Submission ID #: 62782
Scriptwriter Name: Madhulika Pathak
Supervisor Name: Swati Madhu
Project Page Link: https://www.jove.com/account/file-uploader?src=19148558

Title: Analysis of Transforming Growth Factor ß family Cleavage Products Secreted into the Blastocoele of *Xenopus laevis* Embryos

Authors and Affiliations:
Hyung-Seok Kim[1], Jan L. Christian[2*]

[1]Department of Neurobiology, University of Utah
[2]Departments of Neurobiology and Internal Medicine, Division of Hematology and Hematologic Malignancies, University of Utah, School of Medicine

Corresponding Authors:
Hyung-Seok Kim (hskim@neuro.utah.edu)
Jan L. Christian (jan.christian@neuro.utah.edu)

Email Addresses for All Authors:
hskim@neuro.utah.edu
jan.christian@neuro.utah.edu

Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **Yes**

   If **Yes**, can you record movies/images using your own microscope camera? **No**

   If your protocol involves microscopy but you are not able to record movies/images with your microscope camera, JoVE will need to use our scope kit (through a camera port or one of the oculars). Please list the make and model of your microscope.

   Nikon SMZ1B (preferred) or Zeiss Stemi 2000 (if Nikon can't be adapted)

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**

3. **Filming location:** Will the filming need to take place in multiple locations? **No**

Current Protocol Length

Number of Steps: 14
Number of Shots: 39

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Hyung-Seok Kim:** This protocol can be used to study the process by which a wide range of secreted precursor proteins, including TGFß family members, are converted to active proteins following proteolytic cleavage.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested b-roll: LAB MEDIA: Figure 2C and 2D*

1.2. **Hyung-Seok Kim:** The main advantage of this protocol is that it provides a very rapid and inexpensive method to obtained highly concentrated Tgfß cleavage products *in vivo* under physiologic conditions.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Ethics Title Card

1.3. All procedures described are approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Utah.

Protocol

2. Blastocoele Extraction and Analysis of Tgf β Cleavage Products

2.1. To begin with, after the injection, on the following day, remove Ficoll solution **[1]** and any dead or dying embryos **[2]**. Then, rinse the embryos once or twice with MBS *(M-B-S)* **[3-TXT]** and culture the embryos in MBS on the bench at room temperature or at 16 degrees Celsius in the incubator to slow down the development **[4]**.

2.1.1. WIDE: Talent removing the Ficoll solution

2.1.2. Talent removing dead embryos

2.1.3. Talent rinsing the embryos with MBS **TEXT: MBS: Modified Barth's Solution (0.1x)**

2.1.4. Talent keeping the embryos for culture on a bench at room temperature

2.2. Heat and pull the glass capillaries to a fine point using a micropipette puller with the desired settings **[1-TXT].** Using forceps, clip off the tip of a pulled needle **[2]**. To prevent clogging, the opening of the blastocoele aspiration needle should be larger than the microinjection needle **[3-TXT]**.

2.2.1. Talent heating and pulling the glass capillaries using micropipette puller **TEXT: Heat Settings: 1- 67.4 °C, 2- 62 °C; please refer to the text.**

2.2.2. SCOPE: Talent clipping off the tip of the needle *Videographer: This step is important!*

2.2.3. SCOPE: Blastocoele aspirating needle on left and microinjection needle on right **TEXT: Needle: Left- Aspiration, Right-Microinjection** *Videographer: This step is important!*

2.3. Insert the aspiration needle into the needle holder connected to the microinjector **[1]** and attach the needle holder to the micromanipulator **[2]**.

2.3.1. Talent inserting the aspirating needle into needle holder of microinjector

2.3.2. Talent attaching needle holder to a micromanipulator

2.4. Place early to the mid-gastrula stage embryos in an MBS-filled injection tray or dish **[1]**. Insert the needle below the embryo surface near the animal pole **[2]**. Press the fill button on the microinjector while observing the needle and the embryo through dissecting microscope **[3]**.

2.4.1. Talent placing the embryos in MBS-filled injection tray or dish

2.4.2. SCOPE: Talent inserting the needle below the embryo surface **TEXT: Avoid: Needle with a large opening and deep insertion** *Videographer: This step is important!*

2.4.3. Talent pressing the fill button on microinjector

2.5. Over a few seconds, the level of clear fluid rises in the needle, and the embryo collapses and becomes concave **[1]**. Pulse the inject button one or more times **[2]** to eject any cloudy white matter entering the needle containing debris or proteases **[3]**.

