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TITLE:

Modeling Paracrine Noncanonical Wnt Signaling *In Vitro*

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SUMMARY:

The present study outlines a highly reproducible and tractable method to study paracrine noncanonical Wnt signaling events *in vitro.* This protocol was applied to evaluate the impact of paracrine Wnt5a signaling in murine neural crest cells and myoblasts.

ABSTRACT:

Noncanonical Wnt signaling regulates intracellular actin filament organization and polarized migration of progenitor cells during embryogenesis. This process requires complex and coordinated paracrine interactions between signal-sending and signal-receiving cells. Given that these interactions can occur between different types of cells from different lineages, *in vivo* evaluation of cell-specific defects can be challenging. The present study describes a highly reproducible method to evaluate paracrine noncanonical Wnt signaling *in vitro.* This protocol was designed with the ability to (1) conduct functional and molecular assessments of noncanonical Wnt signaling between any two cell types of interest; (2) dissect the role of signal-sending versus signal-receiving molecules in the noncanonical Wnt signaling pathway; and (3) perform phenotypic rescue experiments with standard molecular or pharmacologic approaches.

This protocol was used to evaluate neural crest cell (NCC)-mediated noncanonical Wnt signaling in myoblasts. The presence of NCCs is associated with an increased number of phalloidin-positive cytoplasmic filopodia and lamellipodia in myoblasts and improved myoblast migration in a wound-healing assay. The *Wnt5a-ROR2* axis was identified as a crucial noncanonical Wnt signaling pathway between NCC and second heart field (SHF) cardiomyoblast progenitors. In conclusion, this is a highly tractable protocol to study paracrine noncanonical Wnt signaling mechanisms *in vitro*.

INTRODUCTION:

Noncanonical Wnt signaling is an evolutionarily conserved pathway that regulates cellular

filament organization and directional migration. This pathway has been implicated in multiple biological processes, including embryonic tissue morphogenesis[1–3], lymphatic and vascular angiogenesis[4–7], and cancer growth and metastasis[8–10]. At the cellular level, noncanonical Wnt signaling is carried out through coordinated paracrine interactions between signal-sending and signal-receiving cells. These interactions frequently occur between cells of different lineages or types and involve a diverse molecular network that includes up to 19 ligands and multiple receptors, co-receptors, and downstream signal transduction effectors[11]. Further complicating this signaling process, previous studies have shown that ligand–receptor combinations can vary in a context- and tissue-dependent manner[12,13], and that the same source ligands that drive noncanonical Wnt signaling in signal-receiving cells can be produced by multiple signal-sending cell types[14,15]. Given the cellular and molecular complexity associated with noncanonical Wnt signaling, the ability to study individual and clinically relevant mechanisms *in vivo* has been limited.

Attempts have been made to study noncanonical Wnt signaling using cell culture techniques *in vitro*. For example, wound-healing assays performed in cellular monolayers have been used to functionally assess cellular directional migration[4,16–19]. Immunostaining techniques have been used to perform spatial analyses of surface protein expression to evaluate noncanonical Wnt-induced changes in cellular morphology[7,10], architecture, and asymmetric polarization[18–20]. Although these approaches have provided important tools for characterizing Wnt-related phenotypes in signal-receiving cells, the lack of signal-sending components in these protocols limits their ability to accurately model paracrine signaling mechanisms observed *in vivo*. As a result, there remains a critical need to develop *in vitro* systems that allow robust and reproducible evaluation of paracrine signaling interactions between signal-sending and receiving cells of the noncanonical Wnt pathway, particularly those of different cell types.

To this end, the primary objective of this study was to establish a protocol to model paracrine noncanonical Wnt signaling interactions *in vitro.* We developed a non-contact coculture system that recapitulates signal-sending and signal-receiving components of these interactions and allows the use of standard molecular, genetic, or pharmacologic approaches to independently study specific ligand–receptor mechanisms in the noncanonical Wnt pathway. Mechanisms of NCC-mediated Wnt signaling were examined in myoblasts using established murine cell lines. As proof of principle, this model was used to corroborate findings of prior *in vivo* studies in mice that implicate the *Wnt5a-ROR2* axis as a relevant noncanonical Wnt signaling pathway between NCCs[21] and SHF cardiomyoblast progenitors[3,22,23].

PROTOCOL:

1. Preexperimental expansion and passaging of cells

1.1. C2C12 cell culture:

1.1.1. Prepare 500 mL of C2C12 culture medium by combining Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 1 % penicillin/streptomycin.

1.1.2. Thaw a vial of C2C12 cells in 37 °C water bath. While the C2C12 cells are thawing, add 5 mL of C2C12 medium to a 15 mL conical tube. Immediately transfer the thawed cells to the 15 mL tube using a P1000 pipette.

NOTE: C2C12 cells are murine myoblast cells that have been previously used and validated as a primary cell line for modeling cardiomyoblast progenitors.

1.1.3. Gently mix C2C12 cells in the conical tube using a serological pipette. Then, add the entire volume of thawed cells in C2C12 medium (6 mL) to a 75 cm^2 flask.

1.1.4. Add 6 mL of fresh C2C12 medium to the flask for a total volume of 12 mL. Gently rotate the flask such that the cell and media solution covers the entire bottom of the flask. Place the flask in a cell culture incubator (37 °C, 5% CO_2) to allow the cells to adhere.

1.1.5. Allow the cells to expand in the incubator until they reach ~60% confluency.

NOTE: This can be determined by periodically removing the cells from the incubator and quickly checking their confluency under a microscope. Be sure to minimize the time that cells are removed from the incubator.

1.2. Passaging C2C12 cells:

1.2.1. Warm the C2C12 medium, 500 mL of 1x PBS, and 0.25% trypsin-EDTA in a 37 °C water bath. After the reagents have been warmed, transfer all the reagents to the cell culture hood.

1.2.2. Bring the flask containing the C2C12 cells into the cell culture hood. Gently rinse the flask containing the C2C12 cells with warm 1x PBS twice.

1.2.2.1. Tilt the flask at a 45° angle and aspirate the C2C12 medium with a glass pipette connected to vacuum suction. While maintaining a 45° angle, carefully add 10 mL of warm 1x PBS to the corner of the flask using a serological pipette. Lay the flask flat on the surface and gently move the flask in circular motions to ensure that the 1x PBS washes over the entire monolayer of cells.

NOTE: Be careful not to disrupt the C2C12 monolayer while aspirating the medium.

1.2.3. Remove the 1x PBS after the second wash. Add 1 mL of 0.25% trypsin–EDTA to the flask, gently move the flask as described above to allow the trypsin–EDTA solution to spread over as much of the monolayer as possible and place the flask in the incubator for 2 min.

1.2.4. After 2 min incubation, remove the flask from the incubator and gently tap it to detach any remaining cells.

1.2.5. Add 9 mL of C2C12 medium to the flask with a serological pipette to quench the trypsin.

Using the serological pipette, gently rinse the cells with the medium and collect the 10 mL of trypsinized cell suspension. Add 10 mL of the cell suspension to a fresh 15 mL conical tube and centrifuge at 100 × *g* for 5 min.

1.2.6. Return the conical tube to the cell culture hood and remove the supernatant with a glass pipette connected to vacuum suction. While aspirating the supernatant, take care not to disrupt the cell pellet at the bottom of the conical tube. To do this, leave ~0.2 mL of the supernatant in the conical tube. Resuspend the cells in 10 mL of fresh C2C12 medium.

1.2.7. For additional passaging, add 1 mL of the resuspended cells into a 75 cm^2 flask. Add 11 mL of C2C12 medium to the flask with a serological pipette and place the flask in the incubator.

1.3. STO cell culture:

1.3.1. Prepare STO culture medium by making 500 mL of DMEM with 7% FBS, 1% penicillin/streptomycin, and 2 nM L-glutamine.

1.3.2. Prepare 0.1% gelatin by adding 0.5 g of gelatin powder to a bottle containing 500 mL of tissue-grade or autoclaved water. Add 7 mL of 0.1% gelatin solution to a 75 cm^2 flask. Rotate the flask such that the gelatin solution covers the entire bottom of the flask. Let the flask incubate for 30 min at room temperature in the cell culture hood.

1.3.3. Following 30 min incubation, remove the excess gelatin using a pipette connected to vacuum suction. Let the flasks remain in the incubator for another 30 min prior to use.

1.3.4. Thaw a vial of STO cells in a 37 °C water bath. Using a P1000 pipette, immediately transfer the thawed cells to a 15 mL conical tube containing 5 mL of freshly prepared STO culture medium.

NOTE: STO cells are murine embryonic fibroblasts routinely used as feeder cells in cell culture protocols.

1.3.5. Gently mix the cells in the conical tube using a serological pipette. Add the entire volume (6 mL) of cells to a gelatin-coated 75 cm^2 flask. Rotate the flask to ensure that the cell suspension is well distributed along the bottom of the flask.

1.3.6. Add 6 mL of fresh STO medium to the flask for a total volume of 12 mL. Rotate the flask as described above to ensure that the cells and medium are well distributed along the bottom of the flask. Place the flask in the 37 °C incubator to allow the cells to adhere. Allow the cells to proliferate in the incubator until they reach ~60–70% confluency.

1.4. Passaging STO cells:

1.4.1. Warm STO cell medium, 1xPBS, and 0.25% trypsin–EDTA in a 37 °C water bath.

1.4.2 Gently rinse the flask containing STO cells with warm 1x PBS twice as described in step 1.2.2.1.

1.4.3. Add 1 mL of 0.25% trypsin–EDTA to the flask using a P1000 pipette. Place the flask in the 37 °C incubator for 5 min.

1.4.4. After 5 min incubation, remove the flask from the incubator. Gently tap the flask to detach any adherent cells.

1.4.5. Add 9 mL of STO medium to the flask to quench the trypsin and collect the 10 mL of trypsinized cell suspension as described above. Add 10 mL of the cell suspension to a 15 mL conical tube and centrifuge at 100 × *g* for 5 min.

1.4.6. Remove the supernatant and resuspend the cells in 10 mL of STO medium as described above. For additional passaging, add 1 mL of the resuspended cells into a 75 cm^2 flask. Add 11 mL of STO medium, rotate the flask to ensure even distribution of the cells and medium along the bottom of the flask, and place the flask in the incubator.

1.5. Inactive STO cell culture and O9-1 cell basal medium preparation:

1.5.1. Prepare O9-1 cell basal culture medium by mixing 500 mL of DMEM with 15% FBS, 1% penicillin/streptomycin, 2 nM L-glutamine, 0.1 mM minimum nonessential amino acids, 1 nM sodium pyruvate, and 55 µM beta-mercaptoethanol.

1.5.2. Prepare mitomycin C solution in 1x PBS at a concentration of 0.5 mg/mL by adding 4 mL of 1x PBS directly to a mitomycin C vial. Pipette the solution multiple times with a P1000 pipette. To further dissolve the mitomycin C in 1x PBS, place the vial on a vortex mixer or benchtop rocker for 45 min to 1 h.

1.5.3. Inactivate the STO cells by treating the STO plates with a final concentration of 0.01 mg/mL mitomycin C added to the standard STO medium. Place the STO plates inside the incubator for 2 h, taking care to protect the flask containing mitomycin C from light by minimizing the time spent outside the incubator. Following mitomycin treatment, wash the cells in 1x PBS twice as described in step 1.2.2.1.

NOTE: Mitomycin C inhibits the proliferation of STO cells so that they can be used to generate conditioned medium over several days without becoming overconfluent in the flask.

1.5.4. After removing the 1x PBS, add 12 mL of O9-1 basal culture medium to the inactivated STO cell cultures and incubate them for 24 h. Collect the conditioned, inactivated STO + O9-1 basal culture medium and place it in 50 mL conical tubes wrapped in foil to protect it from light.

1.5.5. Add 12 mL of fresh O9-1 basal culture medium to the inactivated STO cell cultures as described above. Repeat this step by adding fresh O9-1 basal culture medium to the same flanks

containing the inactivated STO cells every 24 h.

NOTE: Following mitomycin C treatment, the inactivated STO cells cannot proliferate; hence, the flasks containing the inactivated STO cells can be reused to generate conditioned medium.

1.5.6. Store the foil-wrapped 50 mL conical tubes containing conditioned, inactivated STO + O9-1 basal culture medium at 4 °C until a total of 500 mL of the medium has been collected.

1.6. O9-1 cell culture:

1.6.1. Prepare O9-1 cell growth culture medium using 500 mL of conditioned STO + O9-1 basal medium, and add 10^3 units of leukemia inhibitory factor (LIF) and 25 ng/mL basic fibroblast growth factor (basic-FGF).

