

Retroviral Gene Transfer in Chondrogenic Limb Bud Micromass Cultures

BioTechniques 24:660-666 (April 1998)

**N. Susan Stott, Yun-Shain Lee
and Cheng-Ming Chuong**
University of Southern California,
Los Angeles, CA, USA

ABSTRACT

We report development of a model of retroviral gene transduction in high-density limb bud cell micromass culture. The replication competent avian retrovirus RCAS BP (A) carrying the human placental alkaline phosphatase gene (RCAS AP) was used as a marker for retroviral infection and spread. The final protocol balances the need to allow time for retroviral integration and gene transduction against loss of chondrogenic potential when limb bud cells are plated at low density. It includes: (i) incubation of the dissociated limb bud cells with RCAS virus for 2 h followed by low-density culture for 48 h to allow retroviral gene expression; and (ii) secondary replating as high-density micromass culture to initiate chondrogenesis. The pattern and level of chondrogenesis in the retrovirus-transduced micromass cultures is similar to regular micromass cultures. At least 40%–50% of cells express the retroviral-transduced genes 24 h after high-density plating. This new approach facilitates ectopic gene expression in micromass culture, enabling molecular dissection of chondrogenesis and serves as a model for gene transduction in other organotypic cultures.

INTRODUCTION

Understanding how single cells collaborate to form a complex organism with many different organs is a central issue in biological research. Organotypic cultures, which mimic the process of organogenesis and can be experimentally manipulated, have been helpful research tools in studying how cells interact with each other and their environment to achieve a complex three-dimensional structure. However, it has not been easy to incorporate genetic approaches into these cultures. Many of these cultures involve the growth of primary cells over a short period of time where stable transfection is non-applicable and where transient transfection may be either too toxic or transfect too few cells. Application of some of the knowledge gained from gene therapy technology (13,29) to organotypic cultures to alter gene expression would make these models much more powerful.

In this report, we describe our efforts to adopt such an approach in one well-established organotypic culture, high-density limb bud micromass culture (1,6,9,40). In this culture system, dissociated epithelium-free mesenchymal cells derived from the distal third of the chick limb bud, embryonic stage E23/24 (14), are plated in 10–15- μ L drops at a density of 2×10^7 cells/mL. The cells initially appear homogenous, but within 24 h, small cell aggregates are formed. An extracellular matrix rich in chondroitin sulfate and proteoglycans accumulates, and by four days, cartilage nodules are present that are positive for type II collagen and Alcian Blue staining, both markers of chondrocyte phenotype. The sequence of cell differentiation in micromass culture parallels that seen in *in vivo* cartilage formation,

making this a good model for the study of chondrogenesis (5).

Micromass culture has been used to study the role of growth factors and adhesion molecules in chondrogenesis (6,21,37,43). These studies have been facilitated by the development of serum-free media (33,34). Progress has also been made in dissecting out some of the signaling pathways involved in chondrogenesis (23,25,41). However, it has been difficult to use this model to dissect the molecular pathways that control chondrogenic differentiation. We have used electroporation to introduce exogenous genes into micromass culture (43); however, the high cell death rate during this procedure and the transient nature of the gene expression make it difficult to achieve a high level of gene expression.

Retroviral-mediated gene delivery has several advantages over transient transfection: (i) the initial infection is not toxic to the cell; (ii) the gene expression is stable; (iii) and the percentage of infected cells will increase over time if the virus is replication-competent. In this report, we devised a two-stage micromass culture, with a low-density plating window to allow retroviral gene transduction in primary limb bud cells before high-density plating. We used the replication competent avian retroviral vector (RCAS) (A) (17,31,35), as this produces high levels of exogenous gene expression in infected chick limb buds *in ovo* (30) and chick chondrocytes in long-term culture (20). We demonstrate that the viral exposed cell cultures retain the capacity to undergo chondrogenesis in high-density culture after 48 h of low-density plating. The low-density plating period provides a window for viral replication and gene expression before the onset of

chondrogenesis. This new strategy provides a new approach to analyze the molecular cascade in chondrogenesis.

