Summary

Unlike peripheral nervous system (PNS), the adult mammalian central nervous system (CNS) has limited ability to regenerate after injury. Several factors are known to either promote or inhibit axonal regeneration. In this work, we used phylogenetic profiling to determine whether neuronal regeneration is an evolutionarily conserved process or not. We utilized PrePhyloPro database to find the correlation and similarity in phylogenetic profiles of 13 genes that are known to be involved in either promoting or inhibiting axonal regeneration. Although axonal regeneration does not seem to be evolutionarily conserved, clusters of potentially linked genes were identified. EPHNB3, SEMA3A, SEMA4D, STAT3 and SOCS3 and MAG showing a high degree of correlation in their phylogenetic profiles. The whole proteome analysis of the phylogenetic profiles identified candidate genes that might interact with the reference genes, based on the clustering of the factors and the high degree of correlation with the phylogenetic profiles of the reference genes. These functional linkage between reference and candidate genes suggests involvement in axonal regeneration.

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LIST OF ABBREVIATION

ATF-3: Activating transcription factor 3

bZip: Basic region leucine zipper

cAMP: cyclic adenosine monophosphate (cAMP)

CDC: Centers for disease control and prevention

cGMP: cyclic guanosine monophosphate

CNS: Central nervous system

CREB: cyclic AMP responsive element binding

CSPG: chondroitin sulfate proteoglycan

DINE: Damaged-induced neuronal endopeptidase

DRD1A: Dopamine receptor D1

DRG: Dorsal root ganglion

EPHNB3: Ephrin B3

ER: endoplasmic reticulum

ERF: Ets2 repressor factor

GALR1: Galanin receptor 1

GAP43: Growth-associated protein 43

GDNF: Glial cell line-derived neurotrophic factor

Gp130: glycoprotein 130

GPI: glycosyl phosphatidylinositol

GPR174: G protein-coupled receptor 174

GRH: Grainhead

GRHL: Grainhead-like family

HMG: high mobility group

Hsp27: Heat shock protein 27

IGFBP7: Insulin-like growth factor binding protein 7

ITIM: Immunoreceptor tyrosine-based inhibitory motifs

JAK/STAT: Janus kinase/signal transducer and activators of transcription

KEGG: Kyoto Encyclopedia of Genes and Genomes

LINGO-1: Leucine-rich repeat and immunoglobulin-like domain-containing nogo receptor-interacting protein

LRSM1: Leucine-rich repeat and sterile alpha motif-containing 1

MAG: myelin-associated glycoprotein

MiRNA: microRNA

NgR: Nogo receptor

NMBR: Neuromedin B receptor

NR4A2: Nuclear receptor subfamily 4 group A member 2

NTN: Neurturin

OLIG2: Oligodendrocyte lineage transcription factor 2

Omgp; oligodendrocyte myelin glycoprotein

OPRD: δ -opioid receptor

PirB: Paired immunoglobulin-like receptor B

PKC: Protein kinase C

PNS: Peripheral nervous system

PSPN: Persephin A

PTEN: Phosphatase and testin homolog

RAG: regeneration-associated gene

Rho: Ras homolog gene family member A

RICS: Rho GTPase activating protein 32

ROCK: Rho-A/Rho kinase

RORB: RAR-related orphan receptor B

SEMA3A: Semaphorin 3A

SEMA3D: Semaphorin 3D

SEMA3S: Class III semaphorins

SEMA4D; Semaphorin 4D

SHP1: Src homology region 2-containing protein tyrosine phosphatase 1

SHP2: Src homology region 2-containing protein tyrosine phosphatase 2

SOCS3: Suppressor of cytokine signaling 3

SOX11: SRY (sex determining region Y) - box 11

SPRR1A: small proline-rich protein 1A

STAT3: signal transducer and activator transcription 3

TBI: Traumatic brain injury

TNF: Tumor necrosis factor

TSPAN19: Tetraspanin 19

VAMP: Vesicle-associated membrane protein 1

VSIG10: V-set and immunoglobulin domain containing 10

WHO: World Health Organization

WNT4: Wingless-type MMTV integration site family

Wnts: Wingless-type MMTV integration site family

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Introduction

The central nervous system (CNS) injuries including the spinal cord and brain are the major health problems in the world (1). As many as 500,000 people around the world suffer from spinal cord injury each year. These people are 2 to 5 times more vulnerable to die prematurely (2; 3). According to the World Health Organization (WHO), traumatic brain injury (TBI) will be the major cause of death and disability by the year 2020 (4). More than 2 million people per year suffer from traumatic brain injuries in US (1). Over 500,000 people suffer from stroke and at least 10,000 people suffer from spinal cord injury annually (1). According to United States Centers for Disease Control and Prevention (CDC), 50,000 people die from TBI each year and 85,000 people live with long-term disability (5).

Injury to the adult central nervous system is highly destructive because central neurons are unable to spontaneously regenerate after injury. (6; 7). Two main obstacles exist for CNS regeneration: myelin-associated inhibitors and glial scar formation (6; 8). The glial scar which acts as a physical barrier to growth is formed by astrocytes after injury. It contains inhibitory molecules that prevent axon growth. The main inhibitory molecules found in glial scar are chondroitin sulfate proteoglycans (CSPGs). These molecules are upregulated by astrocytes after injury. They both bind to the membrane and are secreted to extracellular space. However, it is no receptor identified yet for them (6). Myelin-associated inhibitors are proteins expressed by oligodendrocytes and are distributed in the myelin (8). The most important myelin-associated inhibitors included: Nogo-A, myelin-associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein (OMgp). All these three bind to an axonal Nogo 66 receptor (NgR) (6; 8). There are also shows affinity to paired

immunoglobulin-like receptor B (PirB) (9). We can also name Ephrin B3 and Semaphorin 4D (SEMA4D) as myelin-associated inhibitors (10; 11). Administration of Semaphorin 3A (SEMA3A) inhibitor to rat's injured spinal cord also shows enhancing regenerative responses and functional recovery suggesting the role of SEMA3A in an axon regeneration inhibition (12).

Axon regeneration in the peripheral nervous system

In contrast to CNS, axons in peripheral nervous system readily regenerate after injury. Proximal portion of the axon forms buds that regenerate and re-innervate their distal targets. After injury, the debris is removed by glial cells and macrophages clearing the path for growing axons. In the meantime, the distal portion of the axons undergoes Wallerian degeneration that includes myelin and axons degeneration but not endoneurium. The endoneurium will later serve as a duct to direct axons to their correct target (6; 13).

After nerve injury, numerous regeneration-associated genes (RAGs) are upregulated. These include Activating transcription factor 3 (ATF3), signal transducer and activator transcription 3 (STAT3), SRY-box containing gene 11 (SOX11), growth-association protein 43 (GAP43) and c-Jun (6; 14).

The absence of Nogo and rapid clearance of MAG by macrophage can lead to successful axon regeneration in the peripheral nervous system (PNS). Myelin-associated inhibitors are proteins expressed by oligodendrocytes and are distributed in the myelin (8). Moreover, Schwann cells down regulate all myelin proteins after injury. This can also be a factor for different regeneration capacity between CNS and PNS (8). Another important factor in CNS regeneration failure is that CNS neurons do not upregulate growth-associated genes in the same way as PNS neurons. Therefore, even in the absence of inhibitors, CNS regeneration is limited (6).

Generally, we categorized genes that associated with regeneration in two groups: 1- Genes inhibiting regeneration 2-Genes promoting regeneration. Here, we briefly discuss these genes and product and their association with regeneration.

Genes inhibiting regeneration

Nogo-A

Nogo-A is an isoform of Nogo or reticulon-4 protein and is known as neurite outgrowth inhibitor. It is expressed in oligodendrocytes, neocortical neurons, cerebellum, spinal motor neurons, optic nerves, dorsal root ganglion (DRG) neurons, dentate gyrus, amygdala, thalamus, hypothalamic nuclei and *etc.* Nogo-A is a membrane protein of 1163 amino acids and ~ 190-250 kDa. It has two inhibitory domains: A 66-amino acid domain which expressed on the surface of oligodendrocytes inhibit axon outgrowth through interaction with NgR. The other adjacent 24 amino acids sequence which is not inhibitory itself facilitates binding of Nogo-66 to NgR. It has a specific N-terminal domain which is known to inhibit neurite outgrowth and fibroblast spreading. Nogo-A has seven N-glycosylation sites and multiple potential O-glycosylation sites (15-17).

Two intracellular signaling pathways are linked to Nogo: calcium and Rho-A/Rho kinase (ROCK) pathway. Blocking each of these pathways resulted in inhibition of myelin- or Nogo-induced growth inhibition. Myelin inhibitors trigger growth cone collapse and growth inhibition via RhoA activation and Rac inactivation. The growth cone is a specialized structure at the distal end of neurite that is associated with axonal development and regeneration. This conical expansion actively extends and retracts during neurite outgrowth and axon pathfinding. The inhibitory effect of myelin is modulated through modulating RhoA activation by protein kinase C (PKC). PKC modulates RhoA activation in a calcium-dependent manner. Another mechanism underlying the

role of calcium in neuronal growth and nogo-induced inhibition is that myelin inhibitors binding to NgR1 trigger phosphorylation of epidermal growth factor receptor (EGFR) in a calcium-dependent manner. Moreover, cytoplasmic Ca^{2+} regulates neurite elongation and motility of growth cone by regulating cytoskeletal structure (15; 18-21).

It is shown that genetic deletion of Nogo-A promotes raphespinal and corticospinal tract growth. Using antibodies against Nogo-A caused dorsal root ganglion neurites to grow on myelin and CNS. Nogo-A knockout resulted in moderate enhancement in regenerative sprouting and elongation. Generally, gene deletion or neutralization of NogoA and blockade of NgR or downstream messenger ROCK and Rho increased regenerative sprouting and functional recovery. In addition to the role in inhibiting neuronal regeneration, it is suggested that Nogo-A may have a role in tumor development and regulating apoptosis (22; 23).

Myelin-associated glycoprotein

Myelin-associated glycoprotein (MAG) is a transmembrane protein belongs to the immunoglobulin superfamily. MAG is a sialic acid-binding protein. It is expressed by glia in the peripheral and CNS nerves. MAG has two isoforms: L-MAG which is formed early in development and exist in considerable amounts in adult CNS nerves. L-MAG is moderately expressed in peripheral nerves during development and is almost absent in the adult. The other isoform S-MAG is the abundant form in the adult PNS but not in adult human CNS (16).

MAG has a role in formation and maintenance of myelin sheaths and is found in both CNS and peripheral myelin. It is known as a potent inhibitor of axonal regeneration. It seems that MAG is a bifunctional regulator of axon growth, therefore in addition to the inhibitory role in neurite

outgrowth, it has role in promoting neurite extension depending on the age and type of neurons (24; 25).

Lee *et al.* showed that MAG deletion resulted in increasing sprouting of serotonergic axons while reducing sprouting in CST (26). Wong *et al.* indicated that *in situ* inactivation of MAG in chick retinal optic nerve increases the optic nerve regeneration (27).

