pISSN 2302-1616, eISSN 2580-2909
Vol 10, No. 1, June 2022, pp. 66-76
Available online http://journal.uin-alauddin.ac.id/index.php/biogenesis
DOI https://doi.org/10.24252/bio.v10i1.27378

The dynamics of Wnt-5a and Shh expression in tissue regeneration process of Mice (*Mus musculus*) digit tip

Titta Novianti[1], Febriana Dwi Wahyuni[1], Syafruddin Ilyas[2*]
[1]Biotechnology Study Program, Faculty of Health Sciences, Universitas Esa Unggul
Jl. Raya Arjuna Utara No 9, West Jakarta, Indonesia, 11470
[2]Department of Biology, Faculty of Mathematic and Life Sciences, Universitas Sumatera Utara
Jl. Dr. T. Mansur No.9, Padang Bulan, Medan, North Sumatera, Indonesia, 20222
*Email: syafruddin@usu.ac.id

ABSTRACT. Some genes had a role in the tissue regeneration process. We suggested Wnt-5a and Sonic Hedgehog (Shh) had a role in the tissue regeneration process of digit tip mice after amputation. The genes play a role in the proliferation, migration, differentiation, and morphogenesis of cells in the regeneration of injured tissue. The purpose of this study was to analyze the expression of these genes in the process of tissue regeneration of mice digit tip. The study used 28 male mice, we amputated the phalanges 3rd of digit tip mice, and we analyzed be tissue morphology, gene, and protein expression on day 0, day 1, day 3, day 5, day 10, day 15, and day 25 after amputation. The results of digit tip mice amputated showed a tissue regeneration process at the 3rd phalanges. Organ growth became intact again on day 25. The result demonstrated that Wnt-5a and Shh expression were dynamic during regeneration of digit tip mice. The results of the statistical analysis test showed that the expression of the Wnt-5a and Shh genes by qPCR and ELISA test showed significantly different expressions on each treatment day with the one-way ANOVA test ($p < 0.05$). The results of gene and protein expression graphs show dynamic expressions in each phase of tissue regeneration. We suggested that they had a role in each phase of tissue regeneration. When the Wnt-5a gene expression is high, there is an active process of cell proliferation and migration, and when Shh gene expression increases, Wnt-5a gene expression decreases as if there is an alternation of both gene expressions. When the Shh gene expression increases, the cells carry out the process of tissue morphogenesis so that the tissue forms again.

Keywords: histological analysis; Shh gene; terminal phalanges amputation; tissue regeneration; 18s rRNA gene

Article History: Received 13 February 2022; Received in revised form 17 March 2022; Accepted 20 May 2022; Available online 30 June 2022. Ver: Pre-Press
How to Cite This Article: Novianti T, Wahyuni FD, Ilyas S. 2022. The dynamics of Wnt-5a and Shh expression in tissue regeneration process of Mice (*Mus musculus*) digit tip. *Biogenesis: Jurnal Ilmiah Biologi*. vol 10(1): 66–76. doi: https://doi.org/10.24252/bio.v10i1.27378.

INTRODUCTION

Research on tissue regeneration is still in development to find the right therapy for adult organisms that were injured or amputated organs (Guedelhoefer & Alvarado, 2012). Research on gene expression that plays a role in the tissue regeneration process is a way to develop therapy in amputated organs. The tissue regeneration process includes a 4-step process that requires the activity of different genes and proteins at each stage (Udalamaththa *et al*., 2016; Joven & Simon, 2018). The stages of this regeneration process determine an organism's ability to regenerate tissue to restore its injured or amputated tissue. The first stage in the tissue regeneration process is the inflammatory stage. At this inflammatory stage, genes, proteins, and white blood cells had a play a role in overcoming the inflammatory state (Petrova & Joyner, 2014). The next stage is granulation, cell migration, and proliferation, replacing damaged cells and forming new cells. Genes and cells that are expressed in the wound healing phase are thought to be an attempt by the tissue to heal wounds. The third stage is the regeneration phase in which cells proliferate to form new tissue. The last stage is the maturation stage undergoes a morphogenesis process to form adult tissue to replace amputated or injured tissue (Hutchins *et al*., 2014; Dawson *et al*., 2016).

The tissue regeneration process is very complicated because it involves various genes, proteins, cells, and molecules (Miller *et al*., 2019). Among them, some genes that have a role in the tissue regeneration process are the Wnt-5a and the Sonic Hedgehog (Shh) gene (Iriana et al., 2021). The genes have a role proliferation, migration, and differentiation of cells to form new tissues. Wnt-5a

protein is in embryonic development, cell proliferation, and cell differentiation process and even plays a role in cancer cells proliferation. The signaling of the Wnt-5a molecule is currently still studied intensively to describe the signaling mechanism in physiological and pathological therapeutic to achieve the development of appropriate therapeutic strategies (Rishikaysh *et al*., 2014; Lozito & Tuan, 2016).

Wnt-5a protein is involved in the formation of the lungs, heart, and mammary organs. Wnt-5a also plays a role in regulating stem cell renewal, osteogenesis, and tissue regeneration. Wnt encodes short-range secreted signaling molecules that regulate cell fate, adhesion, shape, proliferation, differentiation, motility, and development of various organ systems. Wnt-5a may play a role in modulating cell fate determination and stem cell differentiation (Gauron *et al.*, 2013; Mastelaro de Rezende *et al*., 2020).

In line with its role in stem cell morphogenesis and differentiation, Wnt-5a is involved in tissue repair and regeneration after injury. A study demonstrated strong induction of Wnt-5a-positive mesenchymal cells after intestinal injury localized to the wound bed (Stulberg et al., 2012). The presence of Wnt-5a provides demarcation of areas of regenerating proliferation through potentiation of TGF-b signaling allows fine-tuning of proper wound regeneration and healing. Increased amounts of Wnt-5a in lung tissue from a mouse model of acute respiratory distress syndrome may be a repair response of damaged lungs to cope with injury. High Wnt-5a expression can stimulate Hh expression (Kumawat & Gosens, 2016).