1.6.2. Prepare 0.1% gelatin-coated flasks as described in steps 1.3.2–1.3.3.

1.6.3. Thaw a vial of O9-1 cells in a 37 °C water bath. Immediately transfer the thawed cells to a 15 mL conical tube containing 5 mL of freshly prepared O9-1 growth medium.

NOTE: The O9-1 cell line is the only stable, multipotent neural crest cell line that has been generated from the mouse. This cell line is commonly used in neural crest *in vitro* experiments.

1.6.4. Gently mix the cells using a serological pipette. Add the entire 6 mL of cells to a gelatin-coated 75 cm^2 flask.

1.6.5. Add 6 mL of O9-1 growth medium to the flask for a total volume of 12 mL. Place the flask in the 37 °C incubator to allow the cells to adhere. Allow the cells to proliferate in the incubator until they reach ~60–70% confluency.

1.7. Passaging O9-1 cells:

1.7.1. Warm O9-1 growth medium, 1x PBS, and 0.25% trypsin–EDTA in a 37 °C water bath.

1.7.2. Gently rinse the plate containing O9-1 cells with warm 1x PBS twice as described in step 1.2.2.1.

1.7.3. Add 1 mL of 0.25% trypsin–EDTA to the flask and place it in the 37 °C incubator for 5 min.

1.7.4. After 5 min incubation, remove the flask from the incubator and tap the flask gently to detach any adherent cells.

1.7.5. Add 9 mL of O9-1 growth medium to the flask to quench the trypsin. Collect 10 mL of the trypsinized cell suspension and add 10 mL of this cell suspension to a 15 mL conical tube. Centrifuge the tube at 100 × *g* for 5 min.

1.7.6. Remove the supernatant and resuspend the cells in 10 mL of O9-1 growth medium. For additional passaging, add 1 mL of the resuspended cells to a 75 cm^2 flask. Add 11 mL of O9-1 growth medium and place the flask containing the cells in 12 mL of medium in the 37 °C incubator.

2. Plating cells in a coculture system

2.1. Plating C2C12 cell chambers:

2.1.1. Following trypsinization (as described in step 1.2), resuspend the C2C12 cell pellet in 10 mL of C2C12 medium. Dilute the cells in a 1:20 ratio in C2C12 medium by removing 0.5 mL of the resuspended C2C12 cells and adding them to a new 15 mL conical tube containing 9.5 mL of fresh C2C12 medium. Gently mix the suspension with a serological pipette.

2.1.2. Add 1 mL of the 1:20-diluted C2C12 cells to each well of a new 4-chambered well using a P1000 pipette and place the 4-chambered well in the incubator.

2.2. Plating O9-1 cell inserts:

2.2.1. Following trypsinization, resuspend the O9-1 cell pellet in 10 mL of O9-1 growth medium. Dilute the cells in a 1:20 ratio in O9-1 growth medium by removing 0.5 mL of the resuspended C2C12 cells and adding them to a new 15 mL conical tube containing 9.5 mL of fresh O9-1 growth medium. Gently mix the suspension with a serological pipette.

2.2.3. Place a single permeable insert (see the **Table of Materials**) inside each well of a new 4-chambered well filled with 1 mL of O9-1 growth medium.

NOTE: This 4-chambered well should be different from that containing the C2C12 cells from step 2.1.3.

2.2.4. Add 300 µL of the diluted O9-1 cell suspension to each insert using a P1000 pipette. Ensure that the bottom of each insert is submerged within the well filled with 1.3 mL of O9-1 growth medium. Place the well in the 37 °C incubator.

2.3 (Optional). Perform siRNA knockdown in the O9-1 cells or C2C12 cells.

2.3.1. Perform gene knockdown by siRNA 18–24 h after plating either O9-1 cell inserts or C2C12 cell chamber wells.

2.3.1.1. Dilute the siRNA and transfection reagent in reduced-serum medium (see the **Table of Materials**) according to the manufacturers' recommendations and the desired concentrations for the experiment. Gently mix the diluted siRNA and transfection reagent (1:1) and incubate the mixture at room temperature for 7 min.

NOTE: In this protocol, 50 nM concentrations were used as this siRNA concentration was determined to result in sufficient knockdown of target gene expression.

2.3.1.2. Add the siRNA-lipid complexes to either the O9-1 cell inserts or the C2C12 cell chamber wells as determined by the experimental design and incubate the cells with the siRNA-lipid complexes for ~36–48 h.

3. Performing wound assay and quantitatively assessing myoblast migration

3.1. Wound assay:

3.1.1. Allow the O9-1 cell inserts and C2C12 cell chamber wells to adhere and proliferate in the incubator until both cells are at ~70–80% confluency before proceeding with this portion of the protocol. If cells grow >90% confluent, do not proceed with the scratch assay, as cells will merely detach from the well.

3.1.2. Warm 1x PBS and C2C12 medium by placing them in a 37 °C water bath.

3.1.3. Remove supernatant medium from the C2C12 chamber well and wash the cells once with 1x PBS. Remove the 1x PBS and immediately scratch the cells with a sterile P10 pipette tip.

3.1.3.1. Pass the sterile P10 pipette tip firmly in a single direction to span the entire length or width of the cell monolayer (e.g., right to left, top to bottom). Be sure to scratch each well containing cells only once.

NOTE: To optimize scratching results, scratch wells of different experimental conditions at a similar level of confluency. To do this, ensure that each 4-chambered well has cells for each required experimental condition (e.g., well #1 negative control, well #2 positive control). In addition, use a new sterile P10 pipette for each scratch and apply a similar amount of force to the pipette each time. Do not attempt to create more than one scratch in each well.

3.1.3.2. After scratching, quickly add 1 mL of 1x PBS back into the well using a P1000 pipette tip. Repeat this process for each well that will be scratched.

NOTE: Given the variability associated with each scratch, it is recommended that multiple wells (n = 3) are used for wound creation in each experimental condition. Work expeditiously as it is critical to minimize the duration for which the cells are without 1x PBS during these steps. After removing 1x PBS from each well, one should not take more than 5 s to generate a wound in each well.

3.1.4. After generating a wound and adding 1x PBS back into each well, image the scratch using a brightfield inverted microscope and use this image as the baseline wound size (time 0). To take images, perform the following steps:

3.1.4.1. Turn on the computer and the microscope (see the **Table of Materials**) by pressing the power button. Place the chamber slide on the stage and rotate the objective dial to 5x magnification.

3.1.4.2. Open the imaging software (see the **Table of Materials**) by double-clicking the software icon on the computer desktop. Click the **Camera** tab on the software home screen. Click the **Live** button to visualize the cells on the **AxioCam IC** tab.

3.1.4.3. Ensure that the light filter is pulled all the way out to allow light to pass to the camera and computer screen. Manually move and/or rotate the chamber slide to position the wound area in the center of the live image on the **AxioCam IC** tab.

3.1.4.4. To take images, click **snap** to open a **new** tab next to the **AxioCam IC** tab that contains the image.

3.1.4.5. To save this still image, click **file** on the top left of the software home page **| save as |** enter the **file name** in the file name box. Save the figure in Carl Zeiss image (***.czi**) format (the default setting), and select **desktop** on the bar on the left to save the file to the desktop as a **.czi** file, which can only be opened in the Zen lite 2012 software program.

3.1.4.6. To save the picture as a .tiff, click **file | save as |** enter **file name** in the file name box. Save the figure as tagged image file (***.tiff**) by clicking the **save as type** button and selecting ***.tiff** from the dropdown menu.

NOTE: The .tiff format can be opened in any image processing software.

3.1.4.7. Manually reposition the chamber slide to take 2–3 more images at other points of the wound in the same well.

NOTE: In total, this will result in 3–4 non-overlapping, high-magnification images of the wound in each well.

3.1.5. Remove the 1x PBS from each well and add 1 mL of C2C12 medium.

NOTE: Be cautious not to pipette too aggressively when removing or adding solutions to the chamber well following wound generation, as this may cause cells to detach from the chamber well. In addition, tilt the chamber well so that aspiration and reintroduction of solutions can be done at the corners of each well to minimize cell monolayer disruption.

3.1.6. Assemble the well insert coculture system by manually placing the inserts containing the O9-1 cells in each well of the chamber well. Gently push the inserts down into the well such that the bottom of the insert sits just above the underlying C2C12 cells. Return the well insert constructs to the incubator.

NOTE: Do not allow the bottom of the insert to physically touch and mechanically disrupt the underlying C2C12 cells.

3.1.7. Allow the cells to migrate for a total of 9–12 h. To determine the optimal migration time, check the cells at 6 h following wound creation, then every 2–3 h thereafter. End the experiment when the cells in control or positive control conditions begin to completely cover the wound.

NOTE: Given the non-contact nature of the construct, the overlying insert does not need to be removed from the wells when checking interval migration progression. Migratory variability will be observed depending on factors such as the cell types used in this assay, cellular density at the time of wound generation, the width of the wound created, and experimental conditions of manipulated cells (e.g., gene knockdown, recombinant protein addition). Concentrations of these reagents should be determined experimentally with guidance from manufacturer recommendations.

4. Immunofluorescence staining and imaging of migrating myoblasts

4.1. Terminating the migration assay and deconstructing the well insert system:

4.1.1. Remove the O9-1 cell inserts after a 9–12 h migration period (or alternative time designated by experimental conditions). Carefully aspirate the C2C12 medium using a P1000 pipette. Add 0.5 mL of 1x PBS to the chamber wells, and take final images of cells following migration.

4.1.2. Carefully aspirate all 0.5 mL of 1x PBS mixed with medium and remove the plastic chambers from the chamber wells using kit instructions, leaving the underlying slide containing the C2C12 cells.

NOTE: Be careful not to disrupt the C2C12 cells adherent to the slide when removing the chamber wells.

4.2. Performing immunostaining:

4.2.1. Immediately place the slide in a slide holder containing 4% paraformaldehyde (PFA) and incubate for 10 min at room temperature. Pour out the 4% PFA and add 0.1% Triton X-100-containing 1x PBS (1x PBST) to the slide holder to wash the slide for 15 min at room temperature. Pour out the 1x PBST and add 1x PBS to the slide holder to wash the slide for 10 min at room temperature. Repeat this step once more for a total of two 1x PBS washes.

4.2.2. Remove the slide from the slide holder. Trace the outer edges of the slide with a hydrophobic pen to create a hydrophobic boundary around the slide to prevent solutions from spilling from the slide. Take care not to disrupt the adherent cells.

4.2.3. Add 1% bovine serum albumin (BSA) blocking solution (diluted in 1x PBS) to the slide (~0.5 mL per slide). Ensure that the solution is contained within the hydrophobic boundary created in step 4.2.4. Incubate the slide for 1 h at room temperature in a humidified slide chamber.

NOTE: Although BSA blocking is not required for phalloidin immunostaining, this step in the protocol allows for coupling with fluorescence antibody staining.

4.2.4. After blocking for 1 h, pour out the blocking solution from the slide, add phalloidin antibody (diluted to 1:200 in 1% BSA blocking solution by adding 5 µL of the antibody to 995 µL of BSA solution) to the slide and incubate at 4 °C overnight. Again, ensure that the solution is contained within the hydrophobic boundary created in step 4.2.4. Given that the phalloidin antibody is conjugated to Alexa Fluor-488 dye, minimize light exposure of the antibody reagent before and after adding to the slide.

4.2.5. The following day, place the slide in a slide holder protected from light exposure (e.g., wrap the slide holder in foil or use a non-transparent slide holder). Wash the cells in the slide holder with 1x PBS for 10 min at room temperature. Repeat the wash for a total of three 10 min washes.

4.2.6. Add 4',6-diamidino-2-phenylindole (DAPI)-containing mounting medium and mount the slides with glass coverslips. Image the cells that have migrated using a standard fluorescence microscope.

REPRESENTATIVE RESULTS:

Effects of NCCs on migratory capacity of murine myoblasts

This assay was first applied to evaluate the impact of NCCs on the migratory capacity of myoblasts. **Figure 1** outlines the schematic model of the assay. To test this impact, scratch assays were performed with myoblasts that were grown in isolation (without NCC inserts) compared to those grown in the presence of inserts. As a positive control, 500 ng/mL of recombinant Wnt5a (rWnt5a) was added to chamber wells with NCC inserts. This concentration of rWnt5a was determined by a dose–response analysis performed in C2C12 cells (**Supplemental Figure S1**). Representative images of NCC inserts are shown in **Supplemental Figure S2**, demonstrating that the NCCs are healthy at this time point. Immunofluorescence demonstrates robust knockdown of Wnt5a at the protein level following incubation with 50 nM of *Wnt5a* siRNA (**Supplemental Figure S3**). Following a 9 h migration period, it was found that the presence of NCCs significantly increased the migratory capacity of myoblasts compared to myoblasts assayed in the absence of NCC inserts (72.6% wound-repopulated area vs 59.1% wound-repopulated area, $p = 0.033$). The addition of rWnt5a to coculture wells accelerated myoblast migration, with some wound areas demonstrating complete recovery by the 9 h time point, as shown in **Figure 2**. As expected, migratory myoblasts in all three conditions exhibited normal migratory cellular morphology, including well-formed and protruding filopodia and lamellopodia and asymmetric polarization of actin cytoskeletal projections (**Figure 2C**).