Oligodendrocyte myelin glycoprotein

Oligodendrocyte myelin glycoprotein (OMgp) is a glycosyl phosphatidylinositol (GPI)-linked protein with 440 amino acids long and ~ 110-120 kDa molecular weight. OMgp is expressed by oligodendrocytes and neurons in CNS. OMgp is mainly found in purkinje cells of the cerebellum, motor neurons of brainstem and pyramidal cells of hippocampus. It is also found in PNS (16).

The expression of OMgp is extensively increased following spinal cord injury. OMgp is known to involve in growth cone collapse and inhibition of neurite outgrowth. It contains a leucine-rich repeat domain which is needed for its function. OMgp is also suggested to play a role in formation and maintenance of myelin sheaths as well as regulation of cell proliferation. OMgp is the minor component of myelin and is localized predominantly in paranodal loops, next to the node of Ranvier (8; 28).

Nogo receptor

The Nogo receptor (NgR) is a common receptor for at least three myelin-associated inhibitors Nogo, MAG and OMgp. NgR is a GPI-linked protein with 473 amino acids in length and ~85kDa molecular weight. It contains a series of leucine-rich repeats (LRRs) followed by C-terminal sequence. Nogo receptor is found in neocortex, amygdala, thalamus, hippocampus,

substantia nigra and caudate putamen. NgR is also presented in non-neuronal cells including macrophages, fibroblasts, glioma cells and some human immune cells. Since Nogo receptor is a GPI-linked protein it is unable to transduce the signal across the membrane, therefore, requires co-receptor. NgR is often assembled in a heterotrimeric complex composed of p75, Leucine-rich repeat and Immunoglobulin-like domain-containing nogo receptor-interacting protein (LINGO-1) and TROY (16; 29).

The Nogo receptor mediates growth cone collapse and inhibit axon regeneration by interacting with Nogo, MAG and OMgp. NgR antagonist NEP-40 treatment resulted in sprouting of serotonergic fibers and enhancement of axonal growth in corticospinal tract. Moreover, blocking NgR was shown to increase raphespinal and corticospinal axonal sprouting into lumbar spinal cord after spinal cord injury. Using neutralizing NgR antibody was also indicated that NgR has a role in neurite outgrowth inhibition (30; 31).

Phosphatase and testin homolog

Phosphatase and testin homolog (PTEN) is a dual specificity phosphatase that have both lipid phosphatase and protein phosphatase activity. PTEN is the tumor suppressor which inactivated by gene deletion or mutation in almost all cancers (32). PTEN protein contains 3 parts: 1- C-terminal C2 domain 2- 50 aminoacid C-terminal tail containing PDZ binding motif and CK2 phosphorylation sites 3- N-terminal phosphatase catalytic domain (33).

Suppressor of cytokine signaling-3

Suppressor of cytokine signaling 3 (SOCS3) is a member of SOCS family which is known as a negative regulator of Janus kinase/signal transducer and activators of transcription (JAK/STAT). SOCS3 protein like other members of family contains a short C-terminal, a central SH2 domain, SOCS box and N-terminal domain. SOCS3 binds the JAKs and directly inhibiting their kinase activity (34).

SOCS3 deletion in adult retinal ganglion cells promotes injured optic nerve to regenerate. This regeneration-promoting effect was shown to be glycoprotein 130 (gp 130) - dependent pathway. Consistently, it was demonstrated that deletion of SOCS3 and PTEN could be a synergistically strategy to promote robust axonal regeneration. SOCS3 deletion by activation of STAT3 pathway and PTEN deletion by activation of mTOR pathway could consider as distinctive but complementary arms of regenerative program (35-37).

Ephrin B3

Ephrin B3 is a member of ephrin family which is known to involve in brain development. EPH family has an important role in the migration of specific populations of cells in developing and adult brain. The EPH and EPH-related receptors contain the largest family of receptor protein tyrosine kinases (38; 39).

Ephrin-B3 is a member of transmembrane ephrin B family that binds to the EphA4 receptor on axons of the corticospinal tract. It is shown to negatively regulate cell proliferation and apoptosis in the adult subventricular zone. Ephrin B3 also regulates synapse and spine formation in cortex and hippocampus. Benson *et al.* proposed ephrin B3 as a myelin-based inhibitor of neurite

outgrowth indicating that ephrin B3/EphA4 is active in myelin inhibition, regeneration and functional recovery after spinal cord injury (10; 40).

Myelin-associated glycoprotein which is a member of immunoglobulin super family is found in both CNS and PNS. MAG seems to be bifunctional. It promotes regeneration in young neurons while inhibiting regeneration in adult neurons. In vitro evidence shows that MAG inhibits neurite outgrowth. However, genetic deletion of MAG shows no observable regeneration in corticospinal tract after injury in mice (6; 8).

Oligodendrocyte myelin glycoprotein is a glycosylphosphatidyl inositol (GPI)-linked protein which is expressed in both PNS and CNS. It interacts with NgR1 and PirB through interaction via leucine-rich repeat resulting neurite growth inhibition (6; 8; 41).

Semaphorin 4D

Semaphorin 4D (SEMA4D), also known as CD100, is a member of class IV membrane-bound proteins semaphorins. It is first identified in the immune system that has a role in activation of B cells and T cells. SEMA4D has two known receptors: Plexin-B1 and CD72. Plexin-B1 with high-affinity for SEMA4D, is expressed at a very low level in the adult brain. CD72, another SEMA4D receptor with lower affinity, which originally recognized in the immune system, expressed widely in adult CNS suggesting an inhibitory activity of SEMA4D in the adult CNS (11; 42).

Moreau-Fauvarque, C. *et al.* indicated that SEMA4D is expressed by myelin and oligodendrocytes in the CNS and is upregulated during myelination period or after spinal cord injury near the site of lesion. They suggest SEMA4D as an inhibitory factor for axonal growth and regeneration but the exact mechanism is still unknown (11).

Recently, it was found that miR-125b, a microRNAs (miRNAs) that conserved between Mexican axolotl salamander (*Ambystoma mexicanum*) and mammals is essential for axonal regeneration and functional recovery in axolotls (43). MiR-125b mediates its action through direct downstream target SEMA4D. Increasing the miR-125 in mammal downregulated SEMA4D and other glial-scar-related genes, therefore promoting regeneration and enhancing functional recovery (43). Consistently, it is indicated that knockdown of SEMA4D in oligodendrocytes improves functional recovery after spinal cord injury (44).

Semaphorin 3A

Semaphorin 3A belongs to the class III semaphorins and is known for its repulsive signals and apoptotic activities. SEMA3A is expressed in cranial and spinal motor neurons, neurons of the amygdala and subiculum, entorhinal cortex stellate cells and olfactory bulb mitral cells (42). In the intact adult brain, SEMA3A is not detectable in glial cells but expressed at low level in the leptomeningeal sheet. The expression of SEMA3A in peripheral and central lesions of motor neurons is in opposite way. SEMA3A expression is decreased in motor neurons after peripheral nerve lesion while it is upregulated after central nerve lesion (42).

Administration of SM-216289, a selective inhibitor of SEMA3A, into lesion site of transected spinal cord of adult rat results in increasing level of regeneration and /or preservation of injured neurons, decreasing apoptotic cell number, robust Schwann cell-mediated myelination and improvement in functional recovery. These results demonstrate the important role of SEMA3A as a regeneration inhibitor (12). Consistently, it is shown that immediate injection of SEMA3A to goldfish eye after nerve injury interferes with several pathways of regeneration including axon growth, retinal ganglion cells survival and myelin debris clearance (45).

Genes promoting regeneration

Activating transcription factor-3

Activating transcription factor 3 is a member of ATF/cyclic AMP-responsive element binding (CREB) family of transcription factors. It is characterized by a basic region leucine zipper (bZip) motif which has a role in binding to ATF/CRE promoter DNA sequence. This region is also necessary to form homo- and hetero-dimer with other bZip protein to regulate transcription. ATF-3 regulates transcription by binding to DNA sites with Jun proteins. It activates transcription if form heterodimer and repress it if form homodimer (46; 47).

ATF-3 is expressed in axotomized neurons. It is upregulated in neurons projecting to injured peripheral nerves. It is also upregulated in some types of CNS neurons after injury. For example, in the retina, expression of ATF-3 is linked linearly to the degree of axonal injury. Retinal ganglion cells have a greater capacity for axonal regeneration than other CNS neurons. ATF-3 expression in neurons and glia are at a very low level but it is increased rapidly following injury. ATF-3 was upregulated by glia where axons are regenerated while it is upregulated at low level where axons are not regenerated. It is suggested that the contribution of ATF-3 in nerve regeneration is due to enhancing the growth state of injured neurons (47; 48).

The transcription factors are essential for axonal regeneration as many genes have to be regulated to regrow the axons. ATF-3 down-regulates fibronectine, which is produced by Schwann cells and induces neurite outgrowth. Heat shock protein 27 (Hsp27), c-jun, small proline-rich protein 1A (SPRR1A), and damage-induced neuronal endopeptidase (DINE) are upregulated by ATF-3. The first two are associated with axonal regeneration, the next has role in promoting neurite outgrowth and the last in sprouting of motor axons (47; 49).

Signal transducer and activator transcription 3

Signal transducer and activator transcription 3 (STAT-3) belongs to STAT family of transcription factors. The 92-kDa protein is consist of 770 amino acid. It comprised of C-terminal transactivation domain, SH2 domain, a linker, DNA-binding domain and N-terminal coiled-coil domain. STAT-3 is activated through phosphorylation of tyrosine 705 and serine 727 at C-terminal domain (50; 51). STAT-3 is known to have role in regeneration after spinal cord and peripheral nerve injury. It is shown that spinal cord injury or axotomy stimulates phosphorylation of STAT-3. Elevation of P-STAT-3 promotes regeneration. STAT-3 also is known to have role in neuroprotection after ischemic brain injury. It also has role in development of nervous system. STAT-3 is suggested to have dual role in regeneration after spinal cord injury. STAT-3 is known to be important for development of glial cells and therefore may contribute to glial scar. As glial scar is a physical barrier for regeneration, STAT-3 deletion in astrocytes may be a plausible way to protect neurons (50).

SRY-box containing gene 11

SRY (sex determining region Y)-box 11 (SOX11) is a member of transcription factor SRY-box family that all contain a DNA-binding high mobility group (HMG) domain and protein specific domains that involve in activation and repression of gene transcription (52; 53).

SOX11 is a transcriptional activator that is highly expressed in embryonic CNS and PNS during axon growth. In PNS, SOX11 significantly expressed in developing trigeminal and dorsal root ganglia. SOX11 is known to be involved in neuron survival and neurite outgrowth. It is demonstrated that the level of SOX11 expression is enhanced following peripheral but not central nerve injury. SOX11 knockdown results in reduction of regeneration capacity. It is shown that

expression of ATF3 is decreased by SOX11 knockdown suggesting that the interaction of SOX11 with ATF3 may involve in axon growth and regeneration (52; 53).

Recently, Wang *et al.* demonstrated the effect of SOX11 overexpression in CNS. They found that overexpression of SOX11 induced regenerative axon growth and sprouting in corticospinal tract while reducing forelimb dexterity. The reason for this negative correlation between increased CST sprouting and functional recovery is not yet clear but it may be because of formation or maintenance of erroneous connections in absence of activity-dependent mechanisms. (54).