The Hedgehog (Hh) signaling pathway is complex. The Hh gene involves many regulatory proteins. Invertebrates, three Hh homologs have been identified: Sonic Hh (Shh), Indian Hh (Ihh), and desert Hh (Dhh) (Iwakura et al., 2013). All three Hh ligands activate the same signal transduction pathway but regulate different organ systems. The Shh gene expresses in the central nervous system, lungs, teeth, intestines, and hair follicles. In adult mouse satellite cells, HH signaling continues to function as a contributing factor in survival and proliferation. Interestingly, upregulation in Shh and Ptch1 transcription has also been detected in fully differentiated mature muscle upon regeneration induction after ischemic injury (Mizukoshi *et al*., 2016; Ding & Wang, 2017). The results of the research by Miller *et. Al.* (2019) showed the role of Shh in the regeneration of the digit tip mice until the process was complete.

The study's purpose was to obtain an accurate description of the expression of Wnt-5a and Shh genes at the phase so that the stimulation of the gene expression can be carried out in organs that have limited regeneration ability.

MATERIALS AND METHODS

Sample of research. We got a research ethics permit from the Esa Unggul University Research Ethics Council with the license number was 0118-19.109/DPKE-KEP/FINAL-EA/UEU/V/2019. The study used 28 *Mus musculus* var. Swiss Webster as research objects, mice are eight weeks old, and the weight is 20 grams. The number of mice was by the Federer formula. The mice were grouped into the control group (which was not amputated) and the amputated treatment group with 6 treatment days. Each group consisted of 4 mice. We obtained the mice from the Research and Development Department of the Indonesian Ministry of Health laboratory. The mice were maintained and treated by laboratory staff from the Department of Research and Development, Ministry of Health of the Republic of Indonesia. The mouse cage is a plastic tub measuring 15 x 30 cm and height is 10 cm, which is covered with perforated wire. The cage is lined with fine sawdust, which is easy to change every 3 days. Drinking water is provided using a glass drinking bottle that can be sucked in at any time. The mice feed was in the form of pellets containing 10% protein, 3% fat, 8% fiber and 12% water content. Feed is stored at a temperature of 15-16^{o} C and stored for a maximum of 4-6 weeks after the packaging is opened.

We anesthetized the mice using ketamine/xylazine (K, 100 mg/kg; X, 10 mg/kg) at a dose of 0.5 g/kg BW, and mice digit tip were amputated at 3rd phalanges using microdissection scissors (FST

15003-0). Amputation of the 3rd phalanges or terminal phalanges of the mouse digit tip can stimulate tissue regeneration ((Lehoczky *et al*., 2011; Miller *et. al.* 2019). We analyzed the tissue regeneration after growth on day 0 (4 hours after amputation), 1, 3, 5, 10, 15, and 25 days after amputation by qPCR analysis, histology, and Elisa assay. Treatment days are determined based on the beginning and end of each phase of the tissue regeneration process. Mice that had regenerated tissue after amputation anesthetized by ketamine/xylazine (K, 100 mg/kg; X, 10 mg/kg) at a dose of 0.5 g/kg BW. The regenerated tissue of mice digit tip was crosswise cutting in the 3rd phalanges. The tissue for the histological assay was put into a 10% formalin solution, the tissue for the qPCR test put into the RNA-ase solution, and the tissue for the ELISA test put in a cool box. All samples were obtained at the laboratory for analysis.

Histology. The study made the histological samples using hematoxylin eosin (HE) staining. Histological preparation was stained with Hematoxylin Eosin (HE) staining and histochemistry: formalin 10%; 70% alcohol; alcohol 80%; alcohol 95%; and 100% alcohol; xylol; paraffin block; hematoxylin-eosin.

qPCR mRNA analysis. The study used an extraction RNA kit from Sigma Aldrich (gene-lute extraction kit RNA) to extracted RNA from tissue regenerated samples. Study do amplification the Wnt-5a and Shh genes by the qPCR kit test (SensiFAST™ SYBR® No-ROX One-Step Kit) that we have designed the primary DNA of the two genes. We used Bio-Rad real-time machine qPCR to analyze the qPCR assays of samples. The analysis includes the steps of DNA synthesis, Reverse transcriptase inactivation, and DNA band propagation for 40 cycles by PCR machine with annealing temperature at 57^0C for Shh and Wnt-5a genes, and 55 ^{0}C for 18S rRNA gene, followed by the melting curve stage. We used the 18s rRNA gene as the reference gene. The negative control used nuclease-free water instead of RNA to exclude false-positive results. The qRT-PCR test results in efficiency values and Cycle Threshold (CT). The analysis of gene expression was assessed by relative quantification so that the relative levels of mRNA expression using the Livak method. We designed DNA primer by BLAST and multiple alignment method. We used Primer3 software to get the pick primer.

DNA Primer of Wnt-5a gene of *Mus musculus* var. Swiss Webster
Forward: AGTGTCATGGAGTGTCTGGC
Reverse: CGGACTGGGGTCGATGTAGA
DNA Primer of Shh gene of *Mus musculus* var. Swiss Webster
Forward: TTGGCCATCTCTGTGATGAA
Reverse: CCACGGAGTTCTCTGCTTTC

The qPCR procedure was as follows: DNA synthesis for 5 min, temperature 42^0C; Reverse transcriptase inactivation 2-5 minutes, temperature 95^0C; PCR cycles carried out 40 for 10 seconds at a temperature of 95^0C; 30 seconds at a temperature of 57^0C for the Wnt-5a and Shh genes, then the melting curve stage for one minute at a temperature of 95^0C, one minute at a temperature of 55^0C, 10 seconds at a temperature of 55^0C.

ELISA test. We used mouse monoclonal antibody Wnt-5a (Santa Cruz, CA; sc-365370), Mouse monoclonal antibody Shh (Santa Cruz, CA; sc- 365112), and the biotin unconjugated Mouse monoclonal anti-mouse Wnt-5a or anti-mouse Shh purchased from Santa Cruz Biotechnology. We obtained the Peroxidase conjugated affinity-purified from Santa Cruz, Streptavidin-peroxidase from Sigma, polyethylene glycol (PEG) polymers from Sigma, and Washing buffer was Hank's balanced salt solution (HBSS+) with Ca^{2+} and Mg^{2+} supplemented with 1% bovine serum albumin (BSA) from Sigma. The binding buffer adds 5% PEG and HBSS+ on the trip plate has high Binding from Greiner Bio-One. A direct binding ELISA was used to determine the binding of the Wnt-5a antibodies to the 96 wells plates. In brief, 50 µL solutions containing anti-human Wnt-5a or anti-mouse Wnt-5a were added to 96 wells plates and incubated overnight at 4°C. The next day, the 96 wells plates were washed twice and blocked with washing buffer. The wells were treated with a 50 µL solution containing enzyme-linked antibody (HRP conjugated F(ab')2 donkey anti-rabbit IgG or donkey anti-

goat IgG at 4 µg/mL) for 30 min at 40^0C and then washed multiple times with washing buffer. After washing, the wells treated by buffer was 200 µL, incubate the wells in the dark at four degrees overnight. We determined the absorbance (OD 450 nm) of the enzyme-substrate reaction solution at different time points (15, 30, 45, 60, and 120 min). We diluted the Wnt-5a and enzyme-linked secondary antibodies in the HBSS solution and washed them with a buffer. We performed every treatment in Duplo wells (Dorn *et al*., 2016).

