Video Article

Analysis of Protein-protein Interactions and Co-localization Between Components of Gap, Tight, and Adherens Junctions in Murine Mammary Glands

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Abstract

Cell-cell interactions play a pivotal role in preserving tissue integrity and the barrier between the different compartments of the mammary gland. These interactions are provided by junctional proteins that form nexuses between adjacent cells. Junctional protein mislocalization and reduced physical associations with their binding partners can result in the loss of function and, consequently, to organ dysfunction. Thus, identifying protein localization and protein-protein interactions (PPIs) in normal and disease-related tissues is essential to finding new evidences and mechanisms leading to the progression of diseases or alterations in developmental status. This manuscript presents a two-step method to evaluate PPIs in murine mammary glands. In protocol section 1, a method to perform co-immunofluorescence (co-IF) using antibodies raised against the proteins of interest, followed by secondary antibodies labeled with fluorochromes, is described. Although co-IF allows for the demonstration of the proximity of the proteins, it does make it possible to study their physical interactions. Therefore, a detailed protocol for co-immunoprecipitation (co-IP) is provided in protocol section 2. This method is used to determine the physical interactions between proteins, without confirming whether these interactions are direct or indirect. In the last few years, co-IF and co-IP techniques have demonstrated that certain components of intercellular junctions co-localize and interact together, creating stage-dependent junctional nexuses that vary during mammary gland development.

Video Link

The video component of this article can be found at https://www.jove.com/video/55772/

Introduction

Mammary gland growth and development occurs mainly after birth. This organ constantly remodels itself throughout the reproductive life of a mammal[1]. The adult mammary gland epithelium is comprised of an inner layer of luminal epithelial cells and an outer layer of basal cells, mainly composed of myoepithelial cells, surrounded by a basement membrane[2]. For a good review on mammary gland structure and development, the reader can refer to Sternlicht[1]. Cell-cell interactions via gap (GJ), tight (TJ), and adherens (AJ) junctions are necessary for the normal development and function of the gland[1,3,4,5,6]. The main components of these junctions in the murine mammary gland are Cx26, Cx30, Cx32, and Cx43 (GJ); Claudin-1, -3, -4, and -7 and ZO-1 (TJ); and E-cadherin, P-cadherin, and β-catenin (AJ)[7,8]. The levels of expression of these different junctional proteins vary in a stage-dependent manner during mammary gland development, suggesting differential cell-cell interaction requirements[9]. GJ, TJ, and AJ are linked structurally and functionally and tether other structural or regulatory proteins to the neighboring sites of adjacent cells, thus creating a junctional nexus[10]. The composition of the junctional nexus can impact bridging with the underlying cytoskeleton, as well as nexus permeability and stability, and can consequently influence the function of the gland[8,9,10,11]. The components of intercellular junctions residing in junctional nexuses or interacting with one another at different developmental stages of mammary gland development were analyzed recently using co-immunofluorescence (co-IF) and co-immunoprecipitation (co-IP)[9]. While other techniques allow for the evaluation of the functional association between proteins, these methods are not presented in this manuscript.

As proteins merely act alone to function, studying protein-protein interactions (PPIs), such as signal transductions and biochemical cascades, is essential to many researchers and can provide significant information about the function of proteins. Co-IF and microscopic analysis help to evaluate a few proteins that share the same subcellular space. However, the number of targets is limited by the antibodies, which must be raised in different animals, and by the access to a confocal microscope equipped with different wavelength lasers and a spectral detector for multiplexing. Co-IP confirms or reveals high-affinity physical interactions between two or more proteins residing within a protein complex. Despite the development of novel techniques, such as fluorescence resonance energy transfer (FRET)[12] and proximity ligation assay (PLA)[13], which can simultaneously detect the localization and interactions of proteins, co-IP remains an appropriate and affordable technique to study interactions between endogenous proteins.

The step-by-step method described in this manuscript will facilitate the study of protein localization and PPIs and point out pitfalls to avoid when studying endogenous PPIs in the mammary glands. The methodology starts with the presentation of the different preservation procedures for the tissues required for each technique. Part 1 presents how to study protein co-localization in three steps: i) the sectioning of mammary glands, ii) the double- or triple-labeling of different proteins using the co-IF technique, and iii) the imaging of protein localization. Part 2 shows how to precipitate an endogenous protein and identify its interacting proteins in three steps: i) lysate preparation, ii) indirect protein immunoprecipitation, and iii) binding partner identification by SDS-PAGE and Western blot. Each step of this protocol is optimized for rodent mammary gland tissues and generates high-quality, specific, and reproducible results. This protocol can also be used as a starting point for PPI studies in other tissues or cell lines.