Importance of NCC-derived Wnt5a for polarized migration of myoblasts

To evaluate the paracrine effect of NCC-derived Wnt5a on myoblast migration, wound-healing assays were performed in myoblasts following the siRNA-mediated knockdown of *Wnt5a* in NCCs. First, *Wnt5a* knockdown efficiency was validated in NCCs by real time-quantitative polymerase chain reaction. Treatment with 50 nM siRNA against *Wnt5a* was found to reduce *Wnt5a* gene expression by 64% compared to negative control (scrambled) siRNA (**Figure 3A**). Using this concentration, O9-1 cell inserts were transfected with either control siRNA or *Wnt5a* siRNA 48 h prior to assembling the coculture. C2C12 cells were grown under normal conditions, and wounds were created at the appropriate confluency. Immediately following wound generation, negative control or *Wnt5a* knockdown NCC inserts were added to each well. After a 10 h migration period, it was found that knockdown of *Wnt5a* in NCCs significantly reduced underlying myoblast migratory capacity compared to myoblasts assayed with control NCCs (39.1% wound-repopulated area vs 74.8% wound-repopulated area, $p < 0.001$). Moreover, myoblasts assayed in the presence of NCCs with knockdown of *Wnt5a* displayed abnormal cytological morphology by immunostaining, including reduced cytoplasmic areas and fewer actin cytoskeletal projections (**Figure 3D**). To rescue myoblast migration, exogenous supplementation of 500 ng/mL of rWnt5a was added to coculture wells containing *Wnt5a* knockdown inserts. The addition of exogenous rWnt5a was found to completely rescue migratory and morphologic defects observed in these myoblasts (**Figure 3C,D**).

Wnt5a signaling through ROR2 in myoblasts as a driver of polarized migration

To better understand the signal-receiving cell mechanisms in this paracrine model, the assay was repeated following the knockdown of the *ROR2* receptor in myoblasts. In this experiment, myoblasts were transfected with 50 nM of *ROR2* siRNA ~40 h prior to wound generation, which was shown to be sufficient to knock down *ROR2* gene expression by 54% (**Figure 4A**). During this time, NCC inserts were grown in parallel under normal conditions. After myoblasts reached appropriate confluency, scratch assays were performed, and coculture well inserts were assembled. After a 10 h migration period in the presence of NCC inserts, *ROR2* knockdown myoblasts demonstrated reduced migratory capacity compared to myoblasts treated with negative control siRNA (48.1% wound-repopulated area vs 75.7% wound-repopulated area, $p = 0.019$) (**Figure 4B,C**). The addition of 500 ng/mL rWnt5a failed to rescue myoblast migratory capacity following *ROR2* knockdown, suggesting that *ROR2* depletion disrupts the ability of myoblasts to receive *Wnt5a* signals (**Figure 4B,C**). Immunostaining for phalloidin corroborated the migratory data and showed that a reduction in phalloidin-positive lamellopodia and filopodia in *ROR2* knockdown myoblasts was not restored by supplemental rWnt5a (**Figure 4D**).

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic model of the assay. Step 1 includes the *in vitro* expansion of C2C12 myoblasts and NCCs using STO feeder cells. Step 2 involves the plating of NCCs and C2C12 cells in the coculture system. Step 3 includes the wound assay performed in underlying C2C12 cells to evaluate cellular migratory capacity. Step 4 involves immunostaining for phalloidin to evaluate cytological architecture and morphology of migrated cells. Abbreviations: NCCs = neural crest cells; Ab = antibody.

Figure 2: Presence of neural crest cells increases myoblast migratory capacity. (**A**) The presence

of neural crest cell (NCC) inserts at the time of wound generation leads to improved myoblast migration. The addition of exogenous recombinant Wnt5a (rWnt5a) to NCC-C2C12 cocultures has the strongest positive effect on myoblast migration. (**B**) Quantification of average myoblast repopulated area at 9 h following wound generation (error bars show standard deviation). (**C**) Phalloidin staining of myoblasts at the wound border 9 h following wound generation. Dashed rectangles show phalloidin-stained myoblasts at the migratory front. A total of n = 3 samples were used for each experimental condition quantified in **B**. Scale bars = 200 µm (for **A** and **C**).

Figure 3: Neural crest cell-derived Wnt5a is necessary for myoblast migration. (**A**) Relative mRNA expression of *Wnt5a* to validate siRNA-mediated knockdown in NCCs. (**B**) Migration of C2C12 myoblasts is significantly reduced following *Wnt5a* knockdown in NCCs. Addition of exogenous rWnt5a is sufficient to rescue this migratory deficit in myoblasts. (**C**) Quantification of average myoblast-repopulated area at 10 h following wound generation (error bars show standard deviation). (**D**) Phalloidin staining of myoblasts at the wound border 10 h following wound generation. Dashed rectangles show phalloidin-stained myoblasts at the migratory front. A total of n = 3 samples were used for each experimental condition quantified in **C**. Scale bars = 200 µm (for **B** and **D**). Abbreviations: NCCs = neural crest cells; siRNA = small interfering RNA.

Figure 4: Wnt5a signals through ROR2 receptors in myoblasts to drive migration. (**A**) Relative mRNA expression of *ROR2* to validate siRNA-mediated knockdown in C2C12 cells. (**B**) Knockdown of *ROR2* in myoblasts reduces their migratory capacity despite the presence of NCCs. Exogenous rWnt5a fails to rescue myoblast migration after *ROR2* knockdown. (**C**) Quantification of average myoblast-repopulated area at 10 h following wound generation (error bars show standard deviation). (**D**) Phalloidin staining of myoblasts at the wound border 10 h following wound generation. Dashed rectangles show phalloidin-stained myoblasts at the migratory front. A total of n = 3 samples were used for each experimental condition quantified in **C**. Scale bars = 200 µm. Abbreviations: NCCs = neural crest cells; siRNA = small interfering RNA.

Supplemental Figure S1: Dose-dependent analysis for recombinant Wnt5a supplementation. Dose-dependent analysis for recombinant Wnt5a supplementation testing 0 ng/mL, 100 ng/mL, and 500 ng/mL found 500 ng/mL of exogenous rWnt5a to be the optimal concentration to drive myoblast migration and phalloidin cytoarchitectural changes during a 12 h migratory period *in vitro.* Scale bars = 200 µm.

Supplemental Figure S2: **Representative images of well inserts**. Brightfield images of well inserts containing O9-1 neural crest cells treated with (**A**) 50 nM negative control siRNA and (**B**) 50 nM *Wnt5a* siRNA**.** Scale bars = 200 µm. Abbreviation: siRNA = small interfering RNA.

Supplemental Figure S3: **Representative images of Wnt5a protein expression in O9-1 cells following siRNA-mediated Wnt5a knockdown**. Immunofluorescence staining of Wnt5a protein in cell culture wells containing O9-1 neural crest cells treated with (**A**) 50 nM negative control siRNA and (**B**) 50 nM *Wnt5a* siRNA**.** Scale bars = 20 µM. Abbreviations: siRNA = small interfering RNA; DAPI = 4',6-diamidino-2-phenylindole.

DISCUSSION:

The noncanonical Wnt/planar cell polarity (PCP) signaling pathway is a critically important cellular signaling pathway that has been implicated in multiple developmental[24,25] and disease processes[24,26]. During embryonic development, noncanonical *Wnt* signaling involves an expansive network of molecular signals from signal-sending cells that ultimately induce changes in morphology, asymmetric organization, and directional migration in signal-receiving cells[11]. Previous studies have shown that the specific ligand–receptor pathways that drive this signaling are diverse, context-dependent, and often vary between cell types[12–15]. Owing to this molecular complexity, the ability to assess paracrine noncanonical *Wnt* signaling interactions using conventional genetic recombination methods *in vivo* has been limited. While *in vitro* systems have increasingly been used as an alternative approach to study noncanonical *Wnt* cellular phenotypes, available protocols focus exclusively on downstream signal-receiving aspects of the pathway and fail to sufficiently model the intercellular and paracrine nature of these signaling events. Therefore, the objective of the present study was to develop a protocol for a non-contact coculture system that recapitulates paracrine Wnt interactions *in vitro*. The focus of this protocol was to model two characteristic aspects of functional noncanonical Wnt signaling *in vitro*, including the organization of intracellular filament proteins and polarized migratory capacity.

As a proof of concept, this protocol was applied to study paracrine mechanisms in the context of heart development. During cardiogenesis, reciprocal signaling events between cardiac NCCs and SHF progenitor cells are crucial for the appropriate maturation of the cardiac outflow tract (OFT)[27–29]. Previous work has shown that during mouse cardiac development, NCC-mediated Wnt/PCP signaling in pharyngeal SHF cells is required for SHF progenitor cell incorporation into the developing OFT and for normal OFT alignment[21]. Schleiffarth et al. and others have shown that genetic knockout of the gene encoding the Wnt/PCP ligand, *Wnt5,* has been shown to disrupt SHF progenitor cell organization and migratory capacity, resulting in a foreshortened and misaligned cardiac OFT[22,23,30]. With established murine NCC (O9-1)[31] and myoblast (C2C12) cell lines, this protocol demonstrates that coculture with NCCs is associated with increased phalloidin-positive cytoplasmic filopodia and lamellipodia and improves myoblast migratory capacity in a wound-healing assay. These molecular and functional endpoints *in vitro* build on previously published protocols for NCC manipulation[31] and closely model the *in vivo* phenotypic changes described in *Wnt5a* global knockout mice, validating the utility of this model.

To determine the specific molecular pathway driving noncanonical Wnt signaling between NCCs and myoblasts, parallel cell-specific knockdown experiments for the genes encoding the candidate ligand, *Wnt5a*, in NCCs and its corresponding receptor, *ROR2*, in myoblasts were performed. As expected, knockdown of molecules in both signal-sending (*Wnt5a* in NCCs) and signal-receiving (*ROR2* in myoblasts) cells independently disrupted Wnt/PCP-related actin cytoarchitectural changes and inhibited myoblast migration. Importantly, phenotypic rescue with recombinant Wnt5a was only observed in the NCC-*Wnt5a* knockdown condition, which supports a mechanism whereby NCC-derived Wnt5a activates PCP signaling in myoblasts through ROR2 receptors. These results are consistent with data from mouse genetic studies that identify the Wnt5a–ROR2 axis as a crucial Wnt/PCP signaling axis between NCCs and SHF cells during embryonic heart development[3,21]. Though not experimentally tested in this protocol, it remains

unclear if SHF-derived Wnt5a provides reciprocal paracrine signals to the neural crest through ROR2 receptors. This hypothesis could be tested using this protocol by repeating the experiments with C2C12 cells on the top and O9-1 cells on the bottom of the well insert construct. If SHF-derived Wnt5a does provide a reciprocal paracrine signal through NCC-ROR2, then one would expect the knockdown of *Wnt5a* in C2C12 cells to inhibit migratory capacity and actin polymerization of the underlying O9-1 cells.

There are several unique strengths of this protocol. First, it is a non-contact well insert system that incorporates the sequential use of wound-healing assays and immunostaining techniques to evaluate functional and molecular Wnt/PCP characteristics in the same population of signal-receiving cells. This not only provides a robust approach to phenotyping noncanonical Wnt-induced intracellular filament organization and polarized migratory changes *in vitro* but also permits more granular assessments of signal transduction mechanisms. While this protocol provides proof of principle regarding Wnt5a–ROR2 molecules, the model also easily lends itself to evaluating the effects of other ligands and receptors in the noncanonical Wnt signaling pathway. One can further adapt the immunostaining protocol to evaluate the expression of multiple potential downstream effector proteins (e.g., RhoA, p-JNK, Daam1, Rac1) that have been shown to transduce noncanonical Wnt signals *in vivo.* In addition, protein levels of these various effector molecules can be correlated with either migratory or actin cytoarchitecture phenotypes. Second, the non-contact nature of the coculture system allows for the independent manipulation of specific signal-sending versus signal-receiving molecules in the Wnt/PCP pathway. In these representative results, it was elected to perform cell-specific siRNA knockdown. However, this protocol is also amenable to the use of pharmacologic inhibitors or genetically modified cell lines to evaluate candidate ligand–receptor pathways for clinical application. Similarly, one can perform phenotypic rescue experiments by adding molecular or pharmacologic compounds to the coculture medium, as was shown with recombinant Wnt5a. Targeting these compounds to selectively rescue signal-sending versus signal-receiving derangements further validates paracrine mechanistic pathways in ways that are not permitted using current *in vivo* model systems.

