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A Functional Assay to Assess Connexin43 Mediated Cell-to-Cell Communication of Second Messengers in Cultured Bone Cells

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Summary

Cell-to-cell transfer of small molecules is a fundamental way by which multicellular organisms coordinate function. Recent work has highlighted the complexity of biologic responses downstream of gap junctions. As the connexin-regulated effectors are coming into focus, there is a need to develop functional assays that allow the specific testing of biologically relevant second messengers. Here, we describe a modification of the classic gap junction parachute assay to assess biologically relevant molecules passed through gap junctions.

Keywords

Osteoblasts; Connexin43; Cell-to-cell Communication; Gap Junctions; Luciferase Reporter Assay; Osteoblast; Transient Transfection; Parachute Assay

1. Introduction

Cell-to-cell transfer of small molecules through gap junctions regulates diverse biologic processes (1,2). In bone, the intercellular communication between gap junction-coupled osteoblast lineage cells is important to tissue function. Recently, a large number of studies have implicated connexin43 (Cx43) containing gap junctions in a complex array of biologic responses that influence bone quality in vivo (3–6). These responses include anabolic and catabolic effects on bone, depending on the cue initiating the cellular response such as mechanical load, disuse or hormonal challenge. As the biologic effects that occur downstream of messages communicated by gap junctions expand, it becomes imperative to be able to develop assays to define the biologically relevant second messengers that are passed between cells.

We have developed a method that allows the assessment of Cx43 communicated signals by modifying a classic assay for probing gap junction function known as the parachute assay. As originally described (7), the parachute assay takes advantage of a population of *donor* cells that are loaded with a gap junction permeable, low molecular weight fluorescent dye

such as calcium green AM ester. The *donor* cells are then seeded onto a confluent monolayer of unlabeled cells, termed the *acceptor* cells. Gap junctional communication is determined by the diffusion of the low molecular weight fluorophore from the *donor* cell to the *acceptor* cell. Later modifications included the use of a non-gap junction transferred dye such as DiI to discriminate the *donor* and *acceptor* cell populations (8).

The fundamental concept of our assay is the same. We specifically activate signaling in the *donor* cell population by introducing a reporter to that signaling event into the *acceptor* cell population and then seed the *donor* cells onto the *acceptor* cells. The degree of gap junctional communication is measured by reporter activation. Thus, this method assesses not just the passive movement of a molecule from cell to cell, but the functional consequences of intercellular communication. The gap junction dependence of the communication of this signal is verified by culturing donor and acceptor cells on transwell chambers and/or by the use of gap junction inhibition.

In the example we present, we transfect the *donor* cell population with a constitutively active fibroblast growth factor receptor 1 (caFGFR1; myr-FGFR-TDII, provided by Dr. Daniel Donoghue, University of California San Diego) (9). FGFR1 is known to activate PLC γ 1, which in turn leads to second messengers accumulation (9,10). The *acceptor* cell population is transfected with a Runx2-luciferase reporter construct. We have previously shown that Cx43 amplifies FGF2-dependent signaling to increase the activity of the transcription factor Runx2 (11,12). Further, we have shown this involves the inositol pyrophosphate second messenger system (13). Obviously, this system can be adapted to numerous second messenger-generating effectors in the *donor* cell and diverse readouts (e.g., signal pathway specific luciferase reporters, fluorophore activation, etc.) in the *acceptor* cell population. Lastly, this system can be easily adapted to other cell types and other gap junctions. Indeed, we have recently used a similar approach to show the delivery of siRNA between mesenchymal stem cells and synovial fibroblasts in culture (14).

2. Materials

For all tissue culture procedures and reagents used with live cells, aseptic technique and sterile solutions are required. All solutions should be made using ultrapure water. Chemicals should be molecular biology grade or ACS grade, as available.

2.1. Cell Culture and Transfection

1. MC3T3-E1 clone 4 cells (ATCC, Manassas, VA, USA) (*see* Note 1,2).
2. P100 tissue culture dish and 24 well multi-well tissue culture plates.

¹This assay can be adapted to other cell types. The MC3T3 cell line used here has a modest amount of endogenous Cx43 expression. In this context, overexpression or knockdown of Cx43 levels can impact the degree of cell-to-cell coupling and downstream signaling. We have also used this assay in UMR106 osteoblast-like osteosarcoma cells with little endogenous Cx43 expression and in ROS17/2.8 cells with robust Cx43 expression. In the case of low endogenous Cx43 abundance as in UMR-106 cells, overexpression of Cx43 is required to detect cell-to-cell communication, while siRNA-mediated knockdown is ineffective. The converse is true in ROS17/2.8 cells.