Growth-associated protein 43

Growth-associated protein 43 (GAP43) is a growth cone protein that expressed in neurons especially during axonal elongation. GAP43 is a strictly conserved protein among vertebrates. The primary structure of GAP43 has two conserved domains with high degree of homology in vertebrates. The first domain is the N-terminal tail that is tightly bound to cytoplasmic face of nerve terminal. A second domain is a calmodulin binding region. *In vivo*, GAP43 mainly exists in presynaptic terminals and sometimes at very low level in dendrites (55; 56).

GAP43 is upregulated during PNS regeneration and also during the primary development of CNS projection. It plays an important role in control of axon growth and modulating formation of new projections. Although neurons in CNS and PNS have different characteristics relating to regeneration, GAP43 expression in the growth state is generally the same in both nervous systems. In CNS, GAP43 becomes detectable in neurons after final cell division [34].

The expression of GAP43 is at the highest level during the first two postnatal weeks that synapse formation and axon terminal branching occurs. In PNS, GAP43 is detectable after final cell

division and exists along the length of axon and their terminals at high level until postnatal day 4. Likewise the development, during regrowth of injured neurons in PNS, GAP43 is upregulated (57).

Blocking GAP43 result in reduction of growth cone formation. Overexpression of GAP43 stimulates nerve sprouting in adult nervous system and the blocking result in inability in sprout-promoting activity. In adult rat retinal ganglion cells, it is demonstrated that expression of GAP43 is increased after injury and this can enhance the injured CNS neurons potential to regenerate (58; 59).

C-jun

The basic leucine zipper protein c-jun is the primary component of transcription factor AP-1. It is the cellular homolog of transforming oncogene of the avian sarcoma virus 17 (v-jun) and encodes a 39kDa nuclear phosphoprotein. It activates through N-terminal phosphorylation of Ser-63 and -73 residues by JNK. However, some actions are phosphorylation-independent (60-62).

C-jun is an important regulator of axonal response and produced immediately after nerve injury. It expressed at high level during peripheral regenerative process. C-jun activation in Schwan cells regulates Wallerian degeneration and control transdifferentiation of myelin and non-myelinated Schwan cells to repair cells (63; 64).

C-jun is essential for neuronal regeneration and survival. It acts as negative regulator of myelination in Schwan cells so that its expression is at low level when the myelination is started. In the absence of c-jun, specifically in Schwan cells, the neuronal regeneration is impaired and the neuronal death is enhanced. The absence of c-jun resulted in impairment of perineuronal sprouting, lymphocyte recruitment and microglial activation. A suggested mechanism for this

effect is that c-jun deficiency resulted in decreased expression of CD44 and galanin that are known to be involved in regeneration (60; 63-65).

Objective and significance of the present study

Assessing the molecular mechanisms inhibiting the axon regeneration can help us in development of therapeutics to promote recovery after axonal injury. Due to devastating effect of CNS injuries on both injured people and public, it is very important to explore the molecular mechanisms of axon regeneration. To gain further insight into this, it is necessary to find genes and proteins involved in neuronal regeneration or inhibition. In this project, we use phylogenetic profiling to find new genes that may be involved in neuronal regeneration.

Phylogenetic profiling

The exponentially increasing number of sequenced genomes, using experimental techniques to find protein functional linkage is costly and time-consuming. Therefore, computational methods is needed to help the experimental methods. Several computational methods are developed to predict the functional linkage between proteins based on the co-occurrence patterns.

Phylogenetic profiling is a bioinformatics technique to identify co-occurrence between a set of genes or proteins across a large number of species. In other words, in this method the evolutionary co-occurrence pattern is used to find the functionally related proteins. The phylogenetic profiling methods are based on the assumption that co-evolve pattern, co-absence and co-presence of genes or protein across organisms is the result of functionally-dependence of the proteins (66).

Phylogenetic profiling compares genome sequences across different species. In the simplest form, it is dual marking of absence or presence of a given protein across a set of organism. This can be for example showing by 0 for absence of a gene or protein and 1 for its presence. This profile then will be compared with other genes or proteins to find out if these genes or proteins cooperate in same cellular system (67). Many studies successfully used this technique to find unknown new members of protein complexes.

The efficiency of phylogenetic profiling depends on four factors: 1) The ability to detect proper homologs in a set of genomes. 2) The method used to represent the phylogenetic distribution of a protein family profile. 3) Selecting the target species: the increasing number of species enhances the accuracy of prediction. 4) Using the appropriate method to score and compare a match between phylogenetic profiles. These methods could be Jaccard coefficients, statistical measures, simple distance measures and mutual information content (67).

Phylogenetic profiling can help us in identifying genes or proteins that are function in same cellular system. This approach is based on the hypothesis that genes work together in the same cellular system are gained and lost together during evolution. This technique is also used for inferring biological roles of unknown proteins (66-68)

The purpose of this study was to identify novel genes or new pathways potentially involved in axonal regeneration. Our hypothesis was that the factors involved in promoting or inhibiting axonal regeneration are likely to have evolved together. We used phylogenetic profiling to test this hypothesis. Examining the phylogenetic profile of genes involved in regeneration can further help us to propose novel genes and mechanisms involved in axonal regeneration.

PrePhyloPro

PrePhyloPro is a user-friendly web-based software to predict whole genomic linkage. The database is a collection of whole genome protein sequences of 276 eukaryotic, 614 bacterial and 82 archaeal species. It is included at least one species in every known Class or Phylum. It also contains mitochondrial and chloroplast proteins. The protein sequences and annotation information from 972 species were retrieved from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. 20,127 human proteins sequences compared with the selected species using BLASTP (69).

The phylogenetic profiling method is named “Top” which is the combination of Pearson correlation coefficient (ranging from -1 to 1, threshold θ_c) and Jaccard similarities (ranging from 0 to 1, threshold θ_j). The “Top” method uses co-occurrence pattern to detect linkage. A phylogenetic tree is also applied displaying model-based method. The phylogenetic tree is created based on small ribosomal RNA (16S/18S) sequences retrieving from SILVA database.

PrePhyloPro has three steps to obtain prediction result. The first step which is choosing algorithm parameters includes top number threshold, BLAST E-value threshold and the reference organisms. The top number is the number of linkage corresponding to each queried protein. For BLAST E-value threshold three options (0.001, 0.0005, and 0.0001) are available. The smaller top number and BLAST E-value get more reliable but less number of linkage. The second step is setting the size of protein names. The third step is uploading a table of query genes in “txt” or “csv” format. The gene ID is retrieved from the “input gene list”. The cyan color is set for genes that promote neuronal regeneration and the violet color is set for inhibitors of neuronal regeneration. In all processes of obtaining phylogenetic profiling using PrePhyloPro, we set linkage number = 100, Blast Value = 0.0001 and Homo sapiens for reference organism.

Methods

To understand the exact mechanism of regeneration we need to study both promoters and inhibitors. Thus, we choose two sets of genes: First, the regeneration associated genes including: ATF-3 (48), STAT3 (70; 71), SRY (sex-determining region Y)-box 11 (SOX11) (53; 54), GAP43 (58; 59), and c-Jun (60; 63). And the second, the genes which is known to inhibit regeneration including: nogo-A (72; 73), myelin-associated glycoprotein gene (MAG) (24), oligodendrocyte-myelin glycoprotein gene (OMgp) (74; 75), nogo-A receptor (NgR) (72), phosphatase and testin homolog (PTEN) (36; 37; 76), suppressor of cytokine signaling 3 (SOCS3) (35-37), ephrin B3 (EPHNB3) (10), semaphorin 4D (SEMA4D) (11) and semaphorin 3A (SEMA3A) (12).

The reference genes were entered in into the PrePhyloPro database to compare their phylogenetic profiling (<http://prephylopro.org/phyloprofile/>). The database determined the Correlation score (Cor score). In addition to showing the correlation degree, the database presented genes with similar phylogenetic profiling from Homo sapiens genome. Each gene may exist in the proposed list of more than one genes. So that, the genes with the maximum number of the repetition were chosen. These include:

Neuromedin B receptor (NMBR), RAR-related orphan receptor B (RORB), dopamine receptor D1 (DRD1A), semaphorin 3D (SEMA3D), G protein-coupled receptor 174 (GPR174), tetraspanin 19 (TSPAN19), Ets2 repressor factor (ERF), δ -opioid receptor (OPRD), wingless-type MMTV integration site family (WNT4), galanin receptor 1 (GALR1), nuclear receptor subfamily 4 group A member 2 (NR4A2), V-set and immunoglobulin domain containing 10 (VSIG10),

oligodendrocyte lineage transcription factor 2 (OLIG2) and insulin-like growth factor binding protein 7 (IGFBP7).

We refer to the reference genes as gene set 1 (G1) and the candidate genes identified by PrePhyloPro database with 4 repetitions as set genes 2 (G2). The combination of G1 and G2 was referred to as set genes 3 (G3). We repeated this process with set genes 3 (G3).

Results

To compare the phylogenetic profiling of genes involved in regeneration, 14 genes that are known to involve in regeneration (either by stimulatory or inhibitory activity) were selected. The reference genes include genes that promote neuronal regeneration (ATF3, STAT3, SOX11, C-JUN and GAP43) and those inhibiting it (NOGO, MAG, OMgp, NgR, PTEN, SOCS3, EPHNB3, SEMA3A and SEMA4D). The reference genes were entered to the PrePhyloPro database exploring the degree of correlation among phylogenetic profiles (Fig. 1, 2). The phylogenetic correlation between the genes is scored from 0 to 1 (from lowest to the highest).

A random set of 14 genes was also considered as negative control (Fig. 3). The average Cor score for reference genes is 0.67 and the average Cor score for random genes is 0.52. The small difference between the two Cor scores might be due to the fact that reference genes are from multiple pathways.

A detailed analysis of the correlation score of the reference genes indicates that 16% have the Cor score above 0.9. The Cor score for 6% of reference genes is between 0.8 to 0.9, 22% between 0.7 to 0.8, 16% between 0.6 to 0.7, 20% between 0.5 to 0.6 and 20% below 0.5 (Fig. 4).

However, just 4% of the random genes have a Cor score above 0.9. The Cor score for 48% of random genes is below 0.5.

Not surprisingly, these results demonstrate that not all genes involved in neuronal regeneration evolve together. Neural regeneration consists of several functional groups that may follow different patterns of evolution. It is noteworthy that one highly correlated subset, including SOCS3, SEMA3A, SEMA4D, MAG, EPHNB3 and STAT3, appeared within this group.

The genes involved in regeneration are clustered in two main group based on their phylogenetic profiling

According to phylogenetic profiling data, the reference genes are clustered in two main groups. Group one including OMgp, NgR, PTEN and SOX11 are originated from a common ancestor and group 2 including SOCS3, SEMA3A, SEMA4D, MAG, EPHNB3, STAT3, GAP43, NOGO, ATF3 and C-JUN are originated from a common ancestor (Fig. 1). It is demonstrated that all reference genes exist in animals. OMgp, NgR, PTEN and SOX11 (group 1) have homologs in both fungi and plants. OMgp and NOGOR are also shown to have some homologs in bacteria suggesting that they might be in the same evolutionary family group. The phylogenetic profiling clustered OMgp and NOGOR in one subgroup and PTEN and SOX11 in another one. The correlation score between the first two is 0.84 and the second is 0.85. OMgp, NgR and PTEN have inhibitory activity regarding neuronal regeneration while SOX-11 has regeneration-promoting activity. Most of the genes in group 2 are just found in animals except for the ATF-3, c-jun and Nogo. ATF-3 and c-jun are also found in fungi. Nogo is shown to have some homologs in both fungi and plants (Fig. 1).

