Investigating the Cellular and Molecular Mechanisms of Wound Healing in Oocytes and Embryos

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Investigating the cellular and molecular mechanisms of wound healing in Xenopus oocytes and embryos

Short title: Wound healing assays in Xenopus

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Abstract

The African clawed frog *Xenopus* has remarkable capacities to heal wounds rapidly and to regenerate complex tissues. Due to its experimental tractability, studies using Xenopus oocytes, embryos and larvae have contributed extensively to our understanding of the molecular and cellular mechanisms underpinning wound healing and tissue regeneration (Li et al. 2016). In this protocol, we describe wound healing assays following mechanical or laser injuries of oocytes and multicellular epithelia in *Xenopus laevis* embryos. We also explain how to perform assays aimed at investigating the cellular and molecular events during wound healing, including gene knockdown and overexpression experiments. In the latter assays, we explore the use of biochemical pulldown assays to investigate the activity of Rho GTPases, as well as the injection of mRNAs encoding fluorescent proteins or probes, followed by quantitative confocal image analyses to assays the dynamics of cytoskeletal components and their regulators.

Materials

Reagents

Marc's Modified Ringer's (MMR) solution (1X recipe: 0.1 M NaCl, 2 mM of KCl, 1 mM MgCl2, 2mM CaCl2, 5 mM HEPES (pH 7.5 at 23ºC)).

Normal Amphibian Medium (NAM) solution (10X recipe: 1.1 M NaCl, 20 mM KCl, 10 mM Ca(NO3)2, 10 mM MgSO4, 1 mM EDTA, 10 mM NaHCO3, 20 mM sodium phosphate pH 7.4)

Freon-113 (CAS no. 76-13-1) (substitutes: carbon tetrachloride (CCl4) (Sigma 319961, CAS no. 56-23-5), or 1, 1, 2, 2-tetrachloroethane (C2H2Cl4) (Sigma 185434, CAS 79-34-5)).

Lysis buffer: 50 mM Tris-HCl pH 7.5, 10 mM MgCl2, 100 mM NaCl, 1% Nonidet P-40, 5% glycerol, 1 mM DTT. 1 tablet of cOmplete mini Protease Inhibitor (Roche 04693124001) and 1 tablet of PhosStop Phosphatase Inhibitor (Roche 04693124001) per 10 ml. Make fresh and keep on ice until use.

GST binding buffer: 25 mM Tris-HCl pH 7.5, 30 mM MgCl2, 40 mM NaCl, 0.5% Nonidet P-40, 1 mM DTT. 1 tablet of cOmplete mini Protease Inhibitor and 1 tablet of PhosStop Phosphatase Inhibitor per 10 ml. Make fresh and keep on ice until use.

GST wash buffer: 25 mM Tris-HCl pH 7.5, 30 mM MgCl2, 40 mM NaCl. Make fresh and keep on ice until use.

Glutathione Sepharose 4B beads (GE Healthcare 17-0756-01).

GTPγS (Sigma-Aldrich G8634)

GDP (Sigma-Aldrich G7127)

Constructs: details of a suggested selection of plasmids including pCS2-GFP/mCherry-moesin, pCS107-Rhoa, pCS107-Pak1-GST, pCS107-rGBD-GST can be found in Li et al. (2013) and Soto et al. (2013). All published plasmids have been deposited in Amaya lab repository and are available upon request.

Antibodies: Rac1/2/3: Cell Signaling #2465; Cdc42: Cell Signaling #2462; RhoA: Santa Cruz SC-179

Equipment

Dumont #5 fine forceps

Petri dishes with or without glass bottom

Refrigerated centrifuge

Dissecting microscope with LED illumination and a 10x-75x zoom range

Confocal microscope with a Micropoint pulse ablating laser

Method

Mechanical wounding assays using Xenopus embryonic epithelia

1. Culture embryos in 0.1 x MMR at either room temperature or 16^{o}C until the blastula or tailbud stages. If gain or loss of function experiments are desired, inject 1-2 cell stage embryos with antisense morpholino oligonucleotides or *in vitro* transcribed mRNAs targeting or encoding the gene products of interest, respectively, prior to raising the embryos to the blastula or tailbud stages. For drug treatment assays, pre-incubate embryos for 30 min before wounding experiments.
2. Transfer embryos to 75% (vol/vol) Normal Amphibian Medium (NAM) containing 0.2% BSA in an agarose dish.
3. For blastula stage epithelial wounding experiments, use clean fine forceps to remove the vitelline membranes approaching from the vegetal side (unpigmented side) of the embryo at Stage 8 to limit damage to the animal (pigmented) side of the embryo.
4. Carefully remove a desired area of the superficial / outer layer (pigmented) of the animal side (as a reference, 8-10 cell diameter area), leaving the deep layer (unpigmented or lightly pigmented) intact (Figure 1A and B).
5. For tailbud stage epithelial wounding experiments, use clean fine forceps to remove vitelline membrane from the embryo.

