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Efficient Ectopic Gene Expression Targeting Chick Mesoderm

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Abstract

The chick model has been instrumental in illuminating genes that regulate early vertebrate development and pattern formation. Targeted ectopic gene expression is critical to dissect further the complicated gene interactions that are involved. In an effort to develop a consistent method to ectopically introduce and focally express genes in chick mesoderm, we evaluated and optimized several gene delivery methods including implantation of 293 cells laden with viral vectors, direct adenoviral injection, and electroporation (EP). We targeted the mesoderm of chick wing buds between stages 19 and 21 (Hamburger and Hamilton stages) and used β-galactosidase and green fluorescent protein (GFP) to document gene transfer. Expression constructs using the CMV promoter, the β-actin promoter, and vectors with an internal ribosomal entry sequence linked to GFP (IRES-GFP) were also compared. Following gene transfer, we monitored expression for up to three days. The functionality of ectopic expression was demonstrated with constructs containing the coding sequences for Shh, a secreted signaling protein, or Hoxb-8, a transcription factor, both of which can induce digit duplication when ectopically expressed in anterior limb mesoderm. We identified several factors that enhance mesodermal gene transfer. First, the use of a vector with the β-actin promoter coupled to the 69% fragment of the bovine papilloma virus yielded superior mesodermal expression both by markers and functional results when compared to several CMV driven vectors. Secondly, we found the use of mineral oil to be an important adjuvant for EP and direct viral injection to localize and contain vector within the mesoderm at the injection site. Lastly, although ectopic expression could be achieved with all three methods, we favored EP confined to the mesoderm with insulated microelectrodes (confined microelectroporation - CMEP) because vector construction is rapid, the method is efficient, and results were consistent and reproducible.

Keywords: Chicken, ectopic gene expression, mesoderm, adenovirus, electroporation, technique
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Introduction

The chick has been an important model in which to study genes that regulate early vertebrate development and pattern formation. Furthermore, the avian embryo is accessible and can be manipulated throughout embryonic development. This accessibility provides a unique opportunity not only to monitor, but also to perturb gene expression. A number of techniques have been used to introduce genes ectopically. Carrier molecules such as lipofectamine can easily be employed to introduce expression plasmids, however, transfection efficiency is low and reagents can be toxic for *in vivo* application (Nagahiro, Mora, Boasquevisque, Scheule, and Patterson, 2000). In contrast, viral vectors provide a robust translation mechanism as well as an efficient route for cellular entry via specific receptors. Retroviruses have been used, but are limited to relatively small genes (2-4 kb), transduce only cells that are actively dividing, and integrate the transgene randomly into the host DNA (de la Pompa and Zeller, 1993). Adenoviruses have high transduction efficiency and allow for integration of up to 8 Kb of genetic material (Horwitz, 1996). The transduced genes are episomal and thus do not disrupt normal gene structure or expression. However, adenoviral vectors do exhibit species and tissue specific (epithelium) preferences (Tomko, Johansson, Totrov, Abagyan, Frisen, and Philipson, 2000). In addition, the generation of viral vector constructs can be time consuming.

Electroporation (EP) recently has been employed for the introduction of genes into the chick embryo (Momose, Tonegawa, Takeuchi, Ogawa, Umesono, and Yasuda, 1999); (Itasaki, Bel-Vialar, and Krumlauf, 1999); (Yasuda, Momose, and Takahashi, 2000); (Atkins, Wang, and Burke, 2000); (DeLise and Tuan, 2000); (Swartz, Eberhart, Mastick, and Krull, 2001); (Swartz ME, Eberhart J, Pasquale EB, Krull CE, 2001). In EP, current creates micropores in the

cell membrane and drives the charged DNA into cells. As a current conductor, mesoderm appears to be less well coupled than epithelium and we found that DNA injected into the mesoderm and electroporated with electrodes that traverse the epithelium had a propensity to transfect the surface epithelium rather than the underlying mesoderm.

In an effort to create a relatively simple technique that would consistently allow focal temporal and spatial expression of ectopic genes in chick mesoderm, we evaluated and modified techniques using adenoviral vectors and EP. To demonstrate the functionality of these techniques, we used constructs containing the coding sequences of Shh, a secreted morphogen, or Hoxb-8, a transcription factor, both of which can induce mirror image digit duplication when expressed in the anterior limb mesoderm.

Materials and Methods

Chick embryos

White leghorn chick embryos (Hyline, Lakeview, CA) were incubated at 39^{o} C in a standard humidified avian incubator. Eggs were windowed and embryos staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951).

Adenoviral Vectors

We used replication-defective recombinant adenoviral vectors containing β-gal (AdV/β-gal) or enhanced green fluorescent protein (AdV/GFP) driven by the Cytomegalovirus (CMV) promoter (Nyberg-Hoffman, Shabram, Li, Giroux, and Aguilar-Cordova, 1997). Replication defective viruses pose a relatively low biohazard threat and maintain focal local expression. The modified human embryonic kidney tumor (293) cell line containing the adenovirus E1 region for viral assembly was used as the permissive host to replicate and package the adenoviral vectors. The 293 cells were grown to 80% confluence in 35 mm culture dishes and then transduced with 2 x10^{9} viral particles per dish (in 2 mls). The dishes were centrifuged (800 x g) to enhance viral entry (Nyberg-Hoffman, Shabram, Li, Giroux, and Aguilar-Cordova, 1997). The vectors were purified to high titers (~ 1X10^{12} viral particles/ml - vp/ml) by standard protocols. We further developed an adenoviral vector containing *Shh* (AdV/*Shh*) using the coding sequence for the secreted portion of Shh (Shh680 - (Riddle, Johnson, Laufer, and Tabin, 1993)), AdTrack and the AdEasy system (He, Zhou, da Costa, Yu, Kinzler, and Vogelstein, 1998).

Implantation of cells laden with adenoviral vectors

For implantation of vector-laden 293 cells, the cells were transduced as above, and after 30 hrs of replication (prior to cell rupture), the cells were stained with neutral red (Fisher Scientific, Pittsburgh, PA) and harvested as cell clusters/ sheets of approximately 200 μm in diameter. The transduced cell clusters were then transplanted into the forelimb bud of stage 21 chick embryos, behind the rapidly dividing progress zone. The embryos were recovered at various times after implantation (0, 30, 60 and 120 hours), and enzymatic activity assayed (β-gal vector) or visualized under fluorescence (GFP vector). Selected embryos underwent frozen section analysis to confirm the secondary transduction of chick cells adjacent to implanted 293 cell clusters.