Considering the role of each gene in regeneration it can be seen that 5 of genes that have a role in inhibiting neuronal regeneration are just found in animals: SOCS3, SEMA3A, SEMA4D, MAG and EPHNB3 (Fig. 1). The other four genes involved in neuronal regeneration (OMgp, NgR, PTEN and Nogo) have homologs in fungi and plants as well as animals. OMgp and NgR also have some homologs in bacteria.

Two of genes that involve in promoting regeneration are just found in animals (STAT3 and GAP43). However, the other three genes promoting regeneration (SOX11, ATF3 and C-JUN) are also found in fungi. SOX11 also have some homologs in plants.

The phylogenetic profile of SOCS3, SEMA3A, SEMA4D, MAG, Ephrin B3 and STAT3 are highly correlated.

Analyzing the correlation score between the reference genes shows that the average Cor score among these 14 genes is 0.67. The minimum Cor score which is assigned to phylogenetic profiling correlation between OMgp and GAP-43 is 0.35 and the maximum Cor score which is assigned to the correlation between SOCS3 and SEMA3A is 1. The average Cor score between genes in group one is 0.76 and between genes in group two is 0.78.

Interestingly, we found that SOCS3, SEMA3A, SEMA4D, MAG, EPHNB3 and STAT-3 are highly similar based on evolutionary aspects (Average Cor score = 0.97, $0.94 \leq \text{Cor} \leq 1$). We named this group as H1. They are just found in the animals and no other kingdoms. Interestingly, the phylogenetic profiling of SEMA3A and SOCS3 is exactly similar (Cor = 1). As expected, SOCS3 that activates STAT-3 has correlated evolution (correlation score = 0.96). Except for STAT3, all genes in H1 group has inhibitory activity for regeneration. These genes have a minor

evolutionary correlation with OMgp, NgR, PTEN and SOX11 (Average Cor score = 0.49, $0.42 \leq \text{Cor} \leq 0.59$).

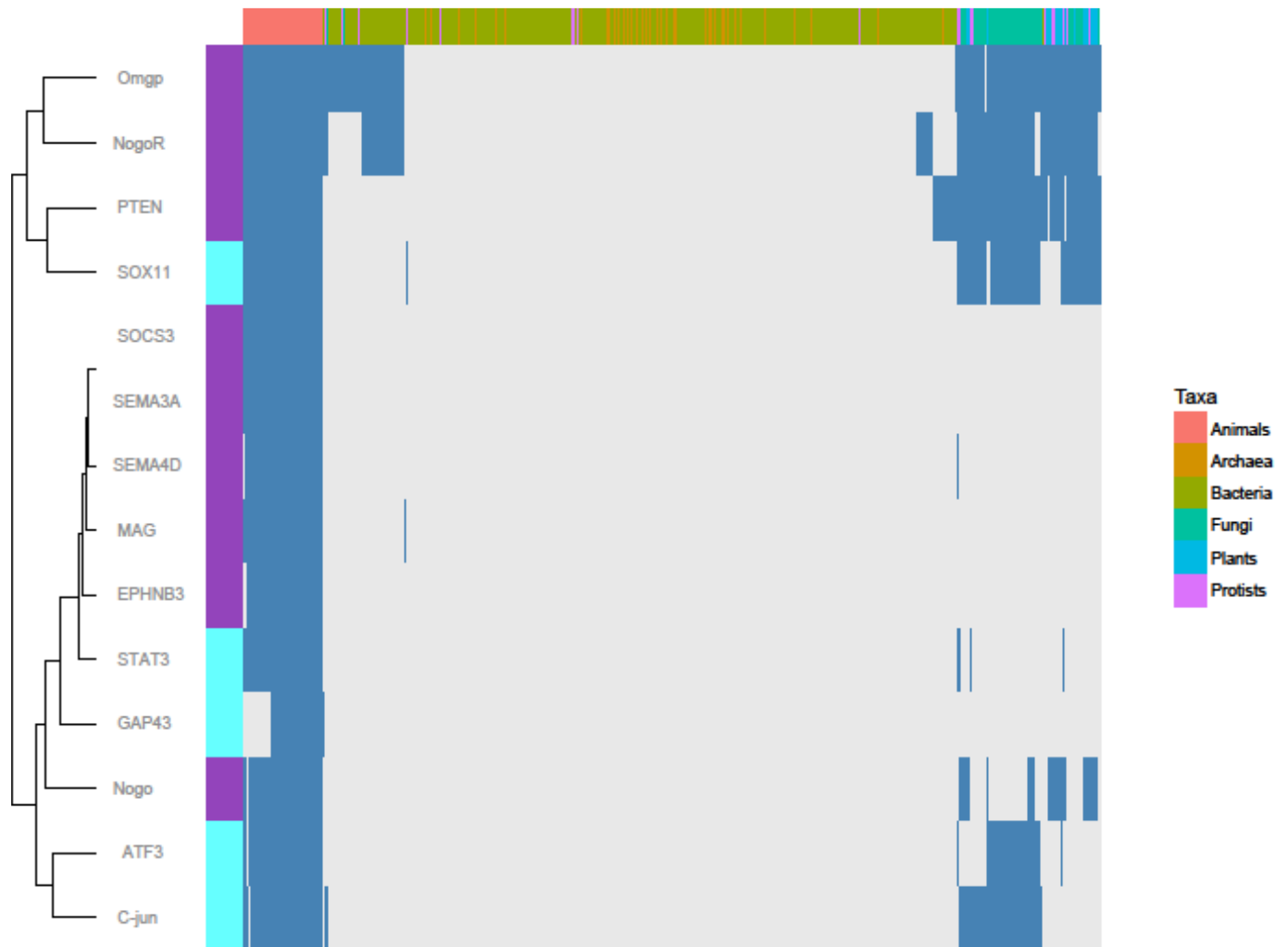


Figure 1. Phylogenetic profiling of reference genes associated with axonal regeneration. The vertical axis shows the reference genes. The horizontal axis shows different kingdom of the creature. The existence of genes in different kingdom is shown in steel blue. The violet color shows the genes inhibit axonal regeneration and the cyan color indicates the genes promote neural regeneration.

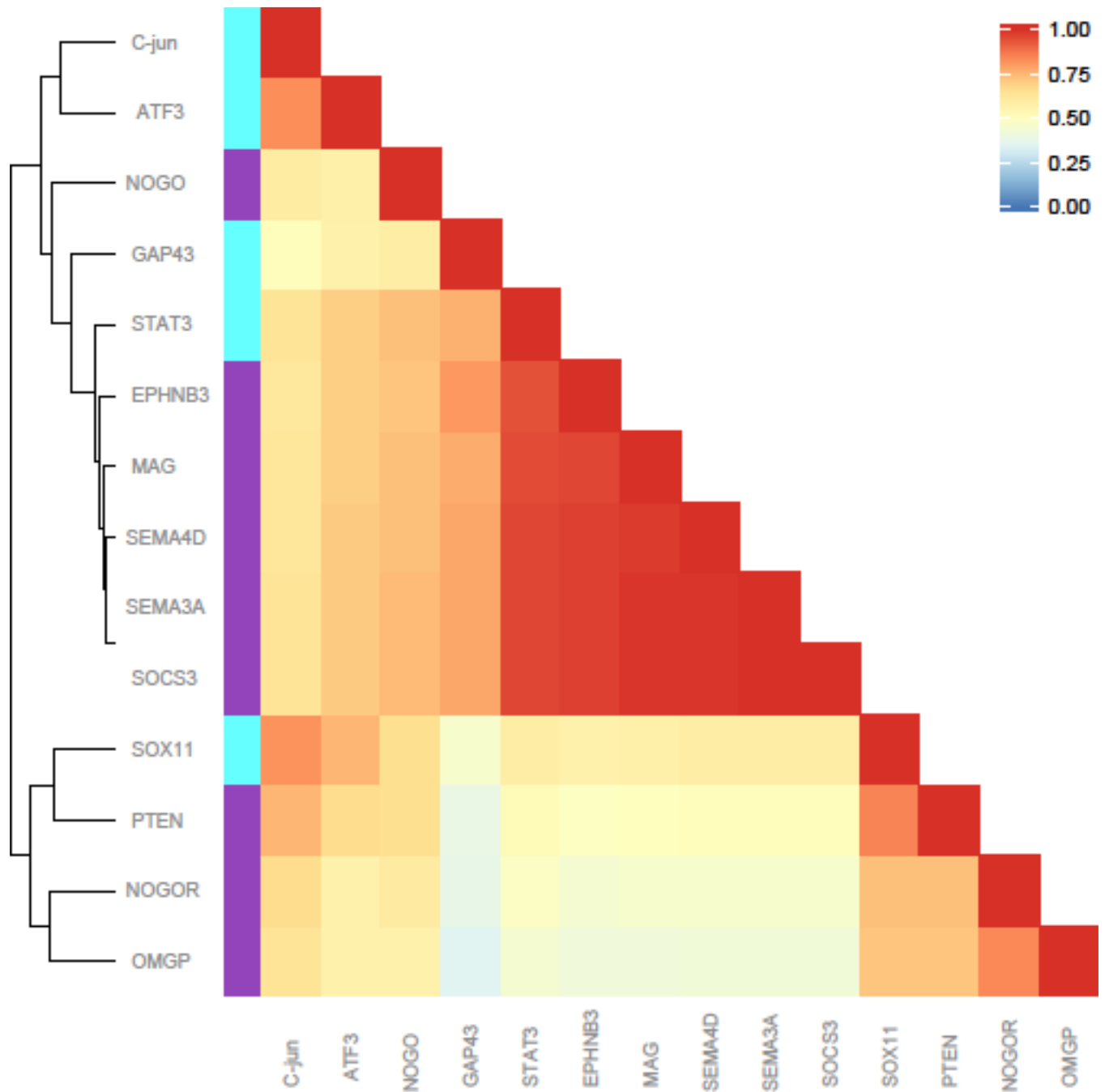


Figure 2. Correlation matrix plot of reference genes that associates with axonal regeneration. The vertical and horizontal axis shows the reference genes. It is clear that the reference genes are categorized in two subgroup, based on the phylogenetic profiling. The colors spectrum shows the correlation score from 0 (blue) to 1 (red). The red color shows that 6 genes are highly correlated in their phylogenetic profile.

