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Departamento de Ciências Médicas

Susana Duarte Barros Lopes Ferreira

Análise in silico **de transcriptomas de medula espinal regenerativa**

In silico analysis of regenerating spinal cord transcriptomes

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biomedicina Molecular, realizada sob a orientação científica da Dr.ª Sandra Vieira professora auxiliar convidada do Departamento de Ciências Médicas, e a coorientação da Dr.ª Raquel Silva, professora auxiliar convidada do Departamento de Ciências Médicas da Universidade de Aveiro

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palavras-chave

Sistema nervoso central, lesão na espinal medula, neuroregeneração, modelos regenerativos, sobre- e sub-expressão de genes e funções, análise bioinformática

resumo

As lesões na medula espinal são uma desordem neurológica comum com um impacto significativo na sociedade moderna do ponto de visto físico, psicosocial e socioeconómico.

Apesar de vários vertebrados serem capazes de regenerar lesões do sistema nervoso central, nomeadamente da medula espinal (ex. Rã, Peixe-zebra, Salamandra), está bem estabelecido que os seres humanos, e outros mamíferos adultos, não o conseguem fazer. Como tal, em consequência de lesões traumáticas no cérebro ou medula espinal, há incapacidade dos axónios crescerem extensivamente no tecido lesado. No entanto, um estudo importante realizado no virar do século por Ramón y Cajal, comprovou que a incapacidade das fibras nervosas regenerarem "deriva de condições externas, da presença ou ausência de fatores auxiliares que são indispensáveis para o processo regenerativo", trazendo assim esperança que a neuroregeneração possa ser alcançada por modulação de condições celulares e moleculares.

Esta dissertação tem como objetivo adquirir uma melhor e mais extensa compreensão dos genes e processos fisiológicos que são cruciais durante a regeneração da medula espinal, usando estudos de expressão genómica de modelos regenerativos, tais como Xenopus laevis, Xenopus tropicalis e Danio rerio, estabelecendo-se simultaneamente um paralelismo com os respetivos ortólogos humanos com o objetivo de encontrar genes candidatos no genoma humano passíveis de serem modulados com vista a alterar o estatuto não-regenerativo dos mamíferos adultos.

keywords

Central nervous system, spinal cord lesion, neuroregeneration, neuroregenerative models, upregulated and downregulated genes and functions, bioinformatics analysis

abstract

Spinal cord injuries are a common neurologic disorder that have devastating impacts on modern society, be it from physical, psychosocial, or socioeconomic point of view.

Although many small vertebrates are capable of regenerating lesions to the central nervous system, namely the spinal cord, (e.g. frog, zebrafish, salamander) it is well established that humans and other adult mammals cannot. As so, failure of axons to grow extensively through damaged central nervous system (CNS) tissues is a common consequence of injury to the brain and spinal cord on adult mammals. However, an important study made at the turn of the century by Ramón y Cajal, proved that the failure of central fibers to regrow “derives from external conditions, the presence or absence of auxiliary factors that are indispensable to the regenerative process”, thus bringing hope that neuroregeneration can be achieved by modulating cellular and molecular conditions.

Through this dissertation, we aim to get a better understanding of the involvement of the genes and physiological processes that are crucial during regeneration of the spinal cord, using genome wide expression studies of regenerative models such as Xenopus laevis, Xenopus tropicalis, and Danio rerio, while drawing parallel to its human orthologues. Being our goal to find perfect gene candidates in the human genome that are predictably capable of being modulated so we can alter the non-regenerative status of the adult mammals.

Abbreviations

ASIA - American Spinal Injury Association
ATP – Adenosine Triphosphate
BDNF – Brain Derived Neurotrophic Factor
BSCB – Blood-Spinal Cord Barrier
BP – Biological Processes
CC – Cellular Components
ChABC – Chondroitinase ABC
CINC - Cytokine-Induced Neutrophil Chemoattractant
CNS – Central Nervous System
CNV – Copy Number Variation
CSPGs – Chondroitin Sulfate Proteoglycans
CTGF – Connective Tissue Growth Factor
DNA – Deoxyribonucleic acid
DREZ – Dorsal Root Entrez Zone
DSPGs – Dermatan Sulphate Proteoglycans
DRG - Dorsal Root Ganglia
DPT – Days Post Transection
ECM – Extracellular matrix
ES – Electrical stimulation
GFAP - Glial Fibrillary Acidic Protein
GAG - Glycosaminoglycan
GWE - Genome wide expression
HSPGs – Heparan Sulphate Proteoglycans
KSPGs – Keratan Sulfate Proteoglycans
MAG – Myelin Associated Glycoprotein
MF – Molecular Functions
MMPs – Metalloproteinases
MPO – Myeloperoxidase
NAD – Nicotinamide Adenine Dinucleotide
NADPH - Nicotinamide Adenine Dinucleotide Phosphate
NMN – Nicotinamide Mononucleotide
NMNAT – Nicotinamide Mononucleotide Adenylyltransferease
NSICSC – National Spinal Cord Injury Statistical Center
NT–3 – Neurotrophin-3
OMgp - Oligodendrocyte-myelin glycoprotein
PPi - Pyrophosphate
PNS – Peripheral Nervous System
P4H – Prolyl 4-Hydroxylase
RNAseq – Ribonucleic Acid Sequencing
SCI – Spinal Cord Injury
SCIRE – Spinal Cord Injury Research Evidence
TGF – Transforming Growth Factor
WD – Wallerian Degeneration

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Chapter 1 – Introduction

Spinal cord injuries (SCI) are amongst the most serious and debilitating health conditions, with devastating impact on a patient's life style since it causes marked neuropathology and limited functional recovery. More often than not, SCI results in a physical disability of the patient, with tremendous impact on one's life quality and the life quality of the family. Spinal cord regeneration is very inefficient in humans, causing paraplegia and quadriplegia. Indeed, humans, and adult mammals in general, have a limited capacity to regenerate, and thus severe injuries result in unsightly scarring, loss of function and disfigurement. However, species across the animal kingdom show a variable range of regenerating ability, with some fish and amphibians being capable of regenerating complete appendages after amputation during the entire lifespan or just during some stages of their lives. For example, *Xenopus laevis and Xenopus tropicalis* have a regenerative and non-regenerative stage. As tadpoles, these species are fully capable of functional recovery after spinal cord injury, while its juvenile form (froglet) loses this capacity during metamorphosis[3]. This remarkable ability shown by regenerative animals is of great interest. Studying model organisms that can regenerate the spinal cord in response to injury could be useful for understanding the cellular and molecular mechanisms that explain why this process fails in humans. As LV Polezhaev stated: "In order to study why regeneration of organs does not occur in those animals which do not possess regenerative capacity, it is necessary to know how the process of regeneration occurs in animals which do possess regenerative capacity"[4]. According to his ideas, in order to initiate regeneration of organs which under normal conditions do not regenerate, it is necessary to manipulate basic mechanisms directly, changing physiological events and the metabolism of the organism and of the organ[4].

Regeneration of the central nervous system has always been a subject of intense investigation, but despite the amount of scientific research and breakthroughs that we were able to achieve, it still feels like something out of reach. This is mainly due to underlying absence of conditions for this process to occur: on one hand, the lack of substances able to sustain and invigorate the slow and scanty growth of the sprouts,

and on the other hand, the scarcity in the paths or systems of interrupted nerve fibers of catalytic agents capable of attracting and directing the axonic current to its destination[2].

Meaningful rehabilitation after SCI encompasses many aspects of acute and chronic patient care, and the underlying basic science of wound healing in the central nervous systems involves fundamental concepts of cellular structure and function, and a functional communication between glial cells and neuronal processes, as well as complex molecular interactions between cells and their extracellular environments. As such, we can infer that should experimental biology accomplish methods for inducing regeneration, it is going to be as a result of a combinatorial approach and by means of multifactorial components. Therefore, it is essential to find and study the plethora of elements required for successful regeneration, and devise strategies with which to move more rapidly toward a clinically efficacious therapy.

1.1 – The Spinal Cord

The nervous system is broadly subdivided into Peripheral Nervous System (PNS) and Central Nervous System (CNS). The CNS consists of the brain and the spinal cord. The PNS is composed by cranial nerves and spinal nerves together with their ramifications and cell bodies that make up the peripheral ganglia[5].

The spinal cord or *medulla spinalis,* forms the elongated, nearly cylindrical, part of the central nervous system which is contained within the vertebral column in the upper two-thirds of the vertebral canal. Its average length in the male is about 45 cm and in the female 42 to 43 cm, while its average weight amounts to 30 grams. It extends from the level of the upper border of the atlas to that of the lower border of the first or second lumbar vertebrae[6]. The spinal cord major role is in controlling the functions of and receiving the input from the trunk and limbs via afferent and efferent connections that are organized in 31 pairs (12 thoracic, 5 lumbar, 5 sacral and 1 coccygeal) of segmentally arranged spinal nerves that attach to the cord as dorsal and ventral rootlets[5].

The spinal nerves are named and numbered according to the vertebrae from which they emerge, and so, cervical nerves 1-7 emerge from their respective 1-7 vertebrae.

Since they are only seven vertebrae, the eighth cervical nerve emerges between the seventh cervical (C7) and the first thoracic vertebrae (T1) (Figure 1).

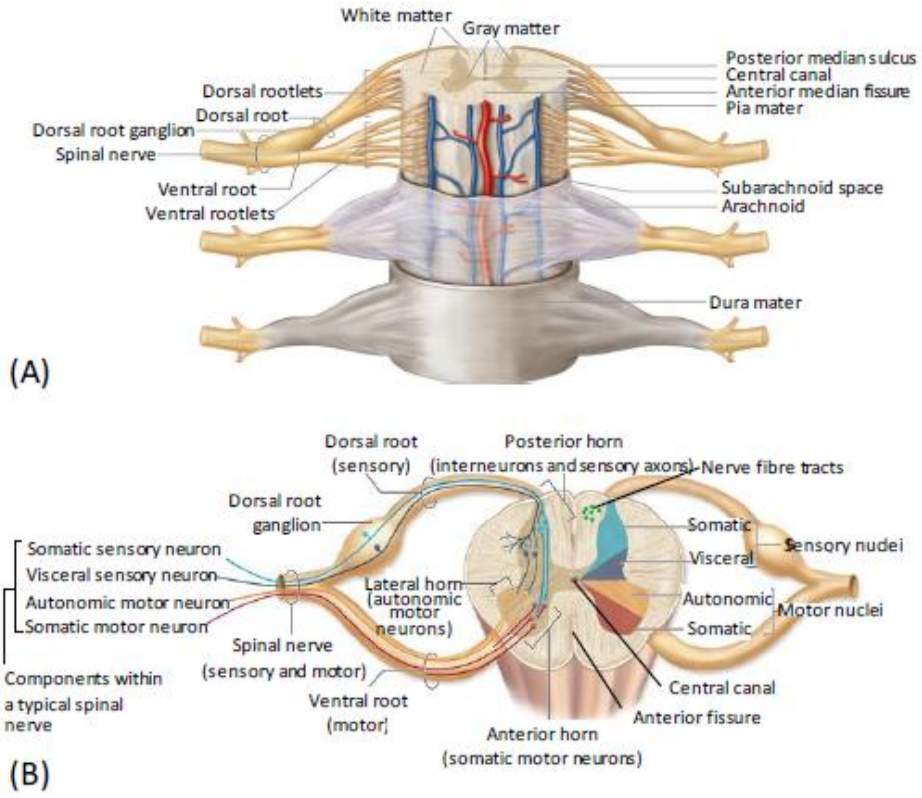


Figure 1 – Representation of a few spinal cord segments with its associated nerve fibers. (A) Ventral perspective; (B) Transected cut (Adapted from [8]).

Each spinal nerve possesses two roots, an anterior and a posterior, which are attached to the surface of the med*ulla spinalis* opposite the corresponding column of gray substance. The posterior or dorsal roots carry primary afferent (sensory) nerve fibers from cell bodies located in dorsal root ganglia, while the anterior or ventral roots carry efferent (motor) fibers from cell bodies located in the spinal grey matter. The sensory nerve roots enter the spinal cord through the dorsal roots, and the motor roots emerge from the cord via ventral roots, at each level[5]. The cell bodies from the afferent nerve fibers, belonging to the peripheral nervous system, are located in the dorsal root ganglion where a single axon bifurcates (Figure 1) giving rise to one branch

that connects with the periphery (e.g. the skin of a foot) and another one that connects with the dorsal horn of the spinal cord[7].

Internally, the spinal cord differentiates into a central core of gray matter surrounded by white matter. The gray matter consists of numerous nerve cells and nerve fibers held together by neuroglia. Throughout the most part, the gray matter presents the appearance of a sponge-like network, but around the central canal and on the apices of the posterior columns it mostly resembles a gelatinous shape.

The grey matter is shaped into a characteristic butterfly form (Figure 2), and consists of two symmetrical portions, one on each half of the medulla spinalis[6].

The white matter consists of medullated nerve fibers imbedded in a sponge-like network of neuroglia, arranged in three funiculi: anterior, lateral and posterior. The anterior funiculus lies between the anterior median fissure and the most lateral of the anterior nerve roots; the lateral funiculus between these nerve roots and the postero-lateral sulcus; and the posterior funiculus between the postero-lateral and the posterior median sulci.

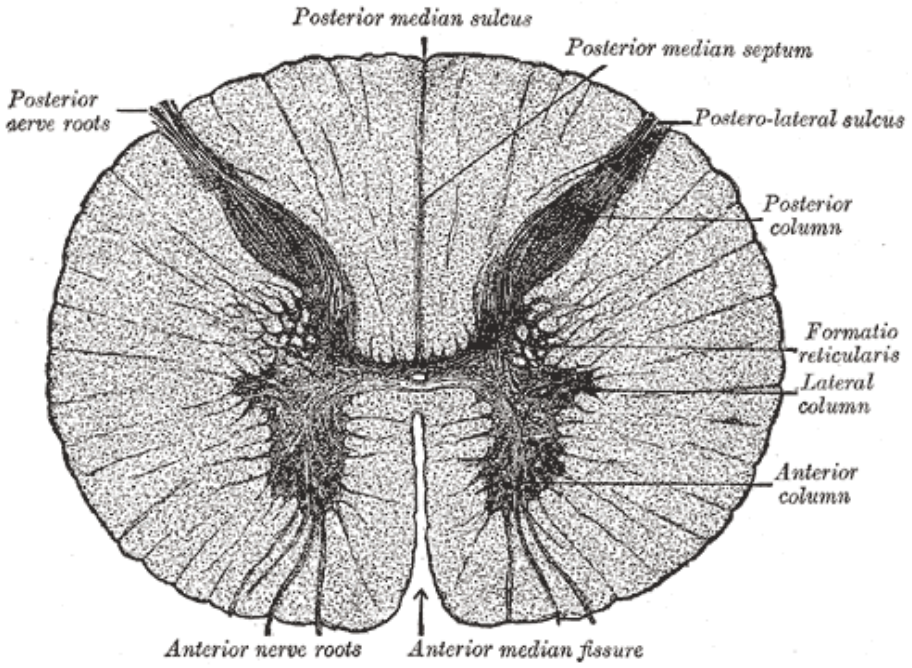


Figure 2 –Transverse \of the *medulla spinalis* in the mid-thoracic region (Taken from [6]) .

1.2 – The Spinal Cord Injury through History

The first descriptions of spinal cord injury originate in ancient Egypt (Third Dynasty, 3000-2500 years BC). During that period, a great amount of human resources was dedicated to construction work, namely the creation of pyramids, which lead to a high incidence of trauma, enabling to describe a great variety of injuries in the human body[9]. In 1862, the Egyptologist Edwin Smith purchased documents from that period, probably written by Imhotep, an Egyptian official, and architect responsible for the step pyramid of Sakkara and a practicing surgeon, which contained among 48 cases of trauma, 6 reporting to spine and/or SCI. One case ("*Treatment instructions concerning a crushed vertebra of the back of his neck*") reports a complete SCI, caused by a fracture in the cervical region, presenting paralysis of both arms and legs (tetraplegia), loss of sensation below the level of injury, and loss of urinary bladder control, concluding that it was "an ailment not to be treated"[9,10]. Another interesting fictional reference to traumatic SCI dates the time of the Trojan War (1100 BC) when according to the epic tale of the Odyssey, Elpenor fell from the palace roof and broke his neck, leaving his soul to the nether world ruler, Hades[9].

Centuries later (460 - 377 BC), Hippocrates analyzed the correlation between vertebral and spinal cord injuries. He observed that if the spinal cord damage had only occurred on one side, a subsequent paralysis would be located on the same side as the damage. He also described clinical conditions of chronic paralysis, such as constipation, bladder problems, pressure sores, and venous stasis of the lower limbs, because of a traumatic spinal cord injury. In order to reduce spinal deformities, Hippocrates created traction devices (Figure 3), where spinal manipulations were carried out. These devices credited him for creating methods for reducing spinal deformities. These extension techniques with the application of traction are still widely used today in the treatment of spinal disorders. Nonetheless, he believed that there was no real treatment for individuals with fractures combined with paralysis and that they were destined to die[11]. Due to this and other efforts in the field of medicine, Hippocrates was considered the "father of medicine" and was able to separate medicine from mythology, as it is summarized in the book *Corpus Hippocraticum*, which he wrote[9].

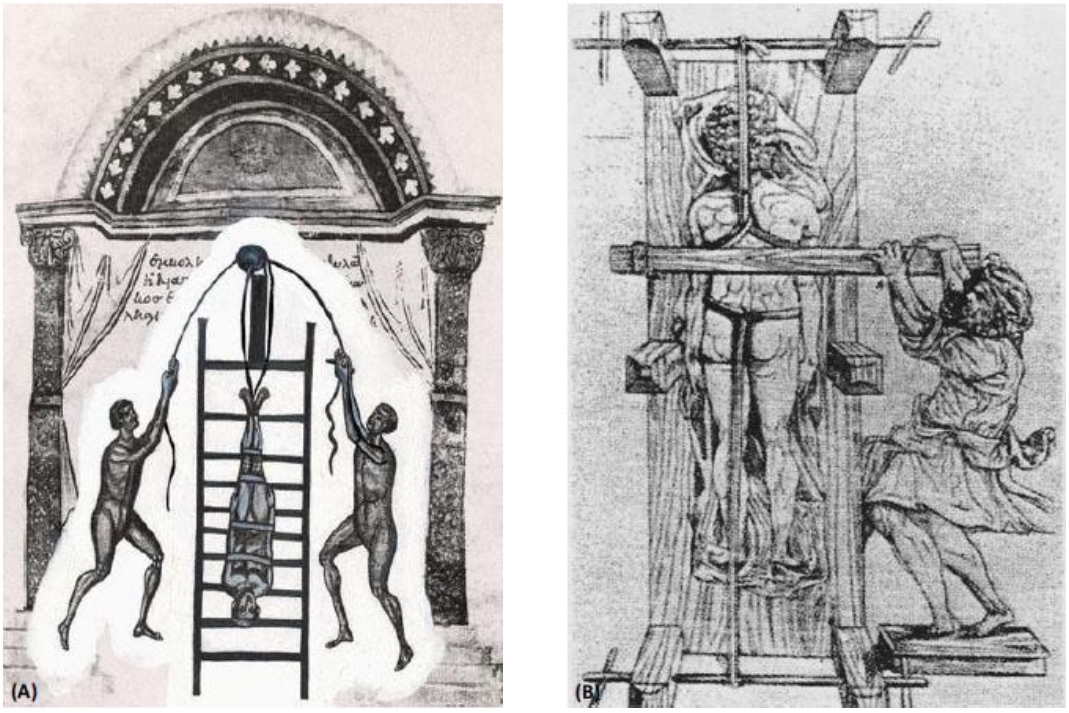


Figure 3 - Hippocratic Ladder (A) and Board (B) devices. To reduce spinal curvatures, the patient was hung upside down while tied on a ladder (A); the weight of the trunk and limbs would act as the pulling force, which would straighten the spine. In the Hippocratic Board (B) the patient was placed in a prone position and stretched from the shoulder area upward, and from the hips downwards; a wooden board would be placed crosswise over the injured area and its compression would lead to deformity reduction. Taken from [9,11]

Some of the greatest advances in the understanding of the spinal cord dysfunction were conducted by another eminent greek physician, Galen of Pergamon (130-200 AD) being considered the father of experimental physiology. Galen, who was initially a physician of gladiators, had a unique opportunity to study traumatic and often traveled to Alexandria to study human anatomy in particular affections to the spinal cord[12]. At that time, he not only described the anatomy of the brain, spinal column, and spinal cord, he proved experimentally in his work "*On Anatomical Procedures"* and "*On affected areas"* that injuries to the spinal cord led both to paralysis and loss of sensation below the lesion. More so, he described four types of spinal deformities, namely kyphosis, lordosis, scoliosis, and succussion[12,13]. Additionally, he noted that a longitudinal incision did not result in any symptoms but a transverse incision at the level of the cervical vertebras would result in paralysis and loss of sensory functions below the level of injury[11].

After nearly a thousand years of medical stagnation, it was only in 1543, that the Flemish anatomist and physician Andreas Vesalius (1514-1564) published one of the most significant medical books ever written, composed of seven volumes, *"De Humani Corporis Fabrica*", showing for the first time drawings of the human nervous system correctly illustrated[14]. Because until that time, the anatomical conception of the human body, as presented by Galen and Hippocrates, was mainly based on studies conducted on cadavers in battlefields, from observation of athletes exercising, and from dissections of animals, once dissection of human bodies was prohibited[12]. For these reasons, the work of Vesalius constitutes the first textbook who presents the human body in a detailed and correct fashion[9].

In 1646, the German surgeon Fabricius Hildanus described a new method to reduce cervical region dislocations: he inserted a clamp through the spinous processes in the neck, after which traction was applied. Almost a century later, in 1762, during the battle of Amenenburg, the surgeon Andre Louis successfully removed a bullet lodged in the lumbar spine of a soldier, and he not only survived but also regained some functional movement in the lower extremities[11].

During the 19th century, debate raged about the efficacy of spinal cord injury and many physicians considered spinal cord operations to be something fruitful: "Laying a patient upon his belly and by incisions laying bare bones and exposing the spinal marrow itself, exceeds all beliefs". This nihilism toward this type of surgical intervention was further increased when Henry Cline in 1814 attempted to perform a decompressive laminectomy in a patient who suffered a thoracic fracture dislocation and complete paralysis, and the patient not only did not experience any kind of clinical improvement but also died nine days later[9,11]. Not all experiments with surgery during this period, however, were so negative, and English surgeons like Astley Cooper and Benjamin Bell still favored this application, despite such poor outcomes accomplished by their colleagues. It was during this period, in 1824, that Bell also included descriptions of the types of neurological derangements that resulted from trauma, such as the distinction between flaccid and spastic paralysis and the concept of spinal shock[11].

During World War I (July 28th 1914-November 11th 1918), mortality following SCI was as high as 80% within the first two weeks following injury. According to American data collected for a brief period of 4 weeks' time, there were 147,651 admissions to the

hospital, whereas 598 of these patients sustained wounds to the spine. During wartime, medical advertisements were constant reminders of the casualties and disabling wounds (Figure 4)[15].

Figure 4 – Advertising devices for invalid transportation during the World War I. Taken from [15]

From there on, the importance of addressing care for spinal cord injury victims was perceived, as the education in this field became paramount in the medical schools' programs. Government funding became a financial base for physical rehabilitation, occupational therapy, and vocational re-education. During the 1920s, neurosurgery was also recognized by professional societies as a specialty in the US and the UK[15].

Thus, from the middle of the 20th century, there were great advances in the technology used in basic science research applied to the study of SCI. Also, during these decades, several associations aroused, which contributed to the diagnosis and comprehensive management of spinal cord injuries through annual meetings, teaching sessions, workshops, and research grants, and with the opening of rehabilitation centers. These centers opened the way for better care of spinal cord injured patients, not only increasing the patient's survival but also leading them to readapt to the society[11].

Among the most recent advances on experiments in nerve injuries, the work of Ramon y Cajal in the mid-1920s is to be mentioned[2]. In his first chapter, he describes in detail the phases of Wallerian degeneration of peripheral nerves, mentioning the particularity that forming fibers avoid capillaries and fat cells, passing around them. Besides the fibers under degeneration, he also observed some emitting newly formed

collaterals, and others ending in growth cones that could penetrate the region of the scar*: In contrast to the clubs and masses lacking a generative capacity, there are others which are active, capable of sprouting, and to which we have given the name of bud or club of growth because their analogy to the cone of growth of embryonic axons*[2,7,8] (Figure 5).

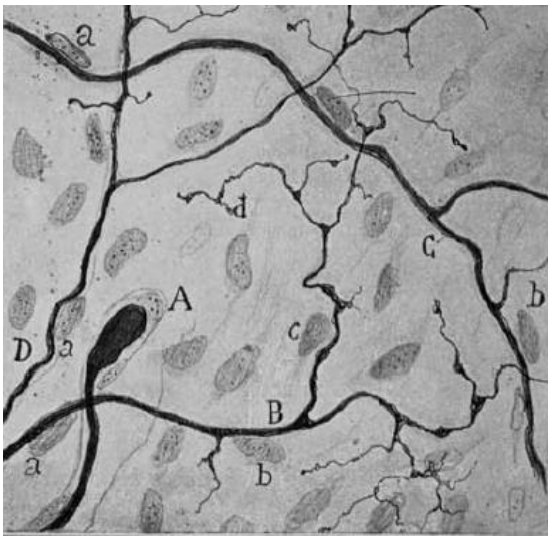


Figure 5 – Portion of the cicatricial nerve segment displaying fibers that are ramified around other cells. Taken from [7]

Nowadays, the care that is given to spinal cord injury patients is much more efficient, although it requires knowledge within a variety of medical fields and also an interest in development and research. Neuroprotection and regeneration are currently the main focus of the latter. Although healing the injured spinal cord is still out of reach, it is now possible to offer several therapeutic measures to improve the health and quality of life of spinal cord injured victims, with the aspiration of providing them a long and fulfilling life.

1.3 – Epidemiology of Spinal Cord Injury

Spinal cord injury remains one of the most important causes of morbidity and mortality in modern society. More than half of all the injuries to the central nervous system, namely in the spinal cord, result from trauma. Vehicle crashes are currently the leading cause of injury with an incidence around 38%, followed by falls, that

represent 30.5%, 13.5% from violence, and 9% as a result of sports injuries[16]. Some diseases can also cause severe injury to the spinal cord such as multiple sclerosis, diabetes, spina bifida and others.

According to the National Spinal Cord Injury Statistical Center's (NSCISC) 2016 database, an estimated 17,000 people suffer traumatic SCI in the United States each year. In Europe, the estimated incidence oscillated from 3.3 to 130.6 individuals with traumatic SCI per million inhabitants a year. This echoes the experience of several countries including Bulgaria, Denmark, Finland, France, Germany, Iceland, Ireland, Italy, Norway, Portugal, Romania, Spain, The Netherlands, Estonia, and Turkey (Table 1 – Annexes). Data from 2005 and forward indicates that the common age for injury is 40.7 years and the majority (80.7%) tend to be represented by males, with a three-fold to four-fold increased incidence when compared with the female gender[16,17].

