

Retroviral Gene Transduction in Limb Bud Micromass Cultures

N. Susan Stott and Cheng-Ming Chuong

1. Introduction

Since Solursh and his coworkers set up the limb bud micromass cultures in 1977 (*1*), this procedure has become a major model to analyze cellular and molecular event involved in chondrogenesis (*2–7*). Recently, RCAS retroviral vectors have been developed that can infect chicken embryos *in ovo*. These have advanced our understanding in limb bud patterning remarkably (*8*). Here we describe a newly developed protocol to merge these two procedures, which will make micromass culture an even more powerful model for the analysis of chondrogenic mechanisms.

Micromass culture has been used to study the role of growth factors, enzyme modulators, drugs, and adhesion molecules in chondrogenesis (*3–7*). These studies have been facilitated by the development of serum-free media (*9*). However, because some signaling molecules are intracellular, it has been difficult to dissect the chondrogenic pathways at a molecular level. To manipulate gene expression in micromass cultures, we have used electroporation to introduce exogenous genes into limb bud cells (*7*). However, this leads to a high mortality rate of cells, so we have been continuing to search for better procedures.

With the advent of gene therapy (*10*), retroviral mediated gene delivery has become a more mature technology. It has several advantages. The initial infection is not toxic to the cell, the gene expression is more stable than other procedures, and the percentage of infected cells will increase over time if the virus is replication competent. It takes approx 18 h after retrovirus infection for the gene to be expressed. Because precartilage condensation formation in micromass cultures starts within 3 h of plating, we would like to have most, if not all, cells expressing the transgene at this stage. To achieve this goal, we have devised a novel two-stage micromass culture, with a low-density plating window allowing retroviral gene transduction into primary limb bud cells, and a regular high-density plating allowing cells to differentiate (*see Note 1*). This strategy was used successfully to demonstrate the dual action of SHH in chondrogenesis (*11*; *see Note 2*) and the arrest effect of Wnt 7a at early stages of precartilage condensation formation (*16*).

2. Materials

1. Fertilized pathogen-free chicken eggs: (SPAFAS Connecticut Hatchery, Preston, CT). These chicken embryos are susceptible to infection by retroviruses carrying the A envelope subgroup (**15**). Chicken embryos were staged as described (**12**).
2. Media:
 - a. Hank's buffered saline solution (HBSS; Gibco-BRL, Gaithersburg, MD).
 - b. Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL) supplemented with 2% fetal calf serum and gentamicin (1:1000).
 - c. Calcium-magnesium free saline (CMF 10X): 80 g of 1.37 M NaCl, 3 g of 0.04 M KCl, 0.5 g of 0.004 M NaH₂PO₄, 0.25 g of 2 M KH₂PO₄, 10 g of 0.12 M NaHCO₃, 20 g of 0.1 M glucose, in 1000 mL distilled water, pH 7.3.
 - d. Defined medium for micromass cultures. Based on Paulsen et al. (**9**). 60% Ham's F-12 (BioWhittaker, Walkersville, MD), 40% DMEM, 5 µg/mL insulin (Sigma Chemical Co., St. Louis, MO), 5 µg/mL transferrin (Sigma), 50 µg/mL ascorbic acid (Sigma), 100 nM hydrocortisone (Sigma).
3. Enzymes: Trypsin, collagenase (Worthington Biochemicals, Freehold, NJ), type-I collagen (UBI).

3. Methods

The outline of the procedure is shown in **Fig. 1**.

1. SPAFAS pathogen-free eggs are used. The distal one third of limb buds from stage 23-24 chicken embryos (**13**) are dissected and pooled. After soaking the limb buds in ice-cold 2X calcium- and magnesium-free medium (CMF) containing 0.25% EDTA, epithelia are removed and discarded. The pooled limb bud mesenchymal cells are subjected to mild dissociation conditions in HBSS containing 0.006% of trypsin and collagenase for 10 min at 37°C. The digestion is stopped with fetal calf serum. The cells are gently triturated, then centrifuged (150g) at 15°C and resuspended to a concentration of 2×10^7 /mL in ice-cold micromass defined medium (DM).
2. For infection with retrovirus, the dissociated limb bud cells are incubated with retrovirus-containing medium (10^6 cells/mL of retroviral medium) at 4°C with gentle shaking for 2 h. After a gentle centrifugation (150g, 3 min), the cell pellets are resuspended in defined medium (*see* **Notes 2, 4, and 6**).
3. Tissue culture dishes are precoated with 100 µg/mL of type-I collagen in 0.02 N acetic acid for 1 h at 37°C and then neutralized with HBSS. For low-density plating, cells are plated at 5.5×10^4 cells/cm² on these dishes in defined medium and cultured at 37°C, 5% CO₂/95% air for 2 d.
4. Cells are washed 2X with HBSS and trypsinized again with 0.006% trypsin and collagenase in HBSS without Ca²⁺ and Mg²⁺. Digestion is stopped with fetal calf serum and the dissociated cells passed through cell microsieving (20 µm) to ensure a single-cell suspension. The cells are plated in 10 µL drops at a density of 2×10^7 /mL on collagen type I precoated 35-mm tissue culture dishes. The cells are allowed to attach for 1.5 h and then 1.5 mL of defined medium is added.
5. From this point on, the cultures proceed similar to normal micromass cultures (*see* **Note 3**). Cells form visible precartilaginous condensations in 1–2 d and cartilage nodules in 3–4 d. These cartilage nodules have been shown to be positive for alcian blue and collagen II immunostaining (**14**).