Protocol

All animal protocols used in this study were approved by the University Animal Care Committee (INRS-Institut Armand-Frappier, Laval, Canada).

1. Identifying Protein Co-localization

1. **From tissue to microscopic slides**
   NOTE: Tissues and sections should be handled on dry ice.
   1. Excise the mammary glands from an animal (for a complete description of this procedure, refer to Plante *et al.*)[14].
   2. Embed the excised tissue in freezing/mounting medium on dry ice. Add enough medium to cover the gland. When the medium is solidified, transfer the tissues to a freezer at -80 °C for later use[9].
   3. Using a cryomicrotome set at ≤ -35 °C, cut the tissues into 7-10 µm-thick sections and place them on microscope slides.
      NOTE: When possible, place two sections on each slide; the left section will be used as a negative control to verify the specificity of the antibodies and the autofluorescence of the tissue, while the right side will be labeled with the antibodies.
   4. Keep the sections at -80 °C for later use.

2. **Co-IF staining**
   1. Retrieve the appropriate microscopic slides from the freezer and immediately fix the sections by immersing them in formaldehyde 4% for 15 min at room temperature (RT).
   2. Then immerse the slides in phosphate-buffered saline (PBS) at RT. Leave the slides in PBS at RT until proceeding to the next step.
   3. Circle each section of the slide using a commercially available hydrophobic barrier or a water-repellent lab pen (see the **Table of Materials**). Be careful not to touch the tissue. Immediately add drops of PBS to the tissue and place the slide in a humid histology chamber for the remainder of the procedure.
      NOTE: The tissue sections must remain moisturized. Alternatively, use a box with a lid and place wet paper towels on the bottom.
   4. Block each tissue section with 100-200 µL of 3% bovine serum albumin (BSA)-Tris-buffered saline (TBS)-0.1% polysorbate 20 (see the **Table of Materials**) for 30 min at RT. While the samples are blocking, prepare the primary and secondary antibody solutions by diluting the antibodies in TBS-0.1% polysorbate 20.
      NOTE: The required concentration for the antibody is provided by the manufacturer; see the **Table of Materials** and **Figures 1 and 2** for examples, as well as Dianati *et al.*[9]. Although it is not necessary to work in the dark when using most fluorophore-conjugated antibodies, avoid exposing the antibody solutions or stained tissues to intense, bright light.
   5. Remove the blocking solution by aspiration and incubate the sections in 100-200 µL of the diluted primary antibody for 60 min at RT. Alternatively, incubate with the primary antibody overnight at 4 °C.
   6. Remove the primary antibody solution by aspiration and wash the sections with 250-500 µL of TBS-0.1% polysorbate 20 for 5 min. Remove the wash solution by aspiration and repeat the wash twice.
   7. Remove the wash solution by aspiration and incubate the sections with 100-200 µL of the appropriate fluorophore-conjugated secondary antibody for 60 min at RT.
   8. Remove the secondary antibody solution by aspiration and wash the sections with 250-500 µL of TBS-0.1% polysorbate 20 for 5 min. Remove the wash solution and repeat twice.
   9. Repeat step 2.5-2.8 using the appropriate combination of primary and secondary antibodies for the subsequent protein(s) of interest.
   10. Remove the wash solution by aspiration and perform the nuclei staining by incubating the section with 100-200 µL of 1 mg/mL 4',6-diamidino-2-phenylindole (DAPI) in TBS-0.1% polysorbate 20 for 5 min at RT.
   11. Remove the DAPI solution by aspiration and mount the slides using a water-soluble, non-fluorescing mounting medium (see the **Table of Materials**) and coverslips. Proceed one slide at a time.
       NOTE: Incubating the nucleus stain for more than 5 min will not change the intensity of the staining. Alternatively, remove the DAPI solution on all slides and incubate the tissues in PBS while mounting the slides.
   12. Place the slides flat in a 4 °C refrigerator for at least 8 h. Proceed to the fluorescence microscopic imaging (see **Figures 1 and 2**).

3. **Microscopic imaging**
   1. Visualize the fluorophore-conjugated secondary antibodies using a confocal microscope equipped with the various lasers required to excite the fluorophores at their specific wavelengths.
      Note: To be able to visualize ducts and alveoli, a 40X objective with a numerical aperture of 0.95 is suggested. An example of the specific settings is provided in **Figure 1**.
   2. Verify the localization of each protein individually by scanning the image one wavelength at the time.
      Note: At this stage, it is important to critically analyze the localization of the junctional proteins. To be able to form junctional nexuses, these proteins must be localized at the plasma membrane.
   3. Determine the co-localization of proteins by merging the images scanned with the lasers at the different wavelengths.
      Note: Protein co-localization can be visualized by the change of color resulting from the emission of two or more fluorophores at the same location and can be measured using the appropriate software (**Figures 1 and 2**; also see Reference 9).