6. Remove, pinch or puncture a desired area of the epithelium in the flank of the embryo (as a reference, 1/3 the length of the trunk of the embryo).

   When regions of the epithelium are removed, it is common to remove some of the yolk cells under the skin. This should have minimal affect on the experiment. However, the size of the wound in step 4 and step 6 should be kept as consistent as possible to ensure consistent results between replicates and reliable comparisons between different treatments.

7. Leave embryos in 75% NAM throughout the experiment.
8. To assess the overall effect of a gene or a drug on wound healing, observe wounded embryos using a dissecting microscope or low magnification (10x or 20x) objectives on a confocal and image every 20 min. Wound closure under a stereoscope can be measured by outlining the leading edge of the epithelium (Figure 1E). Normally the leading edge should be clear to see due to the colour difference between the two epithelial layers, but in case it is difficult to locate the edge, embryos can be fixed at the end of experiments and stained by phalloidin to show the wound edge. For confocal imaging, embryos are injected with mRNAs of proteins that either localize to the cell membrane or bind the F-actin. Both outline the leading edge during wound healing. Completion of wound closure takes a few hours, examples of successful and incomplete healing are shown in Figure 1E. To observe the more rapid dynamics of the cytoskeleton or signalling events, use a 60x objective on a confocal and image every 2 min. Depending on the process under investigation, images may need to be taken more or less often to capture its dynamic nature.

   Embryos can be injected with moesin-gfp to observe actin dynamics (Li et al. 2013), gfp-α-tubulin to observe microtubule dynamics (Woolner and Papalopulu 2012)*, GEM-GECO or C2-mrfp to observe calcium dynamics* (Clark et al. 2009; Soto et al. 2013)*, or gfp/mcherry-caax to label the plasma membrane and thus, outline the cell boundaries* (Li et al. 2013)*.*

Laser wounding assays in Xenopus oocytes and embryonic epithelia

9. For oocyte laser wounding assays, prepare oocytes as described in (Sive et al. 2000). Inject morpholino or mRNAs as desired, and culture the oocytes at 16°C until needed.
10. For embryonic epithelial laser wounding assays, inject embryos with morpholinos or mRNAs at the 1-2 cell stage, culture until Stage 9 in 0.1 x MMR.
11. Mount oocyte or embryos in a glass bottom dish or coverslip-sealed imaging chamber (Figure 1C and D) in 75% NAM. Fit the dish or imaging chamber on a confocal stage.
12. Make laser wounds on the surface of the oocyte or the embryo. The wound healing process can be observed and recorded immediately post laser wounding.

As guidance, we used a Micropoint pulse nitrogen pumped dye laser (Laser Science, Inc.) to wound the specimen in our experiments. A possible combination of pulsed laser settings to ablate the tissue is: 561 nm laser wavelength, power 40 and pulse 10 on Micropoint. The user is advised to adjust the settings and equipment to fulfil the requirement of his/her experiments. Take images as frequently as possible being conscious to ensure minimal photo-damage, while capturing the dynamics of the proteins or signals under investigation.

GST pull down to quantify activation of small Rho GTPases; Rac, Cdc42 and RhoA

13. Inject embryos at 1-2 cell stage with desired mRNAs. Grow embryos to Stage 8 or 9 in 0.1 x MMR.

    To detect Rac1 and Cdc42 activities, inject 500 pg mRNA of pak1-gst. To detect RhoA activity, co-inject 500 pg mRNA of egfp-rhotekinGBD and 125 pg rhoa mRNA (Li et al. 2013).

14. Resuspend and transfer 300 µl Sepharose Glutathione beads into 1.5 ml Eppendorf tubes. Centrifuge at 1,000 rcf for 30 sec at 4°C. Remove the aquaeous phase at the top without disturbing the bead pellet. Wash the beads with 300 µl binding buffer three times at the same centrifuging speed and temperature. Add a final volume of 150 µl binding buffer to resuspend the beads. Place on ice until use.