The number of injuries occurring around the world is alarming (Figure 6). In 2001 the European Council predicted that each year, 85,000 people would be confined to a wheel chair for the rest of their lives, after surviving a traumatic spinal cord injury. According to the same institute (NSCISC), since 2010 the neurologic category most reported to the database is incomplete tetraplegia (40.6%), followed by incomplete paraplegia (18%) and complete tetraplegia (11.6%). Less than 1% of persons experience complete neurologic recovery by hospital discharge[16].

Developing countries have the highest 1-year mortality rates and in some of them, the occurrence of a spinal cord injury is likely to be fatal in the first year following injury. Until recently, the leading cause of death was renal failure; meanwhile, the advances in urologic management have reduced this number of cases. Presently, pneumonia, pulmonary embolism, and septicemia are the main causes of the reduced life expectancy for this population[16]. Nonetheless, life expectancy for patients with spinal cord injuries has been improving over the last 40 years in developed countries when compared to under developed ones[18,19].

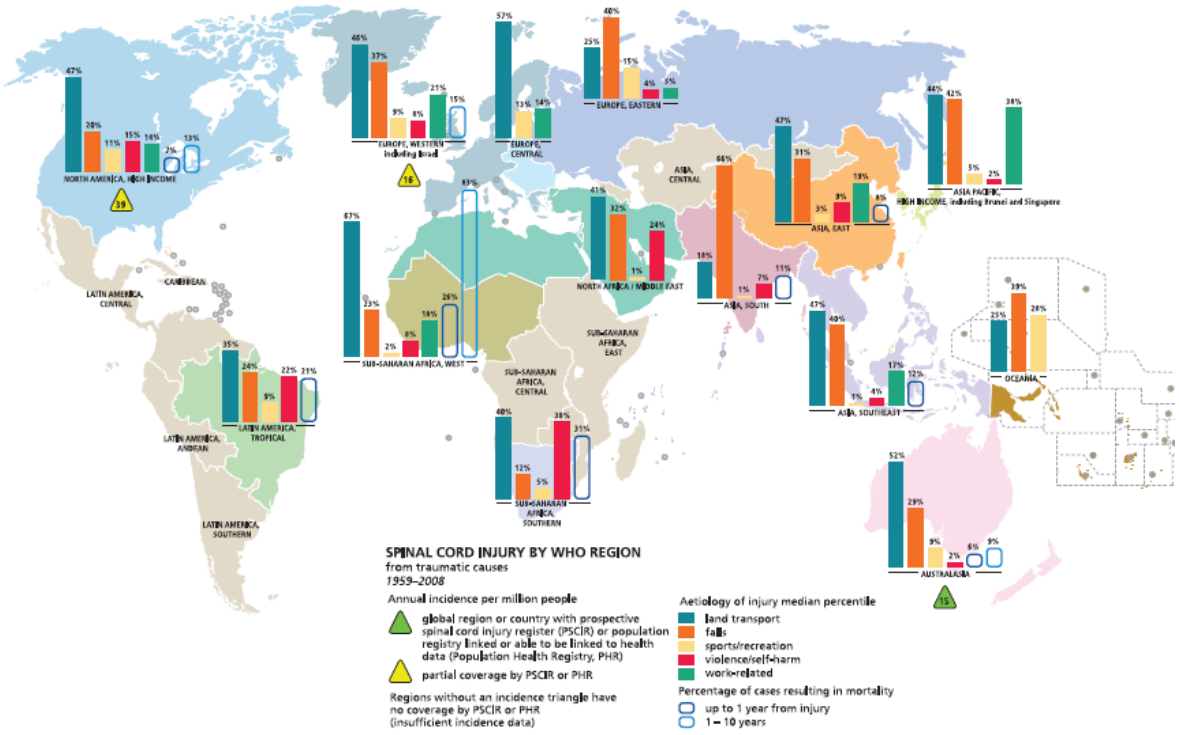


Figure 6 - Global mapping of SCI from traumatic causes by WHO regions 1959–2008. Taken from [18]

1.4 – SCI consequences and classification

Human spinal cord injury often results from a traumatic insult to the spinal column or by contusion injuries. After the trauma, there is a subsequent displacement of the spinal cord due to the pressure from the broken bone, disk fragments, hematoma and swelling inside the closed vertebral canal. The consequent injury has a series of serious effects on the spinal cord segment with the breakdown of connections and networks (Figure 7) and it may compromise either completely or incompletely its major functions, namely the motor, sensory, autonomic and reflex functions[20].

The functional consequences of such an injury are associated with autonomic paralysis including dysfunctional internal organs, limb muscle atrophy, sensory impairment, and chronic pain[17]. After a spinal cord injury, many cells die immediately, as well as progressively, and disconnected myelinated axon segments are phagocytosed by macrophages. Cyst formation (Figure 7) usually occurs in this location, and axons can sprout into trabeculae that are formed from ependymal cells. Many ascending and descending axons are interrupted and fail to regenerate over long distances[1].

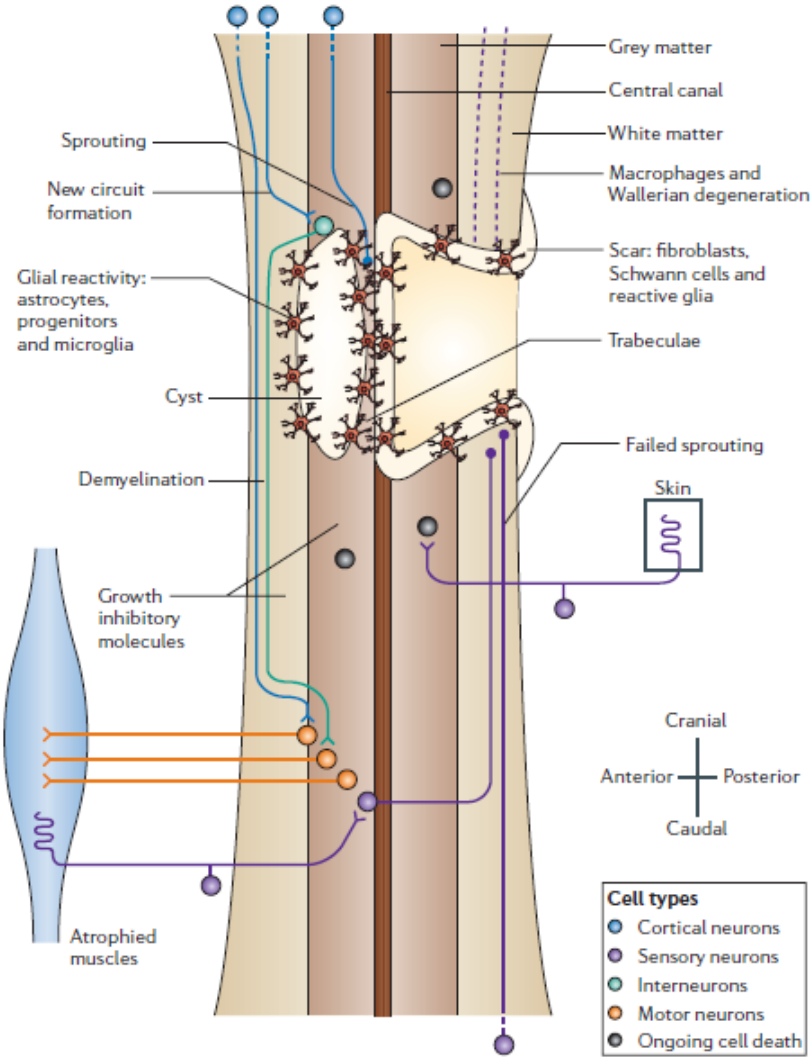


Figure 7 – Representation of a sagittal view through a region of cervical spinal cord injury (SCI), showing a combination of features from different types of injury. From:[1].

Many complications may arise from SCI including loss of bladder and bowel control, increased risk for urinary tract infections, sexual dysfunction, skin breakdown and pressure sores, spasticity, inability or reduced ability to regulate heart rate, blood pressure and body temperature, autonomic dysreflexia, muscle atrophy, blood clots, osteoporosis and cardiovascular disease can result from injury and from the consequent reduced physical activity[21]. Also, clinical reports show that spinal cord injured patients present a lifelong inability to maintain a neutral energy balance, meaning that they tend towards nutritional deficits that result in an underweight body mass. These patients usually represent a higher risk for developing an infection and the due to the insufficient subcutaneous fat mass they are at increased risk of presenting pressure ulcers[21].

1.4.1 - Spinal Cord Injury Classification

Intense inflammatory responses follow spinal cord injuries. It initially undergoes a primary mechanism of injury, followed by a substantial secondary damage as a result of an inflammatory reaction that further promotes more tissue damage, but that is also involved in its repair[22]. Overall, the first largely determines the patient's hospital admission neurological stage/grade, therefore representing a strong prognostic indicator[20], and the latter may contribute to the exacerbation of the damage caused and limit the healing processes, which means this secondary mechanism of injury may lead to overall morbidity and mortality.

The patients are grouped into severity categories (neurologic grades) according to the extent of their primary injury and classified according to the American Spinal Injury Association (ASIA) impairment scale (AIS)[17]. The extent of the neurological injury is stratified into "complete" (ASIA grade A), when no motor functions are preserved from the sacral nerves (S4-5), and "incomplete" (ASIA grade B-D), with ASIA grade E representing a normal neurological status. ASIA grade B reflects an incomplete motor impairment whilst the sensory functions remain intact. In ASIA grade C and D the motor function is preserved below the neurological level, and more than half of key muscle functions below the neurological level of injury (Figure 8)[17].

There are four main characteristic mechanisms of primary injury: a) impact plus persistent compression, b) impact alone with transient compression, c) distraction and d) laceration/transection. The first one most commonly involves impact plus persistent compression, which is frequently seen in burst fractures with retropulsed bone fragments compressing the cord, fracture-dislocations, and acute disc ruptures. The second one involves solely an impact with only transient compressions, as are seen in hyperextension injuries in individuals with underlying degenerative cervical spine disease. The third mechanism for spinal cord injury involves distraction, forcible stretching of the spinal column in the axial plane[20]. Radiological studies may not detect this type of trauma, especially in children which still possess cartilaginous vertebral bodies, ligament laxity, and underdeveloped musculature, or in adults with underlying degenerative spine disease[23]. The last mechanism for spinal cord injury, laceration, may range from minor injury to complete transection and usually results from missile injury, sharp bone fragment dislocation, or severe distraction[20]. A complete

transaction of the spinal cord is rare but may occur from high-energy rotational/translational forces, as it happens in splice fractures on the C3[17].

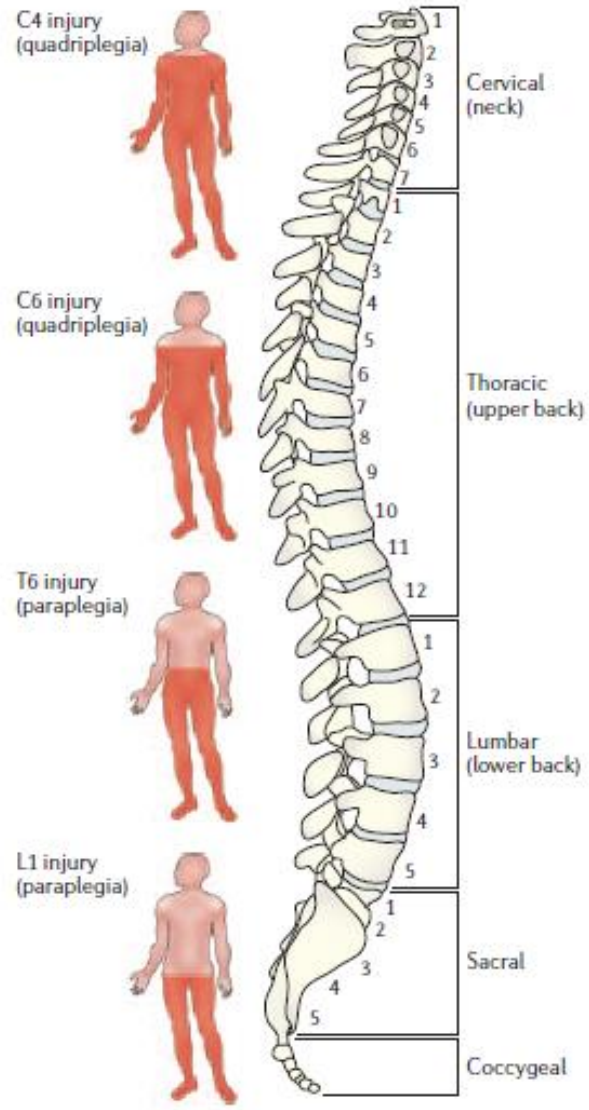


Figure 8 – **The ASIA impairment scale.** Levels of injury and extent of paralysis. Injury to specific levels of the spine can cause spinal cord injuries. Varying degrees of nerve damage often result in paralysis bellow the lesion site. Adapted from [1].

The consequences of a spinal cord injury may vary depending on the type, level and severity of the injury. Complete injuries lead to the loss of function below the level of injury, resulting in the absence of motor and sensory function. In incomplete injuries, some sensation and/or movement below the level of injury is retained. A spinal cord injury at the neck level may result in tetraplegia and impair the ability to breathe. Injuries to the lower spine may cause weakness and loss of sensation in the legs and lower parts of the body, and paraplegia (Figure 8)[21].

1.5 - Regeneration in the Central Nervous System

For a very long time, it was widely accepted the hypothesis firstly postulated by Edwin Smith[10] that an injured spinal cord was an untreatable condition. However, in the late 20s, Ramon y Cajal[2] conducted the above mentioned pioneer study that postulated that the central nervous system had an intrinsic ability to regrow after an injury, though this would not happen spontaneously due to lack of trophic support and the formation of physical barriers, such as glial scars, which would prevent the axons to grow past the lesion site[2].

Since then, there have been several advances in spinal cord injury research, for instance, the work of David and Aguayo in the early 1980s, that showed that CNS axonal processes were able to regenerate for significant distances when their regrowth was supported by long peripheral nerve bridges circumventing the spinal cord lesion[24]. However, once that peripheral nerve graft was gone the nerve fibers were not able to grow past that site, an observation that still holds for most therapeutic approaches, regarding nerve grafts in the field of SCI[25].

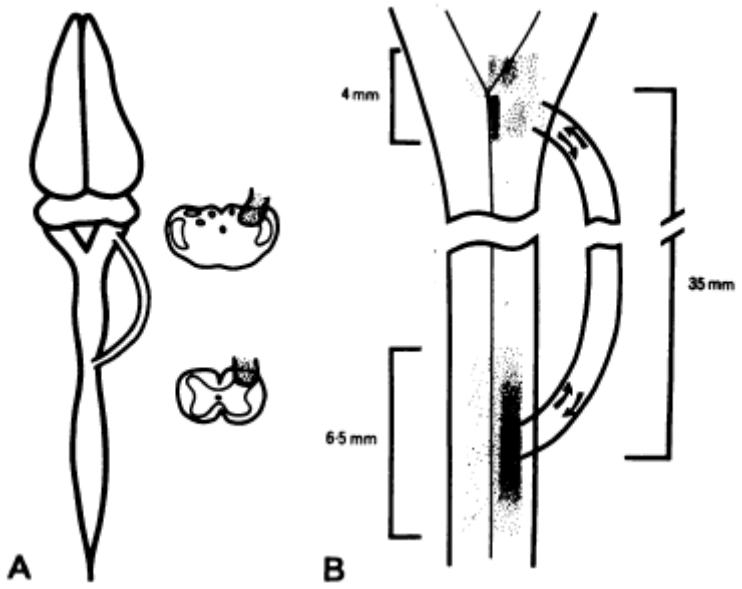


Figure 9 – (A) Diagram of the dorsal surface of the rat CNS, showing a peripheral nerve graft-linking the medulla and the thoracic spinal cord. (B) Approximate rostrocaudal position of CNS neurons (dots). Taken from [24].

In 1985, Schwab and colleagues also saw sympathetic or sensory axons invade and grow through sciatic nerve explants in culture, but never into and through optic nerve explants. This further proves what it is seen *in vivo*, where regenerating peripheral or central neurons grow well inside peripheral nerve transplants but do not grow and elongate within CNS environment[26]. The referred cultures within CNS explants were grown in optimal conditions, leading to the conclusion that even under optimal conditions the axons did not grow, because it is not only trophic factor deficiency's that cause the failure in axon growth. Both living and dead optic nerve were avoided by the growing axons, which shows the presence of inhibitor factors that prevented the growth of these axons[26]. This means, the axonal regeneration process is limited or impeded due to the absence of a combination of factors that should be present simultaneously at the injury site, which may not occur due to some events that imped this environment to be formed, as myelin constituents and the presence of a glial scar that contributes to regenerative failure. Moreover, guidance molecules like semaphorins and ephrins are present at the lesion site and tend to repel regenerating axons in the CNS[27].

1.6 – Wallerian Degeneration

In 1849, Augustus Waller discovered that if a bundle of fibers was to be cut, the portions of fibers which are separated from their cells rapidly degenerate and become atrophied, while the cells and the parts of the fibers connected with them undergo little alteration[6]. This was termed 'Wallerian degeneration' defined as the process of degeneration of the axon distal to the site of transection, in which the entire distal segment of the nerve degenerates, with disintegration of the distal axonal segment, degradation of the myelin sheaths and axon cytoskeleton, macrophage infiltration and subsequent apoptotic death of oligodendrocytes around the lesion site[28]. Hypotheses about what initiates Wallerian degeneration include loss of trophic support from the cell body and activation of calpain by calcium influx[28].

Axons depend on the supply of energy in form of ATP to fuel important processes like axonal transport, maintenance of the resting membrane potential and

neurotransmitter release. When these ATP levels are not met, irreversible damage to the axons occur. Little is known about the regulation of Wallerian degeneration, but the mapping of the *Wld* gene should give some insight. In particular, an important tool to study Wallerian degeneration is the axon-protective Wallerian degeneration slow (WLD^s) mutant mouse which has shown to ameliorate the decline in axonal ATP and NAD levels after neurite transection[29]. WLD^s protein is thought to act in the axon through its NMNAT activity, converting nicotinamide mononucleotide (NMN) and ATP to NAD and PPi[29].

Wallerian degeneration occurs in both PNS and CNS but with some differences (Figure 10). In the PNS, after axonal degeneration, the blood-tissue barrier permeability increases, the myelin sheaths break down, Schwann cells that formerly ensheathed the axons proliferate, align to form longitudinal arrays, and increase their production of neurotrophic factors that can promote axon regeneration; an influx of macrophages further occurs to remove the cellular and myelin debris distal to the site of axonal injury.

Proximal to the injury site, neuronal cell bodies react to injury by inducing expression of growth-related genes. In mammals, Wallerian degeneration (WD) in the PNS is fast, taking about 7 to 14 days, or even earlier. The initiation of WD in the severed optic nerve of the regenerating animal newt (*Triturus viridescens)* showed significant degeneration of nonmyelinated axons as early as six hours after lesion and was almost complete after 48h[30,31].On the other hand, WD in the CNS of mammals is dramatically slower, taking months to years. These differences are probably a result of the failure to clear CNS myelin debris. In the CNS, this failure in myelin debris' clearance may be due to a lack of extensive opening of the BBB, as it occurs in PNS, preventing the entrance of serum opsonins and peripheral macrophages in distal white matter tracks. As a result, the persistence of myelin debris in the CNS contributes to the failure of CNS axons to regenerate[30]. The myelin debris has several inhibitors of axonal regeneration namely Nogo, myelin-associated glycoprotein (MAG), oligodendrocyte-myelin glycoprotein (OMgp), tenascin-R, proteoglycans, and chondroitin sulfate proteoglycans (CSPGs), which contribute to the unsuccessful regeneration of the CNS. Astrocytes at the site of injury also interfere with regeneration, and neurons typically fail to activate

the growth-associated genes[30]. Another key difference between the rate of WD in CNS and PNS involves the differential actions of Schwann cells (SCs) in the PNS and oligodendrocytes in the CNS. While the SC have a pivotal role in both the breakdown of the myelin sheath and the clearance of its debris in the PNS, in contrast, oligodendrocytes have little to none ability to help clear myelin and axons debris in the CNS[30].

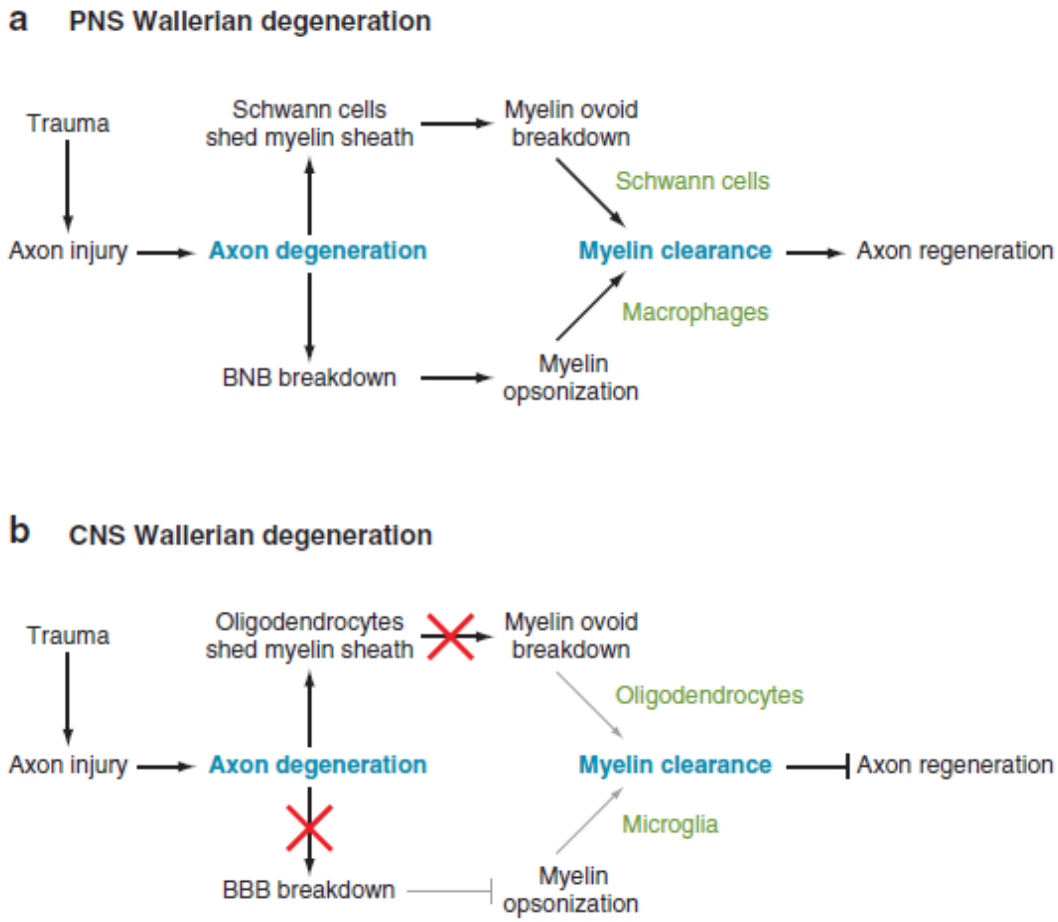


Figure 10 – Comparing Wallerian degeneration (WD) in the PNS and CNS. (*a*) Schematic summary of the histological changes during WD in the PNS leading to myelin clearance. Axon degeneration triggers two parallel responses that result in rapid clearance of myelin debris: firstly, Schwann cells break down myelin into smaller myelin ovoids, then serum-derived opsonins stimulate macrophage clearance of myelin debris. After that, rapid removal of myelin enables axon regeneration in the PNS. BBB, brain-blood barrier. (*b*) During CNS WD neither one of these processes exist. Oligodendrocytes do not clear myelin ovoids, and the lack of extensive opening of the Blood-Brain Barrier (BBB) prevents the entrance of serum opsonins and peripheral macrophages in distal white matter tracks. The persistence of myelin debris in the CNS contributes to the failure of CNS axons to regenerate. Taken from [30]

Why WD is rapid and robust in the PNS but slow and incomplete in the CNS is a longstanding mystery, and deciphering this mystery could account for the failure of CNS axons to regenerate. What we do know is that in the PNS, rapid WD results in an extracellular environment that promote axon regeneration, whereas in mammalian CNS, slow WD results in the prolonged presence of myelin-associated inhibitors that likely contribute to the failure of axons to regenerate[30]. For this reason, it is plausible to infer that triggering rapid CNS myelin clearance may enhance CNS axon regeneration after injury.

1.7 – The Glial Scar

Scarring is a general tissue response after injury that promotes wound healing and physically separates the lesioned tissue from the external environment. It consists of a dense extracellular matrix network, whose backbone is made of collagen IV, and that serves as a binding matrix for numerous other extracellular matrix components and inhibitory molecules like proteoglycans and semaphorins, but also growth-promoting factors[32]. The fibrous scarring is surrounded by the glial scar which is the area of astrogliosis, characterized by high immunoreactivity to glial fibrillary acidic protein (GFAP)[33]. Hence, although the wound healing scar may contribute to seal the injury site from the spared tissue, the glial scar is far more than just a physical barrier. It's a source of factors such as tenascid acid, semaphorins, ephrins, various proteoglycans, that make up the inhospitable biochemical milieu that makes it impossible for axons to regrow[34].

The glial reaction to injury results in the recruitment of microglia, oligodendrocyte precursors, meningeal cells and astrocytes to the lesion site. These cells give both structural and physiological support to neurons, and are also responsible for the response to injury or disease[25,35,36]; in theory, some of these responses should have a beneficial effect: they isolate the injury site and minimize the area of inflammation and cellular degeneration. However, many astrocytes in the lesion area become hypertrophic and acquire a reactive phenotype, releasing inhibitory extracellular

matrix molecules such as chondroitin sulphate proteoglycans (CSPGs)[34]. After injury, CSPGs expression is rapidly upregulated by reactive astrocytes, being its gradient highest at the center of the lesion, and diminishing gradually into the penumbra. Also, its spatiotemporal expression depends on glial boundaries in the developing CNS such as the spinal cord roof plate, optic tectum, and dorsal root entry zone (DREZ)[37,38].

There has been an interesting study by Ramer et al., in which a population of rats, after undergoing rhizotomy and a treatment of neurotrophin 3 (NT-3) to the dorsal root ganglia (DRG), showed that DRG axons were able to overcome CSPG-enriched glial barrier at the DREZ, but when reaching the degenerative white matter myelin their growth was aborted. This supports a hierarchy of inhibitory influences, in which the myelin shows to be more potent than the glial scar[39]. Controversially, DRG neurons microtransplanted into the spinal cord with minimal scarring were able to project their axons over long distances through degenerating white matter tracts, stopping only on contact with CSPGs at the glial scar. These conflicting reports show that both CSPGs and myelin-associated inhibitors (Figure 11) are likely to be involved in regenerative failure, although with some overlap and differences in function due to their different spatial and temporal regulation[38].