Statistical analysis. We used the Kolmogorov-Smirnov test for statistical analysis to test for the normality of semi-quantitative data for the expression of Shh and Wnt-5a genes and proteins. We use the Kolmogorov-Smirnov test for Statistical analysis for data normality test. One-way ANOVA with Tukey-Kramer for multiple comparisons test was applied to evaluate the statistical significance among different treatments on day 0, day 1, 3, 5, 10, 15, and day 25 after amputation. We used A Student's t-test for evaluated the significance data between the two treatments. In both tests, the results are $p < 0,05$ was considered significant.

RESULTS AND DISCUSSION

The growth of digit tips mice was slow from day 0 to day 10 (**Fig.1.** A-E), after day 10 (**Fig.1**. F-G), the growth of digit tip mice shows relatively fast. On day 15, Nail growth appeared to be more intact than the previous day (**Fig.1.**F). On day 25, the digit tip mice looked normal again (**Fig.1.** G). Amputation of the 3rd phalanges or terminal phalanges of the mouse digit tip can stimulate tissue regeneration. The regeneration capacity of the 1st and 2nd phalanges of the digit tip mice is very limited (Lehoczky *et al*., 2011).

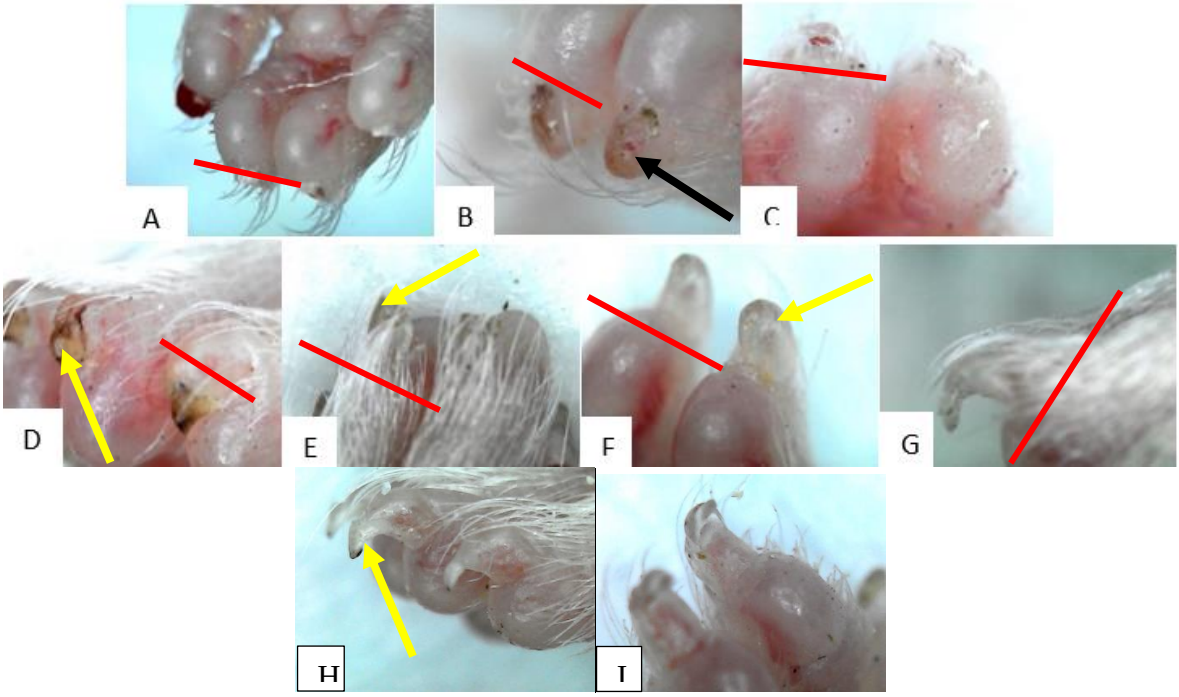

Fig. 1. Morphology of toe growth of mice after amputation (A) on day 0; (B) on the 1st day, the growth of the wound closure at the base of the 3rd phalanges appears; (C) on day 3, nail growth begins to appear; (D) the 5th day of nail growth which was not much different from the 3rd day; (E) 10th day of longer nail growth; (F) day 15 the growth of the nail fast relatively; and (G) day 25 of nail growth as before amputation of the 3rd phalanges. Red line showed the amputated area. Black arrow showed wound closure, and yellow arrow show the nail (H) and (I) control

Histological analysis. Histological showed the cell activity in the amputated tissue area of digit tip mice from days 1-25 (**Fig. 2**). On day 1, there was an area of the wound on the nail tissue and the phalange 3rd. On day 3, the epithelial covered the wound area on the surface of the nail tissue. The connective tissue appears as if pressing the growth of nail tissue so that the tissue in the 3rd phalanges

grows complexly on day 10. On days 15 and 25, the 3rd phalanges and nail tissue grew complete and intact, resembling normal tissue before amputation.

On day 1, this tissue due to the amputation wound bleeding amputation. The marker of bleeding tissue is the spread of red blood cells spread in the tissue. The tissue bleeding is the spread of red blood cells near the amputation area (**Fig. 3**.A). There has been inflammation at these phases as white blood cell identical to inflammation. Connective tissue continues to grow due to the proliferation and differentiation of cells in the tissue on day 3 (**Fig.3**. B-C).