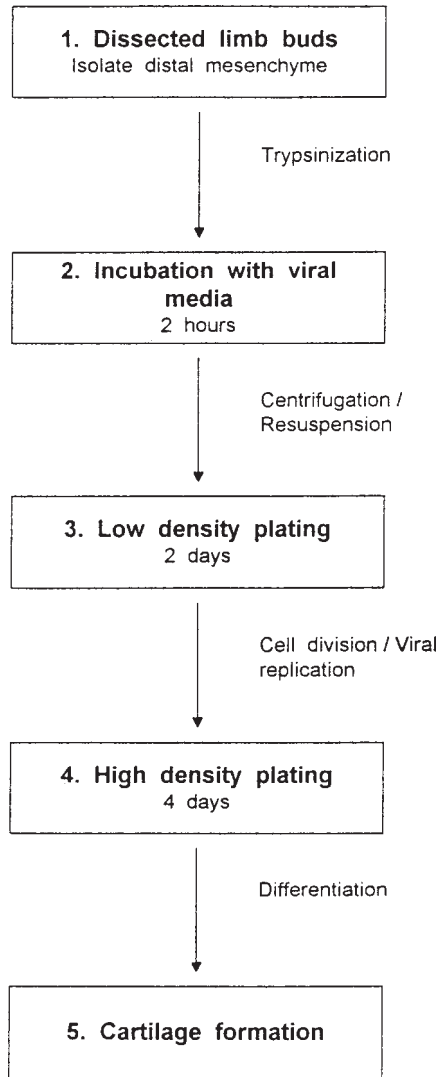


Fig. 1. Flowchart of the protocol.

4. Notes

1. Rationale for the two-stage procedures: The low-density window balances two needs: the need for rapid proliferation and retroviral integration and the need to maintain chondrogenic competence. Low-density culture provides a time window for cell proliferation and viral infection. Fetal calf serum can induce chondrocyte maturation, so we used serum-free medium during this time. To resume chondro-differentiation, we plate cells at high density and with type-I collagen as the substrate. Type-I collagen is used because it is highly expressed in limb bud *in vivo* prior to the precartilaginous condensation phase and may facilitate cell attachment and enhance chondrogenic competence.
2. Retroviral infectivity: Retroviral medium is produced and titered according to published methods (8). Retroviral media is filtered with a 0.45- μ m surfactant-free cellulose acetate

filter (Nalge Nunc International, Rochester, NY) and stored in aliquots at -70°C . The infectivity, judged by staining of antibodies to viral gag, reaches 60–75% of cells after 3 d of high-density plating (13).

3. Effect of this procedure and RCAS on micromass cultures: Infection with RCAS vector did not significantly alter cell numbers as judged by DNA content and ^3H thymidine incorporation (13). Both virus-free and RCAS-infected cultures showed a minor and similar level of reduction in overall chondrogenic differentiation compared with regular, traditional one-stage micromass culture (1). This may be a result of the manipulation during the two-stage protocol. RCAS virus infection per se does not decrease the pattern of chondro-differentiation as judged by Alcian blue staining and immunostaining with antibodies to type-II collagen (13).
4. Optimization of virus mediated gene transduction: We have tried to vary some parameters to achieve better results. The following are our experiences. These are the parameters that could be altered when applying similar procedures to other organotypic cultures.
 - a. The time for virus infection was varied from 1 to 4 h. During this period, infectivity gradually increases but cell viability drops. Two hours of incubation with virus is a good middle point.
 - b. Plating densities between 10^4 and 10^6 cells/cm² were tested for cell survival and growth together with different substrates. A density too high allowed the cells to become confluent and triggered premature chondrogenic differentiation, whereas a density too low decreased cell survival and growth. A plating density of 5.5×10^4 cells/cm² provided the best middle point for cell growth without differentiation.
 - c. We also have tried to vary the length of low-density cultures. This period was varied from 1 to 3 d. With shorter time, infectivity is not high enough and the cell number is low. With longer time, infectivity is high, but cell-survival rate drops and the competence to form cartilage nodules after high-density plating also drops. Thus, 2 d of low-density plating provides the best balance between maintenance of chondrogenic competence and high levels of retroviral gene expression.
5. Because genes in retrovirus take about 18 h to be expressed, direct addition of retroviral media to the micromass cultures would not lead to exogenous gene expression until 18 h later. If the events to be perturbed are late events, one can still get results. This appears to be the case of the suppressive effect of Wnt 7a (14).
6. We typically used RCASBP (A) for limb bud micromass cultures. However, other subgroups of RCAS are available that carry different envelope glycoproteins (8,15). It is also possible to use cells from different specific chicken lines (15) to make a chimeric micromass cultures so that viral resistant cells and viral susceptible cells can be mixed. This allows the analysis for autonomous and nonautonomous effects.
7. Another strategy is to use limb buds from transgenic mice. This would be better as all cells then should contain the transgenes. However, the logistics of timing the mice to be at the right developmental stages and checking the genotype makes this approach less suitable. In comparison, it is easier to get large numbers of chicken embryos of the appropriate stages.
8. It is also possible to apply similar strategies to other organotypic cultures. Differences in cell surface receptor availability (8,10,15) and cellular properties may require some adjustment of various parameters. It is also possible to test different RCAS strains (15).

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