MATERIALS AND METHODS

Materials

Fertilized pathogen-free chicken eggs were purchased from SPAFAS Inc. (Norwich, CT, USA). These chicken embryos are susceptible to infection by retroviruses carrying the A envelope subgroup (2). Chicken embryos were staged as previously described (14). pRCASBP (A) (8,11,17) and pRCASBP (A) Alk-P (10) were gifts from Dr. Steven Hughes (NCI-Frederick Cancer Research Facility); and Drs. C.L. Cepko and D.M. Feket (both of Harvard University, Boston), respectively. The monoclonal antibody against viral Gag protein was a gift from Dr. Bruce Morgan (MGH, Harvard University). The monoclonal antibody to chick type II collagen (2B1) was supplied by Dr. Richard Mayne, Hybridoma Core Facility of the Multipurpose Arthritis Center, University of Alabama. All studies were performed in accordance with local vivaria guidelines.

Production of Retroviral Media

Retroviral medium was produced and titered according to published methods (30,42). Retroviral media was filtered with a 45- μ m, surfactant-free cellulose acetate filter (Nalgene[®]; Nalge Nunc International, Rochester, NY, USA) and stored in aliquots (100 μ L and 1 mL) at -70°C. The retroviral titer ranged from 5×10^5 to 5×10^6 IU/ μ L.

Retroviral Infection in Micromass Culture

Dissociated limb bud cells were prepared from the distal third of the chicken limb buds at embryonic stage 23-24 as previously described (1,21). Epithelium was removed and discarded after soaking the limb buds in ice-cold 2 \times calcium and magnesium-free medium (CMF) with 0.25% EDTA. The pooled limb buds were then subjected to mild dissociation conditions in Hank's balanced salt solution (HBSS) (without Ca²⁺ and Mg²⁺) containing 0.006% of trypsin and collagenase for 10 min at 37°C. The digestion was then stopped

with fetal calf serum (FCS). The cells were gently triturated, then centrifuged (150 \times g) at 15°C and resuspended to a concentration of 2×10^7 /mL in ice-cold defined medium (DM). DM contained 60% Ham's F-12 (BioWhittaker, Walkersville, MD, USA), 40% Dulbecco's modified Eagle medium (DMEM), 5 μ g/mL insulin (Sigma Chemical, St. Louis, MO, USA), 5 μ g/mL transferrin, 50 μ g/mL ascorbic acid, 100 nM hydrocortisone (all from Sigma Chemical). The dissociated limb bud cells were then incubated with retrovirus-containing medium (10^6 cells/mL of retroviral medium) at 4°C with gentle shaking for defined time intervals. After centrifugation (150 \times g), the cells were resuspended in DM and plated at low density (5.5×10^4 cells/cm²) for defined time periods at 37°C, 5% CO₂/95% air. The culture dishes (Falcon[®]; Fisher Scientific, Pittsburgh, PA, USA) had been pre-coated with 100 μ g/mL of type I collagen (UBI) in 0.02 N acetic acid for 1 h at 37°C and then neutralized with HBSS.

At defined time intervals, the low-density cell cultures were washed twice with HBSS, and the cells were trypsinized with 0.006% trypsin and collagenase (Worthington Biochemical, Freehold, NJ, USA) in HBSS without Ca²⁺ and Mg²⁺. The dissociated cells were then passed through Cell Microsieve netting (20 μ m; BioDesign, Carmel, NY, USA) to ensure a single-cell suspension and plated in 10- μ L drops at a density of 2×10^7 /mL on collagen type I (UBI) pre-coated, 35-mm, tissue culture dishes (Corning Costar, Cambridge, MA, USA). The cells were allowed to attach for 1.5 h, and then 1.5 mL of defined medium was added.

Immunocytochemistry

Immunostaining was performed as previously described (25). Cultures were fixed either with 2.5% paraformaldehyde (PFA) in phosphate-buffered saline for 30 min (Gag staining) or Bouin's fixative for 25 min followed by 70% ethanol washes (type II collagen staining). Specimens were incubated with primary antibody overnight, followed by secondary biotinylated horse anti-mouse antibody (Vector Laboratories, Burlingame, CA, USA) or alkaline phosphatase (AP) conjugated anti-rab-

bit IgG (Promega, Madison, WI, USA) and then detected using either VECTA-STAIN[™] ABC kit (Vector Laboratories) and 3-amino 9-ethyl carbazole substrate or nitro blue tetrazolium/5-Bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) substrate (Promega).

Quantitation of Chondrogenesis

Micromass cultures were fixed with 2.5% PFA and stained with 1% Alcian Blue 8GX in 0.1 N HCl, pH 1.0 for 3 h (15,26). Stained cultures were then destained with 70% ethanol. After photographs were taken, the bound Alcian Blue dye was extracted with 0.5 mL 4 M guanidine HCl (pH 5.8) and quantified by measuring absorbance at optical density (OD) 600 nm (22,25).