There are many critical steps of this protocol. First, it is important to ensure that primary cells are expanded and maintained at the appropriate confluency throughout the protocol. If C2C12 myoblasts are allowed to proliferate to 100% confluency, they will begin to fuse and differentiate from myoblasts into myotubes. Hence, these cells must be passaged at the appropriate density as described. Second, given that STO feeder cells are needed to generate conditioned medium to grow O9-1 cells, it is imperative that one appropriately inactivates STO cells with mitomycin C and makes sufficient (at least 500 mL) O9-1 growth medium using inactivated STO cells prior to thawing and plating O9-1 cells. Perhaps the most critical step in this protocol is the generation of appropriate scratches with uniform geometry and width in the myoblast monolayer[32,33]. Step 3.1.4 details several tips for optimizing this part of the protocol. Despite these recommendations, it should be acknowledged that the variability associated with standard 2D scratch assays remains a technical challenge and a limitation of this protocol. Therefore, it is necessary to have multiple technical replicates of each experimental condition.

Finally, although the results presented here were generated using murine NCCs and myoblasts, this protocol can, in principle, be adapted to include any signal-sending and signal-receiving cell of interest. As a result, this system not only has applications for advancing basic mechanisms of paracrine noncanonical Wnt signaling in a variety of developmental contexts, but it can also be used to test therapeutic mechanisms for Wnt/PCP-related disease processes. Examples include pharmacologic screening for drugs that inhibit the Wnt/PCP-induced migratory capacity of malignant cells or restore directional migration of patient-derived terminal cell types with defective PCP signaling capacity at baseline. Beyond the noncanonical Wnt signaling pathway, this protocol can also be adapted to study other paracrine signaling mechanisms and pathways between two cell types. For example, siRNA-mediated knockdown of known secretory molecules in other pathways (e.g., *Notch*, *Bmp/Tgf-β*, *Fgf*) in O9-1 cells can be coupled with immunostaining of proliferative, differentiation, or apoptotic markers in underlying myoblasts.

In conclusion, this protocol establishes a novel and highly tractable experimental protocol to study mechanisms of noncanonical Wnt-related intracellular filament organization and polarized migration *in vitro.* The methods described here improve upon existing techniques by maintaining the intercellular and paracrine nature of Wnt interactions and allow for the independent assessment of signal-sending versus signal-receiving components of this pathway. This protocol can be broadly applied to investigate basic paracrine Wnt/PCP signaling mechanisms between two cell types and screen for new therapeutic compounds targeting Wnt/PCP-related disease processes.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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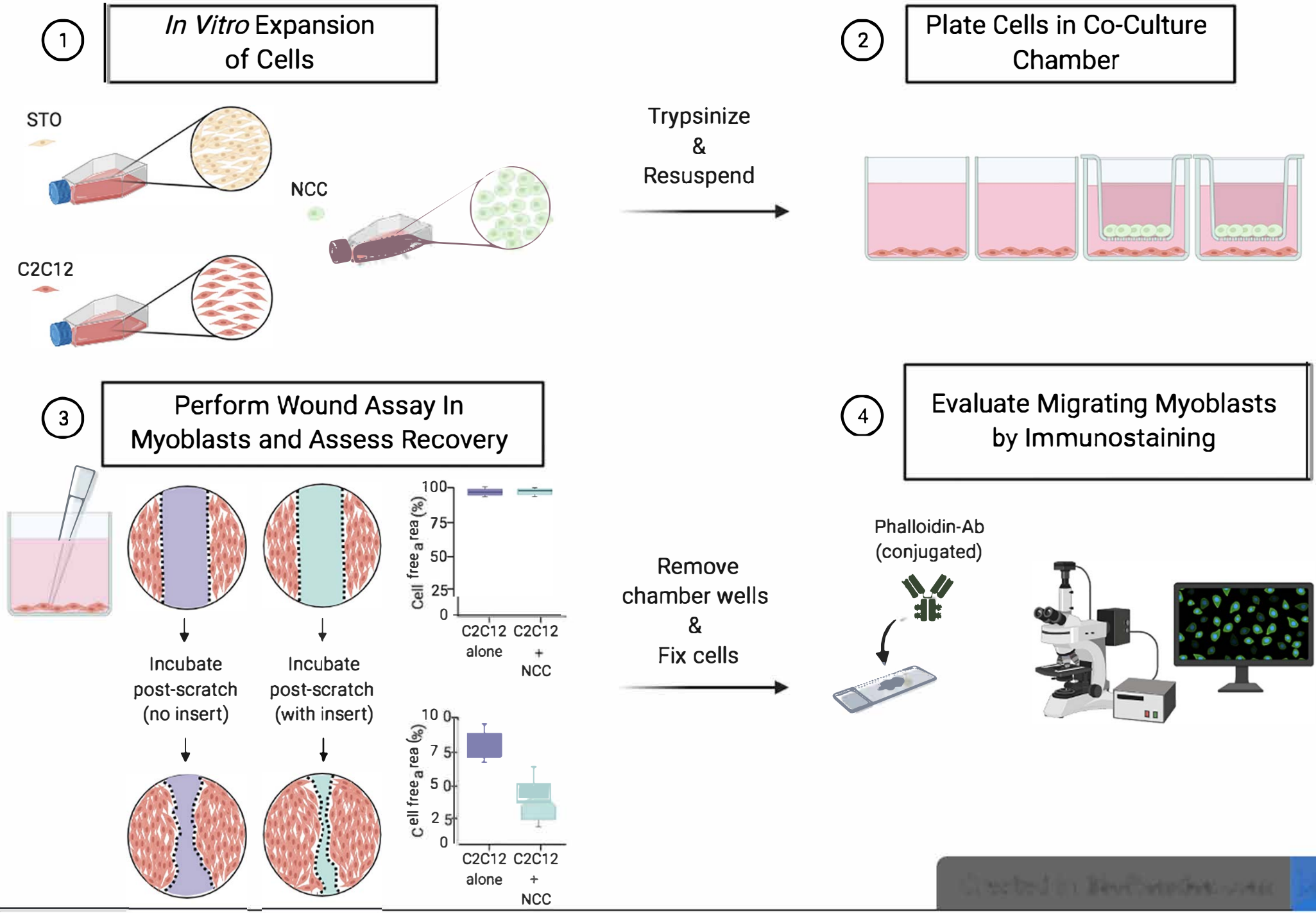
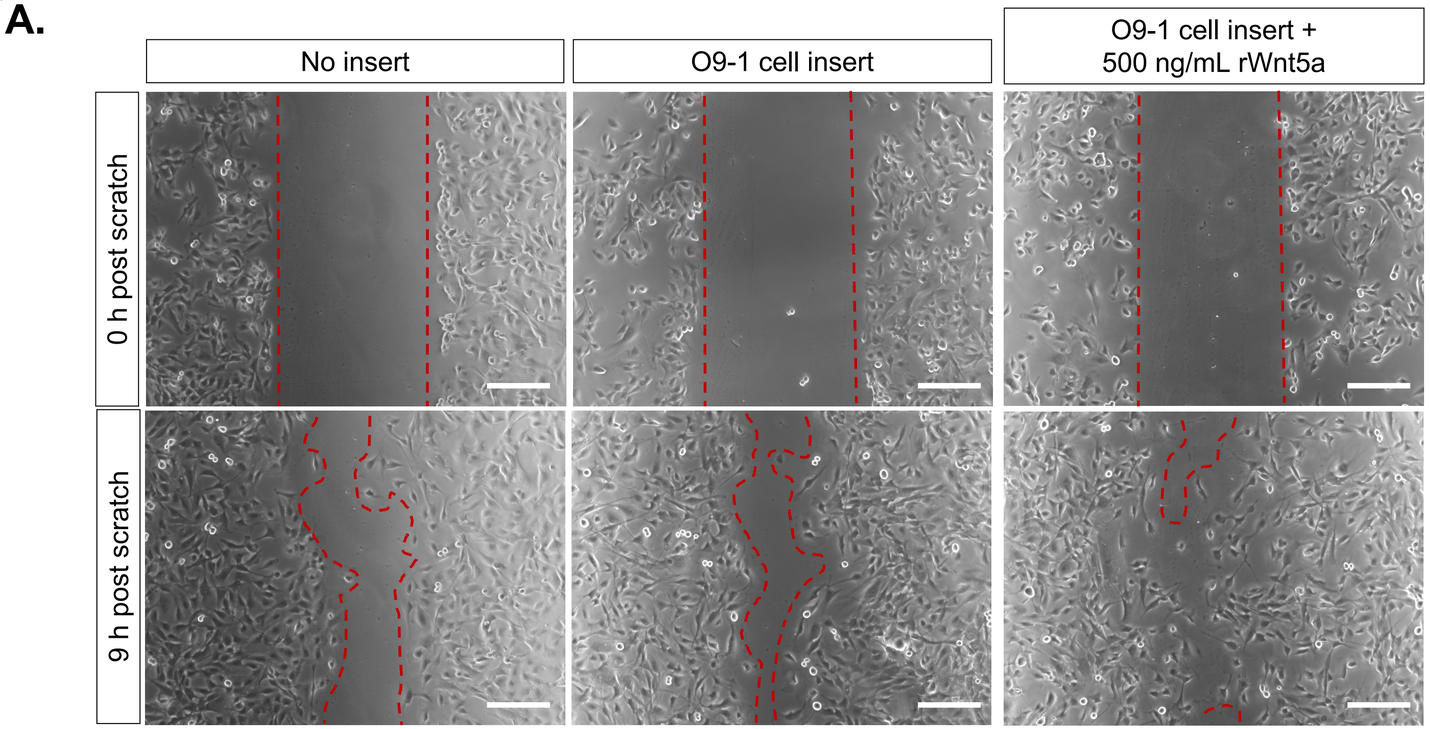
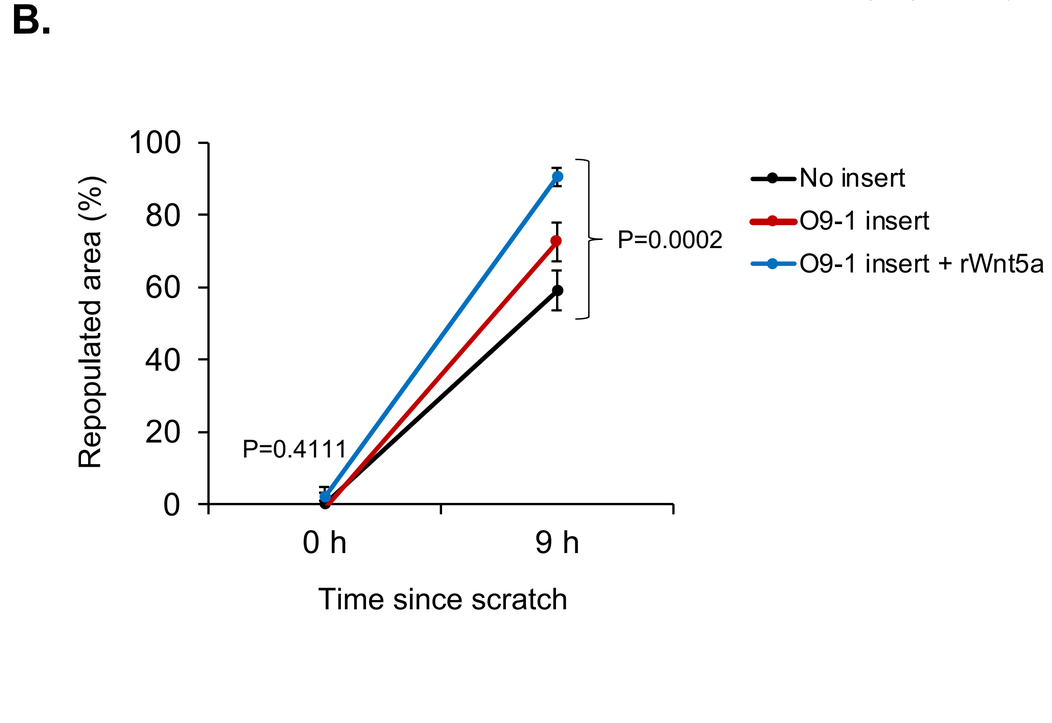
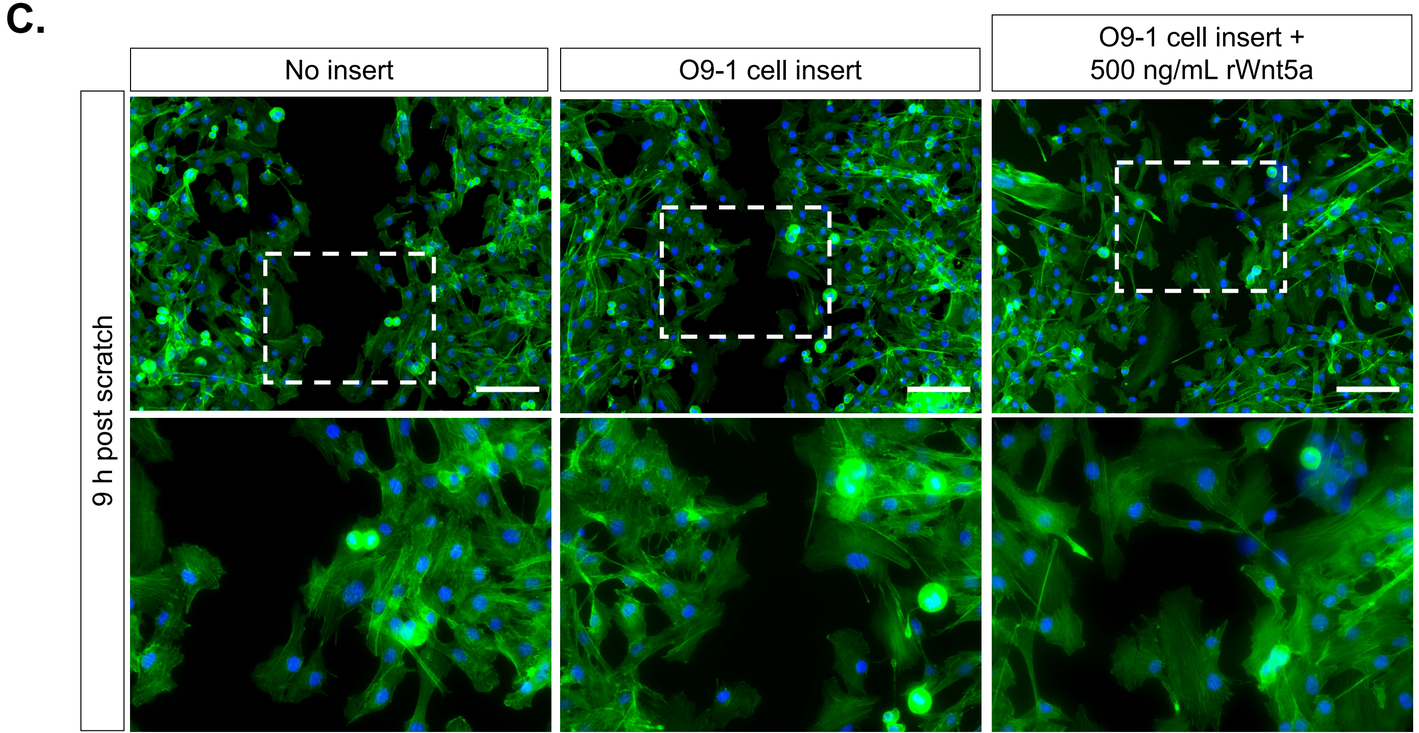
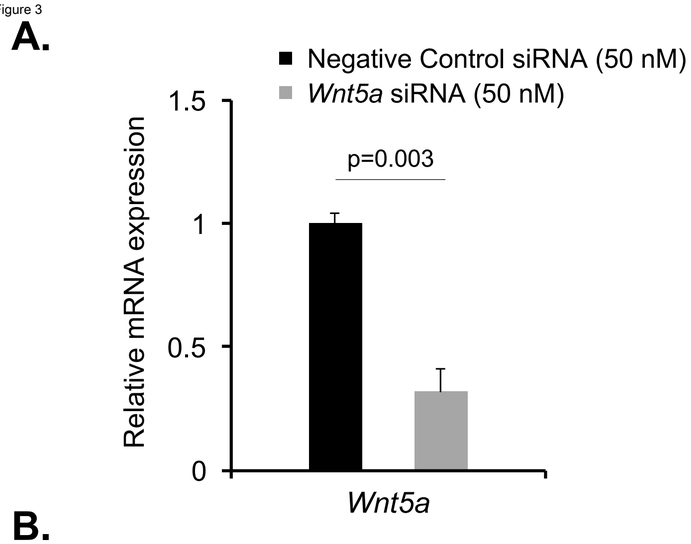


