



## Histology Analysis of Tissue Regeneration Process of Digit Tip Mice (*Mus musculus*) post amputation

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### ABSTRACT

*The ability to regenerate tissue is different for each organism. Mice (*Mus musculus*) able to regenerate the 3rd phalange of a digit. The tissue regeneration process has four phases are the wound-healing phase, the blastema phase, the regeneration phase, and the maturation phase. Each phase has a different process and different activity of cells. Histological analysis is very important to see the activity of each cell in each phase of tissue regeneration. Through histological analysis we can find out the role of each cell in the tissue regeneration process as well as the processes that occur in tissue regeneration. In this study, we analyzed tissue histology in the digit tip mice at each regeneration phase post amputated. The phalanges were amputated on the 3<sup>rd</sup> phalanges of digit tip of 24 male mice which had been previously sedated using ketamine / xylazine. Digit tip were allowed to grow and regenerate, and samples were taken on days 0, 1, 3, 5, 10, 15, 25 after amputation. Histological analysis was performed using Hematoxylin-eosin staining on a sample preparation that had been made into paraffin blocks first. The histological showed that at the beginning of the wound, the tissue rapidly forms a thin epidermal layer to cover the wound. In the wound healing phase, some of embryonal cells proliferated and migrated actively. In the blastema phase, granule cells cluster to form various new tissues. In the regeneration phase, new tissue begins to form, such as blood vessel, muscle, bone, and epidermal tissue. In the regeneration phase on day 15, several new tissues have begun to form, such as blood vessel tissue, muscle, hemorrhoid, bone and epidermis. Finally, in the maturation phase on day 25, the tissue morphology process occurs and perfecting the digit tip mice tissue.*

**Keywords:** *histology, phalanges of digit, mice, tissue regeneration, wound healing*

### INTRODUCTION

The digit tip mice on the P3 (phalange 3) have a high regeneration ability. In the P3 there are epidermal tissue, dermis, nerves, connective tissue, blood vessels, and bones that will regenerate and differentiate to form new tissues after amputation [1]. The beginning of the

wound healing phase is the tissue will become inflamed which is marked by the number of white blood cells in the tissue [2]. Lymphocyte cells have the role of killing germs, stimulating the immune response and stimulating fibroblast cells in the tissue regeneration process. The fibroblast cells have an important role in tissue

regeneration process that acts to form the extracellular matrix [3,4].

The digit tip mice contain several cells and extracellular components which include the P3 of bone tissue, cartilage bones, muscles, blood vessels, and nerves which are surrounded by connective tissue, epidermis and nail tissue. The main difference between the structure of the digit tip mice with the extremities of mammals is that the tip can regenerate after amputation [5]. Similar to the regeneration process of the salamander finger, regeneration of the mouse finger includes several phases including the inflammatory phase, histolysis of the bone, the formation of wound epidermis, blastema phase, and redifferentiation [6].

Regeneration of mice digit tip is an amputation level-specific healing event that diverges from wound healing response. The amputation at the second phalanx (P2) of the digit tip of mice results in a healing response. The healing response includes an in the inflammatory cascade, histolysis, and formation of a wound epidermis. But in P3 amputation, the wound healing process was formed from a blastema and developed a fibrotic scar instead. The fibroblast cells that are from the regeneration-incompetent region are capable of participating in blastema formation. The difference between regenerative and non-regenerative responses involves the microenvironment of tissue regeneration associated with blastema formation [7,8].

The process of closure epidermis in a P2 amputation of mice digits tip is deposit collagen fibrils from fibroblast cells at the distal of the bone stump. The collagen fibrils capped the bone in the neonate phase and adult phase of P2 amputation. Stem-like progenitor cells activated and formed a regeneration blastema over the following to 2 weeks. The source of blastema cells and the relative contribution of skeletal muscle have been studied, but the mechanisms of progenitor cells recruited from the stump have remained unclear [8, 9, 10].

In this study, we analyze the histological of tissue regeneration process of digit tip mice to analysis of embryonal cells are thought to have a

role in the tissue regeneration process. The results of this research can be continued to stimulating tissue regeneration in adult mammalian tissue by utilizing embryonal cells that play a role in the tissue regeneration process.

## METHODS

### Ethical clearance

Ethics permission for this study obtained from the Research Ethics Commission of Universitas Esa Unggul (number 0118-19.109/DPKE-KEP/FINAL-EA/UEU/V/2019).

