ANALYSIS OF GRHL3 EXPRESSION IN THE ZEBRAFISH EPIDERMIS UPON DEFECTIVE INTRACELLULAR TRANSPORT

MASTER'S DISSERTATION THESIS BY:

Ameya C Pofare

Indian Institute of Science Education and Research, Pune

UNDER THE SUPERVISION OF:

Mahendra S Sonawane, PhD

Department of Biological Sciences,

Tata Institute of Fundamental Research, Mumbai

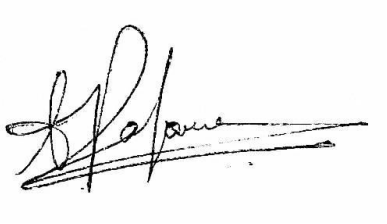
CERTIFICATE

This is to certify that this dissertation entitled “Analysis of grhl3 expression in the zebrafish epidermis upon defective intracellular transport” towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents original research carried out by “Ameya C. Pofare at the Tata Institute of Fundamental Research” under the supervision of “Mahendra S. Sonawane, PhD, Department of Biological Sciences” during the academic year 2017.

Mahendra S. Sonawane, PhD

DECLARATION

I hereby declare that the matter embodied in the report entitled “Analysis of grhl3 expression in the zebrafish epidermis upon defective intracellular transport” are the results of the investigations carried out by me at the Department of Biological Sciences, Tata Institute of Fundamental Research, under the supervision of Mahendra S. Sonawane, PhD and the same has not been submitted elsewhere for any other degree.



Ameya C. Pofare

ACKNOWLEDGEMENTS

I am much grateful to Dr. Mahendra Sonawane for making me a part of this wonderful research group. He has been very supportive and patient with me around. I have learnt enormously during this course of time and this experience has certainly prepared me for the challenges of the future. I am much obliged to Indraneel, Shruti and Shamik for guiding me through the various lab procedures and the many discussions that we had. I would also like to thank Sunny, Sandeep and the rest of the lab members for extending their support at all times.

Along with IISER Pune, I have been part of an equally reputed institution since a year - NCRA. Both the places have been extremely friendly and the personnel here have kept things moving smoothly. I would also like extend my gratitude to DST-INSPIRE for providing the opportunity to interact at various events throughout the country.

Away from my home, the city of Pune had become my new home and the friends I made here were my new family. I had many delightful experiences here and this kept me marching forward throughout. The time spent here was a big green patch in my life and I will cherish these moments forever.

I feel indebted to my family for allowing me to choose this hitherto unheard form of training. These five years were filled with a great many ups and downs and I had the constant support of my family at every challenge. It would not have been possible for me to come this far without their blessings.

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ABSTRACT

The skin functions as a protective barrier for the body. The epidermis is the outermost layer of the skin and is constantly exposed to wear and tear. Some mutants of zebrafish show balling up of epidermal cells wherein the cells shows accumulation of vesicles.

Previous work in the lab has shown increased expression of grainyhead like-3 in balled up cells of myosin Vb mutants (unpublished). Presented work shows more evidence of upregulation of grainyhead like-3 in balled up cells, namely in *clint1* morphants and *romeharsha* mutants. This suggests a strong correlation between the wound healing mechanism of grainyhead like-3 and the recovery of balled up cells.

INTRODUCTION

Multicellular organisms have a distinct outermost cell layer that protects the rest of the organism from external aggressions. Chitinous exoskeleton in arthropods is an example of tissue that serves this purpose. For vertebrates, the skin exists as the outermost tissue encompassing the entire body. The skin functions as a protective layer of the body and defends it from variations in the external environment. It also performs specialised functions like absorption and secretion.

The epidermis is the outermost layer of the skin. It has the ability to robustly sustain most abrasions and it strives to cover the organism at all times. This epidermis thus becomes a dynamic barrier where the tissue highly regulated by apoptosis and proliferation. Any anomaly in this balance can lead to the formation of tumour or atrophy of the tissue. This makes the study of the epidermis of vital importance to the survival and overall well-being of the organism.