There are several inhibitors of axonal growth, including myelin-associated molecules and extracellular matrix-related inhibitors (Figure 11). In the first category, we can include NoGo, which is a member of the reticulon family of membrane proteins and has at least three isoforms (Nogo-A, -B and -C), being that the one best characterized is NoGo-A that is highly expressed in CNS oligodendrocytes[38]. There are several other myelin-associated components that can inhibit axon outgrowth *in vitro*, including myelin-associated glycoprotein (MAG), oligodendrocyte myelin glycoprotein (OMgp), the transmembrane semaphorin 4D (Sema4D/CD100) and ephrin B3, being that the two latest have been described has repulsive guidance cues with roles in axon pathfinding during development and implicated as inhibitors of axon repair in the adult[38].

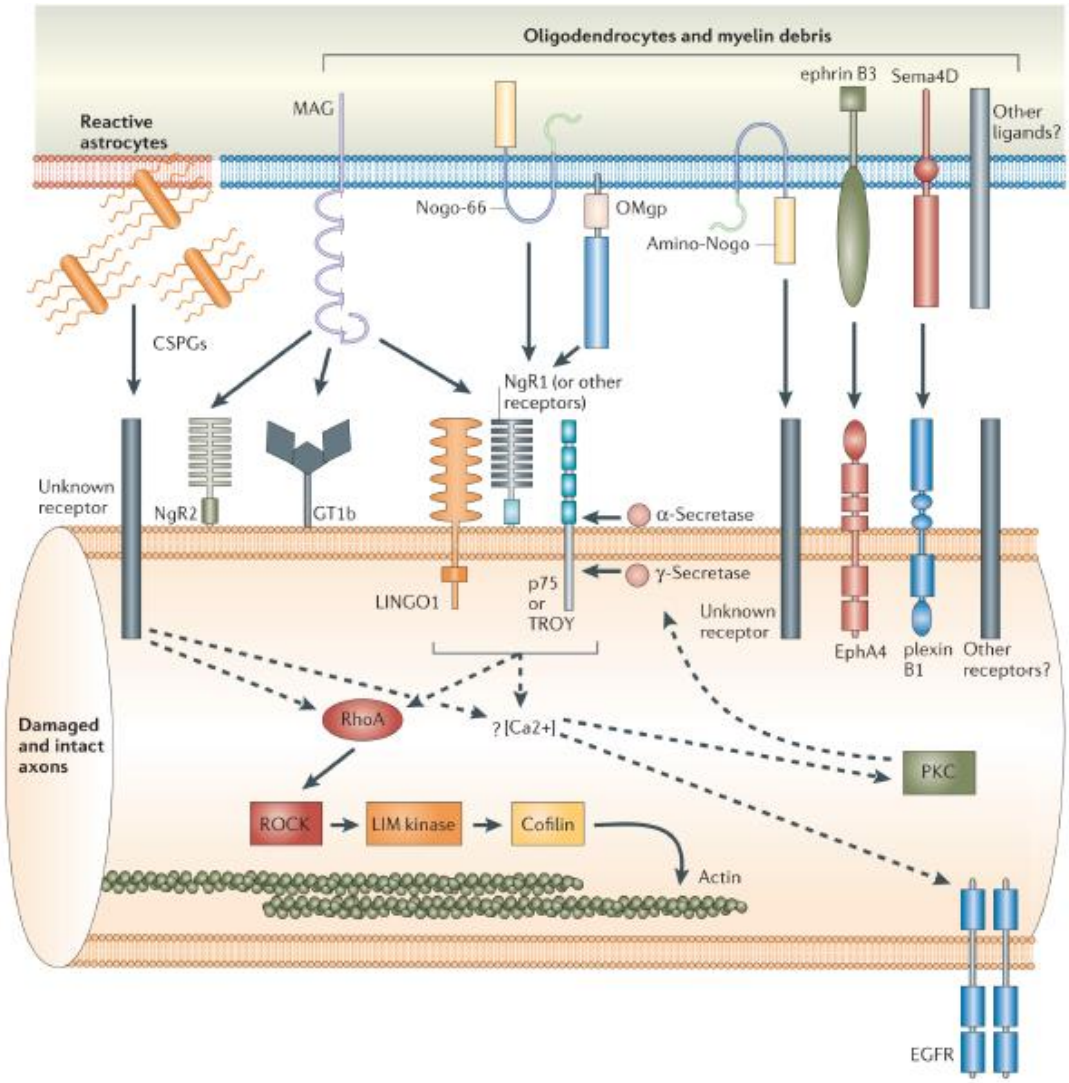


Figure 11 – Glial inhibitors and intracellular signaling mechanisms. One of the most important molecular inhibitors is the CSPGs in the extracellular matrix, which is produced by hypertrophic astrocytes, and myelin-associated glycoprotein (MAG), Nogo-A, oligodendrocyte myelin glycoprotein (OMgp), ephrin B3 and the transmembrane semaphorin 4D. Among the signaling components are the RhoA and the rise of intracellular calcium. Taken from [38].

In addition to the myelin-associated molecules, astrocytes produce a class of molecules known as proteoglycans, namely heparan sulphate proteoglycan (HSPG), dermatan sulphate proteoglycan (DSPG), keratan sulphate proteoglycan (KSPG) and chondroitin sulphate proteoglycan (CSPG). Most studies focus on CSPGs that form a relatively large family (aggrecan, brevican, neurocan, vesican, phosphacan and NG2) and KSPGs. Both are extracellular matrix molecules which consist of a protein core linked by four sugar moieties to a sulphated glycosaminoglycan[34] (GAG) and that seem

to establish limitations for axonal growth in the developing brain and spinal cord including inhibitor effects in extending neurites[40]. After SCI, the expression levels of these ECM inhibitors are up-regulated significantly, as soon as 24 hours post-injury[40]. The glial scar thus forms both a mechanical and a molecular barrier to axonal growth, creating an environment that according to its constituents becomes inhospitable and impedes axonal growth. Beyond it, axons cannot grow and acquire a dystrophic appearance of stalled growth[2].

1.8 – Inflammatory Processes in Spinal Cord Injury

When trauma to the CNS occurs, there is a tendency for researchers to categorically lump mechanisms of brain and spinal cord neuroinflammation together. The problem is that there are significant differences in the composition, magnitude and temporal sequence in which these inflammatory components are expressed in both structures. Schnell et al. proved these different responses by comparing the inflammatory responses provoked by identical injuries inflicted to mouse brain and spinal cord[41]. Following a parasagittal incision to the cortex or a similar incision to the dorsal spinal cord, marked differences in the cellular inflammation are observed: in the brain, neutrophil infiltration is minimal and restricted to the lesion site. As for the spinal cord, twice as many neutrophils infiltrate the lesion within the 24 h with large numbers of cells infiltrating into the surrounding parenchyma. Similarly, activation and recruitment of CNS macrophages were attenuated and restricted in its distribution after brain injury relative to SCI. Lymphocyte numbers were also 2 to 3 times greater in the spinal cord with increased infiltration into surrounding tissue. In the same experiment, neuroinflammation was induced by non-traumatic microinjection of IL-1β or TNFα, and similar changes were observed. In response to these cytokines, the recruitment of neutrophils and macrophages in the CNS was always higher in the spinal cord. After microinjection with IL-1β, lymphocytes infiltrated the spinal cord but never the brain. TNFα microinjections into the brain elicited a response comprised only of CNS macrophages while identical injections to the spinal cord elicited neutrophils and macrophages[41]. These differences may be due to molecular and anatomical

distinctions between the two structures. For example, in the spinal cord, microvascular injury and serum extravasation is increased in magnitude and duration relative to the brain, being also more susceptible to the permeabilizing effects of cytokines[41,42]. There is also a unique pattern of chemokine expression between the two CNS structures, which may explain the differential leukocyte recruitment. Specifically, neutrophil-attracting chemokines (e.g. CINC) are up-regulated to a greater extent in the injured spinal cord than in the brain[43].

Therefore, although there is a tendency to combine the neuroinflammation processes that occur in the brain and in the spinal cord, this is not accurate and it is becoming clearer that the spinal cord should not be considered simply as an extension of the brain[44].

The role of neuroinflammation is nonetheless controversial, as both beneficial and detrimental effects have been observed and associated with microglia/macrophages, lymphocytes, antibodies, and cytokines. Although inflammation is a ubiquitous consequence of CNS trauma, there are fluctuations in the composition, magnitude and temporal sequence in which these inflammatory components are expressed[20].

1.8.1 – Neuroinflammation in Primary Spinal Cord Injury

Contrary to previous belief, the CNS does not constitute an immune privileged system, since it shares many commonalties with other systems. Immediately after SCI, the BBB breakdown and blood-vessel fragmentation leads to an increased expression of leukocyte adhesion molecules on the surface of the endothelial cells and to an over flow of plasma proteins to the parenchyma of the injured tissue[45].

The primary response to a traumatic injury to spinal cord occurs as a result of the mechanical impact to the canal from displaced bone or intervertebral disk, or from an acute twisting of the spinal cord at the time of injury[17]. This primary response leads to a panoply of complex cellular responses, including astrocyte activation, oligodendrocyte death, ependymal cell proliferation, axonal degeneration, demyelination, disruption of neuronal ionic homeostasis, macrophage/microglia invasion, astrocyte hypertrophy and neurons and oligodendrocyte death[44,46-48] (Figure

12). Because of its higher metabolic requirements, the initial mechanical insult tends to primarily damage the central grey matter, in which disruption of blood flow results in local infarction due to hypoxia and ischemia, sparing the great majority of the white matter, especially peripherally, which may be attributed to its softer consistency and greater vascularity[20]. Also, neurons that traverse through the injury site, become physically disrupted and exhibit diminished myelin thickness[20].

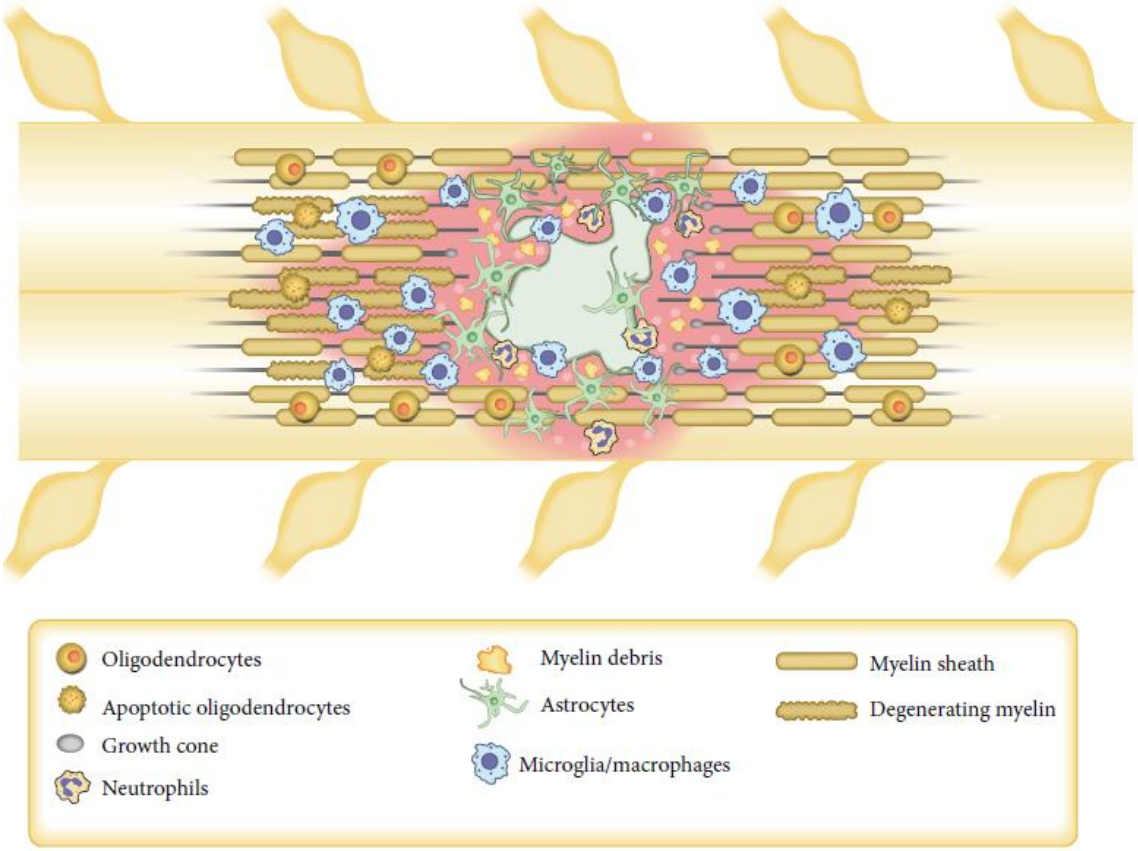


Figure 12 – Schematic representation of a spinal cord injury site. Immediately after injury, is generated a robust hemorrhagic area followed by glial cell activation. Simultaneously, axons that are undergoing degeneration and other neuronal cells that are dead, will stimulate the recruitment of inflammatory cells from the periphery. Neutrophils are the first to arrive at the tissue parenchyma and begin to secrete molecules that eventually worsen tissue and vascular damage. After a while, monocytes infiltrate the spinal cord, where they become macrophages, and persist until several months at the injury site. Neurons not affected by trauma activate their regenerative machinery, only to fail across the injury site formed by the glial scar. Taken from [49].

Within the injured CNS, resident cells are activated in response to the traumatic impact and initiate a synchronized neuroinflammatory response, which includes pro-inflammatory cytokines, chemokines, and complement activation products. Chemotaxis by chemokines and complement anaphylatoxins leads to transmigration of haematogenous inflammatory cells, such as neutrophils, macrophages, and lymphocytes into the injured CNS where they play a role in the removal of cellular and myelin debris as well as in releasing neurotoxins and cytokines that lead to neuronal and oligodendrocyte death [17,50]. These infiltrating leukocytes perpetuate the neuroinflammatory response by the local release of neurotoxic molecules, including reactive oxygen species, nitrogen-derived free radicals, proteases, and other neurotoxic enzymes. Combined, these secondary pathophysiological events ultimately lead to the breakdown of the blood-spinal cord barrier (BSCB), resulting in an uncontrolled leakage of systemic toxic molecules, such as matrix metalloproteases and other inflammatory mediators, into the subarachnoid space in the injured spinal cord.

After a trauma to the spinal cord, the first cells to participate in the inflammatory response, arriving as early as 4h post injury, are neutrophils which activate several mechanisms including production of a variety of oxidative and proteolytic enzymes, which in turn exacerbate neuropathological events, and by enlarging the lesion, potentially worsen neurological dysfunction[22,51].

Microglial cells from the cord also penetrate and invade locally in an attempt to repair the damage. They are normally present 1 day after the injury, leading to increased areas of CD68 immunoreactivity associated with the phagocytic phenotype of macrophages at 1-3 days post-injury[22] Microglia are usually distributed outside areas of necrosis, whereas phagocytic macrophages are mostly in areas of necrosis[22].

Some unneeded damage may result from too many neutrophils accumulating in the area and causing “by-stander” tissue damage[22]. For example, in an ischemia-reperfusion-tissue injury setting, when neutrophils and macrophages are undergoing oxidative burst, they will release proteases and reactive oxygen species that can cause substantial secondary damage by mediating lipid peroxidation and protein nitration, and by activating redox-sensitive signaling cascades and consumption of nitric oxide. Neutrophils and activated microglia are therefore the major sources of NADPH oxidase-derived reactive oxygen species in the injured spinal cord[22]. Although these

compounds are intended for killing pathogens, if released inappropriately, may also contribute to tissue damage[22,52]. These inflammatory mediators increase the expression of endothelial leukocyte adhesion molecules which in turn can damage endothelial cells[53]. This damage is in partly attributed to the overexpression of P-Selectin, which is involved in the pathogenesis of tissue injury induced by ischemia-reperfusion-injury. When certain stimuli are produced, such as thrombin, histamine and oxygen free radicals, P-Selectin - a member of the endothelial leukocyte adhesion molecule family - is rapidly expressed and translocated to the plasma membrane where it functions as a receptor for monocytes and neutrophils[53-55].

All these events combined will eventually culminate in a severe secondary degeneration after injury, one of the reasons why regeneration of the spinal cord fails.

1.8.2 - Neuroinflammation and Secondary Spinal Cord Injury

Secondary mechanisms of injury include an array of disturbances that include neurogenic shock, excitotoxicity, vascular insults such as hemorrhage and ischemia-reperfusion, calcium-mediated secondary injury and fluid-electrolyte disturbances, immunologic injury, disturbances in mitochondrion function, apoptosis, and other miscellaneous processes that result in a complex inflammatory response that can prevail for months or years after the initial trauma[22,20].

The lesion cavity of a CNS injury expands as inflammatory cells interact with the surrounding reactive astrocytes and other reactive glial cells. This lesioned region is associated with upregulation of inhibitory extracellular matrix molecules, such as proteoglycans, which are highly concentrated in the lesion center and in a lower concentration in the penumbra. This intense inflammatory response leads to a cascade of secondary damage to axons not directly affected by trauma, and demyelination of adjacent axons. The gradient of inhibitory molecules upregulated in the areas of intense inflammation provides a non-permissive environment for regeneration, and dystrophic neurons develop in the classic way Cajal described, as sterile end-balls with clubbed endings, which remain characteristic of abortive attempts at regeneration[56].

As previously said, the secondary spinal cord injury caused by neutrophils and macrophages is partially a result of oxidative and proteolytic enzymes, for instance, neutrophils and other phagocytes express myeloperoxidase (MPO)[22,53] an oxidative enzyme that generates a hypochlorous acid that kills pathogens. Along with the oxidative enzymes, matrix metalloproteinases (MMPs) are also released from the inflammatory cells, in particular, MMP-9 that allows penetration of the blood-CNS-barrier[57].

There are numerous others mechanisms of secondary injury, including impairment of spinal cord blood flow[58], electrolyte changes, the release of excitotoxic amino acids, free radicals, and the levels of GFAP, secreted by reactive astrocytes, which begin to increase and form irreversible and reversible scars around the lesion site and in regions distal to the lesion site. This event known a7s astrogliosis is one of the hallmarks of CNS injury. This “vicious-cycle” of self-perpetuating exacerbated neuroinflammation leads to spinal edema, loss of regulation of local and systemic blood pressure, imbalance of activated metalloproteinases, release of cytotoxic neurotransmitters, expansion of the primary traumatic lesion and delayed neuronal cell death, and up to now there is no single pharmacological agent available that can prevent the development of secondary SCI and that induces regenerative processes that would help heal the spinal cord and restore neurological function[59,60].

1.9 - Treatment for Spinal Cord Injury Patients

The dream of achieving spinal cord regeneration in the future entails a large effort from several areas, and it is believed that combinatorial treatments will be required when translating experimental research to find clinical therapies.

There have been some modestly successful attempts in the past for central regeneration, but none of them showed to be fruitful in regards to central axonal regeneration in adult mammals, and the greatest obstacle on this regard seems to be Wallerian degeneration. Several studies have attempted to address this issue. For example, treatment with the enzyme chondroitinase ABC (ChABC), which breaks down

inhibitory sugar chains components of CSPG molecules, promotes regeneration of lesioned axons in the brain and spinal cord[61].

In order to axonal regeneration to become successful, two major obstacles would have to be solved: firstly, the regenerated axons are required to connect with their distal part; secondly, even if they achieve regeneration, it would be at very slow rate and by the time the axons would have reached the target neurons of the central grey matter they would imperially need to re-established their synaptic connections to be functional. Fortunately, the CNS's plasticity would be of some help in this regard. Because severed axons distal to the injury degenerate (caudally in the motor pathways and rostrally in sensory tracts), even if regeneration succeeds to grow a few millimeters into the distal cord, there still may be twenty or more centimeters of pyramidal tracts below the lesion[62].

Since 1903, when Tello and Cajal[63] demonstrated that the CNS could regenerate, experimental science has put a lot of effort into repairing the injured spinal cord. Their work placing peripheral nerve grafts to bridge the injury zone was successful, but sadly, only few evidence in human patients support its translation into the clinics[2,64]. Several studies that have combined growth factor treatment with cellular grafting techniques. For example, infusion of neurotrophins has been shown to enhance regeneration into Schwann cells grafts. Another technique that has also been used is to genetically modify cellular graft tissues (such as fibroblasts and Schwann cells) prior to transplantation to make them secrete neurotrophic factors and this way provide injured axons with a cellular bridge and a source of trophic support[61].

In 1979, Jaffe and Poo studied the effects of a steady electrical field upon the growth of nerve processes *in vitro* and discovered that electrical fields increase neurite outgrowth of chick dorsal root ganglion (DRG) explants[65]. Regarding electrical stimulation, it has also been shown that low-frequency electric stimulation (20 Hz) is capable of inducing alterations within the nerve cells, namely the expression of regeneration-associated genes in motor neurons. After a low-frequency electrical stimulus the intracellular calcium and cAMP level increase, that in turn alter the expression of several genes such as the ones related to axon regeneration including gap-43, bdnf, neuritin, pacap and α1-tubulin, and myelination p0 and par-3[66].

Since then, many mechanisms have been used in experimental medicine to enhance regeneration of the spinal cord, with success to some extent or another. These experimental approaches include placement of molecular, cellular or "synthetic" bridges in the lesion cavity; *in situ* hydrogels[67] and hydrogel tubes[68], stimulation of the injured spinal cord with growth factors; "conditioning" of neurons (which involves activation of transcription programs that directly enhance axonal growth potential of the central branches of DRG after peripheral branches being severed[69]) and proteins into an active growth state. Growth factors modulate neuronal survival, neurite outgrowth, synaptic plasticity, and neurotransmission[70]; among them, the brain-derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF), neurotrophin 3 (NT3) and neurotrophins 4/5 (NT-4/5), and fibroblast growth factor (FGF)[70], have proved to show some efficacy in combination with MSCs, has a potential therapeutic strategy for SCI[70].

As far restorative neurology goes, there are some methods being used towards spinal cord treatment, namely pharmacological (baclofen), epidural electrical stimulation[71], tonic stimulation of the lumbar cord[72] and intensive sensory inputs using the electrified glove and massage.

One approach to SCI treatment can be directed towards the prevention of the formation a collagenous scar. This may be useful in the sense that, although some modest successes have been accomplished in targeting single inhibitors of the scar since a multicity of components are involved in scarring, it seems unlikely that a single key component solves the problem of growth failure for all neuronal populations. Therefore, suppression of a collagenous scar may not influence secretion on inhibitory molecules by astrocytes. Nevertheless, it deprives inhibitors of their binding sites and prevents accumulation at the lesion center[33]. One novel strategy to do so is by using iron chelators since one of the key enzymes of collagen IV biosynthesis is prolyl 4-hydroxilase (P4H), which is dependent on its cofactors iron, ascorbate, and 2-ocoglutarat. Therefore, by introducing an iron chelator we would be able to inhibit P4H and therefore collagen IV scarring[33].

More recent work on spinal cord injury treatments includes a microconnector system (mMS) (Figure 13). This device is a multi-channel system composed of polymethylmethacylate (PMMA) with honey-combed shaped holes and a tube

connected to a vacuum pump to apply negative pressure and suck the tissue into the device. In the case of a chronic spinal cord injury, the lesioned and scarred spinal cord tissue is resected over an area of 4 mm in length. After the microsurgical scar resection, the resulting cavity is filled with polyethylene glycol (PEG 600) which was found to provide an excellent substratum for cellular invasion, revascularization, axonal regeneration and even compact remyelination *in vivo*[73].

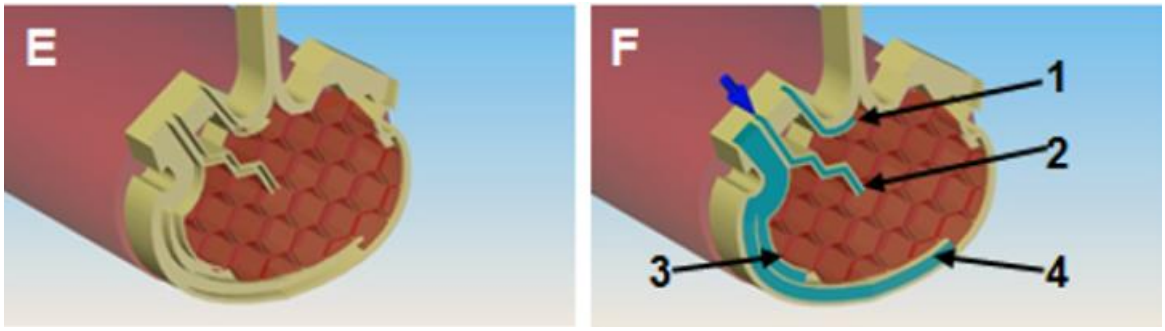


Figure 13 – Design and structure of the mMS device. E – adhesive forces keep the spinal cord stumps in close proximity (micrometers); F – pharmacological substances can be administered in the lumen via 4 internal micro-channels (1-4 black arrows). Taken from [73]

All in all, the literature on SCI suggest that several treatment interventions can promote regeneration of damaged axons, although combinatory treatments prove to be the ones with more extensive axon regeneration[61]. Although the degree of such regeneration remains modest, it may be sufficient to account for some functional recovery[61].

1.10 - Animal Models of Spinal Cord Injury

Most studies of regenerative biology that are intended for biomedical applications have focused on stem cells *in vitro*. However, to gain a full understanding of regeneration, the processes that are involved in it must be studied *in vivo*. For such approach, model organisms are essential tools to provide the necessary knowledge that will eventually allow us to manipulate and control regenerative properties (Annexes - Table 2).

Regeneration in invertebrates has been studied for more than 200 years, and the ones receiving more attention seem to be the diploblast *Hydra vulgaris*, and the triploblast *Schimidtea mediterranea* and *Dugesia japonica*[74]. Regeneration in amphibians, which includes the newt, salamander, and the frogs *Xenopus laevis* and *Xenopus tropicalis*, is thought to be mainly mediated by extensive cellular transdifferentiation. Among the vertebrates, the newt is generally regarded as the "champion" of regeneration: as an adult, it can regenerate several organs, including limbs, the tail, the brain and spinal cord, jaws and the heart. However, newts are difficult to breed under laboratory conditions, and as such, have not been accessible to traditional genetics[74]. Regeneration in mammals is, compared to other vertebrates, far less great. However, there is a continuous renewal of tissues in mammals as part of tissue homeostasis, as for example in haematopoiesis, gametogenesis, and intestinal-tract epithelium and skin renewal.

Xenopus laevis

Xenopus laevis has a regenerative and non-regenerative stage. As a tadpole, it is fully capable of functional recovery after spinal cord injury, while its juvenile form (froglet) loses this capacity during metamorphosis[3]. *X. laevis* has a chromosome number (2n=36) nearly double of that of the Western clawed frog *Xenopus tropicalis* (2n=20) and most other diploid frogs[75]. *X. laevis* is, therefore, an allotetraploid that arose via the interspecific hybridization of diploid progenitors with 2n=18 followed by subsequent genome doubling to restore meiotic pairing and disomic inheritance[76].

On the downside, *X. laevis,* has a large genome, with an estimated size of 3.1 billion bases (Gbp) on 18 chromosomes and a generation time of 1-2 years that comprises 66 stages of development, being the final stage achieved at day 58.