2. Studying PPIs

NOTE: Abdominal mammary glands should be used to study PPIs, as thoracic glands are in close association with the pectoral muscles. Excise the mammary glands (for a complete description of this procedure, refer to Plante *et al.*)[14] and keep them at -80 °C for later use.

1. **Lysate preparation**
    1. Place weighing paper and 2 mL microcentrifuge tubes on dry ice to pre-cool them before proceeding with the next steps.
    2. Take the mammary gland tissue from -80 °C and keep them on dry ice.
    3. Weigh the tissues on the pre-chilled weighing papers and then transfer the tissue to 2 mL microcentrifuge tubes (handle on dry ice). Use between 50 and 100 mg of tissue per sample. Keep the tissue on dry ice until step 2.1.5.
    4. Prepare the required amount of triple detergent lysis buffer supplemented with NaF, $NaVO_3$, and protease/phosphatase inhibitor, as indicated in the **Table of Materials**, using the following formulas. Mice: required buffer (µL) = mouse tissue weight (mg) x 3; Rat: required buffer (µL) = rat tissue weight (mg) x 5.
    5. Add the required amount of ice-cold lysis buffer (calculated in step 2.1.4) to the 2 mL tube containing the tissue.
    NOTE: In steps 2.1.5-2.1.6, proceed with one single tube at a time.
    6. Homogenize the tissue for 30-40 s using continuous homogenization on a tissue grinder; always keep the tube on ice. Adjust the tissue homogenizer to medium speed and gently move the grinder up and down inside the tube.
    7. Repeat steps 1.6 and 1.7 with the other tubes.
    8. Incubate the lysates on ice for 10-30 min.
    9. Centrifuge the tubes at 170 x g for 10 min at 4 °C.
    10. Meanwhile, identify 6-10 microcentrifuge tubes (0.6 mL) for each sample and keep them on ice.
    11. Once the centrifugation is done, check the tubes. Ensure that they contain a top layer of fat, clear, yellow-to-pink lysates (depending on the stage of development) and a pellet.
    12. Create a hole in the lipid layer using a 200-µL pipette tip to access the liquid phase. Change the tip and collect the lysate without disturbing the pellet or aspirating the lipid layer. Aliquot the lysate in pre-labeled tubes on ice (step 2.1.11) and store them at -80 °C.
    13. Use an aliquot to quantify the protein concentration using an appropriate commercially available kit (see the **Table of Materials**).

2. **Indirect immunoprecipitation**
    1. On ice, thaw two aliquots of the total mammary gland lysates prepared previously.
    NOTE: One aliquot will be used for the IP of the target protein, while the other will serve as the negative control.
    2. Collect 500-1,000 µg of the lysate and dilute it in PBS to reach a final volume of 200 µL in each 1.5 mL tube.
    NOTE: The amount of lysate to be used depends on the abundance of the protein of interest and the efficiency of the antibody (see **Figure 3** for an example, as well as the **Table of Materials**). To optimize for each target, different amounts of lysate (*i.e.,* 500, 750, and 1,000 µg) and antibody (*i.e.,* 5, 10, and 20 µg) should be used. Proceed with the following steps (2.2.3-2.3.7.4).
    3. Add the antibody against the antigen of interest to the first tube of lysate and keep it on ice.
    NOTE: The required amount is usually suggested on the instruction sheet provided by each company (see the **Table of Materials**).
    4. In the second tube, prepare a negative control by adding the same concentration of isotype IgG control as the antibody used in step 2.2.3.
    5. Incubate the tubes overnight at 4 °C on a tube roller-mixer at low speed.
    6. The following day, add 50 µL of magnetic beads to new 1.5 mL tubes for pre-washing.
        1. Select either Protein A or Protein G magnetic beads based on the relative affinity to the antibody.
        2. It is important to avoid using aggregated beads; gently mix the bead suspension until it is uniformly re-suspended before adding it to the tubes.
    7. Place the tubes containing the beads on the magnetic stand and allow the beads to migrate to the magnet. Remove the storage buffer from the beads using a 200 µL pipette.
    8. Wash the beads by adding 500 µL of PBS-0.1% polysorbate 20 and vortex the tubes vigorously for 10 s.
    9. Put the tubes back on to the magnetic stand and allow the beads to migrate to the magnet.
    10. Remove the excess wash buffer by pipetting with a 200 µL pipette.
    11. Add the reaction complex (lysate-antibody) from step 2.2.5 to the beads and incubate for 90 min at RT on the roller mixer.
    12. Place the tubes on the magnetic stand and allow the beads to migrate to the magnet. Using a 200 µL pipette, aspirate and discard the lysate and place the tubes on ice.
    13. Wash the beads by adding 500 µL of PBS, placing the tubes on the magnetic stand, and removing the liquid using a 200 µL pipette. Repeat this wash step. During the wash steps, avoid vortexing and keep the samples on ice.
    14. Wash the beads once with PBS-0.1% polysorbate 20 without vortexing and discard the last wash buffer using a 200 µL pipette tip.
    15. To elute, add 20 µL of 0.2 M acidic glycine (pH = 2.5) to the tubes and shake them for 7 min on the roller mixer.
    16. Centrifuge at high speed for a few seconds (quick spin) and collect the supernatant in a fresh ice-cold tube.
    17. Repeat steps 2.2.14 and 2.2.15 for each tube.
    NOTE: The final volume will be 40 µL.
    18. Add 10 µL of 4x Laemmli buffer to the 40-µL eluted sample from step 2.2.16.
    NOTE: The color will turn yellow due to the acidic pH.
    19. Immediately add 1 M Tris (pH = 8), one drop at a time, to the eluted sample from step 2.2.18 until its color turns blue. Proceed to the next tubes.
    20. Boil the samples from step 2.2.18 at 70-90 °C for 10 min. Proceed immediately to gel electrophoresis. Alternatively, transfer the samples to a freezer at -80 °C until loading.