Direct viral injection

Adenoviral vectors (at 1.0 X 10^{12} vp/ml) were mixed with equal volumes of phenol red in PBS (viral cocktail) to visualize the injected vector. Approximately 0.25μl of the viral cocktail was injected directly into the anterior margin of stage 20 wing buds using microneedles (pulled 1 mm diameter glass capillary tubes (World Precision Instruments- inside diameter 0.3 mm) using a Pointe Instrument puller. Mineral oil (Swan, by Perrigo, Allegan, MI) was used as the hydraulic fluid for the syringe. For oil-virus-oil injections, we first aspirated 0.25μl of viral cocktail, followed by ~0.05μl of oil. The mixture was then injected into the limb, with oil entering first, followed by the viral cocktail, then finally sealed with oil.

Construction of the pCX-IG vector

We used the backbone of the pCX-GFP plasmid for our electroporation protocol because it has several features that enhance the expression of the transgene in the chicken model (Ikawa, Kominami, Yoshimura, Tanaka, Nishimune, and Okabe, 1995); (Niwa, Yamamura, and Miyazaki, 1991). Specifically, we isolated the IRES-GFP insert from a previous construct, pIG (Oberg, unpublished), using a primer located upstream of the IRES sequence - pEGFP-5p1 (5'-GATCTCGAGCTCAAGCTTCG-3'), and one targeting the 3-prime end of EGFP sequence - pEGFP-3p1 (5'-GCATGGACGAGCTGTACAAGTAA-3') (both primers synthesized by Sigma Genosys). This construct contains the internal ribosomal entry

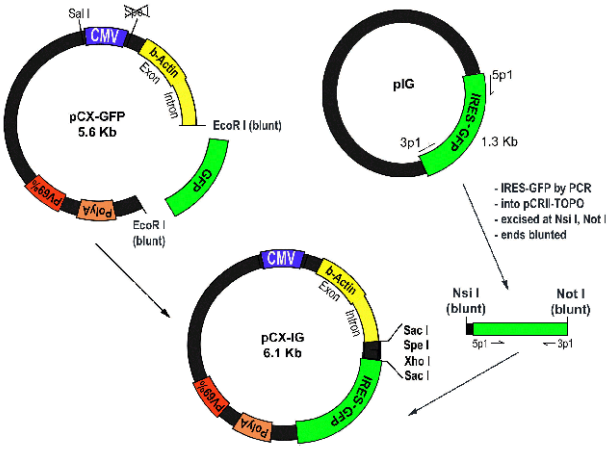

Figure 1. Construction of pCX_IG Vector.
PCX-GFP was modified by eliminating the *Spe*I restriction enzyme site and removing the GFP at *Eco*RI. As described in Methods and Materials, IRES-GFP was isolated via PCR from pIG and inserted into pCRII-TOPO. The IRES-GFP sequence was then excised at *Eco*RI and ligated into pCX backbone. The pCX-IG clone was then selected by *BamH*I restriction analysis which yields a 1.96 kb fragment confirming proper orientation.

sequence (IRES) linked to enhanced green fluorescent protein (EGFP) to heighten the tracking of insert mRNA expression. PCR generated a single 1.3 kb fragment which was inserted into pCRII-TOPO (Invitrogen). pCX-IG was formed by excising the IRES-GFP sequence from pCRII-TOPO at *Eco*RI and ligating it into pCX at *Eco*RI after destroying the *Spe*I site at base 18 (pCX cut with *Spe*I, blunted with Klenow fragment, then recircularized with ligase) (Figure 1). 293 cells were transfected with pCX-IG via lipofectamine plus reagent (Life Technologies) to check construct integrity and functionality. The new construct also contains *Xho*I and *Spe*I in the multiple cloning site (MCS) for unidirectional transgene insertion.

Confined Microelectroporation Microelectrodes

Tungsten needles (0.01 mm diameter - Omega Engineering, Stamford, CT) were sharpened and covered with red and black nail polish. The red polish was applied first, leaving only the distal 100 µm of the needle tip exposed. The outer black polish layer covered all but 30 µm of the red polish. Microelectrode insertion down to the black boundary ensured that the exposed tip is within the mesoderm and the epithelium is insulated from the current pulses (Figure 2). The microelectrodes are separated by a gap of 0.4mm.

Electroporation Protocol

Non-linearized pCX-IG constructs (0.25µl volume with a final concentration of 0.5-1 µg/µl) were injected into the anterior limb bud mesoderm of chick embryos between stage 19 and 21 (Hamburger and Hamilton, 1951) using a glass microneedle. DNA was mixed with phenol red in PBS to visualize the injection volume (*eg* 2µl of Shh-IG (2µg/µl), 1µl of pCX-GFP (1.5µg/µl), 2µl of concentrated phenol red/PBS). Approximately 0.1µl of mineral oil accompanies the DNA to seal the site of injection. Mineral oil is used as the