Xenopus tropicalis

X. tropicalis has a small genome, about 1.7 Gpb on 10 chromosomes, matures in only 4 months and requires less space than *X. laevis*. Also, it has a completely known genome sequenced and is a true diploid. Unlike mammals, teleosts fish and amphibians like adult urodeles and anuran larvae are capable of functional recovery after spinal cord transection. In *Xenopus tropicalis* and *laevis*, regeneration is restricted to larvae or

tadpole stages (stages 50 to 54), while once metamorphosis has concluded, the resulting froglets are unable to regenerate (stages 58 to 66)[3].

Danio rerio

For the past three decades, *Zebrafish* has successfully scaled up on the list of best animal models, due to its hardy nature, the presence of many organs and cell types similar to other mammals and the fact that it's capable of mimicking conditions that affect humans[77]. *Zebrafish* present some characteristics that make it a very effective animal model, such as external fertilization, fast growth, and high number of offspring. For example, the females spawn around 300 eggs per week under optimal conditions, which results in fecundity higher than 300,000 eggs per Kg of the female[78]. They also present optic and embryo transparency, ease to apply classic embryological, early development of the cardiovascular system which translates to a unique opportunity for observation of blood flow and organs[79], and the possibility to apply biochemical and molecular biological techniques[77,80].

What really stands out in *Zebrafish*, though, it's the fact that in adult (90 days to 2 years) it's capable of re-growing injured axons after SCI and re-establish proper connections to recover the most significant functions[81]. Also, many genes and biological mechanisms are conserved from the *Zebrafish* to human. For these reasons, this eukaryotic fish became one of the best models for humans diseases, and it's used for laboratory routine knock-down genes via morpholinos, has become routine work[80].

Chapter 2 – Objectives

Due to all the above mentioned restrains that still exist towards regeneration of central nervous system axons, the purpose of this dissertation was oriented in finding genetic targets whose modulation may help SCI repair. The candidate genes to be chosen correspond to the ones that are more robustly regulated whilst regeneration of the spinal cord in animal models occurs. The neuroregenerative animal models from which data will be obtained pertain to *Xenopus laevis, Xenopus tropicalis* and *Danio rerio*. This should be accomplished after processing and analysis of data from genome wide expression studies of these animals.

With these target genes, we aim to have a better understanding of the regeneration processes and pathways involved, while drawing parallels to its human orthologues and human gene pathways. We also aim to define possible putative genes whose expression might be feasible to manipulate, in order to accomplish repair in the so far non-regenerative adult mammal, thus moving forward into a realistic approach to human spinal cord regeneration therapy.

Chapter 3 – Methods

3.1 – Selection and data recovery

Data were recovered using searches several NCBI tools, such as GEO (Gene expression omnibus) Datasets, Array Express and PubMed. The literature search was repeated without date or language restriction using the terms 'Spinal Cord Regeneration', 'SCI', 'Neuroregeneration', 'Spinal Cord Injury', 'CNS Regeneration', 'Genome Wide Expression Studies AND Spinal Cord Injuries', 'Xenopus laevis AND Spinal Cord Injury', 'Xenopus tropicalis AND Spinal Cord Injury', and 'Zebrafish AND Spinal Cord Injury'. The keywords for PubMed included the above plus RNAseq, genome wide expression or GWE. Because our animal targets were those capable of regeneration at least in some stage of their lives, the search was limited to *Xenopus laevis*, *Xenopus tropicalis,* xenopus, frog, zebrafish, *Danio rerio*, *Triturus viridescens,* newt*,* salamander, and planarian.

From the neuroregenerative animals cited above, five results corresponded to genome wide expression studies focused on regeneration. Two of these referred to regeneration of the tail after transection and were excluded. After this, the remaining three studies were referent to regeneration of the spinal cord in three animal models *Xenopus laevis, Xenopus tropicalis,* and the *Danio rerio.*

3.2 – Data Analysis and Human orthologues

The initial set of data used for *X. laevis* was Additional File 3 – Transcripts that show a different response to spinal cord injury in R- and NR-stages retrieved from PubMed Central, with the PMCID: PMC4046850. In this file, fold change appeared in the form of base two logarithms.

The data used for *X. tropicalis* included the files: E-MEXP-2420.raw.1.zip and E-MEXP-2420.processed.1.zip. The files were obtained from PubMed Central, with the PMCID: PMC3247858. The direct link can be found at http://www.ebi.ac.uk/arrayexpress/.

In the processed data file, each Affymetrix probe name identified three replicates per time point, and so a mean of the gene expression values was calculated.

The initial data used for *D. rerio* included several files with differentially expressed genes according to functional clusters (Table S6-Table S18 from Gene Expression Omnibus, accession number: GSE39295). Also, fold change values were not represented in logarithmic scaling. For this reason, a base two logarithm was applied to all fold changes, followed by individual extraction from each file to compile a single file, with all differentially over/under-expressed genes, for each time point.

For each study, genes were grouped separately into over expressed and under expressed lists according to a fixed logarithmic fold change threshold of 4 for over expressed genes, and -4 for under expressed genes (or log2 > 2 and log2 < -2).

Each study had different control time points (*X. laevis* – day 1, 2 and 6; *X. tropicalis* – 6h, 24h, and 60h; *Danio rerio* – Day 1, 3, 7, 10 and 15), in order to standardize the analysis, time point categories were considered at day 1 and day 2/3 for the Venn Diagrams and for further discussion.

The next step was to map the human orthologues for the final list of genes of the three species: *X. tropicalis, X. laevis* and *Danio rerio.*

Gene identifiers for *Xenopus laevis, Xenopus tropicalis*, and *Danio rerio,* and respective human orthologues were standardized through the Ensembl[88], Biomart, DAVID[85,90], bioDBnet[83], ZebraMine[84] and HumanMine[84], by using the pre-existing gene IDs or the microarray probe names, to convert the gene identifiers into a single Ensembl database identifier.

To convert IDs to other databases and to access human orthologues, several databases were used, including: BioMart, bioDBnet and InterMine. The database used in BioMart was Ensembl and the archives accessed were Ensembl 85: Jul 2016 and Ensembl 86: Oct 2016. This database allowed conversion of Affymetrix probe ID into Ensembl IDs in *Xenopus tropicalis*. It also allowed orthologue extraction through online queries in R for *Xenopus laevis*. BioMart possesses other databases such as Uniprot and HapMap which allow users direct access to a diverse set of data, including the possibility of accessing the data through online queries from R[82].

To retrieve human orthologues for *Xenopus tropicalis*, bioDBnet was accessed (v3.0 2016 July). BioDBnet[83] is an online resource which currently possesses 153 database identifiers covering several aspects of biology including genes, proteins, and pathways.

This online tool allows a dynamic navigation through the existing databases, offering ID conversion and orthologue conversions and has various tools to enhance the quality of the results.

Conversion of *Danio rerio* IDs was performed through the InterMine platform[84]. InterMine is an open-source data warehouse initially designed for *Drosophila* (FlyMine) but has now been adopted for a number of major model organisms such as zebrafish (ZebrafishMine), yeast (YeastMine), rat (RatMine), mouse (MouseMine), *Xenopus tropicalis* (XenMine) and nematode worm (WormMine). Through this tool, we can create large biological databases from a range of heterogeneous data sources, allowing easy integration of new data types. The orthologues for *Xenopus tropicalis* were therefore extracted from this free online database, namely XenMine.

The Ensembl IDs for the human orthologues of the genes differentially expressed in *Danio rerio*' were obtained from bioDBnet[83] and HumanMine[84] using Symbol and Synonyms. Obtaining Ensembl IDs for *Xenopus laevis* required making a script in R.

In the script the input files were: NCBI IDs for *Xenopus laevis* (taken from ftp://ftp.xenbase.org/pub/GenePageReports/NcbiMrnaXenbaseGene_laevis.txt), a list of *Xenopus* gene IDs from XenBase[91] (taken from: ftp://ftp.xenbase.org/pub/GenePageReports/XenbaseGenepageToGeneIdMapping.txt) plus a list human of orthologs with Ensembl IDs from XenBase obtained using Annotate Package (taken from: ftp://ftp.xenbase.org/pub/GenePageReports/XenbaseGeneHumanOrthologMapping.txt).

Next, R was used to merge the lists and get the human gene name. For *Xenopus tropicalis*, Ensembl IDs were retrieved from Biomart. Affymetrix *X. tropicalis* probeset IDs names were uploaded to BioMart using Ensembl Genes 86 database.

3.3 – Gene enrichment analyses

For gene enrichment analysis two softwares were used, namely DAVID and PANTHER.

Each list of differentially expressed genes was analyzed by the Database for Annotation, Visualization and Integrated Discovery (DAVID) gene ontology tool (https://david.ncifcrf.gov/summary.jsp)[85].

DAVID[85] (DAVID Bioinformatics Resources 6.8) allows functional analysis of large gene lists derived from high-throughput approaches. In this dissertation, the most useful tool used in DAVID was the gene-annotation enrichment analysis that increased the probability of identifying the most enriched biological process in a large amount of data. Nonetheless, it is also possible to map a large number of genes in a list and associate them with other biological annotation terms such as GO Terms or Pathways.

Genes were clustered into biological functional groups, and an enrichment score of 2 was set as the threshold above which the clusters were considered statistically significant. Duplicated genes are removed automatically from the listings when uploaded into DAVID. For *D. rerio,* from a total of 243 up-regulated genes uploaded for functional clustering, 167 results were identified as *Danio rerio* and 76 were not mapped. As for down-regulated genes, from a total of 217 genes, 160 were mapped for *D. rerio* and 57 were not included.

For *Xenopus tropicalis* from a total of 1557 up-regulated genes uploaded for functional clustering analysis on DAVID, 376 were mapped as *Xenopus tropicalis* genes, and the remaining were removed, on the other hand, from a total of 1477 down-regulated genes uploaded for functional clustering analysis, 446 were identified as *X. tropicalis* genes and the remaining were not further considered. For *X. laevis* from a total of 624 up-regulated genes, 214 were identified as *X. laevis* genes. As for down-regulated genes, from a total of 296 genes uploaded for functional clustering analysis, only 79 were identified.

For each dataset, the Gene Functional Classification Tool was also used to perform pathway enrichment analysis based on Gene Ontology Terms (GO Terms) for each time point and for up and down-regulated genes. Secondly, broader clusters were created for the sum of all up-regulated and down-regulated genes of every time point, within each study. From all clusters obtained, only the ones with Enrichment Scores above 2 were considered. Also, from each cluster, it was selected the category Biological Processes which included the higher number of genes. For *Xenopus laevis,* GenBank accession numbers were used to upload the lists and for *Xenopus tropicalis and Danio rerio,* Ensembl IDs were used.

The next two analysis were performed using PANTHER[86], a tool also used to perform gene enrichment analysis similar to the ones performed by DAVID. The PANTHER[86]

(PANTHER11.1 April, 2015) software provides methodologies for relating protein sequences and their relationships to the functions of proteins in a large scale. In fact, PANTHER may be used to report the size and sequence diversity of the families and subfamilies. We can also use PANTHER/X ontology to give a high-level representation of gene function across the human and mouse genomes and for ranking missense single nucleotide polymorphisms (SNPs) according to their likelihood of affecting protein another's function. For this dissertation, it was used the Statistical overrepresentation test in the Gene Analysis Tools, to characterize clusters of enriched biological processes in the gene lists available.

Lastly, JVenn software (http://bioinfo.genotoul.fr/jvenn/) was accessed to create the Venn diagrams. Jvenn[87] (2015) is online platform used to easily create Venn diagrams. These diagrams allow better visual comparison between lists. In this dissertation, Venn diagrams were essential for identifying common human orthologues in all the gene lists available.

In the first analysis we used Ensembl IDs from *Danio rerio* and *Xenopus tropicalis* to create pie charts of Biological Processes, obtained through Functional Classification viewed in Pie Chart Tool. This tool was firstly used separately for the time point categories previously considered, namely day 1 and day 2/3 and secondly considering all time points for up-regulated genes and down-regulated genes. Secondly, a GO-term enrichment analysis in PANTHER was used to define which biological processes were enriched. Therefore, an over-representation test with default settings was performed at the same time points, using Bonferroni correction. Only GO terms with a p-value <0.05 were considered.

Lastly, only human orthologues with Ensembl IDs were uploaded to create the Venn diagrams. Venn diagrams were first created for the time points categories mentioned above (Day 1 and day 2 for up and down- regulated genes for all species; and later included the remaining time points (6h, day 3, day 7, day 10 and day 15) to create Venn diagrams which included all up and down-regulated genes from all species. The number of genes in all time points is reduced compared to data uploaded to JVenn program due to automatic elimination of duplicates.

Figures 14-16 schematize fluoxograms of processing and analytical actions taken for each different dataset.

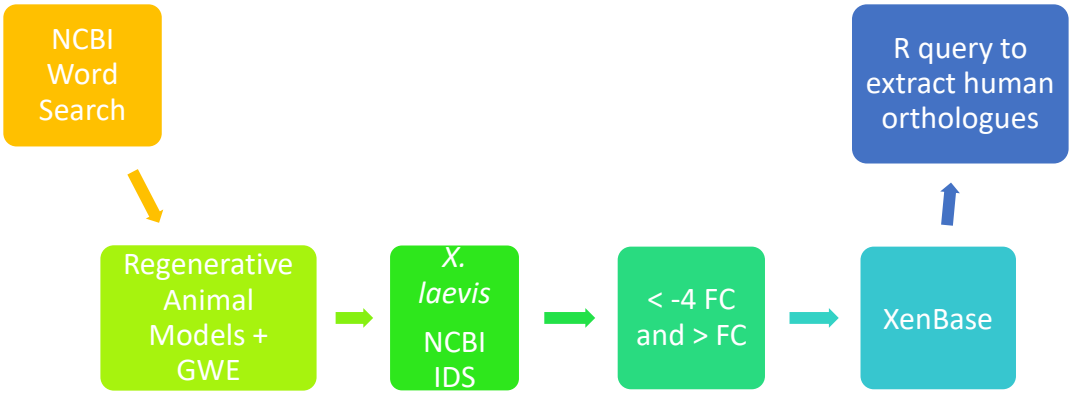


Figure 14. *X. laevis* data processing fluxogram.

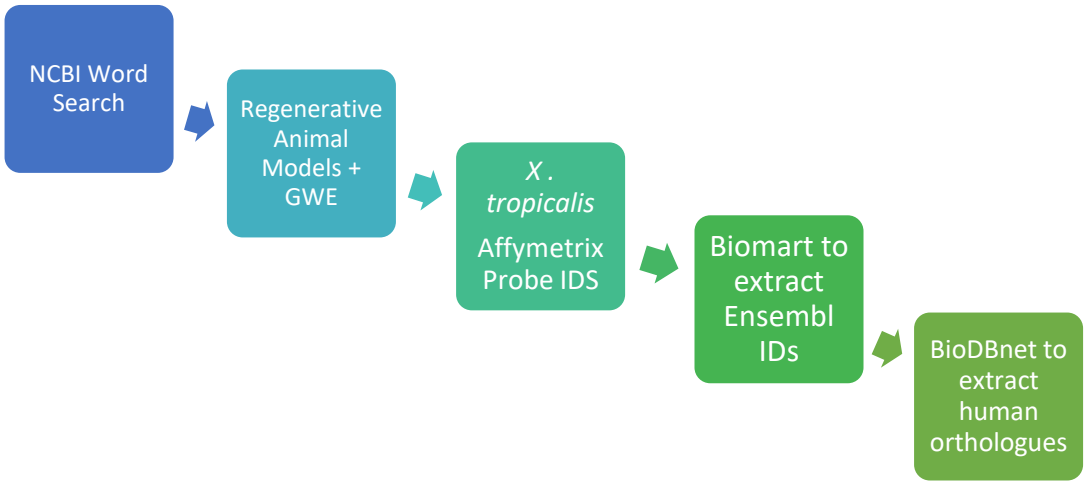


Figure 15. *X. tropicalis* data processing fluxogram.

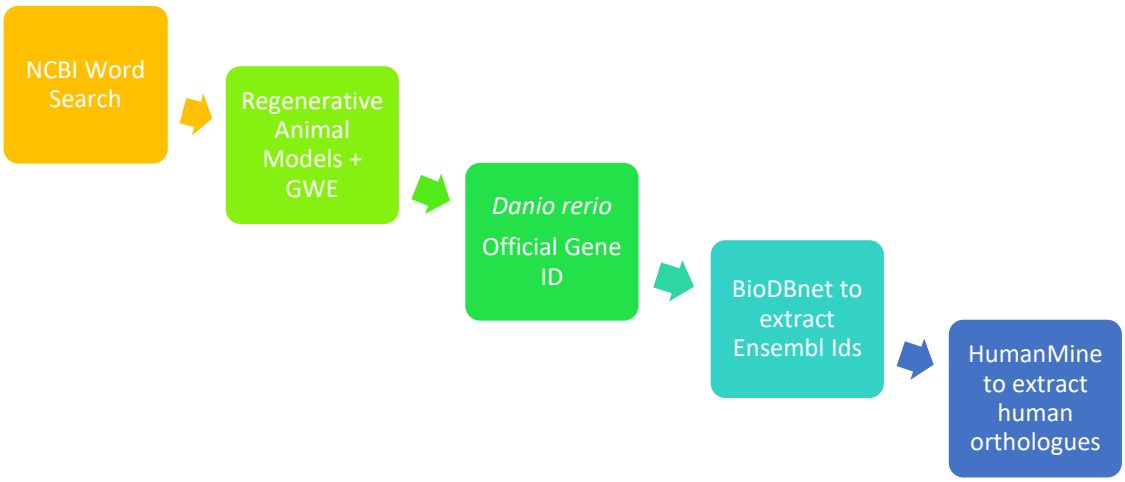


Figure 16. *Danio rerio* data processing fluxogram.

Chapter 4 – Results

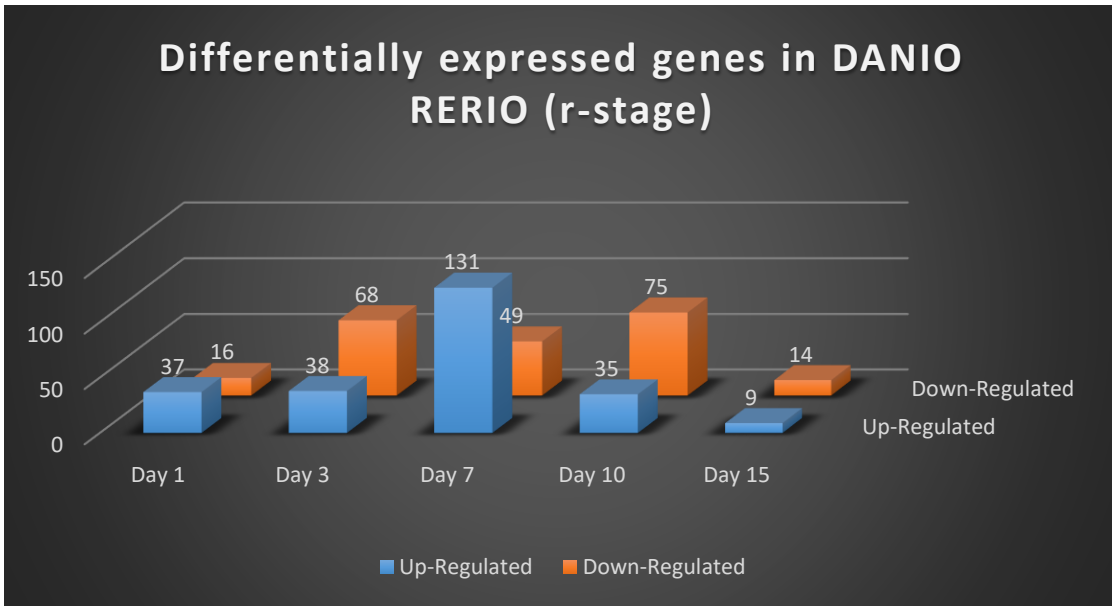
After data treatment, the number of genes from the initial files (Raw data) was significantly reduced and compiled into a file containing genes with known Ensembl IDs *(*Annex file – Complete Data Compilation). The following graphs show differentially expressed genes with attributed Ensembl IDs and grouped into FC > 4 for up-regulated genes, and FC < -4 for down-regulated genes.

4.1 – *Danio rerio*

4.1.1 – Differentially expressed genes

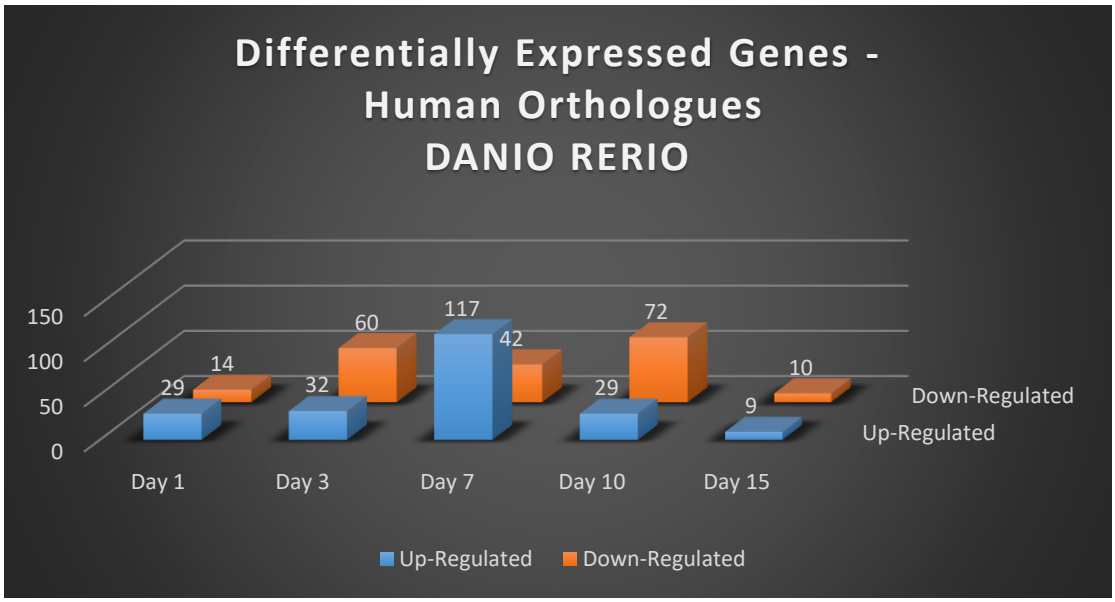
Graphic 1 shows a representation of the number of genes that show differential expression at different time points after SCI in *Danio rerio.* The data represents gene expression in the injured spinal cord at five different time points of regeneration (Day 1, Day 3, Day 7, Day 10, and Day 15). After data collection (please see files in Annex, Table S6 – Table S18), the researchers have observed that a total of 472 genes showed differential expression. From this set of annotated genes 37, 38, 131, 35 and 9 are up-regulated at Day 1, 3, 7, 10 and 15, respectively. On the other hand, 16, 68, 49, 75 and 14 are down-regulated in Day 1, 3, 7, 10 and 15, respectively. The maximum number of genes differentially expressed is found at day 7. It is also evident that the number of up-regulated genes is lower at earlier (day 1 and 3) and later (day 15) phases of regeneration, and higher at mid-phase time points (day 7 and 10). For down-regulated genes the dynamic of the gene expression is similar throughout the five time points, exception made for day 7 that is lower than corresponding up-regulated genes for the same time point.

All graphics are normalized to the same scale to a better visualization and comparison between number of genes before and after conversion to its human orthologues.



Graphic 1 – Distribution of differentially expressed genes both up-regulated and down-regulated after SCI at different time points in *Danio rerio.* Established fold change (FC): >4 for up-regulated genes, <-4 for down-regulated genes.

Graphic 2 shows a representation of the number of known human orthologues that show a differential expression in the various experimental time points in *Danio rerio*. The data represents the respective known orthologues for *Danio rerio* genes differentially expressed after SCI at different time points, namely Day 1, 3, 5, 10 and 15. A total of 414 known human orthologues showed differential expression. From this set of genes, 29, 32, 117, 29 and 9 are up-regulated at Day 1, 3, 7, 10 and 15 respectively. Additionally, 14, 60, 42, 72 and 10 are down-regulated in Day 1, 3, 7, 10 and 15 respectively. Conforming with the previous results, a maximum number of differentially expressed genes is presented at day 7, and up-regulated genes show lower expression at earlier and later phases of regeneration and a higher expression on mid-phase of regeneration. For down-regulated genes, the number of differentially expressed genes follows the same pattern described in graphic 1.



Graphic 2 – Distribution of differentially expressed genes (human orthologues) both up-regulated and down-regulated after SCI at different time points in *Danio rerio*. Fold change >4 and <-4.

4.1.2 – Gene Enrichment Analysis

A GO-Term analysis in DAVID was used to couple genes according to their biological similarities. Gene ontology tools analyses the genes according to three categories: biological processes (GOTERM_BP), that refer to a biological objective to which the gene or gene product contributes; molecular functions (GOTERM_MF), defined as the biochemical activity of a gene product and cellular components (GOTERM_CC) that refer to the place in the cell where a gene product is active[92]. This categorization allows us to turn information into knowledge and extract conclusions about the most significant biological processes occurring at each time point of the phases of regeneration in the three species. Annotation clusters with enrichment scores higher than 2 were considered biologically significant. The biological process chosen to represent the GO Term was the one with the higher number of genes.

Table 1 shows enriched biological processes in *Danio rerio* in Day 1, 3, 7 and 10. No biologically enriched clusters appeared for day 15. At day 1, Proteolysis presents as enriched for up-regulated genes. No enriched biological processes appeared for down-regulated genes at this time point. Transcription regulation is enriched at day 3 in down-regulated genes. No enriched biological processes appeared for up-regulated

genes at this time point. Day 7 presents Transcription regulation and DNA replication as biologically enriched for up-regulated genes. No enriched biological processes appeared for down-regulated genes at this time point. Day 10 also presents Transcription regulation as biologically enriched in up-regulated genes, and DNA replication is enriched in down-regulated genes.

Table 1. Significantly enriched gene ontology categories by biological processes (BP) in *Danio rerio,* day 1 for up-regulated genes, day 3 for down-regulated genes and day 7 for up and down-regulated genes, p-value < 0,05. "% ID" indicates the percentage of genes associated with each GO TERM relatively to the total number of genes that were categorized.

Go Term	**% ID**	**Number of genes**	**Enrichment Score**	**p-value**	**Day & Alteration**
Proteolysis	18.2	4	2.603	3.24E-02	**Day 1 Up-Regulated**
Regulation of transcription, DNA-templated	22.5	11	2.308	4.21E-03	**Day 3**
Regulation of transcription, DNA-templated	22.5	11	2.224	4.21E-03	**Down-Regulated**
Regulation of transcription, DNA-templated	16.7	16	3.835	7.76E-03	**Day 7**
DNA replication	7.3	7	3.310	8.83E-06	**Up-Regulated**
Regulation of transcription, DNA-templated	16.7	16	2.103	7.76E-03	
Regulation of transcription, DNA-templated	42.1	8	2.375	3.58E-04	**Day 10 Up-Regulated**
DNA replication	10.9	6	2.679	8.80E-06	**Down-Regulated**

Table 2 depicts enriched biological processes in the collective of up and down-regulated genes in *Danio rerio*. This analysis will help us get a better understanding of the main biological processes that prove to be important in the process of regeneration among the up-regulated genes for the cumulative time points. Transcription regulation and DNA replication appear to be enriched in up-regulated genes, whereas Transcription regulation, DNA replication, Dorsal/ventral pattern formation and Transport appear to be enriched in down-regulated genes.