DNA Assay

Total DNA was measured using a fluorimetric method (24). The emission at 458 nm was read using a Model F-2000 Fluorescence Spectrophotometer (Hitachi Instruments, San Jose, CA, USA) and compared to a standard curve constructed using calf thymus DNA. The same lot of calf thymus DNA and Hoechst 33258 (Sigma Chemical) was used throughout to minimize variation. Chicken DNA would have been preferable if the absolute amount of DNA is required. However, use of calf thymus DNA is suffice for comparing of relative values.

[³H]Thymidine Labeling

At defined times, cultures were incubated in DM containing 5 μ Ci/mL [³H]thymidine (Amersham, Arlington Heights, IL, USA) for 1 h at 37°C. The culture media was then aspirated, cultures washed with DMEM three times and then incubated in ice-cold 10% trichloroacetic acid for 5 min. The acid precipitable radioactivity was solubilized in 0.5 mL 10% sodium dodecyl sulfate (SDS) and counted in a Model LS 5000CE Scintillation Counter (Beckman Instruments, Fullerton, CA, USA).

Alkaline Phosphatase Assay

AP activity was quantitated according to published methods (32) and normalized to total DNA. For AP histochemical staining, cells/cultures were fixed with 2.5% PFA to reduce endogenous AP activity, then incubated in a

Research Reports

color-detection solution consisting of 100 mM Tris, pH 9.5, 100 mM NaCl, 50 mM MgCl₂ containing 225 µg/mL NBT and 175 µg/mL BCIP substrate (Promega). Color was developed at room temperature. The cultures were mounted with glycerol mounting medium.

RESULTS AND DISCUSSION

A Novel Two-Step Micromass Culture Allows Viral Infection Without Compromising Chondrogenic Differentiation

RCAS-mediated gene transduction *in vivo* has several key requirements. These include a cell surface receptor to which the virus can bind, a period of rapid cellular proliferation to allow integration of the viral genome into the cellular DNA, and a time delay of up to 18 h before viral gene expression is detected, during which time viral mRNA is transcribed and viral proteins translated (30). Many key events occur in micromass cultures within the first 18 h (1,40). Thus, addition of the viral media to the culture at the time of high-density plating, although simple, may not lead to gene expression in time to modulate the early events of chondrogenesis (39).

To solve this dilemma, we have modified micromass culture to include a low-density plating period. The dissociated limb bud cells are prepared as for routine micromass culture but then incubated in retroviral medium for several hours to allow retroviral binding to cell-surface receptors. The limb bud cells are then plated at low density and allowed to proliferate. We reasoned that rapid proliferation of the limb bud cells could occur during the period of low-density plating, thus facilitating stable retroviral integration into the cellular DNA and enhancing retroviral gene expression without permitting differentiation. To optimize the conditions for retroviral infection, we used RCAS AP (10), which carries the human placental alkaline phosphatase gene, as a marker for retroviral gene expression. The exogenous human AP does not influence cell proliferation or differentiation when injected into the developing limb bud (10,30), making this a good marker for retroviral infection.

Table 1. Expression of Virus-Transduced AP Correlated to Duration of Low-Density Plating and Chondrogenic Potential

Hours of Low-Density Plating	First Detectable Exogenous AP Expression after High-Density Plating ^b	Maximum Exogenous AP Expression ^b	% Chondrogenesis at 4 Days after High-Density Plating ^a
24 h	Day 2 1.3×	19×	90
48 h	Day 1 1.3×	20×	90
72 h	Day 1 3.3×	32×	50

^aNormalized to regular micromass culture (100%).
^bNormalized to endogenous background (1×).

A 48-h, Low-Density Window Does Not Impair Chondrogenic Potential

The low-density window balanced two needs: the need for rapid proliferation to facilitate retroviral integration and the need to maintain chondrogenic competence. FCS can induce chondrocyte maturation (4), so we used serum-free medium in the low-density period. Plating densities between 10⁴ to 10⁶ cells/cm² were tested for cell survival and growth together with different substrates. Too high a density allowed the cells to become confluent and triggered premature sporadic chondrogenic differentiation, while too low a density decreased cell survival and growth. A plating density of 5.5 × 10⁴ cells/cm² plus the use of type I collagen substrate to facilitate cell attachment provided the best conditions for cell growth without differentiation during the low-density period.