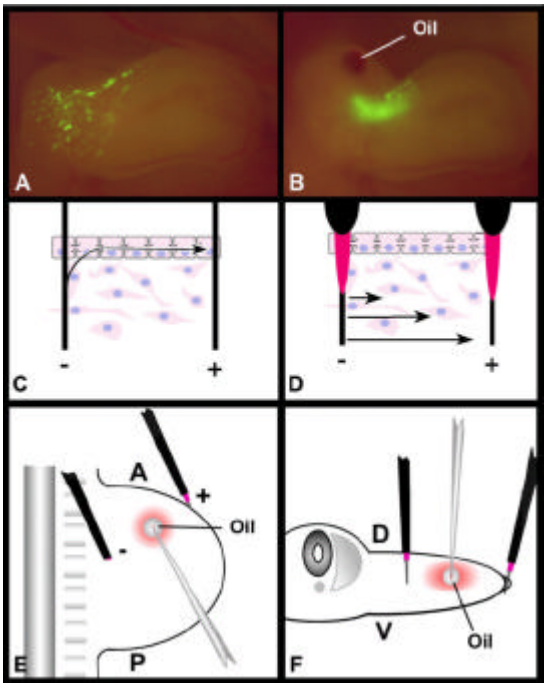

Figure 2. Electroporation and GFP Expression.
Lack of microelectrode insulation results in transfection of the epithelium (A). We hypothesized that when the microelectrodes contact the epithelium, current travels more readily through the coupled epithelial cells rather than the loosely-associated mesoderm (C). With proper insulation, current is confined to the mesoderm (D) allowing for efficient transfection (B). Thus our protocol for confined microelectroporation (CMEP) entailed injection of non-linearized expression plasmid (0.5 - 1.0 µg) mixed with phenol red/PBS for visualization. The insulated microelectrodes were then inserted, the negative (-) microelectrode within the mesoderm and the positive (+) straddling the injected DNA, within the AER or touching the tip of the limb bud (E- dorsal view showing anterior (A) and posterior (P) alignment; F transverse view showing dorsal (D) and ventral (V) alignment).

hydraulic fluid for the syringe. The injection volume is prepared by aspirating 0.25µl of plasmid/phenol red cocktail, followed by ~0.05µl of oil. The mixture is then injected into the limb, with the oil entering first, followed by the plasmid cocktail, and finally by oil (~0.05µl). The microelectrodes are then inserted, straddling the injection site. The insulated negative microelectrode (anode) is inserted near the proximal base of the limb bud mesoderm. The positive electrode (cathode) is placed at the tip of the limb bud, either between the AER and the outer ectodermal cap or just touching the tip of the limb. Insulation of the cathode is not as critical as the insulation of the anode since the current originates from within the mesoderm and thus travels through the mesoderm towards the cathode (Figure 2). Electroporation (EP) was performed using the CUY-21 Square Wave Electroporator (INMS Ltd, Tokyo, Japan;Distributor Protech Intl, Austin, TX) at voltages between 5 and 75Volts with 10 pulses of 25 ms ON/50 ms OFF. After each EP, the microelectrodes were placed into bleach and current pulsed through the electrodes to remove tissue residue. The electrodes were then dipped in RNase-free water (x2) to rinse off the bleach and 10 mM TE buffer.

To check for the effects of confined microeletroporation (CMEP) on chick embryo survival and to determine transfection efficiency, we used pCX-GFP and performed CMEP on the anterior limb mesoderm of 20 consecutive chick embryos at stage 20-21. After 24 hours the chick survival was determined, and the GFP expression present within the limb buds was visualized by a fluorescent dissecting microscope (Lieca MZ-8) and digitally recorded (Sony, DKC-5000). Fluorescent intensity was graded on a scale of 0-4 with 0 being no fluorescence, 1 being detectable fluorescence in the presence of excitation illumination only and 4 being intense fluorescence even when combined with standard light illumination.

To determine how rapidly transgene expression occurs after transfection, we used pCX-GFP and performed CMEP on 9 chick embryos at stage 20-21. We then examined the embryos for expression of GFP by fluorescence microscopy at various time points after CMEP (3, 6, 12, 24, and 72 hrs) and digitally recorded the results.

Because expression of potent marker genes does not necessarily correlate with expression of functional relevant genes, we exploited the capacity of Shh and Hoxb-8 to induce digit duplication when expressed in the anterior margin of the limb bud to test the functional adequacy of CMEP. We constructed an *Shh* expression plasmid by inserting the coding sequence of the secreted portion of chicken Shh (shh680 -(Riddle, Johnson, Laufer, and Tabin, 1993)) into the pCX-IG construct at *Xho*I and *Spe*I within the multiple cloning site, after subcloning the sequence into pCRII-TOPO. Using this pCX-*Shh*-IG construct, we performed CMEP on the anterior limb mesoderm of stage 20 chick embryos. We also generated a *Hoxb-8* expression plasmid by inserting the entire chicken coding sequence (Lu, Revelli, Goering, Thaller, and Eichele, 1997) into pCX-IG (pCX-B8-IG) as described above and carried out similar CMEP on the anterior limb mesoderm of stage 19 chick embryos.

24 hours after CMEP, we examined the embryos under fluorescent microscopy to evaluate transfection efficiency. Embryos with adequate transfection were retained for analysis of gene function. Embryos with adequate transfections were either harvested 30 hours after CMEP, and subjected to *in situ* hybridization for *Shh* or *Hoxb-8* as described by Yamada et al. (Yamada, Szendro, Prokscha, Schwartz, and Eichele, 1999) or allowed to develop until day 10 (stage 35-36) and skeletal structure was determined following 3% TCA fixation, alcian green staining and soft tissue clearing with methyl salicylate.

Results and Discussion

Implantation of 293 cells

Since the 293 cells contain the E1 portion of the adenoviral genome, "replication-incompetent" adenoviral vectors (vectors missing the E1 portion of the genome) transduced in these cells can replicate, be packaged into capsids and induce cell lysis. Implanation of viral-laden 293 cells into chick mesoderm results in secondary transduction following 293 cell lysis (Figure 3). Multiple copies of the released viral vectors can transduce the surrounding chick mesodermal cells, but these replication incompetent vectors cannot replicate, form a capsid, induce cell lysis or perpetuate the transduction. Both β-galactosidase and GFP containing viral constructs were used and demonstrated similar transduction efficiency and duration of ectopic expression. Since diffusion of the colorized product of β-galactosidase can extend beyond the cells containing the marker enzyme, it is possible that some of the expression identified was from residual 293 cells. Thus we used GFP as an intracellular marker to