3. **Downstream application: gel electrophoresis followed by Western blot**
    1. Prepare separating and stacking SDS-PAGE acrylamide gels (1.5 mm thickness) following standard procedures[15].

NOTE: The choice of gel (8-15% acrylamide, gradient: see the **Table of Materials**) should be determined based on the molecular size of the protein to be precipitated and of the potential binding partners to be analyzed. These proteins must be resolved from each other to allow for proper immunodetection.

2. Thaw the immunoprecipitation (IP)-eluted samples (step 2.2.20) on ice.
3. Prepare protein lysates from the same samples (used for the IP procedure above). Use 50 µg of total lysate and add 4X Laemmli sample buffer. Boil the samples at 70-90 °C for 5 min and place on ice until loading.
   NOTE: These samples will be loaded beside the eluted IP sample to demonstrate the presence of precipitated proteins in the total lysate.
4. Load the prepared lysates from step 2.3.3 and the precipitated samples from step 2.2.20 side-by-side in an acrylamide gel and run them in running buffer (10x running buffer: 30.3 g of Tris, 144.1 g of glycine, and 10 g of SDS in 1 L of distilled water) at 100 V for approximatively 95 min, or until the edge of the migrating proteins reaches the bottom of the gel.
5. Transfer the gels to a nitrocellulose or PVDF membrane using a standard protocol[9,15].
6. Block the membrane for 1 h on a rocker on low speed in 5% dry milk-TTBS (20 mM Tris, 500 mM NaCl, and 0.05% polysorbate 20).
7. Identify whether the precipitation was successful.
   1. Probe the membrane using the first antibody against the precipitated protein, diluted in 5% dry-milk-TTBS at the concentration recommended by the manufacturer, overnight at 4 °C on a rocking platform with slow agitation.
      NOTE: See the **Table of Materials** for recommendations.
   2. The following day, wash the membrane 3 times for 5 min each with TTBS on a rocking platform with high agitation.
   3. Incubate the membrane in the appropriate secondary antibody conjugated with horseradish peroxidase (HRP), diluted in TTBS, for 1 h at RT on a rocking platform with slow agitation.
      NOTE: Alternatively, a secondary antibody conjugated with a fluorochrome can be used if an appropriate apparatus to detect the signal is available.
   4. Perform 3 to 6 washes, each for 5 min, with TTBS on a rocking platform with high agitation. Analyze the signal of the secondary antibody by incubating the membrane with a commercially available luminol solution (see the **Table of Materials**) and follow the manufacturer instructions. Detect the signal using a chemiluminescence imaging system (see the **Table of Materials**).
      NOTE: For a detailed protocol on Western blot analysis, see Reference 16.

8. To identify interacting proteins, perform steps 2.3.7.1-2.3.7.4 using the appropriate antibodies on the same blot.
   NOTE: If proteins are interacting, the binding partners will be co-immunoprecipitated with the target protein and will thus be detectable by Western blotting. Step 2.3.8 can be repeated with more antibodies to determine whether other proteins reside in the same proteins complex, as long as the molecular weights of the proteins differ enough to be well-separated on the gel and membrane.
9. To confirm that the identified binding partners are not artifacts, reciprocal IP should be performed.
   NOTE: This is performed by repeating steps 2.2.1-2.2.20 with the same lysate but precipitating one of the binding partners identified in step 3.8. Then, steps 3.1-3.8 are repeated using the primary antibody against the first protein of interest.