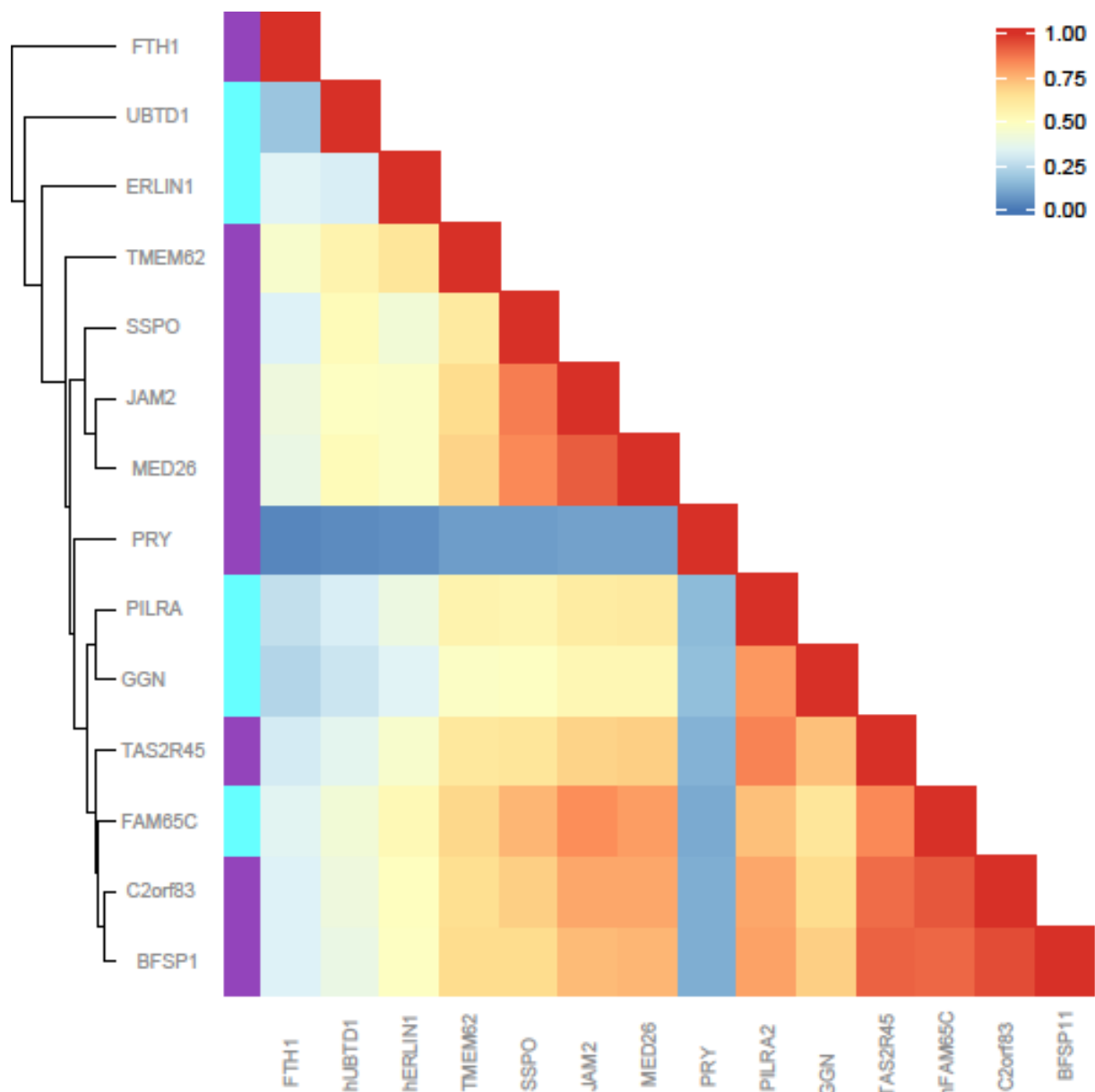


Figure 3. Correlation matrix plot of random genes. The horizontal and vertical axis shows the random genes. The colors spectrum shows the correlation score from 0 (blue) to 1 (red).

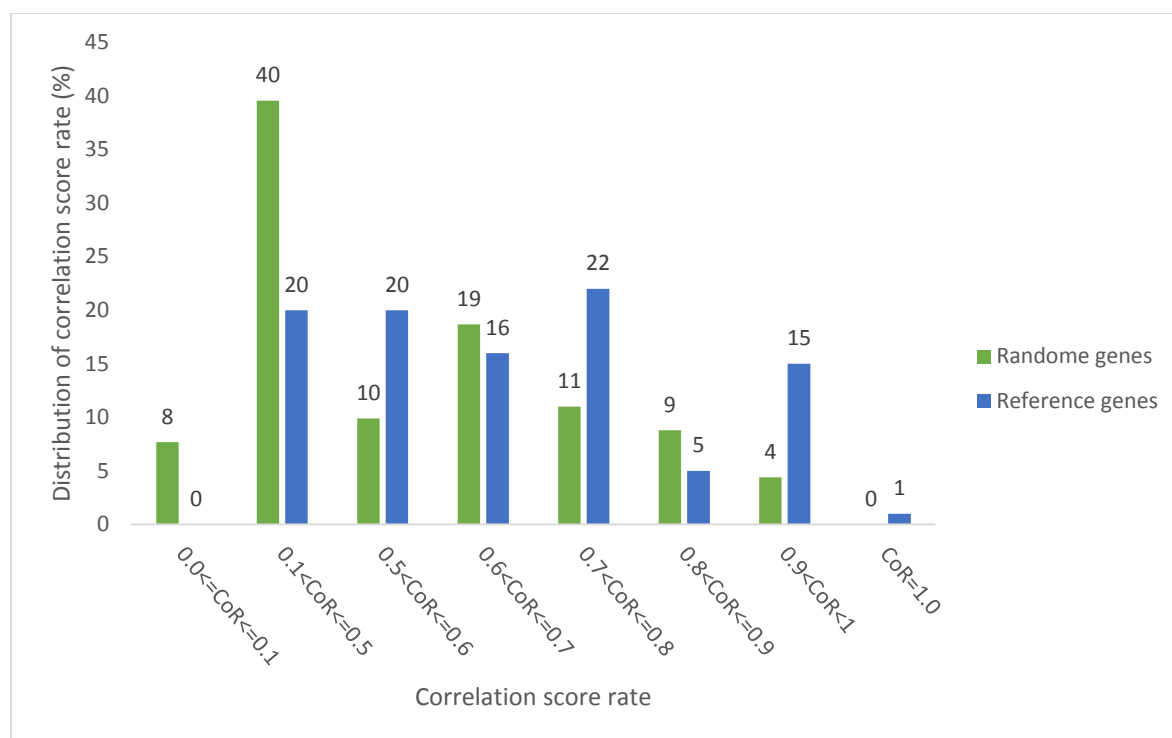


Figure 4. The correlation score range distribution of reference genes and random genes. The horizontal axis shows the correlation rate from 0.0 to 1.0 and it is divided into eight bands. Each vertical bar shows the distribution percentage of reference genes (blue) and random genes (green) falling in the corresponding correlation score rate band.

Prediction of new genes that may involve in neuronal regeneration using PrePhyloPro

There is a hypothesis describing that proteins with similar phylogenetic profiling strongly tend to be functionally linked. In other words, proteins that function in a pathway or make up a multimeric structural complex are likely to evolve in a correlated fashion. Based on this hypothesis, we assume that genes that involve in axonal regeneration may evolve together (77). To examine this, 14 genes that are known to involve in axonal regeneration (either by regeneration-promoting or inhibitory activity) were selected. The reference genes were entered to the PrePhyloPro database exploring the degree of correlation among phylogenetic profiles.

Setting the linkage number = 100, the blast value = 0.0001 and the organism: *Homo sapiens*, the PrePhyloPro suggested 2302 evolutionary homologs with the reference genes. We choose the genes that have the most repetition within the suggested genes (4 repetitions). The genes are: neuromedin B receptor (NMBR), RAR-related orphan receptor B (RORB), dopamine receptor D1 (DRD1A), semaphorin 3D (SEMA3D), G protein-coupled receptor 174 (GPR174), tetraspanin 19 (TSPAN19), Ets2 repressor factor (ERF), δ -opioid receptor (OPRD), wingless-type MMTV integration site family (WNT4), galanin receptor 1 (GALR1), nuclear receptor subfamily 4 group A member 2 (NR4A2), V-set and immunoglobulin domain containing 10 (VSIG10), oligodendrocyte lineage transcription factor 2 (OLIG2) and insulin-like growth factor binding protein 7 (IGFBP7).

To see the phylogenetic profiling correlation of G3, we entered G3 in PrePhyloPro (Fig. 5 and 6.). All the G2 genes show a high degree of phylogenetic correlation with SEMA3A, SEMA4D, MAG and SOCS3. As expected, G2 exist only in animals (Fig. 5).

STAT3, EFNB3, MAG, SOCS3, OLIG2, VSIG10, NR4A2, GALR1, WNT4, OPRD1, ERF, TSPAN19, SEMA3A, GPR174, SEMA3D, DRD1, NMBR, RORB, SEMA4D and IGFBP7 are shown a high degree of correlation in their phylogenetic profiles (Fig. 6). However, SOX11, PTEN, NOGOR and OMgp show a low degree of correlation with the mentioned genes.

