# Description Of The Regeneration Process

## Anatomical modifications and cellular responses

Regeneration is initiated by wound healing, which is a process mediated by local muscle contraction that allows contact between the remaining dorsal and ventral epidermis layers, which happens right after a few minutes post-injury to prevent further damage and invasion of pathogens. After this, the muscle relaxes, leading to the stretch of the epidermis, creating a thin membrane that closes the wound, called wound epidermis (Chandebois, 1980). In the experiences regarding regeneration in Leptoplana sp. it was possible to observe that, 4 days post-injury, there are two epidermis ends close to each other, even though they are not in close contact. This small detachment of the two epidermis layers may be explained by the fact that during the fixation process of the samples there is a sudden muscle relaxation that can draw apart these two ends.

Besides this response to wound healing, as previously mentioned, neoblasts and their proliferative capacity are also essential for the regenerative process (Orii et al. 2005). It has been described in most planarian species, for instance, in Schmidtea mediterranea and in Girardia tigrina (Baguñà, 1976; Saló & Baguñà, 1984) that neoblasts exhibit a coordinated biphasic pattern of mitotic activity during regeneration, where in the first 6 hours post-injury there is a “body-wide peak”, followed by a second peak restricted to the wound site between the second and fourth day after the injury in cases when there is tissue loss. This second peak leads to the production of non-dividing neoblasts in the pre-existing tissue in a specific area close to the wound site known as postblastema. These neoblasts will then migrate and accumulate in the new undifferentiated tissue (blastema) which will allow its growth (Saló & Baguñà, 1984). This second peak also seems to occur in Leptoplana sp., since 4 days post-injury it is possible to notice the presence of neoblasts and the formation of the blastema in the region near the wound site.

The neoblasts accumulated after the blastema formation will then differentiate into the cell types needed to re-establish the lost body parts. This cell differentiation step happens due to the expression of cell-differentiation genes, which are activated 72 hours post-injury (3 days) and continue to be expressed over time (Cebrià et al. 2018). It is possible to notice that, 4 days post-injury, at the wound site, within the regeneration blastema, there are neoblasts with different sizes and in different differentiation stages that are beginning to organize themselves to restore the lost parts of the tissue and produce properly patterned structures. Besides this, the neoblasts seem to not have such a noticeable cellular organization, which may be explained by the fact that they are located in a more anterior region, furthest from the wound site which seems to be in a more advanced wound healing stage, making these neoblasts more advanced in the differentiation process.

This differentiation phase of neoblasts into other cell types is a continuous process over time and it is possible to notice that after one month of regeneration there is a great mass of small cells, namely fibroblasts dispersed in the mesenchymal tissue contrary to what was observed after the fourth day of regeneration in which the cells were much bigger and undifferentiated and their number was much lower. However, although the internal anatomy of these planarians

appears to be practically restored after one month into the regeneration process (except for some artifacts in the epidermis, acquired during the process of sample preparation), the planarians had not yet acquired their original body size and did not appear to be regenerating. Considering that the normal regeneration time of the lost structures in S. mediterranea is about 1-2 weeks (Oviedo et al. 2008), a possible explanation for the fact that this did not happened in this specimen of Leptoplana sp. after one month may be due to stressful conditions, such as the quantity and quality of water since planarians were kept in Petri dishes with an amount of water that might not be enough, low concentration of essential nutrients, or even high salinity due to water evaporation that may have provided some bacteria growth and infections.

Actually, except for the samples fixed for histologic observations, the remaining planarians from the regeneration experiments had trouble keeping their integrity, which is reflected in the survival time graph in which the majority of the individuals and their respective fragments had a very short survival time. Despite this, the results seem to indicate that cutting the animal in 2 fragments is more efficient, which may be related to the fact that there are less structures to regenerate than when the animals are cut in 3. In addition, another interesting result indicates that in some cases, the posterior fragment has a longer survival time than the anterior fragment, which had already been described in the literature for other planarian species (Baguñà and Romero 1981; Oviedo et al. 2003; Calado et al. 2017, pp. 573-574; Pandian, 2020, pp. 69). In an attempt to try to understand this result, it can be thought that the vital organs such as brain, eyes and sensory organs are all situated in the anterior fragment of the body and these organs have a higher energy cost (i.e., they need more energy to maintain their normal function). Therefore, in stressful conditions and lack of food, it becomes difficult to maintain these organs and a process known in the literature as “degrowth” occurs, leading to a decrease in the body size of the animal (Baguñà and Romero 1981; Oviedo et al. 2003), which happened to some individuals in these experiments, that ended up completely disintegrated. On the other hand, in the posterior fragment of the body, these organs are not present, since the animal is still regenerating, so these fragments remain longer, hence they have a longer survival time.

