Role of Epidermal Growth Factor signaling in epidermal homeostasis of *Danio rerio* (zebrafish)

Thesis submitted by-

Sourabh J. Bhide

Indian Institute of Science Education and Research, Pune

Under the Supervision of-

Dr. Mahendra S. Sonawane

Department of Biological Sciences,

Tata Institute of Fundamental Research, Mumbai

Certificate

This is to certify that this dissertation entitled “Role of Epidermal growth factor signaling in epidermal homeostasis in *Danio rerio* (zebrafish)” 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 “Sourabh J. Bhide at the Tata Institute of Fundamental Research” under the supervision of “Dr. Mahendra Sonawane, Department of Biological Sciences” during the academic year 2013-2014.

Dr. Mahendra Sonawane

Declaration

I hereby declare that the matter embodied in the report entitled “Role of Epidermal growth factor signaling in epidermal homeostasis in *Danio rerio* (zebrafish)” 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 Dr. Mahendra Sonawane and the same has not been submitted elsewhere for any other degree.

Sourabh Bhide

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Last but not the least; I am grateful to my parents and my friends for supporting me through my ups and downs during this period.

Abstract

The epidermis covers the entire organism forming a barrier between the external environment and the organism. It averts any mechanical, biological or chemical attack from the environment. Epidermis is not a static but a dynamic barrier wherein the old dying cells are replaced constantly by new ones, so study of epidermal homeostasis is of vital importance. Growth factors like egf and fgf have been known to be involved in the maintenance of epidermal homeostasis. However it is not clear whether any of these factors play any role in the early embryonic epidermis, which is just a bilayered epithelium, and whether there is a layer specific requirement of these factors. Here, I have investigated this issues using zebrafish larval epidermis as a model. In this study, I show that EGF signaling is active in epidermis and has a role in regulating cell proliferation. By blocking the activity of the EGF receptors using an inhibitor I see decreased proliferation specifically in the periderm, the outermost epidermal layer. Also the cross-sectional area of the peridermal cells increases in these embryos as compared to the wild type. Thus, the results indicate that EGF signaling is essential to maintain proliferation and optimal surface area in the periderm.

Introduction

Every organism has to protect itself from the external harsh environment for its survival. Epidermis acts as the first line of defense for the organism against the life threatening external conditions. Epidermis is equipped with tight and adherence junctions as well as desmosomes, which facilitate this tissue to act as a physical barrier against any mechanical insults or intrusions (Katz et al., 2002, Ray et al., 2013) and help retain the water content inside the organism. It is also equipped with ion channels that facilitate ion exchange with the surrounding to maintain the osmotic pressure (Mauro et al., 1993, Bakkers et al., 2002) . It helps in buffering the pH by controlling the ion flux, which is important for many biochemical reactions.

In order to perform these functions the epidermis must cover the organism at all times. During development, the epidermis has to continually grow along with the organism. Also, in adult epidermis, there is a continuous turn-over of cells as the dying cells are incessantly replaced by new ones (Pellettieri and Alvarado, 2007). Thus, epidermis is not a static but a dynamic barrier wherein the proliferation and apoptosis are tightly regulated. Loss in the balance between cell death and cell replacement by proliferation may lead to tumor formation or atrophy of the epidermal tissue(Candi et al., 2008, Koster and Roop, 2004, Weinberg and Hanahan, 2000). Thus, the study of the development and maintenance of the epidermis is of vital importance.

Epidermis development in early Zebrafish embryogenesis

zebrafish has been a popular vertebrate model organism in studying developmental biology. Its ex utero development, large clutch size and transparent embryos makes it even more convenient to study epidermis. thus we choose Zebrafish as a model to elucidate mechanisms in the early development of the epidermis.

In zebrafish embryogenesis during blastula stage, the embryo forms a squamous epithelial monolayer called enveloping layer (EVL). The EVL arises from the exterior cells of the blastula or the blastomers and covers the blastoderm. As the cells divide the

EVL starts covering the yolk from the blastoderm margin. The cells of EVL divide contributing to the monolayer as the embryo grows. It becomes lineage specific by 4 hour past fertilization (hpf). Also by this stage the EVL starts expressing differentiation markers like cytokeratin 8 (Imboden et al., 1997). During epiboly the EVL cells migrate to cover the yolk. Subsequently the EVL forms the external layer of the epidermis covering the entire embryo, which is now called as the periderm. The periderm contains membrane localized components of tight junction complex which is critical for the barrier function of the epidermis. The EVL however is transient and is shed in later stages when intermediate layers begin to appear around 15 dpf and are replaced by basally derived cells (Lee et al., 2014) On the other hand, formation of another layer called epidermal basal layer (EBL) starts forming by gastrula stage after formation of three germ layers. The EBL arises independently from the ventral ectoderm. (Bakkers et al., 2002). Thus, by the end of somite formation (24 hpf) the epidermis of *zebrafish* embryo is bi-layered.

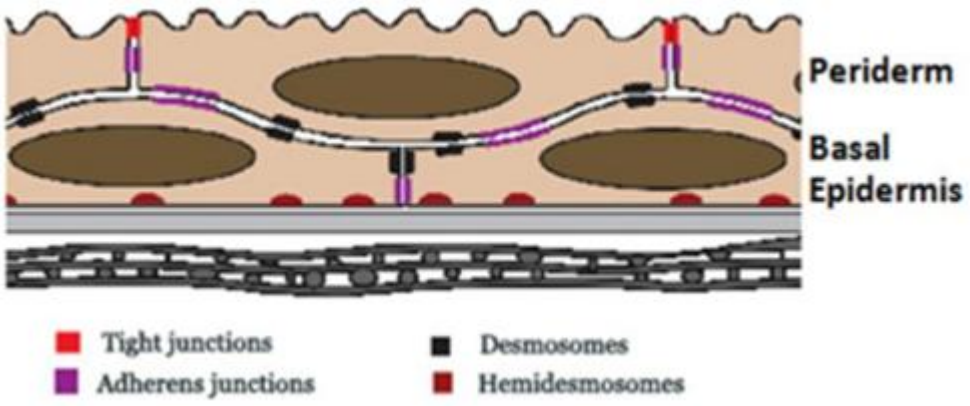

Fig.1. Epidermis of the Zebrafish at early stages. It comprises of a bilayer comprising of the basal epidermis and the periderm. The periderm is equipped with tight junctions and adherent junctions to perform its barrier functions. Besides adherens junctions, basal epidermis has hemisdesmosomes, which help in anchoring epidermis to the extracellular matrix. (reproduced from Sonawane et al,2009)

Epidermal Growth Factor signaling

Epidermal growth factor (egf) receptors belong to a family of receptor tyrosine kinases (RTKs). It has previously shown that they have a role in invertebrate and vertebrate development (Shilo, 2003) . EGF signaling has also been shown in many systems to modulate proliferation, survival, adhesion, migration and differentiation. In *Drosophila,* EGF signaling has been associated to multiple roles in development including patterning of the neuroectoderm, patterning of dorsal midline, determination of dorsal follicle cells and many more (Shilo, 2003). In mouse, EGF signaling has been attributed to the growth, maturation, function and maintenance of epithelial tissues (Sibilia and Wagner, 1995; Lin et al., 2000;Luetteke et al. (1994)). In humans, over expression of EGF receptors has been linked to some of the cancers of epithelial and glial origin like basal cell carcinoma, and squamous cell carcinoma (Arteaga and Engelman, 2014; Hynes and Lane, 2005). They have also been considered as good targets for cancer therapy (Mendelsohn and Baselga, 2000).

In vertebrates, there are four members in the family of EGF receptors viz: Erythroblastic leukemia viral oncogene homolog 1 (ErbB1a/EGFR), Erythroblastic leukemia viral oncogene homolog 2 (ErbB2/c-Neu/HER2), Erythroblastic leukemia viral oncogene homolog 3(ErbB3/HER3) and (ErbB4/HER4) Erythroblastic leukemia viral oncogene homolog 4. There are at least 6 known ligands including EGF, transforming growth factor-α (TNF- α) amphiregulin, epiregulin, betacellulin and neuregulin-1 (nrg1).