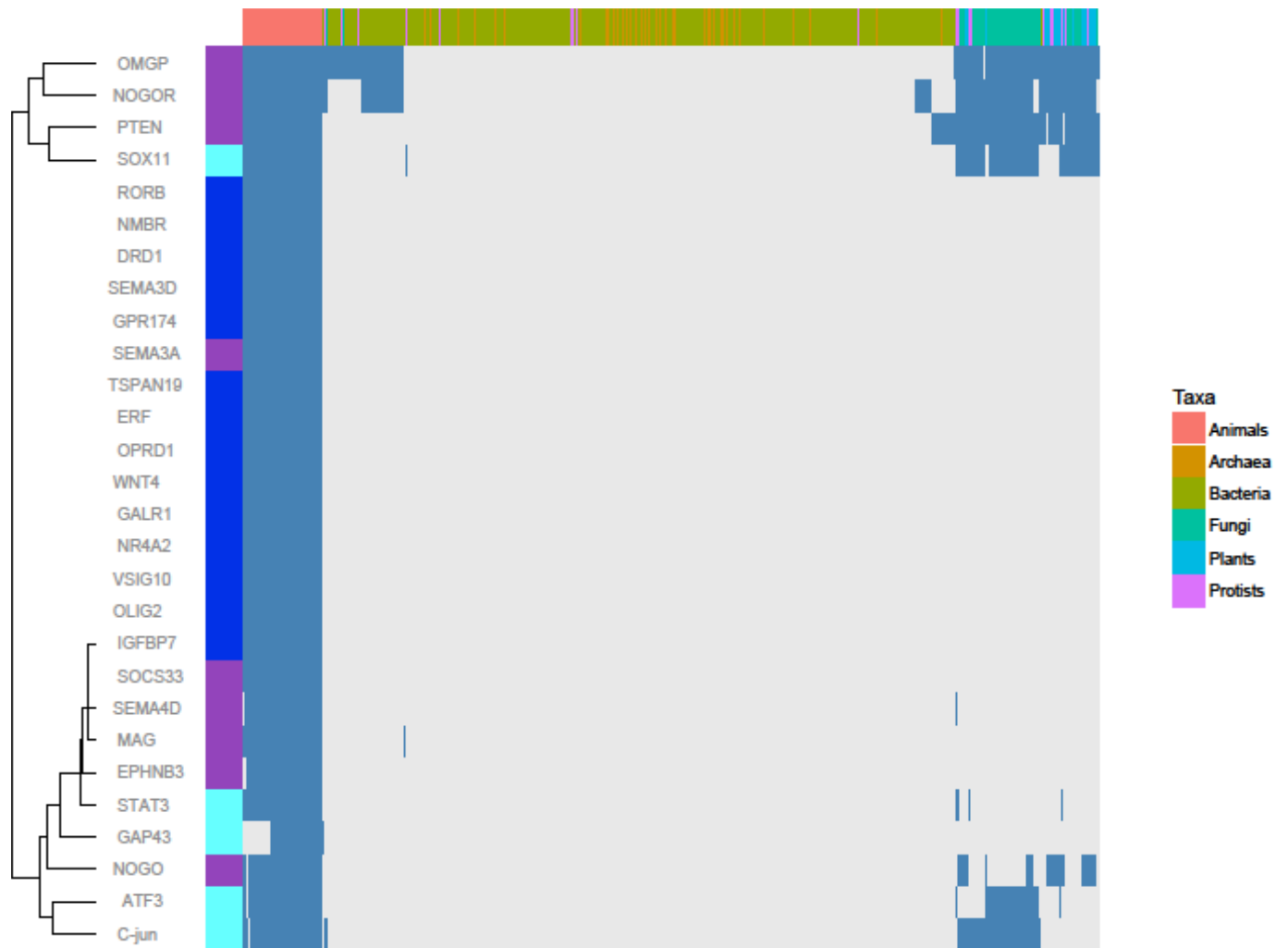


Figure 5. Phylogenetic profile of reference and candidate genes. The vertical axis shows the reference genes and candidate genes. The horizontal axis shows different kingdom of creatures. The existence of genes in different kingdom is shown in steel blue. The violet color shows the genes inhibit axonal regeneration and the cyan color indicates the genes promote neural regeneration. The blue color shows the candidate genes. All the candidate expressed just in animals.

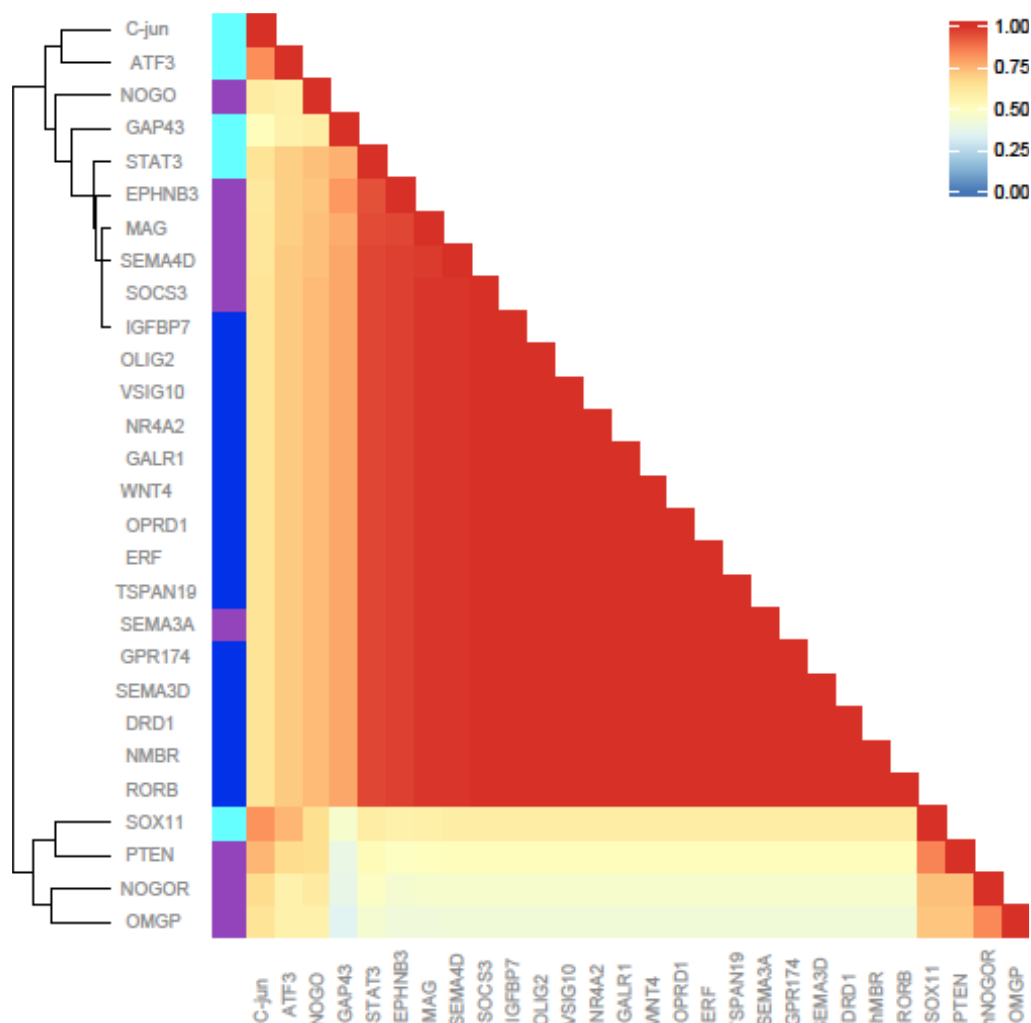


Figure 6. Correlation matrix plot of reference and candidate genes. The horizontal and vertical axis shows the reference genes and candidate genes. The genes in group3 are categorized in two subgroups. All the candidate genes are categorized in same group. The candidate genes are highly correlated based on their phylogenetic profiling.

To assess the possible shared functions of G1, G2 and G3 we did gene ontology enrichment analysis using biological process, cell component and molecular function. The gene ontology of reference genes from 20972 human genes indicates 87 biological processes with significant p-value. The biological process assigned to majority of G1 (12 genes or more) includes cell

differentiation, developmental process, neurogenesis, nervous system development, cell differentiation, anatomical structure morphogenesis and signal transduction. The cellular component of G1 shows MAG and PTEN both present in myelin sheath adaxonal region and Schmidt-Lanterman incisure. There is no similar cellular component for other genes. The molecular function of G1 shows no assigned function. The common known biological process for candidate genes was phospholipase C-activating G-protein coupled receptor signaling pathway which is shared between NMBR, DRD1, OPRD and GALR1. The GO molecular function analysis for candidate genes was shown that receptor activity, molecular and signal transducer activity is common in half of the genes. The cellular component for candidate genes was not same with each other.

The GO ontology for G3 shows common biological processes such as cellular response to a stimulus, cell communication, signaling, organism process, developmental process, cellular process, neurogenesis and etc. in a majority of genes. The GO ontology molecular function for G3 was signal and molecular transducer activity, receptor activity, transmembrane receptor activity, RNA polymerase II transcription factor activity, sequence-specific DNA binding and etc. There is no same cellular component in G3.

Using KEGG PATHWAY Database indicates that SEMA3A, SEMA4D, ephrin B and WNT4 are involved in axonal guidance pathway (Fig. 7). Axon guidance is regarded as a key stage for neuronal network. Axonal guidance factors such as semaphorins, ephrins, netrins and slits are read by growth cone receptors. The downstream signal transduction pathway converges onto the Rho GTPase. This makes the changes in cytoskeletal organization that determine the way the growth cone turns (78).

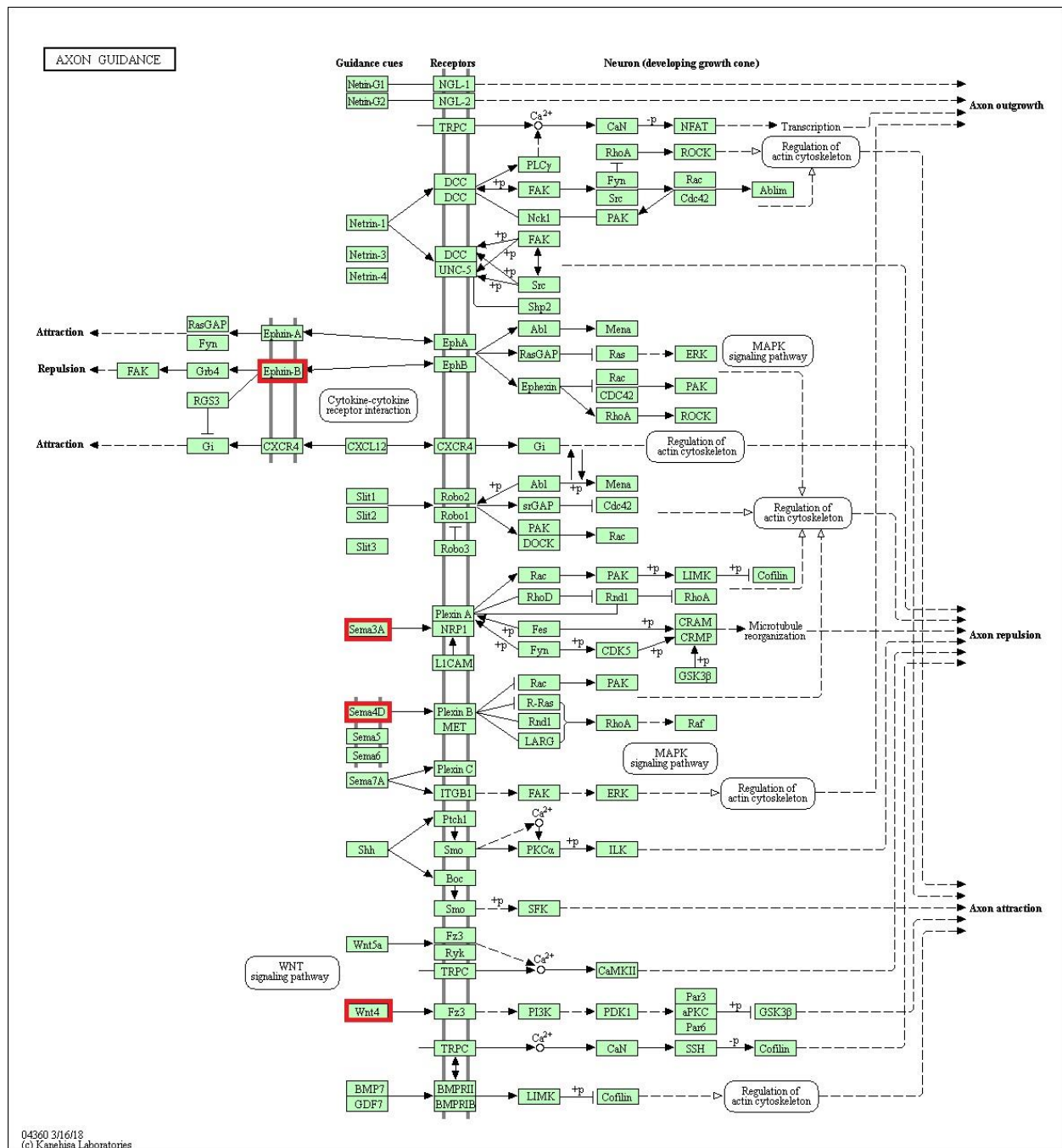


Figure 7. Pathway of axonal guidance. Adapted from: <http://www.genome.jp/kegg/pathway.html> (78)

The genes with high phylogenetic homology in the G1 and G2 are expressed exclusively in animals. As the candidate genes are highly correlated with 4 of reference genes, we, therefore, decided to assess five genes with higher Cor score for each gene in G1 and G2. This resulted in

Persephin A, FOXD4, Grainhead-like family, Leucine-rich repeat containing family, leucine-rich repeat and sterile alpha motif-containing 1, Vesicle-associated membrane protein 1, leucine-rich repeat and Ig domain-containing and Rho GTPase activating protein 32.