²MC3T3 clone 4 cells maintain a relatively stable phenotype in culture, but in our hands passage numbers over 20 can sometimes result in phenotype changes and reduced transfection efficiency.

3. Complete tissue culture medium: α Minimum Essential Medium, 10% fetal bovine serum, 1% penicillin-streptomycin
4. Calcium magnesium-free Hank's balance salt solution (HBSS): 0.138 M sodium chloride, 0.005 M potassium chloride, 0.00044 M potassium phosphate monobasic, 0.00003 M sodium phosphate dibasic, 0.004 M sodium bicarbonate, 0.0056 M glucose. Sterile filter and store at 4°C.
5. Tissue culture grade 0.25% Trypsin EDTA solution (Thermo Fisher Scientific, Waltham, MA, USA). Store at -20°C.
6. Transwell chambers, 5 μ m pore size (Corning Life Sciences, Corning NY USA)
7. Plasmid DNA (*see* Note 3, 4). Here we use a pSFFV-neo (empty vector control for Cx43 overexpression construct), pSFFV-Cx43 (Cx43 overexpression), constitutively active FGFR1 (myr-FGFR-TDII, provided by Dr. Daniel Donoghue, University of California San Diego), pcDNA3 (empty vector control for FGFR1), Runx2 luciferase reporter (p6xOSE2-Luciferase, provided by Gerard Karsenty, Columbia University), and pRL-TK Renilla luciferase (Promega, Madison WI USA).
8. Transfection reagents. Jet Prime transfection reagent (Polypus Transfection, Ilkirch-Graffenstaden, France) (*see* Note 5).
9. Sterile 1.7 ml eppendorf tubes

2.2. Luciferase Reporter Assay

1. Dual-injector Centro LB960 Luminometer (Berthold Technologies, Oak Ridge, TN, USA).
2. HBSS (*see* above).
3. Luciferase Lysis Buffer: 0.025 M Tris base. Adjust pH to 7.8 with phosphoric acid. Add 0.002 M dithiothreitol, 0.002 M cyclohexylenedinitrilotetraacetic acid (CDTA), 10% glycerol, 1% triton-X-100. Sterile filter and store (*see* Note 6)
4. Firefly Luciferase Assay Buffer: 0.020 M Tricine, 0.001 M magnesium carbonate hydroxide pentahydrate, 0.0027 M magnesium sulfate. Adjust pH to 7.8 with sodium hydroxide. 0.0001 M EDTA, 0.0324 M

³High quality plasmid DNA is critical to effective and reproducible transfection results. We routinely use a Hi-Speed Maxi prep kit (Qiagen, Valencia, CA, USA) to prepare our transfection grade plasmids.

⁴When performing overexpression studies, the promoter driving expression of the gene of interest can have a profound impact on function. In our experience, often the robust overexpression from strong promoters (e.g., CMV and CAG) can have paradoxical effects on signaling (e.g., strong overexpression and knockdown produce the same results). We have encountered this problem in MC3T3 with Cx43 overexpression. Instead, the pSFFV-Cx43 vector, which is driven by a weaker promoter, performs very well in our hands. For different promoters and plasmid constructs, determination of the optimal concentration for the intended biological consequence may be necessary (15).

⁵We have used numerous transfection methods in our labs to conduct these assays, including Lipofectamine 2000 (Invitrogen), FuGene 6 (Promega) and calcium phosphate co-transfection.

⁶Commercial alternatives are available. For dual firefly/renilla luciferase assays, we have successfully used Promega's Passive Lysis Buffer or Renilla Luciferase Assay Lysis Buffer.

dithiothreitol, 0.00063 M adenosine 5'-phosphate. Sterile filter. Add 0.0005 M D-luciferin and 0.00043 M coenzyme A, lithium salt. Aliquot 10 ml/tube and store at -80°C . (see Note 7)

5. Renilla Luciferase Assay Buffer 1: 1.1 M sodium chloride, 0.0022 M EDTA, 0.22 M potassium phosphate monobasic, 0.00575 M potassium phosphate dibasic, 0.44 mg/ml bovine serum albumin, 0.0013 M sodium azide. Adjust pH to 5.0 with hydrochloric acid. Store at 4°C .
6. 1000X coelenterazine solution: Make a 1M hydrochloric acid solution in methanol. Dissolve 0.00143 mM coelenterazine in the acid-methanol solution. Store at -80°C in small aliquots.
7. Renilla Luciferase Working Buffer: Dilute coelenterazine solution 1:1000 into Renilla Luciferase Assay Buffer 1 just prior to use. (see Note 8)
8. 96 well opaque assay plate