Representative Results

To determine whether GJ, AJ, and TJ components can interact together in the mammary gland, co-IF assays were first performed. Cx26, a GJ protein, and β-Catenin, an AJ protein, were probed with specific antibodies and revealed using fluorophore-conjugated mouse-647 (green, pseudocolor) and goat-568 (red) antibodies, respectively (**Figure 1B** and **C**). Data showed that they co-localize at the cell membrane of epithelial cells in the mice mammary gland on lactation day 7 (L7), as reflected by the yellow color (**Figure 1D**). Secondly, Claudin-7, a TJ protein; E-cadherin, an AJ protein; and Connexin26 (Cx26), a GJ protein, were probed with their specific antibodies and were revealed with fluorophore-conjugated rabbit-488 (green), mouse-555 (red), and mouse-647 (coral blue; pseudocolor) antibodies, respectively (**Figure 2B-D**). E-cadherin and Claudin-7 co-localization is displayed as a yellow-to-light-orange color, while Cx26 co-localization with E-cadherin and Claudin-7 resulted in white punctuated staining in mice mammary glands on pregnancy day 18 (P18) (**Figure 2E**).

To find out which junctional proteins intermingle and physically tether together at the cell membrane, co-immunoprecipitation was performed using mammary gland tissues from lactating mice (L14). Results showed that Cx43, a component of GJ, interacts with E-Cadherin and Claudin-7, but not with Claudin-3 (**Figure 3A and B**). These results were confirmed by the reciprocal IP; when E-Cadherin was immunoprecipitated, it interacted with Cx43 and Claudin-7 (**Figure 3C**).

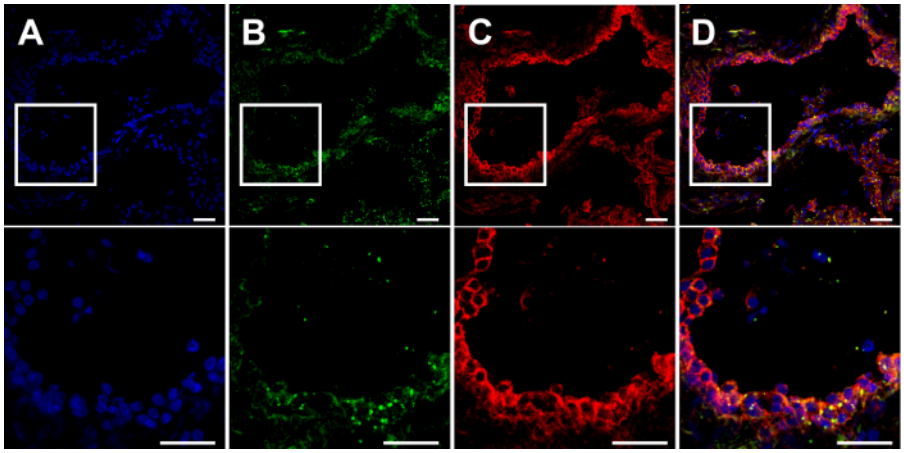

Figure 1: β-Catenin and Cx26 co-localize at the cell membrane in mice mammary glands. Cryosections from mammary glands of mice at lactation day 7 (L7) were cut (7 µm) and processed for immunofluorescent staining. (**A**) Nuclei were stained with DAPI (blue). (**B**) Cx26 (green, pseudocolor) and (**C**) β-Catenin (red) are shown combined with appropriate fluorophore-labeled antibodies. (**D**) A merged image. Images were obtained with a confocal microscope equipped with a spectral detector. DAPI was visualized using the following settings: emission wavelength, 450.0 nm; excitation wavelength, 402.9 nm; laser power, 1.2; detector gain, PMT HV 100; PMT offset, 0. Cx26 (647) was visualized using the following settings: emission wavelength, 700.0 nm; excitation wavelength, 637.8 nm; laser power, 2.1; detector gain, PMT HV 110; PMT offset, 0. β-Catenin (568) was visualized using the following settings: emission wavelength, 595.0 nm; excitation wavelength, 561.6 nm; laser power, 2.1; detector gain, PMT HV 110; PMT offset, 0. Scale bars = 50 µm. Please click here to view a larger version of this figure.