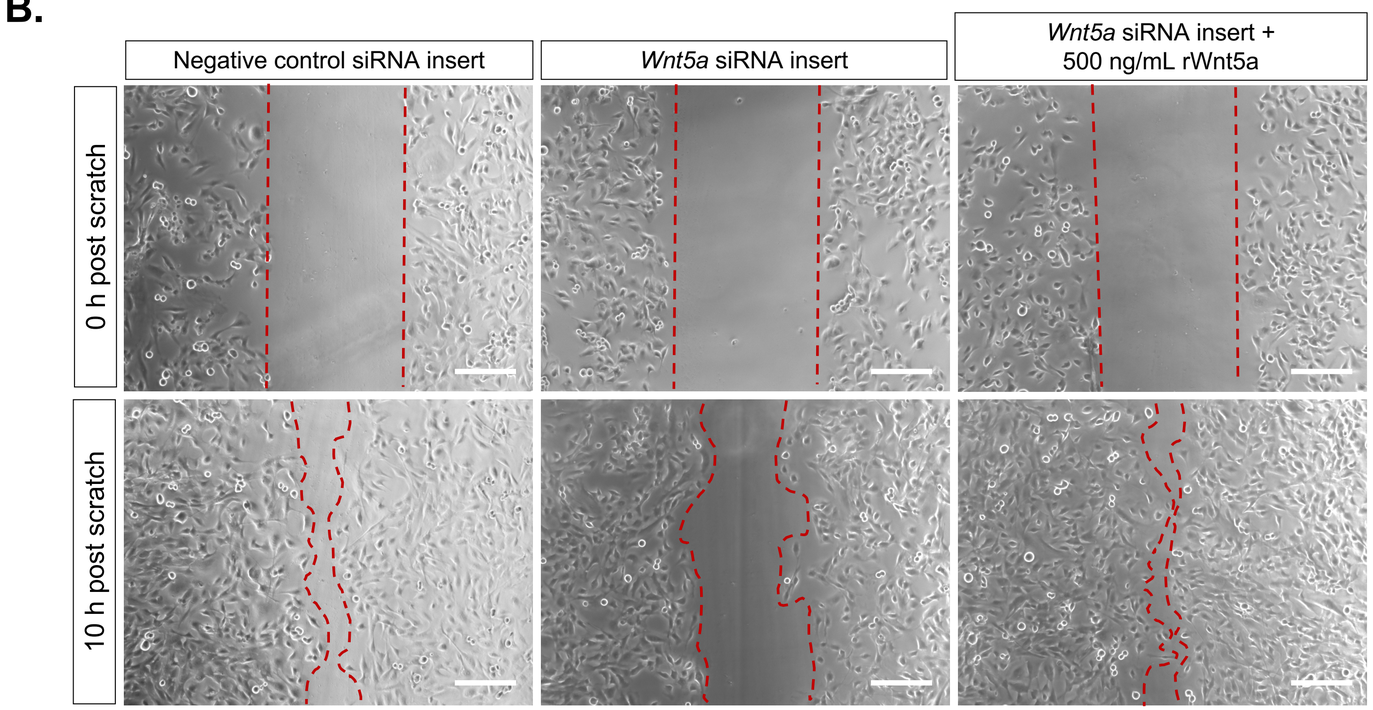
Figure 2

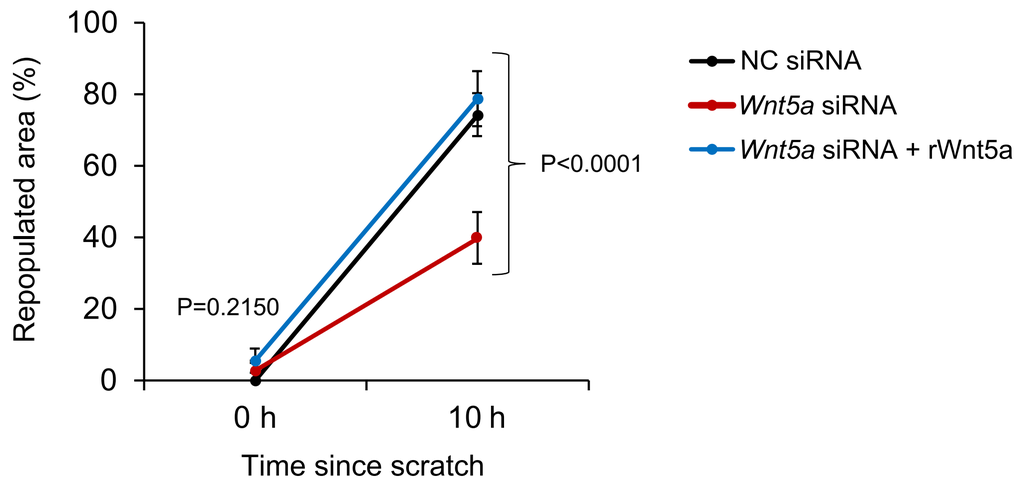


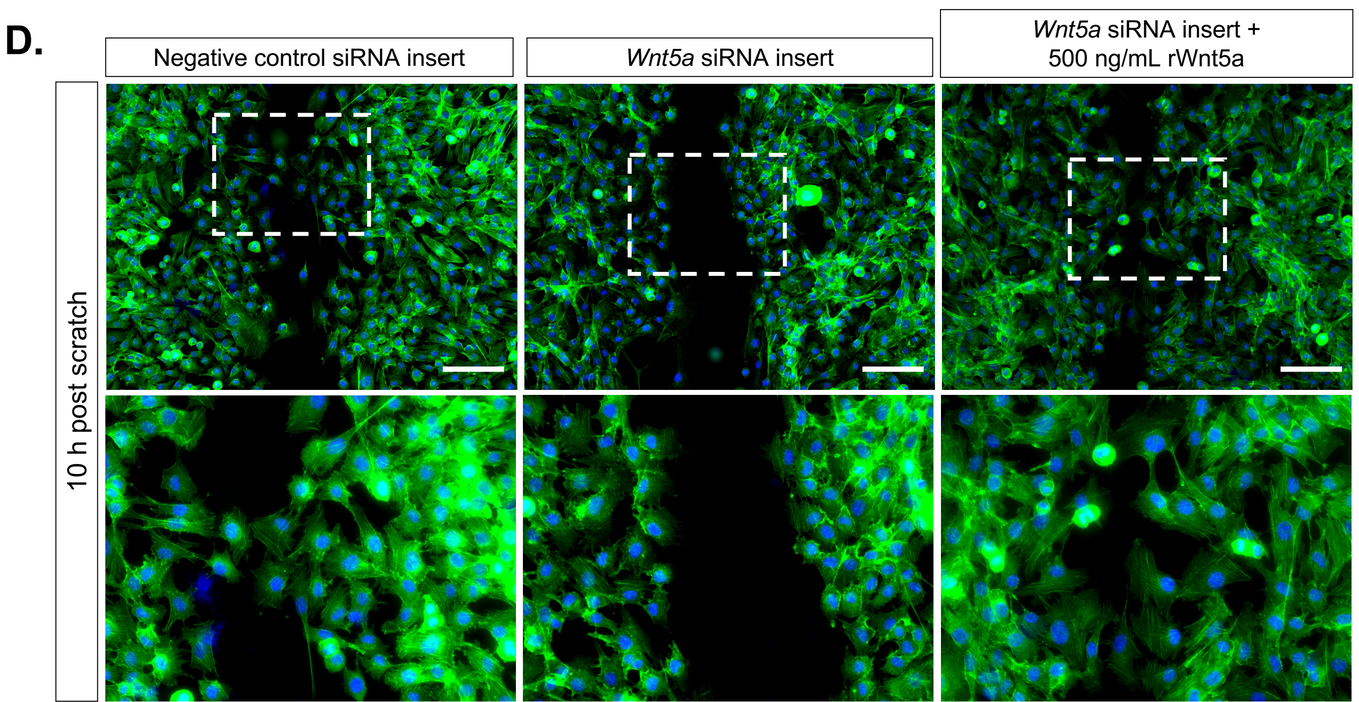


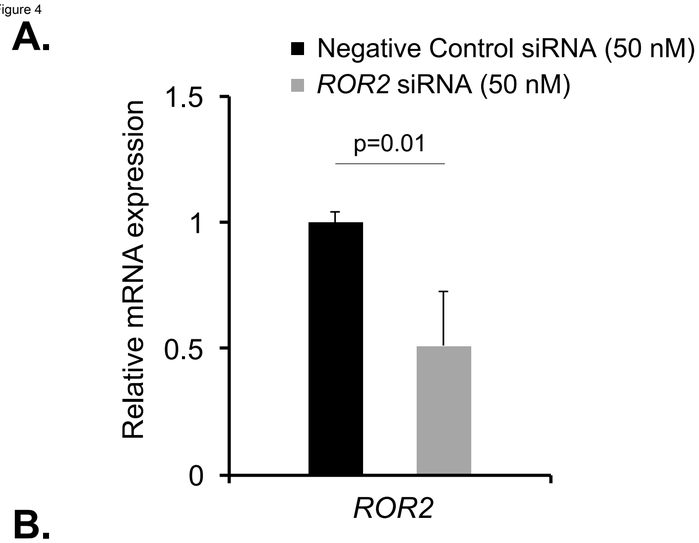


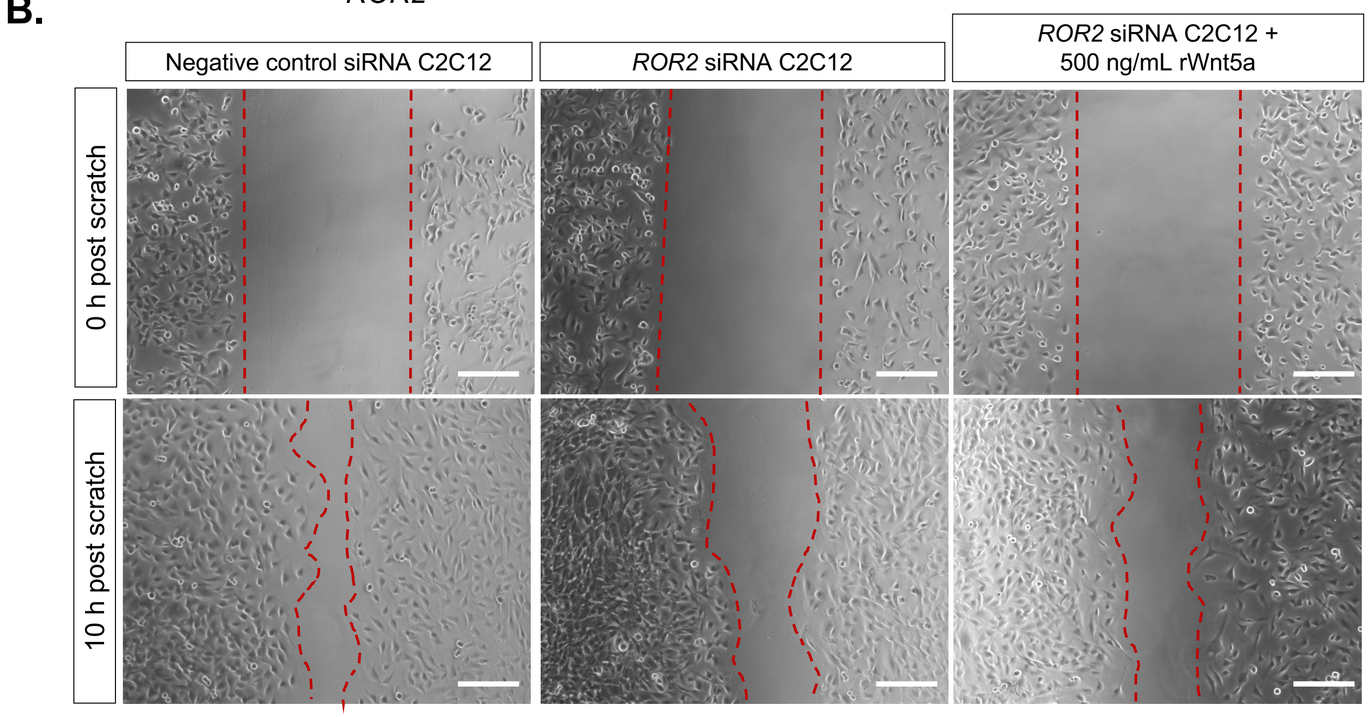


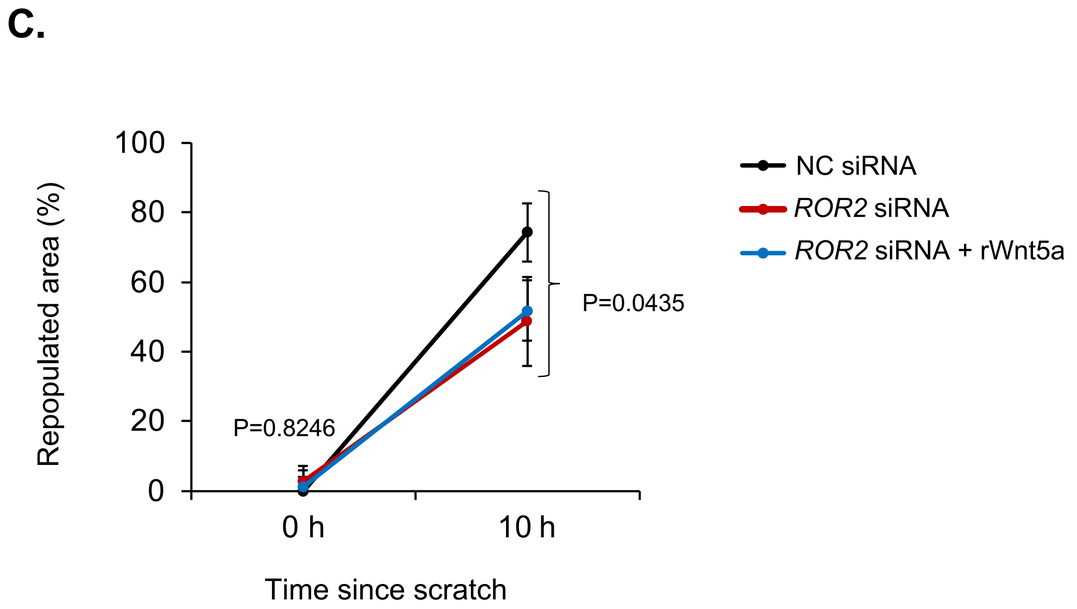


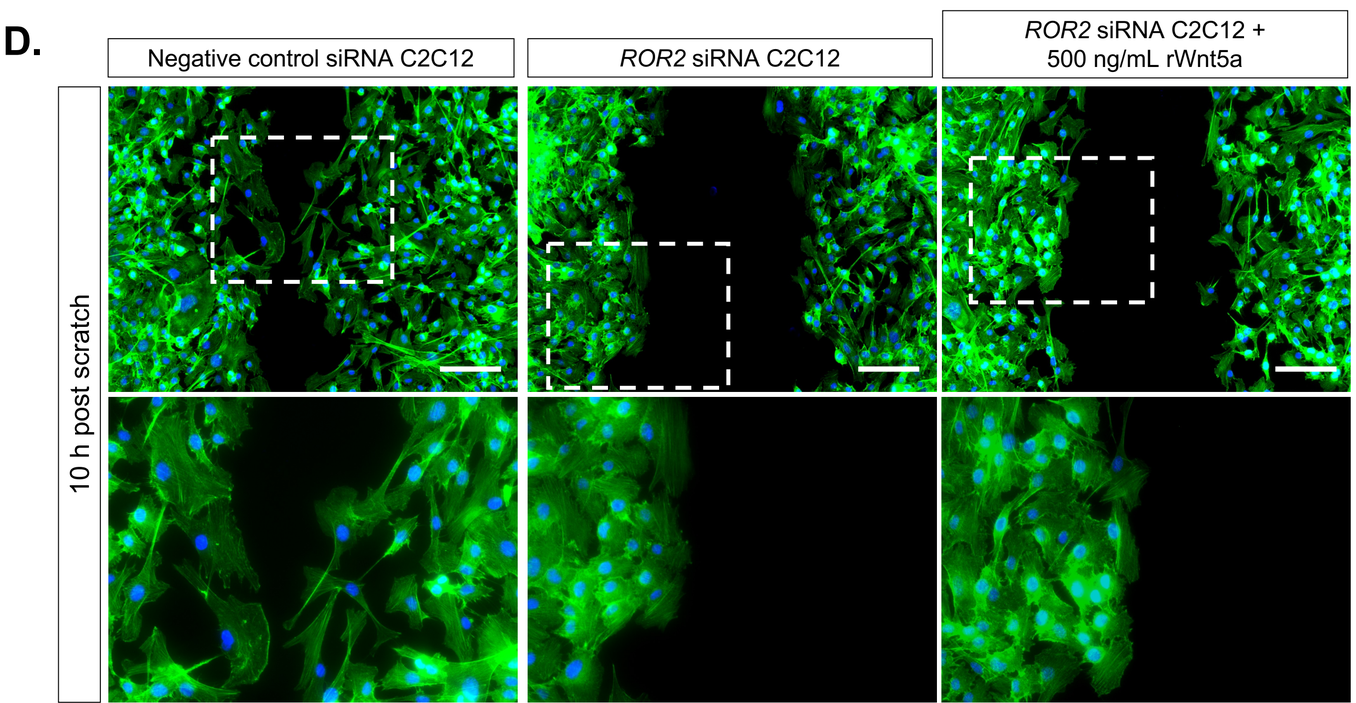












Responses to Reviewers

We thank the journal, the editor and the reviewers for the favorable review of our manuscript. We have extensively revised our manuscript to incorporate all of the suggestions made during the review process and we believe this has immensely improved the quality of our work – we thank the journal for their efforts in this regard. We hope the revised version is acceptable to the journal.

Editorial Changes
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
- The manuscript was thoroughly reviewed and all spelling and grammar issues were corrected.

2. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s) but before punctuation.
- The formatting for all reference numbers was corrected as instructed.

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.
For example: Falcon, OptiMEM, transwell, etc.
- All commercial language was removed from the manuscript.

4. Please do not underline sub-headings in the protocol.
- All sub-headings were removed from the protocol.

5. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
- All personal pronouns have been revised in the manuscript text.

6. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed?
- The “how” question for each step including those that describe how to mix solutions, aspirate/resuspend cell pellets, and plate cells have been added to the manuscript.

Please provide the volume of various solutions and media used in the protocol.
- The volumes for all solutions and media were included in the protocol.

Please write all gene names in italics.
- All gene names have been converted to italics font.

Step 1.5.3: What does “by treated STO plates” mean? The mitomycin C solution was added to the flask containing STO cells in STO media (step 1.4.6)?
- We thank the reviewer for bringing attention to this typographical error. The text has been revised to describe the following steps in correct order: mitomycin C solution is dissolved in 1x PBS (step 1.5.2.), STO cells are inactivated by adding mitomycin C solution to the flask containing STO cells in STO media (step 1.5.3).

Step 1.5.4: How much O9-1 basal media was added?