3. Methods

3.1. Cell Culture and Transfection

1. MC3T3 clone 4 osteoblasts cell lines are maintained in complete tissue culture media at 37°C , 5% CO_2 in a tissue culture incubator. Cells are passaged at 1:4 to 1:10 prior to reaching confluence.
2. One day prior to transfection, *donor* and *acceptor* cells are seeded at 60,000 cells/cm² into a P100 tissue culture treated plate (see Note 9). For *donor* cells, there should be four separate sets of plates: (1) pSFFV-neo, pcDNA3; (2) pSFFV-Cx43, pcDNA3; (3). pSFFV-neo, caFGFR1, (4) pSFFV-Cx43, caFGFR1. For *acceptor* cells, there should be two separate sets of plates: (1) pSFFV-neo, p6xOSE2-Luc, pRL-TK and (2) pSFFV-Cx43, p6xOSE2-Luc, pRL-TK.
3. Donor Cell Prep: Label 4 sterile 1.7 ml eppendorf tubes: (1) pSFFV-neo, pcDNA3; (2) pSFFV-Cx43, pcDNA3; (3). pSFFV-neo, caFGFR1, (4) pSFFV-Cx43, caFGFR1. To each tube add 500 μL JetPrime buffer, then pipet (a) 8 μg of pSFFV-neo or pSFFV-Cx43 and (b) 4 μg of pcDNA or caFGFR1 to each tube, as appropriate. Vortex the samples for 10 sec to mix the reagents. Next add 48 μL JetPrime reagent to each tube (see Note 10). Vortex the samples for 10 sec and incubate at room temperature for 10 min. In a drop wise fashion, pipet the transfection mix onto the cells in the

⁷Commercial alternatives are available, including the DLR assay reagents from Promega. Our reagents perform comparably to this reagent in head-to-head tests in our lab using these cells and cost considerable less.

⁸Our Renilla luciferase working reagent is based on a paper by Dyer et al (16).

⁹These high plating densities provide our best transfection efficiencies, support cell survival and allow the cells to remain in contact, a necessary condition to study gap junctions. In our hands, osteogenic cells do not proliferate very robustly post-transfection. While these cell densities have produced reproducible data for us in these cell types, optimal cell densities may have to be empirically determined for other cell types.

¹⁰For transfection of MC3T3 clone 4 osteoblasts, we have found that a 4:1 JetPrime:DNA ratio is far superior to the manufacturer's recommendation.

appropriately labeled plate. Swirl the plate gently to mix and return to the incubator. After 4 h, replace the media on the cells with fresh complete tissue culture media. Return to the incubator.

4. *Acceptor* Cell Prep: Label 2 sterile 1.7 ml eppendorf tubes: (1) pSFFV-neo, p6xOSE2-Luc, pRL-TK and (2) pSFFV-Cx43, p6xOSE2-Luc, pRL-TK. To each tube add 500 μ l JetPrime buffer, then pipet (a) 8 μ g of pSFFV-neo or pSFFV-Cx43 and (b) 4 μ g of pOSE2 and (c) 1 μ g of pRL-TK plasmid to each tube, as appropriate Vortex the samples for 10 sec. Next add 52 μ L JetPrime reagent to each tube. Vortex the samples for 10 sec and incubate at room temperature for 10 min. In a drop wise fashion, pipet the transfection mix onto the cells in the appropriately labeled plate. Swirl the plate gently to mix and return to the incubator. After 4 h, replace the media on the cells with fresh complete tissue culture media. Return to the incubator.
5. Co-culture with cell-cell contacts: 48 h post-transfection, wash both the *donor* and *acceptor* cell cultures two times with HBSS to remove any residual media, trypsinize the cells from the tissue culture plates with 1 ml 0.25% Trypsin EDTA solution at 37°C for <5 min, until the cells round up. Resuspend the cells in 9 ml of complete tissue culture media, transfer to a 15 ml sterile conical tube and pellet the cells by centrifugation at 500 $\times g$ for 10 min. Resuspend the cell pellet in 10 ml complete tissue culture media. For *acceptor* cells, plate 50,000 cells/well into a 24 well multiwell plate. For each group, plate 3 to 6 replicates. Immediately after seeding the acceptor cells, add the appropriate co-cultured *donor* cells into the same well. For *donor* cells, plate 150,000 cells/well into a 48 well multiwell plate. (see Note 11). (Fig. 1) Return the cells to incubator for 16 h (see Note 12). Then proceed to the luciferase reporter assay (Step 3.2).
6. Co-culture in transwell chambers: Repeat the plate layout as in the preceding step (5), however, the *acceptor* cells get plated in the bottom of the 24 well multiwell plate. Then insert the transwell chamber into the well, and seed the *donor* cells into the insert, precluding direct cell-to-cell contact between the *donor* and *acceptor* cells. Return the cells to incubator for 16 h. Then proceed to the luciferase reporter assay (Step 3.2).