Chondrocytes plated in low-density monolayer culture tend to de-differentiate, losing type II collagen expression (3). To determine the maximum length of time for which limb bud cells could be plated at low density without loss of chondrogenic potential, the cells were cultured at low density for varying time periods and then replated as high-density cultures for four days. High-density cultures derived from cells grown at low density for either 24 or 48 h showed only mild decreases in chondrogenic differentiation at four days (Table 1) with similar levels of exogenous gene expression. However, exogenous gene expression was detected 24 h earlier in the high-density cultures derived from cells cultured for 48 h at low density. Extending the low-density cul-

ture period to 72 h increased the levels of exogenous gene expression but had two significant drawbacks: (i) decreased cell survival in serum-free conditions after 48 h and (ii) a 50% reduction in chondrogenic differentiation in the high-density plating period (Table 1). Thus, 48 h of low-density plating provides the best balance between maintenance of chondrogenic competence and high levels of retroviral gene expression.

Secondary High-Density Plating Initiates Chondrogenesis

High-density limb bud cell micromass cultures are initially homogenous in appearance but form well-defined cartilaginous nodules when grown in serum-free defined medium for four days (33). High-density micromass cultures prepared from RCAS-infected limb bud cells grown at low density for 48 h showed a similar pattern of chondrogenic differentiation. Immunostaining for type II collagen, a marker for chondrogenic differentiation, was negative at 24 h in both regular and RCAS-infected micromass cultures (not shown) but strongly positive at 72 h in both cultures (Figure 1, A and B). Both virus-free and RCAS-infected cultures showed a similar reduction in overall chondrogenic differentiation compared to regular micromass culture, suggesting that virus infection *per se* does not decrease chondrogenesis. Type I collagen substrate was essential in the secondary high-density period to facilitate cell attachment and chondrogenic differentiation. Type I collagen is highly expressed in limb mesenchymal cells *in vivo* before the pre-cartilaginous

condensation phase (7) and may enhance chondrogenic competence.

The low-density plating period did not adversely affect the relative DNA content per culture. The DNA content after four days of high-density culture averaged $1.58 \pm 0.5 \mu\text{g}$ for regular micromass cultures, $1.43 \pm 0.8 \mu\text{g}$ for the virus-free culture exposed to 48 h of low-density plating and $1.42 \pm 0.4 \mu\text{g}$ in the RCAS-infected cultures (average of 4–6 independent experiments \pm standard deviation). The DNA standard used was calf thymus DNA, so the values should be seen as relative indicators of DNA content rather than absolute figures. Cellular proliferation was also not affected by RCAS infection (Figure 2). Infection with RCAS AP also did not affect DNA content or chondrogenic differentiation (not shown).

Optimization of Virus-Mediated Gene Transduction

We identified cells infected by RCAS AP by: (i) immunocytochemical staining for viral Gag protein and (ii) histochemical staining for the viral encoded AP activity. Figure 3, A and B shows a typical staining pattern after 48

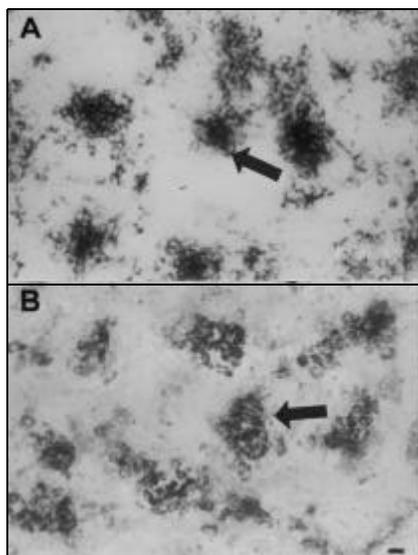


Figure 1. RCAS virus infection does not influence type II collagen expression in micromass culture. (A and B) After 72 h of high-density culture, both regular micromass cultures (A) and RCAS-infected micromass cultures (B) show positive staining for type II collagen in the center of the cartilaginous nodule (closed arrows). Both cultures were plated at a density of 6.6×10^6 cells/mL to facilitate immunostaining. Scale bar = 25 μm .

h of low-density plating. The RCAS AP-infected cells show positive cytoplasmic staining for the Gag protein (Figure 3A), while noninfected cells are negative. In parallel cultures, the RCAS AP-infected cells are darkly stained for AP around the nuclear membrane (Figure 3B), while noninfected cells are weakly positive for endogenous AP. Both staining methods identified a similar percentage of infected cells at 48 h in parallel cultures. Although the *gag* message can be expressed in more possible viral mRNA isoforms, and the reporter gene AP is only expressed in certain spliced forms (10), the results here suggest that, in our cultures, the majority of virus-infected cells do express the exogenous gene carried by the virus.