EGF receptors comprises of a ligand binding extracellular domain, a hydrophobic trans-membrane domain and a cytosolic tail with intrinsic receptor tyrosine kinase activity. Usually they exist as monomers but ligand binding facilitates dimerization. They form both homodimers and heterodimers (Yarden and Sliwkowski, 2001). On dimerization the two cytoplasmic tyrosine kinase domains reposition in order to facilitate trans-phosphorylation of the tyrosine residues. Subsequently, this provides docking sites for the sarcoma (Schmidt-Ruppin A-2) viral oncogene (Src) homology 2 (SH2) or phosphor-tyrosine binding (PTB) domains of intracellular transducers and adaptors proteins. Consequently, multiple pathways can be activated, namely; the Ras-mitogen-activated protein kinase (MAPK) cascade, the phosphoinoside 3-kinase (PI3K)/Atk

pathway, the Signal transducer and activator of transcription (STAT) transcription factors and the Src pathway (Schlessinger, 2002) .

MAPKs cascade, upon activation by the cell surface receptors, results in the regulation of cell proliferation and survival. The activation of EGFRs by auto or trans-phosphorylation recruits Growth factor receptor-bound protein 2 (Grb2) and Son of sevenless (Sos) to form a complex that binds with Src homology 2 domain containing transforming protein (Shc), an adapter protein (Madhani, 2001). The EGFR/Grb2/Sos complex thus formed facilitates interaction of membrane associated Ras with Sos. Ras is a guanine exchange factor, which is activated by exchange of Ras- bound GDP for GTP by this association. Activated Ras in turn activates serine/threonine kinase Raf-1 (Kerkhoff and Rapp, 2001). This phosphorylates Erk-1 and Erk-2, translocating them to the nucleus. Phosphorylated-Erks (pErk) when enters the nucleus regulate various nuclear transcription factors like Elk1, Ets2, c-myc etc. (Bogoyevitch and Court, 2004). Thus, pErk can be considered as a one of the read-outs for active EGF signaling.

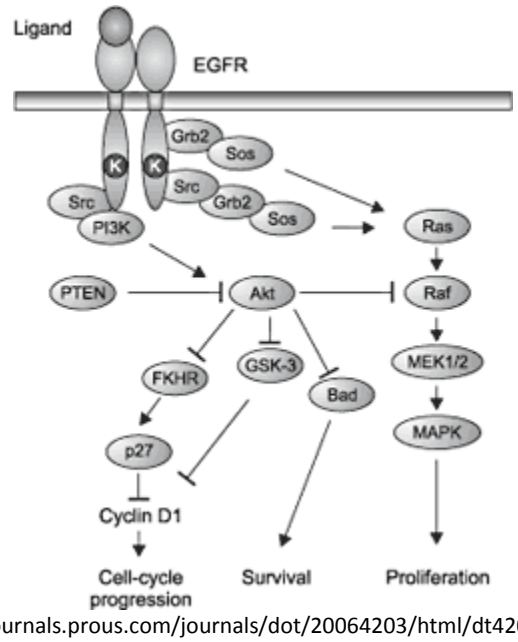

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Fig.2. Epidermal growth factor receptor (egfr) signal transduction. The egfrs dimerize on ligand binding and phosphorylate each other to activate various downstream pathways. They modulate cell proliferation, differentiation, maturation and also apoptosis.

In systems like drosophila, chick and mouse, role of egf signaling in development of various organs and tissues including epidermis has been demonstrated. The majority of the results were obtained using loss of function studies as over-expression of the egf receptors lead to lethality. These studies however elucidate the roles only in the later stages as the promoters used to generate knockouts (k5/k14) express in later stages. Zebrafish offers a simple system wherein one can investigate roles in the early development.

Materials and methods

1. **Zebrafish maintenance**

   For all experiment wild type (Tubingen) fish were maintained at 28.5℃. To obtain embryos, a male and a female were kept in a separate box with a barrier between them overnight. The fish mate and lay after the barrier is removed. The embryos collected, were maintained at 29.5℃ in E3 buffer till appropriate developmental stage of the experiments. For all RNA in situ hybridization experiments wild type albino fish were used.

2. **Glycerol and Methanol upgradation**

   Following steps were followed in serial up-gradation in methanol. For

   downgrading the same steps were followed but in reverse order.

   3x wash with PBS (PBST for embryos) @ RT, 10 min each

   Incubate in 25 % MeOH / 75 % PBS, 10 min @ RT

   Incubate in 50 % MeOH / 50 % PBS, 10 min @ RT

   Incubate in 75 % MeOH / 25 % PBS, 10 min @ RT

Incubate in 100 % MeOH, 30 min @ RT

Change solution to 100 % once more and immediately store @ -20 °C

Following steps were followed in serial glycerol up-gradation

Incubate in 25 % Glycerol/ 75 % PBS, 10 min @ RT

Incubate in 50 % Glycerol / 50 % PBS, 30 min @ RT

Incubate in 75 % Glycerol/ 25 % PBS, 2 hour @ RT

Store at 4 °C.

3. RNA isolation and cDNA synthesis

Wild type (Tu) embryos at 2 dpf were fixed in 500 μl trizol after dechorionation and stored overnight at -80℃. The embryos were crushed using an autoclaved pestle and trizol was added to makeup total volume of 1 ml. 0.2ml chloroform (per 1ml trizol) was added and shaken to mix well for 15 sec. centrifuge at 12000g for 15 min at 4℃. The upper aqueous layer was transferred to a fresh tube. 0.5 ml iso-propanol was added and incubated for 20 min at room temperature. Centrifuge at 12000g for 10 min at 4℃. The aqueous phase was discarded and the pellet was retained. Embryos were washed with 75% ethanol, and then centrifuged at 7500*g for 5 min at 4℃. the aqueous phase was removed and air-dried the pellet. To dissolve the pellet, 50 μl DEPC treated water was added. cDNA was synthesized using Invitrogen cloned AMV first strand synthesis kit. (Catalogue number- 12328-032)

4. Cloning *in situ* probes

Probes for *errb2* was synthesized from the following PCR combinations

ErbB2- forward 5'-TCC TCA GCT GGT GTG TTC AG -3'

reverse 5'-CCC ACA GGA AGA GTT GGG TA -3'

Probes were cloned using Invitrogen TOPO TA cloning kit (dual promoter) in pCR II TOPO vector.(catalogue number-K4610-20)

5. Probe synthesis

Probe synthesis was carried out using Roche DIG labeling kit (SP6/T7) (product number- 11175025910)**,** to transcribe the sense and anti-sense probes. The sense probes were used as controls. The synthesized probes were the purified using purification columns (Biorad). The probes were stored at -80^{o}C.

6. RNA *in situ* hybridization

24, 36 and 48 hpf stages were de-chorionated and fixed in 4% PFA in PBS overnight at 4℃. Embryos were washed twice with PBS for 5 minutes each at room temperature and serially upgraded to 100% methanol and stored at -20℃.

<u>Day1</u>

The embryos were serially downgraded from 100% methanol to PBS. Then were rinsed with PBS to completely wash away any residual methanol. To make the embryos permeable for the probes they were treated with 10μg/ml Proteinase K for embryos beyond 24 hpf stage (30 hpf- 30s, 36 hpf- 45s, 48 hpf- 60 s). Then were fixed in 4%PFA/PBS at room temperature for 20 min. the PFA was washed away with 1x PBST. Then were incubated in pre-hybridization buffer at 67 degree C for at least 2 hour. The DIG labeled probes were pre warmed in the pre hybridization buffer at 67 °C for 15 min and then incubated overnight at 67 °C with the embryos. (Keep the anti-sense and sense control in separate tubes.)

<u>Day 2</u>

Embryos were washed briefly in 50% formamide- 3X SSCT (Tween-20 0.1%) to remove excess probe. Then incubated in the same solution 3 times for 30 min each. Incubated in 50% formamide 2X SSCT 3 times 30 min each. Incubated in 2X SSCT for 30 min, following 0.2X SSCT for 30 mins. (Make sure to use different micropipette tips for sense and antisense tubes) All the SSCT incubations were done at 67°C. Embryos were then incubated for 10 min each 3 times in 1X PBST. Then, were washed with Maleic acid buffer (MAB) 3 times for 10 min each. Blocking solution was made in MAB. To resuspend the powder, it was kept at 67degreeC. Then embryos were incubated in blocking solution for 3 hour. Then the embryos were incubated overnight with anti DIG AP antibody in blocking (1:2000 dilution) at 4 °C.