3.2. Luciferase Reporter Assay

1. Remove the media from the cells and rinse twice in 1.0 mL HBSS. For cells cultured in transwell chambers, the *donor* cells (in the transwell

¹¹We have successfully used *donor:acceptor* cell ratios that span 1:4 to 4:1. A 3:1 ratio was used in the example provided here. We typically find that when you have a sensitive readout for your *acceptor* cells, that a higher *donor:acceptor* cell ratio is beneficial. However, some insensitive readouts, such as the Cx43-dependent siRNA transfer studies we performed (14) require a larger number of *acceptor* cells to reliably detect changes.

¹²We have examined time courses for these effects on luciferase reporter assays, which require signaling, luciferase gene expression, and luciferase protein synthesis, and found that the minimum amount of time required to detect reproducible effects is 4 h. However, typically co-culture for 16–24 h produces a larger effect.

inserts) can be discarded and the *acceptor* cells rinsed with HBSS (*see* Note 13).

2. Add 200 μ L per well Luciferase Lysis Buffer. Incubate at room temperature for 30 min with gentle shaking.
3. Transfer 50 μ L of each lysate into the wells of an opaque 96 well assay plate.
4. Pre-load 10 ml of Firefly Luciferase Assay Buffer into pump 1 of the Berthold Centro LB960 Luminometer. Likewise, pre-load 10 mL of Renilla Luciferase Working Buffer into pump 2.
5. Insert the plate into the luminometer and program it to: (1) dispense 100 μ L from pump 1 (Firefly) with a “by well” measurement operation; (2) delay 2 sec to allow mixing; (3) measurement for 20 sec; (4) dispense 100 μ L from pump 2 (Renilla) with a “by well” measurement operation; (5) delay 2 sec to allow mixing; (6) measurement for 20 sec.
6. Relative luciferase activity can be determined by dividing the firefly luciferase activity by the Renilla luciferase activity. Next, average the replicates together and graph the data.
7. A gap junction communicated signal will result in the activation of the luciferase reporter only when the *donor* cell expresses both the constitutively active signal protein and Cx43 and the *acceptor* cell expresses both Cx43 and the luciferase reporter construct and the cells are co-cultured in direct contact (Fig. 2).

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¹³Calcium ions are potent inhibitors of luciferase activity. Thus, thorough removal of tissue culture media and washing with Ca²⁺ and Mg²⁺ free HBSS is required for optimal luciferase activity.

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DONOR Cells	pSFFV-neo; pcDNA3	pSFFV-Cx43; pcDNA3	pSFFV-neo; caFGFR1	pSFFV-Cx43; caFGFR1
ACCEPTOR Cells	pSFFV-neo; p6xOSE2-Luc	pSFFV-Cx43; p6xOSE2-Luc	pSFFV-neo; p6xOSE2-Luc	pSFFV-Cx43; p6xOSE2-Luc

Figure 1.

Matrix of the co-culture seeding set-up for conducting the parachute assay. In this example, a 3:1 ratio of *donor:acceptor* cells were seeded together in the indicated combinations. Cells of the matrix are labeled with the specific combination of co-transfected plasmids for modulating Cx43 expression as well as constitutively active FGFR1 expression.

Figure 2.

Representative luciferase reporter data. A. MC3T3 clone 4 Cell were co-cultured with direct cell-to-cell contact, as indicated in the cartoon. Expression of the caFGFR1 stimulated expression of the p6xOSE2-Luciferase plasmid only in when both the *donor* and *acceptor* cells expressed Cx43 containing plasmids (red bar). Histograms represent average relative luciferase activity from triplicate samples. Error bars indicated standard deviations. **, p-value <0.01. B. The MC3T3 clone 4 cells were cultured as indicated above, except *donor* cells were seeded into a transwell chamber without direct cell-to-cell contact with the

acceptor cells. In this context, the donor cells were unable to stimulate luciferase activity in the *acceptor* cells, independent of the Cx43 status.

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