2.5.1. SCOPE: The level of fluid rising and the embryo collapsing, with some white matter being aspirated. *Videographer: This step is important!*

2.5.2. Talent pulsing the inject button

2.5.3. SCOPE: White matter being ejected until only clear fluid is retained. *Videographer: This step is important!*

2.6. To detect the cleavage products on immunoblots, aspirate the blastocoele fluid of 10 to 20 embryos or more depending on antibody **[1]**. Pipette 1 microliter of nuclease-free water onto a paraffin piece placed on the injection tray **[2]**.

2.6.1. SCOPE: The level of fluid rising and the embryo collapsing (without white matter to indicate an optimal aspiration) *Videographer: This step is important!*

2.6.2. Talent pipetting water onto paraffin placed in injection tray

2.7. Submerge the needle in the water drop **[1]** and press the inject button **[2]** to dispel the blastocoele fluid into the water **[3]**.

2.7.1. SCOPE: Talent submerging the needle in water drop

2.7.2. Talent pressing the inject button

2.7.3. SCOPE: Fluid emptying out of needle *Videographer: This step is important!*

2.8. Alternatively, to eject the fluid directly onto the parafilm and prevent it from flattening out, pulse the inject button **[1]** to expel the fluid under lower pressure **[2]**. Transfer the harvested blastocoele fluid into a sterile microcentrifuge tube on ice **[3-TXT]** and add nuclease-free water to adjust the final volume to 30 microliters **[4]**.

2.8.1. Talent pulsing the inject button

2.8.2. SCOPE: Blastocoele fluid getting expelled under low pressure *Videographer: This step is important!*

2.8.3. Talent transferring the harvested fluid into sterile centrifuge tube **TEXT: Expected Harvest: ~0.3-0.5 µL blastocoele fluid/embryo.**

2.8.4. Talent adding water to adjust the final volume

2.9. To detect Tgf-β *(T-G-F-beta)* precursor proteins, transfer the blastocoele fluid-depleted embryos to a separate tube on ice **[1]**. Remove excess MBS **[2]** and add 200 microliters of pre-chilled embryo lysate buffer **[3-TXT]**. To fully homogenize the embryos, pipette up and down 10 to 20 times until no clumps remain **[4]**.

2.9.1. Talent transferring the embryos to the tube

2.9.2. Talent removing excess MBS

2.9.3. Talent adding embryo lysate buffer **TEXT: Embryo lysate buffer: 4°C, 10 µL /embryo**

2.9.4. Talent pipetting the embryos up and down

2.10. Centrifuge the homogenized embryos in a refrigerated microcentrifuge at 10 thousand times *g* for 10 minutes **[1]**. Then, remove 160 microliters of the supernatant using a P-200 *(P-two hundred)* pipette and transfer to a new tube on ice, being careful to avoid the white yolk proteins and other cellular debris in the bottom half of the tube **[2]**.

2.10.1. Talent putting the tube for centrifugation

2.10.2. Talent removing the supernatant

2.11. Repeat the microcentrifugation once **[1]** and transfer 128 microliters of the clear supernatant to a new tube on ice **[2]**. At this point, cleared embryo lysates and the blastocoele fluid collected can be stored at minus 80 degrees Celsius for as long as desired **[3]**.

2.11.1. Talent putting the tube for centrifugation

2.11.2. Talent transferring the supernatant to new tube

2.11.3. Talent storing the blastocoele fluid in -80 ^{0}C

2.12. Deglycosylate the cleaved proteins present in blastocoele fluid, modified through the trans-Golgi network, with PNGase *(P-N-G Ace)* F by following the manufacturer's instructions. The deglycosylated products would migrate as a more condensed band on SDS gels, which can aid in accurate identification **[1].**

2.12.1. Talent performing any step of the deglycosylating procedure using PNGase F
Videographer: If possible, try to shot with PNGase F label visible

2.13. To assess the prodomain fragment monomers and unfolded proteins in the blastocoel and lysate, analyze the proteins under reducing conditions by adding 5 microliters of reducing 4 times sample buffer to 15 microliters blastocoele fluid **[1]** and 15 microliters of clarified embryo lysate **[2]**.