Day3

To remove the antibody, the embryos were rinsed briefly with MAB. Then were incubated in MAB 3 times 30 min each. then were incubated in PBS 4 times 15 min each. Fresh staining solution was prepared and embryos were incubated in it 4 times 15 min each. the embryos were carefully transferred to a 12 well plate. 400 μl BM-purple AP precipitating substrate was added to the well. Before adding the container was shaken well and pre warmed for 30 min at 37°C. The well plate was covered by aluminum foil and incubated at 37°C till the coloration was satisfactory.

Later the embryos were fixed in 4% PFA/PBS overnight at 4°C.

Then, they were rinsed with PBS and gradually upgraded to 80% glycerol.

7. BrdU incorporation assay

To mark the cells in synthesis phase, we used BrdU incorporation assay. 10mM BrdU in 2% DMSO dissolved in E3 was used. The embryo were kept in this mixture for 2 hours and then washed with E3 and fixed over night at 4℃ in 4% PFA in PBS. Later the embryos were upgraded in 100% methanol and stored at -20℃. For the drug treatments followed by BrdU assay, the drug was added to the above BrDU solution. The DMSO was adjusted accordingly to make final 2% concentration.

For staining BrdU treated embryos, they were serially down-graded to PBS. Then treat with 4 N HCl for 20 minutes followed by 2 quick washes of PBS. Then we followed the protocol as described as given in immunohistochemistry section.

8. Immunohistochemistry

The embryos were serially downgraded to PBS from either 100 % methanol (for BrdU, Lgl2) or directly from 4 % PFA/PBS (pMAPk). Then washed 5 times 10 min each with 1XPBT and then incubated in 10% NGS in PBT for at least 3 hours at room temperature. Incubated in primary antibody with appropriate dilutions (anti BrdU 1:50, anti pErk 1:50) overnight at 4℃. Next day the embryos were washed with PBST 5 times 30 min each. Incubated the embryos in secondary antibody in 1% NGS in PBT for 4 hour at room temperature on a rotor. Then washed away the secondary antibody with PBT 5 times 15 min each. Fixed in 4% PFA/ PBS for 1 hour at room temperature. Then washed briefly in PBS for 20 min and then serially upgraded in 80% glycerol in PBS. Embryos were stored at 4℃.

antibody name	lot number	company	raised in	dilution used
anti-p63	MAB4135	Millipore; chemicon	mouse	1/100
anti-phospho ERK	02M4759	sigma	rabbit	1/50
anti-BrdU	SM1667P	Acris antibodies	rat	1/50
anti-Lgl2 serum (K128 1.B.4E)		synthesized in house	rabbit	1/400
anti-E-Cadherin	610182	BD transduction labs	mouse	1/100
anti- GFP		Bangalore GeneI	mouse	1/200

Table1. List of antibodies used.

9. Drug treatments

For all the experiments PD168393 (Merck Millipore product number 513033-1MG) drug was used at 10 µM concentration in 1% DMSO in E3. The embryos

were de-chorionated before the treatment. Treatment was started at 18hpf. For control the embryos were immersed in 1% DMSO.

10. Image analysis

Fixed samples were mounted in 80% glycerol with plasticine spacers between the cover slip and slide. Imaging of immunostainings was done over the head (dorsal head periderm) using the Zeiss LSM 510 Meta with Plan-apochromat 63X/1.40 oil or on Zeiss LSM 710 with Plan-apochromat 63X/1.40. A digital zoom of 2X was used for most images, except for area analysis where a 1.5X zoom was used. 1024 by 1024 image dimensions were used, with an averaging of 4.
Bright field images for *in situ* hybridization experiment were taken on OLYMPUS SZX12 with OLYMPUS Camedia c-5050 Zoom camera and DF PLAPO 1X PF lens. 1024 by 1024 image dimensions were used, with an averaging of 4.

11. Area analysis

Confocal stacks were captured at a slice interval of 0.373µM. To quantify surface area, cell outlines were traced in each slice by monitoring the Measure Stack plug-in of ImageJ. The perimeter in each slice was multiplied by the slice thickness (0.373µM) and added along with the area of the first and the last slice to obtain an estimate of total surface area. To quantify the area of the micro-ridges, images were smoothened and thresholded such that edges of the ridges were neatly defined. This was followed by using the Analyze Particle command to detect and measure edge lengths. Detection was monitored and aided by manual detection using Wand (tracing) tool wherever required. The perimeter of all the edges was summed up and multiplied with slice thickness and number of slices the ridges extend to in order to get an estimate of the area sequestered in the membrane folds.

12. 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_20 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_20 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 ddH2O. Adjust pH upto 7.0 with a few drops of concentrated HCl. Adjust volume to 1 L with ddH2O. Sterilize by autoclaving.

To make 2x, 0.2x or 0.05xSSCT, dilute 20xSC with ddH2O and add 2 ml Tween-20 per L.

10xMaleic acid buffer stock:

For 1 L of concentrated stock solution, begin with ~750 ml ddH2O 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 ddH2O 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 MgCl2, 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 MgCl2 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 MgCl2 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

1. Cloning of *erbb2* probe for in situ hybridization

ErbB2 and ErbB3 unlike ErbB1 and ErbB4 do not have both; the ligand binding and the catalytic domain. ErbB2 does not have a ligand binding domain while ErbB3 lacks catalytic domains. ErbB2 is the preferred partner for all other ErbBs to dimerize for signal transduction. Also ErbB2 has been shown to have some functions in the epidermis development as well. Besides, in zebrafish it has been shown to impart the oncogenic phenotype to the epidermis in *pen/lgl2* mutant (Reischauer S et al., 2009). We look first at expression analysis of ErbB2. For the purpose we cloned the probe using the primer combination as mentioned in the materials section. The probe length is 800 bp (Fig.3 a). The product was cloned in pCR II TOPO vector (invitrogen). The vector consists of dual promoters (SP6 and T7) on either strand, which was used to synthesize DIG labeled sense and anti sense RNA probes (Fig.3 b).

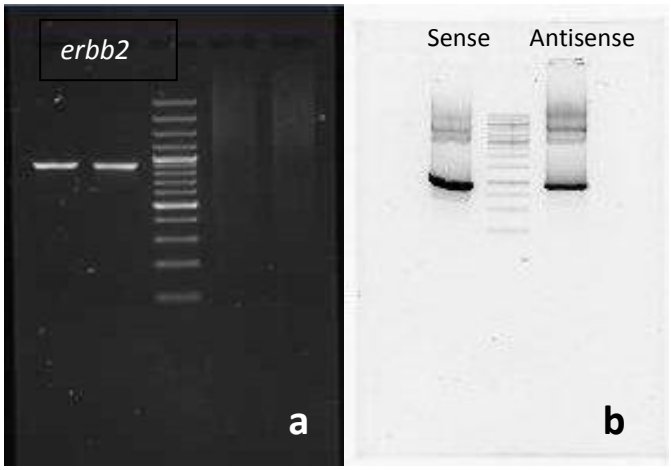
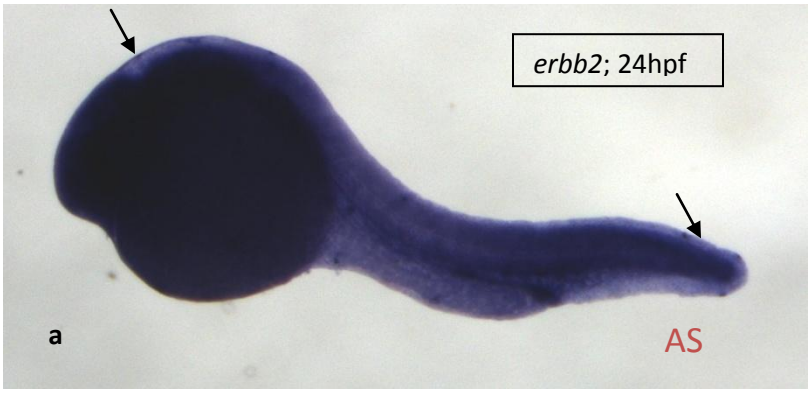


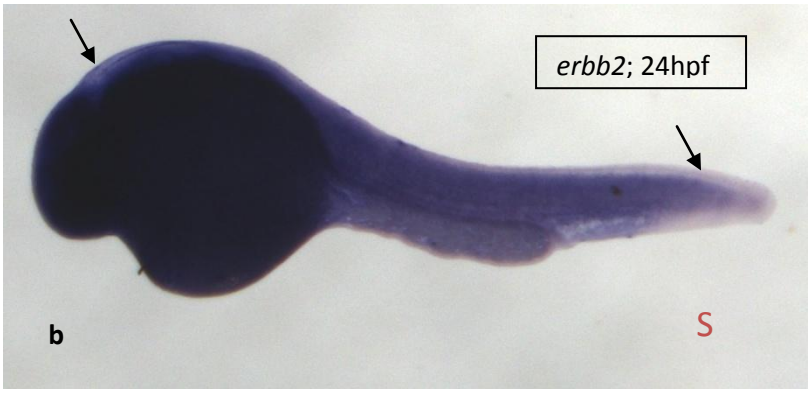
Fig.3. *Erbb2* probe synthesis. (a) shows PCR amplification of *erbb2* along with 100bp ladder. (b) shows synthesis of DIG labeled RNA probe along with 1kb ladder in the middle.