- **Larval zebrafish epidermis is bilayered**

The adult zebrafish epidermis is multi layered. However, the epidermis is devoid of a complex structure during early development. The zebrafish blastoderm is covered during early blastula stages by an enveloping layer (EVL) made up of monolayer of squamous epithelial cells. The EVL proliferates and slowly encompasses the entire surface of the embryo and forms a single layer of cell called the periderm (Kimmel et al, 1995). The second layer of the epidermis is derived from the germ layers post gastrula stage. The epidermal basal layer (EBL) is obtained from the ventral ectoderm and undergoes division to form the basal epidermis (Bakkers et al, 2002). By the end of somite formation (24 hpf), the zebrafish epidermis is bi layered.

The periderm and the basal epidermis have different cell junctions. The periderm has multiple tight junctions and they maintain the barrier function of the epidermis. The basal epidermis has many hemi desmosomes binding the tissue to the layer of extra cellular matrix beneath.

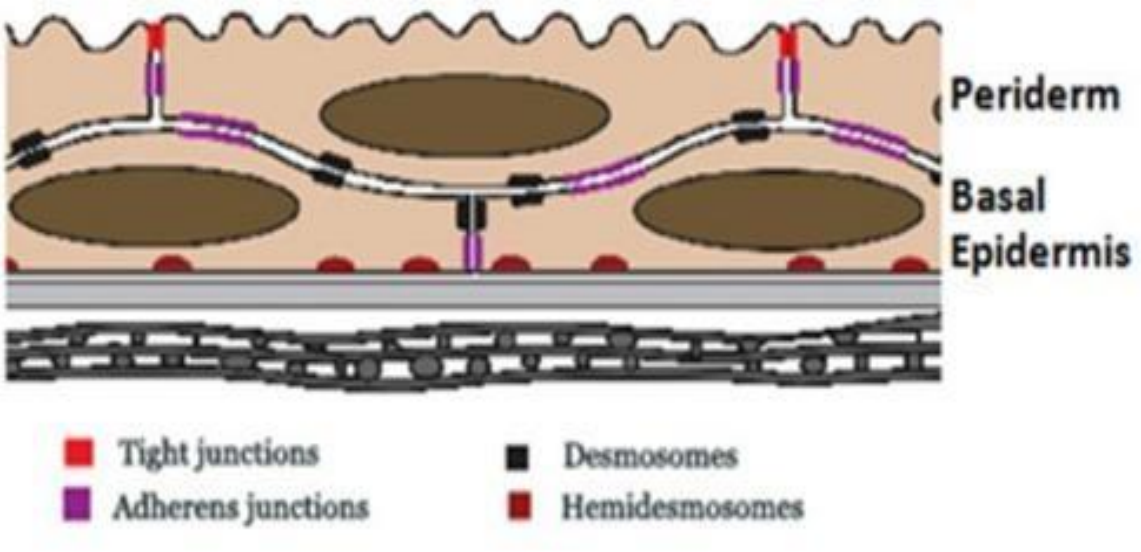

Figure 1: Zebrafish epidermis during early development. The bilayered zebrafish epidermis are connected with various cell junctions in the apical and basal layers. (Sonawane et al, 2005)

- **Balling up phenotype**

Multiple mutant species of zebrafish and their phenotypes have been observed and recorded at the zebrafish information network (ZFIN). A mutant that shows absence of a function of myosin Vb motor protein, the goosepimple (gsp) mutant, has been known to show balling up of

cells(Sonal, Sidhaye et al, 2014). The mutant embryo display an overgrowth of cells in the nape and this phenotype is starkly visible at 48 hpf.

Another mutant displaying the balling up phenotype is the *clint1* mutant (Dodd et al,2009). A mutation in the clathrin interacting adaptor protein brings out this phenotype which is visible from 24 to 48 hpf.