2.13.1. Talent adding reducing buffer to blastocoele fluid

2.13.2. Talent adding reducing buffer to embryo lysate

2.14. To assess the formation of cleaved homodimeric or heterodimeric ligands in the blastocoele, analyze the proteins under non-reducing conditions by adding 5 microliters of non-reducing 4 times sample buffer to the remaining 15 microliters of blastocoele fluid **[1]**. Then, heat it for 5 minutes **[2-TXT]** and place it on ice **[3]**.

2.14.1. Talent adding non-reducing sample buffer to blastocoel fluid

2.14.2. Talent heating the mixture **TEXT: Heating: 100 ^{0}C**

2.14.3. Talent placing it on ice

Results

3. Results: Analysis of Cleaved BMP Ligands in *Xenopus laevis* Blastocoele Fluid

3.1. After using this protocol, the proteins were separated by SDS-PAGE *(S-D-S-page)*, and the immunoblots were probed with antibodies that recognize the myc *(mik)*-epitope tag **[1]**.

3.1.1. LAB MEDIA: Figure 2C, 2D

3.2. Under the reducing conditions, in the lysates from embryos expressing only BMP4 or BMP7 **[1],** a single band corresponding to cleaved BMP4 *(B-M-P-four)* monomers **[2]** and a slower migrating band corresponding to cleaved BMP7 monomers were detected **[3]**. Both bands were detected in embryos co-expressing BMP4 and BMP7 **[4]**.

3.2.1. LAB MEDIA: Figure 2C, 2D

3.2.2. LAB MEDIA: Figure 2C, 2D *Video editor: Please emphasize on lower panel (reducing conditions), lane 1 (BMP4) in both figures*

3.2.3. LAB MEDIA: Figure 2C, 2D *Video editor: Please emphasize on lower panel (reducing conditions), lane 2 (BMP7) in both figures*

3.2.4. LAB MEDIA: Figure 2C, 2D *Video editor: Please emphasize on lower panel (reducing conditions), lane 3 (BMP4+7) in both figures*

3.3. When the proteins were separated under non-reducing conditions, a single mature BMP4 and 7 heterodimer band of intermediate mobility were detected **[1]** along with a trace amount of BMP4 homodimer in the embryos co-expressing BMP4 and BMP7 **[2]**.

3.3.1. LAB MEDIA: Figure 2C *Video editor: Please emphasize on upper panel (non-reducing conditions) lane 3 (BMP4 + 7), upper dark black band (corresponding to red + green schematic on the right)*

3.3.2. Figure 2C *Video editor: Please emphasize on upper panel (non-reducing conditions), lane 3 (BMP4 + 7), lower lighter black band (marked by green + green schematic on the right)*

3.4. BMP4 and BMP7 heterodimer formation was also observed **[1]** when BMP7 protein levels were high **[2]**. However, in this case, the excess BMP7 formed homodimers in embryos co-expressed BMP4 and BMP7 **[3]**. These results demonstrated that BMP4 and 7 preferentially form heterodimers when co-expressed in *Xenopus laevis* embryos **[4].**

3.4.1. LAB MEDIA: Figure 2D *Video editor: Please emphasize on upper panel (non-reducing conditions), lane 3 (BMP4 + 7), lower light black band (corresponding to red + green schematic on the right)*

3.4.2. LAB MEDIA: Figure 2D *Video editor: Please emphasize on upper panel (non-reducing conditions) lane 1 (BMP4) and 2 (BMP7)*

3.4.3. LAB MEDIA: Figure 2D *Video editor: Please emphasize on upper panel (non-reducing conditions), lane 3 (BMP4 + 7), upper dark black band (corresponding to red + red schematic on the right)*

3.4.4. LAB MEDIA: Figure 2C and 2D *Video editor: Please emphasize on upper panel (non-reducing conditions), lane 3 (BMP4 + 7), black band (corresponding to red + green schematic on the right) in both the figures*

Conclusion

4. Conclusion Interview Statements

4.1. **Hyung-Seok Kim:** In this experiment collecting pure blastocoele is the most important step, which requires precise needle handling experience. Just keep trying and fixing errors.

4.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested b-roll: 2.4.2*

4.2. **Hyung-Seok Kim:** This procedure tests whether Bmp heterodimers form and which amino acids are important for this. Knock-in mouse can be generated to ask whether these amino acids are functionally important.

4.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested b-roll: 3.4.4*