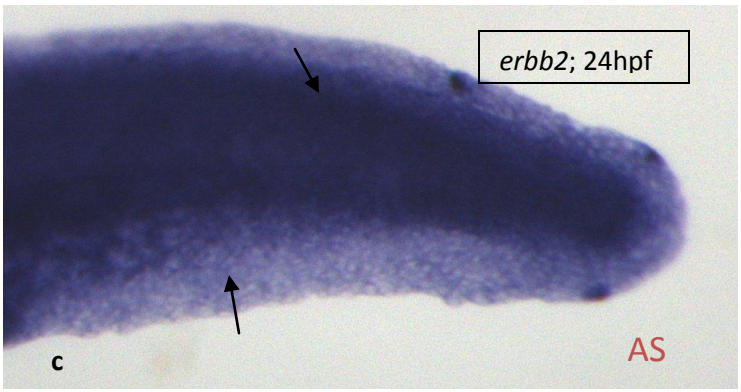
2. Temporal and spatial analysis of *erbb2* expression

We checked if the EGF receptor ErbB2 is expressed in the epidermis during early embryogenesis. We used RNA *in situ* hybridization to visualize the transcripts. DIG labeled antisense probes were synthesized *in vitro*. Anti-DIG antibody was used to visualize probe bound to the RNA transcripts.

ErbB2 transcripts are seen to be present in the epidermis, finfold, brain and the gut. The finfold is formed when an epidermal sheet folds to give it the structure. Thus staining in the finfold is a good indication of expression in the epidermis. This is indicated by arrow in (Fig.4 c,i,m). The staining is strong in early stages and is seen to recede by 48 hpf. Staining is also prominent in the epidermis above brain which is marked with an arrow in (Fig.4 a,e,g,k).



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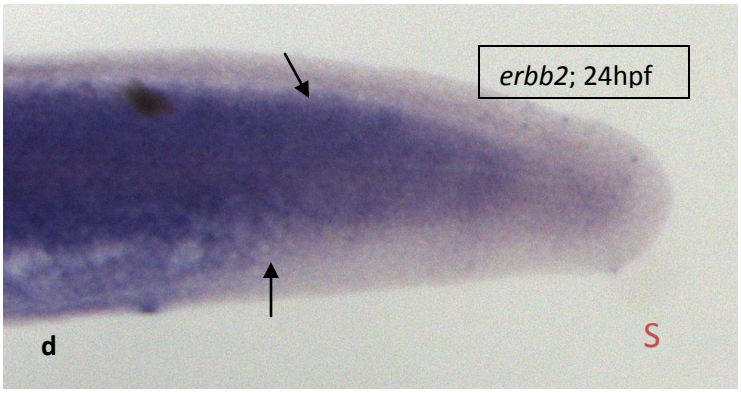
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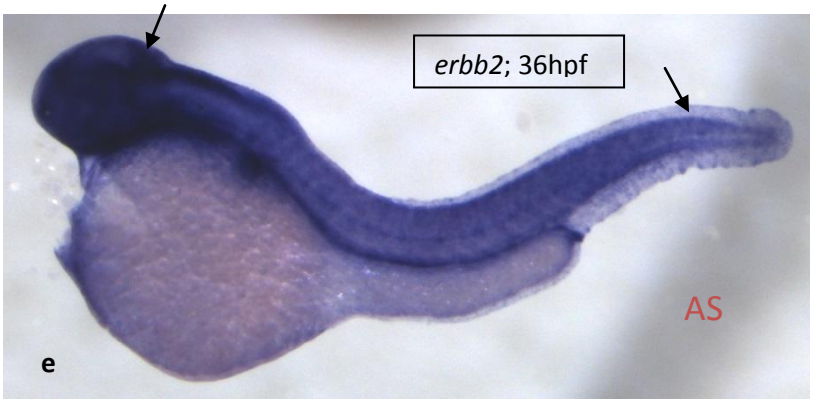
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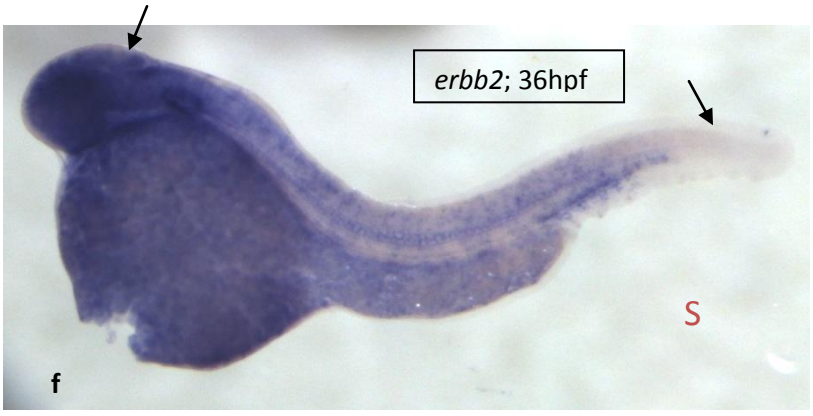
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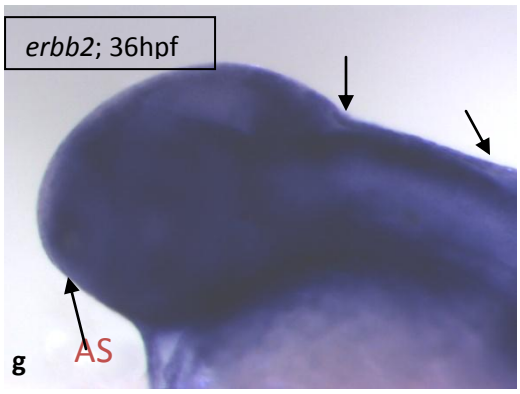
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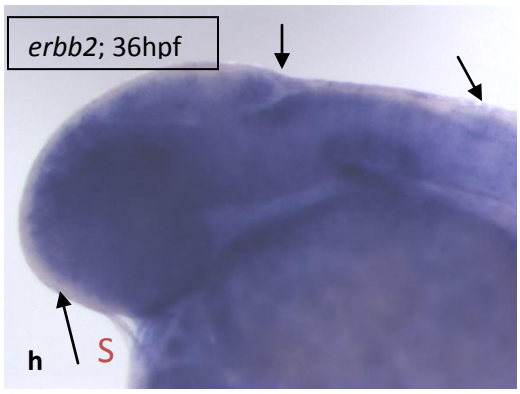
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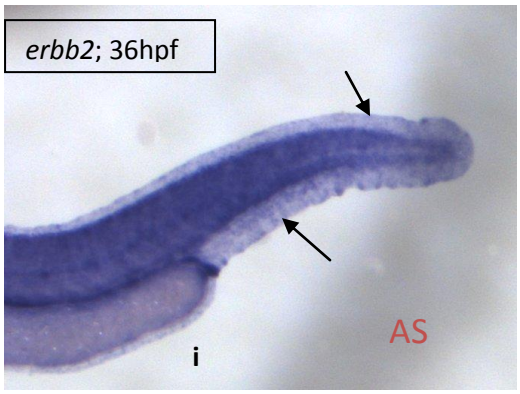
erbb2; 36hpf
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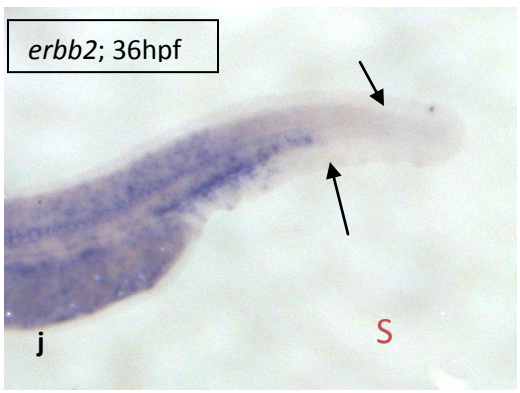
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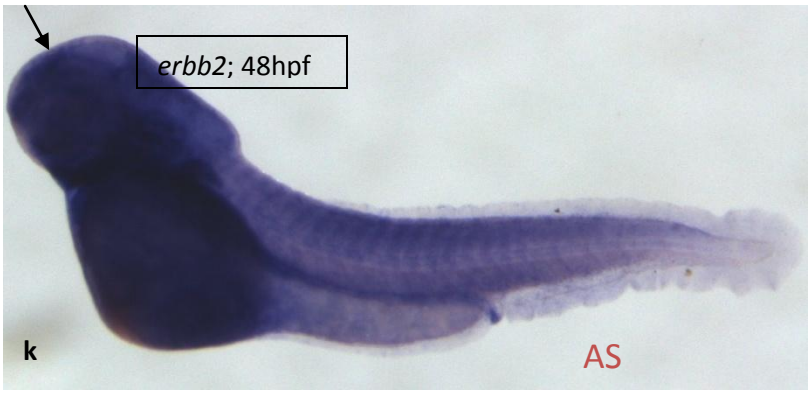
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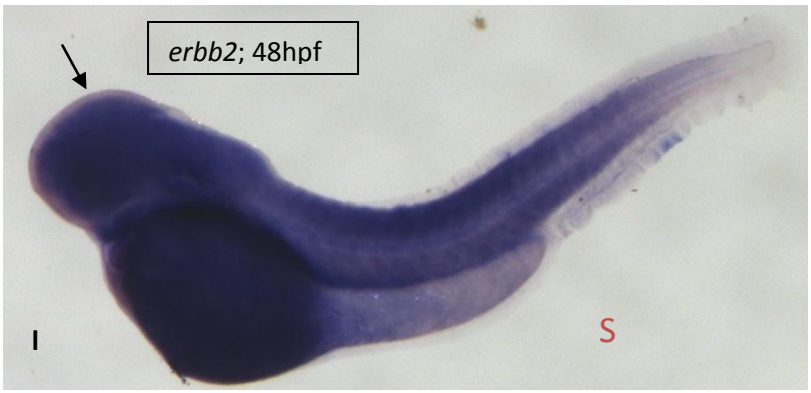
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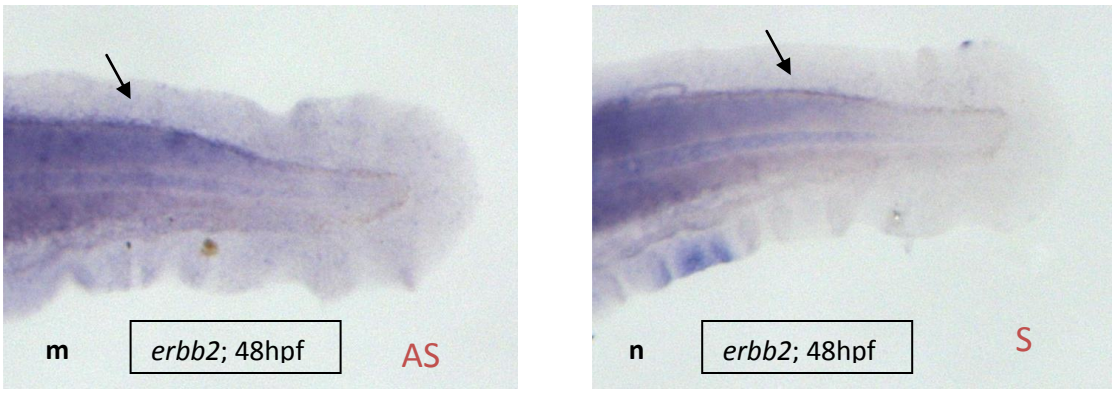