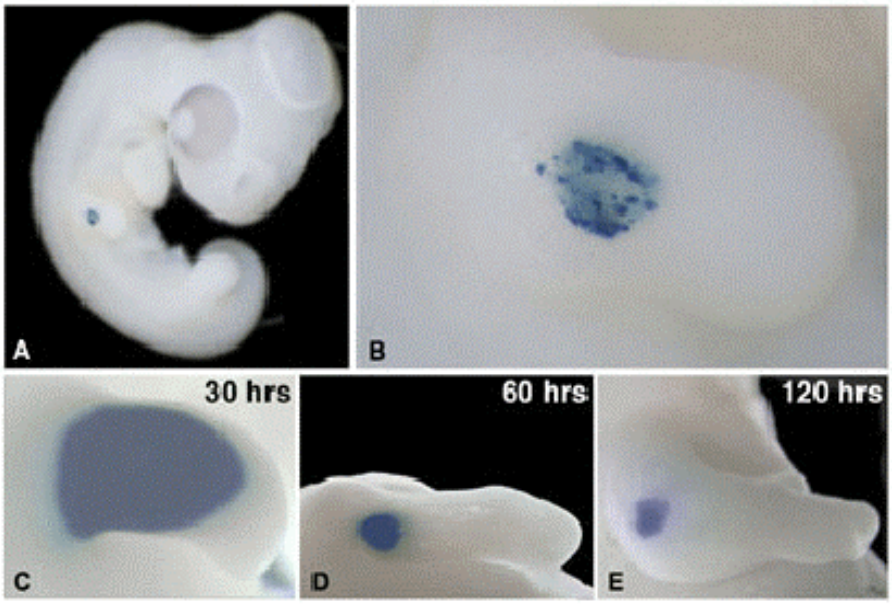

Figure 3. Beta-galactosidase Expression after 293 Cell Implantation.
A) An embryo at stage 24 following implantation of 293 cells laden with adenoviral vector containing betagalactosidase. B) At higher magnification individual cell clusters colorized by enzymatic activity can easily be identified. C) After 30 hours, viral transduction of the surrounding chick mesoderm is evident. D) At 60 hours no apparent further spread was detected. E) Enzymatic activity persists for at least 5 days after implantation.

demonstrate secondary transduction of the chick mesodermal cells. Fluorescence of individual GFP containing stellate mesodermal cells was seen surrounding the implant 30 hours after implantation.

After initial transduction of the surrounding chick mesoderm, little additional spread was detected; however, expression persisted for at least five days following implantation. 60 hours after implantation (Figure 4, B, 10X), expression within the limb was recorded, and then the limb was rapidly frozen and sectioned along the plane indicated for panels "C-E". Under fluorescence microscopy (D, E; 125X), transduction of the chick cells is identified by diffuse and focally intense GFP fluorescence in the stellate mesodermal cells. Cartilage (Crt) also demonstrated some diffuse GFP fluorescence. Histologic evaluation following Hematoxylin staining (Figure 4, C, 125X), revealed a few remnant neutral red stained 293 cells at the site of implantation. One potential problem of this technique was the scattered remnant 293 cells that persisted five days after implantation. Persistent 293 embryonic kidney cells may have the potential to generate and secrete developmentally important bioactive factors that could interfere with ectopic expression of the transgene.

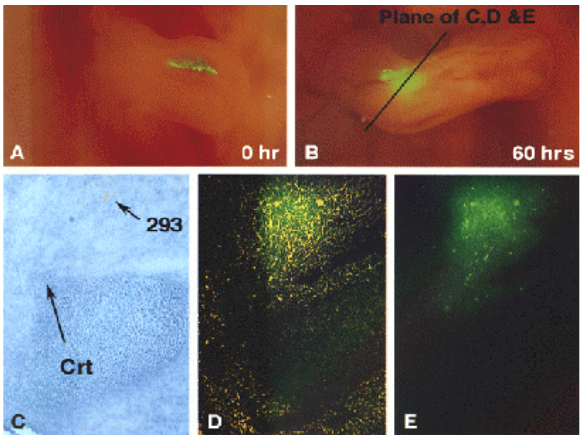

Figure 4. Expression and Localization of GFP in Chick Limb Bud Following 293 Cell Implantation.
A) GFP was visualized by fluorescence after implanting 293 cells laden with adenoviral vectors containing the GFP coding sequence. B) At 60 hours, fluorescence has increased and expression has spread to the surrounding mesodermal cells. After fluorescent imaging, the limb bud was rapidly frozen and sectioned along the indicated plane (black line). C) Sections from B following hematoxylin staining showing residual 293 cells containing neutral red dye. GFP is focally expressed in the mesodermal cells and some diffuse fluorescence is detected in the cartilage (D - fluorescence in the presence of excitation illumination and low-light phase contrast microscopy, E- fluorescence in the presence of excitation illumination only).

Direct Injection of Adenoviral Vectors

Viral vectors were injected into limb mesoderm via a glass microneedle using mineral oil as the hydraulic medium for the syringe. Direct injection, even with a significant volume (up to 1µl) of high titer (10^{13} vp/ml), resulted in insufficient transduction of chick limb mesoderm. Interestingly, several (12 out of 82) chicks showed significant expression limited to the cardiac outflow tract (Figure 5 A,B) following direct injection. This may be related to the elevated levels of cardiac coxsackievirus and adenovirus receptor (CAR) during embryogenesis (Ito, Kodama, Masuko, Yamaura, Fuse, Uesugi, Hirono, Okura, Kato, Hotta, Honda, Kuwano, and Aizawa, 2000). Alternatively, this limited and specific expression pattern may be related to the cell-specific milieu of transcription factors needed to optimize CMV promoter activity (Koedood, Fichtel, Meier, and Mitchell, 1995); (Ewart, Cohen, Meyer, Huang, Wessels, Gourdie, Chin, Park, Lazatin, Villabon, and Lo, 1997).