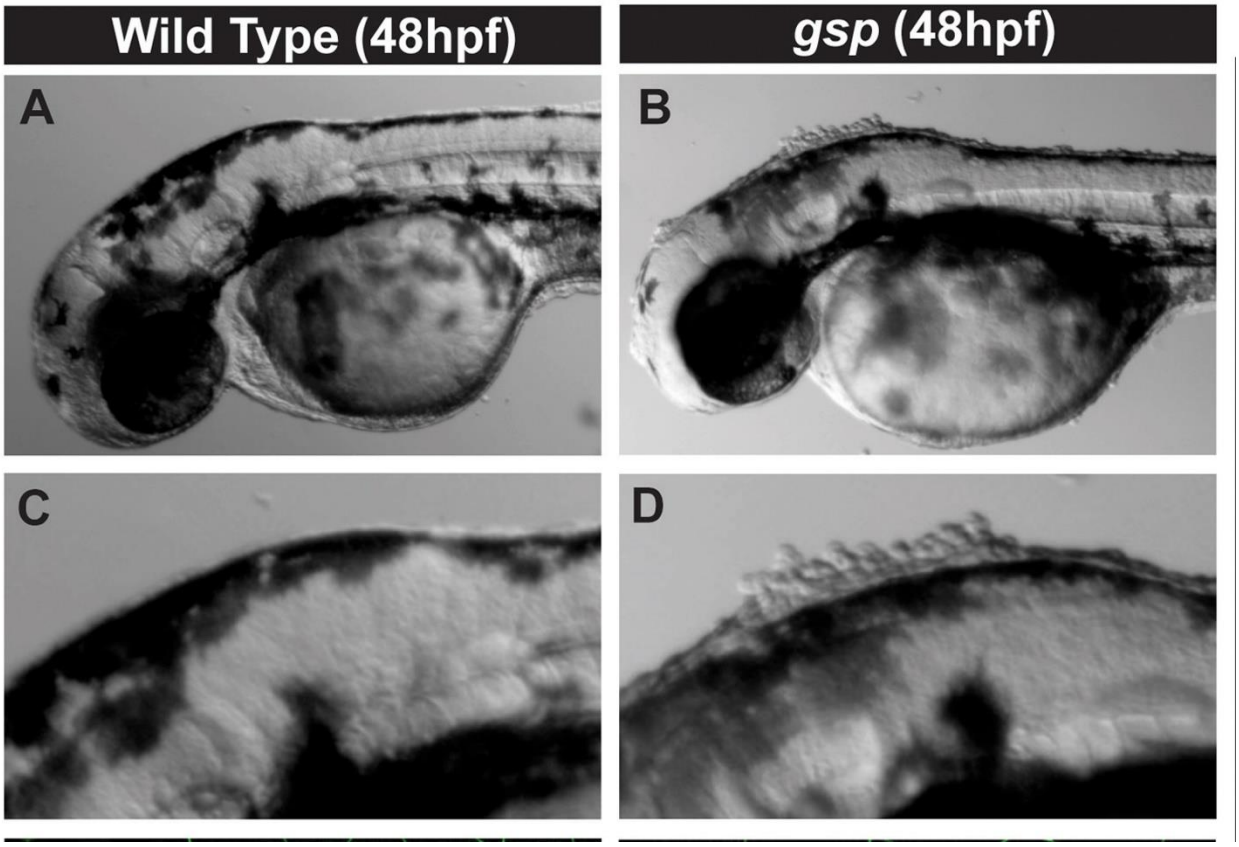

Figure 2: Wildtype embryos and myosin Vb mutants. The balled up cells are visible distinctly in image 3b and 3d (modified from Sonal, Sidhaye et al, 2014).