Table 2. Significantly enriched gene ontology categories by biological processes (BP) in *Danio rerio,* for all up-regulated genes in all time points (Day 1, 3, 7, 10 and 15) and all down-regulated genes in all time points. p-value < 0,05. "% ID" indicates the percentage of genes associated with each GO TERM relatively to the total number of genes that were categorized.

Go Term	**% ID**	**Number of genes**	**Enrichment Score**	**p-value**	**Day & Alteration**
Regulation of transcription, DNA-templated	19.8	33	5.1511	4.17E-06	**Sum of the Up-Regulated genes**
DNA replication	4.2	7	2.8029	2.41E-04	
Regulation of transcription, DNA-templated	16.2	26	4.173	1.15E-03	**Sum of the Down-regulated genes**
Regulation of transcription, DNA-templated	16.2	26	3.2194	1.15E-03	
DNA replication	4.4	7	2.8555	1.87E-04	
Dorsal/Ventral pattern formation	4.4	7	2.7115	7.99E-04	
Transport	6.2	10	2.0568	7.92E-01	

4.1.3 –Biological Processes Analysis

Profile of biological processes differentially regulated after SCI in regenerative stages

The following pie charts (Figures 17-19) show how biological processes are grouped according to time points and per up- or down-regulation of the genes in *Danio rerio*. This analysis will help us get a better understanding of the main biological processes that prove to be important in the process of regeneration. Also, it allows to make a comparative analysis between the biological processes that are more relevant in the up-regulated genes vs. the down-regulated genes.

On day 1, there are 9 biological processes corresponding to up-regulated genes (Figure 17a) and 9 biological processes corresponding to down-regulated genes (Figure 17b). The biological process related to Biological adhesion is only present in up-regulated genes whereas Biogenesis is only present in down-regulated genes.

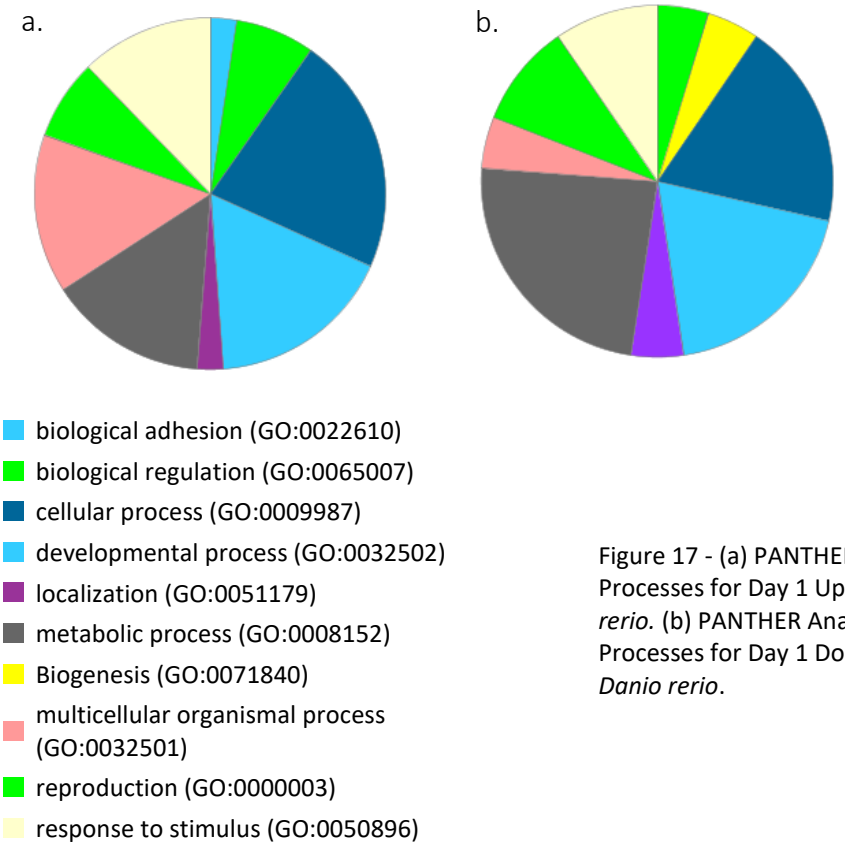


Figure 17 - (a) PANTHER Analysis of Biological Processes for Day 1 Up-Regulated genes in *Danio rerio.* (b) PANTHER Analysis of Biological Processes for Day 1 Down-Regulated genes in *Danio rerio*.

On day 3, there are 12 biological processes corresponding to up-regulated genes (Figure 18a) and 13 corresponding to down-regulated genes (Figure 18b). Biological process related to Biological adhesion is only present in down-regulated genes.
Collectively, there are 12 biological processes groups for up-regulated genes, and 12 for down-regulated genes (Figure 19).

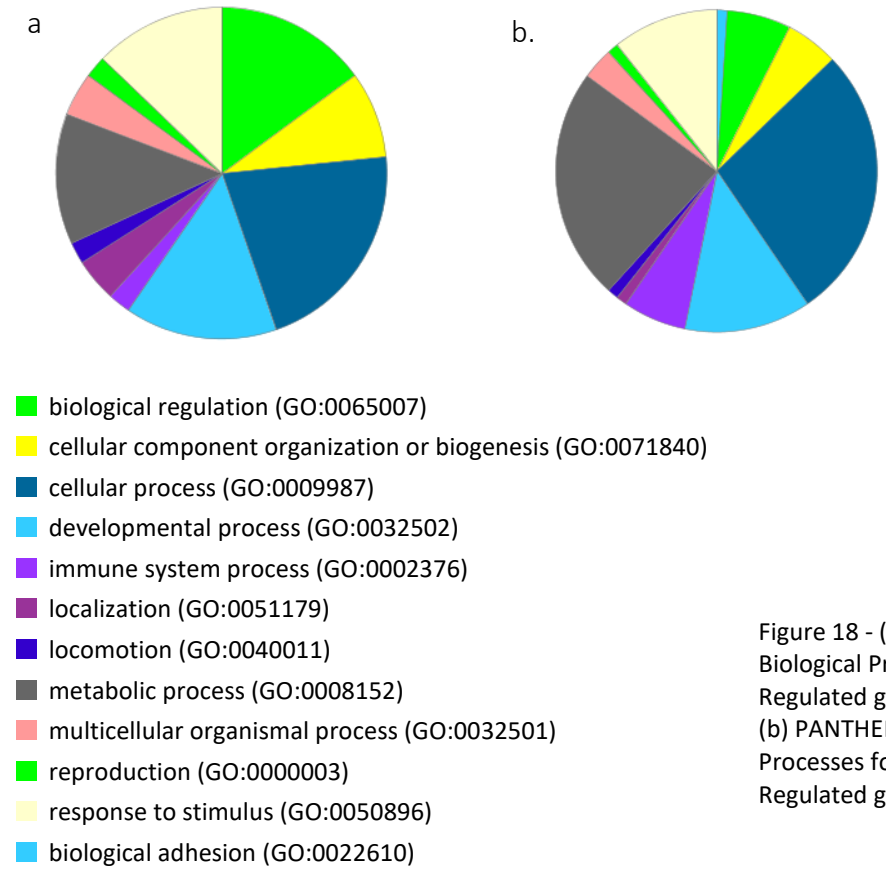
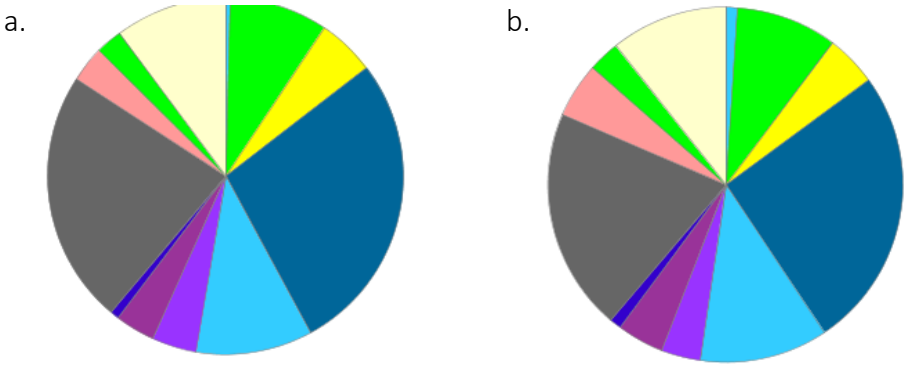


Figure 18 - (a) PANTHER Analysis of Biological Processes for Day 3 up-Regulated genes in *Danio rerio.* (b) PANTHER Analysis of Biological Processes for Day 3 down-Regulated genes in *Danio rerio*.



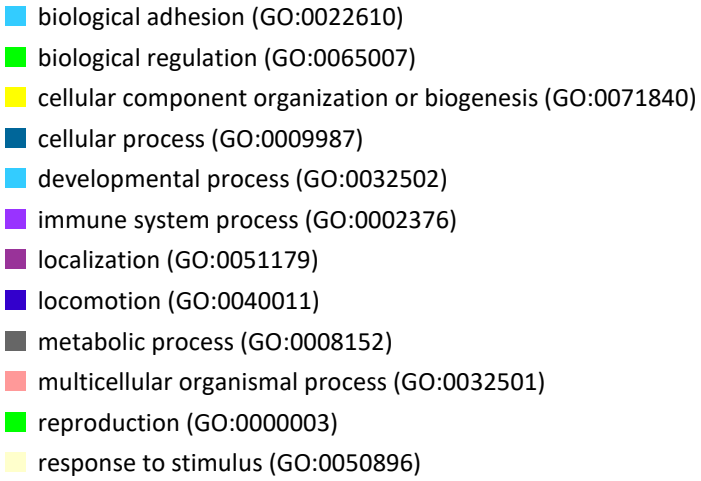


Figure 19 – (a) PANTHER Analysis of Biological Processes for the Sum of up-Regulated genes in *Danio rerio.* (b) PANTHER Analysis of Biological Processes for the Sum of down-Regulated genes in *Danio rerio*.

4.1.2.1 – Gene enrichment analysis

Gene enrichment analysis was performed on all gene lists in order to find the most pertinent biological processes involves in regeneration of the spinal cord.

Regarding biological processes up-regulated genes on day 1, the results revealed that the categories with the largest number of genes were related to response to endogenous stimulus (15%; p-value = 3.44E-02). 6 genes were not classified (30%; p-value = 0). No enriched biological processes appeared for down-regulated genes at the same time point. No enriched biological processes appeared for up-regulated genes on day 3. For day 3 and concerning down-regulated genes, the results revealed that the categories with the largest number of genes were related to: signal transduction (36%; p-value = 2.02E-02), cellular processes (36%; p-value = 2.40E-03) and cell surface receptor signaling pathway (31%; p-value = 3.99E-03). Regarding the biological processes of the sum of all up-regulated genes after SCI in *Danio rerio*, the results (Table 3) showed that the categories with the largest number of genes were related to: cellular processes (55%; p-value = 1.81E-03), developmental processes (25%; p-value = 8.02E-04), system development (18%; 1.24E-04), cell surface receptor signaling pathway (16%; p-value = 1.81E-03) and nervous system development (15%; p-value = 3.44E-05). 25 genes were not classified (19%; p-value = 0).

Table 3 - Significantly enriched gene ontology categories by a biological process for the sum of up-regulated genes in *Danio rerio*. P-value < 0.05. "% ID" indicates the percentage of proteins associated with each GO term relative to the total number of genes that were categorized.

Most Significant Biological Processes					
GO term	**% ID**	**p-value**	**GO term**	**% ID**	**p-value**
Cell surface receptor signaling pathway	16	1.81E-03	Mitosis	9	6.89E-04
Cellular process	55	1.11E-03	Cell cycle	13	3.63E-03
Nervous system development	15	3.44E-05	Muscle organ development	7	1.02E-02
System development	18	1.24E-04	Skeletal system development	6	5.44E-03
Developmental process	25	8.02E-04	Segment specification	5	4.59E-03
Cell differentiation	9	3.04E-03	Pattern specification process	6	2.84E-03

Regarding biological processes for the sum of all down-regulated genes after SCI in *Danio rerio*, the results (Table 4) showed that the categories with the largest number of genes were related to: cellular processes (54%; p-value = 6.31E-03), primary metabolic processes (42%; p-value = 1.15E-02) and system development (15; p-value = 1.20E-02). 27 genes were not classified (21%; p-value = 0).

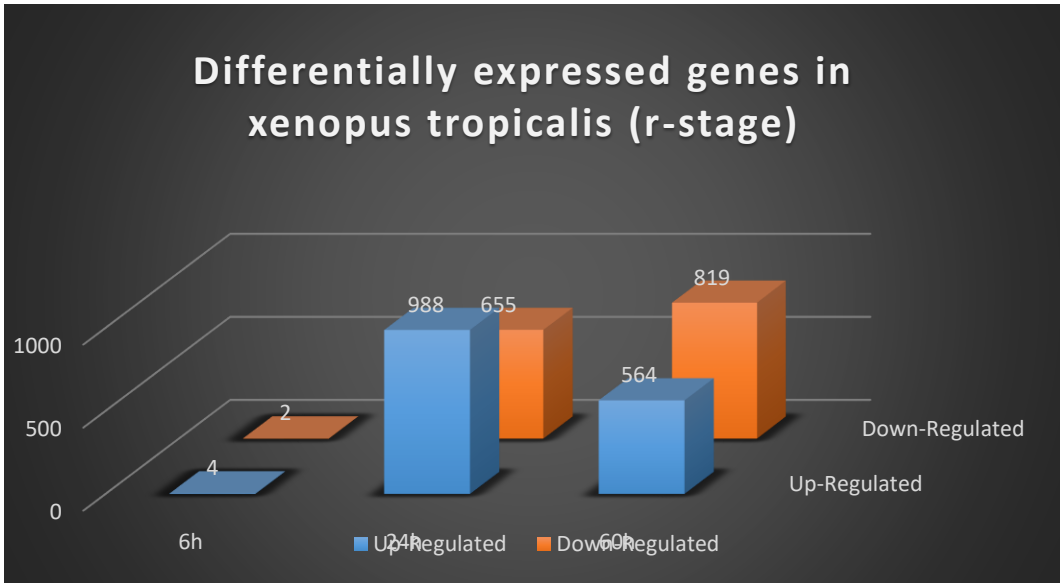
Table 4 - Significantly enriched gene ontology categories by a biological process for the sum of down-regulated genes in *Danio rerio*. P-value < 0.05. "% ID" indicates the percentage of proteins associated with each GO term relative to the total number of genes that were categorized.

Most Significant Biological Processes					
GO term	**% ID**	**p-value**	**GO term**	**% ID**	**p-value**
Primary metabolic process	42	1.15E-02	Mitosis	8	1.66E-02
Pattern specification process	6	2.26E-03	Cell cycle	12	3.32E-02
Skeletal system development	6	3.33E-02	Cellular process	54	6.31E-03
System development	15	1.20E-02	Mesoderm development	9	3.17E-02
Muscle organ development	7	7.99E-03	Nervous system development	12	8.48E-03

4.2 – *Xenopus tropicalis*

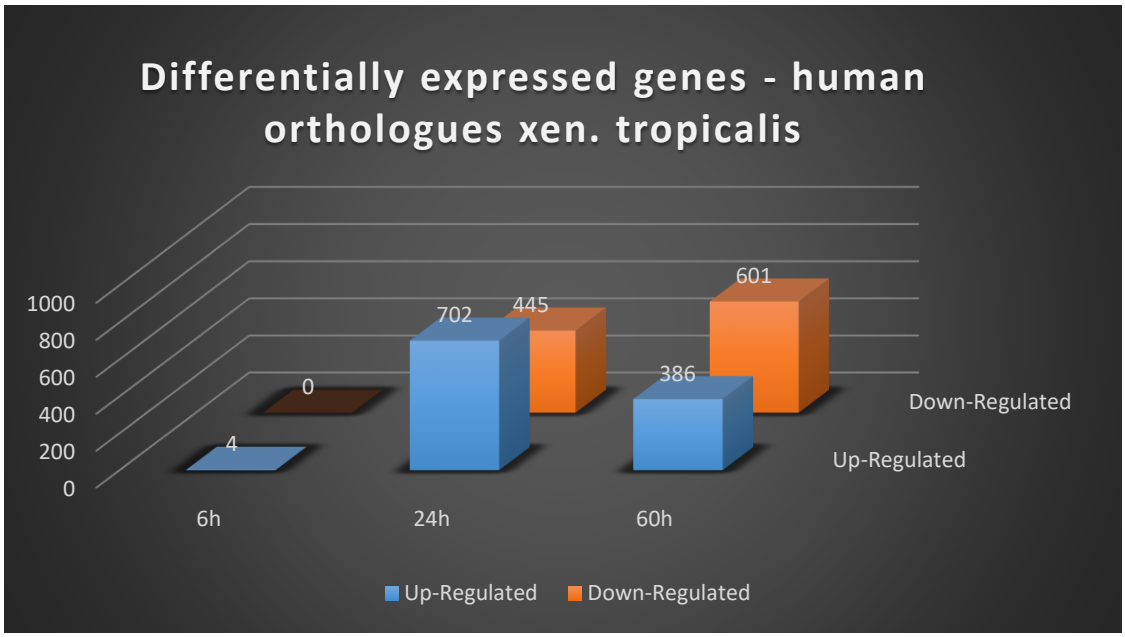
4.2.1 – Differentially expressed genes

Graphic 3 shows a representation of the number of genes that show differential expression in different time points post SCI in *Xenopus tropicalis* during a regenerative stage (pre-metamorphic stages 49-51). The data represents gene expression in the injured spinal cord at three different time points of regeneration: 6h, 24h, and 60h post-injury. After data collection from additional files E-MEXP-2420.raw.1.zip and E-MEXP-2420.processed.1.zip, a total of 3032 genes showed differential expression. From this set of annotated genes 4, 988 and 564 are up-regulated at hour 6, 24, and 60h respectively. On the other hand, 2, 655 and 819 are down-regulated at hour 6, 24, and 60h respectively. A maximum number of genes differentially expressed was found at day 1 (24h). The minimum number of expressed genes appears in an earlier phase (6h). The number of expressed genes either up- and down- regulated tends to increase over time, reaching its peak in an intermediate stage (24h – 1 day) and decreasing in later phases of regeneration (60h – 2.5 days). All graphics are normalized to the same scale for a better visualization and comparison of the number of *Xenopus* genes and the number of its orthologues.



Graphic 3 – Distribution of differentially expressed genes both up-regulated and down-regulated after SCI at different time points in *Xenopus tropicalis*. Established fold change (FC): >4 for up-regulated genes, <-4 for down-regulated genes.

Graphic 4 shows a representation of the number known human orthologues that show a differential expression at different time points during the regenerative stage in *Xenopus tropicalis* after SCI. A total of 2138 known human orthologues showed differential expression. From this set of genes, 4, 702 and 386 are up-regulated at hour 6, 24 and 60h respectively. Complementary, 0, 445 and 661 are down-regulated at hour 6, 24 and 60h, respectively. Conforming with the previous results, the number of differentially expressed genes is very low at early phases of regeneration, with a large increment on intermediate phase (24h), decreasing differential expression in later phases of regeneration (60h).



Graphic 4 – Distribution of differentially expressed genes (human orthologues) both up-regulated and down-regulated after SCI at different time points in *Xenopus tropicalis*. Established fold change (FC): >4 for up-regulated genes, <-4 for down-regulated genes.

4.2.2 – Gene Enrichment Analysis

Table 5 indicates biologically enriched processes 24 and 60 hours after spinal cord injury. After 24 hours, the Epoxygenase P450 pathway is enriched in down-regulated genes. No enriched biological processes appeared for up-regulated genes at this time

point. After 60 hours, Collagen catabolism processes are enriched for up-regulated genes. No enriched biological processes appeared for down-regulated genes.

Table 6 shows enriched biological processes for the collective of up and down-regulated genes. For up-regulated genes, one biological process is enriched, namely Collagen catabolism processes. On the other hand, import of potassium is biologically enriched in down-regulated genes.

Table 5. Significantly enriched gene ontology categories by biological processes (BP) in *Xenopus tropicalis,* for 24h for down-regulated genes, 60h for up-regulated genes p-value < 0,05. "% ID" indicates the percentage of genes associated with each GO TERM relatively to the total number of genes that were categorized.

Go Term	**% ID**	**Number of genes**	**Enrichment Score**	**p-value**	**Day & Alteration**
Epoxygenase P450 pathway	2.1	5	2.097	2.22E-03	**Day 1 (24h) Down-Regulated genes**
Collagen catabolic process	1.3	3	2.237	2.16E-03	**Day 2.5 (60h) Up-Regulated genes**

Table 6. Significantly enriched gene ontology categories by biological processes (BP) in *Xenopus tropicalis,* for all up-regulated genes in all time points (6h, 24h, and 60h) and all down-regulated genes in all time points. p-value < 0,05. "% ID" indicates the percentage of genes associated with each GO TERM relatively to the total number of genes that were categorized.

Go Term	**% ID**	**Number of genes**	**Enrichment Score**	**p-value**	**Alteration**
Collagen catabolic process	0.8	3	2.116	4.83E-03	**Sum of the Up-Regulated genes**
Potassium ion import	0.9	4	2.882	1.12E-02	**Sum of the Down-regulated genes**

4.2.3 – Biological Process Analysis

The following pie charts (Figure 20-22) show how biological processes are grouped according to time points and per up or down-regulation of the genes in *Xenopus tropicalis*. After 24h, there are 12 biological processes corresponding to up-regulated genes (Figure 20a) and 12 biological processes corresponding to down-regulated genes (Figure 20b).

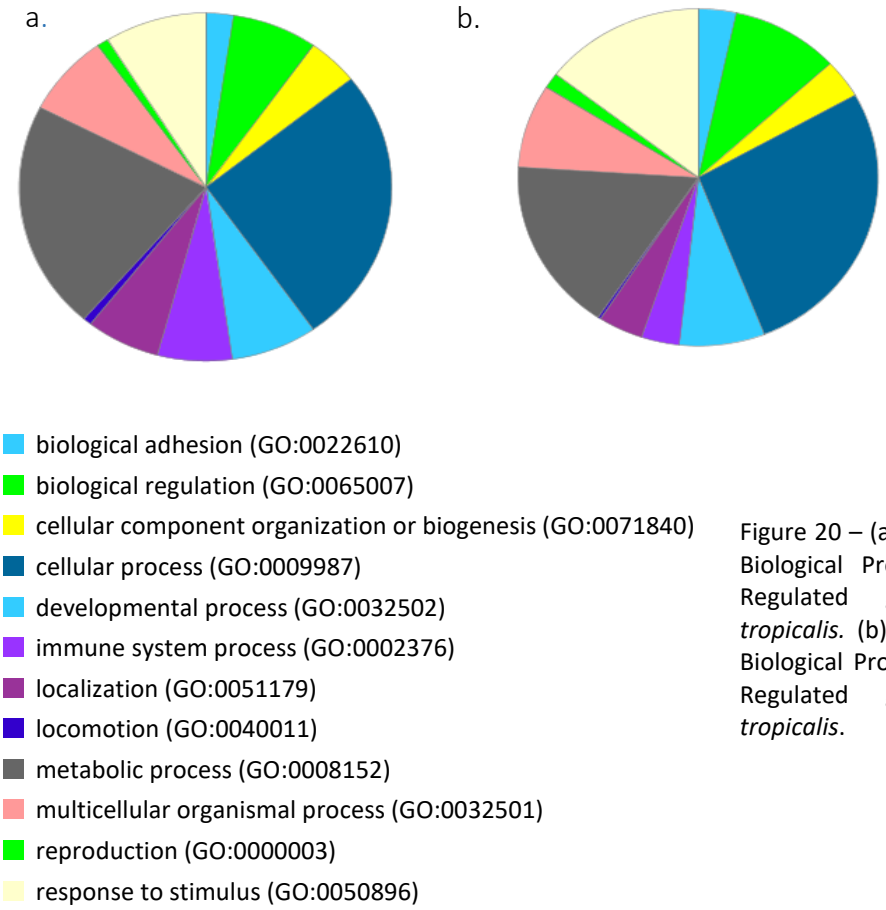


Figure 20 – (a) PANTHER Analysis of Biological Processes for 24h Up-Regulated genes in *Xenopus tropicalis.* (b) PANTHER Analysis of Biological Processes for 24h Down-Regulated genes in *Xenopus tropicalis*.

After 60h, there are 12 biological processes corresponding to up-regulated genes (Figure 21a) and 12 biological processes corresponding to down-regulated genes (Figure 21b).

Collectively, there are also 12 biological processes in up and down-regulated genes for *Xenopus tropicalis* (Figure 22).