### Animal model

This study used 24 male mice (*Mus musculus*), the weight were  $20 \pm 1$  grams, and the old were 8 weeks. We got the animal from Litbangkes-UI laboratory. Animal adapted during 1 month in Laboratory and treatment of animals are carried out in the laboratory of litbangkes-UI experimental animals. The mice cages were exposed for 12 hours with UV light and the cages were covered with fine sawdust, which was changed every day. Mice get food and drink in accordance with the nutrition standards from litbangkes-UI. As the Frederrer formula, we used 8 group of treatments (include the control) and each group consisted of 3 mice. We observed the growth of digit tip mice on day 0, 1, 3, 5, 10, 15, and 25 post-amputation. Mice were anesthetized using combination of ketamine-xylazine (each doze are 0.1.ml. kg BW) before amputated the digit tip. We euthanized the mice after the treatment.

### Histology samples of H&E staining

The regenerated tissue samples from post-amputation of digit tip mice put in 10% formalin. Then dehydration is done in a multilevel alcohol solution and for clarification done on xylol solution. tissue was clarified by xylol and immersion in liquid paraffin.

After the paraffin block was formed, the tissue was cut with a microtome 4-5  $\mu\text{m}$  thick. The cut slices were placed on a slide and ready to be stained with Hematoxylin Eosin staining as follows: deparaffinized and immersed in a 70%,

90% and 100% alcohol solution. The preparation is briefly dried and added with one drop of adhesive solution (Canada balsam) and then covered with a cover glass.

**RESULTS**

Morphological observations of the growth mice digit tip regeneration on 3rd phalanges from days 0, 1, 3, 5, 10, 15, and 25, appear to begin to arise tissue day 5, and increasingly clear from day 10 (Figure 1).

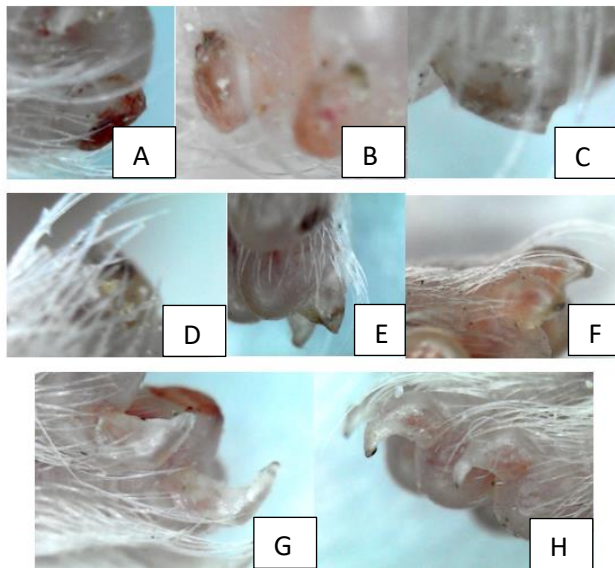


Figure1. The growth of 3rd phalanges digit tip mice; A. Day 0 post amputation ; B. Day 1; C.

Day 3; D. Day 5; E. Day 10, F. Day 15; G. Day 25 DPA; H. Control

The results of statistical analysis (table 1) showed the difference of digit tip length significantly ( $p < 0.05$ ) between day 0-day 1, between day 10-day15, and between day 15- day 25.

Table 1. The differences by Anova test in the growth of digit tip mice on each growth day (day 0-25 post amputation)

(I) length	Std. Error	Sig.
day_0 day_1	6,2197653	,012
day_1 day_3	6,2197653	,282
day_3 day_5	6,2197653	,644
day_5 day_10	6,2197653	,083
day_10 day_15	6,2197653	,000
day_15 day_25	4,8974693	,000

In graphic of Figure 2, there is the significantly different growth tissue of digit tip mice between day 0 and 1, between day 10 and 15, and between day 15 and 25.