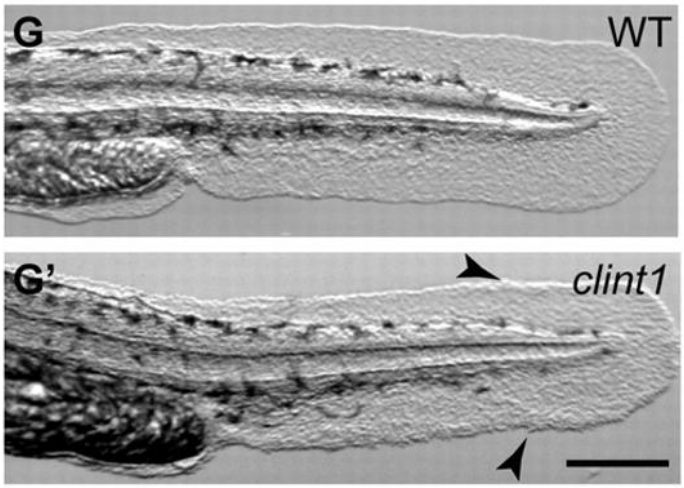

Figure 3: Balling up in clint1 mutants. Balling up of cells is visible in the fin fold of the mutant embryo (Dodd et al 2009).

A new mutant has been identified in an ENU mutagenesis screen and named *romeharsha* (Sonawane et al, unpublished). The mutation has not been identified yet. This mutant shows balling up of cells along the fin fold from approximately 36 to 48 hpf.

- **Grainyhead like-3**

The grainyhead (grh) gene family of genes encodes for a transcription factor in drosophila which has important role in epithelial maintenance. The vertebrate homologs of grh, referred to as grainyhead like (grhl), have been reported in fish, mouse and humans. Wound healing is an extremely vital process for an organism and is found to be conserved across species. Grainyhead like-3 (grhl3) is a transcription factor known for its important role in wound healing. In

drosophila, grhl3 is reported to be upregulated in in damaged chitin structures (Mace et al, 2005). In mice, it is upregulated in the damaged stratum cornuem (Yu et al,2006).

Previous work by Mandar in the lab has shown increased expression of grhl3 in the balled up cells of myosin Vb mutants. By 60 hpf, the balling up phenotype in myosin Vb mutants disappear and we see a rescue of phenotype. In the absence of grhl3, this rescue is not seen and the balled up cells disintegrate from the epidermis and the tissue becomes fragile. This suggests a role of grhl3 in maintaining epithelial integrity.

- **Zebrafish as a model organism**

Figure 4: A zebrafish embryo at 36 hpf. Zebrafish embryos are transparent and facilitate observation of changes in the epidermis. (Source - http://hk.apple.nextmedia.com/international/art/20071017/10298232)

Zebrafish (danio rerio) proves to be an efficient model organism for studying the early epidermal development. This vertebrate model organism has high genomic homology with humans: 70% of

genes in humans have at least one orthologue in zebrafish (Howe K, Clark MD, Torroja CF, et al, 2013). Thus many cellular and molecular processes in humans can be modelled in zebrafish. The zebrafish eggs are fertilised ex-vitro and further development of the embryo happens externally, allowing us to track down the various developmental processes since fertilisation. The transparent zebrafish embryos and the bilayered epidermis facilitates easy observation of the epidermis. It is feasible to conduct experiments with zebrafish is as a large clutch of eggs is obtained from each mating pair. Although development in zebrafish takes longer than other invertebrate model organisms, it still serves as an excellent system to study epidermal development.

AIMS AND OBJECTIVES

- To find out if *grainyhead like-3* upregulation is a general response to balling up of cells or is it specific to *myoVb* mutants.
- To find out the spatial and temporal expression patterns of *grainyhead like-3* in *romeharsha* and *clint1* mutants using in situ hybridization.

STRATEGY

Clone cDNA of grhl3 to make in situ probe (already done in the lab)

Fix samples of clint1 MO (24,30,36,48,72 hpf) and romeharsha (36,48 hpf) at various timepoints

PCR amplify the probe

Prepare DIG labelled RNA probe

In situ hybridization

MATERIALS AND METHODS

1. Zebrafish Maintenance

   All the fish were maintained at a temperature of 28.5°C for the experiments. For collection of eggs, one male and one female fish were kept in a breeding tank overnight, separated by a barrier. The next morning, the barriers were removed and the fish lay. The eggs collected were maintained at a temperature of 29.5°C.