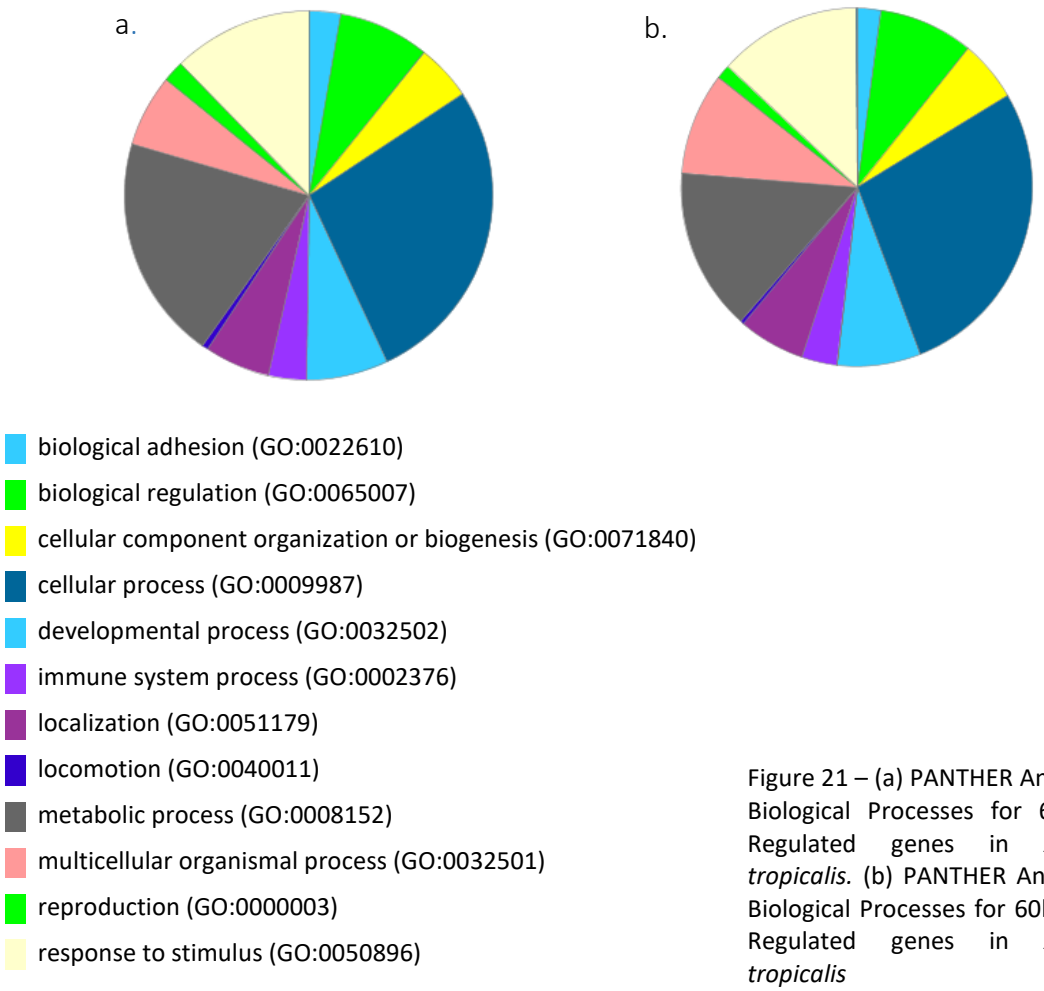


Figure 21 – (a) PANTHER Analysis of Biological Processes for 60h Up-Regulated genes in *Xenopus tropicalis.* (b) PANTHER Analysis of Biological Processes for 60h Down-Regulated genes in *Xenopus tropicalis*

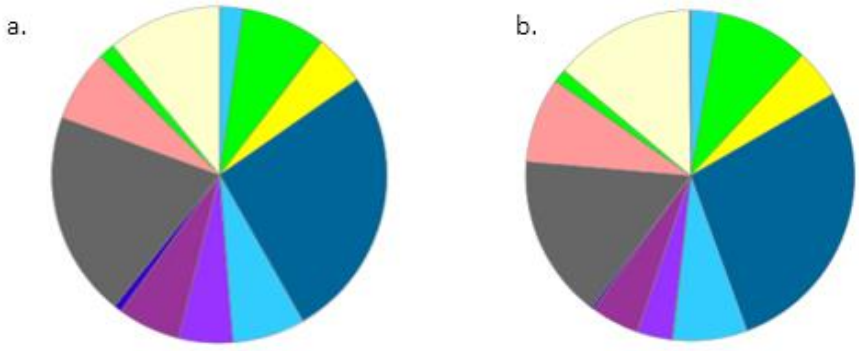


Figure 22 – (a) PANTHER Analysis of Biological Processes for the Sum of Up-Regulated genes in *Xenopus tropicalis.* (b) PANTHER Analysis of Biological Processes for the Sum of Down-Regulated genes in *Xenopus tropicalis*.

4.2.2.1 – Gene enrichment analysis

According to the analysis of the most pertinent biological processes, the results revealed that the categories with the largest number of genes at 24h concerning up-regulated genes, were related to: immune system process (9,3%; p-value = 8.27E-03), and cellular component biogenesis (0,4%; p-value = 4.11E-02). 174 genes were not classified (32.3%; p-value = 0). The results for 24 hours after SCI concerning down-regulated genes (Table 7) revealed that the categories with the largest number of genes were related to: cellular process (53%; p-value = 8.76E-06), response to stimulus (27%; p-value = 6.97E-11), cell communication (22%; p-value = 1.38E-05), signal transduction (20%; p-value = 1.24E-04) and biological regulation (19%; p-value = 1.73E-04). 136 genes were not classified (27%; p-value = 0).

Table. 7 – Significantly enriched gene ontology categories by a biological process at 24h, down-regulated genes in *Xenopus tropicalis*. P-value < 0.05. "% ID" indicates the percentage of proteins associated with each GO term relative to the total number of genes that were categorized.

Most Significant Biological Processes					
GO term	**% ID**	**p-value**	**GO term**	**% ID**	**p-value**
Cell adhesion	6.6	7.24E-04	Multicellular organism process	15	1.38E-03
Biological adhesion	6.6	7.24E-04	Biological regulation	19	1.73E-04
Response to stimulus	27	6.97E-11	Cell communication	22	1.38E-05
Developmental process	15	9.08E-03	Signal transduction	20	1.24E-04
System process	13	6.00E-04	Cellular process	53	8.76E-06

Regarding biological processes of up-regulated genes at 60h after SCI, no biological processes were found enriched. 141 genes were not classified (33%; p-value = 0). Regarding biological processes of down-regulated genes, the results revealed that the categories with the largest number of genes at 60h post injury were related to: cellular processes (53%; p-value = 1.50E-06), response to stimulus (25%; p-value = 4.34E-09), cell communication (21%; 1.31E-04) and signal transduction (18%; p-value = 2.23E-03). 180 genes were not classified (28%; p-value = 0) (Table 8).

Table 8. Significantly enriched down-regulated biological processes at 60h post-SCI in *Xenopus tropicalis*. P-value < 0.05. "% ID" indicates the percentage of proteins associated with each GO term relative to the total number of genes that were categorized.

Most Significant Biological Processes					
GO term	**% ID**	**p-value**	**GO term**	**% ID**	**p-value**
Biological regulation	16	4.98E-02	neurological system process	12	1.12E-05
Signal transduction	18	2.23E-03	system process	15	9.47E-09
Cell communication	21	1.31E-04	single-multicellular organism process	18	6.40E-09
Cellular process	53	1.50E-06	multicellular organismal process	18	7.39E-09
Developmental process	14	3.46E-03	response to stimulus	15	4.34E-09

Table. 9 – Significantly enriched gene ontology categories by a biological process for the sum of down-regulated genes in *Xenopus tropicalis*. P-value < 0.05. "% ID" indicates the percentage of proteins associated with each GO term relative to the total number of genes that were categorized.

Most Significant Biological Processes					
GO term	**% ID**	**p-value**	**GO term**	**% ID**	**p-value**
Regulation of biological process	15	3.54E-05	System process	14	2.25E-09
Biological regulation	17	3.30E-06	Single-multicellular organism process	16	6.77E-09
Signal transduction	19	3.41E-08	Multicellular organism process	16	4.25E-09
Cell communication	21	1.09E-09	Developmental process	13	6.47E-04
Cellular process	52	7.63E-09	Response to stimulus	25	1.41E-15
Neurological system process	11	6.09E-06	Intracellular signal transduction	9	2.47E-03

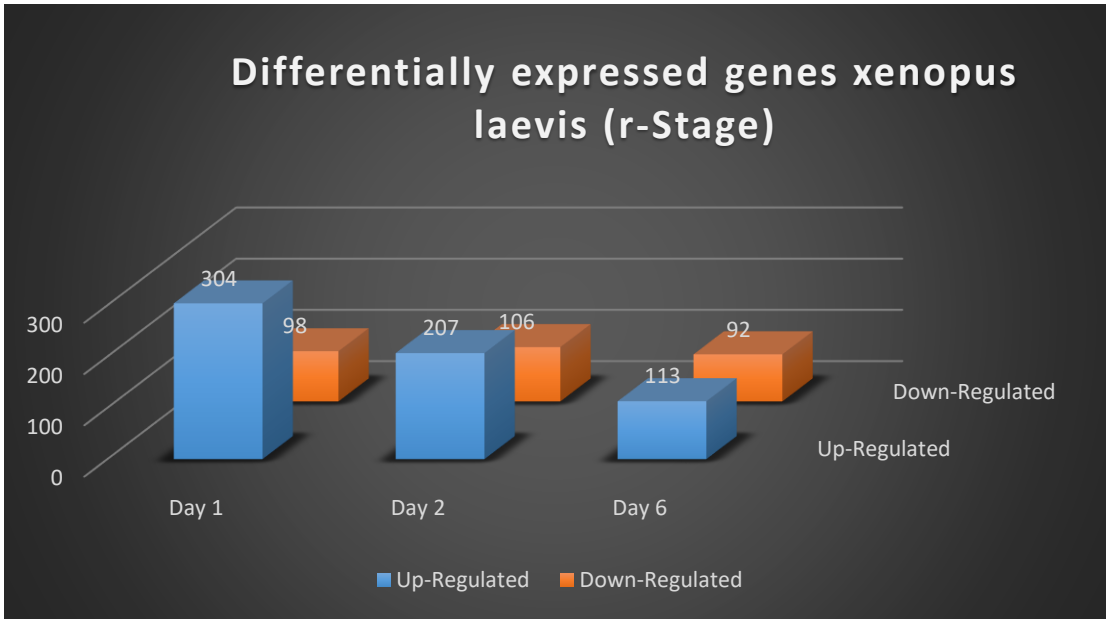
The analysis showed that the biological processes with the largest number of collective up-regulated genes were related to: response to stimulus (22%; p-value = 3.81E-04), signal transduction (17%; p-value = 5.42E-03) and immune system response (9%; 1.59E-03). 234 genes were not classified 32%; p-value = 0). Regarding the down-regulated proccesses, results (Table 9) revealed that the categories with the largest number of collective down-regulated genes were related to: cellular process (52%; p-value = 7.63E-09), response to stimulus (25%; p-value = 1.41E-15), cell communication (21%; p-value = 1.09E-09) and signal transduction (19%; p-value = 3.41E-08).

4.3 - *Xenopus laevis*

4.3.1 – Differentially expressed genes

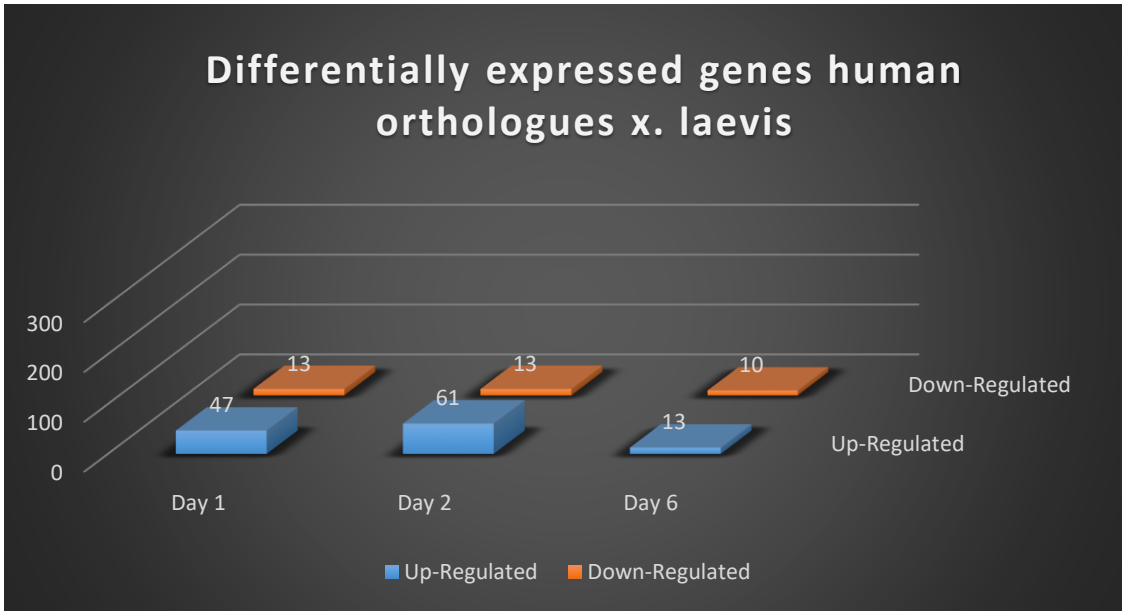
Graphic 5 shows a representation of the number of genes that show differential expression at different time points following SCI in *Xenopus laevis,* during the regenerative stage. Genes were considered as differentially expressed when their fold change was higher than 4. This high fold change cutoff allows a more restrict selection of the genes that play a pivotal role in regeneration of the spinal cord.

The data represents gene expression in the injured spinal cord at three different time points of regeneration (Day 1, Day 2, and Day 6). After data collection from Additional file 3, a total of 920 genes showed differential expression. From this set of annotated genes 304, 207 and 113 are up-regulated at Day 1, 2, and 6 respectively. On the other hand, 98, 106 and 92 are down-regulated in Day 1, 2 and 6, respectively. As expected from the analysis of the lists containing differentially expressed transcripts, the number of transcripts that are up-regulated throughout the three time points is higher than the ones that are down-regulated. Also, while up-regulated genes' expression decreases over time, down-regulated genes maintain a constant pattern.



Graphic 5 - Distribution of differentially expressed genes both up-regulated and down-regulated after SCI at different time points in *Xenopus laevis*. Established fold change (FC): >4 for up-regulated genes, <-4 for down-regulated genes.

Graphic 6 shows a representation of the number known human orthologues with a differential expression in three different time points (day 1, day 2 and day 6) after SCI, during the regenerative stage in *Xenopus laevis*. A total of 157 known human orthologues showed differential expression. From this set of genes, 47, 61 and 13 are up-regulated at day 1, 2 and 6 respectively. Complementary, 13, 13 and 10 are down-regulated at day 1, 2 and 6, respectively. The distribution of differentially expresses genes is somewhat distinct from Graphic 5. According to the data, the maximum peak of differentially expressed genes occurs in day 2 for up-regulated genes, as for the down-regulated genes, the variation is not significant.



Graphic 6 - Distribution of differentially expressed genes (human orthologues) both up-regulated and down-regulated after SCI at different time points in *Xenopus laevis*. Established fold change (FC): >4 for up-regulated genes, <-4 for down-regulated genes.

4.3.2 – Gene Enrichment Analysis

Table 10 shows enriched biological processes for day 1 and day 2. Day 1 presents biological processes such as Cell cycle and Collagen catabolism as enriched in up-regulated genes, and Immune response processes in down-regulated genes. On the other hand, day 2 presents only enriched biological processes for up-regulated genes, namely in Cell division, Microtubule-based movement, and Mitosis.

Table 10. Significantly enriched gene ontology categories by biological processes (BP) in *Xenopus laevis,* day 1 for up and down-regulated genes and day 2 for up-regulated genes, p-value < 0,05. "% ID" indicates the percentage of genes associated with each GO TERM relatively to the total number of genes that were categorized.

Go Term	% ID	Number of genes	Enrichment Score	p-value	Day & Alteration
Cell Cycle	10.4	10	4.786	2.76E-09	**Day 1 Up-Regulated genes**
Collagen Catabolic Processes	3.1	3	3.249	1.69E-03	
Immune *Response*	13.8	4	2.002	3.46E-04	**Down-Regulated genes**
Cell Division	17.4	19	9.352	1.01E-12	**Day 2**
Microtubule-based Movement	8.3	9	8.519	6.40E-10	
Regulation of G2/M transition of mitotic cell cycle	2.8	3	5.028	8.78E-04	**Up-Regulated genes**
Mitotic *chromosome condensation*	3.7	4	3.802	9.52E-05	
DNA replication initiation	4.6	5	2.391	8.73E-05	

Table 11 depicts enriched biological processes for the collective of up and down-regulated genes. Accordingly, biological processes enriched in up-regulated genes include cell division, microtubule-based movement, mitosis, and collagen catabolism processes. On the other hand, immune response processes are enriched in down-regulated genes.

Table 11. Significantly enriched gene ontology categories by biological processes (BP) in *Xenopus laevis,* for all up-regulated genes in all time points (day 1, 2 and 6) and all down-regulated genes in all time points. p-value < 0,05. "% ID" indicates the percentage of genes associated with each GO TERM relatively to the total number of genes that were categorized.

Go Term	% ID	Number of genes	Enrichment Score	p-value	Alteration
Cell Division	9.9	21	8.906	1.01E-11	**Sum of the Up-Regulated genes**
Microtubule-based movement	4.2	9	8.541	1.26E-08	
DNA replication initiation	5.7	12	6.123	4.75E-15	
R*egulation of G2/M transition of mitotic cell cycle*	1.4	3	3.761	1.83E-03	
Mitotic chromosome condensation	1.9	4	3.159	2.85E-04	
Collagen catabolic process	2.4	5	2.813	1.81E-05	
Immune response	8.9	7	5.392	2.18E-06	**Sum of the Down-Regulated Genes**

4. 4 – Analysis of common orthologues using Venn Diagrams

Using JVenn diagrams[87], we were able to obtain differentially expressed genes that are simultaneously present in more than one species at a specific time point (Figure 23 and 24) or in the total experimental periods evaluated, up or down-regulated (Figure 25).

Figure 23a) shows up-regulated genes divided by species. From a total of 407 up-regulated genes, for the same time point, 1 gene was found as present in all three species: MMP9; 7 genes were found to be common for *X. tropicalis and X. laevis*:

DHRS9, OLFM4, CSF3R, ALDH1A3, HSP90AA1, PRSS57 and STEAP4; and 2 shared genes between *Xenopus tropicalis and D. rerio*, namely ADAM9 and IL1B.

Regarding down-regulated genes, illustrated in Figure 23b), the resulting diagram indicates that from a total of 360 down-regulated genes in day 1, uploaded to JVenn program, only 1 gene appeared to be simultaneously present in *X. laevis and X. tropicalis*: CYP26A1, but no common genes were revealed between the three species.

a. b.

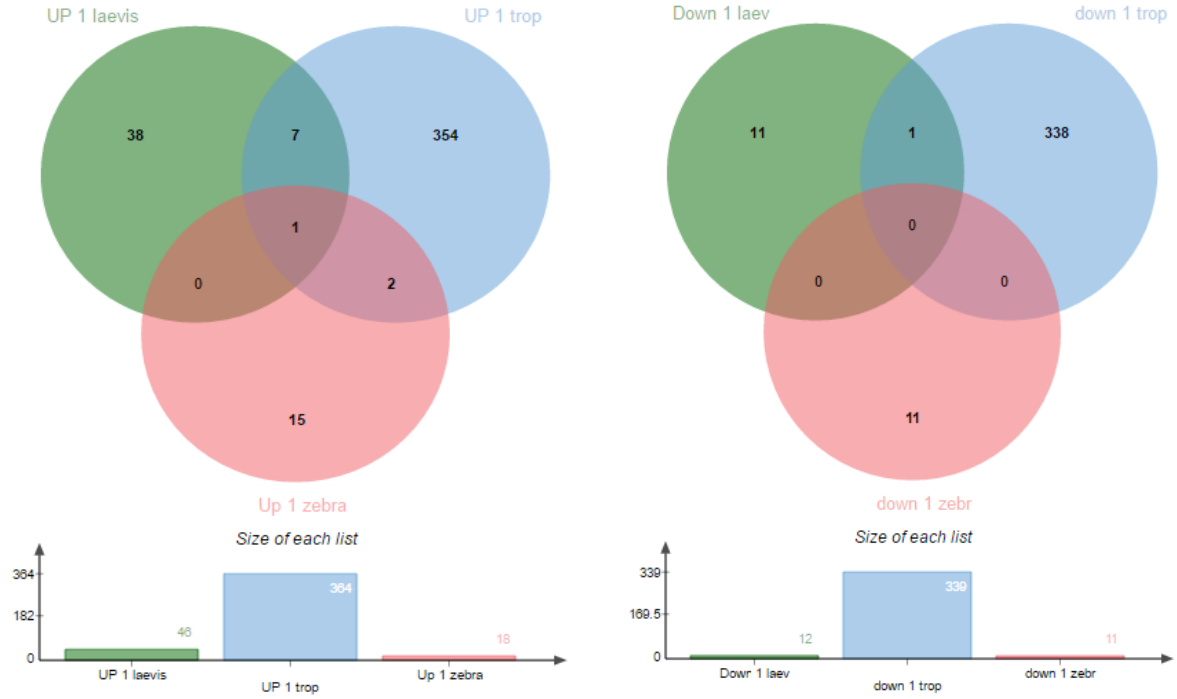


Figure 23 – (a) Venn diagram showing all up-regulated human orthologues detected at day 1 for each species. (b) Venn diagram showing all down-regulated human orthologues detected for day 1 for each species.

Figure 24a) depicts up-regulated genes in day 2 separated by specie. The results indicate 3 common genes between *X. laevis* and *X. tropicalis*: RACGAP1, MMP9, and LRAT, from a total of 258, and no common genes between the three species.

From a total of 511 down-regulated genes in day 2 after SCI, there were no common genes between the three species, 2 common genes differentially expressed shared between *X. tropicalis* and *D. rerio*: HSP90AA1 and PAXIP1, and 1 shared gene between *X. laevis and X. tropicalis*: CYP1A1 (Figure 24b).

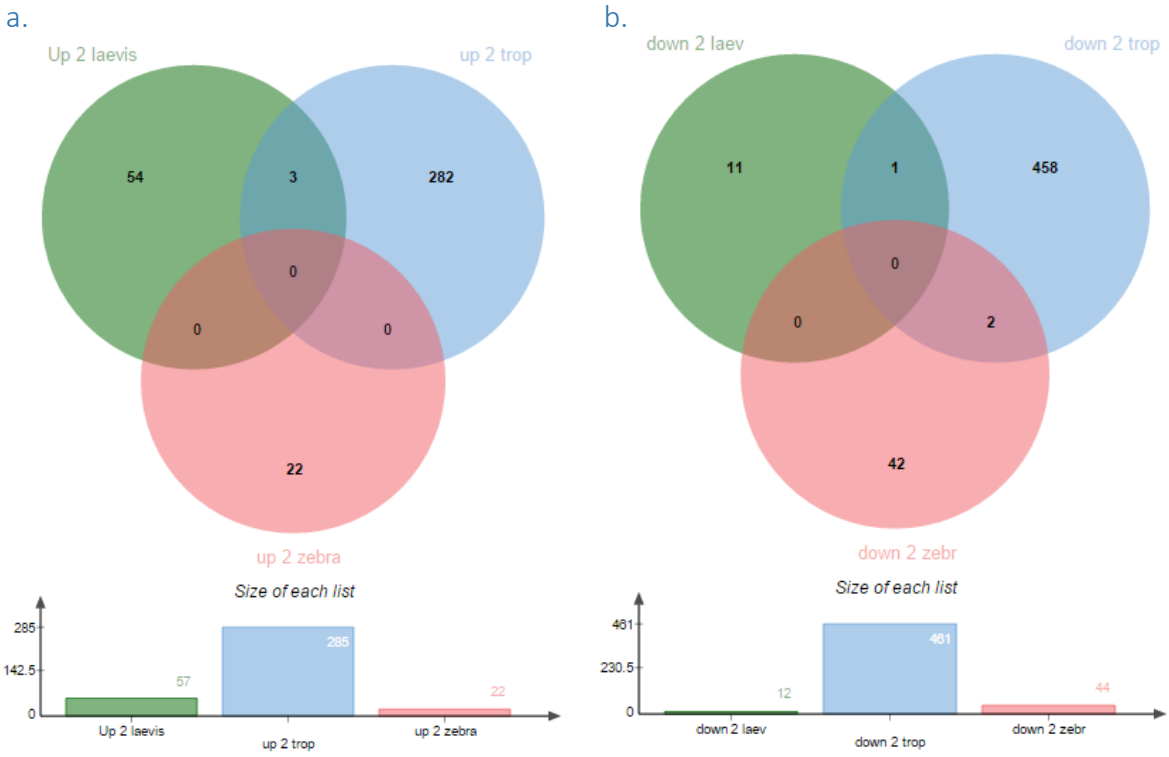
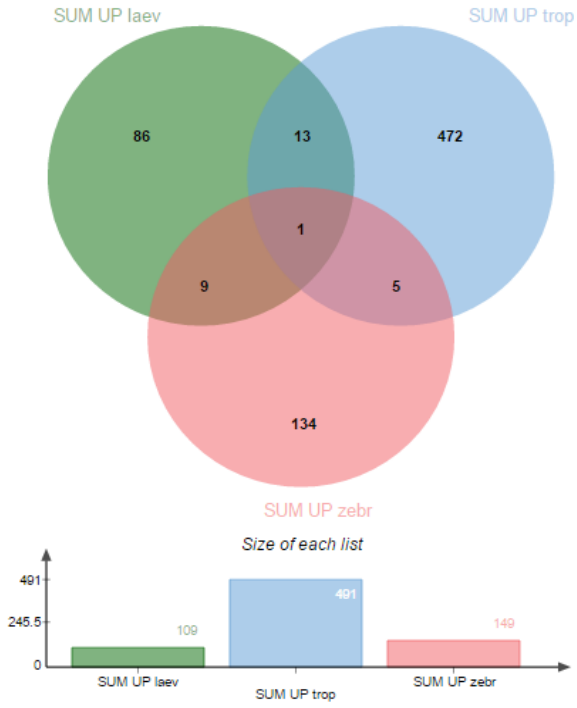


Figure 24 - (a) Venn diagram showing all up-regulated human orthologues detected at day 2 for each species. (b) Venn diagram showing all down-regulated human orthologues detected for day 2 for each species.

Figure 25a) shows collectively all up-regulated genes for all time points in the three species. The resulting diagram indicates that from a total of 692 up-regulated genes, 13 genes were common between *X. laevis* and *X. tropicalis*: DHRS9, OLFM4, CSF3R, ALDH1A3, HSP90AA1, PRSS57, STEAP4, RACGAP1, NCF2, PLEK, SASH3 and KMO; 5 between *Danio rerio* and *X. tropicalis:* IL1B, ADAM9, ST6GAL1, MGAT3 and RUNX2; 9 genes shared by *X. laevis* and *D. rerio*: MCM2, MCM4, SUV39H1, KIF11, LEF1, TYMS, CCNA2, SOCS3, and CDC20; and 1 common gene to all three species: MMP9.

Figure 25b) displays in sum all down-regulated genes from all time points in the three species. The resulting diagrams indicate that from a total of 827 down-regulated genes, 9 genes were common between *X. tropicalis* and *D. rerio*: CDH23, SCN4B, HLA-B, NOS2, HSP90AA1, TRPV4, PAXIP1, FZD3, and KCTD16; 3 genes were common between *X. laevis* and *X. tropicalis*: CYP26A1, CYP1A1 and CCNO, but there were no common down-regulated genes between the three species.

a.



b.

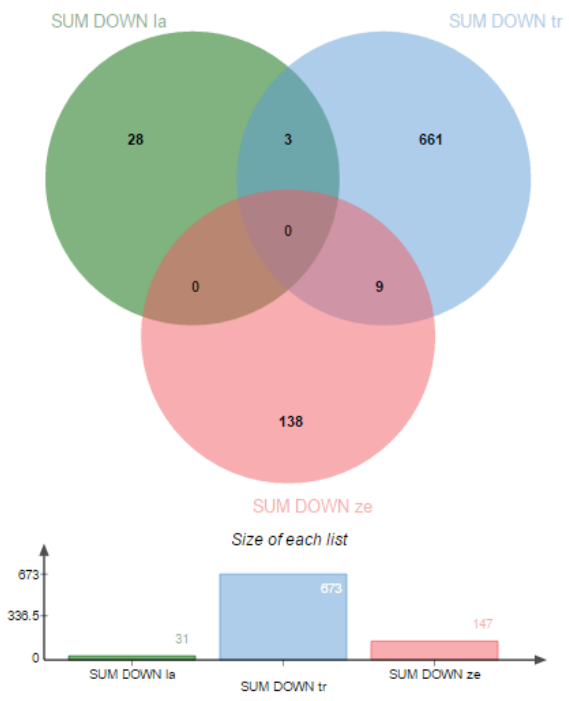


Figure 25 - (a) Venn diagram showing all up-regulated human orthologues detected at all time points for each species. (b) Venn diagram showing all down-regulated human orthologues detected for all time points for each species.