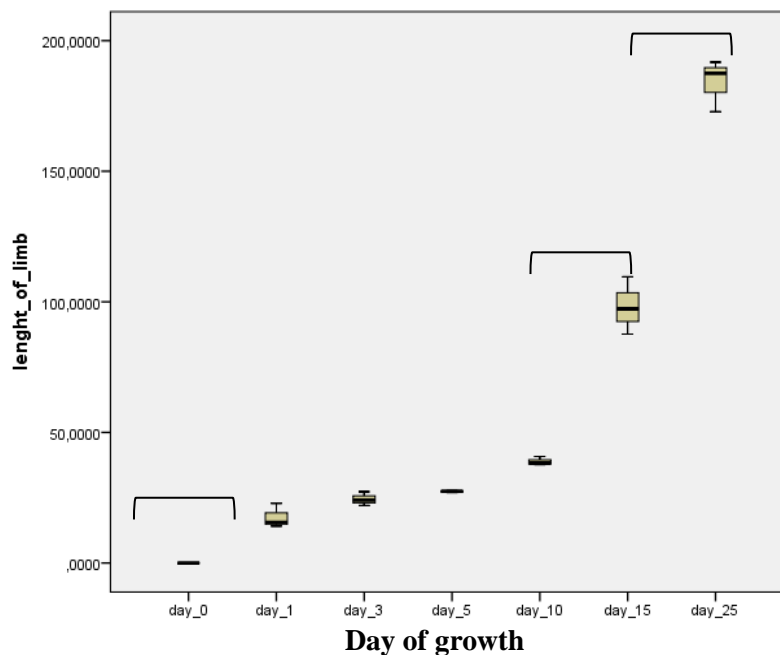


Figure 2. Graphic of growth digit tip mice from day 0-25 post-amputation, there was a significant difference between day 0 and 1; between day 10 and day 15; between day 15 and day 25

Histological observations revealed changes in the embryonal area of cells such as fibroblasts and osteoblasts. In tissue control, these embryonal cells appear in the basal area of the nail tissue of mice (Figure 3). At the wound

phase, this area expands and becomes narrower on day 10, indicating a relatively fast cell division to replace damaged cells with new tissue.

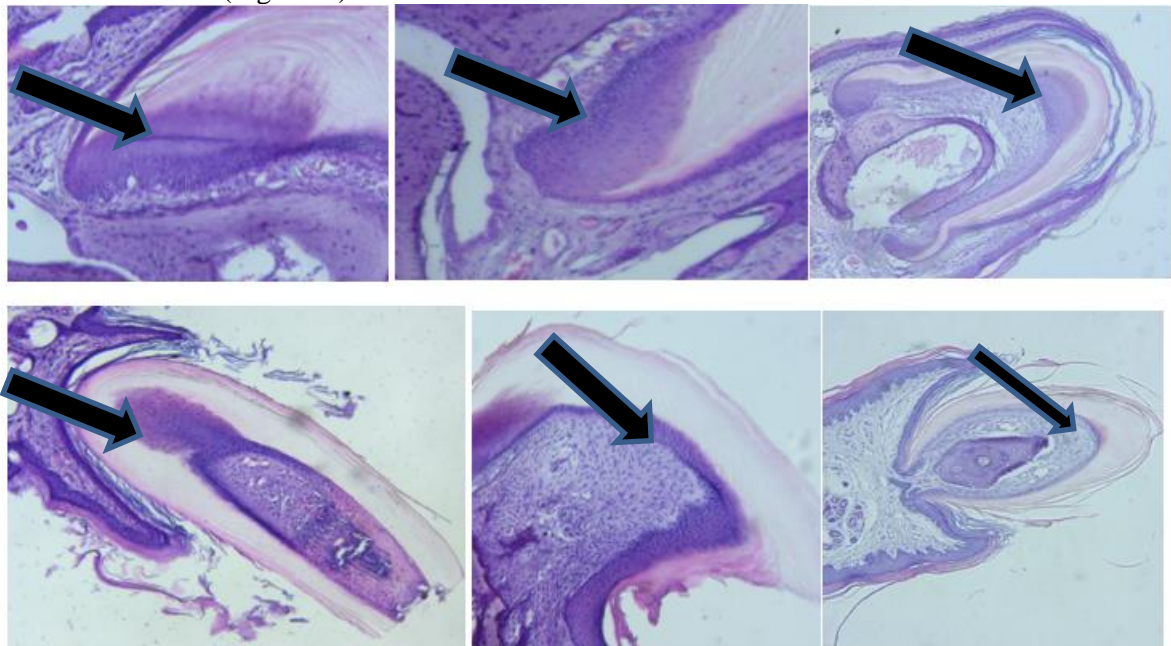


Figure 3. Tissue histology of growth of the digit tip mice after amputation, black arrows show changes in embryonal tissue from day 0 to day 10 and compared with controls; A. Day 0; B. Day 1; C. day 3; D. day 5; E. day 10; F. Control. (Magnified 4 x10)

The embryonal area on day 0 consists of cells such as fibroblasts, monocyte, osteoblast cells, and mesenchymal cells that have a role in the process of cell division and formation of new tissue that will replace damaged tissue (Figure 4).