- 12 mL of O9-1 basal media is added to 75 cm^2 flasks. This volume was included in all references made to O9-1 basal media in 75 cm^2 flasks.

Step 1.5.5: Was this step repeated on the same STO cell culture?
- Yes, this step should be repeated to the same flasks containing inactivated STO cells as inactivated STO cells will not proliferate, thus facilitating their repetitive use. Step 1.5.5. has been revised to explicitly state this.

Step 2.2.3: Briefly describe the inserts used here.
- The inserts were briefly described in step 2.2.3.

Step 2.3.2: What is the concentration used in this protocol?
- The concentration for target siRNAs used in this protocol was 50 nM. This was determined experimentally by a previously conducted dose-dependent analysis. Data supporting the use of this concentration to generate sufficient gene knockdown by PCR is presented in Figure 3A (Wnt5a siRNA) and Figure 4A (ROR2 siRNA).

Step 3.1.4: How was the cell scratching protocol optimized, including the number of times scratching was done, the percentage of cells scratched, the width of each scratch, time taken to complete this step, etc.?
- We thank the editor for this question. Optimizing the scratching protocol is an important component of the protocol’s overall success. To optimize the scratching protocol, it is recommended that one scratches cells of different experimental conditions at similar levels of confluency. To do this, it is recommended that the user allows for each 4-chambered well to have cells under each condition (e.g. well #1 negative control, well #2 positive control, etc.). This will control for variation in confluence between 4-chambered wells. In addition, one should use a new sterile p-10 pipette tip and attempt to apply the same force with each scratch. One should not attempt to create more than one scratch in each well. Given the variability associated with each scratch, it is recommended that multiple replicates for each experimental condition are used (n=3). Finally, one should work expeditiously so as to minimize the time that cells are without 1x PBS during these steps. After removing the 1x PBS from a well, one should not take more than 5 s to generate a scratch in that well. Evaluation of the scratch at the zero-time point should be used as a guide to indicate comparable cell-free zone. All of these recommendations for optimizing the scratching protocol have been included in step 3.1.4.