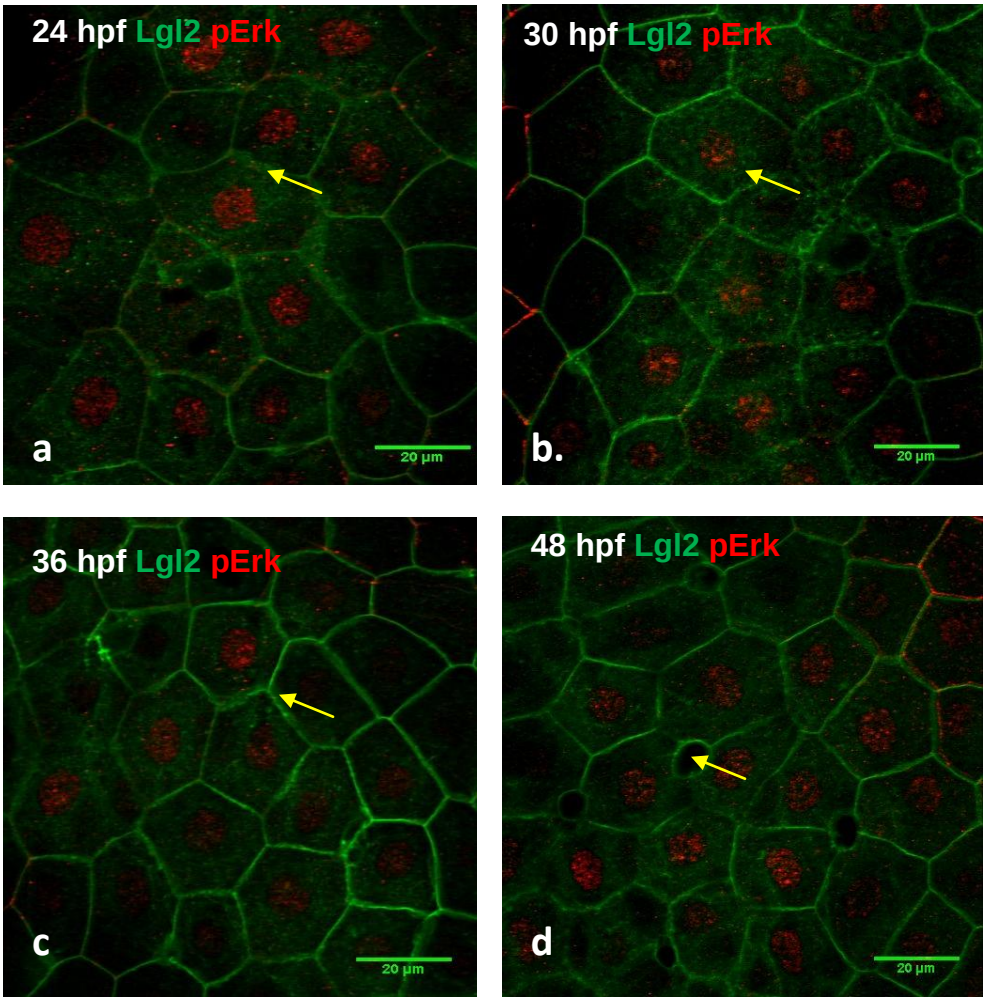
Fig.4. Whole mount *in situ* hybridization analysis showing *erbb2* expression in the epidermis. Views of the whole embryos from 24-48 hpf reveal expression in the epidermis above the head and the finfold as marked by the arrows (k,e,a). Other sites of *erbb2* expression include the brain and the presumptive gut. Close up images of the finfold are shown in (c,i,m) which clearly indicate strong expression in the stages 24 and 36 hpf but mild expression at 48 hpf. The epidermis over the head shows clear expression in zoomed in image (g). [AS-antisense, S- sense]

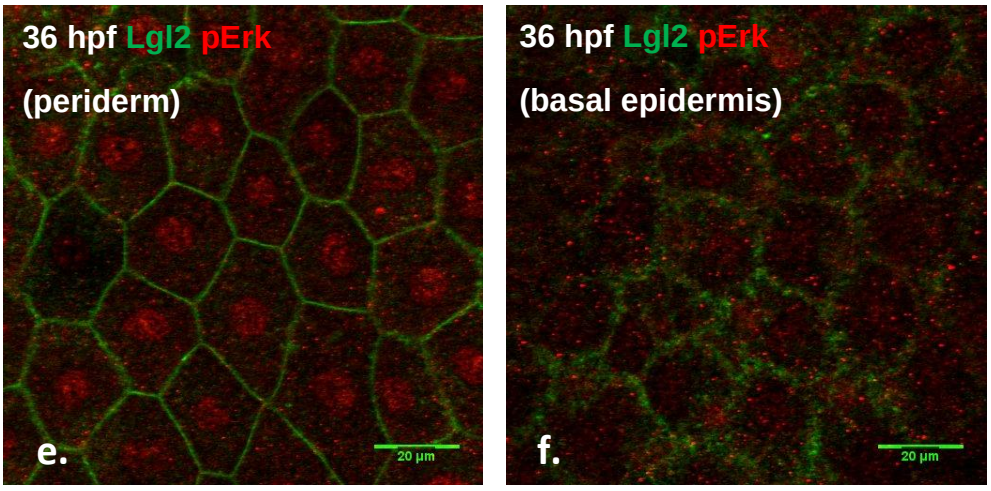
DIC Images for Erbb2 *in situ* hybridization taken on an Apotome microscope, reveal that Erbb2 is expressed in both the layers. Although presence of transcripts in both the layers is evident, it is really very difficult to comment any difference in the expression levels. For that, a careful histological study is required. Since Erbb2 is a receptor, the levels of its expression need not necessarily correlate with its activity. Thus, to check the activity we look at levels of its activated downstream molecule Erk (discussed in results section 3).