Fortuitously, we accidentally injected a bolus of mineral oil into the wing mesoderm while injecting the viral vector and 24 hrs later there was notable GFP expression. Of 82 limb mesoderm injections up to this point, this was the only direct injection that led to significant GFP expression. We subsequently used a vector-oil injection protocol, and accomplished consistent ectopic gene expression (Figure 5 C,D). The mechanism by which oil enhances transduction is not entirely clear. However, injection of oil appears to reduce initial diffusion of the viral-phenol red cocktail and seals the puncture site keeping the vector confined to the site of injection. Furthermore, the mineral oil may concentrate the vector to the periphery of the oil droplet. Injection of oil and vehicle alone does not induce fluorescence and does not affect developmental patterning (data not shown). Oil improved transduction with 95% (22 out of 23) exhibiting fluorescence; however, expression was weak, with only 2 out of 23 consecutive injections attaining adequate expression (score of 3 on a scale of 0-4). To demonstrate functionality of this ectopic expression, we constructed an adenoviral vector containing the coding sequence for the secreted portion of Shh, driven by the CMV promoter (Adv/*Shh*). Injection of the Adv/*Shh*

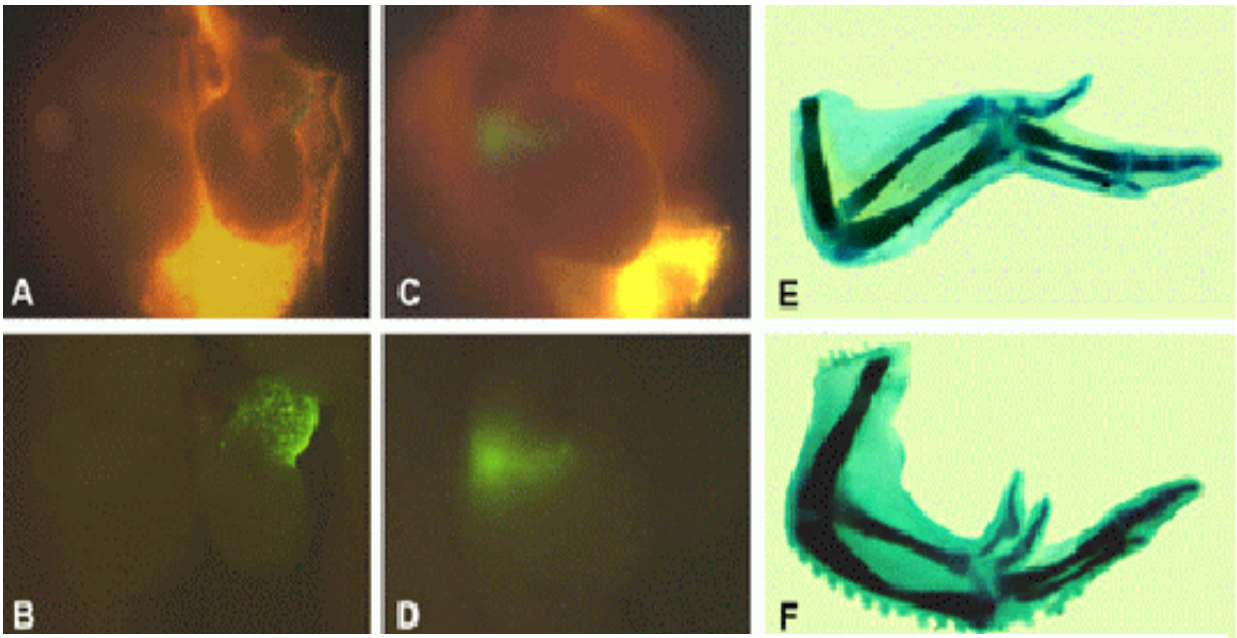

Figure 5. Direct Injection of AdV/GFP.
Direct injection of AdV/GFP alone resulted in no subsequent GFP expression within the limb bud. Occasionally, GFP expression was present within the embryo but localized only to the cardiac outflow tract (A - fluorescence in the presence of excitation illumination and standard light illumination, B- fluorescence in the presence of excitation illumination only). Localized GFP expression is seen 24 hours after injection. (C - fluorescence in the presence of excitation illumination and standard light illumination, D - fluorescence in the presence of excitation illumination only). At day 10, the limbs were stained with alcian green and soft tissue was cleared with methyl salicylate. E) Skeletal structure of day 10 chick limb (st. 35-36) after injection of adenovirus containing GFP only. The digits display the normal orientation (432). F) Injection of adenoviral vector containing the coding sequence for the secreted portion of Shh injected into the anterior limb bud via virus-oil protocol results in duplication of digit 2 (4322').

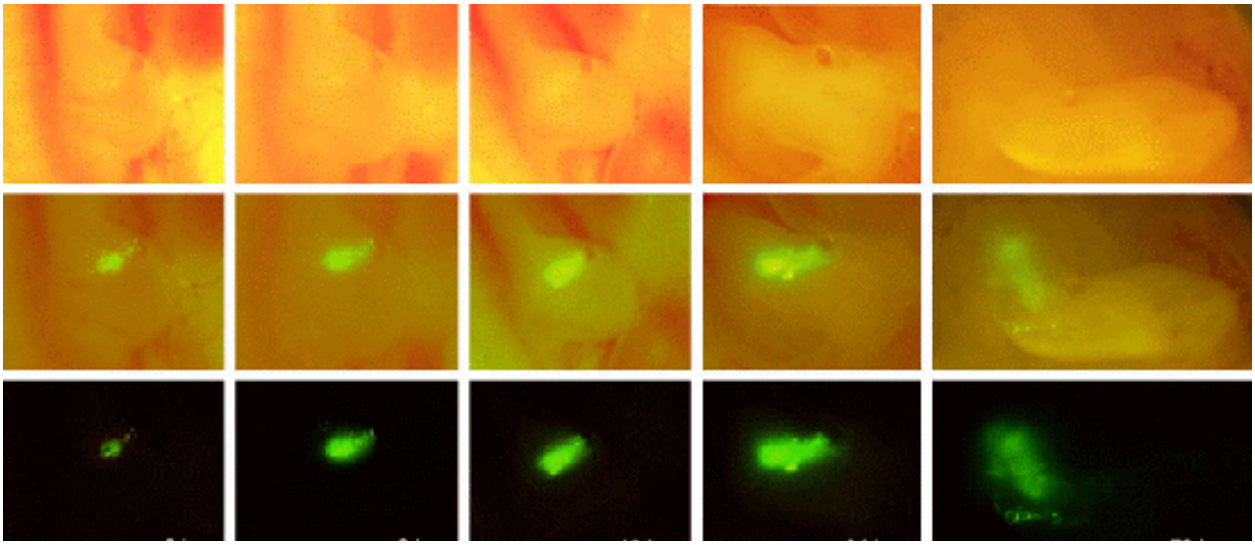

Figure 6. Time Course of GFP Expression in Chick Limb Buds.
Three hours after CMEP, fluorescence of expressed GFP can be detected within the limb bud. The intensity of fluorescence progressively increases indicating accumulation of expressed GFP. By 24 hours, expression and fluorescence peak. By 72 hours, the fluorescence has dissipated somewhat as growth redistributes the transfected cells (top row - standard light illumination; middle row - fluorescence in the presence of excitation illumination combined with standard light illumination; bottom row - fluorescence in the presence of excitation illumination only).

vector into the anterior mesoderm of stage 20 chick limb buds via the vector-oil protocol induced ectopic expression, confirmed by green fluorescence and subsequent digit duplication (Figure 5 F). The extent of mirror duplication was limited to digit 2 and present in only 10% of chicks injected. We attribute the limited expression, translation and extent of duplication to the poor performance of the Cytomegalovirus (CMV) promoter in mesodermal cells (see below).