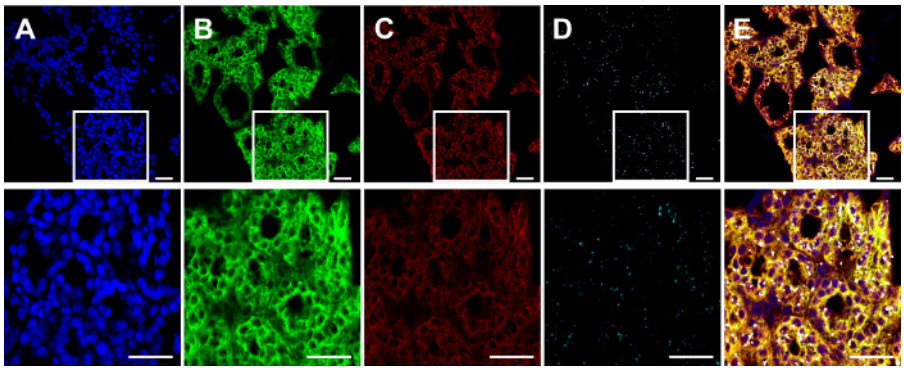

Figure 2: Connexin26 (Cx26), E-cadherin, and Claudin-7 co-localize at the cell membrane in mice mammary glands. Cryosections from mammary glands on pregnancy day 18 (P18) were cut (7 µm) and processed for immunofluorescent staining using (**B**) Claudin-7 (green), (**C**) E-Cadherin (red), and (**D**) Cx26 (coral blue; pseudocolor), combined with the appropriate fluorophore-labeled antibodies. (**A**) Nuclei were stained with DAPI (blue). Images were obtained with a confocal microscope equipped with a spectral detector. DAPI was visualized using the following settings: emission wavelength, 450.0 nm; excitation wavelength, 402.9 nm; laser power, 5.4; detector gain, PMT HV 65; PMT offset, 0. Claudin-7 (488) was visualized using the following settings: emission wavelength, 525.0 nm; excitation wavelength, 489.1 nm; laser power, 5.0; detector gain, PMT HV 12; PMT offset, 0. E-Cadherin (568) was visualized using the following settings: emission wavelength, 595.0 nm; excitation wavelength, 561.6 nm; laser power, 13.5; detector gain, PMT HV 45; PMT offset, 0. Cx26 (647) was visualized using the following settings: emission wavelength, 700.0; excitation wavelength, 637.8; laser power, 5.0; detector gain, PMT HV 55; PMT offset, 0. Scale bars = 50 µm. Please click here to view a larger version of this figure.

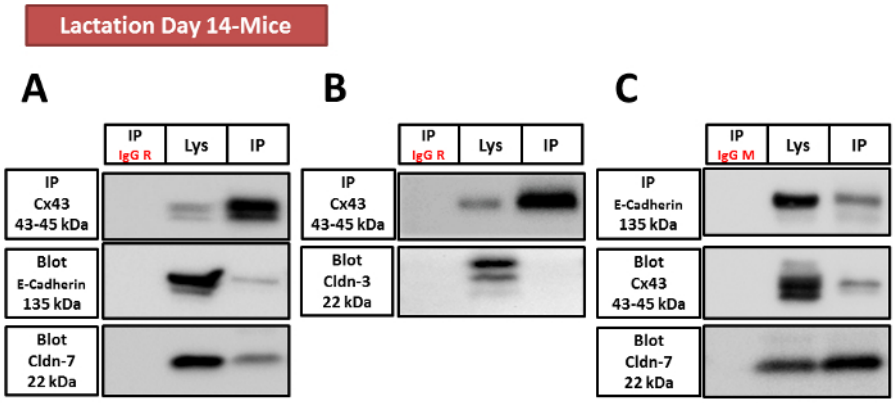

Figure 3: Cx43, E-Cadherin, and Claudin-7, but not Claudin-3, are involved in a protein complex. Cx43 (**A** and **B**) and E-Cadherin (**C**) were immunoprecipitated using 500 mg of total lysates from mammary glands of mice at lactation. IPs and lysates were loaded in gels and transferred on PVDF membranes. Because Claudin-7 and Claudin-3 have the same molecular weight, they could not be analyzed on the same membrane. Thus, two parallel IPs were performed with the same lysate for Cx43, loaded on two gels, and transferred (membranes A and B). Membrane A was first probed with Cx43 to confirm the efficiency of the IP (top panel). Then, the membrane was sequentially probed with E-Cadherin and Claudin-7. Western blot analysis showed that the two proteins interact with Cx43. Membrane B was first probed with Cx43 to confirm the efficiency of the IP (top panel) and then probed with Claudin-3. Western blot analysis showed that Claudin-3 did not IP with Cx43, demonstrating that the two proteins do not interact. Membrane C was first probed with E-cadherin to confirm the efficiency of the IP (top panel). Then, the membrane, was sequentially probed with Cx43 and Claudin-7. Western blot analysis confirmed the interactions between the proteins. Please click here to view a larger version of this figure.