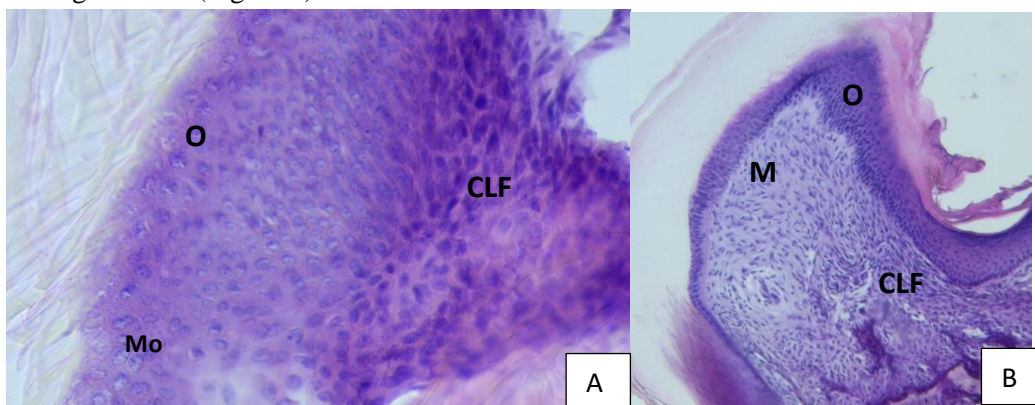


Figure 4. Embryonal tissue that contains fibroblasts like cells (FLC), Monocyte (Mo) and osteoblasts (O) and mesenchymal (M) cells A. Embryonal tissue under the nails of mice (magnified 40 x 10); B. whole tissue under the nails of mice (magnified 4 x 10)

**DISCUSSION**

The growth of digit tip mice tissue showed that was not significantly different in the wound healing phase (days 1-10). However, the growth length of digit tip mice on day 0 and day 1 showed a significant difference. In the first day

of tissue regeneration is an inflammation phase. In the inflammatory phase, there is a high cell division relatively and resulting in a significant difference in the length of digit tip mice. On day 0, inflammation triggers the emergence of

monocyte cells. In the inflammatory phase, monocyte cells dominate the wound area that has a role in killing germs, stimulating the immune response, and stimulating the formation of fibroblast cells [11, 12]. Fibroblast cells were found in the connective tissue of the injured area. The wound area of digit tip was covered by a layer of keratin on day 3, thus allowing to protect the tissue.

Histological analysis of the growth tissue of digit tip mice from day 0 to day 25 showed a different activity in every growth phase. Osteoblast cells located in the basal part of mice nails differentiated to osteocyte cells and formed the new nail tissue. While on day 1 to day 10, there was no significant difference in growth because on that day was occurred the wound healing phase. In the wound-healing phase, the new epidermis layer was formed and closed the wound area in the digit tip mice post-amputation. The closure of the wound area allows embryonal cells to differentiate and form new tissue. Embryonal cells included mesenchymal cells, fibroblast cells, and osteoblast cells, that have a role in tissue regeneration [4, 13]. Osteoblast cells are the embryonal cells that it differentiates to osteocyte cells so that the nail tissue was formed in the digit tip tissue of mice. The high osteoblast cells in the digit tip tissue of mice will facilitate the formation of new nail tissue [8, 13, 14].

Mesenchymal cells will divide and differentiate into muscle, bone, nerve, and connective tissue. Mesenchymal cells are abundant in the embryonal area that has a role in tissue regeneration. In the tissue regeneration process of digit tip mice on day 10 DPA, the dermis and connective tissue begin to appear. Mesenchymal cells were found in various organs that can regenerate the damaged or injured tissue. In lizard tails, mesenchymal cells were found in adipose tissue at the basal tail. Lizards are able to regenerate their tissue tail after performing autotomy [6]. Likewise, zebrafish fins have mesenchymal cells in the basal area of fish fins, thus allowing zebrafish to regenerate tissue from injured fins [15].

The wound healing phase is the initial phase for determining the next tissue growth. In this phase the statistical test results show no significant difference in tissue growth. Shorter observation time spans in the wound healing phase (day 0 to day 10) of tissue growth of digit tip mice, allows for more intense observation. The wound healing phase is the initial phase determining the next tissue growth [9, 10]. In

this phase, the statistical test results show no significant difference in tissue growth. In this phase, the statistical test results show no significant difference in network growth. this showed that in this phase there is no tissue regeneration process, but there is an activated the embryonal cells to divide and differentiate the new cells [8, 9]. Blastema cells will differentiate to form new tissue. In the growth of digit tip mice, there is a significant difference growth after the blastema phase, this indicated that tissue formation will occur after the blastema phase. Statistical tests showed the significant growth differences after the blastema phase, starting from day 10 to day 25. In this phase, various epidermal tissues, blood vessels, dermis, muscles, and new bones were formed.