2. Fish strains

   For experiments, the wild type Tuebingen (Tu) fish and the wild type Tuebingen Albino fish were used. One more strain, the NOO47 *romeharsha* fish were used for in situ hybridization.

3. Embryo Sorting and Dechorionation

   Approximately 12 hours post fertilization, the plate was examined for dead and decaying embryos. The healthy embryos were picked and transferred on to a new plate. 24 hours post fertilization, the embryos were dechorionated manually using forceps. The healthy dechorionated embryos were again transferred to a new plate.

4. Methanol and glycerol upgradation

   The given steps are followed for serial upgradation in methanol. For serial down gradation in methanol the same steps are followed but in reverse order.

   Wash three times with PBS for 10 min @RT

Incubate in 25% methanol - 75% PBS solution for 10 minutes @RT

Incubate in 50% methanol - 50% PBS solution for 10 minutes @RT

Incubate in 75% methanol - 25% PBS solution for 10 minutes @RT

Incubate in 100% methanol solution for 30 minutes @RT

Change the 100% methanol solution once again and immediately store at -20°C

For serial upgradation in glycerol:

Incubate in 25% glycerol - 75% PBS solution for 10 minutes @RT

Incubate in 50% glycerol - 50% PBS solution for 30 minutes @RT

Incubate in 75% glycerol - 25% PBS solution for 2 hours @RT

Store at 4°C

5. Probe synthesis

   DIG labelling kit was used to synthesize probes, with T7 polymerase used for anti-sense and SP6 for sense probes. The sense probes were used as controls for the experiment. The synthesized probes were column purified and stored at -80°C.

6. RNA in situ hybridization

   Embryos are dechorionated and fixed in 4% PFA overnight. The samples are serially upgraded to 100% methanol and stored.

   Day 1:

   The fixed embryos are serially downgraded from 100% methanol to PBS. 10µg/ml proteinase K was used to permeabalise the embryos and fixed in 4% PFA for 20 min.

After washing away the PFA, the samples were incubated in pre-hybridization buffer for 2 hours at 67°C. The DIG labelled probes were pre-warmed in prehybridization buffer for 15 min and then incubated in the samples overnight at 67°C.

Day 2:

The excess probes were given multiple washes sequentially using 50x Formamide-3xSSCT, 50% Formamide- 2x SSCT, 2x SSCT and 0.2 SSCT. The washes were given at 67°C and followed by washed with PBST and maleic acid buffer (MAB). The blocking solution was prepared in MAB at 67°C and the samples were incubated in blocking solution for 3 hours. The embryos were then incubated overnight with anti- DIG AP antibody in blocking solution (1:2000 dilution).

Day 3:

The embryos were rinsed briefly in MAB and incubated in MAB and PBS solutions.

Fresh staining solution is prepared and the embryos are incubated in it. The samples are transferred on to a 12 well plate. 400 µL BM- purple AP precipitating substrate is added to the wells and incubated at 37°C till coloration is visible. The samples are then fixed in 4% PFA and upgraded to 80% glycerol.

7. Imaging

Post in situ hybridization, DIC images of the samples were taken. The images were taken using DISCOVERY V20 SteREO with AxioCamHR3 camera with image dimensions 1388x1040 pixels.

8. Reagents

PBS, phosphate buffered saline:

To make 1 L of 10x stock:

NaCl 80.0 g

KCl 2.0 g

Na_2HPO_4 14.4 g

KH_2PO_4 2.4 g

ddH_2O to 1.0 L

Dilute to 1X working concentration

PBST, phosphate buffered saline + Tween-20:

To make 1 L of 10x stock:

NaCl 80.0 g

KCl 2.0 g

Na_2HPO_4 14.4 g

KH_2PO_4 2.4 g

Tween-20 10.0 ml

ddH_2O to 1.0 L

Dilute to 1X working concentration

Proteinase-K :

Prepare 20mg/ml stock solution in distilled water. Store at -20°C. (Merkmillipore)

Pre hybridization buffer:

Formamide	250 ml
20xSSC12	125 ml
50 mg/ml yeast tRNA	5 ml
10% Tween-20	10 ml
1 M citric acid	4.60 ml
50 mg/ml heparin	500 µl

ddH_2O to 500 ml total

Scale up or down as appropriate. Aliquot and store at -20 °C. Note: Citric acid is used to give a final solution of pH ~5.5-6.0. Verify that this pH has been achieved in the final solution.