It is also important to take into account that the regenerative capacity is different among the different planarian species (Owlarn & Bartscherer, 2016), which may influence and explain the results obtained. In fact, information from Pandian (2020) shows that the order Tricladida, in which the species S. mediterranea is included, has greater regenerative potency than the order Polycladida to which the species under study (Leptoplana sp.) belongs (Pandian, 2020, pp. 70-71). In addition, it is also mentioned that some species of the order Polycladida, such as L. tremellaris, are capable of regenerating the posterior fragment but not the anterior fragment, and from the 2 000 species included in this order, 13 (0.65%) have regenerative capacity, and, as for the order Tricladida, from the 1 000 species that constitute it, 23 (2.3%) have regenerative capacity (Pandian, 2020, pp. 235). However, despite all these observations, in the regeneration experiments with Leptoplana sp. neoblasts have proven their extraordinary ability to replace lost and/or damaged cells which indeed demonstrates that they may have interesting therapeutic applications in terms of regenerative medicine and recovery of damaged cells, tissues, and organs.

## Molecular changes underlying regeneration

All these anatomical and cellular modifications, essential for the regeneration process, occur in response to the expression of a series of genes. It is known that there are constitutive genes

whose expression is practically constant regardless whether the organism's cells are in normal or pathophysiological conditions, however, on the other hand, there are genes whose expression changes depending on the situation, which are denominated differentially expressed genes (DEG). Thus, focusing on these DEG and comparing the results from the Venn diagrams from the first and second sets of comparisons, it is possible to verify that in the second set of comparisons the total number of DEG is much higher than in the first case which makes sense since although the comparison was made between the corresponding regions, the animals were in different conditions, since one of them is intact and the other is in regeneration process. Therefore, although the region under study also affects the gene expression, as suggested by the results of the first set of comparisons, the biggest influence is the condition to which planarians are subjected. The same trend seems to occur regarding the number of DEG involved in regeneration which is also supported by the results of the heatmaps, the expression patterns of most of the DEG involved in regeneration seems to be very similar between the two regions in study which indicates that it is not the body region that has the greatest impact in gene expression but the state in which the planarian is found. In addition, the fact that there are two different regions under study provide greater support to the results obtained, since the gene expression values were quite similar between the pole and the flanking piece, which constitutes an advantage. Therefore, the following analyzes will be based on the results of the DEG found in the comparisons iii and iv, whose expression levels from Table 3.3 indicate that 72-hours after transverse amputation, most of the genes found to be involved in the process of cell differentiation are up-regulated either in the flanking piece or in the pole piece of the regenerating planarians when compared to the intact planarians, which corroborates what was observed in the histological analysis of Leptoplana sp., indicating that on the fourth day of regeneration there was already some stem cells differentiation.

The results of gene expression from these comparisons also indicate that during the regeneration process there is an investment in DNA replication, cell proliferation and growth which is verified through the high expression of some genes involved in these processes, such as FOXK1, PCNA, CycD (CCND1) and MCM2-7 genes. MCM (Mini chromosome maintenance complex) is a component of the pre-replication complex, which in turn is a component of the licensing factor. This MCM complex is a hexamer of six polypeptides (MCM2-7) evolutionarily conserved in all eukaryotes, which constitute a family of DNA helicases that have a role in the initiation and elongation phases of eukaryotic DNA replication (Tye, 1999). MCM is also the target of various cell cycle checkpoints, for instance in the G1/S transition of the mitotic cell cycle (Bochman, 2009). Due to these functions in which MCM complex is involved, it is expected that all these proteins have higher expression levels in regenerating planarians when compared with intact planarians, which is in fact confirmed by the results of Table 3.3, except for MCM6 in the flanking piece, although its expression level is very close to 1.5 (value from which a gene is considered differentially expressed). All these functions in which MCM complex is involved, namely DNA replication and cell cycle regulation, respectively, in which the protein interactions are also represented.

The fact that these MCM proteins are involved in DNA replication, cell proliferation, and cell cycle regulation demonstrates, as it is known, that these processes are closely related since it is essential to have a great control of the cell cycle to prevent an abnormal cell proliferation that may lead to the development of malignancies. Thus, there are some checkpoints that occur throughout the cell cycle, as, for instance, G1/S transition, G2 DNA damage checkpoint, or even cell cycle arrest, which are ensured by the expression of genes such as PCNA, CycD (CCND1), MCM2-7, MCM8, PRIM1, RAD51, and BLM.