Table 12 – List of human orthologues that are simultaneously differentially expressed in the collective sum of up-regulated genes in more than one species, their biological function and reported relations with spinal cord, tissue injury and regeneration.

Gene	Biological Function	Role in spinal cord, tissue injury and regeneration
DHRS9	Involved in Retinol Metabolism[93,94]	Not documented
OLFM4	Expressed in the inflamed colonic epithelium. The encoded protein is an antiapoptotic factor and an extracellular matrix glycoprotein that promotes tumor growth and facilitates cell adhesion[95].	Marked up-regulation (480 fold) after SCI at P28 in *Monodelphis domestica*[96].
CSF3R	The C-terminus of CSF3R is required for SHP1 down-regulation of CSF3-induced STAT activation[97].	Not documented
ALDH1A3/ALDH6	Encodes retinoid signals that participate in vertebrate morphogenesis. Affected tissues include the eye, craniofacial structures, heart, circulatory, urogenital, respiratory system, limbs and the anterior-posterior axis of the central nervous system[98].	ALDH6 was found on a subset ventral of spinal cord interneurons[98]. GEO Profiles: *Mus musculus*, ID: 28010789.
HSP90AA1/Hsp90α	Protection of tissues from environmental insults; repair of damage tissue; promotes cell motility[99].	Hsp90α binds to LRP-1 in extracellular space promoting cell motility and wound closure; repair on non-cutaneous injured tissues[99].
PRSS57	Encodes an arginine-specific serine protease which undergoes proteolytic activation before storage in azurophil granules, in neutrophil cells. Plays a role in defense against microbial pathogens[100].	Not documented
STEAP4	Appears to be involved in responses to nutrients and inflammatory stress, fatty acid, and glucose metabolism[101]. Attenuates high glucose concentrations and inflammation[102].	Not documented
RACGAP1	Plays a regulatory role in the initiation of cytokinesis, control of cell growth, tumor malignancy and differentiation[103].	Not documented
NCF2/p67-phox	Cytoplasmic polypeptide subunit of phagocyte NADPH oxidase that plays a role in innate immunity. Mutations in NCF2 can result in immunodeficiency[104].	Not documented
PLEK/P47	$P47^{phox}$ is a subunit of NADPH oxidase	Regeneration of skeletal

	involved in production of ROS[105];	muscles[105]; PLEK represents a risk for spinal cord atrophy[106].
SASH3	Involved in cell signaling. May function as a signaling adapter protein in lymphocytes. Stimulates cell migration and proliferation[107].	Not documented
LRAT	Implicated in Vitamin-A metabolism and hepatic retinoid stores.	Hepatic retinoid stores levels provided by LRAT are needed for liver regeneration after liver injury[108].
KMO/ DJ317G22.1	The kynurenine pathway is an important mediator of neuropathic pain pathology[109].	Has increased mRNA levels in the spinal cord and DRG after injury. Its inhibition results in reduced neuropathy[109].
IL1B	Involved in the healing process in the immune microenvironment and in the tissue injury response. Impairs MSC proliferation, migration, and differentiation by inhibiting the Akt/GSK-3β/β-catenin pathway[110].	IL1B negatively regulate bone regeneration in the mouse. Inhibits the regenerative capacities of mesenchymal stem cells[110].
ADAM9	Type I trans-membrane proteins involved in proteolysis, adhesion, cell fusion, and in cell signal transduction. Plays a role during embryonic development and tissue formation[111].	Expression of ADAM9 during chicken spinal cord development[111]
ST6GAL1	Involved in glycosylation of macromolecules which in turn is key to ensuring normal cell differentiation and embryogenesis. Contributes to the regulation of pluripotency in human pluripotent stem cells[112]	Not documented
MGAT3	Transporter of gamma-aminobutyric acid (GABA). It is involved in the regulation of the biosynthesis and biological function of glycoprotein oligosaccharides.	Not documented
RUNX2	Is one of the major transcription factors required for osteogenic and osteoblastic differentiation and skeletal morphogenesis and acts as a scaffold for nucleic acids and regulatory factors involved in skeletal gene expression[113].	Not documented
MMP9	Involved in the breakdown of extracellular matrix in physiological processes. Involved in cell migration.	MMP9' pathway is up-regulated is Zebrafish after SCI[81]; altered

		expression detected in spinal cord and surrounding tissues in rat after SCI[114]
MCM2	Involved in the initiation and elongation of eukaryotic genome replication, particularly the formation and elongation of the replication fork[115].	MCM2 is a hub protein in PPI network constructed for SCI in rats. Up-regulation of MCM2 is related to cell efforts in repairing DNA and regenerate themselves[115].
MCM4	Involved in the initiation and elongation of eukaryotic genome replication, particularly the formation and elongation of the replication fork[115].	Not documented
SUV39H1	SUV39H1 plays a role in limiting genomic instability in dividing cells. SUV39H1 downregulation may contribute to the establishment of senescence by increasing genomic instability[116].	SUV39H1 showed differential expression in the regenerating fin of zebrafish[117].
KIF11/Eg5/kinesin-5	Motor protein involved in spindle formation (chromosome positioning, centrosome separation and establishment of a bipolar spindle) during mitosis[118]. Expressed in the course of development during of axonal growth and in lower concentrations in adults[119].	Inhibition of Kinesin-5 increases axonal length in adult mice and enables them to overcome CSPG barrier[119].
LEF1	Downstream effector and transcriptional target of Wnt signaling. Proliferation-associated transcription factor. Directly down-regulated by knockdown of endogenous β-catenin[120].	Target of Wnt signaling, being induced in wound epithelial cells adjacent to the amputation plane 12h post-amputation. Consistently down-regulated at day 1 post-injury[121].
TYMS	Involves in DNA repair and replication.	Not documented
CCNA2	Involved in the regulation of the cell cycle, promoting transition through G1/S and G2/M.	Involved in cell cycle pathways, up-regulated in the hippocampus over of a few months after SCI[122]; decreasing levels of CCNA2 with age contributes to dysfunction of liver regeneration[123].
SOCS3	In the CNS SOCS3 expression in neurons, oligodendrocytes, astrocytes and microglia is regulated by a wide range of cytokines (IL-4,	SOCS3-dependent Jak/STAT pathway plays a critical role during

	-6 and -10, interferon-β and -ϒ) and lipopolysaccharide. Involved in inflammatory responses after neural injury[124]. Suppressor of axon growth[125].	neuronal loss during secondary damage after SCI[124]; SOCS3 negatively regulates mitochondrial STAT3 after SCI[126,127].
CDC20	Involved in cell division, including nuclear movement prior to anaphase and chromosome separation. Plays an essential role in dendrite morphogenesis in postmitotic neurons[128]	After SCI, Csc20 is enriched at the centrosome in neurons which is crucial for Cdc20-depended dendrite development[128].

Table. 13 – List of human orthologues that are simultaneously differentially expressed in the collective sum of down-regulated genes in more than one species, their biological function and reported relations with spinal cord, tissue injury and regeneration.

Gene	Biological Function	Role in spinal cord, tissue injury and regeneration
CDH23	Involved in stereocilia organization and hair bundle formation.	Regeneration of tip links in auditory hair cells[129]
SCN4B	The protein encoded is one of the several sodium channel beta subunits which interact with voltage-gated alpha subunits to change sodium channel kinetics[130]. SCN4β is a novel substrate of β site amyloid precursor protein cleaving enzyme (BACE1) and ϒ-secretase. Plays an important role in the control of electrical signaling and cell adhesion[130]. Acts as a metastasis-suppressor gene preventing hyperactivation of cell migration in breast cancer[131].	Not documented
HLA-B	Plays a central role in the immune system by presenting peptides derived from the endoplasmic reticulum lumen.	Levels of HLA-B was found elevated in patients who suffered from SCI or severe head trauma[132].
NOS2	Reactive free radical which acts as a biological mediator in several processes including neurotransmission and antimicrobial and antitumoral activities.	NOS levels are increased immediately after an injury to the spinal cord; is thought to play a role in secondary auto-destruction of neural tissue following spinal cord injuries[133].

HSP90AA1/ Hsp90α	Protection of tissues from environmental insults; repair of damage tissue; promotes cell motility[99].	Hsp90α binds to LRP-1 in extracellular space promoting cell motility and wound closure; repair on non-cutaneous injured tissues[99].
TRPV4	Calcium channel, involved in the pathogenesis of age-related neurodegenerative diseases. Extensively expressed in the brain, including the hippocampal neurons, hypothalamus, basal ganglia and cerebellum, and in the spinal cord[134].	TRPV4 channels mediate Ka-K-Cl-co-transporter-induced brain edema after TBI[135]; TRPV4 immunoreactivity is increased in the spinal cord, hippocampal formation, thalamus, basal nuclei and cerebellum of aged rats[134].
PAXIP1	Involved in DNA damage response and in transcriptional regulation. Plays a role in early development.	Not documented
FZD3	Receptor for Wnt proteins. Coupled to the beta-catenin canonical signaling pathway[136].	Suppression of Wnt/β-catenin signaling is required for lens regeneration in *Xenopus laevis*[136].
KCTD16	The KCTD subunits are cytosolic proteins that determine the kinetics of the $GABA_B$ receptor response[137].	Not documented
CYP26A1	Encodes a member of the cytochrome P450 superfamily which is involved in the metabolism of various substances in the liver and small intestine[138].	Important in the differentiation of oval cells into hepatoblast-like cells in the injured liver[98]; involved in peripheral nervous system regeneration (optic nerve injury) in the adult frog[139]
CYP1A1	Encodes a member of the cytochrome P450 superfamily which is involved in drug metabolism and synthesis of cholesterol, steroids, and other lipids[98].	Not documented
CCNO	Involved in the regulation of the cell cycle. Disruption of this gene is associated with primary ciliary dyskinesia-19.	Not documented

Chapter 5 - Discussion and Conclusion

The goal of this dissertation was to obtain a better understanding of the extensiveness and implication of gene expression in the regeneration of spinal cord. To achieve this goal, three studies involving regenerative animal models (*Xenopus laevis*, *Xenopus tropicalis*, and *Zebrafish)* were used. The transcriptomic data was manipulated according to our goal standard: finding robust candidate genes that could possibly be modulated in humans to improve spinal cord repair. For this reason, regarding data analysis of up and down-regulated genes, a fold change of > 4 and <-4 (or $Log_2 > 2$ and $Log_2 < -2$) was chosen as a threshold. This does not to replicate the original studies, since the fold changes applied in these were fold change > 2 and <-2.

One of the limitations encountered when doing this dissertation is due to the fact that not many genome-wide expression studies were yet conducted on regenerative animal models when concerning complete spinal cord injuries. On the other hand, many practical limitations also arise from lack of information databases. For example, neither PANTHER nor BioMart possesses *Xenopus laevis* database, which made it more difficult when enrichment analysis was performed, or when a single gene ID tried to be applied to all species.

Another difficulty to overcome was related to the limited human orthologues that are documented. This was more obvious, but not limited to, the case of *Xenopus laevis*, where a large amount of data was lost when the correspondence was made. Data loss is evident in graphs 2, 4 and 6.

Lastly, the major limitation pertains to the fact that all three studies had individual methodologies and therefore the results vary significantly among them in terms of the number of transcripts. This may also be due to the fact that there may have been more extensive losses of genes throughout the several analyses in one study that in the others.

The first conclusion of this study pertaining the first-time point established for the normalization (Day 1) is that gene up-regulation of more than 4-fold represents in average 69% of gene expression, whereas on day 2/3, it only represents 43%.

Also, throughout the other time points, gene up-regulation is always more marked than gene down-regulation, which is common to all three studies. For instance, in *Danio rerio*, gene up-regulation is 11% higher than gene down-regulation; in *Xenopus tropicalis*, gene up-regulation is 5% higher, and in *Xenopus laevis* the percentage is the highest (53%). This suggests that there are more robust differentially expressed genes in up-regulation than down-regulation, demarcating more pathway activation than silencing.

From non-enrichment biological process analysis of *Danio rerio* (Fig. 17-21), we were able to discern the common processes that coherently appear throughout the time points and species. Regarding up-regulated genes, biological processes present include cellular processes, biological regulation, biological adhesion, metabolic processes, developmental processes, response to stimulus and reproduction. Similarly, on down-regulated genes, biological processes executed by differentially expressed genes pertain to cellular processes, biogenesis, developmental processes, response to stimulus and biological adhesion.

After gene enrichment analysis, of danio rerio genes, it was detected a high predominance of genes involved in developmental processes such as “nervous system development”, “muscle organ development” and “skeletal system development”, in up-regulated genes, whereas the cellular processes categories “cell communication“ and “cell cycle” was observed in the down-regulated genes. Categories related to response to stimulus (“immune response”) were more enriched in down-regulated genes. Genes belonging to the cell cycle categories “mitosis”, “cell division” and “DNA replication” were more expressed in up-regulated genes throughout the time points.

We can also conclude from the Venn diagrams that common genes are more abundant when comparing *Xenopus tropicalis* and *Xenopus laevis* than versus the other species. Among the three species, we have encountered 28 common genes in total in the sum of the up-regulated genes and 12 among the sum of down-regulated genes.

Despite some of them not have been documented has being involved in spinal cord regeneration, or even regeneration for that matter (ex: CYP1A1, CCNO, KCTD16, and PAXIP1), several of them have. For example, ADAM9 which been reported to spinal cord regeneration in chicks[111] is a member of the type I trans-membrane proteins, and is involved in proteolysis, adhesion, cell fusion and in cell signal transduction. NOS2’

levels are increased immediately after the injury to the spinal cord being thought to play a role in secondary auto-destruction of neural tissue following spinal cord injuries[133], among others such as HLA-B, CDC20, and SOCS3. Interest may be given to genes HSP9OAA1 and MMP9, which appear in two days separately, initially as up-regulated (day 1) and in later stages as down-regulated (day3). HSP9OAA1 or Hsp90α has been correlated to pathological or stressful conditions leading to its rapid secretion by keratinocytes and dermal fibroblasts. Heat-shock protein 90 alfa will then promote cell motility and wound closure after its binding to the LDL-receptor related protein-1 (LRP-1) in extracellular space[99]. Similarly, MMP-9, an extracellular matrix metalloproteinase, responsible for extracellular matrix breakdown, has been related to altered pathways in Zebrafish[81] and rats[140] after SCI. In fact, MMP-9 altered expression has become somewhat of a signature in SCI events, and along with MMP-2 highly regulates activities during wound healing[140]. MMPs are modulated by physiological inhibitors such as tissue inhibitors of matrix metalloproteinases (TIMPS), α2-macroglobulin being also highly altered by neutrophil infiltration. Another important find regarding MMP9 is pertaining to its involvement in the functional clearance of CSPG *in vitro*. In fact, it was found that areas free of CSPG coincided with MMP-9 expression[141]. Other genes also seem to play important roles in either the inflammatory response after SCI or regeneration of specific organs. For example SOCS3-dependent Jak/STAT pathway plays a critical role during neuronal loss during secondary damage after SCI[124]. STAT3' pathway which when activated translates into higher levels of neurite and dendrite outgrowth, is negatively regulated by SOCS3 after its binding to both the JAK kinase and cytokine receptor. For this reason, inhibition of SOCS3 with IL-6 will lead to increased outcomes in neurite and dendrite outgrowth[124]. SOCS3 functions are nonetheless quite noticeable when regarding infections once SOCS3' expression is rather stimulated by cytokine or innate immune response receptor agonists present in several viruses, bacteria, and parasites[142]. Another very interesting gene is KIFF11 also known as kinesin-5, a homotetrameric motor protein that generates forces between neighboring microtubules. Kinesin-5 seems to act as a brake on cytoplasmic dynein, and its inhibition would result in faster growing axons after injury[119]. CDC20 has also been localized as enriched in centrosomes of neurons playing a crucial role during cell cycle, and particularly anaphase. CDC20 is a

coactivator to anaphase-promoting complex (APC), which is essential in dendrite morphogenesis[143]. Other genes such as SUV39H1 have been related to fin regeneration in *Zebrafish*[116] and LRAT[108] which plays a crucial role during hepatic regeneration after liver injuries.

These group of genes, which in one way or the other seem to guide regenerative animals into achieving what adult mammals cannot: spinal cord regeneration, are the ones that may deserve our attention for future modulation prospects.

Chapter 6 – Bibliography

1 Thuret, S., Moon, L. D. & Gage, F. H. Therapeutic interventions after spinal cord injury. *Nat Rev Neurosci* **7**, 628-643, doi:10.1038/nrn1955 (2006).

2 Ramon y Cajal, S. & May, R. M. *Degeneration & Regeneration of the nervous system*. (1928).

3 Lee-Liu, D. *et al.* Genome-wide expression profile of the response to spinal cord injury in Xenopus laevis reveals extensive differences between regenerative and non-regenerative stages. *Neural Dev* **9**, 12, doi:10.1186/1749-8104-9-12 (2014).

4 Polezhaev, L. V. *Loss and restoration of regenerative capacity in tissues and organs of animals*.

5 Mancall, E. L., MD. (Copyright © 2011 by Saunders, an imprint of Elsevier Inc.).

6 Susan Stranding, P. (Elsevier Limited, 2008).

7 Lobato, R. D. Historical vignette of Cajal's work "Degeneration and regeneration of the nervous system" with a reflection of the author. *Neurocirugia (Astur)* **19**, 456-468 (2008).

8 Herbrandson. (2005).

9 Anders Holtz, R. L. 336 (Oxford University Press, Inc., 2010).

10 van Middendorp, J. J., Sanchez, G. M. & Burridge, A. L. The Edwin Smith papyrus: a clinical reappraisal of the oldest known document on spinal injuries. *Eur Spine J* **19**, 1815-1823, doi:10.1007/s00586-010-1523-6 (2010).

11 Lifshutz, J. & Colohan, A. A brief history of therapy for traumatic spinal cord injury. *Neurosurg Focus* **16**, E5 (2004).

12 Vasiliadis, E. S., Grivas, T. B. & Kaspiris, A. Historical overview of spinal deformities in ancient Greece. *Scoliosis* **4**, 6, doi:10.1186/1748-7161-4-6 (2009).

13 Marketos, S. G. & Skiadas, P. K. Galen: a pioneer of spine research. *Spine (Phila Pa 1976)* **24**, 2358-2362 (1999).

14 Van Hee, R. Andreas vesalius: his surgical activities and influence on modern surgery. *Acta Chir Belg* **116**, 62-68, doi:10.1080/00015458.2016.1140958 (2016).

15 Hanigan, W. C. & Sloffer, C. Nelson's wound: treatment of spinal cord injury in 19th and early 20th century military conflicts. *Neurosurg Focus* **16**, E4 (2004).

16 Center, N. S. C. S. *Spinal Cord Injury (SCI) Facts and figures at a glance*, 2016).

17 Stahel, P. F., VanderHeiden, T. & Finn, M. A. Management strategies for acute spinal cord injury: current options and future perspectives. *Curr Opin Crit Care* **18**, 651-660, doi:10.1097/MCC.0b013e32835a0e54 (2012).

18 Cripps, R. A. *et al.* A global map for traumatic spinal cord injury epidemiology: towards a living data repository for injury prevention. *Spinal Cord* **49**, 493-501, doi:10.1038/sc.2010.146 (2011).

19 New, P. W., Cripps, R. A. & Bonne Lee, B. Global maps of non-traumatic spinal cord injury epidemiology: towards a living data repository. *Spinal Cord* **52**, 97-109, doi:10.1038/sc.2012.165 (2014).

20 Dumont, R. J. *et al.* Acute spinal cord injury, part I: pathophysiologic mechanisms. *Clin Neuropharmacol* **24**, 254-264 (2001).

21 Yoshimura, N. Bladder afferent pathway and spinal cord injury: possible mechanisms inducing hyperreflexia of the urinary bladder. *Prog Neurobiol* **57**, 583-606 (1999).

22 Fleming, J. C. *et al.* The cellular inflammatory response in human spinal cords after injury. *Brain* **129**, 3249-3269, doi:10.1093/brain/awl296 (2006).

23 Pang, D. & Wilberger, J. E. Spinal cord injury without radiographic abnormalities in children. *J Neurosurg* **57**, 114-129, doi:10.3171/jns.1982.57.1.0114 (1982).

24 David, S. & Aguayo, A. J. Axonal elongation into peripheral nervous system "bridges" after central nervous system injury in adult rats. *Science* **214**, 931-933 (1981).
25 Estrada, V. & Müller, H. W. Spinal cord injury - there is not just one way of treating it. *F1000Prime Rep* **6**, 84, doi:10.12703/P6-84 (2014).
26 Schwab, M. E. & Thoenen, H. Dissociated neurons regenerate into sciatic but not optic nerve explants in culture irrespective of neurotrophic factors. *J Neurosci* **5**, 2415-2423 (1985).
27 Hermanns, S., Klapka, N., Gasis, M. & Müller, H. W. The collagenous wound healing scar in the injured central nervous system inhibits axonal regeneration. *Adv Exp Med Biol* **557**, 177-190, doi:10.1007/0-387-30128-3_11 (2006).
28 Coleman, M. P. *et al.* An 85-kb tandem triplication in the slow Wallerian degeneration (Wlds) mouse. *Proc Natl Acad Sci U S A* **95**, 9985-9990 (1998).
29 Godzik, K. & Coleman, M. P. The axon-protective WLD(S) protein partially rescues mitochondrial respiration and glycolysis after axonal injury. *J Mol Neurosci* **55**, 865-871, doi:10.1007/s12031-014-0440-2 (2015).
30 Vargas, M. E. & Barres, B. A. Why is Wallerian degeneration in the CNS so slow? *Annu Rev Neurosci* **30**, 153-179, doi:10.1146/annurev.neuro.30.051606.094354 (2007).
31 Turner, J. E. & Glaze, K. A. The early stages of Wallerian degeneration in the severed optic nerve of the newt (Triturus viridescens). *Anat Rec* **187**, 291-310, doi:10.1002/ar.1091870303 (1977).
32 Klapka, N. & Müller, H. W. Collagen matrix in spinal cord injury. *J Neurotrauma* **23**, 422-435, doi:10.1089/neu.2006.23.422 (2006).
33 Brazda, N. & Müller, H. W. Pharmacological modification of the extracellular matrix to promote regeneration of the injured brain and spinal cord. *Prog Brain Res* **175**, 269-281, doi:10.1016/S0079-6123(09)17518-0 (2009).
34 Silver, J. & Miller, J. H. Regeneration beyond the glial scar. *Nat Rev Neurosci* **5**, 146-156, doi:10.1038/nrn1326 (2004).
35 Toy, D. & Namgung, U. Role of Glial Cells in Axonal Regeneration. *Exp Neurobiol* **22**, 68-76 (2013).
36 Veeravalli, K. K., Dasari, V. R. & Rao, J. S. Regulation of proteases after spinal cord injury. *J Neurotrauma* **29**, 2251-2262, doi:10.1089/neu.2012.2460 (2012).
37 Sun, F. & He, Z. Neuronal intrinsic barriers for axon regeneration in the adult CNS. *Curr Opin Neurobiol* **20**, 510-518, doi:10.1016/j.conb.2010.03.013 (2010).
38 Yiu, G. & He, Z. Glial inhibition of CNS axon regeneration. *Nat Rev Neurosci* **7**, 617-627, doi:10.1038/nrn1956 (2006).
39 Ramer, M. S., Duraisingam, I., Priestley, J. V. & McMahon, S. B. Two-tiered inhibition of axon regeneration at the dorsal root entry zone. *J Neurosci* **21**, 2651-2660 (2001).
40 Jones, L. L. & Tuszynski, M. H. Spinal cord injury elicits expression of keratan sulfate proteoglycans by macrophages, reactive microglia, and oligodendrocyte progenitors. *J Neurosci* **22**, 4611-4624, doi:20026464 (2002).
41 Schnell, L., Fearn, S., Klassen, H., Schwab, M. E. & Perry, V. H. Acute inflammatory responses to mechanical lesions in the CNS: differences between brain and spinal cord. *Eur J Neurosci* **11**, 3648-3658 (1999).
42 Habgood, M. D. *et al.* Changes in blood-brain barrier permeability to large and small molecules following traumatic brain injury in mice. *Eur J Neurosci* **25**, 231-238, doi:10.1111/j.1460-9568.2006.05275.x (2007).
43 Campbell, S. J., Wilcockson, D. C., Butchart, A. G., Perry, V. H. & Anthony, D. C. Altered chemokine expression in the spinal cord and brain contributes to differential interleukin-1beta-induced neutrophil recruitment. *J Neurochem* **83**, 432-441 (2002).
44 Popovich, P. G., Wei, P. & Stokes, B. T. Cellular inflammatory response after spinal cord injury in Sprague-Dawley and Lewis rats. *J Comp Neurol* **377**, 443-464 (1997).
45 Hausmann, O. N. Post-traumatic inflammation following spinal cord injury. *Spinal Cord* **41**, 369-378, doi:10.1038/sj.sc.3101483 (2003).