Discussion and conclusion

There are many factors that are suggested to have a role in axonal regeneration. However, the exact mechanism of regeneration is still a mystery. In order to overcome the inability of mature CNS to regenerate, we need to know the exact mechanism of axonal regeneration, the factors that promote or inhibit axonal regeneration and how these factors are linked to each other. The phylogenetic profiling is a method to find the functionally linked proteins and predict the function of uncharacterized proteins.

From 14 reference genes, the phylogenetic profiles of 6 of them are highly correlated, suggesting functional linkage. These are SOCS3, SEMA3A, SEMA4D, MAG, EPHNB3 and STAT3. Except STAT3, all these genes have inhibitory activity in neural regeneration. There is no evidence for direct interaction of these genes except for SEMA3A and STAT3. Sequence analysis of SEMA3A in human, rat and mouse illustrated a consensus DNA binding motif for STAT3 (79).

PrePhyloPro showed that SOCS3 and SEMA3A are highly correlated based on phylogenetic profile (Cor score =1). We performed a literature search to find evidence for interaction between these genes. Although no direct interaction between SOCS3 and SEMA3A has been reported, there might be indirect interaction between the two genes. It is known that SOCS3 and PTEN regulate two independent pathways that promote axonal regeneration

synergistically. SOCS3 negatively regulates JAK/STAT and PTEN negatively regulates mTOR pathway. Deletion of either SOCS3 or PTEN resulted in enhanced optic nerve regeneration in adult retinal ganglion cells which tapered off after two weeks. However, simultaneous co-deletion of SOCS3 and PTEN causes sustained axonal regeneration (36).

Until now, it has not been reported if SOCS3 and SEMA3A have any interaction with each other. However, it is known that SEMA3A induces PTEN accumulation at the growth cone. Moreover, STAT3 regulates the expression of SEMA3A (79). It is found that mammalian target of rapamycin complex1 (mTORC1) activity is decreased following SEMA3A knockdown in cancer stem-like cells. It is also shown that tumor cells down regulated SEMA3A by activating mTOR pathway (80; 81). The similar phylogenetic profile of SEMA3A and SOCS3 could suggest that SEMA3A may link these two pathway.

Unexpectedly, the phylogenetic profile of Nogo and its receptor was not similar (Cor score= 0.61). They are classified as different evolutionary groups. Nogo receptor was found in animal, fungi, plant and some species of bacteria but Nogo was just found in animal and few species of fungi and plant. Due to close interaction these two protein have, we assume that Nogo receptor undergoes modular evolution.

The theory of modular evolution suggests that proteins evolve partly from rearrangements of their domains. This can result in evolution of novel biological functions in different evolutionary lineages (82-84). The phylogenetic profiling database suggested some genes that have high evolutionary homology with the reference genes including neuromedin B receptor (NMBR), RAR-related orphan receptor B (RORB), dopamine receptor D1 (DRD1A), semaphorin 3D (SEMA3D), G protein-coupled receptor 174 (GPR174), tetraspanin 19 (TSPAN19), Ets2 repressor factor (ERF), δ -opioid receptor (OPRD), wingless-type MMTV integration site family (WNT4), galanin

receptor 1 (GALR1), nuclear receptor subfamily 4 group A member 2 (NR4A2), V-set and immunoglobulin domain containing 10 (VSIG10), oligodendrocyte lineage transcription factor 2 (OLIG2) and insulin-like growth factor binding protein 7 (IGFBP7). These genes show a high degree of correlation in their phylogenetic profiles with SEMA3A, SOCS3, SEMA4D and MAG. We suggested that these genes might be functionally linked to each other. To confirm this, the published articles were assessed to find evidences for interaction of each of these genes or their encoded proteins with the suggested genes or proteins that is shown to have high evolutionary homology.

Neuromedin B receptor is a G protein coupled receptor which belongs to bombesin receptor family. NMBR may have role in regulation of cell growth and neuronal response and is known to have role in cancer. NMBR antagonists reduces the tumor cell growth (85). There is no evidence if neuromedin B receptor has role in neuronal regeneration. However, it is known that NMBR transactivates epidermal growth factor receptor (EGFR) in lung cancer cells (79). Myelin inhibitors and CSPGs produced influx of intracellular calcium which leads to activation of EGFR. Moreover, EGFR suppress myelin inhibitors and chondroitin sulfate proteoglycans in inhibiting neurite outgrowth (86).

The similar phylogenetic profile of NMBR with SEMA3A, SOCS3, SEMA4D and MAG and the interaction with EGFR in lung cells suggests that neuromedin B receptor might interact with EGFR in nervous system and this interaction might play a role in axonal regeneration.

RAR-related orphan receptor beta is expressed in brain, retina and pineal glands. There is no evidence of participation of this gene in neuronal regeneration or any neuronal activity. RORB interacts with NM23-2 which is a metastasis suppressor and also regulates cell proliferation and

promotes neuronal differentiation (87). The involvement of RORB in neuronal development and neuronal regeneration could be a subject of future studies.

Dopamine increases neurite outgrowth and growth cone formation through activation of D1 receptor in embryonic striatal neurons. In addition to the role of D1R in striatal neurons development, dopamine controls neurogenesis during regeneration of dopamine neurons in adult salamander. Many of these effects attributed to D1 dopamine receptor. (88-91). In contrast, some studies ascribed opposite role to dopamine D1 receptors. They showed that activation of D1R reduces neurite outgrowth in frontal cortical cultures. This is further demonstrated that is the result of destabilization of dendritic microtubule associated protein (MAP2) which is due to increased phosphorylation. The different effect of dopamine on neuronal growth is shown that this effect is subtype-specific (92; 93).

Semaphorin family are secreted, transmembrane glycoproteins that were initially identified by their importance in axonal guidance and development of nervous system. The semaphorin family are classified in 8 groups based on the structure, amino acid sequences and phylogenetic tree analysis. Class I and II exist in invertebrates, class III to VII found in vertebrates and class VIII belong to viruses. Semaphorins are mostly expressed in nervous system but are also found in other tissues. Class III semaphorins (SEMA3s) are important modulators of neuronal regeneration, remyelination and revascularization after CNS trauma. Most of the action of semaphorins are ascribed to binding to neuropilins which are the receptor for several members of this family (94; 95).

SEMA3D promotes proliferation of hindbrain neuroepithelial cells during neural crest development suggesting to act downstream of Wnt/TCE signaling. SEMA3D can repel or collapse the growth cone under control of cyclic adenosine monophosphate (cAMP) and cyclic guanosine

monophosphate (cGMP). The conversion behavior from repulsion to attraction and vice versa has potential implication for neuronal regeneration (96; 97).

Tetraspanins are a family of glycoproteins with four transmembrane domains that are known to have a role in cell-cell interactions. Chernousov *et al.* examined the role of five groups of tetraspanin in Schwann cell-axon interaction. They found that two types of tetraspanin CD63 and CD81 are crucial for extension of Schwann cell processes along axons. The role of tetraspanin 19 in Schwann cell-axon interaction is not assessed yet. However, due to the importance of Schwann cells in promoting axonal regeneration and the similar phylogenetic profile of tetraspanin 19 with genes involved in regeneration, it would be worthy to study on the role of tetraspanin 19 on Schwann cells spreading along axons (98-101).

Opioid receptors (δ , μ and κ) are G protein coupled receptors that are involved in various physiological activities such as cell proliferation, neurogenesis and gliogenesis. δ -opioid receptor (DOR) activation is neuroprotective against hypoxic/excitotoxic stress, while its inhibition resulted in neuronal injury. It is found that DOR triggers neurite outgrowth and neuronal differentiation via STAT5-G α i/o signaling pathway (102; 103).

Wingless-type MMTV integration site family (Wnt's) plays an important role in developing and mature nervous system. Wnt proteins are signaling molecules regulating embryonic patterning, cell proliferating and cell determination. They are axon guidance molecules that control synapse formation and maintenance, axon pathfinding, axon remodeling and dendritic morphogenesis. During development, WNT4 attracts ascending sensory axons. Wnts are not detected in the normal adult spinal cord. However, WNT1, WNT4 and WNT5 are shown to induce after spinal cord injury. Inhibiting repulsive Wnt signaling resulted in enhanced sprouting of CST

axons and more robust regeneration suggesting the inhibitory effect of repulsive Wnt signaling following central nervous system injury (104-108).

Nuclear receptor subfamily 4 group A member 2 (NR4A2) is a transcription factor that belongs to the orphan nuclear receptor family 4 subgroup A. NR4A2 has a role in regulating axon outgrowth, neuronal patterning and terminal differentiation. It is responsible for regulating expression of key enzymes for dopamine synthesis. It is involved in maturation, differentiation and survival of dopaminergic neurons (109).

The oligodendrocyte lineage transcription factor 2 (OLIG2) is expressed in the developing CNS and postnatal brain. OLIG2 is known to regulate the genesis and differentiation of oligodendrocytes which are crucial for myelination of axons. It modulates neurogenesis and astrogenesis. Complete knockout of OLIG2 in mice leads to embryonic lethal because of lacking motor neurons (110-113).

GAP43 is shown to have high homology with Persephin A (PSPN) (COR=0.94). PSPN is a neurotrophic factor that promotes the survival of motor neurons and dopaminergic neurons in culture. PSPN inhibits the degeneration of ventral midbrain dopaminergic neurons after 6-hydroxydopamine treatment in vivo. PSPN is expressed in embryonic and adult brain. It is 40% identical to glial cell line-derived neurotrophic factor (GDNF) and neurturin (NTN) (114-117).

Several family members of FOXD4 are shown to have phylogenetic homology with c-jun and ATF3. FOXD4 is a forkhead box family transcription factor. It plays an important role in directing embryonic stem cells towards a neural stem cells lineage in *Xenopus* and mouse embryo. However, little is known about its function in other vertebrates. It downregulates genes

promoting the differentiating of neural plate and upregulates genes that maintain immature neural ectoderm. This function is the same in marine and mammals (118; 119).

The phylogenetic profiling showed that some members of the Grainhead-like family (GRHL1, GRHL2, and GRHL3) has similar phylogenetic profile with ATF3. GRHLs are mammalian homologs of Grainhead in *Drosophila* (GRH). GRH is a developmental transcription factor that positively regulates cell proliferation in post-embryonic development. In mammals, GRHLs involve in epithelial morphogenesis and development. Interestingly, Grainhead-like 2 (GRHL2) is shown to have a role in neurulation (120-122).