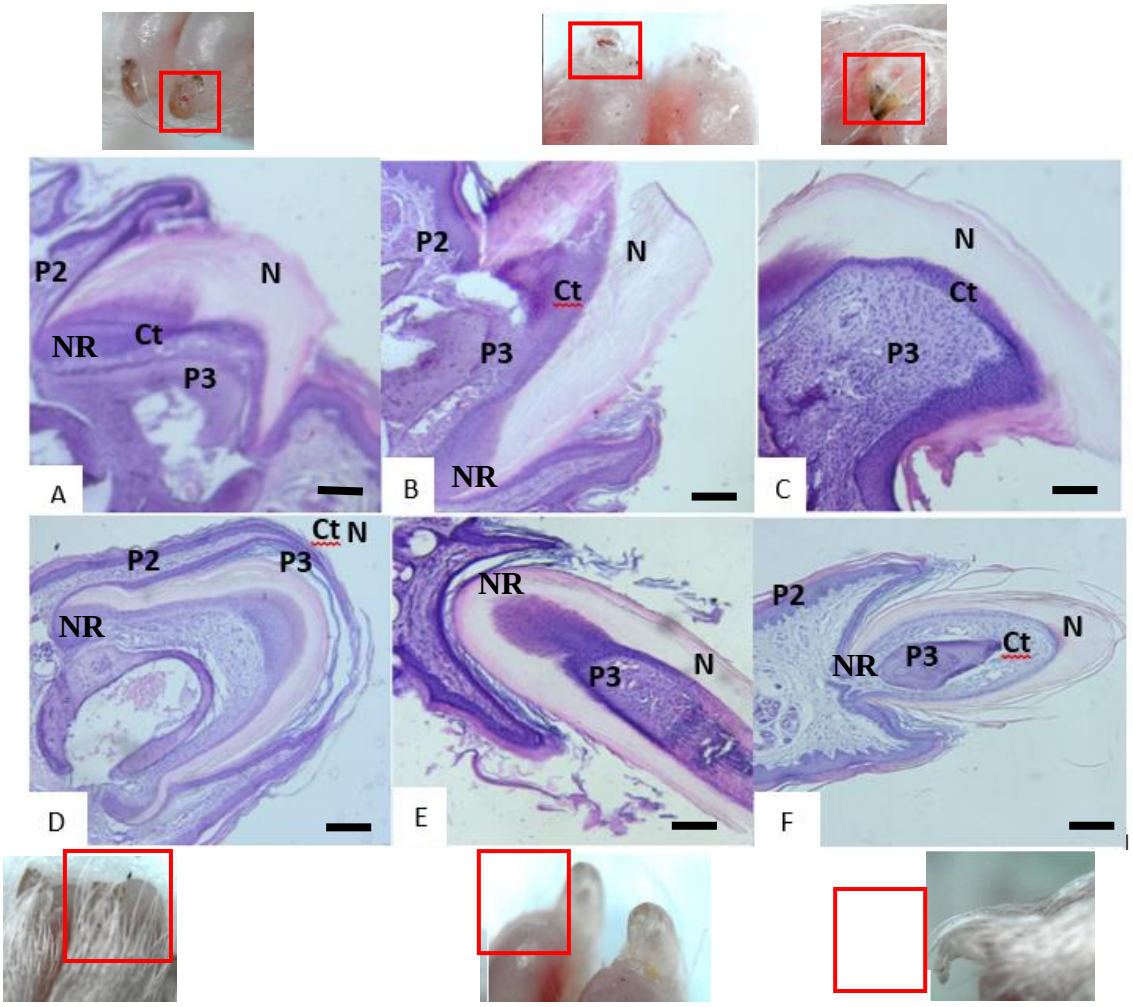

Fig. 2. Histological picture of digit tip mice from days 1-25 showed the tissue regeneration process in the amputated tissue area. (A) On day 1, the nail tissue (N) and the 3rd phalange tissue (P3) were showed the wound area. The 3rd phalange tissue (P3) is adjacent to the Connective tissue (Ct) and the nail root (NR). The 2nd phalanges tissue (P2) was intact; (B). on day 3, epithelial begins to cover the wound area on Nail tissue. The connective tissue (Ct) begins to grow; (C) on day 5, Ct and NR tissues begin to grow and push the nail; (D) on day 10, seen from the front, the histology of nail tissue is growing and complex, the area of Ct is expanding, P3 tissue is growing beside N and forming an epidermal layer; (E) day 15, seen from the front, the growth of N has expanded to beat the P3 network; (F) on day 25, the front view of the amputated toe tissue returned to normal tissue, with a proximal N arrangement, supported by Ct and P3. HE staining. Magnified 100 X (▬ bar 1 mm).

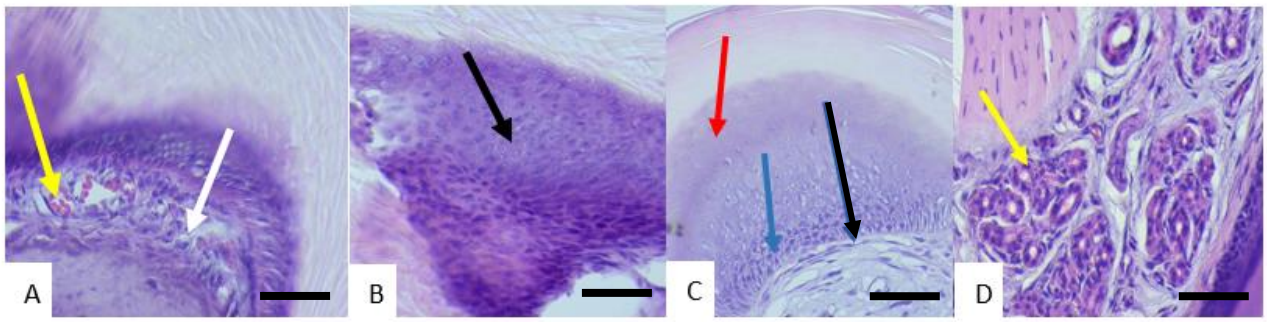

Fig. 3. Cell activity in the nail root tissue and connective tissue day 1 to day 5; (A) Red blood cells leak out of vessels (yellow arrows) in adipose-rich (white arrows) connective tissue on the first day after amputation and the ; (B) the growth of digit tip mice on the third day after amputation and shows progenitor cell proliferation (black arrow); (C) The connective tissue shows the presence of fibroblast-like cells (blue arrows), chondroblast cells (red arrows); and some of cells (black arrows) in basal lamina which will form new nail tissue; (D) New blood vessel (yellow arrow) on day 5, after amputation. HE staining. (Magnification 400 X)(bar ▬ 1 um).