15. Wound Stage 8 or 9 embryos with forceps in 75% NAM. Collect 30-50 embryos per condition or time point in 1.5 ml Eppendorf tubes, remove as much liquid as possible, and place the tubes on ice. Homogenise each tube of embryos in 500 µl freshly prepared and pre-chilled lysis buffer, centrifuge at full speed (>16,000 rcf) for 15 minutes at 4°C.

16. Transfer the supernatant to another pre-chilled Eppendorf tube. Add 200 µl pre-chilled Freon 113 into each tube, vortex 15 seconds, and centrifuge at full speed for 10 minutes at 4°C. Transfer the upper aqueous phase into a new Eppendorf tube (450 µl approximate total volume), take 5% volume (~9 µl) as input.

    Note: If Freon is not available, carbon tetrachloride (CCl4) or 1, 1, 2, 2 tetrachloroethane (C2H2Cl4) can be used as substitutes.

17. Add equal volume (450 µl) binding buffer and 50 µl pre-washed beads into each tube. Incubate on an end-to-end nutator for 30 min (for Rac1 and Cdc42) or 1 h (for RhoA) at 4°C.

18. Centrifuge the tubes at 1,000 rcf for 30 sec at 4°C. Remove the buffer, wash the beads 1 time with 500 µl pre-cooled binding buffer, and 2 times pre-cooled wash buffer. DO NOT pipet but gently invert the tube 3-4 times to mix. Centrifuge at 1, 000 rcf for 30 sec at 4°C at each step.

19. Levels of active Rac, Cdc42 and RhoA are detected using western blot, described in detail in Li et al., (2013).

Discussion

We presented three protocols to assay wound healing in Xenopus oocytes or embryos: mechanical wounding in macroscale, and laser wounding and GST pull down assays at the cellular and molecular level.

Mechanical wounding and observation (steps 1-8) is the most accessible protocol of the three. When combined with gene knockdown/knockout or chemical treatment, it provides a quick and robust assessment whether a certain gene, protein, or signalling pathway is involved in wound healing (Li et al. 2013; Soto et al. 2013; Li et al. 2016). Nonetheless, it does require some practice to make the size and depth of wounds consistent, which may affect the speed and quality of healing.

Laser wounding (steps 9-12) has two major advantages. First, since the wounding laser is normally mounted on or a part of a confocal or multiphoton system, it is normally easy to injury and immediately image using the same system. Second, the observation of the cellular and molecular events can be started seconds or even milliseconds post wounding, which cannot be achieved after mechanical wounding. For this reason, laser wounding has been the method of choice in a variety of experiments to assess the molecular and cellular bases in Xenopus wound healing (Clark et al. 2009; Burkel et al. 2012; Soto et al. 2013; Davenport et al. 2016). Specifically, membrane dynamics, cytoskeletal dynamics, and how they are regulated by small Rho GTPases and their effectors near the wound edge were studied using single cell laser wounding model (Davenport et al. 2016; Burkel et al. 2012). Multicellular cytoskeletal networks and signal propagation to mobilise a sheet of epithelium in multicellular wound healing were studied using embryonic wounding model (Clark et al. 2009; Soto et al. 2013). On the other hand, the challenge of this protocol is to maintain normal tissue growth or vitality over time, particularly in an experiment lasting hours. Therefore, tests and calibrations of tissue health should always be carried out before an experiment.

In the past decade, fluorescence resonance energy transfer (FRET)-based tools to measure activation of small Rho GTPases have been well developed (Fritz and Pertz 2016), especially in cultured cells (Santiago-Medina et al. 2012). However, the use of FRET tools in Xenopus embryo is still limited (Yamashita et al. 2016), making biochemical approaches, such as active Rho GST pulldown assays (steps 13-19), a valid and powerful method to examine the dynamics of these signalling molecules. Also, because the readout of this assay is based on a collection of embryos, individual effect is reduced to the minimum, giving a more robust measurement of the activity of the detected molecules. A limitation of this protocol for the time being is that there is a limit of available antibodies in

Xenopus, thus, detection of endogenous proteins pulled down is not always possible. In this case, overexpression of tagged target proteins can be used as an alternative option for the measurements.