Several members of Leucine-rich repeat containing family is suggested by PrePhyloPro database to have homology with OMgp, NgR and STAT3. Leucine-rich repeats (LRR) is one of the most common and evolvable motifs in many proteins with various functions such as nervous system development and innate immunity. LRR is a structural motif with 20 to 30 amino acids consisting a conserved 11-aminoacid rich in leucine at N-terminal end (123).

LRR proteins are involved in axonal guidance, dendritic arborization and synapse formation during development of nervous system. They regulate axon myelination and neural circuit stabilization in mature nervous system. In zebrafish, there is a transmembrane leucine-rich protein (zfNLRR) that its expression is increased during regeneration suggesting that may function as ligand binding receptor or neuronal-specific adhesion molecule during regeneration after nerve injury. The same protein binding motifs were found in frog, mouse and human (124; 125).

Another gene set that is shown to have similar phylogenetic profiling with OMgp is leucine-rich repeat and sterile alpha motif-containing 1 (LRSAM1). LRSAM1 is an E3 ubiquitin ligase that is involved in receptor endocytosis, viral budding and regulation of cell adhesion

molecules. LRSAM1 is expressed at high level in fetal and adult nervous system as well as muscle tissue. It is expressed highly in peripheral sensory and motor neurons. It is found that LRSAM1 is linked to Huntington's disease by its role in the clearance of protein aggregates. It is also suggested that LRSAM may develop Parkinson's disease in Charcot-Marie-Tooth (CMT) patients. Charcot-Marie-Tooth disease is an inherited motor and sensory neuropathy characterized by peripheral axon degeneration. (126-128).

Vesicle-associated membrane protein 1 (VAMP1) has a similar phylogenetic profile to that of PTEN (Cor score = 0.91). VAMP1 play a role in synaptic vesicle and exocytosis in neurons. It is selectively expressed in the retina and distinct brain regions such as zona incerta and rostral periolivary region. VAMP1 mRNA is transiently reduced after facial neuron axotomy suggesting may have a role in nerve regeneration (129; 130).

Another group of genes that is shown to have similar phylogenetic profiling with OMgp and NgR, belongs to the family of leucine-rich repeat and Ig domain-containing. LINGO1 is a human homolog of Slit-a in drosophila which is an axonal guidance molecule. LINGO1 is a principal component of NgR1/p75^{NTR} complex. It has been suggested that inhibition of neurite outgrowth by Nogo-A, OMgp and MAG is mediated by multi-component complex including NgR1, LINGO1 and a member of tumor necrosis factor (TNF) receptor (p75^{NTR} or TROY). LINGO1 negatively regulates neuronal survival, oligodendrocyte differentiation and myelination and axonal regeneration through inhibiting protein kinase B (Akt) phosphorylation and activating Ras homolog gene family member A (Rho) signaling pathway. Several lines of evidence indicated that blocking LINGO1 promotes axonal regeneration, neuronal survival, oligodendrocytes differentiation and remyelination. To our knowledge, there is no study done on the effect of the other member of LINGO family but it can be assessed in further studies (131-134).

The phylogenetic profiling data also shows that Rho GTPase activating protein 32 (RICS) has high homology with SOX11. RICS is a beta-catenin-interacting protein that has GAP activity against Rac1 and Cdc42. RICS which is primarily expressed in brain is known to involve in synaptic adhesion, regulating neurite extension and dendritic development. RICS modulate dendritic morphogenesis by suppressing RhoA/Rock signaling (135-137).

Calcium signaling in axonal regeneration

Calcium signaling has been implicated in axonal regeneration. Elevated Ca^{2+} or cAMP accelerates growth cone formation in injured axons. Conversely, blocking the voltage-gated calcium channels decreases regenerative growth (138). In growth cone, elevated levels of intracellular Ca^{2+} activates cAMP. cAMP activation reduces the intracellular level of cGMP. Increasing the ratio of cAMP to cGMP activates ryanodine receptors (RyRs). RyRs mediate release of Ca^{2+} from endoplasmic reticulum (ER). The increase in Ca^{2+} induces more cAMP to be formed. cAMP promotes neurite growth, inhibit the inhibitory interaction of growth cone and act as axonal guidance cue. cAMP is shown to induce regeneration of Mauthner axon that has a poor capacity to regenerate after spinal cord injury in Zebrafish. Moreover, increasing Ca^{2+} leads to activation of Ca^{2+} -dependent enzymes like adenylate cyclase. It is known that adenylate cyclase activity increases for several hours after axonal injury. In rat sciatic nerve, the regeneration increased by adenylate cyclase stimulation or exogenous dibutyryl-cAMP (139; 140).

Assessing the reference genes and the candidate genes indicated that some of them are linked to calcium signaling: extracellular gradients of MAG induces calcium-dependent growth cone turning responses. C-AMP signaling activity enhancement increases MAG-induced Ca^{2+} signals that result in the conversion of growth cone repulsion to attraction. NgR and P75

neurotrophin receptor (P75^{NTR}) co-expression is required for MAG-induced Ca²⁺ enhancement in growth cone (18; 141; 142).

Localization of PTEN in ER regulates Ca²⁺ transfer from ER to mitochondria and sensitivity to apoptosis. This ability is the result of inhibition of PI3K/Akt pathway. PI3K/PTEN pathway has an important role in translocation of cation channel TRPC6 to plasma membrane which may affect Ca²⁺ signaling in cells endogenously express TRPC6 (143; 144).

Ephrin B3 modulates NMDA receptors at hippocampal synapses. NMDA receptors are glutamate-gated cation channels that have high permeability to calcium. The influx of Ca²⁺ through NMDA receptors mediates synaptic plasticity, cell growth, apoptosis, kinase and phosphatase activity and protein trafficking (145; 146).

SEMA3A facilitates axonal transport and dendritic development through local calcium signaling and sensitive voltage-gated sodium channels. SEMA3A induced growth cone collapse by increasing the intracellular calcium concentration through R-type calcium channels (147-149).

Depolarization in mature neurons causes STAT3 activation which induces calcium influx through L-type voltage-gated calcium channels. In keratinocytes, it is shown that IL31 induced calcium influx. Calcium entry cause activation of STAT3 therefore, increasing beta-endorphin release. In another study, it is indicated that intracellular calcium chelation inhibited epidermal growth factor-induced STAT3 activation. In human dorsal root ganglion, it is shown that GAP43 become active in response to hypotonicity. Activation of GAP43 induces Ca²⁺ releasing from inositol triphosphate-sensitive intracellular store that regulates actin dynamics at growth cone (150-152).

GAP43 is involved in Ca^{2+} -dependent fusion of synaptic core complex. GAP43 is suggested to act as a calmodulin sponge which absorbs free calmodulin and sequesters submembranous regions and releases free calmodulin in response to activation of protein kinase C. (153-155).

ATF3 is a nuclear calcium-regulated gene protects against dendrotoxicity and facilitates neuroprotection recovery after excitotoxic change. Calcium entry through synaptic NMDA receptors leads to ATF3 expression in hippocampal neurons. ATF3 acts as a transcriptional repressor that protects hippocampal neurons from apoptosis. (156; 157).

C-jun acts as a calcium-regulated transcription activator that mediates transcription activation following activation of L-type voltage-gated calcium channels. The regulation of c-jun activity by calcium signals requires calcium/calmodulin (CaM)-dependent protein kinase IV and CREB-binding protein (CBP) (158).

Assessing the relation of calcium signaling with the candidate genes (G2) indicates that D1 dopamine receptors and δ -opioid receptors are linked to calcium signaling. Calcium signaling is involved in D1-like dopamine receptors modulation of neocortical and hippocampal neuronal excitability and synaptic processes. D1-D2 dopamine heterodimer activates intracellular calcium signaling leading to activation of CaMKII and production of BDNF in striatal neurons (159; 160).

Opioid receptors are also known to negatively modulate calcium channels. As other GPCRs, $G\alpha$ and $G\beta\gamma$ dissociated from each other following opioid receptors activation. Dissociated $G\beta\gamma$ subunit binds to calcium channel resulting in inhibition of calcium signaling. It is reported that opioid receptor activation inhibits N- and P/Q-type calcium channels but not L-channels in rat nucleus tractus solitaries (161; 162).

The link between the G3 genes and protein kinases are also assessed to find if they involve in similar pathway. PKC is known to interact with STAT3 and phosphorylate STAT3 serine 727. PKC is also shown to interact with GAP43. Phosphorylation of GAP43 by PKC regulates postsynaptic gephrin aggregation in developing GABAergic synapses (163-166).

PKC mediates the actions of D1R and regulates D1R signaling. PKC activation augmented agonist-induced D1R signaling. Phosphor-PKC is also regulated by D1R. Activation of renal D1R translocates PKC from intracellular compartments to the plasma membrane. PKC and D1R are shown to colocalize in goldfish retina. PKC is also demonstrated to phosphorylate δ -opioid receptor leading to β -arrestin and catherin-mediated internalization and desensitization of receptor (167-172).

Another protein kinase that is involved in phosphorylation of proteins encoded by G1 and G2 genes is mitogen-activated protein kinase (MAPK). MAPK negatively modulates STAT3 activity by reducing tyrosine phosphorylation or maybe by association. MAPK regulates ATF3 gene expression. MAPK-dependent activation of c-jun is suggested to be involved in neuronal differentiation. Neuromedin B stimulates the activation of an isoform of MAPK known as P42 MAPK (48; 173-177).

Some of the genes involved in regeneration are transcription factors

The genes are known to involve in regeneration belong to different functional groups. One of these groups is transcription factors. ATF3, STAT3, SOX11 and c-jun are among these genes. From the reference genes, it is found that RORB is a transcription factor perhaps in circadian pacemaking system. Another gene is NR4A2 also known as Nurr1 which is an important transcription factor for development and maintenance of midbrain dopaminergic neurons. OLIG2

is a transcription factor that is expressed in brain oligodendroglial tumors. It regulates ventral neuroectodermal progenitor cell fate (178-180).

To conclude, the phylogenetic profiling data suggests that the axonal regeneration does not seem to be an evolutionarily conserved process. However, the data shows that the similarity in the phylogenetic profile of 80% of reference genes are more than 50% and about 43% of the reference genes have high similarity in their phylogenetic profile (more than 80%). The genes with high similarity in their phylogenetic profile are just expressed in animals. This may be due to the fact that these specific genes are evolved together in animals to do specific function in axonal regeneration.

In order to find the best result from phylogenetic profiling, it is better to assess all genes involved in a specific function. Since axonal regeneration is a multifunctional process and several molecules and signaling pathways are involved in and our knowledge about the factors is still incomplete, finding a consecutive evolutionary tree is difficult. To gain this, we need to expand knowledge about different factors that are involved in axonal regeneration.

Interestingly, we find that most of the candidate genes identified by PrePhyloPro are likely to be either directly or indirectly linked to axonal regeneration. They are known to participate in various physiological functions such as neurogenesis, neuronal regeneration, neuronal differentiation, dendritic arborization, axonal guidance, synapse formation, neuronal response, axonal outgrowth, gliogenesis, remyelination and dendritic developments. This confirms the basic notion behind phylogenetic profiling that the genes or proteins with a similar evolutionary profile are likely to be functionally linked. Confirming the method strengthens the potential involvement of the candidate genes in axonal regeneration or related physiological functions.

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