46 Blight, A. R. Delayed demyelination and macrophage invasion: a candidate for secondary cell damage in spinal cord injury. *Cent Nerv Syst Trauma* **2**, 299-315 (1985).
47 Crowe, M. J., Bresnahan, J. C., Shuman, S. L., Masters, J. N. & Beattie, M. S. Apoptosis and delayed degeneration after spinal cord injury in rats and monkeys. *Nat Med* **3**, 73-76 (1997).
48 Shuman, S. L., Bresnahan, J. C. & Beattie, M. S. Apoptosis of microglia and oligodendrocytes after spinal cord contusion in rats. *J Neurosci Res* **50**, 798-808 (1997).
49 Mietto, B. S., Mostacada, K. & Martinez, A. M. Neurotrauma and inflammation: CNS and PNS responses. *Mediators Inflamm* **2015**, 251204, doi:10.1155/2015/251204 (2015).
50 Selmaj, K. W. & Raine, C. S. Tumor necrosis factor mediates myelin and oligodendrocyte damage in vitro. *Ann Neurol* **23**, 339-346, doi:10.1002/ana.410230405 (1988).
51 Donnelly, D. J. & Popovich, P. G. Inflammation and its role in neuroprotection, axonal regeneration and functional recovery after spinal cord injury. *Exp Neurol* **209**, 378-388, doi:10.1016/j.expneurol.2007.06.009 (2008).
52 Downey, G. P., Fukushima, T., Fialkow, L. & Waddell, T. K. Intracellular signaling in neutrophil priming and activation. *Semin Cell Biol* **6**, 345-356 (1995).
53 Taoka, Y. *et al.* Role of neutrophils in spinal cord injury in the rat. *Neuroscience* **79**, 1177-1182 (1997).
54 DiSabato, D. J., Quan, N. & Godbout, J. P. Neuroinflammation: the devil is in the details. *J Neurochem* **139 Suppl 2**, 136-153, doi:10.1111/jnc.13607 (2016).
55 Farooque, M., Isaksson, J. & Olsson, Y. Improved recovery after spinal cord trauma in ICAM-1 and P-selectin knockout mice. *Neuroreport* **10**, 131-134 (1999).
56 Fitch, M. T. & Silver, J. CNS injury, glial scars, and inflammation: Inhibitory extracellular matrices and regeneration failure. *Exp Neurol* **209**, 294-301, doi:10.1016/j.expneurol.2007.05.014 (2008).
57 Noble, L. J., Donovan, F., Igarashi, T., Goussev, S. & Werb, Z. Matrix metalloproteinases limit functional recovery after spinal cord injury by modulation of early vascular events. *J Neurosci* **22**, 7526-7535 (2002).
58 Bingham, W. G. *et al.* Blood flow in normal and injured monkey spinal cord. *J Neurosurg* **43**, 162-171, doi:10.3171/jns.1975.43.2.0162 (1975).
59 Kuffler, D. P. Maximizing neuroprotection: where do we stand? *Ther Clin Risk Manag* **8**, 185-194, doi:10.2147/TCRM.S16196 (2012).
60 Courtine, G., van den Brand, R. & Musienko, P. Spinal cord injury: time to move. *Lancet* **377**, 1896-1898, doi:10.1016/S0140-6736(11)60711-3 (2011).
61 Bradbury, E. J. & McMahon, S. B. Spinal cord repair strategies: why do they work? *Nat Rev Neurosci* **7**, 644-653, doi:10.1038/nrn1964 (2006).
62 Kakulas, B. A. & Kaelan, C. The neuropathological foundations for the restorative neurology of spinal cord injury. *Clin Neurol Neurosurg* **129 Suppl 1**, S1-7, doi:10.1016/j.clineuro.2015.01.012 (2015).
63 Puelles, L. Contributions to Neuroembryology of Santiago Ramon y Cajal (1852-1934) and Jorge F. Tello (1880-1958). *Int J Dev Biol* **53**, 1145-1160, doi:10.1387/ijdb.082589lp (2009).
64 Amr, S. M. *et al.* Bridging defects in chronic spinal cord injury using peripheral nerve grafts combined with a chitosan-laminin scaffold and enhancing regeneration through them by co-transplantation with bone-marrow-derived mesenchymal stem cells: case series of 14 patients. *J Spinal Cord Med* **37**, 54-71, doi:10.1179/2045772312Y.0000000069 (2014).
65 Jaffe, L. F. & Poo, M. M. Neurites grow faster towards the cathode than the anode in a steady field. *J Exp Zool* **209**, 115-128, doi:10.1002/jez.1402090114 (1979).
66 R. Shane Tubbs, E. R., Mohammadali M. Shoja, Marios Loukas, Nicholas Barbaro, Robert J. Spinner. *Nerves and Nerve Injuries: Vol 2: Pain, Treatment, Injury, Disease and Future Directions*. (Academic Press 2015).

67 Jain, A., Kim, Y. T., McKeon, R. J. & Bellamkonda, R. V. In situ gelling hydrogels for conformal repair of spinal cord defects, and local delivery of BDNF after spinal cord injury. *Biomaterials* **27**, 497-504, doi:10.1016/j.biomaterials.2005.07.008 (2006).
68 Katayama, Y. *et al.* Coil-reinforced hydrogel tubes promote nerve regeneration equivalent to that of nerve autografts. *Biomaterials* **27**, 505-518, doi:10.1016/j.biomaterials.2005.07.016 (2006).
69 Parikh, P. *et al.* Regeneration of axons in injured spinal cord by activation of bone morphogenetic protein/Smad1 signaling pathway in adult neurons. *Proc Natl Acad Sci U S A* **108**, E99-107, doi:10.1073/pnas.1100426108 (2011).
70 Awad, B. I., Carmody, M. A. & Steinmetz, M. P. Potential role of growth factors in the management of spinal cord injury. *World Neurosurg* **83**, 120-131, doi:10.1016/j.wneu.2013.01.042 (2015).
71 Harkema, S. *et al.* Effect of epidural stimulation of the lumbosacral spinal cord on voluntary movement, standing, and assisted stepping after motor complete paraplegia: a case study. *Lancet* **377**, 1938-1947, doi:10.1016/S0140-6736(11)60547-3 (2011).
72 Minassian, K. *et al.* Mechanisms of rhythm generation of the human lumbar spinal cord in response to tonic stimulation without and with step-related sensory feedback. *Biomed Tech (Berl)*, doi:10.1515/bmt-2013-4013 (2013).
73 Brazda, N. *et al.* Experimental Strategies to Bridge Large Tissue Gaps in the Injured Spinal Cord after Acute and Chronic Lesion. *J Vis Exp*, e53331, doi:10.3791/53331 (2016).
74 Sánchez Alvarado, A. & Tsonis, P. A. Bridging the regeneration gap: genetic insights from diverse animal models. *Nat Rev Genet* **7**, 873-884, doi:10.1038/nrg1923 (2006).
75 Beck, C. W., Izpisúa Belmonte, J. C. & Christen, B. Beyond early development: Xenopus as an emerging model for the study of regenerative mechanisms. *Dev Dyn* **238**, 1226-1248, doi:10.1002/dvdy.21890 (2009).
76 Session, A. M. *et al.* Genome evolution in the allotetraploid frog Xenopus laevis. *Nature* **538**, 336-343, doi:10.1038/nature19840 (2016).
77 Kinth, P., Mahesh, G. & Panwar, Y. Mapping of zebrafish research: a global outlook. *Zebrafish* **10**, 510-517, doi:10.1089/zeb.2012.0854 (2013).
78 Chakraborty, C., Sharma, A. R., Sharma, G. & Lee, S. S. Zebrafish: A complete animal model to enumerate the nanoparticle toxicity. *J Nanobiotechnology* **14**, 65, doi:10.1186/s12951-016-0217-6 (2016).
79 Chávez, M. N., Aedo, G., Fierro, F. A., Allende, M. L. & Egaña, J. T. Zebrafish as an Emerging Model Organism to Study Angiogenesis in Development and Regeneration. *Front Physiol* **7**, 56, doi:10.3389/fphys.2016.00056 (2016).
80 Bellipanni, G. *et al.* Zebrafish as a Model for the Study of Chaperonopathies. *J Cell Physiol* **231**, 2107-2114, doi:10.1002/jcp.25319 (2016).
81 Zhao, C. *et al.* Genome-wide profiling of AP-1-regulated transcription provides insights into the invasiveness of triple-negative breast cancer. *Cancer Res* **74**, 3983-3994, doi:10.1158/0008-5472.CAN-13-3396 (2014).
82 Yates, A. *et al.* Ensembl 2016. *Nucleic Acids Res* **44**, D710-716, doi:10.1093/nar/gkv1157 (2016).
83 Mudunuri, U., Che, A., Yi, M. & Stephens, R. M. bioDBnet: the biological database network. *Bioinformatics* **25**, 555-556, doi:10.1093/bioinformatics/btn654 (2009).
84 Smith, R. N. *et al.* InterMine: a flexible data warehouse system for the integration and analysis of heterogeneous biological data. *Bioinformatics* **28**, 3163-3165, doi:10.1093/bioinformatics/bts577 (2012).
85 Huang, d. W., Sherman, B. T. & Lempicki, R. A. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* **4**, 44-57, doi:10.1038/nprot.2008.211 (2009).
86 Thomas, P. D. *et al.* PANTHER: a library of protein families and subfamilies indexed by function. *Genome Res* **13**, 2129-2141, doi:10.1101/gr.772403 (2003).

87 Bardou, P., Mariette, J., Escudié, F., Djemiel, C. & Klopp, C. jvenn: an interactive Venn diagram viewer. *BMC Bioinformatics* **15**, 293, doi:10.1186/1471-2105-15-293 (2014).
88 Aken, B. L. *et al.* The Ensembl gene annotation system. *Database (Oxford)* **2016**, doi:10.1093/database/baw093 (2016).
89 Baker, M. Quantitative data: learning to share. *Nat Meth* **9**, 39-41, doi:10.1038/nmeth.1815 (2012).
90 Huang, d. W., Sherman, B. T. & Lempicki, R. A. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res* **37**, 1-13, doi:10.1093/nar/gkn923 (2009).
91 Karpinka, J. B. *et al.* Xenbase, the Xenopus model organism database; new virtualized system, data types and genomes. *Nucleic Acids Res* **43**, D756-763, doi:10.1093/nar/gku956 (2015).
92 Ashburner, M. *et al.* Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nat Genet* **25**, 25-29, doi:10.1038/75556 (2000).
93 Soref, C. M. *et al.* Characterization of a novel airway epithelial cell-specific short chain alcohol dehydrogenase/reductase gene whose expression is up-regulated by retinoids and is involved in the metabolism of retinol. *J Biol Chem* **276**, 24194-24202, doi:10.1074/jbc.M100332200 (2001).
94 Chetyrkin, S. V., Belyaeva, O. V., Gough, W. H. & Kedishvili, N. Y. Characterization of a novel type of human microsomal 3alpha -hydroxysteroid dehydrogenase: unique tissue distribution and catalytic properties. *J Biol Chem* **276**, 22278-22286, doi:10.1074/jbc.M102076200 (2001).
95 Zhang, J. *et al.* Identification and characterization of a novel member of olfactomedin-related protein family, hGC-1, expressed during myeloid lineage development. *Gene* **283**, 83-93 (2002).
96 Saunders, N. R. *et al.* Age-dependent transcriptome and proteome following transection of neonatal spinal cord of Monodelphis domestica (South American grey short-tailed opossum). *PLoS One* **9**, e99080, doi:10.1371/journal.pone.0099080 (2014).
97 Dong, F., Qiu, Y., Yi, T., Touw, I. P. & Larner, A. C. The carboxyl terminus of the granulocyte colony-stimulating factor receptor, truncated in patients with severe congenital neutropenia/acute myeloid leukemia, is required for SH2-containing phosphatase-1 suppression of Stat activation. *J Immunol* **167**, 6447-6452 (2001).
98 Grün, F., Hirose, Y., Kawauchi, S., Ogura, T. & Umesono, K. Aldehyde dehydrogenase 6, a cytosolic retinaldehyde dehydrogenase prominently expressed in sensory neuroepithelia during development. *J Biol Chem* **275**, 41210-41218, doi:10.1074/jbc.M007376200 (2000).
99 Jayaprakash, P. *et al.* Hsp90α and Hsp90β together operate a hypoxia and nutrient paucity stress-response mechanism during wound healing. *J Cell Sci* **128**, 1475-1480, doi:10.1242/jcs.166363 (2015).
100 Kasperkiewicz, P. *et al.* Design of a Selective Substrate and Activity Based Probe for Human Neutrophil Serine Protease 4. *PLoS One* **10**, e0132818, doi:10.1371/journal.pone.0132818 (2015).
101 Grunewald, T. G., Bach, H., Cossarizza, A. & Matsumoto, I. The STEAP protein family: versatile oxidoreductases and targets for cancer immunotherapy with overlapping and distinct cellular functions. *Biol Cell* **104**, 641-657, doi:10.1111/boc.201200027 (2012).
102 Chuang, C. T. *et al.* Steap4 attenuates high glucose and S100B-induced effects in mesangial cells. *J Cell Mol Med* **19**, 1234-1244, doi:10.1111/jcmm.12472 (2015).
103 Yeh, C. M. *et al.* Opposing prognostic roles of nuclear and cytoplasmic RACGAP1 expression in colorectal cancer patients. *Hum Pathol* **47**, 45-51, doi:10.1016/j.humpath.2015.09.002 (2016).
104 Tarazona-Santos, E. *et al.* Evolutionary dynamics of the human NADPH oxidase genes CYBB, CYBA, NCF2, and NCF4: functional implications. *Mol Biol Evol* **30**, 2157-2167, doi:10.1093/molbev/mst119 (2013).

105 Zhang, Q. *et al.* Reactive oxygen species generated from skeletal muscles are required for gecko tail regeneration. *Sci Rep* **6**, 20752, doi:10.1038/srep20752 (2016).
106 Akkad, D. A. *et al.* Multiple sclerosis risk loci correlate with cervical cord atrophy and may explain the course of disability. *Neurogenetics* **16**, 161-168, doi:10.1007/s10048-015-0438-0 (2015).
107 Erdem-Eraslan, L. *et al.* Tumor-specific mutations in low-frequency genes affect their functional properties. *J Neurooncol* **122**, 461-470, doi:10.1007/s11060-015-1741-1 (2015).
108 Shmarakov, I. O., Jiang, H., Yang, K. J., Goldberg, I. J. & Blaner, W. S. Hepatic retinoid stores are required for normal liver regeneration. *J Lipid Res* **54**, 893-908, doi:10.1194/jlr.M029801 (2013).
109 Rojewska, E., Piotrowska, A., Makuch, W., Przewlocka, B. & Mika, J. Pharmacological kynurenine 3-monooxygenase enzyme inhibition significantly reduces neuropathic pain in a rat model. *Neuropharmacology* **102**, 80-91, doi:10.1016/j.neuropharm.2015.10.040 (2016).
110 Martino, M. M. *et al.* Inhibition of IL-1R1/MyD88 signalling promotes mesenchymal stem cell-driven tissue regeneration. *Nat Commun* **7**, 11051, doi:10.1038/ncomms11051 (2016).
111 Lin, J. *et al.* Expression of seven members of the ADAM family in developing chicken spinal cord. *Dev Dyn* **239**, 1246-1254, doi:10.1002/dvdy.22272 (2010).
112 Wang, Y. C. *et al.* Glycosyltransferase ST6GAL1 contributes to the regulation of pluripotency in human pluripotent stem cells. *Sci Rep* **5**, 13317, doi:10.1038/srep13317 (2015).
113 Bendre, A., Büki, K. G. & Määttä, J. A. Fam3c modulates osteogenic differentiation by down-regulating Runx2. *Differentiation* **93**, 50-57, doi:10.1016/j.diff.2016.11.005 (2016).
114 Hou, Y. N. *et al.* Effect of hyperbaric oxygen on MMP9/2 expression and motor function in rats with spinal cord injury. *Int J Clin Exp Med* **8**, 14926-14934 (2015).
115 Chen, G., Fang, X. & Yu, M. Regulation of gene expression in rats with spinal cord injury based on microarray data. *Mol Med Rep* **12**, 2465-2472, doi:10.3892/mmr.2015.3670 (2015).
116 Sidler, C. *et al.* A role for SUV39H1-mediated H3K9 trimethylation in the control of genome stability and senescence in WI38 human diploid lung fibroblasts. *Aging (Albany NY)* **6**, 545-563, doi:10.18632/aging.100678 (2014).
117 Saxena, S. *et al.* Role of annexin gene and its regulation during zebrafish caudal fin regeneration. *Wound Repair Regen* **24**, 551-559, doi:10.1111/wrr.12429 (2016).
118 Johnson, K. *et al.* Kif11 dependent cell cycle progression in radial glial cells is required for proper neurogenesis in the zebrafish neural tube. *Dev Biol* **387**, 73-92, doi:10.1016/j.ydbio.2013.12.021 (2014).
119 Lin, S. *et al.* Inhibition of Kinesin-5, a microtubule-based motor protein, as a strategy for enhancing regeneration of adult axons. *Traffic* **12**, 269-286, doi:10.1111/j.1600-0854.2010.01152.x (2011).
120 Song, H. *et al.* The regenerating spinal cord of gecko maintains unaltered expression of β-catenin following tail amputation. *J Mol Neurosci* **55**, 653-662, doi:10.1007/s12031-014-0405-5 (2015).
121 Chen, C. H. *et al.* Transient laminin beta 1a Induction Defines the Wound Epidermis during Zebrafish Fin Regeneration. *PLoS Genet* **11**, e1005437, doi:10.1371/journal.pgen.1005437 (2015).
122 Wu, J. *et al.* Spinal cord injury causes brain inflammation associated with cognitive and affective changes: role of cell cycle pathways. *J Neurosci* **34**, 10989-11006, doi:10.1523/JNEUROSCI.5110-13.2014 (2014).
123 Enkhbold, C. *et al.* Dysfunction of liver regeneration in aged liver after partial hepatectomy. *J Gastroenterol Hepatol* **30**, 1217-1224, doi:10.1111/jgh.12930 (2015).
124 Liu, X., Williams, P. R. & He, Z. SOCS3: a common target for neuronal protection and axon regeneration after spinal cord injury. *Exp Neurol* **263**, 364-367, doi:10.1016/j.expneurol.2014.10.024 (2015).

125 Benowitz, L. I., He, Z. & Goldberg, J. L. Reaching the brain: Advances in optic nerve regeneration. *Exp Neurol* **287**, 365-373, doi:10.1016/j.expneurol.2015.12.015 (2017).
126 Park, K. W., Lin, C. Y., Benveniste, E. N. & Lee, Y. S. Mitochondrial STAT3 is negatively regulated by SOCS3 and upregulated after spinal cord injury. *Exp Neurol* **284**, 98-105, doi:10.1016/j.expneurol.2016.08.002 (2016).
127 Park, K. W., Lin, C. Y., Li, K. & Lee, Y. S. Effects of Reducing Suppressors of Cytokine Signaling-3 (SOCS3) Expression on Dendritic Outgrowth and Demyelination after Spinal Cord Injury. *PLoS One* **10**, e0138301, doi:10.1371/journal.pone.0138301 (2015).
128 Jin, L. *et al.* Identifying gene expression profile of spinal cord injury in rat by bioinformatics strategy. *Mol Biol Rep* **41**, 3169-3177, doi:10.1007/s11033-014-3176-8 (2014).
129 Indzhykulian, A. A. *et al.* Molecular remodeling of tip links underlies mechanosensory regeneration in auditory hair cells. *PLoS Biol* **11**, e1001583, doi:10.1371/journal.pbio.1001583 (2013).
130 Oyama, F. *et al.* Sodium channel beta4 subunit: down-regulation and possible involvement in neuritic degeneration in Huntington's disease transgenic mice. *J Neurochem* **98**, 518-529, doi:10.1111/j.1471-4159.2006.03893.x (2006).
131 Bon, E. *et al.* SCN4B acts as a metastasis-suppressor gene preventing hyperactivation of cell migration in breast cancer. *Nat Commun* **7**, 13648, doi:10.1038/ncomms13648 (2016).
132 Minaire, P., Betuel, H., Girard, R. & Pilonchery, G. Neurologic injuries, paraosteoarthropathies, and human leukocyte antigens. *Arch Phys Med Rehabil* **61**, 214-215 (1980).
133 Nakahara, S. *et al.* Changes in nitric oxide and expression of nitric oxide synthase in spinal cord after acute traumatic injury in rats. *J Neurotrauma* **19**, 1467-1474, doi:10.1089/089771502320914697 (2002).
134 Lee, J. C. & Choe, S. Y. Age-related changes in the distribution of transient receptor potential vanilloid 4 channel (TRPV4) in the central nervous system of rats. *J Mol Histol* **45**, 497-505, doi:10.1007/s10735-014-9578-z (2014).
135 Lu, K. T., Huang, T. C., Tsai, Y. H. & Yang, Y. L. TRPV4 channels mediate Na-K-Cl-co-transporter-induced brain edema after traumatic brain injury. *J Neurochem*, doi:10.1111/jnc.13920 (2016).
136 Hamilton, P. W., Sun, Y. & Henry, J. J. Lens regeneration from the cornea requires suppression of Wnt/β-catenin signaling. *Exp Eye Res* **145**, 206-215, doi:10.1016/j.exer.2016.01.003 (2016).
137 Cathomas, F. *et al.* Behavioural endophenotypes in mice lacking the auxiliary GABAB receptor subunit KCTD16. *Behav Brain Res* **317**, 393-400, doi:10.1016/j.bbr.2016.10.006 (2017).
138 Hirose, A. *et al.* Analysis of CYP2R1 and CYP26A1 Expression Patterns in Regeneration in Mice with Liver Injury. *Biol Pharm Bull* **39**, 1955-1960, doi:10.1248/bpb.b16-00402 (2016).
139 Duprey-Díaz, M. V., Blagburn, J. M. & Blanco, R. E. Optic nerve injury upregulates retinoic acid signaling in the adult frog visual system. *J Chem Neuroanat* **77**, 80-92, doi:10.1016/j.jchemneu.2016.05.008 (2016).
140 Zhang, H., Adwanikar, H., Werb, Z. & Noble-Haeusslein, L. J. Matrix metalloproteinases and neurotrauma: evolving roles in injury and reparative processes. *Neuroscientist* **16**, 156-170, doi:10.1177/1073858409355830 (2010).
141 Vadivelu, S. *et al.* NG2+ progenitors derived from embryonic stem cells penetrate glial scar and promote axonal outgrowth into white matter after spinal cord injury. *Stem Cells Transl Med* **4**, 401-411, doi:10.5966/sctm.2014-0107 (2015).
142 Carow, B. & Rottenberg, M. E. SOCS3, a Major Regulator of Infection and Inflammation. *Front Immunol* **5**, 58, doi:10.3389/fimmu.2014.00058 (2014).
143 Sivakumar, S. & Gorbsky, G. J. Spatiotemporal regulation of the anaphase-promoting complex in mitosis. *Nat Rev Mol Cell Biol* **16**, 82-94, doi:10.1038/nrm3934 (2015).

Chapter 7 – Annexes

7. 1 – Regenerative capabilities of animal models of regeneration

Table 1. Model systems in regeneration research, and their genetic and genomic tools. (Adapted from: [74]).

Table 1 - Model systems in regeneration research, and their genetic and genomic tools.					
Species or group	**Regenerative Capabilities**	**Microarray**	**Transgenesis**	**Knockout / Knock down**	**Sequenced Genome**
Invertebrates					
Hydra	All tissues and organs	No	Yes	RNAi	No
Planarians	All tissues (neurons, muscles, epithelia) and organs (brain, sensory organs, digestive system, musculature)	Yes	No	RNAi	Yes
Ascidians	All tissues and organs	Yes	Yes	Morpholinos	Yes
Vertebrates					
Newts	Limbs, tail, heart, lens, spinal cord, brain, jaw, retina, hair cells of the inner ear	Yes	Yes	Morpholinos	No
Axolotls	Limbs, tails, heart, spinal cord, brain	Yes	Yes	Morpholinos	No
Frogs	Pre-metamorphic limbs, tail, retina, lens, hair cells of the inner ear	Yes	Yes	Morpholinos	Yes
Zebrafish	Fins, tail, heart, liver, spinal cord, hair cells of inner	Yes	Yes	Mutagenesis, Morpholi	Yes

	ear, lateral line			nos	
Chicks	Hair cell of the inner ear	Yes	Yes	Morpholinos	Yes
Mice	Liver, digit tips	Yes	Yes	Mutagenesis, homologous recombination	Yes

7. 2 – Dataset 1: *Xenopus laevis*

This dataset was obtained from PubMed Central, with the PMCID: PMC4046850. From the supplementary material, the following files were used: Additional file 3: Transcripts that show a different response to spinal cord injury in R- and NR- stages;

In this study was performed full transection of the spinal cord at the midpoint between fore and hind limbs in both tadpoles (stage 50-54; R-stage) and froglets (stage 88; NR-stage). Spinal cord transection severed all innervation between the rostral and caudal regions, leaving an ablation gap between the rostral and caudal stumps. Spinal cords of transacted animals were dissected by isolating a fragment caudal to the lesion site. Equivalent samples were obtained from sham-operated animals, that served as controls, to which the injury was only made to the dorsal skin and muscle, leaving the spinal cord intact.

The gene expression was studied on three different time points, namely 1-day post-transection (DPT), 2 dpt and 6 dpt.

The obtained differentially expressed transcripts results were for R stage: at day 1, there were 868 up-regulated genes and 525 down-regulated genes; at day 2, there were 392 up-regulated genes and 308 down-regulated genes; at day 6 there were 166 up-regulated genes and 189 down-regulated genes. For NR-stage: at day 1, there were 332 up-regulated genes and 238 down-regulated genes; at day 2 there were 377 up-regulated genes and 158 down-regulated genes; at day 6, there were 2580 up-regulated genes and 961 down-regulated genes.

Out of a total of 7,431 transcripts detected in all samples as differentially expressed, a total of 1405 (18,9%) were differentially expressed in both stages, 2199 (29,6%) were regulated exclusively on R stage, and 3827 (51,5%) in the NR-stage.

7.3 – Dataset 2: *Xenopus tropicalis*

This dataset was obtained from PubMed Central, with the PMCID: PMC3247858. Supplementary data was retrieved from Array Expressed Database (Experiment E-MEXP-2420, http://www.ebi.ac.uk/arrayexpress). Additional files downloaded included: E-MEXP-2420.raw.1.zip and E-MEXP-2420.processed.1.zip.

In this study, 20 pre-metamorphic tadpoles (stages 49-51) were amputated at the tail level including the spinal cord, notochord, muscle, and dorsal aorta. In this study, the controls were non-cut tails.

In order to perform RT-qPCR, tail tissues were collected in biological triplicates at several time points: 0h, 6h, 12h, 24h, 36h, 48h and 72h post-amputation.

There was a total of 58,861 targets in the array, which form that pool, only 16,059 had unique RefSeq protein IDs. From the 16,059 genes, approximately 45% of the gene targets showed at least one expression level change of greater or less than 2 fold between successive time points.

7.4 – Dataset 3: *Danio rerio*

This dataset was obtained from Gene Expression Omnibus with the accession number: GSE39295, available from:

http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE39295.

Additional files used for data analysis were retrieved from PMC (PMCID: PMC3896338), and include: Table S6: List of differentially expressed genes related to M1 and M2 type macrophages after SCI in zebrafish; Table S7: List of differentially expressed genes related to cell death and anti-apoptosis after SCI in zebrafish; Table S8: List of differentially expressed genes related to cell migration after SCI in zebrafish; Table S9: List of differentially expressed genes related to cellular dedifferentiation process after SCI in zebrafish; Table S10: List of differentially expressed genes related to cell cycle and cell proliferation regulation after SCI in zebrafish; Table S11: List of

differentially expressed genes related to neurogenesis and neuronal differentiation after SCI in zebrafish; Table S13: List of differentially expressed genes related to anterior-posterior and dorsoventral pattern formation after SCI in zebrafish; Table S14: List of differentially expressed genes related to axonogenesis and axonal guidance after SCI in zebrafish; Table S15: List of differentially expressed genes related to different signaling pathways after SCI in zebrafish; Table S16: List of differentially expressed genes commonly expressed in fin, retina, heart and spinal cord regeneration in zebrafish; Table S17: List of differentially expressed genes related to N-glycan biosynthesis, one carbon folate metabolism and ion channel transport after SCI in zebrafish; Table S18: List of differentially expressed unannotated genes after SCI in zebrafish.

A longitudinal cut at the side of the fish was made to expose the vertebral column and the injury to the spinal cord (crushing) was made at the level of the 15/16th vertebrae. Sham-operated animals served as gene expression normalizers and the tissues affected were only skin and muscle. The gene expression was observed at various time points, namely day 1, 3, 7, 10 and 15 post-injury. Approximately 1 mm length of spinal cord both rostrally and caudally from injury epicenter was dissected out from 50-60 fishes in each batch and pooled for RNA extraction.

In this study, a total of 3,842 differentially expressed genes during spinal cord regeneration in zebrafish was identified.