The initial phase of tissue regeneration begins with the inflammatory as the initial phase of tissue in overcoming wounds. In the wound area in the digit tip mice, inflammation occurs is characterized by the presence of white blood cells in the wound area (**Fig. 3**). The inflammatory phase is accompanied and continued by the stages of cell proliferation and differentiation. In Fig. 3, the connective tissue shows the activity of cells that actively proliferate and differentiate into chondrocyte cells to form nail tissue.

The growth of mice digit tip from day 0 (4 hours after amputation) showed slow growth until day 5 (**Fig 4**). In this phase, cells actively proliferate and differentiate (**Fig. 3**), even no new tissue has yet formed, so the digit tip mice growth in this phase is still slow. Recent studies on mice digit tip regeneration using genetic tracing and single-cell transcriptomic profiling have shown that mice digit tip blastema is heterogeneous and consists of many different cell types. These cell types include endothelial and lymphatic endothelial cells, vascular smooth muscle cells, pericytes, Schwann cells, macrophages, neutrophils, T cells, monocytes, pre-osteoclasts and various types of mesenchymal cells including osteoblasts (Storer & Miller, 2020). The elegant study conducted by Takeo and colleagues demonstrated that early signaling of the Wnt gene in the nail epidermis of mice is required for nail stem cell differentiation under homeostatic conditions and is equally important for mesenchymal responses during regeneration (Takeo *et al*., 2013).

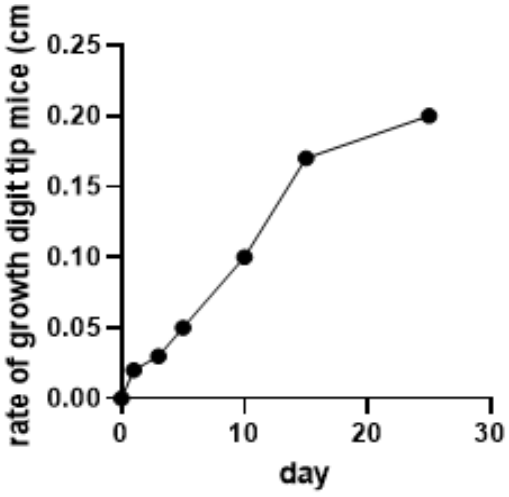

Fig. 4. The graph of mice digit tip growth (cm) from day 0 until day 25 after amputation.

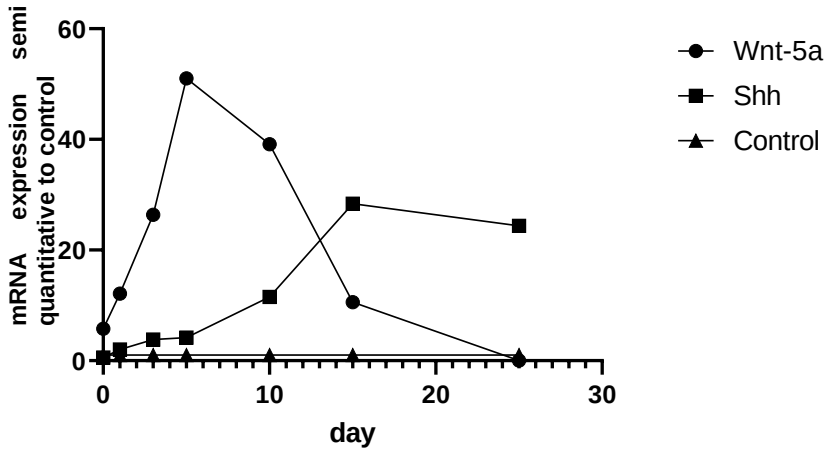

Fig. 5. Gene expression from the qPCR assays showed Shh gene expression at the beginning of autotomy showed a low and increased expression on day 15 and slightly decreased on day 25 while Wnt-5a gene expression began to increase on the first day and reached its peak on the tenth day and decreased until day 2.

qPCR analysis. The results of the qPCR analysis of the Shh and Wnt-5a genes showed the different expression dynamics from each treatment day (**Fig. 5**). The expression of the Wnt-5a gene reached its peak on day 5. After day 5, Wnt-5a expression began to decrease. At the same time on the day, 5 Shh gene expression started to increase and reached its peak on day 15. At the same time, the Wnt-5a gene decreased drastically. On day 25, their expression decreased relatively, Shh gene expression was still relatively high, while Wnt-5a gene expression decreased significantly and was lower than control. The expression of Wnt-5a and Shh genes is different for every growth day, using the one-way ANOVA test ($p < 0,05$) (**Table 1**).

Table 1. One-way ANOVA Test Wnt-5a and Shh genes expression by q-PCR test

		Sum of Squares	df	Mean squares	f	Sig
Wnt-5a	Between groups	4103.415	6	683.903	39.423	.000
	Within groups	121.436	7	17.384		
	Total	4224.651	13			
Shh	Between groups	1553.028	6	258.838	42.472	.000
	Within groups	42.660	7	6.094		
	Total	1595.688	13			

ELISA Test. Expression of Wnt-5a and Shh protein by ELISA test showed the differences in the expression on each day of tissue growth after amputation. Wnt-5a protein was not expressed yet on day 0, but on day 1, Wnt-5a expression was higher than Shh and beta-actin expression. Wnt-5a protein expression started to increase from day 1 until day 3 and reached its peak on day 5. After day 5, protein expression decreased until day 25 (**Fig. 6**).

Shh protein expression increased somewhat slowly compared to Wnt-5a protein. The Shh protein expression on day 1 increased slightly, the expression of Shh protein slowly increased until it reached its peak on day 15, expression decreased slightly on day 25. Using analytical statistics (one-way ANOVA), the expression of Wnt-5a and Shh protein is different for every growth day ($p < 0,05$) (**Table 2**).

Table 2. One-way ANOVA Test Wnt-5a and Shh protein expression by ELISA

		Sum of Squares	df	Mean squares	f	Sig
Wnt-5a	Between groups	.293	6	.049	6.113	.016
	Within groups	.056	7	.008		
	Total	.349	13			
Shh	Between groups	.369	6	.061	1.813	.025
	Within groups	.204	7	.034		
	Total	.573	13			

The expression of Wnt-5a genes and proteins in this phase begins to increase, and Wnt-5a has a role in overcoming inflammation in the wound area. Wnt-5a signaling abnormalities are associated with several human pathologies such as cancer, fibrosis, and inflammation (Kumawat & Gosens, 2016; Dorn *et al*., 2016). However, IL1-β as the inflammatory process in the area inflammation will stimulate the increase of Wnt-5a in myofibroblasts, endothelial cells, and chondrocytes (Rishikaysh *et al*., 2014).