Step 3.1.5: The images were taken with the cells in 1x PBS or without PBS? Please describe all the steps associated with imaging the cells including the microscope setting, magnification used, imaging parameters, etc. Please make sure to provide all the details such as “click this”, “select that”, “observe this”, etc.

- Images were taken with cells in 1x PBS (added back into wells in step 3.1.4). All steps associated with imaging the cells have been added to step 3.1.5.
- The steps for taking pictures are detailed below and were added to the manuscript at step 3.1.5:

1. Turn on computer and the Zeiss Vert.A1 microscope by pressing the power button
2. Place the chamber slide on the stage and rotate the objective dial to 5x magnification
3. Open Zen lite 2012 software by double clicking the software icon on the computer desktop
4. Click “Camera” tab on the software home screen. Click “Live” button to visualize the cells on the AxioCam IC tab
5. Ensure that the light filter is pulled all the way out to allow light to pass to the camera and computer screen
6. Manually move and/or rotate the chamber slide as needed to position the wound area in the center of the live image on the AxioCam IC tab
7. To take picture, click “snap”, this will open a new tab next to the AxioCam IC tab that contains the image.
8. To save this still image, click file on the top left of the software home page → click save as → enter the file name in the file name box. Save figure in carl zeiss image (*.czi) format (this is the default setting) → select desktop on the bar on the left to save the file to the desktop → this saves file as a .czi file, which can only be opened in the Zen lite 2012 software program
9. To save the picture as a .tiff, click file → save as → enter file name in the file name box. Save figure as tagged image file (*.tiff) by clicking the “save as type” button and selecting “*.tiff” from the dropdown menu. This saves file as a .tiff, which can be opened in any image processing software
10. Manually reposition the chamber slide to take an additional 2-3 more pictures at other points of the wound in the same well.
11. In total, this will result in 3-4 non-overlapping high magnification images of the wound in each well.

Step 4.2.4: Does tracing the cell edges help in determining the migration of the cells? If yes, please mention this in the protocol.

- The tracing of the cell edges with the hydrophobic pen is meant to ensure that the antibody solution applied to the slide will not spill/leak from the edge of the slide. This is meant to ensure that the cells are adequately incubated with antibody. This detail has been added to step 4.2.4.

Step 4.2.8: How were the images acquired? Please elaborate the steps for acquiring images. Please make sure to provide all the details such as “click this”, “select that”,

“observe this”, etc. Please mention all the steps that are necessary to execute the action item. Please provide details so a reader may replicate your analysis including buttons clicked, inputs, screenshots, etc. Please keep in mind that software steps without a graphical user interface (GUI) cannot be filmed.
- The images were acquired according to the steps outlined above in 3.1.5. The software does have a GUI and these steps will be amenable to filming.

7. Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate Figure Legend.
- Scale bars have been included in all pictures taken with the microscope and the definition of the scale has been added to the respective Figure Legends.

8. Please provide an n number of samples for all the figures. Please state in the figure legends what do the error bars represent: Standard error or standard deviation, for all figures.
- An n=3 samples were used for all main manuscript figures and error bars represent standard deviation. These details were added to the figure legends for all manuscript figures. For the supplemental figures, representative pictures of n=1 samples for each condition is shown.

9. Please also include the following in the Discussion in detail along with citations:
a) Critical steps within the protocol
b) Any limitations of the technique
- Critical steps of the protocol, include: (1) expanding and maintaining primary cells at appropriate confluency to avoid unwanted differentiation or cell loss; (2) appropriately inactivating STO feeder cells and generating sufficient quantities of conditioned media for O9-1 cell growth prior to initially plating O9-1 cells; (3) making wound scratches of appropriate geometry and width. A thorough elaboration of each of these critical steps was added to the discussion section of the manuscript. The key limitation of this protocol is the variability with 2D scratch wound generation. This was explicitly discussed in the discussion section as well. Two published citations referencing this limitation of 2D wound healing assays were also included. Finally, the importance of adhering to the recommendations provided in this protocol to minimize the adverse impact of this limitation (step 3.1.4) was reiterated in the discussion.

10. With regards to video production, please let us know how you would like to proceed. Please select one of the options below.

10.1. Journal Produced Videos (JPV): If you are within our videographer network, you can choose the Video Produced by JoVE option (https://www.jove.com/authors/faq). Once the manuscript is accepted, we write the script, film the protocol, and produce the video for you. If selecting this option, please provide us with the complete filming address.
10.2. Hybrid filming option (APF). In this case, upon manuscript acceptance, we write the script for you, you will then perform the filming and send us the raw footage straight

out of the camera. Using these video clips, we will then produce the video for you. Please see the attached criteria. You do not need to film till the scripting is done.
- Given the current situation with COVID-19 in the Los Angeles County Area and the university policies surrounding COVID-19, we prefer the hybrid filming option (APF).

Reviewer #1 Comments:

Manuscript summary:
Toubat and Ram Kumar report an in vitro technique to evaluate paracrine noncanonical Wnt signaling between signal sending and signal receiving cells in a co-culture system using established cell lines. The experiments are documented in detail and the combinatorial wounding and co-culture approach is potentially of general interest. However, the following major points need to be addressed.

Scientific points:
1. Please state clearly how generally applicable to paracrine signaling mechanisms this method is. The emphasis on non-canonical Wnt signaling in the title and elsewhere may reduce the impact for a broad readership.
- We thank the reviewer for raising this important point. The goal of this protocol was to create an in vitro assay that can recapitulate multiple biological endpoints that have been described in non-canonical Wnt signaling literature. To accomplish this, we combined a wound-healing assay (polarized directional migration of cells) with immunocytochemical staining of phalloidin (to study cellular architecture changes and actin polymerization). However, as the reviewer correctly notes, this protocol can be adapted to study different candidate paracrine signaling mechanisms between O9-1 and C2C12 cells. For example, one can conduct siRNA-mediated knockdown of known secretory molecules in other developmental pathways (Notch, BMP/TGF-b, FGF, etc.) in O9-1 cells and assess for other biologic endpoints in myoblasts, including proliferative, differentiation, or apoptotic markers. This point has been added to the discussion section.

2. The authors should discuss the relevance for the second heart field where Wnt5a is also made by the mesoderm. What happens if Wnt5a is knocked down in myoblasts or Ror2 in neural crest cells? This should at least be discussed.
- The reviewer is correct that signaling from second heart field cells to the neural crest is also developmentally relevant during outflow tract maturation in the heart. With respect to the non-canonical Wnt signaling pathway, no study to date has specifically determined whether conditional deletion of Wnt5a in second heart field progenitors disrupts paracrine signaling to neural crest cells through ROR2. However, the reviewer appropriately raises this as an intriguing hypothesis that can be studied using this protocol. Our model will lend itself to test this hypothesis as well, by switching O9-1 and C2C12 cells between the insert and the chamber and knocking out genes as described by the reviewer, which is another strength of our model. Thus, as recommended by the reviewer, this point was elaborated in the discussion section of the manuscript.

3. Please clarify if myoblast morphology was different to that in controls on Wnt5a knock-down without adding 09-1 cells.

- Baseline myoblast morphology was similar. However, myoblast polarized morphology was lost following Wnt5a knock-down in O9-1 cells, but was rescued following the addition of rWnt5a.

4. The work of Schleiffarth and colleagues from 2007 (PMID: 17515859) should be included in the discussion.
- We thank the reviewer for bringing attention to this study that was mistakenly omitted from the initial manuscript submission. The study from Schleriffarth et al. (2007) has been included and explicitly mentioned in the discussion.

Technical comments:
5. The authors should clearly state at some point what are the salient differences in their protocol from other wounding and transwell coculture experiments.
- The most important difference between this protocol and other wounding and transwell coculture experiments is that this protocol utilizes a non-contact transwell system that incorporates both wound-healing assays and immunocytochemical techniques to evaluate functional and molecular Wnt/PCP characteristics in the same population of signal-receiving cells. This provides a robust approach to phenotyping non-canonical Wnt-induced intracellular filament organization and polarized migratory changes *in vitro*. Such a system can be used to pursue granular assessments of precise signal transduction mechanisms between any two cell types participating in non-canonical Wnt signaling. These points are explicitly stated in the discussion.

6. In the trypsinization steps (such as 1.2.5), is the centrifugation step necessary, given that the activity of the enzyme is already quenched? Why not just seed by diluting 1 ml with 11 ml of medium in a new flask? For C2 cells, this is sufficient. Does this passaging method really need to be repeated for each cell type?
- The reviewer is correct that the addition of media quenches the enzyme. However, the centrifugation and resuspension steps allow for the removal of any residual enzyme and cellular debris. To minimize the potential negative impact that such debris can have on subsequent plating and passaging, we elect to centrifuge with each trypsinization step. But we candidly admit that this step is indeed optional.

7. Introduce the importance of each cell line by a short phrase, including why STO cells need to be inactivated.
- The importance of each cell line has been added to the protocol when first referencing the respective cell line (step 1.1.2 for C2C12s; step 1.3.4. STO cells; step 1.6.3. O9-1 cells). We also include a brief description of why STO cells need to be inactivated to serve as feeder cells for the generation of conditioned media in step 1.5.3.

8. Concerning the wound assay, please clarify how you distinguish between 80 and 90% confluence. Is there a risk the myoblasts will start to differentiate at high levels of confluence?
- Generally, cell confluence is determined qualitatively in this protocol. We do not describe methods for reliably measuring differences between 80% and 90% confluence here. However, we provide a range, as this is typically done for cell culture protocols,

and is meant to reflect an average of the variability of confluence within the flask. The reviewer is correct that there is a known risk of myoblast differentiation at high levels of confluence. This point was also brought up by the editor (see comments above). Thus, we have included specific mention of the need to keep C2C12 cells from growing over confluent throughout the protocol to avoid differentiation, cellular fusion, and myotube generation in the newly written paragraph highlighting the critical steps of the protocol in the discussion.

9. Please provide details as to the length and width of the scratch (step 3.1.4). Is it easy to monitor the wound size every 2-3 hours with the overlying insert (step 3.1.8)? Does this have to be removed each time?
- Details regarding the scratch and how to optimize this part of the protocol were also requested by the editor (see comments above). Thus, as requested by both the editor and the reviewer, extensive details have been added to step 3.1.4. We thank the reviewer for raising an excellent point regarding the feasibility of monitoring the wound size during the migration assay. This procedure is easy as it does not require removing the overlying insert given the non-contact nature of the construct. This detail has been added to step 3.1.8.

10. How different are C2 and 09-1 culture media? Is one cell type compromised during co-culture?
- C2C12 and O9-1 cell culture media do differ, as O9-1 media contains conditioned media from inactivated STO cells and additional reagents such as LIF, essential amino acids, and b-FGF. It is for this reason that both cell lines are initially expanded in parallel using separate flasks. It is not until the wound generation and cell migration portion of the assay (final 9-10 hrs) that the co-culture system is assembled and the two cell types share the same media. While a comprehensive analysis of cellular changes when co-cultured has not been performed, our data demonstrate that cellular morphology and quantity (proliferation/viability) appear similar in both cell types for the brief period of co-culture needed for our experiments, suggesting that neither cell type is compromised.

11. Finally, though importantly, the authors should be aware that Phalloidin is not an antibody, thus this is not immunocytochemical staining. The blocking step is unlikely to be necessary. The authors could however point out that this step can be coupled with fluorescent antibody staining.
- We appreciate and thank the reviewer for raising this point. We have revised the manuscript to replace “immunocytochemical” with “immunostaining”. We also explicitly state that the blocking step is included to allow for coupling with fluorescence antibodies.

Reviewer #2 Comments:

Manuscript Summary:

There is definite value in a protocol for studying cells specifically responding to another cell line's Wnt5a signal.

Major Concerns:
As this protocol purports to establish a system with two distinct cell lines in co-culture that send and receive Wnt5a signals, there should be a demonstration of such through at very least a western blot showing its expression due to the presence of O9-1 cells as well as its abolition at the protein level in response to siRNA as well. Additionally, please demonstrate error bars and significance for qPCR readings.
- Error bars and p-values representing significance have been added to the qPCR readings. We thank the reviewer for bringing attention to the importance of validating the abolition of Wnt5a protein expression in response to the siRNA. We have conducted an immunofluorescence stain for Wnt5a protein level in O9-1 neural crest cells treated with negative control siRNA and Wnt5a siRNA at the same siRNA concentrations and with the same protocol used for the PCR data. These immunofluorescence data are now included as Supplemental Figure 3.