Confined Microelectroporation - Localizing Transfection to Mesoderm

Pilot electroporation studies demonstrated a tendency for epithelium, rather than mesoderm, to be transfected (Figure 2 A). In an effort to direct and limit current flow through the mesoderm rather than the epithelium, we engineered needle-sharp microelectrodes that were exposed only at their tips (distal 100 μm). In addition we used mineral oil, as with direct viral injection, to seal the injection site and confine the DNA/dye cocktail to the site of injection. Thus, we refer to this technique as confined microelectroporation (CMEP) to denote the restricted current flow, the use of oil to confine the DNA/dye cocktail and the use of microelectrodes during EP (Figure 2 B). Transgenic expression following CMEP is more rapid than with adenoviral vectors and can be easily visualized with GFP as a marker 3 hours

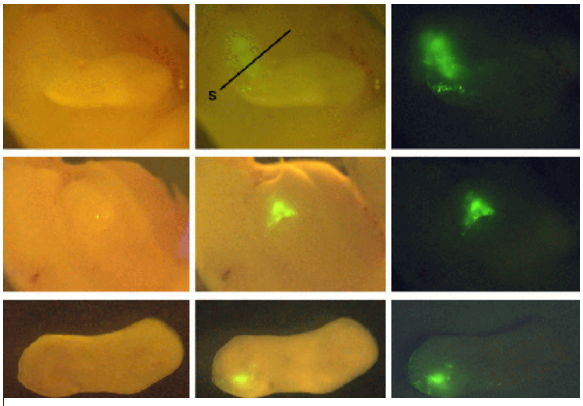

Figure 7. Confirmation of Restricted Mesodermal Expression.
To confirm restricted mesodermal expression, the limb from Figure 6 at 72 hours (top row) was sectioned transversely as indicated (black line -S- in middle image, top row). Expression in the upper limb stump is restricted to tissue deep within the mesoderm (middle row). The amputated limb displays similar restricted mesodermal expression (bottom row). Although a few transfected epithelial cells are evident, greater than 90% of the transfected cells are localized to the mesoderm (left column - standard light illumination; middle column - fluorescence in the presence of excitation illumination combined with standard light illumination; bottom column - fluorescence in the presence of excitation illumination only).

after transfection (Figure 6). Expression is maximal by about 24 hours, remains localized to the site of injection within the mesoderm (Figure 7) and persists for up to 8 days following transfection (data not shown).

Confined Microelectroporation - Optimizing Transfection Efficiency

Optimal voltage was determined empirically from 5-75V, with good transfection evident from 25V through 75V. The size of the electrode and amount of the electrode exposed for current transfer is related to electrode resistance and thus current flow with larger electrodes (with less resistance) being able to generated more current at lower voltages (Current=voltage/resistance). For example, 0.01 mm tungsten electrodes with 100 μm of the tip exposed have a resistance of about 3 ohms, while 100 μm of a 0.4 mm electrode has a resistance of 1.3 ohms. Thus, to deliver the same current the voltage for the smaller electrode must be nearly three times greater. One of the advantages of the CUY-21 electroporation unit is its capacity to measure resistance, allowing voltage to be modified to maintain a desired current flow.

Morphologically, at 75V, a residual white rim of tissue developed adjacent to the positive microelectrode after CMEP, indicating cellular coagulation. Reduction of voltage reduced the apparent coagulation. In limbs receiving CMEP at 50V, only a mild indentation in the anterior margin near the site of positive microelectrode placement was seen (data not shown). At 24 hours, GFP expression was prominent, and when chicks were harvested at day 10, no evidence of structural limb deficiencies were identified.

Previous work by others (Eichele, 1989) (Hinchliffe, Garcia-Porrero, and Gumpel-Pinot, 1981) demonstrated that much of the anterior limb bud at stage 20 could be removed without causing a significant impact on limb development. Although tissue loss initially restricts overall growth of the limb when compared to the opposite wing, growth and development have “caught up” by day 10, and no significant defect remains. Locations other than the anterior limb bud may be less forgiving with loss of organizing or developmentally critical tissue, thus we recommend the lowest possible voltage to accomplish adequate transfection.

Although successful EP induced transfection can be described on a binary scale (i.e. Yes or No) with a single cell being considered a positive outcome, for the technique to be useful in dissecting genetic pathways, ectopic expression must involve an adequate quantity of targeted cells that exhibit adequate transgene expression. We graded fluorescence on a scale of 0-4 with 0 being none, 1 being detectable by excitation illumination only and 4 being bright expression under fluorescence and standard light illumination. We defined a minimum adequate transfection/expression (T/E) as a 100 μM diameter of mesodermal cells (which contains roughly 2,000 cells) with intense (>3+) fluorescence. In twenty sequential CMEP transfections, 94% of the surviving chicks displayed fluorescence, with 82% fulfilling our stated criteria for adequate T/Es (Table 1).

Optimizing the Expression Vector for Electroporation

Chick ID	Flrsnc Rel	Int
1	Y	3
2	Dead	
3	Y	2-
4	Y	3+
5	N	0
6	Y	4
7	Y	4+
8	Y	2
9	Y	4
10	Y	4+
11	Y	3
12	Y	2+
13	Y	4+
14	Dead	
15	Y	3
16	Y	3+
17	Y	3
18	Y	4+
19	Y	4
20	Y	3

Table 1. Evaluation of GFP Expression 24 Hours After CMEP

Of 20 consecutive chicks undergoing CMEP, 2 died (90% survival) with all but one of the surviving chicks demonstrating GFP expression/fluorescence (Flrsnc) at the site of EP (94%). The relative intensity (Rel Int) was graded on a scale of 0-4 with 0 being no fluorescence, 1 being detectable fluorescence in the presence of excitation illumination only and 4 being intense fluorescence even when combined with standard light illumination.