The high expression of Wnt-5a genes and proteins in this phrase indicates the role of these genes and proteins in proliferation and differentiation cells in the tissue regeneration process of digit tip mice. According to Kumawat & Gosens (2016), Wnt-5a predominantly activates b-catenin signaling to relay its diverse cellular effects such as cell polarity, migration, proliferation, cell survival, and immunomodulation (Kumawat & Gosens, 2016). Expression of the Shh gene and protein in this phase is relatively low because the Shh has a role in tissue morphogenesis (Muthu *et al*., 2019).

The peak of the Wnt-5a gene and protein expression occurred on day 5, the increased cell proliferation and differentiation so that the growth of nail and connective tissue appeared to be more widespread. Wnt-5a protein may also play a role in the developing endothelium and increasing the formation of new blood vessels that will support cell proliferation and differentiation *(Lovett-Barr et al*., 2012; Hutchins *et al*., 2014). It has shown a large number of blood vessel formations (Fig.3.D). The high expression of the Wnt-5a gene and protein indicates its role in forming new blood vessels (Baarsma *et al*., 2017; Mastelaro de Rezende *et al*., 2020).

On day 5, the Wnt-5a gene and protein expression increased, the Shh gene and protein expression increased. Wnt-5a expression is stimulating the expression of Wnt/β catenin pathway feedback regulating Hh activity through transcriptional regulation of GLI3 (Vitulo *et al*., 2017). GLI3 is known to be a transcriptional repressor of the Hh signaling pathway in the absence of ligand stimulation (Lingappan & Savani, 2020).

After day 10, tissue regeneration in the toes of the mice showed significant growth. Morphologically, the tissue regeneration of the organ shows a significant rapid growth of nail tissue. In this phase, connective tissue formed the elongated nail tissue. Epidermal tissue starts to cover the phalanges tissue (injury area) (see Fig. 3.B). In this phase, the organ of digit tip mice grows complexly and becomes a tissue-like normal. The effect of the dynamics of Wnt-5a and Shh proteins expression is regulation of the tissue regeneration process. Hh signaling limits Wnt activity that the Wnt pathway regulates Hh activity via transcriptional regulation conserver (Lee *et al*., 2016). The reciprocal inhibition between the Hh and Wnt signaling pathways the balance between cell proliferation and differentiation (Ding & Wang, 2017; Herman *et al*., 2018).

.

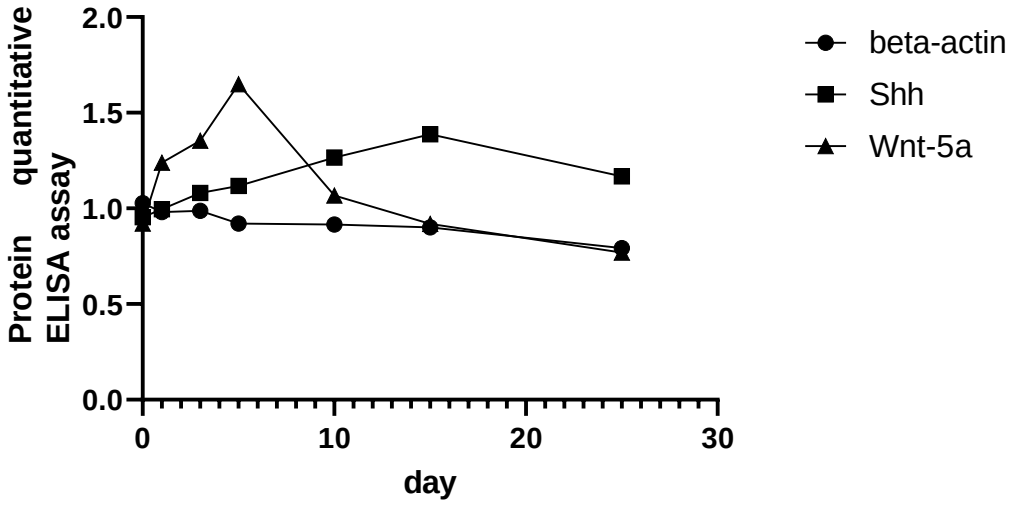

Fig. 6. Expression dynamics of Shh and Wnt-5a proteins. Beta-actin protein as the control. Wnt-5a protein expression increased significantly from day 0 until it reached its peak on day 5, and the expression decreased until day 25. Shh protein expression increased slowly and reached its peak on day 15 when Wnt-5a protein expression decreased. The b-actin protein expression as positive control showed stability.

Pharmacological research has revealed the integration between Hh and Wnt signaling through active and inhibitory drugs (Maimets *et al*., 2022). The Hh pathway acts upstream of Wnt to inhibit Wnt signaling activation; However, Wnt activation may salvage suppressive Hh signaling in regulating amphibian and chick limb regeneration (Moura *et al*., 2014; Mescher, 2017). A synergistic interaction reported that Ihh signaling is active in the early stages of osteoblast maturation during fracture repair. The synergic upregulated Wnt signaling in differentiated osteoblasts. Further studies have shown that deletion of the motor protein of the kinesin in the dental mesenchyme results in Hh suppression and Wnt activation, which affects the development of incisors and molars. This discovery may reveal a link between the two signaling pathways at the gene level (Rishikaysh *et al*., 2014; Lingappan & Savani, 2020).

In mice injury, Shh expression in basal cells is activated and induces increased Wnt protein expression in stromal cells. Increased activity of these signaling circuits may help prevent the further spread of infection and stimulate the restoration of urothelial and stromal cells. This finding demonstrates an interaction between the Hh and Wnt signaling pathways (Willert & Nusse, 2012; Ding & Wang, 2017). We suggested that organisms require an appropriate balance of these signaling pathways to control proliferation and differentiation.

Enhanced Shh signaling will limit canonical Wnt signaling in the lambdoidal region by promoting the expression of genes encoding Wnt inhibitors, which are involved in cleft lip development. Further studies have shown that Wnt signaling may be a downstream pathway of Hh signaling. However, the Wnt gene is also a target of Shh in several developmental systems and is consistent with these observations. Wnt-5a expression in developing hair follicles requires Shh. These results suggest that Wnt-5a may mediate some of the effects of Shh in hair follicle morphogenesis, a hypothesis supported by the fact that Wnt-5a and Shh can regulate proliferation. Homozygous Wnt-5a knock-out exhibit perinatal mortality mice, mainly due to respiratory failure, exhibit extensive developmental abnormalities (Kumawat & Gosens, 2016; Ward *et al.*, 2016).