Discussion

Cell-cell interactions via junctions are required for the proper function and development of many organs, such as the mammary gland. Studies have shown that junctional proteins can regulate the function and stability of one another and activate signal transduction by tethering each other at the cell membrane[10]. The protocols presented in the current manuscript have provided interesting findings about junctional protein differential expression, localization, and interaction during normal murine gland development[9]. Given that junctional protein localization is critical to the function of the proteins, and because they are known to interact with scaffolding proteins and numerous kinases[3], co-IF and co-IP are effective techniques in the field of cell-cell interactions. Not only are these methods essential to enlighten the necessity of junctional nexuses in mammary gland development and their dysregulation in breast cancer, but they can also be used in other tissues and in experiments using cell lines.

The mammary gland is composed of two main compartments: the stroma and epithelium[4]. The adult epithelium is made of two layers of cells. In this approach, the proximity of the potential binding partners was determined using the co-IF technique, and their physical interactions were confirmed using co-IP. Co-IF has been successfully used by others to demonstrate the co-localization of proteins within the same tissue, structure, cell, or intracellular compartment[17,18]. The main advantage of this technique is the visual information it brings about the cellular or subcellular localization of each protein within the different cell types composing the tissue. Although this technique is quite simple, some recommendations must be followed. For instance, to avoid tissues damage, always add the solutions one drop at a time using a 200 µL pipette or a transfer pipette. This will allow for the immersion of the tissue surface without damaging the tissues. Similarly, remove the solutions using a Pasteur pipette placed beside the tissues by gently tilting the slide. Moreover, for antibodies whose storage solution contains glycerol, careful suction of the wash buffers is required to reduce the background. Moreover, the presence of milk proteins, such as caseins, during lactation can interfere with the antibodies by trapping them, resulting in false positives. A critical analysis of the resulting image is thus required, specifically at this stage.

This co-IF technique also has certain limitations or pitfalls. First, it requires specific antibodies. As mentioned previously (step 1.1), it is recommended to use one section (*i.e.,* the one on the right side) on each slide for staining following the steps described above, adding the primary and secondary antibodies sequentially. For the remaining section (*i.e.,* the one on the left side), follow the same procedure, using TBS-polysorbate 20 0.1% instead of the primary antibody solutions. While it is also possible, and even better, to verify the specific binding of the antibody using peptide competition, peptides used to generate commercial antibodies are not always available. It is thus important to verify the specificity of the binding using positive and negative controls (*i.e.,* tissues or cells known to express, or not, the protein of interest). Moreover, antigen fixation sites can be inaccessible, especially for formalin-fixed tissues, thus resulting in the absence of a specific signal. An antigen-retrieval procedure may thus be required for some tissues. A short incubation with detergent can also be performed prior to antigen-retrieval to permeabilize the cell membrane. Second, for multiplexing, antibodies must be raised in different animals. For instance, if anti-rabbit was used in step 1.2.6, anti-mouse could be selected in step 1.2.10, but not another antibody raised in rabbit. Since most commercial antibodies are raised in rabbits, mice, or goats, it is sometimes difficult, or even impossible, to target two proteins at the same time due to the lack of appropriate antibodies. To overcome these limitations, one can either buy commercially available, pre-labeled antibodies or label primary antibodies with fluorophores using commercially available kits. Third, another shortcoming is linked to the excitation and emission of the different fluorophores. To avoid overlap of the signals from two different antibodies, the excitation-emission spectrum of each fluorophore must be separated. Thus, the number of targets that can be analyzed at once will vary according to the configuration of the available microscope. Finally, the quality of the analysis is highly dependent upon the microscope used. More detailed and precise data can be obtained using a confocal microscope compared to an epifluorescent microscope. The use of super-resolution microscopy can reveal protein co-localization in even more detail[19].

Although co-IF brings important insights about the proximity of potential binding partners, it should be complemented by other methods to identify physical interactions between proteins. Among the available methods, co-IP is probably one of the most affordable to perform, as the equipment and material are easily accessible. Using an antibody bound to magnetic beads, one can isolate protein complexes and identify the components present in that complex using typical Western blot analysis. Similar to co-IF, some recommendations should be followed for best practices. For instance, it is recommended to minimize the samples when homogenizing the tissues to reduce the time between steps 2.1.5 and 2.1.9. While an experienced person can process up to 10 samples at a time, a beginner should not handle more than 4-6 samples. Similarly, the number of tubes should be limited when performing the IP protocol for the first time. It is recommended to start with a negative control (*i.e.,* IgG) and a positive control (*i.e.,* a tissue known to contain the protein to be immunoprecipitated) only. The second trial should be dedicated to optimization (see step 2.2.2). Once these steps give satisfying results, samples to be analyzed can be processed. Note that a negative control should always be included in the procedure.