3. Analysis of pErk localization in the developing epidermis and its dependence on EGF signaling.

Presence of EGF receptors does not necessarily mean that the signaling is active. To check the activity of EGF receptors, I analyzed the levels of nuclear pErk. Erks are downstream of MAPK cascade which when activated by phosphorylation, are translocated to the nucleus. Thus, activity of EGF receptors can be estimated from levels of nuclear pErk. The wild type embryos were stained for pErk at developmental stages 24, 30 36 and 48 hpf and Lethal giant larvae 2 (Lgl2) was used as a basolateral marker of the epidermal cells. These

staining revealed that pErk was nuclear at the stages examined, indicating that EGF signaling is active in the periderm at the early developmental stages (Fig.5). The levels of nuclear pErk were not significant in the basal epidermis as compared to the periderm. This is evident in the (Fig.5.e,f) where embryos at 36 hpf were stained for pErk and Lgl2 was used as basolateral membrane marker which marks both the layers: periderm and basal epidermis.





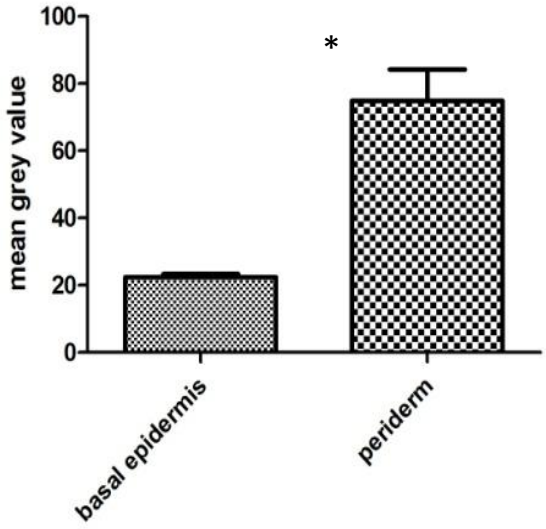


Fig.5. Localization of pErk in the periderm. Activation of EGF receptors results in phosphorylation Erk1 which is translocated to the nucleus and modulates expression of its target genes. Thus nuclear localization of pErk suggests active EGF signaling. The figure represents a time series over 4 developmental stages indicating active EGF signaling in the epidermis. In the figure Lgl2 is used as a baso-lateral marker to specify the periderm. The basal epidermis however, does not show any nuclear localization of pErk. Both the images (Fig.5 e,f) are taken in the same frame but different z-stacks. (*ttest,p,0.01, error bar indicate mean and standard error)

A way to understand any biological system is to perturb it and ask what the consequence of the perturbation is. This perturbation can either be external (mechanical stress, chemical alterations in the external milieu) or internal (mutation or knockdown of a gene function). I have tried to use similar strategy for my masters' project. I inhibited the EGF receptors using an inhibitor drug and assessed its effects. As mentioned earlier, EGF receptors are RTKs, which have a cytosolic kinase domain. I used a drug, PD168393 (PD) which alkylates Cys773 residue of this catalytic domain of EGFR, to inhibit its activation. It irreversibly inhibits the receptors as the modification disables the binding of ATP which is essential for phosphorylation and hence activation of the EGF receptors. I checked the effect of PD treatment on levels of pErk at 36 hpf stage. I observed that the nuclear pErk is considerably lowered in the embryos treated with PD as compared to the control untreated embryo (Fig.6).

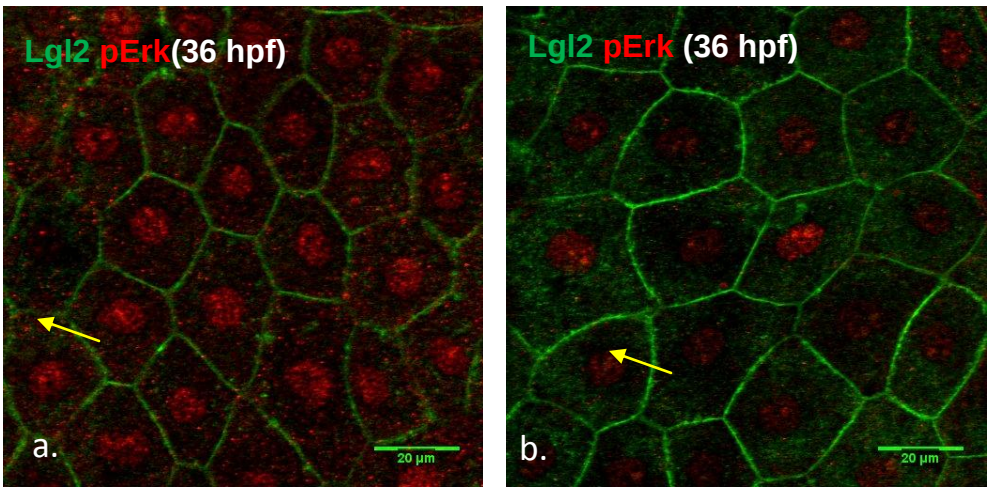


Fig.6. Effect of inhibiting EGFRs on nuclear pErk staining. To check if PD treatment inhibits the EGFRs, levels of nuclear pErk were monitored. The treatment shows a considerable reduction in levels of nuclear pErk as seen in (b) as compared to DMSO control (a).

4. EGF signaling regulates cell proliferation in the periderm.

To check effect of inhibiting EGF receptor activation on proliferation we use Bromodeoxyuridine (BrdU) incorporation assay. BrdU is a synthetic nucleotide analogous to thymidine. While the cell is in the synthesis phase of the cell cycle, BrdU gets incorporated in the DNA. One can detect its presence using immunohistochemistry. I started the PD drug treatment at 18 hpf and continued till 27 hpf. Then treated the embryos with BrdU for 2 hours, then rinsed with E3 buffer and fixed in PFA (detailed protocol in methods section). I quantified the BrdU lablled cells in the periderm of total of 15 embryos each (PD treated and untreated) from three different experiments. I observed that there was a considerable reduction in number of BrdU labeled cells in PD treated as compared to control untreated embryos (Fig.7 a). This effect was seen specifically in the periderm (Fig.8 c, Fig.7 c). To confirm whether there is any effect on the basal epidermis, I performed triple labeling including BrdU, Lgl2 and P63- a transcription factor essential for maintenance of stem cells in the basal epidermis as the third marker (only p63 and BrdU staining shown). The proliferation in basal epidermis was unaffected due to the drug treatment (Fig.8 a-c). These data suggest that EGF signaling is responsible in regulating proliferation in the periderm. The proliferation in PD treated periderm is seen to drop considerably as compared to control (Fig.7 a-c).