In the morphological development of digit tip mice, it appears after the 10th day is increasingly complex to form various tissues. On day 10, the Shh gene and protein expression increased sharply, the Wnt-5a gene and protein expression decreased until day 25. The process indicates that Shh has a role in the tissue morphogenesis process and the formation of intact organs. Rishikaysh *et. al*. (2014) observed that deletion of Wnt-10b, the only Wnt ligand we found expressed during fungiform papillae formation, caused similar effects on Shh expression and fungiform papillae formation as did the Lef1

null mutation. Thus, the deletion of Lef1- or Wnt-10b dependent Wnt/b-catenin signaling severely diminishes the size and (eventually) the number of fungiform papillae and decreases the expression of Shh in fungiform papillae (Rishikaysh *et al.*, 2014). On day 25, the expression of the Shh gene and protein began to decrease. The Shh expression is lower than the Wnt-5a expression. Because of the role of Shh decrease in the tissue morphology, the digit tip mice tissue is complete and returns to normal tissue again.

CONCLUSION

In the early phase of the tissue regeneration process of mice digit tip, cell proliferation and cell migration occurred, along with the high expression of the Wnt-5a gene in that phase. After cell proliferation, followed by cell morphology processes at the same Shh gene expression reached its peak. We suggested that Wnt-5a and Shh have a role in this process. The dynamics of gene and protein expression of Wnt-5a and Shh in the process of tissue regeneration coincided with the process of proliferation, cell migration, and cell morphogenesis in post-amputation digit tip mice.

ACKNOWLEDGEMENTS

Authors thank to the Ministry of Education and Culture, Research, and Technology of the Republic of Indonesia provided the DIKTI Research grant fund for the 2019-2020 PKPT scheme. The number of Contract Funding is 225/SP2H/LT/DPRM/2019. Thanks also to Esa Unggul University for supporting this research to completion. We also thank the University of North Sumatra Medan as a partner in this research.

REFERENCES

Baarsma HA, Skronska-Wasek W, Mutze K, Ciolek F, Wagner DE, John-Schuster G, Heinzelmann K, Günther A, Bracke, K. R., Dagouassat, M., Boczkowski, J., Brusselle, G. G., Smits, R., Eickelberg, O., Yildirim, A., & Königshoff, M. (2017). Noncanonical WNT-5A signaling impairs endogenous lung repair in COPD. *Journal of Experimental Medicine*, *214*(1), 143–163. https://doi.org/10.1084/jem.20160675

Dawson LA, Simkin J, Sauque M, Pela M, Palkowski T, Muneoka K. 2016. *Analogous cellular contribution and healing mechanisms following digit amputation and phalangeal fracture in mice*. *1959*, 39–51. https://doi.org/10.1002/reg2.51

Ding M, Wang X. (2017). Antagonism between hedgehog and wnt signaling pathways regulates tumorigenicity (Review). In *Oncology Letters* (Vol. 14, Issue 6, pp. 6327–6333). Spandidos Publications. https://doi.org/10.3892/ol.2017.7030

Dorn J, Yassouridis A, Walch A, Diamandis EP, Schmitt M, Kiechle M, Wang P, Drecoll E, Schmalfeldt B, Loessner D, Kotzsch M, Magdolen V. 2016. Assessment of kallikrein-related peptidase 5 (KLK5) protein expression in tumor tissue of advanced ovarian cancer patients by immunohistochemistry and ELISA: Correlation with clinical outcome. *American Journal of Cancer Research*, *6*(1), 61–70.

Gauron C, Rampon C, Bouzaffour M, Ipendey E, Teillon J, Volovitch M, Vriz S. 2013. Sustained production of ROS triggers compensatory proliferation and is required for regeneration to proceed. *Scientific Reports*, *3*, 1–9. https://doi.org/10.1038/ncb2659

Guedelhoefer OC, Alvarado AS. 2012. Amputation induces stem cell mobilization to sites of injury during planarian regeneration. *Development*, *139*(19), 3510–3520. https://doi.org/10.1242/dev.082099

Herman PE, Papatheodorou A, Bryant SA, Waterbur CKM, Herdy JR, Arcese AA, Buxbaum JD, Smith JJ, Morgan JR, Bloom O. 2018. Highly conserved molecular pathways, including Wnt signaling, promote functional recovery from spinal cord injury in lampreys. *Scientific Reports*, *8*(1), 1–15. https://doi.org/10.1038/s41598-017-18757-1

Hutchins ED, Markov GJ, Eckalbar WL, George RM, King JM, Tokuyama MA, Geiger LA, Emmert N, Ammar MJ, Allen, A. N., Siniard, A. L., Corneveaux, J. J., Fisher, R. E., Wade, J., DeNardo, D. F., Rawls, J. A., Huentelman, MJ, Wilson-Rawls, J., & Kusumi, K. (2014). Transcriptomic analysis of tail regeneration in the lizard Anolis carolinensis reveals activation of conserved vertebrate developmental and repair mechanisms. *PLoS ONE*, *9*(8). https://doi.org/10.1371/journal.pone.0105004

Iriana S, Asha K, Repak M, Sharma-Walia N. 2021. Hedgehog signaling: Implications in cancers and viral infections. *International Journal of Molecular Sciences*, *22*(3), 1–30. https://doi.org/10.3390/ijms22031042

Iwakura T, Inui A, Reddi AH. 2013. Stimulation of superficial zone protein accumulation by hedgehog and Wnt signaling in surface zone bovine articular chondrocytes. *Arthritis and Rheumatism*, *65*(2), 408–417. https://doi.org/10.1002/art.37768

Lehoczkya JA., Robert B., & Tabin CJ.(2011). Mouse digit tip regeneration is mediatedby fate-restricted progenitor cells. *PNAS*, 108 (51), |20609–20614. https://doi/10.1073/pnas.1118017108

Joven, A., & Simon, A. (2018). Homeostatic and regenerative neurogenesis in salamanders. *Progress in Neurobiology*, *March*, 0–1. https://doi.org/10.1016/j.pneurobio.2018.04.006

