Immunostaining Protocol: P-Smad2 (Xenograft and Mice)

Alexandre Calon[1], Elisa Espinet[1], Sergio Palomo-Ponce[1], Daniele V. F. Tauriello[1], Mar Iglesias[2], María Virtudes Céspedes[3], Marta Sevillano[3], Cristina Nadal[4], Peter Jung[4], Xiang H.-F. Zhang[5], Daniel Byrom[6], Antoni Riera[6], David Rossell[7], Ramón Mangues[7], Joan Massague[7], Elena Sancho[7*] and Eduard Batlle[7*]

[1]Oncology Programme, Institute for Research in Biomedicine, Barcelona, Spain; [2]Department of Pathology, Hospital Universitari del Mar, Barcelona, Spain; [3]IIB Sant Pau, Hospital de la Santa Creu i Sant Pau, Barcelona, Spain; [4]Institut de Malalties Hemato-Oncològiques, Hospital Clínic, Barcelona, Spain; [5]Cancer Biology and Genetics Program, Memorial Sloan-Kettering Cancer Center, New York, USA; [6]Chemistry and Molecular Pharmacology Programme, Institute for Research in Biomedicine, Barcelona, Spain; [7]Biostatistics and Bioinformatics Unit, Institute for Research in Biomedicine, Barcelona, Spain

*For correspondence: elena.sancho@irbbarcelona.org; eduard.batlle@irbbarcelona.org

[Abstract] Metastasis depends on a gene program expressed by the tumor microenvironment upon TGF-beta stimulation. CRC (Colorectal cancer) cell lines did not induce robust stromal TGF-beta responses when injected into nude mice as shown by lack of p-SMAD2 accumulation in tumor-associated stromal cells. To enforce high TGF-beta signaling in xenografts, we engineered CRC cell lines to secrete active TGF-beta. Subcutaneous tumors obtained from HT29-M6TGF-β, KM12L4aTGF-β cells and SW48TGF-β cells contained abundant p-SMAD2$^+$ stromal cells.

Materials and Reagents

1. Paraffin sections (subcutaneous tumors samples or liver metastasis from nude mice respectively injected subcutaneously or intrasplenic with CRC cells)
2. XILOL
   Note: Xylol also referred to as xylene or dimethylbenzene is a solvent used in histology as a clearing agent to remove paraffin from dried microscope slides prior to staining.
3. MilliQ H_2O
4. Wash buffer (Dako, catalog number: K800721)
5. Rabbit anti-P-Smad2 (Cell Signaling Technology, catalog number: 3108)
6. BrightVision poly-HRP anti- Rabbit (Immunologic, catalog number: DPVR-110HRP)
7. Envision FLEX antibody diluent (Dako, catalog number: K8006)
8. Peroxidase Blocking Solution (Dako, catalog number: S202386)
9. ImmPACT DAB (Vector Laboratories, catalog number: SK-4105)

10. DPX mounting media (Sigma-Aldrich, catalog number: 06522)
11. Hematoxylin
12. Citrate buffer (pH 6) (see Recipes)

Equipment

1. Oven
2. Immunostaining apparatus
3. Autoclave

Procedure

1. Stove samples at 65 °C just before starting the immunostaining technique. Remove the samples from the oven when the wax present in sections is completely undone.
2. De-waxing and rehydration: Place slides in a rack to perform following washes (bath).
    a. XILOL: 10 min
    b. XILOL: 10 min
    c. XILOL: 5 min
    d. 100% EtOH: 10 min
    e. 100% EtOH: 5 min
    f. 96% EtOH: 5 min
    g. 90% EtOH: 10-15 times
    h. 80% EtOH: 10-15 times
    i. 70% EtOH: 10-15 times
    j. 50% EtOH: 10-15 times
    k. 25% EtOH: 10-15 times
    l. H_2O MilliQ: 10-15 times
3. Antigen retrieval.
    a. Citrate Buffer (pH 6)
    b. Time: 20 min autoclave (121 °C)
4. 3 washes 5 min with 1 ml 1x wash buffer.
5. Blocking endogenous peroxidase.
    a. 200 ml Peroxidase Blocking Solution
    b. Time: 10 min
6. 3 washes 5 min with 1ml 1x wash buffer.
7. Incubation with primary antibody.
    a. Antibody: Rabbit anti-P-Smad2

b. Dilution 1/200 in Envision FLEX antibody diluent
c. 200 µl/sample
d. O/N 4 °C

8. 3 washes 5 min with 1 ml 1x wash buffer.
9. Incubation with antibody BrightVision.
   a. Antiboby: BrightVision anti-Rabbit
   b. 150 µl/sample
   c. Time: 45 min at room temperature
10. 3 washes 5 min with 1 ml 1x wash buffer.
11. Revealed with ImmPACT DAB.
    a. 200 µl/sample
    b. Time: 10 min
12. 3 washes 5 min with distilled water.
13. Hematoxilin (1 ml) counterstaining, time: 2 min.
14. Rinse in distilled water bath.
15. Rinse in TAP water bath.
16. Dehydration and mounting with DPX.

Representative data

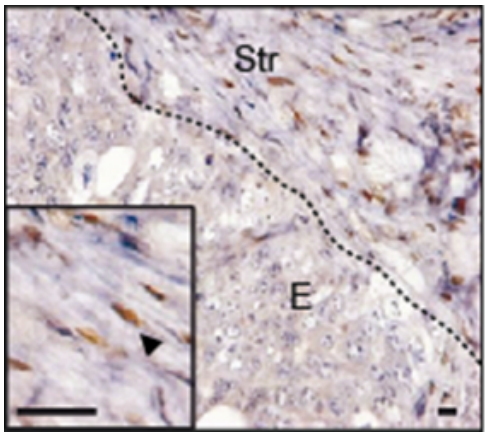

Figure 1. p-SMAD2 staining (arrowhead) in liver metastasis generated after intrasplenic injection of CRC cells. E: epithelial cells, Str: stromal cells. Scale bars = 10 µm.

Recipes

1. Citrate buffer (pH 6)

   Citrate 5.8 g sodium citrate

Set pH 6 with citric acid
2 L MilliQ

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