Time: 2024.07.08-2024.07.14

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1. **Experiment:** Detect the activating signal pathway by Western Blotting
2. **Time:** 2024.07.08-2024.07.14
3. **Member:** Song Zhang, Xudong Tang, Hanyue Liu, Xiaoyuan Chen, Xinyu Zhu, Yinran Luo, Yaqi Gao
4. **Material:** Lysis solution, PBS, protein loading buffer, electrophoresis buffer, transfer buffer, blocking buffer, centrifugal tube, electrophoresis tank, transfer membrane device, NC membrane, β-actin antibody (CST,4970), Phospho-Jak1 antibody(CST,74129), Jak1 antibody (CST,29261), Phospho-Jak3 antibody (CST,5031), JAK3 antibody (CST,80331), Phospho-ERK antibody (CST), ERK antibody (CST), Phospho-STAT1 (CST,9167), STAT1 antibody (CST,14994), Phospho-STAT3 (CST,9145), STAT3(CST,9139), Phospho-AKT (CST,9271), T-AKT(CST,4691)
5. **Method:**

(1) Prepare the protein sample

① The culture medium was discarded and the cells were rinsed three times by 2 ml PBS.

② The cells were added lysis buffer and they are split for 10 mins on ice. The cells are then scraped off and transferred to an Eppendorf tube, and the lysis process was continued for another 20 mins.

③ The sample was centrifuged at 14,000 rpm for 15 mins at 4°C and the supernatant was collected.

(2) SDS-PAGE electrophoresis

① The glass panel was cleaned.

② After aligning, the glass panel was placed securely in the clamp. Once leak testing is complete, they are attached vertically to the rack in preparation for pouring the gel.

③ 10% separating gel was mixed thoroughly by shaking immediately after adding TEMED. When pouring the gel, a 10 mL pipette was used to draw 5 mL of the gel and the gel was slowly released along the edge of the glass plate. Stop pouring when the gel level reaches the middle of the green indicator line. Then, a layer of water was carefully added on top of the gel.

④ When a refractive line appears between the water and the gel, it indicates that the gel has solidified. After another 3 mins, the water was poured off and an absorbent paper was used to dry the remaining water.

⑤ 5% stacking gel was mixed thoroughly by shaking immediately after adding TEMED. The stacking gel was poured to fill the remaining space in the gel mold. Then, the comb is insert into the stacking gel. Waot for the stacking gel to solidify completely. Once solidified, the comb should be gently pulled straight up to remove from the gel.

⑥ The stacking gel was rinsed with water and then placed it into the electrophoresis tank.

⑦ the upper and lower chambers of the electrophoresis tank was filled with 1×Tris-Glycine buffer solution, and then the samples are loaded.

⑧ The electrophoresis tank is connected to the electrophoresis apparatus. The upper chamber is connected to the negative terminal and the lower chamber is connected to the positive terminal. The initial voltage is set to 80V. Once the bromophenol blue indicator has reached the interface between the separating gel and the stacking gel, it is switched to a constant voltage of 120 V and the electrophoresis is run until the dye reaches the bottom of the gel, whereupon the electrophoresis is stopped.

(3) Blotting

① The gel is immersed in the transfer buffer for 10 mins to equilibrate.

② Based on the size of the gel, six pieces of PVDF membrane and filter paper are cut and placed in the transfer buffer for 10 mins to equilibrate. (The PVDF membrane needs to be soaked in pure methanol for 1 minute beforehand.)

③ The transfer membrane is assembled as follows: sponge, 3 layers of filter paper, gel, membrane, 3 layers of filter paper, sponge. After placing each layer, bubbles are removed using a pipette. The gel is placed on the negative side (black side).

④ The transfer tank is placed in an ice bath, and the membrane (with the black side facing the black side of the tank) is inserted. The transfer buffer is added, and the electrodes are connected. The electrophoresis is run at 100 V for 2 hrs with a current setting of 0.25 A.

⑤ After the transfer is complete, the power is turned off, and the hybridized membrane is taken out.

(4) Blocking and Hybridization

① 5% skimmed milk powder is prepared using TBST as the solvent.

② The membrane is immersed in 5% skimmed milk powder and shaken at a low speed on a rocker for 2 hrs to block. After blocking, the 5% skimmed milk powder is discarded, and the membrane is washed three times with TBST at a high speed on a rocker for 5 mins each time.

③ Primary antibody incubation: The primary antibody is added, and the membrane is incubated overnight at 4°C.

④ Secondary antibody incubation: The primary antibody is recovered, and the membrane is washed three times with TBST for 15 mins each time. The corresponding secondary antibody is added, and the membrane is incubated at room temperature on a rocker for 2 hrs. Afterwards, the membrane is washed three times with TBST for 15 mins each time.

(5) Visualization: The A and B luminescent solutions are mixed in a 1:1 ratio to prepare the ECL working solution. After removing the PVDF membrane from the TBST wash solution and blotting excess moisture with filter paper, the membrane is placed on an imager. The ECL working solution is uniformly applied to the membrane, air bubbles are removed, and exposure is initiated.

6. Result:

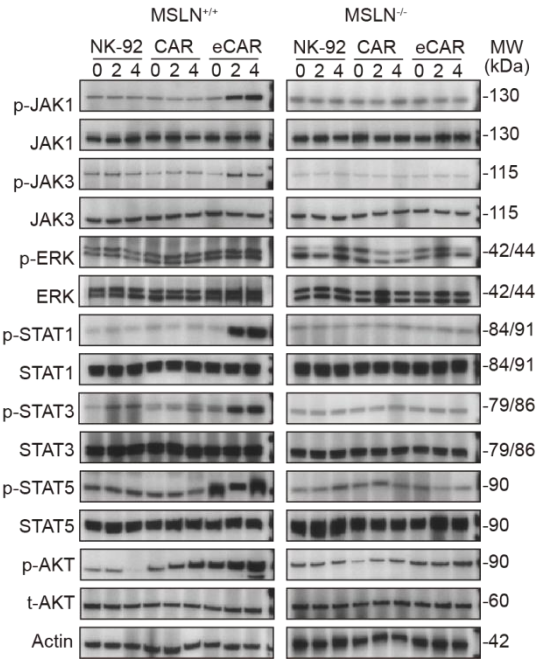

Fig.1 the activating signal pathway detected by Western Blotting