Using these methods, we first assessed the effect of differing incubation times with virus on the percentage of

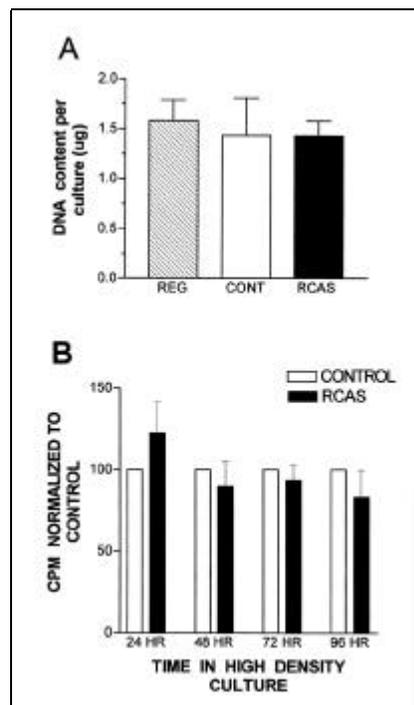


Figure 2. RCAS virus infection does not affect cell proliferation in micromass culture. (A) Histogram showing total DNA content of micromass cultures at four days in different culture conditions. The data are the means of values from cultures performed in 4–6 independent experiments. Bars indicate standard deviations. (B) Histogram showing uptake of [^3H]thymidine in control and RCAS-infected cultures. Data for RCAS-infected cultures are shown as a mean percentage relative to control cultures for four independent experiments. Bars indicate standard deviations.

Research Reports

infected cells. Lengths of incubation greater than 1 h did not significantly increase the percentage of infected cells after 48 h of low-density plating, suggesting that viral binding to the receptor is at maximal levels by 1 h. Cell plating efficiency was markedly decreased after 4 h of incubation time, probably because of decreased cell viability. Cell viability by trypan blue exclusion was found to be 100% at 1 h, >95% at 2 h, 90%–95% at 3 h and between 85%–90% at >3 h ($n = 2$). For subsequent studies, we chose to use 2 h of incubation with virus.

An average of 22.5% of cells (range 10%–30%, $n = 4$) expressed the viral Gag protein after 48 h of low-density plating. This number reflects the initial infection rate, as secondarily infected cells have not yet begun to express viral proteins (30). Other studies using replication-defective retroviruses to infect primary cells have shown similar infection rates of between 10% to 20% (27). Successful retroviral infection requires

the cell to begin replicating soon after the virus is internalized (28), so that the virus can integrate into the cellular genome (12,18,19,38). Since not all cells will enter the cell cycle immediately after plating, viral infection will always be limited by this factor. Differences in cell-surface receptor availability (2) may also lead to inter-experimental variation. Some receptors are sensitive to trypsinization, so we tested milder dissociation conditions than we use for regular micromass culture and found this to be as effective in dissociating the cells.

The RCAS AP-infected micromass cultures showed intense nodular staining for Gag and AP activity after three days of high-density culture (Figure 3, C and D). This intense nodular staining may reflect the higher density of cells in the nodules, as cells between nodules were also stained. Recent studies have suggested that cells in the distal wing/limb buds may not be as homogeneous as previously thought (33), and therefore, the nodular staining could also reflect differing cellular susceptibilities to retroviral infection. We estimated the infection rate to be 40%–50% of cells after one day and 60%–75% of cells after three days of high-density plating.

To quantitate the level of RCAS AP viral infection in the secondary high-density culture, we measured the total AP levels. Control cultures (virus-free or RCAS vector only) expressed endogenous AP at similar levels throughout the culture period, ranging from 50–150 nm paranitrophenyl (PNP)/min/ μg DNA. In contrast, cultures infected with RCAS AP showed increasing AP activity over the four days (Figure 4) consistent with viral spread through the culture.