One of the advantages of the adenoviral system is the capacity for the viral backbone to efficiently enter a cell via the membrane bound CAR receptor. High titer delivery of adenoviral vectors allows multiple copies of the vector to enter the cell via this receptor-mediated transduction and thus, high copy numbers per cells can be achieved. Unfortunately, the adenoviral receptors are better expressed in epithelium, and our target tissue was mesoderm (Tomko, Johansson, Totrov, Abagyan, Frisen, and Philipson, 2000). In contrast, electroporation overcomes receptor-mediated internalization through current-generated membrane micropores. The current also drives the negatively charged DNA into the cell cytoplasm.

However, non-viral transfection generally yields low copy numbers per cell and thus relatively low levels of gene expression. In an effort to enhance intracellular gene expression, we obtained an expression plasmid that contained the 69% fragment of bovine papilloma virus which has been reported to induce cytoplasmic replication of the plasmid in eukaryotic cells (from 100-300 copies/cell) (Ikawa, Kominami, Yoshimura, Tanaka, Nishimune, and Okabe, 1995;Niwa, Yamamura, and Miyazaki, 1991).

We further modified the pCX backbone by the addition of an internal ribosomal entry sequence (IRES) linked to GFP downstream of the insert cloning site (Figure 1). The IRES allows the insert gene and marker gene to be translated from the same transcript. However, ribosomal binding to the IRES is less efficient than at the 5' capped end of the mRNA. Although useful in generating and verifying constructs *in vitro,* as well as, monitoring the transfection of the construct *in ovo* after 48 hrs, the reduced IRES related translation of GFP limited early visualization. We found that co-transfection with 0.3 μg/μl of pCX-GFP aided in early detection of transfection efficiency and functionally correlated with subsequent construct transfection (Figure 8).

This expression vector also contained the chicken β-actin promoter. β-actin is a ubiquitously expressed cytoskeleton protein (Mohun, Brennan, and Gurdon, 1984) and while the CMV promoter appears to target a select subset of cells based on CAR receptor expression and cell-specific transcription factors, the β-actin promoter appears to be useful in a broader population of cells (Sawicki, Morris, Monks, Sakai, and Miyazaki, 1998);(Quitschke, Lin, DePonti-Zilli, and Paterson, 1989). In chick mesoderm, wing buds transfected with GFP driven by the β-actin promoter had a higher percentage of adequate T/Es (90% - 9 of 10) than wings transfected with CMV driven GFP (29% - 6 of 21). Furthermore, expression intensity was 4+ in 5 of the 10 wings with the β-actin-GFP, while none of the wings with CMV driven GFP exhibited more than 3+ score (Figure 8).

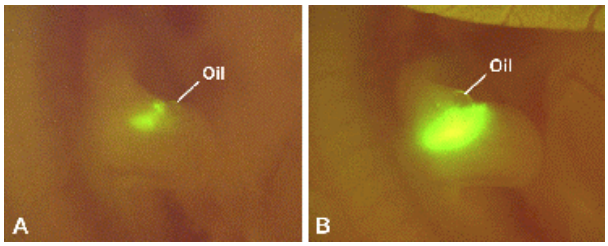

Figure 8. Comparison of CMV and ?ß-actin Promoters Driving GFP Expression.
GFP expression driven by the CMV promoter (A, AdTrackCMV) as compared to the ß–actin promoter (B, pCX-GFP) 24 hrs after transfection. Expression intensity is graded 3 for the wing bud in panel "A" and 4+ for the wing bud in panel "B".

Ectopic Expression by CMEP - Testing Functional Adequacy

To test the functional adequacy of CMEP in ectopic mesodermal gene expression, we inserted the coding sequence to the secreted portion of Shh (Shh680-(Riddle, Johnson, Laufer, and Tabin, 1993) into pCX-IG. The secreted portion of Shh is the active component of the zone of polarizing activity (ZPA), which establishes the anterior-posterior limb pattern. When ZPA grafts with as few as 100 Shh producing cells are transplanted into the anterior limb mesoderm, mirror image distal limb duplication occurs (digit pattern 43234)(Riddle, Johnson, Laufer, and Tabin, 1993). CMEP with the β-actin driven *Shh* construct (1μg/μl in 0.25μl) consistently induced mirror image duplications (Figure 9) (100% in 53 consecutive transfections with adequate T/Es).

In situ hybridization for *Shh* showed mesodermal expression in the expected region of CMEP (data not shown). Interestingly, in several transfections with only focal epithelial or subepithelial expression generating an inadequate T/E by our criteria, digit duplication occurred. We concluded that robust overexpression of *Shh* even in a few well-positioned cells, may be adequate for this secreted factor to induce digital duplication. To test further the functionality of CMEP in ectopic