SSC and SSCT:

Make 20xSSC stock for dilution to working concentrations of 2x, 0.2x, 0.05x and for use in hybridization solution.

20xSSC stock solution (3.0 M NaCl, 0.3 M trisodium citrate):

Dissolve 175.3 g NaCl, 88.2 g trisodium citrate in 800 ml ddH_2O. Adjust pH upto 7.0 with a few drops of concentrated HCl. Adjust volume to 1 L with ddH_2O. Sterilize by autoclaving.

To make 2x, 0.2x or 0.05xSSCT, dilute 20xSC with ddH_2O and add 2 ml Tween- 20 per L.

10xMaleic acid buffer stock:

For 1 L of concentrated stock solution, begin with ~750 ml ddH_2O then add:

1 M maleic acid 116.07 g

1.5 M NaCl 87.66 g

Adjust to pH 7.5 with NaOH pellets initially (~72 g per liter) then 10 M NaOH solution. Buffer will clear around pH 6.0. Desired pH is easy to overshoot! Fill with ddH_2O to 1000 ml.

Use at 1x for washes, etc.

Roche blocking reagent:

To make a 10x stock (10% w/v), dissolve 10 g Roche blocking reagent in 100 ml 1x MAB over moderate heat with stirring. Aliquot and store at -20°C. For working strength, dilute to 1x with MAB.

Staining solution

100 mM Tris HCl pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl, 0.1% Tween-20 Make fresh daily from stocks, to make 200 ml (scale up if necessary):

ddH2O 164 ml

1 M Tris 20 ml

1 M $MgCl_2$ 10 ml

5 M NaCl 4 ml

10% Tween-20 2 ml

1M TRIS (pH=9.5) :

60.55g TRIS in 500ml. Adjust the pH before making up the volume to 500ml.

0.5M MgCl2:

Dissolve 25.25g of $MgCl_2$ in 250ml DEPC treated water.

5M NaCl:

Dissolve 146.1g of NaCl in 250ml DEPC treated water.

4 %PFA:

Dissolve 4% w/v Para formaldehyde suitable volume by constant stirring at 65°C. Then make up the volume. (Do this always in chemical hood.)

DEPC treatment:

Add 0.1% DEPC to the solution for treatment. Keep it at 37°C O/N. Autoclave before use. Take extreme precaution while handling DEPC.

RESULTS

- Fixed samples of romeharsha

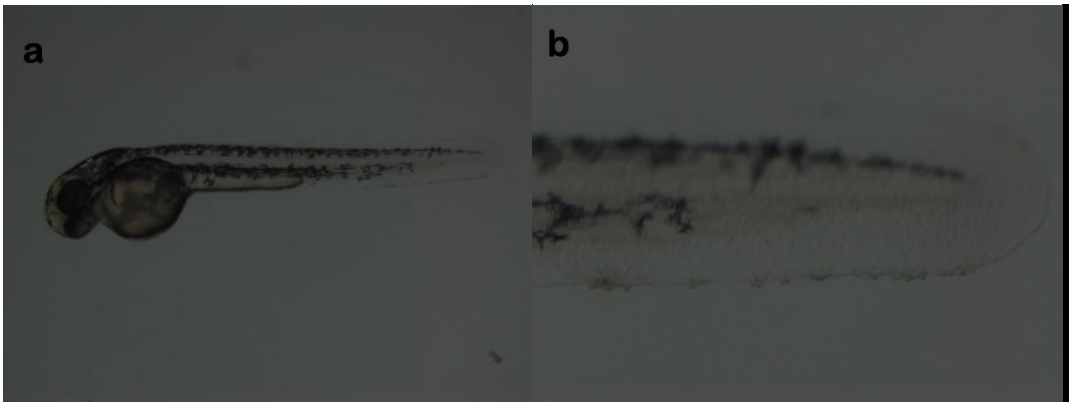

Figure 5: Samples of *romeharsha* at 36 hpf. The figure 5b shows the morphological phenotype of romeharsha with balled up cells along its fin fold.