This study enhances the value of micromass culture by developing novel methodology for retroviral gene transfer into primary limb bud cells. To reconcile the conflicting needs for harvestable cell number, viral infectivity and chondrogenic competence, we recommend a 48-h, low-density plating period followed by high-density plating. Under these conditions, 20%–30% of cells express the exogenous gene at the time of high-density plating. This level of exogenous gene expression is sufficient to produce an altered phenotype in the high-density culture when expressing genes that encode soluble growth factors or extracellular signaling molecules. Changes in phenotype are less apparent but can still be detected with genes encoding transmembrane

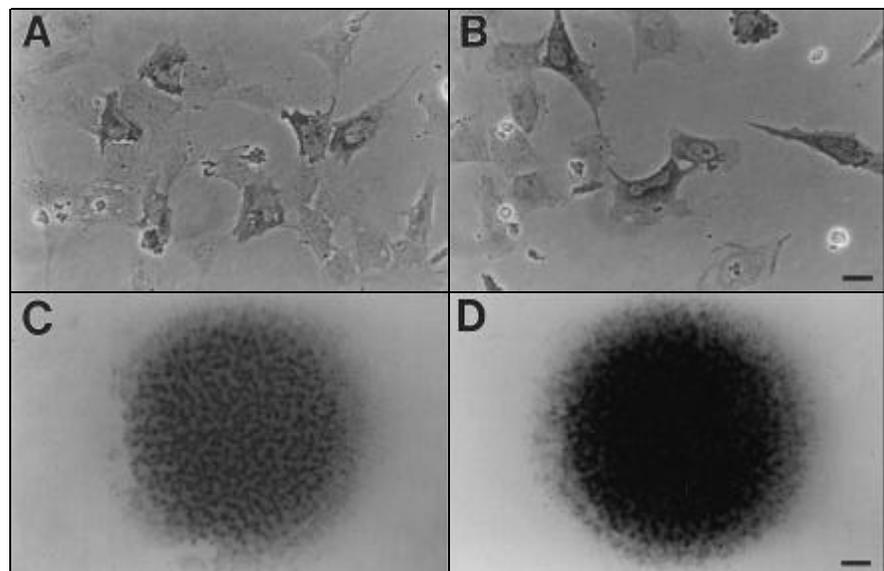


Figure 3. Expression of viral Gag protein and AP in RCAS AP-infected, low-density and high-density cultures. (A and B) After 48 h of low-density plating, cells are immunostained with antibodies against viral Gag protein (A) or histochemically stained for AP (B). Approximately one-third of the RCAS AP-exposed cells show positive staining for Gag protein and AP. Scale bar = 12.5 μm . (C and D) Cells are processed further into secondary high-density high micromass cultures. At 72 h, cultures are immunostained with antibodies against viral Gag protein (C) or histochemically stained for AP (D). Note intense nodular staining for both proteins. Scale bar = 500 μm .

proteins or intracellular signaling factors. We have recently completed two studies, using the genes *sonic hedgehog* (42) and *Ras* (our unpublished data),

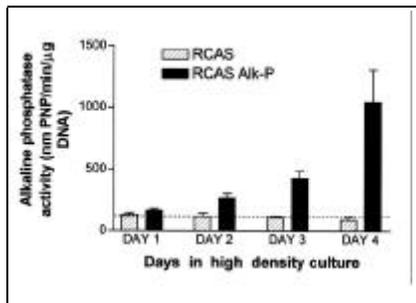


Figure 4. Effect of duration in high-density culture on levels of viral-transduced gene expression. Histogram showing viral-transduced AP activity (nm PNP/min/ μ g DNA) in high-density micromass cultures infected either by RCAS AP or vector only (RCAS). AP activity in cultures infected with RCAS AP is 30% above background 24 h after high-density plating and then increases. Data represents mean \pm standard deviation for three to four independent experiments. Bars indicate standard deviations.

and shown phenotypic changes with both. We used RCASBP (A); however, other subgroups of RCAS are available that carry different envelope glycoproteins (8,30). This allows superinfection of the same cell with two different subgroups of RCAS or limitation of infection to a subset of cells.

This work serves as the beginning of a new approach to dissect the chondrogenic molecular cascade. Strategies similar to those described here can be applied to other organotypic cultures (16,36); however, specific conditions have to be developed for each culture as different cells will vary both in their response to viral vectors and their phenotypic stability.

ACKNOWLEDGMENTS

We would like to dedicate this paper to the late Dr. Michael Solursh, a good scientist and a kind human being. We

thank Dr. Bruce Morgan for the monoclonal antibody to the Gag protein, Drs. Constance Cepko and Donna Fekete for the RCAS alkaline phosphatase viruses and Dr. Stephen Hughes for the RCASBP (A) vector. This work was funded by the NIH, NSF, CTR and the Wright Foundation.