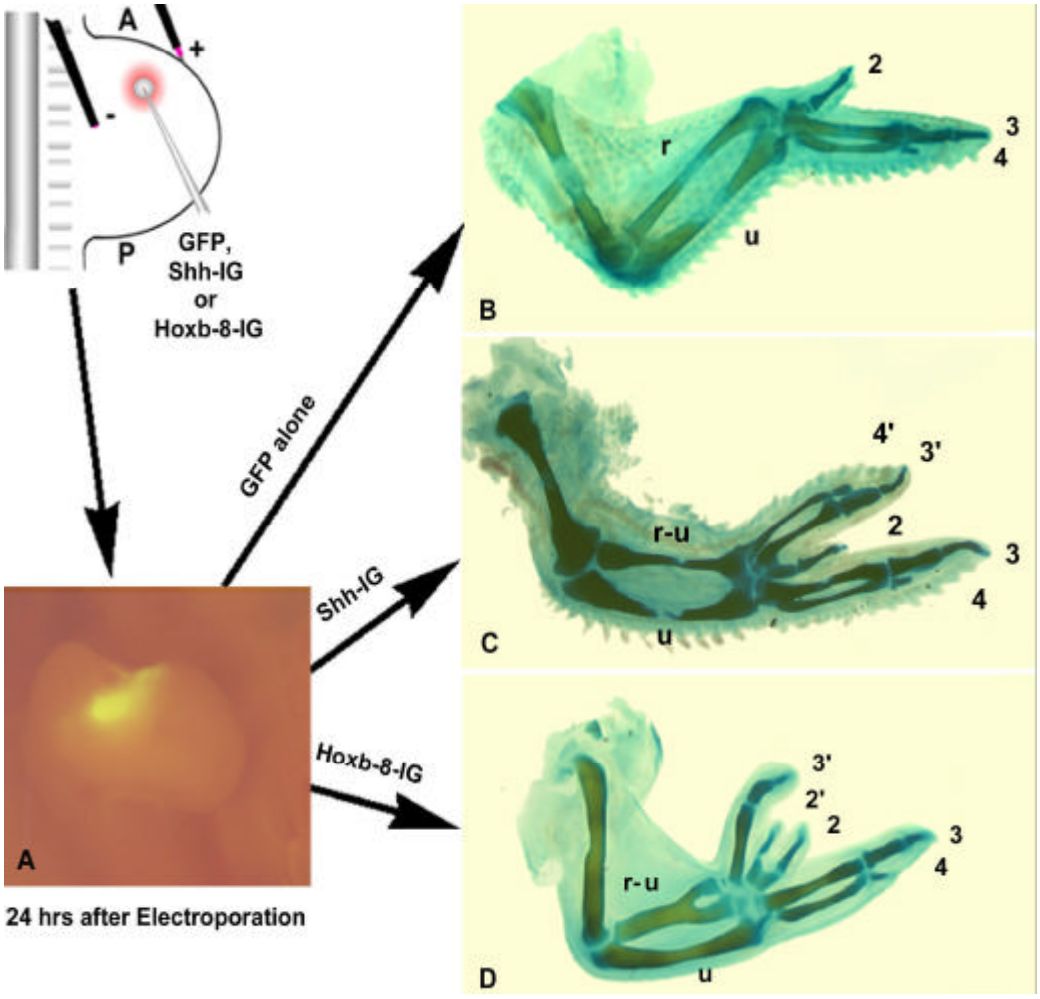

Figure 9. CMEP of *Shh* and *Hoxb-8*: Correlation Between GFP Expression and Functional Morphology with Digit Duplication

24 hours after electroporation with pCX-GFP, pCX-*Shh*-IG or pCX-*Hxb8*-IG, efficiency of transfection was determined by GFP expression (A) chicks with abundant ectopic expression were monitored for changes related to the ectopic transgene expression. With GFP only expression a normal chick wing develops (B) with a single radius (r), ulna (u) and three uniquely identifiable digits (digits 4,3 & 2). Ectopic transfection of *Shh* (C) induced transformation of the radius into an ulna (r-u) and mirror image digit duplication (4323'4'). Ectopic transfection of *Hoxb-8* (D) resulted in transformation of the radius into an ulnar (r-u) with bifurcation of the distal head and duplication of digits 2 and 3 (4322'3').

mesodermal gene expression, we focused on a transcription factor that would require endogenous action within cells to initiate distal limb duplication.

Charite and coworkers (Charite J, de Graaff W, Shen S, and Deschanmps J, 1994) demonstrated that anterior ectopic expression of a homeobox transcription factor, Hoxb-8, within the lateral plate mesoderm was adequate to induce the production of a ZPA within the forelimb mesoderm with subsequent Shh production and distal limb duplication. Thus, we inserted the *Hoxb-8* coding sequence into the pCX-IG expression plasmid (Figure 1). CMEP of this *Hoxb-8* construct into the anterior limb bud of stage 19 chicks induced distal limb duplications (4/9) (Figure 9) indicating that the ectopic expression was indeed functionally significant.

Conclusions

We have explored and optimized several delivery methods to introduce focal ectopic gene expression in chick mesoderm. Implantation of viral-laden 293 cells was capable of confining the viral load to a discrete targeted area. When coupled with a unique oil/vector/oil injection method, direct adenoviral vectors were also able to transduce target mesoderm focally. Electroporation (EP) was also optimized to yield efficient focal ectopic transfection of limb mesoderm. To optimize EP, we insulated tungsten microelectrodes to confine the current to the

targeted mesoderm, then confined the injected DNA/dye cocktail to the site of injection by co-injection with mineral oil similar to our viral injection method. Collectively, we termed these modifications confined microelectroporation (CMEP). We further demonstrated the capacity for focal ectopic expression of functionally significant developmental genes by both adenoviral vectors and CMEP.

To optimize efficiency of transfection or transduction, several parameters of the expression construct were evaluated. CMV as the sole promoter has been commonly used for expression constructs, however, we found the pCX vector, with a CMV enhancer region linked to the chicken β-actin promoter, to be superior to standard CMV driven expression plasmids for mesodermal expression in chicks. In addition, the ability to increase the construct copy number per cell, and thus expression, through efficient transduction efficiency or intracellular replication has been a distinct advantage enjoyed by viral vectors; however, the pCX vector developed by Niwa and coworkers (Niwa, Yamamura, and Miyazaki, 1991) also includes the 69% fragment of the bovine papilloma virus which was shown to impart intracellular replication capacity to a non-viral expression construct.

One other modification to our protocols was the inclusion of GFP as a marker of transfection efficiency. This allowed us to select efficient transfections with adequate localized expression for further evaluation and helped us differentiate between genetic and technical impotency.

Although focal ectopic expression could be achieved by all three methods, we found that the construction time for viral vectors (even with the AdEasy system) significantly exceeded the construction time of expression plasmids using pCX-IG. Furthermore, the biosafety concerns of adenoviral handling limit its widespread use despite the use of replication-defective viral vectors. It is not yet known how the size of the pCX-IG plasmid constructs will affect CMEP transfection efficiency. Thus, we currently favor the CMEP technique as a rapid and efficient means in which to focally express ectopic genes up to 1 kb. We feel that this technique will be a important tool in unraveling the complicated genetic interactions that regulate vertebrate development and pattern formation.

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