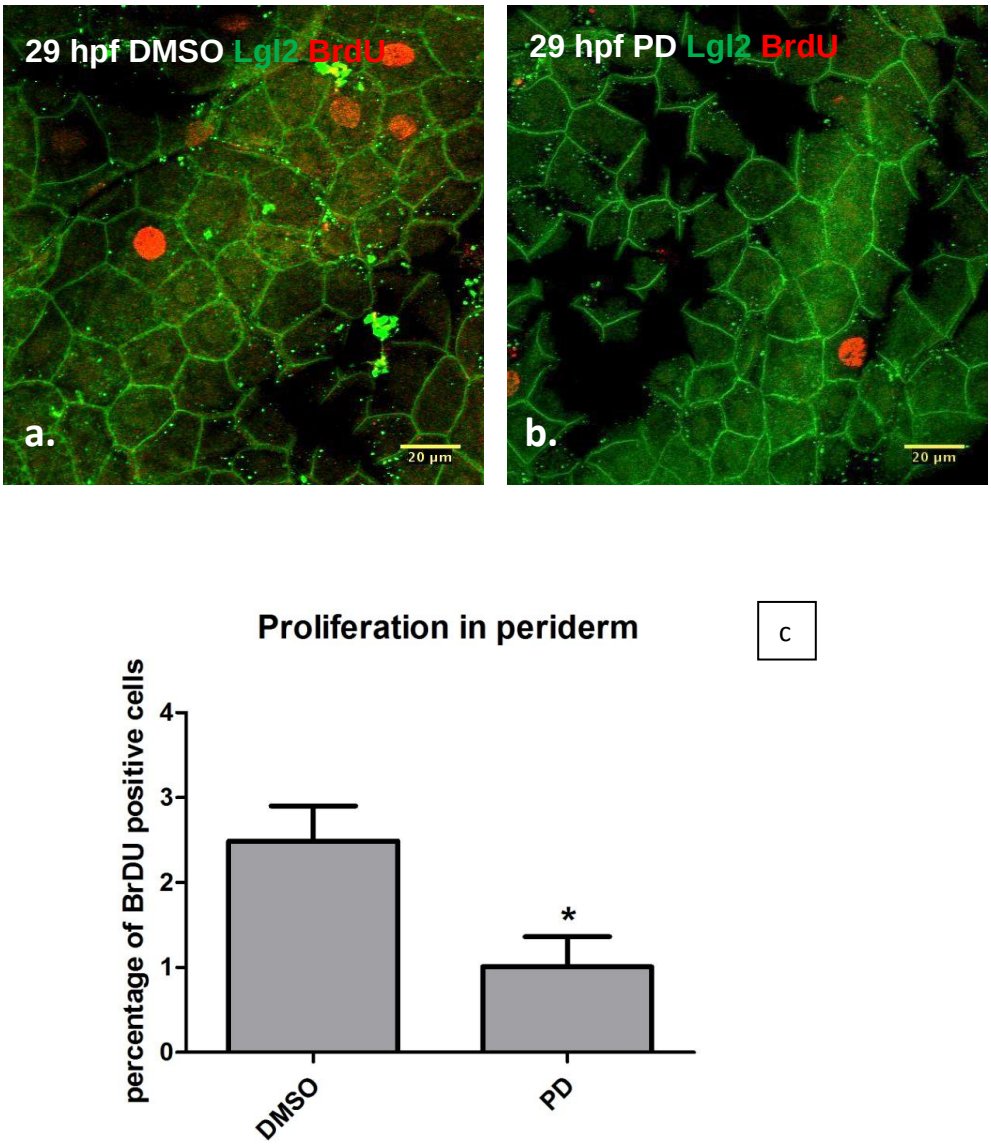


Fig.7. Effect of PD treatment on proliferation in the periderm. BrdU marks all the nuclei in the S-phase during the treatment. After PD treatment number of proliferating cells decreases as compared to the DMSO control (Fig.7.a,b). The graph (Fig.7.c) shows percentage of BrdU positive cells in the treated and control embryos. There is a considerable decrease in number proliferating cells in the periderm (c) in the PD treated embryos (*ttest, $p<0.0001$,error bar represents mean and standard error)

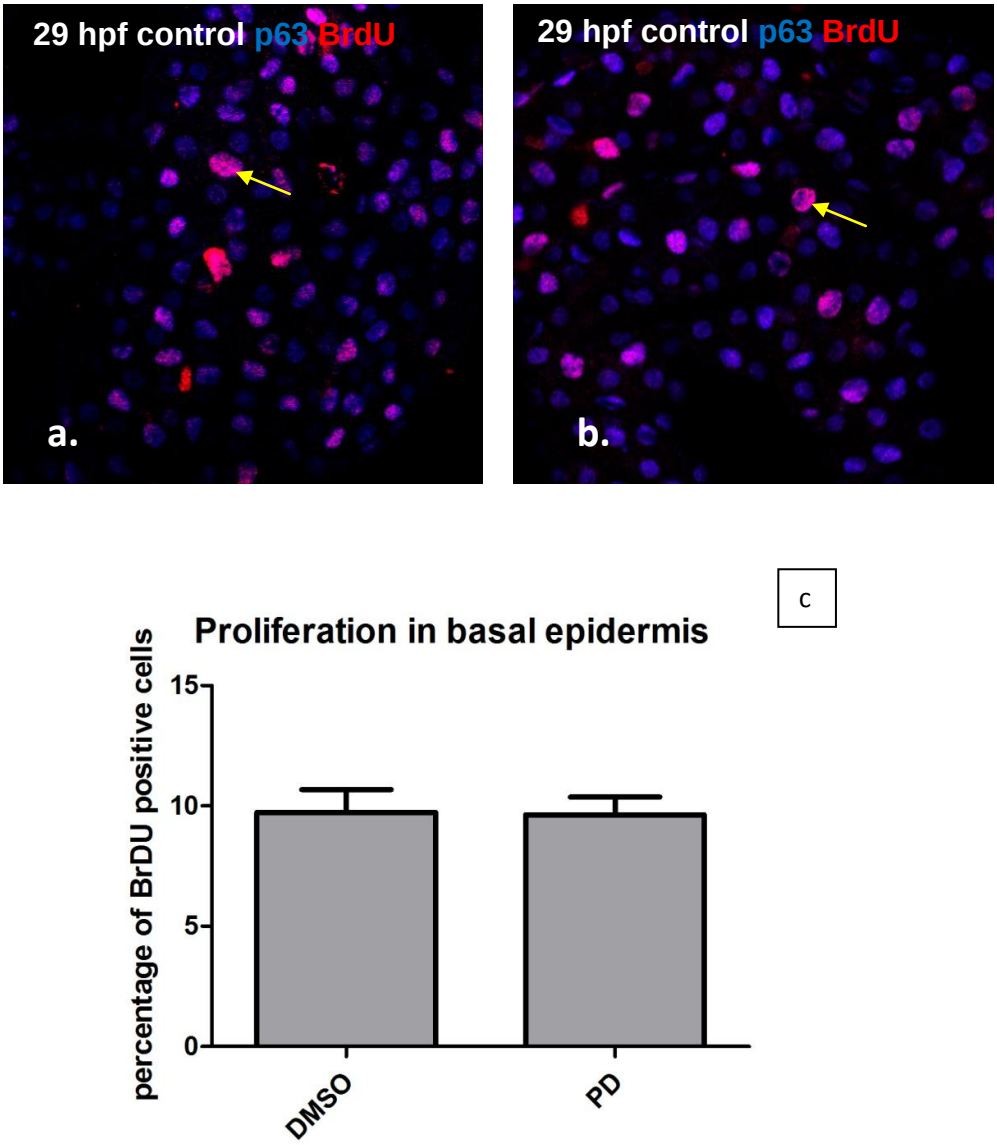
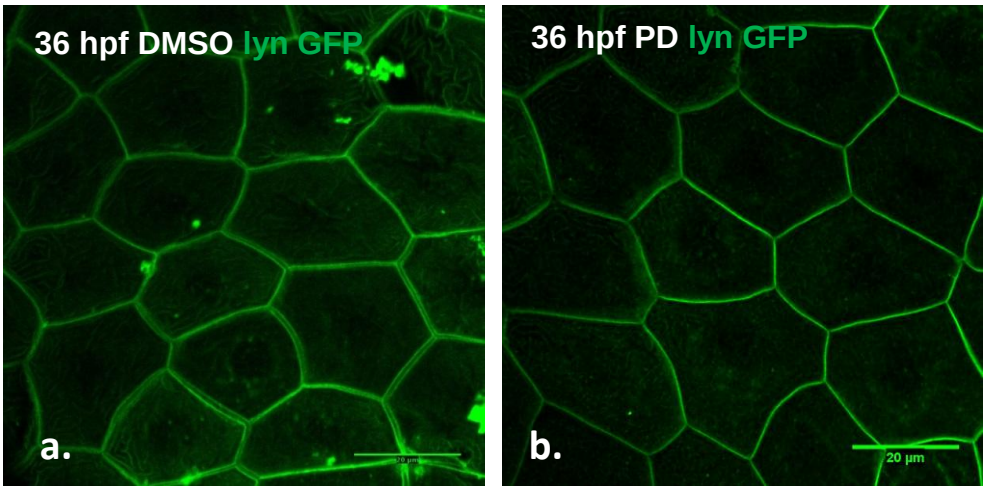


Fig.8. Effect of PD on proliferation in the basal epidermis. The figure represents overlay of p63 and BrdU in the basal epidermis. There is no effect of PD treatment on proliferation in basal epidermis (a,b). Anti-p63 antibody marks the cells of the basal epidermis. The graph (c) represents percentage of BrdU positive cells in the basal epidermis. There is no significant change in the BrdU count in the basal layer after PD treatment (ttest, $p>0.05$, error bar represents mean and standard error).