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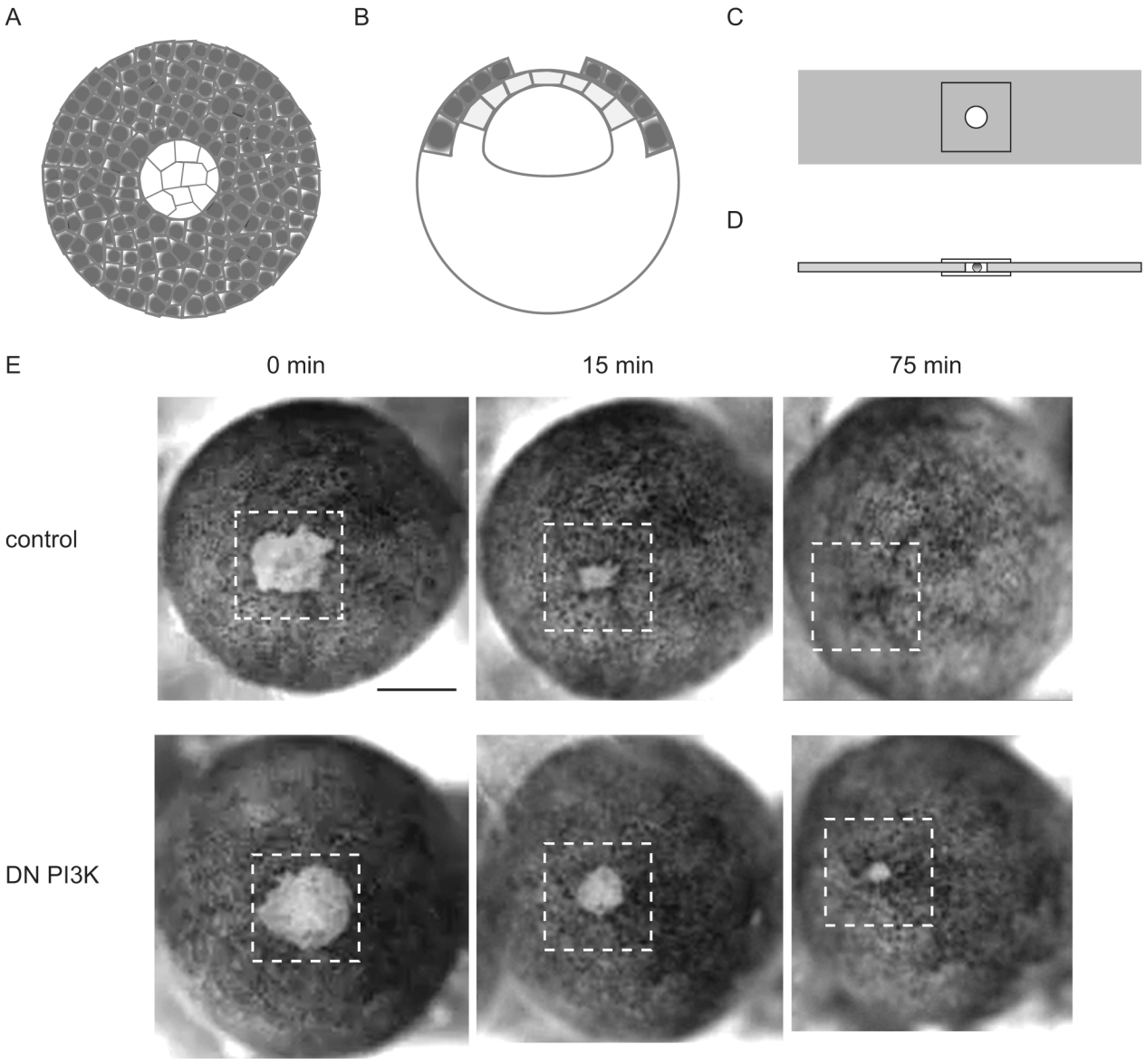
Figure legend

Figure 1. Wounding and observation of embryonic epithelium. A. Top view of a superficial wound on the animal side of a blastula embryo. Pigmented cells are of the superficial layer, and non-pigmented cells are of the deep layer, which is kept intact in the experiment. B. Transverse side view of a superficial wound on a blastula embryo. C. Self-made imaging chamber for laser wounding and observation. The big (grey) slide is made of steel, with a hole in the middle. D. Side view of the chamber, with an embryo mounted inside the hole and sealed from both sides with coverslips. E. Sample pictures of wound healing in control and dominant negative PI3K overexpressing blastula

stage embryos. Note that, while control wounds heal completely by 75 minutes post injury, DN PI3K expressing wounds maintain incompletely healed wounds at 75 minutes post injury. Wounded areas are highlighted in dashed squares. Scale bar – 200μM.


A
B
C
D
E
0 min
15 min
75 min
control
DN PI3K