Kumawat, K., & Gosens, R. (2016). WNT-5A: Signaling and functions in health and disease. In *Cellular and Molecular Life Sciences* (Vol. 73, Issue 3, pp. 567–587). Birkhauser Verlag AG. https://doi.org/10.1007/s00018-015-2076-y

Lee, Y., Lee, J. K., Ahn, S. H., Lee, J., & Nam, D. H. (2016). WNT signaling in glioblastoma and therapeutic opportunities. *Laboratory Investigation*, *96*(2), 137–150. https://doi.org/10.1038/labinvest.2015.140

Lingappan, K., & Savani, R. C. (2020). The wnt signaling pathway and the development of bronchopulmonary dysplasia. *American Journal of Respiratory and Critical Care Medicine*, *201*(10), 1174–1176. https://doi.org/10.1164/rccm.202002-0277ED

Lovett-Barr, M. R., Satriotomo, I., Muir, G. D., Wilkerson, J. E. R., Hoffman, M. S., Vinit, S., & Mitchell, G. S. (2012). Repetitive Intermittent Hypoxia Induces Respiratory and Somatic Motor Recovery after Chronic Cervical Spinal Injury. *Journal of Neuroscience*, *32*(11), 3591–3600. https://doi.org/10.1523/JNEUROSCI.2908-11.2012

Lozito TP, Tuan RS. 2016. Lizard tail skeletal regeneration combines aspects of fracture healing and blastema-based regeneration. *Development*, *143*(16), 2946–2957. https://doi.org/10.1242/dev.129585

Maimets M, Pedersen MT, Guiu J, Dreier J, Thodberg M, Antoku Y, Schweiger PJ, Rib L, Bressan RB., Miao Y., Garcia, KC, Sandelin A, Serup P, Jensen KB. 2022. Mesenchymal-epithelial crosstalk shapes intestinal regionalisation via Wnt and Shh signalling. *Nature Communications*, *13*(1), 1–12. https://doi.org/10.1038/s41467-022-28369-7

Mastelaro de Rezende M, Zenker Justo G. Julian Paredes-Gamero E, Gosens R. 2020. Wnt-5A/B Signaling in Hematopoiesis throughout Life. *Cells*, *9*(8), 6–8. https://doi.org/10.3390/cells9081801

Mescher AL. 2017. *Macrophages and fibroblasts during inflammation and tissue repair in models of organ regeneration*. *March*, 39–53. https://doi.org/10.1002/reg2.77

Miller TJ, Deptula PL, Buncke GM, Maan ZN. 2019. Digit tip injuries: Current treatment and future regenerative paradigms. In *Stem Cells International*. Hindawi Limited. https://doi.org/10.1155/2019/9619080

Mizukoshi K, Koyama N, Hayashi T, Zheng L, Matsuura S, Kashimata M. 2016. Shh/Ptch and EGF/ErbB cooperatively regulate branching morphogenesis of fetal mouse submandibular glands. *Developmental Biology*, *412*(2), 278–287. https://doi.org/10.1016/j.ydbio.2016.02.018

Moura RS, Carvalho-Correia E, DaMota P, Correia-Pinto J. 2014. Canonical Wnt signaling activity in early stages of chick lung development. *PLoS ONE*, *9*(12), 1–27. https://doi.org/10.1371/journal.pone.0112388

Muthu V, Rohacek AM, Yao Y, Rakowiecki SM, Brown AS, Zhao YT, Meyers J, Won KJ, Ramdas S, Brown CD, Peterson KA, Epstein DJ. 2019. Genomic architecture of Shh-dependent cochlear morphogenesis. *Development (Cambridge)*, *146*(18). https://doi.org/10.1242/dev.181339

Petrova R, Joyner AL. 2014. Roles for Hedgehog signaling in adult organ homeostasis and repair. In *Development (Cambridge)* (Vol. 141, Issue 18, pp. 3445–3457). Company of Biologists Ltd. https://doi.org/10.1242/dev.083691

Rishikaysh P, Dev K, Diaz D, Shaikh Qureshi WM, Filip S, Mokry J. 2014. Signaling involved in hair follicle morphogenesis and development. *International Journal of Molecular Sciences*, *15*(1), 1647–1670. https://doi.org/10.3390/ijms15011647

Storer MA, Miller FD. 2020. Cellular and molecular mechanismsthat regulate mammalian digittip regeneration. *Open Biol*.10: 200194.http://dx.doi.org/10.1098/rsob.200194

Stulberg MJ, Lin A, Zhao, H, Holley SA. 2012. Crosstalk between Fgf and Wnt signaling in the zebrafish tailbud. *Developmental Biology*, *369*(2), 298–307. https://doi.org/10.1016/j.ydbio.2012.07.003

Takeo M, Chou WC, Sun Q, Lee W, Rabbani P,Loomis C, Taketo MM, Ito M. 2013. Wnt activation innail epithelium couples nail growth to digitregeneration. *Nature*, 499, 228–232. (doi:10.1038/nature12214)

Udalamaththa VL, Jayasinghe CD, Udagama PV. 2016. Potential role of herbal remedies in stem cell therapy: proliferation and differentiation of human mesenchymal stromal cells. *Stem Cell Research and Therapy*, *7*(1), 1–8. https://doi.org/10.1186/s13287-016-0366-4

Vitulo N, Dalla Valle L, Skobo T, Valle G, Alibardi L. 2017. Transcriptome analysis of the regenerating tail vs. the scarring limb in lizard reveals pathways leading to successful vs. unsuccessful organ regeneration in amniotes. *Developmental Dynamics*, *246*(2), 116–134. https://doi.org/10.1002/dvdy.24474

Ward MA, Pierre JF, Leal RF, Huang Y, Shogan B, Dalal SR, Weber CR, Leone VA, Musch MW, An GC, Rao MC, Rubin DT, Raffals LE, Antonopoulos DA, Sogin ML, Hyman NH, Alverdy JC, Chang EB. 2016. Insights into the pathogenesis of ulcerative colitis from a murine model of stasis-induced dysbiosis, colonic metaplasia, and genetic susceptibility. *American Journal of Physiology - Gastrointestinal and Liver Physiology*, *310*(11), G973–G988. https://doi.org/10.1152/ajpgi.00017.2016

Willert K, Nusse R. 2012. Wnt proteins. *Cold Spring Harbor Perspectives in Biology*, *4*(9), 1–14. https://doi.org/10.1101/cshperspect.a007864.