5. Inhibition of EGF signaling leads to increase in cell size in the periderm.

To quantify the cell surface area I used a transgenic line where lyn-GFP is cloned with *Claudin* promoter. It marks the cell membrane including the vesicles, ridges and the plasma membrane. Fig.9 shows peridermal cells at stages 36 and 48 hpf. PD treated embryos have cells with larger surface area (Fig.9 b,d) as compared to the DMSO control (Fig.9 a,c). We quantified the cell surface area at 36 hpf and 48 hpf (Fig.9 e,f). We treated the embryos in 3 different set and scanned 5 embryos with 5 cells in each set. It was observed that the cell surface area is greater in PD treated as compared to the untreated control.

Increase in the cell surface area after PD treatment becomes significant only by 36hpf and not in earlier stages. But effect on proliferation is clear in early stages (result section 4). Thus decrease in proliferation precedes increase in the surface area. It might be possible that this increase seen after inhibiting EGF signaling might be a compensation for reduction in proliferation



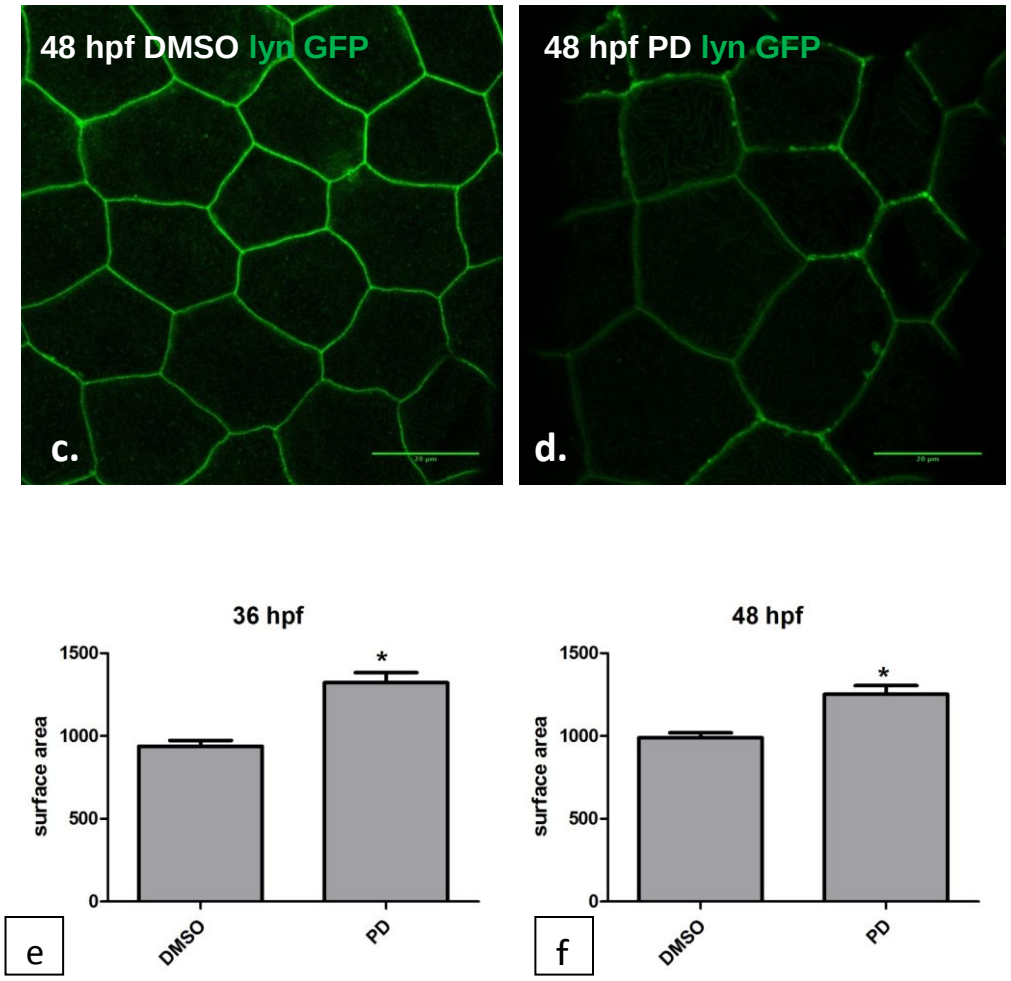


Fig.9. Effect of PD treatment on the surface area of the periderm. lyn GFP marks the membrane in the epidermal cells. The figures show the periderm at 2 stages 36 and 48 hpf. The surface area after PD treatment (b,d) is greater as compared to the control (c,d). The graphs represent the mean surface area of the peridermal cells at 36 hpf (e) and 48 hpf (f) respectively. It represents not just the cross-sectional area but the total surface area. In both the stages the surface area is greater in case of PD treated as compared to DMSO control (a,c). (*ttest, $p<0.0001$, error bars indicate mean and standard error)

Discussion

The mammalian epidermis is a stratified tissue consisting of basal, spinous, granular and cornified layer. The basal layer in adults divides and the progeny contributes to the remaining differentiated layers. As the newly divided cell from basal layer migrates towards the most superficial layer, it undergoes change in the keratin composition and finally differentiates. Many factors like p63, Krupple like factor proteins and inhibitor of NF-kB kinase are crucial for the stratification (Bakkers et al., 2002; Katz et al., 2002). It is seen that the skin regains this structure even after a wound is caused which is possible due to machinery that detects the disruption, regulates the proliferation appropriately and brings the system back to its original form. However, it is not clear how the proliferation is regulated in the epidermis during early development when the tissue has just two layers- basal epidermis and periderm.

In zebrafish embryonic epidermis both the peridermal and basal epidermal cells divide up to 36 hpf (Sonal, Sidhaye et al, unpublished). Beyond this stage peridermal cells cease to divide whereas basal epidermal cells remain proliferative. The mechanisms that regulate the proliferation in these two layers are not clearly understood yet. It is still unclear if similar mechanisms exist for the both layers or there are different mechanisms for the basal layer and the periderm. My results suggest that the regulation of cell proliferation via EGF signaling is specific to only the periderm. Effect of inhibition of egf receptors is seen only in the periderm. It is intriguing that the control of proliferation is distinct in the two closely associated layers of the same tissue and warrants further investigation.

In situ hybridization studies suggest that *erbb2* is expressed in the epidermis. Erbb2 does not have a ligand binding domain; hence it has to interact with other egf receptors for signal transduction. Inhibiting EGF signaling results in decrease in cell number and increase in cell surface area. This suggests that the tissue maintains a balance between its number and cell size. The primary role of the epidermis is to cover

the entire organism. If the cell number reduces due to some reason (using inhibitors) the tissue has a mechanism to detect this and increase the cell size.

Control of cell size and number is not just restricted to the epidermis; all the organs need to have this balance (Stanger, B. Z et al., 2008; Pan, D et al., 2010; Leevers, S. J. & McNeill, H et al., 2005). The mechanisms that modulate this may be similar in all organs. In my opinion the tissue tries to optimize not in accordance to biochemical cues but to a certain physical quantities like tension and shear stress. By this logic every cellular behavior including size control should be tangled with such physical parameters. One hypothesis can be that activation of EGF signaling is coupled with adherens junctions to modulate the balance in cell size and number under physical stress. It has been previously show that E-cadherins interact with RTKs (Renshaw et al., 1997). It is an open question whether E-cadherins can modulate the cell surface area and cell number to maintain the tissue homeostasis via RTKs like EGF receptors. Zebrafish is an excellent model to investigate this issue further.

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