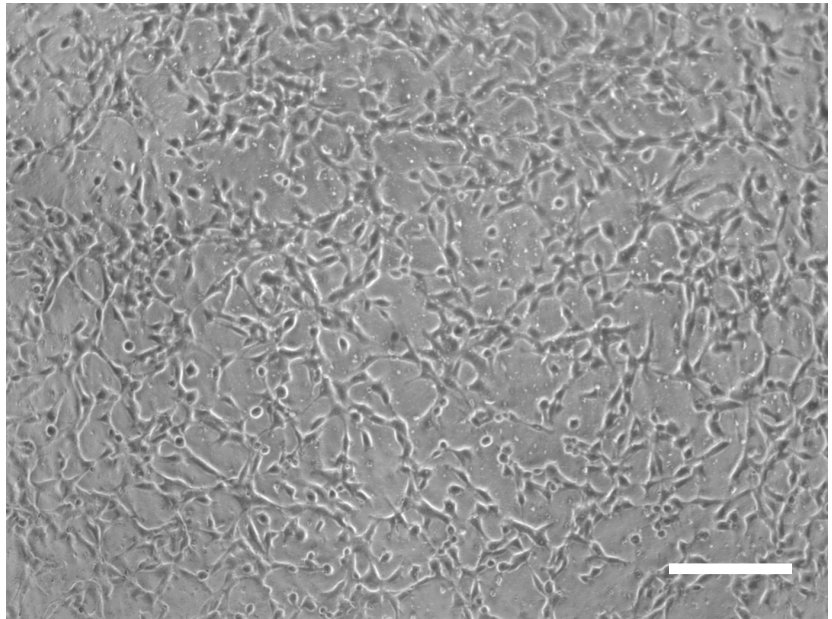
It looks like there is a significant change with rWNT5A in combination with the presence of O9-1 cells, but it seems no p-value for between C2C12 alone vs C2C12 with O9-1, which would be the most important for demonstrating the viability of this system meaning that the 'Wnt5a-secreting' cells are in fact able to enhance repopulation on their own. Without demonstration of a significant difference due to only O9-1 cells, it would call into question the viability of using a scratch assay as a readout for signal response at best, and at worst possibly the use of these two specific cell lines to study Wnt5a response.
- We thank the reviewer for bringing attention to this point. The reviewer is correct that the demonstration of a statistically significant difference in migratory potential of C2C12 cells grown alone vs those grown in the presence of O9-1 cells is critical. In the manuscript text, we reported the absolute difference in the percentage of wound recovery between these two conditions (59% C2C12 cells alone vs 73% in C2C12+O9-1). The p-value shown in the figure reflected a comparison of the wound recovery across all three groups. We apologize for not presenting this in a clear manner and have revised the presentation now. The revised manuscript text now clearly states the p-value ($p=0.033$) for the comparison of C2C12 cells alone vs C2C12+O9-1 cells. To address this concern throughout, we added corresponding p-values for the other pairwise comparisons described in the remaining sections of the results: 39% C2C12 repopulation with Wnt5a knockdown vs 75% control O9-1 cells, $p<0.001$; and 48.1% C2C12 repopulation with ROR2 knockdown vs control C2C12 cells, $p=0.019$.

Minor Concerns:
It is unclear on the significance level of the quantification of area repopulated after scratch assay. Does the $p=0.411$ refer to the difference at the start? Should this not be 0% by definition as the second reading is based on this area for each replicate?
- We thank the reviewer for raising this point. The p-value included at the start of the assay reflects statistical comparison of the the average wound area under each condition. That is to say, a non-significant p-value such as $p=0.411$ at time=0, can be interpreted as the average wound width under each condition is not statistically significantly different at baseline.

Supplemental Figure 1

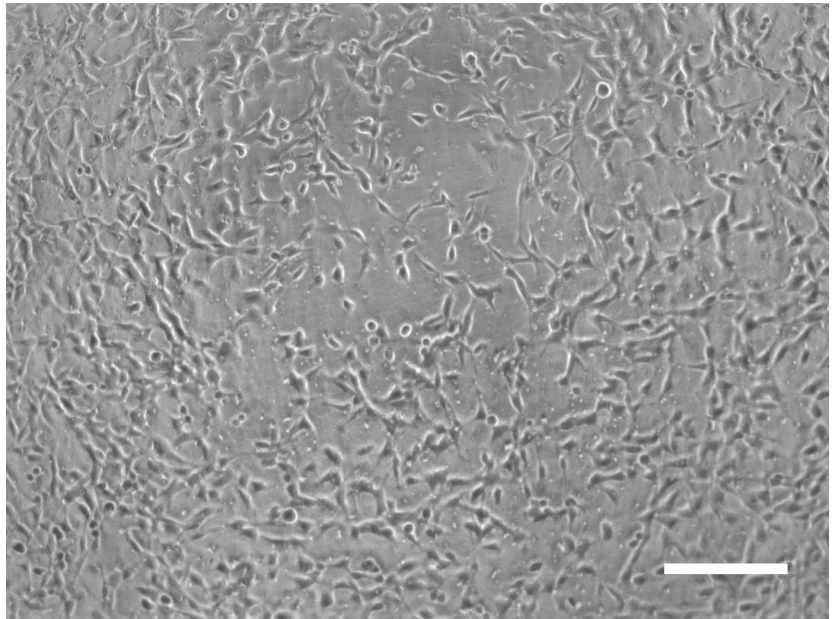
A.

O9-1 insert (treated with 50 nM of negative control siRNA)

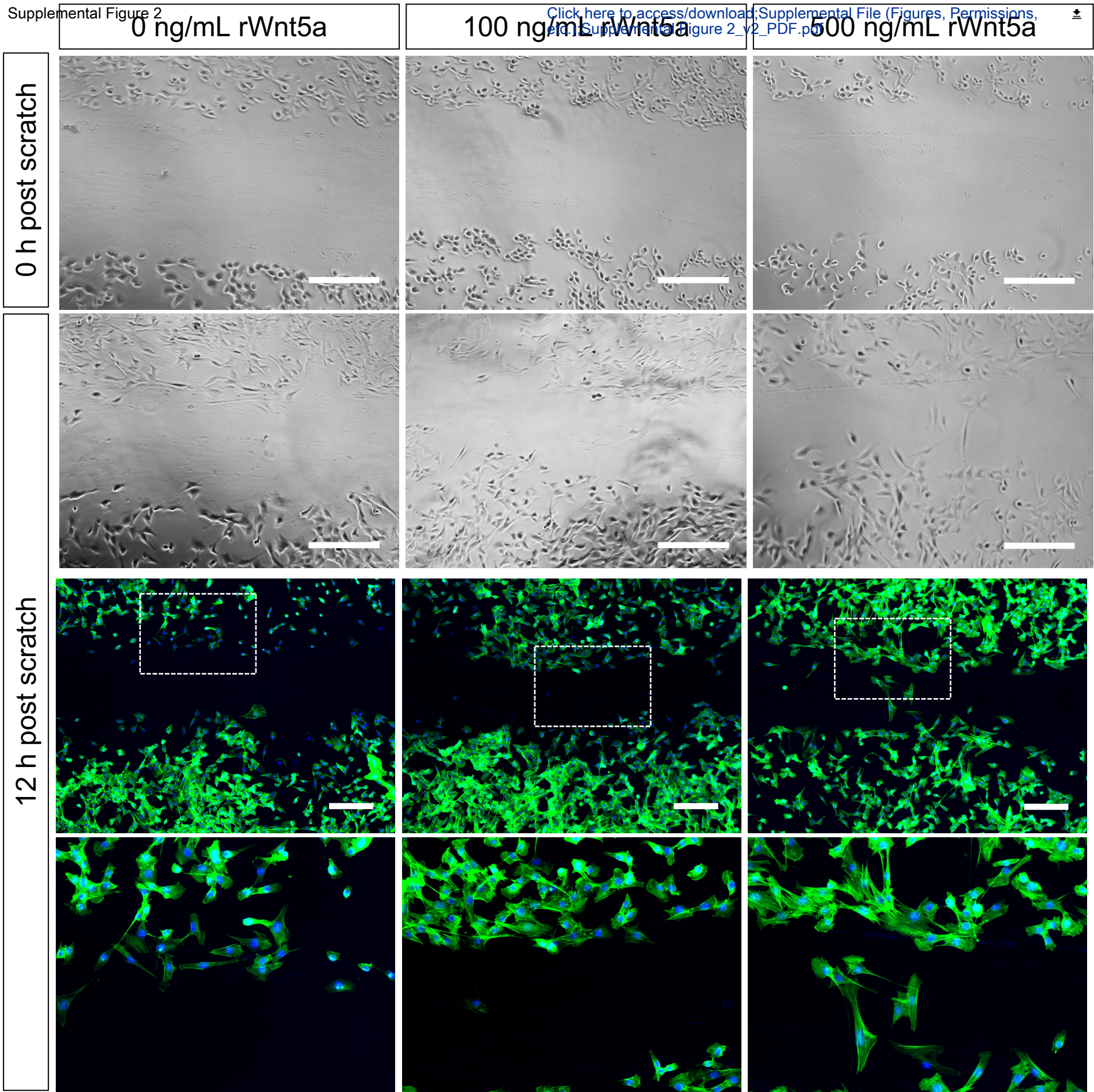


B.

O9-1 insert (treated with 50 nM of *Wnt5a* siRNA)



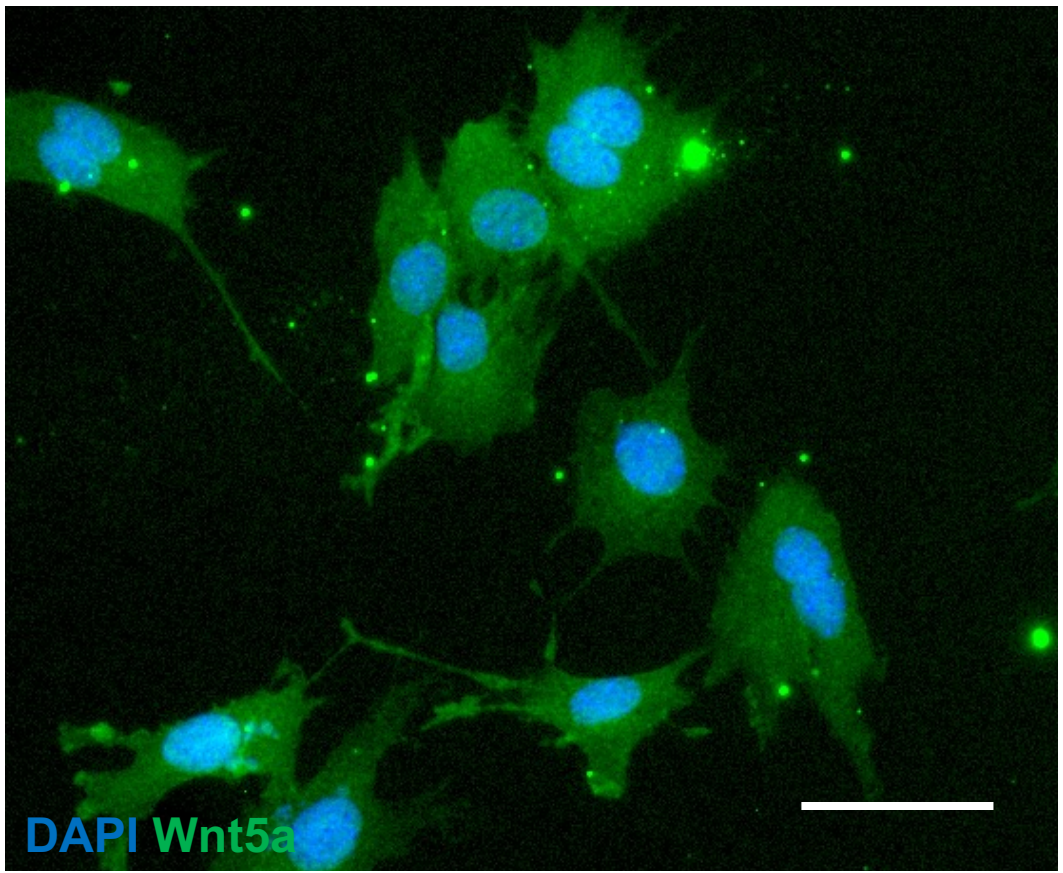
Supplemental Figure 2



Supplemental Figure 3

A.

O9-1 cells (treated with 50 nM of negative control siRNA)



B.

O9-1 cells (treated with 50 nM *Wnt5a* siRNA)