REFERENCES

1. Ahrens, P.B., M. Solursh and R.S. Reiter. 1977. Position-related capacity for differentiation of limb mesenchyme in cell culture. *Dev. Biol.* 60:69-82.
2. Bates, P., A.T. Young and H.E. Varmus. 1993. A receptor for subgroup A Rous sarcoma virus is related to low density lipoprotein receptor. *Cell* 74:1043-1051.
3. Benya, P.D. and J.D. Shaffer. 1982. Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. *Cell* 30:215-224.
4. Bruckner, P., I. Horler, M. Mendler, Y. Houze, K.H. Winterhalter, S.G. Eich-Bender and M.A. Spycher. 1989. Induction and prevention of chondrocyte hypertrophy in cul-

Research Reports

- ture *J. Cell Biol.* 109:2537-2545.
5. **Caplan, A.I.** 1991. Mesenchymal stem cells *J. Orthop. Res.* 9:641-650.
6. **Daniels, K., R. Reiter and M. Solorsh.** 1996. Micromass cultures of limb and other mesenchyme, p. 237-247. *In* M. Bronner-Fraser (Ed.), *Methods in Avian Embryology*. Academic Press, New York.
7. **Dessau, W., H. von der Mark, H.K. von der Mark and S. Fischer.** 1980. Changes in the patterns of collagens and fibronectin during limb-bud chondrogenesis. *J. Embryol. Exp. Morph.* 57:51-60.
8. **Dorner, A.J., J.P. Stoye and J.M. Coffin.** 1985. Molecular basis of host range variation in avian retroviruses. *J. Virol.* 53:32-39.
9. **Downie, S.A. and S.A. Newman.** 1994. Morphogenetic differences between fore- and hind-limb precartilaginous mesenchyme: relation to mechanisms of skeletal pattern formation. *Dev. Biol.* 162:195-208.
10. **Fekete, D.M. and C.L. Cepko.** 1993. Replication-competent retroviral vectors encoding alkaline phosphatase reveal spatial restriction of viral gene expression/transduction in the chick embryo. *Mol. Cell Biol.* 13:2604-2613.
11. **Fekete, D.M. and C.L. Cepko.** 1993. Retroviral infection coupled with tissue transplantation limits gene transfer in the chicken embryo. *Proc. Natl. Acad. Sci. USA* 90:2350-2354.
12. **Fritsch, E.F. and H.M. Temin.** 1977. Inhibition of viral DNA synthesis in stationary chicken embryo fibroblasts infected with avian retroviruses. *J. Virol.* 24:461-469.
13. **Gordon, E.M. and W.F. Anderson.** 1994. Gene therapy using retroviral vectors. *Curr. Opin. Biotechnol.* 5:611-616.
14. **Hamburger, V. and H.L. Hamilton.** 1951. A series of normal stages in development of the chick embryo. *J. Morphol.* 88:49-91.
15. **Hassell, J.R. and E.A. Horrigan.** 1982. Chondrogenesis: a model developmental system for measuring teratogenic potential of compounds. *Teratog. Carcinog. Mutagen.* 2:325-331.
16. **Hoffman, R.M.** 1993. To do tissue culture in two or three dimensions? That is the question. *Stem Cells* 11:105-111.
17. **Hughes, S.H., J.J. Greenhouse, C.J. Petropoulos and P. Suttrave.** 1987. Adaptor plasmids simplify the insertion of foreign DNA into helper-independent retroviral vectors. *J. Virol.* 61:3004-3012.
18. **Humphries, E.H., C. Glover and M.E. Reichmann.** 1981. Rous sarcoma virus infection of synchronized cells establishes provirus integration during S-phase DNA synthesis prior to cellular division. *Proc. Natl. Acad. Sci. USA* 78:2601-2605.
19. **Humphries, E.H. and H.M. Temin.** 1974. Requirement for cell division for initiation of transcription of Rous sarcoma virus. *J. Virol.* 14:531-546.
20. **Iwamoto, M., K. Yagami, P. Lu Valles, B.R. Olsen, C.J. Petropoulos, D.L. Ewert and M. Pacifici.** 1993. Expression and role of c-myc in chondrocytes undergoing endochondral ossification. *J. Biol. Chem.* 268:9645-9652.
21. **Jiang, T.X., J.R. Yi, S.Y. Ying and C.M. Chuong.** 1993. Activin enhances chondrogenesis of limb bud cells: stimulation of precartilaginous mesenchymal condensations and expression of NCAM. *Dev. Biol.* 155:545-557.
22. **Kiernan, J.A.** 1990. *Histological and Histochemical Methods: Theory and Practice*, 2nd ed. Pergamon Press, Oxford.