Every organism that has the ability to regenerate the tissue, in this tissue there are embryonal cells that will form new tissue. In the histology observations of digit tip mice obtained mesenchymal embryonal cells, osteoblasts and fibroblasts that will regenerate to form a variety of new tissue. On day 10, connective tissue, cartilage, and connective tissue were formed. The tissue was grown around the wound area and formed tissue on digit tip mice on day 15 and 25. In the regeneration phase on day 10 to day 15 of the tissue is the blastema formation phase, whereas the actual tissue growth occurs on the 15th day until day 25. The growth curve showed a very sharp increment line and the statistical test results show significantly different growth results.

Mice digit tissue growth which was quite significant after day 10, showed osteoblast activity to divide and differentiate. On the 25th day the embryonic tissue is getting narrower and thinner due to the differentiation of embryonal cells to form new tissue so that the number is getting smaller, it can be compared with controls that have a very narrow embryonal cell area but are ready to divide if injured so new tissue will form.

## CONCLUSION

The histology of tissue regeneration of digit tip mice shows the activity of embryonic cells in the wound healing phase as a determinant of the tissue regeneration process in the next phase and the success of the tissue regeneration process.

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## REFERENCES

- [1] Marrero L, Simkin J, Sammarco M & Muneoka K. Fibroblast reticular cells engineer a blastema extracellular network during digit tip regeneration in mice. *Wiley Regeneration*. 2017; 4: 69-84. DOI:10.1002/reg2.75.
- [2] Mescher AL. Macrophages and fibroblasts during inflammation and tissue repair in models of organ regeneration. *Wiley regeneration*. 2017; 4:9-53. DOI: 10.1002/reg2.77.
- [3] Calve S & Simon H. Biochemical and mechanical environment cooperatively regulate skeletal muscle regeneration. *The FASEB Journal*. 2012: 2538-2548
- [4] Fernando WA, Leininger E, Simkin J, Li N, Malcolm CA, Sathyamoorthi S, Han M, & Muneoka K. Wound Healing and blastema formation in regenerating digit tips of adult mice. *Developmental Biology*. 2011;350: 301-310
- [5] Pintoa R, Godwina JW, Alexander R & Rosenthala NA. Macrophages are required for adult salamander limb regeneration. 2013. *PNAS*; vol. 110 (no. 23): 9415–9420
- [6] Simkin J, Han M, Yu L, Yan M & Muneoka K. The Mouse Digit Tip: From Wound Healing to Regeneration. Article in *Methods in Molecular Biology* (Clifton, N.J.) Source: PubMed. 2013: 414-435. DOI: 10.1007/978-1-62703-505-7\_24
- [7] Wynn TA & Vannella KM. Macrophages in Tissue Repair, Regeneration, and Fibrosis. *Immunity*. 2016; 44: 450-462.
- [8] Panigrahya D, Kalisha BT, Huang S, Bielenberga DR, Lea HD, Yang J, Edini ML et al. Epoxyeicosanoids promote organ and tissue regeneration. *PNAS*. 2013; vol. 110 (33): 13528–13533.
- [9] The blastema and epimorphic regeneration in mammals Ashley W. Seiferta,\*, Ken Muneokab, *Developmental Biology* 433 (2018) 190–199
- [10] Guedelhoefer OC and Alvarado AS. Amputation induces stem cell mobilization to sites of injury during planarian regeneration. *Development*. 2012;139(19): 3510-3520. DOI: 10.1242/dev.082099.
- [11] Lozito TP and Tuan RS. Lizard tail skeletal regeneration combines aspects of fracture healing and blastema-based regeneration. *Development*. 2016; 143(16):2946–2957. DOI: 10.1242/dev.129585
- [12] Londono R, Wenzhong W, Wang B, Tuan RS. Cartilage and Muscle Cell Fate and Origins during Lizard Tail Regeneration. *Frontiers Bioengineering and Biotechnology*. 2017; 5(70):1–9. DOI: 10.1242/dev.129585.
- [13] Reinke JM and Sorg H. Wound repair and regeneration. *European Surgical Research*. 2012; 49(1):35-43. DOI: 10.3390/ijms15011647.
- [14] Shi X and Garry DJ. Muscle stem cells in development, regeneration, and disease. *Genes and Development*. 2006; 20(13): 1692-1708. DOI: 10.1101/gad.1419406
- [15] Guedelhoefer OC and Alvarado AS. Amputation induces stem cell mobilization to sites of injury during planarian regeneration. *Development*. 2012;139(19): 3510-3520. DOI: 10.1242/dev.082099.