This co-IP technique also has potential pitfalls. First, it requires tissues to be homogenized in conditions permitting the preservation of the links between proteins. For membrane proteins, such as junctional proteins, it is also crucial to use a buffer that will preserve the bonds between the proteins while also allowing their solubility. Second, similar to co-IF, it requires high-affinity antibodies for both the target protein and the binding partners. Moreover, because proteins remain in their tertiary conformation and protein complexes are not dissociated by homogenization, if the antibody recognizes part of the target protein that is in close proximity to the binding domain of a partner or that is hidden inside the native structure of the protein, the IP can be compromised. It is thus essential to always verify the efficiency of the IP using Western blotting before concluding the absence of a binding partner. Third, co-IP can generate false-positive results because of the protein either binding directly to the beads or precipitating during the procedure without being part of the complex. To identify these artifacts, an IgG control is required, as well as a reciprocal IP, as described in the methods presented. It is also possible to add a "pre-cleaning" procedure between steps 2.2.2 and 2.2.3 to avoid the unspecific binding of proteins to the beads. To do so, incubate the lysates with 50 µg of beads for 1 h at 4 °C on a roller mixer. Remove the beads with the magnetic stand and proceed to step 2.2.3. Fourth, the heavy and light chains of IgG can be the same size as the proteins of interest or the binding partner, thereby masking the signal. One solution is to dissociate the IgG chains with glycine, as described in this manuscript. It is also possible to purchase secondary antibodies that only recognize native antibodies and therefore do not bind to the denatured light and heavy chains loaded in the membrane (see the **Table of Materials**). The two methods may sometimes have to be combined. Fifth, co-IP allows for the identification of a limited number of binding partners, in part because of the number of antibodies that can be probed on the membrane. It also requires the pre-identification of these binding partners, either by co-IF or through a literature review. Finally, co-IP allows for the identification of the proteins present within a complex, and not for the direct interaction between two proteins.

Results from co-IP can be analyzed in different ways. It is possible to solely identify interacting partners by re-probing the same membrane with different antibodies, as described in this manuscript. It is also possible to quantify this interaction. To do so, the amount of the protein immunoprecipitated is first quantified by probing the membrane with an antibody against this protein. The signal intensity is analyzed using an imaging software, as described in this manuscript. Then, the membrane is re-probed with an antibody against the binding partner, and the signal intensity also quantified. The interactions between the two proteins can then be expressed as a ratio of the amount of the binding partner to the amount of the immunoprecipitated protein. However, to allow for comparison, the different samples must be processed at the same time and loaded on the same membrane. Biological replicates can be proceeded and analyzed similarly, and statistical analysis can be performed.

In the last few years, other techniques were developed to analyze PPIs. For instance, FRET allows for the identification of interacting proteins through energy transfer from one tag to another, only when the proteins are close enough to interact[20]. However, because it requires proteins to be tagged, this technique cannot be used in tissues. Similarly, it is possible to identify PPIs using a protein fused with a bacterial biotin ligase, BirA[21]. This ligase will add biotin to proteins that come in close proximity (*i.e.,* interact) with the chimeric protein. While this method is innovative and unbiased, it cannot be performed in tissues. Alternatively, PLA can be used in tissues. Similar to co-IF, this assay is based on antibody affinity. For this assay, secondary antibodies are tagged with DNA sequences that can interact when they are in close proximity (*i.e.,* upon PPIs) and form a circular DNA molecule[22]. This circular DNA molecule is then amplified and detected using fluorescently labeled complementary oligonucleotides. Although this assay is elegant, it requires many validation steps and still relies on primary antibody affinity and some knowledge of potential interacting partners. Finally, an unbiased alternative to the traditional IP assay has also been developed to identify PPIs. In rapid immunoprecipitation-mass spectrometry of endogenous protein (RIME) assays, IP samples are analyzed by mass spectrometry (IP/MS)[23] instead of Western blot analysis. The main advantage of this high-throughput method is that it provides massive information about all the endogenous interacting proteins using few materials. However, it requires access to a peptide-sequencing instrument[23].

It is important to mention that, after several preliminary tests, each step of this protocol has been optimized for the mammary gland. However, the method can surely be used for other organs after a few modifications. For co-IF, the optimal temperature for mammary gland sectioning, suitable blocking and washing and solutions, and the proper antibody concentrations were all tested. For co-IP, various lysis and elution buffers and methods of extractions were also tested and have a major impact on IP success. In sum, each step of this protocol is important to obtaining high-quality and reproducible results with the least possible background and the most specificity. While other methods are available, co-IF followed by co-IP remain valid and simple methods to evaluate PPIs. These two techniques can be used both in tissues and in cell lines and only require a few validation steps and controls.

Disclosures

The authors have nothing to declare.

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