For in situ hybridization, zebrafish embryos were fixed at various time points. Albino Tuebingen embryos were collected and dechorionated at 24, 30, 36, 48, 72 hpf. The same procedure was done with embryos injected with *clint1* morpholino. Mutant *romeharsha* embryos were sorted and dechorionated at 36 and 48 hpf. The dechorionated embryos were fixed in 4% PFA overnight, upgraded to 100% methanol the next day and stored at -20°C.

- Restriction Digestion of grhl3 TOPO TA plasmid

To make probes for in situ hybridization, Mandar in the lab synthesized cDNA sequence of grhl3 in TOPO TA II vector. After transformation and plasmid purification of this sequence, sense and anti sense probes are required. The TOPO vector is digested independently at two sites using

SpeI and XhoI enzyme. In each case we get a band close to 5 kb. This band is purified and stored at -20°C.

Figure 6: Plasmid map of TOPO TA II vector. The SP6 promoter and T7 promoter are on either sides of the PCR product.

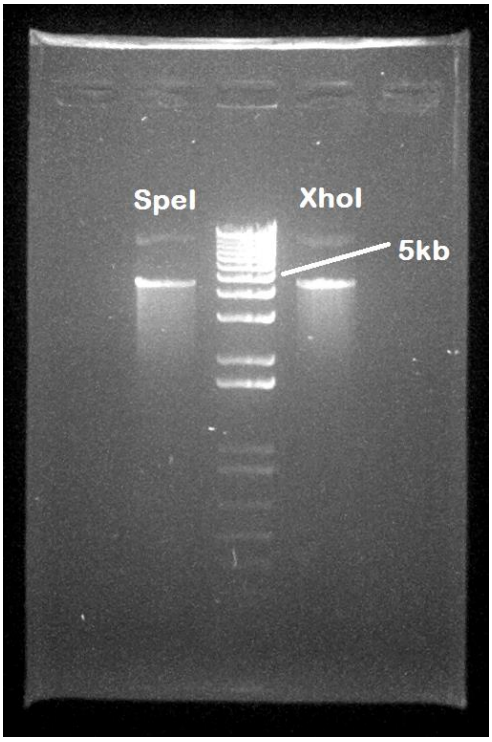

Figure 7: grhl3 probe synthesis. The TOPO TA vector was digested independently using SpeI and XhoI vectors to obtain bands around 5 kb.

DISCUSSION

Previous work in the lab on myosin Vb has confirmed the presence of grhl3 in wound healing response during vesicle accumulation. The expression of grhl3 brings about a rescue of the phenotype in the mutants. The presented work tries to establish this principle in *clint1* morphants and *romeharsha* mutants. Also, knockout of grhl3 in myosin Vb mutants does not show the balling up phenotype and leaves the epidermis very fragile. This tells us that upregulation of grhl3 is a general response to balling up of cells. Furthermore, grhl3 serves an important role in maintaining epidermal integrity.

Grainyhead and its homologues have shown expression in wound healing from invertebrates like drosophila melanogaster to complex mammals like mice. This suggests grainyhead to be a very crucial component of the wound healing machinery, something that is conserved over a wide range of taxa.

Apart from its role in repairing and maintaining epithelial integrity, grainyhead is also linked with various types of cancer. grhl2 role has been implicated in gastric cancer and breast cancer. Overexpression or knockdown of grainyhead has been known to cause lethality. It can be an interesting study to find the role of grhl3 in tumour development in the background of zebrafish.

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