23. **Kosher, R.A. and K.H. Walker.** 1983. The effect of prostaglandins on in vitro limb cartilage differentiation. *Exp. Cell Res.* 145:145-153.
24. **Labarca, C. and K. Paigen.** 1980. A simple, rapid and sensitive DNA assay procedure. *Anal. Biochem.* 102:344-352.
25. **Lee, Y.S. and C.M. Chuong.** 1996. Activation of protein kinase A is a pivotal step involved in both BMP-2 and cyclic AMP-induced chondrogenesis. *J. Cell Physiol.* 170:153-165.
26. **Lev, R. and S.S. Spicer.** 1964. Specific staining of sulfate groups with Alcian blue at low pH. *J. Histochem. Cytochem.* 12:38-41.
27. **Miller, A.D.** 1992. Retroviral vectors. *Curr. Topics Microbiol. Immunol.* 158:1-23.
28. **Miller, D.G., M.A. Adam and A.D. Miller.** 1990. Gene transfer by retrovirus vectors occurs only in cells that are actively replicating at the time of infection. *Mol. Cell Biol.* 10:4239-4242.
29. **Miller, N. and R. Vile.** 1995. Targeted vectors for gene therapy. *FASEB J.* 9:190-199.
30. **Morgan, B.A. and D.M. Fekete.** 1996. Manipulating gene expression with replication competent viruses. *Method Cell Biol.* 51:185-218.
31. **Odorizzi, G. and I.S. Trowbridge.** 1994. Recombinant Rous sarcoma virus vectors for avian cells. *Methods Cell Biol.* 43A:79-97.
32. **Pacifici, M., E.R. Golden, M. Iwamoto and S.L. Adams.** 1991. Retinoic acid induces type X collagen expression in cultured chick chondrocytes. *Exp. Cell Res.* 195:38-46.
33. **Paulsen, D.F., W-D. Chen, L. Pang, B. Johnson and D. Okello.** 1994. Stage and region-dependent chondrogenesis and growth of chick wing-bud mesenchyme in serum-containing and defined tissue culture media. *Dev. Dyn.* 200:39-52.
34. **Paulsen, D.F. and M. Solorsh.** 1988. Microtiter micromass cultures of limb-bud mesenchymal cells. *In Vitro Cell. Dev. Biol.* 24:138-147.
35. **Petropoulos, C.J. and S.H. Hughes.** 1991. Replication competent retrovirus vectors for the transfer and expression of gene cassettes in avian cells. *J. Virol.* 65:3728-3737.
36. **Resau, J.H., K. Sakamoto, J.R. Cottrell, E.A. Hudson and S.J. Meltzer.** 1991. Explant organ culture: a review. *Cytotechnology* 7:137-149.
37. **Roark, E.F. and K. Greer.** 1994. Transforming growth factor beta and bone morphogenetic protein-2 act by distinct mechanisms to promote chick limb cartilage differentiation in vitro. *Dev. Dyn.* 200:103-116.
38. **Roe, T., T.C. Reynolds, G. Yu and P.O. Brown.** 1993. Integration of murine leukemia virus DNA depends on mitosis. *EMBO J.* 12:2099-2109.
39. **Rudnicki, J.A. and A.M.C. Brown.** 1997. Inhibition of chondrogenesis by *Wnt* gene expression *in vivo* and *in vitro*. *Dev. Biol.* 185:104-118.
40. **Solorsh, M., T.F. Linsenmayer and K.L. Jensen.** 1982. Chondrogenesis from single limb mesenchyme cells. *Dev. Biol.* 94:259-264.
41. **Sonn, J.K. and M. Solorsh.** 1993. Activity of protein kinase C during the differentiation of chick limb bud mesenchymal cells. *Differentiation* 53:155-162.
42. **Stott, N.S. and C.M. Chuong.** 1997. Dual action of sonic hedgehog on chondrocyte hypertrophy: retrovirus mediated ectopic sonic hedgehog expression in limb bud micromass culture induces novel cartilage nodules that are positive for alkaline phosphatase and type X collagen. *J. Cell Sci.* 110:2691-2701.
43. **Widelitz, R.B., T.X. Jiang, B.A. Murray and C.M. Chuong.** 1993. Adhesion molecules in skeletogenesis: II. Neural cell adhesion molecules mediate precartilaginous condensations and enhance chondrogenesis. *J. Cell Physiol.* 156:399-411.

Received 18 August 1997; accepted 18 November 1997.

Address correspondence to:

Dr. Cheng-Ming Chuong
Department of Pathology
University Southern California
HMR 204, 2011 Zonal Ave
Los Angeles, CA 90033, USA
Internet: cmchuong@zygote.hsc.usc.edu